EMERGING ENGINEERING APPROACHES IN CANCER IMMUNOTHERAPY

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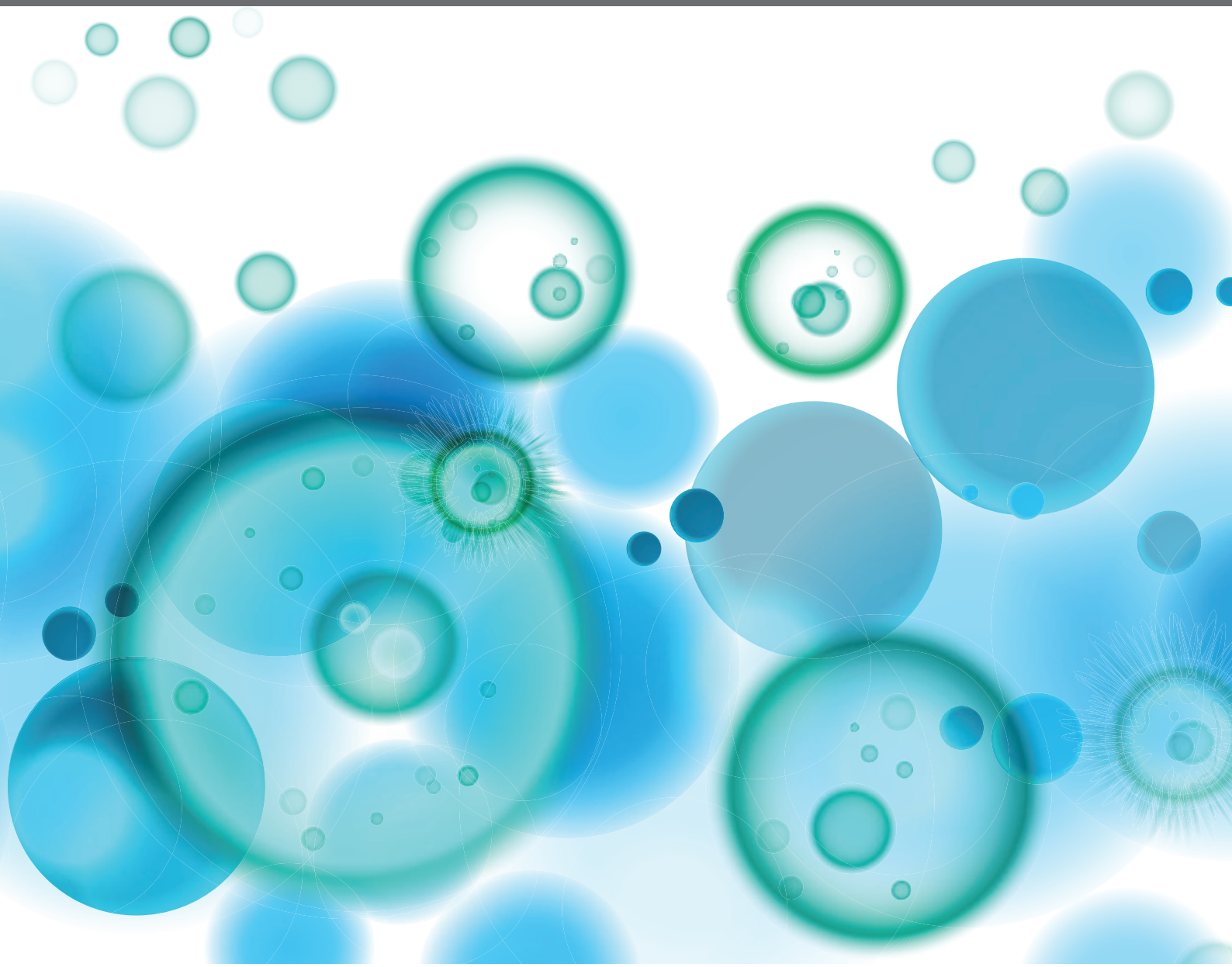

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EMERGING ENGINEERING APPROACHES IN CANCER IMMUNOTHERAPY

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Editorial: Emerging engineering approaches in cancer immunotherapy

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Editorial on the Research Topic
Emerging engineering approaches in cancer immunotherapy

As an unprecedented approach, cancer immunotherapy has transformed cancer treatment. However, only a minority of patients benefits from cancer immunotherapy. In order to improve the efficacy of cancer immunotherapy and reduce the occurrence of immune-related adverse reactions, emerging engineering approaches have been explored for cancer immunotherapy. This invited Research Topic is composed of 16 articles, including 4 original research papers, 8 review articles, 1 minireview article, 1 opinion article, 1 perspective article and 1 case report, contributed by a total of 109 researchers from all over the world (Total views: 53,987; as of July 26, 2022). This Research Topic covers a range of novel engineering approaches for cancer immunotherapy, including engineered T cells therapy (Xu et al.), bacteria-based synergistic therapy (Bao et al.), bioinspired membrane-coated nanoplatform (Mu et al.), and injectable hydrogel delivery system (Liu et al.), and so on.

Cancer has been threatening human beings with incurable, high mortality and high recurrence rate. Compared with non-tumor patients, tumor patients are more susceptible to SARA-Cov-2 and have poor prognosis (Huang et al.). Traditional therapeutic includes surgery, chemotherapy, radiotherapy, etc. To seek better treatment strategies, it is crucial to understand the mechanism of tumor occurrence and development. Overexpressed NPM1 promotes tumor growth. Liu et al. analyzed TCGA and GEO data and found that NPM1 is a prognostic biomarker related to immune infiltration in lung adenocarcinoma (LUAD), and is related to m6A modification and glycolysis. As an effective target for the diagnosis and treatment of LUAD, this provides a new strategy for the therapy of LUAD. Histone acetylation plays a role in regulating tumorigenicity, tumor progression, and tumor microenvironment. Xu et al. comprehensively analyzed 36 histone acetylation regulators in hepatocellular carcinoma

(HCC) for the first time, and found a close correlation between histone acetylation patterns and tumor malignant pathways and tumor microenvironment, which is an important indicator for hepatocytes and provides new strategies for personalized and precise immunotherapy and prognosis of cancer.

So far, immunotherapy has a place in cancer treatment, such as the application of immune checkpoint inhibitors for HCC (Liu et al.). Combining traditional therapies with immunotherapy plays an important role in breast cancer (Zhang et al.). A case report has confirmed that combination of penpulimab and anlotinib can successfully treat extensive-stage small-cell lung cancer (ES-SCLC) (Zhang et al.). Engineered T-cell therapy includes adoptive T-cell therapy (ACT) (Xu et al.), among which chimeric antigen receptor T Cells (CAR-T) therapy has received extensive attention, especially in hematological tumors. Nonetheless, engineered T-cell therapy faces many challenges that hinder its clinical application. To accelerate the development of ACT, suitable experimental models and test platforms can be selected. Xiao et al. demonstrated that immunocompetent microphysiological system (iMPS) could triple-culture three-dimensional (3D) colorectal tumor microtissues, 3D cardiac microtissues, and human-derived natural killer cells in the same microfluidic network, and was able to simulate the *in vivo* state for corresponding tests. This provides new approaches for efficacy and early safety testing of new candidate for ACTs. For a more economically desirable effect, regenerable human induced pluripotent stem cells (iPSCs) were genetically engineered to differentiate into immune cells with enhanced antitumor cytotoxicity, increased persistence and decreased immunogenicity. CAR-T cells derived from iPSCs can be pre-prepared as off-the-shelf products and applied in a large number of patients, offering great promise for the next generation of ACT (Netsrithong et al.). CAR-T therapy can create new complications such as cytokine release syndrome, neurotoxicity, and even fatal cerebral edema. CD28-CAR heterodimerization may be an important cause of severe neurotoxicity (Ferreira et al.). To reduce its systemic toxicity, *in vivo* CAR-T cell therapy induced by gene editing tools can serve as a new generation of CAR-T cell therapy (Xin et al.). The development of CAR-T therapy in solid tumors is still in its infancy. By adopting some nanotechnology, such as nanozymes, RNA vaccines, etc., to help CAR-T cells target and accumulate in solid tumors, or to stimulate CAR-T cells by remodeling the tumor microenvironment, improve the survival rate and proliferation rate of CAR-T cells, and provide new ideas for the application of CAR-T cells in solid tumors (Mi et al.). Tissue resident memory $CD8^+$ T (Trm) lymphocytes exist in various digestive tract cancers. $CD8^+$ Trm cells own strong cytotoxicity, have ability to directly kill epithelial-derived tumor cells, and are important for maintaining the homeostasis of digestive tract mucosa and anti-tumor. But the application of $CD8^+$ Trm cells in gastrointestinal cancers is still in its early stages. Specific drug therapy and cancer vaccine therapy targeting tumor-associated $CD8^+$ Trm cells may become an important direction for precision cancer therapy (Mei et al.).

In addition to engineered T-cell therapy, other approaches have also been used to combat the challenges of cancer immunotherapy. Injectable hydrogel as a unique platform that can target the immunosuppressive tumor microenvironment have the advantages of good biocompatibility, good biodegradability and low toxicity (Liu et al.). Bioinspired membrane-coated nanoplatform have opened up novel research directions for cancer immunotherapy due to superior immune regulation and excellent tumor targeting (Mu et al.). The advantage of bacteria targeting tumor makes them an excellent platform for combination with immunotherapy. Optimizing bacteria-based therapy through strategies such as bioengineering or chemical modification can avoid the safety issues posed by this therapy (Bao et al.).

In general, this Research Topic reports the application of novel engineering approaches in cancer immunotherapy, which provides new ideas and strategies for cancer immunotherapy. Solving the challenges faced in cancer immunotherapy by various means has made an essential contribution to clinical translation and provides new hope for cancer patients.

Author contributions

All authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication.

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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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NPM1 Is a Prognostic Biomarker Involved in Immune Infiltration of Lung Adenocarcinoma and Associated With m6A Modification and Glycolysis

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Background: Overexpression of NPM1 can promote the growth and proliferation of various tumor cells. However, there are few studies on the comprehensive analysis of NPM1 in lung adenocarcinoma (LUAD).

Methods: TCGA and GEO data sets were used to analyze the expression of NPM1 in LUAD and clinicopathological analysis. The GO/KEGG enrichment analysis of NPM1 co-expression and gene set enrichment analysis (GSEA) were performed using R software package. The relationship between NPM1 expression and LUAD immune infiltration was analyzed using TIMER, GEPIA database and TCGA data sets, and the relationship between NPM1 expression level and LUAD m6A modification and glycolysis was analyzed using TCGA and GEO data sets.

Results: NPM1 was overexpressed in a variety of tumors including LUAD, and the ROC curve showed that NPM1 had a certain accuracy in predicting the outcome of tumors and normal samples. The expression level of NPM1 in LUAD is significantly related to tumor stage and prognosis. The GO/KEGG enrichment analysis indicated that NPM1 was closely related to translational initiation, ribosome, structural constituent of ribosome, ribosome, Parkinson disease, and RNA transport. GSEA showed that the main enrichment pathway of NPM1-related differential genes was mainly related to mTORC1 mediated signaling, p53 hypoxia pathway, signaling by EGFR in cancer, antigen activates B cell receptor BCR leading to generation of second messengers, aerobic glycolysis and methylation pathways. The analysis of TIMER, GEPIA database and TCGA data sets showed that the expression level of NPM1 was negatively correlated with B cells and NK cells. The TCGA and GEO data sets analysis indicated that the NPM1 expression was significantly correlated with one m6A modifier related gene (HNRNPC) and five glycolysis related genes (ENO1, HK2, LDHA, LDHB and SLC2A1).

Conclusion: NPM1 is a prognostic biomarker involved in immune infiltration of LUAD and associated with m6A modification and glycolysis. NPM1 can be used as an effective target for diagnosis and treatment of LUAD.

Keywords: NPM1, lung adenocarcinoma, immune infiltration, m6A modification, glycolysis

INTRODUCTION

Recent studies show that lung adenocarcinoma (LUAD) is the second most diagnosed cancer and the leading cause of cancer death worldwide (1). Despite improved diagnosis and treatment strategies for lung disease, LUAD patients still have a high mortality rate and poor prognosis (2). The development of LUAD is a complex multi-step process, which may be closely related to the abnormal expression of some genes. Therefore, a better understanding of the molecular mechanisms of LUAD could provide more accurate biomarkers for tumor diagnosis and treatment.

Nucleophosmin 1 (NPM1) is a multifunctional protein that is mainly localized in nucleoli and shuttles between the nucleus and cytoplasm (3). In recent years, the focus of NPM1 research has gradually shifted from hematological diseases to solid tumors (4, 5). Previous studies have demonstrated that NPM1 is overexpressed in several types of tumors and promotes the occurrence and progression of tumors (6–8). Our previous studies found high expression of NPM1 in LUAD, but failed to investigate the biological function of NPM1 more broadly (9).

Tumor immunotherapy, N6-methyladenosine (m6A) modification and targeted glycolytic pathway are hot spots in cancer therapy, which have been used for a wide variety of applications in the research and treatment of LUAD. However, there have been few studies on the multifaceted analysis of NPM1 in LUAD, especially the relationship between NPM1 with LUAD immunotherapy, glycolysis and m6A modification.

In this study, we downloaded The Cancer Genome Atlas (TCGA) LUAD data sets and Gene Expression Omnibus (GEO) data sets. Bioinformatics analysis was performed using R software package and other online databases to investigate differences in NPM1 expression in different cancers, and cell assay and immunohistochemistry (IHC) were used to verify differences in NPM1 expression between LUAD samples and normal samples. The NPM1 co-expression gene network in LUAD was analyzed from multiple aspects, and the biological functions and signal transduction pathways of these genes were analyzed. Finally, the relationship between NPM1 and tumor immune cell infiltration, m6A and glycolysis related genes was discussed, which is helpful to understand the possible mechanism of LUAD.

MATERIALS AND METHODS

Ethics Statement

The protocol of this study had been approved by the Ethics Committee of Taihe Hospital Affiliated of Hubei University of Medicine (Shiyan, China) and conducted according to the principles stated in the Declaration of Helsinki.

Expression of NPM1 in LUAD

We used Oncomine (www.oncomine.org) (10, 11) online database and TCGA data sets (www.tcga-data.nci.nih.gov/tcga) (12) to analyze the difference of NPM1 expression in different tumors. Oncomine database used Student's t test to compare the expression level of NPM1 in cancer samples and control group, and selected data with fold change > 2 and P value < 0.000001. We also analyzed the LUAD data sets in TCGA (n = 594) and GEO (www.ncbi.nlm.nih.gov/geo; GSE31210, n = 246) (13) data sets to study the difference of NPM1 expression between tumor tissues and normal tissues. The relationship between NPM1 expression level and clinicopathological characteristics of LUAD patients was studied by analyzing the clinical data of LUAD data sets in TCGA database, and the prognostic and diagnostic value of NPM1 in LUAD was evaluated by Cox model and ROC curve. Finally, we verified the differential expression of NPM1 in LUAD and normal samples by qRT-PCR and IHC staining. The specific procedures refer to previous studies (14), and see the **Supplementary Materials** for details.

Enrichment Analysis of NPM1 Gene Co-Expression Network In LUAD

The TCGA LUAD data sets was analyzed using the stat packet of R software to study the co-expression genes related to NPM1 expression. Pearson's correlation coefficient was calculated to test the statistical correlation, and ggplot2 package of R software was used to draw volcano map and heat map for display. Gene ontology (GO) function and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis of co-expressed genes were performed by clusterProfiler package (version: 3.18.0) (15) of R software, and visual analysis of data was performed by ggplot2 software package.

Gene Set Enrichment Analysis

To further understand the underlying mechanism of NPM1, we divided samples from the TCGA LUAD data sets into two groups based on the median expression level of NPM1 and performed GSEA (www.gsea-msigdb.org/gsea/index.jsp) (16) to investigate whether genes in the two groups were rich in meaningful biological processes. The annotated gene set c2.cp.v7.2.symbols.gmt [Curated] was selected as the reference gene set. FDR (qvalue) < 0.25 and P < 0.05 were considered statistically significant.

Correlation Between NPM1 and Tumor Immune Infiltrating Cells

To further explore the potential immunomodulatory mechanism of NPM1 in the regulation of tumor-infiltrating immune cells, we used the TIMER database (www.cistrome.shinyapps.io/timer) (17, 18) to evaluate the correlation between NPM1 expression in TCGA LUAD samples and immune infiltrating cells. Immune infiltrating cells include B cells, neutrophils, CD4+ T cells, macrophages, CD8+ T cells and dendritic cells. We analyzed the relationship between NPM1 copy number variation (CNV) and immune cell infiltration using the somatic copy number alteration (SCNA) module in the TIMER database. R's CIBERSORT (19) software package was used to detect the proportion of 22 immune cells in LUAD samples with high and low NPM1 expression. We further performed Kaplan-Meier curve analysis to investigate the differences in survival between high and low expression levels of NPM1 and immune cell. In addition, we analyzed the association between NPM1 and immune cell marker genes in LUAD samples using TIMER, GEPIA, and TCGA databases. Immune cell markers are selected from the website of R&D Systems (www.rndsystems.com/cn/resources/cell-markers/immune-cells).

Correlations of NPM1 Expression With m6A Modification in LUAD

The R software package was used to analyze the correlation between the NPM1 expression and the m6A related genes expression in the GSE31210 and TCGA LUAD data sets, including ZC3H13, YTHDF3, HNRNPA2B1, IGF2BP1, IGF2BP3, YTHDC2, YTHDF1, FTO, HNRNPC, METTL14, METTL3, WTAP, RBM15, ALKBH5, IGF2BP2, RBMX, RBM15B, YTHDC1, VIRMA and YTHDF2 (20). R software package was used to analyze the proportion of m6A related genes in LUAD samples with high and low NPM1 expression. The Kaplan-Meier curve showed the relationship between he expression of related genes and the prognosis of LUAD. The data were analyzed visually by ggplot2 software package.

Correlations of NPM1 Expression With Glycolysis in LUAD

To further analyze the correlation between NPM1 expression and LUAD glycolysis, R software package was used to analyze the correlation between expression of NPM1 and glycolysis related genes in GSE31210 and TCGA LUAD data sets, including ENO1, G6PD, HK1, HK2, LDHA, LDHB, PDHB, PDK3, PDK4, PGK1, PKM, SLC2A1, SLC2A2 and SLC2A3. The proportion of glycolysis related genes in LUAD samples with high and low NPM1 expression was analyzed by R software package. Kaplan-Meier curves showed the relationship between the expression of related genes and the prognosis of LUAD. The software package ggplot2 was used for visual analysis of the data. To further confirm the idea that NPM1 overexpression affects the glycolysis of LUAD, we retrospectively analyzed images of 40 LUAD patients who underwent ^{18}F-FDG PET/CT scans and analyzed them with IHC scores of the corresponding surgically resected tissues to explore the possibility that NPM1 may influence the glycolysis process of LUAD.

RESULTS

Pan-Cancer Analysis of NPM1 mRNA Expression in Different Databases

We used Oncomine online database and TCGA data sets to analyze the difference of NPM1 mRNA expression between LUAD group and control group. Oncomine database analysis showed that the expression of NPM1 in colorectal cancer (21–24), head-neck cancer (25), kidney cancer (26–28), leukemia (29), liver cancer (30), lung cancer (31, 32), lymphoma (33) and sarcoma (34) was higher than that in normal tissues. The expression of NPM1 in breast cancer (35) was lower than that in normal tissues (**Figure 1A**). **Table 1** summarizes the details of NPM1 expression in various cancers.

We further analyzed the expression of NPM1 mRNA in human tumors using TCGA data sets. **Figure 1B** shows the difference of NPM1 in different tumor tissues and normal tissues. Compared with normal tissues, the expression level of NPM1 was significantly increased in BRCA (breast invasive carcinoma), CHOL (cholangiocarcinoma), COAD (colon adenocarcinoma), ESCA (esophageal carcinoma), GBM (glioblastoma multiforme), HNSC (head and neck squamous cell carcinoma), KIRC (kidney renal clear cell carcinoma), LIHC (liver hepatocellular carcinoma), LUAD (lung adenocarcinoma), LUSC (lung squamous cell carcinoma), PRAD (prostate adenocarcinoma), READ (rectum adenocarcinoma) and STAD (stomach adenocarcinoma), while it was significantly decreased in KICH (kidney chromophobe) and UCEC (uterine corpus endometrial carcinoma).

Expression Levels of NPM1 in LUAD Patients

We analyzed LUAD data sets from TCGA and GEO to investigate the differential expression of NPM1 in LUAD samples and normal samples. Analysis of both TCGA and GEO data showed that the expression level of NPM1 was significantly increased in LUAD samples compared to the control group (**Figures 1C, D**). To further prove the accuracy of the predicted results, qRT-PCR and IHC staining experiments were used to further verify the results. qRT-PCR results showed that the expression level of NPM1 mRNA was significantly increased in human lung adenocarcinoma cell lines compared with normal human lung epithelial cells (**Figure 1G**). IHC staining showed that NPM1 was mainly expressed in the nucleus of LUAD cells. The NPM1 IHC score in tumor sample tissue was significantly higher than that in paracancerous tissue (**Figures 1H, I**). These results suggest that NPM1 overexpression may contribute to the progression of LUAD. To further evaluate the prognostic and diagnostic potential of NPM1 in LUAD, we performed Cox regression model and ROC curve analysis. The results of Cox regression model analysis showed that high expression of NPM1 in LUAD predicted worse survival (HR = 1.51(1.13-2.02), P = 0.006) (**Figure 1E**). The results of ROC analysis showed that NPM1 had a good prediction accuracy for

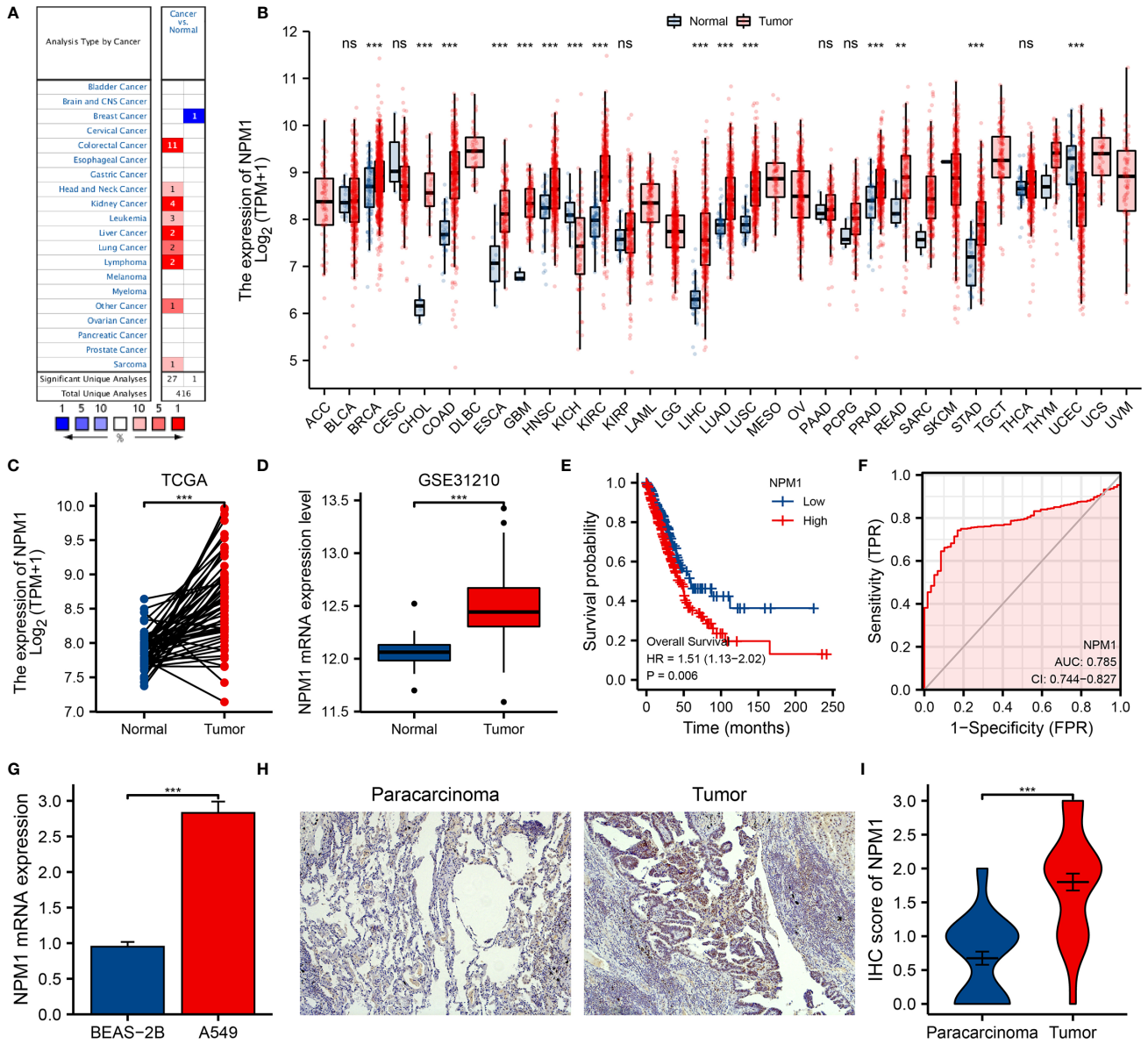

FIGURE 1 | The expression of NPM1 in lung adenocarcinoma (LUAD) and pan-carcinoma. **(A)** NPM1 mRNA expression levels in pan-cancer were measured using Oncomine. **(B)** Pan-cancer data downloaded from the TCGA data sets were used to assess NPM1 mRNA expression levels. **(C)** Difference in expression of NPM1 between LUAD and matched normal tissues in TCGA data sets. **(D)** Difference in expression of NPM1 between LUAD and normal tissues in GSE31210 data sets. **(E)** The survival curve of NPM1. **(F)** ROC curve analysis of NPM1 diagnosis. **(G)** Difference of expression of NPM1 in LUAD cell lines and human normal lung epithelial cell lines. **(H)** Immunohistochemistry assay was used to analyze the expression of NPM1 in LUAD tissues and paracarcinoma tissues. **(I)** The mean NPM1 IHC score in LUAD tissue was significantly higher than that of matched paracarcinoma tissue. *P < 0.05; **P < 0.01; ***P < 0.001; ****P < 0.0001. ns, not significant.

LUAD, and the area under the ROC curve was 0.785 (95%CI: 0.744-0.827) (**Figure 1F**).

To further determine the potential importance of NPM1 in clinical Settings, we analyzed clinical outcomes from TCGA LUAD samples. The results showed (**Figure 2**) that the expression of NPM1 in Stage II group was significantly higher than that in Stage I group. The expression of NPM1 in T4 group was higher than that in T1, T2 and T3 groups. The expression of NPM1 in N0 group was lower than that in N1 and N2 groups. During OS events, NPM1 expression was significantly higher in patients who died than in the surviving group. Similarly, NPM1 expression was significantly higher in patients who died than in the survival group during DSS events.

Enrichment Analysis of NPM1 Gene Co-Expression Network in LUAD

We used the stat package of R software to analyze the co-expressed genes associated with NPM1 expression in the

TABLE 1 | NPM1 expression in cancerous versus normal tissue in ONCOMINE.

Cancer Site	Cancer Type	P Value	t–Test	Fold Change	Reference (PMID)
Breast	Invasive Breast Carcinoma	1.51E-31	-24.245	-34.469	18438415
Colorectal	Colon Adenocarcinoma	6.36E-9	7.443	2.299	11306497
	Colon Adenoma	2.66E-18	13.932	2.737	18171984
	Cecum Adenocarcinoma	6.08E-15	11.581	3.115	TCGA Colorectal
	Colon Mucinous Adenocarcinoma	6.11E-11	9.054	3.475	TCGA Colorectal
	Rectal Adenocarcinoma	3.11E-17	11.654	2.592	TCGA Colorectal
	Colon Adenocarcinoma	1.10E-17	13.451	2.657	TCGA Colorectal
	Colorectal Carcinoma	5.56E-12	8.599	2.158	20957034
	Colon Adenocarcinoma	5.09E-14	8.859	2.209	17640062
	Colon Adenoma	4.12E-8	10.752	2.714	20957034
	Colon Carcinoma	8.28E-7	9.333	2.185	20957034
	Colorectal Carcinoma	8.03E-14	14.326	3.487	20957034
Head-Neck	Oral Cavity Squamous Cell Carcinoma	4.20E-8	6.277	2.232	21853135
Kidney	Hereditary Clear Cell Renal Cell Carcinoma	1.93E-13	11.212	2.078	19470766
	Non-Hereditary Clear Cell Renal Cell Carcinoma	1.27E-9	7.895	2.034	19470766
	Clear Cell Renal Cell Carcinoma	3.22E-7	8.348	2.604	17699851
	Clear Cell Renal Cell Carcinoma	1.38E-11	9.277	2.245	16115910
Leukemia	Pro-B Acute Lymphoblastic Leukemia	1.65E-11	9.502	2.498	20406941
	T-Cell Acute Lymphoblastic Leukemia	1.24E-25	12.948	2.316	20406941
	Acute Myeloid Leukemia	6.46E-25	14.102	2.043	20406941
Liver	Hepatocellular Carcinoma	2.84E-71	23.638	2.632	21159642
	Hepatocellular Carcinoma	2.28E-8	7.333	2.421	21159642
Lung	Lung Adenocarcinoma	1.28E-7	6.128	2.025	17540040
	Squamous Cell Lung Carcinoma	2.29E-11	9.623	2.262	20421987
Lymphoma	Burkitt's Lymphoma	8.78E-8	8.028	3.979	18794340
	Diffuse Large B-Cell Lymphoma	2.77E-7	6.644	3.860	18794340
Sarcoma	Myxoid/Round Cell Liposarcoma	6.64E-7	9.520	2.786	20601955

LUAD data sets of TCGA. Only the data of protein-coding genes were retained. As shown in **Figure 3A**, 5845 genes were positively correlated with the expression of NPM1, and 4625 genes were significantly negatively correlated with the expression of NPM1 ($P < 0.05$). When the threshold selection was cor > 0.7 and $P < 0.05$, four genes showed the strongest correlation, namely RACK1 (cor = 0.747, P = 1.196E-96), BTF3 (cor = 0.734, P = 1.867E-91), RPL26L1 (cor = 0.714, P = 1.273E-84) and NHP2 (cor = 0.704, P = 2.323E-81). The heat map showed the top 50 important genes positively and negatively correlated with NPM1 expression, respectively (**Figures 3B, C**). The detailed description of co-expressed genes is shown in **Supplementary Table 1**.

The GO function and KEGG pathway enrichment analysis of the top 200 co-expressed genes positively correlated with NPM1 expression were performed by R software package. Under the condition of p.adj < 0.05 and qvalue < 0.2, NPM1 co-expressed genes were involved in 156 biological process (GO-BP), 60 cell component (GO-CC), 16 molecular function (GO-MF) and 5 KEGG. The bubble graph demonstrates the top 5 messages for GO-BP, GO-CC, GO-MF and KEGG, respectively. GO functional annotations showed that NPM1 co-expressed genes were mainly involved in the translational initiation, ribosome, and structural constituent of ribosome (**Figures 3D–F**). KEGG pathway analysis demonstrated that the co-expression of NPM1 was primarily associated to the ribosome, Parkinson disease, and RNA transport (**Figure 3G**). **Supplementary Table 2** summarized the details of the GO function and KEGG pathway of NPM1 co-expression enrichment analysis.

Gene Set Enrichment Analysis

To characterize the potential function of NPM1 gene, GSEA was performed on the differential genes. A total of 419 gene sets were found, including mTORC1 mediated signaling (FDR = 0.205, P = 0.036), p53 hypoxia pathway (FDR = 0.205, P = 0.045), signaling by EGFR in cancer (FDR = 0.205, P = 0.039), antigen activates B cell receptor BCR leading to generation of second messengers (FDR = 0.159, P = 0.006), aerobic glycolysis (FDR = 0.163, P = 0.007), methylation (FDR = 0.205, P = 0.035) (**Figure 4**). Detailed enrichment analysis information is shown in **Supplementary Table 3**.

Correlation Between NPM1 and Tumor Immune Infiltrating Cells

We used the TIMER database to analyze the correlation between NPM1 expression and immune infiltrating cells in LUAD. The results showed that the expression of NPM1 was negatively correlated with the expression levels of B cells (r = -0.149, P = 1.03E-3), CD4+ T cell (r = -0.221, P = 8.89E-7) and macrophages (r = -0.117, P = 1.00E-2), while positively correlated with the expression levels of CD8+ T cells (r = 0.104, P = 2.23E-2) (**Figure 5A**). At the same time, we found that NPM1 CNV has a closely association with the degree of infiltration of B cell, CD4+ T cell, macrophages, neutrophils and dendritic cell (**Figure 5B**).

CIBERSORT analysis showed that NPM1 expression level had correlation with tumor immune cell infiltration (**Figure 5C**), including B cell memory ($P < 0.001$), B cell plasma (P = 0.003),

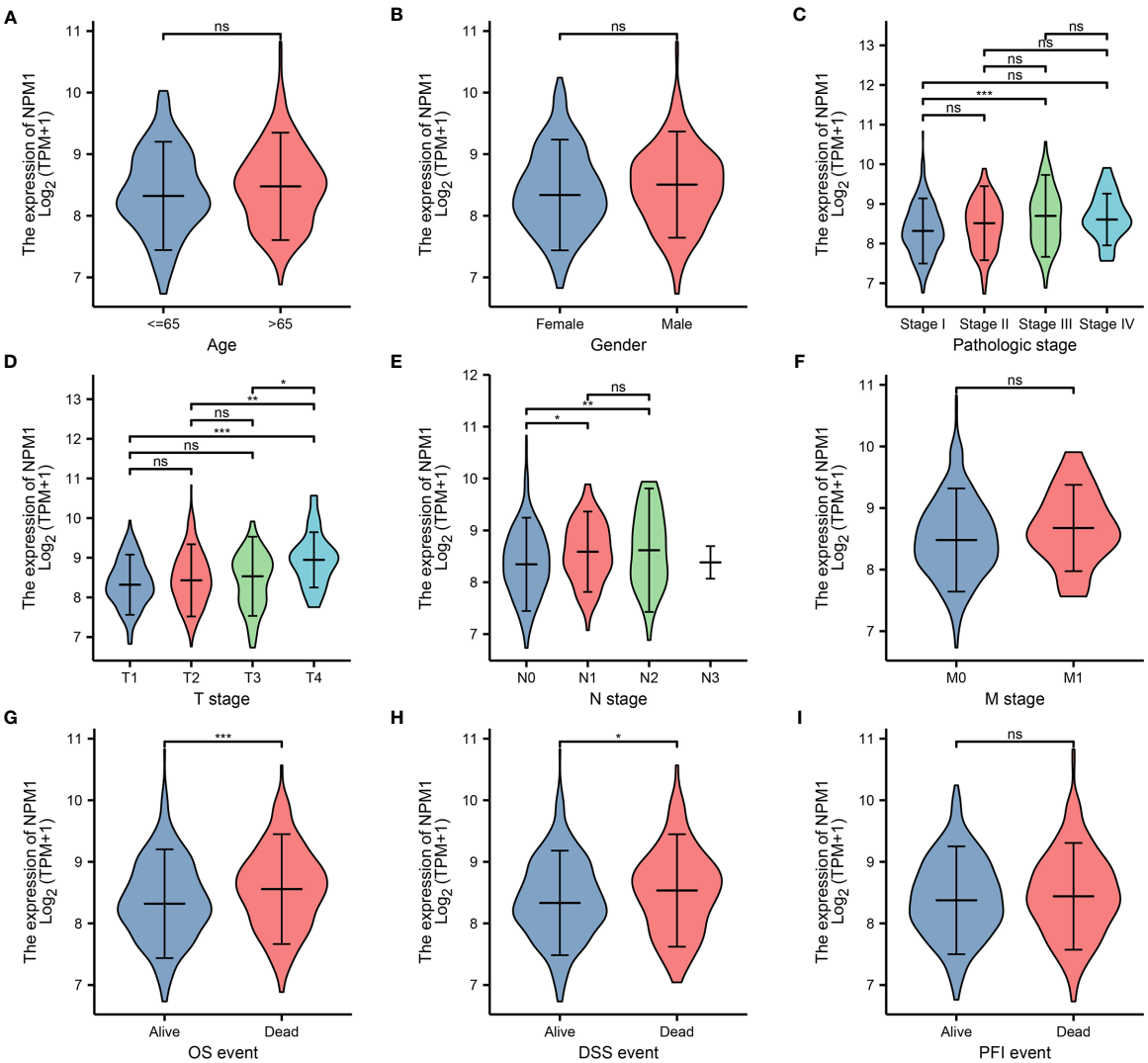

FIGURE 2 | Relationship between NPM1 mRNA expression and clinicopathological parameters in lung adenocarcinoma (LUAD) patients. The NPM1 mRNA expression level was expressed by using ggplot2 package of R software for the patient characteristics of **(A)** age, **(B)** gender, **(C)** pathologic stage, **(D)** T stage, **(E)** N stage, **(F)** M stage, **(G)** OS event, **(H)** DSS event and **(I)** PFI event. *P < 0.05; **P < 0.01; ***P < 0.001; ****P < 0.0001. ns, not significant.

T cell CD4+ memory activated (P = 0.004), T cell regulatory (Tregs) (P < 0.001), T cell gamma delta (P = 0.031), NK cell activated (P = 0.036), Macrophage M0 (P < 0.001), Macrophage M2 (P = 0.007), Myeloid dendritic cell resting (P = 0.032) and Myeloid dendritic cell activated (P < 0.001). We further generated Kaplan-Meier curve using the TIMER database to investigate the differences in survival between high and low expression levels of NPM1 and immune cell. We found B cell infiltration (P < 0.001), dendritic cell infiltration (P = 0.048) and NPM1 expression (P= 0.017) to significantly correlate with LUAD prognosis (**Figure 5D**).

To evaluate the relationship between NPM1 and various immune infiltrating cells of LUAD, TIMER, GEPIA databases and TCGA LUAD data sets were analyzed to analyze the association between NPM1 and immune marker genes of several immune cells (**Table 2**). All three analyses demonstrated that the expression of NPM1 was associated with B cell and NK cell immune marker genes, including CD19, MS4A1, CD79A, B3GAT1, KIR3DL1 and CD7. The scatter plot showed the correlation between NPM1 expression and B cell and NK cell immune marker genes, respectively (**Figure 6**).

Correlations of NPM1 Expression With m6A Modification in LUAD

Modification of m6A plays an important role in the development of LUAD. By analyzing the GSE31210 and TCGA LUAD data sets to investigate the correlation between NPM1 expression and the expression of 20 m6A related genes in LUAD. The results demonstrated that in the GSE31210 and TCGA LUAD data sets,

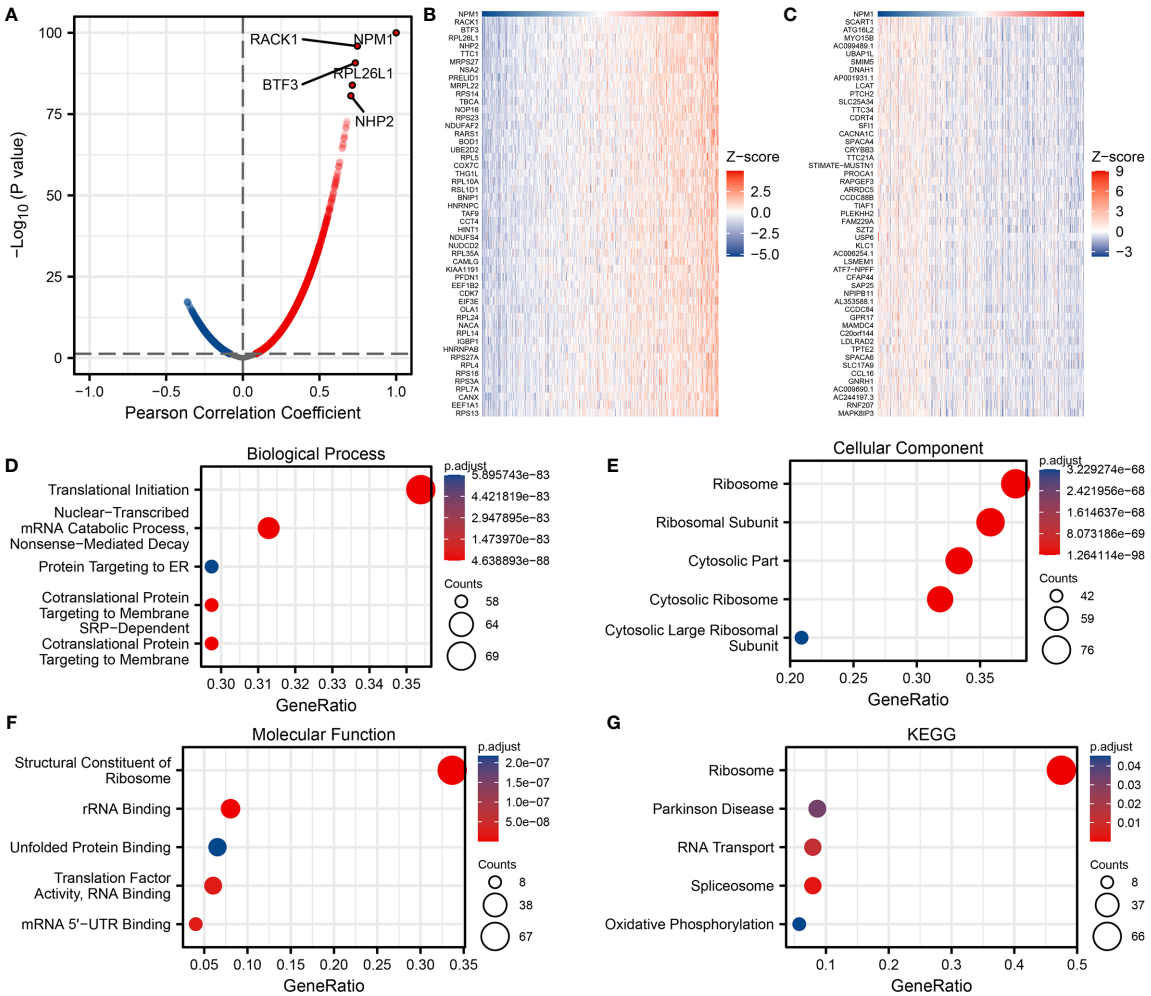

FIGURE 3 | Enrichment analysis of NPM1 gene co-expression network in lung adenocarcinoma (LUAD). **(A)** Volcano map showed co-expression genes associated with NPM1 expression in TCGA LUAD data sets. **(B, C)** Heat maps showed the top 50 co-expression genes positively and negatively correlated with NPM1 expression in the LUAD data sets. **(D–F)** Enrichment analysis of gene ontology (GO) terms for NPM1 co-expression genes. **(G)** Enrichment analysis of Kyoto Encyclopedia of Genes and Genomes (KEGG) terms for terms for NPM1 co-expression genes.

the expression of NPM1 was significantly positively correlated with ALKBH5, HNRNPC, IGF2BP1 and YTHDF2 (**Figure 7A**, $P < 0.05$). In addition, NPM1 expression was significantly positively correlated with HNRNPA2B1, METTL14, RBM15B, RBMX, VIRMA, WTAP, YTHDF1 and YTHDF3 in the TCGA LUAD data sets ($P < 0.05$), while NPM1 expression was negatively correlated with HNRNPA2B1, YTHDC1 and ZC3H13 expression in the GSE31210 data sets ($P < 0.05$).

The scatter plot shows the association between NPM1 and m6A related genes expression (**Figure 7B**). At the same time, TCGA LUAD samples were divided into high and low expression groups according to the expression level of NPM1. We attempted to analyze the m6A related genes differential expression between high and low groups with NPM1 expression to determine whether m6A modification was different between high and low groups with NPM1 expression in LUAD (**Figure 7C**). The results demonstrated that compared with the low expression group, the expressions of HNRNPC, METTL14, RBMX, VIRMA, WTAP, YTHDF2 and YTHDF3 in the high expression group of NPM1 were increased ($P < 0.05$). Venn diagram showed both expression correlation and differential expression of genes, including HNRNPC and YTHDF2 (**Figure 7D**). Kaplan-Meier curve showed that high expression of HNRNPC was strongly associated with poor prognosis of LUAD ($P = 0.001$), while YTHDF2 expression was not associated with poor prognosis of LUAD ($P = 0.295$) (**Figure 7E**). These results suggest that NPM1 may be closely related to the m6A modification of LUAD, especially through its regulation with HNRNPC, and ultimately affect the progression and prognosis of LUAD.

Correlations of NPM1 Expression With Glycolysis in LUAD

Glycolysis of tumor cells plays an important role in the progression of LUAD. By analyzing the GSE31210 and TCGA

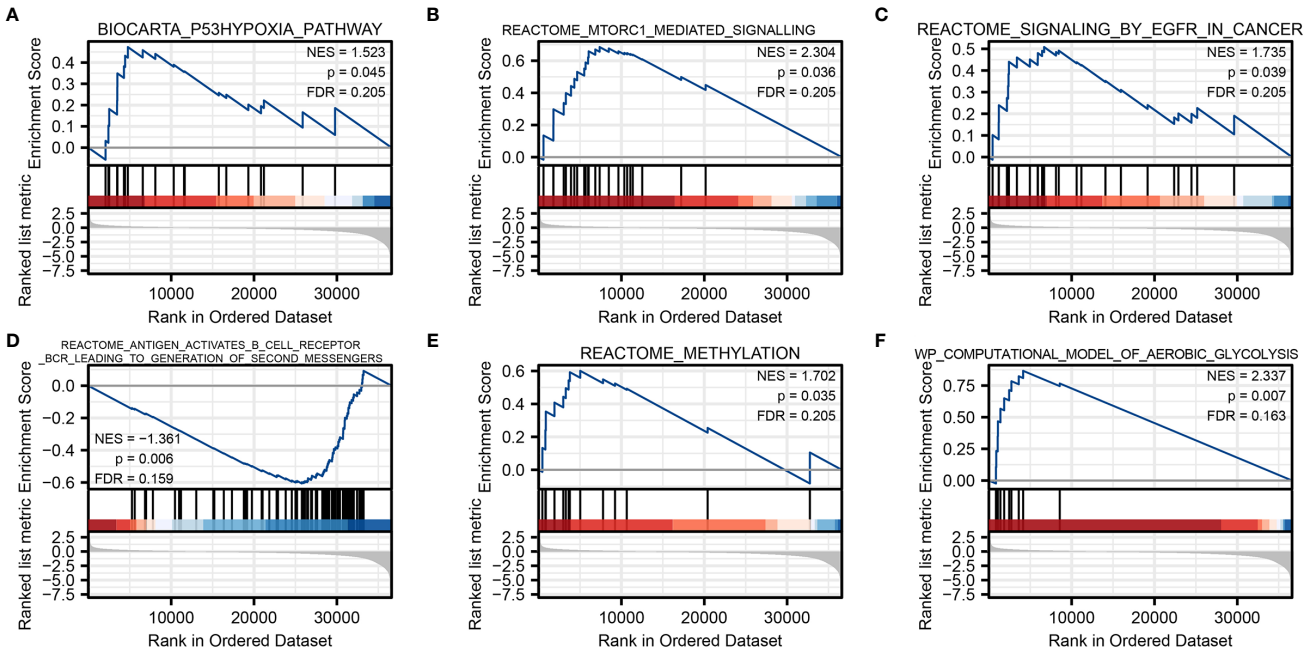

FIGURE 4 | Gene Set Enrichment Analysis. Pathway enriched in the p53 hypoxia pathway **(A)** mTORC1 mediated signaling **(B)** signaling by EGFR in cancer **(C)** antigen activates B cell receptor BCR leading to generation of second messengers **(D)** methylation **(E)** and aerobic glycolysis **(F)**.

LUAD data sets to investigate the correlation between NPM1 and the expression of 14 glycolysis related genes in LUAD. The results showed that the expression of NPM1 was significantly positively correlated with ENO1, G6PD, HK2, LDHA, LDHB, PDK3, PGK1 and SLC2A1 in the GSE31210 and TCGA LUAD data sets (**Figure 8A**, $P < 0.05$). In addition, NPM1 expression was significantly positively correlated with HK1, PDHB, PKM and SLC2A3 in the TCGA LUAD data sets ($P < 0.05$), while NPM1 expression was negatively correlated with PDK4 expression in the GSE31210 data sets ($P < 0.05$).

The scatter plot shows the association between NPM1 and glycolysis related genes (**Figure 8B**). At the same time, we attempted to analyze the differential expression of glycolysis related genes between the high and low groups with NPM1 expression (**Figure 8C**). The results demonstrated that compared with the low expression group, the expression of ENO1, HK1, HK2, LDHA, LDHB, PDHB, PGK1, PKM, SLC2A1 and SLC2A3 were increased in the high expression group of NPM1 ($P < 0.05$). Venn diagram showed both expression correlation and differential expression of genes, including ENO1, HK2, LDHA, LDHB, PGK1 and SLC2A1 (**Figure 8D**). Kaplan-Meier curves showed that high expression of ENO1, HK2, LDHA, LDHB and SLC2A1 was strongly associated with poor prognosis in LUAD ($P < 0.05$), while PGK1 expression was not ($P > 0.05$) (**Figure 8E**).

Further analysis showed a significant correlation between FDG uptake and NPM1 immunohistochemical staining in LUAD patients (**Figure 9**, $P < 0.05$). These results suggest that NPM1 may be closely related to the glycolysis of LUAD, especially through the regulation of ENO1, HK2, LDHA, LDHB and SLC2A1, and ultimately affect the progression and prognosis of LUAD.

DISCUSSION

NPM1 is a highly conserved protein commonly found in eukaryotic cells. It is mainly localized in the nucleus and can shuttle between the nucleus and cytoplasm to participate in nucleocytoplasmic signal transport (3, 4). Studies have shown that the content of NPM1 in tumor cells and growing cells is significantly higher than that in quiescent cells (36, 37). Overexpression of NPM1 can promote the growth and proliferation of various tumor cells (5–8). These results suggest that NPM1 may be a potential target for tumor gene therapy. However, there are few studies on the comprehensive analysis of NPM1 in LUAD.

In the present study, the NPM1 expression in tumors was predicted by bioinformatics analysis, and the expression of NPM1 in LUAD was verified by cell assay and immunohistochemical staining. Through the analysis of Oncomine database, we found NPM1 was overexpressed in 9 types of cancer, and analysis of the TCGA data set found that NPM1 was overexpressed in 13 types of cancer, which was consistent with the results of previous studies (4, 36, 37). Based on the analysis of GEO and TCGA LUAD data sets, the expression level of NPM1 in LUAD tissues was significantly higher than that in normal tissues. The expression of NPM1 in

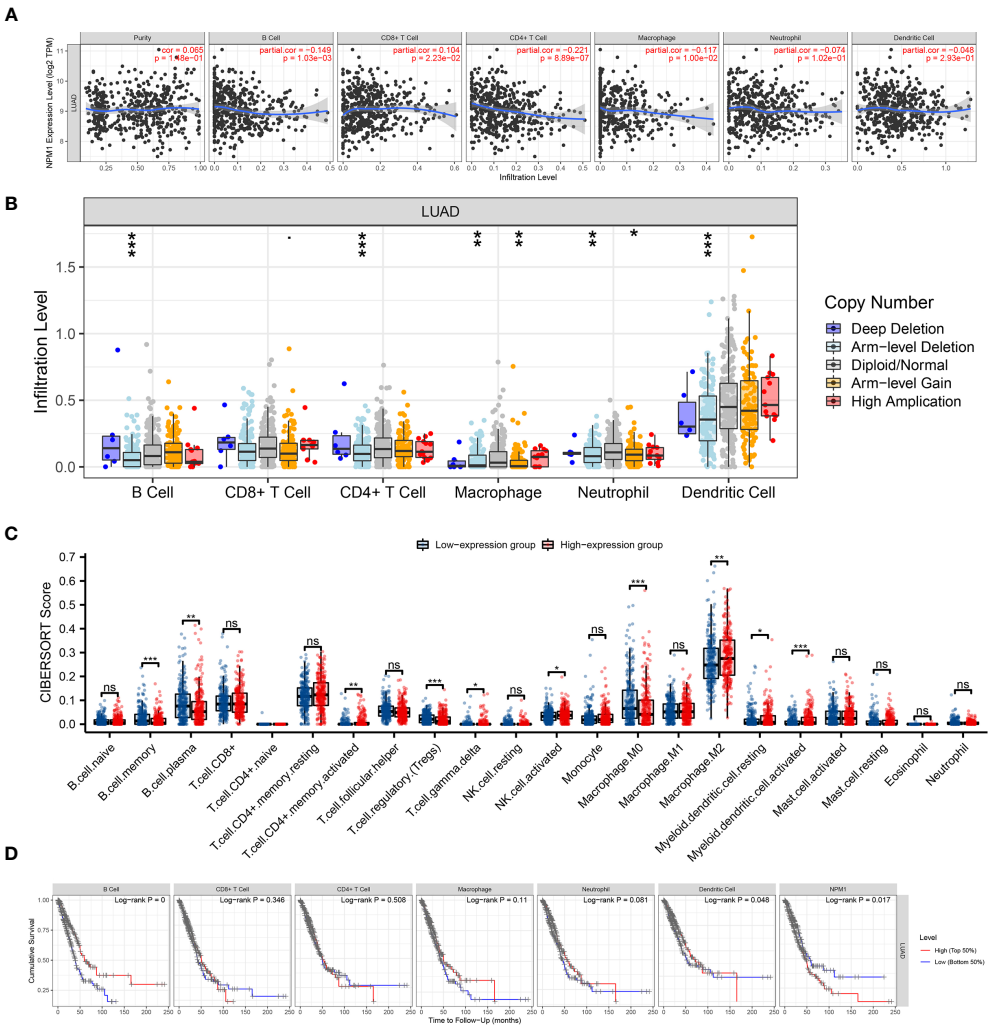

FIGURE 5 | Correlation between NPM1 and Tumor Immune Infiltrating Cells. **(A)** Correlation between the expression of NPM1 and immune infiltrating cells in lung adenocarcinoma (LUAD). **(B)** NPM1 CNV affects the infiltrating levels of B cell, CD4+ T cell, macrophages, neutrophils and dendritic cell in LUAD. **(C)** Changes of 22 immune cell subtypes between high and low NPM1 expression groups in LUAD tumor samples. **(D)** Kaplan-Meier plots of immune infiltration and NPM1 expression levels in LUAD. *P < 0.05; **P < 0.01; ***P < 0.001; ****P < 0.0001. ns, not significant.

LUAD and normal samples was detected by qRT-PCR and IHC, and the analysis results were consistent with the above results. We also used ROC curve to analyze the ability of NPM1 expression to predict LUAD, and found that NPM1 had certain accuracy in predicting the outcome of tumors and normal samples. Previous studies have found that NPM1 expression had certain accuracy in predicting the prognosis of gastric cancer (38) and prostate cancer (39). At the same time, we also found that high expression of NPM1 predicted a worse prognosis in patients with LUAD, suggesting that changing the expression level of NPM1 may improve the prognosis in patients with LUAD. Finally, NPM1 expression was found to be related to tumor grade. In conclusion, NPM1 may serve as a potential diagnostic and prognostic marker for LUAD.

However, current studies on the role of NPM1 in tumor mainly focus on its role in ribosome processing and assembly, centrosome replication and molecular chaperone (4, 36, 37). Other biological functions of NPM1 in LUAD are less studied. In this study, R software package was used to analyze the co-expression genes of NPM1 in LUAD, and it was found that the expressions of RACK1, BTF3, RPL26L1 and NHP2 in LUAD had the strongest correlation with NPM1. Wu et al. (40) found that PHB2 promotes tumorigenesis *via* RACK1 in non-small cell lung cancer. Jeon et al. (41) found that kahweol inhibited the proliferation of NSCLC cells through ERK-mediated signaling pathways and the downregulation of BTF3, while the role of RPL26L1 and NHP2 in LUAD has not been reported. The GO and KEGG function enrichment analysis of 200 co-expressed

TABLE 2 | Correlation analysis between NPM1 and immune cell marker gene in TIMER, GEPIA and TCGA.

Description	Gene markers	TIMER		GEPIA		TCGA	
		Purity		Tumor		Tumor	
		rho	P	rho	P	rho	P
B cell	CD19	-0.197	**1.08E-05**	-0.24	**5.00E-08**	-0.193	**6.86E-06**
	MS4A1	-0.149	**9.01E-04**	-0.18	**6.70E-05**	-0.184	**1.88E-05**
	CD79A	-0.189	**2.39E-05**	-0.26	**1.20E-08**	-0.169	**8.43E-05**
CD8+ T Cell	CD8A	0.013	7.69E-01	0.0061	8.90E-01	0.048	2.67E-01
	CD8B	-0.013	7.68E-01	-0.016	7.30E-01	0.037	3.99E-01
	IL2RA	0.099	**2.81E-02**	0.15	**1.20E-03**	0.111	**1.04E-02**
Tfh	CXCR3	-0.134	**2.79E-03**	-0.14	**2.70E-03**	-0.050	2.51E-01
	CXCR5	-0.168	**1.83E-04**	-0.39	**3.50E-09**	-0.130	**2.63E-03**
	ICOS	-0.006	8.89E-01	0.019	6.70E-01	-0.023	5.99E-01
Th1	IL12RB1	-0.103	**2.23E-02**	-0.082	7.30E-02	-0.070	1.04E-01
	CCR1	-0.019	6.69E-01	0.05	2.70E-01	0.045	2.97E-01
	CCR5	-0.037	4.11E-01	0.0054	9.10E-01	-0.019	6.56E-01
Th2	CCR4	-0.031	4.93E-01	0.02	6.60E-01	-0.061	1.56E-01
	CCR8	0.029	5.18E-01	0.091	**4.60E-02**	0.023	6.03E-01
	HAVCR1	0.080	7.72E-02	0.088	5.20E-02	0.046	2.93E-01
Th17	IL21R	-0.087	5.23E-02	-0.064	1.60E-01	-0.079	6.81E-02
	IL23R	0.012	7.97E-01	0.097	**3.30E-02**	-0.088	**4.15E-02**
	CCR6	-0.095	**3.43E-02**	-0.0089	8.50E-01	-0.103	**1.73E-02**
Treg	FOXP3	-0.054	2.35E-01	-0.057	2.10E-01	0.011	8.00E-01
	NT5E	0.104	**2.12E-02**	0.16	**3.20E-04**	0.162	**1.72E-04**
	IL7R	-0.008	8.53E-01	0.022	6.30E-01	-0.067	1.21E-01
T cell exhaustion	PDCD1	-0.071	1.15E-01	-0.087	5.50E-02	-0.018	6.74E-01
	CTLA4	-0.067	1.39E-01	-0.089	5.20E-02	-0.091	**3.49E-02**
	LAG3	-0.141	**1.74E-03**	-0.19	**2.50E-05**	-0.072	9.61E-02
M1 Macrophage	NOS2	-0.080	7.50E-02	-0.008	8.60E-01	0.007	8.81E-01
	IRF5	-0.223	**5.80E-07**	-0.14	**1.40E-03**	-0.095	**2.83E-02**
	PTGS2	-0.051	2.56E-01	-0.053	2.50E-01	-0.060	1.68E-01
M2 Macrophage	CD163	0.009	8.38E-01	0.048	2.90E-01	0.036	4.09E-01
	MRC1	0.011	8.14E-01	0.12	**1.00E-02**	0.029	5.03E-01
	CD209	0.016	7.15E-01	0.11	**2.10E-02**	0.052	2.30E-01
TAM	CCL2	-0.001	9.74E-01	0.0077	8.70E-01	0.071	1.02E-01
	CD86	-0.021	6.36E-01	0.053	2.40E-01	0.046	2.87E-01
	CD68	-0.055	2.24E-01	0.088	5.30E-02	0.034	4.30E-01
Monocyte	CD14	-0.095	**3.41E-02**	-0.046	3.20E-01	0.050	2.50E-01
	CD33	-0.062	1.66E-01	-0.0073	8.70E-01	0.005	9.12E-01
	ITGAX	-0.197	**9.99E-06**	-0.17	**1.60E-04**	-0.179	**3.32E-05**
Natural killer cell	B3GAT1	-0.138	**2.11E-03**	-0.12	**6.70E-03**	-0.153	**3.79E-04**
	KIR3DL1	-0.158	**4.36E-04**	-0.11	**2.10E-02**	-0.091	**3.62E-02**
	CD7	-0.205	**4.28E-06**	-0.23	**3.50E-07**	-0.096	**2.57E-02**
Neutrophil	FCGR3A	0.034	4.56E-01	0.096	**3.50E-02**	0.092	**3.36E-02**
	CD55	-0.059	1.94E-01	0.058	2.00E-01	0.053	2.21E-01
	ITGAM	-0.090	**4.46E-02**	-0.029	5.20E-01	-0.038	3.81E-01
Dendritic cell	CD1C	-0.069	1.27E-01	-0.008	8.60E-01	0.012	7.77E-01
	THBD	-0.010	8.22E-01	0.087	5.60E-02	0.065	1.33E-01
	NRP1	0.019	6.80E-01	0.12	**9.40E-03**	0.040	3.52E-01

Bold values indicate P < 0.05.

genes positively correlated with NPM1 expression demonstrated that the co-expression of NPM1 was primarily associated to translational initiation, ribosome, and structural constituent of ribosome. KEGG pathway analysis showed that the co-expression of NPM1 was primarily associated to ribosome, Parkinson disease, and RNA transport, which was like the findings of previous studies (4). The GSEA pathway enrichment analysis showed that the differential genes grouped according to NPM1 expression were mainly enriched in the mTORC1 mediated signaling, p53 hypoxia pathway, signaling by EGFR in cancer, antigen activates B cell receptor BCR leading to generation of second messengers, aerobic glycolysis and methylation pathways. Previous studies have shown that the occurrence and development of LUAD are closely related to the first three pathways (42–44).

Immune infiltration of tumor cells is associated with lymph node metastasis and prognosis of LUAD (45, 46). TIMER database analysis showed that the expression level of NPM1 in LUDA was negatively correlated with B cells, CD4+ T cells and macrophages, and positively correlated with the expression level

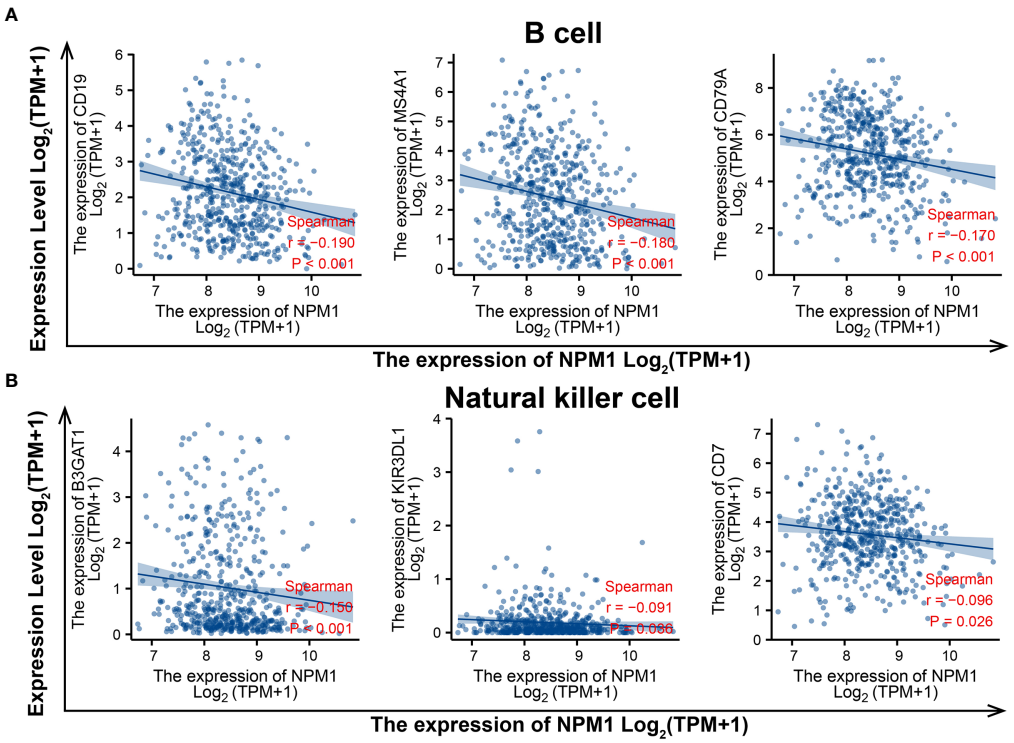

FIGURE 6 | NPM1 expression correlated with B cell and natural killer cell in lung adenocarcinoma (LUAD). Markers include CD19, MS4A1 and CD79A of B cell **(A)** B3GAT1, KIR3DL1 and CD7 of natural killer cell **(B)**.

of CD8+ T cells. In addition, NPM1 CNV was significantly correlated with the infiltration levels of B cells, CD4+ T cells, macrophages, neutrophils and dendritic cells. These results suggest that NPM1 may be involved in the immune response to the tumor microenvironment of LUAD, especially to B cells, CD4+T cells and macrophages. The proportion of 22 tumor immune cells in LUAD was determined by CIBERSORT analysis. We identified 10 types of immune cells, including memory B cells, plasma B cells, activated memory CD4+ T cells, regulatory T cells, gamma delta T cells, activated NK cells, M0 macrophages, M2 macrophages, resting myeloid dendritic cells and activated myeloid dendritic cell, and their expression ratio showed significant differences with different expression levels of NPM1. At the same time, survival analysis also found that LUAD patients with B cell low expression group had a worse prognosis. In addition, through the analysis of TIMER, GEPIA database and TCGA data sets, we found that the expression of NPM1 was significantly negatively correlated with the gene markers of B cells and NK cells, suggesting that NPM1 may affect the immune infiltration of LUAD by affecting the expression of B cells and NK cells. B cells and NK cells are important immune cells of the body, which have a wide range of anti-tumor effects (47–50). Yang et al. (48) found that in lung cancer cells, blocking the transforming growth factor-β signaling pathway enhanced the antitumor effect of NK-92 cell therapy. Germain et al. (49) found that lung cancer patients with high density B cells had a better prognosis. We speculate that the overexpression of NPM1 inhibits the infiltration of B cells and NK cells in LUAD, and ultimately further accelerates tumor progression. We suggest that the high expression of NPM1 in LUAD patients may trigger an anti-tumor immune response, suggesting that NPM1 plays an important role in the immune regulation of LUAD. However, more experiments are needed to further verify our hypothesis, especially the relationship between NPM1 and B cells and NK cells, respectively.

As a part of methylation modification, m6A modification is one of the most common RNA methylation modifications, which can influence the occurrence and development of cancer by regulating cancer-related biological functions (2, 51, 52). Li et al. (51) found that FTO, as an m6A demethylase, is highly expressed in acute myeloid leukemia and plays an important role in carcinogenesis. However, there are few studies on the relationship between NPM1 and m6A in solid tumors. In this study, we found that the expression level of NPM1 was significantly positively correlated with ALKBH5, HNRNPC, IGF2BP1 and YTHDF2. We also found that the expression levels of HNRNPC, METTL14, RBMX, VIRMA, WTAP, YTHDF2 and YTHDF3 were significantly increased in the high NPM1 expression group. Finally, Kaplan-Meier curve analysis showed that LUAD patients with high HNRNPC expression had

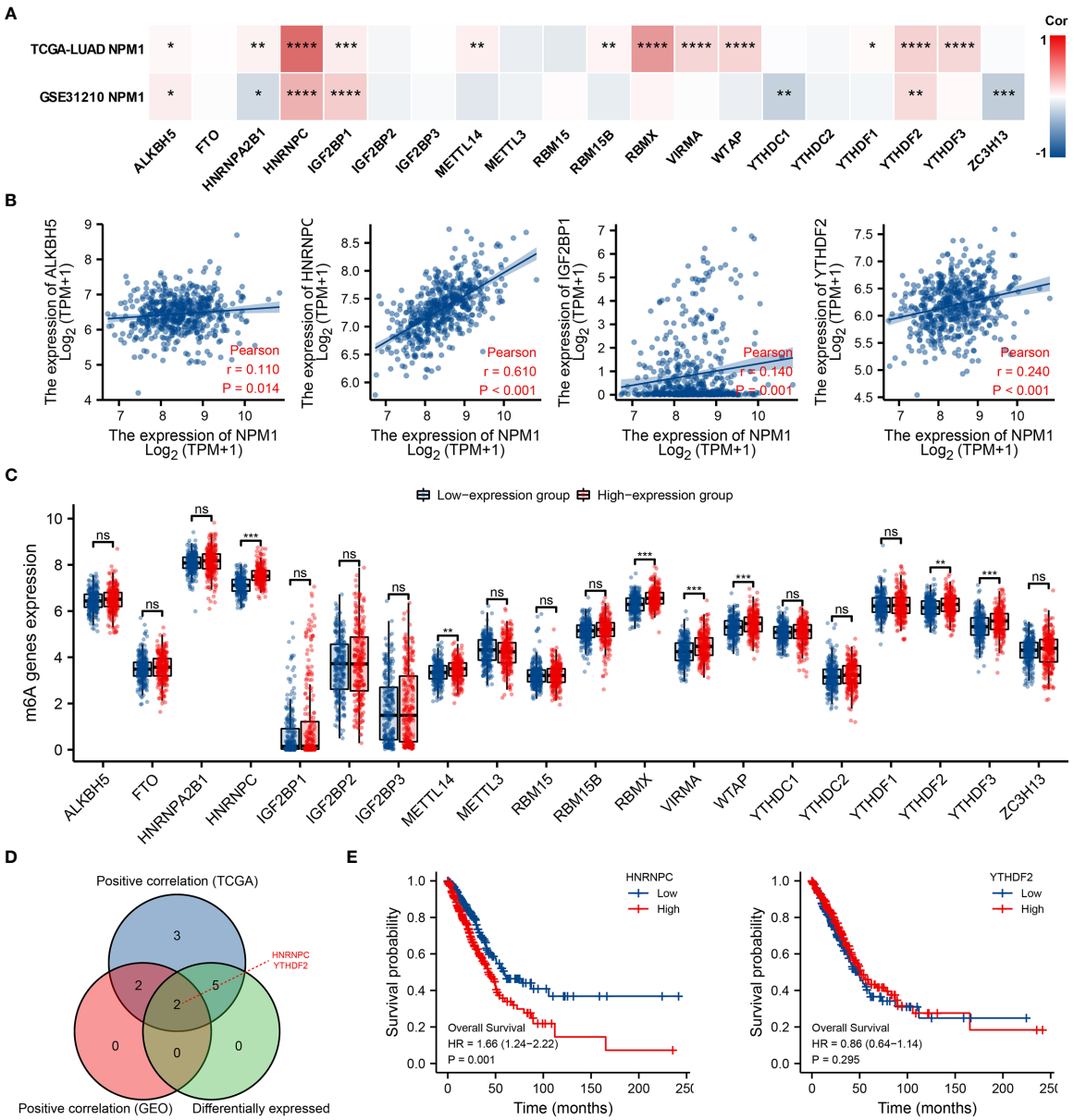

FIGURE 7 | Correlations of NPM1 expression with m6A related genes in lung adenocarcinoma (LUAD). **(A)** GSE31210 and TCGA LUAD data sets analyzed the correlation between the NPM1 and the m6A related genes expression in LUAD. **(B)** Draw a scatter plot to show the correlation between the NPM1 and the m6A related genes expression, include ALKBH5, HNRNPC, IGF2BP1 and YTHDF2. **(C)** The differential expression of m6A related genes between high and low NPM1 expression groups in LUAD tumor samples. **(D)** Venn diagram showed both expression correlation and differential expression of genes, including HNRNPC and YTHDF2. **(E)** Kaplan-Meier curve of HNRNPC and YTHDF2. *P < 0.05; **P < 0.01; ***P < 0.001; ****P < 0.0001. ns, not significant.

a worse prognosis. We believe that the cancer promoting effect of NPM1 gene is related to the modification of m6A, which may affect the methylation level of LUAD through its association with HNRNPC, and ultimately affect the progression of LUAD.

The enhancement of glycolysis is strongly associated to the development of cancer and the poor prognosis. Targeting cancer glycolysis metabolism is a new strategy for cancer treatment (53). Zhu et al. (54) found that NPM1 promoted aerobic glycolysis and tumor progression in patients with pancreatic cancer by inhibiting the fructose-1, 6-bisphosphatase 1. In this study, we found that the expression level of NPM1 was significantly positively correlated with ENO1, G6PD, HK2, LDHA, LDHB, PDK3, PGK1 and SLC2A1.We also found that the expression levels of ENO1, HK1, HK2, LDHA, LDHB, PDHB, PGK1, PKM, SLC2A1 and SLC2A3 were significantly increased in the high expression group of NPM1. Finally, Kaplan-Meier curve analysis

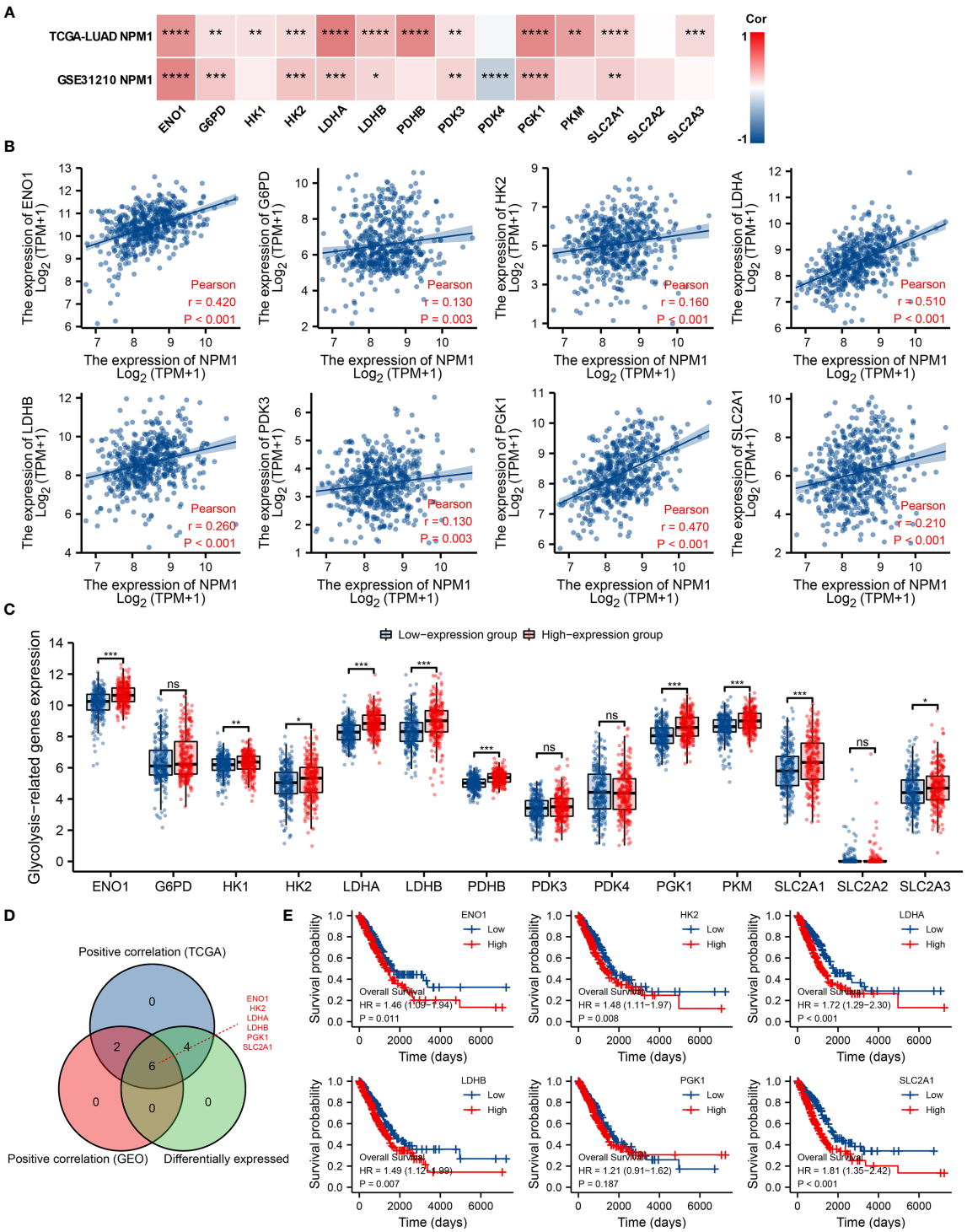

FIGURE 8 | Correlations of NPM1 expression with glycolysis related genes in lung adenocarcinoma (LUAD). **(A)** GSE31210 and TCGA LUAD data sets analyzed the correlation between the NPM1 and the m6A related genes expression in LUAD. **(B)** Draw a scatter plot to show the correlation between the NPM1 and the glycolysis related genes expression, include ENO1, G6PD, HK2, LDHA, LDHB, PDK3, PGK1 and SLC2A1. **(C)** The differential expression of glycolysis related genes between high and low NPM1 expression groups in LUAD tumor samples. **(D)** Venn diagram showed both expression correlation and differential expression of genes, including ENO1, HK2, LDHA, LDHB, PGK1 and SLC2A1. **(E)** Kaplan-Meier curve of ENO1, HK2, LDHA, LDHB, PGK1 and SLC2A1. *P < 0.05; **P < 0.01; ***P < 0.001; ****P < 0.0001. ns, not significant.

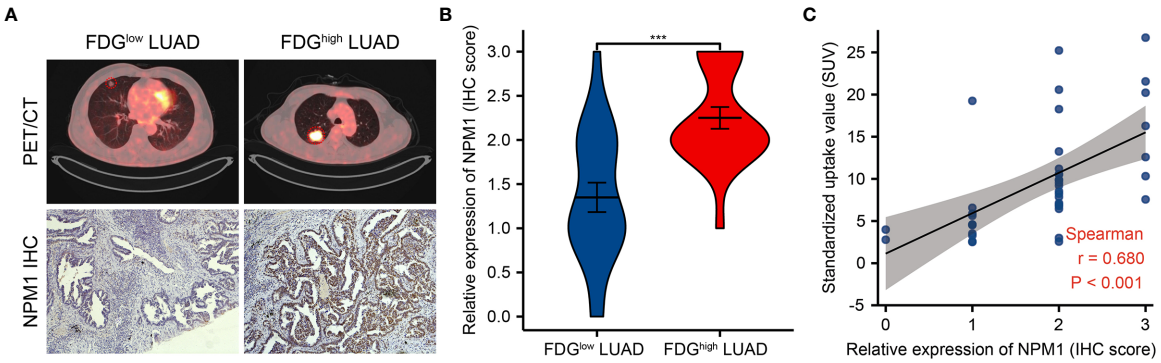

FIGURE 9 | Correlations of NPM1 expression with glycolytic metabolism in lung adenocarcinoma (LUAD). **(A)** Representative PET/CT images and NPM1 immunohistochemical images of LUAD patients with FDG high uptake and FDG low uptake (SUVmax). **(B)** Statistical analysis of NPM1 expression in LUAD patients with FDG high uptake and patients with FDG low uptake. **(C)** Correlation between FDG uptake and NPM1 expression in 40 LUAD patients. *P < 0.05; **P < 0.01; ***P < 0.001; ****P < 0.0001.

showed that LUAD patients with high expression of ENO1, HK2, LDHA, LDHB and SLC2A1 had a worse prognosis. Further analysis found a significant association between FDG uptake and NPM1 immunohistochemical staining in LUAD patients. We suggest that NPM1 may enhance the glycolytic ability of LUAD by promoting the expression of ENO1, HK2, LDHA, LDHB and SLC2A1, and thus promote the occurrence and development of LUAD.

In conclusion, our study confirmed that NPM1 is overexpressed in LUAD, and its expression level is related to clinical case characteristics and prognosis of LUAD patients. The expression level of NPM1 is closely related to the extent of immune cell infiltration, which may reduce the anti-tumor effect by inhibiting the infiltration of B cells and NK cells. NPM1 is associated with m6A modification and glycolysis, and m6A modification may promote the glycolysis and malignant proliferation of LUAD by enhancing the stability of NPM1. NPM1 can be used as a biomarker for the diagnosis, treatment and prognosis of LUAD.

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 Ethics Committee of Taihe Hospital Affiliated of Hubei University of Medicine. Written informed consent for participation was not required for this study in accordance with the national legislation and the institutional requirements.

AUTHOR CONTRIBUTIONS

X-SL conceived the project and wrote the manuscript. X-SL, L-MZ, L-LY and YG participated in data analysis. X-SL, X-YK and X-YL participated in discussion and language editing. Z-JP reviewed the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2021.724741/full#supplementary-material

Supplementary Table 1 | NPM1 co-expressed genes.

Supplementary Table 2 | The GO and KEGG enrichment analysis of NPM1 coexpression genes.

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REVIEW
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Engineered T Cell Therapy for Gynecologic Malignancies: Challenges and Opportunities

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Gynecologic malignancies, mainly including ovarian cancer, cervical cancer and endometrial cancer, are leading causes of death among women worldwide with high incidence and mortality rate. Recently, adoptive T cell therapy (ACT) using engineered T cells redirected by genes which encode for tumor-specific T cell receptors (TCRs) or chimeric antigen receptors (CARs) has demonstrated a delightful potency in B cell lymphoma treatment. Researches impelling ACT to be applied in treating solid tumors like gynecologic tumors are ongoing. This review summarizes the preclinical research and clinical application of engineered T cells therapy for gynecologic cancer in order to arouse new thoughts for remedies of this disease.

Keywords: gynecologic malignancies, engineered T cells, CAR-T, TCR-T, adoptive T cell therapy, immunotherapy

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INTRODUCTION

Gynecologic malignancies are serious threats to women's health worldwide. Although traditional procedures like surgery, radiotherapy and chemotherapy have effectively decreased mortality, researchers are seeking new ideas and strategies to reduce the recurrence and metastasis of tumors, alleviate adverse drug reactions, as well as further improve the life quality of patients.

Adoptive T cell therapy (ACT) is one of the most powerful weapons among a wide range of approaches focusing on our immune system. The basic principle of this treatment refers to reinfusing autologous lymphocytes which are expanded, screened and modified *in vitro* to patients for tumor regression mediated by T cells. Early preclinical research successfully proved that with a genetically transferred synthetic receptor targeting antigen CD19, which is a broad marker commonly expressed by B cell lymphoma cells, reinfused autologous T cells could eliminate

established B cell tumors in mice (1). Based on multiple tried-and-true basic experiments, clinical trials later showed prominent advantages of this kind of engineered T cells named chimeric antigen receptor T cells (CAR-Ts) in patients with hematological malignancies (2–5). Promoted by these significant achievements, adoptive T cell therapy has proved to be the potential adjuvant therapy for tumor treatment.

The application of natural tumor-infiltrating lymphocytes (TILs) obtained from suspension or fragments of the resected tumor is the earliest achievement of ACT. In 24th May, 2019, a TIL product named LN-145 was granted as the breakthrough designation for cervical cancer (6), exhibiting remarkable objective response rate (ORR) and disease control rate (DCR) in treating cervical cancer (7). Although TILs have higher concentration of specific T cells comparing to peripheral T cells, the hostile tumor microenvironment attenuates the long-term survival of functional T cells, as TILs are sensitive to anergy, exhaustion and apoptosis. In addition, the gathering of TILs requires joint efforts of surgeons to obtain fresh tumor samples where effective lymphocytes could be extracted. Groundbreakingly, engineered T cells, including T cell receptor modified T cells (TCR-Ts) and CAR-Ts, currently have a promising advance in tumor immunotherapy since they could be genetically modified in structure to target specific tumor antigens or to express cytokines ameliorating immunosuppressive tumor microenvironment. Two CAR-T products have already been approved by the USA Food and Drug Administration (FDA) for refractory leukemia and lymphoma immunotherapy (8, 9).

In this review, we discuss the application of engineered T cells in gynecologic malignancies in preclinical and clinical trials, and explore further opportunities of implicating this therapy in clinical decision for gynecologic oncology. A brief timeline of milestones associated with this field is arranged (**Figure 1**). Pioneer clinical application of engineered T cells, critical clinical trials carried out for gynecologic cancers and commercial CAR-T agents and related synergist approved by the FDA are included (10–12).

ENGINEERED T CELLS

Based on the gene editing technology, engineered peripheral T cells with specific antigen binding receptors like TCRs or CARs could further facilitate ACT progress compared with TILs. These two therapies have different mechanisms and efficiency preference for treating distinct tumors. Currently, mainstream cell preparation methods include the following steps: (1) obtaining frozen apheresis white blood cell (WBC) product from patients; (2) the selection and enrichment of T cells by corresponding selection beads; (3) activation of T cells *via* addition of stimulating cytokines like interleukin (IL) 2 and

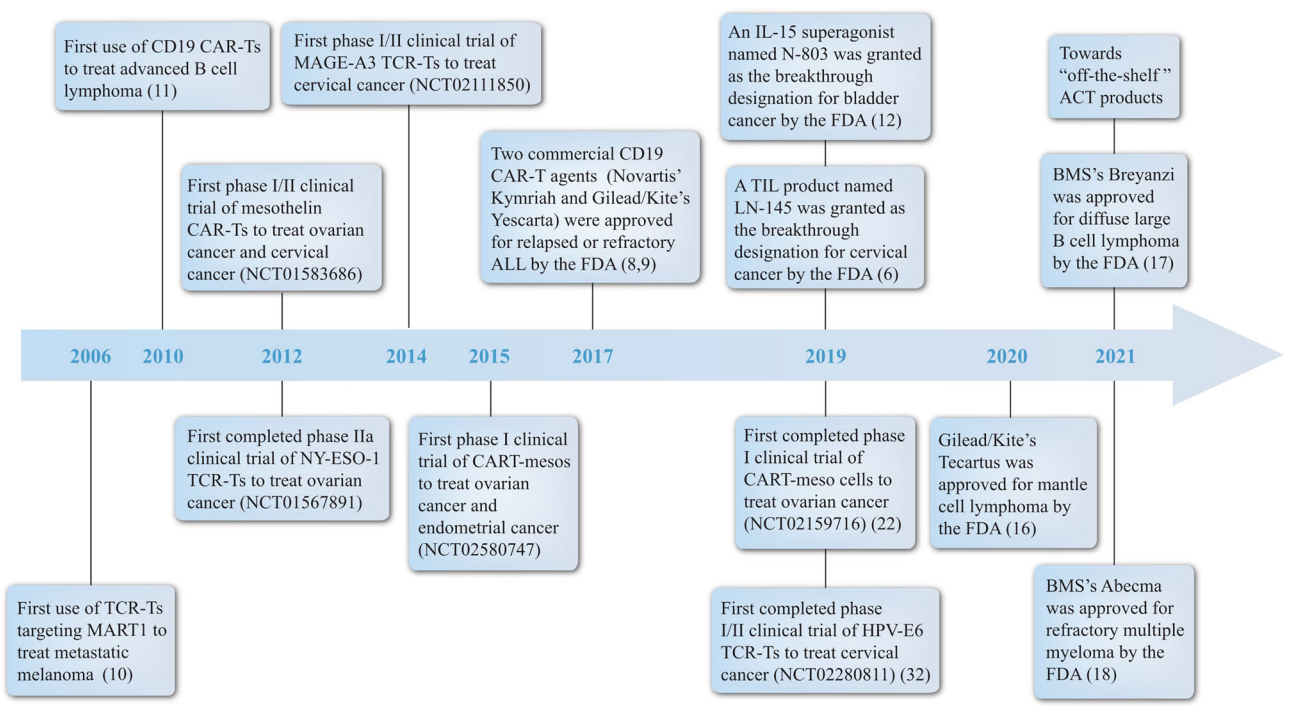

FIGURE 1 | Milestones of ACT. A brief summary of some landmark achievements in ACT development history with a focus on engineered T cells for treating gynecologic malignancies from the year 2006 to 2021. Significant events include: (1) pioneer treatment of metastatic melanoma by TCR-T and B cell lymphoma by CAR-T; (2) the first or the fastest progressing clinical trial of engineered T cells in different gynecologic tumors; (3) the acknowledgement of CAR-T, TIL and IL-15 products by FDA. ACT, adoptive T cell therapy; BMS, Bristol-Myers Squibb; CAR, chimeric antigen receptor; FDA, the Food and Drug Administration; IL, interleukin; TCR, T cell receptor; TIL, tumor infiltrating lymphocyte.

beads like anti-CD3/CD28 beads; (4) transduction of target CAR or TCR genes through lentiviral, retroviral vectors or transposase systems and so on; (5) expanding the number of T cells *in vitro*; (6) cryopreservation.

T Cell Receptor Modified T Cells (TCR-Ts) Therapy

TCRs are specific receptors on the surface of T cells capable of recognizing peptide major histocompatibility complex (pMHC) formed by peptide antigens presented by the MHC on tumor or antigen presenting cells. The killing ability of CD8+ T cells depends on the specific identification of cleaved peptide chains bound to class I human leukocyte antigen (HLA) by TCRs, therefore it is noteworthy that the function of TCRs only works in HLA-appropriate patients. T cell sources derived from individuals or humanized mice with matched HLA alleles and sophisticated techniques are required for the personalized production of TCRs. The alpha and beta chain pair of TCRs can be genetically modified to target tumor antigens and thus T cells transfected with these new TCRs can specifically recognize and eliminate cancer cells. Recently, a non-virus solution using the Sleeping Beauty (SB) transposons system to target unique neoantigens was described (13), which exhibited advantages with lower price and risk of random insertional mutagenesis.

Compared with the antibody-binding-like principle of CAR-Ts, TCR-Ts can recognize target antigens more extensively since they not only identify cell membrane antigens but also intracellular tumor antigens presented by pMHC, inducing a more orderly and durable immunological synapse formation process. Particularly, the targeting of almost 90% solid tumors relies on tumor specific antigens (TSAs) inside tumor cells, while surface antigens are often tumor associated antigens (TAAs) which can also be expressed by normal tissues to affect their function. Besides, TCR-Ts follow the natural signaling pathway to maintain their original regulatory mechanism, being more sensitive to low-copy antigens than CAR-Ts. Consequently, the potential of TCR-Ts dramatically outweighs CAR-Ts in treating solid tumors (14). However, the utility of TCR-Ts in treating solid tumors is progressing slowly. Currently, there is no market approval for any TCR-T products. Several clinical trials are still ongoing.

Chimeric Antigen Receptor T Cells (CAR-Ts) Therapy

The most obvious character of CAR-T cells in contrast to TCR-T cells is that CARs can directly bind antigens in an MHC-independent fashion, therefore they are potentially able to detect most of the surface-expressing targets in patients who have various HLA types. This is particularly important for immunotherapy because tumor cells losing MHC-associated antigens are probable to escape immune surveillance. A CAR is composed of an extracellular antigen-binding domain, most of which is an antibody–derived single-chain variable fragment (scFV), a transmembrane domain and an intracellular signaling domain of the TCR CD3ζ chain to activate T cells (15). The consisting improvements of CAR-T include the introduction of an additional co-stimulatory molecular CD28 or 4-1BB (CD137) intracellular domain (16), and inducers for transgenic cytokines like IL-12 and IL-15 (17) (**Figure 2**).

The landmark of CAR-T therapy is the commercial CD19 specific CAR-T approved by the FDA for relapsed or refractory acute lymphocytic leukemia (ALL). Two commercial agents, tisagenlecleucel (Kymriah, Novartis) (9) and axicabtagene ciloleucel (Yescarta, Kite Pharma) (8) were acknowledged in 2017. After this, brexucabtagene autoleucel (Tecartus, Kite Pharma) (18), lisocabtagene maraleucel (Breyanzi, Bristol-Myers Squibb) (19) and idecabtagene vicleucel (Abecma, Bristol-Myers Squibb) (20) were approved successively by the FDA for marketing, further promoting the clinical implement of CAR-T therapy in hematological malignancies. Among these agents, only Abecma targets B cell maturation antigen (BCMA), others continue to focus on CD19.

STUDIES OF ENGINEERED T CELLS IN COMMON MALIGNANT GYNECOLOGIC TUMORS

Unlike the popularity of CAR-T therapy in hematological malignancies, studies for broader swaths in the field of gynecologic tumors are still in the bud. Antigen selection is crucial in deciding treatment programs which lead to TCR-T or CAR-T therapy and the treatment efficiency. Where the antigen is expressed at the cell and tissue level should be the first consideration by high-throughput, ultra-sensitive mass spectrometry and other means when ACT is carried out. Improvements could be reflected in the optimization of antigen selection for patients with different types of gynecological tumors in the future.

Ovarian Cancer

Ovarian cancer significantly jeopardizes the health of women with high lethality. With advanced surgical treatment and systematic care, the five-year relative survival rate of patients is slightly promoted, but still less than 50% (21).

Armed with the knowledge that the melanoma-associated antigen 4 (MAGE-A4) and the New York esophageal squamous cell carcinoma 1 (NY-ESO-1) are commonly expressed by ovarian cancer cells (26.4% and 3.6% respectively) (22), TCR-T products targeting these two ideal antigens have been designed and applied in clinical research. MAGE-A4^{c1032}T cells are used in HLA-A*02:01 (A2+) patients with MAGE-A4 positive tumors including ovarian cancer in an ongoing phase I multi-tumor study (NCT03132922). In cohort 3/expansion (28 patients), 7 patients with synovial sarcoma had partial response (PR), 11 patients had stable disease (SD), 5 patients had progressive disease (PD) and the remaining 5 were non-evaluable. MAGE-A4 specific TCR-T exhibited therapeutic potential and manageable adverse effects at a dose range of (1.2~10) $\times 10^9$ (23). In further research, a CD8α co-receptor was introduced into CD4+ T cells alongside the engineered TCR (ADP-A2M4CD8). These modified CD4+ T cells could in turn

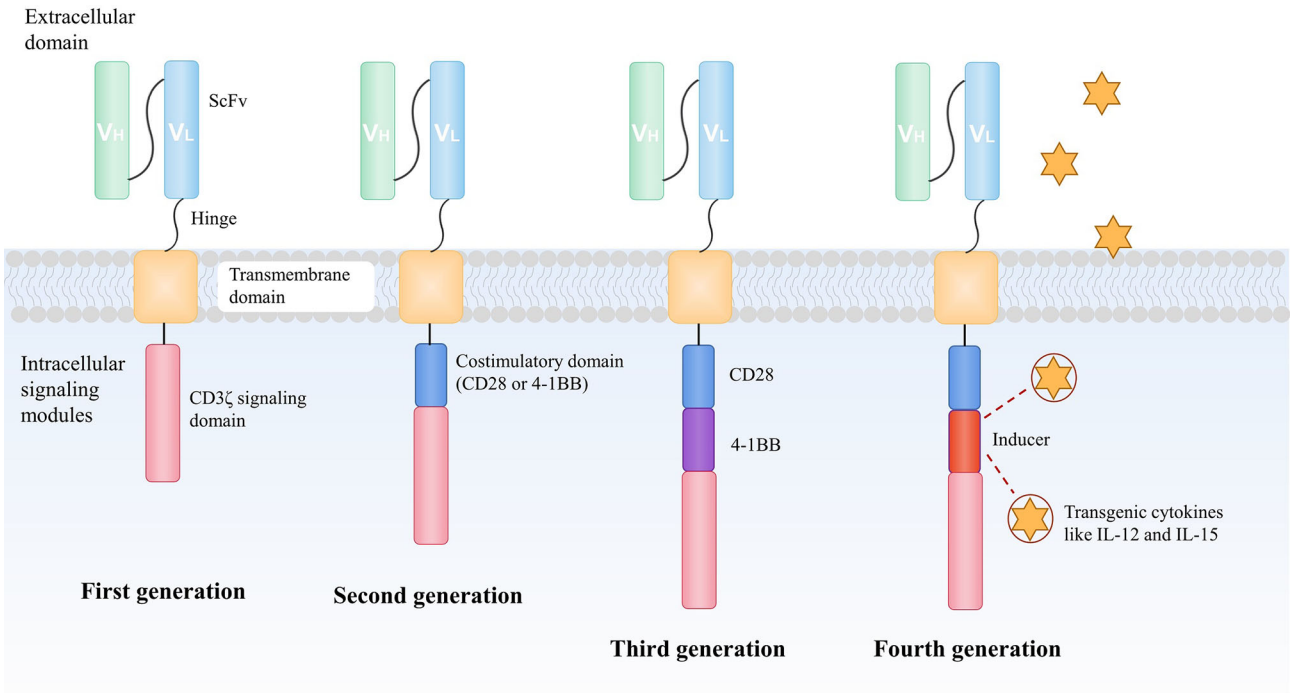

FIGURE 2 | The development of CAR construction. A CAR is composed of an extracellular antigen-binding domain, most of which is an antibody–derived scFV, a transmembrane domain and an intracellular signaling domain of the TCR CD3ζchain to activate T cells. To enhance the antitumor ability of CAR-T, the design of CARs has evolved over recent years. The second generation of CAR consists of an additional co-stimulatory domain, usually CD28 or 4-1BB (CD137) moieties to improve the capacity of persistence and proliferation of T cells. An extra co-stimulatory domain (CD28 and 4-1BB or TLR2) is added in the third-generation of CAR to further augment the efficacy of infused CAR-T cells. In the fourth generation of CAR, the intracellular segment of the cytokine receptor is also added to the CAR, which effectively promotes the expansion of T cells. CAR, chimeric antigen receptor; scFv, antibody-derived single-chain variable fragment; TCR, T cell receptor; TLR, toll-like receptor.

elevate the cytotoxicity and expansion of effector CD8+ T cells (24). NY-ESO-1 is the most broadly researched antigen with a panel of phase I/II clinical studies ongoing (NCT01567891, NCT03159585, NCT03691376, NCT03017131, NCT02869217). TBI-1301 is a cell product which is genetically modified to express NY-ESO-1 specific TCR. Butler et al. conducted a phase Ib clinical trial using TBI-1301 to treat HLA-A*02:01+ or A*02:06+ patients with NY-ESO-1+ solid cancers (NCT02869217). The ovarian patient had SD for 4.7 months and the standard dose infused was 5×10^9 (25). Another study used affinity enhanced autologous NY-ESO-1^{c259}T cells for treating HLA-A*02:01, *02:05, or *02:06 positive recurrent ovarian cancer (NCT01567891). However, so far, no objective tumor response has been recorded for 6 patients who completed the research.

Mesothelin (Msln) is another frontier antigen for ovarian cancer. Anderson et al. conducted a preclinical experiment with Msln specific TCR_{1045} T cells. These T cells exhibited tumor cytotoxicity both in $ID8_{VEGF}$ ovarian cancer cells and in murine model, but the function was on the wane within 21 days. To enhance the antitumor activity, engineered T cells were repeatedly infused to mice and a maintained effect was seen. The time to progression (TTP) for TCR_{1045} plus an irradiated peptide-pulsed splenocyte vaccine was longer than that of using T cells alone or no-treatment group (112 days, 91 days, 77 days) (26).

Findings for targeting mesothelin in CAR-T therapy are also of note. Haas et al. enrolled five patients with mesothelin expressing recurrent ovarian cancer in a phase I study (NCT02159716). The most significant result was seen in ovarian cancer among multiple mesothelin+ tumors involved. Patients received lentiviral transduced CART-meso cells with different doses: two were infused with $(1\sim3)\times10^8/m^2$ cells, and three were infused with $(1\sim3)\times10^7/m^2$ cells, both groups were evaluated as SD for 28 days. Although the function of tumor control was observed, these antitumor responses were transient and limited (27). A case of patient with refractory epithelial ovarian cancer after chemotherapy was reported recently. The patient received two infusions of CAR-Ts encoded by genes specific for mesothelin and the immune checkpoint inhibitors. An antiangiogenic drug inhibiting vascular endothelial growth factor receptor (VEGFR)-2 named apatinib was included in the treatment. The follow-up assessment showed partial response with attenuated diameter of liver metastatic nodules and a 17-month survival (NCT03615313). Only slight adverse reactions were observed (28). Zhao et al. revealed that humanized (hu)

CD19 specific CAR had 6-fold higher affinity compared with murine CAR (29). Murine CAR has different structure domains which tend to trigger adaptive immunity. Once immune recognition of murine scFv is established, the therapeutic effect would be considerably subdued. Improved strategy employing huCART-meso cells to treat cancers commonly express mesothelin is now recruiting candidates (NCT03054298). A research using the fourth generation CAR-Ts for refractory or relapsed ovarian cancer has just been initiated with outcomes remaining to be seen (NCT03814447).

Mucin 16 (MUC16) is a glycosylated mucin widely expressed in ovarian cancer, serving as a promising target for CAR-T therapy. A phase I clinical trial is ongoing with MUC-16ecto CAR-T cells to treat recurrent ovarian cancer (NCT02498912). 5 dose levels are planned for the assessment of the maximum tolerated dose (3×10^5, 1×10^6, 3×10^6, 1×10^7, 3×10^7). Furthermore, these CAR-T cells are modified to secrete IL-12, which could improve T cell persistence and overcome various inhibitions from the tumor microenvironment (30). Nectin is a class of cell adhesion molecule which belongs to the Ca^{2+}-independent immunoglobulin superfamily proteins. Nectin-4 is expressed in various organs during fetal development but barely expressed in adults other than placenta. In ovarian tumor tissues, nectin-4 is overexpressed and plays a key role in tumor cell adhesion, migration, aggregation and proliferation (31). Currently there is a phase I clinical trial using the CAR-T, which involves in various costimulatory domains and cytokines (IL-7 and CCL19, or IL-12) to treat nectin-4 positive ovarian cancer (NCT03932565). Recently, Garcia et al. provided evidence that T cells with CAR targeting Müllerian inhibiting substance type 2 receptor (MISIIR) were tumoricidal both *in vitro* and *in vivo* and no reaction was reported to normal primary human cells. Especially, MISIIR specific CAR-Ts lysed multiple human ovarian and other gynecologic cancer cells, showing potency in treating gynecologic malignancies in the clinic (32).

PRGN-3005 UltraCAR-T was engineered to express MUC-16, membrane bound IL-15 (mbIL-15) to promote persistence of T cells and the kill switch to ensure safety simultaneously. It was applied in a phase I clinical trial for patients with advanced and recurrent platinum-resistant ovarian cancer in 2019 (NCT03907527). This is a seminal gene and cellular therapy which owns a non-viral multigenetic transfer patent to produce UltraCAR-T cells without the need for *in vitro* proliferation, thus shortening the waiting period from several weeks to one day. This landmark study has the potential to allow the therapy accessible to common patients by reducing costs. It also holds promise for subverting the current pattern of CAR-T cell therapy by regulating the immune system and tumor targeting in a more precise fashion (33).

Studies have demonstrated that the combination of ACT and immune checkpoint inhibitor (Pembrolizumab and Nivolumab) can fight against T cell exhaustion induced by immune checkpoints and augment the antitumor activity in the treatment of advanced, recurrent or metastatic programmed cell death protein ligand 1 (PD-L1) expressing gynecologic malignancies (34). Accordingly, a programmed cell death protein 1 (PD-1) gene-knocked out transferred T cell product has been promoted recently *via* gene editing technology (CRISPR-Cas9, lentivirus technology, etc.). A phase I clinical study evaluating the safety and efficiency of PD-1 gene-knocked out CART-meso cells for treating mesothelin positive multiple solid tumors is currently ongoing (NCT03747965). A clinical trial of advanced refractory ovarian cancer using αPD-1 CART-meso cell therapy combined with apatinib was also observed with potential therapeutic effect, which is detailed mentioned above (NCT03615313).

Cervical Cancer

Cervical cancer is one of the most common gynecologic malignancies bothering middle-aged women, especially in developing countries. Although the incidence and mortality of cervical cancer have declined in recent years, the morbidity crowd tends to be younger, which is still worthy of vigilance (35).

The infection with high-risk human papillomavirus (HR-HPV) is a noted driver for the development of nearly all cervical cancers. E6 and E7 oncoproteins are highly expressed by HPV+ cervical cancer cells, becoming attractive therapeutic targets for engineered T cells. Preclinical research revealed that HPV-16 E6 (36)/E7 (37) specific TCR-Ts could detect and kill HLA-A2+ HPV-16+ tumor cells *in vitro* without cross-reactivity against human self-peptides. The antitumor avidity of E7 TCR-Ts against cervical cancer was also verified in a murine model.

A phase I/II study of HLA-A2 restricted E6 TCR-Ts for HPV-associated cancers (NCT02280811) was reported by Doran et al. Other interventions include common conditioning regimen, and systemic aldesleukin. Among 6 cervical cancer patients, 2 of them displayed SD, one for 6 months, another for 4 months. The percentage of E6 T cells in infused cells (range from $(1\sim170)\times10^9$) were 51% and 71% respectively. In the phase I portion, no severe adverse effects were observed (38). A first-in-human, phase I clinical trial of HLA-A2 restricted E7 TCR-Ts to treat patients with metastatic HPV-16+ cancers has just uploaded its report (NCT02858310). Two in five patients with cervical cancer displayed PR for 8 months and 3 months, with T cell portion in infused cells (range from $(1\sim107)\times10^9$) being 97% and 96%, respectively. One patient had SD for 3 months, and no response was observed in the remaining two patients. Researchers also proposed that genetic defects in the key elements of the antigen presentation and interferon response were responsible for treatment resistance of ACT (39). Some patients combined the PD-1 blockade therapy to improve T cell infiltration. In trial NCT03578406, five patients were treated with E6 TCR-T monotherapy: two of them received 5×10^6/kg dose and three received 1×10^7/kg dose. 28 days later, three patients had SD, one patient had PD, one patient was loss to follow-up. In another arm, two patients were infused with 5×10^6/kg and 1×10^7/kg of anti-PD-1 TCR-Ts respectively. The patient with lower dose was assessed as SD at both day 28 and month 2 post-infusion, showing promising efficiency for combining engineered T cell therapy with immune checkpoint inhibitor for cervical cancer patients (40).

New therapeutic targets of CAR products have been widely expanded *via* several preclinical researches which have progressed to the stage of animal experiments. CD47 specific CAR-Ts were proved to effectively kill ovarian, pancreatic, and cervical cancer cell lines and retard pancreatic tumor growth in mice (41). Recently, the antitumor efficiency of CART-meso cells was illustrated in SiHa cells *in vitro* by elevated levels of IL-4、IL-2、IL-5、tumor necrosis factor (TNF) α and interferon (IFN) γ secretion. The capacity in tumor control sustained for about 1 week *in vivo*. Better results were obtained following the second injection of T cells (42). Positive responses were also observed in Hela, SiHa, ME-180 and C-33A cell lines and in murine models through natural killer group 2D (NKG2D)/NKG2D-ligand pathway (43).

Currently, a phase I/II study of CART-meso cells in treating metastatic cancers including cervical cancer and ovarian cancer has been terminated with only one patient assessed as SD for > 3.5 months (NCT01583686). There is an ongoing phase I/II clinical trial using CARs targeting antigens such as GD2, prostate specific membrane antigen (PSMA), MUC-1, mesothelin or other markers positive to cervical cancer (NCT03356795). CD22 is often selected as the target for B cell malignancy. Recently, a phase I study employed CD22 specific CAR-Ts to treat solid tumors, including cervical cancer (NCT04556669). They also introduced the anti-PD-L1 monoclonal antibody to the CAR structure. More clinical evidence regarding the efficiency of CAR-T therapy for cervical cancer is required.

Endometrial Cancer

Endometrial cancer (EC) is the sixth most common cancer in women, and this ranking may rise especially in western countries (44). Although the 5-year survival rate of patients in the early stage is 95%, it would sharply decrease to 16% to patients with advanced or recurrent metastatic tumors (45).

There are not enough reports for the clinical assessment of ACT in EC until now. Only one patient treated with 5×10^9 TBI-1301 showed SD for 3.6 months without cytokine release syndrome (CRS) in a phase Ib clinical trial which has been mentioned above (NCT02869217). On 13 Nov 2020, a phase I/II clinical trial has just been initiated using CAR-Ts targeting alkaline phosphatase, placental (ALPP) for endometrial cancer and ovarian cancer (NCT04627740). The primary outcome measures related adverse events and the secondary outcome measures ORR, progression-free survival (PFS) and the number of transferred T cells.

Vulvar Squamous Cell Carcinoma

High-grade squamous intraepithelial lesion (HSIL) is a precancerous lesion of vulvar squamous cell carcinoma (VSCC) caused by HPV infection (46). The risk of cancer development can be reduced by treating HSIL. TCR-Ts targeting HPV-16 E6 protein thus provide a therapeutic window for HSIL to further prevent VSCC. A related phase I clinical trial was closed due to the lack of perceived clinical activity observed in the study (NCT03197025). A phase II study of HPV-16 E7 TCR-Ts for treating HSIL was also terminated without concrete results (NCT03937791). In a clinical study of E7 specific TCR-Ts mentioned above, vulvar diseases are included (NCT02858310).

THE CHALLENGES WITH ENGINEERED T CELLS IN GYNECOLOGIC ONCOLOGY

Several challenges become apparent when it comes to the promotion of engineered T cells. The major concern with this therapy is the severe adverse effect. TAAs can also be expressed by normal tissues, causing undesired on-target/off-tumor toxicity. CD19 CAR-Ts could induce the deficiency of normal CD19+B cells and cause weakened immunity. Besides, some TCRs or CARs are not specific to target antigen, but cross-react to other self-antigens. Taking MAGE-A3 specific TCR-Ts as an example, in previous studies, there were fatal events associated with injury in MAGE-A13 expressing tissues like the nervous system (47) and titin of cardiac cells (48, 49). MAGE-A13 was marginally expressed but unexpected and deadly destructive. Antigen selection is the first consideration in designing an ACT protocol. It is critical to choose ideal antigens that are tumor-specific, carcinogenic and immunogenic in order to strengthen the antitumor efficiency and reduce related toxicity simultaneously. In clinical trials using TCR-Ts to treat gynecologic malignancies, the target antigens involve: HPV16-E6/E7, NY-ESO-1, MAGE-A3, MAGE-A4, mesothelin. Antigens used as CAR-T therapeutic targets include: mesothelin, CD70, CD22, CD133, GD2, PSMA, MUC1, MUC16, human epidermal growth factor receptor 2 (HER-2), nectin-4, anti-alpha folate receptor (FR-α), ALPP, B7-H3, TnMUC1 (**Table 1**). In recent years, neoantigens have also emerged as a potential therapeutic option for gynecologic tumors since they are induced by somatic point mutations in tumor cells instead of co-expression with normal tissues. Matsuda et al. have successfully generated 3 neoantigen-specific TCRs through whole-exome sequencing (WES) of 7 ovarian tumors and the induction of peripheral blood mononuclear cells (PBMCs) isolated from healthy donors. These T cells could recognize their corresponding neoantigens although cross-reactivity to the wild-type peptide was observed in one of them (50). As an infant in the field of immunotherapy, it warrants further investigation whether these neoantigens will continue to be stably expressed by tumor cells.

CRS is another common threat particularly for CAR-T treatment. The excessive stress reaction of immune system would release superabundant cytokines such as TNF-α、IL-1、IL-6、IL-12、IFN-α、IFN-γ, leading to systemic inflammatory response syndrome (SIRS) and multiple organ failure. Grade 3 and 4 CRS can be life-threatening. In a multicenter clinical trial using CD19 CAR-Ts to treat refractory diffuse large B-cell lymphoma, 20% patients had grade ≥3 CRS events. More seriously, a rare case of fulminant haemophagocytic lymphohistiocytosis was reported (51). In another trial of CD19 CAR-Ts treating refractory ALL, 3 cases

TABLE 1 | Clinical trials of engineered T cells in gynecologic cancer immunotherapy (www.clinicaltrails.com).

Cancer	Type	antigen	Stage and Result	Host	NCT
Ovarian cancer	TCR-T	MAGE-A4	Phase I (recruiting) 7 pts had PR, 11 had SD, 5 had PD	University of Miami, USA	NCT03132922
	TCR-T	NY-ESO-1	Phase IIa (completed with results) No objective effects have been reported	City of Hope National Medical Center, USA	NCT01567891
	TCR-T	NY-ESO-1	Phase I (completed without results)	Zhujiang Hospital of Southern Mediacal University, China	NCT03159585
	TCR-T	NY-ESO-1	Phase I (recruiting)	Roswell Park Cancer Institute, USA	NCT03691376
	TCR-T	NY-ESO-1	Phase I (active, not recruiting)	Roswell Park Cancer Institute, USA	NCT03017131
	TCR-T	NY-ESO-1	Phase Ib (recruiting) One patient had SD for 4.7m with grade 2 CRS	Princess Margaret Cancer Centre, Canada	NCT02869217
	TCR-T	NY-ESO-1	Phase I (unknown)	Shenzhen Second People's Hospital, China	NCT02457650
	TCR-T	Neoantigen	Phase II (suspended)	National Institutes of Health Clinical Center, USA	NCT04102436
	TCR-T	Neoantigen	Phase II (suspended)	National Institutes of Health Clinical Center, USA	NCT03412877
	CAR-T	Mesothelin	Phase I (completed with results) Five patients had SD for 28 days	Abramson Cancer Center of the University of Pennsylvania, USA	NCT02159716
	Hu CAR-T	Mesothelin	Phase I (recruiting)	University of Pennsylvania, USA	NCT03054298
	CAR-T	Mesothelin	Early Phase I (recruiting)	Shanghai 6th People's Hospital, China	NCT03814447
	CAR-T	Mesothelin	Phase I (terminated) Only one patient had SD for > 3.5m	National Institutes of Health Clinical Center, USA	NCT01583686
	CAR-T	Mesothelin	Phase I/II (recruiting)	The Second Affiliated hospital of Zhejiang University School of Medicine, China	NCT03916679
	CAR-T	Mesothelin	Early Phase I (recruiting)	The Second Affiliated hospital of Zhejiang University School of Medicine, China	NCT03799913
	CAR-T	Mesothelin	Phase I (recruiting)	Shanghai East Hospital, China	NCT04562298
	CAR-T	Mesothelin	Phase I (Active, not recruiting)	National Cancer Institute, USA	NCT03608618
	CAR-T	Mesothelin	Phase I (unknown)	Biotherapeutic Department and Pediatrics Department of Chinese PLA General Hospital	NCT02580747
	αPD1-CAR T	Mesothelin	Early Phase I (recruiting)	Shanghai 10th people's Hospital, China	NCT04503980
	αPD1-CAR T	Mesothelin	Phase I/II (recruiting)	Shanghai Cell Therapy Research Institute.	NCT03615313
	CAR-T	MUC16	Phase I (active, not recruiting)	Memorial Sloan Kettering Cancer Center, USA	NCT02498912
	CAR-T	Nectin4/FAP	Phase I (recruiting)	The Sixth Affiliated Hospital of Wenzhou Medical University, China	NCT03932565
	UltraCAR-T	MUC16	Phase I (recruiting)	Fred Hutch/University of Washington Cancer Consortium, USA	NCT03907527
	CAR-T	B7-H3	Phase I (not yet recruiting)	Lineberger Comprehensive Cancer Center, USA	NCT04670068
	CAR-T	ALPP	Phase I/II (not yet recruiting)	Xinqiao Hospital of Chongqing, China	NCT04627740
	CAR-T	FRα	Phase I (recruiting)	University of Pennsylvania Health System, USA	NCT03585764
	CAR-T	CD133	Phase I (completed without results)	Biotherapeutic Department and Pediatrics Department of Chinese PLA General Hospital	NCT02541370
	CAR-T	HER-2	Phase I (recruiting)	Zhongshan Hospital Affiliated to Fudan University, China	NCT04511871
	CAR-T	HER-2	Phase I/II (withdrawn)	Southwest Hospital of Third Millitary Medical University, China	NCT02713984
	CAR-T	CD70	Phase I/II (suspended)	National Institutes of Health Clinical Center, USA	NCT02830724
	CAR-T	TnMUC1	Phase I (recruiting)	The Angeles Clinic and Research Institute, USA	NCT04025216
Cervical cancer	TCR-T	HPV-E6	Phase I/II (completed with results) One patient had SD for 6m, one had SD for 4m	National Institutes of Health Clinical Center, USA	NCT02280811
	αPD1-TCR T	HPV-E6	Phase I (recruiting) Enhanced SD in combination with anti-PD-1 therapy	Qingzhu Jia, Chongqing, China	NCT03578406
	TCR-T	HPV-E7	Phase I/II (recruiting)	National Institutes of Health Clinical Center, USA	NCT02858310
	TCR-T	HPV-E7	Early Phase I (suspended)	National Institutes of Health Clinical Center, USA	NCT04476251
	TCR-T	HPV-E7	Phase I (withdrawn)	National Institutes of Health Clinical Center, USA	NCT04411134
	TCR-CD4+ T	MAGE-A3	Phase I/II (active, not recruiting) One patient had CR for > 29m	National Institutes of Health Clinical Center, USA	NCT02111850
	TCR-T	MAGE-A3	Phase I/II (terminated) One patient had PR after 6w and 12w	National Institutes of Health Clinical Cente, USA	NCT02153905
	CAR-T	Mesothelin	Phase I (terminated)	National Institutes of Health Clinical Center, USA	NCT01583686

(Continued)

TABLE 1 | Continued

Cancer	Type	antigen	Stage and Result	Host	NCT
			Only one patient had SD for > 3.5m		
	αPD1-CAR-T	CD22	Phase I (recruiting)	Fourth Hospital of Hebei Medical University, China	NCT04556669
	CAR-T	GD2, PSMA, MUC1, Msln	Phase I/II (recruiting)	Shenzhen Geno-immune Medical Institute, China	NCT03356795
Endometrial cancer	CAR-T	Mesothelin	Phase I (unknown)	Biotherapeutic Department and Pediatrics Department of Chinese PLA General Hospital	NCT02580747
	CAR-T	ALPP	Phase I/II (not yet recruiting)	Xinqiao Hospital of Chongqing, China	NCT04627740
Vulvar squamous cell carcinoma	TCR-T	HPV-E6	Phase I (terminated)	National Institutes of Health Clinical Center, USA	NCT03197025
	TCR-T	HPV-E7	Phase II (terminated)	National Institutes of Health Clinical Center, USA	NCT03937791
	TCR-T	HPV-E7	Phase I/II (recruiting)	National Institutes of Health Clinical Center, USA	NCT02858310

ALPP, alkaline phosphatase, placental; CAR, chimeric antigen receptor; CR, complete response; CRS, cytokine release syndrome; FAP, fibroblast activation protein; FRα, anti-alpha folate receptor; HER-2, human epidermal growth factor receptor 2; HPV, human papillomavirus; MAGE-A, melanoma-associated antigen; Msln, mesothelin; MUC16, mucin 16; NY-ESO-1, New York esophageal squamous cell carcinoma 1; PD, progressive disease; PD-1, programmed cell death protein 1; PR, partial response; PSMA, prostate specific membrane antigen; SD, stable disease; TCR, T cell receptor.

of death induced by refractory CRS were reported (52). Management methods of CRS include: monoclonal antibodies against IL-6 (siltuximab, clazakizumab) and its receptor (tocilizumab), IL-1 receptor (anakinra), glucocorticoids, alemtuzumab and etc (53). In trial NCT02869217, the patient with ovarian cancer had grade 2 CRS which required tocilizumab to manage.

Tumor heterogeneity is reflected in different sites of the same tumor or its recurrent lesion, being responsible for antigen escape. The loss of target antigen after ACT represents a key mechanism in the recurrence of tumor. Unfavorable feedback has been obtained from CD19-negative relapses. In up to 60% patients with refractory ALL, relapses after receiving CD19 CAR-T therapy could happen due to the loss of CD19 antigen. Once the antigen load is insufficient to activate immunoreaction, patients would become resistant to CAR-T therapy. Efforts were made to overcome this obstacle through establishing a dual CAR-T which could combine an additional antigen like CD123, a stem cell marker expressed in CD19-negative relapses, to prevent possible antigen loss (54).

The immunosuppressive microenvironment is a contributing factor to the proliferation, metastasis and drug resistance of gynecologic tumor cells. Particularly, abdominal cavity metastasis is a common pathological feature of ovarian cancer, and the formation of ascitic fluid provides a favorable microenvironment for affecting tumor growth and invasiveness. It promotes vascular and lymphangiogenesis in tumor tissues and enables tumor cells to evade immune surveillance *via* several pathways: (1) offering ligands for immune checkpoint proteins, such as PD-1 and cytotoxic T lymphocyte associate protein-4 (CTLA-4); (2) providing an immune suppressive setting through cytokines such as IL-10, IL-6, TGF-β vascular endothelial growth factor (VEGF) and so on, extracellular matrix components like matrix metalloproteinases (MMPs) or suppressive cells such as myeloid-derived suppressor cells (MDSCs) and regulatory T cells (Tregs); (3) interaction with multiple active substances in stromal cells, such as tumor-associated macrophages (TAMs), cancer-associated fibroblasts (CAFs), and endothelial cells; (4) creating a physically and chemically hostile metabolic environment that is hypoxia, glucose-deficient, acidic, full of indolamine-1-oxidase and arginase (55).

The application of CAR-T therapy has long been constrained with unsatisfactory results in solid tumors including gynecologic tumors. A major hindrance for the broader use of CAR-Ts is attributed to the resistance of tumor microenvironment. Researchers found that by expressing IL-7 and CCL19 in CAR-Ts in mice, the immune cell infiltration in tumor tissues increased, thus reinforcing antitumor effects (56). In addition, chemokines e.g. CCR2b (57) and CCR4 (58) are factors affecting the progression and metastasis of tumor. Conversely, they can also facilitate the tumor infiltration of CAR-Ts when co-expressed with T lymphocytes. Although attempts in the combination of immune checkpoint blockades and ACT seem to make reversing the inhibitory microenvironment a reality, this strategy is still flawed due to neglect of the systemic network comprised of multiple immune suppressive mechanisms. A more concentrated attack on solid tumors is to use lipid nanoparticles to ferry immune-modulatory agents that are pertinently combined into components of tumor microenvironment. Compared with monotherapy, the level of TAMs, MDSCs and Tregs all reduced (9.4-fold, 4.6-fold, 4.8-fold), and the concentration of antitumor cells like CD8+ T cells and invariant natural killer T cells (iNKTs) increased (6.2-fold, 29.8-fold) (59). It seems to be a promising method with less cost, labor and fewer adverse effects.

The transient persistence of transferred T cells also makes it challenging to achieve optimal clinical results. Increasing the number of long-term memory T cells is a feasible way in obtaining sustained immunity. Stem memory T cells (Tscm) are superiorly potential in self-renewal, proliferation and long-last existence compared with T cells in other stages (60). Exploring approaches to induce Tscm-like T cells has been a hot spot of tumor immunology in recent years. Productive methods include cancer vaccines with regulated TCR signaling (61), co-culture with cytokines like IL-7, IL-15, IL-21 (62), and the addition of co-stimulation domains (63).

THE FUTURE OF ENGINEERED T CELLS IN THE FIELD OF GYNECOLOGIC TUMORS

An essential contributing factor for the broader application of engineered ACT technology is a systematically manufactured process. The whole process should be strictly controlled with quality testing to obviate contamination and satisfy clinical demand. Although multiple CAR-T agents have been permitted into the market, the preparation of T cells before treatment is still performed in a personalized pattern, which is time-consuming for 12 days in average with small scale (64). The protocol is now embracing a more automatic and universal fashion called 'off-the-shelf' ACT manufacture using allogenic T cells that are modified to be mildly immunoreactive to the host (65). Importantly, the depletion of allogeneic TCR, class I HLA molecule of donor T cells with CRISPR-Cas9 system would make 'off-the-shelf' CAR-Ts come true by reducing the risk of graft-versus-host disease (GVHD) (66).

The efficiency of engineered T cells in treating gynecologic tumors is currently not fully supported by sufficient clinical data and warrants further attempts in the clinical setting. Efforts to break barriers discussed above such as antigen selection, toxicities, the immune-unfavorable microenvironment in gynecologic tumors, the persistence of infused cells are making headway. Future investigation should provide update on these topics: (1) carrying forward clinical and preclinical trials; (2) more appropriate antigen binding sites; (3) how to break barriers to produce engineered T cell in a larger scale without toxicity; (4) how to maintain the cytotoxicity of engineered T cells in the tumor microenvironment; (5) synergistic treatment with immune checkpoint inhibitors or other substances. With further work to be done and deeper understanding of ACT, it would present a potential treatment for gynecologic oncology.

Another direction in engineered ACT technology is using natural killer (NK) cells as an alternative to T cells. NK cells have been proved to be safer in terms of CRS and GVHD risks than modified T cells with insensitivity to MHC and the presence of inhibitory receptor as a safety switch (67). A phase I study using mesothelin specific CAR-NK cells to treat epithelial ovarian cancer is ongoing (NCT03692637).

SUMMARY

Engineered T cells therapy for gynecologic cancer would inevitably face the existence of practical challenges such as safety concerns, difficult choices of appropriate antigen, the immunosuppressive tumor microenvironment, the short pharmacological duration and high finical cost. Based on a substantial number of preclinical researches with various models, series of phase I/II clinical trials are exploring the optimal route and dosage of ACT products, or whether a combination with surgery, radiotherapy, chemotherapy, or other immunotherapies would facilitate the treatment of malignant gynecologic tumors with decreased recurrence and metastasis rate, reduced adverse drug reactions, and improved life quality of patients.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication.

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REVIEW
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Advances in Adoptive Cell Therapy Using Induced Pluripotent Stem Cell-Derived T Cells

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Adoptive cell therapy (ACT) using chimeric antigen receptor (CAR) T cells holds impressive clinical outcomes especially in patients who are refractory to other kinds of therapy. However, many challenges hinder its clinical applications. For example, patients who undergo chemotherapy usually have an insufficient number of autologous T cells due to lymphopenia. Long-term *ex vivo* expansion can result in T cell exhaustion, which reduces the effector function. There is also a batch-to-batch variation during the manufacturing process, making it difficult to standardize and validate the cell products. In addition, the process is labor-intensive and costly. Generation of universal off-the-shelf CAR T cells, which can be broadly given to any patient, prepared in advance and ready to use, would be ideal and more cost-effective. Human induced pluripotent stem cells (iPSCs) provide a renewable source of cells that can be genetically engineered and differentiated into immune cells with enhanced anti-tumor cytotoxicity. This review describes basic knowledge of T cell biology, applications in ACT, the use of iPSCs as a new source of T cells and current differentiation strategies used to generate T cells as well as recent advances in genome engineering to produce next-generation off-the-shelf T cells with improved effector functions. We also discuss challenges in the field and future perspectives toward the final universal off-the-shelf immunotherapeutic products.

Keywords: adoptive cell therapy, induced pluripotent stem cells, T cells, chimeric antigen receptor, tumor infiltrating lymphocytes, cancer immunotherapy, off-the-shelf T cells

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INTRODUCTION

Adoptive cell therapy (ACT) of T lymphocytes offers a potential therapy for chronic viral infection and cancers. ACT can be achieved by isolating T cells from the excised tumor mass (tumor infiltrating lymphocytes or TILs), *ex vivo* expanding and reinfusing them into the patient to target viral or tumor antigens (1, 2). However, the process of TIL isolation and expansion limits their clinical applications since it is technically difficult, labor-intensive, costly and difficult to standardize. TILs do not often provide potent anti-tumor effects due to exhaustion of T cells. In addition, identification of antigen-specific T cells in other solid tumors is very challenging (3). To improve specificity and cytotoxicity of ACT, genetic engineering approaches to target the antigens by transduction of antigen-specific T cell receptor (TCR) or chimeric antigen receptor (CAR) gene can be performed. The engineered T cells are then expanded and reinfused into the patient after

lymphodepletion. The TCR-engineered T cells recognize target antigens, which are processed within the cytoplasm and presented by specific human leukocyte antigen (HLA) or major histocompatibility complex (MHC) class I molecules on the surface of the viral-infected cells or cancer cells (4). Several studies reported the use of TCR-engineered T cells to treat patients including NY-ESO-1-directed tTCR and MAGE-A3-directed tTCR for multiple myeloma (MM) (5, 6), and WT1-directed tTCR for acute myeloid leukemia (AML) (7). However, ACT using TCR-engineered T cells is limited by the need to engineer TCR specific for antigen and MHC molecules of the patient.

In contrast, antigen recognition by CAR is mediated by a synthetic hybrid receptor composed of an extracellular antigen-recognition domain, which is a single-chain variable fragment (scFv) derived from the variable regions of a monoclonal antibody (mAb), a transmembrane (TM) domain and intracellular signaling domains such as TCR-derived CD3ζ and co-stimulatory domains (CD28 or 4-1BB) (8). Unlike TCR-engineered T cells, CAR T cells can recognize a specific antigen and eliminate the tumor cells in an HLA-independent manner, therefore, enhancing therapeutic outcomes. Clinical trials using CAR T cell therapy showed a long-term remission in both hematological malignancies and solid tumors (9, 10). To date, the US FDA approved four CD19-directed CAR T cell products: Kymriah™ in 2017 and Yescarta™ in 2018, Tecartus™ in 2020, and recently Breyanzi® in 2021, for the treatment of relapsed or refractory B cell malignancies (1, 2). Despite its remarkable success, ACT using autologous TCR- or CAR-engineered T cells has some unavoidable limitations. The ACT therapy relies on personalized manufacture, which proves very challenging in terms of time and cost to manufacture T cells thereby restrictive for large-scale clinical applications. Moreover, it is also technically difficult to obtain sufficient number of autologous T cells from lymphopenic patients who are heavily pretreated with chemotherapy, or immunodeficient patients, to generate a clinically relevant dose of T cells for therapy (3, 11). In order to obtain sufficient number of cytotoxic T cells (CTLs) for ACT, *ex vivo* expansion to enrich the number of CTLs is required before infusion. This process involves several stimulation steps using various cytokines to increase T cell proliferation. Long-term culture can drive CTLs into an "exhausted state", where CTLs have shortened telomere length, and lose proliferative capacity and effector function, which hinder their clinical practicality (4, 5).

One way to generate an unlimited supply of universal allogeneic CAR T cells for cancer immunotherapy is to use induced pluripotent stem cells (iPSCs) as a starting material. Advances in iPSC technology have made the generation of autologous pluripotent stem cells (PSCs) possible. These cells have unlimited proliferation and can be differentiated into all specialized cell types of the body; therefore, they represent an autologous renewable cell source for regenerative medicine. iPSCs can be derived from various somatic cell sources, mainly skin fibroblasts and peripheral blood, by introducing the Yamanaka factors (OCT4, SOX2, KLF4 and c-MYC) (6, 7). One of the useful applications of iPSCs in regenerative medicine is the production of CTLs for viral or cancer immunotherapy. Previous studies demonstrated that iPSCs generated from T cells retained rearranged TCR genes. Upon differentiation toward T cell lineage, the iPSC-derived T cells re-expressed the same TCR as those of the parental T cells (8, 9). In addition, iPSCs are amenable to genetic modification, so it is possible to engineer the cells to have enhanced specificity and effector functions. Since iPSCs can be expanded unlimitedly, clinical-scale quantities of T cells with the desired antigen specificity can be manufactured. In this review, we provide the basic knowledge and recent advances of iPSC-derived T cell generation for clinical applications starting from the initial cell source for iPSC generation to the applications of iPSC-derived T cell products for cell-based therapy. In addition, we summarize future directions and challenges towards the final universal, off-the-shelf immunotherapeutic products.

T CELL BIOLOGY AND APPLICATIONS IN ACT

T cells play an essential role in the host defense mechanism against pathogens and cancers. They can be distinguished from other types of lymphocytes by the expression of TCR, which binds to the foreign antigen presented on the MHC. This interaction induces the release of cytotoxic granules and expression of Fas-ligand, which results in the target cell apoptosis (10). T cells originate from hematopoietic stem cells (HSCs), which give rise to all blood cell lineages. HSCs in the bone marrow differentiate into common myeloid progenitors (CMPs), which produce granulocyte-macrophage progenitors (GMPs) and megakaryocyte-erythroid progenitors (MEPs), or common lymphoid progenitors (CLPs), which produce lymphoid cells (12). T cell development occurs after CLPs from the bone marrow migrate into the thymus *via* the bloodstream. In the thymus, CLPs receive the Notch signal from cortical thymic epithelial cells (cTECs). During the first step of T cell development, the Notch signal stimulates CLPs to commit to double-negative (DN) cells ($CD8^-/CD4^-$) (13), which can be divided into four subpopulations (DN1 to DN4) based on the expression of CD25 and CD44 (14). From the DN1 to DN4 stages, the precursor cells undergo TCR rearrangement mediated by RAG protein to generate TCR. TCRs are randomly generated and are unique for each precursor cell. After successful TCR rearrangement, the DN4 cells express both co-receptors, CD4 and CD8 (double-positive (DP) cells). During this step, the DP cells undergo a positive selection in the cortex; the DP cells expressing TCRs that are able to bind to MHC molecules plus self-antigens on the cTEC surface with appropriate affinity will be retained (15). The outcomes of the positive selection depend on the signals from TCRs and the co-receptors (CD4 or CD8). If the DP cells have TCRs that are able to bind to MHC class II of cTECs, the DP cells will become CD4 single-positive (SP) cells by downregulating CD8 expression. On the other hand, if the DP cells have TCRs that fit the MHC class I molecule, the DP cells

will downregulate the expression of CD4 and become CD8 SP cells. The DP cells that receive too low TCR signals or no TCR signals for self-antigen-MHC molecules will undergo apoptosis to prevent the generation of useless T cells (16).

Apart from positive selection, TECs also involve in negative selection, the process to eradicate the autoreactive T cells. In this process, the SP cells migrate to the medullar of the thymus where the SP cells encounter more diverse self-antigen MHC provided by medullary thymic epithelial cells (mTECs) and dendritic cells. The SP cells that bind with high affinity to the self-antigen will be eliminated from TCR repertoires by apoptosis (17). The process of negative selection generates mature T cells with a highly diverse TCR repertoire and self-tolerance to enter the bloodstream and circulate to peripheral tissues in response to pathogens (18). The newly generated T cells are considered naïve T cells at this stage because they have not been exposed to an antigen. When the naïve T cells interact with an antigen-presenting cell showing the MHC/peptide complex that can specifically bind to their TCR, T cell activation is initiated. This activation triggers the proliferation of the naïve T cell clone and differentiates the naïve T cells into the effector T cells. During this period, $CD4^+$ and $CD8^+$ T cells exhibit inflammatory cytokine secretion and cytotoxicity toward the transformed cells or infected cells, respectively. If the pathogen is successfully eliminated, the majority of effector T cells will die while the surviving effector T cells will be differentiated further to the memory cells. These cells are inactive and maintained for long-term immunity (19).

In 1987, Rosenberg and colleagues reported the first ACT using TILs to treat patients with metastatic malignant melanoma. TILs were expanded by *in vitro* culture in the presence of recombinant interleukin 2 (IL-2) and transfused into the patients to treat melanoma. The results demonstrated that TILs had autologous tumor-specific cytotoxicity; in addition, TILs from some patients also had limited capacity to kill allogeneic fresh tumor targets suggesting that adoptive transfer of TILs could be a potential approach for the treatment of cancer patients (20). In 1994, a larger number of patients with metastatic melanoma were treated with autologous TILs with IL-2, with or without the administration of cyclophosphamide. However, the results demonstrated that only 5 of 29 patients had complete responses (21). It was subsequently shown that lymphodepletion prior to ACT increased the complete response rate of the therapy (22, 23), and this finding led to a breakthrough in ACT against melanoma. However, TIL treatments in some types of solid cancer, such as breast cancer or cholangiocarcinoma, are not as effective as in melanomas (24), and the number of TILs is often insufficient for the treatment. To enhance the specificity of T cells and efficacy of ACT, TILs from the patients were transduced with transgenic TCR (25). These engineered TILs simultaneously react with two different antigens. Previous studies showed that the infusion of NY-ESO1 TCR-engineered T cells resulted in tumor regression in melanoma and synovial sarcoma patients (26, 27). Although genetic-engineered T cells have been developed against many antigens, their TCRs must bind to the tumor antigen presented on the HLA class I molecule to mediate the specific killing effect. This process often results in poor treatment efficacy since tumors can downregulate HLA class I molecules and co-stimulatory molecules (28, 29). To overcome this problem, CAR technology has been developed. The first generation of CAR invented in 1989 (30, 31) comprises the scFv from the antibody fused with the transmembrane domain of TCR, which contains the transduction signal, CD3ζ chain. In the second and third generations of CAR, the co-stimulatory domains derived from CD28, 4-1BB, or OX40 are added to enhance T cell activation and improve CAR T cell function against the tumors that do not express co-stimulatory molecules (32).

Although the clinical outcomes of CAR T cell therapy have been very impressive, the manufacturing costs for a single infusion of these novel therapies are very costly: $475,000 for Kymriah and $373,000 for Yescarta, making them inaccessible to most patients (33, 34). These prices do not include the hospitalization fees; therefore, the cost for the treatment needs to be reduced in order to make it economically practical and accessible to most cancer patients. Another important limitation of ACT is to find a healthy HLA-matched donor; therefore, some transplant centers focus on developing third-party T cell banks from common HLA donors (35). Other efforts have been made to generate universal allogeneic CAR T cells, which utilize healthy donor T cells for CAR and TCR engineering to increase antigen specificity and avoid graft-versus-host disease (GvHD), respectively (36–41). The treatment using these universal allogeneic CD19 CAR T cells (UCART19) demonstrated great success in two pediatric patients with acute lymphoblastic leukemia (ALL) (40). Recently, the successful results from two multicenter phase 1 studies using UCART19 in patients with relapsed and/or refractory B-ALL emphasize the potential of CAR T cells to induce complete remission in 67% of patients, even in the patients with high disease burden (42). However, there are some concerns regarding the manufacturing process; prolonged *ex vivo* culture can cause T cell exhaustion and reduced effector functions. In addition, there is also batch-to-batch variability during the manufacturing process. Therefore, clinical studies with larger cohorts are required to validate allogeneic CAR T cells (43).

INDUCED PLURIPOTENT STEM CELLS AS A NEW CELL SOURCE FOR ACT

Although ACT of functional CTLs has offered a potential therapy for viral infection and cancers, the *ex vivo* expansion of autologous T cells has proved very challenging. This problem can be overcome by regenerating antigen-specific CTLs through iPSC reprogramming. Previous studies demonstrated that iPSC-derived CTLs could be expanded from 100-fold to 1,000-fold within two weeks of culture compared to 20-fold of the original T cells. These regenerated CTLs also exhibited higher telomerase activity and longer telomere length than the original T cells.

Furthermore, the marker of exhausted T cells, PD-1, was not expressed, whereas the markers of central memory T cells, CCR7, CD27 and CD28, were co-expressed (9). In a more recent study, the regenerated CD8αβ CTLs were expanded up to 10,000-fold and changed their phenotype from a naïve to an effector/memory profile. In this study, 10^4 iPSCs were used to generate 10^9-10^{10} CD8αβ CTLs sufficient for a single transfusion (44). Apart from the regeneration of CTLs, iPSCs also provide an unlimited cell source for other T cells subsets such as regulatory T cells (Tregs) (45). Tregs play a critical role in suppressing cell-mediated immunity leading to the maintenance of immunological tolerance. Patients with autoimmune disorders have been found to have lower levels of Tregs (46). Furthermore, patients with type 1 diabetes (T1D) also have a deficient number of Tregs (47). Therefore, the generation of a large number of functional Tregs followed by ACT to autoimmune patients is required to suppress the hyperactivity of autoreactive T cells. Due to a low frequency of Treg in peripheral blood (~1-2% in humans), several attempts have been made to generate Tregs from iPSCs for use in ACT. The first proof-of-concept study showed that mouse iPSC-derived Tregs could control the development of collagen-induced arthritis in the rheumatoid arthritis mouse model (48). Similarly, the mouse iPSC-derived Tregs could migrate to the pancreas and prevent the destruction of pancreatic β-cells by autoreactive T cells in the T1D mouse model (49). Therefore, a combination of iPSC technology with adoptive immunotherapy or CAR technology may provide a large number of T cells for future clinical applications.

Unlike other differentiated cell types, the generation of functional CTLs with a specific TCR from iPSCs depends significantly on the original somatic cell sources (**Figure 1**). When using non-T cell sources such as fibroblasts or keratinocytes as a somatic cell source, the derived iPSC clones bear the germline TCR gene. After T cell differentiation *in vitro*, the iPSC-derived T lymphocytes are generated with unpredictably rearranged TCR. This process recapitulates normal T cell development where sequential expression of CD7, cytoplasmic CD3, and surface CD3 was observed followed by TCR gene rearrangement of the γδ and αβ loci, respectively (50). However, without autologous TECs, positive and negative selection may not occur. Therefore, these iPSCs can only be used for studying normal T cell development and disease modeling; they are not suitable for clinical use due to the concern about autoreactive T cells. Apart from studying normal T cell development, disease-specific iPSCs can be generated from somatic cells (non-T cells) of patients with inherited diseases affecting the immune system such as X-linked Severe Combined Immunodeficiency (SCID-X1) with the Interleukin-2 receptor gamma chain (*IL-2Rγ*) mutation (51) or recombination-activating gene 1 (*RAG1*) mutations (52) to study abnormal T cell development in these disease models. Genetic correction in these disease-specific iPSCs using genome editing technologies such as TALENs or CRISPR/Cas9 systems with a subsequent *in vitro* differentiation also offers great potentials for future autologous therapy (51).

In 2011, Seki et al. developed the method for iPSC generation from mature human peripheral blood T cells using a Sendai viral vector to avoid transgene insertion (53). This method could generate iPSCs from a small amount (approximately 1 ml) of human peripheral blood samples (54). However, their method used fetal bovine serum (FBS) and mouse embryonic fibroblasts (MEF) as feeder cells, which result in contamination of xenogeneic antigens and zoonotic pathogens. In 2014, the generation of human iPSCs from peripheral blood T cells in a defined culture system was achieved using Sendai viral transduction and various combinations of chemically defined culture medium and coating matrices. For example, the combination of mTeSR1 medium and Matrigel resulted in the highest reprogramming efficiency (0.005%) (55). Overall, the reprogramming efficiencies under the feeder-free system are generally lower than those using the feeder cells. Even though the reprogramming efficiency using blood cells is lower than fibroblasts, blood cells are preferable because the isolation is minimally-invasive and easy to perform.

On the other hand, generation of iPSCs from T cells results in the pre-rearranged TCR gene in the iPSC clones. The rearranged TCR can eliminate the risk of autoreactive TCR since the T cells undergo positive and negative selection in the thymus. However, the specificity of TCR is unknown. In 2013, Themeli et al. reported the generation of CD19 CAR-engineered T-iPSCs that can efficiently be differentiated into CAR T cells against $CD19^+$ malignant B cells *in vitro*. These T-iPSC-derived CAR T cells displayed therapeutic activity by potently inhibiting tumor growth in a mouse model (56). Similarly, Minagawa et al. demonstrated that when the monocyte-derived iPSCs were transduced with a transgenic antigen-specific TCR, these cells exhibited a monoclonal expression of the transduced TCR after T cell differentiation *in vitro*. The iPSC-derived transgenic TCR T cells could also delay tumor progression in xenograft cancer models (57). These two studies showed that even though the iPSCs have no antigen-specific TCR, the specificity of iPSC-derived T cells can be achieved by transduction of CAR or transgenic TCR to generate therapeutic T cells for cancer immunotherapy.

After the concept of T cell production utilizing PSCs has been proposed, Watarai et al. utilized the nuclear transfer technique to reprogram NKT cells. The nuclear transfer ESCs bearing rearranged invariant Vα14-Jα18 TCRα gene were established from the mouse NKT cells (58). This study has proved that the rearranged TCR gene was retained throughout the reprogramming and differentiation process. Advances in the iPSC technology in 2006 led to reprogramming of $CD8^+$ T cells specific to $MART1^+$ melanoma using Sendai viral vectors carrying OSKM factors and SV40 large T antigen at MOI 30. Analysis of TCRα chain mRNA in the $CD8^+$ T cells generated from these iPSCs confirmed that the iPSC-derived $CD8^+$ T cells expressed the same TCRα chain gene as the parental MART1-specific T cells (8). Similarly, Nishimura et al. reported successful reprogramming of antigen-specific T cells into iPSCs. First, the transduction was performed using six retroviral vectors encoding OCT3/4, SOX2, KLF4, c-MYC, NANOG, and LIN28A; however, no iPSC-like colonies were observed. In the second attempt, the reprogramming was performed using the Sendai viral (SeV) vector system consisting of two Sendai viral vectors. The first

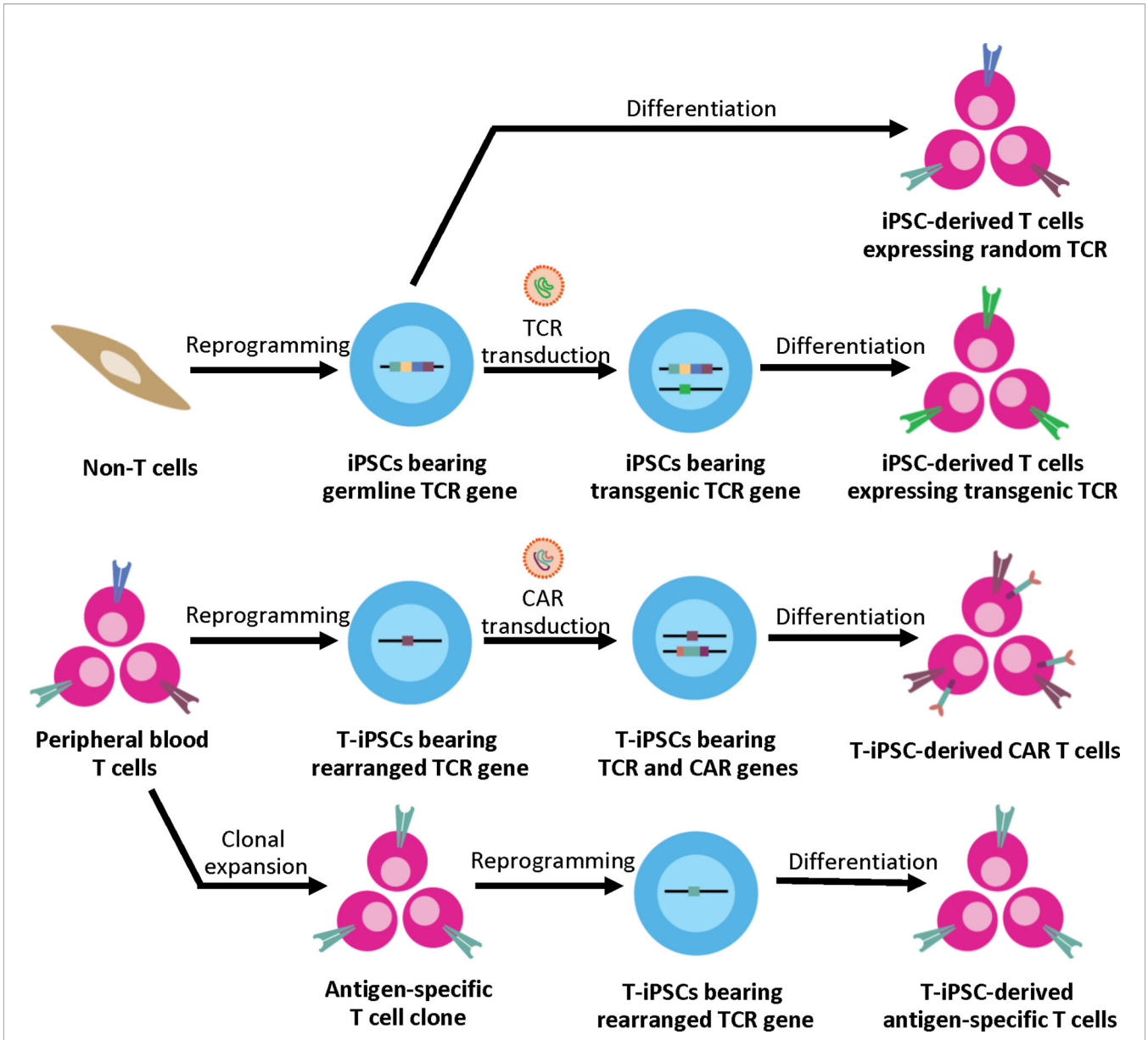

FIGURE 1 | Generation of iPSC-derived T cells from different somatic cell sources. Non-T cell sources contain germline TCR gene, upon T cell differentiation, the iPSC-derived T cells express random TCR. These T cells can be used for studying normal T cell development and disease modeling. For applications in ACT, the exogenous TCR can be introduced to the iPSCs. Upon T cell differentiation, the transgenic TCR generates the CD3 signal, which then leads to allelic exclusion and inhibition of endogenous TCR rearrangement; therefore, the iPSC-derived T cells express the transgenic TCR to target specific antigens. Alternatively, peripheral blood T cells can serve as a cell source for iPSC generation. T cell has the rearranged TCR gene, which is retained throughout the reprogramming and differentiation process. For applications in ACT, T-iPSCs can be engineered with CAR to enhance tumor specificity, or the antigen-specific T cell clone can be used for reprogramming to generate the antigen-specific T cells.

vector encodes OSKM factors and the microRNA-302, while the second vector encodes the SV40 large T (LT) antigen. The iPSC-like colonies appeared on the mouse embryonic fibroblast (MEF) feeder cells within 40 days after transduction (9). The same approach enables reprogramming of several T cell clones specific for Nef antigen in HIV, pp65 antigen in cytomegalovirus (CMV), glutamic acid decarboxylase (GAD) antigen in type 1 diabetes, and alpha-Galactosylceramide (α-GalCer). Importantly, the iPSCs and the parental T cells had identical antigen-recognition sites (CDR3 sequence) on the *TCRA* and *TCRB* genes (9). Recently, the SeV vectors encoding five factors (OSKM + SV40 LT antigen) were used for reprogramming various types of antigen-specific T cells and NKT cells, including WT1-specific T cells, LMP2-specific T cells (44), GPC3-specific T cells (57), b3a2-specific T cells (59) and $V\alpha24^{+}$ invariant natural killer T cells (60, 61).

In contrast to peripheral blood T cells, antigen-specific T cells are mainly effector memory T cells or central memory T cells, which are in the latest stage of development. Effector memory T cells or central memory T cells are prone to apoptosis when stimulated due to their short telomere length (62). Therefore, reprogramming of antigen-specific T cells is very technically challenging. Previous studies showed that the process requires supplementation of the OSKM factors with additional factors such as hTERT and SV40 LT antigen, which have potent anti-apoptotic activity (63), and the use of MEF feeder cells (8, 9). It is worth noting that the hTERT and SV40 large T antigen are known oncogenes; upon insertion into the genome, these factors may cause tumorigenesis. Even though it is not a concern when using the SeV vector system because the viral RNA is diluted and removed from the cells after reprogramming, the SV40 large T antigen might increase double-stranded break (DSB)-associated mutations. Thus, other pluripotency-associated genes, such as NANOG and LIN28, were used instead of SV40 LT antigen in combination with OSKM factors for T cell reprogramming (64). This system called 6-factor (OSKM + NL) offers advantages over the conventional system (OSKM + SV40 LT) by eliminating the oncogenes and is therefore preferable for applications in ACT. In addition, T-iPSCs reprogrammed by a 6-factor system were able to efficiently differentiate into antigen-specific T cells with strong cytotoxicity against cervical cancer. There is no significant difference in cytotoxicity from that of the conventional T-iPSCs (64). Although this 6-factor system successfully generated iPSCs from the antigen-specific T cells, there are two main issues associated with using antigen-specific T cell-derived iPSCs for clinical translation, including clonal variability, which affects T cell differentiation potential (65), and alloreactivity (66). The study demonstrated that approximately 50% of antigen-specific T cell-derived iPSC clones exhibited great T cell differentiation potential (66). There is also a possibility that T cell alloreactivity will occur at 10% even in the case of HLA-matched patients (67, 68). Therefore, to develop an off-the-shelf product from T-iPSCs for use in an allogeneic setting, it is necessary to establish multiple clones of antigen-specific T cell-derived iPSCs and screen for the best clones and other spare clones in case of alloreactivity. It was estimated that eight initial iPSC clones are sufficient to create two powerful T-iPSC clones (66). The generation and screening of eight iPSC clones are time-consuming and expensive, especially from antigen-specific T cell sources. An alternative approach such as introducing TCR or CAR into T-iPSCs would be more practical for developing off-the-shelf ACT.

GENERATION OF T CELLS FROM PLURIPOTENT STEM CELLS

Generation of T cells from PSCs requires two essential stages. First, PSCs need appropriate signals from microenvironments to be committed toward hematopoietic stem cells (HSCs), followed by the Notch signaling for T cell lineage commitment (69). During the first step toward HSCs, PSCs must be differentiated into the definitive mesoderm (ME) and hemogenic endothelium (HE), which then undergoes the process known as an endothelial-to-hematopoietic transition (EHT). During EHT, the HE is rounded up and releases the floating cells with hematopoietic stem/progenitor cell (HSPC) markers, CD34 and CD43, into the medium (70, 71). Two waves of hematopoiesis occur in human embryo development, primitive and definitive. Definitive hematopoiesis can give rise to HSPCs with the potential to develop into T cells (72). The previous study demonstrated that there are no true markers to distinguish between the primitive and definitive HSPCs in the $CD34^+$ $CD43^+$ populations. Therefore, identification of ME by using the phenotypes KDR^+ and $CD235a^-$ is essential (73). After HSC induction, the differentiation process must recapitulate normal T cell development in the thymus where sequential expression of CD7, cytoplasmic CD3, and surface CD3 was observed, followed by TCR gene rearrangement of the γδ and αβ loci, respectively. This section focuses on various approaches that have been used to mimic the microenvironment in the thymus to induce mature T cell differentiation *in vitro* (**Figure 2** and **Table 1**).

Co-Culture System Using Stromal Cells

A simple and well-known method to induce T cell commitment *in vitro* is the co-culture system with mouse stromal cells, OP9, as supporting cells for T cell differentiation. The OP9 cell line can be derived from the mouse bone marrow with a defect in macrophage colony-stimulating factor (MCSF) production (85). The OP9 cells can be expanded *in vitro* for a long time and selectively facilitate HSPC differentiation and lymphoid development (86). In 2002, Schmitt et al. developed a monolayer co-culture system for *in vitro* T cell differentiation using the OP9 cell line overexpressing Delta-like 1 (OP9-DL1), a human homolog of the Notch ligand. Co-culture of mouse HSCs with the OP9-DL1 cells induced $CD4^+$ $CD8^+$ double-positive (DP) T cells and $CD8^+$ SP T cells (87). In 2005, La Motte-Mohs et al. published the first report of the generation of human T cells from $CD34^+$ HSPCs using the OP9-DL1 co-culture system (88). A similar co-culture system has been used to generate T cells *in vitro* using the MS5 and C3H/10T1/2 stromal cell lines expressing DL1. Similar to OP9-DL1, MS5 and C3H/10T1/2 stromal cells overexpressing DL1 support the differentiation of umbilical cord blood $CD34^+$ HSPCs to $CD7^+$ DN cells after 3-4 weeks of co-culture (82, 89). Apart from DL1, Delta-like 4 (DL4) is also known as a ligand for Notch-1 receptor (90). The *in vitro* study showed that DL4 overexpression in stromal cells could support T cell development in a similar manner to DL1 (69, 91). Although there was no significant difference between the yield of T cell differentiation when co-culturing with OP9-DL1 and OP9-DL4, DL4 provided better results at physiological expression levels (92). Further study indicated that DL4 provided a 10-fold greater Notch receptor binding affinity than DL1 (93). As a result, some studies used OP9-DL4 as a feeder cell for T cell differentiation from pluripotent stem cells (50, 52, 72).

The first successful generation of T cells from iPSCs was reported in 2009 by Lei et al., where mouse iPSCs co-cultured with the OP9-DL1 cells in the presence of Flt3L and IL-7 could be differentiated into the $TCR\beta^+$ $CD8^+$ SP T cells. These cells

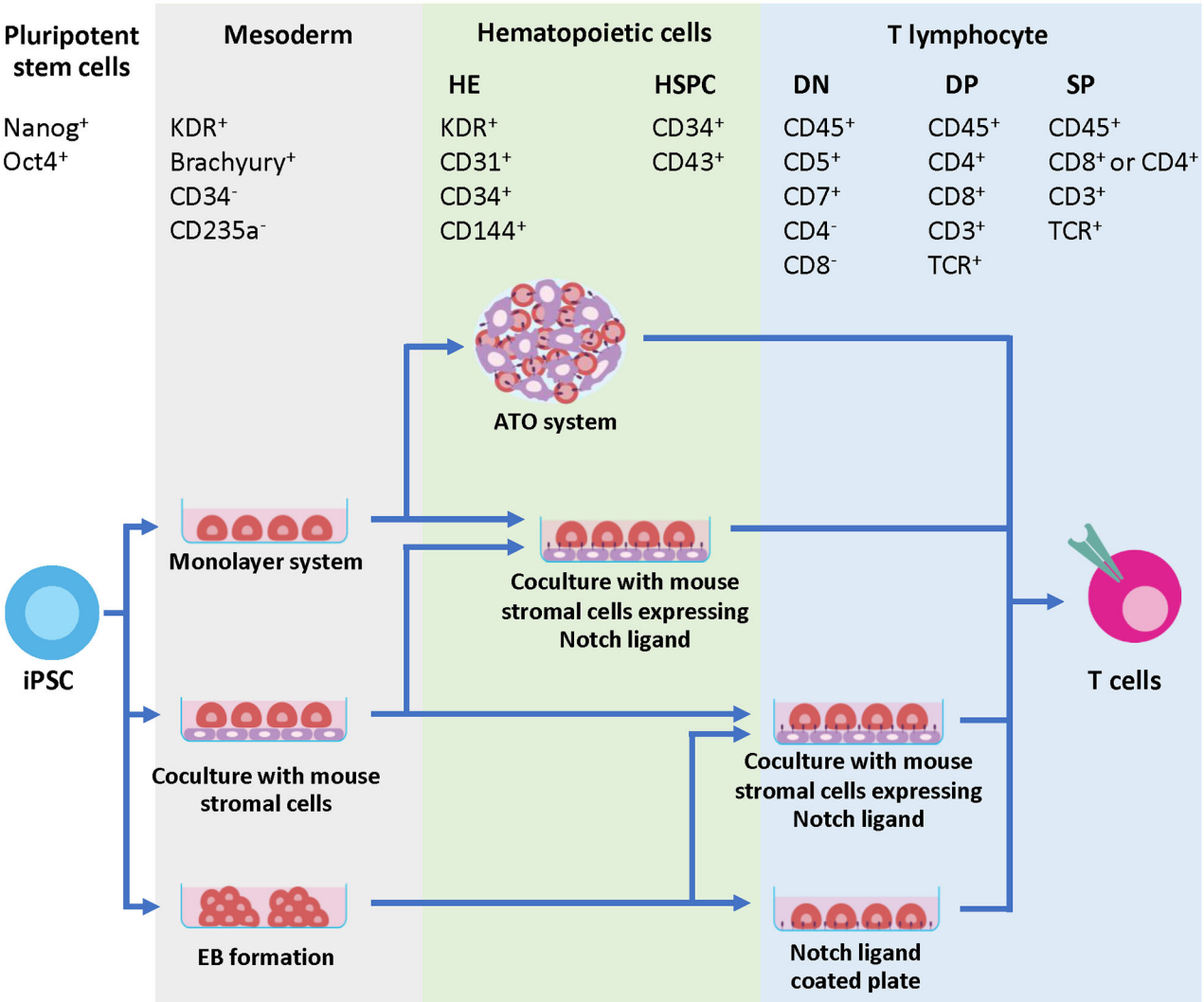

FIGURE 2 | Developmental markers during T cell differentiation and strategies to generate iPSC-derived T cells. The initial step of hematopoietic differentiation can be achieved by various protocols, including feeder-free protocols such as monolayer system, co-culture with mouse stromal cells and EB formation. During this step, the mesodermal (ME) cells expressing Brachyury and KDR are generated. The ME cells are committed further to HE, which express KDR, CD31, CD34 and CD144. During EHT process, $CD43^+$ HSPC emerges from the HE layers. Specification of T cell lineage requires Notch signaling, which can be provided through co-culture with mouse stromal cells such as OP9-DL1 or OP9-DL4. Co-culture of iPSC-derived multipotent HSPCs with these cells in 2D or 3D system efficiently generates mature T cells with phenotypes $CD8^+$ $CD4^-$ TCR^+ and $CD3^+$. Alternatively, the Notch signals can be provided through a coating matrix mixture of retronectin and recombinant DL4 protein.

produced IL-2 and IFN-γ after activation with anti-CD3 antibody, indicating that they are functional T cells. In addition, the iPSC-derived T cells restored the T cell pool in $Rag1^{-/-}$ mice after infusion (94). In contrast to mouse iPSCs, a single-step co-culture system with the OP9-DL1 cells has not been achieved in human iPSCs. To generate T cells, human iPSCs were differentiated to $CD34^+$ HSPCs *via* three methods, embryoid body (EB) formation (56, 72), monolayer system (80, 95), and direct co-culture with the OP9 (96) or C3H10T1/2 cells (9). The $CD34^+$ cells were then transferred onto the OP9-DL1 cells in the presence of Flt3L and IL-7 to further differentiate into pro-T cells, which later required TCR signal to become mature T cells (8, 9, 97). The mouse iPSC-derived pro-T cells can acquire TCR signals from the MHC molecule on the OP9 cells (94, 98). In contrast, the human iPSC-derived DP T cells cannot recognize the mouse MHC molecule on the OP9 cells, so they cannot obtain the TCR signal from co-culturing with the OP9-DL1 cells. Therefore, activation of human pro-T cells using anti-CD3 antibody is required to generate mature T cells (8, 9, 44).

Although $TCR\alpha\beta^+$ $CD8^+$ T cells can be derived from human iPSCs, previous studies showed that human iPSCs could generate only T cells expressing CD8α subunit (CD8αα T cells) and high levels of innate T cell-related markers (such as CD56) (8, 44, 56, 82). The CD8αα T cells differentiated from human iPSCs were

TABLE 1 | Generation of T cells from human iPSCs.

Cell source of iPSCs	Regenerated T cells	T cell differentiation	Functional test	Ref
Non-T cells				
- **Keratinocytes**	Randomly rearranged TCR T cells	Co-culture with OP9-DL4 cells	*In vitro* TCR activation assay	(74)
- **Myeloid cells**	WT1-TCR transduced T cells	Co-culture with OP9-DL1 cells	*In vitro* and *in vivo* specific killing assay	(57)
		Culture onto DL4-coated plate	*In vitro* and *in vivo* specific killing assay	(75)
- **Monocytes**	WT1-TCR transduced T cells	Co-culture with OP9-DL1 cells	*In vitro* and *in vivo* specific killing assay	(76, 77)
- **Fibroblasts**	T cells	Co-culture with OP9-DL1 cells	*In vitro* TCR activation assay	(78)
		Co-culture with MS5-DL4 cells in ATO	N/A	(79)
T cells				
- **PHA-activated lymphocytes**	CD19-CAR transduced T cells	Co-culture with OP9-DL1 cells	*In vitro* and *in vivo* specific killing assay	(56)
- **Purified CD3$^+$ T cells**	T cells	Co-culture with OP9-DL1 cells	*In vitro* TCR activation assay	(80)
- **MART-1 specific CTL clone**	MART-1-specific T cells	Co-culture with OP9-DL1 cells	*In vitro* TCR activation assay	(8)
- **Sorted MART-1-tetramer$^+$ T cells**	MART-1-specific T cells	Co-culture with OP9-DL1 cells	*In vitro* specific killing assay	(66)
- **Nef-specific CTL clone**	Nef-specific T cells	Co-culture with OP9-DL1 cells	*In vitro* specific killing assay	(9, 81)
		Culture onto DL4-coated plate	*In vitro* specific killing assay	(75)
	iC9-transduced Nef specific T cells	Co-culture with C3H10T1/2-DL1 cells	*In vitro* specific killing assay	(82)
- **GAG-specific CTL clone**	GAG-specific T cells	Co-culture with OP9-DL1 cells	*In vitro* specific killing assay	(81)
		Culture onto DL4-coated plate	*In vitro* specific killing assay	(75)
- **GPC3-specific CTL clone**	RAG2 KO GPC3-specific T cells	Co-culture with OP9-DL1 cells	*In vitro* and *in vivo* specific killing assay	(57)
	GPC3-specific T cells	Culture onto DL4-coated plate	*In vitro* specific killing assay	(75)
- **LMP1-specific CTL clone**	LMP1-specific T cells	Co-culture with C3H10T1/2-DL1/4 cells	*In vitro* specific killing assay	(83)
- **LMP2-specific CTL clone**	LMP2-specific T cells	Co-culture with OP9-DL1 cells	*In vitro* specific killing assay	(44)
		Co-culture with C3H10T1/2-DL1/4 cells	*In vitro* and *in vivo* specific killing assay	(83)
	iC9-transduced LMP2-specific T cells	Co-culture with C3H10T1/2-DL1 cells	*In vitro* and *in vivo* specific killing assay	(82)
- **WT1-specific CTL clone**	WT1-specific T cells	Co-culture with OP9-DL1 cells	*In vitro* and *in vivo* specific killing assay	(44, 76, 77)
- **Sorted HPV16-E6 -tetramer$^+$ T cells**	HPV16-E6-specific T cells	Co-culture with C3H10T1/2-DL1/4 cells	*In vitro* and *in vivo* specific killing assay	(64)
- **Sorted HPV16-E7 -tetramer$^+$ T cells**	HPV16-E7-specific T cells	Co-culture with C3H10T1/2-DL1/4 cells	*In vitro* specific killing assay	(64)
- **b3a2-specific Th1 clone**	CD4-transduced b3a2-specific T cells	Co-culture with OP9-DL1 cells	Priming CTLs to increase specific killing *in vitro* and *in vivo*	(59)
- **Expanded TILs from colorectal cancer specimens**	Multiclonal colorectal cancer-specific T cells	Culture onto DL4-coated plate	*In vitro* and *in vivo* specific killing assay	(84)

ATO, artificial thymic organoid; b3a2, junction region of BCR-ABL p210; CAR, chimeric antigen receptor; CTL, cytotoxic T lymphocyte; DL1, delta-like 1; DL4, delta-like 4; GAG, group-specific antigen; GPC3, glypican-3; HPV16-E6, human papillomavirus type 16 early protein 6; HPV16-E7, human papillomavirus type 16 early protein 7; iC9, inducible caspase-9; KO, knockout; LMP1, latent membrane protein 1; LMP2, latent membrane protein 2; MART-1, melanoma antigen recognized by T cells 1; Nef, negative regulatory factor; PB, peripheral blood; PHA, phytohaemagglutinin; RAG2, recombination activating gene 2; TCR, T cell receptor; Th1, T helper type 1; TIL, tumor-infiltrating lymphocytes; WT1, Wilms' tumor 1.

different from the effector T cells in peripheral blood, which are CD8αβ T cells. More importantly, the regenerated CD8αα T cells from iPSCs showed a gene expression pattern similar to those of the innate T cells and exhibited a non-specific killing effect (44, 56).

Recently, Maeda et al. reported a novel method to generate the CD8αβ T cells from human iPSCs. During the differentiation step, the $CD4^+$ $CD8^+$ DP cells were sorted and activated using anti-CD3 antibody to generate CD8αβ T cells similar to the

effector T cells from peripheral blood (44). DNA sequencing revealed that the TCR gene of the iPSC-derived T cells and the parental T cell clone were completely identical, suggesting that antigen specificity of the parental T cells was retained in the iPSC-derived T cells (8, 9, 44). Thus, *in vitro* cytotoxicity of regenerated T cells was comparable to the parental antigen-specific T cells (44). Moreover, the regenerated T cells had a rejuvenated phenotype. The iPSC-derived T cells established from an HIV-1-specific CTL clone could be expanded from 100-fold to 1000-fold within two weeks, whereas the parental T cells could be expanded up to 20-fold. The regenerated CTLs also had a 1.5-fold longer telomere length than parental CTLs (9). Finally, the treatment with the iPSC-derived CD8αβ T cells markedly delayed tumor growth in the mouse model (44, 57, 82). Worth noting that there is no report of the successful generation of $CD4^+$ helper T cells from iPSCs even though T-iPSC was derived from the $CD4^+$ T cell clones (59). Antigen-specific $CD4^+$ T helper cells are essential in controlling immune reactions. These cells can amplify anti-tumor immunity by inducing the activation of tumor antigen-specific CTLs. Therefore, the absence of $CD4^+$ T cells in the iPSC-derived T cell population may lead to insufficient control of tumor growth in patients.

Artificial Thymic Organoid

The three-dimensional (3D) structure of primary thymic stromal cells has been shown to promote positive selection and TCR rearrangement of human T cells *in vitro* (99). In 2017, Seet et al. developed a new method called artificial thymic organoids (ATO) system that combines the 3D organoid culture elements and the expandability of the stromal cell line. The ATO system requires a serum-free medium and the MS5 mouse stromal line expressing human DL1 or DL4 (MS5-DL1 or DL4 cells), which formed small 3D aggregates with human HSPCs by centrifugation. The 3D aggregates were plated onto micropore filters and cultured for six weeks. This ATO system fully recapitulated the T cell development, especially during the TCR rearrangement. At week 6 in ATOs, up to 20% of total cells expressed TCRαβ and CD3, indicating that the cells reached the SP stage without the requirement of anti-CD3 antibody. In addition, CD8 SP T cells and CD4 SP cells isolated from ATOs produced IFN-γ and IL-2 in response to PMA and ionomycin activation (100).

The ATO system was also applied to generate mature T cells from ESCs and iPSC (79). Firstly, the ESCs or iPSCs were induced to mesodermal lineage using BMP4, VEGF and bFGF for three days in the monolayer culture system. The cells were then dissociated into single cells and centrifuged with the MS5-DL4 cells to form aggregates, which were cultured in the hematopoietic induction medium for two weeks followed by the T cell induction medium for 50 days. This approach generated CD8 and CD4 SP T cells, which produced IFN-γ in response to phorbol 12-myristate 13-acetate (PMA) stimulation. Deep sequencing results revealed that the TCRα and β chain rearrangement occurred during the T cell differentiation in the ATO system. Moreover, when using the NY-ESO-1-specific TCR engineered H1 ESC line in the ATO system, nearly 100% of the generated T cells expressed NY-ESO-1-specific TCR. Transduction of NY-ESO-1-specific TCR also inhibited the rearrangement of the endogenous TCRαβ due to allelic exclusion of the TCR gene. Following 14 days of expansion, the ESC-derived TCR-engineered T cells expanded approximately 100-fold and displayed specific cytotoxicity against the NY-ESO-1 expressing target cells *in vitro* and in immunodeficient mice. Interestingly, the studies demonstrated that the ATO system could support the robust differentiation of $CD4^+$ T cells (79, 101). However, the function and potential of $CD4^+$ helper T cells generated from this method have not been clearly investigated.

Feeder-Free Differentiation System

Despite the success in generating T cells, the use of mouse cells as supportive feeders is not compatible with the development of clinical-grade products due to contamination of xenogeneic antigens. Although there have been many attempts to develop human feeder cells to replace the mouse cell lines, the results were unsatisfactory. Human fibroblasts or keratinocytes engineered to express DL4 were insufficient to promote the differentiation of human HSPCs to DN or DP T cells (102, 103). The first attempt to differentiate mouse HSPCs toward T cells under the feeder-free system was performed using the recombinant Notch ligand DL1 fused with Fc domain of human IgG (DL1-Fc)-coated culture dish. This system enabled the generation of the DP T cells that could reconstitute mature T cells in the NOD/SCID mouse model (104). A similar approach to differentiate mouse HSPCs applied the DL4-Fc protein-immobilized culture dish in the medium supplemented with SCF, Flt3L and IL-7. This system efficiently promoted the DP T cell development (105). For a scalable T cell differentiation system, Taqvi et al. immobilized the DL4 protein on microbeads to support T cell development from bone marrow-derived HSPCs. The results showed that the DL4-conjugated bead system was sufficient to induce T cell commitment; however, most differentiated cells were committed to the B cell lineage leading to inefficient T cell generation (106).

Another group developed a novel feeder-free method combining the recombinant VCAM-1 with DL4 proteins. This system synergistically increased the robustness of T cell commitment from cord blood-derived HSPCs in a xenogeneic-free differentiation medium. After two weeks of differentiation, the differentiated cells were arrested at the DP stage with the phenotype of $CD34^-$ $CD7^+$ $CD5^+$ cells. The purified $CD7^+$ cells were further differentiated *in vivo* by intrahepatically injecting into neonatal immunocompromised mice. After 10–12 weeks post-engraftment, functional mature T cells were detected and circulated in the peripheral blood of the immunodeficient mice (107). Recently, Iriguchi et al. reported the success of using a feeder-free system to generate iPSC-derived mature T cells. The iPSC-derived $CD235a^-/CD14^-/CD34^+/CD43^+$ cells were purified and differentiated into the functional antigen-specific T cell lineage under a feeder-free system using immobilized DL4 protein and retronectin. During the differentiation, 3×10^5 iPSCs could give rise to 6.2×10^8 T cells. Importantly, these iPSC-derived T cells demonstrated the anti-tumor function in both *in vitro* and *in vivo* xenograft models (75). Similarly, Ito et al.

demonstrated that this feeder-free protocol could be applied for the generation of tumor-specific T cells from TIL-derived iPSCs. The result showed that the regenerated T cells retained the T cell function and tumor-specific killing. Moreover, there was no additional rearrangement at either the TCRα or TCRβ chains of the T cells generated by this feeder-free protocol (84). However, these two studies still used bovine serum albumin in the medium to obtain a large number of mature T cells; therefore, the development of a complete xenogeneic-free condition for clinical translation of iPSC-derived T cells is still very challenging.

ADVANCES OF IPSC-DERIVED CAR T CELLS FOR OFF-THE-SHELF ACT

The advent of genetic engineering has created the so-called next-generation stem cell-based therapies with enhanced therapeutic efficiencies (108). The most promising therapeutic application in oncology to date has been CAR technology. To date, there are four CD19 CAR T cell products approved by the FDA for the treatment of relapsed or refractory large B cell lymphoma (2), and more than 900 ongoing clinical trials targeting different types of cancers (ClinicalTrials.gov). While CAR T cell therapy holds impressive clinical outcomes, many challenges hinder its applications, including insufficient autologous T cells due to lymphopenia in patients and a high production cost. Human iPSCs have become an attractive cell source for the generation of CAR T cells regarding their self-renewal capacity. In 2013, Themeli et al. reported the first proof-of-concept study showing that the CD19 CAR-engineered iPSCs could be used as a starting cell source for generating the functional CD19 CAR T cells with anti-cancer capability in a xenograft model (56). To broaden the applicability of CAR T cell therapy, many attempts have been made to generate allogeneic CAR T cells devoid of TCR to eliminate the risk of GvHD. These strategies employ genome editing technologies such as zinc finger nucleases (37), TALENs (40) or CRISPR/Cas9 (36) to disrupt TCR expression in primary T cells from healthy donors and introduce CAR specific to cancer antigens. Using CRISPR/Cas9 technology, Sadelain and colleagues generated the engineered T cells with CD19 CAR gene knockin at the TCR α constant (TRAC) locus. The engineered T cells lack the endogenous TCR expression and simultaneously express CD19 CAR under the control of its transcriptional regulatory elements. These engineered TRAC-encoded CD19 CAR T cells exhibited increased anti-tumor activities in the leukemic mouse model regarding the responses and prolonged medium survival compared to the conventional, randomly integrated CD19 CAR T cells. This study emphasized the importance of transcriptional regulation of CAR expression; the use of endogenous regulatory elements resulted in a better-defined T cell product with minimized TCR-induced autoimmunity and alloreactivity as well as delayed exhaustion (36). Although the absence of TCR expression can lower the risk of GvHD, CD3 signaling from CAR can alter the T cell lineage commitment. The presence of all three CD3ζ immunoreceptor tyrosine-based activation motifs (ITAMs) has been shown to compromise the therapeutic potency of CAR T cells. Therefore, the team modified the second and third CD3ζ ITAMs of CAR to be non-functional (1XX) and generated CD19 1XX CAR T cells. These engineered CAR T cells have calibrated ITAM activity with similar strength of CD3 signaling from TCR, thereby exhibiting increased persistence and better therapeutic efficacy in the well-established pre-B acute lymphoblastic leukemia (B-ALL) mouse model compared to the CAR T cells with all three CD3ζ ITAMs or other types of mutants (109).

Despite excellent results obtained in primary CAR T cells, multiplex genome engineering, quality control, and validation are technically challenging. One way to address this issue is to harness the unique characteristics of iPSCs, which are amenable to genetic manipulation and clonal validation. Fate Therapeutics has combined the iPSC technology with CAR to generate the iPSC-derived TCR-less CD19 1XX CAR T cell product to treat B-ALL. Upon T cell differentiation, the iPSCs harboring TRAC-CD19 1XX CAR could give rise to the highest $CD4^+$ $CD8^+$ DP population compared to other types of iPSC-derived CAR T cells. Importantly, the $CD4^+$ $CD8^+$ DP cells could be efficiently differentiated into CD8αβ SP CAR T cells (110). This novel platform, so-called "the first-of-kind off-the-shelf hiPSC-derived CAR19 T cell product FT819" was manufactured under the current Good Manufacturing Practice (cGMP) compliance and applied in the pre-clinical study. The *in vivo* leukemia xenograft mouse studies also showed that FT819 could control tumor burden and prolong survival rate similar to those of the CD19 CAR T cells (111, 112). In addition, the mixed lymphocyte reactions performed with HLA-mismatched peripheral blood mononuclear cells (PBMCs) confirmed the lack of alloreactivity, thereby eliminating the risk of GvHD (113). Recently, Phase I multicenter trial of FT819 has been initiated in up to 300 patients with relapsed/refractory B cell malignancies. Various FT819 dose levels ranging from 30 to 900 million cells will be tested to find the recommended Phase II dose. Three treatment regimens for each type of cancer will be included: Regimen A, FT819 will be given as a single dose; Regimen B, FT819 will be given as a single dose combining with IL-2; and Regimen C, FT819 will be given at three fractionated doses (114).

Besides the risk of GvHD, graft rejection by the recipient's immune cells is another concern. Several groups have generated universal or hypoimmunogenic iPSC lines by eliminating HLA class Ia (HLA-A, -B, and -C) and class II molecules to avoid immune rejection by CD8 T cells and CD4 T cells, respectively, and introducing HLA class Ib (HLA-G or HLA-E) or immune checkpoint molecules (PD-L1 or CD47) to prevent NK cell-mediated lysis or phagocytosis by macrophages (115–122). To date, the main challenge for translating these approaches is how to avoid NK cell-mediated lysis. This can be achieved by suppressing the activating signals or promoting the inhibitory signals. However, there are diverse activating and inhibitory receptors expressed on NK cells of each individual; thus, targeting multiple receptors is necessary to completely prevent the NK cell attacks (123, 124). Previous studies showed that

expression of HLA-E in the HLA-null iPSC-derived $CD45^+$ cells (116) and iPSC-derived retinal pigment epithelial cells (125) could inhibit NK cell-mediated lysis through the interaction with CD94/NKG2A receptors. However, it was shown that approximately 50% of NK cells express NKG2A receptor (126); therefore, HLA-E expressing cells may still be a target for $NKG2A^-$ NK cells (122). More recently, Wang et al. took a step forward by knocking out poliovirus receptor (PVR) or CD155, a ligand for NK cell-activating receptor DNAM-1, in the HLA-E-transduced, HLA-I- and HLA-II-null iPSCs. Upon differentiation toward cytotoxic T cells, the engineered cells could reduce the activation of $DNAM\text{-}1^+$ NK cells, consisting of both $NKG2A^+$ and $NKG2A^-$ populations, and persisted longer than the HLA-intact iPSC-derived T cells *in vitro* and *in vivo* in the presence of allogeneic immunity (119). Therefore, engineering multiple inhibitory/activating signals could lead to a more effective escape from NK cells making the iPSC-derived T cells applicable to a larger number of patients.

Apart from the modification of TCR and/or HLA genes and the introduction of CAR for the generation of universal iPSC-derived CAR T cells, there are attempts to engineer the iPSCs with other molecules to expand the potential of adoptive iPSC-derived CAR T cell therapy. One feature is the expression of a high-affinity, non-cleavable form of antibody receptor CD16 (hnCD16), which allows the scientists to adjust the specificity of the T cell killing through antibody-dependent cellular-cytotoxicity (ADCC) by adding a monoclonal antibody. For example, the iPSC-derived CD19 CAR-hnCD16 T cells could efficiently recognize and kill both $CD19^+$ $CD20^+$ and $CD19^-$ $CD20^+$ tumor cells when combined with anti-CD20 monoclonal antibody (Rituxan) (113). Therefore, this strategy could be applied to target multiple cancer antigens. Another approach to increase the persistence and therapeutic efficacy of iPSC-derived CAR T cells is to engineer a signaling-fusion complex such as IL-7 receptor fusion (IL-7RF), which is a fusion protein of IL-7 receptor and its ligand; therefore, IL-7RF can generate IL-7 signal by itself without exogenous IL-7 support. The addition of IL-7RF led to higher anti-tumor activity compared to the control group in both the *in vitro* and *in vivo* studies (127).

In 2020, a novel TCR (MC.7.G5) was discovered using a genome-wide CRISPR-Cas9 screening. This TCR exhibits a pan-cancer cell recognition potential *via* the invariant monomorphic MHC class I-related protein MR1 molecule. T cells expressing the MR1-restricted TCR (MR1-TCR) could kill a broad range of cancer cells independently of classical MHC molecules. Importantly, these MR1-TCR T cells are inert when being co-cultured with healthy cells from various tissues (128). The discovery of the MR1-TCR offers therapeutic opportunities for many cancers in all individuals. Recently, Nguyen et al. demonstrated the feasibility of the MR1-TCR in the engineered iPSCs, which also express CD19 CAR and hnCD16. Upon T cell differentiation, the engineered iPSC-derived T cells could recognize multiple hematological and solid tumor cell lines. Expression of hnCD16 also enhanced killing of $CD20^+$ Raji cells when combined with Rituximab or $HER2^+$ SKOV3 cells in the presence of anti-HER2 monoclonal antibody (Herceptin). Besides, the CD19 CAR T cells expressing either MR1-TCR or hnCD16 could eliminate CD19-negative lymphoma cells in the co-culture system (129). Altogether, these studies demonstrate the feasibility of iPSCs as a potential renewable cell source of CAR T cells and pave the way for developing off-the-shelf CAR T cell products with enhanced therapeutic efficacy (**Figure 3**).

CHALLENGES AND FUTURE PERSPECTIVES

Adoptive immunotherapy using CAR T cells has shown great success in patients with relapse and refractory B cell malignancies. While autologous T cells provide safety regarding lower risks of adverse side effects such as GvHD, the manufacturing process takes too long for some patients. In addition, the T cell doses largely depend on each individual. This becomes challenging in patients with a low number of T cells. *Ex vivo* expansion of T cells can result in T cell exhaustion, which reduces effector functions. These issues limit the clinical utility. Recently, the treatment using allogeneic T cells from healthy donors has gained more interest since the cells can be prepared and comprehensively validated in advance as off-the-shelf cell products, which can eventually lower the manufacturing cost and time (130). Advances in genome editing technologies have generated various types of engineered T cells with enhanced antigen specificity and persistence, and reduced alloreactivity so the cells can be applied to patients with broader histocompatibility. At present, several clinical trials are being performed to test the safety and efficacy of these engineered T cells, as reviewed in (131).

Meanwhile, iPSCs have been used as a starting cell source for the generation of immune cells for next-generation adoptive immunotherapy. The iPSCs offer advantages such as unlimited proliferation and the ability to differentiate into various cell types, including T cells and NK cells, and ease of multiplexed genome editing. With these properties, the engineered iPSC clones can be isolated, expanded, differentiated, functionally validated and banked in advance (132). However, there are several manufacturing and regulatory hurdles that need to be overcome. For example, the reprogramming methods must be integration-free to avoid potential mutagenesis and transgene reactivation. The process must be performed under cGMP standards (133). At the Center for iPS Cell Research and Application (CiRA), Kyoto University, Japan, the clinical-grade clonal master cell banks were derived from peripheral blood or umbilical cord blood of HLA-homozygous healthy volunteers using episomal plasmid reprogramming (134). Before the secondary cell stock can be used, it is essential to ensure that the cells exhibit normal karyotype and the residual plasmids were absent. Genomic integrity associated with reprogramming and prolonged culture of the established iPSC line, such as chromosomal alterations, copy number variations (CNV), and indel mutations, should be determined using whole-exome sequencing and SNP array, or whole-genome sequencing (134, 135). In addition, if the iPSCs are genetically engineered using

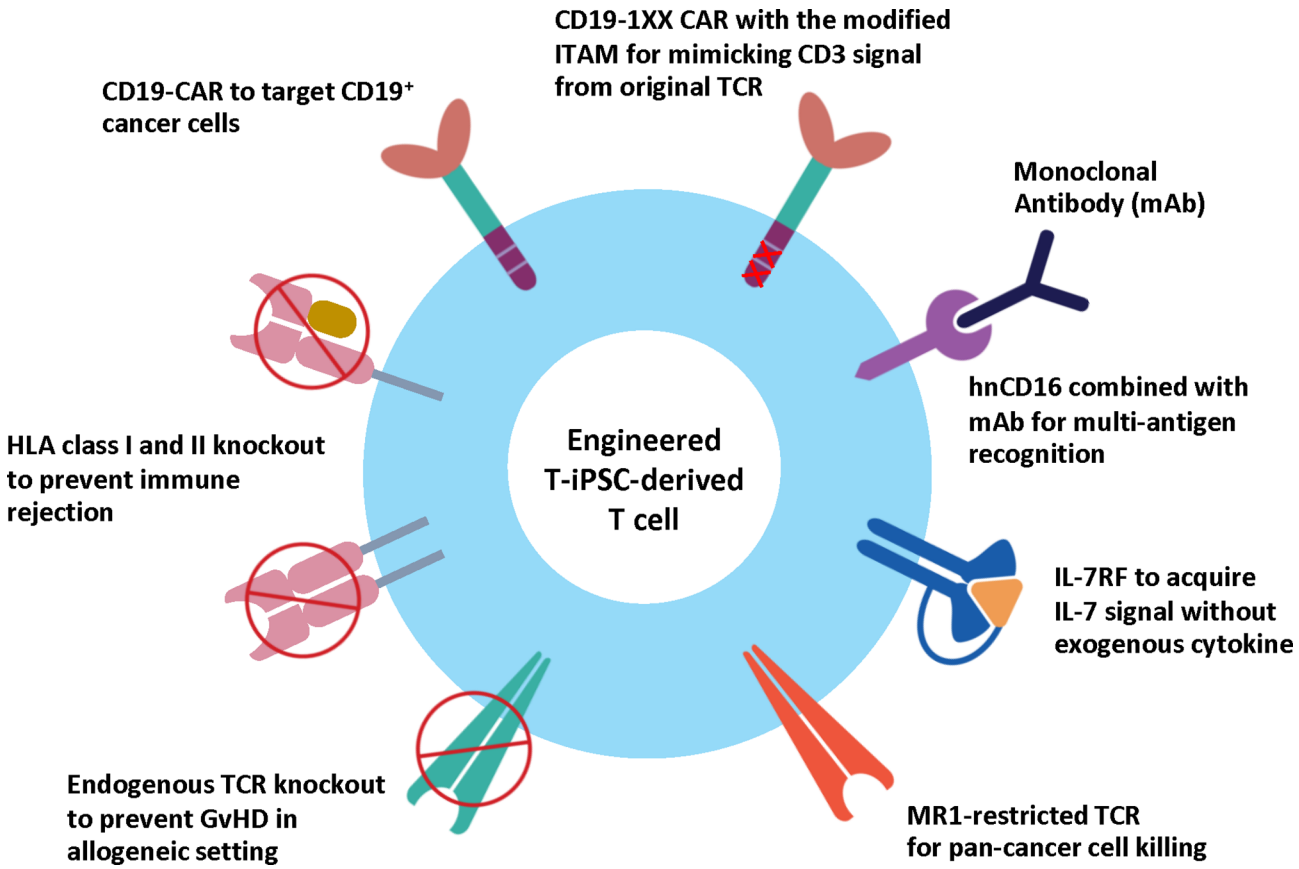

FIGURE 3 | Engineered T-iPSC-derived T cells for next-generation ACT. Genome editing technologies can be used to eliminate the endogenous TCR to reduce the risk of graft-versus-host-disease (GvHD) or HLA molecules to reduce the risk of immune rejection for allogeneic use, or to introduce CAR to specifically target cancer cells. However, the conventional CAR with three ITAM motifs generates higher CD3 signals than endogenous TCR and results in altered T cell differentiation of iPSCs. CD19-1XX CAR construct is the novel CAR construct with mutated second and third ITAM motifs to reduce the CD3 signal. Apart from CAR, the iPSC-derived T cells can be modified to express MR1-restricted TCR to target a wide range of cancer cells. Other strategies to enhance cytotoxic activity and persistence include the expression of hnCD16 and IL-7 RF.

the CRISPR/Cas9 system, the off-target activity from the incorrect binding of sgRNA can often occur and result in insertion-deletion (indel) mutations. Therefore, after clonal selection, it is recommended to conduct the whole-genome sequencing and careful screening of the clones for sterility, mycoplasma, and endotoxin before they are applied in clinics (136).

Apart from the quality control of the established iPSC line, the quality control of the final product, in this case, differentiated T cells, must be performed to evaluate the phenotype and function both *in vitro* and in pre-clinical studies. The differentiation protocol to generate T cells should be developed under a xenogeneic-free system i.e., without serum supplementation or mouse stromal cells as supportive feeders. To date, most published protocols still rely on the use of xenogeneic feeder cells. Although a recent study reported the use of the immobilized-DL-4 protein to generate clinically relevant functional iPSC-derived CD8$\alpha\beta^+$ CAR-T cells (iCART), the therapeutic efficacy of iCART cells was more inferior than that of primary CART cells. This was due to the absence of $CD4^+$ T cells, which also play an important role in the anti-tumor effect of CAR T cell therapy (75). While the 3D ATO platform could produce $CD4^+$ T cells, this approach still requires co-culture with the mouse MS5-DLL4 cell line (137). Therefore, the generation of clinical-scale iPSC-derived functional T cells consisting of both $CD8^+$ and $CD4^+$ cells is necessary (138). Furthermore, the risk of tumor formation after transplantation due to residual pluripotent cells is the most significant concern. Cell sorting should be done to eliminate the contaminating cells as part of a quality check. In addition, the tumorigenicity test using immunodeficient mice such as NOG mice is also required to ensure that the transplanted cells are safe for clinical translation (133, 139). It is worth noting that the cell manufacturing process is far more sophisticated and complicated than pharmaceutical products. Altogether, these challenges are the main hurdles that slow down the clinical translation of iPSC-derived cell products.

As mentioned earlier, genome editing technology has been applied to generate universal iPSC-derived T cells. The removal

of HLA-I can pose a potential safety risk. If the transplanted cells are virally infected or transformed into a tumor, they would not be recognized by the immune cells. Therefore, the solution to these problems is to introduce a suicide gene such as inducible Caspase 9 (iCas9) into the cells. Upon activation by a specific chemical inducer of dimerization (CID), the caspase cascade is induced, and the cells rapidly undergo apoptosis (140). This suicide system was previously tested in the T-iPSCs, and the results showed that the cytotoxic T cells derived from the iC9-expressing T-iPSCs were effective against EBV-induced tumors in the mouse model. Upon administration with CID, the iC9 system was activated, leading to apoptosis of CTLs. The suicide system can also be exploited to eliminate contaminating iPSCs or tumors derived from iPSCs as well as preventing adverse events such as GvHD, cytokine release syndrome, "on-target, off-tumor toxicities" in iPSC-derived T cell therapy (82).

Other concerns observed in CAR T cell therapy could also be considered for developing iPSC-derived T cells. The therapeutic efficacy of CAR T cell therapy mainly depends on the identification of the tumor-associated antigens or neoantigens that are expressed only on the tumor cells and not on the healthy cells. The ideal target antigen will have fewer adverse effects from "on-target, off-tumor toxicities" (141). Furthermore, in solid tumors, the immunosuppressive tumor microenvironments (TME) represent a significant barrier that impairs the function of CAR T cells. Several approaches have been applied to alter the TME from immunosuppressive to pro-inflammatory, including the use of a conditioning regimen prior to T cell infusion, small molecules to interfere with immunosuppressive cells, and blocking antibodies such as anti-PD-1 scFv to inhibit immune checkpoints (142, 143) as well as engineering CAR to express cytokine receptor or to secrete cytokines such as IL-12, IL-18, IL-15 to increase T cell persistence and anti-tumor efficacy (141, 144–147). To date, the CAR T cell therapy for solid tumors in clinical trials has not been effective since T cells cannot penetrate and survive in the TME. To overcome these hurdles, CAR platforms in other immune cells have been explored. One of which is macrophages that have abilities to penetrate the TME, perform phagocytosis and antigen presentation, and interact with other immune cells in the TME. Recently, Zhang et al. incorporated CD19-specific CAR into iPSCs and differentiated them into macrophages (CAR-iMac). Upon activation with leukemia and lymphoma cells, the CAR-iMAC were polarized toward the pro-inflammatory M1 subtype and able to phagocytose the tumor cells in an antigen-dependent manner. Therefore, combining iPSC-derived CAR T cells and CAR-iMac may provide an improved outcome in patients with the heavy burden of solid tumors (148).

CONCLUSION

Advances in iPSC and genome editing technologies offer great promise toward the next-generation ACT where the iPSCs can be engineered to have a more potent cytotoxic function, increased persistence, and less immunogenicity. The iPSC-derived CAR T cells can be prepared and validated in advance as off-the-shelf products to be administered to a large number of cancer patients. Although several hurdles and challenges remain to be overcome, this strategy will provide an infinite supply of true off-the-shelf cell products for cancer immunotherapy.

AUTHOR CONTRIBUTIONS

All authors contributed to the article and approved the submitted version.

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Immunotherapy for Hepatocellular Carcinoma: Current Status and Future Prospects

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Hepatocellular carcinoma (HCC) is the most prevalent primary liver cancer with poor prognosis. Surgery, chemotherapy, and radiofrequency ablation are three conventional therapeutic options that will help only a limited percentage of HCC patients. Cancer immunotherapy has achieved dramatic advances in recent years and provides new opportunities to treat HCC. However, HCC has various etiologies and can evade the immune system through multiple mechanisms. With the rapid development of genetic engineering and synthetic biology, a variety of novel immunotherapies have been employed to treat advanced HCC, including immune checkpoint inhibitors, adoptive cell therapy, engineered cytokines, and therapeutic cancer vaccines. In this review, we summarize the current landscape and research progress of different immunotherapy strategies in the treatment of HCC. The challenges and opportunities of this research field are also discussed.

Keywords: immunotherapy, hepatocellular carcinoma, HCC, immune checkpoint inhibitors, adoptive cell therapy, vaccine, CAR-T, TCR-T

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INTRODUCTION

Hepatocellular carcinoma (HCC) is the fifth most commonly occurring cancer and the third leading cause of cancer death globally (1). In 2020, there were approximately 906,000 new cases and 830,000 deaths of primary liver cancer worldwide, most of which were HCC (comprising 75%-85% of cases) (2). Although surgery is now the most effective treatment for HCC, tumor recurrence is quite common following tumor resection, and the age-standardized five-year relative survival rate for HCC is only 18.1% (3). Due to the difficulty of early diagnosis, the majority of HCC patients are diagnosed as an advanced stage at the initial visit and lose the opportunity for curative treatment such as hepatectomy or radiofrequency ablation, making HCC the second leading cause of cancer-related death in adult males due to the lack of effective therapies (4). The two clinically approved targeted therapy drugs, sorafenib and lenvatinib, could only extend the overall survival by 2 to 3 months (5, 6). Therefore, novel HCC treatment approaches are desperately needed.

Immunotherapy has been proven effective and safe in treating solid tumors, with long-term survival and tolerable toxicity (7, 8). The liver is an immunologically tolerant organ, uniquely capable of limiting hypersensitivity to antigens from food and bacterial products *via* the portal vein, and capable of accepting liver transplants (9). It is suggested that the development of anti-tumor immunity against HCC is synergistically hindered by this tolerogenic property of the liver and the immunosuppressive

tumor microenvironment of HCC. However, the potential of cancer immunotherapy to elicit systemic and durable anti-tumor responses may make it an ideal therapeutic option for HCC, which is characterized by metachronous multicentric occurrence. To date, several immune checkpoint inhibitors (ICIs) targeting cytotoxic T lymphocyte antigen 4 (CTLA-4), programmed cell death protein-1 (PD-1), or its ligand programmed cell death-ligand 1 (PD-L1) have been approved by the U.S. Food and Drug Administration (FDA) for various types of cancers, including HCC (10–12). Other immunotherapeutic strategies, such as adoptive cell therapy, chimeric antigen receptor-modified immune cells, engineered cytokines, and therapeutic cancer vaccines, are matured to clinical trials and bring new hope for HCC patients (13–16). In this review, we first summarize the current landscape of immunotherapy for HCC (**Figure 1**), then discuss this research field's challenges, opportunities, and future directions.

ANTIBODY-BASED THERAPY

Immune Checkpoint Inhibitors (ICIs)

Immune checkpoints are inhibitory immunoreceptors expressed by effector immune cells that prevent them from becoming overactivated. These inhibitory receptors include but not limited to CTLA-4, PD-1, T cell immunoreceptor with Ig and ITIM domains (TIGIT), T cell immunoglobulin and mucin domain containing-3 (TIM3), lymphocyte-activation gene 3 (LAG3), B and T lymphocyte attenuator (BTLA) (17). HCC and other solid tumors use this physiological mechanism to evade anti-tumor immune responses (18). ICIs are monoclonal antibodies that could block the interaction of immune checkpoint proteins with their ligands, thereby enhance the anti-tumor immune response by preventing the inactivation of T cells and restoring immune recognition and immune attack. At present, the targets of ICIs mainly include PD-1, PD-L1, and CTLA-4 (13). PD-1 is a member of the CD28 family, expressed on the surface of most immune cells, mainly on activated T cells, natural killer (NK) cells, regulatory T cells (Treg), myeloid-derived suppressor cells (MDSC), monocytes, and dendritic cells (DC). PD-1 can bind to its ligands PD-L1 and PD-L2, which are expressed in various tumors, including HCC, to transmit inhibitory signals to T cells and induce the immune escape of tumor cells (19).

In 2017, the PD1 inhibitor nivolumab was granted accelerated approval in the United States for the second-line treatment of patients with advanced HCC after treatment with sorafenib. To date, several exploratory studies of ICIs in treating HCC have been conducted. Pembrolizumab and atezolizumab, targeting PD-1 and PD-L1 respectively, have been gradually incorporated into the

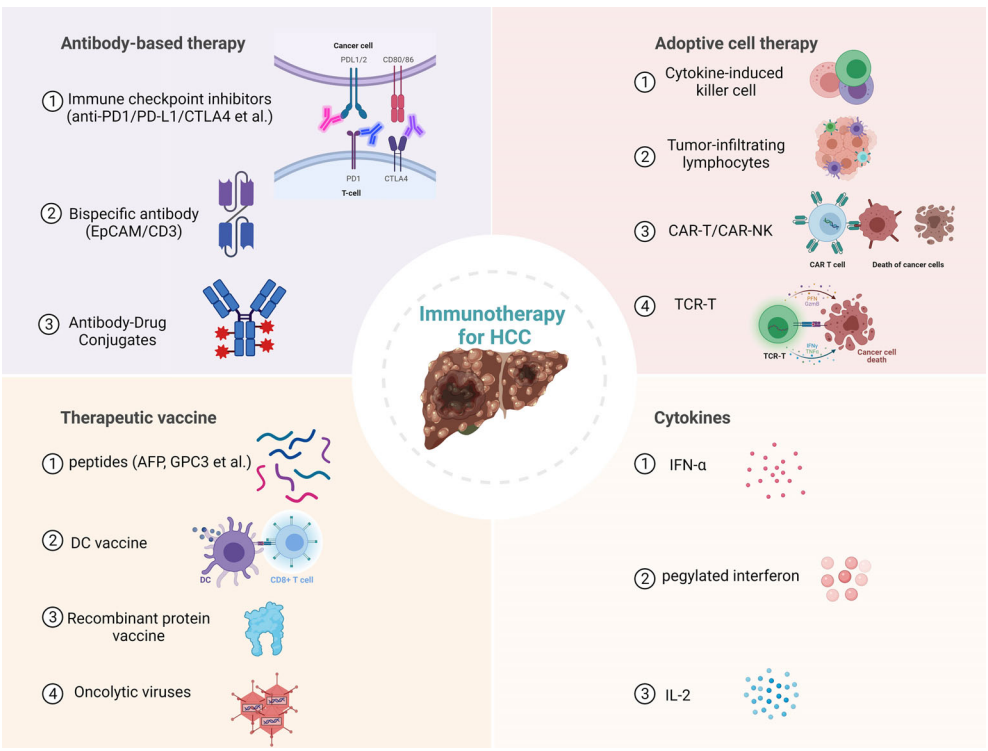

FIGURE 1 | Current immunotherapies for hepatocellular carcinoma (HCC). PD1, Programmed cell death protein 1; PD-L1, Programmed cell death ligand 1; CTLA4, cytotoxic T lymphocyte antigen 4; EpCAM, epithelial cell adhesion molecule; CAR-T, Chimeric antigen receptor T cell; NK, Natural killer cell; TCR-T, T cell receptor engineered T cell; AFP, Alpha-fetoprotein; GPC3, Glypican 3; DC, dendritic cell.

treatment guidelines in many countries and recommended as a clinical treatment option for HCC. Nivolumab and pembrolizumab result in a 15-20% rate of objective remissions (including 1-5% complete remissions) that are durable and associated with prolonged survival. In the CheckMate 040 trial, the median duration of response to nivolumab among 48 patients in the dose-escalation cohort was 17 months, and the 2-year survival rate among responders was greater than 80% (20). KEYNOTE-240, a phase III clinical trial testing pembrolizumab following sorafenib treatment in 413 patients compared with placebo, showed statistically prolonged survival (HR 0.78; P=0.023). The progression-free survival and overall survival curves showed that some patients benefited from pembrolizumab in the long term. Nearly 20% of patients who received pembrolizumab remained progression-free for more than one year, compared with less than 7% in the control group (21). The phase III CheckMate 459 trial compared nivolumab with sorafenib in 743 patients naive to systemic agents, patients who received nivolumab lived longer than those who received sorafenib (median survival 16.4 versus 14.7 months, HR 0.85; P=0.07) (22). Longer follow-up of the CheckMate 459 trial confirmed the ability of nivolumab versus sorafenib to increase the rate of long-term survival (29% versus 21% at 33 months) (23). The latest report in European Society for Medical Oncology (ESMO) 2021 Annual Meeting shows that tislelizumab, a humanized monoclonal antibody (mAb) with high affinity for PD-1 demonstrated durable response in patients with previously systemically treated unresectable HCC and was well tolerated. A global, randomized phase 3 trial is ongoing that compares tislelizumab with sorafenib as first-line treatment in adult patients with unresectable HCC (NCT03412773) (24).

CTLA-4 is another member of the CD28 family that is mainly expressed on activated T cells and dendritic cells and is involved in the negative regulation of the immune response after binding to B7 molecules (25). Ipilimumab and tremelimumab are both CTLA-4 inhibitors, of which Ipilimumab is the first immune checkpoint inhibitor approved by the FDA in 2011 for the treatment of patients with advanced skin cancer (26). Ipilimumab is an IgG1 mAb, while tremelimumab is an IgG2 mAb, with different antibody-dependent cell-mediated cytotoxicity (ADCC) and complement-dependent cytotoxicity (CDC) activities (27). A clinical trial in 2013 showed that tremelimumab could effectively play an anti-HCC effect, with a partial response rate of 17.6% and disease control rate of 76.4% (28). With the in-depth investigation of the mechanism of CTLA-4 inhibitors, some scientists believe that the mechanism of CTLA-4 inhibitors is not through the immune checkpoint but by targeted elimination of Tregs in tumors (29). TIM3 is expressed on tumor-infiltrating lymphocytes (TILs) and tumor-associated macrophages (TAMs) of human HCC and negatively regulates the effector function of T cells, whereas its expression on Treg cells results in enhanced suppressor activity (27, 30, 31). The highly expressed TIM3 is associated with less differentiated HCC (32). LAG3 expression is significantly higher on tumor-specific CD4+ and CD8+ TILs than in other immune compartments in patients with HCC. LAG3 has another functional soluble ligand, fibrinogen-like protein 1, which is synthesized by hepatocytes (33). On March 5, 2019, the sialic acid-binding immunoglobulin-like lectin-15 (Siglec-15) was described as a novel immunosuppressive molecule in Nature Medicine by Professor Lieping Chen (34). The latest research shows that Siglec-15 promotes the migration of liver cancer cells by repressing the lysosomal degradation of CD44 (35). The T-cell immunoreceptor with immunoglobulin and ITIM domains (TIGIT) is another immune checkpoint involved in tumor immune surveillance (36). The TIGIT/CD155 pathway inhibits T cell activation by enhancing IL-10 production and diminishing IL-12 by DCs (37). Taken together, these preclinical data support the investigation of TIM3, LAG3, Siglec-15, and TIGIT inhibitors in HCC in combination with PD1 and PDL1 blockade.

Current clinical trial results show that patients treated with ICIs alone have a lower response rate, so the combined use of ICIs and other treatments will be the future direction. In 2020, the results from IMbrave150, a global, randomized phase 3 trial, showed that atezolizumab in combination with the anti-angiogenic drug bevacizumab significantly reduced the risk of death in patients with advanced unresectable HCC and significantly improved the quality of patient survival (38). The combination of pembrolizumab plus lenvatinib, a tyrosine kinase inhibitor (TKI), showed an overall response rate (ORR) of 46%, with complete response (CR) and partial response (PR) observed in 11% and 35% of included patients with unresectable HCC, respectively (39). Similarly, recent preclinical and clinical studies have proved that the combined application of ICIs with transcatheter arterial chemoembolization (TACE), radiofrequency ablation (RFA), and radiotherapy can also promote the efficacy of anti-tumor immunotherapy (40, 41). In addition, camrelizumab combined with the chemotherapy regimen FOLFOX4 is being investigated as first-line therapy for advanced HCC in a phase Ib/II clinical trial (42). A summary of the past three years of clinical trials associated with ICIs therapy for HCC is listed in **Table 1**.

Bispecific Antibody (BsAb) Therapy

Unlike monoclonal antibodies, BsAbs are prepared mainly by recombinant DNA technology and can specifically bind two antigens or epitopes simultaneously (43). BsAb can directly enhance the activity of immune cells against tumors and can also target immune checkpoints and tumor-associated antigens (TAAs) to reverse immunosuppression in the tumor environment. Therefore, they have more advantages in terms of synergistic effects than monoclonal antibodies and can also mediate a variety of specific biological effects. In most cases, BsAbs recruit and activate immune cells to kill tumor cells by bridging the gap between immune cells and tumor cells (44). Solitomab (AMG110, MT110) is a humanized bispecific EpCAM/CD3 antibody. The anti-EpCAM single-chain variable fragment (scFv) is fused to the anti-CD3 scFv *via* a Gly4Ser linker to form the bispecific T-cell engager (BITE), whose binding to γδ T cells can lead to near-complete lysis of HCC cell lines *in vitro* (45). Another BsAb, Glypican-3 (GPC3)/CD3 BITE, is thought to recruit cytotoxic T lymphocyte (CTL) to eliminate GPC3 + HCC cells (46). In one study, two anti-GPC3 Fab fragments were fused *via* flexible linker peptides to one

TABLE 1 | Clinical trials of ICIs therapy for HCC the last three years (www.clinicaltrails.com).

NCT ID	Phase	Interventions	Country
NCT04943679	1, 2	Anti-PD-1/PD-L1/PEG-IFN-α	China
NCT03638141	2	Durvalumab/Tremelimumab	US
NCT04165174	2	Terepril monoclonal antibody/Apatinib	China
NCT04696055	2	Regorafenib in combination with Pembrolizumab	US
NCT04728321	2	Anti-PD-1/CTLA-4 bispecific antibody AK104/Lenvatinib	China
NCT04444167	1, 2	Anti-PD-1/CTLA-4 bispecific antibody AK104/Lenvatinib	China
NCT04193696	2	Radiation therapy and systemic anti-PD-1 immunotherapy	China
NCT03869034	2	Transarterial Infusion Chemotherapy Combined With PD-1 Inhibitor	China
NCT04974281	1	Anti-PD-1 and Lenvatinib Plus TACE	China
NCT04418401	1	Donafinib Combined With Anti-PD-1 Antibody	China
NCT04814043	2	Anti-PD-1 and lenvatinib plus TACE and chemotherapy	China
NCT04814030	2	Anti-PD-1 Plus Chemoembolization and chemotherapy	China
NCT04273100	2	Anti-PD-1 combined with TACE and lenvatinib	China
NCT03605706	3	Camrelizumab (PD-1 Antibody) in Combination With chemotherapy	China
NCT03839550	2	Apatinib mesylate +PD-1 antibody SHR-1210	China
NCT04564313	1	Anti-PD-1 Antibody Camrelizumab	China
NCT04233840	2	Nivolumab (PD-1 Antibody)	China
NCT04297280	2	Anti-PD-1 Antibody (IBI308) Combined With TACE	China
NCT04229355	3	DEB-TACE plus PD-1 inhibitor	China
NCT03857815	2	Radiation Combined With Anti-PD-1 Antibody (IBI308)	China
NCT04639284	NA	Anti-angiogenic agents plus anti-PD-1/PD-L1 antibodies	China
NCT04518852	2	TACE combined with sorafenib and PD-1 mAb	China
NCT04172571	2	anti-PD-1 antibody AK105 plus anlotinib hydrochloride	China
NCT03939975	2	anti-PD-1 therapy in combination with incomplete thermal ablation	China
NCT04248569	1	DNAJB1-PRKACA Fusion Kinase Peptide Vaccine Combined With Nivolumab and Ipilimumab	US
NCT04802876	2	Spartalizumab (PD-1 inhibitor)	Spain
NCT04191889	2	Hepatic Arterial Infusion combined with Apatinib and Camrelizumab	China
NCT03829501	1, 2	anti-ICOS mAb (KY1044) in combination with anti-PD-L1 mAb (atezolizumab)	US
NCT03652077	1	INCAGN02390 (TIM3 inhibitor)	US
NCT03836352	2	DPX-Survivac, in Combination Cyclophosphamide, Pembrolizumab,	US
NCT03849469	1	XmAb®22841 in Combination with Pembrolizumab	US
NCT04709380	3	Radiotherapy Plus Toripalimab	China
NCT04167293	2, 3	Sintilimab and Stereotactic Body Radiotherapy	China
NCT04157985	3	PD-1/PD-L1 Inhibitors	US
NCT04658147	1	Perioperative Nivolumab With or Without Relatlimab	US
NCT03713593	3	Lenvatinib in Combination With Pembrolizumab	US
NCT04629339	2	INCB086550 (Oral PD-L1 Inhibitor)	Bulgaria
NCT04487704	NA	camrelizumab	China
NCT04114136	2	Anti-PD-1 mAb Plus Metabolic Modulator	US
NCT04785287	1, 2	Anti-CTLA4 mAb, Nivolumab, and Stereotactic Body Radiation	US
NCT04116320	1	Focused Ultrasound Ablation and PD-1 Antibody Blockade	US
NCT04740307	2	pembrolizumab/quavonlimab (MK-1308A) plus lenvatinib	US
NCT04665609	3	Thermal Ablation, Anlotinib and TQB2450 (PD-L1 inhibitor)	China
NCT03867084	3	Pembrolizumab (PD-1 inhibitor)	US
NCT04246177	3	lenvatinib and pembrolizumab in combination with TACE	US
NCT03655613	1, 2	PD-1 inhibitor(APL-501 or nivolumab) + c-Met inhibitor (APL-101)	Australia
NCT04052152	2	Anlotinib Hydrochloride Capsules combined with Sintilimab injection	China
NCT04204577	2	Thermal Ablation, Apatinib and PD-1 Antibody SHR-1210	China
NCT04102098	3	Atezolizumab (Anti-PD-L1 Antibody) Plus Bevacizumab	US
NCT04828486	2	Futibatinib and Pembrolizumab	US
NCT03785210	2	Nivolumab (Anti-PD1), Tadalafil and Oral Vancomycin	US
NCT03949231	3	PD1/PDL1 Inhibitor	China
NCT03680508	2	TSR-022 (Anti-TIM-3 Antibody) and TSR-042 (Anti-PD-1 Antibody)	US
NCT03973112	2	HLX10 in Combination With HLX04	China
NCT04912765	2	Neoantigen Dendritic Cell Vaccine and Anti-PD1 (Nivolumab)	China
NCT03859128	2, 3	Toripalimab (PD-1 Antibody)	China
NCT04926532	1, 2	Toripalimab (PD-1 Antibody) Plus Sorafenib	China
NCT03722875	NA	SHR-1210 (PD-1 Antibody) Plus Apatinib	China
NCT04014101	2	Anti-PD-1 Antibody SHR-1210 Combined With Apatinib Mesylate	China
NCT04947826	2	combination therapy of HAIC with PD-1 antibody and VEGF antibody	China
NCT04411706	2	Anti-PD-1 Antibody combined with apatinib and capecitabine	China
NCT03764293	3	Anti-PD-1 Antibody SHR-1210 Combined With Apatinib Mesylate	China
NCT03793725	2	Anti-PD-1 Inhibitor SHR-1210 in Combination With Apatinib	China

(Continued)

TABLE 1 | Continued

NCT ID	Phase	Interventions	Country
NCT04297202	2	Anti-PD-1 Inhibitor SHR-1210 in Combination With Apatinib	China
NCT04393220	2	Combination of PD-1 and VEGFR-2 Blockade	China
NCT04665362	1	Oncolytic Virus M1Combined With Anti-PD-1 Antibody and Apatinib	China
NCT03966209	1	JS001(PD-1 inhibitor)	China
NCT03732547	2	Anti-PD-1 Antibody Combined With PolyIC	China

NA, Not available.

asymmetric third Fab-sized binding module to form an IgG-shaped TriFab, which could be applied to engage two antigens simultaneously, or for targeted delivery of small and large payloads (47).

ADOPTIVE CELL THERAPY (ACT)

ACT is an immunotherapy that uses the immune cells of the patient or a healthy donor to fight cancer and has recently become an essential tool in the treatment of cancer (48). Compared to antibodies or other targeted drugs, ACT can be activated and replicate ***in vivo*** and has a long-lasting anti-tumor effect. Therefore, ACT is also referred to as a "living" treatment method (49). ACT is considered a highly individualized cancer therapy because most effector cells are derived from the patient. Because expanded or genetically modified effector cells can recognize and attack tumor antigens, ACT is more specific than chemotherapy (50). ACT clinical trials for the treatment of HCC registered at clinicaltrials.gov in the last three years are listed in **Table 2**.

Cytokine-Induced Killer Cell (CIK)

CIK cells are a heterogeneous population of immune cells produced by *in vitro* expansion of human peripheral blood mononuclear cells (PBMC) in the presence of IL-2, IFN-γ, and anti-CD3 monoclonal antibodies (51). CIK cells are mainly composed of natural killer T (NKT) cells, natural killer (NK) cells, and cytotoxic T lymphocytes (CTLs). CIK can recognize tumor cells through the adhesion molecules and lyse tumor cells in a major histocompatibility complex (MHC) independent manner. In a phase I clinical trial, Shi et al. used CIK cells to treat primary HCC and found that the symptoms and characteristics of HCC patients were relieved without significant side effects, indicating autologous CIK cells can efficiently improve the immunological status in HCC patients (52). Clinical trials have also shown that CIK cell therapy can not only be used to treat patients with inoperable primary HCC but also has some effect in treating HCC patients after tumor resection. Takayama et al. reported a clinical trial of CIK treatment in 150 patients with postoperative HCC. They found that the treatment had no significant adverse effects, and the recurrence rate was 18% lower in the treatment group, suggesting that CIK cells therapy could reduce the recurrence rate of patients with postoperative HCC and prolong the recurrence-free survival (53).

Researchers have also made many attempts to combine conventional treatments with CIK cell therapy. TACE combined with CIK cells could prolong progression-free survival in HCC patients compared to TACE alone (54). Wang XP et al. reported that after combined treatment of primary HCC patients with CIK cells and local radiofrequency (RF) hyperthermia, T and NKT cells increased significantly, and alpha-fetoprotein (AFP) decreased from 167.67 ± 22.44 to 99.89 ± 22.05 ng/ml (P = 0.001) (55). Although side effects such as pyrexia, chills, myalgia, and fatigue were associated with CIK therapy in 17% of patients, they were not severe enough to discontinue therapy (56). These data suggest that CIK cells in combination with TACE or RF hyperthermia are safe and effective in treating HCC patients.

Tumor-Infiltrating Lymphocytes (TIL)

TIL is one of the representative components of the host anti-tumor immune responses, which including regulatory T cells (Treg), NK cells, T cells, and B cells (57). Experiments in mice show that TIL is 50-100 times more effective than lymphokine-activated killer (LAK) cells in treating advanced metastatic tumors (58). The feasibility of TIL therapy was demonstrated in a phase I clinical trial in patients with primary HCC (59). Because TILs are isolated from surgical tumor specimens and can recognize multiple antigens, the tumor-inhibitory effect of TIL is stronger than that of therapies targeting single antigens or mutations. Previous studies have shown that TILs in HCC are rare but may have a significant impact on tumor recurrence and patient prognosis (60). In a randomized clinical trial, adoptive TIL therapy was shown to improve recurrence-free survival after liver resection in 150 patients with HCC (53). Patients with HCC and prominent lymphocyte infiltration who underwent surgical resection had a 38.6% lower recurrence rate and a 34.9% higher five-year survival rate than patients without marked lymphocyte infiltration (61). However, it is difficult to isolate TILs from the tumor tissues of HCC patients and expand them *in vitro*. In addition, only a few patients with HCC can tolerate lymphocyte deletion, which is essential before TIL infusion (62).

Chimeric Antigen Receptor T Cell (CAR-T)

CAR-T therapy is novel cancer immunotherapy in which T cells are genetically modified to recognize specific TAA and is the current research hotspot of ACT (63). CAR-T cell therapy has achieved encouraging outcomes in the treatment of hematological malignancies. CAR-T cells targeting CD19 and B-cell maturation antigen (BCMA) have been approved by the U.S. FDA for the treatment of acute B-cell lymphocytic leukemia, certain types of lymphomas, and multiple myeloma (64, 65). Due to the heterogeneity of solid tumors, lack of specific targets, and susceptibility to the tumor microenvironment, CAR -T therapy for liver cancer is still in development (66).

TABLE 2 | Clinical trials of ACT for HCC the last three years (www.clinicaltrails.com).

NCT ID	Target	Phase	Interventions	Country
NCT04121273	GPC3	1	CAR-T	China
NCT02905188	GPC3	1	CAR-T	US
NCT04538313	NA	1, 2	TILs	China
NCT03884751	GPC3	1	CAR-T	China
NCT03899415	HBV antigen	1	TCR-T	China
NCT03980288	GPC3	1	CAR-T	China
NCT03672305	c-Met/PD-L1	1	CAR-T	China
NCT04162158	NA	1, 2	Allogeneic NK cells	China
NCT04368182	AFP	1	TCR-T	China
NCT03971747	AFP	1	TCR-T	China
NCT03993743	CD147	1	CAR-T	China
NCT04011033	NA	2, 3	Autologous iNKT cells	China
NCT03941626	DR5, EGFR vIII	1, 2	CAR-T/TCR-T	China
NCT04951141	GPC3	1	CAR-T	China
NCT04550663	NKG2DL	1	CAR-T	China
NCT03441100	MAGEA1	1	TCR-T	US

ACT, Adoptive cell therapy; GPC3, Glypican 3; CAR-T, Chimeric antigen receptor T cells; TILs, tumor-infiltrating lymphocytes; HBV, hepatitis B virus; TCR-T, T cell receptor engineered T cells; AFP, Alpha-Fetoprotein; iNKT, Invariant natural killer T; DR5, Death receptor 5; EGFR vIII, Epidermal growth factor receptor variant III; NKG2DL, NKG2D ligand; MAGEA1, MAGE family member A1.

In contrast to the T-cell receptor (TCR) structure of conventional T cells, the CAR structure is independent of the major histocompatibility complex (MHC) antigen presentation, avoids restriction by MHC molecules, and solves the problem of tumor immune escape due to downregulation of MHC (67) (**Figure 2**). To date, a growing number of clinical trials have been conducted to demonstrate the value of CAR-T cell therapy in solid tumors.

GPC3 is a heparan sulfate proteoglycan containing 580 amino acids and is overexpressed in HCC but is not present or shows very low expression in normal tissues (68, 69). Gao et al. constructed for the first time CAR-T cells targeting GPC3 and demonstrated that GPC3 CAR-T cells could effectively eliminate the growth of HCC cells *in vitro* and *in vivo* (70). Recently, our lab reported that by splitting the CAR construct into two parts (split GPC-3 CAR-T cells), HCC tumors could be eliminated with a decreased amount of proinflammatory cytokines (71). Another study established patient-derived xenograft (PDX) HCC models and proved that GPC3 CAR-T cells suppressed tumor growth but with varying efficacy due to different expressions of PDL1 on tumor cells (72). This suggests that the combination of CAR-T therapy and ICIs is a feasible strategy to achieve higher efficacy in eradicating PD-L1-positive HCC.

Alpha-fetoprotein (AFP), a secreted glycoprotein, is highly expressed in the fetus but very low in adults. However, when HCC occurs in adults, AFP is re-expressed (73). Conventional CAR-T cells can only recognize tumor surface but not intracellular antigens. Considering that all intracellular antigens are presented by MHC class I molecules, Liu et al. generated some unique CAR-T cells which can selectively bind to the AFP158-166 peptide-MHC complex, then lyse HLA-A*02:01+/AFP+ tumor cells (74). Meanwhile, they conducted a phase I clinical trial (NCT03349255) successfully

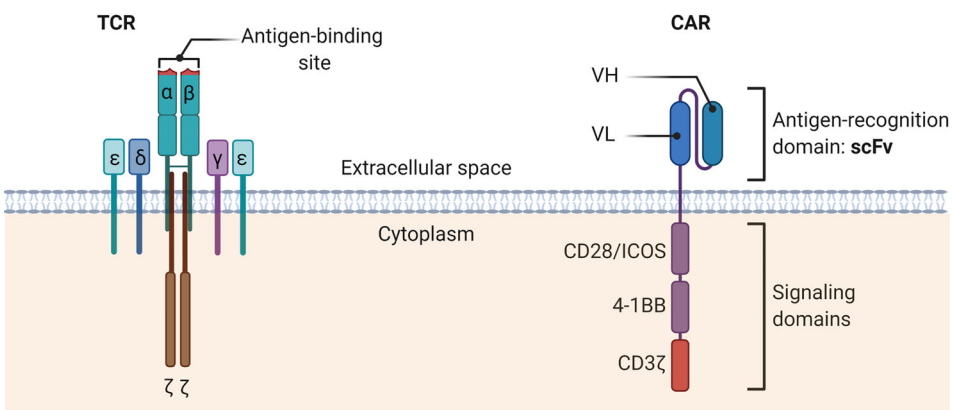

FIGURE 2 | The schematic diagrams of the structures of TCR complex and CAR. The TCR α and β chains bind the MHC-peptide on antigen-presenting cells. Other CD3 molecules, especially the CD3ζ, transmit signals and activate the T cells. TCR, T cell receptor; CAR, Chimeric antigen receptor; scFv, Single-chain variable fragment; VH, heavy chain variable domain; VL, light chain variable domain.

evaluating the safety and efficacy of CAR-T cells in AFP-expressing HCC patients. Therefore targeting intracellular antigens with CAR-T cells is a promising strategy for HCC treatment.

c-Met is a tyrosine kinase receptor that can induce hepatocyte proliferation, survival, and regeneration (75). Overexpression of c-Met can promote the development and progression of HCC. Therefore, c-Met is considered a potential target for the treatment of HCC. Jiang et al. generated CAR-T cells targeting c-Met and PD-L1 and found that dual-targeted CAR-T cells exhibited marked cytotoxicity against c-Met+ PD-L1+ HCC cells (76).

Natural-killer group 2 member D ligands (NKG2DL) are expressed in many primary tumors, including HCC, but not in normal tissues (77). Therefore, NKG2DL may provide a useful target for HCC immunotherapy. Recently, Sun et al. constructed novel NKG2D- CAR-T cells that target NKG2DL expressed on HCC cells and found that NKG2D-CART cells specifically lysed HCC cells with high expression of NKG2DL but did not affect the NKG2DL negative cell line (78). The results of the xenograft model also showed that NKG2D-CAR-T cells could successfully inhibit tumor growth *in vivo*.

CD147, a type I transmembrane glycoprotein, was highly expressed in HCC and other solid tumors (79). Zhang et al. introduced Tet-On inducible CD147-CART cells to treat HCC and found that with the supply of Dox, Tet-On inducible CD147-CART cells could lyse multiple HCC cell lines *in vitro* and effectively inhibit the growth of cancer cells in the HCC xenograft model (80). Recently, a phase I study (NCT03993743) was conducted to assess the safety of hepatic artery infusions (HAI) CD147-CART cells for advanced hepatocellular carcinoma.

Other candidates target antigens for HCC CAR-T therapy involve Mucin 1 (81), EpCAM (82), and CD133 (83–85). However, all of the targets mentioned above are TAAs, which are expressed not only in cancer cells but also in normal cells at low levels, therefore causing on-target, off-tumor toxicities in healthy tissues. Finding new specific antigens and improving the efficacy and safety of CAR-T therapy in HCC is the most important task for future researches.

CAR-NK

In the liver, the proportion of NK cells is significantly higher than in the peripheral blood and spleen. Therefore, NK cell is believed to play an important role in the prevention of HCC and is considered a potential cell therapy resource for the treatment of HCC (86). The strategy used to generate CAR-T cells can also be applied to NK cells to generate CAR-NK cells. In addition, CAR-NK cells can reduce the risk of autoimmune response and tumor transformation because of their shorter lifespan than CAR-T cells (87). Moreover, CAR-NK cells can be produced from a variety of sources, including the NK92 cell line, peripheral blood mononuclear cells (PBMC), umbilical cord blood (UCB), and induced pluripotent stem cells (IPSC). Therefore, CAR-NK cells can be supplied "off-the-shelf", eliminating the need for personalized and patient-specific products, as is the case with current CAR-T therapies, and reducing the risk of syngeneic xenograft reactions and graft-versus-host disease (GVHD) (88).

In 2018, Yu et al. developed GPC3-specific CAR-NK cells and explored their potential in the treatment of HCC (89). In the study, GPC3-specific CAR-NK cells could induce significant cytotoxicity and cytokine production when co-cultured with GPC3+ HCC cells *in vitro*. Furthermore, soluble GPC3 and TGF-β did not inhibit the cytotoxicity, and no significant difference in anti-tumor activity was observed under hypoxic (1%) conditions. In another study, Tseng et al. utilized CD147 as the target antigen and created CD147-specific CAR-T and CAR-NK cells for the treatment of HCC (90). The results showed that CD147-specific CAR-NK cells could effectively kill various malignant HCC cell lines *in vitro* and HCC tumors in xenograft and PDX mouse models. Importantly, GPC3-synNotch-inducible CD147-specific CAR-NK cells selectively kill GPC3+CD147+, but not GPC3-CD147+ HCC cells and do not cause severe on-target/off-tumor toxicity in a human CD147 transgenic mouse model.

One of the major obstacles to CAR-NK immunotherapy is the lack of efficient gene transfer methods in the primary NK cells. Many recent studies have demonstrated successful transduction of expanded NK cells with retroviral vectors, with efficiencies ranging from 27% to 52% after a single round of transduction (91). However, the insertional mutations associated with retroviral transduction and the deleterious effects on primary NK cell viability are among the most important limitations of this method in a clinical setting.

TCR-Engineered T Cell (TCR-T)

TCR-T cells are produced by modifying T cells with the gene of exogenous TCRs to specifically recognize the tumor antigen peptides-MHC complex (92). Since all tumor-derived proteins can be processed by proteasomes and presented by MHC, both the tumor surface and intracellular antigens can be targeted by TCR-T cells. Hence, TCR-T therapy should have broader applications than CAR-T.

Hepatitis C virus (HCV) infects approximately 130-150 million people globally and can lead to associated liver diseases, including HCC (93). Spear et al. generated HCV-specific TCR-T cells by genetically engineering T cells with a high affinity, HLA-A2-restricted, HCV NS3:1406-1415-reactive TCR (94). The results showed that HCV-specific TCR-T cells could induce regression of established HCV+ HCC *in vivo*, suggesting HCV-specific TCR-T therapy may be a plausible option for treating HCV-associated HCC.

A smaller percentage of Hepatitis B virus (HBV)-infection-derived HCC tissues retain the HBV gene expression, which can become TCR-T targets. In 2011, Gehring et al. generated HBV surface antigen-specific TCR-T cells from PBMC of chronic HBV and HBV-related HCC patients (95). These HBV-specific TCR-T cells were multifunctional and capable of recognizing HBV-related HCC tumor cells. In addition, a phase I clinical trial was conducted to evaluate the safety and efficacy of HBV-specific TCR-T in preventing the recurrence of HCC after liver transplantation (96) (NCT02686372).

As mentioned earlier, AFP is another HCC-associated TAA. Recently, Docta et al. reported the identification of a human HLAA2/AFP158-specific TCR (97), and a clinical trial using autologous T cells from HCC patients engineered with this AFP-specific TCR has been initiated and is ongoing (NCT03132792). In 2018, we identified multiple HLA-A2/AFP158-specific TCRs

from HLA-A2 transgenic mice using an immunization strategy with recombinant lentiviral priming and peptide boosting (98). Human T cells equipped with these TCRs showed potent anti-tumor activity *in vitro* and *in vivo*. Furthermore, systematic X-scan data showed that these TCR T cells have minimal or no cross-reactivity against human cells. A clinical trial using these TCRTs to treat HCC patients has been initiated (NCT03971747).

Other candidates target antigens for HCC TCR-T therapy involve GPC3 (99), New York esophageal squamous cell carcinoma 1 (NY-ESO-1) (100), and human telomerase reverse transcriptase (hTERT) (101). However, due to TCR's promiscuity, TCR-T cells may cross-react normal tissue MHC-peptide complex, leading to off-target toxicity. Both mouse and human-derived TCRs can produce off-target toxicity. The melanoma-associated antigen (MAGE)-A3/HLA-A1 TCR, although derived from humans, caused significant cardiac toxicity by targeting the cardiac muscle protein titin (102). On the other hand, although NY-ESO-1 TCRT has shown clinical anti-tumor efficacy, most other TCRTs have not been proven effective for patients. Several factors can be considered to improve the anti-tumor effect of TCR-T therapy, including prolonging the survival period of TCR-T *in vivo*, improving tumor infiltration, and preventing T cell exhaustion.

THERAPEUTIC VACCINE

The therapeutic vaccine is an immunotherapy that introduces tumor antigens into patients in various forms, overcomes the immunosuppressive tumor microenvironment, and then activates the patient's immune system to fight cancer (103). In 2010, Sipuleucel-T (Provenge) became the first therapeutic autologous vaccine approved by the U.S. FDA for the treatment of men with asymptomatic or minimally symptomatic castrate-resistant metastatic prostate cancer (104). At present, therapeutic vaccines used for HCC mainly include peptides, DCs, and oncolytic viruses. A summary of the past three years of clinical trials concerning therapeutic vaccine therapy for HCC is listed in **Table 3**.

In a phase I study, administration of AFP-derived peptides to 15 patients with HCC caused no adverse events and resulted in the generation of T cells with receptors that responded to the peptides. Among the 15 patients, one had a complete response, and eight had a slowing tumor growth. The T cells of the patient who had a complete response expressed a highly functional TCR induced by the peptide vaccines (105). In another phase I clinical trial, a GPC3-derived peptide vaccine was used in 33 patients with advanced HCC and reported that the vaccine was well-tolerated and elicited a high rate of GPC3-specific CTL responses (106). Another phase II study showed that GPC3-positive HCC patients treated with GPC3-derived peptide vaccine as an adjuvant therapy had a significantly lower recurrence rate after one year than patients who received surgery alone (24% vs. 48%, $p = 0.047$) (107). Multidrug resistance-associated protein 3 (MRP3) is a carrier-type transporter, and its high expression is associated with various cancer cells, including HCC (108). A phase I clinical trial evaluated the safety and immunogenicity of an MRP3-derived peptide as a vaccine in 12 HCC patients (109). The vaccination was well-tolerated, inducing MRP3-specific immunity in 72.7% of patients, with the median overall survival (OS) being 14.0 months (95% CI: 9.6–18.5). When the hTERT-derived peptide was used as a therapeutic vaccine in 14 HCC patients, the induction of hTERT-specific T cells correlated with the absence of HCC recurrence, suggesting a possible role of cellular immunity to hTERT in preventing recurrence (110).

DCs are responsible for T-cell stimulation and anti-tumor immune response enhancement (111). A phase I trial of autologous dendritic cell-based immunotherapy was conducted in inoperable primary HCC patients to evaluate the safety and feasibility. Eight HCC patients were enrolled in this trial, and in one patient, the tumor shrank and showed necrotic changes on computed tomography, whereas in two other patients, serum levels of tumor markers decreased after vaccination (112). Another phase II clinical trial results showed that the DCs vaccine pulsed ex vivo with HepG2 cell lysate was safe and well-tolerated with evidence of anti-tumor efficacy (113). Furthermore, infusion of DC in combination with TACE enhances tumor-specific immune responses more effectively than TACE alone, although the effect is insufficient to prevent the recurrence of HCC (114). Further clinical trials are ongoing, but the results have not yet been announced.

Oncolytic viruses are viral particles engineered to lyse tumor cells and induce anti-tumor immune responses. JX-594 (Pexa-

TABLE 3 | Clinical trials of therapeutic vaccines for HCC the last three years.

NCT ID	Target	Phase	Interventions	Country
NCT04251117	Neoantigen	1, 2	personalized neoantigen DNA vaccine (GNOS-PV02) and plasmid encoded IL-12 (INO-9012) in combination with pembrolizumab (MK-3475)	US, New Zealand
NCT04912765	Neoantigen	2	Dendritic Cell Vaccine and Nivolumab	Singapore
NCT04248569	DNAJB1-PRKACA fusion kinase	1	Peptide Vaccine Combined With Nivolumab and Ipilimumab	US
NCT03674073	Neoantigen	1	Dendritic Cell Vaccine Combined With Microwave Ablation	China
NCT04147078	Neoantigen	1	Dendritic Cell Vaccine	China
NCT04317248	NA	2	Multiple Signals loaded Dendritic Cells Vaccine	China
NCT04246671	HER-2	1, 2	TAEK-VAC-HerBy vaccine: Modified Vaccinia Ankara-BN (MVA-BN) virus	US
NCT03942328	Streptococcus pneumoniae	1	Autologous Dendritic Cells and Pneumococcal 13-valent Conjugate Vaccine	US

NA, Not available.

Vec) is currently the main oncolytic virus used in HCC clinical trials (115). JX-594 is a vaccinia virus with disruption of the viral thymidine kinase (TK) gene for cancer selectivity and insertion of human granulocyte-macrophage colony-stimulating factor (hGM-CSF) for immune stimulation (116). Heo et al. reported a randomized phase II clinical trial (NCT00554372) evaluating the feasibility of JX-594 in 30 HCC patients and found that high-dose JX-594 infusion achieved longer median OS compared to the low-dose arm (117). However, in patients previously treated with sorafenib (NCT01387555), the median OS was not significantly different in patients treated with JX-594 (118). Currently, two phase III clinical trials associated with JX-594 in treating advanced HCC is ongoing (NCT02562755, NCT03071094). In summary, although the therapeutic vaccine for HCC shows good prospects, its clinical application still requires further clinical trials to verify its efficacy and safety.

Although therapeutic vaccines have a promising future in treating HCC, some challenges still need to be overcome. First of all, the immunosuppressive tumor microenvironment (TME) of HCC can induce antigen-specific T cell tolerance, resulting in poor vaccine effectiveness. There is a growing need for new therapeutic strategies for HCC vaccines to enhance anti-tumor immune responses by counteracting the immunosuppressive TME. Chemotherapy can enhance the anti-tumor effect of cancer vaccines by overcoming the immunosuppressive TME, improving the cross-presentation of tumor antigens, and increasing the number of effector cells in the TME (119, 120). The combination of appropriately dosed systemic/local chemotherapy with cancer vaccines could be a potentially attractive option for HCC patients. Alternatively, the combination of ICIs and cancer vaccines could be an additional attractive option for HCC patients. Two clinical trials are currently underway using ICIs combined with a kinase peptide vaccine (NCT04248569) or a neoantigen DC vaccine to treat patients with HCC. Another major challenge is that most of the HCC vaccines presented in the current study are based on TAA. TAA is expressed not only on cancer cells but also on normal cells, resulting in an inadequate T-cell immune response and failing to elicit a robust clinical response. Neoantigens are newly expressed antigens in tumors that can be generated from viral proteins, normal cellular proteins, or mutated host genes (121). Since T cells that respond to neoantigens are not negatively selected during thymic maturation and can be primed into potent tumor-killing effector T cells, neoantigens are ideal targets for immunotherapy (122). Given the growing interest in neoantigen-based therapies, many clinical trials of therapeutic vaccines, including three clinical trials for HCC neoantigens, are registered at ClinicalTrials.gov.

CYTOKINES

Cytokines are key components of the immune system and play a critical role in the immune response to cancer. Because the immune system is capable of recognizing and destroying cancer cells, there has been great interest in the use of cytokines for cancer treatment in recent decades (123). Interferon-alpha (IFN-α) was the first cytokine approved by the U.S. FDA for the treatment of hairy cell leukemia (HCL) in 1986 (124). High-dose IL -2 was approved in 1992 for the treatment of metastatic renal cell carcinoma (mRCC) and in 1998 for metastatic melanoma (MM). Since initial approval, IFN-α has been extended to follicular lymphoma, melanoma, mRCC in combination with bevacizumab, and acquired immunodeficiency syndrome (AIDS)-related Kaposi's sarcoma.

A meta-analysis found that IFN-α could decrease mortality and early recurrence rates of HCC following curative treatment but exerted no effect on the late recurrence rate (125). Interestingly, the effect of adjuvant IFN-α on postoperative recurrence differs between HBV-HCC and HCV-HCC cases, indicating different strategies with adjuvant IFN-α should be used to treat HCC with different backgrounds. In another meta-analysis, the effects of adjuvant pegylated interferon (Peg-IFN) therapy on the survival of patients with hepatitis-related HCC after curative treatment were investigated (126). The results showed that adjuvant Peg-IFN therapy could improve recurrence-free survival (RFS) and overall survival (OS) in patients after curative treatment for hepatitis-related HCC without causing severe side effects.

Although IFN-α is gradually being displaced as the first-line anti-tumor drug, the new long-acting Peg-IFN continues to play an important role as a companion drug in HCC treatment (127). A preclinical study using the PDX HCC model has shown that interferon-β (IFN-β), in addition to its antiviral effect, can also exert anti-tumor activity through the JAK-STAT and p53 signaling pathways (128). In addition, IL-2 also has a pleiotropic effect on the immune system, which can increase the proliferation of T cells and activate their anti-tumor action. In patients with inoperable HCC, after treatment with IL-2, the survival rate of patients has increased (129).

However, cytokines as monotherapy has not fulfilled their original promise because parenteral administration of cytokines does not achieve sufficient concentration in the tumor, is usually associated with severe toxicity and induces humoral or cellular checkpoints. To circumvent these obstacles, cytokines are being investigated clinically with newly developed cytokine mutants (superkines), chimeric antibody-cytokine fusion proteins (immunokines), anti-cancer vaccines, and cancer-targeted monoclonal antibodies to enhance their ADCC or to preserve cellular response and anti-cancer efficacy.

CHALLENGES AND OPPORTUNITIES

The liver is an immunomodulatory organ containing a high density of innate and adaptive immune cells (130). Under physiological conditions, the liver is constantly exposed to intestinal antigens derived from food and microbial products. Accordingly, the liver has intrinsic immune tolerance that allows suppression of inappropriate inflammatory responses (131). The tumor immune microenvironment (TIME) is complex and consists of distinct populations of immune cells that influence response to immunotherapy and patient survival. The TIME of HCC is mainly composed of TAMs, MDSCs, cancer-associated fibroblasts

(CAFs), tumor-associated neutrophils (TANs), TILs, DCs, and extracellular matrix (ECM) (132). Compared with other solid tumors, HCC TIME exhibits a more potent immunosuppressive effect, and almost all cell subsets and numerous regulatory mechanisms contribute to HCC progression, posing a major challenge for effective cancer immunotherapy.

In recent years, cancer immunotherapy has made major breakthroughs, and its use in HCC has attracted increasing attention. However, there are still many problems, such as uncertain efficacy, low objective remission rate (OR), numerous side effects, and resistance to the drug even when patients benefit from it. Therefore, improving the tumor immunological microenvironment and balancing the body's immune response to benefit more patients is an urgent problem and a future development direction for HCC immunotherapy.

Reportedly, the OR of PD-1/PD-1 ICIs alone rarely exceeds 40%, and the OR of nivolumab and pembrolizumab in HCC did not exceed 20% (133). On the other hand, immune-related adverse events (IRAE) is an important reason affecting the widespread use of cancer immunotherapy (134). ICIs can cause inflammatory side effects, including hypophysitis, thyroid dysfunction, and diabetes. CAR-T therapy can cause some severe side effects such as cytokine release syndrome (CRS), neurotoxicity, and even death. In addition, 7%-9% of patients cannot be treated with CAR-T due to failure of CAR-T cell production (135). Other challenges in immunotherapy for HCC and other solid tumors include selecting more specific targets for immunotherapy, how to ensure that ACT cells reach the tumor site more effectively, and how to overcome immunosuppression by the tumor microenvironment.

Another major challenge in immunotherapy for HCC is the lack of markers to predict the effect of treatment. The latest report in the ESMO 2021 Annual Meeting shows that the survival of patients with advanced HCC treated with nivolumab was related to the Child-Pugh (C-P) liver function score at baseline (136). However, other methods of immunotherapy are mostly still in the early clinical stage, and there are no good indicators for predicting the therapeutic effect.

HCC is in a complex immunological microenvironment, so a single immunotherapy method or even immunotherapies alone have a lower remission and survival rate, and multitarget combination therapy should be the focus of future development. In a mouse model, four components of the host immunity consisting of a tumor antigen targeting antibody, an ICI, a powerful T cell vaccine, and a T cell-stimulating cytokine were required to eradicate large established tumors (137). Recently, the data of the phase III clinical trial IMbrave150 showed that atezolizumab in combination with the anti-angiogenic drug bevacizumab significantly reduced the risk of death in patients with advanced unresectable HCC and significantly improved patients' quality of life, making it the first first-line combination therapy for patients with unresectable advanced HCC (38). The combined use of ICIs against different targets may produce synergistic effects. Similarly, the combined application of immunotherapy with local therapy, such as radiofrequency ablation, radiotherapy, embolization, can also promote the efficacy of cancer immunotherapy (40, 41).

CONCLUSION

Although current immunotherapy for HCC has achieved some success, it still faces challenges such as low objective remission rate and adverse treatment reactions. Therefore, comprehensive analysis from multiple aspects to formulate personalized precision immunotherapy schemes for HCC patients, effectively evaluating and predicting the efficacy of immunotherapy, and adopting combined treatment strategies are urgent questions to be answered, and also the future trend of HCC immunotherapy research.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication.

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CAR T-Cell Therapy: Is CD28-CAR Heterodimerization Its Achilles' Heel?

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INTRODUCTION

Chimeric antigen receptor (CAR) T-cell therapy has dramatically expanded the success rate of cancer immunotherapy, especially in CD19-expressing blood cancers. Yet, it has also given rise to new complications, notably cytokine release syndrome, neurotoxicity, and, sometimes, fatal cerebral edema. The exact mechanisms of such toxicities across different CD19 CAR T-cell products, however, remain hotly debated. It was recently demonstrated that CARs containing a CD28 transmembrane domain (TMD) can heterodimerize with the endogenous CD28 receptor. Here, we hypothesize that, upon on-target activation, this heterodimerization is responsible for the increased sensitivity of CD19 CAR to $CD19^{low}$ brain mural cells, resulting in increased risk of developing severe neurotoxicity. This hypothesis may only be confirmed with a clinical trial comparing two CD19-CD28-TMD CARs differing only by targeted amino-acid mutations in the CD28 transmembrane domain.

T lymphocytes engineered with anti-CD19 chimeric antigen receptors (CAR) are emerging as powerful treatments for leukemia and lymphoma. The US Food and Drug Administration (FDA) approved two CD19 CAR T-cell products in 2017, which have shown clinical efficacy in the treatment of relapsed/refractory (r/r) acute lymphoblastic leukemia (ALL) and r/r non-Hodgkin lymphoma (NHL). The first CAR product, tisagenlecleucel (KYMRIAH/Novartis Pharmaceuticals Corp., thereafter referred to as CTL019), originally developed by CAR T-cell pioneer Carl June and colleagues, is currently approved for patients up to 25 years of age with r/r ALL and, since 2018, for adults with r/r NHL. In 2017, axicabtagene ciloleucel (YESCARTA/Kite Pharma, Inc., a Gilead Sciences Company, thereafter referred to as KTE-C19), is approved for adult patients with r/r NHL. Since then, two other CD19-CAR T-cell products have been FDA-approved: brexucabtagene autoleucel in 2020 (KTE-C19/TECARTUS/Kite Pharma, Inc., thereafter referred to as KTE-X19, a product differing only from KTE-C19 by an extra-step in the manufacturing process to exclude malignant circulating cells) for adult patients with r/r mantle cell lymphoma, and in 2021 lisocabtagene maraleucel (BREYANZI/Juno Therapeutics, Inc., a Bristol-Myers Squibb Company, thereafter referred to as JCAR-17, a product with the same CAR design as its previous generation JCAR-14) for adult patients with r/r large B-cell lymphoma. Notably, these CAR-T have the same single chain variable fragment (scFv), but different hinge (HD), transmembrane (TMD), and intracellular signaling domains (ICD) (**Figure 1A**).

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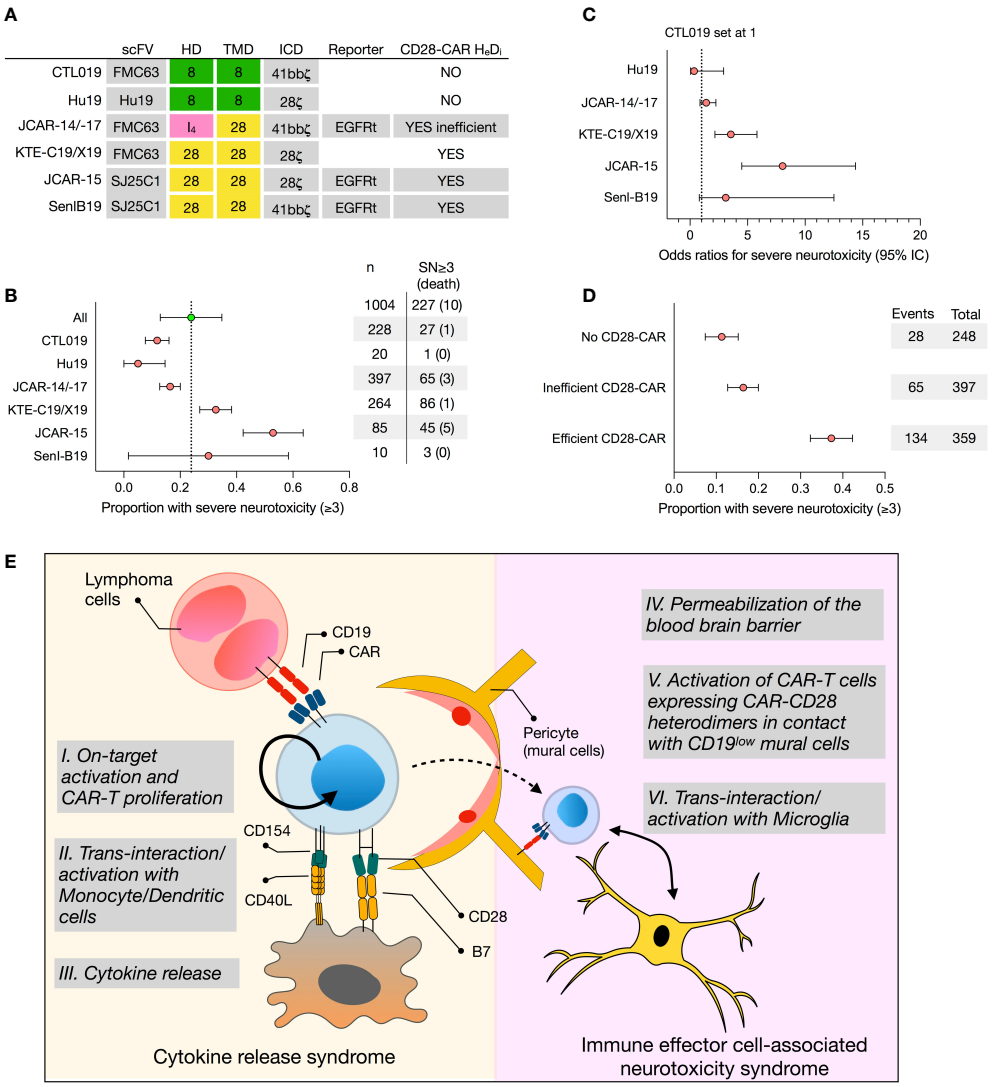

FIGURE 1 | Retrospective analysis of the proportion of severe neurotoxicity of selected CD19 CAR T-cell products and proposed model for CAR T cell-mediated neurotoxicity. **(A)** Construct designs of 5 selected CD19 CAR T-cell products, namely tisagenlecleucel (CTL019), Hu19, JCAR-14/-17, axicabtagene ciluleucel (KTE-C19), JCAR-15, and SenI-B19, differing by their hinge (HD) and transmembrane (TMD) domain. **(B)** Forest plot representing untransformed proportions of severe neurotoxicities (SN, grade 3 or higher) among patients treated with CAR T-cell products. Confidence intervals (95%) were calculated using binary random effect and DerSimonian-Laird methods with OpenMeta (http://www.cebm.brown.edu/openmeta/index.html). **(C)** The odds ratios of grade 3 or higher severe neurotoxicity comparing Hu19, JCAR14-/17, KTE-C19, JCAR15, and Senl B19 CAR-T products with CTL019 (set as reference) are shown. Calculations were made on SPSS Statistics (IBM, New York, NY) and based on a Pearson Chi-Square test and logistic regression tests assuming that clinical monitoring among the different studies and CD19 CAR-T-cell product is comparable. **(D)** Forest plot representing untransformed proportions of severe neurotoxicities comparing CARs with no CD28-CAR heterodimers (Hu19, CTL019), inefficiently formed CD28-CAR heterodimers (JCAR-14/17), and efficiently formed CD28-CAR heterodimers (SenlB19, JCAR-15, KTE C19). **(E)** CAR T cells, following on-target activation (I.), undergo several rounds of proliferation in the absence of antigen. This proliferation, fueled by CD40L-CD40 and B7-CD28 interactions with monocytes and/or dendritic cells (II.), ultimately results in cytokine release syndrome (CRS) (III.). In turn, CRS compromises the blood-brain barrier (IV.), allowing CAR T cells to penetrate the central nervous system (CNS). If CAR-CD28 heterodimers assemble on the cell surface, CAR T cells in the CNS interact with mural cells expressing low levels of CD19 (V.), as well as with microglia expressing co-stimulatory receptors (VI.), triggering immune effector cell-associated neurotoxicity syndrome (ICANS). *H_eD_i, heterodimerization; SN, severe neurotoxicity; HD, hinge domain; TMD, transmembrane domain; ICD, intracellular domain.*

SAFETY CONCERNS OF CAR T-CELL THERAPY

Although CAR T-cell therapy can induce spectacular clinical remission, safety remains an important concern with up to one-third of the patients developing significant toxicities, namely cytokine release syndrome (CRS) and immune effector cell-associated neurotoxicity syndrome (ICANS) (1, 2). By 2018, eighteen patients died after receiving CD19-CAR T-cells (3). CRS is the most commonly observed cause of toxicity coinciding

with the peak of CAR T-cell expansion (4), manifesting as fever, life-threatening hemodynamic instability with multi-organ failure, and, in some cases, fulminant hemophagocytic lymphohistiocytosis. ICANS is the second most common adverse event in CAR T-cell therapy ranging from mild cognitive impairment to an encephalopathic state characterized by confusion, delirium, seizures, and cerebral edema. ICANS can happen concurrently with or independently of CRS, a feature distinct from other organ-specific toxicities (1). The management of CRS and ICANS is currently based on administering anti-IL-6 monoclonal antibodies, sometimes together with corticosteroids. The latter are, however, avoided whenever possible to prevent inhibition of the infused CAR T cells (3). Importantly, ICANS normally resolves within 2-3 weeks after CAR T-cell infusion, although later recurrences are possible (3).

Notably, some CD19-CAR T cells products are more frequently associated with the development of severe ICANS (**Figure 1** and **Supplementary Table 1** and references therein). To address the rate of neurotoxicity among selected CD19 CAR-T cells products, we performed a linear regression analysis of reported severe neurotoxicity observed among 1004 patients treated with CTL019, Hu19, JCAR-14, JCAR-17, KTE-C19, KTE-X19, JCAR-15, and Senl-B19 (**Supplementary Table 1** and **Figures 1B, C**). The odds ratio of having grade 3 or higher severe neurotoxicity was significantly higher for KTE-C19 (3.5, 95% confidence interval (CI), 2.2-5.5) and JCAR-15 (8.0, 95% CI, 4.5-14.4) than with CTL019 (set to 1), JCAR-14/-17 (1.4 95%CI 0.9-2.3), Hu19 (0.3, 95% CI 0.05-2.9) and SenI-B19 (3.1, 0.8-12.5) (**Figures 1B, C**). These results are consistent with a recent meta-analysis (1). Additionally, they were also observed in a single clinical trial comparing side by side CAR T cells produced in the same conditions but engineered with a CAR design matching either CTL019 or KTE-C19 (5). Infusion with the KTE-C19-like product had to be suspended due to the high rate of neurotoxicity events (5). These data echo the unexpectedly high rate of severe ICANS 18/32, 56%) experienced during a phase 2 clinical trial, the ROCKET study, testing CD19-CAR T engineered with a CD28-HD, TMD and ICD (JCAR-15). This trial had to be terminated after the death of five patients from cerebral edema.

IDENTIFYING CAR FEATURES ASSOCIATED WITH TOXICITY

The mechanism behind the observed differences in CAR T-cell toxicity profiles between different products remains hotly debated. First, all main CAR T-cell products (accounting for >80% of infusions) share the same scFv, clone FMC63, ruling out major differences in CAR antigen affinity. Second, severe neurotoxicity was observed with CAR-T cells engineered with a CD28-ζ or 4-1BB-ζ ICD using lentiviral or retroviral transduction protocols (2, 6). Finally, no study found a link between the CD4/CD8 T cell ratio in the final CAR T-cell infusion product and neurotoxicity occurrence, even though the starting cell populations (PBMCs *vs.* enriched CD4 and CD8 T cells) and the expansion protocols (anti-CD3/CD28 beads *vs.* anti-CD3 alone) differed between them. Data from clinical studies show that tumor burden is a risk factor for developing CRS and ICANS (2). Recent preclinical studies showed that recipient's monocytes can be transactivated *via* the CD40-CD40L pathway and responsible for the bulk of IL-1 and IL-6 production during CRS, excluding models based solely on the direct interplay between CAR T cells and tumor cells. Indeed, blocking IL-6 receptor with tocilizumab or using IL-1 receptor antagonist prevents CRS in mouse models, providing a rationale for using these monoclonal antibodies for the treatment of CRS after CAR T cell therapy (7). Another comprehensive analysis found a significant association between elevated pre-treatment disease burden and high peak CAR T-cell expansion, concomitantly with blood brain barrier disruption and central nervous system-specific production of IL-6, IL-8, MCP1, and IP10 (6). There was, however, no significant correlation between severe neurotoxicity and transfused CAR T-cell number or tumor cell presence in the brain. More recently, single-cell RNA sequencing surveys revealed the existence of rare (0.2% of brain cells) CD19-expressing cells in the brain: mural cells, including pericytes and vascular smooth muscle cells, which support vasculature and are critical for the integrity of the blood-brain barrier. This suggests that lysis of brain mural cells by CD19-CAR T cells may be partly responsible for ICANS (8).

Yet, those results do not explain why there is an increased risk of developing ICANS when infusing KTE-C19/KTE-X19 or JCAR-15 as compared to CTL019 (**Figures 1B, C**). Importantly, KTE-C19/KTE-X19 and JCAR-15 share the same hinge, transmembrane, and signaling domain, all derived from the CD28 molecule. It is known that CD28 signaling, as compared to 4-1BB, results in faster and larger magnitude changes in protein phosphorylation, influencing the response and differentiation of effector T cells (9). However, in a recent phase 1 clinical trial, Brudno et al. showed that a humanized CD19 CD28-zeta CAR containing a CD28 signaling domain but a CD8-derived hinge (HD) and transmembrane (TMD) domain resulted in much reduced severe neurotoxicity: only 5% of patients who received Hu19-CD8-CD28-zeta T cells (Hu19) experienced it versus 50% of patients who received KTE-C19 (10). On the other hand, Li and colleagues tested a CD19-CAR with a CD28-TMD/HD but a 4-1BB intracellular costimulatory domain (Senl-B19) and reported 30% of ICANS (11). While it must be acknowledged that both studies included only a limited number of patients, these results suggest that the CD28 signaling domain is not sufficient to provoke neurotoxicity and, more importantly, that the roles of the HD and TMD in CAR T-cell-mediated neurotoxicity are currently underestimated.

THE IMPACT OF THE CAR TRANSMEMBRANE DOMAIN IN CAR T-CELL TOXICITY

Several lines of evidence suggest that the CAR's HD and TMD are not inert and can modulate CAR-T cell activation. Carl June and colleagues first showed that tonic signaling *via* CARs bearing a

CD28-TMD, but not a CD8-TMD, sustained *in vitro* T-cell proliferation up to 3 months in the absence of exogenous IL-2 and following a single TCR stimulation (12). Alabanza et al. found that CD19-CAR T cells produced significant higher levels of inflammatory cytokines upon CD19 recognition if featuring a CD28-TMD/HD instead of a CD8-TMD/HD (13). Crystal Mackall and co-workers demonstrated that swapping the CD8-TMD/HD in a CD19 4-1BB-ζ CAR for a CD28-TMD-HD lowered the antigen density threshold for CAR T-cell activation (14). Finally, we have recently demonstrated that CD28 TMD-containing CARs can recruit and dimerize with endogenous CD28, which normally exists as a homodimer on the cell surface, *via* a four amino acid motif in the TMD (15, 16). Consistent with this, in-depth analysis of the CAR interactome and signalosome revealed that the top interacting partner of a CAR bearing a CD28-TMD/HD is endogenous CD28, and CAR mediated-signaling is associated with phosphorylation of endogenous CD28 (9, 17). This association, through heterodimerization of the CAR with endogenous CD28 receptor *via* the CD28-TMD (15), may result in stronger signal transduction, facilitating CAR T-cell activation in the context of low levels of CAR antigen, such as in low-CD19 mural cells. It is interesting to note that CD28-CAR heterodimerizes inefficiently if the CAR is built with an IgG4-HD. In silico modeling of the hinge-hinge interactions suggested that the membrane proximity of the IgG4 hinge is too short to form CAR-CD28 inter-molecular disulfide bonds for stabilizing the CAR-CD28 heterodimerization, leading to preferential CAR-homodimerization (15). This observation may explain why JCAR-14/-17, engineered with a CD28-TMD and IgG4-HD, caused less ICANS than KTE-C19/KTE-X19 or JCAR-15 (**Figures 1B, C**). The risk of developing ICANS may thus be directly linked to the capacity to form CD28-CAR heterodimers (**Figure 1D**).

DISCUSSION

In conclusion, we hypothesize that, while CAR T cells are specifically activated on-target, they will undergo several rounds of proliferation in the absence of antigen. This proliferation may be fueled by CD40L-CD40 and possibly also by CD28-B7 trans-interactions with monocytes and/or dendritic cells, ultimately resulting in CRS. This process may compromise the blood-brain barrier, facilitating the trafficking of CD19-CAR T cells into the central nervous system. Depending on whether CAR-CD28 heterodimers are efficiently formed and present on the cell surface, CAR T cells could interact with low-CD19 mural cells and with microglia, known to express co-stimulatory receptors, ultimately initiating ICANS (**Figure 1E**). The fitness of the cells as well as the level of CAR expression could directly influence the severity of neurotoxicity. It will be extremely challenging to validate this hypothesis based solely on preclinical mouse models. In our opinion, its best demonstration will come from a clinical trial comparing side by side CD19-CAR T cells differing only by select amino acid mutations in their TMD. Such results may have an important impact on the future design and choice of CD19-CAR T cells for hematological but also autoimmune disease treatment.

AUTHOR CONTRIBUTIONS

LF and YM wrote this manuscript. YM performed the meta-analysis. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

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An Immunocompetent Microphysiological System to Simultaneously Investigate Effects of Anti-Tumor Natural Killer Cells on Tumor and Cardiac Microtissues

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Existing first-line cancer therapies often fail to cope with the heterogeneity and complexity of cancers, so that new therapeutic approaches are urgently needed. Among novel alternative therapies, adoptive cell therapy (ACT) has emerged as a promising cancer treatment in recent years. The limited clinical applications of ACT, despite its advantages over standard-of-care therapies, can be attributed to (i) time-consuming and cost-intensive procedures to screen for potent anti-tumor immune cells and the corresponding targets, (ii) difficulties to translate *in-vitro* and animal-derived *in-vivo* efficacies to clinical efficacy in humans, and (iii) the lack of systemic methods for the safety assessment of ACT. Suitable experimental models and testing platforms have the potential to accelerate the development of ACT. Immunocompetent microphysiological systems (iMPS) are microfluidic platforms that enable complex interactions of advanced tissue models with different immune cell types, bridging the gap between *in-vitro* and *in-vivo* studies. Here, we present a proof-of-concept iMPS that supports a triple culture of three-dimensional (3D) colorectal tumor microtissues, 3D cardiac microtissues, and human-derived natural killer (NK) cells in the same microfluidic network. Different aspects of tumor-NK cell interactions were characterized using this iMPS including: (i) direct interaction and NK cell-mediated tumor killing, (ii) the development of an inflammatory milieu through enrichment of soluble pro-inflammatory chemokines and cytokines, and (iii) secondary effects on healthy cardiac microtissues. We found a specific NK cell-mediated tumor-killing activity and elevated levels of tumor- and NK cell-derived chemokines and cytokines, indicating crosstalk and development of an inflammatory milieu. While viability and morphological integrity of cardiac microtissues remained mostly unaffected, we were able to detect alterations in their beating behavior, which shows the potential of iMPS for both, efficacy and early safety testing of new candidate ACTs.

Keywords: microphysiological system, 3D microtissue, natural killer cell, adoptive cell therapy, efficacy and safety assessment

INTRODUCTION

The lack of treatment options renders cancer one of the major health burdens of our time. The International Agency for Research on Cancer ranks cancer the second leading cause of death, with an estimated global impact of 19.3 million new cancer cases and approximately 10 million cancer deaths in 2020 alone (1). Current standard cancer treatments, i.e., radio- and chemotherapies as well as surgery are still confronted with multiple setbacks. While non-invasive approaches suffer from severe side effects, low efficacy, and therapy resistance, invasive surgery is only applicable for a limited number of localized and contained solid tumors (2). The search for safer and more durable therapies led to the interdisciplinary efforts in the fields of oncology and immunology and the development of cancer immunotherapies. Since the first description of immunotherapy in the 1980s (3), a large number of immunotherapeutic approaches have recently entered clinical evaluation (4). These novel therapies utilize different components of the immune system, such as antibodies or immune cells to instruct the patient's immune system to target the cancer cells (5, 6). Among emerging cancer immunotherapies, adoptive cell therapy (ACT) – a cell-based immunotherapy – holds promise to personalize immunotherapy for each patient's condition. Cytotoxic immune cells, such as $CD8^+$ T cells or natural killer (NK) cells are isolated from patients (autologous) or healthy donors (allogeneic). The cells are expanded *in vitro* and, in some cases, genetically engineered to increase their lifespan and *in-vivo* tumor-killing activity. High numbers of these immune cells are then transferred back into the patient to mediate anti-tumor activity (7). Although ACT offers an alternative treatment option for cancer patients, who are refractory to standard therapies, clinical trials of ACT with satisfactory results have been limited to hematologic malignancies (7, 8). For non-hematologic solid tumors, positive outcomes of such therapies are sporadic. For instance, despite of its success to suppress leukemia (9), NK cell-based ACT did not show any activity against metastatic melanoma in a clinical trial by Parkhurst et al. (10). It is worth mentioning that this clinical trial for ACT, and many other trials, were carried out after substantial *in-vitro* testing. The high anti-tumor activity evidenced in pre-clinical *in-vitro* screenings and the contrasting lack of efficacy afterwards *in vivo* highlight the poor *in vitro-to-in vivo* translatability of complex treatments. Such poor translatability has been attributed mainly to the widespread use of conventional two-dimensional (2D) cell cultures and animal models for pre-clinical evaluations (11).

Traditional 2D cell cultures fail to mimic the architecture and cellular heterogeneity of a solid tumor and cannot realistically recapitulate tumor-immune cell interactions. Likewise, animal models fail to reliably predict the efficacy and safety of immune-cell-based therapies due to critical immunological differences between animals and human beings (12, 13). During the past two decades, human cell-derived 3D tissue models have attracted more attention as tumor models for therapy screening as they overcome problems associated with 2D cell cultures. Under carefully designed culture conditions, tumor cells can form 3D microtissues (MTs) that are spherical, compact, and closely resemble *in-vivo* tumors in terms of structure, metabolism, loss of polarized cell morphology – as found in epithelial tissue-originated tumors, and gene-expression profiles (14).

Microphysiological systems (MPSs) combine advanced tissue models, such as 3D MTs, organoids or bioartificial tissues with microfluidic technology. Such systems are key innovations to further develop and refine advanced tissue models. The microfluidic components within MPSs can be designed to mimic different aspects of a tissue's microenvironment, such as physical and mechanical cues, and allow for interconnection of several tissue models (15). Currently developed MPSs can interconnect up to ten organ models for an experimental duration of up to four weeks (16, 17), making them suitable systems for systemic investigations of inter-tissue communication and for therapeutic testing. A wide range of single- or multi-tissue MPSs have been developed, among which are lung MPSs (18), gastrointestinal MPSs (19), liver MPSs (20), and immunocompetent MPSs (iMPSs).

The majority of reported iMPSs for immune-oncology purposes included either single tumor cells or 3D tumor MTs (TuMTs) that were embedded in hydrogel. Immune cells were added into microfluidic channels adjacent to the hydrogel, which were initially separated from the tumor cells (21). Such a configuration mimics the placement of cell components in the tumor microenvironment (TME). The hydrogel recapitulated the dense interstitial extracellular matrix (ECM) mesh of an *in-vivo* TME that immune cells have to penetrate to reach the tumor cells. Such realistic configurations helps to avoid overestimations of anti-tumor efficacy – which are likely to be obtained with systems that combine immune and tumor cells and enforce mutual interaction (22, 23). Furthermore, 3D constructs and iMPSs can help to mimic processes, such as immune-cell recruitment and migration, tumor infiltration, and TME-relevant immunosuppression (22, 24–26) that cannot be studied with conventional 2D cell cultures. Although it could be shown that TME can influence therapeutic outcomes, the indispensable use of ECM hydrogel limits the experimental readout options to microscopy measurements. Additionally, most studies focused on demonstrating treatment efficacy while the safety assessment of candidate ACTs was neglected. Two major risk factors of ACT include (i) on-target, off-tumor attack of healthy cells by cytotoxic immune cells, and (ii) the high level of soluble inflammatory chemokines and cytokines that are released during tumor recognition and elimination. Cytotoxic immune cells recognize tumor cells *via* pairing between specific sets of their surface receptors and corresponding ligands on the tumor cell surface. However, most of these ligands are also expressed on healthy cells, which can result in accidental on-target, off-tumor attack by these immune cells (27). Moreover, tumor-immune cells interactions can give rise to a complex of inflammatory chemokine and cytokines, eventually creating an inflammatory environment that is harmful to bystander organs (27–29). These adverse effects are difficult to predict even with animal models (30). Currently, most ECM hydrogel-based iMPSs are also not capable to simultaneously assess drug efficacy on the tumor and its toxicity on secondary, healthy organs.

In an effort to narrow the gap between *in-vitro* studies and the *in-vivo* situation, we developed an iMPS, which allows for co-culturing of anti-tumor immune cells and 3D MTs. With this

system, we aim at addressing current limitations of iMPS, such as the local confinement of immune cells in hydrogels, the low experimental throughput due to technical complexity, and missing models of healthy tissues for simultaneous toxicity testing. We used umbilical cord blood (UCB)-derived NK cells, whose anti-tumor activity involves both, direct interaction of NK cells with tumor cells and indirect tumor suppression *via* chemokine/cytokine signaling (31, 32). The 3D tumor model was established from the colorectal tumor cell line HCT116, the cells of which can produce their own ECM (33) and form compact, solid tumor-like MTs (TuMTs). 3D cardiac MTs (CarMTs) – formed from induced pluripotent stem cell (iPSC)-derived cardiac myocytes – were chosen as healthy-tissue model. All organ models were combined in the microfluidic chip that was developed for culturing of suspension cells and several, spatially separated, solid tissue models. Dedicated cell enrichment zones confined NK cells inside the medium reservoirs at the ends of the microfluidic channels (**Figure 1A**). During the experiments, NK cells either stayed in the cell enrichment zones or circulated back and forth along the same microfluidic channel (**Figure 1A**, ii and iii). Medium perfusion was actuated by gravity-driven flow by tilting the microfluidic chips, which ensured a constant exchange of soluble factors between the three tissue types. Different indicators of tumor-NK cell interaction were used: (i) NK cell-induced apoptosis of tumor cells, (ii) an elevated level of inflammatory chemokines [interleukin-8 (IL-8)] and cytokines [interferon-γ (IFN-γ), tumor necrosis factor-α (TNF-α), granulocyte-macrophage colony-stimulating factor (GM-CSF)], produced by TuMTs and NK cells, and (iii) invasion of NK cells into the TuMT volume (**Figure 1B**). To study the health status and detect structural damages of CarMTs, we recorded and analyzed the pattern of their spontaneous beating and measured soluble Troponin I in the cell culture supernatant. Our iMPS can potentially be used for early recognition of ACT-associated cardiotoxicity, particularly for NK cell-based ACT, the causes and consequences of which are still under investigation (34, 35).

MATERIALS AND METHODS

Microfluidic Chip

We modified the Akura™ Flow MPS discovery platform (InSphero, Schlieren, Switzerland), which was originally developed to study inter-tissue communication between 3D MTs (36). The microfluidic chip features two individual microfluidic channels with medium reservoirs at both ends. Each channel can accommodate up to ten fluidically interconnected MTs, which are located in the MT compartments (**Figure 1A**, i). To accommodate NK cells in suspension and to promote their direct interaction with 3D MTs, we adapted the chip by computer numerical control (CNC) micro-milling: (i) We introduced a drop-shaped cell-enrichment zone in the medium reservoirs (**Figure 1A**, ii and iii, left panels). The cell enrichment zone retained NK cells close to the entrance to the microfluidic channel after each tilting cycle and prevented them from accumulating in the low-flow zones in the corners of the reservoirs. For gravity-driven flow-based experiments, each microfluidic channel was supplied with 200 µL of fresh medium every day. This enabled the use of enough cell-culture medium to maintain all tissue models viable during the culturing periods. (ii) To facilitate direct cell-cell interactions between NK cells and MTs, we removed the barrier structures in the MT compartments (**Figure 1A**, ii and iii, right panels) and enlarged the microfluidic channels to a cross-section of 220 µm × 600 µm (height × width). More details on the performed

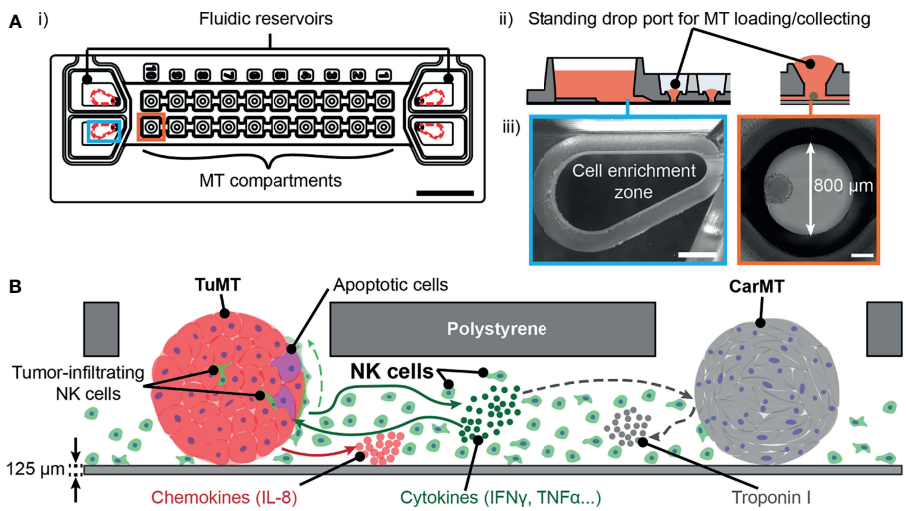

FIGURE 1 | **(A)** i) A schematic drawing of the iMPS, which is based on the Akura™ Flow platform (modifications indicated as red dashed lines). Scale bar: 10 mm. ii) Cross-sectional view of one reservoir and adjacent MT compartments. iii) Bright-field images of the cell enrichment zone inside one reservoir (scale bar: 1 mm) and a MT compartment with a TuMT (scale bar: 200 µm). **(B)** Schematic representation of on-chip cell cultures and possible interactions among components.

modifications are shown in **Figure S1**. Gravity-driven perfusion was induced by tilting the chip back and forth over a tilting angle of ±5° using the Akura™ Flow system (InSphero) inside a standard cell-culture incubator. Each tilting cycle included a 5-min halt at the positions of maximum tilting angle in both directions and a 1 h 40 min halt in a horizontal position. Detailed protocols for MT loading and system operation were also previously described by Lohasz et al. (36) and are demonstrated in **Video S1**.

Cell Cultures

Formation of 3D Tumor and Cardiac MTs

All cell cultures were maintained in a humidified incubator at 37°C and 5% CO_2 (Binder CB 220, Tuttlingen, Binder, Germany). The HCT116 human colorectal carcinoma cell line (ATCC® CCL-247) was purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). In brief, cells were cultured in cell culture flasks using a tumor-growth medium that contains Roswell Park Memorial Institute (RPMI) 1640 medium (BioConcept, Allschwil, Switzerland), 10% heat-inactivated fetal bovine serum (h.i. FBS; Gibco, Thermo Fisher Scientific, Waltham, MA, USA), 2 mM CTS™ GlutaMAX™ supplement (Gibco, Thermo Fisher Scientific), 1 mM sodium pyruvate (Gibco, Thermo Fisher Scientific), 1× non-essential amino acids (NEAA) (Merck, Darmstadt, Germany), and 50 µg/mL Kanamycin (BioConcept). Medium exchange was done every two days, and the cells were sub-cultured when reaching approximately 85% confluence.

The hiPSC line, CW30318CC1 (healthy donor, female), was obtained from the CIRM hPSC Repository funded by the California Institute of Regenerative Medicine (CIRM) *via* FujiFilm Cellular Dynamics (Madison, WI, USA). This cell line was differentiated to cardiac myocytes using the PSC Cardiomyocyte Differentiation Kit (Gibco, Thermo Fisher Scientific). iPCS-derived cardiac myocytes were maintained as monolayers in standard 12-well plates (Greiner Bio-One, Kremsmünster, Austria), pre-coated with Geltrex extracellular matrix (Gibco, Thermo Fisher Scientific) – diluted 1:50 in PBS without Ca^{2+} and Mg^{2+} (Gibco, Thermo Fisher Scientific). Medium exchange was performed twice a week with a cardiac myocyte growth medium that contains RPMI 1640, 2 mM CTS™ GlutaMAX™ supplement, 1× B27 supplement (Gibco, Thermo Fisher Scientific), and 50 µg/mL Kanamycin. No passaging was performed during cardiac myocytes maintenance as the cardiac myocytes hardly divide in culture. Only prior to MT formation, cells were lifted with TrypLE Express enzyme solution (Gibco, Thermo Fisher Scientific) for cell suspension preparation. Here, TrypLE Express enzyme solution was used to preserve the expression of cell surface markers (37).

For 3D MT off-chip production and maintenance, Nunclon™ Sphera™ U-shaped-bottom, 96-well plates (96U-well plates) (Thermo Fisher Scientific) were used. 3D TuMTs were formed from the HCT116 cell line in tumor-growth medium at an initial seeding density of 500 cells/MT. In brief, 100 µL of cell suspension containing 5000 cells/mL were seeded to each well of a 96U-well plate and spun down at 250 ×g for 2 min. TuMTs were ready to use at day 4 post seeding when their diameters reached approximately 400 µm. At this size, the necrotic core did not form yet, and the TuMTs were large enough to not escape the MT compartments.

We formed CarMTs in the cardiac myocyte growth medium using an initial seeding density of 6500 cells/MT. Cardiac myocyte suspension was prepared in cardiac myocyte growth medium, supplemented with 20% h.i. FBS. Then, 200 µL of the prepared suspension were seeded to each well of a 96U-well plate and spun down at 200 ×g for 3 min. After 24 h, a compact cell cluster formed, and the medium was replaced with standard cardiac myocyte growth medium. Spontaneous beating of CarMTs typically started between day 3 and day 4. To ensure reproducibility among experiments, we only used CarMTs from day 5 post seeding, when beating activity was observed in 100% of MTs. Regular microscopy inspection was carried out, and CarMTs with a weak beating activity or abnormal shapes were disqualified. CarMT size attained roughly 380 µm at day 5 post seeding with a slight shrinkage (~10-20 µm in diameter) over time due to compaction. Once formed, CarMTs can be maintained up to one month with medium exchange twice a week. During all preparation steps, all cells were kept at 37°C on a thermostat plate. Both types of MT were imaged with a Cell3iMager Neo plate scanning system (SCREEN Group, Kyoto, Japan) for quality check before each experiment.

NK Cells

Ethical Statement

Anonymized human umbilical cord blood (UCB) samples were collected from healthy newborns of both sexes at the University Hospitals Basel with parental informed consent. Relevant ethical regulations were followed, according to the guidelines of the local Basel ethics committee (vote 13/2007V, S-112/2010, EKNZ2015/335).

Sample Processing and Cell Isolation

After collection, UCB cells were processed by density gradient centrifugation. CD34 positive ($CD34^+$) and negative ($CD34^-$) cells were separated using EasySep CD34 positive selection kit II (StemCell Technologies, Vancouver, BC, Canada) and cryopreserved.

NK cells were isolated from the cryopreserved $CD34^-$fraction (hematopoietic stem cells removed) of human umbilical cord blood (UCB). We used the EasySep NK cells isolation kit (StemCell Technologies) to isolate NK cells and maintained them in NK cell-growth medium (RPMI 1640, supplemented with 10% h.i. FBS, 2 mM CTS™ GlutaMAX™ supplement, 1 mM sodium pyruvate, 1× non-essential amino acids (NEAA), 50 µg/mL Kanamycin, 50 µM β-mercaptoethanol (Gibco, Thermo Fisher Scientific), and 200 U/mL recombinant human interleukin-2 (IL-2; Peprotech, Cranbury, NJ, USA)) for up to two weeks. Fluorescein isothiocyanate (FITC)-conjugated CD45 (clone HI30), Phycoerythrin (PE)-conjugated CD3 (clone UCHT1), and Allophycocyanin (APC)-conjugated CD56 antibodies (clone HCD56) – all were purchased from StemCell Technologies – were used to confirm the purity of NK cells after isolation by flow cytometry (BD Fortessa, BD Biosciences, Franklin Lakes, NJ, USA). Additionally, 2-(4-amidinophenyl)-6-indolecarbamidine dihydrochloride (DAPI) stain (Merck) was used to assess cell viability in flow cytometry analysis. Where

indicated, NK cells were transferred to an NK cell-activating medium that contained 1000 U/mL IL-2 and 20 ng/mL of recombinant human interleukin-15 (IL-15; Peprotech) for 5 days before the experiments with a partial medium exchange at day 3. This pre-treatment was extensively used to enhance the overall proliferation and cytotoxic activity of NK cells against the target tumor (38, 39), especially before on-chip cultures.

Cell Labeling and Live-Cell Imaging

To spatially track NK cells within the chip, we labeled the cells with Cytopainter Cell Proliferation Staining Reagent – Green fluorescence, (Abcam, Cambridge, UK), diluted from 500× stock solution in NK cell-growth medium, for 40 min at 37°C before seeding them into the iMPS. BioTracker NucView Blue 405 Caspase-3 Dye (PBS) (Merck) was added directly into the cell-culture medium with a final concentration of 5 µM to visualize apoptotic cells during the experimental duration. Live-cell imaging was performed on a fluorescence Nikon TiE microscope (Nikon Europe B.V., Amsterdam, Netherlands) every day with a Plan Fluor 10× objective.

Static, Well Plate-Based Cultures of NK Cells and MTs

For static co-culture experiments, we combined NK cells with each type of MT in a 96U-well plate to assess the cytolytic activity and the cytokine release of isolated NK cells. Since HCT116 cells are relatively resistant to NK cell-induced cytolysis at a low effector-to-target (E:T) ratio (40), we used a high E:T ratio of 10:1 based on the initial seeding density of HCT116. First, the culture wells were pre-loaded with 100 µL of NK cell-growth medium, into which pre-formed MTs were transferred by contact transfer. Then, the wells were topped with 100 µL of NK cell suspension prepared in the same medium. For mono-cultures, the wells were filled with equal volumes of NK cell-growth medium without cells. The plate was placed inside a cell-culture incubator for three days without medium exchange. The morphological changes of MTs were monitored daily by bright-field imaging. MTs and the cell-culture supernatant were collected every day for performing viability assays and chemokine/cytokine quantitations. Jurkat cells, clone E6-1 (ATCC) were co-cultured with MTs using the same experimental layout as a negative control for tumor-killing activity. As positive controls for cardiotoxicity, CarMTs were treated with 30 µM Doxorubicin hydrochloride (Dox; Tocris, Bristol, UK) for 3 days in a Nunclon Sphera 96U-well plate before measuring Troponin I levels (41).

On-Chip Cultures in iMPS

TuMTs and CarMTs were transferred to the iMPS chip by using a contact-transfer technique at day 4 and at day 5 post seeding. Each microfluidic channel was loaded with six TuMTs and four CarMTs. Phenol red-free NK cell-growth medium was used for all on-chip cultures. Fluorescently labeled NK cells were spun down at 500 ×g for 5 min at 4°C and resuspended in a pre-warmed medium at a density of 1.67×10^6 cells/mL. Since a local administration of NK cells has proven to increase the amount of NK cells at the tumor site and can lead to better tumor suppression (42), we introduced the NK cell suspension directly into MT compartments through their loading ports. A total amount of 30 µL of NK cell suspension was loaded in 5 µL-dispensing steps into each TuMT-containing MT compartment. The chip was kept in a horizontal position (without perfusion) for 3 hours to prime the interaction between NK cells and MTs. Fluorescence imaging was conducted at the end of the priming period to check the presence of NK cells inside the MT compartments and cell enrichment zones. On-chip cultures were maintained for 3 days in the Akura™ Flow system inside a cell culture incubator. To assess the beating activity of CarMTs, we recorded 20 second-long AVI videos of each CarMT with a frame rate of 100 frames per second at the beginning and at the end of the experiments. Medium was exchanged daily during 3 days, and the removed medium was stored at -20°C for supernatant-based assays. After the co-culturing period, all unbound NK cells were removed from the microfluidic chip, and MTs were either (i) collected from the chip for ATP-dependent viability assays using the CellTiter-Glo 3D cell viability assay (Promega, Madison, WI, USA) or (ii) fixed for high-resolution microscopy.

Immunofluorescence (IF) Staining and High-Resolution Microscopy

MTs were fixed directly on chip after the experiment. In brief, all supernatant was removed from the reservoirs, then all microfluidic channels were flushed twice with 200 µL of phosphate-buffered saline (PBS, with calcium chloride (Ca^{2+}) and magnesium chloride (Mg^{2+}), Merck). Then, 100 µL of 2% formaldehyde in PBS (Merck) were added to the microfluidic channels for 10 min. All channels were flushed again three times with 200 µL of PBS (without Ca^{2+} and Mg^{2+}; Gibco), and MTs were blocked with 5% bovine serum albumin (BSA; Merck) in PBS (without Ca^{2+} and Mg^{2+}) for at least 1 hour. Depending on the experiments, different combinations of the following antibodies were used: Alexa Fluor (AF) 647-conjugated anti-Cytokeratin 18 (CK18; clone C-04; Santa Cruz Biotechnology, Dallas, TX, USA) – 1:50 dilution, AF594-conjugated polyclonal anti-CD69 (Bioss Antibodies, Woburn, MA, USA) – 1:200 dilution, and AF647-conjugated anti-human major histocompatibility complex (MHC) class I chain-related protein A and B (MICA/B) (clone 6D4, BioLegend, San Diego, CA, USA) – 1:50 dilution. All antibodies were diluted in 0.1% BSA in PBS (without Ca^{2+} and Mg^{2+}) and incubated with the MTs overnight at 4°C. The washing step was repeated and, when applicable, nuclear counterstaining was performed using NucBlue™ Live ReadyProbes™ Reagent (Hoechst 33342, Invitrogen, Thermo Fisher Scientific). We used a non-hardening mounting medium [ibidi Mounting Medium (Ibidi, Gräfelfing, Germany)] to fill the whole system before imaging.

We acquired 190 – 200 µm-thick Z-stacks of MTs in 2-µm steps in different culture conditions to detect tumor-infiltrating NK cells using either an inverted Leica SP8 (Leica Microsystem, Wetzlar, Germany) or an inverted Nikon A1 (Nikon Europe B.V.) confocal laser scanning microscope. To inspect the expression of MICA/B NK cell ligand on the surface of TuMTs

and CarMTs, 100 µm-thick Z-stacks of MTs were acquired in 0.4-µm steps using an X-Light v3 inverted spinning disk confocal microscope (Nikon Europe B.V.).

Enzyme-Linked Immunosorbent Assay (ELISA)

Cell-culture supernatant was collected into a low-binding Nunc™ 96-well polypropylene storage microplate (Thermo Fisher Scientific). We centrifuged the plate at 2000 ×g for 10 min to remove cell debris, then transferred all supernatant to a new storage plate of the same type and stored the supernatant at -20 °C until use. We employed a customized bead-based multiplex assay according to the manufacturer's protocol (BioRad, Hercules, CA, USA) to measure IL-8, GM-SCF, IFN-γ, and TNF-α inside the supernatant. Soluble Troponin I and soluble MICA (sMICA) were measured separately using a human cardiac Troponin I ELISA kit (Abcam) and a MICA human ELISA kit (Invitrogen, Thermo Fisher Scientific), respectively, according to the manufacturers' protocol and a Tecan Infinite M1000 Pro plate reader (Tecan, Männedorf, Switzerland).

Data Analysis

Microscope images were processed and analyzed using the Nikon NIS-Elements Advanced Research (Nikon Europe B.V.) or ImageJ software. Beating patterns of CarMTs were analyzed using the Musclemotion macro (43) in ImageJ (National Institution of Health, Stapleton, NY, USA). We used the Bio-Plex Manager software (BioRad) and Microsoft Excel (Redmond, WA, USA) to analyze data obtained from the multiplex assay and the Troponin I ELISA. This data was statistically analyzed with one-way or two-way ANOVA depending on the data set and visualized using GraphPad Prism 7 software (GraphPad Software, San Diego, CA, USA). Data obtained from sMICA ELISA assay was processed and statistically analyzed with GraphPad Prism 7. All statistical results were represented as mean ± standard deviation (SD) with a significance of $P < 0.05$, unless indicated differently.

RESULTS

Static, Well Plate-Based Cultures of NK Cells and MTs

Human NK cells are characterized by the absence of surface markers CD3 and the presence of CD56 ($CD3^-/CD56^+$). Therefore, after isolation, we quantitated the proportion of $CD3^-/CD56^+$ cells in the obtained population using flow cytometry. **Figure S2** shows that the purity of $CD3^-/CD56^+$ cells in our samples was up to 99.2%. The isolated NK cell population also appeared to express CD56 at different relative levels, which reflected the maturity and differentiation state of the NK cells. $CD56^{bright}$ NK cells with high CD56 surface expression are immature and less cytotoxic as compared to fully differentiated $CD56^{dim}$ NK cells with lower CD56 surface expression. These immature $CD56^{bright}$ NK cells, however, can become as potent as their mature, differentiated counterpart through additional cytokine treatment (39), hence the use of NK cell-activating medium in our experiments.

The two selected solid tissue models, TuMTs and CarMTs, were qualitatively assessed for their ectopic expression of membrane-bound MICA/B. MICA/B are the most studied ligands for the NK group 2D (NKG2D) activating receptor, which is universally expressed by NK cells (44). **Figure 2A** shows high expression levels of membrane-bound MICA/B on tumor cells within the optically accessible outer layers of the TuMTs, while MICA/B was poorly expressed in CarMTs. These results are supported by other studies that report high expression levels of MICA/B on the cell surface of tumor cells but not on the surface of normal cells (45). Based on this result, we expected our UCB-derived NK cells to recognize and eradicate tumor cells, while CarMTs should remain mostly unaffected. In static co-cultures of each MT type and NK cells, we closely monitored the size change of the MTs and their chemokine/cytokine production to scrutinize the extent and specificity of NK-cell-mediated tumor-killing activity. Our results indicated that, in static TuMT-NK-cell co-cultures,

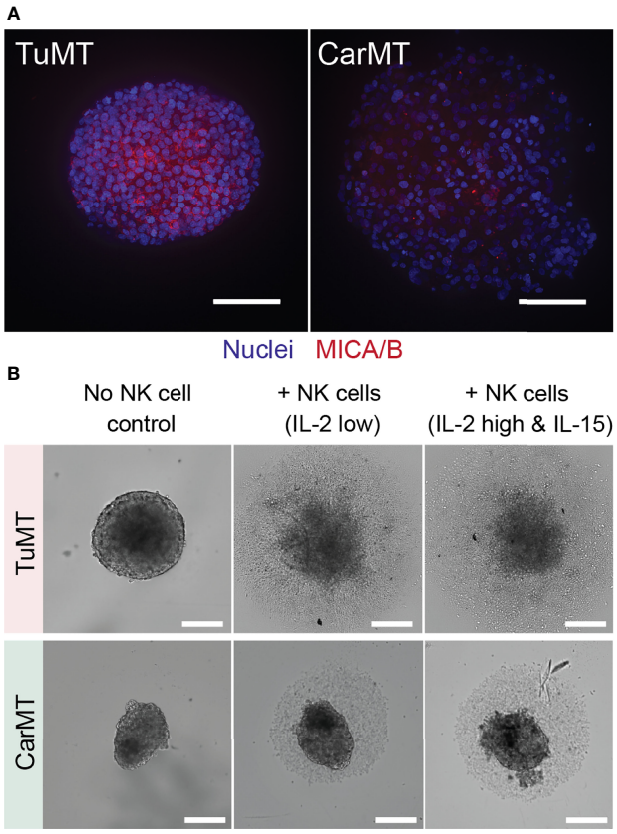

FIGURE 2 | **(A)** Membrane-bound MICA/B expression on TuMTs and CarMTs shown by maximum intensity projection of 100 µm-thick Z-stacks. Scale bars: 100 µm. **(B)** Specific anti-tumor activity of UCB-derived NK cells in static co-cultures in a 96U-well plate. Upper panels: TuMT disintegrated after 72 hours in co-culture with NK cells. Lower panels: CarMT remained intact in co-cultures with UCB-derived NK cells. Scale bars: 200 µm.

UCB-derived NK cells showed specific anti-tumor activity against TuMTs, regardless of the cytokine treatments. As shown in **Figure 2B** (upper panel), TuMTs completely disintegrated after 3 days in TuMT-NK-cell co-cultures. The fast cytolysis of TuMTs occurred within the first day and was confirmed by the low intracellular ATP-dependent viability of the MTs and increased IFNγ concentrations, as compared to the low basal levels in mono-cultures of NK cells or TuMTs (**Figures S3A, B**). As expected, NK cells did not affect the morphology and viability of CarMTs after 3 days in co-culture as shown in **Figure 2B** (lower panel) and **Figure S3C**. IFN-γ levels in CarMT-NK-cell co-cultures were at least 10-fold lower than in co-cultures of NK cells and TuMTs (**Figure S3D**).

Negative control experiments with Jurkat cells, which do not have cytotoxic activity against TuMTs, showed that a certain additional mass of suspension cells did not interfere with the growth of TuMTs (e.g., through nutrient competition). No IFN-γ was detected in this co-culture (data not shown).

iMPS: On-Chip Inter-Tissue Communication and Anti-Tumor Effects of NK Cells

To fully understand the dynamics and effects of each individual tissue model in our iMPS, we included multiple cell culture combinations, categorized into 3 groups as shown in **Table 1**: (i) mono-cultures of each individual tissue model, i.e., TuMTs, CarMTs, and NK cells, (ii) co-cultures of pairs of tissue models, and (iii) a triple culture that included all cell models. Data collected from mono-cultures were used as reference to assess the contributions of each tissue/cell type in the co-cultures and the triple culture, which revealed direct and/or indirect interactions.

Tumor Growth

To obtain a first assessment on how CarMTs and/or NK cells affect TuMT growth in different culture conditions, we tracked the diameter of 18 individual TuMTs per cell culture condition every day during three days. Absolute TuMT size changes were calculated in reference to the size at day 0 of the experiment, at which the MTs were transferred to the chip. As shown in **Figure 3A** and **Table S1**, TuMTs grew steadily and similarly in mono-culture and in co-culture with CarMTs during the three days of the measurements.

In contrast, we observed heterogeneous changes in TuMTs size when adding NK cells to cultures with TuMTs and the triple culture with both MT types (**Figure 3A** and **Table S1**). In those cultures, the average growth of TuMTs was significantly lower than that of TuMTs in mono-cultures and TuMT-CarMT co-cultures (**Figure S4A**). Several TuMTs, especially in the triple cultures, shrank between day 2 and day 3 of the experiment. These shrinking MTs shared a few commonalities: (i) higher NK cell accumulation within the MT compartment and the TuMT itself, (ii) lower viability as shown by higher caspase 3/7 activity through live-cell fluorescence imaging (**Figure 3B**, left panel), and low intracellular ATP content, measured at day 3 of the experiment (**Figure S5**). TuMTs that grew in diameter had none or only a few NK cells on their surface or in the peripheral zone (**Figure 3B**, right panel). This heterogeneous tumor growth suppression can be attributed to (i) different levels of interaction between NK cells and TuMTs during the initial priming period and/or the first day (**Figure S6**), (ii) poor tumor invasion by NK cells, and/or (iii) immune escape of TuMTs (46).

Proteolytic shedding of MICA's ectodomain is one of the major mechanisms used by tumor cells to escape from NK cell-mediated killing (44). The released sMICA has been shown to impair tumor cell recognition and cytotoxic activity of NK cells by direct blockage or by sMICA-induced internalization and degradation of NKG2D receptors (45). After confirming the membrane-bound expression of MICA on TuMTs (**Figure 2A**, left panel), we also measured the sMICA concentration released into the cell culture supernatant for different culture conditions. In agreement with the IF staining results for membrane-bound MICA/B (**Figure 2A**, right panel), we did not detect any sMICA in the mono-cultures of CarMTs or NK cells, as well as in the CarMT-NK cell co-cultures. In contrast, less than 5 pg/mL of sMICA were detected in mono-cultures of TuMTs in a 3-day experiment, which indicates the presence of MICA shedding (**Figure 3C**). Interestingly, MICA shedding was enhanced significantly in TuMT/NK cell co-cultures and in triple cultures, especially at day 3 of the experiment.

Tumor-Infiltrating NK Cells

As an additional endpoint analysis of the experiment, we fixed the MTs directly on-chip and stained them with CD69 and CK18 antibodies. CD69 is an activation marker for NK cells, while CK18 is an epithelium-specific cytoskeletal protein. CK18 plays a role in maintaining tissue integrity and was shown to be overexpressed in colorectal cancer tissues and cell lines, including the HCT116 cell line used in our work (47). It is important to note that only cells that were double positive for Green fluorescence and CD69 staining were qualified as $CD69^+$ NK cells, as NK cells were

TABLE 1 | All cell culture conditions in the microfluidic on-chip cultures.

Culture condition	Abbreviation	Tissue model/combination
Mono-culture	Mono	1. TuMTs 2. CarMTs 3. NK cells
Co-culture	Co	1. TuMTs – NK cells 2. CarMTs – NK cells 3. TuMTs – CarMTs
Triple culture	Triple	1. TuMTs – CarMTs – NK cells

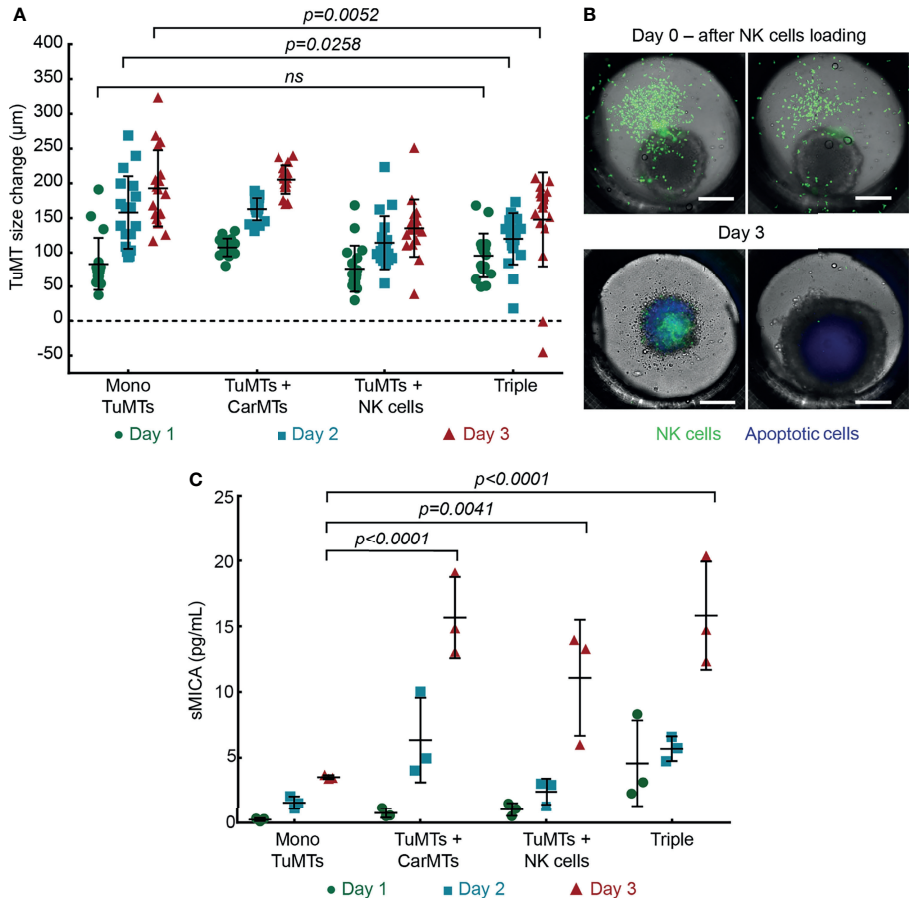

FIGURE 3 | **(A)** TuMT size changes monitored by bright-field imaging. Diameters of individual TuMTs measured at Day 1 (D1), Day 2 (D2), and Day 3 (D3) were normalized to their own diameter at day 0 (n = 18 MTs) (Mono: mono-culture, Triple: triple culture). Detailed statistical comparisons between conditions are shown in **Figure S4A** (ns: not significant). **(B)** Representative fluorescence images reflecting heterogeneous size changes of TuMTs in triple cultures. NK cells were labeled with Green fluorescence cell proliferation staining reagent, while apoptotic cells were labeled with Blue 405 Caspase-3 Dye. Scale bars: 200 µm. **(C)** Quantitation of sMICA released into the supernatant of different cell cultures during a 3-day experiment (n = 3). Detailed statistical comparisons between conditions are shown in **Figure S4B**.

stained with Cytopainter staining reagent prior to being seeded into the iMPS. We searched for tumor-infiltrating NK cells by taking Z-stacks of a total thickness of 190 – 200 µm and a Z-stack size of 2 µm using a confocal microscope. As shown in **Figure 4**, we found only a few NK cells that infiltrated the TuMTs across all examined MTs. Most of these tumor-infiltrating NK cells were $CD69^+$ and resided within the few outermost cell layers of the TuMT.

Chemokine/Cytokine Signaling

We next investigated the chemokine/cytokine signaling in different culture conditions inside our MPS. To evaluate the response of TuMTs to NK cell exposure, we measured IL-8 in the cell culture supernatant in all cell culture conditions. IL-8 level has been proven to increase in many types of solid tumors, including colorectal tumor. An increased serum IL-8 content is currently considered a potential predictive marker of higher grade tumor burden and resistance to chemo- and immune-therapies (48). As shown in **Figure 5A**, mono-cultures of TuMTs produced increasing amounts of IL-8, ranging from 83 ± 17 pg/mL at day 1 to 138 ± 18 pg/mL at day 2, and 147 ± 19 pg/mL at day 3. In contrast to the levels measured for TuMT mono-cultures, IL-8 levels significantly spiked in co-cultures of TuMTs and NK cells. They slightly fluctuated in the TuMT-NK-cell co-cultures but increased steadily in triple cultures – from 320 ± 120 pg/mL at day 1 to 430 ± 100 pg/mL at day 3 – and remained significantly different from those observed in TuMT mono-cultures. Mono-cultures of NK cells and CarMTs consistently produced less than 10 pg/mL of IL-8 (**Figure S7A**).

As an indicator for indirect anti-tumor activity of NK cells, we measured the amount of GM-CSF, IFN-γ, and TNF-α, which were released by NK cells into the cell-culture medium. In the absence of NK cells, all these cytokines of interest were undetectable (**Figures S7B-D**). However, NK cells in mono-culture abundantly produced all three cytokines. The absence of other T cell-associated cytokines, e.g., IL-6, and IL-17 (data not shown), confirmed that NK cells were the only source of these cytokines in our system (**Figures 5B-D**). All cytokine levels dropped slightly over time in mono-cultures

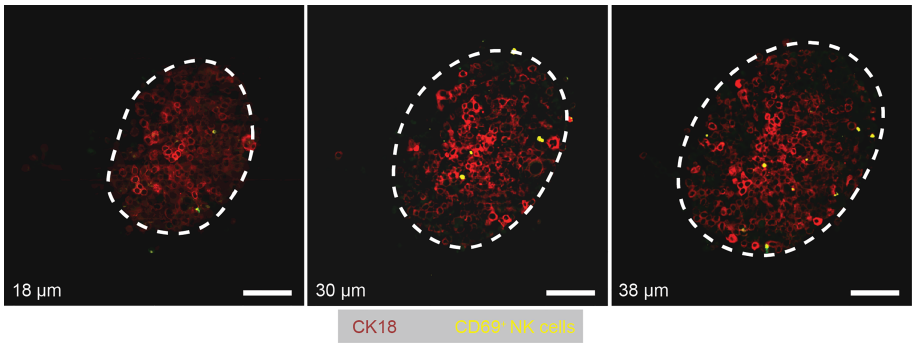

FIGURE 4 | Images showing tumor-infiltrating NK cells at different Z-positions in a TuMT. The Z-depth – in reference to the bottom of the TuMT – is indicated at the bottom left of each image. White dashed lines indicate the outer border of the TuMT in the corresponding Z-plane. Scale bars: 100 µm.

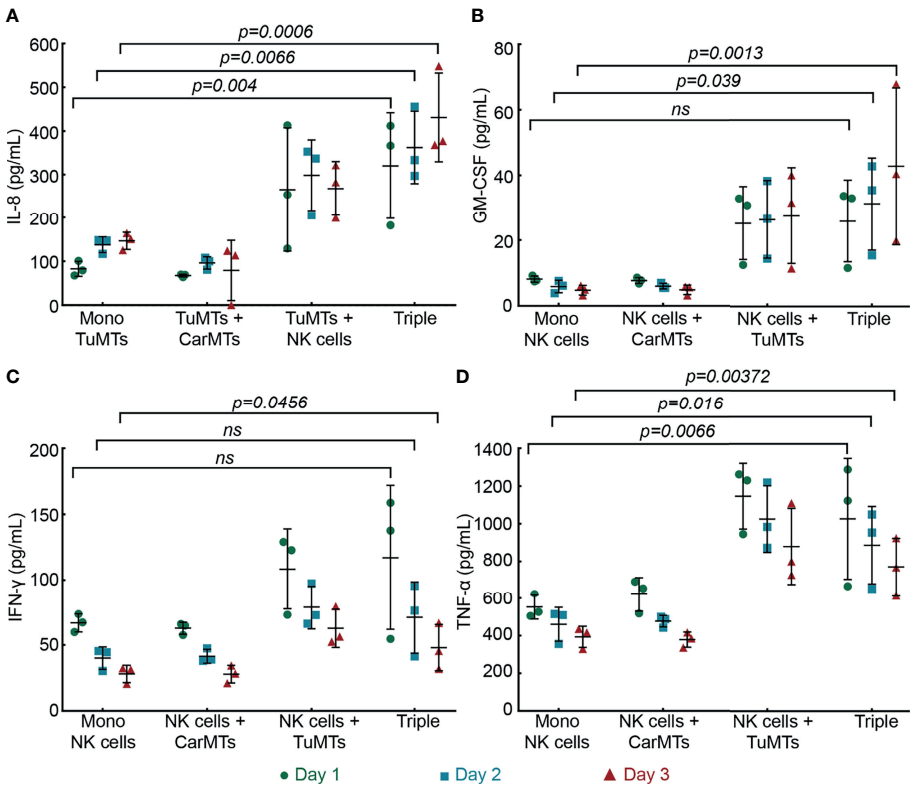

FIGURE 5 | Quantification of the chemokines/cytokines **(A)** IL-8, **(B)** GM-CSF, **(C)** IFN-γ, and **(D)** TNF-α in the supernatant of different cell culture conditions over a 3-day experimental period (n = 3) (Mono, mono-culture; Triple, triple culture) (ns: not significant). Detailed statistical comparisons between conditions are shown in **Figure S8**.

of NK cells, which is commonly observed when IL-15 was withdrawn from the cell culture medium (49, 50). The production of these cytokines was more extensive in co-cultures of TuMTs with NK cells, compared to mono-cultures of NK cells. However, all cytokines displayed different time-dependent dynamics. Over the experimental period, GM-CSF levels increased slightly in TuMT-NK cell co-cultures and triple cultures. In contrast, IFN-γ and TNF-α levels decreased slightly over time in all culture conditions. Interestingly, the IFN-γ level peaked at day 1 and dropped to a basal level within less than 2 days in the triple cultures, while there was no clear trend in TuMT-NK-cell co-cultures with respect to the basal level. TNF-α levels of all

culture conditions that included TuMTs remained higher than of those without tumors until the end of the experiment (**Figure S8**).

Besides NK cells that (i) moved inside the iMPS with the flow (**Video S2**) and (ii) interacted with TuMTs (**Figures 3**, **4**), a portion of NK cells did accumulate inside cell enrichment zones during the experiment. This circumstance offered us the possibility to parallelly investigate the indirect tumor growth suppression of NK cells through soluble mediators, i.e., chemokines/cytokines. Therefore, in a different set of triple cultures, we removed all NK cells inside the cell enrichment zones on day 1. **Figure S9** shows the drop of GM-CSF, IFN-γ, and TNF-α levels after NK cell removal, while IL-8 levels increased over the next two days, as all TuMTs continued to grow, albeit slowly (**Figure S9A**). This experiment further confirmed the dependency of the system on NK-cell-mediated signaling.

NK-Cell-Induced Anti-Tumor Activity Effects on CarMTs

Finally, we investigated the behavior of CarMTs for all described culture conditions by analyzing their physical interaction with NK cells, ATP-dependent viability, soluble Troponin I secretion, and beating patterns. The Troponin I level in patient serum is a clinically used biomarker that indicates cardiac injuries at elevated levels. Hence, we used soluble Troponin I as an indicator for health status of CarMTs in our iMPS. As shown in **Figure 6A** and **Figure S10A**, NK cells infiltrated CarMTs but did not negatively affect the viability of CarMTs under all culture conditions. Additionally, while CarMTs disintegrated after being exposed to 30 µM Dox for 3 days in a well-plate-based test, CarMTs in co-culture with NK cells and in triple culture on-chip remained intact (**Figure S10B**). The average Troponin I level per CarMT was lower than 10 pg/mL under all conditions in our iMPS as compared to the value obtained for Dox-treated MTs (47 ± 19 pg/mL per CarMT), which indicated that there was no structural damage of cardiac myocytes in the CarMTs (**Figure 6A**). Looking at the contraction profiles of the MTs, only a slight arrhythmia was observed in the CarMTs of CarMT-NK-cell co-culture (**Figure S11C**), while the CarMTs of the triple culture exhibited an obviously decreased beating rate (**Figure S11D**). In-depth analyses of the beating patterns of four exemplary CarMTs per culture condition revealed an increased average peak-to-peak time only in the CarMTs of the triple culture (**Figure 6B**). The majority of scrutinized CarMTs in the triple culture showed irregular contraction amplitudes as shown in **Figure 6C**. In fact, under this culture condition, CarMTs experienced highly elevated levels of both tumor-derived and NK cell-derived pro-inflammatory chemokines and cytokines, most importantly IL-8 and TNF-α (**Figures 5A, D**), that have been shown to negatively affect cardiac contractility *in vivo* (51, 52). Meanwhile, in CarMT-

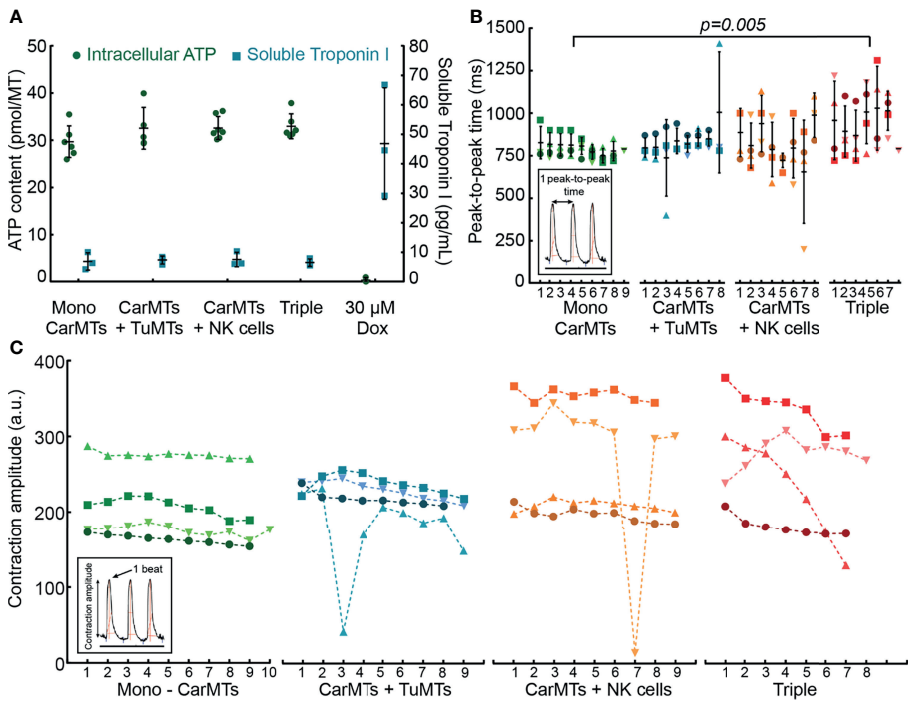

FIGURE 6 | **(A)** ATP-contents of CarMTs (n = 6) indicating viability and average soluble Troponin I, produced by individual CarMTs under different culture conditions (n = 3) (Mono, mono-culture; Triple, triple culture; Dox: Doxorubicin hydrochloride). **(B)** Changes in beating patterns of CarMTs under different conditions, represented as peak-to-peak time between contractions (ms). The figure shows exemplary patterns of four CarMTs per culture condition. The numbering on the X-axis indicates the peak-to-peak interval count of individual CarMTs within a 20-seconds recording window (peak-to-peak intervals are shown in the insert graph.). **(C)** Contraction amplitudes of CarMTs under different culture conditions. The figure shows exemplary patterns of four CarMTs per culture condition. The numbering on the X-axis indicates the beat count of individual CarMTs within a 20-second recording window (beat counting is illustrated in the insert graph). For each culture condition, contraction amplitudes of the same CarMT were connected by a dashed line in chronological order. The same color code was applied for the same CarMT in both **(B, C)**.

TuMT and CarMT-NK cell co-cultures, only one in four of CarMTs exhibited irregular contraction amplitudes, suggesting that detrimental effects on CarMTs may already be inflicted at a lesser extent by TuMTs or activated NK cells, or in other words, by lower levels of TuMT-derived IL-8 (**Figure 5A**) or NK cell-derived TNF-α (**Figure 5D**).

DISCUSSION

Despite the therapeutic potential of immune cell-employed ACT, there is still a large gap between *in-vitro* performance and *in-vivo* efficacy. This discrepancy mainly is due to a limited access to physiologically relevant tumor models and a lack of suitable *in-vitro* platforms for studying interactions between tumor models and immune cells. Interdisciplinary approaches will help to overcome these problems and increase the relevance of *in-vitro* screenings. While 3D tumor models offer more biological relevance (14, 53–56), iMPSs can provide physiological niches and critical cues for tumor models and immune cells to recapitulate physiological interaction (22, 24, 57–59). Although many initiatives show promising results, standardized iMPSs are still missing. Reasons may include the limited scalability of many academic approaches, the use of non-standardized and highly specialized tissue models, differences in screening protocols among laboratories, and the difficulty to transfer existing approaches to a broader community and clinical or industrial settings.

In this work, we developed an iMPS to study direct and indirect effects of anti-tumor NK cells on TuMTs and CarMTs. The inclusion of CarMTs into our iMPS allowed for a simultaneous assessment of potential off-target effects caused by anti-tumor NK cells. Interestingly, while a complete eradication of 3D TuMTs by NK cells was achieved in our static experiment, we observed heterogeneous tumor-killing activities by NK cells in our iMPS. This discrepancy shows how static culture conditions – where all cell components are forced to interact – can lead to an overestimation of ACT efficacy. Direct killing of TuMTs by NK cells was observed in our iMPS by a combination of different features: accumulation of NK cells in direct proximity of the MT, an increase in caspase 3/7 activity in tumor cells, and TuMT growth arrest or shrinkage. We also observed TuMTs that displayed a non-responsive phenotype within the same microfluidic channel. In such non-responsive TuMTs, growth and viability were not affected by the presence of NK cells (**Figure 3** and **Figure S6A**). As shown in **Figure S6B**, growth trajectories of TuMTs were determined by the level of direct interaction between TuMTs and NK cells within the first day of co-culturing rather than the number of NK cells in proximity of the TuMTs during the initial priming period. TuMTs that harbored large numbers of NK cells at day 1 grew slower or were subjected to growth suppression. Meanwhile, TuMTs harboring only a few NK cells at day 1 experienced less growth suppression that was mainly a consequence of the presence of NK cell-derived cytokines. The increased level of sMICA shedded from TuMTs (**Figure 3C**) may contribute to the observed ineffective NK cell-mediated tumor killing activity and heterogeneous tumor growth suppression.

Additionally, as shown in **Figure S6**, once the diameter of a given TuMT surpassed 500 μm, it was more likely to resist NK cell-induced growth suppression. It has been shown in other studies that TuMTs that are larger than 500 μm in diameter typically develop a hypoxic core (60, 61). Hypoxia induces hypoxia-driven adaptive mechanisms that promote tumor heterogeneity and survival while it imposes an immunosuppressive microenvironment on immune cells (62). Although the specific effect of hypoxia on NK cells remains elusive, it was shown to cause NK-cell dysfunction and to impair direct tumor-killing by tumor-infiltrating NK cells (63).

The chemokine/cytokine profiles of the on-chip cultures confirmed the reciprocal signaling between TuMTs and NK cells, indicating their interaction. We observed with all TuMTs that only a few NK cells infiltrated the TuMTs. Similarly, only low numbers of tumor-infiltrating NK cells were reported in different studies (46, 64, 65). Using whole-tissue sections of 112 patients and performing an *in-situ* quantification of immune cells, Halama *et al.* showed that NK cells were scarce in colorectal cancer tissue, even at early stages of the tumor development. NK cell invasion and retention in tumor tissue was low despite a high local level of chemokines, such as IL-8, and increased levels of IFN-γ and TNF-α in comparison to the mucosa adjacent to the tumor tissue (64). In another study, Rios-Doria et al. (66) developed xenograft models from different human tumor cell lines in humanized mice and quantified the presence of different immune-cell types within the tumor. Their results showed high infiltration levels for B-cells and dendritic cells, while tumor-infiltrating NK cells only amounted to between 1% and 5% of total tumor-infiltrating lymphocytes. Interestingly, the low number of NK cells – comparable to the number of tumor-infiltrating NK cells – was shown to induce resistance against NK cell-mediated killing in melanoma-resection-derived melanoma cell lines (67). To reveal the reasons for the resistance against NK-cell-mediated killing in our iMPS, extensive genomic and proteomic analyses will be required in future work.

We attributed the heterogeneous anti-tumor activity of NK cells to (i) different numbers and/or activation states of NK cells that could establish physical interactions with TuMTs within the first day of the experiment, (ii) chances of mutations within TuMTs that lead to immune-editing and eventually escape from NK cell-induced cell apoptosis (68), (iii) the development of tolerance for tumor cells by NK cells (69), or (iv) the activity suppression of NK cells by hypoxia and soluble factors shed from tumor cells (63, 70).

By including gravity-driven flow, our iMPS readily supported indirect, soluble-factor-mediated interaction between all included tissue models. This feature allowed us to simultaneously examine the response of TuMTs to NK cell-mediated killing activity and its impact on healthy CarMTs. A constant exposure of CarMTs to chemokines/cytokines, released by TuMTs-NK cells interaction – as shown in our iMPS – is difficult to realize with medium-conditioning approaches due to the short half-live times of IL-8 and TNF-α (half-live time of IL-8: 24 minutes, half-live time of TNF-α – 18.2 minutes) (71).

Interestingly, we did not detect any structural damages of cardiac myocytes in CarMTs for all our on-chip culture conditions. Nevertheless, the high level of chemokine and cytokine release by both TuMTs and NK cells upon interaction in the triple culture

significantly reduced the beating frequency and altered the contraction amplitude of CarMTs. This observation is in agreement with *in-vitro* and *in-vivo* investigations by Buoncervello et al. (52). In their *in-vitro* analysis, the authors dosed cardiac myocyte cultures with different inflammatory chemokines/cytokines, including IL-8, IFN-γ, and TNF-α for 48 hours. They reported an absence of cell death but various "severe phenotypic changes" in chemokine/cytokine-treated cardiac myocytes, indicating a dysfunction of contractile cytoskeletal elements. They also provided evidence on the link between colorectal tumor-induced heart systolic dysfunction and chronic systemic inflammation in their follow-up *in-vivo* experiment (52). Similar to our results, they did not detect any elevation of Troponin I in animal plasma across all conditions. Not many studies have yet investigated the risks associated with NK cell-based ACT so that NK cells are generally considered to cause less side effects than T-cells (72). However, this consideration may be due to the fact that suitable tissue models and testing platforms that could reveal more subtle adverse effects are still lacking. Moreover, solid tumors can alter the immune response and other signaling pathways in ways that can lead to unexpected damages to other organs. Therefore, more systemic approaches and better tools are needed for researchers to address these open questions.

CONCLUSION

In summary, we presented a simple and user-friendly iMPS that offers: (i) long-term triple culture of 3D TuMTs with anti-tumor NK cells and healthy CarMTs, (ii) microscopy-based observation of direct TuMT-NK cell interaction and evaluation of the spontaneous beating activity of CarMTs, (iii) collection of the cell-culture supernatant for chemokine/cytokine profiling, and (iv) harvesting of all tissue models for endpoint analyses. This proof-of-concept work is aimed at demonstrating the potential and versatility of iMPSs for use in immuno-oncology research, especially for early *in-vitro* validation and safety assessment of therapy approaches. More in-depth investigations regarding the growth inhibition of TuMTs, the specific receptor-ligand interactions involved in NK cell-mediated tumor killing, and more extensive profiling of the signaling-molecule repertoire remain topics for future work.

DATA AVAILABILITY STATEMENT

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

AUTHOR CONTRIBUTIONS

ON and PM conceived the approach and designed the experiments. TS established the pipeline to obtain human UCB samples. WW processed the UCB samples and prepared $CD34^-$ fractions of UCB. JL differentiated cardiac myocytes from human iPSCs and developed protocols for CarMT formation. ON performed all other experiments and analyzed the data. ON, PM, CL, and AH wrote the paper. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2021.781337/full#supplementary-material

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REVIEW
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Immunostimulatory Properties of Chemotherapy in Breast Cancer: From Immunogenic Modulation Mechanisms to Clinical Practice

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Breast cancer (BC) is the most common malignancy among females. Chemotherapy drugs remain the cornerstone of treatment of BC and undergo significant shifts over the past 100 years. The advent of immunotherapy presents promising opportunities and constitutes a significant complementary to existing therapeutic strategies for BC. Chemotherapy as a cytotoxic treatment that targets proliferation malignant cells has recently been shown as an effective immune-stimulus in multiple ways. Chemotherapeutic drugs can cause the release of damage-associated molecular patterns (DAMPs) from dying tumor cells, which result in long-lasting antitumor immunity by the key process of immunogenic cell death (ICD). Furthermore, Off-target effects of chemotherapy on immune cell subsets mainly involve activation of immune effector cells including natural killer (NK) cells, dendritic cells (DCs), and cytotoxic T cells, and depletion of immunosuppressive cells including Treg cells, M2 macrophages and myeloid-derived suppressor cells (MDSCs). Current mini-review summarized recent large clinical trials regarding the combination of chemotherapy and immunotherapy in BC and addressed the molecular mechanisms of immunostimulatory properties of chemotherapy in BC. The purpose of our work was to explore the immune-stimulating effects of chemotherapy at the molecular level based on the evidence from clinical trials, which might be a rationale for combinations of chemotherapy and immunotherapy in BC.

Keywords: breast cancer, chemotherapy, immunotherapy, immunogenic modulation, clinic trial

INTRODUCTION

Breast cancer (BC), a highly heterogeneous disease, is the most common cancer among women (1). The 2021 global cancer statistics showed about 2.3 million newly diagnosed BC and approximately 0.69 million BC deaths, with a higher incidence than lung cancer (2, 3). The survival rates of BC vary widely worldwide, with an estimated five-year survival rate of 80% in developed countries while less than 40% in developing countries (1, 4). BC is generally comprised of luminal A, luminal B, HER2 overexpression, basal-like triple negative breast cancer (TNBC), and other special subtypes

proposed by St. Gallen International Breast Cancer Conference in 2013 (5). Subtype identification provides a fundamental basis for decision making in the therapeutic management of BC (6). Thus, to select the most appropriate systemic therapy for BC, subtype classification is quite necessary (7). Modern therapy of BC involves a combination of surgery of operable tumors, chemotherapy (neoadjuvant/adjuvant), endocrine therapy, targeted therapy, radiotherapy and immunotherapy (8). The initial approach for BC was aggressive surgery in the early 20th century (6). And the types of chemotherapy and their indications have experienced rapid growth since radical mastectomy evolved from more aggressive to less aggressive (9). In 2001, a National Institute of Health consensus panel concluded that owing to a clear survival benefit by adjuvant polychemotherapy, it should be recommended to the majority of women with localized BC regardless of lymph node, menopausal, or hormone receptor status (10). Since then, the status of chemotherapy in the treatment of BC has been established.

It is traditionally recognized that BC is characterized by low tumor mutation burden (TMB) and poorly immunogenic. However, recent evidence revealed that infiltrating lymphocytes (TILs) and programmed cell death-ligand 1 (PD-L1) were expressed in a considerable proportion of HER2+ BC and TNBC patients (11). Cancer immunotherapy aims to provoke an immune response by either enhancing the cytotoxic potential of immune cells or blocking the immunosuppressive tumor microenvironment (12). Immunotherapy has a rich content including immune checkpoint blockade, adoptive cell therapies, adoptive cell therapies vaccines and oncolytic viruses (13). Among these therapy strategies, the United States Food and Drug Administration (FDA) has approved immune checkpoint inhibitors (ICIs) targeting PD-1 (programmed cell death receptor 1), PD-L1 (programmed cell death 1 ligand 1), and CTLA-4 (cytotoxic T-lymphocyte-associated antigen-4) for treatment of solid tumors such as BC (14, 15). Among all subtype of BC, TNBC, the most invasive BC, was regarded as the most immunogenic type due to the presence of tumor neoantigens, and high levels of lymphocytic infiltration, mutation (16). The results of the IMpassion130 trial demonstrated a substantial overall survival (OS) benefit and brought BC into immunotherapy era (17). Thus, considerable effort has been dedicated to combination of standard-of-care chemotherapies with immunotherapy in BC.

Chemotherapy was previously thought to be solely immunosuppressive, but recent data showed that it might also possess immunostimulatory properties. In this mini review, we summarized the updated clinical trials on immunotherapy and chemotherapy combinations in BC. More importantly, we discussed recent literature on the immunomodulatory effects of chemotherapy with a focus on immunostimulatory function.

IMMUNE CHECKPOINT INHIBITORS COMBINED WITH CHEMOTHERAPY IN BC

First, the IMpassion130 (NCT02425891) trial funded by F. Hoffmann–La Roche/Genentech comparing chemotherapy plus placebo versus chemotherapy plus atezolizumab brought BC into the immunotherapy era. In this phase 3 trial, 902 patients with untreated metastatic TNBC were randomly assigned (in a 1:1 ratio) to receive atezolizumab plus nab-paclitaxel or placebo plus nab-paclitaxel. Patients received atezolizumab 840mg or placebo intravenously on days 1 and 15 and received nab-paclitaxel at a dose of 100 mg/m^2 that administered intravenously on days 1, 8, and 15 of every 28-day cycle. This trial displayed a substantial progression-free survival (PFS) benefit in patients with metastatic TNBC either the intention-to-treat population or the PD-L1–positive subgroup. With a median follow-up of 12.9 months, among the ITT population, the median PFS was significantly prolonged after the addition of atezolizumab as compared to chemotherapy alone (7.2 vs 5.5 months); further, in the PD-L1 positive population, the respective PFS benefit was more improved (7.5 vs 5.0 months). Regarding the intention-to-treat analysis, the median OS was 21.3 months (atezolizumab plus nab-paclitaxel) and 17.6 months (placebo plus nab-paclitaxel), while in the PD-L1 positive population, the OS was increased 9.5 months with the addition of atezolizumab (25.0 vs. 15.5 months) (18). The above data has attracted significant interest in clinical scientist, and then a series of ongoing trials that were design for chemotherapy combined with immunotherapy begun to emerge. Subsequent randomized Phase III trial IMpassion131 (NCT03125902) evaluated first-line paclitaxel with or without atezolizumab for unresectable locally advanced/metastatic TNBC. 651 eligible patients were randomized 2:1 to atezolizumab plus paclitaxel or placebo plus paclitaxel. At the primary analysis, no significant improvement of PFS or OS was observed while adding atezolizumab to paclitaxel and the reasons for this remain unclear. At a median follow-up of 9.0 months (atezolizumab-paclitaxel arm) and 8.6 months (placebo-paclitaxel arm), in the PD-L1-positive population, median PFS was 6.0 months and 5.7 months, respectively. Final OS results also showed no difference between arms (atezolizumab-paclitaxel arm 22.1 months versus placebo-paclitaxel arm 28.3 months). Results in the ITT population were in accord with the PD-L1-positive population. Conclusions from IMpassion131 also contrasted with results from the KEYNOTE-355 trial (we will further elaborate below) that evaluated a more extensively chemotherapy backbones (including both paclitaxel and nab-paclitaxel, as well as gemcitabine/carboplatin) with a different immunotherapy agent, pembrolizumab (15). Both IMpassion130 and IMpassion131 excluded patients with early relapse (disease progression within 12 months of chemotherapy for early breast cancer), however IMpassion132 (NCT03371017) is one of the first trials prospectively focusing on the early relapsing TNBC population. The IMpassion132 trial combined atezolizumab with two commonly used non-taxane chemotherapy regimens (gemcitabine plus carboplatin, or single-agent capecitabine), which aimed to determine whether similar improvement observed in the IMpassion130 could be achieved with an alternative chemotherapy backbone in the case of early relapse. This phase III trial is ongoing and the primary end point is OS in the ITT population (19).

KEYNOTE-355 (NCT02819518), compared pembrolizumab plus chemotherapy (nab-paclitaxel; paclitaxel; or gemcitabine plus carboplatin) with placebo plus chemotherapy, showed a significant and clinically meaningful improvement in PFS among patients with locally recurrent inoperable or metastatic TNBC with combined positive score(CPS)of 10 or more. Pembrolizumab combined chemotherapy showed a positive result both in patients CPS≥10 and CPS≥1. Median PFS was 9·7 months and 5·6 months (pembrolizumab–chemotherapy and placebo–chemotherapy, respective) among patients with CPS≥10. Among patients with CPS≥1, median PFS was 7·6 and 5·6 months. Results in the ITT population were 7·5 and 5·6 months. These findings suggested a role for the combination of pembrolizumab and chemotherapy for the first-line treatment of metastatic TNBC (20). Compared to KEYNOTE-355, another ongoing phase III clinical trial KEYNOTE-522 (NCT03036488) mainly focused on patients with early TNBC. A pathological complete response (pCR) at the time of definitive surgery and event-free survival (EFS) in the ITT population were the two primary end points. A total of 1174 patients with previously untreated stage II or stage III TNBC were randomly assigned (in a 2:1 ratio) to the pembrolizumab–chemotherapy group (784 patients) or the placebo–chemotherapy group (390 patients). Patients in pembrolizumab–chemotherapy group received therapy with pembrolizumab plus paclitaxel and carboplatin. Placebo–chemotherapy group received placebo plus paclitaxel and carboplatin, and both groups received doxorubicin–cyclophosphamide or epirubicin–cyclophosphamide. At the first interim analysis of 602 patients, the percentage of patients with a pCR was 64.8% (pembrolizumab–chemotherapy group) and 51.2% (placebo–chemotherapy group). In the PD-L1–positive population, the percentage of patients with a pCR was 68.9% versus 54.9% (pembrolizumab–chemotherapy group versus placebo–chemotherapy group), while the percentage of patients with a pCR was 45.3% versus 30.3% (pembrolizumab–chemotherapy group versus placebo–chemotherapy group) in the PD-L1–negative population. The patients who received pembrolizumab showed a significantly higher pathological complete response percentage than those who received placebo. Across all treatment phases, the incidence of treatment-related adverse events of grade 3 or higher was 78.0% and 73.0%, including death in 0.4% (3 patients) and 0.3% (1 patient), in the pembrolizumab–chemotherapy group and placebo–chemotherapy group, respectively (21).

The above clinical trials including chemotherapy plus atezolizumab or pembrolizumab not only provide powerful evidence for the benefits of chemotherapy combined with immunotherapy, but also provide us new treatment alternatives, which enable more BC patients to benefit from immunotherapy. Several clinical trials have been designed to explore the potentiality of chemotherapy combined with immunotherapy with a variety of patterns. I-SPY2 trial which focus on the BC patients with a high-risk and stage II/III evaluated pCR rates of pembrolizumab combined with neoadjuvant chemotherapy. Both NCT02513472 and NCT03051659 paid attention to the combination of pembrolizumab and eribulin. A summary of completed and ongoing Phase Ib/II and Phase III clinical trials in BC is presented in **Tables 1**, **2**.

ENHANCING THE ANTIGENICITY OR ADJUVANTICITY OF BC CELLS

Impact of Chemotherapy on Tumor Antigenicity

In recent years, in the absence of infection, a novel type of cell death has been shown to be capable of triggering $CD8^+$ T cells-mediated responses against "dying cell" neoantigens through cell stress-related processes, which has become an emerging research interest and has been referred to as "immunogenic cell death" (ICD) (33, 34). Chemotherapy-mediated ICD is also governed by cell stress, where the involved fundamental processes are regulated by cytoprotective pathways such as autophagy and endoplasmic reticulum stress (35, 36). Evidence available indicated that obviously enhanced tumor antigenicity induced by chemotherapeutic drugs might be caused by elevated major histocompatibility complex (MHC) expression and presentation of tumor neoantigens (TNA) or tumor-associated antigens (TAA) (37). Many existing chemotherapeutic agents and ionizing radiation can enhance the tumor antigenicity and the adjuvanticity effects of malignant cells when they elicit ICD and anticancer immunity (38). Anthracyclines, the cornerstone of chemotherapy regimens for BC, have been proven to one initiator or potentiator of ICD process through activation of the NLRP3 inflammasome (39). Previous preclinical studies demonstrated that 5-fluorouracil (5-FU) directly induced the upregulation of membrane-associated carcinoembryonic antigen (CEA) and MHC molecules in BC cell lines (40). Docetaxel and doxorubicin were also shown to promote the expression of antigen-processing machinery components, resulting in increased loading of MHC-I molecules in BC cells (41). Topotecan characterized as topoisomerase I-targeting drug showed immunogenic potential in TNBC cells by stimulating MHC I expression, inducing the secretion of interferon-β and activation of type I IFN signaling (42). Furthermore, an increasing expression of antigen-presenting molecules (MHC-I, MHC-II, and CD1d) was observed after gemcitabine and cyclophosphamide treatment in 4T1 mammary carcinoma cells, and thus promoting the antigen presenting behavior of dendritic cells (DCs) (43–45). The elevated expression of MHC-II and CD86 mediated by novel chemotherapeutic compound was also reported in TNBC cell line MDA-MB-231 (46). There are clear associations between the presence of MHC molecules and clinical outcomes in BC (47). Higher expression of MHC class II (MHC II) pathway genes expressions might predict longer disease-free survival (DFS) and low risk of recurrence for TNBC patients (48). Collectively, the upregulation of MHC-related molecules could remodel the immunopeptidome of cancer cells after chemotherapy, and thus enhancing their antigenicity.

TABLE 1 | Summary of primary phase III clinical trials adding immunotherapy to chemotherapy in breast cancer.

Trial (National Clinical Trial Identifier)	Phase	Interventions	Patients enrolled	Number of patients	Primary endpoint	Key Results	Ref
IMpassion130 (NCT02425891)	III	Nab-paclitaxel ± atezolizumab	Untreated metastatic TNBC unselected for PD-L1	902 (451 treated with atezolizumab)	PFS OS	Median PFS 7.2 months VS 5.5 months(PD-L1+ 7.5 months) Median OS 21.3 months VS 17.6 months (PD-L1+ 25.0months)	(18)
IMpassion131 (NCT03125902)	III	Paclitaxel ± atezolizumab	Inoperable locally advanced/ metastatic TNBC	651 (293 PD-L1 +)	PFS	Median PFS 6.0 months VS 5.7 months(PD-L1+ 7.5 months)	(15)
IMpassion132 (NCT03371017)	III	First-line chemotherapy (capecitabine [mandatory in platinum-pretreated patients] or gemcitabine+ carboplatin) ± atezolizumab	Early relapsing metastatic TNBC	approximately 350	OS	Ongoing	(19)
Impassion031 (NCT03197935)	III	chemotherapy (nab-paclitaxel +doxorubicin + cyclophosphamide) ± atezolizumab	Early-stage TNBC (untreated stage II–III)	333 (165 treated with Chemotherapy+ atezolizumab)	pCR	Ongoing at data cutoff (April 3, 2020) pCR 58% VS 41% pCR 69% VS 49% (PD-L1+)	(22)
KEYNOTE-119 (NCT02555657)	III	pembrolizumab arms VS chemotherapy arms	mTNBC (treatment with anthracycline or taxane before)	622 (312 pembrolizumab)	OS(PD-L1 CPS>=1 or CPS>=10)	Median OS 10·7 months VS 10·2 months (PD-L1 CPS>=1) 12.7months VS 11.6 months (PD-L1 CPS>=10) 9·9 months VS11.8 months (overall population)	(23)
KEYNOTE-355 (NCT02819518)	III	chemotherapy (nab-paclitaxel; paclitaxel; or gemcitabine plus carboplatin) ± Pembrolizumab	Previously untreated locally recurrent inoperable or mTNBC	847 (566 pembrolizumab)	OS、PFS(PD-L1 CPS>=1 or CPS>=10 and ITT populations)	Median PFS 9·7 months VS 5·6 months(PD-L1 CPS>=10) 7.6 months VS 5·6 months (PD-L1 CPS>=1) 7.5 months VS 5·6 months (ITT population)	(20)
KEYNOTE-522 NCT03036488	III	Chemotherapy(paclitaxel +carboplatin) ± pembrolizumab	Early-stage TNBC (untreated stage II–III)	1174	pCR EFS (ITT population)	first interim analysis pCR 64.8% VS 51.2% the incidence of treatment-related adverse events of grade 3 or higher 78.0%VS 73.0%	(21)

Chemotherapy-Induced Alterations of Damage-Associated Molecular Patterns (DAMPs)

At late time point of cell death, tumor cells can transfer "eat me signals" to facilitate immune cells phagocytosis and tumor antigen presentation, resulting in the conversion of dying tumor cells to adjuvanted-endogenous tumor vaccines (49). The nature of DAMPs is the fundamentally dynamic responding to chemotherapy-elicited cell stress that involve in multifaceted influences on extra- and intracellular microenvironments (50). The release of DAMPs often reflects the re-expression of novel membrane-bound, secreted proteins and increased intracellular components, such as type I interferon and adenosine triphosphate (ATP) (51). Among them, high mobility group box 1 (HMGB1), calreticulin (CRT) and surface heat shock protein 90 (HSP90) have been recognized as key ICD-related DAMPs, which were reported to improve antigen uptake and presentation of DC cells, and assist the CD8+ T cells to exert antitumor activity (52–54). These DAMPs induced by chemotherapeutic drugs could promote a state of anti-tumor immunity. However, other studies showed that DAMPs such as HMGB1, CRT, and ATP were also involved in BC progression, metastasis, and drug resistance (55–57). So, DAMPs represent a double-edged sword in BC.

The interactions between HMGB1 and TLR-2, TLR-4, and TLR-9 could also participate in cross-presentation of anti-tumor T lymphocytes *in vivo*, which lead to the activation of DCs and trigger antitumor immune responses (58, 59). In BC patients, the expression of HMGB1 was able to effectively measure the immunogenicity and effectiveness of chemotherapeutic drugs (60). *In vitro*, the level of extracellular HMGB1 was increased in conditioned media after doxorubicin treatment in MB-231 cells (61). Moreover, a significant increase of HMGB1 release was also determined in HCC1143 cells with epirubicin/docetaxel intervention (62). After neoadjuvant chemotherapy (NCT), plasma HMGB1 dramatically increased for BC patients who

TABLE 2 | Summary of phase Ib/II clinical trials adding immunotherapy to chemotherapy in breast cancer.

Trial (National Clinical Trial Identifier)	Phase	Interventions	Patients enrolled	Number of patients	Primary endpoint	Key Results	Ref
NCT01633970	Ib	Nab-paclitaxel ± atezolizumab	Stage IV or locally recurrent TNBC (all patients experienced at least 1 treatment-related adverse event)	33	safety tolerability	73% grade 3/4 adverse events, 21% grade 3/4 adverse events of special interest and no deaths	(24)
KEYNOTE-173 (NCT02622074)	Ib	Pembrolizumab+ chemotherapy	Early-stage TNBC (high-risk)	60	safety RP2D	neutropenia adverse event 73% Immune-mediated adverse events and infusion reactions 30%(grade>=3 10%) two cohorts meet the RP2D threshold	(25)
NCT02513472	Ib/II	Eribulin +pembrolizumab	mTNBC(≤2prior systemic anticancer therapies in the metastatic setting.)	167	safety, tolerability ORR	ORRs 25.8% (stratum1 n=66) 21.8% (stratum2 n=101) ORR PDL-1+ VS ORR PDL-1-: 34.5% VS16.1% (stratum 1) 24.4% VS 18.2% (stratum2)	(26)
ALICE (NCT03164993)	II	Chemotherapy (pegylated liposomal doxorubicin+ cyclophosphamide) ± atezolizumab	mTNBC	75	Safety PFS	Ongoing	(27)
KEYNOTE-086 (NCT02447003)	II	Pembrolizumab	Previously treated mTNBC (prior treatment with anthracycline and taxane)	170 (105 PD-L1+)	ORR safety	ORR 5.3% (PD-L1+ 5.7%)	(28)
NCT03051659	II	Eribulin ± pembrolizumab	HR+/ERBB2-metastatic breast cancer	88	PFS	median PFS 4.1 vs 4.2 months	(29)
I-SPY2 Trial (NCT01042379)	II	NACT (taxane and anthracycline) ± pembrolizumab	Early-stage breast cancer (high risk)	300	pCR	ongoing, estimated pCR rates pCR 44% vs 17% (ERBB2- cohort) pCR 30% vs 13% (HR+/ERBB2- cohort) pCR 60% vs22% (TNBC cohort)	(30)
GeparNuevo (NCT02685059)	II	NACT (nab-paclitaxel + EC) ± pembrolizumab	Early-stage TNBC	174	pCR	pCR 53.4% VS 44.2%	(31)
ICON (NCT03409198)	IIb	Chemotherapy ± ipilimumab and nivolumab	Metastatic HR+ breast cancer	75	Safety PFS	Ongoing	(32)

CPS, combined positive score; EFS, event-free survival; EC, E=epirubicin, C= cyclophosphamide; ERBB2-, ERBB2-Negative; HR+, Hormone Receptor Positive; ITT, intention-to-treat; ORR, objective response rate; OS, overall survival; PD-L1, programmed death-ligand 1; pCR, pathological complete response; PFS, progression-free survival; RP2D, recommended phase II dose; stratum 1, number of prior systemic anticancer therapies is 0; stratum 2, number of prior systemic anticancer therapies is 1–2; TNBC, triple negative breast cancer; mTNBC, metastatic triple-negative breast cancer; NACT, neoadjuvant chemotherapy.

apparently obtain complete pathological complete response or partial remission (62). Another report also demonstrated that upregulated expression levels of HMGB1 and CRT were found after NCT in both BC patients and cell lines. And increase levels of HMGB1 have been shown to predict an improved therapeutic outcome in BC patieants receiving NCT (63, 64). CRT is an essential initiator of ICD signaling that is exposed at the surface of membrane and surrounded by immature and mature DCs (54). In a BC model, docetaxel did not alter the secretion of HMGB1 or ATP. However, exposure to CRT was observed in BC cell lines after docetaxel intervention, and antitumor immunity was reinforced mainly by the increased antigen presenting capacity and translocation of CRT (41). *In vitro* studies indicated that paclitaxel, gemcitabine and doxorubicin-mediated chemotherapy could efficiently kill cancer cells and lead to a high level of DAMP (CRT and HMGB1) (65–67). It has been shown that cyclophosphamide analogues improved tumor immunogenicity by facilitating the release of ICD markers (CRT, HMGB1, and ATP) (43). Altogether, these observations underscore the importance of adjuvanticity for chemotherapy to support the initiation of clinically anti-tumor immunotherapy.

ACTIVATION OF IMMUNE EFFECTOR CELLS

Impact of Chemotherapy on the Innate Immunity

Innate immune cells including DCs, natural killer (NK) cells and macrophages may at least represent as adjuvants to immune checkpoint inhibitors (68). Some chemotherapies drugs have direct implications for DCs and NK cells. *In vitro* studies showed NK cells-mediated cytotoxicity against BC cells was significantly enhanced following epirubicin-based pretreatment indicating the combination of anthracycline-based chemotherapy and NK cells-based immunotherapy was potentially an efficient strategy for BC treatment (69). Initially, cytotoxic chemotherapeutics were demonstrated to induce an overall dysfunction of NK cells responses in localized and metastatic BC patients (70, 71), while the NK cells (CD56) numbers and macrophages (CD14) rapidly returned to normal after adjuvant chemotherapy (72). Another study reported that both epirubicin-based and doxorubicin-based regimen could result in an increased percentage of monocytes and NK cells, but a marked decrease was observed in B-cell numbers (73).

Similarly, advanced BC patients using single-agent paclitaxel or docetaxel led to an enhancement of NK and LAK cytotoxic activity and increase of IFN-γ, IL-2, IL-6, GM-CSF cytokine levels in serum (74, 75). For clinical practice, a reduction in the infiltration of NK cells into tumor tissue has been proposed to be a predictor of chemotherapeutic treatment failure in BC (76, 77). During follow-up after adjuvant therapy, a previous study reported that NK cells cytotoxicity showed significantly elevated at all time-points and did not correlate with the mode of adjuvant radiotherapy or chemotherapy after a one-year follow-up (78). In addition, other studies suggested that the absolute number of activated NK cells was higher in BC patients who achieved pathological complete responses (PCR) after neoadjuvant chemotherapy, which implied that the improvement of NK cell activities was essential requirement for pCR especially in HER2-positive BC patients (79, 80). NCT could induce immune activation and a release from local immunosuppression in the tumor microenvironment, and thus activation of peripheral NK cells might promote the elimination of metastatic tumors in BC (81).

The impacts of chemotherapy on DCs have also been studied in BC. The antitumor efficacy of chemotherapies drugs is essentially determined by DCs that present antigens to tumor-specific T lymphocytes (39). Paclitaxel and doxorubicin were shown to improve the antigen presentation ability of DCs through stimulating the expression of costimulatory molecules and IL-12p70 (82). A study found that DCs in tumor lysate could consistently activate CD8+ CTLs for killing cancer cells in locally advanced BC, indicating DC-based vaccinations might be well suited to treat chemotherapy-resistant BC patients (83). A combination of doxorubicin and cyclophosphamide with autologous DCs was favorable to prolong the survival of T cells and recover immune functions capacity (84). One mechanism might be that this combination enhanced tumor immunogenicity as cryptic vaccines and promoted the adjuvant effects of ICD. Additionally, a recent multi-omics analysis revealed that BC patients with higher level plasmacytoid DCs tended to exhibit a more sensitive immune response and chemotherapies response, which highlighted that the potential benefit from combination of chemotherapy and immunotherapy might be achieved in BC patients with high immune infiltration of plasmacytoid DCs (85). Regarding the associations between DCs and chemotherapy in the clinic, significant efforts have been made. Prior to NAC, a marked unresponsiveness to *in vitro* stimulus was observed for DCs, while NAC could induce a remarkable responsiveness of APC compartments (86). A previous study also described a correlation between circulating DCs level and pCR in BC and their findings suggested that patients with a poor pCR after NAC were characterized by low expression of myeloid-derived DCs and plasmacytoid DCs (87). Altogether, these observations pave the way to translate innate antitumor immunity into innovative immunotherapies for fighting refractory BC.

Impact of Chemotherapy on the Adaptive Immunity

B cells displayed dramatic depletion after chemotherapy and remained persistent low level even 9 months following systemic chemotherapy (88, 89). It has been reported that the percentage of peripheral blood B cells was substantial decreased by FEC (5-fluorouracil, epirubicin, cyclophosphamide) or FDC (5-fluorouracil, doxorubicin, cyclophosphamide) regimens in BC (73). Likewise, vinorelbine, cyclophosphamide and 5-FU were also reported to decrease the number of circulating B cells in which cyclophosphamide had the largest influence over levels of B cells (90). The reason for cytotoxic chemotherapy effect on B cells was partly due to an increased sensitivity of B cells to chemotherapeutic agent *in vitro* compared to T-cells (91). Tumor infiltration of B cells in the tumor microenvironment could serve as a promising biomarker to select BC patients who might benefit from NAC (92). Memory B cells was correlated with pCR to NAT in ER-negative BC tumors, which indicated humoral immunity was essential for mediating response to cytotoxic therapy (93). Also, higher B cells infiltration could potentiate the local cytotoxic immune response and were correlated with better outcomes in hormone receptor-negative BC patients (94).

Substantial evidence suggested that chemotherapy contributed to T-cells independent immune responses. *In vivo* treatment of tumor-bearing mice demonstrated that doxorubicin led to a significant increase in the number of CD4 + T cells, CD8+ T cells and NK cells and promoted expression of interferon γ (IFN-γ) and granzyme B (95). In another pre-clinical experiments, the administration of anthracycline also facilitated the infiltration of CD4+ and CD8+ T cells in TNBC mouse model (96). Several possible mechanisms have been proposed to explain these phenomena. Treatment of doxorubicin promoted cytotoxic T lymphocytes accumulation by a potent production of IFN-γ and IL-17 in a BC mouse model, which suggested that γδ T cells indeed played a sizable role in doxorubicin-induced anti-tumor immune response (97). Low doses of cyclophosphamide were shown to reverse the immunosuppression and strongly enhanced the abundance of tumor infiltrating T cells *via* the secretion of various cytokines and activation antigen-presenting cells (98). Furthermore, high dose of cyclophosphamide could completely eradicate tumor cells, while cyclophosphamide at low doses was able to reduce the number of circulating Tregs but increase the production of tumor-specific T cells (99). In clinical contexts, the percentages of CD3+, CD4+ T cells and Treg cells in blood samples of BC were significantly decreased after 6 cycles of chemotherapy (100). To assess the effect of combination chemotherapy on subsets of immune cells, a study revealed that anthracycline-based regimen could induce an increase of cytotoxic T and NK cells, but a dramatic decrease of B cells in blood (73). A better clinical response during chemotherapy has been linked to higher level of circulating CD8+ T-cell (101). Some studies have addressed the effects and correlations of NAC on effector T cells. After NAC, BC patients with beneficial therapeutic effects often correlated with an increased level CD4+ and CD8+ T-cells, and decreased CTLA-4+ T cells and VEGF (102, 103). It has been previously documented that the expression of CD8/Foxp3 was upregulated in cancer tissues of pCR cases, which implied that activation of antitumor T cell responses was occurred in these tumors (104). Tumor microenvironment characteristics analysis

further revealed that higher level of stromal tumor infiltrating CD8+ T cells and B cells significantly correlated with pCR in NAC (105–107). However, existing studies have focused on the prognostic value of infiltrating of immune effector cells on chemotherapy. Understanding how to maximize the therapeutic potential of chemotherapy-induced immunomodulatory effects remains an open question.

HAMPERING THE FUNCTIONS OF IMMUNOSUPPRESSIVE CELLS

Treg Cells

Treg cells mainly function in preventing excessive immune activation. Blocking or depleting Tregs is therefore a viable therapeutic strategy to enhance antitumor immunity (108). Studies have revealed that the depletion of Treg cells in immune cell infiltrate was associated with a protective anticancer immunity. This also meant that anticancer immunity switched from a silent immune response to an active immune response (109, 110). A study showed that BC patients had more Treg cells than normal individuals. Meanwhile, an increasing level of Treg cells and lower ratio of Th/Tr cells were found in Stage IV BC patients compared to stage I, II, or III BC patients (111). It has been described that the percentage of Treg cells was reduced after 6 chemotherapy cycles among stage II/III BC patients (100). Paclitaxel was shown to not only reduce CD4+Foxp3+ Tregs cells but hinder cytokine production of Tregs (112). The weakening effect of cyclophosphamide on Tregs cells was often observed at low dose (99). Additionally, metronomic cyclophosphamide regimens also led to a profound and effective Treg inhibition in metastatic BC patients (99). Low Treg abundance was determined in TNBC but not in ER-positive or Her2-negative subtype, especially for patients with pCR after NAC, which indicated that Treg abundance might serve as a predictive biomarker for evaluating their NAC effectiveness in TNBC (113).

M2 Macrophages and MDSCs

Tumor-associated M2 macrophages (M2-TAMs) was proposed to promote immune escape and limit the efficacy of immunotherapy. Targeting M2-TAMs synergizes with immune checkpoint blockade has emerged as promising strategies for cancer treatment (114). Docetaxel administration could induce a switch from M2-like phenotype to M1-like phenotype in mammary tumor-bearing mice (115). In another 4T1 BC lung metastasis mice model, nanosystem-based co-delivering doxorubicin was also able to modulate the polarization from M2 macrophages to antitumor M1 macrophages (116). BC patients who fail to respond to anthracycline-containing NAC were predominantly associated with the presence of M2+ macrophage phenotype (117).

Myeloid-derived suppressor cells, a heterogenic population of immature myeloid cells, were characterized by their immunosuppressive effects. Cytotoxic agents against MDSCs represent therefore an appealing therapeutic strategy for cancer therapy but its underlying molecular mechanism remains obscure (118, 119). So far, many cytotoxic chemotherapeutics were shown to have excellent repression on MDSCs in BC (120). In mouse model of BC, an inhibitory effect on MDSC of doxorubicin has been demonstrated in the spleen, blood, and tumor tissues (95). Furthermore, the treatment of doxorubicin could increase the frequency of the effector lymphocytes or NK cells that effectively reduced MDSC ratios (95). The above studies not only suggested the direct cytotoxic effect on cancer cells, but also highlighted the immunomodulatory role of doxorubicin on MDSC. In another animal models, downregulation of splenic CD44+, IL-17A+ MDSCs effect of cisplatin was revealed by single cell mass cytometry in 4T1 metastatic BC model (121). Docetaxel, one chemotherapeutic agent for treating anthracycline-refractory BC, have been reported to suppress the level of MDSCs and stimulate the CTL response in spleens of mice (115). Gemcitabine and cyclophosphamide were also found to be capable of inhibiting the accumulation of MDSCs (43). Beyond that, capecitabine depleted MDSCs and relieved their inhibitory effects on T and NK cells (122). A single arm, pilot study observed that levels of circulating MDSCs increased after doxorubicin and cyclophosphamide treatment but decreased after paclitaxel treatment for BC patients with NAC (123). Compared to patients with Non-pCR following NAC, circulating MDSCs seemed to lower for complete or near pCR BC patients (123). Additional studies have also demonstrated that BC patients with a lower level of circulating MDSCs before treatment preferred to achieve a higher probability of a pCR after the last cycle of chemotherapy (124). However, it is a well-recognized challenge to determine the target against MDSCs owing to its multiface of MDSCs and the complexity of tumor microenvironment. Besides, considerable research efforts are focusing on the total MDSCs populations in BC. Thus, the immunomodulatory effects of chemotherapy on different MDSC subtypes remain to be explored.

Effects of Anticancer Agents on the Immune Checkpoints

In the past, BC was thought to be a "cold" tumor with low immunogenicity and mutation burden. However, studies in recent years have identified high PD-L1 and tumor infiltrating lymphocytes in TNBC and HER-2-positive breast cancers (125, 126). At the preclinical level, doxorubicin was shown to inhibit tumor immunosuppression through down-regulating the expression of immune checkpoints PD-1 and TIM-3 in the tumor tissue (127). In a TNBC murine model, doxorubicin/cyclophosphamide regimen was able to effectively inhibit tumor growth, increase the survival benefit, promote infiltrating of CD8+ T cells and suppress the suppressor molecules PD-L1 expression (128). With regard to PD-L1 expression changes in BC after chemotherapy, a panel of six anti-cancer compounds were experimentally found to induce PD-L1 expression in four BC cell lines through a cellular stress response pathway (129). Study by Samanta et al. demonstrated that doxorubicin, gemcitabine, or paclitaxel induced HIF-dependent, transcriptional activation of CD47, CD73, and PDL1 expression that imparted TNBC cells the ability to evade the immune systems (130). Similar findings have been reported that paclitaxel, etoposide and 5-fluorouracil could induce PD-L1 expression in BC cells and up-regulated PD-L1

promoted PD-L1-specific T cell apoptosis (97). After treating with metronomic cyclophosphamide, BC patients exhibited a higher expression PD-L1 in tumor cells; however, no obvious benefit was observed for CTX regimens combined with concomitant PD-L1 antibody therapy (131). A case report described that level of CD8 and PD-L1 expression on immune cells were increased after capecitabine and gemcitabine-carboplatin-iniparib therapy (132). A clinical trial aimed to identify molecular alterations of immune gene signatures following neoadjuvant chemotherapy of TNBC and they found several immune checkpoints including IDO1, PD-L1 and CTLA4 were upregulated in pre-treatment samples who achieved pCR (133). Collectively, the absence of unifying PD-L1 protocols makes it hard to draw a convincing conclusions from these studies. Besides, PD-L1 levels are generally evaluated in tissues prior to chemotherapy, which might not reflect the real status of the tumor microenvironment after chemotherapy.

CONCLUSION

For many decades, cytotoxic chemotherapeutics are still the cornerstone of BC treatment (134). However, encouraging

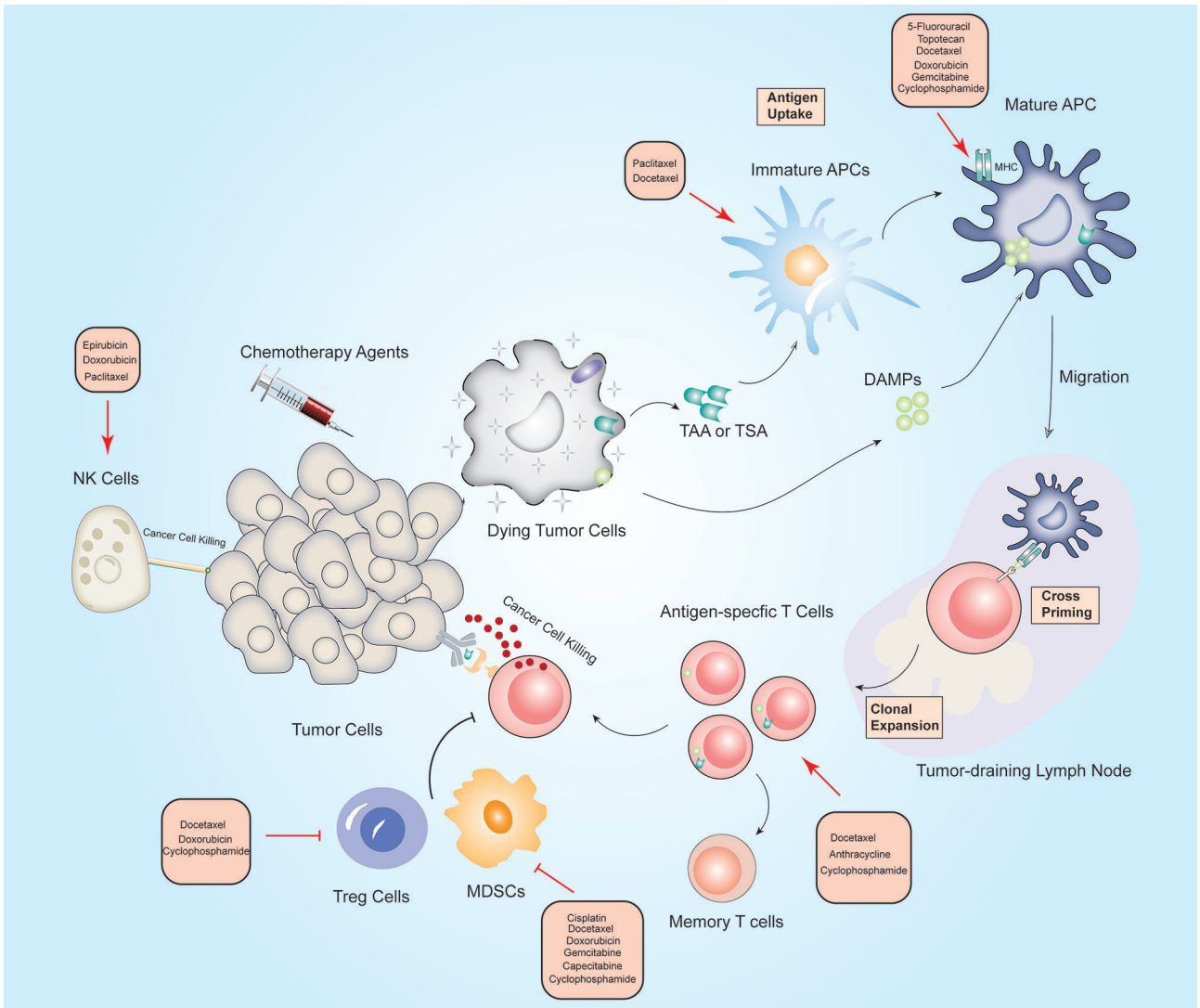

FIGURE 1 | Overview of the immunostimulatory properties of chemotherapy in breast cancer. On-target effects: When tumor cells are exposed to chemotherapeutic drugs, TAA, TSA and DAMPs release by dying tumor cells are engulfed by immature DCs, which promotes APCs maturation. Archived antigen-bearing APCs then migrate to the tumor-draining lymph node, where APCs cross-prime to T cells. Thereafter, antigen-specific T cells undergo clonal expansion, and at least some of them differentiate into memory T cells. Activated T cells then recognize tumor cells and mediate cytotoxic killing of tumor cells. Off-target effects: Chemotherapeutic drugs can activate immune effector cells including natural killer (NK) cells, dendritic cells (DCs), and cytotoxic T cells, and depletion of immunosuppressive cells including Treg cells, M2 macrophages and (myeloid-derived suppressor cells) MDSCs. Red arrows indicate an increased effect and red flat ended lines represent an inhibitory effect. The text boxes near the arrows list the chemotherapy agents that elicit immunomodulatory effects in BC.

advancements in cancer immunotherapy have provided more options for certain subtypes of BC (11, 135). Single chemotherapeutics agents or single immuno-oncological therapy cannot obtain ideal therapeutic effect for advanced BC (136). Thus, combining immunotherapy with the currently-available therapies has shown great promise. Current mini-review summarizes the updated clinical trials on immunotherapy and chemotherapy combinations in BC (**Tables 1**, **2**) and provides an overview of immune-stimulating properties of cytotoxic chemotherapy (**Figure 1**). There remains large room for improvement of synergistic effects of these two combined modalities, so identifying prerequisites for designed immunotherapy combination strategies are of special importance.

ICD is a specific type of cancer cell death characterized by antigen-specific immune responses against the antigens of dying cancer cells (137). Anthracycline and taxanes-containing chemotherapy can promote immunostimulatory activity by increasing the antigenicity or adjuvanticity of cancer cells (138). The ICD effects mediated by chemotherapy have largely centered on chemotherapy-induced alterations of DAMPs (50, 139). Notably, through DAMPs mechanisms, chemotherapy stimulates immune system to recruit DCs and activate the immune responses specific for tumor-relevant antigens. Conversely, fewer studies have looked at the effects of chemotherapeutic drugs on tumor cell antigenicity. Future studies are required to elucidate the molecular mechanism of DAMPs in ICD and provide specific interventions targeting them to facilitate development of chemoimmunotherapeutic regimens. In BC, numerous studies have demonstrated that chemotherapeutic agents can act directly on immune cell subsets to elicit antitumor immunity. Off-target effects of chemotherapy on immune cell subsets mainly involve activation of immune effector cells including NK cells, DCs, and CTLs, and depletion of immunosuppressive cells including Treg cells, M2 macrophages and MDSCs. However, the dynamic alterations of effector immune cells in full course of adjuvant chemotherapy remain unknown.

Cytotoxic chemotherapies may act as upfront measures that are capable of converting "cold" BC tumors into "hot" lesions, which may be successful clearance with ICIs. In the present review we have focused on the immunomodulatory effects of chemotherapy in BC. In addition to chemotherapy, endocrine therapy, targeted therapeutic agents and radiation have also been demonstrated to have analogous immunoregulatory function for BC, in particular for radiotherapy (140, 141). Thus, these therapeutic options should also be suggested for combined immunotherapy based on different intrinsic subtypes of BC. The immunotherapy era provides additional selections for clinicians in BC treatment, but at the same time, many unanswered questions exist regarding combinations with chemotherapy and immunotherapy. How to identify prerequisites of combination treatment given patient's immune status and intrinsic characteristics. Limited information is available on the impact of cytotoxic chemotherapy on immune checkpoints pathways not confined only PD-L1, PD-1 or CTLA4. Lastly, it should be noted that single-agent chemotherapy can act on multiple steps of antitumor immune response, and one chemotherapy regimen may also play two opposite roles in different immune targets.

Therefore, when considering potential applications in clinic, drug dose, timing of administration and appropriate population would need to be carefully considered.

AUTHOR CONTRIBUTIONS

XH and JZ were involved in the design of the work and figures. JZ and SP performed the literature search and wrote the draft. CJ, LH, JD, QS and HJ edited the manuscript and provided the critical revisions. All authors contributed to the article and approved the submitted version.

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The Potential Mechanism of Cancer Patients Appearing More Vulnerable to SARS-CoV-2 and Poor Outcomes: A Pan-Cancer Bioinformatics Analysis

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To explore the potential mechanism of cancer patients appearing more vulnerable to SARS-CoV-2 infection and poor COVID-19 outcomes, we conducted an integrative bioinformatics analysis for SARS-CoV-2-required genes and host genes and variants related to SARS-CoV-2 susceptibility and COVID-19 severity. BLCA, HNSC, KIRC, KIRP, LGG, PCPG, PRAD, TGCT, and THCA patients carrying rs10774671-A (OAS1) genotype may be more likely to have poor COVID-19 outcomes relative to those who carry rs10774671-G, because individuals carrying rs10774671-A will have lower expression of OAS1, which serves as a protective factor against SARS-CoV-2 processes and poor COVID-19 outcomes. SARS-CoV-2-required genes were correlated with TME, immune infiltration, overall survival, and anti-cancer drug sensitivity. CHOL patients may have a higher risk of SARS-CoV-2 infection than healthy subjects. SARS-CoV-2-induced ACE2 and NPC1 elevation may have a negative influence on the immune responses of LUSC and CD8+T infiltration of LUAD, and negatively affect the sensitivity of anti-lung cancer drugs. LUSC and LUAD patients may have a varying degree of adverse outcomes if they are infected with SARS-CoV-2. miR-760 may target and inhibit ACE2 expression. Cancer patients appearing vulnerable to SARS-CoV-2 infection and having poor COVID-19 outcomes may be partly due to host genetic factors and dysregulation of SARS-CoV-2-required genes. OAS1, ACE2, and miR-760 could serve as the treatment and intervention targets for SARS-CoV-2.

Keywords: COVID-19, cancer, rs10774671 (OAS1), SARS-CoV-2, ACE2

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INTRODUCTION

As of 24 August 2021, 2019 novel coronavirus (COVID-19), which is caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), has infected more than 200 million patients, including 4.2 million deaths (https://covid19.who.int/). Recently, some vital host genes required for SARS-CoV-2 infection processes containing initial binding (ACE2), endosomal entry (RAB7A, ACTR2/3, and ARPC3/4), spike protein cleavage, and viral membrane fusion (CTSL, TMPRSS2, TMEM199, ATP6AP1/2, ATP6V0B/C/D1, ATP6V1 families, and TOR1AIP1), endosome recycling (PIK3C3, WDR81, SNX27, VPS26A, VPS29, VPS35, COMMD2/3/4, COMMD3-BMI1, and ACP5), ER-Golgi trafficking (PPID, ERMP1, DPM3, and CHST14), and transcriptional modulators (SPEN and SLTM) were identified by Daniloski et al. (1) and Hoffmann et al. (2) using a genome-scale CRISPR loss-of-function screen or protease inhibitor in human cell lines (**Figure 1A**). By using single-cell transcriptomics, RNA interference knockdown, and small-molecule inhibitors, the loss of endosomal entry pathway genes ATP6AP1, ATP6V1A, CCDC22, NPC1, PIK3C3, and RAB7A was validated to result in

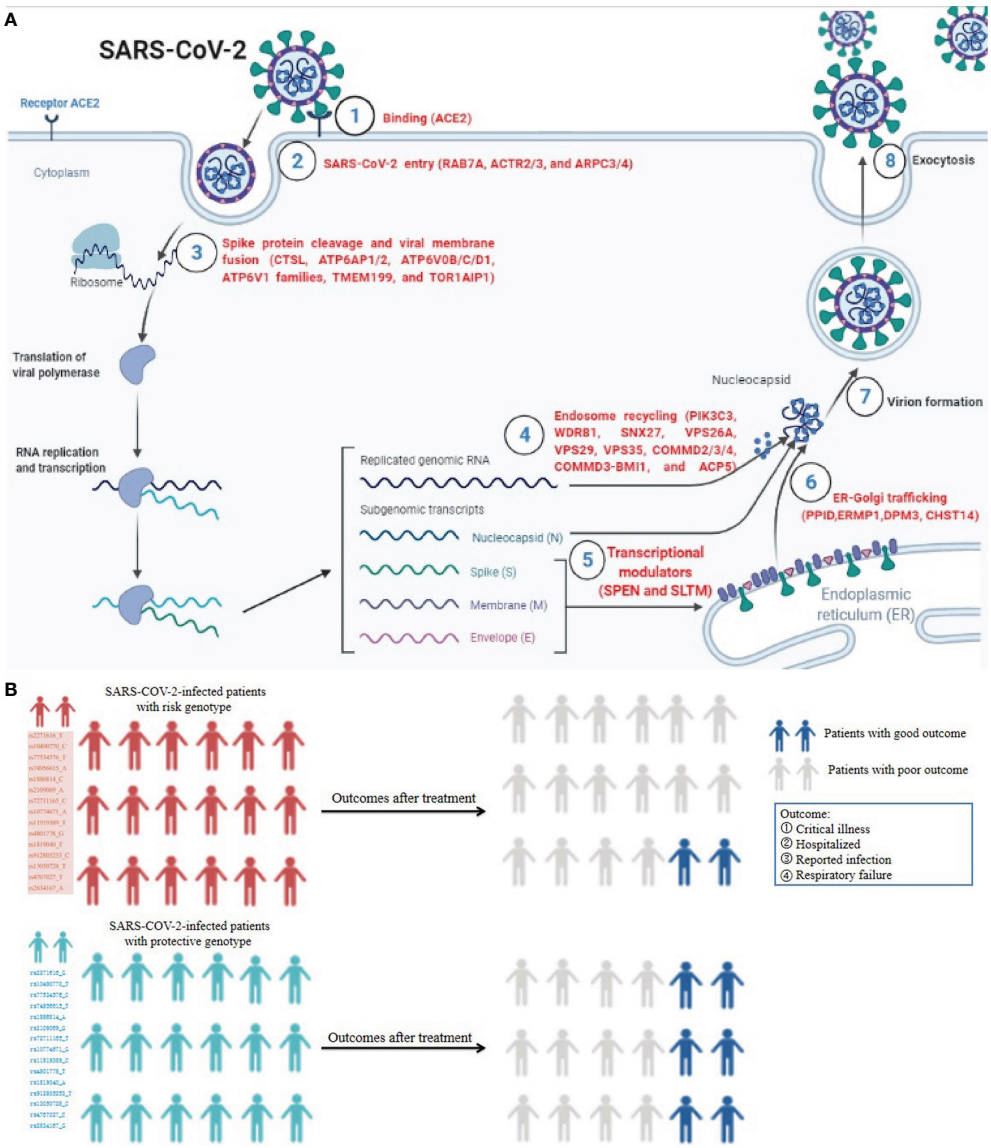

FIGURE 1 | The roles of SARS-CoV-2-required genes in SARS-CoV-2 infection processes and influence of host genetic factor in COVID-19 outcomes. **(A)** The vital host genes required for SARS-CoV-2 infection processes containing initial binding, endosomal entry, spike protein cleavage, and viral membrane fusion, endosome recycling, ER-Golgi trafficking, and transcriptional modulators. **(B)** SARS-COV-2-infected patients with risk genotypes appear more vulnerable to SARS-COV-2 infection and poor outcome, while those carrying protective genotypes appear more vulnerable to lower SARS-COV-2 possibility and good outcome[5-9]. This figure is drawn on the Biorender website at https://biorender.com/.

increased cellular cholesterol, which can block SARS-CoV-2 infection (1–3). Severe acute respiratory syndrome coronavirus (SARS-CoV) and SARS-CoV-2 share 79.5% homologous sequences, and both viruses use similar host genes as receptors to enter human body cells (2). Kong and colleagues (4) indicated that normal lung and lung cancer cell lines infected with SARS-CoV can elevate ACE2 expression, maintaining a high level of expression at 1 and 2 days. Notably, several research teams have demonstrated that SARS-COV-2-infected patients with risk genotypes appear more vulnerable to SARS-COV-2 infection and poor outcomes, while those who carry protective genotypes appear more vulnerable to lower SARS-COV-2 possibility and good outcomes (5–9), indicating host-specific genetic factors play an important role in SARS-CoV-2 susceptibility and COVID-19 outcomes (**Figure 1B**). These findings provide new insights into the mechanisms of pathogenesis of SARS-CoV-2 susceptibility and poor outcomes.

Risk factors for severe events and deaths from SARS-CoV-2 infection include older age, smoking, and medical comorbidities, which are common in cancer patients. Four studies analyzing cancer patients with SARS-CoV-2 infection revealed that they appear more vulnerable to SARS-CoV-2 and show more deteriorating conditions and poor outcomes compared with non-cancer patients (10–13). Bernard et al. (10) and Dai et al. (11) indicated that patients with different cancer types (especially lung cancer and hematological cancer) and late metastatic stage have the highest frequency of severe events. The possible reasons for this may be attributed to cancer-related immunosuppression, known complications, and immunotherapy treatment (11, 13). However, the exact mechanisms remain unclear. Given a large number of cancer patients and the continuing spread of SARS-CoV-2, exploring this molecular mechanism could contribute to the treatment of cancer patients infected with SARS-CoV-2.

This study explores the potential mechanism for cancer patients appearing vulnerable to SARS-CoV-2 and poor outcomes *via* integrative bioinformatics analyses for SARS-CoV-2-required genes (ACE2, TMPRSS2, ATP6AP1, ATP6V1A, CCDC22, NPC1, PIK3C3, and RAB7A), host genes, and variants related to SARS-CoV-2 susceptibility and COVID-19 severity.

MATERIALS AND METHODS

Data Download and Processing

RNA-seq and clinical data of 33 cancer types, and pan-cancer immune signature scores, stemness score, and stemness score data were downloaded from The Cancer Genome Atlas (TCGA) database *via* UCSC Xena (https://xena.ucsc.edu/). Drug susceptibility data including DTP NCI-60 and RNA-seq were obtained from the CellMiner database (https://discover.nci.nih.gov/cellminer/). In addition, RNA-seq datasets (GSE163959 and GSE147507) of human nasal turbinate, lung tissues, A549 cells, and primary human bronchial epithelial cells (NHBEs) with or without SARS-CoV-2 infection were downloaded from Gene Expression Omnibus (GEO) database (https://www.ncbi.nlm.nih.gov/geo/).

Expression Analysis of SARS-CoV-2-Required Genes and Host Susceptibility Genes in Human Tissues and Cells After SARS-CoV-2 Infection

Package edgeR was used to normalize GSE163959 and GSE147507 raw count datasets. The t-test was utilized to compare the expression of SARS-CoV-2-required genes between control and SARS-CoV-2 infected samples. Package pheatmap was utilized to show their expression status. The same analysis was also performed for host-specific genes associated with COVID-19 susceptibility and severity. $P < 0.05$ was considered statistically significant.

Expression Quantitative Trait Locus Analysis for Host Genes and Variants Related to COVID-19 Susceptibility and Severity

PancanQTL web platform was used to comprehensively evaluate the effect of variants related to COVID-19 susceptibility and severity on local gene expression (cis-eQTLs) in 33 cancer types. This platform included the expression and genotype data of 9,196 tumor samples and 5,606,570 cis-eQTL-gene pairs in 33 cancer types from TCGA (14). We then assessed the expression status of host genes related to COVID-19 severity in multiple organs and tumor tissues *via* The Human Protein Atlas database (https://www.proteinatlas.org/).

Evaluating Expression Profiles of SARS-CoV-2-Required Genes Across Human Tissues

To identify the expression profiles of eight SARS-CoV-2-required genes across human tissues, we examined their expression across 21 tissue types using 4,790 RNA-seq datasets from the Genotype-Tissue Expression (GTEx) v8 database (https://www.gtexportal.org/home/datasets).

Differential Expression Analysis for SARS-CoV-2-Required Genes Across 33 Cancer Types

Differential expression analysis for SARS-CoV-2-required genes was performed across 33 cancer types by wilcox.test function. R package pheatmap was used to visualize their differential expression status between cancer samples and non-cancer samples. $P < 0.05$ was considered statistically significant.

Identification of SARS-CoV-2-Required Genes Associated With the Stage and Prognosis of Cancer Patient

Differential expression analysis between SARS-CoV-2-required genes and stage types in pan-cancers was performed using Gene Set Cancer Analysis (GSCA) database (http://bioinfo.life.hust.edu.cn/GSCA/#/expression) (15) and GEPIA 2 database (http://gepia2.cancer-pku.cn/#index) (16). Moreover, we used R packages survival, survminer, and reshape2 to explore the association between the expression of SARS-CoV-2-required

genes and the prognosis of cancer patients. Firstly, based on the survival data, Kaplan–Meier curve was utilized to analyze the overall survival according to the high and low expression values of the gene. We then conducted the univariate Cox regression analysis for the relationship between the overall survival and expressions of SARS-CoV-2-required genes. $P < 0.05$ was considered statistically significant.

Tumor Microenvironment Analysis

Kruskal.test and R packages ggplot2, limma, and reshape2 were used to test the association between immune subtypes and the expressions of these genes according to the immune landscape of cancers (17). The correlation between SARS-CoV-2-required gene expression and tumor microenvironment (TME) was analyzed with Spearman correlation and R packages estimate, limma, and corrplot, according to the ESTIMATE immune, stromal, and estimate scores, which can analyze the infiltration levels of both stromal and immune cells in cancers (18). Furthermore, cancer stem cell-like properties of each patient were obtained from stemness scores based on transcriptomic mRNA (RNAss) and epigenetic DNA methylation (DNAss). The association of stemness scores with SARS-CoV-2-required genes was assessed by spearman analysis.

Immune Infiltration Analysis

GSCA database (15) was used to conduct immune infiltration analysis for SARS-CoV-2-required genes. Additionally, differential expression analysis for interested immune cell types between tumor and adjacent normal tissues was performed in the ImmuCellAI database (http://bioinfo.life.hust.edu.cn/ImmuCellAI/#!/resource) using wilcoxon test (19). Survival analysis was conducted to compare survival curves between high and low immune cell abundance in one cancer by multivariable Cox proportional hazard model. Covariates contained immune cell infiltration and clinical factors (tumor stages, age, and gender). *P* value of the log-rank test as shown in each plot was used to compare the survival curves of the two groups. Kaplan-Meier plot for immune cell infiltration was drawn to visualize the survival difference.

Assessment of Association Between SARS-CoV-2-Required/Susceptibility Genes and Cancer-Related Genes

To explore the association between key SARS-CoV-2-required/susceptibility genes and estimate their influence in cancer-related genes affecting the prognosis of cancers, we performed a survival analysis for SARS-CoV-2-required/susceptibility genes in lung cancer *via* the GEPIA2 database. Based on TCGA lung cancer tissues, we evaluated their correlation using spearman correlation analysis. In addition, we performed a differentially expressed gene analysis between lung cancer tissues and normal tissues *via* the limma package of R soft. $|\log2 \text{ (fold change)}| > 2$ and P value < 0.05 after being adjusted by false discovery rate were applied as the cutoff for differential gene expression screening. We then assessed their correlation with the prognosis of lung cancer by the GSCA database.

Drug Sensitivity Analysis

To identify the relationship between drug sensitivity and SARS-CoV-2-required genes, we evaluated the correlation between the expression of each SARS-CoV-2-required gene and z-score for cell sensitivity data (GI50) by spearman correlation analysis based on DTP NCI-60 and RNA-seq data obtained from the CellMiner database (20). In addition, a similar analysis was also performed *via* the GSCA database based on Genomics of Drug Sensitivity in Cancer (GDSC) and Cancer Therapeutics Response Portal (CTRP). $|Cor| > 0.20$ and $P < 0.01$ were considered as statistically significant.

Prediction and Analysis of Upstream MicroRNAs of SARS-CoV-2-Required Genes

Upstream binding microRNAs of SARS-CoV-2-required genes were predicted based on seven prediction programs, containing RNA22, miRmap, PicTar, microT, PITA, miRanda, and TargetScan in starBase 3.0 database (http://starbase.sysu.edu.cn/), which mainly focus on miRNA-target interactions (21). The predicted microRNAs were obtained according to their appearance in one or more programs. StarBase 3.0 then was used to analyze the correlation of SARS-CoV-2-required genes with microRNAs, and assess the expression status of microRNAs in pan-cancer and normal control tissues. Additionally, survival analysis of microRNAs was also performed.

Statistical Analysis

All statistical analyses were based on R soft version 4.10 and attached packages. Wilcox test was utilized to determine differentially expressed SARS-CoV-2-required genes between normal and tumor samples. Spearman correlation analysis was utilized to assess the correlation between two variables. Log-rank tests and Kaplan-Meier curves were utilized to evaluate the relationship between gene expression and overall survival. $P < 0.05$ was considered statistically significant.

RESULTS

OAS1 May Serve as a Protective Factor Against SARS-CoV-2 Infection and Poor COVID-19 Outcomes

COVID-19 severity-related genes (SLC6A20, LZTFL1, FOXP4, TMEM65, ABO, OAS1, TAC4, DPP9, TYK2, ZBTB11, IL10RB, KANSL1, PLEKHA4, and IFNAR2) and single nucleotide polymorphisms (SNPs) identified by genome wide association studies were summarized in **Table S1** (5–9). To explore the role of genes related to COVID-19 severity in the process of SARS-CoV-2, we performed a differential expression analysis for these genes in human nasal turbinate, lung tissues, A549 cells (non-small cell lung cancer), and normal human bronchial epithelial cells (NHBEs) with/without SARS-CoV-2 infection. As shown in **Figures 2A–D** and **Table S2**, OAS1 and PLEKHA4 expressions were significantly elevated in turbinate, lung tissues, and NHBEs

infected with SARS-CoV-2 compared to the control cells. In up to 14,134 cases and 1.2 million controls, Zhou et al. found that higher plasma OAS1 protein level is related to reduced susceptibility (OR = 0.78, $P = 8 \times 10\text{-}6$), hospitalization (OR = 0.61, $P = 8 \times 10\text{-}8$), and COVID-19 death or ventilation (OR = 0.54, $P = 7 \times 10\text{-}8$) (9). We further explored whether OAS1 is specifically or widely expressed in organs and tumor tissues *via* The Human Protein Atlas (HPA) database (https://www.proteinatlas.org/). We found that OAS1 was broadly expressed in different human organs and tumor tissues, with low organ and cancer specificity (**Figures 2E, F**). These findings suggest that OAS1 may serve as a protective factor against SARS-CoV-2 progress and poor COVID-19 outcomes in the wide organs and tissues.

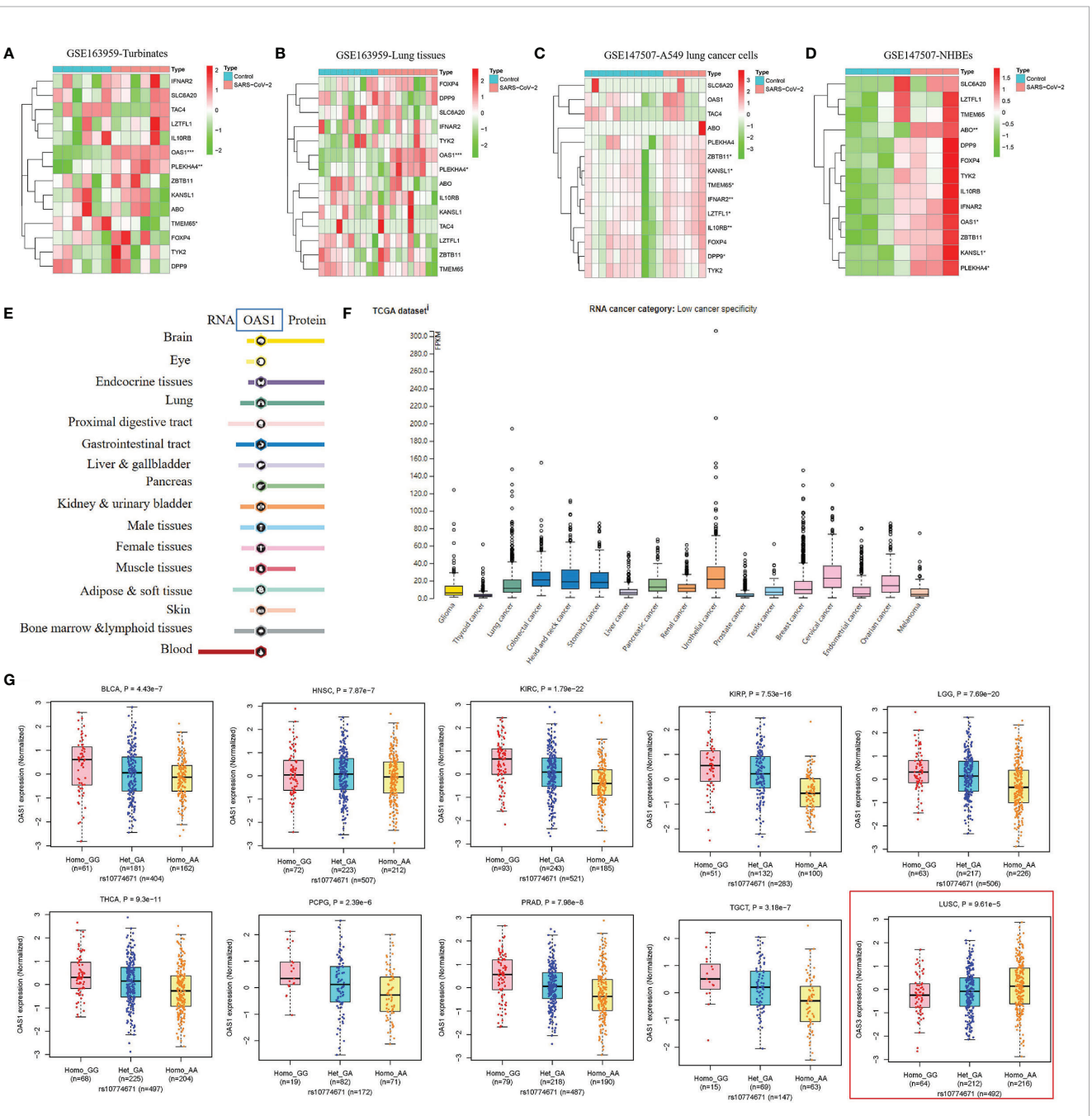

FIGURE 2 | The role of genetic factors in SARS-CoV-2 susceptibility and COVID-19 outcomes. **(A–D)** The response of host genes related to SARS-CoV-2 susceptibility and COVID-19 outcomes in human tissues and cells infected with SARS-CoV-2. **(E, F)** Expression status of OAS1 in human organs and tumor tissues. This analysis was performed in The Human Protein Atlas (HPA) database (https://www.proteinatlas.org/). **(G)** The association of rs10774671-A (OAS1) with OAS1 expression in BLCA, HNSC, KIRC, KIRP, LGG, PCPG, PRAD, TGCT, and THCA tissues.

Cancer Patients Carrying rs10774671-A (OAS1) Genotype May Appear Vulnerable to Poor COVID-19 Outcomes

COVID-19 severity-related SNPs were summarized in **Table S1**. Rs2271616-T (SLC6A20), rs10490770-C, rs11385942-GA (LZTFL1), rs1886814-C (FOXP4), rs72711165-C (TMEM65), rs505922-C (ABO), rs10774671-A (OAS1), rs77534576-T (TAC4), rs2109069-A (DPP9), and rs74956615-A (TYK2) was reported to increase the risk of SARS-CoV-2 susceptibility and poor COVID-19 outcomes such as critical illness, hospitalization, and respiratory failure, while rs11919389-C (ZBTB11), rs912805253-T (ABO), rs2834167-G (IL10RB), rs4767027-C (OAS1), rs1819040-A (KANSL1), rs4801778-T (PLEKHA4), and rs13050728-C (IFNAR2) decrease these risks (5–9). To further explore the effect of these genotypes on expressions of potential key genes, we performed an expression quantitative trait locus (cis-eQTL) analysis for these variants in 33 cancer types. As summarized in **Table S3**, rs4801778-T (PLEKHA4) was positively associated with TULP2 expression in LUAD (β = 0.26, P= 9.57E-05), while negatively associated with HSD17B14 expression in PAAD and PRAD (-0.37 < β < -0.36, 1.20E-08 < P < 8.36E-06). Rs11919389-C (ZBTB11) showed a positive relationship with LOC285359 and LOC100009676 expressions in GBM, LGG, PRAD, TGCT, or THCA (0.20 < β < 0.42, 3.78E-26 < P < 3.04E-05) and had a negative relationship with ZBTB11 and SENP7 expressions in LGG or OV (-0.24 < β < -0.14, 7.65E-05 < P < 9.95E-05). Rs13050728-C (IFNAR2) was positively linked to IFNAR2 expression in BRCA, LGG, and THCA (0.13 < β < 0.19, 5.58E-10 < P < 7.34E-05) and negatively associated with IL10RB expression in LGG (β = -0.16, P = 1.97E-06). In particular, rs10774671-A (OAS1) was positively related to OAS3 expression in LUSC (β = 0.20, P = 9.61E-05) and showed a consistent negative association with OAS1 expression in BLCA, HNSC, KIRC, KIRP, LGG, PCPG, PRAD, TGCT, and THCA (-0.57 < β < -0.23, 1.79E-22 < P < 2.39E-06) (**Figure 2G**). The above findings indicate that BLCA, HNSC, KIRC, KIRP, LGG, PCPG, PRAD, TGCT, and THCA patients carrying rs10774671-A (OAS1) genotype may be more likely to have poor COVID-19 outcomes relative to those carrying rs10774671-G because individuals carrying rs10774671-A will have lower expression of OAS1, which serves as a protective factor against SARS-CoV-2 infection and poor COVID-19 outcomes.

SARS-CoV-2 Affects Expression Levels of SARS-CoV-2-Required Genes

Kong and colleagues found that SARS-CoV can obviously increase ACE2 and TMPRSS2 expression levels in Calu-3 cells during 24-48 hours compared with that at 12 hours (4), indicating this kind of virus may elevate the expression of SARS-CoV-2-required genes in human tissues or cells. To explore the influence of SARS-CoV-2 infection in SARS-CoV-2-required genes, we performed a differential expression analysis for eight SARS-CoV-2-required genes in human nasal turbinates and lung tissues, A549 cells, and NHBEs with/without SARS-CoV-2 infection. We found that ACE2 is significantly elevated in human nasal turbinate infected with SARS-CoV-2 compared with mock infected turbinate (P = 0.002). Furthermore, the expressions of ATP6AP1, NPC1, and PIK3C3 in A549 cells were significantly influenced by SARS-CoV-2 infection compared with the control group. In addition, NHBEs infected with SARS-CoV-2 also showed an obviously increased expression of ACE2, TMPRSS2, NPC1, and RAB7A compared to that in control NHBEs (P < 0.06) (**Figures 3A–D**). These results indicate that SARS-CoV-2 can affect the expression levels of SARS-CoV-2-required genes in human normal tissues or cells and lung tumor cells.

Identification of SARS-CoV-2-Required Gene Expression in Human Tissues

SARS-CoV-2 was reported to invade various tissues such as the lung, nerve, adrenal, esophagus, thymus, pancreas, breast, skin cervix, and lymph node (22), with different susceptibility across these tissues (22, 23). In this study, we analyzed the expression profiles of SARS-CoV-2-required genes in normal tissue types and explored whether this might influence the susceptibility of the corresponding tissue tumor to SARS-CoV-2. Using 4,790 RNA-seq datasets from the Genotype-Tissue Expression (GTEx) v8 database, we evaluated their expression across 21 tissue types. We found that ACE2 and TMPRSS2 had an obvious expression difference between human tissues. ACE2 exhibited the high expression level in the testis, small intestine, and thyroid (6.33 < average TPM < 46.53), a secondary level in the pancreas, lung, ovary, fallopian tube, breast, vagina, and minor salivary gland (1 < average TPM < 2.38), and the low level in blood, muscle, spleen, nerve, prostate, bladder, live, uterus, pituitary, and adrenal gland (0.019 < average TPM < 0.70). TMPRSS2 exhibited a high expression level in the prostate, stomach, lung, thyroid, small intestine, pancreas, liver, and minor salivary gland (12.72 < average TPM < 178.1), the secondary level in vagina, breast, fallopian tube, pituitary, and bladder (1.37 < average TPM < 6.73), and a low level in other tissues. The other six SARS-CoV-2-required genes especially ATP6AP1 and RAB7A showed a broad expression in all tissues (2.18 < average TPM < 312.3) (**Figure 3E**). These findings mean that the thyroid, small intestine, testis, and lung may show a higher SARS-CoV-2 infection risk relative to other tissues because both ACE2 and TMPRSS2 expression were higher.

To further explore whether different cancer types had different SARS-CoV-2 infection risks, we evaluated specific expression profiles of SARS-CoV-2-required genes in the tumor tissues. We observed that ACE2 and TMPRSS2 had a lower expression level across all cancer types, compared to other SARS-CoV-2-required genes (**Figure 3F**). Similar to the normal organ tissues, the corresponding tumor tissues also showed an obvious expression difference for both ACE2 and TMPRSS2, of which BLCA, CECS, CHOL, COAD, ESCA, KIRP, LUAD, LUSC, PAAD, READ, STAD, THCA, and UCEC had the higher expressions of ACE2 and TMPRSS2 compared with other tumor tissue types (**Figure 3G**). These results indicate that the above cancer types might have a higher SARS-CoV-2 infection risk.

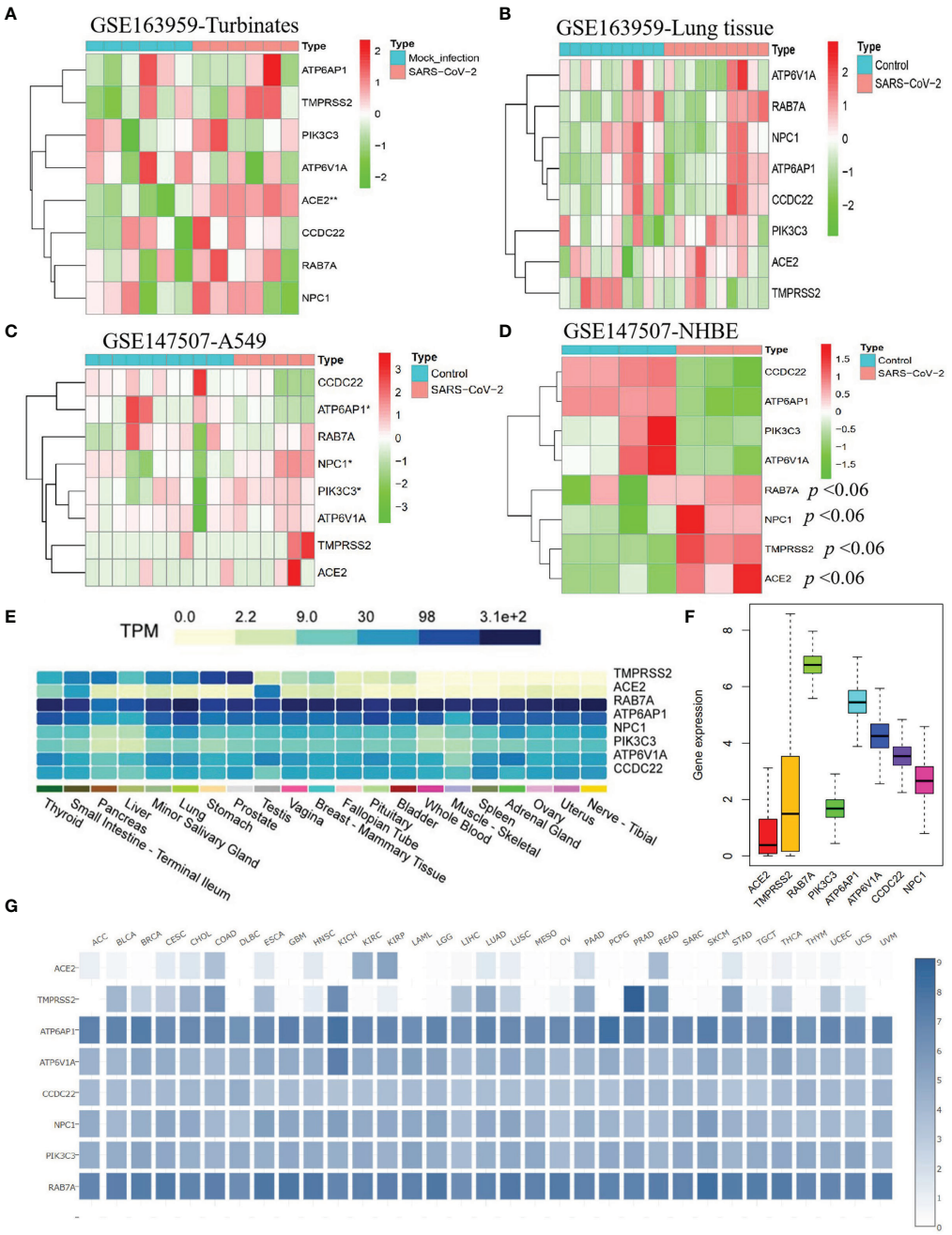

FIGURE 3 | The response of SARS-CoV-2-required genes in human cells and tissues infected with SARS-CoV-2 and their expression status in human tissues and corresponding tumor tissues. The expression profiles of eight SARS-CoV-2-required genes in human nasal turbinate **(A)** and lung tissues **(B)**, A549 cells **(C)**, and primary human bronchial epithelial cells (NHBEs) **(D)** with/without SARS-CoV-2 infection. **(E)** Expression profiles of SARS-CoV-2-required genes in 21 human tissues. **(F)** Mean expression value of SARS-CoV-2-required genes in cancers. **(G)** Expression profiles of SARS-CoV-2-required genes across 33 cancer types.

Identification of SARS-CoV-2-Required Gene Expression in Pan-Cancers

To evaluate whether cancer patients appear more vulnerable to SARS-CoV-2 relative to healthy individuals, we performed a differential expression analysis for SARS-CoV-2-required genes across 18 cancer types that had more than 5 normal samples. We found that these genes showed different expression levels between 18 types of tumor tissues and corresponding control tissues. ACE2 exhibited a significantly higher level in GBM, KIRP, LUAD, and UCEC, as well as an obviously lower level in BRCA, KICH, LIHC, PRAD, and THCA, compared with that in normal tissues ($P < 0.05$) (**Figure 4A** and **Table S4**). Notably,

75% of SARS-CoV-2-required genes including RAB7A, PIK3C3, ATP6AP1, ATP6V1A, CCDC22, and NPC1 showed a consistently significant upregulation in CHOL relative to normal tissues (1.09E-06 < P < 0.0001). In addition, ACE2 and TMPRSS2 appeared to be upregulated in CHOL compared to normal tissues (P > 0.05), indicating a possibly high risk of SARS-CoV-2 infection for CHOL (**Figures 4A, B**). Furthermore, 62.5% of SARS-CoV-2-required genes containing ACE2, TMPRSS2, ATP6AP1, ATP6V1A, and CCDC22 exhibited a significant upregulation in UCEC relative to normal tissue, while only PIK3C3 showed an obvious downregulation in this cancer.

SARS-CoV-2-Required Gene Expressions Affect Pan-Cancer Stage and Prognosis

To explore the role of SARS-CoV-2-required genes in pan-cancer prognosis, we performed the survival and univariate Cox proportional hazards regression analyses for these genes in all cancer types. Survival analysis indicated that ACE2 expression showed a good overall survival in KIRC, OV, and MESO. ATP6AP1 and ATP6V1A showed a positive association with good survival in PAAD and KIRC, respectively. The high CCDC22 or NPC1 expressions had a positive relationship with the poor survival in LIHC or MESO. RAB7A expression showed a poor prognosis in LIHC, UCEC, and PAAD, while exhibited a good prognosis in UVM (P < 0.01) (**Table S5**). Notably, univariate Cox proportional hazards regression analysis suggested that only CCDC22, RAB7A, ATP6V1A, and ATP6AP1 expressions were significantly associated with the overall survival of LAML and showed a high risk for poor prognosis. Moreover, our results also suggested that only NPC1, RAB7A, CCDC22, ATP6V1A, and ATP6AP1 expressions were obviously correlated with overall survival of LIHC and had a high risk for poor prognosis (HR >1, 6.78E-05 < P < 0.08) (**Figure S1A** and **Table S5**). While, only ACE2,

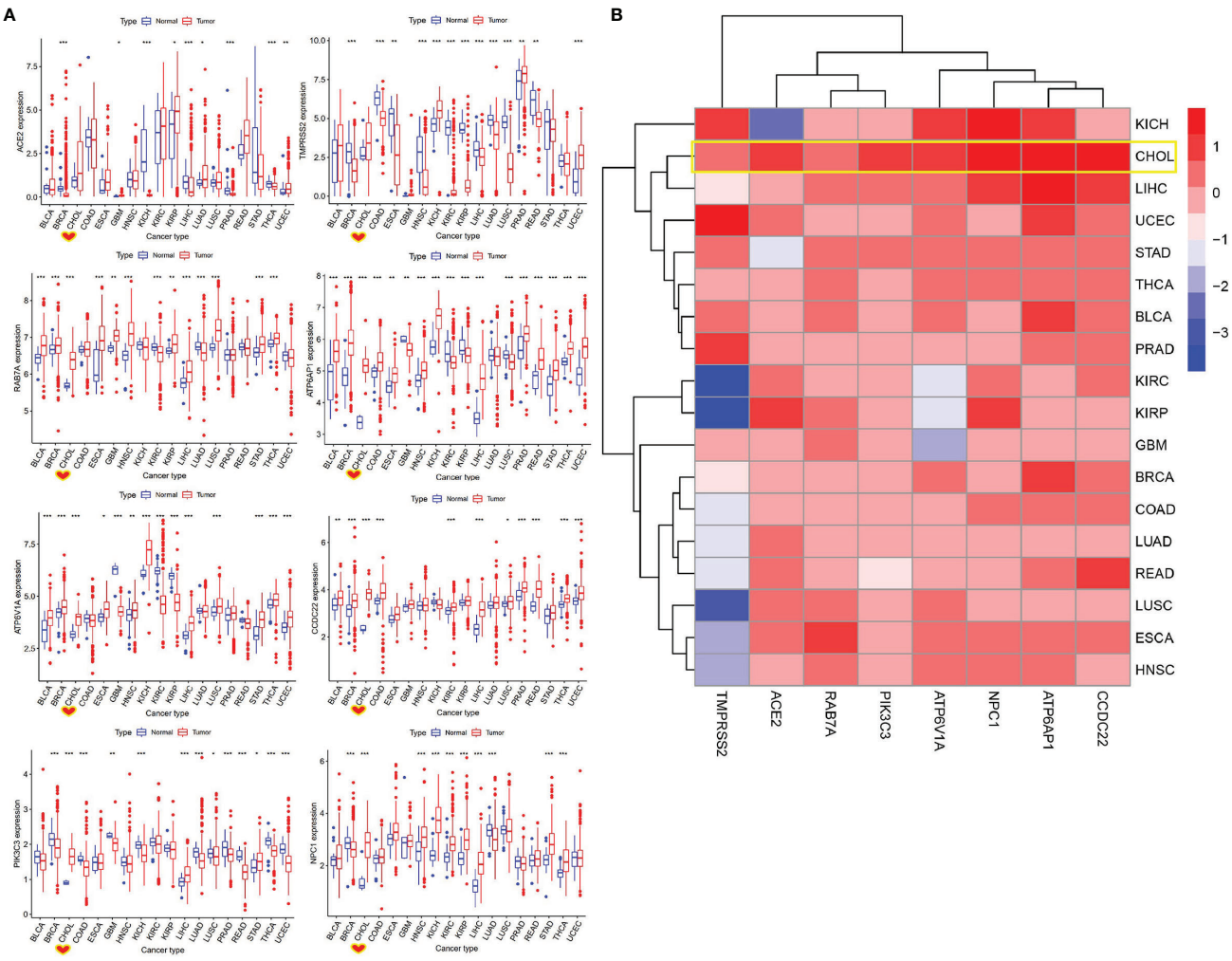

FIGURE 4 | Identification of differentially expressed SARS-CoV-2-required genes between cancer and control samples. **(A)** The expression of SARS-CoV-2-required genes between cancer and control samples. *P < 0.05, **P < 0.01, ***P < 0.001. **(B)** Heatmap of $\log_2$ (fold change) for SARS-CoV-2-required genes between cancer and control samples. Blue represents downregulation, Red presents upregulation.

PIK3C3, ATP6V1A, and ATP6AP1 expressions were obviously linked to the overall survival of KIRC and showed a low risk for poor prognosis (HR <1, 3.26E-09 < P < 0.009). These findings suggest a possibility that patients with LAML or LIHC infected after SARS-CoV-2 may have a poor prognosis.

Dai et al. (24) observed that patients with metastatic cancer (Stage IV) had a higher risk of death, ICU admission, and severe conditions, compared with no cancers or cancers without metastasis. In this study, we assessed the expression status of SARS-CoV-2-required genes in the different stage types of pan-cancers and predicted the potential risk of SARS-CoV-2 infection of cancer patients with high or low stage type. In the GSCA database, we found that multiple SARS-CoV-2-required genes were downregulated in the high stage type (Stage III or IV) of KIRC compared with low stage type (Stage I or II) (**Figure S1B** and **Table S6**). We then confirmed the results in GEPIA 2 database, and observed that ACE2, TMPRSS2, RAB7A, ATP6AP1, ATP6V1A, and PIK3C3 were significantly downregulated in high stage of KIRC compared with low stage (3.68 < F value < 10.4; 1.21E-06 < Pr (>F) < 0.012). NPC1 and CCDC22 also showed a decreased tendency in the high stage relative to the low stage. This means that patients with a low stage of KIRC may have a higher SARS-CoV-2 infection risk than those with a high stage.

SARS-CoV-2-Required Gene Expressions Are Related to Immune Response and the Tumor Microenvironment in Pan-Cancers

The TME comprised of stromal cells, immune cells, fibroblasts, blood vessels, endothelial cell precursors, etc., plays an important role in the initiation and maintenance of tumorigenesis (25) and affects the resistance to chemotherapy and radiotherapy, metastasis, and recurrence of cancer patients (26). To understand the association of SARS-CoV-2-required gene with TME in pan-cancers, we performed a spearman correlation analysis according to the ESTIMATE immune, stromal, and estimate scores. RAB7A, PIK3C3, ATP6AP1, ATP6V1A, and NPC1 expressions were found to show a consistently positive relationship with immune, stromal, and estimate scores in DLBC. Similarly, RAB7A, ATP6V1A, CCDC22, and NPC1 expressions had a consistently positive association with immune, stromal, and estimate scores in LAML. While TMPRSS2, PIK3C3, ATP6AP1, and ATP6V1A expressions exhibited a consistently negative relationship with immune, stromal, and estimate scores in KICH (**Figure S2A** and **Table S7**). Moreover, we further explored their roles in modulating cancer stem cells by measuring RNAss and DNAss. As shown in **Figure S2B** and **Table S7**, SARS-CoV-2-required genes might be linked to cancer stem cells purity in cancers, especially DLBC, TGCT, and THYM. Overall, these findings indicate that these genes could involve TME. The high expression levels of RAB7A, PIK3C3, ATP6AP1, ATP6V1A, CCDC22, and NPC1 were significantly associated with lower tumor purity of patients with hematologic cancer LAML, while TMPRSS2, PIK3C3, ATP6AP1, and ATP6V1A expressions were obviously correlated with higher tumor purity of KICH patients.

Immune subtypes, containing wound healing (C1), INF-gamma dominant (C2), inflammatory (C3), lymphocyte depleted (C4), immunologically quiet (C5), and TGF-β dominant (C6) are closely linked to overall survival and progression-free interval of cancer patients. For cancer patients, the C3 immune subtype shows the best prognosis, while C2 and C1 exhibit poor outcomes. Patients with the C4 or C6 immune subtypes have the least favorable outcome (17). We then explored the correlation of SARS-CoV-2-required genes with immune response. All SARS-CoV-2-required genes were found to be involved in six immune infiltration types in human tumors (P < 0.001) (**Figure S2C**). Moreover, in LUSC, high ACE2 and NPC1 expressions were found to be associated with decreased C3 immune infiltration and correlated with increased C1, C2, and C6 immune infiltration (**Figure S2D**). In contrast, upregulation of RAB7A, ATP6V1A, and PIK3C3 was linked to the increased C3 and the decreased C4 in KICH (**Figure S2E**). These findings indicate that by affecting immune subtypes, ACE2 and NPC1 expressions may associate with a less favorable outcome in LUSC, and RAB7A, ATP6V1A, and PIK3C3 expressions might link to a favorable outcome in KICH.

SARS-CoV-2-Required Gene Expressions Correlate With Immune Cell Infiltration in Pan-Cancers

Immune cell infiltration, including T cell (CD3+/CD8+/CD4+T, memory/effector T cell, and regulatory T cell), T helper 1 (TH1) cell, T helper 17 (TH17) cell, T helper 2 (TH2) cell, natural killer (NK) cell, plays a crucial role in inhibiting tumor cells or providing supports for tumor growth, and associates with a prognosis of 17 human cancers (24, 27–29). We utilized the ImmuCellAI database to perform a differential level analysis for immune cell infiltration between tumor and adjacent normal tissues and conducted a comprehensive analysis for the prognostic value of the major immune cell types across pan-cancers. We observed a broadly different abundance of immune cell types between tumor and adjacent tissues in 17 cancers. As shown in **Figure 5A**, nTreg, iTreg, Tr1, and Th1 were obviously enriched in the nidus of most cancer types. Conversely, several antitumor cells containing NKT, Th2, and Th17 exhibited a lower infiltration in most tumor types than the corresponding adjacent tissues. Especially, most immune cell types such as nTreg, iTreg, Tr1, CD8 naive, Th1, Exhausted, CD8+T, Cytotoxic, GammaDelta, MAIT, Tfh, NKT, CD4+T, Th2, and Th17 were enriched in the nidus or adjacent tissues of LUAD and LUSC. In addition, we found that CD8+T, GammaDelta, and Tfh were correlated with favorable prognoses in most cancers analyzed. Conversely, nTreg, NKT, and TH17 were indicative of poor prognosis, which is consistent with another previous report (24). Notably, CD8+T showed a strong correlation with the good prognosis of LUAD patients (**Figure 5B**). Moreover, eight SARS-CoV-2-required genes had a positive or negative association with the abundance of most immune cell types in different cancer types (FDR < 0.01) (**Table S8**), among which, the expressions of ACE2 and NPC1 were negatively associated with the abundance of CD8+T (**Table S8**). These results suggest a relationship between SARS-CoV-2-required genes and immune cell infiltration and prognosis in pan-cancers. SARS-CoV-2-

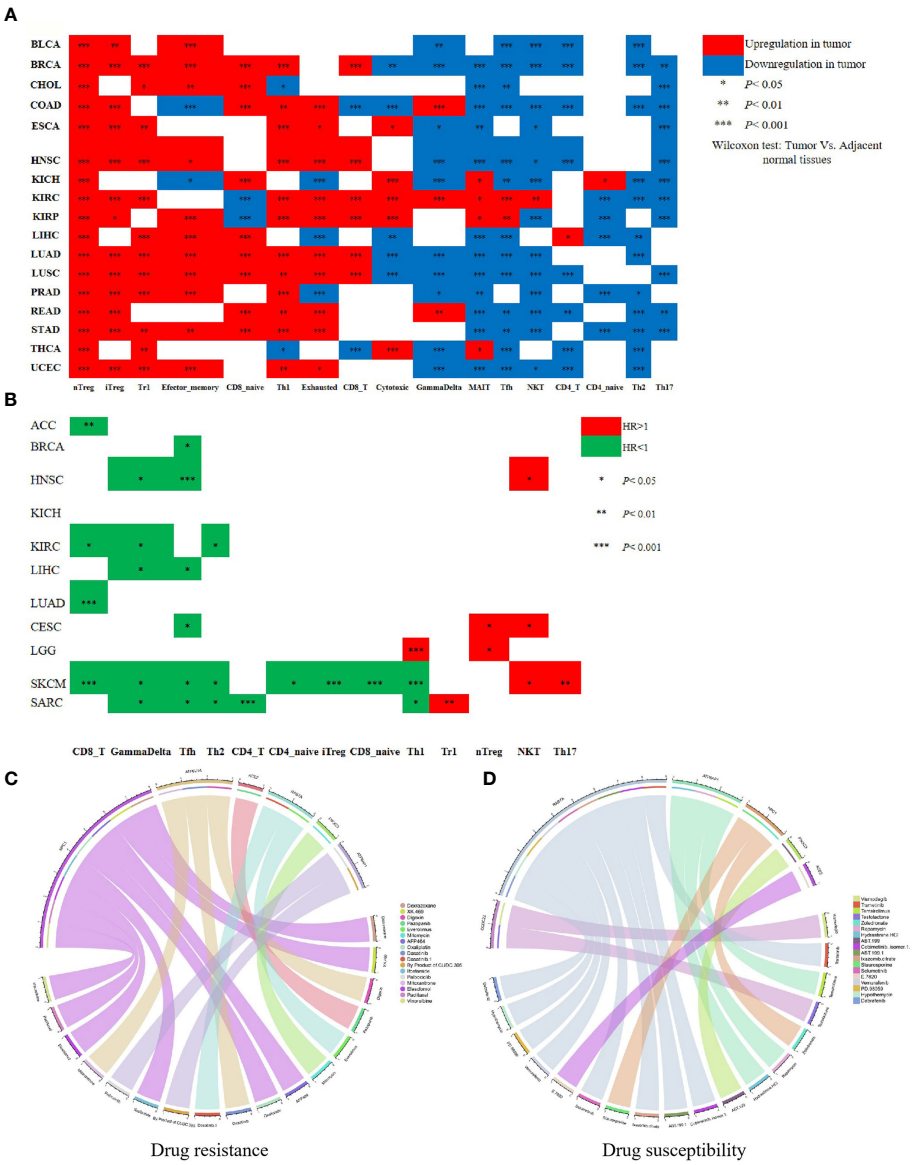

FIGURE 5 | Immune cell infiltration involved in the prognosis of cancer patients and SARS-CoV-2-required genes associated with drug sensitivity. **(A)** The abundance of immune cell types between tumor and adjacent tissue in 17 cancers. **(B)** The relationship between immune cell types and the prognosis of cancer patients. **(C)** The relationship between drug sensitivity and SARS-CoV-2-required genes. **(D)** The positive relationship between drug sensitivity and SARS-CoV-2-required genes. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

induced ACE2 and NPC1 elevation may have a negative influence in CD8+T of LUAD patients, which may result in a poor prognosis.

SARS-CoV-2-Required Genes Affect Anti-Cancer Drug Sensitivity

Anti-cancer drug resistance is implicated in the therapeutic effect and prognosis of cancer patients. In this study, we evaluated the influence of SARS-CoV-2-required genes in anti-cancer drug sensitivity. As summarized in **Table S9**, SARS-CoV-2-required genes showed a broad influence on anti-cancer drug sensitivity. ACE2, RAB7A, PIK3C3, ATP6AP1, NPC1, and ATP6V1A had a negative association with the sensitivity of 16 anti-cancer drugs ($P < 0.05$). Especially, ACE2 expression was significantly associated with the decreased sensitivity of Pazopanib (advanced renal cell cancer and soft tissue sarcoma). NPC1 expression showed an obviously negative relationship with the sensitivity of Dexrazoxane (a cardioprotective agent against the cardiotoxic side effects of chemotherapeutic drugs), Oxaliplatin (carcinoma of the colon or rectum), Ifosfamide (testicular, ovarian, cervical, and bladder cancers, osteocarcinoma, small cell lung cancer, and non-Hodgkin's lymphoma), Elesclomol

(metastatic melanoma), Paclitaxel (Kaposi's sarcoma and cancer of the lung, ovarian, and breast), and Vinorelbine (metastatic non-small cell lung carcinoma) ($P < 0.01$) (**Figure 5C**), suggesting that the upregulation of ACE2 and NPC1 may reduce the curative effect of these drugs. ACE2, RAB7A, CCDC22, ATP6AP1, NPC1, and PIK3C3 exhibited a positive relationship with sensitivity of 18 anti-cancer drugs ($P < 0.01$) (**Figure 5D**).

Correlation Between OAS1, ACE2, TMPRSS2, and Lung Cancer-Related Genes and Their Influence on the Prognosis of Lung Cancer

In this study, we found that several SARS-CoV-2-related genes may affect the prognosis of lung cancer patients. To further explore the potential mechanism of these genes on the prognosis of lung cancer, we performed a correlation and survival analysis for SARS-CoV-2-related genes and lung cancer-related genes in lung cancer. We found that OAS1, ACE2, TMPRSS2 were associated with overall survival of lung cancer (**Figure 6A**). High OAS1 was associated with the poor overall survival of LUAD, while high TMPRSS2 expression showed a good overall survival in LUAD. ACE2 expression had a positive association with overall survival of LUSC (**Figure 6B**). Correlation analysis indicated that OAS1 expression had a negative correlation with TMPRSS2 expression in LUAD. ACE2 expression showed a positive association with TMPRSS2 expression in LUAD. OAS1 expression exhibited no relationship with ACE2 expression in LUAD. For LUSC, there was a positive association between OAS1, TMPRSS2, and ACE2 expressions (**Figure 6C**). Differential gene expression analysis identified 268 downregulated and 69 upregulated differentially expressed genes (DEGs) in LUAD as well as 561 downregulated and 296 upregulated DEGs in LUSC. Among these DEGs, 35 upregulated and 236 downregulated genes showed a consistent association with both LUAD and LUSC (**Table S10**). We then estimated the association of 271 DEGs with OAS1, TMPRSS2, and ACE2 expressions in LUAD and LUSC. We found that

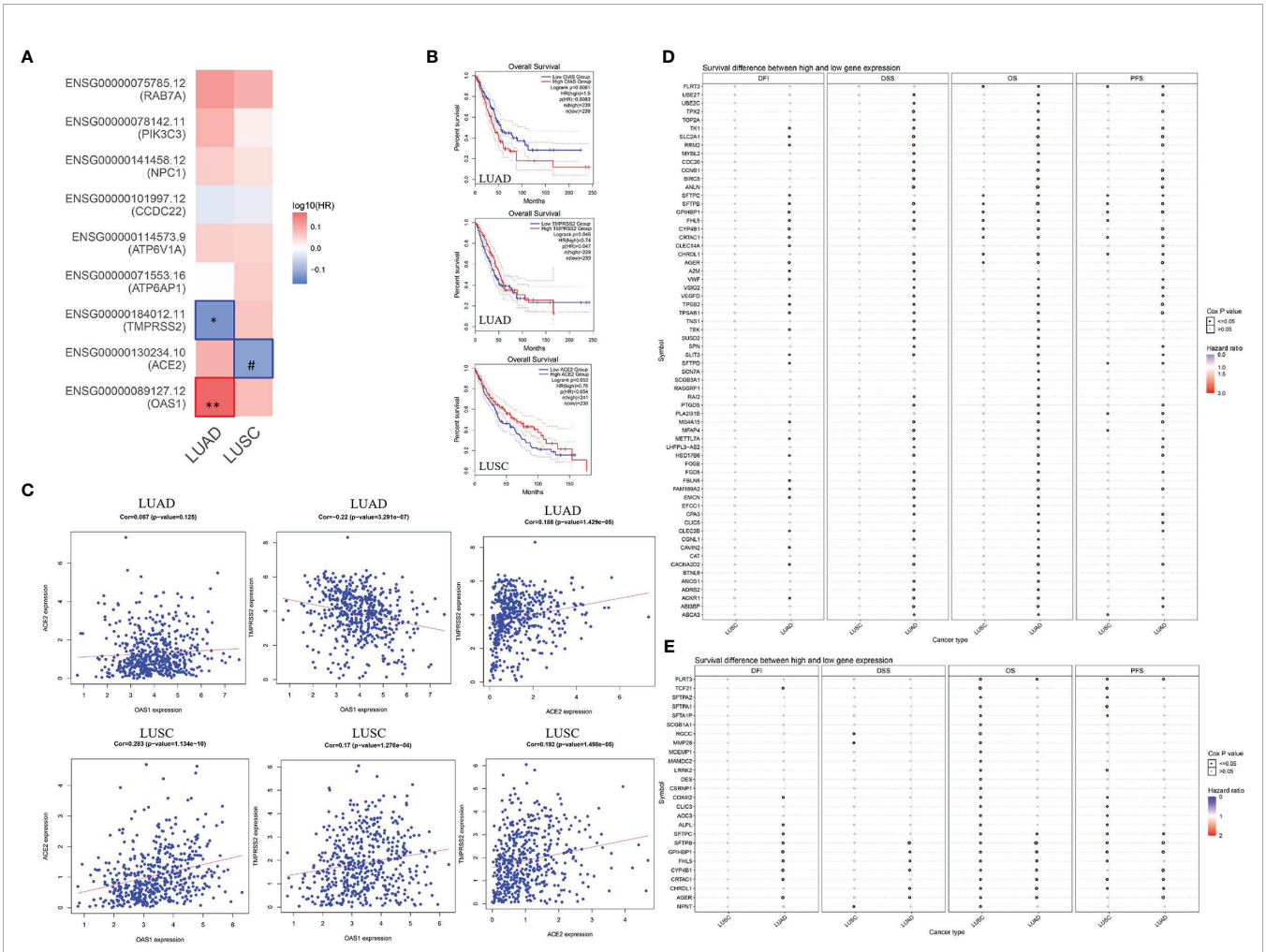

FIGURE 6 | Correlation between OAS1, ACE2, and TMPRSS2, and effect of lung cancer-related genes in the prognosis of lung cancer. **(A, B)** Survival analysis for SARS-CoV-2-required/susceptibility genes in LUAD and LUSC. **(C)** Correlation between OAS1, ACE2, and TMPRSS2. **(D)** Association of lung cancer-related genes with the prognosis of LUAD. **(E)** Association of lung cancer-related genes with the prognosis of LUSC. *$P < 0.05$; **$P < 0.01$; #$0.05 < P < 0.1$.

ACE2 expression had a positive correlation with GPX2 expression and a negative correlation with SLC2A1 expression in both LUAD and LUSC. OAS1 expression was positively related to C1QB expression in both LUAD and LUSC. TMPRSS2 expression was negatively associated with 13 lung cancer-related genes and showed a positive correlation with 130 lung cancer-related genes (**Table S11**). Finally, we carried out a survival analysis for the above 146 lung cancer-related genes in LUAD and LUSC. The results showed that 64 lung cancer-related genes were linked to overall survival of LUAD patients, and 26 lung cancer-related genes were correlated with overall survival of LUSC patients. Among these genes, high SLC2A1 expression was associated with the poor overall survival of LUAD patients, but not in LUSC. Notably, FLRT3, CYP4B1, CHRDL1, SFTPC, SFTPB, and AGER, which had a positive correlation with TMPRSS2, were significantly downregulated in LUAD and LUSC. LUAD and LUSC patients with high FLRT3 expression had poor overall survival. CYP4B1, CHRDL1, SFTPC, SFTPB, and AGER expressions were linked to the good overall survival in LUAD patients (**Figures 6D, E**, and **Table S11**), whereas the opposite was true in patients with LUSC, indicating that these genes may have different effects on prognosis of LUAD and LUSC.

Upstream Regulators of SARS-CoV-2-Required Genes

Non-coding RNA is widely acknowledged to regulate the expression of target genes. To identify the upstream regulators of SARS-CoV-2-required genes and explore the potential treatment and intervention targets for SARS-CoV-2, we used the starBase database to predict microRNAs targeting SARS-CoV-2-required genes. As summarized in **Table S12**, one hundred and forty-six microRNAs were found to be upstream regulators of SARS-CoV-2-required genes. In this study, we focused on the ACE2 and its upstream microRNAs. Total 12 microRNAs containing miR-29a-3p, miR-29b-3p, miR-143-3p, miR-149-5p, miR-29c-3p, miR-432-5p, miR-599, miR-653-5p, miR-760, miR-942-5p, miR-1251-5p, and miR-212-5p were predicted to target ACE2 and showed a significantly negative or positive correlation with ACE2 expression in 30 cancer types ($-0.42 < Cor < 0.46$; $P < 0.05$) (**Figure 7A**). These microRNAs also exhibited significantly differential expression between 17 types of cancer samples and the corresponding control samples (**Figure 7B**) and had a different effect on the prognosis of 25 cancer types (**Figure 7C**). In particular, miR-760, targeted ACE2, had a negative relationship with ACE2 expression (Cor = -0.24; P = 4.55E-06) (**Figure 7D**), and was markedly upregulated in LIHC (FDR= 0.007) (**Figure 7E**). Its upregulation was positively linked to the poor prognosis of LIHC patients (HR=1.78; P = 0.0015) (**Figure 7F**). Conversely, ACE2 was downregulated in LIHC (FDR = 0.0038) (**Figure 7G**), and its upregulation showed a positive association with the favorable prognosis of LIHC patients (HR = 0.65; P = 0.017) (**Figure 7H**). These findings indicate that microRNAs could be the potential regulator of SARS-CoV-2-required genes. Notably, miR-760 may have the potential to serve as a treatment and intervention target for SARS-CoV-2 because of its inhibitory effect on ACE2.

DISCUSSION

Four epidemiological investigations revealed that cancer patients appear more vulnerable to SARS-CoV-2 and show poor outcomes compared with non-cancer patients (10–13). Moreover, several research teams have demonstrated that host-specific genetic factors play an important role in SARS-CoV-2 susceptibility and COVID-19 outcomes (5–9). In this study, we aimed to explore whether SARS-CoV-2-required genes and host genes and variants play a critical role in the SARS-CoV-2 susceptibility of cancer patients and poor COVID-19 outcomes of cancer patients infected with SARS-CoV-2.

Firstly, we evaluated the response of 14 host genes related to SARS-CoV-2 susceptibility and COVID-19 outcomes in multiple cell types of the respiratory system after SARS-CoV-2 infection. We found that SARS-CoV-2 can significantly elevate OAS1 and PLEKHA4 expressions in turbinate, lung tissues, and NHBEs. OAS1 showed a broad expression in different human organs and tumor tissues of the HPA database, with low organ and cancer specificity. Zhou et al. identified that increased plasma OAS1 protein level is positively associated with reduced COVID-19 susceptibility and poor outcomes in 14,134 cases and 1.2 million controls. Collectively, these findings suggested that OAS1 may serve as a protective factor against SARS-CoV-2 infection and poor COVID-19 outcomes in the wide organs and tissues. We further explored the effect of SNPs located on these 14 host genes on expressions of potential key genes *via* expression quantitative trait locus (cis-eQTL) analysis in 33 cancer types. Rs4801778-T (PLEKHA4), rs11919389-C (ZBTB11), rs13050728-C (IFNAR2), and rs10774671-A (OAS1) exhibited a positive or negative regulation in TULP2, HSD17B14, LOC285359, LOC100009676, SENP7, IFNAR2, OAS3, and OAS1 in multiple cancer types. Especially, rs10774671-A (OAS1) showed a consistent negative association with OAS1 expression in BLCA, HNSC, KIRC, KIRP, LGG, PCPG, PRAD, TGCT, and THCA. Taking together, these findings indicate that BLCA, HNSC, KIRC, KIRP, LGG, PCPG, PRAD, TGCT, and THCA patients carrying rs10774671-A (OAS1) genotype may be more likely to have poor COVID-19 outcomes relative to those carrying rs10774671-G because individuals carrying rs10774671-A will have the lower expression of OAS1, which serves as a protective factor against SARS-CoV-2 progress and poor COVID-19 progress outcomes.

Subsequently, we assessed the response of eight SARS-CoV-2-required genes in multiple cell types of the respiratory system after SARS-CoV-2 infection. We observed that SARS-CoV-2 increased ACE2 and NPC1 expression in normal/tumor tissues or cells of the human respiratory system, similar to one previous report (4). We then evaluated expression profiles of SARS-CoV-2-required genes in human normal and pan-cancer tissues. We found that ACE2 and TMPRSS2 showed an obvious expression difference between different human tissues, while other SARS-CoV-2-required genes had a widely high or medium expression

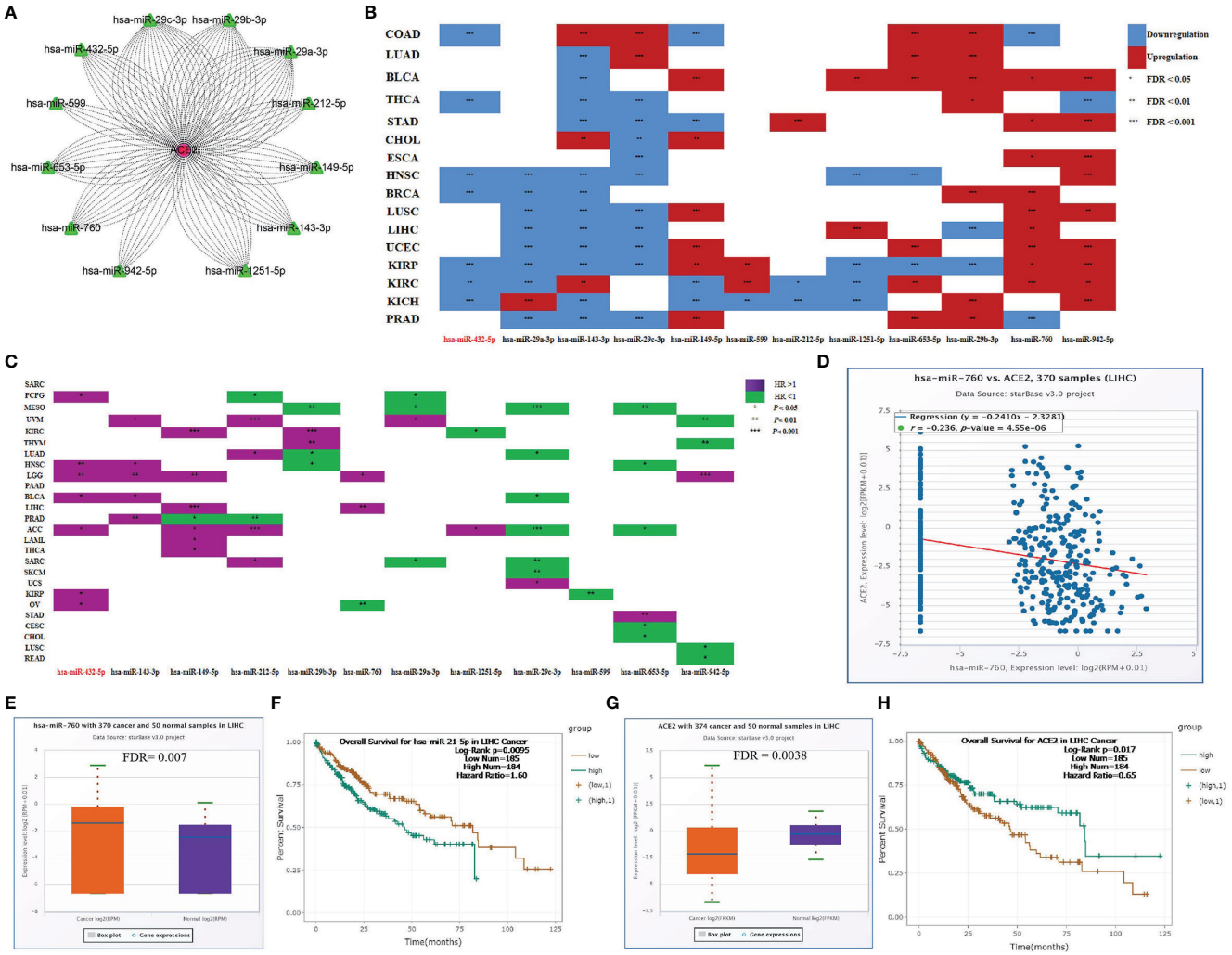

FIGURE 7 | Upstream regulators of ACE2. **(A)** microRNAs targeting ACE2. **(B)** Differentially expressed microRNAs between cancer tissues and control tissues. **(C)** Association between microRNAs and the prognosis of cancers. **(D)** Correlation between miR-760 and ACE2 in LIHC. **(E)** Expression difference of miR-760 between LIHC tissues and control tissues. **(F)** Association between miR-760 and the prognosis of LIHC. **(G)** Expression difference of ACE2 between LIHC tissues and control tissues. **(H)** Association between ACE2 and the prognosis of LIHC. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.1$.

level in all tissues. Compared with other tissues, the small intestine, and thyroid, testis, lung, pancreas, breast, and fallopian had higher expression levels of SARS-CoV-2-required genes. For tumor tissues corresponding to the above organs, BLCA, CECS, CHOL, COAD, ESCA, KIRP, LUAD, LUSC, PAAD, READ, STAD, THCA, and UCEC showed higher expressions of ACE2 and TMPRSS2 compared with other tumor tissue types. These results may mean a higher SARS-CoV-2 susceptibility in these tissues and the corresponding tumor tissues.

We further analyzed the expression profiles for eight SARS-CoV-2-required genes in pan-cancers. Seventy-five percent of SARS-CoV-2-required genes including RAB7A, PIK3C3, ATP6AP1, ATP6V1A, CCDC22, and NPC1 were found to show a consistently significant upregulation in CHOL relative to normal tissues. In addition, ACE2 and TMPRSS2 appeared to be upregulated in CHOL compared to normal tissues. These results indicate that CHOL patients potentially have a higher risk of SARS-CoV-2 infection compared with healthy subjects. In addition, we observed an association of SARS-CoV-2-required genes with the poor or good prognosis of multiple cancer types by survival analysis and univariate Cox proportional hazards regression analysis. Among which, CCDC22, RAB7A, ATP6V1A, and ATP6AP1 expressions were significantly associated with the poor overall survival of LAML, suggesting a possibility that patients with hematological cancer (LAML) after SARS-CoV-2 infection may have a poor prognosis, and might support conclusions by Bernard et al. (1) and Dai et al. (11) that SARS-CoV-2-infected patients with hematological cancer have the highest frequency of severe events including death rates and

ICU admission. By GSCA and GEPIA 2 databases, we confirmed that ACE2, TMPRSS2, RAB7A, ATP6AP1, ATP6V1A, and PIK3C3 were significantly downregulated in the high stage of KIRC compared with the low stage. NPC1 and CCDC22 also showed a reduced tendency in the high stage. These results mean that patients with a low stage of KIRC may have a higher SARS-CoV-2 infection risk than those with a high stage.

We analyzed the relationship of SARS-CoV-2-required genes with TME and immune response in pan-cancers and found that their expression was significantly associated with tumor purity of patients with LAML and KICH. Immune subtypes were reported to involve overall survival and progression free intervals of cancers. C3 (inflammatory) shows the best prognosis in cancer patients, while C2 (IFN-γ dominant), C1 (wound healing), C4 (lymphocyte depleted), and C6 (TGF-β dominant) exhibit the poor outcome (7). In our analysis, the high ACE2 and NPC1 expressions were found to be associated with the decreased C3 immune infiltration of LUSC, and correlate with increased C1, C2, and C6 immune infiltration. Immune cell infiltration plays a crucial role in the prognosis of multiple human cancers (24, 27–29). Similar to the previous report, we found that CD8+T, GammaDelta, and Tfh were correlated with the favorable prognosis in most of the cancer types analyzed; while nTreg cells, NKT, and TH17 cells were indicative of poor prognosis (24), which may be affected by SARS-CoV-2-required genes. Notably, CD8+T showed a positive correlation with the good prognosis of LUAD patients, and the expressions of ACE2 and NPC1 were negatively associated with the abundance of CD8+T. Given that ACE2 and NPC1 were significantly upregulated in the normal tissues and cells or tumor cells of the respiratory system infected after SARS-CoV-2, these findings indicate that LUSC or LUAD patients infected with SARS-CoV-2 may have a worse outcome because SARS-CoV-2-induced ACE2 and NPC1 elevation may have a negative influence in C3 and a positive effect on the C1, C2, and C6 immune infiltration of LUSC, or have a negative influence in CD8+T of LUAD. This also may support the conclusions of Bernard et al. (10) and Dai et al. (11) that patients with lung cancer have a high frequency of severe events.

To further explore the potential mechanism of SARS-CoV-2-related genes on the prognosis of lung cancer, we performed a correlation and survival analysis for these genes and lung cancer-related genes in lung cancer. Our results suggested that OAS1, ACE2, and TMPRSS2 expressions showed a different interaction in LUAD and LUSC and had a different effect on the prognosis of LUAD and LUSC. Notably, OAS1 expression showed a negative association with TMPRSS2 expression in LUAD, while exhibited a positive correlation with TMPRSS2 expression in LUSC, indicating that upregulation of OAS1 may decrease TMPRSS2 expression in LUAD but may increase TMPRSS2 expression in LUSC. We also found that these genes showed a consistent association with 90 lung cancer-related genes having different influences on the prognosis of LUAD or LUSC patients. Especially, five lung cancer-related genes including CYP4B1, CHRDL1, SFTPC, SFTPB, and AGER were consistently downregulated in both LUAD and LUSC had a positive correlation with TMPRSS2, exhibited an opposite effect on the prognosis of LUAD and LUSC. These findings indicate that LUSC and LUAD patients may have a varying degree of adverse outcomes if they are infected with SARS-CoV-2 because of the opposite interaction between OAS1 and TMPRSS2 in LUAD and LUSC as well as the opposite effect of these lung cancer-related genes on the prognosis of LUAD and LUSC.

We evaluated the influence of SARS-CoV-2-required genes in anti-cancer drug sensitivity, a common event influencing the therapeutic effect and prognosis of cancer patients. SARS-CoV-2-required genes were found to show a broad influence in anti-cancer drug sensitivity. Notably, ACE2 and NPC1, elevated in human cells or tissues infected with SARS-CoV-2, were found to be significantly associated with the decreased drug sensitivity (Pazopanib, Dexrazoxane, Oxaliplatin, Ifosfamide, Elesclomol, Paclitaxel, and Vinorelbine) of multiple cancer types including small cell lung cancer and metastatic non-small cell lung carcinoma, suggesting that patients with cancers (especially lung cancers) after SARS-CoV-2 infection may have a poor outcome because of the negative effect of SARS-CoV-2-induced upregulation of ACE2 and NPC1 on these anti-cancer drug sensitivity.

MicroRNA is dysregulated in various cancers *via* different mechanisms, which in return influences cancer hallmarks such as tumor cell proliferation, death inhibition, metastasis, and angiogenesis (30). In the current analysis, 146 microRNAs were found to be the upstream regulators of SARS-CoV-2-required genes. Total 12 microRNAs were predicted to target ACE2, with a significantly negative or positive correlation with ACE2 expression in 30 cancer types. Especially, miR-760, a broadly downgraded tumor suppressor in various cancer types (30–33), may have the potential to serve as a treatment and intervention target for SARS-CoV-2 because of its inhibitory effect on ACE2. Elevating miR-760 could be beneficial for cancer treatment and SARS-CoV-2 prevention.

In conclusion, the findings in this study demonstrate that BLCA, HNSC, KIRC, KIRP, LGG, PCPG, PRAD, TGCT, and THCA patients carrying rs10774671-A (OAS1) genotype may have a higher risk for poor COVID-19 outcomes relative to those who carry rs10774671-G. SARS-CoV-2-required genes were correlated with TME, immune response, and infiltration, overall survival, anti-cancer drug sensitivity of pan-cancers. CHOL patients may have a higher risk of SARS-CoV-2 infection than healthy subjects. As shown in **Figure 8**, lung cancer patients infected with SARS-CoV-2 may have a worse outcome because SARS-CoV-2-induced ACE2 and NPC1 elevation, which in turn promotes further SARS-CoV-2 invasion, may influence the immune subtypes of LUSC and immune infiltration in CD8+T of LUAD, and affect the sensitivity of anti-cancer drug. LUSC and LUAD patients may have a varying degree of adverse outcomes if they are infected with SARS-CoV-2. OAS1, ACE2, and miR-760 could serve as treatment and intervention targets for SARS-CoV-2. Future studies are needed to confirm the results by *in vitro* and *in vivo* experiments.

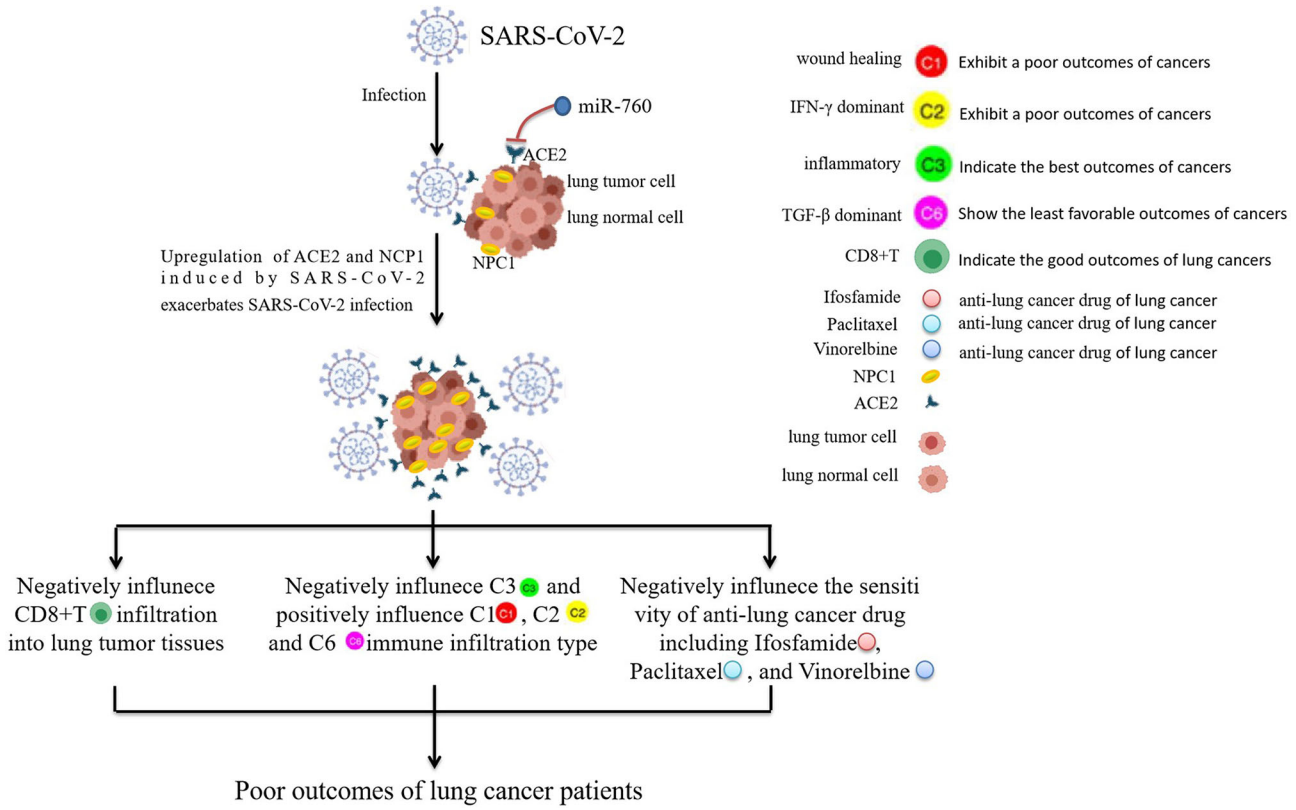

FIGURE 8 | The prediction of the worse outcome for lung cancer patients infected with SARS-CoV-2. Lung cancer patients infected with SARS-CoV-2 may have a worse outcome because SARS-CoV-2-induced ACE2 and NPC1 elevation may have a negative influence on the immune response of LUSC and CD8+T infiltration of LUAD, and negatively affect the sensitivity of anti-cancer drugs including Ifosfamide, Paclitaxel, and Vinorelbine. miR-760 may have the potential to serve as a treatment and intervention target for SARS-CoV-2 because of its targeted inhibition effect on ACE2.

DATA AVAILABILITY STATEMENT

The data generated in this study are publicly available in the TCGA, GEO (GSE163959 and GSE147507), GTEx, HPA, GSCA, GEPIA 2, PancanQTL, starbase 3.0, CellMiner, and ImmuCellAI databases.

AUTHOR CONTRIBUTIONS

LX and XH designed research. XH, HL and HZ drafted the manuscript and revised the paper. XH, HZ, and HL performed analyses. XH, TW, LP, LT, QZ, XG, WL, AC, QD, YZ, HW, MH, DD, and ZL participated in data consolidation and plotting. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2021.804387/full#supplementary-material

Supplementary Figure 1 | The prognosis analysis for SARS-CoV-2-required genes in 33 cancer types and the different assessment of SARS-CoV-2-required genes in different stages of pan-cancers. **(A)** Univariate Cox regression analysis for SARS-CoV-2-required genes. **(B)** SARS-CoV-2-required genes ACE2, TMPRSS2, RAB7A, ATP6AP1, ATP6V1A, and PIK3C3 were downregulated in high stage type of KIRC compared with low stage type identified by GSCA (http://bioinfo.life.hust.edu.cn/GSCA/#/) and GEPIA 2 (http://gepia2.cancer-pku.cn/#index) databases.

Supplementary Figure 2 | SARS-CoV-2-required genes associated with immune subtypes and tumor microenvironment. **(A)** Association of SARS-CoV-2-required gene expression with the ESTIMATE immune, stromal, and estimate

scores. **(B)** Association of SARS-CoV-2-required gene expression with RNAss and DNAss. Association of SARS-CoV-2-required genes with immune subtypes in all cancer patients **(C)**, LUSC patients **(D)**, and KICH patients **(E)**.

Supplementary Table 7 | SARS-CoV-2-required genes associated with immune subtypes and tumor microenvironment.

Supplementary Table 9 | SARS-CoV-2-required genes associated with drug sensitivity.

Supplementary Table 10 | Identification of differentially expressed genes in LUAD and LUSC, and their association with prognosis of lung cancer patients.

Supplementary Table 11 | Correlation between OAS1, ACE2, TMPRSS2, and lung cancer-related genes.

Supplementary Table 12 | Upstream regulators of SARS-CoV-2-required genes.

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Bioinspired Membrane-Coated Nanoplatform for Targeted Tumor Immunotherapy

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Immunotherapy can effectively activate the immune system and reshape the tumor immune microenvironment, which has been an alternative method in cancer therapy besides surgery, radiotherapy, and chemotherapy. However, the current clinical outcomes are not satisfied due to the lack of targeting of the treatment with some unexpected damages to the human body. Recently, cell membrane-based bioinspired nanoparticles for tumor immunotherapy have attracted much attention because of their superior immune regulating, drug delivery, excellent tumor targeting, and biocompatibility. Together, the article reviews the recent progress of cell membrane-based bioinspired nanoparticles for immunotherapy in cancer treatment. We also evaluate the prospect of bioinspired nanoparticles in immunotherapy for cancer. This strategy may open up new research directions for cancer therapy.

Keywords: bioinspired membrane, nanoparticle, tumor targeting, immunotherapy, nanobiotechnology applications

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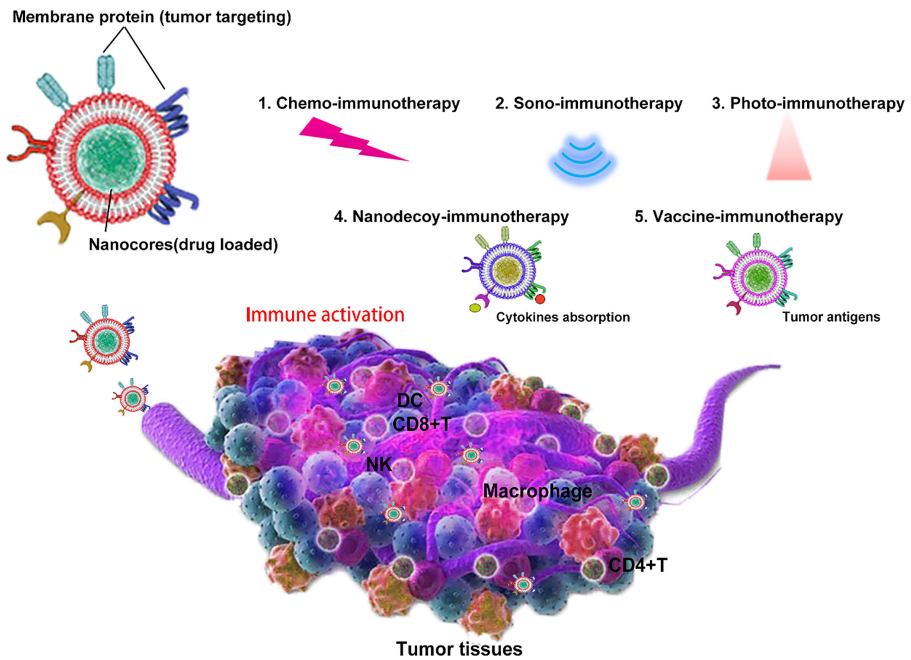
INTRODUCTION

Cancer has been one of the most refractory diseases worldwide, causing millions of deaths with a vast social consumption annually (1, 2). Much progress has been made in cancer treatments, such as surgical excision, chemotherapy, and radiotherapy, and the survival of cancer patients has also been greatly improved (3–5). However, the initial clinical response rate to many tumors did not achieve the desired results, and with the extension of treatment time, the tumor often develops drug resistance and is easy to relapse. Cancer immunotherapy as a new therapy developed rapidly in recent years, which can control and kill tumor cells by stimulating or rebuilding the immune system (6). Nonetheless, it was reported that these treatments could not achieve the ideal therapeutic results and even caused unexpected damages to the human body due to deficient tumor targeting (7, 8). Therefore, there is an urgent need to develop a novel delivery system to address the issues.

Recently, cell membrane-coated nanoparticles have attracted much attention due to their biocompatibility, prolonged half-time, and superior tumor targeting from the source cells. Tumor immunotherapy based on bioinspired nanoparticles is a new therapy developed rapidly in recent years. Despite its potential significance for cancer treatment with excellent immune effect, there is a lack of discussion that focuses on bioinspired nanoparticles. Hence, this study aims to review bioinspired nanoparticles with different functions and strategies, such as nanodecoy-, vaccine-, photo-, sono-, and chemo-immunotherapy (**Scheme 1**), and also discusses the current lack of development and future development prospects.



SCHEME 1 | Bioinspired cell membrane-based nanoparticles in tumor immunotherapy. Cell membrane-coated nanoparticles can be used as nanodecoy-, vaccine-, photo-, sono-, and chemo-immunotherapy for tumor eradication.

CELL MEMBRANE COATING NANOPLATFORM IN THE THERAPY OF CANCER

According to the function of membrane-coated nanoparticles, it was briefly classified into nanodecoy-, vaccine-, photo-, sono-, and chemo-immunotherapy, and the advancement of membrane-coated nanoplatforms (NPs) in tumor treatment is also discussed in this section.

Nanodecoy-Immunotherapy in Cancer Treatment

Tumor cells can produce various cytokines (mainly including GM-CSF, granulocyte-macrophage colony-stimulating factor, and CXCL2, chemokine ligand 2), resulting in an immunosuppressive environment through recruiting myeloid-derived suppressor cells (MDSCs) and thus inhibit the functions of tumor-specific $CD8^+$ T cells and cause tumor cells' immune escape (9, 10). Due to this fact, it remains the major obstacle that limits the efficacy of immunotherapy, such as immune checkpoint therapy. MDSCs (consisting of ~80% PMN-MDSCs, polymorphonuclear and ~20% M-MDSCs, monocytic populations) are responsible for the immunosuppressive tumor microenvironment (TME), which not only primarily suppresses $CD8^+$ T cells' immune response but also directly facilitates tumor growth and metastasis. Target elimination of MDSCs may help improve antitumor immune response, but it often brings about serious side effects. Recently, the pseudoneutrophil cytokine sponges (pCSs), fabricated by coating neutrophil membrane onto PLGA cores to mimic PMN-MDSCs, were reported (10). Inheriting the properties of source cells, pCSs can specifically absorb or neutralize MDSC-related cytokines and hence decrease or disrupt the recruitment of MDSCs and subsequently relieve immune tolerance. When incubated with GM-CSF and CXCL2, pCSs can show a superior binding capacity to them in a dose-dependent manner even compared with RBC@NPs. Inspired by the facts, mice bearing B16F10 were injected pCSs daily for 8 days, then the peripheral lymphoid organs and tumors were collected and analyzed by flow cytometry, and the results showed that pCSs could significantly suppress the expansion of MDSCs in the bone marrow and thus decreased their assembly in peripheral lymphoid organs and tumors. However, in immunodeficient B6/Rag1−/− or NOG mice, pCSs treatments did not limit the progress of melanoma or breast cancer. At the same time, it could delay the growth of the tumors in normal mice. In short, the antitumor activity of pCSs is established on an intact immune system. In murine breast cancer 4T1 and melanoma B16F10 models, pCSs administration can significantly enhance the infiltration of CD^+8 T cells and improve antitumor immune response. Furthermore, in the combination therapy with anti-PD-1, pCSs suppress tumor growth and prolong survival. Collectively, the neutrophil cell membrane-coated NPs can be a novel immune-modulating nanoplatform for effective cancer immunotherapy.

Vaccine-Immunotherapy in Cancer Treatment

Cancer vaccines can drill immune cells to specifically recognize and eradicate cancerous cells while sparing normal cells, which is established by effective tumor-associated antigen delivery (11,

12). However, application of cancer vaccines is rarely reported in clinics. Presently, tumor vaccine development is notoriously limited because tumor antigens are derived from normal antigens with subtle mutation or facile upregulation that is difficult to stimulate cellular immunity (13, 14). In particular, cancer cell membrane-coated nanoparticles have been used in homologous targeting drug delivery because of the entire inheritance of source cells. Therefore, taking advantage of cancer membrane, whose membrane proteins could also be tumor-specific antigens, to activate immune response would be a promising strategy to enhance immunotherapy (15, 16).

In a recent study, B16-F10 cancer cell membrane-coated murine-specific CpG-NPs (CpG-CCNPs) achieved a superior prophylactic and therapeutic efficacy in melanoma therapy (17). In the design, adjuvant CpG-loaded PLGA NPs were synthesized to be the inner cores, which can stimulate the maturation of DCs and the subsequent activation of tumor-specific T cells through TLR-9 signaling. When incubated with bone marrow-derived dendritic cells (BMDCs), the inner cores wrapped with the B16-F10 membrane showed more enhanced endocytosis by BMDCs compared with bare CpG NPs. Consistent with the findings, after subcutaneous injection, CpG-CCNPs can be actively internalized by macrophages and BMDCs in the draining lymph node while B or T cells had relatively less cell uptake due to the nonspecific interactions. DCs can be significantly activated to mature with the confirmation of the upregulation of CD40, CD80, CD86, and MHC-II. Meanwhile, due to the existence of melanoma major antigens such as gp100 and tyrosinase-related protein (TRP)-2 on the surface of CpG-CCNPs, it can strongly generate gp100-specific and TRP-2-specific T cells in the spleen, verifying the previous speculation that the nanoparticles were able to train the immune system against various tumor antigens. When vaccinated with CpG-CCNPs, mice then received B16-F10 cancer cell injection challenges and showed an enhanced tumor-preventing efficacy (86% of mice were tumor free during the 5-month post-challenge) compared with other formulations. Then, the therapeutic efficacy of the CpG-CCNPs was also examined in B16-F10 tumor-bearing mice, and the results revealed that subcutaneous injection of the CpG-CCNPs combined with an intraperitoneal injection of anti-CTLA-4 and anti-PD-1 could inhibit tumor growth and prolong the survival time than other treatments. Besides CpG, the toll-like receptor 7 agonist imiquimod (R837) as a novel adjuvant was also encapsulated into PLGA NPs and then covered with mannose-modified tumor cell membranes (NP-R@M-M). Significantly, the B16-OVA cancer cell membrane was wrapped onto the NPs and then intradermally injected into mice bearing B16-OVA melanoma tumor, and it can effectively trigger the maturation of DCs and subsequent specific T-cell response. Correspondingly, NP-R@M-M (B16-OVA cancer cell membrane coating) alone or combined with anti-PD-1 checkpoint therapy exhibited an enhanced B16-OVA tumor-inhibiting efficacy while sparing 4T1 breast cancer tumor, illustrating the specificity of the tumor nanovaccine. Collectively, the works provided a rational design by applying autologous cancer cell membrane as tumor-specific antigen and combining coating nanotechnology to construct an antitumor nanovaccine platform.

Sono-Immunotherapy in Cancer Treatment

Sonodynamic therapy (SDT) is based on ultrasound (US), and it can produce large amounts of cytotoxic singlet oxygen ($^1O2$) and induce US cavitation and hyperthermia (18). Due to its superiorly deeper tissue penetration, SDT has been developed as a potential alternative to traditional cancer therapy (19). Considering the fact that current SDT agents often show a low SDT efficacy due to insufficient tumor accumulation, a bioinspired membrane-coated nanoplatform would overcome these limitations (20). Moreover, SDT can also be used to activate the antitumor immune response and demonstrate a superior synergistic effect with immunotherapy.

In a recent study, a macrophage membrane-coated nanoplatform, integrating SDT, chemotherapy, and immunotherapy, is fabricated (18). In the design, production of $^1O_2$ *in situ* and targeted delivery carbon monoxide (CO) to TME were combined upon stimulation by the exogenous US and endogenous H_2O_2. Other than physically inducing cancer cell death, the macrophage-coated nanoplatform can also take advantage of these cracked tumor cells to activate tumor-specific $CD8^+$T cells to enhance immunotherapy. Importantly, due to the existence of the macrophage membrane, the nanoplatform can inhibit immune clearance, prolong drug circulation time, and thus enhance tumor suppression. Then, the chemotherapeutic NLG919, an indoleamine 2,3-dioxygenase (IDO) inhibitor, was loaded into the nanoparticles to inhibit tumor metastasis. Collectively, the macrophage membrane-coated nanoplatform represents a promising antitumor strategy by integrating multimode cancer therapies, which would be an alternative to clinics in the future.

Photo-Immunotherapy in Cancer Treatment

Photodynamic therapy (PDT) is a promising cancer therapeutic strategy and has attracted much attention due to its non-invasiveness (21, 22). Local tumors can be inhibited by the reactive oxygen species (ROS) generated by PDT due to photosensitizers and laser irradiation (23), whereas the unwanted photosensitizer leakage from delivery vehicles has largely limited the progress of PDT, and PDT alone is not enough to active systemic immune response to eradicate the metastatic tumor cells (24–26). Therefore, decreasing photosensitizer leakage and improving tumor targeting would reverse the unsatisfactory therapeutic outcomes.

In a recent study, Kim et al. developed a cell membrane nanovesicle-based PDT strategy and efficiently inhibit local tumor growth and suppress its metastasis (27) (**Figure 1**). Notably, KillerRed (KR), a red fluorescence protein with emission spectrum 510–600 nm and a photo-responsive sensitizer with solid ability to generate ROS upon laser irradiation, was selectively expressed on 4T1-Fluc cancer cells, avoiding the leakage mentioned above from vehicles that reversely enhanced PDT therapy. Then, the KR overexpressing cancer membrane was extracted (KR-CCM) and then hybridized with monophosphoryl lipid A (MPLA)-embedded liposomes to form the about 250-nm lipocomplex (Lp-KR-CCM-A). Especially in the design, the 4T1 cancer membrane can improve tumor targeting (about 3.3-fold higher cancer-targeting efficiency than a control liposome) because of homotypic affinity and MPLA can stimulate an immune response

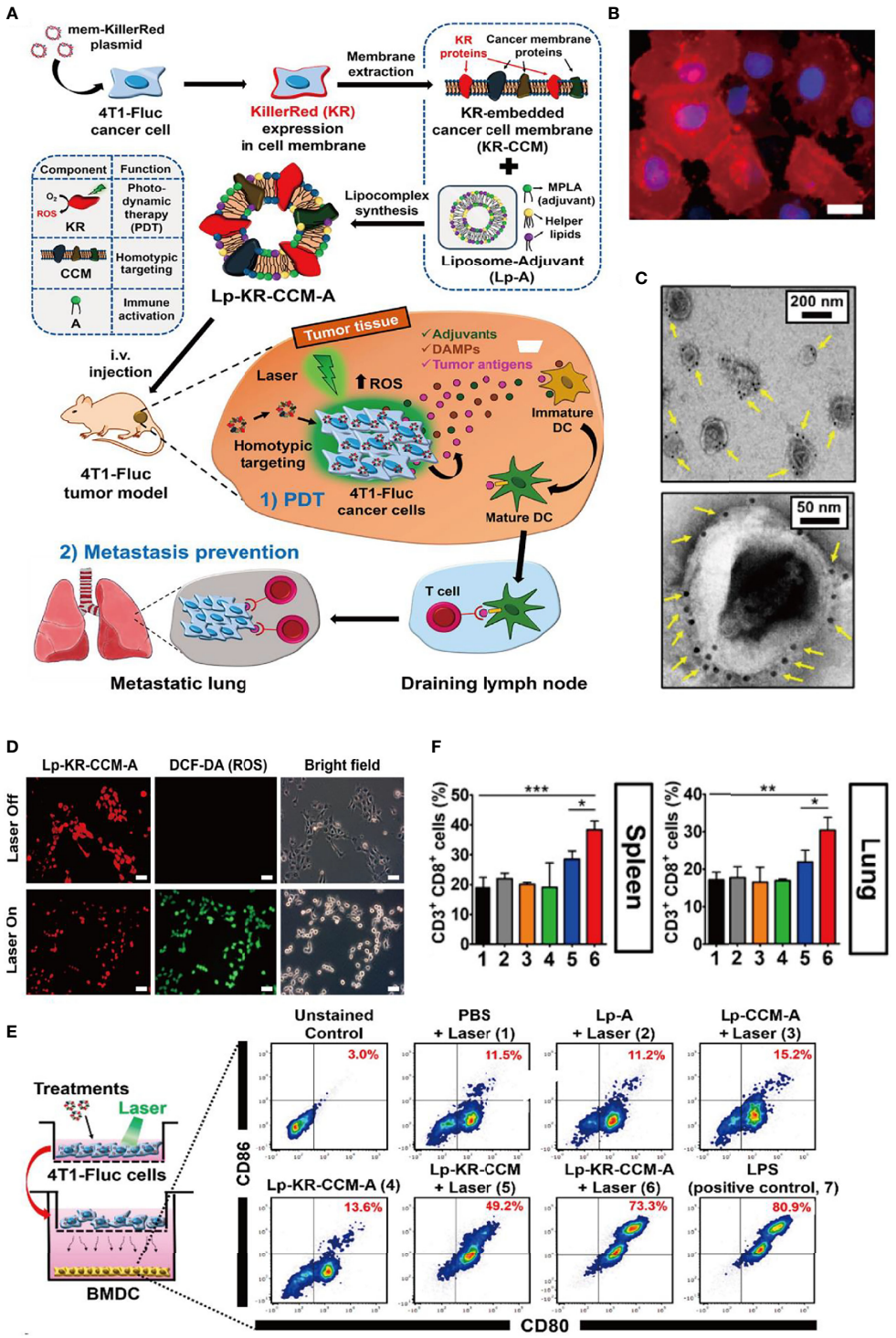


FIGURE 1 | Bioinspired membrane-based nanotherapeutics for photo-immunotherapy. **(A)** Schematic illustration of preparation of Lp-KR-CCM-A and its application in cancer therapy. KR as photosensitizer in Lp-KR-CCM-A and can produce ROS upon laser irradiation to kill cancer cells (PDT) and thus enhance immunotherapy with the help of lipid adjuvant MPLA. **(B)** Representative FL image showing KR expression in 4T1-Fluc cell membrane after transfection with mem-KR plasmid. **(C)** Lp-KR-CCM-A stained with KR antibody-conjugated immuno-gold. **(D)** *In vitro* ROS generation induced by Lp-KR-CCM-A internalized in 4T1-Fluc cells upon laser irradiation for 20 min. DCF-DA was used as an indicator of intracellular ROS. **(E)** *In vitro* BMDC maturation following different treatments and irradiation of 4T1-Fluc cells in a co-culture system. **(F)** Analysis of cytotoxic $CD8^+$T cells (gated on $CD3^+$ T cells) in the spleen and lung after the indicated treatments. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$. Reproduced with permission (27), Copyright 2019, American Chemical Society.

by targeting TLR4. After PDT, the subsequently generated ROS induced cancer cells apoptosis and the released cancer damage-associated molecular patterns (DAMPs) elicited DC maturation to active systemic tumor-specific T cells to attack the metastatic cancer cells in homotypic tumor-bearing mice. In short, the study novelty constructed the biomimetic lipocomplex technology and may improve cell membrane-based cancer therapy.

Chemo-Immunotherapy in Cancer Treatment

Recurrence is one of the significant challenges that cause patient death even after radical surgery in cancer therapy (28). In addition, it has been reported that surgery wound and the resulting inflammatory environment may accelerate recurrence or metastasis. Hence, performing a post-operation consolidation treatment is necessary, and immune checkpoint blockade (ICB) to revert exhausted $CD8^+$T cells has raised much attention (29–31). Despite significant progress, current ICB-based therapies are still restricted by autoimmune disorders and low objective drug response (32). Unwanted binding of PD-1 or PD-L1 antibody to normal tissues with i.v. injection may be one of the main reasons responsible for the compromised efficacy (33). Recently, platelet-based systems have attracted much attention as bioinspired drug delivery vehicles (28, 34). However, it potentially limited its progress and clinical use since blood-separated platelets are anucleated cellular fragments without proliferation potency (35). To address the issues, a strategy of genetic engineering platelet-based cascade amplification immunotherapy was proposed (36). In the design, lentivirus encoding EGFP-PD-1 was used to infect megakaryocyte (MK) progenitor cell line L8057 to express PD-1 stably. Stimulated by PMA, MKs underwent maturation, morphology change, and ultimately produced PD-1-expressing platelets. Due to the intrinsic properties, the purified platelets can actively target the tumor surgery wound or the resulting inflammatory microenvironment, and are then activated to produce microparticles. In the incomplete-surgery B16F10 tumor model, after three times i.v. injection of PD-1 platelets, the growth of residual tumor was significantly suppressed, whereas free platelets treatment could not prevent the recurrence. Through flow cytometry analysis, it was observed that PD-1-expressing platelets could induce more $CD8^+$ T cells to infiltrate the tumors than that of free platelets or PBS, and the infiltrated $CD8^+$ T cells showed enhanced secretion of granzyme B, indicating a reversion of T-cell exhaustion. The superior therapeutic outcomes can be attributed to *in situ* activation of platelet-derived microparticles of PD-1. To verify whether the *in situ* activation resulted in tumor eradication, bare aPDL1-platelet derived microparticles (PMPs) were collected from the platelets in similar research. Moreover, the results illustrated that direct injection of the PMPs could not inhibit the tumor and no more than free antibody. These results can illustrate that *in situ* activations of P–aPD-1/L1 at the tumor surgery wound were crucial for anticancer effect. Moreover, to further evaluate the ability of depletion of Tregs in TME, a model drug cyclophosphamide (CP) was loaded into PD-1-expressing platelets through co-culture or electroporation. In the therapy of the same B16F10 tumor model with incomplete resection, the results showed that CP-PD-1 platelet treatment could decrease Tregs in TME while vastly increasing the frequency of reinvigorated $CD8^+$T cells, demonstrating directly blocking tumor relapse. Collectively, the study identified that gene engineering PD-1 vesicle could be an effective bioinspired multifunctional platform for cancer theranostics, in which targeted therapeutic delivery and immunotherapy were combined.

DISCUSSION AND FUTURE PERSPECTIVE

Cancer immunotherapy changes the treatment pattern of tumors and brings hope for tumor patients, especially those with advanced malignant tumors. However, it also faces many problems, such as low immune response rate, lack of adequate and reliable predictive markers of curative effect, and lack of targeting. Monoclonal antibody immunotherapy, CAR-T, or TCR-T therapy cannot show an excellent therapeutic effect on all individuals and all tumors, and the adverse reactions are not the same. The selection of specific targets and the combined application of multiple therapies can partially solve the problem of mistarget faced by cancer immunotherapy at present (37). With the continuous emergence and innovation of photodynamic, sonodynamic, and other new technologies and methods, immunotherapy based on cell membrane-coated nanoparticles has ushered in rapid development, showing great potential for cancer treatment in the early stage of clinical trials (38, 39). However, the efficacy of cancer treatment still needs to be further improved, and future research needs to find more specific immune targets, such as tumor-specific antigens and new immune checkpoints, to avoid unnecessary targeting and missed toxicity.

In addition, future studies still need to consider the following two aspects: (1) Based on the different types and mechanisms of cell membrane-coated nanoparticle immunotherapy, how can the unique toxicity caused by histocompatibility problems in immunotherapy be avoided? (2) The discovery of cancer drugs depends on preclinical models to determine the priority of drug targets, to study the mechanism of action, the method of administration, the dose and time of treatment, and safety management (40). At present, immunotherapy based on cell membrane-coated nanoparticles is mostly limited to the essential animal experimental stage, and the clinical conversion rate is low, so the construction of a preclinical model close to the human immune environment is the key.

DATA AVAILABILITY STATEMENT

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

AUTHOR CONTRIBUTIONS

DM, PH, YS, and LJ conceptualized and wrote the manuscript. GL corresponded to the article. All authors contributed to the article and approved the submitted version.

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REVIEW
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The Emerging Role of Tissue-Resident Memory CD8$^+$ T Lymphocytes in Human Digestive Tract Cancers

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Malignant digestive tract tumors are a great threat to human public health. In addition to surgery, immunotherapy brings hope for the treatment of these tumors. Tissue-resident memory CD8$^+$ T (Trm) cells are a focus of tumor immunology research and treatment due to their powerful cytotoxic effects, ability to directly kill epithelial-derived tumor cells, and overall impact on maintaining mucosal homeostasis and antitumor function in the digestive tract. They are a group of noncirculating immune cells expressing adhesion and migration molecules such as CD69, CD103, and CD49a that primarily reside on the barrier epithelium of nonlymphoid organs and respond rapidly to both viral and bacterial infection and tumorigenesis. This review highlights new research exploring the role of CD8$^+$ Trm cells in a variety of digestive tract malignant tumors, including esophageal cancer, gastric cancer, colorectal cancer, and hepatocellular carcinoma. A summary of CD8$^+$ Trm cell phenotypes and characteristics, tissue distribution, and antitumor functions in different tumor environments is provided, illustrating how these cells may be used in immunotherapies against digestive tract tumors.

Keywords: CD8$^+$ Trm cells, characteristics, antitumor effects, immunotherapy, digestive tract tumors

INTRODUCTION

Malignant digestive tract tumors are a great threat to human public health. According to 2020 global cancer statistics, digestive tract tumors such as esophageal cancer (EC), gastric cancer (GC), colorectal cancer (CRC), and hepatocellular carcinoma (HCC) rank in the top 10 in cancer incidence and mortality and account for 23.4% of all new cases and 36.7% of deaths (1). The gastrointestinal mucosa is prone to inflammatory lesions and tumors resulting from long-term stimulation by physical and chemical factors and microorganisms (2). When tumors occur, although innate immune cells, as the vanguard, can induce rapid effector responses, powerful adaptive immunity involving various subsets

of T cells, which is then triggered, is the main force to exert antitumor roles (3). As an important member of memory T cells, the tissue-resident memory T (Trm) subset is a group of noncirculating immune cells that reside in peripheral tissues and mediate tumor defense through cytokine secretion in humans and rodents (4–6). Trm cells include $CD8^+$ Trm cells, $CD4^+$ Trm cells, regulatory Trm cells, natural killer Trm cells, and γδ Trm cells, in which $CD8^+$ Trm cells are extensively studied in antitumor research due to their powerful cytotoxic activity. $CD8^+$ Trm cells mainly reside on the barrier epithelium of nonlymphoid organs and respond rapidly to both viral and bacterial infection and tumorigenesis. In human digestive tract mucosa, $CD8^+$ Trm cells play a key role in anti-infection and antitumor immunity because they elicit a rapid immune response after antigen stimulation (7) .

Thus, $CD8^+$ Trm cells play an important role in maintaining homeostasis and resisting tumorigenesis within the digestive tract mucosa. By recognizing homologous antigens, $CD8^+$ Trm cells in the tumor microenvironment (TME) can rapidly secrete cytokines to activate innate immune cells and enhance the expression of chemokines and adhesion receptors, which in turn recruit circulating immune cells needed to exert essential antitumor functions. $CD8^+$ Trm cell infiltration is associated with improved prognosis in common digestive tract tumors, such as EC, GC, CRC, and HCC (8–11).

Many treatments for malignant digestive tract tumors have shifted from traditional chemotherapy to a combination of chemotherapy and immunotherapy. In the TME of most digestive tract cancers, $CD8^+$ Trm cells usually show an exhausted phenotype with the expression of inhibitory immune checkpoints such as programmed cell death protein-1 (PD-1) and T cell immunoglobulin and ITIM domain (TIGIT) (12–14). Although immune checkpoint inhibitors are widely used in the treatment of digestive tract tumors, there is still a high incidence of immune-related adverse events, and many patients do not respond well to immune checkpoint inhibitors due to the absence of prognostic markers, resulting in poor therapeutic outcomes (15–17). Therefore, adequate understanding of how variations in $CD8^+$ Trm cells in the TME affect digestive tract tumor pathogenesis is of great practical significance for clinical treatment. However, until now, the roles of $CD8^+$ Trm cells in digestive tract tumors have not been comprehensively described.

Herein, we review recent progress in understanding of the tissue distributions, biological characteristics and antitumor mechanisms of $CD8^+$Trm cells in EC, GC, HCC and CRC to provide directions for combined precision targeted therapy strategies and prognosis prediction.

BIOLOGICAL CHARACTERISTICS OF $CD8^+$ TRM CELLS

The Origin and Maintenance of $CD8^+$ Trm Cells

Trm cells are differentiated from naive T cells (18). The predominant phenotypes of $CD8^+$Trm cells express CD69, CD103, and CD49a (19–21), but do not express lymphoid homing molecules CCR7 and CD62 L and cannot be recycled (22–24). For tumor immunity, cross-priming by type 1 classical dendritic cell (cDC1) subsets, whose development and/or function depends on basic leucine zipper ATF-like transcription factor 3 (Batf3) transcription, is necessary for optimal generation of Trm cells (25–27). Indeed, Batf3-lineage DCs migrate to the draining lymph node to mediate T cell cross-priming, while another subset remains in the tumor site to produce CXCR3 ligands CXCL9 and CXCL10 (CXCL11 in humans) used to recruit $CD8^+$ effector T cells back to the target tissue (27). After cross-priming by Batf3-driven DCs, naive T cells and central memory T (Tcm) cells can differentiate into precursor Trm (pTrm) cells that enter the blood and circulate into targeted tissues. CD69 is upregulated on pTrm cells after exposure to IFN-α released by macrophages. After reaching the upper cortex, pTrm cells express CD103 and further differentiate in response to TGF-β. Kruppel-like factor 2 (KLF2) is a transcription factor encoding sphingosine-1 phosphate receptor 1 (S1PR1) and CD62 L, two molecules critical for naive T cell recirculation (28). Competition of CD69 and S1PR1 enables T lymphocytes to reside in peripheral tissue and differentiate into Trm cells. At the same time, T cells entering the epithelial tissue upregulate CD103 and downregulate the transcription factor KLF2 in response to TGF-β, promoting the residence of $CD8^+$ T cells (29). TNF-α and type I interferon can upregulate the expression of CD69 on the surface of $CD8^+$ Trm cells (24). In $CD103^-$Trm cells, the memory lymphocyte cluster (MLC) can also provide signals to maintain $CD103^-$Trm residence (23, 24) (**Figure 1**).

Although CD69 expression is upregulated in the early stage of Trm cell development, it cannot be used as a reliable marker of tissue residence because it is also expressed on other immune cells, and T cells expressing CD69 are still able to enter the circulation (30). CD103, also known as α E-integrin and human mucosal lymphocyte antigen, is an integrin expressed on intraepithelial T cells and some peripheral regulatory T cells. By binding to its ligand E-cadherin, CD103 can make antigen-specific T lymphocytes reside in epithelial tissue and is thus considered a reliable marker for Trm cells (23). CD49a, also known as very late antigen-1 (VLA-1), is a member of the integrin family. By binding to collagenase type IV, CD49a can prompt cells to be retained and survive in tissues (31). Furthermore, the maintenance of Trm cells in tissues is dependent on cytokines such as TNF-α, IL-15, TGF-β, and IL-33, while migration and retention are impacted by chemokines such as C-X-C motif chemokine receptor 6 (CXCR6), CCR10, and CXC chemokine ligand 17 (CXCL17) (30).

The Role of $CD8^+$ Trm Cells in the Antitumor Immune Response

Tumor-infiltrating $CD8^+$ T cells are effector T cells that can directly recognize and kill target cells, serving as the immune system's frontline force against tumors. $CD8^+$ T lymphocytes are represented by cytotoxic T lymphocyte (Tc1) subsets, which have antitumor and anti-infection functions by producing high levels of perforin, granzyme B, IFN-γ, and TNF-α (32). Of the immune cells that infiltrate the TME, the infiltration of $CD8^+$ T lymphocytes, especially Tc1 subsets, is usually associated with a

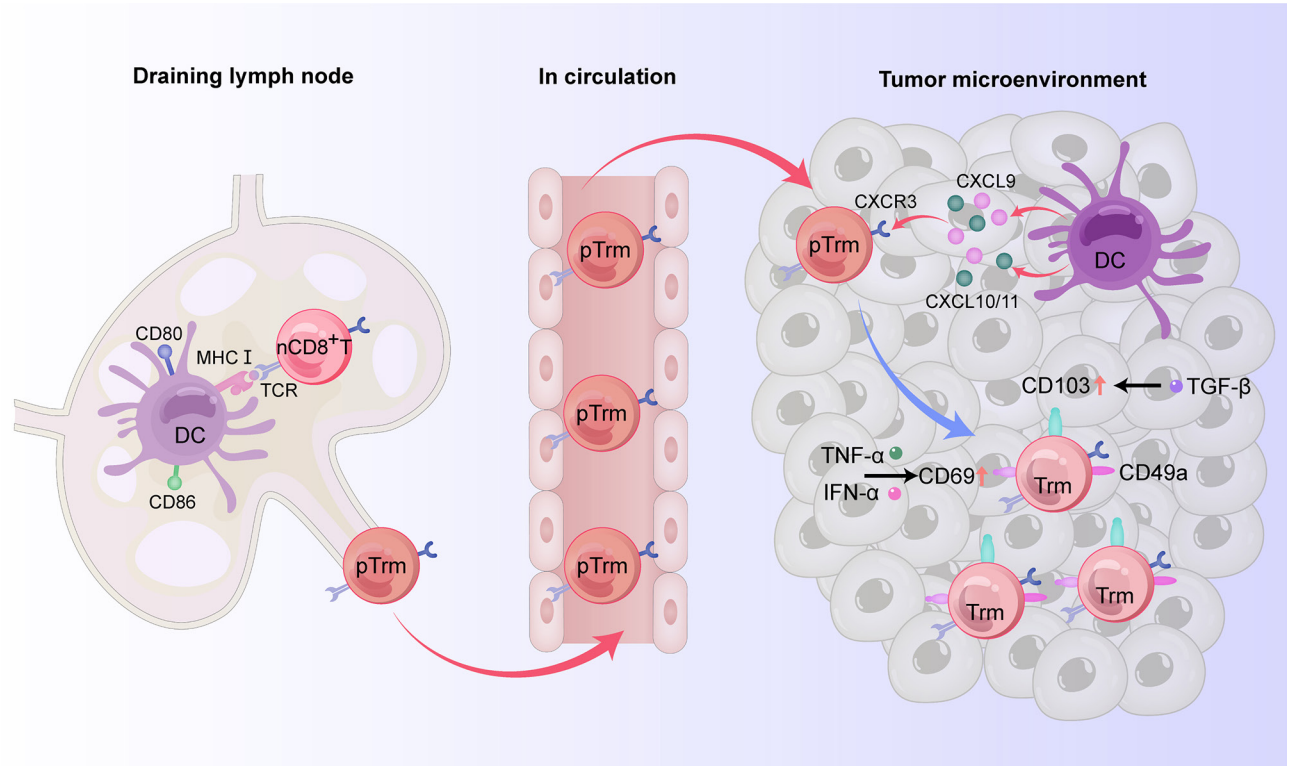

FIGURE 1 | The origin and phenotypes of CD8+ Trm cells in human digestive tract tumors. In the draining lymph node, naive CD8+ T cells can differentiate into precursor Trm (pTrm) cells after cross-priming by Batf3-driven DCs and then enter the blood and circulate into the target tissue. By producing the CXCR3 ligands CXCL9 and CXCL10 (CXCL11 in humans), another subset of DCs remaining in the tumor site recruits pTrm cells into the tumor microenvironment. CD69 is upregulated on pTrm cells after exposure to TNF-α and IFN-α. After reaching the upper cortex, pTrm cells express CD103 and further differentiate in response to TGF-β. In addition to expressing CD69 and CD103, mature CD8+ Trm cells also express the adhesion molecule CD49a, thus possessing resident properties.

more favorable prognosis (33). The antitumor function of CD8+ T cells depends on both differentiation and transport into the TME (34). In the TME of solid tumors, factors such as abnormal chemokine secretion and tumor angiogenesis can hinder the transport and function of CD8+ T lymphocytes (35). When this occurs, CD8+ Trm cells play an extremely important role in the antitumor process (36). Among the various subsets of Trm cells, CD8+ Trm cells are considered the first line of defense for peripheral tissues to inhibit early exposed antigens and have thus received considerable attention. The response of CD8+ Trm cells to re-exposed homologous antigens in the barrier tissue is faster than the response of circulating memory T cells (37, 38), primarily as a result of the critical locations in which they reside. These regions are the most common sites exposed to pathogens such as bacteria and viruses and where epithelial cancers originate. When activated, CD8+ Trm cells can quickly release perforin and granzyme B to directly kill target cells (6, 39) and amplify the activation of a small number of cells into an organ-wide response (40). While Trm cells may have phenotypic heterogeneity based on their location in the epithelia or stroma and the tumor subtype, these cells can promote recruitment of T lymphocytes into the epithelial TME and enhance the early signal transduction of CD8+ T lymphocytes within tumors (41). During tumorigenesis, CD69+CD8+/CD103+CD8+/CD49a+CD8+ T lymphocytes are highly activated, showing better effector function than traditional CD8+ T cells, and are able to control tumor growth (42).

When persistently exposed to tumor antigens, upregulation of inhibitory receptors such as PD-1, cytotoxic T lymphocyte associated antigen-4 (CTLA-4), TIGIT, T cell immunoglobulin- and mucin-domain-containing molecule-3 (TIM3), and lymphocyte activation gene-3 (LAG3) can lead to impaired killing function and exhaustion of CD8+ T cells (43, 44). For example, as esophageal squamous cell carcinoma (ESCC) progresses, changes in the TME are accompanied by an increase in immunosuppressive cells such as regulatory T (Treg) cells, myeloid-derived suppressor cells (MDSCs), and immuno-suppressive DCs, as well as soluble inhibitory molecules such as indole-2,3 dioxygenase (IDO) (45) and fibroblast growth factor 2 (FGF2) (46), resulting in reduced infiltration and functional inhibition of CD8+ T cells (47). In recent years, it has been shown that tissue-resident T lymphocytes can overexpress PD-1 and other immune checkpoint molecules, such as TIGIT, LAG-3, and Tim-3, in some experimental animal and human tumor tissues (36, 48). There are two possibilities for this phenomenon: 1) tumor infiltrating CD8+ T lymphocytes express a variety of

integrins, including CD49a, and remain in the TME in a quiescent/exhausted state, or 2) $CD8^+$ T cells in the TME upregulate the expression of multiple integrins after exhaustion through an undetermined mechanism (**Figure 2**).

The following sections define the characteristics of $CD8^+$Trm cells along with current research evaluating a role for $CD8^+$Trm cells in antitumor therapy for four common digestive tract cancers, EC, GC, CRC, and HCC (**Table 1**).

CHARACTERISTICS OF $CD8^+$ TRM CELLS AND THEIR POTENTIAL USE IN THE TREATMENT OF DIGESTIVE TRACT CANCERS

$CD8^+$ Trm Cells in EC

In 2020, EC ranked seventh in new cases and sixth among cancer-related deaths, with one in 18 deaths caused by EC (1). ESCC, which primarily occurs in Asian countries, accounts for about 90% of all pathological types of EC (63). Since it is directly exposed to foreign antigens in food, the esophageal mucosa has a special immune cell composition that plays an important role in maintaining esophageal homeostasis and mucosal anti-infective and antitumour processes. Strong expression of CD45RO, CD8, CD3, and CD107a in EC tissues indicates that there are cytotoxic memory $CD8^+$ T cells in the stroma of these tumors (64). Although $CD103^+CD8^+$ T cells express PD-1 and TIM-3 in ESCC, they are relatively active cell subsets (12). Cells with the Trm phenotype have higher proliferation ability and express cytotoxicity-related molecules, indicating that there are highly activated antitumor subsets in $CD8^+$ tumor infiltrating lymphocytes (TILs) in the TME.

The role of $CD8^+$ Trm cells in EC is not well understood. Alterations in $CD8^+$ Trm cell phenotypes and biological functions and the significance of these cells to EC prognosis and diagnosis remain obscure. Indeed, we have focused on the role of tissue-resident $CD8^+$ T cells in EC for many years and

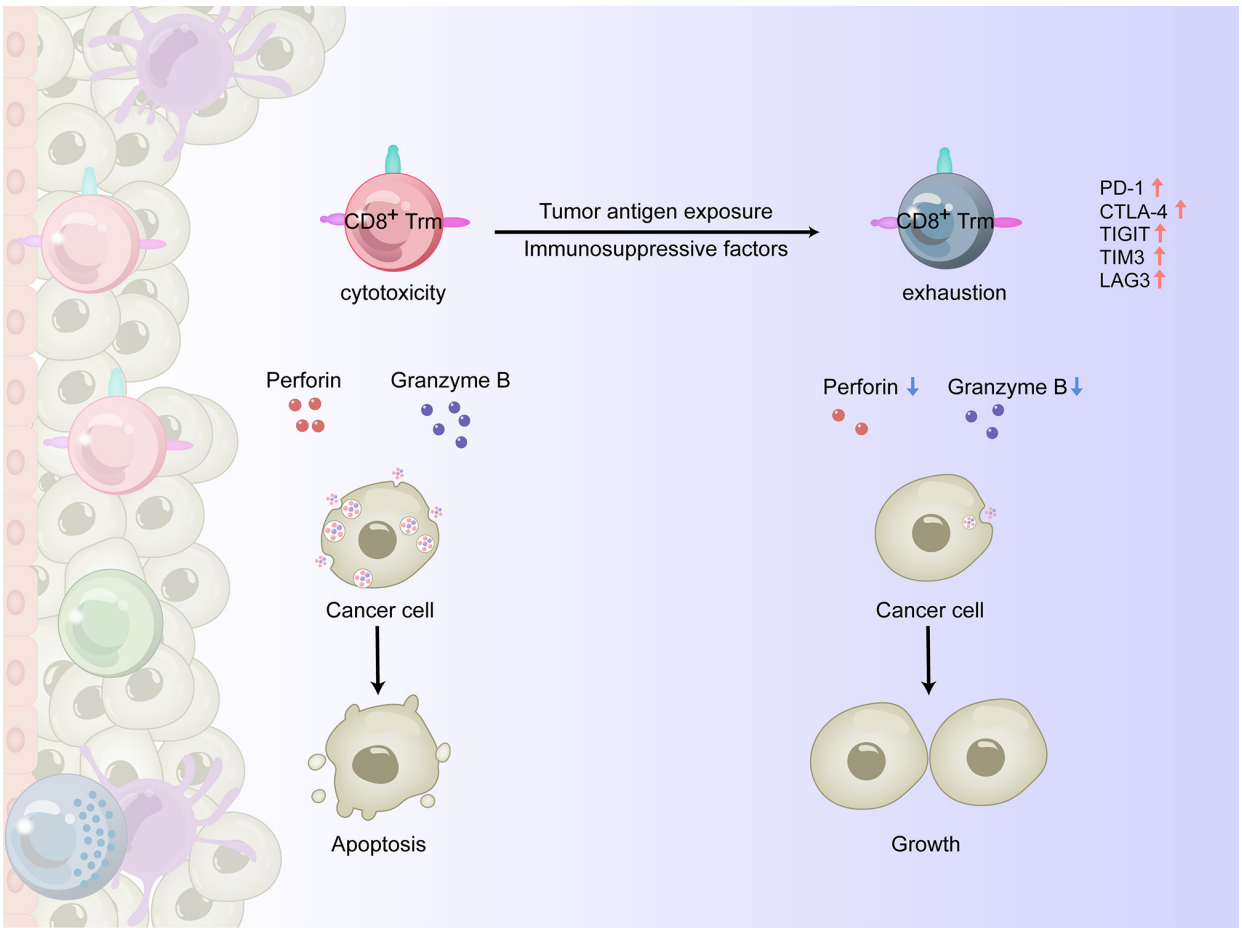

FIGURE 2 | The antitumor effects of $CD8^+$ Trm cells in the TME of human solid tumors. In the process of tumorigenesis, $CD8^+$ Trm cells could be highly activated and show a higher effector function than traditional $CD8^+$ T cells, releasing perforin and granzyme B and killing cancer cells. However, when persistently exposed to tumor antigens and immunosuppressive factors, the upregulation of inhibitory receptors such as PD-1, CTLA-4, TIGIT, TIM3 and LAG3 leads to impaired killing function and exhaustion of $CD8^+$ Trm cells, making them unable to control tumor growth.

TABLE 1 | Characteristics of CD8+Trm cells in human digestive tract tumors.

Tumor types	Phenotypes	Inhibitory receptors	Cytotoxicity	Characteristics	Cytokines	References
EC	CD69 CD103	PD-1 TIGIT TIM-3	+	In addition to expressing inhibitory receptors, CD8+Trm cells in the EC have high proliferation ability and high cytotoxicity-related molecule expression.	IFN-γ IL-2 CD107a	(13, 49)
GC	CD69 CD49a CD103 RUNX3	PD-1 TIGIT CD39	+	CD8+Trm cells in the GC can induce SPEM by producing high levels of IFN-γ, produce high levels of cytolytic enzyme and IFN-γ in the presence of a large amount of various inhibitory receptors, and are related to the formation of TLS.	IFN-γ Granzyme B Perforin CD107a IL-2 TNF-α	(10, 21, 28, 50–53)
CRC	CD69 CD103	PD-1 CD39	+	CD8+Trm cells in the CRC have significant resident properties and tumor reactivity. With a unique methylome pattern and distinct epigenetic properties, they can enhance tissue immunity, improve barrier function, and prevent microbiota-associated diseases.	IFN-γ Granzyme B Perforin	(11, 54–59)
HCC	CD69 CD49a CD103 CD49b CD11c	PD-1 TIM-3 LAG-3 CTLA-4 CD244 CD39	+	As a unique population with low cytotoxicity, hepatic CD8+Trm cells provide long-term protection for human papillomavirus-like virus HPV-induced HCC.	Granzyme B Granzyme K Perforin Granulysin	(60–62)

found that CD49a, PD-1, and TIGIT molecules are highly expressed on $CD8^+$ T cells in the TME of ESCC patients, indicating that there is also a population of tissue-resident $CD8^+$ T cells with high expression of CD49a that shows the immune exhaustion phenotype in the ESCC TME. Multiple components of the ESCC TME can lead to immune exhaustion of $CD103^+CD8^+$ TILs, which can be repaired by αPD-1 blockers.

Clinical studies show that $CD103^+$ $CD8^+$ TILs are linked to the overall survival of ESCC patients (12). Thus, CD103 may be a suitable marker to evaluate the antitumor immune response of $CD8^+$ T cells in ESCC, and infiltration of $CD103^+CD8^+$ TILs in the TME may be used as a biomarker to predict better prognosis in esophageal carcinoma (8, 12). It is worth noting that understanding the phenotype and function of $CD8^+$ Trm cells in the occurrence and development of ESCC and exploring how best to reverse immune exhaustion and restore the antitumor function of CD8+Trm cells is an urgent issue that must be addressed by ESCC immunotherapeutic research. Establishing effective immune intervention strategies that target inhibitory molecules and reverse immune exhaustion will improve precision clinical immunotherapy for ESCC.

$CD8^+$ Trm Cells in GC

GC is one of the most common cancers in the world. In 2020, this disease ranked fifth in morbidity, with more than one million new cases, and fourth in mortality, with an estimated 769,000 deaths (1). *Helicobacter pylori* infection is a major risk factor for the development of chronic gastritis to GC (65, 66), but the exact role of inflammatory components in disease progression remains unclear. Two types of gastric metaplasia, intestinal metaplasia and spasmodic cleavage peptide expression metaplasia (SPEM), are precancerous lesions of human gastric adenocarcinoma (51). The accumulation of $CD8^+$ Trm cells in the gastric mucosa involves the regulation of absent in melanoma 2 (Aim2), one of the key components of the inflammasome. Previous studies show that the lack of Aim2 can promote the accumulation of $CD8^+$ Trm cells in chronic inflammatory gastric mucosa by preventing CD62 L and S1PR1 function (67). While the high levels of IFN-γ produced by gastric $CD8^+$ Trm cells can induce SPEM (68), these cells have antitumor cytotoxicity when a tumor occurs (67).

$CD103^+CD8^+$ Trm cells in GC have similar phenotypes to those in other nonlymphoid tissues, including downregulation of lymph node homing-related molecules such as CD62 L, CCR7, and T cell factor 1 (TCF-1) and upregulation of tissue inhabitation promoting molecules such as CD69, CD49a, and Runt-related transcription factor 3 (RUNX3) (20, 31, 50, 52, 69, 70). Approximately 30% of TILs in GC are $CD69^+CD103^+$ Trm cells, which highly express the inhibitory receptors PD-1, TIGIT, and CD39 (53). However, $CD103^+CD8^+$ T cells can produce high levels of cytolytic enzymes and IFN-γ in the presence of a wide variety of inhibitory receptors (9). Moreover, PD-1 blockade effectively restored the function of $CD103^+CD8^+$ T cells but not $CD103^-CD8^+$ T cells. Thus, $CD103^+CD8^+$ Trm cells represent highly activated T cell subsets in GC and play an important role in inhibiting tumors (9).

Trm cell metabolism in GC tissues does not utilize glucose but relies on fatty acid oxidation to maintain cell survival, such that loss of fatty acids results in Trm cell death. GC cells outperform Trm cells at lipid uptake and may induce Trm cell death. Targeting PD-L1 can promote the survival of Trm cells by reducing the expression of fatty acid binding protein (Fabp)4 and Fabp5 in gastric tumor cells, increasing the expression of Fabp4/5 in Trm cells, and promoting lipid uptake by Trm cells (53). Thus, metabolic reprogramming may be an effective way to prolong the life span of GC Trm cells and enhance antitumor immunity, including $CD8^+$ Trm cell survival. In addition, B cells in the tumor can form cell masses known as tertiary lymphoid structures (TLSs), which can induce immune cells to effectively recognize and attack cancer cells. In the gastric TME, TLSs are positively correlated with tumor-infiltrating CD8+Trm cells. Studies have indicated that Trm cells may be related to the

formation of TLSs, and both may improve the outcomes of targeted therapy for PD-1 inhibitors in GC (71–73).

CD8+Trm Cells in CRC

CRC ranks third in the world in incidence and second in mortality (1). As the organ with the largest interface with its environment, the gut is exposed to billions of antigens every day. The immune system needs to ensure tolerance to non-dangerous antigens and establish a strong immune response against potentially dangerous antigens (74). Immune cells are unevenly distributed in the gut. While $CD8^+$ T cells (especially $CD8^+$ Trm cells), monocytes, and $CD19^+$ B cells are concentrated in the proximal colon, γδ T cells and NK cells are more abundant in the transverse colon, and $CD4^+$ T cells and antibody-secreting cells are enriched in the distal colon and rectum (54). $CD8^+$ T cells in the human intestinal tract are mainly Trm cells, which have CD103 and CD69 phenotypes and provide the first response to infection and tumors on the mucosal surface. TGF-β plays different roles in the formation and maintenance of Trm cells in the intestine. During secondary lymphoid organogenesis, TGF-β inhibits the migration of effector $CD8^+$ T cells to the intestine, while during maintenance, TGF-β promotes the residence of $CD8^+$ T cells (55). The regulatory function of Trm cells in the intestinal tract may be involved in intestinal homeostasis. It has been reported that promoting Trm and dendritic cell interactions can enhance tissue immunity, improve barrier function, and prevent microbiota-associated diseases (56). Due to the distinctiveness of the intestinal tract, $CD8^+$ Trm cells have phenotypic and functional heterogeneity in response to infection and cancer, from pluripotent to differentiated, and show preferential protection at sites of imminent exposure to pathogens or persistent disease (75). In CRC, CD103 and CD69 are associated with immune recognition of Trm cells (57–59). $CD103^+CD39^+CD8^+$ T cells have significant resident properties and tumor reactivity (10), with a unique methylome pattern in which the tumor reactivity markers CD39 and CD103 are specifically demethylated. This process provides these cells with distinct epigenetic properties (76).

CRC can be divided into microsatellite stable CRC (MSS) and high microsatellite unstable CRC (MSI-H). While tumor-infiltrating lymphocytes are abundant in MSI-H, which make up approximately 15% of CRCs, MSS CRC lacks tumor-infiltrating lymphocytes and is thus associated with a less favorable prognosis (77, 78). $CD8^+$ Trm cell numbers were much higher in MSI-H than in MSS. Other studies show that deletion of the IL-15 gene, which is essential to maintaining intestinal Trm cells (79), is associated with poor prognosis, indicating that $CD8^+$Trm cells play an important antitumor role in CRC. However, in MSI-H CRC, the expression of PD1 tended to increase in $CD8^+$Trm cells, indicating that checkpoint inhibition therapy targeting Trm cells in MSI-H CRC may be of great significance (79).

CD8+ Trm Cells in HCC

In 2020, primary HCC was the sixth most frequently diagnosed cancer, with more than 900,000 new cases, and the third leading cause of cancer mortality, with 830,000 deaths (1). This malignant tumor usually occurs in chronic inflammatory liver disease, such as fibrosis or cirrhosis, and is associated with certain risk factors, including hepatitis B virus (HBV), hepatitis C virus (HCV), alcohol abuse, and metabolic diseases (80, 81). Increased infiltration of cytotoxic T, NK, and NKT cells in the liver plays an active antitumor role in primary HCC. To avoid unnecessary activation of innate immune cells during continuous exposure to food and microbial-derived antigens, the liver needs to maintain a relatively immunotolerant environment. When immunogenic stimulation occurs, liver $CD103^+$ dendritic cells express high levels of MHC-II, CD80 and CD86, which result in massive activation of $CD8^+$ T cells (82). For example, HBV induces $IFN\gamma^+CD8^+$ T cells to upregulate CD69 and CD103 and induces liver $CD8^+$ T cells to show the Trm phenotype *in situ* (83). The presence of T cells and cytotoxic cells in TILs correlates with a favorable prognosis of patients with HCC. More than 50% of these tumor-infiltrating lymphocytes express CD69 (84), and about 20-30% are positive for CD103, thus showing resident characteristics. However, unlike other tumors, only about 5% of human hepatic $CD69^+CD8^+$ T cells express CD103 (85). Recent studies have shown that hepatic $CD8^+$ Trm cells adhere to the liver *via* LFA-1, and the residence of $CD8^+$ T cells in the hepatic sinusoid depends on the LFA-1-I/CAM-1 interaction (86). However, chronic tumor antigen stimulation and immunosuppressive cells and their production in the TME can put Trm cells into a "dysfunctional state". Targeting immune checkpoint molecules such as PD-1, TIM-3, LAG-3, and CTLA-4 can restore the dysfunction of Trm cells (87). However, hepatic $CD8^+$ Trm cells are a unique population with low cytotoxicity (60), which may be related to the immunotolerant ecological properties of the liver. Thus, anti-PD-L1 or anti-PD-1 alone may not restore this dysfunction, and other agents, such as IL-2, may have a synergistic effect in improving the antitumor immunity of $CD8^+$ Trm cells in HCC (87). In addition, the development and maintenance of tumor-specific $CD8^+$ Trm cells induced by adenoviral vector immunization vaccine in the liver can provide long-term protection for human papillomavirus-like virus (HPV)-induced HCC and can enhance the formation of $CD8^+$ Trm cells by targeting CTLA-4 (61). Thus, $CD8^+$ Trm cells may also play an active role in tumor vaccine therapy for HCC.

APPLICATION OF CD8+ TRM CELLS IN CANCER IMMUNOTHERAPY

The exhaustion phenotype of $CD8^+$Trm cells in the TME does not prevent antitumor activity from being reactivated. *In vitro* studies of $CD103^+CD8^+$ T cells with high expression of PD-1 in lung cancer have shown that blocking the expression of PD-1 on these immune cells can restore their cytotoxicity against autologous tumor cells, suggesting that anti-PD-1 therapy may restore the killing function of $CD8^+$ Trm cells toward autologous tumors (62). In the last few decades, anti-PD-1/PD-L1 therapies have shown remarkable efficacy in patients with malignant gastrointestinal neoplasms. For instance, the international

randomized phase III KEYNOTE-181 and KEYNOTE-590 studies in EC patients showed that pembrolizumab provided a clinically meaningful overall survival (OS) benefit versus the control group (88, 89). Indeed, clinically meaningful improvements in overall response rate (ORR), progression-free survival and OS were observed in GC patients treated with pembrolizumab plus chemotherapy in the KEYNOTE-059 and KEYNOTE-062 trials (90, 91). However, although anti-PD-1 mAb is a promising approach for advanced GC patients, the response rate is still limited, with an ORR of only about 12.0% and a disease control ratio of about 34.7% (92). Although immunotherapy has produced durable responses in MSI-H CRC, with recent FDA approval of pembrolizumab in the first-line setting of metastatic CRC (93), MSS CRC has long been considered resistant to PD-1/PD-L1 blockade. However, combination therapy, such as co-inhibition of anti-PD-1 and STAT3 or regorafenib, a small molecule tyrosine kinase inhibitor, can elicit an effective antitumor response in a small subset of MSS CRC patients (49, 94). Disappointingly, the ORR of checkpoint inhibitors in HCC patients is only 15-20% (95). Recently, the Nivolumab (CheckMate-459) III phase trial failed to meet the primary endpoint, so an effective immunosuppressive therapy against HCC is still lacking (96).

There is no denying that the use of PD-1 inhibitors to reverse the exhaustion of immune cells such as $CD8^+$ Trm cells, alone or with other checkpoint antibodies, has had controversial results. Due to tumor heterogeneity, a lack of reproducibility of results, and a complex scoring system, PD-L1 is not suitable as a predictive biomarker (97). While methods such as the combined positive score, which detects PD-L1 levels in tumors and lymphocytes, can be used clinically to evaluate patient response to PD-1/PD-L1-related inhibitors, their specificity for evaluating therapeutic impact is poor (98). Therefore, treatment options for patients with unresectable, locally advanced, or metastatic esophageal cancer are still limited, requiring the search for new predictive indicators and immunotherapy strategies (99).

Another way to increase the number of functional $CD8^+$ Trm cells in tumors is by inducing their expansion using tumor vaccines. Studies demonstrate that vaccination can induce Trm cells in the tissue after natural infection and vaccination. For example, intravaginal immunization or systemic perfusion has been shown to boost vaginal mucosa by inducing Trm cells in the reproductive area (100). In addition, encoding respiratory syncytial virus mechanisms or recombinant cytomegalovirus vectors of Bacille Calmette-Guerin vaccine proteins for intranasal vaccination promotes immune cells to develop resident properties (101, 102). The vaccine-specific $CD8^+$ T cell response can provide long-term protection against HPV-induced skin cancer and HCC but is dependent on the induction and accumulation of $CD8^+$ Trm cells by blocking CTLA-4 early after immunization (61). Local radiotherapy by vaccination (103), which changes the expression of selectin, integrin, and chemokines, can also enhance the recruitment of resident $CD8^+$ T lymphocytes in the tissue and tumor site.

PERSPECTIVE

$CD8^+$ Trm cell infiltration plays a critical role in the antitumor immune response in the digestive tract. CD69, CD103, CD39, and CD49a are the key biomarkers of tumor-reactive $CD8^+$ Trm cells and can be used as prognostic molecules for different digestive tract tumors (57, 59). However, $CD8^+$ Trm cells that have infiltrated digestive tract tumors can also express immune checkpoint molecules such as PD-1, CTLA-4, TIGIT, TIM3, and LAG3, which can damage their killing function and cause immune exhaustion (104, 105). While targeted application of immune checkpoint inhibitors has achieved good results, the lack of immune markers and disparate responses to immune checkpoint inhibitors diminish the efficacy of treatment. Determining how best to increase the number and function of tumor-associated $CD8^+$ Trm cells helps to maximize antitumor immunity. There is also great diversity among $CD8^+$Trm cell phenotypes found in different digestive tract organs. For example, while PD-1^{hi} $CD8^+$ Trm cells highly express cell adhesion and tissue positioning markers, including CD69 and integrins CD11c, CD49a, CD49b, and CD103 in HCC (87), $CD103^+CD8^+$ Trm cells express tissue residency-promoting molecules, such as CD69, CD49a, and RUNX3, in gastric cancer (9). CD103 is an important marker of $CD8^+$ Trm in ESCC. ESCC patients with co-expression of PD-L1/TIM3 or PD-L1/TIGIT in $CD8^+$ Trm cells have a lower survival rate than those expressing either marker alone (106). This may explain why only a small number of ECC patients benefit from treatment with PD-1 inhibitors. The absence of predictive indicators results in a high rate of immune-related adverse events in response to drugs targeting PD-1/PD-L1, with only a small number of patients showing positive outcomes. Nevertheless, a novel strategy to solve this problem is developing nanodrug delivery systems with a high drug loading capacity and targeting ability. It has been reported that biodegradable polymers such as poly (ursolic acid) are used as drug carriers for treating CRC and other cancers. The anticancer drug effectively loaded into poly(salicylic acid) nanoparticles shows ultrahigh blood vessel penetration, tumor penetration, and tumor accumulation due to the special prickly nanostructure (107, 108). Thus, the combination of a therapeutic polymer platform and immunotherapy to achieve precise targeted therapy may be a new attractive therapeutic strategy for treating digestive tract cancer.

In conclusion, alimentary tract neoplasms are a serious threat to human health. Immunotherapy for digestive tract tumors still has many problems, including blind treatment, side effects, and disparate individual responses. $CD8^+$ Trm cells exist in various digestive tract tumors and are closely related to disease prognosis. However, current research on the utilization of $CD8^+$ Trm cells in digestive tract tumors is still in the early stages. Thus, a comprehensive understanding of $CD8^+$ Trm cell phenotypes and the characteristics of corresponding immune checkpoint molecules that are expressed in digestive tract tumors will be important to help guide accurate diagnosis and treatment of different tumor types. Specific drug therapy and tumor vaccine therapy that targets tumor-associated $CD8^+$ Trm cells may

become an important direction for antitumor research and tumor precision therapy.

AUTHOR CONTRIBUTIONS

XM performed the study design and drafted the manuscript. HL and XZ participated in the manuscript writing. MC obtained the funding, participated in the paper design, and contributed fruitful discussions. KC conceived the study and participated in the paper design and writing. All authors contributed to the article and approved the submitted version.

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GLOSSARY

AIM	activation inducer molecule
Aim2	absent in melanoma 2
Batf3	basic leucine zipper ATF-Like transcription factor 3
CCR7	chemokine receptor 7
cDC1	classical dendritic cell
CRC	colorectal cancer
CTLA-4	cytotoxic T lymphocyte associated antigen-4
CXCL17	CXC chemokine ligand 17
CXCR6	C-X-C motif chemokine receptor 6
EC	esophageal cancer
ESCC	esophageal squamous cell carcinoma
Fabp	fatty acid binding protein
FGF2	fibroblast growth factor 2
GC	gastric cancer
HBV	hepatitis B virus
HCC	hepatocellular carcinoma
HCV	hepatitis C virus
HPV	papillomavirus-like virus
IDO	indole-2,3 dioxygenase
KLF2	Kruppel-like factor 2
LAG3	lymphocyte activation gene-3
MDSCs	myeloid-derived suppressor cells
MLC	memory lymphocyte cluster
MSI-H	high microsatellite unstable CRC
MSS	microsatellite stable CRC
ORR	overall response rate
OS	overall survival
pTrm	precursor Trm
RUNX3	Runt-related transcription factor 3
S1PR1	sphingosine-1 phosphate receptor 1
SPEM	spasmodic cleavage peptide expression metaplasia
TCF-1	T cell factor 1
Tcm	central memory T
TIGIT	immunoglobulin and ITIM domain
TILs	tumor infiltrating lymphocytes
TIM3	T cell immunoglobulin-and mucin-domain-containing molecule-3
TME	tumor microenvironment
TLSs	tertiary lymphoid structures
Treg	regulatory T
Trm	tissue-resident memory T
VEGF	vascular endothelial growth factor

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REVIEW
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Injectable Hydrogel as a Unique Platform for Antitumor Therapy Targeting Immunosuppressive Tumor Microenvironment

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Cancer immunotherapy can boost the immune response of patients to eliminate tumor cells and suppress tumor metastasis and recurrence. However, immunotherapy resistance and the occurrence of severe immune-related adverse effects are clinical challenges that remain to be addressed. The tumor microenvironment plays a crucial role in the therapeutic efficacy of cancer immunotherapy. Injectable hydrogels have emerged as powerful drug delivery platforms offering good biocompatibility and biodegradability, minimal invasion, convenient synthesis, versatility, high drug-loading capacity, controlled drug release, and low toxicity. In this review, we summarize the application of injectable hydrogels as a unique platform for targeting the immunosuppressive tumor microenvironment.

Keywords: cancer immunotherapy, tumor microenvironment (TME), injectable hydrogels, immunogenic cell death, abscopal effect, controlled drug release

INTRODUCTION

Cancer is a major threat to human health worldwide (1). Cancer immunotherapy has emerged as a promising cancer treatment approach that can inhibit tumor metastasis and recurrence by boosting antitumor immune responses (2, 3). Cancer immunotherapies have revolutionized the treatment of many cancer types in clinical settings. Immunotherapeutic agents include immune checkpoint inhibitors, vaccines, immunologic adjuvants, adoptive cell transfer, and nonspecific immune-stimulating factors (e.g., cytokines) (4). Nevertheless, low T cell infiltration levels, the presence of inhibitory immune cells, and the lack of neoantigens limit response to immunotherapy. Systemic administration of conventional drugs often requires high dosages or multiple injections, which can lead to severe immune-related adverse effects and low patient compliance (5–7). Multiple immunosuppressive factors in the tumor microenvironment (TME) have been shown to affect the delivery of therapeutic agents and efficacy of T cell-based therapies, thus influencing the therapeutic efficacy of cancer immunotherapy (8–10). Therefore, modulating or reprogramming the

immunosuppressive TME can enhance the efficacy of cancer immunotherapy. Many studies and clinical trials aiming to target tumor immunosuppressive microenvironment to eradicate malignant cells are ongoing (10, 11).

Hydrogels with 3D network structures have been widely used in various fields, especially in biomedicine (7, 12–14). Injectable hydrogels have attracted considerable attention as vehicles for sustained drug delivery *in situ* because of their unique advantages, including easy delivery by syringe and minimal surgical wounds (13, 15). Injectable hydrogels can be loaded with various agents, including chemotherapeutic drugs, immunotherapeutic agents, antibodies, vaccines, cytokines, and immune cells (7, 14, 16). Sustained and controlled release of these therapeutic agents by injectable hydrogels can activate systemic antitumor immune responses and inhibit tumor metastasis and recurrence while causing minimal toxicity (7). Herein, we highlight recent advances in reprogramming the immunosuppressive TME using injectable hydrogels to improve the efficacy of cancer immunotherapy (**Figure 1**).

CATEGORIES OF INJECTABLE HYDROGELS

Injectable hydrogels are usually formed by quick sol-gel phase transition or chemical polymerization *in situ*. They can be directly delivered into the target sites by injection (12, 16). Injectable hydrogels can be classified into chemically and physically cross-linked hydrogels based on the gelling mechanism (13, 16). Chemically cross-linked injectable hydrogels are generated by introducing covalent linkages between polymer chains *via* disulfide formation, photo-irradiation, enzymes, Schiff's base reactions, Michael-type addition reactions, or Diels-Alder reactions (16). On the other hand, physically cross-linked injectable hydrogels are formed through intermolecular interactions, such as hydrogen bonds, hydrophobic interactions, ionic cross-linking, and host-guest interaction (16). Injectable hydrogels can also be classified as natural or synthetic hydrogels based on the polymers used for their preparation (7). Natural injectable hydrogels are typically

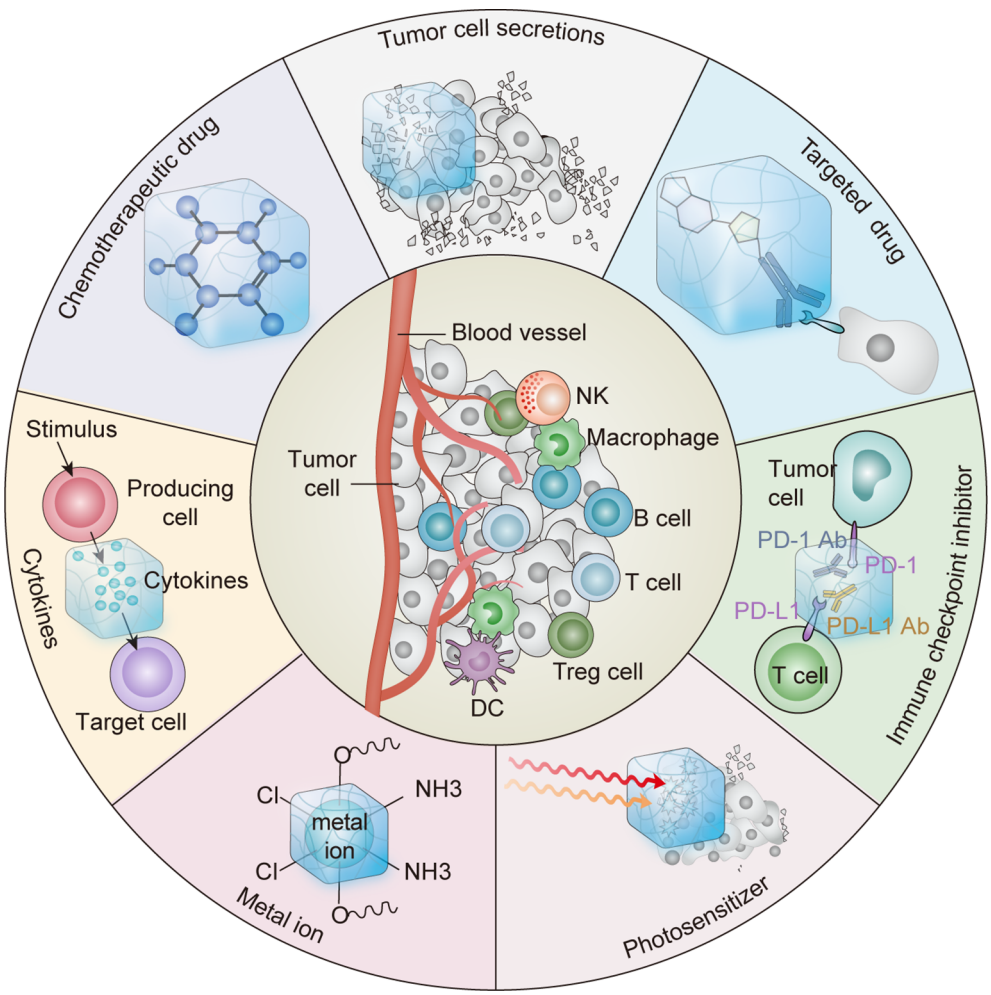

FIGURE 1 | Schematic diagram of antitumor therapy platform using hydrogels as platform to elicit antitumor immune response.

composed of polysaccharides, proteins, and DNA. In contrast, synthetic hydrogels consist of biodegradable polymers, such as polypeptides and polyesters (7). Additionally, injectable hydrogels can be divided into ordinary hydrogels and smart hydrogels according to their responses to external stimuli. Ordinary injectable hydrogels are not sensitive to environmental changes, whereas smart injectable hydrogels can be affected by temperature, pH, enzyme, and photoelectricity (13, 17). Moreover, injectable hydrogels can be biologically functionalized with targeting moieties that have an affinity for unique or overexpressed tumor cell markers for targeted drug delivery applications (18).

Over the past decade, many studies have investigated the antitumor potential of drug-loaded hydrogels (19). The therapeutic potential of hydrogels has also been investigated in patients with cancer. Up to September 2021, four clinical studies related to drug-loaded hydrogels for the treatment of cancer have been registered in the US registry of clinical trials (https://clinicaltrials.gov/). Two completed, open-label, dose escalation clinical studies (NCT02891460, NCT02307487) evaluated the efficacy of mitomycin C-loaded hydrogels (TC-3) in patients with bladder cancer. The results of these studies have not been published yet (**Table 1**).

IMMUNOSUPPRESSIVE STATUS OF TME

TME is an integral part of tumors and can affect the efficacy of cancer treatment (9). At different stages of tumor development, different immune cell types are present in the TME. At an early stage, tumors are infiltrated by antitumor immune cells, including macrophages, natural killer (NK) cells, lymphocytes, and dendritic cells (DCs) (20). However, at later stages of tumor development, antitumor immune responses are hindered by immunosuppressive cells, such as myeloid-derived suppressor cells (MDSCs), regulatory T cells (Tregs), and M2 macrophages (20, 21). The balance between different types of immune cells determines the outcome of antitumor immune responses.

$CD8^+$ cytotoxic T lymphocytes (CTLs) and $CD4^+$ T helper (Th) cells are paramount immune cells for tumor cell elimination (22). Th1 responses, characterized by the production of IFN-γ, TNF-α, and IL-2, are also essential for tumor rejection. However, Th1 responses can also contribute to tumor escape *via* IFN-γ-induced expression of the checkpoint molecule programmed death-ligand 1 (PD-L1) or tumor immunoediting and selection of resistant clones (23). In addition, long-term exposure of tumor antigens to Th1 cells and other T cell subtypes may promote the expression of inhibitory receptors, such as PD-L1, lymphocyte activation gene 3 protein (LAG-3), and T-cell immunoglobulin (Ig) domain and mucin domain protein 3 (TIM-3) (24). Immune checkpoint pathways in cancer cells can cause T-cell dysfunction and immune evasion. Immune checkpoint blockade (ICB), especially antibodies against cytotoxic T-lymphocyte-associated protein 4 (CTLA-4), programmed cell death protein 1 (PD-1), and PD-L1, can reverse immunosuppression and prevent immune evasion (9). ICB has shown remarkable long-term survival benefits in cancer patients with several types of tumors, including melanoma, non-small cell lung cancer, and renal cell carcinoma (16, 25).

However, Tregs, another subset of $CD4^+$ T cells, often inhibit antitumor immune responses and promote tumor growth. Tregs can directly interact with CTLs and NK cells or indirectly inhibit the antitumor activity of CTLs and NK cells by producing immunoregulatory cytokines, such as IL-10 and TGF-β (10). Notably, Tregs have been associated with unfavorable survival in patients with many types of cancer (26). Hence, eliminating Tregs in the TME may enhance antitumor immune responses. Th2 cells can also block T-cell-induced tumor rejection by promoting T-cell anergy, suppressing T-cell-mediated cytotoxicity, and enhancing humoral immunity (10).

Tumor cells promote the recruitment of bone marrow-derived cells (BMDCs), which can differentiate into tumorigenic cell subtypes under certain conditions (20). For instance, tumor-associated macrophages (TAMs) derived from BMDCs promote tumor progression by facilitating angiogenesis, invasion, and metastasis *in vivo* (27). MDSCs, another type of BMDCs, can suppress antitumor immune responses by inhibiting T cells and NK cells and promoting the expansion of Treg populations within the TME (21).

INJECTABLE HYDROGELS TARGETING IMMUNOSUPPRESSIVE TUMOR MICROENVIRONMENT

Targeting Immune Checkpoint Molecules

Immune checkpoint blockade (ICB) immunotherapies, especially antibodies against CTLA-4, PD-1, and PD-L1, have revolutionized cancer treatment (28). However, ICB monotherapies

TABLE 1 | Drugs embedded in hydrogels were used to treat cancers based clinical trials up to September 2021.

Study title	Conditions	Status	Identifier
A Prospective Open Label Comparative Dose Ranging Study Evaluating the Effect of Pre-TURBT Intravesical Instillation of Mitomycin C (MMC) Mixed with TC-3 Gel in Patients with Non Muscle Invasive Bladder Cancer (NMIBC)	Bladder Cancer	Withdrawn	NCT01799499
Safety and Tolerability Study Which Evaluate Intravesical Instillation with Mitomycin C Mixed with TC-3 Drug Retaining Hydrogel Device in Patients with Muscle Invasive Bladder Cancer	Bladder Cancer	Completed	NCT02891460
Safety of Pre-TURBT Intravesical Instillation of Escalating Doses of TC-3 Gel and MMC in NMIBC Patients	Bladder Cancer	Completed	NCT02307487
Safety and Efficacy of Doxorubicin-eluting-bead Embolization in Patients with Advanced Hepatocellular Carcinoma	Hepatocellular Carcinoma	Unknown	NCT02525380

show limited efficacy in most patients and may cause significant toxicity (6, 9, 29). Therefore, more effective and safer combination therapies involving ICB are under development. PD-L1 expressed on the surface of tumor cells and on antigen-presenting cells can interact with PD-1 expressed on activated T cells, promoting T-cell apoptosis, anergy, and exhaustion (30, 31). Blocking the PD-1/PD-L1 pathway with anti-PD-1 or anti-PD-L1 antibodies has demonstrated promising therapeutic efficacy in a variety of tumor types (32–35); however, response rates are only 10%–30% (29, 36). Low neoantigen burden, insufficient infiltration of tumor-specific T cells, and low expression of PD-L1 may contribute to the low response rates in cancer patients treated with ICB (20, 37–41). Moreover, multiple administration cycles of anti-PD-1 antibodies can induce severe immune-related side effects (42–44); local delivery of antibodies can minimize off-target effects and increase drug bioavailability (45).

Wang et al. developed a drug-based supramolecular hydrogel for local delivery of immune checkpoint inhibitors (ICIs) to boost the host's immune system against tumors (**Figure 2**) (46). They first synthesized the amphiphilic prodrug, diCPT-PLGLAG-iRGD, by conjugating a hydrophilic iRGD. This prodrug can spontaneously assemble into supramolecular nanotubes (P-NTs). By mixing a therapeutic dose of anti-PD-1 antibodies and P-NTs, they developed a hydrogel loaded with anti-PD-1 antibodies. Wang et al. found that this formulation could serve as a reservoir for long-term release of camptothecin (CPT) and anti-PD-1 antibodies within the TME, thereby inducing a potent antitumor immune response. They also found that local P-NT-anti-PD-1 treatment in GL-261 brain cancer and CT 26 colon cancer models led to tumor regression in 100% of mice.

The low immunogenicity of some tumor types and the body's decreased immune responses to tumor limit the development of immunotherapy. Immunogenic cell death (ICD), featured by the release of tumor-associated and tumor-specific antigens, danger-associated molecular patterns, and pro-inflammatory cytokines, plays an essential role in cancer immunotherapy (47). Recent evidence suggests that neoadjuvant chemotherapy and the use of biomaterials-based delivery systems both enhance the therapeutic efficacy of immunotherapy owing to the induction of ICD (48, 49). Gu et al. engineered an injectable reactive oxygen species (ROS)-responsive hydrogel co-loaded with gemcitabine

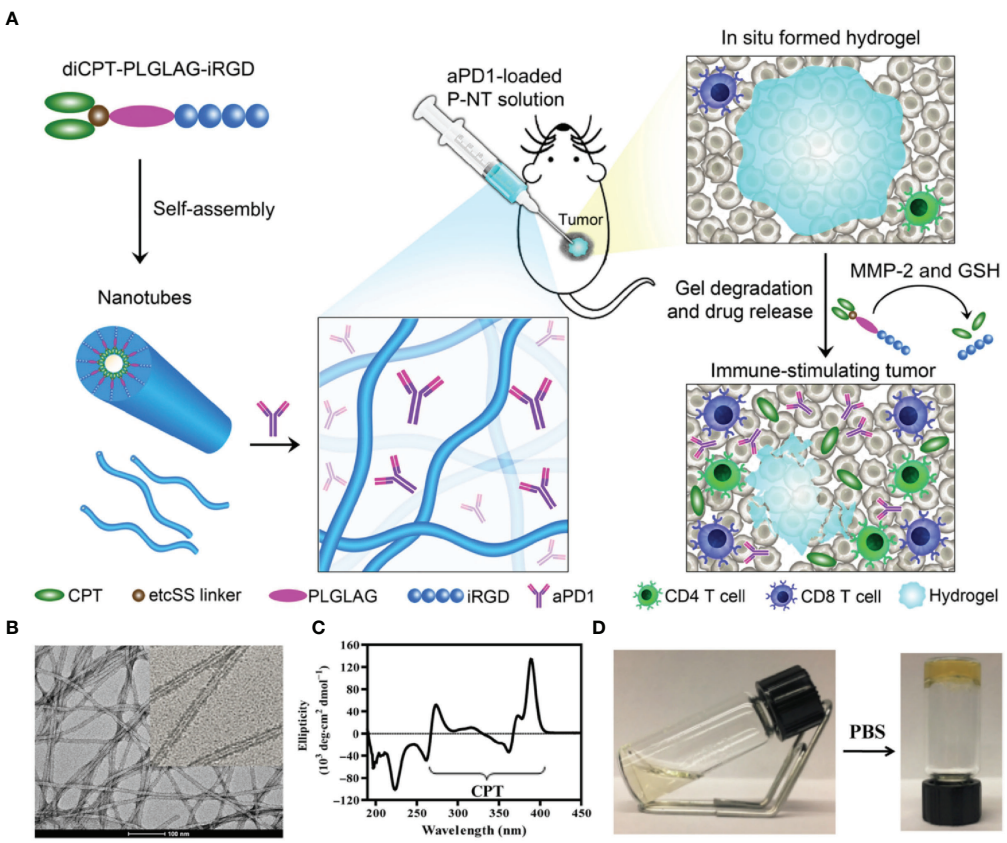

FIGURE 2 | Schematic illustration of the *in situ* formed P-NT-anti-PD-1 hydrogel. **(A)** *In situ* formation of P-NT-anti-PD-1 hydrogel, which enables localized CPT and anti-PD-1 delivery and promotes the activation of CD4 and CD8 T cells in the tumor microenvironment. **(B)** Representative transmission electron microscopy (TEM) image of the networks of the P-NT hydrogel. **(C)** Circular dichroism (CD) spectrum of camptothecin (CPT) solution. **(D)** Photographs of liquid P-NT transformed into hydrogel after the addition of phosphate-buffered saline (PBS). Reprinted with permission from Science Advances (46).

(GEM) and anti-PD-L1 antibodies for *in situ* chemo-immunotherapy (50). As the scaffold consists of ROS-degradable hydrogel and the TME contains high levels of ROS, GEM and anti-PD-L1 antibodies can be specifically released in the TME. In B16-F10 melanoma and 4T1 breast tumor (low-immunogenic) mouse models, local GEM delivery increased tumor immunogenicity and augmented the antitumor efficacy of ICB, thereby promoting tumor regression and suppressing tumor recurrence. To enhance the expression of tumor-associated antigens, Ruan et al. developed an *in situ* formed dual-bioresponsive gel depot for co-delivery of anti-PD-1 antibodies and zebularine (Zeb), a demethylation agent that enhances the expression of tumor-associated antigens (51). Anti-PD-1 antibodies were loaded into pH-sensitive $CaCO_3$ nanoparticles (anti-PD1-NPs) and encapsulated with Zeb in the ROS-responsive hydrogel (Zeb-anti-PD-1-NPs-Gel). Local release of Zeb increased the immunogenicity of cancer cells and decreased immunosuppression. By doing so, Zeb boosted the ability of anti-PD-1 antibodies to induce T cell-mediated antitumor immune responses, inhibiting tumor growth and prolonging survival in mice bearing B16-F10 tumors. In addition to direct use of anti-PD-1 antibodies to block the PD-1/PD-L1 pathway, targeting of a specific pathway that involves PD-L1 transcriptional repressors is also practicable. Li et al. reported a cancer cell membrane-derived hydrogel scaffold loaded with Ca^{2+} channel inhibitor dimethyl amiloride (DMA) and cyclin-dependent kinase 5 inhibitor roscovitine for cancer treatment. In this system, cancer cell membrane, DMA and roscovitine were chosen with the aim of creating an antigen depot, suppressing Ca^{2+}-governed exosome secretion and down-regulating tumor cell PD-L1 expression, respectively (52).

CTLA-4 is expressed on activated Th1 cells and CTLs, and binds to co-stimulatory molecules CD80 and CD86 of antigen-presenting cells, thereby inhibiting the activation and proliferation of T cells (53). Although blocking CTLA-4 signaling unleashes antitumor immune responses, systemic administration of anti-CTLA-4 antibodies may cause severe immune-related adverse events (5, 54–57). Chung et al. evaluated thermosensitive poloxamer 407 (P407) hydrogels as a slow-release system for optimizing anti-CTLA-4 therapy (58). They found that P407 hydrogel-mediated delivery of anti-CTLA-4 antibodies reduced serum antibody levels, mitigated the side effects of ICB, and exerted antitumor effects in mice bearing CT26 tumors. Similarly, Harui et al. found that local administration of hydrogel-encapsulated anti-CTLA-4 antibodies exhibited enhanced efficacy and minimal systemic toxicity in mice with MC-38 tumors (59). Peritumoral administration of 100 μg of anti-CTLA-4 antibodies loaded in hydrogels had similar or greater effects than systemic administration of 600 μg of antibodies. While preserving antitumor activity, serum exposure following the administration of hydrogel-encapsulated anti-CTLA-4 was only 1/16th of that following systemic therapy.

Song et al. developed an injectable PEG-b-poly(L-alanine) (PEA) hydrogel to co-deliver a tumor vaccine consisting of tumor cell lysates (TCLs), granulocyte-macrophage colony-stimulating factor (GM-CSF), and anti-CTLA-4 antibodies and anti-PD-1 antibodies (60). TCLs, GM-CSF, anti-CTLA-4 antibodies, and anti-PD-1 antibodies were encapsulated into the porous PEA hydrogel by mixing these agents with PEA aqueous solution. Sustained release of tumor antigens and GM-CSF promoted the recruitment and activation of DCs *in vivo*, inducing tumor-specific CTL responses. The extended release of ICIs from the hydrogel further enhanced T-cell activation and reduced Treg levels in the TME by blocking PD-1 and CTLA-4 pathways. Notably, the hydrogel-based combination therapy exhibited greater antitumor effects than the vaccine alone or ICB monotherapy in melanoma and 4T-1 mouse models.

Targeting Tumor-Associated Macrophages

Tumor-associated macrophages (TAMs) are a key component of the TME and play a significant role in tumor progression (61, 62). There are two main subtypes of TAMs: classically activated M1 macrophages (M1-TAMs) and alternatively activated M2 macrophages (M2-TAMs). M1-TAMs, which express high levels of IL-12 and IL-23, can scavenge foreign antigens and kill tumor cells (63). Tumor cells typically promote polarization of TAMs toward M2 in TME, facilitating IL-10 production and tumor growth (8). The balance between M1 and M2 TAMs has been associated with drug resistance, angiogenesis, and immunosuppression in tumors (8). Most macrophage-targeting therapies have three goals (9, 64): (1) inhibit macrophage recruitment by blocking the C-C motif chemokine ligand 2 (CCL2)/C-C motif chemokine receptor 2 (CCR2) axis (65, 66); (2) deplete macrophages or block -factor (CSF)-1/CSF-1R signaling (67, 68); (3) reprogram TAMs toward an M1-like phenotype using melittin (69), IFN-γ (70), CD40 agonists (71), or tumor hypoxia-targeting agents (72). As macrophages are present throughout the body, systemic modulation of macrophages can lead to off-target effects and systemic toxicity (73). Furthermore, CCL2/CCR2- and CSF-1/CSF-1R-targeting strategies often result in the development of monocyte and macrophage populations that enhance neoangiogenesis and metastasis (74, 75).

M2-TAM depletion has proved effective in promoting tumor regression by suppressing TAM-associated immunosuppression (8). Although melittin is a potent anticancer agent, its hemolytic effects limit its clinical application. To overcome this obstacle, we developed a melittin-RADA32 hybrid peptide hydrogel. The melittin- and doxorubicin (DOX)-loaded peptide hydrogel (melittin-RADA32-DOX, or MRD hydrogel) exerted potent anti-melanoma effects by modulating the TME (76). Moreover, MRD hydrogels loaded with melittin and DOX exhibited direct cytotoxic effects, specifically depleted M2-like macrophages, and induced robust and long-lasting innate and adaptive immune responses. Notably, a single injection of the formulation significantly reduced the growth of primary melanoma tumors.

External stimuli can stimulate the reprogramming of M2-TAMs into M1-TAMs, which have tumoricidal effects (77). KN93, a specific inhibitor of CAMKII, was found to have a direct tumoricidal activity and the ability to induce macrophage reprogramming (78). To further potentiate these effects of the

melittin-RADA32 hydrogel, we designed a melittin-RADA24 peptide hydrogel loaded with KN93 (MR52-KN93; MRK hydrogel) (79). Compared with free KN93, the MRK hydrogel was more potent in eliminating tumor cells and inducing immunogenic cell death. Moreover, MRK significantly reduced the portion of M2-like TAMs and increased the ratio of M1-like to M2-like TAMs in the TME (**Figure 3**).

The TME is usually acidic due to the presence of hypoxia and glycolytic metabolism (79, 80). Cancer cell-derived lactate plays a critical role in the polarization of macrophages from the M1 phenotype to the M2 phenotype, which promotes tumor growth and metastasis (80). Liao et al. found that methylcellulose hydrogels loaded with lactate oxidase promoted lactate depletion and lactate-mediated repolarization of macrophages (81).

Several recent studies reported the direct involvement of TAMs in tumor resistance to ICB. By comparing the TME of ICB-resistant and ICB-sensitive murine tumors, Muraoka et al. found that TAMs in resistant tumors lacked antigen-presenting activity (82). They also found that cholesteryl-modified pullulan nanogels could efficiently deliver large peptides to TAMs and that upon TLR stimulation, the nanogel system elicited antigen-presenting activity in TAMs (82). By modulating TAMs, this

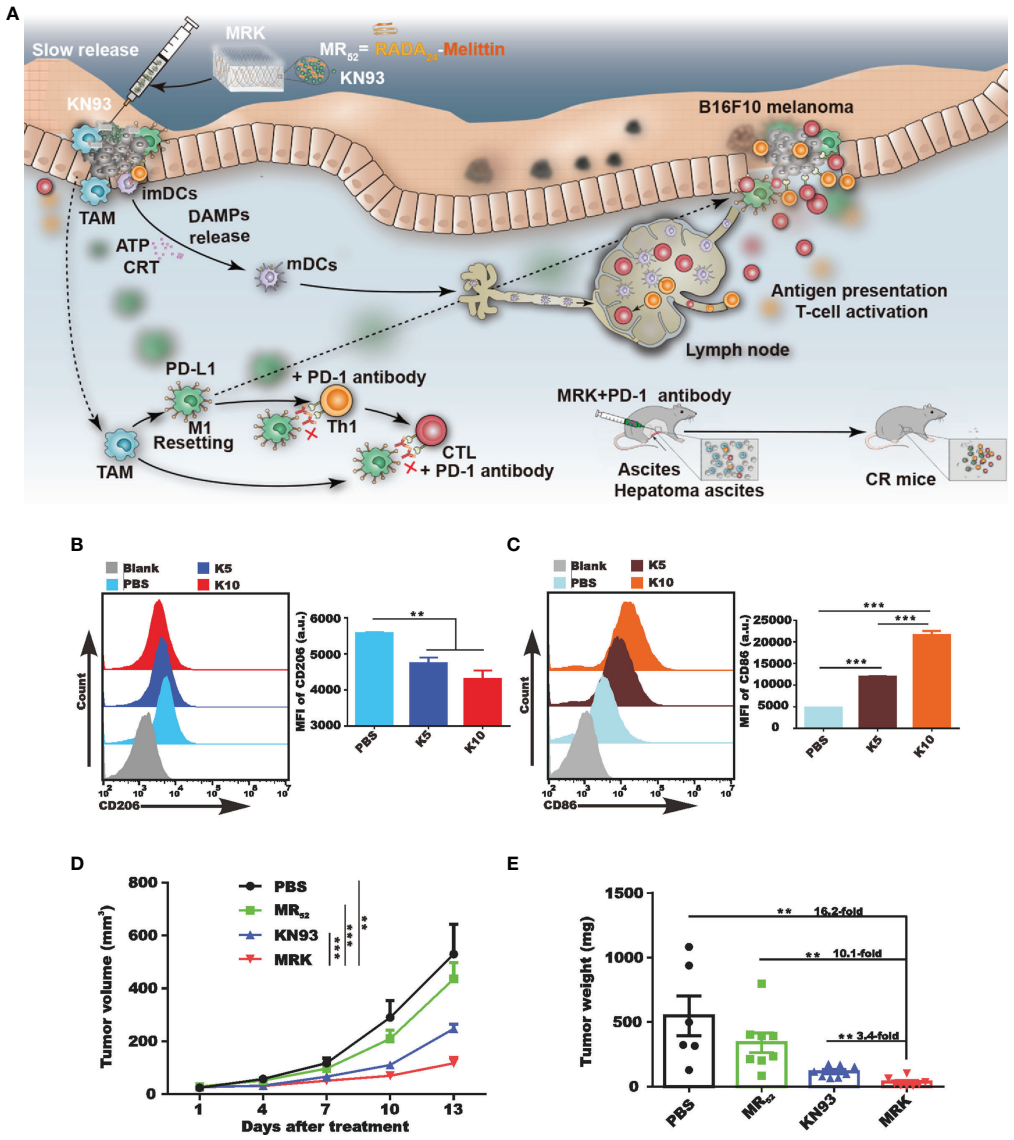

FIGURE 3 | *In vivo* activation of the immune system of tumor-bearing mice by MRK. **(A)** Schematic diagram summarizing the therapeutic effects of the MRK hydrogel alone or combined with anti-PD-1 antibodies. Subcutaneous injection of MRK stimulates dendritic cell maturation and T cell activation in the lymph nodes. Activated T cells eliminate tumor cells. MRK can also stimulate M1-type polarization of tumor-associated macrophages, activating Th1 cells and cytotoxic T lymphocytes. MRK combined with PD-1 alleviates hepatocellular ascites in mice. **(B, C)** Comparison of the production of M2-type macrophages **(B)** and dendritic cells **(C)** in each group. **(D, E)** Tumor volume **(D)** and weight **(E)** in different groups. Reprinted with permission from Theranostics (78). **P < 0.01, ***P < 0.001.

formulation transformed ICB-resistant tumors into ICB-sensitive tumors. These results strongly support targeting TAMs as a promising strategy for enhancing the efficacy of cancer immunotherapy.

Because M1-TAMs can promote tumor rejection, direct injection of M1-TAMs can significantly cause tumor regression *in vivo*; however, the induction of acute inflammatory responses limits the clinical translation of this approach (83). To improve this strategy, Guerra et al. employed a synthetic extracellular matrix (ECM) system consisting of cross-linked PEGdA and Gel-PEG-Cys as a carrier for local delivery of activated M1 macrophages. They found that M1-loaded hydrogels promoted apoptosis in hepatocellular carcinoma cells and tumor regression *in vivo* while exhibiting low immunogenicity, high biocompatibility, and improved release kinetics (84).

Targeting the Tumor Vasculature

Normal vascularization is critical for nutrients and oxygen supply, as well as metabolic waste removal. However, abnormal vascularization characterized by immature, disorganized, and permeable blood vessels creates a hostile TME characterized by hypoxia, low pH, low interstitial fluid pressure, decreased immune cell infiltration and activity, and increased risk of metastasis (85, 86). Furthermore, abnormal vascularization reduces the diffusion of chemotherapeutic drugs and impairs the efficacy of radiotherapy (86). Therefore, vascular normalization could restore tumor perfusion and oxygenation and enhance the efficacy of chemotherapy and radiotherapy (87, 88).

Antibodies against vascular endothelial growth factor (VEGF) have emerged as a promising therapeutic strategy for solid tumors, as tumor growth and metastasis require neoangiogenesis (89). Targeting VEGF signaling induces tumor vasculature normalization, further reprogramming the immunosuppressive TME and increasing the number of tumor-infiltrating lymphocytes (TILs) (90, 91). Bevacizumab, the first approved anti-VEGF drug to inhibit tumor angiogenesis in the United States, has a limited half-life and membrane permeability. To overcome these limitations, Ferreira and coworkers designed a bevacizumab-loaded alginate hydrogel for localized anti-VEGF cancer therapy by mixing alginate solution with bevacizumab and cross-linking it with calcium chloride (92). The tridimensional hydrogel increased drug stability, especially in acid environments, and provided slow and continuous drug release to the tumor and surrounding tissues after local application. Moreover, with the development of photodynamic therapy (PDT), it has shown the potential to trigger local and systemic antitumor immune responses. However, abnormal angiogenesis and hypoxia in TME promote immunosuppression. The immune response after routine PDT is usually insufficient to cause tumor regression, which limits the efficacy of PDT. Based on this, Zhou et al. developed a prolonged oxygen-generating phototherapy hydrogel (POP-Gel) system by combining the photosensitizer-loaded thermosensitive hydrogel with calcium superoxide and catalase to relieve tumor hypoxia. Long-term effective oxygen supply improved the hypoxic state of TME and down-regulated the expression of HIF-1α and VEGF, further inducing a robust antitumor adaptive immune response (93).

RNA interference (RNAi) enables robust and specific gene silencing, providing a promising therapeutic avenue for cancer treatment. However, efficient drug delivery systems for short interfering RNAs (siRNAs) are lacking (94–96). Fujii et al. developed a self-assembled nanogel of cholesterol-bearing cycloamylose with a spermine group (CH-CA-Spe) as a carrier to deliver VEGF-specific siRNAs (siVEGFs) into tumor cells. This system showed low toxicity in patients, efficient intratumor delivery, and high stability *in vivo* (97). The siVEGF-nanogel complex was taken up by tumor cells *via* the lysosomal pathway and suppressed VEGF expression in renal cell carcinoma cells. Intratumoral injections of the complex effectively suppressed tumor growth and neovascularization. The treatment also significantly suppressed MDSC infiltration and IL-17A production in the spleen, suggesting that silencing of VEGF locally in the tumor may modulate systemic immune responses.

Despite promising findings in preclinical models, the efficacy of anti-angiogenic therapies in the clinic has been disappointing, as most patients exhibit innate or acquired resistance to the treatment (98). However, anti-angiogenic therapeutics can increase the efficacy of immunotherapy (99). Additionally, low doses of anti-VEGF antibodies can induce vascular normalization, prevent the differentiation of TAMs toward an immune inhibitory M2-like phenotype, and block VEGF-mediated inhibition of DC maturation (90). Therefore, vascular normalization with anti-angiogenic therapies in combination with other therapies may be an attractive therapeutic strategy. Pal et al. developed a biocompatible self-assembled lithocholic acid dipeptide-derived hydrogel (TRI-Gel), which provided sustained delivery of DOX, anti-angiogenic combretastatin-A4 (CA4), and dexamethasone (100). TRI-Gel therapy inhibited cancer cell proliferation, angiogenesis, and inflammation at the tumor site, thereby suppressing tumor progression and prolonging median survival with reduced drug resistance (100). Yu et al. designed an *in situ* thermo-gelling hydrogel (mPEG-b-PELG) to co-deliver combretastatin A4 disodium phosphate (CA4P) and cisplatin (CDDP) for the local treatment of colon cancer (101). Compared with the free drugs, the CA4P and CDDP co-loaded gel induced less tumor cell death *in vitro*, while its antitumor effect was highest in C26 tumor-bearing mice after peritumoral injection (101).

Starvation therapies can inhibit tumor progression by decreasing nutrient supply indispensable for tumor growth (102, 103). Blood vessel occlusion can permanently occlude blood and nutrition supply to the tumor. However, this strategy is often associated with poor persistence, frequent tumor metastasis and recurrence, and embolism in normal blood vessels. Zhang and coworkers established an extravascular gelation shrinkage-derived internal stress strategy to narrow blood vessels, occlude blood and nutrition supply, reduce vascular density, induce hypoxia and apoptosis, and ultimately promote starvation of the tumor (104). To this end, they engineered an organic-inorganic composite hydrogel

consisting of PEG-SH-modified gold nanorods (GNR-PEG-SH) and thermal-sensitive hydrogel mixture (chitosan (CS)/mPEG-Mal/pNIPAAm-co-AAc; hydrogel-GNR). When irradiated with an 808 nm laser, hydrogel-GNR induced internal stress, which narrowed intratumor and adjoining blood vessels in a GNR-dependent manner. This starvation therapy inhibited tumor progression in both PANC-1 pancreatic cancer and 4T1 breast cancer mouse models. Importantly, this starvation strategy suppressed tumor metastasis and tumor recurrence by reducing vascular density, occluding blood and nutrition supply (**Figure 4**).

Targeting Other Immunoregulatory Cells and Factors

In view of the strong immunosuppressive effect of Tregs in the TME, targeting Tregs has emerged as an attractive strategy to unleash antitumor immune responses and reenforce immune-mediated tumor rejection (10). Tumor-specific Tregs residing at the TME express high levels of CTLA-4 and OX40, and *in situ* injection of anti-CTLA-4 and anti-OX40 together with CpG can deplete tumor-infiltrating Tregs (104). This *in situ* immunomodulation approach activates systemic antitumor immune responses more effectively than systemic immunomodulation strategies (105). The co-delivery of tumor anti-CTLA-4, anti-PD-1, and tumor vaccines using injectable PEG-b-poly(L-alanine) hydrogels increased the efficacy of immunotherapy by reducing the number of Tregs and increasing the number of activated $CD8^+$ T cells in the TME (60). In addition to directly killing tumor cells, some chemotherapeutic agents can regulate the immune system through various mechanisms, including the modulation of Tregs (106–112). Co-delivery of DOX and CpG self-crosslinking nanoparticles (CpG NPs) using injectable α-cyclodextrin/polyethylene glycol hydrogels increased the number of cytotoxic $CD8^+$ T lymphocytes and decreased the numbers of MDSCs, M2-TAMs, and Tregs in the TME (107). Additionally, although chemotherapy alone reduced the number of Tregs to some extent, combination therapy using α-cyclodextrin/

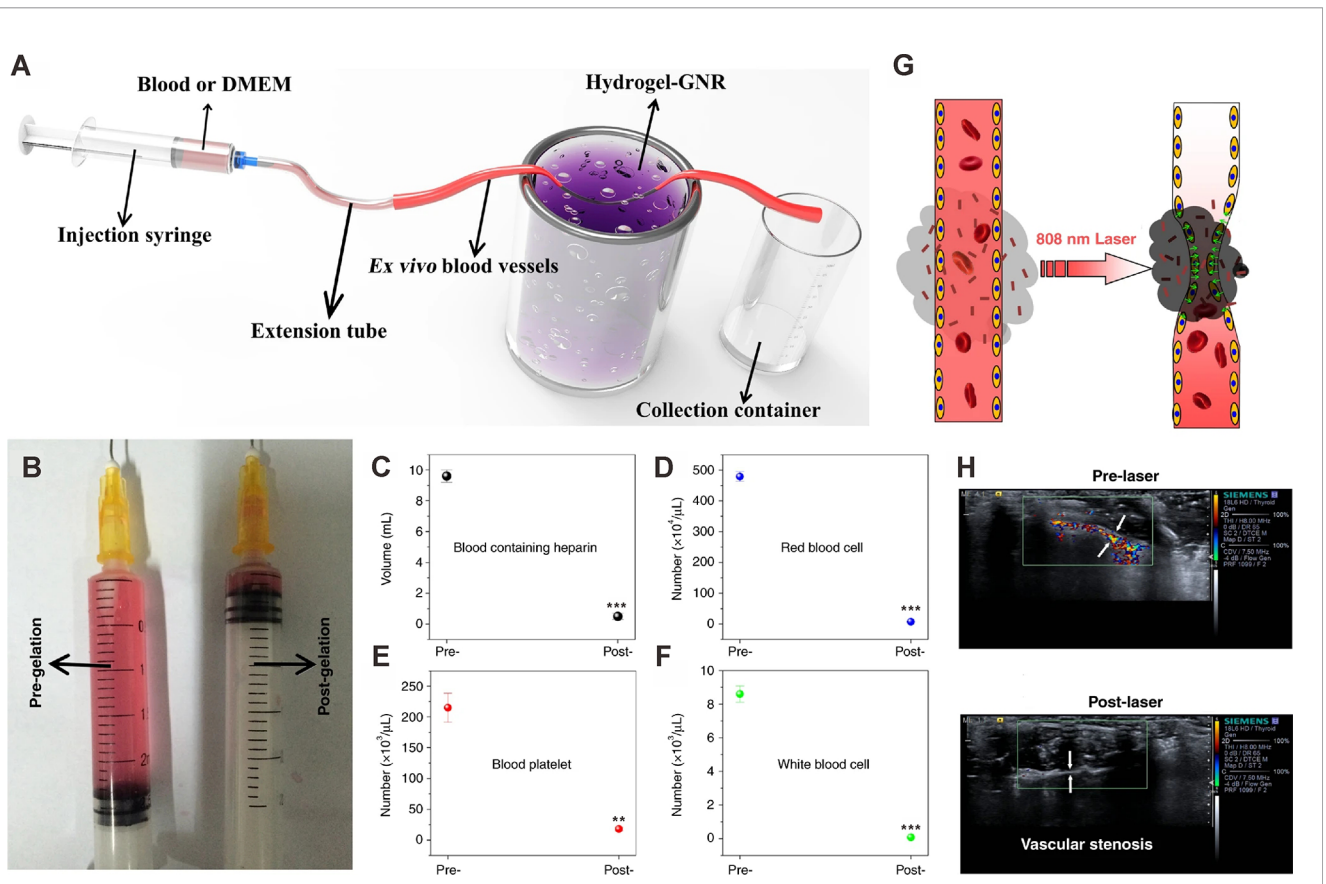

FIGURE 4 | Occlusion of blood supply in ex vivo and *in vivo* artery models. **(A)** Schematic of the experimental apparatus for evaluating vessel occlusion ex vivo in blood vessels (with an inner diameter of 1.00 mm) treated with the gelation shrinkage-induced internal stress platform. **(B)** Traversed volume of DMEM in the ex vivo blood vessel model before (left) and after (right) gelation of hydrogel-GNR. **(C–F)** Collected blood volume **(C)**, red blood cells **(D)**, blood platelet **(E)**, and white blood cells **(F)** traversed through ex vivo blood vessels using rabbit blood containing heparin. **P<0.01 and ***P<0.001 compared to pre-gelation; determined using Student's t-test. **(G)** Schematic representation of the extravascular gelation shrinkage-induced internal stress system irradiated with 808 nm laser. **(H)** CDFI images of abdominal arteries of nude mice treated which hydrogel-GNR. CDFI images were captured before and after irradiation with an 808 nm laser. White arrows indicate the blood vessels of the abdominal artery. Reprinted with permission from Springer Nature (104).

polyethylene glycol hydrogels-CpG NP-DOX remarkably reduced the number of Tregs in the TME (107).

The balance between different immune cell subsets, immune factors, and signaling molecules determine the outcome of antitumor immune response. Intratumoral delivery of immunomodulatory cytokines has been tested in the clinic as a strategy to augment antitumor immune responses (10). To elicit a therapeutic response, sufficient concentrations and long-lasting release of cytokines in TME are necessary, along with a non-toxic concentration of the cytokine outside of TME. GM-CSF, IL-2, IL-12, and IFN-γ are among the several cytokines tested for local cancer treatment based on injectable hydrogels (16). Son et al. demonstrated that GM-CSF improved the function of antigen-presenting cells and enhanced antitumor immune responses (113). Co-delivery of GM-CSF and anticancer drugs using a chitosan-based hydrogel system resulted in a synergistic anticancer effect, as tumor-specific $CD8^+$ T cell responses were significantly enhanced (113). Den Otter et al. developed physically crosslinked dextran hydrogels for the local delivery of IL2. The system exhibited a strong therapeutic effect, enhancing the clinical applicability of IL-2 (114). Kurisawa and coworkers developed an injectable hyaluronic acidtyramine (HATyr) conjugate hydrogel to locally deliver IFN-α2a to treat liver cancer (115). The enzymatically crosslinked HATyr hydrogel released IFN-α2a in the TME and inhibited tumor growth while providing tunable hydrogel stiffness and rapid gelation rate (115). Eonju Oh et al. utilized gelatin-based hydrogels for sustained co-delivery of DCs and oncolytic adenovirus (oAd) co-expressing IL-12 and GM-CSF while preserving the biological activity of the cytokines (116). Compared with single treatment (oAd or DC) or combination treatment without the gel (oAd+DC), oAd+DC/gel treatment resulted in a significantly higher expression of IL-12, GM-CSF, and IFN-γ in tumors through a positive feedback loop. The high levels of IL-12, GM-CSF, and IFN-γ in the TME strongly activated endogenous and exogenous DCs, which migrated to the draining lymph nodes and promoted the activation and infiltration of $CD4^+$ and $CD8^+$ T cells into the tumor, finally leading to robust tumor regression. Interestingly, oAd+DC/gel treatment also alleviated tumor-induced thymic atrophy (**Figure 5**).

Chronic inflammation in TME can promote cancer progression in several ways, and remission of chronic inflammation can help control the tumor (117). The cyclooxygenase 2 (COX2) inhibitor celecoxib has been shown to exert antitumor effects in various human cancers (118, 119). For instance, simultaneous and local administration of anti-PD-1 monoclonal antibodies and celecoxib using alginate hydrogels resulted in stronger antitumor effects than anti-PD-1 or celecoxib alone. In addition, the formulation elicited a potent and sustained antitumor immune response (120). Notably, co-delivery of celecoxib and anti-PD-1 monoclonal antibodies increased the numbers of INF-γ-expressing $CD4^+$ and $CD8^+$ T cells and decreased the numbers of intratumoral Tregs, MDSCs, and PD-L1-positive tumor cells. Furthermore, this co-delivery system enhanced the expression of the anti-angiogenic chemokines CXCL9 and CXCL10 and suppressed the intratumoral production of IL-1, IL-6, and COX2, suggesting reduced inflammation and angiogenesis in the tumor.

CONCLUSION

Numerous injectable hydrogels have been developed over the past years (121). Injectable hydrogels offer many advantages, including good biocompatibility and biodegradability, minimal invasion, convenient synthesis, versatility, high drug-loading capacity, and controlled drug release ability (122). Owing to their unique properties, injectable hydrogels can be used as drug delivery systems, which can locally and continuously release therapeutic agents. Although intratumoral injections suffer from localized treatment and inhomogenous distribution across tumors, injectable hydrogels as drug delivery systems can overcome many limitations of current systemic therapies for cancer, especially systemic toxicity and limited efficacy (123). Compared with intravenous delivery, the intratumoral injection can provide direct contact with tumor cells and immune cells, eliciting a more strong and long-lasting immune response. Besides localized treatment for single tumor, injectable hydrogels can be applied for the treatment of extensive pleural and peritoneal metastasis, such as malignant pleural effusion and malignant ascites. More importantly, in some cases, injectable hydrogels can not only effectively promote ICD of tumor cells and reshape immunosuppressive TME against local tumors but also often generate abscopal effect against distant metastases by activating systemic antitumor immunity (124).

To eradicate cancer cells, effector immune cells must first be activated and overcome the multiple suppressive factors in the TME. Strategies to reverse the immunosuppressive TME include the targeted inhibition of key immunomodulatory factors in the TME using inhibitors of angiogenesis (89), ICIs (60), and agents targeting immunoregulatory cells and factors (113). Off-target effect and treatment resistance greatly weaken the therapeutic effect of single treatment regimen. Therefore, a shift from monotherapy to combination therapies is essential to provide more options of available treatments. The development of novel combination therapies may help enhance the antitumor effects of current therapies and prevent the development of treatment resistance. Hydrogels provide a promising platform for the co-delivery of multiple agents targeting various components of the TME while causing minimal systemic toxicity. In addition, injectable hydrogels can also be combined with conventional treatments, such as radiotherapy and chemotherapy, to transform immunosuppressive TME to a pro-inflammatory state and amplify the antitumor immune response (50, 121, 125).

Despite the advances in injectable hydrogels, there are still several challenges that limit their clinical translation. It is necessary to determine at which stage of tumorigenesis a given treatment is most effective, and whether the effect of treatments depends on the composition of TME at the primary and metastatic sites. Although several combination systems demonstrate synergistic effects, their compositions need to be further optimized to maximize their

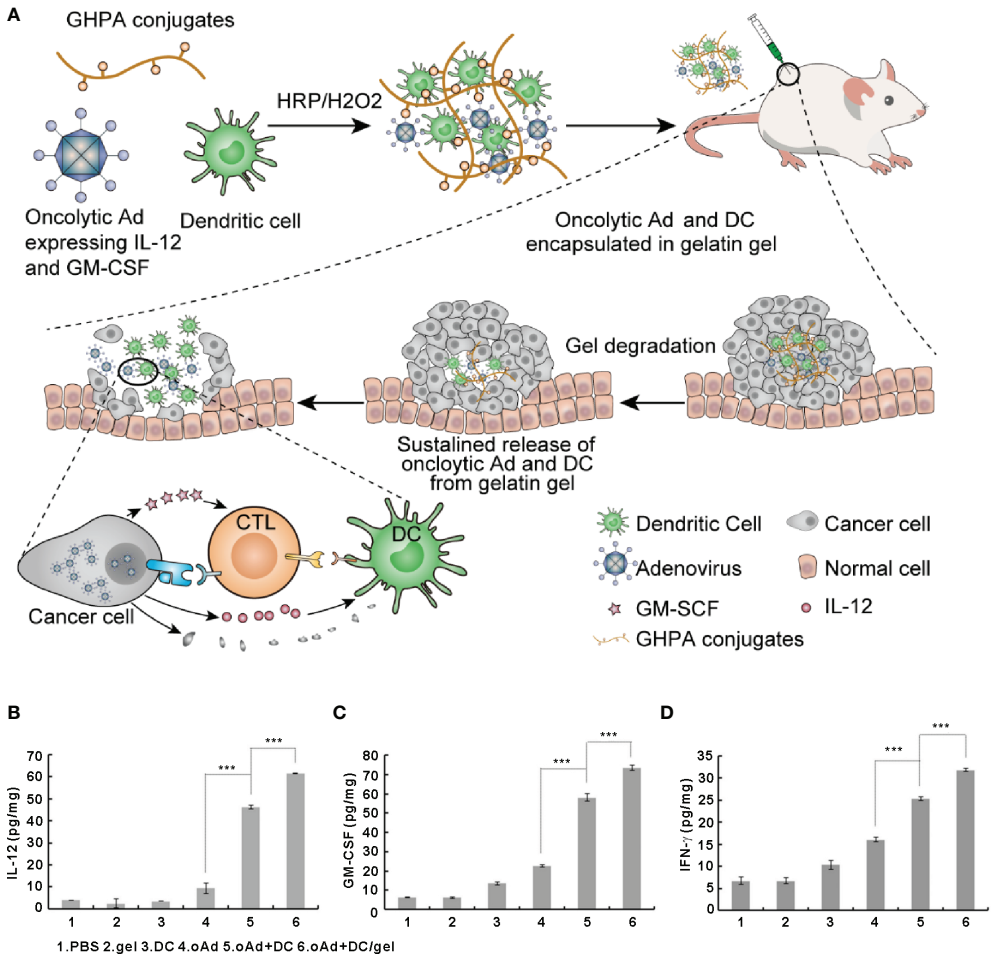

FIGURE 5 | Oncolytic adenoviruses and dendritic cells encapsulated in gelatin gels activate the immune system to eliminate tumor cells and induce the expression of IL-12 and GM-CSF in tumor cells. **(A)** Schematic representation of optimized biodegradable polymeric reservoir-mediated local and sustained co-delivery of dendritic cells and oncolytic adenovirus expressing IL-12 and GM-CSF. **(B–D)** The expression levels of IL-12 **(B)**, GM-CSF **(C)**, and IFN-γ **(D)** in tumors. Reprinted with permission from Elsevier (116). ***P < 0.001.

antitumor efficacy and reduce side effects. Furthermore, future work is required to ensure that in addition to exerting antitumor effects locally and modulating the TME, hydrogels also activate systemic immune responses to prevent metastasis and tumor recurrence. Future multidisciplinary studies are warranted to design injectable hydrogel-based delivery systems for the co-delivery and sequential release of different therapeutic agents to maximize the overall therapeutic efficiency of cancer therapies and accelerate their clinical translation, especially in some late-stage cancers, such as malignant pleural effusion and malignant ascites (126).

AUTHOR CONTRIBUTIONS

YL and YG wrote the manuscript. JH and HJ drafted the outline for the review and revised the manuscript. BY and P-CL checked the format and content of the manuscript. All authors contributed to the article and approved the submitted version.

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Histone Acetylation Regulator-Mediated Acetylation Patterns Define Tumor Malignant Pathways and Tumor Microenvironment in Hepatocellular Carcinoma

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Background: Histone acetylation modification is one of the most common epigenetic methods used to regulate chromatin structure, DNA repair, and gene expression. Existing research has focused on the importance of histone acetylation in regulating tumorigenicity, tumor progression, and tumor microenvironment (TME) but has not explored the potential roles and interactions of histone acetylation regulators in TME cell infiltration, drug sensitivity, and immunotherapy.

Methods: The mRNA expression and genetic alterations of 36 histone acetylation regulators were analyzed in 1599 hepatocellular carcinoma (HCC) samples. The unsupervised clustering method was used to identify the histone acetylation patterns. Then, based on their differentially expressed genes (DEGs), an HAscore model was constructed to quantify the histone acetylation patterns and related subtypes of individual samples. Lastly, the relationship between HAscore and transcription background, tumor clinical features, characteristics of TME, drug response, and efficacy of immunotherapy were analyzed.

Results: We identified three histone acetylation patterns characterized by high, medium, and low HAscore. Patients with HCC in the high HAscore group experienced worse overall survival time, and the cancer-related malignant pathways were more active in the high HAscore group, comparing to the low HAscore group. The high HAscore group was characterized by an immunosuppressive subtype because of the high infiltration of immunosuppressive cells, such as regulatory T cells and myeloid-derived suppressor cells. Following validation, the HAscore was highly correlated with the sensitivity of anti-tumor drugs; 116 therapeutic agents were found to be associated with it. The HAscore was also correlated with the therapeutic efficacy of the PD-L1 and PD-1 blockade, and the response ratio was significantly higher in the low HAscore group.

Conclusion: To the best of our knowledge, our study is the first to provide a comprehensive analysis of 36 histone acetylation regulators in HCC. We found close correlations between histone acetylation patterns and tumor malignant pathways and TME. We also analyzed the therapeutic value of the HAscore in targeted therapy and immunotherapy. This work highlights the interactions and potential clinical utility of histone acetylation regulators in treatment of HCC and improving patient outcomes.

Keywords: histone acetylation, tumor microenvironment, hepatocellular carcinoma, drug sensitivity, immunotherapy

INTRODUCTION

Hepatocellular carcinoma (HCC) is the most common primary liver cancer and ranks as the fifth leading malignancy worldwide (1). Most patients with HCC have poor outcomes because of limited early diagnosis and few available treatment options for advanced-stage HCC (2). Even with active treatment, such as liver transplantation, resection, percutaneous ablation, transarterial chemoembolization, HCC is likely to recur and metastasize, with a 5-year survival rate of less than 20% (3, 4). In addition, both traditional chemotherapy and molecular-targeted agents are impeded by tumor heterogeneity, as well as the intrinsic and acquired drug resistance that can develop in tumors. These characteristics limit the efficacy of systemic therapy in HCC patients (5). Therefore, there is an urgent need to investigate new strategies to improve the clinical outcomes of patients with HCC. Recently, with deeper exploration of the relationship between the immune system and cancer, new therapeutic strategies aimed at mobilizing the host immune system to eradicate tumor cells would advance the cancer therapy field and introduce greater efficacy in curing cancer.

Numerous cancer immunotherapy strategies have rapidly emerged in recent years. The most notable immune-checkpoint inhibition (ICI) treatments consist of agents targeting the inhibitory immune receptors, cytotoxic T-lymphocyte (CTL)-associated protein 4 (CTLA-4/CD152), programmed death protein 1 (PD-1/CD279), and programmed death ligand 1 (PD-L1/B7H1/CD274). These agents have become effective standard therapies in several advanced malignancies, including melanoma (6–8), Merkel cell carcinoma (9), urological cancers (10), non-small cell lung cancer (11), mis-match repair-deficient tumors (12), and Hodgkin's lymphoma. Their response rates range from 25 to 60% in first- and second-line settings (13). Recently, ICI treatment has also been approved for HCC, gastric cancer, triple negative breast cancer, cervical cancer, and head and neck cancer, with response rates closer to 15% (14).

Nonetheless, the efficacy of ICI treatment is still limited because of the ability of cancer tumors to develop primary, adaptive, or acquired resistance to immunotherapy. The resistance of cancer to immunotherapy depends on various factors including the tumor microenvironment (TME), the patient's genetic background, epigenetics, metabolism, and cell stemness (15). At the same time, the multiple factors involved in immunotherapy resistance also provide many more targets that can be attacked by therapeutic agents. To improve the efficacy of immunotherapy, ICI can be combined with other treatments to overcome the immunotherapy resistance.

One such treatment involves histone acetylation. This is one of the most common epigenetic methods used to regulate chromatin structure, DNA repair, and gene expression (16). Histone acetylation is a type of posttranslational modification in which multiple lysine residues at the N-terminus of histones are catalyzed by histone acetyltransferases (HATs). This process is highly dynamic, reversible, and regulated by proteins that can be divided into three categories: "writer", "reader", and "eraser". The "writers" refer to enzymes that transfer acetyl groups to histones, and the "erasers" refer to enzymes that remove acetyl groups from histones. The "readers" are effector proteins that can recognize the modified histones (17). Acetylation neutralizes the positive charge on lysine, weakening the electrostatic association between the histones and the DNA; this makes the DNA becomes more accessible to transcription factors (18).

In general, histone acetylation is associated with elevated transcription whereas histone deacetylation is often associated with gene repression. Previous reports have demonstrated that histone acetylation is closely related to tumorigenesis and can impact certain biological processes of tumor cells, including proliferation (19), apoptosis (20), metastasis (21), and stemness (22). Histone deacetylases (HDACs) are critical regulators of gene expression that enzymatically remove acetyl groups from histones. As such, they are an example of "erasers." Numerous correlative studies have demonstrated aberrant expression of HDACs (HDAC1, HDAC5, and HDAC7) in human tumors, which can serve as molecular biomarkers to distinguish between tumorous and normal tissue (23). HDAC inhibitors (HDACi) can induce acute hyperacetylation of histones and generate the re-expression of tumor-suppressor genes to inhibit tumor growth.

Abbreviations: HCC, hepatocellular carcinoma; MDSC, myeloid-derived suppressor cells; TME, myeloid-derived suppressor cells; HBV, hepatitis B virus; HCV, hepatitis C virus; TACE, transarterial chemoembolization; ICI, immune-checkpoint inhibition; PTM, posttranslational modification; HATs, histone acetyltransferases; HDACs, histone acetyltransferases; HDACi, histone deacetylases inhibitors; TCGA, the cancer genome atlas; ICGC, international cancer genome consortium; GEO, gene expression omnibus; AFP, alpha-fetoprotein; OS, overall survival; DEGs, differentially expressed genes; PFS, progression-free survival; CR, complete response; PR, partial response; SD, stable disease; PD, progressive disease; CMF, 5-fluorouracil; EMT, epithelial-to-mesenchymal transition; CSCs, cancer stem cells; GSVA, gene set variation analysis; ssGSEA, single-sample gene-set enrichment analysis; GDSC, genomics of drug sensitivity in cancer; TIDE, the tumor immune dysfunction and exclusion; ROC, receiver operating characteristic; PCA, principal component analysis; HR, hazard ratio; CNV, copy number variation.

Many HDACi have been proven to have potent anti-tumor effects in several hematological and solid malignancies (24, 25). Recently, researchers have found that histone acetylation is closely related to the TME. Furthermore, numerous studies have demonstrated that HDACi can reshape the TME *via* various mechanisms, enhancing the ability of the immune system to kill tumor cells. Specifically, these mechanisms include upregulating the expression of tumor antigens, enhancing antigen-processing ability, improving the cytolytic activity of CD8+ T cells, and disrupting the immunosuppressive function of IL-10 producing regulatory T cells (26–29). For instance, in preclinical cancer models, HDACi were shown to enhance the efficacy of immune checkpoint blockade using anti-PD1/PDL1 or anti-CTLA4, immunostimulant therapies such as anti-CD40 and anti-CD137, and adoptive T cell immunotherapy (30–34).

Collectively, the above findings indicate that histone acetylation plays an important role in the regulation of the TME, and the molecular agents that target histone acetylation regulators have the potential to disrupt cancer immunotherapy resistance. As a result, combining molecular agents that target histones with immunotherapy could produce additional clinical benefit to patients. However, due to limitations in technical methodology, previous analysis has been confined to a small number of histone acetylation regulators, whereas the antitumor effect of histone acetylation modification is characterized by highly integrated interactions of numerous regulators. Therefore, a comprehensive understanding of how the regulatory network of multiple histone acetylation regulators affects the biological behavior of tumor cells and TMEs would contribute to the development of immunotherapeutic strategies.

In this study, we retrospectively investigated genomic alterations in 1599 HCC samples from the Cancer Genome Atlas (TCGA), International Cancer Genome Consortium (ICGC), and Gene Expression Omnibus (GEO) cohorts. Our objective was to comprehensively evaluate the patterns of histone acetylation modification based on 36 histone acetylation regulators. We found that histone acetylation patterns are distinct in their activation of malignant cancer-related pathways and infiltration of multiple immune cells. We also constructed an HAscore model to quantify the histone acetylation patterns in individual patients based on the differentially expressed genes (DEGs) among them. Finally, we assessed the therapeutic value of the HAscore in targeted HCC therapy and immunotherapy.

MATERIALS AND METHODS

Collection of HCC Datasets and Preprocessing

The workflow of the study is shown in **Figure S1A**. Gene expression data and clinical features of liver cancer samples were retrospectively retrieved from publicly available datasets of the NCBI GEO database (https://www.ncbi.nlm.nih.gov/geo/), TCGA (https://portal.gdc.cancer.gov/), and ICGC (https://dcc.icgc.org/). Specifically, the clinical data we used from the TCGA database included tumor stage, histological grade, vascular tumor cell type, viral hepatitis serologies, Child–Pugh scores, alpha-fetoprotein (AFP), gender, and overall survival (OS) times. In addition, we obtained genomic mutation data (including somatic mutation and copy number variation) of TCGA-LIHC from the UCSC Xena database. In general, nine hepatocellular carcinoma cohorts—TCGA-LIHC, ICGC-LIRI (Japan), ICGC-LICA (France), GSE14520, GSE76427, GSE116174, GSE104580, GSE112790, and GSE121248—for 1599 patients were included for further analysis.

RNA sequencing data, including fragments per kilobase million (FPKM) values and count values, were consistently transformed into transcripts per kilobase million (TPM) values (35). For microarray data from GEO, the normalized matrix files were directly downloaded and normalized by the "normalizeBetweenArrays" method of the R package limma after gene symbol transformation, so that the intensities or log-ratios would have similar distributions across a set of arrays (36). Finally, we used the "ComBat" method of the sva Package (37) to adjust the batch effect caused by non-biotechnological bias.

Two immune checkpoint blockade treatment cohorts with available expression and clinical information were used in our study. First, we obtained the IMvigor210 cohort (http://research-pub.gene.com/IMvigor210CoreBiologies), which consists of advanced urinary tract transitional cell carcinoma treated with atezolizumab, an anti-PD-L1 antibody (38). Second, we obtained the *David Liu* cohort (https://www.nature.com/articles/s41591-019-0654-5), which consists of metastatic melanoma treated with nivolumab or pembrolizumab (39). The gene expression profiles of the pre-therapy biopsy samples were curated and transformed into the TPM format for further analysis.

We searched and collected the following datasets with targeted therapy and chemotherapy from the GEO database: the GSE5851 dataset (advanced metastatic colorectal cancer treated with cetuximab monotherapy); GSE148623 dataset (ductal breast cancer treated with ricolinostat, an HDAC6 inhibitor); and GSE22219 dataset (early primary breast cancer treated with adjuvant cyclophosphamide, methotrexate, and 5-fluorouracil).

Corresponding clinical data were collected from the appropriate GEO dataset metadata and the supplemental files of relevant articles. All baseline information on the available data is summarized in **Table S1**.

Consensus Clustering Expression Pattern of 36 Histone Acetylation Regulators

The literature related to histone acetylation modification was retrieved, and 36 acknowledged histone acetylation genes were curated and analyzed to identify distinct histone acetylation modification patterns (**Table S2**). An unsupervised consensus clustering algorithm was applied to determine robust clustering of liver cancer. We used the R package ConsensusClusterplus to perform the above steps and conducted 1000 repetitions to ensure the stability of the classification (40).

Gene Set Variation Analysis (GSVA) and Functional Annotation

To explain the differences in biological processes between histone acetylation modification patterns, we realized GSVA enrichment analysis by using "GSVA" R packages. This method is commonly used to estimate the variation in pathways and biological process activity in samples of an expression dataset (41). The gene sets of "h.all.v7.4.symbols" were downloaded from the MSigDB database for further GSVA analysis. The 13 most common oncogenic hallmarks, epithelial-to-mesenchymal transition (EMT), and cancer stem cell (CSC) signatures were obtained from the supplementary table prepared by Sanchez-Vega et al. (**Table S3**) (38, 42, 43). Differences were considered statistically significant at P values < 0.05. We used the clusterProfiler R package to perform functional annotation for histone acetylation modification-related genes, with a cutoff value of FDR < 0.05 (44).

Estimation of TME Cell Infiltration

We used the single-sample gene-set enrichment analysis (ssGSEA) algorithm to quantify the relative abundance of each cell infiltration in the HCC TME. The gene sets defining each immune cell type were obtained from the study by Charoentong (**Table S4**) (45). The enrichment scores calculated by ssGSEA analysis were used to represent the relative abundance of the TME infiltrating cells in each sample. The immune-related features were collected from previously published studies (**Table S3**) (46, 47).

Differentially Expressed Genes (DEGs) Among Histone Acetylation Modification Phenotypes

To identify histone acetylation modification-related genes, we classified patients into three distinct histone acetylation modification patterns based on the expression of the 36 histone acetylation modification regulators. DEGs among different modified histone acetylation patterns were determined using limma (36). The significance criteria for determining DEGs were set as adjusted P values < 0.001 and |FC| > 1.5. The adjusted P value for multiple testing was calculated using the Benjamini–Hochberg correction.

Construction of Histone Acetylation Gene Signatures

To quantify the modified histone acetylation patterns of individual tumors, we developed a scoring scheme to quantify the histone acetylation modification level of individual patients and described it as the HAscore. Specifically, 965 DEGs were first identified from different HAclusters, and prognostic analysis was performed for the DEGs using univariate Cox regression model analysis. Subsequently, 591 genes with significant prognoses were selected for further analysis. Next, the patients were classified into several groups for further analysis by adopting an unsupervised clustering method for analyzing prognosis-related DEGs. The consensus clustering algorithm was used to define the number of gene clusters and their stability. We then transformed the expression of these genes into a Z score and conducted principal component analysis (PCA) to construct modified acetylation-relevant gene signatures. Both principal components 1 and 2 (PC1 and PC2, respectively) were selected to act as signature scores. This method focused on the score of the set with the largest block of well-correlated (or anti-correlated) genes, while down-weighting contributions from genes that did not track with other set members. We then adopted a formula like that of previous studies to define the HAscore (48, 49):

$$\text{HAscore} = \Sigma(\text{PC1i} + \text{PC2i})$$

where i is the expression of histone acetylation modification phenotype-related genes

Calculation of the EMT Score

EMT gene signatures were collected from Mak et al. (50), including 25 epithelial and 52 mesenchymal marker genes. Similar to this previous study (50, 51), the EMT score for each sample was evaluated as $\Sigma_i^N \frac{M^i}{N} - \Sigma_j^n \frac{E^j}{n}$, where M and E represent the expression of the mesenchymal and epithelial genes, respectively. Likewise, N and n represent the number of mesenchymal and epithelial genes, respectively.

Correlation Analysis of HAscore and Drug Sensitivity

The Genomics of Drug Sensitivity in Cancer (GDSC) database is the largest public resource for information on drug sensitivity in cancer cells and molecular markers of drug response (52). From here, we collected the transcription profiles of approximately 1000 cancer cell lines, drug response measurements (as AUC of the drug-sensitive curve) in cancer cell lines, as well as targets and pathways of drugs. We performed Spearman correlation analysis to calculate the correlation between drug sensitivity and HAscore and considered |Rs| > 0.3 and FDR < 0.05, estimated by Benjamini and Hochberg adjustment, as significant correlation.

Quantification of the Immune Response Predictor: TIDE

The tumor immune dysfunction and exclusion (TIDE) algorithm proposed by Jiang et al. was used to predict immune checkpoint blockade response by modeling distinct tumor immune evasion mechanisms, including the induction of T cell dysfunction in tumors with high infiltration of CTL and the prevention of T cell infiltration in tumors with low CTL levels by immunosuppressive cells (53). A higher TIDE score indicates that tumor cells are more likely to induce immune escape, thus indicating a lower response rate to ICI treatment. In our study, we used the all-sample average in each study as the normalization control and calculated the TIDE score of each sample using the TIDE tool on the TIDE web application (http://tide.dfci.harvard.edu/), following the developer's instructions.

Statistical Analysis

The data were analyzed using R (version 4.0.0) and R Bioconductor packages. The normality and homogeneity test of

variance were tested using the Shapiro–Wilk normality test and Bartlett homogeneity test, respectively. The Wilcoxon test, Kruskal–Wallis test, and t-test or one-way ANOVA were used to compare the differences as nonparametric or parametric methods. Correlation coefficients were computed using Spearman's and distance correlation analyses. A receiver operating characteristic (ROC) curve was used to verify the validity of the model. Based on the correlation between HAscore and patient survival, the Survminer package was used to determine the best cutoff point of survival information for each cohort. The surv-cutpoint function was used to dichotomize the HAscore, and all potential cutting points were repeatedly tested to find the maximum rank statistic. Then, the patients were divided into high and low HAscore groups according to the maximum selected log-rank statistics to lessen the calculated batch effect. Survival curves for the prognostic analysis were conducted using the Kaplan–Meier method, and log-rank tests were used to assess differences between groups. The chi-squared test or Fisher test was used to analyze the differences in clinical features between the HAscore groups. A univariate Cox regression model was used to generate the hazard ratio (HR) for histone acetylation regulators and histone acetylation-related genes. To verify whether the HAscore was an independent prognostic predictor, we incorporated the HAscore and related clinical parameters into a multivariate Cox regression model analysis. All statistical analyses were two-sided, and statistical significance was set at $P < 0.05$.

RESULTS

Genetic and Transcriptional Alterations of the 36 Histone Acetylation Regulators in HCC

After a systematic review of published articles about histone acetylation, 36 histone acetylation regulatory genes in HCC were identified and incorporated into our analysis, including 9 "writers", 12 "erasers", and 15 "readers", as shown in **Figure 1A** (**Table S2**). Metascape analyses and KEGG enrichment of the 36 histone acetylation regulators were conducted. Significantly enriched biological processes were mainly related to histone modification and cancer-related pathways, as summarized in **Figures 1B** and **S1B**. To determine the genetic alterations of histone acetylation regulators in cancer, we assessed the prevalence of non-silent somatic mutations in the 36 histone acetylation regulators. In the HCC cohort of TCGA, 95 of the 364 (26.1%) samples experienced genetic alterations in histone acetylation regulators, primarily involving missense mutations and splice-site mutations (**Figure 1C**). Among them, the mutation frequencies of BPTF and SMARCA4 were the highest (3%), followed by HDAC9, EP300, BAZ2B, PBRM1, CREBBP, HDAC4, BRD4, and TAF1. In addition, the mutation co-occurrence across histone acetylation regulators was examined, and we found that there was a significant mutation co-occurrence relationship between TAF1 and SMARCA4 (**Figure S1C**). Furthermore, we examined somatic copy number variations (CNVs) of the 36 regulators and found that CNV was widespread among them, and CNV gain was the major alteration (**Figure 1D**). The location of CNV alteration of m6 A regulators on chromosomes is shown in **Figure S1D**. To ascertain whether these genetic variations influenced the expression of histone acetylation regulators in HCC patients, we compared the mRNA expression of these regulators between normal and HCC samples (**Figure 1E**). The results revealed that most genes were upregulated in the HCC samples than in the normal samples, excluding HDAC9, DPF3, and SMARCA2. The genes with higher frequency of CNV gain than of CNV loss were more likely to be upregulated in tumors (such as BPTF, BRD4, and YEATS4). However, the gene expression patterns of some regulators in tumor and non-tumor samples were not consistent with CNV alteration. For example, HDAC1 had a higher frequency of CNV loss than of CNV gain, but the mRNA expression of HDAC1 was upregulated in HCC samples. To investigate the discrepancy between CNV values and mRNA expression, we divided the HCC cohort into four groups based on CNV value (HCC samples with CNV gain, CNV loss, non-significant alteration of CNV, and normal samples). We analyzed the mRNA alterations in different groups of 10 regulators whose mRNA expression was not significantly consistent with CNV pattern (**Figure S1E**). The results showed that mRNA expression was higher in the CNV gain group than in the other three groups, and mRNA expression was lower in the CNV loss group than in the CNV gain and non-significant CNV groups. The above analyses indicate that CNV changes play an important role in regulating the expression of histone acetylation regulators. Furthermore, based on the expression of these 36 regulators, we were able to distinguish HCC samples from normal samples (**Figure 1F**).

This analysis demonstrated that the genetic landscape and expression pattern of histone acetylation regulators between HCC and normal samples are highly heterogeneous, indicating that the imbalanced expression of histone acetylation regulators may play a crucial role in the onset and development of HCC.

Identification of Three Clinical Feature-Related Histone Acetylation Patterns Based on the 36 Regulators

We obtained clinical data and mRNA expression matrices of 1599 HCC samples from nine datasets—TCGA-LIHC, ICGC-LIRI (Japan), ICGC-LICA (France), GSE14520, GSE76427, GSE116174, GSE104580, GSE112790, GSE121248—for further analysis of the expression patterns among the 36 histone acetylation regulators. To explore the prognostic value and expression relationship of histone acetylation regulators, the mRNA sequencing data from the TCGA-LIHC and ICGC-LIRI cohorts with prognostic information were integrated into one meta cohort for univariate Cox regression and Spearman correlation analyses. The results demonstrated that multiple regulators (HDAC2, HDAC1, HAT1, HDAC11, YEATS4, SMARCA4, HDAC5, BRDT, DPF2, HDAC4, KAT7, SMARCA2, BPTF, BRD4, PBRM1, HDAC3, BRD3, DPF1)

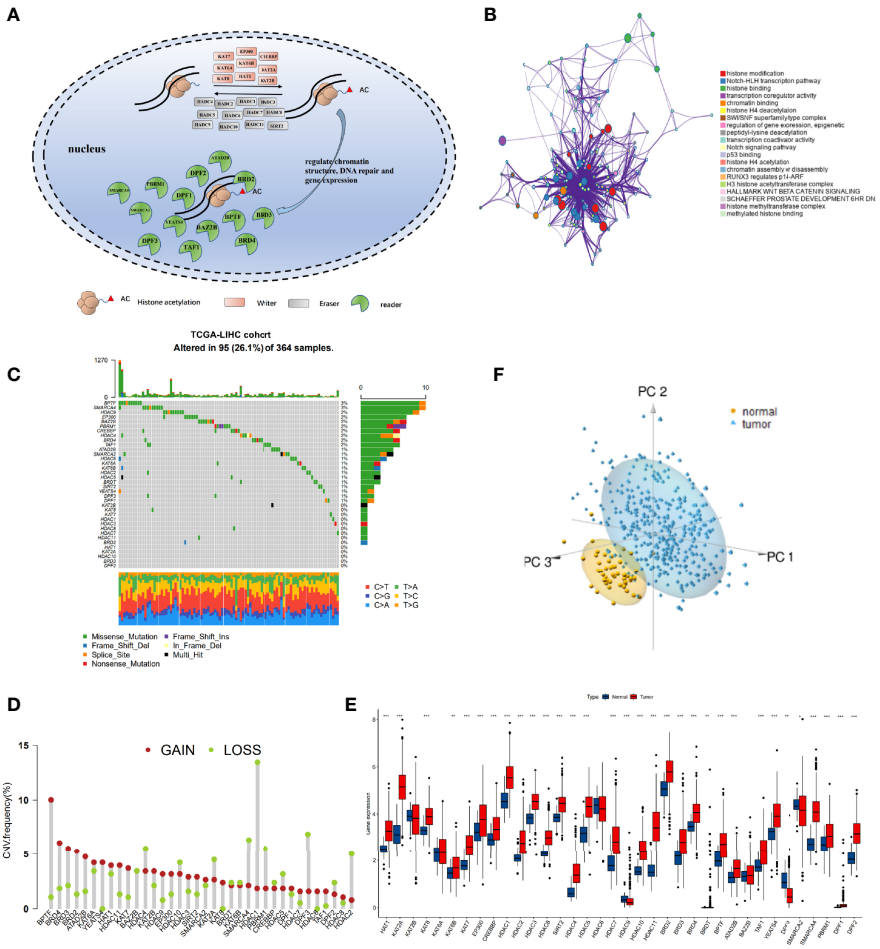

FIGURE 1 | The landscape of genetic alterations of histone acetylation regulators in hepatocellular carcinoma (HCC). **(A)** Summary of the dynamic reversible process of histone acetylation modification mediated by regulators ("writers," "erasers," and "readers") and their biological functions. **(B)** Functional annotations of 36 regulators analyzed by the Metascape enrichment tool. Cluster annotations are shown in the color code. **(C)** The mutation frequency of 36 histone acetylation regulators in TCGA-LIHC cohort. Each column represents individual patients. The barplot on top shows TMB, and the numbers on the right display the mutation frequency of each regulator. The barplot on the right shows the proportion of each variation type. The stacked barplot on the bottom displays the fraction of conversions in each sample. **(D)** The copy number variation (CNV) frequency of histone acetylation regulators in TCGA-LIHC was prevalent. The column represents the alteration frequency. The deletion frequency is a light-green dot; the amplification frequency is a crimson dot. **(E)** Boxplot shows the expression of the 36 histone acetylation regulators between tumor and normal tissues in the TCGA-LIHC cohort. Tumor: red; Normal: blue. (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$). **(F)** Principal component analysis of the 36 histone acetylation regulators to distinguish tumors from normal samples in TCGA-LIHC. Tumor: pale blue; normal: yellow.

were risk factors for HCC, and only SMARCA2 was a protective factor against HCC (**Figure S2A** and **Table S5**). Correlation analysis revealed a significant relationship among the expression of the 36 regulators. Most of them were positively correlated with each other, even though they belonged to different biological groups ("writer", "eraser", or "reader") and had different or opposed bio-functions (**Figure S2B**). The expressions of HDAC10 and HDCA11 ("erasers") were negatively correlated with that of KAT2B ("writer"), and the expression of HDAC11 was negatively correlated with that of DPF3 and SMARC2 ("readers"). These were the only negative correlations between the expressions of the regulators. The comprehensive landscape in the expression network of histone acetylation regulators and their prognostic significance in HCC patients is depicted in **Figure 2A** (**Table S6**). These results indicate that there is a tight cross-talk among the histone acetylation regulators. The writers, erasers, and readers construct a complex network and integrally regulate the histone acetylation modifications, impacting the development of HCC.

To identify the expression pattern of the 36 regulators, the mRNA expression data of 774 HCC samples from the combined datasets (TCGA-LIHC, ICGC-LIRI, and ICGC-LICA cohorts) were classified using ConsensusClusterPlus. Three qualitatively different histone acetylation patterns were identified using unsupervised clustering, including 198 cases in pattern A, 204 cases in pattern B, and 372 cases in pattern C. We termed these patterns HAcluster_A–C (**Figure S2C** and **Table S7**). Clustering of histone acetylation was repeated in the GEO meta cohort

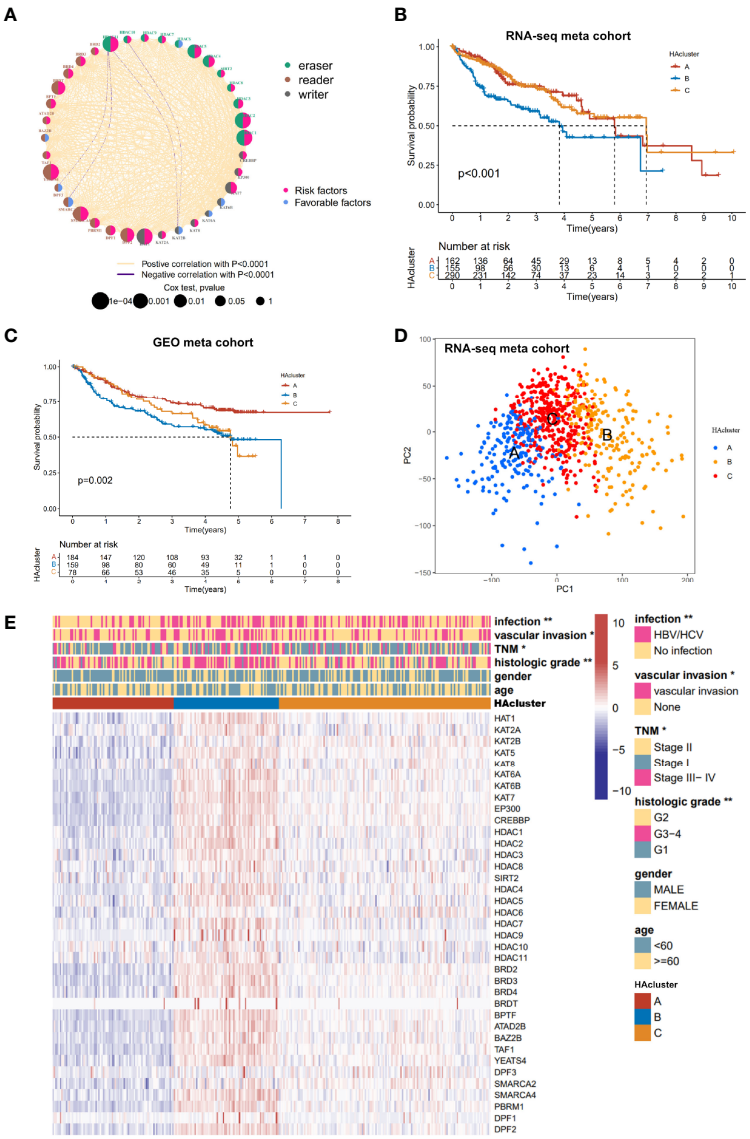

FIGURE 2 | Histone acetylation modification pattern and clinical characteristics of each pattern. **(A)** The interaction among histone acetylation regulators in liver cancer. The circle size describes the effect of each regulator on the prognosis and scale by *P* value. Favorable factors are shown with a pink semicircle on the right. Risk factors are shown with a blue semicircleon the right. Three histone modification types of the 36 histone acetylation regulators are depicted by different colored semicircle on the left. Readers: Indigo; writers: brown; erasers: gray. The red and blue lines represent positive and negative correlations, respectively ($P < 0.0001$). **(B)** Survival analyses of three histone acetylation modification patterns based on 607 patients from the RNA-seq meta cohort (TCGA-LIHC, ICGC-LIRI). **(C)** Survival analyses of three histone acetylation modification patterns based on 421 patients from the GEO meta cohort (GSE14520, GSE76427, GSE116174). **(D)** Principal component analysis of the transcriptome profiles between three histone acetylation modification patterns, indicating a prominent difference on the transcriptome between different HAclusters (based on RNA-seq meta cohort). **(E)** Unsupervised clustering of the 36 histone acetylation modification regulators in the TCGA-LIHC cohort. The HAcluster, viral infection, vascular invasion, TNM stage, histology grade, age, and gender were used as sample annotations. Red represents high expression, and blue represents low expression. Comparison of clinical characteristics proportion analysis between three HAclusters was evaluated by Chi-square test (*$P < 0.05$, **$P < 0.01$).

(GSE14520, GSE76427, GSE116174, GSE104580, GSE112790, and GSE121248), and a similar result was obtained (**Figure S2D**). Notably, the PCA analysis shows that there was a significant difference in the transcriptional profile among the three different histone acetylation patterns, indicating that unsupervised clustering was successful (**Figure 2D**). The prognostic analysis revealed that the survival probability of patients in HAcluster_B was worse than in HAcluster_A and HAcluster_C based on the combined datasets of TCGA-LIHC and ICGC-LIRI cohorts that have prognostic information (**Figure 2B**). The prognosis predictive ability of the HAcluster was re-examined using the combined data from the GEO

database and we obtained similar results (**Figure 2C**). Most histone regulators, including writers, erasers and readers were highly expressed in HAcluster_B, followed by HAcluster_C and HAcluster_A (**Figures 2E** and **S2E**). This indicated that the patients in HAcluster_B have the most active histone acetylation modification and the modification turnover is fast. This may be a risk factor for the prognosis of HCC patients. In addition, the HAcluster was closely correlated with the clinical features of HCC. The viral infection events, vascular invasion, high TNM grade, and high histologic grade were significantly enriched in HAcluster_B, as examined in the TCGA HCC cohort (**Figure 2E**).

Three Histone Acetylation Patterns Associated With Distinct Tumor Molecular Backgrounds and Immune Infiltration

To identify the differences in biological behavior among the three histone acetylation modification patterns, GSVA enrichment analysis based on KEGG gene sets was performed (**Table S8**). Compared to HAcluster_A and HAcluster_C, HAcluster_B was enriched in carcinogenetic activation and stromal pathways, cancer pathways, p53/MAPK/MTOR/NOTCH/WNT/ERBB/TGF_BETA signaling pathways, cell cycle, and apoptosis. On the other hand, HAcluster_A and HAcluster_C were enriched in several biometabolism-related pathways (**Figures 3A, B** and **Table S9**). We confirmed this result by conducting GSVA enrichment analysis based on oncogenic hallmark data obtained by Sanchez-Vega et al. and Mariathasan et al. (**Table S3**) (38, 42); the results showed that HAcluster_B was enriched in most of the malignant pathways, similar to the above analysis (**Figure 3C**). Notably, the activity of angiogenesis, EMT, and cancer stemness was also high in HAcluster_B (**Figure 3C**). As shown in **Figure S3A**, **B**, mRNA expression of stem cell biomarkers in HCC and the EMT score were the highest in HAcluster_B. These analyses indicate that the histone acetylation pattern was closely related to cancer's bio-behavior in HCC, and the high activity of histone acetylation relators could be a crucial factor in improving the degree of malignancy.

Previous studies have reported a significant correlation between TME infiltration of immune cells and modified histone acetylation (54, 55). Therefore, we comprehensively investigated the functional role of the regulatory network composed of histone acetylation regulators in the TME. The ssGSEA algorithm was used to quantify the relative abundance of immune cells infiltrating the TME (**Table S10**). The Spearman correlation analysis showed a strong correlation between regulators and TME-infiltrating immune cells (**Figure 3D**). For example, the expression of "erasers" HDAC7 and HDAC9 were positively correlated with most of the TME-infiltrating immune cells, and there was a positive correlation between activated CD4 T cells and most of the regulators. Additionally, the differences in TME cell infiltration among thethree histone acetylation patterns were analyzed (**Figure 3E**). HAcluster_B was remarkably differences from HAcluster_A and HAcluster_C. The activated dendritic cells and plasmacytoid dendritic cells were higher in HAcluster_B than in HAcluster_A and HAcluster_C, indicating a highly active antigen-presenting function in this group. The natural killer cells were also high in HAcluster_B. However, activated CD8 T cells, the most powerful effectors in the anticancer immune system (56), along with other important tumor killer cells and gamma delta T cells (57) were both lower in HAcluster B than that in HAcluster_A and HAcluster_C. It is known that myeloid-derived suppressor cells (MDSC) (58) and regulatory T cells are immune suppressive cells (59), while type 2 T helper cells are pro-tumorigenic (60). Both MDSC and type 2 T helper cells were significantly higher in HAcluster_B, and regulatory T cells were higher in HAcluster_B; however, this was not statistically significant. These results indicated that HAcluster_B is an immunosuppressive subtype, and its high levels of immunosuppressive cells offset the positive influence of highly-activated antigen pressing cells, which led to a poor prognosis for patients in HAcluster_B. To confirm this hypothesis, we analyzed the activity of immune suppression, immune cytolytic effect, and antigen processing in the three histone acetylation patterns based on the related gene signature data from Bindea et al. and Thorsson et al. (**Table S11**) (46, 47). The results demonstrated that the activities of immune suppression and antigen processing were the highest in HAcluster_B, and the immune cytolytic activity of HAcluster_B was the lowest among the three groups, in agreement with previous analyses (**Figure 3F**).

Construction of a Digital Model for Quantifying Histone Acetylation Patterns of Individual HCC Patients

To gain a comprehensive understanding of the differences in biological features among the three HAculsters, we identified 591 DEGs that were significantly associated with patient prognosis to characterize the HAcluster, based on three HAclusters previously analyzed in the RNA-seq meta cohort (**Figure S4A** and **Table S12**). The GO enrichment of these DEGs showed that their functions were mainly enriched in histone acetylation, cell cycle, RNA splicing, DNA replication, and cell adhesion (**Figure 4A**). We found that patients could be clustered into three phenotype-related subtypes based on these DEGs, named geneCluster_A, geneCluster_B, and geneCluster_C, (**Figure S4B**, **C**). Most DEGs were highly expressed in geneCluster_B, followed by geneCluster_C and geneCluster_A (**Figures 4B** and **S4D**). Most histone acetylation regulators were highly expressed in geneCluster_B (**Figure S4E**). The survival analyses showed that patient prognosis in geneCluster_B was the worst, as analyzed in the RNA-seq meta cohort and GEO meta cohort (**Figures 4C** and **S4F**). To depict and quantify the histone acetylation pattern of individual HCC patients using a convenient and precise method, we constructed a score model based on these phenotype-related DEGs. This model was termed the histone acetylation score (HAscore; see *Materials and Methods*). We found that the HAscore was positively correlated with the mRNA expression of histone acetylation regulators and phenotype-related DEGs. The HAscore in HAcluster_B and geneCluster_B was the highest. The HAscore was moderately high in HAcluster_C and geneCluster_C, and

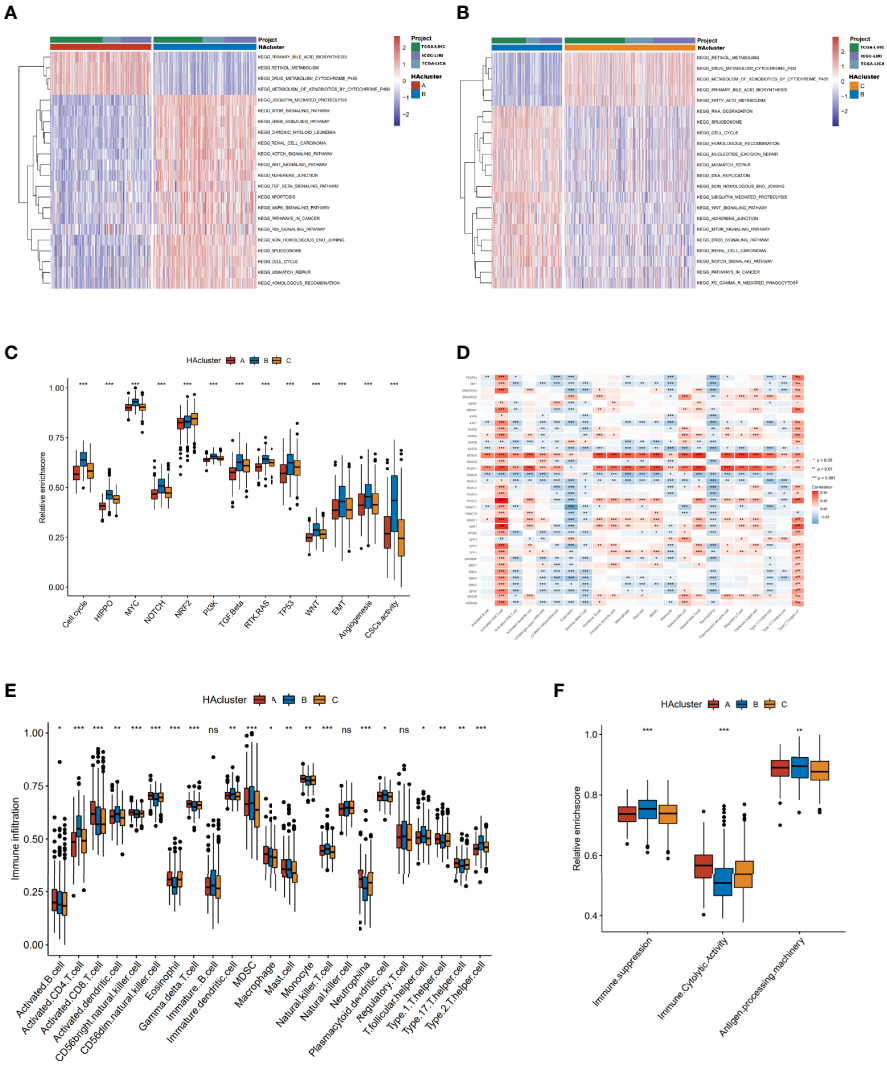

FIGURE 3 | Biological characteristics of histone acetylation patterns. **(A, B)** GSVA enrichment analysis demonstrates the activation states of KEGG biological pathways between distinct HAclusters in RNA-seq meta cohort and the activated group visualized by heatmap. Yellow and blue represent activated and inhibited pathways, respectively. The HAcluster and project of database were used as sample annotations. **(A)** HAcluster A vs HAcluster B; **(B)** HAcluster B vs HAcluster **(C)** Differences in oncogenic pathways among the three distinct HAclusters. **(D)** The correlation between the 36 histone acetylation regulators and TME infiltration cells in RNA-seq meta cohort. Positive and negative correlations are marked in red and blue, respectively. **(E)** Boxplot of abundance of TME-infiltrating cells in three HAclusters, based on the RNA-seq meta cohort. **(F)** Differences in immune-related functional pathways among the three distinct HAclusters. The statistical differences among the three HAclusters were tested by the Kruskal–Wallis test. (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; ns, non-significant).

the lowest in HAcluster_A and geneCluster_A (**Figures 4D, E**). Next, we divided patients into high HAscore and low HAscore groups using the Survminer package and conducted an overlap analysis of these three different classifiers based on a histogram of frequency distribution (analyzed on samples in the RNA-seq meta cohort with prognostic information). The results showed that samples in the high HAscore group were all from geneCluster_B (172 out of 204: 84.3%), while 166 out of 191 (86.9%) samples in HAcluster_B composed the majority of geneCluster_B. In addition, most of the patients in geneCluster A and geneCluster_C belonged to HAcluster_A and HAcluster_C, respectively, and contributed to the main part of the low HAscore group (**Figure 4G**). The above results suggest that these three computational methods of classification have a high degree of coincidence.

Furthermore, we analyzed the prognostic prediction value of the HAscore in patients with HCC. The results demonstrate that the patients in the RNA-seq meta cohort and GEO meta cohort with low HAscores, had a prominent survival benefit (**Figures 4F** and **S4G**). Based on the RNA-seq meta cohort, the AUCs of the time-dependent ROC curves for the HAscore were 0.708, 0.612, 0.624 and 0.573 at 1-, 2-, 3- and 5- year overall survival, respectively (**Figure 4H**). Similar results were obtained from the GEO cohort (**Figure S4H**). Next, we performed multivariate Cox

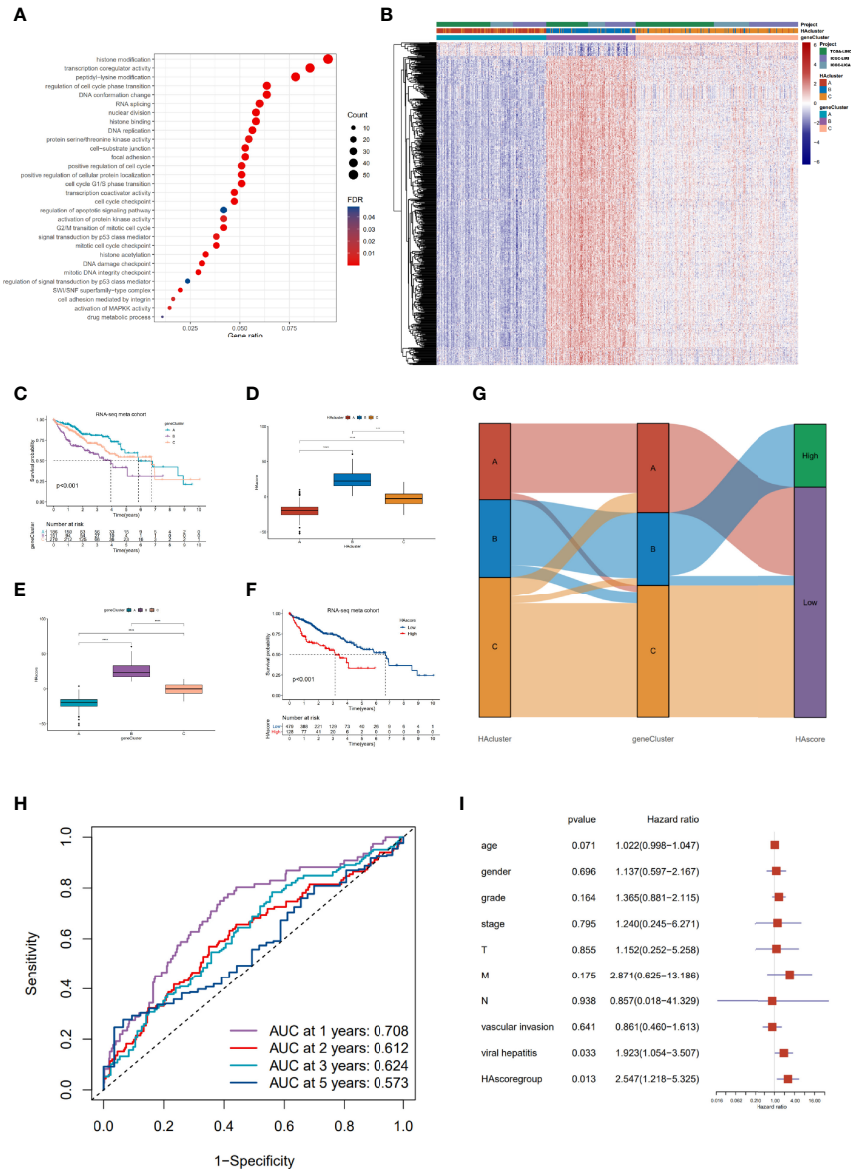

FIGURE 4 | Construction of the characteristic signature of histone acetylation patterns and its prognostic significance. **(A)** GO enrichment analysis for histone acetylation pattern related genes with prognostic significance. The x-axis indicates the gene ratio within each GO term. **(B)** Unsupervised clustering of 591 histone-acetylation-related genes in RNA-seq meta cohort. The HAcluster, geneCluster, and cohorts were used as sample annotations. **(C)** The survival curves of different geneClusters in the RNA-seq meta cohorts (TCGA-LIHC and ICGC-LIRI) were estimated by the Kaplan–Meier plotter (p = 1.62e-05, Log-rank test). **(D)** Differences in the HAscores of the HAclusters in the RNA-seq meta cohorts. **(E)** Differences in the HAscores of the geneClusters in the RNA-seq meta cohorts. The statistical differences were tested by the Kruskal–Wallis test. (****$P < 0.0001$). **(F)** Survival analyses for low and high HAscore groups in the RNA-seq meta cohort (TCGA-LIHC and ICGC-LIRI) using Kaplan–Meier curves ($P = 4.28e-07$, Log-rank test). **(G)** Alluvial diagram demonstrating the changes in the HAcluster, geneCluster, and HAscore groups. **(H)** The predictive value of HAscore in patients from the TCGA-LIHC and ICGC-LIRI RNA-seq meta cohorts (AUC: 0.708, 0.612, 0.624 and 0.573 for 1, 2, 3, 5- year overall survival). **(I)** Multivariate Cox regression model analysis of the factors including HAscore, patient age, gender, TNM status, histology grade, vascular invasion, and viral hepatitis serologies in the TCGA-LIHC cohort.

regression analysis using patient clinical characteristics including age, sex, histologic grade, TNM stage, vascular invasion, and viral infection. We found that the HAscore was a robust and independent prognostic biomarker for evaluating outcomes of patients in the TCGA-LIHC and GSE14520 cohorts (**Figure 4I**, HR = 2.547, 95% CI: 1.218-5.325, $P = 0.013$; **Figure S4I**, HR = 1.647, 95% CI: 1.058-2.563, $P = 0.027$). In addition, survival analyses based on the HAscore were also conducted for stomach adenocarcinoma, bladder urothelial carcinoma, skin cutaneous melanoma, and head and neck squamous cell carcinoma. The results show that the survival prognosis of patients with high HAscores was worse than those of patients with low HAscores

(**Figure S4J**). These results indicate that the HAscore was closely related to prognosis and could be seen as a risk factor for HCC and several other cancers.

Clinical Features, Transcriptional Molecular Characteristics, and TME-Infiltrating Cells Associated With the HAscore

Our analyses have revealed survival prognostic differences between the high HAscore and low HAscore groups. Therefore, we determined to further explore the latent mechanism behind these results. We analyzed the relationship between the HAscore and the characteristics of the sample including clinical characteristics, transcriptional molecular background, and TME. The GSE14520 dataset and the TCGA-HCC cohort with adequate clinical information were used to analyze the correlation between HAscore and clinical characteristics. As shown in **Figures 5A** and **S5C**, the HAscore was higher in the groups with high AFP expression, vascular invasion, viral infection, multiple nodules, advanced histologic grade, TNM staging, and CLIP staging. In the TCGA-LIHC cohort, samples with high AFP expression, viral infection, vascular invasion, advanced histologic grade, and TNM staging were significantly higher in the high HAscore group (**Figure 5B** and **Figure S5D**). In the GES14520 dataset, samples with high AFP expression, advanced TNM staging, and CLIP staging were significantly higher in the high HAscore group (**Figure S5A, B**). Considering that the above-mentioned clinical characteristics were all risk factors for HCC prognosis (3, 61, 62), these results elucidate the fact that patients with a high HAscore had a worse survival prognosis.

Furthermore, the correlation between HAscore and tumor molecular background was analyzed. The results show that nearly all the cancer-related malignant pathways (such as cell cycle, HIPPO, MYC, PI3K, and MYC), excluding the NRF2 signaling pathway, were significantly positively correlated with the HAscore (**Figure 5C** and **Table S13**). The EMT score was also higher in the high HAscore group (**Figure S5E**), indicating that patients with high HAscores had higher activation of the malignant pathway, resulting in a worse prognosis. Next, correlation analysis involving HAscore, tumor-infiltrating immune cells, and immune function was performed (**Figure 5D**). The results demonstrate that the infiltration of pro-tumorigenesis cells, type 2 T-helper cells (P = 1.5e-13), and immunosuppressive cells, including MDSCs (P = 6.1e-05) and regulatory T cells (P = 0.00099), were significantly positively correlated with the HAscore. The immune cytotoxic cells-gamma delta T cells that were significantly negatively correlated with the HAscore (P = 0.02026). The HAscore was also significantly positively correlated with the activity of immune suppression (P = 4.536376e-12) and negatively correlated with immune cytolytic activity (P = 1.827941e-09) (**Figure 5C**). Additionally, in the high HAscore group the enrichment of the number of MDSC, regulatory T-helper cells, and type 2 T-helper cells was significantly higher, whereas that of the number of cytolytic gamma delta T cells was significantly lower (**Figure 5E**). The above results demonstrate that the HAscore was closely correlated with TME, and the high HAscore group was considered an immunosuppressive subtype.

The Predictive Ability of the HAscore Model in the Sensitivity of Anti-Tumor Drugs

Recently, numerous molecular-targeted agents have been developed for the treatment of certain cancers and have had good results. The above analyses reveal that histone acetylation modification is closely related to the functional pathways of cancer, such as cell cycle, DNA replication, the p53 pathway, and the PI3K/mTOR signaling pathway. Thus, the HAscore could have potential value in predicting the related drug response in patients. To test this hypothesis, we assessed the association between the HAscore and the response to drugs in cancer cell lines using the GDSC database. Using the Spearman correlation analysis, we identified 42 correlated pairs in which the AUC of the drug-sensitive curve was significantly positively correlated with HAscore (**Table S14**). These drugs included cetuximab, a monoclonal antibody that inhibits epidermal growth factor receptor (Rs = 0.522, P < 3.15E-61), the MEK inhibitor trametinib (Rs = 0.444, P < 3.15E-61), and the HSP90 inhibitor tanespimycin (Rs = 0.443, P < 3.15E-61). These results suggest that these drugs could be more sensitive in samples with low HAscores. In contrast, 74 correlated pairs were identified in which the AUC of the drug-sensitive curve was significantly negatively correlated with HAscore. These included the HDAC6 inhibitor ACY-1215 (Rs = -0.521, P < 3.15E-61), Wee1 inhibitor MK-1775 (Rs = -0.492, P < 3.15E-61), and Bcl-2 inhibitor sabutoclax (Rs = -0.472, P < 3.15E-61). These results suggest that these drugs could be more sensitive in samples with high HAscores (**Figure 6A**). Additionally, the signaling pathways of the genes targeted by these drugs were analyzed. Notably, the drugs that were sensitive in samples with high HAscores mostly targeted histone acetylation, mitosis, cell cycle, and DNA replication. This result is consistent with our previous analyses, which demonstrated that most histone modification regulators were highly active in the high HAscore group, along with cell cycle and DNA replication. In addition, we found that the drugs that were sensitive in samples with low HAscores mostly targeted the MEK2 and RTK signaling pathways (**Figure 6B**).

To examine whether the HAscore could predict the drug response in patients, we analyzed the relationship between drug response and HAscore based on several datasets that were treated with related anti-tumor agents. In the GSE5851 dataset, an analysis of cetuximab monotherapy in patients with advanced metastatic colorectal cancer reveals that the HAscore of responders was significantly lower than that of non-responders (**Figure 6C**), and the progression-free survival (PFS) of the low HAscore group was significantly longer than that of the high HAscore group (**Figure 6D**). The AUC of drug sensitivity-dependent ROC curves for the HAscore was 0.691 (**Figure 6E**). These results are consistent with our finding that the sensitivity of cetuximab was higher in the low HAscore group. Furthermore, in the GSE22219 dataset, an analysis of a

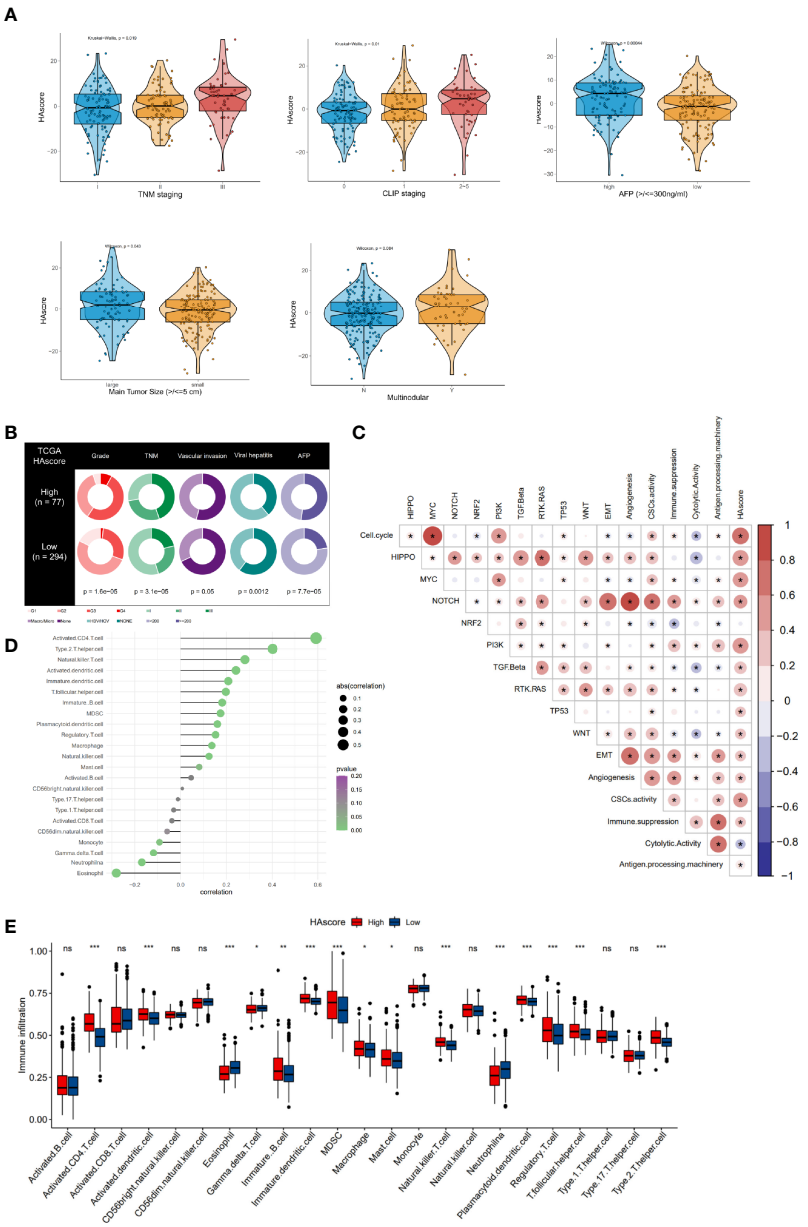

FIGURE 5 | Clinical features, molecular characteristics, and TME infiltrating cells of the distinct HAscore groups. **(A)** Difference in HAscore among distinct clinical features related subgroups in the GSE14520 cohort. The Wilcoxon test was used to test the statistical differences among clinical features related subgroups. **(B)** Clinical features for the high and low HAscore groups in TCGA-LIHC cohort. Chi-squared test or Fisher test was used to test the statistical differences. **(C)** Correlations between the HAscore and the known gene signatures in RNA-seq meta cohort using Spearman analysis. Positive correlation is marked with red and negative correlation with blue. The asterisks represent the statistical *P* value (*$P < 0.05$). **(D)** Correlations between HAscore and TME infiltrating cell abundance in RNA-seq meta cohort using Spearman analysis. The circle size and x-coordinates describe the correlation coefficient. The color of the circle is scaled by *P* value. **(E)** Boxplot of each TME infiltrating cell abundance for high and low HAscore groups in the RNA-seq meta cohort. The statistical differences among the HAscore groups were tested by the Kruskal–Wallis test. (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; ns, non-significant).

cyclophosphamide, methotrexate, and 5-fluorouracil regimen in patients with breast cancer shows that the PFS of patients with high HAscores was significantly longer (**Figure 6F**), consistent with our previous analyses, which showed that methotrexate (Rs = -0.422, $P < 3.15E-61$) and 5-fluorouracil (Rs = -0.386, $P < 3.15E-61$) were more sensitive in high HAscore samples. The above results indicate that ACY-1215 (ricolinostat), an HDACi, was sensitive in the high HAscore sample. The analysis based on the GSE148623 dataset reveals higher HAscores in responders and longer PFS in high HAscore patients (**Figures 6G, H**); however, this was not statistically significant because of the small sample size ($N = 10$). Collectively, these analyses indicate

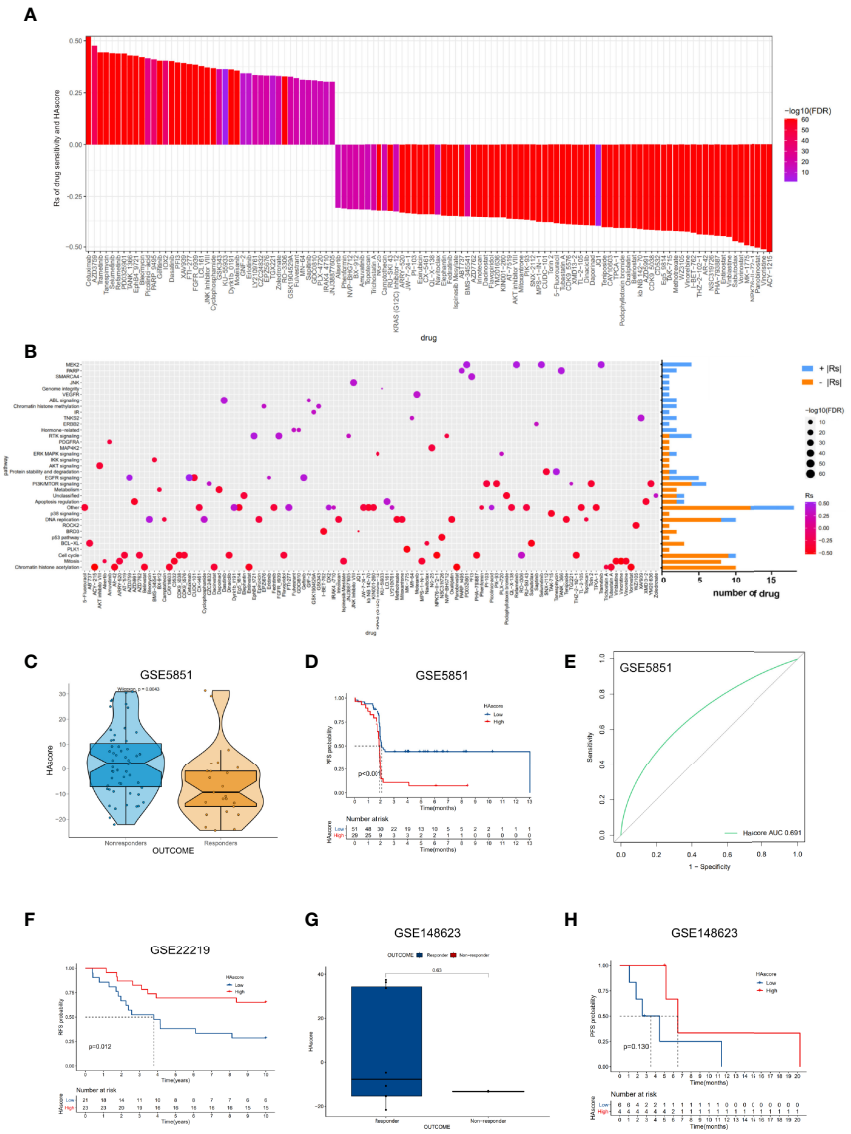

FIGURE 6 | The relationship between HAscore and drug sensitivity. **(A)** The Spearman analysis was used to evaluate the correlation between HAscore and AUC of drug-sensitive curve. The brightness of column indicates the significance of the correlation. The height indicates the values of Rs. **(B)** Signaling pathways targeted by drugs that were closely correlated with HAscore. The horizontal axis shows the drug names, and the vertical axis shows the signaling pathway targeted by the drugs. The bar graph on the right displays the number of drugs in each signaling pathway. The significance of the correlation is shown by the size of the point. **(C, G)** The difference of HAscores between distinct clinical outcomes of related anti-tumor drugs, including cetuximab **(C)** and ricolinostat **(G)**. **(D, F, H)** Kaplan–Meier curves show the overall survival time in high HAscore or low HAscore group after the treatment of related anti-tumor drugs, including cetuximab **(D)**, a cyclophosphamide, methotrexate, and 5-fluorouracil regimen **(F)**, and ricolinostat **(H)**. **(E)** The predictive value of the HAscore to the sensitivity of cetuximab (AUC = 0.691).

that the HAscore has potential value in predicting drug response in patients.

The HAscore Model Predicts Response to Immunotherapy With a PD-L1 or PD-1 Blocker

The emergence of immunotherapies targeting the PD-L1 and PD-1 pathway blockade provides a positive outlook for patients with cancer. However, the benefits of ICI therapy are still limited because of innate or acquired immunotherapy resistance. Thus, many studies have aimed to identify predictors of ICI therapy for appropriate candidates, such as TIDE, which is widely used and strongly recommended to evaluate the immune response in cancer-related studies (63–68). Considering that the HAscore appears to be closely correlated with the TME, we examined the power of the HAscore to predict the response of patients to ICI therapy based on two immunotherapy cohorts. First, we analyzed the relationship between the HAscore and TIDE

based on the TCGA-ICGC and GEO cohorts. The results show that the TIDE scores were significantly higher in the high HAscore group for both cohorts (P <2.2E-16; P = 1.7E-05; **Figures 7A, B**), and the HAscore was positively correlated with the TIDE score (Rs = 0.31; P < 2.2E-16; Rs = 0.15; P = 2.2E-05) (**Figures S6A, B**). In addition, the HAscore was significantly positively correlated with MDSC infiltration (Rs = 0.49; P = 1.37e-47; Rs = 0.67; P = 4.03e-109) and exclusion immune subtype (Rs = 0.46; P = 1.38e-42; Rs = 0.29; P = 1.05e-17) calculated by the TIDE method in TCGA-ICGC and GEO cohorts (**Figures S6C, D**). This result is consistent with our previous finding, which demonstrated that the high HAscore group was an immune suppressive subtype. Further, analysis in the anti-PD-L1 immunotherapy cohort (Imvigor210) shows that patients with a low HAscore had prolonged overall survival time (P = 0.003) (**Figure 7C**) and better therapeutic outcomes. The proportion of patients with complete response (CR) or partial response (PR) to the anti-PD-L1 blocker was 27% in the low HAscore group versus 13% in the high HAscore group (**Figure 7D**, chi-squared P = 0.0133). **Figures 7E, F** show that the neoantigen burden and mutation burden were high in the low HAscore group (P = 0.00022; P = 0.012), and the TIDE score was low in the low HAscore group. This is consistent with the finding that patients with low TIDE score seemed to gain more clinical benefit from IBI therapy (**Figure S6E**). **Figure S6F** shows that the AUC of the sensitivity-dependent ROC curve was 0.606 for the HAscore vs. 0.582 for TIDE score (P = 0.608). The study of the *David Liu* cohort that was treated with anti-PD-1 immunotherapy yielded similar results. **Figure 7G** shows that the OS of patients with low HAscores was significantly longer than that of patients with high HAscores (P < 0.001). Additionally, the proportion of patients with CR or PR to the anti-PD-1 blocker was 43% in the low HAscore group versus 17% in the high HAscore group (**Figure 7H**, Fisher; P = 0.03947). The above results indicate that patients with low HAscores could gain more survival advantage and greater benefit from ICI treatment. Further, the established modified histone acetylation score model could improve the selection of drugs for HCC and the prediction of response to anti-PD-L1 or anti-PD-1 immunotherapy.

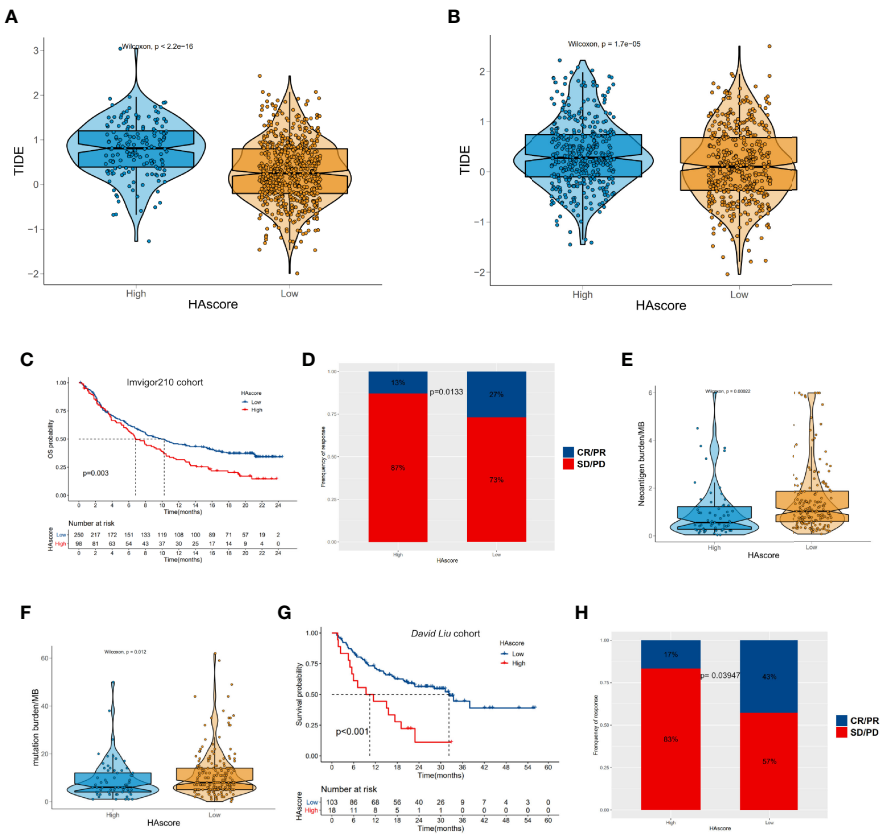

FIGURE 7 | The relationship between HAscore and immunotherapy. **(A, B)** The TIDE scores of individual HCC samples in the high HAscore or the low HAscore groups. **(A)** shows the result from the RNA-seq meta cohort and **(B)** shows the result from the GEO meta cohort. **(C, G)** Kaplan–Meier curves show the overall survival time in the high HAscore or the low HAscore groups after the treatment of PD-L1 pathway blockgade immunotherapy **(C)** or PD-1 pathway blockade immunotherapy **(G)**. **(D, H)** The proportion of patients with different responses to PD-L1 blockage **(D)** or PD-1 blockage **(H)**. **(E, F)** the differences of neoantigen burden **(E)** or mutation burden **(F)** in the high HAscore or the low HAscore group.

DISCUSSION

Ample evidence exists showing that histone acetylation plays an essential role in cancer biological processes such as proliferation, apoptosis, differentiation, EMT, and drug sensitivity (69). Recently, researchers have found that histone acetylation also has an indispensable role in shaping the TME, which is an important factor in determining patient prognosis. However, most studies have focused on a single histone acetylation regulator. Relatively little is known about the relationship between the three types of histone acetylation regulators ("writer," "eraser," and "reader") and their function in cancer. Considering that the histone acetylation regulators function as a tight network, it is necessary to analyze them as a whole in cancer research.

In this study, we analyzed the correlation among 36 histone acetylation regulators and found that the expression levels of nearly all of the regulators were positively correlated with each other; however, the functions of these regulators were different (even opposite). Based on unsupervised clustering of the 36 regulators, we divided the patients into three histone acetylation phenotypes (HAcluster_A, HAcluster_B, and HAcluster_C). Interestingly, their patterns were distinctly expressed in the 36 regulators. Nearly all the regulators had the highest expression in HAcluster_B, the regulators were moderately expressed in HAcluster_C, and the regulators had the lowest expression in HAcluster_A. This indicates that the activity and turnover of histone acetylation was intense in HAcluster_B. Our survival analysis reveals that the OS of patients in HAcluster_B was the worst of the three phenotypes. Furthermore, to better characterize the three histone acetylation phenotypes, we identified differentially expressed genes among them. Based on these genes, we constructed an HAscore model to digitally quantify the histone acetylation phenotype in individual patients. The results show that the HAscore was the highest in HAcluster_B, and the survival prognosis of the high HAscore group was the worst.

To explore the mechanism causing the prognostic difference among patients with different histone acetylation phenotypes, we first analyzed cancer biological features with the three histone acetylation patterns and two HAscore groups. We found that HAcluster_B was characterized by significant activation of the mTOR, ERBB, NOTH, WNT, TGF-β signaling pathways, cell cycle, and apoptosis. The HAscore was also significantly positively correlated with the activation of cell cycle, angiogenesis, EMT, cell stemness, and cancer-related malignant signaling pathways (HIPPO, MYC, NOTH, PI3K, TGF-β, RTK/RAS, TP53, and WNT). The above-mentioned biological functions and signaling pathways play an important role in promoting tumor development. For example, HIPPO (70), NOCTH (71), TGF-β (72) and WNT (73) are crucial signaling pathways that regulate various cancer-related processes, including cell proliferation, invasion, metastasis, and immunologic escape. The abnormal activation of these signaling pathways promotes cancer malignancy and leads to a poor prognosis (74–77).

Cancer stem cells are a subtype of cells that can self-renew by division and generate tumor progeny required for sneaking through and tumorigenesis (78, 79). In addition to their cancer-initiating ability, CSCs play a critical role in modulating other processes such as EMT (80), immunotherapy resistance (81) and drug resistance (82). These four signaling pathways also play key roles in supporting CSC activity (83). In HAcluster_B and the high HAscore group, where the malignant signaling pathways were active; the biomarkers for HCC stem cells were all highly expressed, indicating the high activity of CSCs in these two groups. These findings can partially explain why patients in HAcluster_B or those with high HAscores had the worse survival prognosis.

ICI therapy is a potentially good application in this setting because it mobilizes the autoimmune system to kill cancer cells. Mounting evidence has confirmed that diverse HDACi could alter the biological processes of immune cells and reshape the immune microenvironment, enhancing the tumor-killing effect of the immune system (84–86). In this study, we found that histone acetylation patterns were closely related to TMEs, and there were distinct differences in tumor-infiltrating immune cells among the three histone acetylation patterns. The activated dendritic cells, plasmacytoid dendritic cells, and antigen processing activity were significantly higher in HAcluster_B and the high HAscore groups. The biological processes of antigen processing and presentation play a critical role in improving the cancer-killing effect of immune cells (87). Previous studies have pointed out that HDACi, which improve the level of histone acetylation, could enhance antigen presentation by cancer cells (26, 85, 88). Interestingly, HAcluster_B and the high HAscore group had the highest expression of HATs, which improves histone acetylation levels, and this could be the reason for the high antigen processing and presentation observed in these two groups. Future research will have to confirm this hypothesis. Although antigen processing and presentation are active in HAcluster_B and the high HAscore groups, the immune-suppressive cells, MDSCs, and regulatory T cells were higher in both of them. This indicates that the HAcluster_B and the high HAscore groups were immune-suppressive subtypes, and the pro-immunity effect brought by activated antigen processing and presentation was offset by the immune-suppressive cells. Further functional enrichment analysis confirmed that HAcluster_B was highly enriched in immunosuppressive gene signatures and less enriched in immune cytolytic gene signatures. In addition, the HAscore was positively correlated with immune suppression and negatively correlated with cytolytic activity. These analyses indicate that the immune-suppressive subtype may be a reason for the poor prognosis of patients in the HAcluster_B group or with a high HAscore.

Finally, considering the strong relationship between histone acetylation patterns, cancer-related malignant signaling pathways, and TME, we examined the potential therapeutic effects of the HAscore. We found that it was positively correlated with the sensitivity of drugs targeting histone acetylation, cell cycle, mitosis, DNA replication, BRD3, and ROCK2. In contrast, we found that the HAscore was negatively correlated with the sensitivity of drugs targeting MEK2, PARP, VEGFR, ABL signaling, and histone methylation. These results imply that patients with higher HAscores could benefit more from the positively-correlated drugs while the negatively correlated drugs would be more

suitable for patients with lower HAscores. In addition, we found that the HAscore could also predict the response of patients to anti-PD-L1 or anti-PD-1 immunotherapy. Compared to the patients with high HAscores, patients with lower HAscores were more sensitive to ICI immunotherapy. However, the benefits of ICI treatment are still limited due to the primary, adaptive, and/or acquired resistance to cancer immunotherapy (14). Fortunately, researchers have found that certain molecular-targeted anti-tumor agents can prevent cancer's immunotherapy resistance and combining these anti-tumor agents with ICI immunotherapy could greatly improve patient prognosis rather than a single-drug regimen. For example, researchers have found that the combination of a selective HDAC3 inhibitor with anti-PD-L1 immunotherapy enhanced tumor regression in a syngenic murine lymphoma model (86). Additionally, a phase 2 clinical trial has shown that camrelizumab (a PD-1 monoclonal antibody) combined with apatinib (a VEGFR-2 tyrosine kinase inhibitor) shows promising efficacy and acceptable safety in patients with advanced HCC in both the first-line and second-line settings (89). This result is significantly better than ICI therapy using a single immune-checkpoint inhibitor (90, 91). Our findings provide evidence that the HAscore can be a predictor for the sensitivity of certain targeted drugs combined with ICI therapy. This indicates that there are potential new treatment options for choosing a suitable targeted agent to improve the outcome of immunotherapy in patients with HCC.

CONCLUSION

In this study, we comprehensively evaluated the histone acetylation patterns of 1599 HCC cancer samples based on 36 histone acetylation regulators and identified three distinct histone acetylation patterns. The integrated analysis indicates that the differences in the activation of cancer-related malignant pathways and TME could be the main reason for the distinct prognostic outcomes of the three histone acetylation patterns. Based on the transcriptional differences among histone acetylation phenotypes, we constructed an HAscore model to digitally depict them, and identified the therapeutic utility of the HAscore in targeted therapy and immunotherapy. In summary, our study shows that evaluating the histone acetylation patterns of individual tumors will enhance our understanding of the characteristics of the TME and help develop personalized, combined, and immune-targeted therapeutic strategies for HCC patients. However, there are limitations in this study. The prognostic value of HAscore model on five-year OS of HCC patients is unsatisfactory. In future, more efforts should be paid to improve this model.

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

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

Conception and design: MP and DY. Development of methodology: MP, DY, WL, and YX. Acquisition of data: WL and YX. Analysis and interpretation of data (e.g., statistical analysis, bioinformatic, computational analysis):YX and WL. Writing, review, and/or revision of the manuscript: WL, YX, QL, DY, and MP. Administrative, technical, or material support: MP and DY. Study supervision: YX, WL, MP, and DY. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2022.761046/full#supplementary-material

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REVIEW
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In-Vivo Induced CAR-T Cell for the Potential Breakthrough to Overcome the Barriers of Current CAR-T Cell Therapy

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Chimeric antigen receptor T cell (CAR-T cell) therapy has shown impressive success in the treatment of hematological malignancies, but the systemic toxicity and complex manufacturing process of current autologous CAR-T cell therapy hinder its broader applications. Universal CAR-T cells have been developed to simplify the production process through isolation and editing of allogeneic T cells from healthy persons, but the allogeneic CAR-T cells have recently encountered safety concerns, and clinical trials have been halted by the FDA. Thus, there is an urgent need to seek new ways to overcome the barriers of current CAR-T cell therapy. *In-vivo* CAR-T cells induced by nanocarriers loaded with CAR-genes and gene-editing tools have shown efficiency for regressing leukemia and reducing systemic toxicity in a mouse model. The *in-situ* programming of autologous T-cells avoids the safety concerns of allogeneic T cells, and the manufacture of nanocarriers can be easily standardized. Therefore, the *in-vivo* induced CAR-T cells can potentially overcome the abovementioned limitations of current CAR-T cell therapy. Here, we provide a review on CAR structures, gene-editing tools, and gene delivery techniques applied in immunotherapy to help design and develop new *in-vivo* induced CAR-T cells.

Keywords: CAR-T cells, barriers, *in-situ* editing, gene-editing tool, nano-delivery

INTRODUCTION

Chimeric antigen receptor T cell (CAR-T cell) therapy is a new cell immunotherapy technique that incorporates synthetic receptors into T cells that recognize and kill tumor cells with a cognate targeting ligand (1, 2). CAR-T cell therapy has demonstrated unprecedented response rates in patients with B cell lymphoma since the first approval of CD19-targeted CAR-T cells in the USA (1, 3–5). However, along with the remarkable achievements of CAR-T cell therapy, many systemic toxicities, such as cytokine release syndrome (CRS) and neurotoxicity, have also been frequently reported (2, 6–8). Additionally, the complex manufacturing process of CAR-T cells limits the broader applications of this therapeutic method as a standard clinical treatment (2, 9–11). Therefore, there is an exigent need to develop a new paradigm of CAR-T cells to overcome these barriers and allow this therapeutic method to benefit more patients. To simplify the complex

manufacturing process of CAR-T cells, universal allogeneic CAR-T cells from healthy persons have been tested in clinical trials (12–15). Universal CAR-T cells can be off-the-shelf and then infused into patients like usual medicines, without needing to wait for the isolation of autologous T cells from patients (12, 16); however, last year's death case during the clinical trial of UCARTCS1A from Cellectis raised safety concerns about allogeneic CAR-T cells. The FDA also recently halted all clinical trials on universal CAR-T cells from Allogene due to safety concerns (17). Thus, we need new strategies to overcome the associated toxicity and simplify the manufacturing process of current CAR-T cell therapy. *In-vivo* CAR-T cells induced by nanocarriers loaded with CAR genes and gene-editing tools have shown promising effects for regressing leukemia (18–20). The *in-situ* programming of autologous CAR-T cells can enhance the targeted killing of tumor cells and reduce systemic toxicity such as CRS and neurotoxicity. Additionally, the nanocarriers can be easily manufactured in a standardized method (21) *In-vivo* induced CAR-T cells provide a potential solution to overcome the barriers of current CAR-T cell therapy. Thus, here, we review CAR structure design, gene-editing tools, and gene delivery systems and the future trend of immune cell therapy.

CAR STRUCTURE AND EVOLUTION

The structure of the chimeric antigen receptor (CAR) has a modular design consisting of an antigen-binding domain, a hinge, a transmembrane domain, and an intracellular signaling domain (**Figure 1A**). The antigen-binding domain is usually a single-chain variable fragment (scFv) molecule derived from a monoclonal antibody that can bind to antigens on the surface of malignant cancer cells (4, 22–24). The transmembrane domain is responsible for anchoring the CAR onto the T cell membrane. The intracellular signaling domain generally contains a T cell activation domain derived from the CD3ζ chain of the T cell receptor as well as co-stimulatory domains often comprised of an immunoreceptor tyrosine-based activation motif containing regions of CD28 or 4-1BB (also known as CD137 and TNFRSF9) (25–29). Variations in each component of the CAR structure enable fine-tuning of the functionality and antitumor activity of the resultant CAR-T cell product. Various CAR structures have been designed to improve the safety and efficacy of CAR-T cell therapy. Once the designed CAR genes are integrated into T cells, the scFv on the surface of T cells specifically recognizes tumor-associated antigens and binds CAR-T cells with tumor cells. After that, the intracellular signal domains of CAR-T cells are activated and cause CAR-T cells to proliferate and secrete cytokines that kill tumor cells (30–32).

There have been five generations of CAR structures since the first clinical application of CAR-T cells by Carl June at the University of Pennsylvania and hematologist David Porter at the Children's Hospital in Philadelphia in 2011 (33–35). The first-generation CAR contained an intracellular stimulation region and an extracellular scFv. This generation of CAR-T cells could not continuously proliferate due to the lack of costimulatory molecules (**Figure 1B**) (34). The second-generation CAR added a costimulatory molecule, such as CD28, or 4-1BB (CD137) to enhance the proliferation and reduce the toxicity of CAR-T cells (36). Yescarta™ (Tisagenlecleucel) and Kymriah™ (axicabtagene ciloleucel) are second-generation CAR-T cells that contain CD28 and 4-1BB, respectively (36). The third-generation CAR includes two costimulatory molecules, such as CD27, CD28, tumor necrosis factor superfamily 4 (OX40, also known as CD134), CD137 (4-1BB), or CD244 (37, 38). The fourth-generation CAR is called TRUCKs (T cells redirected for antigen-unrestricted cytokine-initiated killing), which combines the direct antitumor capacities of CAR-T cells with the immune modulating function of the delivered cytokine (34, 39). TRUCKs have entered early-phase clinical trials using a panel of cytokines, including IL-7, IL-12, IL-15, IL-18, IL-23, and their combinations. The fifth generation integrates an additional membrane receptor that controls the activation of CAR-T cells in an antigen-dependent manner (38, 40).

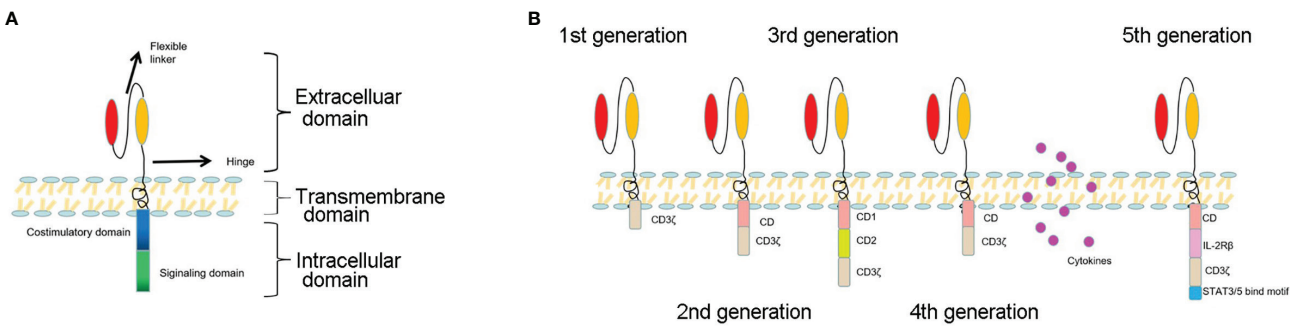

FIGURE 1 | **(A)** The basic structure of a CAR: extracellular domain, transmembrane domain, and intracellular domain. **(B)** The development of the five generations of CARs. The first generation only contained the CD3ζ chain functional energy domain9; the second generation contained CD3ζ+ a costimulatory molecular domain (CD28, 4-1BB, etc.); the third generation contained CD3ζ and two costimulatory molecular domains; the fourth generation included suicide gene editing, immune factor modification, and other integrated and refined regulatory tools; the fifth generation included simultaneous activation of TCR, costimulatory domain, and cytokine triple signaling.

In addition to adding new functional molecules into the CAR structure, many studies have chosen alternative tumor-targeted sites for new CAR structures. CD30 shows very strong expression on malignant cells in Hodgkin's lymphoma, rather than on healthy lymphocytes and hematopoietic stem/progenitor cells (HSPCs). CD30 CAR-T cell therapy has shown superior results in the treatment of $CD30^+$ malignant tumors, while healthy activated lymphocytes and HSPC were unaffected (41). CD20 is a 33–37-kDa non-glycosylated transmembrane phosphoprotein that helps develop and differentiate B cells (42). CD20 is highly expressed in late pre-B cells and mature B cells, but it is not expressed on the surface of HSPCs (43). CD20 CAR T-cell therapy which has shown promise in the treatment of B-cell non-Hodgkin lymphoma is now being considered for patients with relapsed or refractory CD20-positive chronic lymphocytic leukemia. Lym-1 targets the conformational epitopes of human leukocyte antigen D-associated antigens (HLA-DRs) on the surface of human B-cell lymphoma. The binding affinity of Lym-1 with malignant B cells is higher than that of normal B cells (44). Lym-1 CAR-T cells have exhibited potent antitumor effects against B-cell lymphoma. Some alternative targeting sites combine with CD19 to form dual-target CAR T cells. For example, CD37 combined with CD19 was incorporated into one CAR to generate a dual-specific CAR T cell capable of recognizing CD19 and CD37 alone or together (45). CD79b is also a complementary targeting site for CD19. CD19 and CD79 dual-specific CAR-T cells prevented the escape of B-cell lymphoma from a single CD19 CAR-T cell (46, 47). Some alternative targeting sites have co-targeting functions that act on tumor cells and tumor microenvironments. For instance, CD123 was expressed in both Hodgkin lymphoma cells and tumor-associated macrophages so that anti-CD123 CAR-T cells could co-target these two kinds of cells and kill them simultaneously (48). The CAR structure is continually evolving to improve the efficacy of current CAR-T cell therapy (32, 49).

BARRIERS TO CURRENT CAR-T CELL THERAPY

Five CAR-T cell products have been approved by the FDA from 2017 to 2021, as listed in **Table 1**. KYMRIAH™ (Tisagenlecleucel) is the first approved CAR-T cell therapy for adult patients with certain types of B-cell lymphoma (50). Three approved CAR-T cell products, YESCARTA™ (Axicabtagene ciloleucel), TECARTUS™ (brexucabtagene autoleucel), and BREYANZI® (lisocabtagene maraleucel), are also approved for the treatment of B cell lymphoma (51–53). The fifth CAR-T cell product, ABECMA® (idecabtagene vicleucel), is used for multiple myeloma therapy (54). Beyond the five approved CAR-T cell products, a large pipeline of CAR-T cells is being studied in clinical trials (55–57), but current CAR-T therapy has several barriers, such as associated toxicity, immunosuppressive tumor microenvironments, and complex manufacturing processes, which hamper the more widespread implementation of CAR-T therapy (58–60).

The major toxicities associated with current CAR-T therapy include cytokine release syndrome (CRS), immune effector cell-associated neurotoxicity syndrome (ICANS), and on-target/off-tumor toxicity (61–63). CRS is caused by the generation of massive inflammatory cytokines, such as IL-6, IL-10, IL-2, and TNFα, after CAR-T cell treatment. CRS often causes fever, hypotension, hypoxia, organ dysfunction, and even life-threatening adverse reactions (8, 64, 65). The occurrence of severe or life-threatening CRS can reach 25%. ICANS is another common toxicity associated with CAR-T cell therapy and is characterized by neurological abnormalities with aftereffects, usually within 1 week of CAT-cell treatment. The frequent adverse effects caused by ICANS include toxic encephalopathy with aphasia, confusion, and word-finding difficulty (66–68). On-target/off-tumor toxicity is due to the non-special expression of targeting proteins on both normal and malignant cells (69, 70). For instance, when administrating CD19 CAR-T cell in patients with malignant B cells, the on-target/off-tumor effect will lead to B cell aplasia and result in hypogammaglobulinemia due to the eradication of $CD19^+$ B cell progenitors by CD19 CAR T cells (71, 72).

The immunosuppressive tumor microenvironment (MVT) inhibits the activation of CAR-T cells and accelerates the exhaustion of T cells (70, 73). Unfavorable factors in immunosuppressive MVT include hypoxia, various immunosuppressive cells, and the sustained expression of co-inhibitory receptors (74, 75). Hypoxia is defined as a shortage of oxygen in the tumor MVT. Immunosuppressive cells in the tumor MVT contain regulatory T cells (Tregs), tumor-

TABLE 1 | An overview of currently approved CAR-T products.

Category	Approval	Target	Indication
Tisagenlecleucel, tisa-cel	Aug. 2017	CD19	B-cell acute lymphoblastic leukemia (ALL) that is refractory or has relapsed after receiving at least second-line regimens; relapsed or refractory large B^-cell lymphoma (second indication approved in 2018)
Axicabtagene	Oct. 2017	CD19	Treatment in adult patients with relapsed or refractory large B-cell lymphoma (LBCL) Adult patients with relapsed/refractory mantle cell lymphoma (MCL) and B-cell acute lymphoblastic leukemia (ALL)
Ciloleucel, Axi-Cel Brexucabtagene autoleucel, KTE-X19	Jul. 2020	CD19	
Lisocabtagenemaraleticel, L iso-cel	Feb. 2021	CD19	Relapsed/refractory diffuse large B-cell lymphoma (DLBCL)
Idecabtagene Vicleucel, ide-cel	Mar. 2021	BCMA	Patients with relapsed/refractory multiple myeloma who have received four or more previous therapies, including immunomodulators, proteasome inhibitors, and anti-CD38 monoclonal antibodies

associated macrophages (TAMs), and myeloid-derived suppressor cells (MDSCs) (74, 76).

The current manufacturing process of CAR-T cells is a highly complex endeavor, including T cell collection, genetic modification and expansion, and infusion back into patients (77, 78). These multistep technologies and logistics are rife with risks (10). Additionally, the long-term and individualized manufacturing processes pose great challenges for building up standard operating procedures (79). The costly and technology-intensive manufacturing processes of current CAR-T cells make them out of reach for many cancer patients in need of this novel therapy.

GENE-EDITING TOOLS IN CAR-T CELL THERAPY

The gene-editing tools frequently applied to CAR-T cell therapy include zinc-finger nucleases (ZFN), transcription activator-like effector nucleases (TALEN), and clustered regularly interspaced short palindromic repeats-associated 9 (CRISPR-Cas9) technology (80–82). ZFN is the first broadly applied gene-editing tool that includes zinc fingers, a large multimeric protein, wherein each individual finger targets three to four base-pair sequences within genomic DNA (83, 84). Multimeric zinc finger proteins are able to link with the FokI endonuclease to create a ZFN that can cleave site-specific double-stranded DNA and lead to homologous recombination (HR) or non-homologous end-joining (NHEJ) (85). ZFN can achieve effective and specific gene-editing, but it is time-consuming to optimize the targeting protein molecules. TALEN are composed of several TAL units that can recognize base pairs of DNA and link to an endonuclease to generate the site-specific cleavage of DNAs (86, 87). TALEN are more economical than ZFN but still require a long time to optimize the system. CRISPR-Cas9 technology is the most popular gene-editing tool due to its simplicity and efficiency. The CRISPR-Cas9 complex was initially identified as an immune system for cleaving foreign viral DNA in *Streptococcus* pyogenes (88). These CRISPR complexes are first transcribed into RNAs (crRNAs), including bacterial CRISPR sequences, viral sequences (protospacers), and intervening sequences (PAMs) (89). These crRNAs are then complexed with the Cas endonuclease. Once the Cas–crRNA complex recognizes a homologous protospacer and PAM sequence, the Cas endonuclease cleaves the double-stranded DNA, followed by an automatic DNA repair process (88). A short-guide RNA (sgRNA) was introduced into the CRISPR-Cas9 system as the crRNA, making CRISPR-Cas9 an efficient, specific, and simple gene-editing tool (90). The development of gene-editing technology has allowed the precise surgical gene-editing of CAR-T cells to generate exhaustion-resistant T cells *via* removing the PD1 of T cells (91). CRISPR-Cas9 was also used to deplete endogenous antigens, such as CD33 and CD7, in normal cells to reduce the on-target off/tumor toxicity of redirected T cells (92, 93). The CRISPR-Cas9 system has been used in many CAR-T clinical trials involving more than twenty-one target antigens (**Figure 2**) (94–97). CD19 and BCMA account for nearly one-half of the CAR-T clinical trials on these target antigens. To use the CRISPR-Cas9 system more widely to edit CAR-T cells, efficient delivery methods must be developed.

GENE DELIVERY SYSTEMS

Plenty of delivery systems have been used to deliver gene therapy products including the gene-editing tools and CAR genes (**Figure 3**). Viral vectors have the highest transfection efficiency and have been widely used to deliver genes in various applications (96), but they suffer from the immunogenicity and cellular toxicity. Adenovirus-associated viruses (AVV) have a lower risk of toxicity than other viral vectors such as lentivirus, and adenovirus due to insertional mutagenesis (98). However, the AAV vector has a smaller packaging size (~5.0 kb) than other viral vectors (99). Non-viral delivery systems for gene delivery can be classified into either physical or chemical techniques. Physical techniques include electroporation, needle injection, laser irradiation, and gene guns. Electroporation is one of the most widespread application methods, which induces pore formation on cell membranes and the transient permeability of genes using electric pulses (100–102). Physical techniques have attractive effects on gene delivery due to their low immunogenicity, but they cannot target internal organs. Chemical techniques that mainly use nano-delivery systems include cationic lipids or polymer-based nanoparticles, golden nanoparticles, silica nanoparticles and quantum dots, carbon nanotubes, exosomes, ferritin, and cell membranes. Lipid-based nanoparticles are one of the most attractive non-viral vectors for gene delivery as several formulations of these carriers have been approved to use in the clinic (103–105). Especially, lipid-based nanoparticles have recently been successfully used to deliver SARS-CoV-2 mRNA vaccines (106). Lipid nanoparticles have also been used to deliver the CRISPR/Cas9 system to achieve *in-vivo* genome editing at clinically relevant levels (107, 108). Polymer-based nanoparticles are another system suitable for gene delivery applications. Positively charged polymers can form stable polyplexes with genes that disrupt cell membranes and enable endosomal escape (109, 110). The limitation of polymer-based nanoparticles is their toxicity and immunogenicity caused by the interaction of their positively charged surfaces with negatively charged cell membranes and proteins in blood circulation (111, 112). Exosomes are naturally secreted extracellular vesicles with nanometer sizes that are being extensively investigated as gene delivery vectors due to their natural biocompatibility and minimal immune clearance (113, 114); however, more efforts are required to overcome the difficulties in production, isolation, and purification (115). Cell membranes derived from platelets and red blood cells are biomimetic vectors used for gene delivery that have natural biocompatibility and targeting, but their transfection efficiencies need to be improved (116–118). Each of the other chemical nano-vectors has unique characteristics that determine their effects on gene delivery. Some have shown potential efficiency

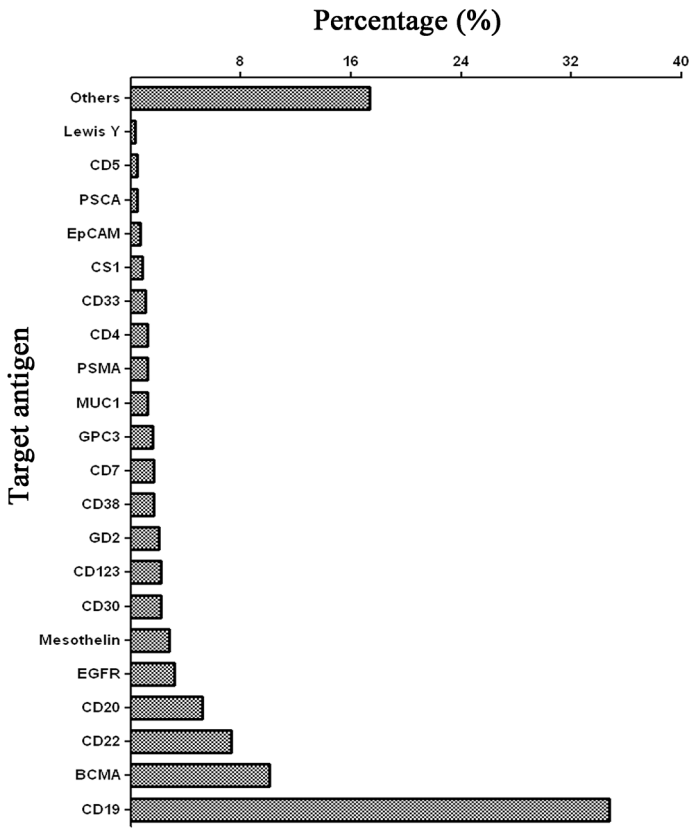

FIGURE 2 | Target antigens of CAR-T cell therapy using CRISPR-Cas9 gene-editing technology registered in ClinicalTrials.gov until June 2021. The data only include clinical trials that are registered in USA.

for the treatment of many diseases, but optimal delivery systems are still unrealized for clinical use.

IN-VIVO CAR-T CELL INDUCTION

The current manufacturing process of CAR-T cells requires dedicated equipment and significant technical expertise and is also labor-intensive and time-consuming. (10, 119, 120). It limits the broader worldwide applications of this technology and drives up the price of CAR-therapy, making it out of reach of many patients (121). To simplify the production process, universal CAR-T cells from allogeneic healthy persons were tested in clinical trials, but, the FDA recently halted all clinical trials on the universal CAR-T cells from Allogene due to safety concerns of allogeneic CAR-T cells. There is an urgent need to develop a safe and simple production process for CAR-T cells. *In-vivo* programming of CAR-T cells by nanoparticles is an elegant and novel approach to simplify and standardize the complex manufacturing process of *ex-vivo* CAR-T cells (122). Additionally, the *in-situ* induction of CAR-T cells effectively reduces the systemic toxicity of CRS and ICANS. Recently, *in-vivo* induced CAR-T cells were accomplished through the nano-delivery of CAR structures or gene-editing tools by the team of Matthias Stephan from the Fred Hutchinson Cancer Research Center (Seattle, USA) (18, 20). They accomplished the stable and transient expression of targeting CAR protein in T cells *via* the infusion of nanoparticles loaded with CAR-DNA and CAR-mRNA, respectively. In these two works, the core of the nano-delivery systems was composed of a cationic polymer, poly(β-amino ester), assembled with a second-generation CAR structure targeted to CD19. The exterior of the nano-delivery system was composed of polyglutamic acid (PGA) conjugated with an anti-CD3 antibody. The polymer nanoparticles carrying CD19-specific CAR genes quickly and specifically edited T-cells *in vivo* and brought about comparable antitumor efficacies to conventional laboratory-manufactured CAR T-cells without inducing systemic toxicity. In addition to the polymer nanoparticles, viral vectors such as lentiviruses and AAV have also been tested for the *in-vivo* generation of CAR-T cells. Christian J. Buchholz and his colleagues first reported that lentiviruses encapsulated with a second-generation anti-CD19 CAR gene induced *in-situ* CAR T cells in immunodeficient NOD-*scid*-IL2Rc^{null} (NSG) mice and showed antitumor activity (123, 124). They also exhibited cytokine release syndrome that is notorious in clinical practice. In their study, CAR-positive NK and NKT cells were unexpectedly detected, which were likely caused by the non-specificity of the lentiviral

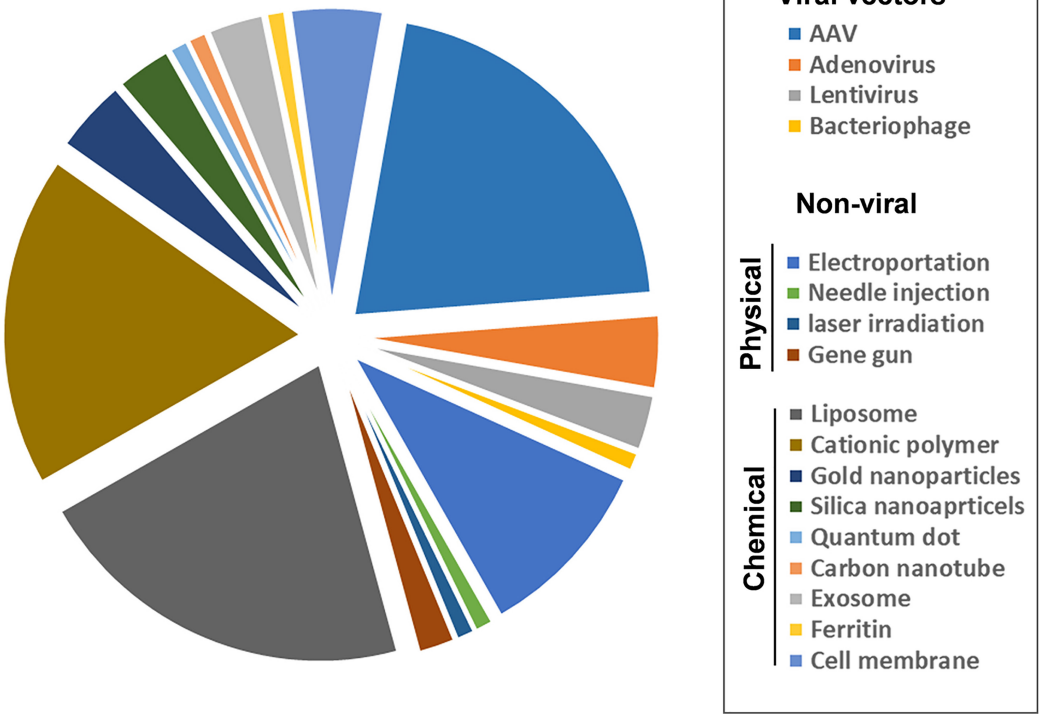

FIGURE 3 | Representation of viral and non-viral nano-delivery systems classified as viral vectors, non-viral (physical and chemical). AAV, adeno-associated virus. The total number of papers is 18,968 obtained from PubMed, and Microsoft Excel was used to obtain the pie graph. The keywords are the name of vectors and gene delivery or CAR gene.

vector. To overcome the non-specificity of the viral vector, Samuel K Lai et al. developed a bispecific binder to redirect the lentiviral vector to T cells for the *in-vivo* specific engineering of CAR-T cells (125). They observed the antitumor activity from the *in-vivo* CAR-T cells engineered by lentivirus, but a relatively low number of CAR-expressing T cells. They considered this to be proof of a valuable and unverified theory of the superior performance and self-renewal capacity of *in-vivo* CAR-T cells compared with that of *ex-vivo* CAR-T cells. However, the toxicity of the *in-vivo* CAR-T cells engineered by the bispecific binder-redirected lentivirus was not included in this work. Among the viral vectors, AVV has a lower risk of toxicity. Xilin Wu et al. recently reported that AAV encoding a third-generation CAR gene could sufficiently reprogram immune effector cells to generate *in-vivo* CAR T cells (126). In this work, they showed a strong proof of concept of AAV-induced *in-vivo* CAR-T cells, but the authors were concerned about the non-specificity of the AAV carrying the CAR gene. Except for the non-specificity of the viral vector, a universal safety concern of viral vectors is the random insertion of genes in the chromosol. Precise and rapid gene editor tools such as CRISPR have been widely used to generate *ex-vivo* CAR-T cells. There are many studies on *in-vivo* gene-editing using CRISPR, but there are still no reports on the application of CRISPR to generate *in-vivo* CAR-T cells. The future applications of combing gene-editing tools and CAR genes will accelerate the clinical adoption of *in-vivo* CAR-T cells.

The nano-delivery of designed CAR-structures and gene-editing tools can induce the *in-vivo* formation of CAR-T cells with multiple functions to overcome the barriers of current CAR-T cells, such as associated CRS and ICANS toxicities, immunosuppressive microenvironment, and complex manufacturing processes (**Figure 4**). Systemic toxicities can be reduced through tumor *in-situ* editing and the expansion of T cells (18, 62). The incorporation of special cytokine genes into a CAR structure enables CAR-T cells to secrete cytokines, flushing the immunosuppressive microenvironment and making it suitable for the survival and proliferation of T cells (127–129). Loading gene-editing tools with CAR structures into nanoparticles can knock out the genes of immune checkpoint blockades to reverse T-cell exhaustion (130–132). More importantly, this approach resolves the difficulty of process standardization and scale-up of the manufacture of *ex-vivo* CAR-T cells (133). The final gene-editor nanoparticles can be conveniently produced, stored, and delivered as usual medicines (**Figure 5**). These studies are just the beginning of the period of *in-vivo* induced CAR-T cells. Their clinical applications still require more efforts to monitor the *in-vivo* editing and expansion status of T-cells.

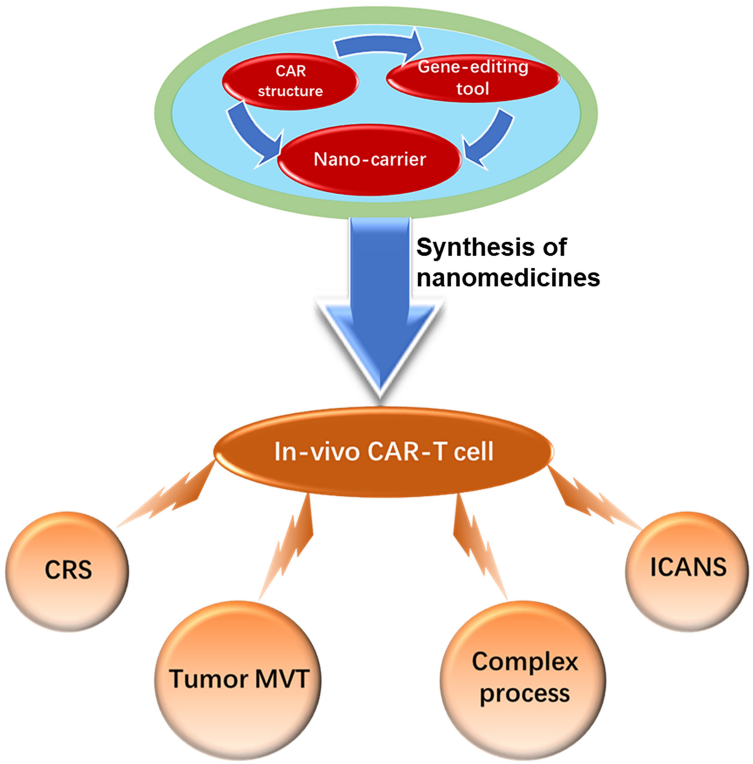

FIGURE 4 | Overcoming the barriers of cytokine release syndrome (CRS), immune effector cell-associated neurotoxicity syndrome (ICANS), tumor microenvironment (MVT), and complex process through *in-vivo* CAR-T cell induced by nanomedicines composed of nano-carrier loaded with the CAR structure or a gene-editing tool.

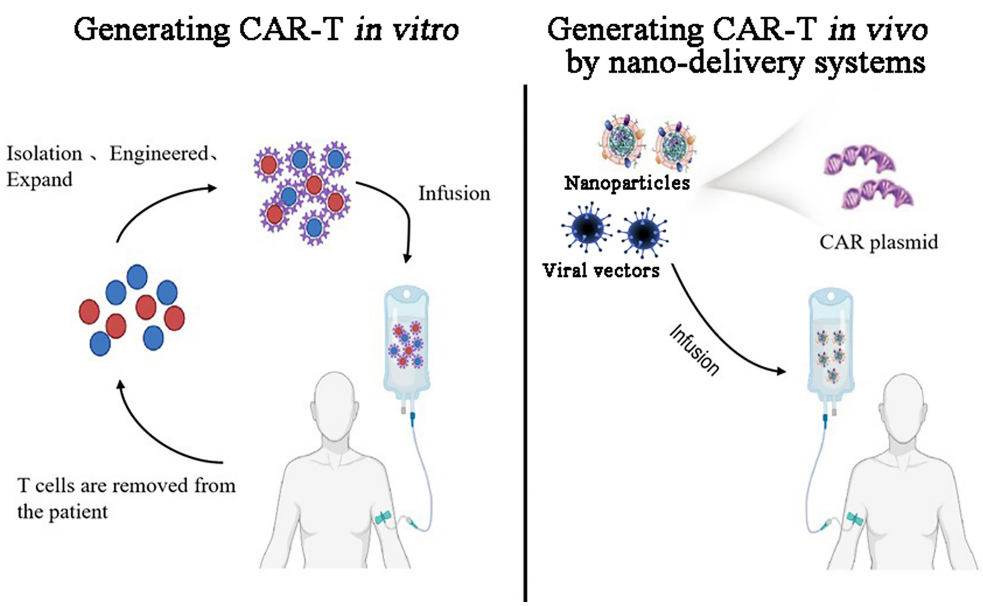

FIGURE 5 | Comparison of generating CAR-T *in-vitro* and generating CAR-T-*in-vivo* by nano-delivery systems: *In-vitro* CAR T cells are first isolated from the patient, proliferated *in-vitro*, and then genetically engineered to screen the successfully edited CAR T cells, which are amplified to a certain number of infusions into the patient. *In-vivo* induced CAR T cells use nanotechnology to encapsulate CAR-expressing plasmids into nano-delivery systems including polymer nanoparticles and viral vectors such as lentivirus and AAV, which are then targeted to tumor regions *in-vivo* to edit T cells *in-situ* at tumor sites to kill tumors.

CONCLUSION AND FUTURE PROSPECTS

Enormous achievements have been made in CAR-T cell therapy in the last decade, and five CAR-T cell products are available in the clinic. However, current CAR-T cell therapy also has some barriers that need to be overcome such as CRS and ICANS toxicity and expensive and complex manufacturing procedures. The *in-vivo* induced CAR-T cells by nanoparticles loaded with CAR genes and gene-editing tools have shown potential breakthroughs to overcome the abovementioned barriers of current CAR-T cell therapy. Although very few studies have reported nanoparticle-induced *in-vivo* CAR-T cells, robust preclinical data have predicted the future of cellular therapy through nano-delivery approaches. The field of *in-vivo* induced CAR-T cell therapy is still in its infancy with many challenges for the translation of this approach into clinical practice. A systematic summary of the nano-delivery systems for inducing *in-vivo* CAR-T cells can guide the design of the nanoparticles and their cargo to optimize their efficacy (134–136). In summary, *in-vivo* induced CAR-T cells are expected to replace current CAR-T cell therapy and become the standard immune-cell therapy for cancers.

AUTHOR CONTRIBUTIONS

TX, LC, CZ, YZ, ZH, and XW performed the discussion. TX and ZH drew the figures. TX, ZH, and XW conceived and wrote the manuscript. All authors contributed to the article and approved the submitted version.

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MINI REVIEW
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Advances in Nanotechnology Development to Overcome Current Roadblocks in CAR-T Therapy for Solid Tumors

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Chimeric antigen receptor T cell (CAR-T) therapy for the treatment of hematologic tumors has achieved remarkable success, with five CAR-T therapies approved by the United States Food and Drug Administration. However, the efficacy of CAR-T therapy against solid tumors is not satisfactory. There are three existing hurdles in CAR-T cells for solid tumors. First, the lack of a universal CAR to recognize antigens at the site of solid tumors and the compact tumor structure make it difficult for CAR-T cells to locate in solid tumors. Second, soluble inhibitors and suppressive immune cells in the tumor microenvironment can inhibit or even inactivate T cells. Third, low survival and proliferation rates of CAR-T cells *in vivo* significantly influence the therapeutic effect. As an emerging method, nanotechnology has a great potential to enhance cell proliferation, activate T cells, and restarting the immune response. In this review, we discuss how nanotechnology can modify CAR-T cells through variable methods to improve the therapeutic effect of solid tumors.

Keywords: nanotechnology, CAR-T, solid tumor, immunity, therapeutic effect

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INTRODUCTION

CAR-T therapy has made remarkable achievements in the research and clinical treatment of cancer, especially in the treatment of B cell malignancies (1–3). Unlike conventional surgery, radiotherapy, chemotherapy, immune checkpoint blocking therapies, targeted drug therapy, and CAR-T cell therapies offer more therapeutic options for patients with previously refractory tumors (4–8). To date, the United States Food and Drug Administration has approved five CAR-T therapies, namely, -Kymriah, Yescarta, Tecartus, Breyanzi and Abecma, -for hematologic malignancies (9). However, CAR-T cell therapy has not achieved satisfactory results in the treatment of solid tumors, such as colon, kidney, and ovarian cancers, for which the best clinical trial outcome is stable disease (10–14).

To improve the efficacy of CAR-T therapy in solid tumors, CAR-T cells must overcome three obstacles. First, the lack of tumor-specific antigens, dense stroma and aberrant vasculature at the tumor site prevent CAR-T cells from efficiently targeting the solid tumor site (15). Second, the tumor immune microenvironment and immunosuppressive mechanisms reduce the antitumor

activity of CAR-T cells in solid tumors. Finally, because of the initial differentiation state of selected T cells, the cumbersome production process of CAR-T cells, and the tumor microenvironment (TME) with low oxygen, acidity and nutrition, the survival and proliferation rates of CAR- T cells *in vivo* were low.

Nanotechnology has multiple features that allow it to address the challenges of CAR T cell therapy in treating solid tumors. With optimal size, high surface area to volume ratio, a variety of shapes and components, as well as surface modification and charge, nanoparticles have a wide range of applications in tumor therapy (16–20). Nanoparticles employed in clinical treatments can be targeted to the site of the lesion with less accumulation in healthy tissue, stronger drug permeability, and retention, and can be rapidly biodegraded and eliminated without pharmacological and toxicological activities (21–23). Therefore, a number of researchers are exploring the use of nanoparticles in combination with CAR-T therapy to improve the efficacy of CAR-T therapy in solid tumors. Herein, we briefly introduce the three major challenges of CAR T cells in solid tumor therapy, and summarize how to combine nanoparticles with CAR T cells from different perspectives to solve the challenges in solid tumor therapy (**Figure 1**).

CURRENT ROADBLOCKS IN CAR-T CELL FOR SOLID TUMORS

Numerous clinical trials of CAR-T cell therapy for solid tumors have been carried out, and a meta-analysis of the efficacy of CAR-T therapy in solid tumors showed an overall response rate

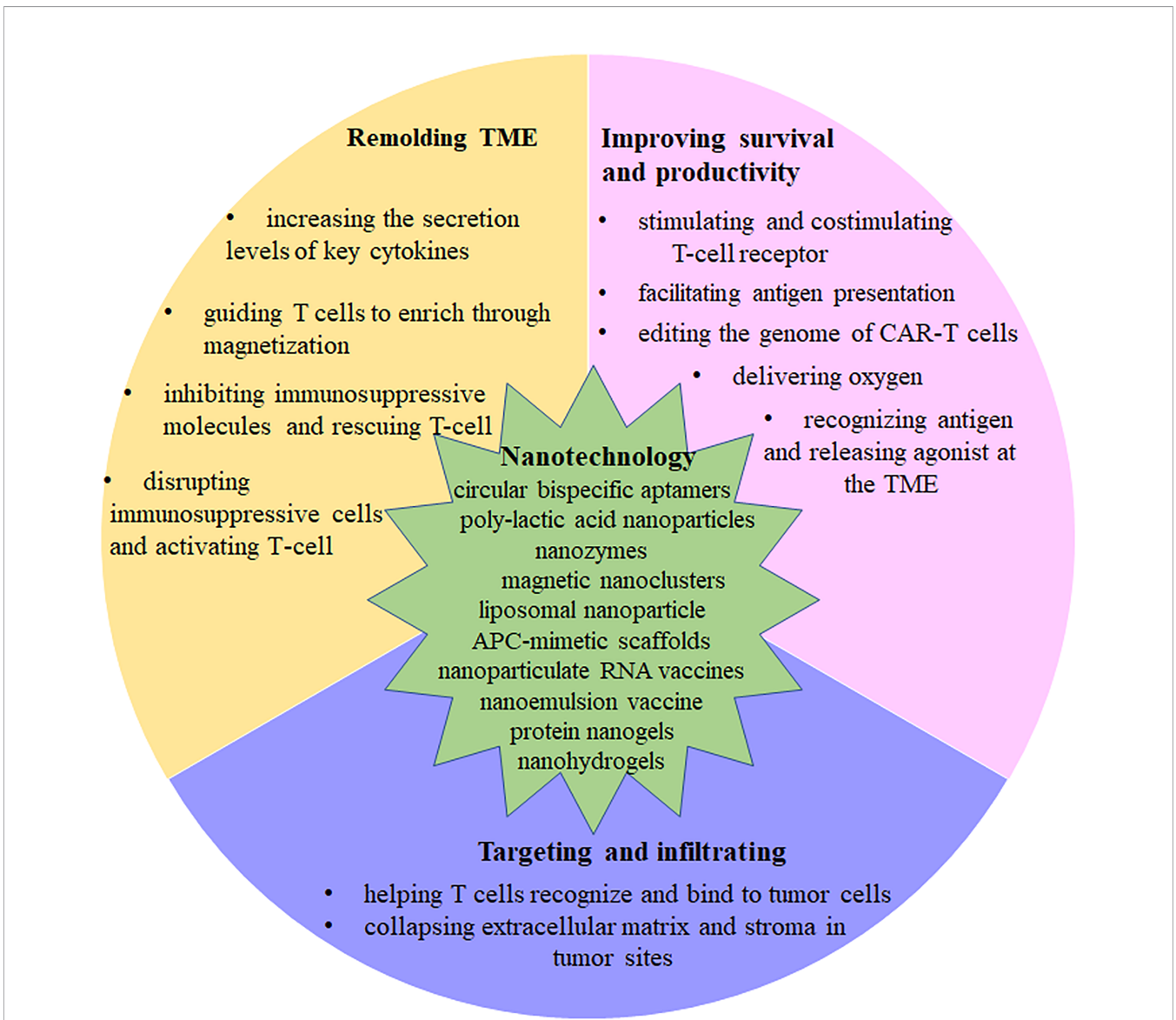

FIGURE 1 | The mechanisms of Nanotechnology affect CAR-T function. Summary of strategies that are discussed in detail in this review.

of 9%, although various therapeutic strategies have been implemented (24). There are three major factors that influence CAR-T therapy, as described below.

Targeting and Infiltrating

CAR T cells are designed to select tumor-associated antigens (TAA) due to the lack of tumor-specific antigens (TSA). In a large number of clinical trials CAR T cell targeting tumor-associated antigens have been found cause damage to normal tissue with low expression of tumor-associated antigens during the process of recognizing and killing tumor cells, which is referred to as the off-target effect (25). Moreover, the reasons behind the success of CAR T cells in the treatment of hematologic tumors is that they can migrate in blood, lymph nodes, and bone marrow to interact with cancer cells (26). By dynamic imaging microscopy on fresh tumor slices from nine patients, Donnadieu et al. (27) investigated T cells with reduced motility in the stroma of human lung tumors, which hinted towards T cells facing difficulties in entering into the tumor due to the presence of obstacles. This makes it easy to understand that there are several other reasons why CAR T cells have difficulty entering solid tumor. Tumor-associated fibroblasts (TAFS) and abnormal vasculature at the tumor site result in compact tumor tissue and a dense extracellular matrix (ECM), which prevent CAR T cell to enter the solid tumor microenvironment (28, 29). The experiments conducted by Peschel et al. (30) confirm the lack of adoptively transferred T cells accumulation in solid tumors, while the infused HER2-specific T cells spread out in the breast cancer patient's bone marrow. In addition, chemokines can induce T cell migration along the direction of increasing chemokine concentration. However, some solid tumors inhibit chemokine secretion and CAR T cells lack receptors that match chemokines secreted by solid tumors (31, 32), such that chemokine receptors on T cells mismatch with tumor secreted chemokines (33–35). Moreover, the low expression of adhesion molecules including ICAM-1 and 2, VCAM-1 and CD34 in tumor endothelial cells (EC) inhibit the effector T-cell from adhering to the EC and being transported to the tumor (36).

Tumor Immunosuppression

Immunosuppression of the solid tumor microenvironment is another significant challenge for CAR-T therapy. The causes of tumor cells escaping the anti-tumor immune response are complex, including the presence of immunosuppressive cells, the presence of immunosuppressive cytokines and the absence of immune activating factors. The presence of immunosuppressive cells such as dendritic cells (DCs), myeloid-derived suppressor cells (MDSCs), regulatory cells (Tregs), and M2 macrophages in solid tumors sites, which secrete suppressive cytokines-such as transforming the growth factor-β (TGF-β), adenosine, interleukin-10 (IL-10), and vascular endothelial growth factor (VEGF) extracellularly-, suppresses the immune system and reduces the anti-tumor activity of CAR-T (37–40). Moreover, the immune checkpoint molecules PD-1 and CTLA4, when combined with the corresponding ligands, inhibit the killing effect of T cells on the tumor and the activation of T cells (41, 42).

Survival and Proliferation

CAR T cells are targeted to the tumor site by a chimeric receptor mediated expressed on the T cell surface, and eliminate cancer cells through cell killing (43). Studies have shown that the long-term survival and proliferation of CAR T cells capable of maintaining normal function *in vivo* played a decisive role in the therapeutic effect (44). However, the expansion of the CAR T cells during the treatment of solid tumors is low *in vivo*. For example, Michael et al. detected a large number of CAR T cells in ovarian cancer patients after 2 days of transfusing *in vitro* gene-edited T cells back into the body, but the increase only lasted for about 1 month, and quickly declined to be virtually undetectable in the majority of patients (13). Even with large doses of CAR T cells, the presence of CAR T cells in the circulatory system was not detected (45). Moreover, clinical data showed that longer CAR-T cell persistence indicates longer delays, in the development of disease progression (46). The factors that influence the survival of CAR T cells in patients are complex, including the differentiation and functional status of CAR T cells, CAR target affinity, CAR immunogenicity, tedious time-consuming production process, immunosuppressive and hypoxic tumor microenvironment (47–49). Various nanotechnology strategies may improve CAR T cell persistence and expansion *in vivo*, which would endow CAR-T therapy with superior antitumor activity in the treatment of solid tumors.

APPLICATION OF NANOTECHNOLOGY IN CAR-T THERAPY IN SOLID TUMORS

Nanotechnology to Aid CAR T Cell Target and Accumulate in Solid Tumors

To overcome the off-target effect caused by tumor-associated antigens, one group designed circular bispecific aptamers to help T cells recognize and bind to tumor cells. The aptamer can simultaneously bind naïve T cells and tumor cells, and then specifically activate T cells in the cell-cell junction complex. This strategy helps T cells pinpoint the tumor site and kill cancer cells. Thus, the targeted treatment of all kinds of cancer is possibly realized by the use of specific anticancer aptamers (50).

In an effort to arm CAR T cells to collapse physical barriers caused by angiogenesis, a dense extracellular matrix and stroma in tumor sites, researchers have proposed numerous of NP-based strategies (51, 52). By combing photothermal therapy with the adoptive transfer of CAR T cells, Gu et al. succeeded in promoting the accumulation and enhancing the conventional CAR-T therapy against solid tumors. The indocyanine green (ICG), a near-infrared (NIR) dye, is wrapped in poly(lactic-co-glycolic) acid (PLGA) nanoparticles. Once exposed to NIR light irradiation, ICG is used as the photothermal agent released into solid tumor (53–55). Mild hyperthermia of the tumor disrupts its compact structure, reduces interstitial fluid pressure (IFP), increases blood perfusion, and releases tumor-specific antigens that could significantly stimulate CAR T cells. After about 20 days, tumor growth was significantly inhibited, and no tumor cells were detected in about one-third of the treated mice (56).

Other researchers fabricated indocyanine green nanoparticles (INPs) conjugated CAR T cells *via* the biorthogonal reaction. After mild photothermal intervention, tumor vessels expanded, blood perfusion increased, the ECM ablated and the tumor tissues became loose. Thus, INPs engineered CAR-T biohybrids accumulated and infiltrated extensively in the tumor, remodeled the TME, restarted the immune response, and boosted the efficacy of CAR-T immunotherapy. This microenvironment photothermal-remodeling strategy provides a promising prospect for CAR-T therapy in solid tumors (57).

Nanotechnology to Remold Tumor Microenvironment to Stimulate CAR T Cells

To reset immunosuppression of cancer environment and promote the activation of CAR T cells, Zhao and colleagues effectively combined the use of the nanozymes method. They synthesized a tumor-targeting HA@Cu_{2-x}S-PEG (PHCN) nanozyme with photothermal and catalytic properties. After irradiation by a near-infrared laser, the tumor extracellular matrix is damaged by converting light energy into local heat (58–60). Moreover, the reactive oxygen species by nanocatalyzed tumor therapy increased the secretion levels of key cytokines, such as the interferon and tumor necrosis factor as well as tumor-specific antigens, thus activating the corresponding CAR T cells at the tumor site (61).

To surmount the obstacle of hostile microenvironment, researchers tend to combine CAR-T therapy with the use of cytokines and/or antibodies. However, one problem is that CAR T cells and cytokines/antibodies disperse preventing their accumulation in the tumor sites (62, 63). Therefore, Xie et al. used a pH-sensitive benzoic–imine bond and inverse electron demand Diels–Alder cycloaddition to link magnetic nanoclusters (NCs) and the PD-1 antibody (aP) together to form NC-Ap. The constructed NC-aP binds to effector T cells due to their PD-1expression. Magnetic resonance imaging (MRI) guided T cells and aP to enrich in solid tumors through magnetization. Because of the acidic tumor microenvironment, the aP is released after the benzoic–imine bond, and then hydrolyzed. Consequently, the adoptively transferred T cells and aP synergistically inhibit solid tumor growth with a few side effects (64).

One of immunosuppressive molecules that inhibits the immune function of $CD4^+$ and $CD8^+$ T cells is adenosine. On the surface of activated T cells, the A2a adenosine receptor (A2aR) expressed and trigged adenosine to accumulate outside the cell, which suppressed T-cell proliferation and inhibited IFN–γ secretion (65, 66). Thus, using nanotechnology to efficiently transport SCH-58261 (SCH), a small molecule inhibitor of A2aR, to CAR T cells in tumors is a promising method. According to their report, Wang et al. used CAR-T therapy and SCH–loaded cross-linked multilamellar liposomes (cMLV) together, which significantly inhibited the tumor growth and improved the survival of treatment groups, the tumor infiltration rate of T-cells, as well as the expression level of IFN–γ *in vivo*. Through rescuing tumor-residing T-cell hypofunction, this method augments CAR T-cell efficacy in solid tumors (67).

The presence of immunosuppressive molecules- such as CTLA-4 and PD-L1 is another important cause of tumor immunosuppression. They enable tumor cells to escape surveillance by inhibiting the activation of immune cells, namely the "immune escape" (68, 69). To reset the suppressive solid tumor microenvironment, inhibitors targeting checkpoint molecules (such as CTLA-4, PD-1 and PD-L1) and CAR-T therapy were used in combination (70, 71). The disadvantages of using immune-checkpoint inhibitors (ICIs) include the emergence of a series of new immune-related adverse events and systemic toxicities (72). Stephan et al. designed a liposomal drug-loaded nanoparticle and decorated it with the tumor-targeting peptide iRGD. In addition, PI-3065, a PI3K kinase inhibitor that disrupts the function of immune-suppressive regulatory T cell subsets and myeloid-derived suppressor cell (40), and 7DW8-5, an immunostimulant-invariant natural killer T cell (iNKT) agonist was placed in the liposome (73, 74). They demonstrated that this new target nanoparticle alters the tumor immunosuppression and evidently enhances the anti-tumor activity of CAR T cells (75).

Nanotechnology to Aid CAR T Cells Survive and Proliferate

The number of tumor-infiltrating lymphocytes is positively related with clinical outcomes of CAR-T therapies (36, 76, 77). T cells obtained from patients are limited, such that amplification *in vitro* may be an effective solution. In the body, the expansion of T cells requires the assistance of antigen-presenting cells (APC), which cannot be achieved *in vitro*. In light of this problem, Mooney et al. utilized mesoporous silica to create micro-rods and added in the APC-secreting factor interleukin-2, which extends the lifespan of T cells. They also coated the high-aspect ratio mesoporous silica micro-rods (MSRs) with supported lipid bilayers (SLBs) and a variety of antibodies that activate T cells, mimicking APC's cell membrane. In cell culture, these rods randomly and automatically form a scaffold structure that allows T cells to move around and expand freely. Results showed that APC-mimetic scaffolds generate more CAR T cells and maintain good killing efficacy compared to conventional expansion systems (78).

The lack of proliferation signals in TME results in a low survival rate of CAR T cells. As emerging therapies, nanoparticulate RNA vaccines deliver liposomal antigen-encoding RNA (RNA-LPX) to activate T cells in cancer patients (79). Recently, Sahin et al. combined CAR-T with the nanoparticulate RNA vaccine to achieve the regulated proliferation of CAR-T cell expansion depending on RNA-LPX dose. The mechanism involves that antigen delivery to antigen-presenting cells in the spleen, lymph nodes, and bone marrow by intravenous injection, followed by the initiation of a toll-like receptor-dependent type-I IFN-driven immune-stimulatory program (80). Moreover, Chan et al. used the tailored nanoemulsion (Clec9A-TNE) vaccine to effectively solve the problem of limited antigen presentation, promote the proliferation of CAR T cells *in vivo*, and augment the efficacy of solid tumor therapy (81).

Conventional manufacturing of CAR-T cells includes several elaborate procedures such as isolation, modification and expansion, resulting a few effective redirected T cells that can be used. Meanwhile, virus transfection and electroporation are commonly used to help T-cells express targeted chimeric antigen receptors (CARs) or T cell receptors. In turn, these methods have drawbacks as they are time-consuming, have a small application scale (82, 83). Stephan et al. designed a new genetic programming named "hit-and run", which transports mRNA nanocarriers into cells through simple mixing and transient expression of the target gene. The mRNA nanocarrier has three prominent advantages: (i) lyophilized mRNA NPs can be used for each application that has no effect on its properties and efficacy. (ii) NP uptake and transfection efficiency did not differ whether T cells proliferated or not. (iii) Lymphocyte-targeted mRNA nanocarriers can edit the genome of CAR-T-cells without influencing on their function. The paramount of this method is that it can simply produce CAR T cells at a clinical scale within a short time and without complex handling procedures *in vitro* (84).

Another novel method was developed to program numerous circulating T cells and effectively remove cancer cells *in situ*. On the surface of the biodegradable poly (β-aminoester)-based nanoparticles, anti-CD3e f(ab′)2 fragments are coupled with it to target T cells. Inside of the nanoparticles, the poly(beta-aminoester) (PBAE) polymer is assembled with microtubule-associated sequences (MTAS) and nuclear localization signals (NLS), which facilitates the gene transfer in the nucleus of the T cells. To maintain CAR expression in T cells, the CD19 CAR plasmid was flanked by the piggyBac transposase gene through a cut-and-paste mechanism. These stable polymer nanoparticles allow simple manufacture and storage, which provides a practical, economical and widely available pathway for CAR-T therapy (85).

The immunosuppression and hypoxia in the solid tumor microenvironment result in the weaking CAR T cells infiltration and proliferation. One research group constructed an injectable hydrogel-encapsulated porous immune-microchip system (i-G/MC) with oxygen reservoirs to intratumorally deliver CAR T cells. In the injectable i-G/MC system, IL-15-loaded alginate microspheres were made into thin immune-MCs (i-MCs), which were connected with HEMOXCell (Hemo; an oxygen carrier)-loaded alginate, and the alginate forms a gel layer by self-assembly (86). The i-MCs were highly porous and interconnected, which facilitates CAR T cell transport. Hemo, a marine extracellular hemoglobin, has a strong oxygen storage capacity and binds up to 156 oxygen molecules (per Hemo molecule). After the i-G/MC was injected into the solid tumor, the hydrogel (gel) layer degraded quickly, Hemo delivered oxygen to TME, as well as CAR T cells, and decreased the expression level of HIF-1α. Results showed that the immune-niche improves hypoxia TEM and promotes survival and infiltration of CAR T cells in solid tumors.

To avoid the side effects of systemically-administered supporting cytokines like interleukins, protein nanogels (NGs) with interleukin (IL)-15 super-agonist were designed. The NGs recognized the specific cell surface antigen and subsequently released the drug at the sites of antigen encounter, for instance, the tumor microenvironment. Most importantly, the NG delivery enhanced the cell proliferation level 16-fold in tumors and administered eight-fold higher doses of cytokine without toxicity (87).

CONCLUSION

In preclinical studies, researchers have proposed a number of strategies to improve CAR T cell function through the use of nanotechnology. However, there are still some fundamental issues to be addressed in the clinical application of CAR T therapy. For example, the carcinogenicity, reproductive toxicity and persistence of magnetic nanoclusters are still unknown and therefore it cannot be used in clinical therapy. The use of near infrared laser will cause damage to human skin, short-term use will appear skin swelling phenomenon, long-term may affect human reproductive function and induce cancer. The safety, immunogenicity and toxicity of nano-vaccines have yet to be verified. Will nano-derivative biodegrades induce non-specific immune responses? Due to the specificity of tumor-associated antigens, the preparation cycle of tailored nanoemulsion vaccine is time consuming and involves high cost….

These questions from clinical studies may seem disappointing, but many studies have highlighted the potential of nanotechnology in combination with CAR T therapies for solid cancers, which giving us great hope for CAR T cells. Currently, there are about 40 CAR-T targets in clinical trials in solid tumors, which has significantly outnumbered hematological tumors. Different from CD19, which is often used as a target for CAR-T therapy in hematologic tumors, the main targets of CART development in solid tumors include Mesothelin, GD2, HER2, GPC3, Claudin18.2(CLDN18.2) and so on. Most CAR-T studies in solid tumors have low response rates in the 0-25% range (88). Recently, the EMA granted prime eligibility to CAR T - cell product candidate CT041, which against the claudin18.2 protein (CLDN18.2) for the treatment of gastric/gastroesophageal junction cancer. Results from a phase I clinical trial published in 2019 show a total objective response rate of 33% in a small group of patients with advanced gastric or pancreatic cancers, with no serious side effects (89). This means that CT041 is expected to become the world's first approved solid tumor CAR T product, thus achieving zero breakthrough in solid tumor treatment.

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JM: Conceptualization; writing-original draft. QY: Writing-review and editing. YM: Conceptualization; writing-review and editing. All authors contributed to manuscript revision, read, and approved the submitted version.

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REVIEW
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Bacteria–Based Synergistic Therapy in the Backdrop of Synthetic Biology

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Although the synergistic effect of traditional therapies combined with tumor targeting or immunotherapy can significantly reduce mortality, cancer remains the leading cause of disease related death to date. Limited clinical response rate, drug resistance and off-target effects, to a large extent, impede the ceilings of clinical efficiency. To get out from the dilemmas mentioned, bacterial therapy with a history of more than 150 years regained great concern in recent years. The rise of biological engineering and chemical modification strategies are able to optimize tumor bacterial therapy in highest measure, and meanwhile avoid its inherent drawbacks toward clinical application such as bacteriotoxic effects, weak controllability, and low security. Here, we give an overview of recent studies with regard to bacteria-mediated therapies combined with chemotherapy, radiotherapy, and immunotherapy. And more than that, we review the bacterial detoxification and targeting strategies *via* biological reprogramming or chemical modification, their applications, and clinical transformation prospects.

Keywords: cancer treatment, immunotherapy, bacterial therapy, chemical modification, synthetic biology

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INTRODUCTION

Recent investigations have shown a decline in cancer mortality (lung cancer, melanoma, and so on) with the combined application of traditional and emerging therapies. Yet, it remains the primary cause of disease-related death worldwide. According to the Big Data techniques, more than 17 million cancer deaths worldwide are predicted by 2030 (1). Traditional antitumor curative treatments such as surgery and chemoradiotherapy inevitably have side effects such as the inability to eradicate cancer cells thoroughly, nonspecific cytotoxicity, and drug/radiotherapy resistance. More importantly, the highly anticipated innovative regimens such as tumor-targeted therapy and immunotherapy face challenges, namely, off-target effects, therapeutic resistance, and insufficient clinical response rate (2–4). These studies above highlight enormous challenges in cancer treatment and illustrate the significance of finding new anticancer therapies.

Encouragingly, a large number of studies have shown that some types of bacteria can selectively migrate to the tumor hypoxic area and stimulate an antitumor immune reaction, thus presented as a promising platform for cancer treatment (5). In 1868, Coley et al. attempted to use *Streptococcus pyrogenes* to infect tumor patients. It was surprising that some patients had witnessed tumor size reduction and some of them even disappeared completely, suggesting that bacterial therapy might be a valuable option (6, 7). However, the approach of Coley was questioned for a long time due to the fatal infections (6). After long-term exploration, the researchers found that specifically gene-

deleted bacterial strain possessed attenuated virulence and high safety. Additionally, they found that living attenuated bacteria possess the unmatched superiorities of active targeting and specific intratumoral colonization (8).

The aforementioned advantages were attributed to the nourishing, hypoxic, and immunosuppressive features of the tumor microenvironment (TME) (9, 10). First, obligate anaerobes (such as *Clostridium* and *Bifidobacterium*) and facultative anaerobes (such as *Salmonella*, *Pseudomonas*, and *Escherichia coli*) preferentially accumulate in the high-density nutrient areas of tumors through their own specific chemical receptors, flagellar movement, and signal transduction proteins (11). Second, an inherent immune escape mechanism exists in TME to avoid the monitoring and elimination of tumor cells. Similarly, obligate or facultative anaerobic bacteria can survive without being cleared by innate immune cells such as macrophages and neutrophils or adaptive immune response (12). In addition, the deformed and damaged vascular network of TME can also promote the intratumoral infiltration and intrusion of anaerobic bacteria (13). Interestingly, a recent study has verified the aforementioned elucidation. The authors detected 1,526 human tumors and their adjacent normal tissues, namely, lung, breast, pancreas, ovary, and brain. Bacteria were found to exist intracellularly in each tumor type, with unique populations in each kind of cancer. Moreover, the bacteria in the tumor are mainly intracellular and present in both cancer cells and immune cells, indicating that they might be important components of TME (14).

In addition to the above advantage of specifically intratumoral colonization, some genetically attenuated bacterial strains are able to secrete cytotoxic proteins and stimulate potent immune reaction to kill tumor cells effectively (15). However, the failed attempts of VNP20009 in phase 1 trial indicated that combination therapy is urgently needed to make the enhancement of tumor targeting and clinical response rate possible (16). For instance, bacteria such as *Listeria monocytogenes*, *Clostridium tetani*, and *Lactobacillus acidophilus* have been applied as immunostimulants and inhibitors of tumor growth when combined with chemoradiotherapy or immunotherapy (17). More importantly, the advancements in genetic engineering combined with chemical synthesis have made bacteria-mediated tumor-targeting therapy a prospective anticancer treatment strategy to reduce systemic toxicity and improve targeting efficiency.

In recent years, a mounting number of research publications on applications of bacteria–based synergistic therapy has been published. To summarize the recent progress of bacteria-based cancer therapy, we searched various keywords *via* search engine PubMed with different keywords in the past 12 years (2010–2022) and found 11,188 related papers. Among them, 7,451 studies were related to the keywords "bacteria and cancer", followed by 1,532 studies on "bacteria and clinical trials", 797 studies related to "bacteria and nanotechnology", 615 documents on "bacteria and genetic engineering", and 608 studies related to "bacteria and immunotherapy". By comparison, the research on the combination of bacteria with chemotherapy, radiotherapy or immunotherapy is still limited (**Figure 1**). In this review, firstly, we discussed the tumor targeting properties of bacteria and the potential mechanism in the introduction. Secondly, we reviewed the bacteria-based combination therapy with chemotherapy, radiotherapy, and immunotherapy respectively. Thirdly, we reviewed the bacteria-mediated chemical modification and biological engineering. Lastly, we summarized clinical applications of bacteria-based cancer vaccines and its challenges in the future.

BACTERIA-MEDIATED COMBINED CANCER TREATMENT

In general, the roles of bacteria in cancer initiation and progression are a double-edged sword. On the one hand, some pathogenic bacteria can induce chronic inflammation and promote tumor development (18). *Helicobacter pylori*, as one prime example, could lead to gastric tumorigenesis through persistent inflammatory stimulation, increased epithelial cell proliferation, and deregulated signaling transduction pathways crucial for

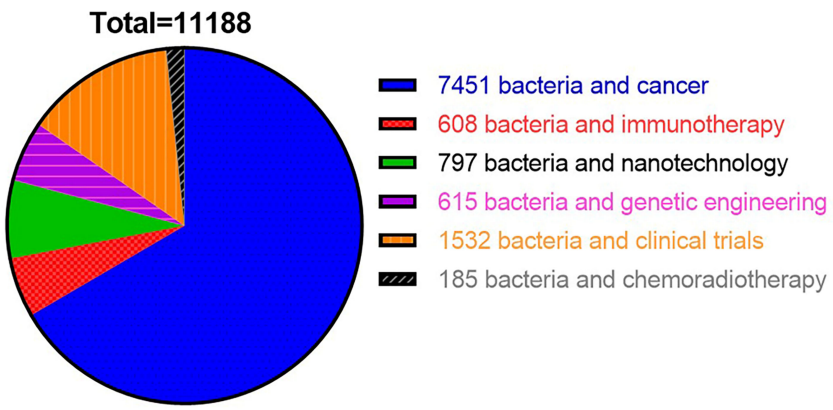

FIGURE 1 | Statistical chart showed overall number of studies published in PubMed from 2012 to 2022 using different keywords.

cancer maintenance (19, 20). On the other hand, some bacteria have shown great potential in treating various tumors. Bacteria can express and secrete a large number of metabolites with different biological activities that can be widely used in clinic, such as actinomycin D, doxorubicin, bleomycin, and mitomycin (21–24). Besides, a variety of enzymes, namely, L-asparaginase and arginine dehydrogenase, produced by bacteria have displayed definite anticancer efficacy (25, 26). The bacterial components and secretions are natural apoptosis inducers and immune agonists especially when employed in combination therapy (27) **(Table 1)**.

Combined Bacteria-Mediated Chemotherapy

Chemotherapy, as a classical systemic treatment, is relatively effective against some types of cancer such as malignant lymphoma, childhood acute leukemia, and chorionepithelioma. Despite all these, it also has its dark side: digestive tract reaction, arrest of bone marrow, immunosuppression, and insufficient tumor targeting, particularly in acidic and hypoxic areas (38, 39). The hypoxic area of the tumor center is usually chemotherapy resistant; from this respect, anaerobes targeting the anoxic area can cooperate with chemotherapy and make up for the shortcomings perfectly (40). For example, *Salmonella*-laden temperature-sensitive liposomes (thermobots) and high-intensity focused ultrasound along with tumor heating (~40–42°C) were used to observe macrophage-related immune alterations and whether they could work in coordination with enhanced colonic chemotherapy: TB1: passively incubated TBS (mean fluorescence intensity (MFI): 8.16 ± 0.014); TB2: TBS with biotin–streptavidin (MFI: 21 ± 0.14). The activity of doxorubicin (Dox)-loaded bacteria and untreated control bacteria was 70–75% compared with *Salmonella*. The results showed that the efficacy of TB1 and TB2 was relatively higher than that in the control group at body temperature, and TB2 showed a higher killing rate than TB1 when heated (~80% vs 60%). In addition, the expression of TNF-α, IL-1β, and IL-10 in each group was significantly higher than that in the untreated control group (316 ± 53 ng/ml vs 58.3 ± 1.15 ng/ml, 84.7 ± 3.93 ng/ml, and 110.48 ± 7.82 ng/ml, respectively). In another study, the acid-unstable conjugate of maleic anhydride (ECN-Ca-Dox) was used to couple Dox with *E. coli Nissle* 1917 (ECN) to accumulate bacteria and release antibiotics. The accumulation of DOX in the tumor was 12.9 and 6.4%, respectively, 3 h and 3 days after the intravenous injection of ECN-Ca-Dox, which was much higher than that in the control group. As expected, the percentage of cell proliferation in the ECN-Ca-Dox group was significantly lower than that in the ECN-sa-Dox group (15.1 ± 1.2% vs 40.6 ± 4.3%); the rate of apoptotic cells in the ECN-Ca-Dox group increased correspondingly (41). The study next verified whether TAPET-CD (an attenuated strain of *Salmonella typhimurium* expressing *E. coli* cytosine deaminase) could convert nontoxic 5-fluorocytosine (5-FC) into active anticancer drug 5-fluorouracil (5-FU). The inhibitory effect of TAPET-CD combined with 5-FC on colon tumors after subcutaneous transplantation was evaluated. High levels of 5-FU were detected in tumors of mice treated with combined therapy, but not in normal tissues. The combined treatment had a higher inhibition of tumor growth than TAPET-CD alone (88–96% vs 38–79%). After receiving a single injection of TAPET-CD, the tumor growth was remarkably inhibited (79% on the 40th day), and the TAPET-CD/5-FC group had a notable antitumor effect (88% on the 47th day) (42).

Besides enhancing tumor killing, some probiotics can also reduce the side effects caused by chemotherapy. Chemotherapy-related intestinal catarrh of colorectal cancer is often caused by 5-FU. However, patients treated with *Lactobacillus* had less diarrhea. Also, no toxic events related to lactic acid bacteria were detected, indicating that the supplementation of *Lactobacillus rhamnosus* could enhance gastrointestinal tolerance and reduce the frequency of severe diarrhea and abdominal discomfort associated with 5-FU chemotherapy (43). One thing to note, however, is that lactic acid bacteria can also cause local infections. In rare cases, probiotics may cause systemic infections through bacteremia, especially in patients with compromised immunity or Crohn disease (44). Therefore, the safety of combined bacteria-mediated chemotherapy *in vivo* is a matter to be considered.

TABLE 1 | Summaries of studies on bacteria with chemotherapy and radiation therapy.

Type of bacteria	Methods	Application	Outcome	References
selenium-enriched *Bifidobacterium longum*	Intraperitoneal injection	Chemotherapy	Prevention of infection in small intestinal mucositis	(28)
Bifidobacterium longum DD98	Preventive medication	Chemotherapy	Alleviation of intestinal and hepatic toxicities	(29)
Salmonella typhimurium A1-R	Targeted infection tumor	Chemotherapy	Quiescent G0/G1cancer cells to cycle to S/G2/M and chemosensitive	(30)
Lactobacillus	Probiotic capsules	Chemotherapy	The vaginal microbiome changes in a normal direction	(31)
Lactobacillus kefiri LKF01	Oral administration	Chemotherapy	Effective in preventing severe diarrhoea	(32)
Bifidobacterium infantis	Mixture of specific monoclonal antibody and radiation	Radiation therapy	Prevention of tumor growth and prolonged survival	(33)
Lactobacillus acidophilus LA-5 plus *Bifidobacterium animalis* subsp.*lactis* BB-12	Oral administration	Radiation therapy	Prevention of incidence and severity of radiation-induced diarrhoea	(34)
Salmonella typhimurium	Infected murine melanoma cells exposed to 8 Gy of γ-radiation	Radiation therapy	H2AX phosphorylation and apoptosis in melanoma	(35)
Salmonella Typhimurium	Modified miRNA expression vector encoding	Radiation therapy	Improved efficacy of radiotherapy	(36)
Heat-killed *Salmonella Typhimurium*	Intraperitoneal injection	Radiation therapy	Alleviation of radiation-induced lung injury	(37)

Combined Bacteria-Mediated Radiotherapy

More than 60% of tumor patients need radiotherapy, yet the radiation resistance of some tumor types and decreased radiotherapy sensitivity of intratumor hypoxic areas largely account for the failure of radiotherapy (45–47). Engineered *E. coli* (5×10^7 colony-forming unit (CFU)) cooperated with different doses of radiation (0, 8, 15, and 21 Gy). The combination of bacteria and 21-Gy radiation significantly reduced the tumor, completely eradicated the CT26 tumor, and dramatically inhibited tumor metastasis (48). In a recent study, Shiao et al. found that fungi and bacteria from the intestinal system in breast cancer and melanoma mouse models exhibited disparate roles. Although fungi depletion by antibiotics boosted responsiveness to radiotherapy, the bacteria exhaustion greatly accelerated tumor growth. Mechanistically, elevated Dectin-1 (intrinsic receptor of fungi infection) expression in tumor cells negatively correlated with the survival of patients with breast cancer after radiation therapy (49).

Probiotics can also exhibit the protective effect of radiotherapy. A new probiotic mixture (Microflorana-F) was tested in a male Wistar rat model of acute radiation-induced enteritis to examine the effect of supplementation of lactic acid bacteria on radiotherapy-related diarrhea in colorectal cancer. After feeding standard food and active/inactive probiotics (the same probiotics but heat inactivated) for 14 days, the changes in endotoxemia and bacterial translocation were observed. Early death (1 week) mainly occurred in rats fed standard food or inactivated probiotics. The level of endotoxin in the irradiated rats fed with standard food and inactivated probiotics increased remarkably, but the aforementioned indexes were notably improved after the addition of an active probiotic mixture (P <0.05). In the culture of blood, portal vein, and bile samples, active probiotics alone markedly reduced the bacterial contamination in all samples (compared with inactivated probiotics and standard feed samples, P <0.01) (50). In addition, probiotic *E. coli Nissle* 1917 bacteria with catalase secretion were used to relieve hypoxia of the tumor center and boost the sensitivity of radiotherapy. This bacterial strain could promote O_2 generation and subsequent reactive oxygen species production after X-ray irradiation, and as expected, notably suppress tumor growth (51).

Bacteria-Mediated Immunoregulation and TME Amelioration

The immunosuppressive properties of TME contribute to the immune escape of tumor cells and clinical efficacy attenuation of immunotherapy. Nevertheless, the bacteria colonized in tumor hypoxic areas are expected to remold TME and improve immune response (52). Once infected by bacteria, a large number of innate immune cells gather in the TME to kill tumor cells directly or secrete pro-inflammatory cytokines. For example, *S, typhimurium* ΔppGpp strain could activate Toll-like receptor (TLR)4 and TLR5 pathways, resulting in the massive infiltration of macrophages and neutrophils in TME and transformation of M2-like macrophages that promoted tumor progression into M1-like macrophages that inhibited tumor development (53, 54). In addition, when bacteria infected tumor cells, the release of ATP and the secretion of inflammatory bodies could trigger an inflammatory storm, which, together with cytokines or chemokines, such as IL-1β and IL-18, and pore-forming protein gasdermin D could promote tumor regression (55). Besides, Chandra et al. found that *L. monocytogenes* promoted the targeting effect of immune cells after infecting tumor cells, increased the production of IL-12 through MDSC subsets, and enhanced the cytotoxic effect of T and NK cells (56). In addition, as an indispensable part of innate immunity, TLRs and various pathogen-associated molecular patterns could be stimulated by the signals of Gram-negative bacteria, namely, lipopolysaccharide (TLR4), flagellin (TLR5), and unmethylated CpGDNA (TLR9) (57).

Apart from the innate immune system, the adaptive immune response also plays a pivotal role in bacteria-mediated antitumor therapy. Once anaerobes such as *Salmonella* entered into the tumor region, B lymphocytes and $CD8^+$ T cells infiltrated and the number of regulatory T cells (Tregs) decreased to stimulate a strong tumor-killing reaction (58). Meanwhile, the anticancer activity is also exerted *via* increased expression of immunostimulatory factors (such as IL-1β, TNF-α, and IFN-γ) and inhibited immunosuppressive factors, namely, arginase-1 (Arg-1), IL-4, transforming growth factor-β (TGF-β), and vascular endothelial growth factor (VEGF) (59). Similarly, *Clostridium* infection recruits granulocytes and cytotoxic lymphocytes to TME and induces various cytokines and chemokines, thus leading to the activation of functional T cells and tumor regression (60, 61).

Tumor growth requires a special blood supply to support the oxygen and metabolic demands. Bacteria can not only eliminate cancer cells directly but also inhibit neovascularization and destroy blood vessels in the tumor tissues. Saccheri et al. found that *Salmonella* infection increased the expression of Cx43, while inhibited hypoxia-inducible factor 1α and VEGF to reduce angiogenesis in a melanoma model (62). Moreover, the upregulation of TNF-α after *Salmonella* infection promotes the permeability of blood vessels of tumor regions and leads to vascular bleeding, which subsequently contributes to the infiltration of cytotoxic immune cells (63). Therefore, apart from competing with tumor cells for nutrients and activating apoptosis or autophagy signaling pathways, bacteria can also stimulate immune responses and improve suppressive TME (64, 65).

CHEMICAL MODIFICATION AND BIOLOGICAL ENGINEERING OF BACTERIAL STRAINS

Besides the synergistic reaction with the aforementioned treatments, bacteria have gained attention because of the advantage of intratumor colonization (66). Although facultative or obligate anaerobic bacteria have shown prime

tumor colonization ability and are considered to be natural tumor-targeting carriers, the tumor-targeting ability and therapeutic safety of bacteria rarely go hand in hand (9, 67, 68). The main reason is that although obligate anaerobic bacteria are relatively safe and can successfully target tumors, they do not directly dissolve the tumor. In contrast, facultative anaerobic bacteria present excessive natural toxicity but may bring about obvious systemic toxicity (20). Therefore, biological engineering and chemical modification technologies are urgently needed for original bacterial strains to enhance tumor-targeting ability and acquire tolerable toxicity during systemic administration.

Chemical Modification of Bacteria

The surface of the bacterial cell wall is electronegative. Thus, positively charged nanoparticles can be self-assembled to the surface of bacterial strains such as *Salmonella* through electrostatic interaction. Hu et al. designed a cationic nanoparticle-coated bacterial carrier assembled with a cationic polymer and plasmid DNA to synthesize nanoparticle-coated attenuated bacteria for an oral DNA vaccine in tumor immunotherapy *in vivo*. The plasmid encoding vascular *VEGFR2* gene and antigen gene could induce antigen-activated T lymphocytes and cytokines, inhibiting tumor angiogenesis and growth (69). Besides, *Bifidobacterium* (BF), a Gram-positive bacteria with a large amount of protein in the cell wall, is also negatively charged on the surface. BF was combined with cationic phase-change nanoparticles (CPNs) by electrostatic adsorption. During high intensity focused ultrasound irradiation on a tumor, BF-CPN particles could increase the energy deposition after liquid–gas phase transition. Also, the upconversion nanorods (CS-UCNR) of the core–shell structure were coated with protonated oleic acid to make its surface positively charged. *Via* electrostatic interaction and anaerobic *Bifidobacterium* UCC 2003, the imaging agent CS-UCNR was loaded and gathered on the tumor site through the anaerobic targeting of bacteria. The combination of anaerobes and functional NPs improved the treatment of tumor hypoxia and provided a novel approach for specific diagnosis and treatment (70).

Reforming bacteria by chemical bonding is another strategy due to high levels of endogenous amino groups on the cell surface. For instance, the nano photosensitizer (indocyanine green nanoparticles, INPs) is covalently bound to the surface of transgenic attenuated *S. typhimurium* strain YB1 through an amide bond. The functional INPs with the reactive carboxylic acid group (-COOH) and the amino group (-NH2) on the bacterial surface could be directly covalently linked to form a biological hybrid micro-swimmer (YB1-INP). The scanning electron microscope images showed that more than 60% INPs were attached to the surface of YB1. YB1-INP migration could be induced by the destruction of tumor tissue and the production of bacterial nutrients after photothermal treatment. The bioaccumulation of YB1-INPs was 14 times higher than that without photothermal intervention. YB1-INPs showed the characteristics of specific intratumor targeting, good photothermal conversion, and efficient fluorescence imaging and could eliminate large solid tumors without recurrence (71). In another study, poly(lactic acid-glycolic acid) copolymer PLGA nanoparticles loaded with low-boiling point perfluorohexane were integrated with anaerobic *Bifidobacterium longum* through amide bonds. The anaerobic targeted bacteria could infiltrate into the tumor deeply, increase energy deposition by affecting the acoustic environment of TME, and change the acoustic features of tumor tissue. This strain could destroy tumor cells with liquid–gas phase transition during irradiation. Thus, the addition of the bacterial anaerobic enhanced the tumor-targeting performance and retention time of administration (72).

Vesicles of cell membranes have received immense attention as delivery vectors in recent years. Nanoscale proteolipid vesicles have unmatched superiorities in drug delivery applications, namely, controllable dimensions, flexible assembly, and tractable surface modification (73). For example, bacteria-secreted outer membrane vesicles (OMVs) of attenuated Gram-negative bacteria *Klebsiella pneumoniae* along with adriamycin were prepared simultaneously and then transported to NSCLCA549 cells. Dox-OMV showed distinct tumor growth inhibition ability, good tolerance, and better pharmacokinetics. The pathogenic characteristics of OMVs containing bacterial antigens enabled macrophages to recruit in the tumor microenvironment and activate immune response (74). Further, the bacterial secretions could also be combined with nanomaterials to enhance antitumor efficacy (75). In addition, the nano-bionic pathogens were prepared by encapsulating cisplatin nanoparticles on the surface of chemotherapeutic drug cisplatin. The biomimetic nanoparticles encapsulated by OMVs were injected into tumor-bearing mice after photothermal therapy and showed a superior tumor clearance effect together with photothermal therapy (76).

Biological Engineering of Bacteria

Bacteria have the unique ability to manipulate genes, and flagella on bacteria that can penetrate tissues make them a desirable platform to be reprogramed. Various bacteria are preferentially clustered in tumors, such as *E. coli Salmonella Bifidobacterium*, and *Clostridium* (57, 77, 78). However, unattenuated live bacteria bring about safety risks and even death due to bacterial toxins when systemically administered. To be on the safe side, the virulence-related genes must be modified *via* transposon, gene site-directed mutation, and so forth (79, 80) (**Table 2**). A new type of tumstatin drug (Tum) delivery system was established by engineering *Bifidobacterium*. The inhibitory effect of Tum transgenic *B. longum* (BL) on tumors in mice was measured. The weight, growth, and percentage of vascular endothelial cells of the transplanted tumor were also observed. After 39 days of oral (OR) administration or injection into tumors (INT) and into vena caudalis (INV), the inhibition rate in the INV-BL-Tum, INT-BL-Tum, and OR-BL-Tum groups on the transplanted tumor was 64.63, 75.21, and 38.56%, respectively. The apoptosis of tumor cells and vascular endothelial cells in the INT-Tum treatment and INV-Tum treatment groups was dramatically higher than that in the control group (P <0.05). All these findings confirmed the tumor-inhibitory effect of the engineered bacteria (87).

TABLE 2 | Studies on engineered bacteria.

Types of bacteria	Methods	Results	Reference no.
Escherichia coli Nissle 1917	Encoding amino acids 45–132 of tumstatin was subcloned into inducible expression vectors and solubly expressed in *Escherichia coli* BL21	Effectively restrain mice bearing B16 melanoma tumor.	(81)
Escherichia coli Nissle 1917	Bearing azurin-expressing plasmids using rabbit anti-azurin polyclonal antibody.	1. B16 melanoma and orthotopic 4T1 breast tumor growth were restrained 2. Pulmonary metastasis was prevented	(82)
Escherichia coli DH5α-lux/βG	Transforming with pRSETB-lux/βG and plasmid extraction was carried out by miniprep method	1. Targeted homing and proliferation in TME 2. Tumor growth was inhibited.	(83)
Salmonella enterica serovar *Typhimurium*	Genetically engineered SalpIL2 was constructed by inserting the human IL-2 gene intovX^{4550} downstream	The safety of an orally in canine osteosarcoma were confirmed	(84)
Salmonella enterica	Modified attenuate *Salmonella enterica* released a recombinant fluorescent biomarker	1. Fluoromarker transport through tumor tissue 2. Previously undetectable microscopic tumors were identified.	(85)
Listeria monocytogenes	Expressing mesothelin (CRS-207) with chemotherapy	1. Anti-tumor immune responses increased 2. Susceptibility of neoplastic cells to immune-mediated killing enhanced.	(86)

VNP20009 is another attenuated *Salmonella* strain. The photothermal agent polydopamine (PDA) was transported to the anoxic and necrotic areas of the tumor with the tumor-targeting ability of VNP20009 to improve the antitumor effect on malignant melanoma. When the concentration of dopamine was 1,000 μg/ml, the temperature of PDA-VNP suspension increased by 23.0°C after irradiation for 300 s. However, under the same conditions, the temperature of deionized water was raised only by 8.2°C. B16F10 cells were irradiated with PDA-VNP prepared using dopamine, and 80.7% of the cells were killed. The number of bacteria in the tumor injected with PDA-VNP prominently exceeded that in other organs. The results displayed that the PDA coated on the surface of VNP20009 did not affect the targeting and colonization ability of bacteria to tumor after photothermal therapy, and the combination therapy was conducive to tumor inhibition (88). Further, Chowdhury et al. recently designed one nonpathogenic *E. coli* strain with nanobody anti-CD47 expression controllable. CD47 is a kind of "Don't eat me" signal mainly expressed on tumor macrophages. This platform effectively activated the infiltration of cytotoxic T lymphocytes, promoted faster tumor regression, inhibited distant metastasis, and delayed the survival time of mice in the experimental group (10).

Advantage and Disadvantages of Bacteria for Tumor-Specific Targeting and Drug Delivery

Preliminary clinical trials of bacterial cancer treatment have not been as successful as expected for several reasons. One possible reason is due to the pathogenicity of bacteria. For example, in a retrospective analysis of intravesical BCG therapy in 258 patients, complications included acute urinary retention, hematuria, and urinary tract infection (1.2% vs 2.7% vs 5.4% respectively). In addition, age is another major risk, with a higher risk of complications over the age of 80 at diagnosis (19.0% vs 7.5%, p = 0.01). Timely intervention should be performed when complications arise, and the risks and benefits of resuming intravesical BCG immunotherapy should be carefully assessed (89). In addition, in a multicenter, phase III, open and randomized controlled trial of *Lactobacillus brevis* CD2 (LBCD2) for the prevention of oral mucositis in patients with head and neck tumors, a total of 68 patients were randomly divided into the intervention group (LBCD2 lozenges) and the control group (sodium bicarbonate mouthwash). Intervention and control measures were discontinued when grade 3 or 4 oropharyngeal mucositis was present during radiotherapy. The results showed that there was no significant difference between the intervention group and the control group (40.6% vs 41.6% respectively, P = 0.974), and the intervention group was similar to the control group in terms of quality of life, pain and dysphagia. However, the risk of enteral nutritional requirements was significantly reduced in the control group (OR = 0.341, 95% CI = 0.127–0.917, p <0.917). This result is related to different RT techniques, preventive measures, bacterial species, research subjects, mucositis score and others, which need to be further studied (90).

In addition to the inherent pathogenicity, another reason dampening bacteria-based therapy is that in animal models, the toxicity is minimal due to the strong targeted colonization of bacteria and the small number of bacteria required. However, when translated into human trials, the number of bacteria used for treatment and the space of necrosis within the tumor need to be calculated and evaluated more accurately (91). Besides, the comprehensive roles of bacteria therapy are quite complicated in different tumor context. Recent studies have found that microbial regions promote the molecular pathogenic mechanism of cancer initiation and development. in the chemotherapy resistance of colorectal cancer patients, *Fusobacterium* was abundant in recurrent colorectal cancer tissues after therapy (92). In addition, castrated-resistant prostate cancer mice and patients have rich intestinal microflora, which makes androgen precursors converted into active androgens and participate in tumor drug resistance (93).

The characteristics of hypoxia in tumor tissue, especially in the central area, make the tumor resistant to radiotherapy, which ultimately leads to poor therapeutic effect (94). Gold nanoparticles (GNPs) with the characteristics of evading immune system and targeting tumor become a suitable radiosensitizer for radiotherapy, but its delivery effect in the anoxic area of the tumor center is not good, so a tool that can targeted transport GNPs is needed to make up for the effect of radiotherapy in the hypoxic area. Previous studies have shown that bacteria can selectively colonize in anoxic sites and active in these areas. *S. typhimurium* as a highly active delivery agent has been reported by many studies on hypoxic regions of tumors. Amirhosein et al. (95) used live attenuated *Salmonella* Typhi Ty21a with folic acid functionalized GNPs (FA-GNPs) to obtain the Golden Bacteria, then injected FA-GNPs into the tail vein of CT-26 tumor-bearing mice, and calculated the ratio of periphery regions of tumors in comparison with central regions of tumors. The result of FA-GNPs injection group and Golden Bacteria group was 1.95 ± 0.13 vs 0.61 ± 0.10. This observation demonstrates that even if GNPs modified with folic acid targeted cancer cells, it still reached the periphery of the tumor rather than the center of the tumor. The main reason is that the vascular system around the tumor is different from that in the central area, the surrounding blood vessels are more mature and dense, intelligent targeting is still unable to use systemic circulation for effective treatment in hypoxia areas. However, the flagellum movement of anaerobic bacteria can be a means of transport in anoxic zone to change this dilemma. The bacteria in this study are safe as an active carrier in tumor-bearing mice. and there was a significant advantage in transporting GNPs to the central hypoxic area of the tumor.

CLINICAL APPLICATION OF BACTERIA-BASED CANCER VACCINE

Bacteria-based cancer vaccine is a crucial application of bacteria toward clinical transformation. Cancer vaccines mainly include four components: vectors, various formulations, cancer adjuvants, and specific antigens. Among these, cancer-specific antigens may be the most concerning section determining the effectiveness and specificity of tumor vaccines (96–98). Through chemical modification and biological engineering as mentioned earlier, bacteria present considerable foreground of clinical application. When the virulence is attenuated, bacteria display great potential of exerting an antitumor effect. The inherent features of bacteria make them effective immunostimulants (99, 100).

Bacillus Calmette–Guérin (BCG), the only recognized and licensed live bacteria for cancer treatment, was an attenuated strain of *Mycobacterium bovis* and was successfully applied by Morales in 1976 to treat superficial bladder cancer (BC) (101, 102). Nowadays, BCG has become a significant choice for treating high-risk superficial BC in most countries (103). A randomized trial compared the efficacy of intravesical maintenance therapy with BCG and radical cystectomy (RC) in treating high-grade non-muscular invasive bladder cancer (NMIBC). Of the 23 patients treated with BCG, 4 developed NMIBC after induction, 3 developed NMIBC after 4 months, and 2 had metastatic cancer. The 20 patients who underwent RC treatment, 5 had no tumor, 13 had highly malignant NMIBC, and 2 were detected with muscle infiltration. The adverse reactions in both groups were mild [15/23 (65.2%) BCG vs 13/20 (65.0%) RC] with similar quality of life. The results showed that a considerable number of patients were suitable for bladder preservation and could contribute to health and quality of life (104).

Recently, Alejandrina et al. attempted to verify the treatment potential of *S. typhimurium* vaccine strain CVD915 with the liver metastasis model of breast cancer and lymphoma models. After 21 days, tumor infiltration was observed in both tumor models. In addition, the expression of tumor-suppressive IL-10 levels and the number of neutrophils and regulatory T cells decreased. In the lymphoma model, about 10% of the mice witnessed the elimination of tumor growth. The tumor-specific Th1 reaction was triggered in the CVD915 group, followed by an increase in the number of $CD4^+$ T cells and dendritic cells. Meanwhile, the number of tumor nodules in the liver decreased by 50%, and the tumor volume decreased by 45% (105).

The use of attenuated *Listeria*, as a bacterial vector for cancer vaccines, has been widely reported in preclinical trials (106, 107). Taking cervical cancer as an example, the cancer-specific antigen human papillomavirus type 16 E7 (HPV-16 E7)was fused with the attenuated sequence Listeriolysin O (LLO) of hemolysin protein to establish therapeutic cervical cancer vaccines. The vaccines based on attenuated *Listeria* delayed tumor progression *in situ* (108) and provided long-term immunity for patients with early-stage cervical cancer (109). This vaccine could induce robust immunity reaction and proliferation of cytotoxic T cells. It had unique advantages such as genomic nonintegration and could be exhausted easily *via* antibiotics to avoid serious side effects (110–112). Therefore, taking advantage of live bacteria as biological carriers to deliver recombinant tumor-specific antigens is an effective approach to develop cancer vaccines.

Andreas et al. studied an oral DNA vaccine VXM01 that induced an immune response against vascular endothelial growth factor receptor 2 (VEGFR2) in patients with stage IV and locally advanced pancreatic cancer (113). The vaccine used a licensed live and attenuated *S. typhimurium* strain Ty21a as vectors. Subsequently, 18 patients with advanced pancreatic cancer receiving VXM01 and 8 patients receiving placebo (isotonic sodium chloride). The oral vaccine was given four times on the first, third, fifth, and seventh days, while the fortified vaccine was given six times per month after the last vaccination. The results showed that 75% (3/4) of the patients in the high-dose group and 66.7% (8/12) in the low-dose group had a 1.8-fold increase in T cell response compared with the placebo group. In addition, patients receiving high-dose VXM01 vaccination showed a generally strong anti-VEGFR2 response (114). Furthermore, the safety and immunogenicity of the recombinant *Lactococcus lactis* vaccine expressing the HPV type 16 E7 oncogene were evaluated in a phase I safety and immunogenicity test of healthy female volunteers (115, 116). A total of 55 qualified volunteers were divided into vaccine and

placebo groups. Compared with the placebo group, a specific IgG immune response could be induced 30 days after vaccination in the 1×10^{9} CFU/ml group compared with the placebo group (5×10^{9} CFU/ml vs 1×10^{10} CFU/ml, $P = 0.0137$ vs $P = 0.0018$). This study showed that the candidate HPV16 E7 oncoprotein oral vaccine produced by *Lactobacillus* was safe and immunomodulatory (**Table 3**).

CHALLENGES AND FUTURE PROSPECTIVE

Although bacteria have potent cytotoxicity (such as Coley's toxins) and powerful intratumor colonization capabilities which make them desirable tumor killing agents and delivery platform, obstacles toward clinical applications has been there all along. One of the main challenges is that they may cause immune side effects due to the inherent immunogenicity. Novel biological and chemical modification can expand bacteria-mediated clinical transformation, because researchers can obtain bacteria with maximal advantages *via* knockout related genes to reduce pathogenicity while retaining functions of bacteria. In addition, timely management of effective antibiotics can also reduce the risk of severe infection. Secondly, in consideration of the dose-dependent toxicity presented in previous clinical trials, it seems difficult for bacteria to be administered multiple times. Thus, limited drug loading capacity is another challenge dampening the applications of attenuated bacteria strains. Interestingly, how to transform bacteria into intelligent "bacterial factory" or "bacterial machine" appears to be emerging research highlights (117, 118). The combined applications of robot technology and biotechnology are expected to promote bacteria to move intelligently to tumor sites and increase drug loading capacity at the same time (**Figure 2**).

CONCLUSIONS

The unmatched advantage of tumor-targeting properties make bacteria the ideal oncolytic agent to kill tumor cells specifically

TABLE 3 | Selected clinical trials investigating bacteria and cancer vaccine.

Trial number	Therapeutic agent	Population	Mode of delivery	Stage of trial	Country
NCT02302170*	*Helicobacter pylori* vaccine	Healthy children aged 6–15 years	Oral vaccination	Phase 3	China
NCT00736476*	*Helicobacter pylori* antigens-vacuolating cytotoxin A, cytotoxin-associated antigen and neutrophil-activating protein	Healthy non-pregnant adults aged 18–40 years	Intramuscular injection	Phase 1/2	Germany
NCT02371447*	Recombinant *Bacillus* Calmette–Guérin (VPM1002BC)	Patients with intermediate to high risk and recurrent NMIBC	Intravenous infusion	Phase 1/2	Switzerland
NCT02243371*	*Listeria* monocytogenes-expressing mesothelin (CRS-207)	Patients with cytologically or histologically-proven, metastatic adenocarcinoma of the pancreas	Intravenous infusion	Phase 2	USA
NCT01838200*	*Bacillus* Calmette–Guérin	Patients with unresectable stage III or stage IV melanoma	Subcutaneous injection	Phase 1	Australia

ClinicalTrials.gov identifier.

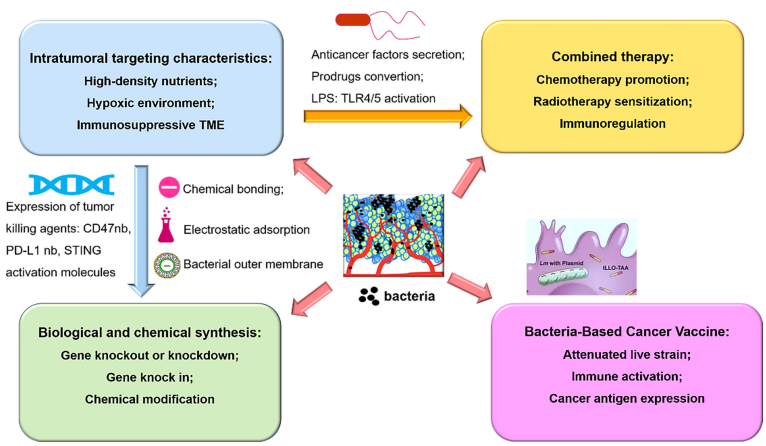

FIGURE 2 | An overview of bacteria-based synergistic therapy, namely, the mechanism of tumor targeting properties, combined therapy with chemoradiotherapy or immunotherapy, chemical modification or biological engineering, and bacteria-mediated delivery of cancer vaccine.

and the excellent platform to deliver multifarious drugs. However, bacteria-mediated therapy alone can hardly eliminate tumor cells completely. As bacteria therapy has two sides, a large number of clinical studies are needed to balance the role of both good and evil of bacteria-based therapy when combined with chemotherapy agents, radiation or immunotherapy. So far, BCG is the only viable curative treatment approved by the FDA up to the present. Although cancer vaccines based on attenuated *Listeria* have entered clinical trials of phase III, there are still some difficulties to be addressed such as bacterial virulence, instability of expression plasmid in bacteria, drug delivery efficiency intracellular. Development of genetic engineering approaches, optimization of chemical modification process and selection of targeted reagents such as tumor specific antigen peptide are of supreme importance in the near future.

AUTHOR CONTRIBUTIONS

YB: Draft writing, reviewing and data processing. YC: Draft supervision and data checking. WLi: Draft reviewing and data checking. WLu: Draft reviewing and data checking. PZ: Draft writing, reviewing and funding acquisition. DQ: Draft supervision and investigation. All authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication.

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CASE REPORT
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Successful Treatment of a Patient With Multiple-Line Relapsed Extensive-Stage Small-Cell Lung Cancer Receiving Penpulimab Combined With Anlotinib: A Case Report

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Small-cell lung cancer (SCLC) is a highly malignant, rapidly developing group of diseases with poor biological behavior. Most patients have extensive-stage SCLC (ES-SCLC) when they are first diagnosed. Standard chemotherapy is prone to relapse in a short period of time, and the patients' median overall survival (OS) can reach only 13 months when chemotherapy is given in combination with PD-L1 inhibitors. To date, no studies have verified the efficacy and safety of the composite treatment of ES-SCLC with penpulimab and anlotinib despite some recognized data and advantages related to this regimen. Penpulimab, a novel PD-1 inhibitor with an IgG1 subtype, has a structural modification of the Fc segment which can prevent the immune cells from being phagocytosed or killed and can steadily avoid tumor immune escape. This case report describes a 71-year-old man who had ES-SCLC for 7 years which progressed after receiving standard systemic chemotherapy combined with radiotherapy. The third-line treatment of four cycles of anlotinib and carilizumab was discontinued because of grade 2 immune-related pneumonia despite the efficacy being evaluated as stable disease. After maintaining 22 months of progression-free survival, the patient relapsed and switched to a safer regimen of penpulimab combined with anlotinib to continue the treatment for four cycles. Partial response evaluation was confirmed twice, and the patient remained in good general condition. The combination of penpulimab and anlotinib can positively regulate the therapeutic effect by simultaneously acting on the tumor microenvironment and promoting blood vessel normalization. In general, this case provides support for the successful possibility of a rechallenge with immune checkpoint inhibitors, the better clinical efficacy of cross-line therapy with anlotinib, and the drug safety of penpulimab, suggesting a beneficial therapy for the clinical treatment of ES-SCLC.

Keywords: penpulimab, anlotinib, ICI rechallenge, small-cell lung cancer, case report

INTRODUCTION

Although extensive-stage small-cell lung cancer (ES-SCLC) is very sensitive to initial treatment, with a tumor remission rate of 60–80%, most patients still experience relapse or drug resistance after initial treatment. SCLC patients have a median overall survival (OS) of only 4–5 months after further chemotherapy (1, 2), and their general prognosis is poor (3). Although the efficiency of treatment depends largely on the time interval between the end of the initial treatment and relapse, an individualized selection of effective later-line treatment options significantly relieves symptoms.

SCLC can produce a better immune response with immune checkpoint inhibitors (ICIs) because of its high mutational burden and immunogenicity. Therefore, the combination of immunotherapy and chemotherapy can significantly increase anti-tumor efficacy and improve prognosis compared to chemotherapy alone (4). Penpulimab (trade name: Anico) is a new type of recombinant humanized anti-PD-1 monoclonal antibody (mAb) with a special subtype of IgG1 structure that is relatively stable. A modified Fc segment and an optimized Fab segment can silence the Fc effect by preventing the phagocytosis or killing of immune cells and reducing fever and infusion reactions. Penpulimab, a novel mAb against PD-1 with anIgG1 subtype, not only enhanced the efficacy of immunotherapy but also greatly improved the safety of the drug. In addition, anlotinib is an oral multi-target tyrosine kinase inhibitor that selectively inhibits vascular endothelial growth factor receptor, fibroblast growth factor receptor, platelet-derived growth factor receptor, c-Kit, and c-Met (5, 6). The ALTER1202 study (7, 8) showed that anlotinib could be a third-line standard treatment for patients with ES-SCLC for whom chemotherapy failed.

In recent years, many positive results have been achieved with anti-PD-1 ICIs combined with anti-angiogenic-targeting regimens, such as lenvatinib plus pembrolizumab for hepatocellular carcinoma (9) and atezolizumab plus bevacizumab for renal cell carcinoma (10). In 31 patients evaluated based on the RECIST 1.1 criteria, the first-line treatment of hepatocellular carcinoma with anlotinib in combination with penpulimab achieved an overall response rate (ORR) of 31%, a disease control rate (DCR) of nearly 83%, and a median progression-free survival (PFS) of 8.8 months, which was comparable to the efficacy of similar anti-angiogenic therapy and immunotherapy combinations. Adverse effects were manageable, and the safety profile was deemed satisfactory (11). Similarly, studies on the treatment of ES-SCLC have repeatedly reported the clinical benefits of this drug combination approach. In a single-arm, open, phase Ib dose exploration study of the treatment of advanced solid tumors with TQB2450 (PD-L1 inhibitor) and allotinib among six patients with SCLC, four cases showed that the treatment had a partial response (PR) efficacy (12). In a phase 2 study of the second-line treatment of SCLC (13), it was observed that the ORR of patients with SCLC who received carilizumab combined with apatinib as a second-line treatment reached 33.9%, and the median OS was 8.4 months. It is worth noting that the median OS was even 8.0 months in resistant patients, suggesting that the combination of ICIs and antiangiogenic drugs is a promising therapeutic strategy for recurrent and advanced SCLC with increased clinical recognition.

As an mAb against PD-1 with a new IgG1 subtype, penpulimab has only been approved for marketing in China. Currently, there are only recurrent or refractory indications for Hodgkin's lymphoma. There are no published or relevant treatment data or case reports for penpulimab as a treatment for SCLC; therefore, its therapeutic efficacy is unclear. The first experimental application of penpulimab for patients suffering from recurrent SCLC and immune-related pneumonia due to other mAbs is shown in this case, along with successful immune rechallenge treatment. The patient achieved continuous remission, and the therapeutic effect was evaluated as PR compared with the initial treatment. Moreover, the patient's general condition remained good without serious adverse reactions.

BACKGROUND

A 71-year-old man was admitted to the hospital for "repeated cough and sputum" in early November 2014. He had a BS of 1.96 m^2, Eastern Cooperative Oncology Group score of 1, atrial fibrillation for more than 10 years, and no smoking history or family history. The chest CT (November 25, 2014) (**Figure 1**) revealed the following: left pulmonary central cancer, obstructive atelectasis in the left upper lobe, obstructive pneumonia, nodules in the left lower lobe, and left hilar lymph node metastasis. The left lower pulmonary veins might have been involved, including multiple millet lesions in the right lung and pleural fluid on the left side. No obvious signs of bone metastasis were observed on bone ECT or PET-CT. The histopathological examination of the fibreoptic bronchoscopy and immunohistochemistry samples revealed heterogeneous cell clusters in the diseased tissue: CD56 (NK-1) (+), CgA (minority +), and TTF-1 (+). The results of the chest CT, histopathology, immunohistochemistry, bronchial brush tablets, and lavage fluid base provided sufficient evidence for the diagnosis of left SCLC, staged as ES.

The patient received chemotherapy with the etoposide and cisplatin for six cycles from December 6, 2014 to March 26, 2015, and lung radiotherapy and prophylactic brain irradiation were continued since April 25, 2015. In early June 2018, the patient suddenly developed a cough and expelled blood-stained sputum. According to the subsequent chest CT (June 8, 2018) (**Figure 1**), there were multiple blurred patches and striped shadows in the lung tissue near the mediastinum of the left lung and the left hilar descending aorta. A lump of about 3.2 × 2.3 cm was seen on the side, which was considered malignant. There were multiple miliary shadows in the left lung and irregular nodules in the lower left lobe, with a diameter of approximately 0.6 cm each. Considering the recurrence of the patient's condition, he was administered the regimen with carboplatin and etoposide for three cycles. After that, the lung lesion was reduced by 25% so that the curative effect was evaluated as SD, and local radiotherapy was continued for one cycle.

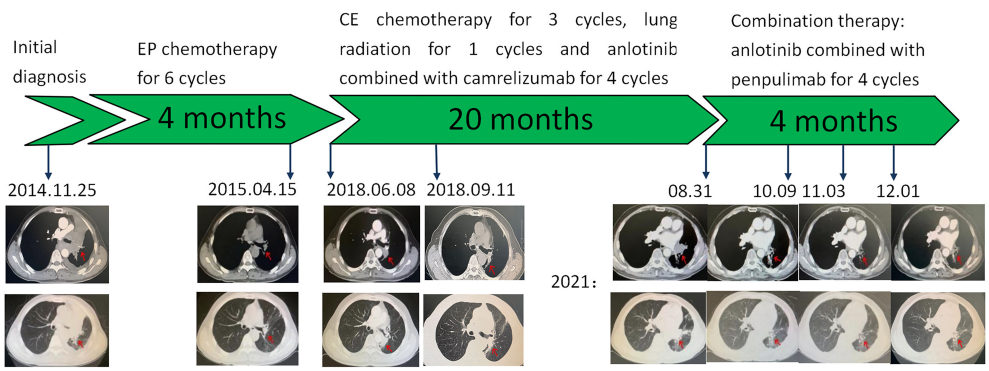

FIGURE 1 | Graphic timeline of the case.

On November 1, 2019, the patient coughed up blood again. After four cycles of treatment with anlotinib and carilizumab, the symptoms improved, and the efficacy was evaluated as SD. However, the patient developed grade 2 immune-related pneumonia, leading to the discontinuation of this regimen. On August 31, 2021, the patient was reexamined *via* lung CT after "coughing up sputum and hemoptysis for 1 week" (**Figure 1**). Multiple nodules and masses were identified in the lower lobe of the left lung; new larger nodules were apparent in the hilar area, approximately 3.8 × 2.3 cm in size. Multiple miliary foci were observed in both lungs. Left pleural effusion and thickened left pleura were also observed. There were multiple small lymph nodes in the left supraclavicular fossa with a shorter diameter of 0.3 cm. The patient's condition recurred. For further treatment, an unprecedented and experimental combination therapy with penpulimab and anlotinib was applied for two cycles. The chest CT (October 09, 2021) (**Figure 1**) revealed the following: The size of the mass in the left lower hilar area was about 3.5 × 2.5 cm and was reduced when compared with the chest CT on August 31, 2021. Small nodules and strips were also seen in the left lower lobe; the largest was about 0.7 × 0.3 cm, and the rest were similar to those previously described. The effect of PR was evaluated based on the examination of the images. After receiving the combination treatment for two cycles, a re-examination *via* chest CT (November 03 and December 06, 2021) (**Figure 1**) showed that the patient's condition was stable, and the curative effect assessment of lesions showed a shrinking trend within the SD range. In summary, the effect was significant, and so far, no immune-related adverse reactions occurred after four cycles of treatment, which further proved the considerable clinical efficacy and drug safety of this regimen. Timeline of the treatment was shown in **Table 1**.

TABLE 1 | Timeline of the treatment.

Time	Major medical examination	Diagnosis or disease evaluation	Treatment
2014.11.25	Chest CT, whole-body bone scan, positron emission tomography–computed tomography, fiber bronchoscopy	Small-cell lung cancer of the left lung at ES stage	Puncture of the lung lesions
2014.12.06–2015.03.26	–		(Etoposide: 200 mg on day 1, day 2, and day 3 + 100 mg on day 4 + cisplatin: 60 mg on day 1 and day 2) for 6 cycles
2015.04.15	Chest CT	Partial response	
2015.04.25	–		Lung radiotherapy and prophylactic cranial radiotherapy
2018.06.08	Chest CT	Local relapse	
2018.06.09–08.12	–		(Etoposide: 200 mg on day 1, day 2, and day 3 + 100 mg on day 4 + carboplatin: 500 mg on day 1) for 3 cycles
2018.09.11	Chest CT	Stable disease	Lung radiotherapy
2019.11.01	Chest CT	Local relapse	
2019.11.02–2020.02.05	–		(Anlotinib: 8 mg from day 1 to day 14 + camrelizumab: 200 mg on day 1) for 4 cycles
2021.08.31	Chest CT	Local relapse	Anlotinib: 8 mg from day 1 to day 14 + penpulimab: 200 mg on day 1
2021.10.09	Chest CT	Partial response	Anlotinib: 8 mg from day 1 to day 14 + penpulimab: 200 mg on day 1
2021.11.03	Chest CT	Stable disease	Anlotinib: 8 mg from day 1 to day 14 + penpulimab: 200 mg on day 1
2020.12.06	Chest CT	Stable disease	Anlotinib: 8 mg from day 1 to day 14 + penpulimab: 200 mg on day 1

DISCUSSION

SCLC is a highly aggressive neuroendocrine tumor with high malignancy, easy metastasis, and rapid progression. Based on the Impower133 and Caspian trials (14, 15), the US Food and Drug Administration recommended atezolizumab or durvalumab combined with platinum as the first-line treatment option for ES-SCLC. Despite the high response rate to initial platinum therapy, almost all patients with ES-SCLC relapse after a short-term treatment with a poor prognosis. Topotecan is a currently approved second-line standard treatment, and navuluzumab or palolizumab can also be used for the treatment of recurrent SCLC. However, the National Comprehensive Cancer Network recommended subsequent systemic and palliative symptomatic treatment after the failure of first- or second-line treatment, suggesting that there is no standard treatment recommendation. Most patients will progress after receiving two or more previous treatment regimens, and there are several limitations with third- and later-line treatment options for patients who cannot receive effective drug treatment, thus affecting their OS. The PFS of this patient after frontline platinum-containing chemotherapy was longer, which suggested that the patient had better drug sensitivity.

Anlotinib inhibits tumor growth through anti-tumor angiogenesis and controls tumor cell proliferation and metastasis (16). In the ALTER-1202 study, anlotinib brought better survival benefits to patients receiving third- and subsequent-line treatment options for SCLC; their median PFS was extended by 3.4 months (hazard ratio, HR: 0.19), and their median OS was prolonged from 4.9 to 7.3 months (HR: 0.53) when compared with the placebo. According to the ALTER-1202 study, the Guidelines for the Diagnosis and Treatment of SCLC in Chinese Society Clinical Oncology recommended anlotinib as a standard choice for the third-line treatment of SCLC. In 2019, the National Medical Products Administration also approved the use of anlotinib for SCLC, providing a standard third-line therapy for patients with SCLC in China.

Other mAbs against PD-1 currently on the market all use IgG4 subtypes, while IgG1 is only applied in penpulimab. MAbs with IgG4 subtypes can give rise to poor stability, Fc–Fc interactions, and antibody drug aggregation and can combine with anti-tumor-specific IgG1 to inhibit natural IgG1 performance and promote tumor immune escape. By comparison, antibodies with IgG1 subtypes are more stable, which can reduce the likelihood of drug aggregation and prevent tumor immune escape. In addition, most of the listed mAbs against PD-1 are unmodified, leading to a reduction in immune cells and affecting the anti-tumor immune response and IL-8 release. In the case of penpulimab, genetic engineering is used to carry out structural modifications to prevent immune cell destruction and phagocytosis, decreasing the release of IL-8 and enhancing the curative effect. Based on the currently available clinical data, there had not been any comparative studies of two mAbs against PD-1. In the AK105-201 study, penpulimab was applied to the treatment of relapsed and refractory classical Hodgkin's lymphoma; the ORR was defined as the primary endpoint and reached 89.4%, and all patients had an OS of 18 months. Remarkably, the incidence of grade 3 adverse events in patients receiving parimizumab was only 4.3%, and there was no grade 4 to 5 immune-related adverse event (irAE) compared with the first-generation PD-1 (17).

In this case, the patient was diagnosed with ES-SCLC upon first presentation, with a disease course of up to 7 years. After four cycles of third-line treatment with anlotinib and carilizumab, the disease was evaluated as SD, but treatment was discontinued because of grade 2 immune-related pneumonia. At the end of August 2021, the patient's lung lesions recurred. Since PFS was maintained for 22 months after anlotinib and carilizumab administration, the patient was initially judged to be someone who could continue to benefit from immunotherapy. Given that the patient's immune-related pneumonia returned to level 1, immunotherapy was reconsidered. An observational, cross-sectional, pharmacovigilance cohort study showed that about 28.8% of initial irAEs reoccurred upon rechallenge treatment with ICIs (18). For patients who consider resuming ICI treatment, it is necessary to reduce the possibility of irAE occurrence, leading to discontinuation. Therefore, the original therapy was replaced with a safer ICI, which, in this case, was penpulimab. After four cycles of treatment with the combined regimen of penpulimab and anlotinib, efficacy was assessed as PR when compared with the baseline. The patient benefited from the therapy continuously, without further adverse effects such as immune-related pneumonia. Therefore, this case not only proves the superior safety of penpulimab but also shows that rechallenge in immunotherapy with ICIs and the trans-line treatment with anlotinib are still effective, bringing great clinical benefit to patients with ES-SCLC. Compared to the successful approval of PD-L1 ICIs, two PD-1 drugs, nivolumab and pembrolizumab, were withdrawn by the FDA for the treatment of SCLC in 2020 and 2021, respectively, due to their limited benefits. In this case, a PD-1 inhibitor combined with anlotinib might improve the tumor remission rate of PD-1 ICIs and is a novel idea to try for SCLC treatment.

In recent years, data on the safety and effectiveness of restarting ICIs after immunotherapy has been interrupted by the presence of many irAEs. A retrospective study indicated that 68 patients with non-small-cell lung cancer (NSCLC) who were administered an ICI stopped their treatment due to irAEs, with only 38 patients then resuming treatment. Subsequently, 18 patients (48%) did not experience irAE recurrence, and the irAEs experienced thereafter were only mild to moderate (12/20, 60%). ICI rechallenge in patients who discontinued treatment due to irAEs may have potential benefits. In the KEYNOTE-010 trial, patients with NSCLC who received pembrolizumab retreatment had an ORR of up to 42.9%; another large European retrospective analysis showed that patients who received an ICI rechallenge had a median OS of between 15.0 to 18.4 months (19, 20). Based on clinical experience, this patient may have sustained an immune benefit and received a successful immune rechallenge therapy. This was achieved by switching to the safer penpulimab after the patient developed grade 2 immune-associated pneumonia while on carilizumab, suggesting that the choice of drug for immune rechallenge could be a break from conventional therapy with the original drug (21).

The main reason for the limited benefit of immunotherapy in patients with SCLC is the lack of biomarkers to predict its efficacy and toxicity. Based on a previous classification, Gay et al. (22) analyzed the RNA sequence data of 81 SCLCs to classify SCLC into four transcriptionally distinct subgroups: ASCL1/SCLC-A, NEUROD1/SCLC-N, POU2F3/SCLC-P, and SCLC-inflamed (SCLC-I). The vast differences in the immune microenvironment of different subtypes of SCLC (23), based on the phenotypic molecular expression of the SCLC-I subtype, showed that it may have a higher response to immunotherapy. Additionally, trends were observed in the follow-up analysis of the IMpower133 study, providing an advantage for SCLC in immunotherapy population selection as potential biomarkers and related mechanisms provide strong evidence for this study. It seems possible to declare that the treatment of SCLC has entered the era of precision therapy (24). Therefore, the main limitation of this study is that early judgment of the immune benefit for patients comes from clinical experience after the assessment of efficacy. We hope that future universal molecular typing of SCLC can pre-screen more populations, though this requires more researchers to conduct more in-depth and extensive research.

CONCLUDING REMARKS

In summary, our case further demonstrates the efficacy and safety of penpulimab combined with anlotinib for the later-line treatment of ES-SCLC, and two regimens of different immunological drugs combined with an anti-vascular-targeting agent achieved ideal survival benefits. However, different outcomes in safety suggest that the selection of immune agents in combination therapy may be a key factor affecting the treatment outcome. After the patient developed grade 2 immune-related pneumonia, choosing the safer penpulimab as a rebooted ICI and combining it with the cross-line therapy of anlotinib improved the survival and did not lead to the development of any irAEs. In summary, this combination is a good treatment method for patients with ES-SCLC. It is expected that, with the continuous development of oncology medicine, relevant clinical trials can be conducted to obtain more scientific and rigorous data to verify these findings in the near future.

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

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

AUTHOR CONTRIBUTIONS

CZ and XC contributed to the conception and design and provided administrative support. ZZ provided necessary information. YL and ZZ took charge of the collection and assembly of data, conducted the disease analysis, provided the summary. All authors contributed to the article and approved the submitted version.

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