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

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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/CCl4) model in hepatocyte-specific Mif knockout (MifΔhep) mice, 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/CCl4 treatment of MifΔhep 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.
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

### 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.

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### 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.
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^{floxed/AlfpCre} mice with a hepatocyte-specific Mif knockout, named Mif^{Δhep} mice. Mif^{floxed/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/CCl4 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 CCl4. Mice (n ≥ 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 CCl4 (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 CCl4-treated (only fibrosis induction) wildtype or Mif^{floxed/AlfpCre} mice served as controls.

Hence, all mice were randomized to receive either DEN/CCl4 or CCl4 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 CCl4 injection, by iso-flurane 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 presdesigned TaqMan Gene Expression Assays (Applied Biosystems, ThermoFisher Scientific, Waltham, MA, USA) and was carried out for Mif, Col1a1, CD74, Pena and Ki67. Data were normalized to 18S and analysed by the 2⁻ΔΔCT 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 D385 (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 ≥ 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 ≥ 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% H₂O₂ 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 ≥ 6 independent experiments per group were evaluated. Data are expressed as means ± SD of n ≥ 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\(^{\text{flx/flox}}\) 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 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/CCl\(_4\) 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\(^{\text{flx/flox}}\)/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 Col1a1 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\(_{\alpha}\), 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\(^{\text{flx/flox}}\)/AlfpCre\(^{-}\) control and Mif\(^{\Delta}\)hep mice. RT-qPCR analysis revealed a fivefold increase in expression of
**FIGURE 1** MifΔhep mice show reduced tumour burden in DEN/CCl₄ 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/CCl₄-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 MifΔhep/C0 mice and CCl₄-treated wildtype mice were used as (staining) controls (left panel). (c) Tumour number is significantly lower in DEN/CCl₄-treated MifΔhep 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 MifΔhepAlfpCre mice compared to a MifΔhep mouse. Data shown are individual values with means ± SD from 10 mice per group.
Ki67 in tumour tissue of control mice when compared to Mif Δhep 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 Pcm1 as another marker of cell cycle progression (Figure 3b). Subsequently, immunohistochemical analysis of Ki67 expression in tumour tissue of Mif Δhep mice compared to control mice was performed. Here, the number of Ki67-positive cells in tumour tissue of Mif Δhep 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
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/CCL4 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/CCL4-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/CCL4 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/CCL4-treated MifΔ/Δ mice, Sirius Red staining and Col1a1 mRNA expression analysis revealed comparable fibrotic area fractions in both CCL4 as well as DEN/CCL4-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/CCL4-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 MifΔ/Δ 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 ≥ 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/CCl₄ 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/CCl₄-treated Cd74⁻/⁻ mice. (c) Tumour size trends to be smaller in DEN/CCl₄ 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 Pcm1 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/CCl₄-treated Cd74⁻/⁻ mice reveals significantly less Ki67 positive cells in tumour tissue of Cd74⁻/⁻ compared with control 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. MifΔhep 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/CCl4-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 overexpressed 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/CCl4 model in mice with a hepatocyte-specific Mif knockout. The DEN/CCl4 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 Miffl/−/−AlfpCre+ mice were characterized by a significantly decreased tumour number and trended towards a decreased tumour size compared to Miffl/−/−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 MifΔhep mice, we evaluated the effects of hepatocyte-derived MIF as well as CD74 expression during DEN/CCl4 treatment in the context of hepatic inflammation and fibrogenesis. The reduced tumour number in both MifΔhep as well as Cd74−/− animals was not related to changes in intrahepatic inflammatory activity.
or severity of tissue injury. Moreover, neither the hepatocyte-specific knockout of Mif nor the global Cd74 knockout influenced fibrosis development during CCl₄- or DEN/CCl₄-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 CCl₄ 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<sup>−/−</sup> mice were injected with carcinogenic DEN followed by weekly, low dose, CCl₄ injections for 21 weeks, whereas Heinrichs et al. performed CCl₄-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<sup>−/−</sup> 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 (Sadah 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.
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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. Bucaula 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 Immunoochemistry, 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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