Received: 16 November 2020 | Revised: 23 June 2021 | Accepted: 25 June 2021

DOI: 10.1111/bph.15622

RESEARCH ARTICLE

Macrophage migration inhibitory factor exerts pro-proliferative and anti-apoptotic effects via CD74 in murine hepatocellular carcinoma

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Funding information
Bundesministerium für Bildung und Forschung, Grant/Award Numbers: FKZ 031L0041, STOP-FSGS-01GM1901A, FKZ 031 L0041; Deutsche Forschungsgemeinschaft, Grant/Award Numbers: DJ100/1-1, Project ID 36842431, Project-ID 403224013, DJ100/1-1

Background and Purpose: Macrophage migration inhibitory factor (MIF) is an inflammatory and chemokine-like protein expressed in different inflammatory diseases as well as solid tumours. CD74—as the cognate MIF receptor—was identified as an important target of MIF. We here analysed the role of MIF and CD74 in the progression of hepatocellular carcinoma (HCC) in vitro and in vivo.

Experimental Approach: Multilocular HCC was induced using the diethylnitrosamine/carbon tetrachloride (DEN/CCl_4) model in hepatocyte-specific *Mif* knockout (*Mif* $^{\Delta hep}$), *Cd74*-deficient, and control mice. Tumour burden was compared between the genotypes. MIF, CD74 and Ki67 expression were investigated in tumour and surrounding tissue. In vitro, the effects of the MIF/CD74 axis on the proliferative and apoptotic behaviour of hepatoma cells and respective signalling pathways were assessed after treatment with MIF and anti-CD74 antibodies.

Key Results: DEN/CCl_4 treatment of *Mif* $^{\Delta hep}$ mice resulted in reduced tumour burden and diminished proliferation capacity within tumour tissue. In vitro, MIF stimulated proliferation of Hepa 1–6 and HepG2 cells, inhibited therapy-induced cell death

Abbreviations: AMPK, AMP-activated protein kinase; BrdU, bromodeoxyuridine; DEN, diethylnitrosamine; ERK1/2, extracellular-signal regulated kinases 1 and 2; GEPIA, gene expression profiling interactive analysis; H&E, haematoxylin-eosin; HCC, hepatocellular carcinoma; HIF-1α, hypoxia-inducible factor 1α; HSC, hepatic stellate cell; ISO-1, MIF antagonist (CAS 478336-92-4); MIF, macrophage migration inhibitory factor; NASH, non-alcoholic steatohepatitis; siRNA, small interfering RNA; TME, tumour microenvironment; TNM, tumour, node and metastasis; TUNEL, terminal deoxynucleotidyl transferase dUTP nick end labeling.

and induced ERK activation. The investigated effects could be reversed using a neutralizing anti-CD74 antibody, and *Cd74*$^{-/-}$ mice developed fewer tumours associated with decreased proliferation rates.

Conclusion and Implications: We identified a pro-tumorigenic role of MIF during proliferation and therapy-induced apoptosis of HCC cells. These effects were mediated via the MIF cognate receptor CD74. Thus, inhibition of the MIF/CD74 axis could represent a promising target with regard to new pharmacological therapies aimed at HCC.

KEYWORDS
apoptosis, CD74, chemokine, hepatocellular carcinoma, proliferation

1 | INTRODUCTION

Hepatocellular carcinoma (HCC) is the most common malignancy of the liver with rising incidence and a 5-year survival rate of only 5.1% (Njei et al., 2015). Its pathogenesis varies based on the underlying aetiology—in this context, the majority of cases develop in patients with pre-existing liver cirrhosis due to alcoholic or fatty liver disease as well as chronic viral hepatitis (Fattovich et al., 2004). The persistent injury of liver tissue favours malignant transformation of hepatocytes that suppresses apoptosis and promotes tumour proliferation and neovascularization (Schwabe & Luedde, 2018; Sia et al., 2017). Currently, a major research goal is to further elucidate the molecular mechanisms underlying hepatocarcinogenesis to improve and individualize treatment. Of note, tumour progression is not only determined intrinsically by the mutational alteration of the tumour cells themselves. In fact, changes in the production of soluble factors such as cytokines and associated signalling pathways within the tumour microenvironment (TME)—a complex network of tumour, stromal and immune cells—have been identified to crucially promote HCC growth (Saviano et al., 2019).

One such cytokine is **macrophage migration inhibitory factor (MIF)**. MIF is a pleiotropic inflammatory cytokine, discovered over 50 years ago as a soluble factor (Bloom & Bennett, 1966). Since then, MIF has been characterized in different pathological conditions including solid tumours, such as colorectal, lung, breast, head and neck and prostate cancer (Kindt et al., 2016). In these circumstances, MIF was identified to drive carcinogenesis by influencing cell proliferation, apoptosis inhibition, immune cell infiltration, metastasis and other oncogenic processes (Choudhary et al., 2013; Dumitru et al., 2011; Verjans et al., 2009). Especially regarding the inhibition of apoptosis, MIF's interaction with its cognate receptor **CD74**, which is the cell surface expressed form of the HLA class II histocompatibility antigen γ chain, has been studied in detail (Leng et al., 2003; Shi et al., 2006). Here, MIF/CD74 signalling was identified to regulate tumour cell survival (Lue et al., 2007). Other studies revealed a MIF/CD74-mediated phosphorylation of extracellular-signal regulated kinases 1 and 2 **(ERK1/2)**, thereby triggering cell proliferation (Leng et al., 2003). Moreover, MIF/CD74 silencing resulted in decreased NF-κB signalling as well as mitochondrial fragmentation followed by increased apoptosis in different human cancer cell lines (De et al., 2018).

Besides its role in promoting tumourigenesis, MIF has been functionally implicated in the progression of chronic liver disease. MIF is expressed in multiple types of both parenchymal and non-parenchymal cells present in the chronically diseased liver: For instance, MIF is secreted by adipocytes and fibroblasts as well as several immune cells, endothelial cells and Kupffer cells upon inflammatory stimuli. Interestingly, as shown by our previous study, hepatocytes represent the main source of MIF within the liver during the progression of non-alcoholic steatohepatitis (NASH) (Heinrichs et al., 2021). In the liver, CD74 is expressed on hepatocytes as well as hepatic stellate cells (HSC) (Maubach et al., 2007). Specifically, the MIF/CD74 axis was identified to exert hepatoprotective effects in a model of NASH, as well as liver fibrosis induced by chronic toxic liver injury (Heinrichs et al., 2011, 2014). The antifibrotic effect was found to be mediated by the CD74/AMP-activated protein kinase **(AMPK)** signalling pathway in HSCs leading to attenuated fibrogenic HSC activation.

What is already known?

- The MIF/CD74 axis has been characterized to promote cancer progression in several solid tumours.

What does this study add?

- MIF exerts CD74-mediated carcinogenic effects during hepatocarcinogenesis and inhibits therapy-induced apoptosis of HCC cells.

What is the clinical significance?

- MIF and CD74 represent potential targets in prospective treatments of hepatocellular carcinoma.

In the present study, we hypothesized that MIF might also play an essential role during hepatic carcinogenesis. We therefore investigated the effects of MIF influence on HCC growth in an experimental model of murine HCC in vivo. Our data provide insights into the functional engagement of MIF with its receptor CD74 during proliferation and apoptosis of hepatoma cells in vitro. Our data represent an important experimental basis to evaluate whether targeting the MIF/CD74 axis could become relevant in prospective approaches to the treatment of HCC

2 | METHODS

2.1 | Animal studies and induction of murine hepatocellular carcinoma

Animal studies were carried out in accordance with the law of the State Agency for Nature, Environment and Consumer Protection of North Rhine-Westphalia, Germany and approved by the local Animal Ethical Committee. Animal studies are reported in compliance with the ARRIVE guidelines (Percie du Sert et al., 2020) and with the recommendations made by the *British Journal of Pharmacology* (Lilley et al., 2020). In general, mice were housed at constant temperature (20-21°C) and humidity (50%) in transparent cages (two to five mice per cage) with nesting material (Nestlets: https://www.plexx.eu/products/nestlets/) and had ad libitum access to water and standard mouse chow (ssniff; Spezialdiäten GmbH, Soest, Germany).

To investigate the role of MIF and CD74 in murine HCC, male mice from different mouse strains were included in our analysis: First, *Mif*-floxed mice were previously established in our laboratory (Fingerle-Rowson et al., 2003) and cross-bred with mice expressing *Cre*-recombinase under the control of a hepatocyte-specific AFP-enhancer-albumin promoter resulting in $Mif^{flox/flox}$AlfpCre^{+} mice with a hepatocyte-specific *Mif* knockout, named $Mif^{\Delta hep}$ mice. $Mif^{flox/flox}$AlfpCre^{-} mice served as controls. In addition, CD74-deficient mice ($Cd74^{-/-}$) (Mun et al., 2013) as well as wildtype C57BL/6 mice obtained from The Jackson Laboratory (Bar Harbor, ME, USA) or Charles River Laboratories (Wilmington, MA, USA) respectively, were used.

To induce hepatocellular carcinoma, the previously established and well-standardized DEN/CCl_4 mouse model was performed in a randomized fashion as previously described (Uehara et al., 2014). Mice were treated with a combination of mutagenic diethylnitrosamine (DEN) and the hepatotoxic agent CCl_4. Mice ($n \geq 6$ per group) received a single i.p. injection of DEN (100 mg kg^{-1} *i.p.*) at day 15 of age followed by repetitive i.p. injections of low dose CCl_4 (0.5 ml kg^{-1} *i.p.*) once a week (week 4–26) resulting in formation of HCC lesions in fibrotic liver tissue as we aimed to resemble the human situation. Untreated and CCl_4-treated (only fibrosis induction) wildtype or $Mif^{flox/flox}$AlfpCre^{-} mice served as controls.

Hence, all mice were randomized to receive either DEN/CCl_4 or CCl_4 only treatment or left untreated to complement the three groups of comparison. During the study protocol, mice were monitored daily using a previously established score sheet that was further evaluated and confirmed by the State Agency for Nature, Environment and Consumer Protection of North Rhine–Westphalia, Germany. No necessity of analgesia occurred during the study protocol. Mice were killed at the age of 26 weeks and 48 h after the last CCl_4 injection, by isoflurane inhalation in a closed chamber where the effective gas concentration (5%) was reached within seconds. Tumour burden (size of the biggest tumour and tumour number per liver) was assessed by the same experimenter in a blinded manner.

2.2 | Measurement of gene expression via RT-qPCR

Isolation of total RNA from snap-frozen liver tissue samples was performed as previously described (Berres et al., 2010). To distinguish between tumour and adjacent tissue-associated effects, liver tissue from tumour bearing mice was separated macroscopically into tumour and surrounding tissue. Quantitative real-time PCR reactions were performed with predesigned TaqMan Gene Expression Assays (Applied Biosystems, ThermoFisher Scientific, Waltham, MA, USA) and was carried out for *Mif*, *Col1a1*, *CD74*, *Pcna* and *Ki67*. Data were normalized to 18S expression and were analysed by the $2^{-\Delta\Delta CT}$ method relative to control gene-of-interest expression. Data are presented as mean ± SD.

2.3 | Immunofluorescence and immunohistochemically staining

For immunohistochemical analysis, 5-μm tissue sections were deparaffinized. Endogenous peroxidase activity was blocked by incubating the sections in methanol with 0.3% hydrogen peroxide for 10 min at room temperature. Heat-induced antigen retrieval was performed with citrate buffer (pH 6.0, Vector) in a steamer for 20 min. To block non-specific staining, Avidin/Biotin Blocking Kit (Vector Laboratories Ltd., Burlingame, CA, USA) was incubated with the section, each for 10 min at room temperature. Sections were stained for 1 h at room temperature with anti-Ki67 antibody, clone D3B5 (Cell Signalling, Frankfurt am Main, Germany), diluted in 1%BSA/PBS. Visualization of primary antibody binding was performed via biotin-labelled secondary antibody and Peroxidase Substrate Kit DAB (Vector Laboratories, Peterborough, UK). Slides were counterstained with haematoxylin. Ki67 positive cells were counted to yield the positive cells:total cells ratio, from three independent magnification fields per tumour per slide). At least one slide per mouse was evaluated and tissue from $n \geq 6$ mice per group was analysed. To histologically assess liver fibrosis, Sirius red staining was performed as previously described (Heinrichs et al., 2011): Sirius red-positive area was quantified per slide using the software Image J/NIH. Our analysis included three independent magnification fields per slide and at least one slide per mouse was evaluated. For both Ki67 immunohistochemistry and Sirius red staining, data were expressed as means ± SD of $n \geq 6$ mice

per group. MIF staining was performed on formalin-fixed tissue with heat-induced antigen retrieval in citrate buffer (pH 6.0) and processed as previously described (Djudjaj et al., 2017). The primary anti-human/mouse MIF antibody was obtained from Sigma–Aldrich (Darmstadt, Germany), secondary antibody anti-rabbit Alexa647 was from Life Technologies (Carlsbad, CA, USA).

2.4 | Cell proliferation assay

The murine and human hepatoma cell lines Hepa 1–6 and HepG2 respectively (CLS Cell Lines Service GmbH, Eppelheim, Germany) were used to investigate the role of MIF in proliferation of immortalized hepatoma cells in vitro. Hepa 1–6 or HepG2 cells were cultured on 96 well plates in DMEM supplemented with 10% FCS and penicillin–streptomycin (100 U ml^{-1}) and were grown 60% confluent. Second, cells were starved on DMEM+0.5% FCS for 2 h. After starvation, cells were incubated with DMEM + 0.5% FCS (control) or stimulated with recombinant murine MIF (rmMIF) diluted in DMEM + 0.5% FCS at different concentrations ranging from 10 to 1000 ng ml^{-1} or as indicated for 24 h. To test, whether the effects of MIF on the proliferative behaviour depends on binding to CD74, both cell lines cells were pre-incubated with either a rat anti-mouse (12 μg ml^{-1}) or anti-human CD74 antibody (10 μg ml^{-1}) or the MIF inhibitor ISO-1 (800 nM) for 60 min and afterwards stimulated with recombinant murine or human MIF in DMEM+0.5% FCS for 24 h. Proliferation was assessed by a colorimetric immunoassay based on the measurement of BrdU incorporation during DNA synthesis (Cell Proliferation ELISA; Diagnostics, Mannheim, Germany) following the manufacturer's instructions.

2.5 | Cell death assays

To evaluate cell death in Hepa 1–6 and HepG2 cells respectively, cells were seeded on object plates and cultured until confluent. Subsequently, cells were incubated with MIF in increasing concentrations from 50 to 500 ng ml^{-1}. In a second approach, cells were incubated with DMSO or **sorafenib** (25 μM or 5 μM for human assays, dissolved in DMSO) after pre-incubation with 300 ng ml^{-1} MIF and/or an anti-CD74 antibody (12 μg ml^{-1} for murine and 10 μg ml^{-1} for human assays) and/or the MIF inhibitor ISO-1 (800 nM). Sorafenib was used as a cytotoxic agent to induce cell death. Cell death rate was assessed by subsequent terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL)-assay to detect DNA fragmentation in apoptotic cells. Therefore, object plates were fixed in 4% paraformaldehyde for 10 min at room temperature. After 3x5 min of washing in PBS, the sections were treated with 3% H_2O_2 in methanol for 10 min at room temperature and with 0.1% Triton in 0.1% sodium citrate for 2 min at 4 °C. The object plates were incubated in TUNEL-Mix (in situ Cell Death Detection Kit, Roche, Mannheim, Germany) overnight. After another washing step, nuclei staining was performed using DAPI and photomicrographs were taken with a fluorescence microscope (Zeiss, Germany). TUNEL-positive cells were counted (positive cells/total cells, three independent magnification fields per slide) and $n \geq 6$ independent experiments per group were evaluated. Data are expressed as means ± SD of $n \geq 6$ independent experiments/group. Determination of cell counting was performed in a blinded manner.

2.6 | Western blot

To test the influence of MIF/CD74 signalling on ERK phosphorylation Hepa 1–6 cells were incubated with rmMIF (100 ng ml^{-1}) with or without pre-incubation with an anti-CD74 antibody (12 μg ml^{-1}, BD Pharmingen, San Diego, CA, USA) in DMEM + 0.5% FCS for 20 min. After treatment, cells were lysed and subjected to SDS-PAGE. ERK activation was revealed with an antibody against phosphorylated ERK (anti-pERK, 1:1000) and total ERK1/2 (anti-ERK1/2, 1:1000) as well as actin detected for standardization. Anti-rabbit horse-radish peroxidase (HRP)-conjugated antibody (1:10000, GE Healthcare, Freiburg, Germany) was used for development and signals quantitated by chemiluminescence using an Odyssey® Fc imager. Unprocessed scans of the Western Blots are supplied in the supplemental data file.

2.7 | Data and statistical analysis

Data are shown as means ± SD. Continuous variables were compared by two-sided *t* test with Welch's correction in case of unequal variances. When comparing more than two group means, one-way ANOVA was applied. In case of statistical significance ($P < 0.05$) and exclusion of significant variance inhomogeneity, one-way ANOVA was followed by post hoc tests. Here, regular lines indicate significance resulting from one-way ANOVA, capped lines indicate a significant difference between two groups as revealed by post-hoc test. Data are expressed as means ± SD of 10 mice per group or ≥5 independent in vitro experiments. The exact number of animals for each experiment are stated in the figure legends. Values of $P < 0.05$ were considered significant. Statistical tests were performed using GraphPad Prism 5.0 (RRID:SCR_002798; GraphPad, San Diego, CA, USA).

2.8 | Materials

Murine recombinant MIF was prepared as previously described (Bernhagen et al., 1994, 2007), has purity of >95% and is endotoxin-free as determined by Limulus assay. Human recombinant MIF was purchased by R&D Systems (Minneapolis, MN, USA). Neutralizing rat anti-mouse CD74 antibody (clone In-1) was obtained from BD Pharmingen (San Diego, CA, USA); neutralizing mouse anti-mouse and -human CD74 antibody (clone LN-2) was obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The MIF antagonist **ISO-1**

(CAS 478336-92-4) was purchased from Sigma–Aldrich (Taufkirchen, Germany). Sorafenib (Bayer Schering Pharma AG, Germany) was dissolved in DMSO and used to induce cell death in hepatoma cells in vitro.

2.9 | Nomenclature of targets and ligands

Key protein targets and ligands in this article are hyperlinked to corresponding entries in the IUPHAR/BPS Guide to Pharmacology (http://www.guidetopharmacology.org) and are permanently archived in the Concise Guide to PHARMACOLOGY 2019/20 (Alexander et al., 2019; McGrath et al., 2015).

3 | RESULTS

3.1 | Macrophage migration inhibitory factor expression is up-regulated in murine tumour tissue and *Mif* $^{\Delta hep}$ mice show reduced tumour burden in the DEN/CCl_4 HCC model

To gain first insights in the potential regulation of MIF expression during murine hepatic carcinogenesis, C57BL/6 wildtype mice were treated with DEN/CCl_4 to induce multilocular HCC. After killing the mice, the liver tissue of tumour bearing mice was macroscopically dissected into tumour- and surrounding tissue and *Mif* expression at the mRNA level in these different tissue types was analysed. *Mif* expression was induced in the tumour tissue of tumour-bearing mice when compared to liver tissue from CCl_4-treated fibrosis control mice or untreated mice and showed a trend towards up-regulated MIF expression within tumours compared to surrounding tissue (Figure 1a). Next, we confirmed an intratumor overexpression of MIF at the protein level using immunohistochemical staining of liver sections of untreated and fibrotic control tissue as well as liver tissue from tumour-bearing C57BL/6 wildtype mice. MIF expression was strikingly increased in the tumour compared to surrounding tissue. Interestingly, our staining analysis revealed that the tumour cells themselves appeared to be the major source of MIF within the tumoral tissue (Figure 1b).

Next, we induced HCC growth in mice with a hepatocyte-specific *Mif* knockout generated by crossing *Mif* $^{flox/flox}$ with AlfpCre^{+} mice, hereinafter indicated as *Mif* $^{\Delta hep}$ mice, and studied the DEN/CCl_4 model. Significantly diminished *Mif* mRNA expression in the liver tissue of DEN/CCl_4 treated *Mif* $^{\Delta hep}$ mice had been previously confirmed (Figure S1). Tumour number and size of the biggest tumours were analysed in explanted livers. Strikingly, the tumour numbers were significantly reduced in mice with impaired hepatocyte-specific MIF expression (*Mif* $^{\Delta hep}$ mice) compared to MIF-proficient control mice (AlfpCre^{-} mice, Figure 1c). Additionally, there was a trend towards a smaller diameter of the biggest tumour in *Mif* $^{\Delta hep}$ mice (Figure 1d). Representative images of tumour-bearing livers of both genotypes illustrating differences in tumour burden are shown in Figure 1e,f.

3.2 | *Mif*$^{\Delta hep}$ mice do not develop reduced fibrosis nor decreased inflammatory response during DEN/CCl_4 treatment when compared to controls

Based on our observation of a significantly decreased tumour burden in *Mif* $^{\Delta hep}$ mice, we next investigated potential pro-carcinogenic MIF-dependent mechanisms in chronic liver disease. One hypothesis was that MIF might potentiate liver injury during DEN/CCl4 treatment by triggering hepatic fibrogenesis and thereby further promote tumour growth. We compared fibrosis staging in DEN/CCl_4-treated *Mif*$^{\Delta hep}$ mice and *Mif*$^{flox/flox}$AlfpCre^{-} control mice. Sirius Red staining revealed comparable fibrotic area fractions in both CCl_4- as well as DEN/CCl_4-treated animals of both genotypes (Figure 2a,b). To confirm this result, we complemented RT-qPCR analysis of *Col1α1* mRNA expression in liver tissue from both control and *Mif* $^{\Delta hep}$ mice. In line with the histological results, we did not detect differences in collagen expression in both genotypes during chronic liver injury (Figure 2c). We concluded that MIF does not exert its pro-carcinogenic effects based on triggering fibrogenesis during DEN/CCl_4-treatment.

Moreover, as chronic CCl_4-treatment induces an inflammatory response, we were curious to evaluate the extent of the intrahepatic inflammatory response as well as possible differences in liver function in control and Mif $^{\Delta hep}$ tumour mice. We therefore determined bilirubin and transaminases concentrations in the serum of treated Mif$^{\Delta hep}$ and control mice. As bilirubin concentrations were not increased in all tested animals, there was no evidence of decreased liver function neither during CCl_4- nor DEN/CCl_4-treatment (Figure S3a). Moreover, the investigated genotypes developed comparable transaminase levels as a correlate of chronic liver injury during CCl_4-treatment (Figure S3b,c). The extent of transaminase increase during DEN/CCl_4-treatment did not differ from that during CCl_4-treatment reflecting chronic and constant liver injury that was not further increased in tumour compared to fibrosis mice. As further shown by representative haematoxylin and eosin (H&E) staining of tumour and surrounding tissue (Figure S3d), flow cytometric (FACS) analysis of CD45-positive immune cells within tumour and surrounding tissue of control and Mif$^{\Delta hep}$ tumour mice (Figure S3e) as well as bulk tissue RT-qPCR analysis of inflammatory markers including ***Tnf-α***, ***Il-6*** and ***Il-10*** (Figure S3f–k) and comparison of liver weights (Figure S3l), we neither noticed necrotic areas nor significant differences in the severity of the inflammatory response within tumour and surrounding tissue when comparing the investigated genotypes.

3.3 | Macrophage migration inhibitory factor promotes liver tumour cell proliferation both in vivo and in vitro

Next, we hypothesized that MIF might up-regulate the proliferation of tumour cells to promote tumour growth. We assessed the expression of Ki67 as a marker of proliferation in the tumour and surrounding tissue of both *Mif*$^{flox/flox}$AlfpCre- control and *Mif* $^{\Delta hep}$ mice. RT-qPCR analysis revealed a fivefold increase in expression of

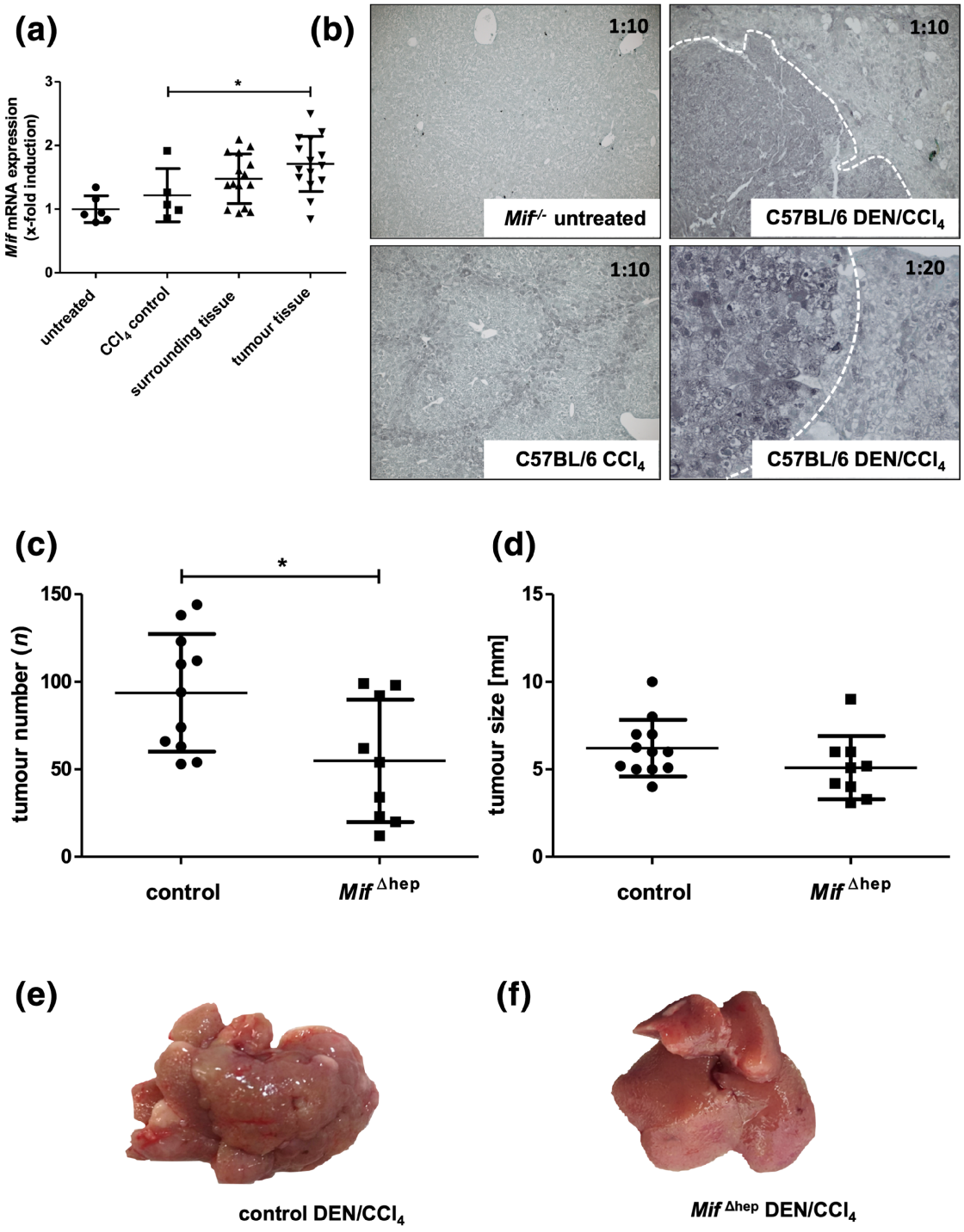

FIGURE 1 *Mif* $^{\Delta hep}$ mice show reduced tumour burden in DEN/CCl_4 model of murine HCC. (a) *Mif* expression is increased in tumour tissue compared to liver tissue of untreated and fibrosis control mice as analysed by RT-qPCR. *$P < 0.05$, significantly different as indicated; one-way ANOVA followed by post hoc test. (b) Immunohistochemical analysis of macrophage migration inhibitory factor (MIF) reveals increased MIF expression in tumour tissue (section to the left of the dotted line; amplification 1:10 and 1:20) of DEN/CCl_4-treated wildtype mice compared to surrounding tissue (section to the right of the dotted line, amplification 1:10). MIF IHC staining in liver tissue of untreated *Mif*$^{-/-}$ mice and CCl_4-treated wildtype mice were used as (staining) controls (left panel). (c) Tumour number is significantly lower in DEN/CCl_4-treated *Mif* $^{\Delta hep}$ mice, compared with control mice. *$P < 0.05$, significantly different as indicated; one-way ANOVA followed by post hoc test. However, in (d), the tumour size is not significantly different. In (e, f), representative pictures of a resected tumour bearing liver of a *Mif*$^{flox/flox}$AlfpCre^{-} compared to a *Mif* $^{\Delta hep}$ mouse. Data shown are individual values with means ± SD from 10 mice per group

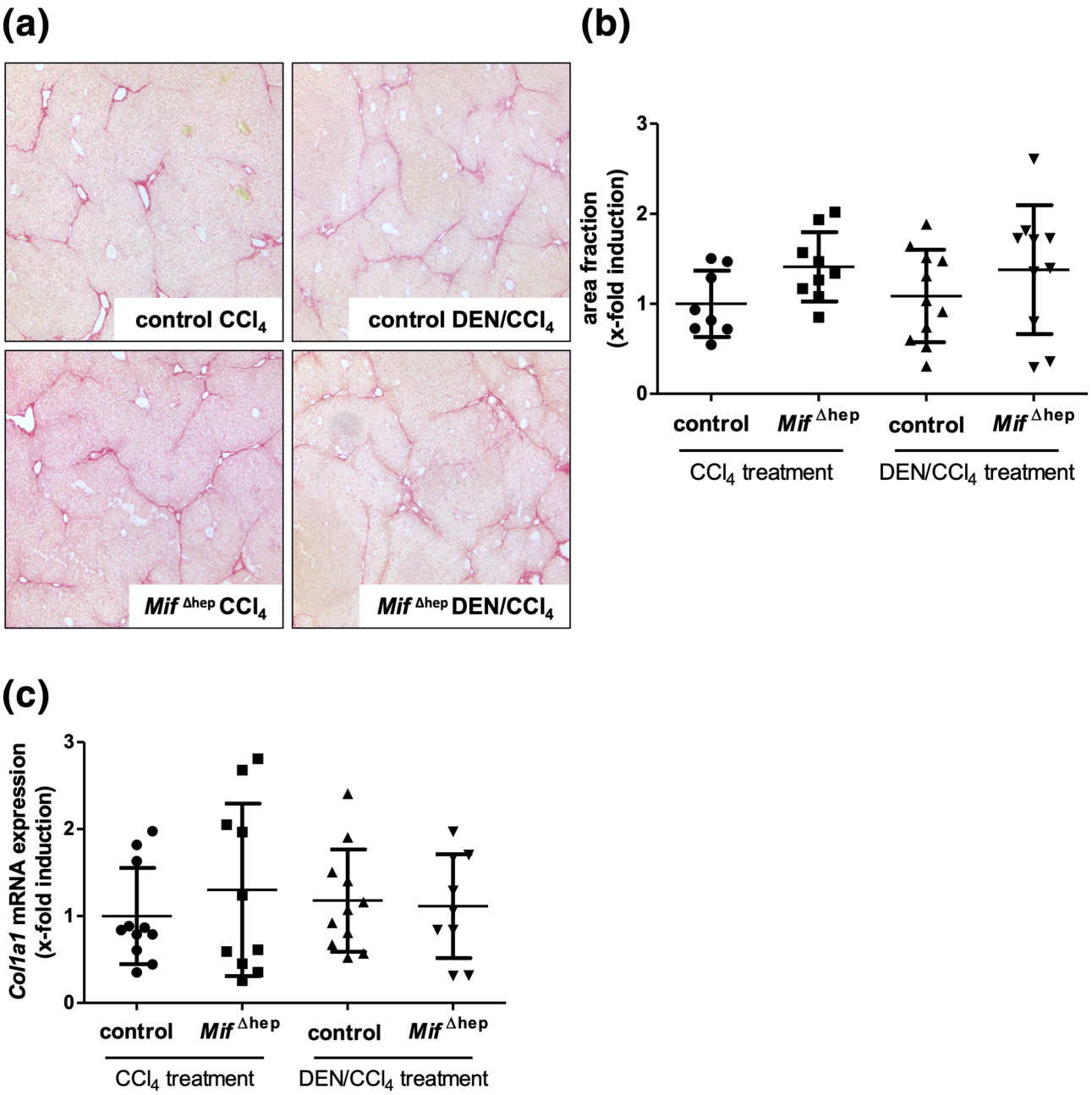

FIGURE 2 *Mif* $^{\Delta hep}$ mice do not develop less fibrosis than wildtype mice during DEN/CCl_4-treatment as surrogate for liver damage. (a) Sirius red staining reveals equivalent Sirius red positive area in *Mif* $^{\Delta hep}$ mice (lower stainings) compared to wildtype mice (upper stainings) treated with CCl_4 only or DEN/CCl_4. Representative stainings are shown. (b) Quantification of Sirius red positive area in liver tissue of CCl_4- or DEN/CCl_4-treated control and *Mif* $^{\Delta hep}$ did not reveal significant differences. (c) *Col1α1* expression at the mRNA level did not differ in *Mif* $^{\Delta hep}$ tumour mice compared with control as RT-qPCR of liver tissue from CCl_4 or DEN/CCl_4-treated control and *Mif* $^{\Delta hep}$ mice reveals. Data shown are individual values with means ± SD from 10 mice per group

Ki67 in tumour tissue of control mice when compared to *Mif* $^{\Delta hep}$ mice, whereas there was no difference when comparing *Ki67* expression in the surrounding tissue of the two genotypes (Figure 3a). Similarly, a significant difference between the compared groups was evident for *Pcna* as another marker of cell cycle progression (Figure 3b). Subsequently, immunohistochemical analysis of Ki67 expression in tumour tissue of *Mif* $^{\Delta hep}$ mice compared to control mice was performed. Here, the number of Ki67-positive cells in tumour tissue of *Mif* $^{\Delta hep}$ mice compared to control mice showed a trend towards a reduced proliferation (Figure 3c,d; representative stainings are shown). To investigate whether the pro-proliferative effect of MIF could be reproduced in vitro, we performed proliferation assays using the murine hepatoma cell line Hepa 1–6. Hepa 1–6 cells were incubated with increasing concentrations of MIF and

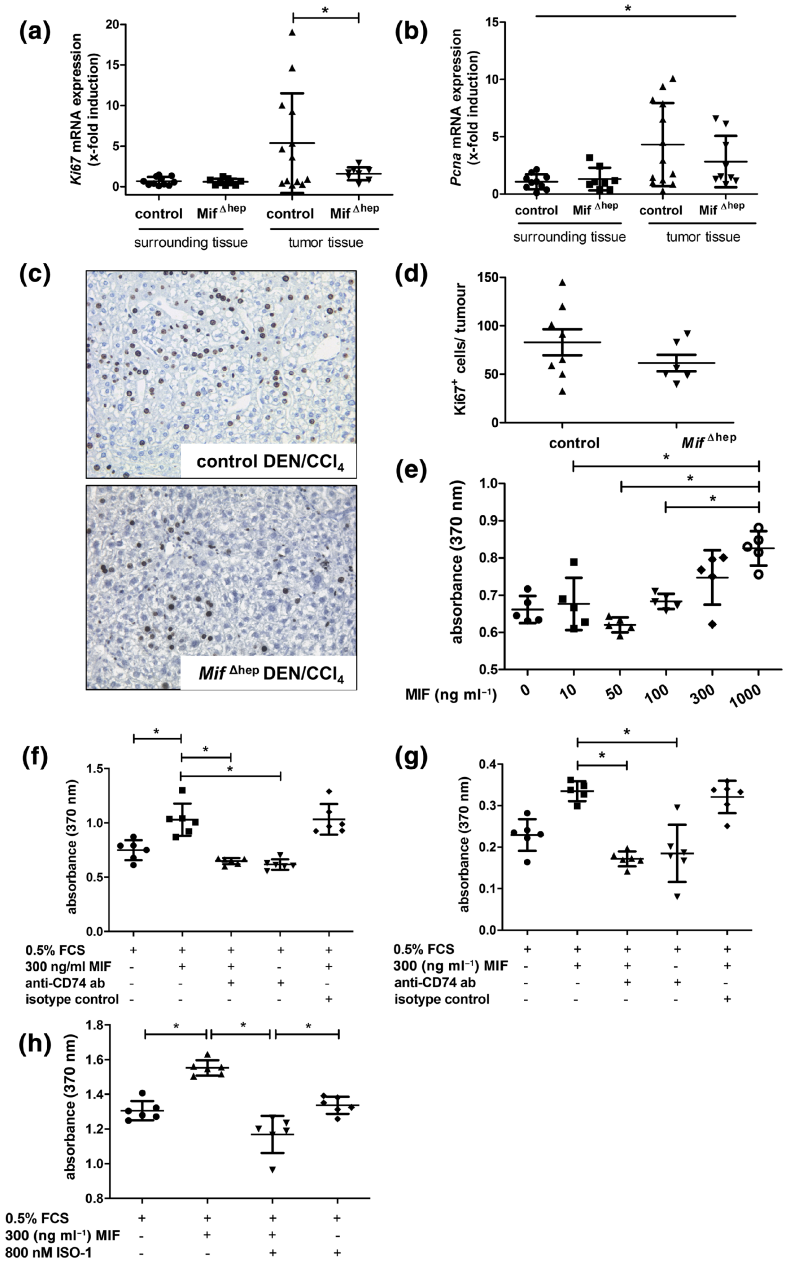

FIGURE 3 Macrophage migration inhibitory factor (MIF) promotes liver tumour cell proliferation both in vivo and in vitro. (a) *Ki67* mRNA expression is significantly decreased in tumour tissue of *Mif* $^{\Delta hep}$ mice compared to *Mif*$^{flox/flox}$AlfpCre^{-} control mice as RT-qPCR of tumour and surrounding tissue reveals. (b) *Pcna* mRNA expression significantly differs between the four compared means of the groups surrounding and tumour tissue respectively of *Mif* $^{\Delta hep}$ mice compared to *Mif*$^{flox/flox}$AlfpCre^{-} mice and trends to be decreased in tumour tissue of *Mif* $^{\Delta hep}$ mice compared to *Mif*$^{flox/flox}$AlfpCre^{-} control mice as RT-qPCR reveals. (c) Representative IHC stainings of Ki67 positive cells in tumour tissue of DEN/CCl_4-treated *Mif* $^{\Delta hep}$ compared to *Mif*$^{flox/flox}$AlfpCre^{-} control mice. (d) Quantification of immunohistochemical analysis of Ki67 positive cells per tumour of DEN/CCl_4-treated Mif $^{\Delta hep}$ compared to Mif$^{flox/flox}$AlfpCre^{-} control mice reveals a trend towards less Ki67 positive cells in tumour tissue of Mif $^{\Delta hep}$ mice. (e) BrdU assay shows a dose-dependent effect on proliferation capacity in Hepa 1–6 cells after incubation with recombinant murine MIF in vitro. (f) Incubation of an anti-CD74 antibody before stimulation of Hepa 1–6 cells with MIF blocks its effect on the proliferation of Hepa 1–6 cells. (g) This effect is confirmed during application of an anti-CD74 antibody before stimulation of human HepG2 cells with MIF. (h) Application of an MIF-specific inhibitor ISO-1 together with recombinant MIF also inhibits MIF's pro-proliferative effect. Data shown are individual values with means ± SD from 8 mice per group or ≥5 independent in vitro experiments. *$P < 0.05$, significantly different as indicated; one-way ANOVA followed by post hoc test

the proliferation activity was quantified by BrdU incorporation. MIF promoted proliferation of Hepa 1–6 cells in a concentration-dependent manner (Figure 3e). Subsequently we investigated whether the pro-proliferative effect of MIF might depend on MIF binding to CD74. The expression of CD74 by Hepa 1–6 cells had previously been reported (Heinrichs et al., 2011). We pre-incubated

Hepa 1–6 cells with an anti-CD74 antibody followed by MIF addition. The proliferation of cells incubated with the anti-CD74 antibody was significantly decreased compared to Hepa 1–6 cells that had been incubated with MIF alone (Figure 3f). This effect was further confirmed in the human hepatoma cell line HepG2 (Figure 3g). Interestingly, the incubation with the anti-CD74 antibody alone also resulted in decreased proliferative capacity, indicating an autocrine effect of HepG2-derived endogenous MIF. In contrast, the corresponding isotype control did not influence the proliferation behaviour when applied in combination with MIF. Moreover, when incubating the Hepa 1–6 cells with MIF in presence of the MIF antagonist ISO-1, the pro-proliferative effect of MIF was significantly decreased (Figure 3h). In summary, these data are consistent with the interpretation that the pro-proliferative function of MIF is mediated by the interaction of MIF and CD74 and represents a MIF-specific effect.

3.4 | Macrophage migration inhibitory factor inhibits therapy-induced apoptosis of tumour cells in vitro in a CD74-dependent manner

We considered that MIF might show further pro-carcinogenic actions on malignant hepatocytes. We therefore investigated MIF's role in apoptosis of Hepa 1–6 cells. Hepa 1–6 cells were incubated with increasing concentrations of MIF and TUNEL staining was performed to quantify spontaneous culture-associated apoptosis. The number of TUNEL-positive cells was reduced after incubation with higher concentrations of MIF (Figure 4a), however, as expected, spontaneous apoptosis in the culture system was a rather rare event in all settings. From the perspective of clinical translation, we next tested whether MIF/CD74 signalling also affected therapy-induced cell death, which could interfere with response to HCC treatment. The multi-kinase inhibitor sorafenib, a standard first-line therapeutic agent for advanced HCC, was applied to induce cell death in Hepa 1–6 cells. Sorafenib led to a death rate of approximately 18% of treated cells. Pre-incubation of Hepa 1–6 cells with MIF protected the cells from sorafenib-induced death, with only 7% TUNEL-positive cells detected (Figure 4b, representative stainings are shown in Figure 4c). Strikingly, the inhibition of MIF/CD74 interaction by an anti-CD74 antibody partly reversed this effect (Figure 4b), suggesting that the MIF/CD74 axis contributed to MIF's anti-apoptotic effect. This effect was only observed when MIF and the anti-CD74 antibody were applied during sorafenib-induced cell death; in contrast, application of MIF and anti-CD74 antibody without chemotherapeutic agent did not result in a significantly increased cell death, compared to MIF only treatment. The effect was further confirmed in HepG2 cells (Figure 4d; representative TUNEL stainings shown in Figure 4e). In accordance, the MIF inhibitor ISO-1 showed a trend towards increased cell death rate during therapy-induced cell death, compared to treatment with sorafenib and MIF alone (Figure 4f).

3.5 | *Cd74*$^{-/-}$ mice show reduced tumour burden in the DEN/CCl_4 model

Based on our results supporting a pro-proliferative and anti-apoptotic, CD74-dependent action of MIF during hepatocarcinogenesis, we analysed whether the absence of the CD74 receptor might influence tumour growth. First, we tested whether CD74 is expressed in tumour and surrounding tissue of DEN/CCl_4-treated C57BL/6 mice. We observed a fivefold up-regulated expression of *CD74* on mRNA level in tumour tissue, compared to untreated and fibrosis control mice (Figure 5a). Subsequently, *Cd74*$^{-/-}$ mice were treated with DEN/CCl_4 to induce HCC growth. Assessment of tumour burden revealed a significantly reduced tumour number in *Cd74*$^{-/-}$ compared to wildtype controls (Figure 5b). Accordingly, there was a strong trend towards smaller tumours in mice lacking CD74 (Figure 5c). Representative images of the tumour bearing livers of both genotypes are shown in Figure 5d,e. Consistent with our results in DEN/CCl_4-treated *Mif* $^{\Delta hep}$ mice, Sirius Red staining and *Col1α1* mRNA expression analysis revealed comparable fibrotic area fractions in both CCl_4-as well as DEN/CCl_4-treated animals of both *Cd74*$^{-/-}$ and control animals (Figure S2). We concluded that neither MIF nor CD74 exerts its pro-carcinogenic effects based on triggering fibrogenesis during DEN/CCl_4-treatment. Moreover, serum bilirubin and transaminase concentrations as well as tissue necrosis and inflammatory infiltrates evaluated by H&E staining as well as flow cytometry-based analysis of CD45-positive cells were comparable in *Cd74*$^{-/-}$ surrounding and tumour tissue compared to surrounding and tumour tissue of control and *Mif*$^{\Delta hep}$ mice (Figure S3).

Interestingly, further investigation of tumour and surrounding tissue using RT-qPCR as well as immunohistochemistry revealed significantly decreased proliferation in both tumour and surrounding tissue of *Cd74*$^{-/-}$ animals compared to wildtype controls, as the expression of *Ki67* and *Pcna* mRNA was significantly reduced in tumour tissue of tumour bearing *Cd74*$^{-/-}$ animals (Figure 5f,g). This result was confirmed by Ki67 staining of tumour tissue of both genotypes (Figure 5h, representative stainings are shown): Quantification of stainings revealed a significantly reduced Ki67-positive cell count in tumour tissue areas of *Cd74*$^{-/-}$ animals (Figure 5i).

3.6 | Macrophage migration inhibitory factor promotes ERK phosphorylation in hepatoma cells in a CD74-dependent manner

In other solid tumours, MIF was shown to promote tumour growth by activating the **Akt** and ERK pathway. We therefore investigated these signalling pathways in our in vitro assays in hepatoma cells to evaluate potential molecular mechanisms that could explain the relevance of MIF/CD74 in hepatic carcinogenesis. Stimulation of Hepa 1–6 cells with recombinant murine MIF for 20 min resulted in a 3.7-fold increase in ERK phosphorylation as revealed by Western Blot analysis (Figure 6). Importantly, addition of the anti-CD74 antibody reversed this effect.

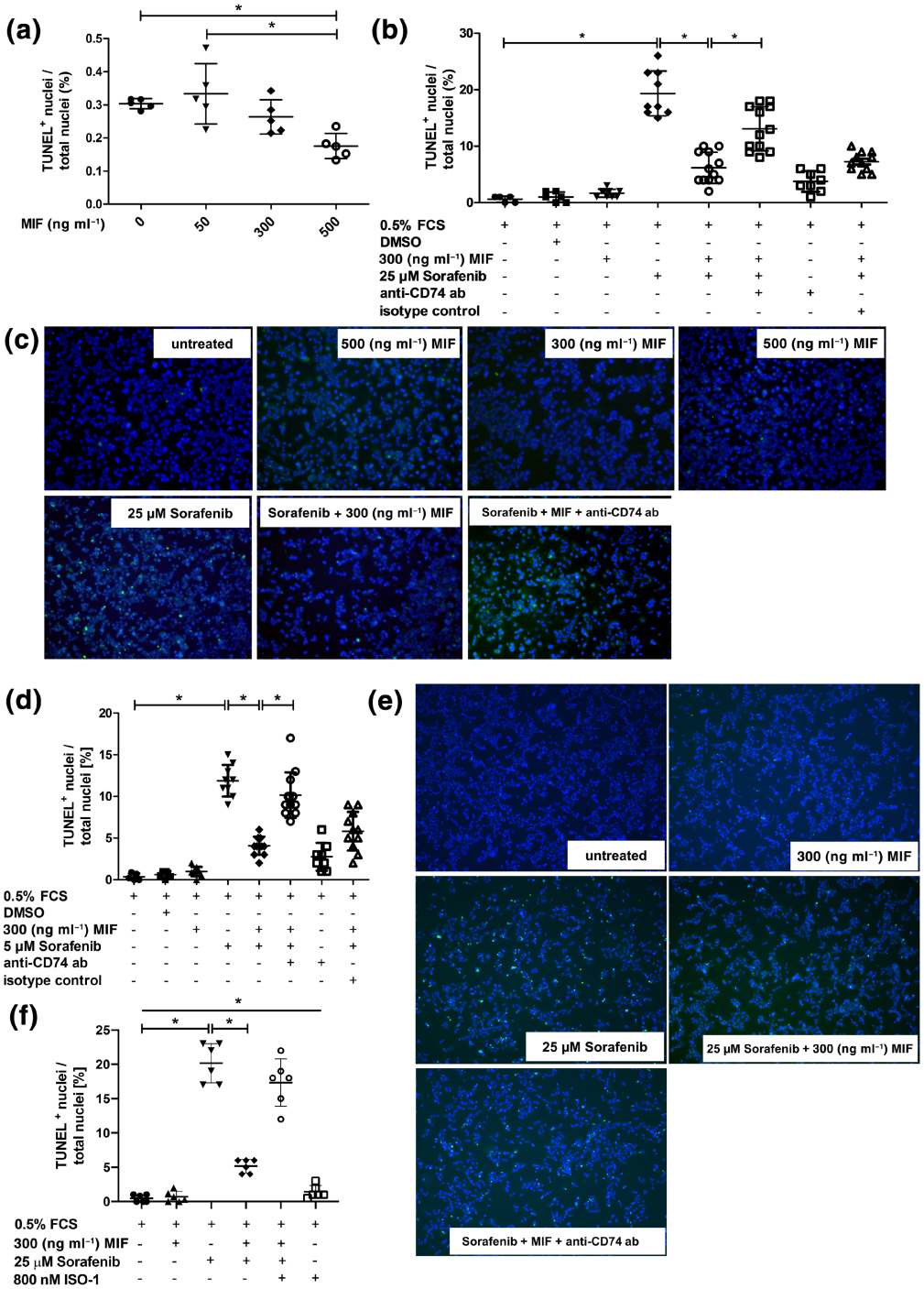

FIGURE 4 Macrophage migration inhibitory factor (MIF) inhibits therapy-induced apoptosis of tumour cells in vitro in a CD74 dependent manner. (a) Incubation of Hepa 1–6 cells with MIF shows a dosage-dependent trend to reduced apoptosis in vitro as shown by TUNEL staining. (b) Sorafenib was used as a cytotoxic agent in Hepa 1–6 cells to induce cell death. Pre-incubation of Hepa 1–6 cells with MIF results in decreased apoptosis in TUNEL staining. The addition of an anti-CD74 antibody reverts this effect. (c) Representative TUNEL stainings of Hepa 1–6 cells without treatment or incubated with MIF ± Sorafenib ± anti-CD74 antibody (ab) are depicted. (d) Pre-incubation of human HepG2 cells with recombinant MIF results in decreased apoptosis in TUNEL staining. The addition of an anti-CD74 antibody reverts this effect. (e) Representative TUNEL stainings of HepG2 cells without treatment or incubated with MIF ± Sorafenib ± anti-CD74 antibody are depicted. (f) Application of an MIF-specific inhibitor ISO-1 together with recombinant MIF also trends to inhibit MIF's anti-apoptotic effect during therapy-induced cell death. Data shown are individual values with means ± SD from $n \geq 5$ independent experiments. $^*P < 0.05$, significantly different as indicated; one-way ANOVA followed by post hoc test

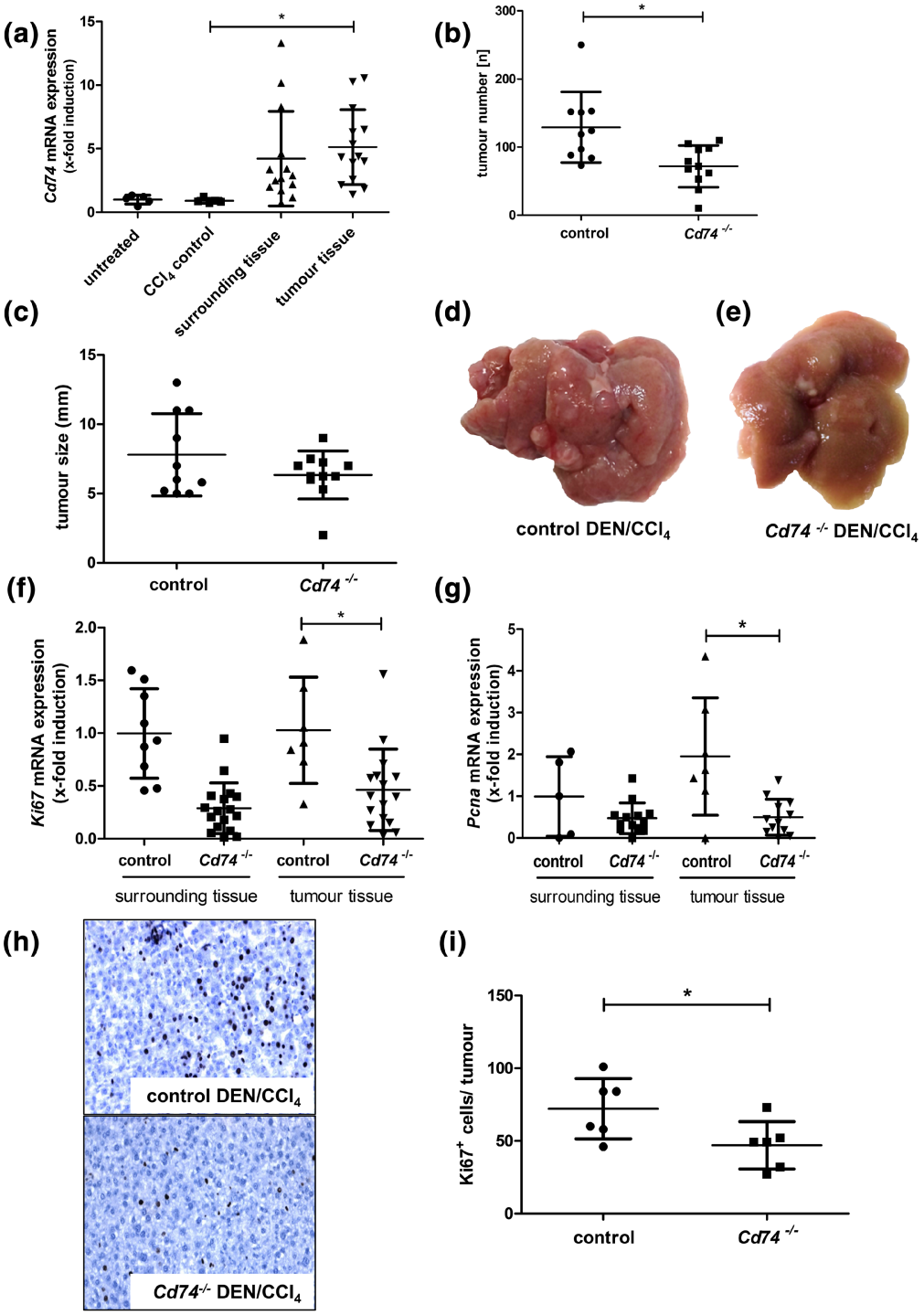

FIGURE 5 *Cd74* $^{-/-}$ mice show a reduced tumour burden in DEN/CCl_4 model compared to wildtype mice. (a) *Cd74* expression is increased in tumour tissue as analysed by RT-qPCR. (b) Tumour number is significantly lower in DEN/CCl_4-treated *Cd74*$^{-/-}$ mice. (c) Tumour size trends to be smaller in DEN/CCl_4 treated *Cd74*$^{-/-}$ compared with wildtype mice. (d,e) Representative pictures of a resected tumour bearing liver of a wildtype compared with a *Cd74*$^{-/-}$ mouse. (f, g) *Ki67* as well as *Pcna* expression on mRNA level is significantly decreased in *Cd74*$^{-/-}$ tumour mice compared with wildtype control as RT-qPCR of surrounding and tumour tissue reveals. (h) Immunohistochemical analysis of Ki67 expression reveals reduced count of Ki67 positive cells in tumour tissue of *Cd74*$^{-/-}$ mice compared with wildtype control mice. Representative stainings are shown. (i) Quantification of Ki67 positive cells per tumour of DEN/CCl_4-treated *Cd74*$^{-/-}$ mice reveals significantly less Ki67 positive cells in tumour tissue of *Cd74*$^{-/-}$ compared withcontrol mice. Data shown are individual values with means ± SD from 10 mice per group. *$P < 0.05$, significantly different as indicated; one-way ANOVA followed by post hoc test

4 | DISCUSSION

In this study, we have shown that MIF exerts a pro-carcinogenic function in an experimental HCC model. *Mif* $^{\Delta hep}$ mice show a reduced tumour burden compared to controls. Additionally, MIF exerts a pro-proliferative and anti-apoptotic function in both murine and human hepatoma cells in vitro. These effects are reversed by inhibition of MIF binding to its receptor CD74. Strikingly, the induction of HCC growth in *Cd74*$^{-/-}$ mice also results in a reduced tumour burden.

Expression of MIF as well as CD74 were increased in tumour tissue of wildtype mice with DEN/CCl_4-induced HCC. These data are consistent with previous studies (Zhang et al., 2011). Interestingly, overexpression of MIF was also shown for human HCC and was further associated with tumour size and relevant disease characteristics such as TNM (i.e., tumour, node and metastasis) stage (Wang et al., 2014) as well as short-term survival (Lu et al., 2018). Moreover, analysis of the GEPIA database revealed an increased expression of CD74 within human hepatocellular carcinoma (HCC) tumour tissue. Similar findings were recently published for CD74 to be over-expressed in HCC due to chronic hepatitis B infection (Lu et al., 2018). However, CD74 has so far not been assessed in terms of a prognostic relevance in HCC.

Immunohistochemical analysis revealed that the tumour cells themselves are the major source of enhanced MIF expression. The fact that hepatocytes, as the main cell type in liver, are primarily responsible for MIF production is supported by observations in alcohol-induced liver injury in humans (Marin et al., 2017) as well as in an experimental model of NASH in mice (Heinrichs et al., 2021). To test our hypothesis that hepatocyte-derived MIF exerts functional relevance during hepatocarcinogenesis, we performed the DEN/CCl_4 model in mice with a *hepatocyte-specific Mif* knockout. The DEN/CCl_4 model represents a chronic and toxic liver injury model reproducing the fibrotic phenotype of HCC that is typically observed in humans (Uehara et al., 2014). These *Mif*$^{flox/flox}$AlfpCre^{+} mice were characterized by a significantly decreased tumour number and trended towards a decreased tumour size compared to *Mif*$^{flox/flox}$AlfpCre^{-} mice. These data emphasize the role of hepatocyte-derived MIF. However, our data do not elucidate whether MIF facilitates tumour growth independent of liver disease aetiology. Therefore, further studies modelling other hepatic pathologies, that is, viral or metabolic liver injury, are needed to address the role of MIF in hepatocarcinogenesis with different aetiologies.

To assess the underlying mechanisms that could explain the reduced tumour number in *Mif* $^{\Delta hep}$ mice, we evaluated the effects of hepatocyte-derived MIF as well as CD74 expression during DEN/CCl_4 treatment in the context of hepatic inflammation and fibrogenesis. The reduced tumour number in both *Mif* $^{\Delta hep}$ as well as *Cd74*$^{-/-}$ animals was not related to changes in intrahepatic inflammatory activity

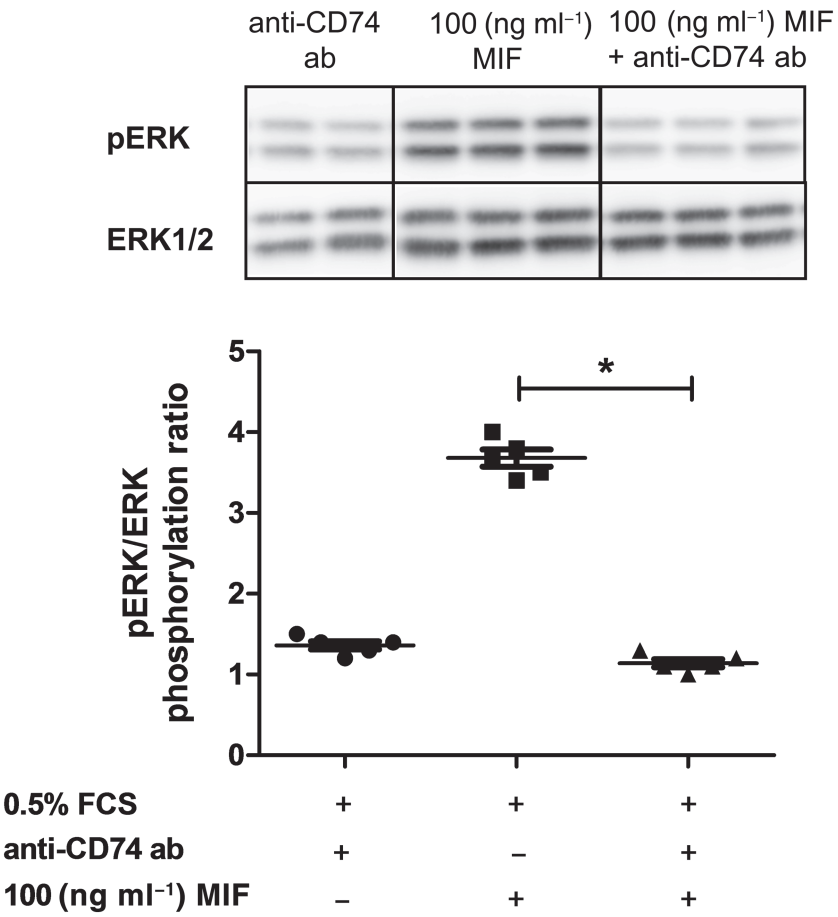

FIGURE 6 Macrophage migration inhibitory factor (MIF) promotes ERK phosphorylation in Hepa 1–6 cells in a CD74-dependent manner. Western Blot analysis of total cell lysates after 20-min incubation with 100-ng ml^{-1} recombinant MIF results in a 3.7 fold increase in ERK phosphorylation. Addition of the anti-CD74 antibody reversed this effect. ERK1/2 is used for standardization. Data shown are individual values with means ± SD from five independent experiments performed. *$P < 0.05$, significantly different as indicated; one-way ANOVA followed by post hoc test

or severity of tissue injury. Moreover, neither the hepatocyte-specific knockout of *Mif* nor the global *Cd74* knockout influenced fibrosis development during CCl_4- or DEN/CCl_4-treatment. These findings are not in line with previous observations describing significant effects of MIF during experimental fibrogenesis. In a model of chronic liver injury induced by repetitive CCl_4 injections, MIF exerted CD74-dependent antifibrotic effects by influencing the proliferation of hepatic stellate cells (Heinrichs et al., 2011). This apparent discrepancy of data might be explained by the methodological differences. In our current model, $Mif^{\Delta hep}$ mice were injected with carcinogenic DEN followed by weekly, low dose, CCl_4 injections for 21 weeks, whereas Heinrichs et al. performed CCl_4-treatment with a twofold higher dose for 6 weeks only. Secondly, in our current study, mice with a hepatocyte-specific *Mif* knockout were used, while Heinrichs et al. performed their experiments in mice with a global *Mif* knockout. Thus, differences in the duration and severity of liver injury as well as the differential MIF expression by hepatocytes, compared to non-parenchymal cells, might influence the hepatic micro-environment resulting in different MIF-related effects. To this end, a previous study by Marin et al. who studied the expression of MIF in alcohol-induced liver injury (Marin et al., 2017), observed that the expression of the MIF receptors including CD74 depends on the aetiology of liver disease—a fact that could further explain different effects of MIF on the progression of different liver diseases.

We hypothesized that MIF might influence proliferation and apoptosis of tumour cells representing important features of hepatocarcinogenesis. In vivo, we observed a reduced number of proliferating cells within the tumour tissue of $Mif^{\Delta hep}$ and a MIF-driven, CD74-dependent proliferation of hepatoma cells in vitro. Interestingly, the incubation with the anti-CD74 antibody alone also resulted in decreased proliferative capacity, indicating an autocrine effect of hepatoma cell-derived "endogenous" MIF. These results are in accordance with previous data from an HCC xenograft model where a small interfering RNA (siRNA)-mediated *Mif* knockdown resulted in reduced proliferation and increased apoptosis of HCC cells (Huang et al., 2014). Hence, both tumour cell-derived MIF as well as "exogenous" MIF, that is, immune cell-derived MIF should be considered in the context of HCC-directed therapies. A pro-proliferative function of MIF also has been shown for other solid tumours, such as bladder cancer (Choudhary et al., 2013) and melanoma (Tanese et al., 2015). Additionally, in a renal carcinoma cell line, a knockdown of CD74 reduced activation of the hypoxia-inducible factor 1α (HIF-1α) pathway associated with induced apoptosis and inhibited invasion (Ji et al., 2014). Further studies need to address which downstream signalling pathways are influenced by MIF/CD74 signalling in hepatocarcinogenesis. Our study indicates that ERK could be such a pathway as ERK phosphorylation in Hepa 1–6 cells was increased during MIF stimulation in a CD74-dependent manner. This is in line with previous studies on MIF's carcinogenic function in other solid tumours, such as pancreatic cancer (Wang et al., 2018). As an inflammatory milieu is also known to induce CD74 surface expression on different class II-negative cell types (Klasen et al., 2018; Tanese et al., 2015) the interaction of MIF and CD74 and intracellular signal transduction could especially be of particular relevance in inflammation-driven tumour growth, such as the chronic liver disease due to viral hepatitis and NASH.

Interestingly, the MIF/CD74 axis was furthermore identified to play a pivotal role in the inhibition of apoptosis as MIF activated NF-κB through CD74, thereby averting apoptosis (De et al., 2018). Here we could show that during cytotoxic agent-induced apoptosis of hepatoma cells, MIF displays a CD74-dependent, anti-apoptotic behaviour as it attenuated sorafenib-induced cell death. Interestingly, this effect was restricted to chemotherapy-induced cell death, as the inhibition of MIF in the absence of sorafenib challenge, did not result in an increased cell death, compared to MIF only treatment. These results are in line with data from breast cancer cell lines after knockdown of CD74, which resulted in decreased apoptosis (Ssadh et al., 2019) and the finding that apoptosis inhibition by MIF/CD74 is intracellularly promoted by the activation of Akt (PKB) in breast cancer cells (Lue et al., 2007).

Our data are of clinical relevance, as we provide insights into how MIF and its cognate receptor CD74 drive hepatocarcinogenesis in vivo and complement the human studies that have previously indicated a prognostic relevance of MIF and CD74 expression in HCC patients (Hira et al., 2005; Wirtz et al., 2021). Nevertheless, further animal studies are needed to evaluate the effects of an anti-CD74 directed therapy during HCC growth, for example, in models of orthotopic HCC transplant in murine livers previously treated with fibrogenic agents (Liu et al., 2020; Reiberger et al., 2015). Moreover, the MIF/CD74 axis should be investigated in human HCC. In this context, MIF inhibitors have already been studied in vitro and in vivo (Varinelli et al., 2015): The MIF inhibitor ISO-1 was investigated in a pancreatic cancer xenograft model in BALB/c nude mice and found to inhibit tumour growth (Cheng et al., 2020). We here demonstrated an anti-proliferative effect of ISO-1, when applied together with MIF in a proliferation assay using a murine HCC cell line in vitro. Available MIF inhibitors are characterized by high bioavailability and low toxicity and may represent appropriate therapeutic agents for potential use in liver cancer patients. CD74 also is a promising target in cancer therapy as a CD74-specific monoclonal antibody, **milatuzumab**, is in advanced clinical testing for lymphoma. As the inhibition of the MIF/CD74 axis resulted in a significant, albeit not complete, attenuation of tumour growth, targeting the MIF/CD74 axis in hepatic carcinogenesis could thus be envisaged as a combination therapy together with established therapeutic regimens. For example, a combination therapy including an anti-MIF/CD74-directed approach together with multi-kinase inhibitors such as sorafenib, could represent an interesting therapeutic strategy in HCC, as the blockade of the MIF/CD74 axis increases sensitivity towards the cytotoxic drug.

In summary, our study is the first to describe novel and encouraging aspects of a pro-tumorigenic role of MIF in experimental hepatocarcinogenesis. We suggest that MIF and its cognate receptor CD74 promote HCC growth in vivo by influencing the proliferative and apoptotic behaviour of malignant hepatocytes. Hence, targeting MIF/CD74 signalling may represent a promising approach for HCC treatment.

ACKNOWLEDGEMENTS

We thank Michael Lacy and Simona Gerra (Division of Vascular Biology, Institute for Stroke and Dementia Research, University Hospital Munich, Germany) for providing purified recombinant murine MIF protein and Dr. Günter-Fingerle-Rowson for his contribution in generating the *Mif-flox/flox* mouse strain. This study was supported by the Deutsche Forschungsgemeinschaft (DFG, German Research Foundation) including SFB1382 (Project-ID 403224013; PB04) to M.-L.B., SFB/TRR57 (Project ID 36842431; P07, P25 and Q1) to M.-L.B., J.B. and P.B., DJ100/1-1 to S.D., BO3755/3-1, BO3755/9-1, BO3755/13-1 to P.B., and Strategic Partnerships within LMUexc to J. B.; and by the BMBF (Bundesministerium für Bildung und Forschung, Federal Ministry of Education and Research, Germany) funded project LiSyM (FKZ 031 L0041) to M.-L.B. and STOP-FSGS-01GM1901A to P.B. and S.D. T.H.W. was further supported by the German National Academic Foundation.

AUTHOR CONTRIBUTIONS

T.H.W., A.S., I.B., P.F., D.H. and S.D. performed all experiments; E.F.B. and M.T.K. helped with the experiments; T.H.W. analysed the data and drafted the manuscript; K.M.S. helped with data analysis; J.B. and R.B. provided transgenic mice and critical research reagents, J.B., C.T., P.B., R.B., and R.W. critically edited the manuscript; M.-L.B. designed the study, supervised the analysis of the data, and edited the manuscript.

CONFLICT OF INTEREST

Dr. Bucala and Dr. Bernhagen are listed as co-inventors on patents describing the potential therapeutic utility of MIF antagonists.

DECLARATION OF TRANSPARENCY AND SCIENTIFIC RIGOUR

This Declaration acknowledges that this paper adheres to the principles for transparent reporting and scientific rigour of preclinical research as stated in the *BJP* guidelines for Design and Analysis, Immunoblotting and Immunochemistry, and Animal Experimentation, and as recommended by funding agencies, publishers and other organisations engaged with supporting research.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request. Some data may not be made available because of privacy or ethical reasons.

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REFERENCE

Alexander, S. P. H., Kelly, E., Mathie, A., Peters, J. A., Veale, E. L., Armstrong, J. F., Faccenda, E., Harding, S. D., Pawson, A. J., Sharman, J. L., Southan, C., Buneman, O. P., Cidlowski, J. A., Christopoulos, A., Davenport, A. P., Fabbro, D., Spedding, M., Striessnig, J., Davies, J. A., … CGTP Collaborators. (2019). THE CONCISE GUIDE TO PHARMACOLOGY 2019/20: Other protein targets. *British Journal of Pharmacology*, *176*(Suppl 1), S1–S20.

Bernhagen, J., Krohn, R., Lue, H., Gregory, J. L., Zernecke, A., Koenen, R. R., Dewor, M., Georgiev, I., Schober, A., Leng, L., Kooistra, T., Fingerle-Rowson, G., Ghezzi, P., Kleemann, R., McColl, S. R., Bucala, R., Hickey, M. J., & Weber, C. (2007). MIF is a non-cognate ligand of CXC chemokine receptors in inflammatory and atherogenic cell recruitment. *Nature Medicine*, *13*, 587–596. https://doi.org/10.1038/nm1567

Bernhagen, J., Mitchell, R. A., Calandra, T., Voelter, W., Cerami, A., & Bucala, R. (1994). Purification, bioactivity, and secondary structure analysis of mouse and human macrophage migration inhibitory factor (MIF). *Biochemistry*, *33*, 14144–14155. https://doi.org/10.1021/bi00251a025

Berres, M. L., Koenen, R. R., Rueland, A., Zaldivar, M. M., Heinrichs, D., Sahin, H., Schmitz, P., Streetz, K. L., Berg, T., Gassler, N., Weiskirchen, R., Proudfoot, A., Weber, C., Trautwein, C., & Wasmuth, H. E. (2010). Antagonism of the chemokine Ccl5 ameliorates experimental liver fibrosis in mice. *The Journal of Clinical Investigation*, *120*, 4129–4140. https://doi.org/10.1172/JCI41732

Bloom, B. R., & Bennett, B. (1966). Mechanism of a reaction in vitro associated with delayed-type hypersensitivity. *Science*, *153*, 80–82. https://doi.org/10.1126/science.153.3731.80

Cheng, B., Wang, Q., Song, Y., Liu, Y., Liu, Y., Yang, S., Li, D., Zhang, Y., & Zhu, C. (2020). MIF inhibitor, ISO-1, attenuates human pancreatic cancer cell proliferation, migration and invasion in vitro, and suppresses xenograft tumour growth in vivo. *Scientific Reports*, *10*, 6741. https://doi.org/10.1038/s41598-020-63778-y

Choudhary, S., Hegde, P., Pruitt, J. R., Sielecki, T. M., Choudhary, D., Scarpato, K., DeGraff, D. J., Pilbeam, C. C., & Taylor, J. A. III (2013). Macrophage migratory inhibitory factor promotes bladder cancer progression via increasing proliferation and angiogenesis. *Carcinogenesis*, *34*, 2891–2899. https://doi.org/10.1093/carcin/bgt239

De, R., Sarkar, S., Mazumder, S., Debsharma, S., Siddiqui, A. A., Saha, S. J., Banerjee, C., Nag, S., Saha, D., Pramanik, S., & Bandyopadhyay, U. (2018). Macrophage migration inhibitory factor regulates mitochondrial dynamics and cell growth of human cancer cell lines through CD74-NF-kappaB signaling. *The Journal of Biological Chemistry*, *293*, 19740–19760. https://doi.org/10.1074/jbc.RA118.003935

Djudjaj, S., Martin, I. V., Buhl, E. M., Nothofer, N. J., Leng, L., Piecychna, M., Floege, J., Bernhagen, J., Bucala, R., & Boor, P. (2017). Macrophage migration inhibitory factor limits renal inflammation and fibrosis by counteracting tubular cell cycle arrest. *Journal of the American Society of Nephrology: JASN*, *28*, 3590–3604. https://doi.org/10.1681/ASN.2017020190

Dumitru, C. A., Gholaman, H., Trellakis, S., Bruderek, K., Dominas, N., Gu, X., Bankfalvi, A., Whiteside, T. L., Lang, S., & Brandau, S. (2011). Tumor-derived macrophage migration inhibitory factor modulates the biology of head and neck cancer cells via neutrophil activation. *International Journal of Cancer*, *129*, 859–869. https://doi.org/10.1002/ijc.25991

Fattovich, G., Stroffolini, T., Zagni, I., & Donato, F. (2004). Hepatocellular carcinoma in cirrhosis: Incidence and risk factors. *Gastroenterology*, *127*, S35–S50. https://doi.org/10.1053/j.gastro.2004.09.014

Fingerle-Rowson, G., Petrenko, O., Metz, C. N., Forsthuber, T. G., Mitchell, R., Huss, R., Moll, U., Muller, W., & Bucala, R. (2003). The p53-dependent effects of macrophage migration inhibitory factor revealed by gene targeting. *Proceedings of the National Academy of Sciences of the United States of America*, *100*, 9354–9359. https://doi.org/10.1073/pnas.1533295100

Heinrichs, D., Berres, M. L., Coeuru, M., Knauel, M., Nellen, A., Fischer, P., Philippeit, C., Bucala, R., Trautwein, C., Wasmuth, H. E., & Bernhagen, J. (2014). Protective role of macrophage migration

inhibitory factor in nonalcoholic steatohepatitis. *The FASEB Journal*, *28*, 5136–5147. https://doi.org/10.1096/fj.14-256776

Heinrichs, D., Brandt, E. F., Fischer, P., Kohncke, J., Wirtz, T. H., Guldiken, N., Djudjaj, S., Boor, P., Kroy, D., Weiskirchen, R., Bucala, R., Wasmuth, H. E., Strnad, P., Trautwein, C., Bernhagen, J., & Berres, M.-L. (2021). Unexpected pro-fibrotic effect of MIF in non-alcoholic Steatohepatitis is linked to a shift in NKT cell populations. *Cell*, *10*, 252. https://doi.org/10.3390/cells10020252

Heinrichs, D., Knauel, M., Offermanns, C., Berres, M. L., Nellen, A., Leng, L., Schmitz, P., Bucala, R., Trautwein, C., Weber, C., Bernhagen, J., & Wasmuth, H. E. (2011). Macrophage migration inhibitory factor (MIF) exerts antifibrotic effects in experimental liver fibrosis via CD74. *Proceedings of the National Academy of Sciences of the United States of America*, *108*, 17444–17449. https://doi.org/10.1073/pnas.1107023108

Hira, E., Ono, T., Dhar, D. K., El-Assal, O. N., Hishikawa, Y., Yamanoi, A., & Nagasue, N. (2005). Overexpression of macrophage migration inhibitory factor induces angiogenesis and deteriorates prognosis after radical resection for hepatocellular carcinoma. *Cancer*, *103*, 588–598. https://doi.org/10.1002/cncr.20818

Huang, X. H., Jian, W. H., Wu, Z. F., Zhao, J., Wang, H., Li, W., & Xia, J. T. (2014). Small interfering RNA (siRNA)-mediated knockdown of macrophage migration inhibitory factor (MIF) suppressed cyclin D1 expression and hepatocellular carcinoma cell proliferation. *Oncotarget*, *5*, 5570–5580. https://doi.org/10.18632/oncotarget.2141

Ji, S. Q., Su, X. L., Cheng, W. L., Zhang, H. J., Zhao, Y. Q., & Han, Z. X. (2014). Down-regulation of CD74 inhibits growth and invasion in clear cell renal cell carcinoma through HIF-1alpha pathway. *Urologic Oncology*, *32*, 153–161. https://doi.org/10.1016/j.urolonc.2012.09.013

Kindt, N., Journe, F., Laurent, G., & Saussez, S. (2016). Involvement of macrophage migration inhibitory factor in cancer and novel therapeutic targets. *Oncology Letters*, *12*, 2247–2253. https://doi.org/10.3892/ol.2016.4929

Klasen, C., Ziehm, T., Huber, M., Asare, Y., Kapurniotu, A., Shachar, I., Bernhagen, J., & el Bounkari, O. (2018). LPS-mediated cell surface expression of CD74 promotes the proliferation of B cells in response to MIF. *Cellular Signalling*, *46*, 32–42. https://doi.org/10.1016/j.cellsig.2018.02.010

Leng, L., Metz, C. N., Fang, Y., Xu, J., Donnelly, S., Baugh, J., Delohery, T., Chen, Y., Mitchell, R. A., & Bucala, R. (2003). MIF signal transduction initiated by binding to CD74. *The Journal of Experimental Medicine*, *197*, 1467–1476. https://doi.org/10.1084/jem.20030286

Lilley, E., Stanford, S. C., Kendall, D. E., Alexander, S. P., Cirino, G., Docherty, J. R., George, C. H., Insel, P. A., Izzo, A. A., Ji, Y., Panettieri, R. A., Sobey, C. G., Stefanska, B., Stephens, G., Teixeira, M., & Ahluwalia, A. (2020). ARRIVE 2.0 and the British Journal of Pharmacology: Updated guidance for 2020. *British Journal of Pharmacology*. https://bpspubs.onlinelibrary.wiley.com/doi/full/10.1111/bph.15178

Liu, M., Zhou, J., Liu, X., Feng, Y., Yang, W., Wu, F., Cheung, O. K. W., Sun, H., Zeng, X., Tang, W., Mok, M. T. S., Wong, J., Yeung, P. C., Lai, P. B. S., Chen, Z., Jin, H., Chen, J., Chan, S. L., Chan, A. W. H., … Cheng, A. S. L. (2020). Targeting monocyte-intrinsic enhancer reprogramming improves immunotherapy efficacy in hepatocellular carcinoma. *Gut*, *69*, 365–379. https://doi.org/10.1136/gutjnl-2018-317257

Lu, M., Xu, Y., He, G., Liu, Q., Zhu, J., & Zhang, C. (2018). The expression of CD74 and macrophage migration inhibitory factor protein is upregulated in hepatitis B virus-related hepatocellular carcinoma. *Translational Cancer Research*, *7*, 1537–1547. https://doi.org/10.21037/tcr.2018.11.21

Lue, H., Thiele, M., Franz, J., Dahl, E., Speckgens, S., Leng, L., Fingerle-Rowson, G., Bucala, R., Lüscher, B., & Bernhagen, J. (2007). Macrophage migration inhibitory factor (MIF) promotes cell survival by activation of the Akt pathway and role for CSN5/JAB1 in the control of autocrine MIF activity. *Oncogene*, *26*, 5046–5059. https://doi.org/10.1038/sj.onc.1210318

Marin, V., Poulsen, K., Odena, G., McMullen, M. R., Altamirano, J., Sancho-Bru, P., Tiribelli, C., Caballeria, J., Rosso, N., Bataller, R., & Nagy, L. E. (2017). Hepatocyte-derived macrophage migration inhibitory factor mediates alcohol-induced liver injury in mice and patients. *Journal of Hepatology*, *67*, 1018–1025. https://doi.org/10.1016/j.jhep.2017.06.014

Maubach, G., Lim, M. C., Kumar, S., & Zhuo, L. (2007). Expression and upregulation of cathepsin S and other early molecules required for antigen presentation in activated hepatic stellate cells upon IFN-gamma treatment. *Biochimica et Biophysica Acta*, *1773*, 219–231. https://doi.org/10.1016/j.bbamcr.2006.11.005

McGrath, J. C., Pawson, A. J., Sharman, J. L., & Alexander, S. P. (2015). BJP is linking its articles to the IUPHAR/BPS Guide to PHARMACOLOGY. *British Journal of Pharmacology*, *172*, 2929–2932. https://doi.org/10.1111/bph.13112

Mun, S. H., Won, H. Y., Hernandez, P., Aguila, H. L., & Lee, S. K. (2013). Deletion of CD74, a putative MIF receptor, in mice enhances osteoclastogenesis and decreases bone mass. *Journal of Bone and Mineral Research: The Official Journal of the American Society for Bone and Mineral Research*, *28*, 948–959. https://doi.org/10.1002/jbmr.1787

Njei, B., Rotman, Y., Ditah, I., & Lim, J. K. (2015). Emerging trends in hepatocellular carcinoma incidence and mortality. *Hepatology*, *61*, 191–199. https://doi.org/10.1002/hep.27388

Percie du Sert, N., Hurst, V., Ahluwalia, A., Alam, S., Avey, M. T., Baker, M., Browne, W. J., Clark, A., Cuthill, I. C., Dirnagl, U., Emerson, M., Garner, P., Holgate, S. T., Howells, D. W., Karp, N. A., Lazic, S. E., Lidster, K., MacCallum, C. J., Macleod, M., … Würbel, H. (2020). The ARRIVE guidelines 2.0: Updated guidelines for reporting animal research. *PLoS Biology*, *18*(7), e3000410. https://doi.org/10.1371/journal.pbio.3000410

Reiberger, T., Chen, Y., Ramjiawan, R. R., Hato, T., Fan, C., Samuel, R., Roberge, S., Huang, P., Lauwers, G. Y., Zhu, A. X., Bardeesy, N., Jain, R. K., & Duda, D. G. (2015). An orthotopic mouse model of hepatocellular carcinoma with underlying liver cirrhosis. *Nature Protocols*, *10*, 1264–1274. https://doi.org/10.1038/nprot.2015.080

Saviano, A., Roehlen, N., Virzi, A., Roca Suarez, A. A., Hoshida, Y., Lupberger, J., & Baumert, T. F. (2019). Stromal and Immune Drivers of Hepatocarcinogenesis. In Y. Hoshida (Ed.), *Hepatocellular carcinoma: Translational precision medicine approaches* (pp. 317–331). Cham (CH).

Schwabe, R. F., & Luedde, T. (2018). Apoptosis and necroptosis in the liver: A matter of life and death. *Nature Reviews Gastroenterology & Hepatology*, *15*, 738–752. https://doi.org/10.1038/s41575-018-0065-y

Shi, X., Leng, L., Wang, T., Wang, W., Du, X., Li, J., McDonald, C., Chen, Z., Murphy, J. W., Lolis, E., Noble, P., Knudson, W., & Bucala, R. (2006). CD44 is the signaling component of the macrophage migration inhibitory factor-CD74 receptor complex. *Immunity*, *25*, 595–606. https://doi.org/10.1016/j.immuni.2006.08.020

Sia, D., Villanueva, A., Friedman, S. L., & Llovet, J. M. (2017). Liver cancer cell of origin, molecular class, and effects on patient prognosis. *Gastroenterology*, *152*, 745–761. https://doi.org/10.1053/j.gastro.2016.11.048

Ssadh, H. A., Abdulmonem, W. A., Rasheed, Z., Madar, I. H., Alhoderi, J., Eldeen, S. K. N., Alradhwan, A., Alasmael, N., Alkhamiss, A., & Fernández, N. (2019). Knockdown of CD-74 in the proliferative and apoptotic activity of breast cancer cells. *Open Access Macedonian Journal of Medical Sciences*, *7*, 3169–3176. https://doi.org/10.3889/oamjms.2019.354

Tanese, K., Hashimoto, Y., Berkova, Z., Wang, Y., Samaniego, F., Lee, J. E., Ekmekcioglu, S., & Grimm, E. A. (2015). Cell surface CD74-MIF interactions drive melanoma survival in response to interferon-gamma. *The Journal of Investigative Dermatology*, *135*, 2775–2784. https://doi.org/10.1038/jid.2015.204

Uehara, T., Pogribny, I. P., & Rusyn, I. (2014). The DEN and CCl4 -induced mouse model of fibrosis and inflammation-associated hepatocellular carcinoma. *Current Protocols in Pharmacology*, *66*. https://doi.org/10.1002/0471141755.ph1430s66

Varinelli, L., Caccia, D., Volpi, C. C., Caccia, C., De Bortoli, M., Taverna, E., Gualeni, A. V., Leoni, V., Gloghini, A., Manenti, G., & Bongarzone, I. (2015). 4-IPP, a selective MIF inhibitor, causes mitotic catastrophe in thyroid carcinomas. *Endocrine-Related Cancer*, *22*, 759–775. https://doi.org/10.1530/ERC-15-0299

Verjans, E., Noetzel, E., Bektas, N., Schutz, A. K., Lue, H., Lennartz, B., Hartmann, A., Dahl, E., & Bernhagen, J. (2009). Dual role of macrophage migration inhibitory factor (MIF) in human breast cancer. *BMC Cancer*, *9*, 230. https://doi.org/10.1186/1471-2407-9-230

Wang, D., Luo, L., Chen, W., Chen, L. Z., Zeng, W. T., Li, W., & Huang, X.-H. (2014). Significance of the vascular endothelial growth factor and the macrophage migration inhibitory factor in the progression of hepatocellular carcinoma. *Oncology Reports*, *31*, 1199–1204. https://doi.org/10.3892/or.2013.2946

Wang, D., Wang, R., Huang, A., Fang, Z., Wang, K., He, M., Xia, J. T., & Li, W. (2018). Upregulation of macrophage migration inhibitory factor promotes tumor metastasis and correlates with poor prognosis of pancreatic ductal adenocarcinoma. *Oncology Reports*, *40*, 2628–2636. https://doi.org/10.3892/or.2018.6703

Wirtz, T. H., Loosen, S. H., Schulze-Hagen, M., Gorgulho, J., Kandler, J., Joerdens, M., Demir, M., Mohr, R., Bruners, P., Kuhl, C., Trautwein, C., Berres, M.-. L., Tacke, F., Luedde, T., & Roderburg, C. (2021). Macrophage migration inhibitory factor predicts an unfavorable outcome after TACE for hepatic malignancies. *Clinical and Translational Science*. https://doi.org/10.1111/cts.13033

Zhang, C., Liang, T., Song, J., Jiang, S., Qu, L., & Hou, G. (2011). Evaluation of macrophage migration inhibitory factor as an imaging marker for hepatocellular carcinoma in murine models. *Scandinavian Journal of Gastroenterology*, *46*, 720–726. https://doi.org/10.3109/00365521.2011.568517

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How to cite this article: Wirtz, T. H., Saal, A., Bergmann, I., Fischer, P., Heinrichs, D., Brandt, E. F., Koenen, M. T., Djudjaj, S., Schneider, K. M., Boor, P., Bucala, R., Weiskirchen, R., Bernhagen, J., Trautwein, C., & Berres, M.-L. (2021). Macrophage migration inhibitory factor exerts pro-proliferative and anti-apoptotic effects via CD74 in murine hepatocellular carcinoma. *British Journal of Pharmacology*, *178*(22), 4452–4467. https://doi.org/10.1111/bph.15622