Chemokines in the pathogenesis and as therapeutical markers and targets of HCV chronic infection, and HCV extrahepatic manifestations

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Abstract

Cytokines and chemokines, hepatitis C virus (HCV) infection-induced, participate in viral control and liver damage. The complex cytokine network, operating during initial infection allows a coordinated and effective development of innate and adaptive immune responses. “HCV interferes with cytokines at various levels and escapes immune response by inducing a T helper (Th)2/T cytotoxic 2 cytokine profile”. A predominance of the Th1 immune response (and related cytokines) has been evidenced in chronic hepatitis C infection and in extrahepatic manifestations. Interferon (IFN)-γ and IFN-γ-inducible chemokine (C-X-C motif) ligand (CXCL)9, -10 and -11 recruit inflammatory infiltrates into the liver parenchyma due to the incapability to control the infection process, resulting in extensive liver damage and liver cirrhosis. “The most important systemic HCV-related extrahepatic diseases — mixed cryoglobulinemia, lymphoproliferative disorders, diabetes and autoimmune thyroid disorders — are associated with a complex dysregulation of the cytokine/chemokine network and involve pro-inflammatory and Th1 chemokines. The therapeutical administration of cytokines such as IFN-α may result in viral clearance during persistent infection and reverts this process” reducing circulating CXCL10 levels. “Several studies have reported interleukin (IL)-28B polymorphisms, and circulating CXCL10, may be prognostic markers for HCV treatment efficacy in HCV infection”. Other studies have also shown that HCV clearance by directly acting antiviral agents therapy decreases circulating CXCL10 levels. “Theoretically agents that selectively neutralize CXCL10 could increase patient responsiveness to traditional IFN-based HCV therapy”, simultaneously reducing inflammatory immune cell activation.

Keywords: chemokines, CXCL10, cytokines, IFN inducible chemokines, HCV extrahepatic manifestations, hepatitis C chronic infection, hepatitis C virus.

1. INTRODUCTION

Chemokines are small proteins participating in leukocyte trafficking [1]; they are divided into two major (C-X-3-C motif and C-X-C motif) and two minor (C-C motif and C motif) subfamilies [2-4]. Only one non-conserved aminoacid residue, that separates the N-terminal cysteines, is present in the C-X-C subfamily. "Interferon (IFN)-γ-inducible C-X-C chemokines [IFN-γ-induced protein 10 (IP-10/CXCL10), monokine induced by IFN-γ (Mig/CXCL9), and IFN-inducible T-cell chemoattractant (I-TAC/CXCL11)]", are members of the C-X-C subfamily. These C-X-C chemokines are preferentially expressed on T helper (Th)1 lymphocytes [5-7]. These chemokines bind to C-X-C chemokine receptors (CXCR), and activate "CXCR3 which is a seven trans-membrane-spanning G protein-coupled receptor expressed in activated Th1 lymphocytes [8], Natural Killer (NK) and B cells, and other cells [9,10]".

In response to IFN-γ, CXCL10 is released by different cells, as activated neutrophils, monocytes, epithelial cells, endothelial cells [11,12] and "attracts activated Th1 lymphocytes into the area of inflammation; its expression is associated with a Th1 immune response [13-15] and is increased in infectious, inflammatory [16,17] and autoimmune diseases [2], and cancer". Participating in leukocyte homing to inflamed tissues, CXCL10 worsens inflammation leading to a significant tissue damage [2].

Increased concentration of CXCL10 in biological fluids or tissues is a marker of Th1 orientated T-cells immune response. Activated Th1 lymphocytes secrete tumor necrosis factor (TNF)-α and IFN-γ, that stimulate CXCL10 release from the above reported cells, reiterating the immune cascade by an amplification feedback loop [18].

Organ specific autoimmune diseases, as type 1 diabetes [19-21], Graves' disease [22,23] or Graves' ophthalmopathy, autoimmune thyroiditis (AT) [24], or rheumatological disorders, as systemic lupus erythematosus, rheumatoid arthritis, systemic sclerosis [25-27], psoriasis or psoriatic arthritis [28], show an increased tissue and/or serum CXCL10 expression.

The Th1 immune response is also involved in various neoplastic disorders [29-32], and in the pathogenesis of infectious diseases [33-36], as of those by Mycobacterium tuberculosis [34], Toxoplasma gondii [35], sepsis [37], and others.

2. Hepatitis C

Hepatitis C is caused by the hepatitis C virus (HCV), affecting 150–200 million people worldwide [38]. HCV causes acute symptoms in 15% of cases [39,40]. About 85% of the subjects exposed to HCV develop a chronic infection (CHC) [40-42], and (among them) about 20% of patients develop cirrhosis (over 30 years; overall in patients with risk factors, such as coinfection with hepatitis B, schistosoma, or HIV, use of alcoholics and male gender). HCV cirrhosis is associated with a 20-fold greater risk of hepatocellular carcinoma (HCC) [43-45]. CHC is also associated with extrahepatic manifestations (HCV-EHMs); "the most common is mixed cryoglobulinemia (MC; usually the type II form; an inflammation of small and medium-sized blood vessels)". Other HCV-EHMs are AT, diabetes mellitus, Sjögren's syndrome, porphyria cutanea tarda, and B-cell lymphoproliferative disorders [40,46].

3. Innate immunity in CHC

Activation of innate immune pathways in hepatocytes following HCV infection leads to infiltration of anti-viral immune competent cells into the liver [47].

The recognition of foreign motifs of DNA, RNA, or protein (namely "pathogen associated molecular patterns") by innate pattern recognition receptors (PRRs) activate cellular innate immune pathways. The innate immune response is determined by the interplay of PRRs [belonging to three PRRs families: "Toll-like receptors (TLRs), "retinoic acid inducible gene 1 (RIG-I)-like receptors" (RLRs), or Nod-like-receptors (NLRs)"], and their downstream signaling pathways.

The positive sense HCV RNA is recognized by two different PRRs in the hepatocyte: RIG-I and TLR3 [48]. TLR3 recognizes longer double-stranded RNAs (generated during viral replication) [48]; activated TLR3 binds the signaling adaptor "Toll/interleukin (IL)-1 receptor (TIR)-domain-containing adapter-inducing IFN-β (TRIF)" [49]. Double-stranded, 5′ tri-phosphate RNAs containing poly-U or poly-A motif binds to RIG-I and activates the mitochondrial antiviral-signaling protein (MAVS) signaling adaptor [49]. TRIF and MAVS signaling activate nuclear factor (NF)-κB, C/EBP-β, and IFN regulatory factors (IRFs), which induce gene transcription [49]. These transcription factors have binding sites in the CXCL10 promoter. It has been shown that HCV can induce NF-κB binding to this site in TLR3-expressing hepatocytes [48].

In hepatocytes HCV activation of IRF3 and IRF7 can induce "type I IFNs (IFN-α and IFN-β) and type III IFNs (IL-28A, IL-28B, IL-29)" with anti-viral activity. These cytokines amplify cytokines and chemokines response in liver cells [activating Janus kinases (JAKs) and other signal transducer and

activator of transcription (STAT) proteins] [50]. Activation of JAK-STAT signaling [through the binding of STAT dimers to IFN-stimulated response elements (ISREs) or IFN-γ activation site elements in their promoters] [50] induces IFN-stimulated genes (ISGs). Type II IFN or IFN-γ (produced by infiltrating NK cells, CD8+ T cytotoxic (Tc) cells, and CD4+ Th1 cells) can also induce STAT1-signaling through these elements [50]. The CXCL10 promoter can respond to all three types of IFN, containing putative ISREs and putative STAT-binding sites [51].

The synthesis of anti-viral type-1 IFN [52] is the primary cell defense mechanism at the beginning of the infection; IFN-α/β can stop viral replication and spread to other cells. However, despite type-1 IFN activation, HCV, at least partially, does not respond to IFN-α/β, and may replicate in the liver; in fact, nonstructural proteins E2, NS3, and NS5A may stop the transcription and "expression of IFN-α/β-induced genes" [53].

NK and Natural killer T (NKT) cells exert their anti-viral action via: a- secretion of IFN-γ [54]; b-direct mechanisms [54]; c- facilitating maturation of dendritic cells (DCs) and development of Th1/Tc1 responses [55]. However, HCV attempts to escape the immune reaction blocking NK and NKT cells functions (through the "interaction between HCV E2 protein and NK-cell CD81 molecule") [56].

During HCV infection the interaction of HCV structural proteins with TLR-2 in monocytes can lead to a decrease in IFN-α production [57], inducing IL-10 and inhibiting IL-12 and IFN-α secretion in DCs [58]; also an increased IFN-α production in patients not responding to exogenous IFN-α has been shown [59].

Several studies have demonstrated that a "progressive liver injury in CHC is associated with increased expression of Th1-associated cytokines" [60].

4. Adaptive response in CHC

HCV CD4+ T cells are determinant in adaptive response. These cells secrete Th1 cytokines (as IFN-γ) that induce the recruitment of neutrophil and macrophage, leading to inflammatory response. However, HCV CD4+ T cells release also Th2 cytokines (IL-10, IL-4), which confine Th1 immune response and induce the humoral response [61]. In HCV infections evolving to resolution a strong and sustained, CD4+ T cell specific Th1 response is present [62]. When HCV infection evolves to the chronic phase "a weak CD4-T specific response, and scarce type-1 cytokine

production", are evident [63].

CD8+ Tc are able to clear viruses through type-1 cytokines (IFN-γ, TNF-α), and/or apoptosis-related cytolytic mechanisms. In CHC specific Tc show reduced type-1 cytokine secretion and have anergic characteristics [64]. It has also been shown that, in CHC, regulatory T cells can release IL-10 and transforming growth factor (TGF)-β, inhibiting cytokine synthesis in T cells and proliferation [65].

T cells are determinant in the regulation of humoral responses secreting cytokines. Such humoral responses are not able to control CHC, however, are important in the pathogenesis of HCV-EHMs [66].

5. CXCR3 chemokines and chronic HCV infection

In CHC, "lymphocytes infiltrating hepatitis C-infected liver express elevated levels of CCR5 and CXCR3, and the CXCR3 ligands CXCL10 and CXCL9 are up-regulated on sinusoidal endothelium [67]. *In vitro*, human hepatic sinusoidal endothelial cells", stimulated with IFN-γ in combination with TNF-α secreted CXCL10 and CXCL9. These results suggested that intrahepatic production of IFN-γ drives the increased expression of CXCL10 and CXCL9, promoting "the continuing recruitment of CXCR3-expressing T cells into the hepatic lobule" in CHC [67].

A further study evaluated the expression of CXCL10 both in serum and in the liver of patients with CHC. Unlike in chronic hepatitis B or in liver disorders unrelated to viral infections, in CHC CXCL10 expression was highly correlated with the amount of transcripts for IFN-γ [68].

It was also shown that hepatocytes expressed CXCL10, but not other cell types in the liver. The predominant CD8 T lymphocytes present in the infiltrate of liver lobule were nearly uniformly CXCR3-positive, and the CXCL10 mRNA expression correlated with lobular necroinflammatory activity, indicating that HCV can induce CXCL10 in the hepatocytes, being determinant in the pathogenesis of CHC [69].

In human hepatocyte-derived cells it was demonstrated that NS5A and core proteins, alone or acting synergistically with cytokines, are able to upregulate CXCL10 and CXCL9 genes [70].

The induction of CXCL10 secretion by HCV in hepatocytes has been confirmed in other studies [71,72].

It has also been shown that "intrahepatic CXCL10 mRNA expression levels were significantly

associated with lobular necroinflammatory grade and HCV genotype 1" [73].

Moreover CXCL10 plasma levels were significantly elevated in patients with advanced fibrosis, suggesting that CXCR3-associated chemokines could be considered noninvasive markers of hepatic fibrosis in HCV genotype 1-infected patients [74]. Other studies confirmed that the CXCR3 chemokines are the most significantly expressed ones in CHC probably being determinant in positioning T cells in the liver [75,76]. Also other studies confirmed that CXCL10 is a marker of liver fibrosis in CHC [77-79].

Furthermore it has been suggested that CXCL10 (induced by HCV) leads to increased hepatocyte turnover and the development of fibrosis, cirrhosis, and HCC [80].

HCV antiviral therapy effect on CXCL10 has been also evaluated. In a first study it was shown that after successful treatment of CHC, the serum level of CXCL10 decreased to the same level as in healthy volunteers [81].

Before, during, and after antiviral therapy chemokine levels were tested in 29 patients infected with HCV genotype 1 [82]. CXCL10 and CXCL9 were elevated, and decreased upon successful antiviral therapy. Patients with HCV who subsequently became non-responders (NR) to therapy had the highest baseline level of CXCL10 (assayed before the start of antiviral treatment); suggesting that CXCL10 plasma concentrations could predict responsiveness or nonresponsiveness to antiviral therapy with pegylated IFN (PEG-IFN), with or without ribavirin (RBV) [82].

"To analyse the possible association of serum CXCL10 levels with different outcomes to antiviral therapy, serum CXCL10 levels were determined in 137 CHC patients treated with PEG-IFN plus RBV. Pretreatment serum CXCL10 levels in patients with sustained viral response (SVR) were significantly lower than in NR (332.4 versus 476.8 pg/mL). Serum CXCL10 concentrations significantly decreased in patients with SVR but not in NR. In patients with genotype 1, baseline viral load and pretreatment serum CXCL10 levels were identified as predictive factors of SVR [83].

In a further study treatment with PEG-IFN α-2b plus RBV of CHC patients increased CCR5(high)/CXCR3(high) expressing CD8+ cells frequency in peripheral blood and decreased CXCL10/CCL3 serum concentration. Achievement of viral control was associated with an increase in CXCR3(high) expressing CD8+ cells during treatment" [84].

RBV "improves the kinetics of the early response to therapy in patients with an adequate initial response to PEG-IFN. Patients administered with the combination therapy had serum CXCL10 higher

at 12 hours than those given with PEG-IFN only; the difference was greatest in patients with an adequate first-phase decrease in HCV RNA. CXCL10-induction correlated with first- and second-phase kinetics and with RBV serum concentrations" [85].

Also other studies confirmed that pretreatment CXCL10 levels predicted for both early virological response (EVR) and SVR, to IFN-α and RBV, and may be useful to evaluate candidates for therapy [86-92].

It has been also shown that serum CXCL10 level is a predictor for SVR in HCV-4-infected patients. The baseline CXCL10 level was significantly lower in responders among HCV genotype-4 patients [93,94].

"Polymorphisms of the IL-28B gene are strongly associated with SVR in patients with CHC treated with PEG-IFN and RBV. CXCL10 levels were evaluated in pretreatment serum from 115 NR and 157 sustained responders in the Study of Viral Resistance to Antiviral Therapy of Chronic Hepatitis C cohort and resulted lower in sustained responders with respect to NR [95]. The positive predictive value of low CXCL10 levels (< 600 pg/mL) for SVR was 69%, whereas the negative predictive value of high CXCL10 levels (> 600 pg/mL) was 67%. Whether the combination of pretreatment CXCL10 levels with IL-28B genotype can be considered predictors of treatment response was evaluated. The IL-28B polymorphism rs12979860 was tested. The CC, CT, and TT genotypes were shown in 30%, 49%, and 21% of patients, respectively, with corresponding SVR rates of 87%, 50%, and 39%. Serum CXCL10 levels within the IL-28B genotype groups provided additional information regarding the probability of SVR ($P < 0.0001$). CT carriers with low CXCL10 had 64% SVR versus 24% with high CXCL10. Similarly, a higher SVR rate was identified for TT and CC carriers with low versus high CXCL10 (TT, 48% versus 20%; CC, 89% versus 79%). The Authors concluded that when IL-28B genotype is combined with pretreatment serum CXCL10 measurement, the predictive value for discrimination between SVR and nonresponse is significantly increased, particularly in non-CC genotypes" [95].

Also other studies confirmed that CXCL10 is a useful biomarker to predict treatment failure in HCV patients in combination with IL-28B [96-100].

HCV treatment is changing over time from IFN-α-based therapies to IFN-α-free regimens comprising directly acting antiviral agents (DAAs), showing increased efficacy and tolerability in clinical trials. Upon DAA therapy treatment failure can commonly occur owing to virologic relapse; however, the

reason why relapse occurs or whether certain subjects are more prone to recurrent viremia is still not known. “A clinical trial using the DAA sofosbuvir plus ribavirin (SOF/RBV) performed detailed mRNA expression analysis in liver and peripheral blood from patients who achieved either a SVR or relapsed. On-treatment viral clearance was accompanied by rapid downregulation of ISGs in liver and blood, independently from treatment outcome. Analysis of paired pretreatment and end of treatment (EOT) liver biopsies from SVR patients showed that viral clearance was accompanied by decreased expression of type II and III IFNs, but unexpectedly increased expression of the type I IFN IFNA2. The above reported data indicate that restoration of type I intrahepatic IFN signaling by EOT may facilitate HCV eradication and prevention of relapse after withdrawal of SOF/RBV” [101].

Also other studies confirmed that HCV clearance by DAAs therapy decreases circulating CXCL10 levels [102-104].

6. CXCR3 chemokines and HCV-associated MC

MC is commonly considered a systemic small vessel vasculitis [105,106] and, considering the presence of polyclonal or oligo-monoclonal immunoglobulin M, two types are known (type 2, IgG + monoclonal IgM; and type 3, IgG + polyclonal IgM).

“MC is considered a ‘benign’ B-cell lymphoproliferative disease, as the expansion of rheumatoid factor-producing B-cells is its underlying disorder” [107]. It is suggested that a determinant role in the pathogenesis of hepatitis C virus-associated mixed cryoglobulinemia (HCV+MC) is the inhibition of B-cells apoptosis, that causes their progressive accumulation [107].

Different studies have shown [108,109] that “the evolution of HCV infection toward MC is associated with a strong Th1 response”.

Moreover, we have demonstrated that “circulating CXCL10 and CXCL11 are higher in patients with HCV+MC than in chronic HCV patients”, and they are more strongly elevated in patients with HCV+MC with respect to healthy subjects, especially if active vasculitis is present. Circulating IFN-γ and CXCL11 are markedly associated, suggesting the importance of a Th1 immune response in the pathogenesis of HCV+MC [110-116].

On the whole CXCL10 and CXCR3 seem to be implicated in the pathogenesis of HCV+MC. “The secretion of CXCL10 by CD4+, CD8+ and NKT cells depends on IFN-γ, which is itself mediated by the IL-12 cytokine family. Stimulated by IFN-γ, CXCL10 is secreted by several cell types as

lymphocytes, hepatocytes, endothelial cells, fibroblasts, etc." In tissues, recruited Th1 lymphocytes could be associated with an increased production of IFN-γ and TNF-α, which in turn stimulates CXCL10 secretion from the cells, perpetuating the autoimmune process through an amplification feedback loop. High levels of CXCL10 in circulation have been found in HCV+MC, especially in patients with clinically active vasculitis. Furthermore, HCV+MC patients with AT have higher levels than those without AT.

7. Thyroid disorders associated with CHC

"Many studies have recently confirmed an association of HCV infection with autoimmune thyroid disorders (AITD) in adults [117,118], and children [119]. The thyroid disorders observed in CHC are characterized by a higher risk of AT in females, increased serum levels of anti-thyroperoxidase antibodies, and increased risk of hypothyroidism. Recently several studies have confirmed a high frequency of AT in patients with MC and CHC [120].

An elevated prevalence of papillary thyroid cancer (PTC) has been observed in patients with CHC, and more recently in MC patients, overall in the presence of AT [18,121]. The increased prevalence of PTC in CHC and MC is clinically relevant as approximately 10-30% of these patients may have an aggressive disease, requiring systemic treatments".

The immuonopathogenesis of thyroid disorders associated with CHC and MC has been studied in many papers. Patients with AT have elevated levels of CXCL10, CXCL9, CXCL11, especially if hypothyroidism is present, and the Th1 immune response is implicated in the induction of AT, Graves' disease and Graves' ophthalmopathy [116]. Moreover, HCV has been demonstrated to be present in the thyroid of chronically infected patients [122,123].

On these bases, it has been hypothesized that HCV thyroid infection could upregulate CXCL10 expression and secretion in infected thyrocytes recruiting Th1 cells, that secrete IFN-γ and TNF-α inducing a further CXCL10 release by thyrocytes, initiating and perpetuating the immune cascade, that leads into the appearance of AT in genetically predisposed subjects [124,125]. Many studies have confirmed the above reported theory and have shown elevated serum CXCL10 in patients with CHC, and MC, in the presence of AT [126].

Interestingly, the fact that in MC patients "circulating CXCL10 is significantly higher in presence of AT, compared to patients without thyroiditis", while CCL2 (the prototype Th2 chemokine) is comparable, indicates that the Th1 CXCL10 chemokine is specifically linked with the appearance of AT in these patients [124,127].

The above reported data highlight the importance of the Th1 immune response in the initiation and perpetuation of AT in patients with CHC and MC, in agreement with the observations in other autoimmune disorders.

Interestingly, the increasing prevalence of HCV somehow parallels the increasing prevalence of autoimmune thyroid disease that has been documented by other studies [128,129].

8. The role of cytokines in the therapy of CHC and MC

IFN-α is the cytokine commonly used in the treatment of CHC. PEG-IFN-α combined with RBV causes SVR in 50% of patients [130]. The principal effect of IFN-α is anti-viral, but it has an immunemodulatory action favoring Th1/Tc1 response restoration, too [131]. RBV, a wide-spectrum antiviral agent administered in combination therapy for CHC, has immunomodulatory effects inducing type-1 IFN production [132]. A sustained viral load reduction, with antiviral drugs, facilitated specific T response recovery and the production of type-1 cytokines in CHC [133,134].

A reduced intrahepatic inflammation is obtained also by the administration of Th1-inducing (as IL-12) [135] or anti-inflammatory (as IL-10) cytokines [136]; even if, such therapies remain experimental.

Very recently, different "genome-wide association studies identified single nucleotide polymorphisms near the IL-28B region (rs12979860 and rs8099917) that are strong pretreatment predictors of early viral clearance and SVR in patients with genotype 1 HCV infection [137,138]. IL-28B encodes IFN-λ3, a type III IFN involved in host antiviral immunity. IFN-λ3 up-regulates ISGs (similarly to IFN-α and IFN-β) but via a different receptor; moreover IFN-λ3 affects the adaptive immune response [139]. Further investigations have implicated IL-28B in the spontaneous resolution of acute infection versus the development of chronic HCV infection, suggesting that IL-28B may be a key factor involved in host immunity against HCV [137,138,140].

Clinical studies assessing safety and efficacy in the treatment of HCV with exogenous IFN-λ3 suggest that IFN-λ3 treatment inhibits HCV, and hepatotoxicity in both healthy volunteers and HCV-infected patients has been shown" [141,142].

Conclusion

Cytokines and chemokines, induced by infection with HCV, are implicated in viral control and liver damage. A coordinated and effective development of innate and adaptive immune responses is permitted by the complex cytokine network, that operates at the beginning of the infection. HCV interferes with cytokines at various levels and escapes immune response through the induction of a Th2/Tc2 cytokine profile. A predominance of the Th1 immune response (and related cytokines) has been shown in CHC and in HCV+EHMs. IFN-γ and IFN-γ-inducible CXCL9, -10 and -11 chemokines recruit inflammatory infiltrates into the liver parenchyma owing to the inability to control the infection process, causing sustained liver damage and eventually liver cirrhosis. "The most important systemic HCV-related extrahepatic diseases — MC, lymphoproliferative disorders, diabetes and AITDs — are linked to a complex dysregulation of the cytokine/chemokine network" and involve pro-inflammatory and Th1 chemokines. Viral clearance may be the outcome of therapeutical administration of cytokines such as IFN-α during persistent infection, reverting this process. "Several studies have reported IL-28B polymorphisms, and circulating CXCL10, as prognostic markers for HCV treatment efficacy in HCV infection. Clinical studies assessing safety and efficacy in the treatment of HCV with exogenous IFN-λ3 are ongoing". Several studies have also shown that HCV clearance by DAAs therapy decreases circulating CXCL10 levels.

"Theoretically agents that selectively neutralize CXCL10 could increase patient responsiveness to traditional IFN-based HCV therapy", simultaneously reducing inflammatory immune cell activation. For example, specific inhibitors of the CXCR3A could prevent excessive activation of CD8+ Tc cells and NK cells that lead to excessive hepatocyte death, limiting Kupffer cells and hepatic stellate cells activation and delaying or preventing development of fibrosis.

Conflict of Interest

The authors have no conflict of interests to declare.

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