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Community series in novel insights into immunotherapy targeting tumor microenvironment in ovarian cancer, volume I

Edited by
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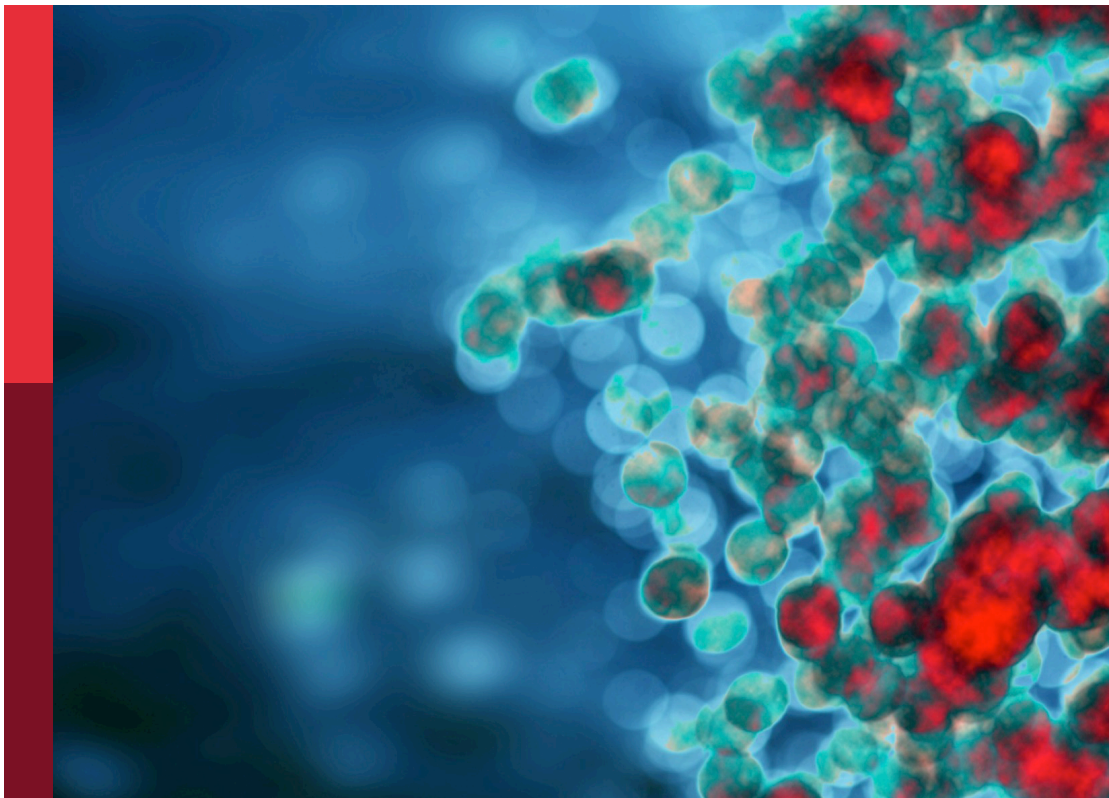

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Community series in novel insights into immunotherapy targeting tumor microenvironment in ovarian cancer, volume I

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Editorial: Community series in novel insights into immunotherapy targeting tumor microenvironment in ovarian cancer: volume I

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KEYWORDS

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Editorial on the Research Topic
Community series in novel insights into immunotherapy targeting tumor microenvironment in ovarian cancer: volume I

Ovarian cancer (OC) is a lethal gynecologic malignancy with an extremely low 5-year survival rate of approximately 50% (1). Although technological advancements have led to a decline in OC mortality over the past decade, it still remains unsatisfactory (2). In addition to traditional treatments such as surgery, radiotherapy, and chemotherapy, immunotherapy has made an indelible mark in the field of anti-tumor therapy (3), particularly as tumor microenvironment (TME) is proposed as an important factor in the initiation and progression of ovarian cancer. Immunotherapies targeting TME seems to be promising approach for OC treatment (4). In this Research Topic, we gathered 12 articles to providing us in-depth evaluations of immunotherapies targeting the dysfunctional immune cells, malignant stroma cells, tumor-promoting soluble factors, and exosomes in the OC microenvironment.

The rapid development of sequencing technology has provided a new and convenient way for us to investigate the molecular and immune changes in OC. In the first article of this topic, Ren et al. used single-cell sequencing to integrate a comprehensive cellular and immunological analysis using paired ascites, tumor and peripheral blood samples. The authors highlighted the key role of immunosuppressive cells, including MDSCs, γδT cells, along with CD8+ effector T cells, in recurrence and chemoresistance ascites. The study also emphasized the chemotherapy induced clonal expansion change of TCR/BCR in peripheral blood. These findings aid to develop new immune-modulatory strategies for patients with relapses or chemo-resistant OC.

OC is usually associated with local and distant metastases, rendering a systemic disease with functional and compositional changes in immune system. Thus, the review provided by Rajtak et al. stands apart from other researchers, as they integrated both local and systemic immunity in OC immunotherapy. The authors raised several points for our attention, including the lack of a large patient cohort both locally and systemically, and mechanisms of tumor cell circulation will be studied in the future.

Besides immune cells, dysfunctional malignant stroma cells and tumor-promoting soluble factors are important therapeutic targets in TME and have attracted great research and clinical interest (5). Novel cancer immunotherapy insights were mined from big data by Chen et al. They figured out a set of potential immunotherapeutic target genes in OC. Single-cell sequencing data shows that some of these target genes are mainly expressed in cancer-associated fibroblasts (CAFs), which are tightly associated with the immunotherapy response of OC patients. This study provides new insights into OC immunotherapy. However, it is unfortunate that the population in this study was mainly white. We hope that multiracial study could be given more attention in the future. Another important stroma cell in TME, cancer-associated mesothelial cells (CAMs), is discussed by Zheng et al.. They summarized the key roles of CAMs in OC progression, prognosis and targeting therapy. This review helped with our continued understanding of CAMs in OC and the development of new and effective therapeutic regimens. As a soluble regulator factor in TME, interferons (IFNs) can influence most of the cells in TME. Liu et al. reviewed the multiple effects of IFNs in OC therapy. They proposed that IFNs can assist anti-ovarian cancer therapy by directly affecting the function and survival of tumor cells and immune cells. Based on the summary of the literature, the authors still had confidence in the treatment of OC with IFNs as therapeutic.

Exosomes are extracellular vesicles measuring 30–100 nm, secreted by living cells. As one of the messengers between tumor cells and their surroundings, they have received significant attention in anti-tumor therapy (6). In this Research Topic, Gong et al. comprehensively described the immunotherapy related biomarkers on exosomes isolated from various body fluids of OC patients and reviewed the vital roles of exosomes in OC immunotherapy and diagnosis. In addition, Luo et al. revealed the anti-tumor effects of NK cell-derived exosomes (NK-EXOs) in OC, demonstrating that the anti-tumor activity of NK-EXOs is not only through the high-efficient up-taken of ovarian tumor cells, but also through reversing NK cells immunosuppression in TME. As a natural stability, low immunogenicity, and tumor targeting vector, NK-EXOs can efficiently deliver DDP to ovarian tumor cells and show a great prospect in OC targeting therapy. This research began with clinical isolation and characterization of exosomes and then verified their findings with a variety of laboratory techniques, making this research very solid.

Apart from exosomes, nanoparticles as another small carrier have been expected to play significant roles in immunotherapy. The nanoparticles can not only directly induce tumor cell death, which promotes antigen presentation and immune activation, but also be used as excellent drug delivery system (DDS) to help with targeting immunotherapy agent delivery (7). Various formulations of DDS have been designed to realize the controlled and targeted immunotherapy agent delivery in OC. In this topic, Peng et al. discussed the research and clinical way to modulate OC microenvironment with DDS. The authors described strategies to improve the efficacy of immunotherapy in OC with DDS, especially by targeting TME. Xu et al. focused on the different nanomaterials used in OC immunotherapy and the promising advances they induced in TME modulating.

Immune checkpoint blockade (ICB) therapy has been a popular anticancer treatment strategy for decades and made an indelible mark in tumor immunotherapy. However, resistance and low-response to ICBs restrict their application in OC treatment, and immune-related adverse events (irAEs) complicate treatment (8). In this Research Topic, we received several reviews focusing on clinical ICB therapy. Wang et al. focused on how the TME component, especially immune cells, influenced the ICB therapy response in OC. To avoid irAEs caused by ICB therapy, Xu et al. reviewed the most up-to-date information on prognostic and predictive biomarkers for ICB therapy and gave valuable advice for guiding precision immunotherapy. Hu et al. provided an overview of various clinically oriented forms of multi-immunotherapy in relation to OC and explored possible combinations of immunotherapies that may be effective, which is of utmost importance to OC clinical multi-immunotherapy.

In recent years, significant progress in lab and clinical research has been achieved, although immunotherapies in OC are still being tested in clinical trials. With the help of all these authors in this topic, we have gained a deeper and broader understanding of TME targeting immunotherapy in OC.

Author contributions

Conceptualization and writing, X-JD and XL; review and editing, H-TW and QZ; supervision, XL and X-WW. All authors have read and agreed to the published version of the manuscript.

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Conflict of interest

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

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Exploration of the underlying biological differences and targets in ovarian cancer patients with diverse immunotherapy response

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Background: Preclinical trials of immunotherapy in ovarian cancer (OC) have shown promising results. This makes it meaningful to prospectively examine the biological mechanisms explaining the differences in response performances to immunotherapy among OC patients.

Methods: Open-accessed data was obtained from the Cancer Genome Atlas and Gene Expression Omnibus database. All the analysis was conducted using the R software.

Results: We firstly performed the TIDE analysis to evaluate the immunotherapy response rate of OC patients. The machine learning algorithm LASSO logistic regression and SVM-RFE were used to identify the characteristic genes. The genes DPT, RUNX1T1, PTPRN, LSAMP, FDCSP and COL6A6 were selected for molecular typing. Our result showed that the patients in Cluster1 might have a better prognosis and might be more sensitive to immunotherapy, including PD-1 and CTLA4 therapy options. Pathway enrichment analysis showed that in Cluster2, the pathway of EMT, TNFα/NF-kB signaling, IL2/STAT5 signaling, inflammatory response, KRAS signaling, apical junction, complement, interferon-gamma response and allograft rejection were significantly activated. Also, genomic instability analysis was performed to identify the underlying genomic difference between the different Cluster patients. Single-cell analysis showed that the DPT, COL6A6, LSAMP and RUNX1T1 were mainly expressed in the fibroblasts. We then quantified the CAFs infiltration in the OC samples. The result showed that patients with low CAFs infiltration might have a lower TIDE score and a higher proportion of immunotherapy responders. Also, we found all the characteristic genes DPT, RUNX1T1, PTPRN, LSAMP, FDCSP and COL6A6 were upregulated in the patients with high CAFs infiltration. Immune infiltration analysis showed that the patients in Cluster2 might have a higher infiltration of naive B cells, activated NK cells and resting Dendritic cells.

Conclusions: In summary, our study provides new insights into ovarian cancer immunotherapy. Meanwhile, specific targets DPT, RUNX1T1, PTPRN, LSAMP, FDCSP, COL6A6 and CAFs were identified for OC immunotherapy.

KEYWORDS

ovarian cancer, immunotherapy response, cancer-associated fibroblasts, machine learning, prognosis

Introduction

Ovarian cancer (OC) represents the seventh most frequent women malignancies around the world (1). Multiple factors contribute to the development of OC, including hormone levels, reproductive factors, genetic susceptibility, environmental exposure, and lifestyle (1). For earl-stage OC, surgery remains the best treatment option and can improve patient long-term survival (2). However, only about 20% of OV patients can be diagnosed and treated early due to unusual symptoms (2). Unfortunately, due to the characteristics of high invasion and metastasis, the prognosis of advanced OC is extremely poor (3).

Combined palliative surgery and chemotherapy are often used to treat advanced OC, aiming to reduce patient pain and prolong survival. In many cases, however, this benefit is limited (4). Despite the use of targeted therapy drugs such as bevacizumab and PARP inhibitors in OC treatment, the 5-year survival rate is still less than 50% (5). Moreover, over the past few decades, survival rates for OC have not been significantly increased (5). There has been considerable progress in immunotherapy in the past ten years, bringing revolutionary changes to the management of solid tumors (6). Although immunotherapy for OC has not been approved yet, with the rapid development of immune checkpoint blockade, cancer vaccine and adoptive cell therapy, there have been a large number of pre-clinical trials of OC immune checkpoint inhibitor therapy, for example, NCT03353831, NCT01772004 and others (7). According to tumor biomarker stratification, identifying sensitive/resistant subgroups might improve immunotherapy response prediction. In light of the experience of other solid tumors and preclinical trials of immunotherapy for OC, these markers mainly include tumor mutation load, PD-L1, tumor infiltrating lymphocytes, homologous recombination defects, and intratumor neoantigen heterogeneity (8). Using these biomarkers to select ideal immunotherapy candidates may be the future of OC treatment.

Researchers have great convenience to investigate further with the rapid development of bioinformatics technology (9). In our study, we performed the TIDE analysis to evaluate the immunotherapy response rate of OC patients. The machine learning algorithm LASSO logistic regression and SVM-RFE were used to identify the characteristic genes. The genes DPT, RUNX1T1, PTPRN, LSAMP, FDCSP and COL6A6 were selected for molecular typing. Our result showed that the patients in Cluster1 might have a better prognosis and might be more sensitive to immunotherapy, including PD-1 and CTLA4 therapy options. Pathway enrichment analysis and genomic instability analysis were performed to identify the underlying biological difference between the different Cluster patients. Single-cell analysis showed that the DPT, COL6A6, LSAMP and RUNX1T1 were mainly expressed in the fibroblasts. Next, we found that the patients with low CAFs infiltration might have a lower TIDE score and a higher proportion of immunotherapy responders.

Methods

Data assessment

A comprehensive retrieval and data quality evaluation of the public database was carried out when the study began. Data quality assessment includes i). Probe numbers; ii). Expression profile magnitude; iii) Clinical information. Finally, the open-accessed data of The Cancer Genome Atlas (TCGA), as well as GSE51088 (10) and GSE53963 (11) from the Gene Expression Omnibus (GEO) database were selected. Detailed, the transcriptional profiling data were "STAR-Counts" form and the clinical information was "bcr-xml" form. The expression profile of GSE51088 and GSE53963 were downloaded from the link of "Series Matrix File(s)" and annotated based on the platform files (GSE51088: GPL7264; GSE53963: GPL6480). Sva package was utilized for data combination and batch effect reduction. The basic information of the enrolled patients was shown in Table 1.

Tumor immune dysfunction and exclusion

TIDE algorithm was performed to predict the underlying immunotherapy response of OV patients (http://tide.dfci.harvard.edu/). All the patients were assigned a TIDE score, in which TIDE > 0 were defined as immunotherapy non-responder and < 0 were defined as immunotherapy responders (12, 13).

TABLE 1 Basic information of enrolled patients.

Clinical Features	Number of patients (n)	Percentage (%)
Age		
<=60	326	55.5%
>60	261	44.5%
Grade		
G1	6	1.0%
G2	69	11.8%
G3	495	84.3%
G4	1	0.2%
Unknown	16	2.7^

The evaluation of the patient's response to PD-1 and CTLA4 therapy was conducted through submap analysis, which is an unsupervised subclass mapping method that reveals common subtypes between independent datasets (https://cloud.genepattern.org/gp).

Machine learning algorithm and molecular subtyping

The machine learning algorithms, including LASSO logistic regression and support vector machine recursive feature elimination (SVM-RFE) were used to identify the characteristic genes (14, 15). Molecular subtyping was conducted based on the ConsensusClusterPlus package in R software.

Pathway enrichment analysis and genomic instability

Gene Set Enrichment Analysis (GSEA) was performed to compare the underlying biological differences between the two groups (16). The reference gene set was Hallmark, c2.cp.kegg.v7.5.1.symbols and c5.go.v7.5.1.symbols gene sets obtained from https://www.gsea-msigdb.org/gsea/downloads.jsp. Genomic instability analysis was evaluated, including the tumor mutation burden (TMB), microsatellite instability (MSI) and tumor stemness (mRNAsi and EREF-mRNAsi). ClueGO analysis is a plug-in of Cytoscape that could decipher functionally grouped gene ontology and pathway annotation networks (17).

Single sample gene set enrichment and immune infiltration analysis

Single sample gene set enrichment analysis (ssGSEA) was used to quantify the infiltration of cancer-associated fibroblasts (CAFs) (18). The reference genes was shown in Supplementary Table S1. CIBERSORT algorithm was used to quantify 22 immune cell infiltration of OC immune microenvironment (19).

Single-cell level

The analysis of the characteristic genes at the single cell level was based on the Tumor Immune Single-cell Hub website (TISCH, http://tisch.comp-genomics.org/). With TISCH, cell-type annotations at the single-cell level are available, allowing exploration of tumor microenvironments (TME) across a variety of cancer types.

Statistical analysis

All the statistical analysis was performed in R software. Kaplan-Meier (KM) survival curve was used to compare the prognosis difference between two groups. The receiver operating characteristic (ROC) curve was utilized to evaluate the prediction ability of specific features. The significance of a difference was determined by the p-value ($p < 0.05$). Student T-tests were performed on data with normal distribution. Non-normal distributions were tested with the Mann-Whitney U test.

Results

Identification of the characteristic gene of immunotherapy response

The flow chart of our whole study was shown in Supplementary Figure S1. TIDE analysis was firstly performed based on the OC patients in TCGA database, in which TIDE > 0 were defined as immunotherapy non-responder and < 0 were defined as immunotherapy responders (Figure 1A). LASSO logistic regression and SVM-RFE algorithms were utilized to screen the characteristic genes of patients in the immunotherapy responder group (Figures 1B-D). Finally, these two algorithms identified 34 characteristic genes (Figure 1E).

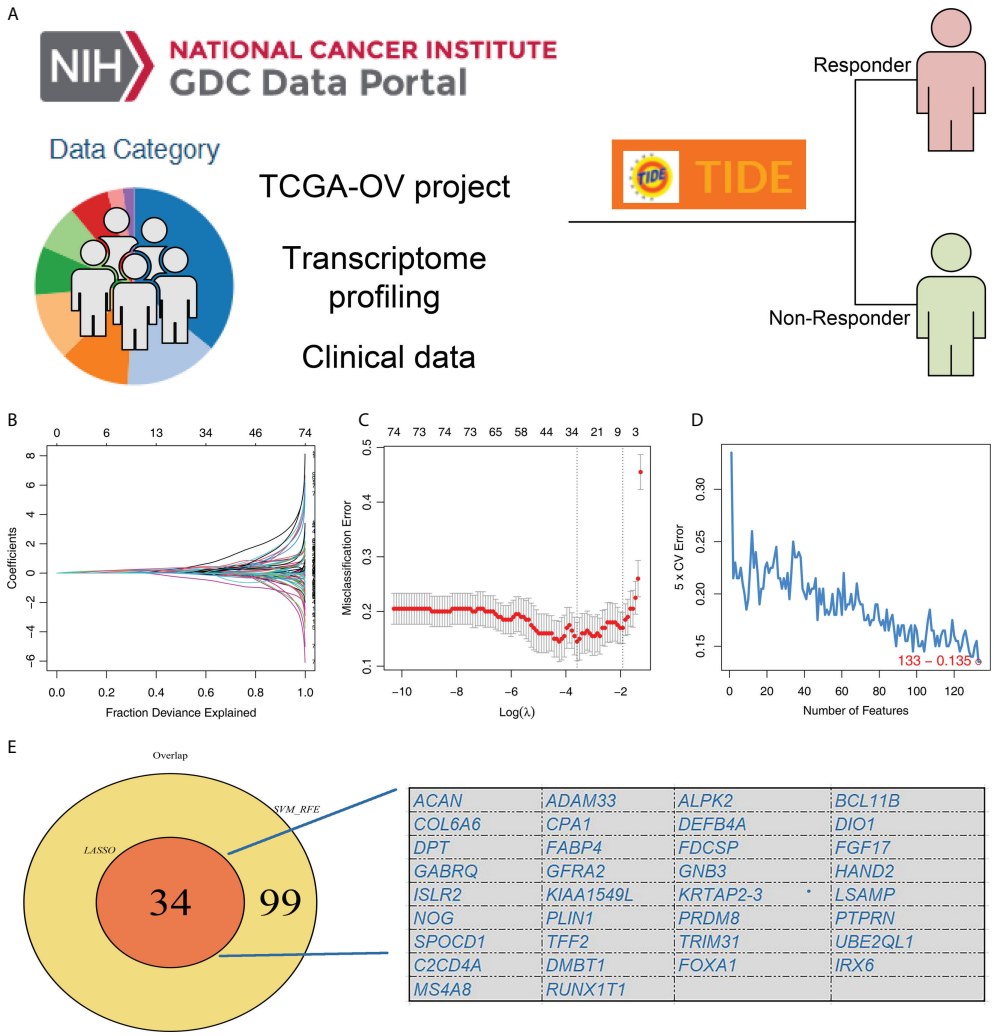
Molecular typing

Our goal is to identify the patients with different prognosis and immunotherapy response rates by clustering samples. Next, we performed the univariate Cox regression analysis and the characteristic genes DPT, RUNX1T1, PTPRN, LSAMP, FDCSP and COL6A6 were identified for molecular typing (Figure 2A).

In detail, the ConsensusClusterPlus package was used for molecular typing in the patients of TCGA database (Figure 2B and Supplementary Figure S2). In all subtypes, dividing patients into two subtypes provides the best differentiation (Figure 2C). The KM survival curve showed that the patients in Cluster2 might have a worse prognosis (Figure 2D). Also, we found that the patients in Cluster2 might have a higher TIDE score than those in Cluster1 (Figures 2E, F). Moreover, DPT, RUNX1T1, PTPRN, LSAMP, FDCSP and COL6A6 all showed a good prediction ability of patients immunotherapy response (Figures 2G-L, DPT, AUC = 0.808; RUNX1T1, AUC = 0.785; PTPRN, AUC = 0.787; LSAMP, AUC = 0.821; FDCSP, AUC = 0.669; COL6A6, AUC = 0.765).

Patients in Cluster1 are more sensitive to immunotherapy

According to the TIDE result, we found that the proportion of immunotherapy responders in Cluster1 is 41.6%, which is greatly higher than the 11.7% in Cluster2 (Figures 3A, B). Submap algorithm indicated that the Cluster1 patients are sensitive to both PD-1 and CTLA4 therapy (Figure 3C). Meanwhile, DPT, RUNX1T1, PTPRN, LSAMP, FDCSP and COL6A6 all showed a higher expression level in immunotherapy non-responders patients (Figures 3D-I). Furthermore, we try to validate our results in the GSE cohorts. GSE51088 and GSE53963 were selected (Figure 3J). Sva package was used for data combination and batch effect reduction (Figure 3K).



ACAN	ADAM33	ALPK2	BCL11B
COL6A6	CPA1	DEFB4A	DIO1
DPT	FABP4	FDCSP	FGF17
GABRQ	GFRA2	GNB3	HAND2
ISLR2	KIAA1549L	KRTAP2-3	LSAMP
NOG	PLIN1	PRDM8	PTPRN
SPOCD1	TFF2	TRIM31	UBE2QL1
C2CD4A	DMBT1	FOXA1	IRX6
MS4A8	RUNX1T1		

FIGURE 1
Identification of the characteristic gene of immunotherapy response. **(A)** TIDE analysis was performed to evaluate the immunotherapy response of TCGA-OC patients, in which TIDE > 0 were defined as immunotherapy non-responder and < 0 were defined as immunotherapy responders; **(B, C)** LASSO logistic regression algorithm; **(D)** SVM-RFE algorithm; **(E)** LASSO logistic regression and SVM-RFE algorithms identified 34 characteristic genes.

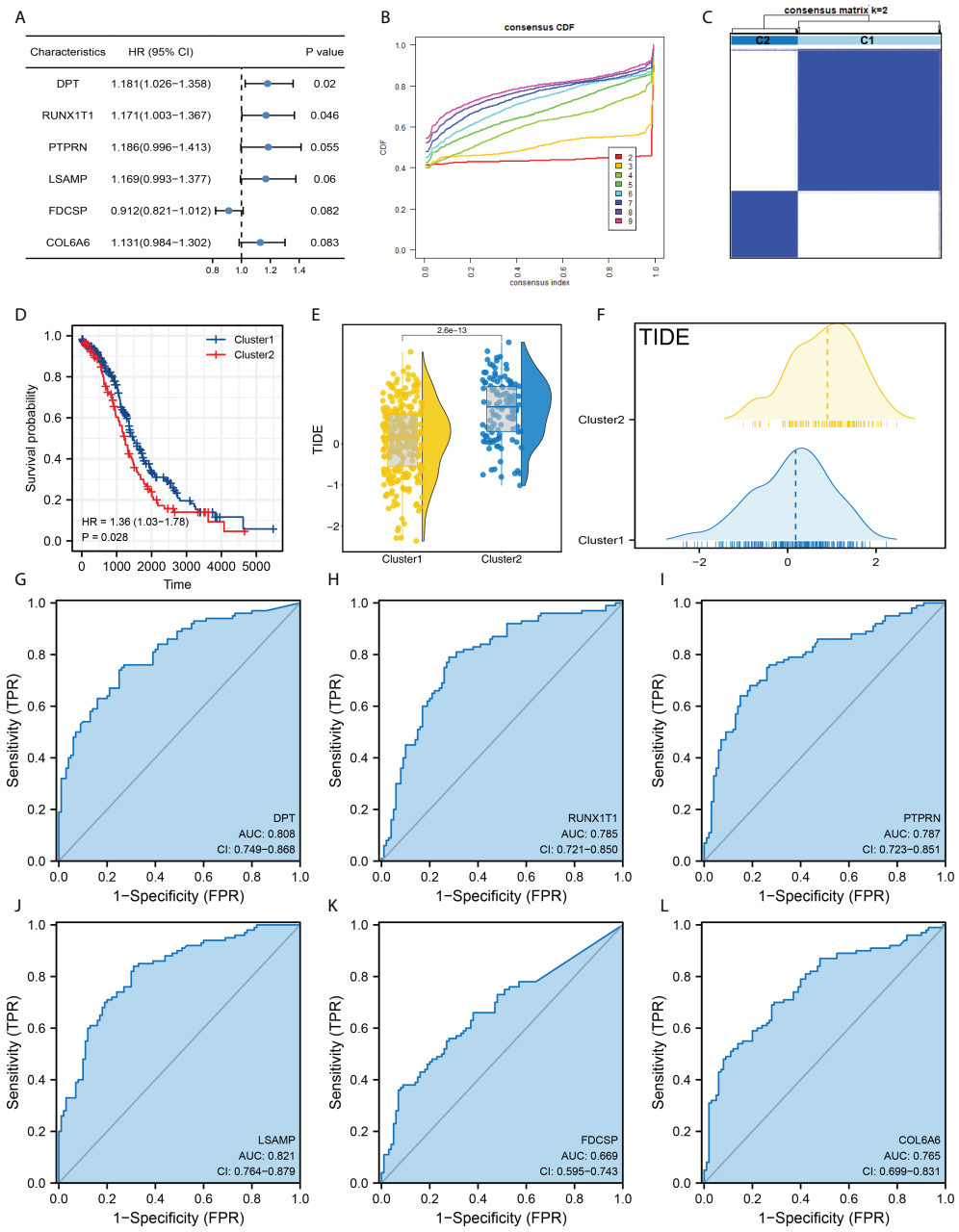


FIGURE 2
Molecular typing based on DPT, RUNX1T1, PTPRN, LSAMP, FDCSP and COL6A6. **(A)** Among all the characteristic genes, DPT, RUNX1T1, PTPRN, LSAMP, FDCSP and COL6A6 were identified for their prognosis correlation ($P < 0.05$); **(B)** ConsensusClusterPlus package was used for molecular typing in the patients of TCGA database; **(C)** Dividing patients into two subtypes provides the best differentiation; **(D)** KM survival curve of patients in Cluster1 and Cluster2; **(E, F)** The patients in Cluster2 had a higher TIDE score than Cluster1; **(G–L)** The prediction ability of DPT, RUNX1T1, PTPRN, LSAMP, FDCSP and COL6A6 on patients immunotherapy response.

Validation in combined GSE cohorts

We next performed the TIDE analysis in the combined GSE cohort (Figure 4A). Same with the result in TCGA, the patients in Cluster1 had a lower TIDE score and a higher proportion of immunotherapy responders than those in Cluster2 (Figures 4B-D and Supplementary Figure S3). KM survival curve showed that the patients in Cluster2 might have a worse survival (Figure 4E). Meanwhile, clinical correlation analysis showed that the patients in Cluster2 might have a more progressive clinical stage, but not pathological grade (Figures 4F, G).

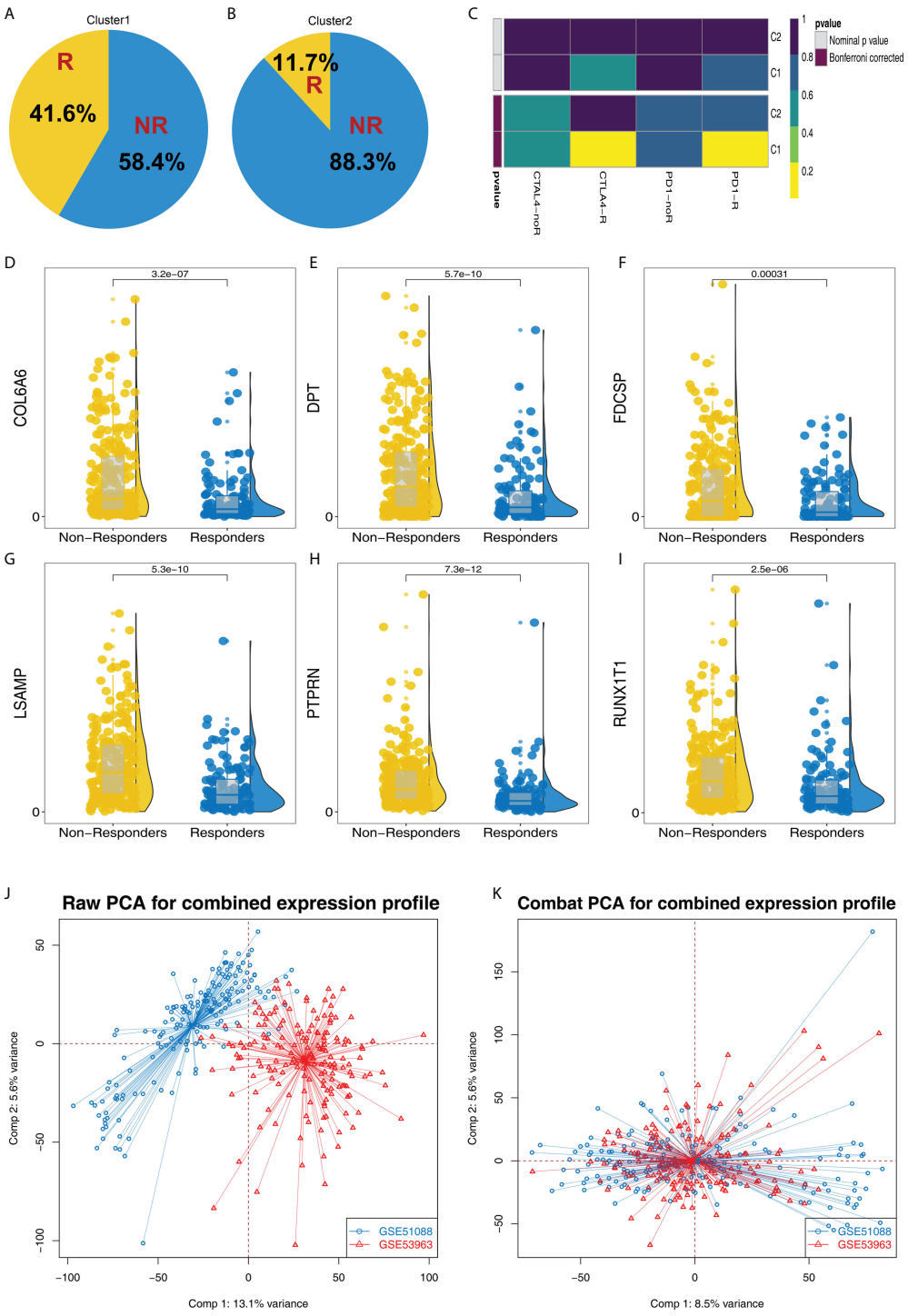


FIGURE 3
Cluster1 and Cluster2 had different immunotherapy response. **(A, B)** The proportion of immunotherapy responders and non-responders in Cluster1 and Cluster2 patients; **(C)** Submap algorithm indicated that the Cluster1 patients are sensitive to both PD-1 and CTLA4 therapy; **(D–I)** The expression level of DPT, RUNX1T1, PTPRN, LSAMP, FDCSP and COL6A6 in immunotherapy responders and non-responders; **(J–K)** Sva package was used for data combination and batch effect reduction of GSE51088 and GSE53963.

Meanwhile, no significant difference was observed between the patients in different age group (Figure 4H).

Pathway enrichment analysis

GSEA analysis showed that in Cluster2, the pathway of epithelial mesenchymal transition (EMT), TNFα/NF-kB signaling, IL2/STAT5 signaling, inflammatory response, KRAS signaling, apical junction, complement, interferon gamma response, allograft rejection were significantly activated (Figure 5A). ClueGO analysis showed that in the Cluster2, the terms of phospholipase C-activating G protein-coupled receptor signaling, regulation of sprouting angiogenesis, neural crest cell migration, sex determination, spleen development, chondrocyte development, roof of mouth development, glycosaminoglycan biosynthetic process, negative regulation of coagulation, monocyte chemotaxis, endocrine process, cell adhesion mediated by integrin, cartilage development and cardiac muscle cell contraction (Figure 5B). Gene ontology (GO) analysis showed that in the Cluster2, the terms of cellular ion homeostasis, negative regulation of cell differentiation, embryonic morphogenesis, metal ion homeostasis, positive regulation of cell death, positive regulation of locomotion, regulation of defense response, taxis, tissue morphogenesis were upregulated (Supplementary Figure S4A). Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis showed that in the Cluster2, the terms of cytokine cytokine receptor interaction, focal adhesion, chemokine signaling pathway, neuroactive ligand-receptor interaction, cell adhesion molecules cams, toll-like receptor signaling pathway, ECM receptor interaction, hematopoietic cell lineage, leukocyte transendothelial migration, leishmania infection were upregulated (Supplementary Figure S4B).

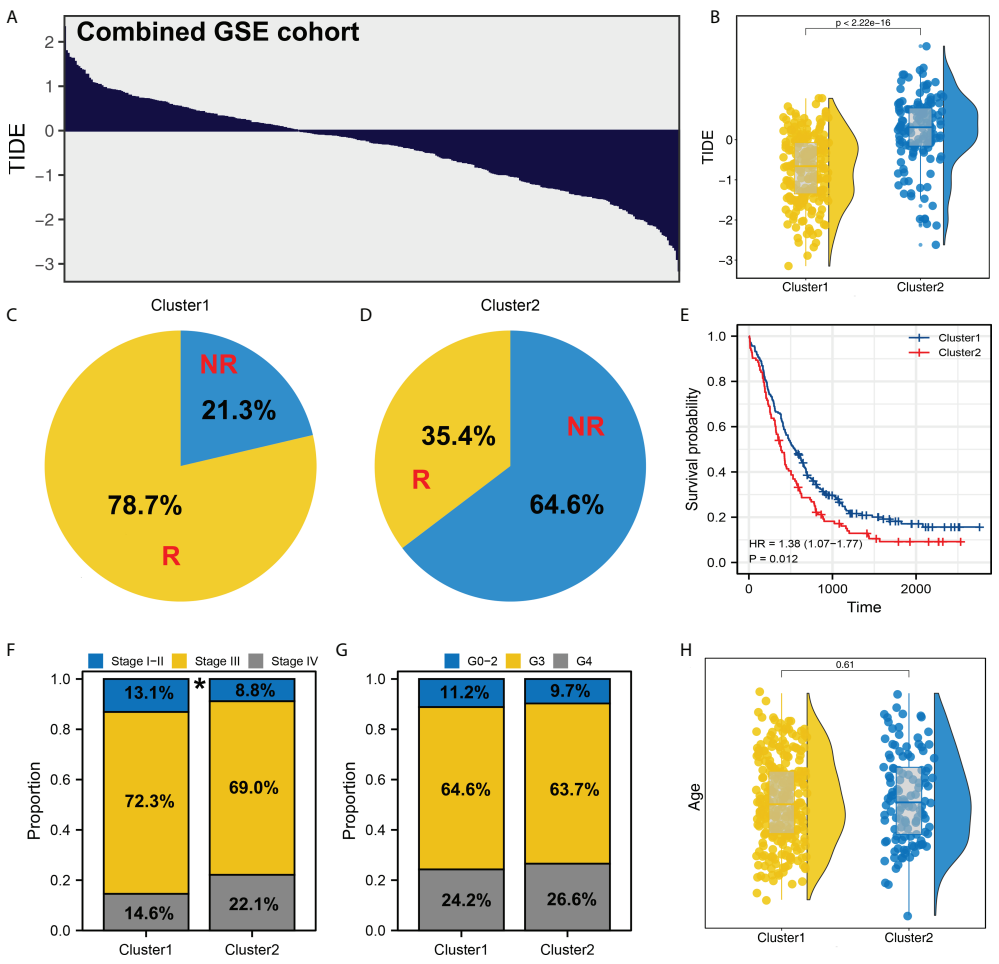


FIGURE 4
Validation in GSE cohort. **(A)** TIDE analysis was performed in the combined GSE cohort; **(B)** In the GEO cohort, Cluster2 also had a higher TIDE score; **(C, D)** The proportion of immunotherapy responders and non-responders in Cluster1 and Cluster2 patients; **(E)** KM survival curve of Cluster1 and Cluster2 patients; **(F–H)** Clinical differences between Cluster1 and Cluster2 (gender, age and grade). * = P < 0.05.

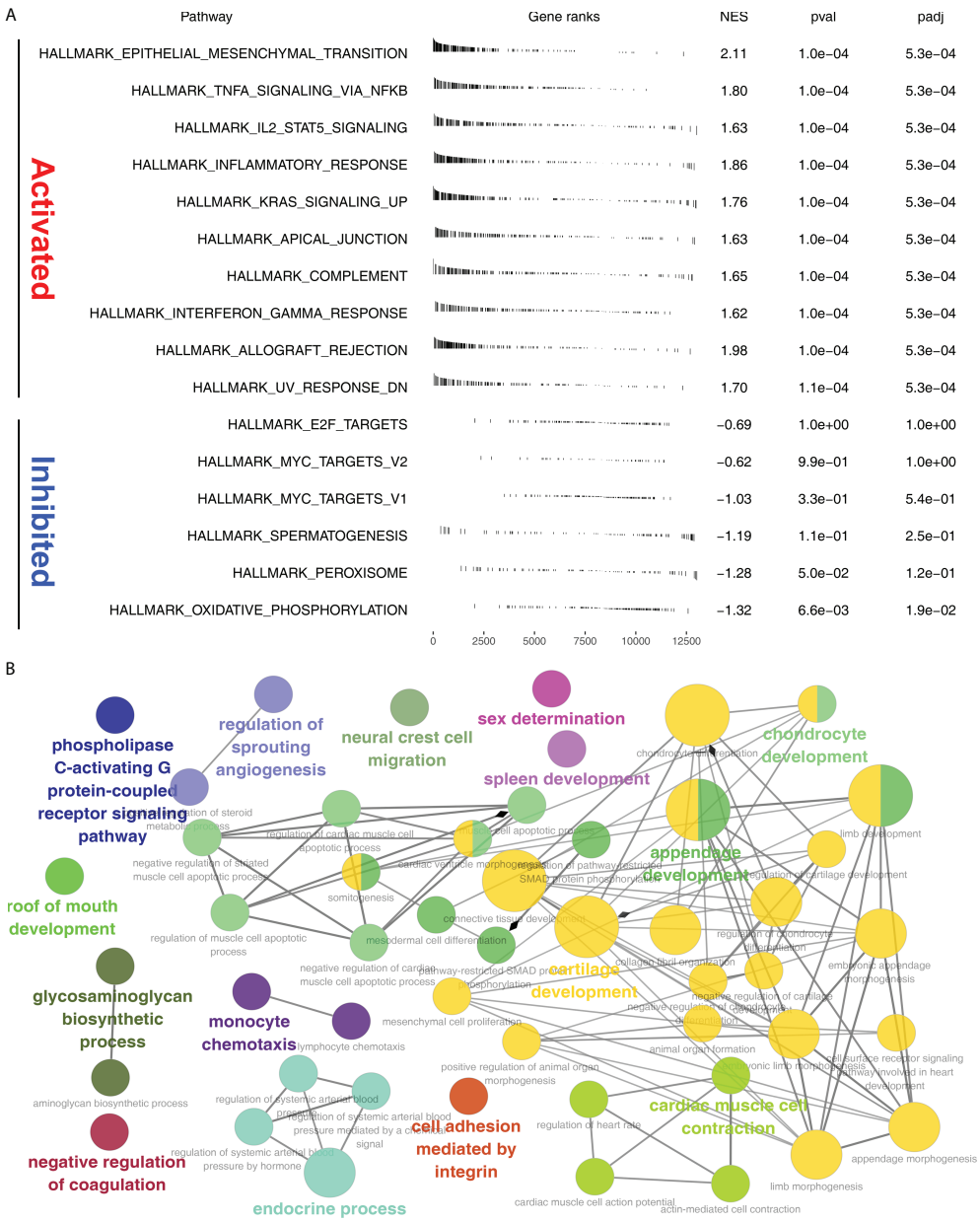


FIGURE 5
Pathway enrichment analysis. **(A)** GSEA analysis of Cluster2 based on the Hallmark gene set; **(B)** ClueGO analysis in Cytoscape software.

Genomic instability analysis

In addition, the copy number profile of the OC patients in TCGA was evaluated, including the gain/loss percentage and the gistic score (Figures 6A, B and Supplementary Figure S5). CNV burden analysis showed the patients in Cluster2 might have a higher burden of copy number loss in the focal level, while no significant difference was observed in the CNV burden of other levels (Figures 6C-F). Moreover, we found that the patients in Cluster2 had a higher TMB_score than that in Cluster1 (Figure 6G). No remarkable difference was found in MSI_score (Figure 6H). However, we noticed that Cluster1 had a higher mRNAsi score (Figure 6I). No significant difference was found in EREG-mRNAsi (Figure 6J).

CAFs is associated with the immunotherapy response of OC

We further explored the characteristic genes in the single-cell level of OC. The result showed that the DPT, COL6A6,

LSAMP and RUNX1T1 was mainly expressed in the fibroblasts both in minor-lineage and malignancy option (Figures 7A, B). Therefore, we think it would be interesting to know if CAFs could affect the immunotherapy response rate in OC patients. Then, we performed ssGSEA analysis to quantify the infiltration level of CAFs in OC patients (Figures 8A, B). In TCGA cohort, the result showed that the patients with low CAFs infiltration might have a lower TIDE score and a higher proportion of immunotherapy responders (Figures 8C, D; 46.8% vs 16.7%). The same conclusion was observed in the GSE cohort (Figures 8E, F, 75.7% vs 47.9%). Notably, the patients in Cluster2 had a higher CAFs infiltration in both TCGA and GSE cohorts, which might partly explain the higher proportion of immunotherapy non-responders in Cluster2 (Figures 8G, H). Interestingly, we found all the characteristic genes DPT, RUNX1T1, PTPRN, LSAMP, FDCSP and COL6A6 were upregulated in the patients with high CAFs infiltration (Figure 8I). Immune infiltration analysis showed that the patients in Cluster2 might have a higher infiltration of naive B cells, activated NK cells and resting Dendritic cells (Figures 8J, K). Pathway enrichment analysis showed that in the patients with high CAFs infiltration, the pathways of EMT, TNF-α signaling, apical junction, IL2/STAT5 signaling, inflammatory response, allograft rejection, KRAS signaling, myogenesis, UV response, complement were activated (Supplementary Figure S6).

Discussion

There is a huge public health impact associated with OC, especially since there are so many forms of OC, each with a unique biology and prognosis (20). Immunotherapy has shown promising application prospects in a variety of solid tumors (21).

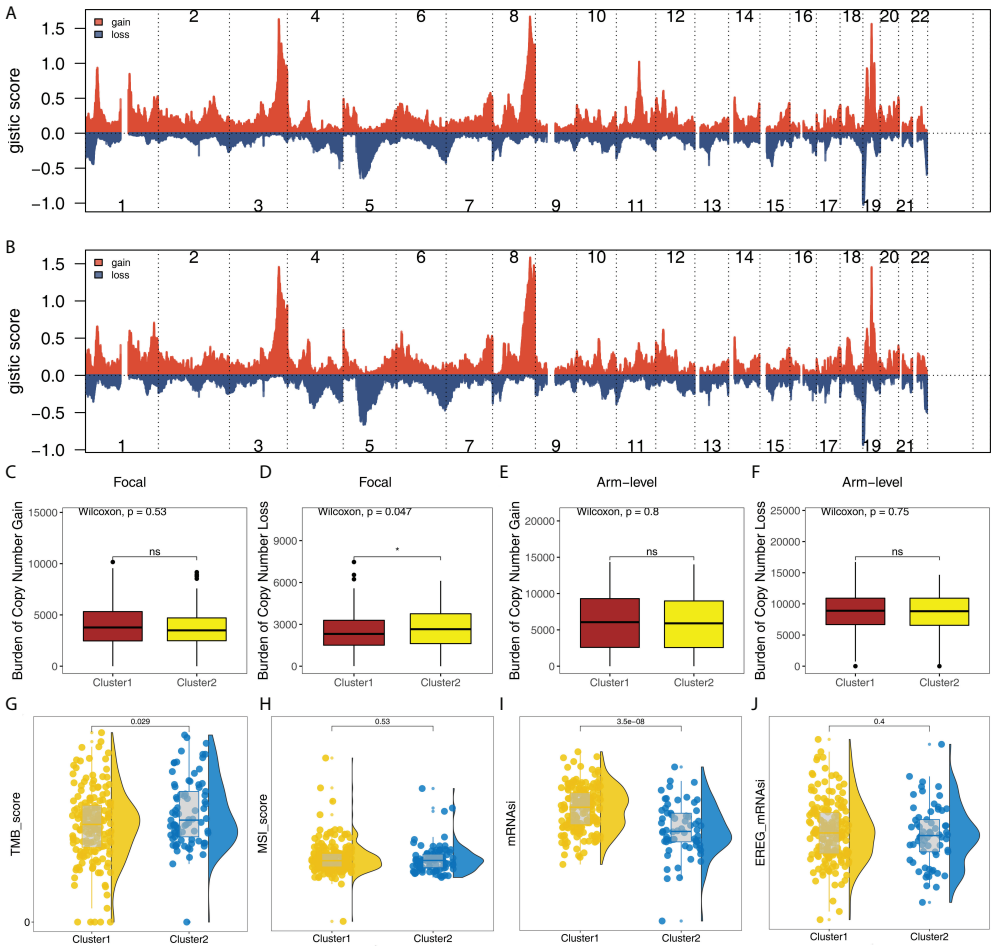


FIGURE 6
Genomic instability analysis. **(A)** The gistic score of copy number profiles of TCGA-OV in Cluster1; **(B)** The gistic score of copy number profiles of TCGA-OV in Cluster2; **(C–F)** The difference of CNV burden in focal gain, focal loss, arm-level gain and arm-level loss in Cluster1 and Cluster2 patients; **(G–J)** The difference of TMB, MSI, mRNAsi and EREG-mRNAsi in Cluster1 and Cluster2 patients. * = P < 0.05. The expanded form of ns = not significant.

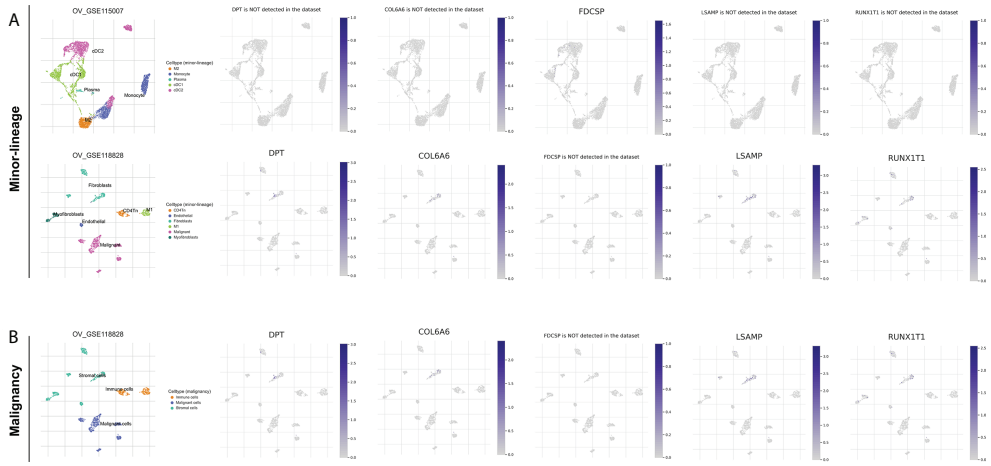


FIGURE 7
Single-cell level of DPT, RUNX1T1, LSAMP, FDCSP and COL6A6 in OC. **(A)** DPT, COL6A6, LSAMP and RUNX1T1 were mainly expressed in the fibroblasts in minor-lineage option; **(B)** DPT, COL6A6, LSAMP and RUNX1T1 were mainly expressed in the fibroblasts in and malignancy option.

Also, in OC, relevant preclinical trials have been carried out with encouraging results. Therefore, prospectively exploring the internal biological mechanisms behind the patients with different response performances to immunotherapy in OC is meaningful.

Here, we performed the TIDE analysis to evaluate the immunotherapy response rate of OC patients. The machine learning algorithm LASSO logistic regression and SVM-RFE were used to identify the characteristic genes. The genes DPT, RUNX1T1, PTPRN, LSAMP, FDCSP and COL6A6 were selected for molecular typing. Our result showed that the patients in Cluster1 might have a better prognosis and might be more sensitive to immunotherapy, including PD-1 and CTLA4 therapy options. Pathway enrichment analysis showed that in Cluster2, the pathway of EMT, TNFα/NF-kB signaling, IL2/STAT5 signaling, inflammatory response, KRAS signaling, apical junction, complement, interferon-gamma response and allograft rejection were significantly activated. Also, genomic instability analysis was performed to identify the underlying genomic difference between the different Cluster patients. Single-cell analysis showed that the DPT, COL6A6, LSAMP and RUNX1T1 were mainly expressed in the fibroblasts. We then quantified the CAFs infiltration in the OC samples. The result showed that patients with low CAFs infiltration might have a lower TIDE score and a higher proportion of immunotherapy responders. Also, we found all the characteristic genes DPT, RUNX1T1, PTPRN, LSAMP, FDCSP and COL6A6 were upregulated in the patients with high CAFs infiltration. Immune infiltration analysis showed that the patients in Cluster2 might have a higher infiltration of naive B cells, activated NK cells and resting Dendritic cells.

During the past two decades, immunotherapy has evolved rapidly and revolutionized treatment options for many cancers. Recently, immune checkpoint inhibitors have been investigated for possible use in reversing immunosuppressive TME, including CTLA4 and PD-1/L1 inhibitors (22). As oncolytic viruses, cancer vaccines, and adoptive cell therapy have advanced rapidly, immunotherapy has also gained much attention in OC therapy. Currently, most types of OC immunotherapy treatment options, like CAR-T and immune checkpoint inhibitors are in clinical trials (23). Although promising approaches have been developed for OC immunotherapy, the immunosuppressive TME still needs to be overcome to improve the effectiveness of immunotherapy (24). In our study, we found that the CAFs was tightly associated with the immunotherapy response of OC patients. Previous studies have explored the role of CAFs in cancer immunotherapy. Through Single-cell analysis, Kieffer et al. identified eight CAFs clusters and they found that PD-1 and CTLA4 proteins were upregulated by cluster 0/ecm-myCAF in regulatory T lymphocytes (Tregs), which increases CAF-S1 cluster 3/TGFβ-myCAF cellular content (25). Obradovic et al. performed scRNA-seq on the cancer tissue obtained from four advanced-stage head and neck squamous cell carcinoma patients treated with the αPD-1 therapy, nivolumab (pretreatment and posttreatment). They revealed that a significant change was observed in the abundance of fibroblasts following treatment with nivolumab and they also identified different CAFs clusters, which have a potential guiding effect (26).

Six characteristic genes were identified, including DPT, RUNX1T1, PTPRN, LSAMP, FDCSP and COL6A6. In OC, Yeh et al. found that in OC, the aberrant TGFβ/SMAD4

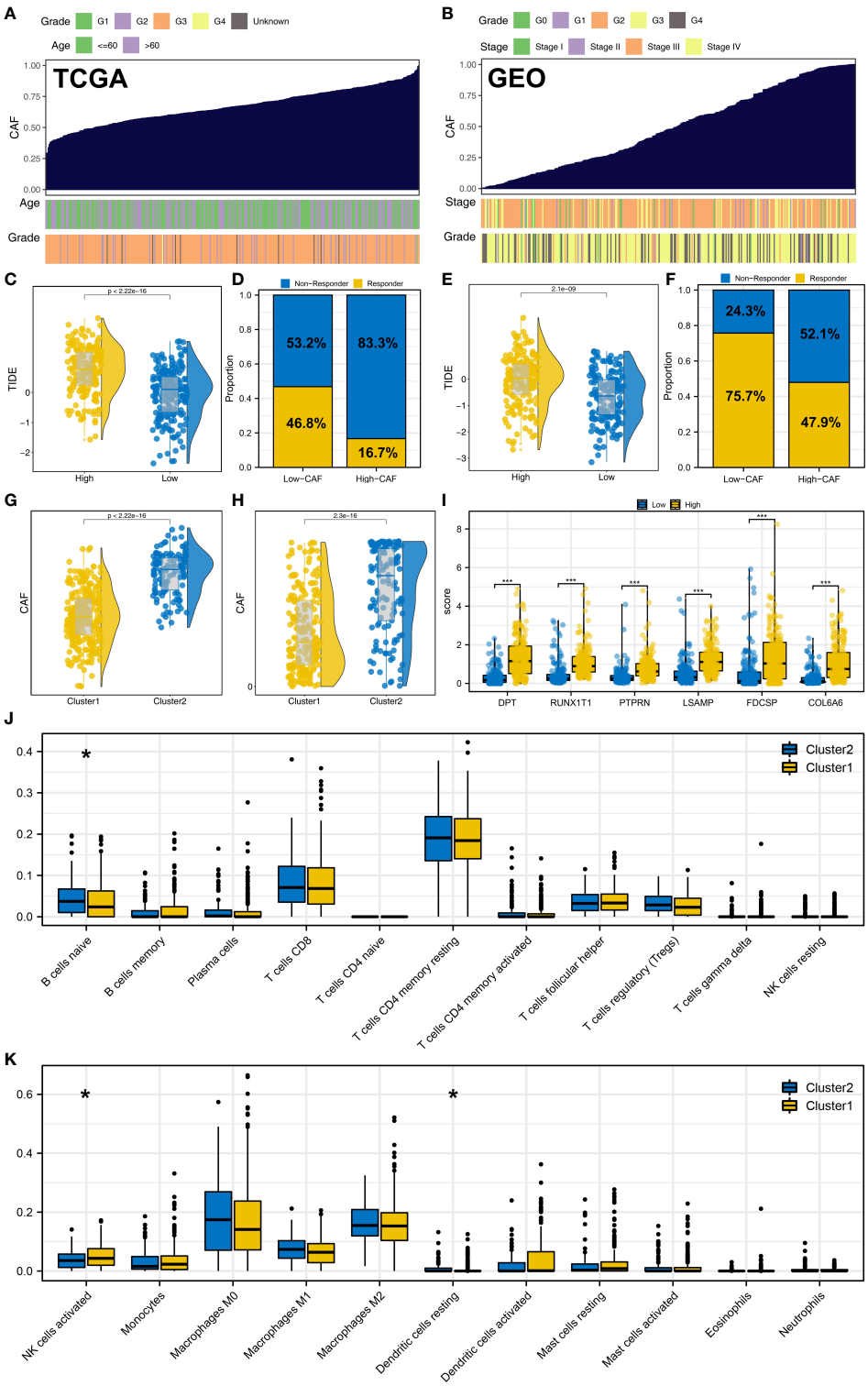


FIGURE 8
CAFs is associated with the immunotherapy response of OC. **(A, B)** ssGSEA algorithm was used to quantify the CAFs infiltration in TCGA and GSE cohorts; **(C, D)** In TCGA, patients with low CAFs infiltration had a lower TIDE score and a higher proportion of immunotherapy responders; **(E, F)** In the GEO cohort, patients with low CAFs infiltration had a lower TIDE score and a higher proportion of immunotherapy responders; **(G)** In TCGA, patients in Cluster2 had a higher CAFs infiltration; **(H)** In the GEO cohort, patients in Cluster2 also had a higher CAFs infiltration; **(I)** DPT, RUNX1T1, PTPRN, LSAMP, FDCSP and COL6A6 were upregulated in the patients with high CAFs infiltration; **(J, K)** Immune infiltration analysis of Cluster1 and Cluster2. * = P < 0.05, *** = P < 0.001.

signaling can induce epigenetic silencing of putative tumor suppressor RUNX1T1 (27). Sun et al. indicated that lncRNA EPB41L4A-AS2 hamper the development of OC by sequestering microRNA-103a and upregulating transcription factor RUNX1T1 (28). Moreover, Wang et al. indicated that FDCSP could facilitate OC metastasis by promoting cancer cell migration and invasion (29). Also, we found that DPT, COL6A6, LSAMP and RUNX1T1 were mainly disturbed in the fibroblast. Kang et al. demonstrated that COL6A6 is expressed in fibroblast and has the potential to be a target of head and neck squamous cell carcinoma (30). In osteosarcoma, Feleke et al. found that LSAMP was highly expressed in the osteoblastic osteosarcoma cells and CAFs, which have the potential to be a therapeutic target (31).

Pathway enrichment analysis showed in Cluster2, the pathway of EMT, TNFα/NF-kB signaling, IL2/STAT5 signaling was significantly activated. EMT plays an important role in promoting tumor malignant biological behavior. In OC, Wu et al. found that ST3GAL1 could contribute to migration, invasion and paclitaxel resistance in OC through EMT induced by TGF-β1 (32). Liang et al. revealed that lncRNA PTAR could promote EMT and invasion in OC by competitively binding miR-101-3p to upregulate ZEB1 expression (33). Immune infiltration analysis showed that Cluster2 had a lower infiltration level of activated NK cells. Research has demonstrated that NK cells can kill ovarian cancer cells effectively. A lower NK cells infiltration might be partly responsible for the worse prognosis of Cluster2.

Several limitations should be noted. Firstly, the population in our analysis was mainly White patients and the underlying race bias is inescapable. Asian and African large-scale sequencing data should be paid more attention in the future. Secondly, there is still no open-accessed genomic data of OC patients with immunotherapy. The response rate predicted by TIDE analysis is still affected by the bioinformatics algorithm and hard to fully reflect the real situation.

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.

Author contributions

Manuscript preparation: JC. Data collection: SC. Data analysis: XD. Chart preparation: LM and YC. Research design: WB and YS. All the authors have read and approved the final draft for submission.

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Conflict of interest

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

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2022.1007326/full#supplementary-material

SUPPLEMENTARY FIGURE 1
The flow chart of the whole study.

SUPPLEMENTARY FIGURE 2
Molucular typing based on DPT, RUNX1T1, PTPRN, LSAMP, FDCSP and COL6A6 in TCGA database.

SUPPLEMENTARY FIGURE 3
Molucular typing based on DPT, RUNX1T1, PTPRN, LSAMP, FDCSP and COL6A6 in GSE database.

SUPPLEMENTARY FIGURE 4
GO and KEGG analysis. **(A)**: GSEA analysis of Cluster2 based on the c2.cp.kegg.v7.5.1.symbols gene set; **(B)**: GSEA analysis of Cluster2 based on the c5.go.v7.5.1.symbols gene set.

SUPPLEMENTARY FIGURE 5
GISTIC plot of Cluster1 and Cluster2. **(A)**: amp_qplot of Cluster1; **(B)**: del_qplot of Cluster2; **(C)**: amp_qplot of Cluster2; **(D)**: del_qplot of Cluster2.

SUPPLEMENTARY FIGURE 6
Pathway enrichment analysis of the CAFs.

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Single-cell sequencing reveals effects of chemotherapy on the immune landscape and TCR/BCR clonal expansion in a relapsed ovarian cancer patient

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Cancer recurrence and chemoresistance are the leading causes of death in high-grade serous ovarian cancer (HGSOC) patients. However, the unique role of the immune environment in tumor progression for relapsed chemo-resistant patients remains elusive. In single-cell resolution, we characterized a comprehensive multi-dimensional cellular and immunological atlas from tumor, ascites, and peripheral blood of a chemo-resistant patient at different stages of treatment. Our results highlight a role in recurrence and chemoresistance of the immunosuppressive microenvironment in ascites, including MDSC-like myeloid and hypo-metabolic γδT cells, and of peripheral $CD8^+$ effector T cells with chemotherapy-induced senescent/exhaustive. Importantly, paired TCR/BCR sequencing demonstrated relative conservation of TCR clonal expansion in hyper-expanded $CD8^+$ T cells and extensive BCR clonal expansion without usage bias of V(D)J genes after chemotherapy. Thus, our study suggests strategies for ameliorating chemotherapy-induced immune impairment to improve the clinical outcome of HGSOC.

KEYWORDS

single-cell sequencing, ovarian cancer, chemotherapy, immune microenvironment, TCR/BCR repertoire, clonal expansion

Introduction

Ovarian cancer (OC) is the ninth most common cause of cancer mortality in women and the second most common cause of gynecologic malignancy death worldwide (1). High-grade serous ovarian cancer (HGSOC), which is one of the most common and lethal pathological types of epithelial OC, poses a challenge to women's health because of its recurrence and chemo-resistance. Platinum-based chemotherapy is the classical first-line treatment regimen for HGSOC and is usually effective initially. However, chemo-resistance eventually develops in about 70% of HGSOC patients after 3 years, leading to cancer relapse and eventually death (2, 3). Immune checkpoint blockade therapy has become a promising modality for a number of malignancies but shows limited benefits for HGSOC (4). Over the past two decades, primary cancer cells, malignant ascites, exfoliated cell clusters (also called "spheroids") and immune cells have been identified in the unique tumor microenvironment (TME) of OC and are strongly associated with intra-abdominal distal organs metastasis, tumor relapse and diverse responses to drugs (5–7). Thus, investigation of the TME and its dynamic response to chemotherapy intervention is vital for elucidating the mechanisms underlying relapse and refractoriness of HGSOC.

Recently, scRNA-seq studies regarding HGSOC have clarified its origins and heterogeneity (8, 9), including its cellular landscape in ascites or metastatic loci (10–13), as well as the correlation between molecular subtypes and prognosis (14). However, several key points of understanding the impact of chemotherapy on HGSOC remain uncovered. First, the influence of chemotherapy on tumor tissue, ascites and PBMCs and the relationship between tumor cells and the TME remain elusive. Second, although the function and subtypes of T cells in HGSOC have been identified and shown to affect prognosis (10, 15), the role of B cells in HGSOC remains uncertain. Third, while V(D)J rearrangement is known to be the basis of immune system diversity that enables responses of T/B cells to antigens (16), dynamics of the TCR/BCR repertoire upon chemotherapy remains unclear in HGSOC. Finally, though the heterogeneity and function of macrophages in HGSOC ascites has been studied (10, 11), the myeloid cell shifts in the TME during platinum-based treatment have yet to be elucidated.

To this end, we utilized scRNA-seq and TCR/BCR sequencing to analyze the cancerous composition and immune community of a tumor lesion, malignancy ascites and peripheral blood from a chemotherapy-resistant relapsed HGSOC patient with progressively shorter progression-free survival (PFS) after several courses of platinum-based chemotherapy. We focused on the intrinsic features of tumor cells and explored the state of myeloid cells and T cells in the ascites. In peripheral blood mononuclear cells (PBMCs), we identified T/B cell subtypes and characterized the dynamics of the TCR/BCR repertoire upon chemotherapy. Our study provides insight into mechanisms of chemo-resistance from the aspect of immunity, thus providing fundamental evidence for implementing immunomodulatory therapies and improving treatment response in HGSOC.

Materials and methods

Collection of patient specimens and HGSOC scRNA-seq data

Specimens were collected from a patient with recurrent HGSOC at Nanfang Hospital. This study was approved by the Ethics Committee of Nanfang Hospital (NO. NFEC-2021-424). Informed consent was obtained from the patient prior to sample collection. During the second debulking surgery, solid tumor tissue was resected, washed in Dulbecco's phosphate-buffered saline (DPBS, ThermoFisher Scientific, USA) and transported in DMEM solution. The ascites fluid was drained with a syringe and preserved in an aseptic 50 ml conical tube. Two specimens were transported on ice for further processing. After the surgery, the patient received the fourth course of platinum-based chemotherapy (six cycles), and PBMC samples were collected before and post this course of treatment. Identified patient information, including the ovarian cancer histology, stage, treatment history, Computed Tomography (CT) and Positron Emission Tomography-Computed Tomography (PET-CT) results, tumor markers and immunological indexes from peripheral blood were collected. 10X Genomics single-cell RNA sequencing data GSE154600 of five HGSOC patients (17), were download from GEO database (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE154600). The dataset includes five HGSOC samples with different chemotherapy responses (*T59, T76, T77* chemo-resistant and *T89, T90* chemo-sensitive).

Preparation of single-cell suspensions

Within 6 hours after isolation, solid specimens were enzymatically dissociated into single cells. Briefly, the tissue was minced with a scalpel and enzymatically digested using 2 mg/mL Collagenase I (Worthington Biochemical) and 2 mg/mL Collagenase IV (Worthington Biochemical) for 30 minutes in a shaker (250 rpm) at 37°C. The digestion was terminated with DMEM + 5% fetal bovine serum (Thermo Fisher Scientific). The cell suspensions were sequentially filtered through 100 μm and then 70 μm cell strainers. Red blood cells were lysed by incubating the cell suspensions in RBC Lysis Solution (Sigma-Aldrich) for 3–10 minutes at 4°C. After centrifugation and resuspension, the concentrations of the single-cell suspensions were adjusted to 7–12×10^5 cells/ml with 5% fetal bovine serum DMEM. Ascites was centrifuged for 10 min at 4°C, and the remaining pellet was resuspended in PBS, filtered, subjected to

RBC lysis, and resuspended as described for the tumor samples. Peripheral blood was collected into heparin tubes (Becton, Dickinson and Company) and processed within 2 hours of collection. PBMCs were isolated by density gradient centrifugation using Ficoll-Paque Plus medium and washed with Ca/Mg-free PBS. The isolated cells derived from above samples were used for single cell sequencing.

Preparation of single cell RNA-seq, TCR-seq and BCR-seq libraries

The suspensions of live cells in sterile-filtered PBS (Corning) with 0.04% BSA (Sigma Aldrich) were used as input for the 10× Chromium controller system (10× Genomics Inc.). Using 10× GemCode Technology, the cells were barcoded to separately index each cell's transcriptome by partitioning them into Gel Bead-in-EMulsions (GEMs). Barcoded Single Cell 50 Gel Beads, RT Master Mix with cells and Partitioning Oil were combined on a microfluidic chip, and GEMs were generated. The GEM RT reactions were activated in a thermocycler (53°C for 45 min, 85°C for 5 min, 4°C hold overnight). Post RT incubation, the GEMs were disrupted and the first-strand cDNA was recovered. cDNA amplification was performed by PCR to generate sufficient material. According to the manufacturer's instructions, scRNA-seq libraries of tumor tissue and ascites were generated using Chromium™ Single Cell 3' Library (v3 chemistry) reagents. For PBMC samples, scRNA-seq libraries were processed using the Chromium™ single cell 5' library & gel bead kit and coupled TCR/BCR libraries were obtained using the Chromium™ single cell V(D)J enrichment kit (10× Genomics). Libraries of scRNA-seq were sequenced on the Illumina Novaseq 6000.

Immunohistochemistry staining

The tumor tissues were collected from Nanfang Hospital. IHC staining was carried out with anti-CD8 antibody (18187-1-AP, 1:200 dilution; Proteintech, Rosemont, USA). The immunostaining results were examined independently by two researchers. Paraffin-embedded ovarian cancer tissues were cut into 4 μm thick sections. Histological evaluation was done with hematoxylin and eosin (H&E). Immunohistochemical staining was performed to confirm the presence of CD8 cells. Briefly, sections were deparaffinized and rehydrated using xylene and serial dilutions of EtOH in distilled water. Tissue sections were incubated in citrate buffer, pH 6 and heated in a microwave oven. Anti-CD8 (18187-1-AP, 1:200 dilution; Proteintech, Rosemont, USA) antibody was applied on tissue sections with one-hour incubation at room temperature in a humidity chamber. Antigen-antibody binding was detected with the labeled polymer-HRP Envision system (DAKO, K4007) and DAB+ chromogen (DAKO, K3468) system. Tissue sections were briefly immersed in hematoxylin for counterstaining and were covered with cover glasses.

Single cell seq data processing

Pre-processing of scRNA-seq fastq files was conducted using Cell Ranger v4.0.0 (10× Genomics). ScRNA-seq reads were aligned to GRCh38, and a count matrix of cell barcodes for downstream analysis was generated using the Cell Ranger count function with parameter–expect-cells = 3000. The raw count matrix for each sample was obtained from the Cell Ranger count filter matrix output (18). The pipeline generates a UMI count matrix, which is processed using Seurat software (4.0.5). Integrated analysis of multimodal single-cell data was achieved using previously established methods (19). The quality of cells was assessed based on three metrics parameters to remove low-quality cells and multiple-like microdropletsCells meeting the following criteria are reserved: (1) The number of total UMI counts per cell (≥500); (2) The number of detected genes per cell (≥500); and (3) The proportion of mitochondrial genes (≤25%). The remaining cells were subjected to further analyses.

Integration, dimension reduction and unsupervised clustering

Core scRNA-seq analysis was performed using Seurat v4.0.5. The counts for each library were normalized using the NormalizeData function. The most highly variable genes were selected using the FindVariable function in Seurat and a PCA matrix with 20 components employing variable genes by using the RunPCA function implemented in the Seurat package. To integrate datasets into a mutual space from different tissues for unsupervised clustering, we used the harmony algorithm, followed by PCA-reduced dimensionality. Then, the mutual nearest neighbor (MNN) was calculated. The shared nearest neighbor (SNN) algorithm, which is the default algorithm for clustering in the pipeline of Seurat, was used for clustering. It includes two steps corresponding to the two functions. First, FindNeighbors was used to calculate the K-nearest neighbors (KNN) of each cell and construct the SNN graph image. Second, FindClusters was used to find cell clusters according to the SNN graph results ("graph-based clustering"). Cells were reclustered separately according to specified parameters without engaging the other cell types. After clustering based on gene expression patterns employing the FindClusters function, cells were visualized with the RunTSNE function in Seurat. Cluster identification was performed at a resolution that best separated the different cell types. Clusters were annotated based on the expression of known marker genes of each cell type. To identify clusters within each major cell type, we performed a second round of clustering for specified cell populations. To discover the

relationship among specific samples, the expression matrix was integrated, clustered, and annotated again. The procedure of each round of clustering was the same as the first round, starting from the expression matrix, including finding the most highly variable genes, calculating the PCA matrix, as well as performing integration analysis using the harmony algorithm, dimensional reduction and unsupervised clustering analysis by Seurat.

Identification of differentially expressed genes and gene set enrichment analysis

We applied the FindAllMarkers function (test. use = Wilcox) in Seurat to identify marker genes of each cluster. For a given cluster, positive markers were compared with other cell groups. The signification threshold was set as $P<0.05$ and $|\log_2$ foldchange$|>0.25$. GO (Gene Ontology Enrichment Analysis) and KEGG analyses of differentially expressed genes were conducted using R package clusterProfiler (20). Specific gene sets were obtained from the Molecular Signature Database (MSigDB; https://www.gsea-sigdb.org/gsea/downloads.jsp). To characterize subclusters of epithelial cells (tumor cells), we performed single-sample gene set enrichment analysis (ssGSEA) of 50 hallmark gene sets (h.all.v7.1.symbols.gmt downloaded from MSigDB) for each subcluster and single cells using R package GSVA. Heatmaps were used to display the results of GSVA based on the average expression, and violin plots were used to display the pathway enrichment results based on the expression of each tumor cell. The pre-ranked gene set enrichment analysis method (R package fgsea) that was designed for GSEA of single-cell sequencing was also conducted to compare functional differences in macrophage populations between the tumor and ascites samples. Genes were ranked by the average log-fold change calculated by the FindMarkers function in Seurat.

G2/M phase identification

For G2/M phase analysis in the tumor compartment, we calculated a G2/M score for each tumor cell using the CellCycleScoring function in Seurat. The per-cell scores were added to the metadata matrix to assess the cell phase of the subclusters in tumor cells, and the stage of the cell phase of each cell was displayed as a t-SNE plot.

Trajectory analysis of single cells

We used the R package Monocle2 (v2.20.0) to perform pseudotime analysis to project high-dimensional transcriptomic data to one dimension that characterizes the relationship between monocytes and macrophages from tumor tissue, ascites and PBMCs. The matrix in the scale of raw UMI counts derived from Seurat objects were converted into new objects by the newCellDataSet function. Genes with mean expression ≥0.1 were used in the trajectory analysis. Selected genes with q-value < 0.01 between the cell groups were applied for dimensional reduction using the reduceDimension function with the parameter reduction_method= "DDRTree" and max_components=2. The trajectory plots were visualized using the plot_cell_trajectory function.

CellChat analysis

Cell communication analysis was performed between epithelial cells and macrophages in tumor and ascites tissues. R package CellChat (v1.1.2), which contains ligand-receptor interaction databases, analyze the intercellular communication networks between different cell clusters from scRNA-seq data (21).First, CellChat was used to evaluate the major signaling inputs and outputs among all epithelial cells and macrophages subclusters in tumor tissue. Next, netVisual_bubble function was utilized to show the significant ligand-receptor interactions between subclusters included.

inferCNV analysis

CNVs analysis of six tumor samples were performed by R package inferCNV(v1.8.1). The inferCNV cutoff parameter was set to 0.1 and HMM option was set to TRUE. The CNVs of tumor cells were calculated by raw expression data compared to myeloid subclusters from each sample. For inferCNV, 400 cells per subcluster were pseudorandomly chosen. CNVs values of each cell were finally limited as -1 to 1. The CNVs score of each cell was calculated as quadratic sum of CNV region.

Flow cytometry analysis

Flow cytometry analysis on patient peripheral blood samples was conducted at three times during the fourth cycle of chemotherapy (T1: before the second chemotherapy began; T2: two days after the sixth chemotherapy; T3: fourteen days after the sixth chemotherapy). Single cell suspensions were stained with antibodies for surface markers. Cells were washed and resuspended in FACS Buffers (PBS+0.5% HI-FBS) until data collection. Flow cytometry was performed with LSR II flow cytometer (BD Bioscience), and data analysis was conducted by FlowJo software. MultitestTM 6-color TBNK (Cat:644611), Fluorescein isothiocyanate (FITC)-conjugated anti-CD4 (Cat:340133), Fluorescein isothiocyanate (FITC)-conjugated anti-CD3 (Cat:349201), allophycocyanin (APC)-conjugated anti-CD25 (Cat:662525), chlorophyll protein complex-(PerCP)-conjugated anti-CD3 (Cat:652831), chlorophyll

protein complex-(PerCP)-conjugated anti-CD45 (Cat:561047), allophycocyanin (APC)-cyanine 7-conjugated anti-CD4 (Cat:341115), phycoerythrin (PE)-cyanine 7-conjugated anti-CD8(Cat:1292923), phycoerythrin(PE)-conjugated anti-CD25 (Cat:652834), Fluorescein isothiocyanate (FITC)-conjugated anti-CD45RA (Cat:662840), and hemolysin for flow cytometry were purchased from BD Biosciences, USA. Absolute number of tubes were purchased from BD Biosciences, USA. Phycoerythrin (PE)-conjugated anti-CD127 (Cat: P010034-B) were bought from Jiangxi CELGENE Biotechnology corporation, P.R. China.

Cytokine assay

Interleukin level assessment on patient peripheral blood samples was conducted at three times during the fourth cycle of chemotherapy (before the second chemotherapy began, two days after the sixth chemotherapy and fourteen days after the sixth chemotherapy). Utilizing an ELISA kit (Biosource, Invitrogen, USA) in accordance with the manufacturer's instructions, inflammation markers including tumor necrosis factor-α(TNF-α), interferon-gamma (IFN-γ), interleukin-2 (IL-2), interleukin-4 (IL-4), interleukin-6 (IL-6), interleukin-10 (IL-10) and interleukin-17 (IL-17) concentrations were measured.

TCGA data analysis

We evaluated the function of core IFN-associated genes (obtained from hallmark gene sets (h.all.v7.1.symbols.gmt) of MSigDB in HGSOC. The TCGA ovarian carcinoma (OV) data were used to predict the correlation of selected genes and patient survival. The gene expression data (counts matrix) and the clinical data were downloaded from UCSC Xena (http://xena.ucsc.edu/). Transcriptional matrices with paired clinical data were selected for analysis. Signatures were dichotomized into high-expression and low-expression groups based on the median GSVA values of per TCGA sample. Quartiles were plotted using R packages survival and survminer, and the p-value of the IFN signatures was calculated using the log-rank test.

Processing of single cell TCR and BCR sequencing libraries

The TCR and BCR sequences for T/B cells were collected from single-cell RNA-Seq data provided by 10× Genomics. Gene quantification and TCR/BCR clonotype assignment were performed using Cell Ranger (v.4.0.0) vdj pipeline with GRCh38 as reference. In this way, we obtained a TCR/BCR diversity metric, containing clonotype frequency and barcode information.

For the TCR, cells with no obvious TCR forms were excluded first, and TCR α/β chains were then obtained with reference to previous work (22). The target TCR α/β chains were defined as follows: (1) TCR barcodes could be found in T cells population from single cell mRNA sequencing data; (2) TCR with only one productive TCR α and β chain were retained. If multiple TCR α or β chains were identified in a T cell, only cells with dominant forms of α and β were retained.

For the BCR, similar filtration steps were conducted. Only cells with productive, paired heavy chain (IGH) and light chain (IGK or IGL) were reserved. After filtration, there were 6201 TCR-positive T cells and 1631 BCR-positive B cells in two blood samples.

Single cell TCR and BCR clonotype analysis

Clonotype analysis of TCR was conducted using the scRepertoire toolkit (23) based on TCR-seq libraries. Each unique TRA(s)-TRB(s) pair was defined as a clonotype in TCR, while each unique IGH(s)-IGK/IGL(s) pair was considered as a clonotype in BCR. If one clonotype was present in at least two cells, the cells possessing this clonotype were regarded as clonal.

For TCR and BCR clonotype analyses, the clonal homeostasis and clonal space occupied by clonotypes of specific proportions were first identified, and the proportion of clonal space occupied by specific clonotypes was visualized using the *clonalHomeostasis* and *clonalProportion* functions. Next, using the *clonalDiversity* function, the diversity across cell clusters was measured using Shannon, Inverse Simpson, Chao, and abundance-based coverage estimator (ACE) indices. Based on the *clonalOverlap* function, the clonotype overlap between cell clusters was then calculated and visualized using *Morisita* index methods. With *quantContig* function, unique clonotypes were scaled to the size of the sample library. Furthermore, the distribution of CDR3 amino acid sequences (whole, TRA, and TRB) was then identified using the *lengthContig* function. Moreover, we chose the top ten most-expanded clonotypes as dominant clonotypes and used *alluvialClonotypes* function to examine their dynamics in T/B cells after chemotherapy.

Single-cell TCR/BCR V(D)J sequencing and analysis

V(D)J sequence assembly, and paired clonotype calling was performed using CellRanger vdj with -reference = refdata-cellranger-vdh-GRCh38-atlas-ensembl-4.0.0 for each sample. Subsequent work was conducted based on the basic statistic

function in R. We first calculated the usage of TRAV/J, TRBV/J, IGHV/J and IGLV/J gene segments. Next, we identified the percentage of each gene segments used. V-J pairs of TCR α/β chains and corresponding frequencies were later confirmed. For the CDR3 amino acid (aa) length, we measured the frequency of TCR/BCR segments with the same aa length, to explore the distribution of the CDR3 aa length.

Survival analysis

Analysis of the association of interferon-associated genes with survival times in TCGA-OV datasets downloaded from UCSC Xena was conducted using the Survival Package, and p-values were calculated using the log-rank test.

Statistical analysis and data visualization

All statistical analyses were performed in software R. Significance was defined as a p value less than 0.05. The Wilcox-test in the Findmarker function in Seurat was performed to distinguish differential expressed genes between different clusters. Pairwise wilcoxon tests were calculated to compare the expression of specific genes between different samples or cell subclusters. The usage bias of V(D)J genes in TCR/BCR was identified by FDR (adjusted p values) using the Fisher test (< 0.05). Clinical statistical analyses (Supplementary Figure S6) were visualized using Graphpad PRISM (version 8.1.0).

Results

The cellular composition of a solitary lesion and ascites from a relapsed chemo-resistant ovarian cancer patient

To elaborate the characteristics of ovarian cancer patients who experience a gradual transition from chemo-sensitivity to -resistance and repeated tumor recurrence despite having received standard and extensive treatment, we evaluated a stage IIIC HGSOC patient. The patient initially underwent primary optimal surgical debulking followed by paclitaxel combined with nedaplatin and experienced the first recurrence after 17 months, indicating platinum-sensitive recurrent relapsed ovarian cancer (24). Unfortunately, she experienced three additional relapses indicated by re-ascending serum CA125/HE4 and imaging, and her PFS became shorter within each recurrence (from 7 months to 4 months to 2 months), suggesting that she developed chemo-resistance (Figure 1A). After three complete courses of platinum/taxol-based chemotherapy, she encountered a third relapse and accepted secondary cytoreductive surgery. To dissect the cellular composition and function of the TME at a key transient period from chemo-sensitiveness to chemo-resistance, as well as the impact of multi-cycles chemotherapy on the immune system, we collected a solitary mass from the vaginal cuff, peritoneal cavity ascites, and PBMCs for further study, with informed consent from the patient and approval of local institutional ethical review board.

To characterize the cellular components of these samples, we generated and analyzed single-cell transcriptomic profiles using 10× Genomics platform (Figure 1B). Based on known cell type markers, we identified and classified 5 cell types displayed by *t*-distributed stochastic neighbor embedding (t-SNE) as follows: tumor cells (*EPCAM, PAX8, WT1*), myeloid cells (*CD14, AIF1, CSF1R*), NK/T cells (*CD2, CD3E, CD3D, GZMA, GNLY, NKG7*), B lymphocytes (CD19, *CD79A, MS4A1*), and cancer-associated fibroblasts (CAFs) (*PDPN, DCN, THY1*) (Figures 1C, D). Similar with previous HGSOC single-cell sequencing reports (8, 11, 25), epithelial cancer cells were the most abundant cellular components followed by myeloid cells in tumor tissues. Contrary to previous research (8), immune cells dominates in ascites but rarely are found in the tumor in our study. CAFs were sparse in both the tumor tissue and ascites (Figure 1E). Moreover, T cells were less abundant in the tumor compared with the ascites (Figure 1E). Thus, these findings are suggestive of potential roles for both epithelial cells and immune cells in recurrence.

Functional and biological features of epithelial tumor cells from the relapsed lesion or ascites

We next analyzed the inherent features of cancer cells from the relapsed solitary tumor and ascites. Nine clusters of epithelial malignancy cells were identified (Figure 2A and Supplementary Figure S1A). The fallopian tube epithelium (FTE) markers *PAX8* and *KRT7* (9) were overexpressed in all subclusters, suggesting that the tumor may originate from FTE (Figure 2B). Furthermore, the C3-EOC-MKI67 (EC3) subpopulation displayed higher expression of chemotherapy resistance-related genes (*FEN1, NEK2, TOP2A* and *MKI67)* (Figure 2C) (8). Using the CellCycleScoring function of Seurat, we determined that the EC3 subgroup of cells were mainly in the S and G2/M phases, indicating that they were characterized by hyperproliferative status (Figure 2D). To functionally annotate the malignant epithelial clusters, we conducted Gene Set Variation Analysis (GSVA) based on hallmark gene sets from Molecular Signatures Database (MSigDB) (Supplementary Figure S1B). The EC3

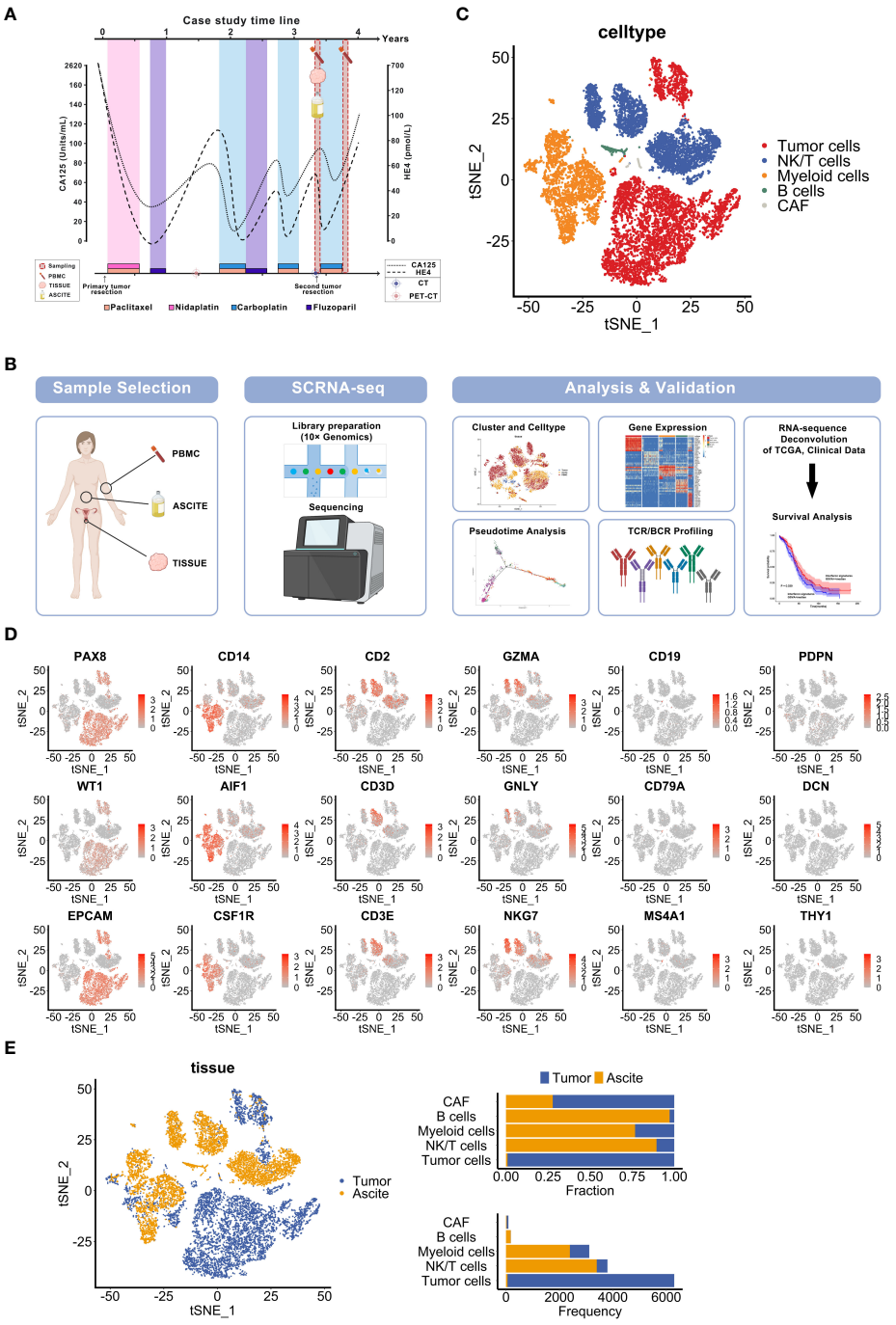

FIGURE 1
Single-cell sequencing to characterize the diverse cellular components of specimens derived from a recurrent ovarian cancer patient. **(A)** Overview of the clinical course and sample collection time of an HGSOC patient. The curved lines indicate changes of tumor biomarkers (CA125, HE4). The timepoints of chemotherapy treatment are shown in the label. **(B)** Overview of the sample collection, profiling strategy and analysis workflow. **(C)** t-SNE visualization of diverse cell types in sample *Tumor* and *Ascite*, colored by each cell type. **(D)** t-SNE plots show cell-type marker genes expression level. **(E)** t-SNE visualization of cells from samples *Tumor* and *Ascite*, colored by sample origin (left panel). Fraction and frequency of cells (x axis) from tumor tissues and ascites in each cell type (y axis) (right panel).

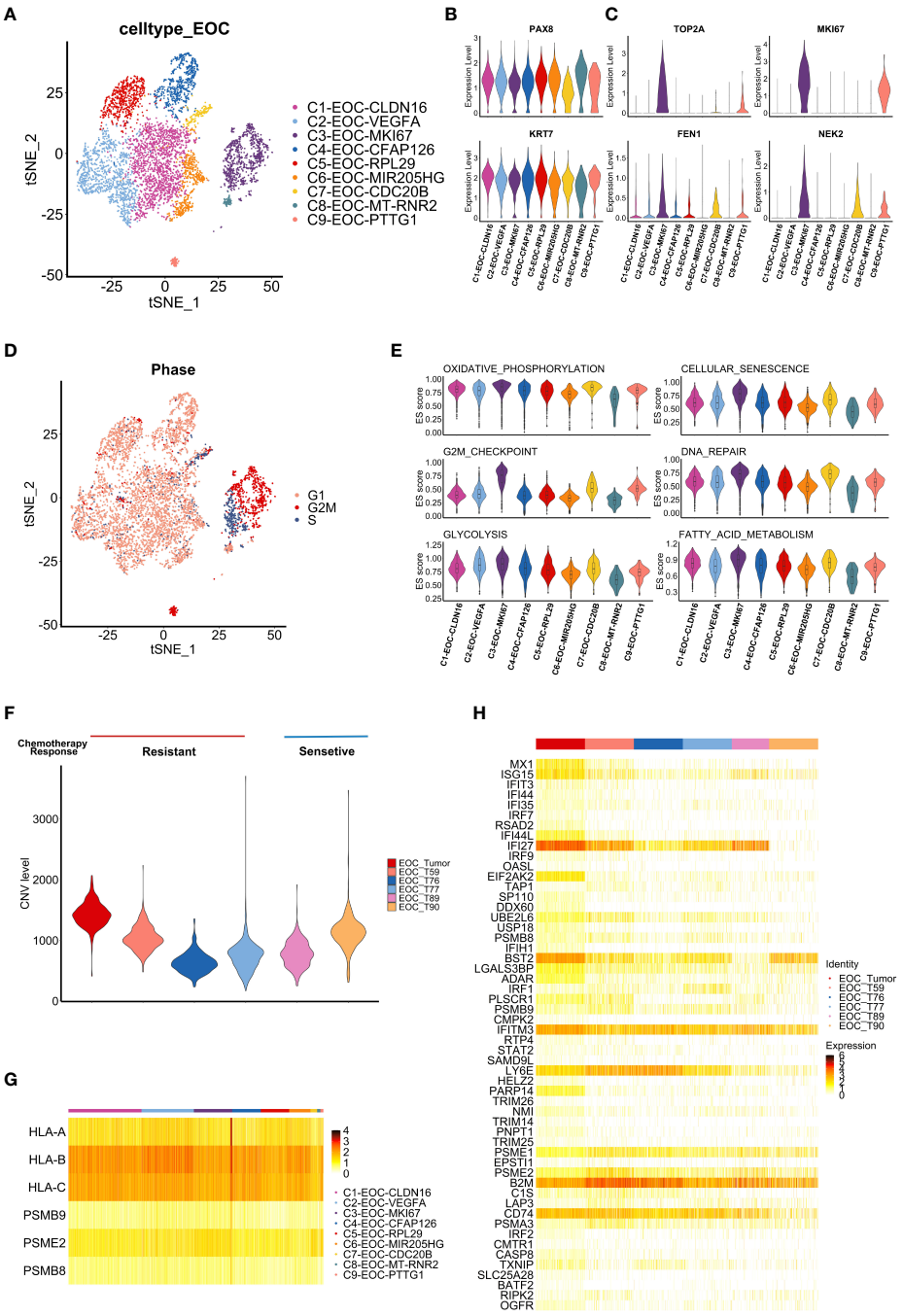

FIGURE 2
Tumor-intrinsic features uncovered by single cell analysis. **(A)** t-SNE visualization of tumor cell subclusters from samples *Tumor* and *Ascites*, colored by tumor cell subclusters. **(B, C)** Violin plots display the expression of fallopian tube epithelium (FTE) markers **(B)** and chemotherapy resistance-related genes **(C)** in each tumor cell subclusters. Distribution of the per cell signature expression Is based on normalized data. **(D)** t-SNE visualization of cell cycle phases of tumor cells in sample *Tumor* and *Ascites*, colored by cell cycle phase. **(E)** Violin plots shows the enrichment level of specific pathways among each tumor cell subcluster. Distribution of the per cell signature expression was based on the GSVA scores. **(F)** Violin plot shows CNV level among tumor cells from our data and five additional HGSOC samples. (*Tumor, T59, T76, T77*: chemo-resistant; *T89, T90*: chemo-sensitive) **(G)** Heatmap shows the expression of antigen presentation related genes in tumor cell subclusters from sample *Tumor*. **(H)** Heatmap shows the expression of interferon response pathway-associated genes in tumor cells from six HGSOC samples (*Tumor, T59, T76, T77, T89, T90*).

population exhibited relatively higher enrichment in several pathways, including oxidative phosphorylation, cellular senescence, G2/M checkpoint, DNA repair pathways, glycolysis, and fatty acid metabolism (Figure 2E). These results suggest that EC3 cells, with features of hyperproliferation, hypermetabolism and chemo-resistance, may account for the progression and recurrence of ovarian cancer.

To validate our findings, scRNA-seq datasets of five HGSOC patients (*T59, T76, T77, T89, T90*) were downloaded from GEO database (GSE154600) (17), which contains respective chemotherapy response. After the integration, dimension reduction and unsupervised clustering mentioned in methods, the same cell types were identified (Supplementary Figure S1C). Tumor cells in chemo-resistant samples showed higher expression of chemoresistance and proliferation related genes (*FN1, LCN2, CD44, FEN1*) (Supplementary Figure S1D) (8, 26–28). Given the association between the malignant tumor and large-scale chromosomal alterations, copy-number variation (CNV) of epithelial ovarian cancer (EOC) cells in six samples were contrasted with myeloid cells (Supplementary Figure S1E). Result of CNV analysis showed that our sample (EOC_Tumor) displayed elevated CNV levels (Figure 2F). Moreover, we explore whether the EC3 cluster is a characteristic cluster in chemo-resistant tumors. The top 10 expressed genes in this cluster were selected to assess the correlation among six samples (Supplementary Figure S1F). Results showed that our sample (EOC_Tumor) harbored higher similarity with two chemo-resistant samples (*T76* and *T77*) in expression profile contrast to two chemo-sensitive samples (*T89* and *T90*) (Supplementary Figure S1G). In conclusion, these results suggest that MKI67 positive cancer cells may contribute to chemotherapy resistance in HGSOC.

Next, we evaluated the expression of antigen presentation-related genes in cancer cells. Similar with the previous report (10), *HLA-B* and *HLA-C* had commonly obvious expression among subclusters (Figure 2G). Furthermore, interferon (IFN) pathway-associated genes were uniformly enriched among most subclusters of tumor cells. Genes associated with the IFN response (e. g. *IFI27, IFITM3, LY6E*), which represents core genes of the IFN pathway, were significantly elevated in tumor cells (Supplementary Figure S1H). To validate our findings, we characterized the expression of these genes in GEO database (GSE154600) and obtained the similar expression profile (Figure 2H). To further predict potential functions of IFN-associated genes in HGSOC, we performed survival analysis based on these genes using the OV-TCGA dataset, which suggested that high expression of IFN-associated genes is related to a better prognosis (log-rank method, P= 0.039) (Supplementary Figure SI). Thus, enrichment of the IFN expression profile (Figure 2H) in relapsed tumor may suggest stronger immune response and good prognosis in this patient. However, the progressively shorter PFS3 of this patient calls for further investigation on tumor immune microenvironment (TIME).

Dissection of the components and role of myeloid cells in TIME

To better elucidate TIME, we analyzed 17301 immune cells from samples collected before the fourth course of chemotherapy, including 910 cells from the tumor, 5949 cells from ascites and 10442 cells from PBMCs. Four major cell types were identified based on previously characterized markers (8, 10), including T cells (*CD3D, CD3G, CD2*), NK cells (*NKG7, GNLY, KLRD1, KLRF1*), B cells (*MS4A1, CD19, CD79A, CD79B*) and myeloid cells (*CD14, AIF1, CSF1R*) (Supplementary Figures S2A–C). T cells and myeloid cells were the dominant immune cells in the ascites and tumor (Supplementary Figure S2D), which is consistent with other studies (8, 10, 29).

We next performed cluster analysis of the myeloid cells and revealed 12 clusters (Figure 3A). Based on previous report (30), we applied genes predominantly expressed in blood-derived monocytes (*S100A8, S100A9 and CSF3R*) and classical monocytes markers (*CD14, CD16 and FCN1*) together as monocytes markers. Consistently (30), high expression of these six markers in monocytes reflect that monocytes are probably educated by TIME. The cluster populations were primarily comprised of six monocyte clusters with high expression of *S100A8, S100A9, RPS2P5, CDKN1C*, and *MKI67*, and three macrophage clusters with high expression of *ADAP2, MARCO* and APOE (Supplementary Figures S2E, F). Of note, MKI67 monocytes and APOE macrophages were mainly derived from tumor, while ADAP2 and MARCO macrophages were mainly from ascites (Figure 3B). Using markers identified in a previous report (31), we found that APOE macrophages exhibited TAM-like signatures (*TREM2, APOE*), whereas ADAP2 macrophages highly expressed MDSC-like signatures (*S100A8, FCN1*) (Figure 3C). In addition, these two clusters showed high expression of M2-like signatures (*CD163, MRC1*), while ascites-derived MARCO macrophages highly expressed MDSC-like signatures with both M1- (*CD68, CD86*) and M2-like signatures (Figure 3C). Next, we explored the trajectory of myeloid cells from different sites by pseudo-time analysis. Except for MKI67 monocytes, PBMC-derived monocytes bifurcated to ascites-resident macrophage populations (ADAP2 and MARCO macrophages) and tumor-resident populations (MKI67 monocytes and APOE macrophages) (Figure 3D), suggesting that peripheral monocytes may migrate to ascites and tumors, and be educated as different subtypes in the TIME.

To characterize the different functions of macrophages in the ascites and tumor, we compared KEGG pathways that were enriched in different subpopulations. Compared with the APOE cluster, both the ADAP2 and MARCO clusters showed lower enrichment of cytokine receptor interactions (Figures 3E, F), indicating impaired activation and cytotoxicity of macrophages in ascites. Moreover, we investigated expression of CCL/CXCL ligand in tumor clusters (Supplementary Figures S3A, B) and CCR/CXCR receptors in myeloid clusters (Supplementary

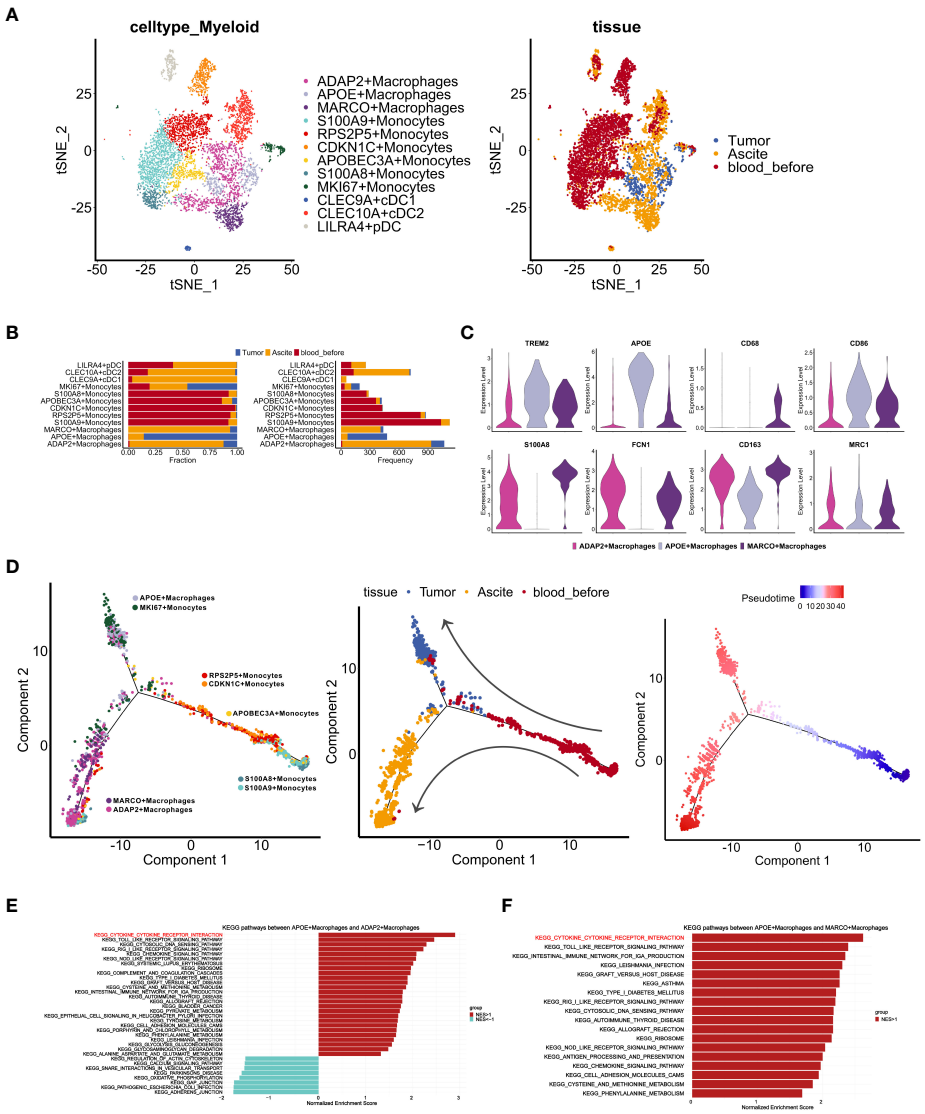

FIGURE 3
Characteristics of myeloid cells in distinct TMEs of ascites, tumor and PBMCs. **(A)** t-SNE visualization of myeloid cells profiles from three samples (*Tumor, Asicite, blood_before*) before the fourth course of chemotherapy, colored by myeloid cell subclusters (left panel) and sample origins (right panel). **(B)** Fraction and frequency of myeloid cells (x axis) from samples (*Tumor, Asicite, blood_before*) in each myeloid subcluster (y axis). **(C)** Violin plots display the expression TAM- (*TREM2, APOE*), MDSC- (*S100A8, FCN1*) and M1-like (*CD68, CD86*), M2-like (*CD163, MRC1*) signatures expression among three macrophage subclusters (ADAP2+ Macrophages, APOE+ Macrophages, MARCO+ Macrophages). **(D)** Pseudotime analysis of monocytes and macrophages from samples (*Tumor, Asicite, blood_before*), colored by each myeloid subcluster (left panel), derived-samples (middle panel) and pseudotime trajectory (right panel). **(E, F)** Gene set enrichment analysis between APOE subcluster and ADAP2 subcluster **(E)**, APOE and MARCO subcluster **(F)** using KEGG gene sets. Pathway enrichment is expressed as normalized enrichment score (NES).

Figures S3C, D). *CXCL16-CXCR6*, known tumor cell-immune cell crosstalk in immune infiltrated tumors (10), showed rare co-expression (Supplementary Figures S3B, D), suggesting the lack of immune cells recruitment mediated *via CXCL16*. To further inspect the interaction between tumor cells and myeloid cells, we performed communication analysis using R package CellChat. We observed top-ranking ligand-receptor pairs of macrophage migration inhibitory factor (MIF) in cancer cells and (CD74 +CD44) in macrophages (Supplementary Figures S3E, F). Contributing to anti-inflammatory, and immune evasive phenotypes in malignant disease (32), MIF was also reported to be elevated in ovarian cancer cells (33). In addition, Midkine (MDK)-LRP1 pairs, which promotes immunosuppressive macrophage differentiation (34), markedly exist from epithelial

clusters to macrophages (Supplementary Figures S3E, F). Together, these results suggest an immune-suppressive state of macrophages in this patient.

In order to confirm whether above characteristics in TIME are unique to drug-resistant tumors, we integrated GSE154600 and our data to identify T cells, B cells, and myeloid cells in all samples. The results showed that proportion of myeloid cells was higher in chemo-resistant tumors, especially in our sample (Supplementary Figure S3G). Myeloid cells were selected for further clustering and macrophages, monocytes and DC cells were identified (Figure S3H). As expected, expression of the M2 signatures (*CD163, MRC1*) was higher in chemo-resistant samples while M1 signatures (*CD68, SOCS3*) (35) expression was low. Immune-suppressive genes (*GPNMB, TREM2*) (36) had elevated level in chemo-resistant samples as well (Supplementary Figure S3I). In summary, these results indicate that macrophages with immune-suppressed phenotype may be a character of chemo-resistant HGSOC.

The inhibitory status of γδT cells contributes to the immunosuppressive environment in ascites

To clarify the role of T cells in TME, we clustered T cells based on the expression of surface markers of cells from tumor, ascites, and PBMCs (Figures 4A, B). Seven T cell clusters were characterized as follows: activated T cells (*PRF1*), memory T cells (*S100A4, GPR183*), naïve T cells (*SELL, LEF1, CCR7*), Tregs (*CTLA4, FOXP3, FOXO1*), cytotoxic T lymphocytes (CTL) (*GZMA, NKG7, GZMH, GZMB*), mucosal-associated invariant T cells (MAIT) (*SLC4A10, TRAV1-2*) and γδT cells (*TRGV9, TRDV2*) (Figure 4C). The TC2-XIST (TC2), TC4-FOSB(TC4) and TC8-BCL2 (TC8) clusters were mostly derived from ascites, while other clusters were mainly from PBMCs (Figure 4B). Notably, TC2 and TC4 clusters were characterized by low expression of T cell markers (Figures 4C, D), such as *RORC, TRDC* and *ZBTB16* (37).

Since γδT cells are characterized by negative expression of *CD4* and *CD8* (38), we annotated TC4 as Vδ2 γδT cells and TC2 as non-Vδ2 γδT cells using R package *SingleR* (Figure 4E), suggesting that TC2 might represent a new subcluster of T cells. Furthermore, gene set enrichment analysis of the TC2 cluster revealed significant enrichment in genes of chromatin organization regulation, thus implicating its potential roles in shaping the immune community of T cells in ascites (Figure 4F). Interestingly, significantly enriched pathways in the TC2 cluster included the apoptosis, RIG-I-like receptor signaling, lysine degradation, and sulfur metabolism pathways, while the ribosome and oxidative phosphorylation (OXPHOS) pathways displayed low level enrichment (Figure 4G). Given that the OXPHOS pathway is a characteristic metabolic phenotype of T cells within the TIME (39), its low level enrichment, along with high enrichment of the apoptosis pathway and low enrichment of the ribosome function pathway, indicate a weakened immune function in TC2.

Chemotherapy induced senescence and TCR clonal expansion of T cells derived from PBMC

Immunohistochemistry (IHC) results showed that $CD8^+$ T cells were more abundant in recurrent tissue than primary lesions (Supplementary Figure S4A), suggesting that local $CD8^+$T cells infiltration in tumor tissue is dynamic during progression of HGSOC. To investigate the peripheral T cell status, which may reflect the systemic immune response (40), we analyzed T cells from PBMCs before and after chemotherapy. Immune cells were classified into populations of myeloid cells, T cells, NK cells and B cells based on known markers (Supplementary Figures S4B–E), among which T cells were the most abundant population of immune cells (Supplementary Figure S4D). We also classified T/B cells according to the TCR/BCR distribution (Supplementary Figure S4E). Consistent with our scRNA-seq results (Supplementary Figure S4F), an increase of NK cells proportion after chemotherapy was detected by flow cytometry (Supplementary Figures S6B, C).

In peripheral blood-derived T cells, we identified eleven subsets based on canonical markers (Figures 5A–C). The $CD4^+$ cells included memory $CD4^+$ T cells ($S100A4^+GPR183^+$), regulatory $CD4^+$ T cells (T_{reg}) ($FOXP3^+IL2RA^+$) and naïve $CD4^+$ T cells ($CCR7^+SELL^+$) (constituted of CD4-C1-naïve-LTB and CD4-C2-naïve-LEF1). Five subsets of $CD8^+$ T cells, including a naïve $CD8^+$ T cell subset ($CCR7^+$ $SELL^+$) and four effector $CD8^+$ T cell subsets (constituted of CD8-C1-effector-NKG7, CD8-C2-effector-GNLY, CD8-C3-effector-GZMB and CD8-C5-effector-ZNF683), expressed high levels of *GZMA* and *NKG7*. In addition, a MAIT subset ($SLC4A10^+TRAV1\text{-}2^+$) and a γδT subset ($TRGV9^+TRDV2^+$) were defined. Expression of exhaustion markers *LAG3*, *CD244* and *EOMES* were detected in all $CD8^+$ T cell clusters (Figure 5C), among which the $CD8^+$-C2-effector-GNLY group harbored the most extensive TCR clonal expansion (Figures 5D, E).

We further conducted cellular proportion analysis before and after chemotherapy. Among $CD8^+$ T cells, C3-effector-GZMB (4.1% vs 3.7%) and C5-effector-ZNF683 (0.8% vs 0.7%) populations increased while C1-effector-NKG7 (14.7% vs 16.0%) and C2-effector-GNLY (8.4% vs 9.4%) populations decreased after chemotherapy (Figure 5F). Importantly, $CD8^+$ GZMB T cells and $CD8^+$ ZNF683 T cells are thought to be exhausted or exhausted-like cells, despite their ascribed cytotoxic function (10, 41). Therefore, changes of cellular proportion in $CD8^+$ T cell subsets indicate the tendency towards an exhaust state, which may reflect the cumulative effects of chemotherapy.

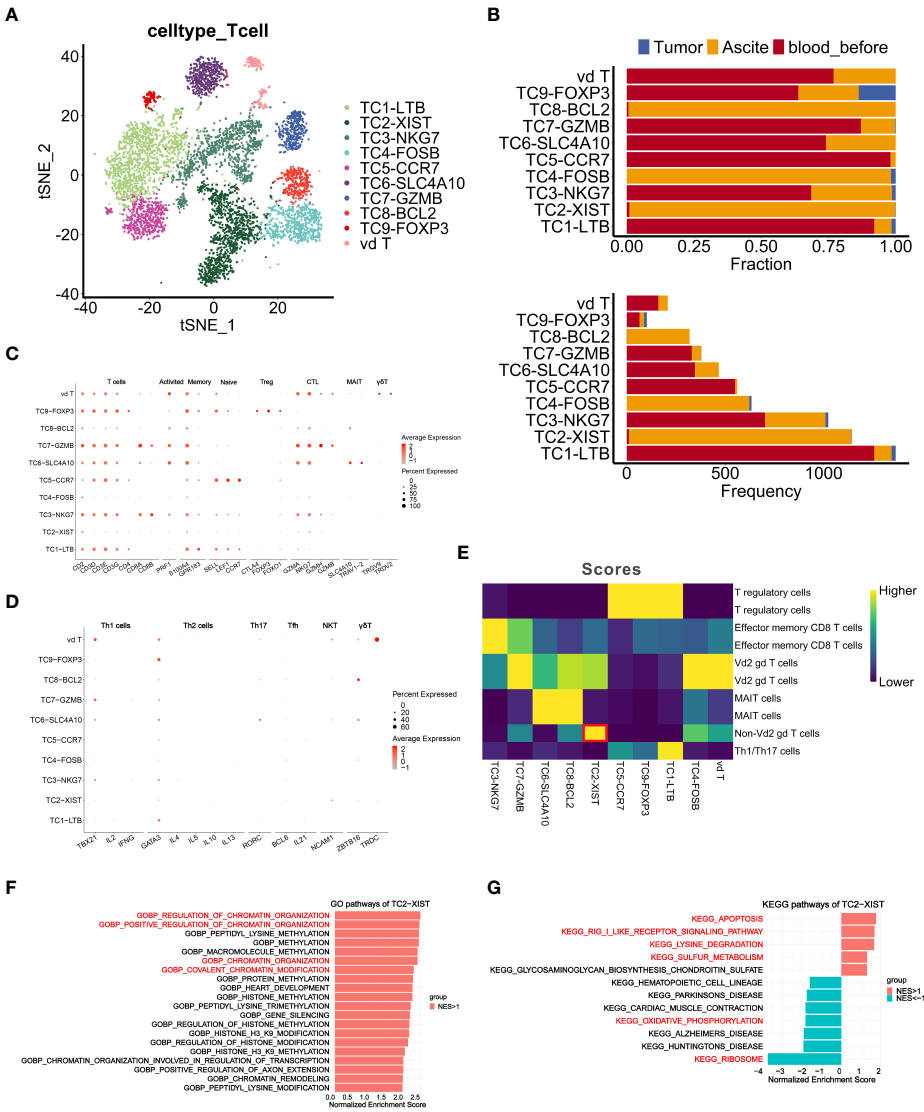

FIGURE 4
Characteristics and dynamics of T cells from samples before chemotherapy at single-cell resolution. **(A, B)** t-SNE visualization of T cell clusters from samples (*Tumor, Ascite, blood_before*) before chemotherapy, colored by the identified T cell subclusters. **(B)** Fraction and frequency of T cells (x axis) from samples (*Tumor, Ascite, blood_before*) in each T cell subcluster (y axis). **(C, D)** Dot plots show expression level of signature genes in each T cell subcluster. **(E)** Heatmap shows the classification result of each T cell subclusters using singleR. **(F, G)** Gene set enrichment analysis of T cell subcluster TC2-XIST using GO gene sets **(F)** and KEGG gene sets **(G)**. Pathway enrichment is expressed as normalized enrichment score (NES).

To investigate whether chemotherapy affects the activation or exhaustion status of T cells *via* regulation of co-stimulatory molecules, which play important roles in the T cell response to antigenic stimuli (42), we compared the expression of co-stimulatory molecule receptors in PBMC-derived T cell clusters before and after chemotherapy. Low expression of immune checkpoint *PD-1* (*PLDCD1*) and *CTLA4* were observed (Supplementary Figure S5A), suggesting poor immune checkpoint blockade status, which is consistent with the low sensitivity of ovarian cancer to immune checkpoint therapy (43, 44). Among the co-stimulatory molecules, upregulation of *CD27*, which participates in the generation of memory $CD8^+$ T cells (42), was observed in all $CD4^+$ T cell clusters except for the CD4-C4-Treg-FOXP3 cluster; *TNFRSF14*, which enhances the tumor-specific immune response (45), increased in all $CD4^+$ T cell clusters except for the CD4-C2-naive-LEF1 cluster; and *LAG3*, a marker of exhaustion (46), showed no significant change in $CD4^+$ T cell clusters (Supplementary Figures S5B–E). We noted that most $CD4^+$ T cell clusters generally showed a higher secretion of pro-inflammatorymolecules (*CD27*, *TNFRSF14*) after

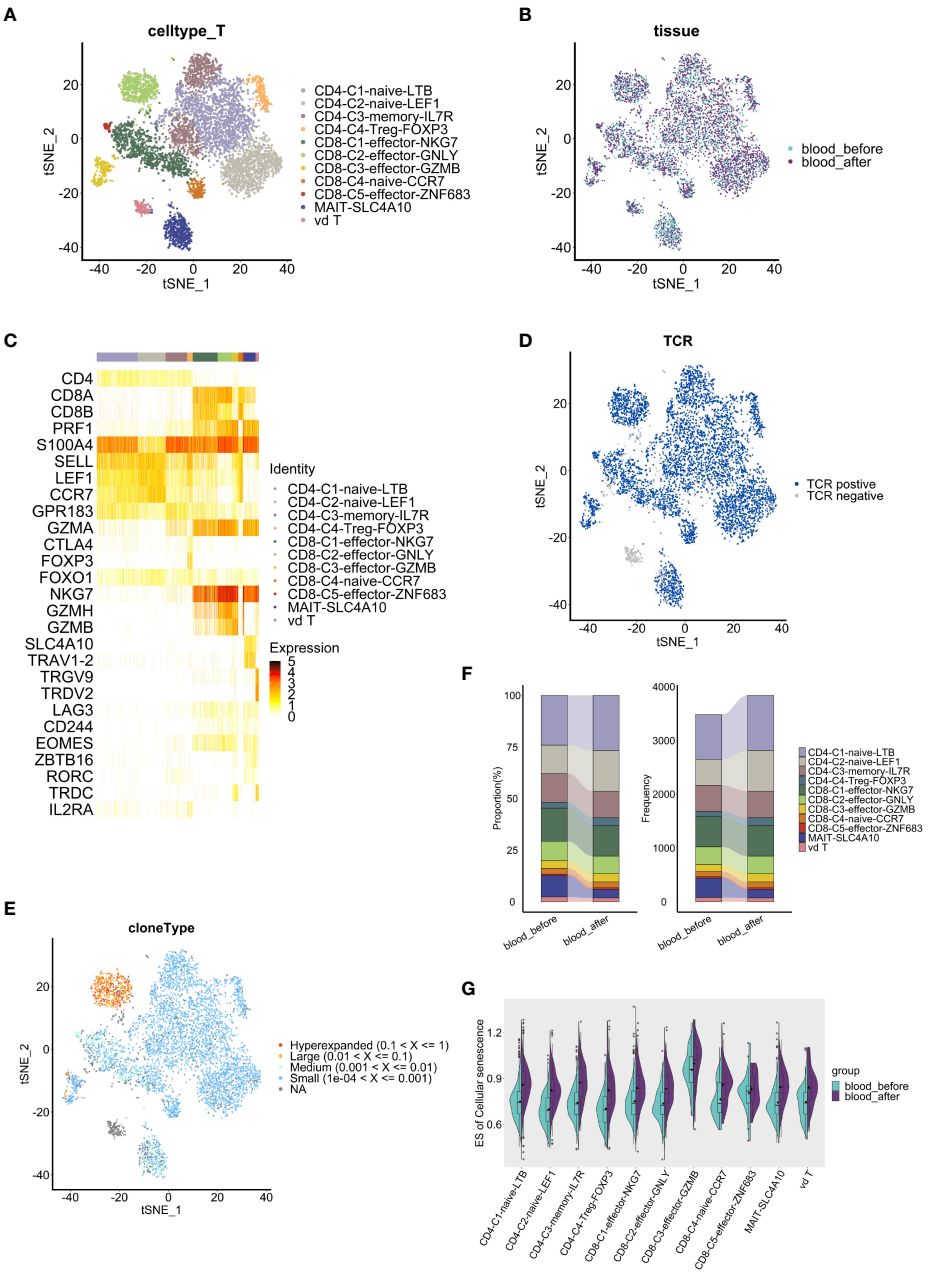

FIGURE 5
Comparative analysis of T cell features and dynamics in peripheral blood. **(A, B)** t-SNE visualization of T cells, colored by subclusters **(A)** and samples **(B)**. **(C)** Heatmap shows the expression level of marker genes in each T cell subcluster. **(D)** t-SNE visualization of TCRs identified in T cells. **(E)** t-SNE visualization of clonal expansion detected in T cells. **(F)** Proportion(left panel) and frequency(right panel) of T cell subclusters (y axis) in two blood samples(x axis). **(G)** Split violin plots show the enrichment level of cell senescence grouped by T subclusters and colored by samples. The results above are generated by comparison between samples (*blood_before, blood_after*).

chemotherapy, indicating an activated state, while $CD8^+$ T cell clusters did not show the same pattern. Among $CD8^+$ T cells, higher expression of *CD27* was only observed specifically in the CD8-C2-effector-GNLY cluster, while elevated expression of *TNFRSF14* was observed in the CD8-C1-effector-NKG7 and CD8-C2-effector-GNLY clusters. Of note, we found a significant higher expression of exhaustion marker *LAG3* in most $CD8^+$ effector T cell and γδT cell clusters (Supplementary Figures S5F–J), suggesting that impaired $CD8^+$ effector T cells, which were promoted towards a more exhausted state by chemotherapy, are

likely to have contributed to recurrence and a shorter PFS in this patient.

Next, to investigate whether chemotherapy can promote T cell senescence, we employed the GSVA method to compare the enrichment level of cellular senescence gene sets (obtained from KEGG, hsa04218) among PBMC-derived T cell clusters. T cell senescence is characterized by the accumulation of dysfunctional and terminally-differentiated cells (47), and a senescence-related gene set was significantly enriched in the CD8-C3-Effector-GZMB T cell cluster (Figure 5G). Of note, all T cell clusters, including the CD8-C3-Effector-GZMB cluster, gained a higher enrichment of cellular senescence-related genes after chemotherapy, strongly implying that chemotherapy promotes and accelerates T cell senescence (Figure 5G). Therefore, our results suggest that chemotherapy induces senescence-like T cell including CD8-C3-Effector-GZMB T cells, which may serve as a dysfunctional subpopulation with exhausted phenotype in HGSOC (10).

To prove our findings, flow cytometry and cytokines assay were performed (Supplementary Figure S6A). The levels of interleukin-6 (IL6), a classical senescence-associated secretory phenotype (SASP) and pro-inflammatory factor, were increased during the treatment period, while TNF-α and IFN-γ displayed a declined level (Supplementary Figure S6E). IL6/IL10 ratio increased gradually (Supplementary Figure S6F), implying a pro-inflammatory status in circulating immune system. However, despite once elevated, the proportion of $CD8^+$ T effector cells ($CD3^+CD8^+$ $CD25^+$) decreased after chemotherapy (Supplementary Figures S6B, C). Similarly, $CD8^+$ T effector cells/T_{reg} cells ($CD4^+CD25^+CD127^-$) ratio increased initially and then decreased after the treatment finished (Supplementary Figure S6D), suggesting a weakened antitumor activity. These results collectively indicate an initially activated but eventually suppressed phenotype of peripheral T cells after chemotherapy, probably caused by T cell senescence.

Whether chemotherapy induces changes in TCR clonal expansion remains unclear. Therefore, we analyzed the dynamic of TCR repertoire during chemotherapy. Notably, we observed that the quantity and proportion of unique T cell clonotypes, which accounted for more than 70% of all clonotypes, increased after chemotherapy (Figure 6A). Only 154 unique clonotypes were shared before and after chemotherapy (Figure 6B). Similar trends were observed among $CD4^+$ and $CD8^+$ T cell subsets (Supplementary Figures S7A, C). These data strongly indicate that TCR clonal expansion was changed by chemotherapy. Interestingly, higher diversity indices (Shannon, Simpson, Chao and ACE index) were observed in $CD4^+$ T cells compared to $CD8^+$ T cells. The clonal overlap within $CD4^+$ T cell clusters was not apparent while a strong overlap within $CD8^+$ T cell clusters exists, especially between CD8-C1-effector-NKG7 and CD8-C2-effector-GNLY cells (Supplementary Figures S7E, F). Moreover, the relative abundance of highly expanded clonotypes decreased, and the low clonal index clonotypes occupied more repertoire space after chemotherapy (Figure 6C and Supplementary Figures S7B, D), suggesting that the TCR clonal expansion change may be explained by clonotypes with low clonal indices. Therefore, chemotherapy appears to have induced TCR clonal expansion in all T cells, and the influence on $CD4^+$ TCR was more apparent.

As the complementarity determining region3 (CDR3) is the TCR region that directly contacts the antigen, thus playing a significant role in the interaction between the TCR and peptide-MHC complex (48), we next investigated whether chemotherapy changed the distribution of CDR3 within the α/β chains in different clonotypes. The distribution of amino acid (aa) length in the CDR3 α/β chain was mostly consistent, with 27aa comprising the most frequent length, both before and after chemotherapy (Figures 6D, E). Notably, the proportion of the CDR3 region with the same length slightly changed in $CD4^+$ T cells (Supplementary Figures S7G, H) but remained almost unchanged in $CD8^+$ T cells (Supplementary Figures S7J, K) after chemotherapy. Furthermore, clonotypes of dominant CDR3 sequences were reduced, and the CVSGDIPTF_CASSSRYSGNTIYF sequence disappeared after chemotherapy (Figure 6F). In $CD4^+$ T cells, the clonotypes with a proportion of dominant sequences decreased significantly after chemotherapy (Supplementary Figure S7I), while the clonotypes in $CD8^+$T cells remained almost unchanged, with the percentage of several dominant clonotypes increased slightly (Supplementary Figure S7L). These results suggest that chemotherapy changes TCR clonal expansion, while the influence on $CD8^+$ T cells is not as apparent as on $CD4^+$ T cells.

V(D)J rearrangement is the basis of TCR/BCR diversity, enabling immune responses of T/B cells to numerous antigens (16). Therefore, we further analyzed the bias of V-J pairs in alpha and beta chains before and after chemotherapy. Interestingly, TRAV5-TRAJ47, TRAV1-2-TRAJ33 and TRAV17-TRAJ54, the three most highly used V-J pairs of alpha chains, remained unchanged while other less-used pairs were changed much more after chemotherapy (Figure 6G). Among the beta chains, TRBV7-8-TRBJ2-5, TRBV20-1-TRBJ2-7 and TRBV20-1-TRBJ2-1 were the three most used V-J pairs before and after chemotherapy, while other less-used pairs were significant changed (Figure 6H). Furthermore, usage bias of V/J genes in T cell clonotypes was observed after chemotherapy (Supplementary Figure S7M). Collectively, based on clonotype and CDR3 analyses, these findings suggest that the TCR repertoire changes may be related to low-expanded clonotypes with low-frequency V-J pairs.

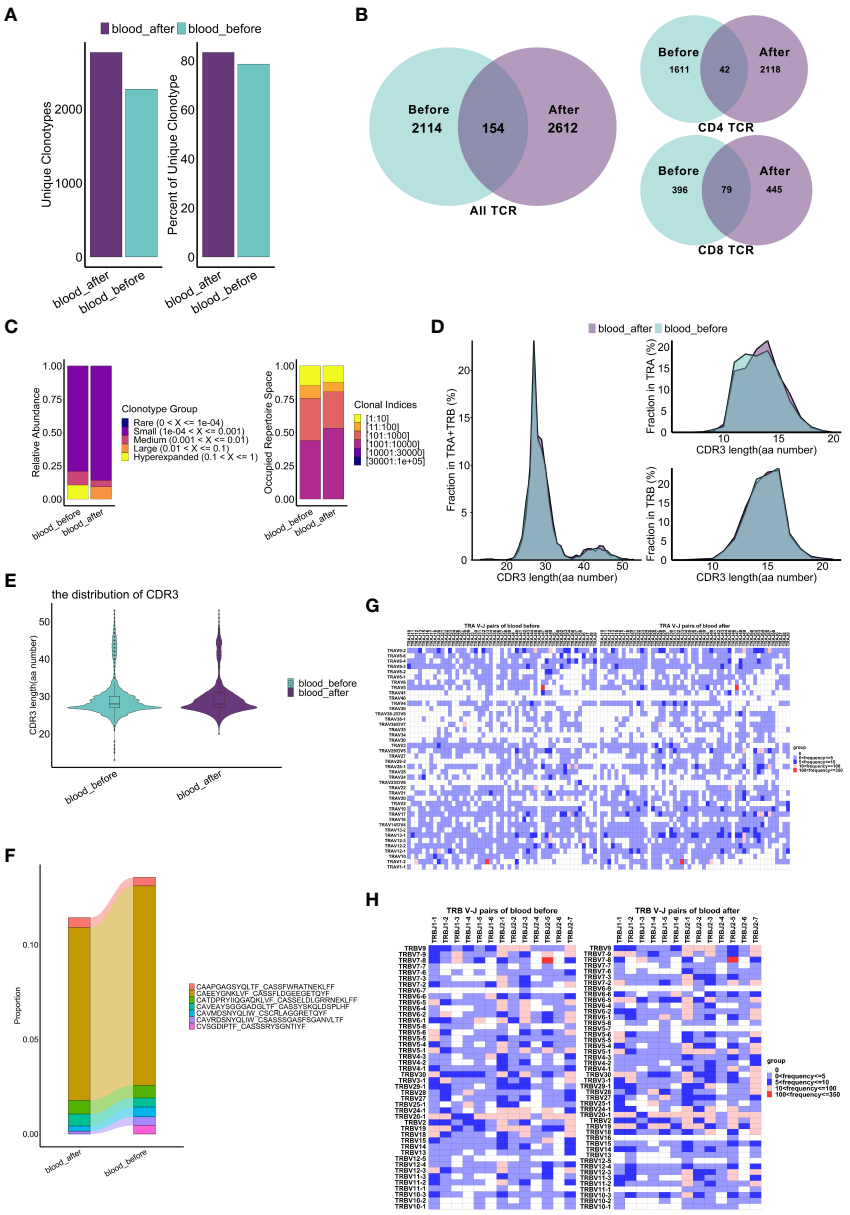

FIGURE 6
Comparative analysis of TCRs pre and post chemotherapy in peripheral blood. **(A)** Bar graphs show quantity and percentage of unique clonotypes. **(B)** Venn diagram showing the common and specific TCR of T cells (whole T cells, $CD4^+$ T cells and $CD8^+$ T cells). **(C)** Clonal homeostatic space representations (clonal space occupied by clonotypes of specific proportions) (left panel) and the relative proportional space occupied by specific clonotypes (right panel) of TCRs across samples. **(D)** Curve graphs show CDR3 aa length distribution of TCRs (TRA: α chains, TRB: β chains, aa: amino acid). **(E)** Violin plots show the CDR3 aa length distribution of TCR. **(F)** Dynamics of dominant CDR3 sequences of TCRs across samples pre and post chemotherapy, colored by the types of dominant sequences. **(G, H)** Heatmaps show frequency of V-J pairs in α chains **(G)** and β chains **(H)** among two samples. The results above are generated by comparison between samples (*blood_before, blood_after*).

Chemotherapy induces B cell activation and changes BCR clonal expansion

Recently, different subsets of B cells have been reported to play important roles during the dynamic progression of tumors (49). For example, the $ICOSL^+$ subset of B cells has been shown to emerge after chemotherapy and may enhance the immune response in breast cancer (50). Furthermore, IgA derived from tumors has been shown to antagonize the growth of OC by governing coordinated responses of tumor cells, T cells and B

cells (51). To assess the influence of chemotherapy on peripheral B cell phenotype and function, we analyzed scRNA-seq and scBCR-seq data. A total of 1690 B cells were obtained, and 1631 cells with full-length productive paired IGH-IGK/IGL chains were retained for further analysis. Based on the expression of canonical markers, the B cells were categorized into three distinct subsets: naïve B cells (*IGHD*), memory B cells (*CD27, IGHA1, IGHG1*) and plasma cells (*CD38, XBP1*) (Figures 7A, B). Comprised of IgM, IgD, IgG and IgA isotypes, naïve B cells accounted for majority of peripheral B cells. All B cells were median-expanded (Figure 7A). The percentages of plasma cells (5.10% vs 3.92%) and memory B cells (22.45% vs 20.54%) increased, and the percentage of naïve B cells decreased (72.45% vs 75.54%) after chemotherapy (Figure 7C), suggesting that neoantigens induced by chemotherapy may cause naïve B cells to differentiate into plasma or memory B cells. Several key genes related to NF-κB signaling(CD74), MAPK signaling(FOS, DUSP1) pathways, were markedly upregulated in both naïve and memory cells after chemotherapy, suggesting that chemotherapy may induce B cell activation, proliferation and maturation. (52, 53) (Figure 7D). Using R package Clusterprofiler we found that a variety of inflammatory response pathways were significantly enriched in naïve B cells after chemotherapy, while protein synthesis and RNA catabolism pathways were enriched in memory B cells (Figure 7E).

Next, we explored the dynamics of BCR repertoires during chemotherapy. Interestingly, we observed a consistent proportion of unique clonotypes before and after chemotherapy, and no unique clonotypes were shared (Figure 7F), suggesting significant changes in BCR clonal expansion may be primarily attributed to the chemotherapy. Of note, no apparent increase was observed in the relative abundance of clonotypes and the occupied space of corresponding clonal indices (Figure 7G), which defers from the results of TCR analysis (Figure 6C). In addition, there were no significant differences in the CDR3 length distribution, while the proportion of CDR3 with the same length was less after chemotherapy (Supplementary Figures S8A, B). Furthermore, a mild difference in distribution was observed in memory B cells but not naïve B cells (Supplementary Figure S8D). Notably, completely different CDR3 dominant sequences (Supplementary Figure S8C) and usage bias of the V-J gene segments in memory B cells relative to naïve B cells after chemotherapy (Supplementary Figures S7E, F) were observed. In summary, chemotherapy promoted peripheral B cell activation and changed clonal expansion of the BCR repertoire, potentially contributing to the response to neo-antigens induced by chemotherapy.

Discussion

HGSOC is characterized by disseminated abdominal spread, easy of recurrence, and chemoresistance in advanced-stage patients. Malignant abdominal ascites provides a complex cancerous and immunological microenvironment for tumor progression and recurrence. Single-cell sequencing provides a vital method to better understand the fundamental mechanisms of cancer relapse and chemoresistance. In this study, we revealed the intratumor heterogeneity, immunosuppressive features in ascites, and dynamic changes of immune status of PBMC in a relapsed chemo-resistant HGSOC patient after chemotherapy. Furthermore, we demonstrated that chemotherapy remodel TIME in peripheral blood and change the clonal expansion of TCR/BCR. These findings highlight the impact of chemotherapy on TIME, which may contribute to future development of novel immune-modulatory strategy for relapsed chemo-resistant ovarian cancer patients.

We first investigated whether intrinsic properties of tumor cells contribute to chemoresistance. FTE markers (*PAX8, KRT7*) were highly expressed in all subclusters of epithelial cells, indicating that the tumor may originate from fallopian tube (8). Of note, EC3 subcluster showed high expression of chemoresistance related genes and was comprised of a large proportion of G2/M cells, along with an elevated metabolism level, which is associated with progression and platinum-based chemoresistance in HGSOC (54, 55). High heterogeneity and high proliferation ability of epithelial cells were probably caused by CNVs (56, 57). Compared with those in sensitive HGSOC samples, chemo-resistant recurrent epithelial cells showed higher CNVs level, implying that EOC_Tumor may be in a more malignant state. Since cancer somatic mutations can generate neoantigens (58), an obvious upregulation of antigen presentation genes across all cancer cell clusters suggests clonal expansion of TCR or BCR to neoantigens. Consistently, IFN-associated genes, were highly expressed in cancer cells from both GSE154600 and our case, which might predict better prognosis. However, a shortened PFS and platinum-free interval (PFI), along with an increased frequency of chemotherapy of this patient still needs more investigation.

Then we further investigate whether status of TIME contribute to chemo-resistance of HGSOC. Previous study has shown that the high expression of M2 marker in macrophages is associated with poor prognosis of ovarian cancer (59), and upregulated M2 marker is considered to imply immune-suppressive phenotype (60). Our patients showed high expression of M2 signatures in both tumor-infiltrated and ascites-resident macrophages, indicating that M2 TAMs polarization may promote chemo-resistance. Our findings also suggest that peripheral monocyte/macrophage subsets may migrate to the ascites or tumors and be educated to perform different functions in the TIME. Integrating GSE154600 and our data, we affirmed our findings that chemo-resistant tumors may share signatures of immunosuppressive myeloid phenotype. In addition, the predominant co-expression of *GPNMB* in myeloid cells (Supplementary Figure S3I) and CD44 in cancer cells (Supplementary Figure S1C) in chemo-resistant samples may

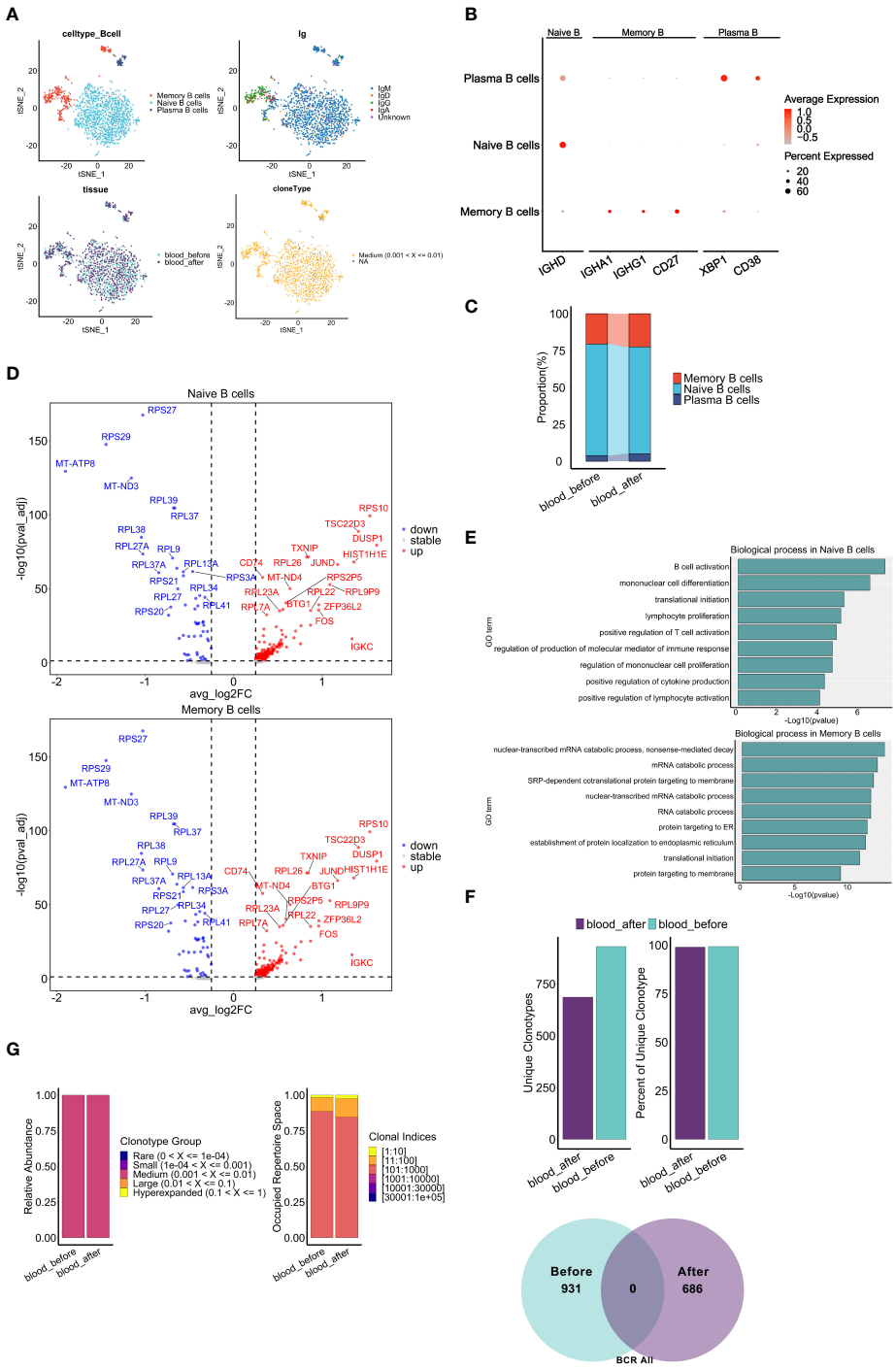

FIGURE 7
Characteristics of B cell subclusters before and after chemotherapy. **(A)** t-SNE visualization of B cells colored by cell types (top left), BCR isotypes (top right), derived-samples (bottom left) and clonal expansion status(bottom right). **(B)** Dot plots show expression level of marker genes of B cell types. **(C)** Proportion of B cell subclusters (y axis) in two blood samples(x axis). **(D)** Volcano plots show DEGs of naive B cells (top panel) and memory B cells (bottom panel) after chemotherapy compared with those before chemotherapy. **(E)** Gene set enrichment analysis of naive B cells (top panel) and memory B cells (bottom panel) after chemotherapy. The analyses were based on the Msigdbr GO database. **(F)** Bar graphs top panel and venn diagram bottom panel show the change of frequency and fraction of unique clonotypes, colored by collection time. **(G)** Clonal homeostatic space representations (clonal space occupied by clonotypes of specific proportions) (left panel) and the relative proportional space occupied by specific clonotypes (right panel) of BCR across samples pre and post chemotherapy. The results above are generated by comparison between samples (*blood_before, blood_after*).

provide us with the mechanism underlying chemo-resistance. Macrophages-secreted GPNMB induces cancer stemness *via* CD44 on cancer cells (61), suggesting that enhanced cancer cell stemness may explain the shorter PFS of this patient, despite high expression of IFN and antigen presentation-related genes. Given that cancer cell and TIME are cross-talked, dual target both parts simultaneously may overcome chemoresistance. The role of γδ T cells in tumor is still unclear and the residency of γδ T cells may play pro- or anti-tumorigenicity (42). Besides, low-activated and immunosuppressive ascites-derived γδ T cells were observed in epithelial ovarian cancer (62), and low metabolism level of T cells can lead to antitumor dysfunction (63). Similarly, we found that ascites-derived γδ T cells had decreased metabolic pathways and increased apoptosis pathways, indicating its immunosuppressive status. These observations suggest that immunosuppressive TME may play an essential role in chemo-resistant HGSOC.

So far, the impact of chemotherapy on phenotype and function of peripheral T/B cells in HGSOC still requires elucidation. Our findings revealed that that chemotherapy promote the transformation of T cells to an exhaustive and dysfunctional status, which interact with enriched M2-like TAM to lead to immune dysfunction, as previous reported (60). In addition, our data showed that chemotherapy leads to T cell senescence, in line with increased IL-6 in peripheral blood, which are hallmarks of cellular senescence (Supplementary Figure S6) (64). Since senescent T cells compose suppressive TME (65), our findings indicate chemotherapy induced immune-suppressive transformation in peripheral blood circulation. Furthermore, our research on TCR reveals a clonal expansion and V(D)J rearrangement, which is not exactly consistent with other study which found that overall repertoire diversity remains stable after the chemotherapy (66). Besides, our results also indicates that chemotherapy leads to the activation, proliferation and maturation of peripheral B cells, suggesting that chemotherapy-induced neoantigens may play a pivotal role in anti-tumor response of B cells through collaboration with T cells (67).

The limitations of this study should be noted here. First, lack of large-number paired clinical resources of relapsed chemo-resistant samples developed from chemo-sensitive, including tumor, ascites and PBMC, leads to inadequate clarification of our conclusion. Second, elucidating mechanism of chemoresistance in HGSOC requires *in vitro* and *in vivo* experiments.

In summary, through integrating cross-sectional analysis of single-cell RNA, TCR and BCR profiles from paired ascites, tumor and peripheral blood samples, we provided important insight into the TME in an HGSOC patient with several cycles of relapse and chemo-resistance. We revealed the variable changes in clonal expansion of the TCR and BCR, laying the foundation for understanding of host anti-tumor immune mechanisms and immune reconstruction induced by chemotherapy. Our research also provides an in-depth exploration of cancerous and immune environments of HGSOC with relapsed platinum-resistance, which may facilitate the development of novel chemotherapy in combination with anti-senescence agents to improve the prognosis and overall survival of ovarian cancer patients.

Data availability statement

The dataset presented in this study is publicly accessible in the GEO database, accession number GSE213243.

Ethics statement

The studies involving human participants were reviewed and approved by the Ethics Committee of Nanfang Hospital (NO. NFEC-2021-424). The patients/participants provided their written informed consent to participate in this study. Written informed consent was obtained from the individual(s) for the publication of any potentially identifiable images or data included in this article.

Author contributions

Conceptualization, YN. Software: RL and JX. Methodology: YR, RL and YN. Formal analysis and statistics: RL, JX and LG. Investigation: YR, RL and SC. Resources: FM. Writing– original draft: YR, HF and RL. Writing– review & editing: YR, HF, RL, YN, YL and FM. Visualization: RL, YR, HF, JX and LG. Supervision: YN, YL, and FM. All authors contributed to the article and approved the submitted version.

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Conflict of interest

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

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2022.985187/full#supplementary-material

SUPPLEMENTARY FIGURE S1
Characteristics of tumor cells revealed at single-cell resolution. **(A)** Fraction and frequency of tumor cells (x axis) from samples (*Tumor, Ascite*) in each subcluster (y axis). **(B)** Heatmap displays the enriched pathways in tumor cell subclusters performed by GSVA analysis. **(C)** t-SNE plot displays main cell types from five HGSOC samples (*T59, T76, T77, T89, T90*). **(D)** Violin plots display expression of selected chemoresistance related genes in tumor cell populations of six samples (*Tumor, T59, T76, T77, T89, T90*). The distribution of the per cell signature expression was based on normalized data. **(E)** Heatmap displays large-scale CNVs of epithelial tumor cells compared to myeloid cells using inferCNV. The annotation on the right indicates the corresponding sample sources. The red represents CNV amplifications and blue represents CNV deletion. **(F)** Heatmap displays top 10 differentially-expressed genes (DEGs) of EC3 in each tumor cell subcluster. **(G)** Expression profiles of top 10 DEGs (shown in Supplementary Figure 1F) were examined by Spearman correlation coefficient between epithelial tumor cells in six HGSOC samples (*Tumor, T59, T76, T77, T89, T90*). **(H)** Violin plots show the enrichment level of interferon-associated signature genes (shown in Figure 2H) among each cell type. Distribution of the per cell signature expression was based on the GSVA scores. **(I)**Kaplan-Meier curve for TCGA-OV cohorts based on expression of interferon-associated signature genes (shown in Figure 2H). The groups are distinguished by median enrichment scores. P value is calculated with log-rank test.

SUPPLEMENTARY FIGURE S2
The landscape of immune cells in ascites, tumor and peripheral blood. (**A** and **B**) t-SNE plots display main cell types from three samples (Tumor, Ascite, blood_before) before chemotherapy, colored by immune cell clusters **(A)** and the origins **(B)**. **(C)** Dot plots display the expression level of signature genes in each immune cell cluster. **(D)** Frequency and fractions of each immune cell cluster among three samples (*Tumor, Ascite and blood_before*). (**E** and **F**) Heatmaps show selected markers(E) and DEGs (F) in each myeloid cell cluster.

SUPPLEMENTARY FIGURE S3
Characteristics of myeloid cells in distinct TMEs and chemokine expression in tumor cell subtypes. **(A, B)** Dot plots show expression level of chemokines ligand family CCL **(A)** and CXCL **(B)** in tumor cell subclusters. **(C, D)** Dot plots show expression level of chemokines receptors family CCR **(C)** and CXCR **(D)** in macrophage cell subclusters. **(E, F)** Ligand-receptor interactions from tumor cell subclusters to APOE+ Macrophages **(E)** and ADAP2+ Macrophages **(F)** in samples (*Tumor, Ascite*). P values are represented by the size of each circle. The color gradient shows the level of interaction. **(G)** Fraction of immune cells from six samples (*Tumor, T59, T76, T77, T89, T90*). **(H)** t-SNE plot displays myeloid cell types from six samples (*Tumor, T59, T76, T77, T89, T90*). **(I)** Boxplots of immune phenotype related gene changes (*CD163, MRC1, CD68, SOCS3, TREM2, GPNMB,* respectively) across macrophage cells from six samples (*indicates a p value < 0.01, ** indicates a p value < 0.001, *** indicates a p value < 0.0001, NS indicates no significance)..

SUPPLEMENTARY FIGURE S4
Landscape of immune cells in peripheral blood collected pre and post the fourth course of chemotherapy. **(A)** Representative H&E and CD8 IHC images for primary (pr_T) and relapsed (re_T) tumor regions shown at ×4 magnification, scale bar 600 μm; ×10 magnification, scale bar 300 μm; and ×20 magnification, scale bar 200 μm. **(B)** t-SNE visualization of immune cell clusters from samples (*blood_before, blood_after*). **(C)** Heatmap shows the expression level of marker genes in myeloid cells, T cells, NK cells and B cells. **(D)** Fraction and frequency of immune cells (x axis) from samples (*blood_before, blood_after*) in each cell type (y axis). **(E)** t-SNE visualization of TCR (top panel) and BCR (bottom panel) distrubtion in all immune cells. **(F)** Proportion of immune cells among samples (*blood_before, blood_after*).

SUPPLEMENTARY FIGURE S5
Comparative analysis of co-stimulatory molecules on T cell clusters in peripheral blood. **(A)** t-SNE visualization of the expression level of *CTLA4* and *PDCD1* in T cells from PBMCs. **(B–E)** Boxplots show the expression level of *CD27, TNFRSF14, TNFRSF1A* and *LAG3* in each $CD4^+$ T cell cluster pre and post chemotherapy. **(F–I)** Boxplots show the expression level of *CD27, TNFRSF14, TNFRSF1A* and *LAG3* in each $CD8^+$ T cell cluster pre and post chemotherapy. **(J)** Boxplots show the expression of *CD27, TNFRSF14, TNFRSF1A* and *LAG3* in γδ T cell cluster pre and post chemotherapy. The results above are generated by comparison between samples (*blood_before, blood_after*).

SUPPLEMENTARY FIGURE S6
Immune function assay of peripheral blood during the treatment of chemotherapy. **(A)** Workflow of the flowcytometry assessing peripheral immune cells and cytokine assay in peripheral blood. Sorting standard of immune cell populations are shown. **(B, C)** Representative flow cytometry plots **(B)** and line charts **(C)** of the proportion of NK cells, $CD8^+$ T_{eff} and T_{reg} in peripheral blood during the treatment of chemotherapy. **(D–F)** Line charts display the change of $CD8^+$ T_{eff}/T_{reg} ratio **(D)**, cytokines concentration **(E)** and IL-6/IL-10 ratio **(F)**. (T1: Before the second chemotherapy began; T2: Two days after the sixth chemotherapy; T3: Fourteen days after the sixth chemotherapy when the sample blood_after was sequenced).

SUPPLEMENTARY FIGURE S7
Comparative analysis of TCRs in $CD4^+$ and $CD8^+$ T cells across samples pre and post chemotherapy. (**A**, **C**) Quantity and percentage of unique clonotypes for $CD4^+$ T cells **(A)** and $CD8^+$ T cells **(C)** between samples pre and post chemotherapy. (**B**, **D**) Clonal homeostasis and clonal proportion of $CD4^+$ T cells (**B**) and $CD8^+$ T cells **(D)** between samples pre and post chemotherapy. (**E**, **F**) Clonotypes diversity measures based on subclusters (left panel) using Shannon, Inverse Simpson, Chao and ACE index. Clonotypes overlap quantifications by clusters (right panel) in $CD4^+$ T cells **(E)** and $CD8^+$ T cells **(F)**. (**G**, **J**) Curve graphs show TCR CDR3 aa length distribution of TRA (upper right) and TRB (bottom right) and both (left) in $CD4^+$ T cells **(G)** and $CD8^+$ T cells **(J)** across samples pre and post chemotherapy. (TRA: α chains, TRB: β chains, aa: amino acid). (**H**, **K**) Violin plots show CDR3 aa length distribution in $CD4^+$ T cells **(H)** and $CD8^+$ T cells **(K)**. (**I**, **L**) Dynamics of dominant CDR3 sequences of TCRs in $CD4^+$ T cells **(I)** and $CD8^+$ T cells **(L)**. **(M)** Bar graphs show the fraction of V and J genes in α chains and β chains among T cells. Genes with significant

changes are labeled red. (*indicates a FDR < 0.01, ** indicates a FDR < 0.001, *** indicates a FDR < 0.0001, **** indicates a FDR < 0.00001). The results above are generated by comparison between samples (*blood_before, blood_after*).

SUPPLEMENTARY FIGURE S8

Comparative analysis of BCRs in naïve and memory B cells across samples pre and post chemotherapy. **(A)** Curve graphs show CDR3 aa length distribution of IGL (upper right), IGH (bottom right) and both (left) of all BCRs across samples pre and post chemotherapy. **(B)** Violin plots show the CDR3 aa length distribution of IGH chains plus IGL chain. **(C)** Dynamics of dominant CDR3 sequences of BCRs across samples pre and post chemotherapy, colored by types of dominant sequences. **(D)** Violin plots show distributions of CDR3 length of naïve B cells (left) and memory B cells(right) across samples pre and post chemotherapy. (**E** and **F**) Bar graphs show the fraction of immunoglobulin IGHV (upper left), IGHJ (upper right), IGLV/IGKV (bottom left), and IGLJ/IGKJ (bottom right) genes in naïve B cells **(E)** and memory B cells **(F)**. Genes with significant changes are labeled red. (*indicates a FDR < 0.01, ** indicates a FDR < 0.001, *** indicates a FDR < 0.0001, **** indicates a FDR < 0.00001). The results above are generated by comparison between samples (*blood_before, blood_after*).

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The role of cancer-associated mesothelial cells in the progression and therapy of ovarian cancer

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Ovarian cancer is currently one of the most common malignant tumors in females with poor survival rates around the world, killing about 200,000 women each year. Although great progress has been made in treatment, most patients receiving first-line therapy experience tumor recurrence. The tumor microenvironment plays an important role in regulating the progression and prognosis of ovarian cancer. Cancer-associated mesothelial cells are the main cell population in the tumor microenvironment, which affect the progression, prognosis and chemical resistance of ovarian cancer. Cancer-associated mesothelial cells can also interact with other microenvironmental components, such as exosomes, macrophages, and adipocytes. Some studies have developed drugs targeting cancer-associated mesothelial cells in ovarian cancer to evaluate the therapeutic efficiency. In this review we highlighted the key role of cancer-associated mesothelial cells in the progression and prognosis of ovarian cancer. We also described the progress of cancer-associated mesothelial cells targeted therapy for ovarian cancer. Continued insight into the role of cancer-associated mesothelial cells in ovarian cancer will potentially contribute to the development of new and effective therapeutic regiments.

KEYWORDS

ovarian cancer, cancer-associated mesothelial cells, tumor progression, chemoresistance, tumor therapy

Introduction

Ovarian cancer (OC) is a common malignant cancer among women in the world and has a poor prognosis (1). According to the latest world health organization data, 313959 patients are diagnosed with OC and 207252 die from it worldwide annually (2). Current frontline treatment for OC consists of initial debulking surgery and subsequent

consolidation chemotherapy. 70-80% of patients experience recurrence after standard frontline treatment, making the five-year survival reach about 45% (3). The high mortality and recurrence rates in OC patients are mainly due to chemotherapy resistance and widespread abdominal metastasis. Recently, immune therapeutics have been introduced to the ovarian cancer treatment landscape, including but not limited to immune checkpoint inhibition (ICI), tumor antigen vaccines and engineered immune cells (4). However, some clinical trials testing ICI in OC have not delivered positive results (5). More effective treatment is urgently in need.

Tumor microenvironment (TME) plays a key role in tumor progression and response to standard chemotherapy. A ton of basic and preclinical studies suggests that co-treatment targeting TME improves therapeutic effect (6). Cancer-associated mesothelial cells (CAMs) are a major part of the OC microenvironment, contributing to cancer progression and chemoresistance. This review summarizes how CAMs obviously influence the progression and prognosis of OC and reviews several targeted therapies for CAMs.

The effects of CAMs in OC

The peritoneum, the omentum and the serous membranes of the small intestine and large intestine are covered by monolayer mesothelial cells. These cells are the primary barrier to prevent the dissemination of OC cells. Like other epithelial cell layers in the body, such as cervical epithelium, the mesothelial cells act as a protective barrier to protect the underlying tissue from OC cells and limit access to the retroperitoneum. A previous study found that primary human mesothelial cells inhibited the initial adhesion and invasion of at least two OC cell lines and three different early passage human OC cell cultures (7). However, in patients with OC, cancer cells can secrete a series of cancer-promoting factors to induce the mesothelial-mesenchymal transition (MMT) of normal mesothelial cells (8). We defined mesothelial cells differentiated by OC cells stimulation as cancer-associated mesothelial cells (CAMs). Compared with mesothelial cells, CAMs undergo obvious morphological changes, and the polarity of cytoskeleton becomes disordered. CAMs also showed significant epithelial-mesenchymal transition (EMT) characteristics, such as the increase of fibronectin, α-SMA and vimentin and reduction of E-cadherin. CAMs no longer have a protective effect, but secrete chemokines to promote the peritoneal metastasis and chemoresistance of ovarian cancer cells.

CAMs and peritoneal metastasis in OC

During the process of peritoneal metastasis of OC, CAMs promote the adhesion and invasion of OC cells to the peritoneum through regulating the expression of multiple chemokines. A recent study found that the expression of intelectin-1 (ITLN1) in CAMs and serum ITLN1 levels in OC patients were significantly lower than those in healthy women. ITLN1 fused with lactotransferrin (LTF) and dampened the binding of LTF to its receptor on the surface of OC cells, low-intensity lipoprotein-receptor-related protein 1(LRP1). ITLN1 attached to LRP1 and transcriptionally increased the expression of MMP1, which contributed to the invasion and metastasis of cancer cells. Simultaneously, ITLN1 inhibited the invasion ability of OC cells by suppressing LTF-induced calcium mobilization and stress fiber formation. In addition, ITLN1 increased recombinant glucose transporter-4 (GLUT4) production in adipocytes, which contributed to increased glucose uptake by adiposes and decreased glucose uptake by tumor cells, thereby the proliferation of OC cells was inhibited. *In vivo* experiments, treatment with recombinant ITLN1 inhibited OC growth (9). In ovarian cancer, hypoxic microenvironment induced CAMs and cancer cells to stabilize HIF-1 and HIF-2. HIF signaling upregulated collagen prolyl 4-hydroxylases (P4HA1, P4HA2 and P4HA3), lysyl hydroxylases (PLOD1 and PLOD2) and lysyl oxidase (LOX) to facilitate the crosslinking and deposition of extracellular collagen type I in mesothelial cells, finally contributed to OC cells metastasis (10). CAMs can also secret several cytokines to promote the metastasis of OC. CAMs were reported to increase the secretion of IL-8 (11) and CCL2 (12). IL-8 secreted by CAMs induced the overexpression of pyruvate dehydrogenase kinase-1 (PDK1) in OC cells *via* CXCR1. PDK1 upregulated the expression of α5 and β1 integrin to enhance the adhesion to fibronectin and mesothelial cells. PDK1 also activated JNK signaling to induce IL-8 production in OC cells (11). In addition, IL-8 bound to CXCR1 and CXCR2 on endothelial cells situated on subperitoneal tissue to promote tumor neovascularization (13). CCL2 facilitated the trans-mesothelial migration and invasion of OC cells *via* activating p38-MAPK pathway through CCR2 (12). Pericellular hyaluronic acid (HA) secreted by CAMs can bind to CD44v3-Vav2 complex on OC cells to activate RhoGTPase (Rac1) pathway signaling, in turn, promoted the activation of cytoskeleton, finally facilitating cancer cells invasion. Simultaneously, HA bound to CD44v3-$p185^{HER2}$ complex to promote $p185^{HER2}$ tyrosine kinase (TK) activation, and then the adaptor molecule Grb2 was recruited. Grb2 not only activated Ras pathway signaling to regulate cancer cells growth, but also interacted with Vav2 to activate Rac1 pathway signaling (14). High levels of Wnt5a deriving from CAMs in ascites fluid boosted the metastasis of OC cells *via* activating its downstream effector Src family kinase Fgr (15). A previous study found that CAMs generated lysophosphatidic acid (LPA) *via* cytosolic phospholipase A2 (cPLA2) and calcium-independent phospholipase A2 (iPLA2) activity to activate extracellular signal-regulated kinase (ERK) and Akt pathway in OC cells, in turn, boosted OC cells to adhere to collagen I, finally promoted the metastasis of OC (16).

The intricate crosstalk between CAMs and cancer cells facilitates the metastasis of OC. Transforming growth factor-β (TGF-β) derived from OC cells induced the phenotypic changes of mesothelial cells to CAMs (17). CAMs increased the secretion of vascular endothelial growth factor (VEGF) in a TGF-β-dependent manner. VEGF secreted by CAMs acted on endothelial cells situated in subperitoneal space and boosted their migratory potential and tube formation ability, thereby promoting tumor neovascularization (18). TGF-β also activated RAC1/SMAD3 pathway *via* TGF-βRII to induce CAMs to upregulate fibronectin expression. Fibronectin in extracellular matrix binds to α5 and β1 integrin on OC cells to support the metastasis (19). Moreover, extrinsic TGF-β derived from OC cells induced the extra secretion of TGF-β from CAMs, leading to a cumulative effect of TGF-β (20). A previous study showed that OC cells secreted hepatocyte growth factor (HGF) to induce MCs to differentiate into CAMs (21). Hepatocyte growth factor (HGF) derived from OC cells also promoted the premature senescence of normal mesothelial cells by inducing mitochondrial oxidative stress *via* activating several signaling pathways including p38-MAPK, AKT and NF-κB (22). Senescent mesothelial cells upregulated the expression of fibronectin (FN) (23) and downregulated the expression of junctional proteins, such as connexin 43, E-cadherin, occludin and desmoglein, leading to destruction of the integrity of the peritoneal mesothelium and makes it easier for the invasion of ovarian cancer (24). Senescent mesothelial cells also secreted angiogenic agents such as CXCL1, CXCL8 and VEGF to stimulate subperitoneal tumor neovascularization (25). In addition, OC cells overexpressed plasminogen activator inhibitor-1 (PAI-1) and transcription factor DLX4 to induce the expression of IL-8/CXCL5 and IL-1β/CD44 *via* activating NF-κB signaling, further enhancing tumor-mesothelial cell interactions and facilitating the metastasis (26, 27).

A schematic illustration of the interaction between CAMs and OC cells to promote metastasis is shown in Figure 1.

CAMs and chemoresistance in OC

Chemoresistance is a primary drawback in the treatment of OC. Multiple studies have demonstrated that HA-CD44 interaction facilitated chemoresistance in various cancers *via* several signaling, such as breast cancer and multiple myeloma (28, 29). In OC, the binding of HA to CD44-Nanog complex activated the expression of Nanog-special target genes Rex1 and Sox2. Nanog activation was determined to be closely related to maintaining the stem cell properties of cancer cells. Some activated Nanog interacted with STAT3 to upregulate the expression of multidrug resistance-1 (MDR1) gene, which contributed to the chemoresistance of cancer cells. In addition, HA facilitated the interaction of ankyrin-MDR1 (P-gp) with CD44 and the complex led to chemotherapeutic drugs efflux in OC cells (30). Similarly, another study found that HA induced the expression of membrane ATP binding cassette (ABC) transporter proteins in OC cells to increase chemo resistance (31).

Recently, a study found that CAMs can secret osteopontin (OPN) to media chemoresistance and stemness in OC. OC cells induced CAMs to upregulate the expression and secretion of OPN in a TGF-β dependent manner. OPN activated HA/CD44/PI3K-AKT signaling pathway to promote the expression of ABC transporter proteins and regulate BCL-2/BAX ratio, finally contributed to boosting chemoresistance (32). Another mechanistic study indicated that the overexpression of FN in CAMs also reduced platinum-sensitivity in OC cells by activating Akt signaling pathway (33). Moreover, the OC spheroids display enhanced resistance to anti-cancer drugs compared to monolayers, while CAMs promoted spheroid formation by OC cells and induced their motility (34, 35). Chemoresistance OC cells showed a higher ability to adhere and grow on mesothelium, which enhances the dissemination and invasion of cancer cells. A schematic illustration of CAMs promoting chemoresistance in OC is shown in Figure 2A.

CAMs and prognosis of OC

Peritoneal metastasis and chemoresistance dramatically influence the prognosis of OC. Secretion of CAMs such as OPN and ITLN1 have been demonstrated to predict overall survival rates in mice (9, 32). A recent scRNA-seq study, which analyzed 18,403 cells gathered from seven untreated patients with high-grade serous tubo-ovarian cancer, identified 6 cellular phenotypes associated with prognosis (36). It was found that concentration of CAMs was correlated with poor outcome. A prospective observational cohort study found that the expression of vascular cell adhesion molecule-1 (VCAM-1) on the CAMs negatively correlated with progression-free and overall survival in OC (37). Moreover, patients with consistently high VCAM-1 expression were more likely to develop platinum resistance than patients expressing low VCAM-1.

CAMs and microenvironment in OC

The highly inhibitory immune microenvironment is considered to be one of the dominant reasons for tumor progression and treatment failure in OC patients. As a key part of the tumor microenvironment, CAMs interact with other cells in the microenvironment to regulate the progression of OC. A schematic illustration of CAMs interacting with other cells in the microenvironment in OC is shown in Figure 2B.

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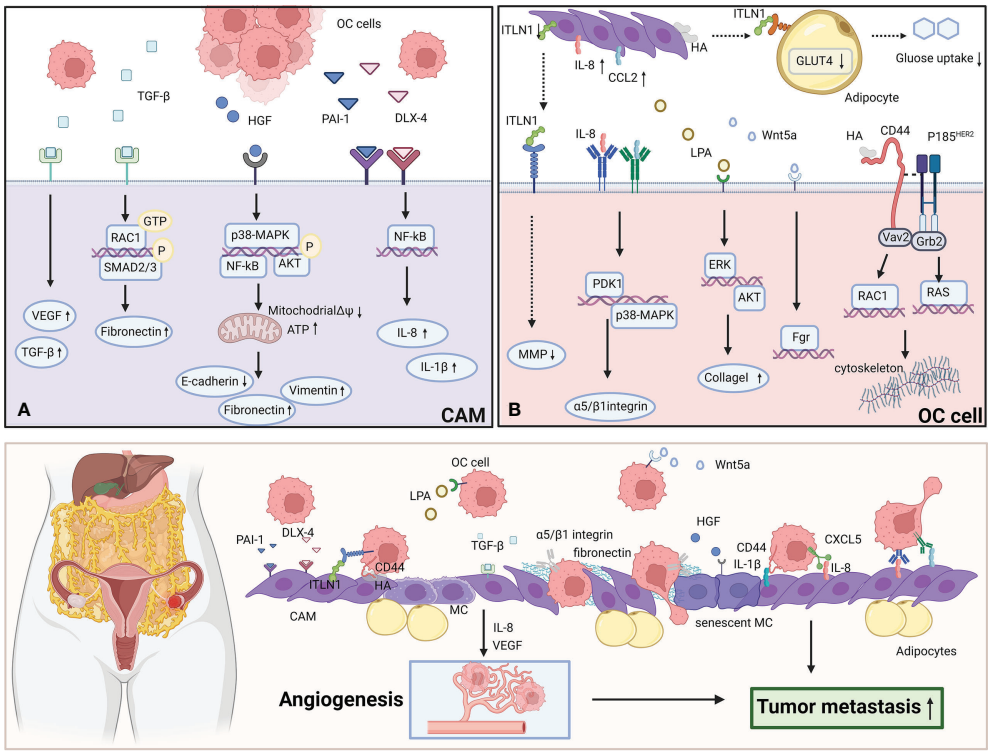

FIGURE 1
CAMs interact with OC cells to promote the metastasis. **(A)** OC cells secret TGF-β, HGF, PAI-1, DLX-4 to effect CAMs *via* various signaling pathways. TGF-β activates RAC1/SMAD3 pathway *via* TGF-βRII to induce CAMs to upregulate fibronectin expression. HGF promotes the premature senescence of normal mesothelial cells by inducing mitochondrial oxidative stress *via* activating several signaling pathways including p38-MAPK, AKT and NF-κB. PAI-1 and DLX4 induce the expression of IL-8/CXCL5 and IL-1β/CD44 *via* activating NF-κB signaling. **(B)** CAMs overexpress ITLN1, IL-8, CCL2, LPA, Wnt5a and HA to effect OC cells by activating several signaling pathways. IL-8 induces the overexpression of PDK1 in OC cells *via* CXCR1.PDK1 upregulates the expression of α5 and β1 integrin to enhance the adhesion to fibronectin and mesothelial cells. CCL2 facilitates the trans-mesothelial migration and invasion of OC cells *via* activating p38-MAPK pathway through CCR2. Wnt5a boosts the metastasis of OC cells *via* activating its downstream effector Src family kinase Fgr. LPA activates ERK and Akt pathway to boost OC cells to adhere to collagen I. HA can bind to CD44v3-Vav2 complex on OC cells to activate Rac1 and Ras pathway signaling. The figure was created with BioRender.com.

CAMs and exosomes

Exosomes are 30-100nm membrane vesicles of endocytosis origin, mediating cell-cell communication and antigen presentation *via* transferring proteins, mRNAs, and microRNAs (38). Recent reports displayed that tumor-derived extracellular exosomes played an important role in communication between CAMs and OC cells to induce immunosuppression, thereby promoting the direct adhesion and invasion of cancer cells to CAMs. For example, OC-derived exosomes carrying CD44 reprogramed mesothelial cells to a more EMT phenotype, which facilitated cancer adhesion and invasion (39). Similarly, *via* co-culturing exosomal annexin A2 (ANXA2) derived from OC cells with human peritoneal mesothelial cells, researchers found that ANXA2 activated PI3K/AKT/mTOR pathway to promote MMT and the degradation of the extracellular matrix of mesothelial cells, finally facilitating establishing pre-metastasis microenvironment of OC (40). In addition, some researchers proposed that OC-derived extracellular vesicles containing MMP1 mRNA induced the apoptosis of mesothelial cells, which exposed the underlying tissue and facilitated peritoneum colonization (41).

CAMs and macrophages

CAMs can secrete Wnt5a to regulate macrophage polarization. High levels of Wnt5a in ascites fluids activated the Src family kinase Fgr to enhance the immunosuppressive immune landscape of OC and promote peritoneal colonization. The knockout of Wnt5a contributed to an increase in M1 macrophages and a decrease in M2 macrophages in a mouse model of ovarian cancer (15). Generally, M1 macrophages

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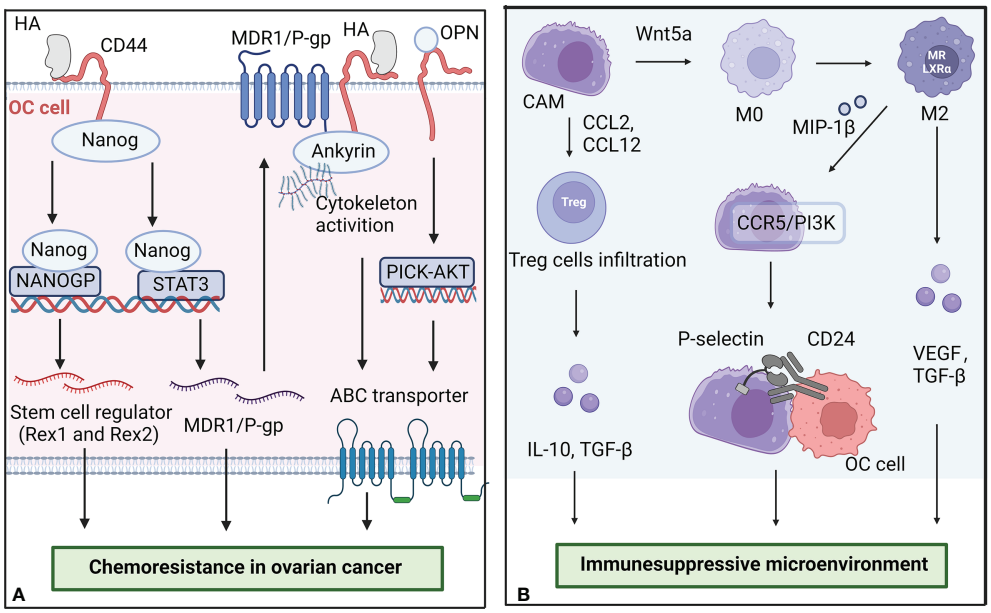

FIGURE 2
The role of CAMs in chemoresistance and the formation of immunesuppressive microenvironment. **(A)** CAMs secret HA and OPN to promote the chemoresistance of OC cells. The binding of HA to CD44-Nanog complex activated the expression of Nanog-special target genes Rex1 and Sox2. Some activated Nanog interacted with STAT3 to upregulate the expression of multidrug resistance-1 (MDR1) gene. OPN activated HA/CD44/PI3K-AKT signaling pathway to promote the expression of ABC transporter proteins. **(B)** CAMs interact with other cells in the microenvironment to promote the formation of immunesuppressive microenvironment in OC. CAMs can secrete Wnt5a to regulate macrophage polarization and increase T regulatory cell infiltration. M2 macrophages promote the adhesion of CAMs and OC cells by overexpressing MIP-1β. The figure was created with BioRender.com.

secrete proinflammatory cytokines such as TNF-α, IL-1 and IL-6, which promotes anti-tumor immune response and enhances immune monitoring, while M2 macrophages mediate immunosuppressive response and promote chronic inflammation and tumor invasion mainly by secreting inhibitory cytokines such as TGF-β, VEGF and MMPs. Moreover, Wnt5a expression increased CCL2, CCL12, CXCL10, and CXCL12 production, which correlating with T regulatory cell and tumor-associated macrophage infiltration.

CAMs influence macrophage polarization, while macrophages can also regulate CAMs to promote the adhesion and invasion of ovarian cancer. M2 macrophages can secrete MIP-1β to activate CCR5/PI3K signaling and then increase P-selectin production by CAMs. P-selectin binds to CD24 on the OC cells surface, leading to increased adhesion of cancer cells (42). Anti-P-selectin antibody and small molecular inhibitor were demonstrated to inhibit OC cells adhesion *in vivo* and *in vitro*.

CAMs and cancer-associated fibroblasts (CAFs)

During peritoneal metastasis, tumor cells can induce the transformation of mesothelial cells into CAFs. A previous study found the presence of CAFs expressing mesothelial markers at the site of tumor implantation in patients with peritoneal dissemination (8). By single-cell RNA sequencing and spectral tracing assays, a recent study demonstrated that antigen-presenting CAFs were derived from mesothelial cells. Further study revealed that IL-1 and TGF-β can induce mesothelial cells to downregulate mesothelial features and acquire fibroblastic features during tumor progression. Antigen-presenting CAFs induced regulatory T cells formation and expansion in an antigen-specific manner, which contributes to tumor immune escape (43).

CAMs and adipocytes

OC cells are prone to metastasize to the omental fat pad (44). Omental adipocytes release and transport free fatty acids to maintain the high energy requirements of cancer cells (45). In diet-induced obesity mice, the density of microvilli on peritoneal mesothelial cells was significantly increased, which contributes to early cell-cell adhesive events in metastatic colonization in ovarian cancer (46). Moreover, the downregulation of ITLN1 in CAMs inhibited the insulin-dependent glucose uptake in mature adipocytes *via* suppressing the expression of GLUT4, which

increased the glucose available to OC cells and promote the tumor growth (9).

Targeting CAMs in therapy of OC

CAMs interact with OC cells *via* expressing some specific markers, such as MSLN, FN and HA. Some studies have developed antibodies targeting these markers to block the interaction of CAMls with OC cells, thereby inhibiting the progression of OC and improving the prognosis of OC survivors.

MUC16 - Mesothelin

MUC16 is a glycoprotein that is overexpressed by OC cells. The shedding of MUC16 from the surface of OC cells to circulation is the basis of serum assay CA125 in clinical. Mesothelin (MSLN) is a differentiation antigen mainly expressed on CAMs, OC cells and mesothelioma cells. MUC16–MSLN interaction mediated the attachment and adhesion of OC cells to CAMs. Blocking the MUC16–MSLN interaction can effectively inhibit cancer cell adhesion and invasion.

Initially, antibodies against MUC16 were developed for the immunotherapy of OC, such as murine IgG1 oregovomab (mAb B43.13, OvaRex) (47). However, the treatment with oregovomab monotherapy failed to prolong the survival of patients with advanced OC in a phase III clinical trial (48). The combination therapy with oregovomab plus carboplatin/paclitaxel effectively improved overall and progression-free survival (49). Some clinical trials of oregovomab in combination with other drugs are also underway, such as bevacizumab and niraparib (ClinicalTrials.gov identifier: NCT04938583; NCT05335993). Later, Antibody–drug conjugates (ADCs) targeting the repetitive MUC16 epitopes were developed (50), which were revealed to have higher antitumor activity, such as 3A5-MMAE (monomethyl auristatin E) (51). Constructing chimeric antigen receptor (CAR) targeting to the retained extracellular domain of MUC16 (MUC-CD) has also been demonstrated to exhibit efficient antitumor activity *in vitro* and *in vivo* (52). Recently, a human bispecific T-cell engaging antibodies (REGN4018) bridging MUC16-expressing cells with CD3 T cells was developed (53). In preclinical studies and toxicology studies, REGN4018 displayed potent antitumor activity and good tolerability. Moreover, the combination of bispecific T-cell engaging antibodies and anti-VEGF enhanced the efficacy (54). Using an oncolytic adenovirus carrying a MUC16- bispecific T-cell engagers (BiTEs) can activate and retarget CTLs to enhance the anti-tumor effect (55). Currently, Phase 1/2 trails are recruiting patients (ClinicalTrials.gov identifier: NCT03564340; NCT04590326).

MORAb-009, a chimeric antibody targeting MSLN, is being investigated in multiple clinical studies. Patients with MORAb-009 treatment exhibited more stable disease and clear increase in serum MUC16, which suggesting MORAb-009 disturbs the MSLN–MUC16 interaction (56). Anti-MSLN antibody-drug conjugate anetumab ravtansine is composed of a human anti-MSLN IgG1 and a maytansine derivative tubulin inhibitor DM4, which shows selective and potent antitumor activity in xenograft tumor models (57). In a phase I multicenter trial, anetumab ravtansine displayed a manageable safety, favorable pharmacokinetics and preliminary antitumor activity in patients with mesothelin-expressing solid tumors (58). Additionally, A randomized phase II Study is underway (ClinicalTrials.gov identifier: NCT03587311). Anti-MSLN CAR-T cells are in progress with some clinical trials (ClinicalTrials.gov identifier: NCT04562298; NCT04503980). In orthotopic mouse models of OC, MSLN-directed CAR T cells provided antitumor immunity and significantly prolonged survival (59). Clinical trials of MUC16-MSLN targeted drugs are described in detail in Table 1.

FN - α5β1 integrin

FN secreted by CAMs is one of the most abundant extracellular matrix proteins in the peritoneal microenvironment. OC cells can adhere to FN *via* α5β1 integrin and directly induce phosphorylation of focal adhesion kinase (FAK), further leading to activation of mitogenic pathways supporting tumor growth (60). Blocking antibodies against α5β1 integrin effectively inhibited OC cells adhesion to mesothelial cells. Volociximab is a high-affinity, chimeric antibody directed against human α5β1 integrin. However, in a phase II, single-arm study, volociximab treatment failed to achieve sufficient clinical activity in patients with recurrent, platinum-resistant ovarian cancer (61). The disappointed result may be related to the use of a single agent intervention in recurrent and advanced OC patients. Combination therapy with volociximab in low-volume residual disease after cytoreductive surgery or as maintenance therapy to prevent recurrence of ovarian cancer may be effective. Moreover, a previous study reported that resveratrol decreased cellular α5β1 integrin level to inhibit ovarian cancer cell adhesion to CAMs *in vitro* (62).

Tissue transglutaminase (TG2) is a transpeptidase that promotes the formation of FN - α5β1 integrin complexes by interacting with FN. A function-inhibiting antibody against the TG2 FN-binding domain suppressed complexes formation and blocked the proliferation of cancer stem cells (63). Compound ITP-79 inhibited the binding of TG2 peptide to the 42-KDA FN fragment in a dose-dependent manner, thereby disrupting FN -

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TABLE 1 Summary of clinical trials using MUC16-mesothelin (MSLN) and FN - α5β1 integrin targeted agents.

Target	Agent	Type of clinical trial	Patient population	Enrollment	Status	ClinicalTrials.gov Identifier
HGF	Rilotumumab	Phase II	Patients with recurrent or persistent ovarian cancer	31	Completed	NCT01039207
MUC16	Oregovomab	Phase II	Patients with ovarian cancer (FIGO Stage III or IV)	102	Terminated	NCT00034372
	Oregovomab	Phase III	Patients with ovarian cancer (FIGO Stage III or IV)	354	Terminated	NCT00050375
	Oregovomab	Phase II	Patients with ovarian, fallopian tube, or peritoneal cancer	102	No known	NCT00004064
	Oregovomab	Phase II	Patients with residual disease from stage III or stage IV ovarian epithelial, fallopian tube, or peritoneal cancer following surgery and chemotherapy	400	No known	NCT00003634
	Oregovomab +Carboplatin + Paclitaxel	Phase II	Patients with advanced ovarian cancer	97	Completed	NCT01616303
	Oregovomab +Bevacizumab +Paclitaxel +Carboplatin	Phase I/II	Patients with BRCA wild type platinum sensitive recurrent ovarian cancer	54	Recruiting	NCT04938583
	Oregovomab +Nivolumab	Phase I/II	Patients with epithelial cancer of ovarian, tubal or peritoneal origin	13	Terminated	NCT03100006
	Oregovomab +Poly ICLC	Phase I	Patients with CA125-associated, advanced ovarian cancer (FIGO Stage III/IV)	10	Terminated	NCT03162562
	Oregovomab +Paclitaxel + Carboplatin +Placebo	Phase III	Patients with advanced epithelial ovarian cancer following optimal debulking surgery	602	Recruiting	NCT04498117
	Oregovomab + Nivolumab + Chemotherapy	Phase I/II	Patients with epithelial cancer of ovarian, tubal or peritoneal origin	31	Recruiting	NCT04620954
	Oregovomab +PLD	Phase II	patients with PARP inhibitor-resistant ovarian cancer	28	Recruiting	NCT05407584
	Oregovomab +Niraparib	Phase II	Patients with platinum sensitive recurrent ovarian cancer.	10	Recruiting	NCT05335993
	DMUC5754A	Phase I	Patients with platinum-resistant ovarian cancer or unresectable pancreatic cancer	77	Completed	NCT01335958
	REGN4018	Phase I/II	Patients with recurrent ovarian cancer	554	Recruiting	NCT03564340
	REGN4018	Phase I/II	Patients with recurrent ovarian cancer	326	Recruiting	NCT04590326
mesothelin (MSLN)	MORAb-009	Phase I	Patients with mesothelin-positive cancers: ovarian, pancreatic, mesothelioma, non-small cell lung cancer.	24	Completed	NCT00325494
	MORAb-009	Phase I	Patients with mesothelin-positive cancers: ovarian, pancreatic, mesothelioma, non-small cell lung cancer.	6	Completed	NCT01521325
	MORAb-009	Early phase I	Patients with mesothelin-positive cancers: ovarian, pancreatic, mesothelioma, non-small cell lung cancer.	7	Terminated	NCT01413451
	Anetumab Ravtansine +Pegylated Liposomal Doxorubicin	Phase I	Patients with ovarian cancer	65	Completed	NCT 02751918
	Anetumab Ravtansine + Bevacizumab + Paclitaxe	Phase I	Patients with refractory ovarian, fallopian tube, or primary peritoneal cancer	96	Active, not recruiting	NCT03587311
	LCAR-M23 (CAR-T cell)	Phase I	Patients with relapsed and refractory epithelial ovarian cancer	34	No known	NCT04562298
	αPD1-MSLN-CAR T cells	Early phase I	Patients with MSLN-positive advanced solid tumors: ovarian cancer, cholangiocarcinoma, colorectal cancer	10	Recruiting	NCT04503980

(Continued)

TABLE 1 Continued

Target	Agent	Type of clinical trial	Patient population	Enrollment	Status	ClinicalTrials.gov Identifier
α5β1 integrin	Volociximab +Liposomal Doxorubicin	Phase I/II	Patients with advanced epithelial ovarian cancer or primary peritoneal cancer relapsed after prior therapy with Plat/ Taxane-based chemo	138	Completed	NCT00635193
	Volociximab	Phase II	Patients with platinum-resistant, advanced epithelial ovarian or primary peritoneal cancer	16	Terminated	NCT00516841

α5β1 integrin complexes and blocking the adhesion of cancer cells to mesothelial cells (64). FN - α5β1 integrin complexes targeting strategies need to be further optimized and tested for safety, tolerability and efficacy in clinical trials in the future. Clinical trials of FN - α5β1 integrin targeted drugs are described in detail in Table 1.

HA-CD44

The binding of HA derived from CAMs to CD44 expressed on OC plays a significant part in promoting tumor metastasis and chemoresistance. Formerly, HA-based drugs have been shown to have anticancer activity in human OC nude mouse xenograft models (65). A study showed that CD44 targeting HA nanoparticles successfully delivered MDR1 siRNA into OC cells, and the nanoparticles combined with paclitaxel improved the sensitivity of MDR cells to paclitaxel and overcome the chemoresistance of OC (66). Subsequently, various HA-conjugated nanomedicines were developed to delivery chemotherapeutic agents such as Granzyme B, paclitaxel and FAK siRNA (67, 68). Some clinical trials have demonstrated the safety and tolerance of HA-based nanoconstructs in colon cancer (69). In the future, clinical trials are needed to further explore the efficacy of CD44 targeting HA-conjugated nanomedicines in the treatment of OC.

HGF

HGF derived from OC promoted the premature senescence of normal mesothelial cells. Senescent mesothelial cells facilitated mesothelial clearance and tumor angiogenesis. A separate study showed HGF led to chemoresistance of OC by upregulating the MET/PI3K/Akt pathway (70). Rilotumumab (AMG 102) is an anti-HGF monoclonal antibody developed to neutralize the biological activity of HGF, thus blocking the HGF/MET pathway. However, in a phase II clinical trail, rilotumumab monotherapy showed limited benefit in patients suffering recurrent or persistent OC (71). This implies that HGF inhibitor combined with other therapeutic strategies may potentially improve efficacy and overcome chemoresistance.

Other drugs mechanism effect CAMs for OC treatment

Several studies have shown that drugs normally used to treat tumors can also modulate CAMs, such as vitamin D, metformin, tamoxifen and so on.

Vitamin D

Some epidemiological studies suggest that low circulating level of vitamin D is related to poor outcome in patients with various cancers (72, 73). In a meta-analysis of randomized controlled trials, vitamin D supplementation therapy can significantly reduce cancer-related mortality (74). A recent study found that vitamin D inhibited the EMT of mesothelial cells to suppress tumorigenesis in OC (75). Mechanistically, vitamin D inhibited thrombospondin-1 expression by suppressing Smad-dependent TGF-β signaling through VDR-SMad3 competition, which blocking the interaction between CAMs and cancer cells. In particular, the stabilized mesenchymal state of CAMs was restored to its normal epithelial state of preventing cancer cell adhesion and growth by adding vitamin D. Moreover, vitamin D was confirmed to reduce MMPs secretion in cancer-associated mesothelial cells. The inhibition of TGF-β signaling and MMPs secretion can enhance the efficacy of immune checkpoint inhibitors (76, 77). These results displayed that the combination of vitamin D and chemotherapy may be effective in advanced ovarian cancer.

β-Escin

β-Escin is the main active component in horse chestnut seed extract, and its anticancer activity has also been reported in various cancers. A study using a three-dimensional quantitative high-throughput screening platform (3D-qHTS) to screen 2420 naturally extracted compounds found thatβ-escin can effectively suppress migration and viability of OC cells *in vitro*. In further mechanistical study, β-escin treatment regulated HIF1α stability and reduced the expression of fibronectin, laminin-C1, tenascin,

and collagen1-a2 in CAMs in mouse, which contributing to the decreased ability of OC cells to adhere and invade (78).

Metformin

Metformin is a common drug used to treat type 2 diabetes. Recent epidemiological studies have shown that metformin has antitumor effects. A prospective phase II clinical trial found that metformin treatment was well tolerated in nondiabetic OC patients and contributed to better median overall survival (OS) (79). Metformin may target multiple immune cells in OC, such as T cells, myeloid-derived suppressor cells (MDSCs), neutrophils and macrophage (80–83). Recently, metformin was reported to alter CAMs in the omental microenvironment (84). Metformin inhibited the expression of tricarboxylic acid (TCA) enzyme succinyl CoA ligase (SUCLG2), activated prolyl hydroxylases (PHDs), finally leading to the inhibition of TGF-β-driven metabolic upregulation of HIF1α in CAMs. The degradation of HIF1α contributed to reducing CCL2 and IL-8 production, thereby blocking the invasion of OC cells to mesothelial cells.

Acacetin

Acacetin is a natural flavonoid widely found in vegetables. Previous studies suggested that acacetin showed anti-cancer efficacy in various cancers. In a mouse model of gastric cancer, acacetin treatment delayed the development of peritoneal metastasis *via* inhibiting PI3K/Akt/Snail signal pathway (85). Recently, emerging evidence has confirmed that acacetin inhibits CAMs-evoked malignant characteristics and reduces PCNA and MMPs secretion, which suppressing the proliferation and invasion of OC cells (86). Mechanically, acacetin can suppress LPA secretion in CAMs and further block the activation of receptor for advanced glycation end-products (RAGE)-PI3K/AKT signaling in OC cells. Moreover, acacetin decreased the secretion of pro-inflammatory cytokine IL-6 and IL-8 production in CAMs.

L-carnosine

L-carnosine is a dipeptide widely distributed in human tissues, and it has anti-senescence and anti-cancer properties. Some studies showed that L-carnosine prolonged the replication life of somatic cells and inhibited the growth of cancer cells *in vitro* and *in vivo* (87). Interestingly, a previous report found that L-carnosine retarded senescence of human peritoneal mesothelial cells and suppressed progression of OC cells (88, 89). As mentioned earlier, mesothelial cells are peculiarlysusceptible to oxidative stress, which facilitates their senescence. L-carnosine can reduce mitochondrial oxidative stress by improving the cell's ability to produce ATP, thereby leading to a compensatory reduction in mitochondrial biogenesis and superoxide production. Moreover, L-carnosine decreases various pro-cancerogenic factors secretion by CAMs, such as IL6, IL8, GRO1, PAI 1 and TGFβ1.

HSVTK-modified CAMs

CAMs can be also used as drug carriers to enhance antitumor effects. A previous study engineered CAMs with the herpes simplex virus thymidine kinase/ganciclovir (HSVTK/GCV) system (90). Engineered CAMs can deliver the HSVTK bystander effect to human OC cells and induce the apoptosis of cancer cells. Intraperitoneal administration of HSVTK-expressing CAMs resulted in reduced tumor growth and prolonged survival in mouse model of OC. Moreover, distribution studies showed that engineered CAMs were preferentially located in tumor sites.

Tamoxifen

Tamoxifen is an estrogen receptor modulator that has been shown to be used in the treatment of chronic peritoneal diseases. In mice peritoneal dialysis model, tamoxifen blocked TGF-β1-induced MMT of normal mesothelial cells, thereby inhibiting peritoneal fibrosis (91). Tamoxifen also inhibited GSK-3β/β-catenin signal pathway to attenuate peritoneal fibrosis (92). In the future, tamoxifen can be used as a prevention against mesothelial cells transformation in improving the treatment of OC peritoneal metastasis (93).

Eficiencies and prospects

OC is a fatal disease with a high recurrence rate and a low 5-year survival. Immunotherapy has a lower successful ratio in OC compared with other immunogenic tumors, such as non-small cell lung cancer (NSCLC) and melanoma. In the last two decades, improvements in surgical approaches and the development of chemotherapeutic agents have led to improved survival rates in patients with advanced ovarian cancer. However, cytotoxic drug therapy is non-selective and usually results in transient antitumor responses and significant toxicity. The vast majority of women with ovarian cancer develop drug resistance after receiving first-line chemotherapy (94). There is increasing evidence that TME plays an important role in shaping tumor heterogeneity and drug resistance (95). Some studies have analyzed the feasibility of modifying TME as a treatment for OC. Exploring immunotherapies targeting the components of TME, such as dysfunctional immune cells, exosomes, CAMs and

metabolites, would help to develop immunotherapies in OC. Mesothelial cells are the major components of OC microenvironment. They are arranged in the viscera and wall of peritoneal cavity, and are widely present in malignant ascites. Some studies have found that CAMs are closely related to the intraperitoneal metastasis, chemical resistance and tumor recurrence of OC (32, 33). In addition, some therapies that attempt to target CAMs have proved effective. For example, in a preclinical study, therapeutic targeting of CAMs-derived OPN enhances cisplatin response by increasing drug concentrations and DNA damage in OC cells (32). Blocking the interaction of CAMs with OC cells by using neutralizing antibody or aptamers has also been shown to be effective *in vivo* and *in vitro*. The review highlighted the key role of CAMs in the progression and prognosis of OC. We also described the progress of CAMs targeted therapy for OC. As the understanding of the mechanisms by which the TME effects OC progression and metastasis continue to improve, new therapeutic targets will be identified and validated, potentially contributing to the development of new and effective therapeutic regiments.

Author contributions

AZ wrote original draft, drew the figure and made the summarizing table. YW, YZ and TZ corrected the draft and wrote the final version. All authors have read and agreed to the published version of the manuscript.

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Conflict of interest

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

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Efficacy evaluation of multi-immunotherapy in ovarian cancer: From bench to bed

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Ovarian cancer, one of the most common gynecological malignancies, is characterized by high mortality and poor prognosis. Cytoreductive surgery and chemotherapy remain the mainstay of ovarian cancer treatment, and most women experience recurrence after standard care therapies. There is compelling evidence that ovarian cancer is an immunogenic tumor. For example, the accumulation of tumor-infiltrating lymphocytes is associated with increased survival, while increases in immunosuppressive regulatory T cells are correlated with poor clinical outcomes. Therefore, immunotherapies targeting components of the tumor microenvironment have been gradually integrated into the existing treatment options, including immune checkpoint blockade, adoptive cell therapy, and cancer vaccines. Immunotherapies have changed guidelines for maintenance treatment and established a new paradigm in ovarian cancer treatment. Despite single immunotherapies targeting DNA repair mechanisms, immune checkpoints, and angiogenesis bringing inspiring efficacy, only a subset of patients can benefit much from it. Thus, the multi-immunotherapy investigation remains an active area for ovarian cancer treatment. The current review provides an overview of various clinically oriented forms of multi-immunotherapy and explores potentially effective combinational therapies for ovarian cancer.

KEYWORDS

ovarian cancer, immunotherapy, multi-immunotherapy, immune checkpoint inhibitor, adoptive cell therapy, cancer vaccine, oncolytic virus

1 Introduction

Ovarian cancer is the most lethal gynecological malignancy, of which epithelial ovarian cancer (EOC) is the most prevalent subtype. Most EOC patients are diagnosed with advanced stage accompanied with tumor spread to the peritoneal cavity. Current frontline treatments include debulking surgery, platinum-taxane maintenance chemotherapy, and recently developed targeted agents and immunotherapy. Despite aggressive treatment, the

5-year survival rate for women diagnosed with stage III or IV disease is still less than 25% (1). Most patients would suffer a recurrence after the initial response to therapy and almost all of them resistance to chemotherapy and leading to the death.

Growing evidence suggests that ovarian cancer is immunogenic cancer. There has been a significant increase in understanding of molecular and genetic changes in the ovarian cancer microenvironment. Thus, various immunotherapies target the tumor microenvironment (TME) and attempt to address the challenges posed by the highly immunosuppressive TME (2). Current immunotherapy for ovarian cancer includes immune checkpoint blockade, adoptive cell therapy, cancer vaccine, oncolytic virus and so on (Figure 1). Despite several of them achieving inspiring efficacy in the clinic, such as PARP inhibitors. Only a tiny fraction of patients benefited from them, and most of them would eventually suffer a recurrence or progression. With the limited efficacy brought by studies testing single-agent immunotherapy in recurrent ovarian cancer, optimism has resurfaced around the possibility that combinational therapy would deliver the better outcome expected by the community. In this review, we summarize the progress of clinical developments in multi-immunotherapies for ovarian cancer and briefly discuss the future directions of combinational therapies in ovarian cancer.

2 Tumor microenvironment in ovarian cancer

The TME comprises the extracellular matrix (ECM) and stromal cells. The ECM consists of water, proteoglycans, minerals, and fibrous proteins secreted by resident cells in an interlocking network (3). The ECM plays a critical role during tumorigenesis, affecting cell migration, invasion, and metastasis. Besides, stromal rearrangement plays a supportive role during the malignancy progresses and eventually, the tumoral and stromal changes aggravate each other and promote a dynamic reciprocity cycle (4). The matrix-centric, stromal-targeted cancer therapies developed as the ECM is altered at the biochemical, architectural, biomechanical, and topographical levels (5). Stromal cells in the TME include cancer-associated adipocytes, mesothelial cells, fibroblasts, and immune cells. Immune cells include tumor-infiltrating lymphocytes (TILs), Tregs, neutrophils, macrophages, dendritic cells (DCs), natural killer (NK) cells, myeloid-derived suppressor cells (MDSCs), polymorphonuclear neutrophils (PMNs), and so on (6, 7) (Figure 2). The tumor-permissive TME is achieved by reprogramming host cells to support tumor phenotypes and functions (6). The metastatic tropism of cancer cells to the omentum, characterized by highly vascularized immune cell structures called milky spots, plays a critical role in the generation of the metastatic TME in the intraperitoneal cavity (6). In addition, not only components in the TME communicate and impact each other, but also ovarian cancer cells communicate with TME through various signaling pathways, such as STATs family pathway, IL-6 pathway, and NF-KB pathway (1). Several factors are associated with response to immunotherapy, including T cell exhaustion, PD-L1 status, microsatellite instability, mismatch repair deficiency, Tumor mutation burden (TMB), CD8+ positivity, T cell infiltration and so on (8). Thus, immunotherapies target TME developed, current immunotherapies target ovarian cancer TME including CAFs targeting therapy, anti-angiogenesis therapy, immune

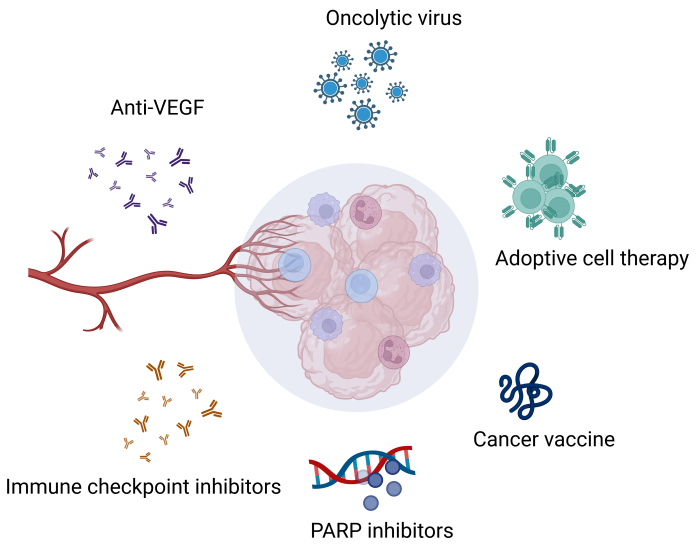

FIGURE 1
Immunotherapies in ovarian cancer. Created with BioRender.com.

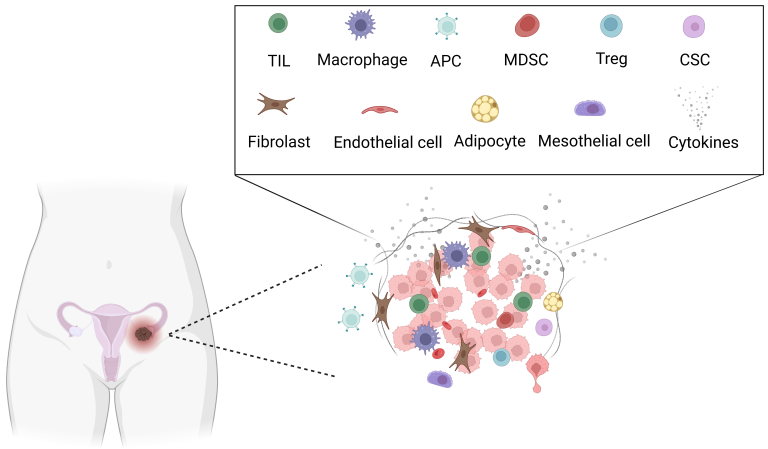

FIGURE 2
Tumor microenvironment in ovarian cancer. Created with BioRender.com. TIL: Tumor-infiltrating lymphocytes (TILs), APC: Antigen-presenting cell, MDSC: Myeloid-derived suppressor cells (MDSCs), Treg: Regulatory T, CSC: cancer stem cell.

checkpoint inhibitors (ICIs), oncolytic virus and so on (9). Tumors responsive to ICIs are usually called hot tumors, which depends on T cells' infiltration. On the contrary, cold tumors usually do not respond to ICIs, which is characterized by poor T cell infiltration (10). Besides, the effectiveness of immunotherapy is associated with baseline immune responses and unleashing of pre-existing immunity. Thus, combinational immunotherapies may boost weak antitumor immunity, enhance tumor antigens cross-presentation, and promotes T cell priming and infiltration (11).

3 Targeting DNA repair-based combination immunotherapies

There are at least five recognized pathways that exist for DNA repair: direct repair, mismatch repair (MMR), nucleotide excision repair (NER), base excision repair (BER), and double-strand break (DSB) recombinational repair. DSB occurs by non-homologous end-joining and high-fidelity homologous recombination repair, which is much more error prone (12). Besides, germline aberrations in critical DNA repair and DNA-damage response (DDR) genes contribute to cancer susceptibility syndromes, including BRCA1, BRCA2, BLM, FANCA, TP53, RAD51C, and MSH2. After exposure to carcinogens, the generation of DNA damage increases the risk of cancer. Therefore, genomic instability is a recognized hallmark of cancer (13). Various agents are developed to target different processes during DNA repair, including PARP inhibitors, NER inhibitors, BER inhibitors, DDR kinases inhibitors, inhibitors targeting termini recognition, end bridging, DNA-end processing, and DNA ligation, inhibitors targeting homology directed repair and Rad51 (14). We will focus on PARPi-based combinational therapies, as it is most widely studied in ovarian cancer.

3.1 PARPi-based combination immunotherapies

The poly (ADP-ribose) polymerase (PARP) is a recognized sensor of DNA damage, which is known for its role in DNA BER and DNA single-strand breaks (SSB) repair. The role of PARP in DSB repair is less elucidated (13). PARP inhibitors have been a new targeted treatment for ovarian cancer, particularly in women with BRCA1 and BRCA2 mutation or patients without a functional homologous recombination repair pathway (15). Homologous recombination deficient cells are susceptible to PARP inhibitors. BRCA1 and BRCA2 are tumor suppressor genes. They are associated with fundamental roles in DNA repair by forming a homologous recombination repair complex (16). Several PARP inhibitors are approved by the US Food and Drug Administration (FDA) or studied in clinical trials, including olaparib, niraparib, rucaparib, veliparib, and talazoparib (17). On March 27, 2017, niraparib was approved by the US FDA. The approval is based on the results of NOVA (NCT01847274) (18). On April 6, 2018, the US FDA approved rucaparib for the maintenance treatment. The approval relies on ARIEL3 (NCT01968213) (19, 20). Based on the results of SOLO-1 (NCT01844986), on December 19, 2018, the US FDA approved olaparib for the maintenance treatment of adult patients with germline or somatic BRCA-mutated (gBRCAm or sBRCAm) who exhibited either a complete or partial response to first-line platinum-based chemotherapy (21). Nevertheless, a recent clinical trial indicated that the efficacy of platinum-based subsequent chemotherapy seems to be reduced in BRCA1/2-

mutated patients with platinum-sensitive relapsed ovarian cancer (PSROC) compared to patients who haven't received PARPi therapy (22). Despite the inspiring benefits PARPi brought, lots of limits still exist. Future studies should focus more on combinations that can enhance the effect of PARPi, benefit patients with non-HRD tumors, mitigate toxicity, and overcome PARPi resistance (23). Therefore, the combination of PARPi and other immunotherapies are developed, especially antiangiogenic agents and immune checkpoint inhibition.

3.1.1 PARPi combined with antiangiogenic agents

Angiogenesis plays a vital role in normal ovarian physiology as well as in ovarian cancer pathogenesis. Tumor progression and growth largely depend on angiogenesis, as tumor could not grow beyond 1-2 mm if the neovascularization cannot meet the requirements of nutrients and oxygen. Thus, antiangiogenic agents have been incorporated into the therapy regimen for ovarian cancer. Vascular endothelial growth factor (VEGF) and VEGF receptor (VEGFR) are primarily explored in clinical settings, and this pathway contributes to malignant ascites and tumor progression (24). Besides, it is also shown that overexpressed VEGF is correlated with tumor staging and prognosis (25). Plenty of angiogenesis inhibitors are being investigated, including Bevacizumab, Aflibercept, Nintedanib, Cediranib, Pazopanib, Sunitinib, Sorafenib, and Trebananib (26). Approved by the FDA, Bevacizumab exhibited modest efficacy, and most patients developed acquired resistance. Therefore, the combination of PARPi and angiogenesis inhibitors are reasonable and meaningful.

There are two purposes for combining PARPi and angiogenesis inhibitors. Firstly, PARPi could decrease angiogenesis (27). Secondly, both VEGF3 inhibitors and hypoxia induce the downregulation of HRD proteins (28, 29). On May 8, 2020, the indication of olaparib was expanded to combination therapy with bevacizumab for first-line maintenance treatment of HRD-positive advanced ovarian cancer (30). The approval was based on the PAOLA-1 trial, which revealed that combined therapy of bevacizumab and olaparib provided a significant progression-free survival (PFS) benefit in HRD-positive patients, regardless of whether the patient had the BRCA mutation (31). More combinational strategies are being studied. In a patient-derived ovarian cancer xenografts (OC-PDXs) model, the combination of PARPi Olaparib and VEGFR inhibitor cediranib reduced the growth of all OC-PDXs independent of BRCA status (32). In 2014, a phase 2 study revealed that Cediranib plus Olaparib could prolong PFS (33). Later, a phase 3 clinical study NRG-GY004 showed that combining Cediranib and Olaparib did not prolong PFS compared with chemotherapy and resulted in reduced patient-reported outcomes (PRO) (34). Besides, other combinational strategies are being investigated too. Compared to monotherapy, niraparib plus bevacizumab significantly increased the PFS of platinum-sensitive recurrent ovarian cancer, while a more extensive scale phase 3 clinical trial is planned (35, 36). More preclinical and clinical studies are needed to provide information about the most appropriate combination strategy and which subset of patients in what clinical setting benefit most.

3.1.2 PARPi combined with immune checkpoint inhibitors

In addition to antiangiogenic agents, PARPi was combined with other targeted immunotherapies, such as PD-1/PD-L1 inhibitors, WEE-1 inhibitors, ataxia-telangiectasia-mutated-and-Rad3-related kinase (ATR) inhibitors, MEK inhibitors, and so on (37). Plenty of studies regarding PARPi and PD-1/PD-L1 combinational therapy are completed or ongoing. Olaparib, niraparib, rucaparib, and talazoparib are combined with anti-PD-1 antibodies (nivolumab, pembrolizumab) and anti-PD-L1 antibodies (durvalumab, atezolizumab, avelumab) (38). PARPi and PD-1/PD-L1 antibodies demonstrated synergistic antitumor activities in animal models regardless of BRCA mutation status, which is achieved by blockade of single-stranded DNA damage repair and activation of the STING-dependent immune response. Moreover, PARPi induces an immunostimulatory micromilieu in ovarian cancer, thereby complementing the activity of PD-1/PD-L1 blockade (39, 40). A phase 2 clinical trial revealed that a combination of olaparib and durvalumab showed modest efficacy whereas blockade of VEGF/VEGFR would be necessary to improve the combination (41). PARPi was also combined with many other ICB in ovarian cancer, such as inhibitors target phosphatidylinositol-4,5-bisphosphate 3-kinase (PI3K) (42, 43), V-akt murine thymoma viral oncogene homolog (AKT) (44), ATR (45, 46), heat shock protein 90 (HSP90) (47, 48), checkpoint kinase 1 (CHK1) (49), cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) (50), salt-inducible kinase 2 (SIK2) (51), insulin-like growth factor-1 receptor (IGF-1R) (52). However, most of the combinations are still in preclinical or phase 1 clinical studies, and a larger scale of clinical studies is needed to further evaluate the efficacy. In addition, the natural compound alantolactone (ALT) could inhibit the thioredoxin reductase, thus inducing ROS accumulation and oxidative DNA damage in cancer cells. A combination of pro-oxidative agent ALT and Olaparib induced tumor regression, which broadened the application of PARP inhibitors (53).

Other agents targeting DNA repair are much less investigated in ovarian cancer. Some studies report their application in other types of cancers as previously reviewed (14). More data are needed on ovarian cancer.

4 Adoptive cell therapy-based combination immunotherapies

Adoptive cell therapy (ACT) mainly refers to chimeric antigen receptor (CAR)-modified T cells, T-cell receptor (TCR)-engineered T cells, natural TILs, CAR-NK cells, and

CAR-macrophages. ACT has achieved a remarkable revolution in the hematological tumor. Nevertheless, for solid tumors, including ovarian cancer, ACT seems insufficient to elicit significant antitumor activity. In ovarian cancer, CAR-T cells target folate-receptor alpha (FRα), mesothelin, MUC-1, and HER2 have been widely investigated. However, no satisfactory therapeutic efficacy has been observed so far. The low avidity and heterogeneous expression of targetable membrane antigens and difficulties in CAT-T cell infiltration and survival are the key obstacles (54). Novel targets or combinational therapies are expected to solve these problems. For instance, CAR-T cells targeting the Mullerian inhibiting substance type 2 receptor (MISIIR), B7-H3, Epithelial cell adhesion molecule (EpCAM), C-X-C chemokine receptor 1 (CXCR1), or C-X-C chemokine receptor 2 (CXCR2), 5T4 significantly controlled tumor growth *in vivo* (55–59). Apart from CAR-T therapy, other ACT, including TCR-T and CAR-NK, are also under investigation. TCR-T therapy is MHC restricted and relies on the presentation of the MHC complex. Unlike CAR-T therapy, whose target antigens are only cell surface proteins, TCR-T could recognize both intracellular antigen fragments and surface proteins as long as MHC molecules present them. In ovarian cancer, TCR-T targeting melanoma-associated antigen 4 (MAGE-A4) and New York esophageal-1 (NY-ESO-1) are in early clinical trials (60). CAR-NK targeting folate receptor alpha (αFR) (61), glypican-3 (GPC3) (62), human leukocyte antigen G (HLA-G) (63), CD44 (64), CD24 (65), CD133 (66), MSLN (67) have achieved therapeutic efficacy in preclinical studies. More clinical data are needed to verify their efficacy in ovarian cancer patients.

4.1 Bispecific CAR-T cells

As we mentioned, a common mechanism of tumor escape from single-target CAR-T cells is the downregulation and mutational loss of the targeted antigen. Thus, targeting multiple antigens may improve the efficacy of CAR-T cells. Several bispecific CAR-T products are under investigation. For instance, Zhen et found that folate receptor 1 (FOLR1) and mesothelin (MSLN) are specifically highly expressed in ovarian cancer cells by screening the GEO database. Therefore, they established tandem CAR-T cells target both FOLR1 and MSLN, and the tandem CAR-T cells exhibited enhanced antitumor activity and prolonged mouse survival compared to single-target CAR-T cells (68). Besides, MSLN CAR-T-secreting anti-CD40 antibody had a more powerful cytotoxic effect on ovarian tumor (69). Dual targeting tumor-associated glycoprotein 72 (TAG-72) and CD47 are effective in ovarian cancer model (70). CAR-T cells targeting PDL1 and MUC16 also demonstrated more potent antitumor efficacy than single-target CAR-T cells (71). Dual CAR-T cells targeting NKG2D and PD-1 ligands exhibited inspiring efficacy in treating metastatic peritoneal tumors (72). In the clinic, CAR-T cells targeting MSLN and PD-1 combined with apatinib exhibited potent therapeutic efficacy in one patient with refractory EOC (73). To summarize, most bispecific CAR-T therapies in ovarian cancer are still in the preclinical stages. Future studies should search for more specific and practical targets in the clinic.

4.2 CAR-T combined with other immunotherapies

According to the modest efficacy of CAR-T in ovarian cancer, several agents are applied to enhance CAR-T cells' efficacy. Firstly, the efficacy of ICIs limited by a lack of a tumor-reactive microenvironment. CAR-T cells may provide the necessary tumor-targeting immune infiltrate. Conversely, ICIs counteract the immunosuppressive environment that undermines optimal CAR-T cell efficacy (74). Thus, combining ICI with CAR-T could be a promising strategy. By loading anti-HER2 or anti-EGFR bispecific antibodies, CD19-CAR-T and activated T cells showed comparable specific cytotoxicity against ovarian cancer cells (75). In addition, arm CAR-T cells with therapeutic cytokines. For instance, IL-12 secreting 4H11-28z CAR-T cells showed enhanced proliferation and antitumor ability compared to 4H11-28z CAR-T cells only (76). Besides, pretreatment of ovarian cancer cells with histone deacetylase inhibitor sodium valproate (VPA) could upregulate NKG2DL expression in ovarian cancer cells expressing low to moderate NKG2DL. Consequently, chimeric NKG2D CAR-T cells exhibited better efficacy by enhanced immune recognition (77). In some papers, upregulation or downregulation of certain receptors could enhance CAR-T cells' efficacy. Co-expressing of CXCR2 enhanced homing and efficacy of CAR-T cells targeting the integrin αvβ6 (78). Besides, adenosine 2A receptors (A2aRs) disruption improved the efficacy of CAR-T cells targeting MSLN (79). As we mentioned before, poor T cell infiltration contributes to the failure of CAR-T therapy. Therefore, to improve T cell infiltration in ovarian cancer, a vascular disrupting agent (VDA) called combretastatin A-4 phosphate (CA4P) was combined with CAR-T cells and results indicated that CA4P enhanced the efficacy of CAR-T cells and could be an effective antitumor agent candidate in treating solid tumor (80). In addition, a substantial body of work suggests that the accumulation of adenosine in the TME contributed to the failure of immunotherapies. As a result, adenosine deaminase 1 (ADA) overexpression improved CAR-T cells' antitumor ability in ovarian cancer (81). In summary, CAR-T-associated combinational therapy is still preclinical studies, and more reasonable and effective combinational strategies are being exploited.

4.3 Other ACT combinational therapies

CAR-NK, TCR-T and CAR-macrophage therapy are alternate cell-based therapies. Cancer-testis antigens (CTA) are developed as targets for TCR-T, including MAGE-A4 and

NY-ES0-1 (60). CAR-NK offers some significant advantages compared to CAR-T, such as better safety, multiple cytotoxic mechanisms, and high feasibility for "off-the-shelf" manufacturing (82). CAR-NK against human leukocyte antigen G (HLA-G) inhibited tumor growth *in vitro* and *in vivo*, and such efficacy was enhanced when combined with chemotherapeutic agents (63). Besides, CXCR1 expression could enhance the antitumor efficacy of NKG2D CAR-NK, which provided a novel strategy for improving the therapeutic efficacy of NK cells (83). CAR-Macrophage own unique advantages. CAR-macrophage could significantly immerse in the TME, and direct kill tumor cells as well as enhance T cell function. In addition, CAR-macrophage has fewer non-tumor toxicities compared to CAR-T (84). Most CAR-macrophage therapies are in the preclinical stage, including CAR-macrophage targeting CD19, CD22, HER2, CCR7 and so on. Only several phase 1 clinical trials for solid tumors are ongoing (85). In ovarian cancer, reports of CAR-NK, TCR-T, and CAR-macrophage are rare. More data from preclinical and clinical studies are needed to prove the safety and antitumor efficacy.

5 Cancer vaccine-based combination immunotherapies

A single application of cancer vaccine in ovarian cancer is under exploration, such as peptide vaccine, whole tumor cell vaccine, cancer stem cells (CSCs), antigen-presenting cell (APC) vaccine, DNA/RNA vaccine, bacteria vaccine and so on. Most of them augment antitumor immunity in ovarian cancer patients. Nevertheless, clinical data only revealed modest efficacy in most patients. Therapeutic efficacy in more patients is testable (86–92). Despite most cancer vaccines only achieving moderate efficacy in other malignancies, combining cancer vaccines and other immunotherapies may broaden its application and elevate efficacy. For instance, murine ovarian cancer cell ID8 was spray dried and made into a microparticulate vaccine. The microparticulate ovarian cancer vaccine exhibited the most efficacious in inhibiting tumor growth when administered with interleukins (93). Adding immunomodulator agents such as IL-12 may augment the efficacy of cell-based cancer vaccine (94). In a phase 2 trial, a multiepitope FRα vaccine called TPIV200 was combined with PD-L1 inhibitor durvalumab in treating advanced platinum-resistant ovarian cancer. The combination was safe and elicited robust FRα-specific immune responses (95). Dual blockade of PD-1 and CTLA-4 enhanced efficacy of the GVAX vaccine in ovarian cancer models through activation of CD4 and CD8 T cells, secretion of cytokines, and inhibition of Treg cells (96). Besides, immunostimulatory adjuvant could elevate the efficacy of cancer vaccines. For instance, cowpea mosaic virus (CPMV) co-delivered with irradiated ovarian cancer cells elicited prophylactic efficacy and immunologic memory responses in mice models (97). 21 recurrent high-grade serous ovarian cancer (HGSOC) patients were treated with a polyvalent antigen-KLH plus OPT-821 vaccine and bevacizumab. Results indicated that the combinational therapy was well-tolerated. Although immunogenic responses were not associated with improved survival, researchers discovered that increased IL-18 correlated with improved PFS while increased PDGF was associated with worse OS (98). Gemogenovatucel-T (Vigil) is an autologous whole tumor cell vaccine transfected with GM-CSF gene and silenced of furin, the critical convertase responsible for activation of TGFβ-1 and TGFβ-2. The vigil was well-tolerated, but the primary endpoint was not met (99). A combination of vigil and a PD-L1 blocking antibody atezolizumab was safe. Further clinical exploration was justified (100). Apart from peptide and irradiated tumor cell vaccine, DC vaccine was combined with ex vivo-stimulated autologous T cells. Six patients were enrolled in this study. They received bevacizumab plus autologous DC pulsed with tumor lysate supernatants, followed by lymphodepletion and adoptive transfer of autologous vaccine-primed and CD3/CD28-stimulated T cells. Four patients benefit from the therapy, including two partial responses (PR) and two stable disease (SD) (101). Combining human monocytes and IFN-α2a and IFN-γ mediated potent antitumor effect in ovarian cancer (102). Immuno-modulators, including anti-CD40Ab and TLR3 ligand—poly(I:C), could enhance the antitumor effect of a DNA vaccine encoding MSLN and antigen-specific connective tissue growth factor (CTGF) (103). CPMV *in situ* vaccination combined with CD47-blocking antibody promoted macrophage activity and enhanced T cell function in ovarian cancer model (104). To summarize, most cancer vaccines could not wholly eradicate established tumors. They exhibit better therapeutic effects when tumor volume is small and the vaccine is given in an adjuvant setting (105).

6 ICI-based combination immunotherapies

6.1 Bispecific ICIs

Dual inhibition of PD-1/PD-L1 exhibited better efficacy in ovarian cancer compared to single-target. Bispecific targeting of PD-1 and PD-L1 induced superior cellular changes in T and NK cells compared to monospecific targeting (106). Besides, A soluble form of the PD-1 receptor (sPD-1) neutralized both PD-L1 and PD-L2 and achieved better efficacy. PD-L2 blockade facilitates ICB resistance through incomplete blockade of the PD-1 signaling pathway (107).

More inhibitors simultaneously target two signaling pathways to enhance the antitumor effects. APCS-540, a newly developed inhibitor targeting glycogen synthase kinase 3 beta

(GSK3B) and histone deacetylases (HDACs), inhibited tumor growth and prolonged survival in an ovarian cancer model (108). Another inhibitor, Istiratumab, bispecific targets IGF-1R and epidermal growth factor receptor 3 (ErbB3). Istiratumab could be a candidate for treating chemotherapy-resistant ovarian cancer (109). Besides, MSC2363318A is a newly developed inhibitor targeting AKT1, AKT3, and P70S6K. Yes-associated protein (YAP1) could be a marker that predicts ovarian tumors' sensitivity to MSC2363318A (110). HKMTI-1-005 simultaneously inhibited the histone methyltransferase G9A and EZH2, which elicited antitumor efficacy in HGSOC (111). Several papers focus on the pro-tumorigenic microenvironment induced by chemotherapy. Tumor cell debris produced by platinum- and taxane-based chemotherapy stimulates a "surge" of macrophage-derived proinflammatory cytokines and bioactive lipids. A dual cyclooxygenase-2 (COX-2) and soluble epoxide hydrolase (sEH) inhibitor PTUPB decreased proinflammatory cytokines and lipids in the TME and delayed ovarian tumor growth (112).

6.2 Dual blockade

When certain ICI works, it is possible that a compensatory signaling pathway was induced, providing an idea of the dual blockade. As one of the most widely applicated inhibitors, PD-1/PD-L1 inhibitors are combined with various inhibitors. Dual blockade of CXCL12-CXCR4 and PD1-PDL1 enhanced antitumor effects compared with the single blockade, which was associated with increased effector T cells infiltration and function, increased memory T cells, and decreased Treg cells in the TME (113). Dual blockade of PD-1 and CTLA-4 elicited antitumor efficacy in preclinical studies (114). A combination of PD-1 inhibitor Nivolumab and CTLA-4 inhibitor Ipilimumab in EOC patients resulted in superior responses and longer PFS (115). PD-1 inhibitor LY3300054 and CHK1 inhibitor prexasertib combinational therapy were tolerable and demonstrated preliminary efficacy in HGSOC patients (116). PD-L1 inhibitor atezolizumab and VEGF inhibitor bevacizumab achieved durable responses and/or disease stabilization in some platinum-resistant ovarian cancer patients (117). High expression of CXCL13 predicted a more prolonged survival and facilitated the maintenance of CXCR5+CD8+ T cells. Besides, CXCL13, combined with anti-PD-1 therapy, significantly retarded ovarian tumor growth (118). Combining cyclin-dependent kinases 4 and 6 (CDK4/6) inhibitor abemaciclib and anti-PD-1 therapy may have a better promise for poorly immune-infiltrated ovarian cancer (119).

Despite that more than 60% of ovarian cancers are positive for the estrogen receptor (ER), ER-targeted treatment in ovarian cancer was disappointing. Src is also activated in most ovarian cancers. It was found that estrogen could activate Src to phosphorylate p27, thus promoting its degradation and increasing cell-cycle progression. Combinational ER and Src blockade therapy by fulvestrant and saracatinib increased cell-cycle arrest, induced autophagy, and inhibited ovarian cancer growth *in vivo* (120, 121). Apart from Src inhibitor, MEK inhibitor selumetinib could also reverse antiestrogen resistance in ER-positive HGSOC. Besides, MAPK overexpression predicted poor prognosis and may help identify MEK inhibitor-responsive cancer (122).

Although the EGFR signaling pathway is usually activated and associated with a poor prognosis, clinical results of EGFR inhibition in recurrent ovarian cancer patients are disappointing. An article revealed that STAT3 activation might contribute to resistance to EGFR inhibition. Therefore, combined inhibition of EGFR and JAK/STAT3 had synergistic antitumor effects, whereas combinational inhibition of other pathways, including AKT/mTOR, MEK, and SRC, was relatively less effective (123). 12 patients received intraperitoneal cisplatin, intraperitoneal TLR3 ligand rintatolimad, and oral COX-2 blocker celecoxib. The study revealed that the combination was safe and tolerable. A phase 2 clinical trial would be tested (124). The insulin growth factor 1 (IGF-1) expression was elevated in two ovarian cancer models treated with bevacizumab. Dual blockade of IGF-1 and VEGF resulted in increased tumor growth inhibition (125). Delta-like ligand 4 (Dll4), one of the Notch ligands, is overexpressed in ovarian cancer. Dual blockade of Dll4 and VEGF markedly reduced ovarian cancer cell growth (126). Overexpression of BCL2L1 was associated with platinum resistance to multiple anti-cancer agents in ovarian cancer. Dual inhibition of FGFR4 and BCL-xL demonstrated potent efficacy and tolerable toxicity (127). Forkhead domain inhibitor-6 (FDI-6) is a forkhead box protein M1 (FOXM1). FDI-6 inhibition elicited the upregulation of N-Ras, phosphoprotein kinase Cδ (p-PKCδ), and HER3. Combination FDI-6 with tipifarnib (N-Ras inhibitor), rottlerin (p-PKCδ inhibitor), or sapitinib (HER3 inhibitor) decreased the survival of cancer cells (128). Src and MAPK are activated in HGSOC. Dual blockade of Src and MAPK by saracatinib and selumetinib inhibited ovarian tumor growth and targeted tumor initiating stem-like cells (129). Dual inhibition of DNA methylation and histone H3 lysine 9 dimethylation by 5-aza-CdR and G9Ai increased viral mimicry and served as a basis for this combination strategy (130). Combined inhibition of MEK and BCL-2/X_L had therapeutic efficacy in HGSOC models, and BIM protein was a biomarker of responsiveness (131). Dual inhibition of PI3K/mTOR and RAS/ERK by PF-04691502 and PD-0325901 showed robust synergistic antitumor efficacy (132).

Targeting agents participating in cancer cell metabolism are being explored. Dual inhibition of glycolysis and glutaminolysis could be a promising therapeutic strategy in ovarian cancer (133). Similarly, A triphenylphosphonium-modified terpyridine platinum (II) complex (TTP) inhibited multiple mitochondrial and glycolytic bioenergetics, thus inducing a hypometabolic state in several cancers, including ovarian cancer (134).

Besides EOC, research on other types of ovarian cancer was much less. The PI3K and murine double minute 2 (MDM2) upregulation predict a worse outcome in clear cell ovarian carcinoma (CCOC). Dual inhibition of PI3K and MDM2 by DS-7423 and RG7112 significantly reduced CCOC growth (135).

6.3 ICIs combined with other immunotherapies

Although ICIs have changed the practice of cancer treatment and prognosis, the application of ICIs for ovarian cancer is limited. Adding cytotoxic cytokines or neutralizing immunosuppressive cytokines may augment the efficacy. IL-10 in the TME sustained the immunosuppression in ovarian cancer. Therefore, IL-10 neutralization enhanced the antitumor efficacy of PD-1 blockade, and the combinational therapy prolonged survival and decreased tumor burden through T cell and B cell immunity in mice (136). Besides, active immunotherapy precedes administrated of ICI. Thus, promoting T cell maturation and resistance to the cytotoxic effects of the Bcl-2 inhibitor (137).

7 Oncolytic virus-based combination immunotherapies

Oncolytic viruses are gene-modified or naturally occurring viruses that selectively replicate and destroy cancer cells without harming the normal tissues (138). Adenovirus, herpes simplex virus (HSV), poxvirus, and measles virus are the most well-known oncolytic viruses in cancer therapy (105, 139). The oncolytic virus is combined chiefly with ICB in ovarian cancer. For example, oncolytic Maraba virus and PD-1 blockade combination mediated heterogeneous radiologic patterns through non-invasive MRI scanning (140). Plant virus CPMV nanoparticles conjugated with anti-PD-1 peptide had superior efficacy against metastatic ovarian cancer compared to adding free anti-PD-1 peptide (141). Oncolytic vaccinia virus therapy in ovarian cancer induced expression of PD-L1 in cancer cells and immune cells. Therefore, combining therapy of oncolytic vaccinia virus and PD-L1 blockade could synergistically enhance therapeutic efficacy (142).

Moreover, oncolytic viruses could be genetically modified to express exogenous cytokines or proteins. A modified Vaccinia Ankara vaccine expressing wild-type human p53 (p53MVA) promoted T cell responses, and combination with gemcitabine or other agents was expected to exhibit superior clinical responses (143). In addition, the oncolytic vaccinia virus (VV) engineered to express a fusion protein of IL-15 and IL-15Ralpha was named vvDD-IL15-Rα. A combination of vvDD-IL15-Rα and PD-1 blockade exhibited a dramatic tumor regression (144). Mice were pretreated with three homologous thrombospondin type 1 repeat domains (3TSR) alone or followed by combination with a fusogenic oncolytic Newcastle disease virus (NDV). 3TSR could normalize tumor vasculature, thus enhancing NDV delivery and trafficking of immune cells to the tumor core. The combinational therapy resulted in a most significant reduction in tumor volume and ascites accumulation (145).

Oncolytic viruses are also combined with other immunogenic agents. The oncolytic vaccinia virus (OVV) was enhanced by MEK inhibitor PD0325901 and trametinib in doxorubicin-resistant ovarian cancer (146). Microtubule destabilizing agents (MDAs) could sensitize tumors to oncolytic virus therapy. The combination of trastuzumab emtansine and oncolytic vesicular stomatitis virus (VSVΔ51) demonstrated that a viral-sensitizing molecule could enhance oncolytic virus efficacy (147). Infection of RNA virus induced upregulation of heat shock protein 70 (HSP70). HSP70 increased measles virus cytotoxicity. HSP90 inhibitors could upregulate HSP70, therefore increasing the efficacy of measles virotherapy (148). Furthermore, modulating interferon modulators by JAK1/2 inhibitor ruxolitinib could overcome partial resistance of an oncolytic vesicular stomatitis virus variant pseudotyped with the nonneurotropic glycoprotein (VSV-GP) (149).

The combination of two types of viruses demonstrated enhanced efficacy. For example, infection with Semliki Forest virus-ovalbumin (SFV-OVA) followed by infection with vaccinia virus-ovalbumin (VV-OVA) induced an enhanced antitumor efficacy through a combination of viral oncolysis and antigen-specific immunity (150).

A limitation of recombinant oncolytic virus therapy is the viral clearance by neutralizing antibodies. Therefore, a study found that cyclooxygenase-2 (Cox-2) inhibitors may circumvent this limitation. Cox-2 inhibitors successfully inhibited the generation of neutralizing antibodies and exhibited more effective antitumor efficacy when combined with the vaccinia virus in ovarian cancer (151). Another obstacle to viral therapy is that oncolytic viruses are large particles. Thus, it is difficult to efficient extravasation from tumor blood vessels. A study proved that the oncolytic sindbis virus target tumor cells by the laminin receptor. Therefore, modulating vascular leakiness by VEGF or metronomic chemotherapy could enhance specific targeting and delivery of sindbis viral vectors (152). Combination of adeno-associated virus (AAV) expressing 3TSR and Fc3TSR and bevacizumab extended mice survival, suggesting a further investigation of such a combination (153). The application of adenoviruses is limited by rapid, systemic cytokine release and consequently inflammatory toxicity. To overcome this obstacle, researchers used β3 integrin to significantly reduce toxicity without compromising antitumor efficacy (154).

8 Chemotherapy-based combination immunotherapies

Chemotherapy combined with cytoreductive surgery is the mainstay treatment for ovarian cancer. Although the majority of

people initially respond to platinum-based chemotherapy, most patients would suffer a recurrence within 5 years. Currently, most clinical studies regarding immunotherapies are applied to patients who previously received chemotherapy, as we discussed before (37). Resistance to platinum agents and PARP inhibitors is one of the main obstacles to ovarian cancer therapy (155). Thus, it's urgent to explore novel targets or combinational strategies. RNA sequencing and panel DNA sequencing revealed that neoadjuvant chemotherapy induces genomic and transcriptomic changes, and combined treatment of AP-1 or SIK2 inhibitors with carboplatin or paclitaxel showed synergistic effects (156). RNA sequencing analysis also suggested that stress promoted chemoresistance, which provided targets to overcome chemo resistance (157). In addition, targeting LRRC15 could inhibit metastatic dissemination through β1-integrin/FAK signaling (158). Apart from preclinical studies, several clinical trials revealed that MEK inhibitor trametinib, Wee1 inhibitor adavosertib, and CDK4/6 inhibitor ribociclib showed preliminary efficacy in ovarian cancer (159–161). Overall, a single application of immunotherapy is unlikely to have a dramatically effect in ovarian cancer. Understanding the interplay between signal pathways may provide a better combined therapy of chemotherapy and immunotherapy.

9 Immunotherapy enhancement strategy

9.1 Nanoparticles-based combination immunotherapies

Poor aqueous solubilities limited the application of several drugs. Nanoplatforms could help solve the barrier. Diblock copolymer nanoplatforms were used to formulate micelles through the solvent evaporation method. A dual drug loaded micelles (DDM) containing chetomin and everolimus targeted HIF and mTOR. The DDM significantly inhibited angiogenesis and induced apoptosis compared to the individual micells (162). Besides, ovarian tumor cells overexpress low-density lipoprotein receptors (LDLr). Thus, LDL-encapsulated cholesterol-conjugated heat shock protein 27 (HSP27) and human epidermal growth factor receptor 2 (HER2) dual inhibitor specifically targeted and inhibited ovarian cancer cells (163).

9.2 Radiotherapy-based combination therapy

Radiotherapy was nearly abandoned in ovarian cancer due to its modest efficacy and toxicity. However, recent studies revealed that a low dose of radiotherapy might reprogram the tumor microenvironment and reverse tumor immune desertification and resistance to immunotherapy (164). Low-dose radiotherapy plays a role in immune modulation and tumor microenvironment reprogramming rather than direct tumor killing. Although radiotherapy could promote antitumor immunity, including tumor antigen presentation and T cell recruitment, immune suppressive cells, including Tregs and MDSCs, are also activated. Therefore, radiotherapy combined with immunotherapy may promote the activity of favorable immune cells and elevate antitumor efficacies (164). Low dose radiotherapy (LDRT) triggered T cell infiltration in an IFN-dependent manner in ovarian cancer patients with immune-desert tumors when combined with immune checkpoint blockade (165). In a preclinical setting, radiation therapy combined with immunostimulatory CPMV elicited significant tumor retardation and increased TIL in the TME (166). Radiotherapy combined with immunotherapy in other types of cancers, including melanoma, lung cancer, and colon cancer, is under plenty of preclinical and clinical studies, providing a basis for application in ovarian cancer (164).

10 Conclusion and future perspectives

Ovarian cancer, especially epithelial ovarian cancer, is typically diagnosed at an advanced stage. Patients who experience a recurrence within six months after the end of platinum-based chemotherapy are characterized by poor prognosis, which needs a novel and effective treatment modality (167). Multi-immunotherapies are expected to prolong the survival and improve the prognosis, plenty of clinical trials are investigating their efficacy in ovarian cancer (Table 1). Immunotherapy could be strengthened through several points. Firstly, it is recommended that all women with newly diagnosed ovarian cancer should be offered genetic testing. Approximately 10%-20% of ovarian cancers are related to germline mutations. Besides, relatives of women with genetic mutations are recommended to have gene testing (168). In addition, several preclinical and early clinical data suggested that toll-like receptor 7 (TLR7) and TLR8 agonists could activate DCs, monocytes, macrophages, and fibroblasts. TLR7/8 agonists also promoted proinflammatory cytokines and chemokines secretion, including IL-6. Thus, activation of TLR7/8 may be a potential target (169). Moreover, RNA-associated therapy aroused researchers' attention. Long non-coding RNAs (lncRNAs) are critical regulators in ovarian cancer occurrence and progression (170). RNA-binding proteins (RBPs), a class of endogenous proteins that bind to mRNA, regulate a series of pathological processes in ovarian cancer (171). Therefore, both lncRNAs and RBPs could be a potential therapeutic target (172–178). Non-coding RNA miR-146b simultaneously inhibited EGFR and IL6-STAT3 signal pathways, resulting in a more

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TABLE 1 Clinical trials of multi-immunotherapy in ovarian cancer.

Number	Clinical trial identifier	Targets	Responsible party	Status
1	NCT04024878	Nivolumab: PD-1 inhibitor NeoVax: 20 peptides and Poly-ICLC	Dana-Farber Cancer Institute	Recruiting
2	NCT05479045	Nivolumab: PD-1 inhibitor NY-ESO-1 Peptide vaccine	Georgetown University	Not yet recruiting
3	NCT02737787	Nivolumab: PD-1 inhibitor WT1 Vaccine NY-ESO-1 Vaccine	Memorial Sloan Kettering Cancer Center	Active, not recruiting
4	NCT05044871	Tislelizumab: PD-1 inhibitor Pamiparib: PARP inhibitor Bevacizumab: Anti-VEGF antibody	Tongji Hospital	Not yet recuiting
5	NCT03806049	Dostarlimab: PD-1 inhibitor Niraparib: PARP inhibitor Bevacizumab: Anti-VEGF antibody	Nordic Society of Gynaecological Oncology - Clinical Trials Unit	Withdrawn
6	NCT03602859	Dostarlimab: PD-1 inhibitor Niraparib: PARP inhibitor	Tesaro, Inc.	Active, not recruiting
7	NCT03955471	Dostarlimab: PD-1 inhibitor Niraparib: PARP inhibitor	Tesaro, Inc.	Terminated
8	NCT05467670	Pembrolizumab: PD-1 inhibitor ALX148: CD47 inhibitor	University of Pittsburgh	Not yet recuiting
9	NCT03596281	Pembrolizumab: PD-1 inhibitor Bevacizumab: Anti-VEGF antibody	Cancer Campus, Grand Paris	Active, not recuiting
10	NCT02537444	Pembrolizumab: PD-1 inhibitor Acalabrutinib: Bruton tyrosine kinase inhibitor	Acerta Pharma BV	Completed
11	NCT05188781	Pembrolizumab: PD-1 inhibitor Anlotinib: TKI	The Affiliated Hospital of Qingdao University	Completed
12	NCT03734692	Pembrolizumab: PD-1 inhibitor Rintatolimod: TLR-3 agonist	University of Pittsburgh	Recruiting
13	NCT03275506	Pembrolizumab: PD-1 inhibitor Bevacizumab: Anti-VEGF antibody	ARCAGY/GINECO GROUP	Active, not recruiting
14	NCT04361370	Pembrolizumab: PD-1 inhibitor Olaparib: PARP inhibitor Bevacizumab: Anti-VEGF antibody	Yonsei University	Enrolling by invitation
15	NCT05271318	Pembrolizumab: PD-1 inhibitor TILT-123: oncolytic adenovirus	TILT Biotherapeutics Ltd.	Recruiting
16	NCT04417192	Pembrolizumab: PD-1 inhibitor Olaparib: PARP inhibitor	National Cancer Center Hospital East	Recruiting
17	NCT05116189	Pembrolizumab: PD-1 inhibitor Bevacizumab: Anti-VEGF antibody	Merck Sharp & Dohme LLC	Recruiting
18	NCT04068974	Camrelizumab: PD-1 inhibitor Apatinib: VEGFR inhibitor	Peking Union Medical College Hospital	Recruiting
19	NCT05145218	TQB2450: PD-1 inhibitor Anlotinib: TKI	Chia Tai Tianqing Pharmaceutical Group Co., Ltd.	Recruiting
20	NCT03574779	TSR-042: PD-1 inhibitor Niraparib: PARP inhibitor Bevacizumab: Anti-VEGF antibody	Tesaro, Inc.	Recruiting
21	NCT03294694	PDR001: PD-1 inhibitor Ribociclib: CDK inhibitor Fulvestrant: ER downregulator	Dana-Farber Cancer Institute	Terminated
22	NCT02891824	Atezolizumab: PD-L1 inhibitor Bevacizumab: Anti-VEGF antibody	ARCAGY/GINECO GROUP	Active, not recruiting
23	NCT03695380	Atezolizumab: PD-L1 inhibitor Niraparib: PARP inhibitor Cobimetinib: MEK inhibitor	Hoffmann-La Roche	Recruiting
25	NCT03394885	Atezolizumab: PD-L1 inhibitor Bevacizumab: Anti-VEGF antibody	Duke University	Completed

(Continued)

TABLE 1 Continued

Number	Clinical trial identifier	Targets	Responsible party	Status
26	NCT03353831	Atezolizumab: PD-L1 inhibitor Bevacizumab: Anti-VEGF antibody	AGO Research GmbH	Active, not recruiting
27	NCT03292172	Atezolizumab: PD-L1 inhibitor RO6870810: BET inhibitor	Hoffmann-La Roche	Terminated
28	NCT02915523	Avelumab: PD-L1 inhibitor Entinostat: HDAC inhibitor	Syndax Pharmaceuticals	Completed
29	NCT03642132	Avelumab: PD-L1 inhibitor Talazoparib: PARP inhibitor	Pfizer	Completed
30	NCT03558139	Avelumab: PD-L1 inhibitor Magrolimab: Anti-CD47 antibody	Gilead Sciences	Completed
31	NCT02943317	Avelumab: PD-L1 inhibitor Defactinib: PYK2 inhibitor	Verastem, Inc.	Terminated
32	NCT03704467	Avelumab: PD-L1 inhibitor M6620: ATR inhibitor	EMD Serono Research & Development Institute, Inc.	Completed
33	NCT03737643	Durvalumab: PD-L1 inhibitor Olaparib: PARP inhibitor Bevacizumab: Anti-VEGF antibody	AstraZeneca	Recruiting
34	NCT04742075	Durvalumab: PD-L1 inhibitor Olaparib: PARP inhibitor UV1: Peptide vaccine	Nordic Society of Gynaecological Oncology - Clinical Trials Unit	Recruiting
35	NCT02431559	Durvalumab: PD-L1 inhibitor Motolimod: TLR8 agonist	Ludwig Institute for Cancer Research	Completed
36	NCT02764333	Durvalumab: PD-L1 inhibitor TPIV200: A Multi-Epitope Anti-Folate Receptor Vaccine	Memorial Sloan Kettering Cancer Center	Completed
37	NCT03899610	Durvalumab: PD-L1 inhibitor Tremelimumab: CTLA-4 inhibitor	Yonsei University	Recruiting
38	NCT03699449	Durvalumab: PD-L1 inhibitor Olaparib: PARP inhibitor Cediranib: VEGFR inhibitor Tremelimumab: CTLA-4 inhibitor	Yonsei University	Recruiting
39	NCT03249142	Durvalumab: PD-L1 inhibitor Tremelimumab: CTLA-4 inhibitor	ARCAGY/GINECO GROUP	Active, not recruiting
40	NCT04015739	Durvalumab: PD-L1 inhibitor Bevacizumab: Anti-VEGF antibody Olaparib: PARP inhibitor	ARCAGY/GINECO GROUP	Active, not recruiting
41	NCT03430518	Durvalumab: PD-L1 inhibitor Eribulin: microtubule-targeting agent	Icahn School of Medicine at Mount Sinai	Completed
42	NCT04644289	durvalumab: PD-L1 inhibitor Olaparib: PARP inhibitor	AGO Research GmbH	Recruiting
43	NCT05422183	Envafolimab: PD-L1 inhibitor Lenvatinib: TKI	Zhongda Hospital	Not yet recruiting
44	NCT05130515	Niraparib: PARP inhibitor Anlotinib: TKI	Sun Yat-Sen Memorial Hospital of Sun Yat-Sen University	Not yet recruiting
45	NCT03783949	Niraparib: PARP inhibitor Ganetespib: Hsp90 inhibitor	Universitaire Ziekenhuizen Leuven	Active, not recruiting
46	NCT05198804	Niraparib: PARP inhibitor ZN-c3: Wee1 inhibitor	K-Group Beta	Recruiting
47	NCT05183984	Niraparib: PARP inhibitor Bevacizumab: Anti-VEGF antibody	ARCAGY/GINECO GROUP	Recruiting
48	NCT03895788	Niraparib: PARP inhibitor Brivanib: VEGFR and FGFR inhibitor	Hunan Cancer Hospital	Unkonwn
49	NCT04826198	Niraparib: PARP inhibitor AsiDNA: DNA Repair Inhibitor	Gustave Roussy, Cancer Campus, Grand Paris	Recruiting

(Continued)

TABLE 1 Continued

Number	Clinical trial identifier	Targets	Responsible party	Status
50	NCT04149145	Niraparib: PARP inhibitor M4344: ATR inhibitor	University of Alabama at Birmingham	Not yet recruiting
51	NCT03944902	Niraparib: PARP inhibitor CB-839: Glutaminase inhibitor	University of Alabama at Birmingham	Terminated
52	NCT04734665	Niraparib: PARP inhibitor Bevacizumab: Anti-VEGF antibody	Yonsei University	Recruiting
53	NCT04376073	Niraparib: PARP inhibitor Anlotinib: TKI	Sun Yat-sen University	Recruiting
54	NCT04267939	Niraparib: PARP inhibitor Elimusertib: ATR inhibitor	Bayer	Recruiting
55	NCT03326193	Niraparib: PARP inhibitor Bevacizumab: Anti-VEGF antibody	Tesaro, Inc.	Active, not recruiting
56	NCT02354131	Niraparib: PARP inhibitor Bevacizumab: Anti-VEGF antibody	Nordic Society of Gynaecological Oncology - Clinical Trials Unit	Completed
57	NCT05009082	Niraparib: PARP inhibitor Bevacizumab: Anti-VEGF antibody	AGO Study Group	Not yet recruiting
58	NCT05170594	Fluzoparib: PARP inhibitor Bevacizumab: Anti-VEGF antibody	The Second Affiliated Hospital of Shandong First Medical University	Recruiting
59	NCT04517357	Fluzoparib: PARP inhibitor Apatinib: VEGFR inhibitor	Jiangsu HengRui Medicine Co., Ltd.	Recruiting
60	NCT05479487	Fluzoparib: PARP inhibitor Apatinib: VEGFR inhibitor	Fudan University	Not yet recruiting
61	NCT04229615	Fluzoparib: PARP inhibitor Apatinib: VEGFR inhibitor	Jiangsu HengRui Medicine Co., Ltd.	Active, not recruiting
62	NCT04669002	Olaparib: PARP inhibitor EP0057: NDC	Ellipses Pharma	Recruiting
63	NCT02889900	Olaparib: PARP inhibitor Cediranib: VEGFR inhibitor	AstraZeneca	Completed
64	NCT03117933	Olaparib: PARP inhibitor Cediranib: VEGFR inhibitor	University of Oxford	Active, not recruiting
65	NCT03278717	Olaparib: PARP inhibitor Cediranib: VEGFR inhibitor	NCT03278717	Recruiting
66	NCT02681237	Olaparib: PARP inhibitor Cediranib: VEGFR inhibitor	University Health Network, Toronto	Completed
67	NCT04729387	Olaparib: PARP inhibitor Alpelisib: PI3K inhibitor	Novartis Pharmaceuticals	Recruiting
68	NCT02340611	Olaparib: PARP inhibitor Cediranib: VEGFR inhibitor	University Health Network, Toronto	Completed
69	NCT02855697	Olaparib: PARP inhibitor Cediranib: VEGFR inhibitor	The Christie NHS Foundation Trust	Completed
70	NCT03314740	Olaparib: PARP inhibitor Cediranib: VEGFR inhibitor	Mario Negri Institute for Pharmacological Research	Unkonwn
71	NCT01623349	Olaparib: PARP inhibitor BKM120: PI3K inhibitor BYL719: PI3K inhibitor	Dana-Farber Cancer Institute	Completed
72	NCT02571725	Olaparib: PARP inhibitor Tremelimumab: CTLA-4 inhibitor	New Mexico Cancer Care Alliance	Active, not recruiting
73	NCT05494580	Pamiparib: PARP inhibitor Surufatinib: TKI	Sun Yat-sen University	Not yet recruiting
74	NCT00130520	Bevacizumab: Anti-VEGF antibody Erlotinib: EGFR inhibitor	University of Arizona	Completed
75	NCT04938583	Bevacizumab: Anti-VEGF antibody Oregovomab: Anti-CA125 antibody	Korean Cancer Study Group	Recruiting

(Continued)

TABLE 1 Continued

Number	Clinical trial identifier	Targets	Responsible party	Status
76	NCT01551745	Bevacizumab: Anti-VEGF antibody Vigil™ Vaccine	Gradalis, Inc.	Completed
77	NCT01202890	Bevacizumab: Anti-VEGF antibody Lenalidomide: Immunomodulatory drug	New Mexico Cancer Care Alliance	Terminated
78	NCT01091259	Bevacizumab: Anti-VEGF antibody Irinotecan: Topoisomerase inhibitor	NYU Langone Health	Completed
79	NCT05113368	Regorafenib: Multi-kinase inhibitor Fulvestrant: ER degrader	Case Comprehensive Cancer Center	Not yet recruiting
80	NCT04625270	VS-6766: Dual RAF/MEK Inhibitor Defactinib: FAK Inhibitor	Verastem, Inc.	Recruiting
81	NCT01936363	Pimasertib: MEK inhibitor SAR245409: PI3K inhibitor	EMD Serono	Completed
82	NCT04998760	ATG-008: mTORC1/2 inhibitor ATG-010: Selective inhibitor of nuclear export compound	Chongqing University Cancer Hospital	Not yet recruiting
83	NCT05057715	VCN-01: Oncolytic adenovirus huCART-meso Cells	University of Pennsylvania	Recruiting
84	NCT02019524	E39: peptide vaccine J65: peptide vaccine	San Antonio Military Medical Center	Completed
85	NCT00003386	BCG vaccine autologous tumor cell vaccine	Sidney Kimmel Cancer Center at Thomas Jefferson University	Terminated
86	NCT02055690	Pazopanib: VEGFR inhibitor Fosbretabulin: Microtubule-targeting agent	The Christie NHS Foundation Trust	Terminated
87	NCT00408590	carcinoembryonic antigen-expressing measles virus oncolytic measles virus encoding thyroidal sodium iodide symporter	Mayo Clinic	Completed
88	NCT00799110	Dendritic Cell/Tumor Fusion Vaccine GM-CSF	Beth Israel Deaconess Medical Center	Active, not recruiting
89	NCT00181688	Iressa: EGFR inhibitor Arimidex: Aromatase inhibitor	Massachusetts General Hospital	Completed

PD-1, Programmed Cell Death Ligand 1; NY-ESO-1, New York esophageal squamous cell carcinoma-1; WT1, Wilms' tumour 1; PARP, Poly (ADP-ribose) polymerase; VEGF, Vascular endothelial growth factor; TKI, tyrosine kinase inhibitor; TLR, Toll-like receptors; ER, Estrogen receptor; CDK, Cyclin-dependent kinase; PD-L1, Programmed cell death ligand 1; MEK, Mitogen-activated protein kinase; BET, Bromodomain and extraterminal domain; HDAC, Histone deacetylase; PYK2, Proline-rich tyrosine kinase 2; ATR, Ataxia-telangiectasia and Rad3-related protein; CTLA-4, Cytotoxic T-lymphocyte-associated protein 4; Hsp90, Heat shock protein 90; Wee1, Wee1-like protein kinase; FGFR, Fibroblast growth factor receptor; NDC, Nanoparticle-drug conjugate; PI3K, Phosphoinositide 3-kinase; EGFR, Epidermal Growth Factor Receptor; CA125, carbohydrate antigen 125; RAF, Rapidly accelerated fibrosarcoma; FAK, Focal adhesion kinase; mTOR, Mechanistic target of rapamycin.

excellent suppression of ovarian cancer cell migration (179). Another non-coding RNA, HOTAIR, was overexpressed in ovarian cancer stem cells (OCSCs). Inhibition of HOTAIR and DNA methylation help eradicate OCSCs and block disease recurrence (180). In addition, several natural agents could target multiple signaling pathways. For instance, berberine was proved to target both EGFR and ErbB2. Berberine inhibited migration and invasion of ovarian cancer cells (181).

To conclude, multi-immunotherapies of ovarian cancer are far from fully elucidated. Future studies should focus on fully recognizing immunogenic characteristics, developing biomarkers, and selecting eligible patients. Multi-immunotherapy is supposed to combine immunotherapies rationally while minimizing toxicities.

Author contributions

XYH wrote the initial draft of manuscript. CB and XZ revised the manuscript. TY reviewed and approved content. All authors contributed to the article and approved the submitted version.

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Conflict of interest

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

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Therapeutic implications of the tumor microenvironment in ovarian cancer patients receiving PD-1/PD-L1 therapy

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Epithelial ovarian cancer (EOC) ranks as the second most common cause of gynecologic cancer death. The conventional treatment for patients with EOC is postoperative therapy along with platinum chemotherapy. However, a more efficient treatment regimen is of great need for these patients diagnosed with advanced disease (FIGO stages III–IV), whose survival is approximately 29%. Immunotherapy seems to be an encouraging therapeutic strategy for EOC. Given the crucial role in the complicated interactions between tumor cells and other cells, the tumor microenvironment (TME) influences the response to immunotherapy. In this review, we discuss feasible strategies for EOC immunotherapy by exploiting the reciprocity of cancer cells and the constituents of the TME.

KEYWORDS

tumor microenvironment, immunotherapy, immune cells, stromal cells, combination therapy

Abbreviations: EOC, epithelial ovarian cancer; TME, tumor microenvironment; PD-1, programmed cell death 1; PD-L1, programmed cell death ligand 1; ECM, extracellular matrix; DCs, dendritic cells; TAMs, tumor associated macrophages; NK, natural killer; ICIs, immune checkpoint inhibitors; Tregs, regulatory T cells; IL-10, interleukin-10; IL-35, interleukin-35; TGF-β, transforming growth factor-β; IDO, indoleamine-2,3-dioxygenase; PGE2, prostaglandin E2; LAG3, lymphocyte activation gene 3; VEGF-A, vascular endothelial growth factor A; EGF, epidermal growth factor; MDSCs, myeloid-derived suppressor cells; ARG1, arginase 1; CAFs, cancer associated fibroblasts; CAAs, cancer-associated adipocytes; HGF, hepatocyte growth factor; PDGF, platelet derived growth factor; FGF-2, fibroblast growth factor 2.

1 Introduction

Epithelial ovarian cancer (EOC) recognized by its high occurrence and poor prognosis (1), ranks as the second most common cause of gynecologic cancer death (2). The conventional treatment for EOC patients is postoperative therapy along with platinum chemotherapy (3). However, survival is dismal since over two-thirds of patients are diagnosed with advanced disease (FIGO stages III–IV) (4), and the survival rate for advanced stages is about 29% (5). Thus, a more effective treatment is of great need for these patients. Currently, immunotherapy is an encouraging treatment for various cancers (6). Immunotherapy agents are used to activate effector and cytotoxic T cells that respond to cancer cells through natural mechanisms, many of which are suppressed during cancer progression (7). Poor response to immunotherapy in ovarian tumors was associated with low expression of programmed cell death ligand 1 (PD-L1) (8). Therefore, it is urgent to explore the cells in the TME and their effects on the response of immune checkpoint inhibitors (ICIs).

The tumor microenvironment (TME), which is made up of vessels, immune infiltration and extracellular matrix (ECM), promotes cancer growth, invasion and metastasis (9). Understanding the interplay between cancer cells and various immune cells in the TME such as T lymphocytes, dendritic cells (DCs), tumor associated macrophages (TAMs) and natural killer (NK) cells, could explain the pathogenesis and explore novel therapies for EOC (10) (11). Immune editing, defined as the dual function of the immune system, can suppress and/or promote tumor growth (12). Studying the dual function of immune cells in the TME can suppress the key pathways that inhibit antitumor responses, and promising therapies will be discovered (13). PD-1 and CTLA-4 expressed on T cells are the basis of immune checkpoint immunotherapy (14). Additionally, immunosuppressive molecules in the TME such as indoleamine-2,3-dioxygenase (IDO), interleukin-10 (IL-10) and prostaglandin E2 (PGE2), can also be targets of immunotherapies (15).

In this review, the effect of the TME in immunotherapies and progress in EOC immunotherapy will be discussed.

2 Tumor microenvironment

2.1 Suppressive immune cells

2.1.1 Regulatory T cells

T-lymphocytes in the TME contain tumor infiltrating lymphocytes (TILs) and regulatory T cells (Tregs). Tregs have been shown to weaken antitumor immunity indicating poor prognosis in patients with EOC (16). Studies have revealed that increases in tumor Treg cells represent a low survival rate of EOC (16), while other studies show their association with a pleasing clinical outcome biomarker in colorectal cancer (17). Immunosuppressive mechanisms regulated by Tregs leading to immunological tolerance and ignorance of cancer are as follows: 1) releasing soluble or membranous repressive cytokines such as interleukin-10 (IL-10), interleukin-35 (IL-35) and transforming growth factor-β (TGF-β), which can kill effector T cells (18). 2) high expression of granzymes and perforin mediates the cytolysis of NK cells and cytotoxic CD8+ T cells (19). 3) interfering with effector T cell metabolism by reducing IL-2, which is competitively consumed by T cells, and increasing adenosine which is an inhibitory molecule (20). 4) inducing DC tolerance by expressing CTLA-4 and ligands CD80 and/or CD86 on DCs that can generate immunosuppressive tryptophan metabolites, lymphocyte activation gene 3 (LAG3) molecules can suppress MHC II molecules on DCs (21). In view of the key immunosuppressive effect of Tregs, several agents have been explored that directly target markers such as CTLA-4 and IL-2 (20, 22).

2.1.2 Tumor associated macrophages

Tumor-associated macrophages (TAMs) are recruited from monocytes in blood and resident peritoneal macrophages, and these are major infiltrating immune cells in the TME (23). Given the important heterogeneity and plasticity, TAMs contain two groups: anti-tumorigenic M1 type and pro-tumorigenic M2 type (24). In the TME, the most pro-tumorigenic M2-like phenotype (25) is critical for cancer angiogenesis, invasion and metastasis through different kinds of cytokines, chemokines, growth factors, and proteases (26, 27). Vascular endothelial growth factor A (VEGF-A), a pro-angiogenetic chemokine and protease secreted by TAMs promotes tumor angiogenesis (26, 28). By producing epidermal growth factor (EGF), TAMs promote cancer cell proliferation (29). Moreover, TAMs exhibit immunosuppressive effects through secreting IL-10, TGF-β, CCL2 and arginase (30, 31). Current studies targeting TAMs mainly include: 1) suppressing M2-like TAMs *via* inhibiting the recruitment of TAMs and exhausting TAMs 2) activating M1-like TAMs by strengthening the repolarization of M2 macrophages into M1 macrophages (32, 33). For inhibition of the recruitment of TAMs, the CCL2/CCR2 axis barricade which is has been found to be helpful in a mouse ovarian cancer model (34), and disrupting the CXCL12/CXCR4 axis which prolongs the survival of a tumor mouse model (35) seem to be an encouraging therapy. The decrease in TAMs caused by inhibiting the CSF-1/CSF-1R pathway has been proven to strengthen the tumor suppressive effect of docetaxel (36). In EOC, trabectedin can effectively deplete macrophages by inducing apoptosis of macrophages and thus play an antitumor role in ovarian cancer (37). Paclitaxel treats ovarian cancer by reprogramming the M2 to the M1 phenotype through

TLR4 in gene expression analysis (38). Furthermore, another macrophage-directed therapy targets PD-L1 on TAMs which is involved in tumor immune escape mechanisms (39, 40).

2.1.3 Myeloid-derived suppressor cells

Myeloid-derived suppressor cells (MDSCs) are a subpopulation of immunoregulatory immature myeloid cells that increase in multiple pathologic situations (41). MDSCs have been implicated in suppressing T cells and impacting other cells in the TME (42). The mechanisms of suppressing T cells include the following: 1) accelerating the depletion of T cell essential amino acids such as L-cysteine, L-arginine and L-tryptophan which are critical for T cell activity (43–45). 2) expressing PD-L1 interacting with PD-1 on T cells to suppress the antitumor effect of T cells and thus promote immune evasion (46). 3) producing ROS and NO which are toxic to T cells (47). Moreover, MDSCs boost the activation of Tregs *via* IL-10 and TGFβ in the need of CD40 (48, 49). MDSCs can be targeted by various strategies: 1) reduction of MDSCs, 2) inhibition of the recruitment of MDSCs, 3) suppression of MDSC function and 4) induction of MDSCs to differentiate into non-suppressive cells (50). Thus, immunotherapies in combination with targeting MDSCs could be a major strategy. Phosphodiesterase-5 (PDE-5) inhibitors targeting arginase 1 (ARG1) and iNOS restabilize the immunosuppressive response of T cells (51). Synthetic triterpenoids activate the Nrf2 gene which modulates antioxidant enzymes and nitroaspirin, inhibiting iNOS and ROS production and thus relieving the oxidative stress caused by MDSCs (52, 53). Furthermore, STAT3 inhibitors combined with immune checkpoint blockade have been shown to be beneficial in lymphoma (54). In addition, blocking COX-2 which is correlated with the expression of ARG1 can also be a promising approach to attenuate MDSC function (55). A schematic illustration of how suppressive immune cells affect the antitumor immune response in the TME is shown in Figure 1.

2.2 Activated immune cells

2.2.1 T lymphocytes

TILs contain CD8+ T and CD4+ T lymphocytes, especially CD8+ TILs which represent a good prognosis of EOC (56). CD8 + T cells recognize and kill pathogenic infections or cancer cells through perforin and granzyme (57). In addition to destroying cancer cells directly, CD8+ T cells suppress tumor vascularization *via* secreting IFN-γ which suppresses the development of cancer. Emerging evidence has revealed that CD8+ T cells in the TME are often beneficial to survival in ovarian cancer patients (58). Furthermore, CD4+ T cells are divided into different subtypes which include T helper 1 (Th1) cells, a group of cells that provide cytokines such as IL-2 and IFN-γ to support the antitumoral effect of CD8+ T cells (59). Moreover, high expression of CCL5 released by CD4+ T cells benefits the activation of DCs and thus induces an antitumor response (60). Hence, increases in Th1 cells within the TME are related to significant outcomes in a variety of cancers (61). As mentioned above, not all T cells function as antitumor effectors such as Tregs and Th17 cells. Thus, immunotherapies targeting the main impaired antitumor T effector cells are considered

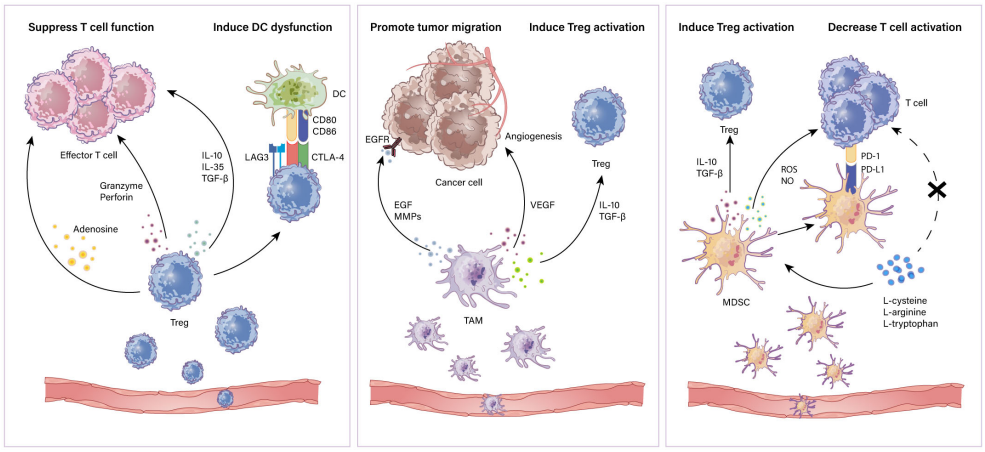

FIGURE 1
Immunosuppressive cells in the TME. Immune suppressive cells mainly contain Tregs, TAMs and MDSCs. Tregs restrain antitumor effector T cells including (i) secretion of repressive cytokines such as IL-10, IL-35 and TGF-β; (ii)induction of cytolysis *via* releasing granzymes and perforins; (iii) metabolic disturbance through adenosine production. Also, Tregs induce antigen presenting cell dysfunction for a tolerant phenotype. TAMs can (i) secrete EGF and MMPs to promote tumor progression; (ii) produce VEGF to aid in tumor angiogenesis; (iii) secrete IL-10 and TGF-β to induce Treg activation. MDSCs suppress T cells through (i) secretion of IL-10 and TGF-β which induce Tregs; (ii) production of NO and ROS which induce cytolysis and inhibit T cell activation; (iii) depletion of essential amino acids which play a crucial role in T cell activation and proliferation.

optimistic therapeutic approaches (62, 63). Recently, major advances have been made in developing PD-1 therapy which can potentiate the efficacy of CD8+ T cell based immunotherapy (64). T cells express PD-1 while other cells such as Tregs, TAMs, and cancer cells express PD-L1. Consequently, blockade targeting PD-1 or PD-L1 can suppress activation of T cell and breakdown immune tolerance and thus potentially mobilize immunity in tumors (65, 66).

2.2.2 Natural killer cells

NK cells are the most efficient antineoplastic effectors and do not need any prior sensitization or HLA-independent tumor target recognition (67, 68). NK cells encompass two main populations with CD56bright/CD16– functioning to produce IFN-γ and TNF-α cytokines, while CD56dim/CD16+ killing tumor cells directly *via* releasing perforin/granzyme or through TRAIL pathways (69). However, NK cells usually exhibit dysfunction in the TME, such as reduced proliferation, decreased secretion of cytotoxic molecules and abnormal expression of immune checkpoints (70). Studies have revealed that PD-L1 on tumor cells can inhibit PD-1 expression on NK cells, thereby promoting immune escape from cancer (71). A series of molecules such as IDO and PGE2 secreted by fibroblasts were shown to suppress the expression of the activating receptor NKG2D and thus mediate immune escape (72). Several immunotherapeutic strategies based on NK cells are currently being explored including adaptive NK cells, cytokines, antibodies and ICIs (68). Remarkably, adaptive NK cell therapy induced by various cytokines exhibits enhanced antitumor effects in ovarian cancer (73). Currently, therapy based on cytokines such as IL-2 and IL-15 is shown to vigorously increase NK cells (74, 75). Although antibody-based immunotherapy is not the gold standard treatment for ovarian cancer patients, antigens on tumor cells including NY-ESO-1 and MUC1 have attracted great attention (76). Furthermore, evidence has revealed that PD-1 and CD96/TIGIT inhibitors potentiate the tumor lysis mediated by NK cells (77).

2.2.3 Dendritic cells

DCs capture and process antigens to T cells and secrete inflammatory cytokines to induce pathogen-specific T-cell effects (78). Generally, DCs submit exogenous antigen peptides to CD4+ T cells through MHC II molecules and endogenous antigens to CD8+ T cells with MHC I molecules, and strengthen CD4+ and CD8+ T cell activity *via* presenting exogenous antigens (79, 80). However, the tumor microenvironment inhibits DC maturation through immunosuppressive factors including VEGF, IL-6, IL-10 and TGF-β, as well as repressive molecules including IDO (81, 82). Nevertheless, upregulating suppressive receptors such as PD-L1 and LAG3 induces T cell exhaustion thereby limiting the immune response (83). IDO produced by DCs restrains the function of NK cell and CD8+ T cell leading to an abated immune response (84). Therapeutic schemes targeting DCs have attracted great attention. Clinical studies revealed that IDO1 inhibitors combined with chemotherapy or ICIs elicit tumor regression (85). Collectively, potential DC based immune therapy seems to be an encouraging target against ovarian cancer. Furthermore, with such an effective ability for Ag presentation and T cell activation, DCs were tested as cancer vaccines that can produce "trained" DCs carrying tumor antigens and thus potentially induce strong antitumor T-cell effects (86, 87). In particular, it is well documented that DC vaccines combined with ICIs may result in synergistic effects (88, 89). A schematic illustration of how immune active cells exhibit dysfunction in the TME is shown in Figure 2.

2.3 Tumor-associated stromal cells

2.3.1 Cancer-associated fibroblasts

CAFs derived from mesenchymal stem cells are crucial components of stromal cell types (90, 91). CAFs produce proteins, paracrine cytokines and various ECMs that contribute to shaping the tumor microenvironment (92, 93). Classical growth factors secreted by CAFs include the following: 1) TGF-β regulates the interaction between cancer and stroma thereby facilitating tumor initiation and progression (94). 2) Epidermal growth factor (EGF) maintains the expression of ATC integrin α5 (ITGA5) which can promote the early peritoneal spread of HGSOC (95). 3) Hepatocyte growth factor (HGF) contributes to proliferation *via* the c-Met/PI3K/Akt and GRP78 pathways (96). 4) CXCL12, IL6, and VEGFA induced by CAFs result in the epithelial-to-mesenchymal transition (EMT) which can promote peritoneal metastasis of ovarian cancer (97). 5) CAFs activate MMPs to assist in the growth, invasion, and metastasis of tumors (98). 6) Lipoma-preferred partner (LPP) which has been proven to increase the focal adhesion and stress fiber formation of ECs contributes to ovarian cancer chemoresistance (99). Therapeutic strategies targeting CAFs fall into several aspects, one of which involves TGF-β inhibitors which were shown to improve the overall survival of EOC in a mouse model with peritoneal metastasis (100). Imatinib, an inhibitor targeting PDGF-D produced by fibroblasts was found to suppress ovarian cancer cell growth (101).

2.3.2 Cancer-associated adipocytes

Cancer-associated adipocytes (CAAs) are documented to promote stromal reshaping and the invasion of cancer cells through interacting with ovarian cancer cells in the TME (102). It has been proven that metastases appear in the omentum which is made up of adipocytes in most patients with ovarian cancer (103). Adipocytes secrete various molecules such as metabolites, MMPs, enzymes and growth factors supporting tumor cell

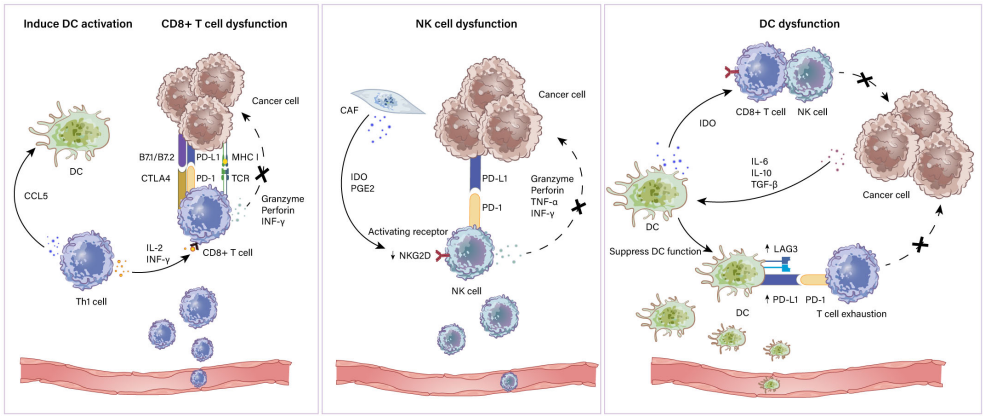

FIGURE 2
Immunoactivated cells in the TME. Immune activated cells include CD8+ and CD4+ T lymphocytes, NK cells and DCs. Th1 cell which is a subtype of CD4+ T cells express CCL5 to induce DC activation and produce IL-2 and IFN-γ to assist CD8+ T cells. CD8+ T cells recognize antigens expressed on MHC class I on cancer cells leading to the cytotoxic killing of cancer cells *via* granzymes and perforins. CD8+ T cell dysfunction is mediated by elevated inhibitory ligand PD-L1 and B7 molecules on cancer cells. NK cell dysfunction mechanisms include (i) PGE2 and IDO derived from CAFs downregulate the expression of activating receptor NKG2D on DCs; (ii) inhibitory ligand PD-L1 and B7 molecules expressed by cancer cells restrain NK cell function by combining with PD-1 and CTLA-4. The mechanism of impaired DC function includes (i) overexpression of PD-1 ligands and LAG3 on DCs results in T cell exhaustion; (ii) IL-6, IL-10 and TGF-β produced by cancer cells alter DC maturation and migration capacity.

progression (104). Furthermore, coculture studies found that CAAs boost the oxidation of cancer cells, indicating that CAAs supply energy to maintain the ovarian cancer cells proliferation (105). Additionally, CAAs produce adipokines that can promote tumor development. Among them, leptin stimulates the migration and invasion abilities of ovarian cancer cells through the JAK/Stat3, PI3K/Akt and RhoA/ROCK pathways (106). FABP4, a lipid chaperone protein is a key regulator in ovarian cancer metastasis (107). Molecules including IL-6, IL8 and TNF-α secreted by CAAs have also been proven to aid in the growth and invasion of breast tumor cells (108). Collectively, the reciprocation between CAAs and cancer cells results in the metastasis of tumor cells. Overall, therapeutic strategies specifically targeting lipid metabolism and transport such as FABP4 inhibitors in ovarian cancer are full of hope (107).

2.3.3 Endothelial cells

Endothelial cells (ECs) impact the process of cancer growth and invasion (109). Angiogenesis is known to be a process regulated by the interplay of angiogenic activators and inhibitors. During tumor progression, oxygen deficiency and accumulation of metabolic products lead to hypoxia and acidity in the TME (110). Hypoxia in the TME induces the production of hypoxia-inducible factors (HIFs), which promote pro-angiogenic factors secreted by ECs, thereby promoting angiogenesis (111). In the course of angiogenesis, factors produced by cancer cells contain VEGF, platelet derived growth factor (PDGF), fibroblast growth factor 2 (FGF-2) and angiopoietins (112). VEGF, a chemokine secreted into malignant ascites contributes to the genesis of tumor blood *via* signaling with VEGF receptor-2 (113). To stabilize and increase the maturation of endothelial cell channels, pericytes express PDGFR-β which can interact with PDGF-B (114). Furthermore, FGF-2 promotes the production of MMPs, collagenase and plasminogen activator resulting in vascularization (115). In addition, FGF expression has been proven to be responsible for resistance to VEGF targeted therapies (116). Angiopoietin 1 and 2 (Ang1/2) has been found to promote the proliferation and survival of ECs *via* binding to the Tie-2 receptor (117). Thus, considerable attention is being paid to exploring therapeutic strategies to block the angiogenic signaling pathway. Bevacizumab as the most studied anti-VEGF monoclonal antibody has been demonstrated to positively increase PFS with cisplatin-based chemotherapy in several randomized phase III trials and has been recognized as the standard treatment in EOC (118, 119). Tyrosine kinase inhibitors (TKIs) such as pazopanib and cediranib are promising VEGFR targeting agents for ovarian cancer patients (120, 121). Trebananib, an inhibitor that targets non-VEGF signaling has meaningful effects on PFS when used in combination with paclitaxel in recurrent ovarian cancer through binding to Ang1/2 (122).

3 Novel combination approaches

It is obviously that ICIs have revolutionized immunotherapy and brought concrete benefits to many patients. However, the

response rate is unsatisfactory with different anti-PD-1 or PD-L1 agents since EOC is known to have a high immunosuppressive TME and low expression of PD-L1 (8). Novel combination therapies are currently evolving. Inhibitor of PD-1 in combination with CTLA-4 increased the frequency of tumor infiltration by effector T cells as well as uniquely decreased the frequency of Tregs in tumors (123). Evidence from clinical trials found that this combination blockade therapy is effective. The NRG-GY003 study showed that in recurrent epithelial ovarian cancer, nivolumab combined with ipilimumab led to an effective response rate of 31.4% while nivolumab alone is 12.2%. In addition, the mPFS was 2 months in the monotherapy group and 3.9 months in the combination treatment group (124). Combining nivolumab with ipilimumab produced higher response rates and longer PFS in EOC than nivolumab alone, but is still limited, so more combination clinical studies such as NCT02834013 and NCT03508570 are underway.

In a mouse model of intraperitoneal ovarian cancer, compared with single drug therapy of AMD3100 (CXCR4 antagonist) and αPD-1, the antitumor efficacy of combined therapy in inhibiting tumor growth and prolonging the survival time of mice was significantly improved (35). This provides strong preclinical evidence for ovarian cancer combination therapy, but to date, no clinical trials related to ovarian cancer have been carried out. It is worth noting that in a phase IIa trial, disease control with BL8040 plus pembrolizumab was 34.5% in 29 patients. 22 patients were treated with BL8040 plus pembrolizumab plus chemotherapy. The results exhibited an effective response rate of 32% and a disease control rate of 77% (125). These data all suggest that the combination of CXCR4 antagonists and PD-1 inhibitors can amplify the antitumor effect of chemotherapy.

In recent years, transmembrane protein triggering receptor expressed on myeloid cells 2 (TREM2) has gained great attention in anti-PD-1 resistant therapy since its enriched expression on TAMs in EOC (126). In a model of invasive ovarian cancer *in situ*, anti-TREM2mAb therapy can drive effective antitumor immunity (126). This finding indicates that TREM2 is a potential immunotherapy target when ICIs are ineffective and TAMs are rich in the tumor microenvironment. A phase I clinical trial (NCT04691375) to evaluate the single drug anti-TREM2 and anti-TREM combined with pembrolizumab in solid tumors which include ovarian cancer is underway.

Evidence has shown that PD-1 blockade in combination with a VEGF-A inhibitor can potentiate antitumor efficiency *via* increases in CD8+ and CD4+ T cells, and decreases in MDSCs and Tregs (127, 128). In a single-arm phase 2 clinical study, 38 women with recurrent epithelial ovarian cancer were screened for intravenous treatment with nivolumab and bevacizumab. The results showed that the ORR was 28.9%, among which 40.0% were platinum sensitive patients and 16.7% were platinum resistant patients (129). These data indicate that the combining nivolumab with bevacizumab is effective and feasible in ovarian cancer patients, especially those who are sensitive to platinum.

Poly-(ADP)-ribose polymerase inhibitors (PARPi), known as synthetic lethal agents in tumors with BRCA1/BRCA2 mutations are rapidly evolving (130). Combining PARPis with PD-1/PD-L1 blockades is a promising therapy that can synthetically enhance the antitumor effect (131). A phase II clinical study of MEDIOLA found that 32 patients received olaparib combined with durvalumab, and the overall disease control rate was 81% (132). The final result of the study showed that the OS of 31 patients treated with olaparib combined with durvalumab and bevacizumab was 31.9 months compared with that of 23.2 months in the two-drug group. In addition, the DCR of the two-drug group at 56 weeks was 9.4%, and that of the three-drug group was 38.7%, which indicated that the three-drug treatment mode was superior to the two-drug treatment mode for platinum-sensitive recurrent non-gBRCA ovarian cancer patients (133). The three-drug regimen has been applied to a third-phase clinical study of first-line maintenance therapy (NCT03737643).

4 Discussion

Immune checkpoint immunotherapy has been the most prominent therapeutic strategy for successfully treating different kinds of cancers. However, the response rate in EOC is low since the immunosuppressive tumor microenvironment could limit the efficiency of ICIs. It is urgent to improve the effect of immunotherapy for EOC. Novel targets have been described and targeting approaches combined with ICs have already impacted the clinical outcomes of ovarian cancer. Targeting immune subtypes such as TAMs, Tregs, CAFs or angiogenesis could contribute to potentiating the antitumor effect of ICs. However, there are still many details to explore and discuss. In summary, the constituents within the TME should all be considered to explore novel combinations that contribute to achieving maximal benefits in EOC.

Author contributions

YW and LZ wrote the original manuscript. YB drew the figure. XM and LW corrected the original draft. All authors contributed to the article and approved the submitted version.

Conflict of interest

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

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The current landscape of predictive and prognostic biomarkers for immune checkpoint blockade in ovarian cancer

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Immune checkpoint blockade (ICB) therapy has evoked a prominent shift in anticancer therapy. Durable clinical antitumor activity to ICB has been observed in patients with ovarian cancer (OC). However, only a subset of patients derive clinical benefit, and immune-related adverse events (irAEs) caused by ICB therapy can lead to permanent tissue damage and even fatal consequences. It is thus urgent to develop predictive biomarkers to optimize patient outcomes and minimize toxicity risk. Herein, we review current predictive and prognostic biomarkers for checkpoint immunotherapy in OC and highlight emerging biomarkers to guide treatment with ICB. The prevalent biomarkers, such as PD-L1 expression status, tumor-infiltrating lymphocytes, mutational burden, and immune gene signatures, are further discussed. We provide a state-of-the-art survey on prognostic and predictive biomarkers for checkpoint immunotherapy and offer valuable information for guiding precision immunotherapy

KEYWORDS

ovarian cancer, immune checkpoint blockade, biomarker, immunotherapy response, prognosis

Introduction

Immune checkpoint blockade therapies (ICBs) can circumvent tumor-mediated immune suppression and reinvigorate antitumor immune responses, in contrast with conventional therapeutic strategies that exert direct cytotoxicity against tumor cells (1, 2). Immune checkpoint inhibitors (ICIs) that target the programmed cell death protein-1 (PD-1)/programmed death receptor ligand-1 (PD-L1) axis or cytotoxic T lymphocyte antigen 4 (CTLA4) have achieved impressive success against various cancer types (3). ICIs have

achieved remarkable clinical activity with durable disease control across multiple advanced tumors (4). Accordingly, several ICIs have been approved by the United States Food and Drug Administration (FDA) for patients with malignancies, including melanoma, lung cancer, triple-negative breast cancer (TNBC), colorectal cancer, gastric cancer, renal cell cancer, head and neck squamous cell cancer, bladder cancer, lymphoma and so on (5). Albeit substantial advancements in clinical therapy, only a minority of patients receiving ICIs derive benefits. In addition, ICB therapy is significantly restricted by the occurrence of immune-related adverse events (irAEs), resulting from immune hyperactivation and subsequent immune homeostasis disturbance. Severe adverse events can lead to permanent disorders and can be lethal in some cases (6). Therefore, there is intense interest in developing predictive and prognostic biomarkers for ICI therapy to better understand the benefits and risks driven by ICB and effectively select patients.

Manipulating the immune environment with ICIs is an attractive therapeutic approach for antitumor therapy in ovarian cancer (OC) (Figure 1). There has been considerable progress in utilizing ICB therapy for OC over the past few years (Table 1; Supplementary Table S1). However, there is still confusion regarding patient selection and the choice of therapeutic regimen for patients with OC, underscoring the need for effective biomarkers to predict response and remission. In this review, we attempt to summarize published original research and clinical trials involving biomarker assessment in OC receiving ICI therapy and discuss ongoing efforts to develop predictive biomarkers of responsiveness and outcomes.

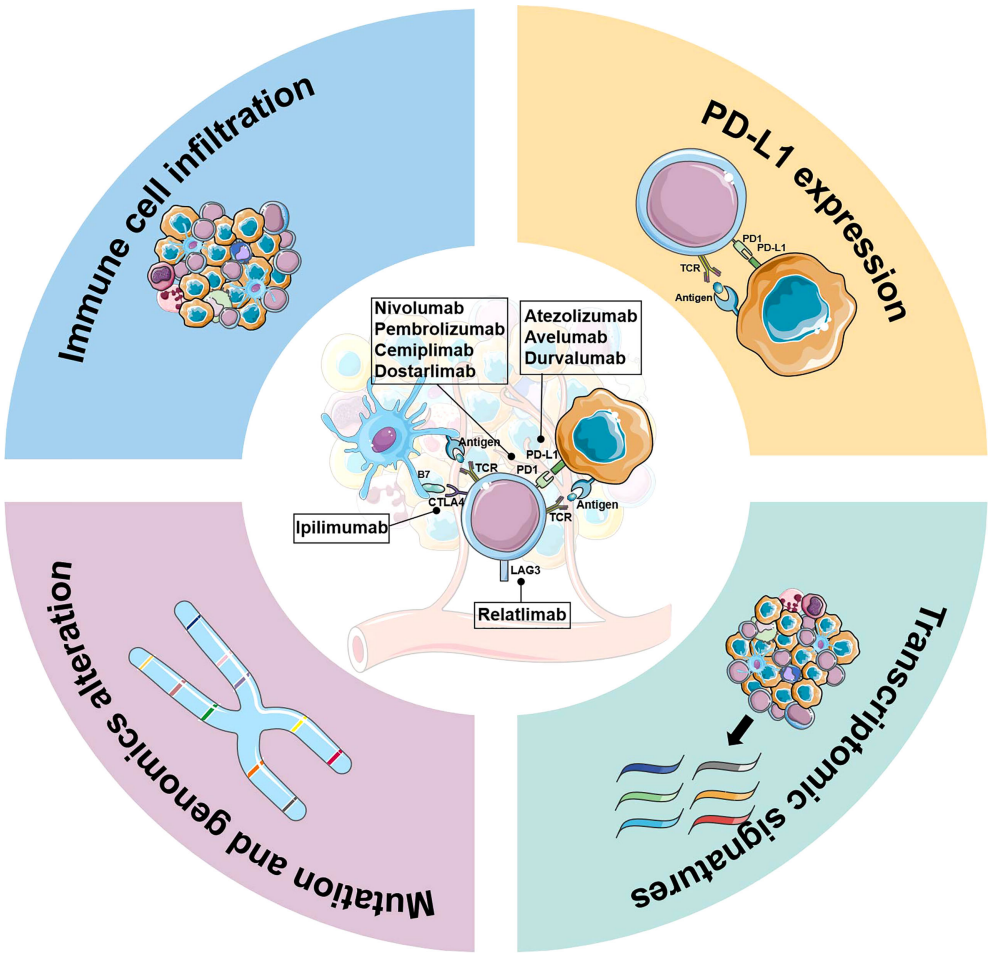

FIGURE 1
Biomarker development for immune-checkpoint inhibitor therapy in ovarian cancer. Key elements in biomarker development for immune checkpoint inhibitors therapy are briefly described, including PD-L1 expression, genomics alterations, immune cell infiltration, and transcriptomic signatures.

PD-L1 expression

Direct measurement of PD-L1 expression is a logical biomarker for predicting response to anti-PD-1/PD-L1 therapies. PD-L1 immunohistochemistry (IHC) assay is now FDA-approved as a companion diagnostic biomarker to select patients most likely to benefit from ICI treatment for multiple cancer types, such as non-small cell lung cancer(NSCLC), metastatic TNBC, and melanoma (5).

The predictive value of PD-L1 expression was assessed in OC patients treated with anti-PD-1/PD-L1 antibodies (Table 1). KEYNOTE-100 (NCT02674061) investigated the clinical activity of pembrolizumab in patients with recurrent advanced OC and introduced PD-L1 stain score as a predictive biomarker, in which patients with higher PD-L1 expression (combined positive score≥10) had an increased overall response rate (ORR) and prolonged overall survival (OS) with pembrolizumab (8). More recently, Sanborn et al. evaluated the efficacy and safety of varlilumab plus nivolumab in patients with advanced solid tumors (10). Significantly, an absolute increase of 5% or more in tumor PD-L1 expression induced by treatment tended to improve progression-free survival (PFS) in OC (7.4 months *vs.* 3.5 months, p=0.07), whereas baseline pretreatment PD-L1 expression was not associated with ORR (10). Prespecified biomarker analysis in the JAVELIN-200 trial revealed a trend for prolonged PFS with the addition of avelumab to pegylated liposomal doxorubicin (PLD) compared with PLD alone among OC patients with PD-L1-positive tumors (12). Nevertheless, several trials yielded inconsistent or even contradictory results regarding the role of PD-L1 expression as a marker for predicting response to ICB and clinical outcomes in OC. Liu et al. (15) obtained the opposite results in evaluating the predictive and prognostic value of PD-L1 expression in recurrent OC patients receiving nivolumab and bevacizumab. Even patients with PD-L1-negative tumors (10/22) had higher therapeutic activity than those with PD-L1-positive expression (2/14) (15). In addition, several studies have shown that the expression of PD-L1 was not predictive of ICI outcome and prognosis in OC patients (36, 38–43). Potential reasons for these paradoxical results include the inability to accurately reflect PD-L1 status due to PD-L1 expression transiency and heterogeneity, differences in the disease status of patients, the poor uniformity between various detection assays, and the lack of standardized criteria and thresholds for assessing positivity (3, 44, 45). Therefore, PD-L1 status is likely insufficient to determine the suitability of ICI therapy for OC patients. Further refinement of the use of PD-L1 expression status as a robust biomarker for checkpoint immunotherapy is warranted.

Tumor-infiltrating immune cells

TIICs can serve as an index to monitor the tumor microenvironment (TME) and play an increasingly important role in the immune response against cancer (46). Therefore, TIICs have also been speculated to be surrogate biomarkers for ICB immunotherapy in many types of cancer, including OC (Table 1). A comprehensive analysis of immune cells in patients with epithelial ovarian cancer (EOC) revealed a positive correlation between the infiltration of immune cells and the clinical outcome of EOC (16). The density of tumor-infiltrating lymphocytes (TILs), specifically $CD8^+$ T cells, is a solid positive prognostic indicator for multiple cancer types regardless of ICI therapy. In fact, CD8 expression in tumors was predictive of clinical benefit with avelumab plus PLD treatment in OC (12). Of note, patients with dual PD-L1-positive and CD8-positive tumors seemed to benefit more from combination treatment than subgroups defined by only one of these biomarkers (12). Another potential predictor of ICI response is tumor-infiltrating mast cells (TIMs) within a tumor (Table 1). In high-grade plasmacytoid ovarian cancer (HGSOC), stromal TIMs (sTIMs) abundance was negatively associated with the ICB response (18). Remarkably, tumors with low sTIMs had enhanced effector functions of $CD8^+$ T cells (18). This finding was corroborated in short-term HGSOC organoids. The effector molecules (GZMB and IFN-γ) on $CD8^+$ T cells were marginally increased in organoids derived from low sTIMs tumors, compared to organoids from high sTIMs tumors (18). Overall, the abundance of sTIMs predicts a dismal prognosis in HGSOC patients treated with anti-PD-1 therapy.

Except for the spatial position and density of TIICs, their phenotype and activation status also impact the clinical benefit of ICIs (3). The immune-inflamed phenotype is usually accompanied by the expression of PD-L1 on infiltrating immune cells and tumor cells, which is associated with a better response to ICI therapy (3). In a trial investigating combination regimens with anti-PD-L1 antibody in women's cancers, a trend toward a positive association of treatment response with the degree of PD-L1-positive TILs was observed (39). In contrast, melanoma patients with PD-L1-positive TILs had a significantly worse prognosis than those with PD-L1-negative TILs (P = 0.008) (47). Further investigations are needed to determine whether PD-L1-positive TILs are suitable to serve as predictors of ICB effectiveness. In addition, other non-neoplastic cells in the TME are also non-negligible, which are probably of biological significance. Therefore, increased awareness of the role of these distinct TME compartments is needed for comprehensive biomarker development to predict ICB response and prognosis.

Mutation and genomics alterations

Tumor development and progression generally occur along with the acquisition and accumulation of mutations (45). Neoantigens generated by mutations may lead to T-cell infiltration, thereby better response to immunotherapy (48). In fact, several studies have attempted to evaluate somatic mutations as biomarkers for predicting ICB response in OC

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TABLE 1 Predictive and prognostic biomarkers for checkpoint immunotherapy in ovarian cancer.

Categories	Biomarker	Association with favorable clinical outcome	Predictive versus prognostic	Tissue type for biomarker assessment	Possible assay type for biomarker assessment	Trial	Treatment	References
PD-L1	tumor PD-L1 expression	positive	predictive	tumor	IHC	NCT02674061	pembrolizumab	(7)
	tumor PD-L1 expression	positive	predictive	tumor	IHC	NCT02674061	pembrolizumab	(8)
	tumor PD-L1 expression	positive	predictive	tumor	IHC	NCT02674061	pembrolizumab	(9)
	tumor PD-L1 expression	positive	both	tumor	IHC	NCT02335918	varlilumab + nivolumab	(10, 11)
	PD-L1 expression both in tumor cells and immune cells	positive	both	tumor	IHC	NCT02580058	avelumab vs. avelumab + PLD vs. PLD	(12)
	tumor PD-L1 expression	potentially positive	predictive	tumor	–	NCT03558139	magrolimab + avelumab	(13)
	tumor PD-L1 expression	potentially positive	predictive	tumor	IHC	NCT02865811	pembrolizumab + PLD	(14)
	tumor PD-L1 expression	negative	predictive	tumor	IHC	NCT02873962	nivolumab + bevacizumab	(15)
TIICs	immune cell infiltration	positive	prognostic	tumor	RNA-seq	–	–	(16)
	CD8 expression	positive	both	tumor	IHC	NCT02580058	avelumab vs. avelumab + PLD vs. PLD	(12)
	immune score	positive	both	tumor	NanoString	NCT02657889	niraparib + pembrolizumab	(17)
	stromal tumor infiltrating mast cells (sTIMs)	negative	prognostic	tumor	IHC	–	–	(18)
Mutation and genomics alteration	the ratio of peripheral $CD8^+PD1^+Ki67^+$ T cells to TMB	positive	prognostic	blood	DNA sequencing	NCT03029598	carboplatin + atezolizumab	(19)
	ARID1A loss/ mutation	positive	predictive	tumor	DNA sequencing	–	–	(20)
	mutational signature 3	positive	both	tumor	DNA sequencing	NCT02657889	niraparib + pembrolizumab	(17)
	fraction of genome altered (FGA)	positive	both	tumor	DNA Sequencing	–	–	(21)
Transcriptomic signature	APOBEC3A expression	positive	both	tumor	qPCR	–	–	(22)
	immune-related genes	positive	prognostic	tumor	RNA-seq	–	–	(23)
	signal transducer and activator of transcription 1 (STAT1)	potentially positive	predictive	tumor	qPCR	–	–	(24)
	CAPG expression	negative	both	tumor	RNA-seq	–	–	(25)
	LAYN expression	negative	both	tumor	RNA-seq	–	–	(26)
	TGF-β score	negative	prognostic	tumor	RNA-seq	–	avelumab/ nivolumab/ pembrolizumab	(27)
	NAD^+ metabolism-related genes (NMRGs)	negative	both	tumor	RNA-seq	–	–	(28)

(Continued)

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TABLE 1 Continued

Categories	Biomarker	Association with favorable clinical outcome	Predictive versus prognostic	Tissue type for biomarker assessment	Possible assay type for biomarker assessment	Trial	Treatment	References
	m6A-related gene signature	potentially negative	both	tumor	qPCR	–	–	(29)
	CXCL9	positive	prognostic	tumor	IHC	–	–	(30)
	CXCL11	positive	both	tumor	RNA-seq	–	–	(31)
	CXCL13	positive	both	tumor	IHC; IF	–	–	(32)
		potentially positive	both	tumor	RNA-seq	–	–	(33)
Peripheral blood biomarkers	increased IFNγ production	positive	predictive	blood	RNA-seq	NCT02484404	durvalumab + olaparib	(34)
	increased levels of CA-125	negative	predictive	blood	CA-125 test	–	–	(35)
	reduced levels of CA-125	potentially negative	predictive	blood	CA-125 test	NCT01772004	avelumab	(36)
	elevated VEGFR3 levels	negative	predictive	blood	RNA-seq	NCT02484404	durvalumab + olaparib	(34)
	ctDNA	negative	both	blood	bespoke ctDNA assays	NCT02644369	pembrolizumab	(37)

IHC, immunohistochemistry; IF, immunofluorescence; TIICs, tumor-infiltrating immune cells; PLD, pegylated liposomal doxorubicin; CA-125, cancer antigen-125; ctDNA, circulating tumor DNA; +, combination therapy; -, not available; /, or.

(Table 1). ARID1A mutation or loss was associated with immune microenvironmental factors in clear cell ovarian cancer (CCC), suggesting that ARID1A status has potential as a biomarker to guide decisions concerning patient selection for ICB therapy in CCC (20). The phase I/II trial (NCT02657889) reported two novel biomarkers for the combination of poly (adenosine diphosphate-ribose) polymerase (PARP) and PD-1 inhibitors in the treatment of platinum-resistant OC (17). Mutational signature 3 reflected homologous recombination deficiency (HRD) status, and positive immune score (IS) was a surrogate of interferon-primed exhausted $CD8^+$ T cells in TME. Specifically, the presence of one or both of the above alternative markers was associated with significantly prolonged PFS (HR = 0.32), while concurrent absence showed no response to PARP/PD-1 inhibitors(ORR= 0%) (17).

Another metric, known as tumor mutation burden (TMB), is a strong predictor of ICB efficacy. Unfortunately, its predictive performance in OC is disappointing. No significant correlation was found between TMB and immunotherapy response in recurrent OC (21). Furthermore, BRCA1/2 mutations and HRD status also did not predict the clinical benefit of ICI in heavily pretreated patients with OC (21). Notably, additional exploratory analyses identified the fraction of genome altered (FGA) as a promising biomarker of response to ICI in OC, which can characterize global copy number alterations. High FGA was significantly associated with improved OS (HR = 0.49; log-rank P = 0.01) and PFS (HR = 0.54; log-rank P = 0.014) after ICI therapy in OC (21). The optimal cutoff for defining high *vs.* low FGA is unclear; therefore, the predictive capacity of FGA warrants further validation.

TMB was also explored in the phase I/II trial (NCT03029598), which evaluated pembrolizumab and carboplatin for recurrent or refractory ovarian, fallopian tube, or primary peritoneal cancer (19). Stratification by the ratio of peripheral $CD8^+PD1^+Ki67^+$ T cells to tumor burden at baseline yielded a significant survival advantage. Patients with a low ratio (<0.0375) had a median OS of only 8.72 months, while those with a high ratio (≥0.0375) had a significantly longer median OS of 18.37 months (p=0.0099). However, no significant survival difference was observed when using $CD8^+PD1^+Ki67^+$ T cell (p=0.53) or tumor burden alone (p=0.24) as stratification criteria (19). Overall, TMB alone does not clearly discriminate responders from non-responders in OC patients treated with ICIs.

Transcriptomic signatures

Gene expression analysis can uncover global tumor and microenvironment features, providing promise for predicting the clinical benefit of checkpoint inhibitor strategies. Multiplex characterization of the TME and gene expression signatures have been proposed as effective methods to dissect the immune contexture and cancer cell-intrinsic features. According to TME information derived from transcriptome data of OC, Li et al. (23) established immune cell infiltration (ICI) scores and an immune-related gene prognostic model to predict the clinical benefits of OC patients undergoing immunotherapy. Signal transducer and activator of transcription 1 (STAT1) has been demonstrated to be associated with TME. A recent study found that STAT1 expression was positively correlated with PD-L1

expression and had the potential to predict the response to ICB in patients with EOC (24). Integrins are transmembrane receptors that mediate the connection between cells and their external environment (49–51).

Several immune-related gene signatures have been confirmed to predict the immunotherapeutic response in OC. The TGF-β regulated signaling pathway was noted to contribute to immunotherapy resistance in OC (27). A significant negative correlation between the TGF-β score and ICI-PFS was observed in OC, with an ICI-PFS of 16.6 months in the low TGF-β score group compared to 2.65 months in the high TGF-β score group ($p = 0.0012$). As the most common RNA modification, N6-methyladenosine (m6A) plays a key role in epigenetics (52). A risk model based on m6A-related targets has an excellent clinical prognostic stratification effect in advanced OC. Importantly, the high- and low-risk groups divided by this model have significant differences in TME contexture, suggesting that this model may be able to predict immunotherapy response in OC (29).

Chemokines have essential roles in modulating immune homeostasis and inflammatory responses (53). Accumulating findings suggest that chemokines can influence cancer cell proliferation, invasion, angiogenesis, and therapy resistance by recruiting immune cells and modulating the TME (54, 55). The prognostic and predictive values of the CXC chemokine family have been addressed in the setting of OC, including CXCL9, CXCL11, and CXCL13 (Table 1). Tumors with high CXCL9 expression had significantly prolonged OS, implying the feasibility of CXCL9 expression as a novel prognostic marker for high-grade serous ovarian cancer (HGSC) (30). Similarly, Fan et al. (33) found a significant positive correlation between the expression of CXCL13, FCRLA, PLA2G2D, and MS4A1 and a better prognosis of OC. Meanwhile, these potential therapeutic genes could reflect OC immune status and allow better predictions of who will respond to ICI. Furthermore, Yang etal. (32) examined the therapeutic effects of CXCL13 and PD-1 blockade in human HGSC tumors and mouse models. They found that CXCL13 can augment the efficacy of PD-1 checkpoint blockade in HGSC by shaping the antitumor microenvironment. CXCL13 can facilitate $CXCR5^+CD8^+$ T-cell recruitment to tertiary lymphoid structures. Furthermore, the combination of CXCL13, CD8, and CXCR5 was confirmed as a potential prognostic indicator or response biomarker for ICB therapy in patients with HGSC. CXCL11 expression has been demonstrated as a biomarker for predicting the response to anti-PD-1/PD-L1 therapy in a clinical trial of OC (31). In OC patients with HRD, tumors with high CXCL11 expression had a more robust immune response to PD-L1 blockade than those with low CXCL11 expression. Notably, the tumor-infiltrating immunophenotype and neoantigen burden were significantly elevated in CXCL11-high tumors.

In addition, several genes have been demonstrated to be associated with immunotherapy efficacy and prognosis in OC (Table 1). For example, Capping Actin Protein, Gelsolin-Like (*CAPG*) (25) and Layilin (*LAYN*) (26) appeared to be indicators of ICI outcome. Tumors with high *CAPG* or *LAYN* expression showed a significantly shorter survival time. In a study, the predictive significance of NAD^+ metabolism-related genes (NMRGs) on immunotherapy response in patients with OC was examined. The high-risk score obtained by the NMRG-based model was also associated with a poorer prognosis (28). Apolipoprotein B mRNA editing enzyme catalytic subunit 3A (APOBEC3A) has been recognized as an indicator of genomic instability and may aid in predicting the prognosis and response to immunotherapy in OC (22).

Peripheral blood biomarkers

In recent years, there has been great interest in developing blood-derived predictive biomarkers of ICI response, owing to its convenient and non-invasive sampling (56). Cancer antigen 125 (CA-125) is an important tumor biomarker specific to OC (57); thus, several studies have carried out exploratory research on the predictive role of CA-125 in OC patients treated with ICIs (Table 1). A phase II trial (NCT02608684), designed for evaluating the combination of pembrolizumab and chemotherapy in platinum-resistant OC, found CA-125 to be a reliable marker that reflected response and progression (42). In a retrospective study of EOC patients treated with ICI (35), the magnitude of increase in CA-125 levels within the first 12 weeks of treatment was significantly smaller in patients with clinical benefit than in those without benefit, suggesting a possible predictive role for the degree of CA-125 increase. In a phase 1b study of avelumab in patients with heavily pretreated OC, 12 patients with an objective response, of whom all 7 patients evaluable for CA-125 levels showed decreased CA-125 concentrations (36).

Dynamic monitoring of circulating tumor DNA (ctDNA) in plasma samples offers a meaningful direction for biomarker identification for immunotherapy in OC patients (37). A satisfying finding was that ctDNA concentration was related to clinical response and benefit, although the effect sizes were modest (37). Additionally, in a phase II trial of olaparib combined with durvalumab for OC, increased IFNγ production and elevated VEGFR3 levels in blood samples showed positive and negative correlations with PFS, respectively ($p=0.023$; $p=0.017$) (34).

Conclusion and future directions

The clinical trials and original research outlined above have shown that classical biomarkers derived from the TME and tumor intrinsic features, such as PD-L1 expression, TMB, TIICs, and transcriptomic signatures, were correlated with ICI response and outcome in OC. Although these findings are intriguing, the implementation of these classical biomarkers has been hampered by inconsistencies and limitations. Promisingly, new biomarkers often designed as substitutes or complements to conventional biomarkers are constantly emerging, such as microbiome, tertiary lymphoid structures (TLSs), and tumor-associated antigens (TAAs).

The potential of microbiome and its derived metabolome as biomarkers for predicting the efficacy of immunotherapy has been validated in melanoma (58), lung cancer (59), hepatobiliary cancer (60), and colorectal cancer (61). Several studies have demonstrated that clinical outcomes of immunotherapy for solid tumors are strongly correlated with the presence of TLSs, suggesting that TLSs may be a valid predictive indicator in the future (62). Elevated levels of carcinoembryonic antigen (CEA) have also been reported to negatively correlate with the prognosis of resected NSCLC patients receiving ICB therapy (63). More recently, a comprehensive predictive model for ICB response was developed across 16 different cancer types, which included the features of peripheral blood such as platelets, neutrophil-to-lymphocyte ratio, albumin, and hemoglobin (HGB) (64). These studies provide new perspectives to develop new biomarkers for OC patients treated with ICB therapy. The predictive values of these biomarkers in OC remain to be validated in routine clinical settings.

As evidenced by the fact that a single biomarker is often insufficient to determine the suitability of ICI therapy for OC patients, the combination of different biomarkers may be more valuable in predicting the clinical prognosis and therapeutic response to immunotherapy. Indeed, it has been proposed that the incorporation of dynamic and static biomarkers could improve decision-making to design tailored immunotherapy strategies. Moreover, the development of relevant biomarkers for the toxicity prediction of ICB therapy has become a research hotspot and is expected to offer effective ways to uncouple immunotherapy toxicity from its antitumor activity.

Author contributions

YX and XH: conceptualization and writing-original draft preparation. FZ: visualization. YX, FZ, HW, JJ and XH: writing-review and editing. XH and JJ: supervision and funding acquisition. All authors have read and agree to the published version of the manuscript.

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Conflict of interest

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

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2022.1045957/full#supplementary-material

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Targeted drug delivery system for ovarian cancer microenvironment: Improving the effects of immunotherapy

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Immunotherapies have shown modest benefits in the current clinical trials for ovarian cancer. The tumor microenvironment (TME) in an immunosuppressive phenotype contributes to this "failure" of immunotherapy in ovarian cancer. Many stromal cell types in the TME (e.g., tumor-associated macrophages and fibroblasts) have been identified as having plasticity in pro- and antitumor activities and are responsible for suppressing the antitumor immune response. Thus, the TME is an extremely valuable target for adjuvant interventions to improve the effects of immunotherapy. The current strategies targeting the TME include: 1) eliminating immunosuppressive cells or transforming them into immunostimulatory phenotypes and 2) inhibiting their immunosuppressive or pro-tumor production. Most of the effective agents used in the above strategies are genetic materials (e.g., cDNA, mRNA, or miRNA), proteins, or other small molecules (e.g., peptides), which are limited in their target and instability. Various formulations of drug delivery system (DDS) have been designed to realize the controlled release and targeting delivery of these agents to the tumor sites. Nanoparticles and liposomes are the most frequently exploited materials. Based on current evidence from preclinical and clinical studies, the future of the DDS is promising in cancer immunotherapy since the combination of agents with a DDS has shown increased efficacy and decreased toxicities compared with free agents. In the future, more efforts are needed to further identify the hallmarks and biomarkers in the ovarian TME, which is crucial for the development of more effective, safe, and personalized DDSs.

KEYWORDS

ovarian cancer, TME (tumor microenvironment), drug delivery system (DDS), immunotherapy, chemotherapy

Introduction

Ovarian cancer is the leading cause of gynecological cancer-associated death (1). Epithelial ovarian cancer, especially high-grade serous ovarian carcinoma, is the most common histologic subtype. Most patients newly diagnosed with ovarian cancer can benefit from the conventional first-line treatment that mainly consists of debulking surgery and platinum-based chemotherapy. However, due to difficulties in the early detection of this disease, the majority of patients with ovarian cancer are initially diagnosed at the advanced stage (most frequently with extrapelvic peritoneal metastasis), which is known for its high recurrence rate and poor prognosis. Although first recurrences are frequently sensitive to chemotherapy, patients with recurrent disease will eventually face the problem of chemotherapy resistance. Thus, novel adjuvant therapies, such as targeted therapy and immunotherapy, are needed in order to provide new therapeutic opportunities for these patients. The use of certain targeted therapies, such as anti-angiogenic agents and poly(ADP-ribose) polymerase inhibitors (PARPi), have been approved by the US Food and Drug Administration (FDA) for patients with advanced-stage or recurrent ovarian cancer either in combination with chemotherapy or in maintenance monotherapy. In contrast, no immunotherapeutic agents have been approved by the FDA in ovarian cancer.

The immunotherapeutic strategies currently investigated in clinical trials for ovarian cancer include: 1) immune modulators, such as immune checkpoint inhibitors (ICIs) and immune regulatory cytokines; 2) cancer vaccines (e.g., dendritic cell vaccination); and 3) chimeric antigen receptor-modified T (CAR-T) cell therapy, as a representative variant of adoptive cell therapies (ACTs) (2–9). Data from important clinical trials on these therapies were reviewed (Table 1). Despite the rapid development of immunotherapies in basic research, the immunotherapy response rates among ovarian cancer patients remain modest, as shown by these clinical trials. The tumor microenvironment (TME) is considered a vital factor in the antitumor efficacy of immunotherapies (10). The TME refers to an intricate ecosystem of different immune cells, endothelial cells (ECs), stromal cells, and the extracellular matrix (ECM), as well as their networking interactions with tumor cells (11). The TME plays an important role in cancer development, progression, and metastasis (12). A drug delivery system (DDS), defined as a formulation or a device that enables a therapeutic substance to selectively reach its site of action, can enhance the efficacy and reduce the side effects of drugs, which makes it a promising strategy to improve the effects of cancer immunotherapy by targeting the TME (13). In this review, we discussed the strategies to improve the efficacy of immunotherapy in ovarian cancer with DDS, especially for those targeting the TME.

Abbreviations: ICIs, immune checkpoint inhibitors; ACTs, adoptive cell therapies; TME, tumor microenvironment; DDS, delivery systems; PARPi, poly(ADP-ribose) polymerase inhibitor; FDA, Food and Drug Administration; CAR-T, chimeric antigen receptor-modified T; NKs, natural killer cells; DCs, dendritic cells; TGF-β, transforming growth factor beta; TAMs, tumor-associated macrophages; MDSCs, myeloid-derived suppressor cells; APCs, antigen-presenting cells; MHC, major histocompatibility complex; CTLs, cytotoxic lymphocytes; CAFs, cancer-associated fibroblasts; CTLA-4, cytotoxic T lymphocyte antigen-4; PD-1, programmed cell death protein-1; TAAs, tumor-associated antigens; ECM, extracellular matrix; EPR, enhanced permeability and retention; PEG, polyethylene glycol; PLD, pegylated liposomal doxorubicin; TLR, Toll-like receptor; IL, interleukin.

Role of the TME in immunotherapy

In the TME, tumor cells coexist and interact with immune cells [e.g., macrophages, neutrophils, dendritic cells (DCs), natural killer (NK) cells, and lymphocytes] and non-immune cells (e.g., fibroblasts and ECs) (14). The TME is shaped by tumor cells to promote tumor development and to respond to stress, stimulation, and treatment. The total TME cannot be simply explained as a unitary "antitumor" or "pro-tumor" environment, but rather a dynamic and plastic system with characteristics such as hypoxia, nutrient deficiency, inflammation, immunosuppression, and angiogenesis. The patterns of the TME in solid tumors are tightly associated with the clinical outcomes of cancer patients (15, 16).

Immune cells

Most solid tumors are infiltrated by myeloid and lymphoid lineage-derived immune cells within the TME playing significant roles in the antitumor response or tumor progression.

Tumor-associated macrophages (TAMs) are a major subpopulation of the myeloid lineage-derived cells in the ovarian TME playing critical roles in the crosstalk between the TME and tumor cells. TAMs are highly plastic, with two functional phenotypes. Depending on the TME, TAMs can differentiate into either the pro-inflammatory M1 macrophages with antitumor activity or the anti-inflammatory M2 macrophages with pro-tumor activity. M1 macrophages possess cytotoxicity and stimulate immunity. In ovarian cancer, TAMs are predominantly M2 macrophages, secreting immunosuppressive cytokines and taking part in regulating T cells, remodeling the ECM, and angiogenesis (17).

Neutrophils are of the myeloid lineage cells and comprise the major subpopulation among polymorphonuclear leukocytes, representing the first line of innate immunity against pathogens. The detection of neutrophils within the TME is an indirect parameter of cancer-related inflammation. Tumor-associated neutrophils can exert antitumor (N1 phenotype) or pro-tumor (N2 phenotype) functions, depending on the related stimulating factors and cytokines within the TME.

TABLE 1 Clinical trials of immunotherapy in ovarian cancer.

Immunotherapy	ID	Phase	*N*	Drugs	Conclusion	Reference
ICI	NCT02580058 JAVELIN 200	III	361	1) Avelumab; 2) Avelumab + PLD; 3) PLD	No benefit	(2)
	NCT03038100 IMagyn050	III	1,300	1) Atezolizumab + PC + bevacizumab; 2) Placebo + PC + bevacizumab	No benefit	(3)
	NCT02718417 JAVELIN 100	III	988	1) PC; 2) PC + avelumab, avelumab maintenance; 3) PC, avelumab maintenance	Terminated	–
	NCT02608684 PemCiGem	II	24	Pembrolizumab + standard treatment	No benefit	(4)
	NCT02811497	II	28	Durvalumab + DNA hypomethylating agent	No benefit	(5)
	NCT02865811	II	26	Pembrolizumab + PLD	Clinical benefit	(7)
	NCT02431559	II	40	Durvalumab + PLD	Clinical benefit	(6)
	NCT03899610	II	23	Durvalumab + tremelimumab + chemotherapy	Clinical benefit	(8)
ICI+PARPi+VEGFi	NCT03740165 KEYLYNK-001	III	1,086	1) Pembrolizumab + olaparib; 2) Pembrolizumab + placebo; 3) Placebo + PC + bevacizumab	Recruiting	–
ICI+PARPi+VEGFi	NCT03737643 DUO-O	III	1,056	1) Durvalumab + olaparib; 2) Durvalumab + placebo; 3) Placebo + PC + bevacizumab	Recruiting	–
CAR-T	NCT02498912	I	18	MUC16-CAR-T cells	Recruiting	–
	NCT02159716	I	19	MSLN CAR-T cells	Patients showed stable disease	–
	NCT03585764	I	18	FRαCAR-T cells	Recruiting	–
	NCT05225363	I	33	Tumor-associated glycoprotein 72 (TAG72) antigen CAR-T cells	Recruiting	–
	NCT03907527	I	71	PRGN-3005 UltraCAR-T cells (co-express a CAR-targeting MUC16 and IL-15)	Recruiting	–
Vaccine	NCT02764333	II	27	FRα vaccine (TPIV 200) + durvalumab	Clinical benefit	–
	NCT02346747	II	91	Gemogenovatucel-T vaccine (Vigil) + chemotherapy	Clinical benefit	(9)
	NCT00001827	II	21	P53 vaccine + IL2	Terminated	–

ICI, immune checkpoint inhibitor; PARPi, poly(ADP-ribose) polymerase inhibitors; VEGFi, vascular endothelial growth factor inhibitors; CAR-T, chimeric antigen receptor-modified T; PLD, pegylated liposomal doxorubicin; MSLN, mesothelin; PC, paclitaxel+carboplatin.

Myeloid-derived suppressor cells (MDSCs) are a heterogeneous population of immature myeloid cells that differ in morphology and function from terminally differentiated mature myeloid cells (e.g., macrophages, neutrophils, and DCs). When activated and accumulating in peripheral lymphoid tissues and the tumors, they are implicated in suppressing immunity and promoting tumor progression. The different function and differentiation of MDSCs are related to the different phenotype of the TME (18).

DCs, well known as the most powerful or professional antigen-presenting cells (APCs), are crucial in immune responses and represent the "bridge" between the innate and adaptive immune systems (19). There are both myeloid and lymphoid DCs. After capturing antigens, DCs process them and present peptides to T cells *via* the major histocompatibility complex (MHC), subsequently initiating a series of T-cell activity. Analogous to TAMs, tumor-infiltrating DCs are of plasticity. They can be immunogenic or tolerogenic depending on the TME. $DEC205^{+}CD11c^{+}MHC\text{-}II^{low}$ immature DCs act on tumor vascularization and immunosuppression. The performance of DCs varies at different stages of tumor development (20). As shown in mouse models of ovarian cancer, tumor growth was prevented by infiltrating DCs at the early stage. However, at the advanced stage, immunosuppressive phenotypes of DCs were found in the TME (21).

NK cells are innate lymphoid cells and effector cells of the innate immune system. These cells do not rely on human leukocyte antigen (HLA)-mediated recognition of neoantigens. The expressed receptors (such as CD16, NKG2D, and natural cytotoxicity receptor) on NK cells mediate the killing of tumor cells (22). NK cells also exert effects on the adaptive immune response to cancer through secreting inflammatory cytokines. Defects in NK cell function, such as aberrant receptor expression or inability to effectively secrete cytotoxic molecules, are possible mechanisms of tumor immune escape (23).

Lymphocytes are important components of the TME. B lymphocytes can mediate innate immunity, secrete antibodies, and act as professional APCs. Within the TME, both the pro- and antitumor activities of B lymphocytes have been identified in solid tumors as different subsets playing diverse roles. T lymphocytes are pivotal in adaptive immunity. $CD4^{+}$ and

$CD8^+$ T cells are mature T cells in the TME (24). After antigen presentation, T cells are activated and start to differentiate into various effector subsets. $CD4^+$ T cells perform a wide variety of functions and are best known as T helper (Th) cells, including Th1, Th2, and Th17, and regulatory T cells (Tregs). Tregs inhibit the activation of immune response and are crucial in the mechanism of tumor immune escape. $CD8^+$ T cells, known as cytotoxic T lymphocytes (CTLs), work by specifically recognizing and killing tumor cells (25). Besides CTLs, gamma-delta (γδ) T lymphocytes can kill ovarian cancer cells when activated by positive signals. There are several activating receptors (e.g., NKG2D) and inhibitory receptors that regulate γδ T-cell killing. The presence of tumor-infiltrating lymphocytes (TILs) has been reported as a positive prognostic factor in a number of solid cancers, including ovarian cancer (26–29).

Non-immune cells

Cancer-associated fibroblasts (CAFs) are an important type of stromal cells in the TME and produce various components in the ECM. Normal fibroblasts can prevent the emergence of neoplastic lesions and inhibit tumorigenesis. CAFs, on the contrary, play a role in immune suppression and angiogenesis, showing pro-tumor function (30). Malfunctioning blood vessels and excessive ECM within the TME impair blood flow and limit the delivery of oxygen, nutrients, and antibodies and immune cells. This results in hypoxia and low pH and induces the production of molecules with immunosuppressive activities, such as vascular endothelial growth factor (VEGF). Angiogenesis, which refers to the formation of new blood capillaries from preexisting vasculature, generating the tumor-associated neovasculature, addresses the need to transport nutrients and oxygen, as well as metabolic wastes and carbon dioxide, in the TME (31). This creates a vicious cycle in which angiogenesis can induce immunosuppression in the TME, while certain suppressive immune cells can induce angiogenesis (32). ECs are the cells lining the vessels within the TME, which play an important role in angiogenesis.

Immunosuppressive modulators

Transforming growth factor-beta (TGF-β) is one of the most important immunosuppressive cytokines. TGF-β proteins are produced by many cell types, including all white blood cell lineages, in a latent form. Activated TGF-β complexes with other factors and binds to TGF-β receptors, physiologically maintaining immunological self-tolerance and suppressing cancer. However, within the TME, aberrant TGF-β activation and signaling promote tumor progression by stimulating epithelial–mesenchymal transition, angiogenesis, CAF activation, and immunosuppression (33). TGF-β also regulates the generation and functions of many immune cell types, including promoting the expansion of Tregs and inducing the polarization of the pro-tumor N2 phenotype of neutrophils (34).

Immune checkpoint molecules are inhibitory receptors that are expressed on immune cells, negatively regulating immune response in the TME. Cytotoxic T lymphocyte antigen-4 (CTLA-4) and programmed cell death protein-1 (PD-1) are the two checkpoint inhibitors garnering the most attention. CTLA-4 is a negative regulator of T cells that counteracts with the co-stimulatory molecule CD28. PD-1 is expressed by T cells and binds to one of the two ligands [programmed death-ligand 1 (PD-L1) and PD-L2] that are expressed on tumor and immune cells (16, 35). The PD-1/PD-L1 pathway is an important axis for restricting tumor immunity.

Phenotype of cancer-immune TME

The cancer–immunity cycle mainly consists of the following processes: 1) release and presentation of tumor-associated antigens (TAAs); 2) priming and activation of T cells; 3) trafficking of T cells to tumors; 4) infiltration of T cells into tumors; and 5) recognition and killing of tumor cells by T cells (36). The cancer-immune TME in solid tumors has been categorized as "hot" (high immunogenicity) or "cold" (low immunogenicity), which mainly depends on the status of immune cell infiltration within the tumor space. This difference in the cancer-immune phenotype of the TME suggests that hot tumors exhibit stronger responses to immunotherapy than do "cold" tumors (37). The cancer-immune TME can be categorized into three main phenotypes (Figure 1) (13): 1) immune-desert type, which shows low immunoactivity due to immunological ignorance (lack of neoantigens), the induction of tolerance, or a lack of appropriate T-cell priming or activation. Tumors of this phenotype are the least responsive to ICIs; 2) immune-excluded type, which is characterized by immune cell trafficking in the tumor periphery due to a limited chemokine state or the barriers of vessels, stroma, and ECM. Tumors of this phenotype are potentially more sensitive to ICIs than those of the immune-desert phenotype; 3) inflamed type, which refers to a dysfunctional antitumor immune response with the infiltration of a number of immune cells (including Tregs, MDSCs, suppressive B cells, and CAFs). $CD8^+$ CTLs are dysfunctional and exhausted. Tumors of this phenotype have the most sensitivity to ICIs.

In most cases, ovarian cancer is considered as a cold tumor and has an immune-desert TME with a low immune cell density either inside or outside of the tumor (38), which is not likely to trigger a strong immune response or respond to immunotherapy. Thus, in order to improve the effects of immunotherapy in ovarian cancer, new strategies are needed to "normalize" the antitumor immunity within the ovarian TME, for example, strategies that target the tumor vasculature, the extravascular barriers, the immunosuppressive status, and the cancer–immunity cycle (13).

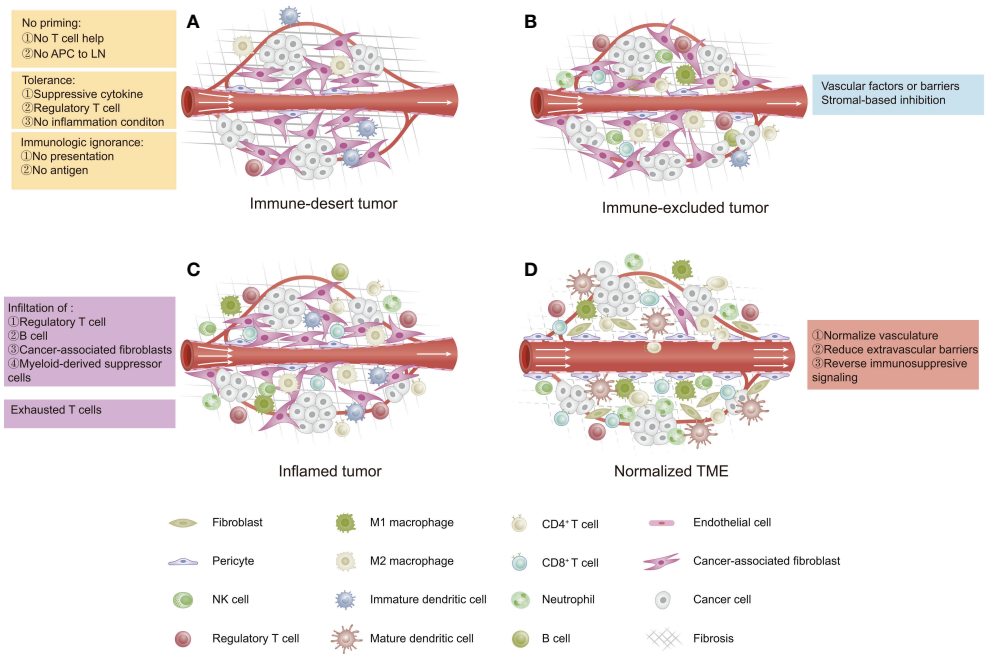

FIGURE 1
Three phenotypes of cancer immunity in the tumor microenvironment (TME). **(A)** Immune-desert type: characterized by a lack of antitumor immune cells due to low immunogenicity. **(B)** Immune-excluded type: characterized by immune cells restricted at the tumor periphery due to tumor vascular barriers and stromal-based inhibition. **(C)** Inflamed type: characterized by immune cells infiltrating the tumor parenchyma and expressing pro-inflammatory cytokines, but a failed antitumor immune response. **(D)** Normalized TME by reversing immunosuppressive signaling, improving tumor perfusion, and reducing barriers.

Role of the DDS in immunotherapy

A DDS is a carrier of a therapeutic substance designed to control its release, improve its solubility and stability, overcome the biological barriers, and target the site. The processes of a DDS include the administration of the therapeutic substance, the release of the active ingredients, and the subsequent transport of the active ingredients to the site of action (39–41). Various materials (organic or inorganic) such as lipids, glycans, and proteins, as well as synthetic polymers, have been utilized for the development and improvement of the DDS (42). According to the particle size, the DDS can be further categorized into nano-, micro-, or macroscale (43). Here, we focused on the DDS at the nanoscale (nanocarrier), which is designed and developed based on the application of nanotechnology (44). Nanocarriers, acknowledged to have enhanced permeability and retention (EPR) effect, help deliver chemotherapeutic or immunotherapeutic drugs selectively to tumors, which results in increased efficacy and reduced systemic toxicity of drugs (45, 46). A wide variety of platforms have been investigated as nanocarriers in preclinical and clinical research, including lipid-based (liposomes), polymer-based (polymeric micelles, dendrimers, and polymeric nanoparticles), drug-conjugated (antibody–drug conjugates), and viral and inorganic nanoparticles (47, 48).

As described above, the clinical use of immunotherapy in many solid cancers is confronted with difficulties related to efficacy and challenges related to safety. With regard to safety, serious adverse effects such as autoimmunity and nonspecific inflammation limit the broad implementation of immunotherapy. For example, systemically administered pro-inflammatory cytokines can lead to autoimmune toxicities and even result in a “cytokine storm.” Thus, a DDS can be utilized to provide safer and more effective cancer immunotherapies (49).

DDS for immune modulators

When it comes to the immune modulatory agents, the DDS can improve the pharmacokinetics and biodistributions of the cytokines and ICIs. Conjugating polyethylene glycol (PEG) has been clinically tried to improve the half-life and stability of pro-inflammatory cytokines (50). In order to reduce the toxicity associated with the systemic administration of drugs, binding cytokines to liposomes or collagen-binding domains can enable the selective delivery of drugs to tumors and draining lymph nodes (51, 52). Matrix-binding molecular conjugates were designed to bind the ICIs to the tumor (53). With this intratumoral and peritumoral delivery, these conjugates remain more localized in the TME than the unmodified ICIs.

DDS for cancer vaccines

With regard to cancer vaccines, a DDS can protect tumor antigens from degradation and enable intracellular delivery (49, 54). For example, lipid-based formulations were designed to improve the instability and inefficient delivery of messenger RNA (mRNA), which were shown to be efficacious in preclinical animal models and in initial clinical studies (55, 56). Furthermore, drug conjugates are utilized to improve the effect of subunit vaccines (such as peptides) in combination with molecular adjuvants by targeting DCs in the lymph nodes. The accumulation of these conjugates in the lymph nodes resulted in increased T-cell priming, improved antitumor efficacy, and reduced systemic toxicity in animal models (57). Other platforms such nanoparticles and dendrimers are also being investigated as carriers in cancer vaccines (58, 59).

DDS for ACTs

A major challenge for ACTs in solid cancers is the localization of T cells at disease sites. Biomaterial-based DDSs, such as polymeric scaffolds, have been investigated to solve this issue (60). Polymeric scaffolds coated with collagen-mimetic peptides bind antigen-specific T cells and deliver them locally within the TME (61). Another challenge for ACTs is that the viability and function of the transplanted cells rapidly decline after administration. High dosages of adjuvant drugs are required to maximize the efficacy of ACTs. T-cell-conjugated nanoparticles, in which an immune-stimulating DDS is conjugated directly to the surface of T cells, were designed to improve the efficacy (62, 63). DDSs activating T cells *in vivo* were also designed, which offered another alternative to conventional ACTs (62). As an example, synthetic/artificial APCs composed of lipids or polymers and functionalized with antigens and surface ligands were designed to mimic APCs in order to activate T cells (64, 65).

DDS for combination therapy

Cancer combination therapy is a promising approach to improving antitumor efficiency and has been investigated in preclinical and clinical studies (66). DDSs can also be exploited in cancer combination treatments and in modulating the immunogenicity in the TME, especially for immunotherapeutic strategies for cold tumors. Tumor cells undergoing selective chemotherapy and radiation can release signals that enhance immunogenicity and induce the activation of T cells locally or systematically, which has been reported to induce immunogenic cell death (ICD) (67). Apart from ICD, chemotherapy is also helpful in normalizing the TME by increasing perfusion and alleviating hypoxia (68). Thus, the combination of chemotherapy and immunotherapy can provide a synergistic effect in antitumor treatment. In this combination therapy, DDS helps achieve the delivery of sustained drug concentrations to enhance the therapeutic effects and reduce the side effects (69). As an example of this combination effect, liposomal DDSs were complexed with PD-L1-blocking signals to form nanoparticles that are targeted to tumor tissue (70). Mice bearing colorectal tumors were injected with both these nanoparticles and the chemotherapy drug (oxaliplatin). The results suggested that oxaliplatin may induce cold tumors to turn into hot tumors, subsequently making them susceptible to immunotherapy, exhibiting reduced toxicity. As another example, twin-like core–shell nanoparticles were developed for synchronous biodistribution and a separate cell targeting delivery of sorafenib (an antiangiogenic agent) and IMD-0354 (a TAM re-polarization agent) to cancer cells and TAMs, respectively, to promote superior synergistic antitumor efficacy and M2 macrophage polarization ability (71). Liposome- and micelle-based chemoimmunotherapies were also designed and studied in animal models (72–74).

DDS targeting the TME in ovarian cancer

A lot of effort has been made to develop new strategies for improving the antitumor efficacy of immunotherapy for ovarian cancer. As described above, the TME in ovarian cancer shows low immunogenicity, which is an obstacle to immunotherapy. The application of a DDS targeting the TME in ovarian cancer has been explored in preclinical and early clinical studies (Table 2, Figure 2).

DDS targeting immune cells

Generally, an increased immune cell infiltration is associated with better prognosis in ovarian cancer. TAMs, as major components within the ovarian TME and playing critical roles in various stages of tumor progression, represent a promising target for cancer drug delivery (88, 89). Signal regulatory protein α (SIRPα) is the surface ligand of CD47 on TAMs. CD47/SIRPα signaling plays an important role in tumor immune escape (75). In a previous study, a virus was used to carry therapeutic genes that blocked the CD47/SIRPα signaling pathway in ovarian cancer. This effectively increased macrophage infiltration into the tumor and enhanced tumor cell killing. Similar to CD47, the CD24 in tumor cells binds the inhibitory receptor on the surface of TAMs to promote the immune escape of ovarian cancer cells. Ovarian cancer

TABLE 2 Drug delivery systems (DDSs) currently developed to target the tumor microenvironment (TME) in ovarian cancer.

Target in the TME	Delivery technology	Effective agents	Combined therapy	Study design	Reference
TAMs (CD47/SIRPα signaling pathway)	Virus	Therapeutic genes	None	Preclinical study	(75, 76)
TAMs (Toll-like receptor)	Liposomes	Resiquimod	PD-1 blockade	Preclinical study	(77)
TAMs (repolarization)	Nanoparticles	MicroRNA-125b	Intraperitoneal paclitaxel	Preclinical study	(78)
TAMs (repolarization)	Nanoparticles	IRF5 mRNA	None	Preclinical study	(79)
M1 macrophages	Nanotubes	Doxorubicin	None	Preclinical study	(80)
DCs	Nanoparticles	Small interfering RNA	None	Preclinical study	(81, 82)
γδ T cells	Liposomes	Aminobisphosphonates	ACTs	Preclinical study	(83)
CAFs and MDSCs	Nanoparticles	Therapeutic genes	None	Preclinical study	(84, 85)
Low immunogenicity	Virus	Peptides	PD-1 blockade	Preclinical study	(86)
Low immunogenicity	Nanoparticles	IL-6	PD-1 blockade	Preclinical study	(87)
Low immunogenicity	Liposomes	Doxorubicin	PD-1 blockade	Early-phase clinical study	(6, 7)

TAMs, tumor-associated macrophages; DCs, dendritic cells; CAFs, cancer-associated fibroblasts; MDSCs, myeloid-derived suppressor cells; ACTs, adoptive cell therapies.

with a decreased CD24 expression was found to be more sensitive to CD47 blockers, indicating co-targeting CD24 and CD47 as a candidate for cancer immunotherapy (76).

In particular, in line with the distinct functions of the two different phenotypes, a high number of classically activated macrophages (M1 macrophages) in the ovarian TME is closely correlated with better prognosis, while increased M2 macrophage infiltration is correlated with poor prognosis (90, 91). Clodronate-loaded liposomes are effective tools for macrophage ablation. Long-term usage of thymoquinone was reported to increase the infiltration of M2 macrophages in the ascites in models of ovarian cancer. When clodronate liposomes were used in combination with thymoquinone, the number of TAMs was significantly reduced while the proportion of M2 macrophages was increased, resulting in the promotion of tumor growth. Toll-like receptor (TLR) 7/TLR8 agonists are potent immunostimulatory molecules that repolarize TAMs. However, these small molecules have poor pharmacokinetic profiles and carry the risk of inducing severe systemic toxicity, which limits their administration *via* intratumoral injection. Anionic liposomes were used to deliver TLR agonists (e.g., resiquimod) administered intraperitoneally in ovarian cancer-bearing mice (77). The results showed the promotion of M1 macrophage polarization and T-cell infiltration in the TME. In addition, the percentage of Tregs was reduced in the TME. These liposome-formulated TLR agonists could also enhance the efficacy of PD-1 blockade. Furthermore, other DDSs were also designed to be administered intraperitoneally. Certain relatively large anionic nanoparticles (>100 nm) have been shown to be able to selectively accumulate in TAMs in a mouse model of metastatic ovarian cancer, while other particles that were smaller than 100 nm, or cationic, or administered intravenously did not show TAM targeting (92). This ability of these nanoparticles opens the possibility of targeting the TAMs in ovarian cancer. Another hyaluronic acid-based nanocarrier encapsulating MiR-125b, a microRNA affecting the phenotype polarization of TAMs, was designed. These nanoparticles specifically targeted TAMs in the peritoneal cavity and repolarized them to the immune-activating phenotype in an ovarian cancer mouse model. Furthermore, these nanoparticles, when combined with intraperitoneal paclitaxel, enhanced the antitumor efficacy of paclitaxel without inducing systemic toxicity (78). Another study using a mouse model of ovarian cancer explored a nanocarrier that could deliver *in vitro*-transcribed mRNA encoding M1-polarizing transcription factors to reprogram TAMs. The infusion of *IRF5* mRNA and IκB kinase beta (IKKβ) nanoparticles reversed the immunosuppressive state of the TAMs by reprograming M2 macrophages into M1 macrophages (79).

Macrophages can also act as carriers themselves. In a mouse model of intraperitoneally metastatic ovarian cancer, engineered doxorubicin-loaded M1 macrophages were designed to transfer drug cargoes into tumor cells *via* a tunneling nanotube pathway. These engineered macrophages were found to penetrate into and accumulate deep within disseminated tumor lesions, resulting in the elimination of metastatic tumors and increase in survival (80).

Immature DCs and MDSCs have been identified as responsible for suppressing the antitumor immune response. These cancer-associated immune cells within the ovarian TME emerge as alternative therapeutic targets complementing current immunotherapies (49). DDSs carrying gene materials or small molecules were engineered to eliminate these cancer-associated immune cells and to transform them into an immunostimulatory phenotype. For instance, linear polyethylenimine-based nanoparticles encapsulating small interfering RNA (siRNA) were described and could be selectively engulfed by tumor-resident DCs when injected into the peritoneal cavity of ovarian cancer-bearing

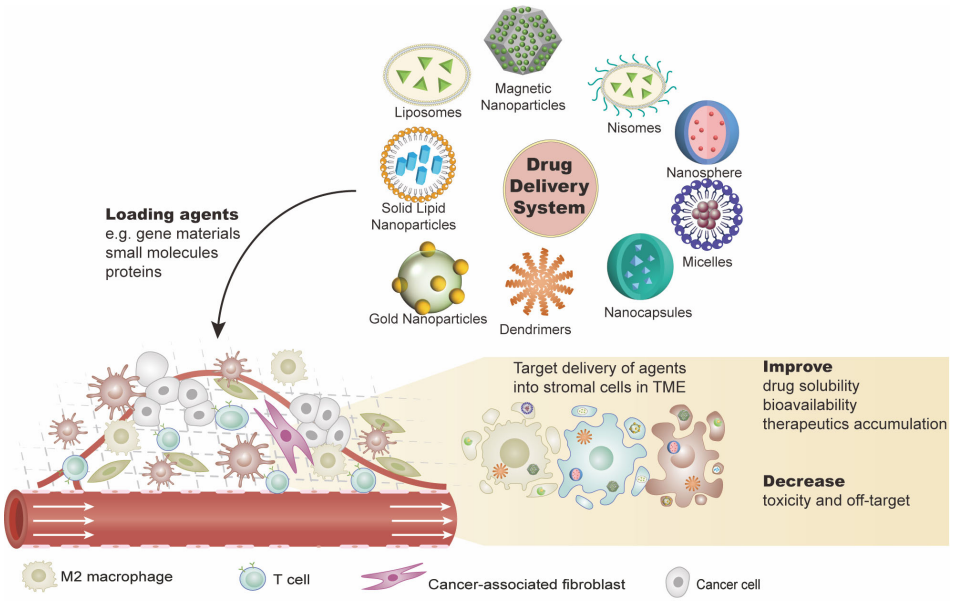

FIGURE 2
Drug delivery systems (DDSs) targeting the ovarian tumor microenvironment (TME). DDSs carry effective agents such as gene materials (e.g., cDNA, mRNA, or miRNA), proteins, or other small molecules (e.g., peptides) into the tumor sites. These agents are expected to work on: 1) eliminating the immunosuppressive cells or transforming them into immunostimulatory phenotypes and 2) inhibiting the immunosuppressive or pro-tumor production of the stromal cell. This combination of agents with DDS not only improves the solubility and stability of the agents but also fulfills the target delivery with reduced toxicities. There are various DDS platforms, such as liposomes and nanoparticles.

mice (81). These nanoparticles induced the activation of DCs. Another basic research indicated that ovarian cancer-associated DCs are also capable of engulfing liposomes carrying plasmid DNA-encoding cytokines in order to support the activities of CTLs (82).

The DDS was also exploited in targeting and modulating lymphocytes. The efficacy of ACT using γδ T cells could be enhanced by aminobisphosphonates such as alendronic acid, the clinical exploitation of which was limited by the inefficient and nonselective uptake of these agents in tumor cells. Aminobisphosphonates were encapsulated within liposomes and investigated in a preclinical study. The results showed that the liposomal alendronic acid rendered advanced tumors susceptible to γδ T-cell-mediated shrinkage and was proven markedly superior when compared with free drug delivered intravenously (83).

DDS targeting non-immune cells

The biological mechanism of CAFs suggests that CAFs represent a therapeutic target in cancer immunotherapy. The current interventions on CAFs mainly include: 1) inhibiting the pro-tumor signaling pathway between CAFs and other stromal cells to reverse tumorigenesis, angiogenesis, and immunosuppression in the TME and 2) inhibiting the production of ECM by CAFs to reduce solid pressure in the TME. For example, fibroblast activation protein (FAP) is a specific marker for CAFs. In a preclinical study of ovarian cancer, upon delivering FAP siRNA to CAFs, the growth of tumor cells was inhibited, with a decreased level of CAFs (84, 85). Many other therapeutic agents such as mRNA and small molecules are good mediators for CAF modulation. Other DDSs, such as a lipid-coated calcium phosphate and lipid–protamine–DNA nanoparticles, were developed as delivery platforms targeting CAFs and have been studied in animal models of pancreatic and bladder cancer (93).

DDS targeting immune modulators

There are various pieces of preclinical evidence that the DDS could exhibit prolonged tumor residence and favorable intratumoral distribution of immune modulators. As one example, cowpea mosaic virus (CPMV) combined with an anti-PD-1 peptide (SNTSESF) was examined as an alternative to the expensive antibody therapies using ICIs. This combination resulted in the increased efficacy of anti-PD-1

peptides in a mouse model of intraperitoneal ovarian cancer. Moreover, an increased potency against metastatic ovarian cancer was only observed when SNTSESF was conjugated to CPMV, but not when given as a free peptide (86). As another example, the hyperactivation of interleukin 6 (IL-6) is a hallmark in the TME of ovarian cancer progression. The effect of IL-6 is achieved *via* activating several signaling pathways such as the RAS–RAF–MAPK and AKT–PI3K–mTORC1 pathways. Dual inhibitor-loaded nanotherapeutics (DiLNs) that can co-deliver PI3K and MAPK inhibitors were also developed. In *in vitro* studies, DiLNs were shown to be stable for over a month and released the drugs in a sustained manner. *In vivo* studies showed that the combination of DiLNs with an anti PD-L1 antibody resulted in superior antitumor effect and longer survival (87).

Pegylated liposomal doxorubicin (PLD) is the first FDA-approved cancer nanomedicine and a paradigm of DDS utilized in ovarian cancer. Besides its use in chemotherapy, PLD can also contribute positive immunomodulatory efforts due to the anthracyline-induced translocation of calreticulin to the cell surface, the upregulation of MHC-I and Fas surface expression, and ICD (94). The efficacy of anti-PD-1 therapy plus PLD has been demonstrated in the early stages of clinical studies. A single-arm, multicenter phase II trial of ovarian cancer indicated that the combination of pembrolizumab (an anti-PD-1 antibody) and PLD was manageable, without unexpected toxicities, and showed preliminary evidence of a clinical benefit. The response rate and survival in this study were both higher than historical comparisons of PLD alone or anti-PD-1 agents alone (7). A similar result was shown in a phase I/II study of durvalumab (an anti-PD-LI antibody) combined with PLD for platinum-resistant recurrent ovarian cancer (6). More clinical trials (e.g., NCT02839707) are ongoing.

Controlled neoantigen release is a major challenge for successful immunotherapy, especially in tumors of the immune-desert phenotype such as ovarian cancer. Many TAAs in solid tumors are not confined to tumor tissues but can also be found in normal somatic tissues, which results in off-target toxicities. Tumor-specific antigens are good candidates for targeting and localizing to the tumor sites in immunotherapy, such as NY-ESO-1 (a cancer–testis antigen). The expression of NY-ESO-1 is restricted in normal somatic tissues, concomitant with a re-expression in solid epithelial cancers (95, 96). NY-ESO-1 vaccines have been designed and investigated in preclinical studies and early phase trials. In ovarian cancer, combination therapies of the NY-ESO-1 vaccine, PLD, and decitabine in 10 patients with recurrent disease showed promising results. Six of the 10 patients had disease stabilization or partial clinical response (97). HLA-A2-restricted peptides presented by tumor cells are candidate antigens for the development of a therapeutic cancer vaccine. A novel liposomal platform called DepoVax^{TM} (DPX; Halifax, NS, Canada) was used to enhance the potency of the HLA-A2-restricted peptide vaccine (DPX-0907). The phase I clinical trial of DPX-0907 exhibited a 61% immune response rate (98). There are many other formulations designed based on the low immunogenicity of TAAs in ovarian cancer, such as a slow-release dendrime of cowpea mosaic virus for *in situ* vaccine delivery (99).

Conclusions and perspectives

The immunosuppressive TME with low immunogenicity is a big obstacle in the implementation of immunotherapy for solid tumors such as ovarian cancer (15, 100, 101). It is believed that a TME-targeted strategy is a valuable adjuvant therapy for ovarian cancer. Given the complexity of the interaction network in the TME, there remains the challenging task of developing drugs or therapies simultaneously targeting multiple pathways. The combined administration of two or more targeted therapeutics, or even the addition of immunotherapeutics and chemotherapeutics, is expected to exhibit a synergistic antitumor effect and improve each other's efficacy. However, toxicity is a major concern.

The application of DDSs in immunotherapy is mainly based on the advantage of selective accumulation in tumor sites relative to normal tissues, which greatly reduces the risk of toxicities. In addition, the peritoneal metastasis and ascites in ovarian cancer make the DDS a potentially valuable approach to carry the load since abundant peritoneal phagocytes can engulf the carriers and accumulate the load inside the tumors, acting as Trojan horses. Various DDS-based strategies have been designed and examined in preclinical studies. Based on the evidence from previous research works, we consider the future of DDSs, especially for nanocarriers, as promising in the immunotherapy for ovarian cancer, not only as a direct delivery platform of immunotherapeutic agents but also as a carrier of genes or functional molecules that can transform the immunosuppressive TME into an immunostimulatory TME. However, not all basic research can result in clinical treatment for patients. In addition to the manufacturing technique and costs, there will be many more concerns when it comes to clinical translation. Furthermore, there is limited information on the long-term biosafety and bioeffect of the component materials themselves in these carriers.

More efforts are needed to further understand the TME in ovarian cancer in order to identify more specific hallmarks and biomarkers that will help in the design and development of more DDSs with better effectivity and biosafety, or even for personalized therapy.

Data availability statement

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

Author contributions

QW and HP performed the literature search and screening. HP drafted the manuscript. QW and XH reviewed and revised the draft. All authors contributed to the article and approved the submitted version.

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Conflict of interest

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

Publisher's note

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Integration of local and systemic immunity in ovarian cancer: Implications for immunotherapy

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Cancer is a disease that induces many local and systemic changes in immunity. The difficult nature of ovarian cancer stems from the lack of characteristic symptoms that contributes to a delayed diagnosis and treatment. Despite the enormous progress in immunotherapy, its efficacy remains limited. The heterogeneity of tumors, lack of diagnostic biomarkers, and complex immune landscape are the main challenges in the treatment of ovarian cancer. Integrative approaches that combine the tumor microenvironment – local immunity – together with periphery – systemic immunity – are urgently needed to improve the understanding of the disease and the efficacy of treatment. In fact, multiparametric analyses are poised to improve our understanding of ovarian tumor immunology. We outline an integrative approach including local and systemic immunity in ovarian cancer. Understanding the nature of both localized and systemic immune responses will be crucial to boosting the efficacy of immunotherapies in ovarian cancer patients.

KEYWORDS

immune cells, ovarian cancer, heterogeneity, tumor microenvironment, immunotherapy, biomarkers, TME, multi-omics

Introduction

Cancer is a heterogeneous disease in which the local and systemic immune responses play an important role in determining tumor growth and clinical outcomes. Over the last decade, immunotherapy revolutionized cancer treatment, yet it exhibits low efficacy in ovarian cancer (OC). OC is the deadliest among gynecological cancers in the world (1).

High mortality is caused by late diagnosis, rapid disease development, relapse, and resistance to therapies (2). Unfortunately, late stages of the disease are associated with local and distant metastases, which render the disease systemic (3) (Figure 1A). Seventy-five percent of patients are diagnosed at advanced stages (stage III or IV); moreover, 75% of these patients die within 5 years (4). Although initial patient responses to cytoreductive debulking surgery and chemotherapy are often sufficient, most patients will develop recurrence of disease within 12–18 months after first-line chemotherapy. In contrast, among patients with early-stage disease (stage I or II), the long-term survival rate (>10 years) is 80%–95% (5).

The main challenges in treating OC include the significant heterogeneity of tumors, lack of diagnostic biomarkers, the complex tumor microenvironment (TME), and the dual role of the immune system. On the one hand, some subtypes of immune cells, e.g., dendritic cells (DCs), cytotoxic T cells, and natural killer (NK) cells, can eradicate tumor cells (immunostimulatory TME). On the other hand, other immune cells, e.g., M2-like macrophages, myeloid-derived suppressor cells (MDSCs), regulatory T cells (Tregs), can have protumor functionality and actively support tumor development (immunosuppressive TME) (6). The paradox of OC immunity is that both TMEs can coexist within the same patients and within the same tumor sites, indicating vast dynamics and variability in immune cell infiltration. Indeed, the coexistence of both immune cell-excluded and immune cell-infiltrated TMEs has been observed in the same tumor sites of the same treatment-naive patients with high-grade serous ovarian cancer (HGSOC) (Figure 1B). This is a major challenge for the successful application of (immuno)therapies that target the TME in OC. Furthermore, it has been shown that chemotherapy promotes local immune activation, indicating that chemotherapy can enhance the immunogenicity of immune-excluded HGSOC tumors (7).

Moreover, the TME is a dynamic niche where cellular components (immune, tumor, and stromal cells) interact with non-cellular components, i.e., secreted molecules (e.g., cytokines, growth factors, metabolites, and others). This complex network of interactions plays a key role in cell survival, invasion, and metastasis and contributes to the escape of the tumor from immune surveillance (8). Indeed, OC predominantly metastasizes along the peritoneum and distant metastatic sites including the lymph nodes, pleura, liver, and

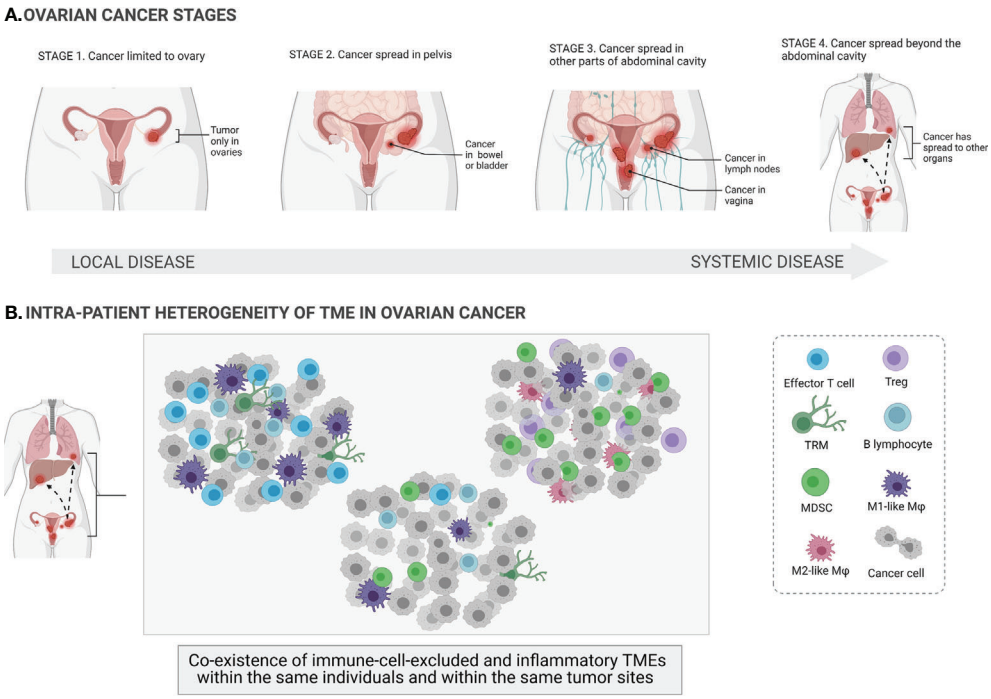

FIGURE 1
Ovarian cancer stages and heterogeneity of the tumor microenvironments. At its earliest stage (stage 1), the tumor (shown in the figure as red masses) is limited to one or both ovaries (local cancer *in situ*) and the ovarian capsule is intact. Once the capsule is disrupted, the tumor spreads beyond the confines of the ovaries (stages 2–4; systemic spread of cancer to other parts of organs) **(A)**. The tumor microenvironment (TME) of ovarian cancer involves a mixture of different immune cells, e.g., effector T cells, regulatory T cells (Tregs), tissue-resident memory T cells (TRM), B cells, macrophages (Mφ), and myeloid-derived suppressor cells (MDSCs). Ovarian cancer is a highly heterogeneous disease, and different TMEs can coexist within the same individuals and within the same tumor sites **(B)**.

lungs. The detailed mechanisms of this metastatic cascade are largely unknown, yet evidence has shown that OC possesses metastatic tropism for the adipose-rich omentum, which has a crucial role in the maintenance of the metastatic TME in the intraperitoneal cavity (8). It is well known that circulating tumor cells (CTCs) can course through the bloodstream as single cells or as cell aggregates, i.e., CTC clusters. Interestingly, these clusters are often observed together with immune cells, which can promote the aggressiveness of the clusters and enhance the capacity to metastasize (9). As metastasis is associated with up to 90% of all cancer deaths (10), more studies on the role of systemic immunity during cancer dissemination in patients will be needed to better understand this process.

Importantly, single-cell RNA sequencing (scRNA-seq) enables deep exploration of immune cell subsets in different types of cancers and the examination of the transcriptional basis of response to therapies. Although tumor-infiltrating T lymphocytes (TILs) are mainly associated with better prognosis and response to immune checkpoint inhibitors (ICIs), scRNA-seq reveals the wide diversity within this cell population, indicating that different TIL states contribute differently to tumor control and response to (immuno) therapies (11). Antigen-specific TILs differentiate into both terminally differentiated T-cell factor 1 $(TCF1)^-$ exhausted effector T (Tex) cells and self-renewing $TCF1^+$ precursor exhausted T (Tpex) cells. It has been shown that Tpex cells are responsible for the long-term maintenance and generation of effector T cells in response to ICIs. Increased Tpex cell level is associated with better patient survival (12). Therefore, targeting Tpex cells can be key for successful immunotherapeutic approaches.

It is well known that effective immune responses involve a coordinated action across different cell types and tissues that create the cancer-immunity cycle. This cycle can be divided into seven major steps, starting with the release of cancer cell antigens (step 1) *via* cancer antigen presentation (step 2), priming and activation (step 3), trafficking of T cells to tumors (step 4), infiltration of T cells into tumors (step 5), recognition of cancer cells by T cells (step 6), and ending with the killing of cancer cells (step 7) (13, 14). However, most studies on tumor immunity focus on local or systemic (peripheral) immune responses, and there is a lack of simultaneous and integrative analysis of different environments, i.e., blood, ascites, and tumor tissue in a large OC patient cohort. There is increasing evidence that both local and systemic (peripheral) immune responses are needed for effective antitumor activity. It has been shown that tumor rejection requires immune cells beyond the TME to facilitate peripheral immune activation (14, 15); even for therapy delivered intratumorally, a systemic immune response was needed for tumor rejection (14). An integrative approach that combines the local tumor niche with systemic immunity is urgently needed to confront the difficulties with treating this disease. Interestingly, using liquid biopsies that analyze cell-free DNA in bodily fluids can serve as useful and noninvasive methods for the selection of targeted immunotherapies and monitoring of cancer progression (16).

In this review, we summarize current knowledge regarding local and systemic immunity in OC. We discuss the clinical relevance of local and systemic immune cells and the soluble mediators involved in disease. Finally, we outline the critical importance of both immune components to more comprehensively understand tumor immunity and to design effective immune-based therapies in OC.

Metastasis and immune heterogeneity in ovarian cancer

More than two-thirds of patients are diagnosed at advanced stages of OC (17), in which the tumor has metastasized beyond the confines of the ovaries. One of the major sites of OC metastasis is the omentum that is composed predominantly of fatty tissue (18). The omentum is a central regulator of peritoneal homeostasis, inflammation, fluid exchange, and angiogenesis and serves as a major source of stem cells and various immune cells (19). Indeed, omental adipose tissue contains a source of immune cell aggregates so-called "milky spots", which contain myeloid cells, B and T lymphocytes, and other immune cells (20). Importantly, omental "milky spots" are the major source of retinoic acid required for the generation of intraperitoneal macrophages that can drive the immunosuppressive TME (8). During OC metastases, the peritoneal TME in which malignant ascites (peritoneal fluid) accumulates represents an immunosuppressive milieu that includes cancer cells, different immune cell types, and numerous tumor-promoting soluble mediators (18, 21, 22). Indeed, not only cellular components of the TME but also soluble signaling factors shape the metastatic niche in the peritoneal cavity, which augments the complexity of the TME. Thus, ascitic fluid provides the opportunity to assess the components of the TME that may serve as valuable clinical biomarkers of the status of disease or to evaluate the potential effect of different therapeutic approaches to assess the antitumor immune response. However, more data are needed to evaluate whether a similar pattern of ascitic fluid components also can be found in the peripheral blood. Using peripheral blood-circulating immune biomarkers can be a valuable approach for designing simple blood tests in clinical practice.

Another major challenge that remains is the high heterogeneity of ovarian tumors that substantially impedes treatment efficacy. Analysis including whole-exome sequencing, RNA-seq, immunohistochemistry, neoepitope prediction, and *in situ* T-cell receptor (TCR) sequencing of metastatic sites of the TME revealed intersite immune heterogeneity. The progressing metastatic sites were characterized by immune cell exclusion, whereas stable and

regressing metastatic sites were infiltrated by both $CD4^+$ and $CD8^+$ T-cell populations and showed oligoclonal expansion of T cells. On the one hand, progressing metastases were characterized by immune suppression and upregulation of Wnt signaling, higher genetic alterations in human leukocyte antigen (HLA) molecules and neoepitope loads. On the other hand, regressing metastases revealed antitumor immune activation with the enrichment of interferon (IFN)-γ, HLA, C-X-C Motif Chemokine Ligand 9 (CXCL9), and TCR signaling (23).

To conclude, single-cell analyses on the protein and RNA level can be a useful tool for analyses of the TME heterogeneity at the single-cell level, leading to a better understanding of cell function. Multiparametric examination of local (i.e., primary tumor tissues, ascites) and systemic (i.e., metastatic tumor sites, peripheral blood) immunity is critical to better understand the biology and immune responses of OC tumors and design potentially effective immunotherapies.

Local (tumor) immunome of ovarian cancer

The tumor niche involves a mixture of different immune cells, e.g., T cells, DCs, NK cells, tumor-associated macrophages (TAMs), MDSCs, and Tregs, which are engaged in both tumor suppression and tumor progression (24). These cells produce various signaling factors such as cytokines, chemokines, growth factors, and other signaling molecules that shape the dynamic communication network and clinical outcome in OC patients (Table 1).

Lymphocytes

Tumor T-cell populations, i.e., $CD8^+$ TILs are associated with better clinical outcomes in OC (25–27, 29, 31–42, 47, 49, 50, 52, 91). First, the presence of $CD8^+$ TILs correlates with improved overall survival (OS) (26, 27, 31–33, 35, 36, 38–40). Second, OC patients with a higher infiltration of $CD8^+$ TILs had prolonged progression-free survival (PFS) (28, 31, 38, 55) and disease-specific survival (DSS) (25, 28, 29, 42, 92) compared to patients with low levels of $CD8^+$ TILs. Third, the infiltration of $CD103^+$ tissue-resident memory T cells (TRM) was associated with better OS (28, 50) and DSS (28, 35, 49, 50).

Next, $CD4^+$ Treg infiltration can be associated with a poor clinical outcome (48, 51). Patients with advanced stage III or IV OC have higher percentages of immunosuppressive $FOXP3^+$ Tregs. Tumor-infiltrating Tregs were associated with reduced survival and high mortality in OC patients (51). However, multidimensional immune profiling revealed that the combination of Cytotoxic T lymphocyte antigen 4 (CTLA-4), Lymphocyte activation gene-3 (LAG-3), and Tregs is associated with improved PFS in HGSOC (93).

Finally, B cells can represent positive or negative prognostic factors. $CD20^+$ B cells from HGSOC positively correlated with DSS (29). Yet, patients with residual disease or another histological subtype demonstrate lack of any significant survival benefit with $CD20^+$ B-cell infiltration (34). In an independent study, it has been shown that tumor-infiltrating $CD20^+$ B cells positively correlate with OS in patients with OC (31). Due to inconsistent results, further studies on B cells in different groups of OC patients will be needed to resolve their clinical relevance in the TME. Nevertheless, recent results from a large HGSOC cohort composed of 534 patients indicated that tumor B cell-derived IgA redirects myeloid cells against extracellular oncogenic drivers, which causes tumor cell death and sensitizes tumor cells to cytolytic killing by T cells (94). In contrast, regulatory B cells (Bregs) promote the conversion of $FoxP3^+$ Tregs from resting $CD4^+$ T cells and support cancer metastasis. Patients with OC showed high frequencies of $IL\text{-}10^+$ B cells in ascites, and their level positively correlated with $Foxp3^+CD4^+$ T cells. These cells also inhibited IFN-γ production by $CD8^+$ T cells, indicating that Bregs can suppress antitumor immune responses (16). Thus, B-cell immune responses in OC may be crucial for (immuno) therapy efficacy.

Dendritic cells

DCs are responsible for antigen presentation, making them intermediaries between the innate and adaptive systems (95, 96). Two main populations of DCs have been reported: the conventional DCs (cDCs) that activate $CD8^+$ T cells *via* cross-presentation and the plasmacytoid DCs (pDCs) that may be engaged in both tumor protection and tumor suppression (97). It has been shown that HGSOC patients possessing mature $LAMP^+$ DCs have better Th1 immune response and favorable OS (55). Similarly, $CD1a^+$ DCs were associated with better survival in OC patients (56). Recently, six DC-related prognostic genes, i.e., *CXCL9*, *UBD*, *CXCL11*, *VSIG4*, *ALOX5AP*, and *TGFBI*, were identified to construct a risk model that could stratify OC patients into two groups with different survival outcomes. In contrast to *VSIG4*, *ALOX5AP*, and *TGFBI*, three genes, i.e., *CXCL9*, *UBD*, and *CXCL11* were associated with better outcomes (98).

Although DCs infiltrate OC, they are usually dysfunctional, have weak antigen presentation activity, and downregulate surface costimulatory molecules (97). Indeed, tolerogenic DCs inhibit antitumor immunity by producing less pro-inflammatory cytokines and more immunosuppressive cytokines. First, intratumoral tolerogenic pDCs secrete less IFN-α, tumor necrosis factor-alpha (TNF-α), Interleukin 6 (IL-6), C-C motif chemokine ligand 5 (CCL5), and macrophage inflammatory protein 1 (MIP1) in OC patients.

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TABLE 1 Tumor tissue immune profiles and their clinical significance in ovarian cancer.

Type of immune cells/ markers	Phenotype	Clinical relevance	Ref
T cells	$CD8^+$ $CD3^+$	↑ $CD8^+$ T cells are associated with prolonged OS, DSS	(25) (26) (27) (28)
		↑ $CD3^+$ and $CD8^+$ T cells are associated with increased DSS	(29)
		↑ $CD3^+$/$CD8^+$ T cells are associated with low stage	(30)
		↑ $CD8^+$ T cells are associated with improved OS	(31) (32) (33) (34) (35) (36)
		↑ $CD8^+$ T cells are correlated with higher histopathological grade and advanced stage	(37)
		↑ $CD8^+$ T cells are associated with better OS, PFS	(38)
	$TIM3^+$ $CD127^+$ $CD4^+ γδT$	↑ $CD8^+$ $TIM3^+$ $CD127^+$ T cells are associated with better OS ↑ $CD4^+$ γδT cells are associated with reduced OS	(39)
		↑ $CD8^+$ is correlated with shorter DFI OS ↑ $CD3^+$ is associated with clinical responsiveness to first-line chemotherapy	(40)
	$CD45^+$ $CD8^+$	High level of $CD8^+$ cells and a high $CD8^+$/$Foxp3^+$ ratio are associated with increased DSS $CD8^+$ $CD45^+$ $Foxp3^+$ cells or a high $CD8^+$/$Foxp3^+$ ratio is associated with an increased DSS in advanced stage High $CD8^+$/Foxp3 ratio is associated with improved OS and PFS The presence of $CD45^+$ and $Foxp3^+$ cells in omental metastases is associated with an increased DSS	(41) (42) (28)
	$CD8^+$/Treg $CD8^+$/$CD4^+$	High $CD8^+$/Treg ratio is associated with better OS High $CD8^+$/$CD4^+$ ratio is associated with better OS	(43)
	$CD45^+$ $PD\text{-}1^+$ $CD8^+$ CD3+	High level of stromal infiltrate of $CD45^+$ and $CD3^+$ cells in the omental lesions is associated with lymph node metastasis High $CD8^+$ infiltrate in the peritoneal lesions compared to the primary tumors is observed in platinum-sensitive tumors High level of stromal PD-1+ cells in the peritoneal lesions is associated with reduced platinum sensitivity	(44)
	TILs	↑ TILs are associated with better OS	(45) (46)
	$CD8^+$ $CD103^+$	High $CD103^+$ TILs are correlated with increased DSS ↑ $CD103^+$ TILs are associated with prolonged OS	(35) (28)
		↑ $CD8^+CD103^+$ TILs are associated with good OS High $CD103^+$ TILs are correlated with increased DSS	(47)
Th17		The number of Th17 cells is decreased in the advanced stages (FIGOIII/IV) vs. FIGO I	(48)
TRM	$CD3^+$ $CD8^+$ $CD103^+$ $PD\text{-}1^+$	$CD103^+$ cells are associated with better DSS $CD103^+$ and $PD\text{-}1^+$ cells are associated with increased DSS	(49) (35)
		$CD3^+$ $CD103^+$ cells are associated with better DSS in patients after primary surgery and adjuvant chemotherapy ↑ $CD103^+$ cells are associated with better OS	(50)
Tregs	$CD4^+$ $CD25^+$ $Foxp3^+$	↑ Tregs are associated with reduced OS	(51)
	$CD4^+$ $CD25^+$ $CD12^-$ $Foxp3^+$	Tregs increase with disease progression ↑ Foxp3 is associated with worse OS	(48) (33) (52)
	$CD4^+$ $Foxp3^+$ Th17	↑ $Treg^+$ Th17 ratio is associated with reduced OS, PFS	(53)
B cells	$CD20^+$	High level associated with better OS and DSS High expression of CD20 correlated with high tumor grade	(54) (31) (29)

(Continued)

TABLE 1 Continued

Type of immune cells/ markers	Phenotype	Clinical relevance	Ref
DCs	LAMP$^+$	↑ LAMP$^+$ cells are associated with longer RFS and OS	(55)
	CD1a$^+$	↑ cells are associated with better survival rate	(56)
pDC	CD4$^+$ CD123$^+$ BDCA2$^+$	↑ pDC is associated with early relapse	(57) (58)
NK	CD16$^+$	↑ associated with worse OS	(59)
	CD56$^+$	↑ associated with better OS	(60)
	CD57$^+$	↑ CD57$^+$ is associated with better OS	(27)
TAMs	CD163$^+$ CD68$^+$	↓ CD163/68$^+$ ratio is associated with better OS and PFS	(61) (46)
	CD163$^+$ COX-2$^+$	↑ COX2$^+$/TAMs ratio is associated with poor OS and RFS	(62)
	CD45RO$^+$	↑ associated with better survival	(63)
	CD206$^+$	↑ CD206$^+$ is associated with poor OS	(64)
	CD163$^+$ CD68$^+$	High M1/M2-like TAMs ratio correlated with improved 5-year prognosis	(65)
	M1 CD14$^+$ CD80$^+$ M2 CD14$^+$ CD163$^+$	High level of M1/M2 ratio is associated with better OS, PFS, and PFI	(66)
MDSCs	Lin- CD45$^+$ CD33$^+$	↑ MDSC is associated with worse OS	(67)
	HLA-DR$^{-/low}$ CD11b$^+$ CD14$^+$ CD15$^-$ M-MDSC	↑ MDSC is associated with worse OS	(68)
	MDSC+VEGF	High VEGF levels correlated with MDSC migration and poor prognosis	(69)
Cytokines and others	IL-8	↑ associated with poor OS and PFS	(70) (71) (72)
	IL-6 IL-10 IFN-γ	↑ IL-6/IL-10 is associated with poor OS ↑ IFN-γ is associated with increased OS	(73) (74)
	IL-22	↑ associated with better OS	(75)
	CCR1	↑ associated with reduced DFS	(76)
	CCR3	↑ CCR3 associated with increased OS	(77)
	CCL18	↑ associated with reduced OS and metastasis	(78)
	CCL28	High CCL28 levels associated with recruitment of Tregs cells and poor disease outcome	(79)
	CXCL2	↑ associated with worse OS	(80)
	CXCL9 CXCL10	↑ associated with better OS	(60)
	CXCL13	↑ associated with longer OS, PFS	(81)
	CXCR5	↑ associated with prolonged survival	(81)
	CXCR6 CXCL16	↑ associated with metastasis	(82)
	CXCR3	↑ associated with a reduced PFS, OS	(83)
	CXCR4	↑ associated with reduced OS and PFS	(84) (85)
	CX3CR1	↑ associated with reduced OS and PFS after chemotherapy	(86)

(Continued)

TABLE 1 Continued

Type of immune cells/ markers	Phenotype	Clinical relevance	Ref
	CD38	High level associated with better OS	(87)
	TGF-β	High level associated with worse OS	(88)
		High TGFβ1 levels associated with $CD8^+$ Treg induction and poor prognosis	(89)
	VEGF	High level associated with poor OS	(72)
	TNF-α	High TNF levels correlated with myeloid cells recruitment and tumor progression	(90)

TIM3, T-cell immunoglobulin and mucin domain 3; IL, interleukin; γδT, gamma/delta T cells; Foxp3, forkhead box P3; TILs, tumor-infiltrating lymphocytes; LAMP, lysosomal associated membrane protein; pDC, plasmacytoid dendritic cells; BDCA2, binding of blood dendritic cell antigen 20; COX2, cyclooxygenase 2; Lin, lineage; HLA-DR, major histocompatibility complex (MHC) II cell surface receptor; IFN-gamma, interferon-gamma; CCR, C-C chemokine receptor; CCL, C-C chemokine ligand; CXCL2, C-X-C motif chemokine ligand; CXCR5, C-X-C motif chemokine receptor; CX3CR1, C-X3-C motif chemokine receptor 1; TGFB1, transforming growth factor beta 1; TNF-α, tumor necrosis factor alpha. ↑ - high; ↓- low.

Second, pDCs induce the secretion of IL-10 from $CD4^+$ T cells, contributing to immune tolerance in these patients. Third, they produce enzymes that negatively regulate effector functions of T cells, i.e., nitric oxide synthase (NOS) and indoleamine 2,3-dioxygenase (IDO). It has been shown that there was a higher level of IDO^+ DCs in tumor-draining LNs compared to the healthy donor LNs (99–101). Moreover, accumulation of pDCs in tumors is associated with early relapse in OC patients (57, 58).

Natural killer cells

NK cells are the first line of defense against the development of cancer and are principal effectors in antibody-dependent cell-mediated cytotoxicity (ADCC), yet the relevance of this population in OC remains controversial. Most reports highlight low infiltration of NK cells within the ovarian tumor, and cells with suppressive activity dominate (102, 103). $CD16^+$ NK cells predicted worse OS (59). In contrast, infiltration of $CD56^+$ NK and $CD57^+$ NK was associated with better OS in OC patients (27, 60).

Myeloid cells

TAMs and MDSCs are the largest groups of myeloid cells in the TME (104).

TAMs can represent two major phenotypical dichotomy, i.e., antitumor M1-like macrophages and protumor M2-like macrophages (5). In OC, TAMs with M2-like phenotype predominantly exist, which drive tumor invasion, angiogenesis, metastasis, and recurrence (74, 105, 106). Indeed, in the malignant ascites of OC, abundant M2-like protumoral TAMs can be found (107). TAM/M2 macrophage frequencies were found to be positively associated with OC stage and ascites volume (107–109). In contrast, M1/M2 ratio was negatively associated with OC stages (65). Both the M1/M2 and M2/TAM ratios have been shown to be positively associated with PFS and OS in OC patients, yet overall, TAM density shows no prognostic relevance (65, 109, 110). M2 density in the ascites is associated with reduced recurrence-free survival (RFS) (74) and PFS (109, 110). It has been shown that $CD163^+Tim4^+$ resident omental macrophages are responsible for the metastatic spread of OC cells, and their genetic or pharmacological depletion inhibits tumor progression and metastatic spread (111). Similarly, using an *in vivo* xenograft OC model, it has been shown that depletion of intraperitoneal macrophages, but not neutrophils or NK cells, reduces the peritoneal metastasis and tumor progression of OC (112).

MDSCs are the key component in immunosuppressive networks (113). Three subsets of these cells exist in humans, i.e., $CD33^+HLA\text{-}DR^{-/low}CD14^+CD15^-$ M-MDSCs that share phenotypic and functional features with monocytes/macrophages, $CD33^+HLA\text{-}DR^{-/low}CD14^-CD15^+$ PMN-MDSCs that are similar to neutrophils, and $CD33^+HLA\text{-}DR^{-/low}CD14^-CD15^-$ early-stage early stage myeloid-derived suppressor cell (eMDSCs) that present more immature cell populations. MDSCs are absent (or present at a very low level) in healthy individuals, whereas they constitutively appear in elevated number in cancers, e.g., in blood, tumor tissue, bone marrow, lymph nodes, and spleen (114, 115). MDSCs were significantly increased in the peripheral blood, ascites, and tumor in OC patients (68, 116, 117). First, tumor-infiltrating $CD33^+$ MDSCs were significantly associated with shorter OS and reduced disease-free interval (DFI) in HGSOC patients (67). Second, IL-6/IL-10 from ascites synergistically expands $CD14^+HLA\text{-}DR^{-/low}$ M-MDSCs in OC patients, and high abundance of ascites/blood-derived MDSCs was associated with a poor prognosis (118). Third, increased MDSCs significantly correlate with decreased intratumoral $CD8^+$ T-cell infiltration and shorter survival (69). Our group demonstrated the existence of all three MDSC subsets in all paired samples from three different environments, i.e., peripheral blood, ascites, and tumor tissue. We observed significantly higher frequencies of M-MDSCs in all three examined environments in OC patients compared to the control group; high levels of both blood-circulating and tumor-infiltrating M-MDSCs were correlated with worse OS in OC patients (68). Thus, it indicates the importance of local and peripheral immune responses.

Soluble factor profile

Soluble mediators released by both immune and cancer cells into the microenvironment can shape the immune response and function as biomarkers (Table 1). The ascites ecosystem can create an immunosuppressive and metastatic environment for OC cells. A key regulator of these processes is transforming growth factor-beta (TGF-β), which promotes survival of OC stem cells, epithelial-to-mesenchymal transition (EMT), and chemoresistance (119). It has been shown that TGF-β is elevated in the ascites of OC patients (120, 121), and blockade of TGF-β signaling limits immune exclusion and improves the chemotherapy response in metastatic OC mouse models (122).

Ovarian tumor–derived soluble factors stimulate neutrophils to create neutrophil extracellular traps (NETs) that promote the OC premetastatic niche. NETs were observed in the omentum of the mouse model of OC and of women with early-stage OC (123).

Using proteomic analysis, 779 proteins in the ascites samples of HGSOC patients have been identified as clinically relevant; CAPG, LCK, and TNFAIP6 have 91.2% correctness in identifying short-term survivors (124). Similarly, multiplex cytokine array analysis of 120 cytokines in the malignant ascites of OC patients showed that high levels of osteoprotegerin (OPG), IL-10, and leptin were associated with shorter PFS (125). However, it is unknown whether the profiles of these soluble markers in the ascites reflect their status in the blood samples.

Multiparametric analysis of local immunome

An increasing number of studies focus on multiparametric analysis of the immune component in cancer patients.

A recent study characterized ascitic fluid using scRNA-seq to profile ~11,000 cells of 11 patients with HGSOC. Results showed significant interpatient variability in the composition of ascites cells, including dichotomous macrophage populations. One population was enriched with major histocompatibility complex (MHC) class II, IFN-γ receptor 1, and M1-associated genes and the other with complement factors, suggesting the existence of both phenotypes in the ascites. Yet, it is unknown whether similar dichotomous macrophage subpopulations exist in the paired tumor and blood samples (126). Moreover, a recent study estimated 22 immune cell subsets from databases with more than 2,000 HGSOC patients who underwent platinum-based chemotherapy. Results showed that a high level of M1 and M0 in tumor tissue was associated with better OS. Neutrophils were associated with poor OS. Among the immunoreactive tumors, the M0 macrophages and the $CD8^+$ T cells were associated with improved OS, whereas the M2 macrophages showed worse OS; programmed death receptor-1 (PD-1) was associated with good OS and PFS in this subtype (127).

Furthermore, three different immune types (A, B, and C) have been identified using the expression of immune-related genes of 307 OC samples. Patients in subtype B had poorer prognosis and lower survival rate. Moreover, the predictive response rate to immunotherapy in type B was significantly higher than that in types A and C; patients in immune type B have a superior response to immunotherapy. Immune subtype B was characterized by low levels of M1 macrophages and Th cells and high levels of Treg-type macrophages and M2 macrophages. IL-6-Janus kinase - signal transducer and activator of transcription 3 (JAK-STAT3) pathway activity was increased in the immune subtype B. In contrast, enrichment of KRAS-downregulation pathway increased in both A and C immune types with superior prognosis (128).

It is well known that different patterns of T-cell accumulation in the tumor niche, i.e., immune infiltrated (a), excluded (b), and desert (c), shape different responses to immunotherapies. scRNA-seq analysis of 15 ovarian tumors showed that predysfunctional $CD8^+$ GZMK T cells are enriched in the excluded tumors, while FCN1 monocytes and immature MARCO macrophages are enriched in desert tumors (129). Yet, it is unknown whether the profiles of immune cells in the tumor niche reflect their status in the ascites or peripheral blood.

Interestingly, recent data of tumor-immune niche single cells, derived from 44 tumors, showed that HGSOC patients with *BRCA1/2* gene mutations had better immune response against tumors and distinct immune cell landscape compared to patients without mutations (130). Thus, different (immuno) therapeutic strategies for these clinical subgroups may be needed.

Using transcriptomic analysis of OC, three immunogenomic subgroups have been proposed, i.e., hyperimmunogenic (a), moderately immunogenic (b), and hypoimmunogenic (c). Activated DCs, M1 macrophages, $CD8^+$ T cells, follicular helper T cells, and $CD4^+$ memory T cells were enriched in the hyperimmunogenic subtype. Intriguingly, this subgroup had the highest expression of *PD-L1* (*programmed death ligand-1*), *PD-1*, and *PD-L2*. Clinically, the hyperimmunogenic subtype had an early International Federation of Gynecology and Obstetrics (FIGO) stage and better survival prognosis and response to immunotherapy compared to those of the moderately immunogenic and hypoimmunogenic subtypes (131).

Finally, three different immunometabolism subtypes of OC were identified, i.e., "immune suppressive-glycan metabolism subtype" with high levels of immunosuppressive cell infiltration and glycan metabolism activation (a), "immune inflamed-amino acid metabolism subtype" with abundant adaptive immune cell infiltration and amino acid metabolism activation (b), and "immune desert-endocrine subtype" with low immune cell infiltration and upregulation of hormone biosynthesis (c). Results showed that "immune inflamed-amino acid

metabolism subtype" was more sensitive to chemotherapy and displayed a significantly better response to immunotherapy compared to "immune suppressive-glycan metabolism subtype" and "immune desert-endocrine subtype" (132). Therefore, immunometabolism subtypes may have a predictive value for (immuno)therapy stratification.

In the future, integration of multiparametric analysis including single-cell analysis on transcriptomic, proteomic, and metabolomic level is needed to understand the heterogeneity of OC and to boost (immuno) therapy efficacy.

Peripheral immunome of ovarian cancer

The tumor niche can influence the systemic immune macroenvironment status, thereby making opportunities for simple and noninvasive blood biomarkers for patient immunostratification and design of immunotherapy. The development of predictive blood-based immune biomarkers for cancer monitoring is of interest; yet, until now, a peripheral immune biomarker that can be used in bedside decision-making in oncology is lacking (15). Nevertheless, human studies demonstrate an association between peripheral immunome and clinical outcome of OC patients (Table 2).

The gold standard markers for monitoring OC patients are Cancer Antigen 125 (CA 125) and Human epididymis protein 4 (HE4). However, their specificity is low. First, CA-125 sensitivity is only 50% in stage I OC (142). Second, a higher level of CA-125 has been reported during menstruation, early pregnancy, endometriosis (143), and peritoneum inflammatory diseases (144). Third, HE4 is better than CA125 in diagnosing patients with OC due to higher specificity, yet HE4 increases with age, smoking, and renal diseases (145).

Recent studies have proposed an analysis of the serum-functional immunodynamic status (sFIS) in OC patients. The concept of this "*in sitro*" (*in vitro* plus *in situ*) assay implies using human myeloid cells that are exposed to patients' serum (*in vitro*) to assess serum-induced (si)-Nuclear Factor Kappa B (NFκB) or IFN/interferon-stimulated gene (ISG) responses (as active signaling reporter activity) within them, thereby mimicking patients' *in situ* immunodynamic status. First, the assay can decode peripheral immunity (by indicating higher enrichment of si-NFκB over si-IFN/ISG responses). Second, it estimates survival trends (si-NFκB or si-IFN/ISG responses associated with negative or positive prognosis, respectively). Third, it coestimates the malignancy risk (relative to benign/borderline ovarian lesions). Data revealed the abundance of protumoral myeloid si-NFκB response^{HIGH}si-IFN/ISG response^{LOW} inflammation in periphery of patients with OC. Interestingly, in the mouse metastatic OC model, the sFIS assay predicted the higher capacity of chemoimmunotherapy (paclitaxel–carboplatin plus anti-TNF antibody combination) in achieving a proimmunogenic peripheral status (si-IFN/ISG response^{HIGH}si-NFκB response^{LOW}), which is aligned with a high antitumor efficacy (146). Thus, the sFIS assay can be beneficial in personalized patient monitoring, immunostratification, and (immuno)therapeutic decision-making in OC.

Moreover, the association of three inflammation-based parameters with the survival of OC patients has been proposed, i.e., lymphocyte/monocyte ratio (LMR) (a), neutrophil/lymphocyte ratio (NLR) (b), and platelet/lymphocyte ratio (PLR) (c). High NLR and PLR and low LMR were independent prediction factors of poor OS and PFS in OC (147).

Recently, it has been proposed that the blood M-MDSCs/DCs ratio is an independent predictive factor for OC survival (116). Furthermore, our study showed a positive correlation of sPD-L1 with PD-L1^{+} M-MDSCs/macrophages in the blood of pretreatment OC patients, yet no prognostic relevance was demonstrated (148). As the efficacy of PD-L1 inhibitors in OC is disappointing, new checkpoint inhibitors or/and precise selection of an appropriate group of patients may be crucial to boost the effectiveness of checkpoint inhibitors.

In many studies, the analysis of blood cytokines in OC patients was either performed individually or combined with just two or three cytokines after individual assessment. As systemic cytokinome networks are complicated in OC patients, an evaluation of the pattern of soluble mediators rather than single individual cytokines can be more informative. A recent study indicated that 12 of 27 serum cytokines correlated with OC histotypes. Two OC histotypes, i.e., HGSOC and clear cell carcinoma (CCC) shared similar cytokinome signatures involved in the "hemotaxis and angiogenesis" and "Th2-type immunity". These results indicate that HGSOC and CCC may share a systemic immunological profile (149).

A better understanding of the network of blood soluble mediators and immune cells might reveal systemic immune characteristics of OC patients.

Immunome in therapy design

A conventional therapeutic strategy in OC is debulking surgery followed by adjuvant platinum and taxane-based chemotherapy that shapes the global immunological landscape (150). It is known that surgery induces an immunosuppressive state to support wound healing and postoperative pain. In OC patients, debulking surgery decreases Tregs in the blood on day 1 postoperatively, with an increase on day 7 postoperatively. Moreover, increased levels of TGF-β also have been observed. In contrast, chemotherapy reduces immunosuppression and promotes immunostimulation in OC patients (151). Understanding these systemic immune consequences is important for designing strategies that augment rather than impede antitumor immune responses, which can include optimal timing, dosing, or combinations.

TABLE 2 Circulatory immune profiles and their clinical significance in ovarian cancer.

Type of immune cells/markers	Phenotype	Clinical relevance	Ref
T cells	$CD45^+$ $CD3^+$	↓ T cells are associated with reduced OS	(133)
Th22	$CD4^+$ $IFN\gamma^-$ $IL17^-$ $IL22^+$	↑ Th22 cells are associated with higher tumor stage	(134)
Th17	$CD4^+$ $IL17^+$ $IFN\gamma^-$	↑ Th17 cells are associated with higher tumor stage	(135)
Tregs	$CD4^+$ $CD25^+$ $Foxp3^+$	High level of Tregs is a significant predictor of OC early relapse	(136)
B cells	$CD45^+$ $CD3^-$ $CD16^-$ $CD56^-$ $CD19^+$	↓ B cells are associated with reduced OS	(133)
DCs	$CD4^+$ $CD123^+$ $BDCA2^+$	High density of pDC correlated with poor disease outcome	(57)
NK	$CD3^-$ $CD16^+$ $CD56^+$	↓ NK is associated with poor OS	(137)
TAMs	$CD14^+$ $CD80^+$ $Glut^+$ $CD14^+$ $CD163^+$	↑ M1/M2 is associated with higher OS, PFS	(66)
MDSCs	$HLA\text{-}DR^-$ $CD14^+$	↑ MDSC is associated with shorter RFS	(118)
	$HLA\text{-}DR^{-/low}$ $CD11b^+$ $CD14^+$ $CD15^-$	↑ M-MDSC is associated with worse OS	(68)
	$CD3^-$ $CD19^-$ $CD56^-$ $HLA\text{-}DR^{-/low}$ $CD14^+$ $CD15^-$	↑ M-MDSC is associated with decreased survival	(116)
Chemokines/cytokines	IL-6 IL-8	↑ IL6 and IL-8 are associated with reduced OS, DFS ↑ IL-8 is associated with poor OS and PFS	(138) (139) (70)
	CXCL1 CXCL2	↑ associated with reduced OS	(140)
	CCR3	↑ associated with increased OS	(77)
	CCL4 CXCL1 CCL20	↑ associated with shorter RFS, OS	(141)
	CCL22	High CCL22 levels correlated with recruitment of Tregs and poor disease outcome	(51)

DCs, dendritic cells; DFI, disease-free interval; DFS, disease-free survival; DSS, disease-specific survival; MDSCs, myeloid-derived suppressor cells; NK, natural killer; OS, overall survival; PD-L1/PD-1, programmed death ligand/receptor-1; PFS, progression-free survival; TAMs, tumor-associated macrophages; Tregs, regulatory T cells; TRM, tissue-resident memory T cells; VEGF, vascular endothelial growth factor; RFS, relapse-free survival. ↑- high; ↓- low.

In recent years, we have witnessed an "immunotherapy tsunami"; however, the results of treatment based on immunotherapy are still unsatisfactory in OC (152). To overcome cancer-related immune dysfunction of cancer, an effective immunotherapy drives peripheral immune response, boosting local and systemic immunity. Multiple strategies have been proposed to modulate the immunome to enhance OC (immuno) therapy efficacy (Figure 2).

Macrophage-targeted strategies

Briefly, macrophage-targeted therapies can be divided into two main strategies, i.e., limiting tumor-promoting M2-like macrophages (a) and activating tumor-suppressing M1-like macrophages (b) (153).

First, several preclinical and clinical trials exploring the restoration of phagocytosis in macrophages using the inhibition of the CD47/signal regulatory protein alpha (SIRPa) pathway have been proposed (154). CD47 acts as a "do not eat me" signal that allows tumor immune evasion (155). CD47 is overexpressed in OC patients and is associated with shorter PFS (156). Thus, CD47/SIRPa signaling pathway can be an attractive target for OC therapy.

Second, it may be of interest to use modified chimeric antigen receptor (CAR)-macrophages (CAR-M) to enhance its phagocytic activity and antigen presentation against tumor cells (157). Two drugs are being tested in clinical trials in OC, i.e., CT-0508, which treats tumor patients with relapsed/refractory human epidermal growth factor receptor 2 (HER) overexpression with anti-HER2 CAR-M (a), and MCY-M11, which uses mRNA-targeted Peripheral blood mononuclear cells (PBMCs) (including CAR-M) to express mesothelin-CAR (b) (158). TAMs are the main population of immune cells in the OC, thus using CAR-M, which can reduce the ratio of TAMs and convert M2-like macrophages to M1-like, can be of great benefit in OC treatment.

Third, the Macrophage-colony stimulating factor (CSF-1)/ Macrophage-colony stimulating factor signaling through its receptor (CSF-1R) axis is the major regulator of macrophage migration and differentiation. Preclinical studies using CSF-1R inhibitor (GW2580) showed reduced tumor volume, ascites, and infiltration of M2-like macrophages in OC mouse models (159). CSF1R inhibition within a triple combination with chemotherapy and antiangiogenic treatment in platinum-resistant OC patients (66, 160).

Finally, supporting M1-like functional activity can be of clinical benefit. IFN-γ, LPS, GM-CSF, and IL-12 polarize macrophages into M1-like cells (95, 161). Interestingly, IL-12 can promote a Th1 response that polarizes macrophages into M1 phenotype. In OC, IL-12 caused reduced tumor growth and even regression. GEN-1 (gene-based IL-12 immunotherapy) has been tested in a few clinical studies (phases I–II) in OC patients (162).

Myeloid-derived suppressor cells-targeted strategies

Our group and others already demonstrated the clinical relevance of MDSCs in OC patients (68, 69, 117, 163). Thus, targeting these cells can be of clinical significance.

A few strategies to target MDSCs have been proposed in cancer patients, e.g., induction of MDSC apoptosis, blocking of MDSC recruitment, inhibition of MDSC immunosuppressive activity, and promotion of the differentiation of MDSCs into mature non-suppressive cells (164).

In mouse studies, the anti-granulocytes (Gr)-1 antibody has been proposed to eliminate MDSCs from the TME. Unfortunately, due to the lack of a Gr-1 homolog in humans, such approach cannot be used in the human clinical setting, and there is an absence of specific inhibitors of human MDSCs. However, it is noteworthy that treatment of OC patients with gemcitabine decreases immunosuppressive MDSCs and increases M1-like macrophages (165).

The efficacy of MDSC-targeting strategies against OC is currently being studied preclinically (164). A better understanding of human MDSC biology is urgently needed to reveal how to selectively target these cells in cancer patients.

Immune checkpoint inhibitors

Blockade of checkpoint inhibitors, i.e., PD-1 and CTLA-4, may rejuvenate the immune system and become increasingly popular in cancer treatment.

Recently, a meta-analysis including 15 clinical trials involving 945 patients was performed to assess the efficacy of anti-PD-1/PD-L1 therapy in OC. The pooled results showed that the overall response rate (ORR) was 19%. Single PD-1/PD-L1 inhibitors showed limited efficacy (ORR was 9%), while combination with chemotherapy showed better efficacy (ORR was 36%). PD-1/PD-L1 inhibitors had a higher ORR in platinum-sensitive OC than in platinum-resistant OC (31% vs. 19%) (166). Similarly, a recent summary of 20 studies where 16 clinical trials targeted PD-1 (nivolumab, pembrolizumab), PD-L1 (avelumab, aterolizumab, durvalumab), and CTLA-4 (ipilimumab, tremelimumab) reported lack of improvement in survival in OC patients, and some trials were terminated early due to toxicity or lack of response (167). In contrast, combining therapy [ICIs with chemotherapy, anti-vascular endothelial growth factor (VEGF) therapy, or Poly(ADP-Ribose) Polymerase (PARP) inhibitors] improved response rates and survival in OC patients, yet it is more toxic (167).

Intrinsic resistance to Immune checkpoint blockade (ICB) remains a challenge. Adoptive transfer of senescence-associated secretory phenotype (SASP)-boosted cells sensitizes OC to anti-PD-1. In the mouse OC model, a reduction of tumor weight and better

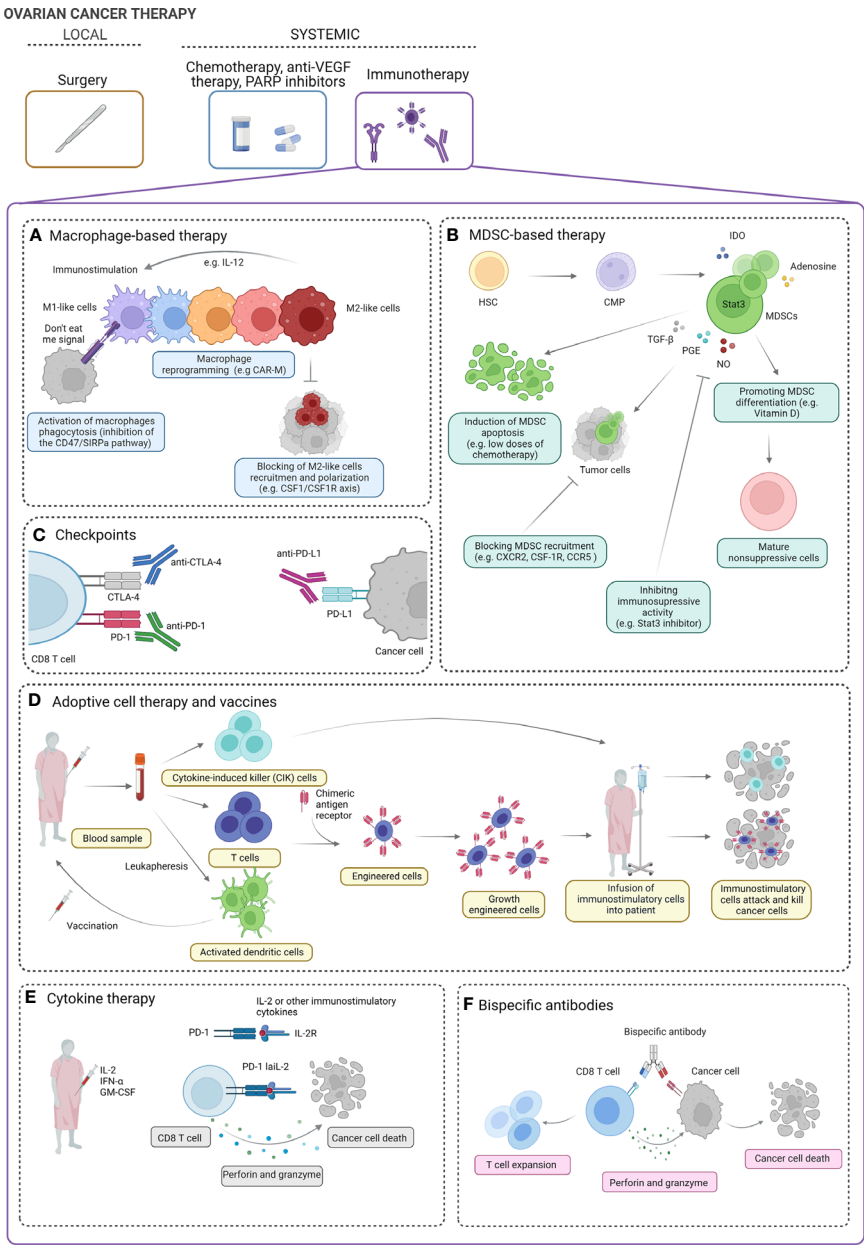

FIGURE 2
Ovarian cancer therapy. The standard treatment for ovarian cancer (OC) includes local intervention (debulking surgery) followed by systemic treatment [chemotherapy, anti-vascular endothelial growth factor (VEGF) therapy, PARP inhibitors]. Several strategies of systemic immunotherapy can be of clinical benefit in OC patients. Macrophage-based strategies can include activation of macrophage phagocytosis, macrophage reprogramming into immunostimulatory M1-like phenotype, and blocking of M2-like cell recruitment **(A)**). Myeloid-derived suppressor cell (MDSC)-based strategies include induction of MDSC apoptosis, blocking MDSC recruitment, inhibiting the immunosuppressive activity of MDSCs, and promoting the differentiation of MDSCs into mature non-suppressive cells **(B)**. Blocking of immunoinhibitory checkpoints (e.g., PD-1, PD-L1, CTLA-4, and others) can boost the immune response and promote ovarian tumor cell killing **(C)**. Cytokine-induced killer (CIK) cells, engineered immune cells (e.g., T cells), and dendritic cell vaccines can be used to boost the antitumor immune response cell activity and enhance cancer cell killing **(D)**. In clinical studies, using IL-2, IFN-α, and GM-CSF has been proposed in OC treatment. In preclinical studies, modified low-affinity IL-2 fusion protein in combination with anti-PD-1 (PD-1-laIL-2) decreases affinity for Tregs and increases avidity to CD8 TILs, which promotes better tumor control and less toxicity than single or combination treatments **(E)**. Bispecific antibodies have affinity for both the tumor-associated antigen and the CD8 effector T cells. In the presence of perforin and granzyme, they effectively target T lymphocytes to elicit antitumor effects **(F)**. IL, Interleukin; M1 and M2, macrophages; CAR-M, chimeric antigen receptor macrophage; CSF-1: macrophage-colony stimulating factor; CSF-1R, macrophage colony stimulating factor signaling through its receptor, SIRPA, signal regulatory protein alpha; HSC, hematopoietic stem cells; CMP, common myeloid progenitor; IDO, indoleamine 2,3-dioxygenase; STAT3, signal transducer and activator of transcription 3; TGF-b, transforming growth factor- beta; PGE, prostaglandin E; NO, nitric oxide; CXCR2, chemokine C-X-C motif receptor 2; CCR5, C-C motif chemokine receptor 5; CTLA-4, cytotoxic T-lymphocyte associated protein 4; PD-1, programmed cell death protein 1; PDL-1, programmed cell death ligand 1; INF-alfa, interferon alpha; GM-CSM, granulocyte macrophage colony-stimulating factor.

immune response, including infiltration of DCs and activated $CD8^+CD69^+$ T cells, have been observed (168). Mechanistically, deep genomic and immune profiling of OC tumors may reveal potential targets that are responsible for the resistance to ICB and lead to the design of more effective clinical trials (167). Clinically, the improved efficacy of anti-PD-1/PD-L1/CTLA-4 therapy would require better patient selection and novel combinations of drugs (169). Interestingly, a high expression of another immune checkpoint, B7-H4, was observed in gynecologic cancers. B7-H4 expression levels inversely correlate with survival in OC patients, making B7-H4 an attractive therapeutic target (170). Finally, non-immune cells, e.g., cancer-associated fibroblasts (CAFs), promote progression and resistance to therapy in OC. Importantly, CAFs shape the immunosuppressive TME milieu and attenuate the efficacy of ICB therapy (171). Therefore, targeting CAFs may be an effective strategy to sensitize OC tumors to ICB therapy.

Adoptive cell therapy and vaccines

In general, adoptive cell therapy (ACT) assumes using autologous or allogeneic antitumor immune cells against cancer.

The effectiveness of cytokine-induced killer (CIK) cell therapy was examined in a group of 646 OC patients after first-line treatment. CIK cells are heterogeneous immunostimulatory host effector cells, including $CD3^+$ $CD56^+$ NKT-like cells (a), CD3-$CD56^+$ NK cells (b), and $CD3^+CD56^-$ antitumor T cells (c). CIK cells proliferate rapidly and can be obtained quickly from cancer patients *via in vitro* culture (a), exhibit strong antitumor activity (b), and possess minimal toxicity (c). The OS rates at 1, 3, and 5 years were respectively 87%, 63%, and 47% for OC patients who received CIK immunotherapy combined with chemotherapy and 65%, 44%, and 31% for control group patients who received chemotherapy alone. Patients with OC who received combined therapy exhibited prolonged OS and better PFS compared to patients with chemotherapy alone (172).

Another approach can be the use of CAR or a tumor antigen-specific TCR. Targets for CAR-T include MUC16 (mucin 16)/Ca 125, mesothelin, and folate receptor-α76–78. Targets for TCR are MAGE-A4 (melanoma-associated antigen 4), WT1 (Wilms' tumor protein 1), and NY-ESO-1 (New York esophageal-1) (173). However, CAR T-cell exhaustion due to persistent antigen stimulation and an immunosuppressive TME is a major limitation to their efficacy in solid tumors (174). Indeed, the immunosuppressive capacity of malignant ascites in OC patients demonstrates its negative effect on adoptively transferred CAR T cells. However, CAR T cells modified to constitutively secrete IL-12 are able to overcome immunosuppression of the TME in a model of ovarian peritoneal carcinomatosis, ultimately improving antitumor activity, and are currently under study in a phase I clinical trial in HGSOC (174, 175).

Moreover, DC vaccination to induce Th17 has been proposed. The development of Th1, Th17, and folate receptor (FR)-α antibodies was observed in most OC patients. Of 18 patients, seven (39%) were recurrence-free with a median follow-up of 49.2 months (176).

Finally, the loss of HLA function is an important escape mechanism for tumors from immunotherapy. Interestingly, large-scale profiling of the immunopeptidome of OC and assessing the HLA-presented antigens can be valuable in designing a new immunotherapy (177). Indeed, HLA ligandomics identified histone deacetylase (HDAC) 1 as an important tumor antigen in HGSOC, indicating HDAC1 as a valuable target for designing new peptide vaccination in OC patients (178).

Cytokine therapy

Cytokines make a bridge between local and peripheral immune responses

IL-2/4/7/12/18, IFN-α/γ, TNF-α, and granulocyte-macrophage colony-stimulating factor (GM-CSF) have been studied in preclinical cancer models, and their antitumor functions have been proposed (179). Although cytokines are easy to administer, their toxicity and lack of specificity may be a limitation for their use in clinical practice.

In a phase II trial, intraperitoneal (IP) IL-2 was administered to OC patients with platinum-resistant or -refractory disease (180). Twenty-five percent of the patients experienced a treatment response with a median survival rate of 2.1 years (181). To avoid lack of selectivity and toxicity, the solution can be delivered as an engineered fusion protein, i.e., a low-affinity IL-2 paired with anti-PD-1 (PD-1–laIL-2). Such conjugate reduced the binding of both IL-2Rα and IL-2Rβ, had lower binding to Tregs, and enhanced avidity to $CD8^+$ TILs, which promoted better tumor control in mice and lower toxicity than single or combination treatments (182). Using IL-2 partial agonists that promote long-lived functional $CD8^+$ T cells can be of interest in designing future clinical trials in OC patients (183, 184).

In a phase II trial, IP IFN-α alternating with cisplatin was administered to 14 OC patients with minimal residual disease as salvage treatment. Fifty percent experienced complete remissions and remained disease-free over a median follow-up of 22 months (185). Moreover, in a phase I/II trial, IP IFN-α together with carboplatin showed a response of 42.8% in OC patients who had previously received intravenous cisplatin-based chemotherapy for recurrent or refractory disease (186).

GM-CSF was evaluated in combination with recombinant IFN-γ 1b (rIFN-γ1b) in a phase II trial of patients with recurrent platinum-sensitive ovarian, fallopian tube, and primary peritoneal cancer. In the group of 59 women, the combination

of GM-CSF and rIFN-γ1b with carboplatin showed a response rate of 56% (187).

Bispecific antibodies

Innovative immunotherapeutic strategy can use bispecific antibodies (BsAb)/fusion proteins that interact with tumor antigens on cancer cells and activate receptors on immune cells. It has been shown that BsAb REGN4018 binding both MUC16 and CD3 inhibits the growth of intraperitoneal tumors in a mouse model of ovarian tumors. The efficacy was shown in both monotherapy and combination of PD-1 and VEGF inhibition (188, 189).

Similarly, BsAb mPEG × HER2 that can easily provide $HER2^+$ tumor tropism to mPEGylated liposomal doxorubicin (PLD) and increase the drug accumulation in cancer cells *via* receptor-mediated endocytosis showed better cytotoxicity and therapeutic efficacy in $HER2^+$ ovarian tumors as compared to non-targeted PLD (190).

So far, BsAb has been approved for the treatment of hematologic malignancies; yet, no BsAb has been approved in OC. However, a few designed BsAb drugs for solid tumors are now undergoing evaluation in phase I/II clinical trials in OC patients, e.g., EpCAM/CD3 (catumaxomab) and delta-like ligand 4/VEGF navicixizumab (OMP-305B83) (191).

Perspectives

The local antitumor immune response cannot exist without coordinated communication with the periphery (15). Therefore, understanding immune responses to cancer should encompass global analysis across the peripheral and local immune system.

First, despite the development of high-throughput single-cell technologies, there are no studies that analyze global OC immunome in a large patient cohort both at the local level (in the tumor microenvironment, ascites) and at the systemic level (in peripheral blood, metastatic tumor sites, etc.). Yet, global immune response changes during tumor development and in response to (immuno)therapy play an important role. Pairing single-cell analyses from the different tumor sites, ascites, and peripheral blood can help the discovery of valuable biomarkers that may be easily analyzed, e.g., in the blood samples, and provide useful information to help stratification of OC patients according to their immune status and management of treatment decision. For example, it would be interesting to study the cancer-immunity cycle for individual OC patients, which allows the matching of specific immunotherapies or combinations of immunotherapies.

Second, since metastases are mainly responsible for cancer-related deaths (10), the future study of mechanistic insight on how tumor cells circulate throughout the body will be crucial. It has been proposed that some immune cells, e.g., neutrophils, support CTCs leading to enhanced metastasis formation (9). However, the role of immunity in the metastatic spread of OC can be even more complex, as recent evidence suggests that CTC release relates to circadian rhythm. Intriguingly, a study shows that more than 78% of all the CTCs obtained were from the human breast cancer samples taken during the resting (sleep) phase (192). The time-dependent nature of CTCs and hence components of the immune system should be considered in future studies on the OC immunity. From the clinical point of view, time-controlled treatment might be needed to achieve maximally effective therapy.

Third, it would also be valuable to explore which anatomic sites drive antitumor immunity and which parameters/immune cells (in peripheral blood) may provide a means for noninvasive monitoring during (immuno)therapy and discovery of new biomarkers. Using cancer liquid biopsies can open new vistas of future work in this field.

Finally, it is worthy to highlight the importance of encouraging and supporting holistic basic research on the global immunome in OC patients, which can help increase the effectiveness of clinical trials.

Overall, global and integrative analysis of both local and systemic immune responses in OC can help understand tumor control and finally increase the effectiveness of (immuno)therapy.

Author contributions

Author contributions: conceptualization, writing, review, editing: AR, MO-L, KŻ, RT, JK, KO. All authors contributed to the article and approved the submitted version.

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Conflict of interest

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

Publisher's note

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Modulating the tumor immune microenvironment with nanoparticles: A sword for improving the efficiency of ovarian cancer immunotherapy

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With encouraging antitumor effects, immunotherapy represented by immune checkpoint blockade has developed into a mainstream cancer therapeutic modality. However, only a minority of ovarian cancer (OC) patients could benefit from immunotherapy. The main reason is that most OC harbor a suppressive tumor immune microenvironment (TIME). Emerging studies suggest that M2 tumor-associated macrophages (TAMs), T regulatory cells (Tregs), myeloid-derived suppressor cells (MDSCs), and cancer-associated fibroblasts (CAFs) are enriched in OC. Thus, reversing the suppressive TIME is considered an ideal candidate for improving the efficiency of immunotherapy. Nanoparticles encapsulating immunoregulatory agents can regulate immunocytes and improve the TIME to boost the antitumor immune response. In addition, some nanoparticle-mediated photodynamic and photothermal therapy can directly kill tumor cells and induce tumor immunogenic cell death to activate antigen-presenting cells and promote T cell infiltration. These advantages make nanoparticles promising candidates for modulating the TIME and improving OC immunotherapy. In this review, we analyzed the composition and function of the TIME in OC and summarized the current clinical progress of OC immunotherapy. Then, we expounded on the promising advances in nanomaterial-mediated immunotherapy for modulating the TIME in OC. Finally, we discussed the obstacles and challenges in the clinical translation of this novel combination treatment regimen. We believe this resourceful strategy will open the door to effective immunotherapy of OC and benefit numerous patients.

KEYWORDS

immunotherapy, ovarian cancer, tumor immune microenvironment, nanoparticles, drug delivery system

Introduction

Ovarian cancer (OC) has a high lethality rate and is the second primary cause of death from gynecologic cancer worldwide (1). Currently, the major treatments for OC are surgery, chemotherapy, and radiotherapy (2, 3). Although patients can achieve short-term remission with these approaches, five-year survival rates are only approximately 30% (4). Recently, immunotherapy has received increasing attention, especially immune checkpoint blockade (ICB), which has emerged as an effective strategy for OC therapy. The anti-PD-1 antibody pembrolizumab has received regulatory approval to treat OC (5).

Although ICB holds tremendous potential for cancer therapy, the current clinical data on OC immunotherapy is not ideal. In general, the limited efficacy of ICB is mainly due to four reasons (1): tumor antigen deficiency (2), insufficient T lymphocyte infiltration, (3) defective tumor antigen processing and presentation mechanisms, and (4) the suppressive tumor immune microenvironment (TIME). Notably, the suppressive TIME is a significant barrier to the immunotherapy of OC. Ovarian tumors contain a large number of immunosuppressive cells, such as M2 tumor-associated macrophages (TAMs), $CD4^+$ regulatory T cells (Tregs), myeloid-derived suppressor cells (MDSCs), and cancer-associated fibroblasts (CAFs), which inhibit the immune response.

In recent years, nanoparticles have been expected to play a significant role in regulating the TIME and improving the efficacy of OC immunotherapy. On the one hand, nanotechnology-mediated photothermal therapy (PTT) and photodynamic therapy (PDT) can induce immunogenic cell death (ICD) of tumor cells, promote antigen presentation, and enhance tumor T cell infiltration (6). For instance, copper sulfide nanoparticles remodeled the TIME by inducing ICD, thus improving the efficiency of immune checkpoint inhibitors (ICIs) in OC. On the other hand, nanoparticles can be used as excellent drug carriers, which can load immunomodulators, such as adjuvants, cytokines, and siRNA, to regulate immunosuppressive cells and inhibit immune checkpoints. For example, Kang Yanan et al. prepared liposomes containing toll-like receptor (TLR) agonists and successfully repolarized M2 TAMs in OC (7).

Above all, nanoparticle-mediated immunotherapy holds great promise in modulating the TIME of OC and improving the treatment outcome. Here, we summarized the composition and function of the TIME in OC and discussed recent advances in immunotherapy to treat OC in preclinical and clinical settings. Moreover, the advantages and progress of nanoparticle-mediated immunotherapy in regulating the TIME and boosting the antitumor immunity of OC are also summarized. Finally, the current limitations and future development strategies in clinical translation of this nanoparticle-mediated immunotherapy have also been discussed.

The tumor immune microenvironment of ovarian cancer

Overview of ovarian cancer

Approximately 90% of ovarian tumors originate in epithelial cells and are called epithelial ovarian cancer (EOC). EOC has been categorized into different subtypes according to histology. The prevalent histologic subtype is high-grade serous ovarian cancer (HGSOC), which accounts for about 80% of cases. Other rarer subtypes include low-grade serous, mucinous, clear cell, and endometrioid tumors. With the development of genomics and single-cell technology, the understanding of the TIME in OC has been deepened (8–12). It has been found that different OC subtypes have distinct macrophage polarization (13). The results of single-cell RNA sequencing revealed that ascites cells in different HGSOC patients differ in composition and functional program including diverse fibroblasts and macrophages (10). In addition, $CD8^+$ and $CD4^+$ T cells have distinct infiltration levels in differentially growing metastases within a single individual (9). Therefore, the TIME of OC is very complex and heterogeneous, which is primarily made up of $CD8^+$ T cells, $CD4^+$ T cells, NK cells, macrophages, MDSCs, etc. Based on their function, these cells can be categorized as activated and suppressive immune cells. Activated immune cells mainly include $CD8^+$ T cells and NK cells. Suppressive immune cells mainly include Tregs, M2 macrophages, MDSCs, etc. In the TIME, activated immune cells play a role in tumor growth inhibition and tumor immunosurveillance. In contrast, suppressive immune cells dampen the function of activated immune cells and promote the growth of tumors (Figure 1).

T lymphocytes

T lymphocytes are the main component of the TIME and are central to adaptive immunity. Mature T cells are classified as $CD3^+$ $CD8^+$ T cells and $CD3^+$ $CD4^+$ T cells, according to their marker gene (14). $CD8^+$ T cells are the prime activated immune cells and are also known as cytotoxic T cells (CTLs). The T cell receptor (TCR) on $CD8^+$ T cells binds to the MHC-I compound on tumor cells, resulting in the production of cytolytic factors (e.g., perforin and granzyme) and inflammatory cytokines (e.g., IL-2 and IL-12) that directly kill tumor cells (15). The mechanism of ICB and adoptive cell therapy (ACT) is to activate $CD8^+$ T cells. An essential prerequisite for the PD-L1 blockade response in OC patients is sufficient T-cell infiltration. Higher infiltrating levels of $CD8^+$ T cells in the TIME indicate a

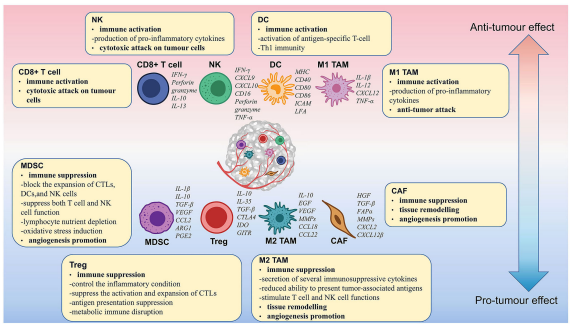

FIGURE 1
Immune cell functions and their interactions in the ovarian cancer tumor microenvironment. M2 TAMs, Tregs, MDSCs, and CAFs suppress the immune response and promote the proliferation, growth, and metastasis of OC. $CD8^+$ T cells, NK cells, mature DCs, and M1 TAMs enhanced the immune response and suppressed tumor growth.

better prognosis in OC patients (16). However, high levels of TGFβ in OC inhibit the function of CTLs (17). Recently, it has been found that the infiltration level of $CD8^+$ T cells in OC is regulated by CXCL9 expressed in antigen presentation cells (APCs) and CCL5 expressed in tumor cells (18).

In the suppressive TIME of OC, dysfunction of $CD8^+$ T cells is another significant cause of immune dysfunction. T-cell dysfunction is caused by inhibiting T-cell mitochondrial biogenesis and the inability to produce sufficient energy intermediates (19). Activation of the IRE1α-XBP1 pathway regulates the T-cell mitochondrial activity and reduces T-cell infiltration and IFN-γ expression. Cytokine IFN-γ levels are linked to TIL infiltration, and increased IFN-γ levels can improve OC patient survival. Downregulation of XBP1 or control of endoplasmic reticulum stress enhances T-cell activity and metabolic adaptation (20). T-cell proliferation is also disturbed by lipid metabolites secreted by tumor cells, including 9-HODE, 5-HETE, and PGD2, which bind to T-cell PPAR and inhibit cell cycle protein E (21).

Natural killer cells

NK cells are innate lymphoid-like cells with potent natural cytotoxicity against tumor cells. NK cells participate in immune regulation *via* a variety of mechanisms, including (1) the expression of CD16 to exert antibody-dependent cytotoxicity (ADCC) and detect target cells encapsulated in antibodies; (2) the production of perforin and granzyme to induce apoptosis in tumor cells directly; and (3) the release of antitumor cytokines such as TNF-α and IFN-γ (22). Various studies have demonstrated the effectiveness of NK cell therapy in patients with OC. Poznanski et al. found that expansion of patient-derived $CD56^{superbright}$ $CD16^+$ NK cells could exert potent cytotoxicity against autologous tumors in an autologous-derived xenograft mouse model of OC patients (23). Recently, Sun et al. showed that intravenous injection of NK cells isolated from the peripheral blood of OC patients inhibited the systemic metastasis of OC and increased the survival rate (24).

The most critical cytotoxic receptors for NK cells involved in immune surveillance are the NKG2D receptor, the CD16 receptor, and natural cytotoxic receptors for NKG receptors, such as NKp30 (25). In contrast, the proinflammatory cytokine MIF transcriptionally downregulates the NK cytotoxicity receptor NKG2D and decreases the cytotoxicity of NK cells (26). Furthermore, chronic receptor-ligand interactions reduce the expression of NK-cell surface receptors, impairing NK-cell cytolytic function and IFN-γ secretion ability (27). Greppi et al. found that OC cells released B7-H6 suppressed the expression of NKp30 on NK cells (28). TGF-β also inhibits NKp30 expression and dampens NK-cell-induced dendritic cell (DC) killing (27). In addition, OC ascites contain high levels of IL-18 and TGF-β, which can suppress the expression of CD16 and ADCC in NK cells (22). In OC, NK cells can also affect T cells to interfere with tumor progression. NK cells promote $CD8^+$ T-cell recruitment in OC by upregulating CCL5, CXCL9, and CXCL10 *via* the CCR5 mechanism (29).

T regulatory cells

Tregs are a heterogeneous subpopulation of $CD4^+$ T cells that express CD25, CTL antigen 4 (CTLA-4), and the transcription factor FoxP3 (30). Tregs are classical immunosuppressive cells that exert immunosuppressive effects and maintain immune self-tolerance *in vivo*. Treg to $CD8^+$ T cell ratios in tumors negatively correlate with survival in OC patients (31). Blocking Treg differentiation, migration, or immunosuppressive functions can reinforce the antitumor immune responses. Moreover, macrophage-secreted CCL22 in the peritoneal cavity can promote Treg migration (32). The hypoxic tumor microenvironment (TME) also favors the metabolic reprogramming of Tregs leading to Treg proliferation. Accumulated Tregs upregulate the secretion levels of IL-10 and promote angiogenesis and immune tolerance of tumors (33).

Tregs can establish a suppressive TIME through multiple mechanisms. On the one hand, Tregs can release immunosuppressive cytokines, such as IL-35, IL-10, and transforming growth factor β (TGF-β), which inhibit the function of $CD8^+$ T cells and promote tumor cell growth (34). On the other hand, Tregs inhibit the TCR signaling pathway of $CD4^+$ CD25- conventional T cells (Tcons), suppress calcium (Ca^{2+}) signaling in Tcons, and reduce the activation of NFAT and NF-κB in Tcons (34). Moreover, the perforin and granzyme

released from Tregs directly kill other immune cells, such as DCs, monocytes, and $CD8^+$ T cells (34). Multiple receptors expressed on Tregs isolated from OC are associated with TCR involvement, including PD-1, ICOS, and 4-1BB. These receptors make Tregs more sensitive to anti-CD3/anti-CD28 stimuli and have a more potent inhibitory capacity (35). Treg-expressed CD73 and CD39 convert pro-inflammatory ATP to adenosine to mediate immunosuppression (36). $CD4^+$ Tregs can differentiate into $CD4^+$ effector T cells upon the activation of glucocorticoid-induced tumor necrosis factor receptor family-related receptor (GITR). Therefore, stimulation of GITR is expected to eliminate Treg-mediated suppression (37).

The secretion of high levels of proinflammatory cytokines (e.g., TNF and IL-6) in OC malignant ascites promoted high expression of tumor necrosis factor receptor 2 (TNFR2) in Tregs. $TNFR2^+$ Tregs enhanced the expression of immunosuppressive molecules, including TGF-β, CD39, CD73, GARP PD-L1, and CTLA-4 (38, 39). The upregulated CTLA-4 in Tregs inhibits the activation and proliferation of effector T cells (40). Tregs in OC induce B7-H4 expression, deliver inhibitory activity to APCs, and blunt the antitumor immune response (41). Abnormal hyperactivation of signal transducer and activator of transcription-3 (STAT3) in tumor-infiltrating immune cells positively regulates the number of Tregs and MDSCs. Therefore, targeting the IL6/JAK/STAT3 signaling pathway is a feasible strategy to alleviate the immunosuppressive TME (42).

Tumor-associated macrophages

TAMs are the largest immune cell population in the TIME of OC, accounting for 39% of the immune cellular repertoire. There are two main types of TAMs based on phenotype: proinflammatory M1-like and anti-inflammatory M2-like (43). M2 macrophages are associated with tumor immunosuppression in OC. In a study of 140 OC patients, Macciò and his colleagues found that a high density of M2 macrophages led to poor overall survival (OS) and prognosis. OS and the M1/M2 ratio were positively associated (13). Polarization and recruitment of M2 macrophages are key factors in OC progression and metastasis. The TIME can shift macrophages from the M1 to the M2 phenotype, creating a suppressive TIME. Ying et al. found that MiR-222-3p in the exosomes of EOC cells induces M2 phenotypic polarization through activation of the STAT3 pathway (44). Collagen triple helix repeat containing 1 (CTHRC1) is an OC secretory protein that induces M2-like polarization in TAMs by activating the STAT6 signaling pathway (45). Another reason for the poor survival and prognosis of OC is the recruitment of M2 macrophages. OC overexpressed UBR5, an E3 ligase, can recruit and activate TAMs by regulating multiple cytokines and chemokines, such as CCL2 and colony-stimulating factor 1 (CSF-1) (46). CSF-1 is a major macrophage survival factor. Targeting TAMs with anti-CSF-1R antibodies is a new therapeutic strategy for OC (47–49). Multiple cell signaling pathways can induce protumor and immunosuppressive properties in M2 TAMs, such as JNK, IL-13, IL-4, AMPK, PPARγ, IRF-3, IRF-4, and C/EBPβ (50).

The most common metastatic route for OC cells is the body cavity route, forming spheroids for metastasis. M2 macrophages are enriched in the omentum, which is the primary site of choice for OC metastasis. M2 macrophages secrete EGF to activate tumor cell EGFR and upregulate the VEGF/VEGFR signaling pathway to promote tumor cell proliferation and migration. In addition, EGF upregulates the expression of ICAM-1 and αMβ2 integrin in TAMs and facilitates the interaction between TAMs and tumor cells to form spheroids (51, 52). TAMs also secrete multiple cytokines and chemokines to reshape the suppressive TIME of OC and promote OC progression. El-Arabey et al. reported that TAMs promote the growth, migration, chemoresistance, and epithelial-mesenchymal transition (EMT) of TP53-mutated HGSOC cell lines by exosomes releasing GATA3 (43). Macrophage secretion of TNF-α induces MIF and EMMPRIN into tumor cells in an NF-κB- and JNK-dependent manner. Subsequently, macrophages release various MMPs to enhance tumor invasion, migration, and vascularization (53). TAMs also secrete IL-6 and IL-10, which activate the STAT3 pathway and promote tumor proliferation (54). TAMs secrete several chemokines, including CCL17, CCL22, and CCL18. These chemokines recruit Tregs and Th2 subsets and promote T-cell differentiation toward a Th2 phenotype (55).

Myeloid-derived suppressor cells

MDSCs are a heterogeneous group of nonterminally differentiated myeloid cells with immunosuppressive properties. Consistent with other immunosuppressive cells, the infiltration of MDSCs is related to shorter OS in OC patients (56). High concentrations of several cytokines (e.g., IL-6, IL-10, IL-1β, VEGF, PGE2, and TNF-α) in the ascites of OC patients induce the accumulation of MDSCs (57). Growth factors G-CSF and GM-CSF promote the production of MDSCs by activating STAT3 and STAT5 signaling pathways and downregulating IRF-8 (58). Multiple chemokines (e. g, CCL1, CCL5, CCL7, CXCL8, and CXCL12) drive the recruitment of MDSCs to OC tumor sites *via* the CCR2, CXCR4, and CCR5 axes. Triggering of the CXCL12-CXCR4 pathway is controlled by the tumor-associated inflammatory mediator PGE2, and targeting PGE2 has the potential to block the migration of MDSCs into ascites (59).

Notably, MDSCs can increase the stem cell-like properties of OC cells. Li et al. found that induction of the CSF2/p-STAT3 signaling pathway by MDSCs could enhance the stemness of EOC cells (60). Cui et al. demonstrated that MDSCs induced microRNA101 expression and suppressed CtBP2, thereby

enhancing the stem cell-like properties of OC (61). The immunosuppressive properties of MDSCs are dependent on PGE2-induced DNA methyltransferase 3 alpha (DNMT3A) upregulation and hypermethylation of myeloid genes (62). MDSCs have re-editable properties similar to those of macrophages. STAT3 inhibition and TLR signaling modulation can repolarize MDSCs and activate their immune function (63).

MDSCs generate a suppressive TIME by inhibiting the activities of activated immune cells. Previous studies found that MDSCs inhibit the activity and proliferation of NK cells and block the antigenic expression of DCs (56). In addition, MDSCs produce TGF-β, IDO, IL-10, and nitric oxide (NO) to reduce the proliferation and cytotoxicity of NK cells and exert immunosuppressive functions (64). The effects of MDSCs on NK cells were manifested by downregulating the expression of surface natural cytotoxicity receptors (NCRs), NKG2D, and DNAM-1 (65). Meanwhile, MDSCs can polarize M1 macrophages to the M2 phenotype and induce Treg amplification. MDSCs induce the activation and accumulation of M2 macrophages and stimulate the production of more IL-10. In turn, IL-10 can upregulate immunosuppressive factors, such as PD-L1 and Arg-1, inducing the activation and amplification of MDSCs (66). HIF-1 in the hypoxic environment of OC redifferentiates MDSCs into TAMs and promotes tumor progression (67). In addition, MDSCs secreted TGF-β and IL-10 can stimulate Treg migration and differentiation through CD40-CD40 L interactions (68).

MDSCs suppress the T-cell-mediated antitumor immune responses in the TIME of OC by multiple mechanisms (1): Depleting nutrients required by lymphocytes: MDSCs inhibit T-cell proliferation by upregulating ARG-1 to consume arginine and isolate 1-cysteine (69); (2) Restricting T-cell recruitment and inducing T-cell apoptosis by expressing Galectin9, AMPKα-1, and ADAM17 (70); (3) Regulating NO and ROS production and stimulate oxidative stress; (4) Production of peroxynitrite (PNT) inhibits the TCR signaling pathway: MDSCs produce peroxynitrite, which nitrates complexes in the TCR-CD8 complex when direct contact with T cells. Meanwhile, MDSCs disrupt the binding of $CD8^+$ T cells to specific peptide-major histocompatibility complex (pMHC) dimers and inhibit T cell antigen recognition (71). (5) Secreting TGF-β enhances T-cell immunosuppressive phenotypic differentiation, such as promoting the differentiation of Th17, Th2, and Tregs (72). (6) Enhancing the expression of PD-L1 on tumor cells by the AKT/mTOR signaling pathway in a PGE2-dependent manner (73).

Cancer-associated fibroblasts

CAFs are another major cell subpopulation in OC masses and play a crucial role in OC progression (74). Tumor cells are protected from immune surveillance by an extracellular layer of dense extracellular matrix (ECM) rich in fibronectin. However, this protective shell is produced by abnormal remodeling of the ECM and excessive deposition of fibroblasts. OC cells construct a strong protective barrier by reprogramming fibroblasts with miRNAs, mainly downregulating miR-214 and miR-31 and upregulating miR-155 (75). Overexpression of STAT4 in EOC cells depends on tumor-derived Wnt7a to induce the production of CAFs (76). NNMT regulates CAF differentiation, reduces histone methylation and s-adenosylmethionine, and supports OC proliferation, growth, and metastasis (77).

Fibroblasts are a key component of the basement membrane and peritoneum of the greater omentum in OC. CAFs recruit ascites tumor cells expressing high levels of alpha5-integrin (ITGA5) to form heterogeneous spheroids called MUs. Additionally, CAFs secrete EGF to maintain the MU structure by maintaining ITGA5 expression, which aids in the trans-somatic metastasis and OC peritoneal dissemination of HGSOC (78). Many markers activate CAFs, such as the extremely heterogeneous αSMA and FAP, and these markers help us develop new therapeutic targets for cancer (79, 80).

CAFs can remodel the ECM by secreting various cytokines as well as produce multiple paracrine signals with OC cells to induce OC cell growth, migration, and invasion. CAFs secrete DKK3 and activate YAP/TAZ and β-linked protein, the former inducing CAF tumorigenesis and the latter promoting OC invasion (81). CAF-derived POSTN promotes EMT by activating the PI3K/AKT pathway. It also promoted TGF-β1-induced activation of fibroblasts and invasion and migration of OC cells (82). TGF-β receptor type II and SMAD signaling upregulate VCAN and activate the NF-kB signaling pathway in CAFs. Alterations in these signaling pathways increase matrix metalloproteinase 9, CD44, and hyaluronic acid-mediated motor receptor expression in CAFs and promote the progression of advanced OC (83). The hepatocyte growth factor (HGF) is a key growth factor derived from CAFs. HGF stimulates OC cell growth and drug resistance by activating the c-Met/PI3K/Akt and GRP78 pathways (84). Fibroblast growth factor-1 (FGF-1) is another crucial factor in CAFs. FGF-1 regulates tumor progression by phosphorylating FGF-4, increasing the expression of Snail1 and MMP3, and activating the MAPK/ERK pathway (85). In addition, CAFs promote OC metastasis by secreting VEGF-A and tenascin-c (86).

Moreover, CAFs recruit immune cells and remodel the TIME *via* several cytokines and chemokines. Taki et al. found that CAFs produce the chemokines CXCL1 and CXCL2, which recruit MDSCs in OC (59). In addition, CXCL12β expression in CAF cells promotes the migration and differentiation of Tregs (87). CAFs interact with multiple immune components to regulate the immune activity of innate and adaptive cells and suppress antitumor immunity. Interleukin (IL)-1β is a major immunosuppressant in the TME and is significantly associated with CAF-expressed PS1. PS1 positively correlates with IL-1β

levels under the regulation of the WNT/β-linked protein pathway. Inhibition of PS1 expression increases the proliferation and migration of CTLs and DCs (88). Browning et al. found that CAFs produce IL-6, a cytokine with protumorigenic function, which leads to severely poor prognosis and chemoresistance in OC patients (54).

Current progress of immunotherapy in ovarian cancer

During the past years, various immunotherapies, including ICB, cancer vaccines, ACT, and cytokines, have been approved for OC treatment (Table 1). In this section, we will expatiate the progress of these treatments in OC.

Immune checkpoint blockade

Effective immunotherapy relies on antigen presentation, inhibition of immunosuppressive cells, and activation of effector T cells. Among them, T-cell-mediated immune responses are crucial and modulated by inhibitory and stimulatory signals. Immune checkpoints regulate T cell activities and are closely related to tumor immunity. Currently, ICIs targeting CTLA-4 and programmed cell death protein 1 (PD-1) or PD-1 ligand (PD-L1) have achieved breakthrough results in clinical trials (89). Anti-PD-L1 antibodies (avelumab, durvalumab, and atezolizumab), anti-PD-1 antibodies (nivolumab and pembrolizumab), and anti-CTLA-4 antibodies (ipilimumab) have received FDA approval for the treatment of several malignancies represented by melanoma and non-small cell lung cancer (5, 90). However, the objective response rates (ORR) for single-agent ICIs in OC are only 6-15% (91). For example, in a phase II study of ipilimumab for patients with platinum-sensitive OC, the best overall response rate (BOR) was just 15% (NCT01611558). In addition, the BOR for platinum-resistant OC patients treated with nivolumab was 15%. In this clinical trial, 40% of the patients had grade 3 or 4 treatment-related side events (92). Besides, in a phase II clinical study of pembrolizumab in advanced recurrent OC (NCT02674061), the ORR was also less than 10% (93).

Due to the unsatisfactory efficacy of single-agent ICIs in OC, combination therapy has recently received much attention. Several studies have combined ICB with polyadenosine diphosphate ribose polymerase (PARP) inhibition, chemotherapy, and antiangiogenic therapy to improve the efficacy of OC immunotherapy (91). For example, pembrolizumab was combined with the PARP inhibitor niraparib for recurrent OC treatment. In this clinical trial, the ORR was 18%, and the illness control rate was 65% (94). Besides, the ORR for OC patients treated with durvalumab plus the anticancer drug trabectedin was 21.4% (95). In addition, patients with platinum-resistant OC responded well to pembrolizumab plus pegylated liposomal doxorubicin. In a clinical trial, 52.2% of the patients achieved a clinical benefit, and 26.1% experienced an overall response (96). Moreover, a phase II trial of nivolumab plus bevacizumab in recurrent OC patients was conducted. The results showed that platinum-sensitive patients had an ORR of 40.0% and platinum-resistant patients had an ORR of 16.7% (97). However, although combination therapy represents an approach to improving the efficacy of ICB, ICIs still demonstrated limited clinical activity for OC patients. The core reason is the suppressive TIME in OC, which leads to insufficient CTL activity. According to Grzywa TM et al., TAMs can decrease the amount of L-arginine in the TME and decrease T cell activation (98).

Cancer vaccines

Cancer vaccines can promote antigen presentation by APCs and enhance the anti-tumor activities of antigen-specific CTLs. They have additional advantages in establishing immune memory and preventing tumor recurrence (99). At present, cancer vaccines, represented by DC vaccines, have achieved successful clinical results in the immunotherapy of various malignancies, including OC, melanoma, and prostatic cancer (100). DC vaccines that target MUC1 and NY-ESO-1 have been used to treat patients with OC. In a phase II clinical study of the MUC1-targeted DC vaccine for EOC patients, MUC1 T-cell-specific responses were observed but did not result in substantially increased progression-free survival (PFS) (101). Multiple antigens have been incorporated into cancer vaccines considering the negative effects of immune escape. However, this strategy still does not improve clinical outcomes for OC. For example, combining a multivalent conjugate vaccine (MUC1-TN, GLO-H, GM2, TF) with an adjuvant for patients with OC in the second or third clinical complete remission following chemotherapy did not prolong OS or PFS compared to adjuvant alone (102).

Taken together, although cancer vaccines can induce strong immune responses, their current clinical outcomes in OC have not been satisfactory. The main reason is the weak immunogenicity and suppressive TIME in OC. According to Schumacher et al., neoantigen recognition is uncertain in OCs, because of the inadequate mutational load and tumor heterogeneity (89, 103). At present, some strategies have been proposed to solve this dilemma. On the one hand, cancer vaccines can be combined with other treatment strategies, such as ICB. For example, patients with advanced platinum-resistant OC treated with the multiepitope FRα vaccine plus durvalumab achieved durable survival, with partial response rates of 3.7% and stable disease (SD) rates of 33.3% (104). On the other hand, incorporating as many tumor antigens as possible into cancer

TABLE 1 Clinical trials of immunotherapy for ovarian cancer.

	Interventions	Number	Phase	Status	Efficacy
Immune Checkpoint Blockade	Ipilimumab	NCT01611558	Phase 2	Completed	BOR: 15%
	Nivolumab	UMIN000005714	Phase 2	Completed	BOR: 15% ORR: 10% (1 mg/kg), 20% (3 mg/kg) PFS: 3.5 months OS: 20.0 months
	Pembrolizumab	NCT02674061	Phase 2	Completed	ORR: 7.4% (received 1-3 prior lines), 9.9% (received 4-6 prior lines) DCR: 37.2% (received 1-3 prior lines), 37.4% (received 4-6 prior lines)
	Pembrolizumab + Niraparib (PARP inhibitor)	NCT02657889	Phase 1/2	Completed	ORR: 18% DCR: 65%
	Durvalumab + Trabectedin	NCT03085225	Phase 1	Active, not recruiting	tumor shrinkage rate: 43% ORR: 21.4% 6-month PFR: 42.9%
	Pembrolizumab + Pegylated liposomal doxorubicin	NCT02865811	Phase 2	Active, not recruiting	CBR: 52.2% ORR: 26.1%
	Nivolumab + Bevacizumab (antiangiogenic agent)	NCT02873962	Phase 2	Recruiting	ORR: 40.0% (platinum-sensitive participants), 16.7% (platinum-resistant participants) PFS: 8.1 months
Cancer Vaccines	MUC1-targeted DC vaccine	NCT01068509	Phase 2	Completed	PFS:13 months (first clinical remission), >42 months (second clinical remission)
	Multivalent conjugate vaccine (MUC1-TN, GLO-H, GM2, TF) + OPT-821 (saponin-based immunoadjuvant)	NCT00857545	Phase 2	Completed	HR of PFS: 0.98 OS: 47 months
	Multiepitope FRα vaccine + durvalumab	NCT02764333	Phase 2	Completed	SD: 33.3% PR: 3.7%
	Oxidized whole-tumor lysate DC vaccine	NCT01132014	Early phase 1	Completed	SD: 52.0% PR: 8.0% 2-year OS: 100% (responders), 25% (nonresponders)
Adoptive cell therapy	TIL + lymphodepleting chemotherapy (cyclophosphamide and fludarabine) + IL-2	NCT02482090	Phase 1	Completed	3-month SD: 66.7% 5-month SD: 33.3% decrease in target lesions: 33.3%
	TIL + cyclophosphamide	/			CR: 14.3% PR: 57.1%
	TIL + lymphodepleting chemotherapy (cyclophosphamide and fludarabine) + IL-2 + Ipilimumab + Nivolumab	NCT03287674	Phase 1/2	Completed	12-month SD: 83.3% PR: 16.7%
	CAR-T targeting mesothelin	NCT02159716	Phase 1	Completed	BOR: 73.3%
Cytokines	Recombinant IL-2	/	Phase 2	Completed	ORR: 25.0%
	α-Recombinant interferon	/	Phase 3	Completed	CR: 36% PR: 9% PD: 55%
	IFN-γ + cisplatin + cyclophosphamide	/	Phase 3	Completed	CR: 68% 3-year PFR: 51% 3-year OS: 74%
	IL-2 + OK-432 + platinum- and Taxol-based chemotherapy	Case report	/	Completed	recurrence rate: 53.8% (immunochemotherapy), 88% (traditional chemotherapy)
	IL-18 + pegylated liposomal doxorubicin	NCT00659178	Phase 1	Completed	SD: 38% PR: 6%
	IL-2 + 13-cis-retinoic acid	/	Phase 2	Completed	5-year PFS: 29% OS: 38%

BOR, Best Overall Response Rate; ORR, Objective Response Rate; PFS, Progression-Free Survival; OS, Overall Survival; DCR, Disease Control Rate; PFR, Progression-free Rate; CBR, Clinical Benefit Rate; HR, Hazard Ratio; SD, Stable Disease; PR, Partial Response; CR, Complete Response.

vaccines is a promising strategy to solve the heterogeneity of OC and improve treatment effectiveness (105). Tanyi JL et al. constructed an oxidized whole-tumor lysate DC vaccine for treating patients with platinum-treated recurrent OC. After administration, the vaccine stimulates T-cell responses and patients experience prolonged survival (106).

Adoptive cell therapy

ACT is an immunotherapeutic regimen that harnesses autologous or allogeneic anticancer lymphocytes to promote tumor regression (105). ACT is mainly divided into three types: expanded natural TILs, chimeric antigen receptor T cells (CAR-T), and T-cell receptor engineered T cells (TCR-T) (107). ACT has achieved striking clinical success in various cancers, such as B-cell leukemias and melanoma. For example, patients with advanced melanoma responded favorably to ACT, with complete tumor shrinkage (108). However, despite several attempts, ACT has not achieved the desired effect for OC patients. Patients with recurrent or advanced EOC were treated with TILs following a single intravenous injection of cyclophosphamide. The results show that only 14.3% of the patients experienced a complete response, and 57.1% experienced a partial response (109). Besides, in a phase I clinical study of CAR-T targeting mesothelin (CAR-T-meso) for patients with OC, the CAR-T-meso cells showed limited clinical activity and short persistence (110).

The poor antitumor activity of ACT in OC is largely associated with the suppressive TIME. At present, several immunotherapies, such as ICB and cytokines, which can regulate the TIME, have been combined with ACT for OC to improve therapeutic activity. The combination of IL-2 with TILs was used to treat six patients with progressive platinum-resistant metastatic OC. There were 4 patients with SD for 3 months and 2 patients for 5 months (111). In another clinical trial, 83.3% of patients with late-stage metastatic HGSOC who received TILs, IL-2, ipilimumab, and nivolumab had SD for up to 12 months. The addition of ipilimumab improved T-cell proliferation positively impacted the T-cell phenotype and boosted CD8 T-cell tumor reactivity (112).

In conclusion, ACT has shown excellent potential in OC treatment, but its successful clinical application still faces obstacles. The physical barriers in OC limit the accessibility of CAR-T cells to tumor cells. Local CAR-T cell administration will offer solutions to this problem and improve antitumor efficiency (113). In addition, the small number of targeted antigens and their heterogeneous expression in ovarian tumors predispose them to antigen escape. Novel CAR-T cells simultaneously targeting multiple TAAs may increase the effectiveness of ACT in OC patients (114).

Cytokines

A large class of tiny biomolecules known as cytokines plays a crucial role in cell signaling. Among them, IFNs, ILs, and chemokines have all been extensively utilized as immunomodulators to treat cancer (115). For instance, IFN-α has achieved FDA approval and is used to treat leukemia in clinical settings (115). In addition, IL-2 can cause complete and long-lasting tumor regression in patients with metastatic melanoma and renal cancer (116). Recently, cytokine-mediated immunotherapy has been evaluated in OC clinical studies but has not yet achieved excellent outcomes. A clinical study found that OC patients had a low response rate to a single intravenous injection of recombinant IL-12 (117). Besides, the overall response rate for platinum-resistant OC patients receiving intraperitoneal administration of IL-2 was 25% (118). Moreover, intraperitoneal injection of α-recombinant interferon (rIFN-α2) resulted in complete remission in 36% of patients with EOC but also induced significant toxic side effects (119). In addition, an adenoviral vector expressing IFN-β was used to treat two OC patients. One of the patients with distant metastasis and malignant pleural effusion achieved a complete response (120).

At present, cytokine-based immunotherapy has been combined with various antitumor therapies such as chemotherapy and antiangiogenic therapy to improve clinical efficacy. In OC patients, combining IFN-γ with first-line chemotherapy improved PFS while causing acceptable toxicity (121). IL-2 was combined with picibanil (OK-432) and traditional chemotherapy drugs for patients with advanced OC. These patients had a lower recurrence rate than patients receiving chemotherapy alone (122). In a clinical trial, patients with OC were treated with IL-18 plus pegylated doxorubicin liposomes. The results show that 6% of patients had a partial objective response, and 38% had an SD (123). In addition, the combination of 13-cis retinoic acid, which has antiangiogenic activity, with low-dose IL-2 was used to treat advanced OC patients. The patients had a 5-year PFS rate of 29% and an OS rate of 38%, with an increased number of lymphocytes and NK cells (124).

Above all, cytokines as excellent immunomodulators have shown exciting potential in combination therapy of OC. However, their low stability and short half-life essentially limit their application in the clinic. These issues are considered to be overcome by utilizing nanoparticle-based drug delivery systems.

Nanomaterials for remodeling the immune microenvironment to enhance cancer immunotherapy

During the past decades, multiple nanoparticles have been applied in OC treatment or synergized immunotherapy, such as liposomes, polymeric micelles, silica-based nanoparticles (SNPs), and metal-based nanomaterials (Table 2). Nanoparticles not only serve as a vehicle to carry anticancer drugs but also as a regulator to modulate the TIME (Figure 2). In this section, we will discuss the role of nanoparticles in remodeling the TIME of OC and improving the efficacy of immunotherapy.

Application of liposomes in enhancing cancer immunotherapy

Liposomes are spherical bilayer nanoparticles composed of cholesterol and phospholipids, which have been used as drug vehicles due to their excellent encapsulation efficiency, targeting ability, biosafety, and biocompatibility (137). The remarkable properties of liposomes result in their FDA approval for use in

TABLE 2 Nanoparticles for regulating TIME and improving immunotherapy.

Nanoparticles	Immunotherapy	Targeting	Payload	Mechanism	Advantages	Ref
Liposome	PDT + ICB	PD-L1	IR775, metformin	PDT induces ICD; metformin downregulates PD-L1	Codelivery of hydrophilic and hydrophobic drugs	(125)
Liposome	Cytokines	TAMs	Resiquimod	TLR7/8 agonists repolarize TAMs	Administered intraperitoneally selective accumulation in TAMs	(7)
Liposome	ICB	Tregs	Indoximod prodrug, mitoxantrone	Indoximod inhibits the IDO-1 pathway and Treg expansion; mitoxantrone induces ICD	Codelivery of hydrophilic and hydrophobic drugs	(126)
Acid-sensitive polymeric nanoparticles	ICB + PDT	PD-L1	siPD-L1, carboplatin prodrug, digitoxin	Carboplatin prodrug initiates the caspase cascade; digitoxin elicits ICD; PD-L1 silencing overcome immune suppression	Environmentally responsive release and escape from the endocytic pathway	(127)
Biodegradable polymeric nanoparticles	Cytokines	TAMs	IRF5/IKKβ encoding mRNAs	IRF5 induces macrophage polarization; IKKβ activates IRF5	Reprogramming TAMs and safety for repeated dosing	(128)
PLG-g-mPEG nanoparticles	Cytokines	TAMs	Cisplatin, Resiquimod	TLR7/8 agonists repolarize TAMs	Passive targeting and drugs codelivery	(129)
Fusogenic lipid-coated MSNP	Repolarize TAMs	TAMs, PI3k	siRNA against PI3kγ, peptide LyP-1	Peptide LyP-1 targets TAMs; PI3kγ downregulation reprograms TAMs	Extremely high gene load and transfection efficiency, selective homing and transfection, avoidance of the endocytic pathway	(130)
Folic acid modified MSNP	Cytokines	T cells and DCs	CCL2	CCL2 recruits immune cells into the tumor tissue	Selective target-localizing ability and safety	(131)
SNPs	Repolarize TAMs	TAMs	/	Relatively large (>100 nm) anionic nanoparticles administered intraperitoneally selectively accumulate TAMs	Administered intraperitoneally selective accumulation in TAMs	(132)
Ferumoxytol capped ultra-large pore MSNP	ICB	PD-1	Anti-PD-1 antibody	Immune checkpoint inhibition	Sustained release and improved tumor specificity of ICIs	(133)
Copper chalcogenide nanoparticles	ICB + PTT	PD-1	Anti-PD-1 antibody, TLR9 agonist CpG	PTT induces ICD; TLR9 agonist CpG elicits activation of innate immune cells and adaptive immunity	Photothermal therapy with high penetration depth	(134)
Fe3O4 nanoparticles coated with a hybrid membrane consisting of ID8 ovarian cancer cell membrane and red blood cell membrane	PTT + PDT	/	Indocyanine green (ICG)	PTT induces ICD; red blood cell membrane coating improves the circulation time and stability; ID8 OC cell membrane coating support homologous homing properties	Prolonged circulation lifetime and high tumor specificity	(135)
Targeting peptide-modified gold nanoparticles	Inhibit TAMs	TAMs	siRNA against VEGF	siRNA inhibits the VEGF pathway in M2 TAMs and tumor cells, stimulating a host immune response	Selective gene silencing	(136)

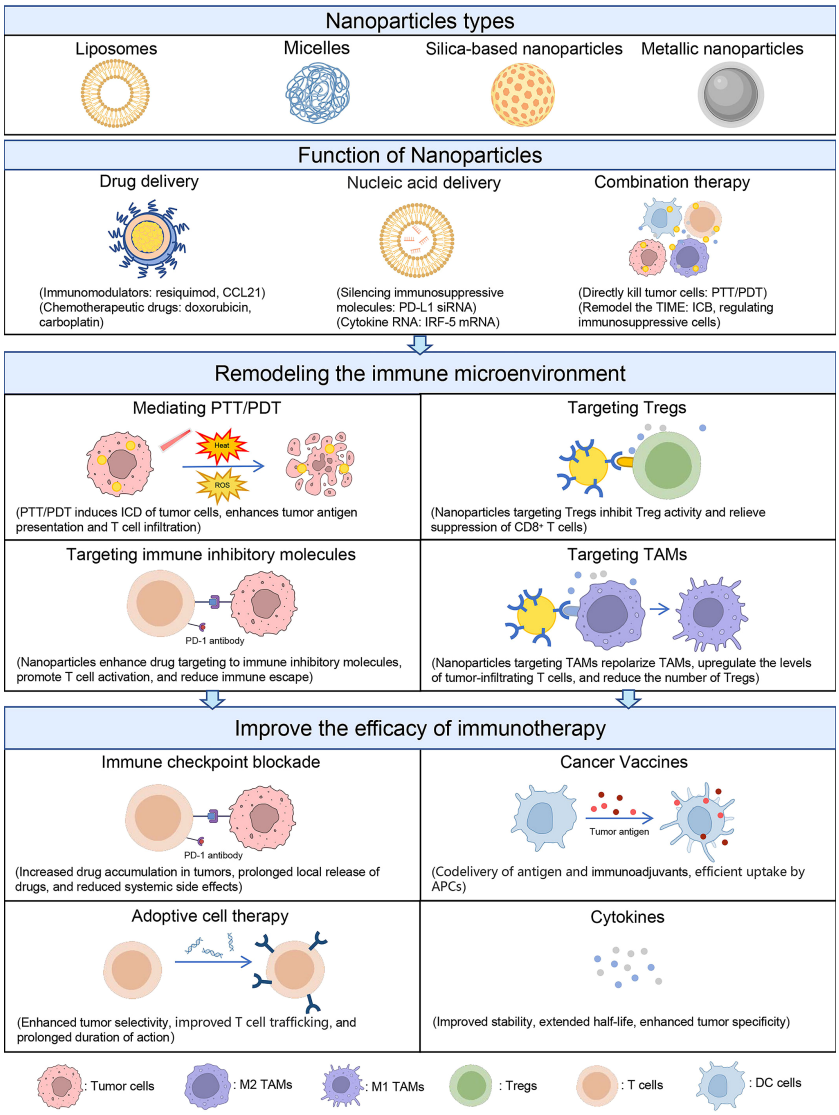

FIGURE 2
Schematic of nanoparticle-mediated immunotherapy regulating the TIME. Nanoparticles are mainly classified as liposomes, micelles, SNPs, and metallic nanoparticles. These nanoparticles have the functions of delivering drugs, delivering nucleic acids, and mediating combination therapy. Based on these functions, nanoparticles can regulate TIME in four ways: (1) mediating PTT and PDT to induce ICD in tumor cells; (2) improving drug targeting to immunosuppressive molecules; (3) targeting Tregs; and (4) targeting TAMs. By reversing the immunosuppressive state of TIME, nanoparticles can enhance the efficacy of immunotherapy such as ICB, ACT, tumor vaccines, and cytokines.

clinical cancer treatment (138). In recent years, immunomodulators, such as adjuvants, photosensitizers, and tumor antigens, have been encapsulated in liposomes to regulate the TIME. For example, Xiong W et al. encapsulated the photosensitizer IR775 and metformin into liposomes. Under laser irradiation, the photosensitizer IR775 generates reactive oxygen species, which induce ICD in bladder and colon cancer cells and enhance antigen presentation (139). After PDT, the upregulation of IFN-γ amplifies the expression of PD-L1 on tumor cells (140, 141). The coencapsulation component metformin mediates the downregulation of PD-L1, which alleviates T cell exhaustion, synergistically enhancing the antitumor effect of PDT.

Since TAMs are the main immune cell population in OC, remodeling TAMs is a prospective strategy to improve the poor clinical outcomes of OC immunotherapy. TLR 7 and TLR 8 agonists, such as liquimod and resiquimod, serve as strong immunostimulatory molecules and have the ability to remodel TAMs (142). However, these small molecule drugs have serious toxicities when administered systemically. As an excellent target

drug-delivery system, liposomes provide a way to deliver drugs into TAMs and remodel the TIME. For example, by loading resiquimod into liposomes, the drugs are efficiently delivered into TAMs and transformed M2 macrophages to the M1-type. Under treatment, the levels of tumor-infiltrating T cells were upregulated, while the percentage of Tregs in the TIME was reduced. When combined with PD-1 blockade, resiquimod-loaded liposomes significantly improve the antitumor efficiency of anti-PD-1 antibodies in OC (7).

Reducing the number of Tregs in the TIME is beneficial for promoting antigen presentation as well as T-cell recruitment and proliferation. Indoleamine 2,3-dioxygenase (IDO) is a metabolic immune regulator that can induce the expansion of Tregs (143). Kuo-Ching Mei et al. co-deliver cholesterol-conjugated indoximod prodrug, an inhibitor of the IDO-1 pathway, and chemotherapeutic agent mitoxantrone by liposomes into tumors. As a result, the number of $Foxp3^+$ Tregs was obviously decreased, which in conjunction with chemotherapeutic drug-induced ICD, significantly boosted the immunotherapy response in multiple solid tumors (144). Therefore, liposomes encapsulating IDO pathway inhibitors can effectively reprogram the TIME by reducing the number of Tregs. This strategy is also expected to be successful in OC immunotherapy. Because a study has shown that IDO is widely expressed in 56% of ovarian tumors and is associated with decreased TIL numbers (125).

Application of polymeric micelles in enhancing cancer immunotherapy

Polymeric micelles generally consist of a lipophilic core and a hydrophilic outer shell. Micelles have become widely used as drug carriers due to their biosafety, biocompatibility, surface modification, tumor targeting, and environmental responsiveness (145). These excellent biological properties have enabled micelles to be FDA-approved for the delivery of anticancer drugs (146). Recently, polymeric micelles have been used for drug delivery, bioimaging, and immunomodulation. Various immunomodulators, such as immunostimulants, immunoadjuvants, photosensitizers, and nucleic acids, have been entrapped into micelles to modulate the TIME (147). For example, FA-modified poly (ethylene glycol)-chitooligosaccharide lactate (COL) micelles were used as HIF-1a siRNA carriers. The micelles are efficiently taken up by cells *via* receptor-mediated endocytosis and significantly induce the transfection and gene knockout of HIF-1a *in vitro*, which effectively inhibits the proliferation of OC (148).

ICB can sensitize T cell-mediated tumor killing and has shown advantages in OC treatment. Genetic interventions, such as PD-L1 siRNA, are emerging as an effective strategy to suppress immune checkpoint signaling. However, the low transfection efficiency of gene therapy restrains its application as promising immunotherapy (126, 149). Cationic polymer micelles as excellent nucleic acid delivery vectors offer an attractive approach to boost genetic immunotherapy and improve the ICB response. Recently, Teo, P. Y. et al. loaded PD-L1 siRNA into folate (FA) or FA-polyethylene glycol (PEG)-modified PEI nanoparticles. The positively charged cationic polymer micelles facilitate the uptake of PD-L1 siRNA by interacting with negatively charged cell membranes. After administration, PD-L1 siRNA successfully transfected OC cells, effectively blocked PD-1/PD-L1 interaction, and enhanced the efficiency of ICB for OC (150). Recently, Ling, Xiang et al. constructed a pH-responsive nanocoordination polymer to deliver siPD-L1. This micelle was endocytosed into endocytic vesicles and ruptured when the endocytic vesicles transform into acidic endolysosomal, disrupting the organelle membrane and releasing siPD-L1 into the cytoplasm. Then PD-L1 was successfully knocked out for immune checkpoint inhibition, which remodeled the TIME and enhanced immune activation in OC (151).

Repolarizing M2 TAMs to the M1 phenotype is an effective strategy to remodel the TIME in OC and enhance antitumor immunity. Although various immunomodulators have been shown to repolarize TAMs, there are still many difficulties in repolarizing TAMs, such as poor targeting and the instability of immunomodulators. Due to the good surface modification, the polymeric micelles can be chemically bonded with diversified active targeting ligands to achieve specific targeting to TAMs. For example, mannose-modified polymeric micelles were used to deliver mRNA encoding IRF-5 and its activating kinase IKKβ. These polymeric micelles target mannose receptors overexpressed in M2 TAMs, delivering their payload exclusively to M2 TAMs. Following treatment, the immunosuppressive and tumor-promoting effects of M2 TAMs were successfully reversed (152). In addition, Yin Wen and his colleagues loaded the TLR7/8 agonist resiquimod and cisplatin onto poly(l-glutamate)-graft-methoxy polyethylene glycol (PLG-g-mPEG) nanoparticles. Benefiting from the protective function of these micelles, TLR agonists were successfully delivered and induced repolarization of macrophages, resulting in a synergistic anticancer effect of chemotherapy and macrophages in OC (153).

Application of silica-based nanoparticles in enhancing cancer immunotherapy

SNPs are one of the most important nanomaterials applied in biomedical applications because of their excellent biocompatibility, biosafety, easy synthesis, and surface modification (154, 155). SNPs are mainly divided into three types: spheres, core-shells, and mesoporous SNPs (MSNPs)

(156). Typically, MSNPs have many empty pores and large surface areas, which endow them as good candidates for drug delivery, bioimaging, and immune regulation. Immunomodulators, such as adjuvants, photosensitizers, cytokines, and siRNA, can be loaded into SNPs to regulate the TIME. For example, LyP-1 peptide-modified SNP-loaded siRNA against PI3K-γ can target TAMs and significantly knock down PI3K-γ expression (the knockdown efficiency is 81%), which leads to TAM polarization and remodels the TIME of OC (157). In addition, the surface modifiability of SNPs endows them can be wrapped with polymers and tumor-targeted peptides. These modified SNPs can enhance the drug delivery ability and avoid the toxicity of anticancer drugs. In our previous study, we developed tumor cell-targeted MSNPs by conjugating the indicated PAA and PEG on their surface (154). The MSNPs can selectively deliver MEK inhibitors into tumor cells instead of T-cells. MSNP encapsulation avoids the cytotoxicity of MEK inhibition on T cells and improves the antitumor efficiency of anti-PD-1 antibodies. The results suggest that MSNPs can avoid small molecule drug-induced immune toxicity and coordinate tumor-targeted therapy and immunotherapy.

Cytokines are classical immunoregulators that have been applied to treat OC. However, their rapid biodegradation and short half-life limit their clinical application. Uniform and large pore diameters as well as the easy surface modification ability endow MSNPs with high loading capacity, making them a candidate vehicle for carrier cytokines. In addition, the enhanced permeability and retention (EPR) effect of nanoparticles causes the carried cytokines to accumulate in TME and enhances their antitumor efficiency. Wimalachandra DC et al. developed an FA-modified SNP to load CCL21. Upon injection, CCL21-loaded SNPs accumulated at the TME of OC, which further recruited immune cells into the tumor tissue (127). Haber et al. found that negatively charged SNPs with a particle size larger than 100 nm administered intraperitoneally selectively accumulated in TAMs in mouse ovarian tumors (128). These results demonstrated that SNPs could serve as a candidate drug delivery system to remodel TAMs and enhance the anti-OC immune response by loading immune regulation agents.

ICB has been demonstrated to be an effective immunotherapy strategy and approved for the clinical treatment of OC. However, systemic toxicity and low local concentrations still need to be addressed. MSNPs have extremely high drug loading and can achieve controlled drug release by surface modification, making them ideal candidates for the delivery of ICIs. Bongseo Choi and his colleagues loaded an anti-PD-1 antibody into the pores of MSNPs and blocked the pores with iron oxide ferumoxytol, finally realizing the sustained release of PD-1 at the tumor site. This MSNP-mediated local ICB treatment after chemotherapy effectively promotes T cell infiltration and reduces Treg numbers in the TIME. This result indicates that MSNPs can achieve sustained release of ICIs and improve the duration of action and tumor specificity of ICIs (129).

Application of metallic nanoparticles in enhancing cancer immunotherapy

Metallic nanoparticles are a kind of novel nanomaterial composed of pure metals (e.g., gold, silver, copper, iron, platinum, etc.) or their compounds (e.g., hydroxides, oxides, sulfides, etc.) (158). In recent years, metallic nanoparticles have been widely used for bioimaging and cancer treatment because of their excellent optical polarizability, electrical conductivity, biocompatibility, chemical properties, and potent photothermal properties induced by near-infrared (NIR) lasers (159). Metallic nanoparticles can mediate tumor PTT and PDT. PTT/PDT is an effective strategy to remodel the TIME and improve the efficiency of immunotherapy (160, 161). The mechanism of metallic nanoparticle-mediated PTT is that metal elements absorb light energy and convert it into heat to destroy malignant cells. Moreover, metallic nanoparticle-mediated PTT and PDT induce ICD in tumor cells, releasing tumor antigens and damage-associated molecular patterns (DAMPs) to stimulate the tumor-specific immune response and enhance immunotherapy (130). For example, gold nanoparticle-mediated PTT has been widely used alone or in combination with immunotherapy, chemotherapy, and targeted therapy to treat malignant tumors. In our previous study, we constructed a MAPK pathway inhibitor-loaded silica-modified gold nanocage (AuNCs) for synergistic melanoma therapy with an anti-PD-1 antibody. AuNC-mediated PTT along with MAPK-targeted therapy effectively kills tumor cells and enhances T-cell infiltration. This treatment regimen significantly improved the antitumor efficiency of PD-1 immunotherapy in the immune "cold" tumor and abscopal tumor models (131).

Although metallic nanoparticles mediated PTT/PDT has achieved gratifying antitumor efficiency in shallow tumors (e.g., melanoma), it has not reached the desired therapeutic effect in OC. In an OC mouse model, PTT alone did not inhibit tumor growth or prolong survival (132). The reason is that (1) the complex suppressive TIME and (2) the OC tumors located in a deep part of the human body prevent a laser from irradiating the tumor. Recently, Qizhen Cao and his colleagues developed copper monosulfide (CuS) nanoparticles to mediate a pulsed wave (PW) laser that can treat OC. CuS nanoparticles mediate photothermolysis, resulting in tumor cell death and improving the antitumor efficiency of PD-1 immunotherapy by promoting antigen presentation and T-cell infiltration (133). In addition, Xiong J et al. developed Fe_3O_4 nanoparticles coated with hybrid biomimetic membranes, which were formed by the fusion of red blood cell membranes and mouse-derived ID8 OC cell

membranes. These metallic nanoparticles can extend the circulation half-life as well as homologous target ID8 OC cells and show synergistic PTT. Under this treatment, the tumor-specific antigens were released, which further improved the efficiency of immunotherapy by activating $CD8^+$ CTLs and decreasing $Foxp3^+$ Tregs (162).

Since the TIME in OC is also an important reason for limiting the effectiveness of PTT, combining strategies to modulate the TIME is a promising strategy to improve the effectiveness of PTT. The surface modifiability of metallic nanoparticles allows them to be coated with polymers and serve as vehicles for immunomodulators, such as adjuvants, cytokines, and siRNA, to regulate the TIME. For example, João Conde et al. constructed targeting peptide-modified gold nanoparticles encapsulated with siRNA against vascular endothelial growth factor (VEGF). Gold nanoparticles can selectively silence VEGF expression in tumor cells and TAMs, inhibiting immunosuppressive M2 TAMs (163). This strategy to remodel the TIME is expected to improve the antitumor effectiveness of PTT in OC.

Application of other nanoparticles in enhancing cancer immunotherapy

Besides the nanoparticles introduced above, many other nanoparticles, such as carbon-based nanomaterials (CNMs) and metal-organic frameworks (MOFs), have also been reported to enhance cancer immunotherapy. CNMs, including carbon nanotubes, carbon dots, and carbon nanohorns, have gained attention in biological applications (164). Among them, carbon nanotubes have been explored as photothermal transduction agents and drug delivery carriers due to their surface modification, enhanced cellular internalization, electronic and optical properties, and biocompatibility (165). Carbon nanotubes can mediate PTT and induce ICD in tumor cells, and serve as delivery vehicles for tumor antigens and immunoadjuvants (166). For example, Yong Li et al. constructed annexin A5- modified single-walled carbon nanotubes for synergistic metastatic breast cancer therapy with an anti-CTLA-4 antibody. The nanoparticle-mediated PTT enhanced the abscopal response of ICB and increased the 100-day survival of tumor-bearing mice (167).

MOFs are novel nanoparticles composed of metal ions or clusters and organic ligands (134). The properties of MOFs, such as high porosity, large surface areas, surface modification, and luminescence characteristics, endow them as good candidates for drug delivery and diagnosis agents (135). Recently, various immunomodulators, including immunoadjuvants, tumor antigens, photothermal agents, and sonosensitizers, have been encapsulated in MOFs to modulate the TIME and synergize with immunotherapy (136, 161, 168, 169). For instance, Jiali Luo et al. developed cancer cell membrane-coated triphenylphosphonium decorated MOFs. The MOF-loaded sonosensitizer facilitated antigen presentation by mediating sonodynamic therapy. Co-delivered TLR agonist R387 promoted DC maturation. When combined with ICB, this nanoplatform finally reversed the suppressive TIME and enhanced the antitumor efficacy of immunotherapy (168).

Conclusion and prospects

With a more in-depth understanding of the TIME, immunotherapy, especially ICB, tumor vaccines, ACT, and cytokines, has gained extensive attention in the treatment of OC. Although these immunotherapies have achieved excellent results in a variety of tumors, they are not ideal for the treatment of OC. This is mainly due to the suppressive TIME in OC, including Tregs, TAMs, MDSCs, and CAFs, which inhibit the antitumor immune response. Therefore, using smart strategies to transform the suppressive TIME into an antitumor state is of great significance for increasing the effectiveness of OC immunotherapy and extending patient survival.

In recent years, with the development of nanomedicine, immunotherapy combined with nanomaterials to modulate immune stimulation has achieved excellent preclinical and clinical efficacy. Mainstream nanoparticles include liposomes, micelles, SNPs, and metallic nanoparticles. Based on the excellent properties of nanoparticles in biocompatibility, drug loading, targeting capability, surface modification, and photothermal conversion, they have been widely used for regulating immune response and immunotherapy. On the one hand, nanoparticles can deliver immunomodulators that regulate the TIME of OC. On the other hand, photosensitizer-loaded nanoparticles or metallic nanoparticles can mediate PDT/PTT to induce the ICD of tumor cells, promoting antigen presentation and T-cell infiltration. These advantages make nanoparticles promising candidates for modulating the TIME and improving OC immunotherapy.

However, the toxicity, specific tumor targeting, and effectiveness of nanoparticles also need to be considered in clinical translation. In terms of toxicity, nanoparticles can not only interact with organism's cells or blood cells but also produce toxic ions by the dissolution of nanomaterials. Through these mechanisms, nanoparticles exert toxicity leading to the damage of cells or vital enzymatic functions (170). Several strategies hold promise for reducing these toxic effects of nanoparticles. The nanoparticle can carrier negative surface charges and attenuate nanoparticle-cell interaction through modification with ligands, such as PEG (171). Covering the shell material or minimizing the surface area can

reduce the dissolution of toxic ions (170). For specific tumor targeting, the poor manifestation of the EPR effect in the clinic and the nanoparticle-protein complex formed in systemic circulation cause off-target effects. Specifically, the differentials between animal models and human tumors and the complex TEM contribute to the failure of EPR-mediated targeting delivery in clinical translation. Several strategies, including enhancement of vascular permeability and depletion of tumor extracellular matrix, provide a chance to minimize the gaps between theoretical expectation and clinical outcome (172). Otherwise, serum proteins and opsonins are easily adsorbed on nanoparticle surfaces, which is probable to mask targeting ligands. Based on a deeper understanding of the interactions between nanoparticles and organisms, consideration of the protein corona effect when designing nanoparticles could improve the targeting efficiency (173). In terms of effectiveness, inefficient and unstable drug loading results in low drug concentrations in the TME and insufficient therapy. The limited light penetration depth also restricts the anti-tumor effects of photosensitive nanoparticle -mediated PTT. To improve the antitumor efficiency, nanoparticles with high pore volume and novel loading strategies have been developed that are beneficial for the enrichment of drugs at tumor sites (174). Some other approaches, including improving the photothermal conversion efficiency and developing NIR-II window PTT, are promising strategies to enhance the efficiency of PTT (175).

In this review, we discussed the impact of the TIME in OC on immunotherapy and mentioned the role of nanoparticles in modulating the TIME and improving the immunotherapeutic efficacy of OC. Using multiple approaches to overcome current shortcomings, we expect to leverage nanoparticle-based drug delivery systems to provide opportunities for the clinical application of OC immunotherapy.

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Conflict of interest

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

Publisher's note

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The role of interferons in ovarian cancer progression: Hinderer or promoter?

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Ovarian cancer (OC) is a common gynecologic malignancy with poor prognosis and high mortality. Changes in the OC microenvironment are closely related to the genesis, invasion, metastasis, recurrence, and drug-resistance. The OC microenvironment is regulated by Interferons (IFNs) known as a type of important cytokines. IFNs have a bidirectional regulation for OC cells growth and survival. Meanwhile, IFNs positively regulate the recruitment, differentiation and activation of immune cells. This review summarizes the secretion and the role of IFNs. In particular, we mainly elucidate the actions played by IFNs in various types of therapy. IFNs assist radiotherapy, targeted therapy, immunotherapy and biotherapy for OC, except for some IFN pathways that may cause chemo-resistance. In addition, we present some advances in OC treatment with the help of IFN pathways. IFNs have the ability to powerfully modulate the tumor microenvironment and can potentially provide new combination strategies for OC treatment.

KEYWORDS

interferons, ovarian cancer, tumor microenvironment, immune cell, immunotherapy, biotherapy

Introduction

Ovarian cancer (OC) has an insidious onset and a bad prognosis. And OC screening is not effective in reducing mortality (1). Nearly half of patients are diagnosed at stage III, when survival rates sharply decrease. The current main treatments are chemotherapy and surgery. Patients initially respond to treatment, but most patients ultimately relapse with resistant OC. Worse still, the global number of incident cases and deaths of OC increased as data showed from 1990 to 2019 (2). Therefore, new treatments or effective combinations need to be further investigated.

Findings suggest that the tumor microenvironment (TME) plays an important role in the development of OC. And an increasing amount of attention has been given to TME as a therapeutic potential in recent years. Interferons (IFNs) are an important class of pleiotropic cytokines in the OC microenvironment and are divided into three subtypes. In OC, one studies type-I-IFN (IFN-I) and type-II-IFN (IFN-II). Most cells in OC can secrete them, and various types of immune cells and cancer cells make a larger contribution. Accordingly, IFNs can regulate almost all cells in the OC microenvironment. IFNs directly affect not only tumor cell production, cell cycle, stemness property, migration, and drug resistance, but also immune cell recruitment, differentiation, activation, and immune activity by regulating cellular gene expression. Ultimately, IFNs have a huge impact on tumor progression. IFN treatment has a good clinical response in hematologic malignancies (hairy cell leukemia and chronic myeloid leukemia) and certain solid tumors (melanoma, renal cancer and AIDS-related Kaposi's sarcoma) through itself or as part of combination treatment (3–7). Although IFNs have received long-standing concerns in the study and treatment of OC, clinical studies of IFNs in OC have not yet achieved breakthroughs. A summary of studies on the mechanisms of different OC treatments revealed that many treatments need to function through IFNs, and perturbation of IFN signalling pathway could lead to the inability of some drugs. Therefore, make good use of IFN responses are beneficial to the treatment of OC. With the deepening of immunotherapy in recent years, the cooperation between IFNs and immunotherapy seemed to be effective. This review summarizes many associations between OC and IFNs, expecting to provide a reference for improving the cold OC microenvironment.

Secretion and regulation of IFNs in the microenvironment of OC

The TME in OC is extremely complex that various types of cytokines and cells exist. The main sources of IFNs are immune cells in the microenvironment. For example, pDCs (plasmacytoid dendritic cells) are the prime origin of IFN-I, $CD8^+$ T cells, NK cells and Th1 $CD4^+$ T cells are the main source of IFN-γ. And macrophages, cancer cells and etc. also secrete IFNs under the regulation of the microenvironment (8, 9) (Figure 1).

Tumor-infiltrating lymphocytes

Tumor-infiltrating lymphocytes (TILs), such as NK cells, $CD4^+$ and $CD8^+$ T cells play important roles in the OC microenvironment by secreting IFNs with immunity. Ascites contains a large number of $CD4^+$ and $CD8^+$ T cells, both of which can produce large amounts of IFN-γ. Compared to T cells in the blood of healthy body, however, T cells secretion ability of IFN-γ was relatively lower in blood, ascites and tumor tissue of patients with epithelial ovarian cancer (EOC) (10). Normally, $CD8^+$ T cells eradicate tumor cells by secreting granzyme B, TNF and IFN-γ after TCR attachment. Yet these TCRs in TMA cannot be awakened and recognized causing decrease in IFNs and development of OC (11, 12). Percentage of γδ T cells (innate lymphocytes with unbound MHC) in OC tissues was significantly higher than the cells in normal ovarian tissues. In contrast, patients had lesser levels of IFN-γ secretion by γδ T cells

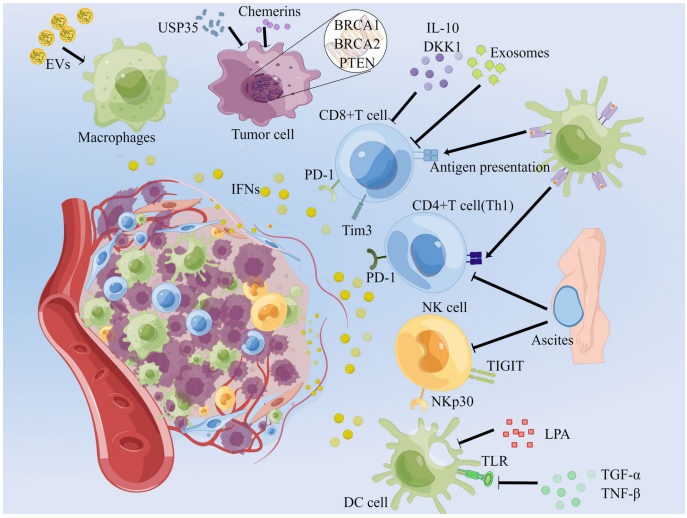

FIGURE 1
Secretion and regulation of IFNs in the OC microenvironment. Normal arrows represent the up-regulators of IFNs secretion, and T-shaped arrows imply barriers. In the OC microenvironment, EVs enriched in nucleic acids stimulate macrophages to secrete IFNs; unstable genes in OC cells result in the secretion of IFNs; $PD\text{-}1^+$ $Tim3^+$ tumor-infiltrating $CD8^+$ T cells are able to continuously secrete IFN-γ; blocking the PD-1 of $CD4^+$ T, $CD8^+$ T cells and TIGIT of NK cells can reduce IFN-γ; and promoting the activating receptor NKp30 plays the inverse effect. EVs: extracellular vesicles, DKK1: dickkopf-related protein 1, LPA: lysophosphatidic acid. This picture is drawn with the help of Figdraw.

in both peripheral blood and cancer tissues compared with the healthy and benign OC patients (13). Furthermore, when $CD8^+$ T cells were exhausted due to continuous exposure to tumor antigens in the OC microenvironment, they would express the immune checkpoint molecules PD-1, and Tim3. Cells with such characteristics had the ability to consistently produce IFN-γ (14). Th1 $CD4^+$ T cells produced high levels of IFN-γ in response to antigen stimulation (15). NK cells from OC ascites had the same ability to produce IFN-γ as healthy donor peripheral blood-NK cells. TILs would tend to secrete a great deal of IFNs to exert their antitumor effects under physiological conditions. Nevertheless, some external disturbances would modulate their secretory effects. Malignant ascites in OC patients inhibited glucose uptake and caused defective N-linked protein glycosylation in T cells, which triggered IRE1α-XBP1 activation that inhibited mitochondrial activity and IFN-γ expression (16). $CD8^+$ T cells co-cultured with B cells in ascites exhibited significant suppression of IFN-γ production, which was later found to be associated with IL-10 expression and low CD80/CD86. When IL-10 depletion was stimulated with CD28, IFN-γ secretion would be upregulated (17). This phenomenon was also present in other cancers in which IL-10 affected the signaling pathway and expression of IFN-γ (18). Mutation in the Wnt pathway was a hallmark of the endometrioid and clear cell subtypes of EOC. Dickkopf-related protein 1 (DKK1) overexpression associated with Wnt mutation decreased IFN-γ secretion from $CD8^+$ T cells (19). There was a decreasing expression of the NK cell activating receptor NKp30 in 50% peritoneal fluid of patients with serous tissue type OC. The fewer NKp30 may be associated with B7-H6 and cause a decrease in IFN-γ (20). TIGIT is an inhibitory receptor on the surface of NK cells. when it was blocked, the ability of NK cells' secretion of IFN-γ to would be increased in OC (21). $CD4^+$ and $CD8^+$ T cells isolated from OC patients were subjected to a significant increase in IFN-γ after the use of PD-1 blocking antibodies (22). Overexpression of Pyruvate dehydrogenase kinase 1 (PDK1) in OC cells impaired IFN-γ secretion in $CD8^+$ T cells by upregulating PD-L1, and an increase in intra-tumor of IFN-γ was observed with DCA (a PDK inhibitor) and anti-PD-L1 antibodies (23). Exosomes in the ascites of OC patients had an inhibition on IFN-γ secretion for $CD4^+$ and $CD8^+$ T cells, but the suppression can only be maintained within 24-48 hours after disconnection from exosomes (24). Although tumor antigens can stimulate TILs to secrete IFNs, the ability of secretion IFNs for TILs in OC tissues was impaired in general, which may be an important reason for the suppressive immune microenvironment in OC.

Antigen presenting cells

Antigen-presenting cells significantly contribute to IFNs production, especially dendritic cells (DCs). PDCs produce large amounts of IFN-I upon stimulation. TLR7 and TLR9 of pDCs were activated to produce IFN-α for tumor-killing (25, 26). Nevertheless, the IFNs secretion function of pDCs is repressed under some conditions. The ligands of the TCR9 and TCR9 were blocked in OC by the action of soluble factors TGF-β and TNF-α cooperation, causing a decrease of IFNs (27). In addition, recent studies have found that lysophosphatidic acid (LPA) was abundant in malignant ascites. It was derived from ATX, which was released by OC cells. LPA triggered the biosynthesis of PGE2 in multiple subtypes of DCs, which inhibited IFN-I signal transduction through the involvement of autocrine EP4. This signal down-regulated multiple IFN stimulated genes (ISGs), which reduced activation and infiltration of $CD8^+$ T cells and NK cells (28). Aberrant epigenetic modifications of TP53 in OC can generate aberrant repeat genes. EVs (extracellular vesicles) can enrich these repeat RNAs, which were then sensed by pattern recognition receptors and induced IFN-I responses in human primitive monocyte and macrophage cell lines (29).

OC cells

Cancer cells are usually accompanied by genetic instability, which causes the production and accumulation of abnormal RNA or DNA, and ultimately induces IFN responses. BRCA mutations are common in OC. It is found that BRCA1 loss allowed OC cells to exhibit a cell-autonomous inflammatory state leading to chromatin reorganization and transcriptional reprogramming. By increasing the sensitivity of the dsDNA sensing pathway and enhancing the supply of cytoplasmic dsDNA to STING (stimulator of interferon genes), DNA sensing and inflammatory (DS/IFN) pathway was overexpressed, thereby causing IFN responses (30, 31). Tumor-prone cells carrying BRCA2 inactivation underwent loss of chromosomal integrity and accumulated cell membrane DNA in the form of micronuclei. Micronucleus bound DNA sensor cGAS and activated the IFN responses (32). In addition to BRCA mutations, PTEN mutation also affected IFN signaling. PTEN deficiency failed to activate the IFN signaling pathway, thus promoting a tumor immunosuppressive microenvironment (33). Furthermore, Jiawei Zhang et al. found that the ubiquitinase USP35 was upregulated in OC tissues, and the upregulation may inhibit IFN-I expression in cancer cells through the STING-TBK1-IRF3 pathway (34). Chemerin is a pleiotropic adipokine that has an important role in the immune system. It was found that co-culture of Chemerin and OC cell lines increased the level of IFN-α approximately fourfold in the culture medium, thereby activating IFN-α responsive genes, such as IFI27, OAS1 and IFIT1, IFI44L, its upstream regulator IRF9 and etc. (35). Although OC cells induce IFN responses and promote immune responses due to their "self-deficiency", the IFN responses are almost ineffective. Immune cells are damaged by OC TAM, which makes it difficult for these immune cells to survive and respond to the stimulation of IFNs. In summary, IFNs can be expressed by a variety of cells in the OC

microenvironment. Apart from cells mentioned above, tumor-associated fibroblasts and endothelial cells also express IFN-I. The relative contribution of each cell to total IFNs levels may depend on the quantity and quality of each cell type within the tumor and is regulated in multiple layers within the OC microenvironment.

The roles of IFNs in ovarian cancer immunity

In early studies, researchers focused on the direct effects of IFNs on OC cells and ascites. With the increasing understanding of tumor immunity, people gradually began to realize the powerful regulatory role of IFNs on TME. And researches on finding the association between IFNs and tumor cells, immune cells and TME are gaining ground.

The effects of IFNs on ovarian cancer cells

To date, the role of IFNs on OC cells is highly controversial. On the one hand, IFNs can adversely affect the survival of OC cells, including their proliferation, metastasis, apoptosis and immune activation. On the other hand, IFNs favor the survival of OC cells, such as helping their growth, metastasis, and drug resistance (Figure 2).

In early studies, it was believed that IFNs had a positive effect on OC treatment. IFNs can induce apoptosis of OC cells directly through the death receptor-mediated pathway and mitochondrial pathway. And the expression of immune-related receptors on ovarian cancer cells is promoted, thus helping immune cells to detect OC cells. In experiments with inoculated NIH-OVCAR-3 cells to nude mouse, IFN-β was found to induce strong expression of Apo2L/TRAIL (Apo2L/tumor necrosis factor-associated apoptosis-inducing ligand) which can combine with death receptors DR4 (TRAIL-R1) and DR5 (TRAIL-R2) inducing apoptosis in OC cells (36, 37). IFN-β also increased human inositol hexakisphosphate kinase 2 (IP6K2) expression by post-transcriptionally regulation, which promoted apoptosis in the nucleus (38). High levels of ISG12A mRNA were found in stage III plasmacytoid OC. ISG12A mRNA impacted apoptosis through the mitochondrial intrinsic pathway, and the expression level of ISG12A in hepatocellular carcinoma was positively correlated with TRAIL-induced apoptosis (39–41). IFN-γ inhibited STAT3 and STAT5 protein phosphorylation pathways in a concentration-dependent manner by upregulating SOC1. In this way, OC cells proliferation, migration, cell cycle and invasion were impeded (42). IFN-α and IFN-γ blocked tumor cell growth and proliferation by reducing RNA synthesis, amino acid uptake and protein synthesis (43). Cell cycle arrest and cytotoxicity in OC cells were caused by HIFN-γ through activation of p53 and p21, leading to cell death (44). Cooperation of granzyme B, IFN-γ, and others from $CD8^+$ T cells and NK cells directly killed OC cells (11, 45). In addition, IFN-β signaling oppressed telomerase activity and reverse transcriptase transcription in OC through the p21WAF1 pathway, which ultimately induced apoptosis in human OC cells (46). Studies have shown that pDCs induce immunosuppression and promote tumor growth in human OC and myeloma. Although the molecular mechanisms by which

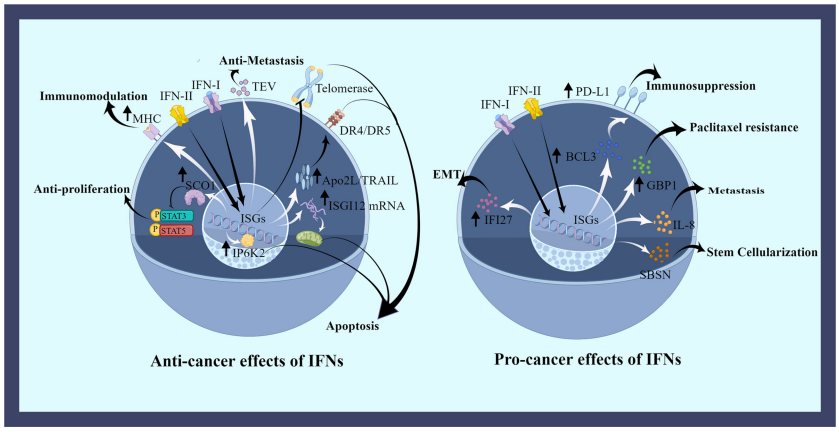

FIGURE 2
The effects of IFNs on ovarian cancer cells. IFNs have both favorable and unfavorable roles in OC cells. IFNs regulate OC cells by promoting the expression of some proteins or inhibiting some pathways. IFNs induce apoptotic effects in the nucleus, mitochondria, and cell membrane. Cell cycle is dysregulated by disrupting phosphorylation. The uptake of regulated tumor extracellular vesicles (TEVs) is inhibited by IFNs counteracting metastasis. And IFNs promote the expression of tumor antigens to aid immune killing. Many negative effects of the elevated protein expression caused by IFNs have been demonstrated, including OC cells metastasis, drug resistance, stemness, epithelial mesenchymal transition (EMT), and immunosuppression. This picture is drawn with the help of Figdraw.

pDCs acquires these properties were unknown, it was interesting to note that human pDCs activated by CpG-containing DNA inhibited the growth of myeloma cells and induced apoptosis *via* producing IFN-α. Nevertheless, direct contact between myeloma cells and pDCs would degrade TLR9 of pDCs, which greatly reduced IFN-α expression, and promoted tumor progression (47). In Moreover, major histocompatibility complex (MHC) and tumor antigen expression on the surface of tumor cells were regulated by IFNs. However, this was related to the heterogeneity of cancer cells; some OC cells exposed to IFN-γ upregulated their cell surface MHC molecules and thus were killed by T cells, while some OC cells did not respond after stimulation by IFN-γ, and such hypo differentiated cells were killed by NK cells (48). IFNs affected not just the apoptosis and immunity of cancer cells, but also their metastasis. Regulated tumor extracellular vesicles (TEVs) secreted by tumors were able to degrade IFNAR1, thus inhibiting IFN-I, impairing ISGs expression and aiding in the formation of pre-metastatic ecological niches. And the uptake of TEVs were reduced by sustained IFN signaling, then metastatic process was compromised (49).

As people have studied more, however, it has been found that certain IFN signaling simultaneously contributes to the deterioration of OC cells. IFN-I responses were stimulated in tumor-prone cells of BRCA2 inactivated. Upregulation of ISGs resulted in cell cycle resumption. IFN responses help dying OC cells resume cell cycle and continue to proliferate (32). IFN-γ stimulated the release of full-length GBP1 in SKOV3 and OVCAR5 *via* a non-classical secretory pathway. GBP1 is a guanylate-binding protein with GTPase activity, which impedes cell production and angiogenesis. It inhibited tumor cell growth in breast cancer, and the expression of GBP1 in OC was associated with paclitaxel resistance, predicting a significantly shorter progression-free survival in OC (50–53). IFI27 was an overexpressing protein induced by IFN-α in OC tissues. It induced epithelial mesenchymal transition of OC cells and promoted migration and invasion of cancer cells (54). IFN-γ had the similar role, inducing IL-8 expression through JAK1/STAT1 signaling and p65 NFκB-mediated, thus helping OC cells to migrate (55, 56). In Addition to that, the synergistic effect of IFN-γ/JAK and ERK signaling pathways induced the expression of skin-specific protein suprabasin (SBSN) in OC cells, which reduced the adhesion of cancer cells and made them more resistant to apoptosis of lost nests, aiding OC cells metastasis and stem-cell-like property (57). PD-L1 expression can be induced either by OC cells or lymphocytes (58, 59). IFN-γ in OC cells amplified PD-L1 expression *via* JAK1/STAT1 and IRF1 signaling in a dose-dependent manner (60–62). Also, IFN-γ induced PD-L1 expression was also associated with Bcl3. IFN-γ was an agent that increased the expression of Bcl3 in OC cells, leading to increased transcriptional activity of PD-L1. PD-L1 expression was significantly reduced in OC cells stably transfected with Bcl3 shRNA (63, 64).

In summary, IFNs' effects are variable. On the one hand, OC cells can be killed with IFNs through direct or indirect effects. And on the other hand, they support their survival. IFNs not only help the formation of cancer cells, but also their metastasis, immune escape and drug resistance.

The effects of IFNs on immune cells in ovarian cancer microenvironment

It is well known that the presence of IFNs implies a pro-inflammatory tumor immune microenvironment. And a high level of IFNs generally facilitates better performance of immune cells. Abnormalities in tissues cause IFN responses that attracts immune cells and enhances their immune function to fight or clean up abnormal substances (65). IFNs initiate and promote immune responses that help the infiltration of immune cells, differentiation toward anti-tumor cells, activation of immune cells, and presentation of antigens, which are conducive to improving the warmth in the immune microenvironment of OC (Figure 2). BRCA1-deficient OC cells underwent chromatin reorganization and transcriptional reprogramming, a process that caused sensitization of the dsDNA-sensing pathway and excessive accumulation of cytoplasmic dsDNA, which then provoked an IFN response *via* the STING pathway, an inflammatory state that can aid in the recruitment of T cells and activation of DCs in tumor (30, 31, 66). $CD4^+$ T (Th1) cells produced high levels of IFN-γ in response to antigenic stimulation, and then the secreted IFN-γ further enhanced Th1 cell development and stimulated macrophages to produce reactive oxygen nitrogen species and TNF-α to kill OC cells (15). Among patients with recurrent metastatic OC inoculated into mRNA-encoded folate-receptor-α transfected autologous DCs, the phenomenon of increased $CD8^+$ and $CD4^+$ T cells was observed due to the rise in the production of IFN-γ (67). EVs with abundant RNA induced IFN-I responses in human primitive monocyte and macrophage cell lines, which induced MHC-I expression for surveillance of the immune system and enriched immune promoting cells (29). Macrophages were induced to differentiate into M1-types with antitumor activity by lipopolysaccharide (LPS) *via* IFN-γ, and IFN signaling in ascites-associated macrophages was associated with good clinical outcomes in a subset of OC patients (68). It is believed that the cooperation between CCL5 and CXCL9 (IFNγ-inducible chemokine) could contribute to immune activation in OC. CCL5 is expressed by cancer cells and CXCL9 is produced by immune cells in cancer tissue. First, CCL5 produced by cancer cells attracted T cells into the tumor tissue, then T cells activated cancer antigens, then tumor antigens induced CXCL9 to secrete by immune cells dependent on the release of IFN-γ, and finally CXCL9 promotes further tumor infiltration by T cells. CCL5 and CXCL9 were linked by IFN-γ in a positive cycle that helped the establishment of hot tumors (69).

Correspondingly, abnormal factors that cause IFN signaling to be reduced or inhibited are detrimental to the infiltration and action of immune cells in cancerous tissue. PERK is an intermediate kinase of the unfolded protein response (UPR), which is activated at an elevated rate in malignant cells. It promoted immunosuppression of tumor Myeloid-derived suppressor cells (MDSCs) by stimulating the transcription factor NRF2. PERK also directly limited IFN-I responses by inducing phosphorylation-driven degradation of IFNAR1. The presence of PERK promoted immune suppression mediated by myeloid-derived suppressor cells (70). Jiawen Zhang et al. found that the ubiquitinase USP35 was upregulated in OC tissues. Because USP35 is a negative regulator of STING-related IFN-I signaling, its high level was negatively correlated with $CD8^+$ T cells, macrophage, neutrophil and DC infiltration in OC patients (34). In response, USP30 is a deubiquitinating enzyme on the outer mitochondrial membrane. The researchers found that sustained killing ability of TILs was reduced in USP30-deficient mouse, and its deletion led to mitochondrial abnormalities that affected the translation process of IFN-γ (71). More interestingly, when TILs express low levels of IFNs, this class of cells may have a suppressive effect on the TME. A study of OC patient specimens revealed that the percentage of γδ T cells was significantly higher in OC tissue than in marginal OC tissue and normal ovarian tissue. Also, there was a positive correlation between the higher number of γδ T cells in OC tissues and the advanced clinicopathological characteristics of OC patients. This is due to the comparatively low level of IFN-γ secreted by γδ T cells, a class of cells with weak cytotoxic effects and immunosuppressive activity in the OC microenvironment (13). Analysis of mRNA in peripheral blood lymphocytes stimulated by exosomes from malignant ascites revealed that malignant vesicles contributed to the formation of an immunosuppressive microenvironment within OC through IFN responses (72).

The roles of interferons in OC treatment

In most cases, the treatment of OC triggers IFN responses, which further enhance these therapeutic effects by modulating the immune system. IFN responses also may impede therapeutic effects at same time. With the exception of chemotherapy, which has little to do with immune activation of OC, other treatments act through immune activation and are associated with IFN response (Figure 3).

Radiation therapy

Radiotherapy (RT) is a common and effective modality for treatment of OC to control cancer by inducing tumor cell death through DNA damage. Coincidentally, DNA damage activates cytoplasmic nucleic acid sensor pathways that induce inflammatory signals such as IFN responses, which reshape the immune environment of the TME (73). DNA damage from RT formed micronuclei and chromatin bridges in irradiated tumor cells and activated the cGAS/STING pathway, leading to IFN-I production. Thus, the antitumor effect of RT was abolished in IFN-I non-responsive hosts (74–76). This pathway was dependent on IFN-I signaling on

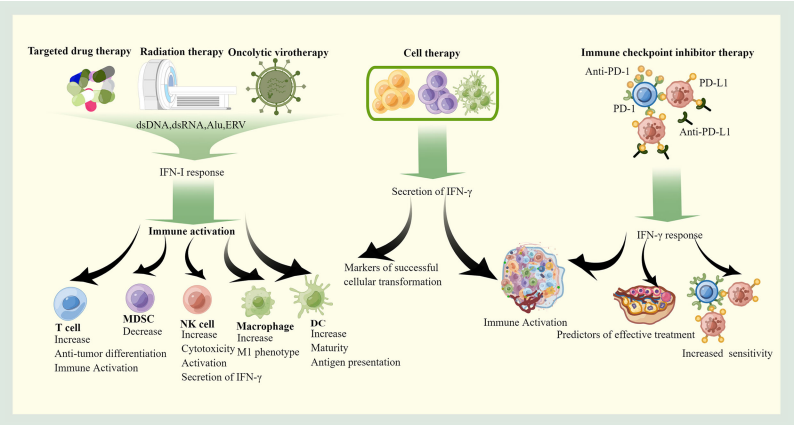

FIGURE 3
The roles of IFNs in therapy. Targeted therapy, radiotherapy and Oncolytic virotherapy stimulate the immune system of OC mainly through the IFN-I pathway, which promotes the recruitment and activation of pro-immune cells. Cell therapy can autonomously secrete IFN-γ to exert anti-cancer effects, and immune checkpoint inhibitor therapy requires indirect induction of IFN-γ production through feedback from immune checkpoints. The produced IFN-γ helps the formation of immune-friendly microenvironment, and high IFN-γ is associated with good prognosis. This picture is drawn with the help of Figdraw.

DCs. IFN signal caused enhanced antigen uptake and presentation by DCs, which initiated naive T cells into an effector phenotype. STING-deficient mouse had much weaker CD8 T-cell response after radiation exposure than WT mouse, and IFN-β treatment was able to rescue the cross-initiation of cGAS or STING deficient DCs (77). In addition, radiotherapy-produced dsRNAs were involved in the IFN-I responses through the RLR family (78, 79). Notably, the RT-induced intrinsic IFN-I responses in cancer cells based on the dose and regimen. The DNA exonuclease Trex1 was able to attenuate the IFN-I responses by decreasing DNA that accumulated in the cytoplasmic matrix during RT. Therefore, it was important to adjust the RT dose that was just below the threshold for activation of Trex1 (80, 81). IFN-γ was also increased after RT. In the RT setting, IFN-γ have no significant direct function on tumor cells. However, comparison the TME under RT in IFN-γ deficient and wild mouse revealed that IFN-γ upregulated VCAM-1 expression of adhesion molecules on tumor vessels in the condition of local radiation, while increased IFN-γ induced chemokines CXCL9 and CXCL10, which contribute to the flow of activated T cells to irradiated tumors (82). In addition, RT enhanced the ability of T cells killing malignant cells in an IFN γ-dependent manner reducing the tumor burden (83). Combination therapy for high-dose RT ICB for OC have tried. Due to the diffuse spread of OC throughout the abdominal cavity, the abdominal viscera are exposed to the high toxicity risk of conventional RT. Follow-up studies found that RT with low-dose radiotherapy could also reprogram the TME (84, 85). Recent studies have shown that 1 Gy irradiation in whole abdominal RT in mouse with advanced OC was sufficient to induce important transcriptional changes *in vivo*, including IFN-α and IFN-γ responses, as well as cross-presenting DCs, which promoted T-cell infiltration (86). IFNs produced by the induction of RT create a positive TME for therapy.

Chemotherapy

The current mainstream chemotherapy is based on paclitaxel and platinum for OC. However, some patients develop chemotherapy resistance in the late stage of treatment, which has been found to be tightly associated with the IFN responses. Part of the IFN responses resist chemotherapy resistance, while part of the responses contributes to the development of chemotherapy resistance. The release of glutathione and cysteine from tumor fibroblasts reduced the nuclear accumulation of platinum in OC cells, leading to resistance to platinum chemotherapy. $CD8^+$ T cells secrete IFN-γ to control fibroblast glutathione and cysteine, thereby reducing fibroblast-mediated platinum resistance *in vivo* (87). Comparison of cisplatin-resistant OC cells with non-resistant cells indicated that ISGI15 (IFN-I induced) was reduced in resistant cells. Free ISIG15 increased the sensitivity of OC cells to cisplatin, and a decrease of ISGI15 was associated with poor prognosis (88). A study analyzing the metabolome and proteome in normal and resistant group OC cells revealed perturbations of IFN signaling in resistant group cancer cells. Tyrosine kinase 2 (TYK2), an enzyme that plays a key role in IFN-I signaling, was reduced in abundance in CAOV3 CBPR cells (89). This conclusion was similarly validated by clinical data showing that patients with high IFN-γ in the immune microenvironment had a better prognosis in platinum-treated patients (90). Furthermore, it has been shown that cancer cells pretreated by IFN-β were more likely to expose calreticulin and aided immunogenic cell death (ICD) when receiving platinum treatment through the IRF1 pathway (91). However, due to the numerous downstream regulatory pathways of IFNs, some pathways may contribute to drug resistance in OC cells. IFI16 belongs to the IFN-inducible PYHIN-200 gene family. Upregulation of IFI16 expression was observed in paclitaxel-resistant and adriamycin-resistant cell lines of OC. Though its real role had not been elucidated yet, it was speculated that it may involve in the regulation of drug-resistant gene expression (92). Meanwhile, extensive preclinical data suggested that many chemotherapies, including oxaliplatin, cisplatin, paclitaxel and 5-fluorouracil, promoted the upregulation of co-inhibitory ligands such as PD-L1. And this is usually a consequence of IFN-I or IFN-γ signaling, which together render drug ineffectiveness (93).

Targeted drug therapy

Targeted drugs usually need to trigger IFN responses or depend on the IFN pathway to act. We will describe some drugs in targeted OC therapy that are associated with IFNs, such as poly (ADP) -ribose polymerase (PARP) inhibitors and epigenetic modulators.

Small molecule inhibitors of PARP (PARPi) have been approved for clinical use in the treatment of BRCA1 and BRCA2-deficient OC (94). The use of PARPi blocked the repair of single-stranded DNA damage in BRCA2-deficient cells, further promoting the exacerbation of high levels of DNA damage inherent in BRCA2-deficient cells. Antigen-presenting cells (APCs) sensed these accumulated dsDNA fragments, which drove the activation of IFN-I signaling, which would contribute to better anti-cancer immune action of PARPi (66, 95). A follow-up study found that PARPi was sensed by the cGAS-STING pathway *via* DNA and stimulated IFN-I production. And this process was independent of BRCA mutations, implying that ovarian cancer cells were triggered IFN-I responses by the use of PARPi (96). However, the secretion of total IFN-γ in immune cells treated with this drug was reduced. It was because of the significant increase in the activity of STAT3, which was a negative regulator of IFN-γ. Such negative regulation caused tumor resistance and immunosuppression (97). This also means that relying on

direct regulation of IFNs by PARPi for may not be effective for anticancer effects due to the bidirectional regulation of PARPi for IFNs. Drugs affecting epigenetics such as AZA (dnmti 5-azacytidine), histone deacetylase inhibitor (HDACi), and etc. caused transcription of repeat elements, forming dsRNA and provoking IFN responses. In the OC model, a clear upregulation of inverted Alu repeats was found with AZA. Inverted Alu repeats can combine with MDA5 (dsRNA sensors), which provoked IFN signaling (98, 99). Additionally, AZA also acted as a DNA methyltransferase inhibitor, which can bind to both DNA and RNA, inhibiting the RNA methyltransferase. Thus, AZA demethylated RNA, and unmethylated RNA activated IFN-I. Through IFN-I signaling, AZA increased immune-promoting cells in the TME while reducing ascites and tumor burden, prolonging survival. If IFNAR1 was blocked, then IFN-I signaling would be restricted and the antitumor effect of AZA would also be limited (100). Stimulation of IFNs by AZA was enhanced in P53-mutant mouse (101). The combination of DNMTi and HDAC6i amplified IFN-I more than either one, reversing the immunosuppressive TME but also increasing PD-L1 expression on the cell surface. Overall, they perform only modest effects on survival (102). IFN-dependent antitumor immunity was stimulated by an increase in endogenous retroviral elements (ERV) induced by inhibition of histone lysine specific demethylase 1 (LSD1). SP-2577 is an LSD1 inhibitor. SP-2577 promoted ERV expression in ovarian cancer cells and activated the dsRNA-induced IFN pathway, which facilitated cytokine expression and infiltration of immune cells in ovarian hypercalcemic organoids (103). CX-5461, an RNA polymerase I inhibitor, led to the accumulation of cytoplasmic dsDNA activating the cGAS-STING-TBK1-IRF3 innate immune pathway, which induced IFN-I and promoted the secretion of IL-6 and CXCL10, contributing to T cell infiltration (104). These drugs not only act directly on cancer cells, but also stimulate the IFN through their subsequent chain reaction, affecting the immune microenvironment and causing a continuous anti-cancer effect.

Immunotherapy

Research on immunotherapy is promising in the treatment of OC, especially for immune checkpoint blockade therapy. Immune checkpoints and IFNs are closely linked due to the fact that changes in immune checkpoints trigger IFN responses and IFN responses also modulate immune checkpoints. At the same time, IFNs serve as predictors of immune checkpoint blockade treatment status. Clinical studies have found that the release of IFN-γ was significantly increased when TILs isolated from OC patients were co-incubated with PD-1 antibodies, reversing the immunosuppressed OC microenvironment to some extent (22, 105). *In vivo* imaging to track intra-tumor factor changes after a PD-1 treatment, and single-cell sequencing validation suggest that the process of successful anti-PD-1 cancer immunotherapy performs as follows. T cells secreted IFN-γ to activate DCs to release IL-12, and then IL-12 stimulated T cells to continue to secrete IFN-γ and activated other immune cells, and then a positive circulating immune response was established within the tumor, ultimately leading to tumor killing (106). At the same time, IFN-I helped carboplatin-treated HGSC sensitize to immune checkpoint blockade therapy *via* STING pathway, and studies have also found that tumor cell resistance to immune checkpoint blocking drugs was associated with reduced sensitivity to IFN-γ signaling or loss of IFN signaling (107, 108). IFNs do not merely have an important effect on PD-1, but also have a close relationship with PD-L1. When PD- L1 knockout (KO) and control OC cells were inoculated intraperitoneally into syngeneic mouse, the PD- L1-KO group showed a significant increase of $CD4^+$ T cells, $CD8^+$ T cells, NK cells and $CD11c^+$ (M1-like) macrophages compared to the control group, and Th1-type cytokines such as IFN-γ were also significantly increased (109). PD-L1 blockade was also capable of increase IFN-γ secretion, and the phenomenon was more pronounced after combination with the pyruvate dehydrogenase kinase inhibitor dichloroacetate (DCA) (23, 110–112). More importantly, it has been shown that resistance to immune checkpoints and loss of IFN-γ signaling were related. Interruption of IFN-γ signaling prevented the induction of PD-L1 expression, rendering PD1-PD-L1 blockade ineffective (113, 114). A statistical review of clinical study data revealed that basal serum IFN-γ levels were associated with disease control rates and overall survival in cancer patients. Patients with high IFN-γ showed better performance in immune checkpoint inhibitor therapy (115). The analysis also suggested that IFN-γ, or IFN-α, IFN-γ, IL-2 combined with TNF-α secretion could predict the efficacy of PD-1 inhibitors in cancer patients (116, 117).

In addition to the immune checkpoints mentioned above, there are some immunomodulatory drugs which also exert anti-cancer effects by modulating the TME through IFN signaling. IL-15 super agonist (N-803) increases NK cell proliferation and IFN-γ production, overcoming the defective NK cell function caused by the immunosuppression of OC. Co-culture with N-803, NK cells and OC-like organs revealed that N-803 increased IFN-γ secretion from NK cells and further increased IFN-γ induced CXCL10 secretion enhancing NK cell cytotoxicity against OC (110, 118). CDK4/6 expression was higher in OC tissues than in normal ovarian tissues. High CDK4/6 expression resulted in an immunosuppressed state in OC and was associated with poor prognosis for OC patients. The CDK4/6 inhibitor palbociclib activated immunity in OC by increasing the secretion of IFN-γ and the expression of ISGs, which upregulated the expression of antigen-presenting molecules (119).

Cell therapy

Cell therapies such as CAR-T therapy, NK cell treatment, and DC vaccines have been extensively studied in OC clinical trials. CAR-T cells enhance cytotoxic effects on malignant cells by specifically recognizing surface antigens on tumors and secreting cytokines like IFN-γ. And then, IFN-γ can inhibit tumor progression by promoting the secretion of downstream cytokines, infiltration of T cell and avascular necrosis (120, 121). 5T4 is a tumor-associated antigen that is actively expressed on the cell surface of most solid tumors, including OC. Effective transduction of patient T cells with anti-5T4 CAR and antigen-specific secretion of IFN-γ was produced by the co-culture of CAR-T cells and matched autologous tumor catabolites. And IFN-γ production correlated with tumor cell surface 5T4 expression levels (122, 123). In addition, CAR-T therapy modulated the differentiation of T cells through IFN-γ affecting tumor immunity (124). One study constructed CAR-T cells with a lentiviral vector, which released a large number of cytokines, such as IL-2, IFN-γ and TNF-α, to activate T cells and NK cells to promote massive release of factors. The CAR-T demonstrated a strong killing ability against OVCAR-3 cells *in vitro* (125). However, results in hematological malignancies showed that IFN-γ was not required for the efficacy of CAR-T, and that IFN-γ inhibition reduced the secretion of other toxic factors and improved the efficacy and clinical durability of CAR-T (126). IFN also can be used alone or in combination with other compounds to mature DCs in ex vivo production (127). Because of the secretion of IFN-γ of DCs and NK cells is one of the indicators of successful construction. Injecting these cells into the body induced therapeutic immunomodulatory effects by Continuously releasing IFN-γ (67, 128–130). In conclusion, IFNs play an important role in cell therapy. The function of IFNs is not only as participant in cell construction, but also as indispensable worker in treatment.

Oncolytic virotherapy

Oncolytic viruses (OV) can exert a therapeutic effect by direct lysis of tumor cells, or transporting therapeutic genes. OC cell lines are capable of making an IFN-I response to induce an antiviral state upon viral infection, which is in the contrast to other cancer cell lines. In the treatment of OV, there is a mutual resistance in treatment of OV between the virus and the IFNs. On the one hand, IFNs block and clear the OV in the organism so that the OV is ineffective against the tumor. On the other hand, the OV stimulates the IFN response, which activates immunity to operate against the tumor. The IFN response inhibited the vesicular stomatitis virus-glycoprotein (VSV-GP), shifting cancer cells to an antiviral state and making them resistant to VSV-mediated tumor lysis. This inhibition can be reversed as IFN signaling was regulated with the Jak1/2 inhibitor ruxolitinib (131). Despite the fact that IFN responses impede the action of OV, OV-induced IFN secretion may recruit more immune cells. Lysing adenovirus delivering TNF-α and IL-2 was found to increase IFN-γ secretion in isolated OC tissues, accompanied by T cell activation, promoting infiltration of tumor lymphocytes in the OC microenvironment (132). Defective IFN signaling may be more conducive to OV sensitivity. Cells with the disruption of innate antiviral defenses associated with IFN were more vulnerable to viruses. In detail, cell sensitivity to viral infection was associated with expression level of IFNAR, genes induced by IFNs, pattern recognition receptors and JAK/STAT pathway (133). And this was proved in many malignant cells (134–136). More interestingly, IFN-I secreted by pDCs enhanced the lytic activity of non-replicating HSV-1d106S (137).

Application of IFN response

Researches on the utilization of IFNs for the treatment of OC have been under way for decades. Indeed, effective treatment with IFNs in OC has been tried, comprising the use of IFNs for the direct treatment of OC, the combination of IFN therapy with other therapies, and treatment with the help of recovery the IFN response in OC. Some clinical trials related to IFN response in OC treatment in recent years are shown in Table 1.

Due to the powerful effects of IFNs on tumor cells and immune microenvironment, many works have been attempted to apply IFNs directly to OC therapy. But the metabolic characteristics of IFNs and the prevalent expression of IFN receptors have limited their application. Attempting local administration and modification of IFNs to improve their bioavailability and targeting is a research trend. For example, coupling IFNs with polyethylene glycol, hyaluronic acid, or aluminum salts not only prolonged the duration of action but also improved drug targeting, allowing them to be trapped in the peritoneal cavity or in the tumor (138–141). In addition, bone marrow mononuclear cells (iPS-ML) have been genetically modified to produce IFN-β. This class of cells reduced cancer cells in an iPS-ML/IFN-β dose-dependent way when co-cultured with OC cells. When injected into OC mouse with ascites, iPS-ML/IFN-β infiltration into the cancerous tissues was observed and cancer-associated ascites was dramatically reduced (142).

In addition to structural modification and exogenous introduction of IFNs to treat ovarian cancer, combining IFNs with other modalities to adjuvant therapy, especially with immunotherapy to improve the suppressive immune microenvironment of OC is a prospective therapeutic tool for establishing hot tumors. The TLR4 agonist MPLA stimulated IFN-I signaling in combination with IFN-γ. Activated macrophages and cytotoxic T cells by IFN-I reversed immunosuppression, prolonged the median survival of tumor-bearing mouse and inhibited metastatic progression of OC

TABLE 1 The clinical trials based on IFN response in OC.

Type	Combination Therapy	Disease	Phase	Status	Reference	Remark
IFN-α	Carboplatin+Paclitaxel+TILs		I/II	Recruiting	NCT04072263	IFN α-2b
	Cisplatin+Celecoxib+ DC Vaccine		I/II	Suspended	NCT02432378[147]	A cocktail of rintatolimod and IFN-α
	Denileukin Diftitox	EOC	II	Terminated	NCT01773889	PEG-IFN α-2b
		EOC	I/II	Terminated	NCT00085384	PEG-IFN α-2b
	Gemcitabine+P53 SLP Vaccin	Recurrent OC	I/II	Completed	NCT01639885	PEG-IFN α-2b
	Radiolabeled Monoclonal Antibody +Paclitaxel		I	Completed	NCT00002734	Recombinant IFN-α
	Carboplatin +Doxorubicin+ Tocilizumab	Recurrent OC	I/II	Completed	NCT01637532	PEG-IFN α-2b
	IL-2+Sargramostim		II	Completed	NCT00003408	Recombinant IFN-α
IFN-β	Recombinant Adenovirus-hIFN-β	Cancer	I	Completed	NCT00066404	
IFN-γ	Carboplatin And Paclitaxel		III	Terminated	NCT00047632	IFN γ-1b
	Tumor Vaccine	Recurrent and Epithelial OC	I	Completed	NCT00004032	
	GM-CSF+Carboplatin		II	Completed	NCT00501644	
IFN-α IFN-γ	Autologous Monocytes		I	Terminated	NCT02948426[141]	PEG-IFN α-2b
Activator of STING	Pembrolizumab	Advanced Solid Tumor	I	Recruiting	NCT04609579	SNX281
IFN	Recombinant L-IFN Adenovirus Injection		I	Recruiting	NCT05180851	

IFN, interferon; OC, ovarian cancer; EOC, Epithelial ovarian cancer; TILs, Tumor infiltrating lymphocytes. Blanks in the disease column refer to ovarian cancer. Blanks in the remark column refer to subtypes of interferon that are not specified.

(143). The binding of IL-4-PE, IFN and IFN caused increased cell death for both OC cells *in vitro* and *in vivo*, increasing tumor-bearing mouse survival. Mechanistically, the synergistic antitumor effect was dependent on IFN signaling, and key proteins activated by both IFNs and IL-4-PE had a critical role in the apoptotic pathway (144). Monocytes have been shown to be cytotoxic to tumor cells in the absence of pro-inflammatory cytokines. In mouse models, stimulation of monocytes with IFN-α and IFN-γ resulted in a significant reduction in tumor volume and an increase in overall survival, which was achieved by modulating intra-tumor immunity (145). During clinical trials, autologous monocytes were stimulated with IFNs *in vitro* and then injected into the peritoneal cavity of patients with advanced chemotherapy-resistant OC. The results showed that IFN α-2a or IFN γ-1b had potent antitumor effects *in vitro* and *in vivo*, and their effects were multiplied with the addition of monocytes (146). Combining adriamycin and IFN-β, IFN-β can promote DOX-mediated cell death (147). A recent study used IFN-γ to transport DOX in the form of nanoparticles. Such nanoparticles greatly increased apoptosis at the cellular level (148).

The blockage of some IFN signaling promotes the development of cold tumors in OC, hence enhancing IFN signaling would facilitate the development of hot tumors as well. PARP7 is a member of the mono-PARP class of enzymes and blocks IFN response by inhibiting nucleic acid sensing. RBN-2397 is a potent and selective inhibitor of PARP7. In preclinical models, RBN-2397 restored IFN-I signaling in tumors, inhibited cancer cell proliferation and induced adaptive immunity, leading to tumor regression (149). In later experiments in patients with advanced solid tumors, an increase in the expression of ISGs and an enrichment of the immune response gene, accompanied by an increment in $CD8^+$ T cells, was observed on tumor biopsies after the use of RBN-2397 (150). Moreover, it was found that Gal-3 secreted by tumor cells or stromal cells bound to N-glycans, forming a glycoprotein/Gal-3 lattice that accumulates in the TME and intercepts glycosylated soluble factors, particularly IFN-γ. As IFN-γ diffusion was restricted in OC, CXCL9/10 concentration decreased, which facilitated "cold" tumor phenotypes by limiting T-cell infiltration. In DCs-rich plasma OC models, the combination of G3-C12@PLGA (Gal-3 antagonist) and anti-PD- 1 peptide was effective. G3-C12@PLGA not only maintained CXCL9/10 concentrations in tumor tissues for a long time by releasing IFN-γ and continuously recruiting $CD8^+$ T cells into tumors, but also

helped anti-PD-1 peptide to function better. The combination of the two significantly inhibited the increase of ascites, reduced the metastasis of peritoneal tumors, prolonged the survival of model mice, and offered the possibility of a cure for OC (151).

Conclusion and outlook

Currently, we are still in an accumulation phase where people are studying the detailed mechanisms of IFNs in OC, both in terms of its action on OC and its regulation by OC. Although the IFN responses have a bidirectional role for OC development, high levels of IFNs are more prognostic, which implies that there is a game in the TME in which the protein produced by the IFNs that favors therapy ultimately dominates. The role of IFNs is a hinderer to an extent in the OC development.

Based on the summary of the literature, there is still confidence in the use of IFNs for the treatment of OC. OC is a cold tumor, and IFNs can change it to a hot tumor, allowing a more active immune environment that is conducive to immunotherapy. And this also provides ideas for other cold tumors. The combination therapy with IFN and cell therapy is a promising research direction. The use of IFNs in OC, however, is worthy of serious deliberating, taking into consideration of the time, dose, and site of use, which are closely related to the therapeutic effect. In addition, due to the numerous response sites of IFNs, the side effects of IFNs should be considered. Selective activation of downstream targets or inhibition of some negative loci may be feasible approaches. At the same time, IFNs serve as presites for polygenic regulation, and we should also consider gene mutations to prevent ineffectiveness or false activation. In conclusion, the roles of IFNs in OC are complex and meaningful. And the combination of IFNs with other therapies is still a field deserving exploring.

Author contributions

TL wrote the manuscript. YL, XW, XY, YF and YZ performed the work of review. HG and ZH designed the work of review. All authors contributed to the article and approved the submitted version.

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Conflict of interest

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

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Exosomes: A potential tool for immunotherapy of ovarian cancer

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Ovarian cancer is a malignant tumor of the female reproductive system, with a very poor prognosis and high mortality rates. Chemotherapy and radiotherapy are the most common treatments for ovarian cancer, with unsatisfactory results. Exosomes are a subpopulation of extracellular vesicles, which have a diameter of approximately 30–100 nm and are secreted by many different types of cells in various body fluids. Exosomes are highly stable and are effective carriers of immunotherapeutic drugs. Recent studies have shown that exosomes are involved in various cellular responses in the tumor microenvironment, influencing the development and therapeutic efficacy of ovarian cancer, and exhibiting dual roles in inhibiting and promoting tumor development. Exosomes also contain a variety of genes related to ovarian cancer immunotherapy that could be potential biomarkers for ovarian cancer diagnosis and prognosis. Undoubtedly, exosomes have great therapeutic potential in the field of ovarian cancer immunotherapy. However, translation of this idea to the clinic has not occurred. Therefore, it is important to understand how exosomes could be used in ovarian cancer immunotherapy to regulate tumor progression. In this review, we summarize the biomarkers of exosomes in different body fluids related to immunotherapy in ovarian cancer and the potential mechanisms by which exosomes influence immunotherapeutic response. We also discuss the prospects for clinical application of exosome-based immunotherapy in ovarian cancer.

KEYWORDS

exosome, ovarian cancer, immunotherapy, tumor microenvironment, biomarker

1 Background

Ovarian cancer is one of the three major gynecological malignancies, accounting for approximately 2.5% of all female cancers (1). The 5-year survival rate for early-stage I ovarian cancer is 70%, compared to less than 29% for advanced stage III or IV (1). Currently available treatments for ovarian cancer mainly include chemotherapy, radiotherapy, surgery, and targeted therapy (2). Among them, chemotherapy and radiotherapy are the most effective means to treat ovarian cancer in clinical practice; however, they have disadvantages including

adverse reactions, drug resistance, and long-term complications (3). In the context of significant advances in drug screening technology (4), there has been increasing interest in the development of oncology drugs that harness new cancer treatment strategies to overcome these problems. Cancer immunotherapy is a therapeutic method to control and eliminate tumors by regulating the immune function of tumor cells (5). Cancer immunotherapy can enhance the immune system and facilitate a durable response, which is suitable for a variety of cancers and can harness the immune system to reactivate the anticancer immune response that overcomes tumor escape (6). Treatments include adoptive cell transfer, nonspecific immune stimulation, vaccination strategies, and immune checkpoint blockade (2).

In recent years, exosome-based immunotherapy for ovarian cancer has become a research hotspot. Exosomes refer to small membrane vesicles with a diameter of 30–100 nm, which contain complex RNA, proteins, lipids, sugars, and nucleic acids (7, 8). Exosomes act on receptors on the cell membrane or directly fuse with the membrane of target cells to participate in local and distant information conduction (9). Exosomes can also be used as potential biomarkers for ovarian cancer (10). Meanwhile, exosomal miRNAs are biomarkers for the diagnosis and prognosis of ovarian cancer (11). Indeed, increased cytoplasmic expression of CD24 is a marker of reduced survival in patients with serous adenocarcinoma of ovarian cancer and is one of the biomarkers of epithelial ovarian cancer (12). In addition, claudin-4 protein is released by ovarian cancer cells and is highly expressed in the peripheral circulation of of ovarian cancer patients. Therefore, exosomes are valuable as screening biomarkers for the detection, diagnosis, and prognosis of ovarian cancer (13).

Exosomes are widely present in the tumor microenvironment (TME), which consists of surrounding non-malignant cells, non-cellular components, extracellular matrix (ECM), and signaling molecules (14). Exosomes are a double-edged sword in the TME, playing an important role in the mutual regulation of tumor and immune cells (Figure 1). Cancer cells can provide an appropriate microenvironment for the development of cancer by regulating immune cells with exosomes, such as *via* cell proliferation, drug resistance, angiogenesis and metastasis, and immune regulation (15). Meanwhile, exosomes secreted by cancer cells can change different types of stromal cells, and promote the growth and invasion of cancer cells, as well as tumor angiogenesis (16). In contrast, immune cells activate immune responses in the TME through exosomes (17). Exosomes exhibit immunogenicity and cell transfer function (18). Exosomes show high antitumor activity in a variety of tumors, promote the expansion of regulatory T cells, inhibit the proliferation and activation of CD8+ T cells, and play an immunosuppressive role. Researchers have found that dendritic cells (DCs) and tumor-secreted exosomes enable antigen presentation and T cell stimulation by expressing numerous major histocompatibility complex class I molecules (MHC-I) and tumor markers, and trigger CD8+ T cell-dependent antitumor responses *in vitro* and *in vivo* (19). Therefore, exosomes have great potential in

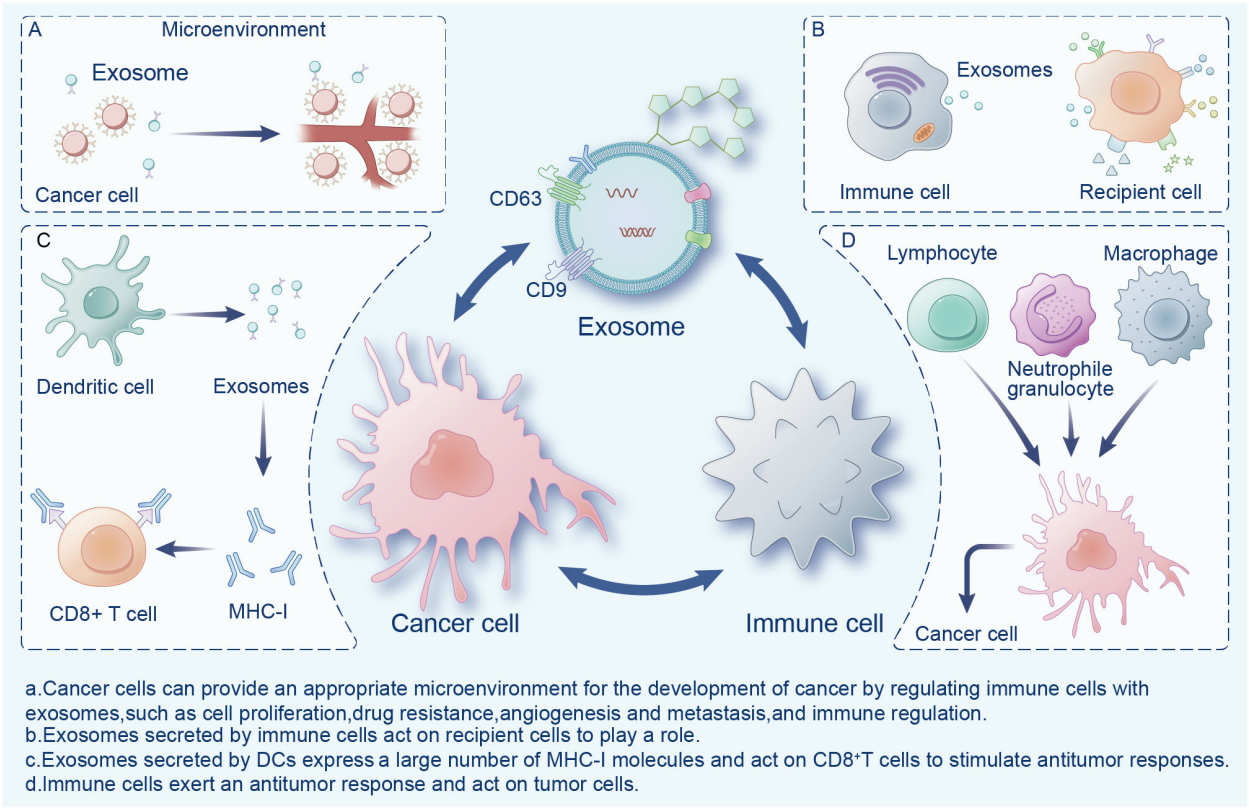

FIGURE 1
Interactions between exosomes, cancer cells, and immune cells in the tumor microenvironment.

cancer immunotherapy and may become the most effective vaccine to stimulate the anti-cancer immune response and serve as a vehicle for targeted anti-gene drugs (20, 21).

This review focuses on exosomes as biomarkers in tumor diagnosis, their role in the TME, and as immunotherapy tools for ovarian cancer.

2 Exosomes in tumor diagnosis

Exosomes are widely found in various body fluids (including ascites, blood, urine, emulsion). In the last decade, exosomes have been suggested to have potential as immunotherapy markers due to their particularity (22, 23). First, exosomes may be superior to some traditional diagnostic methods in terms of sensitivity and specificity, and exosomes contain a variety of bioactive molecules, resulting in less interference (24). Second, exosomes are highly stable and do not degrade in the extracellular environment. Finally, exosomes are widely present in various body fluids. Indeed, the serum exosomes of patients with ovarian cancer contain significantly more circ-0001068 (a novel biological marker) than those of healthy volunteers (25). Studies have found that exosomes in ascites are related to tumor invasion, metastasis, and survival time, and exosomes are highly expressed in ascites (26). Additionally, exosomes from ovarian cancer ascites containing CD147 could be used to monitor treatment response (27). Currently, exosome-based diagnostic kits for clinical diagnosis have been approved by the US Food and Drug Administration (28). This section summarizes exosomes in different body fluids (Figure 2).

2.1 Exosomes in ascites

Various factors contribute to the composition of cancer ascites, including tumor cells, fibroblasts, immune cells, and non-cellular items, such as cytokines, proteins, and exosomes (29), which together regulate the malignant phenotype and biological behavior of tumor cells (30). Numerous tumor-derived exosomes accumulate in the ascites of cancer patients, and ascites-derived exosomes present as novel substances of cancer-rejecting immune antigens, which opens

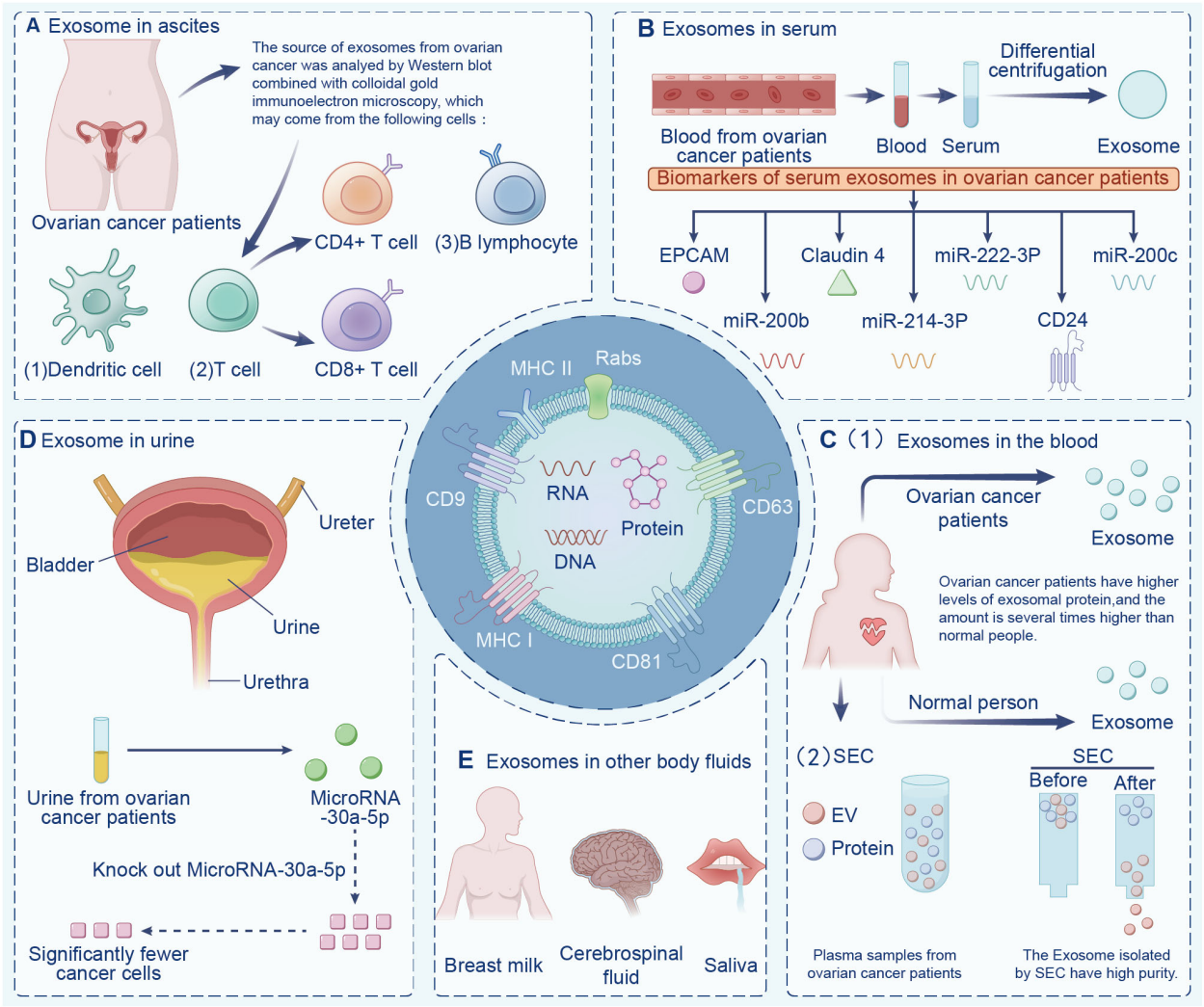

FIGURE 2
Exosomes in different body fluids. Exosomes are widely found in a variety of body fluids, including **(A)** ascites, **(B)** serum, **(C)** blood, **(D)** urine, and **(E)** in other fluids have the potential to be immune markers due to their particularity.

up a new direction in the field of cancer immunotherapy (31). The complexity of ascites determines the multi-origin of ovarian cancer exosomes. Indeed, the source cells of ovarian cancer exosomes may include T cells, B cells, DCs, and ovarian cancer cells (32). Meanwhile, exosomes play crucial roles in tumor immune escape by inducing apoptosis of immune cells (33, 34). Other studies have found that L1 cell adhesive molecules can effectively inhibit the spread of ovarian cancer cells, and CD24 protein is a biomarker of poor prognosis of ovarian cancer (35, 36). Additionally, exosomes has been shown to significantly promote the migration of ovarian cancer cells and increase chemotherapy resistance under a hypoxic environment (37). In this way, exosomes can serve as potential biomarkers of ovarian cancer cells' proliferation, metastasis, and immune escape (38).

Peng et al. isolated exosomes from ascites of patients with ovarian cancer to stimulate PBMCs, and tested the cytotoxicity of PBMCs on ovarian cancer cells (39). Even though exosomes themselves did not affect the invasion and metastasis of ovarian cancer cells, they could impair the cytotoxicity of PBMC in the presence of DCs, thereby achieving anti-tumor immunity. Secretions isolated from patients with ovarian cancer without chemotherapy or radiotherapy using ultracentrifugation and the fluid secretion of body surface markers were analyzed. The results showed significant levels of CD63 and CD9 expression on the surfaces of exosomes in ovarian cancer ascites. Furthermore, the researchers demonstrated that ascites exosomes affect the invasion and metastasis of ovarian cancer cells. Exosomes from patient ascites transferred miR-6780b-5p to ovarian cancer, thereby promoting the metastasis, invasion, and proliferation of ovarian cancer cells (26). Two cargo proteins, CD24 and EpCAM, were found in exosomes from malignant ascites of patients with ovarian cancer. Studies have demonstrated that CD24 is a diagnostic biomarker for poor prognosis of ovarian and other cancers (40). Therefore, exosomes possess potential value in the diagnosis, metastasis, and progression of patients with ovarian cancer.

2.2 Exosomes in serum

New advances have been made in the early diagnosis of ovarian cancer. Recent studies have illustrated that exosomes derived from ovarian cancer contain miRNA, EpCAM, CD24, and other molecules (41–43). Several cancers are characterized by overexpression of EpCAM, which is associated with the proliferation of epithelial cells during tumorigenesis and development (44–46). Glycosylphosphatidylinositol (GPI) links CD24 to the cell surface, and is present in multi-vesicular bodies in the cytoplasm. The quantity of CD24 is closely correlated with the amount of EpCAM in the cytoplasm (47, 48). Exosomes release CD24 into the extracellular microenvironment, which can serve as a tumor marker and predict prognosis for ovarian cancer (49). Increased expression of CD24 indicates an increased invasion rate, poor prognosis, and reduced survival rate of patients with ovarian cancer (42). EpCAM has been detected in exosomes isolated from serum of patients with ovarian cancer, which confirmed the presence of diagnostic miRNAs in exosomes (50). A further study showed that serum exosomes from patients with ovarian cancer contained higher levels of mRNA and miRNA than those from healthy people (51), which provided a basis for tumor-derived exosomes to participate in the transport of genetic material between cells. This also demonstrates that diagnostic miRNAs in serum exosomes of patients with ovarian cancer can be used for the diagnosis of ovarian cancer (52, 53).

A study by Shen et al. found a positive correlation between tumor stage and claudin-4 expression in serum exosomes from patients with ovarian cancer (54). In ovarian cancer tissues with a high level of malignancy, Yang et al. extracted exosomes from serum and found miR-214-3p was highly expressed, which might serve as a biomarker for ovarian cancer diagnosis and prognosis in serum exosomes (55, 56). In patients with ovarian cancer, serum exosome miR-222-3p was more strongly expressed than in healthy women (57, 58). Patients with intermediate and advanced ovarian cancers had higher levels of exosomal miR-200b and miR-200c expression than those with early-stage ovarian cancers (59, 60). It is common for patients with ovarian cancer to develop malignant ascites as their disease progresses; thus, non-invasive detection based on serum exosome miRNA profile has potential value as a new biomarker for early screening and diagnosis of ovarian cancer. Ovarian cancer serum contains significantly more exosomes than benign ovarian tumor serum and normal serum (61). Additionally, patients with advanced ovarian cancer have been found to have significantly more proteins in their exosomes than those with early ovarian cancer (62, 63). It is reasonable to assume that exosome protein contents can be used as a biomarker to identify ovarian cancer stages.

2.3 Exosomes in plasma

Different proteins and Rnas have different effects on immunotherapy. Under normal physiological conditions, programmed cell death protein 1 (PD-1) prevents autoimmunity and keeps T-cell responses within the required physiological range to prevent excessive inflammatory responses from harming the body. But in cancer, PD-1 protects tumor cells from anti-tumor T cell responses, leading to tumor immune escape (64). Cytotoxic T lymphocyte-associated protein 4 can act as an immune checkpoint and down-regulate immune response. It is currently considered as a promising immunosuppressive drug. MiRNA-424 in extracellular vesicles of tumors inhibits CD28-CD80/86 co-stimulatory pathways in T cells and dendritic cells, leading to resistance to immune checkpoint blocking. Modified extracellular vesicles that knock down this miRNA can enhance the efficacy of cancer immune checkpoint suppression therapy (65). The composition of plasma is complex and contains various proteins and RNA which may affect tumor immune response. Serum is a fluid collected after blood clotting, which screens out fibrinogen and clotting factors, and increases clotting products. In the process of coagulation, platelets secrete a large number of exosomes, which affects the accuracy of research results (66).

The overall protein level of exosomes in the plasma of patients with ovarian cancer is higher than that of benign tumors or healthy people, and the expression of miRNA in the exosomes in cancer cell lines, tumor tissues, and plasma has been shown to be significantly different (67). The plasma samples contain abundant soluble proteins (such as albumin and fibrinogen) as well as lipoprotein particles and exosomes. Circulating immunoglobulins in plasma bind to tumor-

derived exosomes, inducing antibody responses to tumor antigens and weakening complement-mediated cytotoxicity against tumor cells (68). Plasma exosome PD-L1 enables cancer cells to evade antitumor immunity. Exosomes deliver PD-L1 from the original cancer cells to other cell types with low or no expression of PD-L1, inhibiting systemic antitumor immunity (64). In addition, specific circulating mirnas (such as miR-21-5p, miR-24-3p, etc.) in the whole plasma and plasma exosomes can be used as predictive biomarkers of anti-PD-1/PD-L1 therapeutic response (69). Plasma lncRNA HOTAIR has been shown to promote the development of tumor and influence the poor prognosis of tumor (70). Due to the complex composition of plasma samples, the influence of free proteins on exosome separation cannot be ignored (71). Researchers found that when compared to conventional biomarkers, exosomes can be considered to have far greater stability (72), as well as being available at considerably higher volumes in the plasma of patients with ovarian cancer compared to healthy people (73, 74).

If the exosomes isolated from plasma contain a large number of free heterotrimeric proteins, subsequent proteomic data analysis will be seriously affected. Not only is the number of detected exosomal proteins limited, but the reduced number of detected proteins leads to a decrease in the abundance of most of the major proteins, which affects the subsequent differential analysis and validation. At present, how to best isolate exosomes is a great challenge. Among the existing exosome separation technologies (75), most researchers prefer differential centrifugation. However, the number of proteins detected after the separation of plasma exosomes by differential centrifugation is less than 300, and the heterotrimeric proteins cannot be effectively removed. Another exosome separation technique, molecular size-based exclusion chromatography (SEC), can obtain exosomes with high purity, which is sufficient for subsequent nucleic acid studies (76, 77). Therefore, the SEC method is being increasingly favored by exosome researchers. However, the SEC method can lead to lipoprotein impurity contamination and has room for improvement.

2.4 Exosomes in urine

The study found that ovarian cancer has unique metabolic characteristics in urine, so urine can be used as the basis for clinical diagnosis and classification of ovarian cancer (78). MiR-15a was significantly up-regulated and let-7a was down-regulated in the urine of ovarian cancer patients, showing potential as a specific diagnostic marker for ovarian cancer (79). The sensitivity and specificity of HMGA1 in ovarian cancer urine are high, and the detection of HMGA1 level in urine can be used as the basis for the diagnosis of serous ovarian cancer (80). Serum biomarker CA125 is an FDA-approved biomarker for ovarian cancer, and urine HE4 is the first marker after CA125 to be approved by the FDA for the diagnosis of ovarian cancer (81). Urinary mesothelin is also a good diagnostic marker for ovarian cancer (82). However, the negative news is that mucinous ovarian cancer does not express HE4, but CA125. In other words, these markers are limited and can only be used as diagnostic markers for specific types of ovarian cancer. In addition to these substances, all types of ovarian cancer urine contains rich and easily enriched exosomes with stable structure. Urinary exosomes are small vesicles secreted into the urine by renal epithelial cells (83) *via* two mechanisms: one is the direct shedding or budding of cell membrane, and the other is the fusion of intracellular multivesicular bodies with the plasma membrane, in which the specific exocytotic vesicles secreted by plasma membrane are urine exosomes. The separation methods of urine exosomes include simple high-speed centrifugation (84), sucrose gradient high-speed centrifugation, and reagent precipitation (85). Isolated urine exosomes have been found to have signature proteins and corresponding particle sizes by immunoelectron microscopy and nanotracer analysis.

Proteins of urine exosomes are derived from glomeruli, renal tubules, prostate, and bladder cells, indicating that exosomes in urine are secreted by cells of the kidney and other urinary organs (86, 87). In addition to proteins, urine exosomes also contain nucleic acids. Indeed, it has been found that urine exosomal RNA is more advantageous than total urine mRNA as a marker of kidney disease (88). Because the membrane structure of urine exosomes can reduce the degradation of RNA enzymes, their stability is higher (89). Additionally, RNA quality analysis and high-throughput sequencing of urinary exosomes revealed that the most important RNA in urinary exosomes is small RNA (90), including miRNA, which is a small non-coding RNA that plays a regulatory role in mRNA processing. RNA, especially miRNA, not only has important applications in the field of renal biomarkers (91), but also suggests the value of exosomes as a basis for biological therapy. MiR-92a is significantly up-regulated in the urine of patients with ovarian cancer, and can be used as a diagnostic marker for ovarian cancer (15). In addition, urinary exosome miR-106b was significantly down-regulated in ovarian cancer samples, showing certain diagnostic potential (92). Urinary exosome miRNA-21 has been widely studied as an emerging biomarker for the diagnosis of prostate cancer, which induces cancer cell proliferation and invasion by regulating the expression of multiple tumor related genes (93). Urinary exosome miR-4516 also marks premature ovarian failure (94).

Exosomes promote the development of ovarian cancer by regulating the biological behavior of tumor cells. Zhou et al. found that microRNA-30a-5p was highly expressed in urine exosomes of patients with ovarian cancer, and once the miR-30a-5p gene was knocked down, the proliferation and migration of ovarian cancer cells could be significantly inhibited (56, 95).

3 Roles of exosomes in the TME

Increasing experiments have proved that cancer cells secrete more exosomes than normal cells. Different exosomes carry different proteins, miRNAs, and other substances (96). Exosomes in ovarian cancer play an important role in tumor occurrence and development (97). We will describe the role of exosomes secreted by different cells in the TME (Figure 3, Table 1).

3.1 Exosomes released by tumors

Studies have found that exosomes carrying relevant molecules (including proteins and miRNAs) can be released from tumor cells and stromal cells in the TME, and interact with immune cells in TME

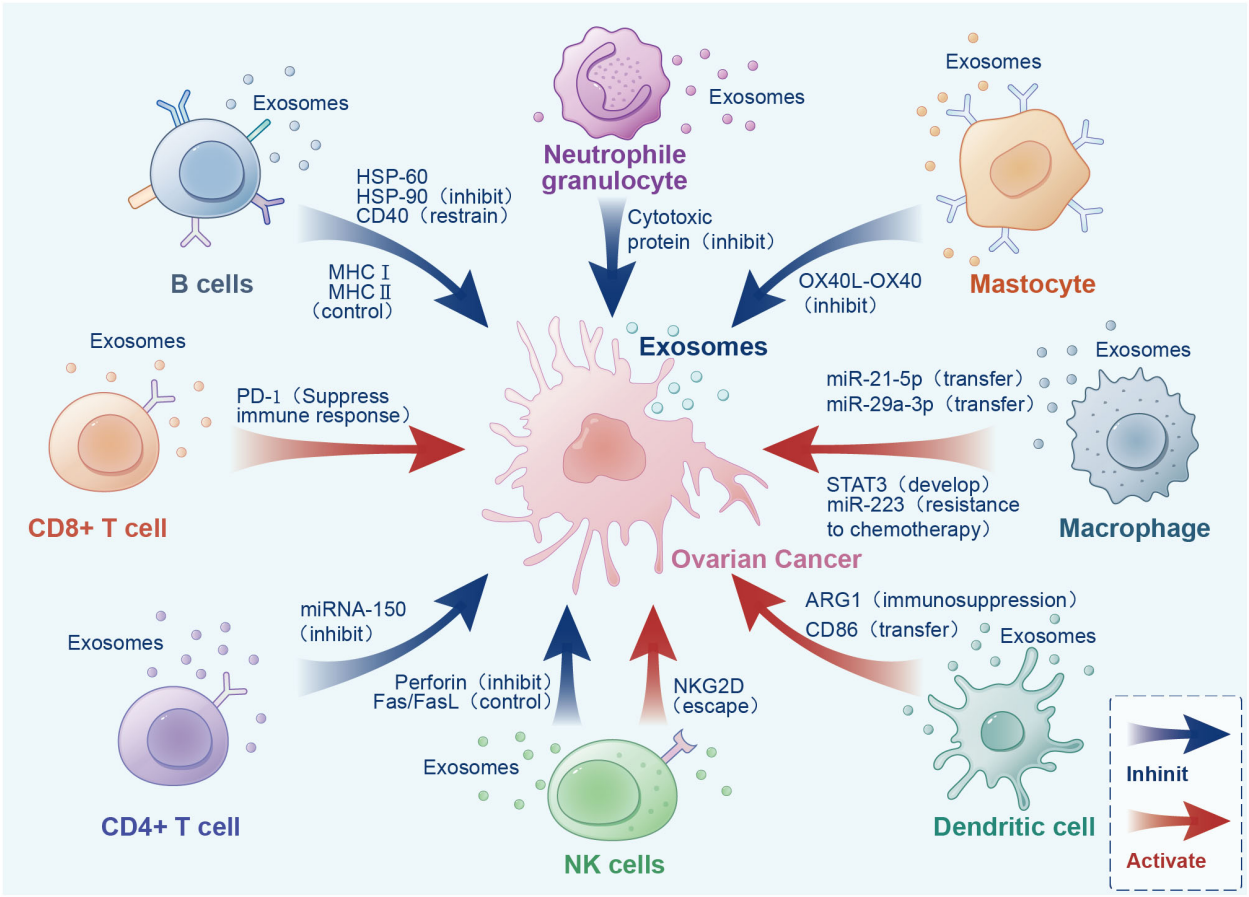

FIGURE 3
Bidirectional effects of exosomes from different sources in the tumor microenvironment.

TABLE 1 Role of exosomes from different cell sources in the tumor microenvironment.

Source of exosomes	Material	Object	Role	Reference
CD8+T cells	mRNA, miRNA	Malignant tumor	Affects tumor development	(98)
	miR-765/PLP2	UCEC	Inhibition of estrogen to promote UCEC	(99)
CD4+T cells	FasL	T cells	Induced T cell apoptosis and immune disorders	(100)
	miRNA-150	Lymphoid tissue	Serum markers	(101)
B cells	DC vaccine	T cells	Inhibition of immune suppressive cytokine production	(102)
NK cells	Perforin Fas/FasL	Tumor cells	Cytotoxicity, tumor cell apoptosis	(103)
	Immune components	Tumor cells	Immunomodulatory, reverse tumor immune suppression	(104)
DCs	MHC I, MHCII	TME	The immune response is dysfunctional	(105)
Neutrophil granulocyte	Cytotoxic protein	Tumor cells	Induction of apoptosis	(106)
	Adriamycin	Glioma	No limitations, promising treatments	(107)
Macrophages	miRNA	Ovarian cancer	Immune suppression, promote cancer development	(108)
	miR-193a-5p	Tumor cells	Promote tumor invasion	(109)
	IncRNA	Cancer cells	Aerobic glycolysis, anti-apoptosis ability of cells	(110)
Mast cells	KIF protein	Tumor	Promote cancer cell proliferation	(111)

UCEC, Endometrial cancer.

to conduct information transmission (112). Exosomes released by tumors provide a suitable microenvironment for tumor cells, but also inhibit the metastasis, occurrence, and development of tumor cells (38, 113).

Ovarian cancer, unlike other cancers, invades the abdominal cavity through ascites (114). Early ascites contains isolated tumor cells, various immune cells, mesothelial cells, and tumor-associated exosomes. These exosomes carrying protein signals specific for ovarian cancer can be isolated from the ascites and serum of patients with ovarian cancer (1). These exosomes can be used as biomarkers for early diagnosis of ovarian cancer. Exosomes secreted by ovarian cancer not only reveal the role of early malignant tumors, but also promote metastasis (115).

A common prerequisite for ovarian cancer metastasis is the formation of a premetastatic niche, which is the microenvironment formed at the distal sites by factors including exosomes secreted by ovarian cancer (116). The premetastatic niche requires immune suppression and evasion, angiogenesis, cancer-associated fibrocytes and tumor macrophages that reshape the stroma of the primary site (117). Studies have also shown that exosomal miRNAs play an indicative role in the pre-transfer niche (118). Exosomes translocate exfoliated miRNAs to tumor cells and their associated macrophages (TAM) and mesothelial cells to regulate the gene expression of target genes (119). However, ovarian cancer metastasis still faces an important barrier to the immune system. The mechanism of anti-tumor immune response in patients with ovarian cancer is disrupted precisely because exosomes suppress the immune response against the tumor (120). Exosomes secreted by ovarian cancer can induce T-cell arrest to achieve immune escape from cancer cells (121). Cytokines are closely associated with tumor progression and immune response (122, 123), and there is evidence that IL-6 promotes distant metastasis in ovarian cancer (124). In other cancers, exosomes have been found to induce IL-6 production in monocytes through a Toll-like receptor (TLR). IL-6 is then involved in signaling and transcription of activator 3 (STAT3) in immune cells, stromal cells, and tumor cells to complete immune escape and realize cancer cell metastasis (125). Additionally, exosomes evade immune surveillance by inhibiting NK cell function (126), inhibiting dendritic cells differentiation (127), and promoting myeloid inhibitory cell differentiation (128). In the omental TME, exosomes secreted by stromal cells contain miR-21, which can change the malignant phenotype (cancer cell movement and invasion) of metastatic ovarian cancer cells, indicating a new direction for the inhibition of ovarian cancer metastasis (129).

At the same time, exosomes from ovarian cancer can induce apoptosis of DCs, hematopoietic stem cells, and peripheral blood lymphocytes in the microenvironment, and inhibit anti-tumor immune response (130). In a study on exosomes from ovarian cancer researchers prepared two sets of culture groups, one with exosomes from malignant ovarian cancer ascites and the other with peritoneal lotions from benign ovarian cancer patients. Normal peripheral blood lymphocytes were added for co-culture and then lymphocytes were extracted for low gene expression analysis. The results showed that 26 immunosuppressive genes were overexpressed in lymphocytes of the malignant ovarian cancer ascites culture group compared to the benign ovarian cancer group, indicating that exosomes inhibit the immunity of lymphocytes through direct interaction with leukocytes (1). Exosomes have also been shown to silence immune cells in the TME, while their phosphatidylserine has been shown to inhibit T cell activation and shorten the growth phase of ovarian cancer (131).

3.2 Exosomes derived from T cells

Different T cells have different cell surface differentiation antigens (CD), which can be divided into CD4+ and CD8+ subsets. CD4+ T cells recognize exogenous antigenic peptides presented by MHC class II molecules, while CD8+ T cells recognize endogenous antigenic peptides presented by MHC class I molecules (132). The number and ratio of T lymphocyte subsets can be used as important indices of cellular immune function (133) in the context of viral infection, cancer, autoimmune diseases, and organ transplant, playing an important role in guiding treatment and prognosis (134). Such as malignant tumors, hereditary immunodeficiency diseases, AIDS, and CD4+T lymphocyte depletion in patients on immunosuppressive drugs (135). An increase in CD8+T cells may indicate autoimmune disease or chronic viral infection, such as chronic active hepatitis or tumor (136). Additionally, if the ratio of CD4/CD8 after transplantation is increased compared to that before transplantation, the patient may have suffered a rejection reaction (137). In the field of tumor immunotherapy, exosomes derived from T lymphocyte subsets have attracted extensive attention based on the various indicative effects of CD8+ and CD4+ T cells.

3.2.1 CD8+ T cell-derived exosomes

The cells in the TME directly affect the occurrence, development, and metastasis of tumors through their interactions (138). CD8+ T cells play an indispensable role in the TME, and CD8+ T cells infiltrating tumor tissues are associated with the prognosis of human malignancies (139). CD8+ T cells can not only kill tumors (140, 141), but also induce the production and release of specific substances (mRNA, miRNA, protein, and lipid) by acting on recipient tumor cells, which can affect tumor development (98) (Table 2).

Endometrial cancer (UCEC) is one of the most common gynecological malignancies, with approximately 200,000 cases diagnosed worldwide annually (161). Despite the rapid development of drug therapy, the prognosis of UCEC is getting worse, and the 5-year survival rate of advanced patients is less than 30% (162). A previous study investigated the mechanism of UCEC development, and revealed the inhibitory effect of CD8+ T cell-derived exosomes on UCEC development (99). CD45RO-CD8+ T cell-derived exosomes inhibit UCEC development through the ERβ/miR-765/PLP2/Notch pathway, and these exosomes interact with the miR-765/PLP2 axis to inhibit estrogen promotion of UCEC development.

Other studies have found that miR-150 contained in CD8+ T cell-derived exosomes can act on macrophages, which in turn act on regulatory T cells. miR-150 is transferred to effector T cells to inhibit cell proliferation and the occurrence of specific immune responses (163). Some research teams have studied ovalbumin-specific TCR transgenic OT-I mice (164), and found that the exosomes derived

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TABLE 2 Genes associated with immunotherapy in ovarian cancer.

Name	Composition	Target point (object)	Potential targets	Role	Source	Ref.
miRNA	miR-222-3p	Macrophages	Diagnostic markers	TAMs M2 polarization	Serum	(15)
	miR-92a	–	Diagnostic markers	–	Urine	
	miR-30a-5p	–	Highly specific diagnostic markers	Inhibits proliferation and metastasis of OC cells	Urine	
	miR-let-7	–	–	Inhibits cell proliferation	OC cell line	
	miR-NAs	Between the skin cells	–	Tumor cell spread	OC cells	
	miR-330–3p	Mesenchymal ovarian cancer cells	Inhibition of tumor development	Enhanced mesenchymal phenotype	Plasma cells	(142)
	miR-21	Adjacent cancer cell	Diagnosis and treatment of metastatic and recurrent ovarian cancer	Inhibits apoptosis of ovarian cancer cells	CAA/CAF	(129)
	miR-233	EOC cell	Predictors of tumor invasion, metastasis, and recurrence	Induced chemoresistance	Macrophages	(143)
	miR-6126	Tumor cells	Regulates ovarian cancer progression	Tumor suppressor factor	Malignant cells	(144)
IncRNA	UCA1	Cancer cells	Biomarker	Inhibition of cell metastasis	Urothelial carcinoma	(145)
	H19	mRNA	Markers of ovarian cancer development	Apoptosis of OC cells was induced	–	(146)
	HOTAIR	LSD 1/REST	Predictive and diagnostic biomarkers	Promotes OC cell proliferation	Plasma	(70)
	MALAT1/ NEAT2	miRNA	Potential markers of ovarian cancer metastasis	Promotes cell proliferation, migration, and invasion	–	(147)
	MEG3	pcDNA	Biomarkers for the diagnosis of advanced cancer	Regulation of tumor suppressors	–	(148)
	NEAT1	Tumor cells	Prognostic markers for ovarian cancer	Prediction of patient survival	–	(149)
	XIST	Tumor cells	Early diagnosis, potential target of antitumor therapy	Promotes the proliferation and invasion of cancer cells and regulates the carcinogenesis of ovarian cancer	–	(150)
circRNA	CDR1as	miR-135b-5p	Ovarian cancer diagnosis and treatment	Tumor suppressor and promotes the expression of HIF1AN	Ovarian tissue	(151)
	circKRT7	miR-29a-3p	Evolutionary driver of malignancy in ovarian cancer	Promotes cancer cell proliferation and metastasis	–	(152)
	circPLEKHM3	miR-9/BRCA 1/ KLF 4/AKT 1	Therapeutic targets, prognostic markers of ovarian cancer	Tumor suppressive effect	–	(153)
	cicrCELSR1	miR-1252	Promotes ovarian cancer	PTX drug resistance was affected and apoptosis rate was increased	–	(154)
	Hsa-circ-0078607	miR-518a-5p/ Fas	Predicting adverse clinical outcomes in ovarian cancer	Inhibits ovarian cancer development	–	(155)
	circ-0061140	miR-361-5p	Ovarian cancer treatment indicators, miRNA sponge	Promotes ovarian cancer development	–	(155)
mRNA	CHAC1 mRNA	Cancer cells	Markers of increased risk of ovarian cancer recurrence	Affects ovarian cancer cell migration	–	(156)
	MUC16 mRNA	Tumor tissue	Markers of poor prognosis in ovarian cancer	Suggests an abnormal increase in MUC16	–	(157)
	MACC1 mRNA	Tumor tissue	Prognostic markers for ovarian cancer	Affects ovarian cancer migration, invasion and expression	–	(158)
	GSK3β mRNA	Tumor cells	Predicts chemotherapy sensitivity	Inhibits the development of ovarian cancer	–	(159)
	CEBPA mRNA	Cancer cell cytoplasm	Ovarian cancer diagnosis, evaluation, prognostic markers	Affects the pathogenesis of ovarian cancer	–	(160)

from CD8+ T cells of mice carried ovalbumin specific TCR and FasL. These exosomes can regulate the pMHC-I expression and the FasL/Fas interaction *in vitro* by inducing DC apoptosis. We have also found that CD4+T cell-derived exosomes from ovalbumin-specific TCR OT-II mice also carried ovalbumin-specific TCR and FasL and could inhibit CD8+ CTL responses. The team demonstrated the immunomodulatory effects of CD8+ T cell and CD4+ T cell-derived exosomes using transgenic mice, but the factors responsible for inhibition have not yet been identified. We can speculate that exosomes derived from CD8+ and CD4+ T cells carrying FasL may affect immune cells through antigen-specific functions. Additional experimental data suggest that FASL-mediated apoptosis of T cells carried by exosomes is associated with tumor escape (165). Ovarian cancer-derived exosomes may impair anti-tumor immunity by carrying FasL/Fas (166), and FasL on ascites-derived exosomes in patients with epithelial ovarian cancer, as well as TRAIL, affects the presence of membranous forms of related ligand and partially explains lymphocyte apoptosis (39). Cells in the ascites of epithelial ovarian cancer lack the membranous form of FasL and are unable to make cell-to-cell contact, thus inhibiting the mechanism of Fas-induced cancer cell death. Meanwhile, exosomes promote tumor cells to attack immune cells carrying Fas by releasing complete secreted intracellular FasL, which is conducive to the immune escape of cancer cells (167).

Seo et al. found that exosomes derived from activated CD8+ T cells can regulate the cells surrounding the tumor and inhibit the development of malignant tumors (168). CD8+ T cells' exosomes inhibit cancer development by killing the surrounding mesenchymal cells, and destroying the tumor stroma (169). Meanwhile, exosomes also act on other anticancer CD8+ T cells. Primarily, IL-12 stimulates changes in the number and size of derived exosomes by acting on CD8+ T cells, and promotes the production of granzyme B and interferon-γ by bystander CD8+ T cells (170). Li et al. found that T cell-derived exosomes can act directly on malignant tumors and exert anticancer effects. Qiu et al. showed that active T cell-derived exosome PD-1 (protein) effectively prevented T cell-mediated immune responses by binding to PD-L1 on cancer cells (171).

3.2.2 CD4+ T cell-derived exosomes

CD4+ T cell-derived exosomes play a variety of roles in the TME and cellular responses. Exosomes derived from active CD4+ T cells contain various proteins (e.g., lysosomal-associated membrane protein 1, CD4, TCR) that can inhibit the antitumor immune response and cytotoxicity of CD8+ T cells, as well as inhibit the proliferation of CD4+ T cells. Indeed, exosomes derived from CD4+CD25+FOXP3+ T cells contain the anti-inflammatory mediator CD73, which inhibits the proliferation of CD4+ T cells. Exosomes containing FasL have also been derived from human B cell-derived lymphoblastoid cell lines and CD4+ T cells to induce apoptosis of target T cells (100).

Some research teams believe that CD4+ T cell-derived exosomal miRNA-150 represents the best potential biomarker for lymphocyte activation. miRNAs from exosomes derived from CD4+ T cells are significantly different from the intracellular miRNAs of other cells, and the signal of lymphocyte activation can be transmitted to serum miR-150, suggesting that miRNA-150 released from CD4+ effector T cells could be used as a serum biomarker of lymphocyte activation (101).

Exosomes secreted from CD4+ T cells carry antigenic MHC-II peptide complexes, which can act as "mini APCs" to directly or indirectly act on T cells and contribute to T cell activation (172). Regulatory T cells are known to inhibit immune cell activation, proliferation, and cytokine secretion in a non-MHC-restricted manner (e.g., DC, NK). However, CD4+CD25+ regulatory T cells can negatively regulate autoimmune responses, and exosomes derived from these cells can also exert immunosuppressive effects (173).

3.2.3 CAR-T immunotherapy

At present, the research on genetically engineered T cells expressing chimeric antigen receptor (CAR) is developing rapidly. Many studies have demonstrated that allogeneic T cells or somatic cells expressing T cell receptors (TCRs) or chimeric antigen receptors (CARs) can be used for cellular immunotherapy, and are expected to become a promising therapy for the treatment of hematological and non-hematological malignancies in the future (174–176).

CAR-T cell-based cellular immunotherapy, also known as CAR-T therapy, can induce rapid and long-lasting clinical responses (177). CAR-T cell-derived exosomes are considered potential new antitumor therapies because of their high inhibitory effect on tumor growth and safety (178). The downside is that CAR-T therapy has a high potential for side effects such as acute toxicity (174).

The therapeutic mechanism of CAR-T therapy is to target cancer cells with specific T cells that are extensively cytotoxic (179). The CAR consists of a target binding domain and a transmembrane signaling domain. The target binding domain is an extracellular domain formed by CAR-T cell-specific expression, while the transmembrane domain is the intracellular domain that provides activation signals to T cells. In general, the targeting specificity of CARs is achieved through antigen recognition regions in the form of single-stranded variable fragments (scFv) or binding receptors or ligands in the extracellular domain, whereas T cell activation functions are achieved through the intracellular domain (180–182).

CAR-T therapy produces toxicity that is different from that of conventional chemotherapy, monoclonal antibody (mAb), and small-molecule targeted therapies (183). The two most common toxic effects of CAR-T immunotherapy are cytokine release syndrome (CRS), characterized by high fever, hypotension, or multiorgan toxicity, and CAR-T-associated encephalopathy syndrome (CRES), which is characterized by a toxic encephalopathy state and usually presents with symptoms such as paranoia and confusion (183, 184). Researchers have observed strong CAR T cell responses in patients with hematological malignancies, but limited CAR T cells in solid tumors. The reason may be that there are obstacles in the TME of solid tumors, such as up-regulation of inhibitory receptors (IR), which can react with homologous ligands of CAR-T cells, such as PD1 and CTLA-4, to inhibit the therapeutic response of CAR-T therapy (185). Meanwhile, exosomes derived from CAR T cells have been found to carry CAR on their surfaces (186). The exosomes carrying CAR did not express PD1, and the antitumor effect of

exosomes was not impaired after recombinant PD-L1 treatment (187). CAR-derived exosomes have been shown to be safer than CAR-T therapy in preclinical *in vivo* models of cytokine release syndrome. Researchers believe that exosomes could be used to create biomimetic nanovesicles, which could be a new and effective strategy for cancer treatment (174).

Cytotoxic T lymphocyte (CTL)-derived exosomes contain CD3, CD8, and TCR, which can unidirectionally deliver lethal content to target tumor cells (188). The conjugation formed by the interaction of TCR with antigen/MHC has been found to mediate the death of target cells, and the activation of TCR promoted CTL to derive exosomes. Lethal compounds in exosomes (including granzyme, lysosomal enzymes, and perforin) activate the killing of target cells (189, 190). Some studies have demonstrated that TCR/CD3 and other complexes exist on CTL-derived exosome membranes (191).

Based on the biological characteristics of exosomes, CAR-T cell-derived exosomes play a direct role in immunotherapy. CAR-T cell-derived exosomes are functionally and structurally similar to synthetic drug vectors similar to liposomes, so CAR exosomes can be used as cancer targeting agents (176, 192). However, exosomes directly isolated from CAR-T cell culture medium may be heterogeneous and lose their targeted therapeutic effect because the antibody-derived scFv in the CAR structure determines the targeting specificity of CAR T cells (174).

3.3 Exosomes derived from B cells

Research on the promotion or inhibition of immune cells in the TME against the tumor has been gradually deepened. However, while the role of T cells has made some progress, the function of B cells is still unclear. Recent studies have shown that B cells play an important role in anti-tumor immunity (193), and numerous B lymphocyte populations (naive B cells, memory B cells, activated memory B cells) have been found in the TME. B cells are the second adaptive immune cell population found in TME (194, 195). B cells have been known to be carcinogenic for decades, but recent studies have linked their presence to improved prognosis in patients with cancer (196).

DC vaccines with exosomes as antigens have been shown to stimulate the clonal expansion response of T cells by pulsed diffuse B lymphocyte-derived exosomes, thereby promoting the secretion of IL-6 and TNF-α, while inhibiting the production of immunosuppressive cytokines IL-4 and IL-10 (102). However, it is puzzling that exosomes derived from B cells instead induce apoptosis of CD4+ T cells (197). Exosomes derived from heat-shocked B cells are rich in HSP60 and HSP90, and also express high levels of MHCI, MHCII, CD40, and other immunogenic molecules, and then induce antitumor effects of CD8+ T cells through these markers (125). Subsequent studies have shown that exosomes as antigens of DC vaccines have limited anti-tumor efficacy in clinical immunostimulation trails. There is increasing experimental evidence that exosomes exert immune escape effects. Mechanistically, tumor-derived exosomes may promote B lymphocyte responses (e.g., amplification of immunosuppressive B cell populations), thereby facilitating cancer cells evasion from immune surveillance (68).

3.4 Exosomes derived from NK cells

Clinical studies on NK cells have found that they show rapid immunity against metastatic or hematologic malignancies, as well as possessing antitumor properties (198–200). Exosomes derived from NK cells have been shown to have tumor-homing ability in a variety of tumor animal models (201), that is, exosomes can be observed in tumors within minutes to hours. Exosomes are then ingested by tumor cells inside tumor tissues, where they kill tumor cells through a variety of mechanisms. Recently, there has been a major breakthrough in the study of NK cell-derived exosomes.

NK cell-derived exosomes have two main functions (202). The first is the cytotoxic effect. The exosomes derived from NK cells contain a variety of bioactive molecules, such as cytotoxic proteins and microRNAs (203). Additionally, exosomes derived from NK cells can also be used as a carrier of anti-tumor drugs. Exosomes take advantage of the targeting of related tumors to reach tumor tissues quickly and precisely, and then increase drug concentrations. The cytotoxic proteins contained in exosomes, such as perforin and Fas/FasL, can cause apoptosis of tumor cells, but do no harm to normal cells (103, 204). Indeed, exosomes derived from NK cells are cytotoxic to melanoma cells but have no effect on normal cells (205). In this way, we can use FasL inhibitor to reduce its toxic effect on melanoma cells. Researchers have studied the principle of NK-exosomes killing melanoma cells (206), and tested the tumor-suppressive effect of NK-exosomes *in vivo* using a mouse model. It was found that the tumor size of the NK exosome-treated group was significantly smaller than that of the control group, indicating that NK cell exosomes induced the apoptosis of melanoma cells *in vitro*. The cytotoxic effect of NK-exosomes is expected to be used in the immunotherapy of cancer (207). Meanwhile, microRNAs contained in exosomes can down-regulate the expression of related genes, thereby inhibiting cell proliferation and inducing apoptosis of tumor cells (208, 209). NK exosomes also contain a variety of immune components, which can exert immunomodulatory effects by targeting the immune system through the paracrine pathway or circulatory system, and can reverse tumor immune suppression (104, 210). Basic experiments have found that NK-exosomes can stimulate immune cells (211). Additionally, NK-exosomes can reduce the immunosuppressive effect of tumor cells, which may be related to their ability to inhibit the expression of programmed death receptor (PD-1) on T cells (212).

As mentioned above, NK-exosomes are cytotoxic to tumor cells but harmless to normal cells. Indeed, in 2002, Italian scientists first discovered that NK cell-derived exosomes expressing FasL (apoptosis-related factor ligand) and perforin molecules were able to kill several types of cancer cell lines (213). However, when NK-exosomes were used against normal cells, no cytotoxicity was observed. This selective killing effect is another advantage of NK-exosomes (214, 215), as we know that traditional chemoradiotherapy methods will inevitably cause damage to normal cells while removing tumor cells. The second advantage of NK-exosomes is that they have fewer side effects. Cell therapy (including infusion) based on NK cells can cause cytokine release syndrome (CRS), referred to as a "cytokine storm," which can trigger a variety of common factors, lead to suspension of treatment, and in some cases, may even be life-threatening (216). However, NK-exosomes have only a small

chance of exploding this side effect. The third advantage is that NK-exosomes can penetrate the "protective barrier" of cancer cells. Immune cells such as NK cells cannot easily cross the "natural barriers" in human tissues, such as the blood–brain barrier, blood–testosterone barrier, and placenta, due to various factors, including the size of the cells themselves. However, cancer cells can nest in those areas and escape immune attack. NK-exosomes are nanoscale in size and contain the same cancer-killing molecules as NK cells, but they are much smaller and better able to penetrate into tumors, conferring them advantages over using cell-based therapies (178).

The characteristics and advantages of NK-exosomes have led to numerous studies on their clinical application in tumor therapy. However, researchers have struggled to isolate functional NK-exosomes on a large scale. Further study has shown that NK cells can be incubated in exosome-free medium for 48 h, before using polymer precipitation combined with density gradient centrifugation to separate EVs (217). However, this method is time extensive. Therefore, a novel microfluidic system has been proposed by the Cancer Research Center team, who found that NK cells could be captured on a graphene oxide microfluidic chip they developed. These NK cells were then incubated on the chip for a period of time, prompting them to release exosomes, which were then captured by tiny magnetic beads from ExoBeads coated with exosome-specific antibodies. The beads were removed from the chip and then NK exosomes were separated from them using a different process (218, 219). This microfluidic system holds promise for use in NK-exosome-based immunotherapy.

However, NK cells can also use tumor-derived exosomes to induce cancer cells to evade immune surveillance. Hepatocellular carcinoma cells secrete CircUHRF1 to promote the expression of mucin domain 3 (Tim-3) and T cell immunoglobulin and inhibit the secretion of IFN-γ and TNF-α by NK cells to achieve immunosuppression (66). In ovarian cancer, NK cells ingest exosomes in ascites and perform phosphatidylserine (PS) treatment on the surface of exosomes to internalize exosomes and induce ovarian cancer cells to evade immune surveillance (220). Additionally, there is a bidirectional effect between NK cells and exosomes in inducing immune escape. It has been demonstrated that NKG2D ligand (NKG2DL) released by exosomes in the extracellular environment mediates cancer cell immune escape using two pathways (221). NKG2D belongs to the C-type lectin-like activated receptor, which is expressed on NK cells, CD8+T cells, and some autoreactive or immunosuppressive CD4+T cells, and can detect and recognize cancer cells. MICA is the most polymorphic in NKG2DL. By expressing MICA*008, exosomes induce and activate NK cells to exhibit an immunosuppressive function, causing sustained downregulation of NKG2D after long-term stimulation, thus destroying the NKG2D mediating function. However, the release of NKG2DLs in the extracellular environment controls the cell surface expression mechanism and directly induces cancer cells to evade the immune surveillance of NKG2D. Tumor-derived exosomes utilize a T cell-independent mechanism to inhibit the killing effect of NK cells on cancer cells. Interleukin-2 (IL-2) plays an important role in the proliferation and differentiation of NK cells. Indeed, tumor-derived exosomes induce IL-2 reactivity to regulatory T cells and inhibit its access to cytotoxic cells, thus facilitating the escape of cancer cells. This dual mechanism of action reveals the role of exosomes in evading tumor immune surveillance (222).

3.5 Exosomes derived from dendritic cells

With the advance of research, DCs have been found to play an indispensable role in the TME. DCs are rich in alpha-fetoprotein, which can activate acquired and innate immune responses and have unique antigen presentation (absorption and expression of tumor antigens) capacity (223). They occupy a high position in tumor immunity and have been applied in the direction of cancer immunotherapy. Exosomes derived from DCs (DEX) have been found to activate the antigenic specificity of cells, induce an anti-tumor immune response, and restore the TME at the same time (224). Compared to immature DCs, mature DCs possess stronger capability in secreting exosomes that induce antigen-specific immune responses. Exosomes derived from mature DCs are 50–100 times more effective than exosomes from immature DCs when exerting immune effects *in vitro* and *in vivo* (225). DC exosomes can also be used as carriers to transmit DC antigens (226). Conversely, tumor-derived exosomes can be used as the intermediate of CTL cross-initiation (227). Exosomes take up tumor antigens and pass them to DCs to control their presentation to MHC-I molecules and induce CD8+T cells to produce effective anti-tumor effects. Meanwhile, exosomes from the ascites of metastatic patients with ovarian cancer interact with DCs to induce tumor-specific cytotoxicity and effectively kill cancer cells. Exosomes deliver tumor-specific antigens to DCs in cord blood, thus stimulating the proliferation and differentiation of resting T cells and inducing cytotoxicity to kill ovarian cancer cells, which may be a promising immunotherapy for ovarian cancer (228).

However, DCs have a poor absorption rate of tumor antigens and low immunogenicity of antigens. Under the inhibition of T cells, DC-derived exosomes are ineffective in tumor treatment (229). Moreover, DC-based immunotherapy is limited by an insufficient immune response, which makes eradication of solid tumors difficult (230). Under further study, new progress has been made, and it has been found that DC-derived exosomes are ideal antigens for DC vaccines (212).

DCs not only have antigen presentation function, but also have anticancer effects by stimulating a large number of exosomes (231). DC-derived exosomes contain MHC I, MHC II, CD86, and HSP70–90 mixtures, which activate CD4+ and CD8+ T cells (232). *In vivo*, tumor peptide-pulsed DC-derived exosomes have been shown to induce specific cytotoxicity of T cells and inhibit or eradicate mouse tumor cell growth in a T-cell-dependent manner. A vaccine regimen based on DC-derived exosomes can replace DC adoptive therapy to a certain extent (233). It is well known that the effector function of CD8+ T cells decreases (a process of depletion) upon sustained antigen stimulation, resulting in a dysfunctional immune response in the TME (105). Studies have found that DC vaccine induces anti-tumor immunity by the following mechanism: on the premise of exosomal CD80 stimulation and IL-2 secretion, the exosomal peptide MHCI begins to express, which transmits signals to CD8+ T cells to activate cell proliferation, thus inducing efficient anti-tumor immunity (2).

Additionally, exosomes derived from DCs containing alpha-fetoprotein (AFP) have been shown to induce IFN-Y-expressing CD8+ T cells in HCC mice, resulting in increased IFN-y and IL-2 and decreased CD25+Foxp3+ Tregs, IL-10, and TGF-B content (234). At present, there are different opinions on the relationship between MHC-containing DEX and T-cell responses. Most believe that DEX

containing MHC activates T cell responses, while others believe that in the presence of intact antigens, DEX containing MHC is not associated with T cell responses (235). Therefore, the immune effects of exosome-based DC vaccines still need to be studied. The immunosuppressive effect of exosomes on DCs also requires attention. Czystowska et al. reported the discovery of an exosome that carries a specific substance (ARG1) and inhibits immunogenesis in the ascites and plasma of patients with ovarian cancer. Exosomes carrying special substances are transported to the draining lymph nodes and then taken up by DCs, thereby blocking their induction mechanism and ultimately inhibiting the proliferation of antigen-specific T cells and causing immunosuppression (236). Additionally, exosome-mediated IFITM2 protein (transmembrane protein 2) transport to DCs leads to inhibitory activation of the IFN-α (interferon) pathway, which reduces IFN-α synthesis and blocks the anti-HBV (hepatitis B virus) efficacy of IFN-α. As a result, the IFN pathway treated with exogenous IFN-α appears a response barrier. This study provides a new explanation for the clinical phenomenon of poor response to IFN-α treatment in CHB (chronic hepatitis B) patients (237). Moreover, a previous study showed that tumor-derived exosomes inhibited DC differentiation by acting on DCs, blocking their immune function, and showed a strong immunosuppressive effect, which may be one of the main mechanisms of immune monitoring of tumor escape (127).

3.6 Exosomes derived from neutrophils

Neutrophils are among the most abundant white blood cells in the immune system and are involved in forming the first line of defense in the innate immune response (238). Neutrophils play important roles in angiogenesis, immunosuppression, and cancer metastasis (239). Some research teams have suggested that neutrophils are involved in the mechanism of promoting cancer metastasis, and confirmed the feasibility of neutrophils as a potential marker of diagnosis and prognosis and a clinical therapeutic target (240). Consequently, the study of exosomal vesicles derived from neutrophils has also been put on the agenda.

Zhang et al. (106) demonstrated that exosomes derived from neutrophils (N-Ex) can induce apoptosis of tumor cells by transmitting cytotoxic proteins and activating the caspase signaling pathway. The research team developed a simple and efficient preparation method for N-Ex and NNVs, which can be used as a safe vehicle for tumor target therapy. They attempted to modify N-Ex with superparamagnetic iron oxide nanoparticles (SPIONs) and found that the modified exosomes significantly improved the efficacy of tumor target therapy. Neutrophils have also been used to produce high-yielding exosome-like nanovesicles (NNVs). Zhang et al. found that engineered SPION-NNVs can be widely and efficiently used in clinical transformation, which has great application significance in the field of drug targeted delivery and tumor therapy. As persistent inflammation is a major feature of the TME, targeted therapy of the inflammatory TME is a research hotspot (241, 242). Some researchers have developed the NEs-Exos system for glioma using N-Ex as delivery vehicles for doxorubicin (107). This treatment system does not have the limitations of conventional chemotherapy and is a promising treatment approach. Studies of N-Ex have further revealed that the activity of other immune cells, such as macrophages and T cells, can be affected by exosomes. At the same time, Li et al. found that N-Ex affected the formation of pathological blood vessels by inhibiting the proliferation and migration of endothelial cells (243). Some research teams have elucidated the potential oncogenic mechanism of exosomes in gastric cancer (244). It was found that gastric cancer cell-derived exosomes (GC-Ex) induced neutrophil activation and extended survival time. Meanwhile, the derived exosomes contain HMGB1 protein, which activates the NF-κB pathway through the interaction with toll-like receptor 4 (TLR4), promotes the autophagy of neutrophils, and ultimately induces the migration of gastric cancer cells. Other studies have shown that activation of TLR4 can stimulate the release of highly immunosuppressive exosomes, promote tumor development, and help tumor cells evade immune surveillance (245). However, the therapeutic mechanism of neutrophil-derived exosomes in ovarian cancer has not yet been elucidated.

3.7 Exosomes derived from macrophages

Macrophages account for approximately half of the total tumor cells (246). In the TME, the vast majority of macrophages are programmed to promote primary tumor development and metastasis (247). However, they also participate in the regulation of anti-tumor adaptive immune response and inhibit tumor growth. Ascites is an obvious indicator of ovarian cancer, which contains a large number of specific macrophages, and these tumor-associated macrophages (TAM) have certain clinical value (248). Many studies have demonstrated that TAM-derived exosomes are involved in the regulation of immune responses and cancer biology.

TAM-derived exosomes release miRNAs that act on CD4+ T cells and induce Treg/Th17 imbalance, and then directly form an immunosuppressive microenvironment to promote the development of ovarian cancer (108, 249). Studies have found that M2 macrophages secrete large amounts of exosomes with immunosuppressive activity, thereby increasing drug resistance and promoting tumor development (250). Another team found that M2-TAM-derived exosomes promote the formation of vascular mimicry in tumor cells and promote tumor development and metastasis, thereby increasing tumor aggressiveness (109). This is because miR-193a-5p carried by exosomes can specifically adsorb and down-regulate the protein expression of TIMP2 to promote the formation of vascular mimicry. Xenotransplantation models have shown that M2 macrophages-derived exosomes carrying miR-155-5p can upregulate IL-6 and affect its stability by disrupting ZC3H12B-mediated mechanisms, that may induce immune escape and tumor formation in colon cancer (251). Macrophage-derived exosomes provide miRNA delivery to ovarian cancer cells, which in turn modulates the tumor immune mechanism in ovarian cancer. These exosomes are enriched in miR-29a-3p, a member of the miR-29 family, that functions essentially during lymphocyte differentiation. High levels of miR-29a-3p expression inhibit PD-L1 expression in ovarian cancer cells by downregulating the FOXO3-AKT/GSK3β axis, leading to immune escape of OC cells and ultimately promoting the proliferation of ovarian cancer cells (252). Xu et al. conducted experiments on exosomes secreted by TAM and found that when exosomes were used as carriers to deliver antigens to DC, T cell immune responses were significantly enhanced (2).

These results suggest that TAM-derived exosomes can serve as potential carriers for the exchange of cellular components between immune cells and enhance immune responses. A recent study found that exosomes secreted by TAMs contain HIF-1α stable long non-coding RNA (HISLA), which has the ability to regulate aerobic glycolysis and anti-apoptosis of cancer cells (110). This study demonstrates that RNA-interference-mediated silencing of HISLA may be a potentially powerful means to inhibit glycolytic processes in cancer cells, and they demonstrate that targeting TAMs-specific lncRNAs has great potential in cancer therapy. Nevertheless, further studies are needed to explore the interactions between TAM-derived exosomes and other immune cells and their relevance in ovarian cancer immunotherapy.

3.8 Exosomes derived from mast cells

Exosomes derived from mast cells (MCs) play a biological logic role in RNA and protein transfer, cell-to-cell communication, and immune regulation (125). It has been suggested that the transfer of miRNAs from MC-derived exosomes to target cells may affect intestinal barrier function (253). However, recent studies have found that lung cancer cells can absorb MC-derived exosomes, which then promote the proliferation of cancer cells by transferring KIT protein (111). The relationship between MC-derived exosomes and lung epithelial tumor cells has been explored, and morphological analysis revealed a phenotype resembling an epithelial-to-mesenchymal transition in A549 cells, which receive signals from exosomes (254). At the same time, the transcriptional analysis revealed that EMT-related phosphorylation cascades were significantly increased in epithelial cells treated with MC-exosomes (255). Other studies have found that MC exosomes can change the biological functions of DCs, T cells, and B cells. MC-exosomes induce antigen-specific immune responses by enabling T cells to produce antigen presentation capabilities (256). CD63+ and OX40L+ exosomes derived from MCs promote the proliferation and differentiation of CD4+ Th2 cells through the interaction of OX40L-OX40 (257). At present, the role of MC-exosomes in the TME is still under study, but they are expected to be a powerful means for the treatment of ovarian cancer in the future.

4 Exosomes as immunotherapy for ovarian cancer

Immune cells can be manipulated ex vivo to adjust the function of T cells (258), B cells, and NK cells, as well as to impart tumor destruction effects. Meanwhile, exosomes derived from stem cells also play a significant role in the field of cancer immunotherapy (259). Numerous studies have shown that stem cell-derived exosomes promote tumor growth and metastasis. Indeed, exosomes derived from mesenchymal stem cells in gastric cancer tissues promote the proliferation and metastasis of cancer cells by transferring miRNA into human gastric cancer cells, thus promoting the development of gastric cancer, suggesting that stem cell-derived exosomes can be used as a new biomarker for gastric cancer (260). The mesenchymal stem cell biomarker (MSC marker) CD105 is expressed by tumor-initiating cell subsets in renal cell carcinoma, and its derived exosomes promote cancer development. During tumor development, derived exosomes accelerate the formation of pre-metastatic niches by promoting cancer cell proliferation and migration, gene remodeling, and triggering angiogenic switches (261). Additionally, exosomes derived from internalized adipose-derived mesenchymal stem cells inhibit the proliferation of SKOV-3 and A2780 ovarian cancer cells in ovarian cancer tissues. Exosomes are involved in inhibiting the development of ovarian cancer by activating apoptosis signals and blocking the cancer cell cycle. Adipose-derived mesenchymal stem cell-derived exosomes carry miRNA to participate in cancer cell inhibition or progression, suggesting that exosomal miRNA plays an important role in the mechanism of ovarian cancer inhibition (262). The role of stem cell-derived exosomes in tumor immunotherapy is multifaceted. Although there have been studies on exosomes derived from stem cells in different tumors, studies on exosomes in the immunotherapy of ovarian cancer are limited, and they are still of great research value.

Exosomes in the serum of patients with ovarian cancer can promote the role of regulatory T cells and inhibit the effect of immune system on tumors by expressing a variety of immunosuppressive factors, such as TGF-β1 and IL-10 (263, 264). Additionally, exosomes isolated from the ascites of patients with ovarian cancer can promote apoptosis of peripheral blood lymphocytes and DCs (265, 266). Data have also shown that EpCAM and CD44 are highly expressed in ascites exosomes, serving as a theoretical and experimental basis for the application of exosomes in ovarian cancer immunotherapy (267).

As ovarian cancer is immunogenic, exosome-based immunotherapy is an attractive field of research (268, 269). In 1996, Raposo et al. first published a report on the function of exosomes in acquired immunity. Subsequently, they conducted a number of studies on the use of exosomes as non-antigenic carriers *in vivo* to stimulate T cells to produce specific immune responses so as to achieve long-term and tumor-specific immune protection (270, 271). Big data on the survival rate of patients with ovarian cancer show that the 5-year survival rate of patients with T-cell infiltration is significantly higher than that of patients without (272, 273). DC cell-derived exosomes have great application prospect in the field of ovarian cancer immunotherapy and their role in tumor antigen vaccine should not be ignored. DCs present antigens to specific T cells to activate T cell proliferation and destroy cancer cells. In addition to activating T cells, exosomes derived from mature DCs can also induce other antigen-presenting cells to activate T cells. However, exosomes derived from immature DCs have the opposite effect and increase cancer cell tolerance (274). Therefore, the DC maturation state determines whether the relevant exosomes launch immune attacks or induce tolerance. DC-derived exosomes recreate the TME while activating the cell's antigen-specific immune response (224). Derived exosomes are also ideal antigens for DC vaccines (212). DC exosome vaccine may replace DC adoptive therapy (233), which has potential clinical application prospects. Phase I clinical trials of DC-derived exosomes have been conducted, focusing on the feasibility of exosome-presenting protein-loaded histocompatibility complexes (275–277). Researchers have hypothesized that exosomes in ascites combined with TLR3 stimulants might prolong progression-free survival in patients with high-grade ovarian cancer (278). Tumor antigen-specific T cells are naturally present in patients with ovarian cancer, and infiltrated T cells have an excellent therapeutic effect in the prognosis of advanced ovarian cancer. Combined chemotherapy/immunotherapy with TLR3 agonists using ventral water derived exosomes carrying tumor-associated antigens activates and amplifies antigen-specific T cell immunotherapy

mechanisms against tumor-induced immunosuppression in advanced ovarian cancer (279).

The stability of exosomes themselves is excellent, and they exist stably in the circulation of human body fluids without causing immune rejection. Studies have shown that exosomes can also increase the stability and bioavailability of a variety of drugs, and enable efficient uptake by intestinal epithelial cells and immune cells. Indeed, the combination of exosomes with curcumin can improve the solubility, stability, and bioavailability of the drug, indicating that the success rate of ovarian cancer treatment can be improved by using exosomes as immunotherapy drugs to target cells/organs. Exosomes also have immunomodulatory biological properties. Exosomes derived from DCs can activate T cells and NK cells to enhance the killing effect on tumor cells, while those released by NK cells include FASL, perforin, and NKG2D, which can kill tumor cells *in vitro* (280). Exosomes can enhance the immune response by enhancing antigen presentation or directly activating immune cells and exerting anti-tumor immune effects. Exosomes can also induce immune tolerance, including exosomes of tumor cells carrying TRAIL, galectin9, or FASL molecules, which can induce apoptosis of CD8+T cells. Indeed, FasL expression in melanoma TEXs can induce apoptosis of T cells *in vivo* (281). Ovarian cancer TEXs inhibit T cell CD3-ξ and JAK3 signaling, thereby preventing T cell activation (282). FrarIgsmyr et al. found that FasL produced by syncytial trophoblasts was released in the form of exosomes, which induced apoptosis of effector cells expressing Fas (283). Ovarian cancer exosomes contain a variety of specific proteins, and their contents change during the development of ovarian cancer, which can be used as potential biomarkers (56, 284).

In recent years, researchers have used the relationship between exosomes and the immune system to combine traditional chemotherapy with immunotherapy to develop immunotherapy for tumor treatment (285, 286). This immunotherapy targets tumor-derived exosomes as potential antigens and uses TLR3 agonists to generate long-lasting T-cell immune effect and destroy the immune tolerance of the tumor (15, 287). Another study provided a new idea for immunotherapy of ovarian cancer, showing that exosomes derived from metastatic ovarian cancer deliver tumor-specific antigens to DCs, which then stimulate T cells to differentiate and induce cytotoxicity (228, 288).

Despite the lack of relevant data on exosome-based immunotherapy for ovarian cancer, this research direction has attracted increased attention.

5 Discussion

Conventional treatment of ovarian cancer can lead to drug resistance, adverse reactions, and long-term complications (2). Exosomes have great potential in the field of ovarian cancer immunotherapy as potential therapeutic markers for cancer, or as a more effective, rapid, and safe vehicle for the delivery of antitumor drugs. Exosome-based immunotherapy can activate the immune system and eliminate tumor cells (289). Exosomes have immunogenicity and molecular transfer ability and most can participate in the immune response (290). Indeed, exosomes derived from CD8+ T cells and CD4+ T cells have antigen-specific functions, which affect immune cells. The essence of the effect of exosomes in different body fluids is different. Exosomes in ascites contain miR-6780b-5p (26), those in serum contain EpCAM, Claudin4 (46, 54), and those in urine contain microRNA-30a-5p (56). In other words, a variety of exosome-based approaches can be employed to treat ovarian cancer. Exosomes are not only derived from different body fluids, but also from different cells in the TME, which have different effects on the immune response. Some cells may promote and enhance the occurrence of immune response, while others may inhibit and weaken the strength of immune response. Exosomes can form a pre-metastatic niche by acting on immune cells, and their transfer of miR-21 can change the malignant phenotype of ovarian cancer cells, which is a potential treatment for metastatic ovarian cancer (291, 292). However, exosomes derived from ovarian cancer induce T-cell arrest, which allows cancer cells to achieve immune escape (121, 293). Meanwhile, cancer cells may produce more exosomes than normal cells, and the amount of exosomes produced by different cancer cells is also different (294).

Despite the increased interest in exosome research, there remain many issues to be addressed. At present, most of the immunotherapy methods based on exosomes are in the experimental stage and lack large-scale clinical trials. Additionally, exosome isolation technology is a major difficulty. As mentioned above, the number of plasma and serum exosomes in cancer is much higher than that in healthy people, which is a promising diagnostic biomarker for ovarian cancer (53). As a whole, our conclusions are mainly based on plasma samples, while the components of plasma samples are very complex, and the effect of free proteins on exosome separation cannot be ignored (71). Differential centrifugation is the most commonly used separation method (295). However, the isolated exosomes will contain more free heterologous proteins, and the detection of the number of exosomal proteins will be limited. At the same time, the types of other proteins detected will also be reduced, resulting in inaccurate difference analysis. The feasibility of exosome separation technology is very important. Some researchers have designed molecular SEC, which can isolate exosomes with high purity (296). However, it also has certain disadvantages, which can lead to contamination by some lipoprotein impurities. Therefore, a perfect exosome separation technology is urgently needed. The activation of T cell responses by DEX containing MHC is also controversial, and the immune mechanisms of exosome-based DC vaccines require further investigation (235). Additionally, the interaction between TAM-derived exosomes and other immune cells and the relevance of immunotherapy in ovarian cancer require further study.

More importantly, exosomes have a role in immune evasion surveillance, as allies of immune escape (120). Increasing data show that exosomes play a key role in the crosstalk between cancer cells and the immune system, supporting the escape of immune surveillance by inhibiting the function of T cells and NK cells (126, 222), and the activation of monocytes, inhibiting the differentiation of DCs (127), promoting the differentiation and increase of myeloid suppressor cells (128), and inhibiting antigen-specific and non-antigen-specific antitumor responses (297). Additionally, studies have shown that DC vaccines with exosomes as antigens have limited anti-tumor efficacy in clinical immunostimulation tests (68). These findings provide new insights into the mechanisms by which cancer-derived exosomes evade immune surveillance and highlight the limitations of exosome-based cancer immunotherapy. It may be possible to effectively reduce immunosuppression by targeting tumor exosomes to expand the prospects of immunotherapy. Alternatively, exosomes can be used as potential diagnostic biomarkers to selectively eliminate cancer-derived exosomes and enhance the efficacy of immunotherapy. Before using exosomes in the clinical immunotherapy of ovarian cancer,

we need to investigate the side effects of exosomes in various aspects to ensure that their effective properties are fully utilized. However, the immunotherapeutic potential of exosomes is enormous.

These studies will help to explore the application of exosomes in ovarian cancer immunotherapy, so as to accelerate their application in clinical practice.

Author contributions

QW and XG conceived the study. XG, HC, DS, and ZX drafted the manuscript. XG and QW performed the literature search and collected the data. AT, ZX, and QW helped with the final revision of this manuscript. All authors reviewed and approved the final manuscript.

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Conflict of interest

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

Publisher's note

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NK cell-derived exosomes enhance the anti-tumor effects against ovarian cancer by delivering cisplatin and reactivating NK cell functions

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Exosomes are membranous vesicles actively secreted by almost all cells and they deliver certain intracellular molecules, including nucleic acids, proteins, and lipids, to target cells. They are also considered to be good carriers for drug delivery due to their biocompatibility, high permeability, low immunogenicity, and low toxicity. Exosomes from immune cells were also reported to have immunomodulatory activities. Herein we evaluated the application of exosomes derived from expanded natural killer cells (eNK-EXO) for the treatment of ovarian cancer (OC). We demonstrate that eNK-EXO express typical protein markers of natural killer (NK) cells, can be preferentially uptaken by SKOV3 cells, and display cytotoxicity against OC cells. Furthermore, eNK-EXO loaded with cisplatin could sensitize drug-resistant OC cells to the anti-proliferation effect of cisplatin. In addition, we show that eNK-EXO could activate NK cells from immunosuppressive tumor microenvironment, the mechanism of which is explored by transcriptional analysis. In summary, eNK-EXO exhibit anti-tumor activity against OC on its own, could be used to deliver cisplatin and enhance its cytotoxic effect against drug-resistant OC cells and also reverse the immunosuppression of NK cells, which may lead to great prospect of using eNK-EXO in the treatment of OC in the clinic. Our work also builds a strong foundation for further evaluation of eNK-EXO in other solid tumor therapies.

KEYWORDS

ovarian cancer, exosomes, natural killer cells, cisplatin, immunomodulatory

Introduction

Ovarian cancer (OC) has the highest mortality rate among gynecological malignancies, and most patients are not diagnosed until late stages due to insidious or nonspecific early clinical symptoms (1). Surgery combined with platinum-based chemotherapy has been the primary treatment for OC. Although most patients are sensitive to platinum-based first-line chemotherapy, more than 80% of patients relapse within 18 months of initial treatment and become resistant to almost all chemotherapy drugs (2). Therefore, primary or secondary resistance has become the main contributing factor to the high mortality rate of OC. Unfortunately, the mechanisms for drug resistance in OC remain unclear. In recent years, increasing evidence has shown that the occurrence of acquired drug resistance of tumor cells is closely related to tumor microenvironment (TME) (3–6). In OC, the tumor is mainly confined to the abdominal cavity, where the peritoneal fluid provides mobile and accessible dynamic environment between OC cells and stromal cells, making OC more prone to recurrence, metastasis, and drug resistance (7). Therefore, the development of novel and efficient therapeutic strategies resolving the drug resistance of OC is expected to improve the prognosis of OC patients (8, 9).

Exosomes are nano-scale bilayer vesicles actively secreted by various cells and contain diverse biomolecules (10). Recently, it has been discovered that exosomes mediate near and long-distance cell-to-cell communication in both healthy and diseased cells, affecting all aspects of cell biology (11, 12). Different cells exert cell-to-cell communication by releasing exosomes carrying different components, such as nucleic acids, lipids, proteins, and metabolites (13). These exosomes are taken up by the recipient cells and information is delivered through material exchange or release of contents (13). In recent years, the potential application of exosomes in anti-cancer therapy have attracted increasing attentions (12). Exosomes derived from NK cells (NK-Exo) encapsulate perforin, granzyme, microRNA (e.g., miR-186, miR-3607, etc.), and other tumor-killing substances during biogenesis, thus exhibiting cytotoxic effects on a variety of tumor cells, including breast cancer, melanoma, and neuroblastoma (14–18). Moreover, NK-Exo express typical NK cell markers (e.g. CD56) and cytotoxicity receptors (e.g.,NKG2D) (10, 19), and can deliver the chemotherapy drug paclitaxel for breast cancer therapy (20). A previous study showed that exposure to an immunosuppressive environment mimicked by TGF-β and IL-10 did not attenuate the original affinity and anti-tumor activity of NK-Exo (10, 16). It could be that NK-Exo retain their anti-tumor activity because of lacking the signalling and metabolic pathways that respond to inhibitory TME (21). Therefore, impeded by many inherent limitations of NK cell-based therapies, such as insufficient tumor targeting, limited NK cell infiltration in TME, and inhibition of NK cell function by TME (22), scientist found that NK-Exo may provide an alternative for cancer treatment, especially in solid tumors, as a "cell-free" immunotherapeutic strategy.

Based on the advantages of drug delivery and immunomodulatory activity of exosomes, we hypothesized that exosomes derived from expanded NK cells (eNK) can deliver tumor therapeutic drugs and reverse the immunosuppression of NK cells. In this study, we demonstrate the therapeutic effect of exosomes derived from eNK cells (eNK-EXO) against OC *in vitro* by itself and with loaded cisplatin (DDP). eNK-EXO not only sensitize OC cells to the cytotoxic effect of DDP, but also reactivate NK cells after being suppressed by TME. These findings suggest that eNK-EXO could potentially reverse immune suppression by reactivating defective NK cells in TME, enhancing the therapeutic effect of anti-cancer drugs.

Materials and methods

Human samples

The cord blood mononuclear cells (CBMC) were isolated from the cord blood of 3 healthy donors by density gradient centrifugation. eNK were obtained by co-culturing CBMC with irradiation-inactivated K562 engineered cells for 14 days in the presence of IL-2 (23). The human ascites mononuclear cells were isolated from the ascites of 2 patients with malignant OC by Ficoll gradient density centrifugation, and the NK cells in ascites (AS_NK) were obtained using EasySep™ Human NK Cell Isolation Kit (Stemcell Technologies). AS_NK and eNK cells were maintained in RPMI 1640 medium supplemented with 5% human plasma and 1% penicillin/streptomycin (Gibco, Gaithersburg, MD, USA). All cells were cultured at 37°C in a humidified atmosphere of 5% CO_2-95% air. The studies involving human samples were reviewed and approved by the Ethical Committee of GuiZhou Medical University, Guizhou, China (2022–82). The patients/participants provided their written informed consent to participate in this study.

Cell culture and treatment

The human OC cell line SKOV3, OV-90 and COC1/DDP (cisplatin resistant) were obtained from the China Center for Type Culture Collection. NK92/MI cells and human ovarian epithelial cells IOSE80 were obtained from Procell Life Science&Technology Co., Ltd. SKOV3, OV-90, COC1/DDP and IOSE80 were cultured in McCoy's 5a medium, DMEM/F12 medium (Gibco, Gaithersburg, MD, USA), RPMI 1640 medium (Gibco), and DMEM/High Glucose medium (HyClone, USA), respectively. All cultural media were supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin (Gibco). NK92/MI was cultured in MEM-Alpha medium (Gibco) supplemented with 12.5% FBS, 12.5% horse serum, 0.2mM Inositol, 2-Mercaptoethanol and 0.02 mM folic acid (Sigma-Aldrich, St. Louis, MO, USA). All cells were cultured at 37°C in a humidified atmosphere of 5% CO_2-95% air.

To obtain NK cells treated with OC ascites (AS-t-NK), the ascites collected from 7 patients (Supplementary Table S1) with malignant OC were aliquoted into 50-mL conical tubes and centrifuged at 300 g for 10 min to separate cell pellets from supernatant (24). eNK cells were then cultured in RPMI 1640 medium containing 10% OC ascites for 24 h.

Preparation of eNK-EXO and eNK-EXO-DDP

eNK-EXO were isolated using differential ultracentrifugation method (10). eNK cells were cultured in conditioned medium containing 5% exosome-free FBS for 48 h and the supernatants were centrifuged for 10 min at 300 g and 10 min at 2000 *g* to remove cells and large debris. Next, the supernatant was centrifuged

at 10000 g for 30 min and filtered through a 0.22 μm filter (Millipore) to remove large vesicles before further centrifugation at 100000 g for 70 min with P28S rotor (Hitachi, CPN100NX, Japan). The pellets were then resuspended in PBS and centrifuged again at 100000 g for 70 min. The exosome pellet was resuspended in 100 μL PBS. All of the above steps were performed at 4°C. The obtained exosomes were quantified by BCA protein assay (Beyotime, China) and stored at -80° C to use within two months.

To prepare DDP loaded eNK-EXO (eNK-EXO-DDP), we used electroporation to load DDP (Qilu Pharmaceutical, China) into eNK-EXO (25). We first mixed 250 μg of eNK-EXO, 250 μg of DDP and 100 μL electroporation buffer in a 200 μL electroporation cuvette, and then the mixed sample was electroporated at 450 V for 120 ms (Celetrix) (26, 27). The obtained eNK-EXO-DDP was used immediately.

Transmission electron microscopy

To examine exosomes under transmission electron microscopy, the purified exosomes (>10^9/mL) from the supernatant of NK cells were suspended in PBS and mounted onto the copper grid. Excess liquid was gently removed with filter paper. Uranyl acetate was then loaded onto the copper grid for 1 min and excess liquid was gently removed with filter paper. Samples were air dried and observed with the HT-7700 transmission electron microscope (Hitachi, Japan) at 100 kV.

Nano-flow cytometry analysis

The particle concentration, size distribution and phenotypes of eNK-EXO were analyzed by nFCM (NanoFCM, China) according to reported protocols (28, 29). Briefly, two single photon counting avalanche photodiodes were used for the simultaneous detection of the side scatter and fluorescence of individual particles. The instrument was calibrated for particle concentration using 200 nm PE and AF488 fluorophore-conjugated polystyrene beads and for size distribution using Silica Nanosphere Cocktail (NanoFCM Inc., S16M-Exo). Any particles that pass by the detector within a 1-min interval were recorded in each test. All samples were diluted to attain a particle count within the optimal range of 2000~12,000/min. Using the calibration curve, the flow rate and side scattering intensity were converted to the corresponding vesicle concentration and size on the NanoFCM software (NanoFCM Profession V1.0).

For immunofluorescent staining, the following antibodies were purchased from Biolegend: APC-conjugated mouse anti-human CD16 (clone 3G8), FITC-conjugated mouse anti-human CD107a (clone H4A3), PE-conjugated mouse anti-human CD56 (clone MEM-188) and PE-conjugated mouse anti-human CD69 (clone FN50). An aliquot of eNK-EXO samples was suspended in 20 μL of PBS with a particle concentration of 1×10^9 particles/mL, mixed with 20 μL antibody and incubated for 60 min at 37°C. After incubation, the mixture was washed with PBS and centrifuged at 100,000 *g* for 70 min at 4°C. The pellet was then resuspended in 50 μL of PBS for phenotype analysis.

Exosome uptake

For the uptake assays, eNK-EXO were labeled with PKH67 (Sigma-Aldrich, USA) and centrifuged at 100,000 g for 70 min to remove any free dye. SKOV3 cells were co-cultured with 20 μg of PKH67-labeled eNK-EXO for 6 h. Then the cells were washed in PBS and fixed in 4% paraformaldehyde, the cell nuclei were stained with 10 μg/mL DAPI. For blocking experiments, SKOV3 cells were co-cultured with 20 μg of PKH67-labeled eNK-EXO for 6 hours in the presence of anti-human CD63 (clone EPR5702; 1:1000; Abcam) or anti-human CD81 (clone D3N2D; 1:1000; Cell Signaling Technology). The cells were then treated with the same steps as described above. For selective uptake detection, SKOV3 cells were labeled by 10 μM DiR (Perkin Elmer, USA) and co-cultured with IOSE80 cells at a ratio of 1:1. The cells were then incubated with 20 μg of PKH67-labeled eNK-EXO for 6 h at 37°C and treated with the same steps as described above. All the images above were acquired by fluorescence microscope (Olympus BX51) and the fluorescence intensity of intracellular eNK-EXO was analyzed using Image J 1.53a software (National Institutes of Health, USA).

Cell viability assays

To evaluate the cytotoxicity of eNK-EXO against tumor cells, SKOV3, COC1/DDP and ISOE80 cells were seeded at 1×10^4 cells per well in 96-well plates and co-cultured with different concentrations of eNK-EXO (10, 20, 40, 60, 80, 100 μg/mL) for 24 h at 37°C. In order to evaluate whether exosomes derived from other cells are cytotoxic against OC cells, we tested the cytotoxicity of 10 μg/mL exosomes derived from mesenchymal stem cells (MSC-exo) on SKOV3 and OV-90 ovarian cancer cells and IOSE80 ovarian epithelial cells as a control. To evaluate the cytotoxicity of eNK-EXO-DDP against tumor cells, SKOV3, COC1/DDP and OV-90 cells were seeded at 1×10^4 cells per well in 96-well plates. Equal amounts of eNK-EXO, DDP and eNK-EXO-DDP (10μg/mL) were added to tumor cells and co-cultured at 37°C for 24 h. 10 μL of detection reagents from Cell Counting Kit-8 (CCK-8; Dojindo, Japan) were added to cultured cells and incubated at 37°C for 2 h before optical densities (ODs) were measured at 450 nm. Measurements were performed in triplicate for each experiment, and all experiments were repeated three times. Cell viability was calculated by the following formula:

$$\text{Survival rates\%} = (OD_{experiment} - OD_{blank})/(OD_{control} - OD_{blank}) \times 100\,\%$$

To evaluate the cytotoxicity of NK cells under various treatment, NK cells, NK92/MI cells and AS-t-NK cells were first treated with or without 80 μg/mL eNK-EXO for 24 h. SKOV3 cells were seeded at 1×10^4 cells per well in 96-well plates, then NK cells, NK92/MI cells, and AS-t-NK cells with or without eNK-EXO treatment were added at an effector-target ratio(E:T) of 1:1 and 2:1, respectively. After 5 h incubation, 10 μL CCK-8 were added into each well for 2 h and the ODs were measured at 450 nm. Measurements were performed in

triplicate for each experiment, and all experiments were repeated three times. Killing rate was calculated by the following formula:

$$\text{Killing rates\%} = \left[1 - (OD_{experiment} - OD_{effect})/OD_{control}\right] \times 100\,\%$$

EdU cell proliferation assay

Cell proliferation was determined by EdU Cell Proliferation Kit (RiboBio, China). SKOV3 cells were incubated with 50 μM EdU for 5 h, then fixed with 4% formaldehyde and stained according to the manufacturer's instructions. The images were acquired by fluorescence microscope (Olympus BX51). The number of proliferating cells and total cells was determined by red and blue signals, respectively. The proliferation rates were calculated by dividing the numbers of proliferating cells by the numbers of total cells.

Flow cytometry

SKOV3, COC1/DDP cells were pre-treated with 10 μg/mL of eNK-EXO, DDP or eNK-EXO-DDP for 24 h. Cell apoptosis was evaluated by flow cytometry analysis using Annexin V-FITC/PI apoptosis detection kit (Absin, China). Briefly, cells were washed in PBS twice and suspended in 1× binding buffer, incubated in the dark with FITC-Annexin-V for 10 min, followed by PI for 5 min before flow cytometry analysis. Cell cycle assay was carried out using cell cycle detection kit (KeyGEN Biotech, China). Briefly, cells were fixed by 70% ethanol overnight, washed in 1×PBS and treated with RNase-containing propidium iodide for 30 min before flow cytometry analysis. To measure cell proliferation, SKOV3 cells were labeled with 2.5 μM CFSE dye solution for 20 min in the dark and then cultured with the same conditions as above for 48 h after removing free dye. Stained cells were collected on a FC500 flow cytometer (Beckman Coulter, USA) and the data was analyzed by the FlowJo-10 software.

Western blot analysis

eNK-EXO treated cells were lysed in 1×RIPA buffer with protease inhibitor PMSF. The lysate was mixed with loading buffer and boiled for 10 min. Proteins were separated on a 12% SDS-PAGE gel, transferred to PVDF membranes and blocked by 5% BSA for 2 h. The membranes were incubated with primary antibodies overnight at 4°C. After being washed in 1×TBST, the membranes were incubated with HRP-conjugated goat anti-rabbit IgG (1:5000; Absin) secondary antibodies at room temperature for 2 h. Primary antibodies used above include anti-CD63 (clone EPR5702; 1:1000; Abcam), anti-CD81 (clone D3N2D; 1:1000; CST), anti-TSG101 (clone EPR7130 (B); 1:1000; Abcam), anti-Calnexin (1:1000; Biodragon), anti-CD56 (clone E7X9M; 1:1000; CST), anti-perforin (1:1000; Biodragon), anti-Granzyme B (1:1000; Biodragon), anti-cleaved caspase 3 (clone 5A1E; 1:1000; CST), anti-cleaved caspase 7 (clone D6H1; 1:1000; CST), anti-PARP (1:1000; CST) and anti-cleaved PARP (clone D64E10; 1:1000; CST). Anti-β-actin (1:500; Santa Cruz) was used to normalize relative expression of target proteins. The images were visualized by chemiluminescence (Bio-Rad Laboratories, USA).

Enzyme-linked immunosorbent assay

To measure the production of perforin, TNF-α, CXCL9, CXCL10 and CXCL11, the supernatant was collected and centrifuged to remove remaining cells. The levels of perforin and the above cytokines were evaluated by human perforin ELISA Kit (Enzyme-linked Biotechnology Co. Ltd., Shanghai, China), human TNF-α ELISA Kit (Multisciences, Hangzhou, China), Human CXCL9 ELISA Kit (Multisciences, Hangzhou, China), Human CXCL10 ELISA Kit (Multisciences, Hangzhou, China), and Human CXCL11 ELISA Kit (Multisciences, Hangzhou, China) according to the manufacturers' instructions, respectively. The ODs were measured at 450 nm and the concentrations of perforin and the cytokines were determined according to their corresponding standard curves.

RNA-seq

Total RNAs were extracted using TRIzol reagent (Invitrogen, CA, USA) and reverse transcribed into cDNA. The libraries were constructed using VAHTS Universal V6 RNA-seq Library Prep Kit. The transcriptome sequencing and analysis were conducted by OE Biotech Co., Ltd. (Shanghai, China). The libraries were sequenced on an illumina Novaseq 6000 platform and 150 bp paired-end reads were generated. Fragments Per Kilobase of exon model per Million mapped reads (FPKM) of each gene was calculated and the read counts of each gene were obtained by HTSeq-count. Principal component analysis (PCA) analysis were performed using R (v 3.2.0) to evaluate the biological duplication of samples. Differential expression analysis was performed using the DESeq2, q value< 0.05 and foldchange > 2 or foldchange< 0.5 was set as the threshold for significantly differential expression gene (DEGs). Hierarchical cluster analysis of DEGs was performed using R (v 3.2.0) to demonstrate the expression pattern of genes in different groups and samples. Based on the hypergeometric distribution, KEGG pathway enrichment analysis of DEGs were performed to screen the significant enriched term using R (v 3.2.0).

Statistical analyses

All experiments were repeated at least three times. All statistical analyses were performed using GraphPad Prism 8, and the data are expressed as the mean± SEM. A P value< 0.05 was considered statistically significant by using the Student's *t*-test: **p*< 0.05, ***p*< 0.01 and ****p*< 0.001.

Results

Isolation and characterization of exosomes from *ex vivo* NK cell culture

We previously established a method to obtain eNK cells from PBMC by co-culturing with K562-mBIL-21 feeder layer cells (Lifeark, China) for 2 weeks (23). Compared with other allogeneic cell sources, such as bone marrow and peripheral blood, cord blood (CB) is readily

available as a frozen, "off the shelf" product, which provides more advantages as the starting material for cell therapies. CB is a rich source of immature NK cell population and the NK cells expanded from CB can mature into potent NK cells with higher proliferation rate and cytotoxicity than that expanded from peripheral blood (30–33). It can be reliably produced for clinical use (34). Thus, we can obtain a large number of highly cytotoxic exosomes from eNK cells culture supernatants. In this study, we used mononuclear cells derived from CB to obtain a large number of eNK cells. As shown in Figure 1A, on day 14, the culture contained over 95% eNK cells ($CD3^-$, $CD56^+$, $CD16^+$). In order to prepare eNK-EXO, we continued to culture eNK cells in RPMI-1640 with 5% exosome-free FBS. eNK-EXO were then isolated as described in methods. Particle size and concentration analysis by nFCM revealed that the particles were homogeneous in size with an average of 73.2 ± 28.5 nm in diameter (Figure 1B). Morphology imaging of eNK-EXO by transmission electron microscopy showed a typical "saucer shape" with a particle size of about 80 nm, which was consistent with nFCM results (Figure 1C). Further characterization of eNK-EXO by western blot analysis indicated the existence of exosome markers such as CD81, CD63, and TSG101, while the negative marker calnexin was only detected in cell lysates (Figure 1D). Therefore, according to the size, concentration, morphology, and protein markers, we concluded that the eNK-EXO isolated from eNK cell culture by our protocol were of good quality and can be used for following studies.

eNK-EXO are cytotoxic against OC cell lines

To further characterize the isolated eNK-EXO, we first determined whether they contained typical markers and cytotoxic proteins derived from eNK cells. As shown in Figure 2A, nFCM analysis showed the presence of CD69 (a marker for NK cell activation) and CD107a (a marker for NK cell degranulation) in eNK-EXO, as well as CD16 and CD56, markers of NK cells. Western blot analysis also confirmed the presence of these two cytotoxic proteins, perforin and granzyme B, in the isolated eNK-EXO (Figure 2B). These results were consistent with previous reports on exosomes isolated from NK cell culture medium (19). NK cells are known to exert their cytolytic effect through the release of effector molecules such as perforin and granzymes, which may contribute to the cytotoxicity of eNK-EXO against tumor cell lines.

NK-Exo were reported to cause lysis of breast cancer and melanoma cell lines (15, 18). However, as far as we know, whether eNK-EXO have cytotoxic effect on OC cells has not been studied. Therefore, we evaluated the cytotoxicity of eNK-EXO against OC cell lines by two separate assays. Firstly, the results of the CCK-8 cell viability assay showed that eNK-EXO caused lysis of SKOV3 and COC1/DDP OC cells in a dose-dependent manner, but not IOSE80, the human ovarian epithelial cells (Figure 2C). Meanwhile, MSC-exo showed no cytotoxicity on SKOV3, OV-90 and IOSE80 cells (Supplementary Figure S1). Secondly, EdU proliferation assay showed that eNK-EXO inhibit the proliferation of SKOV3 cells in a dose-dependent manner (Figures 2D, E). The results of two assays cross-validated the cytotoxicity of eNK-EXO against OC cell lines and were safe against healthy ovarian epithelial cells.

eNK-EXO are preferentially uptaken by OC cell lines

To evaluate whether eNK-EXO can be taken up by OC cells, PKH67-labeled eNK-EXO were incubated with SKOV3 cells for 6 hours and fluorescence images were taken to measure cellular uptake of eNK-EXO (Figure 3A). Quantification of fluorescence intensity showed significant uptake of eNK-EXO by SKOV3 cells and the uptake rate could reach 60% in 6 hours (Figure 3B). To investigate which receptors are responsible for the uptake, we pretreated SKOV3 cells with anti-CD63 or anti-CD81 antibodies and discovered that either blocking CD63 or CD81 could cause a significant decrease in

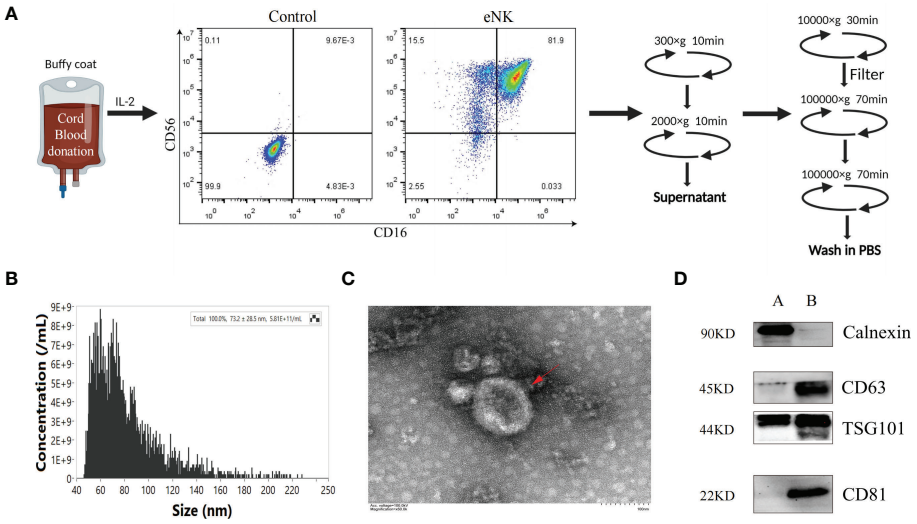

FIGURE 1
Preparation and Characterization of eNK-EXO. **(A)** Work flow of eNK-EXO isolation. **(B)** Particle size distribution curve of eNK-EXO by NanoFCM. **(C)** Transmission electron microscopy images of eNK-EXO, Bar =100 nm. **(D)** Western blot analysis of CD81 (22 kDa), CD63 (45 kDa), TSG101 (44 kDa) and Calnexin (90 kDa) expression on eNK-EXO. NK cell lysate was used as control. (A: cell lysate, B: eNK-EXO).

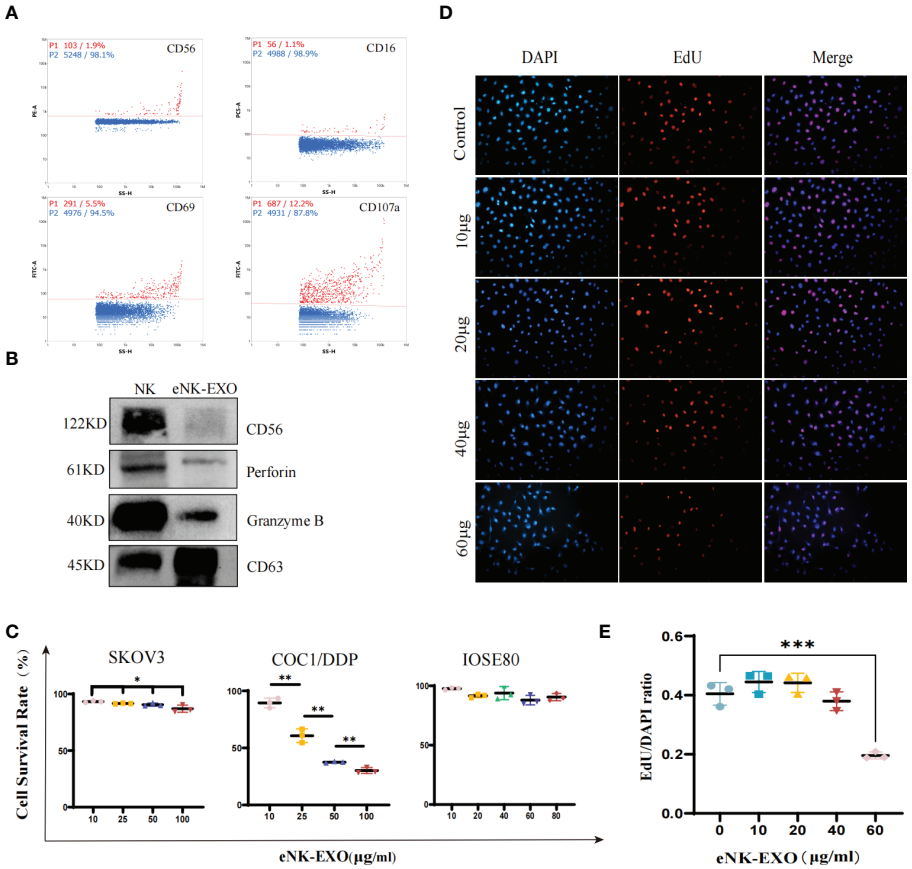

FIGURE 2
Functional characterization of eNK-EXO *in vitro*. **(A)** Expression of eNK-EXO surface markers CD56, CD16, CD69 and CD107a by NanoFCM. Red dots represent positive signals. **(B)** Western blot analysis of CD56 (122KD), perforin (61KD) and GranzymeB (40KD) expression. NK cell lysate was used as control. **(C)** CCK-8 assay results of eNK-EXO against SKOV3, COC1/DDP and IOSE80 cells (n=3, mean ± SEM, t-test, *$p< 0.05$, **$p< 0.01$). **(D, E)** EdU assay results of eNK-EXO against SKOV3 (20×, scale bar = 100 μm). Red signals represent newly proliferating cells, whose proportion was quantified and shown (n=3, mean ± SEM, ***$p< 0.001$) **(E)**.

the proportion of cells that took up PKH67-labeled eNK-EXO (Figures 3C, D). These results indicated that CD63 and CD81 might play an important role in the uptake of eNK-EXO by SKOV3 cells, however, other mechanisms may also be involved.

Based on the result that eNK-EXO were cytotoxic to OC cells but had no cytotoxic effect on normal cells (Figure 2C), we hypothesized that it could be attributed to the different uptake rates between tumor cells and normal cells. To test our hypothesis, DiR-labeled SKOV3 cells and IOSE80 cells were co-cultured at a ratio of 1:1 and treated with PKH67-labeled eNK-EXO for 6 hours. As shown in Figure 3E, preferential uptake of eNK-EXO by SKOV3 cells over IOSE80 was clearly seen as green fluorescence of eNK-EXO readily appeared in DiR-labeled SKOV3 cells while little was seen in IOSE80 (Figure 3E).

The anti-tumor effect of eNK-EXO loaded DDP *in vitro*

To date, dozens of studies have been carried out on the use of exosomes as drug-delivery vehicles (35, 36). Motivated by the excellent performance of exosomes in tumor therapeutic drug delivery, we next explored the potential of using eNK-EXO as delivery vehicles for OC chemotherapeutic drug DDP for OC treatment (Figure 4A).

We assayed the anti-tumor effect of eNK-EXO-DDP, eNK-EXO and free DDP alone against SKOV3, OV90, and COC1/DDP ovarian cancer cells in parallel. As shown in Figure 4B, compared with eNK-EXO and DDP treated alone, the cell survival rate of three OC cell lines decreased significantly after eNK-EXO-DDP treatment in the CCK-8 assay, especially in DDP-insensitive OV-90 cells and DDP-resistant COC1/DDP cells (Figure 4B). These results indicate that eNK-EXO could sensitize OC cells to the effect of DDP. Next, we used flow cytometry and EdU proliferation assay to detect the inhibitory effect of eNK-EXO-DDP on the proliferation of OC cells. Flow cytometry showed that eNK-EXO-DDP significantly suppressed the proliferation of SKOV3 cells, the fluorescence intensity of CFSE decreasing with each generation of cell proliferation (Figure 4C). However, due to the efflux of fluorescent dyes by tumor cells (37, 38), we can't detect the double peak of fluorescence intensity, and the inhibition of proliferation was judged by the degree of rightward shift of the single peak. Moreover, EdU staining also confirmed that eNK-EXO-DDP has a potent inhibitory effect on OC cells (Figure 4D).

DDP exerts its antitumor activity by binding to genomic DNA or mitochondrial DNA to block the production of DNA and arrest DNA

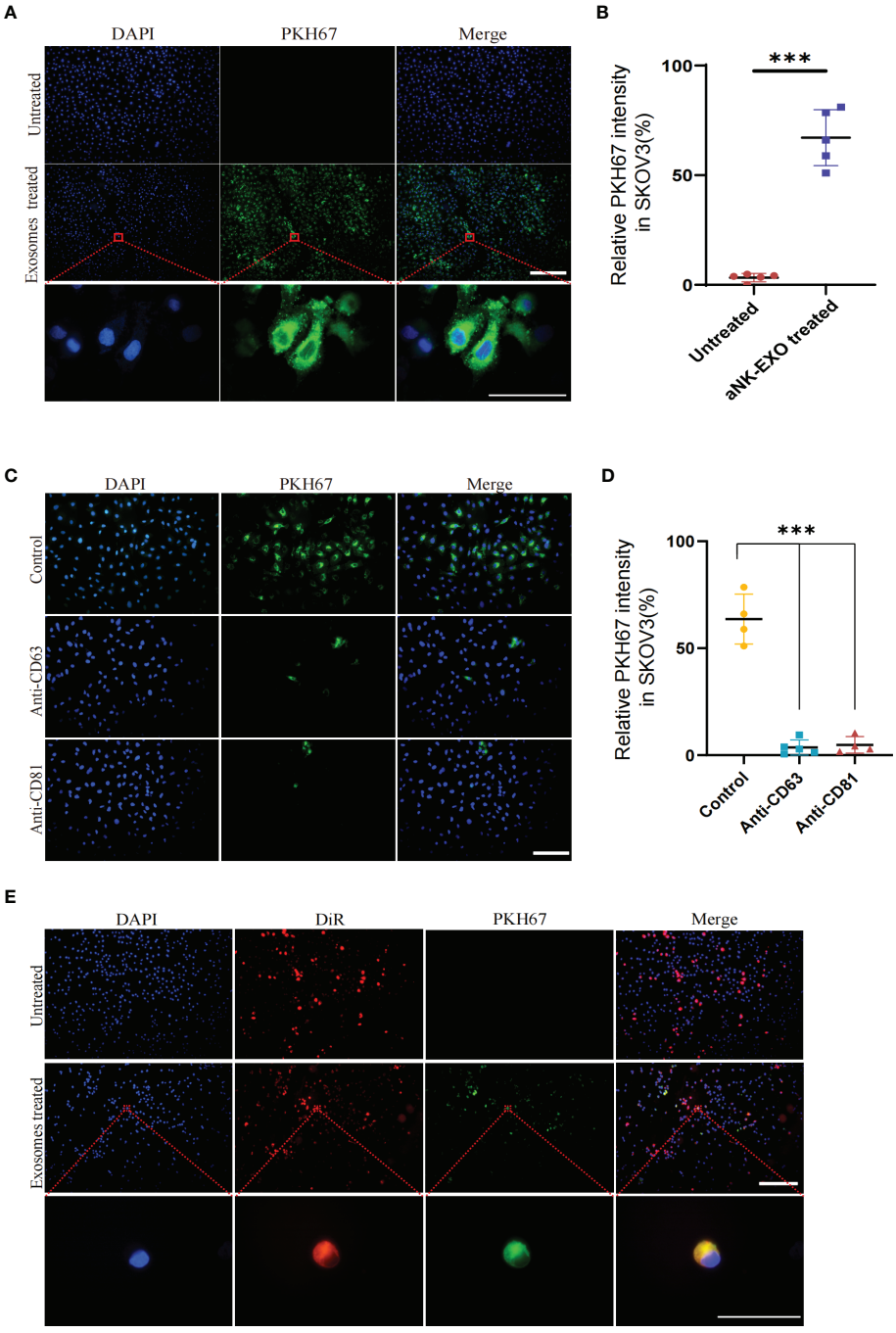

FIGURE 3
Cellular uptake results of eNK-EXO by OC cells. **(A)** eNK-EXO uptake by SKOV3 cells. SKOV3 cells were stained blue by DAPI and eNK-EXO were stained green by PKH67. **(B)** eNK-EXO uptake quantified by green fluorescence intensity (10×, Scale bar=200 μm; 100×, Scale bar=40 μm, n=5, mean ± SEM, ***$p< 0.001$). **(C)** eNK-EXO uptake by SKOV3 in the presence of anti-CD63 or anti-CD81. SKOV3 cells were stained blue by DAPI and eNK-EXO were stained green by PKH67 (20×, scale bar=100 μm). **(D)** eNK-EXO uptake quantified by green fluorescence intensity (n=4, Mean ± SEM, t-test, ***$p< 0.001$). **(E)** Uptake of PKH67-labeled eNK-EXO in the co-culture system of IOSE80 cells and DiR-labeled SKOV3 cells (10×, scale bar=200 μm; 10×, scale bar = 40 μm).

replication which finally led to apoptosis (4, 39–41). We asked if the anti-tumor effect of eNK-EXO-DDP was the result of DDP activity. Cell cycle analysis showed considerable G2/M arrest in eNK-EXO-DDP treated cells (Figure 4E). Apoptosis analyzed by flow cytometry also confirmed that eNK-EXO-DDP can induce apoptosis in SKOV3 cell (27.41% ± 3.12% vs12.29% ± 1.84%) (Figures 4F, G) and the proportion of apoptotic cells was comprised of both late apoptotic (upper right quadrant) and early apoptotic cells (bottom right quadrant). eNK-EXO-DDP treatment could induce significant apoptosis in COC1/DDP cells as well (44.72% ± 10.13% vs 24.56% ± 6.87%). Western blot confirmed the increased expression of cleaved PARP, cleaved caspase-3, and cleaved caspase-7 in two OC cell lines

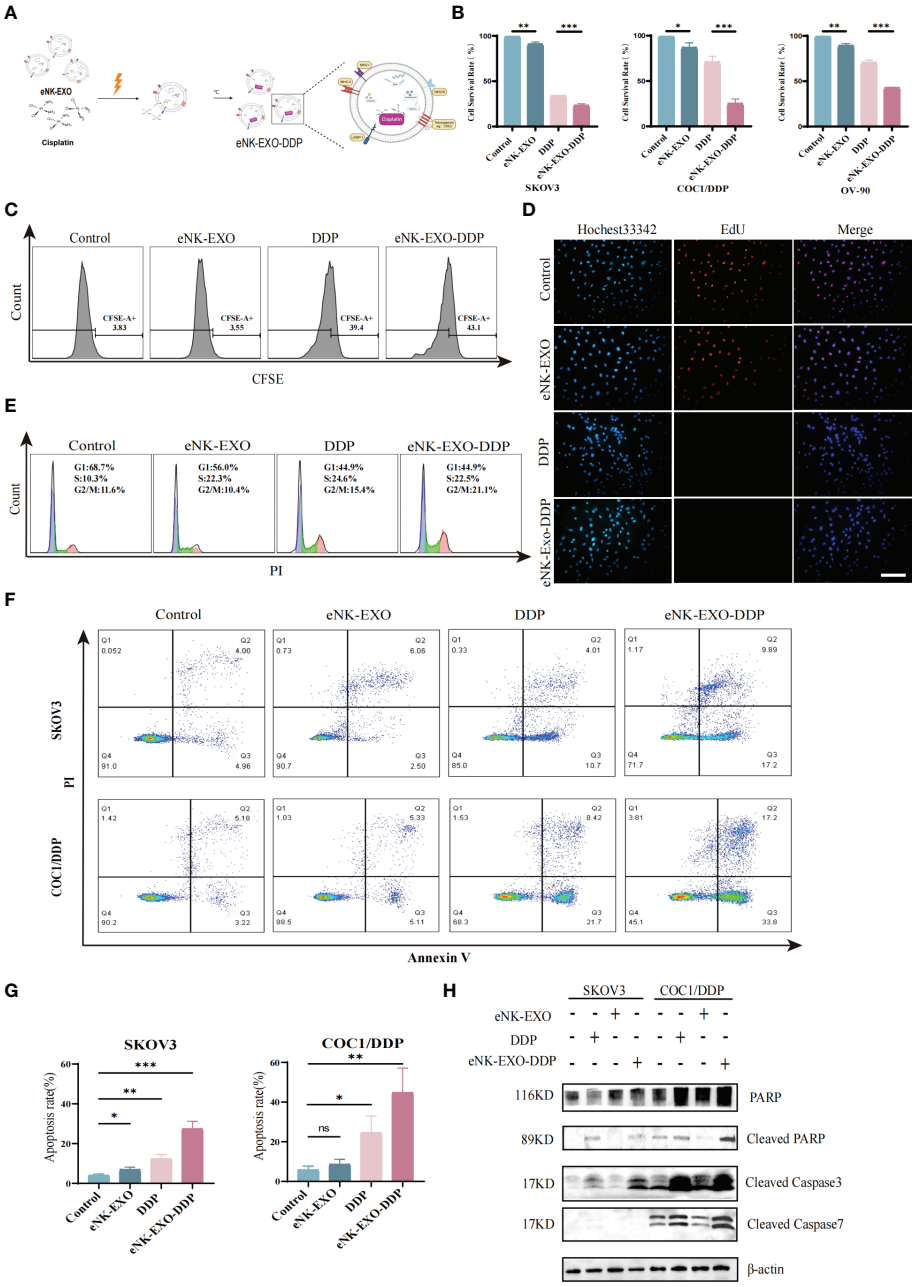

FIGURE 4
Anti-tumor activity of eNK-EXO-DDP *in vitro*. **(A)** Schematic diagram showing the preparation of eNK-EXO-DDP. **(B)** CCK-8 assay showing the cytotoxicity of eNK-EXO-DDP against SKOV3, COC1/DDP and OV-90 cells (n=3, mean ± SEM, ***p*< 0.01, ****p*< 0.001). **(C)** Flow cytometry analysis of anti-proliferation activity of eNK-EXO-DDP against SKOV3 cells. **(D)** EdU assay result of anti-proliferation activity of eNK-EXO-DDP against SKOV3 cells, Scale bar=100 μm. **(E)** Cell cycle analysis of SKOV3 cells after eNK-EXO-DDP treatment by flow cytometry. **(F)** Apoptosis analysis of SKOV3 cells after eNK-EXO-DDP treatment by flow cytometry. **(G)** Statistical analysis of apoptosis rate is based on three experiments, mean ± SEM, **p*< 0.05, ***p*< 0.01, ****p*< 0.001. **(H)** Western blot analysis of PARP, cleaved PARP, cleaved caspase 3 and cleaved caspase 7 in SKOV3 and COC1/DDP cells after different treatments.

upon eNK-EXO-DDP treatment (Figure 4H). These results suggest that eNK-EXO-DDP inhibits cell proliferation by inducing cell cycle arrest and apoptosis in OC cells, demonstrating the ability of eNK-EXO to deliver chemotherapeutic drugs to OC cells and also sensitize the cells to drug treatment. However, eNK-EXO alone did not show an anti-tumor effect, which may be because the dose of eNK-EXO used in the above experiment is only 10 μg/mL, which is not enough to exert its anti-tumor effect. As shown in Figures 2B–E, the anti-tumor effect of eNK-EXO at this concentration is almost negligible.

eNK-EXO enhances the cytotoxicity of NK cells

Based on the immunomodulatory activity of exosomes, we investigated the effect of eNK-EXO on NK cells. ELISA results showed that NK cells treated with eNK-EXO released more perforin and TNF-α than control (Figure 5A). Cytotoxicity of NK cells and NK92/MI cells pre-treated with eNK-EXO was also significantly improved when co-cultured with SKOV3 cells at a 2:1

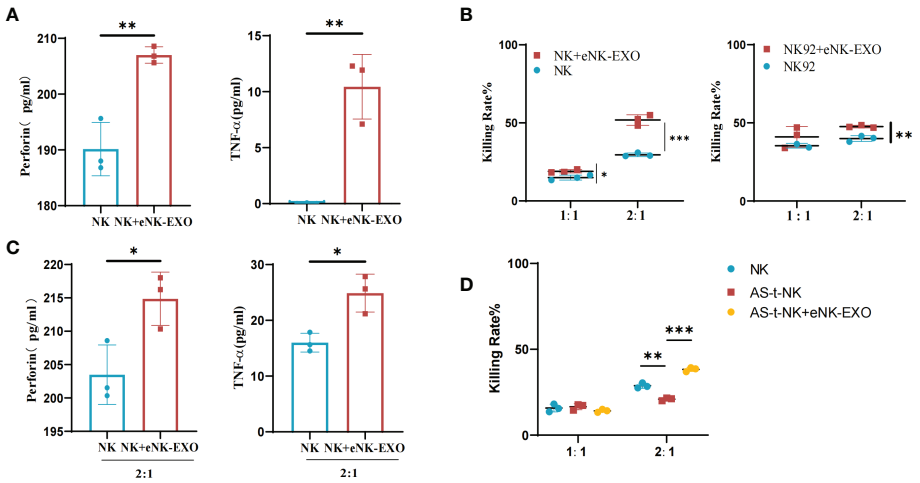

FIGURE 5
Immunomodulatory effects of eNK-EXO *in vitro*. **(A)** ELISA measurement of perforin and TNF-α concentrations in the growth medium of eNK-EXO treated NK cells (n=3, mean ± SEM, $^{**}p< 0.01$). **(B)** CCK-8 assay showing the cytotoxicity of eNK-EXO treated NK cells and NK92/MI cells against SKOV3 cells (n=3, mean ± SEM, t-test, $^{*}p< 0.05$, $^{**}p< 0.01$, $^{***}p< 0.001$). **(C)** ELISA measurement of perforin and TNF-α concentrations in the growth medium of SKOV3 cells co-cultured with eNK-EXO treated NK cells (n=3, mean ± SEM, $^{*}p< 0.05$). **(D)** CCK-8 assay showing the cytotoxicity of AS-t-NK cells and eNK-EXO treated AS-t-NK cells against SKOV3 cells (n=3, mean ± SEM, $^{**}p< 0.01$, $^{***}p< 0.001$).

E:T ratio as measured by CCK-8 (Figure 5B). Increased TNF and perforin release by NK cells were also detected by ELISA when NK cells were pre-treated with eNK-EXO and co-cultured with SKOV3 at a 2:1 E:T ratio (Figure 5C).

To mimic NK cells in OC TME, we treated NK cells with 10% ascites collected from 7 different ovarian cancer patients. The obtained AS-t-NK cells were assayed for their cytotoxicity against SKOV3 by CCK-8. As shown in Figure 5D, the killing effect of AS-t-NK cells on SKOV3 cells was significantly inhibited at a 2:1 E:T ratio. However, treating with eNK-EXO reversed the inhibitory effect and resulted in an even higher cytotoxicity of AS-t-NK cells than that of eNK cells (Figure 5D). Therefore, current data suggest that eNK-EXO may be able to re-activate the cytotoxic function of NK cells once impaired in TME.

RNA sequencing reveals that NK cells treated with eNK-EXO or isolated from OC ascites have a vastly different transcriptional landscape compared to NK cells

Global transcriptional analysis by RNA-seq was performed to understand the molecular mechanisms underlying the functional difference between NK cells, AS_NK cells and eNK-EXO treated NK cells. Triplicated samples for three groups were subjected to RNA-seq analysis. PCA showed that 3 NK cell samples, 3 AS_NK cell samples, and 3 eNK-EXO treated NK cell samples clustered together (Figure 6A), suggesting that most of the observed transcriptional changes are related to the conditions under which the NK cells were treated, with relatively low variability within groups. Then, we performed a pairwise comparison of each group to identify DEGs between groups (Figure 6B). A heatmap was generated for samples and genes derived *via* hierarchical clustering (Figure 6C). Hierarchical clustering across all samples showed that the samples can be classified into two main groups, NK cells vs the other two groups. By comparing AS_NK cells to NK cells, upregulation of 4933 genes and downregulation of 2742 genes were observed while by comparing eNK-EXO treated NK cells to NK cells, upregulation of 196 and downregulation of 32 genes were observed. An adjusted *p* value threshold of<0.05 was assigned by DESeq2 algorithm (Figure 6D). KEGG pathway enrichment analysis revealed that the most enriched DEGs sets in AS_NK vs NK were genes involved in natural killer cell mediated cytotoxicity, and the most enriched DEGs sets in eNK-EXO treated NK vs NK cells are genes involved in cytokine-cytokine interaction, IL-17 signaling pathway, chemokine signaling pathway and TNF signaling pathway (Figure 6E).

A detailed look at DEGs related to the natural killer cell-mediated cytotoxicity pathway in KEGG database revealed considerably lower expression of the following genes *GZMA, CD244 (2B4), CD48, NCR1 (NKP46), NCR2 (NKP44), NCR3 (NKP30), KLRC1 (NKG2A), KLRC2 (NKG2C), KLRC3 (NKG2E), KLRC (NKG2F), KIR2DL1, KIR3DL1* and *KIR3DL3* in AS_NK cells compared to the NK cells (Figure 7A). Among them, *NCR3, KLRC1* and *ITGAL* were most significantly down-regulated (Figure 7B), which may explain the altered cytotoxicity and functional defects of AS_NK cells.

For eNK-EXO treated NK cells, which exhibited increased cytotoxicity than control NK cells, RNA-seq analysis showed four most significantly up-regulated pathways, the upregulated genes from which were shown in the scatter plot (Figure 7C). In addition, heatmaps were generated based on each gene's expression (Figure 7D). Compared with NK cells, most significantly up-regulated genes in eNK-EXO treated NK cells include CXC motif chemokine ligand 10 (*CXCL10*), *CXCL11* and C-C Motif Chemokine Ligand 2 (*CCL2*) (Figures 7D, E). Remarkably, the data revealed a substantial increase in the transcription levels of chemokine ligands including *CXCL5*, *CXCL8*, *CXCL9* and *CX3CL1* (Figure 7E). Chemokine ligands play a major role in the four up-regulated pathways. For example, CXCR3 is expressed on activated NK cells

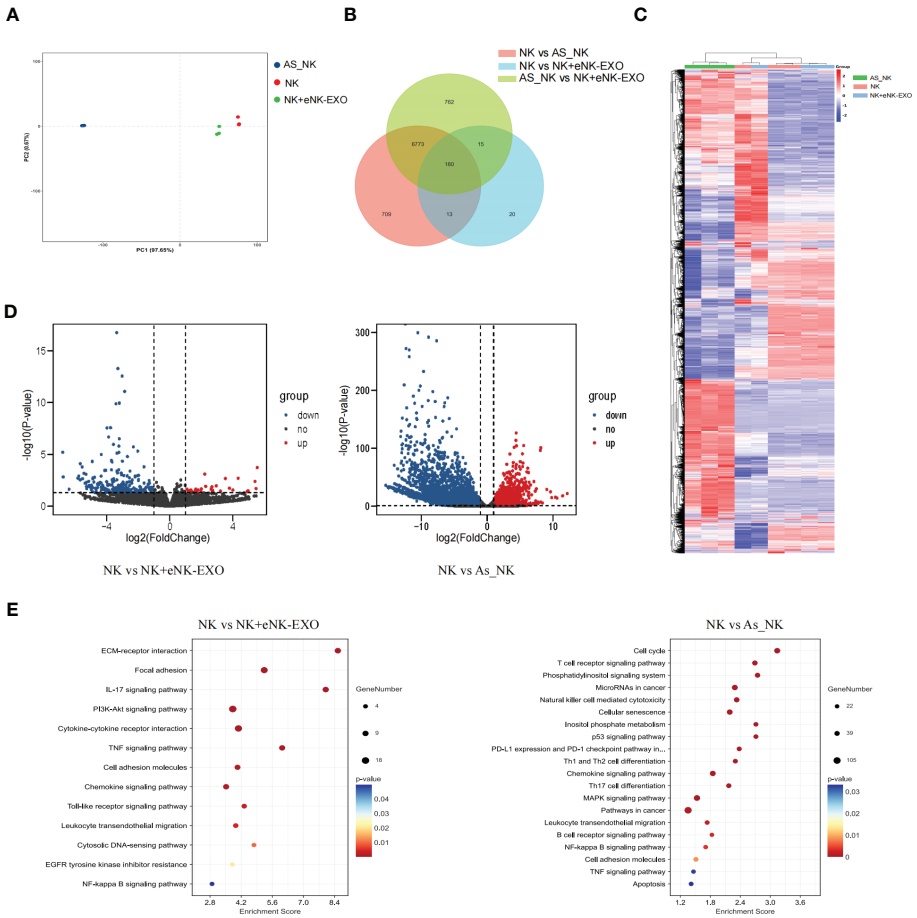

FIGURE 6
Global transcriptional analysis of AS_NK and eNK-EXO treated NK cells. **(A)** Principal component analysis of eNK, AS_NK, and eNK-EXO treated NK cells. **(B)** Pairwise comparison of all groups. **(C)** Clustering heatmap of all the DEGs in three groups (color scale represents relative gene transcription level). **(D)** Volcano plots showing the distribution of significance [-$\log_{10}$(*p*-value)] and the fold changes in the transcription levels of DEGs [log2(fold change)] within different groups. **(E)** Bubble chart showing the functional analysis of DEGs when different groups were compared.

and functions to enhance NK cell cytotoxicity and promote NK cell proliferation and homing to tumor sites, through binding to its ligands (22, 42–45). CXCR3 ligands, CXCL9, CXCL10 and CXCL11 were up-regulated in NK cells treated with eNK-EXO (Supplementary Figure S2). Taken together, eNK-EXO treatment resulted in altered expressions of multiple chemokine ligands on NK cells, which may be one of the mechanisms that lead to phenotypic changes and enhanced cytotoxicity.

Discussion

Immunotherapies have gained increasing attention in cancer research over the past few decades. NK cell transplantation in the treatment of a variety of solid and haematological tumors is being widely evaluated in clinical trials (reference). However, adoptive therapy with NK cells is often limited by storage conditions, cell transportation and TME inhibition from solid tumors (46). Compared with NK cells, NK-Exo are more convenient to store and transport. Exosomes are usually stored at 20°C for the short term and -80°C for long term storage. The freeze-drying method is also used to preserve exosomes, facilitate its development as a therapeutic drug. Besides, NK-Exo may retain anti-tumor activity because of their lacking the signalling and metabolic pathways that respond to inhibitory TME (21). However, special care should be taken to ensure the quality and quantity of anti-tumor contents in NK-Exo which are unstable and related to the source and state of original NK cells.

In this study, we demonstrate that eNK-EXO have typical NK cell markers and contents, and can kill SKOV3, OV90 and COC1/DDP ovarian cancer cells in a dose-dependent manner. Interestingly, eNK-EXO at the same concentration have no cytotoxic effect on normal cells, indicating its safety profile as cancer therapy. A recent study showed that an antibody blocking the exosome surface marker CD63 could suppress the cytotoxicity of EXO301 (exosomes isolated from oncolytic adenovirus infected HCT116 cells) (47). Therefore, we used anti-CD63 and anti-CD81 antibodies in the cellular uptake experiment, and found that either blocking CD63 or CD81 could significantly decrease the uptake of eNK-EXO by SKOV3 cells. This suggests that the death of ovarian cancer cells is initiated by the internalization of eNK-EXO. Moreover, since eNK-EXO killed OC cells but not normal cells in this study, we hypothesized that OC cells

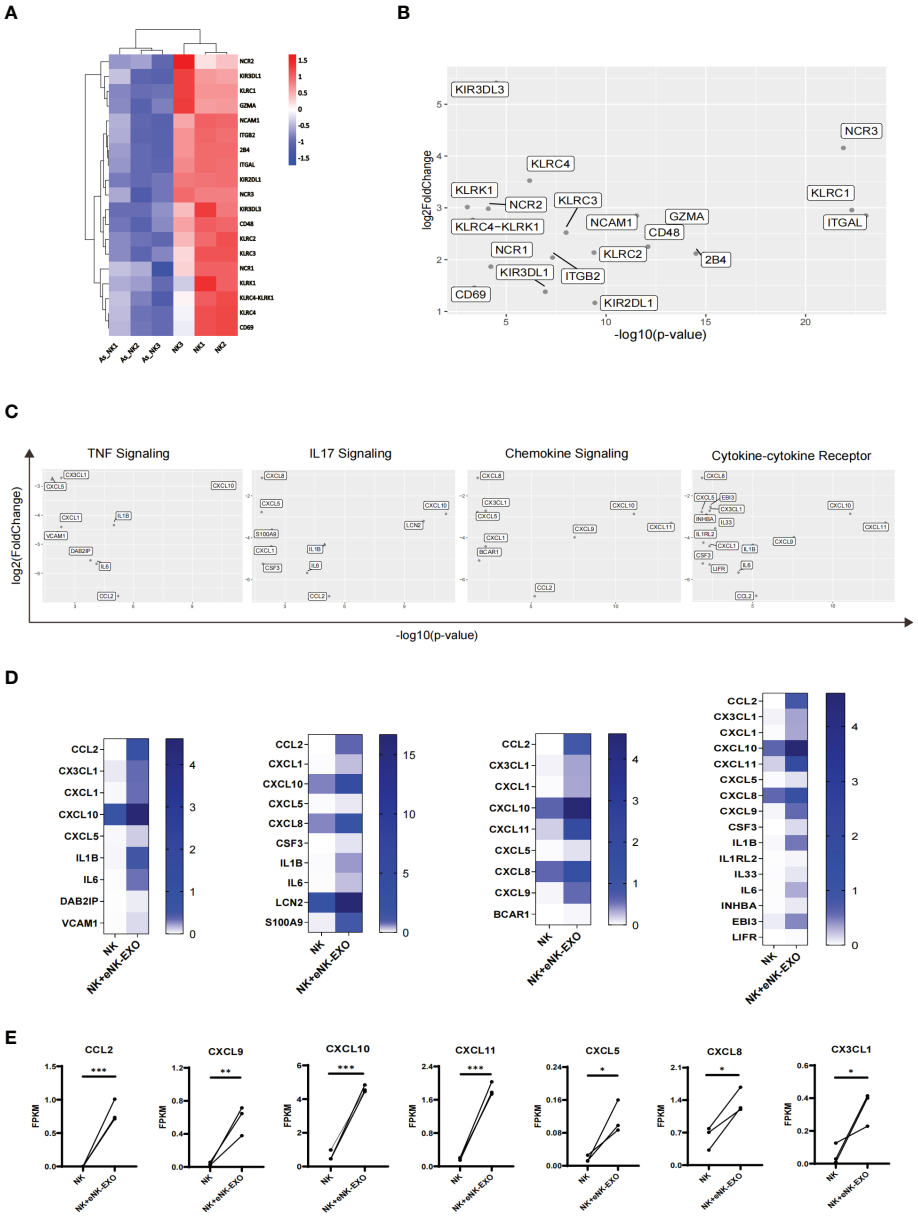

FIGURE 7
Transcriptional alterations in genes related to pathways that control NK cell function. **(A)** Heatmap of transcription levels of identified DEGs associated with NK cell cytotoxicity. Each column represents an individual in the indicated group. **(B)** Scatter plot displaying genes related to NK cell cytotoxicity. Axis values represent the distribution of significance and changes of expression in AS_NK cells compared to NK cells. **(C)** Scatter plot displaying genes related to signaling pathways. Axis values represent the distribution of significance and changes of expression in eNK-EXO treated NK cells compared to NK cells. **(D)** Heatmaps displaying FKPM values of genes graphed in **(C)**. **(E)** Line graphs showing the transcriptional changes in genes related to activation, migration or tumor killing activity of NK cells. The paired t-test was used to determine significance (* $p< 0.05$, ** $p< 0.01$, *** $p< 0.001$).

and normal cells have different uptake efficiency of eNK-EXO and confirmed it by the co-culture experiment. However, the detailed mechanism of the differential uptake of eNK-EXO is still unclear. Exosomes could be internalized by directly interacting with extracellular receptors or by direct fusion with the plasma membrane, or a combination of both (36, 48, 49). Further studies are required to understand which mode of internalization contributes to the observed difference in eNK-EXO uptake between SKOV3 and IOSE80 cells.

Exosomes have natural stability, low immunogenicity and excellent tissue/cell penetration (22), and is expected to become an advanced platform for drug/gene delivery (12). Owing to their unique properties, exosomes are currently under evaluation as vehicles for the transport of tumor therapeutic drugs (12). For example, intravenous administration of MSCs is often retained in the lung or liver, while MSC-exo can avoid this problem while still maintaining the therapeutic function of their originating cells (35, 36). 16 clinical studies are investigating the application of MSC-exo in various diseases (50). For tumor treatment, MSC-exo containing siRNA targeting oncogenic *Kras*^{G12D} mutations are being investigated in clinical trials to treat pancreatic cancer (NCT03608631) (51, 52). Based on the advantages of using exosomes as drug delivery carrier

and the anti-tumor activity of eNK-EXO (53, 54), we evaluated eNK-EXO as a carrier for tumor therapeutic drugs. eNK-EXO-DDP effectively killed OC cells including DDP-insensitive OV90 cells and DDP-resistant COC1/DDP cells, suggesting that eNK-EXO could sensitize tumor cells to DDP. The resistance of tumor cells to DDP may be related to reduced drug uptake, reduced drug inflow or increased drug efflux, drug target changes, and DNA repair (41, 55). In our study, we demonstrated that eNK-EXO-DDP induced cell cycle arrest and apoptosis in OC cells. Therefore, we speculate that the enhanced killing effect of eNK-EXO-DDP on OC cells, especially on COCI/DDP cells is caused by increased uptake of eNK-EXO-DDP by OC cells.

Exosomes have emerged as mediators to reshape the cellular programs of recipient cells. Previous studies have shown exosomes derived from dendritic cells (DCs) and NK cells can exert immunomodulatory effect on target cells. DCs-derived exosomes not only express functional transmembrane MHC and co-stimulatory molecules that enable them to indirectly stimulate adaptive T-cell response, but also express functional transmembrane TNF superfamily ligands (TNFSFLs), including TNF, FasL, and TRAIL, enabling them to directly activate NK cells (56). NK-Exo also contain a variety of proteins involved in immune regulation and can induce the expression of HLA-DR and costimulatory molecules on the surface of monocytes, up-regulate the expression of CD25 and down-regulate the expression of PD-1 on T cells (10), and increase the percentage of $CD56^{+}$ total NK cells and $CD56^{bright}$ and $CD56^{dim}$ subpopulations of NK cells (10, 57). In addition, the above immunomodulatory effects were not affected in the mimicked immunosuppressive environment (10). In our study, eNK-EXO treated eNK cells and NK92 cells showed enhanced tumor-killing activity and increased release of TNF-α and perforin. Interestingly, eNK-EXO treated AS-t-NK cells regained their cytotoxicity against OC cells, which was even stronger than that of NK cells without ascites treatment. The possible molecular mechanism underlying this phenomenon might be explained by the DEGs in the natural killer cell mediated cytotoxicity pathway revealed by RNA-seq analysis, however, how the differential expression of these genes contributes to their varied cytotoxicity required further investigations.

In addition, RNA-seq analysis of eNK-EXO treated NK cells shows significant upregulation of many genes for cytokines that are involved in the regulation of NK cells proliferation, cytotoxicity, and migration, including *CXCL8, CXCL9, CXCL10, CXCL11, CCL2, CXCL5*, and *CX3CL1*. Among them, *CXCL9, CXCL10*, and *CXCL11*, which are significantly up-regulated, are ligands of chemokine receptor CXCR3 (42, 58). CXCR3 is expressed on activated NK cells and participates in physiological processes, such as enhancing NK cells cytotoxicity, promoting NK cell proliferation, and homing to tumor sites, through binding with its corresponding ligands (44, 45). Therefore, we hypothesized that eNK-EXO treated NK cells could enhance their anti-tumor activity and increase the infiltration of NK cells in TME, and change "cold tumor" to "hot tumor" to enhance the effect of tumor immunotherapy. Although the eNK-EXO treated AS_NK cells were not included in the RNA-seq analysis, from the data we have, eNK-EXO can enhance the anti-tumor activity of AS-t-NK cells *in vitro* and up-regulate the expression of genes related to the function of NK cells. Therefore, eNK-EXO could potentially contribute to reversing immune suppression by rescuing defective NK cells in TME.

Taken together, we demonstrated the potential application of eNK-EXO in anti-tumor therapy of OC *in vitro* (Figure 8). Being cytotoxic by itself, eNK-EXO can not only deliver and enhance the killing effect of DDP on OC cells but also reverse the immunosuppression of NK cells, thus enhancing its anti-tumor activity. To our knowledge, this is the first report on eNK-EXO-based drug delivery and improvement of immunosuppressive capacity for OC therapy. However, our study were limited to the cellular level due to large amount of exosomes required for *in vivo* study. Much work is still required to clarify the detailed mechanisms by which eNK-EXO enhances the cytotoxicity of NK cells. Nonetheless, our work may represent a starting point for further evaluation of eNK-EXO in solid tumor therapies.

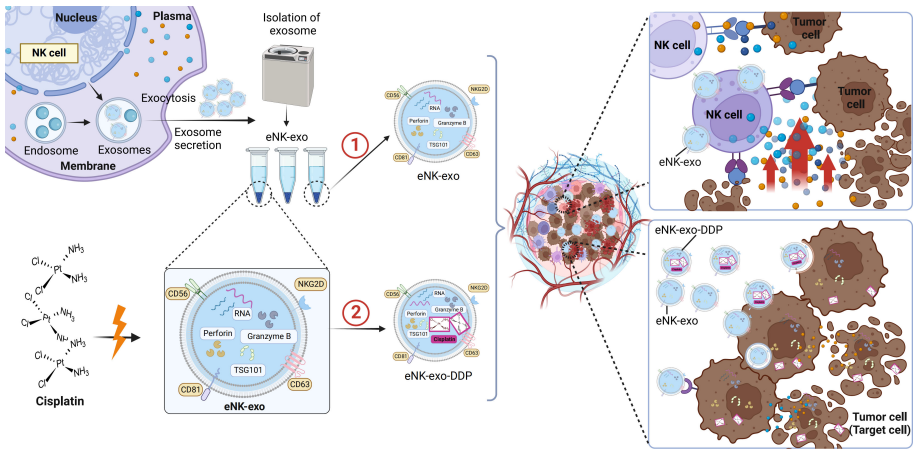

FIGURE 8
Schematic illustration of eNK-EXO enhance the anti-tumor effects against ovarian cancer by delivering chemotherapeutic drugs and reactivating NK cell functions. ① eNK-EXO containing typical NK cell markers and cytotoxic substances derived from eNK cells show cytotoxicity against OC cells *in vitro*. ② eNK-EXO loaded with cisplatin could sensitize OC cells to the effect of cisplatin and enhance NK cell's anti-tumor activity.

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 human participants were reviewed and approved by the Ethical Committee of GuiZhou Medical University (NO.2022-82). The patients/participants provided their written informed consent to participate in this study.

Author contributions

XZ and ZZ designed the study,wrote and critically reviewed the manuscript. HL and JD performed the experiments and wrote the manuscript. AY, YHZ and SL was responsible for the data collection and statistical analysis. XL expanded and characterized NK cells. ZY and QC isolated AS_NK cells from ascites. NY and YY prepared the figures. TG, DZ, YCZ, JZ, WO and WY provided the CB of donors, ascites of patients and patient informed consent. All authors contributed to the article and approved the submitted version.

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Conflict of interest

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2022.1087689/full#supplementary-material

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