Cannabinoid CB2 Receptors Protect Against Alcoholic Liver Disease by Regulating Kupffer Cell Polarization in Mice

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Activation of Kupffer cells plays a central role in the pathogenesis of alcoholic liver disease. Because cannabinoid CB2 receptors (CB2) display potent anti-inflammatory properties, we investigated their role in the pathogenesis of alcoholic liver disease, focusing on the impact of CB2 on Kupffer cell polarization and the consequences on liver steatosis. Wild-type (WT) mice fed an alcohol diet showed an induction of hepatic classical (M1) and alternative (M2) markers. Cotreatment of alcohol-fed mice with the CB2 agonist, JWH-133, decreased hepatic M1 gene expression without affecting the M2 profile. In keeping with this, genetic ablation of CB2 enhanced hepatic induction of M1 gene signature and blunted the induction of M2 markers. CB2 also modulated alcohol-induced fatty liver, as shown by the reduction of hepatocyte steatosis in JWH-133-treated mice and its enhancement in CB2−/− animals. Studies in isolated Kupffer cells and cultured macrophages further demonstrated that CB2 inhibits M1 polarization and favors the transition to an M2 phenotype. In addition, conditioned-medium experiments showed that preventing M1 polarization in CB2-activated macrophages protects from lipid accumulation in hepatocytes. Heme oxygenase-1 (HO-1) mediated the anti-inflammatory effects of CB2 receptors. Indeed, alcohol-fed mice treated with JWH-133 showed increased hepatic expression of macrophage HO-1, as compared to vehicle-treated counterparts. In keeping with this, JWH-133 induced HO-1 expression in cultured macrophages, and the HO-1 inhibitor, zinc protoporphyrin, blunted the inhibitory effect of JWH-133 on lipopolysaccharide-induced nuclear factor-kappa B activation and M1 polarization. Altogether, these findings demonstrate that CB2 receptors display beneficial effects on alcohol-induced inflammation by regulating M1/M2 balance in Kupffer cells, thereby reducing hepatocyte steatosis via paracrine interactions between Kupffer cells and hepatocytes. These data identify CB2 agonists as potential therapeutic agents for the management of alcoholic liver disease. (HEPATOLOGY 2011;54:1217-1226)

Macrophages are a highly heterogeneous, plastic population that undergo pleiotropic coordinated responses to tissue damage through distinct programs of activation, known as classical (M1) or alternative (M2).[1,2] The classical M1 activation process is mainly driven by bacterial molecular patterns, including endotoxin/lipopolysaccharide (LPS), or by Th1 cytokines, such as interferon gamma,

Abbreviations: Arg1, arginase 1; ALT, alanine aminotransferase; AST, aspartate aminotransferase; Ca^{2+}, calcium; CB2, cannabinoid CB2 receptors; CCL, chemokine (C-C motif) ligand; CD, control diet; CD163, cluster of differentiation 163; CM, conditioned medium; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; HO-1, heme oxygenase-1; IL, interleukin; ITS, insulin, transferrin, selenium; LPS, lipopolysaccharide; Mg^{2+}, magnesium; Mgl1, macrophage galactose-type C-type lectin 1; Mrc2, mannose receptor C type 2; mRNA, messenger RNA; NF-κB, nuclear factor-kappa B; NOS2, nitric oxide synthase 2; oxLDL, oxidized low-density lipoprotein; TLR4, Toll-like receptor 4; TNF-α, tumor necrosis factor alpha; WT: wild type; ZnPP, zinc protoporphyrin.

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and results in high proinflammatory and bactericidal potential.[1,2] Thus, the macrophage switch to an M1 phenotype plays a key role in the pathogenesis of a variety of chronic inflammatory diseases, including atherosclerosis,[2] inflammatory bowel disease,[3] or insulin resistance associated with obesity.[4] In contrast, Th2 cytokines, such as interleukin (IL)-4 and IL-13, promote macrophage polarization into an alternative M2 phenotype.[2] M2-polarized macrophages promote the resolution of inflammation and are involved in tissue repair and remodeling.[1,2] Indeed, recent reports indicate that alternative M2 macrophages show antidiabetic properties[5,6] and beneficial effects on atherosclerosis,[2] muscle repair,[7] and infectious colitis.[3]

Chronic alcohol abuse, a leading cause of liver-related morbimortality in Western countries, is associated with several patterns of liver injury, ranging from isolated fatty liver to alcoholic hepatitis, cirrhosis, and hepatocellular carcinoma.[8] Compelling evidence indicates that Kupffer cells play a key role in the early events of alcoholic liver disease. Thus, gadolinium-induced depletion of Kupffer cells prevents steatosis and liver inflammation in rodent models of chronic alcohol feeding.[9] The currently accepted model stipulates that alcohol-induced enhancement of gut permeability to bacterial LPS/endotoxin increases the translocation of endotoxin to the liver that activates Kupffer cells after binding to Toll-like receptor 4 (TLR4).[8,10] Alcohol also sensitizes Kupffer cells to LPS by increasing oxidative stress and primes Kupffer cells to respond to LPS by up-regulating a number of proinflammatory mediators, including cytokines and chemokines, as well as their cognate receptors.[11,12] Among the panel of secreted cytokines, tumor necrosis factor-alpha (TNF-α) is considered as a major mediator of alcohol-induced liver injury, as shown in a number of clinical studies,[8,13] and on the basis of experimental data demonstrating the substantial reduction of hepatic steatosis, as well as liver inflammation and injury, in TNF-R1 deficient mice and in rats treated with TNF-α antibodies.[14,15] These findings have prompted an evaluation of the effect of TNF-α antibody treatment in patients with severe alcoholic hepatitis, an entity associated with elevated short-term mortality. Unfortunately, direct blockade of TNF-α has proved deleterious, owing to a high rate of infectious events in these patients.[16] Therefore, other strategies need to be envisioned, and interventional tools able to favor the anti-inflammatory M2 phenotype in the liver warrant consideration as potential protective agents for the management of alcohol-induced liver injury.

Cannabinoid CB2 receptors are G-protein-coupled receptors predominantly expressed by cells of the immune system, including macrophages. These receptors are constituent elements of an endocannabinoid system with pleiotropic effects that also comprise CB1 receptors, highly lipophilic ligands known as endocannabinoids, and mediators responsible for their synthesis, metabolism, and catabolism.[17,18] A number of studies have demonstrated that CB2 receptors display potent anti-inflammatory properties, although pro-inflammatory effects have occasionally been described.[17,19,20] Thus, CB2 receptors reduce inflammation in models of atherosclerosis[21] and in a variety of neuroinflammatory disorders, including multiple sclerosis, Alzheimer's disease, or amyotrophic lateral sclerosis.[22] In addition, *in vitro* studies have shown that CB2-receptor activation impairs several macrophage functions, such as oxidized low-density lipoprotein (oxLDL)-induced inflammatory response, oxidative stress, migration, and antigen processing.[22] In the liver, recent data indicate that CB2 receptors are induced after acute or chronic injury, both in Kupffer cells and in liver fibrogenic cells.[23-25] Remarkably, endogenous activation of these receptors has been shown to limit liver injury in several instances. Thus, CB2 receptors reduce hepatocyte apoptosis and promote liver regeneration in response to an acute insult,[24] inhibit the inflammatory process associated with acute ischemia/reperfusion,[26] and prevent the progression of hepatic fibrosis in response to chronic liver injury.[23] In addition, endogenous production of the nonselective CB1/CB2 ligand, 2-arachidonoylglycerol, is increased in the liver in response to chronic alcohol feeding.[27] However, though recent studies have reported the steatogenic and fibrogenic properties of CB1 receptors,[27,28] there are no data as to the functional relevance of CB2 receptors during chronic exposure to ethanol. We show here that during alcohol-induced liver injury, activation of Kupffer-cell CB2 receptors

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inhibits classical M1 polarization and favors a transition to an M2 alternative phenotype by a mechanism involving heme oxygenase-1 (HO-1) induction, thereby protecting from the deleterious inflammatory response to chronic ethanol feeding. In addition, our data indicate that activation of macrophage CB2 receptors reduces the development of alcohol-induced fatty liver by paracrine effects on hepatocytes. These findings identify CB2 agonism as a potential therapeutic approach for the management of alcohol-induced liver injury.

Materials and Methods

Additional methods are available in the Supporting Information.

Animals. Female, (8-10 weeks old) mice were used. Experiments included wild-type (WT) C57Bl/6J mice (Janvier, Le Genest, France) and mice with a targeted mutation of the *Cnr2* gene.[29] Homozygous CB2−/− animals were obtained from heterozygous CB2+/− mice that were back-crossed with WT C57Bl/6J animals over 10 generations, and further intercrossed to obtain homozygous animals. Animals were housed in temperature- and humidity-controlled rooms, kept on a 12-hour light-dark cycle, and provided unrestricted amounts of food and water. Animal procedures were conducted in accord with French government policies (Comité d'éthique COMETH authorization no.: 10-0048).

Chronic Exposure to Ethanol. WT and CB2−/− mice were fed for 17 days with a liquid diet adapted from Lieber-De Carli, as described by Gustot et al.[30] Briefly, the ethanol diet was obtained by adding absolute ethanol to a solution of caseine, oils (e.g., safflower, corn, and olive oils), and powders (e.g., maltodextrin, vitamins, xanthan gum, choline bitartrate, mineral mix, methionine, and cellulose) in distillated water. The final concentration of ethanol in this liquid diet is 6.3% (vol/vol), and ethanol accounts for 28% of total caloric intake. The control diet (CD) was obtained by replacing the ethanol by an equivalent quantity of maltodextrin. WT and CB2−/− mice were randomized into ethanol- (n = 15 for WT and CB2−/−) and CD-fed (n = 6 for WT and CB2−/−) groups, then adapted to control liquid diet *ad libitum* for 7 days. Ethanol-fed groups were allowed free access to a 6.3% (vol/vol) ethanol diet for 10 days. Control mice were pair-fed with isocaloric control diet over the entire feeding period. Three independent experiments were performed with the same number of animals and treatments.

The impact of the CB2 agonist, JWH-133, was evaluated in WT mice administered a daily intraperitoneal injection of JWH-133 (3 mg/kg; n = 15) or its vehicle (n = 15) during the 10-day feeding with ethanol. JWH-133 was freshly dissolved in a vehicle solution containing 1 drop of Tween 80 in 0.1 mL of dimethyl sulfoxide, sonicated, and further diluted 50 times in NaCl 9‰.

Body weight and food intake were measured daily for all experiments. The liver was removed, weighed, and either fixed in buffered formalin or snap-frozen in liquid nitrogen. All samples were stored at −80°C until use.

Mouse Kupffer Cells. A Kupffer cell–enriched fraction was obtained from WT and CB2−/− mice after perfusion with liberase and differential centrifugation in Percoll. Briefly, the livers were perfused *in situ* with an isotonic calcium (Ca^{2+})- and magnesium (Mg^{2+})-free saline solution containing 10 mM of Ca^{2+} and 15.4 μg/mL of liberase for 10 minutes. After digestion in 10 mM of Ca^{2+}, 15.4 μg/mL of liberase, 10 μg/mL of DNAse I, and 200 μg/mL of pronase, hepatocytes were pelleted, and the supernatant containing nonparenchymal cells was further centrifuged at 400*g*, resuspended in RPMI with 2% fetal bovine serum (FBS), and separated by centrifugation on a 25%-50% Percoll gradient. The Kupffer-cell fraction located at the interface of the 25%-50% Percoll layer was seeded in RPMI containing 10% FBS and 10 mM of HEPES. This procedure routinely yielded 2×10^6 cells/liver with a purity higher than 65%, as determined by F4/80 immunostaining. Adherent Kupffer cells were treated with 1 ng/mL of LPS or 5 ng/mL of IL-4 for 6 hours.

RAW264.7 Macrophages. Cells were seeded in Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% FBS. After 24 hours, cells were serum-starved and treated with 5 μM of JWH-133 or vehicle for an additional 24-hour period. When indicated, cells were treated with 1 ng/mL of LPS or 5 ng/mL of IL-4 during the last 6 hours.

Culture and Treatment of Hepatocyte Cell Line AML-12. Cells were cultured in DMEM/F12 supplemented with 10% FBS, ITS (insulin, transferring, selenium) (5 μg/mL of insulin, 5 μg/mL of transferrin, and 5 ng/mL of selenium) and 40 ng/mL of dexamethasone. Cells were incubated for 24 hours with a conditioned medium (CM) obtained from RAW264.7 cells treated with 5 μM of JWH-133 or vehicle for 24 hours and 1 ng/mL of LPS for the last 6 hours.

Statistical Analysis. Results are expressed as mean ± standard error of the mean and were analyzed by Mann-Whitney or Kruskal-Wallis, as appropriate,

Table 1. General Characteristics of Mice Fed Alcohol and Control Diet

	WT CD	CB2−/−CD	WT Alcohol	CB2−/−Alcohol	WT CD	WT Alcohol	JWH-133-Treated WT Alcohol
Body weight before alcohol (g)	21.6±0.3	21.8 ± 0.1	21.4 ± 0.2	21.7 ± 0.3	19.6 ± 0.4	19.3 ± 0.2	20.1 ± 0.2
Body weight 10 days after alcohol (g)	21±0.4	21.5 ± 0.1	20.8 ± 0.3*	20.8 ± 0.4*	19.6 ± 0.4	19.4 ± 0.2	19.4 ± 0.2
Alcohol consumption (g/kg/day)	–	–	18.1 ± 0.4	17 ± 0.3	–	21.0 ± 1.0	19.9 ± 0.6
Serum ethanol level (g/L)	–	–	1.3 ± 0.1	1.2 ± 0.2	–	ND	ND
AST (IU/L)	92 ± 12	94 ± 17	209 ± 16*	218 ± 35*	96 ± 6	244 ± 22*	214 ± 28*
ALT (IU/L)	26 ± 4	25 ± 3	84 ± 13*	88 ± 20*	21 ± 3	38 ± 6*	38 ± 6*

*$P < 0.05$, as compared to control-diet–fed mice.

Abbreviations: WT, wild type; CD, control diet; ALT, alanine aminotransferase; AST, aspartate aminotransferase; ND, not determined.

using Prism 4.0 software (GraphPad Software, Inc., San Diego, CA). $P < 0.05$ was taken as the minimum level of significance.

Results

CB2 Receptors Regulate Kupffer Cell Polarization in Alcohol-Fed Mice. Kupffer cell polarization was evaluated in parallel groups of female WT and CB2−/− mice and in WT mice concurrently treated with the CB2 agonist, JWH-133. Animals were fed either a Lieber-De Carli alcohol diet, modified according to Gustot et al.[30] or a paired isocaloric diet. General characteristics of experimental groups are depicted in Table 1. Body weights were similar between groups at the end of the experiment (Table 1), and ethanol-fed groups showed no differences in daily alcohol intakes, serum ethanol levels, and serum transaminases at time of sacrifice (Table 1).

As anticipated, alcohol-fed WT mice displayed significant hepatic induction of M1 markers, including TNF-α and the chemokines, chemokine (C-C motif) ligand 3 (CCL3), CCL4, and IL-6 (Fig. 1). In addition, there was also a parallel induction of genes characteristic of an M2 gene signature, such as arginase 1 (Arg1), mannose receptor C type 2 (Mrc2), and cluster of differentiation 163 (CD163) (Fig. 1). Chronic alcohol feeding did not increase F4/80 and CCR2 messenger RNA (mRNA) expression in either group of animals (Fig. 1), therefore ruling out the possibility that alterations in hepatic M1/M2 marker expression might be related to infiltrating by blood monocytes. These findings show that chronic alcohol feeding promotes polarization of Kupffer cells toward a mixed M1/M2 phenotype.

Treatment of alcohol-fed WT mice with the CB2 receptor agonist, JWH-133, inhibited the induction of M1 genes, as shown by the decrease in IL-6, TNF-α, nitric oxide synthase 2 (NOS2), CCL3, and CCL4 expressions, compared to vehicle-treated animals (Fig. 1A), whereas the M2 response was unaffected (Fig. 1A). CB2−/− mice displayed opposite alterations in the M1 response to alcohol, characterized by enhanced hepatic induction of several M1 markers, including IL-1β, IL-6, TNF-α, and NOS2, compared to WT counterparts (Fig. 1B). In addition, CB2 receptor invalidation also blunted M2 response to alcohol feeding, as reflected by unchanged expressions of Arg1, Mrc2, macrophage galactose-type C-type lectin 1 (Mgl1), and CD163 mRNAs, as compared to control diet-fed mice (Fig. 1B). Altogether, these data indicate that endogenous or exogenous activation of CB2 receptors prevents alcohol-induced M1 polarization of Kupffer cells. In addition,

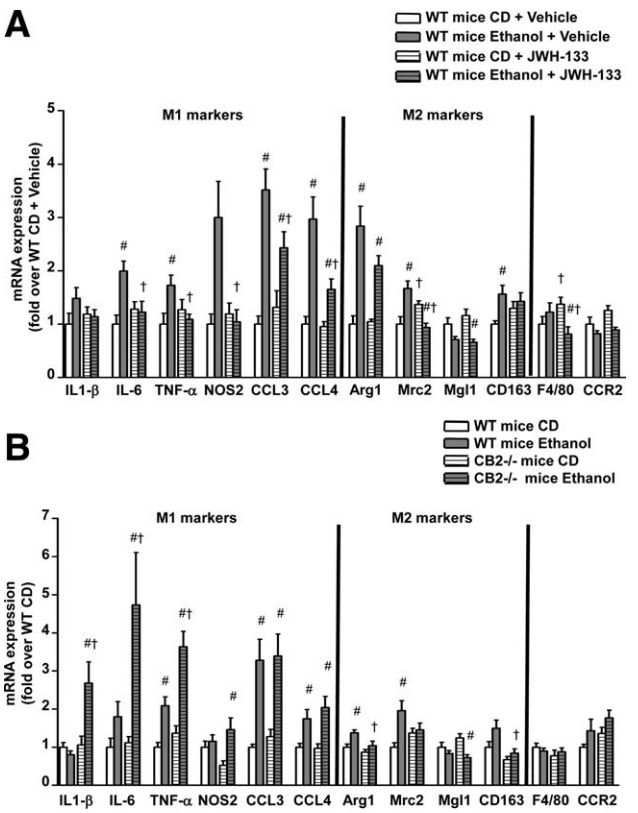

Fig. 1. Endogenous or exogenous activation of CB2 receptors prevents alcohol-induced classical M1 polarization of Kupffer cells and favors alternative M2 polarization *in vivo*. mRNA expression of M1 and M2 genes was characterized in (A) ethanol- or CD WT mice treated with JWH-133 (n = 20) or vehicle (n = 20). #$P < 0.05$ for ethanol versus control diet; †$P < 0.05$ for JWH-133 versus vehicle. (B) Ethanol- or CD-fed WT (n = 63) and CB2−/− (n = 58) mice. #$P < 0.05$ for ethanol versus control diet; †$P < 0.05$ for CB2−/− versus WT.

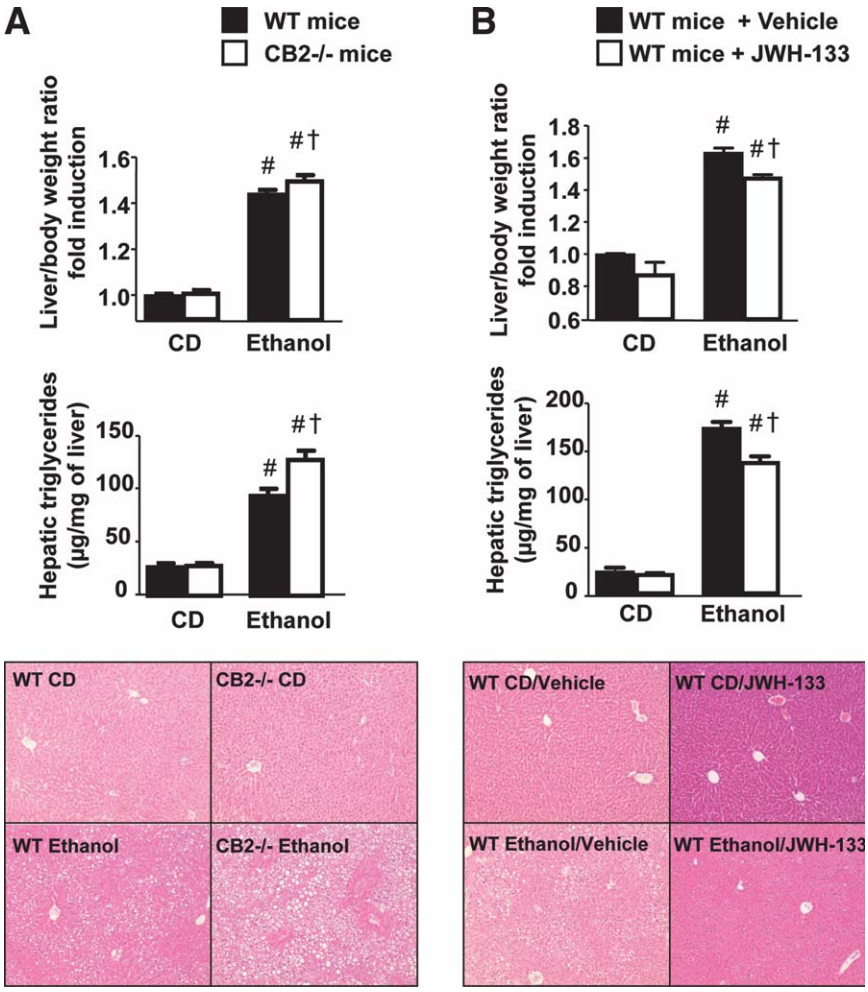

Fig. 2. Endogenous or exogenous activation of CB2 receptors prevents alcohol-induced hepatic steatosis. Liver to body weight ratio, hepatic triglyceride content, and hematoxylin-eosin staining (original magnification, ×200) (A) in CD-fed or alcohol-fed WT (n = 63) and CB2−/− mice (n = 58). #$P < 0.05$ for ethanol versus control diet; †$P < 0.05$ for CB2−/− versus WT. (B) CD-fed or alcohol-fed WT mice treated with JWH-133 (n = 20) or vehicle (n = 20). #$P < 0.05$ for ethanol versus control diet; †$P < 0.05$ for JWH-133 versus vehicle.

findings in CB2-deficient animals also suggest that CB2 receptors promote a switch of Kupffer cells toward an alternative M2 phenotype.

Beneficial Impact of CB2 Receptors on Alcohol-Induced Fatty Liver. We next investigated the impact of CB2 receptor modulation on alcohol-induced fatty liver, a characteristic feature of alcoholic liver disease. CB2-deficient, alcohol-fed mice displayed more severe steatosis, higher levels of liver triglycerides, and a more pronounced hepatomegaly, compared to alcohol-fed WT counterparts (Fig. 2A). Consistent with these data, alcohol-fed WT mice exposed to JWH-133 showed a lesser increase in liver/body weight ratio and in hepatic triglyceride accumulation (Fig. 2B). These findings demonstrate that endogenous and exogenous activation of CB2 receptors reduces the development of alcohol-induced fatty liver.

Antisteatogenic Effects of CB2 Receptors Result From Paracrine Effects of Macrophage CB2 Receptors on Hepatocytes. Considering the predominant expression of CB2 receptors in immune cells, culture experiments were designed to evaluate the role of macrophage CB2 receptors in the regulation of macrophage polarization and hepatocyte steatogenesis.

In a first series of experiments, we characterized the impact of CB2 Kupffer cells isolated from WT or CB2−/− mice and in the macrophage cell line, RAW264.7. M1 and M2 polarization was induced by incubation with LPS and IL-4, respectively. In keeping with *in vivo* findings, CB2−/− Kupffer cells exhibited an enhanced M1 response to LPS, compared to WT Kupffer cells, as shown by a greater induction of TNF-α, IL-1β, and CCL4 induction and a tendency to increase IL-6 expression (Fig. 3A). In addition, the alternative response of CB2−/− Kupffer cells to IL-4 was reduced, as demonstrated by the lower induction of Mrc2, C-type lectin domain family 7 member A (Clec7A) and a tendency to decrease Mgl1 (Fig. 3B). In contrast, JWH-133 reduced M1 gene expression (Fig. 4A) and corresponding cytokine secretion (Fig. 4B) in RAW264.7 cells, without affecting the alternative M2 response elicited by IL-4 (Fig. 4C). There was no effect of JWH-133 on TLR4 mRNA expression (data not shown), suggesting that CB2 receptor

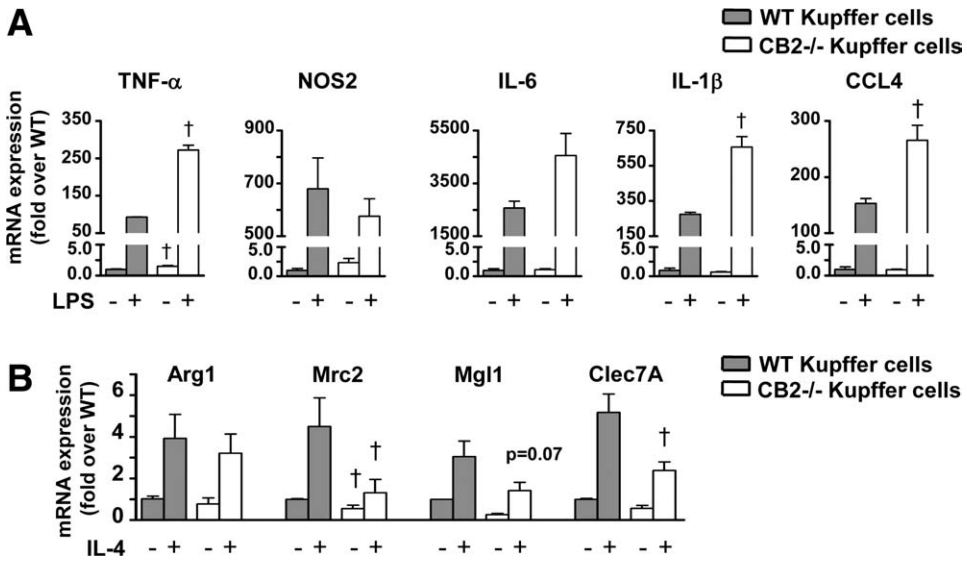

Fig. 3. CB2 receptor invalidation favors M1 polarization and reduces alternative M2 polarization of Kupffer cells *in vitro*. Characterization of M1 and M2 gene-expression profile in Kupffer cells isolated from WT and CB2−/− mice after incubation for 6 hours with 1 ng/mL of LPS (A) with 5 ng/mL of IL-4 (B) (n = 3). †$P < 0.05$ for CB2−/− versus WT.

activation does reduce the sensitivity of macrophages to LPS. Overall, these data indicate that activation of macrophage CB2 receptors reduces the proinflammatory M1 response to LPS and contributes to the alternative M2 response to IL-4, in keeping with results obtained from *in vivo* experiments.

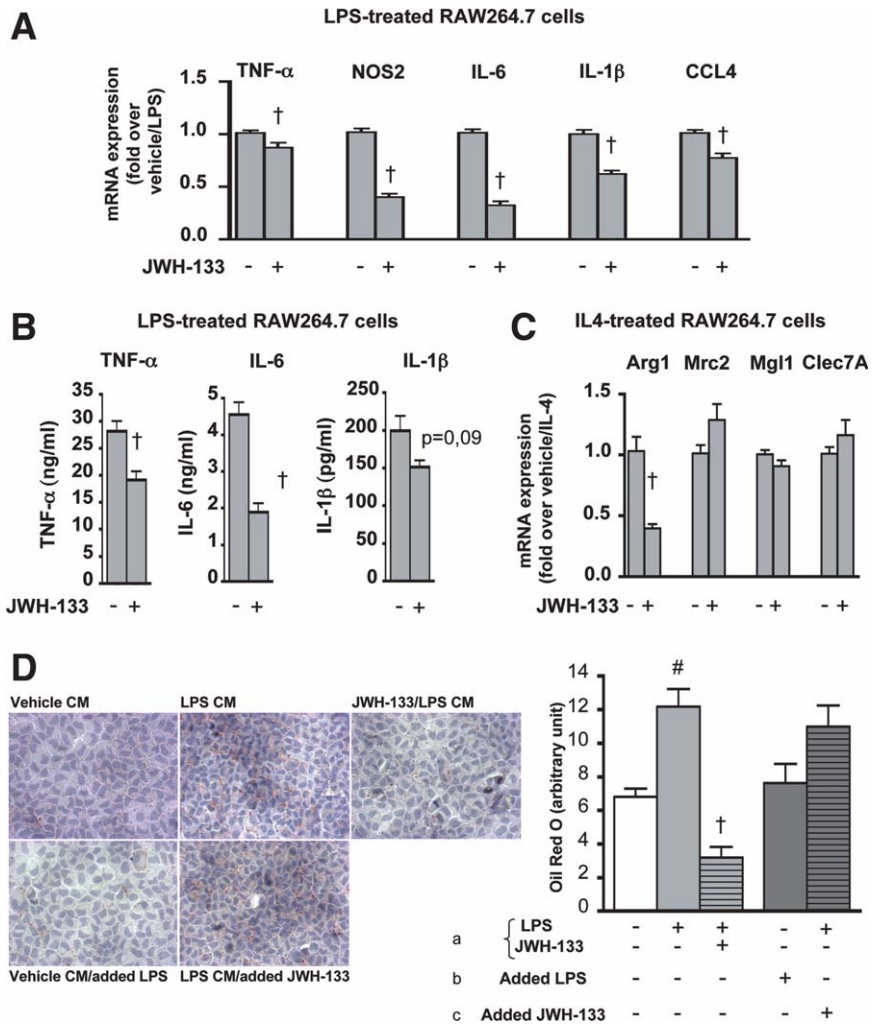

Fig. 4. *In vitro* activation of macrophage CB2 receptor inhibits the proinflammatory M1 phenotype and the steatogenic responses via paracrine effects on hepatocytes. RAW264.7 cells were treated with vehicle or 5 μM of JWH-133 for 18 hours and further incubated with 1 ng/mL of LPS for (A) 6 hours or (B) 24 hours or (C) with 5 ng/mL of IL-4 for 6 hours. (A) M1 gene expression, (B) quantification of secreted cytokine levels, and (C) M2 gene expression (n = 3) †$P < 0.05$ for JWH-133 versus vehicle. (D) Oil Red O staining and quantification from AML-12 hepatocytes exposed to a) the CM from RAW264.7 cells treated with the indicated effectors, b) 1 ng/mL of LPS added directly to the CM from vehicle-treated RAW264.7 cells, c) 5 μM of JWH-133 directly added to the CM from LPS-stimulated RAW264.7 cells. #$P < 0.05$ for LPS versus H_2O; †$P < 0.05$ for JWH-133 versus vehicle.

We next evaluated the impact of CB2 receptor activation in macrophages on fat accumulation in hepatocytes. To that aim, CM was collected from LPS-exposed RAW264.7 cells incubated in the absence or presence of JWH-133. Hepatocytes cultured in the presence of the CM obtained from LPS-exposed RAW264.7 cells displayed enhanced lipid accumulation, as compared to control, as illustrated by Oil Red O staining quantification (Fig. 4D). Fat accumulation was reduced by 74% in hepatocytes exposed to CM prepared from JWH-133-treated, LPS-exposed RAW264.7 macrophages (Fig. 4D). LPS added directly to AML-12 cells had no effect on fat accumulation (Fig. 4D). Moreover, the addition of JWH-133 to AML-12 cells had no protective effects against steatosis elicited by the CM of LPS-treated RAW264.7 cells, therefore ruling out direct effects of the compounds on hepatocytes (Fig. 4D). Altogether, these data demonstrate that macrophage CB2 receptors reduce fat accumulation in hepatocytes via paracrine effects.

HO-1 Mediates Anti-inflammatory Properties of CB2 Receptors. HO-1 is a stress-inducible protein highly expressed in Kupffer cells that displays potent protective anti-inflammatory and cytoprotective effects in the liver against alcohol-induced liver injury,[31] endotoxemia,[32] or ischemia-reperfusion injury.[33] We, therefore, investigated whether HO-1 might mediate CB2-induced anti-inflammatory effects in alcohol-fed mice and, first, characterized the impact of JWH-133 treatment on Kupffer-cell HO-1 protein expression by double immunohistochemistry combining antibodies to HO-1 and F4/80. Alcohol-fed mice treated with JWH-133 displayed a strong HO-1 protein increase in Kupffer cells, compared to alcohol-fed control animals (82% ± 2% versus 57% ± 3%, $P < 0.05$; Fig. 5A). In keeping with *in vivo* findings, JWH-133 induced HO-1 mRNA and protein expression in isolated Kupffer cells and in RAW264.7 macrophages, either alone or in combination with LPS (Fig. 5B,C).

We next investigated the impact of zinc protoporphyrin (ZnPP), a specific competitive inhibitor of HO-1 activity, on LPS-treated RAW264.7 macrophages exposed to JWH-133. Strikingly, ZnPP blunted the inhibitory effect of JWH-133 on LPS-induced nuclear factor-kappa B (NF-κB) translocation into the nucleus (Fig. 6A). In addition, ZnPP also prevented the inhibitory effect of JWH-133 on IL-6 and IL-1β expressions, whereas its effect on TNF-α did not reach statistical significance (Fig. 6B). These data demonstrate that CB2 activation induces HO-1 in macrophages, thereby limiting NF-κB activation and the proinflammatory M1 response.

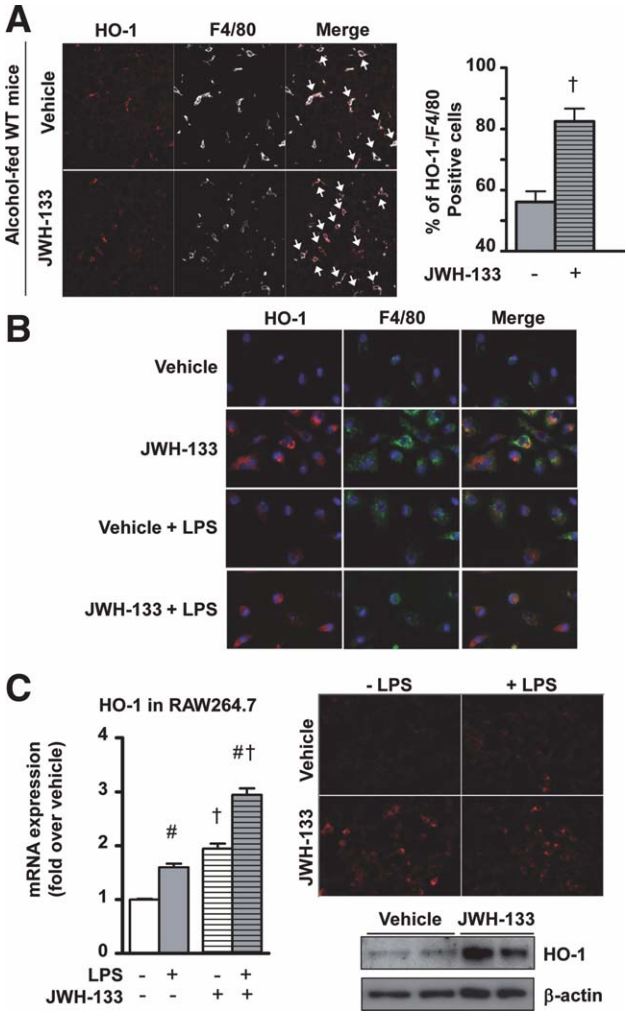

Fig. 5. CB2 receptor activation induces HO-1 expression in macrophages both *in vivo* and *in vitro*. (A) Representative images of HO-1 (red) and F4/80 (white) immunochemical labeling, and percentage of HO-1-positive macrophages in liver tissue sections from ethanol-fed WT mice treated with JWH-133 or vehicle (confocal microscopy; original magnification, ×400); double-positive cells are indicated by the arrows (†$P < 0.05$ for JWH-133 versus vehicle). (B and C) Kupper cells (B) or RAW264.7 cells (C) were treated with 5 μM of JWH-133 or vehicle for 18 hours and further stimulated with 1 ng/mL of LPS or vehicle for 6 hours; HO-1 mRNA expression was quantified by reverse-transcriptase polymerase chain reaction analysis and HO-1 protein expression by immunocytochemistry and western blotting analysis. †$P < 0.05$ for JWH-133 versus vehicle; #$P < 0.05$ for LPS versus H_2O.

Discussion

The present study demonstrates that during alcoholic liver disease, activation of CB2 receptors expressed in Kupffer cells reduces proinflammatory M1 response and favors M2 polarization, thereby eliciting antisteatogenic effects via paracrine interactions with hepatocytes (Fig. 7).

Sustained inflammation constitutes the initial hepatic response to chronic alcohol consumption.[8,12] Experimental and clinical studies have shown that

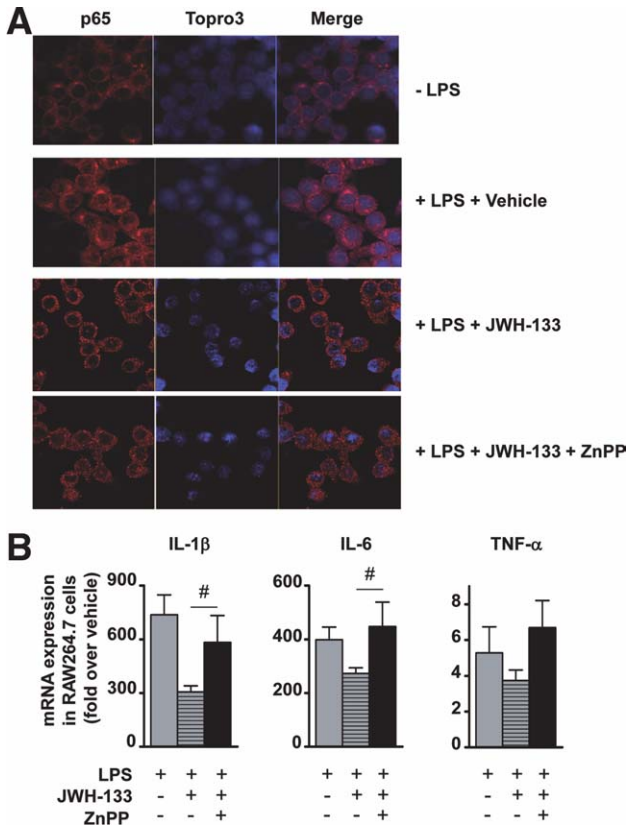

Fig. 6. Anti-inflammatory properties of CB2 receptors are mediated through an HO-1-dependent mechanism. RAW264.7 cells were pretreated with 2 μM of ZnPP or vehicle with or without 5 μM of JWH-133 for 18 hours and further incubated with LPS. (A) Representative immunochemical labeling of p65 NF-κB in RAW264.7 cells 1 hour after the addition of LPS (confocal microscopy; original magnification, $\times$800). (B) mRNA expression of IL-1β, IL-6, and TNF-α in RAW264.7 cells 6 hours after the addition of LPS. #$P < 0.05$ for ZnPP versus vehicle.

Kupffer cells play a pivotal role in this process. Thus, Kupffer cells undergo activation by gut-derived LPS and release several inflammatory mediators, such as TNF-α or IL-1β, suggesting that they may adopt a proinflammatory M1 profile.[8,11,34] In the present study, we provide evidence for a mixed M1/M2 response of Kupffer cells in alcohol-fed animals. Indeed, alcohol triggers hepatic induction of proinflammatory mediators characteristic of a classical M1 profile, including cytokines, such as TNF-α and IL-6, or chemokines, such as CCL3 and CCL4. At the same time, alcohol feeding also enhances liver expression of alternative M2 markers, such as Arg1, Mrc2, and CD163. As yet, mechanisms regulating M1/M2 Kupffer-cell polarization remain largely unexplored. Recent studies in experimental models of obesity have shown that the transcription factor, peroxisome proliferator-activated receptor delta, promotes the transition of Kupffer cells to an M2 phenotype, thereby reducing liver inflammation and fatty liver.[5,6] Adiponectin primes peritoneal and adipose tissue macrophages into an M2 phenotype[35] and reduces TNF-α production in Kupffer cells isolated from alcohol-fed mice exposed to LPS.[31] Our results identify CB2 receptors as a novel regulator of Kupffer-cell polarization. Indeed, *in vivo* and *in vitro* experiments demonstrate that genetic deletion of CB2 receptors is associated with a marked hepatic induction of the M1 signature in response to chronic alcohol feeding and a parallel loss of the M2 alternative response. These findings, therefore, suggest that endogenous CB2 receptors are responsible for M2 response to alcohol feeding. Interestingly, the CB2 agonist, JWH-133, blunts the induction of the M1 classical signature without affecting M2 response to alcohol. Whether the lack of enhancement of M2 markers in animals treated with the CB2 agonist may be the result of partial agonist properties of the compound or to constitutive activity of CB2 receptors remains to be determined.[36,37] Nevertheless, these data demonstrate that, during chronic alcohol exposure, CB2 receptors shift the M1/M2 balance toward a predominant alternative M2 response.

Besides their anti-inflammatory properties on Kupffer cells, CB2 receptors also prevent the development of alcohol-induced fatty liver. Recent studies have demonstrated that cross-talk between Kupffer

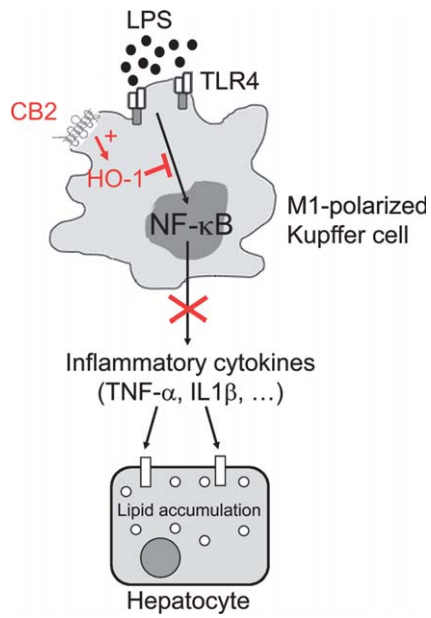

Fig. 7. Schematic representation of the beneficial effects of CB2 receptor activation on alcohol-induced hepatic inflammation and steatosis. CB2 activation induces HO-1 in Kupffer cells, thereby limiting NF-κB activation and LPS-induced secretion of proinflammatory M1 cytokines, leading to the reduction of fat accumulation in hepatocytes via paracrine effects.

cells and hepatocytes is determinant in the control of hepatic steatosis. In rodents exposed to an alcohol diet or a high-fat diet, depletion of Kupffer cells blunts the development of fatty liver.[9,38-40] Furthermore, cocultures of M1-polarized Kupffer cells with hepatocytes promote lipid accumulation into parenchymal cells.[5,6,38,39] In keeping with these data, we show that CM obtained from JWH-133- and LPS-stimulated macrophages reduces lipid accumulation in hepatocytes, compared to CM prepared from macrophages exposed to LPS alone. These data indicate that Kupffer-cell CB2 receptors decrease hepatocyte steatosis after inhibition of M1 polarization. Of note, recent studies have shown that IL-1β and TNF-α, two proinflammatory Kupffer-cell–derived cytokines, promote steatosis.[38-40] We show that liver expression of IL-1β and TNF-α decreases in alcohol-fed mice concurrently treated with JWH-133 and increases in CB2-deficient counterparts. A similar pattern of regulation was also found in our *in vitro* experiments, therefore suggesting that the reduction in Kupffer-cell production of IL-1β and TNF-α may contribute to the protective effects of CB2 receptors on hepatocyte lipid accumulation.

HO-1 is the rate-limiting enzyme in the catabolism of heme into biliverdin, free iron, and carbon monoxide. HO-1 is a stress-inducible protein with potent-protective effects against hepatocyte damage,[41] liver inflammation,[31,33] and fibrogenesis.[42,43] Recent studies have shown that up-regulating HO-1 in Kupffer cells by means of overexpression or by pharmacological activators prevents alcohol-induced release of inflammatory mediators by Kupffer cells.[31,41] However, characterization of HO-1 inducers in Kupffer cells remains poorly documented. Our data identify HO-1 as a novel target of CB2 receptors, as shown both *in vitro* and *in vivo*. Moreover, *in vitro* studies also provide a link between HO-1 induction in Kupffer cells and the anti-inflammatory properties of CB2 receptors, as shown by the abolition of CB2-mediated effects on NF-κB activation and M1 polarization by the specific HO-1 inhibitor, ZnPP. Interestingly, in addition to limiting M1 polarization, recent data also suggest that HO-1 is selectively expressed by M2 macrophages[44] and may drive Kupffer-cell polarization toward an anti-inflammatory phenotype,[33] suggesting that HO-1 is a master regulator of Kupffer-cell phenotype. In keeping with this, our data identify HO-1 as a downstream-signaling pathway, by which CB2 regulates M1/M2 balance in response to chronic alcohol exposure.

We previously reported the antifibrogenic properties of CB2 receptors in experimental models of liver fibrosis.[23] These beneficial properties have been ascribed both to direct effects on hepatic myofibroblasts[23] and to a reduction of inflammatory infiltration of the liver.[45] Recent data suggest that M1-polarized macrophages may promote the progression of liver fibrosis by releasing inflammatory mediators that activate liver fibrogenic cells.[46-48] Moreover, mice carrying a specific deletion of the M2 marker, Arg1, in macrophages are prone to liver fibrosis.[49] Altogether, these data suggest a critical role of the M1/M2 Kupffer-cell balance in the control of fibrosis progression. Whether antifibrogenic properties of CB2 receptors may also involve the inhibition of M1 polarization warrants further investigation.

In conclusion, this study demonstrates that activation of CB2 receptors display beneficial effects on alcohol-induced inflammation and fatty liver. The mechanism involves paracrine interactions between Kupffer cells and hepatocytes. In light of the previously demonstrated hepatoprotective[24] and antifibrogenic[23] effects of CB2 receptors and of their beneficial impact on liver regeneration,[24] our data strongly suggest that CB2 agonists may provide meaningful advances for the management of alcoholic liver disease.

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