

CXCR3 Ligands induce Expression of CXCL1 (KC/murine IL8 homolog) in Mouse Hepatic Stellate Cells

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Abstract

Background and aim: Liver injury leads to infiltration of immune cells and a subsequent activation of hepatic stellate cells. Chemokines are ubiquitous chemotactic proteins which are involved in inflammatory pathways. It has been recently suggested that chemokines can also induce the expression of other chemokines and thereby indirectly regulate immune cell recruitment. We therefore investigated the ability of the chemokine CXCL9 to induce CXCL1, an important neutrophil chemoattractant.

Methods: The ability of CXCR3 ligands CXCL9 and CXCL10 to induce CXCL1 expression was analyzed in immortalized (GRX) and primary hepatic stellate cells isolated from wild-type and CXCR3^{-/-} mice. Cells were treated with different concentrations of chemokines in the absence and presence of pertussis toxin. Furthermore, mice were treated systemically with CXCL9 and hepatic CXCL1 levels as well as neutrophil infiltration were assessed.

Results: Treatment of GRX cells with CXCL9 leads to a dose dependent induction of CXCL1 protein expression. This stimulatory effect was validated in primary hepatic stellate cells ($P < 0.01$). In contrast, stimulation of stellate cells with CCL2 did not result in CXCL1 induction. Increased CXCL1 expression in response to CXCL9 was completely abolished by co-incubation with pertussis toxin and in stellate cells derived from CXCR3^{-/-} mice, the canonical receptor for CXCL9 and CXCL10. Notably, systemic treatment of mice with CXCL9 led to elevated hepatic CXCL1 levels and was associated with enhanced neutrophil infiltration into the liver.

Conclusions: The study describes a chemokine-chemokine pathway in hepatic stellate cells which leads to augmented infiltration of neutrophils into the liver during acute liver injury.

Introduction

Liver fibrosis is the common outcome of many chronic liver diseases, including hepatitis B (HBV) or C virus (HCV) infection, alcoholic and non-alcoholic steatohepatitis (ASH/NASH) or autoimmune diseases [1]. From a pathophysiological point of view, fibrogenesis is the result of an exaggerated wound healing processes, resulting in tissue scarring and an excessive accumulation of extracellular matrix (ECM) proteins within the liver. During this process, hepatic stellate cells are considered to play a major role in matrix production [2]. Upon injury, stellate cells differentiate from a quiescent, vitamin A storing phenotype into activated myofibroblasts which are the main producers of matrix proteins within the liver [3]. Therefore hepatic stellate cells represent a promising target for antifibrotic therapies [4]. However, apart from their matrix producing capabilities, myofibroblasts also secrete many cytokines and chemokines, enabling these cells to direct the positioning of immune cells within the liver [5].

Chemokines are ubiquitous 8- to 12-kDa heparin-binding secreted proteins which are subdivided into four families defined by the number of amino acids between the N-terminal cysteine residues (CC, CXC, CCX, and CX3C) [6]. Furthermore, chemokines are classified depending on whether they are induced by inflammation or are constitutively expressed and involved in homeostatic immune regulation [7]. Many members of the CC- and CXC group are expressed within liver injury where they influence inflammation and fibrosis by chemotaxis of immune cells [8], stellate cell activation [9], angiogenesis and vascular remodeling [10,11]. Specifically, CXCR3-associated chemokines appear to play an important role in pulmonary and kidney fibrosis [12,13]. Furthermore, CXCL9 (monokine induced by interferon- γ , MIG) and its receptor CXCR3 display antifibrotic properties in liver disease in mice and humans [14], rendering this chemokine pathway an interesting target for diseases with fibrotic

tissue injury. Apart from these immune-mediating functions, cationic chemokines such as CXCL9 and CXCL10 also display direct defensin-like antimicrobial activity [15]. In human monocytes, CXCL9 shares some immunomodulatory capabilities of defensin-like cationic host defense peptides and induces the expression of a variety of immunity genes and cytokines in a CXCR3-independent manner. This was especially shown for CXCL1 (KC) [16]. Thus, this particular chemokine might have direct [14], as well as indirect effects on the infiltration of immune cells during the liver disease.

Based on this scientific background we here assess whether CXCL9 specifically induces the chemokine CXCL1 in hepatic stellate cells and whether these immune-regulatory effects are mediated by a G-protein dependent pathway and are functionally relevant *in vivo*.

Materials and Methods

Animal experiments

Wild-type C57Bl/6 mice were purchased from Charles River

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laboratories (Sulzfeld, Germany). CXCR3^{-/-} mice were bred for more than 10 generations on the C57Bl/6 background as previously described [17]. Liver injury in wild-type mice was induced by administration of 0.8 ml/kg body weight carbon tetrachloride (CCl₄) i.p. Mice were treated concomitantly with 1µg recombinant murine CXCL9 (R&D systems) or vehicle i.p. daily.

Isolation and culture of mice hepatic stellate cells

For screening experiments the murine GRX hepatic stellate cell line was used for *in vitro* experiments [18, 19]. Confirmation experiments were performed in primary murine hepatic stellate cells. These cells were isolated from wild-type C57Bl/6 or CXCR3^{-/-} mice by a collagenase-ponase two step perfusion method as described [3]. Cell purity was assessed by bright field microscopy and was estimated at 80 – 90 %. Experiments were performed after 3 – 4 day culturing of primary cells; medium was changed daily to clear cell debris and dead cells. Cells were cultured in DMEM with high-glucose; L-glutamine and sodium pyruvate (PAA Laboratories) supplemented with 10% fetal calf serum (Biochrom KG), 100 IU/ml penicillin, and 100 µg/ml streptomycin (PAA Laboratories). The medium was changed 1 day after initial plating, while cultures were maintained at 37°C, 5% CO₂.

Stimulation of cells with chemokines

Prior to stimulation cells were plated with a density of 0,3 x 10⁶ cells/well. Cells were kept in serum free DMEM medium for 24 hours; next 100 or 1000 ng CXCL9/ml (R&D systems) with or without 10 ng pertussis toxin (Sigma) was added. Experiments were repeated with 100 or 1000 ng murine recombinant CXCL10 and CCL2 (both from R&D systems) as indicated. The supernatant and cells for RNA isolation were harvested after 6 or 24 hours for further analysis.

Chemokine determination

CXCL1 concentration in supernatant of cultured cells or whole liver homogenates was assessed by a sandwich enzyme-linked immunosorbent assay (ELISA) using the mouse CXCL1/KC DuoSet ELISA development kit (R&D Systems Inc., MN, USA, catalog number DY453). Total messenger RNA (mRNA) was isolated from cell lysates or whole liver homogenates and reverse-transcription polymerase chain reaction was performed as described previously. Quantitative real time PCR was performed with SYBR green and β-actin as internal control for CXCL1, CCL3, CCL5. Primers for real time PCR were designed using information from the qPrimerDepot database from <http://mouseprimerdepot.nci.nih.gov/>.

Assessment of neutrophil infiltration *in vivo*

Liver cryosections were fixed with 4% paraformaldehyde for 20 min at room temperature. Next cryosections were incubated with a primary rat anti-mouse anti-Ly6G antibody (Invitrogen, Germany) overnight. Immunofluorescence staining was performed with donkey anti-rat AlexaFluor 488 antibody (Molecular Probes, Invitrogen). Photomicrographs were taken with a fluorescence microscope (Zeiss, Germany) and Ly6G+ cells (green) were counted in three different view fields.

Statistical analysis

All data are presented as mean +/- SEM. Differences between multiple groups were compared using 1-way analysis of variance

(ANOVA) with post hoc test for comparison of individual groups. Two independent groups were compared with Student's t-test. All analyses were performed with GraphPad Prism 5.0 (GraphPad Software, La Jolla, CA).

Results

CXCL9 specifically induces CXCL1 expression in stellate cells

We first evaluated the effect of CXCL9 stimulation on chemokine expression in immortalized GRX hepatic stellate cells which share many characteristics with primary hepatic stellate cells [19]. Stimulation of these cells with 100 and 1000 ng CXCL9 for 24 hours resulted in a dose dependent increase of CXCL1 mRNA expression in the stellate

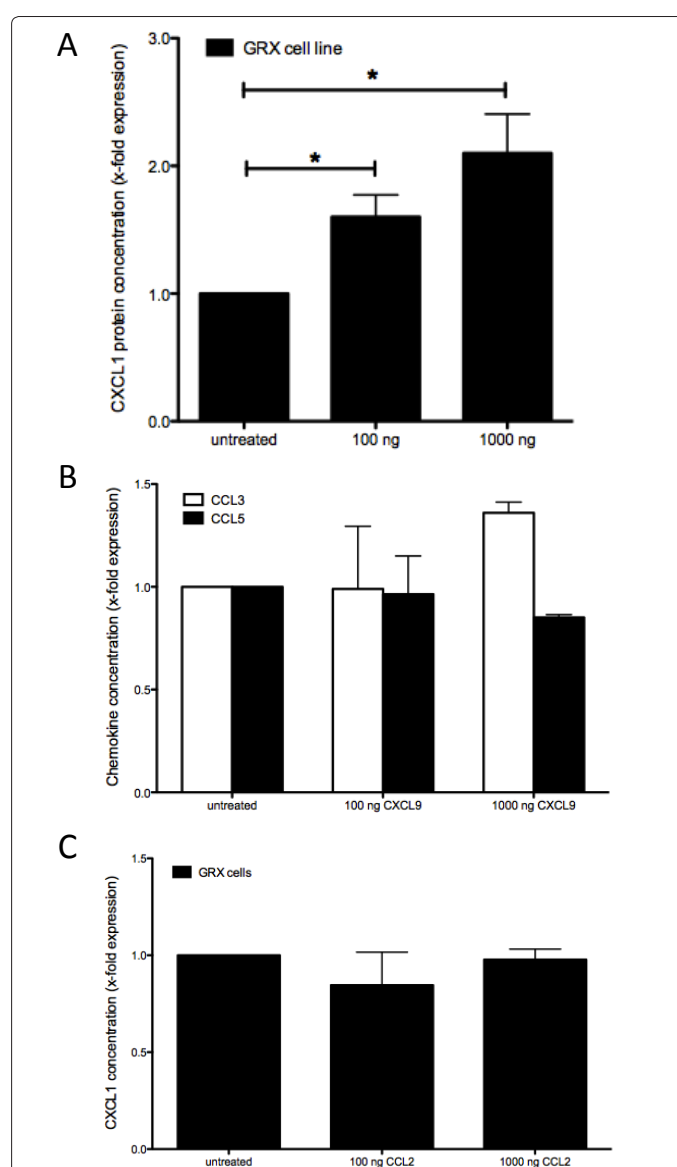


Figure 1: CXCL9 induces CXCL1 expression in immortalized hepatic stellate cells. Whereas CXCL9 induces CXCL1 expression in GRX cells in a dose dependent manner (A), no significant effect was observed for induction of CCL3 and CCL5 (B). Incubation of GRX cells with CCL2 does not lead to a significant induction of the chemokine CXCL1 (C). Three independent experiments were performed, measurements were taken in duplicates, error bars represent SEM values, * p<0.05.

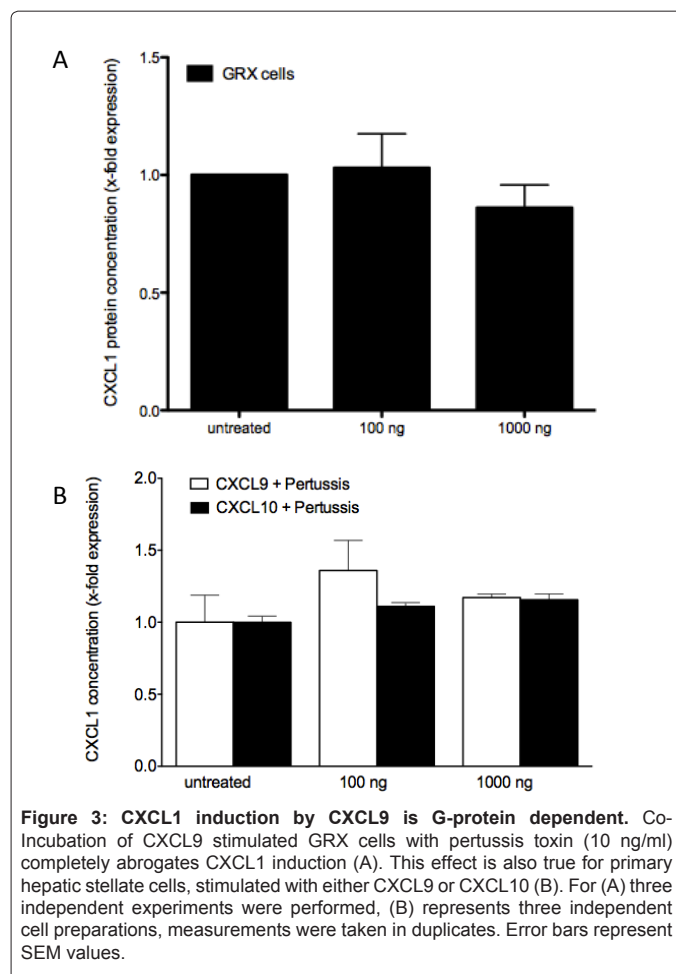
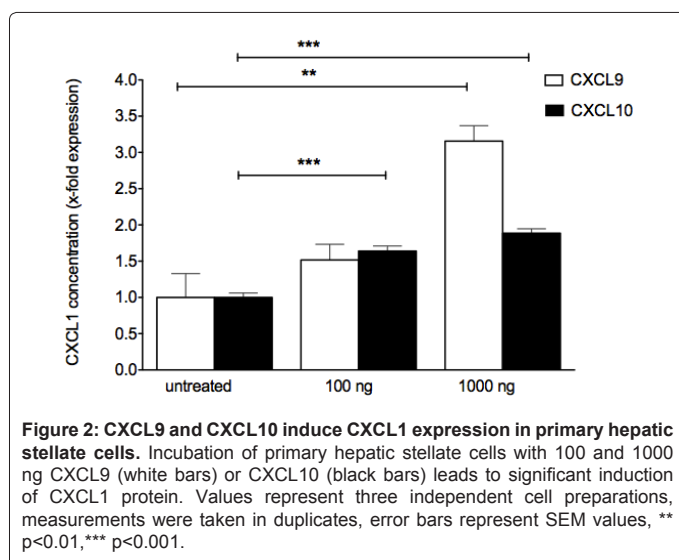
cells (data not shown). These results were confirmed for the CXCL1 protein concentration in the supernatant. In response to incubation with 100 ng CXCL9 the CXCL1 concentration increased 1.6 times, after incubation with 1000 ng CXCL9 a 2.1-fold increase was measured (both $P < 0.05$, Figure 1A). In contrast to CXCL1, other chemokines such as CCL2 or CCL5 were not significantly changed in response to CXCL9 stimulation (Figure 1B). The effects on CXCL1 expression appeared to be specific for CXC chemokines since the more neutrally charged chemokine CCL2 did not show a stimulatory effect on CXCL1 mRNA under these experimental conditions (Figure 1C).

CXCL9 does also induce CXCL1 expression in primary hepatic stellate cells

Because results obtained in cell lines might differ from results gained in primary cells we next isolated primary hepatic stellate cells (HSC) from 12 weeks old C57Bl/6 wild-type mice for confirmation of our *in vitro* results. Overall, incubation of primary cells with 100 and 1000 ng CXCL9 resulted in similar effects compared to the GRX cell line. CXCL1 expression increased 3.1 times after incubation with 1000 ng CXCL9 ($P < 0.01$, Figure 2, white bars). We next evaluated whether the effects of CXCL9 on CXCL1 expression are specific for this particular chemokine or if related chemokines can also induce CXCL1. Indeed, incubation of primary stellate cells with CXCL10 resulted in a dose dependent increase of CXCL1 in the culture supernatant (Figure 2, black bars).

CXCL1 induction by CXCL9 is G-protein dependent

Although the similar effects of CXCL9 and CXCL10 on CXCL1 secretion suggested an important role of their shared chemokine receptor CXCR3, we first formally tested whether CXCL1 induction is G-protein dependent. Upon binding to chemokine receptors $G\alpha$ and $G\beta - \gamma$ subunits of the heterotrimeric G proteins dissociate, leading to calcium flux and activation of the phosphatidylinositol 3-kinase and the small Rho GTPases signaling pathways [20]. The inhibition of these responses is achieved by treatment with pertussis toxin [21,22]. Indeed, CXCL1 induction by CXCL9 in GRX cells (Figure 3A) as well as its induction by CXCL9 and CXCL10 in primary HSCs (Figure 3 B) was abolished by co-incubation with 10 ng pertussis toxin. Thus, a G-protein



dependent mechanism is responsible for the chemokine stimulatory effects on CXCL1 expression.

Induction of CXCL1 by CXCL9 is CXCR3 receptor dependent

As CXCL9 and CXCL10 share the chemokine receptor CXCR3, we next assessed whether this canonical receptor mediates the stimulatory effects leading to CXCL1 secretion of the stellate cells. To this end, we isolated primary stellate cells from CXCR3^{-/-} mice and stimulated these cells with CXCL9. As expected in the light of the G-protein mediated effect, incubation of primary CXCR3 deficient hepatic stellate cells with 100 ng/ml or 1000 ng/ml CXCL9 did not lead to an induction of CXCL1 protein expression (Figure 4). Thus, the induction of CXCL1 by CXCL9 is mediated by its canonical receptor CXCR3.

High systemic CXCL9 levels lead to increased neutrophil infiltration into injured liver *in vivo*

We further evaluated whether these *in vitro* effects are also operating *in vivo*. CXCL1 is a strong chemoattractant for neutrophils [23], which are known to infiltrate a toxically damaged liver [18]. We therefore assessed the infiltration of neutrophils into the liver of mice treated with a single dose of CCl₄ and concomitant application of CXCL9 or vehicle. Notably, systemic application of CXCL9 indeed resulted in a 2-fold increase in CXCL1 levels within the liver (Figure 5). In line with our *in vitro* results, systemic application of CXCL9 was

associated with a significantly higher number of neutrophils within the liver compared to vehicle treated mice (Figure 6). Interestingly, this increased infiltration of neutrophils in response to CXCL9 was only present in mice with induction of liver injury, but not in animals without CCl₄ challenge. Thus, an activation of stellate cells appears to be a prerequisite for the effects of the CXCL9/CXCL1 pathway described in our study.

Discussion

Although chemokines have been traditionally considered to mainly govern the recruitment of immune cells to sites of tissue injury, recent studies have discovered many more functions of these molecules beyond chemotaxis outside [24] and inside the liver [25]. These chemokine functions are subject to many regulatory mechanisms, including translational and post-translational regulation as well as changed receptor activities [26]. Another interesting function of certain chemokines is their defensin-like function, which mediates direct

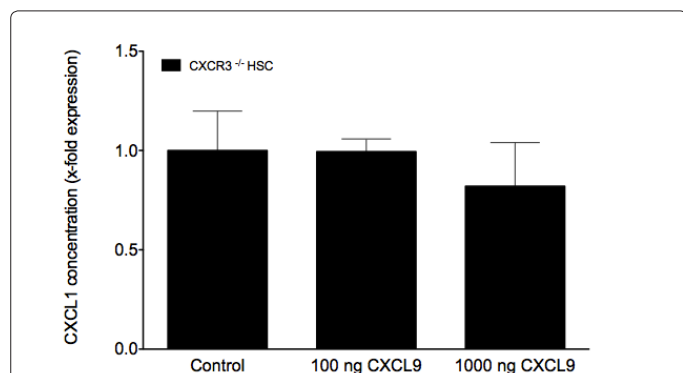


Figure 4: Induction of CXCL1 by CXCL9 is CXCR3 dependent. Primary hepatic stellate cells from CXCR3 deficient mice were treated with 100 or 1000 ng CXCL9. CXCL1 induction in response to CXCL9 treatment was completely blocked by CXCR3 deficiency, demonstrating that this effect is specifically mediated by the CXCR3 receptor. Values represent three independent cell preparations, measurements were taken in duplicates, error bars represent SEM values.

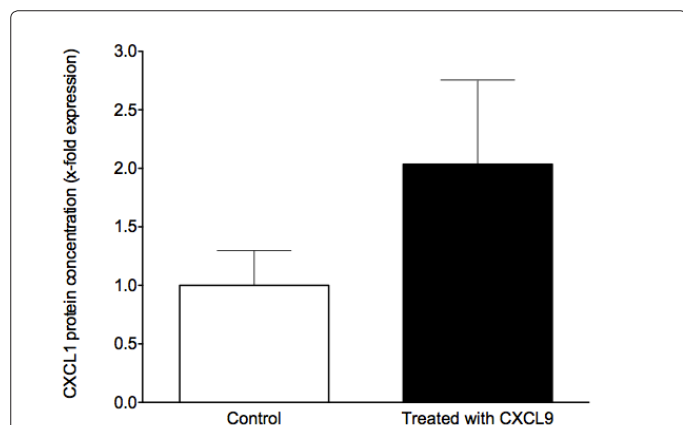


Figure 5: High systemic CXCL9 levels lead to CXCL1 expression *in vivo*. C57Bl/6 wild-type mice were subjected to acute liver injury by a single dose of CCl₄. In addition CXCL9 was administered i.p. Treatment with CXCL9 resulted in a 2-fold increase in CXCL1 protein levels within the liver. Values represent analyses of three mice per group, measurements were done in duplicates, error bars represent SEM values. Statistical comparison of the groups revealed a trend ($P = 0.3$).

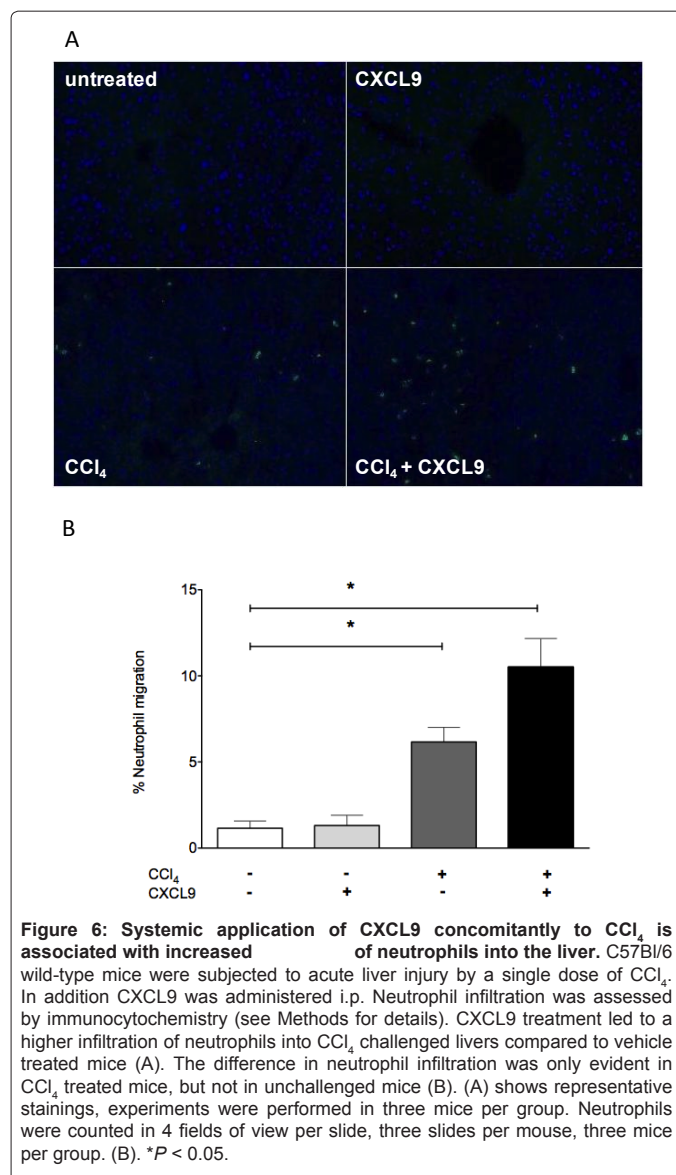


Figure 6: Systemic application of CXCL9 concomitantly to CCl₄ is associated with increased of neutrophils into the liver. C57Bl/6 wild-type mice were subjected to acute liver injury by a single dose of CCl₄. In addition CXCL9 was administered i.p. Neutrophil infiltration was assessed by immunocytochemistry (see Methods for details). CXCL9 treatment led to a higher infiltration of neutrophils into CCl₄ challenged livers compared to vehicle treated mice (A). The difference in neutrophil infiltration was only evident in CCl₄ treated mice, but not in unchallenged mice (B). (A) shows representative stainings, experiments were performed in three mice per group. Neutrophils were counted in 4 fields of view per slide, three slides per mouse, three mice per group. (B). * $P < 0.05$.

antimicrobial effects [15]. Interestingly, a recent report demonstrated also indirect antimicrobial effects of the chemokine CXCL9. In this study CXCL9 induced the expression and secretion of CXCL1 from human monocytes in G-protein independent manner [16]. As CXCL1 is a major chemoattractant for neutrophils, these features of CXCL9 might contribute to its antimicrobial effects *in vitro* and *in vivo*. In a recent study, we have characterized effects of CXCL9 within the liver and could show that its cognate receptor CXCR3 is expressed by hepatic stellate cells [14]. Furthermore, acute liver injury is associated with increased hepatic levels of CXCL9 [27], although the cellular sources of CXCL9 have not yet been clarified. Nevertheless, in the light of immune modulatory effects of hepatic stellate cells [5,28], we designed the current study to assess whether CXCL9 and its related chemokine CXCL10 do also induce CXCL1 in stellate cells and whether this might also be G-protein independent.

Indeed, CXCL9 and CXCL10 induced CXCL1 expression in immortalized and primary hepatic stellate cells. Nevertheless, in

contrast to monocytes [16], this effect of CXCL9 was G-protein dependent and was mediated by their cognate receptor CXCR3. We did not formally test the effects of CXCL11, a third CXCR3 ligand, in these experiments, but it might be hypothesized that this chemokine displays similar properties under these experimental settings. Thus, overall different signaling pathways appear to be operational in monocytes and stellate cells. The biological significance of this finding is yet unclear and should be the subject of future studies. However, it might be speculated that certain effects of CXC chemokines are mediated by non-classical chemokine receptors, which are more abundant on monocytes compared to stellate cells [29].

In contrast to the latter study, we also assessed whether the CXCL9-CXCL1 chemokine axis is also operational *in vivo* within the liver. To this end, we treated mice with recombinant CXCL9 and analyzed the CXCL1 concentration within the liver. Indeed, systemic CXCL9 application increased hepatic CXCL1 concentrations 2-fold. More importantly, the increased CXCL1 concentration was associated with an increased infiltration of neutrophils into the liver. As these cells are known to be important host effector cells during the resolution of liver damage [30], these results are in line with our earlier findings of a beneficial role of CXCL9 during liver tissue injury [14]. However, the new information obtained in the current study is that the effects of CXCL9 are not necessarily direct, but might also be indirect due to induction of other important chemokines. Interestingly, these indirect effects appear to be limited to CXC chemokines but not mediated by more neutrally charged CC chemokines, such as CCL2, which are also strongly expressed in injured liver [31]. Whether the CXCL9-CXCL1 mediated neutrophil infiltration does enhance necrotic cell debris removal [32], or whether this pathway is involved in anti-bacterial defense for the immunosuppressive sequels of acute liver injury [33] needs to be defined in further studies. Furthermore, future studies are warranted to test whether the observed effects are limited to CXCL9 or are also true for the other CXCR3 ligands, as suggested from our *in vitro* results. Another important aspect for further investigation is the distinction of CXCL9 effects on stellate cells compared to the effects on monocytes, which appear to be CXCR3 independent [16], within the liver.

In summary, we here describe a chemokine-chemokine pathway in hepatic stellate cells, which appears to be relevant during liver injury *in vivo* and leads to the augmented infiltration of neutrophils into the liver. This pathway might therefore be considered as a therapeutic target during liver injury.

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