ORIGINAL ARTICLE

Inhibition of monoacylglycerol lipase, an anti-inflammatory and antifibrogenic strategy in the liver

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ABSTRACT

Objective Sustained inflammation originating from macrophages is a driving force of fibrosis progression and resolution. Monoacylglycerol lipase (MAGL) is the rate-limiting enzyme in the degradation of monoacylglycerols. It is a proinflammatory enzyme that metabolises 2-arachidonoylglycerol, an endocannabinoid receptor ligand, into arachidonic acid. Here, we investigated the impact of MAGL on inflammation and fibrosis during chronic liver injury.

Design C57BL/6J mice and mice with global invalidation of MAGL ($MAGL^{-/-}$), or myeloid-specific deletion of either MAGL ($MAGL^{Mye-/-}$), ATG5 ($ATG^{Mye-/-}$) or CB2 ($CB2^{Mye-/-}$), were used. Fibrosis was induced by repeated carbon tetrachloride (CCl_4) injections or bile duct ligation (BDL). Studies were performed on peritoneal or bone marrow-derived macrophages and Kupffer cells.

Results $MAGL^{-/-}$ or $MAGL^{Mye-/-}$ mice exposed to CCl_4 or subjected to BDL were more resistant to inflammation and fibrosis than wild-type counterparts. Therapeutic intervention with MJN110, an MAGL inhibitor, reduced hepatic macrophage number and inflammatory gene expression and slowed down fibrosis progression. MAGL inhibitors also accelerated fibrosis regression and increased Ly-$6C^{low}$ macrophage number. Antifibrogenic effects exclusively relied on MAGL inhibition in macrophages, since MJN110 treatment of $MAGL^{Mye-/-}$ BDL mice did not further decrease liver fibrosis. Cultured macrophages exposed to MJN110 or from $MAGL^{Mye-/-}$ mice displayed reduced cytokine secretion. These effects were independent of the cannabinoid receptor 2, as they were preserved in $CB2^{Mye-/-}$ mice. They relied on macrophage autophagy, since anti-inflammatory and antifibrogenic effects of MJN110 were lost in $ATG5^{Mye-/-}$ BDL mice, and were associated with increased autophagic flux and autophagosome biosynthesis in macrophages when MAGL was pharmacologically or genetically inhibited.

Conclusion MAGL is an immunometabolic target in the liver. MAGL inhibitors may show promising antifibrogenic effects during chronic liver injury.

Significance of this study

What is already known on this subject?

- Cirrhosis lacks treatment, and liver transplantation is considered as the only option for end-stage liver disease, which justifies past and ongoing massive efforts to identify potential therapeutic antifibrotic targets.
- Sustained inflammation originating from resident and infiltrating immune cells drives the fibrogenic process during liver injury, and contributes to its resolution, and targeting macrophages is an attractive antifibrogenic strategy.
- Monoacylglycerol lipase (MAGL) is a proinflammatory enzyme that produces arachidonic acid from the endocannabinoid 2-arachidonoylglycerol and serves as a connecting metabolic hub between endocannabinoid and lipid signalling network.

What are the new findings?

- MAGL invalidation in myeloid cells is sufficient to reduce inflammation and fibrosis progression.
- Therapeutic intervention with MAGL inhibitors slows down fibrosis progression and reverses established fibrosis.
- In vivo and in vitro, anti-inflammatory and antifibrogenic effects of MAGL inhibitors are mediated by autophagy in macrophages.

How might it impact on clinical practice in the foreseeable future?

- MAGL inhibitors are promising antifibrogenic compounds that may prove useful during fibrosis progression and in the context of fibrosis regression.

INTRODUCTION

Liver fibrosis is the consequence of a sustained wound healing response to chronic liver injury regardless of the cause, and ultimately leads to cirrhosis, a major public health problem worldwide.[1 2] In western countries, the prevailing causes of fibrosis and cirrhosis include chronic alcohol consumption and non-alcoholic fatty liver disease associated with obesity and type 2 diabetes.[3 4] Liver transplantation is considered as the only option for end-stage liver disease, which justifies past and ongoing massive efforts to identify

potential therapeutic antifibrotic targets. The fibrogenic process is initiated by reiterative hepatocyte or biliary cell death, inflammation and oxidative stress, which lead to proliferation and accumulation of fibrogenic myofibroblasts at the site of injury.[1 2 5] Advances in the understanding of liver fibrosis pathogenesis have underscored that sustained inflammation originating from resident and infiltrating immune cells drives the fibrogenic process during liver injury, and contributes to its resolution.[1 2 6 7] In particular, it is well established that hepatic macrophages, including Kupffer cells and infiltrating macrophages derived from monocytes, play a key role in the initiation and progression of fibrosis, as demonstrated in mice models of chronic liver injury.[8 9] These data have been corroborated by in vitro studies showing that hepatic macrophages promote activation and survival of hepatic stellate cells.[8 9] In addition, a distinct subpopulation of restorative macrophages promotes fibrosis resolution during the recovery phase of chronic liver injury.[10–12] Altogether, these data suggest that targeting macrophages to control fibrosis progression and to promote resolution may constitute an attractive antifibrogenic strategy.

Monoacylglycerol lipase (MAGL) is the rate-limiting enzyme in the degradation of monoacylglycerols.[13] MAGL preferentially hydrolyses monoacylglycerols to glycerol and fatty acids, with highest expression in brain, white adipose tissue and liver.[13 14] In addition to its role in lipid metabolism, MAGL is a pivotal component of the endocannabinoid system, as it is an endocannabinoid-degrading enzyme converting 2-arachidonoylglycerol (2-AG), an endogenous ligand for the cannabinoid receptors CB1 and CB2, into arachidonic acid. Interestingly, we have previously uncovered the key role of CB1 and CB2 in the pathogenesis of chronic liver diseases, by demonstrating that CB2 receptors display beneficial anti-inflammatory and antifibrogenic effects, whereas CB1 receptors are profibrogenic.[15–19] MAGL terminates 2-AG signalling, and constitutes the major source of arachidonic acid and proinflammatory prostaglandins in the liver or in the brain, where the contribution of phospholipase A2 is minor.[20] Accordingly, inhibition of MAGL has emerged as an anti-inflammatory and protective option in several experimental models of chronic inflammatory diseases, by a mechanism that relies on a shift of lipid metabolism in inflammatory cells, from arachidonic acid and proinflammatory arachidonic acid-derived prostaglandins towards 2-AG.[21 22]

In the present study, we investigated whether MAGL may constitute a novel immunometabolic fibrogenic target in the context of chronic liver injury. We show that mice with either total or myeloid cell-specific deletion of MAGL display reduced liver inflammation and are resistant to fibrosis. In addition, therapeutic intervention with MAGL inhibitors slows down fibrosis progression and accelerates fibrosis regression in experimental models. Mechanistically, we demonstrate that inhibition of MAGL reduces the production of inflammatory cytokines from macrophages independently of CB2 receptors, but *via* an autophagy-dependent pathway.

EXPERIMENTAL PROCEDURES

Additional materials and methods are included in the online supplementary information.

Animals

MAGL-deficient mice ($MAGL^{-/-}$) backcrossed for >10 generations on a C57BL/6J background and *Mgll-loxP/loxP* mice ($MAGL^{flox/flox}$) were generated as previously described.[23 24] $ATG5^{flox/flox}$ mice were obtained from Dr Noboru Mizushima (University of Tokyo, Japan). We generated $CB2^{flox/flox}$ mice, and developed $CB2^{Mye-/-}$ and $ATG5^{Mye-/-}$ mice as we described previously.[25 26] Mice were kept in pathogen-free animal facilities and fed ad libitum. C57BL/6J mice from Janvier (Le Genest-Saint-Isle, France) served as wild-type (WT) mice for $MAGL^{-/-}$ mice, and $MAGL^{flox/flox}$, $CB2^{flox/flox}$ or $ATG5^{flox/flox}$ littermates for $MAGL^{Mye-/-}$, $CB2^{Mye-/-}$ and $ATG5^{Mye-/-}$ mice, respectively.

Generation of $MAGL^{Mye-/-}$ mice

$MAGL^{Mye-/-}$ mice were generated by crossing *Mgll-loxP/loxP* to *LysM-Cre* mice (Jackson Laboratory, Charles River France, L'Arbresle, France), and backcrossing the resulting double heterozygotes (*LysM-Cre+/-*, *Mgll+/loxP*) with *Mgll-loxP/loxP* mice to produce myeloid-specific MAGL knockout mice (*LysM-Cre+/-*, *Mgll-loxP/loxP*) labelled as $MAGL^{Mye-/-}$ and WT littermates (*LysM-Cre-/-*, *Mgll-loxP/loxP*) labelled as $MAGL^{flox/flox}$ mice.

Experimental models of liver fibrosis and fibrosis regression

Experiments were performed in accordance with protocols approved by the Paris-Nord ethical committee C2EA 121 (authorisation number 02529.02).

CCl_4-induced fibrosis

Liver fibrosis was induced in male mice aged 11–14 weeks twice a week by intraperitoneal injection of 0.6 mL/kg (body weight (BW)) carbon tetrachloride (CCl_4, Sigma-Aldrich 87030) diluted 1/10 in mineral oil (MO, Sigma-Aldrich M-5310) for 6 weeks, as previously described.[18] Control animals received MO. Mice were sacrificed 24 hours after the last injection of CCl_4.

Fibrosis regression

Mice were administered 0.6 mL/kg (BW) CCl_4 for 6 weeks and MAGL inhibitors (either JZL184 (Cayman Chemical, Ann Arbor, Michigan, USA, 13158,[27]) or MJN110[28]) or vehicle, were injected 2 hours prior to the last CCl_4 injection and daily until sacrifice at day 1 or day 4 following the last CCl_4 injection. Drugs were diluted in Emulphor:ethanol:phosphate buffered saline; 1:1:18 (vehicle) and injected intraperitoneally at a dose of 15 mg/kg for JZL184 and 10 mg/kg for MJN110. Control animals received MO and drug vehicle.

Bile duct ligation

Bile duct ligation (BDL) was performed on male mice aged 11–13 weeks, as previously described.[19] Mice were administered MJN110 (10 mg/kg, intraperitoneally) or vehicle starting at day 7 following surgery, and daily until sacrifice at day 14. Sham animal underwent laparotomy.

Statistical analysis

All data are expressed as mean±SEM. Statistical analyses were performed using Mann-Whitney U test with Prism software (GraphPad, La Jolla, CA). A p value <0.05 was considered to be statistically significant.

RESULTS

Mice bearing a global or a myeloid-specific invalidation of MAGL are resistant to liver fibrosis

We first investigated the impact of MAGL on experimental fibrosis, owing to the use of mice globally invalidated for MAGL ($MAGL^{-/-}$). $MAGL^{-/-}$ mice chronically exposed to CCl_4 were more resistant to fibrosis than WT counterparts, as evidenced by decrease in sirius red staining (figure 1A). They also displayed increased gene expression of the fibrolytic metalloproteinase *Mmp13*, and lower number of fibrogenic cells, as reflected by

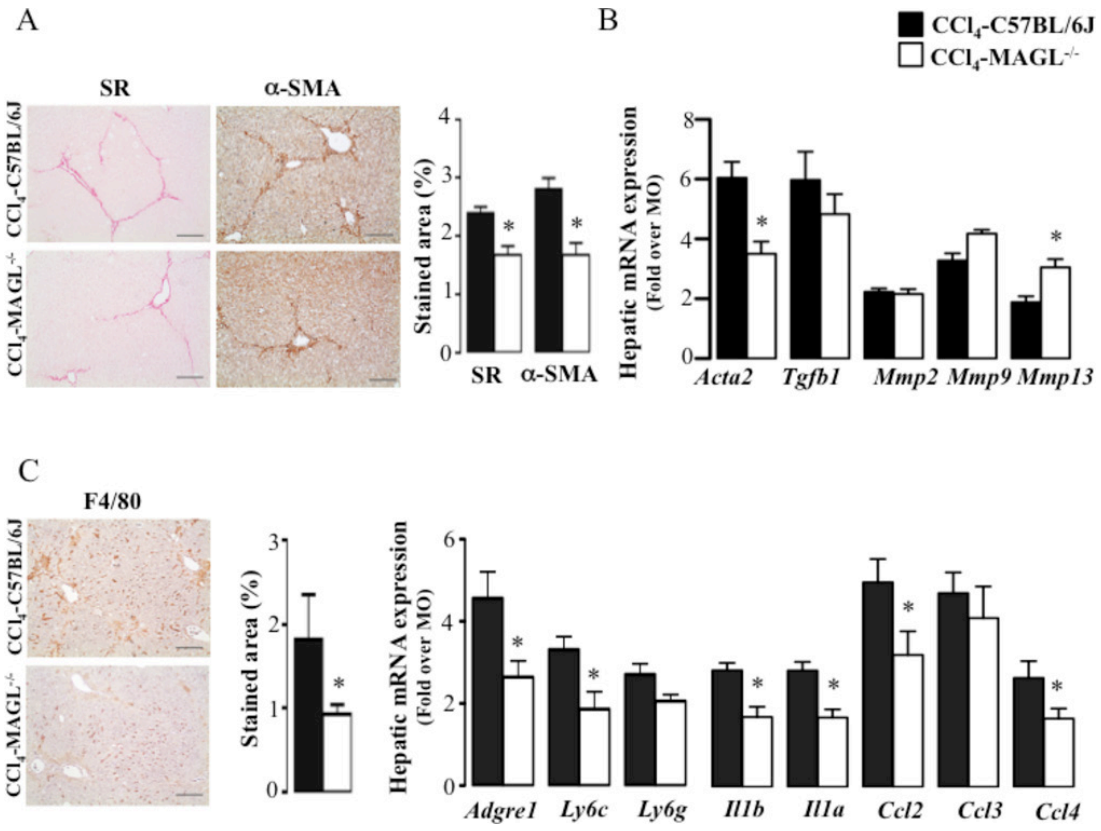

Figure 1 MAGL$^{-/-}$ mice display reduced liver inflammation and injury after chronic carbon tetrachloride (CCl_4) administration. Mineral oil (MO, n=3) or CCl_4 were injected for 6 weeks in C57BL/6J (n=9) and MAGL$^{-/-}$ mice (n=10). (A) Representative images, quantification of sirius red (SR) staining and of α-SMA immunostaining in liver tissue. (B) Hepatic fibrogenic gene expression. (C) Typical images and quantification of F4/80$^+$ cells and hepatic inflammatory genes. Results are expressed as fold over MO-injected C57BL/6J. *p<0.05 for MAGL$^{-/-}$ vs wild-type mice. Scale bar 200 µm. MAGL, monoacylglycerol lipase.

reduced α-smooth muscle actin (α-SMA) staining and decreased gene expression of *Acta2* (figure 1A,B). Moreover, as compared with WT animals, CCl_4-exposed MAGL$^{-/-}$ mice showed reduced number of F4/80$^+$ macrophages (figure 1C), associated with decreased hepatic expression of macrophage genes, *Adgre1 (F4/80)* and *Ly6c* and lower expression of the proinflammatory cytokines, *Il1b* and *Il1a* and the chemokines *Ccl2* and *Ccl4*. These data revealed the proinflammatory and profibrogenic properties of MAGL in the liver, and suggested that MAGL in monocyte/macrophage may constitute an interesting target. In keeping with this hypothesis, mouse bone marrow-derived macrophages and Kupffer cells displayed reduced lipopolysaccharide (LPS)-stimulated production of prostaglandin E_2 (PGE_2), cytokines and chemokines when exposed to the MAGL inhibitor, MJN110 (figure 2A).

In order to further investigate the contribution of the monocyte/macrophage MAGL in liver fibrosis, we generated mice lacking MAGL in the myeloid lineage (MAGL$^{Mye-/-}$) by crossing MAGL$^{flox/flox}$ with transgenic mice overexpressing the recombinant Cre under the control of the lysozyme M promoter. The efficiency of the deletion was confirmed by decreased MAGL expression in peritoneal macrophages from MAGL$^{Mye-/-}$ mice, both at the protein and gene levels (figure 2B). In addition, basal levels of 2-AG were increased in macrophages from MAGL$^{-/-}$ and MAGL$^{Mye-/-}$ mice (figure 2B). Macrophages isolated from MAGL$^{Mye-/-}$ mice also showed significant reduction in PGE_2 and thromboxane B_2 (TXB_2) on LPS stimulation as compared with WT littermates (figure 2B) and were resistant to LPS-induced release of interleukin (IL)-1β, IL-1α, CCL2 and IL-6 (figure 2C). Moreover, MJN110 significantly inhibited cytokines, chemokines (figure 2C), PGE_2 and TXB_2 production from LPS-stimulated peritoneal macrophages (see online supplementary figure S1A). This effect was not due to cytotoxic effects of the compound since no difference in macrophage viability was observed between vehicle and MJN110-treated macrophages (see online supplementary figure S2A). Moreover, the specificity of MJN110 was demonstrated by its lack of additional anti-inflammatory effects in MAGL-deficient macrophages (see online supplementary figure S2B).

In keeping with the antifibrogenic effects observed in MAGL$^{-/-}$ mice, MAGL$^{Mye-/-}$ mice chronically exposed to CCl_4 displayed significant reduction in sirius red staining (figure 3A). As compared with MAGL$^{flox/flox}$, they also showed decreased expression of hepatic *Acta2* and *Mmp9*, and a tendency to reduced *Timp1*, together with increase in *Mmp13* (figure 3A). Moreover, there was a reduction in the number of F4/80$^+$ macrophages, associated with decreased hepatic gene expression of *Adgre1*, *Ly6c*, *Ly6g*, *Il1b*, *Il1a*, *Ccl2* and *Ccl3* (figure 3B). Similar reduction in fibrogenic and inflammatory parameters was observed in MAGL$^{Mye-/-}$ mice subjected to BDL (figure 3C,D). Flow cytometry analysis of hepatic leucocytes also showed a decrease in the frequency of CD11b$^+$Ly6-C^{high} (figure 3D).

MAGL inhibitors reduce fibrosis progression

We next investigated whether therapeutic intervention with MAGL inhibitors slows down fibrosis progression. To that aim, BDL mice were administered every day MJN110, starting

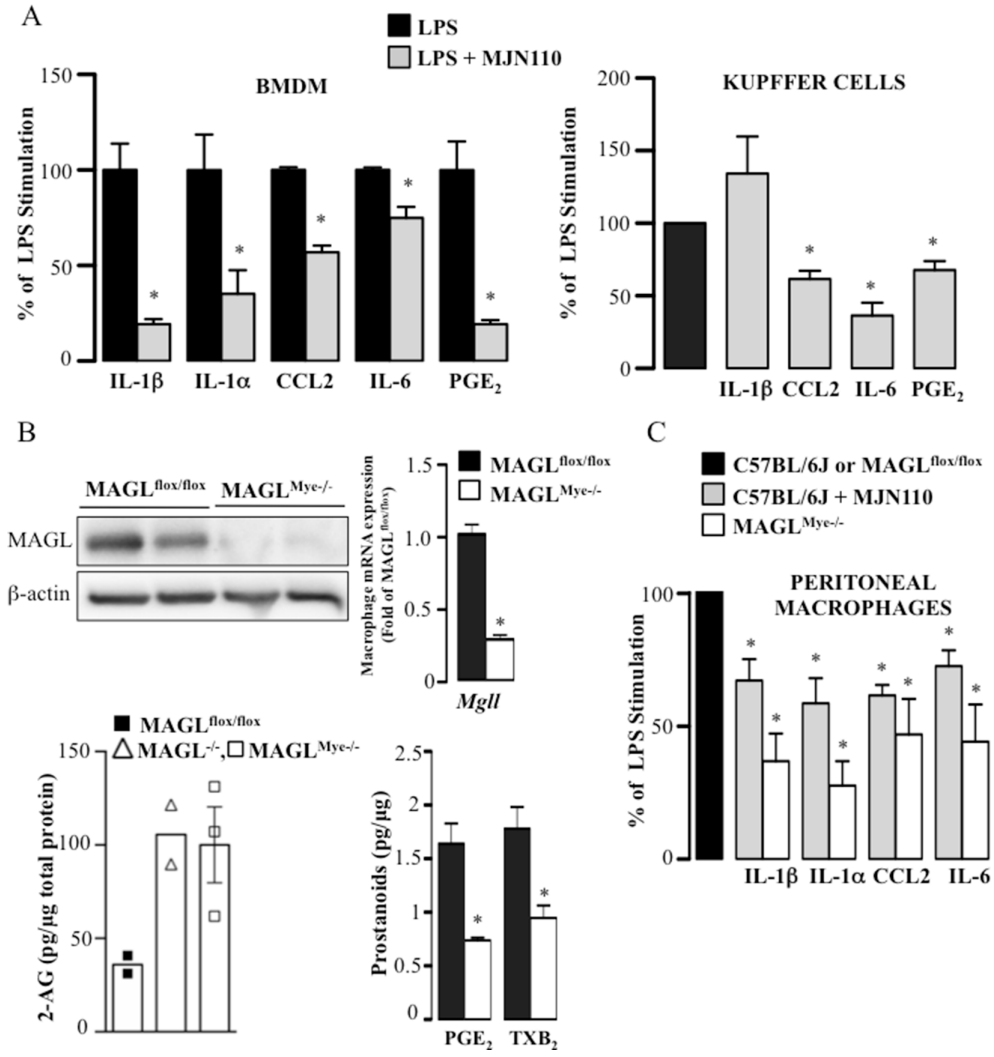

Figure 2 Monoacylglycerol lipase (MAGL) inhibition/invalidation reduces the production of inflammatory mediators in macrophages and increases the production of 2-arachidonoylglycerol (2-AG). (A) Bone marrow-derived macrophages (BMDM) and Kupffer cells were isolated from C57BL/6J mice. ELISA analysis was performed on cells exposed to 10 ng/mL of lipopolysaccharide (LPS) and/or 1 µM MJN110. Data are expressed as % LPS stimulation (n=5, Kupffer cell preparations and quintuplicate determinations for BMDM, n=2 experiments). *p<0.05 for MJN110 vs vehicle. (B) Peritoneal macrophages were isolated from $MAGL^{flox/flox}$, $MAGL^{-/-}$ and $MAGL^{Mye-/-}$ mice. Representative western blot analysis of MAGL (n=6), *Mgll* expression, quantification of 2-AG production (n=2–3 experiments) and prostaglandin E_2 (PGE_2) and thromboxane B_2(TXB_2) release on LPS stimulation (n=3 experiments). *p<0.05 for $MAGL^{-/-}$or $MAGL^{Mye-/-}$ vs wildtype (WT). (C) ELISA analysis in supernatants from LPS-stimulated peritoneal macrophages. Data are expressed as % LPS stimulation (n=10 for MJN110 and n=4 for $MAGL^{Mye-/-}$). *p<0.05 for MJN110 or $MAGL^{Mye-/-}$ vs WT. IL, interleukin.

7 days after surgery. The compound lowered sirius red staining in MJN110-exposed as compared to vehicle-exposed C57BL/6J mice on BDL and reduced hepatic fibrogenic gene expression, including *Tgfb*, *Pdgfrb*, *Timp1* and *Mmp2* (figure 4A). They also showed decreased number of $F4/80^+$ macrophages and inflammatory gene expression (figure 4B).

Antifibrogenic effects of MAGL inhibition are exclusively macrophage-dependent, whereas hepatoprotective properties are macrophage-independent

It has been shown that other liver cell types, including hepatocytes and hepatic stellate cells also express MAGL.[29] Because these cells are also key actors of the fibrogenic process,[1 2] we evaluated whether only macrophages or also additional liver cells contribute to the antifibrogenic properties of MAGL inhibition/inhibitors. To that aim, $MAGL^{Mye-/-}$ BDL mice were administered every day MJN110 starting 7 days after surgery, and the extent of fibrosis was compared with vehicle-treated counterparts. As shown in figure 4C, the extent of sirius red staining inhibition or of fibrogenic gene expression in $MAGL^{Mye-/-}$ BDL mice was similar to $MAGL^{Mye-/-}$ BDL mice exposed to MJN110.

MAGL inhibitors have also been reported to display hepatoprotective properties,[29] and we compared the extent of hepatocyte apoptosis by quantifying the number of cleaved caspase-3-positive hepatocytes in liver tissue sections from mice pharmacologically or genetically invalidated for MAGL, and from mice with myeloid-specific deletion. As shown in figure 4D, mice with global invalidation of MAGL or treated with MJN110 showed reduced cleaved caspase-3 staining in hepatocytes following chronic intoxication with CCl_4 or BDL, although they did not show differences in serum alanine transferase levels (see online supplementary figure S3). Strikingly, there was no difference in the number of cleaved caspase-3-positive hepatocytes in the liver of $MAGL^{Mye-/-}$ mice as compared

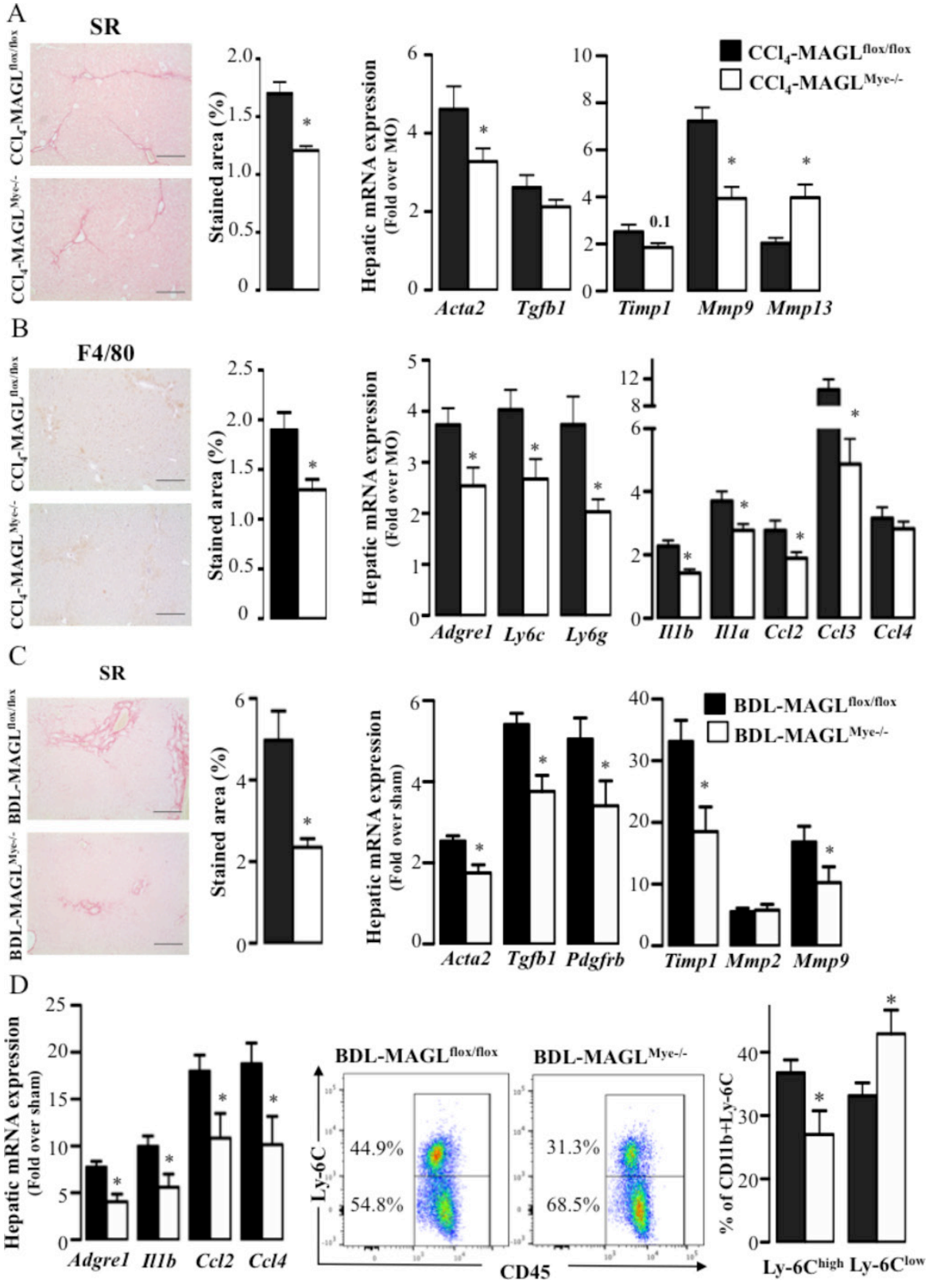

Figure 3 Mice with specific deletion of monoacylglycerol lipase (MAGL) in myeloid cells are protected against inflammation and fibrosis. (A–B) Mineral oil (MO, n=5) or carbon tetrachloride (CCl_4) were injected for 6 weeks in $MAGL^{Mye-/-}$ (n=15) and $MAGL^{flox/flox}$ (n=10) mice. (A) Representative images, quantification of sirius red (SR) staining and hepatic fibrogenic gene expression. (B) F4/80 immunodetection, quantification of $F4/80^+$ cells and hepatic inflammatory gene expression. (C–D) $MAGL^{Mye-/-}$ (n=9) and $MAGL^{flox/flox}$ (n=15) mice were subjected to bile duct ligation (BDL). (C) Representative images of SR staining quantification and hepatic fibrogenic gene expression. (D) Hepatic expression of inflammatory genes, representative dot plots of intrahepatic leucocytes and summary data of flow cytometry analysis. Results are expressed as fold over sham $MAGL^{flox/flox}$ mice. *p<0.05 for $MAGL^{Mye-/-}$ vs $MAGL^{flox/flox}$ mice. Scale bar 200 µm.

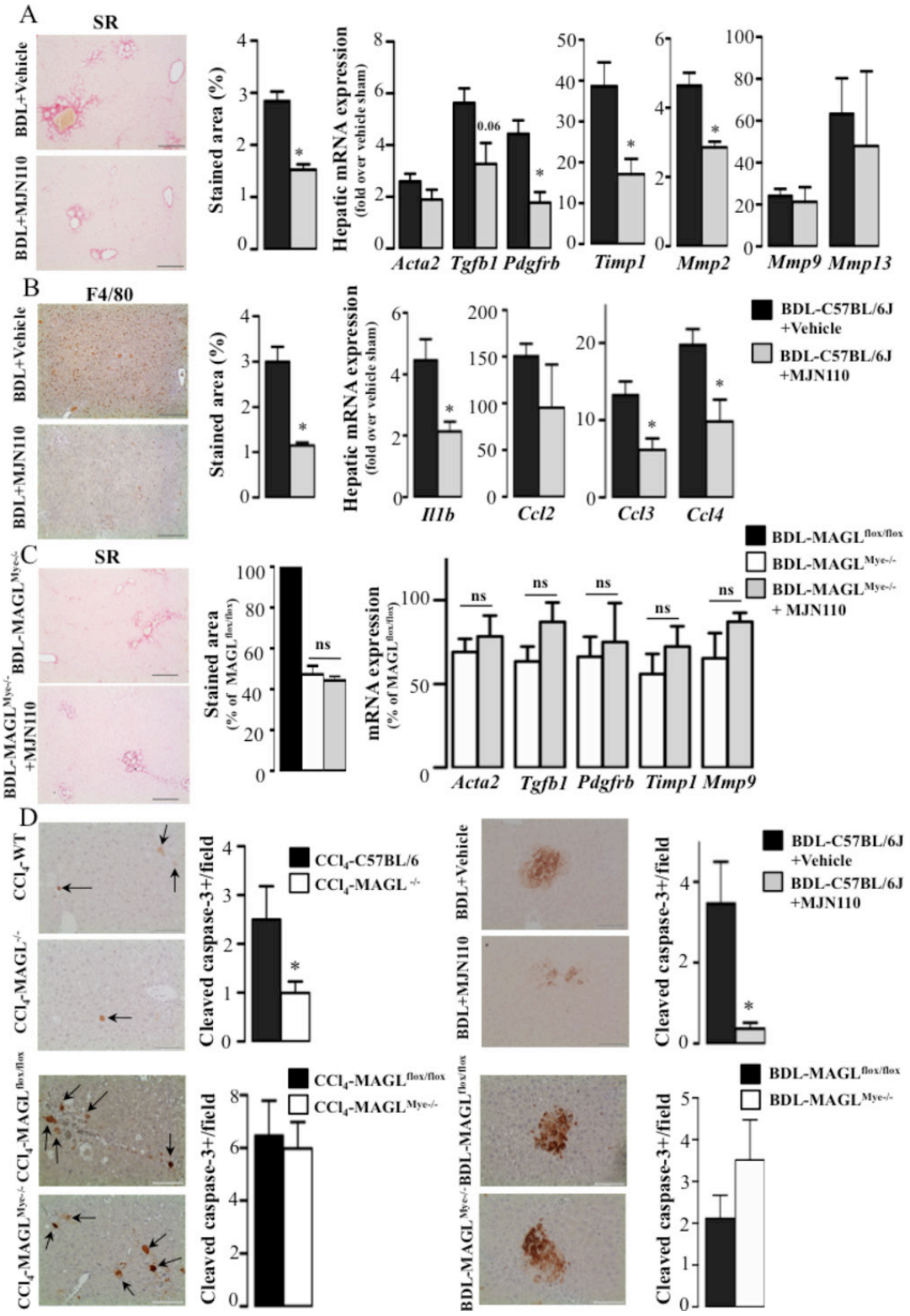

Figure 4 Antifibrogenic effects of monoacylglycerol lipase (MAGL) inhibition are exclusively macrophage-dependent, whereas hepatoprotective effects are macrophage-independent. C57BL/6J, MAGL$^{flox/flox}$ or MAGL$^{Mye-/-}$bile duct ligation (BDL) mice were daily administered MJN110 (n=5) or vehicle (n=4) for 7 days, 1 week after BDL. (A–B) *MAGL inhibition by MJN110 slows down fibrosis progression.* (A) Representative images and quantification of sirius red (SR) staining and hepatic fibrogenic gene expression. (B) F4/80 immunodetection, quantification of F4/80^{+} cells and hepatic inflammatory gene expression. Results are fold over sham, mean±SEM, *p<0.05 for MJN110 vs vehicle. (*C) Antifibrogenic effects of MAGL inhibition are exclusively macrophage-dependent.* Representative images and quantification of SR staining and hepatic fibrogenic gene expression in MAGL$^{Mye-/-}$ BDL mice treated with MJN110 (n=6) or vehicle (n=9). Scale bar 200 µm. Results are expressed as % of staining area or % expression of MJN110 or vehicle of MAGL$^{flox/flox}$. (D) *Hepatoprotective effects are macrophage-independent*. Hepatocyte immunodetection of cleaved caspase-3 and quantification of cleaved caspase-3-positive hepatocytes in BDL and CCl_4-treated mice. *p<0.05 for MAGL$^{-/-}$ or MAGL$^{Mye-/-}$ vs wild-type (WT) or MJN110- vs vehicle. Scale bar 100 µm.

with WT counterparts, whether exposed to CCl_4 or subjected to BDL (figure 4D).

Taken together, these data demonstrate that invalidation of MAGL in myeloid cells exclusively accounts for the decrease in liver fibrosis and does not protect against hepatocyte injury.

MAGL inhibitors promote fibrosis resolution

We also evaluated whether pharmacological inhibition of MAGL accelerates fibrosis regression. Mice were injected with CCl_4 for 6 weeks, fibrosis regression was analysed 4 days after the last CCl_4 injection and expression of proinflammatory and profibrogenic genes quantified 1 day after the last CCl_4 injection. As expected, liver fibrosis was increased and remained elevated 4 days after the last CCl_4 injection. However, mice daily administered with MJN110 and JZL184, another MAGL inhibitor, displayed lower sirius red and α-SMA staining areas (figure 5A,B), associated with decreased expression of hepatic *Acta2*, *Tgfb1 Timp1 and Mmp2* gene expression (figure 5C). Compared with vehicle-treated animals, C57BL/6J mice exposed to JZL184 showed lower *Adgre1* and *Ly6c* gene expression and a concordant inhibition of hepatic cytokine and chemokine gene expression (figure 5D). The flow cytometry analysis of hepatic leucocytes showed that administration of JZL184 resulted in a decrease of the percentage of Ly-6C^{high} and an increase in Ly-6C^{low} cells (figure 5E). Altogether, these results demonstrate that MAGL inhibition accelerates fibrosis regression.

Anti-inflammatory properties of MAGL inhibitors in macrophages are independent of CB2 receptor activation and alpha/beta hydrolase containing domain 6

We evaluated whether the anti-inflammatory effects of MAGL invalidation/inhibition are mediated by CB receptors, and focused on the anti-inflammatory receptor CB2, since CB2 receptor activation in macrophages is anti-inflammatory in the liver,[16 25] whereas CB1 receptors show opposite inflammatory properties.[30] Using macrophages from CB2$^{Mye-/-}$ mice or WT littermates, we observed that inhibition of LPS-stimulated IL-1β, IL-6 and CCL2 production by MJN110 was similar in CB2$^{Mye-/-}$ and WT macrophages (figure 6A). Moreover, inhibition of cytokine and chemokine production triggered by MJN110 or MAGL invalidation was not modified when CB2 receptors were genetically or pharmacologically invalidated (figure 6A). These data demonstrate that the anti-inflammatory effects of MJN110 are not mediated by the CB2 receptor signalling in macrophages. The role of alpha/beta hydrolase containing domain 6 (ABHD6), which converts 2-AG into arachidonic acid,[31] was also ruled out by the lack of effect of WWL70, a selective ABHD6 inhibitor, on LPS stimulation (see online supplementary figure S1B). Under these conditions, WWL70 showed a reduction in PGE_2 and TXB_2 release, which confirmed the efficiency of WWL70 in blocking monoacylglycerol hydrolysis (see online supplementary figure S1A).

Anti-inflammatory properties of MAGL inhibitors in macrophages rely on autophagy activation

We have previously demonstrated that autophagy in macrophages is anti-inflammatory and antifibrogenic.[25 26] We therefore hypothesised that autophagy may underlie the anti-inflammatory effects of MAGL inhibitors. Lipidation of LC3 (LC3-II) was increased in macrophages exposed to MJN110 or isolated from MAGL$^{Mye-/-}$ mice (figure 6B). The lysosomal inhibitor chloroquine further enhanced LC3 lipidation in both conditions, suggesting enhanced autophagic flux (figure 6B). We further investigated whether MJN110 interferes with the autophagic process by affecting autophagosome biosynthesis. Autophagosome-lysosome fusion was blocked with chloroquine, and autophagosome biosynthesis was followed by kinetic analysis of LC3-II accumulation in the presence of MJN110 or vehicle. Blocking autophagosome-lysosome fusion enhanced LC3-II accumulation by MJN110 after 18–24 hours, as compared with 8–12 hours incubation (figure 6C). These data indicated that inhibiting MAGL activity enhances autophagosome biosynthesis.

The role of autophagy was further explored owing to the use of mice deficient in the autophagy gene ATG5 in myeloid cells (ATG5$^{Mye-/-}$ mice).[26] As anticipated,[26] macrophages from ATG5$^{Mye-/-}$ mice were more sensitive to LPS stimulation than WT counterparts (figure 6D). However, whereas LPS-stimulated macrophages from WT-ATG5$^{flox/flox}$ mice showed the expected reduction in IL-1β, IL-1α, CCL2 and IL-6 on exposure to MJN110, the inhibition was lost in LPS-stimulated macrophages from ATG5$^{Mye-/-}$ mice (figure 6D). The involvement of autophagy in the anti-inflammatory and antifibrogenic effects of MAGL inhibitors was further assessed in ATG5$^{Mye-/-}$ BDL mice and their WT counterparts exposed to daily administration of MJN110 starting 7 days after surgery. As shown in figure 6E, the reduction in the number of macrophages and fibrogenic cells, and the decrease in fibrosis area observed in ATG5$^{flox/flox}$ BDL mice exposed to MJN110 were totally lost in ATG5$^{Mye-/-}$ BDL mice. Together these data demonstrate that the anti-inflammatory and antifibrogenic effects of MAGL inhibitors are mediated by autophagy.

DISCUSSION

Liver fibrosis is a wound-healing response to viral, metabolic, toxic, immune-mediated chronic liver injuries. Irrespective of these underlying aetiologies, fibrosis will progress to cirrhosis that is the main risk factor for the development of liver cancer. Tremendous advances have been made in the understanding of mechanisms that underlie the fibrogenic response to chronic liver insult. However, there is no as-yet approved antifibrotic drug to treat patients with liver fibrosis or slow down its progression to cirrhosis. In the present study, we demonstrate that inhibitors of MAGL may open new avenues as antifibrogenic agents.

Reprogramming lipid metabolism in parenchymal and non-parenchymal cells has emerged as a key concept that may be considered to limit fibrosis progression and/or promote its regression. Indeed, changes in lipid metabolism drive hepatocyte injury, and control immune cell phenotype and fibrogenic cell activation. In this context, we hypothesised that MAGL may represent an interesting target, since this endocannabinoid-degrading enzyme serves as a key metabolic hub connecting the endocannabinoid and the eicosanoid signalling network, by hydrolysing 2-AG into arachidonic acid.[20] These data suggest that inhibition of MAGL may limit the production of proinflammatory prostaglandins in the setting of inflammatory disorders.[22 32] MAGL inhibitors reduce inflammation and colon alterations in a model of colitis,[33] reduce macrophage content and improve atherosclerotic plaque alterations,[34] attenuate neuroinflammation in astrocytes[22] and display anti-inflammatory and protective effects during acute lung or muscle injury.[35 36] Surprisingly, very little is known about the role of MAGL in the liver. Recent data have shown that MAGL inhibition limits LPS-induced inflammation in the liver and in the brain[20] and that global genetic and pharmacological inhibition of MAGL protects against inflammation and liver lesions induced by ischaemia/reperfusion injury.[29] Interestingly, MAGL invalidation improves adipose tissue inflammation and

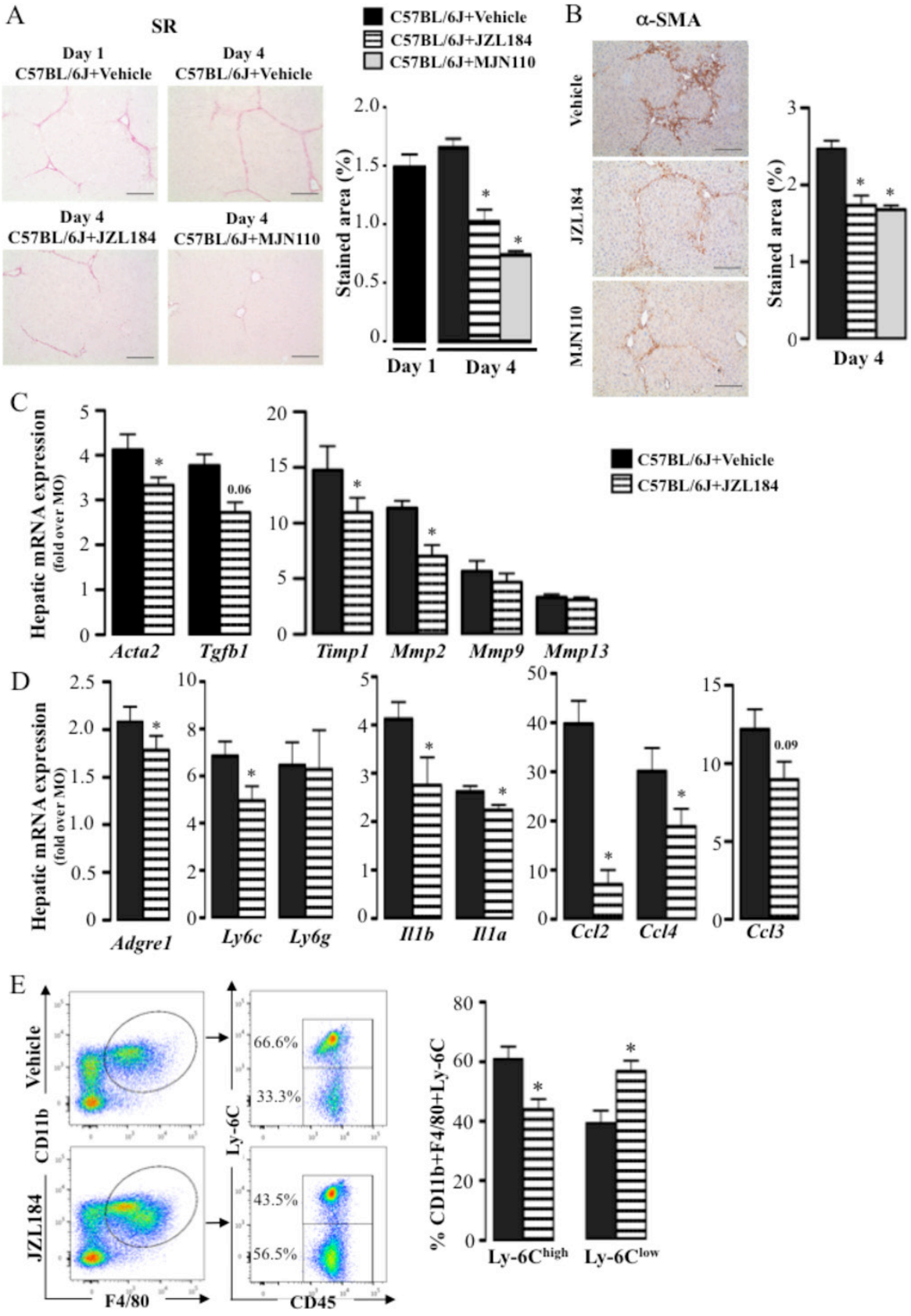

Figure 5 Monoacylglycerol lipase (MAGL) inhibitors accelerate fibrosis regression. (A–B) Representative images and quantification of sirius red (SR) staining (A) and α-SMA immunostaining (B), 1 and 4 days after cessation of carbon tetrachloride (CCl_4) administration. Scale bar 200 µm. (C–D) Hepatic gene expression of fibrogenic (C) and inflammatory (D) genes 1 day after MAGL inhibitor administration. Results are expressed as fold over mineral oil (MO)-injected mice (n=5). *p<0.05 for mice treated with JZL184 or MJN110 vs vehicle (n=8–10 mice/group). (E) Representative dot plots of intrahepatic leucocytes isolated at day 3 after CCl_4 cessation and summary data of flow cytometry analysis. *p<0.05 of MAGL inhibitor *vs* vehicle, n=6 mice/group.

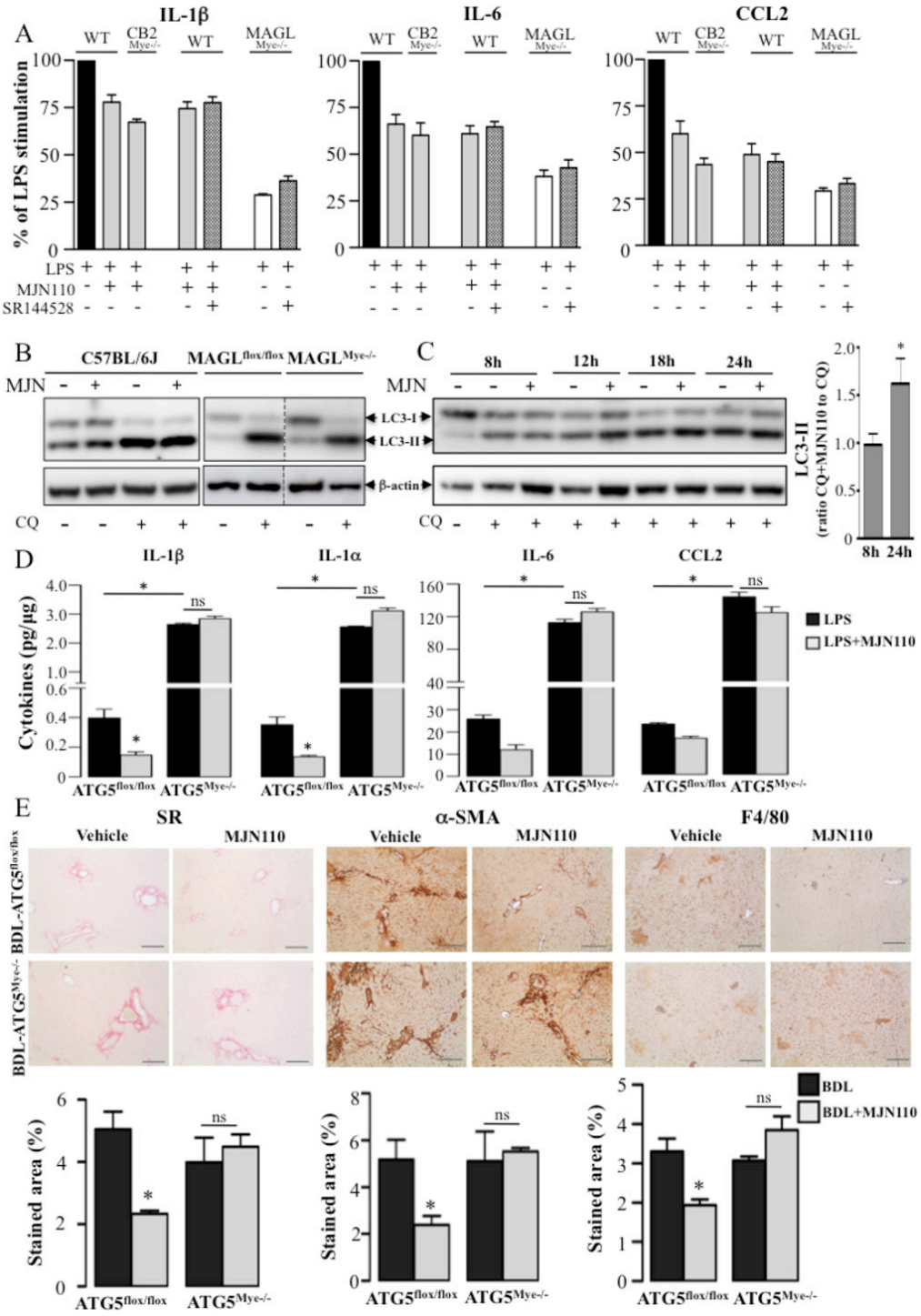

Figure 6 Anti-inflammatory properties of monoacylglycerol lipase (MAGL) inhibition are CB2-independent but rely on autophagy. Peritoneal macrophages were isolated from MAGL$^{Mye-/-}$, CB2$^{Mye-/-}$ and their respective wild-type (WT) counterparts (MAGL$^{flox/flox}$, CB2$^{flox/flox}$), and exposed 10 ng/mL of lipopolysaccharide (LPS) and/or 1 µM MJN110, 1 µM SR144528 or vehicle, for 24 hours. (A) ELISA analysis in the supernatants of macrophages. Data are expressed as % LPS and are the mean±SEM of quintuplicates. *p<0.05 for each treatment compared with WT-LPS. Similar results in n=3 experiments. (B) Western blot analysis of LC3 protein in peritoneal macrophages isolated from C57BL/6J, MAGL$^{flox/flox}$ or MAGL$^{Mye-/-}$, exposed for 24 hours to 1 µM MJN110 or vehicle, with or without 30 µM chloroquine (CQ). For MAGL$^{Mye-/-}$ mice, samples were run on the same gel but were non-contiguous. Similar results in three experiments. (C) Kinetic analysis of LC3-II formation in C57BL/6J peritoneal macrophages exposed to 1 µM MJN110 or vehicle for indicated times, in the presence of 30 µM CQ. Bars show LC3-II expression normalised to β-actin, expressed as ratio of MJN110+CQ to vehicle+CQ (n=4). *p<0.05 for 24 vs 8 hours. (D) ELISA analysis in the supernatants of macrophages from ATG5$^{Mye-/-}$ mice and ATG5$^{flox/flox}$ littermates exposed to 10 ng/mL of LPS and/or 1 µM MJN110 or vehicle for 24 hours. Data are mean±SEM of quintuplicates, similar results in three experiments. *p<0.05 vs WT-LPS. (E) Involvement of autophagy in the inhibitory effect of MJN110 in vivo. Mice were subjected to bile duct ligation (BDL) and, 1 week after BDL, MJN110 (WT, n=4; ATG5$^{Mye-/-}$ mice, n=6) or vehicle (ATG5$^{flox/flox}$, n=7; ATG5$^{Mye-/-}$ mice, n=5) was administered every day for 7 days. Representative images and quantification of sirius red (SR) staining, α-SMA and F4/80^{+} cells. Results are expressed as % of stained area. *p<0.05 for MJN110 vs vehicle. IL, interleukin; ns, not significant.

insulin resistance in obese mice, and reduces triglyceride content in the liver,[37] suggesting that MAGL inhibitors may also provide protection against non-alcoholic fatty liver disease. Our data highlight novel functions of MAGL inhibition, based on genetic and pharmacological MAGL invalidation approaches. Indeed, owing to mice bearing global invalidation of MAGL, we show that MAGL inhibition strongly decreases the fibrogenic response and hepatic myofibroblast accumulation associated with chronic liver injury. Importantly, we also unravel the therapeutic potential of MAGL inhibitors, by showing that administration of MJN110 1 week after BDL slows down fibrosis progression. In addition, MAGL inhibitors delay fibrosis progression and promote fibrosis regression. Indeed, administration of JZL184 following cessation of chronic CCl_4 administration accelerates fibrosis regression and decreases the number of profibrogenic Ly-6C^{high} macrophages while the number of Ly-6C^{low} restorative macrophages is increased. These results highlight the central role of macrophage MAGL in the fibrogenic response, both during fibrosis progression and regression. In keeping, mice bearing a specific deletion of MAGL in myeloid cells are protected against CCl_4-induced liver inflammation and fibrosis, to a similar extent as global knockout animals. These data are further reinforced by in vitro studies in macrophages showing concordant reduction of inflammatory cytokine production and expression, following exposure to LPS. They are also in line with recent data showing that macrophages isolated from transgenic mice with specific overexpression of MAGL in myeloid cells display a proinflammatory M1 phenotype in response to LPS.[38] Although our data demonstrate that MAGL in macrophage undoubtedly participates in hepatic inflammation and fibrosis, other liver cell types expressing MAGL could have been involved. Indeed, in addition to macrophages, MAGL is also expressed in hepatocytes and hepatic stellate cells.[29] However, reduction of fibrosis observed in MAGL$^{Mye-/-}$ BDL mice was not further decreased on administration of MJN110, demonstrating that MAGL in myeloid cells fully account for the profibrogenic effects of MAGL in the liver. Interestingly, the hepatoprotective effects of MAGL invalidation, described in acute models of liver injury,[29] were also confirmed in models of chronic liver injury, as shown by the decrease in the number of cleaved caspase-3-positive hepatocytes in mice globally invalidated for MAGL either genetically or pharmacologically. However, protection against hepatocyte death was not observed in myeloid-specific MAGL-deficient BDL mice, demonstrating that MAGL in non-myeloid cells promotes hepatocyte apoptosis. Whether MAGL inhibition in hepatocytes accounts for hepatoprotection remains to be determined, considering that MAGL is highly expressed in these cells.[29 38]

Among endocannabinoids, 2-AG is considered as a natural ligand for CB receptors, among which CB2 receptors display anti-inflammatory properties in macrophages and antifibrogenic functions in the liver.[15 16] We found that anti-inflammatory effects of MAGL inhibitors were preserved in macrophages derived from myeloid-specific CB2 receptor-deficient mice or macrophages treated with the CB2 receptor antagonist, demonstrating that enhanced CB2-mediated signalling does not account for the effects of MAGL inhibition. In addition, the involvement of CB1-mediated signalling is unlikely, since CB1 receptors show profibrogenic and proinflammatory functions in the liver.[2 17 19 30] Although cannabinoid receptor-dependent anti-inflammatory effects of MAGL inhibitors have been described,[29 33 34 39 40] cannabinoid receptor-independent inhibition of proinflammatory eicosanoid production has also been demonstrated.[20 22 29 37 41 42] We also excluded the involvement of additional pathways described for 2-AG degradation like the hydrolytic enzyme ABHD6, which also showed anti-inflammatory properties in macrophages.[31]

A candidate mechanism that could govern anti-inflammatory and antifibrogenic effects of MAGL inhibitors is autophagy in macrophages. Autophagy is a lysosomal degradation process of cellular constituents that limit inflammatory cytokine release from macrophages.[43] In the liver, we have recently reported that macrophage autophagy is an anti-inflammatory and antifibrogenic pathway, as shown in mice deficient in ATG5 in macrophages that display exacerbated hepatic inflammation and fibrosis.[26] Our data demonstrate that genetic or pharmacological invalidation of MAGL leads to enhanced LC3 lipidation and autophagic flux in isolated macrophages, by a mechanism involving increase in autophagosome biosynthesis. Moreover, the anti-inflammatory effects of MJN110 are lost in ATG5-deficient macrophages and in ATG5$^{Mye-/-}$ BDL mice, together with the reduction of fibrosis. Altogether, these results demonstrate that autophagy mediates the anti-inflammatory properties of MAGL inhibitors, and contributes to its antifibrogenic effects. They also suggest that MAGL inhibitors may provide a mean to stimulate autophagy in macrophages to decrease inflammation in the context of inflammatory disorders.

In conclusion, our study unravels MAGL as a novel immunometabolic target and demonstrates that MAGL inhibitors may be considered as promising antifibrogenic compounds during fibrosis progression and in the context of fibrosis regression.

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