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Pharmacological inhibition of CCR2/5 signaling prevents and reverses alcohol-induced liver damage, steatosis and inflammation in mice

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Abstract

Kupffer cell (KC) and macrophage (MØ) activation contribute to steatosis, inflammation and fibrosis in alcoholic liver disease (ALD). We found increased frequency of MØ, T cells and expression of *Ccr2* and *Ccr5* in the livers of patients with ALD and increased circulating chemokines, CCL2 and CCL5 in alcoholic hepatitis patients. We hypothesized that inhibition of CCL2 signaling with the dual CCR2/5 inhibitor, cenicriviroc (CVC), would attenuate ALD. In a mouse model of ALD, liver injury (ALT) and steatosis were prevented by CVC whether administered as “prevention” throughout the alcohol feeding or as “treatment” started after the development of ALD. Alcohol-induced increases in early liver fibrosis markers (Sirius-red, hydroxyproline and collagen-1) were normalized by both modes of CVC administration. We found that “prevention” and “treatment” with CVC reversed alcohol-related increases in liver mRNA and protein expression of TNF α , IL-1 β , IL-6 and CCL2. CVC administration regimens prevented the increase in infiltrating MØ (F4/80^{lo} CD11b^{hi}) and reduced proinflammatory Ly6C^{Hi} MØ in livers of alcohol-fed mice. CVC increased liver T cell numbers and attenuated *Il-2* expression without an effect on CD69⁺ or CD25⁺ T cell expression. *In vitro*, CVC inhibited CCL2-induced increases in hepatocyte *Fasn* and *Adrp* while it augmented *Acox-1*, *Pgc1 α* and *Ucp-2* expression, suggesting mechanisms for attenuated hepatocyte steatosis. We found that CCL2 and CCL5 sensitized hepatocytes to LPS-induced liver injury (TNF α , ALT and LDH release). Alcohol feeding induced

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Author Contributions

AA and PL completed the CVC injections and the Lieber-DeCarli diet feeding. KK and DC assisted in the animal feeding. AA, PL, AIV, KK and DC performed the animal sacrifice and liver cell isolations. AA, PL, BG and YC stained and acquired the flow cytometry samples. AA, PL, AIV, CC, Adejumo, BS and JM performed biochemical assays. AA and PL performed *in vitro* experiments. AA and PL analyzed and interpreted the data. AA, GS, EL and PV conceptualized the project and designed the experiments. AA, PL and GS wrote the manuscript and BG, EL and PV provided critical review. GS provided overall study supervision.

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apoptosis (PARP and caspase-3 cleavage) and pyroptosis (gasdermin D cleavage) in livers and CVC prevented both these forms of cell death. Together, our data demonstrate preclinical evidence for CCR2/CCR5 inhibition with CVC as a potent intervention to ameliorate alcohol-induced steatohepatitis and liver damage.

Keywords

Cenicriviroc; alcoholic liver disease; macrophage; T cell; chemokine

Introduction:

Alcoholic liver disease (ALD) is an increasing health concern worldwide (1). The clinical pathophysiology of ALD involves a progressive spectrum of steatosis, steatohepatitis, fibrosis, cirrhosis, and increased risk of hepatocellular carcinoma (2). While simple fatty liver, induced by both binge and chronic alcohol consumption, can be reversed after withdrawal, more severe forms of liver injury can develop in 35% of heavy alcohol drinkers (3). Currently, there is no definitive treatment for progressive ALD, except for abstinence, and the current standard of care with prednisolone for acute alcoholic hepatitis has limited efficacy, many contraindications and side effects (4). Thus, an urgent need to explore novel therapies for ALD is apparent.

The pathomechanism of ALD has been attributed to direct effects of alcohol and its metabolites inducing hepatocyte cell death, steatosis, and through macrophage infiltration increased cytokine/chemokine receptor signaling and proinflammatory cytokine induction in the liver (5). The C-C chemokine receptor types 2 and 5 (CCR2 and CCR5), and their respective ligands, C-C chemokine ligand types 2 (CCL2; also known as monocyte chemoattractant protein-1 [MCP-1]) and CCL5 (also known as regulated on activation, normal T cell expressed and secreted [RANTES]) are implicated in liver inflammation and macrophage (MØ) recruitment to the liver (6). In response to animal models inducing hepatocyte injury, such as acetaminophen, carbon tetrachloride or bile duct ligation, resident liver MØ (Kupffer cells) secrete CCL2 promoting monocyte recruitment and migration to the liver, where they mature into pro-inflammatory MØ (7). CCR2 and CCR5 mediate the intrahepatic immune-cell interactions that promote activation and migration of peripheral monocytes to the liver (8, 9). Activation of inflammatory cells and upregulation of several soluble inflammatory mediators, including the ligands of these key receptors, CCL2 and CCL5, are features of ALD (10). In animal studies of ALD, mice with CCL2 deletion or pharmacological blocking of CCL2 showed protection (11, 12). Similarly, animal models lacking CCL5 display lower immune-cell activation and reduced liver fibrosis (13). Therefore, blockade of these pathways and dual inhibition of CCR2 and CCR5 is a promising approach for the treatment of ALD.

Cenicriviroc (CVC) is a novel, oral, dual CCR2/CCR5 antagonist with nanomolar potency against both receptors, and a long plasma half-life (30–40h in humans) (14) with bioavailability and bioactivity in both oral and injectable administration as demonstrated in preclinical rodent studies (15–17). Phase 2 studies 652-2-201 (NCT01092104) and

652-2-202 (NCT01338883), conducted in human immunodeficiency virus (HIV)-infected participants, have shown CCR2 blockade, associated with a reciprocal increase in CCL2 levels, and CCR5 blockade, demonstrated by reduction in HIV-1 RNA levels (14, 18, 19). In addition, marked reductions in circulating biomarker of monocyte activation, soluble cluster of differentiation 14 (sCD14), were observed with CVC treatment in HIV-infected adults (study 652-2-202) and in adults with NASH and liver fibrosis (652-2-203; CENTAUR; NCT02217475) over up to 48–52 weeks of therapy (18, 20). A *post hoc* analysis of phase 2b clinical study 652-2-202 (NCT01338883) revealed reductions in fibrosis indices AST to Platelet Ratio Index (ARI) and Fibrosis-4 (FIB-4), which were significantly correlated with decreases in sCD14 in HIV-infected subjects treated with CVC at 48 weeks (21). Anti-inflammatory and anti-fibrotic effects of CVC have been demonstrated in multiple animal models of acute liver injury and fibrosis (15, 16, 22). The efficacy, safety and pharmacokinetics of CVC in adults with NASH and liver fibrosis compared to placebo have been evaluated in the 2-year CENTAUR study; results of the Year 1 primary analysis showed that CVC was well tolerated and resulted in twice as many participants achieving 1 stage improvement in fibrosis and no worsening of steatohepatitis versus placebo in the ITT population (20, 23). CVC is currently undergoing Phase 3 evaluation for the treatment of liver fibrosis in adults with NASH (AURORA; NCT03028740). Therefore, CVC is an attractive candidate for the treatment of ALD through antagonism of CCR2/CCR5 receptors.

Here, we hypothesize that CCR2/5 blockade using CVC will inhibit macrophage infiltration, lower inflammation and reduce steatosis, thereby alleviating hallmarks of ALD in mice. We employ the Lieber-DeCarli model of ALD and administer CVC throughout the alcohol regimen for 6 weeks (hereafter referred to as “prevention”) or for the final 3 weeks (“treatment”) of alcohol administration. Our results show that CVC significantly improves the hallmark phenotypes of ALD including hepatocyte damage, macrophage infiltration and inflammation in the liver. Our novel *in vivo* findings presented here suggest that CCR2/5 antagonism using CVC is a potential therapy for ALD.

Methods:

Human cirrhotic and normal healthy liver samples

The Liver Tissue Cell Distribution System (LTCDS, Department of Pediatrics, University of Minnesota, Minneapolis, MN) provided 10 human liver samples from patients with end-stage alcoholic cirrhosis who received liver transplantation. Alcoholic cirrhosis was diagnosed by LTCDS and was based on a history of alcohol drinking and liver histology. Control human liver tissue was the non-involved surrounding tissue obtained from patients undergoing partial hepatectomy for liver cancer. The protocol for using these liver samples has been approved by the Liver Tissue Cell Distribution System of the University of Minnesota and the IRB of University of Massachusetts Medical School. An informed consent in writing was obtained from each patient and the study protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki as reflected in *a priori* approval by the IRB.

Animal feeding and CVC administration

The study protocol was approved by the Institutional Animal Use and Care Committee of the University of Massachusetts Medical School. All the methods were carried out in accordance with the approved guidelines. Eight-week-old C57BL/6J female mice were divided into alcohol- and pair-fed (control) groups as well as two CVC alcohol-fed groups. Alcohol-fed mice received 5% ethanol for six weeks in Lieber-DeCarli liquid diet *ad libitum* as previously described (24). Pair-fed animals received a calorie-matched liquid diet. Some alcohol-fed mice received daily subcutaneous injections of 15mg/kg BW CVC for 6 weeks (“prevention” cohort) while others received the same dose daily for 3 weeks (beginning at week 4 through 6 of alcohol feeding; “treatment” cohort). 10% hydroxypropyl- β -cyclodextrin, 5% solutol HS15 and 85% sterile water was used as a vehicle to dissolve CVC into solution. Some mice from each cohort were cheek-bled weekly for liver enzyme measurements. At sacrifice, blood and liver tissues were collected for further assays.

In vitro experiments

For *in vitro* experiments, mouse primary hepatocytes were isolated as described above, plated on collagen-coated plates and allowed to rest for at least 4 hours. The media was changed and cells were then stimulated with either 200ng/mL CCL2 (MCP-1; PeproTech, Rocky Hill, NJ, USA) or 20ng/mL CCL5 (RANTES; PeproTech) for 24 hours. Some groups received either 10nM, 20nM or 100nM CVC. After changing the media, 10ng/mL LPS was added for 18 hours after which culture supernatants were collected for ELISA, ALTs and LDH assay. Cell lysates were prepared for caspase-3 assay.

Bone marrow-derived macrophages were treated with either 10nM, 20nM or 100nM CVC and then 10ng/mL LPS was added for 6 hours. Cell supernatants were collected for ELISA measurement of proinflammatory cytokines.

Statistical Analysis

Statistical significance was determined using either two-tailed t-test or ordinary one-way ANOVA with Tukey’s multiple comparison post-test to compare the means of multiple groups. Data are shown as mean $\pm$ SEM and were considered statistically significant at $p < 0.05$. GraphPad Prism 6.02 (GraphPad Software Inc., La Jolla, CA, USA) was used for analysis.

Additional methods are provided in the Supplemental Material.

Results:

Alcoholic liver disease patients have elevated CCR2/5 signaling axis and increased macrophage markers in the liver

CCL2 (or MCP-1) and CCL5 (or RANTES) are chemokines that bind to their respective receptors, CCR2 and CCR5, and activate downstream signaling (25). To investigate the clinical significance of the CCR2/5 signaling axis in ALD, we first measured the presence of chemokines, CCL2, CCL3, CCL4 and CCL5 in the serum of patients with alcoholic hepatitis (Fig. 1A). We found that CCL2 and CCL5 were significantly elevated in patients

compared with healthy controls, while both CCL3 and CCL4 were unchanged in patients (Fig. 1A). Next, we measured the mRNA expression of ligands *Ccl2*, *Ccl3*, *Ccl4*, *Ccl5*, *Ccl8* and *Ccl13* as well as *Ccr2* and *Ccr5* in the peripheral blood mononuclear cells (PBMCs) of patients with alcoholic hepatitis and found that both *Ccl2* and *Ccl3* were increased in patient PBMCs compared with controls and that *Ccl5* was downregulated while the remaining ligands and receptors were unchanged (Fig. 1B). Having observed upregulation of CCR2/5 ligands in patients with alcoholic hepatitis, we next investigated if this axis is also activated in patients with alcoholic cirrhosis. Liver biopsies from alcoholic cirrhosis patients revealed increases in mRNA expression of *Ccl2*, *Ccl3*, *Ccl4*, *Ccl5* and *Ccl8* as well as *Ccr2* and *Ccr5* compared to control livers (Fig. 1C). Thus, CCR2/5 signaling components are elevated in both patients with alcoholic hepatitis and alcoholic cirrhosis.

Macrophages play an important role in inflammation and progression of the liver injury in alcoholic liver disease (ALD) and cirrhosis and respond to CCR2 chemoattraction (7). The gene expression of characteristic MØ markers, including *Cd11b*, *Cd68* and *Cd14*, were significantly upregulated in the alcoholic cirrhotic livers compared to controls (Fig. 1D). Additionally, CCL5 plays a significant role in activation and migration of T cells, particularly in viral infection (26) and the T cell marker *Cd3* was upregulated in patients with alcoholic cirrhosis (Fig. 1D). Further, we observed increased MØ infiltration in the human alcoholic livers indicated by immunostaining for the MØ markers CD11b, CD68 and CD14 and T cell marker CD3 (Fig. 1E). Together, our data demonstrate elevation of CCR2/5 ligands and receptors in alcoholic liver disease (alcoholic hepatitis and alcoholic cirrhosis) and increases in MØ and T cells in human alcoholic livers.

Administration of the dual CCR2/CCR5 antagonist, cenicriviroc, alleviates chronic alcohol-induced liver injury in mice

Having confirmed elevation of the CCR2/5 signaling axis in patients with ALD, we tested the efficacy of cenicriviroc (CVC), a dual CCR2/5 antagonist, in a mouse model of alcoholic liver disease that is reliable for preclinical testing of potential interventions (27, 28). Administration of CVC for 6 weeks during alcohol administration in the “prevention” cohort showed significant protection from alcohol-induced ALT increases with significant attenuation at 2–4 weeks and normalization of ALT to the level of pair-fed controls at weeks 5–6 (Fig. 2A, left). Furthermore, when CVC was started at week 4 of the alcohol feeding in the “treatment” cohort (after alcohol-induced liver injury was present), we found rapid normalization of ALT levels that remained at the level of pair-fed mice for the final 3 weeks of the alcohol feeding (Fig. 2A, right). ALT was measured from serum taken by weekly cheek bleeding of a subset of mice within each group.

We found a significant improvement in liver pathology in both “prevention” and “treatment” administration of CVC in ALD in mice (Fig. 2B). Liver steatosis, estimated by Oil Red O staining was significantly lower after both regimens of CVC administrations compared to alcohol-fed without CVC treatment (Fig. 2C). Liver triglyceride measurement further confirmed the inhibitory effect of CVC on alcohol-induced liver steatosis (Fig. 2E). Chronic alcohol exposure leads to early signs of mild fibrogenesis in mice and to significant fibrosis and cirrhosis in human patients. Liver fibrosis was assessed using Sirius Red staining (Fig.

2D), hydroxyproline measurement (a surrogate for collagen accumulation; Fig. 2F) and collagen mRNA expression in the liver (Fig. 2G). We found a significant reduction in alcohol-induced liver fibrosis markers in CVC-treated mice both in the “prevention” and “treatment” cohorts (Fig. 2D, F, G). Importantly, this protection was not due to different alcohol exposures as there was no difference in caloric diet intake of alcohol between the CVC and vehicle injected (Supplementary Fig. 1). These results indicate that inhibition of CCR2/5 signaling protected mice from alcohol-induced increase in parenchymal injury and steatosis, improved liver pathology and reduced markers of fibrosis in mice.

CVC reduces pro-inflammatory cytokine production and macrophage activation in ALD

Pro-inflammatory cytokines produced by MØ accelerate the progression of alcoholic liver disease (29). Administration of CVC “prevention” and “treatment” significantly reduced liver mRNA expression of *Tnfa*, *Il-6* and *Ccl2* although alcohol-induced *Il-1β* increase was unaffected by CVC (Fig. 3A). The liver protein levels of IL-1β and IL-6 were significantly increased by alcohol feeding while both TNFα and CCL2 trended toward an increase. CVC reduced IL-1β protein while TNFα and IL-6 trended toward a decrease in alcohol-fed CVC treated mice. Tissue CCL2 protein levels did not change after CVC administration (Fig. 3B), consistent with previous data in animal models with CVC treatment (15). Soluble CD14 (sCD14) serum protein level, an indicator of monocyte activation, was significantly increased after alcohol treatment and reduced after CVC administration in the prevention cohort (Fig. 3C). These data indicate that CVC protects from proinflammatory signaling in the liver as well as macrophage activations.

CVC reduces macrophage infiltration to the liver in ALD

Macrophages are the primary contributors to alcohol-induced inflammation in the liver (7). MØ respond to CCL2 chemotaxis, which is mediated via CCR2. We found that expression of *Ccr2* mRNA by qPCR (Fig. 4A) and F4/80⁺ cells, measured by immunohistochemistry (Fig. 4B), were significantly increased in the livers after alcohol feeding compared to pair-fed controls (Fig. 4C). Furthermore, the alcohol-induced increase in *Ccr2* mRNA and F4/80⁺ cell number were dramatically normalized to the level of controls by CVC “prevention” or “treatment” (Fig 4. A–C).

To examine the state of resident and infiltrating macrophages in the liver following alcohol consumption, we analyzed the CD45⁺F4/80⁺CD11b⁺ population of liver mononuclear cells via flow cytometry (7) (Fig. 4D). We detected no significant difference in the total liver CD45⁺F4/80⁺CD11b⁺ macrophage population and no changes in CD45⁺F4/80^{hi}CD11b^{lo} liver resident Kupffer cells (Fig. 4E). However, alcohol feeding induced a robust increase in the number of CD45⁺F4/80^{lo}CD11b^{hi} infiltrating MØ that was prevented both in the “treatment” and “prevention” CVC treated mice (Fig. 4E).

Recent evaluation of infiltrating hepatic macrophages has revealed subpopulations of proinflammatory macrophages induced by alcohol. CD45⁺F4/80^{lo}CD11b^{hi} infiltrating MØ can be subdivided based on Ly6C expression (30). Ly6C^{Lo} infiltrating MØ express an anti-inflammatory and tissue repair gene profile compared to Ly6C^{Hi} infiltrating MØ that exhibit a proinflammatory gene profile (30). We found that the proportion of CD45⁺F4/80^{lo}CD11b^{hi}

infiltrating MØ that are Ly6C^{Lo} is reduced following chronic alcohol, while Ly6C^{Med} and Ly6C^{Hi} were unchanged (Fig. 4F). However, treatment with CVC increased the proportion of infiltrating MØ that are Ly6C^{Med} and significantly reduced proinflammatory Ly6C^{Hi} from approximately 25% of all infiltrating MØ to below 10% (Fig. 4F). These data show that CVC decreases infiltrating macrophages and dramatically reduces the proportion of proinflammatory macrophages, corroborating the decrease in proinflammatory cytokine expression (Fig. 3).

CVC blocks activation of T cells in alcoholic liver disease

CCL5 is induced in the liver upon alcohol consumption and CCR5 mediates the intracellular signaling cascade of CCL5. We next evaluated the effect of dual CCR2/5 inhibitor CVC on T cells in the liver. We found a significant increase in *Ccr5* mRNA in livers of mice following chronic alcohol feeding (Fig. 5A). CVC significantly lowered *Ccr5* expression in livers in both “prevention” and “treatment” cohorts (Fig. 5A). Immunostaining for CD3 on mouse liver sections revealed a significant increase in CD3⁺ cells in the liver after alcohol that was unchanged by six-week CVC “prevention,” but modestly reduced by “treatment” CVC administration (Fig. 5B–C). *Il-2* expression was increased by alcohol but attenuated by both CVC regimens (Fig. 5F). Alcohol did not have an effect on expression of *Foxp3*, a key transcription factor of regulatory T cells (31), or *Ifnγ* expression (Fig. 5D).

Interestingly, FACS analysis of isolated liver leukocytes revealed that while alcohol consumption did not impact the proportion of CD3⁺ T cells in the liver, CVC administration led to a significant increase in liver T cells (Fig. 5E). Further flow cytometric analysis revealed that CD69⁺ activated T cells were not significantly altered by alcohol (Fig 5F), while CD25⁺ T cells were increased by alcohol consumption (Fig. 5G) (32). CVC in both administration methods tended to reduce in CD25 expression in T cells compared to alcohol alone (Fig. 5G).

CVC blocks alcohol-induced steatosis in primary hepatocytes

Excess alcohol consumption leads to hepatic steatosis caused by the direct effects of alcohol and alcohol metabolites through modulation of hepatic fatty acid synthesis and reduction in fatty acid oxidation (33, 34). Multiple mechanisms have been identified that lead to steatosis in ALD, of which CCL2 is one of the mediators identified (11). Consistent with previous reports that showed CCR2/5 expression on hepatocytes in HCV infected patients (35, 36), we confirmed by flow cytometry that primary hepatocytes in mice express CCR2 and CCR5 and their surface expression was upregulated after chronic alcohol exposure (Fig. 6A). Next, we assessed the effect of CVC on fatty acid metabolism gene expression in primary hepatocytes *in vitro*. We found that CCL2-induced steatosis was attenuated by CVC at multiple levels: the fatty acid synthesis gene, *Fasn*, was down-regulated after CVC treatment in presence of CCL2 (Fig. 6B), while the FA oxidation genes such as *Acox-1*, *Pgc1a* were significantly upregulated after CVC treatment *in vitro* suggesting a direct effect on steatosis (Fig. 6B).

CVC blocks cell death in primary hepatocytes and the liver of alcohol-fed mice

While the robust effect of CVC on inflammation and MØ recruitment can be explained by inhibition of CCR2 receptor signaling, the mechanism by which CVC normalized alcohol-induced hepatocyte injury is less clear. Thus, we hypothesized that CCR2/5 inhibition may have direct protective effects on hepatocytes in ALD. Given the dramatic improvement of ALT levels after CVC administration (Fig. 2A) we sought to test if CCL2 or CCL5 signaling can initiate a hepatocyte response and if CVC can protect against hepatocyte cell death. We found that CCL2 (Fig. 6C) or CCL5 (Fig. 6D) treatment resulted in sensitization of primary hepatocytes to LPS and resulted in TNF α release. CCL2 or CCL5 treatment of primary hepatocyte plus administration of LPS also led to an increase in ALT and LDH release and caspase-3 activity (Fig. 6C,D). Importantly, all of these effects of CCL2 or CCL5 were inhibited by CVC in a dose-dependent manner (Fig. 6C,D). These results indicate that both CCL2 and CCL5 sensitize hepatocytes to LPS-induced injury and that CCR2/5 inhibition with CVC can prevent damage in primary mouse hepatocytes.

Multiple cell death mechanisms could be involved in hepatic injury *in vivo* following chronic alcohol consumption. Therefore, we investigated two prominent pathways including caspase-3-dependent apoptosis and gasdermin D-induced pyroptosis (37). We found that alcohol increased PARP cleavage (clv), compared to pair-fed mice and both CVC administration methods reduced cleaved PARP (Fig. 6E). Because PARP cleavage is upstream of caspase-3 activation, we next measured caspase-3 cleavage and found it was similarly induced by alcohol feeding and this increase was abrogated in CVC treated animals. Although we were unable to detect any change in caspase-11, we did observe an alcohol-induced increase in cleaved gasdermin D (GSDMD), indicative of pyroptosis (Fig. 6E). Together, these data suggest that chronic alcohol induces both apoptosis as well as pyroptosis in the liver and that treatment with CCR2/5 blockade reduces this injury, consistent with observations in primary hepatocytes and circulating markers of liver injury including ALT.

CVC treatment blocks LPS-induced cytokine release from macrophages

The efficacy of CVC in preventing and treating hallmarks of alcoholic liver disease may be related to primary effects on hepatocytes (Fig. 6A–D) or by interrupting macrophage chemotaxis and activation. To investigate the macrophage-specific effect of CVC, we isolated bone marrow-derived macrophages from wild-type mice and treated them *ex vivo* with LPS to induce a proinflammatory response. LPS treatment significantly induced the release of TNF α and IL-6. LPS-induced TNF α release was unaffected by 5mM CVC treatment but was blocked with 20 and 100mM CVC (Fig. 6F). CVC similarly blocked LPS-induced IL-6 release at 100mM (Fig. 6F). These data in primary macrophages, in conjunction with the *ex vivo* primary hepatocyte treatments and *in vivo* following chronic alcohol suggest multiple cell targets for CVC's protective mechanisms in this model of alcoholic liver disease.

Discussion:

Chronic inflammation, steatosis and hepatocyte injury are major components of the pathology and contributors to the clinical manifestations of alcoholic liver disease (5). A hallmark of ALD inflammation is an increase in the chemokines CCL2 and CCL5 (MCP-1 and RANTES, respectively) serving to recruit peripheral macrophages and T cells (6). The infiltration of circulating mononuclear cells (both MØ and T cells) into the liver has been established as a feature of both animal and human alcoholic liver disease and is associated with disease severity (7). Our present study demonstrates that cell recruitment through alcohol-induced CCR2/5 signaling contributes significantly to the phenotype of alcoholic liver disease including hepatocyte injury, development of steatosis and significant cytokine upregulation (Fig. 1–3). Hepatic protection was prominent using both a “prevention” dosing model, in which animals received daily CVC from the start of alcohol, as well as the “treatment” model where CVC began after ALD was already established (Fig 2). Secretion of CCL2 leads to chemoattraction of macrophages by binding to the CCR2 receptor, while CCL5 chemoattracts T cells through CCR5 (6). Blockade of the CCR2/5 receptors inhibits the characteristic alcohol-induced infiltration of peripheral MØ and T cells as well as lymphocyte activation markers (Fig. 3–5). We found that CVC mitigates the metabolic shift in hepatocytes toward lipogenesis induced by CCL2 and reduces cell death pathways including caspase-3 apoptosis and gasdermin D associated pyroptosis. CVC administration also reduces the damage induced by LPS, a gut-derived signal that causes significant liver inflammation and injury in alcoholic liver disease, on primary hepatocytes as well as LPS-induced proinflammatory cytokine release from primary macrophages (Fig. 6).

CCL2 is well known to be upregulated in hepatocytes as well as liver macrophages in alcoholic liver disease (7). Activation of TLR4 by binding to one of its ligands, gut-derived LPS, in the setting of chronic alcohol induces CCL2 expression which can then act as a chemoattractant of peripheral cells as well as a local signaling molecule (30). The infiltration of peripheral macrophages leads to significant damage in alcoholic hepatitis by contributing to fibrosis and cirrhosis (38, 39). Circulating monocytes exhibit a proinflammatory phenotype in patients with alcoholic liver disease and consistent with this, we found increased soluble CD14 levels in ALD that was attenuated by CVC administration. MØ have been shown to increase production of TNF α and other cytokines, as well as to increase responsiveness to LPS (40–42). MØ depletion has been shown to be effective at protecting from alcoholic liver disease in animal models (27, 43). Here, we provide evidence for effective pharmacologic inhibition (“prevention”) and reduction (“treatment”) of infiltrating macrophages into the liver in a mouse model of ALD.

Macrophage expression of CCR2 is not binary and some circulating lymphocytes may infiltrate tissue using non-CCR2 receptor mediated methods (44). Our data suggests that human patients with alcoholic liver disease (hepatitis or cirrhosis) have elevations in circulating chemokines CCL2 and CCL5 and increased ligand/receptor expression in both PBMCs and liver biopsy samples. Patient serum was isolated by whole blood coagulation followed by centrifugation which can cause platelet activation and degranulation (45). This may be responsible for the release of CCL5 into the serum, although CCL2 was also significantly elevated in patients with alcoholic hepatitis and its release is not associated

with platelet degranulation. CCL3 and CCL4 were not increased in patients suggesting there may be specific upregulation of certain chemokines in the progression of alcoholic liver disease.

We therefore explored pharmacologic inhibition of CCR2/5 signaling in a mouse model of chronic alcoholic liver disease and show that blockade of CCR2/5 with CVC is sufficient to block alcohol-induced proinflammatory cytokine expression and macrophage infiltration, and support a CCR2-dependent mechanism for macrophage migration in chronic alcohol use. Injection of CVC (15mg/kg) successfully reduced the infiltration of F4/80^{lo}CD11b^{hi} macrophages to the liver by approximately 67% in both “prevention” and “treatment” dosing (Fig. 4). Liang et al. showed that injection of CVC (20mg/kg) reduced macrophage infiltration by roughly 33% in a mouse model of muscular dystrophy (17) and similar results have been obtained when CVC is delivered orally. For example, Mossanan et al. have shown oral CVC (albeit at a higher dose of 100mg/kg) reduced in APAP-induced liver macrophage infiltration by approximately 40–60% in mice (16). Previous research by Wang et al. has characterized the functional activity of infiltrating macrophages and found that Ly6C expression differentiated proinflammatory MØ (Ly6C^{Hi}) from anti-inflammatory MØ (Ly6C^{Lo}) (30). Here, we show that CVC treatment significantly reduced Ly6C^{Hi} MØ in addition to the reduction in total infiltrating MØ to liver. Whether these population changes are due to CVC protection of hepatocyte cell death and damage or if they are due specifically to reduced chemotactic signaling is not completely clear, however Ly6C^{Hi} MØ highly express CCR2 (30) suggesting that inhibition of CCR2 signaling in these proinflammatory cells likely enhances the hepatoprotective role of CVC.

T cells have long been implicated in the pathogenesis of alcoholic liver disease in which key chemokines, including CCL5, are upregulated in the liver (46, 47). Here, we describe the upregulation of CCR5 in the livers of mice fed alcohol chronically. T cells were detected via both immunohistochemistry and flow cytometry in these livers although the inhibition of CCR5 by CVC treatment did not clearly inhibit T cells in the liver. T cells of alcohol-fed mice have suppressed expression of CD62L (L-selectin), which may contribute to a trend toward decreased T cell numbers found in the liver (48). CD69 and CD25 transmembrane proteins are found on activated T cells (31, 32). Interestingly, patients with alcoholic hepatitis have been found to have reduced numbers of circulating CD25⁺ T cells (49). In our study, we found that alcohol increased the number of CD25⁺ T cells within the liver where they may be homing to serve a particular function. We did not detect an increase in expression of the Treg transcription factor, *Foxp3*, in the liver mononuclear cell fraction, suggesting that the detected increase in CD25⁺CD3⁺ cells represented activated T cells. CVC administration did not statistically affect CD25 expression, but did reduce *IL-2* expression in both administration models. These data suggest that inhibition of CCR2/5 may be able to reduce activated T cells, consistent with reports that CCR5 may be involved in T cell activation in other disease states such as viral infection (26). Despite the limited effect on T cell numbers, CVC successfully alleviated features of ALD significantly reduced infiltrating of proinflammatory Ly6C⁺ macrophages indicating that T cell numbers may be less important than the protective effect of blocking CCR2 and CCR5 in hepatocytes and macrophages (Fig. 6).

Interestingly, we have previously investigated the role of CCL2 and CCR2 signaling in hepatocyte fatty acid oxidation (11). Using CCL2 and CCR2 KO mice, our group showed that induction of genes related to lipid metabolism was absent in CCL2 KO mice and was independent of CCR2. In the present study, using a CCR2/5 dual inhibitor, we show both that steatosis as well as fatty acid synthesis gene induction can be reduced while fatty acid oxidation can be induced by blockade of the receptors. While CCR5 blockade has not been previously studied and may be a beneficial component of CVC protection, previous results do suggest the critical role played by CCL2 in liver lipid regulation (11).

The applicability of these findings in the pre-clinical mouse model to human alcoholic liver disease and acute alcoholic hepatitis awaits future investigations. Based on observations in human acute alcoholic liver disease where monocyte/macrophage activation is a major element of the disease process, inhibition of macrophage activation would be a feasible approach. While the role of T cells is yet to be fully understood in alcoholic hepatitis and ALD, dysfunctional T cells and T cell exhaustion have been described (50). Thus, dual CCR2/5 antagonism may have beneficial applications in treatment of alcoholic liver disease.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations:

KC	Kupffer cell
MØ	macrophage
ALD	alcoholic liver disease
CVC	ceficiviroc
CCR2	C-C chemokine receptor type 2
CCR5	C-C chemokine receptor type 5
CCL2	C-C chemokine ligand types 2
CCL5	C-C chemokine ligand types 5
MCP-1	monocyte chemoattractant protein-1
RANTES	regulated on activation, normal T cell expressed and secreted
HIV	human immunodeficiency virus
ALT	alanine aminotransferase

TNFα	tumor necrosis factor alpha
IL	interleukin
sCD14	soluble cluster of differentiation 14
ARI	AST to Platelet Ratio Index
FIB-4	Fibrosis-4
LTCDS	The Liver Tissue Cell Distribution System
LMNCs	liver mononuclear cellular
Ct	cycle threshold
H&E	hematoxylin and eosin
Fasn	fatty acid synthase
Acox-1	acyl-coenzyme A oxidase 1
Adrp	adipose differentiation-related protein
Pgc1α	peroxisome proliferator-activated receptor gamma coactivator 1-alpha
Ucp-2	uncoupling protein 2
LPS	lipopolysaccharide
fl	full
cl	cleaved
PARP	poly ADP-ribose polymerase
Casp	caspase
GSDMD	gasdermin D

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Figure 1. CCR2 and CCR5 expression and immune cell infiltration to the liver is increased in human alcoholic cirrhosis patients.

A) Chemokines CCL2, CCL3, CCL4 and CCL5 were measured from the serum of patients with alcoholic hepatitis or from healthy controls. B-C) Expression of chemokines *Ccl2*, *Ccl3*, *Ccl4*, *Ccl5*, *Ccl8* and *Ccl13* as well as chemokine receptors *Ccr2* and *Ccr5* were measured by qPCR from peripheral blood mononuclear cells (PBMCs) from patients with alcoholic hepatitis (B) and in liver tissue from patients with alcoholic cirrhosis (C) and compared with healthy controls. D) mRNA expression of macrophage markers *Cd11b*, *Cd68* and *Cd14* and T cell marker *Cd3* was measured by qPCR in the livers of controls and patients with alcoholic cirrhosis. E) Liver tissue from controls and patients with alcoholic cirrhosis was immunohistochemically stained for the macrophage markers CD11b, CD68 and CD14 and the T cell marker CD3. * $p < 0.05$ by student's *t*-test

Figure 2. Inhibition of CCR2/5 signaling alleviates chronic alcohol-induced liver injury. Mice were fed a liquid diet containing 5% alcohol or pair-fed (PF) diet for 6 weeks. Some mice were injected subcutaneously with the CCR2/5 inhibitor Cenicriviroc (CVC) daily for the entire 6 weeks (6 wks) or during the final 3 weeks (wks 4–6) of ethanol feeding. Control mice were injected with vehicle. A) Serum ALT was measured weekly to track liver damage caused by chronic alcohol consumption. The Prevention Cohort received CVC daily beginning at the start of ethanol feeding while the Treatment Cohort began receiving injections at week 4. B) Livers from pair-fed (PF), alcohol-fed or alcohol-fed plus CVC treated mice were stained with hematoxylin and eosin. C) Oil Red O staining was completed on mouse livers to visualize and quantify lipid accumulation. D) Sirius Red staining was completed to visualize and quantify fibrosis development. E) Liver triglycerides were also measured. F) Liver hydroxyproline, a marker of collagen content, was measured using a

colorimetric assay. G) Liver expression of *collagen* mRNA was measured by qPCR. *
p<0.05 by student's *t*-test in (A) and one-way ANOVA in (B-G).

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Figure 3. CVC inhibits pro-inflammatory cytokine expression induced by alcohol.

A-B) Liver mRNA expression of pro-inflammatory cytokines *Tnfa*, *Il-1β*, *Il-6* and *Ccl2* were measured by qPCR (A) and liver protein was measured by ELISA (B). C) Serum soluble CD14 (sCD14) was measured by ELISA from mice fed alcohol, alcohol plus CVC and pair-fed mice. * $p < 0.05$ by one-way ANOVA.

Figure 4. CVC decreases the infiltration of peripheral macrophages into the liver.

A) Liver mRNA expression of *Ccr2*, a target of CVC, was measured by qPCR. B-C) Liver sections were formalin fixed and stained by immunohistochemistry for macrophage surface marker F4/80 to identify and quantify hepatic macrophages. D) Liver mononuclear cells were isolated via tissue digestion followed by density gradient, surface staining for CD11b and F4/80 for differentiating liver macrophages by flow cytometry. Cells were gated based on size, singlets, an amino-reactive dye for living cells and for CD45⁺ positivity. E) Flow cytometry was used to quantify total liver macrophages (F4/80⁺CD11b⁺), resident Kupffer cells (F4/80^{hi}CD11b^{lo}) as well as infiltrating liver macrophages (F4/80^{lo}CD11b^{hi}). F) Infiltrating F4/80^{lo}CD11b^{hi} macrophages were further differentiated by Ly6C^{Lo}, Ly6C^{Med} and Ly6C^{Hi} expression from pair-fed, alcohol-fed and alcohol-fed CVC “treatment” mice. HPF: high-power field. * p<0.05 by one-way ANOVA.

Figure 5. CVC reduces the infiltration and activation of T cells into the liver following chronic alcohol.

A) Liver mRNA expression of *Ccr5*, another target of CVC, was measured by qPCR. B-C) Liver sections were formalin fixed and stained by immunohistochemistry for T cell surface receptor CD3 to identify and quantify hepatic T cells. D) Liver mononuclear cells were isolated via tissue digestion followed by density gradient and mRNA expression of T cell-associated genes *Il-2*, *Foxp3* and *Ifnγ* were measured by qPCR. E-G) Flow cytometry was used to quantify total liver T cells (E; CD3⁺), activated CD69⁺ T cells (F) as well as CD25⁺ T cells (G). Cells were gated for lymphocytes, singlets, an amino-reactive dye for living cells and for CD45⁺/CD3⁺ positivity. * p < 0.05 by one-way ANOVA.

Figure 6. CVC protects from induction of steatosis, inflammation and cell death in primary hepatocytes.

A) Expression of receptor proteins CCR2 and CCR5 was measured on primary mouse hepatocytes from animals fed alcohol or a calorie-matched diet. Both were elevated by chronic alcohol consumption. B) To assess the influence of signaling through CCR2, primary hepatocytes were isolated and cultured *in vitro* with either recombinant CCL2 alone, CVC alone or CCL2 with CVC. mRNA expression of genes associated with fatty acid metabolism revealed the effects of CCL2 and CVC. C-D) Either CCL2 (C) or CCL5 (D), ligands of CCR2 and CCR5 respectively, were used in combination with varying doses of LPS and CVC to observe the induction of TNF α as well as indicators of cell death including alanine aminotransferase (ALT), lactate dehydrogenase release and caspase-3 activity. CVC protected hepatocytes from damage in a dose-dependent manner. E) Western blot measurement of liver lysates from alcohol-fed mice without and with CVC administration as well as pair-fed mice was completed and probed for full (fl) and cleaved (clv) poly ADP-

ribose polymerase (PARP), caspase-3, caspase-11 and gasdermin D (GSDMD). F) Primary mouse bone-marrow derived macrophages were treated *in vitro* with CVC (5nM, 20nM and 100nM) then LPS (10ng/mL) and proinflammatory cytokines TNF α and IL-6 were measured from cell supernatants. * $p < 0.05$ by student's *t*-test or by one-way ANOVA.