OVERCOMING THE IMMUNE MICROENVIRONMENT OF HEPATOCELLULAR CANCER

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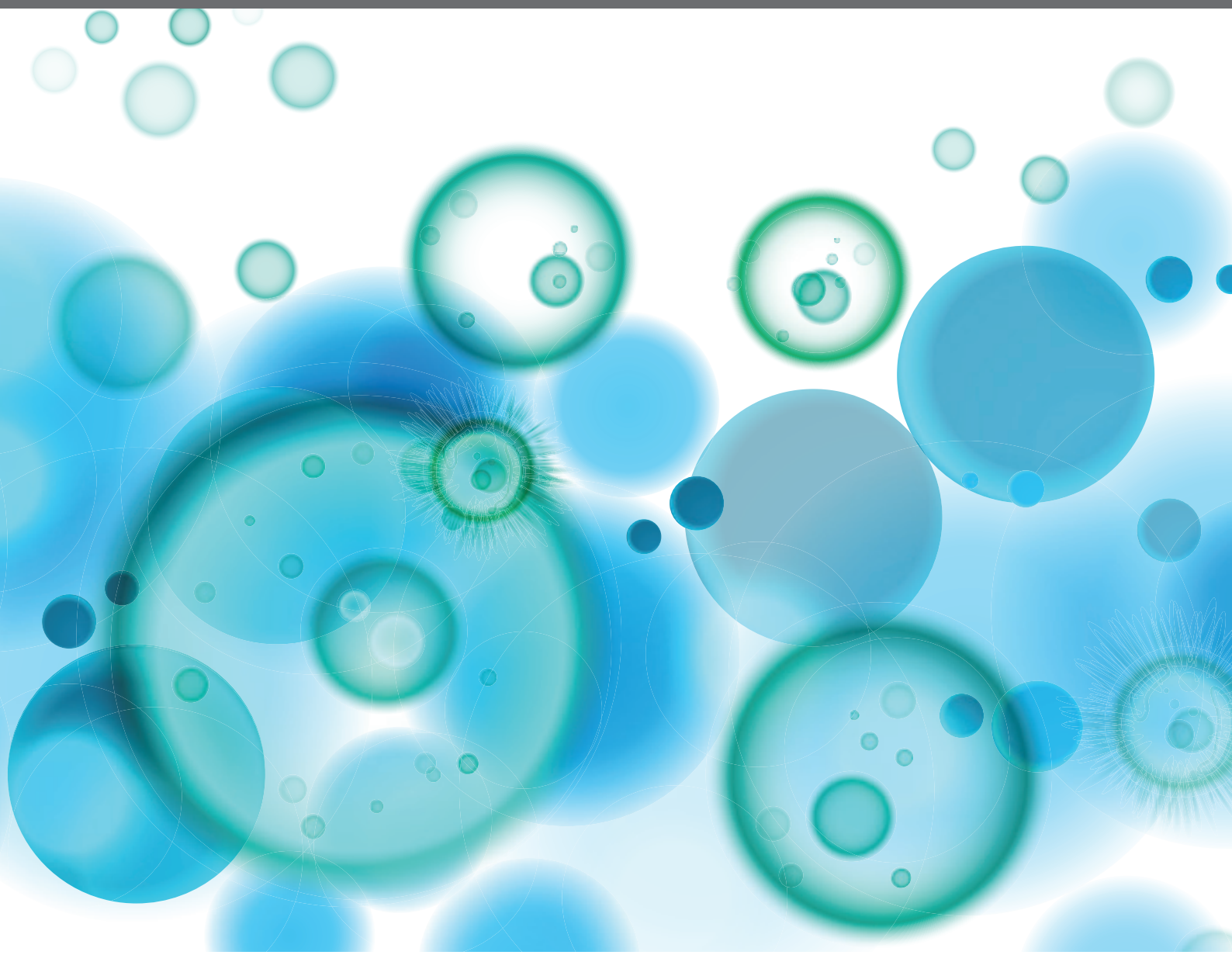

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OVERCOMING THE IMMUNE MICROENVIRONMENT OF HEPATOCELLULAR CANCER

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EDITORIAL
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Editorial: Overcoming the Immune Microenvironment of Hepatocellular Cancer

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Editorial on the Research Topic

Overcoming the Immune Microenvironment of Hepatocellular Cancer

Hepatocellular carcinoma (HCC) is a leading cause of global cancer-related deaths and cases are predicted to rise in the coming decades. In the vast majority of patients, HCC develops in the context of chronic liver disease, which provides the setting for a complex tumour microenvironment characterised by constant induction of cell death with compensatory hyperproliferation, chronic inflammation, maladaptive wound healing, and fibrosis. While this inflammatory microenvironment provides an overall pro-carcinogenic milieu, the hepatic immune system also participates in tumour surveillance and anti-tumour immune responses. Targeting the tumour microenvironment is therefore a critical strategy in both treating advanced HCC and preventing tumour recurrence in patients undergoing curative therapies. The recent approval for the use of immunotherapy for treating HCC, specifically the combination of immune checkpoint blockers with anti-VEGF agents, has helped to confirm the long held belief that targeting the immune microenvironment could be an effective approach to treating this tumour. While immuno-oncological therapeutic options generally provided survival benefit for advanced, non-resectable HCC with manageable side effects compared to previous medical approaches, treatment efficacy is still not satisfying and patient stratification is not well defined. The aim of this article collection is to highlight new pathways that may help in developing novel immunotherapeutic approaches for HCC and to explore the optimal use of immunotherapies in the context of the expanding arsenal of therapies that are now becoming available for advanced HCC.

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EXPLORING HOW TUMOUR CELLS DRIVE THE IMMUNE MICROENVIRONMENT IN HCC

The last few years have seen a major interest in the study of the tumour microenvironment (TME). It is now clear that tumours not only have intrinsic effects that promote cell survival/proliferation but can also influence the extrinsic tissue microenvironment and drive a programme of immune evasion. Lu et al. report that the Na+/K+-ATPase, ATP1B3, is upregulated in HCC tissue and HCC

cell lines and proteomic analysis of publically available databases confirmed a correlation with tumour immune infiltrates. Further functional assays demonstrate that ATP1B3 contributes to key tumourigenic pathways including cell proliferation and migration, and raise the possibility of targeting ATP1B3 in patients with HCC. In another article, Jiang et al. focus on the contribution of Tank-binding kinase 1 (TBK1) in regulating CD8 T cell infiltration in HCC. Using a model of HCC developed on the background of chronic liver inflammation, they demonstrate that an inhibitor of TBK1 alters the cytokine milieu within the tumour and promotes CD8 T cell infiltration leading to suppression of tumour growth. The need to take into account other characteristics of the tumour microenvironment, specifically hypoxia, is highlighted by the analysis carried out by Mo et al. Based on large HCC genomic datasets, they identified a link between hypoxia-associated genes and immunosuppressive features in tumour samples. The hypoxia gene signature may be a potential tool for stratifying patients for immunotherapy but also sheds light on the need to overcome alternative TME pathways in order to boost the efficacy of immunotherapy.

BOOSTING COMMUNICATION IN THE HCC MICROENVIRONMENT

Whilst T cells are the critical effector arm of the immune system in attacking and preventing tumours, they are educated by a range of innate and adaptive immune populations. In the context of HCC, the liver microenvironment itself has a vital role in influencing T cell function by way of its resident non-parenchymal cell populations including Kupffer cells, liver sinusoidal endothelial cells, and hepatic stellate cells. In their review detailing the landscape of HCC, Giraud et al. cover the factors that initiate tumour development in the liver and subsequent tumour progression. They highlight the complex cross-talk between the immune system and hepatic microenvironment and summarise the clinical trials that are now taking place building on the knowledge that has been gathered in the field. Lurje et al. focus on the critical role of antigen presentation in the cross-talk between immune cells and how promoting effective antigen presentation could switch the TME from an immunosuppressive to an immunostimulatory state. They review the rationale for *in situ* vaccination and cover the mechanisms of antigen presentation and the range of approaches that are being undertaken to harness the TME.

IMMUNOTHERAPY AS THE NEW STANDARD OF CARE FOR HCC

The advent of immunotherapy has opened up new options for patients with HCC, yet the outlook in patients with advanced disease remains poor and there still remain significant questions regarding the stratification of patients for immunotherapy. Sharma and Motedayen Aval highlight the issues surrounding second line agents in patients with advanced HCC. They provide a summary of approaches being considered for novel combination therapies to overcome the resistance to – or lack of efficacy – of immune checkpoint inhibitors. Looking at the experience with renal cell carcinoma they also discuss the sequential use of Tyrosine Kinase inhibitors following immunotherapy. In another article, Mohr et al. review the journey towards the use of immune checkpoint inhibitors in HCC but also highlight the questions that still need answering regarding the optimal use of these therapies in the setting of current treatment algorithms. Such open questions include optimal treatment options in non-viral induced HCC or for patients with advanced stage cirrhosis (e.g. Child B or C). Moreover, they summarise the future directions with immune checkpoint inhibitors including combinations with novel immunotherapies as well as elucidating the role of immunotherapy in the neoadjuvant setting and combining with loco-regional therapy and trans-arterial chemoembolization.

Immunotherapy has been an exciting breakthrough in HCC yet the tumour microenvironment of HCC still remains a major challenge to therapeutic success. Understanding the optimal use of immunotherapies in the clinical setting and the identification of new therapies to boost the efficacy of current strategies will hopefully lead to major improvements in survival for patients with HCC.

AUTHOR CONTRIBUTIONS

All authors made a contribution to writing the editorial and approved it for publication.

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ORIGINAL RESEARCH
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Hypoxic Characteristic in the Immunosuppressive Microenvironment of Hepatocellular Carcinoma

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Background: Generally, hepatocellular carcinoma (HCC) exists in an immunosuppressive microenvironment that promotes tumor evasion. Hypoxia can impact intercellular crosstalk in the tumor microenvironment. This study aimed to explore and elucidate the underlying relationship between hypoxia and immunotherapy in patients with HCC.

Methods: HCC genomic and clinicopathological datasets were obtained from The Cancer Genome Atlas (TCGA-LIHC), Gene Expression Omnibus databases (GSE14520) and International Cancer Genome Consortium (ICGC-LIRI). The TCGA-LIHC cases were divided into clusters based on single sample gene set enrichment analysis and hierarchical clustering. After identifying patients with immunosuppressive microenvironment with different hypoxic conditions, correlations between immunological characteristics and hypoxia clusters were investigated. Subsequently, a hypoxia-associated score was established by differential expression, univariable Cox regression, and lasso regression analyses. The score was verified by survival and receiver operating characteristic curve analyses. The GSE14520 cohort was used to validate the findings of immune cell infiltration and immune checkpoints expression, while the ICGC-LIRI cohort was employed to verify the hypoxia-associated score.

Results: We identified hypoxic patients with immunosuppressive HCC. This cluster exhibited higher immune cell infiltration and immune checkpoint expression in the TCGA cohort, while similar significant differences were observed in the GEO cohort. The hypoxia-associated score was composed of five genes (ephrin A3, dihydropyrimidinase like 4, solute carrier family 2 member 5, stanniocalcin 2, and lysyl oxidase). In both two cohorts, survival analysis revealed significant differences between the high-risk and low-risk groups. In addition, compared to other clinical parameters, the established score had the highest predictive performance at both 3 and 5 years in two cohorts.

Conclusion: This study provides further evidence of the link between hypoxic signals in patients and immunosuppression in HCC. Defining hypoxia-associated HCC subtypes may help reveal potential regulatory mechanisms between hypoxia and the immunosuppressive microenvironment, and our hypoxia-associated score could exhibit potential implications for future predictive models.

Keywords: hepatocellular carcinoma, hypoxia, score, immunotherapy, tumor microenvironment

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INTRODUCTION

As the major subtype of liver cancer, hepatocellular carcinoma (HCC) is diagnosed in more than half a million people worldwide each year (1). Characterized by high metastasis and poor prognosis, HCC is one of the most common causes of cancer death (2). Curative treatments, including liver transplantation, liver resection, and ablation are preferred for HCC (3); however, most patients are not suitable for curative treatment, since they are usually diagnosed at advanced stages. In addition, although systemic therapy with sorafenib is the first-line chemotherapeutic treatment for patients with advanced HCC, most patients are highly refractory to this therapy (4). Therefore, there is an urgent need to investigate novel treatments to improve the prognosis of most patients with HCC.

Malignant tumor tissues include not only tumor cells, but also supportive tumor-associated healthy cells (stromal cells and immune cells), which comprise the tumor microenvironment (TME) (5). The TME has recently attracted increasing attention, as it provides a novel context for tumor diagnosis and prognosis (6). The TME also provides essential cues to maintain stemness and promote the seeding of tumor cells at sites of metastasis (5). The estimation of stromal and immune cells in malignant tumors using expression data (ESTIMATE) algorithm can be used to estimate and quantify the TME. Many studies (6–8) have shown that stromal score, immune score, and tumor purity measurements based on the TME can serve as prognostic tumor biomarkers. For HCC, immunohistochemical scoring of CD38 molecule in the TME can be used to predict responsiveness to anti-programmed cell death 1/CD274 molecule (i.e., anti-PD-1/PD-L1) immunotherapy (9). In addition, a TME-based risk score was shown to be an effective prognostic predictor for HCC (10). However, the HCC TME is complex, with diverse populations of innate and adaptive immune cells that influence tumor immune evasion and the response to immunotherapy (11). Furthermore, the HCC TME is characterized by the presence of multiple immunosuppressive factors (12). Therefore, it is necessary to explore and elucidate the roles of intrinsic cellular factors and extrinsic factors in patients with immunosuppressive HCC TME.

Hypoxia is a typical characteristic of the TME, and drives the aggressiveness of many tumors (13–15). In the process of adapting to the hypoxic TME, cancer cells acquire invasive and metastatic properties (16). Interestingly, this hypoxia-associated signature has impressive pan-cancer predictive potential (17). Hypoxia regulates the mitochondrial activity of HCC cells through the hypoxia-inducible factor (HIF)/hes related family bHLH transcription factor with YRPW motif 1/PTEN induced kinase 1 pathway (13). Another study found that the hypoxia-induced microRNA miR-3677-3p promoted the proliferation, migration, and invasion of HCC cells by suppressing sirtuin 5 (18). Moreover, hypoxia inducible lipid droplet associated promotes HCC immune escape from natural killer cells through the interleukin 10/signal transducer and activator of transcription 3 signaling pathway (19). These studies demonstrate that hypoxia plays a crucial role in HCC immunotherapy. However, the underlying mechanism remain to be investigated.

In this study, we first used 29 immune-associated gene sets to identify patients with immunosuppressive TME of HCC through hierarchical clustering. Next, using the same algorithm and clustering method, we identified a hypoxic cluster among the immunosuppressive patients. Patients in the hypoxia group had higher immune cell infiltration and immune checkpoint expression, suggesting increased sensitivity to immunotherapy. Furthermore, we established a hypoxia-related score to predict the prognosis of patients with immunosuppressive HCC. Our results indicate that the presence of TME hypoxia is a potential biomarker of HCC immunotherapeutic response and prognosis.

MATERIALS AND METHODS

Data Collection

Gene expression and clinical data were retrieved from The Cancer Genome Atlas (TCGA; https://portal.gdc.cancer.gov/), Gene Expression Omnibus (GEO; https://www.ncbi.nlm.nih.gov/geo/) and International Cancer Genome Consortium (ICGC; https://dcc.icgc.org/) databases. Three independent cohorts (TCGA-LIHC, GSE14520, and ICGC-LIRI) were employed in our research, with the TCGA-LIHC cohort used as a training dataset and the other two cohorts used as a validation dataset. The hypoxia-associated gene set (Hallmark-hypoxia) was obtained from the MSigDB database (https://www.gsea-msigdb.org/gsea/index.jsp). Hypoxia-associated genes were defined as genes experimentally shown to be upregulated in response to low oxygen levels.

Sparse Hierarchical Clustering and Cluster Validation

First, single sample gene set enrichment analysis (ssGSEA), a special type of GSEA that can estimate a score for each case, was performed using the "GSVA" package, to calculate enrichment scores based on 29 immune-related gene sets. Genes in the immune-related gene sets are shown in **Supplementary File 1**. Second, using the "sparcl" package in RStudio, sparse hierarchical clustering analysis was performed to identify TME with different immunological features based on the ssGSEA results. The "sparcl" package uses a novel framework for sparse clustering, in which observations are clustered based on an adaptively chosen subset of features (20). After cluster identification, the expression levels of major histocompatibility complexes, T cell inhibitors, and T cell stimulators were used to verify the distinct immunological landscapes of the identified clusters. Meanwhile, to verify the TME of different clusters, we estimated the stromal and immune scores of each case using the "ESTIMATE" package. ESTIMATE is a tool for predicting tumor purity, as it detects the presence of infiltrating stromal/immune cells in tumor tissues (21). The ESTIMATE algorithm is based on ssGSEA and generates three final scores: the stromal score (which represents the presence of stromal cells in tumor tissues), the immune score (which represents the infiltration of immune

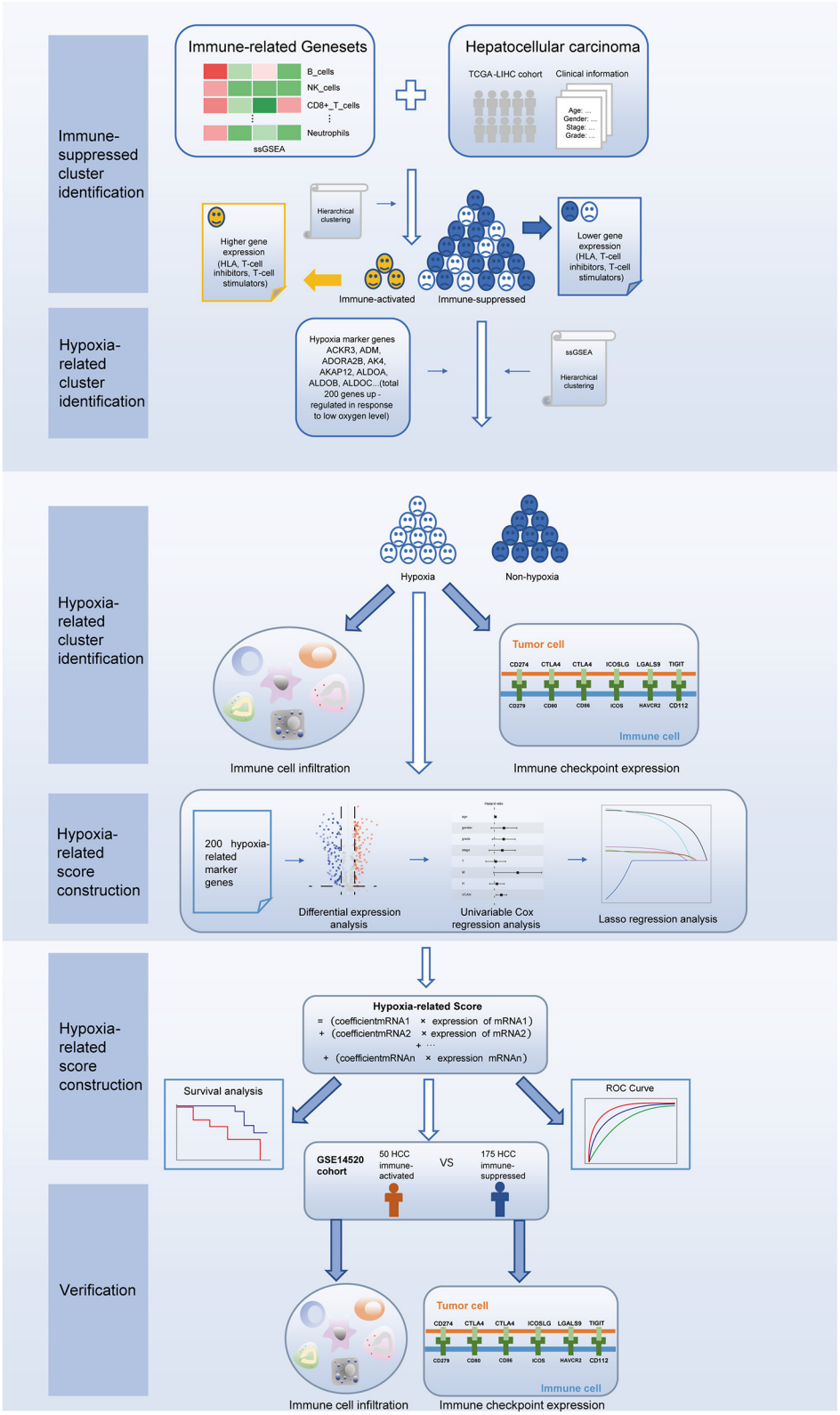

FIGURE 1 | Study flowchart.

TABLE 1 | Baseline characteristics of patients in two cohorts.

Clinical characteristics		Number	Percent (%)
TCGA-LIHC ($n = 377$)			
Survival status	Survival	249	66
	Death	128	34
Age (1 patient missing)	≤65 years	235	62.5
	>65 years	141	37.5
Gender	Female	122	68
	Male	255	32
TNM Stage (24 patients missing)	I	175	50
	II	87	24.6
	III	86	24.4
	IV	5	1
Grade (5 patients missing)	G1	55	14
	G2	180	48
	G3	124	33
	G4	13	5
T classification (3 patients missing)	T1	185	49
	T2	95	26
	T3	81	22
	T4	13	3
ICGC-LIRI ($n = 260$)			
Survival status	Survival	214	82.4
	Death	46	17.6
Age	≤65 years	98	37.7
	>65 years	162	62.3
Stage	I	40	15.4
	II	117	45
	III	80	30.8
	IV	23	8.8

cells in tumor tissues), and the ESTIMATE score (which infers tumor purity). To further explore the hypoxic TME of patients in the immunosuppressive cluster, the expression levels of 200 hypoxia-related marker genes were used to identify different hypoxic clusters by hierarchical clustering. Clusters with different TME immunological characteristics and with different hypoxic characteristics were visualized using tree diagrams. Next, to further investigate correlations between hypoxia and immunotherapy, we examined differences in immune cell infiltration and immune checkpoint gene expression between the clusters. Immune cell infiltration was estimated using the "TIMER2.0" and "MCP-counter" methods. TIMER2.0 (http://timer.comp-genomics.org/) is a comprehensive resource for the systematic analysis of immune cell infiltration, which analyzes six types of immune cells (B cells, $CD4^+$ T cells, $CD8^+$ T cells, neutrophils, macrophages, and dendritic cells). MCP-counter predicts the abundance of 10 cell populations (eight types of immune cells, endothelial cells, and fibroblasts) based on the transcriptomic profiles of human tissues. Immune checkpoint genes (22, encoding both ligands and receptors) were obtained from previous studies (22).

Generation of Hypoxia-Related Score in the Immunosuppressive Cluster

To further elucidate the underlying association between TME hypoxia and clinical HCC immunotherapy, we established a score based on hypoxia-related marker genes. First, differential expression analysis was performed to select the marker genes. Genes with both $P < 0.05$ and $|\log_2 \text{fold change}| > 2$ were considered significantly differentially expressed. A volcano plot was used to visualize the differentially expressed genes. Subsequently, we performed univariate Cox regression analysis to further explore the prognostic genes. Genes in the univariate analysis were eligible for further selection if $P < 0.01$. Lasso regression analysis was performed to establish the hypoxia-related score. In this analysis, a lasso penalty was used to account for shrinkage and variable selection. The optimal value of the lambda penalty parameter was defined by performing 10 cross-validations. The formula for calculating hypoxia-related score was as follows: $score = (coefficient\ of\ mRNA1 \times expression\ of\ mRNA1) + (coefficient\ of\ mRNA2 \times expression\ of\ mRNA2) + \cdots + (coefficient\ of\ mRNAn \times expression\ mRNAn)$. Furthermore, to investigate the correlation between the hypoxia-related score and overall survival, we performed survival analysis using the "survival" package. The patients were divided into two groups (high-risk or low-risk group) based on the median of risk score. Correlations between the established score and other clinical parameters (age, gender, stage, tumor grade, tumor size, distant metastasis, lymph node metastasis, alpha fetoprotein, albumin, and prothrombin time) was also investigated. To further verify the hypoxia-related score, a receiver operating characteristic (ROC) curve was constructed to examine the prognostic accuracy. Meanwhile, univariate and multivariate Cox regression analyses were performed to verify whether the hypoxia-related score was an independent prognostic marker of HCC.

Validation of Hypoxia-Related Classification and Scoring

To ensure the reliability of the established classification and score and validate the immunologic landscapes of the clusters, we repeated the clustering using the GSE14520 cohort. In addition, immune cell infiltration and immune checkpoint expression between hypoxia-related clusters were estimated and compared. Furthermore, the established score was validated by ICGC-LIRI cohort. After identifying the immunosuppressive patients, survival analysis and ROC curve were performed again.

RESULTS

Identification and Validation of an Immunosuppressive HCC Cluster

The procedures in this study are summarized in **Figure 1**. Clinical information for the LIHC and LIRI cohorts is presented in **Table 1**. No relevant clinical information of GSE14520 cohort was found in GEO database. Two clusters were generated by ssGSEA and hierarchical clustering (**Figure 2A**). Each branch in tree diagram represented the case of LIHC cohort. The red

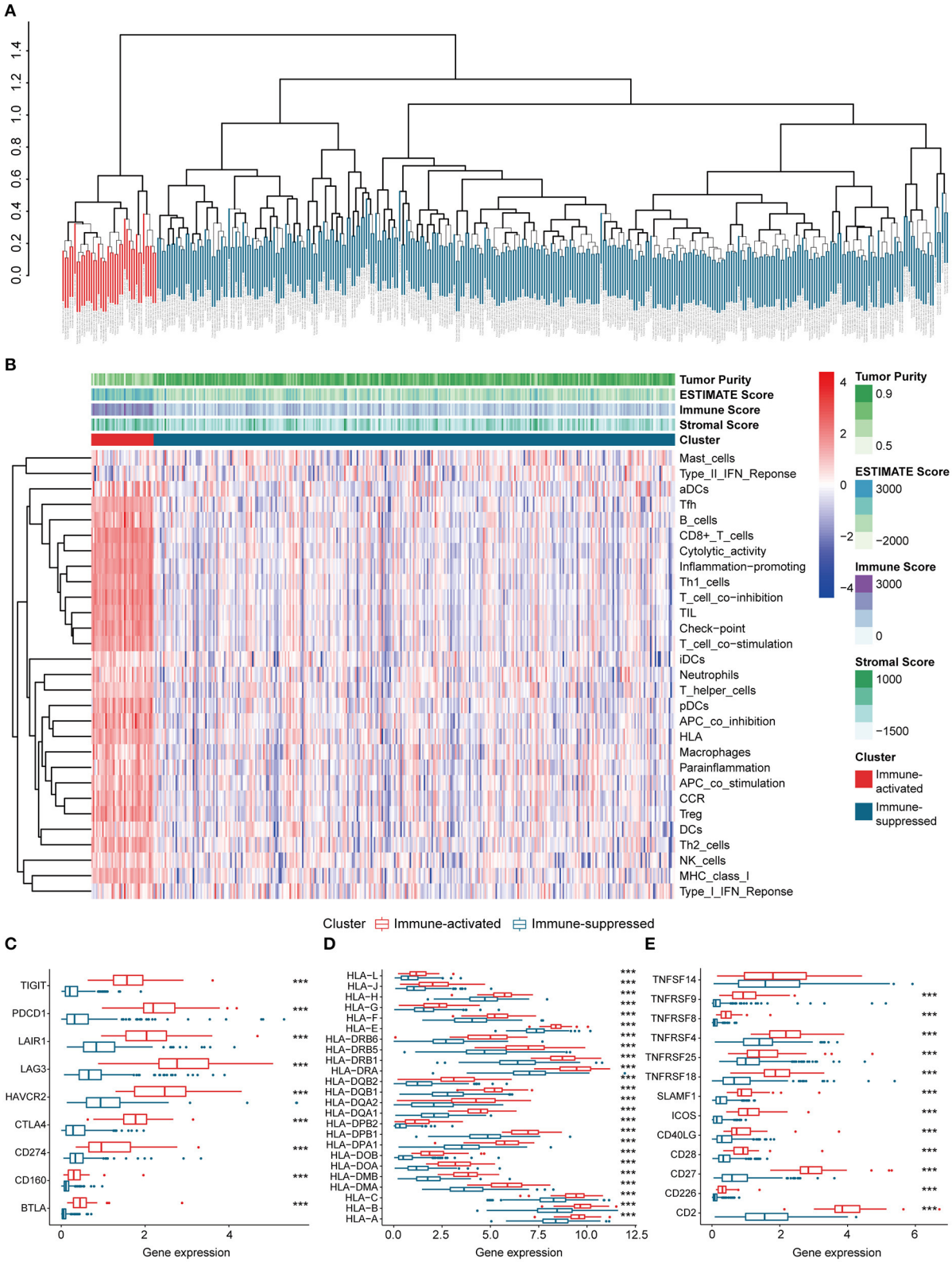

FIGURE 2 | Identification and validation of immune-associated clusters **(A)** Hierarchical clustering of the immune-activated (red) and immune-suppressed (blue) clusters. Each branch in the tree diagram represents one case in the LIHC cohort. **(B)** Heatmap of immune-associated clusters, ssGSEA results, and ESTIMATE score. **(C–E)** Expression of T cell inhibitors **(C)**, major histocompatibility complexes **(D)**, and T cell stimulators **(E)** in each cluster.

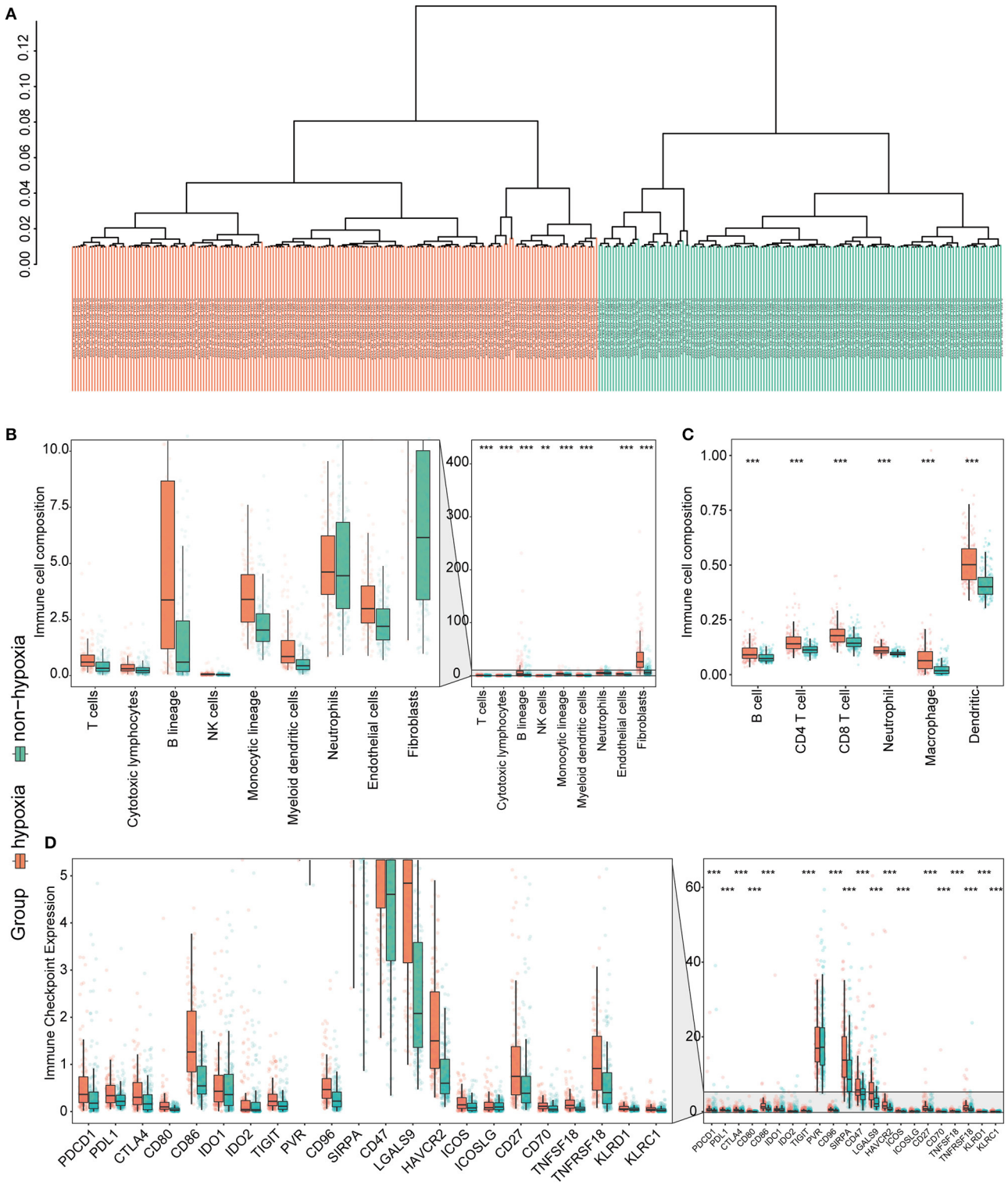

FIGURE 3 | Identification and validation of hypoxia-associated clusters in the immunosuppressive cluster. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. **(A)** Hierarchical clustering of hypoxic (orange) and non-hypoxic (green) patients. **(B,C)** Immune cell infiltration in the hypoxia-associated clusters based on the MCP-counter **(B)** and TIMER2.0 **(C)** methods. **(D)** Immune checkpoint gene expression in each cluster.

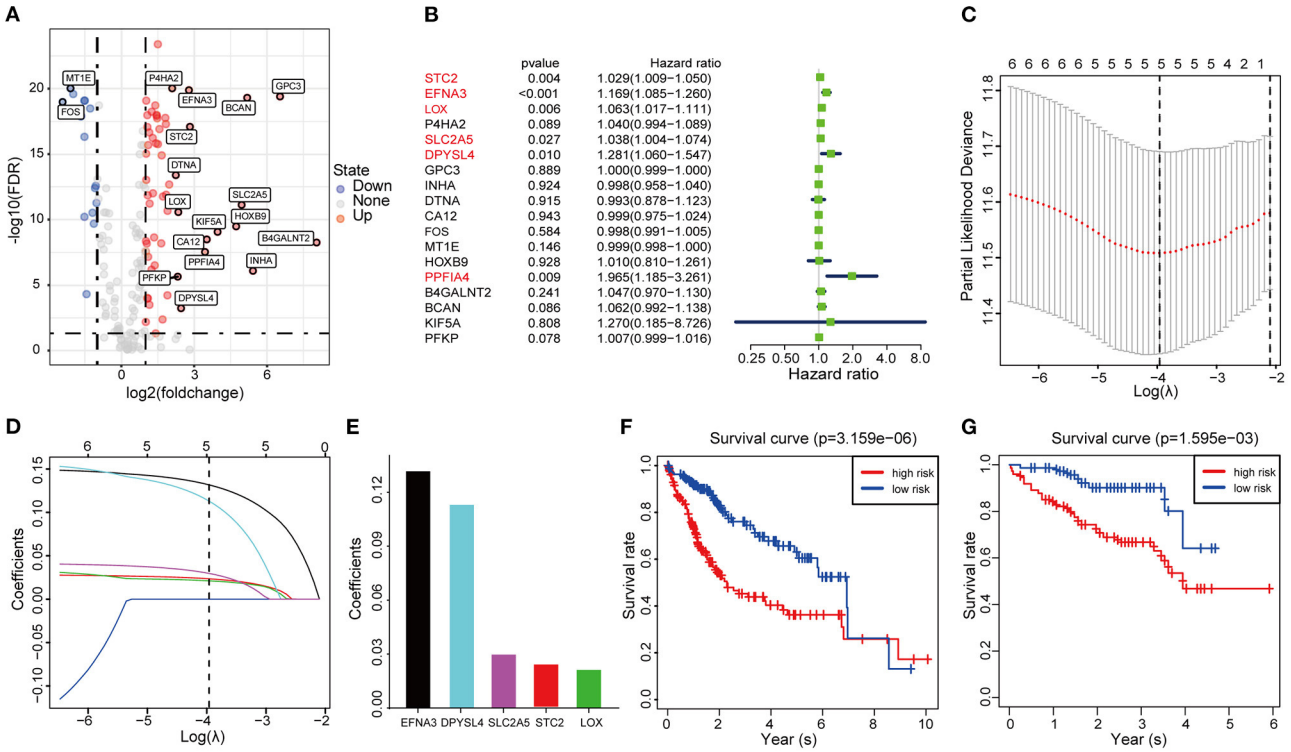

FIGURE 4 | Construction and validation of the hypoxia-associated signature **(A)** Volcano plot of differentially expressed genes. **(B)** Univariable Cox regression analysis of differentially expressed genes. **(C)** Partial likelihood deviance for the lasso regression. **(D)** Lasso regression analysis. **(E)** Coefficients of the included genes. **(F,G)** Survival analysis based on TCGA cohort and ICGC cohort.

one represented the immune-activated cluster while the blue one represented the immunosuppressive cluster. There were 40 cases in the immune-activated cluster, while the remainder comprised the immune-suppressed cluster. **Figure 2B** shows the ESTIMATE and ssGSEA scores of the 29 immune-related gene sets in the two clusters. Compared with the immune-activated cluster, the patients in the immunosuppressive cluster presented relatively lower immune score, lower stromal score, higher tumor purity, and lower levels of immune-related gene sets. Patients in the immunosuppressive cluster also exhibited significantly lower expression levels of T cell inhibitors (**Figure 2C**), major histocompatibility complexes (**Figure 2D**), and T cell stimulators (except for TNF superfamily member 14; **Figure 2E**).

Identification and Verification of a Hypoxia-Related Immunosuppressive HCC Cluster

Considering the crucial role of hypoxia in the TME, we characterized the hypoxia observed in cases in the immunosuppressive cluster. Using the hierarchical clustering method and 200 hypoxia marker genes, hypoxic and non-hypoxic clusters were generated (**Figure 3A**). Patients in the hypoxia cluster exhibited higher immune cell infiltration by both the MCP-counter (**Figure 3B**) and TIMER2.0 (**Figure 3C**) methods (both $P < 0.05$). As illustrated in **Figure 3D**, the majority of immune checkpoint genes were expressed at higher levels in the hypoxia group (with the exception of indoleamine 2,3-dioxygenase 1, indoleamine 2,3-dioxygenase 2, and inducible T cell co-stimulator ligand).

Generation of the Hypoxia-Related Score

Considering the heterogeneity of hypoxia, we next quantified the hypoxic characteristics of different cases. To do this, we established a novel scoring system to evaluate the hypoxic characteristics of patients with immunosuppressive HCC. First, we performed differential expression analysis to identify differentially expressed hypoxia marker genes. Volcano plots indicated that 18 genes (metallothionein 1E; Fos proto-oncogene, AP-1 transcription factor subunit; prolyl 4-hydroxylase subunit alpha 2; ephrin A3; brevican; glypican 3; stanniocalcin 2; dystrobrevin alpha; lysyl oxidase; solute carrier family 2 member 5; kinesin family member 5A; homeobox B9; carbonic anhydrase 12; beta-1,4-N-acetyl-galactosaminyltransferase 2; PTPRF interacting protein alpha 4; inhibin subunit alpha; phosphofructokinase, platelet; and dihydropyrimidinase like 4) were eligible for further analysis (**Figure 4A**). Univariate Cox analysis (**Figure 4B**) and lasso regression analysis (**Figures 4C,D**) identified a score composed of five genes: ephrin A3, dihydropyrimidinase like 4, solute carrier family 2 member 5, stanniocalcin 2, and lysyl oxidase. The coefficients of these genes are presented in **Figure 4E**. Survival analysis of

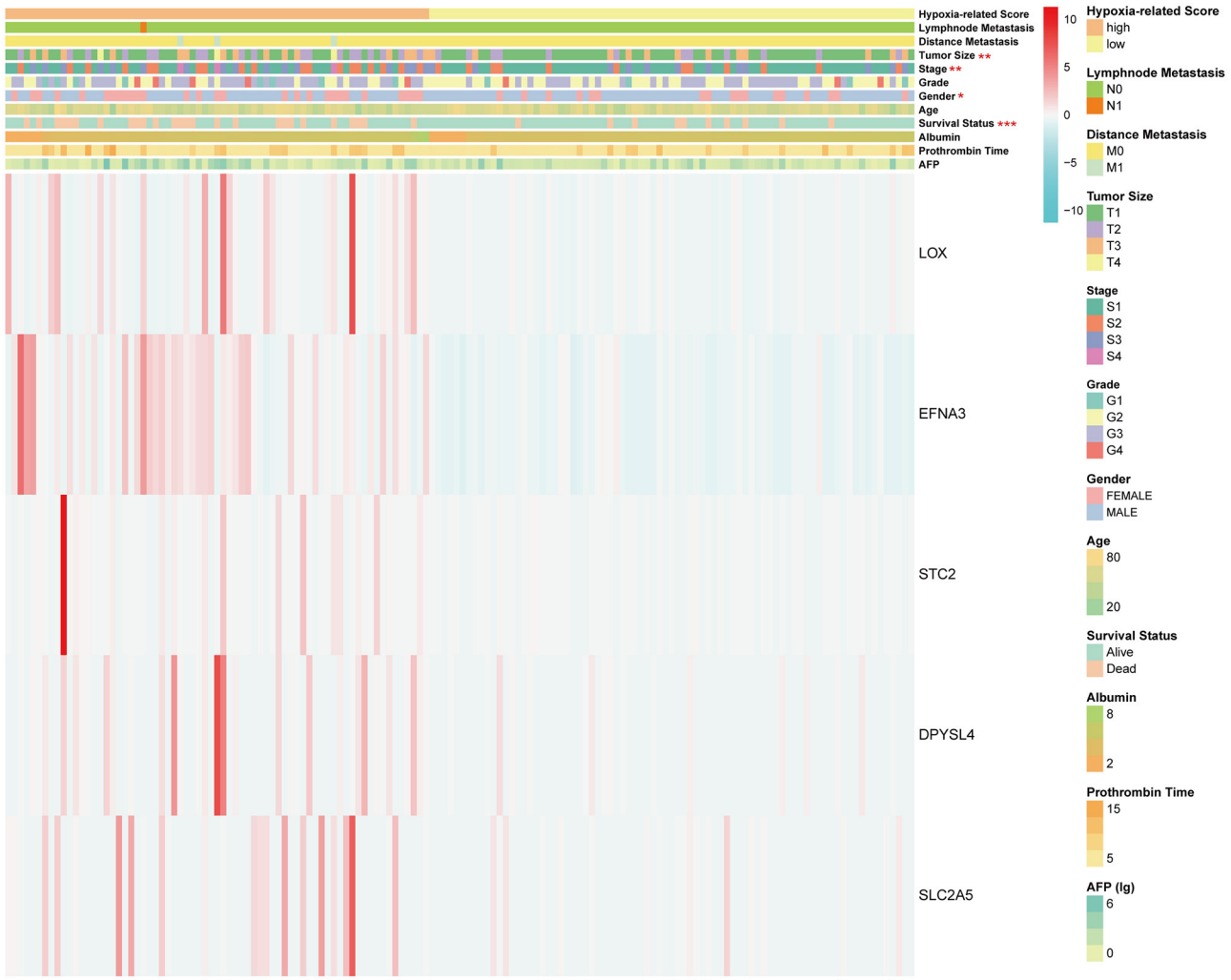

FIGURE 5 | Heatmap of hypoxia-related score. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$. The units of some outcomes are following: AFP (ug/L), prothrombin time (seconds) and albumin (g/dL).

two cohorts demonstrated that the higher the score, the worse the overall survival (**Figures 4F,G**). Furthermore, the heatmap in **Figure 5** indicates that the included genes were highly expressed in the hypoxia group. Hypoxia-related score were also significantly correlated with survival status, gender, tumor stage, and tumor size. In addition, when compared to other clinical parameters, the hypoxia-related score had the highest predictive value at both 3 and 5 years in two cohorts (**Figures 6A–D**). Univariate and multivariate Cox regression analysis indicated that the hypoxia-related score could serve as an independent prognostic marker in patients with immunosuppressive HCC (**Figures 6E,F**).

Verification Using the GSE14520 Cohorts

To validate the hypoxia-related subtype and verify the differences in the immune landscape, we used the independent GEO cohort (GSE14520) for patient clustering. The identified immunosuppressive cluster and hypoxia-related clusters were the same as those in the LIHC cohort (**Figures 7**, **8**). Significant differences in immune cell infiltration were observed between the hypoxia and non-hypoxia groups, and CD86 molecule, poliovirus receptor (PVR), CD96 molecule, signal-regulatory protein alpha (SIRPA), CD47 molecule and galectin 9 (LGALS9) were significantly correlated with the hypoxia cluster.

DISCUSSION

The TME has significant influence on HCC (23), as it contains non-malignant cells that can promote tumor cells proliferation and metastasis. The immunosuppressive features of tumors not only induce cancer progression, but are also a challenge for effective immunotherapy (23). Consequently, the identification of TME-associated biomarkers for HCC is urgently needed. In this study, we identified patients with immunosuppressive HCC using 29 immune-related gene sets and hierarchical

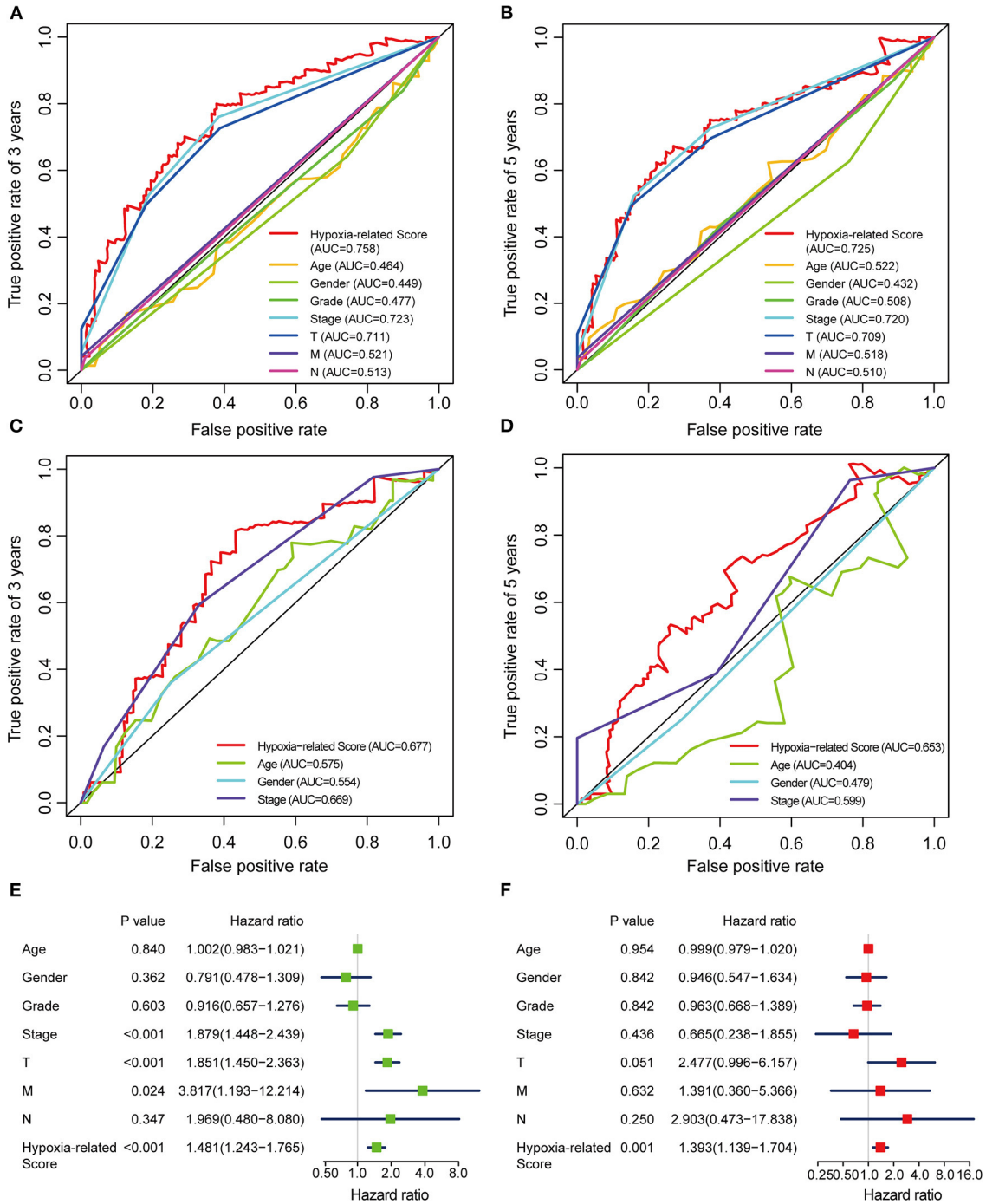

FIGURE 6 | ROC and Cox regression analysis of the hypoxia-related score **(A,B)** ROC analysis of the hypoxia-related score based on TCGA cohort at 3 **(A)** and 5 **(B)** years. **(C,D)** ROC analysis of the hypoxia-related score based on ICGC cohort at 3 **(C)** and 5 **(D)** years. **(E)** Univariable Cox regression analysis. **(F)** Multivariable Cox regression analysis. "T" represents tumor size, "M" represents distance metastasis, "N" represents lymph node metastasis.

clustering. The proportions of patients in the immune-activated and immune-suppressed groups were consistent with the generally immunosuppressive nature of HCC. A significant difference in immune-associated gene expression was also observed, which verified the reliability of identifying patients with immunosuppressive HCC. Subsequently, we investigated the underlying relationship between hypoxia and immunosuppression. Hypoxia is an intrinsic characteristic of solid tumors because of an imbalance between the growth of tumor cells and their nutrient supply (24). The hypoxic TME stimulates HIF-driven transcription, which results in cell proliferation and metastasis (25). Meanwhile, it has been

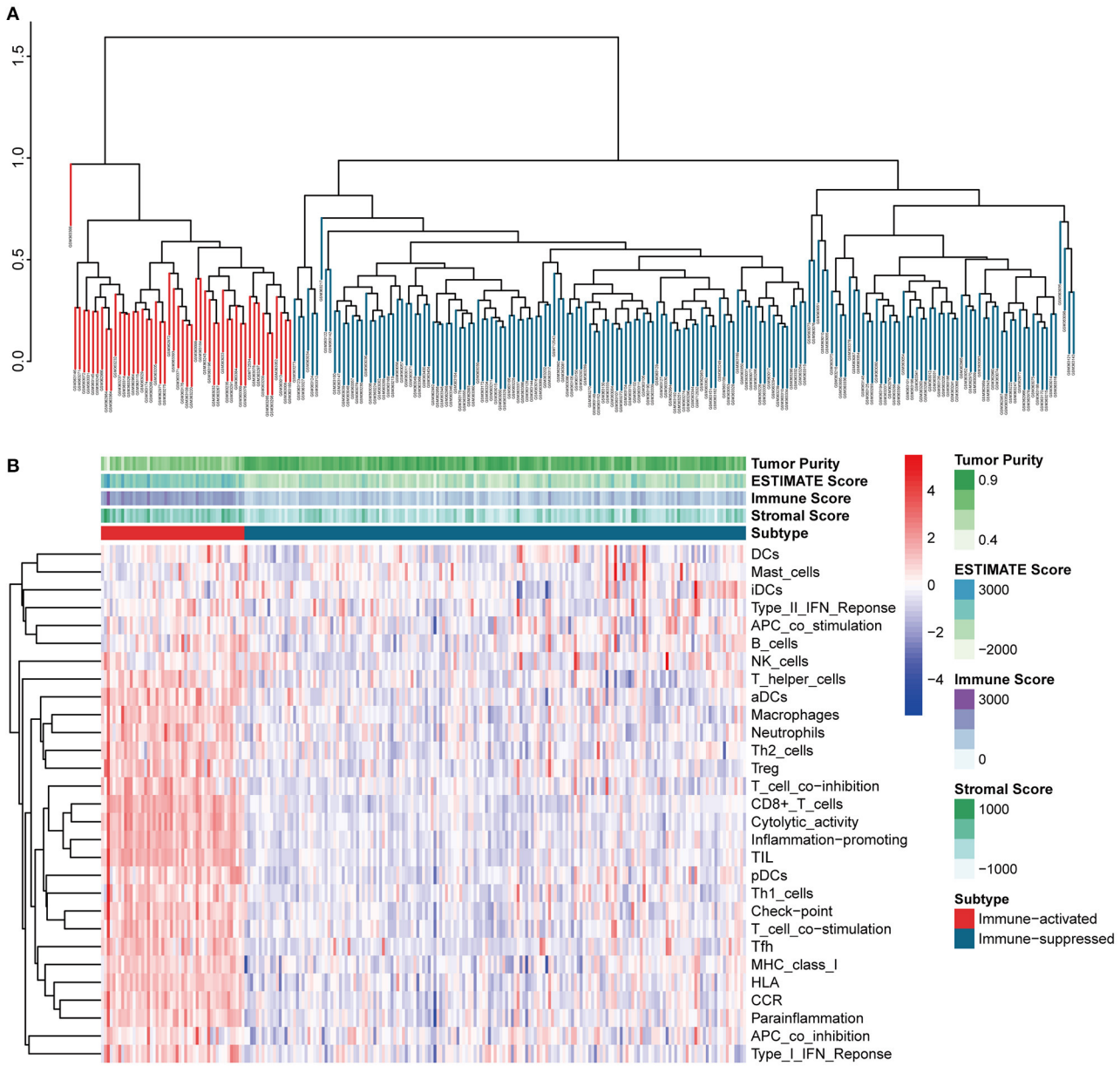

FIGURE 7 | Identification of immune-associated clusters in the GEO cohort **(A)** Hierarchical clustering of immune-activated (red) and immune-suppressed (blue) clusters. Each branch in the tree diagram represents one case in the GSE14520 cohort. **(B)** Heatmap of immune-associated clusters, ssGSEA results, and ESTIMATE score.

reported that hypoxia contributes to HCC cell proliferation, migration, and invasion (26), and accelerates malignant progression by impacting the crosstalk between stromal, tumor, and immune cells in the TME. For example, hypoxia can promote the recruitment of innate immune cells and interfere with the functions of adaptive immune cells (27). Therefore, we hypothesized that hypoxia could have an influence on patients with immunosuppressive HCC.

The hypoxia-associated genes employed in our research were all experimentally demonstrated to be upregulated in hypoxic conditions. Using these genes, we divided the patients with immunosuppressive HCC into two clusters. Hypoxic patients with immunosuppressive HCC exhibited higher immune cell infiltration and immune checkpoint expression, indicating an underlying correlation between hypoxia and the success of immunotherapy. A previous study (28) found that dynamic regulation of HIF1 activity is essential for normal B cell development. HIFs can also induce "don't-eat-me" signals that prevent phagocytosis and purinergic signaling, allowing tumor cells to evade immune surveillance (29). Based on the features of hypoxic patients with immunosuppressive HCC, hypoxia may be an effective

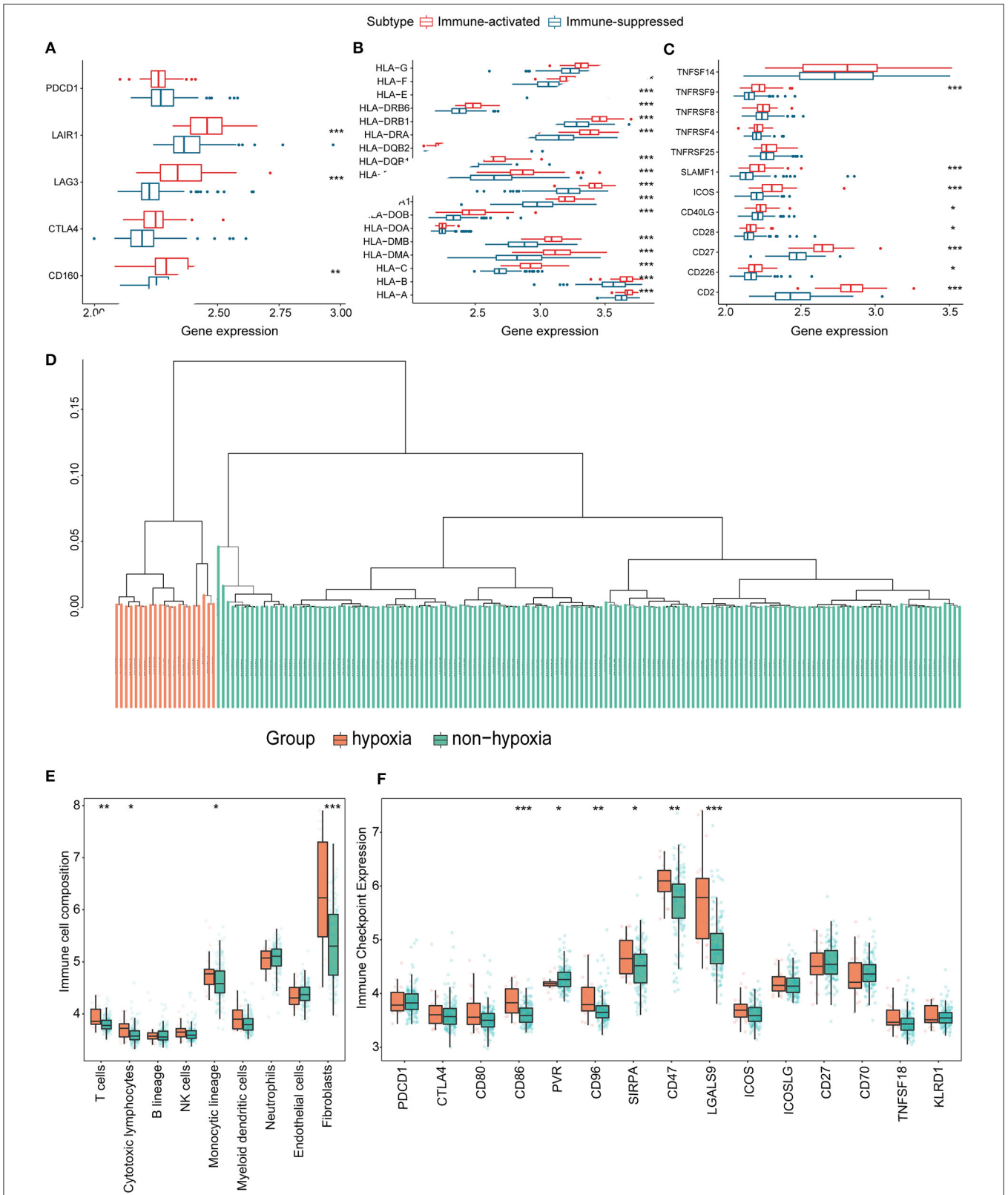

FIGURE 8 | Identification and validation of hypoxia-associated clusters in the GEO cohort. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$. **(A–C)** Expression of T cell inhibitors **(A)**, major histocompatibility complexes **(B)**, and T cell stimulators **(C)** in each immune-associated cluster. **(D)** Hierarchical clustering tree of the hypoxia (orange) and non-hypoxia (green) clusters. **(E)** Immune cell infiltration in each hypoxia-associated cluster using the MCP-counter method. **(F)** Immune checkpoint gene expression in the hypoxia-associated clusters.

biomarker to predict immunotherapeutic responses in patients with immunosuppressive HCC.

Immune subsets demonstrate different immunological functionality. It has been reported that B lymphocytes show numerous tumor-promoting characteristics (30). Another type of immune cells, NK cells, show protective and long-lasting immunity against diverse tumor types through direct cytotoxic activity or interacting with other immune cells (31). In our research, the infiltration of cells with immunosuppressive effect in hypoxia group like monocytic lineage and cancer-associated fibroblasts (both in TCGA and GEO) are higher than that in the non-hypoxia group, suggesting that hypoxia may aggravate the degree of immunosuppression. Classical monocytes mainly exhibit pro-tumor functions, such as differentiation into pro-tumor tumor-associated macrophages (TAMs), metastatic cell seeding, suppression of T cell function, recruitment of Tregs and so on (32). In terms of cancer-associated fibroblasts (CAFs), they are the main source of collagen-producing cells in the TME. CAFs provide mechanical support for tumor tissues and regulate the growth and invasion of tumor cells by remodeling the structure of the extracellular matrix (33). Therefore, we supposed that the more distinct infiltration of monocytes and CAFs played a crucial role in the immunosuppressive TME caused by hypoxia. Even the infiltration of cells with anti-tumor immune response like T cells (both in TCGA and GEO) are also higher in the hypoxia group, the function of T cell may be weakened due to hypoxia. It has been reported that hypoxia, adenosine, lactic acid and low pH impaired the ability of dendritic cells to stimulate T cell responses (34). The different infiltration level of the cells and differentially expressing immune checkpoint genes confirmed the difference of TME and immunotherapeutic response between two groups.

Furthermore, we constructed a novel scoring system (the hypoxia-associated score) to evaluate the hypoxic characteristics of patients with immunosuppressive HCC. The score included five genes (ephrin A3, dihydropyrimidinase like 4, solute carrier family 2 member 5, stanniocalcin 2, and lysyl oxidase) that were all highly expressed in the high-risk group and significantly correlated with worse prognosis. So far, only two of these genes (*STC2* and *LOX*) have been experimentally verified. Umezaki et al. concluded that LOX induced epithelial-mesenchymal transition and could be used to predict intrahepatic metastasis in HCC (35). Wang et al. (36) reported that high expression of STC2 may be associated with HCC occurrence, development, and prognosis. Although no evidence has been found to support the three other genes, they may be novel predictors in HCC. In addition, through the ROC plot and Cox regression analysis, the hypoxia-associated score presented their clinical potential and may serve as an independent predictive biomarker of HCC.

To our knowledge, this is the first study to identify a hypoxia-associated subtype of patients with immunosuppressive HCC. In contrast to a previous study (24), our study presented the following different points. Firstly, the sources of hypoxia-associated genes were distinct. Their study identified the relevant genes by differential expression analysis while we employed the hypoxia-associated genes from Molecular Signature Database. The hypoxia-associated gene set in our research was identified from four datasets (GSE18494, GSE30797, GSE33607, GSE9649) and validated from one dataset (GSE14762), which made the hypoxia-associated genes involved in our research more reliable and specific. Secondly, about 90% patients in our study were identified as the immunosuppressive cluster, which presented the different features (higher tumor purity, lower immune score, higher gene expression of immune checkpoints, and more antigen presentation) compared with the immune-activated cluster. We believe that it is necessary to identify the specific immunosuppressive cluster as the topic for a future study in HCC. Furthermore, the reliable hypoxia-associated genes and comprehensive methodology used in our study enabled the identification of a robust signature. We propose that this signature represents a novel biomarker to predict the immunotherapeutic responses of these patients in the clinic. Nevertheless, there are some limitations to our study. First, many other complicated mechanisms influence the development and progression of HCC, and there may be an intrinsic weakness in using a single characteristic to construct a predictive model. Second, no more available clinical information can be found in TCGA database, so the established prognostic model cannot take into account the clinical environment. Further, our evidence is based on bioinformatics methodology and should be considered preliminarily until its verification through more wet lab experiments and clinical trials.

In conclusion, our study illustrates the crucial role of hypoxia in patients with immunosuppressive HCC. The defined hypoxia-associated subtype may help reveal regulatory mechanisms between hypoxia and the immunosuppressive microenvironment, and its related score exhibits potential implications for future predictive models.

DATA AVAILABILITY STATEMENT

Publicly available datasets were analyzed in this study. This data can be found here: TCGA database (https://portal.gdc.cancer.gov/), GEO database (https://www.ncbi.nlm.nih.gov/geo/), and ICGC database (https://dcc.icgc.org/).

AUTHOR CONTRIBUTIONS

ZM and SZ designed the manuscript. ZM and DL wrote and completed the manuscript. ZM and DR completed the data download and analysis. 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.2021.611058/full#supplementary-material

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ORIGINAL RESEARCH
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TANK-Binding Kinase 1 (TBK1) Serves as a Potential Target for Hepatocellular Carcinoma by Enhancing Tumor Immune Infiltration

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Background: Numerous cancer types present the aberrant TANK-binding kinase 1 (TBK1) expression, which plays an important role in driving inflammation and innate immunity. However, the prognostic role of TBK1 and its relationship with immune cell infiltration in hepatocellular carcinoma (HCC) remain unclear.

Methods: The expression and prognostic value of TBK1 was analyzed by Tumor Immune Estimation Resource (TIMER), Kaplan-Meier plotter and Gene Expression Profiling Interactive Analysis (GEPIA), Clinical Proteomic Tumor Analysis Consortium (CPTAC) and further confirmed in the present cohort of patients with HCC. The association between TBK1 and HCC immune infiltrates, and its potential mechanism were investigated *via* analyses of the Tumor Immune Estimation Resource, tumor-immune system interactions database (TISIDB), CIBERSORT, STRING, and Metascape. The effect of TBK1 on immune infiltrates and the therapeutic value of targeting TBK1 were further investigated in a HCC mouse model by treatment with a TBK1 antagonist.

Results: The level of TBK1 expression in HCC was higher than that measured in normal tissues, and associated with poorer overall survival (GEPIA: hazard ratio [HR]=1.80, P=0.038; Kaplan–Meier plotter: HR=1.87, P<0.001; CPTAC: HR=2.23, P=0.007; Our cohort: HR=2.92, P=0.002). In addition, high TBK1 expression was found in HCC with advanced TNM stage and identified as an independent poor prognostic factor for overall survival among patients with HCC. In terms of immune infiltration, tumor tissues from HCC patients with high TBK1 expression had a low proportion of $CD8^+$ T cells, and TBK1 expression did not show prognostic value in HCC patients with enriched CD8+ T cells. Furthermore, TBK1 expression was positively correlated with the markers of T cell exhaustion and immunosuppressive cells in the HCC microenvironment. Mechanistically, the promotion of HCC immunosuppression by TBK1 was involved in the regulation of

inflammatory cytokines. *In vivo* experiments revealed that treatment with a TBK1 antagonist delayed HCC growth by increasing the number of tumor-infiltrating CD8+ T cells.

Conclusions: The up-regulated expression of TBK1 may be useful in predicting poor prognosis of patients with HCC. In addition, TBK1, which promotes the HCC immunosuppressive microenvironment, may be a potential immunotherapeutic target for patients with HCC.

Keywords: TANK-binding kinase 1, immune infiltration, inflammation, targeted therapy, hepatocellular carcinoma

INTRODUCTION

Hepatocellular carcinoma (HCC) is the most common primary liver malignancy and the fourth leading cause of cancer-related death worldwide (1). More than 50% of patients with HCC are diagnosed with advanced disease (2). Immunotherapy represents a promising strategy for many types of advanced cancer (3). The US Food and Drug Administration approved the use of checkpoint inhibitors (nivolumab and pembrolizumab) as a treatment option for advanced HCC (4). However, as a typically inflammation-associated cancer (5), HCC shows a unique immunosuppressive microenvironment enhanced by inflammation-related stromal cells and cytokines (6). This results in lower response and acquired resistance to checkpoint inhibitors (7). Therefore, it is urgent to identify novel therapeutic targets correlated with the HCC immunosuppressive microenvironment.

TANK-binding kinase 1 (TBK1) is a member of the inhibitor of nuclear factor-κB kinase (NF-κB) family (8). Upon receptor-mediated pathogen detection, TBK1 phosphorylation promotes the activation of the NF-κB pathway in the innate immune response (9). An initial study linking TBK1 to cancer found that TBK1 supports oncogenic Ras transformation with coupling innate immune signaling to tumor cell survival (10). Previous studies also demonstrated aberrant TBK1 expression and its pro-tumor effects in multiple cancers, including the promotion of migration and invasion in melanoma (11), AXL-induced epithelial–mesenchymal transition in pancreatic cancer (12), and tamoxifen resistance by increasing the transcriptional activity of estrogen receptor α in breast cancer (13). However, the underlying functions and mechanisms of TBK1 in HCC progression remain uncertain.

Recently, it was reported that TBK1 restrains the activation and migration of T cells, which are the main type of lymphocytes involved in the antitumor immune response (14, 15). Moreover, TBK1 contributed to tumor immunosuppression by down-regulating the expression of co-stimulatory molecules and decreasing T cell-priming activity in dendritic cells (16). However, another study yielded contrary results indicating that TBK1 participated in the activation of stimulator of the interferon genes pathway, enhancing antitumor immunity in the tumor microenvironment (17). Moreover, TBK1 was identified as a promoter of resistance to immunotherapy (9). Of note, inhibition of TBK1 effectively blocked the release of immune-suppressive cytokines and improved the therapeutic efficacy of anti-programmed death-ligand 1 (anti-PD-L1) (18). These findings prompted us to investigate the effects of TBK1 on the immune microenvironment and its potential value in the treatment of HCC.

In the present study, we investigated the correlation of TBK1 expression with prognosis and immune infiltration in patients with HCC. Mechanistically, we constructed TBK1-related gene networks and analyzed their function using bioinformatics tools. Importantly, the roles of TBK1 in HCC progression and immune infiltration were further explored *in vivo* (in immunodeficient and immunocompetent mice) using the TBK1 antagonist GSK8613. Our data revealed that TBK1 predicted poor prognosis in patients with HCC and may be a therapeutic target by attenuating tumor immunosuppression.

MATERIALS AND METHODS

UALCAN and Gene Expression Omnibus (GEO) Database Analysis

UALCAN is a comprehensive and interactive resource for analyzing cancer data (http://ualcan.path.uab.edu/index.html) (19). It provides access to publicly available cancer databases, including The Cancer Genome Atlas (TCGA) and MET500 data set. Moreover, it enables researchers to identify the up- or down-regulated genes in tumors compared with normal tissues, and compare the expression of genes of interest in subgroups, as defined by individual cancer stages, tumor grade, gender, age, nodal metastasis status, TP53 mutation status, and tumor histology. GEO2R is an interactive web tool that enables researchers to analyze the different expression of genes in two or more groups of samples across experimental conditions in a GEO series (20). In the present study, we investigated the levels of TBK1 mRNA expression in different types of cancer and corresponding normal tissues using UALCAN and GEO2R.

Gene Expression Profiling Interactive Analysis (GEPIA), Kaplan–Meier (KM) Plotter, and Clinical Proteomic Tumor Analysis Consortium (CPTAC) Database Analysis

The online database GEPIA is an interactive web server for the analysis of RNA sequencing expression data from the TCGA and Genotype-Tissue Expression projects, which include 9,736 tumors and 8,587 normal samples (21). The KM plotter is an

online available tool for exploring the effect of 54,675 genes on survival in 21 types of cancer. Sources for the databases include the GEO, TCGA, and European Genome-phenome Archive (22). We performed the survival analysis based on TBK1 mRNA expression in 33 different types of cancer using GEPIA and in 21 different types of cancer using the KM plotter. According to the mRNA expression of markers of CD4, CD8 and B cell in HCC tissues, the KM-plotter tool divided the HCC cohort from TCGA into enriched and decreased infiltration of the three types of cell. We used the KM-plotter to investigate the survival time of HCC patients based on the content of CD4, CD8 and B cell (https://kmplot.com/analysis/index.php?p=service&cancer=pancancer_rnaseq). The tool of "auto select best cutoff" (all possible cut off values between the lower and upper quartiles are computed, and the best performing threshold is used as a cutoff) in GEPIA and KM plotter were used to determine the cut-off values in the survival curves (mRNA level). CPTAC is a database established by The National Cancer Institute to promote the understanding of the molecular basis of cancer by applying large-scale proteomic and genomic analyses, or proteogenomics (23). Survival analysis based on TBK1 protein expression in HCC was also performed *via* the CPTAC database. The proteomic data of TBK1 in CPTAC (≤ 0.00368 defined as TBK1 low expression; > 0.00368 defined as TBK1 high expression) were analyzed to select the cut-off value in survival curves (protein level).

Tumor Immune Estimation Resource (TIMER) Database and Tumor-Immune System Interactions Database (TISIDB) Analysis

TIMER is a comprehensive resource for investigating the interactions between genes of interest and tumor immune interactions in more than 30 types of cancer (https://cistrome.shinyapps.io/timer/) (24). It has incorporated 10,897 samples across 32 types of cancer from TCGA to estimate the abundance of immune infiltrates. The TISIDB is a web portal for the analysis of tumor and immune system interaction; it integrates heterogeneous data types, including literature mining results from the PubMed database, high-throughput screening data, RNA sequencing data of patients with immunotherapy, and TCGA (25). In the present study, we investigated the correlation of TBK1 expression with tumor immune infiltration using TIMER and with tumoral activated $CD8^+$ T cells through the TISIDB in the HCC data set. The abundance profile of tumor-infiltrating immune cells in HCC samples from TCGA was calculated using the CIBERSORT computational method (26).

Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) Pathway Enrichment Analysis

Metascape is an online portal that integrates multiple bioinformatics knowledge bases to provide a comprehensive gene list annotation and analysis resource, especially for functional enrichment, gene annotation, and construction of protein-protein interaction networks (27). Here, we used Metascape to analyze the molecular and functional characteristics of TBK1 and its related genes

Reagents and Chemicals

TBK1 inhibitor GSK8612 were purchased from Selleck Chemicals (S8872). For *in vitro* experiments, GSK8612 were dissolved in DMSO (Sigma-Aldrich, MO, USA) and further diluted to the required concentration. For *in vivo* experiments, GSK8612 suspension was prepared in 0.5% carboxymethyl cellulose sodium normal saline solution. Antibodies to TBK1 were purchased from Proteintech. Antibodies to α-SMA, CD8α, phospho-TBK1 (p-TBK1) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were purchased from Cell Signaling Technology.

Cell Proliferation and Migration Assay

Hepa1-6 and H22 cell line were gifts from Dr. Limin Zheng (School of Life Sciences, Sun Yat-Sen University, Guangzhou, China). Hepa1-6 cells were cultured in DMEM supplemented with 10% inactivated fetal bovine serum and 1% penicillin-streptomycin (Gibco, USA). Hepa1-6 cells were seeded at 1,000 cells per well in 96-well microplates and incubated in normal growth medium for 24 h. Subsequently, the cells were treated with DMSO or GSK8612 for an additional 24, 48, or 72 h. Cell viability was measured using the Cell Counting Assay Kit-8 (CCK-8; Dojindo, Kumamoto, Japan) according to the manufacturer's instructions. Cell migration assays were performed on transwell chambers with 8-μm pore-size filters. Cells were trypsinized and resuspended in serum-free medium with DMSO or GSK8612. 250 μl of cell suspension (1×10^5 cells) was added to the upper chambers in a transwell insert, and the upper chambers were then placed into the wells of a 24-well plate. 750 μl culture medium containing 20% fetal bovine serum (FBS) was added to the lower chamber. After transwell inserts were cultured at 5% CO2 at 37°C for 24 h, cells on the top of the membrane were removed with a cotton swabs. Cells attached on the underside of the membrane were fixed and stained with 0.1% crystal violet. After washing with phosphate-buffered saline (PBS), the number of cells was counted in three random microscopic fields under the microscope.

Histological and Immunohistological Analysis of Liver Sections

Liver and tumor tissues were fixed with 10% formalin, embedded in paraffin and cut into 2 mm sections for staining with hematoxylin-eosin (H&E), Sirius red and immunohistochemistry according to standard procedures (28). For immunohistochemistry (IHC), tumor sections were stained with the appropriate antibodies, and both the intensity and extent of immunostaining were taken into consideration when analyzing the data. The intensity was scored as 0 for negative, 1 for weak staining, 2 for moderate staining and 3 for strong staining. The extent of staining was scored as 0, 0.25, 0.50, 0.75, and 1.00 for less than 5%, 6%–25%, 26%–49%, 50%–74%, and 75%–100% positively stained cells, respectively. The final quantitation of each staining was obtained by multiplying these two values (intensity score × extent score) (29). TBK1

expression was classified as high expression if the score was higher than 1.5; if the score was 1.5 or less, the case was classified as low expression. Two different pathologists who specialize in liver cancer evaluated the results of IHC.

Western Blotting

The total cellular protein and tissue protein was extracted by RIPA Lysis Buffer (Thermo Fisher Scientific, MA, USA) and RIPA Lysis Buffer (Thermo Fisher Scientific) containing protease inhibitors and phosphatase inhibitors (Thermo Fisher Scientific). The protein concentrations of the cell lysates were measured using a Pierce™ BCA Protein Assay Kit (Thermo Fisher Scientific) and equalized before loading. Equal amount of protein extracts from HCC cells or tissues were separated by SDS–PAGE, and transferred onto polyvinylidene fluoride membranes (Sigma-Aldrich, MO, USA). Immunoblot analyses were carried out using the appropriate antibodies, and the bands were visualized using an SuperSignal™ West Pico PLUS chemiluminescence Substrate (Thermo Fisher Scientific).

Flow Cytometry

Fresh mouse liver tissues were finely chopped and dissociated into single-cell suspensions. After removal of red blood cells and liver cells, the leukocytes were further purified using a magnetic-activated cell-sorting separator with CD45 magnetic beads (Miltenyi Biotec, CA, USA). After incubation with V450-labeled CD3, PerCP-Cy™-labeled CD4, and V500-labeled CD8 (BD Biosciences, CA, USA), tumor-infiltrated T cells were detected by a flow cytometer (BD LSRFortessa X-20). Gating strategy for $CD4^+$ and $CD8^+$ T-cell in HCC tissues: lymphocytes were gated by forward and side scatter properties, and then $CD4^+/CD8^+$ T-cells were gated for further analysis (30).

Enzyme-Linked Immunosorbent Assay (ELISA)

The HCC tissues from mouse model collected above were weighed and homogenized at 4°C. Homogenates were centrifuged at 14,000xg for 10 min at 4°C. Supernatants were transferred to clean microcentrifuge tubes for detection. Specific ELISA kits (Jiangsu Meimian industrial, Jiangsu, China) were used to quantitate IL-6 according to the manufacturer's instructions.

In Vivo Treatment Studies

Male immunodeficient (BALB/c nude) and immunocompetent (C57BL/6) mice (aged 4–6 weeks) were subjected to carbon tetrachloride (CCl_4) gavage (40% in 100 μl of olive oil per mouse, volume/volume) for 4 weeks to induce the inflammatory liver microenvironment. Subsequently, mice were injected with 25 μl of HCC cell/Matrigel solution (containing 1×10^6 Hepa1–6 cells) in the subcapsular region of the liver, and were divided into the control or treatment groups (31). On day 3 following inoculation with tumor cells, the TBK1 antagonist GSK8612 was administered orally at the dose of 5 mg/kg for 7 days. Mice were sacrificed 10 days after HCC implantation. The mice were maintained in the laboratory for animal experimentation in a specific pathogen-free environment with laminar air-flow conditions, a 12-h light-dark cycle, and at a temperature of 22°C–25°C. All animals had free access to standard laboratory mouse food and water. Animal experiments were approved by the Bioethics Committee of Jinan University (China) and performed according to established guidelines.

Patients and Specimens

Liver samples (n=139) from patients with HCC who underwent hepatectomy were collected in the First Affiliated Hospital of Jinan University. Patient samples were collected and used with the informed written consent of the patient. All liver samples were obtained under protocols approved by the First Affiliated Hospital of Jinan University Office for Protection of Human Subjects.

Statistical Analysis

The Student's *t* test was used to compare values between two groups and the ANOVA was employed to compare between subgroups with more than two groups. Overall survival (OS) was calculated by KM survival analysis and log-rank tests. Data were expressed as the mean ± standard deviation of at least three biological replicates. $P < 0.05$ denoted statistical significance. All analyses were performed using the SPSS software (Version 23.0; IBM, Armonk, NY, USA).

RESULTS

TBK1 Expression Was Up-Regulated in HCC Tissues

TIMER and UALCAN were used to analyze the transcriptome-sequencing data from TCGA data set to evaluate the differences in TBK1 expression between tumor and normal samples. The results obtained from TIMER revealed that TBK1 expression was up-regulated in nine types of cancer, including liver hepatocellular carcinoma (LIHC), whereas it was down-regulated in only one type of cancer (**Figure 1A**). Moreover, the results obtained from UALCAN indicated that TBK1 expression was significantly increased in bladder urothelial carcinoma (BLCA), breast invasive carcinoma (BRCA), cholangiocarcinoma (CHOL), colon adenocarcinoma (COAD), esophageal carcinoma (ESCA), head and neck squamous cell carcinoma (HNSC), kidney renal clear cell carcinoma (KIRC), kidney renal papillary cell carcinoma (KIRP), LIHC, lung adenocarcinoma (LUAD), lung squamous cell carcinoma (LUSC), and stomach adenocarcinoma (STAD) (**Figure 1B**).

We further confirmed the expression of TBK1 in multiple human cancers using microarray data sets from GEO. Higher TBK1 expression was found in the subtype of breast cancer, cervical cancer, colorectal cancer, gastric cancer, head and neck cancer, kidney cancer, leukemia, liver cancer, and pancreatic cancer compared with that measured in normal tissues or cells. Meanwhile, TBK1 expression was lower in the subtype of brain cancer (**Table 1**). In addition, the protein level of TBK1 expression in HCC and liver tissues were also determined with immunohistochemistry staining. TBK1 was mainly expressed in

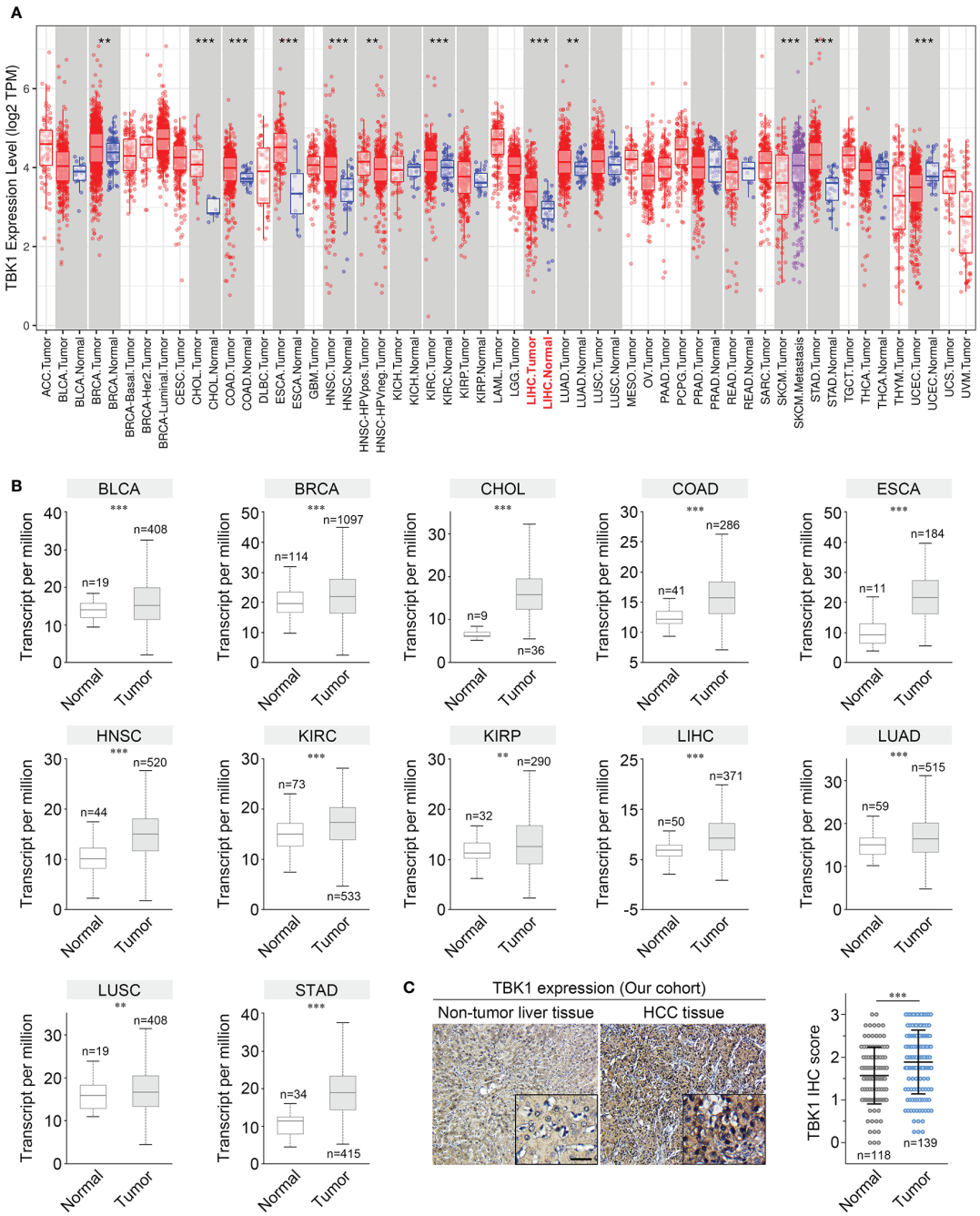

FIGURE 1 | TANK-binding kinase 1 (TBK1) expression levels in human cancer. The levels of TBK1 mRNA expression in different types of human cancer were determined using Tumor Immune Estimation Resource (TIMER) **(A)** and UALCAN **(B)**. **(C)** Representative images of immunohistochemistry (IHC) staining with a TBK1 antibody on HCC tissues (n = 138) and corresponding normal tissues (n = 118) in our cohort. ACC, Adrenocortical carcinoma; BLCA, Bladder urothelial carcinoma; BRCA, Breast invasive carcinoma; BRCA-Basal/Her2/Luminal, Breast invasive carcinoma-Basal/Her2/Luminal; CESC, Cervical squamous cell carcinoma and endocervical adenocarcinoma; CHOL, Cholangiocarcinoma; LIHC, Liver hepatocellular carcinoma; COAD, Colon adenocarcinoma; READ, Rectum adenocarcinoma; DLBC, Lymphoid neoplasm diffuse large B-cell lymphoma; LAML, Acute myeloid leukemia; ESCA, Esophageal carcinoma; GBM, Glioblastoma multiforme; LGG, Brain Lower Grade Glioma; HNSC, Head and neck squamous cell carcinoma; HNSC- HPVneg, Head and neck squamous cell carcinoma-HPVneg; KICH, Kidney chromophobe; KIRC, Kidney renal clear cell carcinoma; KIRP, Kidney renal papillary cell carcinoma; LUAD, Lung adenocarcinoma; LUSC, Lung squamous cell carcinoma; MESO, Mesothelioma; OV, Ovarian serous cystadenocarcinoma; PAAD, Pancreatic adenocarcinoma; PCPG, Pheochromocytoma and Paraganglioma; PRAD, Prostate adenocarcinoma; SARC, Sarcoma; SKCM, Skin cutaneous melanoma; SKCM-Metastasis, Skin cutaneous melanoma- Metastasis; STAD, Stomach adenocarcinoma; TGCT, Testicular Germ Cell Tumors; THCA, Thyroid carcinoma; THYM, Thymoma; UCEC, Uterine Corpus Endometrial Carcinoma; UCS, Uterine Carcinosarcoma; UVM, Uveal Melanoma. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

TABLE 1 | Significant changes in TANK-binding kinase 1 (TBK1) expression in cancer versus normal tissue in GEO the database.

Cancer	Subtype	Fold change	*P* value	Adjusted *P* Value	Reference (PMID)	GEO accession number
Breast	Ductal Breast Carcinoma *in situ*	1.434	<0.001	0.009	19187537	GSE14548
Brain	Oligodendroglioma	−1.569	<0.001	<0.001	16616334	GSE4290
Cervical	Cervical Squamous Cell Carcinoma	1.428	<0.001	<0.001	18191186	GSE7410
	Cervical cancer	4.287	<0.001	<0.001	17510386	GSE6791
Colorectal	Rectal carcinoma	1.504	<0.001	<0.001	18171984	GSE8671
Gastric	Gastric mixed adenocarcinoma	1.727	<0.001	<0.001	19081245	GSE13911
Head and neck	Nasopharyngeal carcinoma	1.651	<0.001	<0.001	17119049	GSE12452
Kidney	Clear cell renal cell carcinoma	1.784	<0.001	<0.001	17699851	GSE6344
	Renal pelvis urothelial carcinoma	1.649	<0.001	<0.001	16115910	GSE15641
Leukemia	T-cell prolymphocytic leukemia	2.543	<0.001	0.012	17713554	GSE5788
Liver	Hepatocellular carcinoma	1.512	0.006	0.037	22689435	GSE50579
Pancreatic	Pancreatic ductal adenocarcinoma	1.656	<0.001	<0.001	19260470	GSE15471

The data sets used in the current study has been published in relevant references and can be obtained by GEO accession.

hepatocytes and HCC cells, and were also detected in stromal cells. In line with the results obtained from TCGA and GEO databases, the findings of this study indicate that TBK1 expression was significantly increased in HCC tissues (P<0.001) (**Figure 1C**).

TBK1 Expression Has Prognostic Significance for Patients With HCC

We performed a survival analysis based on TBK1 mRNA expression by GEPIA in 33 types of cancer to estimate the influence of TBK1 expression on prognosis in patients with cancer. Although the analysis of relapse-free survival (RFS) in patients with HCC did not reach statistical significance, HCC patients with high TBK1 expression had significantly shorter OS (HR=1.800, P=0.038) (**Figure 2A**). In addition, high levels of TBK1 expression were correlated with poorer prognosis of OS in BRCA, ESCA, kidney chromophobe (KICH), KIRP, brain lower grade glioma (LGG), LUAD, Ovarian serous cystadenocarcinoma (OV), pancreatic adenocarcinoma (PAAD), and uveal melanoma (UVM). On the contrary, low levels of TBK1 expression were

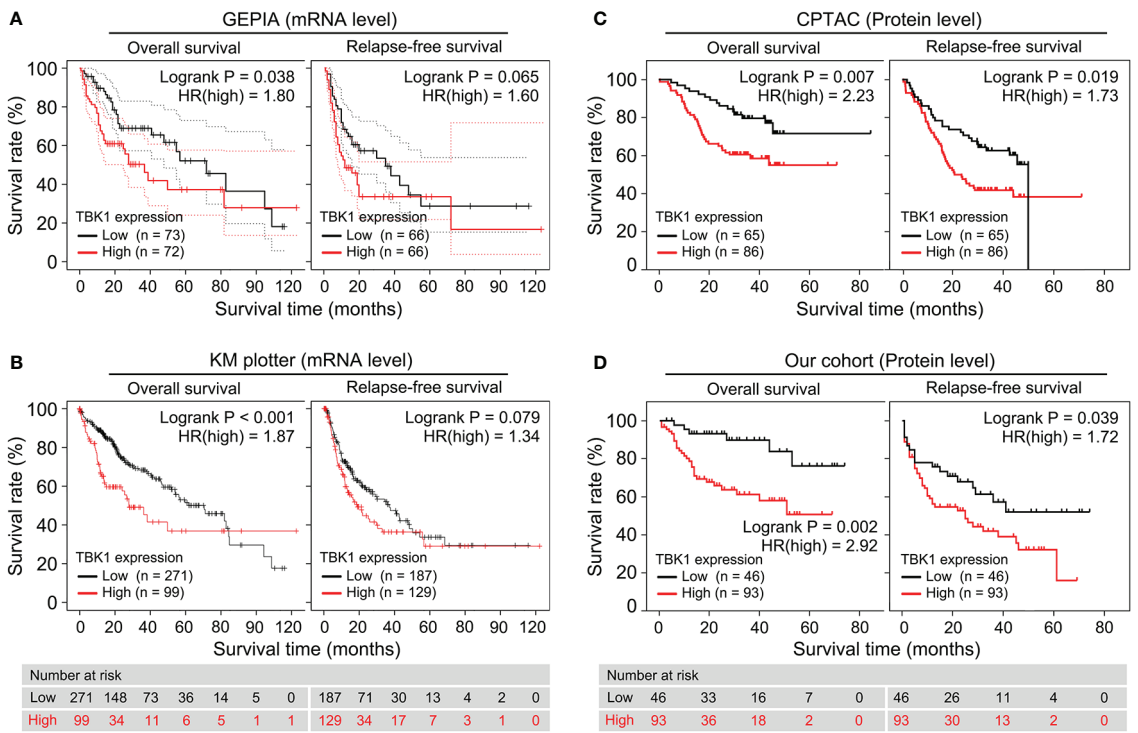

FIGURE 2 | High TANK-binding kinase 1 (TBK1) expression predicted poor prognosis in patients with hepatocellular carcinoma (HCC). **(A, B)** Gene Expression Profiling Interactive Analysis (GEPIA) and the Kaplan–Meier (KM) plotter were used to construct the survival curves of overall survival (OS) and relapse-free survival (RFS) based on the TBK1 mRNA expression in patients with HCC. **(C, D)** The KM survival curves based on TBK1 protein expression in patients with hepatocellular carcinoma (HCC) were determined using Clinical Proteomic Tumor Analysis Consortium (CPTAC) database and our cohort.

correlated with poorer prognosis of OS in rectum adenocarcinoma (READ), thymoma (THYM), and uterine carcinosarcoma (UCS) (**Supplementary Figure 1**).

Next, the prognostic potential of TBK1 in different types of cancer was validated by a pan-cancer analysis of 21 types of cancer *via* the KM plotter. Consistent with the results obtained from GEPIA, the KM plotter indicated that high TBK1 expression was correlated with poorer OS (HR=1.870, $P < 0.001$), but not with RFS (**Figure 2B**). Moreover, the findings of the pan-cancer analysis suggested that increased levels of TBK1 expression were associated with worse OS in ESCA, KIRC, LUAD, Pheochromocytoma and Paraganglioma (PCPG), and THYM; however, they were linked to better OS in BLCA, sarcoma (SARC), and thyroid carcinoma (THCA) (**Supplementary Figure 2**).

Furthermore, the association between the levels of TBK1 protein expression and OS or RFS were investigated in the CPTAC database and our cohort. The analysis demonstrated that the protein levels of TBK1 expression were significantly correlated with poorer OS (CPTAC: HR=2.23, $P = 0.007$; Our cohort: HR=2.92, P=0.002) and RFS (CPTAC: HR=1.73, P=0.019; Our cohort: HR=1.72, P=0.039) in patients with HCC (**Figures 2C, D**).

TBK1 Expression Correlated With Clinicopathological Characteristics and Was Identified as the Independent Prognostic Factor for OS Among Patients With HCC

We analyzed the TBK1 expression based on eight widely recognized clinicopathological parameters of the HCC data set from TCGA, including age, gender, alpha-fetoprotein (AFP), tumor stage, tumor grade, T classification, vascular invasion, liver fibrosis, and the value of platelet-to-albumin ratio. Compared with normal liver tissues, TBK1 expression was markedly increased in HCC classified as Stages I–IV or Grades 1–4. In addition, higher TBK1 expression was found in Stage III

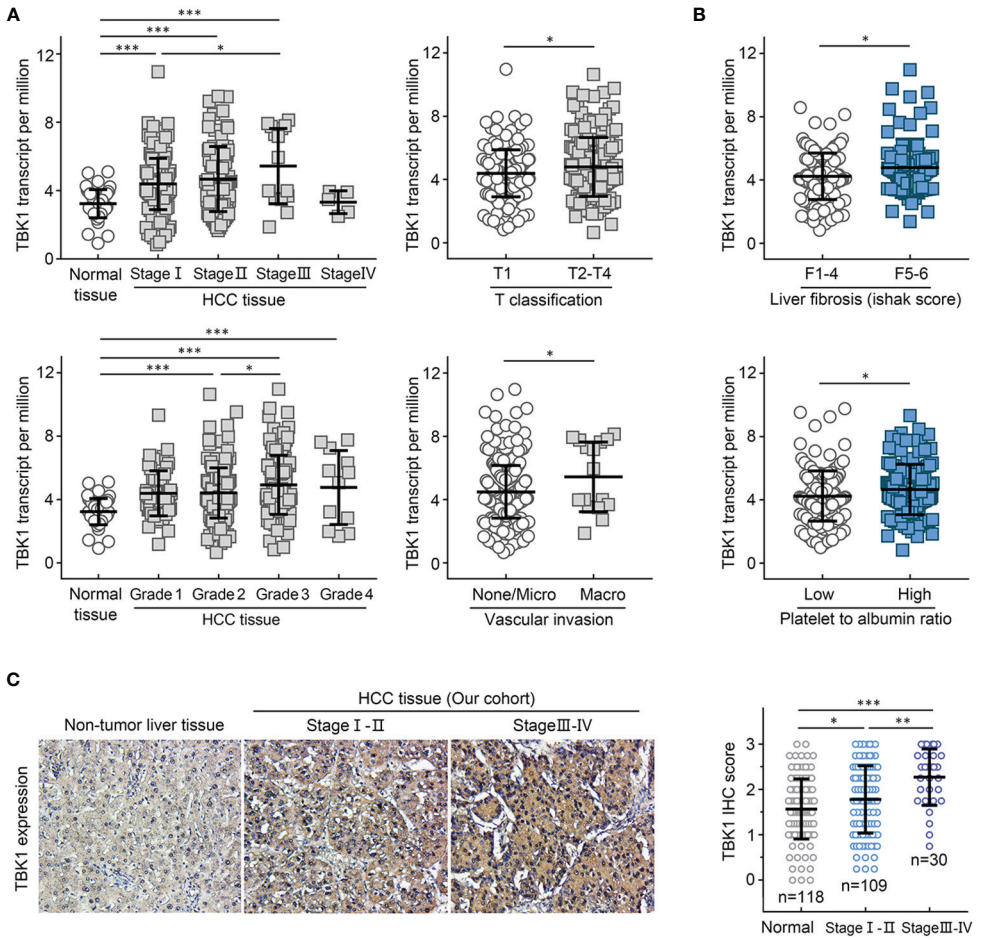

FIGURE 3 | TANK-binding kinase 1 (TBK1) expression was associated with clinicopathological characteristics of patients with hepatocellular carcinoma (HCC). The HCC data set from The Cancer Genome Atlas (TCGA) was used to analyze the levels of TBK1 expression based on the clinical parameters of HCC (TNM stage, grade, T classification, and vascular invasion) **(A)**, and inflammation indicators of patients with HCC (liver fibrosis and platelet-to-albumin ratio) **(B)**. **(C)** The expression levels of TBK1 in different stages of patients with HCC from the present cohort (n=139). *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

HCC versus Stage I and Grade 3 HCC versus Grade 2 (**Figure 3A**). Moreover, patients with a more advanced T classification ($P = 0.020$), severer vascular invasion ($P = 0.031$), higher degree of liver fibrosis ($P = 0.017$), and higher value of platelet-to-albumin ratio ($P = 0.027$) tended to have higher mRNA expression levels of TBK1 (**Figures 3A, B**). Meanwhile, there was no significant association between TBK1 expression and age, sex, or AFP value in patients with HCC (data not shown). We further examined the correlation of levels of TBK1 protein expression in HCC patients with the mentioned clinicopathological characteristics in the present cohort. The analysis demonstrated that increased TBK1 expression was associated with higher degree of platelet-to-albumin ratio, liver fibrosis and tumor stage (**Supplementary Table 1**, **Figure 3C**). These data suggested that HCCs with higher TBK1 expression were more aggressive.

Furthermore, the HCC data set from TCGA and the present cohort were used to determine the independent prognostic potential of TBK1 expression for OS by univariate and multivariate Cox regression analyses. In the HCC data set from TCGA, the univariate analysis indicated that vascular invasion (HR = 1.982, $P = 0.029$), advanced stage (HR = 2.066, $P = 0.022$), and high TBK1 expression (HR = 2.784, $P = 0.002$) significantly contribute to the poor OS. Importantly, the multivariate analysis demonstrated that high expression of TBK1 was an independent risk factor for poor OS in patients with HCC (HR = 2.473, $P = 0.009$) (**Table 2**). In addition, the analysis of present cohort by Cox regression consistently showed the independent prognostic potential of TBK1 expression for OS in patients with HCC (**Supplementary Table 2**). The above results indicated that high levels of TBK1 expression led to poor prognosis and may promote tumor progression in patients with HCC.

Poor Prognosis of HCC Patients With High TBK1 Expression Was Attributed to the Decreased Levels of Tumor-Infiltrating CD8$^+$ T Cells

Liver fibrosis and the platelet-to-albumin ratio (**Figure 3B**) are important indicators of liver inflammation, which results in impaired antitumor immune response (5, 32). Therefore, the association between TBK1 expression and degree of immune infiltration in HCC was further investigated in this study. We analyzed the correlation between TBK1 expression and immune marker genes (33) of B, T, and natural killer (NK) cells, which have been identified as important immune effector cells exerting the antitumor response in HCC (14, 34). The data indicated that TBK1 expression was significantly correlated with two markers of T cells (CD3D and CD3E), one marker of B cells (CD19), and one marker of NK cells (KIR2DL3) (**Figure 4A**). Moreover, we further investigated the correlation between TBK1 expression and immune markers of different functional T cells including CD4$^+$ T cells, CD8$^+$ T cells, Th1 cells, Th2 cells, Tfh cells, and Th17 cells. The results revealed that the TBK1 expression level was significantly correlated with most immune marker sets of T cell in HCC (**Supplementary Figure 3A**). The landscape of tumor-infiltrating immune cells was obtained using the CIBERSORT algorithm, and 22 types of immune cell profiles in patients from the HCC data set of TCGA were constructed to further confirm the association of TBK1 expression with the

TABLE 2 | Univariate and multivariate Cox regression analyses of TANK-binding kinase 1 (TBK1) mRNA expression for overall survival (OS) in patients with hepatocellular carcinoma (HCC) from The Cancer Genome Atlas (TCGA) data set.

Characteristics	OS (n=169)			
	Univariate analysis		Multivariate analysis	
	Hazard	*P* value	1Hazard	*P* value
Age (year)				
≥60 *vs.* <60	1.696 (0.926–3.106)	0.087		
Gender				
Male *vs.* female	0.606 (0.335–1.098)	0.099		
Platelet to albumin ratio				
High *vs.* Low	1.264 (0.688–2.322)	0.450		
Liver fibrosis				
Cirrhosis vs non-cirrhosis	1.117 (0.603–2.031)	0.744		
AFP				
≥400 *vs.* <400	1.319 (0.677–2.571)	0.415		
Vascular invasion				
Yes *vs.* No	1.982 (1.074–3.658)	**0.029**	1.544 (0.818–2.917)	0.180
Tumor grade				
3+4 *vs.* 1 + 2	1.584 (0.882–2.846)	0.123		
Tumor stage				
III+IV *vs.* I+II	2.066 (1.110–3.846)	**0.022**	1.923 (1.027–3.601)	**0.041**
TBK1 expression				
High *vs.* Low	2.784 (1.438–5.395)	**0.002**	2.473 (1.253–4.881)	**0.009**

The parameter including age, gender, platelet-to-albumin ratio, liver fibrosis, alpha-fetoprotein (AFP), vascular invasion, tumor grade, tumor stage, and TBK1 expression in HCC were used for univariate Cox regression analyses and significant parameters were included in further multivariate Cox regression analyses.
Bold values denote statistical significance at the p < 0.05 level.

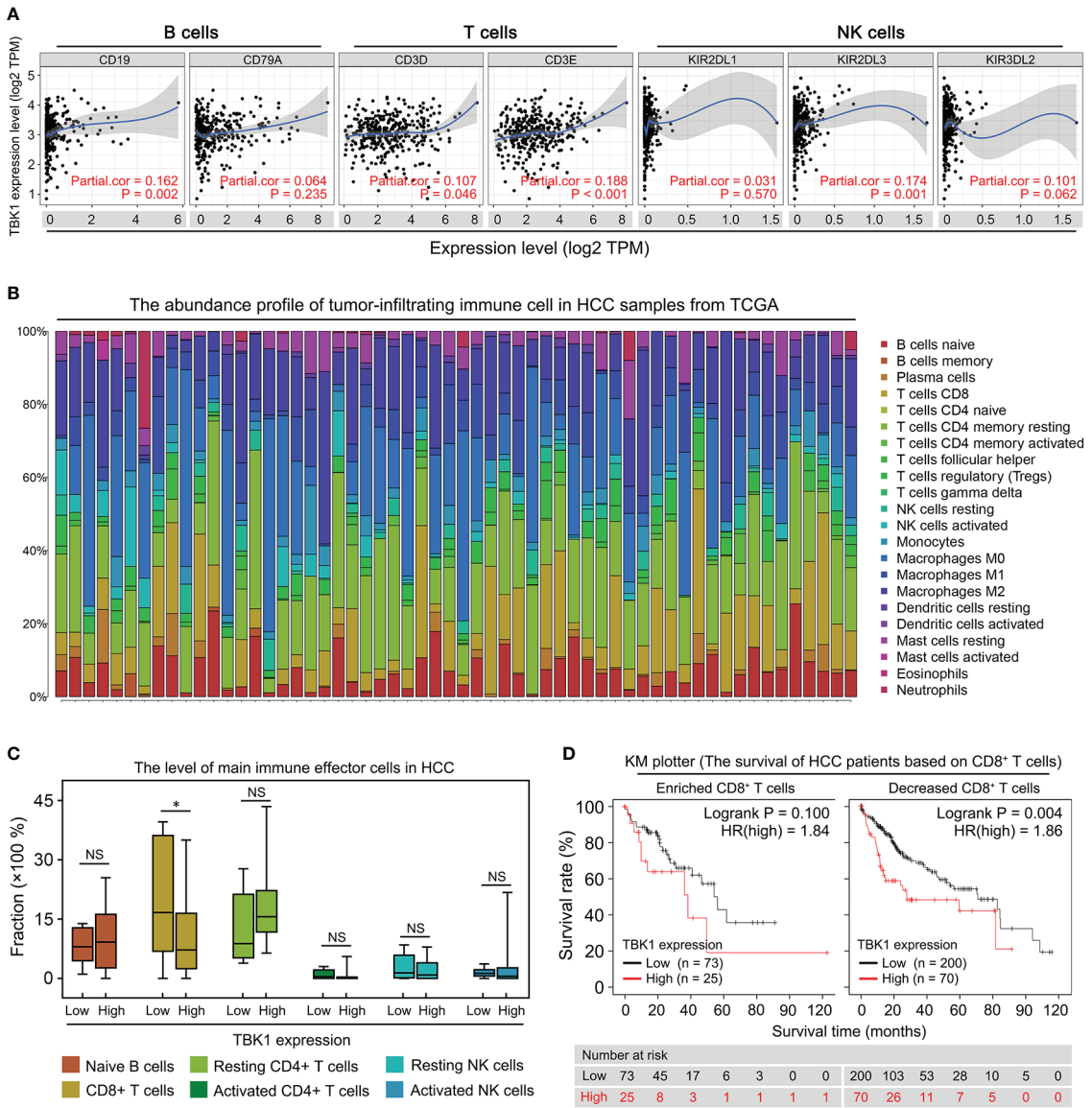

FIGURE 4 | Correlation of TANK-binding kinase 1 (TBK1) expression with tumor immune infiltration in patients with hepatocellular carcinoma (HCC). **(A)** Tumor Immune Estimation Resource (TIMER) was used to analyze the correlation of TBK1 expression with the markers of immune effector cells [B, T, and natural killer (NK) cells]. **(B)** 22 tumor-infiltrating immune cells in HCC samples were estimated using the CIBERSORT algorithm. **(C)** The proportion of main immune effector cells in HCC tissues with high and low TBK1 expression. **(D)** Kaplan–Meier overall survival (OS) curve of high and low TBK1 expression in HCC based on the number of tumor-infiltrating $CD8^+$ T cells. NS, not significant; $^*P < 0.05$.

immune effector cells in this disease (**Figure 4B**). The analysis demonstrated that patients with high TBK1 expression had significantly higher proportions of $CD8^+$ T cells. However, there were no significant differences detected in the infiltration levels of B, $CD4^+$ T, and NK cells (**Figure 4C**).

We performed a prognosis analysis of TBK1 expression in the immune cells subgroup *via* the KM plotter to examine whether the poor prognosis of HCC patients with high TBK1 expression is related to immune infiltration. The results showed that TBK1 overexpression in HCC samples with enriched or decreased B cells, and enriched or decreased $CD4^+$ T cells was a significant indicator of poor prognosis (**Supplementary Figures 3B, C**). However, high TBK1 expression predicted poor prognosis in patients with decreased $CD8^+$ T cells, but not in those with enriched $CD8^+$ T cells (**Figure 4D**). The above data suggested that high TBK1 expression in HCC contributed to tumor progression and poor prognosis at least partly owing to the decreased number of $CD8^+$ T cells.

TBK1 Expression Is Significantly Correlated With the HCC Immunosuppressive Microenvironment

The decreased number and impaired function of $CD8^+$ T cells are mostly resulted by the immunosuppressive molecules and cells in tumor microenvironment (35, 36). Therefore, we used TIMER to investigate the correlation of TBK1 expression with immunosuppressive molecules, the immune checkpoints, involved in T cell exhaustion (37). The analysis suggested that the level of TBK1 expression was positively correlated with the PD-L1 (r = 0.592, $P < 0.001$), hepatitis A virus cellular receptor 2 (HAVCR2; r=0.397, $P < 0.001$), programmed cell death protein 1 (PD-1; r = 0.146, $P = 0.006$), and cytotoxic T lymphocyte-associated antigen-4 (CTLA4; r = 0.161, $P = 0.003$) (**Figure 5A**). The expression of these immune checkpoints is rapidly up-regulated upon T cell activation, and contributes to the deterioration of T cell function (36). Subsequently, we analyzed the correlation of TBK1 expression with the activation of $CD8^+$ T cells by TISIDB, and found that the activated $CD8^+$ T cell was negatively correlated with TBK1 expression in LIHC data set (r = - 0.211, $P < 0.001$) (**Figure 5B**). Moreover, myeloid-derived suppressor cell (MDSC), tumor-associated macrophage (TAM) and regulatory T cell (Treg) are the main immunosuppressive cells in HCC microenvironment (38). The data from TIMER demonstrated the immune marker sets (37, 39) of MDSC (CD33, ITGAM, FUT4), TAM (CCL2, CD68, IL-10) and Treg (FOXP3, CCR8, STAT5B) were significantly correlated with the TBK1 expression (**Figure 5C**).

TBK1 Is Involved in the Functional Network of Inflammatory Cytokines

TBK1-related genes with similar expression patterns were examined using the STRING (functional protein association networks) to better understand the underlying mechanisms of the effects of TBK1 expression on immune infiltration. According to the results, we incorporated the up-regulated top 40 proteins-encoding genes that mostly correlated with TBK1 expression for further analysis. The 40 protein-encoding genes are shown below: RELA, IRF3, TAX1BP1, RNF135, TRAF3, OPTN, UBC, IFI16, IKBKE, TRAF2, SQSTM1, LY96, TICAM2, SIKE1, TICAM1, IRF7, IKBKG, NLRP4, TANK, NLRC3, DDX3X, ZBP1, TRAF5, IFIH1, AZI2, DDX58, PRKDC, DTX4, DDX41, CALCOCO2, TRIM25, TNFAIP3, PTPN11, TMEM173, TLR3, EXOC2, TLR4, MAVS, STAT6, and TRAF6 (**Supplementary Figure 4**).

Subsequently, the biological functions and pathway enrichment of TBK1, and its related genes were predicted and explored by GO and KEGG approaches using Metascape (**Figure 6A**). Network of GO and KEGG enriched terms colored according >to clusters and P-values were also shown (**Figures 6B, C**). The results suggested that the majority of biological functions and

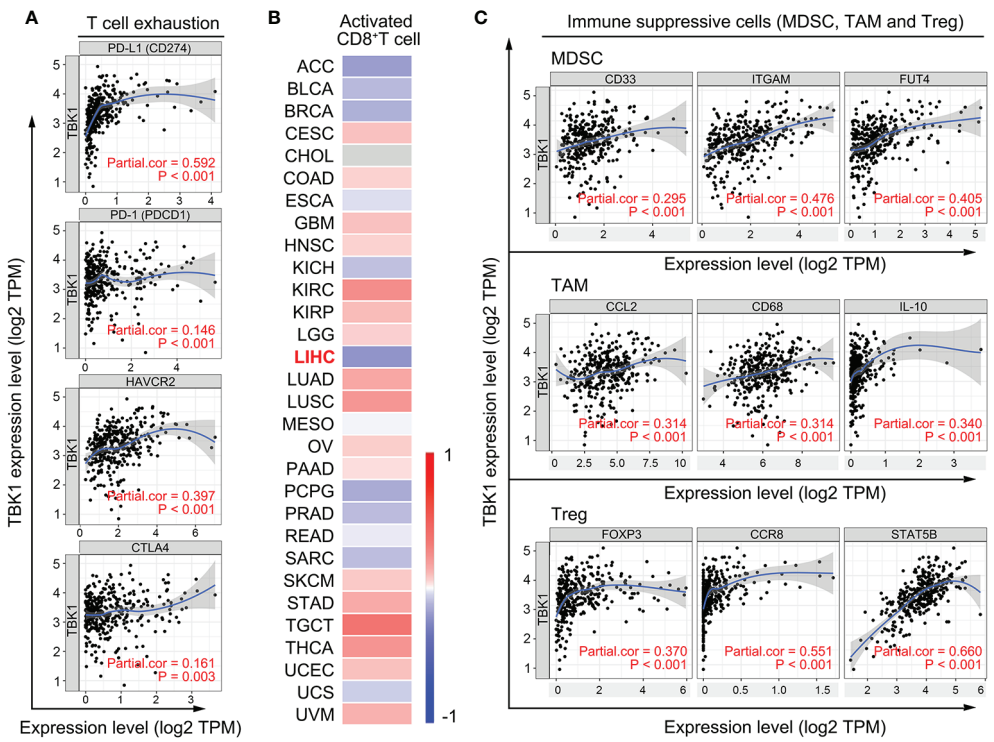

FIGURE 5 | TANK-binding kinase 1 (TBK1) expression is significantly correlated with the markers of immunosuppressive molecules and cells. **(A)** Correlation of TBK1 expression with immunosuppressive molecules (PD-L1, PD-1, HAVCR2, and CTLA4) involved in T cell exhaustion. **(B)** Correlation of TBK1 expression with activated $CD8^+$ T cells in different types of cancer. **(C)** Correlation of TBK1 expression with markers of immunosuppressive cells (MDSC, TAM, and Treg).

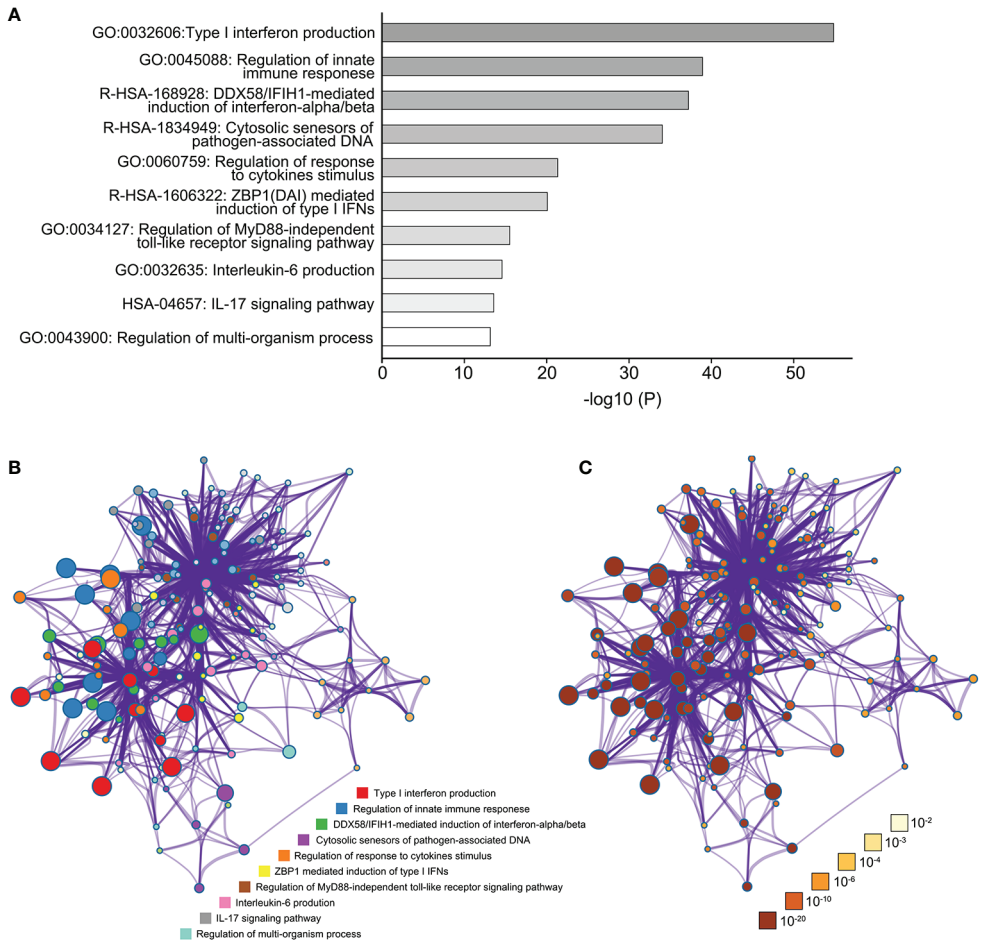

FIGURE 6 | The function network of TANK-binding kinase 1 (TBK1) and TBK1-related genes. **(A)** The gene ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) enriched terms colored according to *P*-values. **(B)** Network of GO and KEGG enriched terms colored according to clusters. **(C)** Network of GO and KEGG enriched terms colored according to *P*-values.

pathways were involved in the inflammatory response of anti-infection (GO:0045088: Regulation of innate immune response; R-HSA-168928: DDX58/IFIH1-mediated induction of interferon-alpha/beta; R-HSA-1834949: Cytosolic sensors of pathogen-associated DNA; GO:0034127: Regulation of MyD88-independent toll-like receptor signaling pathway). These results were consistent with the property of TBK1 gene. More importantly, the production and regulated pathway of inflammatory cytokines were enriched in the function network of TBK1 and its related genes (GO:0032606: Type I interferon production; GO:0032635: IL-6 production; HSA-04657: IL-17 signaling pathway). Type I interferon, IL-6, and IL-17 promote the up-regulation of immunosuppressive molecular and accumulation of immunosuppressive cells in cancer (40–42). Therefore, the results suggested that TBK1-regulated inflammatory cytokines may promote the immunosuppressive microenvironment of HCC, as a clear example of inflammation-related cancer.

TBK1 Antagonist Attenuates HCC Progression by Enhancing Tumor Immune Infiltration

A previous study reported that TBK1 resulted in tumor immunosuppression and may be therapeutically beneficial to patients, in an effort to augment tumoral T-cell infiltration. However, more investigations on the role of TBK1 in immune-competent animals with tumor are warranted (8). Therefore, we further assessed whether TBK1 promotes HCC progression by decreasing immune infiltration, and investigated the potential immunotherapeutic value of targeting TBK1 by treatment with a TBK1 antagonist.

We detected the level of TBK1 activation (Phosphorylated TBK1, p-TBK1) in human HCC tissues and non-tumor liver tissues by western blotting, indicating that p-TBK1 was significantly up-regulated in HCCs compared with non-tumor liver tissues (**Supplementary Figure 5A**). Most cases of human HCC arise in fibrotic or cirrhotic livers which is characterized

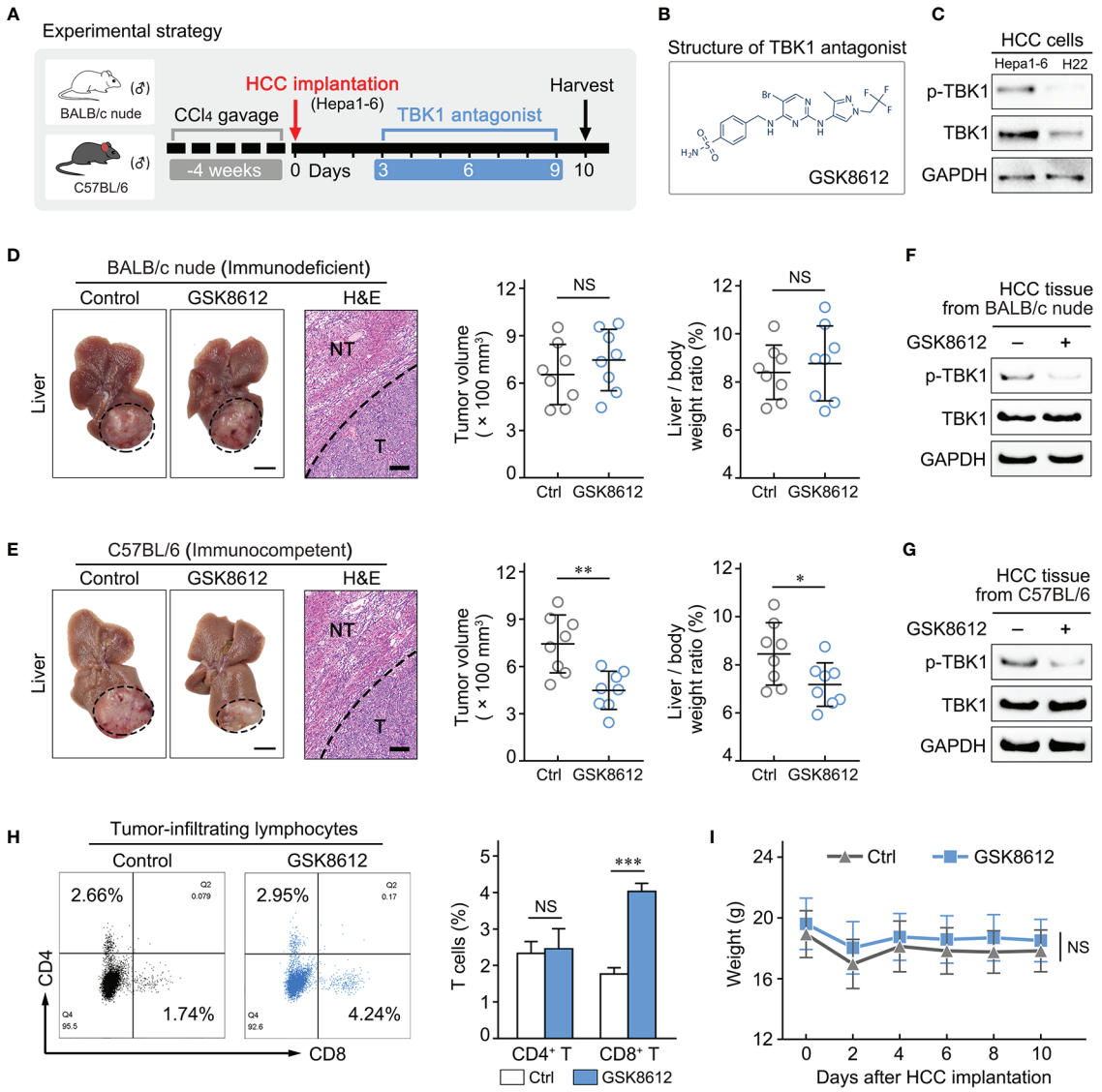

FIGURE 7 | Treatment with a TANK-binding kinase 1 (TBK1) antagonist delayed hepatocellular carcinoma (HCC) growth by increasing the number of tumor-infiltrating CD8+ T cells. **(A)** Experimental design to investigate the effect of the TBK1 antagonist on tumor progression in the orthotopic HCC mouse models with chronic liver inflammation. **(B)** Expression of TBK1 and p-TBK1 in the mouse-derived HCC cell lines (H22 and Hepa1–6). **(C)** Structure of the TBK1 antagonist GSK8612. **(D, E)** Statistical analysis of tumor volume and the liver/body weight ratio, as well as representative images of tumor morphology and hematoxylin-eosin (H&E) staining of liver tissue *in vivo* at the endpoint. **(F, G)** The effect of GSK8612 on TBK1 activation in HCC tissues was detected by western blotting. **(H)** Levels of tumor-infiltrating $CD4^+$ and $CD8^+$ T cells in HCC tissues obtained from immunocompetent mice. **(I)** Weight changes in immunocompetent mice treated with or without GSK8612. Thin scale bars, 5 mm. Bold scale bars, 200 μm. NT, Non-tumor liver tissue; T, Tumor; NS. not significant; $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

chronic unresolved inflammation. Thus, an orthotopic HCC model that recapitulates the pathological features of human HCC (**Supplementary Figure 5B**) were established using BALB/c nude (immunodeficient) and C57BL/6 mice (immunocompetent) with chronic liver inflammation (**Figure 7A**). The HCC mouse models treated with GSK8612, a novel and highly selective TBK1 antagonist, were sacrificed and liver tissues were harvested for further analysis (**Figures 7A, B**). Two strains of mouse-derived HCC cell lines were tested for TBK1 and p-TBK1 expression, and Hepa1–6 cells with higher level of TBK1 activation were used in the current study (**Figures 7A, C**). We found that the GSK8612 did not have an effect on HCC growth in BALB/c nude mice, whereas it significantly attenuated HCC growth in C57BL/6 mice (**Figures 7D, E**). Western blotting demonstrated a decreased TBK1 activation in HCC tissues of immunodeficient and immunocompetent mice after treatment with GSK8612 (**Figures 7F, G**). The degrees of infiltration of $CD4^+$ and $CD8^+$ T cells in the tumors of immunocompetent mice were examined and indicated that the number of tumor-infiltrating $CD8^+$ T cells was markedly increased after treatment

with the TBK1 antagonist (**Figure 7H**). In addition, the TBK1 antagonist resulted in the decreased level of α-SMA$^+$ myofibroblasts in non-tumor liver tissues and IL-6 in tumor tissues demonstrated by IHC staining and ELISA (**Supplementary Figure 5C, D**). However, the difference of CD8$^+$ T cells in non-tumor liver tissues with or without therapy was not observed (**Supplementary Figure 5C**). The increased level of tumor-infiltrating CD8$^+$ T cells after treatment with GSK8612 were also confirmed by IHC staining (**Supplementary Figure 5C**). Besides, there were no significant differences in body weight observed between the two groups (**Figure 7I**). Meanwhile, we investigated the effects of GSK8612 on Hepa1-6 proliferation and migration *in vitro*. The results of CCK8 and Transwell assay showed that the growth rate and migratability of Hepa1-6 were not significantly affected by GSK8612 (**Supplementary Figures 5E, F**). These data suggested that TBK1 contributes to HCC progression by promoting immunosuppression and is a potential therapeutic target in patients with HCC.

DISCUSSION

As an atypical inhibitor of the NF-κB protein kinase, TBK1 mediates the inner immune response induced by signals from pattern-recognition receptors (PRRs) detecting pathogen-associated molecular patterns (9). Besides, TBK1 possesses important functions in the regulation of immune tolerance and adaptive immune responses. Recent studies investigating the function of TBK1 have expanded their focus on cancers, demonstrating the promoting effect of TBK1 on tumor immunosuppression and therapeutic potential of targeting TBK1 (18, 43). In the present study, we reported that variations in the levels of TBK1 expression were associated with prognosis in different types of cancer. In addition, high TBK1 expression was found in more aggressive tumors and identified as an independent poor prognostic factor for OS among patients with HCC. More importantly, TBK1 expression was positively correlated with a decreased number of tumor-infiltrating CD8+ T cells and increased immunosuppressive markers in patients with HCC. Treatment with the TBK1 antagonist attenuated the HCC progression *in vivo* by enhancing the infiltration of CD8+ T cells in the tumor. Thus, the present study demonstrated the prognostic value of TBK1 expression and immunotherapeutic potential of targeting TBK1 in patients with HCC.

The critical role of TBK1 in tumorigenesis and aberrant TBK1 expression in cancer were reported in previous studies (43–45). In this study, data from TCGA and GEO databases consistently demonstrated the up-regulated levels of TBK1 expression in BRCA, HNSC, KIRC, LIHC, and STAD compared with those measured in normal tissues. Furthermore, GEPIA and the KM plotter indicated the significant value of TBK1 expression as a prognostic biomarker in 17 types of cancer. Differentially expressed genes are involved in count for the molecular mechanisms of biological conditions (46). Therefore, the up-regulated TBK1 expression, which is predictive of poor prognosis, may contribute to tumor progression especially in BRCA, KIRC, and LIHC. Consistent with our results, other studies reported that ectopic TBK1 expression accelerated the growth of BRCA by phosphorylating estrogen receptor α (13), and hyperactivated TBK1 was essential for maintaining p62 stability and the oncogenic phenotype of KIRC (47). However, there is limited knowledge regarding the effect of TBK1 on HCC progression. We further investigated the association with clinicopathological parameters and prognostic potential of TBK1 expression to provide more insight into the pathologic role of TBK1 in HCC progression. The results indicated higher expression of TBK1 in patients with more advanced TNM stage, and identified high TBK1 expression as an independent risk factor for poor OS in patients with HCC. These findings suggest that TBK1 could be used as the prognostic biomarker for patients with HCC, and may play an important role in HCC progression.

HCC occurs mostly in a background of chronically inflamed liver, which enhances the induction of antigen-specific tolerance and suppression of immune response to HCC (48). Owing to the correlation of TBK1 expression with inflammation indicators (liver fibrosis, platelet-to-albumin ratio), the present study investigated the effects of TBK1 expression on HCC immune infiltration. The lower number of tumor-infiltrating CD8$^+$ T cells results in impaired host immune defense against HCC progression and poor prognosis (49, 50). Our data further revealed a decreased number of CD8$^+$ T cells in HCC with high TBK1 expression, and no significant prognostic value of TBK1 expression in HCC patients with enriched tumoral CD8+ T cells. Thus, it is reasonable to hypothesize that high TBK1 expression leads to HCC progression and poor prognosis by reducing the infiltration of CD8$^+$ T cells. In addition, recent studies revealed the effect of TBK1 on promoting immunosuppression in lung and cervical cancer (43, 51). This study also observed that TBK1 expression was significantly correlated with the marker genes of the HCC immunosuppressive microenvironment (**Figure 5**), and its potential mechanism was involved in inflammatory cytokines (type I interferon, IL-6, and IL-17). It has been reported that IL-6 promotes the polarization of monocytes recruited by tumor cells into TAM (52) and the amplification of MDSCs in tumor microenvironment (42), IL-17 enhanced the expression of PD-1 and HAVCR2 in tumor-infiltrated CD8$^+$ T cells (53). Although Type I interferon exerts a direct inhibitory effect on tumor growth, it is able to induce immunosuppression through Treg, MDSC accumulation, and PD-L1 up-regulation in a manner of sustained stimulation (54, 55). Collectively, these data suggest that TBK1 induces HCC immunosuppression by sustaining the inflammatory phenotype and promotes HCC progression.

Owing to variable effects on the immune microenvironment in the state of chronic liver inflammation (48), it is important to explore the role of TBK1 in HCC immune infiltration *in vivo*. This study utilized a TBK1 antagonist to treat the orthotopic HCC model established using BALB/c nude and C57BL/6 mice with chronic liver inflammation. The results indicated that treatment did not delay HCC growth in BALB/c nude mice, which is characterized by defective immune responses especially for the

T cell-mediated response (56). However, treatment significantly attenuated HCC progression in immunocompetent C57BL/6 mice, accompanied by increased tumoral $CD8^+$ T cell infiltration. These data confirmed the role of TBK1 in HCC promotion by decreasing immune infiltration. The HCC cell promotes the inflammatory environment *via* releasing inflammatory cytokines and recruiting the tumor-associated macrophages which amplified the inflammatory response (57–59). In addition, HCC-derived cancer-associated fibroblast contributes to the production of PD-$L1^+$ neutrophils by IL-6, impairing the T-cell function and fostering immunosuppression (60). We now report the decreased level of IL-6 in HCC tissues treated by TBK1 antagonist (**Supplementary Figure 5D**) as well as the TBK1 expression in HCC and tumor stroma (**Supplementary Figures 5G, H**). These data suggested that TBK1 antagonist may modulate the immunosuppressive microenvironment by inhibiting the secretion of inflammatory cytokines in HCC cells and cancer-associated fibroblasts. Furthermore, consistent with the other study (61), our results showed the suppression of the activation of hepatic stellate cells and liver fibrosis by TBK1 antagonist (**Supplementary Figure 5C**). Due to the promoting effect of hepatic fibroinflammatory condition on the tumor immunosuppression (5), it is possible that the TBK1 antagonist attenuated the HCC immunosuppression by reducing the fibrosis and inflammatory environment of liver. Previous studies demonstrated the potential value of TBK1 as an immunotherapeutic target for the treatment of cancer (16, 18). Nevertheless, the application of small molecules targeting TBK1 was restricted by its selectivity (8). The recently developed GSK8612, a novel potent and highly selective TBK1 antagonist (62), was used in this study and presented an inhibitory effect on HCC. In addition, the absence of significant weight loss indicative of adverse drug reactions (63) in treated mice partially demonstrated the safety of GSK8612 (**Figure 7I**). These results propose that targeting TBK1 by GSK8612 has potential value as immunotherapy for HCC. Recent reports showed that anti-PD-1/anti-PD-L1-based combination therapy represented a promising strategy for HCC (64), and targeting TBK1 boosted the efficacy of anti-PD-1/anti-PD-L1 in various types of cancer (16, 18). Hence, further studies are warranted to investigate the efficacy of immunotherapy, combining the targeting of TBK1 with administration of immune checkpoint inhibitors, for HCC.

In summary, we demonstrated that increased expression of TBK1 may be useful in predicting the poor prognosis of patients with HCC. Moreover, this study revealed the effect and mechanism of TBK1 on promoting HCC by decreasing immune infiltration, and potential value of targeting TBK1 as an immunotherapy strategy for HCC.

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

The animal study was reviewed and approved by Bioethics Committee of Jinan University (China). 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

YJ, MC, and JH conceived and designed the study. YJ, SC, QL, and JiL performed the experiments. WL, JuL, and ZL collected the clinical samples of each patient. YJ and JH analyzed the data and designed the figure. YJ and JH drafted the manuscript. MW and MC revised 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.2021.612139/full#supplementary-material

Supplementary Figure 1 | GEPIA indicated the prognostic significance of TBK1 expression for OS in 12 types of cancer. **(A–L)** Kaplan–Meier curves of OS based on TBK1 expression in diverse types of cancer.

Supplementary Figure 2 | The KM plotter indicated the prognostic significance of TBK1 expression for OS in 8 types of cancer. **(A–H)** KM curves of OS comparing the high and low expression of TBK1 in different types of cancer.

Supplementary Figure 3 | The correlation of TBK1 expression with the marker of T cells and prognostic potential of TBK1 expression in patients with HCC based on immune infiltration. **(A)** TIMER was used to analyze the correlation of TBK1 expression with the markers of $CD4^+$ and $CD8^+$ T cells as well as Th1, Th2, Tfh and Th17 cells. Comparison of Kaplan–Meier OS curves of high and low TBK1 expression in HCC based on tumor-infiltrating B cells **(B)** or tumor-infiltrating $CD4^+$ T cells **(C)**.

Supplementary Figure 4 | Co-expressed genes with TBK1 among patients with HCC.

Supplementary Figure 5 | TBK1 antagonist improved the immune infiltrates in HCC and attenuated liver fibrosis and tumor inflammation. **(A)** The expression of TBK1 and p-TBK1 in human HCC tissues and non-tumor liver tissues. **(B)** The pathological features of liver tissue from HCC mouse model. **(C)** Representative images of IHC staining with α-SMA and CD8 in liver tissues from control and treatment group (Left panel); statistical analysis of their IHC score (Right panel).

(D) The level of IL-6 in HCC tissues were examined by ELISA. **(E)** The CCK8 and **(F)** Transwell assays used to measure the effect of GSK8612 on Hepa1-6 proliferation and migration. **(G)** TBK1 expression in tumor stroma of human HCC tissues. **(H)** In the liver tissues of C57BL/6 mouse model, TBK1 expression in tumor stroma indicated by Sirius red staining. NT = Non-tumor liver tissue, T = Tumor, NS = not significant; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

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Beyond First-Line Immune Checkpoint Inhibitor Therapy in Patients With Hepatocellular Carcinoma

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Until recently, the treatment landscape for hepatocellular cancer (HCC) was dominated by tyrosine kinase inhibitors (TKIs) which offered an overall survival (OS) benefit when used both in the first-and second-line setting compared to best supportive care. However, the treatment landscape has changed with the introduction of immune checkpoint inhibitors (ICIs) for the treatment of HCC with significant improvement in OS and progression free survival reported with combination atezolizumab and bevacizumab compared to sorafenib in the first-line setting. Nonetheless, the response to ICIs is 20–30% and invariably patients will progress. What remains unclear is which therapeutics should be used following ICI exposure. Extrapolating from the evidence base in renal cell carcinoma, subsequent therapy with TKIs offers both a response and survival benefit and are recommended by European guidelines. However, there are a number of novel therapies emerging that target mechanisms of ICI resistance that hold promise both in combination with ICI or as subsequent therapy. This paper will discuss the evidence for ICIs in HCC, the position of second-line therapies following ICIs and research strategies moving forward.

Keywords: HCC, second-line therapy, tyrosine kinase inhibitors, survival, immunotherapy

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INTRODUCTION

Hepatocellular cancer (HCC) is the fifth most common cause of cancer and the third leading cause of cancer related death worldwide (1). The majority of HCC develops on a background of chronic liver disease secondary to chronic hepatitis B and C, alcohol excess or non-alcoholic liver disease (2). The presence of chronic liver disease has a direct impact on liver function and often limits therapies that can be extended to patients (3). Whilst curative in the early stages, the majority of patients (>70%) will present with advanced stage cancer, and even in those receiving curative therapy with surgery or ablation, the majority will relapse within 5-years and the mainstay of treatment in this setting is that of systemic therapy (2, 4).

For over 20 years the research field has been dominated by molecular targeted agents, the majority inhibiting angiogenesis through blockade of vascular endothelial growth factor receptor (VEGFR) (2). Both in the first and second-line setting, the efficacy of these agents has been modest, with improvements in overall survival (OS) of only 2–3 months and poor objective response rates (5–9), underscoring a need for more efficacious therapeutics in this disease space. In recent years there has been an increasing appreciation of the role of the immune microenvironment

in liver carcinogenesis (10). Being at the junction of the arterial and portal systemic blood flow, the liver has an important immunoregulatory role (11). The liver constitutes the largest reticulo-endothelial system (RES) in the human body, with specialized immune cells including Kupffer cells, innate T-cells, natural kills cells and liver sinusoidal endothelial cells (12). Cirrhosis results in persistent inflammation and damage to the RES leading to impaired immune surveillance and dysregulation of the immune environment, resulting in DNA damage, hepatocyte necrosis and cancer (13). A rich immune infiltrate is observed in the tumor microenvironment (TME) but this infiltrate comprises of predominantly "exhausted" pro-inflammatory T-cell (regulatory T-cells, T-regs) populations that express co-inhibitory receptors such as cytotoxic T-lymphocyte-associated protein 4 (CTLA-4), programmed cell death protein 1 and its ligand (PD-1/PDL-1), T-cell immunoglobulin, mucin-domain containing-3 (TIM-3), and myeloid derived suppressor cells (MDSCs) (14, 15). Together with the secretion of immunoregulatory cytokines, immune tolerance results which is associated with poor prognosis (16, 17). Hence, there is a strong rationale for the use of immunotherapies (ICI) in HCC. The pressing question moving forward is which agent to use in the second-line setting, with tyrosine kinase inhibitors (TKIs) currently recommended post-ICI (18, 19). The aim of this review is to summarize the evidence for ICIs in HCC with a particular focus on combination ICI-therapy and to explore the therapeutic options following ICI. To inform treatment decision-making, we will revisit the current therapeutic portfolio in HCC and discuss future treatment directions.

IMMUNOTHERAPEUTIC STRATEGIES IN HCC

The goals of ICI can broadly be defined as either unmasking a current immune response or stimulating a new or different one (11). The majority of phase III studies have been performed using therapeutics that target molecules such as CTLA-4 and the PD-1/PDL-1 axis in an effort to unmask an immune response (10).

Single Agent Immunotherapeutic Strategies

The first ICI to be approved by the FDA for the management of HCC was nivolumab, an anti-PDL-1 antibody following the publication of CheckMate 040 (20). This was a phase I/II, uncontrolled, open labeled study that evaluated nivolumab, initially in a dose escalation, and then in a subsequent dose expansion cohort, enrolling patients with Child Pugh A and B cirrhosis who had previously received sorafenib ($N = 262$) (20). The study reported an overall response rate (ORR) of 20% with a 9-months survival rate of 74% (95% CI: 67–79%) which led to the phase III randomized controlled trial, Checkmate 459, in which nivolumab was tested against sorafenib in the first-line setting (21). The study failed to meet its primary endpoints of OS; median OS for nivolumab was 16.4 months (95% CI: 13.9–18.4) vs. 14.7 months (95% CI: 11.9–17.2) for sorafenib (HR 0.85, 95% CI: 0.72–1.02, $p = 0.075$) (21).

A similar fate awaited the much anticipated Keynote-240 study, a phase III study that randomized patients to either pembrolizumab or placebo following sorafenib therapy (22). Pembrolizumab is a highly selective humanized IgG4/κ monoclonal antibody that directly inhibits the binding of PD-1 to its ligands, PD-L1 and PD-L2. Despite an ORR of 17% in the phase II Keynote-224 study (23), the phase III study failed to meet either of its co-primary endpoints (OS or PFS). The reported median OS was numerically longer for pembrolizumab, 13.9 vs. 10.6 months for placebo, HR 0.78, 95% CI: 0.61–0.99, $p = 0.024$, but did not meet the pre-specified criteria for statistical significance over placebo (24). Of interest, following progression 41.7% of patients in the pembrolizumab group and 47.4% in the placebo group received subsequent anti-cancer treatment. On *post-hoc* analysis, the median OS was longer in the pembrolizumab group vs. placebo when survival was adjusted for subsequent anti-cancer therapies (13.9 vs. 9.3 months; HR, 0.67; 95% CI, 0.48–0.92; nominal one-sided $p = 0.0066$) (23). 24.8% of patients received TKIs following pembrolizumab and whilst not reported, the efficacy of individual TKIs in this sub-study would be of key interest.

Despite the absence of a clear role for single agent ICIs either in the first or second-line management of HCC, there are a number of other agents under investigation. Durvalumab, an anti-PDL1 IgG1 monoclonal, has been evaluated as part of a phase I/II study in an expansion cohort of 40 HCC patients with Child-Pugh Class A, 93% of whom were sorafenib experienced. An ORR of 10% was reported with a median OS of 13.2 months and a 56% 1-year survival rate (25). Other drugs being investigated include camrelizumab (26), cemiplimab (27) (NCT03916627), and tislelizumab, a humanized IgG4 antibody to PD-1, the efficacy of which is currently being explored in the phase III RATIONALE-301 study compared with sorafenib in the first-line setting (NCT 03412773) (28).

In addition to PD-1 and PDL-1, single agent CTLA-4 inhibitors have been investigated in HCC, although not in the context of large phase III studies. The frist CTLA-4 inhibitor to be studied in HCC was tremelimumab, a fully human IgG2 monoclonal antibody (29). The study investigated the efficacy of tremelimumab 15 mg/kg IV every 90 days in 21 patients with Hepatitis C-associated HCC and reported a response rate of 17.6% and time to tumor progression (TTP) of 6.48 months (95% CI: 3.95–9.14) (29). The reported median OS was 8.2 months and the probability of survival at 1 year was reported to be 43%. Duffy and colleagues investigated the combination of tremelimumab and ablation with the intention of inducing synergistic immunogenic cell death. Tremelimumab was administered as six infusions, 3.5 and 10 mg/kg 4-weekly followed by 3-monthly maintenance. Sub-total tumor ablation was given at day 36. Five out of 19 evaluable patients achieved a partial response, translating into a TTP of 7.4 months and OS of 12.3 months (30). Both studies demonstrated evidence of anti-viral activity with falling HCV RNA load and expansion of HCV-specific T-cell responses (29). There is a paucity of phase III data for anti-CTLA-4 monotherapy and long term efficacy data is wanting as is its efficacy across diverse etiologies of chronic liver disease.

Immunotherapy Combination Studies

Extrapolating from the improved clinical outcomes observed in other malignancies, there are a number of clinical trials investigating the efficacy of combination therapy with both PD-1 and CTLA-4 inhibitors (**Table 1**). The rationale for this combination is that whilst the PD/PDL-1 pathway inhibits the effectiveness of the CD8+ T-cell response, CTLA-4 differentially suppresses the action of antigen presenting cells and T-regs. Thus, by targeting both pathways, there is the expectation of both an increase in the number of activated CD8+ cells infiltrating the tumor and an enhancement of anti-tumor activity.

Cohort 4 of the Checkmate-040 was designed to test the efficacy of varying doses of combination therapy of the CTLA-4 inhibitor, ipilimumab, and nivolumab in patients with advanced stage HCC following progression on sorafenib (arm A: nivolumab 1 mg/kg + ipilimumab 3 mg/kg, arm B: nivolumab 3 mg/kg + ipilimumab 1 mg/kg every 3 weeks for 4 doses followed by nivolumab maintenance (240 mg flat dose every 2 weeks), arm C: nivolumab 3 mg/kg + ipilimumab 1 mg/kg every 6 weeks until discontinuation due to progression or toxicity) (31). Arm A showed the greatest improvement in OS compared to arm B and C and has received accelerated approval in the United States; median OS 22.8 months (95% CI, 9.4-not reached) in arm A vs. 12.5 months (95% CI, 7.6–16.4) in arm B and 12.7 months (95% CI, 7.4–33.0) in arm C (31).

The phase III HIMALAYA study randomizes patients to receive combination therapy with tremelimumab and the PDL-1 inhibitor, durvalumab, durvalumab alone, or sorafenib in the first-line setting (NCT03298451). This trial was instigated based on promising phase I/II results that illustrated an ORR of 15% with disease control rates at 16 weeks of 57% in patients with unresectable HCC treated with durvalumab and tremelimumab with an acceptable safety profile. The authors reported that 20% of patients experienced grade ≥3 related adverse events the most common being an asymptomatic rise in AST (10%) (32).

RATIONALE FOR COMBINATION THERAPY OF ICIs AND MOLECULAR TARGETED AGENTS

The TME in HCC is hypoxic and as a consequence, is characterized by the presence of tortuous, leaky neoangiogenic vessels (33). Hypoxia has been shown to impair the function of immune effector cells and modulate the function of innate immune cells toward immunosuppression (33). Moreover, PD-1 and PD-L1 are unregulated in the hypoxic TME as a mechanism to evade anticancer immune responses, with upregulation of PD-L1 expression observed on MDSCs, dendritic and endothelial cells, as well as on tumor cells (34). Excessive production of VEGF and other pro-angiogenic factors in response to hypoxia creates a pro-tumor microenvironment by impacting on the number and function of T-regs, tumor associated macrophages, and MDSCs resulting in an immunosuppressive environment (33).

The TKI, sorafenib, targets multiple kinases including the VEGF receptor (9). Preclinical work in HCC, illustrates that the TKI, sorafenib, induces hypoxia and over-expression of PDL-1 within the tumor, resulting in accumulation of T-reg and M2-macrophages (35, 36). Moreover, in an elegant study by Shigeta and colleagues, dual blockade with anti-PD-1/VEGFR-2 therapy significantly inhibited HCC growth and improved survival *in vivo* (37). The authors illustrated that dual therapy resulted in an increase in cytotoxic T-cell infiltration and activation, an increase in M2 tumor-associated macrophages and a reduction in T-regs (37). Normalization of vessel architecture with dual therapy was also observed lending preclinical support for the use of combination ICI and anti-angiogenic therapy in the clinical setting.

Clinical Data for the Combination of ICIs and VEGF/VEGFR Axis Inhibitors

The first clinical trial of combination therapy to show a survival benefit in HCC was IMBrave 150 (38). In this open label, phase III study, patients with advanced stage disease were randomized to receive a combination of atezolizumab and bevacizumab or sorafenib. Patients were included if they had preserved liver function, ECOG 0-1 and an absence of main portal trunk invasion. The co-primary endpoints of OS and PFS were both achieved such that the OS at 12 months was 67.2% (95% CI, 61.3–73.1) with combination therapy compared with 54.6% for sorafenib (95% CI, 45.2–64.0) (HR 0.58, 95% CI, 0.42–0.79, $p < 0.001$). PFS was 6.8 months (95% CI: 5.7–8.3) for atezolizumab plus bevacizumab vs. 4.3 months (95% CI: 4.0–5.6) with sorafenib (HR0.59; 95% CI: 0.47–0.76, $p < 0.0001$). Of key interest, quality of life was maintained with atezolizumab plus bevacizumab compared to sorafenib in this essentially palliative population (38). Despite the promise of the trial, some outstanding questions remain. Whilst treatment related adverse events were similar in both treatment groups, discontinuation rates were higher with combination therapy, but no further details were given by the authors. Moreover, the trial does not report rates of cirrhosis which may impact on rates of drug induced adverse events in particular hepatitis, and any real-world data of the combination therapy will be of interest (38).

Numerous combination studies are currently open testing a myriad of permutations with various TKIs and ICIs (**Table 1**). The recently published phase Ib study of combination therapy of pembrolizumab and lenvatinib in patients with unresectable HCC reported no dose limiting toxicities in both the safety run-in ($N = 6$) and expansion phase (39). The authors report an ORR of 46.0% (95% CI: 36.0–56.3%), median PFS of 9.3 months (95% CI: 5.6–9.7 months) and OS of 22 months (95% CI: 20.4–not evaluable, months) (39). This combination is now being evaluated in a phase III vs. single agent lenvatinib (40). Similarly, the combination of regorafenib with pembrolizumab (NCT03347292) and cabozantinib with atezolizumab are being investigated in the first-line setting (41).

TABLE 1 | Emerging immunotherapy combinations for the treatment of hepatocellular cancer.

Trial name/identifier	Setting	Treatment	Phase	Primary endpoints
First-line				
GO30140/NCT02715531	Advanced HCC	Bevacizumab + atezolizumab	Ib	Safety, ORR, PFS
NCT03006926	Advanced HCC	Lenvatinib + pembrolizumab	Ib (dose-escalation and dose-expansion)	Dose escalation: Safety, DLTs Dose expansion: ORR, DCR
NCT03418922	Advanced HCC	Lenvatinib + nivolumab	Ib (part 1 + part 2)	Part 1: DLTs, safety Part 2: Safety
CheckMate 040/NCT01658878	Advanced HCC	Cabozantinib + nivolumab +/– ipilimumab	I/II (dose-escalation, dose-expansion)	Safety, ORR
NCT04039607(CheckMate9DW)	Advanced HCC	Nivolumab + ipilimumab *vs.* sorafenib or lenvatinib	III	OS
NCT03347292	Advanced HCC	Regorafenib + pembrolizumab	Ib (dose-escalation and dose-expansion	Safety, DLTs
LEAP-002/NCT03713593	Advanced HCC	Lenvatinib + pembrolizumab *vs.* lenvatinib + placebo	III, randomized, double-blinded	PFS, OS
COSMIC-021/NCT03170960	Advanced solid tumors, HCC	Cabozantinib + atezolizumab	Ib (dose-escalation and dose-expansion)	Dose escalation: MTD, Recommended dose Dose expansion: ORR
COSMIC-312/NCT03755791	Advanced HCC	Cabozantinib + atezolizumab *vs.* sorafenib *vs.* cabozantinib	III randomized, open-label	PFS, OS
NCT03298451 (HIMALAYA)	Advanced HCC	Durvalumab *vs.* durvalumab + tremelimumab (regimen 1) *vs.* durvalumab + tremelimumab (regimen 2) *vs.* sorafenib	III	OS
NCT04180072	Advanced HCC + chronic HBV infection	Atezolizumab + bevacizumab	II	Best ORR
NCT02519348	Advanced HCC	Durvalumab alone *vs.* tremelimumab alone vs. durvalumab plus tremelimumab (regimen 1 vs. regimen 2) vs. durvalumab bevacizumab	II	Number patients experiencing AEs and DLTs
NCT03764293	Advanced HCC	Camrelizumab + apatinib vs. sorafenib	III	OS, PFS
NCT03439891	Unresectable, locally advanced or metastatic HCC	Nivolumab + sorafenib	II	MTD, ORR
NCT03211416	Advanced or metastatic HCC	Pembrolizumab + sorafenib	Ib/II	ORR
NCT03841201	Advanced HCC	Nivolumab + lenvatinib	II	ORR, safety/tolerability
NCT04310709 (RENOBATE)	Unresectable HCC	Nivolumab + regorafenib	II	Response rate
Second line				
NCT03895970	Advanced hepatobiliary tumors	Lenvatinib + pembrolizumab	IIb	ORR, DCR, PFS
CheckMate 040/NCT01658878	Advanced HCC	Cabozantinib + nivolumab ± ipilimumab	I/II	Safety, ORR
CAMILLA/NCT03539822	Advanced GI tumors, HCC	Cabozantinib + durvalumab	Ib	MTD
REGOMUNE/NCT03475953	Advanced GI tumors, HCC/	Regorafenib + avelumab	I/II (part 1 and part 2)	Part 1: Recommended phase II dose of regorafenib Part 2: ORR
NCT02572687	Advanced solid tumors, HCC, AFP ≥1.5x upper limit of normal	Ramucirumab + durvalumab	I	DLTs
NCT02082210	Advanced solid tumors, HCC	Ramucirumab + emibetuzumab	I/II	Part A: DLTs Part B: ORR
NCT02423343	Advanced solid tumors, HCC and AFP ≥200 ng/mL	Galunisertib + nivolumab	Ib/II (dose escalation and cohort expansion)	Ib: MTD

(Continued)

TABLE 1 | Continued

Trial name/identifier	Setting	Treatment	Phase	Primary endpoints
NCT04014101	Advanced HCC	Camrelizumab + apatinib	II	ORR
NCT04170556 (GOING)	HCC	Nivolumab + regorafenib	I/II	Rate of AEs, rate of death
Other				
CaboNivo/NCT03299946	Locally advanced HCC	Cabozantinib + nivolumab	Ib	Safety, number of patients who complete preoperative treatment and proceed to surgery
NCT03682276 (PRIME-HCC)	Prior to liver resection in HCC	Nivolumab + ipilimumab	I/II	Delay to surgery, incidence of AEs
NCT03222076	Resectable HCC	Nivolumab vs. nivolumab plus ipilimumab (regimen 1) vs. nivolumab + ipilimumab (regimen 2)	II	Incidence of AEs
NCT03510871	HCC	Nivolumab + ipilimumab	II	Percentage of subjects with tumor shrinkage after therapy
NCT03847428 (EMERALD-2)	HCC with high risk of recurrence	Durvalumab + bevacizumab *vs.* durvalumab + placebo vs. placebo alone	III	RFS
NCT03839550	HCC with high risk of recurrence after radical resection	Camrelizumab + apatinib vs. hepatic arterial infusion of chemotherapy	II	RFS
NCT04191889	C-staged HCC in BCLC CLASSIFICATION	Camrelizumab + apatinib and hepatic arterial infusion of FOLFOX chemotherapy regimen	II	ORR

AEs, adverse events; BCLC, Barcelona Clinic Liver class; DCR, disease control rate; DLTs, dose limiting toxicities; FOLFOX, oxaliplatin and 5-fluorouracil; MTD, maximum tolerated dose; ORR, objective response rate; OS, overall survival; PFS, progression free survival; RFS, relapse free survival.

THE ROLE OF TYROSINE KINASE INHIBITORS POST-ICI

Whilst IMBrave150 illustrated an OS and ORR benefit of combination therapy over sorafenib in the first-line setting, data on long-term survivorship and response to subsequent therapies is not yet available (38). Similarly, anti-PD-1 monotherapy (20, 22) and dual checkpoint inhibition with anti-CTLA-4 (31) were approved by the FDA on the basis of response rates rather than evidence of convincing OS benefit. The majority of advanced HCC patients will invariably progress and a looming question is what should be used in the second-line setting following combination ICI therapy. The recently updated European Society of Medical Oncology position regorafenib, cabozantinib, and ramucirumab as therapeutic options following failure of atezolizumab and bevacizumab, a stance that has been adopted by a number of healthcare systems (18, 19), and is supported by a recent network analysis (42). Evidence of efficacy of TKIs following ICI in HCC is limited. A *post-hoc* analysis of 14 patients in the CELESTIAL study who received cabozantinib third line following ICI reported a median OS of 7.9 months (95% CI 5.1–NE) which was comparable to that of patients that had received two prior regimens, median OS 8.5 months (95% CI 7.4–9.7) (43). In another small study of 30 patients with HCC who received TKIs following immunotherapy (combination nivolumab and ipilimumab ($N = 2$), single agent nivolumab ($N = 7$), pembrolizumab ($N = 4$) and durvalumab ($N = 1$), the authors report a median OS, defined from the commencement of TKI till death from any cause, of 602 days (95% CI: 124–not reached) (44). It is unclear from the published abstract if immunotherapy was administered as a single agent or combination and the full publication is awaited. Currently, there are no publications or studies considering the utility of TKIs following combination therapy.

Prior to the introduction of immunotherapy into the therapeutic armamentarium, sorafenib and lenvatinib offered a survival benefit of 2 months for patients with inoperable HCC (7, 9). For those patients who failed first-line therapy with sorafenib, three second-line options were available; regorafenib, cabozantinib and ramucirumab (5, 6, 8). None of these agents have been assessed following lenvatinib failure. *Post-hoc* exploratory analysis of the RESORCE study illustrated that sequential treatment with sorafenib and regorafenib resulted in a median OS of 26 months from start of sorafenib compared to 19 months in those that received sorafenib followed by placebo (45). Similar results were observed in a *post-hoc* analysis of the CELESTIAL trial that illustrated patients who had received prior sorafenib, cabozantinib significantly improved OS, 24.5 months compared to 18.8 months in those receiving placebo (46). In addition, *post-hoc* analysis of the REFLECT data that illustrates an OS benefit of second-line therapy, OS 20.8 vs. 17.0 months (HR 0.87; 95% CI 0.67–1.14) (47). Subgroup analysis illustrated that OS was greatest in those patients who had initially responded to either lenvatinib, 25.7 months (95% CI 18.5–34.6), or sorafenib 22.3 months (95% CI 14.6–not evaluable).

TABLE 2 | Novel targets for molecular therapies in hepatocellular cancer.

NCT	Trial name	Phase	Status	Outcome (if known)
TGF-B inhibitors				
NCT02423343	A Study of Galunisertib (LY2157299) in combination with nivolumab in advanced refractory solid tumors and in recurrent or refractory NSCLC, or Hepatocellular Carcinoma	I/II	Completed	N/A
NCT01246986	A Study of LY2157299 in participants with hepatocellular carcinoma	II	Completed	Median TTP 2.7 months (95% CI: 1.5-2.9) in Part A ($n = 109$) and 4.2 months (95% CI: 1.7-5.5) in Part B ($n = 40$).
NCT02240433	A Study of LY2157299 in participants with unresectable Hepatocellular Cancer (HCC)	Ib	Completed	Recommended dose of galunisertib 150 mg twice daily for 14 days in combination with sorafenib 400 mg BD in Japanese patients.
NCT02906397	Galunisertib (LY2157299) Plus Stereotactic Body Radiotherapy (SBRT) in Advanced Hepatocellular Carcinoma (HCC)	I	Active, not recruiting	N/A
NCT02947165	Phase I/Ib Study of NIS793 in combination with pdr001 in patients with advanced malignancies.	I/Ib	Active, not recruiting	N/A
NCT02178358	A Study of LY2157299 in participants with advanced hepatocellular carcinoma	II	Active, not recruiting	N/A
Bifunctional immunotherapy				
NCT02517398	MSB0011359C (M7824) in metastatic or locally advanced solid tumors	I	Active, not recruiting	No data on HCC but on other tumor lines.
NCT02699515	MSB0011359C (M7824) in subjects with metastatic or locally advanced solid tumors	I	Active, not recruiting	No data on HCC but on other tumor lines.
TIM-3 inhibitors				
NCT03652077	A Safety and Tolerability Study of INCAGN02390 in Select Advanced Malignancies	I	Active, not recruiting	N/A
NCT03680508	TSR-022 (Anti-TIM-3 Antibody) and TSR-042 (Anti-PD-1 Antibody) in patients with liver cancer	II	Recruiting	N/A
NCT03489343	Sym023 (Anti-TIM-3) in patients with advanced solid tumor malignancies or lymphomas	I	Completed	N/A
NCT03099109	A study of LY3321367 alone or with LY3300054 in participants with advanced relapsed/refractory solid tumors	I/Ib	Active, not recruiting	The RP2D for LY3321367 combination therapy is 1,200 mg IV infusions Q2W for cycles 1–2; 600 mg infusions Q2W starting at cycle 3 onward.
NCT03311412	Sym021 monotherapy, in combination with Sym022 or Sym023, and in combination with both Sym022 and Sym023 in patients with advanced solid tumor malignancies or lymphomas	I	Recruiting	N/A
NCT02608268	Phase I-Ib/II study of MBG453 as single agent and in combination with PDR001 in patients with advanced malignancies	I/IIb	Active, not recruiting	No data on HCC but on other tumor lines
NCT03744468	Study of BGB-A425 in combination with tislelizumab in advanced solid tumors	I/II	Recruiting	N/A
NCT02817633	A Study of TSR-022 in participants with Advanced Solid Tumors (AMBER)	I	Recruiting	No data on HCC but on other tumor lines.

(Continued)

TABLE 2 | Continued

NCT	Trial name	Phase	Status	Outcome (if known)
NCT03307785	Study of Niraparib, TSR-022, bevacizumab, and platinum-based doublet chemotherapy in combination with TSR-042	Ib	Active, not recruiting	N/A
WNT inhibitors				
NCT02069145	Dose escalation study of OMP-54F28 (Ipafricept) in combination with sorafenib in patients with HCC	I	Completed	N/A
NCT03645980	DKN-01 inhibition in advanced liver cancer	I/II	Recruiting	N/A
NCT01608867	A dose escalation study of OMP-54F28 (Ipafricept) in subjects with solid tumors	I	Completed	Ipafricept was well-tolerated, with RP2D of 15 mg/kg Q3W. Prolonged SD was noted in desmoid tumor and germ cell cancer patients.
Anti-LAG-3				
NCT04567615	A study of relatlimab in combination with nivolumab in participants with advanced liver cancer who have never been treated with immuno-oncology therapy after prior treatment with tyrosine kinase inhibitors	II	Not yet recruiting	N/A
MET inhibitors				
NCT03655613	APL-501 or nivolumab in combination with APL-101 in locally advanced or metastatic HCC and RCC	I/II	Recruiting	N/A
CD105				
NCT02560779	Trial of TRC105 and sorafenib in patients with HCC	Ib/II	Completed	N/A
NCT01375569	TRC105 for liver cancer that has not responded to sorafenib	II	Completed	TRC105 is well tolerated in this HCC population post-sorafenib ($N = 8$). Evidence of antiangiogenic activity but unlikely that the study will proceed to second stage.
NCT01306058	Sorafenib and TRC105 in hepatocellular cancer	I/II	Completed	Recommended dose of TRC105 was 15 mg/kg, PR 25%.
HIF1A inhibitors				
NCT02564614	A Study of Hypoxia-inducible Factor 1a (HIF1A) Messenger Ribonucleic Acid (mRNA) Antagonist (RO7070179), to demonstrate proof-of-mechanism in adult participants with Hepatocellular Carcinoma (HCC)	Ib	Completed	Recommended dose 10 mg/kg, 1PR, 1SD
IDH1 inhibitors				
NCT03684811	A study of FT 2102 in participants with advanced solid tumors and gliomas with an IDH1 mutation	I/II	Active, not recruiting	N/A
NCT02465060	Targeted therapy directed by genetic testing in treating patients with advanced refractory solid tumors, lymphomas, or multiple myeloma (The MATCH Screening Trial)	II	Recruiting	N/A
NCT02421185	Study to evaluate the safety, pharmacokinetics, and pharmacodynamics of JNJ-42756493 (Erdafitinib) in participants with advanced Hepatocellular Carcinoma	I/II	Completed	N/A

(Continued)

TABLE 2 | Continued

NCT	Trial name	Phase	Status	Outcome (if known)
NCT04194801	A Phase Ib/II study of Fisogatinib(BLU-554) in subjects with Hepatocellular Carcinoma	I/II	Recruiting	N/A
NCT02508467	A phase 1 study of fisogatinib (BLU-554) in patients with Hepatocellular Carcinoma	I	Active, not recruiting	BLU-554 is well-tolerated at the recommended dose of 600 mg and demonstrates important clinical activity in FGF19 IHC+ advanced HCC pts who have failed prior systemic therapy.
NCT02834780	Phase 1 study to evaluate the safety, pharmacokinetics and pharmacodynamics of H3B-6527 in participants with advanced Hepatocellular Carcinoma	I	Active, not recruiting	1,000 mg QD RP2D. 2 of 17 pts with HCC achieved PRs and an additional 7 with SD were on treatment for ≥5 months.

AE, adverse event; BD, twice a day; CI, confidence interval; HR, hazard ratio; IRC, Independent review committee; IV, intravenous; NSCLC, nonsmall-cell lung carcinoma; ORR, objective response rate; OS, overall survival; PFS, progression free survival; Q2W, once every 2 weeks; QD, four times a day; RP2D, recommended phase II dose; SD, stable disease; TTP, primary endpoint.

Given that all therapeutics that have previously shown activity in HCC in phase III trials target VEGFR and angiogenic signaling to some extent, it can be expected that all these agents could be successfully combined with ICI (5–9). Which TKI would be more efficacious following ICI remains to be elucidated. Extrapolating from renal cell carcinoma, another tumor driven by angiogenesis, sequential TKI use following ICI therapy is associated with incremental OS benefit, leading to international guidelines to recommend the use of any multi-targeted TKI that has not been used in the first-line setting in combination with ICI, an approach that is gaining traction in HCC (44, 48, 49). Another therapeutic approach is the evaluation of novel therapies that target ICI resistance mechanisms or alternate signaling pathways in HCC (**Table 2**).

MECHANISMS OF ICI RESISTANCE IN HCC AND TREATMENT STRATEGIES

Resistance to ICIs can either be primary or acquired, and the mechanisms that drive this process are an evolving field. What is clear is that "cold" tumors do not respond to ICI whilst "hot" tumors do. Cold tumors are characterized by an infiltrate of MDSCs, T-regs, low tumor mutational burden and poor antigen presentation, resulting in an inability to mount an immune response toward the tumor (50). A number of novel therapeutics are currently being developed to essentially transform a "cold" tumor microenvironment into a "hot" tumor and to enhance the endogenous T-cell response. Of these, a number are being trialed in HCC including TIM-3, and lymphocyte activation gene 3 (LAG-3) antagonists, and inhibitors of transforming growth factor β (TGFβ) receptor ligands, and tumor necrosis factor (TNF) receptor (51).

TIM-3 is a transmembrane protein expressed on exhausted CD8+ cells that is expressed with other co-inhibitory receptors such as PD-1 and CTLA-4. The combination of TSR-022, a TIM-3 antagonist, TSR-042, a novel anti-PD-1 is currently the subject of a phase II study in HCC (NCT03680508). Similarly, lymphocyte activation gene 3 (LAG-3) suppresses T-cells activation and cytokine secretion, thereby ensuring immune homeostasis and is currently the subject of clinical trials (**Table 2**).

The tumor growth factor-β (TGFβ) signaling pathways play a key role in cellular invasion and proliferation, driving hepatocarcinogenesis (52). In addition, TGFβ signaling in the TME has been shown to result in tumor T-cell exclusion and poor response to PD-1/PD-L1 blockade, and there is rationale to combine TGFβ with ICIs (53). Galunisertib, an oral small molecule inhibitor of the TGFβ receptor I (TGFβRI) kinase, has been evaluated in phase II study of 149 patients with HCC who had progressed following sorafenib (54). Enrollment was stratified according to AFP>1.5ULN with a median OS of 7.3 months (95% CI: 4.9–10.5) in those patients with an AFP < 1.5ULN and 16.8 months (95% CI: 10.5–24.4) with AFP >1.5ULN (54). Galunisertib in combination with nivolumab is currently being investigated in HCC and other solid tumors (NCT02423343). OX40 is a member of the TNF receptor family

that is highly expressed on activated immune cells. On ligand binding, T-cell survival, proliferation and effector function is enhanced (55). MEDI0562 is an agonistic, humanized IgG monoclonal antibody directed at OX40 that has undergone phase I evaluation with acceptable toxicity (56). It is anticipated that the combination of MEDI0562 with ICI may enhance the immunomodulatory effects.

CONCLUSION

Currently, for patients that receive either sorafenib or lenvatinib first-line there is a clear benefit with second-line therapy from the RESORCE, CELESTIAL, REACH 2 studies. There is no randomized evidence supporting the use of second-line ICIs following sorafenib or lenvatinib despite the prolonged survival benefit observed in the KEYNOTE-240 study. Promising results are observed with the combination of nivolumab and ipilumumab in the second-line setting which has been approved by the FDA. There is evidence that combination atezolizumab and bevacizumab improves OS in the first-line setting but there are no clear answers as to what to use second-line. What is clear is that the survival for patients with advanced HCC is improving and whilst the correct sequence and drug combination is not yet clear, the survival gains are reasons for enthusiasm. The next few years will herald an exciting time for drug development in HCC both in terms of novel therapeutics but also their accompanying biomarkers which are sorely needed.

AUTHOR CONTRIBUTIONS

RS and LA designed and wrote the manuscript.

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REVIEW
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Hepatocellular Carcinoma Immune Landscape and the Potential of Immunotherapies

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Hepatocellular carcinoma (HCC) is the most common liver tumor and among the deadliest cancers worldwide. Advanced HCC overall survival is meager and has not improved over the last decade despite approval of several tyrosine kinase inhibitors (TKi) for first and second-line treatments. The recent approval of immune checkpoint inhibitors (ICI) has revolutionized HCC palliative care. Unfortunately, the majority of HCC patients fail to respond to these therapies. Here, we elaborate on the immune landscapes of the normal and cirrhotic livers and of the unique HCC tumor microenvironment. We describe the molecular and immunological classifications of HCC, discuss the role of specific immune cell subsets in this cancer, with a focus on myeloid cells and pathways in anti-tumor immunity, tumor promotion and immune evasion. We also describe the challenges and opportunities of immunotherapies in HCC and discuss new avenues based on harnessing the anti-tumor activity of myeloid, NK and γδ T cells, vaccines, chimeric antigen receptors (CAR)-T or -NK cells, oncolytic viruses, and combination therapies.

Keywords: immunotherapy, immune checkpoint inhibitors, tumor microenvironment, tumor-associated macrophages, immunosuppression, inflammation, cirrhosis, NASH

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PREFACE

The liver is a critical hub of metabolism, glucose storage, lipid and cholesterol homeostasis, detoxification and processing of xenobiotics, endocrine regulation of growth signaling, blood volume regulation, and immune surveillance. These essential functions are coordinated by multiple cell types: the hepatocytes, which make up 80% of the liver volume; the cholangiocytes, which line the biliary ducts and are the second most abundant parenchymal cells of the liver; the liver sinusoidal endothelial cells (LSECs), which line the hepatic sinusoidal walls and display specialized functions in scavenging, antigen presentation and leukocyte recruitment [reviewed in (1)]; the hepatic stellate cells (HSCs), the body's largest storage site of vitamin A at quiescent state; and the liver-resident immune cells, which are particularly enriched in this important immune organ. The liver is continuously challenged with microbial- and danger-associated molecular patterns (MAMPs and DAMPs) and non-self-peptides derived from dietary and gut-derived microbial antigens. Its capacity to deal with these insults is reflected by its particular immune environment. Indeed, the liver hosts the largest population of tissue-resident macrophages, known as Kupffer cells (KCs). It also exhibits a high frequency of tissue-resident lymphocytes, namely natural killer (NK) cells, NKT cells, conventional αβ T cells, unconventional γδ T cells and B cells. The liver's diverse immunotolerance mechanisms limit the development of chronic liver diseases, including cirrhosis and liver cancers.

Hepatocellular carcinoma (HCC), which accounts for approximately 90% of the incidence of all primary liver cancers, is the 5th most prevalent cancer worldwide and the 4th leading cause of death globally (2). Both environmental and genetic risk factors contribute to the etiology of HCC. The most notable environmental and potentially preventable risk factors include oncogenic virus infection with hepatitis B virus (HBV) or hepatitis C virus (HCV), alcohol abuse, and the metabolic syndrome related to obesity and diabetes mellitus [reviewed in (3)]. In addition, some rare monogenic diseases and several single nucleotide polymorphisms (SNPs) predispose individuals to HCC [reviewed in (4)] (**Figure 1A**). HCC incidence has doubled in the last three decades in the US, presumably due to high prevalence of HCV infection in the mid 1900's and increasing obesity-related non-alcoholic fatty liver disease (NAFLD) progressing to non-alcoholic steatohepatitis (NASH). Accordingly, suppression of HBV/HCV infections may improve HCC clinical outcomes, but few patients with HCC are cured of their hepatic infections due to treatment cost, compliance and toxicity issues, and NAFLD is expected to become the major risk factor for developing HCC in developed countries in the near future (5). In very early or early-stage HCC (stage 0/A, according to the Barcelona Clinic Liver Cancer [BCLC] staging system), the most effective therapeutic option remains surgical resection, liver transplantation or percutaneous local ablation. In this early stage, the median overall survival (mOS) is >60 months with a 5-year survival of 60–80%, but the 5-year recurrence rate is up to 70% [reviewed in (6)]. However, the large majority of HCCs are diagnosed at an intermediate (stage B) or an advanced stage (stage C), when the mOS is ∼11–20 months with a 5-year survival of 16%.

The therapeutic options for these stages are limited to locoregional treatments, including transarterial chemoembolization (TACE) or radioembolization with yttrium 90 (90Y)-microspheres, and systemic treatment with multi tyrosine kinase inhibitors (TKi), such as Sorafenib (7) or Lenvatinib (8), according to international guidelines (9). While approved as a first-line therapy, these TKi improve mOS by 3 months (7, 8, 10) and are associated with significant side effects (11). In patients that progress following first line TKi treatment, the second-line options have been, until recently, alternative TKi, primarily regorafenib (12) and cabozantinib (13), or the fully human monoclonal antibody targeting vascular-endothelial growth factor (VEGF) receptor type 2 (VEGF-R2) ramucirumab (14). More recently, immune checkpoint inhibitors (ICI) have emerged as an alternative therapy in HCC and two anti-PD-1 drugs, nivolumab and pembrolizumab, have been approved in the USA based on two trials (15, 16) as a second line treatment for patients with advanced HCC refractory to sorafenib. The overall response rate (ORR) of nivolumab was reported to be 23% in sorafenib-naïve patients and 16-19% in sorafenib-experienced patients, with a mOS of 15 months. However, this did not reproduce in the phase III trial checkmate 459, in which the ORR to nivolumab in sorafenib-naïve patients was 15%, with a mOS of 16 months, i.e., not different from that with sorafenib. Further, in a recent trial, pembrolizumab monotherapy did not statistically impact HCC patients mOS and progression-free survival (PFS), as a second-line treatment (17). The combination of Regorafenib (angiogenesis inhibitor) and nivolumab has next been proposed as a second line treatment in sorafenib non-responders. This year, the combination of atezolizumab (anti-PD-L1) and bevacizumab (anti-VEGF) has obtained approval as a new first line therapy, as it improved mOS > 17 months (18) (**Figure 1B**). However, despite this therapeutic advance, ∼75% of HCC patients do not respond to these immunotherapies for unclear reasons. While there is evidence that boosting the activity of tumor-specific T cells might benefit patients with HCC, the underlying chronic inflammation renders this cancer's tumor microenvironment (TME) somewhat unique, and highlights the urgent need to further explore this organ-specific immunity, identify biomarkers to select patients who are likely to respond to such treatments, and develop new immunotherapies combinations.

Abbreviations: ACT, adoptive cell therapy; ADCC, antibody-dependent cell cytotoxicity; BCLC, Barcelona Clinic Liver Cancer; CAR, chimeric antigen receptors; DAMPs, danger-associated molecular patterns; EC, endothelial cells; ECM, extracellular matrix; EMT, epithelial-to-mesenchymal transition; fDC, follicular dendritic cell; HBV, hepatitis B virus; HCC, hepatocellular carcinoma; HCV, hepatitis C virus; HSC, hepatic stellate cells; ICI, immune checkpoint inhibitor; IFNg, interferon gamma; IHC, immunohistochemistry; ILCs, innate lymphoid cells; IKC, immune killer cells; irAEs, immune-related adverse events; KC, Kupffer cells; LIHC, liver hepatocellular carcinoma; LSEC, liver sinusoidal endothelial cells; MAGE-A, melanoma antigen gene A; MAMPs, microbial-associated molecular patterns; MAPK, mitogen-activated protein kinase; MDSCs, myeloid-derived suppressor cells; MNPs, mononuclear phagocytes; mOS, median overall survival; mregDCs, mature DCs enriched in immunoregulatory molecules; mTOR, mammalian target of rapamycin; NK cells, natural killer cells; cNK cells, conventional NK cells; LrNK cells, liver-resident NK cells; NKT cells, natural killer T cells; NSCLC, non-small cell lung cancer; ORR, overall response rate; pDC, plasmacytoïd dentritic cells; PFS, progression-free survival; PI(3)K, Phosphoinositide 3-kinase; RTK, receptor tyrosine kinase; SART, squamous cell carcinoma antigen recognized by T cells; scRNAseq, single cell RNA sequencing; SNPs, single nucleotide polymorphisms; TAA, tumor-associated antigens; TACE, transarterial chemo-embolization; TAMs, tumor-associated macrophages; TCR, T-cell receptor; Tex, exhausted T cells; TIC, tumor-initiating cells; TILs, tumor-infiltrating lymphocytes; TKi, tyrosine kinase inhibitors; TLS, tertiary lymphoid structures; TME, tumor microenvironment; Treg, regulatory T cells; TSA, tumor-specific antigens.

THE LANDSCAPE OF PARENCHYMAL, STROMAL AND IMMUNE CELLS IN THE HEALTHY VS. CIRRHOTIC LIVER

Prior to delving into the immune landscape and immunosuppressive mechanisms of HCC, we briefly overview the architecture of the liver and its immune system under physiological conditions, and highlight specific changes occurring in cirrhosis. Anatomically, the human liver is composed of eight functional segments organized into hepatic lobules containing their portal vein, hepatic artery and bile duct triads (**Figure 2A**). Around 80% of the blood supply is delivered from the gut via the portal vein, while the remaining 20% flows through the hepatic artery. Upon mixing, the blood equilibrates and drains across the lobule through the hepatic sinusoids into the central veins, while the bile flows in the opposite direction via bile canaliculi. Such an organization creates oxygen and

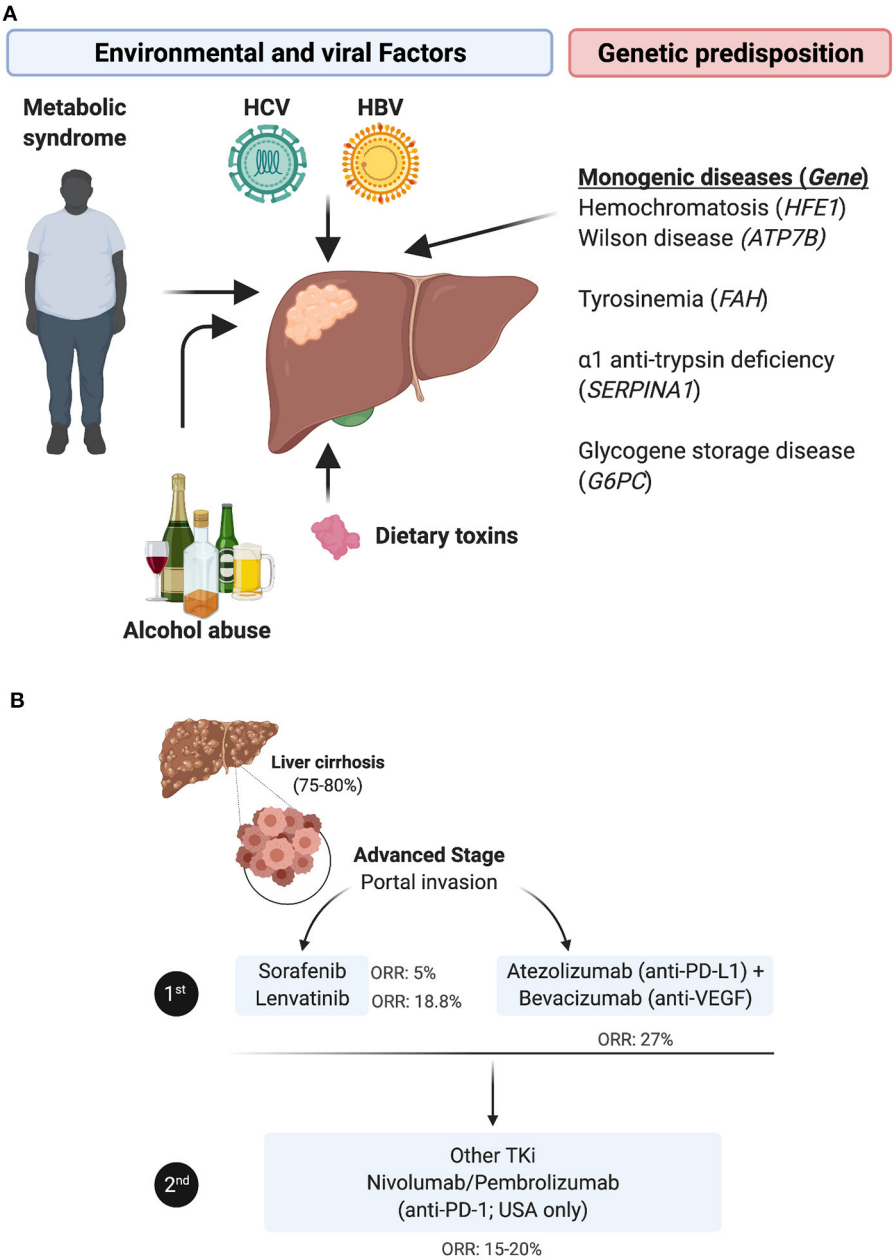

FIGURE 1 | HCC etiologies, genetic predisposition and current standard of care for the advanced stage. **(A)** HCC etiologies include chronic infection with HBV or HCV, alcohol abuse, dietary toxins and/or the metabolic syndrome linked to obesity and type 2 diabetes. In rare cases, HCC stems from a monogenic disease e.g., hemochromatosis, caused by mutations in the homeostatic iron regulator gene *HFE1*; Wilson disease involving mutations in the ATPase copper transporting beta gene *ATP7B*; tyrosinemia, resulting from mutations in the gene encoding fumarylacetoacetate hydrolase *FAH*, α1-trypsin deficiency caused by mutations in serpin family A member 1 *SERPINA1;* or glycogen storage disease, in which the glucose-6-phosphatase gene is mutated. **(B)** The standard of care for treating patients with advanced HCC has been revised with the approval of immune checkpoint inhibitors. In first line, patients are administered TKi, mainly sorafenib or lenvatinib, or given the newly approved combination of bevacizumab (anti-VEGF) + atezolizumab (anti-PD-L1). In second line, patients refractory to TKi are treated with other TKIs, whereas anti-PD-1 ICI, nivolumab or pembrolizumab, have only been approved in the USA as an option for second line (despite the lack of superior efficacity in phase III trials compared to TKi).

metabolic gradients, referred to as liver zonation, controlled in part by WNT/β-catenin signaling (**Figure 2B**). Liver sinusoids are lined by a fenestrated monolayer of LSECs that lack a basement membrane, allowing the blood to directly reach the underlying hepatocytes, organized in two-layered plates. The luminal side of LSECs interacts with liver resident immune cells, such as KCs, whereas their basal side, facing the space of Disse, interacts with hepatocytes and HSCs (**Figure 2C**). The liver has

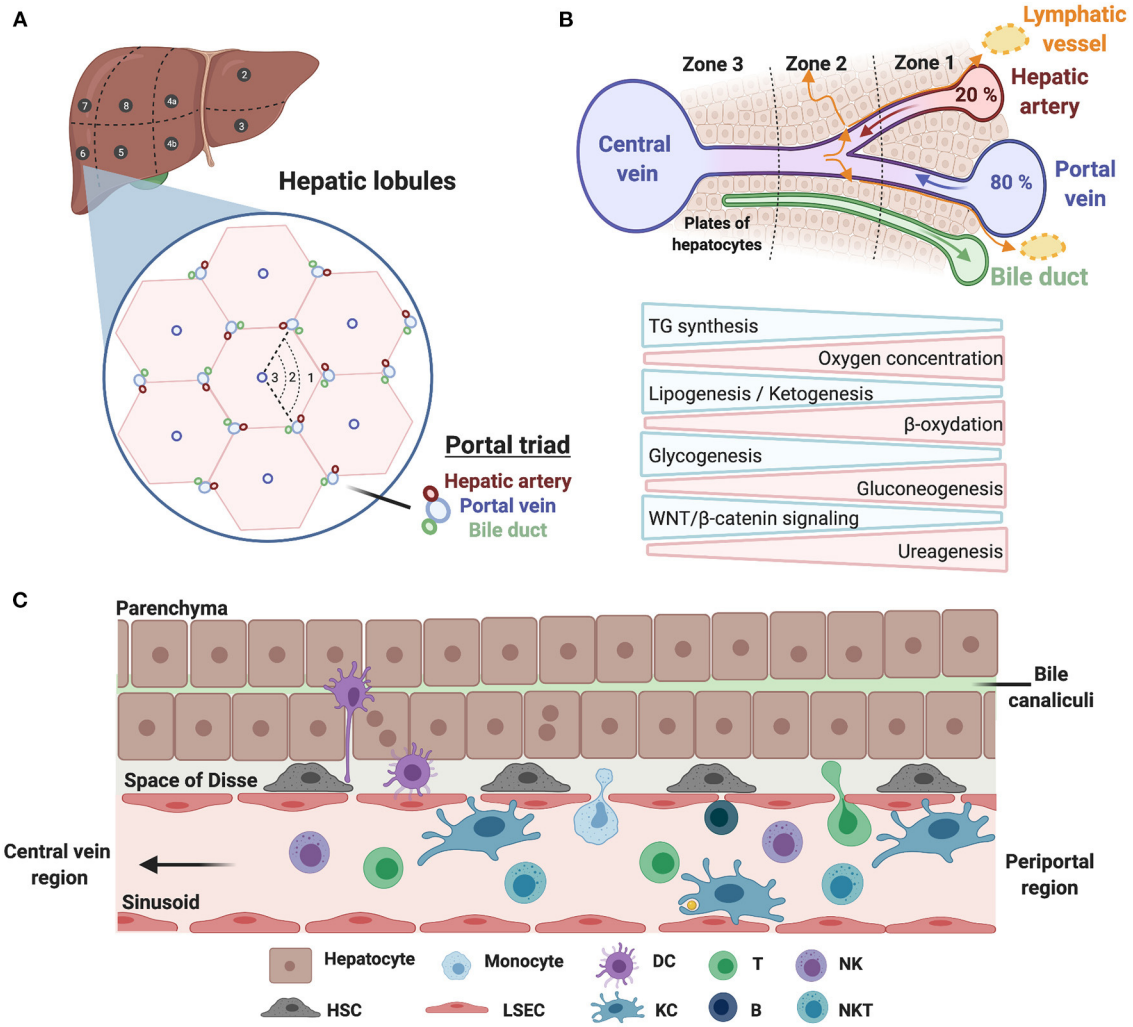

FIGURE 2 | Architecture of the human liver and its immune system. **(A)** Schematic illustration of the human liver anatomy namely its 8 segments, hepatic lobules, and triads of portal vein/hepatic artery/bile duct. **(B)** The liver zonation. Oxygen and metabolic gradients define three liver zones with specialized hepatocytes functions. **(C)** A zoom on hepatic cellular interactions across the sinusoids, the space of Disse and the hepatocyte plates. Liver sinusoidal endothelial cells (LSECs) line the liver sinusoid by forming a fenestrated monolayer. Their basal side interacts with hepatocytes and hepatic stellate cells (HSCs) in the space of Disse, whereas their luminal side interacts with liver-resident leukocytes, including Kupffer cells (KCs).

long been considered as a site of immune tolerance. This was based on early findings that transplanted allogeneic liver was significantly better tolerated than other organs, and patients required low levels of immunosuppression [reviewed in (19)]. Liver immune tolerance stems from complex interactions among liver-resident cells and peripheral leukocytes, and involves poor or incomplete activation of $CD4^+$ and $CD8^+$ T cells, elevated expression of immune checkpoints and an immunosuppressive environment mediated by IL-10 and TGFβ [reviewed in (20)]. KCs that function to preserve tissue homeostasis through their phagocytic and antigen presentation activity are important players in maintaining immune tolerance. Interestingly, a recent paper from the Germain group unraveled that microbiota sensing by LSECs imposes a chemokine gradient around the portal triads resulting in discriminate abundance of KCs and other immune cells (e.g., NKT cells) in periportal regions. Functionally, such an "immune zonation" is critical in limiting local infection and associated inflammatory tissue damage and in preventing the systemic spread of bacteria (21). Besides KCs, hepatic NK cells are capable of directly killing stressed cells, and mediate antibody-dependent cell cytotoxicity (ADCC) upon engagement of CD16 (FcγRIIIA). Their activity is regulated by a dynamic equilibrium between activating [NKG2D, NKp46 (NCR1), NKp44 (NCR2), and NKp30 (NCR3)] and inhibitory (KIR and NKG2A) receptors. In addition to producing various cytokines, chemokines, and growth factors, they maintain

immune tolerance through expression of immune checkpoints. Liver-resident NK cells (LrNK) differ from conventional NK (cNK) cells with respect to their origin, phenotypes and functions. Notably, LrNK cells share functional properties with innate lymphoid cells (ILCs) commonly found in mucosal tissues. NKT cells, which also express the NK cell marker CD56, actively patrol the liver and contribute to the clearance of pre-malignant senescent hepatocytes (22). They are recruited via the chemokine receptor CXCR6 interacting with CXCL16, secreted by LSECs and KCs, and are activated upon engagement of the glycolipid receptor CD1d. Last, $CD19^+$ B cells exert their functions through antibody production, antigen presentation and immune cell regulation.

Liver injury, caused by viral infection or chronic steatohepatitis related to alcohol or metabolic disorders, triggers an inflammatory cell death, leading to DAMP release and the influx of immune cells. Chronic inflammation activates HSCs, the main actors in liver fibrosis that produce extracellular matrix (ECM) components, forming the so-called "scar tissue." Liver cirrhosis, which affects 1% of the world population, represents the soil where most HCC cases develop. Indeed, continuous cellular stress, repetitive cycles of necrosis and compensatory regeneration of parenchymal cells and chronic inflammation elicit cellular senescence and mutagenesis leading eventually to HCC development. Furthermore, a reduction of sinusoid porosity (defenestration), associated with collagenization of the space of Disse, was shown to impede immunosurveillance [reviewed in (23)].

The recent use of high-dimensional single cell approaches (e.g., mass cytometry and single cell RNA sequencing [scRNAseq]) in humans has unraveled the cellular landscape of the healthy (24, 25) and cirrhotic (26) livers and uncovered subtype heterogeneity for all major liver populations. According to two reports by Aizarani et al. (24) and MacParland et al. (25), in which parenchymal and non-parenchymal cells from dissociated human normal liver tissue were analyzed, the healthy liver is predominantly populated by leukocytes, which make up 45% of all liver cells, out-numbering hepatocytes (ALB^{high}) that account for ∼35% of the cells in this organ. This is followed by endothelial cells, including LSECs ($CD34^-$ $CLEC4G^+$ $CLEC4M^+$) and macrovascular endothelial cells ($CD34^+$ $PECAM^{high}$) that account for ∼7.5% and ∼2.5% of hepatic cells, respectively. HSCs ($RGS5^+$ $ACTA2^+$) are found at <1% of the cells in this organ whereas cholangiocytes ($EPCAM^+$ $KRT19^{high}$ $CTFR^{high}$ ALB^{low}) occupy ∼9% of the liver cellular landscape (24, 25) (**Figure 3**). Interestingly, among the $EpCAM^+$ cholangiocytes, a putative bipotent liver progenitor population was identified by Aizarani et al. (24) based on the expression of intermediate levels of the intracellular calcium signal transducer *TACTSD2/TROP2* ($TROP2^{int}$). This population was shown to give rise to $ASGR1^+$ hepatocyte-biased cells ($TROP2^{low}$) or $KRT19^{high}$ $CFTR^{high}$ ALB^{low} cholangiocytes ($TROP2^{hi}$) (24). Furthermore, to model liver zonation, Aizarani et al. (24) applied diffusion pseudotime analysis and showed that hepatocytes and LSECs gene expression is highly zonated. LSECs in the periportal zone expressed genes involved in hormone signaling and metabolism, whereas pericentral and mid zone LSECs and hepatocytes were enriched in gene expression related to platelet activation, immune regulation and scavenging.

Among the leukocytes, the ratio of lymphocytes to mononuclear phagocytes (MNPs) is 3:1, with the former occupying ∼35% and the latter 10% of total liver cells. The lymphocytic compartment includes ∼11% αβ T cells, ∼6.7% γδ T cells, ∼12.3% NK + NKT cells, and ∼ 5% B cells (25). Among the innate immune cells, NK cells cluster in three groups, NK1 ($XCL1^+$ $CCL3^+$), NK2 ($XCL2^+$ $CD160^+$ $KLRD1^+$) and cytotoxic NKs ($GNLY^+$ $FGFBP2^+$ $SPON2^+$), whereas MNPs consist of three subsets, including two $CD68^+$ KC clusters, KC1 ($CD1C^+$ $FCER1A^+$) and KC2 ($MARCO^+$ $LILRB5^+TIMD4^+$) and a liver resident inflammatory macrophage subset (LYZ^+ $CD74^+$). The 3 classical dendritic cell (DC) subsets were also identified, namely conventional DCs, cDC1 ($CD1C^+$ $CLEC9A^+$) and cDC2 ($FCER1A^+$ $CD1E^+$), and plasmacytoid DCs ($LILRA4^+$ $CLEC4C^+$ $GZMB^+$) (**Figure 3**).

In the cirrhotic liver, scRNAseq uncovered all major immune cell populations and revealed a decrease in $CD8^+$ T cells, associated with an increase in $CD4^+$ T cells, as compared to the healthy liver. Re-clustering of MNPs identified four subgroups, annotated as KC1 ($CD163^+$ $MARCO^+$ $TIMD4^{hi}$), KC2 ($CD163^+$ $MARCO^+$ $TIMD4^{low}$), scar-associated macrophages ($TREM2^+$ $CD9^+$), tissue monocytes ($MNDA^+$ $S100A12^+$ $FCN1^+$) (**Figure 3**). The $MARCO^+$ population decreases in cirrhosis compared to the healthy liver while $TREM2^+$ $CD9^+$ scar-associated macrophages, derived from circulating monocytes, expand early in the course of the disease. This latter population of cells is conserved in humans and mice and displays pro-fibrogenic properties (26). Deep clustering of mesenchymal cell populations uncovered a cluster of $PDGF\alpha^+$ cells that also expand in cirrhosis, expressing high levels of fibrillar collagens and pro-fibrogenic genes. RNA velocity experiments indicated a trajectory from human HSCs to these scar-associated mesenchymal cells, and ligand/cognate receptors analysis combined with functional studies, pointed to TNFRSF12A, PDGFRA, and Notch signaling as important regulators of mesenchymal cell function in the human liver fibrotic niche (26).

Collectively, these single cell analyses revealed context-dependent cellular phenotypic diversity, opening the field to exploring potential mechanisms involved in HCC progression from cirrhosis. For instance, the fibrotic context is associated with the emergence of scar-associated mesenchymal cells and scar-associated macrophages with pro-fibrogenic properties. Future functional studies are needed to determine the value of targeting these cell subsets or specific molecular effectors therein as therapeutic strategies in HCC.

HCC SUBTYPES ACCORDING TO MOLECULAR AND IMMUNE CLASSIFICATIONS

Molecular Classification of HCC

Progression from cirrhosis to HCC is mediated by a step-wise accumulation of somatic mutations and copy number

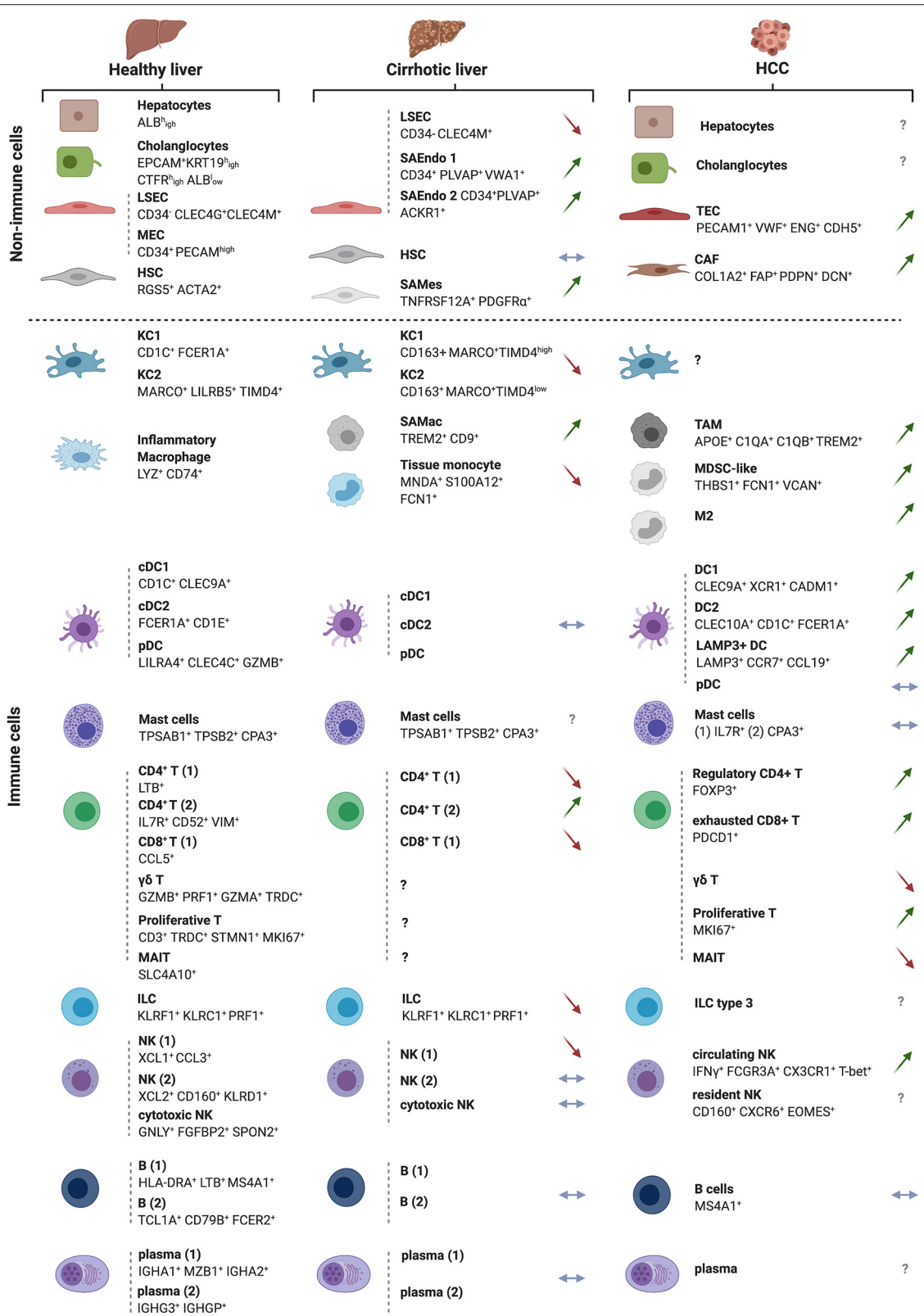

FIGURE 3 | The landscapes of the normal, cirrhotic and HCC-bearing livers. All immune and non-immune cell types identified by high-resolution single cell analyses of the human healthy and cirrhotic livers and of HCC are illustrated along with their discriminatory markers. Arrows depict direction of change in cirrhosis or HCC vs.

(Continued)

FIGURE 3 | the normal liver, with green arrows indicating an expansion, red arrows a depletion and blue horizontal arrow no change in the examined cell subset. The cellular landscapes of the healthy and cirrhotic livers were from (26). The information on the HCC landscape was from (27), but with complementary information from the following studies: γδ T cells and M2 macrophages (28), cancer-associated fibroblasts (CAFs) and tumor-endothelial cells (TECs) (29). New subsets of cells arising in the cirrhotic condition are also depicted and labeled as 'scar-associated' cells: SAEndo: scar-associated endothelial cells; SAMes: scar-associated mesenchymal cells; SAMac: scar-associated macrophages, as in (26). The HCC analyses were on sorted CD45^{+} immune cells. Symbols for genes and associated proteins are defined in **Supplementary Table 1**.

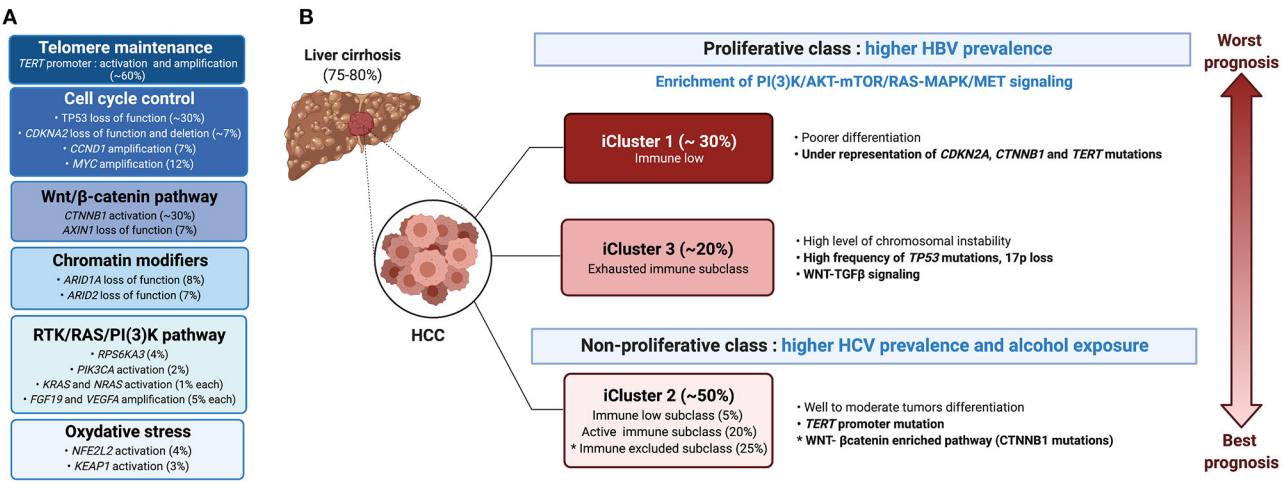

FIGURE 4 | Molecular and immunological classifications of HCC. **(A)** The main oncogenic events associated with HCC are presented in descending order of incidence, the most common being defects in telomere maintenance, followed by alterations in cell cycle control, and activation of the WNT/β-catenin pathway. **(B)** Based on molecular features, HCC patients can be grouped into either the proliferative or non-proliferative class, the first with higher prevalence of HBV infection and a bad prognosis, whereas the second including cases with HCV infection or alcohol abuse and having a better prognosis. Integration of genomic, expression and epigenetic data by the TCGA research network (32) identified a different classification, namely iClusters 1-3. iCluster 2 which represents 50% of the patients and is related to the non-proliferative class, includes three sub-classes: an "active immune subclass," an "immune excluded" subclass and an "immune low" subclass. An "immune exhausted" subclass is found within iCluster 3 of the proliferative class, whereas iCluster 1 is characterized by an "immune low" signature.

variations in driver genes (30). The most frequent alteration is the reactivation of the telomerase reverse transcriptase (*TERT*), a key event observed in 20% of high-grade dysplastic lesions and up to 60% of early HCC (31). Besides *TERT* promoter mutations that impact telomere maintenance, 10 pathways were found to be recurrently altered in HCC, including pathways involved in cell cycle control (*TP53*, *CDKNA2*, *CCND1*), oxidative stress (*NFE2L2*, *KEAP1*), and chromatin modification (*ARID1A*, *ARID2*), but also the Wnt/β-catenin pathway (*CTNNB1, AXIN1*) and the RTK/RAS/PI3K pathway (*RPS6KA3*, *PIK3CA*, *KRAS*, *NRAS*, *FGF19*, *VEGFA*) (32) (**Figure 4**). The TGFβ pathway is additionally involved in HCC progression, with some tumors presenting aberrant activation of this pathway, whereas others harboring inactivating mutations in genes required for TGFβ signal transduction e.g., the *SPTBN1* gene (33). Last, ∼20% of HCC express markers of progenitor cells, e.g., epithelial cell adhesion molecule (EpCAM) and cytokeratin 19 (CK19) and arise from either progenitors or dedifferentiated hepatocytes (12).

Earlier studies classified HCC into two main transcriptome-based classes, based on genetic, epigenetic and phenotypic features: HCC of the proliferative class, which displayed a poor clinical outcome, and HCC of the non-proliferative class, with a better outcome. The proliferative class was associated with the HBV etiology, and characterized by the activation of PI3K–AKT–mTOR, RAS–MAPK, and MET signaling along with chromosomal instability (34). The non-proliferative class, which is more prevalent in alcohol- or HCV-related HCC, regrouped heterogeneous tumors, including a subclass characterized by mutations in *CTNNB1*, the gene encoding β-catenin. More recent classification by Schulze et al. (35) and the Cancer Genome Atlas (TCGA) network (32) revised the molecular landscape of HCC (**Figure 4**). Three integrative clusters were identified: whereas, iCluster 1 and iCluster 3 distinguished two subclasses of the proliferative class, iCluster 2 overlapped with the non-proliferative class. iCluster 1 is associated with poorer differentiation, higher tumor grade, the presence of macrovascular invasion and overexpression of proliferation (*PLK1*, *MKI67*) and progenitor cells (EPCAM and AFP) gene markers, while iCluster 3 is characterized by high frequency of *TP53* mutation, 17p loss and activation of WNT-TGFβ signaling. On the other end, iCluster 2 regroups heterogenous moderately differentiated tumors characterized by *TERT* promotor mutations. Identification of the mutational landscape of HCC unveiled several druggable targets in >25%

of the cases (35). However, a potential limitation of tumor cell-based therapy is a notable inter- and intra-tumoral heterogeneity, mediated in part by non-neutral selection of mutations conferring a selective advantage (30) and subclone evolution (36). Using scRNAseq of liver cancers, Ma et al. (29) identified links between intra-tumoral heterogeneity (ITH), tumor micro-environment (TME) and survival outcome. They discriminated ITH according to the average expression of 10 cancer stemness genes, namely *EPCAM, CD24, CD44, CD47, KRT19, PROM1, ALDH1A1, ANPEP, ICAM1* and *SOX9*. This allowed them to derive diversity scores based on transcriptomic profiles, grouping the tumors into Div-high and Div-low groups. The Div-high group displayed poorer mOS and PFS, expressed higher levels of hypoxia-inducible factor 1-alpha (HIF1α)-dependent VEGFA and displayed a marked TME reprogramming. Concordantly, NOTCH and VEGF signaling, together with fetal-associated endothelial cells ($PLVAP^{+}$ $VEGFR2^{+}$) found in tumors, have been demonstrated to reprogram the $CD14^{+}$ monocytes into fetal-like immunosuppressive tumor-associated macrophages (TAMs ($FOLR2^{high}$ $CD163^{high}$) (37).

Immunological Classification of HCC

Immunological classification of HCC has been proposed by different groups using gene expression profiling (38) and protein level approaches based on multiplex immunohistochemistry (IHC) analysis (38) and mass cytometry (CyTOF) (28). Using deconvolution of 8 datasets, Llovet and colleagues analyzed a total of 956 HCC samples and reported that ∼25% of HCC cases expressed an immune gene signature (39). Such an "immune class" was found to be associated with better mOS, and expressed PD-1 and PD-L1, tertiary lymphoid structures (TLS) markers and determinants of cytolytic T cells activity e.g., an IFNγ signature. Further stratification identified two TME-based sub-classes within the immune class, dubbed the "active immune" and the "exhausted immune" subclasses. The "active immune" sub-class was enriched in T cell response effectors (IFNγ and granzyme B signatures), whereas the "exhausted immune" sub-class included signatures of T cell exhaustion, immunosuppressive macrophages and TGFβ signaling. A third immunological class, referred to as "immune excluded" was distinguished in ∼25% of HCC patients, based on the expression of immune genes, particularly an immunosuppressive signature, in the tissue surrounding the tumor, but with little immune gene expression in the tumor core. Such an "immune excluded" class was associated with a bad prognosis and overlapped with a subset of tumors in TCGA iCluster 2 with an activated WNT-β-catenin pathway (39) (**Figure 4B**). The immunological environment of HCC and its association with the molecular classification was further analyzed by Kurebayashi et al. (38) using multiplex immunohistochemistry. The authors classified HCC into three immune-subtypes based on the numbers of infiltrating immune cells: "Immune-high," "immune-mid" and "immune-low." Consistent with Sia et al. (39), the "immune-high" subtype, which was enriched in T cells and B-/plasma cells, was associated with a good prognosis (38). Zhang et al. (28) expanded this analysis and defined three HCC groups, namely the "immunocompetent," "immunosuppressive," and "immunodeficient" subtypes. The immunocompetent subtype, characterized as $CD45^{high}$ $FOXP3^{low}$, had normal T cell infiltration including high infiltration of γδ T cells. On the contrary, the immunosuppressive subtype, marked by a $CD45^{high}$ $FOXP3^{high}$ staining, exhibited high frequencies of immunosuppressive cells (regulatory T and B cells and immunosuppressive macrophages) and molecules (PD-1, PD-L1, TIM-3, CTLA-4, VEGF, TGFβ, and IL-10). Finally, the $CD45^{low}$ immunodeficient subtype showed a reduced infiltration of lymphocytes (28). While these studies demonstrated marked heterogeneity in HCC tumors and their associated TME with broad classification of patients, in depth characterization of the immune landscape of HCC at high resolution is expected to refine patients stratification and identify putative immune-therapeutic targets.

The Immune Landscape of HCC

The immune landscape of HCC has been more recently explored using single cell approaches. In general, a progressive depletion of intrahepatic LrNK cells, cytolytic T cells and γδ T cells and an enrichment of regulatory T cells (T_{reg}) and macrophages occur in HCC (28, 28, 32, 40–42) (**Figures 3**, **5**). While tumor-infiltrating $CD8^{+}$ T cells are significantly correlated with better prognosis (38, 43), T_{reg} are associated with a poorer mOS (44). RNA velocity analysis indicated a directional flow from proliferative to exhausted $CD8^{+}$ T cells in HCC (27). Exhaustion is characterized by the expression of a range of inhibitory receptors, including PD-1, TIM-3, LAG3, TIGIT, and LAYN [reviewed in (45)], and with reduced effector functions via TOX-mediated epigenetic and transcriptional alterations (46–48). However, not all exhausted $CD8^{+}$ T cells are the same, as two subsets can be discriminated: $PD\text{-}1^{+}$ $TCF1^{+}$ "precursors" that self-renew and give rise to $PD\text{-}1^{+}$ $TCF1^{-}$ "terminally differentiated" exhausted T cells (49–51). Notably, the presence of the precursors, but not the terminally differentiated exhausted T cells, is associated with a better response to anti-PD-1. Similarly, NK cells display an exhausted phenotype, expressing high levels of immune checkpoints such as PD-L1, PD-1, LAG3, TIM-3, CD155, and CD96 (52, 53). Further, they produce immunosuppressive cytokines such as TGFβ and IL-10 and less IFN-γ (52–56). The role of B lymphocytes in the development of HCC and their prognostic value is still debated. Their ADCC and antigen-presentation functions are countered by their ability to induce immunosuppression. In surgically resected HCC, $CD20^{+}$ B cells are associated with a better prognosis (38, 57), especially when they are in close proximity of tumor-infiltrating T cells (58). However, their prognostic value in the context of TLS depends on whether these are found intra-tumorally or in the surrounding tissue [reviewed in (59)]. Notably, TLS presence in the adjacent non-tumoral liver tissue was associated with an increased risk for late recurrence and a poor mOS in 82 patients with surgically resected HCC (60). Mechanistically, such ectopic TLS harbored progenitors/cancer stem cells (expressing CD44v6) and a tumor-promoting environment characterized by a persistent NF-κB activation favoring tumor outgrowth, as demonstrated in a mouse model (60). Concordantly, alymphoid conditions suppressed $CD44v6^{+}$ HCC-initiating cells and

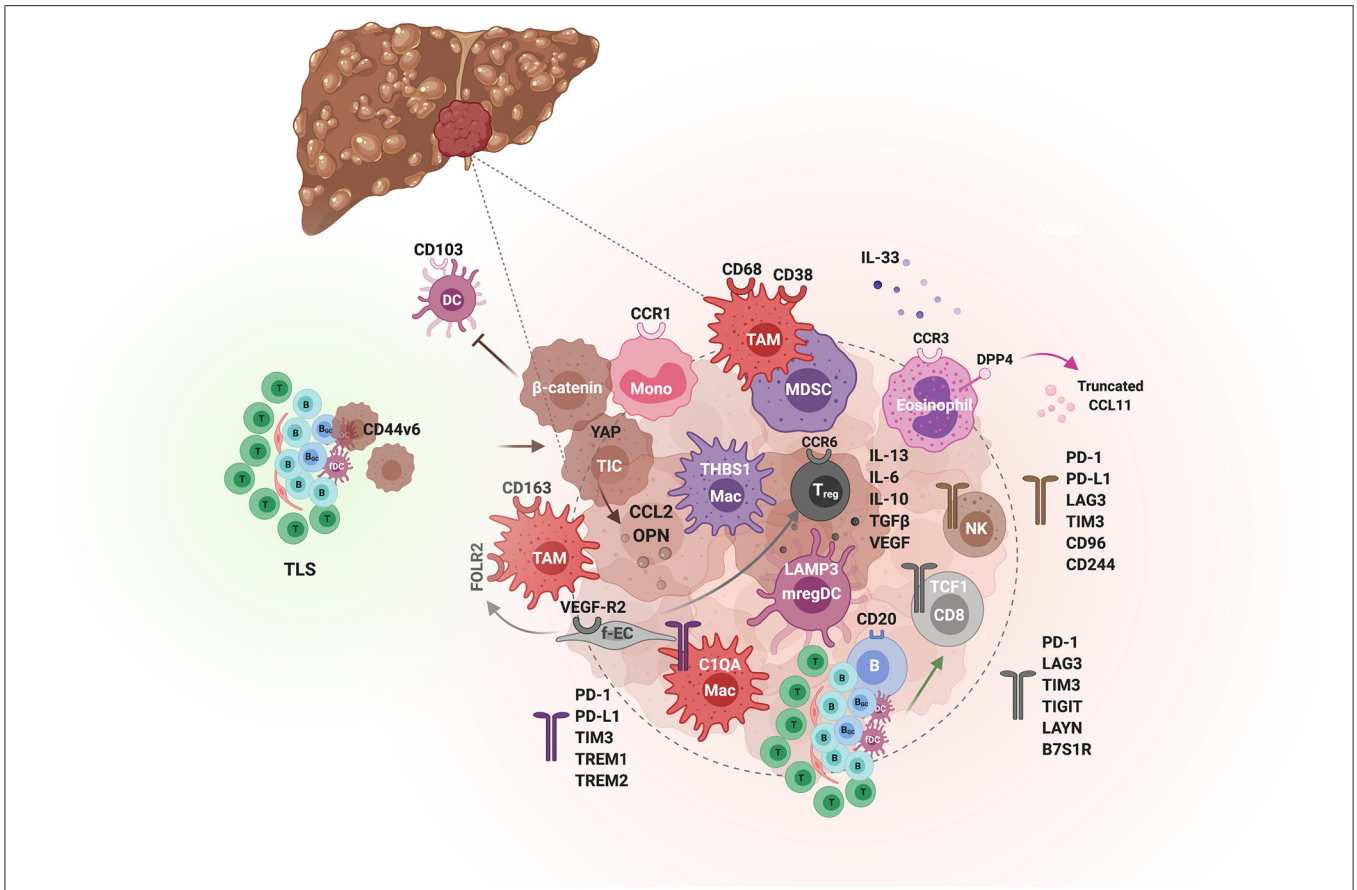

FIGURE 5 | The tumor immunological microenvironment of HCC. Schematic illustration of the different actors demonstrated to contribute to immunosuppression or immune activity in the TME of HCC. Tumor-initiating cells (TIC) and tumor cells (hues of brown) orchestrate the immunological environment by secreting inflammatory cytokines e.g., osteopontin (OPN) and chemokines e.g., the monocyte chemoattractant CCL2, which promote tumorigenesis through the recruitment of monocytes and their differentiation to tumor-associated macrophages (TAMs). In addition, β-catenin-activated tumor cells can inhibit anti-tumor immunity by blocking $CD103^+$ DCs tumor infiltration. Spatially, TAMs are enriched in the peri-tumoral area, express CD68, CD163, CD38, and the folate receptor (FOLR2), induced by fetal-associated endothelial cells (fEC). Two subsets of tumor-enriched macrophages can be distinguished by single cell analyses, $THBS1^+$ macrophages and $C1QA^+$ macrophages, with an MDSC and TAM signature, respectively. The latter is associated with a poor prognosis in HCC. The immunosuppressive activity of TAMs is mediated by various immune checkpoints, including PD-1, PD-L1, TIM-3, TREM1 and TREM2. Besides TAMs, the TME is enriched in Treg but depleted of other T cells and NK cells, which when present exhibit an exhausted phenotype. Among the CD8 T cells, The PD-1^+ $TCF1^+$ "precursors" are associated with a good response to anti-PD-1. TLS found in the adjacent non-tumoral liver tissue are associated with a poor prognosis as they can harbor progenitor/cancer stem cells (expressing CD44v6) and promote tumorigenesis by impairing the clearance of senescent hepatocytes. In contrast, intratumoral TLS and $CD20^+$ B cells are predictive of a lower risk of relapse and a better CD8 T cell anti-tumor activity. Eosinophils also confer a tumorilytic activity, and enhancing their recruitment with inhibitors of the CCL11 peptidase DPP4 improved the efficacy of anti-PD-1+anti-CTLA4 immunotherapy.

prevented hepatocarcinogenesis (60, 61). Additionally, B cells favored HCC progression by limiting the resolution of senescence-mediated liver fibrosis (62). In contrast, it was recently shown that intra-tumoral TLS were predictive of a lower risk of early relapse after surgical resections, as analyzed in 273 patients (63). In contrast, $CD68^+$ $CD163^+$ TAMs, which accumulate at the tumor margin, and $CCR1^+$ monocytes, are associated with bad mOS (64–66). TAMs are recruited to HCC in response to CCL2. The expression of the *CCL2* gene is controlled by diverse mechanisms including through APOBEC3B-mediated de-repression of epigenetic marks in its promoter (67), and through Yes-associated protein (YAP) transcriptional regulation, as shown in tumor-initiating cells (TICs) (68). Together with IL-13, CCL2 drives the metastasis of MYC/Twist1 tumors (69). TAMs contribute to HCC malignant progression and metastasis through diverse mechanisms, e.g., *via* the production of cytokines such as IL-6 (70) and hepatocyte compensatory proliferation (71), immunosuppression and induction of epithelial-to-mesenchymal transition (EMT) [reviewed in (72)]. The immune checkpoint TIM-3, induced by TGFβ in the tumor microenvironment (TME), is implicated in the pro-tumoral effects of TAMs in HCC (70). Similarly, the receptors Triggering Receptor Expressed on Myeloid cells (TREM)1 (73) and TREM2 (74) have been demonstrated to promote the dysfunction and

apoptosis of cytotoxic $CD8^+$ T cells in HCC, while enhancing the recruitment of $CCR6^+$ $Foxp3^+$ Tregs. Platelets, key effectors of immune-mediated tissue damage, have also been implicated in HCC. Using different mouse models of dietary-inducing NASH and data from human patients, Malehmir et al. (75) demonstrated enhanced platelets influx, aggregation and activation in liver sinusoids in NASH. This was mediated by their interaction with KCs, involving hyaluronic acid/CD44 binding and the platelet receptor glycoprotein 1b alpha (GPIbα). Anti-platelet treatments, including aspirin, or specific blockade of GPIbα blunted the development of NASH, through limiting CD8 T lymphocytes, NKT cells and KC recruitment. In the HBsAg transgenic mouse model of HCC, platelets were similarly shown to promote the recruitment of HBsAg-specific CD8 T cells that elicit cycles of hepatocyte killing and inflammation leading to fibrosis. Inhibition of platelet activation potently reduced the development of HCC in this model (76).

scRNAseq of sorted $CD45^+$ cells from the tumor, adjacent liver, hepatic lymph nodes, blood, and ascites of 16 treatment-naive HCC patients recovered all of the major cell populations such as T, B, NK and myeloid cells, but also few minor cell populations including mast cells and ILCs (27) (**Figure 3**). All types of T cells (including Treg, exhausted T cells [Tex] and proliferative T cells) were enriched in the tumors, as previously reported (77). Four clusters of NK cells, enriched in the tumor, were identified, including two circulating NK clusters ($IFN\gamma^+$ $FCGR3A^+$ $CX3CR1^+$ $T\text{-}bet^+$) and two LrNK clusters ($CD160^+$ $CXCR6^+$ $EOMES^+$). However, their respective roles in tumorigenesis and patients prognosis have not been addressed. A diverse repertoire of functionally distinct myeloid cells were identified, particularly, two subsets of macrophages within the tumors: THBS1+ macrophages enriched in myeloid-derived suppressor cell (MDSC) genes (S100A genes, FCN1 and VCAN) and C1QA+ macrophages, enriched in tumor associated macrophage (TAM) genes APOE, C1QB and TREM2 (**Figures 3**, **5**). Only the latter was associated with a poor prognosis in the TCGA liver hepatocellular carcinoma (LIHC) cohort. In parallel, 3 intra-tumoral clusters of DCs were distinguished, namely cDC2 (highly expressing CD1C, FCER1A, and CLEC10A), cDC1 (highly expressing CLEC9A, XCR1 and CADM1) and a non-classical $LAMP3^+$ DCs (highly expressing CCR7, LAMP3, CD80 and CCL19) with migration capacity toward the lymph nodes. Interestingly, ligand-receptor pairs analysis indicated that the $LAMP3^+$ DCs are the subset that would interact with Tex cells and T_{regs}. This LAMP3 population seems to correspond to mregDCs, a DC cluster annotated in human lung cancer as a population involved in tumor antigen uptake and expressing immunoregulatory molecules (78, 79).

HCC Patients Response to ICIs

PD-1 is primarily expressed on the surface of activated T cells, but also on NK/NKT cells (54), B cells (80) and myeloid cells including monocytes, DCs, MDSCs and TAMs (81). Its induction in response to cytokine signaling is tightly regulated at the epigenetic and post-transcriptional levels (48, 81, 82). Recently, two studies used multiparametric flow cytometry and multiplex IHC to show that higher intratumoral frequency of $PD\text{-}1^{high}$ $CD8^+$ T cells (83) and $CD38^+$ $CD68^+$ macrophages (84) was strongly associated with improved response to ICI in patients with advanced HCC. PD-L1 is expressed by DCs, monocytes, macrophages, B cells, NK cells, LSECs, and tumor cells. Its expression is induced by hypoxia (73), among other mechanisms. PD-L1 expression on tumor-infiltrating immune cells is associated with a better prognosis while the prognostic value of its expression on neoplastic cells is controversial (39, 43). Further, the response of patients with HCC to Nivolumab (anti-PD1) was not found to be associated with PD-L1 expression on tumor cells, implicating other PD-L1 expressing cells in this response (15). In murine models with transplantable HCC, PD-L1 expression on myeloid cells mediated the anti-PD-L1 response (85).

Previous studies in melanoma and non-small cell lung cancer (NSCLC) have attributed the response to ICIs to tumor mutational burden (86, 87), levels of neo-antigens (88) or tumor-specific antigens (89, 90), the presence of TLS [reviewed in (59)] or specific oncogenic pathways (91, 92). Nonetheless, the mechanisms involved in patients response to ICIs, particularly in HCC, remain for the most part unclear. For example, the mutational burden did not correlate with ICI response in HCC (93), and neither the mutational load nor the presence of neoantigens was associated with the immune class, which predicted a favorable response to ICI therapy (39). Instead, the activation of β-catenin was associated with resistance to ICI, as demonstrated in a mouse model (94) (**Figure 4**). Using a *MYC;p53*$^{-/-}$ HCC mouse model, Ruiz de Galarreta et al. (94) demonstrated that β-catenin promoted immune escape by preventing the recruitment of $CD103^+$ DCs, impairing antigen-specific T cells-mediated anti-tumor immunity. Accordingly, activating mutations in *CTNNB1* correlate with resistance to ICI monotherapy with either anti-PD-1 or anti-PD-L1, as shown in a prospective sequencing analysis of 27 evaluable advanced HCC patients, in which none of the 10 patients with WNT pathway alterations achieved clinical benefit, whereas around half of the non-WNT pathway–altered patients showed durable stable disease (93). Nevertheless, these results also show that 50% of ICI non-responders harbor mechanisms unrelated to β-catenin activation. Treating fibrosis using a TGFβ neutralizing antibody in the STAM™ mouse model fibrosis-associated HCC, triggered a redistribution of $CD8^+$ lymphocytes into the tumors, which re-invigorated anti-tumor response (95). These results are consistent with those of Mariathasan et al. (96), who reported that TGFβ attenuated the response to PD-L1 blockade by restricting intra-tumoral T-cell infiltration. Since TGFβ alterations are found in a subset of HCC patients (33), agents that block this pathway should be tested in this group, highlighting the need for personalized medicine. Similarly, the immunosuppressive molecule VEGF was found to be enriched in a subset of HCC patients, particularly those with Div-high tumors, supporting the use of the anti-PD-L1 (Atezolizumab) + anti-VEGF (Bevacizumab) combination. However, the cellular subsets, putative signaling pathways, and associated biomarkers required for an effective patient's response to this new combination therapy require

further exploration. The etiology of HCC might contribute to the heterogeneity in patients' response to immunotherapy. Indeed, Lim et al. (44) reported that the TME of HBV-related HCC is more immunosuppressive than that of non-viral HCC. Particularly, PD-1^{high} Tregs and PD-1^{+} $CD8^{+}$ resident memory T cells were more prominent in HBV-related HCC, suggesting that PD-1 blockade might be a suited strategy for this etiology. In contrast, immunotherapies that target $CD244^{+}$ NK cells and Tim-3^{+} $CD8^{+}$ T cells, enriched in non-viral HCC, may be more effective in those patients (44).

ICI Combination Therapies

Several pre-clinical studies and ongoing clinical trials (**Table 1**) are exploring the potential of combining different ICIs. For e.g., a phase III trial is currently testing the combination of durvalumab (anti-PD-L1) and tremelimumab (anti-CTLA-4) as a first line therapy (NCT03298451). The combinations of ICIs together with ablation (97), chemo-radioembolization or targeted therapies (TKi or anti-VEGF) in the adjuvant or neoadjuvant setting are also being explored (**Table 1**). For e.g., two trials are testing the combination of pembrolizumab + Lenvatinib (NCT 03006926) or of pembrolizumab + regorafenib (NCT03347292) for first line therapies. Radioembolization was reported to elicit an immune response, both locally and systemically, leading to enhanced infiltration of TIM-3^{+} tumor-infiltrating lymphocytes (TILs), NK, and NKT cells (98). It is thus plausible that an ICI targeting TIM-3 might enhance the clinical response of radioembolization or other interventions in HCC patients. A phase II trial is currently testing cobolimab, a TIM-3 binding antibody, in combination with anti-PD-1 on the response of patients with locally advanced or metastatic liver cancer (NCT03680508). Similarly, combining multiple strategies targeting inhibitory receptors (PD-1, TIM-3, LAG3, CTLA4, TREM1, TREM2) and/or their ligands (PD-L1, B7 superfamily member1 [B7S1]) have shown synergistic effects in restoring TILs anti-tumoral immune responses in pre-clinical studies (73, 74, 99–102) and enhancing NK cell infiltration and activity (52–56, 103). Additional strategies include the inhibition of TAM recruitment, their polarization to an immunosuppressive phenotype or their function in hampering anti-tumor immunity or promoting tumorigenesis. The pro-inflammatory protein osteopontin (OPN) produced by cancer cells has been implicated in cancer promotion and metastasis, through the stimulation of CSF1 signaling in TAMs. Blockade of the CSF1/CSF1R pathway enhanced the efficacy of anti PD-L1 in OPN-overexpressing HCC, by reducing macrophage recruitment (102). Blockade of the CCL2/CCR2 axis was also shown to inhibit the recruitment of TAMs leading to enhanced infiltration of $CD8^{+}$ T cells and improved anti-tumor immunity (104). However, this approach should be considered with caution as some macrophages exert anti-tumoral activity. Indeed, Eggert et al. (105) reported that the CCL2-CCR2 axis promotes the clearance of senescent hepatocytes preventing HCC outgrowth in mice. Among the TAM targets that recently surfaced as critical inhibitors of anti-tumor immunity are the receptors TREM1 and TREM2. Blockade of TREM1 (73) or TREM2 (74) attenuated immunosuppression and $CD8^{+}$ T cell dysfunction boosting the efficacy of anti-PD-1/PD-L1 immunotherapy. An alternative approach to skew TAM functions is through vaccination. Using a mouse model, a recent study demonstrated that a *Listeria*-based HCC vaccine enhanced the efficacy of PD-1 blockade by skewing the TAMs to an anti-tumoral phenotype (106). Consistent with the improved patients response to the anti-PD-L1 + anti-VEGF combination therapy, it has been recently demonstrated using murine models of HCC that this approach fortified hepatic vessels and overcame resistance to either monotherapy (107). Last, a less studied immune cell population in the context of anti-tumor immunity are the eosinophils, which were recently shown in a murine model of HCC to promote tumor-cell killing through degranulation and contribute to the efficacy of the anti-PD1 + anti-CTLA4 combination immunotherapy. Their recruitment in response to the cancer-cell secreted alarmin IL-33, is mediated by the chemokine CCL11, and enhanced with the administration of sitagliptin, an inhibitor of dipeptidyl peptidase DPP4 (CD26) that cleaves CCL11. These results suggest that combined modulation of both type 1 and 2 immune responses may improve therapeutic management of HCC (108).

THE FUTURE OF IMMUNOTHERAPIES IN HCC BEYOND ICI

Besides ICI, several immunotherapeutic strategies for HCC patients are emerging, such as targeted therapies promoting ADCC, adoptive cell therapy (ACT), including the transfer of autologous CD8 T cells, iNKT cells, γδ T cells, cytokine-induced immune killer cells (IKC), chimeric antigen receptor (CAR)-T cells, oncolytic viruses and vaccines (**Figure 6**). For a number of these strategies, tumor-specific antigens (TSA) or tumor-associated antigens (TAA) are targeted. In HCC, these include α-fetoprotein (109–111), hTERT (112), glypican-3 (GPC3) (113–115), p53 (116), melanoma antigen gene A (MAGE-A) (117), squamous cell carcinoma antigen recognized by T cells (SART) (118), and NY-ESO-1 (119). More recently, the oncogenic phosphatase PRL3 was confirmed as a TAA, as it was shown to be expressed in tumors, but not in patient-matched normal tissue, across 11 cancers. A humanized antibody targeting this TAA, PRL3-zumab, was shown to enhance the intra-tumoral recruitment of B cells, NK cells and macrophages, suggesting that this antibody might promote tumor killing by ADCC (120). Similarly, the elevated expression of GPC3 in >70% of HCC, and its association with poor prognosis (121), has led to the development of several immunotherapeutic strategies, including the humanized monoclonal antibody codrituzumab (122), bi-specific antibodies (123), CAR-T cells (124), antibody-drug conjugates (125), and vaccines (126). GPC3-CAR-T cells have been shown to be polyfunctional and capable of eliminating HCC in a transplantable orthotopic mouse model (127), and there are currently at least 5 phase I clinical trials recruiting patients with HCC to test GPC3-CAR-T cells (**Table 1**; ClinicalTrials.gov, December 2020). Another CAR-T cell tested in multiple solid tumors including HCC is the EpCAM-CAR-T, as registered

TABLE 1 | Clinical trials of immunotherapies for HCC.

Immunotherapy	Identifier	Study title	Interventions	Number enrolled	Primary completion
Phase III clinical trials					
ICI + combinations					
ICI as Adjuvant (Stage A)	NCT03867084	Safety and Efficacy of Pembrolizumab (MK-3475) vs. Placebo as Adjuvant Therapy in Participants With Hepatocellular Carcinoma(HCC) and Complete Radiological Response After Surgical Resection or Local Ablation (MK-3475-937/ KEYNOTE-937)	Biological: Pembrolizumab Drug: Placebo	950	June 2025
	NCT03383458	A Study of Nivolumab in Participants With Hepatocellular Carcinoma Who Are at High Risk of Recurrence After Curative Hepatic Resection or Ablation (CheckMate 9DX)	Biological: Nivolumab Other: Placebo	530	Jan 2023
	NCT04102098	A Study of Atezolizumab Plus Bevacizumab vs. Active Surveillance as Adjuvant Therapy in Patients With Hepatocellular Carcinoma at High Risk of Recurrence After Surgical Resection or Ablation (IMbrave050)	Drug: Atezolizumab Drug: Bevacizumab	662	Mar 2023
	NCT03847428	Assess Efficacy and Safety of Durvalumab Alone or Combined With Bevacizumab in High Risk of Recurrence HCC Patients After Curative Treatment (EMERALD-2)	Drug: Durvalumab Drug: Bevacizumab Other: Placebo	888	Sept 2022
	NCT03859128	Toripalimab or Placebo as Adjuvant Therapy in Hepatocellular Carcinoma After Curative Hepatic Resection (JUPITER 04)	Biological: TORIPALIMAB INJECTION (JS001)	402	Oct 2022
ICI + TACE (Stage B)	NCT04229355	DEB-TACE Plus Lenvatinib or Sorafenib or PD-1 Inhibitor for Unresectable Hepatocellular Carcinoma	Drug: DEB-TACE plus Sorafenib Drug: DEB-TACE plus Lenvatinib Drug: DEB-TACE plus PD-1 inhibitor	90	Dec 2022
	NCT04246177	Safety and Efficacy of Lenvatinib (E7080/MK-7902) With Pembrolizumab (MK-3475) in Combination With Transarterial Chemoembolization (TACE) in Participants With Incurable/Non-metastatic Hepatocellular Carcinoma (MK-7902-012/E7080-G000-318/LEAP-012)			
	NCT03949231	Infusion of Toripalimab Via Hepatic Arterial vs. Vein for Immunotherapy of Advanced Hepatocellular Carcinoma	Drug: Toripalimab	200	Jan 2022
	NCT03755739	Trans-Artery/Intra-Tumor Infusion of Checkpoint Inhibitors for Immunotherapy of Advanced Solid Tumors	Drug: Checkpoint inhibitor (CPI) such as Pembrolizumab	200	Nov 2033
	NCT04268888	Nivolumab in Combination With TACE/TAE for Patients With Intermediate Stage HCC	Drug: Nivolumab and TACE/TAE Procedure: TACE/TAE	522	June 2025
	NCT0378957	A Global Study to Evaluate Transarterial Chemoembolization (TACE) in Combination With Durvalumab and Bevacizumab Therapy in Patients With Locoregional Hepatocellular Carcinoma (EMERALD-1)	Drug: Durvalumab Drug: Bevacizumab Other: Placebo Procedure: Transarterial Chemoembolization (TACE)	600	Aug 2021
ICI + stereotaxic radiotherapy (Stage B)	NCT04167293	Combination of Sintilimab and Stereotactic Body Radiotherapy in Hepatocellular Carcinoma (ISBRT01)	Radiation: stereotactic body radiotherapy Drug: Sintilimab	116	Nov 2021
Monotherapy (Stage C)	NCT02576509	An Investigational Immuno-therapy Study of Nivolumab Compared to Sorafenib as a First Treatment in Patients With Advanced Hepatocellular Carcinoma	Drug: Nivolumab Drug: Sorafenib	743	May 2019

(Continued)

TABLE 1 | Continued

Immunotherapy	Identifier	Study title	Interventions	Number enrolled	Primary completion
	NCT03412773	Phase 3 Study of Tislelizumab vs. Sorafenib in Participants With Unresectable HCC	Drug: Tislelizumab Drug: Sorafenib	674	June 2021
ICI + αVEGF (Stage C)	NCT03434379	A Study of Atezolizumab in Combination With Bevacizumab Compared With Sorafenib in Patients With Untreated Locally Advanced or Metastatic Hepatocellular Carcinoma [IMbrave150] (IMbrave150)	Drug: Atezolizumab Drug: Bevacizumab Drug: Sorafenib	480	Feb 2021
	NCT03794440	A Study to Evaluate the Efficacy and Safety of Sintilimab in Combination With IBI305 (Anti-VEGF Monoclonal Antibody) Compared to Sorafenib as the First-Line Treatment for Advanced Hepatocellular Carcinoma.	Drug: Sintilimab Drug: IBI305 Drug: Sorafenib	566	Dec 2022
ICI + TKi (Stage C)	NCT03713593	Safety and Efficacy of Lenvatinib (E7080/MK-7902) in Combination With Pembrolizumab (MK-3475) vs. Lenvatinib as First-line Therapy in Participants With Advanced Hepatocellular Carcinoma (MK-7902-002/E7080-G000-311/LEAP-002)	Drug: lenvatinib Biological: pembrolizumab Drug: saline placebo	750	May 2022
	NCT03764293	A Study to Evaluate SHR-1210 in Combination With Apatinib as First-Line Therapy in Patients With Advanced HCC	Drug: SHR-1210 Drug: Apatinib Drug: Sorafenib	510	Dec 2021
	NCT03755791	Study of Cabozantinib in Combination With Atezolizumab vs. Sorafenib in Subjects With Advanced HCC Who Have Not Received Previous Systemic Anticancer Therapy (COSMIC-312)	Drug: Cabozantinib Drug: Sorafenib Drug: Atezolizumab	740	June 2021
ICI + ICI (Stage C)	NCT03298451	Study of Durvalumab and Tremelimumab as First-line Treatment in Patients With Advanced Hepatocellular Carcinoma (HIMALAYA)	Drug: Durvalumab Drug: Tremelimumab (Regimen 1) Drug: Tremelimumab (Regimen 2) Drug: Sorafenib Drug: Durvalumab (Regimen 1) Drug: Durvalumab (Regimen 2)	1324	Dec 2020
	NCT04039607	A Study of Nivolumab in Combination With Ipilimumab in Participants With Advanced Hepatocellular Carcinoma (CheckMate 9DW)	Drug: Nivolumab Drug: Ipilimumab Drug: Sorafenib Drug: lenvatinib	650	Mar 2023
ACT	NCT02678013	RFA+Highly-purified CTL vs. RFA Alone for Recurrent HCC	Procedure: RFA Procedure: RFA+highly-purified CTL	210	Jan 2020
	NCT02709070	Resection+Highly Purified CTL vs. Resection Alone for HCC	Procedure: resection Procedure: highly-purified CTL	210	Mar 2020
	NCT03592706	Autologous Immune Killer Cells to Treat Liver Cancer Patients as an Adjunct Therapy	Biological: IKC (Immune Killer Cells) Procedure: TACE (Transcatheter Arterial Chemoembolization)	60	Feb 2021
OV	NCT02562755	Hepatocellular Carcinoma Study Comparing Vaccinia Virus Based Immunotherapy Plus Sorafenib vs. Sorafenib Alone	Biological: Pexastimogene Devacirepvec (Pexa Vec) Drug: Sorafenib	600	Dec 2020

(Continued)

TABLE 1 | Continued

Immunotherapy	Identifier	Study title	Interventions	Number enrolled	Primary completion
Vax	NCT02232490	Liver Cancer Immunotherapy: Placebo-controlled Clinical Trial of Hepcortespenlisimut-L	Biological: hepcortespenlisimut-L Biological: Placebo	120	Nov 2019
Phase II clinical trials					
ICI + combinations					
Neoadjuvant (Stage A)	NCT03222076	Nivolumab With or Without Ipilimumab in Treating Patients With Resectable Liver Cancer	Biological: Ipilimumab Biological: Nivolumab	30	Sept 2022
	NCT03510871	Nivolumab Plus Ipilimumab as Neoadjuvant Therapy for Hepatocellular Carcinoma (HCC)	Drug: nivolumab, ipilimumab	40	Dec 2022
	NCT03630640	Neoadjuvant and Adjuvant Nivolumab in HCC Patients Treated by Electroporation	Drug: Nivolumab Injection [Opdivo]	50	Sept 2020
	NCT03682276	Safety and Bioactivity of Ipilimumab and Nivolumab Combination Prior to Liver Resection in Hepatocellular Carcinoma	Biological: Ipilimumab Biological: Nivolumab	32	Dec 2020
	NCT04174781	Neoadjuvant Therapy for Hepatocellular Carcinoma	Drug: Sintilimab Injection Drug: TACE	61	Nov 2020
ICI + TACE (Stage B)	NCT03638141	CTLA-4 /PD-L1 Blockade Following Transarterial Chemoembolization (DEB-TACE) in Patients With Intermediate Stage of HCC (Hepatocellular Carcinoma) Using Durvalumab and Tremelimumab	Drug: Durvalumab Drug: Tremelimumab (Cohort A dose) Drug: Tremelimumab (Cohort B dose)	30	Nov 2020
	NCT04273100	PD-1 Monoclonal Antibody, Lenvatinib and TACE in the Treatment of HCC	Combination Product: PD-1 mAb combined with TACE and lenvatinib	56	Dec 2020
	NCT03817736	Sequential TransArterial Chemoembolization and Stereotactic RadioTherapy With ImmunoTherapy for Downstaging Hepatocellular Carcinoma for Hepatectomy	Procedure: TACE Radiation: SBRT Drug: Immune Checkpoint Inhibitor	33	Feb 2022
	NCT04522544	Durvalumab (MEDI4736) and Tremelimumab in Combination With Either Y-90 SIRT or TACE for Intermediate Stage HCC With Pick-the-winner Design	Drug: Tremelimumab Drug: Durvalumab Procedure: Y-90 SIRT Procedure: TACE	84	Mar 2024
	NCT04518852	TACE, Sorafenib and PD-1 Monoclonal Antibody in the Treatment of HCC	Combination Product: TACE combined with sorafenib and PD-1 mAb	60	July 2022
	NCT03937830	Combined Treatment of Durvalumab, Bevacizumab, Tremelimumab and Transarterial Chemoembolization (TACE) in Subjects With Hepatocellular Carcinoma or Biliary Tract Carcinoma	Drug: durvalumab Drug: Doxorubicin-Eluting Beads Procedure: TACE (and 2 more...)	22	Dec 2022

(Continued)

TABLE 1 | Continued

Immunotherapy	Identifier	Study title	Interventions	Number enrolled	Primary completion
	NCT03817736	Sequential TransArterial Chemoembolization and Stereotactic RadioTherapy With ImmunoTherapy for Downstaging Hepatocellular Carcinoma for Hepatectomy	Procedure: TACE Radiation: SBRT Drug: Immune Checkpoint Inhibitor	33	Feb 2022
	NCT04268888	Nivolumab in Combination With TACE/TAE for Patients With Intermediate Stage HCC	Drug: Nivolumab and TACE/TAE Procedure: TACE/TAE	522	June 2025
	NCT03259867	Combination of TATE and PD-1 Inhibitor in Liver Cancer	Drug: Opdivo Injectable Product or Keytruda Injectable Product Combination Product: Trans-arterial tirapazamine embolization	80	Oct 2020
	NCT04191889	A Trial of Hepatic Arterial Infusion Combined With Apatinib and Camrelizumab for C-staged Hepatocellular Carcinoma in BCLC Classification	Combination Product: Hepatic Arterial Infusion combined with Apatinib and Camrelizumab	84	Dec 2021
	NCT03397654	Study of Pembrolizumab Following TACE in Primary Liver Carcinoma (PETAL)	Drug: Pembrolizumab Combination Product: Trans-arterial chemoembolization	26	Mar 2020
ICI + radioembolization Thermal ablation and radiotherapy (Stage B)	NCT03033446	Study of Y90-Radioembolization With Nivolumab in Asians With Hepatocellular Carcinoma	Radiation: Y-90 Radioembolization Drug: Nivolumab	40	Dec 2019
	NCT03380130	A Study of the Safety and Antitumoral Efficacy of Nivolumab After SIRT for the Treatment of Patients With HCC (NASIR-HCC)	Drug: Nivolumab Device: SIR-Spheres	40	Oct 2019
	NCT03753659	IMMULAB - Immunotherapy With Pembrolizumab in Combination With Local Ablation in Hepatocellular Carcinoma (HCC)	Drug: Pembrolizumab Procedure: Radio Frequency Ablation (RFA) Procedure: Microwave Ablation (MWA) (and 2 more...)	30	Mar 2022
	NCT04193696	RT+ Anti-PD-1 for Patients With Advanced HCC (RT+PD-1-HCC)	Drug: Radiation therapy and systemic anti-PD-1 immunotherapy for patients with advanced hepatocellular carcinoma	39	June 2020
	NCT03864211	Thermal Ablation Followed by Immunotherapy for HCC	Procedure: Thermal ablation Drug: Toriplimab	120	Mar 2021
	NCT04167293	Combination of Sintilimab and Stereotactic Body Radiotherapy in Hepatocellular Carcinoma (ISBRT01)	Radiation: stereotactic body radiotherapy Drug: Sintilimab	116	Nov 2021

(Continued)

TABLE 1 | Continued

Immunotherapy	Identifier	Study title	Interventions	Number enrolled	Primary completion
	NCT03316872	Study of Pembrolizumab and Radiotherapy in Liver Cancer	Drug: Pembrolizumab Radiation: Stereotactic Body Radiotherapy (SBRT)	30	Apr 2020
Monotherapy Stage C)	NCT01693562	A Phase 1/2 Study to Evaluate MEDI4736	Drug: MEDI4736	1022	Feb 2020
	NCT03389126	Phase II Study of Avelumab in Patients With Advanced Hepatocellular Carcinoma After Prior Sorafenib Treatment (Avelumab HCC)	Drug: Avelumab	30	Dec 2019
ICI + TKi (Stage C)	NCT01658878	An Immuno-therapy Study to Evaluate the Effectiveness, Safety and Tolerability of Nivolumab or Nivolumab in Combination With Other Agents in Patients With Advanced Liver Cancer	Biological: Nivolumab Drug: Sorafenib Drug: Ipilimumab Drug: Cabozantinib	1097	Aug 2020
	NCT03841201	Immunotherapy With Nivolumab in Combination With Lenvatinib for Advanced Stage Hepatocellular Carcinoma	Drug: Lenvatinib Drug: Nivolumab	50	July 2021
	NCT04183088	Regorafenib Plus Tislelizumab as First-line Systemic Therapy for Patients With Advanced Hepatocellular Carcinoma	Drug: Tislelizumab+ regorafenib for part 1;Tislelizumab+ regorafenib for group 1 of part 2; Placebo+regorafenib for group 2 of part 2.	125	Mar 2024
	NCT04310709	Combination of Regorafenib and Nivolumab in Unresectable Hepatocellular Carcinoma	Drug: Regorafenib/Nivolumab	42	May 2022
	NCT03439891	Sorafenib and Nivolumab in Treating Participants With Unresectable, Locally Advanced or Metastatic Liver Cancer	Other: Laboratory Biomarker Analysis Biological: Nivolumab Drug: Sorafenib	40	Sept 2022
	NCT03170960	Study of Cabozantinib in Combination With Atezolizumab to Subjects With Locally Advanced or Metastatic Solid Tumors	Drug: cabozantinib Drug: atezolizumab	1732	Dec 2020
	NCT03899428	Immune Checkpoint Therapy vs. Target Therapy in Reducing Serum HBsAg Levels in Patients With HBsAg+ Advanced Stage HCC	Drug: Durvalumab Drug: Sorafenib Drug: Lenvatinib (and 2 more...)	30	Dec 2021
	NCT04442581	Cabozantinib and Pembrolizumab for the First-Line Treatment of Advanced Liver Cancer	Drug: Cabozantinib S-malate Biological: Pembrolizumab	29	Sept 2023
	NCT04523662	Study on the Effectiveness and Safety of Carrelizumab Combined With Apatinib Mesylate and Radiotherapy in the Treatment of Advanced Liver Cancer	Drug: Camrelizumab Apatinib Mesylas	27	Aug 2022
	NCT04212221	MGD013 Monotherapy and Combination With Brivanib Dose Escalation and Expansion Study in Advanced Liver Cancer Patients	Drug: MGD013 monotherapy Drug: MGD013 in combination with Brivanib Alaninate	300	Dec 2022
	NCT03463876	A Trial of SHR-1210 (an Anti-PD-1 Inhibitor) in Combination With Apatinib in Patients With Advanced HCC(RESCUE)	Drug: SHR 1210+apatinib	190	June 2019
ICI + ICI	NCT03228667	QUILT-3.055: A Study of Combination Immunotherapies in Patients Who Have Previously Received Treatment With Immune Checkpoint Inhibitors	Drug: N-803 + Pembrolizumab Drug: N-803 + Nivolumab Drug: N-803 + Atezolizumab (and 7 more...)	636	June 2021

(Continued)

TABLE 1 | Continued

Immunotherapy	Identifier	Study title	Interventions	Number enrolled	Primary completion
	NCT03311334	A Study of DSP-7888 Dosing Emulsion in Combination With Immune Checkpoint Inhibitors in Adult Patients With Advanced Solid Tumors	Drug: DSP-7888 Dosing Emulsion Drug: Nivolumab Drug: Pembrolizumab	84	Nov 2021
	NCT03228667	QUILT-3.055: A Study of Combination Immunotherapies in Patients Who Have Previously Received Treatment With Immune Checkpoint Inhibitors	Drug: N-803 + Pembrolizumab Drug: N-803 + Nivolumab Drug: N-803 + Atezolizumab (and 7 more...)	636	June 2021
	NCT04430452	Hypofractionated Radiotherapy Followed by Durvalumab With or Without Tremelimumab for the Treatment of Liver Cancer After Progression on Prior PD-1 Inhibition	Biological: Durvalumab Radiation: Hypofractionated Radiation Therapy Biological: Tremelimumab	30	Aug 2022
	NCT04547452	Combination of Sintilimab and Stereotactic Body Radiotherapy in Advanced Metastatic HCC	Radiation: Stereotactic body radiation therapy Drug: Anti-PD-1 antibody drug named Sintilimab	84	July 2022
	NCT03655613	APL-501 or Nivolumab in Combination With APL-101 in Locally Advanced or Metastatic HCC and RCC	Biological: APL-501 Drug: APL-101 Biological: Nivolumab	119	Sept 2020
	NCT04380545	Nivolumab, Fluorouracil, and Interferon Alpha 2B for the Treatment of Unresectable Fibrolamellar Cancer	Drug: Fluorouracil Biological: Nivolumab Biological: Recombinant Interferon Alpha 2b-like Protein	15	July 2021
	NCT02519348	A Study of Durvalumab or Tremelimumab Monotherapy, or Durvalumab in Combination With Tremelimumab or Bevacizumab in Advanced Hepatocellular Carcinoma	Biological: Durvalumab + tremelimumab Biological: Durvalumab Biological: Tremelimumab Biological: Durvalumab + Bevacizumab	433	Nov 2020
	NCT03755739	Trans-Artery/Intra-Tumor Infusion of Checkpoint Inhibitors for Immunotherapy of Advanced Solid Tumors	Drug: Checkpoint inhibitor (CPI) such as Pembrolizumab	200	Nov 2033
	NCT02940496	Pembrolizumab With or Without Elbasvir/Grazoprevir and Ribavirin in Treating Patients With Advanced Refractory Liver Cancer	Drug: Elbasvir/Grazoprevir Other: Laboratory Biomarker Analysis Biological: Pembrolizumab Drug: Ribavirin	30	Dec 2021
	NCT03836352	Study of an Immunotherapeutic, DPX-Survivac, in Combination With Low Dose Cyclophosphamide & Pembrolizumab, in Subjects With Selected Advanced & Recurrent Solid Tumors	Other: DPX-Survivac Drug: Cyclophosphamide Drug: Pembrolizumab	184	Dec 2022

(Continued)

TABLE 1 | Continued

Immunotherapy	Identifier	Study title	Interventions	Number enrolled	Primary completion
	NCT03544723	Safety and Efficacy of p53 Gene Therapy Combined With Immune Checkpoint Inhibitors in Solid Tumors.	Drug: Ad-p53	40	June 2022
	NCT03680508	TSR-022 (Anti-TIM-3 Antibody) and TSR-042 (Anti-PD-1 Antibody) in Patients With Liver Cancer	Drug: TSR-022 and TSR-042	42	Oct 2022
CAR-T	NCT03941626	Autologous CAR-T/TCR-T Cell Immunotherapy for Solid Malignancies	Biological: CAR-T/TCR-T cells immunotherapy	50	Dec 2020
	NCT03638206	Autologous CAR-T/TCR-T Cell Immunotherapy for Malignancies	Biological: CAR-T cell immunotherapy	73	Mar 2023
	NCT03013712	A Clinical Research of CAR T Cells Targeting EpCAM Positive Cancer	Biological: CAR-T cell immunotherapy	60	Dec 2018
ACT	NCT03093688	Clinical Safety and Efficacy Study of Infusion of iNKT Cells and CD8+T Cells in Patients With Advanced Solid Tumor	Biological: Infusion of iNKT cells and CD8+T cells	40	Dec 2021
	NCT04502082	Study of ET140203 T Cells in Adults With Advanced Hepatocellular Carcinoma (ARYA-1)	Biological: ET140203 autologous T cell product	50	Jan 2023
	NCT03998033	Study of ET140202 T Cells in Adults With Advanced Hepatocellular Carcinoma	Biological: ET140202 autologous T cell product	50	July 2022
	NCT03592706	Autologous Immune Killer Cells to Treat Liver Cancer Patients as an Adjunct Therapy	Biological: IKC (Immune Killer Cells) Procedure: TACE (Transcatheter Arterial Chemoembolization)	60	Feb 2021
	NCT02856815	Safety and Efficacy of "Immune Cell-LC" in TACE Therapy	Biological: Immuncell-LC	78	October 30, 2020
OV	NCT03071094	A Trial to Evaluate the Safety and Efficacy of the Combination of the Oncolytic Immunotherapy Pexa-Vec With the PD-1 Receptor Blocking Antibody Nivolumab in the First-line Treatment of Advanced Hepatocellular Carcinoma (HCC)	Biological: Pexastimogene Devacirepvec (Pexa Vec) Drug: Nivolumab	30	Sept 2020
Vax	NCT03067493	RFA Combined With Neo-MASCT for Primary HCC: a Phase II Trial	Biological: Neo-MASCT	98	Mar 2021
Phase I clinical trials					
ICI + combinations					
Neoadjuvant (Stage A)	NCT03682276	Safety and Bioactivity of Ipilimumab and Nivolumab Combination Prior to Liver Resection in Hepatocellular Carcinoma	Biological: Ipilimumab Biological: Nivolumab	32	Dec 2020
ICI + TACE (Stage B)	NCT03143270	A Study to Test the Safety and Feasibility of Nivolumab With Drug Eluting Bead Transarterial Chemoembolization in Patients With Liver Cancer	Drug: Drug Eluting Bead Transarterial Chemoembolization Drug: Nivolumab	14	Apr 2022
ICI + radioembolization (Stage B)	NCT03099564	Pembrolizumab Plus Y90 Radioembolization in HCC Subjects	Drug: Pembrolizumab Device: Y90 radioembolization	30	July 2020
	NCT02837029	Nivolumab and Yttrium Y 90 Glass Microspheres in Treating Patients With Advanced Liver Cancer	Other: Laboratory Biomarker Analysis Biological: Nivolumab Radiation: Yttrium Y 90 Glass Microspheres	27	July 2019

(Continued)

TABLE 1 | Continued

Immunotherapy	Identifier	Study title	Interventions	Number enrolled	Primary completion
	NCT01658878	An Immuno-therapy Study to Evaluate the Effectiveness, Safety and Tolerability of Nivolumab or Nivolumab in Combination With Other Agents in Patients With Advanced Liver Cancer	Biological: Nivolumab Drug: Sorafenib Drug: Ipilimumab Drug: Cabozantinib	1097	Aug 2020
	NCT03474640	Safety, Tolerability and Pharmacokinetics of an Anti-PD-1 Monoclonal Antibody in Subjects With Advanced Malignancies	Biological: Toripalimab, Recombinant Humanized anti-PD-1 Monoclonal Antibody	258	Aug 2022
	NCT03655613	APL-501 or Nivolumab in Combination With APL-101 in Locally Advanced or Metastatic HCC and RCC	Biological: APL-501 Drug: APL-101 Biological: Nivolumab	119	Sept 2020
	NCT04564313	Safety and Efficacy of Camrelizumab (Anti-PD-1 Antibody) in Recurrent HCC After Liver Transplantation	Drug: Camrelizumab treatment	20	July 2021
	NCT02940496	Pembrolizumab With or Without Elbasvir/Grazoprevir and Ribavirin in Treating Patients With Advanced Refractory Liver Cancer	Drug: Elbasvir/Grazoprevir Other: Laboratory Biomarker Analysis Biological: Pembrolizumab Drug: Ribavirin	30	Dec 2021
	NCT03203304	Stereotactic Body Radiotherapy (SBRT) Followed by Immunotherapy in Liver Cancer	Drug: Nivolumab Drug: Ipilimumab	50	Aug 2021
	NCT04220944	Combined Locoregional Treatment With Immunotherapy for Unresectable HCC.	Drug: Sintilimab Procedure: Microwave Ablation Procedure: TACE	45	June 2021
	NCT03864211	Thermal Ablation Followed by Immunotherapy for HCC	Procedure: Thermal ablation Drug: Toriplimab	120	Mar 2021
New ICI	NCT04374877	Study of SRF388 in Patients With Advanced Solid Tumors	Drug: SRF388	122	July 2021
CAR-T	NCT04121273	GPC3-targeted CAR-T Cell for Treating GPC3 Positive Advanced HCC	Biological: CAR-T cell immunotherapy	20	Oct 2021
	NCT02905188	Glypican 3-specific Chimeric Antigen Receptor Expressing T Cells for Hepatocellular Carcinoma (GLYCAR)	Genetic: GLYCAR T cells Drug: Cytoxan Drug: Fludarabine	14	Dec 2021
	NCT03198546	GPC3-T2-CAR-T Cells for Immunotherapy of Cancer With GPC3 Expression	Biological: GPC3 and/or TGFβ targeting CAR-T cells	30	Aug 2020
	NCT03941626	Autologous CAR-T/TCR-T Cell Immunotherapy for Solid Malignancies	Biological: CAR-T/TCR-T cells immunotherapy	50	Dec 2020
	NCT03638206	Autologous CAR-T/TCR-T Cell Immunotherapy for Malignancies	Biological: CAR-T cell immunotherapy	73	Mar 2023
	NCT03013712	A Clinical Research of CAR T Cells Targeting EpCAM Positive Cancer	Biological: CAR-T cell immunotherapy	60	Dec 2018

(Continued)

TABLE 1 | Continued

Immunotherapy	Identifier	Study title	Interventions	Number enrolled	Primary completion
ACT	NCT03093688	Clinical Safety and Efficacy Study of Infusion of iNKT Cells and CD8+T Cells in Patients With Advanced Solid Tumor	Biological: Infusion of iNKT cells and CD8+T cells	40	Dec 2021
	NCT04032392	Immunotherapy of Advanced Hepatitis B Related Hepatocellular Carcinoma With γδ T Cells	Biological: autologous γδ T cells	20	July 2021
	NCT04502082	Study of ET140203 T Cells in Adults With Advanced Hepatocellular Carcinoma (ARYA-1)	Biological: ET140203 autologous T cell product	50	Jan 2023
	NCT03998033	Study of ET140202 T Cells in Adults With Advanced Hepatocellular Carcinoma	Biological: ET140202 autologous T cell product	50	July 2022
	NCT03132792	$AFP^{c332}T$ in Advanced HCC	Genetic: Autologous genetically modified $AFP^{c332}T$ cells	45	June 2021
	NCT03441100	TCR-engineered T Cells in Solid Tumors: IMA202-101	Drug: IMA202 Product Device: IMA_Detect	15	June 2022
	NCT03319459	FATE-NK100 as Monotherapy and in Combination With Monoclonal Antibody in Subjects With Advanced Solid Tumors	Drug: FATE-NK100 Drug: Cetuximab Drug: Trastuzumab	100	Oct 2021
	NCT03841110	FT500 as Monotherapy and in Combination With Immune Checkpoint Inhibitors in Subjects With Advanced Solid Tumors	Drug: FT500 Drug: Nivolumab Drug: Pembrolizumab (and 3 more...)	76	Mar 2022
Agonists/Cytokines	NCT02315066	Study Of OX40 Agonist PF-04518600 Alone And In Combination With 4-1BB Agonist PF-05082566	Drug: PF-04518600 Drug: PF-04518600 plus PF-05082566	176	Dec 2020
	NCT03655002	IRX-2, Cyclophosphamide, and Nivolumab in Treating Patients With Recurrent or Metastatic and Refractory Liver Cancer	Drug: Cyclophosphamide Biological: Cytokine-based Biologic Agent IRX-2 Biological: Nivolumab	28	June 2022
OV	NCT03071094	A Trial to Evaluate the Safety and Efficacy of the Combination of the Oncolytic Immunotherapy Pexa-Vec With the PD-1 Receptor Blocking Antibody Nivolumab in the First-line Treatment of Advanced Hepatocellular Carcinoma (HCC)	Biological: Pexastimogene Devacirepvec (Pexa Vec) Drug: Nivolumab	30	Sept 2020
Vax	NCT04248569	DNAJB1-PRKACA Fusion Kinase Peptide Vaccine Combined With Nivolumab and Ipilimumab for Patients With Fibrolamellar Hepatocellular Carcinoma	Drug: DNAJB1-PRKACA peptide vaccine Drug: Nivolumab Drug: Ipilimumab	12	Mar 2024

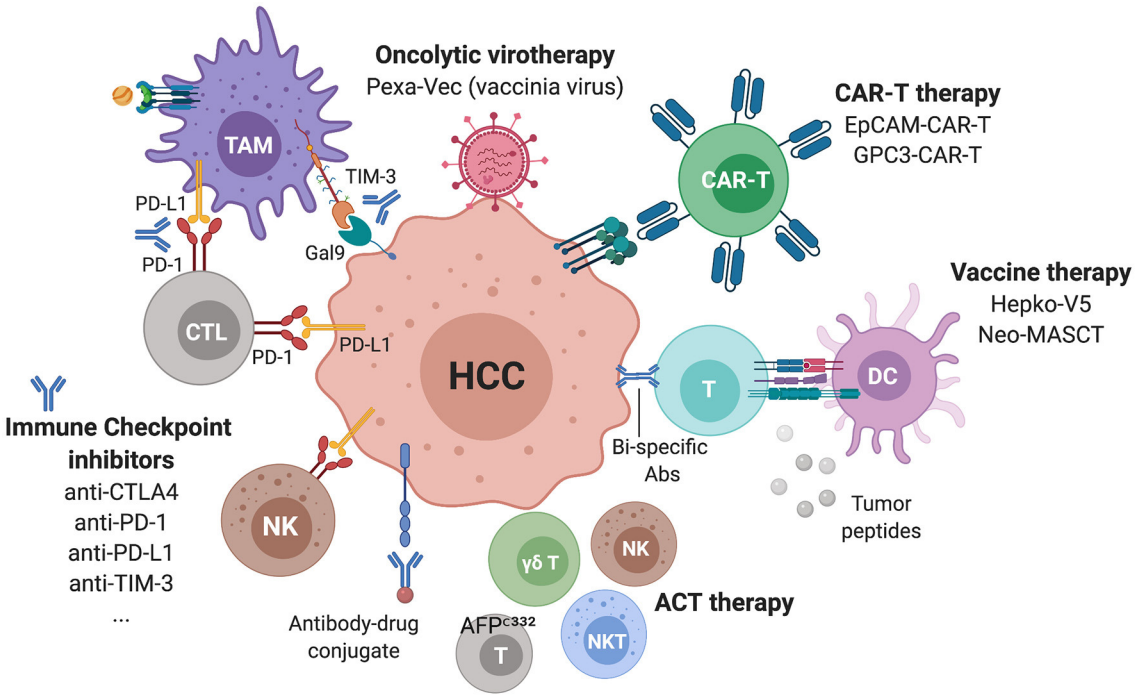

FIGURE 6 | Immunotherapies in ongoing clinical trials for advanced HCC. Several ICIs targeting checkpoints on lymphocytes but also NK and myeloid cells are currently being assessed as monotherapies or in combinations. Additional strategies include CAR-T cells, oncolytic viruses, vaccines, antibody-drug conjugates and bi-specific antibodies.

in a phase I/II trial (NCT03013712). In a similar approach, a phase I trial is testing the transfer of autologous genetically modified AFP^{c332}T cells, T cells expressing an enhanced TCR Specific for α-fetoprotein in HLA-A2 positive patients with advanced HCC (NCT03132792). In the oncolytic viruses sphere, a phase III trial (NCT02562755) is testing a vaccinia virus-based immunotherapy, Pexastimogene Devacirepvec (Pexa-Vec), in patients with advanced HCC, based on promising results from the phase IIb trial TRAVERSE (128). Pexa-Vec is also being tested in combination with nivolumab in the first-line treatment of advanced HCC in a phase II trial (NCT03071094). As for vaccines, a phase III trial (NCT02232490) is evaluating the benefit of hepcortespenlisimut-L (Hepko-V5), an oral allogeneic vaccine derived from patients' blood, given in an experimental arm vs. placebo, to patients with advanced HCC. Similarly, a phase II trial (NCT03067493) is testing the Neoantigen Multiple Target Antigen Stimulating Cell Therapy (Neo-MASCT) vaccine, which consists of 18 cycles, each including one DC subcutaneous injection and one CTL infusion.

CONCLUSIONS

HCC comprises a heterogeneous set of cancers with different etiologies, mutations and immune microenvironments, as demonstrated by broad molecular and immunological classifications. The advent of recent technologies including single cell approaches is now allowing high resolution characterization of the immune landscapes of HCC and is expected to uncover novel immunotherapeutic targets and approaches tailored to patients. ICI combination therapies are expected to dramatically improve the systemic therapy of advanced HCC. However, the prioritization of different combinations requires additional understanding of liver-specific immunity and the validation of therapeutic targets in suitable pre-clinical models of HCC taking into consideration the genetic heterogeneity of tumor cells and the cirrhotic or NASH environments. Further, an in-depth characterization of the biomarkers leading to improved patients' response to the various such combinations will contribute to better selection of patients and ameliorate the outcome. Last, a critical issue not discussed here is the management of immune-related adverse events (irAEs) often elicited by immunotherapies and that should be considered in designing and implementing immunotherapies. It is hoped that with the rapidly evolving field of oncoimmunology and trials in different cancer types, we will learn valuable lessons for future drug discovery in HCC.

AUTHOR CONTRIBUTIONS

JG and MS conceived the structure of this review. DC contributed to the description of the immune landscape of the liver and of HCC and prepared **Figure 3**. J-FB contributed to the review of clinical activity in HCC. All authors revised the manuscript and approved the final 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.2021.655697/full#supplementary-material

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REVIEW
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Lessons From Immune Checkpoint Inhibitor Trials in Hepatocellular Carcinoma

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The implementation of immune checkpoint inhibitors (ICI) into the clinical management of different malignancies has largely changed our understanding of cancer treatment. After having proven efficacy in different tumor entities such as malignant melanoma and lung cancer, ICI were intensively tested in the setting of hepatocellular carcinoma (HCC). Here they could achieve higher and more durable response rates compared to tyrosine-kinase inhibitors (TKI), that were sole standard of care for the last decade. Most recently, ICI treatment was approved in a first line setting of HCC, for cases not suitable for curative strategies. However, only a subset of patients benefits from ICI therapy, while others experience rapid tumor progression, worsening of liver function and poor prognosis. Efforts are being made to find immune characteristics that predict tumor responsiveness to ICI, but no reliable biomarker could be identified so far. Nevertheless, data convincingly demonstrate that combination therapies (such as dual inhibition of PD-L1 and VEGF) are more effective than the application of single agents. In this review, we will briefly recapitulate the current algorithms for systemic treatment, discuss available results from checkpoint inhibitor trials and give an outlook on future directions of immunotherapy in HCC.

Keywords: hepatocellular carcinoma, immunotherapy, checkpoint inhibitor treatment, clinical trials, liver cirrhosis

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INTRODUCTION

Hepatocellular carcinoma (HCC) is the fifth most common malignancy in men (7.9% of all cancers) with 523,000 new cases per year worldwide and the seventh most common malignancy in women (6.5% of all cancers) with 226,000 new cases (1, 2). Although the incidence and prevalence in western world countries is lower compared to Asia, HCC represents a major medical and socioeconomic problem worldwide, being one of the leading causes of cancer-related deaths (2, 3).

The vast majority of HCC arises in the context of liver cirrhosis, that means in a setting of chronic inflammation and continuous liver injury. By constant induction of cell death and compensatory hyperproliferation, but also *via* provoking an immunogen microenvironment, chronic inflammation leads to a pro-carcinogenic milieu (4). Following the prevalence of major risk factors for liver cirrhosis, the incidence of HCC has been steadily increasing over the last

decades. It was only most recently that a reversal of this trend was observed in western world countries (5). The increase of HCC cases in the USA and Europe in the last decades has been mainly attributed to the hepatitis C epidemic in the 1970s and 1980s. Moreover, the fast-growing number of obesity and metabolic syndrome, leading to nonalcoholic fatty liver disease (NAFLD) and steatohepatitis (NASH), is likely to condition a future increase of liver cirrhosis and also HCC - despite the foreseeable decline of hepatitis C-related HCC. In a few countries (e.g., Thailand, Japan, Singapore) the HCC incidence could be stabilized or reduced by hepatitis B vaccination programs (6).

Despite significant advances in diagnosis and tumor therapy, the prognosis of HCC remains poor, especially in advanced stages. This is particularly due to the fact that HCC often occurs in functionally compromised livers or is only diagnosed when curative therapies such as resection, transplantation, or local ablative techniques are no longer possible. These patients are left to palliative treatment options only, including systemic tumor therapy. With the introduction of tyrosine-kinase inhibitors (TKI) and recently immune checkpoint inhibitors (ICI), pharmacological treatment options for patients with advanced HCC have greatly improved. Nevertheless, their efficacy is still not satisfying. Thus, there is an unmet need for novel treatment options to further improve patients' prognosis.

In this review, we will briefly recapitulate the current algorithms for systemic treatment, discuss available results from checkpoint inhibitor trials and give an outlook on future directions of immunotherapy in HCC.

CURRENT AND EMERGING THERAPEUTIC OPTIONS FOR HCC

Continuous viral (e.g., chronic hepatitis B, C, delta co-infection), toxic or metabolic liver injury leads to chronic liver inflammation and conditions the transformation toward fibrosis and cirrhosis. The proinflammatory environment of liver cirrhosis provides an ideal breeding ground for the development of hepatocellular carcinomas.

In this context, close surveillance for all patients with cirrhosis has been recommended in international guidelines. Nevertheless, numerous primary liver tumors are still diagnosed at tumor stages that are no longer curative (intermediate or advanced stages of HCC according to the Barcelona Clinic of Liver Cancer (BCLC) staging system) (7). According to current guidelines these patients should be treated with systemic therapy. However, pharmacological treatment of HCC is challenging as HCCs show important tumor heterogeneity and arise from a distinct microenvironment, with regard to different etiologies of liver injury and different degrees of liver dysfunction. Considering the individual tumor microenvironment could be particularly relevant for immune-stimulating ICI strategies, as this might aggravate inflammatory and fibrogenic processes, e.g. in NASH (8).

HCC has long been considered to be refractory to systemic therapy. Trials with classical chemotherapy such as platinum derivatives or gemcitabine did not lead to a significant improvement in survival but proved to be very toxic against a background of impaired liver function. In 2008, the SHARP trial established sorafenib, which simultaneously inhibits tumor growth by targeting the Raf-MEK-ERK cascade as well as angiogenesis by targeting vascular endothelial growth factor (VEGFR) 2, platelet-derived growth factor receptors (PDGFR) and KIT as a novel standard treatment in patients with advanced HCC (9). Although sorafenib showed greater efficacy in certain subgroups, such as patients with hepatitis C virus infection or elevated neutrophil-lymphocyte ratio (NLR), its overall moderate efficacy and poor toxicity profile limited its use in clinical practice (10, 11). In 2018, lenvatinib, another TKI targeting VEGFR 1-3, fibroblast growth factor receptor (FGFR) 1-4, PDGFR, RET and KIT (12), was tested as non-inferior to sorafenib in the REFLECT trial and represented an alternative to the latter in the first line treatment of patients with advanced HCC or intermediate HCC refractory to loco-ablative treatments. Just recently, donafenib, another TKI was suggested as a third TKI suitable for first line therapy of HCC. In a phase II/III trial donafenib was associated with a longer median overall survival (OS) when compared with sorafenib (12.1 vs. 10.3 months, $p = 0.0363$), no significant differences were observed in the median progression free survival (3.7 vs. 3.6 months, $p = 0.2824$), objective response rate (4.6% vs. 2.7%, $p = 0.2448$), and disease control rate (30.8% vs. 28.7%, $p = 0.5532$) (13).

OS with TKI treatment was approximately one year in both the SHARP and REFLECT trials, with a progression free survival (PFS) of approximately 4 months. After disease progression under a TKI, re-administration of a TKI was tested in the RESORCE study and the CELESTIAL study, with regorafenib and cabozantinib respectively. Both substances target the VEGFR 1-3, as well as the MET and AXL pathway (14), and have been approved for use in patients refractory to sorafenib (15, 16). In addition to these classical TKI, Ramucirumab, a novel antibody directed against VEGFR 2 has demonstrated efficacy when used in patients with elevated serum alpha-fetoprotein (AFP) levels (17). In summary, TKI built the standard-of-care treatment for patients with advanced HCC or intermediate stage HCC, refractory to, or unsuitable for loco-ablative treatments. However, moderate efficacy and unfavorable toxicities conditioned the need for better treatment options.

The introduction of ICI into the clinical management of different malignancies has changed our view on how to treat

Abbreviations: AFP, alpha-fetoprotein; BCLC, Barcelona Clinic of Liver Cancer; CTLA-4, cytotoxic T-lymphocyte-associated protein 4; DCR, disease control rate; DOR, duration of response; EORTC QLQ, European Organization for the Research and Treatment of Cancer Quality-of-Life Questionnaire; FGFR, fibroblast growth factor receptor; HCC, hepatocellular carcinoma; ICI, immune checkpoint inhibitors; irAE, immune related adverse events; LRT, locoregional therapies; NLR, neutrophil-lymphocyte ratio; NAFLD, nonalcoholic fatty liver disease; NASH, nonalcoholic steatohepatitis; ORR, objective response rate; OS, overall survival; PDGFR, platelet-derived growth factor receptors; PD-1, programmed cell death 1 protein; PD-L1, programmed death-ligand 1; PFS, progression free survival; TTD, time to deterioration; TTSD, time to symptom deterioration; TACE, transarterial chemoembolization; TKI, tyrosine-kinase inhibitors; VEGFR, vascular endothelial growth factor receptor.

cancer. Immune checkpoints are "control points" of the immune system. They are based on surface receptors that, together with their ligands, prevent the immune system from attacking the body's own cells. In many malignant tumors, proteins that target immune checkpoints are upregulated. This allows the tumor cells to escape from attacks of the immune system (immune evasion). As shown in **Figure 1**, ICI block inhibitory immune checkpoints and thus trigger a defense response of the immune system toward tumor tissue. Immunotherapies seemed promising in patients with primary liver cancer, since cirrhotic livers feature an immunosuppressive environment that protect cancer cell from being recognized by the immune system, which, in turn, may be overcome by ICI (18). ICI were and are being tested in many different studies in the context of HCC.

SINGLE-AGENT IMMUNOTHERAPY

Immunotherapy has become a new and promising pillar in the treatment of HCC. So far mainly monoclonal antibodies inhibiting programmed cell death 1 protein (PD-1), programmed death-ligand 1 (PD-L1), or cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) were used in clinical trials of ICI.

Nivolumab blocks PD-1 and was tested in the setting of HCC in the non-comparative CheckMate 040 study (19). Patients with Child-Pugh A, pretreated with sorafenib (n = 182) or treatment-naïve (n = 80), received nivolumab in this phase I/II study in a dose-escalation (0.1–10 mg/kg every 2 weeks (Q2W)) and in a dose-expansion phase (3 mg/kg Q2W). Primary endpoints were safety and tolerability for the escalation phase and objective response rate (ORR) for the expansion phase. ORR and disease control rate (DCR) were 20% and 64%, respectively. 91% of responders had responses lasting 6 months or longer, and 55% had responses lasting 12 months or longer. Median OS duration was 28.6 months in sorafenib naïve patients and 15 months in patients pretreated with sorafenib. Additionally, a cohort of 49 patients with Child-Pugh B received a 240 mg flat dose of nivolumab Q2W. Interestingly, the safety profile of nivolumab in these patients was comparable to that observed in patients with Child-Pugh A. In a Child-Pugh B setting, nivolumab monotherapy also demonstrated durable responses with ORR of 10% and DCR of 55% (20).

Based on that data, phase III CheckMate 459 study compared nivolumab 240 mg Q2W (n = 371) to sorafenib (n = 372) in a first line setting. The differences in OS failed to meet statistical significance. The 33-months OS was 29% for nivolumab vs. 21% for sorafenib (21). Nevertheless, overall improvements in median OS (16.4 vs. 14.7 months), ORR (15% vs. 7%, respectively), and CR rate (4% vs. 1%, respectively) were considered clinically meaningful (22). The excellent survival in both arms is probably attributable to the subsequent therapy that patients received (49% for nivolumab and 53% for sorafenib, with 20% of patients treated with sorafenib receiving subsequent immunotherapy), which probably contributed to the study's negative results (23). Moreover, a slower deterioration of liver function as evidenced by albumin or bilirubin levels and Child–Pugh scores was observed under nivolumab therapy.

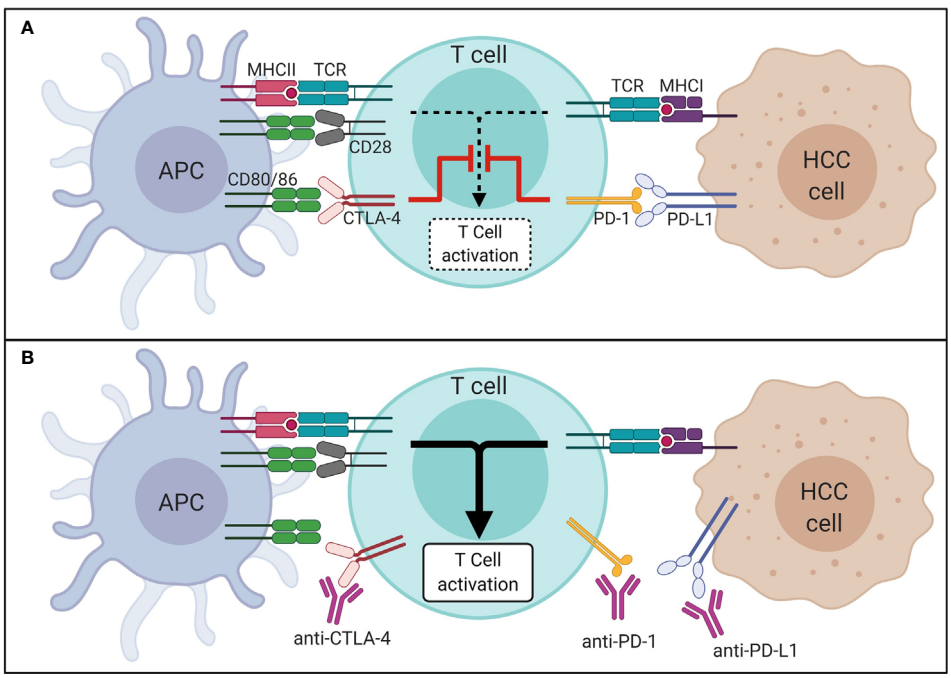

FIGURE 1 | Mechanism of immune checkpoints in HCC in absence **(A)** and presence **(B)** of ICI. APC, antigen presenting cell; CTLA-4, cytotoxic T-lymphocyte-associated protein 4; HCC, hepatocellular carcinoma; ICI, immune checkpoint inhibitor; MHC, major histocompatibility complex; PD-1, programmed cell death 1 protein; PD-L1, programmed death-ligand 1; TCR, T cell receptor.

To establish potential associations between HCC immunobiology and clinical outcomes, inflammatory gene expression signatures were assessed retrospectively from the CheckMate 040 population (24). Tumor responses were observed regardless of tumor cell PD-L1 status. Median OS was 28.1 vs. 16.6 months for patients with tumor PD-L1 ≥1% vs. <1% (p = 0.03). Tumor inflammation measured by CD3 or CD8 showed a non-significant trend toward improved OS (p = 0.08), whereas macrophage markers were not associated with OS. Tumor PD-1 and PD-L1 expression were associated with improved OS (p = 0.05 and p = 0.03, respectively). These analyses suggest that anti-tumor immune response may play a role in the treatment benefit of nivolumab in HCC.

In the keynote-224 (phase II, n = 104) and keynote-240 study (phase III, n = 413) the PD-1 inhibitor pembrolizumab was applied after sorafenib failure or intolerance. Patients received a fixed dose of 200 mg every 3 weeks (Q3W). In the phase II trial ORR (primary end point) was 18%, DCR was 61%, and OS was 12.9 months (25). The phase III trial compared pembrolizumab vs. placebo and failed to reach prespecified level of statistical significance for OS (13.9 vs. 10.6 months, respectively) and PFS (3.0 vs. 2.8 months, respectively) (26). Nevertheless, ORR was significantly higher with pembrolizumab (18% vs. 4%, p = 0.00007), and median duration of response (DOR) was 13.8 months with pembrolizumab. Survival in the sorafenib control arm was again very long, attributable to the exclusion of macrovascular invasion, better management of patients, and the availability of subsequent therapies, including immunotherapies, that were not available at trial initiation (23). While failing statistical significance, a clinical benefit of durable responses for patients who achieved a response to treatment could be demonstrated in both studies.

Along with nivolumab and pembrolizumab, camrelizumab, another PD-1 antibody, was evaluated in a phase II trial with 3 mg/kg every 2 or 3 weeks (n = 109 vs. 108, respectively). ORR was 15%, OS probability at 6 months was 74%, median OS was 13.8 months (27). Treatment-related serious adverse events occurred slightly higher in the every 2 weeks group (15% vs. 7%). Immune-related adverse events of any cause occurred in 80% in the every 2 weeks group and 87% in the every 3 weeks group. Overall, camrelizumab had a safety profile similar to other PD-1 ICIs, except for higher occurrence of reactive cutaneous capillary endothelial proliferation.

Similar results were obtained when applying durvalumab 10 mg/kg Q2W to pretreated HCC patients in a phase I/II trial (n = 40). ORR was 10%, median OS was 13.2 months (28).

In a phase Ia/Ib study tislelizumab's dose was evaluated with 200 mg Q3W. ORR in pretreated HCC patients was 12% (29). A phase III trial is comparing tislelizumab with sorafenib in treatment naïve patients, primary endpoint is OS (NCT03412773).

COMBINATION STRATEGIES FOR IMMUNOTHERAPY

Dual blockade of PD-(L)1 and VEGF has the potential to increase antitumoral activity through joint mechanisms (30). This was the rationale for the phase 1b study assessing efficacy and safety of atezolizumab 1200 mg Q3W alone (n = 59) and combined with bevacizumab 15 mg/kg Q3W (n = 60) in a first line setting. Longer median PFS was associated with combination therapy compared to sole application of the ICI (5.6 vs. 3.4 months, p=0.011) (31). In the phase III IMbrave 150 trial a fixed dose of atezolizumab 1200 mg and bevacizumab 15 mg/kg Q3W (n = 336) was compared with sorafenib (n = 165) in a 2:1 ratio in therapy naïve patients with unresectable HCC and Child–Pugh score ≤ 6. Coprimary endpoints were OS and PFS. Underlying etiology for liver cirrhosis was predominantly viral hepatitis B and C. Macrovascular invasion was frequent and most patients were staged as BCLC C. Median PFS was 6.8 months in the combination group and 4.3 months in the sorafenib group (HR: 0.59, p < 0.0001). OS at 12 months was 67% vs. 55%, respectively. Median OS was not reached in the combination arm. Grade 3 or 4 adverse events occurred in 57% and 55%, respectively. Except for hypertension, other high-grade toxic effects were infrequent (32). Besides symptoms of impaired liver function, patients with HCC frequently suffer from diverse conditions that limit their daily lives and make systemic therapy a challenge. Against this background, the effect on patients' quality of life is an increasingly important endpoint in the contemplation and evaluation of new therapies. The IMbrave 150 trial included the prespecified endpoints of time to deterioration (TTD) of quality of life, physical functioning, and role functioning, assessed by the European Organization for the Research and Treatment of Cancer Quality-of-Life Questionnaire (EORTC QLQ-C30). EORTC QLQ-C30 addresses these issues on a 100-point scale, with a drop of at least 10 points considered to be clinically meaningful (33). In both arms > 90% of patients completed the questionnaire, highlighting the quality of the analysis. Compared with sorafenib, the combination of atezolizumab/bevacizumab delayed TTD of patient-reported QOL (median TTD 11.2 vs. 3.6 months; physical functioning (median TTD 13.1 vs. 4.9 months), role functioning (median TTD 9.1 vs. 3.6 months)). Moreover, immunotherapy delayed TTD in patient-reported appetite loss, fatigue, pain, and diarrhea when comparing to sorafenib. A lower proportion of patients receiving the combination therapy experienced clinically meaningful deterioration in each of these symptoms when compared to TKI. In line with these results, a recent analysis demonstrated that the combination therapy showed similar efficacy regardless of age (34). In older patients, aged ≥ 65 years, the median OS was not reached in the atezolizumab/bevacizumab arm vs. 14.9 months in the sorafenib arm. In older patients PFS (7.7 vs. 4.8 months, respectively) and ORR (26% vs. 13%, respectively) also favor the application of combination therapy. Frequency and severity of adverse events were similar between the 2 age groups and consistent with the known safety profiles of atezolizumab/bevacizumab. Notably, no additional risks or toxicities were reported in older patients. Considering safety and efficacy data, these findings support an overall clinical benefit in patients with unresectable HCC. The combination of atezolizumab/bevacizumab was recently approved by European authorities

and is being incorporated in guidelines as first-line therapy in advanced HCC.

Another strategy to induce a stronger immune response and enhance the clinical efficacy of ICI monotherapy, was to simultaneously block two different immune checkpoints. In the setting of non-small-cell lung carcinoma and melanoma high doses of anti CTLA-4 in combination with a PD-(L)1 inhibitor resulted in an initial proliferation and increase of peripheral T cells (35, 36). In the phase I/II CheckMate 040 trial nivolumab (anti PD-1) and ipilimumab (anti CTLA-4) were administrated in different doses and regimens to patients previously treated with sorafenib (n = 148). The primary endpoint ORR was 31% with a median DOR of 17 months. Thus, this combination led to an ORR twice that of nivolumab monotherapy. DCR was 49%, OS at 24 months was 40% (37). These results led to the currently recruiting phase III CheckMate 9DW trial, comparing nivolumab 1 mg/kg Q3W plus ipilimumab 3 mg/kg for 4 doses to sorafenib or lenvatinib in therapy naïve patients with a Child-Pugh sore ≤ 6 (NCT04039607, planned n = 1084). Primary endpoint is OS, secondary endpoints are ORR, DOR, and time to symptom deterioration (TTSD). In the phase I/II Study 22 trial patients with sorafenib failure or intolerance received durvalumab (anti PD-L1) and/or tremelimumab (anti CTLA-4) either as monotherapy or as combination therapy with different dosages (n = 40 + 332). Best median OS with 18.7 months could be achieved with the combination of a single priming dose of tremelimumab 300 mg combined with durvalumab 1500 mg being continued in a Q4W regimen, the ORR was 24% (38). Pharmacodynamic biomarker analyses showed that CD8+ lymphocyte expansion was associated with treatment response. The durvalumab/tremelimumab combination is currently being compared to sorafenib in the phase III Himalaya trial in a first line setting (NCT03298451, planned n = 1200). Primary endpoint is OS, secondary endpoints are TTP (time to progression), PFS, ORR, DCR, DOR, and safety.

A synergistic effect is expected, when combining immunotherapy and directly targeting TKIs. The phase Ibkeynote-524 trial tested lenvatinib (12 mg if ≥ 60 kg, 8 mg if < 60 kg) plus pembrolizumab 200 mg Q3W (n = 104). ORR was 46% with a median DOR of 12.6 months. Median PFS was 8.6 months (39). Based on these findings, a double-blind randomized phase III trial is comparing the combination of lenvatinib/pembrolizumab vs. lenvatinib alone in therapy naïve patients with Child-Pugh score A (NCT03713593, planned n = 750). Primary endpoints are OS and PFS, secondary endpoints are ORR, DOR, DCR, TTP, and safety.

Other combinations are being tested in phase III trials in the setting of patients with advanced HCC who did not previously receive systemic therapy, e.g., atezolizumab/cabozantinib vs. sorafenib (NCT03755791, planned n = 740), or camrelizumab/apatinib vs. sorafenib (NCT03764293, planned n = 510). In this context, another CheckMate 040 cohort compared nivolumab/cabozantinib vs. the triple combination of nivolumab/ipilimumab/cabozantinib applied in different regimens. Investigator-assessed ORR was 17% in the nivolumab/cabozantinib arm and 26% in the nivolumab/ipilimumab/cabozantinib arm. DCR was 81% vs. 83%, and median PFS was 5.5 vs. 6.8 months, respectively. Median OS was not reached in either arm. No new safety signals were observed in either arm, demonstrating than even very intensive combinations are feasible in patients with HCC (40).

As described above, the concept of combination therapies is to increase the efficacy of ICI by further stimulating the immune response, meaning to "make cold tumors hot". Apart from pharmacological combinations, locoregional therapies (LRT) or transarterial chemoembolization (TACE) can be combination partners in this context. Besides local tumor control, they affect tumor immunity through complex mechanisms (41). LRT and TACE cause immunogenic cell death leading to the release of various tumor antigens. Moreover, they were demonstrated to enhance the number of dendritic cells in the HCC tumor microenvironment (42), leading to an increased antigen presentation and an enhanced response due to the activation of T-cells (43). Corroborating this concept, different trials are ongoing which are summarized in **Table 1**.

With autoimmune related adverse events (irAE), ICI therapy brought a novel spectrum of side effects, that was completely different than that known from chemotherapies. Risks of irAEs were widely reported as manageable and toxicity rates were generally lower than in TKI groups. Nevertheless, compared with cytotoxic agents, the possibility of identifying clinically relevant toxicity of ICI in early-phase clinical trials is relatively low (43% vs. 70%) (44). irAEs may develop long after the typical period of safety evaluation in oncology trials, and rather small sample sizes may not detect rare but life-threatening toxicity. In the IMbrave 150 study bleeding complications were observed in 7% of the atezolizumab/bevacizumab group vs. 4.5% in the sorafenib group. Although bleeding risk was not increased compared with that observed in previous anti-VEGF trials, a careful hepatologic management is necessary. The safety of ICI in the setting of advanced cirrhosis and their efficacy in different etiologies of liver injury remain open questions and need to be addressed in future trials.

IMMUNOTHERAPY IN A (NEO) ADJUVANT SETTING

HCC resection is in most cases not a definitive cure of malignancy, as recurrence rate after hepatectomy is high (45). Tumor recurrence after HCC resection is approximately 70% within 5 years, whereas up to 50% show early recurrence within the first 2 years, which is associated with tumor characteristics such as a large tumor, an incomplete tumor capsule, and venous or microvascular invasion (46). Nevertheless, neoadjuvant or adjuvant therapies are not recommended as they have not been proven to improve the outcome. The phase III Storm trial evaluated sorafenib as an adjuvant treatment, but concluded that it is not an effective intervention in such a setting (47). Therefore, adjuvant strategies in patients with HCC remain an unmet medical need.

Characteristics of the immune contexture have been shown to correlate with recurrence and outcome. The density of CD3 and CD8 T cells in the tumor and its margins is a prognostic marker for recurrence (48). The presence of T cells and cytotoxic cells as well as

TABLE 1 | Ongoing clinical trials of immune checkpoint inhibitors (ICI) in combination with locoregional therapies (LRT) or transarterial chemoembolization (TACE).

ICI	LRT/TACE	N	Primary Outcome	Secondary Outcome	Identifier (Name)	Phase
durvalumab ± bevacizumab	TACE	600	PFS	OS, QoL,	NCT03778957 (EMERALD-1)	III
pembrolizumab + lenvatinib	TACE	950	PFS, OS	ORR, DCR, DOR, TTP, safety		III
PD-1 mAb, lenvatinib	TACE	56	ORR	PFS, TTP, DCR, DOR, OS	NCT04273100 (PLTHCC)	II
camrelizumab	TACE	60	PFS	TTP, OS, ORR, DCR, DOR, safety	NCT04483284	II
durvalumab + tremelimumab	cryoablation, RFA, TACE	50	PFS	safety	NCT02821754	II
durvalumab + tremelimumab	radiation	70	ORR	safety, OS, DCR, PFS, DOR, TTP	NCT03482102	II
durvalumab + tremelimumab	Y-90 SIRT, TACE	84	ORR	PFS, OS, safety, ORR, QoL	NCT04522544 (IMMUWIN)	II
nivolumab	TACE	49	ORR	PFS, TTP, OS, DOR, TTFS, QoL	NCT03572582 (IMMUTACE)	II
nivolumab	Y-90 SIRT	40	ORR	TTR, DOR, TTP, PFS, OS, QoL, safety	NCT03033446	II
pembrolizumab	RFA, MWA, brachytherapy, TACE	30	ORR	TTR, RFS, OS, safety, biomarkers	NCT03753659	II
nivolumab	deb-TACE	14	safety	-	NCT03143270	I
pembrolizumab	TACE	26	safety	PFSR	NCT03397654	Ib

DCR, disease control rate; DOR, duration of response; MWA, microwave ablation; ORR, objective response rate; OS, overall survival; PD-1 mAb, programmed cell death 1 protein monoclonal antibody; PFS, progression free survival; PFSR, progression free survival rate; QoL, quality of life; RFA, radio frequency ablation; RFS, recurrence free survival; TTFS, time to failure of strategy; TTP, time to progression; TTR, time to response.

the absence of macrophages and Th2 cells positively correlates with patient survival and does not differ between different etiologies and HCC stages (49). High expression of PD-L1 by tumor or immune cells is associated with a more aggressive tumor and is a predictor of recurrence (50). Altogether, there is a strong rationale for adjuvant immunotherapy and several clinical trials are investigating the role of ICI and antiangiogenic agents in an adjuvant setting. An overview of ongoing clinical trials is given in **Table 2**.

ICI have the potential to achieve significantly higher ORR than TKI. Better tumor responses lead to tumor size reduction and may make secondary resectability feasible. Thus, ICI based regimens may even open the field of neoadjuvant strategies for HCC. The combination of ipilimumab and nivolumab is currently being evaluated as a neoadjuvant therapy in patients undergoing hepatic resection, assessing tumor shrinkage and OS after resection (see **Table 2**).

CONCLUSION AND FUTURE DIRECTIONS

Although ICI monotherapy could achieve good responses in some patients, they could not demonstrate superiority to TKI based therapies. Most recently, the atezolizumab/bevacizumab combination was associated with an unparalleled benefit of survival. Thus, immunotherapy is likely to have a huge impact on the management of HCC due to the ability to produce durable and clinically relevant responses. With the combination of different agents, higher response rates and longer overall survival may be achieved. Nevertheless, a significant percentage of HCC do not respond to immunotherapy and an immunologic classification is urgently needed to guide treatment decisions. In both CheckMate 040 and CheckMate 459, PD-L1 expression was not correlated with tumor response and patient prognosis (24). Experimental markers such as circulating tumor cells, cell free DNA, miRNA have been studied in the context of HCC (51). However, these do not currently play a role in routine clinical practice.

ICI brought a novel spectrum of immune related adverse events. Dual blockade of CTLA-4 and PD-1 or PD-L1 results in enhanced toxicities, especially when higher doses of CTLA-4 inhibitors are used. Combinations with TKI or anti-VEGF carry the risk of higher toxicities in cirrhotic patients that demand a close surveillance and hepatologic management. The selection of patients is crucial regarding safety. Alternative strategies than immunotherapy may be preferred in the setting of liver

TABLE 2 | Ongoing clinical trials of (neo)adjuvant immunotherapy for hepatocellular carcinoma (HCC).

ICI	Controls	Setting	Identifier (Name)	Phase
atezolizumab + bevacizumab	active surveillance	adjuvant after curative resection or ablation	NCT04102098 (IMbrave 050)	III
durvalumab ± bevacizumab	placebo	adjuvant after curative resection or ablation	NCT03847428 (EMERALD-2)	III
nivolumab	placebo	adjuvant after curative resection or ablation	NCT03383458 (CheckMate 9DX)	III
pembrolizumab	placebo	adjuvant after curative resection or ablation	NCT03867084 (KEYNOTE-937)	III
nivolumab ± ipilimumab	–	perioperative in (potentially) resectable HCC	NCT03222076	II
nivolumab + ipilimumab	–	neoadjuvant prior to resection	NCT03510871	II
nivolumab + ipilimumab	–	neoadjuvant prior to resection	NCT03682276 (PRIME-HCC)	I/II
nivolumab + cabozantinib	–	neoadjuvant prior to resection	NCT03299946	I

transplantation or uncontrolled autoimmune disease. Nevertheless, ICI have the potential to stabilize quality of life for patients with HCC. A longer time to deterioration of health-related quality of life was demonstrated under ICI when compared to TKI (33). To reach optimal benefit of immunotherapy, biomarkers to predict response are urgently needed. Furthermore, the therapeutic sequence of different classes and the combination of available agents needs to be identified. Despite all remaining challenges, checkpoint inhibitors have already today revolutionized the treatment of HCC.

AUTHOR CONTRIBUTIONS

All authors drafted the manuscript and provided intellectual input. All authors contributed to the article and approved the submitted version.

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Integrative Transcriptomic, Proteomic and Functional Analysis Reveals ATP1B3 as a Diagnostic and Potential Therapeutic Target in Hepatocellular Carcinoma

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The Na+/K+-ATPase (NKA), has been proposed as a signal transducer involving various pathobiological processes, including tumorigenesis. However, the clinical relevance of NKA in hepatocellular carcinoma (HCC) has not been well studied. This study revealed the upregulation of mRNA of ATP1A1, ATP1B1, and ATP1B3 in HCC using TCGA, ICGC, and GEO database. Subsequently, ATP1B3 was demonstrated as an independent prognostic factor of overall survival (OS) of HCC. To investigate the potential mechanisms of ATP1B3 in HCC, we analyzed the co-expression network using LinkedOmics and found that ATP1B3 co-expressed genes were associated with immune-related biological processes. Furthermore, we found that ATP1B3 was correlated immune cell infiltration and immune-related cytokines expression in HCC. The protein level of ATP1B3 was also validated as a prognostic significance and was correlated with immune infiltration in HCC using two proteomics datasets. Finally, functional analysis revealed that ATP1B3 was increased in HCC cells and tissues, silenced ATP1B3 repressed HCC cell proliferation, migration, and promoted HCC cell apoptosis and epithelial to mesenchymal transition (EMT). In conclusion, these findings proved that ATP1B3 could be an oncogene and it was demonstrated as an independent prognostic factor and correlated with immune infiltration in HCC, revealing new insights into the prognostic role and potential immune regulation of ATP1B3 in HCC progression and provide a novel possible therapeutic strategy for HCC.

Keywords: Na^+/K^+-ATPase (NKA), hepatocellular carcinoma (HCC), ATP1B3, biomarker, immune

INTRODUCTION

Hepatocellular carcinoma (HCC) is a primary liver cancer with high mortality and is the most common malignancy (1), which occurs frequently in Asia, Africa, southern Europe and China (2). Although early surgical resection and liver transplantation are effective treatments for HCC (3), the 5-year recurrence rate for HCC remains poor because of its high recurrence and metastasis rates (4). Therefore, useful prognostic and therapeutic indicators are urgently needed.

The ion transporter Na^+/K^+-ATPase (NKA) is a transmembrane protein that transports Na^+ and K^+ across cell membranes (5), which is essential for the cellular electrochemical gradient (6), ion homeostasis (7), cell adhesion (8), and intracellular signaling (9). The functional NKA consists of α subunits and β subunits. So far, 4 NKA α-subunits (α1, α2, α3, and α4) and 4 β-subunits (β1, β2, β3, and β4) have been identified. The abnormal NKA could lead to a variety of diseases, including hypokalaemic periodic paralysis and CNS symptoms (10), cardiovascular disorders (11), atherosclerosis (12), Alzheimer (13). Recent studies showed that NKA was dysregulated in multiple cancers and involved in the progression of these cancers (14). For example, Mathieu et al. (15) showed that the NKA α1 subunit is highly expressed in human melanoma and involved in cell migration and apoptosis. Lee et al. (16) reported that the NKA β1 subunit is low-expressed in medulloblastoma. Bechmann et al. (17) revealed that NKA α1, α3, and β1 subunits were highly expressed in colorectal cancers and associated with tumor metastases. Nevertheless, the clinical relevance of NKA in HCC remains unclear.

In this study, we investigated the expression of NKA α/β subunits in HCC using 6 independent public datasets. We demonstrated ATP1B3 as a prognostic factor which is correlated with immune infiltrating in HCC. Functional analysis revealed ATP1B3 as a potential oncogene of HCC, indicating that ATP1B3 as a diagnostic and potential therapeutic target in HCC.

MATERIALS AND METHODS

NKA Expression in Different Datasets

The expression levels of NKA α/β subunits in HCC were identified from ICGC (https://icgc.org/daco) and TCGA (https://cancergenome.nih.gov/) datasets (18). Then, the expression levels of ATP1A1, ATP1B1, and ATP1B3 were verified in three independent GEO datasets (GSE45436, GSE76427 and GSE102079) download from https://www.ncbi.nlm.nih.gov/gds (19).

The transcription levels of NKA genes in various cancers were detected in the GEPIA database (http://gepia.cancer-pku.cn/) (20) and ONCOMINE database (https://www.oncomine.org/) (21). The thresholds were set as: logFC > 1 and $p < 0.01$.

Survival Analysis

The prognostic value of ATP1A1, ATP1B1, and ATP1B3 for HCC in the TCGA database were appraised by the Kaplan-Meier plotter database (http://kmplot.com/analysis/) (22) and then validated using the ICGC database using R software (version 3.5.2).

The Relationship Between ATP1B3 and Clinical Characteristics of HCC

The expression of ATP1B3 in HCC patients with different clinical characteristics was analyzed using R software and then validated using the UALCAN database (http://ualcan.path.uab.edu) (23). The significance of differential gene expression was assessed by t-test and one-way ANOVA. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

LinkedOmics Database Analysis

The co-expressed genes of ATP1B3 in HCC was detected using LinkedOmics (http://www.linkedomics.org/login.php). Co-expressed genes can be analyzed statistically and displayed in the volcano, Heat maps. Gene set enrichment analysis (GSEA) can also be used in LinkedOmics functional modules to perform Gene Ontology (GO) term annotation, KEGG pathway analysis, and target enrichment of kinases, miRNAs, and transcription factors' (TF) (24). Pearson test was used to evaluate the significant correlation of co-expressed genes. FDR < 0.01 was significant expression, $p < 0.05$ was significantly related genes.

Correlations of ATP1B3 Expression With Immune Infiltration in TIMER and GEPIA

The association between ATP1B3 and immune cells infiltration in HCC was confirmed using the TIMER database (http://cistrome.org/TIMER/). It provides the infiltration of 6 types of immune cells to assess the abundance of immune infiltration (25, 26). Furthermore, the expression of ATP1B3 in immune subtypes and molecular subtypes in HCC was identified using the TISIDB database (http://cis.hku.hk/TISIDB/). It integrates a large amount of tumor immunity-related data, including 988 genes related to anti-tumor immunity, and can analyze the data of 30 TCGA cancer types to calculate the gene expression of immune subtypes and molecular subtypes in different tumors (27).

Next, the correlations between ATP1B3 and immune markers expression in HCC was investigated using the TIMER and GEPIA databases. These immune markers have been referenced previously (28). The correlation between ATP1B3 and each immune gene markers was presented using scatterplots, Pearson test was used for statistical significance evaluation, and log2 RSEM was adopted to regulate gene expression levels. ATP1B3 was plotted on the y-axis, while marker genes are plotted on the x-axis.

Proteomics Database Analysis

The expression and prognosis of ATP1B3 protein were generated using the CPTAC proteomics database (https://cptac-data-portal.georgetown.edu/cptacPublic/). Moreover, the proteomics and phospho-proteomics data from 316 HCC patients were download from Gao's work (29). These data can well verify the relationship between proteins and survival and clinical, and find

candidate proteins that can be used as tumor biomarkers (30, 31).

Cell Lines and Culture

HCC cell lines (Huh7 and HCCLM3) and human normal liver cell (LO2) were obtained from American Type Culture Collection (ATCC, Manassas, VA, USA). Huh7 and HCCLM3 were cultured in RPMI-1640 medium (BI, Israel) containing 10% FBS (BI, Israel) at 37° C in 5% CO_2. And LO2 cultured in DMEM (BI, Israel) medium containing 10% FBS (BI, Israel) at 37° C in 5% CO_2.

qRT-PCR and Western Blot

The protein and mRNA expression levels of ATP1B3 in the HCC cells and normal liver cell were detected by Western blots and qRT-PCR, respectively, as described previously by us (32–34). The Anti-ATP1B3 antibody was purchased from Santa (sc-135998, 1:50), the Anti-Tubulin antibody was purchased from Elabscience (E-AB-20036, 1:2000), and the ATP1B3 primer used for the amplification was as follows: 5′-TGATCCAACTTC GTATGCAGGG-3′ and 5′-ACATGCAACATAAA CTGGACCC-3′ (Sangon Biotech, China).

Transfection of siATP1B3

50 nM of siATP1B3 was transfected into HCC cells by using Lipofectamine™ 2000 (Invitrogen) according to the manufacturer's instruction (35). The ATP1B3 siRNA was 5′-CUCAUAAUGGAAUGAUAGATT-3′ and 5′-UCUA UCAUUCCAUUAUGAGTT-3′ (TSINGKE, China).

Cell Migration Assay

Transwell migration assay and wound healing assay were performed as described previously (36, 37). The assay was performed three times in triplicate.

Plate Clone Formation and MTT Assay

Cell proliferation was monitored by Plate clone formation and MTT assay as described previously by us (38, 39). The assay was performed three times in triplicate.

Cell-Cycle and Cell Apoptosis Assay

Cell-cycle and cell apoptosis were performed by flow cytometry analysis as described previously (35, 40). The assay was performed three times in triplicate.

Clinical Samples and Immunohistochemistry (IHC)

Fifteen formalin-fixed, paraffin-embedded HCC and paired adjacent liver tissues were collected from Xiangya Hospital of Central South University from September 2019 to January 2020. Our study was approved by the ethics committee of Xiangya Hospital, Central South University.

According to our previously described (41, 42), IHC and an immunoreactive score of ATP1B3 (Anti-ATP1B3 antibody: 67554-1-Ig, proteintech, 1:1000) were conducted on the formalin-fixed and paraffin-embedded tissue sections.

Statistical Analysis

Statistical obtained from TCGA were all analyzed by R-3.6.1. The differential expression of the 8 NKA genes in the TCGA and ICGC cohort were evaluated using the "limma" and "vioplot" package, and the heat map was generated using the heatmap package of the R software. The survival package was used for the survival analysis of the sample from ICGC. The relationship of ATP1B3 expression and clinical characteristics were assessed applying logistic regression. Univariate and multivariate analysis revealed the relationship between ATP1B3 and the clinical factors, the immune cell infiltration with OS of HCC using the "survival" R package. The ROC curves, with AUC values quantified with the survival ROC package. Other data were calculated statistically using SPSS software ver20.0 (SPSS, Inc, Chicago, IL, USA) and GraphPad Prism 7.0 (GraphPad Software, La Jolla, CA, USA). $p < 0.05$ was considered statistically significant.

RESULTS

NKA Genes Expression in HCC

We first analyzed the mRNA level of 8 NKA genes (ATP1A1-4, ATP1B1-4) in HCC using TCGA and ICGC (LIRI-JP) datasets. Among these, the mRNA expression of ATP1A1, ATP1B1, and ATP1B3 were evidently increased in HCC compared to normal tissue in TCGA with logFC >1 and p<0.01 (**Figure 1A** and **Table S1**). Although ATP1A2, ATP1A4, and ATP1B2 were differently expressed between HCC tissue and normal tissue, ATP1A2, ATP1B2 and ATP1A4 mRNA levels were very much low in both HCC and normal tissue, and ATP1B2 expression was slightly reduced in HCC compared to liver tissue with |log2FC| <0.5. Similar results were also observed in ICGC (LIRI-JP) datasets (**Figure 1B** and **Table S2**). Subsequently, the expression levels of three genes (namely ATP1A1, ATP1B1, and ATP1B3) were also validated in 3 independent GEO datasets (GSE45436, GSE76427, and GSE102079) (**Figure 1C**). Finally, the Oncomine database and GEPIA database showed that the mRNA expression of ATP1B1 and ATP1B3 are widely upregulated in various cancers, including Leukemia, Lung cancer, Lymphoma, Head and neck cancer and so on (**Figures S1**–**S3**).

Prognostic Value of ATP1A1, ATP1B1, and ATP1B3 in HCC

We next investigated the prognostic value of ATP1A1, ATP1B1, and ATP1B3 for HCC using the Kaplan-Meier plotter. The HCC patients with high ATP1A1 showed worse overall Survival (OS: HR = 1.66 (1.14-2.4), $p = 0.007$), Progression-Free Survival (PFS: HR = 1.61 (1.13-2.31), $p = 0.0082$), Relapse Free Survival (RFS: HR = 1.73 (1.16-2.59), $p = 0.007$) and Disease Free Survival (DSS: HR = 1.92 (1.06-3.48), $p = 0.029$) in **Figure 2A**. High ATP1B3 was related to worse prognosis in HCC (OS: HR = 2.3 (1.59-3.34), $p = 5.8E-6$; PFS: HR = 1.39 (1.03-1.86), $p = 0.029$; RFS: HR = 1.43 (1.02-1.98), $p = 0.034$; DSS: HR = 2.01 (1.29-3.15), $p = 0.0018$). Similar results were also observed in the ICGC database (**Figure 2B**). Moreover, the univariate and

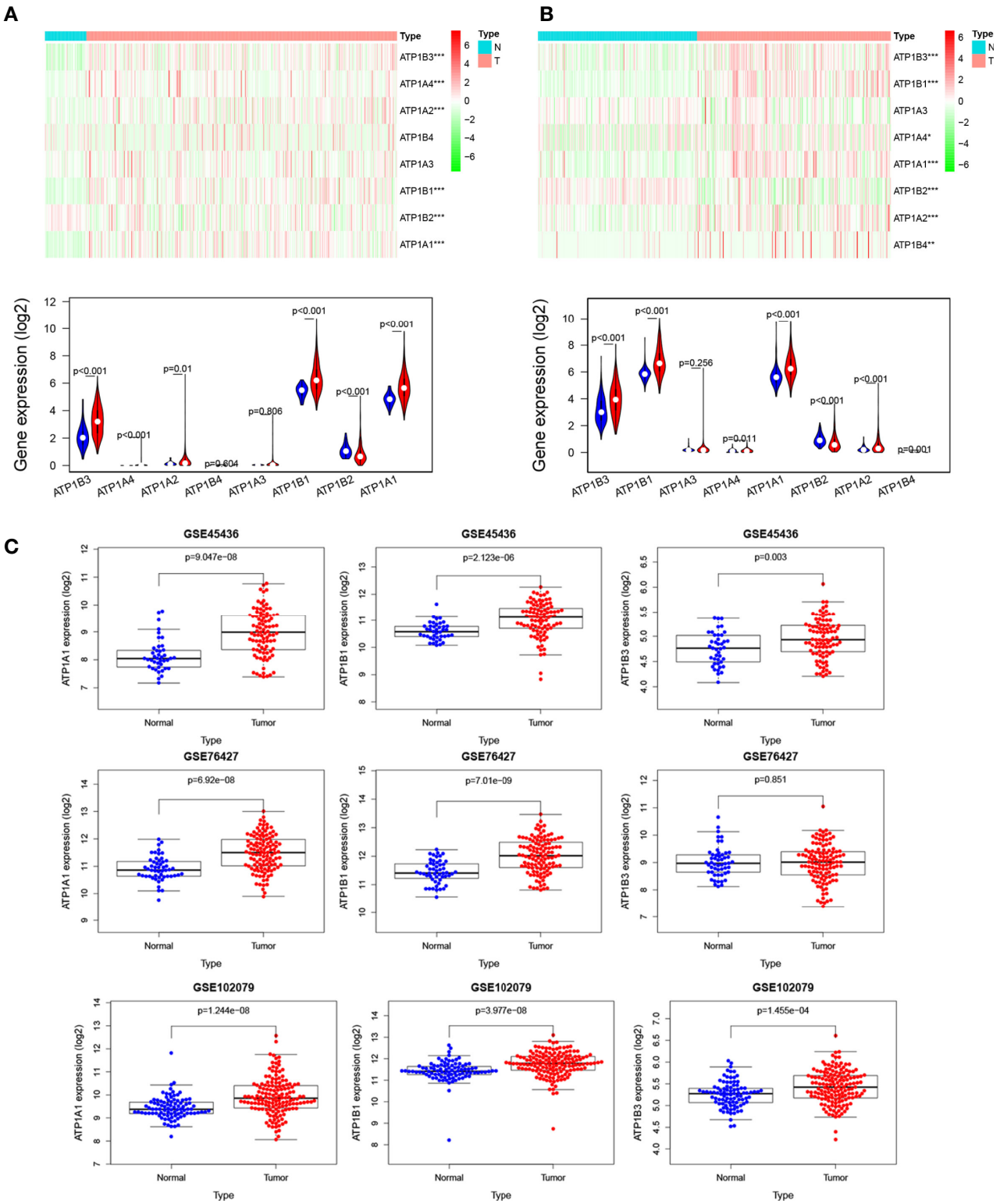

FIGURE 1 | NKA genes expression in HCC. The mRNA levels of NKA genes in HCC from **(A)** TCGA database. Up, heatmap. Down, Violin plot, Red: HCC tissue; Blue: normal tissue. **(B)** ICGC database. Up, heatmap. Down, Violin plot, Red: HCC tissue; Blue: normal tissue. **(C)** The mRNA levels of ATP1A1, ATP1B1, and ATP1B3 in HCC from four independent GEO datasets (GSE45436, GSE76427, GSE64041, and GSE102079).

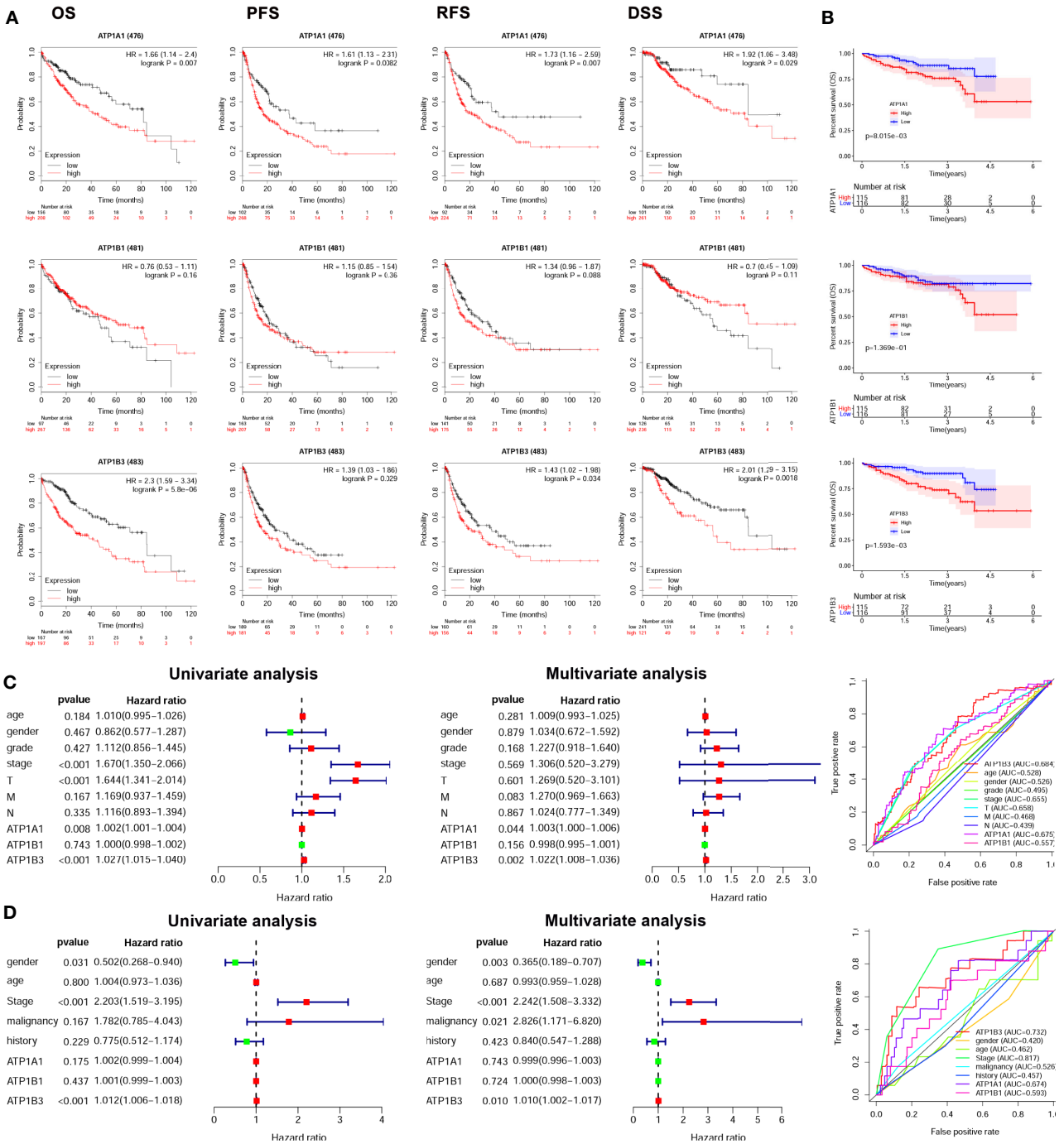

FIGURE 2 | ATP1A1, ATP1B1, and ATP1B3 mRNA are associated with prognosis of HCC patients. **(A)** The survival curves of OS, PFS, RFS, and DSS with high/low ATP1A1, ATP1B1 and ATP1B3 in TCGA HCC cohorts using the Kaplan-Meier plotter (OS, n=364; RFS, n=316; PFS, n=370; DSS, n=362). The high and low mRNA expression is splitting by best cutoff. **(B)** The survival curves of OS with high/low ATP1A1, ATP1B1, and ATP1B3 in ICGC HCC cohorts, the high and low mRNA expression is splitting by median. Univariate and multivariate analysis and ROC curve revealed the relationship between ATP1A1, ATP1B1, ATP1B3, and the clinical factors with overall survival of HCC in **(C)** TCGA database and **(D)** ICGC database. (T, stage T; N, stage N; M, stage M).

multivariate analysis showed that only ATP1B3 was an independent prognostic factor for OS of HCC using both TCGA and ICGC database (**Figures 2C, D**). Finally, the AUC values of ATP1B3 for the OS model from TCGA and ICGC database were 0.684 and 0.732 respectively, which were more sensitivity and specificity than the clinical factors (**Figures 2C, D**, right). These results indicated that ATP1B3 was an independent prognostic biomarker for HCC.

ATP1B3 Is Correlated With Clinicopathological Characteristics in HCC

Based on the clinical data extracted from TCGA-LIHC, we found that high ATP1B3 was associated with higher stage, higher grade, and more dead (p=0.01, p=0.03, and p=0.008) (**Figure 3A**). Consistent with these results, high ATP1B3 was associated with higher stage, higher grade, and more dead in the ICGC database (**Figure 3B**). These results were also confirmed by the UALCAN (**Figure S4**).

We next explored the association between ATP1B3 expression and the clinicopathological characteristics of HCC patients using Kaplan-Meier Plotter (**Table 1**). For OS, high expressed ATP1B3 related with poor OS in all stage, grade I/II/III, T 1/2/3, none-vascular invasion, grade (male/female), White and Asian race, no-Alcohol consumption, both with or without Hepatitis virus. For PFS, ATP1B3 expression was significantly hazardous to HCC patients with stage I, grade II, T 1, none-vascular invasion, female, Asian, and Hepatitis virus (**Table 1**).

ATP1B3 Co-Expression Networks in HCC

We analyzed the ATP1B3 co-expression networks in HCC using LinkedOmics. As shown in **Figure 4A**, a total of 9,531 genes expression were significant correlations with ATP1B3 expression (FDR < 0.01) with 2,564 (green dots) negatively correlated genes and 6,967 positively correlated genes (red dots). The top 50 positively and negatively co-expressed genes were shown in the heat map (**Figures 4B, C** and **Table S3**). Among these, 39 of 50 positive genes and 21 of 50 negative genes were associated with OS of HCC with a high/low hazard ratio (HR) ($p < 0.05$) (**Figures 4D, E**).

GO annotation revealed that these genes participate in various immune response, including leukocyte cell-cell adhesion, leukocyte migration, antigen processing and presentation, leukocyte proliferation. In contrast, various metabolic processes were inhibited, including steroid metabolic process, antibiotic metabolic process, fatty acid metabolic process, and dicarboxylic acid metabolic process (**Figure 4F** and **Table S4**). KEGG pathway analysis showed the enrichment in immune and metabolic

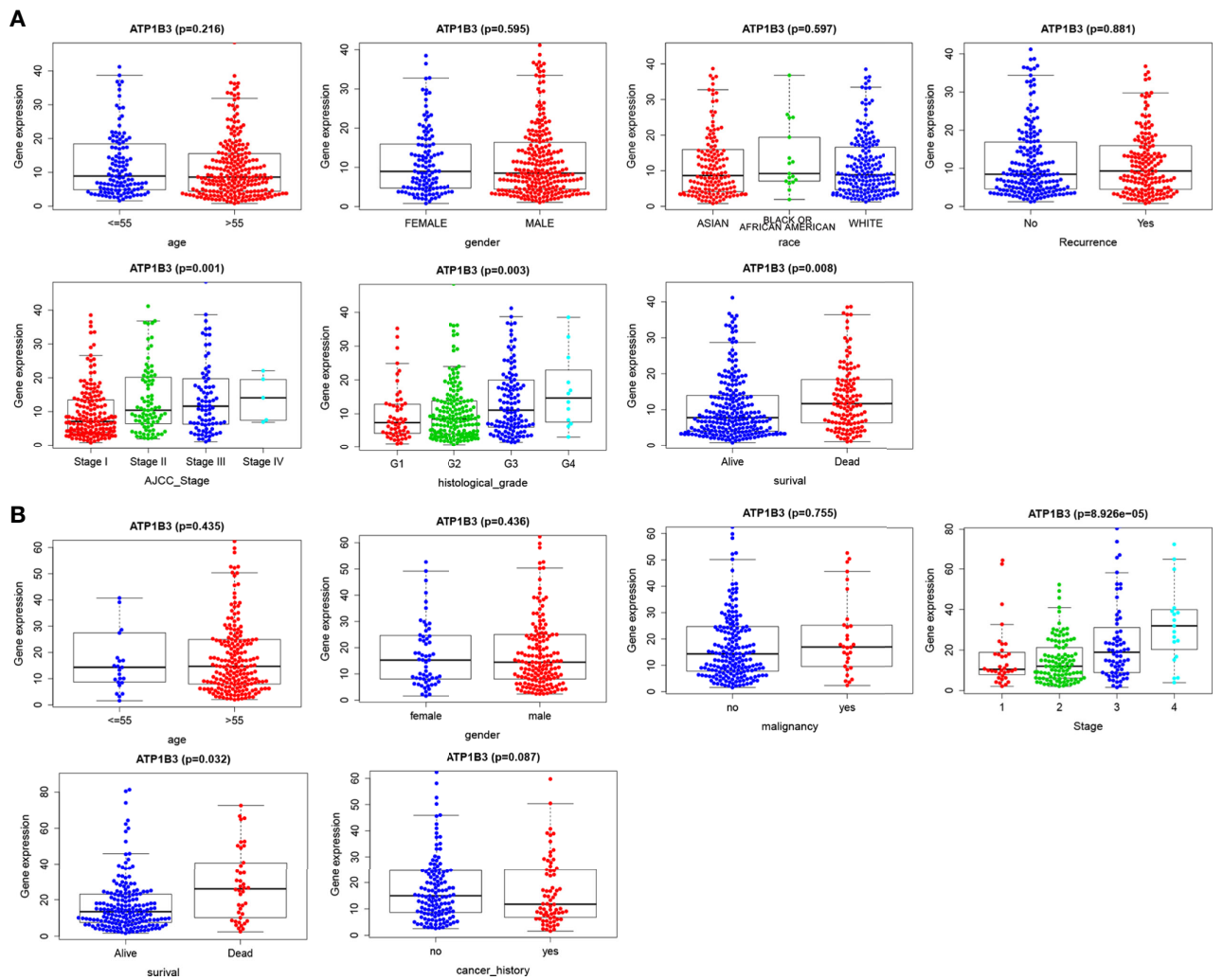

FIGURE 3 | ATP1B3 is correlated with clinicopathological characteristics of HCC patients. **(A)** TCGA database. **(B)** ICGC database.

TABLE 1 | Correlation of ATP1B3 mRNA expression with OS (n = 364) and PFS (n = 370) in liver hepatocellular carcinoma with different clinicopathological features.

OS (364)	Number	HR	*p* value	PSF (370)	Number	HR	*p* value
Stage				Stage			
I	170	2.58 (1.4-4.77)	0.0017	I	170	1.84(1.08-3.14)	0.0225
I+II	253	2.32(1.44-3.75)	0.0004	I+II	254	1.4(0.91-2.13)	0.1201
II	83	2.4(0.9-6.38)	0.0706	II	86	0.65(0.36-1.17)	0.1469
II+III	166	2.1(1.25-3.53)	0.0043	II+III	167	0.81(0.54-1.21)	0.3001
III	83	2.44(1.27-4.67)	0.0056	III	83	1.45(0.82-2.54)	0.1968
III+IV	87	2.54(1.34-4.83)	0.0032	III+IV	88	1.39(0.8-2.4)	0.242
IV	4	–	–	IV	5	–	–
Grade				Grade			
I	55	0.67(0.25-1.77)	0.4166	I	55	1.65(0.73-3.73)	0.2218
II	174	2.72(1.57-4.72)	0.0002	II	175	1.83(1.18-2.85)	0.0063
III	118	2.9(1.58-5.34)	0.0003	III	119	1.42(0.86-2.34)	0.1682
IV	12	–	–	IV	12	–	–
AJCC_T				AJCC_T			
I	180	2.43(1.35-4.38)	0.0023	I	180	1.7591.04-2.93)	0.0329
II	90	2.66(1.02-6.99)	0.0384	II	92	0.68(0.38-1.21)	0.1827
III	78	2.65(1.21-5.8)	0.0116	III	78	1.48(0.77-2.84)	0.2418
IV	13	–	–	IV	13	–	–
Vascular invasion				Vascular invasion			
none	203	2.73(1.62-4.61)	8.90E-05	none	204	1.86(1.19-2.9)	0.0058
micro	90	1.9(0.82-4.37)	0.1261	micro	91	0.59(0.3-1.12)	0.1013
macro	16	–	–	macro	16	–	–
Gender				Gender			
male	246	3.33(1.98-5.58)	1.40E-06	male	246	1.35(0.93-1.96)	0.1113
female	118	2.49(1.17-5.32)	0.0146	female	120	1.75(1.05-2.94)	0.0312
Race				Race			
white	181	1.62(0.99-2.64)	0.0517	white	183	1.41(0.94-2.12)	0.0914
black or african	17	–	–	black or african	17	–	–
asian	155	4.38(2.1-9.12)	1.70E-05	asian	155	1.81(1.13-2.91)	0.0129
Alcohol consumption				Alcohol consumption			
yes	115	1.78(0.9-3.51)	0.0936	yes	115	1.46(0.87-2.47)	0.1536
no	202	2.4(1.52-3.78)	0.0001	no	204	1.54(0.98-2.44)	0.0613
Hepatitis virus				Hepatitis virus			
yes	150	2.68(1.32-5.42)	0.0045	yes	152	3.2(1.54-6.64)	0.0009
no	167	189(1.15-3.11)	0.0109	no	167	1.49(0.91-2.43)	0.11

*Short bars appear due to limited sample size for parameters and hazard ratio cannot be calculated. OS, overall survival; PFS, progression-free survival. *, p < 0.05.*

pathways, including rheumatoid arthritis, Fc gamma R-mediated phagocytosis, Leishmaniasis, and so on (**Figure 4G** and **Table S5**). These findings demonstrated that ATP1B3 is involved in the immune response and the metabolic regulation of HCC.

ATP1B3-Related Networks in HCC

To address the ATP1B3-related network in HCC, we analyzed the transcription factors (TF), miRNAs, and kinases in ATP1B3 co-expressed genes. The top 3 most significant related kinases are LCK proto-oncogene (LCK), p21 (RAC1) activated kinase 1 (PAK1), LYN proto-oncogene (LYN) (**Tables 2** and **S6**). No ATP1B3 co-expressed miRNA was enriched by GSEA (**Table S7**). The most significant ATP1B3 co-expressed TF was belong to the SRF transcription factor family **(Table S8)**, including CFL1, CAP1, SUSD1, FOSL1, KCNMB1.

The Association of ATP1B3 and Immune Infiltration in HCC

Basing on the GO analysis, we next detected the correlations of ATP1B3 and immune cells in HCC using the TIMER. We found that ATP1B3 was correlated with tumor purity (r = -0.353, p = 1.33E-11) and the B cells infiltration (r =0.266, p = 5.52E-7), CD8+ T infiltration (r = 0.249, p = 3.25E-6), CD4+ T infiltration (r = 0.169, p = 1.65E-3), Macrophage infiltration (r = 0.356, p = 1.25E-11), Neutrophil infiltration(r = 0.301, p = 1.21E-8) and Dendritic cell infiltration (r = 0.328, p = 5.46E-10) (**Figure 5**). Particularly, ATP1B3 CNV has evidently correlated with immune infiltration including B cells, CD8+ T cells, macrophages and neutrophils (**Figure 5**). Moreover, Univariate analysis showed that ATP1B3, Neutrophil and Macrophage were significantly associated with OS in HCC, and multivariate analysis showed that ATP1B3 and CD8+ T cells were independent factors of OS in HCC (**Figure 5**). Furthermore, ATP1B3 was also observed differently expressed in immune subtypes (**Figure 5**) and molecular subtypes (**Figure 5**) in HCC using TISIDB database. In addition, **Figure S5** showed that ATP1B3 was associated immune cells infiltration in pan-cancer.

The Correlation Between ATP1B3 and Immune Markers and Immune-Related Cytokines in HCC

Next, we investigated the ATP1B3 crosstalk with immune cells, basing on the correlations between ATP1B3 and immune-related gene expression in HCC using the TIMER (**Figure 6** and **Table 3**) and GEPIA databases (**Table 4**). The results revealed that ATP1B3

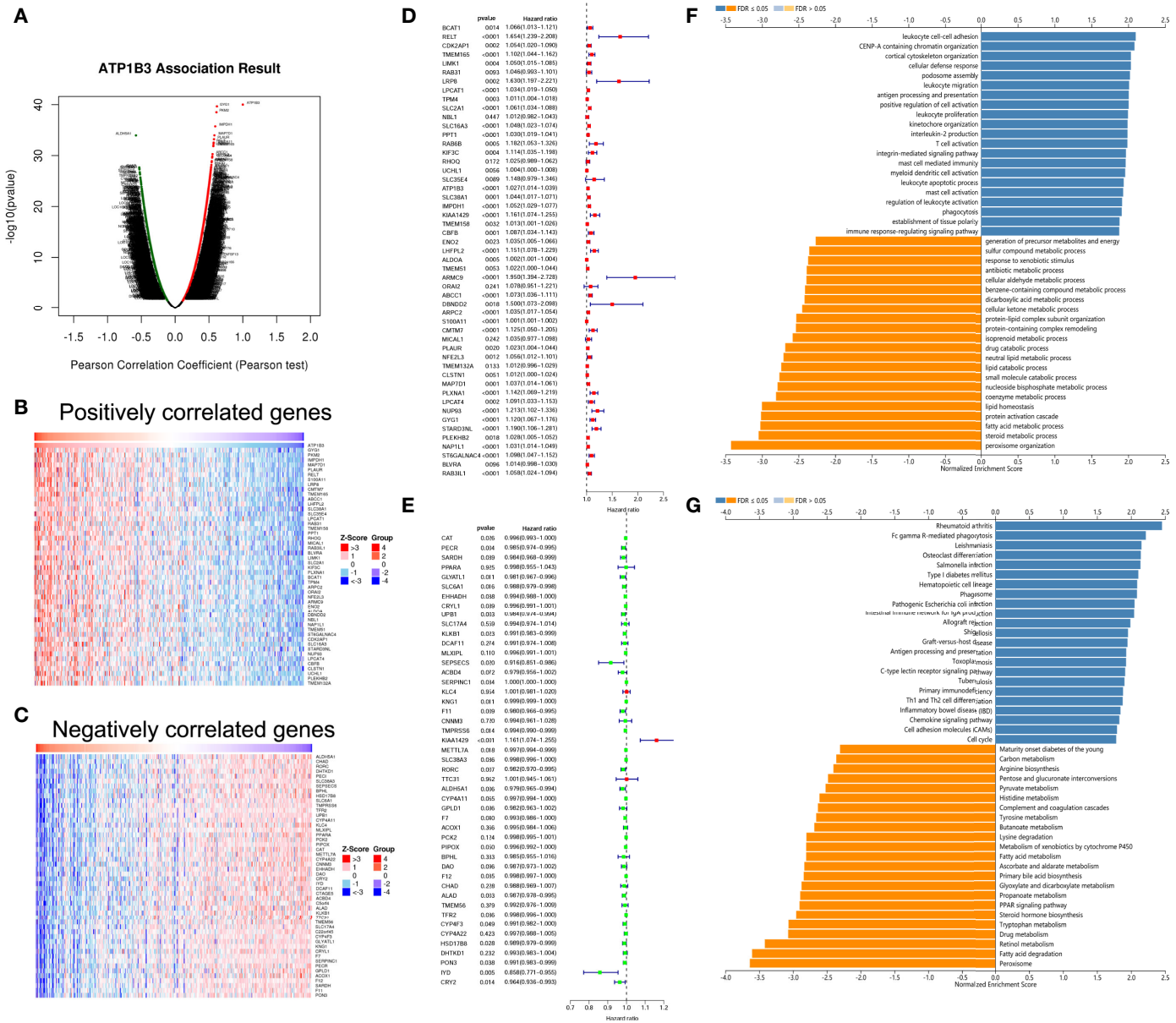

FIGURE 4 | ATP1B3 co-expression genes in HCC using the LinkedOmics. **(A)** The volcano plot of ATP1B3 co-expression genes. Heat maps revealed the top 50 positively **(B)** and negatively **(C)** co-expressed genes of ATP1B3 in HCC. Cox analysis revealed the prognosis of 50 positively **(D)** and negatively **(E)** co-expressed genes of ATP1B3 in HCC. The GO enrichment **(F)** and KEGG pathways **(G)** of ATP1B3.

TABLE 2 | The Kinases, miRNAs and transcription factors-target networks of ATP1B3 in HCC.

Enriched Category	Geneset	Leading Edge Number	*p* Value	FDR
miRNA_target	GCAAGAC,MIR-431	22	0	0.13008
	ACACTCC,MIR-122A	36	0.002242	0.15456
	GGGGCCC,MIR-296	33	0.00231	0.19513
	AGGAAGC,MIR-516-3P	32	0.002151	0.21191
	GCGCTTT,MIR-518B,MIR-518C,MIR-518D	8	0.057221	0.26356
	TACGGGT,MIR-99A,MIR-100,MIR-99B	13	0.048223	0.27066
	GGCCAGT,MIR-193A,MIR-193B	33	0.018182	0.2822
	AACTGAC,MIR-223	23	0.017978	0.28409
	GTGGTGA,MIR-197	32	0.02069	0.29278
	GTGTGAG,MIR-342	31	0.021327	0.30936
Transcription_Factor_target	V$SRF_01	25	0	0.003348
	V$HNF4_01	73	0	0.004539
	RGAGGAARY_V$PU1_Q6	218	0	0.005022
	V$ELF1_Q6	92	0	0.006428
	V$PEA3_Q6	110	0	0.00703
	GGGNNTTTCC_V$NFKB_Q6_01	60	0	0.008035
	RGTTAMWNATT_V$HNF1_01	23	0	0.009078
	V$CP2_02	121	0	0.011048
	V$AP1_Q6_01	95	0	0.011717
	V$HNF1_01	57	0	0.012347
Kinase_target	Kinase_LCK	26	0	0.038989
	Kinase_PAK1	21	0	0.041155
	Kinase_LYN	30	0	0.049819
	Kinase_PRKCB	37	0	0.05361
	Kinase_ITK	5	0.010959	0.070999
	Kinase_PRKG1	13	0.005076	0.073375
	Kinase_PRKCG	16	0.002268	0.078845
	Kinase_SYK	19	0	0.081382
	Kinase_ROCK1	17	0	0.092708
	Kinase_PLK1	45	0	0.094946

*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$

expression was positively correlated with the makers of CD8+ T, T cell, M1 Macrophage, B cell, TAM (tumor-associated macrophage), DCs, Th1 (T helper cell 1), Tfh (Follicular helper T cell), and T cell exhaustion. Moreover, ATP1B3 was also associated with HCC-related cytokines and chemokines. Our research shows that the expression of ATP1B3 is positively correlated with IL10, IL22, IL34 and negatively correlated with IL27 (**Figure 6B**). These findings revealed the potential association between ATP1B3 and immune cell infiltration in HCC. As HCC is associated a higher level of inflammation, it is relatively evident that every marker upregulated to such HCC initiation and progression will be correlated to inflammation markers. So further experiments are needed for this speculation.

ATP1B3 Protein Expression and Prognosis in HCC

To further confirm the function of ATP1B3 in HCC, we analyzed the expression and prognosis of ATP1B3 protein levels using the CPTAC proteomics database. The ATP1B3 protein level was elevated in tumor tissue compared to normal tissues (**Figure 7A**), and its' expression was associated with the high differentiated tumor and medical history of liver cirrhosis (**Figure 7B**). HCC patients with high-expressed ATP1B3 showed poor OS (p =0.07) (**Figure 7C**). Moreover, the univariate analysis proved that ATP1B3, tumor size, and differentiation were significantly associated with OS in HCC, and multivariate analysis showed that tumor size and differentiation were independent factors of OS in HCC using the CPTAC database (**Figure 7D**). The relationship between ATP1B3 and 50 top negative/positive co-expressed genes were confirmed using the CPTAC database in **Figures S6** and **S7**. The correlation between ATP1B3 and immune gene was also confirmed using the CPTAC database in **Figure S8**. Moreover, the proteomics and phospho-proteomics levels of ATP1B3 of 316 HCC patients were analyzed using Gao's data (29). As shown in **Figure 7E**, the protein level of ATP1B3 was elevated and the phosphorylation of ATP1B3 was downregulated in tumor tissue compared to paratumor tissues. And ATP1B3 protein level was associated with TNM stage (p= 0.06) (**Figure 7F**). HCC patients with high-expressed ATP1B3 shows worse OS (P=0.002) (**Figure 7G**). Moreover, the univariate analysis proved that ATP1B3, TNM, and age were significantly associated with OS in HCC, and multivariate analysis showed ATP1B3 was an independent factor of OS in HCC (**Figure 7H**).

ATP1B3 Related Potential Drug in HCC

Drug sensitivity plays a crucial role in HCC treatment. We next analyzed the correlation of ATP1B3 expression to sorafenib-therapy and PD-1 immunotherapy using GSE109211 and GSE120714 database. We found that HCC patients with sorafenib-resistant have higher ATP1B3 expression compared to HCC patients with sorafenib-sensitive (**Figure 8**). However,

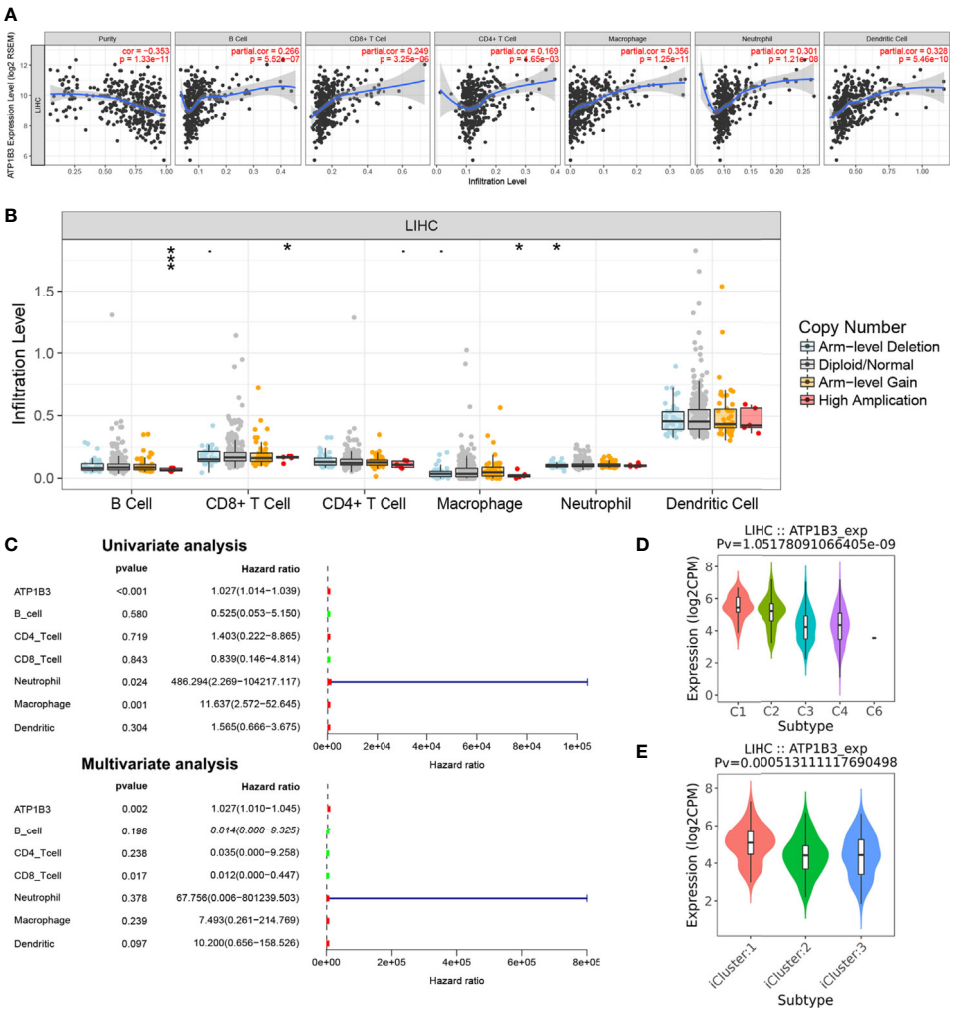

FIGURE 5 | The association of ATP1B3 and immune infiltration level in HCC using the TIMER. **(A)** The correlation between ATP1B3 expression level and immune infiltration. **(B)** The relationship between ATP1B3 CNV and immune infiltration. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. **(C)** The prognosis of ATP1B3 and immune cells for OS of HCC. **(D)** Associations between ATP1B3 expression and immune subtypes in HCC. C1 (wound healing); C2 (IFN-gamma dominant); C3 (inflammatory); C4 (lymphocyte depleted); C5 (immunologically quiet); C6 (TGF-b dominant). **(E)** Associations between ATP1B3 expression and molecular subtypes in HCC.

no significant difference in ATP1B3 expression was observed between with/without PD-1 immunotherapy in HCC patients (**Figure 8B**). To further investigated the potential drug for HCC patients with high ATP1B3 expression, we analyzed the role of 34 chemicals on ATP1B3 expression using GSE69844 (**Table S9**). We found that 10 μM and 100 μM Progesterone could slightly reduce ATP1B3 expression in HepaRG cells (**Figure 8C**). These results demonstrate that Progesterone may be an expected drug for the treatment of HCC patients with high-expressed ATP1B3. This needs to be further confirmed by experiments.

ATP1B3 Expression Is Increased in the HCC Cells and HCC Tissues

We further confirmed the expression of ATP1B3 in HCC cells and HCC tissues using qPCR, western blot and IHC. As shown in **Figure 9A**, ATP1B3 is upregulated in HCC tissues compared with paratumor tissues. Similarly, compared with human normal liver cells (LO2), both protein expression and mRNA expression levels of ATP1B3 were upregulated in HCC cells (Hhu7 and HCCLM3) (**Figures 9B, C**).

Silenced ATP1B3 Represses HCC Cell Proliferation, Migration and Induces HCC Cell Apoptosis

To investigate the role of ATP1B3 in HCC, we transfected ATP1B3 siRNA (Hhu7-siATP1B3 and HCCLM3-siATP1B3) into Hhu7 and HCCLM3 cells to knockdown ATP1B3 expression (**Figures 10A, B**), and then analyzed the effects of silenced ATP1B3 on HCC cells proliferation, migration, invasion and cycle, apoptosis. MTT and plate clone formation assay suggested that silenced ATP1B3 significantly inhibited HCC cells proliferation (**Figures 10C, D**). Transwell migration assay

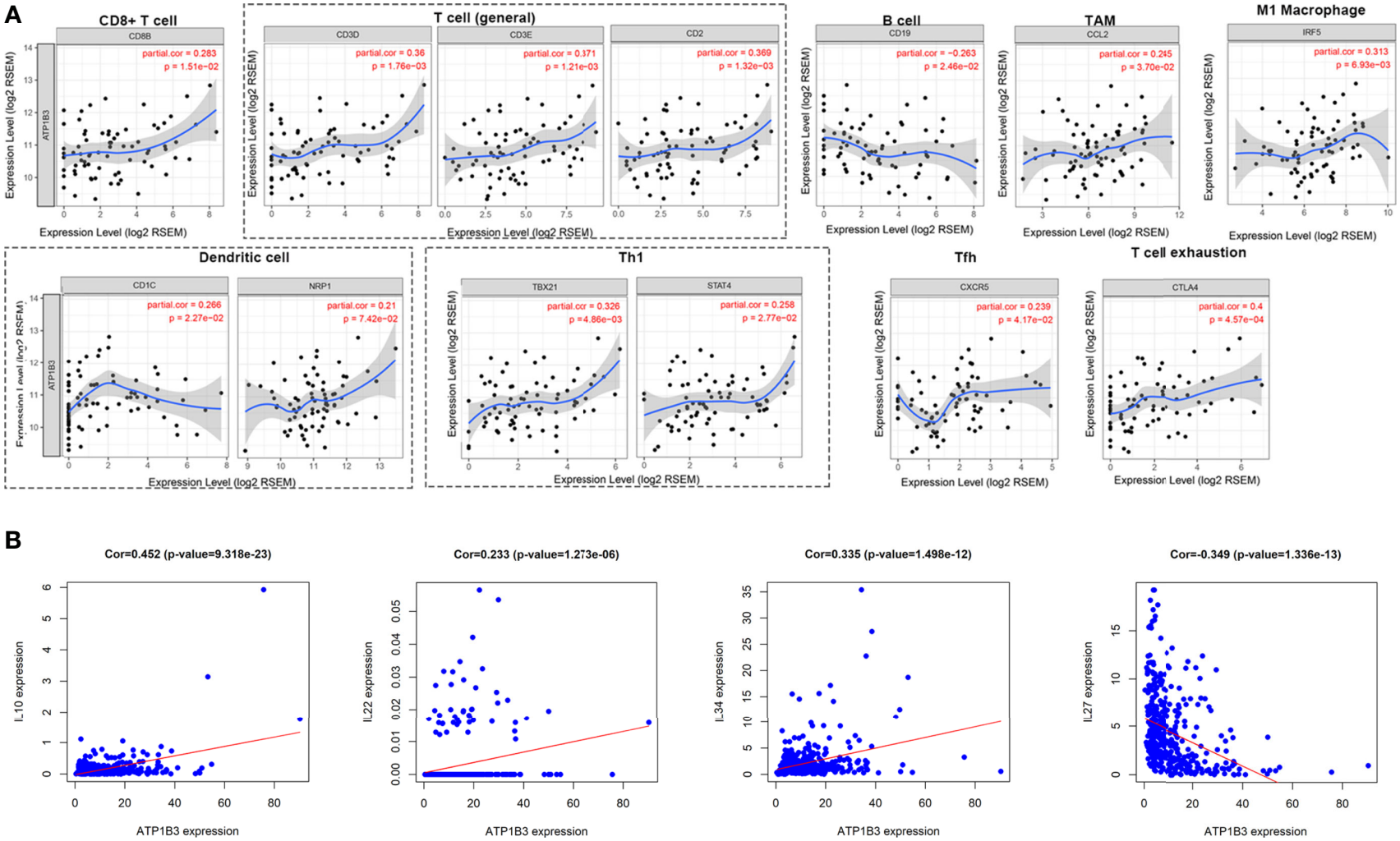

FIGURE 6 | Correlation between ATP1B3 and immune markers and immune-related cytokines in HCC. **(A)** The correlation between ATP1B3 and immune-related marker genes in HCC (TIMER). **(B)** The correlation between ATP1B3 and HCC-related cytokines.

TABLE 3 | Correlation analysis between ATP1B3 and relate genes and markers of immune cells in TIMER.

Description	Gene markers	HCC			
		None		Purity	
		Core	*p*	Core	*p*
CD8+ T cell	CD8A	0.193695	0.087216	0.226704	0.053766
	CD8B	0.24628	0.028676	0.283328	0.015142
T cell (general)	CD3D	0.295098	0.008287	0.360033	0.001756
	CD3E	0.30925	0.005732	0.371476	0.001213
	CD2	0.29944	0.007545	0.368986	0.001316
B cell	CD19	-0.21158	0.06123	-0.2629	0.024633
	CD79A	0.206353	0.068175	0.190102	0.107196
	CD20/KRT20	0.069475	0.542921	0.107764	0.364148
	CD38	0.17554	0.121766	0.178714	0.130333
Monocyte	CD86	0.219279	0.052356	0.224556	0.056138
	CD115/CSF1R	0.193744	0.087135	0.219431	0.062145
TAM	CCL2	0.213121	0.059468	0.244564	0.037045
	CD68	0.123539	0.277431	0.081046	0.495473
	IL10	0.08718	0.44488	0.082802	0.486149
M1 Macrophage	iNOS/NOS2	0.036588	0.748873	-0.00432	0.971037
	IRF5	0.301388	0.007149	0.313443	0.006929
	COX2/PTGS2	0.178607	0.115283	0.222918	0.058005
M2 Macrophage	CD163	0.080185	0.481597	0.075294	0.526662
	VSIG4	0.097347	0.392623	0.086679	0.465895
	MS4A4A	0.134494	0.236785	0.129154	0.276146
Neutrophils	CD66b/ CEACAM8	0.056755	0.619325	0.086414	0.467261
	CD11b/ITGAM	0.165847	0.143872	0.17351	0.142092
	CCR7	0.178271	0.115981	0.174791	0.139127
Natural killer cell	KIR2DL1	0.124381	0.274769	0.089358	0.452167
	KIR2DL3	0.184795	0.103019	0.225681	0.054885
	KIR2DL4	0.124151	0.275663	0.092833	0.434706
	KIR3DL1	-0.13216	0.245613	0.092833	0.434706
	KIR3DL2	0.231732	0.039888	0.213627	0.069564
	KIR3DL3	0.045013	0.693647	0.06811	0.566946
	KIR2DS4	0.190579	0.092498	0.21482	0.067984
Dendritic cell	HLA-DPB1	0.197785	0.08065	0.229444	0.050859
	HLA-DQB1	0.19131	0.091238	0.181263	0.124851
	HLA-DRA	0.126144	0.267366	0.128596	0.278246
	HLA-DPA1	0.154333	0.17412	0.160538	0.174852
	BDCA-1/CD1C	0.292641	0.008867	0.266351	0.022742
	BDCA-4/NRP1	0.205964	0.068706	0.210243	0.074208
	CD11c/ITGAX	0.054771	0.630979	-0.00886	0.94072
Th1	T-bet/TBX21	0.250883	0.025738	0.326148	0.004863
	STAT4	0.230101	0.041346	0.257719	0.027716
	STAT1	0.086246	0.448998	0.063854	0.591474
	IFN-γ/IFNG	0.093085	0.414531	0.142996	0.227475
	TNF-α/TNF	0.175729	0.121358	0.211978	0.071798
Th2	GATA3	0.206938	0.067385	0.200184	0.089491
	STAT6	-0.09837	0.387653	-0.0571	0.631374
	STAT5A	0.161587	0.154563	0.160109	0.17602
	IL13	-0.06835	0.549514	-0.07901	0.506375
Tfh	BCL6	0.14389	0.205385	0.154364	0.19225
	CXCR5	0.208307	0.065442	0.239045	0.041672
	ICOS	0.186286	0.100222	0.20881	0.076248
	BCL-6	0.14389	0.205386	0.154364	0.19225
Th17	STAT3	0.063218	0.579202	0.039726	0.738612
	IL17A	0.180941	0.110532	0.169988	0.150491
Treg	FOXP3	0.040141	0.724896	0.025344	0.831455
	CCR8	0.125941	0.268745	0.082091	0.489913
T cell exhaustion	STAT5B	0.067259	0.555148	0.060167	0.613102
	TGFβ/TGFB1	0.174513	0.123859	0.161097	0.173335
	PD-1/PDCD1	0.182633	0.107183	0.228417	0.051933
	CTLA4	0.365268	0.000933	0.399865	0.000457
	LAG3	0.176874	0.118796	0.16538	0.162032
	TIM-3/HAVCR2	0.060467	0.595846	-0.00268	0.982079
	GZMB	0.196774	0.082188	0.185335	0.11646

*HCC: hepatocellular carcinoma; CHOL: cholangiocarcinoma; TAM: tumor-associated macrophage; Th: T helper cell; Tfh: Follicular helper T cell; Treg, regulatory T cell; Cor, R value of Spearman's correlation; None, correlation without adjustment. Purity, correlation adjusted by purity. *p < 0.05, **p < 0.01, ***p < 0.001.*

and wound healing assay suggested that silenced ATP1B3 significantly inhibited HCC cells migration (**Figures 10E, F**). Flow analysis suggested that silenced ATP1B3 induced HCC cells apoptosis (**Figure 10G**) and blocked cell cycle in G0/G1 phase (**Figure 10H**). Moreover, we detected the EMT markers in ATP1B3 silenced HCC cells by western blot. The results showed that silenced ATP1B3 significantly upregulated E-cadherin expression, and downregulated N-cadherin and vimentin expression. These results proved that ATP1B3 promoted EMT in HCC (**Figure 10I**). Moreover, we have tried to detect the effects of ATP1B3 on cell proliferation in LO2 by MTT (**Figure 10J**) and plate clone formation (**Figure 10K**). We found that ATP1B3 silencing had no significant effect on the proliferation of healthy liver cells. In conclusion, these results proved that ATP1B3 could promote the tumorigenicity of HCC.

DISCUSSION

Na^+/K^+-ATPase (NKA) is a multifunctional transmembrane protein that plays a crucial role in cell adhesion (14), cell movement (43), cell proliferation and apoptosis (8), and signal transduction (44). Emerging studies have shown the abnormal expression (6) and the prognosis of NKA in various cancers (9). However, the clinical relevance of NKA in HCC remains limited. In this paper, multiple public databases are used for the first time to comprehensively analyze the expression of NKA subunits in HCC and its correlation with HCC prognosis and reveal its possible mechanism in HCC.

NKA was reported to be dysregulated in multiple cancers (14). For example, NKA α1 subunit (ATP1A1) is upregulated in non-small cell lung cancer (NSCLC) (45), esophageal squamous cell carcinoma (ESCC) (46), renal cell carcinoma (47), glioma (48), but downregulated in prostate cancer (49). NKA β1 subunit (ATP1B1) is downregulated in human epithelial cancer cells (50–52). A few studies report the abnormal expression of NKA in HCC. For example, Shibuya et al. (53) and Li et al. (54) pointed

TABLE 4 | Correlation analysis between CCL14 and marker genes of immune cells in GEPIA.

	Gene markers	Cancer		Normal		GTEx	
		Core	p	Core	*p*	Core	*p*
	CD8B	0.34	1.40E-11	0.5	0.00022	0.36	0.00013
T cell (general)	CD3D	0.29	1.30E-08	0.38	0.006	0.4	1.60E-05
	CD3E	0.28	7.50E-08	0.38	0.0065	0.32	6.00E-04
	CD2	0.29	9.20E-09	0.32	0.023	0.4	1.70E-05
B cell	CD19	0.069	0.18	0.12	0.43	0.22	2.10E-02
TAM	CCL2	0.28	7.00E-08	0.87	4.40E-16	0.85	0
M1 Macrophage	IRF5	0.25	1.30E-06	0.37	0.0088	0.19	4.50E-02
Dendritic cell	BDCA-1/CD1C	0.16	0.016	0.24	0.088	-0.03	7.60E-01
	BDCA-4/NRP1	0.43	0	0.36	0.01	0.44	1.50E-06
	T-bet/TBX21	0.23	9.60E-06	0.41	0.0028	0.34	2.60E-04
Th1	STAT4	0.12	0.021	0.37	0.008	0.19	5.00E-02
Tfh	CXCR5	0.39	4.70E-15	0.22	0.13	0.14	1.60E-01
T cell exhaustion	CTLA4	0.29	1.20E-08	0.42	0.0024	0.53	2.10E-09

*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

out that ATP1A3 overexpression in HCC is related to the antitumor activity of bufalin. It can be used as a therapeutic target for bufalin. L Zhuang et al. (4) showed that ATP1A1 was upregulated in HCC, and its function as an oncogene by promoting proliferation and migration of HCC cells. Whereas the potential prognostic role of NKA in HCC remains unclear. Consistent with previous study, we found that ATP1A1 and ATP1A3 were upregulated in HCC from TCGA database. Moreover, ATP1B3 were also significantly upregulated in HCC with logFC > 1 and $p < 0.01$ using TCGA, ICGC, and GEO datasets. The prognostic analysis revealed that ATP1B3 was an independent factor for the OS of HCC based on transcriptomic data from TCGA, ICGC, and GEO.

ATP1B3 encodes the β3 subunit of NKA and regulates cell adhesion (55). The study has displayed that ATP1B3 expression was increased in gastric cancer tissues and was closely related to related to gastric cancer patients' clinical characteristics (51). Here, we found that ATP1B3 high expression was associated with clinical characteristics of HCC patients including stage and grade. Subsequently, the expression and prognosis of ATP1B3 protein in HCC were also confirmed using the CPTAC database and proteomics and phospho-proteomics data from Gao's work. The results indicated that ATP1B3 is a useful biomarker for diagnosis and prognosis of HCC prognosis. Furthermore, we validated that ATP1B3 is increased in HCC cells and tissues. Meanwhile, we also proved that silenced ATP1B3 repressed HCC cell proliferation, migration and induced HCC cell apoptosis. In brief, these results suggest that ATP1B3 could be an oncogene and promote tumorigenicity of HCC.

To investigate the potential mechanism of ATP1B3 in HCC, we analyzed the co-expressed genes of ATP1B3. The results showed that they were mainly involved in various immune responses, simultaneously inhibiting the metabolism of steroids and fatty acids. At the same time, the ATP1B3 expression was positively related to kinase expression, including LCK and LYN, which have been reported to play a crucial part in regulating B cell receptor signaling (52, 56). As previously reported that NKA regulates Src family kinase activity (including FYN and LYN) (57). Recruitment of NKA-LYN complex in macrophages promotes atherosclerosis (12). The NKA α-1/Src complex activates a variety of pro-inflammatory factors/chemokines and mediates pro-inflammatory effects (58). Pieces of evidence have proven the involvement of NKA in the inflammatory response (59). Therefore, we speculated that ATP1B3 might be involved in the immune regulation of HCC.

Immune infiltration is a significant factor in the tumor microenvironment, which plays a crucial part in the development and prognosis of tumors (60). Various immune cells contributed to the immune microenvironment of HCC including macrophages, neutrophil, dendritic cell, adaptive immune CD4+, CD8+ T-lymphocytes, and NK cells (61). Studies showed that infiltrated macrophages were polarized M2-TAM (tumor-associated macrophages), which act as immune suppressor cells and lead to reduction and exhaustion of CD8+T cells in HCC (62). Tregs were proved to be increased in HCC and impede immune surveillance (63). Despite the effect of NKA on tumor immunity has not been widely reported, it is known that knockdown of NKA α1 in macrophages can inhibit cardiotonic steroid (CTS)-induced macrophage infiltration and the accumulation of immune cells *in vivo* (64). We found that ATP1B3 was significantly correlated with tumor purity and B cell infiltration, $CD8^+$ T infiltration, $CD4^+$ T infiltration, Macrophage infiltration, Neutrophil infiltration, and Dendritic cell infiltration. Also, ATP1B3 and $CD8^+$ T cells were found to be independent factors of HCC. In addition, we also found that ATP1B3 expression was positively correlated with the makers of CD8+ T, T cell, B cell, TAM, M1 Macrophage, DCs, Th1, Tfh, and T cell exhaustion. These immune cells are regulated by various cytokines and chemokines in the tumor environment, leading to different functions (65). Our research shows that the expression of ATP1B3 is positively correlated with IL10, IL22, IL34, and negatively correlated with IL27. IL10 was reported to inhibit the cytotoxicity of NK cells through the STAT3 signaling pathway, thereby promoting the recurrence and metastasis of HCC (66). In addition, IL22 is highly expressed in HCC and is related to the growth and malignancy of HCC tumors (67). IL-34

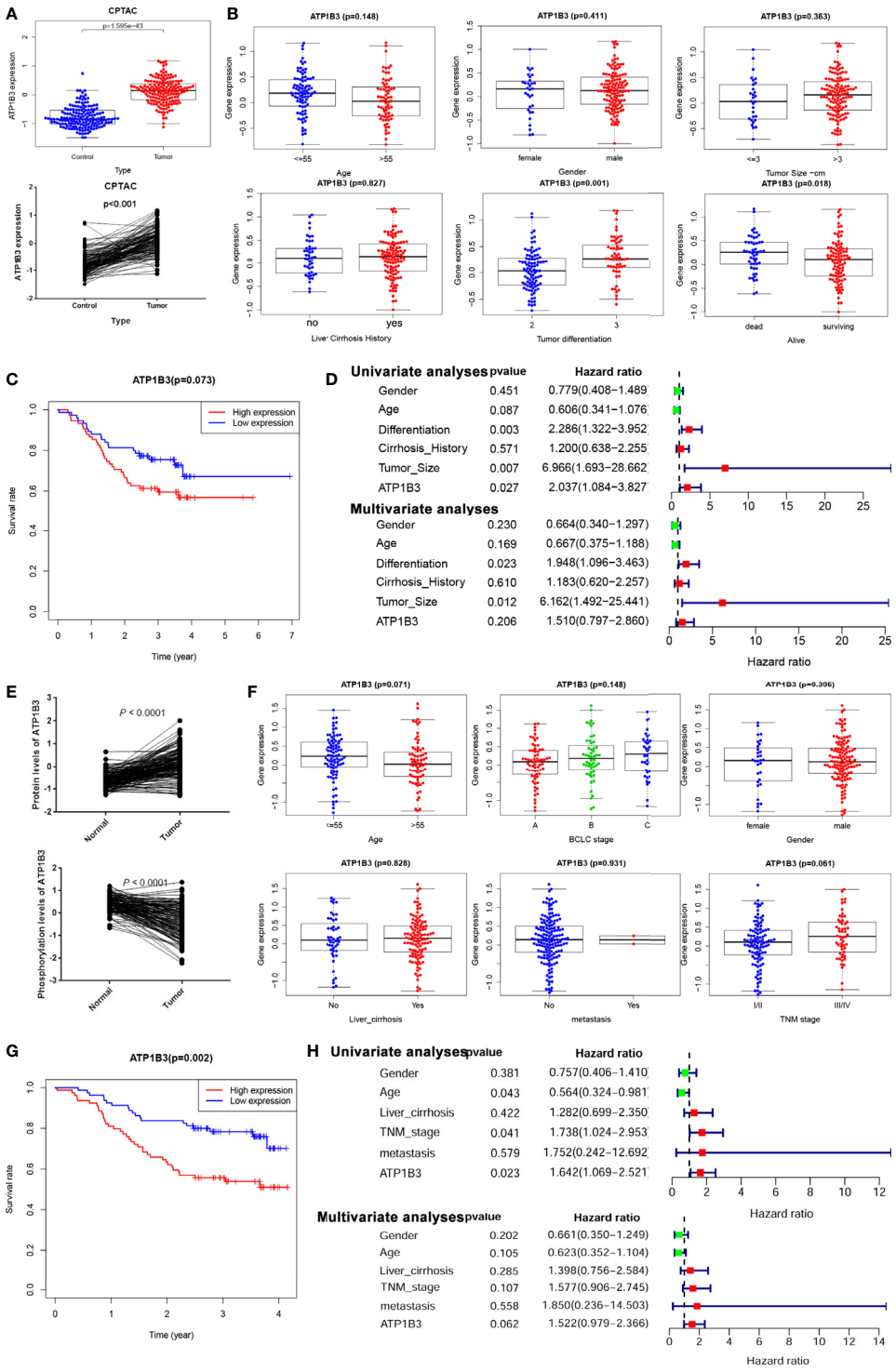

FIGURE 7 | ATP1B3 protein expression and prognosis in HCC. **(A)** ATP1B3 protein levels in HCC using CPTAC proteomics database. **(B)** The association between ATP1B3 protein expression and clinical features in HCC using CPTAC proteomics database. **(C)** The survival curves of OS with high/low ATP1B3 in CPTAC HCC cohorts. **(D)** Univariate and multivariate analysis revealed the relationship between ATP1B3 and the clinical factors with OS of HCC in the CPTAC database. **(E)** The protein level and the phosphorylation level of ATP1B3 in the proteomics and phosphor-proteomics data. **(F)** The association between ATP1B3 protein expression and clinical features in the proteomics and phosphor-proteomics data. **(G)** The survival curves of OS with high/low ATP1B3 in the proteomics and phosphor-proteomics data. **(H)** Univariate and multivariate analysis revealed the relationship between ATP1B3 and the clinical factors with OS of HCC in the proteomics and phosphor-proteomics data.

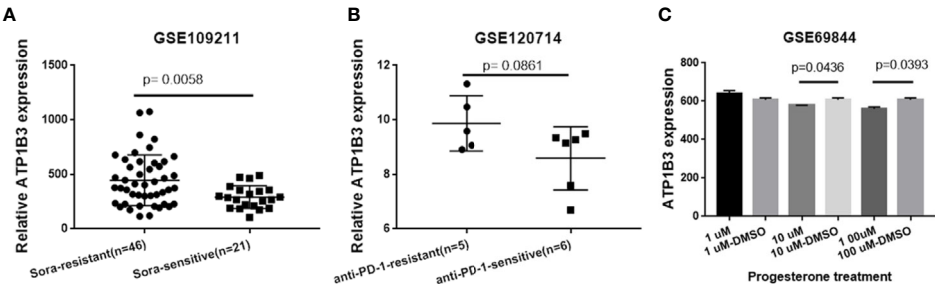

FIGURE 8 | ATP1B3 related potential drug in HCC. **(A)** ATP1B3 expression in sorafenib-resistant/-sensitive HCC patients. **(B)** ATP1B3 expression in anti-PD1 immunotherapy-resistant/-sensitive HCC patients. **(C)** The GSE69844 dataset revealed that Progesterone could reduce ATP1B3 expression in HepaRG cells.

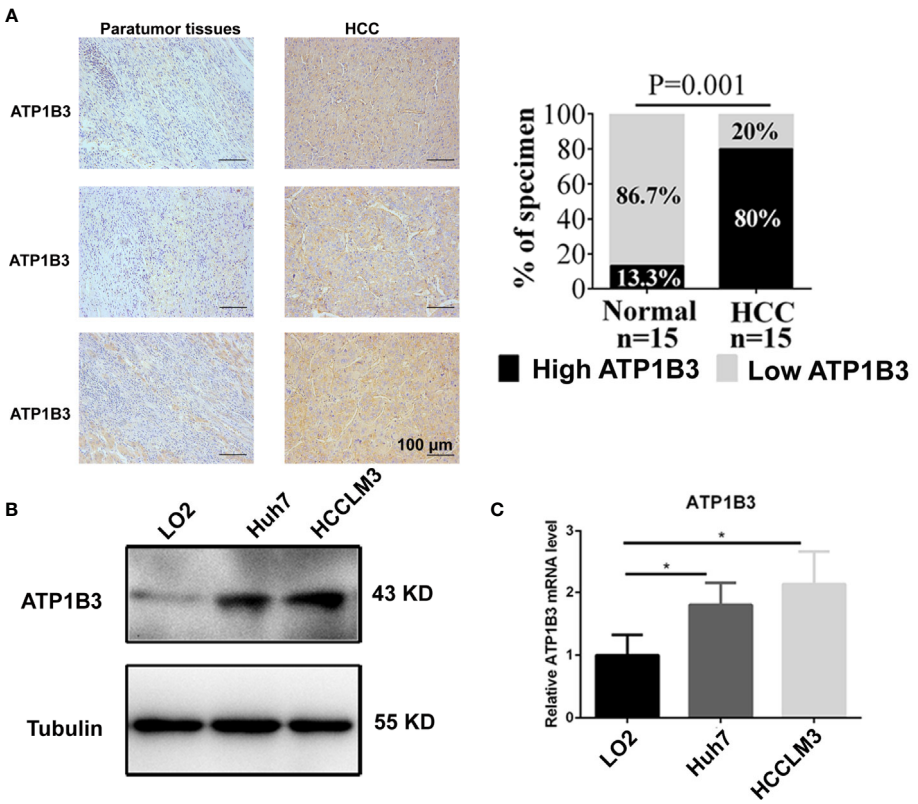

FIGURE 9 | ATP1B3 expression in the HCC cells and HCC tissues. **(A)** The protein levels of ATP1B3 in HCC tissues and paratumor tissues were detected by IHC. Scale bars= 100 μm. **(B)** The protein expression level of ATP1B3 in HCC cells and normal liver cell were detected by Western Blot. **(C)** The mRNA expression level of ATP1B3 in HCC cells and normal liver cell were detected by qPCR. *$p < 0.05$.

promotes the proliferation and migration of HCC through CSF1-R and CD138 (68). In addition, the DC-derived cytokine IL27 can exert anti-tumor activity by activating NK cells (69). These results imply that ATP1B3 may involve in immune infiltration by regulating immune-related cytokine in HCC.

Basing on the potential therapeutic and prognostic role of ATP1B3 on HCC patients, we analyzed the drug sensitivity of HCC patients with different expressed ATP1B3. Our result revealed that HCC patients with sorafenib-resistant have higher ATP1B3 expression compared to HCC patients with sorafenib-sensitive, suggesting that ATP1B expression is associated with sorafenib-resistant in HCC patients. Subsequently, 34 chemicals analysis results showed that 10 μM and 100 μM Progesterone slightly reduced ATP1B3 expression in HepaRG cells, indicating that Progesterone may be a combined drug strategy for sorafenib in the treatment of HCC. Moreover, studies showed that Na/K-

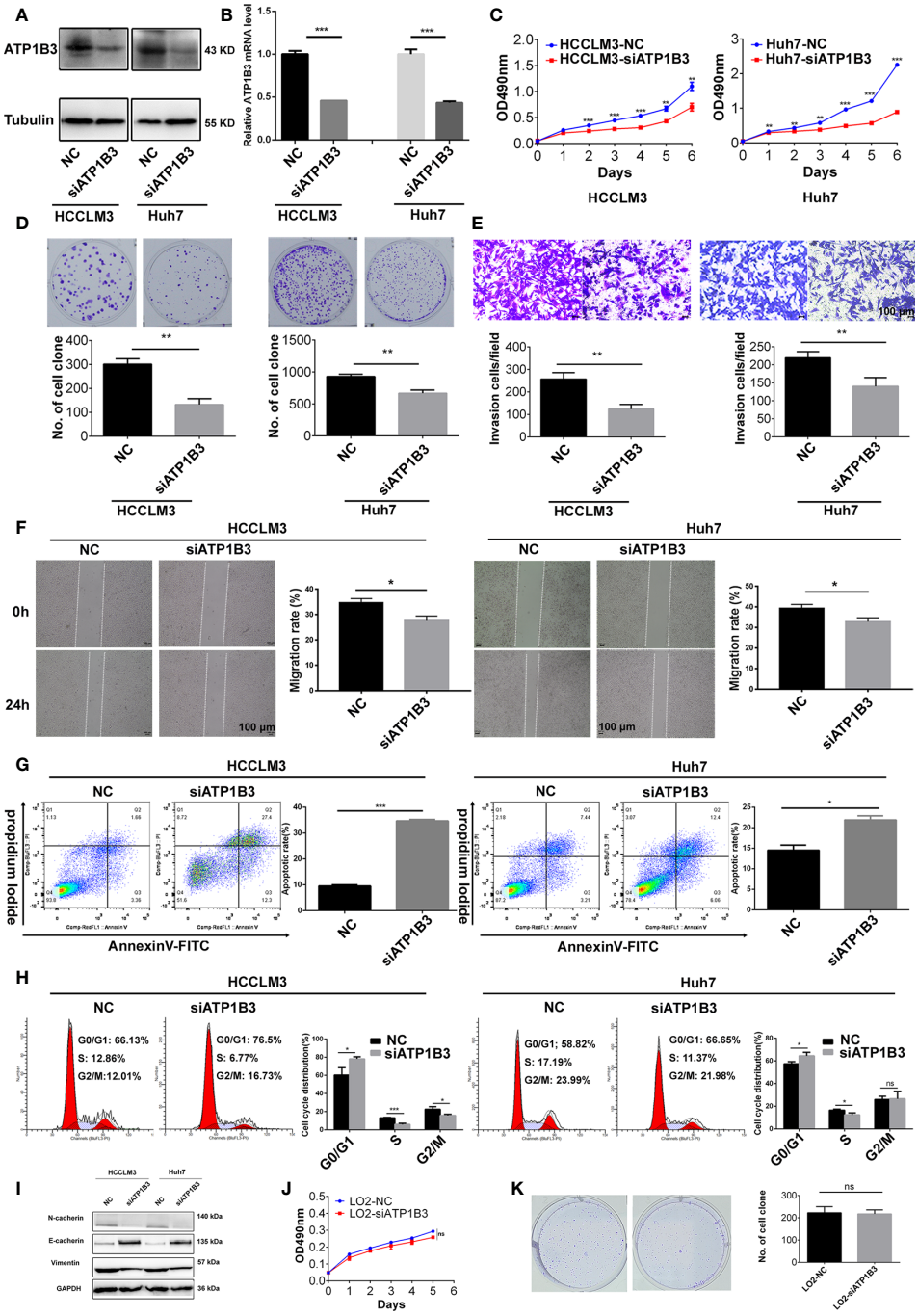

FIGURE 10 | ATP1B3 promotes HCC cell proliferation, migration and inhibits HCC cell apoptosis. Western Blot **(A)** and qPCR **(B)** showed that the expression of ATP1B3 were silenced by siRNA in Hhu7 and HCCLM3, respectively. MTT **(C)** and plate clone formation **(D)** analysis revealed the cell proliferation regulated by ATP1B3. Transwell migration **(E)** and scratch wound healing **(F)** assay revealed the migration ability regulated by ATP1B3. Apoptosis **(G)** and Cell cycle **(H)** assay revealed the regulation of ATP1B3 on cell apoptosis and cell cycle using flow cytometry. **(I)** The EMT markers in ATP1B3 silenced HCC cells. The effects of ATP1B3 on cell proliferation in LO2 by MTT **(J)** and plate clone formation **(K)**. Scale bars= 100 μm, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

ATPase is a target for anticancer drugs perillyl alcohol (POH), and Cardioprotection drug DRRSAb, indicating their potential therapeutic effect for HCC (11, 70). However, the treatment effect of these drugs for HCC has yet to be proved.

CONCLUSIONS

In summary, our results indicate that ATP1B3 is upregulated and promote the tumorigenicity of HCC, and it is also an

independent prognostic biomarker for the diagnosis of HCC with a potential immunomodulatory role, providing a novel prognostic biomarker and potential therapeutic target for HCC.

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by The ethics committee of Xiangya Hospital, Central South University. Written informed consent for participation was not required for this study in accordance with the national legislation and the institutional requirements.

AUTHOR CONTRIBUTIONS

Conceptualization: YZ and SL. Methodology: SL, SC and XP. Investigation: SL and XP. Writing – Original Draft: YX and SL. Writing – Review and Editing: YZ, SL and RC. Funding Acquisition: YZ, RC and XP. 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.2021.636614/full#supplementary-material

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REVIEW
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In Situ Vaccination as a Strategy to Modulate the Immune Microenvironment of Hepatocellular Carcinoma

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Hepatocellular Carcinoma (HCC) is a highly prevalent malignancy that develops in patients with chronic liver diseases and dysregulated systemic and hepatic immunity. The tumor microenvironment (TME) contains tumor-associated macrophages (TAM), cancer-associated fibroblasts (CAF), regulatory T cells (Treg) and myeloid-derived suppressor cells (MDSC) and is central to mediating immune evasion and resistance to therapy. The interplay between these cells types often leads to insufficient antigen presentation, preventing effective anti-tumor immune responses. *In situ* vaccines harness the tumor as the source of antigens and implement sequential immunomodulation to generate systemic and lasting antitumor immunity. Thus, *in situ* vaccines hold the promise to induce a switch from an immunosuppressive environment where HCC cells evade antigen presentation and suppress T cell responses towards an immunostimulatory environment enriched for activated cytotoxic cells. Pivotal steps of *in situ* vaccination include the induction of immunogenic cell death of tumor cells, a recruitment of antigen-presenting cells with a focus on dendritic cells, their loading and maturation and a subsequent cross-priming of CD8+ T cells to ensure cytotoxic activity against tumor cells. Several *in situ* vaccine approaches have been suggested, with vaccine regimens including oncolytic viruses, Flt3L, GM-CSF and TLR agonists. Moreover, combinations with checkpoint inhibitors have been suggested in HCC and other tumor entities. This review will give an overview of various *in situ* vaccine strategies for HCC, highlighting the potentials and pitfalls of *in situ* vaccines to treat liver cancer.

Keywords: hepatocellular carcinoma (HCC), immunotherapy, *in situ* vaccine, dendritic cells (DC), tumor microenvironment

INTRODUCTION

Liver cancer is the fourth leading cause of death worldwide, causing almost 800,000 deaths annually (1, 2). Hepatocellular carcinoma (HCC) is the most frequent primary liver malignancy, accounting for approximately 80% of primary liver cancers (3). The most common etiology of HCC is chronic liver disease, caused by viral infection, alcohol-related liver disease (ALD) and nonalcoholic steatohepatitis (NASH) (4, 5). The overall prognosis of patients with HCC remains poor, despite the establishment of screening programs, advancements in surgical and interventional therapies as well as systemic treatment options (1, 6–8).

Only a small fraction of patients is diagnosed at disease stages still amenable to curative therapies such as orthotopic liver transplantation, liver resection and interventional ablation (9, 10). In intermediate and advanced tumor stages, the majority of patients receive palliative treatment, including interventional strategies, as well as systemic pharmaceutical therapies. The latter have been shaped considerably over the last years, mainly through the discovery of multikinase inhibitors such as Sorafenib in 2008, which was the first drug to improve the survival of HCC patients, however, prolonging overall survival (OS) by less than three months (11). Since then, several other multikinase inhibitors like Lenvantinib, Regorafenib and Cabozantinib gained regulatory approval, however, also showing only modest improvement of patient survival. Immunotherapies such as checkpoint inhibitors represent the most important breakthrough in cancer therapy in the past two decades and were also explored for therapy of advanced HCC (12). However, the response rates to immune checkpoint inhibition as a monotherapy [e.g. Nivolumab, anti-programmed death (PD)-1] in HCC were still very low (about 15-20%), and strongly dependent on the tumor immune status (13, 14). In this regard, defective antigen cross-presentation by dendritic cells (DC), the most important professional antigen-presenting cells, and an exhaustion of the cytotoxic T cell response promote tolerance to the tumor and resistance to checkpoint inhibition (15). Thus, strategies activating the DC-CD8+ T cell axis to restore a CD8+ antitumor response have the potential to improve patients' outcomes and are intensely investigated. First evidence that combination therapies can improve response to checkpoint blockade in HCC has been provided by a phase III study investigating the combination of the checkpoint inhibitor atezolizumab (anti-programmed death ligand (PD-L) 1) and bevazicumab [anti-vascular endothelial growth factor (VEGF)], which reported outcomes superior to Sorafenib in HCC (12).

Cancer vaccines have been proposed as a strategy to induce or reactivate antitumor immune responses (16). Their mechanism is based on isolating patient-derived DCs, pulsing them with tumor-associated antigens (TAAs) and maturation signals, followed by their reinfusion (17). However, the great variability of tumor antigens and the lack of universal TAAs have prevented their clinical use until now (18). Moreover, the inherent logistical difficulties of preparing individualized vaccines *ex vivo* limits their application. Similarly, T-cell transfer of CD8+ T cells is associated with a simultaneous homeostatic inhibition of T cells, yielding overall disappointing clinical results in solid tumors like HCC (19, 20).

Inducing and stimulating an immune response specifically at the tumor site is referred to as *in situ* vaccine, a concept that takes advantage of the whole repertoire of TAAs available at the tumor site (21). Thus, the intratumoral or systemic injection of immunomodulators can induce presentation of TAAs by antigen-presenting cells (APCs) and, subsequently, the activation of a cytotoxic T cell response with the generation of both effector and memory CD8+ T cells. Several prerequisites for a successful antitumor immune response have been identified: i) The availability of TAAs in a sufficiently immunogenic setting to trigger phagocytosis and activate DCs; ii) an efficient antigen presentation with co-stimulatory signals to successfully cross-prime CD8+ cytotoxic T cells; and iii) a cytotoxic T cell response that overcomes inhibitory signals from the tumor and TME. Collectively, this will result in adaptive antitumor responses with local and systemic effects (**Figure 1**) (22).

Different strategies can support and enhance all steps of this treatment process, which will be described in detail in this article. This review aims to give a comprehensive overview of *in situ* vaccines for the treatment of HCC in the context of the underlying immune dysfunction and immunosuppressive TME. Both preclinical and clinical *in situ* vaccine strategies and techniques will be discussed, highlighting opportunities as well as potential limitations and pitfalls of this immunotherapeutic approach.

Liver and HCC Immunology

Many challenges in treating hepatic malignancies originate from the tolerogenic nature of hepatic immune responses and are aggravated by distinct immunosuppressive effects conferred by the tumor and its TME (23, 24). The liver is in continuous contact to non-self antigens from the portal tract and hepatic immune tolerance is the ordinary response to non-self structures, unless they are accompanied by distinct danger signals (25).

Abbreviations: ALD, alcohol-related liver disease; APC, antigen-presenting cell; BCG, Bacillus Calmette-Guerin; CAF, cancer-associated fibroblasts; CCL, C-C chemokine ligand; CCR, chemokine receptor; cDC, conventional dendritic cell; CXCL, CXC-ligand; DAMP, damage-associated molecular pattern; DC, dendritic cell; Flt3L, Fms-like tyrosine kinase 3 ligand; FOLFOX, 5-fluorouracil plus oxaliplatin; GM-CSF, granulocyte-macrophage colony-stimulating factor; HCC, hepatocellular carcinoma; HMGB1,high mobility group box 1; HSP, heat shock protein; HSV, herpes simplex virus; ICD, immunogenic cell death; IFN, interferon; IL, interleukin; iNHL, indolent non-Hodgkin lymphoma; irAEs, immune-related adverse events; MDA5, melanoma differentiation-associated gene 5; MDSC, myeloid-derived suppressor cells; MMP, matrix metalloproteinase; mRNA, messenger RNA; NASH, nonalcoholic steatohepatitis; NDV, Newcastle disease virus; NK, Natural Killer; OS, overall survival; PAMP, pathogen associated molecular pattern; PD, programmed death; pDC, plasmacytoid dendritic cell; PD-L, programmed death ligand; polyIC, polyinosinic:polycytidylic acid; PRR, pattern recognition receptor; RAGE, receptor for advanced glycation endproducts; RIG-I, retinoic acid-inducible gene-I; TAA, tumor-associated antigen; TACE, Transarterial Chemoembolization; TAM, tumor-associated macrophage; Th, T helper; TIM-3, T-cell immunoglobulin and mucin-domain containing-3; TLR, toll-like receptor; TME, tumor microenvironment; TNF, tumor necrosis factor; TRAIL, tumor necrosis factor-related apoptosis-inducing ligand; Treg, regulatory T cell; T-VEC, talimogene laherparepvec; VEGF, vascular endothelial growth factor; VSV, vesicular stomatitis virus.

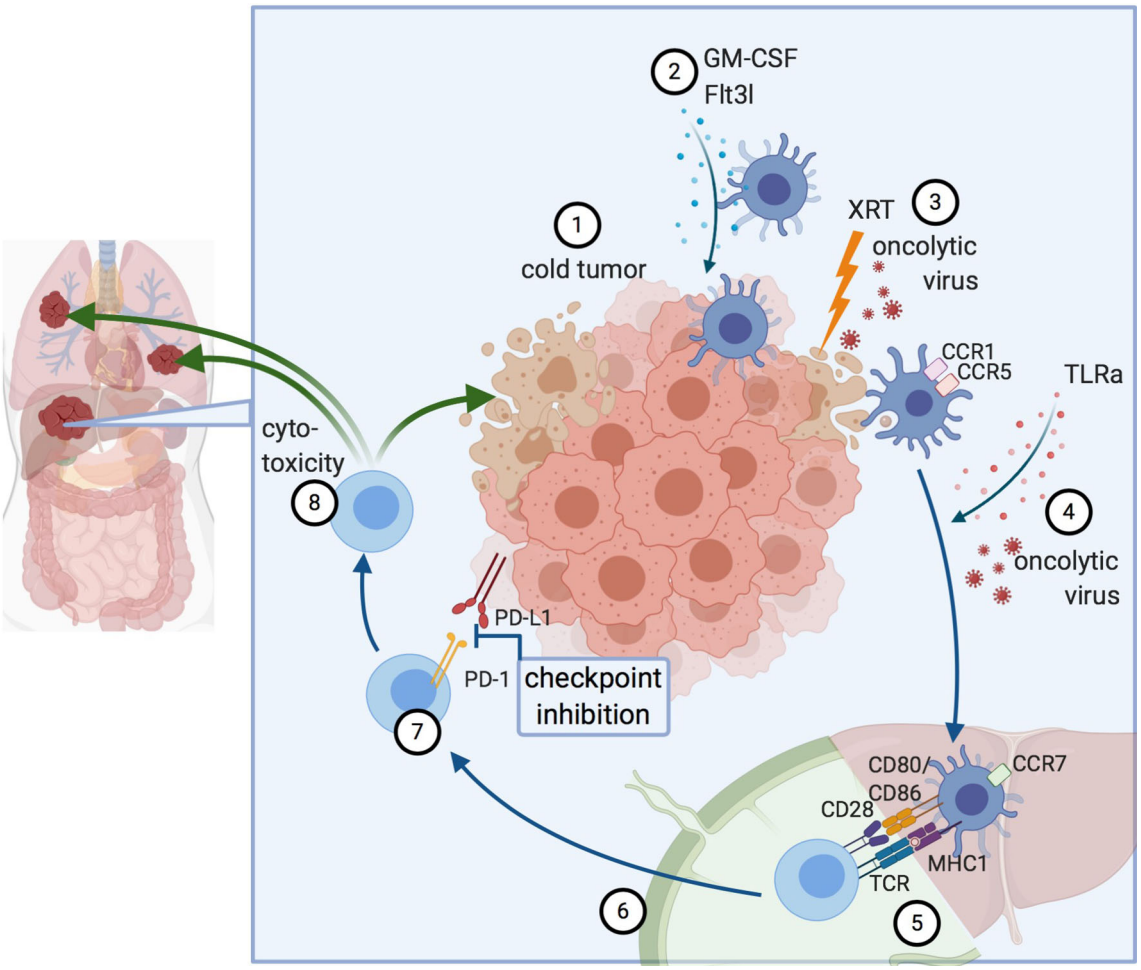

FIGURE 1 | Principles of *in situ* vaccines. 1) Cold tumor devoid of DCs and T cells. 2) DC recruitment to the tumor. 3) Induction of immunogenic cell death, for example by radiation or oncolytic viruses. 4) Maturation signals for DCs lead to 5) Antigen presentation and cross-priming of CD8+ T cells. This can occur either in the liver itself (in tertiary lymphoid organs forming near liver tumors) or the draining lymph node. 6) Activated T cells migrate to the tumor. 7) Abrogation of inhibitory signaling e.g. *via* checkpoint inhibition. 8) Cytotoxicity against the treated tumor and by abscopal effects against other lesions, as well. Created with biorender.

Antigen presentation in the liver can be performed by professional APCs such as DCs as well as liver-specific APCs, e.g. hepatic stellate cells, Kupffer cells, liver sinusoidal endothelial cells and even hepatocytes (26). Due to an overlap of markers, e.g. Kupffer cells and other macrophages in the mouse liver can express the "DC marker" CD11c or MHC-II molecules, it is particularly challenging to dissect the contribution of different myeloid APCs in the liver. This is even more difficult in diseased liver, as liver injury (or tumor development) commonly leads to a strong recruitment and accumulation of myeloid cells in the liver (27).

DCs, the most important professional APCs, usually have an immature phenotype in the liver. They can interact with T cells directly in the liver or migrate to the draining lymph node to present antigens there (28). While the exact significance of the place of antigen presentation – directly in the liver, especially in proximity to portal tracts, or after DC migration to lymph nodes – is not entirely clear in HCC, it has been shown that DC-mediated T cell activation can occur in both localizations (29, 30).

The main subsets of DCs include conventional (cDC) and plasmacytoid DCs (pDC). Type 1 conventional DCs (cDC1) are capable of cross-presenting extracellular antigens in a MHCI-restricted manner to CD8+ cells (31), a process that, depending on the state of DC maturity and concomitant expression of costimulatory molecules or tolerogenic signals can result either in T cell cross-tolerance or in an efficient T cell cross-priming with ensuing cytotoxic activity (32). The latter makes the cDC1-CD8+ T cell interaction essential for tumor recognition and the initiation of antitumor immune responses.

While mutated neoantigens are rarely presented on HCC cells (33), TAAs such as alpha-fetoprotein (AFP), glypican-3 (GPC-3) or New York esophageal squamous cell carcinoma-1 (NY-ESO-1) are oftentimes overexpressed in HCC and phagocytosed by APCs (34, 35). Nevertheless, DCs are often unable to effect successful T cell cross-priming, with multiple underlying

mechanisms of dysfunction, including DC immaturity or "semi-maturity" (36), the induction of a tolerogenic DC phenotype by tumor-derived factors (37) and the expression of immune checkpoints (38, 39). These mechanisms culminate in DCs that either fail to activate specific T cell responses or even promote specific immune tolerance, leading to a suppression of CD8+ T cell responses and to cancer immunosurveillance failure (37).

The TME of HCC is composed of immune cells such as tumor-associated macrophages (TAM), myeloid-derived suppressor cells (MDSC), regulatory T cells (Tregs), inflammatory DCs, as well as stromal cells like cancer-associated fibroblasts and significantly contributes to cancer immune evasion (40, 41). Typical effects include the disruption of essential DC functions like DC maturation, phagocytosis and migration as well as the inhibition of T cell responses (42), but also the promotion of angiogenesis and tumor growth (43). Furthermore, the TME supports Th17 responses, with a resulting aggravation of the underlying chronic liver inflammation on the one hand, and, on the other hand, with proangiogenic effects (44). The HCC TME is considered to be one of the central determinants of therapy resistance, for example against Sorafenib (45). The principle of overcoming the inhibitory effects of tumor cells and their TME by harnessing the DC-T cell axis has evolved into several promising therapeutic approaches, including *in situ* vaccines (**Figure 2**) (17).

Inducing Immunogenic Cell Death

Immunogenic cell death (ICD) is a stress-induced, regulated type of cell death that triggers an adaptive immune response (46). It is characterized by the release of cellular antigens, which are taken up and presented by APCs and immune activation depends on sufficient antigenicity and adjuvanticity (47, 48). Antigenicity is determined by the quality and quantity of TAAs, while adjuvanticity is determined by the simultaneous release of danger signals such as damage-associated molecular patterns (DAMPs) (49). Immunostimulatory DAMPs include a release of endoplasmatic reticulum proteins like calreticulin and heat shock proteins (HSP), the toll-like receptor (TLR) 4 and TLR9 agonist high mobility group box 1 (HMGB1) and ATP, leading to DC recruitment and activation at the site of tumor cell death (50). For *in situ* vaccination, ICD provides an elegant method to harness the whole breadth of available cancer antigens for an immune response. In preclinical and clinical settings, various endogenous and exogenous stimuli can trigger ICD, including several conventional chemotherapeutic agents (51), radiation therapy (52) as well as therapeutic oncolytic viruses (53), which

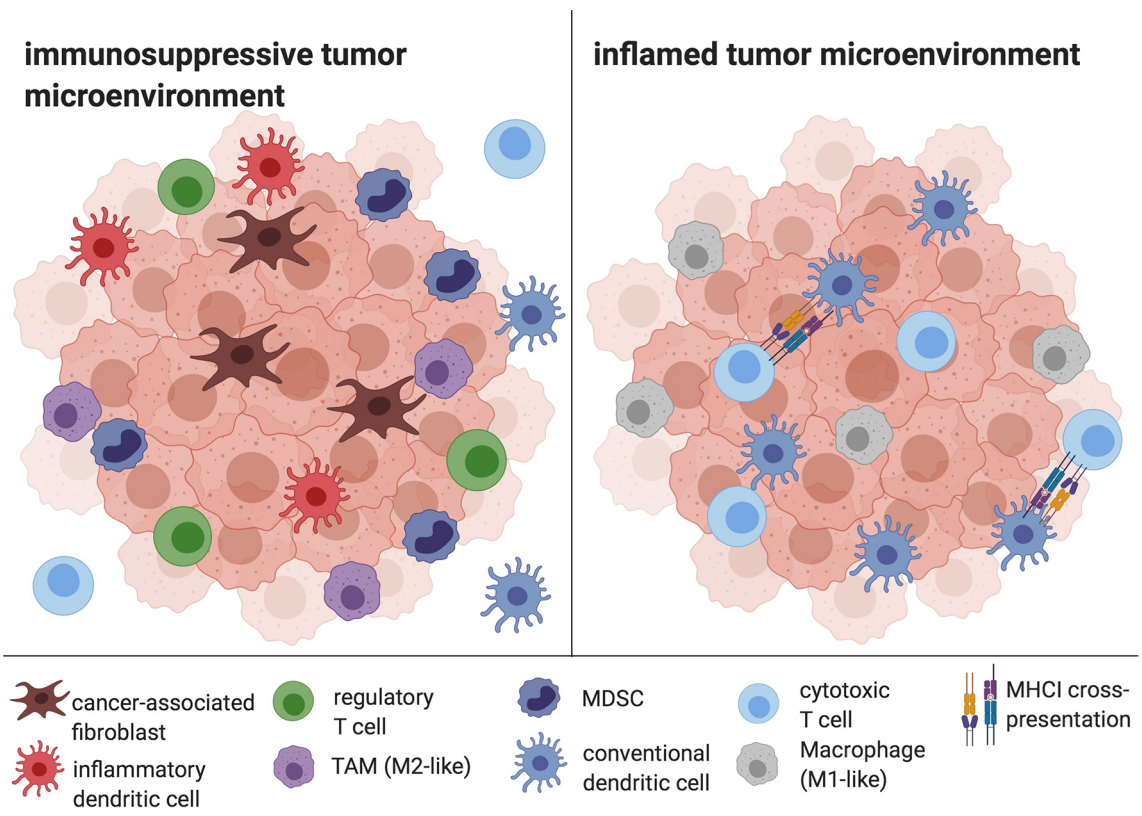

FIGURE 2 | The HCC TME. Cancer-associated fibroblasts, infDCs, TAMs, Tregs and MDSC mediate immune evasion and prevent APC and CD8+ T cell infiltration and efficient antigen presentation. In contrast, an inflamed tumor microenvironment is characterized by the depletion of tolerogenic cells and the infiltration of DCs, CD8+ cells and M1-like macrophages, enabling antigen cross-presentation and cytotoxic activity. Created with biorender.

have already been described for *in situ* vaccination approaches and will be discussed in detail below. Nevertheless, triggers such as radiotherapy might also induce immunosuppressive changes in the TME (54) which has to be taken into account while developing *in situ* vaccination protocols for HCCs.

Oncolytic Viruses

Oncolytic viruses exhibit a tropism for malignant cells, selectively infecting tumor cells while sparing normal cells. They replicate inside and lyse cancerous cells, releasing TAAs in an immunogenic fashion with simultaneous release of DAMPs and pathogen associated molecular patterns (PAMPs) (53, 55). The concomitant expression of different transgenes can mediate additional immunomodulatory effects. For example, human granulocyte-macrophage colony-stimulating factor (GM-CSF) has been integrated into the viral genome to accompany viral replication with GM-CSF production to recruit APCs and promote their maturation (see also section on *Recruiting and Activating APCs*). Subsequent cross-priming of CD8+ T cells induces a cytotoxic response with ensuing systemic effects, and, ideally, accompanied by a memory response with long-lasting immunity (56). Various viral strains have been described as potential antitumor vaccines, each conferring different (side-) effects (21).

The first oncolytic virus to gain regulatory approval in the USA as well as in the European Union and Australia was talimogene laherparepvec (T-VEC), a modified herpes simplex virus (HSV) 1 expressing GM-CSF for intralesional injection of advanced malignant melanoma (57). A phase Ib/II trial combining intratumoral T-VEC with Pembrolizumab is currently investigating the injection of T-VEC into HCC and hepatic metastases (MASTERKEY-318, NCT02509507). Based on these advances, an HSV-1-based oncolytic vector (Ld0-GFP) was engineered to trigger ICD both *in vitro* and in mice models, where Ld0-GFP induced tumor eradication in over 60% of established hepatomas (58).

To date, the oncolytic pox virus vaccine JX-594 expressing the transgenes GM-CSF and β-galactosidase is the oncolytic virus with the most clinical evidence in HCC (see **Table 1**) (23). Observed effects of JX-594 application included a T cell response against vaccinia, β-galactosidase and TAAs such as MAGE-A1, MAGE-A3 and survivin in a subset of patients (53). Further, polyclonal antibody-mediated cytotoxicity was also suggested as a driver of antitumor activity (60). A disruption of tumor vasculature, mediated by a selective infection of tumor-associated vascular endothelial cells in murine tumors and human HCC, has been identified as an additional mechanism of action. As such, vaccinia exploits high cellular thymidine kinase levels to replicate, a process that is enhanced in tumor-associated vasculature *via* VEGF and other mediators (64). Encouraging clinical results were achieved with intralesional injections of JX-594 in 10 patients with advanced primary and metastatic liver tumors in a phase-I setting over a decade ago (59). A subsequent dose-finding study in subjects with advanced HCC suggested an improved OS for intravenous high-dose JX-594 application (60). However, a Phase IIb trial in sorafenib-experienced patients did not show a superior OS of the JX-594-group compared to best supportive care (53). Hoping that JX-594 therapy may induce a T-cell response that overcomes an immunosuppressive TME and increases sorafenib responsiveness, the PHOCUS phase III trial (NCT02562755) compared sorafenib treatment with vaccinia virus-based immunotherapy, followed by sorafenib. The results of an interim futility analysis, however, led to the termination of the study because it was considered unlikely that the trial would meet the primary endpoint, OS (65). A phase I/IIa trial combining JX-594 with Nivolumab as first-line treatment of advanced HCC is still ongoing (NCT03071094).

A different approach in oncolytic viruses harnesses the high telomerase activity of malignant tumors to selectively infect tumor cells. The oncolytic adenovirus variant Telomelysin successfully induced ICD, recruitment of CD8+ T cells and inhibition of intratumoral Foxp3+ lymphocyte infiltration. When combined with PD-L1 blockade, Telomelysin A caused systemic tumor regression in subcutaneous murine pancreatic and colon cancer models (66). Currently, a phase I study (NCT02293850) is recruiting patients with HCC to evaluate safety and efficacy of Telomelysin.

New virological engineering methods have yielded several novel, elegant concepts of oncolytic virus therapy (see **Table 2**). For example, engineering hybrid vectors has been proposed to circumvent distinct side effects of the individual viral strains. Thus, a recombinant virus from vesicular stomatitis virus (VSV) and Newcastle disease virus (NDV) (r-VSV-NDV) combined the rapid replication of VSV with the efficient ICD-induction of a fusogenic virus while avoiding the safety and environmental concerns associated with the parental vectors. Mice with orthotopic HCC tumors showed prolonged survival under r-VSV-NDV therapy, with an enhanced safety profile compared to the parental strains (67). Another recent development in oncolytic virus therapy not yet investigated in humans is the integration of programmable and modular synthetic gene circuits into an adenovirus vector. A hierarchical assembly method combines tumor lysis with a controlled expression of the immune effectors GM-CSF and interleukin (IL)-2, as well as single-chain variable fragments against the checkpoint inhibitors PD-1 or PD-L1. Both *in vitro* and *in vivo* xenograft models showed antitumor efficacy and HCC tumor regression. Mice treated with the synthetic oncolytic adenovirus were protected from HCC tumor rechallenge and had significantly increased intra-tumoral lymphocytes, as well as a significantly higher proportion of interferon (IFN)-γ producing and Ki67+ CD8+ T cells (74).

HMGB1 and HMGN1

The nonhistone chromatin-binding proteins HMGB1 and high-mobility group nucleosome binding domain 1 (HMGN1) are involved in the regulation of cell death and survival. In the extracellular milieu, HMGB1 and HMGN1 function as alarmins that contribute to the immunogenicity of cell death. HMGB1 is released from damaged cells due to the permeabilization of nuclear and plasma membranes and binds to receptors on immune cells such as TLR2, TLR4 and receptor for advanced glycation endproducts (RAGE) (49), while HMGN1

TABLE 1 | Clinical Trials on *in situ* vaccines in HCC/solid tumors.

Number	Type of cancer	Phase	Substance	Name	Application	Combination Therapy	Patients	Status	Oncological Findings	Immunological Findings	Year	Ref.
Oncolytic Virus												
NCT02509507	HCC, liver metastases	I/IIb	oncolytic herpes virus expressing GM-CSF	Talimogene Laherparepvec (T-VEC)	IT	Pembrolizumab IV	206	recruiting			2015	
NCT00629759	HCC, liver metastases	I	oncolytic pox virus (thymidine kinase deleted vaccinia virus) + GM-CSF	Pexastimogene Devacirepvec (Pexa-Vec, JX-594)	IT		14	completed	30% partial response, 60% stable disease, 10% progressive disease (RECIST) 80% objective response by Choi criteria	induction of white blood cells and cytokine release (IL-6, IL-10, TNF-α) development of anti-JX-594 antibodies	2006	(59)
NCT00554372	HCC	IIa	oncolytic pox virus + GM-CSF	JX-594	IT		30	completed	15% objective response, 50% intrahepatic disease control rate (mRECIST) 62% Choi response rate OS significantly higher in high-dose compared to low-dose group	induction of antitumoral immunity (*in vitro* antibody-mediated complement-dependent cytotoxicity against HCC cell lines) induction of cytotoxic T cell activity to vaccinia peptides & JX-594 transgene product	2007	(60)
NCT01171651	HCC (Sorafenib naive)	II	oncolytic pox virus + GM-CSF	JX-594	IV/IT		25	completed	significant decrease of tumor perfusion in both injected and non-injected tumors	n.a.	2010	(61)
NCT01387555	HCC (PD under Sorafenib)	IIb	oncolytic pox virus + GM-CSF	JX-594	IV/IT	Best supportive care	129	completed	no improvement of OS, response rate, time to progression compared to best supportive care alone	T cell proliferation T cell response to vaccinia peptides & TAAs	2011	(53)
NCT02562755	HCC (Sorafenib naive)	III	oncolytic pox virus + GM-CSF	JX-594	IT	Sorafenib PO	459	completed			2015	
NCT03071094	HCC	I/IIa	oncolytic pox virus + GM-CSF	JX-594	IT	Nivolumab IV	30	active, not recruiting			2017	
NCT02293850	HCC	I	oncolytic adenovirus expressing hTERT promotor	Telomelysin (OBP-301)	IT		18	recruiting			2014	
TLR agonists												
NCT02556463	solid tumor	I	TLR 7/8 agonist	MEDI9197	IT	Durvalumab IV and/or radiotherapy	53	terminated	no objective clinical response (19 and 28% disease control rates)	increased intratumoral CD8+ & PD-L1+ cells induction of type 1 and 2 IFN & TH1 response increased TLR7/8 downregulated genes	2015	(62)
NCT02668770	solid tumor	I	TLR9 agonist	Levitolimod (MGN1703)	SC/IT	Ipilimumab IV	55	active, not recruiting			2016	
Interleukins												
NCT01417546	solid tumor	I	fusion protein of IL-12	NHS-IL12	SC		83	recruiting	6% partial response, 40% stable disease, 54% progressive disease (RECIST)	IgG isotype antibodies *in vitro* induction of antibody-dependent cellular cytoxicity	2011	(63)
NCT03946800	solid tumor	I	IL-12 mRNA	MEDI1191	IT	Durvalumab IV	87	recruiting			2019	

(Continued)

TABLE 1 | Continued

Number	Type of cancer	Phase	Substance	Name	Application	Combination Therapy	Patients	Status	Oncological Findings	Immunological Findings	Year	Ref.
NCT02960594	solid tumor (high risk of relapse after curative therapy)	I	DNA-based vaccine encoding IL-12	INO-9012	IM	other DNA vaccines	93	completed			2016	

IM, intramuscular; IT, intratumoral; mRECIST, modified 'Response Evaluation Criteria in Solid Tumors'; mRNA, messenger RNA; SC, subcutaneous.
Patients included in the studies had locally advanced/metastases not suitable for resection and had progressive disease under standard therapies or contraindications, if not otherwise indicated.

predominantly binds to TLR4. Both HMGB1 and HMGN1 convey pro-inflammatory effects including DC activation, Th1 polarization and the enhancement of T cell antitumor responses (81, 82).

The prominent role of HMGB1 in this context was illustrated in murine anti-tumor vaccination models, where HMGB1 blockade abrogated therapeutic effects both *in vivo* and *in vitro* (83, 84). Because of its ability to activate DCs, synthetic HMGB1 peptides have been investigated as adjuvants to enhance the immunogenicity of vaccines, both against infectious agents and tumors (85–87). Concerns about the intratumoral application of HMGB1 in malignant tumors are based on the observation that reactive oxygen species, which are often elevated in the TME, can oxidize HMGB1 and neutralize its immunostimulatory activity (88). Furthermore, the immune checkpoint receptor T-cell immunoglobulin and mucin-domain containing-3 (TIM-3) on tumor-associated DCs was able to abrogate therapy-induced immunogenicity of cell death by interacting with HMGB1. The inhibition of uptake of nucleic acids from dying, chemotherapy-treated tumor cells into DC endosomes resulted in lower immunogenicity (89). Of note, HMGB1 expression is elevated in tumors and serum of HCC patients and its expression inversely correlates with survival (90, 91). A contribution of HMGB1 and its receptor(s) to HCC carcinogenesis has been suggested by several sources (92, 93) and *in vitro* data showed that HMGB1 enhanced the ability of proliferation, migration and invasion of HCC cells (94). So far, to our knowledge, HMGB1 has not been explored as an adjuvant for *in situ* vaccines for HCC, probably owing to its Janus face in tumorigenesis, TME immunosuppression and DC-T cell crosstalk.

In 2012, Yang et al. first reported that extracellular HMGN1 significantly contributes to T cell antitumor immunity, with a central role in antigen-specific immune responses (95). Since then, HMGN1 has been successfully explored as an HCC vaccine adjuvant, both in *ex vivo* settings (96) and in *in situ* concepts. Thus, the intratumoral delivery of HMGN1, the TLR7/8 agonist Resiquimod and checkpoint inhibitors cured established subcutaneous hepatomas and protected the mice against tumor rechallenge (see also *TLR7/8*) (77).

Bacteria and Their Products

While the dysregulation of the gut microbiota in HCC and chronic liver disease has received significant attention (97), few authors have explored bacterial immunotherapy for HCC. The most established bacterial immunotherapy for solid tumors is Bacillus Calmette-Guerin (BCG). It is routinely used intravesically in bladder cancer as an immunogenic adjuvant treatment after resection of high-grade, early-stage tumors (98, 99), while the search for targeted treatments for these tumors are still ongoing (100, 101). BCG induces multifaceted immunological effects. Multiple BCG component agonists mediate an innate response by activating TLR2, 4 and Dectin-1 and 2, host sensors on diverse immune cells including CD14+ monocytes and neutrophils (102). Pattern recognition receptors (PRR) on APCs are activated by BCG, leading to TLR activation and antigen presentation with CD4+ and CD8+ cell activation (102). Furthermore, BCG confers a direct cytotoxic effect on

TABLE 2 | *In vivo* studies on *in situ* vaccines in HCC.

Substance (Name)	Application	Tumor model	Findings		Ref.
			oncological	immunological	
Oncolytic virus					
HSV-1 based oncolytic vector (Ld0-GFP)	IT/IV	SC xenograft nude mice model (Huh7, Hep3B) syngeneic HCC mouse model orthotopic HCC mouse model (H22)	inhibited tumor growth/tumor size reduction	n.a.	(58)
VSV-NDV hybrid vector with glycoprotein exchange	IV	transgenic AST mice (liver-specific albumin promoter, loxP-flanked stop cassette, SV40 large T antigen oncogene) immune-deficient NOD-SCID mice	prolonged OS in tumor-bearing mice safe in immune-deficient mice	tumor-specific viral syncytium formation leads to tumor ICD	(67)
oncolytic adenovirus encoding TRAIL and IL-12	IV	orthotopic xenograft (Hep3B) in athymic nude mice	tumor regressions/ necrosis	apoptosis promotion, activation of caspase-3 and -8 IFN-γ upregulation NK cell and APC infiltration VEGF and CD31 (tumor microvessel) repression	(68)
Bacteria/bacterial products					
Clostridium novyi-NT spores with iron oxide nanoclusters	Rats: IT Rabbits: *via* the hepatic artery	Rats: N1-S1 inoculation Rabbits: VX2 tumor (orthotopic inoculation)	spore delivery to tumor is feasible	oncolytic activity	(69)
Chemotherapeutics					
Icaritin + Doxorubicin + Lenvantinib	IV Lenvantinib orally	hemisplenic hepatoma (Hepa1-6) mouse model	synergistic inhibition of tumor growth protection against tumor rechallenge	upregulated CD8+ and CD4+ T cells, activated DC cells and memory T cells downregulated MDSC, Treg, and M2-like macrophages	(70)
Flt3L					
radio-inducible suicide gene therapy (+CD40-L/) + Flt3-L gene therapy	IP	orthotopic hepatoma (BNL transfected with radiation-inducible promoter-controlled HSV-TK) in mice	increased OS, inhibition of tumor growth and cure protection against tumor rechallenge	upregulated activated CD8+ T cells, upregulated CD4+ T cells and NK cells Th1 polarization	(71, 72)
defective adenovirus expressing Flt3L + 5FU	Adenovirus: IT 5FU: IP	SC hepatoma (Hepa1-6) in mice	tumor growth inhibition cure of established tumors tumor-specific immunity can be adoptively transferred between animals by transfusing CD3+CD8+ T cells	elevated intratumoral DCs NK cells and lymphocytes	(73)
GM-CSF					
Adenovirus with synthetic gene circuits with GM-CSF/checkpoint blockade expression	IT	xenograft nude mice model (Huh7, HepG2) s.c. hepatoma (Hepa1-6) model	inhibited tumor growth protection against tumor rechallenge	increased IFN-γ+ and Ki-67+ cells among the tumor infiltrating CD8+ T cells	(74)
Adenovirus encoding GM-CSF/IL-12	hepatoma: IT DEN-induced tumors: *via* hepatic artery	Mouse: orthotopic hepatoma (BNL) Rat DEN model	Synergistic tumor regression	CD8+ T cells, NKT cells, and macrophages exert antitumor functions, elevated IFN-γ	(75)
TLR agonists					
TLR9 agonist + anti-PD-1/ anti-PD-L1	IP	SC and orthotopic hepatoma (Hepa1-6) in mice	Synergistic inhibition of tumor growth	TLR9 signaling promotes PD-L1 transcription	(76)

(Continued)

TABLE 2 | Continued

Substance (Name)	Application	Tumor model	Findings		Ref.
			oncological	immunological	
HMGN1 + TLR7/8 agonist (R848/resiquimod) + Anti-CTLA4/anti-PD-L1/ Cytoxan	HMGN1, R848, Anti-CTLA4, anti-PD-L1: IT Cytoxan: IP	SC hepatoma (Hepa1-6)	cured hepatomas, protection from tumor rechallenge	tumor-specific CD8+ T cells, elevated CXCL9, CXCL10, and IFN-γ expression in the tumor, tumor T cell infiltration	(77)
Interleukins					
Lipid nanoparticles delivering IL-12 mRNA	IV	*LAP-tTA/tet-O-hMYC* transgenic mice (MYC-driven HCC)	reduced tumor burden and prolonged OS	increased splenic volume and inducted IFNγ mRNA recruitment of CD44+ CD3+ CD4+ Th cells	(78)
radiation + adenoviral vector encoding IL-12	IT	SC or orthotopic hepatoma (BNL, BNL-P2)	tumor regressions and systemic effects against distant tumors	upregulated MHC class II, CD40 and CD86 on tumor-infiltrating DCs; Reduction of MDSCs and ROS Activated intratumoral CD8+ T and NK cells	(79)
Checkpoint Inhibitors					
Radiation + anti-PD-L1	Injection (not specified)	IM inoculation (HCa-1)	combination treatment significantly suppressed tumor growth, significantly improved OS	radiation upregulated tumor PD-L1 expression increasing apoptosis, decreasing tumor cell proliferation, restoration of CD8+ T cell functions	(80)

IM, intramuscular; IP, intraperitoneally; IT, intratumoral; IV, intravenous; mRNA, messenger RNA; SC, subcutaneous.
Experimental animals are wild type mice, if not otherwise indicated.

cancer cells, inducing oxidative stress and resulting in ICD, reflected in the release of HMGB1 (103). The possibility of localized intravesical administration has corroborated its role in bladder cancer, but BCG has also been investigated for the treatment of other cancer entities, with data in HCC limited to case reports, such as the successful therapy combination of BCG, IL-2 and melatonin (104).

Based on the rationale that gram-positive bacteria activate DCs *via* TLR2 signaling and that anaerobic bacteria could thrive in the hypoxic TME, bacteriolytic therapy with *Clostridium* species has been suggested as a potential inductor of tumor ICD (105). While intravenous administration causes severe side effects, rat and rabbit models confirmed that both intratumoral injection and intra-arterial transcatheter infusion of *C. novyi* into hepatic malignancies are feasible (69, 105). *In vitro* assays showed that *C.novyi*-treatment resulted in oncolysis and a significantly decreased metabolic activity of rodent HCC cell lines (69).

Radiotherapy

Radiotherapy directly induces DNA damage and is a widely used cancer treatment in both curative and palliative settings (106). While whole liver toxicity limits the application of external-beam radiotherapy for HCC treatment (107, 108), selective approaches like stereotactic body radiation therapy, radioembolization and selective internal radiation therapy constitute valid local clinical treatment options for HCC, but their efficacy is limited by extrahepatic spread and tumor manifestation outside the irradiated field (6, 109). While abscopal effects are limited to case reports in HCC (110, 111), a growing body of evidence points towards the induction of ICD and a modulation of the TME through radiotherapy (54, 106). Thus, the effects of radiotherapy have not only been linked to DNA damage, but also to TAA release, DAMP secretion, TLR4 activation on DCs and ensuing cross-presentation to CD8+ T cells (83, 112). Furthermore, an upregulation of the chemotactic C-C chemokine ligand (CCL)5 and CXC-ligand (CXCL)16 pathway and an increased infiltration of CD8+ T cells and Natural Killer (NK) cells into the tumor were observed in HCC patients undergoing Yttrium-90 radioembolization, along with an increase of APCs and CD4+ and CD8+ cells in peripheral blood (113). However, radiotherapy also confers subsequent dosage- and fractionation-dependent immunosuppressive effects on the TME (54). This includes recruitment of Tregs to the TME and a "M2-like polarization" of TAMs (54), as well as an increased tumor PD-L1 expression and a heightened fraction of exhausted PD-1+/TIM-3+ CD8+ T cells (80, 113). In this regard, the combination with systemic immunomodulators and checkpoint inhibitors is a pervasive strategy to re-establish immunosurveillance (54) that so far has only been explored preclinically (80) and in small nonrandomized settings with encouraging results (114, 115). Murine colon carcinoma tumor models showed that low-dose radiotherapy-mediated tumor PD-L1 expression is induced by CD8+ T cell IFNγ signaling and peaks at 72 hours after treatment. Here, combination treatment with checkpoint inhibitors (anti-PD-1 or anti-PD-L1, respectively) was most effective when administered

concomitantly (116). Several *in situ* vaccine regimens have harnessed radiotherapy as an inducer of ICD in HCC, including promising combinations of radiotherapy with IL-12 (see also section on *Optimizing Cross-Presentation and T Cell Priming*) (79).

Chemotherapy and Transarterial Chemoembolization (TACE)

Several chemotherapeutic agents are more effective in immunocompetent hosts because they induce ICD and favorably modulate the TME (117, 118). While many pathway inhibitors and chemotherapy regimens do not confer a survival benefit in HCC and negatively impact liver function in chronic liver disease while conveying considerable side effects (107, 119–122), exploring chemotherapeutic agents as triggers of ICD may require dosage adaption and addition of immunomodulators (123). Therefore, chemotherapeutics without a positive clinical effect in conventional HCC therapy may still be implemented for *in situ* vaccine concepts to, firstly, trigger ICD and, secondly, to modulate the TME. Furthermore, the local application of chemotherapy in combination with embolizing agents – transarterial chemoembolization (TACE) – has emerged as a selective and valid treatment option (124).

Several sources have confirmed that chemotherapy agents can induce ICD in cancer cells. *In vitro* experiments showed that anthracyclines promote ICD in tumor cells by inducing the translocation of calreticulin, HSP70 and 90 to the cell surface and promoting HMGB1 release (125). Their stimulation of TLR3 results in a rapid type I IFN production, with subsequent CXCL10 release (126). Doxorubicin, widely implemented in TACE, induced ICD in HCC cell lines, however, with a weak effect on immune cells (70, 127). This effect was augmented by adding the mitophagy-inducing drug icatirin, which resulted in protection from tumor rechallenge. Synergistic effects of icatirin and doxorubicin furthermore included a remodeling of the TME, with an upregulation of CD8+ and CD4+ T cells, memory T cells and activated DCs, while the numbers of MDSCs, Tregs, and M2-polarized macrophages decreased (70). The cytokine profile showed decreased levels of CCL2, TGFβ, IL-4, IL-6, IL-10 and increased levels of IFNγ, tumor necrosis factor (TNF)-α, and IL-12, with the latter a potent inducer of a T helper (Th)-1 phenotype (70, 128). Similarly, oxaliplatin, also clinically used for TACE, has been shown to promote ICD *in vitro* and to induce DC maturation as well as increase CD8+ T cells in an HCC inoculation mouse model (129). A recent study has linked therapeutic resistance to oxaliplatin-based TACE to the density of infiltrating TAMs, since HCC cells co-cultured with macrophages showed higher oxaliplatin-resistance *in vitro*. Furthermore, HuH7 xenografts co-implantated with THP-1 derived macrophages responded significantly less to oxaliplatin treatment in a murine tumor model (130).

Modulating the TME can contribute to the success of *in situ* vaccination of solid tumors, and several chemotherapeutic agents are able to restore an efficient antitumor response by depleting or changing immunosuppressive cell populations. An early report showed that low-dose cyclophosphamide selectively depleted CD4+CD25+ Tregs, restoring peripheral T cell proliferation and NK cell killing activities (131). Additionally, cyclophosphamide-induced ICD expanded the cDC1 compartment and facilitated cross-priming of T cells (132). At the same time, cyclophosphamide was also reported to expand CD11b+ Ly6C^{hi} CCR2^{hi} MDSCs that inhibited long-term tumor control in a murine lymphoma model through the PD-1-PD-L1 axis (133).

Depletion of MDSCs has been attributed to several chemotherapeutics, including doxorubicin (134), cisplatin (135) and oxaliplatin (136). Oxaliplatin treatment also increased intratumoral T-cell infiltration (including Tregs) in mice (137), while other studies suggested that platinum-based therapies promote TAMs by enhancing M2 polarization (138).

A serious immunological concern regarding systemic chemotherapy is systemic immune suppression because of myelo-and lymphopenia, especially when dosage approaches the maximum tolerated dose (139). However, in clinical reality, routine regimens usually employ significantly lower doses and do not impair systemic vaccination immune responses, as demonstrated by Wumkes et al. in cohorts of chemotherapy-treated patients with solid tumors who had adequate responses to influenza vaccination (140).

Several chemotherapeutic agents have already been harnessed to improve the efficacy of immune checkpoint inhibitors in other tumor entities. Cisplatin was able to sensitize triple negative breast cancer to PD-1 blockade (141), while 5-fluorouracil plus oxaliplatin (FOLFOX) combined with checkpoint blockade showed strong synergistic effects, because FOLFOX induced PD-1+ cytotoxic T cell infiltration (142). In a syngeneic HCC mouse model, the combination of oxaliplatin and anti-PD-1 antibodies inhibited tumor growth better than the respective monotherapies (129).

Probably due to the minor role of systemic chemotherapy in HCC treatment, only few studies have explored chemotherapy within *in situ* vaccination models. Intratumoral application of an adenovirus expressing Fms-like tyrosine kinase 3 ligand (Flt3L) together with 5-fluorouracil in a murine hepatoma model induced complete remission of established tumors (see **Table 2**) (73).

Sorafenib

Besides exerting anti-proliferative and anti-angiogenic effects by inhibiting VEGFR, PDGFR and RAF (143), the multikinase inhibitor sorafenib can also induce autophagy-mediated ICD. As such, sorafenib mediates ferroptosis, a regulated form of ICD that results from a decreased antioxidant capacity, coupled with iron overload and massive lipid peroxidation (144). Sorafenib-induced ferroptosis was shown to be accompanied by a HMGB1 release with subsequent inflammation (145), underlining the potential of Sorafenib-induced cell death in *in situ* vaccine concepts.

At the same time, dose-dependent effects of Sorafenib on antitumor immunity have been noted, with high-dose Sorafenib reported to increase the proportion of PD-1 expressing CD8+ T cells and resulting in less intratumoral T cell infiltration in a woodchuck hepatitis virus-induced HCC model (146). In vitro,

subclinical Sorafenib doses selectively increased CD4+ CD25- effector T cell activation and blocked Treg function in PBMCs from HCC patients (147). This concept has been applied to a murine adoptive T cell therapy, where low-dose Sorafenib both enhanced function and migration of transferred CD8+ T cells and decreased the number of MDSCs and Tregs in the TME (148).

Recruiting and Activating APCs

Flt3L

Flt3 is essential to the regulation of homeostatic DC development in the bone marrow and lymphoid organs and the upkeep of sufficient numbers of peripheral DCs (149–151). Administration of recombinant Flt3L leads to an additional mobilization from the macrophage DC progenitor compartment (149), an effect that has been confirmed in both healthy volunteers and cancer patients (152–154). Furthermore, Flt3L injection combined with polyinosinic:polycytidylic acid (polyIC), a TLR3 agonist, induced the expansion and activation of CD103+ DC progenitors (cDC1) in a murine melanoma model, leading to an increased sensitivity to checkpoint blockade (155).

Oncolytic viruses expressing Flt3L have been investigated in an animal model of *in situ* vaccination (71). Kawashita et al. demonstrated that radio-inducible suicide gene therapy, using a cytotoxic expression vector of herpes simplex virus thymidine kinase controlled by a radiation-inducible promoter, was significantly enhanced in its efficacy by addition of a recombinant adenovirus expressing human Flt3 ligand (Adeno-Flt3L) in a hepatoma mouse tumor model. Adeno-Flt3L led to a Th1-polarized immune response with activation of cytotoxic CD8 + T cells. Additional boosting of the antitumor response was achieved with the addition of Adeno-CD40L to enhance DC maturation, with mice that had cleared the tumor being protected from subsequent tumor rechallenge (71). Clinically, Flt3L- based *in situ* vaccines have been investigated in several malignancies, such as colon carcinoma and indolent non-Hodgkin lymphoma (iNHL) (84, 156). Thus, an *in situ* vaccine regimen consisting of Flt3L, radiotherapy and a TLR3 agonist induced systemic CD8+ T cell antitumor responses in a mouse model of iNHL and renewed the susceptibility to checkpoint blockade. Furthermore, a clinical trial exploring this *in situ* vaccination regimen (NCT01976585) reported durable clinical remissions in patients with iNHL. Immunological effects of this combination included the induction of TAA-laden, cross-presenting DCs and tumor infiltration of activated CD8+ T cells with upregulated PD-1 expression, which were responsive to anti-PD1 targeting (84).

Flt3L application dramatically expands cDC and pDC populations in peripheral lymphoid organs such as the liver. In a mouse model, Flt3L-induced DC expansion enhanced fibrosis regression in a matrix metalloproteinase (MMP)-9-dependent manner, implying its potential benefits even in cases of chronic injury and fibrotic remodeling (157). Therefore, Flt3L for HCC therapy may offer the opportunity to harness intrinsically elevated DAMPs to then induce the maturation of the recruited DC populations. Potentially, this may abrogate the need for DC-directed adjuvants, warranting the exploration of Flt3L in HCC.

GM-CSF

GM-CSF is a cytokine driving the differentiation, proliferation and activation of macrophages and DCs, with a polarization towards cDC1 and Th1 responses (158). The intra-tumoral application of GM-CSF has been validated in several solid tumors as a technique to attract and stimulate DCs. The systemic application is associated with considerable toxicities, and several trials have demonstrated the feasibility of intralesional injection in solid tumors such as malignant melanoma (159). A large trial in over 800 patients with resected malignant melanoma reported that GM-CSF monotherapy failed to confer clinical benefits in the adjuvant setting and did not enhance the response to an antitumor vaccine (160). Accordingly, the intralesional application of GM-CSF encoding agents has gained increased interest. The oncolytic pox virus vaccine JX-594 with the transgenes GM-CSF has been investigated for HCC with heterogeneous results (discussed in 3.1) (60). Another concept is the intra-tumoral injection of combination treatments with a GM-CSF and IL-12 encoding adenovirus. Here, GM-CSF monotherapy did not show significant therapeutic effects but was able to augment the efficacy of the IL-12 agonist. While IL-12 monotherapy only induced antitumoral NK cells, the addition of intratumoral GM-CSF succeeded in recruiting activated CD8+ T cells, NKT cells, and macrophages and achieved a higher rate of tumor regressions (see also *IL-12*) (75).

GM-CSF has also been implicated in HCC carcinogenesis, with an immunosuppressive effect on the TME. Accordingly, HCC patients presented with elevated GM-CSF levels in comparison to healthy controls (161). Ilkovitch et al. showed that GM-CSF injection in healthy adults leads to an expansion of MDSCs in the liver, effecting a heightened PD-L1 expression on Kupffer cells and an impaired IFN-γ production by activated T cells (162). In mice orthotopically implanted with Hepa1-6 cells, GM-CSF expression by tumor cells led to an infiltration with MDSCs, while neutralization of GM-CSF and IL-6 abrogated HCC progression in this model, with decreased MDSC and TAM infiltration (161). Though the effect of GM-CSF may be dependent on its spatiotemporal distribution in the TME, the observed effects may pose a potential pitfall of GM-CSF application in vaccine concepts.

Alarmins for DC Recruitment and Activation

Adjuvants to enhance DC immunogenicity hold promise to attract DCs to the tumor, augment antigen presentation, and polarize the ensuing response towards Th1 and cytotoxic T cells. A major group of agents harnessed to this aim are alarmins – endogenous intercellular signals that activate defense mechanisms and provoke an immune response via, amongst others, chemokine receptors (CCR) or TLRs (163). Besides their manifold influences on the innate immune response, some alarmins confer distinct effects on DC recruitment and maturation. As a consequence, DCs mature, upregulating CCR7, a process that facilitates their interaction with CCL19 and CCL21 and thus enables them to home to local lymph nodes (164). Some of the following chemotactic mediators and

alarmins have been used individually, while others are integrated in multimodal *in situ* vaccination concepts.

When examining alarmins in the context of HCC and chronic liver disease, it should be noted that many of these pathways are severely dysregulated in this setting. Along other mechanisms of chronic inflammation, an increased gut permeability with translocation of intestinal bacterial components (PAMPs) typically causes a chronic TLR4-mediated inflammatory response and contributes to hepatocarcinogenesis (165). As several immunostimulatory agents proposed as adjuvants for *in situ* vaccines overlap with the preexisting chronic liver inflammation and with tumor-promoting pathways, a careful examination of these pathways is warranted in the context of HCC.

TLR3

Agonists of the TLR3 receptor include double-stranded RNA and single-stranded viral RNA with incomplete stem structures (166). TLR3 is highly expressed in the endosomal compartment of cDC1, and its stimulation induces cytokine and chemokine production, DC activation and maturation *via* the TLR3/TICAM-1 pathway and antigen cross-presentation (167, 168).

Modulation of the TME has been described as a potential effect of TLR3 signaling. Injection of polyIC, a dsRNA analog, resulted in a change of macrophage populations, converting "tumor-supporting macrophages" to "tumor suppressors". The latter were characterized by M1-like polarization, TNF-α production and tumoricidal properties (169). However, polyIC is a ligand for multiple other PRRs besides TLR3, including protein kinase R, retinoic acid-inducible gene-I (RIG-I) and melanoma differentiation-associated gene 5 (MDA5), leading to severe systemic side effects (168, 170). TLR3 stimulation with attenuated systemic cytokine production was achieved using various other substances, including synthetic dsRNA derivatives (170) or dsRNA coupled to nanoparticle-based delivery systems (171). The former led to a Th1 polarization, reflected in elevated IL-12 production and CD8+ T-cell priming (172).

TLR3 receptor expression has been associated with improved patient survival in HCC and linked to chemokine-mediated intratumoral lymphocyte infiltration (173). In this line, loss-of-function polymorphisms of TLR3 were highly prevalent in HCC-bearing populations in comparison to controls (174). Moreover, a recent study by Bonnin and Fares et al. found that downregulation of TLR3 mediates resistance to apoptosis in HCC cells and is a potent escape mechanism. Interestingly, transgenic mice with an absence of TLR3 expression exhibited accelerated hepatocarcinogenesis without an altered tumor immune infiltrate (175).

While, to the best of our knowledge, no clinical trial currently investigates TLR3 agonists for HCC therapy, preliminary data from the NCT01976585 trial, an *in situ* vaccine approach including polyICLC (HiltonolTM) combined with checkpoint blockade in patients with indolent non-Hodgkin lymphomas showed encouraging response rates (84). Ongoing trials investigate the application of TLR3 agonists in other malignancies, among others, in advanced colorectal cancer in combination with pembrolizumab (NCT04119830), in malignant melanoma (NCT04093323) and in the neoadjuvant setting in malignant pleural mesothelioma (NCT04345705) (176).

TLR4

TLR4 is a receptor with a wide range of activating agents, including HMGB1, LPS, HSP60 and 70, that confers a broad variety of effects (177). While systemic LPS administration causes severe side effects, intra-tumoral applications have been suggested previously (178). Several studies reported that TLR4 agonists have been successfully harnessed as adjuvants in several models of other tumor entities like malignant melanoma and clinically, in BCG immunotherapy (179, 180). While *in vitro* activation of the surface TLRs 1/2 and 4 and the endosomal TLRs 3 and 9 has a similar activating effect on splenic DCs, *in vivo* data showed that stimulation of the surface TLRs 1/2 and 4 suppressed CD8+ T cell responses (181). Furthermore, TLR2 and TLR4 signaling increased the fraction of CD11c+ cDC2, which were defective in priming CD8+ T cells, and elevated IL-10 secretion and PD-L1 and PD-L2 expression on DCs (181, 182). An appealing explanation for this observation is that because endosomal TLRs are activated in viral infection, they promote cross-presentation, while this mechanism is not necessary in most bacterial infections, sensed by the surface TLRs 1/2 and 4 (181).

Similarly, LPS stimulation in the liver activated cDC2, the most prevalent DC subset in the liver, with ensuing IL10 secretion and almost no increase of proinflammatory cytokines. As a result, an increased production of Tregs from naive CD4+ cells and a promotion of a Th2 responses was reported (183). Tregs were also recruited *via* CXCL10/CXCR3 and TLR4 signaling in a rodent liver transplantation model, promoting HCC recurrence after ischemia-reperfusion injury (184). Furthermore, TLR4 signaling has been linked to HCC invasion, multidrug resistance, tumor angiogenesis and metastases, and TLR4 antagonists suggested as therapeutic modalities for HCC (185–187). To our knowledge, the role of *in situ* vaccine concepts with TLR4 agonists has not yet been clinically explored in HCC (188).

TLR9

Endosomal CpG motifs are recognized by TLR9, and the receptor can be targeted with nucleotides or nucleotide derivatives (188, 189). As a result, antigen-presenting cells are activated and CD8+ T cells differentiate into a terminal state of CD127lowKLRG1high effector cells with initial antitumor efficacy, but a limited lifespan (190). The latter observation may partially explain initially promising, but short-lasting clinical antitumor effects of TLR9 agonists (190).

A downregulation of TLR9 due to the single nucleotide polymorphism of the TLR9 promoter -1486T/C has been previously implied in impaired innate immunity (191), and also recently been associated with an increased risk of HCC recurrence after liver transplantation (192). At the same time, activated TLR9 signaling in tumor cells not only falls short of inducing an antitumor immune response, but even facilitates HCC survival. A synergy of HMGB1 and TLR9 was shown to up-regulate mitochondrial biogenesis of HCC cell lines and in murine HCC models under hypoxic conditions, promoting tumor survival and proliferation (193).

Several clinical trials investigating TLR9-agonist therapy reported negative results in small-cell lung cancer and in metastatic head and neck squamous cell carcinoma (194, 195).

Subsequent murine studies showed additive treatment effects of a TLR9 agonist in combination with anti-PD-1 or anti-PD-L1 therapy in hepatoma cell lines and HCC (see **Table 2**) (76, 190). Of note, TLR9 agonism enhanced PD-L1 expression *via* PARP1 and STAT3, facilitating immune escape in the absence of checkpoint inhibition, but leading to synergistic effects in combination treatment (76). Moreover, in murine HCC models of anti-PD-1 nonresponders, TLR9 agonists were able to achieve durable remissions with systemic antitumor effects. CD8+ T cell proliferation with the generation of CD127highKLRG1low long-lived memory precursors and infiltration and the presence of IFN-γ and TNF-α signaling were observed after the combination of TLR9 agonist and checkpoint inhibition (190). Clinical studies of checkpoint inhibition combined with TLR9 agonists are underway for other cancer entities like malignant melanoma and B cell lymphoma (NCT02668770, NCT02254772).

A virus-like particle encapsulating a CpG-A TLR9 agonist (CMP-001) has recently been reported to cause tumor regression in syngeneic hepa1–6 mouse models of HCC, with a greater antitumor activity of CMP-001 monotherapy than that of sorafenib or PD-L1 blockade (196). While, to our knowledge, no clinical study is currently accruing patients for CMP-001 treatment in HCC, encouraging clinical data has been recently reported in malignant melanoma. As such, CMP-001 reversed PD-1 blockade resistance patients with progressive disease, resulting in an overall response rate of 23.5% (NCT02680184) (197), while the treatment combination of CMP-001 and Nivolumab (anti-PD-1) yielded an encouraging pathological response rate of 70% in the neoadjuvant setting in advanced melanoma (NCT03618641). This study observed an increased intra-tumoral infiltration of CD8+ T cells and CD303+ pDCs as well as elevated numbers of circulating activated PD1+/Ki67+ CD8+ T cells in patients with favorable response (198).

TLR7/8

The small molecules Imiquimod (TLR7 agonist) and resiquimod (TLR7/8 agonist) are widely recognized topical drugs applied for benign and malignant epithelial tumors (199) and cutaneous hematological malignancies (200). TLR7/8 stimulation results in an expansion of effector T cells, as well as an activation of DCs and NK cells (200). In preclinical HCC models, TLR7/TLR8 stimulation led to the maturation of DCs and to the promotion of IFNI/IL12-mediated activation of NK cells. Thus, the cytolytic activity of NK cells against HCC cells was significantly augmented *in vitro* and in HepG2 xenograft-bearing nude mice in the presence of monocyte-derived DCs (201). In a murine Hepa1-6 hepatoma model, a regimen consisting of HMGN1, resiquimod and a checkpoint inhibitor resulted in the elimination of established tumors and protected the mice against tumor rechallenge. The authors noted increased Hepa1-6-specific cytotoxic CD8+ T cells, CXCL9, CXCL10, and IFN-γ upregulation as well as an increased tumoral infiltration of T cells (77).

A recently published study investigated the combination of the TLR7 and 8 agonist MEDI9197 with PD-L1 inhibition with or without radiation therapy in various solid tumors, including one patient with HCC. While this regimen resulted in systemic and intratumoral immune activation with a Th1 and type 1 IFN gene expression signatures, intratumoral CD8+ T cell infiltration and tumor PD-L1 expression, none of the 52 included patients showed an objective response to treatment. Furthermore, while the use for superficial lesions was feasible, adverse effects were frequent when MEDI9197 was injected in visceral or deep-seated lesions, including death from hemorrhagic shock after injection into a liver metastasis (62).

Optimizing Cross-Presentation and T Cell Priming

HSPs

HSPs are a family of proteins classified by molecular weight that chaperone the folding and translocation of proteins under cellular stressors such as infection, inflammation, toxins and hypoxia (202). The signaling effects of HSPs are highly dependent on its localization and binding partners. While high levels of intracellular membrane-associated Hsp70 in cancer cells are anti-apoptotic, extracellular soluble Hsp70 can trigger innate and adaptive immune responses. The ability of HSP to chaperone TAAs and facilitate their uptake by APCs with subsequently endorsed cross-presentation is central to their immunogenic effects. Furthermore, HSPs recruit leukocytes, polarize Th cell responses towards Th1 cells, activate NK cells as well as induce the maturation of DCs (163, 203). While reliable evidence that tumor-derived HSP-peptide complexes are able to enhance cross-presentation of TAAs has been brought forward by several studies, the exploration of their immunogenic effects may be warranted to boost *in situ* vaccination strategies.

IL-12

IL-12 is a potent regulator of adaptive T cell responses that activates cytotoxic T and NK cells, downregulates Th2 responses and induces a polarization towards Th1 responses (128, 204). Furthermore, IL-12 modulated the TME by converting monocytes into tumoricidal "M1-like" macrophages that inhibit HCC growth *in vitro* and in xenograft mouse models (205). While elevated IL-12 levels in HCC patients were associated with favorable clinical outcomes, the systemic application of IL-12 incurred dose-limiting toxicities, directing research efforts towards more sophisticated IL-12 delivery systems (78, 206). Delivering IL-12 *via* a messenger RNA (mRNA) lipid nanoparticle resulted in a reduced tumor burden in MYC-oncogene driven murine HCC. An increased infiltration of activated CD44+CD3+CD4+ Th cells into the tumor and an increased IFNγ production were observed in this model (78). An oncolytic adenovirus encoding human tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) and IL-12 genes showed antitumor efficacy *in vitro* and in a murine xenograft model, with ensuing IFN-γ production and infiltration of NK cells and APCs. Furthermore, the combination led to a remodeling of the tumor microvasculature, with a repressed VEGF production, a decreased CD31 expression and reduced microvessel density (68). The adenovirus-mediated gene transfer of IL-12 and GM-CSF showed synergistic effects in orthotopic murine liver tumors and chemically induced multifocal liver tumors. Tumor regressions and a boost of IFN-γ signaling, as

well as an enrichment for CD8+ T cells, NKT cells and macrophages in the TME was reported (75).

Several clinical studies are currently investigating IL-12 therapy for solid tumors, including the application of an anti-DNA antibody-based fusion protein of IL-12 (NCT01417546), mRNA encoding for IL-12 and checkpoint blockade (NCT03946800), as well as an IL-12 DNA therapy combined with hTERT (NCT02960594) (see **Table 1**).

IL-2

Over 20 years ago, the systemic application of IL-2 was reported to achieve treatment responses in patients with metastatic renal cell carcinoma and malignant melanoma (207, 208). Since then, IL-2 has gained considerable attention for its potential to recruit and activate cytotoxic CD8 T cells and NK cells, to cause T cell proliferation and to induce polarization of the TME towards a Th1response. At the same time IL-2 activates and stimulates the proliferation of immunosuppressive Tregs *via* their CD25 receptor (209). Recently, an engineered IL-2 variant with abolished CD25 binding was reported to keep up its effects on CD8 T cells and NK cells, while evading the stimulatory impact on Tregs (210).

Several studies have suggested a protective effect of IL-2 against HCC development and recurrence. The high expression level genotype +114 TT was associated with a lower risk of HCC development in a hepatitis B positive cohort, while high peritumoral IL-2 levels were associated with a lower risk of tumor recurrence (211, 212). An ultra-low dose regimen of systemic IL-2 showed a moderate treatment efficacy in patients with advanced HCC, with an overall response rate of 16% (213). Severe dose-limiting toxicities (e.g. vascular leak syndrome) of systemic IL-2 therapies have prompted the investigation of intra-tumoral and vehicle-driven applications of IL-2 (214). The combination of radiotherapy with the intra-tumoral application of an adenovector encoding IL-12 showed significant tumor regressions with abscopal effects in both subcutaneous and orthotopic hepatoma models. The combination treatment resulted in a reduction of MDSCs, increased functionally activated CD8+ T cells in tumor tissues and enhanced DC maturation (79).

Ensuring Anti-Tumor Efficacy

Inhibition of Immune Checkpoints

Checkpoint inhibitors have substantially shaped the therapy of many malignancies in advanced disease stages, such as malignant melanoma, mismatch repair-deficient colorectal carcinoma and non-small cell lung cancer (215–217). Tumors responsive to checkpoint inhibition have in common a high tumor mutational burden, which directly implicates a high neoantigen burden with immunogenic effects on DCs and T cells (218).

In 2017 and 2018, the FDA granted accelerated approval for Nivolumab and Pembrolizumab in HCC, based on data from the CheckMate 040 and Keynote 224 trials, respectively. Both showed similar response rates of 15-20% (14, 219–221). A more recent development was the approval of the combination of atezolizumab (anti-PD-L1 antibody) and bevacizumab (anti-VEGF antibody) as first-line therapy for patients with unresectable or metastatic HCC, due to its superior efficacy compared to sorafenib in a phase III clinical trial (12).

The observation that only a subset of patients exhibits durable tumor responses to checkpoint inhibition therapy can be explained with the concepts of "cold" and "hot tumors". "Hot tumors" are characterized by a pre-existing adaptive immune response with CD8+ T cell infiltration, IFN-γ signaling and efficient presentation of tumor antigens. Checkpoint blockade then activates this pre-existing response. Thus, the clinicopathological features of low tumor T cell infiltration, low PD-1 T cell and PD-L1 expression, insufficient neoantigens and low mutational burden as well as the absence of IFN-γ signaling have been linked to a primary resistance to checkpoint inhibition (222). The response to anti-PD1 and anti-CD137 therapy has also been clearly linked to the presence of cross-priming cDC1 (223).

A genomic profiling study from the Barcelona working group noted that approximately 27% of HCCs have a high infiltration of immune cells with respective PD-1 and PD-L1 expression and active IFN-γ signaling (224). The majority of patients in this group showed an active adaptive T-cell response, while the remaining three-quarters of HCC patients did not exhibit positivity for markers predictive of successful checkpoint inhibitor response (224), corroborating the observation from clinical studies, where the response rate of HCC patients to checkpoint inhibition was about 15-20% (13, 14). As such, there is an urgent need to find immunomodulatory treatment options for the remaining majority of patients. Several *in situ* vaccination regimens of *in vivo* HCC models have reported additive effects with checkpoint blockade, e.g. for radiotherapy (80), TLR7/8 agonists (77) and TLR9 agonists (190).

The TME clearly contributes to evasion from checkpoint blockade; for example, TAMs are capable of capturing monoclonal antibodies directed against PD-1 by engaging with the Fc domain, terminating their activating effect on T cells (225). Increased numbers and activity of Tregs can further contribute to an insufficient checkpoint blockade by direct or indirect (production of the anti-inflammatory cytokines IL10 and TGF-β) mechanisms of T cell inhibition (226). In this regard, immunomodulation by *in situ* vaccines is a promising strategy to modulate the TME prior to checkpoint therapy.

PERSPECTIVES AND PITFALLS

The primary aim of cancer immunotherapy is to elicit a lasting, durable antitumor immunity based on an effective CD8+ T cell response. Because they harness the entire breadth of TAAs and direct the subsequent immune response, *in situ* vaccines are a highly individual therapy that ideally employs a standardized approach (21). In HCC, there are several disease-specific characteristics that each constitute significant challenges for therapy. These include an elevated risk of recurrence after surgical or locoregional therapy, impaired liver function, chronic hepatic injury and risk of carcinogenesis (227). These specific challenges warrant an intense immunological investigation with a potential to implement *in situ* vaccines here. HCCs typically arise in a fibrous environment and show prominent neovascularization, with a malformed vasculature that inhibits CD8+ T cell infiltration and hampers CD8+

effector functions (228). The underlying liver fibrosis may further impair trafficking of immune cells with impaired antigen recognition due to fibrovascular remodeling (229). Given the clinical efficacy of the Atezolizumab plus Bevacizumab combination and the prominent role of angiogenesis in HCC biology, the exploration of VEGF inhibition to normalize the tumor vasculature may be also warranted for HCC *in situ* vaccination concepts (12, 230). Another challenge in orchestrating a hepatic antitumor response may lie in the inherently tolerogenic direction of hepatic immune responses and hepatic DCs, especially (183). This may be further aggravated by the fact that antigen presentation can also be performed by numerous other hepatic cell types, including liver sinusoidal endothelial cells, hepatocytes, macrophages and Kupffer cells, and contributes to immune tolerance after antigen presentation (231, 232).

As the induction of ICD alone mostly fails to create an effective antitumor response due to insufficient antigen or danger signal release (233), the development of higher-order combination protocols to ensure additional recruitment and activation of APCs as well as the overcoming of the immunosuppressive TME represents the key to success (35). The downside of these approaches might be a more frequent occurrence of immune-related adverse events (irAEs) (234). Although the underlying mechanisms are still not completely understood, the activation of tissue-resident cytotoxic T cells, increased cytokine levels and the formation of auto-antibodies most likely contribute to impaired self-tolerance (235). Since most HCC patients already suffer from chronic inflammatory conditions of the liver, the appearance of liver auto-antigens and activation of CD8+ T cells due to *in situ* vaccination may trigger hepatic irAEs. Although clinical data are still sparse, it has been indicated that HCC patients treated with immune checkpoint inhibitors show higher proportions of hepatic irAEs compared to other treated tumor patients (236). Currently, only limited experimental and clinical evidence is available for *in situ* vaccination in HCC, and the results from upcoming clinical trials are eagerly awaited.

The advent of immunotherapy in multiple solid tumors including HCC has prompted the development of new therapeutic combinations that modulate the TME and the systemic antitumor response. Besides exploring new strategies to optimize the efficacy of standard immunotherapies, it is essential to find approaches that target and guide all essential steps of antitumor immunization. *In situ* vaccines may provide an opportunity to elicit lasting responses against HCC and to overcome the TME.

AUTHOR CONTRIBUTIONS

Conceptualization: LH. Investigation: IL, WW, and LH. Writing — Original draft preparation: IL and WW. Writing — Review and Editing: RM, CR, FT, and LH. Supervision: LH. Funding Acquisition: FT. All authors contributed to the article and approved the submitted version.

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