Research Article
Viral Hepatitis

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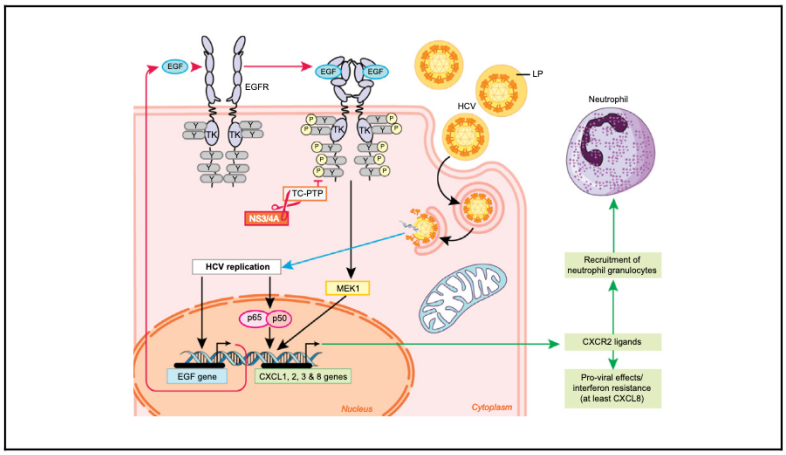
HCV modifies EGF signalling and upregulates production of CXCR2 ligands: Role in inflammation and antiviral immune response

Graphical abstract



Highlights

- HCV enhances the expression of EGF and of CXCR2 ligands in its host cell.
- Upregulation of CXCR2 ligand expression by HCV is mediated via induction of EGF.
- In addition, NF-κB plays a role in HCV-related upregulation of CXCR2 ligand expression.
- HCV enhances the effects of EGF by enhancing EGF-induced signal-transduction.
- In response to EGF stimulation HCV infected cells recruit neutrophil granulocytes.

Authors

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Lay summary

In most cases hepatitis C virus (HCV) results in chronic infection and persistent viral replication, taking decades until development of overt disease. To achieve such a course, the respective virus must have developed mechanisms to circumvent antiviral response, to modulate the inflammatory response and to utilise the infrastructure of its host with moderate effect on its viability. The present study provides novel data indicating that HCV induces epidermal growth factor production in its host cell, enhancing epidermal growth factor-inducible expression of chemokines that bind to the CXCR2 receptor and recruit neutrophile granulocytes. Importantly, chemokines are critical mediators determining the pattern of immune cells recruited to the site of injury and thereby the local inflammatory and immunological milieu. These data strongly suggest that HCV triggers mechanisms that enable the virus to influence the inflammatory and immunological processes of its host.

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HCV modifies EGF signalling and upregulates production of CXCR2 ligands: Role in inflammation and antiviral immune response

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See Editorial, pages 564–566

Background & Aims: To affect immune response and inflammation, the hepatitis C virus (HCV) substantially influences intercellular communication pathways that are decisive for immune cell recruitment. The present study investigates mechanisms by which HCV modulates chemokine-mediated intercellular communication from infected cells.

Methods: Chemokine expression was studied in HCV_{cc}-infected cell lines or cell lines harbouring a subgenomic replicon, as well as in serum samples from patients. Expression or activity of mediators and signalling intermediates was manipulated using knockdown approaches or specific inhibitors.

Results: HCV enhances expression of CXCR2 ligands in its host cell via the induction of epidermal growth factor (EGF) production. Knockdown of EGF or of the p65 subunit of the NF-κB complex results in a substantial downregulation of HCV-induced CXCR2 ligand expression, supporting the involvement of an EGF-dependent mechanism as well as activation of NF-κB. Furthermore, HCV upregulates expression of CXCR2 ligands in response to EGF stimulation via downregulation of the T-cell protein tyrosine phosphatase (TC-PTP [PTPN2]), activation of NF-κB, and enhancement of EGF-inducible signal transduction via MEK1 (MAP2K1). This results in the production of a cytokine/chemokine pattern by the HCV-infected cell that can recruit neutrophils but not monocytes.

Conclusions: These data reveal a novel EGF-dependent mechanism by which HCV influences chemokine-mediated intercellular communication. We propose that this mechanism contributes to modulation of the HCV-induced inflammation and the antiviral immune response.

Lay summary: In most cases hepatitis C virus (HCV) results in chronic infection and persistent viral replication, taking decades until development of overt disease. To achieve such a course, the respective virus must have developed mechanisms to circumvent antiviral response, to modulate the inflammatory response and to utilise the infrastructure of its host with moderate effect on its viability. The present study provides novel data indicating that HCV induces epidermal growth factor production in its host cell, enhancing epidermal growth factor-inducible expression of chemokines that bind to the CXCR2 receptor and recruit neutrophile granulocytes. Importantly, chemokines are critical mediators determining the pattern of immune cells recruited to the site of injury and thereby the local inflammatory and immunological milieu. These data strongly suggest that HCV triggers mechanisms that enable the virus to influence the inflammatory and immunological processes of its host.

Introduction

As a leading cause of chronic liver disease worldwide, in more than 70% of infected individuals HCV establishes a persistent infection characterised by continuous replication and high serum titres. The high propensity for persistence and the insidious course of disease is suggestive of powerful mechanisms allowing HCV to subvert host antiviral immunity, to modify the inflammatory response, and to utilise host cell infrastructure without affecting cell viability. This enables the virus to avert development of overt disease over decades, despite ongoing viral replication. At least in part, this is because HCV is tightly interconnected to host intercellular and intracellular signalling pathways.[1,2] Hence, investigating the interaction of HCV with its host also provides a possibility to illuminate pathways that are relevant for the regulation of different processes of the host, such as growth factor signalling, control of cell growth, differentiation or cell death, inflammatory processes, and antiviral immunity. Amazingly, HCV achieves this molecular piracy with only 10 viral proteins, proteolytically released from one single precursor protein, which is encoded by a positive stranded viral RNA genome. These proteins are multifunctional and required for virus particle formation as well as RNA replication, but also for a broad interaction with different signal transduction pathways of the host.[1] For example, the viral protease NS3/4A on the one hand is central to the processing of the viral precursor protein to generate mature viral proteins. On the other hand, it mediates proteolytic cleavage of different signalling molecules of the host cell such as mitochondrial antiviral-signalling protein (MAVS)[3] and TRIF (TICAM1)[4]

Keywords: Chemokine signalling; Epidermal growth factor; EGF receptor; Hepatitis C; Non-structural 3/4A protease; T-cell protein tyrosine phosphatase; Virus-host interaction.
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involved in antiviral signalling, or the ubiquitously expressed T-cell protein tyrosine phosphatase (TC-PTP [PTPN2]).[5,6] Being an endogenous negative regulator of the EGF receptor (EGFR),[7] cleavage of the latter results in a sensitisation of the EGFR and subsequent signal transduction, as well as in a constitutive and ligand independent activation of AKT kinase.[5] Consistently, TC-PTP expression is reduced in individuals with chronic HCV infection[5,8] and in mice with liver-specific expression of the viral NS3/4A protease.[5,6] These animals not only show an enhanced ligand-induced activation of EGFR and its downstream signalling intermediates but also an altered composition of immune cells in their liver tissue.[6,9] This indicates that HCV or HCV-encoded proteins can markedly influence intercellular communication signals crucial for immune cell recruitment. Among these, chemokines are the most important factors that control recruitment of immune cells and determine the local immunological and inflammatory environment. Indeed, the production of several chemokines, including CCL2 or CXCL8, is influenced by HCV replication as well as by isolated expression of HCV-encoded proteins.[10] Thereby CXCL8 belongs to a group of chemokines that are recognised by the chemokine receptor CXCR2 and play an important role in the recruitment of neutrophil granulocytes,[11] which most likely contribute to the increased serum concentrations of inflammatory cytokines such as TNFα, which can be observed in individuals with chronic HCV infection.[12] The upregulation of TNFα appears to also be responsible for protection against the deleterious effects of lipopolysaccharide observed in transgenic mice with liver-specific expression of NS3/4A.[13] However, the mechanisms involved in the regulation of chemokine production by HCV are incompletely understood and are the subject of the research described in the present manuscript.

Materials and methods

Materials

A complete list of the antibodies as well as the cell culture media and inhibitors used in this study is provided in the supplementary materials. The antibodies used for immunoblotting are compiled in the supplementary materials. Recombinant EGF was obtained from Sigma Aldrich (St. Louis, Missouri, USA). AG1478 was purchased from Calbiochem (Bad Soden, Germany) and U0126 from Promega (Mannheim, Germany). Human EGF ELISA Kit was purchased from Abcam (Cambridge, UK) and eBioscience™ Human IL-8 ELISA Ready-SET-Go!™ Kit, 2nd Generation from Fisher Scientific (Schwerte, Germany).

Cell culture

The human hepatoma cell lines HuH7, HuH7.5, and HepG2 as well as the cell lines harbouring either the subgenomic HCV genotype (GT) 1b-derived replicon (HuH9.13, HuH5.15) or the HCV NS3/4A protease (HepG2 NS3/4A) were cultured and tested for mycoplasma as outlined in the supplementary material.

RNA isolation and real-time PCR

The methods used for RNA isolation and reverse transcription quantitative PCR (RT-qPCR) are outlined in the supplementary material. Specificity of RT-qPCR was controlled by no template and no reverse-transcriptase controls. Semiquantitative PCR results were obtained using the ΔΔCT method and quantification cycles were normalised to *SDHA* which was used as a reference gene.[14,15] Each sample was analysed twice by RT-qPCR. The mean value from the respective measurements was taken as n = 1.

Transfection procedure and small interfering RNA

HuH cells were transiently transfected using EGF-, MEK 1-, p65-, and TC-PTP-specific small interfering RNA (siRNA) from Thermo Scientific Dharmacon (Lafayette, CO, USA) according to the manufacturer's instructions and as described in the supplementary material.

Immunoblotting and immunodetection

Protein extracts from cell culture were prepared and analysed by immunoblotting as outlined in the supplementary material.

Infection of HuH7.5 cells with the HCV strain JC1 (HCV_{cc})

HuH7.5 cells were infected with HCV_{cc} (strain JC1)[16,17] 24 h after seeding with a multiplicity of infection of 1. Cells were used for experiments 72 h after infection. The percentage of infected cells was routinely determined at the time of analysis (72 hpi) by assessment of NS5A expression using immunocytochemistry (Fig. S1) as outlined in the supplementary material and was at 90% on average for acute infection.

Immunofluorescence staining of infected cells

Cells were stained as outlined in the supplementary material and analysed with an LSM 510 Meta confocal laser scanning microscope (Zeiss, Jena, Germany).

Isolation of neutrophil granulocytes from human buffy coats

Neutrophil granulocytes were isolated from freshly collected buffy coats obtained from healthy blood donors (University of Düsseldorf, Blood Transfusion Service) by a density gradient centrifugation over Ficoll-Paque gradient (GE Healthcare). The granulocyte- and erythrocyte-containing layer was washed with PBS w/o Ca^{2+}/Mg^{2+}. Erythrocytes were lysed by resuspension in water for injection for 45 s. Isotonia was restored by addition of 5 M NaCl. Lysed erythrocytes were removed by centrifugation for 8 min at 900 rpm and four wash steps with PBS w/o Ca^{2+}/Mg^{2+}. Neutrophils were cultivated in RPMI 1640 plus GlutaMAX™ media supplemented with 10% heat inactivated FCS, 100 U/ml penicillin and 0.1 mg/ml streptomycin.

Isolation of monocytes from human buffy coats

Human peripheral blood mononuclear cells (PBMCs) were isolated from freshly collected buffy coats obtained from healthy blood donors (University of Düsseldorf, Blood Transfusion Service) by a density gradient centrifugation over Ficoll-Paque gradient (GE Healthcare). Monocytes were isolated from PBMCs by MACS technology using human CD14 microbeads (Miltenyi Biotec) according to the manufacturer's instructions. CD14+ monocytes were cultivated in RPMI 1640 plus GlutaMAX™ media supplemented with 10% heat inactivated FCS, 100 U/ml penicillin and 0.1 mg/ml streptomycin.

Statistical evaluation

Statistics were calculated using the SPSS Statistics software from IBM (Ehningen, Germany). The significance was calculated using the Mann-Whitney-U Test. Data are expressed as fractions of the normalised value of the control, which was set to 1. Data are presented as mean + SEM (n ≥3). *P* values smaller than 0.05 were considered significant. Data were marked with *for $p \leq 0.05$, ** for $p \leq 0.01$, or *** for $p \leq 0.001$.

For further details regarding the materials and methods used, please refer to the CTAT table and supplementary information.

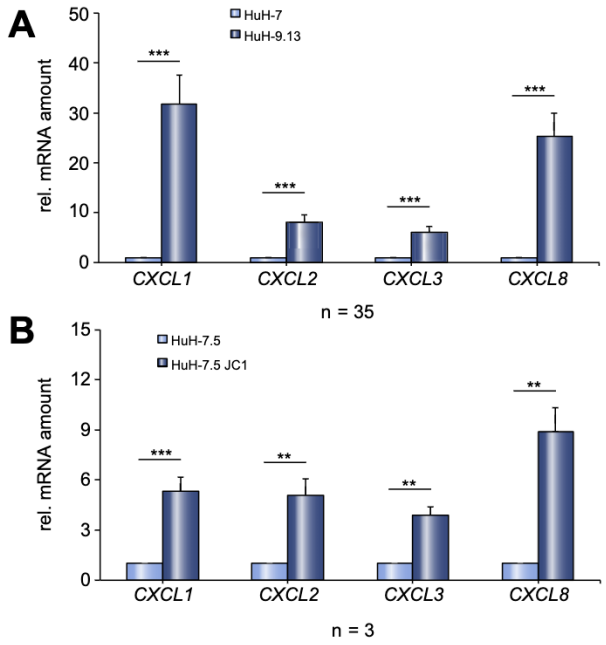


Fig. 1. HCV induces expression of CXCR2 ligands. Abundance of *CXCL1*, *CXCL2*, *CXCL3* and *CXCL8* mRNA was analysed by RT-qPCR (A) in HuH9.13 replicon or (B) HuH7.5 cells infected with the HCV_{cc} strain JC1 and compared to respective controls. (A) and (B) Semiquantitative PCR results were obtained using the ΔΔCT method and quantification cycle were normalised to *SDHA*, which was used as reference gene,[14] as outlined in the material and methods section. Results are expressed as fractions of the normalised value of the control, which was set to 1 and data are presented as mean + SEM of n ≥3 independent experiments as indicated. Significances were calculated using the Mann-Whitney-U Test. Data were marked with * for $p \leq 0.05$, ** for $p \leq 0.01$, or *** for $p \leq 0.001$. HCV, hepatitis C virus; RT-qPCR, reverse transcription quantitative PCR.

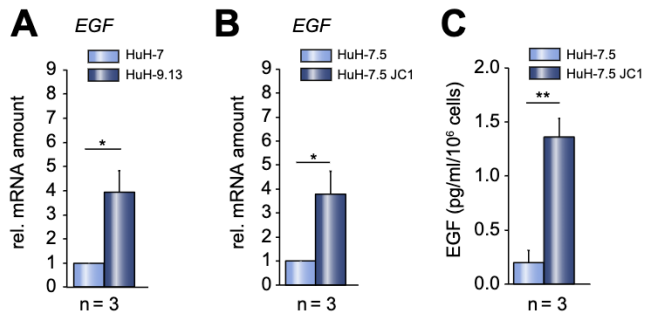


Fig. 2. HCV triggers enhanced EGF expression. (A) Abundance of *EGF* mRNA in HuH9.13 replicon and HuH7 control cells was analysed by RT-qPCR. For (B) and (C) HuH7.5 cells were infected with the HCV_{cc} strain JC1 or left uninfected for control. Seventy-two hours after infection, either total RNA extracts were prepared and analysed by RT-qPCR for the abundance of *EGF* mRNA (B) or supernatants were collected and analysed for secretion of soluble EGF by using ELISA (C). (A) and (B) Data were calculated as outlined in the legend to Fig. 1. For (A) to (C) data are presented as mean + SEM of n = 3 independent experiments as indicated. Significances were calculated using the Mann-Whitney-U Test. Data were marked with * for $p \leq 0.05$, ** for $p \leq 0.01$, or *** for $p \leq 0.001$. EGF, epidermal growth factor; HCV, hepatitis C virus; RT-qPCR, reverse transcription quantitative PCR.

Results

HCV induces expression of CXCR2 ligands

Using a proteome profiler chemokine array for preliminary screening (Fig. S2) and subsequent extensive analysis of transcript expression by RT-qPCR the CXCR2 ligands CXCL1 and CXCL8 were identified as chemokines that are upregulated in cell lines harbouring the HCV subgenomic replicon genotype (GT)1b (HuH9.13) when compared to matching controls. Validation experiments confirmed that apart from these two chemokines, HCV also triggers the expression of CXCL2 and CXCL3 (Fig. 1A). Consistently, an enhanced expression of these chemokines was also detectable in HuH7.5 cells infected with the HCV_{cc} strain JC1 (GT2a) when compared to uninfected controls (Fig. 1B). To exclude a possible effect of HCV or EGF on expression of *SDHA*, used as the reference gene for normalisation of the RT-qPCR results, the reference genes *HPRT1*, *HBMS (SMG9)*, *GAPDH* and *TBP*[14,15] were tested as well (Fig. S3).

Enhancement of CXCR2 ligand expression by HCV is mediated by upregulation of EGF expression

By using a subgenomic HCV replicon we have previously shown that HCV enhances EGF signalling.[5] Consistently, both in HCV_{cc}-infected cells (Fig. S4A–D) as well as in cells containing a subgenomic HCV replicon (Fig. S5A–D), an enhanced activation of EGFR, ERK1 (MAPK3), ERK2 (MAPK1), $p38^{MAPK}$ (MAPK14) and AKT was observed. Quantification of the amount of *EGF* transcript in HCV replicon-containing cells and HCV_{cc}-infected cells revealed an upregulation of *EGF* mRNA compared to the respective control cells (Fig. 2A, B), as well as an increased abundance of EGF protein (Fig. 2C). Most interestingly, knockdown of *EGF* expression in HCV replicon-harbouring cells (Fig. 3A to D and I) or in cells infected with the HCV_{cc} strain JC1 (Fig. 3E to H and J) by siRNA resulted in a significant reduction of the HCV-enhanced expression of the CXCR2 ligands *CXCL1*, *CXCL2*, *CXCL3* and *CXCL8* (Fig. 3). However, CXCR2 ligand expression was not significantly affected if *EGF* knockdown was performed in control cells (data not shown). These data strongly suggest that upregulation of CXCR2 ligand expression upon HCV infection involves the enhancement of EGF expression. Notably, in line with these *in vitro* results and previous reports,[18,19] enhanced serum concentrations of EGF and of the CXCR2 ligand CXCL8 can also be observed in individuals with chronic HCV infection when compared to controls (Fig. 4A to C). Although these data do not provide evidence that there is a causal link between increased serum levels of EGF and upregulation of CXCR2 ligands in patients with chronic HCV infection, they support the *in vivo* relevance of the data reported herein. This assumption is, at least in part, further supported by the positive correlation between viral load and CXCL8 serum levels in patients with chronic HCV infection, as determined by the Pearson correlation coefficient (Fig. 4D). Likewise, serum levels of EGF and CXCL8 (Fig. 4E) tend to positively correlate in patients with chronic HCV infection, although not statistically significant, but not in respective control patients (correlation coefficient: −0.1831; two-tailed value of $p = 0.6372$, data not shown). There was also no statistically significant correlation between viral load and EGF (correlation coefficient: 0.2869; two-tailed value of $p = 0.4909$, data not shown).

HCV enhances the expression of CXCR2 ligands in response to EGF stimulation

The data outlined above indicate that HCV upregulates the release of CXCR2 ligands in an EGF-dependent manner. To study whether HCV also modulates expression of these ligands in response to stimulation with exogenous EGF, cell lines harbouring a subgenomic HCV replicon (cell clones HuH9.13 and HuH5.15) (Fig. 5A–E and Fig. S6) or infected with the HCV_{cc} strain JC1 (Fig. 5F–J) were stimulated with EGF and analysed relative to matching controls. Interestingly, compared to control cell lines

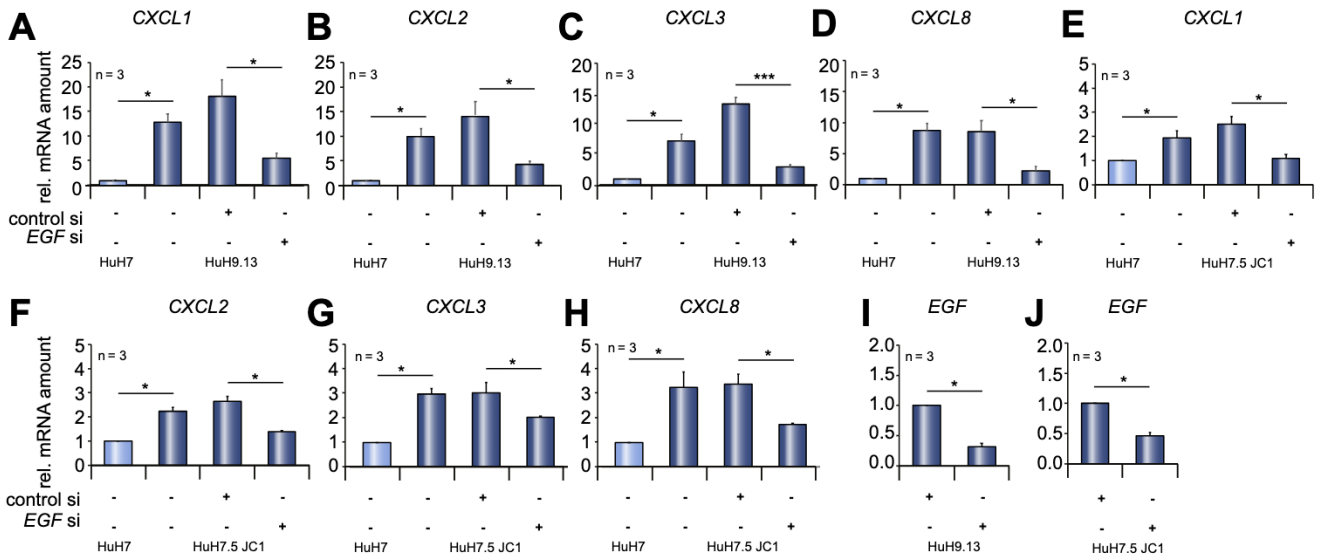
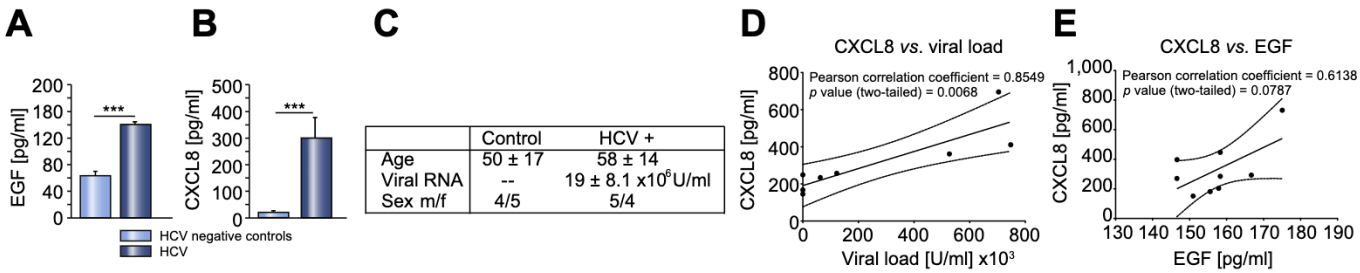


Fig. 3. Upregulation of CXCL8 expression and the other CXCR2 ligands involves EGF-dependent signalling. For (A) to (D) HuH9–13 cells (grey bars) were left untreated for control or transfected with control siRNA or EGF-specific siRNA as indicated and outlined in the materials and methods section. Seventy-two hours after transfection the amounts of (A) *CXCL1*, (B) *CXCL2*, (C) *CXCL3*, (D) *CXCL8*, and (I) *EGF* transcripts were quantified by RT-qPCR. (E) to (H) HuH7.5 cells were left untreated for control or transfected with control siRNA or EGF-specific siRNA as indicated 24 h prior to infection with the HCV_{cc} strain JC1 (grey bars). 48 h after infection the amounts of (E) *CXCL1*, (F) *CXCL2*, (G) *CXCL3*, (H) *CXCL8* and (J) *EGF* transcripts were quantified by RT-qPCR. The data of untreated Huh7 (A to D) or Huh7.5 (E to H) cells (black bars) are depicted for control of the basal expression levels of the transcripts of *CXCL1*, *CXCL2*, *CXCL3* and *CXCL8*. For (A) to (J) results of at least three independent experiments are depicted. Data were calculated as outlined in the legend to Fig. 1 and data are presented as mean + SEM of n = 3 independent experiments. Significances were calculated using the Mann-Whitney-U Test. Data were marked with * for $p \leq 0.05$, ** for $p \leq 0.01$, or *** for $p \leq 0.001$. EGF, epidermal growth factor; HCV, hepatitis C virus; RT-qPCR, reverse transcription quantitative PCR.



	Control	HCV +
Age	50 ± 17	58 ± 14
Viral RNA	--	19 ± 8.1 x10^6 U/ml
Sex m/f	4/5	5/4

Fig. 4. EGF and CXCL8 levels are elevated in sera of HCV-infected patients. Sera from HCV negative donors (HCV negative controls) or patients with chronic HCV infection (HCV) were analysed for the serum concentrations of (A) soluble EGF and (B) soluble CXCL8 using ELISA. The table (C) provides information on average age, viral load, and sex distribution of the different groups. More detailed information on genotype, viral load, cirrhosis and co-morbidities are summarised in Table S2. The graphs presented in (D) and (E) represent the Pearson correlation coefficient for correlation of CXCL8 serum levels with (D) viral load and (E) EGF serum levels. For A and B significances were calculated using the Mann-Whitney-U Test. Data were marked with * for $p \leq 0.05$, ** for $p \leq 0.01$, or *** for $p \leq 0.001$. EGF, epidermal growth factor; HCV, hepatitis C virus.

(black bars), expression of the CXCR2 ligands *CXCL1*, *CXCL2*, *CXCL3* and *CXCL8* and in particular transcript and protein abundance of CXCL8 were enhanced in cells containing replicating HCV (Fig. 5). Evaluation of the enhancing effect of HCV on induction of CXCL8 expression in response to different concentrations of EGF demonstrated that already 1.25 ng/ml EGF resulted in an approximately tenfold increase of CXCL8 expression in cell lines harbouring the subgenomic HCV replicon, while in respective control cells the increase was only threefold (Fig. S7).

HCV-mediated upregulation of CXCL8 expression and enhanced EGF-inducible expression of CXCL8 in HCV replicating cells involves activation of EGF signalling and NF-κB

To further characterise the pathway involved in the enhancement of EGF-inducible CXCR2 ligand expression in HCV containing cells, the effect of EGFR signalling inhibition by pharmacological or siRNA-based treatment was assessed in cell lines harbouring an HCV replicon or infected with the HCV_{cc} strain JC1. The data presented above indicate that the CXCR2 ligand family member that was most prominently influenced by HCV is CXCL8. Therefore, these analyses were focused on CXCL8 expression and could identify activation of EGFR tyrosine kinase as well as activation of MEK1 and NF-κB as signalling events, which are involved in the regulation of basal and/or EGF-inducible CXCL8 expression by HCV. Thus, inhibition of EGFR (Fig. 6A, B) or the downstream target MEK1 (Fig. 6C, D) using the specific inhibitors AG1478 (EGFR) and U0126 (MEK1 and MEK2 [MAP2K2]) blocked EGF downstream signalling (Fig. S8) as well as expression of CXCL8 in EGF-treated HuH9.13 cells or cells infected with the JC1 virus. In line with this, knockdown of MEK1 expression (Fig. S9A), but not of EGF itself (Fig. S9B), using specific siRNA resulted in a downregulation of enhanced expression of CXCL8 in HuH9.13 cells stimulated with EGF. However, inhibition of EGFR signalling also affected EGF-inducible CXCL8 expression in HuH7.5 control

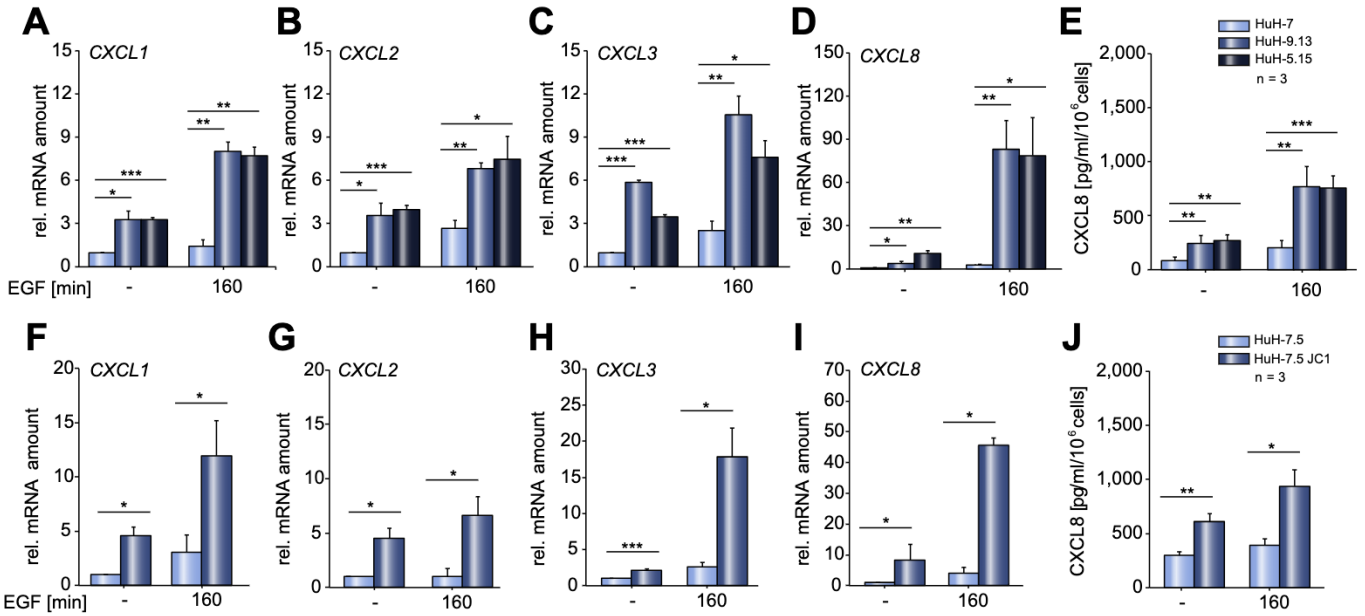


Fig. 5. HCV enhances EGF-inducible CXCR2 ligand expression. For (A) to (E) HuH9.13 or HuH5.15 cells harbouring the HCV replicon and HuH7 control cells and for (F) to (J) HuH7.5 cells either infected with the HCV_{cc} strain JC1 or left uninfected for control were treated with 40 ng/ml EGF for 160 min as indicated on the bottom of each panel. (A) to (D) and (F) to (I) Total RNA extracts were prepared and the abundance of CXCL1 (A and F), CXCL2 (B and G), CXCL3 (C and H) and CXCL8 (D and I) mRNA was determined by RT-qPCR. Data were calculated as outlined in the legend to Fig. 1 and are presented as mean + SEM of n = 3 independent experiments. For (E) and (J) respective supernatants were collected and analysed for production and release of soluble CXCL8 using ELISA. Significances were calculated using the Mann-Whitney-U Test. Data were marked with * for $p \leq 0.05$, ** for $p \leq 0.01$, or *** for $p \leq 0.001$. EGF, epidermal growth factor; HCV, hepatitis C virus; RT-qPCR, reverse transcription quantitative PCR.

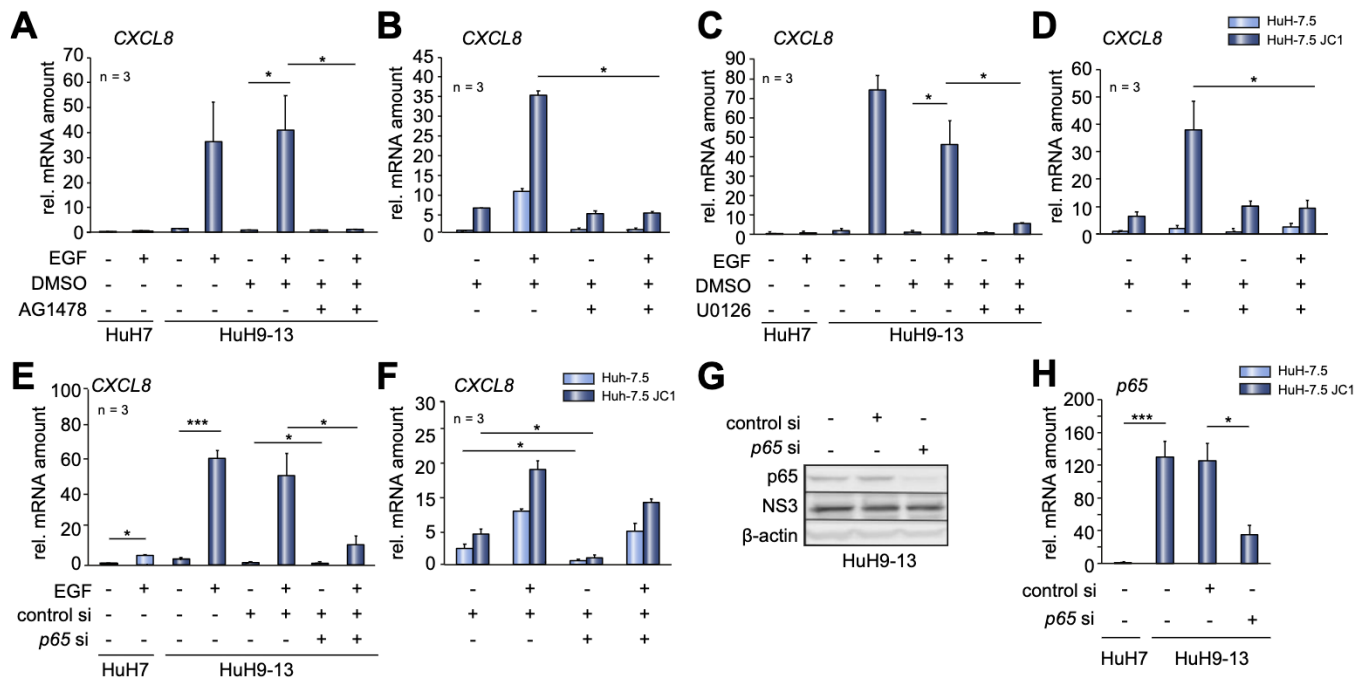


Fig. 6. HCV-mediated upregulation of CXCL8 expression and enhancement of EGF-inducible CXCL8 expression involve activation of EGF signalling and NF-κB. For (A) to (D) either HuH9.13 replicon cells (A and C) or HuH7.5 control cells and HuH7.5 cells infected with the HCV_{cc} strain JC1 (B and D) were pretreated with 10 μM AG1478 (A and B) or 10 μM U0126 (C and D) for 2 h, as indicated, followed by stimulation with 40 ng/ml EGF for 160 min. For (E) and (F) HuH9.13 replicon cells or HuH7.5 control cells were transfected with control siRNA or p65 siRNA as indicated. For (F) HuH7.5 cells were either infected with 1 multiplicity of infection of the HCV_{cc} strain JC1 24 h after transfection or were left uninfected. For (E) and (F) 72 h after transfection, cells were stimulated with 40 ng/ml EGF for 160 min. For (A) to (F) and (H) amounts of (A to F) *CXCL8* and (H) *p65* transcripts were quantified by RT-qPCR. Data were calculated as outlined in the legend to Fig. 1 and data are presented as mean + SEM of n = 3 independent experiments. For (G) total protein was extracted and expression of p65 as well as the viral NS3 were determined by immunoblot. β-actin expression was assessed as loading control. Significances were calculated using the Mann-Whitney-U Test. Data were marked with * for $p \leq 0.05$, ** for $p \leq 0.01$, or *** for $p \leq 0.001$. EGF, epidermal growth factor; HCV, hepatitis C virus; RT-qPCR, reverse transcription quantitative PCR.

cells (Fig. 6B) supporting the notion that HCV enhances mechanisms that regulate CXCL8 expression in response to EGF and does not induce the activation of a specific pathway on its own. Apart from this, knockdown of the p65 subunit of the NF-κB signal transducing complex was also sufficient to abrogate the enhancing effect of HCV on basal as well as inducible CXCL8 expression in HuH9.13 replicon cells (Fig. 6E). While in uninfected and HCVcc-infected HuH7.5 cells, knockdown of p65 mainly affected the basal expression of CXCL8 (Fig. 6F). These data indicate that NF-κB is involved in regulation of basal CXCL8 expression in uninfected HuH7.5 cells and plays a role in the enhancement of CXCL8 expression by HCV. This assumption is further supported by the observation that inhibition of IKK2 (IKBKB) using the IKK2 inhibitor was also able to efficiently reduce basal and EGF-inducible expression of CXCL8 in Huh9.13 cells (Fig. S10). In contrast, although enhanced in response to HCV infection[1] (Fig. S4B, D) and blocked by inhibition of EGFR (Fig. S8), activation of $p38^{MAPK}$ or AKT appears to be dispensable for the effect of HCV on basal and EGF-inducible CXCL8 expression. Accordingly, neither inhibition of AKT nor inhibition of $p38^{MAPK}$ by respective small compound inhibitors, or by knockdown of *AKT* expression using specific siRNA, affected expression of CXCL8 in HuH9.13 cells (Fig. S11). Of note, the inhibitors used do not impair cellular viability as suggested by the results of an WST assay (Fig. S12).

NS3/4A and subsequent downregulation of TC-PTP are involved in enhanced activation of EGFR signalling and upregulation of basal and inducible expression of CXCL8

We have recently demonstrated that HCV enhances EGFR signalling via NS3/4A-mediated proteolytic cleavage of TC-PTP at position aa 123/124 and aa 216/217. Correspondingly, when compared to respective controls, infection of Huh7.5 cells with the HCV_{cc} strain JC1 (Fig. S4E), as well as the presence of the subgenomic HCV replicon (Fig. S5E), reduces the abundance of TC-PTP, which is the major negative regulator of EGFR tyrosine kinase activity.[7] Hence, it is conceivable that NS3/4A expression and subsequent cleavage of TC-PTP is involved in HCV-mediated enhancement of CXCL8 expression. We therefore analysed the effect of isolated expression of the NS3/4A protease, as well as the influence of targeted disruption of TC-PTP expression, on CXCL8 expression. The data indicate that EGF-inducible signal transduction (Fig. S13) and CXCL8 expression (Fig. 7A) are enhanced in HepG2 cells stably expressing NS3/4A when compared to mock-transfected control cells. These data indicate that expression of NS3/4A alone is suitable to enhance EGF-inducible expression of CXCL8 and that, in line with a recent report from our group,[5] subsequent downregulation of TC-PTP by HCV might be involved in mediating the effects of HCV on CXCL8 expression. In line with this assumption, basal and EGF-inducible expression of CXCL8 (Fig. 7B) as well as EGF-inducible activation of EGFR and subsequent downstream signalling via activation of ERK1/2, $p38^{MAPK}$ and AKT was upregulated in HuH7 cells upon targeted disruption of TC-PTP expression using TC-PTP specific siRNA (Fig. S14).

In the presence of HCV, EGF triggers the release of a chemokine pattern that mediates recruitment of neutrophils but not of monocytes

A major action of chemokines is induction of directed cellular migration. Thereby CXCR2 ligands are mainly involved in the recruitment of neutrophils.[11] To determine whether, in the presence of HCV, EGF-induced chemokine release is able to trigger migration of inflammatory cells such as neutrophils or monocytes, migration assays were performed. As depicted (Fig. 8A), supernatants conditioned by the control cell line HuH7 do not affect migration of neutrophils, when compared to control media or media conditioned by EGF-treated HuH7 cells. In contrast, media conditioned by EGF-treated cell lines harbouring the subgenomic HCV replicon (HuH9.13) can enhance the migratory activity of neutrophils (Fig. 8A) but not of CD14+ monocytes (Fig. 8B).

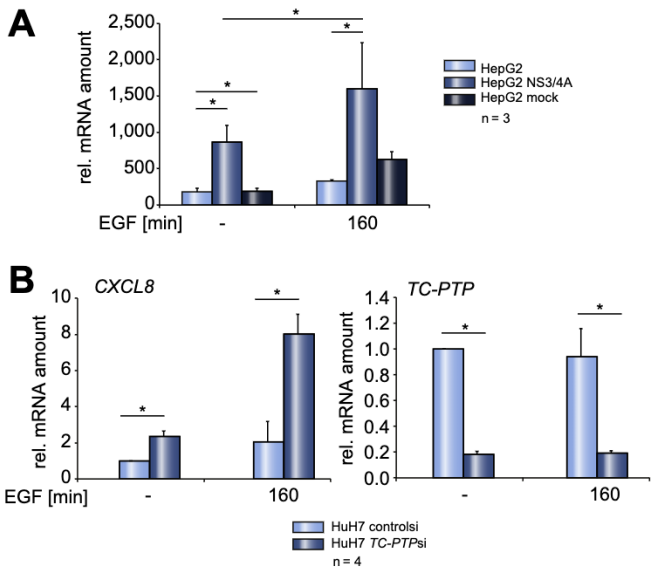


Fig. 7. NS3/4A as well as downregulation of TC-PTP enhance CXCL8 expression in response to EGF. For (A) HepG2 either stably transfected with HCV NS3/4A or untransfected as well as mock-transfected HepG2 cells were treated with 40 ng/ml EGF for 160 min. For (B) HuH7 cells were transfected with control siRNA or TC-PTP-specific siRNA as outlined in the materials and methods section and treated with 40 ng/ml EGF for 160 min. For (A) and (B) amounts of CXCL8 (A and B) and TC-PTP (B) transcripts were quantified by RT-qPCR. Data were calculated as outlined in the legend to Fig. 1 and data are presented as mean + SEM of n ≥3 independent experiments as indicated. Significances were calculated using the Mann-Whitney-U Test. Data were marked with * for $p \leq 0.05$. EGF, epidermal growth factor; HCV, hepatitis C virus; RT-qPCR, reverse transcription quantitative PCR.

Discussion

Previous data indicate that during infection with HCV, viral host cell entry and replication are tightly interconnected with growth factor signalling.[5,20–22] Hence, EGFR serves as a co-receptor for viral entry and its virus-induced activation as well as EGFR downstream signalling are required for viral entry and also influence replication.[5,20–22] To optimise the function of this pathway for its own purpose, HCV interferes with surface expression of ErbB growth factor receptor family members and the expression of its endogenous negative regulator.[5,20]

The current study provides novel data indicating that HCV enhances the release of members of the chemokine family, that specifically recognise the chemokine receptor CXCR2 (Fig. 1) via a mechanism that involves HCV-mediated upregulation of EGF expression (Fig. 3). Thereby, HCV enhances CXCR2 ligand expression, which is further upregulated by EGF, except for CXCL8, while EGF-mediated induction of gene expression is clearly enhanced in the presence of HCV. Furthermore, HCV

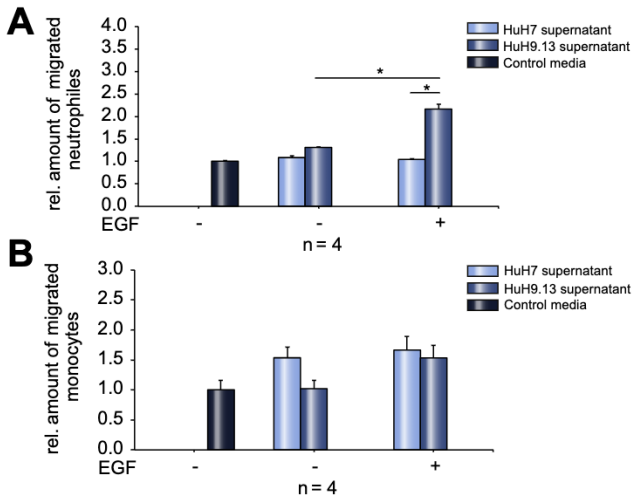


Fig. 8. In the presence of HCV, EGF triggers the release of a mediator pattern that mediates recruitment of neutrophils but not of monocytes. (A) and (B) HuH7 control cells and HuH9.13 replicon cells were treated with 40 ng/ml EGF for 8 h. Supernatants were transferred in the basal compartment of a transwell system. Cell-free culture media was used as control. Neutrophil granulocytes (A) and monocytes (B) were extracted from human buffy coats as described in the Material and Methods section and cultivated in cell culture inserts (pore size 3 μm). A total of 40 μL WST-1 reagent was added to the basal compartment for 3 h. Analysis was performed photometrically at 450 nm and 620 nm. The results are expressed as fractions of the normalised value of the control, which was set to 1, and data are presented as mean + SEM of n = 4 independent experiments. Significances were calculated using the Mann-Whitney-U Test. Data were marked with * for $p \leq 0.05$. EGF, epidermal growth factor; HCV, hepatitis C virus.

enhances the ability of EGF (Fig. 5) and EGFR-mediated signalling (Figs. S4 and S5) to induce CXCR2 ligand expression in its host cell, resulting in the release of a mediator pattern, that mediates recruitment of neutrophil granulocytes (Fig. 8).

Several reports indicate that HCV triggers expression of CXCR2 ligands, particularly of CXCL8, *in vivo* and *in vitro*.[18,23–27] Consistently, when compared to uninfected individuals or patients in whom HCV infection has been successfully eliminated, CXCL8 serum levels are increased in patients with chronic HCV infection[18,28] and positively correlate with viral load (Fig. 4B and D). Various HCV-encoded proteins including NS2,[24] NS4A, NS4B,[26] and NS5A[23] have been reported to be involved in HCV-induced CXCL8 expression. Thereby, the action of these different viral proteins appears to converge at the activation of the transcription factors NF-κB and/or AP1[23,24,26] but also involves regulation at the level of transcript stability.[27] The data presented herein indicate that, in addition to NS2, NS4A, NS4B/B, and NS5A, the virus encoded protease NS3/4A is also involved in HCV-mediated upregulation of CXCR2 ligand expression (Fig. 7A). Thereby, NS3/4A mediates its effect on EGF signalling via cleavage of the ubiquitously expressed tyrosine phosphatase TC-PTP,[5] which is an endogenous negative regulator of the EGF receptor.[7] Consequently, its HCV-mediated degradation results in an enhancement of EGF-inducible signal transduction,[5] an effect that can be mimicked by knockdown of TC-PTP protein expression using specific siRNA (Fig. S14 and [5]). As demonstrated herein, downregulation of TC-PTP expression also mimics the effect of HCV on CXCL8 expression (Fig. 7B), indicating that HCV-mediated sensitisation of EGF signalling and enhanced CXCR2 ligand expression are interlinked. In line with this, HCV infection as well as the isolated expression of the HCV-encoded NS3/4A protease downregulate TC-PTP expression (Fig. S4, Fig. S5 and [5]) and substantially enhance EGF-inducible CXCR2 ligand expression (Figs. 5 and 7A, Figs. S6 and S7). Of note, stimulation of cell lines harbouring the HCV replicon with EGF results in the release of a chemokine pattern into the supernatant, which triggers migratory activity of neutrophil granulocytes but not of monocytes (Fig. 8). Because CXCR2 ligands are major chemoattractants of neutrophil granulocytes,[11] it can be concluded that HCV preferentially enhances CXCR2 ligand expression in response to EGF stimulation.

The data further indicate that HCV not only enhances EGF signalling and expression of certain target genes but also upregulates the expression of EGF itself (Fig. 2), which in turn mediates the upregulation of CXCR2 ligand expression in the infected host cell suggesting an EGF-mediated circuit (Fig. 3). The induction of EGF expression by HCV itself may also contribute to the increased serum levels of EGF observable in patients with chronic infection ([19] and Fig. 4A), although the analysis of the data presented herein suggests that there is no significant correlation between viral load and EGF expression. Thus, there might be additional reasons for increased EGF serum levels observable in HCV-infected individuals. However, there is at least a tendency for a positive correlation between serum levels of EGF and CXCL8 in patients with chronic HCV infection (Fig. 4E) suggesting that in these patients EGF and CXCL8 levels may at least in part also be interlinked *in vivo*.

In its host cell, HCV activates EGFR[21] and enhances EGFR-mediated signalling via AKT, MEK1/2, ERK1/2, and $p38^{MAPK}$, for example (Figs. S4 and S5 and [5]). From these different signalling events, the activation of EGFR and MEK1/2 appears to be important for EGF-inducible expression of the CXCR2 ligand CXCL8 in HCV_{cc}-infected cells while activation of AKT and $p38^{MAPK}$ seem to be dispensable. Consistently, inhibition of EGFR tyrosine kinase activity or of MEK1/2, using the EGFR-specific inhibitor AG1478 (Fig. 6A and B) or either the MEK1/2 inhibitor U0126 (Fig. 6C and D) or MEK1-specific siRNA (Fig. S9), inhibited EGF-inducible CXCL8 expression in cell lines harbouring the HCV replicon or infected with the HCV_{cc} strain JC1. Interestingly, although EGF itself does not affect activation of NF-κB in HCV_{cc}-infected cells (data not shown), the data suggest that the constitutive activation of NF-κB in the presence of HCV proteins or upon infection with HCV[13,23,26] is required for the enhancing effect of HCV on basal and EGF-inducible chemokine expression. Correspondingly, knockdown of the NF-κB subunit p65 using specific siRNA (Fig. 6E), or inhibition of NF-κB activation (Fig. S10), result in a substantial decrease of basal and inducible expression of CXCL8 in cells harbouring a subgenomic replicon. Of note, knockdown of p65 in HuH7.5 cells prior to infection with the HCV_{cc} strain JC1 (Fig. 6F) not only prevented HCV-mediated enhancement of basal CXCL8 expression, but also resulted in a significant reduction of basal CXCL8 expression in HuH7.5 control cells. Hence, these data suggest that HCV-enhanced induction of chemokine expression by EGF in infected host cells does not solely depend on EGFR and sensitisation of EGFR, but also on an HCV-mediated alteration of intracellular signalling, which is independent from EGF/EGFR. This includes constitutive activation of transcription factors such as NF-κB, which are involved in the transcriptional control of *CXCL8* gene expression in response to different stimuli, including inflammatory mediators,[29] but also to different HCV proteins.[23–26]

Most interestingly, the mechanisms delineated herein seem to counteract the HCV-mediated interruption of virus- or double-stranded DNA-inducible RIG1-mediated cytokine and chemokine expression[3,30,31] by NS3/4A-mediated cleavage of CARDIF (also termed MAVS or VISA). In particular, they suggest that HCV itself at least partially (or selectively) leads to the expression of distinct chemokines, such as CXCL8. In this context, it is interesting to note that CXCL8 confers resistance to type I interferons[18,23,32,33] and has implications for viral replication.[34] This association of CXCL8 with resistance to type I interferons is further supported by the observation that the baseline level of CXCL8 is an independent risk factor for the achievement of sustained virological response in patients with chronic HCV.[35]

The *in vivo* relevance of these observations remains to be established. However, as demonstrated previously[18,19,28] and corroborated herein (Fig. 4), serum concentration of CXCR2 ligands such as CXCL8[18,28,35] and of the growth factor EGF[19] are upregulated in patients with chronic HCV infection and at least in the case of CXCL8 positively correlate with viral load (Fig. 4D). This, and the fact that successful eradication of HCV in patients with chronic HCV infection results in a decrease or even normalisation of the serum levels of both CXCL8[28] and EGF,[19] suggests that these alterations are mainly induced by the virus.

Concerning the pathogenetic relevance of these data with respect to liver injury, it should be noted that in patients with chronic hepatitis C, hepatic expression levels of the CXCR2 ligand CXCL8 were strongly associated with the severity of portal inflammation and with the presence of bile duct lesion.[36] Based on the data presented it can be speculated that this, at least in part, is due to an EGF-dependent mechanism by which HCV enhances production of CXCL8, which promotes hepatic inflammation by recruiting neutrophils to the infected tissue. This may even be enhanced under conditions that result in an increased production of EGF.

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Conflict of interest

The authors declare no conflicts of interest that pertain to this work.

Please refer to the accompanying ICMJE disclosure forms for further details.

Authors' contributions

CG: performance of experiments, analysis and interpretation of data, proofreading of manuscript. KR: performance of experiments, analysis and interpretation of data, proofreading of manuscript. NS: performance of experiments, analysis and interpretation of data, proofreading of manuscript. SS: performance of experiments, analysis and interpretation of data, critical revision of manuscript. CE: performance of experiments, proofreading of manuscript. UA: performance of experiments, proofreading of manuscript. HHB: patient samples, proofreading of manuscript. RB: analysis and interpretation of data, proofreading of manuscript. DH: analysis of data, proofreading of manuscript. JGB: study conception and design, analysis and interpretation of data, writing and drafting of the manuscript, critical revision of manuscript.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.jhep.2018.04.005.

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Author names in bold designate shared co-first authorship

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