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## Hepatic macrophages but not dendritic cells contribute to liver fibrosis by promoting the survival of activated hepatic stellate cells in mice

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### Abstract

Although it is well established that hepatic macrophages play a crucial role in the development of liver fibrosis, the underlying mechanisms remain largely elusive. Moreover, it is not known whether other mononuclear phagocytes such as dendritic cells contribute to hepatic stellate cell (HSC) activation and liver fibrosis. Here we show for the first time that hepatic macrophages enhance myofibroblast survival in an NF- $\kappa$ B-dependent manner, and thereby promote liver fibrosis. Microarray and pathway analysis revealed no induction of HSC activation pathways by hepatic macrophages but a profound activation of the nuclear factor-kappa B (NF- $\kappa$ B) pathway in HSCs. Conversely, depletion of mononuclear phagocytes during fibrogenesis *in vivo* resulted in suppressed NF- $\kappa$ B activation in HSCs. Macrophage-induced activation of NF- $\kappa$ B in HSC *in vitro* and *in vivo* was mediated by IL-1 and TNF. Notably, IL-1 and TNF did not promote HSC activation but promoted survival of activated HSC *in vitro* and *in vivo* and thereby increased liver fibrosis, as demonstrated by neutralization in co-culture experiments, and genetic ablation of IL-1 and TNF receptor *in vivo*. Co-culture and *in vivo* ablation experiments revealed only a minor contribution to NF- $\kappa$ B activation in HSCs by dendritic cells, and no contribution of dendritic cells to liver fibrosis development, respectively.

**Conclusion**—Promotion of NF- $\kappa$ B-dependent myofibroblast survival by macrophages but not dendritic cells provides a novel link between inflammation and fibrosis.

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## Keywords

Kupffer cell; NFkB; Interleukin 1; Tumor necrosis factor; M1

The development of liver fibrosis constitutes one of the major complications of chronic liver disease with many clinical consequences such as the development of esophageal varices and ascites being directly related to the presence of liver fibrosis (1). The hepatic wound healing response is a concerted action of multiple resident and non-resident cell types that not only provides a scaffold for structural stability but also involves the removal of cellular debris by infiltrating hepatic macrophages (HM) and the regeneration of functional parenchyma (2,3). Hepatic stellate cells (HSCs) are considered the main fibrogenic cell type in the liver, and are responsible for the production of various types of extracellular matrix (ECM) (2,3). HSCs undergo a well-characterized activation process during which they lose their characteristic vitamin A and lipid stores, and obtain a myofibroblastic phenotype (2,3). The activation of HSCs is controlled by multiple soluble mediators including TGF $\beta$  and PDGF, and is part of a complex cellular network that controls the hepatic wound healing response. Previous studies have demonstrated that multiple cell populations including HM, myeloid-derived suppressor cell, B cells, T cells and natural killer cells influence the development of liver fibrosis (4-12). Among those, HM exert a profound effect on HSCs and hepatic fibrosis as shown by genetic or pharmacologic models of macrophage depletion (6,7,13). At the same time, HM also contribute to fibrosis resolution through MMP13 and matrix remodeling (6,14,15). However, the mechanisms by which HM promote liver fibrosis remain largely elusive. Dendritic cells (DC) are developmentally closely related to macrophages, and exert a profound effect on liver fibrosis regression (16) and the cytokine microenvironment during fibrogenesis (12), but their contribution to liver fibrosis development remains unknown.

In the present study, we uncovered the promotion of HSC/myofibroblast survival as novel and to-date unknown mechanism through which macrophages promote fibrosis. Moreover, we demonstrate for the first time that neither classical DC (cDC) and plasmacytoid DC (pDC) contribute to fibrogenesis.

## Experimental Procedures

### Mice and liver fibrosis induction

C57BL/6 mice, Balb/c mice, CD11c-DTR-eGFP mice (in C57Bl/6 background), Tnfrsf1a/Il1r1-deleted (“dKO”) mice (in B6.129S background) and B6.129S mice were purchased from Jackson Laboratories (Bar Harbor, ME) and housed in a specific pathogen-free facility. Collagen-GFP reporter mice have been described (17). Hepatic fibrosis was induced in 8-12 week-old male mice by ligating the common bile duct (BDL) for 5-15 days as described (18,19), by 4-20 intraperitoneal injections of carbon tetrachloride (0.125  $\mu$ l/g to 0.5  $\mu$ l/g body weight, all dissolved in corn oil at a ratio of 1:3), or by 18 weeks treatment with 300 mg/l thioacetamide in drinking water as described (13). Some mice received single or repeated intraperitoneal injections of 200  $\mu$ l liposomal clodronate (5 mg/ml) or liposomal vehicle as described (13). All animal procedures were approved by the Columbia University or Mount Sinai School of Medicine Institutional Animal Care and Use Committee, and are in accordance with the “Guide for the Care and Use of Laboratory Animals” by the National Institutes of Health.

### Dendritic cell depletion

All cDC depletion studies were performed in CD11c-DTR chimeric mice that express CD11c-DTR only in bone marrow and its progeny. In the BDL fibrosis model, cDC

depletion was achieved by 2 intraperitoneal injections of diphtheria toxin (25 ng/g body weight) or PBS at days 4 and 6. In the CCl<sub>4</sub> fibrosis model, depletion of cDC was achieved by intraperitoneal diphtheria toxin injection every 72 hrs, 25 ng/g for the first two weeks followed by 10 ng/g for the last two weeks. For the depletion of pDC, C57B6 mice were injected with pDC-depleting antibody 120G8 or isotype control (500 µg/mouse IP dissolved in 200 µL saline) every 48 hrs during the last two weeks of CCl<sub>4</sub>-induced fibrosis.

### Statistical analysis

All data are expressed as means ± standard deviation. For comparison of two groups, two-sided unpaired t-test or Mann-Whitney test were used. For multiple group comparisons, ANOVA with Tukey posthoc analysis was performed. For correlation, Pearson correlation co-efficient was calculated. A p-value < 0.05 was considered statistically significant.

Additional procedures are described in the Supplementary Method Section.

## Results

### Macrophages activate NF-κB in HSCs *in vitro* and *in vivo*

HSCs activate in a complex *in vivo* environment, characterized by the presence of multiple resident and recruited cell populations including macrophages. To identify signaling pathways through which hepatic macrophages (HM) exert profibrogenic effects, we determined, by microarray analysis, which genes and signaling pathways are activated in HSCs co-cultured with F4/80-positive HM from fibrotic livers (Suppl.Fig.1). Microarray analysis revealed that co-culture of HSCs with HM in a contact-independent manner resulted in a profound influence on gene expression shifting the pattern towards those observed in *in vivo*-activated HSCs isolated either from bile duct-ligated or CCl<sub>4</sub>-treated mice (Fig.1A-B), as previously postulated by us (18). IPA pathway analysis of the more than 1400 genes with significant and >2-fold change (Suppl.Table 1) revealed liver fibrosis and inflammatory responses as the most significant toxicological and biological functions (Suppl.Fig.2A-B), and the NF-κB pathway as center component of the highest ranked network (Fig.1C). Accordingly, NF-κB-regulated genes were significantly overrepresented among genes with more than 10-fold induction ( $\chi^2$  test, p<0.00001). Out of the 82 genes with more than 10-fold upregulation, 18 have been described as NF-κB-dependent (Fig.1D, Suppl.Table 2). We confirmed these results by real-time qPCR, and found that co-culture with HM for 24h or for five days increased the expression of known NF-κB-regulated genes including Il6, Saa3, Cxcl5, Cxcl14, Serpinb2, Ch25h and Mmp13 (Fig.2A,C). Surprisingly, HM did not induce classical HSC activation markers such as Col1a1, Col1a2 or Acta2 mRNA and did not change αSMA protein levels in HSCs (Fig.2C). We confirmed that all NF-κB-dependent genes, including Timp1, were suppressed in the presence of adenoviral IκB superrepressor (Fig.2A) or by short-term treatment with IKK inhibitor Bay 11-7085 at very low non-toxic concentrations (Suppl.Fig.3). NF-κB activation was further confirmed by p65 immunohistochemistry (Fig.2D) and immunoblot (Fig.2E) demonstrating p65 translocation, p65-S536 phosphorylation and IκBα degradation in HSCs treated with conditioned media from HM but not after treatment with control media. Similar observations were made in an NF-κB reporter assay, in which co-culture with HM induced a greater than 15-fold increase in NF-κB-driven luciferase activity (Fig.2F). Based on these results, we focused on the NF-κB pathway in subsequent analyses of mechanisms by which HM affect HSCs and fibrogenesis. Next we determined whether HM alter NF-κB-dependent gene expression in HSCs in the fibrotic liver employing a depletion approach. For this purpose, we analyzed gene expression in FACS-sorted ultrapure HSCs isolates that were immediately lysed after isolation and thus provide a “snapshot” of HSC gene expression in the fibrotic liver. NF-κB dependent gene expression was highly upregulated in HSCs activated *in vivo*

in comparison to quiescent HSCs (Fig.2G). Macrophage depletion by repeated liposomal clodronate injection efficiently reduced F4/80-positive and CD11b- and F4/80-double positive macrophages, and ameliorated liver fibrosis following BDL and CCl<sub>4</sub> treatment (Suppl.Fig.4). Notably, macrophage depletion strongly suppressed the expression of the NF- $\kappa$ B dependent genes that were upregulated by HM in our co-culture system (Fig.2G). We further excluded that liposomal clodronate directly affects NF- $\kappa$ B activation by NF- $\kappa$ B reporter assay and or cell death in cultured HSCs (Fig.2H,I).

### IL-1 and TNF mediate macrophage-induced NF- $\kappa$ B activation in HSCs

Next, we investigated mechanisms through which HM induce NF- $\kappa$ B activation in HSCs. First, we tested the contribution of IL-1 and TNF to HM-induced NF- $\kappa$ B in HSCs, based on (i) their known potent activation of NF- $\kappa$ B, (ii) the presence of the IL-1 receptor in the NF- $\kappa$ B network identified by IPA analysis (Fig.1C), and upregulated M1 markers iNOS and Cox2 in HM from BDL mice (Suppl.Fig.1C). HM induced NF- $\kappa$ B to the same degree as rmIL-1 $\beta$ , and to a higher degree than rmTNF $\alpha$  (Fig.3A). TNF and IL-1 neutralization in co-cultures by antagonistic TNF-receptor I-Fc and IL-1-receptor I-Fc chimera demonstrated potent inhibition of NF- $\kappa$ B-driven luciferase activity by IL-1 neutralization, and moderate inhibition by TNF neutralization (Fig.3B). Combined TNF and IL-1 neutralization almost completely blocked the HM-induced NF- $\kappa$ B-driven luciferase activity. Similarly, IL-1 and TNF neutralization also blocked HM-induced upregulation of NF- $\kappa$ B dependent genes Ch25h, Cxcl5, Saa3, Serpinb2, Il6 and Mmp13 with IL-1 neutralization exerting pronounced and TNF neutralization exerting moderate effects (Fig.3C). Again, combined TNF and IL-1 neutralization resulted in almost complete suppression of NF- $\kappa$ B-dependent gene expression. Conversely, IL-1 $\beta$  and TNF $\alpha$  upregulated all NF- $\kappa$ B target genes in HSCs that were induced by HM with the exception of Cxcl14 (Fig.3F). Moreover, converting the HM population that consisted of a mixed M1/M2 phenotype (Suppl.Fig.1C) as in previous studies (20), to an inflammatory M1 phenotype by treatment with LPS and IFN $\gamma$  further increased the expression of NF- $\kappa$ B dependent genes in HSCs (Fig.3D). Conversely, converting the HM population to an M2 phenotype by combined treatment with IL-10 and IL-4 suppressed the expression of NF- $\kappa$ B-dependent genes in HSCs (Fig.3E).

### IL-1 and TNF do not induce HSC activation but enhance HSC survival *in vitro* and *in vivo*

As one main function of the NF- $\kappa$ B pathway is the protection from cell death, we next determined whether HM-induced NF- $\kappa$ B prevented HSC death, or whether it directly promoted HSC activation. In contrast to previous studies (21), IL-1 $\beta$  and TNF $\alpha$  failed to directly induce HSC activation (Fig.3F-G). Co-culture of HSCs with HM strongly suppressed cell death induced by prolonged cell culture in low-serum media (Fig.4A), a well-established method of inducing HSC death (22,23) that showed signs of apoptosis such as caspase 3 cleavage (Fig.4A). The protective effects of HM were almost completely abolished by the combined neutralization of IL-1 and TNF (Fig.4A). Conversely, rmIL-1 $\beta$  was as efficient as HM in rescuing HSC from cell death (Suppl.Fig.5). Furthermore, neutralization of IL-1 and TNF did not reduce viability of HM, and HM supernatant could also suppress HSC death (data not shown), again emphasizing that HSCs and not HM are the relevant targets of IL-1 and TNF. To determine whether this survival pathway was also responsible for the decreased fibrosis observed in macrophage-depleted mice, we performed TUNEL assays in clodronate- or vehicle-treated collagen-GFP reporter mice following BDL to detect apoptosis in GFP-positive collagen-producing myofibroblasts. In mice receiving liposomal clodronate during fibrogenesis, we detected a five-fold increase in GFP- and TUNEL-double positive cells using confocal microscopy (Fig.4B). Notably, the rate of TUNEL-positive HSCs in vehicle-treated mice were very similar to peak rates in previous studies that investigated the contribution of HSC apoptosis to fibrosis resolution (22,24), thus underscoring the relevance of this dramatic increase in HSC apoptosis for liver fibrosis.

We found no effect of liposomal clodronate on HSC viability thereby excluding that liposomal clodronate directly induces HSC apoptosis in fibrotic livers (Fig.2I). We also observed reduced IL-1 $\beta$  and TNF $\alpha$  mRNA in fibrotic livers from clodronate-treated mice (Fig.4C). To test the *in vivo* relevance of this pathway, we first investigated how deficiency of IL-1 $\beta$ , the predominant activator of NF- $\kappa$ B in our co-culture experiments, affects liver fibrosis. In contrast to previously published studies, we found no statistically significant difference in BDL-induced liver fibrosis between IL1R1 knockout and wild-type mice, and further confirmed this data in the CCl<sub>4</sub> and thioacetamide models of liver fibrosis (Suppl.Fig.6). If IL-1 signaling promoted liver fibrosis by increasing NF- $\kappa$ B-dependent HSC survival rather than direct HSC activation, it would be likely that TNF $\alpha$ , the other major NF- $\kappa$ B activating cytokine produced by macrophages, could still achieve NF- $\kappa$ B activation in HSCs and thus compensate for the loss of IL-1 signaling in this model. Based on the hypothesis that absence of both IL-1 and TNF signaling would be required to reduce HSC survival and liver fibrosis, we performed BDL in TNFR1/IL1R1 double knockout mice (dko) and wild-type control mice. Compared to wild-type mice, dko mice showed significantly reduced hepatic fibrosis after 5 or 15 days of BDL (Fig.5A-B) and a five-fold increase in apoptotic TUNEL and desmin double-positive HSCs without significant differences in hepatic injury (Fig.5B) supporting our hypothesis that suppression of both IL-1 and TNF signaling are required to affect HSC survival and liver fibrosis. Moreover, we found a significant reduction of NF- $\kappa$ B-dependent genes including Il6, Cxcl5, Saa3, Serpinb2 and Timp1 in ultrapure unsorted HSCs from dko mice thus confirming that NF- $\kappa$ B activation in HSC was mediated by TNF and IL-1 (Fig.5C). Our microarray analysis revealed an upregulation of two Trail decoy receptors, murine Trail decoy receptor 1 (Tnfrsf23) and murine Trail decoy receptor 2 (Tnfrsf22) in HSCs co-cultured with HM and in HSCs from BDL and CCl<sub>4</sub> livers (Fig.5D, Suppl.Table 2). Notably, Trail-mediated apoptosis is major contributor to HSC cell death induced by hepatic natural killer cells *in vitro* and *in vivo* (11,25). Neutralization of TNF or IL-1 prevented the upregulation of Tnfrsf22 and Tnfrsf23 mRNA by HM in co-culture experiments (Suppl.Fig.7A). Moreover, depletion of HM by liposomal clodronate or dko of TNFR1 and IL1R1 reduced Tnfrsf22 and Tnfrsf23 expression *in vivo* (Suppl.Fig.7B).

### Dendritic cells do not contribute to HSC activation and fibrosis *in vivo*

Liposomal clodronate does not affect HSC number (13) or biology (Fig.2 H,I), but may deplete dendritic cells (DC), a highly endocytotic cell population as demonstrated by our FACS analysis (Fig.6A). DC contribute to liver inflammation (12) and to the regression of liver fibrosis (16), but their role in the development of liver fibrosis is not known. To test whether DC may contribute to HSC activation and liver fibrogenesis, we first performed a co-culture of DC and HSCs. Similar to HM, DC did not activate HSCs but upregulated the expression of NF- $\kappa$ B dependent genes, and NF- $\kappa$ B-driven luciferase reporter activity through an IL-1 and TNF-dependent manner (Fig.6B). However, activation of NF- $\kappa$ B was considerably lower than the induction we observed in HM co-culture. Based on these results, we next determined whether ablation of DC may have contributed to the reduced fibrogenesis in clodronate-treated mice. In our first approach, we performed BDL in diphtheria toxin-treated or PBS-treated bone marrow-chimeric CD11c-DTR-eGFP mice. Bone marrow chimerism avoids the known side effects of diphtheria toxin treatment observed after long-term treatment in global CD11c-DTR-eGFP mice (26). We did not observe a significant difference in BDL-induced fibrosis as determined by Sirius Red staining and qPCR for fibrogenic genes  $\alpha$ SMA, Col1a1 and TIMP1 (Fig.6C-D). We confirmed this data employing CCl<sub>4</sub> injection for induction of liver fibrosis, again using bone marrow-chimeric CD11c-DTR-eGFP mice. Similar to the BDL model, we did not observe significant differences in liver fibrosis between PBS and diphtheria toxin-treated mice (Fig.6E). As a third approach, we used antibody-mediated ablation of pDC. Again, we



did not observe a reduction of CCl<sub>4</sub>-induced liver fibrosis (Fig.6F). Importantly, we achieved considerable depletion of cDC and pDC by above methods (Suppl.Fig.8). Similar to previous studies (27), we observed neutrophilia in CD11c-DTR mice (Suppl.Fig.9) but consider this unlikely to exert a profound effect on fibrosis based on previous studies (28). Thus, our data suggest that neither class of DC significantly contributes to liver fibrogenesis *in vivo*.

## Discussion

Hepatic fibrogenesis involves multiple resident and recruited cell populations. HSCs represent the center component of this wound healing response but other population including macrophages are known positive modulators of fibrogenesis. Here, we uncover a novel function of macrophages, the promotion of HSC/myofibroblast survival. A second novel finding of our study lies in the discovery that DC do not contribute to liver fibrosis.

Employing microarray and pathway analysis, we discovered that NF- $\kappa$ B, the best characterized anti-apoptotic signaling pathway (29,30) and an important regulator of liver injury and fibrosis (31), was a key pathway activated in HSCs by HM. The relevance and physiologic nature of the employed *in vitro* co-culture system is validated by the finding that this system achieves HSC gene expression patterns highly similar to those found *in vivo*-activated HSCs, and that all gene expression changes and functional consequences of NF- $\kappa$ B activation were confirmed *in vivo*. Activation of the NF- $\kappa$ B pathway was further established by reporter assays, western blots, immunofluorescence and qPCR. Most importantly, NF- $\kappa$ B was activated in HSCs from fibrotic livers, and macrophage depletion reduced NF- $\kappa$ B activation in HSCs. The activation of NF- $\kappa$ B in HSCs in liver fibrosis is consistent with a previous study, but points towards macrophages instead of angiotensin II as the main trigger of NF- $\kappa$ B activation in HSCs (32). Surprisingly, co-culture with macrophages and macrophage-secreted cytokines such as IL-1 $\beta$  and TNF $\alpha$  did not promote HSC activation, and is consistent with the reported minor or insignificant inductions of  $\alpha$ SMA and Col1a1 mRNA (33) and absence of increased  $\alpha$ SMA protein expression in most studies that co-cultured human, and murine HSCs with macrophages (33,34). Only one previous study found a profound and significant activation of rat HSCs by HM (35). In our study, macrophage-induced NF- $\kappa$ B activation rendered activated HSCs more resistant to cell death *in vitro* and *in vivo*, thereby promoting the persistence of activated HSCs and fibrosis. Although the rate of 1% HSC apoptosis in fibrotic livers appeared low, it reflects the rapid removal of apoptotic cells *in vivo* (as opposed to their accumulation *in vitro*), and is virtually identical to peak apoptosis rates reported by Iredale et al. (22). Thus, the observed increase to 5% HSC apoptosis is biologically highly significant, reducing the number activated myofibroblasts and limiting fibrogenic responses as reported (11,22,32,36). It is likely that increased NF- $\kappa$ B activation protects activated HSCs from both intrinsic and extrinsic inducers of cell death. Accordingly, our study also found that HM induce the expression of Trail decoy receptors in HSCs in an NF- $\kappa$ B-dependent manner. This finding is of interest as natural killer cells, which are particularly enriched in the liver and activated during liver injury, significantly contribute to killing of activated HSCs during liver fibrosis in a Trail-dependent manner (11,37,38).

Our study identified IL-1 and TNF as main factors of HM-mediated NF- $\kappa$ B activation and cytoprotection in HSC. Notably, we observed no effect of IL-1 $\beta$  or TNF $\alpha$  on HSC activation. The key role of HM-derived IL-1 and TNF in NF- $\kappa$ B activation and protection from HSC death was not only found *in vitro*, but also *in vivo* as demonstrated by the profound decrease in NF- $\kappa$ B responsive genes in unplated, ultrapure HSCs isolates from TNFR1/IL1R1 double-knockout mice, and increased apoptosis of desmin-positive cells in TNFR1/IL1R1 dko livers after BDL. Previous studies have demonstrated reduced

fibrogenesis in mice deficient in TNFR1 or IL1-R (39,40). In contrast to these studies, we could not observe reduced liver fibrosis in IL-1R knockout mice in three different models of liver fibrosis. This is consistent with the notion that both TNF $\alpha$  and IL-1 $\beta$  are powerful NF- $\kappa$ B activators, that they can likely functionally substitute each other.

Our study employed F4/80-positive HM from bile duct-ligated livers for co-culture experiments and therefore exposed HSCs to a mixture of resident and recruited macrophages typically found during fibrogenesis. Accordingly, our data show that these HM have a mixed M1/M2 phenotype as previously reported (20). Based on our observations that converting HM into M1 phenotype increased, and into M2 phenotype reduced their ability to induce NF- $\kappa$ B-dependent gene expression in HSCs, we conclude that the inflammatory/M1 HM subpopulation contributes to NF- $\kappa$ B activation and HSC survival. It should be emphasized that the M1/M2 classification does not fully account for diverse and often overlapping biological functions of macrophage populations, in particular in the liver (20). It is conceivable that different HM populations collaborate for the induction of fibrosis *in vivo*, with inflammatory M1-type HM promoting HSC survival and M2-type HM affecting HSCs through other pathways. We did not find a significant impact of Gr1 status in HM on NF- $\kappa$ B activation in HSCs (data not shown) suggesting that both recruited and resident macrophages are capable of promoting NF- $\kappa$ B activation in HSCs. Clodronate did not affect HSC activation directly nor did it alter NF- $\kappa$ B activation in HSCs. Moreover, our results employing DC depletion additionally excluded DC as potential contributors to clodronate effects as we did not see a contribution of this cell type to liver fibrosis.

Dendritic cells are key regulators of inflammation and the cytokine milieu in the fibrotic liver (12). Moreover, DC contribute to the regression of liver fibrosis through an MMP9-dependent mechanism (16). However, the contribution of DC to fibrogenesis is unknown. Although we found that CD11c-positive DC induce a moderate degree of NF- $\kappa$ B activation in HSCs via TNF and IL-1 production, we did not observe a role for pDC or cDC in promoting liver fibrosis in BDL- and CCl<sub>4</sub>-induced liver fibrosis. Most likely, the much lower number of DC in the liver in comparison to HM and the lower potency of NF- $\kappa$ B activation by DC renders the contribution of DC-derived TNF and IL-1 to the overall pool and NF- $\kappa$ B-mediated HSC survival insignificant. In this regard, the ratio of DC to HSCs in our co-culture experiments is at least one or two magnitudes higher than the ratio that can be achieved in a fibrotic liver. Another possible explanation may be the critical role of DC in NK cell activation, cells with well established anti-fibrogenic potential (11,41). None of the available CD11c-DTR based ablation strategies can achieve a completely selective depletion of cDC without affecting the composition of other immune cells (26,27). Even recent transgenic mouse models that avoid early neutrophilia after DC depletion still lead to neutrophilia after two days (27). Although neutrophilia represents a confounding factor, we consider it unlikely that neutrophilia affects fibrogenesis based on previous studies that did not show effects on liver fibrosis (28). These data suggest that DC interact with and regulate other immune cells, or that increased granulopoiesis after ablation causes these secondary effects. Nonetheless, the ablation strategies employed in our study (i) avoid common side effects described for DC ablation (26) due to our use of CD11c-DTR chimeric mice and 120G8 antibody, (ii) address the role of both cDC and pDC, and (iii) investigate the role of cDC in two common models of liver fibrosis.

Our study contains several limitations: First, as we observed more than 1400 HM-regulated genes, it is likely that genes besides NF- $\kappa$ B-regulated genes affect HSC responses. Further studies are required to unravel the relevance of NF- $\kappa$ B-independent genes and pathways regulated by HM. These may include additional mediators secreted from HM such as IL-6 and TGF $\beta$  (35,42). Accordingly, our IPA analysis revealed Stat1/3/5 as HM-activated pathways. Second, our studies were performed in mouse models, and further studies are

required to determine whether HM-induced NF- $\kappa$ B activation plays a role in human fibrogenesis. As patients develop fibrosis slowly over decades, pathways that promote long-term myofibroblast survival may be particularly relevant. IL-1 and TNF inhibitors may be considered for antifibrotic therapies but may cause severe side effects. In conjunction with previous studies (32,43), our data support the concept that targeting NF- $\kappa$ B pathway in HSCs and subsequent induction of HSC apoptosis may be a more suitable anti-fibrogenic strategy. In summary, our study shows that HM provide a novel link between inflammation, HSC survival and liver fibrosis, and suggest that inflammatory signaling pathways may provide additional targets for antifibrotic therapies in the liver. Future studies need to determine whether macrophage-mediated promotion of myofibroblast survival also promotes fibrosis in other organs.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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## List of Abbreviations

<b><math>\alpha</math>-SMA</b>	$\alpha$ smooth muscle actin
<b>BDL</b>	bile duct ligation
<b>CCl<sub>4</sub></b>	Carbon tetrachloride
<b>DC</b>	dendritic cells
<b>ECM</b>	extracellular matrix
<b>HM</b>	hepatic macrophages
<b>HSC</b>	hepatic stellate cell
<b>qPCR</b>	quantitative real-time PCR
<b>cDC</b>	classical dendritic cell
<b>dko</b>	double knockout
<b>FACS</b>	fluorescence-activated cell sorting
<b>GFP</b>	green fluorescent protein
<b>IL</b>	interleukin
<b>IPA</b>	Ingenuity Pathway Analysis
<b>mRNA</b>	messenger RNA
<b>NF-<math>\kappa</math>B</b>	nuclear factor kappa B
<b>PBS</b>	phosphate-buffered saline
<b>pDC</b>	plasmacytoid DC
<b>TNF</b>	tumor necrosis factor



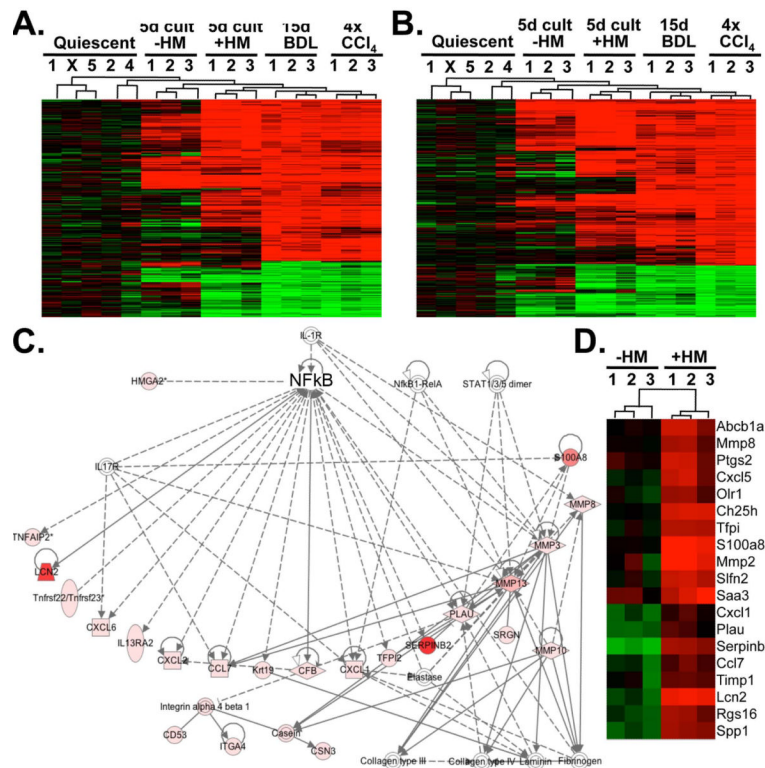
**TUNEL** terminal deoxynucleotidyl transferase–mediated deoxyuridine triphosphate nick-end labeling

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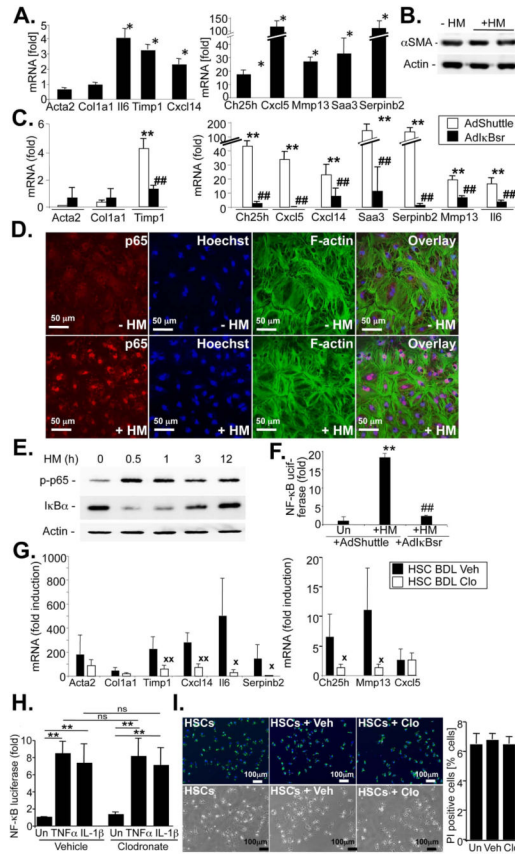
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**Figure 1. Microarray and pathway analysis reveal NF- $\kappa$ B and not fibrogenic activation as the predominant effect of hepatic macrophages on HSCs**

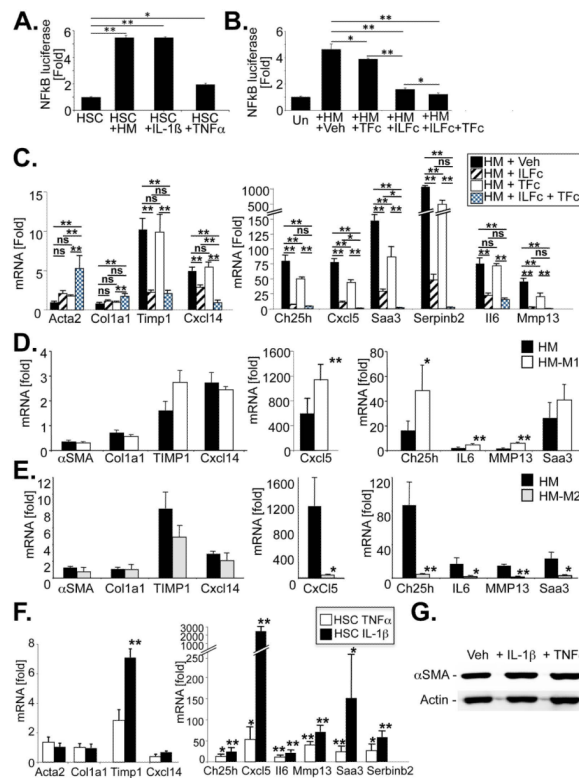
**A-B.** Heatmap from microarrays showing quiescent HSCs, 5 day *in vitro*-activated HSCs, 5 day *in vitro*-activated HSCs co-cultured with HM, HSC from mice bile duct-ligated for 15 days and HSCs from mice after 4 CCl<sub>4</sub> injections. The heatmap (left panel) includes all genes that were significantly (adjusted p-value <0.05) and at least 4-fold up-or downregulated in HSCs from BDL livers (A). The heatmap (right panel) includes all genes that were significantly (adjusted p-value <0.05) and at least 4-fold up-or downregulated in HSCs from CCl<sub>4</sub> livers (B). **C.** Shown is the highest-ranking network from IPA analysis. **D.** Heatmap of NF- $\kappa$ B-responsive genes with significant (adjusted p-value <0.05) and at least 10-fold induction in response to co-culture with HM.



**Figure 2. Hepatic macrophages induce NF- $\kappa$ B but not myofibroblastic activation in HSCs *in vitro* and *in vivo***

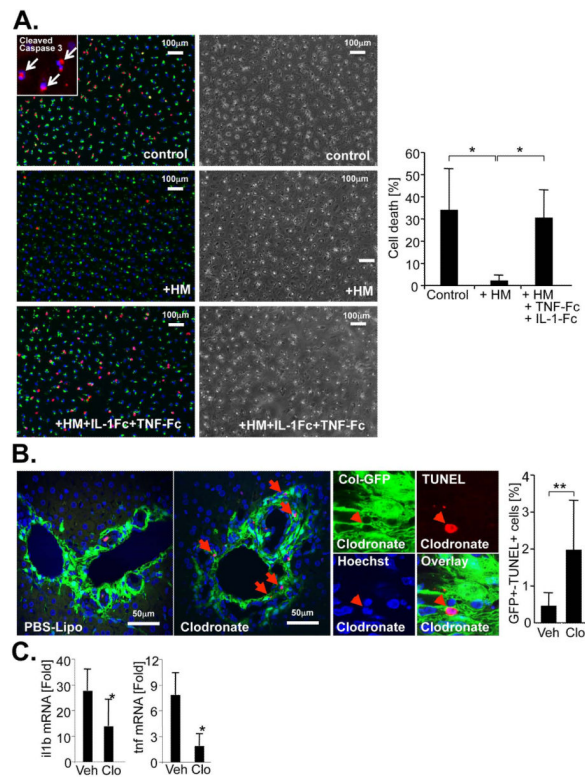
**A-B.** Hepatic stellate cells were co-cultured with HM for 5 days (A), followed by determination of NF- $\kappa$ B responsive genes by qPCR, or western blot for  $\alpha$ SMA (B). **C.** HSCs were infected with AdIkBsr or an empty control adenovirus followed by co-culture with HSCs for 24h. mRNA expression of HSC activation markers and NF- $\kappa$ B responsive genes was determined by qPCR. **D.** HSCs were stimulated with conditioned media from HM for 1h. NF- $\kappa$ B activation was determined by immunofluorescent p65 staining (red fluorescence) in HSCs co-stained for F-actin (green fluorescence). Nuclei were marked by Hoechst (blue fluorescence). **E.** HSCs were stimulated with conditioned media from HM for various times. NF- $\kappa$ B activation was determined by immunoblot for S536-phosphorylated p65 and I $\kappa$ B $\alpha$ . **F.** HSCs were transduced with an adenoviral NF- $\kappa$ B reporter. After co-culture with HM for 24h, luciferase activity was determined and is expressed as fold induction compared to HSCs cultured with empty inserts. **G.** Gene expression patterns were determined in ultrapure unsorted HSCs isolated from 15 day bile duct-ligated vehicle-treated mice (n=6 isolations) and clodronate-treated mice (n=4 isolations) mice by a combination of gradient centrifugation and FACS sorting without additional plating. mRNA expression of fibrogenic genes and NF- $\kappa$ B dependent genes was determined by qPCR and expressed in comparison to quiescent HSCs isolated from control wt mice (n=3 isolations). **H-I.** Hepatic stellate cells were treated with 50  $\mu$ l of 5mg/ml solution per ml of media or liposomal vehicle for 12h. TNF $\alpha$ - and IL-1 $\beta$ -induced NF- $\kappa$ B reporter activity (H.) and cell death, showing Hoechst stained nuclei (blue), retinoids (green) and propidium iodide positive nuclei (red) were determined (I). \*\*p<0.01 in comparison to HSCs cultured without HM. p<0.05 in comparison to HSCs cultured in the absence of HM ## p<0.01 in comparison to AdShuttle-infected HSCs co-cultured with HM.  $\square$  p<0.05,  $\blacksquare$  p<0.01 vs HSC BDL Veh.





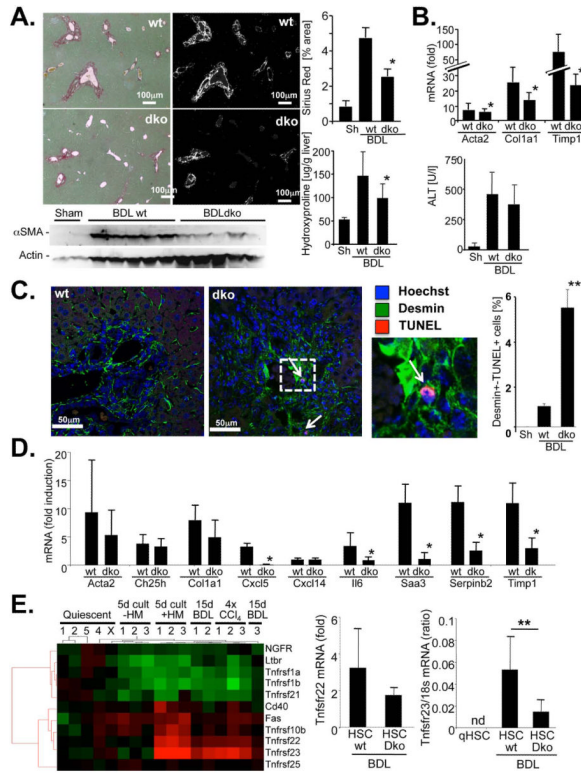
**Figure 3. Macrophage-derived IL-1 and TNF promote NF-κB activation but not myfibroblastic activation in HSCs**

**A.** NF-κB-driven luciferase activity was determined in HSCs that were co-cultured with HM, or treated with IL-1β (5 ng/ml) or TNFα (30 ng/mL) for 24h. **B.** NF-κB-driven luciferase activity was determined in HSC co-cultured with HM in the presence of vehicle (0.1% BSA in PBS), antagonistic TNFRI-Fc chimera (TFC, 0.5 μg/ml), antagonistic IL-1RI-Fc chimera (IL-Fc, 0.5 μg/ml) or both for 24h. **C.** mRNA expression of HSC activation markers or NF-κB responsive genes was determined by qPCR in HSC co-cultured with HM in the presence or absence of TNFRI-Fc chimera, IL-1RI-Fc chimera or both for 24h. **D-E.** Macrophages isolated from BDL livers were treated with treated 16 hours with LPS (10 ng/mL) and interferon gamma (10 ng/mL) to convert them into M1 phenotype (D), or with IL-4 (10 ng/ml) and IL-10 (10 ng/ml) to convert them into M2 phenotype (E). Following 5 washes, M1 or M2 macrophages were co-cultured with HSCs. Co-culture induced expression of NF-κB-dependent genes was determined by qPCR. **F.** HSCs were treated with rmIL-1β (5 ng/ml) or rmTNFα (30 ng/ml) for 6h followed by analysis of HSC activation markers and NF-κB dependent genes by PCR. **G.** αSMA levels were analyzed by immunoblotting after treating HSCs with rmIL-1β or rmTNF for 5 days. \*p<0.05, \*\* p<0.01



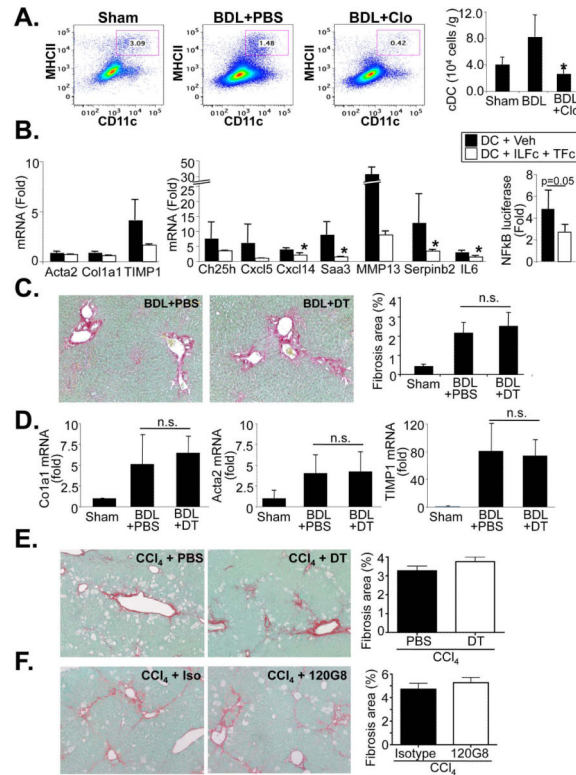
#### Figure 4. Hepatic macrophages protect HSCs from cell death

**A.** HSCs were starved in 0.1% FBS in the presence or absence of HM, and the absence or presence of antagonistic TNFRI-Fc and IL-1RI-Fc chimera. Cell death was determined by propidium iodide staining (red). Nuclei were visualized by Hoechst (blue). Vitamin A-containing HSC lipid droplets were visualized by autofluorescence (pseudocolored in green). Shown are representative images and a quantification of the average of three independent HSC and HM isolations. Insert shows cleaved caspase 3 immunofluorescent staining (red) and Hoechst (blue). **B.** To determine apoptosis in collagen-expressing myofibroblasts, collagen-GFP reporter mice were treated with vehicle (n=5) or clodronate (n=4) followed by BDL for 9 days and TUNEL staining of liver sections. Cell death of collagen-expressing myofibroblasts was visualized by confocal microscopy and quantified by counting cells double-positive cells for GFP (green) and TUNEL (red). Nuclei were visualized by Hoechst staining (blue). **C.** Hepatic levels of IL-1 $\beta$  and TNF $\alpha$  mRNA were determined by qPCR in bile duct-ligated vehicle (n=17) and clodronate-treated (n=10) mice. \*p<0.05; \*\*p<0.01.



**Figure 5. TNF and IL-1 mediate NF-κB activation and protection from cell death in HSCs during liver fibrosis**

**A.** TNFRI and IL1-RI dko (n=5) and wild-type mice (n=10) underwent BDL for 14 days. Hepatic fibrosis was assessed by Sirius Red staining and polarized microscopy, αSMA immunoblotting and hydroxyproline assay. **B.** TNFRI and IL1-RI dko (n=7) and wild-type mice (n=9) underwent BDL for 5 days. Hepatic fibrogenesis was assessed qPCR for profibrogenic genes, hepatic injury by ALT measurement. **C.** Following 2 weeks of BDL, HSCs were isolated from mice TNFRI and IL1-RI dko (n=5 isolations) or wild-type mice (n=5 isolations) by gradient centrifugation and Vitamin A fluorescence-based FACS without plating. mRNA expression of fibrogenic and NF-κB dependent genes was determined by qPCR and expressed in comparison to quiescent HSCs isolated from wild-type mice (n=3 isolations). **D.** To determine apoptosis in HSCs, liver sections from 14 day bile-duct ligated TNFRI and IL1-RI dko (n=5) and wild-type mice (n=5) were stained for desmin (green fluorescence) and by TUNEL (red fluorescence). Nuclei were marked by Hoechst staining (blue fluorescence). Cells positive for both desmin and TUNEL were quantified. \*p<0.05; \*\*p<0.01. **E.** Heatmap of TNF receptor family members including Trail and Trail decoy receptors under different conditions, determined by microarray analysis (left panel). Expression of Trail decoy receptors Tnfrsf22 and Tnfrsf23 as determined by qPCR in ultrapure un plated HSCs isolated from 14 day bile duct-ligated wild-type mice (n=5 isolations) and double-deficient for TNFRI and IL1-RI mice (“dko”, n=5 isolations) by a combination of gradient centrifugation and FACS analysis without additional plating (center and right panel). Data is expressed as fold induction in comparison to quiescent HSCs isolated from control wt mice (n=3 isolations) or as ratio between the investigated mRNA and 18s mRNA if mRNA expression was not detectable in qHSC. \*p<0.05; \*\*p<0.01. nd: non-detectable.



**Figure 6. Dendritic cells moderately induce NF- $\kappa$ B dependent gene transcription in HSCs but do not contribute to BDL- and CCl<sub>4</sub>-induced liver fibrosis**

**A.** Bile duct ligated mice were injected every 5 days for a total of 3 injections with PBS (n=4) or clodronate (n=3) followed by quantification of cDC using CD11c and MHCII flow cytometry FACS plots show percentage of double-positive cells, the bar graph shows cell numbers per gram liver. **B.** mRNA expression of HSC activation markers (left panel), NF- $\kappa$ B responsive genes (middle panel) or NF- $\kappa$ B-driven luciferase activity (right panel) were determined by qPCR and NF- $\kappa$ B reporter assay, respectively, in HSC co-cultured with DC in the presence of antagonistic TNFRI-Fc and IL-1RI-Fc chimera (both 0.5  $\mu$ g/ml) or vehicle (0.1% BSA in PBS) for 24h. **C-D.** Liver fibrosis was induced by BDL in CD11c-DTR bone marrow-chimeric mice. Chimeric male CD11c-DTR were treated with two injections of PBS (n=5) or diphtheria toxin (25 ng/g body weight) (n=6). Mice were sacrificed after 7 days. Deposition of fibrillar collagen was determined by Sirius Red staining (left upper panel) and morphometric quantification (right upper panel) (C). Expression of profibrogenic genes Acta2, Col1a1 and TIMP1 was determined by qPCR. Fold induction was calculated to sham-operated control (D). **E.** Chimeric CD11c-DTR mice (n=4 each group) were used for DC depletion (25 ng/g first two weeks, 10 ng/g last two weeks) and simultaneous liver fibrosis induction using CCl<sub>4</sub> (0.5  $\mu$ l/g, three time per week). Fibrosis was determined by Sirius Red staining. **F.** For pDC depletion C57Bl/6 mice (n=4) were injected every 48 hours with 120G8 antibody (500  $\mu$ g/mouse) or isotype control during last two weeks of CCl<sub>4</sub> induced liver fibrosis (0.5  $\mu$ l/g, three time per week for four weeks). Fibrosis was determined by Sirius Red staining. \* p<0.05