p38γ and p38δ reprogram liver metabolism by modulating neutrophil infiltration

Bárbara González-Terán1,†, Nuria Matesanz2,†, Ivana Nikolic1,†, María Angeles Verdugo1,2, Vinatha Sreeramkumar3, Lourdes Hernández-Cosido3,4, Alfonso Mora1, Georgiana Crainiciuc1, María Laura Sáiz1, Edgar Bernardo1, Luis Leiva-Vega1, Elena Rodríguez1, Victor Bondía1, Jorge L Torres5,6, Sonia Perez-Sieira7,8, Luis Ortega3,4, Ana Cuenda2, Francisco Sanchez-Madrid1, Rubén Nogueiras7,8, Andrés Hidalgo1, Miguel Marcos5,6 & Guadalupe Sabio1,∗

Abstract

Non-alcoholic fatty liver disease (NAFLD) is a major health problem and the main cause of liver disease in Western countries. Although NAFLD is strongly associated with obesity and insulin resistance, its pathogenesis remains poorly understood. The disease begins with an excessive accumulation of triglycerides in the liver, which stimulates an inflammatory response. Alternative p38 mitogen-activated kinases (p38γ and p38δ) have been shown to contribute to inflammation in different diseases. Here we demonstrate that p38δ is elevated in livers of obese patients with NAFLD and that mice lacking p38γ/δ in myeloid cells are resistant to diet-induced fatty liver, hepatic triglyceride accumulation and glucose intolerance. This protective effect is due to defective migration of p38γ/δ-deficient neutrophils to the damaged liver. We further show that neutrophil infiltration in wild-type mice contributes to steatosis development by means of inflammation and liver metabolic changes. Therefore, p38γ and p38δ in myeloid cells provide a potential target for NAFLD therapy.

Keywords  diabetes; inflammation; obesity; steatosis; stress kinases

Subject Categories  Immunology; Metabolism; Molecular Biology of Disease

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Introduction

Non-alcoholic fatty liver disease (NAFLD) is the leading cause of chronic liver disease in Western countries and estimates of its worldwide prevalence range from 6 to 35% (Vernon et al, 2011). NAFLD refers to a wide spectrum of liver damage, ranging from simple steatosis caused by intracellular triglyceride accumulation to inflammation (non-alcoholic steatohepatitis [NASH]), fibrosis, and cirrhosis (Marchesini et al, 2003). NAFLD is a main cause of cryptogenic cirrhosis and may also predispose to hepatocarcinoma (Farrell & Larter, 2006).

The pathogenesis of NAFLD is strongly associated with insulin resistance, obesity and type 2 diabetes (Fabbriini et al, 2010). However, the mechanisms involved in the accumulation of triglycerides in the liver and subsequent hepatocellular damage are multifactorial and not completely understood. Metabolic deregulation and hepatic steatosis have been linked to stress signaling (Sabio & Davis, 2010; Sabio et al, 2010), and the activation of stress kinases in steatosis and obesity suggests a role for these proteins in this disease (Sabio & Davis, 2010). The stress-activated protein kinase group consists of two subfamilies: p38 mitogen-activated kinases (p38 MAPKs) and c-Jun N-terminal kinases (JNKs). While the role of JNKs in the development of steatosis has been widely studied (Sabio et al, 2008, 2009), less is known about the role of the p38 MAPK signaling pathway. In mammals, four p38 MAPK isoforms have been identified: p38α, β, γ, and δ. Despite biochemical evidence of specific roles for the individual isoforms, redundancy and embryonic lethality have impeded attempts to establish their distinct functions in vivo (Sabio & Davis, 2014). Embryos lacking p38α die due to defects in placental development (Adams et al, 2000; Allen et al, 2000; Tamura et al, 2000), but mice lacking p38β, γ, and δ are viable without any obvious defects under basal conditions (Beardmore et al, 2005; Sabio et al, 2005). Kinases p38γ and δ were recently shown to control inflammation by regulating...
macrophage production of tumor necrosis factor (TNF)-α (Risco et al., 2012; Gonzalez-Teran et al., 2013) and T-cell activation (Criado et al., 2014); moreover, p38δ also influences neutrophil inflammatory responses in the lung (Ittner et al., 2012).

Since chronic inflammation is central to the progression of NAFLD, we aimed to define the role of p38γ and p38δ in the development of this disorder. We detected elevated liver expression of p38δ in a cohort of obese patients with NAFLD and found that p38γ and p38δ are responsible for the development of steatosis and NASH in three animal models of NAFLD: mice fed a high-fat diet (HFD), mice fed a high-fat fructose diet (HFF), and mice fed a methionine–choline-deficient (MCD) diet. Lack of p38γ and p38δ in myeloid cells impaired neutrophil migration to the liver and thus protected against steatosis and further hepatic damage. These results highlight the importance of p38 kinases and neutrophils in NAFLD and open a new avenue for the treatment of this disease.

Results

p38γ and p38δ are overexpressed in NAFLD

Analysis of liver biopsies from obese NAFLD patients (body mass index [BMI] > 35 kg/m²) revealed elevated mRNA expression of MAPK13 (p38delta) compared with non-obese individuals without NAFLD, and a similar tendency was detected for MAPK12 (p38gamma) (Fig 1A). Further, among individuals with a BMI < 35 kg/m², hepatic MAPK12 and MAPK13 mRNA was elevated in individuals with liver steatosis compared with control individuals without liver disease (Fig 1B). Western blot analysis confirmed higher liver expression of p38δ protein in obese individuals with steatosis (Fig 1C). To corroborate these results in a mouse model of steatosis, we studied the expression and activation of p38γ and p38δ in livers from mice fed a methionine–choline-deficient (MCD) diet, which induces macrovesicular steatosis and is widely used in NASH research (Anstee & Goldin, 2006). MCD diet increased the mRNA expression of p38δ (Fig 1D) and induced the activation of p38γ and p38δ after 1 week (Fig 1E). This activation remained high during the 3 weeks of the diet (Fig 1E and F). These results indicate a possible role of p38γ and p38δ in the development of steatosis.

Mice lacking p38γ and p38δ are protected against MCD-induced steatosis

To study how these kinases affect the development of fatty liver, we fed a MCD diet to WT mice and mice lacking p38γ (p38γ−/−), p38δ (p38δ−/−), and both p38γ and p38δ (p38γδ−/−). Compared with MCD-diet WT mice, MCD-diet p38γ−/− and p38δ−/− mice showed only a slightly milder liver steatosis as evaluated by H&E and Oil Red staining (Appendix Fig S1A); in contrast, the development of steatosis and inflammation was strongly attenuated in p38γδ−/− mice (Fig 2A and Appendix Fig S2A). These findings were confirmed by biochemical analysis of hepatic triglyceride content (Fig 2B and Appendix Fig S1B). Moreover, whereas MCD diet increased serum levels of alanine transaminase (ALT) in WT, p38γ−/−, and p38δ−/− mice, the level in p38γδ−/− mice was significantly lower, indicating milder liver necrosis (Fig 2C and Appendix Fig S1C). The appearance of steatosis protection only in mice doubly deficient for p38γ and p38δ probably reflects the previously described partial functional redundancy between the two isoforms (Risco et al., 2012; Gonzalez-Teran et al., 2013).

An early event in MCD-induced choline deficiency is the appearance in liver of oxidized lipids, DNA, and proteins. Assay of thiobarbituric acid reactive substances in the livers of MCD-fed animals detected lower oxidized lipid content in p38γδ−/− mice than in WT animals, correlating with lower levels of hydrogen peroxide in the double-knockout mice (Fig 2D). Liver fibrosis is a hallmark of NASH. Livers of MCD-diet WT mice expressed higher levels of Col1a1 and Acta2 than MCD-diet p38γδ−/− mice (Fig 2E), correlating with higher Masson’s trichrome staining (Fig 2F). These results demonstrate that p38γδ−/− mice are protected against MCD-diet-induced steatosis and NASH.

Inflammation plays a key role in the pathogenesis of NAFLD, and the development of hepatic steatosis is associated with increased liver infiltration by myeloid cells (Tiniakos et al., 2010). p38δ/δ kinases regulate inflammation through the control of TNF-α production in macrophages and Kupffer cells (Risco et al., 2012; Gonzalez-Teran et al., 2013), and p38δ modulates neutrophil motility in lung disease (Ittner et al., 2012), prompting us to examine the mRNA expression levels of myeloid cell markers and pro-inflammatory cytokines in mice fed the MCD diet. Liver expression...
**Figure 1.**

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Figure 2. p38γδ−/− mice are protected against steatohepatitis and fibrosis.

A. Representative H&E- and Oil Red-stained liver sections prepared from WT and p38γδ−/− mice fed a ND or the MCD diet for 3 weeks. Scale bar: 50 μm.

B, C. Liver triglycerides (B) and plasma transaminase activity (ALT) (C) measured in WT and p38γδ−/− mice after 3 weeks of MCD diet.

D. TBARS and hydrogen peroxide detected in liver samples from mice fasted overnight after the 3-week MCD diet.

E. qRT–PCR analysis of Col1a1, Acta2, and Timp1 mRNA expression. mRNA expression was normalized to the amount of Gapdh mRNA.

F. Representative Masson’s trichrome-stained liver sections prepared from WT and p38γδ−/− mice fed a ND or the MCD diet for 3 weeks. Scale bar: 50 μm.

Data information: Data are means ± SEM (n = 5–10). *P < 0.05; **P < 0.01; ***P < 0.001 (one-way ANOVA coupled to Bonferroni’s post-tests).
levels of the myeloid cell marker F4/80 and the cytokines Tnfa and Il6 were significantly lower in p38γδ−/− mice than in WT mice (Appendix Fig S2B). However, analysis of M1 and M2 macrophage-differentiation markers revealed no differences in M1 (Il10, Il23) and M2 markers (Il10, Il13 or Arg) between WT and p38γδ−/− mice (Appendix Fig S2C).

**Effect of myeloid cell expression of p38γ and p38δ on MCD-induced steatosis**

To elucidate the role of myeloid-expressed p38γδ in the development of steatosis, we analyzed mice lacking p38γδ in myeloid cells. These mice have complete deletion of p38γ and p38δ in macrophages, and neutrophils infiltrated in liver and spleen while only partial deletion of p38δ was observed in dendritic cells (Appendix Fig S3A). Control mice expressing Cre recombinase (Lyzs-Cre mice) developed the typical hepatic steatosis in response to the MCD diet, with associated liver accumulation of triglycerides and hepatocyte necrosis indexed by serum ALT (Fig 3A–C). In contrast, the response of p38γδLyzs-KO mice to the MCD diet was milder for all three parameters (Fig 3A–C), demonstrating a protection similar to that seen in global p38δ-deficient animals (Lyzs-KO mice) developed the typical hepatic steatosis in response to the MCD diet (Appendix Fig S2B). However, analysis of M1 and M2 macrophage-differentiation markers revealed no differences in M1 (Il10, Il23) and M2 markers (Il10, Il13 or Arg) between WT and p38γδ−/− mice (Appendix Fig S2C).

To confirm that the protection against liver steatosis in MCD-diet induced obesity, but protect against steatosis and diabetes Myeloid-specific p38γδ deficiencies do not affect diet-induced obesity, but protect against steatosis and diabetes

The protection of p38γδ myeloid KO mice against steatosis and liver inflammation, together with the low levels of myeloid cell markers in the livers of these animals suggested a possible effect on liver infiltration in animals fed a MCD diet or HFD. Characterization of liver-infiltrating leukocyte subsets in mice fed either diet revealed that the diet-induced increase in liver-infiltrating neutrophil counts (CD11b+Gr-1high) was significantly bigger in Lyzs-Cre mice than in p38γδ−/− mice (Fig 7A), and similar results were observed in radiation chimeras restored by bone marrow from Lyzs-Cre mice versus p38γδ−/− mice (Appendix Figs S4C and S5). This result correlated with lower levels of circulating neutrophils in p38γδ−/− mice after both diets (Fig 7B and C).

Figure 3. p38γδ−/− mice are protected against steatohepatitis induced by MCD diet.

Lyzs-Cre and p38γδ−/− mice were fed a ND or a MCD diet for 3 weeks.
A. Representative H&E- and Oil Red-stained liver sections. Scale bar: 50 µm.
B. Liver triglycerides (B) and plasma ALT (C) at the end of the diet period.
D. Measurement of plasma TNF-α and IL-6.
E. qRT–PCR analysis of myeloid cell markers and cytokine mRNA expression from liver tissue; mRNA expression was normalized to the amount of Gapdh mRNA.
F. qRT–PCR analysis of M1 and M2 polarization cell markers from liver-infiltrated macrophages. mRNA expression was normalized to the amount of Gapdh mRNA.

Data information: Data are means ± SEM (n = 5-10). *P < 0.05, **P < 0.01, ***P < 0.001 (one-way ANOVA coupled to Bonferroni’s post-tests).
Figure 3.

A. Lyz-Cre  

ND  

MCD  

p38γ/δ Lyz-KO  

ND  

MCD  

B. Triglycerides  

Triglycerides (mg/g tissue)  

***  

**  

Lyz-Cre  

p38γ/δ Lyz-KO  

ND  

MCD  

C. ALT  

ALT (IU/L)  

***  

**  

Lyz-Cre  

p38γ/δ Lyz-KO  

ND  

MCD  

D. TNFα  

TNFα (pg/ml)  

***  

***  

Lyz-Cre  

p38γ/δ Lyz-KO  

ND  

MCD  

E. IL-6  

IL-6 (pg/ml)  

*  

Lyz-Cre  

p38γ/δ Lyz-KO  

ND  

MCD  

F. Liver-sorted macrophages  

Relative amount  

F4/80  

iNOS  

Csf1r  

Ym1  

III10  

II13  

Lyz-Cre  

p38γ/δ Lyz-KO  

ND  

MCD  

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Figure 4. p38γ and p38δ deficiency in myeloid cells improves glucose metabolism in an obesity model.
Lyzs-Cre and p38γ/δLyzs-KO mice were fed a ND or a high-fat diet (HFD) for 10 weeks.

A Body weight measured at the indicated times during HFD treatment.
B Fat mass and lean mass determined by MRI at the end of the diet period.
C, D Liver mass and white fat (WF) mass.
E Respiratory exchange quotient, energy expenditure, and locomotor activity, detected in metabolic cages.

Data information: Data are means ± SEM (n = 5–10). *P < 0.05; **P < 0.01; ***P < 0.001 (one-way ANOVA coupled to Bonferroni’s post-tests).
Neutrophils are the first immune cell type to respond to inflammation and can induce a chronic inflammatory state by promoting macrophage recruitment and interacting with antigen-presenting cells (Mantovani et al., 2011; Talukdar et al., 2012). Neutrophil levels by defensin 1–3 and neutrophils activation by nitrotyrosine staining, a measure of NO production, were elevated in the livers of obese...
Figure 6. The EMBO Journal Vol 35 | No 5 | 2016

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patients with NAFLD (Appendix Fig S6A and B). To test the possible role of neutrophil p38γ/δ expression in the etiology of inflammation-induced liver steatosis, we first compared the capacity of WT and p38γ/δ-neutrophils to migrate to steatotic liver. For this, we performed a competitive cell migration assay that allows direct and simultaneous comparison of the migration of multiple cell subsets in the same mouse. MCD-diet WT mice were i.v. injected with a 1:1 mix of p38γ/δ-neutrophils (6 × 10⁶ cells in total). WT neutrophils arrived at the steatotic liver 1 h after injection, but recruitment of p38γ/δ-neutrophils was markedly curtailed (Fig 7D and E). The more extensive recruitment of WT neutrophils appears to be not due to better survival since neutrophils from both genotypes showed the same survival ratio; however, we cannot rule out some role of p38γ/δ in neutrophil survival (Appendix Fig S7A).

Using intravital microscopy (IVM), we further quantified TNF-α-stimulated neutrophil migration in the microcirculation of the cremaster muscle of mice reconstituted with bone marrow (BM) from Lyzs-Cre or p38γ/δLyzs-KO mice (Movies EV1 and EV2). Numbers of rolling neutrophils were slightly higher in mice receiving p38γ/δLyzs-KO BM, accompanied by higher rolling velocity and contrasting with lower numbers of adherent neutrophils (Fig 7F). This higher rolling velocity is consistent with increased expression of L-selectin observed in neutrophils lacking p38γ/δ. Moreover, the defective adhesion might be explained by the lower expression in p38γ/δLyzs-KO neutrophils of CD11b, an integrin that regulates neutrophil adhesion and migration (Appendix Fig S7B). These results show that neutrophil adhesion and recruitment are compromised in the absence of p38γ/δ. Neutrophil rolling under flow conditions is mediated by L-selectin (Abbasi et al, 1993). To test the involvement L-selectin in the impaired rolling of p38γ/δLyzs-KO neutrophils, we assayed neutrophil migration under flow conditions on plates coated with the CD11b ligand ICAM-1 and the L-selectin ligand E-selectin. Our results indicated that p38γ/δLyzs-KO neutrophils presented a higher rolling velocity than Lyzs-Cre neutrophils (Appendix Fig S7C).

To investigate whether the altered migration capacity of p38γ/δLyzs-KO neutrophils is due to an autonomous effect or a defective production of chemokines, we performed a parabiosis experiment. Efficiency of parabiosis was evaluated by using congenic markers to distinguish blood cells in parabiotic pairs, in which one partner was CD45.1⁺. Parabiotic exposure of p38γ/δLyzs-KO mice to the circulation of WT (CD45.1) mice, both fed the MCD diet, was enough to worsen the steatosis phenotype of p38γ/δLyzs-KO (Appendix Fig S8A). The exacerbated steatosis correlated with a higher proportion of CD45.1 (WT) neutrophils in p38γ/δLyzs-KO livers compared to the proportion observed in livers from Lyzs-Cre mice (Appendix Fig S8B). There were no differences in macrophage infiltration (Appendix Fig S8B), indicating that the wild-type circulation specifically increases liver neutrophil infiltration in p38γ/δLyzs-KO mice. Neutrophils thus appear to be crucial to the steatosis protection in p38γ/δLyzs-KO mice.

Neutrophil-specific p38δ deficiency protects against steatosis

The most abundant p38 isoform in neutrophils is p38δ (Ittner et al, 2012). To test the implication of neutrophil p38δ in liver steatosis, we crossed p38δ-floxed mice with Mrp8-Cre mice (Passegue et al, 2004) to generate mice lacking p38δ specifically in neutrophils (p38δMrp8-KO mice; Appendix Fig S9A). H&E and Oil Red staining of liver sections revealed that these mice were partially protected against MCD-induced steatosis (Appendix Fig S9B). Moreover, p38δMrp8-KO mice had below-normal levels of MCD-induced ALT (Appendix Fig S9C). This protection was associated with low
Figure 7.

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Figure 8. Neutrophil depletion protects against steatosis.
Osmotic minipumps containing saline or Ly6G antibody were implanted subcutaneously in Lyzs-Cre and p38γδLyzs-KO mice. These animals were fed a ND or MCD for 3 weeks.

A Neutrophils and monocytes as a percentage of circulating leukocytes, measured in total blood.

B Representative H&E- and Oil Red-stained liver sections after 3 weeks of treatment. Scale bar: 50 μm.

C, D Liver triglyceride (C) and plasma transaminase activity (ALT) (D) at the end of the diet period.

E Total RNA was extracted from livers, and chemokine and cytokine mRNA levels were determined by qRT-PCR. mRNA expression was normalized to the amount of Gapdh mRNA.

Data information: Data are means ± SEM (n = 5–10). *P < 0.05; **P < 0.01; ***P < 0.001 (one-way ANOVA coupled to Bonferroni’s post-tests).
Figure 9.

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neutrophil infiltration of the liver compared with MCD-diet-fed Mrp8-Cre mice (Appendix Fig S9D). These results indicate that the protection against steatosis is at least partially due to the expression of p38δ in neutrophils.

**Neutrophil depletion protects against steatosis development**

Early neutrophil accumulation triggers monocyte migration and inflammation (Savill et al., 1989), and diminished neutrophil accumulation can ameliorate NAFLD (Nathan, 2006). To clarify whether defective neutrophil migration contributes to the milder hepatic mulation can ameliorate NAFLD (Nathan, 2006). To clarify whether defective neutrophil migration contributes to the milder hepatic

Our analysis shows that loss of p38δ in neutrophils deficient mice was identified by comparing gene expressions by hematopoietic cells drive steatosis in both diet-induced steatosis models. Neutrophils were recently shown to be important mediators of alcoholic fatty liver disease (Bertola et al., 2013), and p38δ is known to control neutrophil inflammatory response in lung by regulating PKD1 activity (Ittner et al., 2012). Our analysis shows that loss of p38γ and p38δ in neutrophils might be responsible for the protection observed in the KO animals. Livers from mice lacking p38γ and p38δ had lower neutrophil infiltration, and neutrophils lacking these kinases could not be recruited to the liver in a competition assay, indicating a cell autonomous effect. The low adhesion and higher rolling velocity detected in neutrophils lacking p38γ and p38δ are broadly consistent with previous reports using p38δ−/− animals (Ittner et al., 2012). We also observed lower

**Discussion**

The alternative p38 MAPKs p38γ and p38δ regulate inflammatory processes through several mechanisms. Here, we demonstrate that expression of both kinases in myeloid cells is necessary for the development of liver steatosis and inflammation in animal models of NAFLD. Further, these kinases control neutrophil migration to the liver, and hepatic neutrophils contribute to liver steatosis by promoting liver inflammation and lipogenic metabolism. Deletion of p38γ/δ expression in the myeloid compartment curtails neutrophil recruitment to the liver, protecting animals against diet-induced steatosis and associated liver damage, and effect that is also partially mediated by the lack of p38δ in neutrophils. These findings indicate a major role of p38γ/δ in controlling neutrophil recruitment during inflammation and suggest that inhibition of neutrophil trafficking is a potential treatment route for steatosis.
The same level of protection against diet-induced steatosis observed by deletion in neutrophils (Gonzalez-Teran et al., 2013; Han et al., 2013). A possible explanation is that p38\(\gamma\)/δ neutrophils might increase the activation or expression of p38\(\gamma\) as a compensatory mechanism. It is also possible that p38\(\gamma\) in another myeloid type (e.g. resident macrophages) contributes to neutrophil migration by controlling cytokine and chemokine production. However, the results of competition migration assays in which resident macrophages are WT, and of the parabiosis experiment, argue against this possibility. Further experiments will be needed to determine the specific roles of p38\(\gamma\) and p38\(\epsilon\) in different myeloid subsets and how these two isoforms can compensate each other. However, a role of neutrophils is clear because lack of p38\(\gamma\) only in neutrophils is enough to protect against steatosis.

On the other hand, the fact that neutrophil-specific deletion of p38\(\gamma\) has a more marked effect on phenotype than the whole-body p38\(\gamma\) KO might indicate that p38\(\gamma\) has an opposing role in another tissue and thereby modulates biological actions in a tissue-specific fashion. Opposing effects in different tissues have been shown for the stress kinase JNK: deletion of this kinase in the liver induces steatosis whereas deletion in fat is protective (Sabio et al., 2008, 2009).

Our data point out an important role of p38\(\gamma\)/δ in neutrophils. The same level of protection against diet-induced steatosis observed in p38\(\gamma\)/δ\(^{–/–}\) mice was also achieved in mice depleted of neutrophils with anti-Ly6G antibody. Moreover, deletion of p38\(\gamma\) in neutrophils reduces hepatic neutrophils infiltration and partially protected against steatosis. These observations strongly suggest that neutrophil recruitment to the liver is essential for the initiation and progression of NAFLD and that neutrophil p38\(\gamma\)/δ expression contributes to the progression of this disease. The central role for neutrophils in NAFLD is consistent with their roles in ethanol-induced liver damage (Bertola et al., 2013) and macrophage recruitment to damaged tissue in obesity (Mansuy-Aubert et al., 2013) and with the description of neutrophil elastase as an important mediator of obesity-induced diabetes (Taluksdar et al., 2012; Mansuy-Aubert et al., 2013). The importance of infiltrating neutrophils in liver inflammatory responses is also indicated by the underexpression of inflammatory genes in MCD-diet-fed p38\(\gamma\)/δ\(^{–/–}\) and neutrophildpleted mice and by the increased lipid oxidation and reduced lipogenesis in the neutrophil-depleted mice.

The data from mouse models correlate well with the overexpression p38\(\gamma\) and p38\(\epsilon\) in livers of individuals with NAFLD, regardless of BMI, which could indicate the involvement of these kinases in the development of steatosis. This elevated p38\(\gamma\) expression could be due to increased neutrophil influx to these livers, as neutrophils are known to express high levels of p38\(\gamma\) (Ittner et al., 2012; Gonzalez-Teran et al., 2013). Accordingly, we also observed elevated neutrophil activity in the livers of obese patients with NAFLD. It would be interesting to characterize the cell-type contribution to p38\(\epsilon\) and p38\(\gamma\) expression in human liver. However, we cannot rule out important roles for p38\(\gamma\) and p38\(\epsilon\) expressed in other cell types involved in steatosis development.

In summary, our findings indicate that neutrophil infiltration triggers the development of NAFLD and that p38\(\gamma\)/δ regulate this process by controlling neutrophil infiltration. Therefore, inhibition of p38\(\gamma\)/δ might represent a novel therapeutic target for NAFLD in humans, with the potential to limit injury and possibly prevent progression to NASH and cirrhosis.

Materials and Methods

Study population and sample collection

The study population included a group of obese adult patients with body mass index (BMI) ≥ 35 kg/m² and a liver biopsy compatible with NAFLD. Participants were recruited from patients undergoing elective bariatric surgery at the University Hospital of Salamanca. As controls, we included individuals with BMI < 35 kg/m² who underwent laparoscopic cholecystectomy for gallstones. These individuals were divided into two groups according to the presence of NAFLD: (i) controls without NAFLD (n = 11) if they had no laboratory- or histopathological evidence of NAFLD or other liver diseases; (ii) controls with NAFLD (n = 9) if they had a liver biopsy compatible with NAFLD. Therefore, three groups of subjects were included in the study: obese patients (BMI ≥ 35 kg/m²) with NAFLD, controls with BMI < 35 without liver disease, and controls with BMI < 35 kg/m² and with NAFLD. Baseline characteristics of these groups are listed in Appendix Table S1.

Participants were excluded if they had a history of alcohol use disorders or excessive alcohol consumption (> 30 g/day in men and > 20 g/day in women), chronic hepatitis C or B, or if laboratory and/or histopathological data showed causes of liver disease other than NAFLD. The study was approved by the Ethics Committee of the University Hospital of Salamanca and all subjects provided written informed consent to undergo liver biopsy under direct vision during surgery.

Data were collected on demographic information (age, sex, and ethnicity), anthropomorphic measurements (BMI), smoking and alcohol history, coexisting medical conditions, and medication use. Before surgery, fasting venous blood samples were collected for determination of complete cell blood count, total bilirubin, aspartate aminotransferase (AST), alanine aminotransferase (ALT), total cholesterol, high-density lipoprotein, low-density lipoprotein, triglycerides, creatinine, glucose, and albumin.

A portion of each liver biopsy was fixed in 10% formalin and stained with hematoxylin–eosin and Masson’s trichrome for standard histopathological analysis. The remaining portion was stored at −80°C for later protein was extraction. The presence of NAFLD was diagnosed using standard criteria, and severity of the disease was established using the NAFLD activity score (NAS) described by Kleiner (Kleiner et al., 2005).

Mice

Mice deficient for p38\(\gamma\) (B6.129-Mapk12tm1) and p38\(\epsilon\) (B6.129-Mapk13tm1) were crossed with B6.129P2-Lyz2tm1(cre)Jo/J or with B6.Cg-Tg(S100A8-cre,-EGFP)1Ilw/J mice backcrossed for 10 generations to the C57BL/6J background (Jackson Laboratory). Genotype was confirmed by PCR analysis of genomic DNA.
Radiation chimeras were generated by exposing recipient mice to 2 doses of ionizing radiation (625 Gy) and reconstituting them with \(2 \times 10^7\) donor BM cells by injection into the tail vein. Proper reconstitution was checked in B6.SJL (CD45.1) control mice transplanted with CD45.2 BM mononuclear cells by immunostaining reconstitution was checked in B6.SJL (CD45.1) control mice

**Hepatic peroxidation**

Liver extracts were prepared by sonication (15 cycles) in cytoplasmic lysis buffer [25 mM Tris–HCl (pH 7.5), 10 mM NaCl, 1 mM EDTA, 100 mM MgCl₂, 1% NP-40, 0.1 mM phenylmethylsulfonyl fluoride, and 10 µg/ml aprotinin and leupeptin]. Malondialdehyde and hydrogen peroxide were assayed with the TRABS Assay Kit (Cayman) and the Amplex Red Hydrogen Peroxide/Peroxidase Assay Kit (Invitrogen).

**Glucose tolerance test**

Glucose tolerance test was performed as described (Mora et al, 2005).

**Isolation of liver-infiltrating mononuclear leukocytes**

Mouse livers were collected, and a single-cell suspension was obtained and passed through a 70-µm strainer. Leukocytes were collected from the interphase of centrifuged Ficoll gradients.

**Flow cytometry**

Isolated liver-infiltrating leukocytes were counted with a CASY Cell Counter (57) and then labeled by surface staining (Streptavidin-PerCP/biotin-conjugated anti-CD11b and APC-conjugated anti-Gr-1; Invitrogen). Flow cytometry was performed with a FACScan cytofluorometer (FACS Canto BD), and data were collected and analyzed with FlowJo software.

**Intravital microscopy**

Intravital microscopy of the cremaster muscle after TNF-α injection (0.5 µg, intrascrotal injection) was performed as reported (Sreeramkumar et al, 2013) using an Axio Examiner Z.1 workstation (Zeiss, Germany). Fluorescently conjugated anti-Ly6G (1 mg/mouse) was injected immediately before acquisition to specifically identify neutrophils. Recorded videos were analyzed using Slidebook software (Intelligent Imaging Innovations). At least 30 venules were analyzed from 3 mice per group.

**Competitive cell migration assay**

Lyzs-Cre and p38\(\gamma/\gamma^{Lyzs-KO}\) neutrophils were isolated from bone marrow by labeling with biotin-conjugated anti-Ly6C/G antibody (BD Pharmigen) and magnetic streptavidin microbeads (Miltenyi Biotec) and then separating them on MACS MS columns (Miltenyi Biotec). Isolated Lyzs-Cre neutrophils were stained with DiO and p38\(\gamma/\gamma^{Lyzs-KO}\) neutrophils were stained with DiD (Vybrant Cell-Labeling Solution, Molecular Probes). Cell viability was checked by DAPI staining followed by FACS. The labeled cells were then mixed at a 1:1 ratio and injected (6 \times 10⁶ cells) into MCD-diet WT mice. After 1 h, liver-infiltrating mononuclear leukocytes were isolated and directly detected by FACS. Fluorescent neutrophils were also detected by confocal microscopy in OCT-cryopreserved liver sections.

**Statistical analysis**

Differences between experimental groups were examined for statistical significance by two-tailed Student’s t-test or one-way ANOVA coupled to Bonferroni’s and Newman–Keuls post-test. Characteristics of patients and controls were compared by means of Mann–Whitney U-test for quantitative variables and \(\chi^2\) or Fisher’s tests for qualitative variables.

For more Materials and Methods, see the Appendix.

**Expanded View** for this article is available online.

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**Author contributions**

BG-T, NM, IGN, and GS designed the study; BG-T, NM, IGN, MAV, VS, AM, GC, MLS, EB, LL-V, ER, VB, and GS performed experimental analysis; metabolic cages were performed by RN and SP-S; intravital microscopy was performed by VS and AH; in vitro neutrophil migration was performed by MLS and FS-M; MM designed and coordinated human study; MM, LH–C, JLT, and LO recruited subjects and were responsible for sample and data collection; AC provided reagents and BG-T, NM, IGN, and GS wrote the manuscript. All authors contributed to the revision of the manuscript and approved the final version.

**Conflict of interest**

The authors declare that they have no conflict of interest.
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