

# Chapter 9

## Hepatitis C Virus (HCV)-Induced Inflammation: The Role of Cross-Talk Between HCV-Infected Hepatocytes and Stellate Cells

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**Abstract** Hepatitis C virus (HCV) is a major public health problem, as 170 million people worldwide are currently chronically infected with the virus. HCV infection leads to chronic inflammation, which is the initial step toward fibrosis and is a significant risk factor for developing hepatocellular carcinoma. HCV-induced liver inflammation involves several events, such as modification of cytokine and chemokine pathways, oxidative stress, and induction of steatosis. Recent studies have revealed that not only HCV-infected hepatocytes but also neighboring cells, such as lymphocytes, Kupffer cells and hepatic stellate cells (HSCs), play important roles in HCV-induced inflammation. In the current study, we found evidence of cross-talk between HCV-infected hepatocytes and HSCs, revealed by the production of cytokines and chemokines. Upon co-culture of HSCs with HCV-infected hepatocytes in vitro, HSCs stimulated HCV-infected hepatocytes to produce pro-inflammatory cytokines and chemokines, including interleukin (IL)-6, IL-8, macrophage inflammatory protein (MIP)-1 $\alpha$  and MIP-1 $\beta$ . This cross-talk is likely to be a key feature of inflammatory diseases caused by HCV infection.

**Keywords** HCV • Hepatic stellate cell • Inflammation

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## 9.1 Introduction

Many cancers arise from sites of infection and chronic inflammation. In 1863, Rudolf Virchow first suggested that the origin of cancer was at sites of chronic inflammation and tissue injury (Balkwill and Mantovani 2001). A role for inflammation in tumorigenesis is generally accepted, and it is evident that an inflammatory microenvironment is an essential component of all tumors (Mantovani et al. 2008).

Cytokines and chemokines are the most important players in inflammation and cancer, as they can either promote or inhibit tumor development. Cancer cell growth and survival are regulated by tumor necrosis factor (TNF)- $\alpha$ , transforming growth factor (TGF)- $\beta$ , and interleukin (IL)-6 (Becker et al. 2005; Jing et al. 2011), and tumor progression is promoted by IL-6, IL-17, and IL-23 (Wang et al. 2009b). By contrast, IL-12, IL-21, and interferon (IFN)- $\gamma$  are anti-tumor cytokines (Weiss et al. 2007).

Hepatitis C virus (HCV) chronically infects approximately 170 million persons worldwide, causing liver fibrosis, which can evolve into cirrhosis and hepatocellular carcinoma (HCC). During development of fibrosis, hepatic inflammation appears to be the major source of pathology. Fibrosis causes an imbalance in extracellular matrix (ECM) production and degradation (Hernandez-Gea and Friedman 2011). Activated hepatic stellate cells (HSCs) are a major source of ECM production. Following liver injury, such as that caused by HCV infection, quiescent HSCs become activated and convert into highly proliferative myofibroblast-like cells, which express the inflammatory and fibrogenic mediators that are responsible for ECM accumulation within the microenvironment (Hernandez-Gea and Friedman 2011).

However, many of the molecular mechanisms underlying the relationship between HCV infection and HSCs remain unclear. Understanding the mechanisms of HCV-related inflammation and development of disease is important for prediction of disease progression and development of new therapeutic approaches.

## 9.2 Hepatitis C Virus Infection Triggers Liver Inflammation

Chemokines and inflammatory cytokines are key regulators of immunity and inflammation during HCV infection, and HCV infection is frequently associated with chronic liver inflammation. Intrahepatic levels of chemokines and cytokines are elevated in HCV infection (Wald et al. 2007; Zeremski 2007). Increased chemokine and cytokine production reflects the development of HCV-related disease, which may include fibrosis, HCC, and hepatitis C-associated cirrhosis. However, the pathogenesis of HCV-related disease is only partially understood. In particular, the HCV core and some non-structural proteins have been demonstrated to be potent inducers of inflammation *in vitro*.

The HCV core has been shown to bind to the endoplasmic reticulum and membranes of lipid vesicles (Chang et al. 2007a, 2008; Miyanari et al. 2007). Such

binding may modulate gene transcription, cell proliferation, and cell death and may be involved in the pathogenesis of HCC (Chou et al. 2005; Tanaka et al. 2008).

In *in vitro* models, ectopic expression of the HCV core in a human hepatoma cell line induces the expression of the transcription factor nuclear factor (NF)- $\kappa$ B, which plays a central role in the inflammatory response to HCV in the liver (Kim et al. 2001; Marusawa et al. 1999; Yoshida et al. 2001). Dendritic cells (DCs) expressing the HCV core protein are induced to produce several inflammatory cytokines, such as IFN- $\gamma$ , TNF- $\alpha$ , IL-6, IL-2, and IL-12 (Li et al. 2006).

Furthermore, the extracellular HCV core, which is detectable in the blood of HCV-infected patients (Kanto et al. 1995; Sabile et al. 1999), activates p38, extracellular signal-regulated protein kinase (ERK), c-Jun N-terminal kinase (JNK), activator protein (AP)-1, and NF- $\kappa$ B in monocytes and macrophages via activation of the Toll-like receptor (TLR) 2 pathway (Chang et al. 2007b; Dolganiuc et al. 2003, 2004). Activation of these molecules triggers production of pro- and anti-inflammatory cytokines, such as TNF- $\alpha$  and IL-10, by monocytes and macrophages (Dolganiuc et al. 2004). Increased IL-10 inhibits the production of IL-12 in human macrophages and DCs (Dolganiuc et al. 2003). DCs exposed to the extracellular HCV core prevent the generation of CD4<sup>+</sup> T helper (Th) 1 responses (Waggoner et al. 2007). Modulation of immune cell function by the HCV extracellular core involves the activation of signal transducer and activator of transcription 3 (STAT3) in monocytes, macrophages, and DCs (Tacke et al. 2011).

Moriya et al. generated transgenic mice expressing the HCV core protein. These mice developed hepatic steatosis, one of the characteristic histopathological features of chronic hepatitis C, and HCC (Moriya et al. 1998, 2001). Although these transgenic mice did not exhibit inflammation, chronic HCV infection in patients can progress to HCC through inflammation. Thus, the transgenic mouse model may not reflect true clinical progression. To overcome this problem, the HCV core was expressed in mice using the tetracycline system (Chang et al. 2009). These mice expressed intermediate levels (slightly higher than physiological levels) of the HCV core in the liver, but these levels were sufficient to induce not only steatosis but also inflammation and fibrosis.

Clinical and experimental evidence suggests that oxidative stress plays a role in HCV-induced liver disease (Lai 2002; Rockey 2000). Patients with chronic HCV infection produce TNF- $\alpha$  (Larrea et al. 1996; Mühlbauer et al. 2003), a cytokine that can generate oxidative stress by stimulating the release of reactive oxygen species (ROS) (Kizaki et al. 1993). The mitochondrial localization of the HCV core contributes to oxidative stress, and expression of the HCV core stimulates mitochondrial ROS production (Li et al. 2007; Otani et al. 2005). Transgenic mice expressing the HCV core develop oxidative stress and liver steatosis (Korenaga et al. 2005; Okuda et al. 2002). Moreover, products from oxidative stress are found in the serum of patients with HCV-induced chronic liver disease, and antioxidants have some beneficial effects in these patients (Idéo et al. 1999; Vendemiale et al. 2001).

The HCV NS3 protein protease triggers an inflammatory reaction mediated by the TLR pathway. Similar to the effects of the HCV core protein, the recombinant NS3 protein also induces the TLR2-mediated production of inflammatory cytokines,

such as IL-6, IL-8 and TNF- $\alpha$ , in monocytes and macrophages (Chang et al. 2007b; Dolganiuc et al. 2003, 2004). Moreover, the levels of TNF- $\alpha$  and IL-1 $\beta$  are markedly increased in Kupffer cells when the cells are stimulated with recombinant NS3 protein (Hosomura et al. 2011). This increase is inhibited by a TLR4 antibody. In contrast, a recent report has indicated that the NS3 protein directly induces TGF- $\beta$ 1 and collagen expression in HSCs by binding to the TGF- $\beta$  type I receptor. This activity contributes to the development of liver fibrosis (Sakata et al. 2013).

Accumulating evidence indicates that the HCV NS5A protein may play an important role in the pathological changes that occur in the liver. NS5A induces TLR4 expression in the liver, and TLR4 expression has been observed in livers of NS5A-TG mice and HCV patients (Machida et al. 2009). NS5A-induced TLR4 expression aggravates alcoholic steatohepatitis. ROS levels in NS5A-TG mice are significantly higher than those of littermate controls (Wang et al. 2009a). In addition, NF- $\kappa$ B and STAT3 are highly activated in the livers of NS5A-TG mice. NS5A stimulates cyclo-oxygenase (COX)-2 expression, which is implicated in inflammation and fibrogenesis through production of various prostaglandins (Núñez et al. 2004).

Recently, an alternative mechanism of HCV-induced inflammation was reported; NS5B, the viral RNA-dependent RNA polymerase (RdRp), was shown to catalyze the production of small RNA species, triggering an innate immune response and leading to the production of both IFN and inflammatory cytokines (Yu et al. 2012).

Inflammation is induced not only by HCV proteins but also by HCV RNA. Retinoic acid-inducible gene 1 (RIG-I) and TLR3 are cellular sensors that recognize HCV double-stranded RNA (dsRNA), resulting in production of chemokines, such as IL-8, RANTES (regulated on activation, normal T cell expressed and secreted), macrophage inflammatory protein (MIP)-1 $\alpha$ , and MIP-1 $\beta$  (Li et al. 2012; Wagoner et al. 2007). Recent reports have indicated that HCV RNA triggers IL-1 $\beta$  expression in hepatocytes, macrophages, and Kupffer cells (Burdette et al. 2012; Chen et al. 2014; Negash et al. 2013). In addition, HCV uptake induces a potassium efflux that activates the NLRP3 inflammasome, which results in IL-1 $\beta$  processing and secretion (Negash et al. 2013). Production of IL-1 $\beta$  stimulates production of pro-inflammatory cytokines and chemokines that are associated with liver disease (Negash et al. 2013).

### **9.3 The Role of Hepatic Stellate Cells (HSCs) in the Progression of Liver Fibrosis**

Liver fibrosis is the hallmark of all chronic liver diseases. A number of intrahepatic cell populations are involved in liver fibrogenesis. Immune cells, such as Kupffer cells and recruited lymphocytes, are important for the inflammatory phase of the fibrogenic response. HSCs (previously known as Ito cells, lipocytes, or fat-storing cells) are the predominant cellular source of ECM components and play a key role in development of liver fibrosis. In the normal liver, HSCs are quiescent, long-lived

cells that store vitamin A. Activation or trans-differentiation of HSCs is regulated by growth factors, such as TGF- $\beta$ , and is associated with pathological conditions, including liver injury, cirrhosis and cancer (Friedman 2008a, b), as a result of the expression of collagen. Activated HSCs may contribute to the growth and progression of HCC by their collagenolytic and angiogenic actions (Corpechot et al. 2002; Olaso et al. 2003; Musso et al. 1997; Torimura et al. 2004). In chronic liver injury, HSCs differentiate into myofibroblast-like cells, which exhibit marked expression of  $\alpha$ -smooth muscle actin (SMA). Myofibroblast-like cells have a high fibrogenic capacity in the chronically diseased liver. A previous report has indicated that TLR4 contributes to myofibroblast activation and fibrogenesis in the liver (Seki et al. 2007). TLR4-dependent modulation of TGF- $\beta$  signaling provides a link between pro-inflammatory and pro-fibrogenic signals. Indeed, HCV-infected patients have higher plasma levels of lipopolysaccharide (LPS) than healthy control patients (Sandler et al. 2011). LPS-stimulated Kupffer cells have a crucial role in hepatic fibrogenesis by stimulating HSCs (Duffield et al. 2005; Rivera et al. 2001). Activated HSCs express TLR4 and are highly responsive to LPS, leading to expression of inflammatory cytokines (Brun et al. 2005; Mühlbauer et al. 2004; Paik et al. 2003). TLR4 is thus a candidate for hepatic fibrosis therapy.

Recently, IL-33, an IL-1-related cytokine, has been found to exhibit pro-fibrotic properties in the liver (McHedlidze et al. 2013). Innate lymphoid cells express the IL-33 receptor (ST2 and IL-1R4) (Chackerian et al. 2007), respond to IL-33 and produce large amounts of the Th2 cytokines IL-5, IL-6, and IL-13 (Moro et al. 2010; Neill et al. 2010). IL-13 triggers the activation and trans-differentiation of HSCs in an IL-4Ra- and STAT6-dependent manner (McHedlidze et al. 2013).

HCV infection is a major cause of chronic liver disease, and a proportion of patients develop progressive fibrosis. HSCs mediate HCV-related liver fibrosis, and HCV infection directly or indirectly induces the activation of HSCs. For example, IL-8 produced by hepatocytes expressing the HCV core induces  $\alpha$ -SMA expression in HSCs (Clément et al. 2010). HCV E2-CD81 interaction in HSCs activates ERK/mitogen-activated protein kinase (MAPK) signaling and expression of matrix metalloproteinase (MMP)-2, a major enzyme involved in the degradation of normal hepatic ECM, resulting in the progression of HCV-related hepatic fibrogenesis (Mazzocca et al. 2005; Ming-Ju et al. 2011). In contrast, CX3CR1, which is more highly expressed in patients with HCV and other chronic liver diseases than in healthy control patients (Isse et al. 2005), suppresses tissue inhibitor of metalloproteinase (*TIMP*)-1 mRNA in HSCs. These processes are risk factors for development of liver fibrosis (Wasmuth et al. 2008).

## 9.4 Cross-Talk Between Hepatocytes and HSCs

Although a critical role of HSCs in the progression of liver fibrosis is well-understood, the functional impact of the cross-talk between hepatocytes and HSCs remains largely unexplored. A recent study has demonstrated cross-talk between

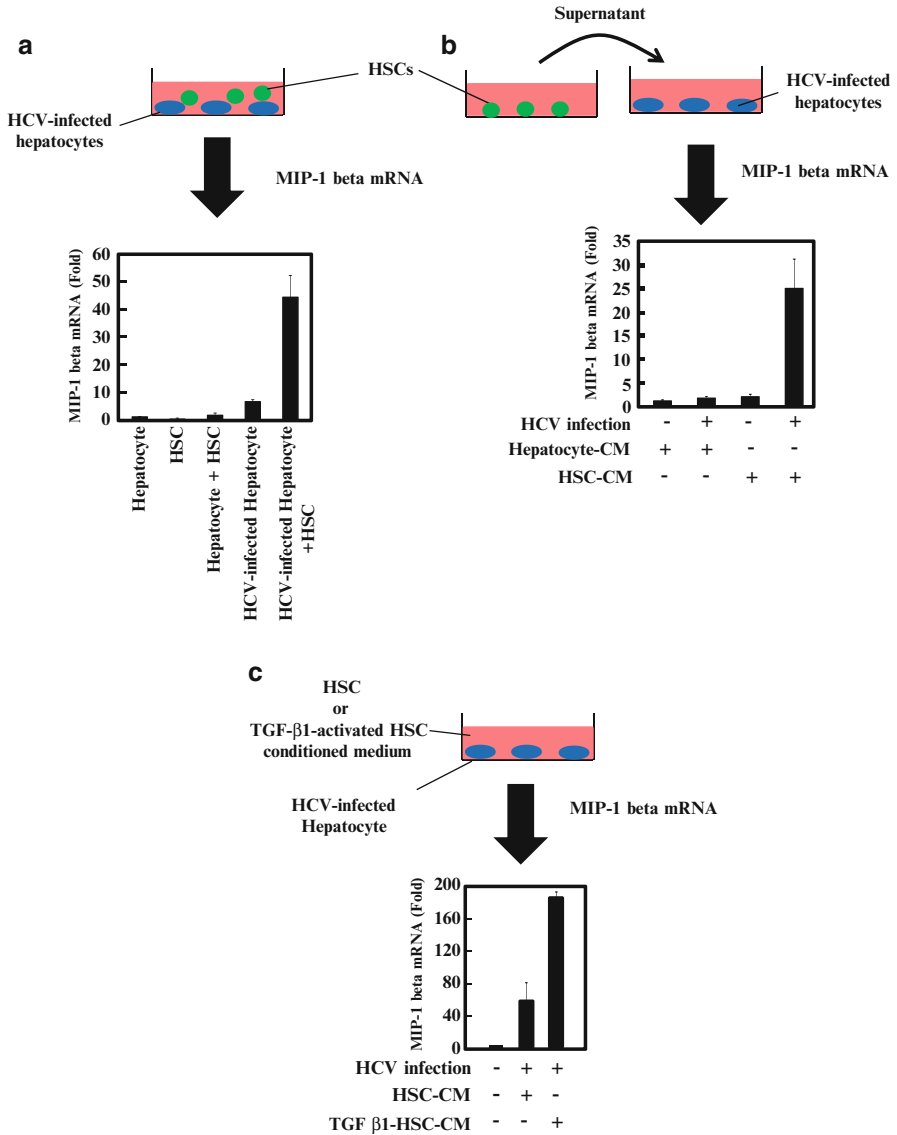
tumor hepatocytes and activated HSCs in a co-culture model (Coulouarn et al. 2012). When hepatocytes (HepaRG cells) were co-cultured with activated HSCs (LX2 cells), hepatocyte expression of pro-inflammatory cytokines (such as IL-1 $\beta$  and IL-6) and chemokines [such as IL-8 and C-C chemokine ligand 2 (CCL2)] was enhanced. In mouse models of alcoholic liver disease, alcohol feeding significantly upregulated IL-1 $\beta$  mRNA in the livers and enhanced the secretion of IL-1 $\beta$  in the serum. IL-1 $\beta$ -mediated signaling induced the expression of pro-inflammatory cytokines, such as TNF- $\alpha$  and IL-6. IL-1 $\beta$  exerts its pathogenic effects by upregulating lipid synthesis in hepatocytes (Miura et al. 2010), activating HSCs (Zhang and Yao 2012), and maintaining macrophages in an inflammatory state (Hou et al. 2000). By contrast, deficiency of the IL-1 $\beta$  pathway prevents the increase in pro-inflammatory cytokines. These results suggest that the induction of IL-1 $\beta$  plays an important role in the development of alcoholic steatosis, inflammation, injury, and fibrosis (Petrasek et al. 2012).

Moreover, the enhanced expression of chemoattractant chemokines may contribute to the establishment of a permissive microenvironment by recruiting immune cells.

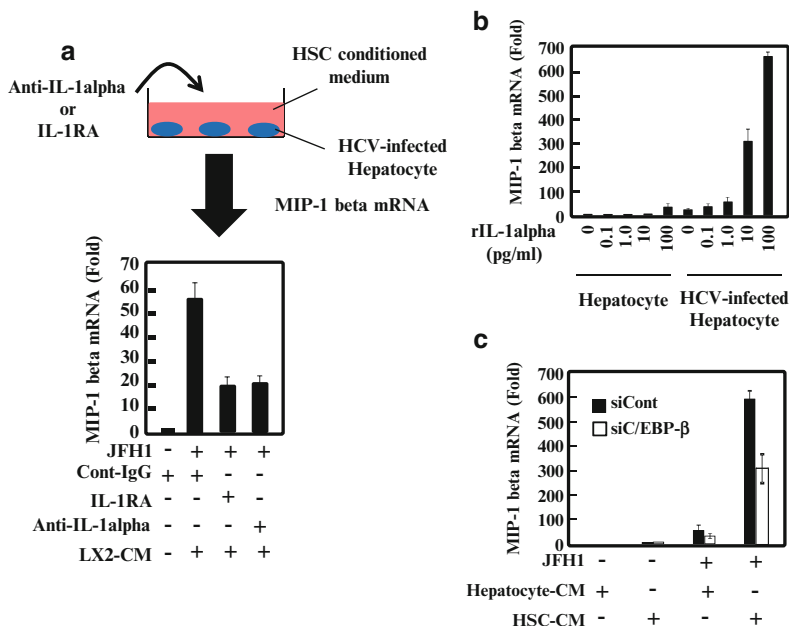
The increase in IL-8 expression affects the intrahepatic immune response due to T cell chemotaxis to the liver. Because IL-8 is elevated in alcoholic hepatitis (McClain et al. 1999), it is tempting to speculate that induction of IL-8 may exacerbate the deleterious effects of ethanol on the liver, contributing to the increased pathological activity in the liver.

The interaction between hepatocytes and HSCs induces the activation of intracellular signaling pathways, such as AKT and ERK, in HSCs (Sancho-Bru et al. 2010). These pathways have been implicated in many biological functions of HSCs, and it also mediates key events in tumor progression, such as angiogenesis. Indeed, the expression of angiogenesis-related genes, such as *MMP2*, *MMP9* and *VEGFA*, is induced in HSCs by hepatocyte–HSC cross-talk (Coulouarn et al. 2012; Th  ret et al. 1997). These findings suggest that the dynamic interaction between hepatocytes and HSCs may lead to enhancement of ECM remodeling and angiogenesis, resulting in the progression of HCC.

By co-culturing HSCs with HCV-infected hepatocytes, we have recently shown that HSCs can act as inflammatory mediators in HCV-infected cells (Nishitsuji et al. 2013). Intrahepatic chemokines, such as RANTES, MIP-1 $\alpha$ , MIP-1 $\beta$  and IP-10, are elevated in HCV patients (Harvey et al. 2003), and these chemokines have been linked to a high degree of liver inflammation (Zeremski 2007). We examined whether the interaction of HSCs with HCV-infected hepatocytes triggered the production of MIP-1 $\beta$ . When HSCs were co-cultured with HCV-infected hepatocytes, but not uninfected hepatocytes, MIP-1 $\beta$  was induced (Fig. 9.1a). Moreover, the conditioned medium of HSCs could stimulate MIP-1 $\beta$  in HCV-infected hepatocytes (Fig. 9.1b). Other pro-inflammatory cytokines and chemokines, such as IL-6, IL-8 and MIP-1 $\alpha$ , are also induced by the interaction between HCV-infected hepatocytes and HSCs (Nishitsuji et al. 2013). Of note, treatment with TGF- $\beta$ , which activates HSCs, augmented inflammation in HCV-infected hepatocytes (Fig. 9.1c). IL-1 $\alpha$  in the conditioned medium of HSCs was involved in the HSC-mediated induction of inflammatory cytokines and chemokines, as it was neutralized by an anti-IL-1 $\alpha$



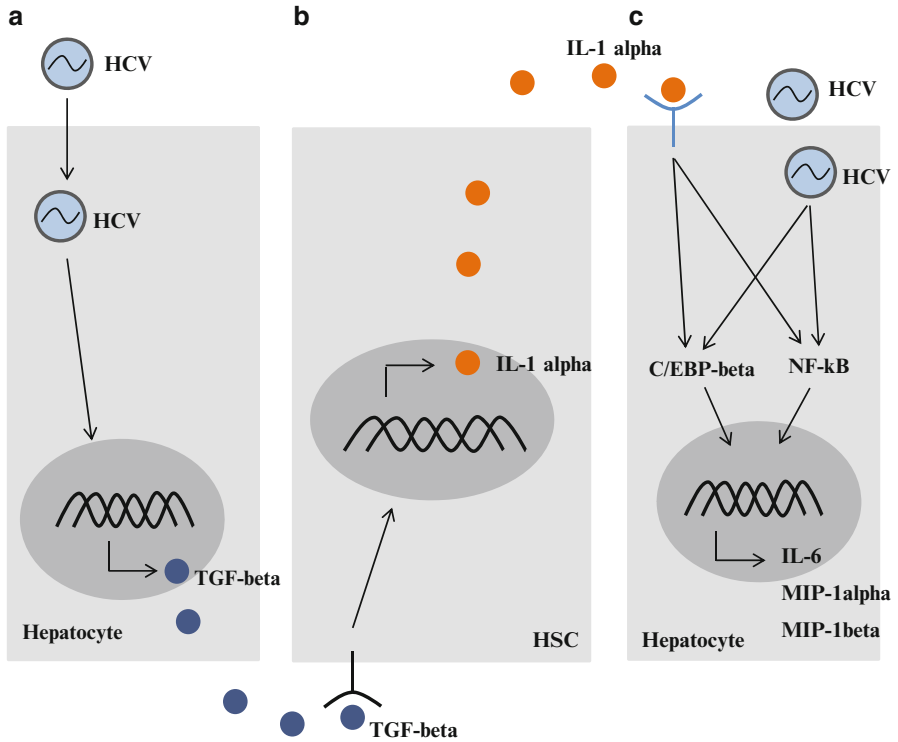
**Fig. 9.1** Cross-talk between hepatitis C virus (HCV)-infected hepatocytes and hepatic stellate cells (HSCs) induces macrophage inflammatory protein (MIP)-1 $\beta$  expression. **(a)** Hepatocytes or HCV-infected hepatocytes were cultured alone or in the presence of HSCs for 24 h. The expression of MIP-1 $\beta$  was measured by quantitative real-time polymerase chain reaction (qRT-PCR). **(b)** Hepatocytes or HCV-infected hepatocytes were treated with conditioned medium from hepatocytes (Hepatocyte-CM) or HSCs (HSC-CM) for 24 h. The expression of MIP-1 $\beta$  was measured by qRT-PCR. **(c)** HSCs were treated with transforming growth factor (TGF)- $\beta$ 1 for 24 h. Hepatocytes or HCV-infected hepatocytes were treated with HSC-CM or TGF- $\beta$ 1-stimulated HCS-CM for 24 h. The expression of MIP-1 $\beta$  was measured by qRT-PCR



**Fig. 9.2** Interleukin (IL)-1 $\alpha$  and CCAAT (cytosine–cytosine–adenosine–adenosine–thymidine)-enhancer-binding protein (C/EBP)- $\beta$  contribute to the hepatitis C virus (HCV)-infected hepatocyte response to hepatic stellate cells (HSCs). (a) HCV-infected hepatocytes were cultured with HSCs together with an isotype control, anti-IL-1, or IL-1 receptor antagonist (IL-1RA) for 24 h. Macrophage inflammatory protein (MIP)-1 expression was analyzed by quantitative real-time polymerase chain reaction (qRT-PCR). (b) Hepatocytes or HCV-infected hepatocytes were treated with various amounts of recombinant IL-1 (0, 0.1, 1.0, 10, or 100 pg/ml) for 24 h. MIP-1 expression was analyzed by qRT-PCR. (c) Hepatocytes and HCV-infected hepatocytes were transfected with control small-interfering RNA (siRNA) or small-interfering C/EBP (siC/EBP)- $\beta$ . After 24 h of transfection, the cells were treated with conditioned medium from hepatocytes (Hepatocyte-CM) or HSCs (HSC-CM) for 24 h. MIP-1 expression was analyzed by qRT-PCR

antibody or IL-1 receptor antagonist (Fig. 9.2a). In addition, recombinant IL-1 $\alpha$  was also capable of inducing inflammatory cytokines and chemokines in HCV-infected hepatocytes (Fig. 9.2b). In HCV-infected patients, IL-1 $\alpha$  mRNA levels are higher than those in healthy control patients (Kasprzak et al. 2004; Wilkinson et al. 2010). Further, IL-1 $\alpha$  induces the acute-phase response and autoactivation of Kupffer cells in the liver (Winwood and Arthur 1993). IL-1 $\alpha$  may contribute to HCV-related chronic inflammatory disease. Importantly, uninfected hepatocytes are unaffected by HSC stimulation. It is interesting that HCV-infected hepatocytes, but not uninfected hepatocytes, respond to IL-1 $\alpha$  produced by HSCs. To investigate this finding, we searched for transcription factors related to inflammatory cytokines (e.g., IL-6, MIP-1 $\alpha$ , and MIP-1 $\beta$ ) and identified CCAAT (cytosine–cytosine–adenosine–adenosine–thymidine)-enhancer-binding protein (C/EBP)- $\beta$ , which stimulates





**Fig. 9.3** Cross-talk between hepatitis C virus (HCV)-infected hepatocytes and hepatic stellate cells (HSCs). (a) HCV infection induces the expression of growth factors, such as transforming growth factor (TGF)- $\beta$ . (b) When quiescent HSCs are activated by growth factors, activated HSCs secrete inflammatory cytokines, such as interleukin (IL)-1 $\alpha$ . (c) HCV-infected cells are stimulated by IL-1 $\alpha$ . An increase in inflammatory cytokine production accelerates inflammation in the liver

production of inflammatory cytokines and chemokines in HCV-infected hepatocytes. Indeed, knockdown of C/EBP- $\beta$  in HCV-infected hepatocytes reduced the response to HSCs (Fig. 9.2c). A recent study has demonstrated that the HCV NS5A protein induces C/EBP- $\beta$  expression (Qadri et al. 2012). Another group proposed that ER stress induced by HCV infection leads to the generation of mature sterol regulatory element-binding protein 1 (SREBP-1) (Joyce et al. 2009). The mature SREBP-1c can induce C/EBP- $\beta$  expression (Le Lay et al. 2002). C/EBP- $\beta$  is therefore a key molecule in the cross-talk between HCV-infected hepatocytes and HSCs.

These results suggest that TGF- $\beta$ , which is induced by HCV infection, stimulates quiescent HSCs, and activation or trans-differentiation of HSCs leads to the expression of IL-1 $\alpha$ , resulting in increased levels of inflammatory cytokines and chemokines in HCV-infected hepatocytes (Fig. 9.3).

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