学位論文

Downregulation of 15-PGDH enhances MASH-HCC development via fatty acid-induced T-cell exhaustion

（15−PGDH の下方制御は脂肪酸代謝の機能不全による T 細胞の枯渇を通じて MASH−HCC の発生を促進する）

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Downregulation of 15-PGDH enhances MASH-HCC development via fatty acid-induced T-cell exhaustion

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Conflicts of Interest

The authors declare that they have no competing interests to disclose.

Running Title

15-PGDH downregulation induces T-cell exhaustion via lipid accumulation in MASH-HCC

Keywords: Chronic inflammation, MASH-HCC, NASH-HCC, PGE2, COX2, 15-PGDH, CD8+ T-cell exhaustion

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Data availability statement

The whole-exome sequencing data were deposited in the DDBJ database under accession number DRA016752. The RNA-sequencing data were deposited in the DDBJ database under accession number DRA016740. All datasets generated during the current study are available from the corresponding authors upon request.

Highlights

1. 15-PGDH downregulation in the background liver is correlated with MASH-HCC occurrence.
2. The accumulation of PGE2 mediated by 15-PGDH downregulation promotes hepatocyte proliferation.
3. 15-Pgdh downregulation in hepatocytes promotes lipid accumulation in the TME.
4. Lipid accumulation in the TME reduces mitochondrial activity and causes CD8+ T-cell exhaustion.

Abstract

Background and Aims: Hepatocellular carcinoma (HCC) mainly develops from chronic hepatitis. Metabolic dysfunction-associated steatohepatitis (MASH) has gradually become the main pathogenic factor for HCC due to the rising incidence of obesity and metabolic diseases. 15-Hydroxyprostaglandin dehydrogenase (15-PGDH) is the enzyme that degrades prostaglandin 2 (PGE2), which is known to exacerbate inflammatory responses. However, the role of PGE2 accumulation caused by 15-PGDH downregulation in the development of MASH-HCC has not been determined.

Methods: We utilized the steric animal model (STAM) to establish a MASH-HCC model using WT and 15-Pgdh+/− mice to assess the significance of PGE2 accumulation in MASH-HCC development. Additionally, we analyzed clinical samples obtained from MASH-HCC patients.

Results: We showed that PGE2 accumulation in the tumor microenvironment (TME) induced ROS production in macrophages and the expression of cell growth-related genes and antiapoptotic genes. On the other hand, the downregulation of fatty acid metabolism in the background liver promoted lipid accumulation in the TME, causing a decrease in mitochondrial membrane potential and CD8+ T-cell exhaustion, which led to enhanced MASH-HCC development.

Conclusions: 15-PGDH downregulation inactivates immune surveillance by promoting the proliferation of exhausted effector T cells, which enhances hepatocyte survival and proliferation and leads to MASH-HCC development.

Impact and Implications: The suppression of PGE2-related inflammation and subsequent lipid accumulation leads to a reduction in the severity of MASH and inhibition of subsequent MASH-HCC progression.

Introduction

Hepatocellular carcinoma (HCC) accounts for approximately 90% of all liver cancers, and despite breakthroughs in medical and surgical treatment in recent years, its 5-year survival rate is still only 18%, making HCC the second deadliest cancer after pancreatic cancer[1]. More recently, the terms metabolic dysfunction associated fatty liver disease (MAFLD) and metabolic dysfunction associated steatohepatitis (MASH) have been introduced to replace the previous nomenclature of nonalcoholic fatty liver disease (NAFLD) and nonalcoholic steatohepatitis (NASH). The adoption of these new terms, MASLD and MASH, is widely supported due to their non-stigmatizing nature and potential to enhance awareness and patient identification[2]. Moving forward, we will use the terms MAFLD and MASH to refer to what was previously recognized as NAFLD and NASH. MAFLD is a common etiology of HCC affecting approximately a quarter of the global population due to the rising incidence of obesity and metabolic diseases[3]. MAFLD is often linked with other ailments, such as chronic metabolic liver disease, and it can progress to more severe forms of liver disease, such as MASH, which is accompanied by severe hepatocellular injury with steatosis characterized by inflammation and ballooning. At least 25% of MAFLD cases are believed to develop into MASH[4,5].

Many reports have shown that chronic precancerous inflammation or tumor microenvironment (TME)-related inflammation caused by tumor cells plays a "combustion-supporting" role in cancer development, which is one of the characteristics of cancer progression[3,6]. Long-term chronic inflammation can lead to the accumulation of inflammatory cells and mediators in hepatic tissues. Prostaglandin E2 (PGE2) is an important mediator involved in inflammation and is synthesized from arachidonic acid by cyclooxygenase 2 (COX-2). PGE2 participates in various inflammatory activities in the human body[7] and contributes to tumor initiation and progression[8]. 15-Hydroxyprostaglandin dehydrogenase (15-PGDH) is known to be an enzyme that degrades PGE2 and a tumor suppressor located upstream of the COX2/PGE2 signaling pathway[9].

Although several studies have shown the functions of 15-PGDH in cancer tissue [10,11], the impact of 15-PGDH on the occurrence of MASH-HCC remains unknown.

Moreover, the advent of immunotherapy, which reactivates antitumor T cells, has revolutionized cancer treatment[12]. Although nivolumab and pembrolizumab have been approved for the treatment of advanced HCC, a recent study revealed that in MASH-HCC, anti-PD-1 drugs conversely promote HCC development due to the impairment of immune surveillance and induction of MASH-related aberrant CD8+ T-cell activation, which causes tissue damage[13]. Although MASH-HCC progression undoubtedly involves an inflammatory response and inflammatory signaling, it is unclear whether PGE2 signaling has an important role in CD8+ T-cell activation during MASH-HCC development. The present study was conducted to explore the relationship between PGE2 accumulation caused by 15-PGDH downregulation and inflammatory signaling-mediated MASH-HCC development.

Material and Methods

Patients and tissue samples

A total of 1273 HCC patients who underwent radical hepatectomy at Kumamoto University Hospital (Kumamoto, Japan) between April 2000 and December 2015 were initially enrolled. Among the 1273 patients, 1125 HCC patients with viral hepatitis and 48 patients who reported drinking ≥ 20 g/day for women and ≥30 g/day for men were excluded based on the MAFLD/MASH criteria. Finally, 100 patients with MASH-HCC were eligible for this study. Hematoxylin and eosin (H&E) staining and immunohistochemical (IHC) staining were performed on liver resection specimens from eligible patients. Preoperative blood test data and survival information were obtained from patient medical records. All patients signed an informed consent form prior to participation in this study, and the study was approved by the Medical Ethics Committee of Kumamoto University (IRB approval number: 1291).

Animal statement and steric animal model (STAM) mouse model

All mouse experiments were designed based on the Declaration of Helsinki and approved by the Kumamoto University Ethics Committee for Animal Experiments (Approval number: A28-052). Efforts were made to meet the scientific goals of this study with the minimum number of animals. Mice were randomly assigned to control and experimental groups for subsequent drug treatment. MASH-HCC was induced in male mice by a single subcutaneous injection of 100 μg STZ (Catalog #S0130, Lot #031M1287V; Sigma–Aldrich) solution (50 mg/mL) 2 days after birth, followed by feeding a high-fat diet (HFD; 57 kcal% fat, Catalog #HFD32; CLEA Japan Inc, Japan) available ad libitum from 4 to 15-20 weeks of age; the mice were sacrificed at 15 and 20 weeks old. The sequence of the 15-Pgdh-deficient mouse gRNA we used to generate a 15-Pgdh-deficient line was as follows: 5'-TGCTCCATGGCGCCAAGGTA-3'; the target site was located between exon one and intron 1 of the 15-Pgdh gene[14]. Homozygous mice carrying the 15-Pgdh mutant allele were crossed with wild-type (WT) mice to obtain 15-Pgdh–heterozygous mice. We utilized 15-Pgdh+/- littermates and 15-pgdh+/+ littermates as the WT control group.

Evaluation of the MAFLD activity score (MAS)

The activity of MASH/MAFLD was evaluated by the MAFLD activity scoring system (MAS). Briefly, the MAS comprises 4 semiquantitative items: steatosis (0-3), lobular inflammation (0-2), hepatocellular ballooning (0-2), and fibrosis (0-4)[15]. MAS was generated by evaluating H&E-stained slides.

Immunostaining and scoring methods

Paraffin-embedded sections (4 μm) obtained from MASH-HCC patients or STAM mice were deparaffinized and soaked in distilled water. Autoclave-induced antigen retrieval was performed. Endogenous peroxidase activity was blocked using 3% hydrogen peroxide. Sections were incubated with primary antibodies against 15-PGDH (ab187161, Abcam), CD86 (#19589, Cell Signaling Technology), CD163 (ab182422, Abcam), Ki-67 (AB_11219741, Spring Bioscience) overnight at 4°C. The sections were subsequently incubated with a biotin-free HRP enzyme-labeled polymer of the Envision Plus detection system (Dako, Tokyo, Japan) for 1 hour at room temperature. Positive reactions were visualized using a diaminobenzidine solution, followed by counterstaining with Mayer’s hematoxylin. All IHC staining was scored as both the intensity and percentage of cell staining. The average intensity of positively

stained cells was given an intensity score from 0 to 3 (0: none, 1: weak, 2: moderate, and 3: strong expression). The average proportion of positively stained cells was estimated and given a percentage score on a scale from 1 to 6 (1 = 0-5%, 2 = 6-20%, 3 = 21-40%, 4 = 41-60%, 5 = 61-80%, and 6 = 81-100%). The two scores were multiplied to characterize 15-PGDH, CD163, CD86, PD-1 and Ki-67 expression as low (0-7) or high (8-18). Sirius Red staining was performed to assess liver fibrosis. Briefly, sections were stained with a 0.1% Sirius Red-1.3% picric acid solution (MUTO PURE CHEMICALS CO. LTD., 33061) and quickly rinsed with acetic acid (0.5%). The positively stained area was measured as a percentage. All scoring assessments were performed independently by two investigators.

Oil Red O staining

Adipocytes and neutral fat were stained with an Oil Red O stock solution (MUTO PURE CHEMICALS CO. LTD, 40491). Briefly, frozen mouse liver sections (15 μm) were incubated with 1 ml of Oil Red O solution (Oil Red O stock solution: distilled water = 6:4) for 15 minutes at room temperature, washed with 60% isopropanol, stained with a hematoxylin solution to visualize cell nuclei, and finally mounted with a water-based mounting medium.

Preparation of single-cell suspensions

The protocol for cell isolation and fluorescence-activated cell sorting (FACS) analysis using mouse samples was described in a previous study[16]. In brief, livers were surgically removed from 15-week-old STAM mice, minced, and dissociated in medium containing Dri Tumor & Tissue Dissociation Reagent (BD) according to the manufacturer's protocol. The cell suspensions were passed through a 70-μm cell strainer (BD Falcon), hemolyzed with VersaLyse (Beckman Coulter), washed with PBS containing 2% FBS and 2 mM EDTA, and used in subsequent experiments.

Flow cytometry

All cells were adjusted to a density of $1x10^6$ cells/100 μL and stained with Fixable Viability Stain 510 (BD Biosciences) prior to surface antibody staining. Purified rat anti-mouse CD16/CD32 (Mouse BD Fc Block™, BD Biosciences) was used for subsequent Fc blocking. Antibodies for surface staining were suspended in PBS containing 2% FBS and 10% mouse serum and incubated

for 30 minutes at 4°C. The samples were then fixed using fixation buffer (BD Biosciences). For intracellular staining, cells were permeabilized with 1x eBioscience™ Permeabilization Buffer (Thermo Fisher Scientific) for 15 minutes at room temperature, and then antibodies for intracellular staining were suspended in the same permeabilization buffer and incubated for 30 minutes at 4°C. Data acquisition was performed using a BD FACSVerse™ (BD Biosciences), collecting 50,000 FVS510-CD45+ events per sample when possible. FlowJo™ software version 10 (BD Biosciences) was used to analyze the acquired data. The antibodies are listed in Supplementary Table 1.

Quantitative reverse transcription (RT)-PCR

Total RNA was extracted from dissociated cells using the miRNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. Complementary DNA (cDNA) was reverse transcribed from the isolated total RNA using SuperScript III, RNaseOUT, Recombinant Ribonuclease Inhibitor, Random Primers and Oligo(dT)12–18 Primer (Thermo Fisher Scientific). mRNA expression was quantified using SYBR Green. Reactions were performed using a LightCycler 480 System II (Roche Diagnostics). All qRT–PCR data are displayed as the means ± standard errors (SEs) of the mean. The primer sequences are listed in Supplementary Table 2.

Western blot analysis

Homogenized cells from the background liver in STAM mice were lysed with RIPA buffer containing a protease and phosphatase inhibitor cocktail (Thermo Fisher Scientific). The lysate was sonicated, debris was removed by centrifugation, and the supernatant was collected as the whole-cell lysate. Protein samples were subjected to SDS–PAGE, transferred to PVDF membranes, and blotted with primary antibodies (ab187161, Abcam) in Can Get Signal Solution 1 (Toyobo) at 4°C overnight. Signals were detected after incubation with anti-rabbit or anti-mouse secondary antibodies in Can Get Signal Solution 2 at room temperature for 1 hour using an ECL Detection System (GE Healthcare, Little Chalfont, Buckinghamshire, UK). Band signals were quantified with ImageJ software (ImageJ, RRID: SCR_003070).

ELISA

The protein levels of PGE2 were measured by a PGE2-EIA Monoclonal Kit (Cayman Chemical Company) using conditioned medium. The assay procedure followed the manufacturer's protocol.

Monocyte isolation, macrophage induction, and reactive oxygen species (ROS) assay

Peripheral blood mononuclear cells (PBMCs) were isolated from fasting blood samples collected from healthy human donors. Subsequently, monocytes were isolated from the PBMCs by immunomagnetic negative selection using the EasySep™ Human Monocyte Enrichment Kit (STEMCELL Technologies). To induce M0 macrophages, isolated monocytes were suspended in RPMI 1640 medium containing 10% heat-inactivated FBS and 50 ng/mL M-CSF (PeproTech) and incubated at 37°C with 5% CO2. During differentiation culture, the medium was replaced with the same medium every 2-3 days, and M0 macrophages were induced in 7 days. The induced cells were detached with EDTA-trypsin on the 7th day and incubated overnight after seeding at $1x10^6$ cells/mL. On day 8, the medium was changed to medium containing 100 pM PGE2 (Cyman No. 14010), and after treatment for 6 hours, ROS were labeled using the DCFDA - Cellular ROS Assay Kit (Abcam) according to the manufacturer's protocol. Labeled ROS were measured and analyzed via flow cytometry and FlowJo software as described above.

DNA isolation, whole-exome sequencing and sequence alignment

Genomic DNA was isolated from background liver tissue using a DNeasy blood & tissue kit (Qiagen) according to the manufacturer's instructions. Exome capture libraries were prepared following the instructions of the SureSelectXT mouse all exon target enrichment system (Agilent Technologies). The exome libraries were sequenced using a 125-base pair paired-end read protocol on an Illumina HiSeq 2500. Sequencing reads were trimmed using Cutadapt (v1.18) and Trimmomatic (v0.39) and aligned to the reference genome GRCm38 (mm10) using BWA (v0.7.17). Aligned reads were marked as duplicates using Picard (v 2.18.21) and SAMtools (v1.11), followed by base recalibration using GATK (v4.1.1.0). The whole-exome sequencing data were deposited in the DDBJ database under accession number DRA016752

Variant identification and mutational signature analyses

Single-nucleotide variants (SNVs) and indel variants were called using Mutect2 (v4.1.1.0). No normal tail DNA samples corresponding to the background liver samples were obtained in this analysis. Therefore, normal liver samples from untreated 15-pgdh+/- mice (n=3) were used as a control sample for comparison to the samples from 15-pgdh+/- (n=3) and 15-pgdh+/- (n=6) STAM mice for SNV/indel detection with Mutect2. SNVs/indels were filtered and annotated using SnpShift & SnpEff (v5.1). GRCm38.74 obtained using SnpEff was used for the annotation file. Nonsynonymous SNVs were confirmed by visual inspection of aligned reads with IGV (v2.4.14). Mutation signature profiling and fitting to SBS was performed using MutationalPatterns (v3.6.0)[17].

RNA sequencing

RNA sequencing was performed following the established protocol from a previous study conducted at Kumamoto University (Kumamoto, Japan). The sequencing procedure was carried out by the Liaison Laboratory Research Promotion Center at Kumamoto University. Total RNA was isolated using the RNeasy Mini Kit (catalog no. 74106, Qiagen), and the concentration and purity of the RNA were assessed using an Agilent 2100 bioanalyzer (Agilent). Only samples with an RNA integrity number (RIN) greater than 8.0 were selected for sequencing. For the sequencing analysis, a NextSeq 500 platform (Illumina) was utilized, and the obtained data were converted to the Fastq format for further analysis. The quality of the data was determined by Trim galore (v0.5.0). The filtered reads were then mapped to the GRCm38 reference genome using STAR (v2.6.0). Transcripts per million mapped reads (TPM) values were calculated using RSEM. The RNA-sequencing data were deposited in the DDBJ database under accession number DRA016740. Use R package: DESeq2 (1.36.0), edge (ver3.38.2) to analyze the differentially expressed genes (DEG) between two groups of STAM mice and use R package ggplot2 (3.3.6) and Complex Heatmap (2.13.1) visualize the difference analysis results. P-values < 0.05 and |log2 FC|> 1.5) were set as cutoff criteria and considered to indicate statistical significance. After performing ID conversion on the input molecule list (ID conversion library package: org.Hs.eg.db), the cluster Profiler package (ver.4.4.4) is used for enrichment analysis and use ggplot2 (ver.3.3.6) for visualization (R (4.2.1)).

Analysis of Liver Cell Atlas and published datasets

The Liver Cell Atlas is a public database based on published liver single-cell RNA sequencing datasets for humans and mice generated in the laboratories of Charlotte Scott and Martin Guilliams from the VIB-UGent Centre for Inflammation Research, Belgium (https://www.livercellatlas.org/index.php). The 15-PGDH gene (official symbol; HPGD) was selected, and the distribution of mRNA expression in all human and mouse liver cells was analyzed.

Fluorescently labeled long-chain fatty acid (LCFA) treatment

Mice were injected intraperitoneally with 50 μg of fluorescently labeled LCFAs, specifically BODIPY FL C16 (Thermo Fisher Scientific), in 50 μl of DMSO. The mice were sacrificed 1 hour after the treatment, and single-cell suspensions were prepared from the mouse background liver as described above. CD8+ T cells were then isolated from the single-cell suspensions using the EasySep™ Mouse CD8+ T Cell Isolation Kit (STEMCELL Technologies), and BODIPY-C16+CD8+ T cells were quantified by flow cytometry.

Palmitoleic acid (Palm) treatment, cell counting and cell trace assay

CD8+ T cells were freshly isolated from single-cell suspensions of splenocytes from WT mice using the EasySep™ Mouse CD8+ T-Cell Isolation Kit (STEMCELL TECHNOLOGIES). The isolated CD8+ T cells were cultured in 96-well microplates in complete medium supplemented with Palm or a mixture of Palm and BSA-Oleate (Sigma–Aldrich) at 100 μM from day 1 to day 7. The CFSE assay was performed using the CellTrace™ Violet Cell Proliferation Kit (Thermo Fisher Scientific) according to the manufacturer's protocol. Cell Trace Violet fluorescence detection and cell counting were performed by flow cytometry. The data were analyzed using FlowJo. During this culture experiment, T-cell activity was maintained with mouse IL-2 (BioLegend) (100 μg/mL for the cell counting assay, 50 μg/mL for the cell trace assay) and Dynabeads™ Mouse T-Activator CD3/CD28 for T-Cell Expansion and Activation (Thermo Fisher Scientific).

Measurement of the T-cell mitochondrial membrane potential

T cells isolated from the spleen or liver were treated with or without Tissue Dissociation Reagent (BD) for 10 minutes and then stained with 2 μM JC-1 and 10 μM CCCP as a positive control for 30 minutes at room temperature for measurement of the mitochondrial membrane potential. FACS analyses were

performed using a Symphony (BD Biosciences). The data were analyzed using FlowJo.

Statistical analysis

All experiments were performed in triplicate, and the data shown are representative of consistent results. Data are presented as the mean ± SE. The Mann–Whitney U test was used to compare continuous variables between two groups. Categorical variables were compared using the $\chi 2$ test. Correlations were evaluated by Pearson correlation coefficient analysis. Survival curves were generated using the Kaplan–Meier method, and the log-rank test was performed for survival analysis. Statistical significance was defined as p values lower than 0.05, and all data met the assumptions of the statistical test used for distribution and variance. Variance was not statistically significant in any of the results. No statistical method was used to predetermine the sample size for any of the experiments (in vitro or in vivo). All statistical analyses were performed with SPSS (Version 26, IBM Corp.) and Prism software (Version 9.0.1, GraphPad).

Results

MASH-HCC patients with 15-PGDH downregulation in the background liver showed a higher NAS and an increased recurrence rate

We first selected 100 patients with MASH-HCC in a total cohort of 1273 HCC patients who underwent radical hepatic resection by excluding 1125 patients with viral hepatitis and 48 patients with a long-term history of alcohol consumption. Then, we performed IHC staining of liver tissues from the 100 MASH-HCC patients to evaluate the expression levels of 15-PGDH using the tumor area or peritumor area (background liver) of each sample. Based on the expression level of 15-PGDH, both the tumor area and the background liver area of the 100 MASH-HCC patients were classified into 4 groups: tumor:background = high:high (Group 1, n=56), high:low (Group 2, n=100), low:high (Group 3, n=117), and low:low (Group 4, n=144) (Supplementary Table 1, Figure 1A, Supplementary Figure 1A).

We subsequently assessed the relationships between MASH-HCC patients' 5-year overall survival (OS) or recurrence-free survival (RFS) and the expression levels of 15-PGDH in the tumor area or background liver by Kaplan–Meier analysis. Groups 2, 3, and 4, which were defined as 15-PGDH–low expression regardless of the tumor and background liver, exhibited significantly shorter OS than that of Group 1. Groups 2 and 3 showed no significant differences in OS. On the other hand, Group 2, which was defined as 15-PGDH–low only in the background liver, showed a significantly shorter RFS than that of Group 3 (Group 2 RFS: 12.0% vs. Group 3 14.53%, P=0.0179). This finding suggests that only when defined based on the background liver is PGDH downregulation associated with MASH-HCC occurrence (Figure 1B).

Given the finding for RFS by Kaplan–Meier analysis, we further examined the importance of 15-PGDH expression in the background liver of MASH-HCC patients. We first examined MAFLD activity in the background liver between the 15-PGDH–high and 15-PGDH–low groups of MASH-HCC patients using the NAS. The expression level of 15-PGDH was inversely correlated with the NAS, and the liver fibrosis level evaluated by Sirius Red staining was higher in the 15-PGDH–low group than in the 15-PGDH–high group (Figure 1C and 1D). Moreover, the levels of AST, ALT, and TG in preoperative blood samples were significantly higher in the 15-PGDH–low group than in the 15-PGDH–high group (Figure 1E). These findings suggest that 15-PGDH downregulation in the background liver is correlated with MASH-HCC occurrence and promotes MAFLD activity.

Moreover, according to the evaluation of the expression of 15-PGDH in steatosis, fibrosis (background liver) and tumor sites, we found that the expression levels of 15-PGDH in patients vary depending on their inflammatory status. To further investigate this notion, we conducted an analysis of 15-PGDH mRNA expression using the public dataset from a previous study[18]. This analysis focused on HCV-infected patient datasets to assess the association with inflammation status more clearly. The results of this analysis demonstrated a gradual decrease in 15-PGDH expression according to the METAVIR Activity Grade (inflammation and fibrosis score). This finding provides additional support for the association between the downregulation of 15-PGDH and inflammation (Supplementary Figure 1B). Based on these findings, we emphasize the importance of considering the inflammation status derived from the downregulation of 15-PGDH.

MAFLD activity and MASH-HCC incidence were increased in STAM 15-Pgdh+/− mice

To elucidate the significance of 15-PGDH downregulation in MASH-HCC development, we first investigated the distribution of 15-PGDH mRNA expression in all human and mouse liver cells from the Liver Cell Atlas, which is a public database based on published liver single-cell RNA sequencing datasets for humans and mice. In the human liver, the 15-PGDH gene is primarily expressed in hepatocytes, with only a small portion of expression originating from several immune cells, such as T cells and basophils. On the other hand, in mice, the 15-pgdh gene is expressed broadly, although hepatocytes are still one of the major sources of its expression (Supplementary Figure 2A, 2B, 2C and 2D). In our previous study, we examined the impact of 15-pgdh from macrophages in a mouse model of pancreatic cancer. 15-PGDH deficiency in macrophages caused tumor proliferation and fibrosis elevation[19]. Therefore, we utilized 15-pgdh-deficient mice (15-pgdh+/-, 15-Pgdh−/−) with whole-body depletion, including hepatocytes and immune cells, which could be more influenced by 15-pgdh. As we described in a previous report, 15-Pgdh−/− mice are born with ductus arteriosus, and indomethacin treatment rescues these mice after birth. However, the successful rescue rate is extremely low[19]. Almost all 15-Pgdh−/− mice died after two to four weeks during the process of MASH induction; therefore, we decided to examine MASH-HCC development in 15 pgdh+/− mice in the current study. Moreover, we used the STAM to establish a MASH-HCC model using WT and 15-Pgdh+/− mice following a previous report[20] (Figure 2A). Western blot analysis confirmed that the expression of 15-Pgdh in liver tissues from STAM 15-Pgdh +/− mice was significantly downregulated (Figure 2B). Then, we examined the accumulation of PGE2 in both types of STAM mice by ELISA. As expected, PGE2 significantly accumulated in STAM 15-pgdh+/− mice (Figure 2C). The number of visible tumors and liver weight of STAM 15-Pgdh+/− mice with MASH-HCC induction for 20 weeks were significantly increased compared to those of STAM WT mice (Figure 2D). On the other hand, 15-Pgdh+/− mice fed only a HFD for 20 weeks were observed to have a significantly increased liver weight but not an increased occurrence of HCC (Supplementary Figure 2E). Moreover, from a pathological perspective, the proportion of poorly differentiated tumors was increased in STAM 15-Pgdh+/− mice compared to STAM WT mice, and MASH-

like HCC was detected in the liver tissues of only STAM 15-Pgdh+/− mice (Figure 2E).

Moreover, we assessed the MAFLD activity of the background liver. Consequently, we found that the MAFLD activity score (MAS) and fibrosis level were higher in the STAM 15-Pgdh+/− mice than in the STAM WT mice (Figure 2F and 2G). Biochemical examination of peripheral blood samples revealed that the levels of AST, ALT, triglycerides (TG), and blood glucose (BG) were significantly higher in STAM 15-Pgdh+/− mice than in STAM WT mice (Figure 2H). These findings strongly support the hypothesis that the accumulation of PGE2 through 15-PGDH downregulation in the background liver promotes MAFLD activity and the incidence of MASH-HCC.

Increased gene expression related to cell growth and antiapoptotic processes could enhance hepatocyte growth in STAM 15-Pgdh+/− mice

To identify the molecular mechanisms by which 15-PGDH downregulation promotes MASH-HCC development, we focused on the relationship between PGE2 accumulation and inflammatory responses in the liver. Based on recent evidence that in chronic inflammation, macrophage mitochondria can produce large amounts of ROS, which can induce DNA damage and M2 polarization[21,22], we hypothesized that the accumulation of PGE2 caused by 15-PGDH downregulation induces ROS production in macrophages followed by DNA damage in hepatocytes. Thus, we first examined whether the accumulation of PGE2 could induce an increase in mitochondrial ROS production by macrophages in vitro. We extracted monocytes from peripheral blood samples collected from healthy human donors and induced M0 macrophages. We then treated M0 macrophages with PGE2 and detected ROS levels using DCFDA (Figure 3A). The results showed that the ROS level in macrophages treated with PGE2 was significantly higher than that in untreated macrophages (Figure 3B). We next evaluated macrophages in the background liver of MASH-HCC patients using an M1-like macrophage-specific marker (CD86) and an M2-like macrophage-specific marker (CD163). The results indicated a significant increase only in M2-like macrophages in 15-PGDH–low MASH-HCC patients compared to 15-PGDH–high patients (Figure 3C and Supplementary Figure 3A). Correspondingly, we observed a significant increase only in M2-like macrophages (CD163) in STAM 15-Pgdh+/- mice compared to STAM WT mice (Figure 3D and Supplementary Figure 3B). Moreover, our analysis revealed a

correlation between CD163 expression and the downregulation of 15-PGDH in MASH-HCC patients, whereas no such correlation was observed with CD86 expression (Supplementary Figure 3C and 3D). These results suggest that PGE2 accumulation is involved in M2-like macrophage polarization in the background liver of MASH-HCC patients.

ROS are a well-known factor responsible for DNA damage and subsequent mutation. Therefore, we next performed whole-exome sequencing using DNA samples extracted from the background liver of STAM mice to investigate whether DNA mutation in hepatocytes is a determining factor in HCC occurrence (Figure 3E). By comparing the number of mutated genes between STAM WT and STAM 15-Pgdh+/− mice, we found that the tumor mutational burden was not notably different between the two strains, although some previously reported cancer-related signatures (SBS11 and SBS29)[23] were increased in STAM 15-Pgdh+/− mice (Figure 3F and 3G).

Given that the mutational burden in hepatocytes was not affected by 15-Pgdh downregulation, we next conducted a comprehensive gene expression analysis by RNA sequencing of background liver tissues from STAM WT and 15-Pgdh+/− mice. Pathway analysis using differentially expressed genes (DEGs) revealed that gene sets related to cell growth and negative regulation of apoptotic signaling pathways were enriched in STAM 15-Pgdh+/− mice compared to STAM WT mice (Figure 3H). Moreover, the expression of 10 genes selected as the top upregulated genes in each of the cell growth- and antiapoptotic process-related pathways was markedly higher in the STAM 15-Pgdh+/− mice than in the STAM WT mice (Figure 3I and 3K). We further confirmed the increased expression of several key genes related to cell growth and antiapoptotic processes by qRT–PCR (Figure 3J and 3L). Supporting the gene expression analysis findings, the number of Ki-67-positive hepatocytes in the background liver was significantly higher in STAM 15-Pgdh+/− mice than in STAM WT mice (Figure 3M). These results suggest that 15-Pgdh downregulation could promote hepatocyte proliferation along with increased gene expression related to cell growth and antiapoptotic processes.

The accumulation of fatty acids in the TME caused CD8+ T-cell mitochondrial dysfunction in STAM 15-Pgdh+/− mice

Based on the Gene Ontology (GO) enrichment analysis results for downregulated genes identified by RNA sequencing, we notably found that

pathways associated with hepatic fatty acid and lipid metabolism capacity were significantly downregulated in the background liver of STAM 15-Pgdh+/− mice (Figure 4A). All 13 genes involved in the fatty acid metabolic process were markedly decreased in the background liver of STAM 15-Pgdh+/− mice compared with that of STAM WT mice (Figure 4B). We also confirmed the downregulation of the key genes involved in fatty acid elongase activity (*Elovl3*), fatty acid beta-oxidation (*Fabp1*), palmitoyl-CoA hydrolase activity (*Acot11*), and the prostaglandin metabolic process (*Akr1c14*) (Figure 4C).

Given these findings, we next compared the accumulation of neutral lipids in the liver between STAM WT and 15-Pgdh+/− mice. Oil Red O staining revealed that neutral lipid accumulation was significantly higher in the stromal area of STAM 15-Pgdh+/− mice than in that of STAM WT mice, suggesting an excess amount of neutral lipids in the TME (Figure 4D). Moreover, we intraperitoneally injected STAM 15-Pgdh+/− mice with fluorescently labeled LCFAs (Bodipy-C16), and after one hour, we analyzed CD8+ T cells isolated from background liver tissue by flow cytometry. In both STAM WT mice and especially STAM 15-Pgdh+/− mice, CD8+ T cells in liver tissue acquired significantly higher amounts of Bodipy-C16 than control splenic CD8+ T cells (Figure 4E). Although infiltrating CD8+ T cells can use LCFAs to fuel fatty acid oxidation (FAO) in low-glucose environments, such as pancreatic ductal adenocarcinoma (PDAC)[24], excessive accumulation of intracellular fatty acids is known to damage cells via lipotoxicity. The levels of palmitoleic acid (Palm) and oleic acid (Ole) are significantly increased compared to the various other lipid classes in livers with MASH[25]. In particular, Palm has been reported to induce mitochondrial dysfunction in CD8+ T cells[26]. Therefore, we isolated CD8+ T cells from the spleens of WT mice and treated the cells with Palm and Ole. Consequently, we found that both Palm and Ole treatment induced cell cycle arrest and decreased the proliferation of cultured CD8+ T cells in a cell trace assay. However, the effect of Palm was stronger than the effect of Ole (Figure 4F and 4G and Supplementary Figure 4A and 4B). Based on these results, we next considered how the accumulation of fatty acids affects the mitochondrial functions of CD8+ T cells in STAM 15-Pgdh+/− mice. We assessed mitochondrial functionality with a JC-1 assay and found a significant reduction in the mitochondrial membrane potential in CD8+ T cells in the background liver of STAM 15-Pgdh+/− mice (Figure 4H). These results indicate that accumulated lipids have a harmful impact on the mitochondrial functions of

CD8+ T cells.

Immune surveillance is inactivated through CD8+ T-cell exhaustion in 15-Pgdh+/− mice

Based on these findings, we next investigated whether the reduction in the mitochondrial membrane potential influences CD8+ T-cell impairment. A recent study reported that fatty acid accumulation in the TME of PDAC reduces CD8+ T-cell infiltration and impairs the mitochondrial function of CD8+ T cells, which induces CD8+ T-cell exhaustion[27,28]. Therefore, we hypothesized that 15-Pgdh downregulation in the background liver reduces hepatic immune surveillance via the accumulation of fatty acids. To explore our hypothesis, we first compared PD-1 expression between STAM 15-Pgdh+/− mice and STAM WT mice. Notably, the expression of PD-1 in CD8+ T cells in the liver of STAM 15-Pgdh+/− mice was significantly increased, while that in other immune cells (CD4+ T cells, NK cells, and NKT cells) was not different, suggesting that CD8+ T cells could be affected by 15-Pgdh downregulation to reduce immune surveillance (Figure 5A and 5B). To further investigate what function of CD8+ T cells was restricted, we examined the ability to secrete effector molecules such as TNF-a and IFN-g. The results showed that the expression of TNF-a and IFN-g in CD8+ T cells in the liver was decreased in 15-Pgdh STAM +/− mice compared to STAM WT mice (Figure 5C and 5D), supporting the hypothesis that CD8+ T-cell exhaustion is induced by 15-Pgdh downregulation. Consistent with the findings of the in vivo experiments, staining of tissue sections of the background liver revealed that the expression of PD-1 was significantly higher in the 15-PGDH–low patient group (Figure 5E). The above findings suggest that 15-Pgdh downregulation inactivates immune surveillance by promoting the proliferation of exhausted effector T cells, which enhances hepatocyte survival and proliferation and leads to MASH-HCC development.

Discussion

The global incidence of MAFLD/MASH has increased significantly, and recent epidemiological investigations have indicated that in the absence of liver cirrhosis, MAFLD/MASH is the leading cause of HCC[29]. Over the past decade, it has been shown that the expression of COX-2 in acute and chronic liver diseases is abnormally elevated, and this inflammatory enzyme has been

related to cancer progression[30]. In this study, we demonstrated the significance of COX-related signaling; in particular, we focused on the accumulation of endogenous PGE2 and activation of COX/PGE2 signaling. The accumulation of endogenous PGE2 silences certain tumor suppressor genes and DNA repair genes, such as cannabinoid receptor 1 (CNR1) and O-6-methylguanine-DNA methyltransferase (MGMT), through DNA methylation to promote tumor growth in colon cancer[31]. Our previous studies found that interleukin-1β (IL-1β) secreted by tumor-associated macrophages could downregulate the expression of 15-PGDH, which is an enzyme that degrades PGE2 in pancreatic ductal cancer. Moreover, in vivo experiments performed with a model lacking the 15-Pgdh gene demonstrated increased expression of aldehyde dehydrogenase 1 (Aldh1), which was found to be negatively correlated with 15-Pgdh and significantly associated with a poor prognosis in PDAC patients[16,32]. Moreover, the analysis using a public database from a previous paper[18] revealed that the level of 15-PGDH gradually decreased in an inflammation-dependent manner. Inflammation triggers liver damage, and PGE2 is known to modulate tissue regeneration, stimulating hepatocyte proliferation[33]. This phenomenon represents a characteristic feature of the hepatocarcinogenesis process in HCC known as multistep hepatocarcinogenesis. Hepatocytes that have been damaged due to inflammation or other factors undergo regeneration and proliferation, ultimately serving as the origin of HCC [34]. Together with these previous studies, our current findings delineate a molecular mechanism underlying the promotion of PGE2 accumulation by downregulation of 15-PGDH in the background liver, leading to tumor progression in MASH-HCC.

Another important function of PGE2 is its ability to promote the accumulation of PD-1+ T cells. PGE2 has an important function in controlling the balance among Th1, Th2, and Treg cells, resulting in the promotion of an immunosuppressive niche and tumor growth in pancreatic carcinoma[35]. To date, blockade of the PD-1 immune checkpoint has been developed as a therapeutic modality in HCC, with anti-PD-1 antibodies, such as nivolumab and pembrolizumab, being approved, and the combination of an anti-PDL1 antibody (atezolizumab) with an anti-VEGF antibody (bevacizumab) is a first-line treatment for advanced HCC[14,36,37]. However, less than 30% of HCC patients respond to immunotherapy[38]. It was recently reported that the levels of aberrant PD-1+CD8+ T cells, which could not perform immune surveillance and exerted tissue-damaging functions in a TNF-dependent manner, were increased by

anti-PD-1 treatment in MASH-HCC. This immune environment may contribute to weakening the efficacy of anti-PD-1 treatment and even lead to HCC development[13,39].

Previously, lipotoxicity was shown to be closely associated with MASH severity. Free cholesterol deposited in hepatocyte mitochondria causes hepatocyte apoptosis and necrosis by activating JNK1 signaling[40]. Moreover, clinical trials focusing on hepatic TG homeostasis have been conducted to normalize lipid metabolism and reduce the risk of severe MASH[41]. On the other hand, recent studies have revealed that lipotoxicity is involved in one aspect of dysfunctional immune surveillance. The accumulation of LCFAs in the advanced pancreatic cancer TME impairs mitochondrial function and triggers the downregulation of very-long-chain acyl-CoA dehydrogenase (VLCAD), which induces the accumulation of LCFAs in intrapancreatic CD8+ T cells[27]. Another report indicated that lipid accumulation increased the expression of CD36, which promotes the uptake of oxidized low-density lipoproteins (OxLDL) in T cells, and this induced lipid peroxidation and downstream activation of p38 kinase, followed by CD8+ T-cell exhaustion[27]. These reports suggest that lipid accumulation in the TME directly contributes to CD8+ T-cell impairment. Supporting this recent evidence, the study findings revealed that phenotypic changes in lipid metabolism in the background liver influenced the TME, which led to a reduction in the mitochondrial activity of exhausted CD8+ T cells in the MASH-HCC mouse model.

Given that PGE2 accumulation enhances MASH-HCC development by affecting hepatocytes and CD8+ T-cell function, it would be beneficial to consider therapeutic intervention. For instance, daily use of aspirin has been found to reduce the risk of adenoma recurrence in patients with a history of colorectal adenoma in randomized controlled trials[42,43,44,45]. Recently, inhibition of the COX-2 inhibitor celecoxib was shown to attenuate MAFLD activity by elevating serum adiponectin levels and Adipo-R1 and Adipo-R2 expression levels in the rat liver[46]. Moreover, we investigated the significance of COX2/PGE2 signaling in promoting MASH-HCC development and leading to inactivation of immune surveillance. Together with these previous studies, our present findings suggest that COX-2 inhibitors may represent a potential novel therapy for use in combination with conventional immunotherapy for MASH.

In summary, the downregulation of 15-PGDH leads to the accumulation of PGE2 in the TME, which induces ROS production in macrophages and the

expression of cell growth-related genes and antiapoptotic genes. On the other hand, the downregulation of fatty acid metabolism in hepatocytes during MASH development promotes lipid accumulation in the TME, which leads to reduced mitochondrial activity and an exhausted phenotype in CD8+ T cells. Aberrant hepatocytes that escape immune surveillance exacerbate the development of MASH-HCC. Given these findings, the suppression of PGE2-related inflammation in addition to immune checkpoint blockade may lead to less severe MASH and inhibition of subsequent MASH-HCC progression.

Abbreviations

HCC, hepatocellular carcinoma; MASH, metabolic dysfunction associated steatohepatitis; 15-PGDH, 15-hydroxyprostaglandin dehydrogenase; PGE2, prostaglandin E2; TME, tumor microenvironment; MAFLD, metabolic dysfunction associated fatty liver disease; NAFLD, nonalcoholic fatty liver disease; NASH, nonalcoholic steatohepatitis; COX-2, cyclooxygenase 2; H&E, hematoxylin and eosin; IHC, immunohistochemical; STAM, steric animal model; HFD, high-fat diet; WT, wild-type; MAS, MAFLD activity score; FACS, fluorescence-activated cell sorting; RT, reverse transcription; cDNA, complementary DNA; SEs, standard errors; ROS, reactive oxygen species; PBMCs, peripheral blood mononuclear cells; SNVs, single-nucleotide variants; LCFA, long-chain fatty acid; Palm, palmitoleic acid; OS, overall survival; RFS, recurrence-free survival; TG, triglycerides; DEGs, differentially expressed genes; GO, Gene Ontology; FAO, fatty acid oxidation; PDAC, pancreatic ductal adenocarcinoma; Ole, oleic acid; CNR1, cannabinoid receptor 1; MGMT, O-6-methylguanine-DNA methyltransferase; IL-1β, interleukin-1β; Aldh1, aldehyde dehydrogenase 1; VLCAD, very long chain acyl-CoA dehydrogenase; OxLDL

Author Contributions

Conception and design: T.Y. and T.I.; data acquisition: X.H., N.Y., A.Y., and T.U.; data analysis and interpretation (e.g., RNA sequencing and computational analysis): X.H., T.Y., N.Y., A.Y., T.U., Y.N., K.Y., and T.I.; manuscript writing, review, and/or revision: X.H., T.Y., K.Y., and T.I.; administrative, technical, or

material support: T.S., K.A., T.U., A.N., L.B., L.F., F.W., J.Z., Y.T., and H.W.; and study supervision: K.I., T.F., H.N., K.T., Y.M., H.B., and T.I.

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Figure Legends

Figure 1. Low expression of 15-PGDH in the background liver indicates an increased recurrence rate for HCC and elevated MASH activity in MASH-HCC patients.

A. Representative IHC staining for 15-PGDH in the 4 groups (tumor:background = high:high (Group 1), high:low (Group 2), low:high (Group 3), low:low (Group 4)). Scale bars, 100 μm.

B. Overall survival (OS) and recurrence-free survival (RFS) of MASH-HCC patients stratified into 4 groups.

C. Representative H&E staining of the background liver of MASH-HCC patients with high or low expression of 15-PGDH and the correlation between the MAFLD activity score (MAS) and 15-PGDH expression score.

D. Representative Sirius Red staining of the background liver of MASH-HCC patients with high or low expression of 15-PGDH and quantitative comparison of the Sirius Red-positive areas between the groups of MASH-HCC patients with high or low expression of 15-PGDH in the background liver (n=10/group).

E. Pearson's correlations of 15-PGDH expression in the background liver with preoperative serum AST, ALT, and TG levels in MASH-HCC patients.

ns, not significant, $*p < 0.05$, $**p < 0.01$ Mann–Whitney U test

Figure 2. MASH-HCC incidence and MASH activity are accelerated in STAM 15-Pgdh+/- mice.

A. Schematic timeline of the development of the steric animal model (STAM) with wild-type and 15-Pgdh+/- mice.

B. Western blot analysis of 15-Pgdh protein levels in liver tissue from STAM WT and 15-Pgdh+/- mice. The graph represents normalization of 15-Pgdh to total B-actin (n=3/group).

C. PGE2 expression was measured by ELISA in STAM WT and 15-Pgdh+/− mice.

D. Representative images of liver tumors from STAM WT and 15-Pgdh+/− mice. The graph represents the number of visible tumors and the ratio of liver weight to body weight (LW/BW) in each group (n=12). Scale bars, 100 μm.
E. Representative H&E staining of well-differentiated HCC from STAM WT mice and moderately to poorly differentiated/MASH-like HCC from STAM 15-Pgdh+/- mice. The graph represents the percentage of tumors grouped by tumor differentiation stage in each group. The STAM WT group included the evaluation of 12 tumors from STAM WT mice (n=6). The STAM 15-Pgdh+/- group included the evaluation of 11 tumors from STAM 15-Pgdh+/- mice (n=4). Scale bars, 100 μm.
F. Representative H&E staining of the background liver of STAM WT and 15-Pgdh+/− mice. The graph represents the MAFLD activity scores (MASs) in each group (n=10). Scale bars, 100 μm.
G. Representative Sirius Red staining of the background liver of STAM WT and 15-Pgdh+/− mice. The graph represents the quantification of Sirius Red-positive areas for each group (n=5). Scale bars, 100 μm.
H. AST, ALT, TG and blood glucose (BG) levels in peripheral blood from STAM WT and 15-Pgdh+/− mice collected at 20 weeks of age (AST, ALT, TG: n=5; BG: n=6).
ns, not significant, $*p < 0.05$, $**p < 0.01$ Mann–Whitney U test.

Figure 3. Hepatocyte proliferation is enhanced by regulating proproliferative and antiapoptotic gene expression in STAM 15-Pgdh+/− mice.
A. Schematic diagram of monocyte isolation from the peripheral blood of healthy donors, followed by macrophage induction and subsequent PGE2 treatment.
B. Flow cytometry histogram showing DCFDA staining of monocyte-differentiated macrophages treated with PGE2.
C. Representative IHC staining for CD163 in the background liver of MASH-HCC patients with high or low expression of 15-PGDH (n=10/group). The graph represents the number of CD163-positive cells per mm3 in four randomly selected areas. Scale bars, 100 μm.
D. Representative IHC staining for CD163 in the background liver of STAM WT and 15-Pgdh+/− mice (n=6/group). The graph represents the number of

CD163-positive cells per mm3 in four randomly selected areas. Scale bars, 100 μm.

E. Schematic diagram of DNA extraction from the background liver of STAM WT and 15-Pgdh+/− mice for whole-exome sequencing (WES).

F. The number of gene mutations in the background liver of STAM WT and 15-Pgdh+/− mice determined by WES.

G. Mutational landscapes of the background liver of STAM WT and 15-Pgdh+/− mice by the COSMIC mutational signature.

H. Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analyses of differentially expressed genes in the background liver of STAM 15-Pgdh+/- mice.

I. Heatmap of the top 10 upregulated genes involved in proproliferation-related pathways in the background liver of STAM WT and 15-Pgdh+/− mice.

J. Validation of the upregulation of selected genes involved in proproliferation-related pathways in the background liver of STAM mice by qRT–PCR.

K. Heatmap of the top 10 upregulated genes involved in the anti-apoptotic process-related pathways in the background liver of STAM WT and 15-Pgdh+/− mice.

L. Validation of the upregulation of selected genes involved in the antiapoptotic process-related pathways in the background liver of STAM mice by qRT–PCR.

M. Representative IHC staining for Ki-67 in the background liver of STAM WT and 15-Pgdh+/− mice (n=6/group). The graph represents the number of Ki-67-positive cells per mm3 in four randomly selected areas. Scale bars, 100 μm.

$*p < 0.05$, $**p < 0.01$ Mann–Whitney U test

Figure 4. CD8+ T-cell dysfunction is induced by the accumulation of fatty acids in the background liver of STAM 15-Pgdh+/- mice.

A. GO enrichment analysis of up- and downregulated genes involved in the fatty acid and lipid metabolism capacity of the background liver of STAM 15-Pgdh+/- mice.

B. Heatmap of 13 enriched genes located upstream of the fatty acid metabolic process in the background liver of STAM 15-Pgdh+/- mice.

C. Validation of the upregulation of selected genes involved in the fatty acid metabolic process in the background liver of STAM mice by qRT–PCR.

D. Representative image of Oil Red O staining of the stromal area in STAM WT and 15-Pgdh+/− mice. Stromal area was identified using Sirius Red staining.

The graph represents the quantification of Oil Red O-positive areas for each group (n=5/group). Scale bars, 25 μm.

E. Quantification of BODIPY-C16 uptake in infiltrating CD8+ T cells in the background liver of STAM mice after intraperitoneal injection of fluorescently labeled LCFAs (BODIPY-C16). Spleen, control.

F. Cell trace analysis of CD8+ T cells isolated from the spleens of WT mice treated with palm (n=2).

G. Cell proliferation of CD8+ T cells isolated from the spleens of WT mice treated with Palm (n=2).

H. Representative histograms of JC-1 analysis of CD8+ T cells in the background liver of WT and STAM mice. The graph represents quantification of the ratio of red to green JC-1 fluorescence (percentage) (n=3/group).

Palm, palmitic acid, $*p < 0.05$, $**p < 0.01$, $***p < 0.001$ Mann–Whitney U test

Figure 5. Immune surveillance was suppressed through CD8+ T-cell exhaustion in STAM 15-Pgdh+/− mice and MASH-HCC patients

A. Flow cytometric analysis of PD-1 expression in infiltrating CD4+ and CD8+ T cells in the background liver of STAM mice. Contour plots showing the gating of CD4+ and CD8+ T cells (left). Representative histograms of PD-1 expression in infiltrating CD4+ and CD8+ T cells and quantification of PD-1+ cells in CD4+ or CD8+ T cells (middle, right).

B. Flow cytometric analysis of PD-1 expression in infiltrating NK cells and NKT cells in the background liver of STAM mice. Contour plots show the gating of NK cells and NKT cells (left). Representative histograms of PD-1 expression in infiltrating NK cells and NKT cells and quantification of PD-1+ cells in NK cells and NKT cells (middle, right).

C. Flow cytometric analysis of TNF-α expression in CD8+ T cells in the background liver of STAM mice. Contour plots showing the gating of TNF-α+ immune cells and quantification of TNF-α+ cells in immune cells.

D. Flow cytometric analysis of IFN-γ expression in CD8+ T cells in the background liver of STAM mice. Contour plots showing the gating of TNF-α+CD8+ T cells and quantification of TNF-α+ cells in CD8+ T cells.

E. Representative IHC staining for PD-1 in the background liver of MASH-HCC patients (n=10/group). The graph represents the number of PD-1-positive cells per mm3 in four randomly selected areas. Scale bars, 100 μm.

ns, not significant, $*p < 0.05$, $**p < 0.01$, $***p < 0.001$ Mann–Whitney U test