**Induction of chemokines by hepatitis C virus proteins: Synergy of the core protein with
interleukin-1 β and interferon- γ in liver bystander cells**

Sara **Abouelasrar Salama**¹, Mieke **Gouwy**¹, Alexandra **De Zutter**¹, Noémie **Pörtner**¹, Lotte
Vanbrabant¹, Nele **Berghmans**¹, Mieke **De Buck**¹, Sofie **Struyf**^{1†}, Jo **Van Damme**^{1†}

¹Laboratory of Molecular Immunology, Department of Microbiology, Immunology and
Transplantation, Rega Institute for Medical Research, University of Leuven, Leuven, 3000,
Belgium.

[†]Equal contribution

Running title

Modulation of cytokine signalling by HCV proteins

Corresponding author

Prof. S. Struyf

Rega Institute for Medical Research, KU Leuven, Herestraat 49, box 1042, 3000 Leuven

Tel +32 16 322 422

Email: Sofie.Struyf@kuleuven.be

Keywords

Chemokines, interferon- γ , interleukin-1 β , Hepatitis C virus, liver endothelial cells

Abstract

Chronic HCV infection accounts for a large proportion of hepatic fibrosis and carcinoma cases observed worldwide. Mechanisms involved in HCV-induced hepatic injury have yet to be fully elucidated. Of particular interest is the capacity of HCV to regulate inflammatory responses. Here, we reveal modulation of cytokine activity by the HCV proteins NS3, glycoprotein E2 and core protein for their ability to induce chemokine expression in various liver bystander cells. Chemokines sustain chronic liver inflammation and relay multiple fibrogenic effects. CCL2, CCL3, CCL20, CXCL8 and CXCL10 were differentially expressed after treatment of monocytes, fibroblasts and liver sinusoidal endothelial cells (LSECs) with HCV proteins. In comparison to NS3 and glycoprotein E2, core protein was a stronger inducer of chemokines in liver bystander cells. IFN- γ and IL-1 β synergized with core protein to induce CCL2, CCL20, CXCL8 or CXCL10 in fibroblasts or LSECs. These findings reveal new mechanisms of hepatic injury caused by HCV.

Introduction

Hepatitis C virus (HCV), a single-stranded positive-sense RNA virus, is of the *Hepacivirus* genus and belongs to the Flaviviridae family. Translation of the HCV genome, which is 9.6 kb in length, gives rise to a polyprotein that is approximately 3000 amino acids in length. The generated polyprotein is processed by cellular and viral proteases giving rise to structural (core protein and glycoproteins E1/E2) and non-structural (NS1 to NS5B) proteins. Once HCV infection is acquired, the majority of patients develop chronic HCV, which is characterized by hepatic fibrosis and hepatocellular carcinoma (Ansaldi et al 2014). Due to the recent development of direct-acting antiviral agents (DAAs), which function by inhibiting viral enzymes, HCV recovery rates have remarkably improved (Omar et al 2018). However, recent studies have reported the emergence of complications associated with the use of DAAs. For instance, resistance-associated substitutions have been identified in treatment-naïve patients, particularly in the *NS3*-, *NS5A*- and *NS5B*-coding regions (Li et al 2017).

Chemokines play a pivotal role in the antiviral response against HCV. CXCR3 ligands, CXCL9 to CXCL11, recruit CXCR3-expressing Th1 cells, cytotoxic T-cells and natural killer (NK) cells to the site of infection. CCR1 and CCR5 expressed on the surface of T-cells also play a role in the recruitment of these effector cells (Brass et al 2014). The CCR6 receptor, whose ligands include CCL20, is expressed by B cells, dendritic cells (DCs), NK and T-cells (Schutyser et al 2003). CCL20 plays a role in fine-tuning T cell responses by regulating the balance between Th17 and T_{reg} polarization (Ranasinghe et al 2018). Furthermore, hepatic recruitment of monocytes relies on the expression of the CCR2 ligand CCL2 (Brass et al 2014). Chemokine expression is primarily regulated by cytokines. Interleukin-1 β (IL-1 β), a major inflammatory cytokine, upregulates various chemokines such as CCL2, CXCL1, CXCL2, CXCL3, CXCL8 and CXCL10 (Luo et al 2015; Sheng et al 2005). Tumour necrosis factor- α (TNF- α), another

principle inflammatory cytokine, induces a similar chemokine profile as IL-1 β (Sheng et al 2005; Tourniaire et al 2013). Furthermore, the production of T-cell chemoattractants, CXCL9-11, occurs mainly in response to interferon- γ (IFN- γ) (Groom et al 2011).

Interestingly, HCV has been found to modulate cytokine expression as a way to evade the immune response. The viral NS3/4A protease complex cleaves mitochondrial antiviral signaling protein (MAVS) and TIR-domain-containing adapter-inducing interferon- β (TRIF), which are signaling molecules downstream of RIG-I-like receptor and Toll-like receptor 3 (TLR3) activation, respectively (Ferreira et al 2016; Li et al 2005). In this manner, the virus inhibits the production of type I and III IFN, thus promoting its own survival. Another HCV protein, the core protein, was shown to inhibit IFN production in human plasmacytoid DCs (pDCs) in response to TLR activation via reduced expression of interferon regulatory factor 7 (IRF7) (Stone et al 2014). Not only does HCV influence cytokine expression, but the virus also possesses the capacity to modulate the signaling pathways of cytokines once they are expressed. HCV core protein effects signaling by type I IFN via the Janus kinase/signal transducer and activator of transcription (JAK-STAT) pathway by inducing a dysfunctional increase in non-phosphorylated STAT1 in pDCs (Stone et al 2014). In another study, glycoprotein E2 was found to promote cell survival of Raji cells by overriding FasL-induced cell death via the upregulation of anti-apoptotic proteins, Bcl-2 and Bcl-XL (Chen et al 2011).

In the present paper, we investigate the chemokine inductive profiles of three HCV proteins, the core protein, glycoprotein E2 and NS3, in various liver bystander cells. We observed differential induction of multiple chemokines by HCV proteins. Furthermore, we reveal the capacity of HCV core protein to enhance cytokine activity regarding the induction of chemokine expression in liver bystander cells.

Materials and Methods

Reagents

Recombinant HCV Core protein (MBS596029; Sequence ID BAM73001.1), NS3 protein (MBS596033; Sequence ID AIZ00641.1) and glycoprotein E2 (MBS434003; Sequence ID AAR22408.1) derived from genotype 1b were obtained from MyBiosource (San Diego, CA, USA). The lipopolysaccharide (LPS) content of the recombinant HCV proteins was as low as 0.02 ng/100 µg of core protein, 0.007 ng/100 µg of NS3 and 0.7 ng/100 µg of glycoprotein E2. The maximal level of LPS reached in the highest tested concentrations of the viral proteins did not influence any of the activities reported here (data not shown). LPS (O111:B4) derived from *E.coli* and dsRNA (P1530) were obtained from Sigma-Aldrich (Overijse, Belgium). Mouse monoclonal IgG2a isotype control (MAB0031) and antibodies against human CCL2 (MAB679, BAF279), CXCL10 (BAF266) and CXCL8 (MAB208) were obtained from R&D Systems (Minneapolis, MN, USA). Recombinant human CCL3 (270-LD) and recombinant human IFN-γ (285-IF) were obtained from R&D Systems. Capture monoclonal anti-human CXCL10 (500-P93), recombinant human IL-1β (200-01B) and recombinant human TNF-α (300-01A) were obtained from Peprotech (London, UK). Mouse monoclonal anti-TLR2 IgG2a antibody (16-9922-82) was obtained from ThermoFischer Scientific (Merelbeke, Belgium). TAK-242 (HY-11109) was obtained from MedChemExpress (Monmouth Junction, NJ, USA).

Chemotaxis assay

The migration of PBMCs was determined in the Boyden microchamber (Neuro Probe, Gaithersburg, MD, USA) as described (De Buck et al 2013).

Cell cultures

Peripheral blood mononuclear cells (PBMCs) and CD14⁺ monocytes were isolated from human 1-day-old buffy coats, derived from healthy donors (Blood Transfusion Center,

Mechelen, Belgium), via density gradient centrifugation and via positive selection (MACS, Miltenyi Biotec, Bergisch Gladbach, Germany) (De Buck et al 2013). Human embryonic skin muscle fibroblasts (E6SM fibroblasts) were grown in minimal essential medium (MEM; Lonza, Verviers, Belgium) supplemented with 10% fetal calf serum (FCS). Primary human liver sinusoidal microvascular endothelial cells (LSECs; Cell Systems, Kirkland, WA) were grown in endothelial cell growth medium (EGM; Lonza) supplemented with EGM 2-MV BulletKit (Lonza).

Induction experiments and chemokine quantification

E6SM fibroblasts were seeded in 48-well plates (15,000 cells/cm², 450 µl/well) in culture medium (Van Damme et al 1989). At 90% confluency, fibroblasts were stimulated with different concentrations of inducers or kept untreated (control) for 72 h at 37°C, 5% CO₂. CD14⁺ monocytes were seeded in 48-well plates (1x10⁶ cells/cm², 450 µl/well) in RPMI 1640 medium supplemented with 0.5% human serum albumin (HSA; Belgian Red Cross, Brussels, Belgium). For TLR2 neutralization, CD14⁺ monocytes were pre-incubated with an anti-TLR2 antibody for 10 min at 37°C, 5% CO₂ prior to stimulation. Monocytes were stimulated for 24 h. LSECs were grown to 90% confluency in 48-well plates (15,000 cells/cm², 500 µl/ well) coated with 0.1% gelatin (in PBS) in growth medium and stimulated for 48 h. For TLR4 neutralization, LSECs were pre-incubated with a TLR4 antagonist, TAK-242, for 10 min, at 37°C, 5% CO₂ prior to stimulation. Cell supernatants were collected, underwent centrifugation (250g, 5 min) and were stored at -20°C until further analysis by ELISA. Human CCL3 and CCL20 were measured with a specific ELISA kit according to the manufacturer's instructions (R&D Systems). Human CCL2, CXCL8, and CXCL10 ELISAs were developed in our laboratory (Paul Proost et al 2006; Schutyser et al 2000).

Flow cytometry

Fibroblasts were seeded in 6-well plates at a density of 15,000 cells/cm². Following a stimulation period of 72 h, the cells were detached via treatment with trypsin for 15 sec. Thereafter, trypsin was neutralized and the cells were allowed to recover for a period of 1 h at room temperature in culture medium. In order to exclude dead cells from the analysis, cells were incubated in Zombie Aqua viability dye (BioLegend, San Diego, CA, USA) in PBS for 15 min at room temperature. To block the Fc receptors, the cells were washed and incubated for 10 min at 4°C with FACS buffer [PBS + 2% FCS + 2 mM ethylenediamine tetraacetic acid (EDTA)]. Afterwards, the cells were stained with the anti-human antibodies mentioned hereafter for 30 min at 4°C. Fluorescein isothiocyanate (FITC)-labelled IL-1R2 antibody (clone REA744) was obtained from Miltenyi Biotech (Bergisch Gladbach, Germany). Phycoerythrin (PE)-labelled IL-1R1 (clone 150503) and allophycocyanin (APC)-labelled IFN- γ R2 (catalogue number FAB773A) were obtained from R&D Systems. Brilliant violet 421 (BV421)-labelled IFN- γ R1 (clone GIR-208) was obtained from BD Biosciences (Heidelberg, Germany). The cells were subsequently washed twice and fixed with 0.4% formaldehyde in FACS buffer. Acquisition was carried out using an LSRFortessa X-20 cell analyzer (BD Biosciences) and data analysis was performed using FlowJo software (Tree Star, Ashland, OR, USA).

Statistical analysis

The data did not meet the assumptions of normality; therefore, non-parametric tests were used to perform statistical analysis. The data were initially analyzed using the Kruskal–Wallis test for comparison of multiple groups. Afterwards, the Mann-Whitney U-test and Wilcoxon signed-rank test were carried out to perform pairwise comparisons using Statistica 13.0 software (StatSoft, Dell, Aliso Viejo, CA, USA). Statistical significance was established at a p-value of less than 0.05. The synergy between an HCV protein and IFN- γ or IL-1 β in chemokine

induction was statistically tested by comparing the chemokine production level in response to combined stimulation, to the sum of the levels measured after treatment with the single stimuli.

Results

1) HCV proteins fail to induce a direct chemotactic response on peripheral blood mononuclear cells (PBMCs)

We evaluated whether HCV proteins can directly stimulate the migration of human PBMCs in the Boyden microchamber chemotaxis assay (figure 1). HCV proteins were found to lack PBMC chemotactic activity. This is evidenced by the low chemotactic indices (CI) observed at the highest tested concentrations of HCV proteins (the core protein at 10 µg/ml; CI 0.4 ± 0.2 , NS3 at 10 µg/ml; CI 1.2 ± 0.2 and E2 at 10 µg/ml; CI 0.4 ± 0.1). In contrast, CCL3 provoked pronounced chemoattraction of PBMCs at a concentration of 1 ng/ml (CI 4.5 ± 0.5).

2) Distinct chemokine expression in CD14⁺ monocytes induced by HCV proteins

Dolganiuc et al. have previously shown cytokine induction in monocytes by HCV proteins (Dolganiuc et al 2003). To confirm and directly compare the chemokine induction capacity of HCV proteins in CD14⁺ monocytes, we carried out induction experiments. We observed a relatively potent induction of CCL2 in response to the core protein (4.8 ± 1.9 ng/ml at 10 µg/ml) which is apparent when compared to CCL2 production in response to the bacterial product LPS (5.8 ± 2.2 ng/ml at 5 µg/ml). On the other hand, NS3 (0.8 ± 0.3 ng/ml at 10 µg/ml) did not induce considerable expression of CCL2 in monocytes (figure 2a). Further, we also measured CCL3 and CXCL8 expression in CD14⁺ monocytes in response to stimulation with HCV proteins. The core protein and NS3 induced significant expression of CCL3 (the core protein 10 µg/ml, p-value 0.03; NS3 10 µg/ml, p-value 0.03). Treatment with 10 µg/ml of the core protein resulted in the expression of 60.5 ± 22.0 ng/ml of CCL3. In contrast, NS3 was less potent; 10 µg/ml induced the expression of 0.3 ± 0.1 ng/ml of CCL3 (figure 2b). Furthermore, the core protein induced dose-dependent expression of CXCL8 in CD14⁺ monocytes (figure 2c). Treatment with 10 µg/ml of the core protein resulted in the expression of 153.5 ± 25.4

ng/ml of CXCL8. In contrast, NS3 was less potent; 10 µg/ml triggered the production of 11.4±5.9 ng/ml of CXCL8 (figure 2c).

3) Induction of CCL2 and CXCL8 by HCV proteins in fibroblasts and primary human liver sinusoidal microvascular endothelial cells (LSECs)

In addition to CD14⁺ monocytes, we analyzed CCL2 and CXCL8 expression in response to HCV proteins in fibroblasts and LSECs (figure 3). Compared to the potent chemokine inducers IL-1β, TNF-α, and dsRNA, the viral proteins were not powerful CXCL8 inducers in fibroblasts. However, at 5 µg/ml, glycoprotein E2 was found to significantly induce low quantities of CXCL8 (1.2±0.4 ng/ml; p-value 0.02) in fibroblasts (figure 3b). Similarly, the viral proteins were very weak inducers of CCL2 in fibroblasts. However, stimulation with the core protein resulted in a dose-dependent increase in CCL2 expression (figure 3a; Mann-Whitney U-test, p-value 0.09; Wilcoxon signed-rank test, p-value 0.04). In contrast, the core protein, although weaker than IL-1β, TNF-α and dsRNA, was a significant inducer of CCL2 (15.0±1.5 ng/ml; p-value 0.005) and CXCL8 (16.3±1.4 ng/ml; p-value 0.01) in LSECs at 25 µg/ml (figure 3c-d). Besides CXCL8 and CCL2, we measured the expression of the CXCR3 ligand CXCL10, but we observed no expression in fibroblasts or LSECs after treatment with the viral proteins (data not shown).

4) Synergy between HCV core protein and IFN-γ to produce CCL2, CXCL8 and CXCL10 in LSECs

The NS5a protein has been previously shown to synergize with a cytokine mixture (IL-1β, TNF-α and IFN-γ) to induce chemokine expression in the human Chang liver cell line (Apolinario et al 2005). To determine whether HCV proteins influence chemokine expression in response to IFN-γ, we carried out induction experiments with combinations of IFN-γ and HCV proteins (the core protein, NS3, and glycoprotein E2) on LSECs. Interestingly, a combination of the core protein with IFN- γ induced a dose-dependent enhancement of CCL2, CXCL8 and CXCL10

expression (figure 4). Being a principal inducer of CXCR3 ligands, IFN- γ alone stimulated significant expression of CXCL10 (7.0 ± 1.8 ng/ml at 200 ng/ml; p-value 0.005), but not of CCL2 or CXCL8 (figure 4). Remarkably, the production of CXCL10 increased more than 10-fold (up to 135.4 ± 52.0 ng/ml) when the core protein was combined with IFN- γ . For CXCL8, a production-level up to 33.0 ± 4.6 ng/ml was reached when 25 μ g/ml of the core protein was combined with 200 ng/ml of IFN- γ , compared to 16.3 ± 1.3 ng/ml of CXCL8 obtained following treatment with 25 μ g/ml of the core protein alone. The levels obtained for CCL2 with these stimuli were very comparable. NS3 and glycoprotein E2 did not influence the inductive capacity of IFN- γ in LSECs (data not shown).

5) Synergy between HCV core protein and IFN- γ to produce CCL2, CCL20, CXCL8 and CXCL10 in fibroblasts

The occurrence of synergy between IFN- γ and HCV proteins was also determined in fibroblasts (figure 5). In contrast to what was observed on LSECs, we did not detect the expression of CXCL10 in response to IFN- γ alone in fibroblasts. Nevertheless, we observed synergy between IFN- γ and the core protein in the expression of CXCL10 (approximately 10-fold upregulation). Furthermore, synergy was also exceedingly pronounced for induction of CCL2, CCL20 and CXCL8 in response to combinations of IFN- γ and the core protein. Nevertheless, the maximal production levels reached were lower than those observed in LSECs (figure 4). NS3 and glycoprotein E2 did not influence chemokine expression in response to IFN- γ in fibroblasts (data not shown).

6) Core protein and IL-1 β synergistically induce CCL20 expression in fibroblasts

To determine whether the HCV proteins influence chemokine expression in response to IL-1 β , we carried out induction experiments with combinations of IL-1 β and HCV proteins on fibroblasts and LSECs. A dose-dependent synergistic effect was observed between the core

protein and IL-1 β in the production of CCL20 by fibroblasts (figure 6b). For CCL20, statistical significance was reached, but for CXCL8, synergy was not obtained in all experiments. Rather an overall additive effect was observed. This is possibly due to the fact that IL-1 β is a strong CXCL8 inducer in fibroblasts (figure 6a). The production levels reached were higher when the core protein was combined with IL-1 β than with IFN- γ ; for CCL20 the maximal production level was 14.0 $\pm$ 0.1 ng/ml after stimulation with the core protein plus IL-1 β in fibroblasts, versus 0.3 $\pm$ 0.1 ng/ml after stimulation with the core protein plus IFN- γ in LSECs. On the contrary, no synergy was observed between the viral proteins and IL-1 β in LSECs. Furthermore, glycoprotein E2 and NS3 did not influence IL-1 β activity in fibroblasts and LSECs (data not shown).

7) HCV proteins utilize TLRs during chemokine induction

Previous studies have suggested that HCV proteins utilize TLRs for cytokine induction in various cell types (Dolganiuc et al 2004; Hosomura et al 2011; Rajalakshmy et al 2015; Swaminathan et al 2014). To determine whether TLR2 is activated by the core protein and NS3 in CD14⁺ monocytes, receptor neutralization was carried out using an anti-TLR2 antibody. Although not statistically significant, TLR2 neutralization decreased the CXCL8 inductive capacity of NS3 by 23% (217.9 $\pm$ 94.0 ng/ml vs. 165.0 $\pm$ 69.3 ng/ml; p-value 0.45) (figure 7a). On the other hand, TLR2 neutralization did not influence chemokine expression in response to the core protein. However, the core protein has also been described to utilize TLR4 (Uraki et al 2015). Therefore, LSECs were stimulated with either core protein alone or in combination with IFN- γ in the presence of the TLR4 antagonist TAK-242. TAK-242 dramatically inhibited the CXCL8-inducing capacity of the core protein (6.2 $\pm$ 0.6 ng/ml vs. 0.6 $\pm$ 0.1 ng/ml; p-value <0.0001). Furthermore, synergy with IFN- γ was completely inhibited following treatment with TAK-242 (97% inhibition; p-value 0.0002) (figure 7b).

8) HCV core protein does not regulate the expression of IFN- γ and IL-1 β receptors in fibroblasts

To reveal the molecular mechanism behind the synergy between the HCV core protein and IL-1 β or IFN- γ , we verified cytokine receptor expression, as Hosui et al. demonstrated upregulation of IFN- γ R2 on murine liver cells expressing HCV core protein (Hosui et al 2003). To determine whether synergy in chemokine induction (figure 5 and 6) between the core protein and IFN- γ or IL-1 β is in fact due to receptor modulation by the core protein, we assessed the expression levels of IL-1R1, IL-1R2, IFN- γ R1 and IFN- γ R2 on fibroblasts using flow cytometry. No significant upregulation of IL-1R1, IL-1R2, IFN- γ R1 and IFN- γ R2 was observed on fibroblasts following stimulation with 25 μ g/ml of the core protein (figure 8).

Discussion

In the present paper, we demonstrate varying chemokine induction profiles of HCV proteins on distinct cell types. Certainly, such distinct chemokine expression is to be anticipated amongst different cell types due to the variable expression of receptors for these viral proteins. Various receptors have been described to be utilized by HCV proteins. However, the majority of studies have attributed the cellular effects of most HCV proteins to TLR activation. The core protein has been reported to utilize TLR2 in addition to TLR1 and TLR6 as co-receptors (Chang et al 2007 and Dolganiuc et al 2004). To a lesser extent, the core protein has also been described to interact with TLR4 (Uraki et al 2015). In another study, the core protein was described as a globular C1q receptor ligand (Song et al 2016). Similarly, NS3 has been demonstrated to activate TLR2 and TLR4 (Hosomura et al 2011; Rajalakshmy et al 2015). In line with this, we observed a reduced chemokine inductive capacity for the core protein in LSECs in the presence of a TLR4 antagonist. Similarly, the synergy between the core protein and IFN- γ for chemokine induction in LSECs was drastically diminished following TLR4 antagonization. These data suggest that the core protein and IFN- γ activate their individual receptors leading to amplified downstream signalling which is translated into enhanced chemokine expression. Chemokine induction by HCV core protein in CD14⁺ monocytes was independent of TLR2, in contrast the CXCL8 production in response to NS3 stimulation was somewhat reduced in the presence of a neutralizing anti-TLR2 antibody.

In this study, we investigated the cytokine induction spectrum of three different HCV proteins. One could question the relevance of these recombinantly expressed viral proteins as inducers. However, as an envelope protein, E2 is certainly visible to extracellular sensors (Dubuisson 2007). In addition, circulating core protein has been detected in serum from patients and has been used in several studies to investigate stimulation of cytokine

production by HCV (Cividini et al 2003; Dolganiuc et al 2003, 2004; McLauchlan 2000; Tanaka et al 1996). Finally, HCV has been demonstrated to induce a specific form of programmed cell death, namely, pyroptosis, which is an inflammatory form of cell death whereby the infected cell lyses and releases its contents into the extracellular environment (Kofahi et al 2016). Therefore, pyroptosis leads to the extracellular occurrence of otherwise intracellular HCV proteins.

We observed differential expression of CCL2 and CXCL8 in CD14⁺ monocytes, fibroblasts, and LSECs in response to the HCV core protein, glycoprotein E2, and NS3. On the other hand, we did not observe expression of CCL20 and CXCL10 after stimulation with one of these viral proteins as a single stimulus. CXCL8 is probably the most frequently reported chemokine to be expressed in response to HCV proteins. Glycoprotein E2 was found to induce the expression of CXCL8 in primary human thyroid cells and human umbilical vein endothelial cells (Hammerstad et al. 2016; Urbaczek et al 2014). In addition, NS5a induces the expression of CXCL8 in monocyte-derived DCs (Wertheimer et al 2007). In contrast to CXCL8, CCL2 expression in response to HCV proteins has only been reported in human hepatic stellate cells (HSCs) (Bataller et al 2004). Previous studies have documented the expression of additional chemokines in response to HCV proteins. Expression of CCL5 mRNA in the Chang liver cell line was shown to occur in response to constitutively expressed HCV core protein (Ruggieri et al 2007). Brenndörfer et al. demonstrated an increase in hepatic expression of murine CCL17 and CCL22 in response to the transgenic expression of the HCV protease complex NS3/4A (Brenndörfer et al 2012).

Not only do HCV products directly induce the expression of chemokines, but they also possess the capacity to synergize with cytokines to enhance said chemokine expression. Helbig et al. documented an increase in CXCL11 mRNA expression under IFN- γ plus TNF- α stimulation in

HCV-infected Huh-7 cells. Further experiments revealed that synthetic dsRNA mediated a synergistic effect with IFN- γ and TNF- α by enhancing signaling via the RIG-I/IRF3 pathway (Helbig et al 2009). Apolinario et al. previously showed that the core protein and NS5a could synergize with a cytokine mixture, consisting of IL-1 β , IFN- γ and TNF- α , to enhance the expression of T-cell specific chemokines in Chang liver cells (Apolinario et al 2005). We demonstrated, for the first time, a several-fold increase in chemokine expression in response to IFN- γ , in the presence of the core protein, in fibroblasts and LSECs. It is worth noting the magnitude of this increment, particularly in the case of LSECs. In those cells, we observed a 10-, 13- and 19-fold increase in CCL2, CXCL8 and CXCL10 expression, respectively. Although not to the same intensity as that seen with IFN- γ , we detected direct synergy between the core protein and IL-1 β in the induction of CCL20 expression in fibroblasts.

The observed synergy promotes speculation on how the virus may benefit from such a drastic increase in chemokine expression. A plausible explanation is that the virus may have evolved to prolong its host's survival by reducing its own pathogenicity, thus ensuring its host remains in a healthy condition for a longer duration of time. Initially, the presence of these chemokines would serve to be beneficial, as it would contribute to viral elimination via the recruitment of various leukocytes. However, persistent exposure to certain chemokines may result in hepatic injury. A hallmark of chronic HCV infection is the inflammatory damage that is inflicted upon the liver, thus leading to the development of fibrosis and cirrhosis. Of note, the concentrations we measured in the culture supernatants are biologically relevant. Indeed, the minimal effective concentration of these chemokines (CCL2, CCL3, CCL20, CXCL8 and CXCL10) in chemotaxis assays falls within the range of 1-10 ng/ml (Van Damme et al 1992; Van Damme et al 1989; Proost et al 2001; Schutyser et al 2003).

The occurrence of synergy between HCV proteins and cytokines *in vivo* and the implications it would bring about on disease progression is intriguing. LSECs line the hepatic sinusoidal space and are thus within close proximity of hepatocytes and HSCs. As a result, upregulated chemokine expression is to be expected within the hepatic environment. Upon hepatic injury, HSCs are activated and differentiate into proliferative myofibroblasts that drive fibrosis (Tacke et al 2012). CCL2 contributes to the activation of HSCs through multiple pathways. CCL2 brings about a fibrotic state by inducing angiogenesis in the portal veins. This angiogenic effect is moderated via CCL2-mediated macrophage infiltration (Ehling et al 2014). Subsets of murine macrophages have been defined and fall within at least two categories, LY6C^{hi} and LY6C^{low}, which display opposing effects in terms of their fibrogenic capacities. The fibrogenic subset, LY6C^{hi} macrophages, activates HSCs via the expression of various cytokines including IL-1 β , CCL2, CCL3, CCL5 and platelet-derived growth factor (Tsuchida et al 2017). Furthermore, the role of the CCL2 receptor, CCR2, has been established in the process of fibrosis. Murine models of hepatic inflammation demonstrated the upregulation of CCR2 in HSCs. Furthermore, upregulation of CCR2 corresponded to several fibrogenic findings in HSCs such as enhanced migration and upregulation of fibrogenic mediators such as reactive oxygen species (Seki et al 2009). CXCL8 is another chemokine found to promote fibrosis. Upregulated CXCL8 is observed in patients with chronic liver disease and correlates with activated HSCs and enhanced expression of α -smooth muscle actin (Clément et al 2010; Zimmermann et al 2011). Furthermore, NK cells have been described to relay an anti-fibrotic effect via induced apoptosis of HSCs. Hintermann *et al.* revealed an inhibitory role of CXCL10 on the anti-fibrotic effect of NK cells (Hintermann et al 2010). CCL20, whose expression is upregulated in other viral infections, was recently found to display fibrogenic effects. Affò et al. demonstrated a chemoattractant effect of HSCs towards CCL20 *via* ERK signaling. Furthermore, silencing of

CCL20 resulted in the downregulation of LPS-induced liver damage thus suggesting that CCL20 may play a role in hepatic fibrosis (Affò et al 2014). The presence of chemokines, such as CCL2 and CCL3, in the hepatic vicinity would induce the recruitment of monocytes that would further escalate the inflammatory response via their own chemokine expression, thus creating an amplification feedback loop. The potential pathogenic activities of chemokines in HCV-induced liver disease are summarized in figure 9. Taken together, our data suggest that LSECs might promote HCV-induced hepatic injury via the production of chemokines that recruit leukocytes and activate HSCs in both a direct and indirect manner.

Figure 1: Chemotactic response of PBMCs to HCV proteins. Varying concentrations of HCV core protein (0.4-10 µg/ml), nonstructural 3 (NS3) protein (0.4-10 µg/ml), glycoprotein E2 (0.4-10 µg/ml) and CCL3 (1 ng/ml) were added to the lower compartment of a Boyden microchamber. Freshly isolated PBMCs were added to the upper compartment and allowed to migrate for 2 h. The chemotactic response is shown as the mean CI + SEM. Results are derived from three independent experiments.

Figure 2: Expression of (a) CCL2, (b) CCL3 and (c) CXCL8 by CD14⁺ monocytes in response to HCV proteins. Freshly isolated CD14⁺ monocytes were induced with lipopolysaccharide (LPS) (5 µg/ml) or varying concentrations of the core protein (0.4-10 µg/ml) and nonstructural 3 (NS3) protein (0.4-10 µg/ml). Following an incubation period of 24 h, cell supernatants were collected and (a) CCL2 (b) CCL3 and (c) CXCL8 expression was determined via specific ELISAs. Results are represented as the mean chemokine expression + SEM and are derived from four independent experiments. Significant upregulation in comparison to control is indicated by asterisks (Mann-Whitney U-test; *p-value<0.05).

Figure 3: Expression of CCL2 and CXCL8 by fibroblasts and LSECs in response to HCV proteins. (a-b) Fibroblasts or (c-d) LSECs were induced with interleukin-1 β (IL-1 β) (10 ng/ml), double-stranded RNA (dsRNA) (100 μ g/ml), tumor necrosis factor- α (TNF- α) (100 ng/ml) or varying concentrations of the core protein (1-25 μ g/ml), nonstructural 3 (NS3) protein (1-25 μ g/ml) and glycoprotein E2 (0.2-5 μ g/ml). Following an incubation period of 72 h, cell supernatants were collected and (a and c) CCL2 and (b and d) CXCL8 expression was determined via specific ELISAs. Results are represented as the mean chemokine expression + SEM and are derived from six independent experiments. Significant upregulation in comparison to control is indicated by asterisks (Mann-Whitney U-test; *p-value<0.05, **p-value<0.01).

Figure 4: Synergy between the core protein and IFN- γ in chemokine expression in LSECs. LSECs were induced with combinations of varying concentrations of interferon- γ (IFN- γ) (20-200 ng/ml) and core protein (1-25 μ g/ml). Following an incubation period of 72 h, cell supernatants were collected and (a) CCL2, (b) CXCL8 and (c) CXCL10 expression was determined via specific ELISAs. Results are represented as the mean chemokine expression level + SEM and are derived from six independent experiments. Significant upregulation in comparison to control is indicated by asterisks (Mann-Whitney U-test; *p-value<0.05, **p-value <0.01). Significant synergy between the combination of IFN- γ and the core protein in comparison to the sum of single inducers is indicated by daggers (Mann-Whitney U-test; †p-value<0.05, ††p-value<0.01).

Figure 5: Synergy between the core protein and IFN-γ in chemokine expression in fibroblasts. Fibroblasts were induced with combinations of varying concentrations of interferon-γ (IFN-γ) (20-200 ng/ml) and core protein (1-25 μg/ml). Following an incubation period of 72 h, cell supernatants were collected and (a) CCL2, (b) CCL20, (c) CXCL8 and (d) CXCL10 expression was determined via specific ELISAs. Results are represented as the mean chemokine expression level + SEM and are derived from six independent experiments. Significant upregulation in comparison to control is indicated by a dollar sign (Wilcoxon signed-rank test; $^{\$}p$ -value<0.05). Significant synergy between the combination of IFN-γ and the core protein in comparison to the sum of single inducers is indicated by a dagger (Mann-Whitney U-test; $^{\dagger}p$ -value<0.05).

Figure 6: Core protein and IL-1β synergistically induce CCL20 expression in fibroblasts. Fibroblasts were induced with combinations of varying concentrations of interleukin-1β (IL-1β) (0.1-1 ng/ml) and core protein (5-25 μg/ml). Following an incubation period of 72 h, cell supernatants were collected. (a) CXCL8 and (b) CCL20 expression was determined via specific ELISAs. Results are represented as the mean chemokine expression level + SEM and are

derived from seven independent experiments. Significant upregulation in comparison to control is indicated by asterisks (Mann-Whitney U-test; *p-value<0.05, **p-value<0.01). Significant synergy between the combination of IL-1 β and the core protein in comparison to the sum of single inducers is indicated by a dagger (Mann-Whitney U-test; † p-value<0.05).

Figure 7: The effect of TLR neutralisation on the chemokine-inducing capacity of NS3 and HCV core protein. (a) CD14 $^{+}$ monocytes were incubated with peptidoglycan (PGN; 50 ng/ml), core protein (2 μ g/ml) and nonstructural 3 (NS3) protein (10 μ g/ml) with or without pre-incubation with an anti-TLR2 antibody (5 μ g/ml) or an IgG isotype control antibody (5 μ g/ml). Following an incubation period of 24 h, cell supernatants were collected and CXCL8 expression was determined via a specific ELISA. Results are represented as the mean chemokine expression level + SEM and are derived from four independent experiments. (b) LSECs were incubated with LPS (50 ng/ml), core protein (25 μ g/ml), IFN- γ (200 ng/ml) or a combination of core protein and IFN- γ with or without pre-incubation with the TLR4 antagonist TAK-242 (20 μ g/ml) or 0.005% DMSO (TAK-242 vehicle). Following an incubation period of 48 h, cell supernatants were collected and CXCL8 expression was determined via a specific ELISA. Results are represented as the mean chemokine expression level + SEM (n=5-9). (a and b) Significant upregulation in comparison to control is indicated by an asterisk (Mann-Whitney U-test; *p-value<0.05). Significant synergy between the combination of IFN- γ and the core protein in comparison to the sum of single inducers is indicated by a dagger (Mann-Whitney U-test; † p-value<0.05).

Significant reduction following treatment with anti-TLR2 antibody or TAK-242 is indicated by a dollar sign (Mann-Whitney U-test; $^{\$}p$ -value<0.05).

Figure 8: The effect of HCV core protein on the expression of IFN- γ and IL-1 β receptors on fibroblasts. Fibroblasts were treated with 25 μ g/ml of core protein or left untreated. Following an incubation period of 72 h, the expression of (a) IFN- γ R1, (b) IFN- γ R2, (c) IL-1R1 and (d) IL-1R2 was determined using flow cytometry. One representative experiment out of four independent experiments is shown. White histograms: unstained control; grey histograms: stained control; black histograms: stained cells treated with 25 μ g/ml of core protein.

Figure 9: An overview of the inflammatory damage inflicted by expressed chemokines.

Acknowledgments

This work was supported by the Research Foundation of Flanders (FWO-Vlaanderen) and C1 funding [C1 Project number C16/17/010] of the KU Leuven. MG is a research expert funded by the Rega foundation. MDB is a postdoctoral research fellow of the FWO-Vlaanderen.

Author disclosure statement

No competing financial interests exist.

References

- Affò S, Morales-Ibanez O, Rodrigo-Torres D, Altamirano J, Blaya D, Dapito DH, Millán C, Coll M, Caviglia JM, Arroyo V, Caballería J, Schwabe RF, Ginès P, Bataller R, Sancho-Bru P. 2014. CCL20 mediates lipopolysaccharide induced liver injury and is a potential driver of inflammation and fibrosis in alcoholic hepatitis. *Gut* 63(11): 1782–1792.
- Ansaldi F, Orsi A, Sticchi L, Bruzzone B, Icardi G. 2014. Hepatitis C virus in the new era: perspectives in epidemiology, prevention, diagnostics and predictors of response to therapy. *World J Gastroenterol* 20(29): 9633–9652.
- Apolinario A, Majano PL, Lorente R, Nunez O, Clemente G, Garcia-Monzon CI. 2005. Gene Expression profile of T-cell-specific chemokines in human hepatocyte-derived cells: evidence for a synergistic inducer effect of cytokines and hepatitis C virus proteins. *J Viral Hepat* 12(1): 27–37.
- Bataller Ramó, Paik YH, Lindquist JN, Lemasters JJ, Brenner DA. 2004. Hepatitis C virus core and nonstructural proteins induce fibrogenic effects in hepatic stellate cells. *Gastroenterol* 126(2): 529–540.
- Brass A, Brenndörfer ED. 2014. The role of chemokines in hepatitis C Virus-mediated liver disease. *Int J Mol Sci* 15(3): 4747–4779.
- Brenndörfer ED, Brass A, Söderholm J, Frelin L, Aleman S, Georg Bode J, Sällberg M. 2012. Hepatitis C virus non-structural 3/4A protein interferes with intrahepatic interferon- γ production. *Gut* 61(4): 589–596.
- Chang S, Dolganiuc A, Szabo G. 2007. Toll-like receptors 1 and 6 are involved in TLR2-mediated macrophage activation by hepatitis C virus core and NS3 proteins. *J Leukocyte*

Biol 82(3): 479–487.

Chen Z, Zhu Y, Ren Y, Tong Y, Hua X, Zhu F, Huang L, Liu Y, Luo Y, Lu W, Zhao P, Qi Z. 2011.

Hepatitis C virus protects human B lymphocytes from fas-mediated apoptosis via E2-CD81 engagement. PLoS ONE 6(4): e18933.

Cividini A, Cerino A, Muzzi A, Furione M, Rebucci C, Segagni L, Gatti M, Barnaba V, Mondelli

MU. 2003. Kinetics and significance of serum hepatitis C virus core antigen in patients with acute hepatitis C. J Clin Microbiol 41(5): 2144–2146.

Clément S, Pascarella S, Conzelmann S, Gonelle-Gispert C, Guilloux K, Negro F. 2010. The

hepatitis C virus core protein indirectly induces alpha-smooth muscle actin expression in hepatic stellate cells via interleukin-8. J Hepatol 52(5): 635–643.

De Buck M, Gouwy M, Proost P, Struyf S, Van Damme J. 2013. Identification and

characterization of MIP-1 α /CCL3 isoform 2 from bovine serum as a potent monocyte/dendritic cell chemoattractant. Biochem Pharmacol 85(6): 789–97.

Dolganiuc A, Kodys K, Kopasz A, Marshall C, Do T, Romics L, Mandrekar P, Zapp M, Szabo G.

2003. Hepatitis C virus core and nonstructural protein 3 proteins induce pro- and anti-inflammatory cytokines and inhibit dendritic cell differentiation. J Immunol 170(11): 5615–24.

Dolganiuc A, Oak S, Kodys K, Golenbock DT, Finberg RW, Kurt-Jones E, Szabo G. 2004.

Hepatitis C core and nonstructural 3 proteins trigger toll-like receptor 2-mediated pathways and inflammatory activation. Gastroenterol 127(5): 1513–1524.

Dubuisson J. 2007. Hepatitis C virus proteins. World J Gastroenterol 13(17): 2406–2415.

Ehling J, Bartneck M, Wei X, Gremse F, Fech V, Möckel D, Baeck C, Hittatiya K, Eulberg D,

- Luedde T, Kiessling F, Trautwein C, Lammers T, Tacke F. 2014. CCL2-dependent infiltrating macrophages promote angiogenesis in progressive liver fibrosis. *Gut* 63(12): 1960–1971.
- Ferreira AR, Magalhães AC, Camões F, Gouveia A, Vieira M, Kagan JC, Ribeiro D. 2016. Hepatitis C virus NS3-4A inhibits the peroxisomal MAVS-dependent antiviral signalling response. *J Cell Mol Med* 20(4): 750–757.
- Groom JR, Luster AD. 2011. CXCR3 ligands: redundant, collaborative and antagonistic functions. *Immunol Cell Biol* 89(2): 207–215.
- Hammerstad SS, Stefan M, Blackard J, Owen RP, Lee HJ, Concepcion E, Yi Z, Zhang W, Tomer Y. 2016. Hepatitis C virus E2 protein induces upregulation of IL-8 pathways and production of heat shock proteins in human thyroid cells. *J Clin Endocrinol Met* 102(2): jc.2016-3403.
- Helbig KJ, Ruszkiewicz A, Lanford RE, Berzsenyi MD, Harley HA, McColl SR, Beard MR. 2009. Differential expression of the CXCR3 ligands in chronic hepatitis C virus (HCV) infection and their modulation by HCV in vitro. *J Virol* 83(2): 836–846.
- Hintermann E, Bayer M, Pfeilschifter JM, Luster AD, Christen U. 2010. CXCL10 promotes liver fibrosis by prevention of NK cell mediated hepatic stellate cell inactivation. *J Autoimmun* 35(4): 424–435.
- Hosomura N, Kono H, Tsuchiya M, Ishii K, Ogiku M, Matsuda M, Fujii H. 2011. HCV-related proteins activate kupffer cells isolated from human liver tissues. *Dig Dis Sci* 56(4): 1057–1064.
- Hosui A, Ohkawa K, Ishida H, Sato A, Nakanishi F, Ueda K, Takehara T, Kasahara A, Sasaki Y,

- Hori M, Hayashi N. 2003. Hepatitis C virus core protein differently regulates the JAK-STAT Signaling pathway under interleukin-6 and interferon- γ stimuli. *J Biol Chem* 278(31): 28562–28571.
- Kofahi HM, Taylor NGA, Hirasawa K, Grant MD, Russell RS. 2016. Hepatitis C virus infection of cultured human hepatoma cells causes apoptosis and pyroptosis in both infected and bystander cells. *Sci Rep* 6: 37433.
- Li K, Foy E, Ferreon JC, Nakamura M, Ferreon ACM, Ikeda M, Ray SC, Gale M, Lemon SM. 2005. Immune evasion by hepatitis C virus NS3/4A protease-mediated cleavage of the toll-like receptor 3 adaptor protein TRIF. *Proc Natl Acad Sci USA* 102(8): 2992–2997.
- Li Z, Chen ZW, Li Hu, Ren H, Hu P. 2017. Prevalence of hepatitis C virus-resistant association substitutions to direct-acting antiviral agents in treatment-naïve hepatitis C genotype 1b-infected patients in Western China. *Infect Drug Resist* 10: 377–392.
- Luo H, Liu Y, Li Q, Liao L, Sun R, Liu X, Jiang M, Hu J. 2015. A20 regulates IL-1-induced tolerant production of CXC chemokines in human mesangial cells via inhibition of MAPK signaling. *Sci Rep* 5: 18007.
- McLauchlan J. 2000. Properties of the hepatitis C Virus core protein: a structural protein that modulates cellular processes. *J Viral Hep* 7(1): 2–14.
- Omar H, El Akel W, Elbaz T, El Kassas M, Elsaeed K, El Shazly H, Said M, Yousif M, Gomaa AA, Nasr A, AbdAllah M, Korany M, Ismail SA, Shaker MK, Doss W, Esmat G, Waked I, El Shazly Y. 2018. Generic daclatasvir plus sofosbuvir, with or without ribavirin, in treatment of chronic hepatitis C: real-world results from 18 378 patients in Egypt. *Aliment Pharmacol Ther* 47(3): 421–431.

- Proost P, Schutyser E, Menten P, Struyf S, Wuyts A, Opdenakker G, Detheux M, Parmentier M, Durinx C, Lambeir AM, Neyts J, Liekens S, Maudgal PC, Billiau A, Van Damme J. 2001. Amino-terminal truncation of CXCR3 agonists impairs receptor signaling and lymphocyte chemotaxis, while preserving antiangiogenic properties. *Blood* 98(13): 3554–3561.
- Proost P, Struyf S, Loos T, Gouwy M, Schutyser E, Conings R, Ronsse I, Parmentier M, Grillet B, Opdenakker G, Balzarini J, Van Damme J. 2006. Coexpression and interaction of CXCL10 and CD26 in mesenchymal cells by synergising inflammatory cytokines: CXCL8 and CXCL10 are discriminative markers for autoimmune arthropathies. *Arthritis Res Ther* 8(4).
- Rajalakshmy AR, Malathi J, Madhavan HN. 2015. Hepatitis C virus NS3 mediated microglial inflammation via TLR2/TLR6 MyD88/NF- κ B pathway and toll like receptor ligand treatment furnished immune tolerance. *PLoS ONE* 10(5): e0125419.
- Ranasinghe R, Eri R. 2018. Pleiotropic immune functions of chemokine receptor 6 in health and disease. *Medicines* 5(3): 69.
- Ruggieri A, Franco M, Gatto I, Kumar A, Rapicetta M. 2007. Modulation of RANTES expression by HCV core protein in liver derived cell lines. *BMC Gastroenterol* 7: 21.
- Schutyser E, Struyf S, Menten P, Lenaerts JP, Conings R, Put W, Wuyts A, Proost P, Van Damme J. 2000. Regulated production and molecular diversity of human liver and activation-regulated chemokine/macrophage inflammatory protein-3 α from normal and transformed cells. *J Immunol* 165(8): 4470–4477.
- Schutyser E, Struyf S, Van Damme J. 2003. The CC chemokine CCL20 and Its receptor CCR6. *Cytokine Growth Factor Rev* 14(5): 409–426.

Seki E, de Minicis S, Inokuchi S, Taura K, Miyai K, van Rooijen N, Schwabe RF, Brenner DA.

2009. CCR2 promotes hepatic fibrosis in mice. *Hepatology* 50(1): 185–197.

Sheng WS, Hu S, Ni HT, Rowen TN, Lokensgard JR, Peterson PK. 2005. TNF-alpha-induced chemokine production and apoptosis in human neural precursor cells. *J Leukocyte Biol* 78(6): 1233–1241.

Song X, Yao Z, Yang J, Zhang Z, Deng Y, Li M, Ma C, Yang L, Gao X, Li W, Liu J, Wei L. 2016. HCV Core protein binds to gC1qR to induce A20 expression and inhibit cytokine production through MAPKs and NF-κB signaling pathways. *Oncotarget* 7(23): 33796–33808.

Stone AEL, Mitchell A, Brownell J, Miklin DJ, Golden-Mason L, Polyak SJ, Gale MJ, Rosen HR. 2014. Hepatitis C virus core protein inhibits interferon production by a human plasmacytoid dendritic cell line and dysregulates interferon regulatory factor-7 and signal transducer and activator of transcription (STAT) 1 protein expression. *PLoS ONE* 9(5): e95627.

Swaminathan G, Pascual D, Rival G, Perales-Linares R, Martin-Garcia J, Navas-Martin S. 2014. Hepatitis C virus core protein enhances HIV-1 replication in human macrophages through TLR2, JNK, and MEK1/2-dependent upregulation of TNF-α and IL-6. *FEBS letters* 588(18): 3501–3510.

Tacke F, Weiskirchen R. 2012. Update on hepatic stellate cells: pathogenic role in liver fibrosis and novel isolation techniques. *Expert Rev Gastroenterol* 6(1): 67–80.

Tanaka E, Kiyosawa K, Matsumoto A, Kashiwakuma T, Hasegawa A, Mori H, Yanagihara O, Ohta Y. 1996. Serum levels of hepatitis C virus core protein in patients with chronic hepatitis C treated with interferon alfa. *Hepatology* 23(6): 1330–1333.

- Tourniaire F, Romier-Crouzet B, Lee JH, Marcotorchino J, Gouranton E, Salles J, Malezet C, Astier J, Darmon P, Blouin E, Walrand S, Ye J, Landrier JF. 2013. Chemokine expression in inflamed adipose tissue is mainly mediated by NF- κ B. *PLoS ONE* 8(6): e66515.
- Tsuchida T, Friedman SL. 2017. Mechanisms of hepatic stellate cell activation. *Nat Rev Gastroenterol* 14(7): 397–411.
- Uraki S, Tameda M, Sugimoto K, Shiraki K, Takei Y, Nobori T, Ito M. 2015. Substitution in amino acid 70 of hepatitis C virus core protein changes the adipokine profile via toll-like receptor 2/4 signaling. *PloS ONE* 10(6): e0131346.
- Urbaczek AC, Ribeiro L, Ximenes VF, Afonso A, Nogueira CT, Generoso WC, Alberice JV, Rudnicki M, Ferrer R, Fonseca L, Costa P. 2014. Inflammatory response of endothelial cells to hepatitis C virus recombinant envelope glycoprotein 2 protein exposure. *Mem Inst Oswaldo Cruz* 109(6): 748–756.
- Van Damme J, Proost P, Lenaerts JP, Opdenakker G. 1992. Structural and functional identification of two human, tumor-derived monocyte chemotactic proteins (MCP-2 and MCP-3) belonging to the chemokine family. *J Exp Med* 176(1): 59–65.
- Van Damme J, Decock B, Conings R, Lenaerts JP, Opdenakker G, Billiau A. 1989. The chemotactic activity for granulocytes produced by virally infected fibroblasts is identical to monocyte-derived interleukin 8. *Eur J Immunol* 19(7): 1189–94.
- Wertheimer AM, Polyak SJ, Leistikow R, Rosen HR. 2007. Engulfment of apoptotic cells expressing HCV proteins leads to differential chemokine expression and STAT signaling in human dendritic cells. *Hepatology* 45(6): 1422–1432.
- Zimmermann HW, Seidler S, Gassler N, Nattermann J, Luedde T, Trautwein C, Tacke F. 2011.

Interleukin-8 is activated in patients with chronic liver diseases and associated with hepatic macrophage accumulation in human liver fibrosis. PLoS ONE 6(6): e21381.