



Influence of inflammasome pathway activation in macrophages on the matrix metalloproteinase expression of human hepatic stellate cells

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ABSTRACT

Inflammasomes are protein complexes that produce IL-1 β in response to damage or pathogens. As such, inflammasomes are involved in several types of hepatic fibrosis. However, the mechanisms by which these complexes drive the liver's fibrogenic status remain unclear. We co-cultured differentiated macrophages (the THP-1 cell line or human monocyte-derived macrophages (MDMs)) with human hepatic fibroblasts (either the LX-2 cell line or primary human hepatic stellate cells (HSCs)). The inflammasome pathway was activated with lipopolysaccharide (LPS) and monosodium urate (MSU) crystals, and the HSCs' responses were analyzed. Our results show that co-culture of HSCs with THP-1 cells upregulated transcription of the genes coding for metalloproteinase (MMP)-3 and MMP-9. After inflammasome pathway activation, the HSCs' phenotype was the same in the presence of THP-1 cells or MDMs (*i.e.* upregulation of MMP-3, MMP-9, and the pro-inflammatory cytokine IL-1 β). We found that two cytokines were involved in these changes: IL-1 β regulated MMP-3 and IL-1 β mRNA expression, whereas TNF- α regulated MMP-9 mRNA expression. Experiments with primary cells revealed that a general inflammatory environment is responsible for the downregulation of pro-fibrotic markers. Our present results suggest that inflammasome pathway activation in macrophages leads to a pro-inflammatory environment for HSCs leading to MMP/TIMP imbalance and enhanced fibrolytic properties.

1. Introduction

Fibrogenesis is a common, widespread, pathophysiological response that occurs in many tissues after chronic or repetitive insult caused by infections, autoimmune reactions, or mechanical trauma [1–3]. The initial damage can be amplified by an inflammatory response and then fibrosis (characterized by extracellular matrix deposition, scar formation, and even organ failure [3]).

Inflammasomes are protein complexes that recognize a diverse set of inflammation-inducing stimuli, including pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs) [4,5]. It controls the activation of the proteolytic enzyme caspase-1, leading to the maturation of the pro-inflammatory cytokines interleukin (IL)-1 β and IL-18 [6,7]. Among, the inflammasome containing the NOD-like receptor (NLR) protein (NLRP)3 is thought to have a major role in many inflammatory disease.

The inflammasome has been implicated in fibrotic disease in various tissues. It reportedly contributes to lung fibrosis [8], and has a role in

several animal models of liver fibrosis. For example, levels of transforming growth factor (TGF)- β 1 and collagen type I α 1 are abnormally low following carbon tetrachloride- or thioacetamide-induced liver fibrosis in NLRP3- or adaptor ASC-deficient mice [9].

The liver contains both parenchymal cells (hepatocytes, which account for the majority of the cells) and immune cells (macrophages, neutrophil leukocytes, dendritic cells (DCs), T cells, natural killer (NK)/NKT cells, and B lymphocytes). Innate immune cells (including monocytes, macrophages, neutrophils, and DCs) express inflammasomes but there is also evidence to suggest that inflammasomes are functionally active in non-immune cells - including hepatocytes [10–14], hepatic stellate cells (HSCs) [13,15], endothelial cells [13,16] and myofibroblasts [17]. HSCs express components of the inflammasome, and activation of primary mouse stellate cells or LX-2 HSCs with monosodium urate (MSU) crystals was associated with elevated TGF- β and collagen type I expression, actin reorganization, and the inhibition of HSC chemotaxis in an NLRP3-dependant manner [15].

However, liver-resident macrophages (Kupffer cells, KCs) produce

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Table 1
Primers used in this study for real-time PCR assay.

Human gene name	Forward sequences 5' -> 3'	Reverse sequences 5' -> 3'
GAPDH	ATGACATCAAGAAGGTGGTG	CATACCAGGAAATGAGCTTG
NLRP1	GGA CTGACGATGACTTCTGG	ATCACA AAGCAGAGACCCG
NLRP3	GTGTTTCGAATCCC ACTGTG	TCTGCTTCTCAGCTACTTTCTG
NLRC4	CAGTCCCCTCACC ATAGAAG	TCAAGTTACCC AAGCTGTCAG
NLRP6	TCTTCATCCACTCTTTCAGGC	CTCAGAAAGGTCTCGGCAG
AIM2	TGAAACCCCGAAGATCAACAC	CCGAGTACTTCCATTTTCCAG
MMP3	GACAAAGGATACAACAGGGAC	TGAGTGAGTGATAGAGTGGG
MMP9	CGAACTTTGACAGCGACAAG	CACTGAGGAATGATCTAAGCCC
COL1A1	CCGGCTCTGCTCTT TAGCG	CGTTCTGTACGCAGGTGATGGTGG
ACTA2	CATCTCATCTCCCTT GAG	ATGAAGGATGGCTGGAACAG

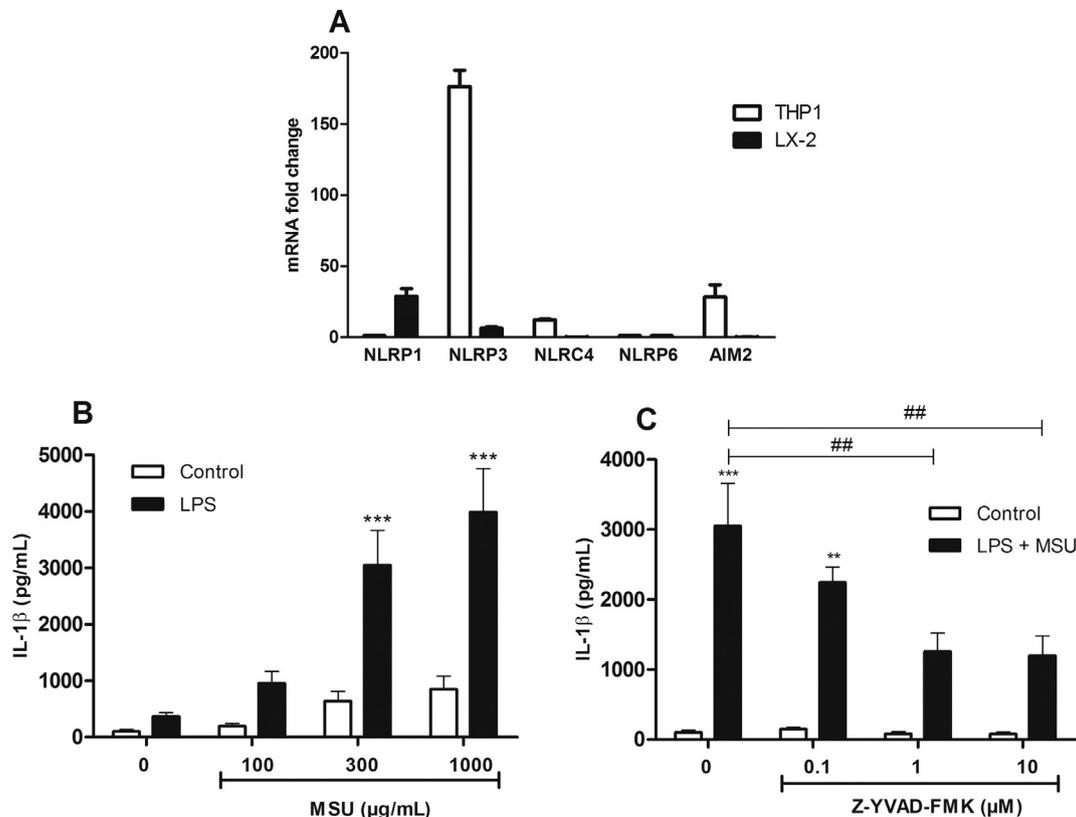


Fig. 1. mRNA expression in the inflammasome pathway in THP1 and LX2 cells, and analysis of the THP-1 cells' responses. mRNA expression in the inflammasome pathway was evaluated in THP-1 and LX2 cells (A). PMA-differentiated THP-1 cells were treated for 18 h with 100 ng/mL LPS and then for 6 h with MSU crystals (100, 300 and 1000 μg/mL). IL-1β levels were quantified using an ELISA (B). One hour before stimulation with MSU crystals, cells were pretreated with increasing concentrations of Z-YVAD-FMK (0.1, 1 and 10 μM). Supernatants were collected, and IL-1β levels were quantified using an ELISA (C). The results are expressed as the mean ± SEM of three independent experiments. **p < 0.01, ***p < 0.001, relative to a control. ##p < 0.01, relative to the LPS + MSU 300 μg/mL condition.

significant amounts of IL-1β [18] and express most of the NLRs. KCs have been identified as the main source of lipopolysaccharide (LPS)-induced IL-1β and IL-18 release [19,20]. Accordingly, caspase-1-deficient KCs were not able to secrete mature IL-1β or IL-18 upon LPS stimulation [21].

The interaction of HSCs with pro-inflammatory cells (such as KCs) is a crucial event in HSC activation and the fibrosis process [22–24]; macrophage migration is an important component of the hepatic wound-healing process that promotes HSC activation and extracellular matrix (ECM) deposition. Indeed, it has been reported that KCs and myofibroblasts are co-localized in portal areas and fibrotic septa - suggesting that these cells work together [24].

IL-1β exerts its effects through paracrine signalling, which is considered to be the primary mode of interaction between immune cells and parenchymal cells in the liver [25,26]. In animal models, liver fibrosis is associated with elevated IL-1 levels are elevated; furthermore,

IL-1R-deficient mice display significantly less liver fibrosis [9]. In this context, the expression of matrix metalloproteinases (MMPs) and tissue inhibitors of metalloproteinases (TIMPs) is modulated by IL-1, which modifies fibrosis and tissue remodelling [9].

The structurally related extracellular zinc endopeptidase MMPs are able to cleave one or several constituents of the ECM [27]. This activity enables the MMPs to modulate a range of biological processes - especially those related to immunity, tissue repair and/or tissue remodelling. Several studies have demonstrated the involvement of MMPs in pulmonary and/or hepatic fibrosis [28]. Along with the TIMPs, MMPs are also considered to the main factors in ECM turnover during hepatic fibrosis [29,30]. However, the exact pathophysiological mechanisms of pulmonary and hepatic fibrosis remain unclear.

Furthermore, the exact mechanisms by which IL-1R signalling promotes fibrosis and the cell type or types that might produce IL-1β have yet to be fully defined. Another important question is whether the pro-

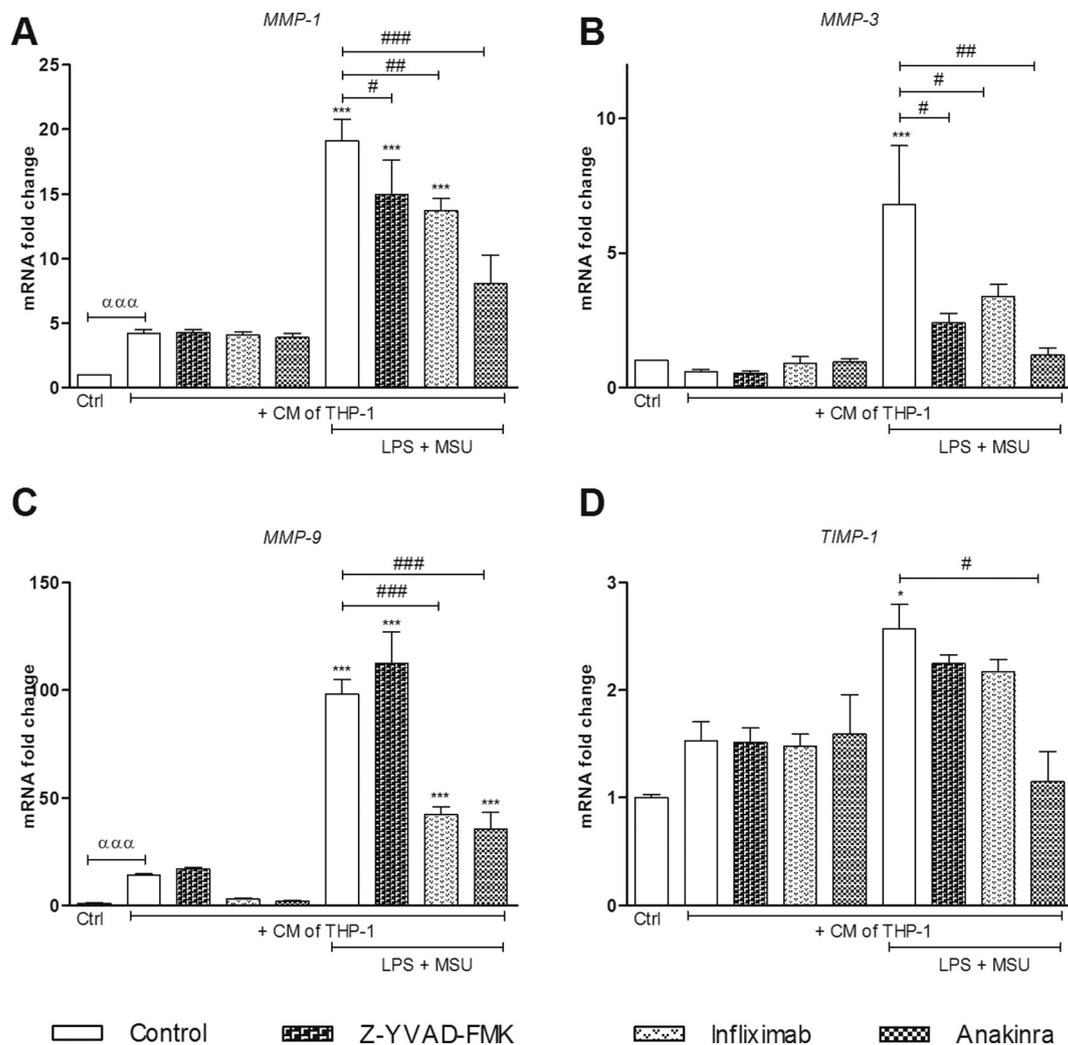


Fig. 2. The roles of TNF- α , IL-1 β and inflammasome activation in the response of LX-2 cells to CM from THP-1 cells. mRNA expression levels of MMP-1 (A), MMP-3 (B), MMP-9 (C), and TIMP-1 (D) were measured in LX-2 cells. The LX-2 cells were stimulated (or not) for 24 h with CM obtained from THP-1 cells that had been activated (or not) with LPS and MSU crystals. In some experiments, the anti-TNF- α monoclonal antibody infliximab (100 μ g/mL), the IL-1 receptor antagonist anakinra (1 μ g/ml), or the caspase-1 inhibitor Z-YVAD-FMK (10 μ M) were added 1 h prior to stimulation of the LX-2 cells with CM. The results are expressed as the mean \pm SEM of three independent experiments. $\alpha\alpha\alpha$ p < 0.001, relative to the control experiment (blank). ***p < 0.001, relative to the control experiment (black) in the absence of LPS + MSU. #p < 0.05, ##p < 0.01, ###p < 0.001, relative to the LPS + MSU condition.

fibrogenic effects of inflammasome activation and IL-1 β signalling are due to (i) the direct activation of matrix-producing cells (such as myofibroblasts) by DAMPs, or (ii) DAMP-induced activation of immune cells (such as macrophages) and thus indirect activation of myofibroblasts through an IL-1 β -mediated pathway. To investigate this question, we co-cultured human hepatic fibroblasts (either the LX-2 cell line or primary human HSCs) with differentiated macrophages (the THP-1 cell line or human monocyte-derived macrophages) in which the inflammasome pathway had been activated by treatment with a combination of LPS and MSU crystals.

2. Materials and methods

2.1. Reagents

Recombinant human granulocyte macrophage colony-stimulating factor (rhGM-CSF) was purchased from R&D Systems (Abingdon, UK). LPS from *E. coli* 055:B5, phorbol 12-myristate 13-acetate (PMA), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), gelatin solution and Nycodenz were obtained from Sigma-Aldrich (St. Louis, MO, USA). Z-YVAD-FMK was purchased from Calbiochem (Darmstadt,

Germany). Infliximab was obtained from MSD (Courbevoie, France), and anakinra was obtained from Swedish Orphan Biovitrum (Levallois-Perret, France). Dulbecco's Modified Eagle's Medium (DMEM), Roswell Park Memorial Institute (RPMI) medium 1640, antibiotics, L-glutamine, sodium pyruvate, trypsin-EDTA, and HEPES were purchased from Invitrogen (Eugene, OR, USA). Foetal calf serum (FCS) was obtained from Lonza (Levallois, France). Bovine serum albumin (BSA) was purchased from Eurobio (Les Ulis, France).

2.2. Crystal preparation

MSU crystals were prepared by recrystallization from uric acid, as previously described [31]. Briefly, 1.68 mg of MSU was dissolved in 0.01 M NaOH preheated to 70 $^{\circ}$ C (pH 7.1–7.2). The solution was slowly and continuously agitated at room temperature until crystals formed. The crystals were washed twice with 100% ethanol, dried, autoclaved, and weighed under sterile conditions. Immediately prior to experiments, the crystals were resuspended in phosphate-buffered saline (PBS), sonicated and examined under a phase microscope.

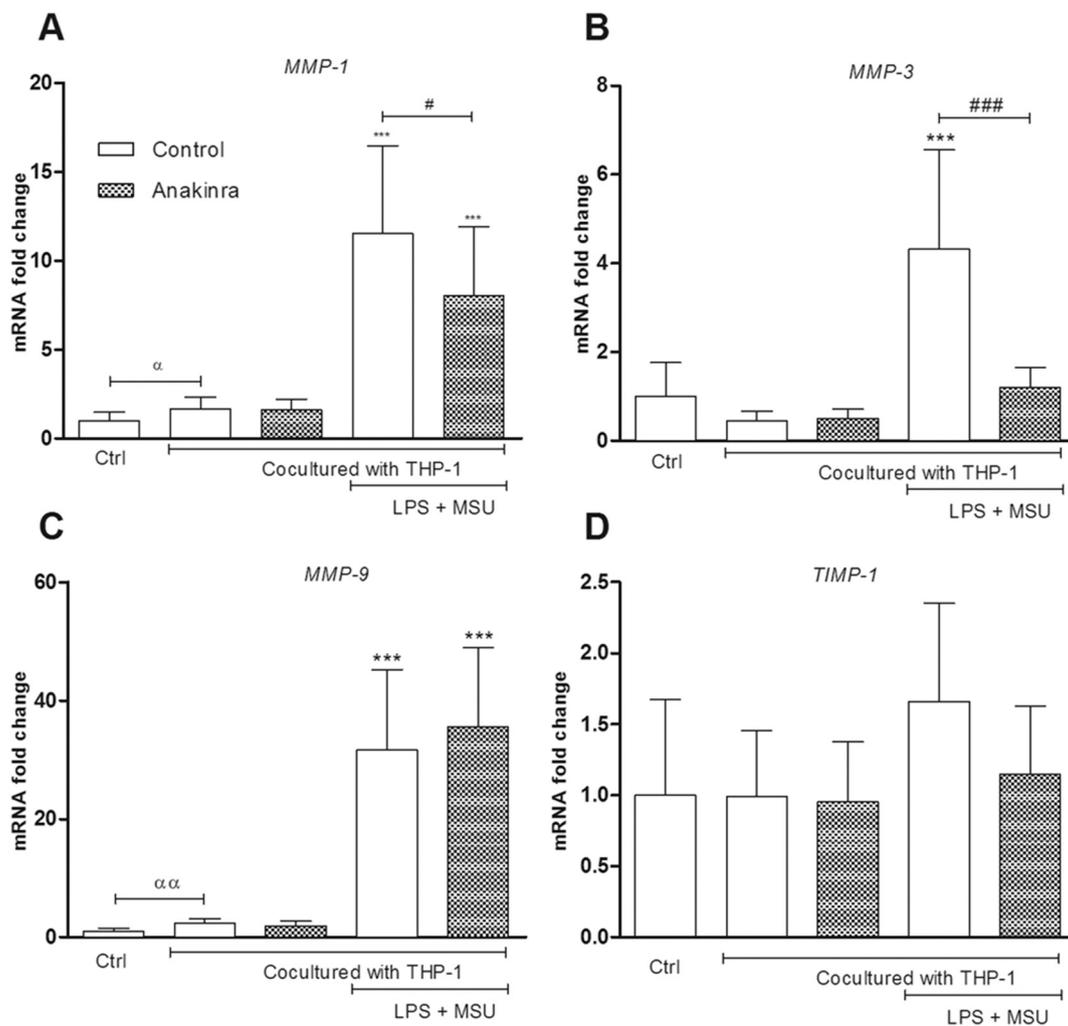


Fig. 3. Role of IL-1 β in the response of LX-2 cells co-cultured with THP-1 cells. mRNA expression levels of MMP-1 (A), MMP-3 (B), MMP-9 (C), and TIMP-1 (D) were measured in LX-2 cells. LX-2 cells were co-cultured (or not) with differentiated THP-1 cells that had been activated (or not) with LPS and MSU crystals. In some experiments, LX-2 cells were pretreated with the IL-1 receptor antagonist anakinra (1 μ g/ml) 1 h before the start of the co-culture. The results are expressed as the mean \pm SEM of three independent experiments. α p < 0.05, $\alpha\alpha$ p < 0.01, relative to the control experiment (blank). ***p < 0.001, relative to the control experiment (black) in the absence of LPS + MSU. #p < 0.05, ###p < 0.001, relative to the LPS + MSU condition.

2.3. Cell culture

THP-1 cells were purchased from the American Type Culture Collection (Manassas, VA, USA) and maintained in RPMI 1640 medium supplemented with 10% FCS, 50 IU/mL penicillin, 50 μ g/mL streptomycin, 2 mM L-glutamine and 1 mM sodium pyruvate at 37 $^{\circ}$ C in a humidified 5% CO₂ incubator. The cells were differentiated by treatment with 10 ng/mL PMA for 3 days, followed by 24 h in the absence of PMA in RPMI 1640 medium supplemented with 2% FCS prior to the experimental treatments.

LX-2 cells were provided by S.L. Friedman (Mount Sinai School of Medicine, New York, NY, USA) [32] and were maintained in DMEM medium supplemented with 10% FCS, 50 IU/mL penicillin, 50 μ g/mL streptomycin, 2 mM L-glutamine, and 1 mM sodium pyruvate at 37 $^{\circ}$ C in a humidified 5% CO₂ incubator. The cells used in experiments were obtained from passage 16 to passage 26.

Primary human MDMs were obtained by differentiating the peripheral blood mononuclear cells (PBMCs) in buffy-coat donations (French Blood Agency, *Etablissement Français du Sang*, Rennes, France), as previously described [31]. The experiments complied with ethical requirements of the French legislation on blood transfusion safety (government act 93-5 dated January 4th 1993) and were approved by the French Blood Agency (Rennes, France). Monocytes from healthy

donors were enriched using a human CD14 microbead separation kit (Miltenyi Biotec, Bergisch Gladbach, Germany). Human macrophages were differentiated from monocytes by a 7-day incubation with 50 ng/mL rhGM-CSF in RPMI medium supplemented with 10% FCS, 50 IU/mL penicillin, 50 μ g/mL streptomycin, 2 mM L-glutamine, and 0.1 mM HEPES.

Primary human HSCs were isolated from histologically normal biopsy specimens after partial hepatectomy in adult patients undergoing liver resection for metastases, as previously described [33]. These samples were obtained from the Rennes Biological Resource Centre (*Centre de Ressources Biologiques Santé*, Rennes, France). The research complied with French and European legislation and regulatory guidelines, and was approved by the biological resource centre's ethics committee (reference: BB-0033-00056). The liver tissue was dissociated by perfusion with a pronase-collagenase solution (Biopredic International, Rennes, France). HSCs were purified from the first supernatant of the liver perfusion (enriched in non-parenchymal cells) after hepatocyte isolation. This method relies on the fact that HSCs are naturally rich in vitamin A, which enables their single-step purification on a Nycodenz density gradient. To this end, the supernatant was centrifuged and the pellet was re-suspended to a final volume of 7 mL in cold Grey's balanced salt solution (GBSS). Five millilitres of calcium-free GBSS + Nycodenz 29% was then added. After mixing, 1 mL of

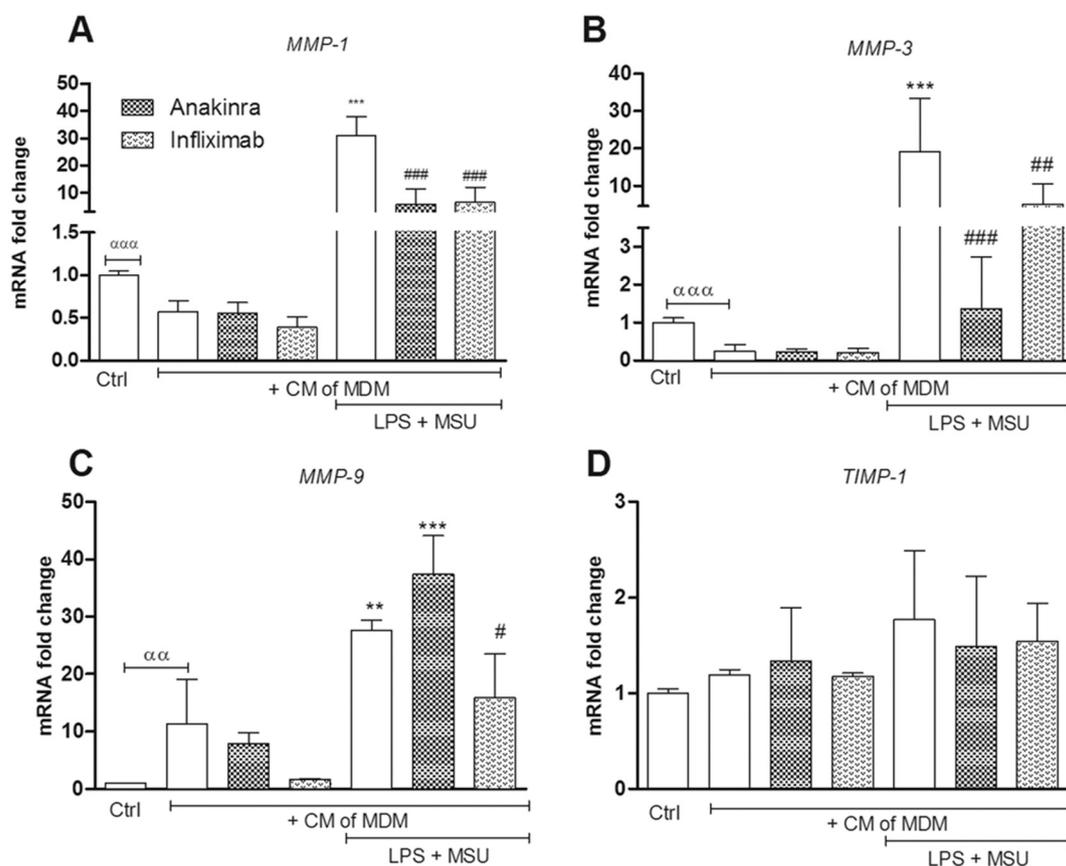


Fig. 4. Role of IL-1 β or TNF- α in the response of LX-2 cells to CM from human monocyte-derived macrophages. mRNA expression levels of MMP-1 (A), MMP-3 (B), MMP-9 (C), and TIMP-1 (D) in LX-2 cells. LX-2 cells were stimulated for 24 h (or not) with CM obtained from human MDMs activated (or not) for 18 h with 100 ng/mL LPS and then for 6 h with 300 μ g/mL MSU crystals. In some experiments, MDMs were pretreated with the IL-1 receptor antagonist anakinra (1 μ g/ml or 100 μ g/mL) or the anti-TNF- α monoclonal antibody infliximab (100 μ g/mL) for 1 h prior to the stimulation of LX-2 cells with CM. The results are expressed as the mean \pm SEM of three independent experiments. $\alpha\alpha\alpha$ p < 0.01, $\alpha\alpha\alpha\alpha$ p < 0.001, relative to the control experiment (blank). ** p < 0.01, *** p < 0.001, relative to the control experiment (black) in the absence of LPS + MSU. # p < 0.05, ### p < 0.001, relative to the LPS + MSU condition.

calcium-free GBSS + BSA 0.3% was placed gently on the cell suspension surface. The tube was then centrifuged for 15 min at 1400g, to allow the HSCs to collect at the interface between the Nycodenz and the BSA; other resident cell types fell to the bottom of the tube. Lastly, HSCs were washed once in GBSS and seeded on tissue culture dishes exposed to vacuum gas plasma (Falcon™, BD Biosciences) in the same medium as LX-2 cells. Primary HSC cultures were maintained at 37 °C in a humidified 5% CO₂ incubator, with a passage every 20 to 25 days. Purification and culture on plastic dishes is known to spontaneously activate HSCs [35].

2.4. Treatments

Non-adherent THP-1 cells or PBMCs were seeded at a density of 1×10^5 per cm² prior to differentiation. LX-2 cells were seeded at 3×10^4 per cm², and grown in DMEM supplemented with 10% FCS until confluence was reached. The medium was then replaced with fresh medium supplemented with 2% FCS for 24 h. Thereafter, cells were incubated for 18 h in the presence or absence of LPS (100 ng/mL). MSU crystals were added for 6 h with or without an hour of pretreatment with Z-YVAD-FMK (a caspase-1-specific inhibitor).

2.5. Co-cultures and conditioned medium studies

Two types of co-cultures were grown in a mixture of 20% DMEM and 80% RPMI 1640 medium supplemented with 2% FCS (determined after an MTT cytotoxicity assay). The first method used conditioned media (CM) from differentiated THP-1 cells or differentiated PBMCs

treated with LPS (or not) for 18 h and then with MSU crystals for 6 h (with or without an hour of pretreatment with Z-YVAD-FMK). The supernatant was removed, centrifuged for 10 min at 10000 rpm (to remove MSU crystals and debris), and added to cultures of LX-2 cells or HSCs that had been pretreated (or not) with anakinra (an IL-1R antagonist) or infliximab (a monoclonal antibody against TNF- α) for 1 h. After 24 h of contact with the CM, the LX-2 cells or HSCs were harvested. In the second method, THP-1/LX-2 cells were co-cultured using six-well plates and 1 μ m pore-size Transwell inserts (BD Biosciences), which allow diffusion of media components but prevent cell migration. The LX-2 cells were plated on the bottom, and the THP-1 cells were plated and differentiated on the insert. After differentiation, inserts containing THP-1 were placed on LX-2 cells, which were pretreated (or not) for 1 h with anakinra, and filled with the same medium (with or without LPS) to create a gravity gradient of the released mediators. A 3:1 macrophage/fibroblast ratio was chosen because this corresponds to the physiological ratio in the liver. After 18 h of incubation with LPS, MSU crystals were added (with or without an hour of pretreatment of THP-1 cells with Z-YVAD-FMK). LX-2 cells were harvested 24 h after MSU stimulation of the THP-1 cells.

2.6. Extraction of total RNA, and real-time PCR

Total RNA was isolated from cells using the SV Total RNA Isolation System (Promega, Madison, WI, USA). RNA quantity and purity were assessed with a Nanodrop ND-1000 spectrophotometer (Nyxor Biotech, Paris, France). Total RNA (1 μ g) was reverse-transcribed into cDNA using the High-Capacity cDNA kit (Applied Biosystems, Foster City, CA,

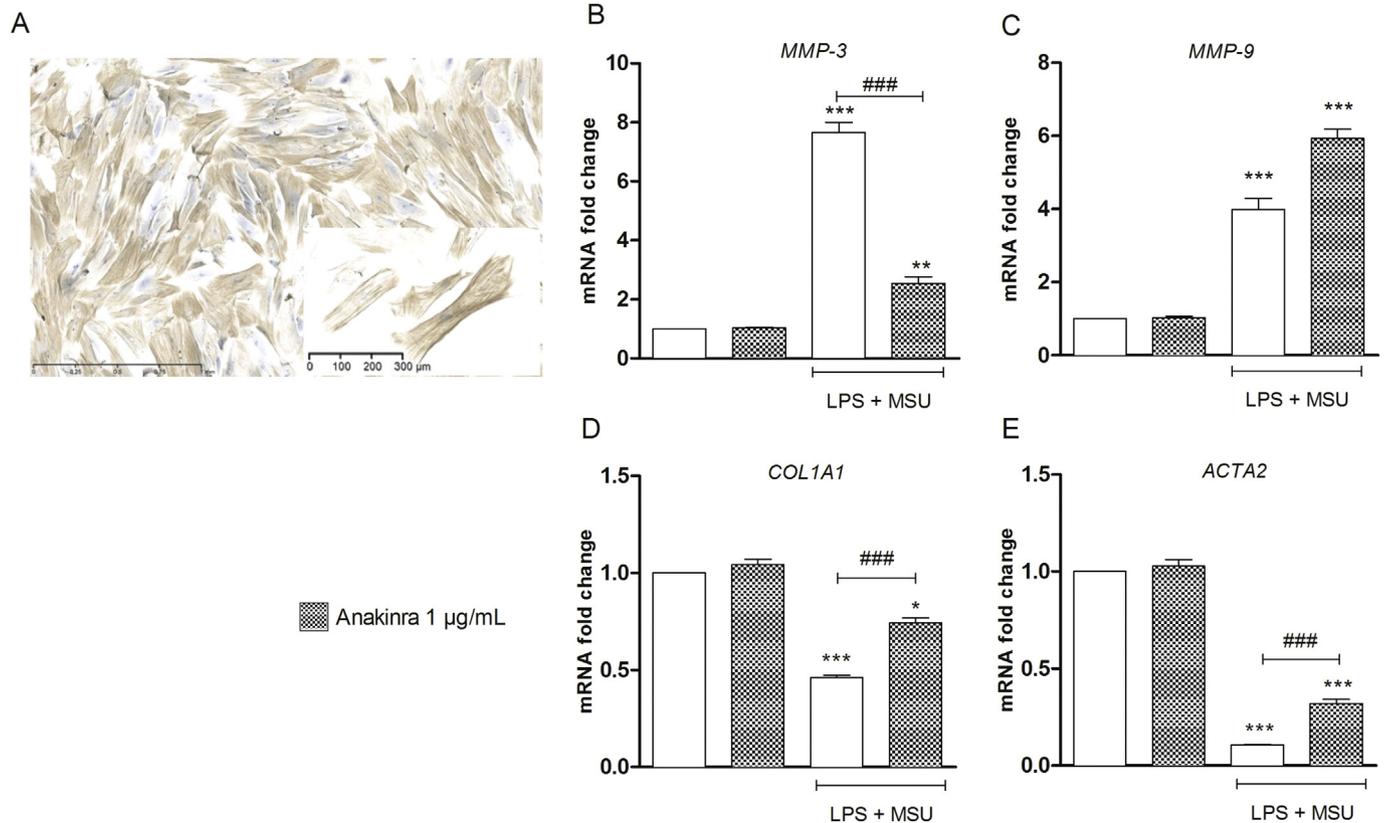


Fig. 5. Role of IL-1 β in the response of primary human HSCs to CM from THP-1 cells. (A) α -SMA immunostaining of 7-day cultures of primary human HSCs purified from patients, and mRNA expression levels of MMP-3 (B), MMP-9 (C), COL1A1/collagen I α 1 (D), and ACTA2/ α -SMA (E), in primary human HSCs. HSCs were stimulated for 24 h (or not) with CM obtained from PMA-differentiated THP-1 cells activated (or not) for 18 h with 100 ng/mL LPS and then for 6 h with 300 μ g/mL MSU crystals. In some experiments, 1 μ g/mL of anakinra was added one hour before stimulation of the HSCs with CM. The results are expressed as the mean \pm SEM of three independent experiments. ** p < 0.01, *** p < 0.001, relative to the control experiment (black) in the absence of LPS + MSU. ### p < 0.001, relative to the LPS + MSU condition.

USA). Real-time quantitative RT-PCR was performed with the SYBR Green fluorescent dye method using the SYBR Green PCR Master Mix (Applied Biosystems) and the 7900HT Fast Real-Time PCR system (Applied Biosystems). Primer pairs for each transcript (Table 1) were chosen with IDT software (<http://eu.idtdna.com/scitools/Applications/RealTimePCR/>) and “blasted” with NCBI (<http://www.ncbi.nlm.nih.gov/BLAST/>). Amplification curves were read with SDS 2.3 software (Applied Biosystems), using the comparative cycle threshold method. The steady-state level of mRNA for each gene of interest was normalized against the value for GAPDH mRNA.

2.7. ELISA of IL-1 β

Media were collected and stored at -20°C until use. The level of IL-1 β in the supernatant was measured using a Duoset[®] ELISA kit (R&D Systems), according to the manufacturer's procedure. Briefly, media were added to plates coated with the capture antibody and incubated at room temperature for 2 h, followed by incubation with biotinylated antibody. Streptavidin-conjugated horseradish-peroxidase was added to the plates. After the addition of substrate, enzyme activity was detected with an ELISA microplate reader (POLARstar Omega, BMG Labtech). The ELISA's sensitivity for human IL-1 β was 3.91–250 pg/mL.

2.8. Alpha smooth muscle actin (α -SMA) immunostaining

HSCs were seeded onto Lab-Tek[®] II chamber slides[™] (Dominique Dutscher, Issy-les-Moulineaux, France) and cultured for 2 days. The cells were washed in PBS, fixed in formaldehyde 4% at 4°C for 30 min, washed again three times with PBS, and then permeabilized with

saponin 0.05% in PBS for 30 min. Immunocytochemistry staining was performed on the Discovery XT automated system using a Ventana DABMap detection kit (Roche Diagnostics, Basel, Switzerland). The preset detection procedure had been optimized for the Discovery system. The Ventana High Temperature Liquid Coverslip (Roche Diagnostics) was applied throughout the automated protocol when appropriate. Between steps, the slides were rinsed with Ventana Tris Based Reaction buffer (Roche Diagnostics). Endogenous peroxidase was blocked by incubation with inhibitor-D 3% H_2O_2 for 4 min at 37°C . After rinsing, slides were incubated at 37°C for 32 min with a 1/500-diluted mouse antibody against α -SMA (Dako Cytomation, Glostrup, Denmark). Signal enhancement was carried out using the Ventana DABMap kit (Roche Diagnostics) with biotinylated horse anti-mouse immunoglobulin G (H + L, Vector Laboratories, Burlingame, CA, USA). Slides were counterstained for 4 min with hematoxylin and then rinsed. After removal from the instrument, slides were manually dehydrated and cover-slipped. Images were captured using a scanner and processed with NIS-Elements AR software (version 4.00.03, Nikon, Paris, France).

2.9. Statistical analysis

The results were expressed as the mean \pm standard error of the mean (SEM) of three independent experiments. Intergroup differences in treatment effects were probed with a one-way analysis of variance with Tukey's post-hoc test for multiple comparisons. The significance of intergroup differences for other parameters was determined using a two-way analysis of variance and Bonferroni's post-hoc test. All analyses were carried out using Prism software (version 5.0, GraphPad Software, La Jolla, CA, USA). The threshold for statistical significance

was set to $p < 0.05$, and all tests were two-sided.

3. Results

3.1. THP-1 cells (but not LX-2 cells) express the NLRP3 inflammasome and release IL-1 β

When considering the various NLRPs, THP-1 cells particularly expressed the NLRP3 inflammasome and AIM2 mRNA, whereas LX-2 cells expressed NLRP1 and (weakly) NLRP3 mRNA (Fig. 1A). Exposure to MSU crystals induce the significant production of IL-1 β after THP-1 cells had been primed with LPS for 18 h (Fig. 1B); the production of IL-1 β was significantly lower after 1 h of treatment with the caspase-1 inhibitor Z-YVAD-FMK, in a dose-dependent manner (Fig. 1C).

3.2. Exposure to CM and co-culture with THP-1 cells induce changes in gene expression in LX-2 cells, and these changes depend on IL-1 β /TNF- α release

Conditioned media from THP-1 cells stimulated with LPS and MSU crystals were found to induce the upregulation of MMP-1, MMP-3 and MMP-9 mRNA expression by LX-2 cells, relative to treatment with CM obtained from non-stimulated THP-1 cells (Fig. 2A–C). In contrast, there was no difference in the mRNA expression of TIMP-1 was noted (Fig. 2D).

Pretreatment of LX-2 cells with infliximab significantly reduced the effects of CM on the mRNA expression of MMP-3 (Fig. 2B) and MMP-9 (Fig. 2C). Pretreatment of LX-2 cells with anakinra significantly reduced the effects of CM on the mRNA expression of MMP-1 (Fig. 2A), MMP-3 (Fig. 2B), MMP-9 (Fig. 2C) and TIMP-1 (Fig. 2D). Moreover, MMP-1 and MMP-3 expression were also significantly reduced by pretreatment with Z-YVAD-FMK (Fig. 2A and B) - suggesting that gene expression in LX-2 cells is modulated by caspase-1-dependent inflammasome activation.

Co-culture of LX-2 cells with LPS- and MSU-stimulated THP-1 cells also upregulated MMP-1, MMP-3 and MMP-9 expression, relative to LX-2 cells co-cultured with non-stimulated THP-1 cells (Fig. 3A–C).

The effects of co-culturing LX-2 cells with LPS- and MSU-stimulated THP-1 cells on MMP-1 and MMP-3 expression were partially reduced by pretreatment of the LX-2 cells with anakinra (Fig. 3A and B).

3.3. Conditioned media from MDMs induces changes in gene expression in LX-2 cells, and these changes depend on IL-1 β /TNF- α release

Conditioned media obtained from LPS- and MSU-stimulated MDMs were found to induce the upregulation of MMP-1, MMP-3 and MMP-9 expression but not TIMP-1 expression, relative to LX-2 cells treated with CM from non-stimulated, differentiated-MDM cells (Fig. 4). The effects on MMP-1 and MMP-3 were reduced by pretreatment with anakinra and infliximab (Fig. 4A and B), whereas the effect on MMP-9 expression was only reduced by pretreatment with infliximab (Fig. 4C).

3.4. Conditioned media from THP-1 cells induces changes in gene expression in primary human HSCs, and these changes are dependent on IL-1 β release

Following the purification of quiescent primary human HSCs, we checked that they had fully differentiated into myofibroblasts by measuring the expression of α -SMA (Fig. 5A). Media conditioned by LPS- and MSU-stimulated THP-1 cells were found to induce the upregulation of MMP-3 (Fig. 5B) and MMP-9 (Fig. 5C) expression, and the downregulation of COL1A1 (Fig. 5D) and ACTA2 (Fig. 5E) expression, relative to primary human HSCs treated with CM obtained from non-stimulated THP-1 cells. The upregulation of MMP-3 expression was reduced by pretreatment with anakinra (Fig. 5B).

4. Discussion

The inflammasome pathway's involvement in liver fibrosis has been reported in many different animal models [35]. However, the precise mechanism by which the inflammasome drives the MMP/TIMP imbalance and fibrogenesis has yet to be characterized. The present study investigated (i) the influence of factors secreted into the medium, and (ii) a model in which macrophages (the THP-1 cell line or blood MDMs) and human hepatic myofibroblasts (the LX-2 cell line or primary HSCs) were co-cultured. In previous work, we found that LX-2 cells were probably involved in the fibrogenic and inflammatory responses induced by several cytokines [36]. We observed that even though LX-2 cells express markers of myofibroblast activation at baseline, they are still able to respond to TGF- β 1 stimulation and thus develop pro-fibrotic and anti-inflammatory phenotypes. However, the direct incubation of LX-2 cells with pro-inflammatory mediators IL-1 β or TNF- α elicits the opposite effect (i.e. a pro-inflammatory phenotype, with down-regulation of the myofibroblast differentiation marker α -SMA), and also stimulates a pro-fibrotic response with the upregulation of MMP-1, MMP-3 and MMP-9. These data suggested that activated HSCs (i) are important factors in hepatic inflammation, and (ii) contribute to the regression or perpetuation of fibrosis by regulating MMP expression.

Inflammasome proteins are expressed in a wide variety of liver cells. Importantly, the liver expresses higher levels of caspase-1 than many other tissues [12]. It has also been reported that myofibroblasts express inflammasome pathway components [17]. A challenge with LPS drastically enhances the expression of NLRs in cultured HSCs - suggesting that inflammasome activation is one of the features that enables these cells to contribute to immune responses in the liver [37]. Here, we found that LX-2 cells expressed NLRP1 mRNA more strongly than other NLRs or AIM2, and also that co-stimulation of LX-2 cells with LPS and MSU crystals induced the release of IL-1 β . However, LX-2 cells generally release low levels of IL-1 β . Indeed, KCs are reportedly the liver cells that express the highest levels of inflammasome pathway components and IL-1 β [14–18]. Our present results are in line with these literature data; we found that differentiated THP-1 cells mostly express NLRP3 (along with other NLRs, and AIM2) on the mRNA level. Moreover, co-stimulation with LPS and MSU crystals elicited the significant release of IL-1 β . This effect was dependent on activation of the inflammasome pathway, since pretreatment with the caspase-1 inhibitor Z-YVAD-FMK significantly decreased the release of IL-1 β but did not modify gene expression following LPS stimulation.

Due to the HSCs' anatomic position in the space of Disse (between the fenestrated endothelium of the sinusoids and the hepatocytes), these cells are optimally positioned for interaction with many types of liver cell. In the fibrotic liver, macrophages consistently migrate towards activated myofibroblasts in areas of scar tissue [22,25,38]. The interactions between KCs and HSCs, however, are known to be very complex as a result of heterogeneity in the individual cell populations. Although co-culture activation alone only partially reproduced the characteristic changes that affect HSCs during liver cirrhosis, co-culture of HSCs with KCs more closely resembled the *in vivo* situation [39]; this finding also indicates the relevance of macrophage-HSC interactions *in vivo*. Crosstalk between macrophages and liver fibroblasts leads to changes in function and in the patterns of cytokines secreted by each cell type [40,41]. Accordingly, our experiments revealed that under basal conditions, CM from the THP-1 cell line or MDMs was unable to induce a pro-inflammatory state in LX-2 cell line - probably because the latter were exposed for a relatively short time (24 h). In contrast, the co-culture of THP-1 cells on inserts with LX-2 cells under basal conditions induced the mRNA upregulation of IL-1 β - probably because the cells shared the same medium for longer (42 h). Following activation of the inflammasome pathway in macrophages (using both CM and inserts for co-culture), IL-1 β and TNF- α were found to be responsible for the pro-inflammatory state observed in LX-2 cells and HSCs.

Chronic inflammation in the liver is responsible for an abnormally

high accumulation of ECM components; the latter include collagens and proteoglycans, which are major factors in the formation of transformed tissue. MMPs and TIMPs are the main regulators of ECM turnover in hepatic fibrosis [30]. HSCs express ECM components, MMPs and TIMPs over different timeframes, and are thought to have a key role in the development of hepatic fibrosis [42]. Liver macrophages can express several MMPs that are involved in matrix degradation and thereby favour the resolution of liver injury and fibrosis [38,43]. We found that following activation of the inflammasome pathway, MMP-1, MMP-3 and MMP-9 mRNA levels were upregulated in LX-2 cells and in HSCs. TIMPs are specific endogenous inhibitors that bind to the active site of the MMPs in a stoichiometric 1:1 M ratio, thereby blocking access to ECM substrates. Four TIMPs (TIMP-1, -2, -3 and -4) have been identified in vertebrates, and their expression is regulated during development, tissue remodelling, and also inflammation [44].

Given that MMPs degrade various components of the ECM, tight regulation of the MMPs' activity is essential for preventing excessive matrix degradation. We did not observe no differences in TIMP-1 mRNA levels, which suggests that these inhibitors did not regulate MMP activity in our experiments. We found that *in vitro* inflammasome activation in macrophages was associated with a change in the MMPs/TIMP ratio. This led to an antifibrotic phenotype to HSCs, with down-regulation of their mRNA expression levels of α -SMA and collagen type I α 1. This finding is in line with a previous study in which the NLRP3 inflammasome in mouse macrophages was involved in ECM deposition via the activation of HSCs [45]. However, it was not clear whether these effects were dependent on IL-1 β and/or TNF- α , given that pretreatment with anakinra, Z-YVAD-FMK or infliximab failed to significantly dampen the effects in LX-2 cells (although anakinra pretreatment of primary human HSCs was effective).

In conclusion, we characterized the complex interactions between pro-inflammatory macrophages and HSCs in the liver. Although direct paracrine signals seem to be active during the early phases of the injury, indirect mechanisms also link inflammation and MMP/TIMP imbalance. The hypothesis whereby activation of the inflammasome pathway and the release of IL-1 β have an indirectly pro-fibrotic role was supported by our observation that the macrophage-induced activation of NF- κ B in HSCs *in vitro* and *in vivo* was mediated by IL-1 β and TNF- α . IL-1 β and TNF- α did not promote HSC activation but did enhance the survival of activated HSCs *in vitro* and *in vivo*, and may influence liver tissue remodelling.

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Conflict of interest

The authors declare no competing interests.

Authors contributions

Concept and design: Sacha Robert, Vincent Lagente, and Elisabeth Boichot; experiments and procedures: Sacha Robert, Thomas Gicquel, Aude Bodin, Alain Fautrel, Emiliano Barreto, and Tatiana Victoni; manuscript drafting: Sacha Robert, Thomas Gicquel, Vincent Lagente, and Elisabeth Boichot.

References

- [1] T.A. Wynn, Common and unique mechanisms regulate fibrosis in various fibroproliferative diseases, *J. Clin. Invest.* 117 (2007) 524–529.
- [2] J.J. Tomasek, G. Gabbiani, B. Hinz, C. Chaponnier, R.A. Brown, Myofibroblasts and mechano-regulation of connective tissue remodelling, *Nat. Rev. Mol. Cell Biol.* 3 (2002) 349–363.
- [3] U.E. Lee, S.L. Friedman, Mechanisms of hepatic fibrogenesis, *Best Pract. Res. Clin. Gastroenterol.* 25 (2011) 195–206.
- [4] T. Kawai, S. Akira, The role of pattern-recognition receptors in innate immunity: update on Toll-like receptors, *Nat. Immunol.* 11 (2010) 373–384.
- [5] O. Takeuchi, S. Akira, Pattern recognition receptors and inflammation, *Cell* 140 (2010) 805–820.
- [6] K. Schroder, J. Tschopp, The inflammasomes, *Cell* 140 (2010) 821–832.
- [7] B.K. Davis, H. Wen, J.P.-Y. Ting, The inflammasome NLRs in immunity, inflammation, and associated diseases, *Annu. Rev. Immunol.* 29 (2011) 707–735.
- [8] P. Gasse, C. Mary, I. Guenon, N. Noulin, S. Charron, S. Schnyder-Candrian, et al., IL-1R1/MyD88 signaling and the inflammasome are essential in pulmonary inflammation and fibrosis in mice, *J. Clin. Invest.* 117 (2007) 3786–3799.
- [9] R.G. Gieling, K. Wallace, Y.-P. Han, Interleukin-1 participates in the progression from liver injury to fibrosis, *Am. J. Physiol. Gastrointest. Liver Physiol.* 296 (2009) G1324–G1331.
- [10] T. Csak, M. Ganz, J. Pespisa, K. Kodys, A. Dolganiuc, G. Szabo, Fatty acid and endotoxin activate inflammasomes in mouse hepatocytes that release danger signals to stimulate immune cells, *Hepatology* 54 (2011) 133–144.
- [11] W. Yan, Y. Chang, X. Liang, J.S. Cardinal, H. Huang, S.H. Thorne, et al., High-mobility group box 1 activates caspase-1 and promotes hepatocellular carcinoma invasiveness and metastases, *Hepatology* 55 (2012) 1863–1875.
- [12] A.S. Paszkowski, B. Rau, J.M. Mayer, P. Möller, H.G. Beger, Therapeutic application of caspase 1/interleukin-1beta-converting enzyme inhibitor decreases the death rate in severe acute experimental pancreatitis, *Ann. Surg.* 235 (2002) 68–76.
- [13] J. Masumoto, S. Taniguchi, J. Nakayama, M. Shiohara, E. Hidaka, T. Katsuyama, et al., Expression of apoptosis-associated speck-like protein containing a caspase recruitment domain, a pyrin N-terminal homology domain-containing protein, in normal human tissues, *J. Histochem. Cytochem. Off. J. Histochem. Soc.* 49 (2001) 1269–1275.
- [14] A.A. Negash, H.J. Ramos, N. Crochet, D.T.Y. Lau, B. Doehle, N. Papic, et al., IL-1 β production through the NLRP3 inflammasome by hepatic macrophages links hepatitis C virus infection with liver inflammation and disease, *PLoS Pathog.* 9 (2013) e1003330.
- [15] A. Watanabe, M.A. Sohail, D.A. Gomes, A. Hashmi, J. Nagata, F.S. Sutterwala, et al., Inflammasome-mediated regulation of hepatic stellate cells, *Am. J. Physiol. Gastrointest. Liver Physiol.* 296 (2009) G1248–G1257.
- [16] A.B. Imaeda, A. Watanabe, M.A. Sohail, S. Mahmood, M. Mohamadnejad, F.S. Sutterwala, et al., Acetaminophen-induced hepatotoxicity in mice is dependent on Th9 and the Nalp3 inflammasome, *J. Clin. Invest.* 119 (2009) 305–314.
- [17] R. Rawat, T.V. Cohen, B. Ampong, D. Francia, A. Henriques-Pons, E.P. Hoffman, et al., Inflammasome up-regulation and activation in dysferlin-deficient skeletal muscle, *Am. J. Pathol.* 176 (2010) 2891–2900.
- [18] 7–23, S.L. Friedman, M.J. Arthur, Activation of cultured rat hepatic lipocytes by Kupffer cell conditioned medium. Direct enhancement of matrix synthesis and stimulation of cell proliferation via induction of platelet-derived growth factor receptors, *J Clin Invest*, 84 1989, pp. 1780–1785.
- [19] K. Matsui, T. Yoshimoto, H. Tsutsui, Y. Hyodo, N. Hayashi, K. Hiroishi, et al., Propionibacterium acnes treatment diminishes CD4+ NK1.1+ T cells but induces type 1 T cells in the liver by induction of IL-12 and IL-18 production from Kupffer cells, *J. Immunol.* 166 (1997) 97–106.
- [20] M. Imamura, H. Tsutsui, K. Yasuda, R. Uchiyama, S. Yumikura-Futatsugi, K. Mitani, et al., Contribution of TIR domain-containing adapter inducing IFN- β -mediated IL-18 release to LPS-induced liver injury in mice, *J. Hepatol.* 51 (2009) 333–341.
- [21] E. Seki, H. Tsutsui, H. Nakano, N. Tsuji, K. Hoshino, O. Adachi, et al., Lipopolysaccharide-induced IL-18 secretion from murine Kupffer cells independently of myeloid differentiation factor 88 that is critically involved in induction of production of IL-12 and IL-1 β , *J. Immunol.* 166 (2001) 2651–2657.
- [22] J.S. Duffield, S.J. Forbes, C.M. Constandinou, S. Clay, M. Partolina, S. Vuthoori, et al., Selective depletion of macrophages reveals distinct, opposing roles during liver injury and repair, *J. Clin. Invest.* 115 (2005) 56–65.
- [23] E. Seki, S. De Minicis, C.H. Osterreicher, J. Kluwe, Y. Osawa, D.A. Brenner, et al., TLR4 enhances TGF- β signaling and hepatic fibrosis, *Nat. Med.* 13 (2007) 1324–1332.
- [24] C. Liu, Q. Tao, M. Sun, J.Z. Wu, W. Yang, P. Jian, et al., Kupffer cells are associated with apoptosis, inflammation and fibrotic effects in hepatic fibrosis in rats, *Lab. Invest.* 90 (2010) 1805–1816.
- [25] C. McClain, S. Barve, S. Joshi-Barve, Z. Song, I. Deaciuc, T. Chen, et al., Dysregulated cytokine metabolism, altered hepatic methionine metabolism and proteasome dysfunction in alcoholic liver disease, *Alcohol. Clin. Exp. Res.* 29 (2005) 180S–8S.
- [26] B. Gao, E. Seki, D.A. Brenner, S. Friedman, J.I. Cohen, L. Nagy, et al., Innate immunity in alcoholic liver disease, *Am. J. Physiol. Gastrointest. Liver Physiol.* 300 (2011) G516–G525.
- [27] T. Vu, Z. Werb, Gelatinase B: structure, regulation, and function, in: W.C. Parks, R.P. Mecham (Eds.), *Matrix Metalloproteinases*, Academic Press, San Diego, 1998, pp. 115–148.
- [28] A. Pardo, S. Cabrera, M. Maldonado, M. Selman, Role of matrix metalloproteinases

- in the pathogenesis of idiopathic pulmonary fibrosis, *Respir. Res.* 4 (2016) 17–23.
- [29] S. Robert, T. Gicquel, T. Victoni, S. Valença, E. Barreto, B. Bailly-Maitre, E. Boichot, V. Lagente, Involvement of matrix metalloproteinases (MMPs) and inflammasome pathway in molecular mechanisms of fibrosis, *Biosci. Rep.* 36 (4) (2016) 15.
- [30] Y.P. Han, Matrix metalloproteinases, the pros and cons, in liver fibrosis, *J. Gastroenterol. Hepatol.* 21 (Suppl. 3) (2006) S88–S91.
- [31] T. Gicquel, S. Robert, P. Loyer, T. Victoni, A. Bodin, C. Ribault, et al., IL-1 β production is dependent on the activation of purinergic receptors and NLRP3 pathway in human macrophages, *FASEB J. Off. Publ. Fed. Am. Soc. Exp. Biol.* 29 (2015) 4162–4173.
- [32] L. Xu, A.Y. Hui, E. Albanis, M.J. Arthur, S.M. O'Byrne, W.S. Blaner, et al., Human hepatic stellate cell lines, LX-1 and LX-2: new tools for analysis of hepatic fibrosis, *Gut* 54 (2005) 142–151.
- [33] O. Rostan, F. Robert-Gangneux, M. Lambert, M. Samson, J.P. Gangneux, Human hepatic stellate cells in primary culture are safe targets for *Leishmania donovani*, *Parasitology* 140 (2013) 471–481.
- [34] S.L. Friedman, Hepatic stellate cells: protean, multifunctional, and enigmatic cells of the liver, *Physiol. Rev.* 88 (2008) 125–172.
- [35] G. Szabo, J. Petrasek, Inflammasome activation and function in liver disease, *Nat. Rev. Gastroenterol. Hepatol.* 12 (2015) 387–400.
- [36] S. Robert, T. Gicquel, A. Bodin, V. Lagente, E. Boichot, Characterization of the MMP/TIMP imbalance and collagen production induced by IL-1 β or TNF- α release from human hepatic stellate cells, *PLoS One* 11 (2016) e0153118.
- [37] S.G. Boaru, E. Borkham-Kamphorst, L. Tihaa, U. Haas, R. Weiskirchen, Expression analysis of inflammasomes in experimental models of inflammatory and fibrotic liver disease, *J. Inflamm. Lond. Engl.* 9 (2012) 49.
- [38] J.A. Fallowfield, M. Mizuno, T.J. Kendall, C.M. Constantinou, R.C. Benyon, J.S. Duffield, et al., Scar-associated macrophages are a major source of hepatic matrix metalloproteinase-13 and facilitate the resolution of murine hepatic fibrosis, *J. Immunol. Baltim. Md.* 1950 178 (2007) 5288–5295.
- [39] S. De Minicis, E. Seki, H. Uchinami, J. Kluwe, Y. Zhang, D.A. Brenner, et al., Gene expression profiles during hepatic stellate cell activation in culture and in vivo, *Gastroenterology* 132 (2007) 1937–1946.
- [40] R. Bonecchi, F. Facchetti, S. Dusi, W. Luini, D. Lissandrini, M. Simmelink, et al., Induction of functional IL-8 receptors by IL-4 and IL-13 in human monocytes, *J. Immunol. Baltim. Md.* 1950 (164) (2000) 3862–3869.
- [41] E. Song, N. Ouyang, M. Hörbelt, B. Antus, M. Wang, M.S. Exton, Influence of alternatively and classically activated macrophages on fibrogenic activities of human fibroblasts, *Cell. Immunol.* 204 (2000) 19–28.
- [42] R.C. Benyon, M.J. Arthur, Extracellular matrix degradation and the role of hepatic stellate cells, *Semin. Liver Dis.* 21 (2001) 373–384.
- [43] A. Pellicoro, R.L. Aucott, P. Ramachandran, A.J. Robson, J.A. Fallowfield, V.K. Snowdon, et al., Elastin accumulation is regulated at the level of degradation by macrophage metalloelastase (MMP-12) during experimental liver fibrosis, *Hepatol. Baltim. Md.* 55 (2012) 1965–1975.
- [44] K. Brew, Tissue inhibitors of metalloproteinases: evolution, structure and function, *Biochim. Biophys. Acta* 1477 (2000) 267–283.
- [45] S. Jianga, Z. Zhanga, J.H. Zhenga, X. Lia, Y.L. Yaoa, Y.L. Wua, S.Z. Songa, P. Sunb, J.X. Nana, L.H. Liana, Potentiation of hepatic stellate cell activation by extracellular ATP is dependent on P2X7R-mediated NLRP3 inflammasome activation, *Pharmacol. Res.* 117 (2017) 82–93.