Journal for ImmunoTherapy of Cancer

MAF1 inhibits hepatocarcinogenesis by fostering an immunostimulatory tumor microenvironment

Di Cao,[1,2] Yue-Ning Wang,[1] Chao-Yue Sun,[3] Haojiang Li,[1] Ge Ren,[4] Yu-Feng Zhou,[1] Mei-Yin Zhang,[1] Shuo-Cheng Wang,[1] Shi-Juan Mai ,[1] Hui-Yun Wang [1]

To cite: Cao D, Wang Y-N, Sun C-Y, *et al*. MAF1 inhibits hepatocarcinogenesis by fostering an immunostimulatory tumor microenvironment. *Journal for ImmunoTherapy of Cancer* 2025;**13**:e009656. doi:10.1136/jitc-2024-009656

► Additional supplemental material is published online only. To view, please visit the journal online (https://doi.org/10.1136/jitc-2024-009656).

DC and Y-NW contributed equally.

Accepted 06 December 2024

[1]State Key Laboratory of Oncology in South China, and Guangdong Provincial Clinical Research Center for Cancer, Sun Yat-sen University Cancer Center, Guangzhou, China
[2]Department of Radiology, Sun Yat-Sen University Cancer Center, Guangzhou, China
[3]College of Biological and Pharmaceutical Engineering, West Anhui University, Lu'an, China
[4]Department of Health Technology and Informatics, Hong Kong Polytechnic University University Learning Hub, Kowloon, Hong Kong

Correspondence to
Dr Hui-Yun Wang;
wanghyun@mail.sysu.edu.cn

Dr Shi-Juan Mai;
maishj@sysucc.org.cn

ABSTRACT

Background The biological significance of MAF1, a tumor suppressor, in carcinogenesis and immune response of hepatocellular carcinoma (HCC) remains unreported. Understanding the underlying mechanisms by which MAF1 enhances anti-tumor immunity in HCC is crucial for developing novel immunotherapy strategies and enhancing clinical responses to treatment for patients with HCC.

Methods Mice were subjected to hydrodynamic tail vein injections of transposon vectors to overexpress AKT/NRas, or c-Myc, with or without wild-type (WT) or mutant-activated (–4A) MAF1, or short-hairpin MAF1 (shMAF1). Liver tissues and tumors were harvested and analyzed using histology, immunohistochemistry, immunoblotting, quantitative reverse-transcription PCR, and flow cytometry. MAF1 was overexpressed or knocked down in HCC cells via lentiviral transfection. Cell lines were analyzed using RNA sequencing, immunoblotting, dual luciferase reporter, and chromatin precipitation assays.

Results Both MAF1-WT and MAF1-4A proteins significantly inhibit hepatocarcinogenesis in mice, with the mutant form exhibiting a stronger suppressive effect. Although MAF1 knockdown alone does not induce abnormalities in the mouse liver, it accelerates c-Myc-induced carcinogenesis. Our results provide the first in vivo evidence that MAF1 plays a tumor suppressor role by activating PTEN to suppress the AKT-mammalian target of rapamycin signaling pathway during hepatocarcinogenesis in physiologically relevant tumor models. More importantly, we found that MAF1 not only enhances the intratumoral infiltration of $CD8^+$ T cells by increasing CXCL10 secretion but also enhances their functional activity by inhibiting PDL1 transcription in mouse liver cancer, which were confirmed in human HCC or in vitro experiments. Furthermore, PDL1 overexpression accelerates mouse hepatocarcinogenesis by antagonizing the tumor-suppressive role of MAF1.

Conclusions Our study uncovers a novel anti-tumor immunity of MAF1 in hepatocarcinogenesis and human HCC. These findings suggest that the stimulated MAF1 could potentially improve immunotherapy in combination with immune checkpoint inhibitors in HCC patients, especially in those with an absence of T cells in HCC tissues.

WHAT IS ALREADY KNOWN ON THIS TOPIC

⇒ Immune checkpoint inhibitors (ICIs) have now been widely applied in cancer therapy. However, only approximately 20% of patients benefit from this immunotherapy, the main reasons for which are the absence of T cells in cancer tissues and the presence of an inhibitory microenvironment.

WHAT THIS STUDY ADDS

⇒ Wild-type and mutant-activated MAF1s significantly inhibit hepatocarcinogenesis in mice, with the mutant form exhibiting a stronger suppressive effect.

⇒ MAF1 expression increases the intratumoral infiltration of $CD8^+$ T cells by elevating CXCL10 secretion and enhances their functional activity by inhibiting PDL1 transcription in mouse liver cancer.

⇒ PDL1 overexpression accelerates hepatocarcinogenesis by counteracting MAF1's tumor-suppressive role.

HOW THIS STUDY MIGHT AFFECT RESEARCH, PRACTICE OR POLICY

⇒ MAF1 may potentially improve immunotherapy in combination with ICIs in hepatocellular carcinoma patients, especially in those with an absence of T cells in HCC tissues.

INTRODUCTION

Hepatocellular carcinoma (HCC) is the third leading cause of cancer death worldwide and has a dismal survival rate.[1] Although HCC patients in the early stage (accounting for less than 30% of all HCC patients) have a favorable outcome after treatment with liver resection, transplantation, and image-guided ablation,[2] most individuals with advanced HCC have a very poor survival rate due to the lack of surgical options and are not sensitive to chemotherapy and radiotherapy. Currently, advanced HCC patients primarily receive targeted therapy involving molecularly targeted drugs (MTD), such as receptor tyrosine kinases (RTKs) inhibitors like sorafenib[3 4] and regorafenib,[5] and/or immunotherapy using immune checkpoint inhibitors (ICIs) like anti-bodies for PD1, its ligand PDL1, and CTLA-4. However, the clinical outcomes with RTK inhibitors or ICIs have been less than desirable so far.[3 6] Therefore, it is of paramount importance to unravel the

molecular mechanisms of HCC to develop novel targeted therapeutic drugs, immunotherapy, and more effective strategies to enhance the survival of HCC patients.[7]

MAF1 is recognized as a key repressor of polymerase III (Pol III)-dependent transcription when cells are affected by nutrient deprivation, drug treatments, and environmental stresses.[8 9] Growing evidence has shown that mTORC1 can directly phosphorylate MAF1, controlling its subcellular localization and transcriptional activity.[10–12] Moreover, MAF1 can inhibit the transcription of lipogenic genes such as FASN and ACC1 via repression of RNA Pol II, thereby regulating lipid metabolism.[13] We recently demonstrated that MAF1 expression is markedly reduced in HCC, negatively correlating with disease progression and prognosis in HCC patients.[14] Furthermore, we found that MAF1 suppresses the growth of both HCC cells and xenograft tumors by binding to the PTEN promoter, enhancing PTEN promoter acetylation and activity, which resulted in the downregulation of AKT-mammalian target of rapamycin (mTOR) activity.[14] Although MAF1 is known as a tumor suppressor and plays an important role in many cancers, its involvement in carcinogenesis has not been demonstrated.

Reports have shown that reduced nuclear MAF1 leads to an increase in RNA Pol III-dependent transcriptions, which are acquired by dendritic cells to stimulate T cell functions.[15] Wu J. *et al* reported that reduced nuclear MAF1 increased the expression of RNA5SP transcripts, which are associated with hepatic IFN production and inflammation in alcohol-associated hepatitis.[16] These data suggest that MAF1 is a potential factor in controlling immunity and inflammation in pathological situations. The complex regulation, activity, and biological consequences of MAF1 in different cell types can vary, which needs further investigation. Currently, few reports demonstrate the involvement of MAF1 in tumor immunity.

In this study, we first investigated the role of Maf1 in hepatocarcinogenesis driven by the AKT/NRas or c-Myc gene in mice delivered by hydrodynamic injection (HDI). We then found that overexpression of MAF1 increased the infiltration and activation of $CD8^+$ T cells in liver tumors in the mouse model. Further in vitro experiments indicated that MAF1 expression stimulated the migration and activation of $CD8^+$ T cells by enhancing CXCL10 expression and directly suppressing the transcription of PDL1 in HCC cells, respectively. Our study provides the first in vivo evidence that MAF1 inhibits carcinogenesis by activating anti-tumor T cell response through enhancement of CXCL10 secretion and inhibition of PDL1 transcription.

METHODS

Plasmids and reagents

The plasmids used in this study, including pT3-EF1a, pT3-EF1a-AKT, pT3-EF1a-NRas, pT3-EF1a-c-Myc, pT3-N90-beta-catenin, and phosphorylated cytomegalovirus–sleeping beauty (pCMV-SB), were described previously.[17] Additionally, pT3-EF1a-Maf1-WT, pT3-EF1a-Maf1-4A, pT3-EF1a-shMaf1, and pT3-EF1a-PDL1 were cloned using a standard molecular cloning approach.

Experiments in mouse hepatocarcinogenesis model by hydrodynamic injection

Wild-type (WT) FVB/N (RRID: MGI:3528175) mice were purchased from Charles River Laboratories (Beijing, China). The mouse hepatocarcinogenesis model driven by oncogenes was established with plasmids delivered through the tail vein by HDI according to the protocols described previously.[18–20] To determine the role of Maf1 in hepatocarcinogenesis, high doses of Maf1-WT (40 µg) or Maf1-4A (40 µg) with low doses of AKT (4 µg), NRas (4 µg), and pCMV-SB (1.92 g) were injected into mice; or pT3-EF1a-shMaf1 (40 µg) with pT3-EF1a-c-Myc (10 µg) and pCMV-SB (2 µg) were injected into mice via the tail vein. To explore the role of PDL1 in hepatocarcinogenesis, pT3-EF1a-PDL1 was also injected into the mice along with either AKT/NRas/Maf1-WT or AKT/NRas/Maf1-4A. Mice injected with pT3-EF1a were used as controls. Mice were housed and monitored according to protocols approved by the Institutional Animal Care and Use Committee of Sun Yat-Sen University.

Hepatocellular carcinoma (HCC) patient samples and data

Tumor tissues were collected from 98 patients with HCC who underwent hepatic resection at the Sun Yat-Sen University Cancer Center from 2004 to 2012 for immunohistochemical analyses. This study was approved by the ethics committees of Sun Yat-Sen University Cancer Center. HCC transcriptome data, derived from 374 human HCC tumors in The Cancer Genome Atlas (TCGA) database, were downloaded from the UCSC Xena website (https://xena.ucsc.edu) (UCSC Xen, RRID: SCR_018938). This data was used to analyze the correlation between Maf1 mRNA and CD8A, tumor necrosis factor (TNF), Gamma interferon (IFNG), Granzyme B (GZMB) mRNA, and Th1/IFNγ signature (online supplemental table S1).

Cell culture, plasmids, and construction of stable cell lines

We confirm that all cell lines used in this study have been authenticated using short tandem repeat (STR) profiling to ensure their identity and purity. The authentication process was conducted by an accredited facility, and the results were regularly monitored to guarantee the reliability of our experiments. Hep3B (RRID: CVCL_0326), Huh7 (RRID: CVCL_0336), and HepG2 (RRID: CVCL_0027) cell lines were maintained in high-glucose DMEM (Invitrogen, USA) supplemented with 10% fetal bovine serum (FBS) (Gibco, Australia). SNU449 (RRID: CVCL_0454) cell line was cultured in RPMI 1640 Medium (Invitrogen, USA) plus 10% FBS. Cells were incubated at 37°C in a humidified chamber containing 5% CO2. Lentiviral plasmid pCDHs, carrying full-length sequences of MAF1-WT, MAF1-4A, or short hairpin RNAs (shRNAs) targeting MAF1 (sequences are listed in online supplemental table S3), were purchased

from Shanghai GenePharma. After a 48-hour infection period with lentivirus and 5 mg/mL polybrene, stable cells with expression of MAF1s or shRNA were selected with 4 µg/mL puromycin for 5 days. Following selection, the cells were cultured in a medium containing 2 µg/mL puromycin. Small interference RNA (siRNA) targeting PDL1 (sequences are listed in online supplemental table S3) and control siRNAs were purchased from Guangzhou RiboBio (www.ribobio.com). These were transfected into HepG2 cells using Lipofectamine™ 3000 (Invitrogen, USA) according to the manufacturer's protocol.

Statistical analysis

Statistical analysis was performed by SPSS (V.11.0; SPSS, Inc., Chicago, IL; RRID: SCR_002865) and GraphPad Prism software (V.9.0; GraphPad Prism Inc., San Diego, CA; RRID: SCR_002798). The measurement data are expressed as mean±SD. Comparisons of averages of two groups were performed with the two-tailed unpaired t-test. Comparisons of averages among three or more groups were performed with analysis of variance. Unpaired t-test with Welch correction, paired t-test, Mann-Whitney test, χ^2 test, or Fisher's exact test was applied as necessary. Survival analyses were conducted using the Kaplan-Meier method and the log-rank test. The correlation between MAF1 expression and CD8A, TNF, IFNG, and GZMB mRNA or Th1/IFNγ gene signature was analyzed using Pearson's correlation test. $p<0.05$ was considered statistically significant (indicated in corresponding figures with one asterisk for $p<0.05$, two asterisks for $p<0.01$, three asterisks for $p<0.001$, and four asterisks for $p<0.0001$).

RESULTS

Maf1 inhibits AKT/NRas-induced mouse hepatocarcinogenesis via suppressing AKT/mammalian target of rapamycin (mTOR) signaling pathway

MAF1 plays a key role as a transcription repressor in the nucleus and is regulated or inhibited by mTOR through phosphorylation. For example, when we treated HCC cells with rapamycin (an mTOR inhibitor), MAF1 proteins translocated from the cytoplasm to the nucleus (online supplemental figure S1A). Studies have shown that four conserved amino acid residues in MAF1 (online supplemental figure S1B) can be phosphorylated by mTOR.[21] To better understand the relationship between the function and phosphorylation of MAF1, we constructed a mutated form of MAF1 in which the four conserved residues were mutated from phosphorylatable residues (serine and threonine) to non-phosphorylatable alanine (MAF1-4A) (online supplemental figure S1C). In vitro experiments demonstrated that AKT expression (online supplemental figure S1D) or TSC1 deletion (online supplemental figure S1E), which activated mTOR kinase, significantly induced translocation of MAF1-WT proteins from nucleus to cytoplasm, but not for MAF1-4A proteins. These results suggest that mTOR-mediated phosphorylation/dephosphorylation can modulate MAF1's function and translocation between cytoplasm and nucleus in HCC cells.

To understand the expression level of endogenous Maf1 proteins in several mouse liver tumor models using relevant oncogenes delivered by HDI, we detected Maf1 expression. The results showed that Maf1 is generally downregulated in HCC tissues in these mouse models (online supplemental figure S2).

Then, we further investigated the role of wild type (Maf1-WT) and mutated-type (Maf1-4A) in a mouse hepatocarcinogenesis model driven by AKT/NRas. In this model, Maf1s were delivered by HDI (figure 1A). As expected, we found that all mice injected with AKT/NRas (control mice) died within 7 weeks due to a lethal liver tumor burden, while mice co-injected with AKT/NRas and Maf1-WT (Maf1-WT mice) died within 30 weeks, and mice with AKT/NRas and Maf1-4A (Maf1-4A mice) survived for more than 35 weeks (figure 1B). At the 7th week, no liver tumor was observed in the mice expressing Maf1-WT or Maf1-4A, in contrast to the large liver tumors present in all control mice. By the 30th week, all Maf1-WT mice had died from lethal liver tumors, whereas Maf1-4A mice only developed small liver tumor nodules (figure 1C,D first row). Under the microscope, typical liver tumors with hematoxylin and eosin (H&E) staining, noticeable cytoplasmic fat droplets with oil red staining, and brown-black PCNA by immunohistochemistry (IHC) were observed in the control mice at the seventh-week post-HDI. In contrast, minimal changes were observed in both Maf1-WT and Maf1-4A mice at the same time point (figure 1D). By the 30th week, noticeable liver tumors were watched in all Maf1-WT mice, while only small HCC nodules with moderate staining for steatosis and PCNA were observed in Maf1-4A mice (figure 1D). In the Maf1-WT mice, a higher degree of mixed hepatocellular/cholangiocellular carcinoma was observed under microscope. Furthermore, we also investigated the role of Maf1-WT and Maf1-4A in a mouse hepatocarcinogenesis model driven by c-Myc/CTNNB1 (online supplemental figure S3A); the results showed that Maf1 can also significantly attenuate the development of c-Myc/CTNNB1-driven liver tumors (online supplemental figure S3B–D). These results clearly demonstrate that Maf1 is a powerful tumor suppressor in mouse hepatocarcinogenesis, which is consistent with our previous findings from human HCC tissues and in vitro and in vivo experiments.[14] Noticeably, the anti-tumor role of Maf1-4A is much stronger than that of Maf1-WT in the mouse models.

We previously demonstrated that MAF1 suppresses human HCC by activating PTEN to inhibit the AKT-mTOR signaling pathway.[14] Therefore, we tested the mechanism in this mouse hepatocarcinogenesis model. As shown by western blot analysis (figure 1E), high levels of Maf1 and PTEN proteins were observed in WT and mutated Maf1 mice, while the control mice had much lower levels of PTEN and undetectable Maf1 in the 7th week. Furthermore, p-AKT and p-RPS6 levels were very low or nearly undetectable in Maf1-WT and Maf1-4A mice

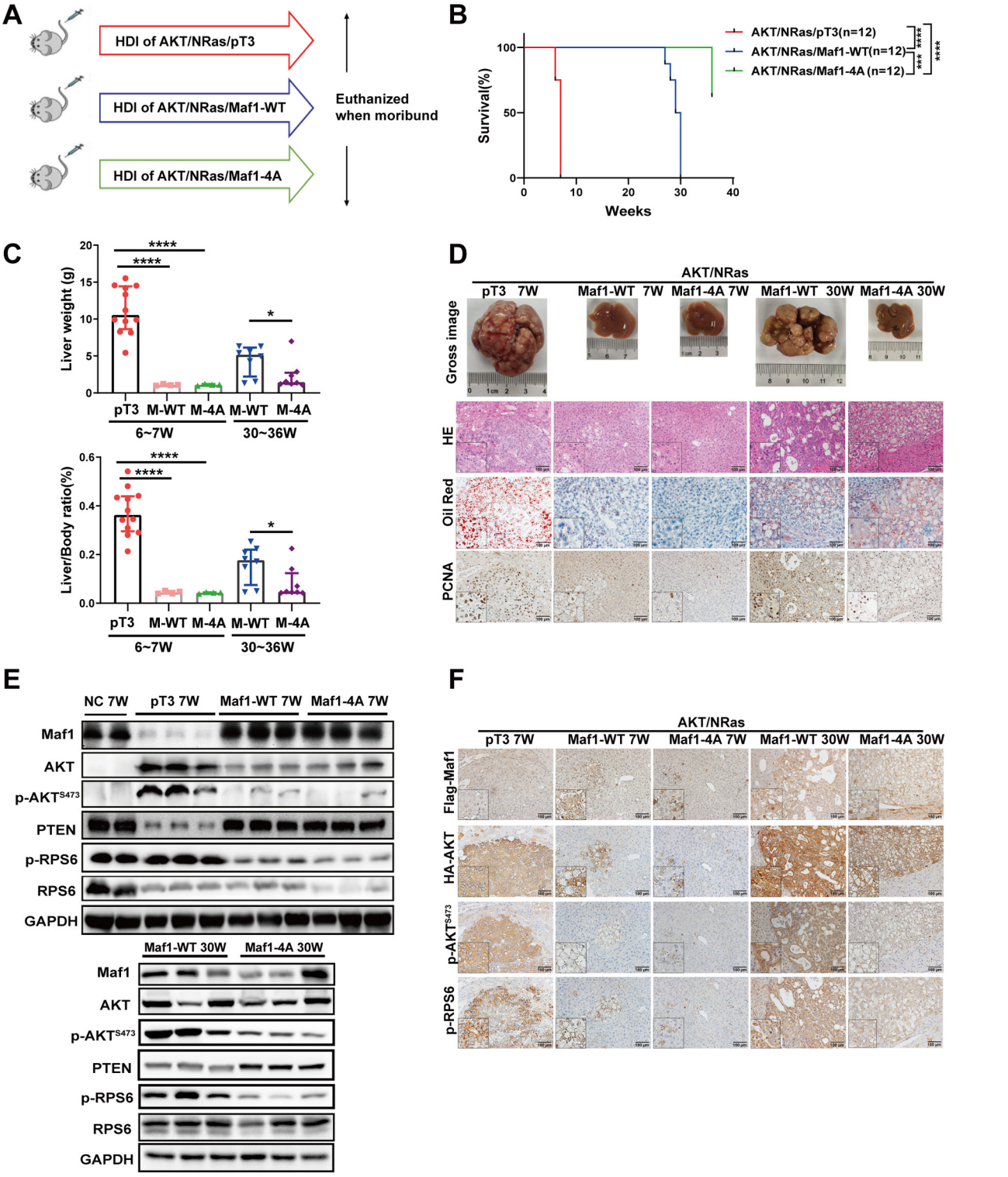

Figure 1 Maf1-WT and Maf1-4A effectively inhibit AKT/NRas-induced hepatocarcinogenesis. (A) Animal study design. (B) Survival curves of AKT/NRas/pT3, AKT/NRas/Maf1-WT, and AKT/NRas/Maf1-4A mice. (C) Histograms of liver weight and liver-to-body weight ratio of different groups at various times. (D) Mouse liver and liver cancer gross images, H&E staining, oil red staining, and immunohistochemical staining of PCNA at the 7th and 30th weeks. (E) Western blot analysis of Maf1, AKT, p-AKT, PTEN. pRPS6, and RPS6 proteins extracted from mouse HCC tissues at 7th week (up panel) and 30th week (low panel). GAPDH was used as a loading control. (F) Immunohistochemical staining of Flag-Maf1, HA-AKT, p-AKT, and p-RPS6 in mouse HCCs. Scale bar: 100 µm (200×); Inset images: 400×. *, p<0.05; ***, p<0.001****; p<0.0001. GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HDI, hydrodynamic injection; H&E, hematoxylin and eosin staining; HCC, hepatocellular carcinoma; NC, mice without any treatment; WT, wild type.

compared with the control mice (figure 1E). By the 30th week, phosphorylated AKT and RPS6 could be observed in both Maf1-WT and Maf1-4A mice, but their levels were markedly weaker in Maf1-4A mice compared with Maf1-WT mice (figure 1E). Correspondingly, the PTEN levels were higher in Maf1-4A mice than in Maf1-WT mice (figure 1E). These results demonstrate that Maf1 indeed activates PTEN, thereby leading to feedback inhibition of AKT/mTOR signaling pathway. Furthermore, immunohistochemical staining (figure 1F) revealed that Maf1 was mainly observed in the nuclei of hepatocytes of Maf1-4A mice and located in both nuclei and cytoplasm of hyperplasic foci of Maf1-WT mice at the 7th week. Similarly, the expression levels of total and p-AKT, and p-RPS6 in the cytoplasm (figure 1F) were consistent with the results obtained from western blot analysis on the control mice and Maf1-WT and Maf1-4A mice at the 7th and 30th weeks, respectively. In summary, these findings suggest that non-phosphorylated Maf1 is the active form of Maf1 that activates PTEN to significantly attenuate the development of AKT/NRas-driven liver tumors by inhibiting the AKT/mTOR signaling pathway.

Knockdown of Maf1 accelerates c-Myc-induced hepatocarcinogenesis by activating the AKT/mammalian target of rapamycin (mTOR) signaling pathway

Subsequently, we sought to investigate whether knockdown of Maf1 alone can induce hepatocarcinogenesis in mice. To do this, we first tested the efficiency of three siRNAs against Maf1 (shMaf1) in Hepa 1–6 cells, a mouse hepatoma cell line, and found that siMaf1-3 had the best knockdown effect (online supplemental figure S4). Then, we synthesized shMaf1 based on the siMaf1-3 sequence. With this shMaf1, we tried to figure out whether Maf1 downregulation could induce hepatoma in the mouse (online supplemental figure S5A). However, the knockdown of Maf1 alone did not cause any tumor development in the mouse liver up to 40 weeks post-injection (online supplemental figure S5B–D). IHC analysis showed that Maf1 downregulation could elevate the expressions of PCNA (online supplemental figure S5D), p-AKT, and p-S6 (online supplemental figure S5E) in the hepatocytes of shMaf1 mice compared with control mice. These results suggested that the loss of Maf1 alone is not sufficient to drive hepatocarcinogenesis but can activate the AKT/mTOR signaling pathway.

Recently, a report indicated that mTORC1 activation is essential for c-Myc-driven hepatocarcinogenesis in mice.[22] Thus, we investigated whether Maf1 knockdown can accelerate the development of c-Myc-induced liver tumors in mice (figure 2A). Not unexpectedly, the survival of experimental mice co-injected with c-Myc and shMaf1 was significantly shorter than that of control mice injected with c-Myc alone (figure 2B), and all experimental mice developed lethal liver tumors and died within 15 weeks, while only four out of eight control mice produced smaller and fewer liver tumors during the same period (figure 2C,D). The liver neoplastic lesions in both experimental and control mice were confirmed by H&E staining, and notable lipid droplets and dark-brown PCNA in the experimental mice were revealed by oil red staining and IHC staining (figure 2D), respectively. This indicates that Maf1 downregulation accelerates aberrant lipid metabolism and cell proliferation in mouse HCC. IHC also showed that Maf1 downregulation markedly elevated the expression of p-AKT and p-RPS6 in the liver tumors of experimental mice compared with those in control mice (figure 2E). Furthermore, western blot confirmed these results obtained from IHC analysis and also demonstrated that Maf1 downregulation resulted in PTEN reduction in experimental mice (figure 2F). Overall, these findings indicate that knockdown of Maf1 accelerates c-Myc-induced hepatocarcinogenesis in mice by downregulation of PTEN to activate the AKT/mTOR signaling pathway.

Maf1 increases intratumoral infiltration of $CD8^+$ T cells and activates the function of $CD8^+$ T cells in mouse liver cancer

At present, immunoreaction is widely recognized as a key factor in the development and progression of cancers. Some reports have shown that Maf1 is involved in organismal immunity.[15 16] However, there have been no reports on the role of Maf1 in tumor immunity. Therefore, we aim to evaluate the effect of Maf1 on the immunoreaction in HCC. To achieve this, we first ectopically expressed MAF1 in Hep3B cells (online supplemental figure S6A), an HCC cell line with relatively low MAF1 level, and treated with or without IFNγ, and then examined the RNA expression profile using RNA sequencing (RNA-seq). Gene set enrichment analysis on the RNA-seq data suggested that MAF1 expression significantly modulated the leukocyte transendothelial migration signaling pathway and T cell receptor signaling pathway without IFNγ stimulation (online supplemental figure S6B) and PDL1 expression and PD1 checkpoint pathway in cancer and T cell receptor signaling pathway with IFNγ stimulation (online supplemental figure S6C), implying that MAF1 may regulate the immune response in HCC.

To confirm that Maf1 regulates immune response in HCC, we collected liver cancer tissues from the aforementioned mouse HCC model and analyzed the relationship between Maf1 expression and tumor-infiltrating immune cells using flow cytometry. The representative polychromatic dot plots showing the gating strategy used to identify immune cell content in mouse liver are shown in online supplemental figure S7. The result showed that Maf1 expression notably increased the ratios of $CD8^+$ T cells among $CD45^+$ T cells infiltrated in HCC tissues in AKT/NRas/Maf1-WT or AKT/NRas/Maf1-4A mice compared with control mice (figure 3A). Conversely, knockdown of Maf1 resulted in lower infiltration of $CD8^+$ T cells in shMaf1 mice compared with control mice (figure 3B). To directly visualize the relationship, we performed IHC on mouse liver samples and found that Maf1-WT or Maf1-4A overexpression increased the number of infiltrated $CD8^+$ T cells (figure 3C), while knockdown of Maf1 decreased

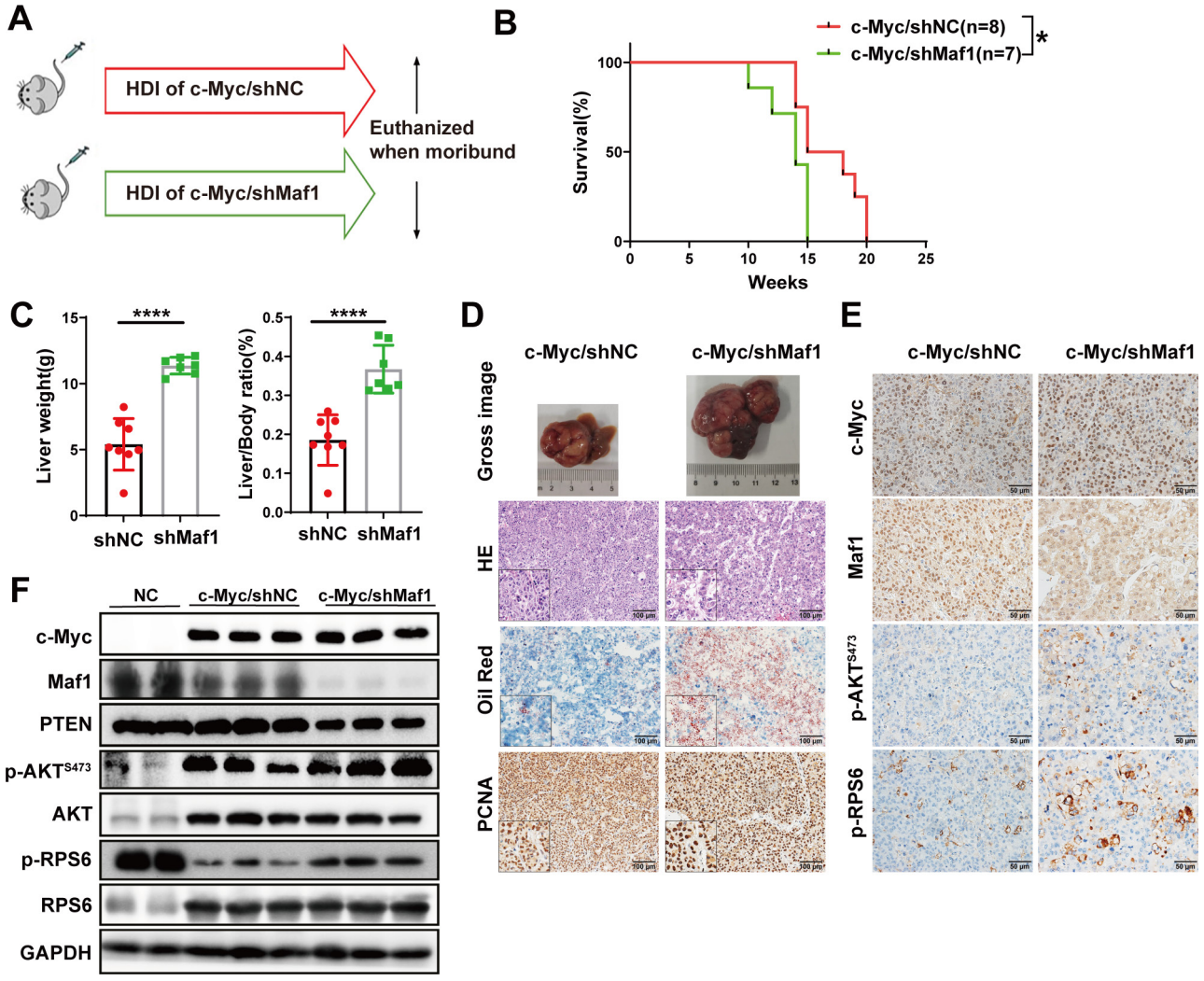

Figure 2 Knockdown of Maf1 accelerates c-Myc-induced hepatocarcinogenesis. (A) Animal study design. (B) Survival curves of c-Myc/shNC and c-Myc/shMaf1 mice. (C) Histograms of liver weights and liver-to-body weight ratios of the two mouse groups. (D) Mouse liver cancer gross images, H&E staining, oil red staining, and immunohistochemical staining of PCNA. Scale bar: 100 µm (200×). Inset images: 400×. (E) Immunohistochemical staining of c-Myc, Maf1, p-AKT^{S473}, and p-RPS6 in mouse HCCs. Scale bar: 50 µm (400×). (F) Western blot analysis of c-Myc, Maf1, PTEN, p-AKT, AKT, p-RPS6, and RPS6 extracted from mouse HCC tissues. *, p<0.05; ****, p<0.0001. NC, mice without any treatment; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; H&E, hematoxylin and eosin staining; HCC, hepatocellular carcinoma; HDI, hydrodynamic injection.

the number of infiltrated CD8$^+$ T cells (figure 3D) in the liver tumors. Besides, we also found that Maf1 overexpression enhances CD45$^+$ cells, CD4$^+$ T cells and NK1.1$^+$ T cells infiltration in mouse liver cancers (online supplemental figure S8A–C). These results demonstrate that the expression of Maf1, especially Maf1-4A, attracts CD8$^+$ T cell infiltration in HCC. Then, we wondered how MAF1 induces the infiltration of CD8$^+$ T cells in HCC. Chemokines such as CXCL10, CXCL9, CCL5, CCL4, and CCL3 are critical factors for the chemotaxis of T cells. Therefore, we detected the expression of these chemokines in mouse liver tumors with quantitative reverse-transcription-PCR (qRT-PCR). We found that overexpression of Maf1-WT or Maf1-4A could increase the mRNA expression of CXCL10, CXCL9, CCL5, and CCL4 (online supplemental figure S9A), while Maf1 knockdown decreased the mRNA expression of CXCL10, CXCL9, and CCL5 (online supplemental figure S9B). These results indicate that Maf1, especially Maf1-4A, may attract T cell infiltration via enhancing the expression of chemokines CXCL10, CXCL9, and CCL5 in mouse liver cancer.

Subsequently, we explored whether Maf1 affects the immune functions of T cells infiltrated in liver cancer. IFNγ is considered a marker of activated CD8$^+$ cytolytic T cells,[23] and PD1 is a marker of immune-suppressed CD8$^+$ T cells. Therefore, we detected the two markers in CD8$^+$ T cells with flow cytometry. We observed that overexpression of Maf1 increased percentages of CD8$^+$IFNγ$^+$ and decreased percentages of CD8$^+$PD1$^+$ cells in CD8$^+$ T cells in Maf1-WT and Maf1-4A mice compared with control mice (figure 3E,F). Conversely, knockdown of Maf1 resulted in lower percentages of CD8$^+$IFNγ$^+$ and higher percentages of CD8$^+$PD1$^+$ cells in c-Myc/shMaf1 mice compared with control mice (online supplemental figure S10A,B).

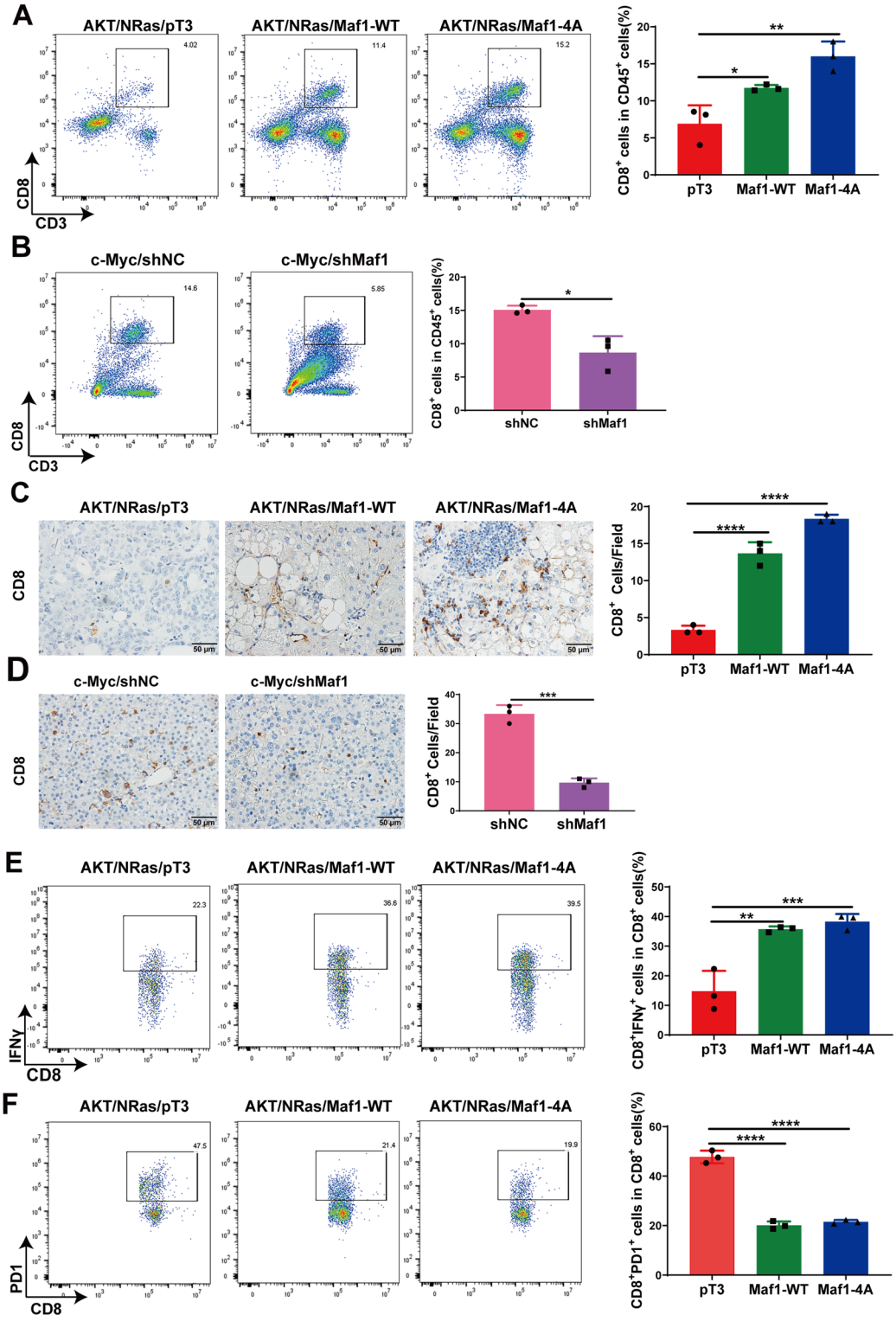

Figure 3 MAF1 overexpression enhances CD8$^+$ T cell infiltration and activates CD8$^+$ T cell functions in mouse liver cancers. (A–B) Representative flow cytometry dot plots of CD8$^+$ T cell in CD45$^+$ cells isolated from mouse liver cancers and histograms of percentages of CD8$^+$ cells in different groups (n=3 for each group). (C–D) Representative pictures of CD8 immunohistochemical staining in mouse liver cancers and histograms of the number of CD8$^+$ T cell in the liver cancers of different groups. The number of CD8$^+$ T cells was counted for five randomly selected fields of digital images for each sample (n=3 for each group). (E) Representative flow cytometry dot plots of IFNγ$^+$CD8$^+$ T cells in CD8$^+$ T cells and histogram of percentages of IFNγ$^+$CD8$^+$ T cells in liver cancers of different groups. (F) Representative flow cytometry dot plots of PD1$^+$CD8$^+$ T cells in CD8$^+$ T cells and histogram of percentages of PD1$^+$CD8$^+$ T cells in liver cancers of different groups. The data are presented as the mean±SD of triplicate determinations. *, p<0.05; **, p<0.01; ***, p<0.001; ****, p<0.0001.

Further animal experiments demonstrated that the tumor-suppressive effect of MAF1 in AKT/NRas-induced liver tumors could be counteracted by CD8 depletion (online supplemental figure S11A–C). Fluorescence-activating cell sorter (FACS) analysis confirmed the effective depletion of $CD8^+$ T cells following anti-CD8 anti-body treatment in the mice (online supplemental figure S11D). These results suggest that Maf1 expression can activate the anti-tumor immunoreaction by increasing the intratumoral infiltration of $CD8^+$ T cells and activating $CD8^+$ T cells, thereby impeding HCC development and progression in mice.

MAF1 expression is positively associated with T cell infiltration in human hepatocellular carcinoma (HCC) tissues

To validate the relationship between MAF1 expression and $CD8^+$ T cells in human HCCs, we analyzed the gene expression dataset from 374 HCC patients in the TCGA-LIHC database. Although the correlation coefficient is slightly marginal, the results demonstrated a positive association between MAF1 expression and the expression of CD8A (a marker of cytotoxic T lymphocytes), TNF, IFNG, as well as GZMB (markers of $CD8^+$ T cell cytolytic activity) in human HCC tissues (figure 4A, online supplemental figure S12). More importantly, MAF1 expression in HCC tissues was positively correlated with most genes of the Th1/IFNγ gene signature (figure 4B and online supplemental table S1), whose primary function is to stimulate the anti-tumor immune response. To confirm the relationship between MAF1 expression and $CD8^+$ T cell infiltration, we examined the protein expressions of MAF1 (figure 4C) and CD8 (figure 4D) using IHC in a cohort of 98 human HCC tissues. We divided the 98 patients into high- and low-MAF1 groups according to their MAF1 expression levels. The high-MAF1 group showed significantly higher infiltration of $CD8^+$ T cells than the low MAF1 group ($p<0.05$) (figure 4E). These results are consistent with those obtained from the mouse HCC model. The findings indicate that MAF1 expression is positively associated with $CD8^+$ lymphocyte infiltration in human HCC tissues, thereby enhancing anti-tumor immunoactivity.

MAF1 expression in hepatocellular carcinoma (HCC) cells induces migration and activation of $CD8^+$ T cells in vitro

To further confirm the role of MAF1 expressed by HCC cells in the regulation of the immune activity of T cells in vitro, we first co-cultured human T cells in the inserts and HCC cells in the well-bottoms using a membrane transwell system (online supplemental figure S13A). Human $CD8^+$ T cell migration rates were determined by FACS assay at the 18th hour after co-culture. The results indicated that MAF1 overexpression in Hep3B cells (online supplemental figure S13B) significantly increased the migration rate of $CD8^+$ T cells, with MAF1-4A showing a stronger effect than MAF1-WT (online supplemental figure S13C); conversely, MAF1 knockdown in HepG2 cells (online supplemental figure S13B) notably decreased $CD8^+$ T cell migration (online supplemental figure S13C). This finding demonstrates that MAF1 expression in HCC cells can indeed induce the migration (chemotaxis) of $CD8^+$ T cells.

Next, we aimed to determine which chemokine(s) mediates the chemotaxis of T cells induced by MAF1. Quantitative RT-PCR analysis showed that only high expression of CXCL10 was detected in Hep3B cells with MAF1 overexpression (online supplemental figure S13D), while the expressions of CXCL9, CCL5, CCL4, and CCL3 were nearly undetectable in the HCC cells (data not shown). When MAF1 was knocked down in HepG2 cells, CXCL10 expression significantly decreased in the HCC cells (online supplemental figure S13D). Furthermore, an ELISA confirmed a higher protein concentration of CXCL10 in the culture supernatant of Hep3B cells expressing MAF1-WT or MAF1-4A compared with that in the supernatant of the control cells and a lower concentration of the protein in the supernatant of HepG2 cells with MAF1 knockdown compared with the control cells (online supplemental figure S13E). Furthermore, we also observed the effect that the cultured medium for MAF1-overexpression cells recruited more T cells compared with that for control cells could be counteracted by an anti-CXCL10 neutralizing anti-body (online supplemental figure S13F). In addition, the effect of cultured medium for MAF1-knockdown cells on T cell recruitment could be rescued by overexpression of CXCL10 (online supplemental figure S13F). These results demonstrated that MAF1, especially MAF1-4A, could enhance CXCL10 expression in HCC cells, thereby stimulating the chemotaxis and infiltration of T cells into HCC tissues.

Finally, we aimed to confirm that MAF1 can activate T cell functions using an in vitro experimental approach. To this end, we co-cultured human T cells with HepG2 cells ($HLA\text{-}A2^+$, online supplemental figure S14) and then collected the T cells to analyze the levels of IFNγ using a FACS assay. The results showed that the co-culture with HepG2 cells expressing MAF1-WT or MAF1-4A significantly increased the ratio of $CD8^+/IFN\gamma^+$ T cells compared with control cells ($p<0.05$) (figure 5A). In contrast, co-culture with HepG2 cells expressing shMAF1 resulted in lower ratio of $CD8^+/IFN\gamma^+$ T cells compared with control cells ($p<0.05$) (figure 5B). Furthermore, after co-cultured with HCC cells that overexpressed MAF1 or MAF1-4A in the co-culture system, T cells exhibited significantly stronger killing power against HCC cells (figure 5C); conversely, after co-cultured with HCC cells that had MAF1 knockdown, T cells demonstrated significantly weaker killing power against HCC cells (figure 5D), suggesting that MAF1 expression in HCC cells can enhance the tumor-cell-killing power of human T cells in the microenvironment. These findings demonstrated that the expression of MAF1, especially MAF1-4A, in HCC cells can increase the chemotaxis and activation of $CD8^+$ T cells in vitro, consistent with the results obtained from mouse models and human HCC tissues.

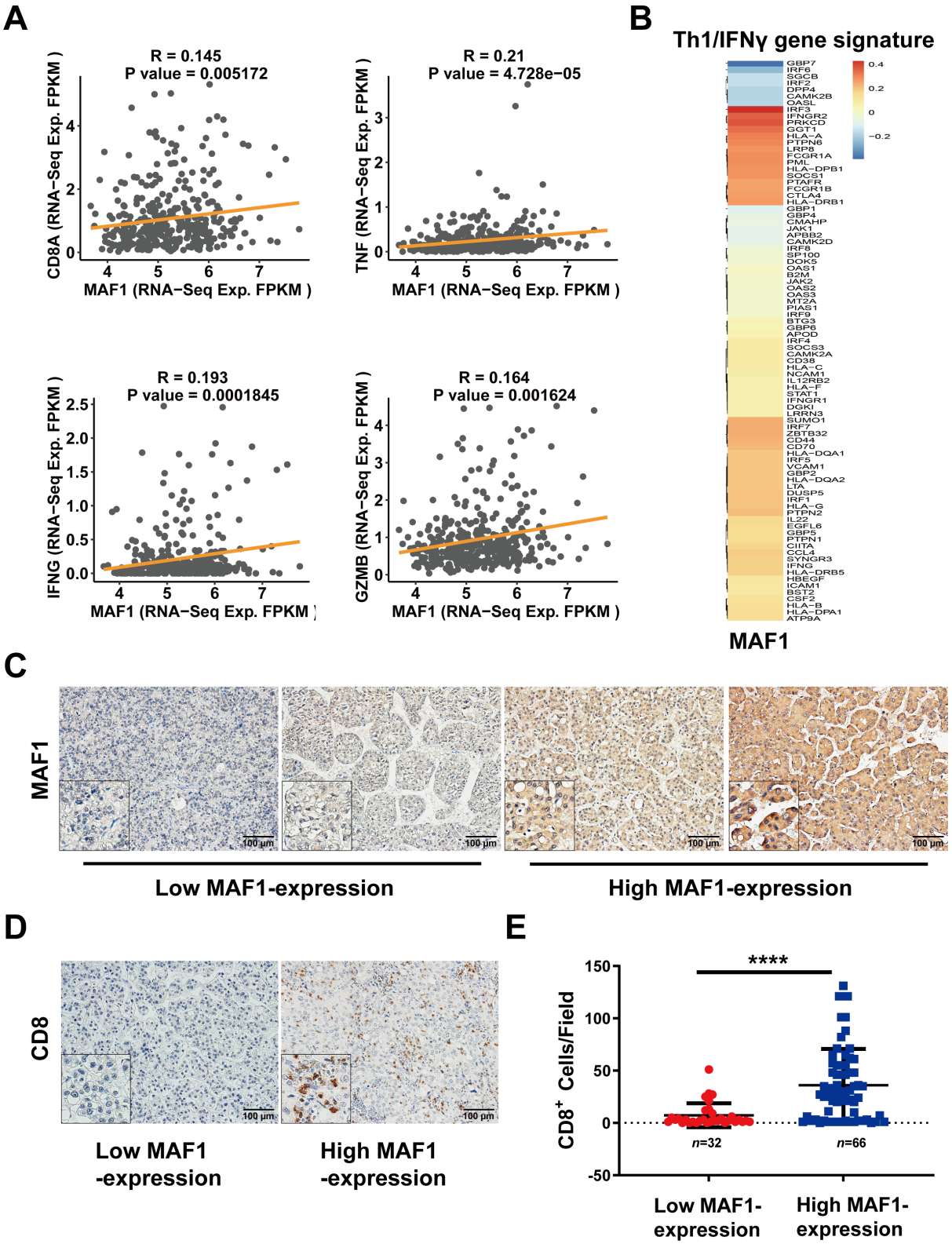

Figure 4 MAF1 expression positively correlates with CD8$^+$ T cell number in human hepatocellular carcinomas (HCCs). (A) Scatter plot for the correlations between MAF1 expression and CD8A, TNF, GZMA, or GZMB expressions in TCGA-LIHC samples. The Pearson correlation coefficient and P values are displayed in each plot. (B) Heatmap for the correlations between MAF1 and Th1/IFNγ gene signature in TCGA-LIHC samples. The color bar represents the Pearson correlation coefficients. (C) Immunohistochemical staining for MAF1 in human HCC tissues collected from Sun Yat-Sen University Cancer Center. Scale bar: 100 µm (200×). (D) Immunohistochemical staining for CD8-positive cells in human HCC tissues with low or high MAF1 expression, collected from the same Center. Scale bar: 100 µm (200×). (E) Scatter plot depicting the number of CD8-positive T cells in human HCC tissues with low or high MAF1 expression. ****, p<0.0001. TCGA-LIHC, The Cancer Genome Atlas Liver Hepatocellular Carcinoma.

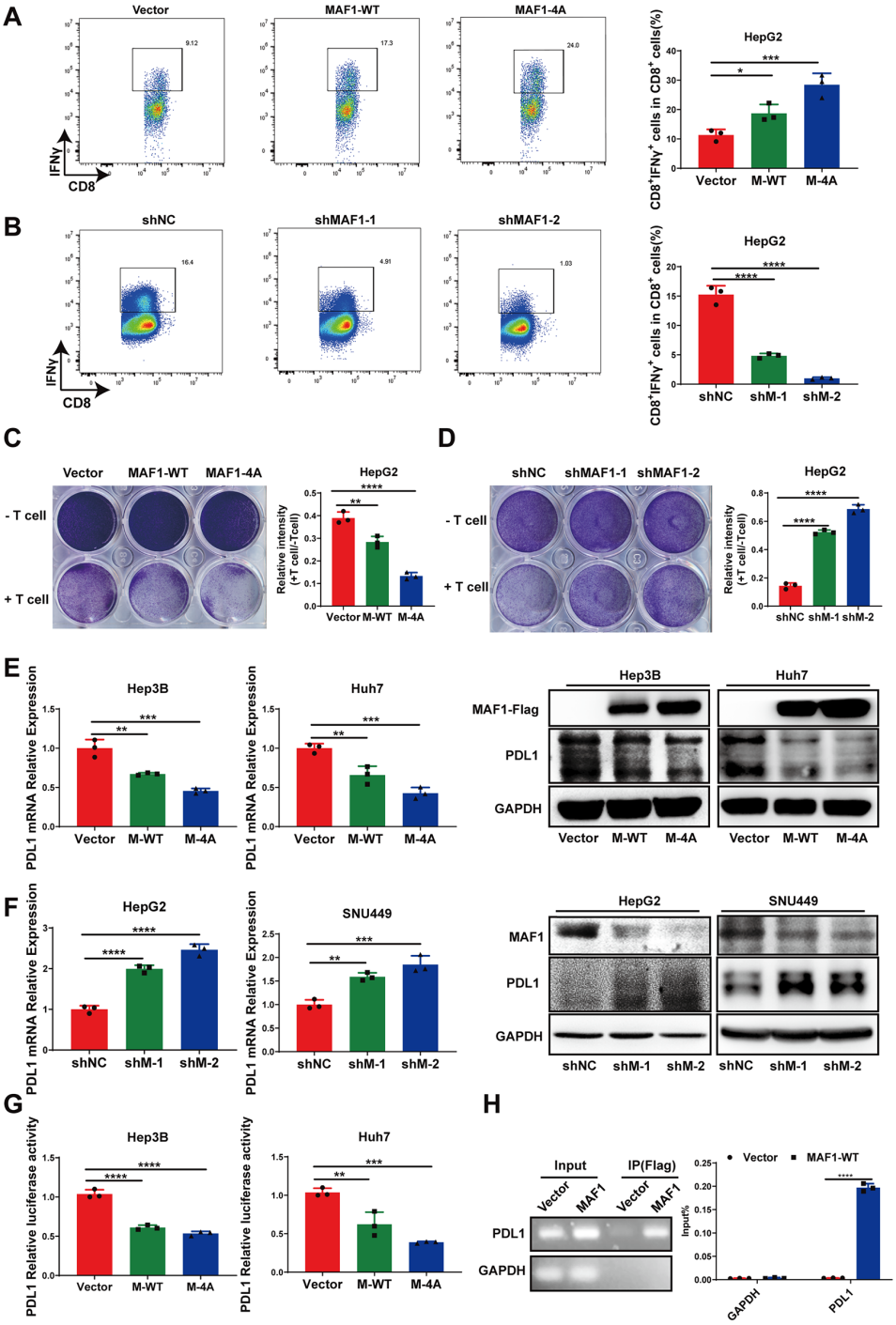

Figure 5 MAF1 increases activation of CD8$^+$ T cells by suppressing PDL1 transcription in liver cancer cells. (A) T cells were activated (IFNγ expression increase in CD8$^+$ T cells) when co-culturing with HepG2 cells stably expressing MAF1-WT or MAF1-4A at a ratio of 10:1 for 24 hours. IFNγ$^+$CD8$^+$ T cells were measured by flow cytometry. (B) T cells were inactivated (IFNγ expression decrease in CD8$^+$ T cells) when co-culturing with HepG2 cells stably downregulating MAF1s at a ratio of 10:1 for 24 hours. IFNγ$^+$CD8$^+$ T cells were measured by flow cytometry. (C–D) After co-cultured with HCC cells overexpressing vector, MAF1 or MAF1-4A (C), or with HCC cells downregulating MAF1 (D) for 48 hours, T cells were washed, and the surviving tumor cells were visualized with crystal violet, and relative fold ratios of surviving cell intensities are shown. (E) PDL1 mRNA and protein expressions were analyzed by qRT-PCR and immunoblotting in Hep3B and Huh7 cells stably expressing MAF1-WT and MAF1-4A. (F) PDL1 mRNA and protein expressions were analyzed by qRT-PCR and immunoblotting in SNU449 and HepG2 cells downregulating MAF1s. (G) Luciferase activity of PDL1 promoter was measured by dual luciferase reporter assay in Hep3B and Huh7 cells stably expressing MAF1-WT or MAF1-4A. (H) PDL1 promoter was bound by ectogenous MAF1 in Hep3B cells stably expressing Flag-MAF1-WT, as showing by chromatin immunoprecipitation and PCR (left panel) or quantitative PCR (right panel). GAPDH was used as a control in the experiment. The data are presented as the mean±SD of triplicate determinations. *, p<0.05; **, p<0.01; ***, p<0.001; ****, p<0.0001. IFNG, gamma interferon;*ns*, not significant. GAPDH, glyceraldehyde-3-phosphate dehydrogenase; qRT-PCR, quantitative reverse-transcription-PCR.

MAF1 activates the function of T cells by suppressing the transcription of PDL1 in vitro

We next sought to understand how MAF1 expression activate T cells. It is well-known that PDL1 is a major immune checkpoint protein that binds to its ligand PD1 on the surface of T cells, thereby inhibiting the anti-tumor immune responses of T cells. Therefore, we hypothesized that MAF1 activated the function of T cells by inhibiting PDL1. As anticipated, ectopic expression of MAF1 resulted in a notable decrease in the mRNA, total protein, and cell surface protein levels of PDL1 in HCC cells (figure 5E and online supplemental figure S15), while MAF1 knockdown led to increased mRNA, total protein, and cell surface protein expressions of PDL1 in HCC cells (figure 5F and online supplemental figure S15). These results suggested that MAF1 may regulate the transcription of PDL1 gene. Then, we performed a dual-luciferase reporter assay to investigate whether MAF1 can bind to the PDL1 promoter. The result showed that MAF1 overexpression caused a marked decrease in the luciferase activity in Hep3B and Huh7 cells (figure 5G), implying that MAF1 can bind to the promoter of PDL1 and inhibit its transcription. Chromatin immunoprecipitation followed by PCR analysis revealed a much higher DNA content of PDL1 promoter in MAF1-overexpressed Hep3B cells compared with that in the control Hep3B cells (figure 5H, online supplemental figure S16), indicating that MAF1 protein indeed binds to PDL1 promoter. These findings indicated that the transcription repressor MAF1 can bind to the PDL1 promoter and suppress its transcription.

Subsequently, we aimed to figure out the role of PDL1 in MAF1-induced T cell activation in HCC cells. To this purpose, we overexpressed PDL1 in MAF1-overexpressing HepG2 cells (figure 6A) and knockdown PDL1 in MAF1-downregulated HepG2 cells (figure 6B). Then, we co-cultured them with human T cells. The results showed that PDL1 overexpression in HepG2 cells expressing MAF1 significantly reduced the expression of IFNγ initiated by MAF1 in $CD8^+$ T cells (figure 6C,E). Conversely, the knockdown of PDL1 in MAF1-downregulated HepG2 cells enhanced IFNγ expression, which was reduced by MAF1 downregulation, in $CD8^+$ T cells (figure 6D,F). Taken together, these findings indicate that MAF1, especially MAF1-4A, can stimulate T cell activation by inhibiting the transcriptional expression of PDL1.

To confirm whether the interaction of Maf1 with PDL1 occurred in the mouse HCC model and human HCC, we first performed IHC and western blot to measure the expression of PDL1 in the aforementioned mouse HCC model. Consistent with the observations in vitro, as demonstrated by IHC and western blot, overexpression of Maf1-WT or Maf1-4A indeed caused an evident decrease in PDL1 protein expression in the liver and tumor tissues of Maf1 mice at the 7th week (online supplemental figure S17A,B), while Maf1 knockdown led to increased PDL1 protein expression in shMaf1 mice compared with control mice at 12–15 weeks (online supplemental figure S17C,D). Finally, we performed IHC staining of MAF1 and PDL1 proteins in 98 HCC specimens. As expected, tumors with high MAF1 expression had significantly lower PDL1 expression than those with low MAF1 expression (online supplemental figure S17E), and MAF1 protein was negatively related with PDL1 protein in 98 human HCC tissues (online supplemental table S2). Collectively, our findings demonstrate that MAF1 activates the anti-tumor function of T cells via suppressing transcription of PDL1 in HCC.

MAF1 promotes anti-tumor immunity through the downregulation of PDL1 expression in vivo

Numerous reports have demonstrated that PDL1 plays a critical role in the immune response and immunotherapy of cancers. However, only a few studies have shown the involvement of PDL1 expression in carcinogenesis including hepatocarcinogenesis.[24–26] Our aforementioned results showed that Maf1 expression resulted in the activation of immune cells by repressing PDL1 expression in HCCs, including human HCC and hepatocarcinogenesis in mice. To further elucidate the role of PDL1 in Maf1-mediated anti-cancer immune response during hepatocarcinogenesis in mice, we established an AKT/NRas-driven HCC model and simultaneously co-injected either Maf1-WT, Maf1-4A or combinations of Maf1s (Maf1-WT or Maf1-4A) and PDL1, via the tail vein (figure 7A). We discovered that all the mice delivered with Maf1-WT/PDL1 or Maf1-4A/PDL1 rapidly developed a lethal liver tumor and had to be euthanized by 16 weeks and 22 weeks post-injection, respectively, while the mice delivered with Maf1-WT or Maf1-4A survived to around 30 weeks and 36 weeks post-injection, respectively (figure 7B), indicating that Maf1 inhibits hepatocarcinogenesis partly through the downregulation of PDL1 expression in vivo. Under microscopy, typical liver cancer was observed in mice with Maf1-WT/PDL1 or Maf1-4A/PDL1, while only steatosis was observed in mice with Maf1-WT and Maf1-4A in the same period (16 weeks) (figure 7C). Furthermore, overexpressed PDL1 was validated by IHC in the cytoplasm of HCC cells in mice with Maf1-WT/PDL1 or Maf1-4A/PDL1 (figure 7C). Not unexpectedly, the infiltrated $CD8^+$ T cells, which increased due to Maf1 expression, were significantly reduced in the liver cancer of mice with Maf1-WT/PDL1 or Maf1-4A/PDL1, especially in the former group, compared with mice expressing Maf1-WT or Maf1-4A (figure 7D, first row), which was confirmed by IHC (figure 7E). More importantly, overexpression of PDL1 resulted in a marked decrease in IFNγ expression (figure 7D, middle row) and a striking increase in PD1 expression (figure 7D, last row) in $CD8^+$ T cells within HCC tissues of Maf1/PDL1 mice. Overall, our findings demonstrate that MAF1 increases the infiltration and activation of T cells by downregulating the expression of PDL1 in an AKT/NRas-driven HCC model in mice.

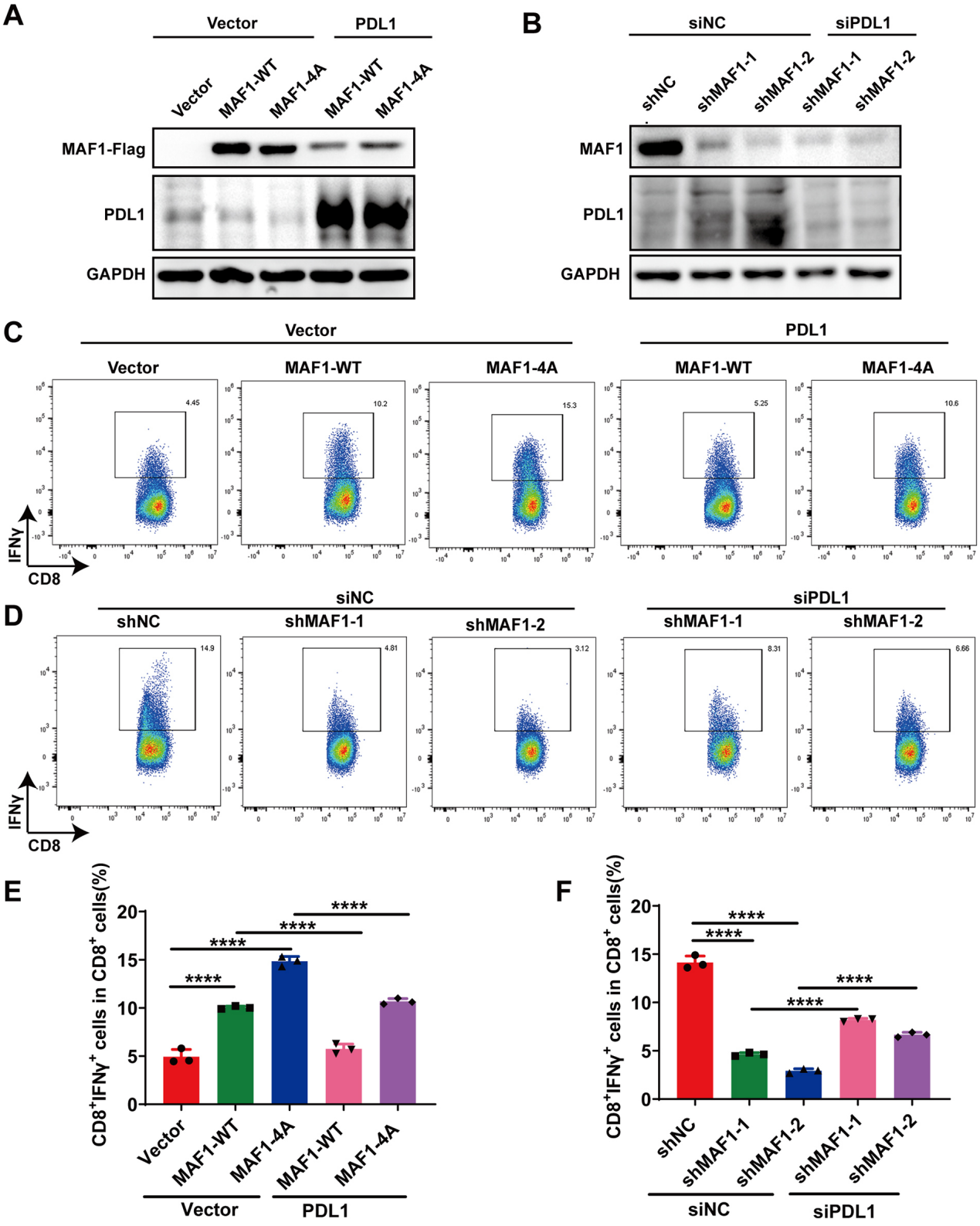

Figure 6 MAF1 in HCC cells induces activation of CD8$^+$ T cell through inhibition of PDL1. (A) MAF1 and PDL1 expressions were determined by immunoblot analysis in HepG2 cells overexpressing MAF1-WT or MAF1-4A or co-overexpressing MAF1 and PDL1. (B) MAF1 and PDL1 expressions were measured by immunoblot analysis in HepG2 cells with downregulation of MAF1 or downregulation of MAF1 and PDL1. (C) Representative dot plots of IFNγ$^+$CD8$^+$ T cells in CD8$^+$ T cells co-cultured with HepG2 cells expressing vector, MAF1-WT, or MAF1-4A or co-expressing MAF1 and PDL1, which were analyzed by flow cytometry. (D) Representative dot plots of IFNγ$^+$CD8$^+$ T cells in CD8$^+$ T cells co-cultured with HepG2 cells expressing shNC, shMAF1-1, and shMAF1-2 or co-expressing shMAF1 and siPDL1. (E) Histograms with statistical analysis for the results displayed in (C). (F) Histograms with statistical analysis for the results displayed in (D). The data are presented as the mean±SD of triplicate determinations. ****, p<0.0001. GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

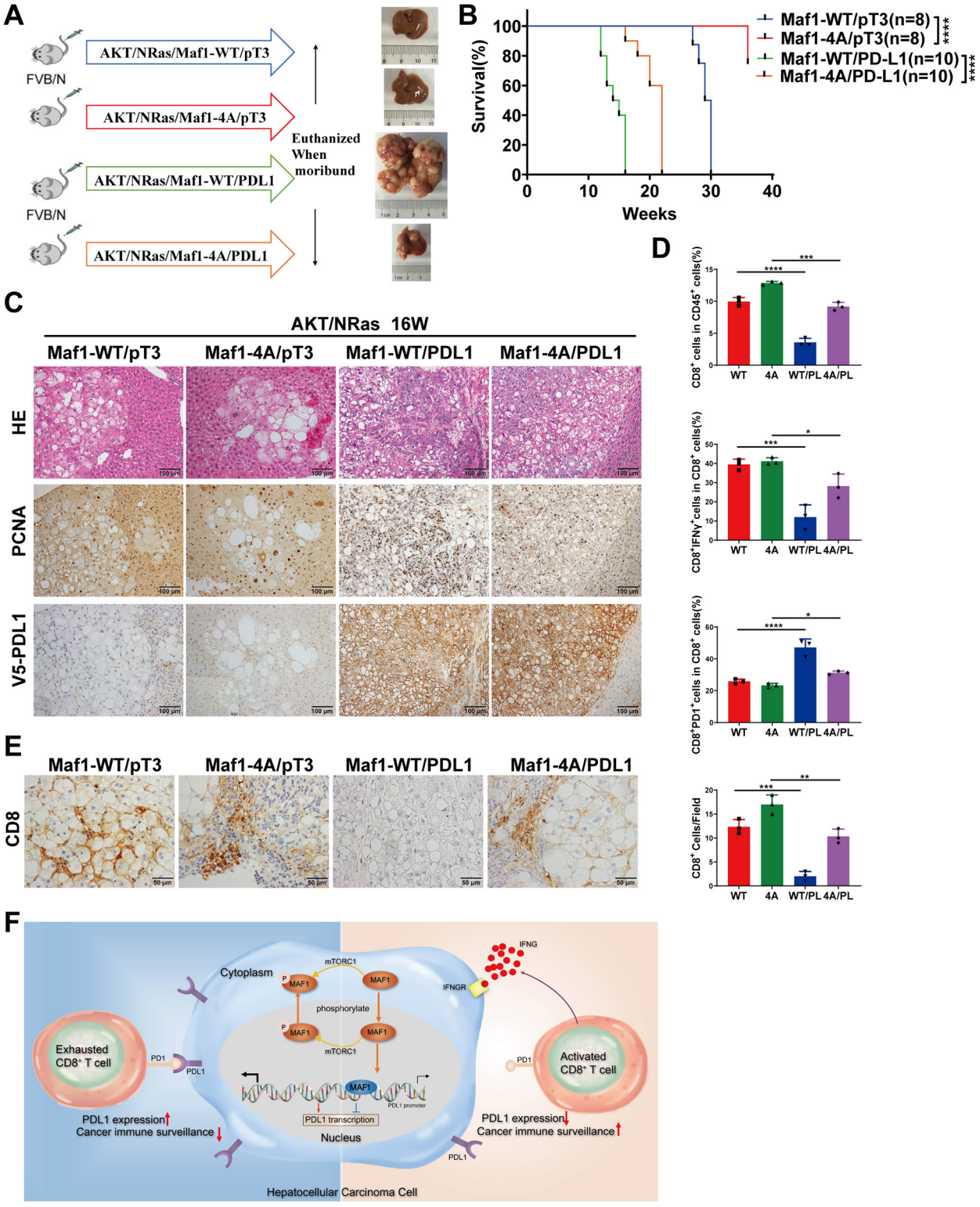

Figure 7 MAF1 promotes anti-tumor immunity through the downregulation of PDL1 expression in vivo. (A) Animal study design. (B) Survival curves of mice treated with AKT/NRas/Maf1-WT/pT3, AKT/NRas/Maf1-4A/pT3, AKT/NRas/Maf1-WT/PDL1, and AKT/NRas/Maf1-4A/PDL1. (C) H&E staining and immunohistochemical staining of PCNA, V5-PDL1 in mouse livers with steatosis (left panel 1 and 2) and HCCs (right panel 1 and 2) at 16th week. (D) Histograms with statistical analysis for the percentages of $CD8^+$ T cell in $CD45^+$ cells, $IFN\gamma^+CD8^+$ T cells in $CD8^+$ T cells, and $PD1^+CD8^+$ T cells in $CD8^+$ T cells isolated from mouse HCCs and livers at 16th week (n=3 for each group). (E) Representative images of CD8 immunohistochemical staining in mouse livers and statistical analysis (n=3 for each group). (F) Proposed model of the role of MAF1 in regulating PDL1 expression and anti-tumor immunity in HCC. Scale bar: 100 µm (200×) or 50 µm (400×). The data are presented as the mean±SD of triplicate determinations. *, p<0.05; **, p<0.01; ***, p<0.001; ****, p<0.0001. H&E, hematoxylin and eosin staining; WT, wild type.

DISCUSSION

In this study, we demonstrate for the first time that overexpression of Maf1-WT, particularly Maf1-4A, significantly inhibits the development and progression of liver tumors induced by AKT and NRas oncogenes. In the inhibitory effect, Maf1-4A exhibits significantly stronger tumor-suppressor activity than Maf1-WT in liver carcinogenesis, suggesting that Maf1-4A is an activated form of Maf1. At the molecular level, our results show that Maf1 increases the infiltration and activation of $CD8^+$ T cells through inhibition of PDL1 transcription. Moreover, this study demonstrates that Maf1 suppresses hepatocarcinogenesis by restraining Akt/mTOR signaling in a physiologically relevant tumor model. These new findings provide the first in vivo evidence that Maf1 is a liver tumor suppressor.

Maf1 effectively inhibits mouse hepatocarcinogenesis driven by AKT/NRas. Histologically, we found the HCC lesions in the AKT/NRas/Maf1-WT group as mixed cholangiocarcinoma tumors and HCCs. The studies by Fan *et al*[27] and Evert *et al*[28] have highlighted Notch signaling as a pivotal oncogenic pathway in intrahepatic cholangiocarcinoma (ICC) development. However, the potential of Maf1 to modulate Notch signaling in hepatocytes, thereby contributing to the transformation to ICC in the AKT/NRas murine model, remains an open question. Further exploration into the molecular crosstalk between Maf1 and Notch signaling pathways will provide critical insights into the pathogenesis of ICC and may be a new focus of future research endeavors.

Currently, tumor immunity is widely considered as one of the most crucial factors in the development, progression, and treatment of cancer. Studies have shown that tumor suppressor genes are also involved in tumor immunity.[25 29] However, no reports have indicated whether MAF1 plays a role in tumor immunity. Here, for the first time, we reveal that Maf1 inhibits hepatocarcinogenesis in mice not only by suppressing the AKT/mTOR signaling pathway but also by activating anti-tumor immunoreaction. In mouse liver cancer driven by AKT/NRas, Maf1 expression resulted in the infiltration and activation of $CD8^+$ T cells by increasing the secretion of chemokines (such as CXCL10) in cancer cells and by enhancing IFN-gamma secretion in T cells, which was confirmed by in vitro experiments. The positive correlation between MAF1 expression and the infiltration of $CD8^+$ T cells was verified in 98 human HCC tissues. In in vitro experiments, we confirmed that MAF1 expression can stimulate the activation of $CD8^+$ T cells by repressing the transcription of PDL1. We demonstrated here that mTORC1 governs an MAF1 phosphorylation switch that regulates MAF1 DNA-binding activity, thereby controlling MAF1's regulation of PDL1 transcription and the anti-tumor immune response (figure 7F). Given that cytotoxic T cells (primarily $CD8^+$ T cells) have been characterized as a crucial part of anti-tumor immunity in human liver cancers,[30 31] understanding the mechanisms governing $CD8^+$ T cell infiltration and activation will offer opportunities for rational design of systemic immunotherapies including combination immunotherapy. The present study provides evidence that MAF1 stimulation can enhance the anti-tumor immunity in HCC, which may aid in designing new combination immunotherapy for advanced HCC patients.

PDL1 is not only a critical immune checkpoint protein that plays a vital role in tumor immunity, but it is also an extremely important target in immunotherapy for cancer. A considerable body of research shows that various PDL1 inhibitors (mainly anti-bodies against PDL1) were widely used in immunotherapy for a variety of cancers. However, only a few studies indicate that PDL1 expression is altered during carcinogenesis in several tumors including liver cancer.[24–26 32 33] In this study, we elucidated for the first time that exogenous PDL1 expression can promote hepatocarcinogenesis in mice by inhibiting the activation of $CD8^+$ T cells in liver cancers. Mice with PDL1 overexpression survived significantly short due to exhaustion of $CD8^+$ T cells compared with control mice. These results indicate that a combination of MAF1 stimulation with PDL1 inhibition may help prevent the development and progression of HCC.

In general, MAF1 expression is reduced in human HCC. The mechanisms leading to the decreased MAF1 in HCC are still not clear. According to the previous study, Maf1 is positively regulated by PTEN via modulation of PI3K/AKT/FoxO1 signaling, and MAF1 expression is reduced with loss of PTEN in HCC.[13] With IHC examination, PTEN loss can be observed in up to 53% of patients diagnosed with HCC.[34] Furthermore, PTEN mutation or deletion, both of which are the mechanisms that lead to loss expression or impaired function of PTEN, may be discerned in a proportion of patients (~20% to 30%).[35 36] These studies suggest that MAF1 downregulation may be due to the PTEN loss in HCC, which activates the PI3k/Akt/mTOR axis that negatively regulates MAF1 expression. Therefore, PI3k/Akt/mTOR axis may be druggable by corresponding inhibitors to restore MAF1 expression, which may enhance ICI therapeutic efficacy in HCC.

In summary, our current study provides the first in vivo evidence that tumor suppressor Maf1 can suppress hepatocarcinogenesis in mice by inactivating the AKT/mTOR signaling pathway. It also identifies for the first time that Maf1 can enhance the anti-tumor immune response by stimulating the infiltration and activation of $CD8^+$ T cells via elevating CXCL10 expression and inhibiting PDL1 transcription. Furthermore, we demonstrate for the first time that PDL1 overexpression promotes liver carcinogenesis in the mouse by causing $CD8^+$ T cell exhaustion. These findings not only provide evidence that MAF1 stimulation (PIK3CA, AKT, or mTOR inhibitors)[13] or combination with PDL1 inhibition or degrader may prevent the development and progression of human HCC but also offer a new immunotherapeutic strategy for patients with advanced HCC.

Contributors HYW and DC conceived the study and designed the experiments. DC, YNW, CYS, and YZ performed the experiments and analyzed the data. HL and GR analyzed the clinical data and samples. MZ and SW helped with a portion of the experiment design and statistical analysis. DC, HYW, YNW, and SM wrote the

paper. HYW is the guarantor for this study. All authors read and approved the final manuscript.

Funding This study was supported by the National Natural Science Foundation of China (Grant No: 82273051, 81972640, 81772991, 81772884, and 81730081).

Competing interests None declared.

Patient consent for publication Not applicable.

Ethics approval All patients' samples were obtained according to the Declaration of Helsinki, and each patient signed a written informed consent for all the procedures. This work was approved by the Institutional Research Ethics Committee of Sun Yat-Sen University Cancer Center, China (ethics approval number: GZR2019-286). All animal experiments were performed following a protocol approved by the Institutional Animal Care and Use Committee of Sun Yat-Sen University Cancer Center, China (ethics approval number: L102012019220Q).

Provenance and peer review Not commissioned; externally peer-reviewed.

Data availability statement Data are available upon reasonable request.

ORCID iDs
Shi-Juan Mai http://orcid.org/0000-0003-0017-8194
Hui-Yun Wang http://orcid.org/0000-0001-6728-4376

REFERENCES

1 Forner A, Reig M, Bruix J. Hepatocellular carcinoma. *Lancet* 2018;391:1301–14.
2 Pascual S, Herrera I, Irurzun J. New advances in hepatocellular carcinoma. *World J Hepatol* 2016;8:421–38.
3 Llovet JM, Ricci S, Mazzaferro V, *et al*. Sorafenib in advanced hepatocellular carcinoma. *N Engl J Med* 2008;359:378–90.
4 Cheng A-L, Kang Y-K, Chen Z, *et al*. Efficacy and safety of sorafenib in patients in the Asia-Pacific region with advanced hepatocellular carcinoma: a phase III randomised, double-blind, placebo-controlled trial. *Lancet Oncol* 2009;10:25–34.
5 Bruix J, Qin S, Merle P, *et al*. Regorafenib for patients with hepatocellular carcinoma who progressed on sorafenib treatment (RESORCE): a randomised, double-blind, placebo-controlled, phase 3 trial. *Lancet* 2017;389:56–66.
6 Park H, Park H, Baek J, *et al*. Target Therapy for Hepatocellular Carcinoma: Beyond Receptor Tyrosine Kinase Inhibitors and Immune Checkpoint Inhibitors. *Biol (Basel)* 2022;11:585.
7 Ehrlich SM, Liebl J, Ardelt MA, *et al*. Targeting cyclin dependent kinase 5 in hepatocellular carcinoma--A novel therapeutic approach. *J Hepatol* 2015;63:102–13.
8 Pluta K, Lefebvre O, Martin NC, *et al*. Maf1p, a negative effector of RNA polymerase III in Saccharomyces cerevisiae. *Mol Cell Biol* 2001;21:5031–40.
9 Upadhya R, Lee J, Willis IM. Maf1 is an essential mediator of diverse signals that repress RNA polymerase III transcription. *Mol Cell* 2002;10:1489–94.
10 Michels AA, Robitaille AM, Buczynski-Ruchonnet D, *et al*. mTORC1 directly phosphorylates and regulates human MAF1. *Mol Cell Biol* 2010;30:3749–57.
11 Wei YH, Tsang CK, Zheng XFS. Mechanisms of regulation of RNA polymerase III-dependent transcription by TORC1. *EMBO J* 2009;28:2220–30.
12 Rideout EJ, Marshall L, Grewal SS. Drosophila RNA polymerase III repressor Maf1 controls body size and developmental timing by modulating tRNAiMet synthesis and systemic insulin signaling. *Proc Natl Acad Sci U S A* 2012;109:1139–44.
13 Palian BM, Rohira AD, Johnson SAS, *et al*. Maf1 is a novel target of PTEN and PI3K signaling that negatively regulates oncogenesis and lipid metabolism. *PLoS Genet* 2014;10:e1004789.
14 Li Y, Tsang CK, Wang S, *et al*. MAF1 suppresses AKT-mTOR signaling and liver cancer through activation of PTEN transcription. *Hepatology* 2016;63:1928–42.
15 Reverendo M, Argüello RJ, Polte C, *et al*. Polymerase III transcription is necessary for T cell priming by dendritic cells. *Proc Natl Acad Sci U S A* 2019;116:22721–9.
16 Wu J, Kim A, Wu X, *et al*. 5S rRNA pseudogene transcripts are associated with interferon production and inflammatory responses in alcohol-associated hepatitis. *Hepatology* 2023;77:1983–97.
17 Chow EK-H, Fan L, Chen X, *et al*. Oncogene-specific formation of chemoresistant murine hepatic cancer stem cells. *Hepatology* 2012;56:1331–41.
18 Calvisi DF, Wang C, Ho C, *et al*. Increased lipogenesis, induced by AKT-mTORC1-RPS6 signaling, promotes development of human hepatocellular carcinoma. *Gastroenterology* 2011;140:1071–83.
19 Ho C, Wang C, Mattu S, *et al*. AKT (v-akt murine thymoma viral oncogene homolog 1) and N-Ras (neuroblastoma ras viral oncogene homolog) coactivation in the mouse liver promotes rapid carcinogenesis by way of mTOR (mammalian target of rapamycin complex 1), FOXM1 (forkhead box M1)/SKP2, and c-Myc pathways. *Hepatology* 2012;55:833–45.
20 Chen X, Calvisi DF. Hydrodynamic transfection for generation of novel mouse models for liver cancer research. *Am J Pathol* 2014;184:912–23.
21 Shor B, Wu J, Shakey Q, *et al*. Requirement of the mTOR kinase for the regulation of Maf1 phosphorylation and control of RNA polymerase III-dependent transcription in cancer cells. *J Biol Chem* 2010;285:15380–92.
22 Liu P, Ge M, Hu J, *et al*. A functional mammalian target of rapamycin complex 1 signaling is indispensable for c-Myc-driven hepatocarcinogenesis. *Hepatology* 2017;66:167–81.
23 Alspach E, Lussier DM, Schreiber RD. *Interferon Gamma and Its Important Roles in Promoting and Inhibiting Spontaneous and Therapeutic Cancer Immunity*, 11. Cold Spring Harb Perspect Biol, 2019.
24 Zhang Q, Pan J, Xiong D, *et al*. Aerosolized miR-138-5p and miR-200c targets PD-L1 for lung cancer prevention. *Front Immunol* 2023;14:1166951.
25 Kang C, Song C-H, Kim N, *et al*. The Enhanced Inhibitory Effect of Estrogen on PD-L1 Expression Following Nrf2 Deficiency in the AOM/DSS Model of Colitis-Associated Cancer. *Front Oncol* 2021;11:679324.
26 Ke M-Y, Xu T, Fang Y, *et al*. Liver fibrosis promotes immune escape in hepatocellular carcinoma via GOLM1-mediated PD-L1 upregulation. *Cancer Lett* 2021;513:14–25.
27 Fan B, Malato Y, Calvisi DF, *et al*. Cholangiocarcinomas can originate from hepatocytes in mice. *J Clin Invest* 2012;122:2911–5.
28 Evert M, Dombrowski F, Fan B, *et al*. On the role of notch1 and adult hepatocytes in murine intrahepatic cholangiocarcinoma development. *Hepatology* 2013;58:1857–9.
29 Cui Y, Guo G. Immunomodulatory Function of the Tumor Suppressor p53 in Host Immune Response and the Tumor Microenvironment. *Int J Mol Sci* 2016;17:1942.
30 Flecken T, Schmidt N, Hild S, *et al*. Immunodominance and functional alterations of tumor-associated antigen-specific CD8+ T-cell responses in hepatocellular carcinoma. *Hepatology* 2014;59:1415–26.
31 Mizukoshi E, Nakamoto Y, Arai K, *et al*. Comparative analysis of various tumor-associated antigen-specific t-cell responses in patients with hepatocellular carcinoma. *Hepatology* 2011;53:1206–16.
32 Tan HY, Wang N, Zhang C, *et al*. Lysyl Oxidase-Like 4 Fosters an Immunosuppressive Microenvironment During Hepatocarcinogenesis. *Hepatology* 2021;73:2326–41.
33 Fu Y, Mackowiak B, Feng D, *et al*. MicroRNA-223 attenuates hepatocarcinogenesis by blocking hypoxia-driven angiogenesis and immunosuppression. *Gut* 2023;72:1942–58.
34 Subbiah IM, Falchook GS, Kaseb AO, *et al*. Exploring response signals and targets in aggressive unresectable hepatocellular carcinoma: an analysis of targeted therapy phase 1 trials. *Oncotarget* 2015;6:28453–62.
35 Hu T-H, Huang C-C, Lin P-R, *et al*. Expression and prognostic role of tumor suppressor gene PTEN/MMAC1/TEP1 in hepatocellular carcinoma. *Cancer* 2003;97:1929–40.
36 Kawamura N, Nagai H, Bando K, *et al*. PTEN/MMAC1 mutations in hepatocellular carcinomas: somatic inactivation of both alleles in tumors. *Jpn J Cancer Res* 1999;90:413–8.