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SYK-3BP2 pathway activity in parenchymal and myeloid cells is a key pathogenic factor in metabolic steatohepatitis

**Short title:** SYK-3BP2 pathway in chronic liver inflammation.

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**Abbreviations:**

MAFLD, Metabolic associated fatty liver diseases; 3BP2, c-Abl Src homology 3 domain-binding protein-2; SYK, spleen tyrosine kinase; PAMP, Pathogen Associated Molecular Pattern; DAMP, Damage Associated Molecular Pattern; TLR4, Toll-like receptor 4; LPS, lipopolysaccharide (LPS); MCDD, methionine and choline deficient diet; ALT, alanine aminotransferase; TREM, triggering receptors expressed on myeloid cells.

**Word count**

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**Synopsis**

This work reports that SYK-3BP2 pathway activity enhances the LPS-TLR4 responses in both hepatocytes and resident liver macrophages. This initiates the onset of local inflammation and contributes to liver accumulation of leukocytes. Targeting of 3BP2 or SYK prevents metabolic steatohepatitis features.

**ABSTRACT**

**Background & aims:** SYK signaling pathway regulates critical processes in innate immunity but its role in parenchymal cells remains elusive in chronic liver diseases. We investigate the relative contribution of SYK and its substrate 3BP2 in both myeloid cells and hepatocytes in the onset of metabolic steatohepatitis.

**Methods:** Hepatic SYK-3BP2 pathway was evaluated in mouse models of MAFLD and in obese patients with biopsy proven-metabolic associated fatty liver diseases (MAFLD)(n=33). Its role in liver complications was evaluated in \textit{Sh3bp2} KO and myeloid-specific \textit{Syk} KO mice.
challenged with MCDD and in homozygous $Sh3bp2^{KI/KI}$ mice with and without SYK expression in myeloid cells.

**Results:** Here, we report that hepatic expression of 3BP2 and SYK correlated with metabolic steatohepatitis severity in mice. 3BP2 deficiency and SYK deletion in myeloid cells mediated the same protective effects on liver inflammation, injury and fibrosis priming upon diet-induced steatohepatitis. In primary hepatocytes, the targeting of 3BP2 or SYK strongly decreased the LPS-mediated inflammatory mediator expression and 3BP2-regulated SYK expression. In homozygous $Sh3bp2^{KI/KI}$ mice, the chronic inflammation mediated by the proteasome-resistant 3BP2 mutant promoted severe hepatitis and liver fibrosis with augmented liver SYK expression. In these mice, the deletion of SYK in myeloid cells was sufficient to prevent these liver lesions. The hepatic expression of SYK is also upregulated with metabolic steatohepatitis and correlates with liver macrophages in biopsy-proven MAFLD patients.

**Conclusion:** Collectively, these data suggest an important role for the SYK-3BP2 pathway in the pathogenesis of chronic liver inflammatory diseases and highlight its targeting in hepatocytes and myeloid cells as a potential strategy to treat metabolic steatohepatitis.

**Keywords:** SYK, 3BP2, TREM1, TLR4, MAFLD, NASH, fibrosis
INTRODUCTION

Metabolic associated fatty liver diseases (MAFLD)\(^1\), are the most common chronic liver diseases worldwide with a global prevalence of 25%\(^2\). The burden of MAFLD is increasing in parallel with the global obesity epidemic\(^3\) and still presents an unmet medical need with no approved pharmacological therapies. MAFLD is a broad term that encompasses the full spectrum of fatty liver diseases from simple hepatic steatosis to metabolic steatohepatitis, fibrosis/cirrhosis and hepatocellular cancer. Obesity and metabolic syndrome are also associated with the development of MAFLD. Similarly, type 2 diabetes mellitus, insulin resistance, hypertension and weight gain are risk factors for MAFLD progression\(^4\)\(^5\). However, the inverse relationship also exists, with MAFLD being a risk factor for many metabolic diseases, including type 2 diabetes\(^6\) and cardiovascular diseases\(^7\).

In addition to genetic and environmental factors, the interaction of gut and adipose tissue with the liver enhances liver metabolic disorders (steatosis and insulin resistance), chronic inflammation and injury-mediated fibrosis\(^8\)\(^9\). In MAFLD liver, a large number of innate immune cells are involved in the onset of the chronic inflammation, including resident and recruited macrophages and neutrophils, but also parenchymal hepatocytes\(^8\)\(^9\). Stressed hepatocytes, such as fatty and sub-lethal hepatocytes, take on an immune function by sensing excessive metabolite levels and pathogen- and damage-associated molecular patterns (PAMPs and DAMPs, respectively), which in turn elicit inflammatory events associated with metabolic dysfunction\(^10\). Macrophages also play a central role in the development and progression of MAFLD\(^11\)\(^12\). Liver-resident macrophages (Kupffer cells) can initiate inflammation and help to recruit blood-derived monocytes. The polarization of both resident and recruited macrophages into pro-inflammatory phenotypes subsequently promotes MAFLD progression. The gut-derived endotoxins, lipids and molecules associated with hepatocellular damage and death (DAMPs, extracellular vesicles, apoptotic bodies etc.) are the main factors contributing to macrophage and hepatic stellate cell (HSC) activation and the regulation of chronic hepatitis and fibrogenesis. In order to study pathways that are conserved in both hepatocytes and macrophages and involved in the steatosis-steatohepatitis transition, we focused on the role of the Spleen tyrosine kinase (SYK) pathway in the regulation of inflammatory mediators.

SYK is a cytoplasmic non-receptor tyrosine kinase, and SYK-dependent signaling is known to regulate critical processes in innate and adaptive immunity downstream Immunoreceptors (including B cell receptors, T cell receptors and Fc receptors), C-type lectins receptors, integrins and Toll-like receptors (TLRs)\(^13\)\(^16\). In the liver, SYK is highly expressed in HSC and inflammatory cells including macrophages\(^17\)\(^18\). SYK is also overexpressed in balloon hepatocytes containing Mallory Denk bodies\(^19\), a hallmark of chronic liver diseases such as
MAFLD and alcoholic liver diseases (ALDs). Recent studies have reported a pathogenic role for SYK in liver fibrosis as previously reported in certain fibrotic-related diseases including scleroderma, renal fibrosis and rheumatoid arthritis. SYK inhibitors blocked HSC activation in vitro and in animal models of hepatic fibrosis, including toxin challenge (carbon tetrachloride (CCl₄), thioacetamide (TAA), N-nitrosodiethylamine (DEN))- and bile duct ligation-induced liver fibrosis models. In addition, SYK inhibitors potently attenuated liver fibrosis and hepatocellular carcinoma (HCC) development in DEN and DEN/CCl₄ animal models. Interestingly, SYK deletion in the myeloid compartment conferred protection against fibrogenic progression upon TAA-mediated liver injury. The regulation of macrophage activity by SYK in the context of liver injury, and exacerbated by DAMPs, could provide an attractive mechanism in chronic liver diseases. This exacerbated activation of macrophages by TLR ligands (DAMPs, PAMPs and saturated lipids) was found to be a central pathogenic mechanism for chronic inflammation and MAFLD. In addition, TREM1, a member of the triggering receptors expressed on myeloid cells (TREM) family that mediates the inflammatory response through TLR4, DNAX-activation protein of 12 kDa (DAP12) and SYK, has been implicated in liver diseases. In line with the important role of the SYK pathway in inflammatory diseases, gain-of-function mutations in SYK, leading to increased phosphorylation and hyper activated downstream signaling, were recently associated with immune dysfunction and systemic inflammation. Among the components of the SYK pathway, we have previously shown that 3BP2 (also known as SH3 binding protein 2, SH3BP2), a cytoplasmic adapter that interacts with SYK, was necessary for TLR-mediated activation of macrophages in response to PAMPs and DAMPs. Indeed, 3BP2-deficient macrophages exhibited dramatically reduced inflammatory responses to microbial challenge and reduced phagocytosis. Remarkably, mutations in SH3BP2 that were found in the human genetic disease Cherubism have been associated in mice with systemic auto-inflammation, SYK hyper phosphorylation and elevated TLR signaling in myeloid cells carrying homozygous Sh3bp2 cherubic mutations. Furthermore, we found that a hemizygous Sh3bp2 cherubic mutation enhanced macrophage responsiveness to TLR ligands. Importantly, both deletion of SYK in myeloid cells and targeting the SYK/3BP2 axis ameliorated the chronic inflammation driven by the mutation of 3BP2 in mice.

Although SYK and its signaling partner 3BP2 are expressed in liver tissue, many details are still elusive, such as their relative local expression in response to liver status and their relative pathogenic roles in hepatocytes and macrophages throughout the development of liver steatosis and progression to steatohepatitis. We thus investigated these open questions in different mouse models of MAFLD, human MAFLD samples, and transgenic animals.
deficient in 3BP2 or SYK signaling or with sustained activation of 3BP2 signaling (cherubic
3BP2 mutants).

RESULTS

Hepatic expression of 3BP2 and SYK correlated with liver complications in mouse
models of MAFLD. We first evaluated the hepatic expression levels of Sh3bp2, Syk and
macrophage marker Emr1 (F4/80) in a dietary mouse model of obesity and hepatic steatosis.
After 33 weeks of high-fat diet (HFD), the wild-type C57BL/6 (Wt) mice developed obesity,
hepatic injury as evaluated by ALT activity, and severe hepatic steatosis (Figures 1A-C).
The hepatic expression levels of Syk and Emr1 were increased upon 33 weeks of HFD
(Figure 1D) and hepatic expression of Syk correlated with hepatic steatosis, ALT activity and
hepatic expression of Emr1 (Figure 1E). The hepatic expression levels of Sh3bp2 tended to
increase with liver complications (Figure 1D). To subsequently investigate the behavior of
the 3BP2 and SYK in response to the progression and the severity of the MAFLD, we
monitored hepatic expression in wild-type mice fed a diet deficient in methionine and choline
(MCDD) for 2 and 7 weeks. The duration of the MCDD challenge correlates with the severity
of the steatohepatitis associated with the aggravation of liver injury and fibrogenesis. At 2
weeks, mice developed moderate hepatic steatosis with mild hepatic inflammation (as
evaluated by the inflammatory foci number) and liver injury (Figures 2A-C). The long-lasting
MCDD challenge (7 weeks) aggravated the liver complications with severe steatosis,
inflammation and liver injury (Figures 2A-C). Hepatic Sh3bp2 and Syk expression levels
were progressively increased with the MAFLD severity (Figure 2D). Hepatic Sh3bp2 and Syk
also strongly correlated with hepatic steatosis, inflammatory foci number, liver injury and
hepatic expression of Emr1 (Figure 2E). Upon seven weeks of MCDD challenge, the
increased Syk expression was due to the upregulation of both isoforms of Syk (Syk-b (S) and
Syk (L) (the latter being the main isoform expressed in liver) (Figure 2F). The hepatic
expression levels of SYK were also increased upon MCDD at the protein level (Figure 2G).
We then evaluated which cell types in the liver were responsible for 3BP2 and SYK
expression. Sh3bp2 and Syk expression at the mRNA level was detected in hepatocytes and
non-parenchymal fraction from non-pathogenic liver in lean mice (Figure 2H). Sh3bp2 was
expressed at higher levels than Syk in hepatocytes. In addition, Sh3bp2 and Syk were highly
expressed in non-parenchymal cells compared to Syk expression in hepatocytes with higher
expression of Syk than Sh3bp2. Therefore, these results indicate that 3BP2 and SYK could
impact the functions of both hepatocytes and non-parenchymal cells. In addition, the
upregulation of 3BP2 and SYK with MAFLD severity suggests a potential role in the steatosis-steatohepatitis transition.

3BP2 deficiency partially prevented liver injury, hepatic steatosis and steatohepatitis induced by MCDD challenge. The role of 3BP2 in liver injury and steatohepatitis induced by MCDD was then investigated using mice deficient for 3BP2. The MCDD challenge was associated with elevated hepatic *Sh3bp2* expression in Wt mice (Figures 3A). The *Sh3bp2*-/- mice displayed less liver damage as shown by the lower ALT activity levels (Figure 3B). Furthermore, histological analyses demonstrated less hepatic steatosis and inflammatory foci in the livers of MCDD-fed *Sh3bp2*-/- mice compared with livers of MCDD-fed Wt mice (Figures 3C-D). The decreased inflammatory foci number was associated with a partial prevention of the recruitment of inflammatory macrophages (F4/80$^{+}$ CD11b$^{\text{high}}$ cells) and neutrophils (Ly6G$^{+}$ Ly6C$^{-}$ cells) into the liver as evaluated by flow cytometric analysis (Figures 3E-F). This was associated with less hepatic expression of *Emr1* (encoding for F4/80) and the adhesion molecule *Sele* (encoding for E-SELECTIN) involved in neutrophil recruitment into the liver (Figure 3G). In addition, MCDD-fed *Sh3bp2*-/- mice displayed less hepatic inflammation as evaluated by expression of inflammatory markers including *Cd44*, *Tnf*, *Ccl2* and *Syk* compared to MCDD-fed Wt mice (Figure 3G). This effect of the hepatic inflammation was also confirmed by the decreased hepatic expression of CD44 and SYK at the protein level (Figure 3H) and decreased serum level of CCL2 (Figure 3I). In addition, the hepatic expression of *Trem1*, an important amplifier of TLR4 pathway via SYK activity decreased in MCDD-fed *Sh3bp2*-/- mice compared to MCDD-fed Wt mice (Figure 3G). In line with this, the expression of *Dap12*, the transmembrane adaptor required for TREM1 function, also decreased (Figure 3G). Since hepatic injury and inflammation are key actors in the fibrogenic process, the 3BP2 deficiency also prevented the priming of hepatic fibrosis as evaluated by the expression of *Tgfb1*, *Timp1* and *Col1a1* (Figure 3G). The protective effect of 3BP2 deficiency on liver complications was also associated with decreased expression of oxidative stress markers *Cyba* (encoding for P22PHOX) and *heme oxygenase-1* (HMOX1) and increased *Ppar-alpha*, the master regulator of beta-oxidation (Figure 3G). Altogether, we show that, by regulating hepatic steatosis, inflammation and liver injury, 3BP2 plays an important role in the onset of MAFLD.

The SYK/3BP2 axis significantly contributed to the upregulation of inflammatory mediators in LPS-stimulated hepatocytes. Since hepatocytes are key actors in the early onset of local inflammation, we investigated the role of 3BP2 and SYK in the expression of inflammatory mediators in hepatocytes. Indeed, hepatocytes sense pathogen products such as LPS and contribute to the upregulation of liver cytokines and chemokines leading to the
recruitment of inflammatory cells into the liver. In the steady state, the silencing of Sh3bp2 strongly decreased the expression of Syk in primary mouse hepatocytes. On the other hand, the downregulation of Syk did not impact Sh3bp2 expression (Figure 4A). In response to LPS, the expression of Sh3bp2 and Syk was upregulated (LPS vs PBS: Sh3bp2=1.62±0.07, p=0.0001; Syk=1.54±0.13, p=0.0074, n=4) and the silencing of Sh3bp2 prevented LPS-stimulated Syk expression (data not shown). In addition, the downregulation of Sh3bp2 or Syk strongly decreased the upregulation of inflammatory mediators including Tnf, Ccl2, Iil1b and Iil6 in response to LPS (Figure 4B). In line with this, SYK inhibition by SYK inhibitor R406 (as evaluated by the phosphorylation levels of SYK and ERK) (Figure 4C) strongly prevented the upregulation of these inflammatory mediators in response to LPS (Figure 4E). Interestingly, R406 treatment also reduced the expression of Sh3bp2 and Syk in the steady state (Figure 4D). These data indicate that 3BP2 regulates SYK expression in challenged hepatocytes and that the SYK/3BP2 axis contributes to the expression of inflammatory mediators in response to PAMPs (LPS).

The knock-down of SYK in myeloid cells partially prevented liver injury and inflammation induced by MCDD challenge. We investigated the contribution of SYK to the development of the liver complications mediated by MCDD feeding. Since SYK deficiency leads to perinatal lethality in mice and because SYK was preferentially expressed in liver non-parenchymal cells (Figure 2H), we focused on the role of SYK in myeloid cells. The Syklox/lox LysMcre/cre mice (mSykKO mice) displayed a significant decrease in SYK expression in hepatic inflammatory monocytes (Ly6G Ly6C high cells) and neutrophils (Ly6G+ Ly6C- cells) without disruption of the SYK expression in lymphocytes as evaluated by flow cytometric analysis (Figure 5A). After 4 weeks of MCDD feeding, the mSykKO mice displayed lower hepatic expression of Syk than MCDD-fed Wt mice (Figures 5B and 5H at the gene and protein levels, respectively) without impacting the sh3bp2 expression level (Figure 5G). In addition, the MCDD-fed mSykKO mice exhibited less liver damage, as shown by the lower ALT levels (Figure 5C). Furthermore, histological analyses showed less accumulation of inflammatory foci in the livers of MCDD-fed mSykKO mice compared with livers of MCDD-fed Wt mice without changing in hepatic steatosis (Figures 5D-E). The down regulation of SYK in myeloid cells strongly prevented the recruitment of infiltrating neutrophils (Ly6G+ Ly6C- cells) and macrophages (F4/80+ CD11bhigh cells) into the liver (Figure 5F). Moreover, these were associated with reduced hepatic expression of inflammatory markers such as Cd44 (Figures 5G and 5H, at the gene and protein levels respectively) and Sele (E-SELECTIN) (Figure 5G). In line with the potential role of SYK in the regulation of macrophage activation/polarization, the hepatic expression of M1 markers, Ilgax (CD11C) and Tnf, decreased, while the expression of the M2 marker, Clec10a (CD301), did not change in...
MCDD-fed mSykKO mice compared to MCDD-fed Wt mice (Figure 5G). As reported for 3BP2 deficiency (Figure 3G), the knock down of SYK in myeloid cells strongly decreased the hepatic expression of Trem1 and Dap12, the priming of the fibrosis (Tgfb1 and Timp1) and oxidative stress (Cyba and Hmox1) (Figure 5G). This effect of the inflammation was also confirmed by the decreased serum level of CCL2 (Figure 5I). These results indicate that the conditional ablation of SYK in myeloid cells is sufficient to reduce liver inflammation, injury and, in turn, the priming of fibrosis in MCDD mice.

**SYK deletion in myeloid cells prevented severe hepatitis and fibrosis in homozygous Sh3bp2KI/KI mice.** To further demonstrate the key role of the SYK/3BP2 pathway in the development of chronic liver diseases, we re-visited this pathway in the livers of cherubic mice. Cherubism is an auto-inflammatory autosomal dominant bone dysplasia associated with point-mutations of the Sh3bp2 gene leading to the accumulation of proteasome-resistant mutant 3BP2. The accumulation of 3BP2 mutant proteins exacerbated activities of intracellular signaling components, including SYK in myeloid cells.31, 35, 42-44. We evaluated the liver status in homozygous cherubic animals (Sh3bp2 G418R knock-in mice (Sh3bp2KI/KI mice), equivalent to the G420R mutation in cherubism patients), which developed systemic inflammation without regression of the lesions with age.33. The 14-week-old Sh3bp2KI/KI mice already displayed cherubic manifestation (kyphosis) and facial inflammation (closed eyes) (Figures 6A-B) in addition to elevated liver weight relative to body weight (Figure 6C) and AST to ALT ratio (Figure 6D). We also found that the Sh3bp2KI/KI mice developed severe hepatitis, as evaluated by the accumulation of large inflammatory foci in the livers without hepatic steatosis (Figures 6E-F). The SYK deletion in myeloid cells in cherubic mice (mSykKOSh3bp2KI/KI mice) prevented all these clinical and biological abnormalities at the early and late age (mean age: 16 versus 43-week-old mice) (Figures 6A-D and data not shown). Interestingly, the SYK deletion in myeloid cells in Sh3bp2KI/KI mice decreased the hepatic expression of SYK at the mRNA and protein levels (Figures 6G-H). The SYK deletion in myeloid cells in Sh3bp2KI/KI mice also prevented the hepatic inflammatory foci accumulation even in old mice (Figures 6E-F). This was also associated with a lower hepatic expression of macrophage marker Emr1 and inflammatory markers including Cd44, Tnf, Ccl2, Trem1 and Dap12 (Figure 6G). This effect of inflammation was also confirmed by the decreased hepatic expression of CD44 at the protein level (Figure 6H) and decreased serum level of CCL2 (Figure 6I). In addition to the prevention of liver inflammation, SYK deficiency in myeloid cells was also associated with the prevention of hepatic fibrosis as evaluated by collagen staining (picrosirius red stain, Figure 6E) and lower gene expression of fibrosis markers like Tgfb1, Timp1 and Col1a1 (Figure 6G). Finally, the hepatic expression of Sh3bp2 and Syk correlated with the liver inflammation and fibrosis (Figure 6G). Altogether, we reported that
the sustained activation of 3BP2-SYK pathway mediated by 3BP2 G418R mutant is thus associated with the development of significant fibrotic-hepatitis.

**Hepatic expression of SYK increased with metabolic steatohepatitis in obese patients.** We examined the relationship between hepatic 3BP2 and SYK expression with MAFLD in human liver biopsies from morbidly obese patients who had undergone bariatric surgery. Patients were classified into 3 groups: without MAFLD, with hepatic steatosis and with metabolic steatohepatitis and severe steatosis (assessed on the basis of histopathological features; Table 1). Liver mRNA levels of *SH3BP2* did not change with obesity and MAFLD (data not shown). By contrast, liver mRNA levels of *SYK* were strongly upregulated with metabolic steatohepatitis (Figure 7A). This expression correlated with NAFLD activity score (NAS), ALT activity and hepatic expression of macrophage marker *CD68* (Figure 7B). Consistent with our animal studies, the hepatic expression of SYK could be a local marker of hepatic inflammation in MAFLD patients.

**DISCUSSION**

Here, we report that hepatic SYK and 3BP2 contribute to the onset of hepatic inflammation by acting on both parenchymal and myeloid cells. In concert with liver macrophages, the SYK/3BP2 axis in hepatocytes could initiate the local inflammation and contribute to the significant accumulation of bone-marrow-derived macrophages and neutrophils in the liver, a hallmark of metabolic steatohepatitis and severe hepatitis. Indeed, SYK/3BP2 regulates the LPS-TLR4 pathway in both hepatocytes and macrophages. The genetic deletion of 3BP2 or SYK in a context of metabolic steatohepatitis or deletion of SYK in myeloid cells in a context of a pro-inflammatory 3BP2 mutation significantly reduced liver inflammation, injury and fibrosis (priming and/or collagen deposition). We also report that hepatic SYK is a marker of liver inflammation and correlates with hepatic macrophage recruitment in mouse and human metabolic steatohepatitis. We also provide evidence that an important mechanism by which 3BP2 participates to metabolic steatohepatitis is the regulation of the expression of its signaling partner SYK, leading to exacerbated SYK-mediated inflammatory pathway in liver. Together, these data provide novel evidence that targeting the SYK/3BP2 axis is a potential therapeutic approach in chronic liver diseases.

We first show that the SYK/3BP2 pathway in liver parenchymal cells plays an important role in the expression of inflammatory mediators in response to LPS. Remarkably, 3BP2 targeting lowers SYK expression and decreases the expression of TNFα, CCL2, IL1β and IL6 in response to LPS (Figure 4). While SYK inhibition slightly decreases the expression of 3BP2, the targeting of SYK (silencing or inhibition) also significantly prevents the upregulation of these inflammatory mediators upon LPS stimulation (Figure 4). In addition to its detoxifying
and metabolic roles, hepatocytes also sense pathogens including bacteria products (for example, LPS) via their membrane and cytoplasmic pattern recognition receptors (PRRs)\textsuperscript{10}. Importantly, it has been reported that deficiency or down-regulation of TLR4 in hepatocytes resolved the hepatic inflammation mediated by HFD and associated with decreased insulin resistance, oxidative stress and hepatic steatosis\textsuperscript{45, 46}. These studies support the notion that TLR4 signaling in liver parenchymal cells plays a pivotal role during the early stage of MAFLD by initiating local inflammation. Our results emphasize the importance of the hepatocyte SYK/3BP2 signaling pathway to control the LPS/TLR4-mediated responses.

Among the inflammatory mediators, hepatocytes are a source of the chemokine CCL2, whose expression is modulated by the SYK/3BP2 pathway (\textbf{Figure 4}). The CCL2/CCR2 pair is a key player in the recruitment of inflammatory monocytes into the injured liver and drives hepatic fibrosis\textsuperscript{47, 48}. To illustrate this, it has been reported that pharmacological inhibition of CCL2 or CCR2 in murine models of MAFLD and chronic liver injury (chronic CCl4 treatment) reduced monocyte recruitment into the liver and ameliorated liver inflammation, injury and fibrosis\textsuperscript{49, 50}. Previous studies, including our own, have also reported that hepatic expression of CCL2 was upregulated in metabolic steatohepatitis patients\textsuperscript{9, 51}. Therapeutic treatments with a dual antagonist of chemokine receptors CCR2/CCR5 are currently under clinical investigation for patients with fibrotic metabolic steatohepatitis\textsuperscript{9}.

This indicates that regulation of the influx of bone marrow-derived monocytes into the liver is an important event in the onset of liver inflammation and MAFLD progression\textsuperscript{9, 49, 52}. In addition to therapeutic chemokine targeting, we show here that global deficiency of 3BP2 and SYK deletion in myeloid cells both prevented the recruitment of macrophages and neutrophils into the liver associated with reduced hepatic expression of CCL2 but also CD44 and E-selectin (\textbf{Figures 3 and 5}). CD44 and E-Selectin (CD62E) are also important drivers of leukocyte recruitment into the inflamed liver\textsuperscript{9}, and we have previously demonstrated that their hepatic expression was strongly upregulated in metabolic steatohepatitis patients\textsuperscript{51, 53}. From our experimental data, we also found that CD44, which interacts with extracellular matrix components including E-selectin, regulates the recruitment of macrophages and neutrophils into the liver. CD44 also regulates the macrophage activation mediated by TLR4 ligands including DAMPs, LPS and saturated fatty acids in a context of steatohepatitis\textsuperscript{53}. This pro-inflammatory polarization of resident and recruited liver myeloid cells can also be dependent on the SYK/3BP2 pathway. Indeed, we have recently reported that 3BP2 is necessary for LPS-induced activation of signaling pathways in macrophages\textsuperscript{27-29, 31}. In addition, it has been shown that SYK inhibition prevents the pro-inflammatory polarization of macrophages\textsuperscript{41}. An important additional amplifier of TLR4-mediated hepatic inflammation is the TREM1/DAP12 pathway\textsuperscript{23-25}. Interestingly, we here reported that the prevention of liver inflammation and injury mediated by the targeting of 3BP2 or SYK strongly decreased
TREM1/DAP12 expression. Given the central place of the SYK pathway in both TLR4 and TREM-1 signaling, this suggests the establishment of an inflammatory loop in which SYK and its partner 3BP2 exacerbate liver inflammation.

The chronicity of liver inflammation is also an important driver of the progression of liver complications such as liver injury and fibrosis. From our different mouse models, the down-regulation of 3BP2 or SYK in myeloid cells reduces both liver injury and fibrosis (fibrosis priming and/or collagen accumulation) along with a significant decrease in hepatic inflammation (Figures 3, 5 and 6). In line with these results, SYK deletion in the myeloid compartment confers protection against fibrogenic progression upon TAA-mediated liver injury. Of the mechanisms related to liver injury, TNFα and sustained oxidative stress are reported to mediate hepatocyte death in chronic liver and inflammatory diseases. Interestingly, 3BP2 and SYK mutant mice display a reduction in liver expression of TNFα and markers of oxidative stress (P22phox and Ho-1), coupled with reduced liver injury (Figures 3 and 5). In addition to promoting liver inflammation and associated injury, the SYK pathway can also directly regulate the activity of the HSC. Indeed, it has been shown that SYK inhibitors prevented HSC activation in vitro and in animal models of hepatic fibrosis. However, the protective effects of SYK targeting seem to be more related to an effect on myeloid cells than on HSC since its deficiency in HSC (LratCreSyk−/−) did not mitigate the severity of fibrosis mediated by TAA treatment.

Interestingly, we also show that SYK deletion in myeloid cells prevents the hepatic inflammation in cherubic mice expressing the 3BP2 G418R mutant (Sh3bp2 G418R knock-in mice, equivalent to the G420R mutation in cherubism patients). Indeed, SYK deletion in these mice mediates a drastic reduction in inflammatory foci number, macrophage recruitment and in hepatic expression of TNFα, CCL2 and CD44. These protective mechanisms were also associated with a restoration of hepatic 3BP2 and SYK expression to normal in Sh3bp2 G418R knock-in mice (Figure 6). Interestingly, both the protective effects of SYK deletion in the accumulation of inflammatory foci and the upregulation of hepatic TNFα expression have been recently reported in another mouse model of cherubic mice (Sh3bp2 P416R knock-in mice). We further show in our Sh3bp2 G418R knock-in mice that the selective knock down of SYK in myeloid cells is also sufficient to prevent the development of liver fibrosis with the quasi-absence of collagen deposition and changes to TGFβ, TIMP1 and COL1α1 expression (Figure 6). Since the SYK inhibitor treatment in cherubic mice reduces systemic and hepatic inflammation, the inhibition of SYK activity could also ameliorate liver fibrosis in mouse cherubism.

We also report that the 3BP2 deficiency reduces liver steatosis upon MCDD challenge (Figure 3). While additional studies to better understand the role of 3BP2 in hepatocyte metabolism and steatosis development are needed, the 3BP2 deficiency is associated with
the prevention of decreased PPARα upon MCDD challenge (Figure 3G). It is well established that PPARα plays a key role in the regulation of hepatocyte metabolism including fatty acid uptake, beta oxidation and triglyceride turnover. Mice with genetic deletion of PPARα fed MCDD developed more severe steatosis and necro-inflammation compared to wild-type mice. To the contrary, treatment with PPARα agonist, Wy-14643, can prevent and reverse MCDD-induced hepatic steatosis, inflammation and liver injury in a PPARα-dependent manner, likely via increased fatty acid oxidation. Interestingly, the treatment of primary hepatocytes with the SYK inhibitor, R406, also enhances PPARα and PGC1α expression (Ppara: x1.8±0.2, p=0.029; Pgc1a: x4.6±1.0, p=0.013, n=4). Altogether, this suggests that SYK and its signaling partner 3BP2 could also be important regulators of hepatocyte metabolism and liver steatosis development.

Finally, we find that the hepatic expression of SYK is upregulated with metabolic steatohepatitis and correlates with the presence of liver macrophage in obese patients (Figure 7). This is not dependent on hepatic fibrosis since our patients with liver biopsy-proven steatosis or metabolic steatohepatitis have only minimal scarring (F1: portal fibrosis without septa). Interestingly, different groups recently reported an association between the hepatic levels of SYK and liver fibrosis/cirrhosis in patients with HBV infection, HCV infection, alcohol abuse, cholestasis and metabolic steatohepatitis. Altogether, these results could indicate that liver SYK expression is a relevant new biomarker for metabolic steatohepatitis and its progression to cirrhosis.

In summary, the harmful functions of the SYK/3BP2 signaling pathway in chronic liver diseases occur at different stages of the disease (from steatosis to fibrosis) and in different liver cells (hepatocytes, macrophages, HSC, etc.). In the early stages, by enhancing LPS- and TLR4 responses in hepatocytes and resident macrophages, the SYK/3BP2 pathway could initiate the onset of local inflammation and the upregulation of factors involved in the recruitment of monocytes and neutrophils into the liver. The increase in pro-inflammatory polarization of recruited macrophages by SYK and 3BP2 could fuel a feed-forward mechanism whereby the amplified local inflammation subsequently contributes to liver injury and fibrosis. Inhibition of the SYK/3BP2 pathway in both hepatocytes and liver non-parenchymal cells (macrophages and HSCs) could represent a new therapeutic strategy to dampen inflammation, cell death and disease progression in chronic liver diseases.

**MATERIALS AND METHODS**

**Animals and study design.** Transgenic mice: Mice had a C57BL/6 background. Sh3bp2°/° (Sh3bp2K0K0) mice have previously been described. Sh3bp2°/° mice were generated by the ICS (Institut Clinique de la Souris, Illkirch, France, [http://www-mci.u-strasbg.fr](http://www-mci.u-strasbg.fr)) by knocking-in (KI) a GGA-to-CGA mutation in the Sh3bp2 exon 9, resulting in a Gly418-to-Arg substitution.
in 3BP2 protein, as previously described 31. LysM<sup>cre/cre</sup> (B6.129P2-Lyz2<sup>tm1(cre)lo/J</sup>) mice were provided by the Jackson Laboratory (Bar Harbor, ME, USA). The Syk conditional knockout mouse (Syk cKO) model was generated by the ICS (internal reference IR00002283 / K504) by floxing exon 2 (containing the ATG starting site) and exon 3 of the Syk locus. Myeloid Syk conditional mutant mice (mSyk<sup>KO</sup>) were obtained by crossing the Syk cKO strain with the LysM<sup>cre/cre</sup> transgenic mice. HFD: 7-week-old wild-type (Wt) mice (Janvier-Labs) were acclimated to our animal facilities under a 12/12h light/dark cycle at a temperature of 21 ± 2 °C and were fed ad libitum either a high-fat diet (HFD: 45% fat D12451; Research Diet) or control diet (Ctrl D) for 33 weeks. MCDD: Averaging 19-week-old male wild-type (Wt), Sh3bp2<sup>ko</sup> and Syk<sup>lox/lox</sup>LysM<sup>cre/cre</sup>(mSyk<sup>KO</sup>) C57BL/6 male mice were acclimated to our animal facilities under a 12/12h light/dark cycle at a temperature of 21 ± 2 °C and were fed ad libitum either a methionine- and choline-deficient diet (MCDD) or control diet (Ctrl D) for 2, 4 and 7 weeks, as indicated (diets from SSNIFF (MCDD # E15653-94; control diet # E15654-04). Characterization of Sh3bp2<sup>KI/KI</sup> mice: Wild-type (mean age=29 Wks, n=6, 16% of male), Sh3bp2<sup>ki/ki</sup> (mean age=14 Wks, n=6, 33% of male), young mSyk<sup>KO</sup> Sh3bp2<sup>ki/ki</sup> (mean age=16 Wks, n=15, 58% of male) and old mSyk<sup>KO</sup> Sh3bp2<sup>ki/ki</sup> (mean age=43 Wks, n=6, 50% of male) were analyzed. At the end of the corresponding challenge, blood was collected and mice were immediately sacrificed, after which the liver was removed. One part of the liver was immediately frozen in liquid nitrogen and stored at -80°C until analysis. The second part was fixed in buffered formalin, paraffin-embedded, sectioned, and stained with Hematoxylin-Eosin and picrosirius red, as indicated. The remaining part was used for a flow cytometry analysis. The guidelines of laboratory animal care were followed, and the local ethical committee approved the animal experiments (CIEPAL: Comité Institutionnel d'Ethique Pour l'Animal de Laboratoire, national agreement n° 28)(NCE/2013-108) (mouse models of MAFLD: APAFIS#5100-201512110477413 v6)(Sh3bp2 project : APAFIS#6999-2016110416248139 v3)(SYK project : APAFIS#16775-2018090516097783 v2)(Authorization of the C3M animal facility: B06-088-20).

Human samples: Morbidly obese patients (Nice) (gene expression: n=33) were recruited through the Department of Digestive Surgery and Liver Transplantation (Archet 2, University Hospital, Nice, France) where they underwent bariatric surgery for their morbid obesity. Bariatric surgery was indicated for these patients in accordance with French guidelines. Exclusion criteria were: presence of hepatitis B or hepatitis C infection, excessive alcohol consumption (>20g/d) or another cause of chronic liver disease as previously described 53, 61, 62. The characteristics of the study groups are described in Table 1. Before surgery, fasting blood samples were obtained and used to measure alanine and aspartate transaminases (ALT and AST, respectively), glucose, insulin and HbA1c. Insulin resistance was calculated
using the homeostatic model assessment index (HOMA-IR) \(^{63}\). Surgical liver biopsies were obtained during surgery and no ischemic preconditioning was performed. Hepatic histopathological analysis was performed according to the scoring system of Kleiner et al. \(^{64}\). Three histopathological features were semi-quantitatively evaluated: grade of steatosis (0, <5%; 1, 5%-30%; 2, >30%-60%; 3, >60%), lobular inflammation (0, no inflammatory foci; 1, <2 inflammatory foci per 200x field; 2, 2-4 inflammatory foci per 200x field; 3, >4 inflammatory foci per 200x field) and hepatocellular ballooning (0, none; 1, few balloon cells; 2, many cells/prominent ballooning). NAFLD activity score (NAS), which is the unweighted sum of scores of steatosis, lobular inflammation, and hepatocellular ballooning, was used to grade activity. Presence of metabolic steatohepatitis was defined as NAS \(\geq 5\). **Control subjects**: liver tissue was obtained from 5 lean subjects (5 women; age, 44±9 years; BMI, 21±1.9 kg/m\(^2\)) undergoing partial hepatectomy for benign tumors (neighbor tissues from four adenoma and one focal nodular hyperplasia; CHU Henri Mondor, Department of Pathology, AP-HP - Université Paris Est Créteil, Créteil, France). Three subjects underwent a left lobectomy or a bisegmentectomy without ischemic preconditioning and two patients underwent a right hepatectomy with a potential ischemic preconditioning (no data). Liver samples did not display any hepatic steatosis, inflammation or fibrosis. All subjects gave their informed written consent to participate in this study in accordance with French legislation regarding Ethics and Human Research (Huriet-Serusclat law). The “Comité Consultatif de Protection des Personnes dans la Recherche Biomédicale de Nice” approved the study (07/04:2003, No 03.017).

**Serum transaminases assay and CCL2 levels.** Serum transaminases activity (AST/ALT) was determined using an *in vitro* test with pyridoxal phosphate activation on Roche/Hitachi cobas c systems (ASTPM, ALTPM, cobas, Meylan, France). Roche/Hitachi cobas c systems automatically calculate the concentration of each sample. Serum levels of CCL2 were evaluated with an ELISA (# DY479, Bio-Techne), as described in the manufacturer’s instructions (dilution to the fifth).

**Flow cytometric analysis.** A fraction of the mouse liver was crushed on a 100 µm cell strainer and washed with RPMI 1640 medium. The cellular suspension was centrifuged at 630 g for 5 min at 4°C. The cell pellet was resuspended and centrifuged on a density cushion of Percoll (40% and 70%) at 780 g for 20 min at RT. The non-parenchymal cell (NPC) fraction between 40% and 70 % Percoll was collected, centrifuged at 630 g for 5 min at 4°C and resuspended in PBS containing 3% fetal bovine serum (FBS), 5 mM EDTA. Cell suspensions were incubated with purified anti-FcgRII/III mAb blocking antibody before being stained at 4°C for 10 min to block Fc receptors. Cells were then stained with fluorochrome-coupled
antibodies for 30 min at 4°C against CD45 (clone 30F11), F4/80 (clone BM8), CD11b (clone M1/70), Ly6G (clone 1A8) or Ly6C (clone AL21). All antibodies were purchased from BD Biosciences, eBioscience and Bio Rad ABD Serotec. For intracellular staining, cells were fixed and permeabilized using the the BD Cytofix/Cytoperm (Ref 554714) according to the manufacturer’s instructions. Samples were first stained for cell surface markers as described above and then for intracellular markers using anti-SYK antibodies (clone D3Z1E XP, PE conjugate, Cell Signaling Technology (CST)). Samples were acquired using a fluorescence activated cell sorting (FACS) Canto II flow cytometer, and the data were analyzed using the FACS Diva software version 8.0.1 (BD Biosciences) or FlowJo V10 software (Treestar).

**Hepatic parenchymal and non-parenchymal cell isolation:** Mouse hepatocytes were isolated with a two-step collagenase procedure. Briefly, mouse livers were perfused with HEPES buffer containing 8 g/l NaCl, 33 mg/l Na₂HPO₄, 200 mg/l KCl and 2.38 g/l HEPES, pH 7.5, supplemented with 0.5 mM EGTA for 3 min at 3 ml/min, then with HEPES buffer for 3 min at 3 ml/min and finally with HEPES buffer supplemented with 1.5 g/l CaCl₂ and 0.026% collagenase type IV (Sigma-Aldrich; C5138, Saint-Quentin-Fallavier, France) for 7 min at 3 ml/min. Livers were then carefully removed and minced in Williams’ E medium (Thermo Fisher Scientific Inc.) supplemented with 10% FBS (Thermo Fisher Scientific Inc.), 100 units/ml penicillin, 100 mg/ml streptomycin, 2 mM L-glutamine and 0.02 UI/ml insulin (Umulin, Lilly France, Neuilly-sur-Seine, France). The cell suspension was then filtered (100 µm) and hepatocytes were pelleted after two centrifugations at 50 g for 5 min. The supernatants were collected at each step, which contained the NPC. Pelleted hepatocytes were resuspended in 39% Percoll solution, centrifuged at 50 g for 5 min at RT and collected at the bottom of the tube. Viability was evaluated by trypan blue exclusion (Sigma-Aldrich).

**Primary hepatocyte treatments.** Down regulation of 3BP2 and SYK was achieved using Lipofectamine RNAiMAX technologies (MSS216132, Mouse Sh3bp2; HSS238073, mouse Syk; Life Technologies) according to the manufacturer’s instructions. Following a 48-hour transfection, cells were incubated with Williams’ E medium supplemented with 0.5% bovine serum albumin for 1 hour and then treated with LPS (50 µg/ml) for 6h. For the R406 inhibitor, cells were incubated with 1) Williams’ E medium supplemented with 0.5% bovine serum albumin overnight and then successively treated with R406 (10 µM) for 2 h and then LPS (50 µg/ml) for 5 min as indicated (Western Blot analysis); 2) Williams’ E medium supplemented with 0.5% bovine serum albumin without or with R406 (10 µM) for 1 h and then treated with LPS (50 µg/ml) for 6 h (gene expression analysis).
Real-time quantitative PCR analysis: Total liver RNA was extracted using the RNeasy Mini Kit (74104, Qiagen, Hilden, Germany) and treated with Turbo DNA-free DNase (AM 1907, Thermo Fisher Scientific Inc.) following the manufacturer’s protocol. The quantity and quality of the RNA samples were determined using the Agilent 2100 Bioanalyzer with RNA 6000 Nano Kit (5067-1511, Agilent Technologies, Santa Clara, CA, USA). Total RNA (1 µg) was reverse transcribed with the High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific Inc.). Real-time quantitative PCR was performed in duplicate for each sample using the StepOne Plus Real-Time PCR System (Thermo Fisher Scientific Inc.) as previously described

53, 65, 66. TaqMan gene expression assays were purchased from Thermo Fisher Scientific Inc.: RPLP0 (Hs99999902_m1)(Mm99999223_gH); B2m (Mm00437762_m1); Syk (Mm01333032_m1; Hs00895377_m1); Sh3bp2 (Mm00449397_m1; Hs00610068_m1); Syk (L) (Mm00441647_m1); Syk (S) (Mm01333035_m1); Emr1 (Mm00802529_m1); Cd44 (Mm01277163_m1); Tnf (Mm00443258_m1); Ccl2 (Mm00441242_m1); Sele (Mm01310197_m1); Itgax (Mm00498698_m1); Clec10a (Mm00546124_m1); Tgfβ1 (Mm03024053_m1); Timp1 (Mm00441818_m1); Col1a1 (Mm00801666_g1); Cyba (Mm00514478_m1); Hmox1 (Mm00516005_m1); CD68 (Hs00154355_m1), Iil6 (Mm00446190_m1), Il1b (Mm00434228_m1), Trem1 (Mm01278455_m1) and Dap12 (Mm00449152_m1). Gene expression was normalized to the β2m (β2 microglobulin, mouse) or RPLP0/36b4 housekeeping genes and calculated based on the comparative cycle threshold Ct method (2−ΔΔCt). Gene expression was normalized to the RPLP0/36b4 housekeeping gene (human and mouse) and calculated based on the comparative cycle threshold Ct method (2−ΔΔCt).

Immunoblotting: Cells or frozen tissues were solubilized in lysis buffer (20 mM Tris, pH 7.4, 150 mM NaCl, 10 mM EDTA, 150 mM NaF, 2 mM sodium orthovanadate, 10 mM pyrophosphate, protease inhibitor cocktail, 1% Triton X-100 and okadaic acid) for 45 min at 4°C. Lysates were cleared (14 000 rpm, 15 min). Proteins were quantified (BCA Protein assay kit, 23225 Thermo Fisher Scientific Inc.), separated by SDS-PAGE and immunoblotted as previously described

53. The proteins were probed with Anti-SYK (#2712, CST), Anti-CD44 (AF6127, Bio-Technne), Anti-phospho SYK (Tyr525/526) (#2710, CST), Anti-p44/42 MAPK (#4695, CST), Anti-phospho-p44/42 MAPK (Thr202/Tyr204)(#9101, CST) and anti-HSP60 (K-19) (sc-1722, Santa Cruz Biotechnology) antibodies at 1 µg/ml (CST) or at 1/1000 (Santa Cruz Biotechnology) as indicated.

Statistical analysis: Statistical significance between two human or mouse study groups was determined using the non-parametric Mann–Whitney test. Data from cell lines were analyzed
using the Student t-test. Spearman’s correlation test was used for the correlative analysis. Results were considered significant for p < 0.05.

ACKNOWLEDGMENTS
Our thanks to i) the INSERM U1065 animal facility staff and Dr V. Corcelle, ii) the INSERM U1065 flow cytometry platform, iii) Dr Jeanne Tran-Van-Nhieu (HU Henri Mondor, Department of Pathology, AP-HP - Université Paris Est Créteil, Créteil, France) for providing the liver tissues from lean subjects and iv) Pr Robert Rottapel (Princess Margaret Cancer Center, University of Toronto, Toronto, Canada) for providing the Sh3bp2 KO mice.
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tissue in severely obese patients independently from metabolic syndrome, Type 2 diabetes, and NASH. Am J Gastroenterol 2006;101(8):1824-33.


FIGURE LEGENDS

Figure 1: Hepatic expression of SYK correlated with hepatic steatosis, liver injury and macrophage marker expression in HFD-fed mice. Wild-type mice fed a control diet (Ctrl D) or HFD for 33 weeks (8-9 mice/group). Body weight (A), serum ALT activity (B) and hepatic steatosis (on H&E stained liver tissue section) (C) have been evaluated. (D) Hepatic expression of Sh3bp2, Syk and Emr1 was evaluated in mice as indicated by mRNA level. The gene expression values were normalized to the mRNA levels of 36b4. Results are expressed relative to the expression level in controls. (E) Correlation between hepatic expression of Syk with hepatic steatosis (%), ALT activity and hepatic Emr1 expression were analyzed using the Spearman’s correlation test. (A-D) Results are expressed as means ± SD and statistically analyzed using the Mann–Whitney test.

Figure 2: Hepatic expression of 3BP2 and SYK correlated with the severity of steatohepatitis in mice. Wild-type mice fed a control diet (Ctrl D) or MCDD for 2 and 7 weeks (4-8 mice/group). Serum ALT activity (A) and hepatic steatosis (B) and inflammatory foci accumulation (C) (on H&E stained liver tissue section) have been evaluated. (D) Hepatic expression of Sh3bp2, Syk and Emr1 was evaluated at the mRNA level. The gene expression was normalized to the mRNA levels of β2m. Results are expressed relative to the expression level in Ctrl D-fed Wt mice for 2 weeks. (E) Correlation between hepatic expression of Sh3bp2 and Syk with hepatic steatosis (%), ALT activity, inflammatory foci number and hepatic Emr1 expression were analyzed using the Spearman’s correlation test. (F) Hepatic expression of Syk-b (S) and Syk (L) was evaluated at the mRNA level in Wt mice fed Ctrl D or MCDD mice for 7 weeks. The gene expression was normalized to β2m mRNA.
levels and expressed relative to Syk-b (S) level in Ctrl D-fed Wt mice. (G) Hepatic expression of SYK and HSP60 was evaluated at the protein level in mice fed a control diet (Ctrl D) or MCDD for 7 weeks (2–4 mice/group). (H) Primary hepatocytes and non-parenchymal cells were isolated from lean mice (n=5). Sh3bp2 and Syk mRNA expression levels were analyzed. The gene expression values were normalized to β2m mRNA and expressed relative to Syk mRNA levels in hepatocytes. (A-D, F, H) Results are expressed as means ± SD and statistically analyzed using the Mann–Whitney test. **, p<0.01 versus control.

Figure 3: 3BP2 deficiency partially prevented liver injury and steatohepatitis induced by MCDD challenge. Wild-type (Wt) and Sh3bp2−/− mice fed a control diet (Ctrl D) or MCDD for 4 weeks (5-12 mice/group). (A) Hepatic Sh3bp2 expression was evaluated in Wt Ctrl D and MCDD mice at the mRNA level. (B) The serum levels of ALT were evaluated. (C-D) H&E staining of liver tissue sections samples from Wt and Sh3bp2−/− after MCDD as indicated. Representative images (C) and quantification of hepatic steatosis and inflammatory foci (D) are shown. (E-F) Hepatic non parenchymal cells were stained for CD45, F4/80, CD11b, Ly6G and Ly6C and analyzed by flow cytometry (5-11 mice/group). (E) Details regarding the gating strategy for the assessment of infiltrating macrophages (F4/80+ CD11bhigh cells) and neutrophils (Ly6G+ Ly6C− cells) into the liver as evaluated by flow cytometric analysis. (F) Results are expressed as means ± SD and statistically analyzed using the Mann–Whitney test. (G) Hepatic mRNA expression levels of Syk and markers of inflammation, fibrosis, oxidative stress and beta-oxidation were analyzed by real-time quantitative PCR (5-12 mice/group). Data are presented as relative mRNA levels normalized to B2m mRNA levels. Data are expressed as means in Heat Map and statistically analyzed using the Mann–Whitney test. *P<0.05, compared with Wt Ctrl D mice. §P<0.05, compared with Sh3bp2−/− Ctrl D mice, §P <0.05, compared with Wt MCDD mice. (H) Hepatic expression of CD44, SYK and HSP60 was evaluated at the protein level in Wt and Sh3bp2−/− mice fed a control diet (Ctrl D) or MCDD for 4 weeks (1-2 mice/group). (F) The serum levels of CCL2 were evaluated (2-10 mice/group). (A, B, D, F, I) Results are expressed as means ± SD and statistically analyzed using the Mann–Whitney test.

Figure 4: The 3BP2/SYK pathway contributed to the expression of inflammatory mediators in LPS-stimulated hepatocytes. (A, B) Primary mouse hepatocytes were transfected with Si Ctrl (n=4), Si Sh3bp2 (n=4) or Si Syk (n = 3) for 48 h, as indicated. Cells were then incubated with medium supplemented with 0.5% BSA for 1 h and treated with LPS (50 µg/ml) or PBS for 6 h. (C) Primary mouse hepatocytes were incubated overnight with medium supplemented with 0.5% BSA and then successively treated with R406 (10 µM) for 2 h and LPS (50 µg/ml) for 5 min (representative immunoblots are shown). (D-E) Primary
mouse hepatocytes were incubated with medium supplemented with 0.5% BSA without or with R406 (10 µM) for 1 h and then treated with LPS (50 µg/ml) or PBS for 6 h. (A, B, D, E) Sh3bp2, Syk, Tnf, Ccl2, Il1b and Il6 mRNA expression levels were analyzed by real-time quantitative PCR as indicated. The gene expression values were normalized to RpLp0 mRNA levels. Results are expressed as means ± SD. Data were statistically analyzed using the Student's t-test. *, p < 0.05 versus Si Ctrl or Ctrl.

Figure 5: SYK deletion in myeloid cells partially prevented liver injury and inflammation induced by MCDD challenge. Wild-type (Syk\textsuperscript{Wt}) and Syk\textsuperscript{flox/flox} LysM\textsuperscript{cre/cre} (mSyk\textsuperscript{KO}) mice fed a control diet (Ctrl D) or MCDD for 4 weeks (4-7 mice/group). (A) Evaluation of SYK expression in hepatic inflammatory monocytes (Ly6G\textsuperscript{−} Ly6C\textsuperscript{high} cells), neutrophils (Ly6G\textsuperscript{+} Ly6C\textsuperscript{−} cells) and lymphocytes (small cells) by flow cytometric analysis (4 mice/group). (B, G) Hepatic mRNA expression levels of Syk, Sh3bp2 and markers of inflammation, fibrosis and oxidative stress were analyzed by real-time quantitative PCR (4-7 mice/group). Data are presented as relative mRNA levels normalized to β2m mRNA levels. Data are expressed as means ± SD (B) or as means in Heat Map (G) and statistically analyzed using the Mann–Whitney test. *P<0.05, compared with Syk\textsuperscript{Wt} Ctrl D mice. §P<0.05, compared with mSyk\textsuperscript{KO} Ctrl D mice. (C) The serum levels of ALT were evaluated. (D) H&E staining of liver tissue sections from Syk\textsuperscript{Wt} and mSyk\textsuperscript{KO} after MCDD as indicated. Representative pictures and quantification of hepatic steatosis are shown. (E) Quantification of inflammatory foci. (F) Liver non-parenchymal cells were stained for CD45, F4/80, CD11b, Ly6G and Ly6C and analyzed by flow cytometry (4-7 mice/group). (H) Hepatic expression of CD44, SYK and HSP60 was evaluated at the protein level in Syk\textsuperscript{Wt} and mSyk\textsuperscript{KO} mice fed a control diet (Ctrl D) or MCDD for 4 weeks (1-3 mice/group). (I) The serum levels of CCL2 were evaluated (3-6 mice/group). (A-F, I) Results are expressed as means ± SD and statistically analyzed using the Mann–Whitney test.

Figure 6: SYK deletion in myeloid cells prevented severe hepatitis in homozygous Sh3bp2\textsuperscript{KI/KI} mice. Wild-type (mean age=29 Wks, n=6, 16% of male), Sh3bp2\textsuperscript{KI/KI} (mean age=14 Wks, n=6, 33% of male), young mSyk\textsuperscript{KO} Sh3bp2\textsuperscript{KI/KI} (mean age=16 Wks, n=15, 58% of male) and old mSyk\textsuperscript{KO} Sh3bp2\textsuperscript{KI/KI} (mean age=43 Wks, n=6, 50% of male) mice were characterized. (A) Age of the mice. (B) Facial appearance (white arrows) (open eyelids reflect lack of facial inflammation) and kyphosis (yellow arrows) of 14-week-old Sh3bp2\textsuperscript{KI/KI} mice and 41-week-old mSyk\textsuperscript{KO} Sh3bp2\textsuperscript{KI/KI} mice are shown. (C) Liver to body weight. (D) Serum AST/ALT ratio. (E) H&E and picrosirius red staining of liver samples from Sh3bp2\textsuperscript{KI/KI} and old mSyk\textsuperscript{KO} Sh3bp2\textsuperscript{KI/KI} mice as indicated. Representative pictures are shown. (F) Quantification of inflammatory foci. (G) Hepatic mRNA expression levels of Syk, Sh3bp2,
markers of inflammation (Emr1, Cd44, Tnf, Ccl2, Trem1, Dap12) and fibrosis (Tgfb1, Timp1 and Col1a1) were analyzed by real-time quantitative PCR (6 mice/group). Data are presented as relative mRNA levels normalized to β2m mRNA levels. Data are expressed as means in Heat Map and statistically analyzed using the Mann–Whitney test. *P<0.05, compared with Wt mice. †P<0.05, compared with Sh3bp22KO mice. (H) Hepatic expression of CD44, SYK and HSP60 was evaluated at the protein level in Wt, Sh3bp22KO and old mSykKO Sh3bp22KO. (F) The serum levels of CCL2 were evaluated (5-6 mice/group). (A, C, D, F, I) Results are expressed as means ± SD and statistically analyzed using the Mann–Whitney test.

Figure 7: Hepatic SYK expression increased with metabolic steatohepatitis in obese patients. (A) The liver SYK and CD68 mRNA expression levels were analyzed by real-time quantitative PCR in lean patients (n=5), in severely obese patients without MAFLD (n=6), with hepatic steatosis (n=16) and with severe hepatic steatosis and metabolic steatohepatitis (n=11). The gene expression values were normalized to RPLP0 mRNA levels. Results are expressed relative to the expression level in lean subjects (means ± SD) and statistically analyzed using the Mann–Whitney test. (B) Correlation between hepatic SYK expression (fold change) with NAFLD activity score (NAS), ALT activity and hepatic expression of CD68 (fold change) were analyzed using the Spearman’s correlation test.
**A**

- BW
  - Ctrl D
  - HFD

**B**

- ALT
  - Ctrl D
  - HFD

**C**

- Steatosis (%)
  - Ctrl D
  - HFD

**D**

- mRNA level (relative expression)
  - Sh3bp2
  - Syk
  - Emr1

**E**

- Syk (fold)
  - Steatosis (%)
  - ALT (U/L)
  - Emr1 (fold)
E

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Spearman's rank correlation test.

F

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G

IB:
- αSYK
- αHSP60

Total Lysates (7 weeks)

H

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<td>Non parenchymal fraction</td>
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** indicates statistical significance.
C

MCDD

Wt

Sh3bp2−/−

D

0.0002 0.0004

ALT

0.0002

0.0003 0.0175 0.0002

Stearosis

0.0003 0.0005 0.0002

Number/10 fields

0.0002 0.0175 0.0002

Inflammatory foci

F

0.0005 <0.0001

Gated on CD45+ cells

0.0005 <0.0001

Infiltrating macrophages (F4/80+ CD11bhigh)

0.0005 <0.0001

Neutrophils (Ly6G+Ly6C−)

H

IB:

CD MCDD CD MCDD

αCD44 100

αSYK 70

αHSP60 55

Total Lysates

I

Serum CCL2 (pg/ml)

0.0040

0.00085

0.0303

Markers of inflammation

Syk 1.01 7.65* 1.16 4.59

Emr1 1.02 7.46* 1.45 2.65

Cd44 1.01 8.66* 1.66 5.43

Tnf 1.02 15.70* 2.55 10.33

Cc2 1.08 22.34* 1.24 12.13

Sele 1.37 30.22* 1.29 16.58

Trem1 1.28 22.83* 1.13 14.65

Dap12 1.01 8.29* 0.90 5.84

Markers of fibrosis

Tgfb1 1.01 5.26* 1.36 3.74

Timp1 1.11 36.86* 1.84 21.76

Col1a1 1.11 20.15* 2.06 9.98

Markers of oxidative stress

Cyba 1.01 10.36* 1.21 5.52

Hmox1 1.05 7.35* 1.05 4.85

Markers of β-oxidation

Ppara 1.02 0.60* 1.06 0.85

Exclusion of duplicate

Gating on CD45+ leucocytes

Inflammatory monocytes

Monocytes

Neutrophils

Infiltrating macrophages
Spearman’s rank correlation test.
Table 1. Characteristics of the obese patients

<table>
<thead>
<tr>
<th></th>
<th>Without MAFLD</th>
<th>Steatosis</th>
<th>NASH</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>n</strong></td>
<td>6</td>
<td>16</td>
<td>11</td>
</tr>
<tr>
<td><strong>Age (years)</strong></td>
<td>37.8 ± 12.7</td>
<td>36.6 ± 9.2</td>
<td>42.5 ± 10.3</td>
</tr>
<tr>
<td><strong>BMI (kg/m²)</strong></td>
<td>42.9 ± 1.1</td>
<td>43.2 ± 4.4</td>
<td>44.6 ± 6.1</td>
</tr>
<tr>
<td><strong>ALT (IU/L)</strong></td>
<td>14.0 ± 3.6</td>
<td>31.8 ± 10.9*</td>
<td>94.6 ± 78.6**</td>
</tr>
<tr>
<td><strong>AST (IU/L)</strong></td>
<td>18.1 ± 4.3</td>
<td>23.4 ± 5.4*</td>
<td>59.1 ± 43.1**</td>
</tr>
<tr>
<td><strong>Fasting insulin (mIU/L)</strong></td>
<td>11.1 ± 7.4</td>
<td>15.7 ± 12.0</td>
<td>39.3 ± 28.5**</td>
</tr>
<tr>
<td><strong>Fasting glucose (mmol/L)</strong></td>
<td>4.8 ± 0.3</td>
<td>5.3 ± 0.5*</td>
<td>7.9 ± 4.5*</td>
</tr>
<tr>
<td><strong>HbA1c (%)</strong></td>
<td>2.3 ± 1.3</td>
<td>3.8 ± 3.0</td>
<td>13.3 ± 10.8**</td>
</tr>
<tr>
<td><strong>NAFLD Activity Score (n)</strong></td>
<td>0 (6)</td>
<td>1(1) 2 (5) 3 (10)</td>
<td>5 (11)</td>
</tr>
<tr>
<td>Grade of steatosis (n)</td>
<td>0 (6)</td>
<td>1 (1) 2 (6) 3 (9)</td>
<td>3 (11)</td>
</tr>
<tr>
<td>Lobular inflammation (n)</td>
<td>0 (6)</td>
<td>0 (15) 1 (1)</td>
<td>1 (11)</td>
</tr>
<tr>
<td>Hepatocellular ballooning (n)</td>
<td>0 (6)</td>
<td>0 (16)</td>
<td>1 (11)</td>
</tr>
</tbody>
</table>

Without MAFLD: patients with normal liver histology; Steatosis: patients with steatosis; NASH: patients with severe steatosis and NASH. Data are expressed as mean ± SD and compared using the non-parametric Mann Whitney test: * P<0.05 compared with “Without MAFLD” and # P<0.05 compared with “Steatosis”.
FIBROTIC HEPATITIS

pro-inflammatory and fibrogenic mediators

Leukocyte recruitment

Pro-inflammatory macrophages

Hepatocytes

3BP2/SYK

TLR

LPS

Monocytes

Neutrophils

LPS

3BP2/SYK

TLR/TREM1

LPS

CMGH

CELLULAR AND MOLECULAR GASTROENTEROLOGY AND HEPATOLOGY