Liver fibrosis induction and partial hepatectomy. Liver fibrosis was induced by eight injections of CCl₄ (0.5 ml/kg i.p., twice weekly). Two-thirds partial hepatectomy was performed as described¹, followed by evaluation of liver-body weight ratio.

Evaluation of liver fibrosis. Hepatic fibrosis was determined by Picrosirius red staining and morphometric analysis using polarized light.² Pictures were obtained with an Olympus 71IX microscope using a 10x or 20x lens. Quantification of respective staining was performed using Photoshop software, analyzing at least ten images per mouse.

Acute liver injury models. Acute liver injury was either induced by a single injection of CCl₄ (0.5 ml/kg body weight, diluted in corn oil, i.p.) or treatment with concanavalin A (12 mg/kg i.v.). Mice were sacrificed 48h following CCl₄ treatment or 24h following concanavalin A treatment.

Immunohistochemical and immunofluorescent staining. Immunohistochemistry was performed on paraffin-embedded liver sections as previously described using primary antibodies against CB1 (Cayman Chemical), CD3 (Life Technology), CD4 (Abcam), CD45 (BD Biosciences), cleaved-caspase 3 (Cell Signaling), F4/80 (AbD Serotec), Ki-67 (Dako), pERK (Cell Signaling) and the Vectastain ABC kit (Vector Labs).³ Pictures were obtained with an Olympus 71IX microscope using a 10x or 20x lens and quantified using Image J and Photoshop software, analyzing at least ten images per mouse. Co-staining of CB2 (Abcam) with F4/80 and CD45 was done by dual
immunofluorescence and subsequent confocal microscopy on a Nikon A1 confocal laser microscope (Nikon Instruments, Melville, NY, USA).

**Determination of endocannabinoids.** Endocannabinoid levels in liver tissue were measured by liquid chromatography/mass spectrometry as previously described.\(^4\)

**Western blot analysis.** ERK, FAAH and pERK protein levels were determined by western blot using rabbit anti-ERK antibody (Cell Signaling), mouse anti-FAAH antibody (Abcam) and mouse anti-pERK antibody (Cell Signaling) followed by incubation with horse radish peroxidase-labeled antibody and chemiluminescent detection, respectively. Blots were reprobed with mouse antibody to β-actin (Sigma).

**Microarray analysis.** To determine the expression of endocannabinoid components, liver samples from normal mouse livers and DEN+CCl\(_4\)-induced HCC’s were compared for the expression of *Cnr1, Cnr2, Dagla, Daglb, Napepld, Trpv1, Faah and Mgll*, using previously described datasets (GSE33446). All arrays in this study were normalized together using GCRMA.\(^5\) Log fold changes between conditions using contrasts in Linear Models for MicroArrays (LIMMA).\(^6\) Both GCRMA and LIMMA are implemented in Bioconductor/R.

**Primary cell isolations.** Primary hepatic stellate cells, macrophages, and hepatocytes were isolated from mice using retrograde liver perfusion and isolation techniques as previously described.\(^7,8\) F4/80-positive hepatic macrophages were isolated after collagenase/pronase perfusion, followed by a 16.95% Nycodenz gradient and subsequent positive selection of F4/80-positive cells by magnetic-activated cell sorting
(MACS) using biotinylated F4/80 antibody (eBioscience) and anti-biotin MACS beads (Miltenyi Biotec).

**HCC cell lines and culture.** Hepa1-6 mouse HCC cell line and Hep3B, HepG2, HuH-7 and PLC/PRF/5 human HCC cell lines were used. Hepa1-6, HuH-7 and PLC/PRF/5 were cultured in Dulbecco’s Modified Eagle Medium (DMEM, Gibco) supplemented with 10% fetal bovine serum (FBS, Gibco), 1% antibiotic/antimycotics (Gibco), and 0.1% gentamycin (Gibco). Hep3B and HepG2 were grown in Eagle’s Minimum Essential Medium (EMEM, ATCC) supplemented with 10% FBS, 1% antibiotic/antimycotics, and 0.1% gentamycin. Cells were seeded on cell culture dishes (Falcon) and sub-cultured by trypsin-EDTA treatment. Medium was renewed 2-3 times a week. All cells were maintained at 37°C in a humidified 5% CO2 atmosphere. Cells were harvested and kept in Buffer RLT (Qiagen) for the RNA isolation.

**RNA isolation and qPCR analysis.** RNA was isolated by a combination of Trizol, subsequent column purification and DNase treatment (Roche Applied Science, Penzberg, Germany). In case of cells, column purification and DNase treatment (Roche Applied Science) were utilized without prior Trizol treatment. Following reverse transcription (High-Capacity cDNA Reverse Transcription kits, Applied Biosystems, NY, USA), quantitative real-time PCR was performed on an ABI 7300 cycler using ABI Taqman primer and probe sets for murine and human samples. Expression of murine and human samples was normalized to 18s and fold-induction was calculated using relative standard curves.
SUPPLEMENTARY REFERENCES


Supplementary figure 1
Supplementary figure 2
Day 15
1×DEN (25 mg/kg i.p.)

Wild-type

TRPV1<sup>ko</sup>

Supplementary figure 3
Supplementary figure 5
Supplementary figure 7
Supplementary figure 8
Supplementary figure 9

A

B
**A**

![Bar charts showing mRNA fold change for Emr1 and Cd20 with significance levels.](chart1)

**B**

![Images of immunohistochemistry for CB2wt and CB2ko in control, non-tumor, and tumor conditions.](chart2)

Supplementary figure 10
Figure A: Immunohistochemical staining of control, Wt, and CB2 knockout (ko) cells untreated and treated with ConA. Quantitation of CD3+ cells shows a statistically significant difference between control and CB2ko (p=0.117).

Figure B: Immunohistochemical staining of control, Wt, and CB2 knockout (ko) cells untreated and treated with ConA. Quantitation of CD4+ cells shows a statistically significant difference between control and CB2ko (p=0.07).

Figure C: mRNA expression levels of Cd3e, Cd20, Emr1, Cd4, Ccl5, Ccl17, and Ccl27 in control, Wt, and CB2 knockout (ko) cells untreated and treated with ConA. p-values for statistical significance are provided for each comparison.
Ccl5 mRNA (fold)  
Control | Wt | CB2\(^{ko}\) | 1 x CCL4 | Macrophages  
P = 0.248

Ccl20 mRNA (fold)  
Control | Wt | CB2\(^{ko}\) | 1 x CCL4 | Macrophages  
P = 0.149

Ccl17 mRNA (fold)  
Control | Wt | CB2\(^{ko}\) | 1 x CCL4 | Macrophages  
P = 0.149

Ccl27 mRNA (fold)  
Control | Wt | CB2\(^{ko}\) | 1 x CCL4 | Macrophages  
P = 0.126

Cxcl5 mRNA (fold)  
Control | Wt | CB2\(^{ko}\) | 1 x CCL4 | Macrophages  
P = 0.149

Cxcl9 mRNA (fold)  
Control | Wt | CB2\(^{ko}\) | 1 x CCL4 | Macrophages

Cxcl10 mRNA (fold)  
Control | Wt | CB2\(^{ko}\) | 1 x CCL4 | Macrophages  
P = 0.083

Supplementary figure 13
Supplementary figure 14
SUPPLEMENTARY FIGURE LEGENDS

Supplementary figure 1. Serum endocannabinoid levels in HCC patients. Endocannabinoid levels in serum of healthy controls (n=5) and patients with HCC (n=5). ns, not significant; AEA, anandamide; 2-AG, 2-arachidonoylglycerol; PEA, palmitoylethanolamide; LEA, linoleoylethanolamide; OEA, oleoylethanolamide; SEA, stearoylethanolamide.

Supplementary figure 2. FAAH-deficient mice show normal responses to acute DEN injection. Wild-type (n=15) and FAAH$^{ko}$ mice (n=15) were injected with DEN (100 mg/kg) followed by determination of Il6, Ccl2, P21 and Tnf mRNA by qPCR 48 hours later. *p<0.05. ns, not significant

Supplementary figure 3. TRPV1 does not contribute to HCC development. (A) Wild-type mice (n=6) and TRPV1$^{ko}$ mice (n=8) were injected with DEN (25 mg/kg i.p.) at the age of 15 days and sacrificed 11 months after DEN. Shown are representative images. (B) Tumor number, largest tumor size, and liver/body weight ratio of wild-type and TRPV1$^{ko}$ mice. ns, not significant

Supplementary figure 4. Determination of inflammation and apoptosis in CB1-deficient and FAAH-/CB1-double-deficient mice. (A) Tnf and Il-6 mRNA levels were determined in non-tumor and tumor tissue from wild-type, CB1-deficient and FAAH/CB1 double-deficient mice. (B) Cleaved caspase-3 staining was quantified in non-tumor and tumor sections from wild-type and CB1-deficient livers. ns, not significant
Supplementary figure 5. Determination of proliferation and Erk activation in FAAH-deficient and CB1-deficient mice. (A-B) Wild-type (n=5) and FAAH-deficient (n=8) mice underwent two-thirds partial hepatectomy and were euthanized 48h later, followed by determination of liver body weight ratio (A) and Ki-67 immunohistochemistry (B). (C) Expression of pERK was determined by immunohistochemistry in non-tumor and tumor tissue of wild-type and CB1-deficient mice, followed by morphometric quantification. (D-F) pErk was determined by western blot analysis in non-tumor and tumor livers of wild-type, CB1-deficient, FAAH-deficient, and CB1-/FAAH-double-deficient (“dko”) mice. * p<0.05. ns, not significant

Supplementary figure 6. FAAH-deficient mice display increased CCl₄-induced liver fibrosis. (A-B) Wild-type (n=13) and FAAH-deficient mice (n=9) were treated with 8 injections of CCl₄. Liver fibrosis was determined by Sirius red staining and morphometric quantification (A) and qPCR for Col1a1 (B). *p<0.05

Supplementary figure 7. Determination of proliferation, apoptosis, fibrogenesis and inflammation in CB2-deficient mice. (A-B) Proliferation was determined by Ki-67 staining and morphometric quantification (A) and qPCR (B) for mKi67 and Ccnb2 in non-tumor and tumor sections from wild-type (n=15) and CB2KO (n=15). (C) Cleaved caspase-3 staining was quantified in non-tumor and tumor sections from wild-type and CB2KO livers. (D) Fibrogenesis was determined by qPCR for Acta2 and Col1a1 in non-tumor and tumor sections from wild-type and CB2KO. (E) Inflammation was determined by qPCR for Il-6, Ccl2 and Tnf mRNA levels with Col1a1 in non-tumor and tumor sections from wild-type and CB2KO. *p<0.05. ns, not significant; N,
normal; NT, non tumor; T, tumor

**Supplementary figure 8. CB2 is predominantly expressed in CD45- and F4/80-positive cells in the liver.** (A-B) Non-tumor and tumor sections (n=8) were co-stained for CB2 and CD45 (A) or CB2 and F4/80 (B), followed by confocal microscopy and quantification of expression of each marker in liver as well as quantification of co-localization. *p<0.05, **p<0.01

**Supplementary figure 9. CB2 is highly expressed in liver macrophages but not in tumor cell lines.** (A) Murine CB2 expression was determined by qPCR in liver tissue (n=4), primary murine liver macrophages (n=5 isolations), hepatocytes (n=3 isolations), hepatic stellate cells (n=4 isolations) and in murine HCC cell lines Hepa 1-6. (B) Human CB2 expression was determined by qPCR in liver tissue (n=4), and in murine HCC cell lines Hep3B, HepG2, Huh7 and PCR/PRF/5. *p<0.05, **p<0.01

**Supplementary figure 10.** (A) *Emr1* and *Cd20* mRNA levels were determined in wild-type and CB2$^{ko}$ mice by qPCR. (B) F4/80 expression was determined in non-tumor tissue and tumor tissue of wild-type (n=15) and CB2 knockout mice (n=15) as well as in normal tissue of wild-type and CB2 knockout mice. ns, not significant.

**Supplementary figure 11.** T cell recruitment and expression of T cell-recruiting chemokines following acute liver injury by concanavalin A. Wild-type (n=6) and CB2-deficient (n=7) mice were treated with concanavalin A and sacrificed 24h later. (A-B). CD3 expression (A) and CD4 expression (B) were determined by immunohistochemical staining and quantification. (C) Expression of T cell markers
Cd3e and Cd4, B cell marker Cd20, macrophage marker F4/80 (encoded by Emr1) and chemokines was determined by qPCR.

**Supplementary figure 12.** T cell recruitment and expression of T cell-recruiting chemokines following acute liver injury by CCl4. Wild-type (n=6) and CB2-deficient (n=5) mice were treated with CCl4 and sacrificed 48h later. (A-B). CD3 expression (A) and CD4 expression (B) were determined by immunohistochemical staining and quantification. (C) Expression of T cell markers Cd3e and Cd4, B cell marker Cd20, macrophage marker F4/80 (encoded by Emr1) and chemokines was determined by qPCR. *p<0.05, **p<0.01

**Supplementary figure 13.** Reduced expression of T cell-recruiting chemokines in CB2-deficient macrophages isolated from CCl4-treated mice. Wild-type (n=4) and CB2-deficient mice (n=4) were treated with one injection of CCl4 and sacrificed 48h later. Expression of chemokines was determined by qPCR. *p<0.05

**Supplementary figure 14.** CB2 expression is not altered in CB1-deficient mice. (A-B) CB2 expression was compared between wild-type (n=13) and CB1-deficient (n=9) mice by immunohistochemistry (A) and qPCR (B).