Research Article
NAFLD and Alcohol-Related Liver Diseases

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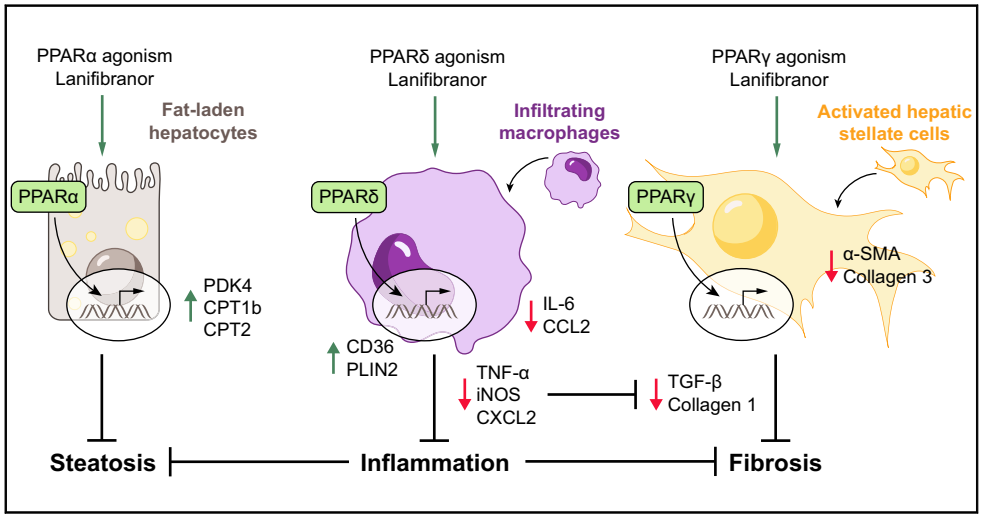
Differential effects of selective- and pan-PPAR agonists on experimental steatohepatitis and hepatic macrophages

Graphical abstract



Highlights

- PPARs are beneficial regulators of metabolism, making them promising drug candidates in NAFLD.
- The pan-PPAR agonist lanifibranor reduces steatosis, inflammation and fibrosis in two NAFLD mouse models.
- Pan-PPAR agonism indirectly inhibits hepatic macrophage infiltration.
- Human and murine macrophages display a metabolically activated phenotype in NAFLD.
- Lanifibranor decreases pro-inflammatory activation of macrophages via PPARδ agonism.

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Lay summary

Peroxisome proliferated-activated receptors (PPARs) are essential regulators of metabolism and inflammation. We demonstrated that the pan-PPAR agonist lanifibranor ameliorated all aspects of non-alcoholic fatty liver disease in independent experimental mouse models. Non-alcoholic fatty liver disease and fatty acids induce a specific polarization status in macrophages, which was altered by lanifibranor to increase expression of lipid handling genes, thereby decreasing inflammation. PPAR isoforms have differential therapeutic effects on fat-laden hepatocytes, activated hepatic stellate cells and inflammatory macrophages, supporting the clinical development of pan-PPAR agonists.

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Differential effects of selective- and pan-PPAR agonists on experimental steatohepatitis and hepatic macrophages☆

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Background & Aims: Peroxisome proliferator-activated receptors (PPARs) are essential regulators of whole-body metabolism, but also modulate inflammation in immune cells, notably macrophages. We compared the effects of selective PPAR agonists to those of the pan-PPAR agonist lanifibranor in non-alcoholic fatty liver disease (NAFLD), and studied isoform-specific effects on hepatic macrophage biology.

Methods: Lanifibranor or selective PPARα (fenofibrate), PPARγ (pioglitazone) and PPARδ (GW501516) agonists were therapeutically administered in choline-deficient, amino acid-defined high-fat diet (CDAA-HFD)- and Western diet (WD)-fed mouse models of NAFLD. Acute liver injury was induced by carbon tetrachloride (CCl_4). The role of PPARs on macrophage functionality was studied in isolated hepatic macrophages, bone marrow-derived macrophages stimulated with palmitic acid, and circulating monocytes from patients with NAFLD.

Results: Lanifibranor improved all histological features of steatohepatitis in CDAA-HFD-fed mice, including liver fibrosis, thereby combining and exceeding specific effects of the single PPAR agonists. Its potent anti-steatotic efficacy was confirmed in a 3D liver biochip model with primary cells. Infiltrating hepatic monocyte-derived macrophages were reduced following PPAR agonist administration, especially with lanifibranor, even after short-term treatment, paralleling improved steatosis and hepatitis. Lanifibranor similarly decreased steatosis, liver injury and monocyte infiltration in the WD model. In the acute CCl_4 model, neither single nor pan-PPAR agonists directly affected monocyte recruitment. Hepatic macrophages isolated from WD-fed mice displayed a metabolically activated phenotype. Lanifibranor attenuated the accompanying inflammatory activation in both murine palmitic acid-stimulated bone marrow-derived macrophages, as well as patient-derived circulating monocytes, in a PPARδ-dependent fashion.

Conclusion: Pan-PPAR agonists combine the beneficial effects of selective PPAR agonists and may counteract inflammation and disease progression more potently. PPARδ agonism and lanifibranor directly modulate macrophage activation, but not infiltration, thereby synergizing with beneficial metabolic effects of PPARα/γ agonists.

Lay summary: Peroxisome proliferated-activated receptors (PPARs) are essential regulators of metabolism and inflammation. We demonstrated that the pan-PPAR agonist lanifibranor ameliorated all aspects of non-alcoholic fatty liver disease in independent experimental mouse models. Non-alcoholic fatty liver disease and fatty acids induce a specific polarization status in macrophages, which was altered by lanifibranor to increase expression of lipid handling genes, thereby decreasing inflammation. PPAR isoforms have differential therapeutic effects on fat-laden hepatocytes, activated hepatic stellate cells and inflammatory macrophages, supporting the clinical development of pan-PPAR agonists.

Introduction

Non-alcoholic fatty liver disease (NAFLD) has become the most common chronic liver disease worldwide. NAFLD, particularly its inflammatory form non-alcoholic steatohepatitis (NASH), can progress to fibrosis, cirrhosis and hepatocellular carcinoma.[1] Insulin resistance is a central pathophysiological mechanism and interconnects NAFLD with its comorbidities such as visceral obesity, type 2 diabetes and atherosclerosis.[2] At present, lifestyle modification is difficult to achieve and sustain, and approved pharmacological therapy is lacking.[3]

Peroxisome proliferator-activated receptors (PPARs) are nuclear receptors that bind fatty acids and their derivatives, and integrate metabolic and inflammatory signalling pathways, making them attractive therapeutic targets for NAFLD.

The 3 isoforms, PPARα, PPARγ and PPARδ(β), have different tissue distributions and functions. PPARα exerts its main actions in the liver, where it transcriptionally drives genes regulating gluconeogenesis, β-oxidation, ketogenesis and lipid transport.[4] The

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hepatic expression of PPARα, but not PPARγ or δ, correlates with the presence of NASH and its histological features.[5] In animal models, PPARα deletion is associated with a worsening of hepatic steatosis whereas the selective PPARα agonist Wy-14,643 reversed NASH and fibrosis.[6,7] PPARγ is the predominant isoform in adipose tissue; it controls glucose metabolism, lipogenesis and adipocyte differentiation, and upregulates adiponection.[8,9] By promoting the storage of fatty acids as triglycerides, PPARγ acts as an insulin sensitizer and prevents ectopic fat accumulation. Indeed, although the hepatocyte-specific deletion of PPARγ decreased hepatic steatosis in genetically obese mice, whole-body insulin resistance was aggravated.[10] The role of PPARδ is less clear, although it promotes hepatic fatty acid oxidation and limits inflammation.[8]

Macrophages have emerged as key mediators of inflammation-mediated insulin resistance.[2] During liver injury, monocytes massively infiltrate the liver and differentiate into proinflammatory monocyte-derived macrophages (MoMFs), which replace the resident Kupffer cells (KCs) as the dominant macrophage population.[11,12] Macrophages are able to respond to a variety of stimuli, including metabolic ones, which can induce specific polarization states.[13,14] PPARγ negatively regulates this proinflammatory polarization, while both PPARγ and PPARδ are involved in anti-inflammatory polarization. Interestingly, deletion of either PPAR isoform in myeloid cells exacerbated insulin resistance and hepatic steatosis.[15–17]

Their multiple beneficial actions in metabolism and inflammation indicate that pharmacological agonists of PPAR(s) represent attractive therapeutic approaches in NAFLD. Fibrates, synthetic agonists of PPARα, have not shown a consistent beneficial effect in NAFLD, although large trials are lacking. In the PIVENS trial, the PPARγ agonist pioglitazone improved hepatic steatosis, lobular inflammation and ballooning, but not fibrosis.[18] However, a meta-analysis on the use of pioglitazone in NASH indicated beneficial effects on advanced fibrosis.[19] Single PPARδ agonists have faced safety concerns,[8] and in a recent phase II trial, the PPARδ agonist seladelpar failed to reduce liver fat as quantified by MR imaging (NCT03551522). Thus, the focus has shifted towards the development of dual and pan-PPAR agonists. The dual PPARα/δ agonist elafibranor has demonstrated beneficial effects on NASH resolution in patients with highly active disease without a clear antifibrotic signal in a phase II clinical trial.[20] The pan-PPAR agonist lanifibranor reduced disease severity in 2 preclinical NAFLD models, the methionine/choline-deficient diet and in foz/foz mice fed a high-fat diet.[21]

Despite advances in our understanding of the beneficial actions of PPARs on insulin sensitivity and NAFLD, the relative potency of the different PPAR agonists in the treatment of NAFLD and their effect on macrophages have not been elucidated.

In this study, we assessed the therapeutic potential of lanifibranor in comparison with single PPAR agonists in two murine models of NASH and fibrosis. Furthermore, we examined the functional consequences of PPAR agonism on macrophage biology as a factor contributing to PPAR-mediated attenuation of steatohepatitis.

Materials and methods

Part of the materials and methods are described in a supplementary file.

Liver injury models and pharmacological treatment

Seven-week old C57BL/6J wild-type mice (Janvier Labs, Le Genest-Saint-Isle, France) were housed in a specific-pathogen-free environment at the animal facilities of the University Hospitals of Aachen and Ghent. Mice were given free access to food and water and housed in a 12 h light/dark cycle.

All *in vivo* experiments were approved by and conducted in agreement with the appropriate institutional and governmental authorities. Reporting was conforming to the ARRIVE guidelines for animal experiments.

Steatohepatitis was induced by feeding 8-week old male mice either a choline-deficient, L-amino acid-defined, high-fat diet enriched with 2% cholesterol (CDAA-HFD) (E15673-940, Ssniff, Soest, Germany) for up to 12 weeks, or a WD rich in saturated fat, sucrose and cholesterol (TD.08811 + 1% cholesterol, Ssniff) for 16 weeks.

After 6 weeks of CDAA-HFD and 10 weeks of WD feeding, mice were randomized to receive either vehicle (methylcellulose 1% + poloxamer 0.1%), the PPARα agonist fenofibrate (100 mg/kg/day), the PPARγ agonist pioglitazone (30 mg/kg/day), the PPARδ agonist GW501516 (10 mg/kg/day) or the pan-PPAR agonist lanifibranor (30 mg/kg/day) once daily via oral gavage for a period of up to 6 weeks, while diet feeding was continued.

Acute liver injury was induced by a single intraperitoneal injection of carbon tetrachloride (CCl_4) (Merck, Germany), dissolved in corn oil, at 0.6 ml/kg body weight, as previously described.[22] PPAR agonists or vehicle were given by oral gavage directly after induction of liver injury and 24 h later. Mice were sacrificed 36 h after the CCl_4 injection.

Statistical analysis

Statistical analysis was performed using Graphpad Prism 6 (Graphpad Software Inc., La Jolla, CA, USA) and SPSS 25.0 (SPSS Software, IBM Corp., NY, USA). Differences between groups were compared using a one-way ANOVA with *post hoc* testing. A two-sided p value <0.05 was considered statistically significant. Continuous variables are presented as mean ± SD.

Results

Pan-PPAR agonism combines the differential effects of selective PPAR agonists on steatohepatitis and fibrosis progression

To investigate the efficacy of single and pan-PPAR agonists in the treatment of progressive steatohepatitis, we employed the CDAA-HFD model, which induces severe inflammation and liver fibrosis. Mice were fed the CDAA-HFD for 12 weeks, and PPAR agonist treatment was administered during the last 6 weeks of diet feeding (Fig. 1A). Adequate dosing was confirmed by differential PPAR target engagement. Specifically, pioglitazone and lanifibranor increased serum levels of adiponectin (Fig. 1B), a main PPARγ target, whereas fenofibrate and lanifibranor increased the hepatic expression of the PPARα target genes pyruvate dehydrogenase kinase (*Pdk*) 4, carnitine palmitoyltransferase (*Cpt*)1b and *Cpt*2 (Fig. S1A). Fibrates are indicated for the treatment of hypertriglyceridemia, whereas thiazolidinediones have indirect effects on circulating lipids.[23] In our study, lanifibranor decreased serum triglyceride levels, which also tended to decrease upon fenofibrate and pioglitazone treatment (Fig. 1B). The PPAR agonists were well tolerated, and no significant effects on body weight or adipose tissue weight were observed, while the liver-to-body weight ratio was lower in mice treated with lanifibranor and pioglitazone (Fig. S1B).

Importantly, treatment with lanifibranor reversed steatohepatitis, as evidenced by highly significant reductions in the

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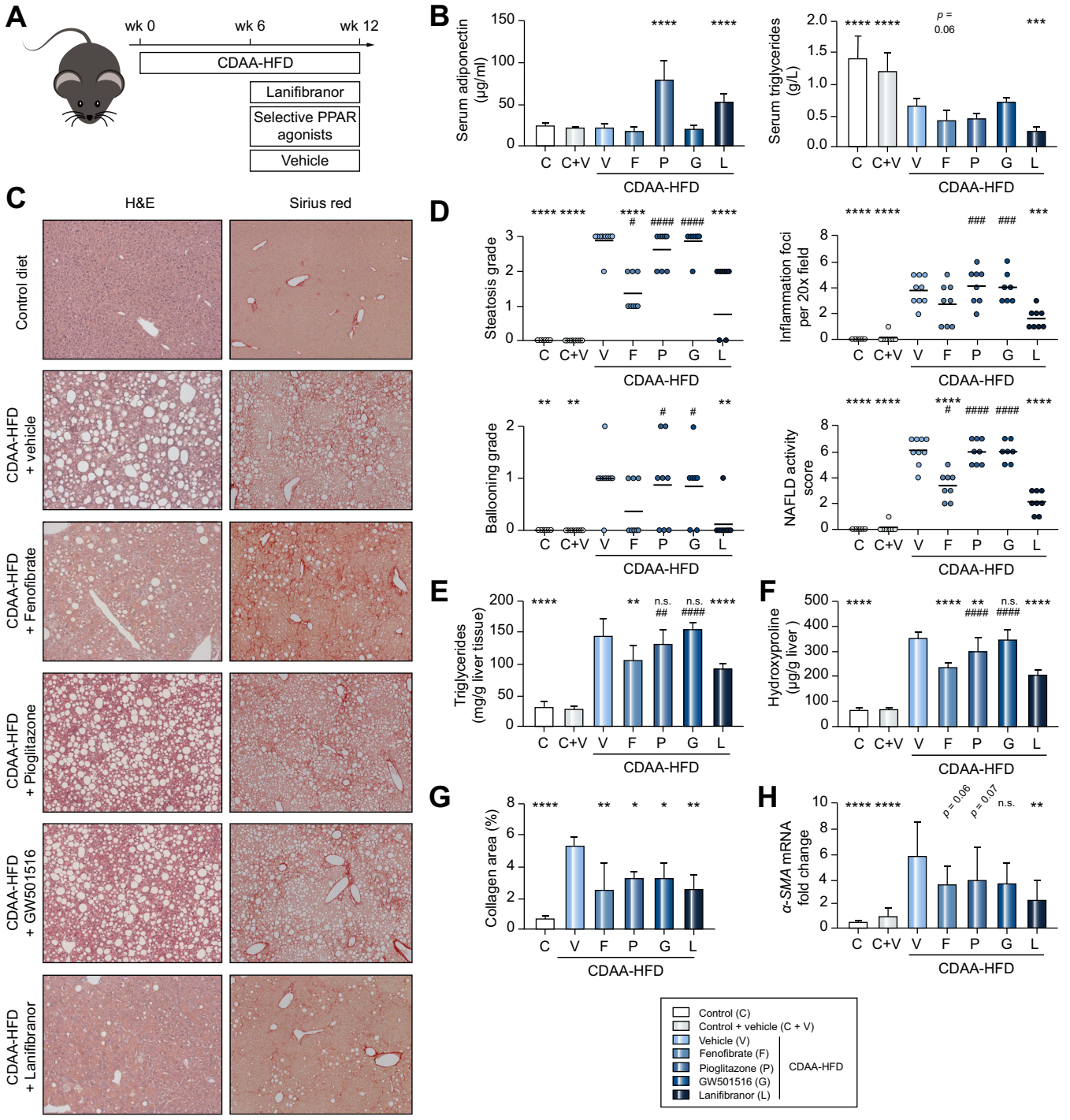

Fig. 1. Therapeutic administration of PPAR agonists ameliorates steatohepatitis and fibrosis. (A) Starting after 6 weeks of experimental steatohepatitis diet, CDAA-HFD diet-fed mice were treated once daily in a therapeutic setting for 6 weeks with single PPARα, γ and δ agonists and the pan-PPAR agonist lanifibranor. (B) Serum adiponectin and triglyceride levels. (C) Representative H&E and Sirius red staining (magnification 100×). (D) Scoring of histological features of steatosis, lobular inflammation and ballooning, and NAFLD activity score. (E) Quantification of liver triglyceride content. (F–G) Liver fibrosis was assessed by quantification of hepatic hydroxyproline content (F) and the fraction of Sirius red positive area (G). (H) Expression of *Acta2* (αSMA). Data are presented as mean ± SD (n = 6 for the control group and n = 8 for the other groups). **p* <0.05; ***p* <0.01; ****p* <0.001; *****p* <0.0001 (one-way ANOVA with *post hoc* test). # denotes the level of significance *vs.* lanifibranor-treated mice. Data are pooled from 2 independent experiments. CDAA-HFD, choline-deficient, amino acid-defined high-fat diet; NAFLD, non-alcoholic fatty liver disease.

NAFLD activity score as well as the subcomponent scoring of steatosis (validated by reduced hepatic triglyceride content), lobular inflammation and hepatocellular ballooning (Fig. 1C–E). Fenofibrate improved these scores, especially steatosis, to a lesser extent, whereas pioglitazone and GW501516 did not significantly impact the histological disease severity. Alanine

aminotransferase (ALT) levels also tended to be lower in lanifibranor-treated mice (Fig. S1C). Lanifibranor ameliorated liver fibrosis, with significant reductions in collagen area, liver hydroxyproline, and reduced expression of fibrogenic mediators (Fig. 1C, F–H and Fig. S1D). The single PPAR agonists improved fibrosis to a lesser extent, with fenofibrate exerting a stronger effect in mice than the PPARγ and δ agonists.

Pan-PPAR agonism inhibits macrophage accumulation in the steatohepatitis/fibrosis model

As liver MoMF are key drivers of NASH and fibrosis progression,[2,12] we studied the impact of PPAR agonism on the hepatic immune cell composition. PPAR agonists, especially lanifibranor, reduced the number of intrahepatic macrophages, as assessed by F4/80 immunohistochemistry as well as F4/80 and *Ccr2* mRNA expression (Fig. 2A). We validated these findings using flow cytometry and observed a marked decrease in the proportion of infiltrating MoMFs upon lanifibranor treatment, which was significantly more pronounced than upon treatment with either single PPAR agonist (Fig. 2B). Lanifibranor similarly reduced infiltrating monocytes, whereas KCs, which are depleted in experimental NASH,[24,25] were not affected (Fig. 2B). Notably, the intrahepatic lymphocyte populations remained unaffected by treatment, or were only relatively increased due to the sharp decrease in MoMFs (Fig. S2A).

Treatment with all PPAR agonists decreased the proportion of circulating blood monocytes to levels comparable with the controls, in part through a reduction in immature Ly6C$^+$ monocytes (Fig. 2C). Blood granulocytes and lymphocyte subsets were unaltered (Fig. S2B).

The reduction in liver MoMFs was accompanied by reduced expression of the proinflammatory mediators *Tnfα, iNos, Il6* and *Cxcl2*, which was most pronounced following lanifibranor treatment (Fig. 2D).

These data indicate that pan-PPAR agonism inhibits MoMF infiltration to a significantly larger extent than obtained by stimulation with single PPAR isoforms, which might contribute to reducing the hallmarks of disease, such as triglyceride accumulation, steatohepatitis and fibrosis.

Reduced NASH activity and macrophage infiltration are early consequences of lanifibranor treatment in experimental steatohepatitis

To examine the pathophysiological sequence of steatohepatitis amelioration upon treatment with PPAR agonists, we analysed the effects of short-term treatment, in which PPAR agonists were administered for 2 weeks in mice that had been subjected to 6 weeks of CDAA-HFD (Fig. 3A). Adequate dosing was again confirmed by PPAR isoform-specific target engagement (Fig. S3A). Similar to the long-term treatment, lanifibranor and, to a lesser degree, fenofibrate, ameliorated NASH as shown by a reduced grading of steatosis, lobular inflammation and ballooning, and thus the overall NAFLD activity score (Fig. 3B–C; Fig. S3B). This was accompanied by a significant reduction in hepatic lipid content and expression of inflammatory cytokines in lanifibranor-treated mice, whereas fenofibrate did not improve these variables. Pioglitazone and GW501516 did not have significant effects on either of these disease markers nor liver histology. Not surprisingly for short-term treatment, liver fibrosis was only modestly, yet significantly, improved by lanifibranor and not by single PPAR agonists (Fig. 3B–D; Fig. S3C).

Lanifibranor decreased the number of hepatic macrophages after just 2 weeks of treatment, as demonstrated on F4/80 immunohistochemistry and hepatic *Ccr2* gene expression (Fig. 3B,E). Strikingly, the proportion and absolute number of hepatic MoMFs were reduced specifically by lanifibranor compared to the single PPAR agonists, which was accompanied by a significant decrease in the overall number of leukocytes (Fig. 3F–G; Fig. S3D). In agreement with the long-term treatment, hepatic monocytes were decreased as well, to the greatest extent by lanifibranor, whereas the liver lymphocyte compartment remained unaffected and the number of blood leukocytes was decreased by all PPAR agonists (Fig. 3F–G; Fig. S3E).

PPAR isoforms combine to improve NAFLD in an obese mouse model

Although the CDAA-HFD diet induces severe inflammation and progression to fibrosis, obesity and insulin resistance do not develop in this model.[26] We therefore investigated the PPAR agonists in a NAFLD mouse model with concomitant obesity and metabolic syndrome. Mice were fed a WD rich in fat, sucrose and cholesterol for 10 weeks, after which daily treatment was administered for 6 weeks (Fig. 4A). Fenofibrate, GW501516 and lanifibranor caused mild weight loss, which was not observed following pioglitazone administration (Fig. 4B). This was associated with a significant decrease in adipose tissue weight (Fig. 4C). In accordance with known beneficial metabolic effects, fenofibrate and lanifibranor decreased serum triglycerides (Fig. 4C). Lanifibranor improved liver injury, as evidenced by decreased serum ALT levels (Fig. 4D).

Histologically, WD feeding caused a less severe NAFLD phenotype compared to CDAA-HFD feeding, with marked hepatic steatosis, mild to moderate inflammation yet absent ballooning and fibrosis. Lanifibranor decreased both steatosis and inflammation. The former was mostly related to PPARα (although PPARδ agonism had a partial effect), while the latter could be linked to a PPARδ effect. As such, the composite NAFLD activity score was most impacted by lanifibranor (Fig. 4E).

Both PPARα and PPARδ agonists reduced the infiltration with monocytes and MoMF, with lanifibranor having the most significant effects. Moreover, the absolute number of leukocytes in the liver were decreased following PPARα, PPARδ and lanifibranor treatment, to a similar level as chow diet-fed control mice (Fig. 4F).

Lanifibranor improves steatosis in a 3D liver biochip but not 2D primary hepatocyte culture

Hepatic triglycerides rapidly decreased after lanifibranor treatment in experimental steatohepatitis in mice (Fig. 3D), in line with the well-documented beneficial metabolic effects of different PPAR agonists.[8,27] To confirm this mode of action, we tested the anti-steatotic efficacy of lanifibranor *in vitro*. Cultured primary murine hepatocytes were stimulated for 24 h or 48 h with a mixture of oleic acid (OA) and palmitic acid (PA) in the presence or absence of lanifibranor. Unexpectedly, lanifibranor treatment did not attenuate hepatocyte fatty acid accumulation. Indeed, treatment moderately elevated intracellular lipids in control hepatocytes (Fig. 5A).

We then examined hepatocyte steatosis in a 3D liver biochip, which more closely mimics the *in vivo* liver anatomy by assembling the different parenchymal and non-parenchymal cell types. We advanced a previously reported culture system that had been

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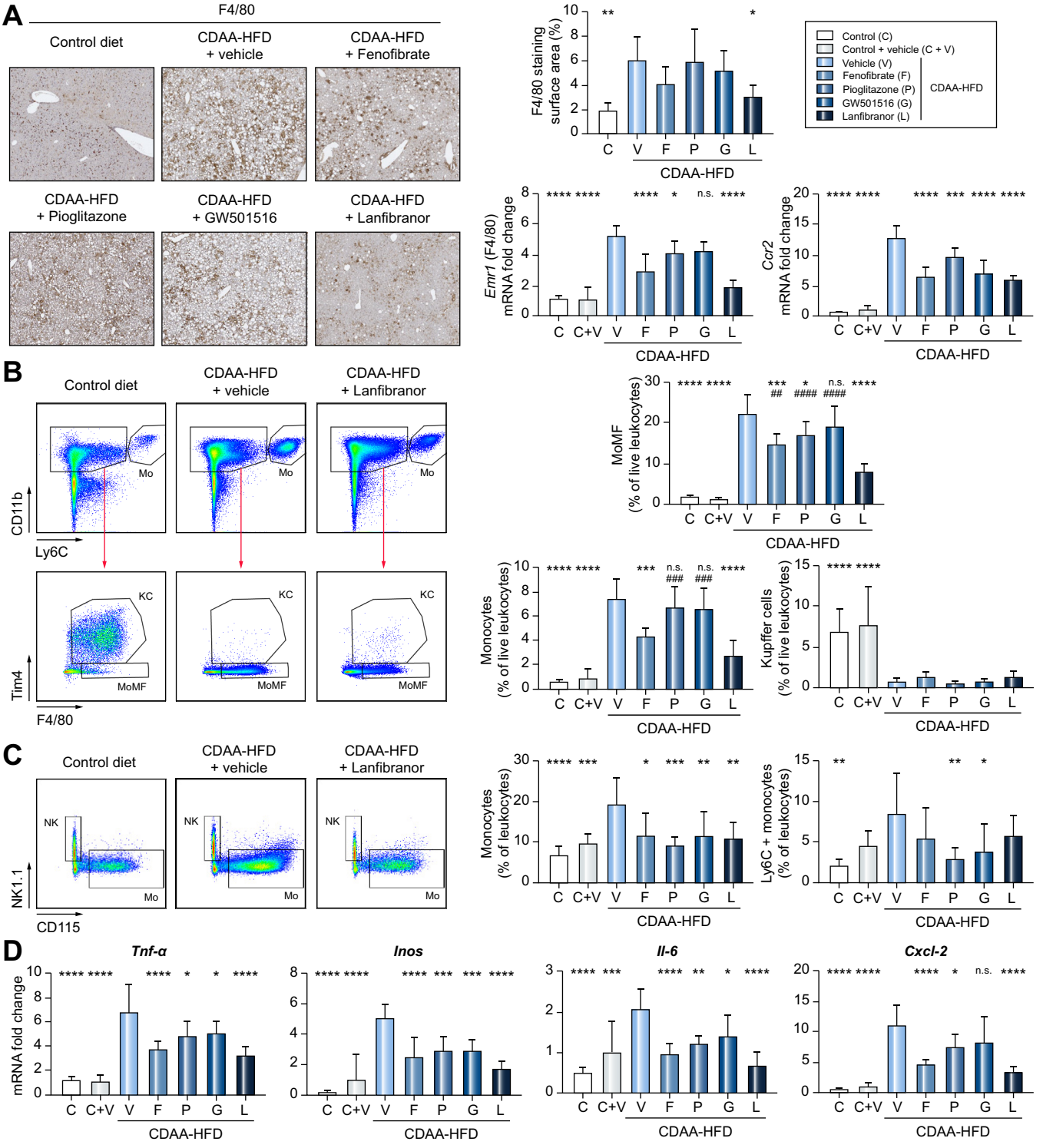

Fig. 2. Lanifibranor treatment decreases macrophage infiltration and inflammation in the CDAA-HFD model. (A) F4/80 immunohistochemistry with representative sections (magnification 100×) and quantification. Expression of *Ccr2* and *Emr1* (F4/80) in liver tissue. (B) Representative flow cytometric plots and quantification of liver MoMFs, monocytes and Kupffer cells. (C) Representative flow cytometric plots and quantification of blood monocytes and Ly6C^{+} blood monocytes. (D) Hepatic expression of proinflammatory markers. Data are presented as mean ± SD (n = 6 for the control group and n = 8 for the other groups). $^{*}p$ <0.05; $^{**}p$ <0.01; $^{***}p$ <0.001; $^{****}p$ <0.0001 (one-way ANOVA with *post hoc* test). $^{\#}$ denotes the level of significance *vs.* lanifibranor-treated mice. Data are pooled from 2 independent experiments. CDAA-HFD, choline-deficient, amino acid-defined high-fat diet; MoMFs, monocyte-derived macrophages.

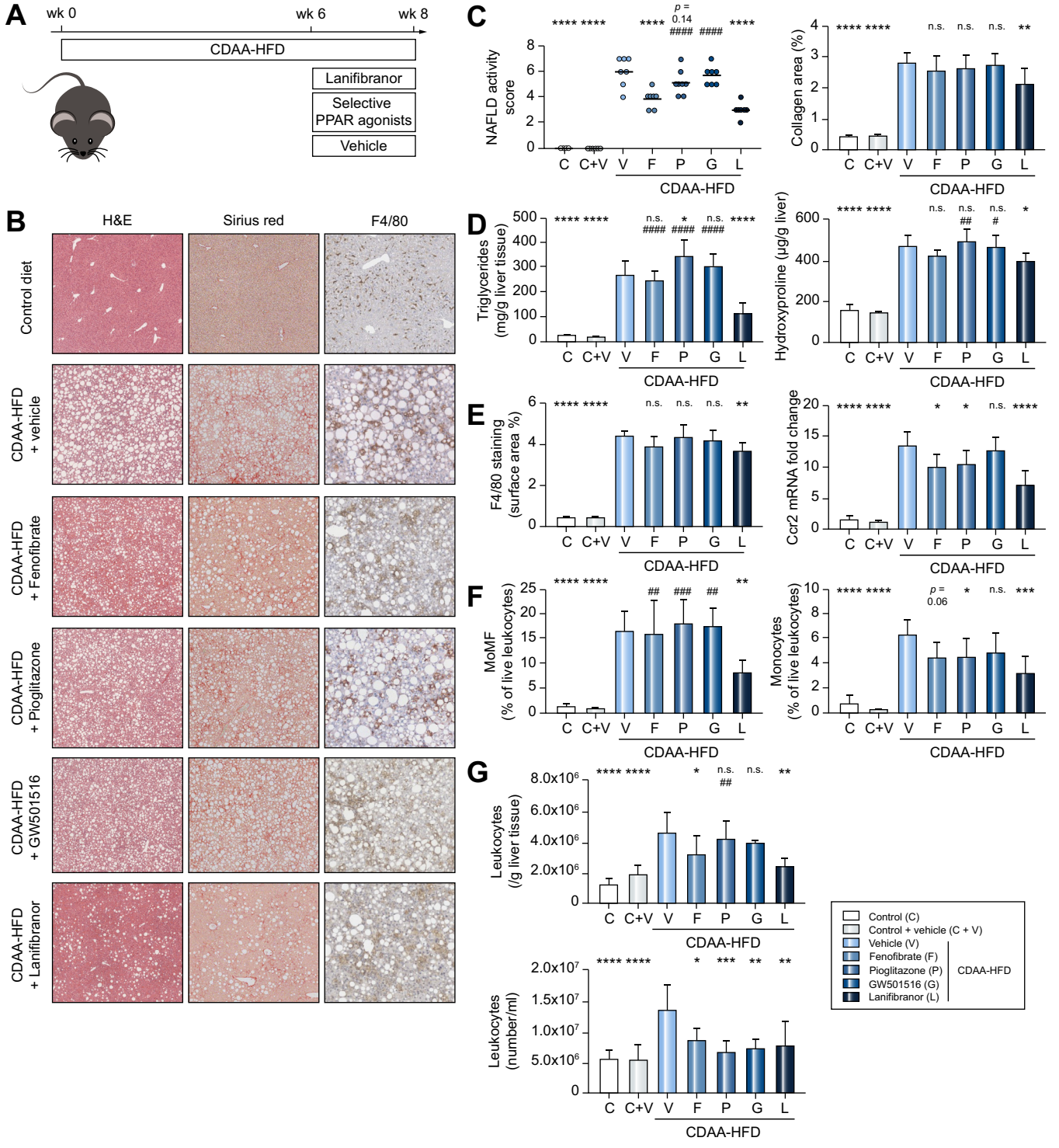

Fig. 3. Short-term pan-PPAR agonist treatment reduces steatohepatitis and macrophage accumulation. (A) Starting after 6 weeks of experimental steatohepatitis diet, CDAA-HFD diet-fed mice were treated once daily in a therapeutic setting for 2 weeks with single PPAR agonists or the pan-PPAR agonist lanifibranor. (B) Representative H&E, Sirius red (magnification 100×) and F4/80 (magnification 200×) immunohistochemistry stainings. (C) NAFLD activity score and quantification of liver fibrosis as the fraction of Sirius red positive area. (D) Quantification of liver triglyceride and hydroxyproline content. (E) F4/80 positive area fraction and *Ccr2* gene expression. (F) Liver MoMFs and monocytes, determined by flow cytometry with gating described as in Fig. 2. (G) Absolute numbers of liver leukocytes per gram of liver tissue and blood leukocytes per ml of blood. Data are presented as mean ± SD (n = 6 for both control groups and n = 7–8 for the CDAA-HFD other groups). *p <0.05; **p <0.01; ***p <0.001; ****p <0.0001 (one-way ANOVA with *post hoc* test). $^{\#}$ denotes the level of significance *vs.* lanifibranor-treated mice. Data are pooled from 2 independent experiments. CDAA-HFD, choline-deficient, amino acid-defined high-fat diet; MoMFs, monocyte-derived macrophages.

established with cell lines[28] by seeding primary murine hepatocytes and stellate cells into a "hepatic chamber", whereas primary endothelial and KCs were seeded into the "portal chamber", with both chambers being separated by a porous membrane (Fig. 5B). The addition of a PA/OA mixture led to fatty acid accumulation in hepatocytes, which was reduced by approximately 33% upon simultaneous treatment with lanifibranor (Fig. 5C).

The discrepancy between the 2D and 3D culture may indicate that PPAR activation in non-parenchymal cells, which interact with and influence hepatocytes, is required to exert its anti-steatotic actions.

Lanifibranor counteracts stellate cell activation

Since lanifibranor ameliorated fibrosis in the CDAA-HFD model, we examined its effects on primary human stellate cells *in vitro*. Lanifibranor reduced the production of α-SMA by stellate cells activated either by TGF-β stimulation or by the stiffness of the culture plastic (Fig. 5D). We obtained similar dose-dependent results in a therapeutic setting, when stellate cells had been activated on stiff plastic by 7 days of culture before the addition of lanifibranor (Fig. 5E).

PPAR agonists do not directly inhibit monocyte infiltration

Although PPARs have been studied in the context of NAFLD, their effects on hepatic macrophages are less clear. As we observed strong effects on hepatic macrophage accumulation (Figs. 2–3), we focused on the impact of PPAR agonism on macrophage biology.

We first investigated whether PPAR agonists directly inhibit leukocyte infiltration into the liver, by means of a single injection of CCl_4. The acute hepatocyte damage following CCl_4 provokes a massive macrophage recruitment to the injured liver,[22] thereby serving as an *in vivo* model to assess monocyte chemotaxis to the liver (Fig. 6A). Liver injury was assessed by serum ALT levels (Fig. 6B) and was accompanied by massive leukocyte infiltration, which was unaffected by treatment with either PPAR agonist (Fig. 6C–D). Importantly, the increased infiltration of monocytes and MoMFs was not attenuated by lanifibranor (Fig. 6C–F).

To validate this conclusion, we performed an *in vitro* chemotaxis assay in which bone marrow leukocytes were stimulated to migrate in a transwell chamber by the chemokine CCL2. In accordance with the CCl_4 experiment, the addition of lanifibranor did not change spontaneous or CCL2- induced migration of monocytes (Fig. 6G).

Thus, in contrast to chemokine and chemokine receptor antagonists undergoing evaluation in NAFLD, for instance CCR2/5 inhibitors,[29] lanifibranor does not directly inhibit hepatic MoMF recruitment.

PPARδ activation inhibits macrophage fatty acid-induced proinflammatory activation

Using single-cell RNA sequencing techniques, we recently identified the distinct 'metabolically activated' macrophage (also termed MMe) phenotype in the hepatic and bone marrow compartments in mice fed a Western-style diet.[13] Upon isolation from livers of mice fed the WD, MoMF displayed this particular gene expression profile, characterized by an increased expression of lipid metabolism genes (*Cd36*, perilipin-2 (*Plin2*)) and inflammatory markers (*Ccl2*, *Trem2*). Lanifibranor treatment reduced expression of the latter while further enhancing expression of lipid metabolism-related genes (Fig. 7A).

It was previously reported that PPARγ was able to counter MMe inflammatory activation, if induced *in vitro* through stimulation with FFAs.[30] Notably, both isolated MoMFs and cultured bone marrow-derived macrophages (BMDMs) expressed high levels of PPARγ and δ, and low levels of PPARα, which remained unaltered after WD feeding or PA stimulation, respectively (Fig. 7A, Fig. S4A–B). Stimulation with the saturated fatty acid PA induced the expression of inflammatory genes as well as genes characteristic of MMe polarization, such as *Plin2* and *Cd36,* but not lysosomal-associated membrane protein 2 (*Lamp2*) (Fig. 7B; Fig. S4C). Treatment with lanifibranor, but not single PPAR agonists, further increased the expression of *plin2* and *cd36* (Fig. 7B), which enable macrophages to handle excess fat.[31] Only lanifibranor reduced the inflammatory gene expression induced by PA stimulation (Fig. 7B), indicating that the synergistic involvement of multiple PPAR isoforms is required to decrease inflammation and improve lipid handling in macrophages. Of these, PPARδ seems the major isoform involved, as the administration of a PPARδ antagonist (GSK0660) either in isolation or combined with lanifibranor increased the expression of *Il6* and impaired that of lipid metabolism genes (Fig. 7C). PPARα antagonism primarily affected lipid metabolic genes, whereas the effects of PPARγ antagonism were minor (Fig. S4D). Importantly, multiplex immunohistochemistry staining confirmed the expression of PPARδ in hepatic macrophages in experimental NAFLD *in vivo* (Fig. 7D; Fig. S4E).

Circulating monocytes are metabolically activated in patients with fibrosing NAFLD

The MMe phenotype is highly conserved between animal models and humans. Adipose tissue macrophages isolated from the omental and subcutaneous adipose tissue from obese individuals exhibited a similar upregulation of inflammatory and metabolic genes as macrophages from HFD-fed mice, whereas markers of 'classical' M1 activation were not induced.[30] To ascertain if the reversal of this phenotype by lanifibranor *in vitro* is relevant to human NAFLD, we investigated whether circulating monocytes in human patients are comparably polarized and whether this correlated to the severity of NAFLD.

The clinical and biochemical patient characteristics (n = 26) are documented in Table S1. Lean healthy volunteers (n = 6; median Fibroscan value 5.0 kPa, median controlled attenuation parameter 199 dB/m) served as controls. We performed flow cytometric and RNA expression analysis of classical monocytes ($CD14^+$ $CD16^-$) as the most abundant subset, which remained proportionally stable between controls and increasing stages of NAFLD (Fig. 8A). PPARδ was the most abundant isoform, and its expression was not influenced by the presence of NAFLD (Fig. 8B).

Monocytes isolated from patients with NAFLD expressed elevated levels of the inflammatory cytokines IL-6, TNF-α and IL-1β, which reached statistical significance in patients with fibrosis (Fig. 8C). Notably, these genes tended to be downregulated in patients with decompensated cirrhosis. Closely mirroring PA-stimulated BMDMs, as well as MoMF isolation from livers of mice fed the WD, *CD36* and *PLIN2* were upregulated in these monocytes as well (Fig. 8D). Next, we isolated monocytes from healthy controls and patients with NAFLD without fibrosis and cultured these for 24 h in the presence or absence of lanifibranor

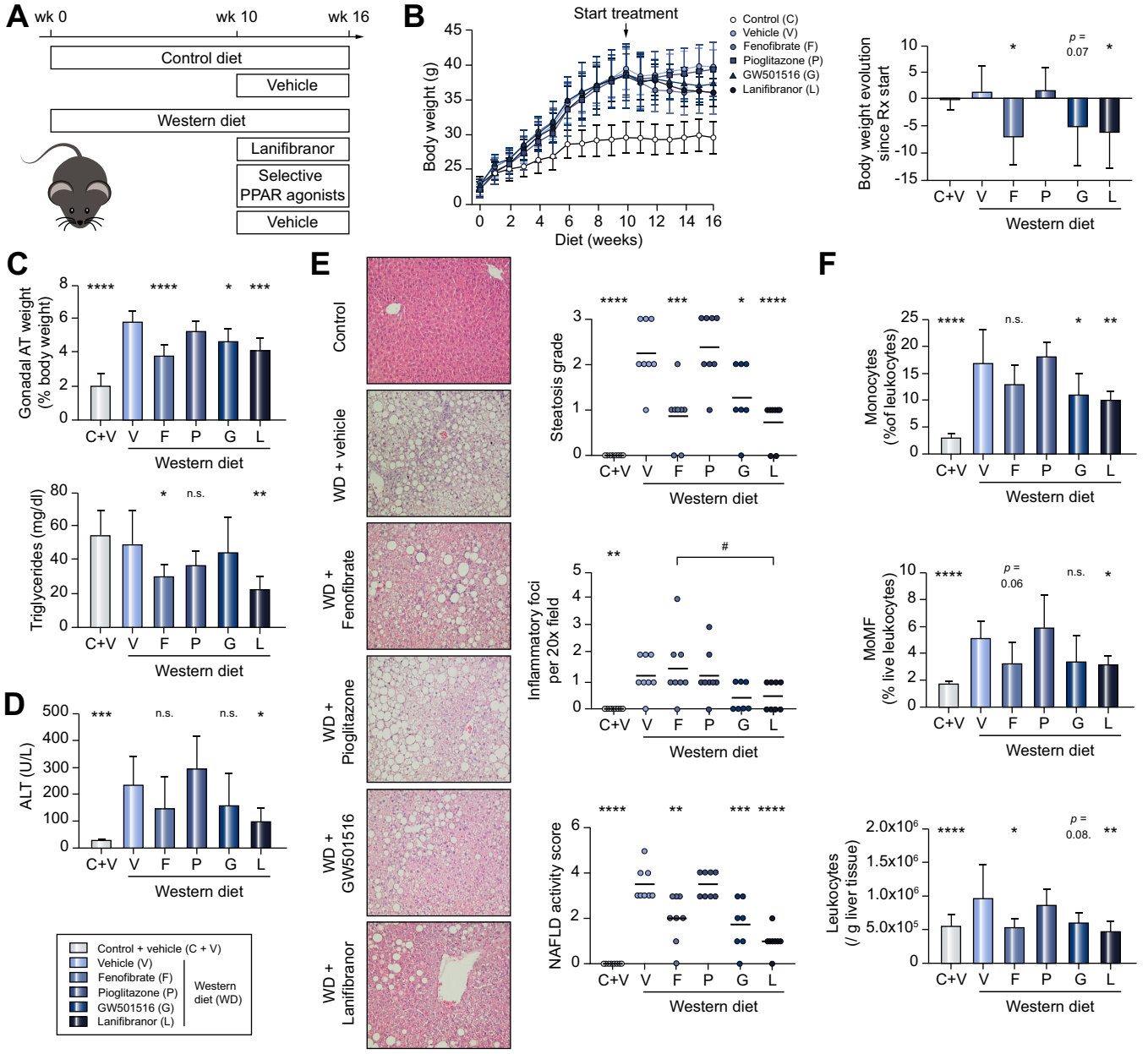

Fig. 4. PPAR agonists reverse NAFLD and macrophage infiltration in an obese mouse model. (A) After 10 weeks of WD feeding, mice were treated once daily in a therapeutic setting for 6 weeks with single PPARα, γ and δ agonists and the pan-PPAR agonist lanifibranor. (B) Body weight evolution (C) Gonadal adipose tissue weight and serum triglyceride levels. (D) Serum ALT levels. (E) Representative H&E staining (magnification 100×) and scoring of histological features of steatosis and lobular inflammation, and NAFLD activity score. (F) Quantification of infiltrating monocytes and MoMF, and absolute number of liver leukocytes. Data are presented as mean ± SD (n = 7–8 per group). *p <0.05; **p <0.01; ***p <0.001; ****p <0.0001 (one-way ANOVA with *post hoc* test). ALT, alanine aminotransferase; NAFLD, non-alcoholic fatty liver disease; MoMFs, monocyte-derived macrophages; WD, Western diet.

or GW501516. Both compounds decreased the expression of *CCL2* and increased that of *PLIN2*, the former more efficiently following lanifibranor (Fig. 8E).

Discussion

PPAR agonists have long been interesting drug candidates for NAFLD given their multiple (beneficial) effects on metabolic pathways. Indeed, the PPAR-α/δ agonist elafibranor induced resolution of NASH without fibrosis worsening in a *post hoc* analysis of a relatively large phase II clinical trial.[20] PPARs not only perform a plethora of metabolic functions, both in the liver as well as systemically, but also modulate inflammatory signalling pathways.[4] In this paper, we investigated the therapeutic potential of lanifibranor in NAFLD mouse models and explored the PPARδ-dependent regulation of macrophage activation in NASH.

Macrophages are central regulators of inflammation-induced insulin resistance in the liver, adipose tissue and sites of ectopic lipid accumulation.[32] In the liver, self-sustaining, yolk sac-derived KCs can be distinguished from the more immunogenic MoMFs, which derive from infiltrating monocytes during active liver injury. Apart from aggravating inflammation, MoMFs

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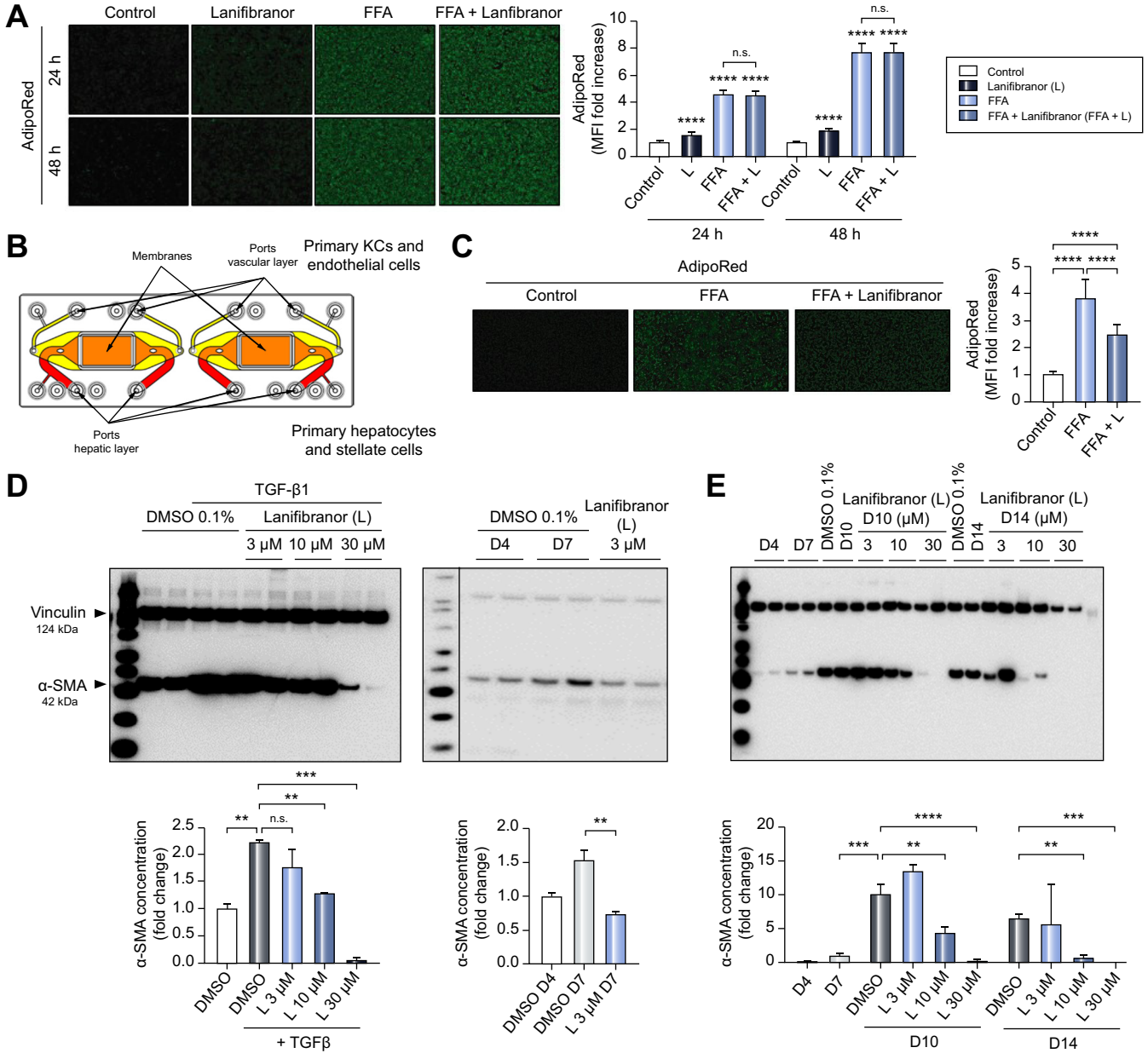

Fig. 5. Lanifibranor attenuates lipid accumulation and stellate cell activation. (A) 2D single layer culture of primary mouse hepatocytes. AdipoRed assay fluorescence images and quantification of MFI in hepatocytes treated as indicated for 24 h or 48 h. (B) Schematic of the 3D liver biochip that consists of hepatic and vascular layers separated by a porous PET-membrane. The "hepatic chamber" contains primary hepatocytes and hepatic stellate cells from mice, the "portal chamber" contains primary KCs and liver sinusoidal endothelial cells from mice. (C) AdipoRed assay fluorescence images and quantification of MFI of hepatocytes in a biochip treated as indicated for 48 h in biochips. (D–E) Primary human hepatic stellate cells were activated by TGF-β or prolonged culture on plastic plates. α-SMA concentration was determined by Western blot and normalized to Vinculin. Data are presented as mean ± SD. $^{**}p$ <0.01; $^{***}p$ <0.001; $^{****}p$ <0.0001 (one-way ANOVA with *post hoc* test). KCs, Kupffer cells; MFI, mean fluorescence intensity.

stimulate liver disease progression trough the secretion of fibrotic and angiogenic factors, and promote stellate cell survival.[2,29] Collectively, the rapid and specific inhibition of hepatic MoMF accumulation by lanifibranor, preceding the regression of liver fibrosis, suggests this cell type may be a major target through which pan-PPAR agonism ameliorates NAFLD, in addition to or in synergy with beneficial metabolic effects. Interestingly, CCl_4 experiments suggested that lanifibranor treatment reduced hepatic monocyte accumulation only indirectly. As such, PPAR agonists could potentially be combined with drugs affecting monocyte infiltration, for instance antagonists of the CCL2/CCR2 chemokine axis.[2,29]

The activation pattern of hepatic macrophage subsets is shaped by the integration of signals from overnutrition, the gut, metaflammation and from the local environment of a steatotic liver. These cues act on the highly plastic macrophages to induce unique polarization states that extend beyond the classical M1-M2 concept. Adipose tissue macrophages display an Mme phenotype in obesity, characterized by the increased expression of proinflammatory cytokines, albeit to a milder degree than in

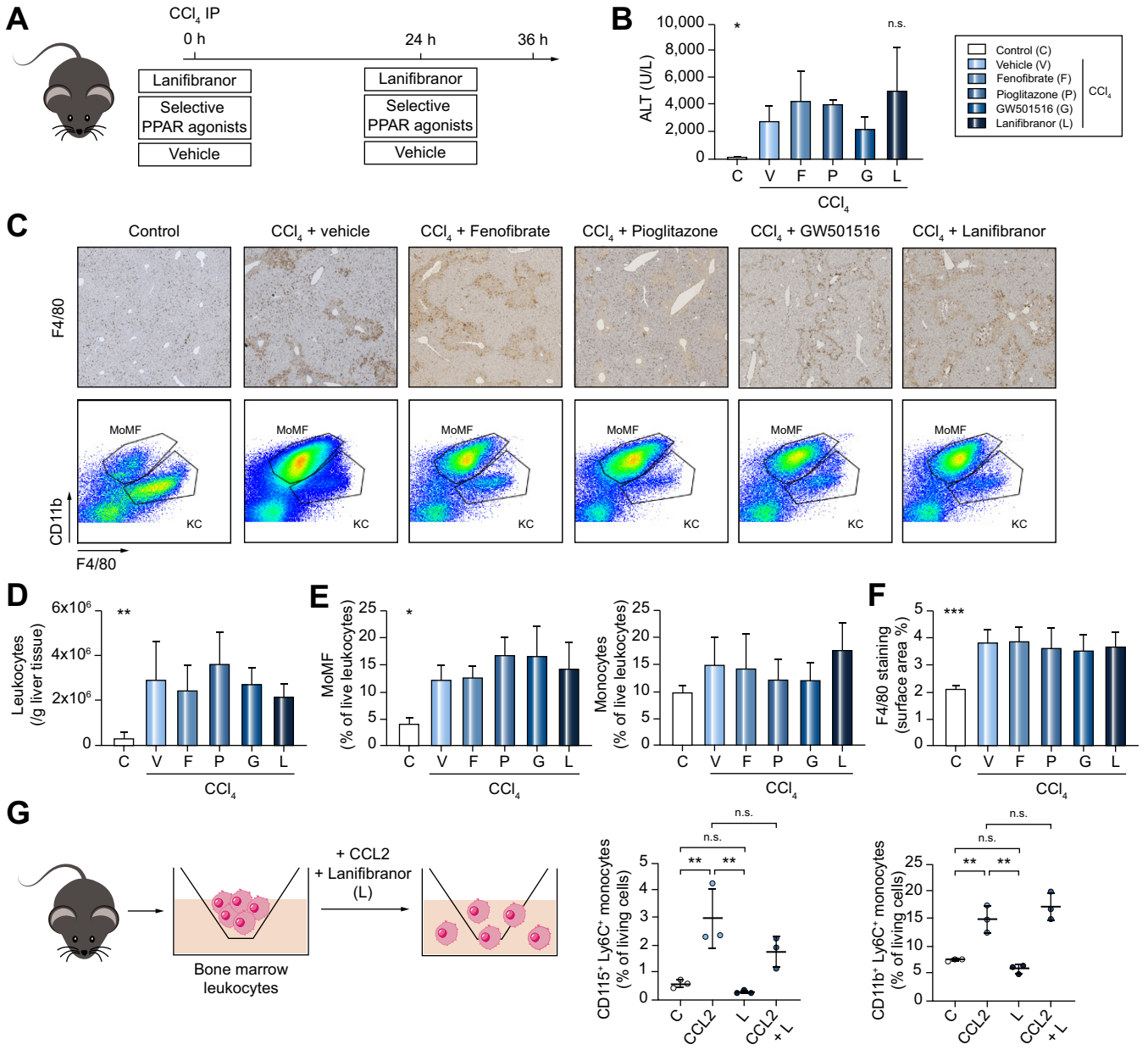

Fig. 6. PPAR agonists do not impact leukocyte infiltration into injured liver. (A) Mice received a single intraperitoneal injection with CCl_4 and were treated at 0 h and 24 h with a vehicle, single or pan-PPAR agonist and sacrificed after 36 h. (B) Serum ALT levels. (C) Representative F4/80 immunohistochemistry staining (magnification 100×) and flow cytometric plots. (D) Absolute number of liver leukocytes, per gram of liver tissue. (E) Quantification of liver MoMFs and monocytes. (F) Quantification of F4/80 staining positive area fraction (n = 5 per group). (G) Bone marrow cells were harvested for a transwell migration assay. Quantification of migrated monocytes following stimulation with CCL2 and/or lanifibranor treatment. Data are presented as mean ± SD *p <0.05; **p <0.01; ***p <0.001; ****p <0.0001 (one-way ANOVA with *post hoc* test). ALT, alanine aminotransferase; CCl_4, carbon tetrachloride; MoMFs, monocyte-derived macrophages.

M1 macrophages, as well as genes involved in lipid metabolism and lysosome biogenesis.[30,33] We now report that human circulating $CD14^+$ $CD16^-$ classical monocytes exhibit a similar polarization status in patients with NAFLD, especially in more advanced stages of fibrosis. Notably, this proinflammatory pattern was absent in patients with decompensated cirrhosis, possibly due to immune exhaustion at this stage.[34]

We recently identified a highly similar MMe phenotype in hepatic macrophages as well as bone marrow myeloid cells isolated from Western-style diet-fed mice,[13] which could be reproduced *in vitro* by the saturated fatty acid PA. Here, we found that lanifibranor reduced the expression of proinflammatory mediators while upregulating genes involved in lipid metabolism. Our data suggest the possibility of uncoupling these key MMe functions as a strategy to reduce insulin resistance and NAFLD. Follow-up experiments with isoform-specific PPAR inhibitors pointed to PPARδ as the main, but not exclusive, mediator of these therapeutic effects. Stimulation of human monocytes *in vitro* remarkably mimicked the findings in PA-stimulated BMDMs.

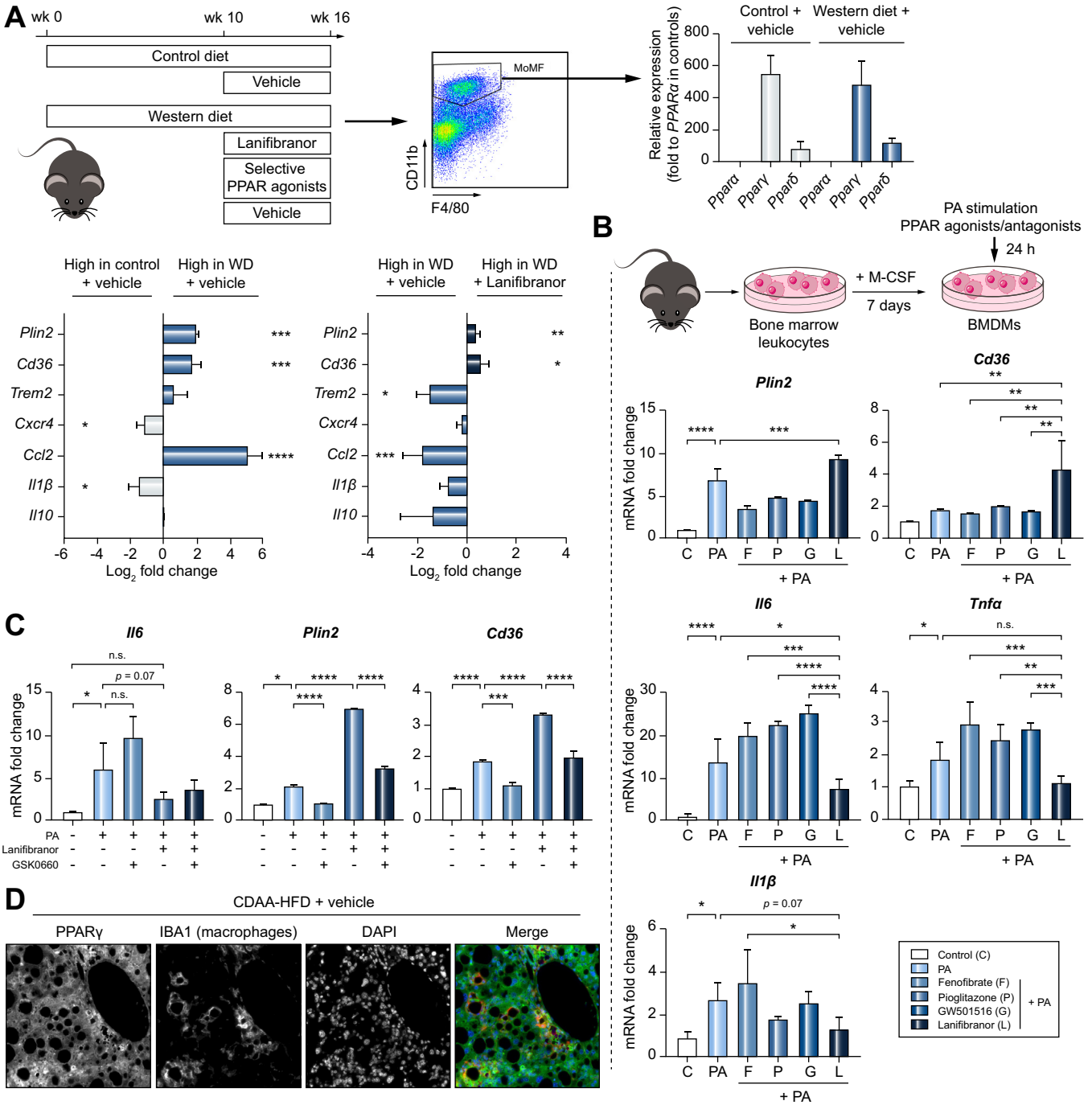

Fig. 7. Lanifibranor alters metabolic macrophage activation through PPARδ. (A) MoMFs were isolated from mice fed the WD as in Fig. 4. Gene expression analysis of PPAR isoforms and macrophage activation markers. (B–C) BMDMs were stimulated with PA and PPAR agonists and/or the PPARδ antagonist GSK0660. mRNA expression of lipid metabolism and proinflammatory genes following treatment with PPAR agonists (B) or lanifibranor and GSK0660 (C). Data are presented as mean ± SD (n = 3 per group). (D) Immunofluorescent staining for PPARδ, the macrophage marker IBA1 and DAPI, with overlay image. *p <0.05; **p <0.01; ***p <0.001; ****p <0.0001 (one-way ANOVA with *post hoc* test). BMDMs, bone marrow-derived macrophages; M-CSF, monocyte-colony stimulating factor; MoMFs, monocyte-derived macrophages; WD, Western diet.

These data reveal the striking similarity between hepatic macrophages *in vivo*, fatty acid-stimulated macrophages *in vitro* and circulating monocytes in patients with NAFLD, reinforcing the concept that metabolic programming in innate immune cells regulates inflammation in obesity and NAFLD.

Here, we show that lanifibranor ameliorated all histological features of NASH in mice. The pan-PPAR agonist lanifibranor thereby synergistically combines differential effects of single PPAR agonists in experimental steatohepatitis. Our data suggest that the amelioration of liver steatosis was mostly achieved via

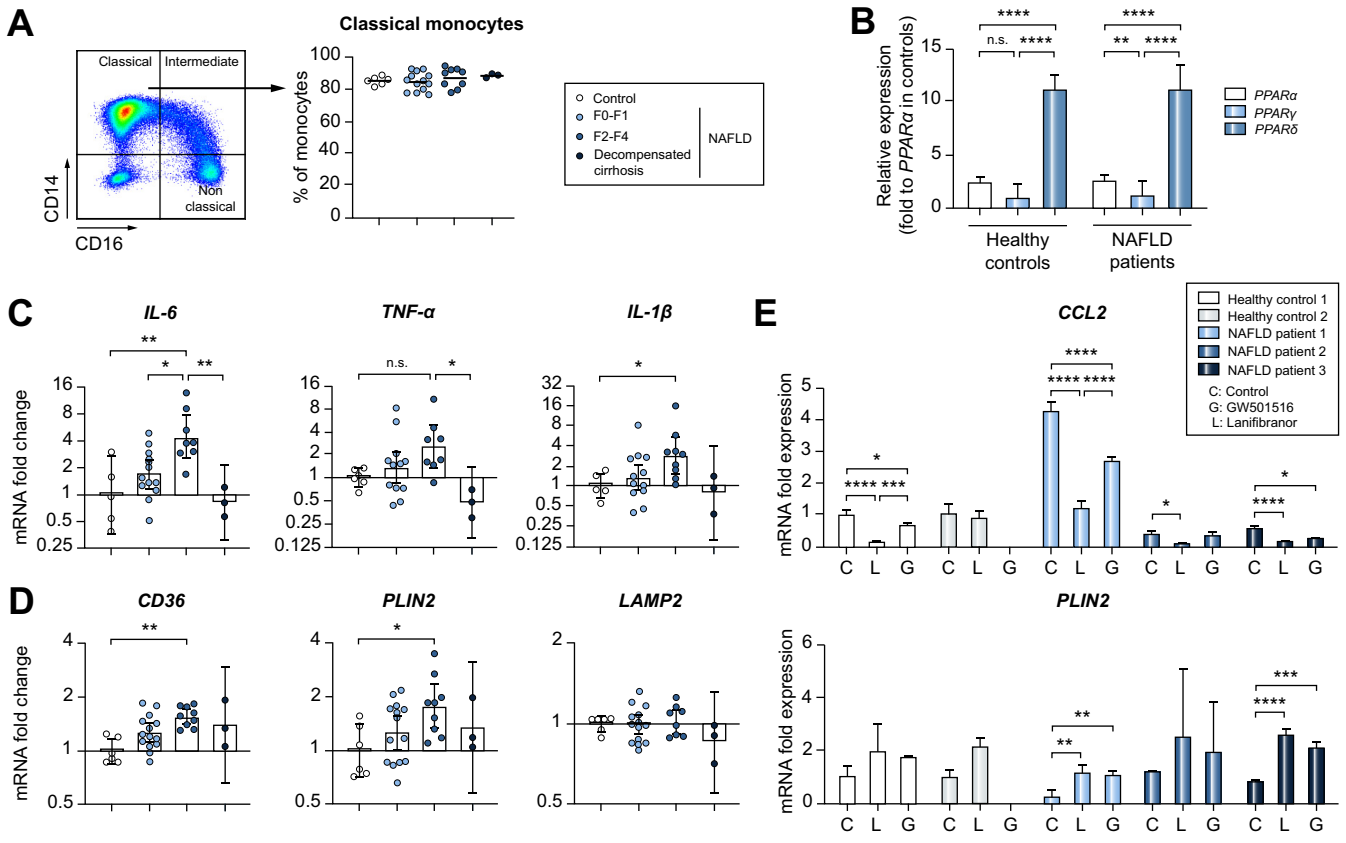

Fig. 8. Monocytes isolated from patients with NAFLD display the metabolic activation phenotype. Peripheral blood mononuclear cells were isolated from whole blood, obtained from healthy controls (n = 6) and patients with NAFLD (n = 26), and subsequently stained for monocyte-specific markers. (A) Representative flow cytometric plot and quantification of the classical monocyte subset ($CD3^-$, $CD20^-$, $CD56^-$, $CD14^+$, $CD16^-$ cells). (B–D) mRNA expression of PPAR isoforms (B), proinflammatory (C) and lipid metabolism (D) genes in classical monocytes. (E) Monocytes were isolated from whole blood and stimulated with lanifibranor. mRNA expression of *PLIN-2* and *CD36*. Data are presented as geometric mean ± 95% CI. **p* <0.05; ***p* <0.01; ****p* <0.001; *****p* <0.0001 (one-way ANOVA with *post hoc* test). NAFLD, non-alcoholic fatty liver disease.

PPARα activation, whereas PPARδ controlled hepatic inflammation and macrophage activation. Furthermore, lanifibranor improved liver fibrosis through the combined results of decreases in steatosis, liver damage and macrophage-mediated inflammation, as well as a direct deactivation of stellate cells, which is mainly driven by PPARγ.[4]

Importantly, the functions of human and mouse PPAR isoforms, in particular PPARα and PPARγ, are species dependent. For instance, the hepatic PPARα expression is lower in humans than mice, and PPARα affects hepatic glycolysis-gluconeogenesis only in the latter.[35] These differences could explain why PPARα disproportionally improves murine NAFLD compared to human NAFLD, while its effects in clinical studies are minor.[8] Conversely, whereas PPARγ agonists (pioglitazone) are potent in humans,[19] we (and others) discerned very limited to no effect on murine NAFLD. Nevertheless, in this study, the pan-PPAR agonist lanifibranor exceeded the species-linked positive effects of PPARα/PPARγ, which may be attributed to a PPARδ-dependent alteration of macrophage polarization. In line with this observation, the PPARα/δ agonist elafibranor retained some of its effect in *Ppara* knockout mice.[36]

Related to this, the current mouse models for NASH are unfortunately not optimal, and the specific research question often dictates which model may be best suited to study certain aspects of NASH.[37] In this study, we first employed the CDAA-HFD NAFLD model, which has the benefit of inducing severe inflammation and progression to fibrosis without the disadvantage of cachexia as observed in the methionine/choline-deficient diet.[26,38] Although this model does not display some particular features of metabolic disease (obesity, insulin resistance), these results are complemented by findings in the WD model, since lanifibranor decreased steatosis, liver injury and macrophage accumulation in both models.

In summary, we provide evidence for a strong therapeutic response to the pan-PPAR agonist lanifibranor in a preclinical model of steatohepatitis and fibrosis, and have identified *in vivo* and *in vitro* effects on hepatic macrophage accumulation and activation. While our work deepens the understanding of the myriad roles that PPAR isoforms play in the coordination of metabolism and inflammation during NAFLD, the translation into a clinical benefit for patients with NASH requires further work. Lanifibranor is currently being evaluated in a phase IIb clinical trial in adults with NASH and high inflammatory activity (NATIVE trial, NCT03008070), which will ultimately determine the therapeutic potential of pan-PPAR targeting on inflammation and fibrosis.

Abbreviations

ALT, alanine aminotransferase; BMDM, bone marrow-derived macrophages; CCl_4, carbon tetrachloride; CDAA-HFD, choline-

deficient, amino acid-defined high-fat diet; KCs, Kupffer cells; M-CSF, monocyte-colony stimulating factor; MFI, mean fluorescence intensity; Mme, 'metabolically activated' macrophage; MoMFs, monocyte-derived macrophages; NAFLD, non-alcoholic fatty liver disease; NASH, non-alcoholic steatohepatitis; OA, oleic acid; PA, palmitic acid; PPAR, peroxisome proliferator-activated receptor; WD, Western diet.

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Conflict of interest

VA, ED, CE and GW are employees of Inventiva. Work in FT's laboratory has received funding by Allergan, Galapagos, Inventiva and Bristol Myers Squibb.

Please refer to the accompanying ICMJE disclosure forms for further details.

Authors' contributions

FT designed and supervised the research. SL and TP performed the animal experiments and analysed the data. CP, AF and FH designed and conducted the biochip experiments. JH, CP, AG, VA, ED and CE conducted experiments. SL, KDM and AG collected and analysed human samples. GW and LD provided important technical support and intellectual input. SL, TP and FT wrote the manuscript. All authors reviewed and approved the manuscript.

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Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jhep.2020.04.025.

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Author names in bold designate shared first authorship

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