Review

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The role of Kupffer cells in hepatitis B and hepatitis C virus infections

Arjan Boltjes[†], Dowty Movita[†], André Boonstra[‡], Andrea M. Woltman[*,‡]

Dept. of Gastroenterology and Hepatology, Erasmus MC University Medical Center, Rotterdam, The Netherlands

Summary

Globally, over 500 million people are chronically infected with the hepatitis B virus (HBV) or hepatitis C virus (HCV). These chronic infections cause liver inflammation, and may result in fibrosis/cirrhosis or hepatocellular carcinoma. Albeit that HBV and HCV differ in various aspects, clearance, persistence, and immunopathology of either infection depends on the interplay between the innate and adaptive responses in the liver. Kupffer cells, the liver-resident macrophages, are abundantly present in the sinusoids of the liver. These cells have been shown to be crucial players to maintain homeostasis, but also contribute to pathology. However, it is important to note that especially during pathology, Kupffer cells are difficult to distinguish from infiltrating monocytes/macrophages and other myeloid cells. In this review we discuss our current understanding of Kupffer cells, and assess their role in the regulation of anti-viral immunity and disease pathogenesis during HBV and HCV infection.

The characteristics of Kupffer cells

Kupffer cells (KC) are tissue-resident macrophages residing in the liver. They are located in the liver sinusoids, and are the largest population of innate immune cells in the liver [1–3]. Due to their abundance and localization, KC are crucial cellular components of the intrahepatic innate immune system that are specialized to perform scavenger and phagocytic functions, thereby removing protein complexes, small particles, and apoptotic cells from blood [1–3]. Together with the sinusoidal endothelial cells, KC are the first barrier for pathogens to enter the liver via the portal vein [4]. This is extremely important, since venous portal blood is rich in pathogen-derived products, such as lipopolysaccharide, and pathogens from the gut, which need to be eliminated from the circulation to avoid systemic immune activation.

The specialized function of KC is reflected by the phenotype: they were identified in the early 1970s as peroxidase-positive cells with cytoplasm containing numerous granules and vacuoles, and occasional tubular, vermiform invaginations [5–8]. At present, human KC are identified by immunohistochemistry or flow cytometry using antibodies directed against CD68, CD14, and CD16 [9–11]. However, it is important to mention that these markers are not unique for human KC and macrophages from other tissues, but are also expressed on monocytes, which are also considered a source of precursor cells for KC, and/or dendritic cells [12]. Different from their human counterpart, rat KC are commonly identified by antibodies against CD68 or CD163 (ED1 and ED2, respectively) [13], and mouse KC using the F4/80 marker [14]. However, also the rat and mouse markers are not unique for KC, but are shared with other leukocytes.

The ambiguity in the identification of KC that exists under steady state conditions is even more challenging under pathological conditions, in which cellular infiltrates are observed consisting of inflammatory monocytes and/or dendritic cells that share certain surface markers. In rat studies, large and small KC were shown to be present in a distinct area within the liver, i.e., in the peri-portal, and peri-venous and mid-zonal area, respectively [10,15–19], and 2 subpopulations of KC have been isolated from rat liver tissue: $ED1^{+}ED2^{-}$ and $ED1^{+}ED2^{+}$ cells [16,17]. Similarly, some studies have identified 2 subpopulations of mouse KC: $F4/80^{+}CD68^{+}$ and $F4/80^{+}CD11b^{+}$ cells from mouse liver tissue [20]. It is likely that these populations either illustrate distinct differentiation phases rather than distinct KC subpopulations, or that they identify infiltrating monocytes instead of resident tissue macrophages. In studies from our group, we defined only one KC population in mouse liver tissue on the basis of F4/80 and CD11b expression [21]. This was in line with a study in humans where only a single population of KC was identified as $CD14^{+}$, $HLA\text{-}DR^{+}$, $HLA\text{-}ABC^{+}$, $CD86^{+}$, and $DC\text{-}SIGN^{+}$ cells, with low expression of CD1b, CD40, and CD83 [9]. It is preferable to identify KC not solely based on the available markers, but also on their morphology and phagocytic ability as their hallmark

Keywords: Kupffer cells; Macrophages; HBV; HCV; Viral hepatitis; Anti-viral immunity; Liver inflammation; Liver damage.

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* Corresponding author. Address: Dept. of Gastroenterology and Hepatology, Erasmus MC University Medical Center, 's Gravendijkwal 230, 3015 CE Rotterdam, The Netherlands. Tel.: +31 10 703 2759; fax: +31 10 703 2793.

E-mail address: a.woltman@erasmusmc.nl (A.M. Woltman).

† These authors contributed equally to this work.

‡ These authors shared senior authorship.

Abbreviations: HBV, Hepatitis B virus; HCV, Hepatitis C virus; KC, Kupffer cells; NK cells, natural killer cells; PRR, pattern recognition receptor; TLR, Toll-like receptor; RLR, RIG-like receptor; NLR, NOD-like receptor; CLR, C-type lectins; SR, Scavenger receptor; ROS, reactive oxygen species; LCMV, lymphocytic choriomeningitis virus; MCMV, murine cytomegalovirus; MHV, mouse hepatitis virus; IFN, Interferon; HCC, hepatocellular carcinoma; HBcAg, Hepatitis B core antigen; HBsAg, Hepatitis B surface antigen; HBeAg, Hepatitis B early antigen; HSPG, Heparan sulfate proteoglycan; MR, Mannose receptor; EGFR, Epidermal growth factor receptor; LDL, Low-Density Lipoprotein; ISG, Interferon-stimulated gene.

function. In this review, KC are identified as $CD68^+$, $CD14^+$, and/or $CD11b^+$ cells (human), $ED1^+$ and/or $ED2^+$ cells (rat) and $CD68^+$, $F4/80^+$ and/or $CD11b^+$ cells (mouse), according to the original studies. Under steady state condition, the majority of tissue-resident macrophages in the mouse liver have a yolk sac origin and are self-maintained. Upon serious challenge, tissue resident KC can be replaced by precursor cells from bone marrow as well as monocytes, which develop into tissue-resident macrophages [22]. Since the distinction between tissue-resident KC and tissue-infiltrating monocyte/macrophages is difficult, and since most studies did not discriminate between these cells with a different origin, we will use the term "KC" to describe both cells.

Studies on human KC are being performed using cells obtained from liver tissue or from liver graft perfusate. Liver graft perfusate is preserved in a different manner than liver tissue. Also, tissue-derived KC are commonly isolated using collagenase, a processing step not included for perfusate, which increases the amount of extracellular debris and may induce phenotypic and functional changes. The source of liver material as well as the method to process the samples are important to take into account when interpreting results on the phenotype and function of KC from the various studies.

Macrophages are specialized in sensing and responding to pathogens and equipped with specific pattern recognition receptors, including scavenger receptors, Toll-like receptors (TLR), RIG-like receptors (RLR), NOD-like receptors (NLR) and C-type lectins. These receptors are expressed by tissue-derived as well as *in vitro*-generated macrophages (reviewed in [23]). However, only few of them have been described for KC and it is not clear whether the others are expressed by KC. Scavenger receptors and C-type lectins are important receptors mediating phagocytosis, which are expressed by human, rat, and mice KC [24–26]. The phagocytic ability of human KC has been shown in relation to removal of erythrocytes, apoptotic cells, and debris [27,28]. In line with that notion, we and others have shown that rat and mouse KC are strongly phagocytic and possess a high level of basal reactive oxygen species (ROS) production [20,21]. Upon *in vivo* administration of dextran particles, *E. coli* or gadolinium chloride, rat and mouse KC take up these particles, produce high levels of ROS, and demonstrate high lysosomal activity [17,18,20,21]. Human KC were shown to express TLR2, TLR3, and TLR4 [9,29]. The expression of other TLR, as well as NLR and RLR have not been described, but cannot be excluded since the murine counterparts were found to express functional TLR1-TLR9 and RIG-I [25,30]. In human and rodents, ligation of TLR on tissue-derived and *in vitro*-generated macrophages resulted in cytokine production [31]. However, to date, studies on the ability of KC to produce cytokines upon TLR ligation resulted in divergent conclusions. For instance, we and others show that KC from human liver tissue and perfusate release IL-10, IL-1β, IL-6, IL-12, IL-18, and TNF upon TLR2, TLR3, and TLR4 ligation *ex vivo* [9,32,33] and [Boltjes, unpublished data]. Similarly, Kono *et al.* showed that liver tissue-derived rat KC produce superoxide, TNF, and IL-6 upon TLR4 ligation *ex vivo* [17]. However, examination of mouse KC isolated from liver tissue by our group and others demonstrated weak induction of TNF and IL-12p40 upon *ex vivo* stimulation with agonist for TLR4, TLR7/8, or TLR9 [20,21], whereas no data are available on the cytokine-producing ability of liver perfusate-derived rat or murine KC. Thus, more studies using highly purified KC with a well-defined phenotype need to be conducted to obtain conclusive data on the TLR responsiveness of KC.

A weak ability of KC to produce cytokines might be related to their tolerogenic function in a steady state condition. KC are frequently exposed to gut-derived antigens. Instead of exerting inflammatory responses, human and murine KC constitutively express TGF-β and PD-1, possess high levels of negative regulators downstream the TLR pathway and secrete IL-10 upon LPS stimulation [20,21,32,34–36]. More importantly, the ability of murine KC to produce pro-inflammatory cytokines upon TLR4, TLR7/8, and TLR9 is by far weaker than that of peritoneal macrophages [21]. This observation suggests that KC play a crucial role in maintaining liver homeostasis in a steady state condition. Additionally, our mouse study and others show that KC are superior in the ability to take up particles and have a higher basal ROS production, in comparison to splenic and peritoneal macrophages, which highlight their function to remove particulates from the circulation [21,37].

Key Points

- Kupffer cells contribute to immune activation and anti-viral immunity upon infection with HBV or HCV
- Both HBV and HCV are able to exploit the function of Kupffer cells
- The receptors and molecular mechanisms involved in the interaction between Kupffer cells and HBV or HCV, or its components, need to be elucidated
- Kupffer cells and/or liver-infiltrating macrophages contribute to tissue damage and play a role in the regulation of fibrosis, cirrhosis, and hepatocellular carcinoma during chronic viral hepatitis
- The contribution of liver-resident Kupffer cells *vs.* liver-infiltrating macrophages in the regulation of viral immunity and disease pathogenesis is hampered by the lack of distinctive phenotypical markers

The role of KC during LCMV infections

Besides their barrier [4] and janitor function [38,39], KC have been shown to play a role in the response to pathogens, including viruses. Studies on the importance and anti-viral immune functions of KC in HBV and HCV infections are difficult to perform, since these viruses only infect and replicate in humans and non-human primates, and immunocompetent small animal models for viral hepatitis are not yet available (reviewed in [40,41]). As an alternative approach several mouse infection models, including lymphocytic choriomeningitis virus (LCMV), murine cytomegalovirus (MCMV), mouse hepatitis virus (MHV) and adenovirus models, have provided information on the role of KC in viral infection. However, in contrast to HBV and HCV where infection and replication is restricted to hepatocytes, these hepatitis mouse models also infect other cells and even other organs. Of these models, MHV and LCMV have been shown to replicate in KC [42,43]. LCMV, MHV, and adenovirus particles can be taken up from the circulation by murine KC via scavenger and

complement receptors, which may limit infection [44–47]. It has been shown that failure in clearing LCMV, MHV, and adenovirus particles during the acute phase results in "spill-over" infection of hepatocytes, prolonged infection, and exacerbated immunopathology [47–49]. Studies using these mouse models have been instrumental in our understanding of the effects on KC during the early phases of virus infections. A number of studies have also evaluated KC during persistent infection in mice. These studies are conducted using specific isolates of LCMV, the clone 13 and WE strains. The development of persistent infection with a high rate of replication of LCMV is similar to HBV and HCV, and important mechanistic pathways identified in LCMV infected mice, were later confirmed to be operational during chronic viral infections in patients. However, in contrast to HBV and HCV, murine LCMV infections are not restricted to the liver, and LCMV replication can also be found in the spleen, lung, and kidney. The long-term consequences of human viral hepatitis, such as fibrosis, are absent in mice, although virus-induced liver damage is observed [44,50]. The effect of chronic LCMV infection on NK cells and virus-specific T cells has been extensively examined, however only few studies have focussed on KC. In contrast to HBV or HCV, active replication of LCMV in the liver, as evidenced by the detection of viral RNA and antigen, has been demonstrated in KC as well as in hepatocytes [43,51,52]. During the first 2 weeks following LCMV infection, an increase of the number of F4/80$^+$ cells is observed, followed by normalization of their numbers [19]. Although differences in MHC class-I expression levels were observed within the F4/80 population by immunohistochemistry, the relative contribution of infiltrating monocytes *vs.* enhanced activation of resident KC is difficult to determine.

An elegant study by Lang *et al.* showed that clodronate-mediated depletion of KC resulted in rapid LCMV dissemination due to the inability to capture virus, which led to replication within hepatocytes and subsequently severe CD8$^+$ T cell-mediated liver damage [44]. The study further showed that KC responded to type I IFN by inducing the expression of interferon-stimulated genes, and that mice lacking IFNAR specifically on macrophages exhibited strongly enhanced viral titers. However, recently a detrimental influence of granulocytes and macrophages in spleen and liver was reported by their ability to produce reactive oxygen species (ROS) following viral infection, although ROS production by liver F4/80$^+$ cells was low [53]. Importantly, the effect of ROS was an impairment of the immune response, and in the absence of ROS mice exhibited lower viral titers and less liver damage. In a different experimental mouse model, which makes use of transgenic intrahepatic expression of the HBV large envelope protein, ROS activity was observed in KC, and these mice exhibited a chronic necroinflammatory liver disease, resembling human chronic active hepatitis [54].

The findings from the LCMV mouse model clearly show the complexity of the anti-viral response in the liver since KC can both contribute to promote and suppress viral eradication and liver pathology. In the following section, we will focus on the interaction of KC with HBV and HCV, and the functional consequences.

The role of KC during HBV and HCV infections

Both HBV and HCV are transmitted predominantly via percutaneous and sexual exposure, while perinatal exposure is often seen for HBV only [55–57]. Infection with these viruses can either resolve spontaneously or develop into chronic liver disease with continuous viral replication in hepatocytes [56–58]. Chronic hepatitis poses an increased risk for liver fibrosis and cirrhosis, hepatic failure, and hepatocellular carcinoma (HCC) [58,59]. Patients with a self-limiting HBV or HCV infection show sustained, vigorous, and multi-epitope-specific CD4$^+$ or CD8$^+$ T cell and B cell responses, whereas in chronic HBV and HCV these responses are weak and/or transient [60–63]. This demonstrates that clearance of the infection is dependent on strong multi-epitope-specific T and B cell responses, which is only possible following effective innate immune responses [63,64]. Here, we will firstly address the role of KC in the interaction and recognition of HBV and HCV, and their role in the induction of a pro-inflammatory response. Pro-inflammatory mediators are important for inhibition of viral replication, the induction of resistance to infection of neighboring cells, and attraction and activation of other immune cells, and consequently contribute to the development of effective virus-specific immunity. Secondly, we will discuss KC-virus interactions that may inhibit the development of effective viral immunity, facilitate viral persistence or promote liver damage.

Interaction of KC with HBV and HCV

HBV is a 3.2 kb partially double-stranded DNA envelope-virus which replicates via RNA intermediates. Hepatitis B core protein (HBcAg)-encapsulated viral DNA and hepatitis B envelope protein (HBsAg) form a complete viral or Dane particle. HBV particles, HBsAg, and hepatitis B early antigen (HBeAg; a truncated form of HBcAg) are secreted by infected hepatocytes and can be detected in serum of HBV patients [58,65].

Evidence for productive HBV infection of cells other than hepatocytes is lacking. Also, detailed information on the presence of HBV (proteins) in KC *in vivo* or the uptake of HBV or its proteins by human KC *ex vivo* has not been reported. Although no information is available on KC, studies using THP-1 monocytic cells, monocytes, and dendritic cells have shown binding of HBV or HBV proteins, leading to their activation. For instance, TLR2 and heparan sulfate proteoglycan (HSPG) were suggested to be responsible for HBcAg recognition on THP-1 cells, and HBcAg-induced activation of THP-1 cells resulted in production of IL-6, IL-12p40, and TNF [66]. However, since HBcAg is only found within infected hepatocytes or viral particles, it is unclear whether HBcAg interacts with KC, via HSPG and/or another extracellular receptor like TLR2. Also, other receptors expressed by KC are known to interact with HBV proteins as demonstrated in other cell-systems (Table 1). For instance, HBsAg can interact with human blood monocytes in a CD14-dependent fashion [67], and with dendritic cells via the mannose receptor [68], which are both receptors known to be also expressed on KC [69]. Finally, complex formation of HBsAg with albumin may lead to enhanced uptake of HBsAg from the circulation by KC and endothelial cells [70].

HCV contains a 9.6 kb positive-strand RNA genome that translates into the structural proteins, core, and E1 and E2 envelope proteins, and the non-structural proteins NS1–NS5. After replication, they form a small-enveloped virus particle containing the newly synthesized RNA genome [71,72].

Compared to HBV, there is a better understanding of the entry receptors on hepatocytes used by HCV. In addition to claudin1, occludin, epidermal growth factor receptor (EGFR), and ephrin

Table 1. Surface molecules and secreted inflammatory mediators facilitating KC roles in HBV/HCV infection.

	HBV		HCV	
	Mediators	[Ref.]	Mediators	[Ref.]
Binding/uptake				
	HSPG	[79]	HSPG	[79]
	CD14	[9]	SR-B1	[82]
	mannose receptor	[69]	LDL-receptor	[81]
			DC-SIGN	[9, 85]
Pattern recognition receptors				
			TLR2	[84, 87]
			TLR4	[88]
Cytokines				
	IL-1β	[89]	IL-1β	[84, 100]
	IL-6	[89]	TNF	[84]
	TNF	[89]	IL-10	[84]
	TGFβ	[91]		
Chemokines				
	CXCL8	[89]		
Co-stimulatory molecules				
			CD40	[94]
			CD80	[94]
			MHC class II	[94]
Immune inhibition or promotion of tolerance				
	TGFβ	[91]	PD-L1	[84, 137]
	PD-L2	[30]	IL-10	[84]
	galectin-9	[135]	galectin-9	[120]
Liver damage				
	IL-6	[89]	TRAIL	[84]
	TRAIL	[84]	granzyme B	[105, 106]
	FasL	[106]	perforin	[105, 106]
	granzyme B	[105]		
	perforin	[105]		
	ROS	[54]		
	galectin-9	[135]		
	TGFβ	[91]		

type-A receptor-2, HCV infects hepatocytes by attaching to HSPG, low-density lipoprotein (LDL) receptor, scavenger receptor (SR)-B1 and CD81. Some, but not all, receptors are expressed by KC (Table 1) [73–82]. It has been reported that incubation of human liver cells with HCV-E2 resulted in HCV-E2 binding to KC in a CD81-dependent manner [83], but also DC-SIGN, a C-type lectin not expressed by hepatocytes, has been demonstrated to bind HCV on KC [84–86].

Although it is unlikely that HCV can replicate in KC, activation of KC by HCV and its proteins has been demonstrated. HCV core and NS3 stimulate human liver perfusate-derived $CD14^+$ KC and monocyte-derived macrophages via TLR2 to produce pro-inflammatory IL-1β, IL-6, and TNF and immunosuppressive IL-10 [84,87]. Recently, it was shown that TLR4, in density gradient- and adherence-isolated liver-derived human KC, mediates NS3 recognition, resulting in TNF production [88]. However, HCV core and NS3 are not secreted at significant levels by infected hepatocytes, posing little relevance to extracellular recognition of HCV by KC via these TLR. Alternatively, phagocytosis of infected hepatocytes by KC may allow intracellular exposure to viral RNA, but so far no evidence exists.

Stimulatory effects of HBV or HCV on KC function

There are only few publications that show a stimulatory effect of HBV or HBV proteins on the function of KC. Hösel *et al.* showed that HBV particles and HBsAg induce IL-1β, IL-6, CXCL8, and TNF production by human $CD68^+$ cell-enriched non-parenchymal cells via NF-κB activation [89] and subsequently inhibit HBV replication in primary hepatocytes. This inhibitory effect was mainly ascribed to IL-6, but also TNF inhibited HBV replication in a non-cytopathic manner [90]. In contrast, Li *et al.* demonstrated that rat $ED1^+$ adherent KC exposed to HBV virions hardly expressed IL-1β, IL-6, or TNF, but produced the immunoregulatory cytokine TGFβ [91].

During chronic HCV infection, KC are increased in numbers in the liver [92,93], and exhibit an activated phenotype with higher mRNA expression levels of the activation markers CD163 and CD33 in livers of chronic HCV patients *vs.* controls [94,95]. Recently, it was reported that in response to HCV human KC release IL-1β and IL-18 *in vitro* [96]. In line with these findings, stimulation of $CD14^+CD68^+$ cells from liver perfusate with UV irradiated cell culture-derived HCV induced IL-1β production. To support this data, *in vivo* co-expression of IL-1β and CD68 was observed using immunofluorescence on liver tissues from patients with chronic HCV [97]. Besides intrahepatic IL-1β, also elevated serum IL-1β levels were detected in patients as compared to healthy individuals [97].

Although a direct effect of HCV-exposed KC on HCV replication is unknown, it was recently reported that KC-derived TNF increased the permissivity of hepatoma cells to HCV. In this study, LPS as well as HCV induced KC to produce TNF, thereby indirectly promoting HCV infection [33]. On the other hand, HCV- or TLR-ligand-induced KC-derived cytokines, such as IL-6, IL-1β, and IFNβ [84,87,97,98], were found to inhibit HCV replication in the HCV replicon model [98–100], implying that KC are also capable of displaying antiviral activity upon HCV exposure.

In addition, release of chemokines and cytokines by KC has an indirect effect on the immune response in the liver by recruitment and activation of infiltrating leukocytes, as also discussed by Heydtmann *et al.* [101]. This may result in a complex interaction between factors produced by liver parenchymal cells, liver resident immune cells including KC, and infiltrating leukocytes. KC are able to activate NK cells and NKT cells, both present at relatively high numbers in the liver, via the production of pro-inflammatory cytokines [9]. In turn, NK and NKT cells produce cytokines such as TNF and IFNγ and are cytotoxic in nature [9,102]. Upon HBV exposure, KC were found to produce CXCL8 [89], which potentially attracts NK and NKT cells during the early phase of HBV infection. KC are also able to recruit dendritic cells to the liver, which involved C-type lectins interactions [103]. This enhanced dendritic cell recruitment may initiate and promote virus-specific T cell responses. In contrast to dendritic cells, KC are less efficient in priming naïve T cells. Nevertheless, mouse KC have been shown to present antigen to $CD4^+$ and $CD8^+$ T cells, inducing these to proliferate and produce IFNγ [104,105]. The relatively high expression of CD40, CD80, and MHC class II found on

CD68$^+$ cells in chronic HCV patients [94] might point towards possible antigen presentation by intrahepatic macrophages.

Although lymphocytes such as NK cells and CD8$^+$ T cells are potent effector cells responsible to kill virus infected cells, KC have been reported to express cytotoxic molecules such as TRAIL, Fas-ligand, granzyme B, perforin, and ROS, enabling them to lyse infected hepatocytes [106–108]. However, since KC act in an antigen-nonspecific manner and hence can lyse hepatocytes irrespective of their infection state, it is tempting to speculate that KC cause more damage to the organ due to their cytotoxic capacity than that they provide protective immunity to the host.

In summary, only limited information exists on the direct interaction between HBV and HCV with KC *in vivo* and *ex vivo*. Macrophages are able to bind HBV or HCV or virus-related proteins *in vitro*, triggering surface and/or intracellular receptors. However, receptors used for these purposes need to be further investigated. Several studies indicate that KC may play a role in controlling HBV and HCV infections by inhibiting viral replication, either directly via the production of cytokines or via their interaction with other cells, as well as in shaping the inflammatory response towards the induction of virus-specific immunity. However, more research is required to get a better insight into the role of KC in regulating intrahepatic immunity.

Suppressive effects of HBV and HCV on KC function

Besides the contribution of KC to viral clearance, viruses may actively interfere with the pro-inflammatory functions of KC to evade host immunity. Various studies show that HBV and HCV are able to interfere with TLR pathways, RIG-I signaling and subsequent pro-inflammatory activities of hepatocytes and immune cells [109–113], but studies describing the effect on human KC are limited. Only one study described that type I IFN production and TRAIL expression by human perfusate-derived KC were suppressed by HCV core protein via disruption of the TLR3/TRIF/TRK1/IRF3 pathway [84]. In addition, numerous studies on monocytes have demonstrated modulation of cytokine production by HCV proteins, and altered TLR responsiveness of monocytes obtained from chronic HCV patients [114–116].

Concerning HBV, pretreatment of non-parenchymal cells including KC, with HBV-Met cell-derived supernatants, HBsAg, HBeAg, or hepatitis B virions almost completely abrogated TLR-induced anti-viral activity, i.e., IFNβ production, interferon-stimulated gene (ISG) induction, IRF3, NF-κB, and ERK1/2 expression [117]. Accordingly, incubating human monocytes with HBeAg or HBsAg inhibited TLR2-induced phosphorylation of p38 MAPK and JNK MAPK, and subsequent production of IL-6, TNF, and IL-12 [29,118,119]. *In vivo*, TLR2 expression by KC and peripheral blood monocytes in HBeAg-positive chronic HBV-infected individuals was lower than that in HBeAg-negative patients and controls. Moreover, TLR2 ligation induced less IL-6 and TNF in those HBeAg-positive patients [29]. These alterations may be related to the inhibitory effect of HBeAg on TLR2 signaling demonstrated *in vitro*. In addition, also TLR3 expression was found to be lower on PBMC from chronic HBV patients compared to control patients as well as on liver cells, including KC [120]. Antiviral therapy of chronic HBV patients with entecavir or pegylated IFN-α partially restored TLR3 expression, but it is unclear whether this is a direct viral effect.

Tolerogenic effects of HBV and HCV related to KC

As mentioned above, KC are constantly exposed to pathogen-derived products from the gut. To prevent excessive inflammation and pathology of the liver, continuous activation of KC is avoided as these cells become refractory to subsequent endotoxin challenge, a phenomenon known as endotoxin-tolerance [121,122]. This contributes to the well-described tolerogenic milieu in the liver. Besides modulation of TLR-signaling pathways, also expression of anti-inflammatory mediators, such as IL-10 and TGFβ, and other soluble and membrane-bound inhibitory molecules are underlying the intrahepatic tolerance [35,105,122,123].

A number of studies have reported that HBV and HCV components affect the production of immunoregulatory cytokines, and consequently promote the tolerogenic milieu of the liver. In this respect, it has been reported that HBV particles preferably induced TGFβ production by rat KC instead of pro-inflammatory cytokines [91]. One of the activities of TGFβ is that it plays a role in maintaining tolerance towards self-antigens by selectively supporting the differentiation of FoxP3$^+$ regulatory T cells [124,125]. Furthermore, HCV core protein induces IL-10 production by human KC [84,87]. Elevated intrahepatic IL-10 levels may suppress pro-inflammatory cytokine production by intrahepatic cells, frustrate KC-NK cell interaction [9,126] and antigen presentation to T cells and their activation [105,127–133]. Interestingly, chronic HBV and HCV patients showed higher plasma levels of IL-10 than uninfected individuals [134,135], which could be the result of a direct viral effect on KC and/or other cells, or the result of a negative feedback mechanism resulting from ongoing liver inflammation. Recently, the role of KC was examined in an established HBV-carrier mouse model. In this model, KC as well as IL-10 were involved in the establishment of antigen-specific tolerance towards peripheral HBsAg vaccination [136].

KC express membrane-bound inhibitory ligands that could facilitate a tolerogenic milieu in the liver. For instance, under steady state conditions, KC are known to express PD-L1, which is a ligand for PD-1 and known to impede T cell function by inhibiting proliferation and cell division [36]. Immunohistochemical analyses of liver biopsies from chronic viral hepatitis patients revealed that CD68$^+$ macrophages expressed increased levels of PD-L2 compared to control liver tissue [30,123,137]. Similar results were reported for galectin-9 with enhanced expression by CD68$^+$ cells by immunohistochemistry, which was confirmed by flow cytometry [137]. Interestingly, enhanced serum levels of galectin-9 were observed in patients with biochemical evidence of highly active chronic HBV-related liver disease (ALT >100 U/L) as compared to patients with relatively low ALT levels (<50 IU/L) or healthy controls. Also comparison of plasma galectin-9 levels in patients with chronic HCV showed higher levels in patients compared to healthy individuals [123]. Furthermore, co-localization of CD68 and galectin-9 was observed in the peri-portal regions of the livers of virtually all the patients with HCV infection, regardless of grade of inflammation or stage of fibrosis, but not in normal control livers [123].

These inhibitory ligands are known to inhibit T cell function upon cell-cell contact via interaction with PD-1 and Tim-3, respectively [138], which is of relevance since both PD-1 and Tim-3 are reported to be upregulated on HBV- and HCV-specific intrahepatic and peripheral blood-derived CD8$^+$ T cells and

associated with T cell dysfunction and exhaustion during chronic viral hepatitis [123,139,140]. Intrahepatic expression levels of PD-L1, PD-L2, and PD-1 correlated with liver inflammation in chronic HBV [30]. Although it has been shown that HCV core protein can induce PD-L1 expression on human perfusate-derived KC [84], it is not clear whether the upregulation of inhibitory ligands on intrahepatic macrophages and its correlation with inflammation are direct effects of HBV or HCV, or are components of negative feedback mechanisms that develop as a consequence of persistent inflammation.

Thus, several studies indicate that both HBV and HCV compromise anti-viral immunity to a certain extent by (1) interfering with signaling of pathogen recognition receptors and the production of pro-inflammatory cytokines by KC and (2) increasing the tolerogenic capacities of KC resulting in the elevated expression of anti-inflammatory mediators. As persistent inflammation in general is accompanied by negative feedback mechanisms, the KC-related anti-inflammatory signals observed during chronic viral hepatitis could be explained by direct viral effects, immune regulation as part of the ongoing inflammatory response, or a combination. However, also immune activating functions of KC have been described upon HBV/HCV interaction. These seemingly contradictory functions probably indicate a critical balance influenced by the extent to which receptors are triggered (or over-triggered) and also by the type of KC receptors that are triggered. Therefore, not only the concentration of virus (proteins), but also the time since infection may strongly affect KC function. Whether also age influences KC function as one of the mechanisms explaining the self-limiting hepatitis often seen in HBV-infected adults, whereas young children usually develop chronic infection, has to be investigated.

Role of KC in viral hepatitis-related liver damage

Liver fibrosis

One of the consequences of sustained low-grade injury induced by persistence of HBV and HCV in the liver is fibrosis, which is characterized by excess collagen deposition and accumulation of extracellular matrix. HBV and HCV may induce fibrinogenesis by activating hepatic stellate cells directly or indirectly by inducing cellular injury, apoptosis, and necrosis, which triggers a wound healing response. KC are thought to be involved in fibrogenesis by the release of various pro-fibrinogenic factors, such as ROS and certain cytokines, such as IL-6, TNF, IL-1, PDGF, and TGFβ, that induce activation of hepatic stellate cells [141]. In addition, KC produce enzymes that are important or the breakdown of matrix, such as collagenases and metalloproteinases, but they also regulate the production of these factors by other cells, leading to disturbance of the homeostatic mechanisms involved in extracellular matrix deposition [142]. Recent studies in experimental animal models demonstrate that these activities are only partially conducted by liver-resident macrophages, but largely depend on recruitment of monocytes as precursors of macrophages into the inflamed and damaged liver [143,144].

Although, in patients with viral hepatitis, no causative role has been demonstrated for KC in the development of liver fibrosis, increased numbers of $CD14^+CD68^+$ KC were found around the regions of damage and fibrosis [134]. These increased numbers were associated with liver injury [93,141,145,146]. A detailed study by Liaskou *et al.* observed that in liver tissue from non-viral hepatitis patients with end-stage liver disease a specific monocyte subpopulation accumulated in the liver, which was able to conduct phagocytic activity and to release inflammatory and profibrinogenic cytokines [147]. Interestingly, a study in HBV replication-competent transgenic mice showed an opposite effect of KC by demonstrating that they did not contribute to liver damage, but prevented liver injury by removal of apoptotic hepatocytes during viral hepatitis [39]. In this model, clodronate-mediated depletion of KC resulted in higher numbers of necrotic hepatocytes and elevated serum ALT levels. In line with this, in a different mouse model, liver-infiltrating monocyte/macrophages mediated regression of fibrosis via phagocytosis of cellular debris [148].

Liver damage and ultimately the induction of fibrosis may, at least in part, be attributed to cytokines produced by KC. Moreover, during viral hepatitis KC have also been found to express cytotoxic molecules, like TRAIL, Fas-ligand, granzyme B, perforin, and ROS, that enable them to kill infected as well as non-infected "bystander" hepatocytes [106–108]. Fas-ligand expression by KC was increased in chronic HBV patients and associated with elevated ALT levels, while granzyme B and perforin expression by KC was increased in both chronic HBV and HCV patients [106,107]. Interestingly, a direct contribution of KC to the pathogenesis of hepatitis has also been reported for viral infections by viruses that infect other organs and are not detected in the liver itself [149]. In influenza infection, KC were indicated as the effector cells killing hepatocytes in an as yet unidentified manner, leading to damage-associated hepatitis. KC can kill hepatocytes either directly via Fas-dependent apoptotic pathways or indirectly by interacting with $CD8^+$ (and possibly $CD4^+$) T cells through stimulation of cytokine secretion and other mediators, such as ROS [149].

Hepatocellular carcinoma

Chronic HBV/HCV and cirrhosis are major risk factors for the development of hepatocellular carcinoma [150]. Although HCC development has been extensively studied in mice and rat, only few studies have directly assessed the importance of KC in HCC development in chronic HBV settings, and no studies are available from chronic HCV settings. Dying hepatocytes, likely resulting from anti-viral activities since HBV and HCV are considered non-cytopathic, will activate neighboring cells, including KC [151], to produce cytokines and growth factors, such as hepatocyte growth factor, IL-6, and TNF, which will further amplify the inflammatory response and drive the compensatory proliferation of surviving hepatocytes [152]. Ongoing cycles of hepatocyte death and regeneration increase the chances of spontaneous mutations and DNA damage [153] eventually resulting in HCC. In HBV-transgenic mice, KC and/or infiltrating macrophages produced high levels of ROS, resulting in extensive oxidative DNA damage in neighboring proliferating hepatocytes and development of HCC [54]. HBV/HCV also activate KC to produce these types of pro-inflammatory mediators, which may support the development of HCC [84,89]. Additionally, the immunoregulatory mediators expressed by KC, either as a direct virus-KC interaction or as a consequence of the inflammatory response, may also inhibit tumor-specific immune responses. For instance, galectin-9 expressed on intrahepatic macrophages caused senescence of $CD4^+$ and $CD8^+$ $Tim3^+$ T cells, and may explain part of the

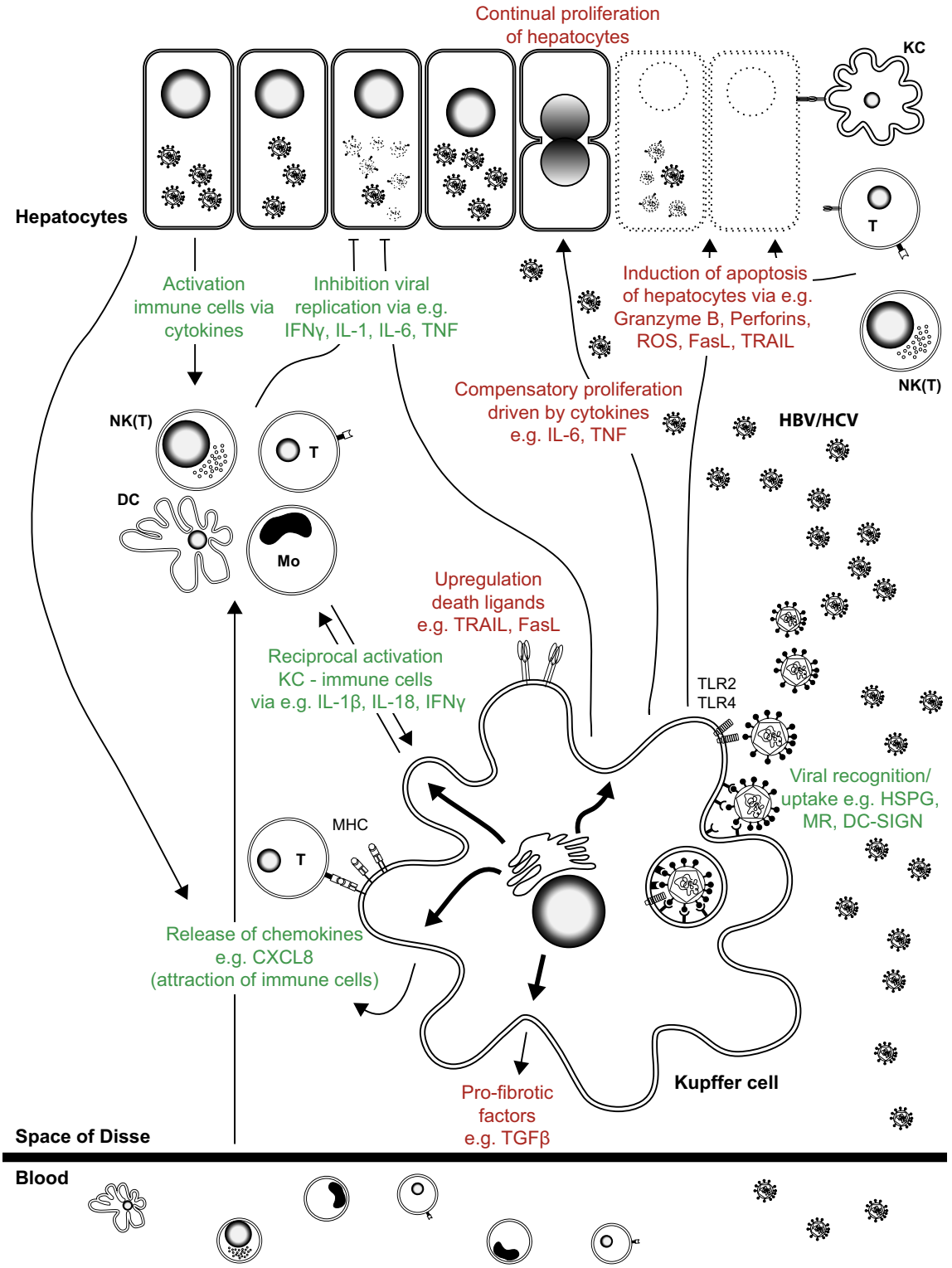

Fig. 1. The role of KC in anti-viral immunity and tissue damage during HBV and HCV infection. Exposure of KC to HBV or HCV will lead to direct activation of KC that, together with infected hepatocytes, release cytokines and chemokines, which are responsible for the attraction of other leukocytes. Activation of infiltrating immune cells leads to further production of cytokines that indirectly activate KC. The secreted cytokines may inhibit viral replication (green text). However, persistent exposure of KC to HBV or HCV will continuously activate KC leading to the ongoing release of cytokines and chemokines attracting and activating more leukocytes. Likewise, continuous activation of infiltrating leukocytes leads to ongoing production of cytokines that indirectly activate KC. Some of the cytokines secreted are pro-fibrotic factors. Additionally, KC and other immune cells are able to induce apoptosis of infected as well as uninfected hepatocytes, and release cytokines, which drive compensatory proliferation of hepatocytes. The ongoing cycles of hepatocyte death and regeneration increase the chances of spontaneous mutations and DNA damage, which may eventually result in HCC (red text).

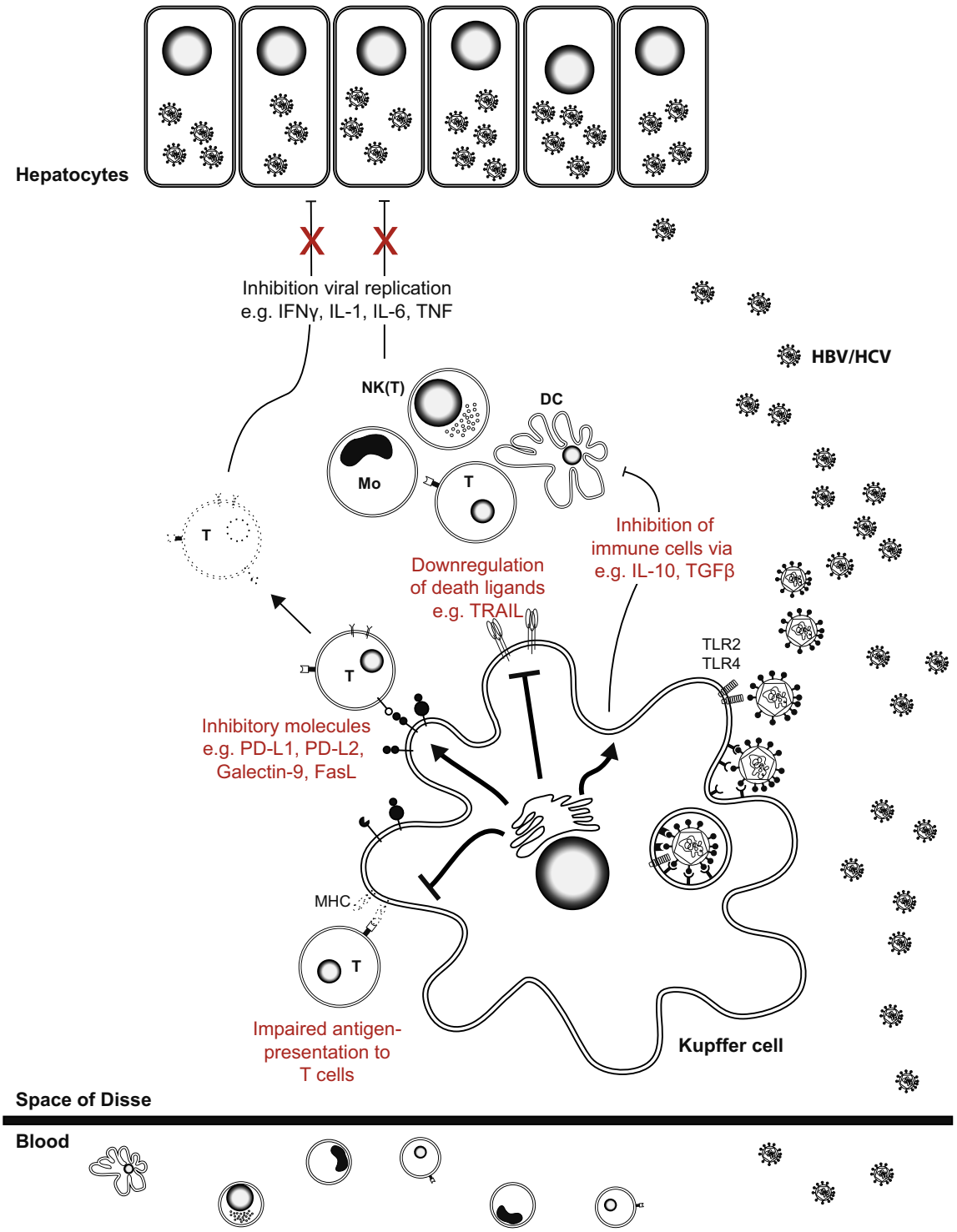

Fig. 2. Role of KC in immune regulation and viral persistence during HBV and HCV infection. Exposure of KC to HBV or HCV will lead to their activation and the release of anti-inflammatory cytokines and expression of inhibitory molecules. Combined with impaired antigen presentation by KC, these regulatory mechanisms will interfere with KC function and that of other immune cells, frustrating anti-viral immunity.

mechanism leading to the development of HCC [154]. Furthermore, one of the HBV-derived proteins, HBxAg, also has direct tumorigenic effects [155]. Hepatocyte regeneration, either influenced by KC or not, allows HBxAg integration in DNA of hepatocytes, which is one of the processes involved in the development of HCC (reviewed in [153]). Whether HBxAg directly interacts with KC is not described.

In conclusion, KC play a central role in liver damage during hepatitis, having all the tools to induce inflammation, cell death, fibrosis, and ultimately HCC, but further research during

HBV/HCV infection remains to be carried out to determine the exact contribution of KC to liver damage in viral hepatitis.

Perspectives

Currently, our understanding of the role of KC in viral hepatitis is incomplete. The detailed contributions of liver-resident KC *vs.* liver-infiltrating macrophages to various processes of disease pathogenesis are difficult to determine, because of the highly overlapping characteristics of these cells. Nevertheless, we can appreciate several possible anti-viral roles of KC, including binding and/or uptake of virus leading to immune recognition and the production of pro-inflammatory mediators resulting in (1) inhibition of viral replication in hepatocytes, (2) activation of neighboring cells, and (3) attraction, activation, and interaction with other immune cells, which will further increase the anti-viral and inflammatory response (Fig. 1). These immune activating roles of KC are beneficial to combat HBV and HCV in the early phases after infection, but may also contribute to tissue damage and the development of fibrosis, cirrhosis, and HCC during chronic viral hepatitis (Fig. 1). Furthermore, also immune regulatory functions of KC have been described, either as a consequence of direct virus-KC interaction, or as part of the complex tolerogenic liver environment and the ongoing inflammatory response upon HBV and HCV-infection, which may counteract the development of effective anti-viral immunity and support viral persistence and related disease pathogenesis (Fig. 2).

With our growing appreciation of the roles of intrahepatic macrophages in both protective and harmful responses, intrahepatic macrophages form an interesting but complex cellular target for treatment options in viral hepatitis. The versatile features assigned to KC may partly belong to infiltrating monocytes/macrophages and therefore future efforts should focus on identifying phenotypical and/or functional characteristics discriminating KC from infiltrating macrophages. Furthermore, the function of KC and other intrahepatic macrophages will largely depend on type, level, and duration of receptors triggered pushing the balance towards either protective or harmful responses. Identification of receptors and underlying molecular mechanisms involved in virus-cell interactions and insight into mechanisms involved in wanted and unwanted responses of the different macrophage populations that exert distinctive functions during the early and later phases of HBV/HCV infection are needed to move the field forward.

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Conflict of interest

The authors declared that they do not have anything to disclose regarding funding or conflict of interest with respect to this manuscript.

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