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CHEMOKINES IN LIVER INFLAMMATION AND FIBROSIS

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TABLE OF CONTENT

1. ABSTRACT

Chemokines may be involved in the tissue response to injury regulating the influx of leukocytes, and modulating a number of other critical biologic actions, including angiogenesis, neoplastic growth, myo-fibroblast activation, and the response to viral infections. In the liver, up-regulated expression of different members of the chemokine system may be induced by almost all types of injury, and there is often a clear relation between the chemokine pattern activated by different types of injury and the predominant subclasses of leukocytes which infiltrate the liver. Neutralization of specific chemokines by passive immunization or the use of animals deficient in specific chemokines or chemokine receptors has indicated a causal relation between up-regulation of chemokines and leukocyte infiltration.

Inflammation is part of the liver wound healing response, that in chronic conditions leads to the development of fibrosis and cirrhosis. Hepatic stellate cells, which play a leading role in the development of fibrosis following their transition to myofibroblasts, express different chemokines. Chemokine expression by stellate cells is regulated by soluble mediators, in particular pro-inflammatory cytokines, as well as growth factors, proteases, and products of oxidative stress. In addition, stellate cells also respond to chemokines with biologic actions relevant for tissue repair, such as cell migration or induction of other chemokines. These data indicate that chemokines in the liver may modulate the progression of liver fibrosis through actions on hepatic stellate cells.

2. OVERVIEW OF THE CHEMOKINE SYSTEM

The word chemokine is the result of the fusion of 'chemotactic cytokines' and indicates a family of cytokines with the ability to stimulate cell migration (1). Chemokines were identified in the late 80s when interleukin-8 (CXCL8) and MCP-1[1] (CCL2) were discovered (2). Because these molecules showed a certain degree of selectivity in the recruitment of neutrophils and monocytes, respectively, chemokines were regarded as selective chemoattractants of leukocyte populations in different conditions of tissue inflammation. Further studies have shown that the chemokine family comprises a high number of ligands and receptors, that selectivity of leukocyte recruitment is not always the rule and that many other cell types are targets of

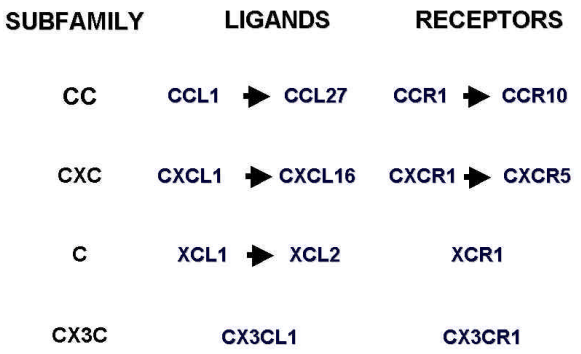

Figure 1. The chemokine system. Chemokines are divided into four subgroups based on the primary structure, and specifically the position of conserved cysteine residues. Each ligand is designated with the letters of the subgroup, the letter 'L' and an Arabic number. The receptors follow a similar nomenclature with the letter 'R'.

he action of the chemokines. More than 40 chemokines have been identified in humans and have been sub-divided into four subgroups based on conserved cysteine residues (1). The CC chemokines are characterized by adjacent cysteines, while molecules of the CXC subgroup have an amino acid in between. The C chemokines, which include the lymphotactins, have only one conserved cysteine, and finally fractalkine (CX3CL1) is the only CX3C chemokine so far identified, and has three aminoacids between two conserved cysteines (Figure 1). The rapid discovery of chemokines by different research groups has led to a very complicated nomenclature and to the identification of the same molecule with as many as six different names or acronyms. For this reason, a new nomenclature system for chemokines has been proposed, based on the one already in use for the receptors (3). Thus, all members of each subgroup are named after their primary structure and a number. The complexity of the chemokine system is paralleled by the large number of receptors. Chemokine receptors are belong to the seven transmembrane domain superfamily of G protein-coupled receptors and are grouped into C, CC, CXC, and CX3C subfamilies based on the ability to bind chemokines of the related subgroup (4). All chemokine receptors bind one or more ligands in the same subgroup, i.e. CC receptors bind exclusively CC ligands, with the only exception of mouse, but not human, CCL21 that activates CCR7 and CXCR3 (5,6). The duffy antigen receptor for chemokines (DARC) has the ability to bind a large number of chemokines of all classes, and is a receptor for the malarial parasite *P. vivax* (7). DARC is localized on the membrane of red blood cells and the interaction with chemokines does not elicit any signaling. Finally, a group of chemokine receptors is encoded by viral genes, and the importance of these molecules in the modulation of viral-host interactions has been recently reviewed (8).

Recent investigation, including data deriving from genetically modified animals, has enormously expanded the fields in which the chemokine system is implicated, much beyond the control of inflammation (Figure 2). The areas of development, angiogenesis, cancer, wound repair, fibrosis, response to infection, leukocyte differentiation and homing have all been shown to be affected by members of the chemokine system, with relevant implications for the pathogenesis and treatment of diseases (1,9). Accordingly, the chemokine system has been shown to have an impact also on the regulation of different functions within the liver. Thus, the recognition of the role of the chemokine system in the recruitment and activation of different leukocyte populations has been paralleled by the identification of several other biologic functions which play a role in different conditions of damage and repair. This review will focus on the 'classical' actions of chemokines in different inflammatory conditions and will briefly review the importance of this system in other areas of liver disease. I have divided the review according to the different clinical situation in which chemokines could be important. The data obtained in animal models of liver injury and repair have been discussed in the section that was deemed more appropriate, but it should be kept in mind that animal models do not always represent a model system for a single specific disease. Finally, the significance of the chemokine system in the pathogenesis of liver fibrosis will be discussed, and the relations between chemokines and hepatic stellate cells will be analyzed in detail. Because most of the cited studies refer to the old chemokine nomenclature, I have maintained the original names to make it easier to refer to the older literature. Table 1 indicates the different names used for the chemokines discussed in this review, together with the name suggested by the new nomenclature.

3. ALCOHOLIC LIVER DISEASE

Alcoholic hepatitis is characterized by an intense inflammatory infiltrate which may be associated with other pathologic aspect of this disorder, including steatosis and/or cirrhosis. Inflammatory cells are mainly represented by neutrophils and the release of elastase and other factors upon activation of these cells is believed to contribute to the pathogenesis of liver damage. Because of the quantitative relevance of neutrophilic infiltration, and of the presence of systemic neutrophilia in most patients with alcoholic hepatitis, attention of many groups has concentrated on the group of CXC chemokines which contain a glutamic acid-leucine-arginine (ELR) motif, and are selective neutrophil chemoattractants via interaction with the receptors CXCR1 and CXCR2, and much emphasis has been directed to interleukin-8 (IL-8). Sheron *et al* (10) compared the serum and plasma levels of this chemokine in patients with different types of alcoholic liver disease and with non-alcohol-related liver injury. In patients with alcoholic hepatitis the highest levels of circulating IL-8 were observed, especially in those with a poor prognosis. These findings were paralleled by the observation that in tissue homogenates from patient with alcoholic hepatitis, IL-8 concentrations were almost tenfold higher than in other alcoholic liver diseases. These data have been subsequently confirmed by several other groups, which also showed the reversibility of the elevation of IL-8 levels during the remission of alcoholic hepatitis (11). The levels of IL-8 in the serum of patients with alcoholic hepatitis is generally correlated with the severity of the

Table 1. Correspondence between the old and new chemokine nomenclature, with the cognate receptors bound

Subfamily	Old nomenclature	New nomenclature	Receptor(s) bound
CXC	GRO-α, KC[a,b]; MIP-2[a,b]	CXCL1	CXCR2
	GRO-β	CXCL2	CXCR2
	GRO-γ	CXCL4	CXCR2
	ENA-78	CXCL5	CXCR1, CXCR2
	IL-8; CINC[b]	CXCL8	CXCR1, CXCR2
	Mig	CXCL9	CXCR3
	IP-10; Crg-2[b]	CXCL10	CXCR3
	I-TAC	CXCL11	CXCR3
	SDF-1	CXCL12	CXCR4
CC	MCP-1	CCL2	CCR2
	MIP-1α	CCL3	CCR1, CCR5
	MIP-1β	CCL4	CCR1, CCR5
	RANTES	CCL5	CCR1,CCR3,CCR5
	MCP-3	CCL7	CCR1,CCR2,CCR3
	MCP-2	CCL8	CCR1, CCR2, CCR3, CCR5
	Eotaxin	CCL11	CCR3, CCR5
	TARC	CCL17	CCR4
	PARC	CCL18	Unknown
	ELC	CCL19	CCR7
	SLC	CCL21	CCR7
C	Lymphotactin-α	XCL1	XCR1
	Lymphotactin-β	XCL2	XCR1
CX3C	Fractalkine	CX3CR1	CX3CR1

[a] KC and MIP-2 are related to each of the three human GRO proteins (CXCL1-3) [b] Indicates a mouse or rat protein Note: Only the chemokines discussed in this review are included.

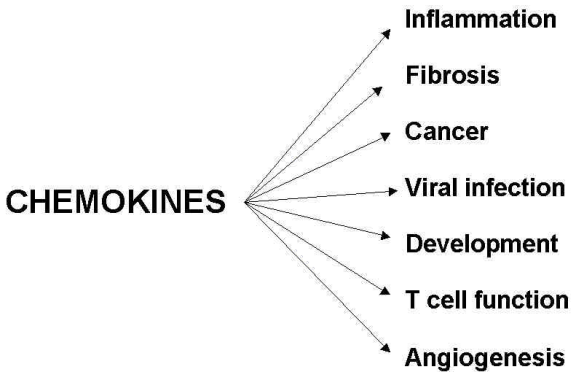

Figure 2. Biologic actions and pathophysiologic conditions regulated by the chemokine system.

disease and with the amount of infiltrating neutrophils (10-13). Interestingly, long-term follow-up of patients with alcoholic hepatitis demonstrated that the subjects with the highest levels of IL-8 were more likely to die within two years (13).

Immunostaining of liver specimens from patients with alcoholic hepatitis showed a diffuse increase in IL-8 signal (10), part of which could be due to production by hepatocytes. This is supported by the observation that ethanol-treated hepatocytes in culture release chemotactic factors of different molecular weights, and that one of these factors shows immunoreactivity when tested with antibodies against IL-8 (14). In addition, both hepatocytes and Kupffer cells in primary culture were found to express CINC, the rat homologue of IL-8, when tested against cytokines or lipopolysaccharide (LPS) (15). Alcohol did not have direct effects on the release of this chemokine by either cell type, but conditioned medium of ethanol-treated hepatocytes markedly stimulated the relase of CINC by Kupffer cells, thus suggesting that ethanol favors the interaction between these cell types to produce the neutrophil chemoattractant. Interestingly, there appears to be a different pattern of chemokine expression during different types of alcoholic liver disease (16). In fact, while hepatocytes express high amounts of chemokine mRNA during alcoholic hepatitis, non-parenchymal cells are the major sites of chemokine expression during alcoholic cirrhosis. These findings are well in agreement with the observation of predominant inflammation in the parenchyma or in the septae during alcoholic hepatitis or cirrhosis, respectively. Direct evidence of the role played by CXC chemokines, and IL-8 in particular, in mediating neutrophil recruitment and tissue damage is provided by the observation that overexpression of CINC in the liver by adenoviral-mediated transfer is associated with severe hepatic inflammation and increase in liver enzymes (17). IL-8 may not be the only CXC chemokine involved in the pathogenesis of alcoholic liver disease. Increased levels of GRO-α, another ELR-CXC chemokine, have been reported in patients with alcoholic hepatitis, and correlated with neutrophil infiltration (18).

Studies carried out on circulating mononuclear cells also confirm the activation of the IL-8 system during alcoholic liver damage. In this setting, a possible

contribution to the stimulation of IL-8 release may be provided by oxidative stress, because lipid peroxidation products were found to markedly increase the release of IL-8 by mononuclear cells (19). This was later confirmed by Nanji *et al.*, who demonstrated increased lipid peroxidation and activation of nuclear factor-?B (NF-?B) in rats treated with alcohol and a diet rich in unsaturated fat (20). NF-?B plays a critical role in regulating transcription of pro-inflammatory genes, including numerous chemokines, and in the groups of rats showing activation of this transcription factor, increased expression levels of CC and CXC chemokines was also observed (20). It is well established that in women, the risk of developing more severe alcoholic liver disease is higher. Interestingly, when female or male rats were treated with alcohol and unsaturated fat for four weeks, female animals developed a more severe liver damage, that was associated with higher levels of lipid peroxidation, NF-?B activation and chemokine expression (21). Thus, chemokines and other pro-inflammatory mediators may contribute to determine gender-related susceptibility to liver damage induced by ethanol. Despite accumulating evidence indicating the role of ELR-CXC chemokines in the pathogenesis of alcoholic liver injury, it has been recently reported that the expression of the chemokines MIP-2 and CINC is reduced in rats administered ethanol and subsequently intoxicated with lipopolysaccaride (22), as established by plasma levels and tissue mRNA. This observation indicates that a blunted increase in pro-inflammatory chemokines caused by alcohol may be part of the increased susceptibility to infections observed in patients with alcoholic liver disease.

Mononuclear cells also produce higher levels of other chemokines during alcoholic hepatitis, including monocyte chemoattractant protein-1 (MCP-1) and macrophage inflammatory protein-1α (MIP-1α), which may be responsible for the recruitment of monocytes and activated lymphocytes during alcoholic liver disease (16, 23, 24). Together with the observation that chemokine levels are higher in the hepatic veins than in the peripheral circulation, these data show that both hepatic and extrahepatic sources contribute to the elevated serum chemokine levels observed in alcoholic hepatitis.

4. ISCHEMIA-REPERFUSION INJURY AND TRANSPLANT REJECTION.

Reducing the damage generated by liver ischemia and reperfusion is critical for liver surgery and for the outcome of liver transplantation. Both warm and cold ischemia are associated with inflammatory infiltration, particularly by neutrophils, increased expression of pro-inflammatory cytokines, and oxidative stress (25). In addition, liver ischemia-reperfusion injury is often associated with damage to other organs, including the lung, where a neutrophil-dependent microvascular injury develops, and tissue damage is dependent on the hepatic release of tumor necrosis factor-α (TNF-α) (26). Because of the neutrophilic infiltration and its possible role in the pathogenesis of injury, it is not surprising that also in this context, considerable attention has focused on the role of ELR-CXC chemokines. Epithelial neutrophil-activating protein-78 (ENA-78) binds and activates CXCR1 and CXCR2, and its expression was found to be increased in the lung of mice undergoing liver ischemia-reperfusion (27). Injection of neutralizing antibodies directed against TNF-α reduced the levels of ENA-78, and passive immunization against ENA-78 significantly reduced the extent of pulmonary damage, indicating that this chemokine is implicated in the pathogenesis of lung injury. The same group extended these observations to the liver, reporting that hepatic damage is also related to elevated ENA-78 expression, which is in turn dependent on an increase in TNF- expression (28). Along these lines, MIP-2 and KC, homologues of the human GRO-α, -ß, and -γ, are two additional ELR-CXC chemokines involved in the development of liver injury induced by ischemia-reperfusion, because neutralization of either one of these molecules resulted in reduced neutrophil infiltration and hepatocellular damage in a rat model of partial liver ischemia (29). Interestingly, while up-regulation if MIP-2 expression was an early event after reperfusion, KC was up-regulated at later time points and its expression was observed also in the lobes that were not made ischemic. Similarly to what previously reported for ENA-78, expression of MIP-2 and KC after liver ischemia-reperfusion also occurred in the lung, where these chemokines participate in the recruitment of neutrophils and in the pathogenesis of edema (30).

It should not be overlooked that also in this condition TNF-α expression plays a pivotal role, and represents one of the major mechanisms of induction of ENA-78 and other chemokines. This contention is supported by recent work showing that when partial ischemia-reperfusion was associated with injection of LPS, increased hepatic production of TNF-α was accompanied by more severe liver and lung injury and by higher levels of ENA-78 and MIP-2 (31). Similarly, in rats with alcoholic steatosis, ischemia-reperfusion led to a greater increase in TNF-α and CINC levels and in the degree of hepatic damage. Thus, increased production of pro-inflammatory mediators, including chemokines, may also be involved in the pathogenesis of primary graft nonfunction of steatotic liver allografts (32). Bajt *et al.* (33) compared the effects of systemic injection of different pro-inflammatory mediators on the hepatic neutrophil sequestration in sinusoids and postsinusoidal venules. While administration of the ELR-CXC chemokine MIP-2 resulted in transient accumulation of neutrophils in the sinusoids, injection of TNF-α or IL-1 exerted a more sustained effect and also caused adherence in the post-sinusoidal venules due to their capacity to upregulate adhesion molecules. The involvement of jun-N-terminal kinase (JNK) in the induction of the hepatic pro-inflammatory response in this setting has been recenltly suggested by the observation that hypothermia reduces cytokine and chemokine expression after ischemia reperfusion and blocks JNK activation (34).

As for many pro-inflammatory conditions in which cytokines and chemokines are involved, NF-?B activation provides the cells with a potent stimulus to increase the transcription of the genes involved in the response to injury. It is therefore not unexpected that conditions associated with inhibition of NF-?B activation

may result in a reduction of damage in different types of liver injury, including the one caused by ischemia-reperfusion. In fact, in a mouse model of partial ischemia-reperfusion, administration of recombinant interleukin-10, a potent anti-inflammatory cytokine, suppressed NF-?B activation and significantly reduced neutrophil recruitment and hepatocellular injury (35). Other cytokines that reduce the reperfusion damage, such as IL-13, have also been show to down-regulate chemokine expression. However, in this case, the protective effect was independent of NF-?B inhibition, and was rather related to activation of the transcription factor STAT6 (36). Some chemokines without the ELR motif are biological antagonists of the ELR-CXC molecules with respect to the modulation of angiogenesis (37). In particular, gamma-interferon-inducible protein-10 (IP-10) and monokine induced by gamma-interferon (Mig) are ligands of CXCR3 and are up-regulated in different conditions of hepatic injury (see below). When mice undergoing partial ischemia-reperfusion where treated with interferon (IFN)-γ, a marked reduction in the levels of ELR-CXC chemokines and an increase in IP-10 and Mig was associated with reduced liver and pulmonary damage (38). However, the recruitment of neutrophils was not modified, suggesting that the protective effect exerted by IFN-γ, and possibly by non-ELR chemokines is independent of changes in neutrophil infiltration. A critical step in the recruitment of neutrophils is represented by up-regulation of adhesion molecules on the endothelium, another event mediated by activation of NF?B. Secretion of the the CC chemokine MCP-1 by activated Kupffer cells could contribute to neutrophil infiltration via induction of ICAM-1 (39). Conversely, expression of MCP-1 by macrophages may be potentiated by the infiltration of neutrophils via release of elastase and production of reactive oxygen species (40). Chemokines have also been shown to cross-talk with selectins, as demonstrated by the observation that partial liver ischemia after injection of antibodies directed against L-selectin or in animals deficient in P-selectin reduce liver damage and neutrophil recruitment, and lowers serum MIP-2 levels (41,42).

Chemokines have also been implicated in the process of liver transplant rejection. Macrophage inflammatory protein (MIP) -1α e -1ß activate the receptors CCR1 and CCR5 and may be involved in the recruitment of activated T lymphocytes. These chemokines were markedly over-expressed by infiltrating leukocytes and endothelial cells in the liver of patients showing signs of transplant rejection (43). Interestingly, the levels of MIP-1α and -1β were reduced by institution of effective immunosuppressive therapy, while remained high in livers undergoing chronic rejection. CINC, the rat homologue of IL-8 was studied in a rat model of allograft rejection in the presence or absence of immunosuppressive therapy (44). Rats with untreated allografts showed increased neutrophil infiltration in the liver, together with higher mRNA levels of CINC, which was mainly expressed by mononuclear cells. Recently, increased expression of the chemokine receptors CXCR3, CXCR4, and CCR5 has been shown in liver-infiltrating lymphocytes during transplant rejection (45). The expression of the ligands for the different receptors was cell-specific, because ligands of CXCR3 and CCR5 were mainly expressed by endothelial cells, whereas biliary cells expressed SDF-1, the ligand for CXCR4. The differential expression of these chemokines may provide a mechanism to direct the action of lymphocytes and the resulting damage to different areas of the hepatic lobule.

5. VIRAL HEPATITIS

Chronic viral hepatitis due to HCV or HBV infection represents one of the major causes of cirrhosis and contributes to a substantial number of liver transplants each year. Because inflammation is a risk factor for progression to cirrhosis, significant attention has focused on the mechanisms of inflammatory cell recruitment in this condition. Most of the available data have been obtained in patients with HCV-related chronic hepatitis or cirrhosis, and it is presently not completely understood to what extent these results may be extended to chronic hepatitis due to other viruses. We provided one of the first reports of an activation of the chemokine system in chronic viral hepatitis in 1993, by showing a marked increase in hepatic MCP-1 gene expression (46). MCP-1 is a potent chemoattractant for several inflammatory cells, including monocytes and activated T lymphocytes, and is considered a prototypic pro-inflammatory chemokine. Several lines of evidence support the hypothesis that MCP-1 may be relevant for the recruitment of inflammatory cells within the injured liver. Mice lacking CCR2, the only high affinity receptor so far identified for MCP-1, have smaller lesions in a model of granulomatous liver injury (47), and expression of MCP-1 temporally precedes the recruitment of monocytes/macrophages in a rat model of acute liver injury (48). In a group of patients with chronic viral hepatitis, mostly related to HCV infection, the number of CD68-positive cells infiltrating the portal tract was directly correlated with the expression of MCP-1 in the same areas (49). Together, these data indicate that MCP-1 may have a role in the recruitment of monocuclear cells to the liver in different conditions of injury, including viral hepatitis. However, it should be kept in mind that neutralization of the biologic activity of MCP-1 does not always result in a reduction of hepatic damage. In a model of acute liver injury due to acetaminophen intoxication, mice lacking CCR2 have a more marked damage than wild type littermates (50). This effect was associated with increased hepatic levels of TNF-α and IFN-γ, the neutralization of which led to an attenuation of liver damage in knock-out animals. Although CCR2 is expressed on activated T lymphocytes irrespective of their Th1 or Th2 polarization (see below), CCR2 deficiency switches the balance towards a Th1-type cytokine pattern, that mediates the damage in the acetaminophen model. Further studies are necessary to establish the effects of CCR2 and/or MCP-1 deficiency in other experimental models of liver injury.

Recent investigation has led to the finding that several chemokine-receptor systems may be responsible for the recruitment of different subsets of lymphocytes in conditions of inflammation, and this may have an impact in the immunopathogenesis of chronic viral hepatitis. Upon differentiation, lymphocytes undergo a transition in the expression pattern of chemokine receptors that renders

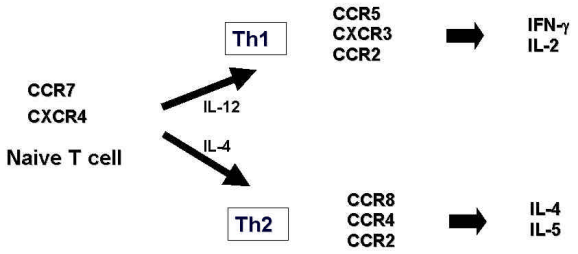

Figure 3. Chemokine receptors in polarized lymphocytes. Polarization towards a Th1 or Th2 phenotype is associated with expression of different subsets of chemokine receptors.

them more susceptible to migrate to areas where the cognate chemokine ligands are expressed. In particular, the migration of polarized $CD4^+$ lymphocytes has been shown to be associated with acquisition of specific patterns of chemokine receptors (Figure 3). T cells polarized toward a Th1 phenotype express CXCR3 and CCR5, while Th2-polarized lymphocytes express CCR8 and CCR4 (3,51). The expression of other chemokine receptors, such as CCR2 (the receptor for MCP-1/CCL2) is maintained along the differentiation pathway, although CCR2 activation may be associated with a shift toward a Th2 polarization. The system comprised by the receptor CXCR3 and its ligands, IP-10, Mig, I-TAC has been studied in detail in some recent work principally conducted in patients with hepatitis C. Expression of IP-10 is markedly stimulated by interferon-γ, and IP-10, together with Mig and I-TAC, binds and activate CXCR3, a receptor expressed by activated T lymphocytes polarized toward a Th1 phenotype. Increased serum levels of IP-10 were first reported by Narumi *et al.* in a group of patients with chronic hepatitis C, where higher IP-10 levels predicted a lower chance of responding to antiviral treatment, and levels of this chemokine were decreased in patients responding to the treatment (52). *In situ* hybridization experiments showed that hepatocytes represent a major site of IP-10 expression, especially in areas of periportal necrosis, where the inflammatory and fibrogenic response is more evident. IP-10 was also found to be one of the differentially expressed genes comparing HCV-infected livers to non-infected samples using a subtraction hybridization approach (53). The critical role of Th1 cytokines in mediating the expression of CXCR3 ligands in the liver is supported by the observation that expression of Mig and Crg-2, the murine homologue of IP-10, is up-regulated by IL-2/IL-12 or IFN-γ *in vivo* and *in vitro* (54). These chemokines were also the major contributors to chemotactic activity towards T lymphocytes and NK cells, as shown by chemotaxis assays using supernatants of hepatocytes isolated from cytokine-treated mice (54). As a counterpart to these observations, high levels of expression of CXCR3 were found in liver-infiltrating T lymphocytes from patients with hepatitis C, where CXCR3 was more expressed than in lymphocytes from peripheral blood (55). In the same study, the contribution of sinusoidal endothelial cells to the expression of the CXCR3 ligands IP-10 and Mig was demonstrated. In fulminant hepatitis induced by administration of concanavalin A or by sequential treatment with killed *P. acnes* and LPS, liver-infiltrating lymphocytes efficiently migrated towards IP-10 gradients (56), confirming that in models of hepatic inflammation which involve T cells, CXCR3-binding chemokines are critical for the recruitment of activated lymphocytes. Up-regulation of Crg-2 has been recently reported in other models of experimental liver injury, including D-galactosamine or CCl_4 intoxication, or bile duct ligation (57). Interestingly, Crg-2 was also increased after partial hepatectomy, and injection of rIP-10 increased hepatocyte growth through a mechanism not related to direct mitogenesis (57). A possible explanation for the effects of IP-10 in this context has been recently shown by the observation that exposure of hepatocytes to IP-10 increases the expression of CXCR2, which in turn would mediate the proliferative response (58). Accordingly, during injury induced by acetaminophen intoxication, IP-10 exerted a hepatoprotective effect that was blocked by anti-CXCR2 antibodies (58). These data indicate a cross-talk between different chemokine systems which targets the function of parenchymal cells, thus showing an example of a non-inflammatory biologic activity of chemokines within the liver.

CXCR3-binding chemokines have been shown to contribute to antiviral defense *in vivo*, as also recently demonstrated in mice deficient in IP-10 (59,60). Moreover, in mice deficient in MIP-1α, reduced expression of IFN-γ and Mig was observed upon infection with murine cytomegalovirus (61). This reduced cytokine response was associated with impairment in the recruitment of natural killer cells to the liver and in the resistance to viral infection, showing that a cascade of mediators comprising MIP-1α, IFN-γ, and Mig is critical to produce an effective tissue antiviral defense. The relevant role played by interferons in general and specifically by IFN-γ in mediating the expression of Mig during inflammation is confirmed by studies carried out in animals deficient in IFN-γ and infected with vaccinia virus (62). In this model, liver expression of Mig was markedly reduced, and the residual expression was dependent on the action of IFN-α/ß. On the other hand, in an elegant study recently conducted using transgenic mice that replicate hepatitis B virus (63), transfer of cytotoxic T lymphocytes that produce IFN-γ was associated with a marked increase in the expression of Crg-2 and Mig by the liver. However, neutralization of the biologic activity of these chemokines reduced the recruitment of inflammatory cells to the liver without limiting the anti-viral effect of cytotoxic T lymphocytes. These data suggest that while IFN-γ is responsible for the anti-viral effect during HBV infection, inhibition of the action of CXCR3-binding chemokines may result in reduced tissue damage, with possible implications for immunotherapy in HBV-infected individuals.

The possible role of IL-8, a CXC chemokine, has also received attention in patients with viral hepatitis and cirrhosis. Transfection of the HBV X protein was associated with increased gene expression of IL-8, that was dependent on multiple transcriptional pathways, including NF-?B (64). Elevated plasma levels of IL-8 have been

reported in patients with post-hepatitis cirrhosis, and correlated with the severity of liver inflammation or dysfunction and with Child-Pugh class (65,66). Intrahepatic gene expression of IL-8 was found to directly correlate with histological activity index, the degree of portal inflammation, and the presence of bile duct lesions (67), and was increased also in patients with advanced cirrhosis (68). More recently, the HCV protein NS5A was shown to up-regulated the expression of IL-8 and to inhibit the antiviral action of interferon *in vitro* (69), and serum levels of IL-8 were significantly higher in patients not responding to interferon treatment (70). In an interesting recent study, Fas-induced hepatocyte apoptosis increased the expression of CXC chemokines such as MIP-2 and KC, which were responsible for liver neutrophilic infiltration (71). This effect, that was dependent on activation of caspases and of the transcription factor AP-1, indicates a direct mechanism of chemokine production and inflammation secondary to cell death within the liver.

A number of other chemokine systems have been found to be modulated in patients with viral hepatitis. Increased expression levels of MIP-1α and MIP-1ß have been shown in patients with hepatitis C, and the expression of the cognate receptor CCR5 on infiltrating T lymphocytes was higher in patients than in normal liver (55,72). High levels of MIP-1α were associated with increased expression of IFN-γ and IL-18, further supporting the presence of a Th1-shifted immune response in the liver of patients with chronic hepatitis C (72). Other CC chemokines, such as PARC and RANTES, are expressed at high levels in the liver of patients with hepatitis C, in the portal tract and in areas of piecemeal necrosis, respectively (73). Expression of these chemokines was adjacent to areas of infiltration with naïve and activated T cells, these latter being predominant in the periportal areas. These data suggest that PARC may contribute to the recruitment of naïve T cells to the portal tract, while RANTES, which attracts activated T cells, directs them to the areas of piecemeal necrosis (73). In chronic hepatitis C, lymphocytes infiltrating the portal tract, often organize in lymphoid aggregates (74). Secondary lymphoid organ chemokine (SLC) is expressed predominantly in the T cell zone of lymph nodes, and its absence, or deficiency in its cognate receptor, CCR7, causes a profound disruption in the architecture of the lymph node (75). In the *P. acnes* model of granulomatous liver inflammation in the mouse, formation of granulomas in the perisinusoidal space was accompanied by formation of a 'portal tract-associated lymphoid tissue' (PALT). Immunostaining for SLC may be detected within PALT, while MIP-1α is the prevalent chemokine where granulomas are formed (76). Analysis of the effects of neutralizing antibodies suggests that MIP-1α expression is required for the recruitment of dendritic cell precursors, while SLC is responsible for attraction of activated dendritic cells to the portal tract and the formation of PALT. These results have been recently extended to human liver disease, with the observation of SLC expression in the PALT present in the liver of patients with primary sclerosing cholangitis (77). A higher percentage of lymphocytes expressing CCR7 was also found, and liver-infiltrating lymphocytes migrated to recombinant SLC *in vitro*. Another system that has been recently studied in viral hepatitis is the one comprised by MIP-3α and CCR6, a receptor expressed by memory T cells (78). A higher percentage of liver-derived $CD4^+$ T cells expressed CCR6 than cells collected from peripheral blood, and the cells expressing CCR6 often co-expressed the Th1 marker CCR5. Expression of MIP-3α was localized in areas of piecemeal necrosis, and was likely dependent on activated dendritic cells. The serum levels of MIP-3 were recently measured in a group of patients with chronic hepatitis C, where they were higher than in control subjects (79). Interestingly, MIP-3α increased in patients that responded to antiviral treatment with interferon, while remained unchanged in non-responders.

6. CHEMOKINES AND LIVER CANCER

The connection between the chemokine system and cancer dates back to 1989, when Graves *et al.* showed expression of chemotactic factors for monocytes by different malignant cells (80). Tumor-derived chemokines are important for the characteristic recruitment of leukocytes, such as tumor-associated macrophages and lymphocytes, to the tumor environment and hence contribute to the promotion of a specific host anti-tumor immune response. However, the action of recruited leukocytes against tumor cells may be counteracted by several tumor-favoring processes mediated by chemokines. In fact, some cytokines derived from cancer cells or tumor-associated leukocytes act as growth factors for tumor cells. Yoong *et al.* (81) have analyzed the expression of several CC and CXC chemokines in specimens of hepatocellular carcinoma, showing immunostaining for Mig, IL-8, MIP-1α, MIP-1β. Expression of these chemokines was accompanied by high levels of expression of CXCR3 and CCR5 by tumor-infiltrating lymphocytes, suggesting a causal relationship between chemokine expression in the neoplastic tissue and lymphocyte recruitment. This was also supported by the fact that the chemotactic activity of hepatoma cell-conditioned medium for tumor-infiltrating lymphocytes was inhibited by antibodies against CXCR3 or CCR5 (81).

Besides this 'classical' role of chemokines as leukocyte chemoattractants, these molecules have other ways of modulating tumor biology. Numerous chemokines have been shown to regulate tumor-dependent angiogenesis, and in particular ELR-CXC chemokines are generally pro-angiogenic, while some non-ELR CXC chemokines, particularly the CXCR3 ligands IP-10 and Mig are anti-angiogenic (37). These mechanisms are likely to play a relevant role also in the liver (82,83). Recently, chemokines have also been shown to play a pivotal role in the direct chemoattraction of neoplastic cells in different malignancies, including breast cancer, where expression of selected ligands may drive metastasis to target organs. The interaction between SDF-1α and CXCR4 may be relevant for metastasis of breast cancer cells to lymph nodes, lung, liver and bone marrow (84).

Another aspect that may be connected with the development of liver cancer, is the regulation of several

biologic actions in hepatocytes, including proliferation, by chemokines, which may participate in the process of liver regeneration after injury or surgical hepatectomy. ELR-CXC chemokines are probably the most active in modulating hepatocyte functions. IL-8 was shown to dose-dependently up-regulate the expression of acute phase proteins by isolated hepatocytes and in hepatoma cells (85). More important, IL-8, ENA-78, and MIP-2 increase proliferation of isolated rat hepatocytes, and expression of these chemokines is up-regulated after two-third hepatectomy (86). In addition, immuno-neutralization of ENA-78 or MIP-2 was associated with a slower rate of recovery of liver size following hepatectomy (86). These results were confirmed in a model of acute liver injury, such as that induced by acetaminophen intoxication (87). Administration of IL-8, ENA-78 or MIP-2 to intoxicated mice reduced hepatic injury even if made 10 hours following drug challenge. The hepatoprotective effect was likely mediated by CXCR2, because these chemokines stimulated proliferation of acetaminophen-treated hepatocytes through an action on this receptor.

SDF-1α is expressed in the liver by mesothelial cells and biliary epithelial cells during embryonic development, and contributes to the recruitment of cells responsible for antenatal B cell lymphopoiesis (88). Knock-out mice for SDF-1α or for the cognate receptor CXCR4 die perinatally and display profound defects in the hematopoietic and nervous systems (89). Interestingly, mRNA expression of SDF-1α was found to be decreased in a number of gastrointestinal cancers, including hepatocellular carcinoma, compared to non-involved adjacent tissue (90). Moreover, hepatoma cells show impaired signal transduction upon binding of SDF-1α to CXCR4, although the exact mechanism of this defect has not been elucidated (91). Recently, our group evaluated the expression of fractalkine, a molecule of the CX3C subfamily that is expressed as a secretory and cell-associated chemokine and of its receptor CX3CR1, in the liver of patients transplanted for fulminant hepatitis. Interestingly, both fractalkine and its receptor were expressed on regenerating parenchymal cells, suggesting the possibility that this axis is involved in the biology of regenerating cells after a massive acute liver damage (92).

7. OTHER CONDITIONS OF LIVER INJURY

The paradigma of 'selective' inflammatory cell recruitment by chemokines, that in many cases is an oversimplification, is actually well fitting in patients with drug-induced liver injury. In this context an eosinophilic infiltrate is typical, and expression of eotaxin, a potent chemoattractant for eosinophils, was described in all patients with drug-induced injury (93). Chemokines such IL-8 are up-regulated during rat endotoxemia, and contribute to hepatic inflammation (94). MCP-1 expression is also increased after endotoxin administration, but in this case, neutralizing antibodies against this chemokine markedly increased animal mortality, up-regulated TNF-α and IL-12 levels, and reduced the anti-inflammatory cytokine, IL-10 (95). Conversely, injection of recombinant MCP-1 protected mice form LPS-related mortality, demonstrating that in this setting, MCP-1 shifts the cytokine balance toward a less inflammatory environment. This contention is also supported by results obtained in a murine model of septic peritonitis, where neutralizing anti-MCP-1 antibodies increased the tissue levels of TNF-α and reduced those of IL-13 (96). Moreover, stem cells factor, which is a protective cytokine in this model, exerts its effects, at least partially, through an induction of MCP-1 production (97). Conversely, in the model of massive liver injury caused by LPS administration to *P. acnes*-primed mice, thymus and activation-regulated chemokine (TARC) was expressed within the granumoma that forms after *P. acnes* injection and mediated the recruitment of CCR4-expressing $CD4^+$ T cells (98). Injection if neutralizing anti-TARC antibodies reduced the expression of TNF-α and Fas ligand and significantly protected the mice from liver injury. The fact that chemokines are a relevant mechanism in the pathogenesis of liver damage during endotoxemia is supported by the observation that mice lacking the Duffy antigen/receptor for chemokines (DARC) have significantly increased inflammatory infiltrates than wild type animals (99). It is believed that this receptor may have the function of a 'sink' for the excess of chemokines that spill over in the circulation during inflammation.

Chemokines have also been indicated as mediators of inflammation during diseases of the biliary system. In this context, an interesting observation is represented by the fact that MCP-1 is expressed by biliary epithelial cells (BEC) even in the normal liver (49), and that expression of MCP-1 and IL-8 in cultured human BEC may be up-regulated by pro-inflammatory cytokines, including TNF-α and IL-1 (100). These data suggest that amplification of inflammation due to release of chemokines by BEC may be implicated in the pathogenesis of allograft rejection and inflammatory liver diseases, and serum levels of IL-8 were found to be increased in a group of patients with primary sclerosing cholangitis (101). An increase in chemokine expression has been described also in patients with primary biliary cirrhosis. In particular, immunostaing for MCP-1, MCP-2 and MCP-3 was present in the portal tract and was attributable to monocyte-derived cells (102). Liver injury associated with non-suppurative destructive cholangitis is also observed during graft-versus-host disease, and in a mouse model, MIP-1α was expressed in several hepatic cells, including BEC (103). When neutralizing antibodies against CCR5, the receptor recognized by MIP-1α, were injected, reduced infiltration of CD8+ T cells was accompanied by a reduction in liver injury, indicating the contribution of this chemokine to the pathogenesis of liver injury in this setting (103).

8. HEPATIC STELLATE CELLS AND FIBROSIS

The pivotal role of hepatic stellate cells (HSC) in the deposition of extracellular matrix and the development of fibrosis is well established. These cells undergo a process known as activation in which they acquire a phenotype characterized by the increased ability to produce extracellular matrix component, proliferate, migrate, and undergo contraction upon exposure to soluble mediators (reviewed in 104). The characteristics of activated HSC

make them more suitable to participate in the repair process after an injury to the liver, and indicate that these cells are not simply the final effectors of a fibrogenic stimulus, but that may modulate all steps of the hepatic wound healing response. In particular, HSC may modulate liver inflammation with several mechanisms (reviewed in 105), including expression of chemokines. In 1993, we provided evidence that secretion of MCP-1 by HSC accounts for most of the chemotactic activity for monocytes observed in HSC-conditioned medium (46). Remarkably, the ability of HSC to express MCP-1 *in vitro* is maintained in the *in vivo* situation. Indeed, separation of different liver cell populations in rats chronically intoxicated with CCl_4 showed that the majority of MCP-1 expression occurs in HSC (106). In addition, we demonstrated that activated HSC contribute to MCP-1 secretion during chronic liver disease in humans, using a combination of immunohistochemistry and *in situ* hybridization (49). Proinflammatory cytokines, including IL-1, TNF-α, and IFN-γ are the most potent stimuli for MCP-1 protein secretion (46,107,108), and rapidly induce a rise in MCP-1 mRNA levels. Increased MCP-1 expression may also be observed in response to lipopolysaccharide (LPS) (107,108). The ability of soluble mediators to increase the expression of MCP-1, as well as of other chemokines, is dependent on the activation state of HSC. In quiescent HSC, MCP-1 may be induced by TNF but not by LPS, while activated HSC become responsive to LPS, and are more sensitive to the effects of TNF (108). This latter observation is likely related to the fact that cytokine-induced activation of NF-κB is increased in activated HSC (109). Another factor that has been shown to modulate chemokine expression in HSC is represented by oxidative stress and products related to lipid peroxidation (110). Generation of hydrogen peroxide, a reactive oxygen species, may be obtained treating HSC with hypoxanthine (or xanthine) and xanthine oxidase, and results in an up-regulation of MCP-1 expression (107,111). Similar effects, although less pronounced, are associated with exposure of HSC to 4-hydroxy-nonenal, a non-oxidant product of oxidative stress (111). Along these lines, it has been recently shown that malondialdehyde-acetaldehyde protein adducts induce the secretion of MCP-1 and MIP-2 (112). These adducts have been found in ethanol-fed rats and in alcoholic patients, and the action of these molecules may be relevant in the pathogenesis of alcoholic liver disease. Transforming growth factor (TGF) -β, which plays a major role in the stimulation of extracellular matrix production by HSC, has been shown to stimulate MCP-1 expression and secretion (107). In addition, adhesion of HSC to different types of extracellular matrix increases the expression of MCP-1 through an integrin-dependent mechanism (113). These latter findings indicate possible mechanisms for the perpetuation of inflammation and fibrogenesis in chronic liver damage. In fact, TGF-β secreted by activated HSC may stimulate MCP-1 and matrix production in an autocrine fashion, and changes in the composition of the matrix surrounding the HSC may provide a greater availability of integrin ligands resulting in further induction of this chemokine. Thrombin, a potent mitogen for mesenchymal cells, including HSC, has also been shown to stimulate MCP-1 expression (114). Thrombin activates members of the family of protease activated receptors (PAR), that may be activated using peptides mimicking the N-terminus of the receptor (115). PAR-1 was the first thrombin receptor to be been identified, and is widely expressed in the liver during injury (116). Activation of this receptor by specific peptides was shown to stimulate MCP-1 expression in HSC (114).

Recent data indicate that expression of MCP-1 may be down-regulated by agonist of PPAR-γ, a transcription factor which is expressed at high levels in quiescent HSC and is dramatically reduced upon transition to the activated phenotype (117). Reduced signaling through this pathway may be one of the molecular mechanisms underlying the high levels expression of MCP-1 in activated HSC, even in unstimulated conditions. Production of arachidonic acid metabolites contributes to the expression of MCP-1 induced by pro-inflammatory cytokines in HSC. Exposure of these cells to cyclooxygenase inhibitors reduced, but did not abolish, the secretion of MCP-1 in the supernatants of cultured HSC stimulated with IL-1 or TNF-α, but not IFN-γ (118). HSC have also been shown to express CD40, a molecule belonging to the TNF receptor superfamily which is activated by a specific ligand expressed on immune cells (119). Incubation of cultured HSC with cells transfected with CD40 ligand caused activation and nuclear translocation of NF-?B and increased the secretion of MCP-1 and IL-8. These data uncover another system regulating the interaction between the immune system and HSC, and that could contribute to the perpetuation of liver fibrosis.

Other studied have focused on the ability of HSC to produce ELR-CXC chemokines. Sprenger *et al.*, (120) have first reported that HSC secrete neutrophil chemoattractants. Antibodies directed against MIP-2 significantly, although partially, reduced the chemotactic effect, and immunoreactive MIP-2 was detected in HSC supernatants using a specific ELISA (120). Also in this case, the ability to express MIP-2 at the gene and protein levels was dependent on the activation state of HSC (120). A likely explanation for the critical role played by HSC activation is the relationship between cytokine stimulation and activation of nuclear factor-κB. DNA binding of NF-κB is present in activated but not in quiescent HSC (121). Moreover, cytokines such as IL-1 and TNF-α induce NF-κB activation only in activated HSC, and this parallels the ability of these cytokines to induce expression of NF-κB dependent genes such as MIP-2 (109). These considerations may be extended to other genes involved in inflammation and expressed by HSC in an activation-dependent fashion, including MCP-1 and CINC. Maher and co-workers (122) have provided evidence that HSC contribute to CINC expression, which becomes evident after activation of HSC on plastic. The increase in CINC mRNA in activated HSC was associated with secretion in the conditioned medium of immunoreactive CINC, the levels of which were up-regulated by incubation with IL-1 or TNF. Moreover, *in vivo* activation of HSC induced by chronic CCl_4 administration or bile duct ligation was accompanied by increased CINC gene expression (122).

The fact that HSC are responsible for the secretion of numerous chemokines underscores the tight relation between inflammation and fibrosis. This aspect becomes even more evident considering that chemokines exert direct biologic actions on HSC. Our group has reported that MCP-1 stimulates the migration of cultured human HSC and activates intracellular signaling (123). Interestingly, RT-PCR analysis of HSC RNA did not show any detectable transcripts by for the chemokine receptor CCR2, which is expressed in leukocytes and binds MCP-1 with high affinity. Therefore, another yet unidentified chemokine receptor is responsible for the biologic actions of MCP-1 on HSC. Along these lines, we have recently shown that human HSC express the chemokine receptor CXCR3 after activation in culture (124). Expression of this receptor is present in other cells, such as vascular smooth muscle cells or glomerular mesangial cells, implicated in the wound healing response in other organs. In HSC, exposure to CXCR3 ligands resulted in the stimulation of cell migration and in the activation of several intracellular signaling pathways, including Ras/ERK and PI3K/Akt (124). Preliminary data indicate that these pathways may also inhibit apoptosis of HSC (125), which is a mechanism involved in the reversal of fibrosis that may be observed after removal of liver injury (126). Thus, through multiple actions on inflammatory cells and on cells responsible for tissue repair, chemokines are likely to establish a vicious circle responsible for the maintenance of chronic inflammation and fibrosis.

9. PERSPECTIVES

The relevance of the chemokine system in different areas of pathophysiology is witnessed by the explosion of information that has become available in the most recent years. There is little doubt that this system is outstandingly important in mediating recruitment of inflammatory cells to the liver in different conditions of injury, although the regulatory role of these molecules appears to be far more complex than initially thought. In this respect, it will be necessary to further understand the relations between expression of different chemokines and the immunopathogenesis of viral hepatitis. This point may have an impact on potential new strategies for the treatment of patients chronically infected with HBV or HCV.

Another rapidly expanding area is that of the interaction between chemokines and cancer. Besides the regulation of cancer-related accumulation of inflammatory cells, hot fields of investigation are the regulation of angiogenesis by chemokines and the direct effect of chemokines on cancer cells, especially as a mechanism for spreading to other tissues. In the field of fibrogenesis, multiple lines of interaction exist betweeen chemokines and the biology of HSC, including the amplification of local inflammatory response by HSC and direct pro-fibrogenic effects of chemokines, that may establish a vicious circle leading to chronic damage and repair. Data from genetically modified animals are needed to weigh the relative importance of chemokines to that of other soluble mediators of fibrogenesis. Finally, some aspects of chemokine biology that may be of outstanding relevance for liver pathophysiology have still to be explored. As an example, several viruses express chemokine-related genes that may have an impact on the course of infection. The increasing attention dedicated to these topics by scientists in the field of liver disease is likely to increase our knowledge of the mechanisms of damage and repair of liver tissue and the pathophysiology of liver disease.

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Abbreviations:

BEC, biliary epithelial cells
CINC, cytokine-induced neutrophil chemoattractant
Crg-2, chemokine responsive to gamma-interferon-2
DARC, duffy antigen receptor for chemokines
ELR, glutamic acid-leucine-arginine
ENA-78, epithelial neutrophil-activating protein-78
ERK, extracellular signal-regulated kinase
GRO, growth-related oncogene
HBV, hepatitis B virus
HCV, hepatitis C virus
HSC, hepatic stellate cell(s)
ICAM-1, intercellular adhesion molecule-1
IFN, interferon
IL, interleukin
IP-10, gamma-interferon-inducible protein-10
JNK , jun-N-terminal kinase
LPS, lipopolysaccharide
MCP, monocyte chemoattractant protein
Mig, monokine induced by gamma-interferon
MIP, macrophage inflammatory protein
NF-?B, nuclear factor-?B
PALT, portal tract-associated lymphoid tissue
PAR, protease activated receptor(s)
PARC, pulmonary and activation-regulated chemokine
PI3K, phosphatidylinositol 3-kinase
RANTES, regulated upon activation, normal T-cell expressed and secreted
SDF-1, stromal cell-derived factor-1
SLC, secondary lymphoid organ chemokine
STAT, signal transducer and activator of transcription
TGF-β, transforming growth factor-ß
TNF-α, tumor necrosis factor-α

Key Words: Fibrosis, Chemokines, Chemokine Receptors, Liver, Hepatitis, Alcohol, Ischemia-Reperfusion, Hepatic Stellate Cells, Hepatocellular Carcinoma, Bile Duct Epithelial Cells, Cirrhosis, Inflammation, Review

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