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Cannabinoid Receptor 1 Promotes Hepatocellular Carcinoma Initiation and Progression via Multiple Mechanisms

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Abstract

Hepatocellular carcinoma (HCC) has high mortality and no adequate treatment. Endocannabinoids interact with hepatic CB1 receptors (CB1R) to promote hepatocyte proliferation in liver regeneration via inducing cell-cycle proteins involved in mitotic progression, including Forkhead Box M1 (FOXM1). Because FOXM1 is highly expressed in HCC and contributes to its genesis and progression, we analyzed the involvement of the endocannabinoid/CB1R system in murine and human HCC. Postnatal diethylnitrosamine (DEN) treatment induced HCC within 8 months in wild-type mice, but fewer and smaller tumors in CB1R^{-/-} mice or in wild-type mice treated with the peripheral CB1R antagonist JD5037, as monitored in vivo by serial magnetic resonance imaging. Genome-wide transcriptome analysis revealed CB1R-dependent, tumor-induced upregulation of the hepatic expression of CB1R, its endogenous ligand anandamide, and a number of tumor promoting genes, including the GRB2 interactome as well as FOXM1 and its downstream target the tryptophan-catalyzing enzyme indoleamine 2,3-dioxygenase (IDO2). Increased IDO2 activity and consequent induction of immunosuppressive Treg cells in tumor tissue promote immune tolerance.

Conclusion—The endocannabinoid/CB1R system is upregulated in chemically induced HCC resulting in the induction of various tumor promoting genes, including IDO2, and attenuation of these changes by blockade or genetic ablation of CB1R suppresses the growth of HCC and highlights the therapeutic potential of peripheral CB1R blockade.

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SUPPLEMENTAL INFORMATION

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Liver cancer is the third most prevalent cancer with 695,000 deaths worldwide (1). The American Cancer Society's estimates for 2013 are about 30,640 new cases of liver cancer in the United States and about 21,670 deaths from these cancers. (2). The 5-year survival rate of individuals with liver cancer is only 8.9% despite aggressive conventional therapy, marking this malignancy the second most lethal after pancreatic cancer (3). Hepatocellular carcinoma (HCC) is the most common type of liver cancer and it accounts for 4 out of 5 liver tumors. The most common underlying factor is cirrhosis caused by viral hepatitis, chronic alcoholism or obesity (4, 5), although HCC can also develop in the absence of cirrhosis. Most treatments are inadequate and there is also a lack of biomarkers for early diagnosis of HCC.

Diethylnitrosamine (DEN)-induced liver cancer in mice is the most widely used animal model of HCC, with a gene expression profile similar to that in a subset of human HCC with low survival (6). The near 100% effectiveness of DEN treatment to induce HCC with a reproducible time-course has made this model uniquely suited to analyze mechanisms of HCC.

Endocannabinoids are lipid mediators that interact with the same G protein-coupled receptors – CB1R and CB2R – that mediate the effects of the psychoactive ingredient of marijuana, Δ^9 -tetrahydrocannabinol. CB1R expression is very high in the brain and much lower yet functionally relevant in peripheral tissues including the liver, whereas CB2R expression is restricted primarily to immune and hematopoietic cells (7). Activation of hepatic CB1R stimulates *de novo* lipogenesis and induces hepatic insulin resistance (7, 8), and their expression is induced by chronic alcohol intake, high-fat diets and liver fibrosis (9–11), conditions known to predispose to cirrhosis and HCC. Hyperactivation of the biosynthesis of the endocannabinoid anandamide and induction of CB1R following partial hepatectomy in mice contribute to the early stages of liver regeneration via inducing key cell cycle proteins involved in mitotic progression, such as FOXM1 (12). FOXM1 is also upregulated in HCC and contributes to tumor progression and various aspects of tumorigenesis (13), and CB1R is induced in HCC (14). Such observations suggest a functional link between the endocannabinoid/CB1R system and FOXM1 in the development and progression of HCC.

We explored the possible such a functional link by monitoring the development and growth of DEN-induced HCC in wild-type mice treated with vehicle or a peripheral CB1R antagonist as well as in CB1R^{-/-} mice. Furthermore, we identified tumor-induced, CB1R-mediated changes in hepatic gene expression by analyzing the transcriptome of normal and cancerous liver tissue from individuals with HCC and from wild-type and CB1R^{-/-} mice. The results highlight the role of CB1R in inducing multiple tumor promoting pathways, including the G_{i/o}/CREBP/FOXM1/Bin1/IDO2 (indoleamine 2,3-dioxygenase 2) pathway that promotes tumor immune tolerance.

Materials and Methods

Animals

Procedures were approved by the Institutional Animal Care and Use Committee and performed in accordance with the NIH Guide for the Care and Use of Laboratory Animals. C57BL/6J mice were from Jackson Laboratories. CB1R^{-/-} and CB1R^{+/+} littermates were obtained by breeding heterozygotes that had been backcrossed to a C57BL/6J background (8). Mice were housed under 12 hour light-dark cycle, fed standard rodent chow and water ad libitum. Two week-old male CB1R^{+/+} and CB1R^{-/-} mice were injected intraperitoneally with 25 mg/kg of diethylnitrosamine (DEN, Sigma) (15) and were subjected to MRI at 8 months to verify the presence and measure the size of the tumor. DEN-treated CB1R^{+/+} mice were then started on the peripheral CB1R antagonist JD5037 (3mg/kg/day by oral gavage) or vehicle for 8 weeks followed by a repeat MRI scan and then sacrificed for obtaining HCC and adjacent non-tumor tissue for *in vitro* analyses.

Human HCC Biopsy Samples

Paraffin embedded blocks of human HCC and normal liver samples were purchased from Capitol Biosciences, Liver Tissue Cell Distribution System, UMN and Matrix Biological, LLC (Washington, DC). Twelve sets of non-tumor and corresponding tumor samples were obtained, with the donors de-identified. All patients were free from hepatitis A, B or C infection.

In vivo Monitoring of HCC by MRI

Mice were anesthetized with 5% isoflurane in 30% oxygen/70% nitrogen. The isoflurane was then reduced to 1–2% and anesthesia was maintained via a nose cone. Core body temperature was monitored rectally by a fiberoptic probe (FISO™) and maintained by blowing heated air into the magnet bore (Bair Hugger™). Breath rate was kept at 35–45 bpm by adjusting the isoflurane level. MRI imaging was performed on a 7T Bruker Bioscan with a 9 cm shielded gradient system. Following an initial locator scan, a block of 16 consecutive, coronal planes covering the mouse liver were scanned using a RARE sequence with the following parameters: slice thickness 1mm, 6×4cm FOV, 256×256 matrix, RARE factor 4, 4 averages, and TR/TE 2000/10.3. Scanning was gated to the breathing cycle so that data were only acquired in between breaths. The captured images were analyzed and quantified using NIH ImageJ software. For each animal, all 16 coronal planes were subjected to morphometric analysis and the plane showing the highest relative area occupied by HCC tissue, expressed in pixels, was used for statistical analyses. Mean values and standard errors were calculated from measurements obtained from 15–30 mice per treatment group.

RESULTS

CB1R Modulate the Incidence and Progression of DEN-induced HCC in Mice

The presence and size of HCC in DEN-treated mice was assessed at 8 months of age by MRI. As illustrated in Fig. 1A/B, the cross-sectional area of tumor tissue was significantly higher in wild-type than in CB1R^{-/-} liver. CB1R^{+/+} mice were then divided into two groups,

one group receiving vehicle and the other 3 mg/kg/day of JD5037 (16) for 8 weeks. Two months later, the size of tumors was significantly smaller in JD5037-treated than in vehicle-treated mice (Fig. 1C/D). Thus, genetic ablation or pharmacological blockade of CB1R attenuates HCC progression.

Activation of the Endocannabinoid/CB1R System in Murine and Human HCC

CB1R were readily detectable by immunohistochemistry in HCC from CB1R^{+/+} mice, but were barely detectable in the adjacent normal liver tissue (Figure 2A). The specificity of CB1R staining was verified by its elimination by blocking peptide or in livers from CB1R^{-/-} mice. CB1R mRNA was similarly increased in HCC versus adjacent non-tumor tissue from vehicle-treated but not from JD5037-treated mice (Figure 2B). CB1R immunoblots revealed a similar pattern of change at the protein level (Fig. 2C). Additionally, tumors from CB1R^{+/+} mice contained significantly more anandamide (AEA) compared to non-tumor tissue, and AEA levels were attenuated in HCC samples from JD5037-treated mice. There was no difference in AEA content in non-tumor vs HCC tissue from CB1R^{-/-} mice (Fig. 2D). Increased AEA in tumors is likely due to increased synthesis, as indicated by the increased expression of the AEA biosynthetic enzyme *N*-arachidonoyl phosphatidylethanolamide-specific phospholipase D (NAPE-PLD) in both mouse and human HCC tissue (Fig. S1).

CB1R activation contributed to increased cell proliferation in HCC versus non-tumor tissue, as deduced from a marked increase in Ki67 staining and its attenuation by CB1R but not by CB2R blockade (Fig. S2A), and from similar changes in *in vivo* BrdU uptake, quantified *ex vivo* by ELISA (Fig. S2B). No difference in AEA levels in DEN-treated vs control mice was detected in heart, kidney, brain and lung, except for a small increase in the spleen (Fig. S3). Tissue levels of the endocannabinoid 2-AG remained unchanged in DEN-treated vs control mice in all tissues including the liver (data not shown).

Human HCC had increased levels of immunoreactive CB1R relative to tumor-free areas (Figure 3A), which was confirmed by Western blots (Fig. 3B) and paralleled by increased *CNR1* gene expression (Fig. 3C). Similar to mice, AEA content was higher in human HCC versus tumor-free tissue (Fig. 3D). The diagnosis of HCC in these samples was verified in H&E counterstained sections highlighting nuclear atypia, involving enlarged nuclei with irregular nuclear membranes and prominent nucleoids as well as aberrant architecture (absence of sinusoids and portal areas), as illustrated in Fig. S4.

Transcriptome Profile of Human and Murine HCC

Gene expression profiles of tumor vs non-tumor tissue from four human HCC samples were obtained by RNAseq. The uniformly high percentage (86–94%) of matched reads documented the high quality of the sequencing data (Table S1). Induced genes were further screened for CB1R-dependent regulation by analyzing RNA sequencing data from pooled non-tumor and tumor liver tissue from CB1R^{+/+} and CB1R^{-/-} mice and JD5037-treated CB1R^{+/+} mice, as detailed in Methods. CB1R expression itself was markedly induced in both human and mouse tumors, and the increase in mouse tumors was abrogated in animals treated with JD5037 (Fig. S5).

CB1R-dependent Induction of FOXM1 in Human and Murine HCC

Among the genes overexpressed in HCC in a CB1R-dependent manner, we further analyzed the regulation of FOXM1, a transcription factor induced in HCC (13). Immunofluorescent staining of FOXM1 protein in human HCC samples indicates induction and nuclear localization (Fig. 4A), which was further validated by real-time PCR and Western blotting (Fig. 4B and C). FoxM1 was also overexpressed in tumor samples from CB1R^{+/+} but not CB1R^{-/-} mice (Fig. 4E and F). Nuclear localization of FoxM1 was observed in CB1R^{+/+} tumor tissue (Fig. 4D).

The mechanism of CB1R-dependent *FOXM1* expression was further analyzed in cultured Hepg2 liver tumor cells (Fig. 5A). *FOXM1* was robustly induced by 1–5 μ M AEA, which was abrogated by pertussis toxin, indicating the involvement of G_{i/o}-coupled signaling via cAMP. Because of the presence of a canonical cAMP response element (CRE) in the *FOXM1* promoter (Fig. 5B), we tested the role of cAMP in the regulation of *FOXM1* transcription using *FOXM1* promoter/luciferase constructs. AEA at 1 μ M markedly stimulated luciferase activity, blocked in the presence of 100 nM JD5037 and abrogated using a CRE deletion construct (Fig. 5B).

A number of cell cycle-related genes, many of them downstream targets of FoxM1, were also induced in tumors in a CB1R-dependent manner, most prominently the cyclin-dependent kinase inhibitor 1a and 3 (*Cdkn1a*, *Cdkn3*), as well as factors promoting angiogenesis, such as vascular endothelial growth factor C (*VegfC*) and platelet-derived growth factor B (*Pdgfb*) (Table S2). As a functional correlate of these latter, angiogenesis was increased in CB1R^{+/+} HCC vs non-tumor tissue, as reflected by an increase in isolectin⁺ vascular endothelial cells (Fig. S6).

Additional genes induced in a CB1R-dependent manner included genes linked to tumor promotion, but also some with tumor inhibitory activity, as listed in Table S2. There were also genes down-regulated in HCC by a CB1R-dependent manner, and these also included both tumor promoters and tumor suppressors (Table S2).

CB1R-dependent Induction of the Tryptophan Catabolic Enzyme IDO2 in HCC

Two isoforms of the enzyme indoleamine 2,3-dioxygenase, IDO1 and IDO2, as well as tryptophan 2,3-dioxygenase (TDO) catalyze the breakdown of the essential amino-acid tryptophan, which leads to immune suppression, a well-documented pathway of immune tolerance in cancer (17). Based on its transcriptome profile, IDO2 was one of the most robustly induced genes in human HCC, whereas IDO1 and TDO expression remained unchanged. IDO2 induction in human HCC was confirmed by immunohistochemistry, immunoblots and real-time PCR (Fig. 6A–C). Ido2 protein and mRNA were also increased in HCC compared to tumor-free tissue from CB1R^{+/+} but not CB1R^{-/-} mice (Fig. 6D,F,G). Hepatocyte-specific expression of Ido2 is indicated by its co-localization with albumin (Fig. 6E). HCC also increased Ido2 enzyme activity in wild-type but not in CB1R^{-/-} mice (Fig. 7A). In primary cultured mouse isolated hepatocytes, 10 μ M anandamide caused a 4-fold increase in *Ido2* mRNA, blocked by 100 nM JD5037 and absent in hepatocytes from CB1R^{-/-} mice, indicating CB1R involvement (Fig. 7B).

The tumor suppressor amphiphysin 2 (Bin1) negatively regulates IDO2 expression (18). *Bin1* was markedly down-regulated in both human and mouse HCC relative to tumor-free tissue (Fig. S7).

FOXM1 Regulates IDO2 which Promotes Tumor Growth by Altering Microenvironment

To test whether the CB1R-dependent induction of *Ido2* is via FoxM1, we analyzed the interaction of FoxM1 with the *Ido2* promoter in HCC and tumor-free tissue from CB1R^{+/+} and CB1R^{-/-} mice. CHIP analyses using FoxM1 antibody followed by PCR of the *IDO2* promoter region demonstrated a clear, CB1R-dependent regulation of *Ido2* expression by FoxM1 in HCC (Fig. 7C). Cancer promotion by IDO is thought to be due to immunosuppression secondary to the breakdown of tryptophan in the tumor microenvironment, which renders effector T cells inactive and dendritic cells immunosuppressive. Indeed, immunosuppressive Treg cells identified by the surface markers CD4, CD25 and FoxP3 were upregulated as early as 8 weeks following DEN treatment in the liver of wild-type but not CB1R^{-/-} mice (Fig. 7D).

Increased Ido2 activity contributes to tumor growth in mice

To test the contribution of increased Ido2 activity to tumor growth, 8-month old wild-type mice with DEN-induced HCC were treated for 6 weeks with the Ido2 inhibitor 1-methyl-L-tryptophan provided in the drinking water at 500 mg/kg/day. Vehicle-treated mice displayed marked progression of tumor growth, which was completely blunted in 1-methyl-L-tryptophan treated mice, as verified by repeat MRI scan at the end of treatment (Fig. S8).

CB1R-GRB2 Interaction in HCC

The GRB2 interactome is implicated in cancer development (19), and GRB2 is also associated with the progression of HCC (20). Transcriptome analyses of human HCC indicated up-regulation of the expression of *GRB2* and its downstream target *RAS*, which was confirmed by Western blotting (Fig. S9A). A similar up-regulation was evident in HCC samples from CB1R^{+/+} but not from CB1R^{-/-} liver (Fig. S9B). Furthermore, GRB2 interacted directly with CB1R, as indicated by their co-immunoprecipitation (Fig. S9C). In addition, flow cytometric cell cycle analyses of mouse HCC cells with CB1R surface expression demonstrated a direct correlation between the level of CB1R expression and the number cells in S-phase (Fig. S9D). These data suggest that surface expression and activation of CB1R is a marker of dividing tumor cells.

DISCUSSION

Previous studies have documented tumor inhibitory effects of plant-derived cannabinoids and their analogs, which could be abrogated by CB1R blockade (21), although the alternative involvement of CB2R (22–24), TRPV1 receptors (25), or receptor-independent effects has also been reported (26). These tumor inhibitory effects occur invariably at micromolar concentrations, well above the CB1 binding constants of the compounds, and thus may be due to down-regulation rather than activation of CB1R. In contrast, endocannabinoids acting at nanomolar concentrations close to their K_d values for CB1R promote the proliferation of a variety of tumor cells, which suggests their involvement in

promoting tumor growth (27), also supported by documented tumor inhibitory effects of CB1R antagonists, although off-target action of the antagonists could not be excluded (28). Here we have documented dramatic reductions in the size and number of chemically induced HCC in mice either by genetic ablation or pharmacological blockade of CB1R. This strongly supports the tumor-promoting role of endocannabinoids acting via hepatic CB1R, which are significantly overexpressed in both mouse and human HCC. These findings also agree with earlier observations that activation of hepatic CB1R by newly synthesized anandamide promotes hepatocyte proliferation in the early stages of liver regeneration (12). Reversal of the tumor-induced overexpression of both CB1R and anandamide by CB1R blockade is also in agreement with earlier findings in the steatotic (11, 29), diabetic (30) or regenerating liver (12), and is compatible with autoinduction of both the receptor and the synthesis of its endogenous ligand, a common feature of tumor promoting growth factors (31).

The strategy of analyzing the transcriptome of tumor and adjacent tumor-free liver tissue from both wild-type and CB1R^{-/-} mice and from wild-type mice treated with a CB1R antagonist helped identifying tumor-related genes regulated by CB1R. The putative link between HCC and the genes thus identified was further validated using human HCC specimens. Among the genes up-regulated in HCC in a CB1R-dependent manner, both *Cnr1* (the gene encoding CB1R itself) and *FoxM1* are also upregulated in the regenerating liver (12). The hepatic level of anandamide was also markedly increased in the tumor, which support an increase in CB1R signaling.

The transcription factor FOXM1 is exclusively expressed in proliferating cells, where it activates the transcription of cell cycle proteins involved in G1/S and G2/M transition and is essential for M phase progression (13). FOXM1 is essential for the development of HCC (32), and silencing FOXM1 expression inhibits HCC growth (33). Here we show that tumor promotion by FOXM1 may also involve altering the tumor microenvironment through increased expression of IDO2. Direct regulation of IDO2 expression by FOXM1 is indicated by the binding of FOXM1 to the promoter region of *IDO2*, likely resulting in transcriptional activation. Increased IDO2 expression may additionally result from down-regulation of *Bin1*, a known transcriptional inhibitor of *IDO2* (18), in both human and mouse HCC. In turn, CB1R up-regulates *FOXM1* gene transcription via a G_{i/o}/cAMP/CREBP pathway as indicated by the anandamide-induced increase in FOXM1 expression in HepG2 cells and its abrogation by PTX or by deletion of a canonical CRE site from the *FOXM1* promoter (Fig. 5).

The growth of HCC is facilitated by the diminished anti-tumor immune response of natural killer (NK) cells, which has been linked to increased expression and activity of Treg cells (34). Tryptophan depletion and/or the accumulation of tryptophan metabolites such as kynurenine is a well-established mechanism whereby tumors suppress the anti-tumor activity of NK cells by boosting the formation and activity of Treg cells (35). The observed increase in Treg cells in liver tumor tissue is compatible with tryptophan depletion and could contribute to suppression of an antitumor immune response. Of the three enzymes involved in tryptophan catabolism (IDO, IDO2 and TDO) only IDO2 was induced in HCC, which supports its key role in suppressing the anti-tumor immune response. Tumor promotion by

IDO2 also involves increased angiogenesis (36), indicated by the CB1R-dependent increase in the expression of the pro-angiogenic genes *Pdgfb* and *Vegfc* and the parallel increase in vascular endothelial cells in HCC compared to non-tumor tissue. The CB1R-mediated induction of IDO2 contributes to the progression of DEN-induced HCC, as indicated by the inhibition of tumor growth by chronic *in vivo* treatment with the IDO inhibitor 1-methyl-L-tryptophan.

The role of proinflammatory immune cells and cytokines in HCC progression has been well documented (37, 38). Recently, a unique set of CD14⁺CTLA4⁺ dendritic cells isolated from peripheral blood of HCC patients was found to suppress CD4⁺ T cell proliferation *in vitro* via increased expression of interleukin (IL)-10 and IDO (39). Here we show that hepatocytes themselves express IDO2 that is increased in HCC or in normal hepatocytes via CB1R activation, and could thus contribute to the immune suppressive microenvironment in HCC.

In addition to the CB1R/Bin1/FOXM1/IDO2 interactome targeting immune suppressive and pro-angiogenic mechanisms, CB1R are involved in inducing a number of other tumor-related genes in HCC. Of these, the GRB2 interactome plays a critical role in regulating receptor tyrosine kinases, cell motility, and EGF-regulated pathways involved in cancer progression, invasion and metastasis (19, 40). As documented here, CB1R interacts directly with GRB2 in both HCC and tumor-free samples, and its downstream effector RAS, another key cell cycle regulator, is also increased in HCC in a CB1R-dependent manner. Interestingly, genes up-regulated in HCC included not only tumor promoter but also tumor inhibitory genes, and the reverse was also true regarding down-regulated genes, which included some tumor promoters (Table S2). While these latter changes may represent compensatory mechanisms, they may also account for the often contradictory data in the literature on tumor promoting or anti-tumor effects of cannabinoids.

In summary, the present findings reveal a tumor promoting function of the endocannabinoid/CB1R system in HCC via inducing a number of tumor promoting pathways, including immune suppression in the tumor microenvironment through activation of the FOXM1/Bin1/IDO2 interactome. These findings raise the possibility that peripheral CB1R blockade may have a role in the treatment of HCC.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

HCC	hepatocellular carcinoma
CB1R	Cannabinoid receptor 1
DEN	diethylnitrosamine
FOXM1	Forkhead Box M1
IDO2	indoleamine 2,3-dioxygenase 2
AEA	arachidonoyl ethanolamide or anandamide
CREBP	cyclic AMP response element binding protein

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Figure 1. Genetic or Pharmacological Ablation of CB1R Attenuates HCC Progression
A. Representative MRI scans in DEN-treated, 8 months-old CB1R^{+/+} and CB1R^{-/-} mice. **B.** Total tumor area in CB1R^{-/-} and CB1R^{+/+} mice was assessed and statistically analyzed as described in Methods. * P<0.05 n=25–30 mice/group. **C.** Representative MRI scans from DEN-treated CB1R^{+/+} mice treated with JD5037, 3 mg/kg/day, or vehicle from 8 to 10 months. **D.** Quantifications of the data in C was done as described for panel B. * P<0.05 vehicle vs JD5037 treatment, n=15–20 mice/group.

Figure 2. The CB1R/Endocannabinoid System is Activated in Murine HCC

A. Immuno-histochemical analyses of CB1R in non-tumor and tumor area of mice liver. Blocking peptides and tumors from CB1R^{-/-} mice were used as negative control. Induction of CB1R demonstrated by RT-PCR (**B**) and Western blotting (**C**). **D.** Induction of the AEA in tumor vs non-tumor tissue. *P<0.05; n=6–10 mice/group. **E.** Flow-cytometric measurements of CB1R surface expression in suspensions of single cells isolated from tumor and non-tumor region of CB1R^{+/+} mice.

Figure 3. Induction of CB1R and AEA in Human HCC

A. Immunohistochemical analyses of CB1R in non-tumor and tumor area of human liver samples. Blocking peptides were used as negative control, scale bar: 100 μ m. CB1R protein detected by Western blot (**B**) and *CNR1* expression quantified by RT-PCR (**C**). **D.** Increased AEA content in HCC vs. normal human liver tissue. * $P < 0.05$ vs non-tumor tissue. $n = 8-12$ subjects/group.

Figure 4. CB1R-dependent Up-regulation of FOXM1 in Murine and Human HCC

A–C: Human HCC. **A:** Immunofluorescence analyses of FoxM1 in liver biopsy samples. Nuclear Stain (green) and anti-Foxm1 (red) were visualized with Sytox Green and Texas Red-conjugated secondary Abs, respectively. Tumor-induction of FOXM1 by RT-PCR (**B**) and immunoblot analyses (**C**). * $P < 0.05$ tumor vs non-tumor; $n = 8–12$ /group. **D–F: Murine HCC.** **D:** Immunofluorescence analyses of FOXM1 in tumor vs. non-tumor areas of CB₁R^{+/+} vs CB₁R^{-/-} liver. Nuclear Stain (red) and anti-Foxm1 (green) were visualized with

PI and FITC-conjugated secondary Abs. Similar changes observed by RT-PCR (**E**) and immunoblot analyses (**F**). * $P < 0.05$, tumor vs non-tumor; $n = 8-12$ /group.

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Figure 5. Mechanism of CB1R-Induced *FOXM1* Expression

A: FOXM1 protein in HepG2 cells is increased by anandamide (AEA) in the absence but not presence of pertussis toxin. Cells were incubated with 1–5 μ M anandamide for 1 h either in the absence or presence of 100 ng/mL PTX. Cell extracts were analyzed by Western immunoblotting. **B:** FOXM1 promoter activity is increased by AEA in a CRE-dependent manner. Promoter activity was quantified using a luciferase reporter system. The site of deletion of a canonical CRE site is schematically illustrated.

Figure 6. IDO2 is Up-regulated in Human and Murine HCC

A–C: Human HCC. **A:** Immuno-histochemical analyses of IDO2 protein in tumor vs non-tumor liver tissue (top), with hepatocyte-specific expression of IDO2 indicated by colocalization with hepatocyte-specific antigen (HSA –green, IDO2 red). **B:** RT-PCR and **C:** immunoblot analyses of IDO2; * $P < 0.05$ tumor vs non-tumor; $n = 8–12$ /group. **D–F:** Tumor and non-tumor tissue from CB₁R^{+/+} and CB₁R^{-/-} mice were analyzed as described for human liver, * $P < 0.05$ tumor vs non-tumor; $n = 8–12$ mice/group.

Figure 7. CB1R- and FOXM1-dependent Induction of IDO2 Increases Treg Cells in HCC Tissue
A. CB1R-dependent induction of *Ido2* expression by AEA in mouse isolated hepatocytes. *Ido2* mRNA was quantified by RT-PCR; *P<0.05 relative to vehicle; n=6/group **B.** IDO activity in tumor and non-tumor tissue. **C.** Chromatin immunoprecipitation (CHIP) indicates direct interaction of FOXM1 with *IDO2* promoter in CB1R^{+/+} tumor samples. **D.** Representative flow-cytometry scatter plot indicates CB1R-mediated increase in CD4⁺

CD25⁺/Foxop3⁺ Treg cells in mouse liver 8 weeks following DEN-treatment. *P<0.05 vs vehicle; n=6/group.

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