NASH limits anti-tumour surveillance in immunotherapy-treated HCC

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Abstract

Hepatocellular carcinoma (HCC) can have viral or non-viral causes[1-5]. Non-alcoholic steatohepatitis (NASH) is an important driver of HCC. Immunotherapy has been approved for treating HCC, but biomarker-based stratification of patients for optimal response to therapy is an unmet need[6,7]. Here we report the progressive accumulation of exhausted, unconventionally activated $CD8^+PD1^+$ T cells in NASH-affected livers. In preclinical models of NASH-induced HCC, therapeutic immunotherapy targeted at programmed death-1 (PD1) expanded activated $CD8^+PD1^+$ T cells within tumours but did not lead to tumour regression, which indicates that tumour immune surveillance was impaired. When given prophylactically, anti-PD1 treatment led to an increase in the incidence of NASH-HCC and in the number and size of tumour nodules, which correlated with increased hepatic $CD8^+PD1^+CXCR6^+$, TOX^+, and TNF^+ T cells. The increase in HCC triggered by anti-PD1 treatment was prevented by depletion of $CD8^+$ T cells or TNF neutralization, suggesting that $CD8^+$ T cells help to induce NASH-HCC, rather than invigorating or executing immune surveillance. We found similar phenotypic and functional profiles in hepatic $CD8^+PD1^+$ T cells from humans with NAFLD or NASH. A meta-analysis of three randomized phase III clinical trials that tested inhibitors of PDL1 (programmed death-ligand 1) or PD1 in more than 1,600 patients with advanced HCC revealed that immune therapy did not improve survival in patients with non-viral HCC. In two additional cohorts, patients with NASH-driven HCC who received anti-PD1 or anti-PDL1 treatment showed reduced overall survival compared to patients with other aetiologies. Collectively, these data show that non-viral HCC, and particularly NASH-HCC, might be less responsive to immunotherapy, probably owing to NASH-related aberrant T cell activation causing tissue damage that leads to impaired immune surveillance. Our data provide a rationale for stratification of patients with HCC according to underlying aetiology in studies of immunotherapy as a primary or adjuvant treatment.

Introduction

Potentially curative treatments for HCC, such as liver transplantation, tumour resection, or ablation, are limited to early-stage tumours[1,2]. Multikinase inhibitors and anti-VEGF-R2 antibodies have been approved for use in advanced HCC[1,2]. Immunotherapy, which is thought to activate T cells or reinvigorate immune surveillance against cancer, showed response rates of 15–30% in patients with HCC[5,8–11]. Nivolumab and pembrolizumab (PD1-directed antibodies) have been approved for treatment of HCC[3,4], although phase III trials failed to reach their primary endpoints to increase survival[1,10,11]. A combination of atezolizumab (anti-PDL1) and bevacizumab (anti-VEGF) demonstrated increased overall and progression-free survival in a phase III trial, making it a first-line treatment for advanced HCC[5]. The efficacy of immunotherapy might be affected by different underlying HCC aetiologies, with diverse hepatic environments distinctly regulating HCC induction and immune responses[6]. Hence, we lack biomarkers that correlate with treatment response to allow patient stratification[12,13]. Non-alcoholic fatty liver disease (NAFLD) is an HCC-causing condition that affects more than 200 million people worldwide[14]. Approximately 10–20% of individuals with NAFLD progress over time from steatosis to NASH[14]. Innate and adaptive immune-cell activation[15–17], in combination with increased metabolites and endoplasmic reticulum stress[16,18], are believed to lead to a cycle of hepatic necro-inflammation and regeneration that potentially leads to HCC[19–21]. NASH has become an emerging risk factor for HCC[1,14,19], which led us to investigate the effects of immunotherapy in NASH–HCC[22–24].

Hepatic $CD8^+PD1^+$ T cells increase in NASH

We fed mice with diets that cause progressive liver damage and NASH over 3–12 months (Extended Data Fig. 1a–c), accompanied by an increase in the frequency of activated $CD8^+$ T cells expressing CD69, CD44 and PD1 (Extended Data Fig. 1d–g). Single-cell mapping of leukocytes showed altered immune-cell compositions in mice with NASH (Extended Data Fig. 1h, i) with strongly increased numbers of $CD8^+PD1^+$ cells (Fig. 1a, b, Extended Data Fig. 1j–m, o). Similarly, elevated $CD8^+$ and $PD1^+$ cells were found in a genetic mouse model of NASH[17] (Extended Data Fig. Fig.1n).1n). Messenger RNA in situ hybridization and immunohistochemistry showed that increasing PDL1 expression in hepatocytes and non-parenchymal cells correlated with the severity of NASH (Extended Data Fig. Fig.1p).1p). Mass spectrometric characterization of $CD8^+PD1^+$ T cells from NASH-affected livers indicated enrichment in pathways involved in ongoing T cell activation and differentiation, TNF signalling, and natural killer (NK) cell-like cytotoxicity (Fig. (Fig.1c).1c). Single-cell RNA sequencing (scRNA-seq) of cells expressing T cell receptor β-chains (TCRβ) from the livers of mice with NASH showed that $CD8^+$ T cells had gene expression profiles related to cytotoxicity and effector-function (for example, *Gzmk* and *Gzmm)* and inflammation markers (for example, *Ccl3*) with elevated exhaustion traits (for example, *Pdcd1* and *Tox*) (Fig. 1d, e). RNA-velocity analyses demonstrated enhanced transcriptional activity and differentiation from *Sell*-expressing $CD8^+$ to $CD8^+PD1^+$ T cells (Extended Data Fig. Fig.1q),1q), indicating local differentiation. Thus, mice with NASH have increased hepatic abundance of $CD8^+PD1^+$ T cells with features of exhaustion and effector functions.

The high numbers of T cells in NASH suggest that anti-PD1-targeted immunotherapy may serve as an efficient therapy for NASH–HCC. Thirty per cent of C57BL/6 mice fed a choline-deficient high-fat diet (CD-HFD) for 13 months developed liver tumours with a similar load of genetic alterations to human NAFLD–HCC or NASH–HCC (Extended Data Fig. 2a, b). NASH mice bearing HCC (identified using MRI) were allocated to anti-PD1 immunotherapy or control arms (Fig. (Fig.1f).1f). None of the pre-existing liver tumours regressed in response to anti-PD1 therapy (Fig. 1g, h, Extended Data Fig. Fig.2c).2c). Rather, we observed increased fibrosis, unchanged liver damage, slightly increased incidence of liver cancer and unaltered tumour loads

and sizes after anti-PD1 treatment (Extended Data Fig. 2 d–h). In anti-PD1-treated mice, liver tumour tissue contained increased numbers of $CD8^+/PD1^+$ T cells and high levels of cells expressing *Cxcr6* or *Tnf* mRNA (Extended Data Fig. 2i–n). We found no regression of NASH-induced liver tumours upon anti-PDL1 immunotherapy (Extended Data Fig. 3a–f). By contrast, other (non-NASH) mouse models of liver cancer (with or without concomitant damage) reacted to PD1 immunotherapy with tumour regression[25], suggesting that lack of response to immunotherapy was associated specifically with NASH–HCC (Extended Data Fig. 3g–i). Thus, NASH precluded efficient anti-tumour surveillance in the context of HCC immunotherapy. Similarly, impaired immunotherapy has been described in mouse models with NASH and secondary liver cancer[25,26].

$CD8^+$ T cells promote HCC in NASH

As $CD8^+PD1^+$ T cells failed to execute effective immune surveillance, but rather showed tissue-damaging potential, we reasoned that $CD8^+$ T cells might be involved in promoting NASH–HCC. We depleted $CD8^+$ T cells in a preventive setting in mice with NASH but without liver cancer (CD-HFD fed for 10 months). $CD8^+$ T cell depletion significantly decreased liver damage and the incidence of HCC in these mice (Fig. (Fig.2i,2i, Extended Data Fig. 4a–j, n). Similar results were obtained after co-depletion of $CD8^+$ and $NK1.1^+$ cells (Fig. (Fig.2i,2i, Extended Data Fig. 4a–f, n). This suggests that as well as lacking immune surveillance functions, liver $CD8^+$ T cells also promote HCC in mice with NASH. Next, we investigated the effect of anti-PD1 therapy on HCC development in mice with NASH. Anti-PD1 immunotherapy aggravated liver damage (Fig. (Fig.2g,2g, Extended Data Fig. Fig.7c)7c) and increased hepatic $CD8^+PD1^+$ T cells, with only minor changes in liver $CD4^+PD1^+$ T cells or other immune-cell populations (Extended Data Fig. 4a–o). Anti-PD1 immunotherapy also caused a marked increase in liver-cancer incidence, independent of changes in liver fibrosis (Fig. (Fig.2i).2i). Mice lacking PD1 (*Pdcd1*$^{-/-}$) showed an increase in incidence of, and earlier onset of, liver cancer, along with increased liver damage and elevated numbers of activated hepatic $CD8^+$ T cells with increased cytokine expression (IFNγ, TNF) (Extended Data Fig. 5a–g). In summary, $CD8^+PD1^+$ T cells triggered the transition to HCC in mice with NASH, probably owing to impaired tumour surveillance and enhanced T cell-mediated tissue damage[27]. Despite a strong increase in $CD8^+PD1^+$ T cells within tumours, therapeutic PD1- or PDL1-related immunotherapy failed to cause tumour regression in NASH–HCC.

We used an immune-mediated cancer field (ICF) gene-expression signature associated with the development of human HCC[28] to understand the tumour-driving mechanisms of anti-PD1 immunotherapy. Preventive anti-PD1 treatment was strongly associated with the pro-tumorigenic immunosuppressive ICF signature (for example, *Ifng*, *Tnf*, *Stat3*, *Tgfb1*), capturing the traits of T cell exhaustion, pro-carcinogenic signalling, and mediators of immune tolerance and inhibition. Depletion of $CD8^+$ T cells led to significant downregulation of the high-infiltrate ICF signature and diminished TNF in non-parenchymal cells (Extended Data Fig. 5h, i). Gene set enrichment analysis (GSEA), mRNA in situ hybridization, and histology of tumours developed in NASH mice that were treated prophylactically with anti-PD1 corroborated these data, showing increased $CD8^+$ T cell abundance and enrichment for genes involved in inflammation-related signalling, apoptosis, and TGFβ signalling (Extended Data Fig. 5j–l). Anti-PD1 treatment triggered the expression of p62 (Extended Data Fig. Fig.5m),5m), which has been shown to drive hepatocarcinogenesis[29]. Array comparative genomic hybridization identified no significant differences in chromosomal deletions or amplifications between tumours from anti-PD1-treated mice or control mice (Extended Data Fig. Fig.5n).5n). In summary, hepatic $CD8^+PD1^+$ T cells did not cause tumour regression during NASH, but rather were linked to HCC development, which was enhanced by anti-PD1 immunotherapy.

We next analysed the hepatic T cell compartment for correlations with inflammation and hepatocarcinogenesis. Comparison of $CD8^+PD1^+$ T cells with $CD8^+$ T cells by scRNA-seq showed that the former showed higher expression of genes associated with effector function (for example,

increased *Gzma*, *Gzmb*, *Gzmk*, *Prf1*; reduced *Sell*, *Klf2*), exhaustion (for example, increased *Pdcd1*, *Tox*; reduced *Il7r*, *Tcf7*) and tissue residency (for example, increased *Cxcr6*, low levels of Ki-67) (Extended Data Fig. 6a–c). Notably, there was no difference in the transcriptome profiles of $CD8^+PD1^+$ T cells in NASH mice after anti-PD1 immunotherapy (Extended Data Fig. Fig.6c),6c), indicating that the number of T cells rather than their functional properties were changed. RNA-velocity blot analyses corroborated these data (Fig. (Fig.2a,2a, Extended Data Fig. 6d–f). Similar patterns of markers (for example, *IL7r*, *Sell*, *Tcf7*, *Ccl5*, *Pdcd1*, *Cxcr6*, and *Rgs1*) correlated with latent time and overall transcriptional activity in NASH mice that received either treatment (Fig. 2a, b, Extended Data Fig. 6e, f). Mass spectrometry-based analyses of $CD8^+$ or $CD8^+PD1^+$ T cells isolated from NASH mouse livers confirmed these findings (Fig. (Fig.2c,2c, Extended Data Fig. Fig.6g6g).

We characterized the transcriptome profiles of $PD1^+CD8^+$ T cells by uniform manifold approximation and projection (UMAP) analysis of high-parametric flow-cytometry data, dissecting the $CD8^+PD1^+$ and $CD8^+PD1^-$ subsets (Fig. (Fig.2d).2d). This revealed that $CD8^+PD1^+$ cells expressed high levels of effector (for example, *Gzmb*, *Ifng*, *Tnf*) and exhaustion markers (for example, *Eomes*, *Pdcd1*, Ki-67^{low}). In particular, $CD8^+PD1^+TNF^+$ cells were more abundant upon anti-PD1 treatment (Fig. (Fig.2e).2e). Convolutional neural network analysis and manual gating validated this result (Fig. (Fig.2f,2f, Extended Data Fig. 6j, k). $CD8^+PD1^+$ T cells were non-proliferative in anti-PD1-treated NASH mice; this result was supported by in vitro experiments, in which anti-PD1 treatment led to increased T cell numbers in the absence of proliferation (Extended Data Fig. 6l, m). Notably, $CD8^+PD1^+$ T cells from NASH mice showed reduced levels of FOXO1, which indicates an enhanced tissue-residency phenotype[30], potentially combined with boosted effector function, as indicated by higher calcium levels in $CD8^+PD1^+$ T cells (Extended Data Fig. 6n, o). Single-cell RNA-seq analysis also showed that $CD8^+PD1^+$ T cells from NASH mice had a tissue residency signature (Extended Data Fig. Fig.6b).6b). Thus, upon anti-PD1 immunotherapy in NASH mice, $CD8^+PD1^+$ T cells accumulated to high numbers in the liver, revealing a resident-like T cell character with increased expression of CD44, CXCR6, EOMES and TOX and low levels of CD244 expression, but lacking expression of TCF1/TCF7, CD62L, TBET, and CD127 (Extended Data Fig. 6p–u). In summary, anti-PD1 immunotherapy increased the abundance of $CD8^+PD1^+$ T cells with a residency signature in the liver.

To investigate the mechanisms that drive the increased NASH–HCC transition in the preventive anti-PD1 treatment-setting, we treated NASH-affected mice with combinations of treatments. Both anti-CD8–anti-PD1 and anti-TNF–anti-PD1 antibody treatments ameliorated liver damage, liver pathology and liver inflammation (Fig. (Fig.2g,2g, Extended Data Fig. Fig.7),7), and decreased the incidence of liver cancer compared to anti-PD1 treatment alone (Fig. (Fig.2i).2i). By contrast, anti-CD4–anti-PD1 treatment did not reduce the incidence of liver cancer, the NAFLD activity score (NAS), or the number of TNF-expressing hepatic $CD8^+$ or $CD8^+PD1^+CXCR6^+$ T cells (Fig. 2g–i, Extended Data Fig. Fig.7).7). However, both the number of tumours per liver and tumour size were reduced, suggesting that depletion of $CD4^+$ T cells or regulatory T cells might contribute to tumour control (Extended Data Fig. 8a, b). The incidence of tumours was directly correlated with anti-PD1 treatment, alanine aminotransferase (ALT), NAS, number of hepatic $CD8^+PD1^+$ T cells, and TNF expression (Extended Data Fig. 8c–e). These data suggested that $CD8^+PD1^+$ T cells lacked immune-surveillance and had tissue-damaging functions[27], which were increased by anti-PD1 treatment, possibly contributing to the unfavourable effects of anti-PD1 treatment on HCC development in NASH.

Augmented $CD8^+PD1^+$ T cells in human-NASH

We next investigated $CD8^+$ T cells from healthy or NAFLD/NASH-affected livers. In two independent cohorts of patients with NASH, we found enrichment of hepatic $CD8^+PD1^+$ T cells

with a residency phenotype (by flow cytometry and mass cytometry) (Fig. 3a, b, Extended Data Fig. 9a–j, Supplementary Tables 1,2). The number of hepatic $CD8^+PD1^+$ T cells directly correlated with body-mass index and liver damage (Extended Data Fig. Fig.9b).9b). To investigate similarities between mouse and human T cells from livers with NASH, we analysed liver $CD8^+PD1^+$ T cells from patients with NAFLD or NASH by scRNA-seq. This identified a gene expression signature that was also found in liver T cells from NASH mice (for example, *PDCD1*, *GZMB*, *TOX*, *CXCR6*, *RGS1*, *SELL*) (Fig. 3c, d, Extended Data Fig. 9k, l). Differentially expressed genes were directly correlated between patient- and mouse-derived hepatic $CD8^+PD1^+$ T cells (Fig. (Fig.3d).3d). Velocity-blot analyses identified $CD8^+$ T cells expressing *TCF7*, *SELL* and *IL7R* as root cells, and $CD8^+PD1^+$ T cells as their endpoints (Fig. 3e, f), indicating a local developmental trajectory of $CD8^+$ T cells into $CD8^+PD1^+$ T cells. The amount of gene expression and velocity magnitude, which indicate transcriptional activity, were increased in $CD8^+PD1^+$ T cells from mice and humans with NASH (Fig. (Fig.3e).3e). The expression of specific marker genes (for example, *IL7R*, *SELL*, *TCF7*, *CCL5*, *CCL3*, *PDCD1*, *CXCR6*, *RGS1* and *KLF2*) along the latent time in patients with NAFLD or NASH differed from that seen in control participants (Fig. (Fig.3g),3g), and correlated with the expression patterns seen in $CD8^+$ T cells from NASH mice (Fig. (Fig.3h).3h). Thus, scRNA-seq analysis demonstrated a resident-like liver $CD8^+PD1^+$ T cell population in patients with NAFLD or NASH that shared gene expression patterns with hepatic $CD8^+PD1^+$ T cells from NASH mice.

Different stages of NASH severity are considered to herald the development of liver cancer[31]. Indeed, different fibrosis stages (F0–F4) in patients with NASH correlated directly with the expression of *PDCD1*, *CCL2*, *IP10* and *TNF*, and the degree of fibrosis correlated with the numbers of $CD4^+$, $PD1^+$, and $CD8^+$ T cells (Extended Data Fig. 10a–d, Supplementary Table 3). Moreover, $PD1^+$ cells were absent from healthy livers but present in the livers of patients with NASH or NASH–HCC, but the number of these cells did not differ with the underlying fibrosis level (Extended Data Fig. 10e, Supplementary Tables 4–6). Species-specific effects, such as the absence in mice of cirrhosis or burnt-out NASH (a condition found in some patients with NASH–HCC[32]), and their possible influence on immunotherapy may make it difficult to translate findings from preclinical models of NASH to human NASH. However, in tumour tissue from patients with NASH-induced HCC—treated with anti-PD1 therapy—we found increased numbers of intra-tumoral $PD1^+$ cells compared to patients with HCC and viral hepatitis (Extended Data Fig. 10f). Thus, we found a shared gene-expression profile and increased abundance of unconventionally activated hepatic $CD8^+PD1^+$ T cells in human NASH tissue.

Lack of immunotherapy response in human NASH–HCC

To explore the concept of disrupted immune surveillance in NASH after anti-PD1 or anti-PDL1 treatment, we conducted a meta-analysis of three large randomized controlled phase III trials of immunotherapies in patients with advanced HCC (CheckMate-459[11], IMbrave150[5] and KEYNOTE-240[10]). Although immunotherapy improved survival in the overall population (hazard ratio (HR) 0.77; 95% confidence interval (CI) 0.63–0.94), survival was superior to the control arm in patients with HBV-related HCC (n = 574; P = 0.0008) and HCV-related HCC (n = 345; P = 0.04), but not in patients with non-viral HCC (n = 737; P = 0.39) (Fig. (Fig.4a,4a, Extended Data Fig. 10g, Supplementary Table 7). Patients with viral aetiology (HBV or HCV infection) of liver damage and HCC showed a benefit from checkpoint inhibition (HR 0.64; 95% CI 0.48–0.94), whereas patients with HCC of a non-viral aetiology did not (HR 0.92; 95% CI 0.77–1.11; P of interaction = 0.03 (Fig. (Fig.4a)).4a)). Subgroup analysis of first-line treatment compared to a control arm treated with sorafenib (n = 1,243) confirmed that immunotherapy was superior in patients with HBV-related (n = 473; P = 0.03) or HCV-related HCC (n = 281; P = 0.03), but not in patients with non-viral HCC (n = 489; P = 0.62; Extended Data Fig. 10h–j). We acknowledge that these results were

derived from a meta-analysis of trials that included different lines of treatment and patients with heterogeneous liver damage, and did not differentiate between alcoholic liver disease and NAFLD or NASH. Nevertheless, the results of this meta-analysis supported the notion that stratification of patients according to the aetiology of their liver damage and ensuing HCC identified patients who responded well to therapy.

To specifically characterize the effect of anti-PD(L)1 immunotherapy with respect to underlying liver disease, we investigated a cohort of 130 patients with HCC (patients with NAFLD $n = 13$; patients with other aetiologies $n = 117$) (Supplementary Table 8). NAFLD was associated with shortened median overall survival after immunotherapy (5.4 months (95% CI 1.8–9.0 months) versus 11.0 months (95% CI 7.5–14.5 months); $P = 0.023$), even though patients with NAFLD had less frequent macrovascular tumour invasion (23% versus 49%), and immunotherapy was more often used as a first-line therapy in these patients (46% versus 23%; Fig. Fig.4b).4b). After correction for potentially confounding factors that are relevant for prognosis, including severity of liver damage, macrovascular tumour invasion, extrahepatic metastases, performance status, and alpha-fetoprotein (AFP), NAFLD remained independently associated with shortened survival of patients with HCC after anti-PD1-treatment (HR 2.6; 95% CI 1.2–5.6; $P = 0.017$, Supplementary Table 9). This finding was validated in a further cohort of 118 patients with HCC who were treated with PD(L)1-targeted immunotherapy (patients with NAFLD $n = 11$; patients with other aetiologies $n = 107$) (Supplementary Table 10). NAFLD was again associated with reduced survival of patients with HCC (median overall survival 8.8 months, 95% CI 3.6–12.4 months) compared to other aetiologies of liver damage (median overall survival 17.7 months, 95% CI 8.8–26.5 months; $P = 0.034$) (Fig. (Fig.4c).4c). Given the relatively small number of patients with NAFLD in both cohorts, these data need prospective validation. However, collectively these results indicate that patients with underlying NASH did not benefit from checkpoint-inhibition therapy.

Liver cancer develops primarily on the basis of chronic inflammation. The latter can be activated by immunotherapy to induce tumour regression in a subset of patients with liver cancer. However, the identification of patients who will respond to immunotherapy for HCC remains difficult. Our data identify a non-viral aetiology of liver damage and cancer (that is, NASH) as a predictor of unfavourable outcome in patients treated with immune-checkpoint inhibitors. The better response to immunotherapy in patients with virus-induced HCC than in patients with non-viral HCC might be due to the amount or quality of viral antigens or to a different liver micro-environment, possibly one that does not impair immune surveillance. These results might also have implications for patients with obesity and NALFD or NASH who have cancer at other organ sites (for example, melanoma, colon carcinoma, or breast cancer) and are at risk for liver damage and the development of liver cancer in response to systemically applied immunotherapy. Overall, our results provide comprehensive mechanistic insight and a rational basis for the stratification of patients with HCC according to their aetiology of liver damage and cancer for the design of future trials of personalized cancer therapy.

Methods

Mice, diets, and treatments

Standard mouse diet feeding (ad libitum water and food access) and treatment regimens were as described previously[17]. Male mice were housed at the German Cancer Research Center (DKFZ) (constant temperature of 20–24 °C and 45–65% humidity with a 12-h light–dark cycle). Mice were maintained under specific pathogen-free conditions and experiments were performed in accordance with German law and the governmental bodies, and with approval from the Regierungspräsidium Karlsruhe (G11/16, G129/16, G7/17). Tissues from inducible knock-in mice expressing the human unconventional prefoldin RPB5 interactor were received from N.

Djouder[17,33]. The plasmids for hydrodynamic tail-vein delivery have been described previously[34–37]. For interventional studies, male mice fed a CD-HFD were treated with bi-weekly for 8 weeks by intravenous injection of 25 μg CD8-depleting antibody (Bioxcell, 2.43), 50 μg NK1.1-depleting antibody (Bioxcell, PK136), 300 μg anti-PDL1 (Bioxcell, 10F.9G2), 200 μg anti-TNF (Bioxcell, XT3.11), 100 μg anti-CD4 (Bioxcell, GK1.5), or 150 μg anti-PD1 (Bioxcell, RMP1-14). $PD1^{-/-}$ mice were kindly provided by G. Tiegs and K. Neumann. Mice for Extended Data Fig. Fig.3g3g were treated with anti-PD1 antibody (Bioxcell, RMP1-14) or isotype control (Bioxcell, 2A3) at an initial dose of 500 μg intraperitoneally (i.p.) followed by doses of 200 μg i.p. bi-weekly for 8 weeks. Mice for Extended Data Fig. Fig.3h3h were treated i.p. with anti-PD1 (200 μg, Bioxcell, RMP1-14) or IgG (200 μg, Bioxcell, LTF-2). The treatment regimen for Extended Data Fig. Fig.3i3i was as described elserwhere[38].

Intraperitoneal glucose tolerance test and measurement of serum parameters were as described previously[17].

Magnetic resonance Imaging

MRI was done in the small animal imaging core facility in DKFZ using a Bruker BioSpec 9.4 Tesla (Ettlingen). Mice were anaesthetized with 3.5% sevoflurane, and imaged with T2-weighted imaging using a T2_TurboRARE sequence: TE = 22 ms, TR = 2,200 ms, field of view (FOV) 35 × 35 mm, slice thickness 1 mm, averages = 6, scan time 3 min 18 s, echo spacing 11 ms, rare factor 8, slices 20, image size 192 × 192 pixels, resolution 0.182 × 0.182 mm.

Multiplex ELISA

Liver homogenates were prepared as for western blotting[17] and cytokines or chemokines were analysed on a customized ELISA according to the manufacturer's manual (Meso Scale Discovery, U-PLEX Biomarker group 1, K15069L-1).

Flow cytometry and FACS

Isolation and staining of lymphocytes

After perfusion and mechanical dissection, livers were incubated for up to 35 min at 37 °C with collagen IV (60 U final concentration (f.c.)) and DNase I (25 μg/ml f.c.), filtered at 100 μm, and washed with RPMI1640 (11875093, Thermo Fisher). Next, samples underwent a two-step Percoll gradient (25%/50% Percoll/HBSS) and centrifugation for 15 min at 1,800*g* and 4 °C. Enriched leukocytes were then collected, washed, and counted. For re-stimulation, cells were incubated for 2 h at 37 °C under 5% CO_2 with 1:500 Biolegend´s Cell Activation Cocktail (with brefeldin A) (423304) and 1:1,000 Monensin Solution (420701). Live/dead discrimination was done using DAPI or ZombieDyeNIR according to the manufacturer's instructions with subsequent staining of titrated antibodies (Supplementary Tables 12–14). Samples for flow cytometric-activated cell sorting (FACS) were sorted and samples for flow cytometry were fixed using eBioscience IC fixation (00-8222-49) or FOXP3 Fix/Perm kit (00-5523-00) according to the manufacturer's instructions. Intracellular staining was performed in eBioscience Perm buffer (00-8333-56). Cells were analysed using BD FACSFortessa or BD FACSSymphony and data were analysed using FlowJo (v10.6.2). For sorting, FACS Aria II and FACSAria FUSION were used in collaboration with the DKFZ FACS core facility.

For UMAP and FlowSOM plots, BD FACSymphony data (mouse and human) were exported from FlowJo (v10). Analyses were performed as described elsewhere[39].

Single-cell RNA-seq and metacell analysis (mouse)

Single-cell capturing for scRNA-seq and library preparation were done as described previously[40]. Libraries (pooled at equimolar concentration) were sequenced on an Illumina NextSeq 500 at a median sequencing depth of ~40,000 reads per cell. Sequences were mapped to the mouse genome (mm10), using HISAT (version 0.1.6); reads with multiple mapping positions were excluded. Reads were associated with genes if they were mapped to an exon, using the Ensembl gene annotation database (Ensembl release 90). Exons of different genes that shared a genomic position on the same strand were considered to represent a single gene with a concatenated gene symbol. The level of spurious unique molecular identifiers (UMIs) in the data was estimated by using statistics on empty MARS-seq wells and excluded rare cases with estimated noise >5% (median estimated noise overall for experiments was 2%). Specific mitochondrial genes, immunoglobulin genes, genes linked with poorly supported transcriptional models (annotated with the prefix "Rp-"), and cells with fewer than 400 UMIs were removed. Gene features were selected using Tvm = 0.3 and a minimum total UMI count >50. We carried out hierarchical clustering of the correlation matrix between those genes (filtering genes with low coverage and computing correlation using a down-sampled UMI matrix) and selected the gene clusters that contained anchor genes. We used $K = 50$, 750 bootstrap iterations, and otherwise standard parameters. Subsets of T cells were obtained by hierarchical clustering of the confusion matrix and supervised analysis of enriched genes in homogeneous groups of metacells[41].

Velocity and correlation analyses of scRNA-seq data

Velocyto (0.6) was used to estimate the spliced and unspliced counts from the pre-aligned bam files[42]. RNA velocity, latent time, root, and terminal states were calculated using the dynamical velocity model from scvelo (0.2.2)[43]. Kendall's rank correlation coefficient (τ) was used to correlate the expression patterns of biologically significant genes with latent time.

Preparation for mass spectrometry, data acquisition, and data analysis

After FACS purification, cells were resuspended in 50% (vol/vol) 2,2,2-trifluoroethanol in PBS pH 7.4 buffer and lysed by repeated sonication and freeze–thaw cycles. Proteins were denatured at 60 °C for 2 h, reduced using dithiothreitol at a final concentration of 5 mM (30 min at 60 °C), cooled to room temperature, alkylated using iodoacetamide at 25 mM (30 min at room temperature in the dark), and diluted 1:5 using 100 mM ammonium bicarbonate, pH 8.0. Proteins were digested overnight by trypsin (1:100 ratio, 37 °C), desalted using C18-based stage-tips, dried under vacuum, resuspended in 20 μl HPLC-grade water with 0.1% formic acid, and measured using A380.

We used 0.5 μg of peptides for proteomic analysis on a C18 column using a nano liquid chromatography system (EASY-nLC 1200, Thermo Fisher Scientific). Peptides were eluted using a gradient of 5–30% buffer B (80% acetonitrile and 0.1% formic acid) at a flow rate of 300 nl/min at a column temperature of 55 °C. Data were acquired by data-dependent Top15 acquisition using a high-resolution orbitrap tandem mass spectrometer (QExactive HFX, Thermo Scientific). All MS1 scans were acquired at 60,000 resolution with AGC target of 3×10^6, and MS2 scans were acquired at 15,000 resolution with AGC target of 1×10^5 and maximum injection time of 28 ms. Analyses were performed using MaxQuant (1.6.7.0), mouse UniProt Isoform fasta (Version: 2019-02-21, number of sequences 25,233) as a source for protein sequences. One per cent FDR was used for controlling at the peptide and protein levels, with a minimum of two peptides needed for consideration of analysis. GSEA was performed using ClusterProfiler (3.18)[44] and gene sets obtained from WikiPathway (https://www.wikipathways.org/) and MSigDB (https://broadinstitute.org/msigdb)[45–47].

Histology, immunohistochemistry, scanning, and automated analysis

Histology, immunohistochemistry, scanning, and automated analysis have been described previously[17]. Antibodies used in this manuscript are described in Supplementary Table 12. For immunofluorescence staining, established antibodies were used, coupled with the AKOYA Biosciences Opal fluorophore kit (Opal 520 FP1487001KT, Opal 540 FP1494001KT, Opal 620 FP1495001KT). For mRNA in situ hybridization, freshly non-baked 5 μm formalin-fixed paraffin-embedded sections were cut and stained according to the manufacturer's (ACD biotech) protocol for manual assay RNAscope, using probes PDL1 (420501), TNF (311081) and CXCR6 (871991).

Isolation of RNA and library preparation for bulk RNA sequencing

RNA isolation[17] and library preparation for bulk 3′-sequencing of poly(A)-RNA was as described previously[48]. Gencode gene annotations version M18 and the mouse reference genome major release GRCm38 were derived from https://www.gencodegenes.org/. Dropseq tools v1.12[49] were used for mapping the raw sequencing data to the reference genome. The resulting UMI-filtered count matrix was imported into R v3.4.4. Before differential expression analysis with Limma v3.40.6[50] sample-specific weights were estimated and used as coefficients alongside the experimental groups as a covariate during model fitting with Voom. *t*-test was used for determining differentially ($P < 0.05$) regulated genes between all possible experimental groups. GSEA was conducted with the pre-ranked GSEA method[46] within the MSigDB Reactome, KEGG, and Hallmark databases (https://broadinstitute.org/msigdb). Raw sequencing data are available at European Nucleotide Archive (https://www.ebi.ac.uk/ena/browser/home) under the accession number PRJEB36747.

Stimulation of CD8 T cells

Stimulation of CD8 T cells was as described elsewhere[27].

Flow cytometry of human biopsies

Analysis of patient material (Supplementary Table 1) was performed on liver tissue (needle biopsies or resected tissue, BIOFACS Study KEK 2019-00114), which were obtained from the patient collection nAC-2019-3627 (CRB03) from the biological resource centre of CHU Grenoble-Alpes (nBRIF BB-0033-00069). Tissue samples were minced using scalpels, incubated (with 1 mg/ml collagenase IV (Sigma Aldrich), 0.25 μg/ml DNase (Sigma Aldrich), 10% FCS (Thermo Fisher Scientific), RPMI 1640 (Seraglob)) for 30 min at 37 °C, stopping enzymatic reactions with 2 mM EDTA (StemCell Technologies, Inc.) in PBS. After filtering through a 100-μm cell strainer, cells were resuspended in FACS buffer (PBS, EDTA 2 mM, FCS 0.5%) with Human TruStain FcX (Fc Receptor Blocking Solution) (Biolegend), incubated for 15 min at 4 °C and stained with antibodies (Supplementary Table 13).

Flow cytometry of human samples (Extended Data Fig. Fig.9f)9f) was approved by the local ethical committee (AC-2014-2094 n 03).

High-throughput RNA-seq of human samples

As previously reported, RNA-seq analysis was performed using the data from 206 snap-frozen biopsy samples from 206 patients diagnosed with NAFLD in France, Germany, Italy, and the UK and enrolled in the European NAFLD Registry (GEO accession GSE135251)[51,52]. Samples were scored for NAS by two pathologists[53]. Alternate diagnoses were excluded, including excessive alcohol intake (30 g per day for males, 20 g for females), viral hepatitis, autoimmune liver diseases, and steatogenic medication use. Patient samples were grouped: NAFL ($n = 51$) and NASH with fibrosis stages of F0/1 ($n = 34$), F2 ($n = 53$), F3 ($n = 54$) and F4 ($n = 14$). Collection and use of data

of the European NAFLD Registry were approved by the relevant local and/or national Ethical Review Committee[51]. A correction for sex, batch, and centre effects was implemented. Pathway enrichment and visualization were as described elsewhere[52,54,55].

Immunohistochemistry of NAFLD/NASH cohort

Sixty-five human FFPE biopsies from patients with NAFLD were included (Supplementary Table 3). Sequential slides were immunostained with antibodies against human CD8 (Roche, SP57, ready-to-use), PD1 (Roche; NAT105, ready-to-use), and CD4 (Abcam, ab133616, 1:500). All staining was performed on the VENTANA BenchMark autostainer at 37 °C. Immunopositive cells were quantified at 400× magnification in the portal tract and the adherent parenchyma.

Isolation of cells for scRNA-seq data analysis (human)

Analyses used liver samples from patients undergoing bariatric surgery at the Department of Surgery at Heidelberg University Hospital (S-629/2013). Samples were preserved by FFPE for pathological evaluation and single cells were generated by mincing, using the Miltenyi tumour dissociation kit (130-095-929) per the manufacturer's instructions, filtering through a 70-µm cell strainer and washing. ACK lysis using the respective buffer (Thermo Fischer Scientific A1049201) was performed, and samples were stored in FBS with 20% DMSO until further processing (scRNA-seq analysis and mass cytometry).

Cells were thawed in a 37 °C water bath, washed with PBS + 0.05 mM EDTA (10 min, 300*g* at 4 °C), Fc receptor-block (10 min at 4 °C), stained with CD45-PE (3 µl, Hl30, 12-0459-42) and Live/Dead discrimination (1:1,000, Thermofischer, L34973), washed and sorted on a FACSAria FUSION in collaboration with the DKFZ FACS. Library generation was performed according to the manufacturer's protocol (Chromium Next EM Single Cell 3′GEM, 10000128), and sequencing was performed on an Illumina NovaSeq 6000. De-multiplexing and barcode processing were performed using the Cell Ranger Software Suite (Version 4.0.0) and reads were aligned to human GRCh38[56]. A gene–barcode matrix containing cell barcodes and gene expression counts was generated by counting the single-cell 3′ UMIs, which were imported into R (v4.0.2), where quality control and normalization were executed using Seurat v3[57]. Cells with more than 10% mitochondrial genes, fewer than 200 genes per cell, or more than 6,000 genes per cell were excluded. Matrices from 10 samples were integrated with Seurat v3 to remove batch effects across samples. PCA analysis of filtered gene–barcode matrices of all $CD3^+$ cells, visualized by UMAP (top 50 principal components), and identification of major cell types using the highly variable features and indicative markers were performed. Pairwise comparisons of $CD4^+$ T cells versus $CD4^+PD1^+$ T cells and $CD8^+$ T cells versus $CD8^+PD1^+$ T cells were performed using the results of differential expression analysis by DESeq2 (v1.28.1)[58], setting $CD4^+$/$CD8^+$ T cells as controls. Volcano plots were then generated using EnhancedVolcano (v1.6.0)[59] to visualize the results of differential expression analysis.

Mass cytometry data analysis (human)

Antibody conjugates for mass cytometry were purchased from Fluidigm, generated in-house using antibody labelling kits (Fluidigm X8, MCP9), or as described before[60,61]. Antibody cocktails for mass cytometry were cryopreserved as described before[62]. Isolation of cells is described in 'Isolation of cells for scRNA-seq data analysis (human)'. Cells were thawed, transferred into RPMI + benzonase (14 ml RPMI + 0.5 µl benzonase), and centrifuged for 5 min at 500*g*. The cell pellet was resuspended in 1 ml CSM-B (CSM (PBS 0.5% BSA 0.02% sodium azide) +1 µl benzonase), filtered through a 30-µm cell strainer, adjusted to 3 ml, counted, resuspended in 35 µl CSM-B and incubated for 45 min at 4 °C, and 100 µl CSM-B was added. Cells were pooled and stained with a

surface antibody cocktail (Supplementary Table 15) for 30 min at 4 °C. Dead cell discrimination was performed with mDOTA-103Rh (5 min, room temperature). For intracellular staining, the FOXP3 intracellular staining kit from Miltenyi Biotec was used per the manufacturer's instructions, followed by staining for intracellular targets for 30 min at room temperature. Cells were washed, resuspended in 1 ml of iridium intercalator solution, and incubated for 25 min at room temperature. Cells were washed with CSM, PBS, and MilliQ water, adjusted to a final concentration of 7.5×10^5 cells/ml and supplemented with 4-element EQ beads. The sample was acquired on a Helios mass cytometer and raw data were EQ-Bead-normalized using Helios mass cytometer and Helios instrument software (version 6.7). Compensation was performed in CATALYST (v1.86)[63] and FlowCore (1.50.0). De-barcoding and gating of single, live $CD45^+$ cells were performed using FlowJo (v10.6.2). Then, data from $CD45^+$ cells were imported into Cytosplore 2.3.1 and transformed using the arcsinh(5) function. Major immune cell lineages were identified at the first level of a two-level hierarchical stochastic neighbour embedding (HSNE) analysis with default perplexity and iteration settings. HSNE with the same parameters was run on $CD3^+$ cells to identify T cell phenotypes. Gaussian mean shift clustering was performed in Cytosplore and a heat map of arcsinh(5)-transformed expression values of all antibody targets was generated. Cell type identification was based on the transformed expression values and clusters showing high similarity were merged manually.

Histological and immunohistochemical analysis of NASH–HCC cohort

Four healthy samples, 16 samples from patients with NASH cases, and non-tumoral tissue adjacent to HCC tumours from patients of the following aetiologies were selected: NASH (n = 26), viral hepatitis (n = 19 HCV, n = 3 HBV), alcohol (n = 5), and other (n = 2). All samples were obtained from International Genomic HCC Consortium with IRB approval. After heat-induced antigen retrieval (10 mM sodium citrate buffer (pH 6.0) or Universal HIER antigen retrieval reagent (ab208572) for 15 min (3 × 5 min), the reaction was quenched using 3% hydrogen peroxide. Samples were washed with PBS and incubated with anti-CD8 (Cell Signaling, Danvers, MA) or anti-PD1 (NAT105, ab52587). DAB (3,3′-diaminobenzidine) was used as a detection system (EnVision+ System-HRP, Dako). PD1-positive cases were defined by considering median positivity by immunohistochemistry[64] and using a cutoff of ≥1% of PD1-positive lymphocytes among all lymphocytes present on each slide. Analysis of human samples from the Department of Pathology and Molecular Pathology, University Hospital Zurich (Extended Data Fig. Fig.10),10), was approved by the local ethics committee (Kantonale Ethikkommission Zürich, KEK-ZH-Nr. 2013-0382 and BASEC-Nr. PB_2018-00252).

Search strategy, selection criteria, and meta-analysis of phase III clinical trials

The literature search was done through MEDLINE on PubMed, Cochrane Library, Web of Science, and clinicaltrials.gov, using the following searches: 'checkpoint inhibitors', 'HCC', 'phase III', between January 2010 and January 2020, and complemented by manual searches of conference abstracts and presentations. Single-centre, non-controlled trials, studies with insufficient data to extract HRs or 95% confidence intervals, and trials including disease entities other than HCC were excluded. As conference abstracts were not excluded, quality assessment of the included studies was not performed. Three studies[5,10,11] fulfilled the criteria and were included in the quantitative synthesis (Extended Data Fig. Fig.10).10). The primary outcome of the meta-analysis was overall survival, defined as the time from randomization to death. HRs and CIs related to overall survival were extracted from the papers or conference presentations[5,10,11]. Pooled HRs were calculated using the random-effects model and we used the DerSimonian–Laird method to estimate τ^2, and the generic inverse variance was used for calculating weights [65]. To evaluate heterogeneity among studies, Cochran's Q test and I^2 index were used. $P < 0.10$ in the Q-test was considered to indicate substantial heterogeneity. I^2 was interpreted as suggested in the literature: 0% to 40% might not

represent significant heterogeneity; 30% to 60% may represent moderate heterogeneity; 50% to 90% may represent substantial heterogeneity; 75% to 100% represents considerable heterogeneity. All statistical pooled analyses were performed using RevMan 5.3 software.

A cohort of patients with HCC treated with PD(L)1-targeted immunotherapy

The retrospective analysis was approved by local Ethics Committees. Data from this cohort were published previously[66]. Patients with liver cirrhosis and advanced-stage HCC treated with PD(L)1-targeted immune checkpoint blockers from 12 centres in Austria, Germany, Italy, and Switzerland were included. The χ^2 test or Fisher's exact test were used to compare nominal data. Overall survival was defined as the time from the start of checkpoint inhibitor treatment until death. Patients who were still alive were censored at the date of the last contact. Survival curves were calculated by the Kaplan–Meier method and compared by using the log-rank test. Multivariable analysis was performed by a Cox regression model. Statistical analyses were performed using IBM SPSS Statistics version 25 (SPSS Inc., Chicago, IL).

A validation cohort of patients with HCC treated with PD1-targeted immune checkpoint blockers

A multi-institutional dataset that included 427 patients with HCC treated with immune checkpoint inhibitors between 2017 and 2019 in 11 tertiary-care referral centres specialized in the treatment of HCC was analysed. Clinical outcomes of this patient cohort have been reported elsewhere[67,68]. Inclusion criteria were: 1) diagnosis of HCC made by histopathology or imaging criteria according to American Association for the Study of Liver Disease and European Association for the Study of the Liver guidelines; 2) systemic therapy with immune checkpoint inhibitors for HCC that was not amenable to curative or loco-regional therapy following local multidisciplinary tumour board review; 3) measurable disease according to RECIST v1.1 criteria at commencement of treatment with immune checkpoint inhibitors. One hundred and eighteen patients with advanced-stage HCC were recruited with Child–Pugh A liver functional reserve, and documented radiologic or clinical diagnosis of cirrhosis. Ethical approval to conduct this study was granted by the Imperial College Tissue Bank (reference number R16008).

Statistical analyses

No statistical methods were used to predetermine sample size. The experiments were not randomized and the investigators were not blinded to allocation during experiments and outcome assessment. Data were collected in Microsoft Excel. Mouse data are presented as the mean ± s.e.m. Pilot experiments and previously published results were used to estimate the sample size, such that appropriate statistical tests could yield significant results. Statistical analysis was performed using GraphPad Prism software version 7.03 (GraphPad Software). Exact *P* values lower than $P < 0.1$ are reported and specific tests are indicated in the legends.

Sample sizes, biological replicates and statistical tests

Fig. Fig.1a:1a: PD1, $n = 5$ mice/group; CD8, ND $n = 6$ mice; CD-HFD $n = 6$ mice; WD-HTF $n = 5$ mice. Scale bar, 100 μm. Fig. Fig.1b:1b: $n = 3$ mice/group. Scale bar, 100 μm. Fig. Fig.1c:1c: ND $n = 4$ mice, CD-HFD $n = 6$ mice. Fig. 1d, e: $n = 3$ mice/group. Fig. Fig.1f:1f: tumour incidence: CD-HFD, $n = 19$ tumours/lesions in 25 mice; CD-HFD + anti-PD1, $n = 10$ tumours/lesions in 10 mice. Fig. Fig.1h:1h: ND, $n = 3$ mice; CD-HFD, $n = 13$ mice; CD-HFD + anti-PD1, $n = 8$ mice; intra-tumoral staining: CD-HFD, $n = 11$ mice; CD-HFD + anti-PD1, $n = 8$ mice. Data in Fig. 1a, h were analysed by two-tailed Student's *t*-test. Data in Fig. Fig.1f1f were analysed by two-sided Fisher's exact test.

Fig. 2a, b: n = 3 mice/group. Fig. Fig.2c:2c: $CD8^+$: ND, n = 6 mice; CD-HFD + IgG, n = 5 mice; CD-HFD + anti-PD1, n = 6 mice; $CD8^+PD1^+$: ND, n = 4 mice, CD-HFD + IgG, n = 6 mice; CD-HFD + anti-PD1, n = 6 mice. Fig. 2d, e: ND, n = 4 mice; CD-HFD + IgG, n = 8 mice; CD-HFD + anti-PD1, n = 6 mice. Fig. Fig.2f:2f: CD-HFD + IgG, n = 6 mice; CD-HFD + anti-PD1, n = 4 mice. Fig. Fig.2g:2g: ND, n = 30 mice; CD-HFD, n = 47 mice; CD-HFD + anti-PD1, n = 35 mice; CD-HFD + anti-PD1/anti-CD8, n = 9 mice; CD-HFD + anti-TNF, n = 10 mice; CD-HFD + anti-PD1/anti-TNF, n = 11 mice; CD-HFD + anti-CD4, n = 8 mice; CD-HFD + anti-PD1/anti-CD4, n = 8 mice. Fig. Fig.2h:2h: $CD8^+PD1^+CXCR6^+$: ND, n = 30 mice; CD-HFD, n = 47 mice; CD-HFD + anti-PD1, n = 35 mice; CD-HFD + anti-PD1/anti-CD8, n = 9 mice; CD-HFD + anti-TNF, n = 10 mice; CD-HFD + anti-PD1/anti-TNF, n = 11 mice; CD-HFD + anti-CD4, n = 8 mice; CD-HFD + anti-PD1/anti-CD4, n = 8 mice. Fig. Fig.2j:2j: tumour incidence: CD-HFD, n = 32 tumours/lesions in 87 mice; CD-HFD + anti-CD8, n = 2 tumours/lesions in 31 mice; CD-HFD + anti-CD8/NK1.1, n = 0 tumours/lesions in 6 mice; CD-HFD + anti-PD1, n = 33 tumours/lesions in 44 mice; CD-HFD + anti-PD1/anti-CD8, n = 2 tumours/lesions in 9 mice; CD-HFD + anti-TNF, n = 3 tumours/lesions in 10 mice; CD-HFD + anti-PD1/anti-TNF, n = 3 tumours/lesions in 11 mice; CD-HFD + anti-CD4, n = 3 tumours/lesions in 9 mice; CD-HFD + anti-PD1/anti-CD4, n = 8 tumours/lesions in 9 mice. All data are shown as mean ± s.e.m. Data in Fig. 2e, g, h were analysed by one-way ANOVA and Fisher's LSD test. Data in Fig. Fig.2f2f were analysed by two-tailed Mann–Whitney test. Data in Fig. Fig.2j2j were analysed by two-sided Fisher's exact test.

Fig. 3a, b: control, n = 6 patients; NAFLD/NASH, n = 11 patients. Fig. Fig.3c:3c: control, n = 4 patients; NAFLD/NASH, n = 7 patients. Fig. 3d–h: mouse, n = 3; human, n = 3. All data are shown as mean ± s.e.m. Data in Fig. 3b, f were analysed by two-tailed Mann–Whitney test. Data in Fig. Fig.3d3d were analysed by two-tailed Spearman's correlation.

Fig. Fig.4a:4a: Hazard ratios are represented by squares, the size of the square represents the weight of the trial in the meta-analysis. Cochran's Q-test and I^2 were used to calculate heterogeneity. Fig. Fig.4b:4b: Kaplan–Meier curve displays overall survival of patients with NAFLD versus those with any other aetiology; all 130 patients were included in these survival analyses (NAFLD n = 13; any other aetiology n = 117). Fig. Fig.4c:4c: Kaplan–Meier curve displays overall survival of patients with NAFLD versus those with any other aetiology (NAFLD n = 11; any other aetiology n = 107). Data in Fig. 4b, c were analysed by Kaplan–Meier method and compared using log rank test.

References

1. Llovet JM, et al. Hepatocellular carcinoma. *Nat. Rev. Dis. Primers.* 2021;7:6. doi: 10.1038/s41572-020-00240-3.

2. European Association for the Study of the Liver EASL Clinical Practice Guidelines management of hepatocellular carcinoma. *J. Hepatol.* 2018;69:182–236. doi: 10.1016/j.jhep.2018.03.019.

3. Zhu AX, et al. Pembrolizumab in patients with advanced hepatocellular carcinoma previously treated with sorafenib (KEYNOTE-224): a non-randomised, open-label phase 2 trial. *Lancet Oncol.* 2018;19:940–952. doi: 10.1016/S1470-2045(18)30351-6.

4. El-Khoueiry AB, et al. Nivolumab in patients with advanced hepatocellular carcinoma (CheckMate 040): an open-label, non-comparative, phase 1/2 dose escalation and expansion trial. *Lancet.* 2017;389:2492–2502. doi: 10.1016/S0140-6736(17)31046-2.

5. Finn RS, et al. Atezolizumab plus bevacizumab in unresectable hepatocellular carcinoma. *N. Engl. J. Med.* 2020;382:1894–1905. doi: 10.1056/NEJMoa1915745.

6. Roderburg C, Wree A, Demir M, Schmelzle M, Tacke F. The role of the innate immune system in the development and treatment of hepatocellular carcinoma. *Hepatic Oncol.* 2020;7:HEP17. doi: 10.2217/hep-2019-0007.

7. Pinter M, Scheiner B, Peck-Radosavljevic M. Immunotherapy for advanced hepatocellular carcinoma: a focus on special subgroups. *Gut.* 2021;70:204–214. doi: 10.1136/gutjnl-2020-321702.

8. Duffy AG, et al. Tremelimumab in combination with ablation in patients with advanced hepatocellular carcinoma. *J. Hepatol.* 2017;66:545–551. doi: 10.1016/j.jhep.2016.10.029.

9. Sangro B, et al. A clinical trial of CTLA-4 blockade with tremelimumab in patients with hepatocellular carcinoma and chronic hepatitis C. *J. Hepatol.* 2013;59:81–88. doi: 10.1016/j.jhep.2013.02.022.

10. Finn RS, et al. Pembrolizumab as second-line therapy in patients with advanced hepatocellular carcinoma in KEYNOTE-240: a randomized, double-blind, phase III trial. *J. Clin. Oncol.* 2020;38:193–202. doi: 10.1200/JCO.19.01307.

11. Yau T, et al. CheckMate 459: A randomized, multi-center phase III study of nivolumab (NIVO) vs sorafenib (SOR) as first-line (1L) treatment in patients (pts) with advanced hepatocellular carcinoma (aHCC) *Ann. Oncol.* 2019;30:v874–v875. doi: 10.1093/annonc/mdz394.029.

12. van der Leun AM, Thommen DS, Schumacher TN. $CD8^+$ T cell states in human cancer: insights from single-cell analysis. *Nat. Rev. Cancer.* 2020;20:218–232. doi: 10.1038/s41568-019-0235-4.

13. Sangro B, et al. Association of inflammatory biomarkers with clinical outcomes in nivolumab-treated patients with advanced hepatocellular carcinoma. *J. Hepatol.* 2020;73:1460–1469. doi: 10.1016/j.jhep.2020.07.026.

14. Anstee QM, Reeves HL, Kotsiliti E, Govaere O, Heikenwalder M. From NASH to HCC: current concepts and future challenges. *Nat. Rev. Gastroenterol. Hepatol.* 2019;16:411–428. doi: 10.1038/s41575-019-0145-7.

15. Wolf MJ, et al. Metabolic activation of intrahepatic $CD8^+$ T cells and NKT cells causes nonalcoholic steatohepatitis and liver cancer via cross-talk with hepatocytes. *Cancer Cell.* 2014;26:549–564. doi: 10.1016/j.ccell.2014.09.003.

16. Ma C, et al. NAFLD causes selective $CD4^+$ T lymphocyte loss and promotes hepatocarcinogenesis. *Nature.* 2016;531:253–257. doi: 10.1038/nature16969.

17. Malehmir M, et al. Platelet GPIbα is a mediator and potential interventional target for NASH and subsequent liver cancer. *Nat. Med.* 2019;25:641–655. doi: 10.1038/s41591-019-0379-5. [PubMed] [CrossRef] [Google Scholar]

18. Nakagawa H, et al. ER stress cooperates with hypernutrition to trigger TNF-dependent spontaneous HCC development. *Cancer Cell.* 2014;26:331–343. doi: 10.1016/j.ccr.2014.07.001.

19. Ringelhan M, Pfister D, O'Connor T, Pikarsky E, Heikenwalder M. The immunology of hepatocellular carcinoma. *Nat. Immunol.* 2018;19:222–232. doi: 10.1038/s41590-018-0044-z.

20. Michelotti GA, Machado MV, Diehl AM. NAFLD, NASH and liver cancer. *Nat. Rev. Gastroenterol. Hepatol.* 2013;10:656–665. doi: 10.1038/nrgastro.2013.183.

21. Friedman SL, Neuschwander-Tetri BA, Rinella M, Sanyal AJ. Mechanisms of NAFLD development and therapeutic strategies. *Nat. Med.* 2018;24:908–922. doi: 10.1038/s41591-018-0104-9.

22. Wang Z, et al. Paradoxical effects of obesity on T cell function during tumor progression and PD-1 checkpoint blockade. *Nat. Med.* 2019;25:141–151. doi: 10.1038/s41591-018-0221-5.

23. Shalapour S, et al. Inflammation-induced IgA^{+} cells dismantle anti-liver cancer immunity. *Nature.* 2017;551:340–345. doi: 10.1038/nature24302.

24. Kim CG, et al. Hyperprogressive disease during PD-1 blockade in patients with advanced hepatocellular carcinoma. *J. Hepatol.* 2021;74:350–359. doi: 10.1016/j.jhep.2020.08.010.

25. Chung AS, et al. Immune checkpoint inhibition is safe and effective for liver cancer prevention in a mouse model of hepatocellular carcinoma. *Cancer Prev. Res.* 2020;13:911–922. doi: 10.1158/1940-6207.CAPR-20-0200.

26. Heinrich B, et al. Steatohepatitis impairs T cell-directed immunotherapies against liver tumors in mice. *Gastroenterology.* 2021;160:331–345.e6. doi: 10.1053/j.gastro.2020.09.031.

27. Dudek, M. et al. Auto-aggressive CXCR6^{+} CD8 T cells cause liver immune pathology in NASH. *Nature*10.1038/s41586-021-03233-8 (2021).

28. Moeini A, et al. An immune gene expression signature associated with development of human hepatocellular carcinoma identifies mice that respond to chemopreventive agents. *Gastroenterology.* 2019;157:1383–1397.e11. doi: 10.1053/j.gastro.2019.07.028.

29. Moscat J, Karin M, Diaz-Meco MT. p62 in cancer: signaling adaptor beyond autophagy. *Cell.* 2016;167:606–609. doi: 10.1016/j.cell.2016.09.030.

30. Kerdiles YM, et al. Foxo1 links homing and survival of naive T cells by regulating L-selectin, CCR7 and interleukin 7 receptor. *Nat. Immunol.* 2009;10:176–184. doi: 10.1038/ni.1689.

31. Taylor RS, et al. Association between fibrosis stage and outcomes of patients with nonalcoholic fatty liver disease: a systematic review and meta-analysis. *Gastroenterology.* 2020;158:1611–1625.e12. doi: 10.1053/j.gastro.2020.01.043.

32. van der Poorten D, et al. Hepatic fat loss in advanced nonalcoholic steatohepatitis: are alterations in serum adiponectin the cause? *Hepatology.* 2013;57:2180–2188. doi: 10.1002/hep.26072.

33. Tummala KS, et al. Inhibition of de novo NAD^{+} synthesis by oncogenic URI causes liver tumorigenesis through DNA damage. *Cancer Cell.* 2014;26:826–839. doi: 10.1016/j.ccell.2014.10.002.

34. Rudalska R, et al. In vivo RNAi screening identifies a mechanism of sorafenib resistance in liver cancer. *Nat. Med.* 2014;20:1138–1146. doi: 10.1038/nm.3679.

35. Kang T-W, et al. Senescence surveillance of pre-malignant hepatocytes limits liver cancer development. *Nature.* 2011;479:547–551. doi: 10.1038/nature10599.

36. Carlson CM, Frandsen JL, Kirchhof N, McIvor RS, Largaespada DA. Somatic integration of an oncogene-harboring Sleeping Beauty transposon models liver tumor development in the mouse. *Proc. Natl Acad. Sci. USA.* 2005;102:17059–17064. doi: 10.1073/pnas.0502974102.

37. Zender L, et al. Generation and analysis of genetically defined liver carcinomas derived from bipotential liver progenitors. *Cold Spring Harb. Symp. Quant. Biol.* 2005;70:251–261. doi: 10.1101/sqb.2005.70.059.

38. Shigeta K, et al. Dual programmed death receptor-1 and vascular endothelial growth factor receptor-2 blockade promotes vascular normalization and enhances antitumor immune responses in hepatocellular carcinoma. *Hepatology.* 2020;71:1247–1261. doi: 10.1002/hep.30889.

39. Brummelman J, et al. Development, application and computational analysis of high-dimensional fluorescent antibody panels for single-cell flow cytometry. *Nat. Protocols.* 2019;14:1946–1969. doi: 10.1038/s41596-019-0166-2.

40. Jaitin DA, et al. Massively parallel single-cell RNA-seq for marker-free decomposition of tissues into cell types. *Science.* 2014;343:776–779. doi: 10.1126/science.1247651.

41. Baran Y, et al. MetaCell: analysis of single-cell RNA-seq data using K-nn graph partitions. *Genome Biol.* 2019;20:206. doi: 10.1186/s13059-019-1812-2.

42. La Manno G, et al. RNA velocity of single cells. *Nature.* 2018;560:494–498. doi: 10.1038/s41586-018-0414-6.

43. Bergen V, Lange M, Peidli S, Wolf FA, Theis FJ. Generalizing RNA velocity to transient cell states through dynamical modeling. *Nat. Biotechnol.* 2020;38:1408–1414. doi: 10.1038/s41587-020-0591-3.

44. Yu G, Wang L-G, Han Y, He Q-Y. clusterProfiler: an R package for comparing biological themes among gene clusters. *OMICS.* 2012;16:284–287. doi: 10.1089/omi.2011.0118.

45. Slenter DN, et al. WikiPathways: a multifaceted pathway database bridging metabolomics to other omics research. *Nucleic Acids Res.* 2018;46:D661–D667. doi: 10.1093/nar/gkx1064.

46. Subramanian A, et al. Gene set enrichment analysis: a knowledge-based approach for interpreting genome-wide expression profiles. *Proc. Natl Acad. Sci. USA.* 2005;102:15545–15550. doi: 10.1073/pnas.0506580102.

47. Liberzon A, et al. Molecular signatures database (MSigDB) 3.0. *Bioinformatics.* 2011;27:1739–1740. doi: 10.1093/bioinformatics/btr260.

48. Parekh S, Ziegenhain C, Vieth B, Enard W, Hellmann I. The impact of amplification on differential expression analyses by RNA-seq. *Sci. Rep.* 2016;6:25533. doi: 10.1038/srep25533.

49. Macosko EZ, et al. Highly parallel genome-wide expression profiling of individual cells using nanoliter droplets. *Cell.* 2015;161:1202–1214. doi: 10.1016/j.cell.2015.05.002.

50. Ritchie ME, et al. limma powers differential expression analyses for RNA-sequencing and microarray studies. *Nucleic Acids Res.* 2015;43:e47. doi: 10.1093/nar/gkv007.

51. Hardy T, et al. The European NAFLD Registry: a real-world longitudinal cohort study of nonalcoholic fatty liver disease. *Contemp. Clin. Trials.* 2020;98:106175. doi: 10.1016/j.cct.2020.106175.

52. Govaere O, et al. Transcriptomic profiling across the nonalcoholic fatty liver disease spectrum reveals gene signatures for steatohepatitis and fibrosis. *Sci. Transl. Med.* 2020;12:eaba4448. doi: 10.1126/scitranslmed.aba4448.

53. Kleiner DE, et al. Design and validation of a histological scoring system for nonalcoholic fatty liver disease. *Hepatology.* 2005;41:1313–1321. doi: 10.1002/hep.20701.

54. Huang W, Sherman BT, Lempicki RA. Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources. *Nat. Protocols.* 2009;4:44–57. doi: 10.1038/nprot.2008.211.

55. Walter W, Sánchez-Cabo F, Ricote M. GOplot: an R package for visually combining expression data with functional analysis. *Bioinformatics.* 2015;31:2912–2914. doi: 10.1093/bioinformatics/btv300.

56. Dobin A, et al. STAR: ultrafast universal RNA-seq aligner. *Bioinformatics.* 2013;29:15–21. doi: 10.1093/bioinformatics/bts635.

57. Stuart T, et al. Comprehensive integration of single-cell data. *Cell.* 2019;177:1888–1902.e21.

58. Love MI, Huber W, Anders S. Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. *Genome Biol.* 2014;15:550. doi: 10.1186/s13059-014-0550-8.

59. Blighe, K., Rana, S. & Lewis, M. EnhancedVolcano: publication-ready volcano plots with enhanced colouring and labeling.

60. Mei HE, Leipold MD, Maecker HT. Platinum-conjugated antibodies for application in mass cytometry. *Cytometry A.* 2016;89:292–300. doi: 10.1002/cyto.a.22778.

61. Mei HE, Leipold MD, Schulz AR, Chester C, Maecker HT. Barcoding of live human peripheral blood mononuclear cells for multiplexed mass cytometry. *J. Immunol.* 2015;194:2022–2031. doi: 10.4049/jimmunol.1402661.

62. Schulz AR, et al. Stabilizing antibody cocktails for mass cytometry. *Cytometry A.* 2019;95:910–916.

63. Chevrier S, et al. Compensation of signal spillover in suspension and imaging mass cytometry. *Cell Syst.* 2018;6:612–620.e5. doi: 10.1016/j.cels.2018.02.010.

64. Veloza L, et al. Clinicopathological evaluation of the programmed cell death 1 (PD1)/programmed cell death-ligand 1 (PD-L1) axis in post-transplant lymphoproliferative disorders: association with Epstein-Barr virus, *PD-L1* copy number alterations, and outcome. *Histopathology.* 2019;75:799–812. doi: 10.1111/his.13857.

65. DerSimonian R, Laird N. Meta-analysis in clinical trials revisited. *Contemp. Clin. Trials.* 2015;45:139–145. doi: 10.1016/j.cct.2015.09.002.

66. Scheiner B, et al. Programmed cell death protein-1 (PD-1)-targeted immunotherapy in advanced hepatocellular carcinoma: efficacy and safety data from an international multicentre real-world cohort. *Aliment. Pharmacol. Ther.* 2019;49:1323–1333. doi: 10.1111/apt.15245.

67. Fessas P, et al. Post-registration experience of nivolumab in advanced hepatocellular carcinoma: an international study. *J. Immunother. Cancer.* 2020;8:e001033. doi: 10.1136/jitc-2020-001033.

68. Pinato DJ, et al. Immunotherapy in hepatocellular cancer patients with mild to severe liver dysfunction: adjunctive role of the ALBI grade. *Cancers.* 2020;12:1862.
doi: 10.3390/cancers12071862.