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# Cytokines and chronic liver disease

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**Abstract**

From an immunological point of view, the healthy liver has been usually associated with the phenomenon of tolerance. A microenvironment of regulatory cytokines produced by liver Kuppfer cells and liver sinusoidal endothelial cells has contributed, together with resident dendritic cells, to generate a tolerogenic environment in this tissue. In this review we discussed the intrahepatic responses to different sorts of liver injury, such as hepatotrophic viruses, alcohol or putative self-antigens. In each case we analyzed the impact of different cytokines in the clinical outcome of the different pathological situations.
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## 1. Structural organization of the liver

Any study on liver diseases needs to take into account its particular structural organization. Hepatocytes represent about two-thirds of the total cell population in the liver. The remaining population of nonparenchymal cells is diverse and includes Kupffer cells (KC), the sinusoidal endothelial cells (LSEC), stellate cells (fat storing), liver resident dendritic cells and lymphocytes (Fig. 1). LSEC form a fenestrated monolayer which allows contact between hepatocytes and lymphocytes. This contact can either be produced through hepatocyte microvilli protruding into the lumen or by lymphocyte pseudopod extensions penetrating into the space of Disse and making contact with cells underlying extracellular matrix and hepatocytes [1]. Kupffer cells and dendritic cells (DC) are derived from circulating monocytes [2], and upon arrival to the liver, reside within the liver sinusoidal vascular space, predominantly in the periportal area. This location allows KC to clear passing blood from endotoxins, and to phagocytose debris and microorganisms. Together with LSEC and resident DC, KC cells represent the liver antigen presenting cells (LAPC). These three types of LAPC seem to be crucial for the maintenance of tolerance under noninflammatory conditions [3].

Lymphocytes in the liver are scattered throughout the parenchyma and portal tracts, and include conventional T cells ($CD4^+$ and $CD8^+$) and unconventional T cells [4]. The latter comprises two major populations, one which expresses NK cell markers. The first cell type known as NKT cells, is characterized by the co-expression of T cell markers and NK receptors, are CD1-reactive and arise in the thymus. The NKT cells can also be subdivided into two subsets: 1 – the classical NKT which can be CD4 positive or CD4/CD8-double negative T cells and displays a very restricted T cell repertoire (Vα24 and Vβ11 TCR chains in humans) and 2 – the nonclassical NKT cells that encompass TCRαβ or TCRγδ, this subset does not use the T receptor Vα24 and does not express the CD8β chain. The second mayor population of unconventional T cells, not expressing NK cells markers, includes the TCRγδ T cells. The high frequency of these cells makes the liver one of the richest source of unconventional T cells in the body. Natural killer cells are also present at a high

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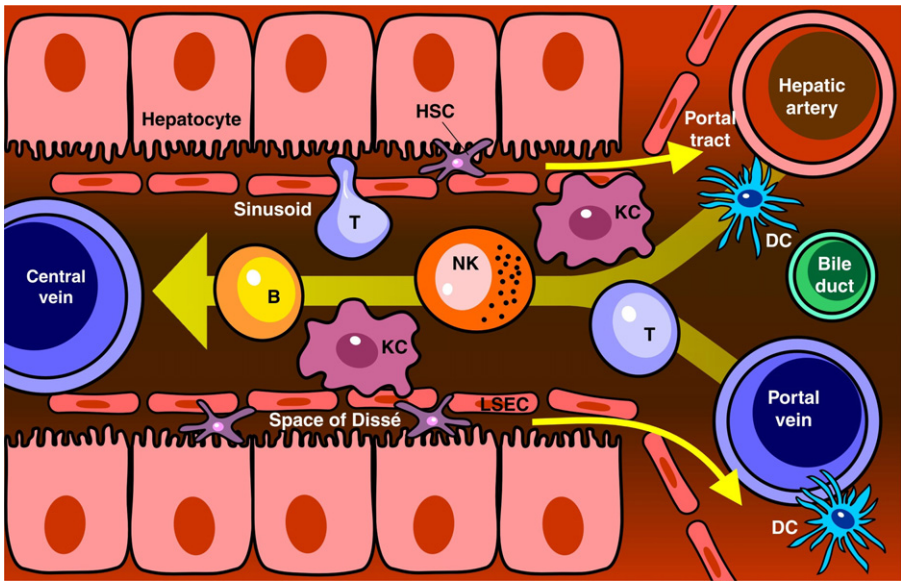


Fig. 1. Architecture of the liver: sinusoids, hepatocytes and immune cells. Sinusoidal endothelial cells (LSEC) form a fenestrated monolayer within the sinusoidal endothelium. This discontinuous structure allows contact between hepatocytes and lymphocytes. The contact can either be produced through hepatocyte microvilli protruding into the lumen or by lymphocyte pseudopod extensions penetrating into the space of Disse. The space of Disse contains hepatic stellate cells (HSC, fat storing). Kupffer cells (KC) reside within the liver sinusoidal vascular space, predominantly in the periportal area. Together LSEC and resident dendritic cels (DC) represent the liver antigen presenting cells (LAPC). Lymphocytes are scattered throughout the parenchyma and portal tracts, and include conventional and unconventional T cells. A low frequency of B cells and abundance of NK are also characteristic of the liver immune microenvironment.

frequency in the liver, and seem to control the migration and expansion of liver NKT cells [5].

## 2. Factors which determine tolerance within the liver

The classic model of primary T cell activation established that bone marrow-derived immature DC acquire antigen (Ag) from sites of infection, migrate to regional secondary lymphoid tissues, differentiate, and activate naïve T cells [6].

Immature DC residing within non-lymphoid organs such as liver or the respiratory tract, are deficient in cell surface costimulatory molecules and can exhibit tolerogenic properties [7]. In the absence of immune suppression, liver allografts can induce donor-specific tolerance to fully MHC-mismatched mouse [8].

After tolerogenic liver-derived DC precursors were first described in mice [9], the phenomenon of liver tolerance was associated to a preferential production of interleukin-10 (IL-10) within the liver [10], particularly by liver resident DC [11] and KC [12].

Unlike DCs, KC are not known to migrate out of the liver, and could potentially acquire and present Ag within their microenvironment [13]. In liver transplantation, KC recovered from chronically accepted hepatic allografts have increased FasL expression and a greater ability to induce apoptosis of alloreactive T cells, regulate IL-10 and transforming growth factor (TGF)-β secretion and significantly prolong the survival of hepatic allografts [14]. Tumor necrosis factor (TNF)-α together with IL-10 seem to diminish T cell activation, through down-regulation of receptor-mediated antigen uptake and MHC class II-expression on LSEC and DC.

An additional mechanism for the liver low response to Ag derives from the establishment of peripheral self-tolerance caused by the steady-state migration of immature bone marrow-derived DCs to regional lymph node (RLN). In the absence of activating stimuli and during normal tissue turnover, these immature DC capture antigen from dying cells, retain their immature phenotype and migrate to the RLN [15]. In addition to those potentially self-antigen specific energized T cells, immature DC reaching RLN might be also responsible for inducing T regulatory (Treg) cells [16].

In comparison with other lymph nodes, liver RLN contains a regular number of immature myeloid DC but a relative paucity of plasmocytoid DCs [17] that might also contribute to the weakness of cellular immune responses induced in the liver.

In a microenvironment dominated by the presence of IL-10 secreted by KC and DC, and the self-tolerance induced by immature DCs, the presence of NKT cells might also contribute to the liver tolerance [18]. Although immunomodulatory characteristics of NKT cells were previously described [19], their role in liver tolerance remains uncertain.

## 3. The induction of intrahepatic immune responses

The current view of $CD8^+$ T cell activation assumes that, to penetrate an infected organ, cytotoxic $CD8^+$ cells must

first be primed, usually within the RLN [20]. For this purpose, a professional APC must be infected and then migrate to the regional lymph node to present antigen via the classical endogenous MHC class I presentation pathway.

In the development of intrahepatic immunity, no definitive evidence is available to answer the question whether direct presentation of antigen can efficiently occur in the liver or whether it is confined to the draining lymph nodes.

Direct presentation by KC and LSEC would require that these cells acquire the Ag through cross-presentation, a phenomenon that allows exogenous antigen access to MHC class I processing pathway. The mechanism of cross-presentation has been in part clarified. It involves protein fragments ''chaperoned'' by heat-shock proteins as the necessary and sufficient source of antigen transferred to antigen presenting cells for priming $CD8^+$ T cell responses [21]. Other mechanisms may also be involved.

As already mentioned, KC are mainly involved in the induction of liver tolerance [14,22]. LSEC employ similar molecular mechanisms for cross-presentation as DC, but the outcome of this presentation seems to lead to $CD8^+$ T cell tolerance rather than to immunity. LSEC have a high capacity for Ag uptake, but a low or absent expression of MHC class II, CD86, and CD11c. Consistent with this phenotype, LSEC alone are insufficient to activate naïve T cells [23]. As uptake of circulating Ag into LSEC occurs efficiently *in vivo*, it is likely that cross-presentation by LSEC contributes to $CD8^+$ T cell tolerance observed in situations where soluble antigen is present in the circulation [24].

T cell-mediated protection against liver-trophic viruses depends on constant supply of activated effectors $CD8^+$ T cells to maintain immune responses that control emergence, spread and expansion of the virus. The origin of naïve and activated $CD8^+$ cells in the liver has been the object of great controversy [25].

Direct presentation and T cell activation by hepatocytes were demonstrated to be possible. Experiments based on models of liver transplantation [26] or models based on transgenic expression of Ag [27] have suggested that $CD8^+$ T cells activated by Ag expressed on hepatocytes undergo abortive activation leading to premature apoptosis. These observations lead to the introduction of the concept that the liver is a ''graveyard'' or a killing field [25]. This idea of a graveyard for activated lymphocytes had implications in oral tolerance, which is induced through portal vein infusion of antigenic cells, and the same concept could be applied to the tolerance to allogeneic liver allografts, and to the persistence of some liver pathogens including hepatitis C virus (HCV). The graveyard concept assumes that immune tolerance might be a result of an antigenic intrahepatic priming, while the expression of the same Ag in liver-draining lymph nodes is believed to result in effective immunity.

In a recent report Bowen et al. [28] suggested that the balance between immunity and tolerance is established by competition for primary activation of $CD8^+$ T cells between the liver and secondary lymphoid tissues, with the immune outcome determined by the initial site of activation. By using a transgenic mouse model in which antigen is expressed within both liver and lymph nodes, it was demonstrated that naïve $CD8^+$ T cells activated within the lymph nodes were capable of mediating hepatitis. By contrast cells undergoing primary activation within the liver exhibited defective cytotoxic function and shortened half-life and did not mediate hepatocellular injury.

In contrast, another report [29], provided new insights into the role of intrahepatic priming. This work demonstrated that exogenous Ag expressed only by hepatocytes resulted in clonal expansion, interferon (IFN)-γ synthesis, and cytotoxic effector function. This interesting study indicates that local activation of naive $CD8^+$ T cells can cause full $CD8^+$ T cell activation, and exclude hepatocellular presentation as the cause of the failure of CTL effector function against some liver pathogens such as hepatitis C. According to this study, abortive $CD8^+$ T cell activation by hepatocytes does not explain ''liver tolerance'' as previously postulated [30].

## 4. Intrahepatic immune responses to liver-trophic viruses

The hepatitis B virus (HBV) and hepatitis C virus (HCV) infections are the most common causes of liver disease worldwide. Although both viruses induce immune-mediated acute and chronic necroinflammatory liver disease, the natural history and the outcome of HBV and HCV infections differ profoundly. Whereas HBV vertical transmission of mother to neonate always results in chronic hepatitis, infection during adulthood typically does not; instead, it results in lifelong protective immunity [31]. Conversely, one characteristic of the HCV infection is that it readily establishes chronic hepatitis in 60–80% of infected adults [32].

### *4.1. Role of cytokines of the innate immunity*

Microarray analysis performed in liver biopsies from experimentally infected chimpanzees revealed striking differences in the response during the first weeks that follow infection. While HBV appears to avoid the induction of strong innate immune responses [33], HCV induces the expression of type-I interferon (IFN) [34]. Although HCV replicons are highly sensitive to type-I IFNs *in vitro* [35], this expression does not correlate with the *in vivo* outcome of infection, since HCV frequently succeeds in establishing chronic hepatitis. In spite of this apparent lack of response at the early phase of infection, HBV DNA can be cleared from the serum and liver before an adaptive immune response is detected. This observation is indicating that an innate immune response might be produced, although below the level of detection. Also, clearance in HBV could be mediated by IFN-γ secreted by activated NKT [36].

Although barely detectable in the normal liver, production of TNF-α is one of the earliest events in many types of

liver injury. Activation of TNF receptors leads to induction of death signals. Since hepatocytes can minimize these death signals and survive, it was suggested that liver injury requires at least two ''hits'': one that increases the exposure of TNF-α, and another that interferes with the normal ability of hepatocytes to protect themselves from TNF-α-induced cell death (reviewed in [37]).

Hepatitis C virus core protein increases the apoptosis induced by TNF-α and as a consequence, hepatocytes become more vulnerable to the higher levels of TNF-α found in chronic HCV patients [38–40]. Untreated HCV patients with high TNF-α serum levels also have high histopathological index [41]. This is in line with the presence of a low producer TNF genotype associated with a self-limited HCV infection [40]. In contrast, TNF-α polymorphism appears not to play a role on the outcome of HCV infection [38,42–44].

The ultimate *in vivo* effect of TNF-α on hepatocytes is strongly influenced by other cytokines present in the liver tissue, as it is the exacerbated liver injury induced in mice in the absence of the anti-inflammatory effects of IL-10 (see also anti-fibrotic effect of IL-10 and other cytokines in fibrosis and cirrhosis).

Induced by virus, or by IL-1 and TNF-α, IL-6 acts on hepatocytes as the major trigger for the production of acute phase proteins (APP) that modulate liver fibrosis either through inhibition of proteases (i.e. collagenase) capable of degrading extra cellular matrix proteins or by binding to other cytokines. Serum IL-6 levels are positively correlated with the duration of HCV infection and with the viral load [45]. While in HCV-infected individuals, the low producer genotype is associated with viral clearance [46], IL-6 gene polymorphisms were found not to associate with any parameter of the chronic disease or with the clinical outcome of a liver transplantation [44,47]. Elevated amounts of IL-8 are also related to liver inflammation and fibrosis [48,49]. One possible source of this cytokine may be the endothelial liver cells since the HUVEC (human umbilical vein endothelial cells) produce IL-8 upon *in vitro* stimulation with HCV-like particles.

A large immunogenetic study has recently demonstrated the role of NK cells in HCV infection [50]. In this study individuals sharing specific combination of NK-cell receptor with given HLA-C alleles were associated with HCV clearance and clinical recovery. However, HCV has the ability to interfere with NK-cell activity. For instance, HCV-infected individuals have NK cells with a decreased secretion of cytokines and an impaired capacity to activate DC [51]. Furthermore, it was demonstrated *in vitro* that HCV envelope protein (E2) can block NK cell activity [52].

### 4.2. Role of the adaptive immunity

In patients who spontaneously recovered from an HBV or HCV infection, specific multi-epitope $CD4^+$ and $CD8^+$ T cell-mediated responses can be detected [53,54]. In contrast, immune responses of patients with chronic HCV or HBV are usually late, transient and with an insufficient T cell response [55,56]. *In vivo* depletion of either $CD4^+$ or $CD8^+$ T cells on experimentally-infected chimpanzees prevent HCV and HBV clearance and clinical recovery [57–59]. Several proinflammatory Th1 cytokines such as IFN-γ, IL-2 and TNF-α are believed to participate in virus elimination. In contrast, IL-10 inhibits the Th1 effector mechanisms [60].

#### 4.2.1. Role of cytokines in HCV infection

A vigorous NK cell and Th1 cell-mediated immune response seems to be a key factor in the protection against HCV infection [50,61]. However, one characteristic of the HCV infection is that it readily establishes chronic hepatitis in 60–80% of infected adult individuals [32]. Several factors have been postulated to explain inefficient clearance of HCV in such a large population. $CD4^+$ and $CD8^+$ T cells responses are generated during acute HCV infection in individuals who become chronic, but an impaired ability to proliferate induce the progressive disappearance of HCV-specific T cells from peripheral blood [62].

In addition to host genetic factors, such as cytokine polymorphisms (see below), impairment of DC function was described in some patients [63]. A potentially important additional factor includes the recently described role of the T-regulatory (Treg) cells. Treg cells are known to contribute to the maintenance of immunological self-tolerance, the control of immune responses against pathogens, tumor antigens and/or alloantigens. It was recently reported that Tregs are essential in modulating the specific anti-HCV response, through the participation of virus-specific $CD4^+CD25^+$ Treg cells [64] and/or intrahepatic IL-10 secreting-$CD8^+$ Treg cells [65]. However, as already mentioned, the mechanisms whereby HCV causes acute liver injury and initiates the cascade of events leading to the establishment of persistent infection and development of chronic liver disease are still not clearly established [29,30]. Many additional factors including age, gender, alcohol consumption, body mass index, steatosis and HIV or HBV co-infection affect disease outcome, but are insufficient to explain it.

Several reports postulated that IFN-γ participates in viral clearance. The levels of IFN-γ produced by antigen-stimulated T cell lines derived from self-limited patients were higher than the obtained from chronic patients [66]. In the nearly universal recurrent HCV infection after liver transplantation, the genetic ability to produce high levels of IFN-γ was associated with the absence of recurrence in transplanted patients, whereas transplanted patients carrying a gene polymorphism associated with low production of IFN-γ where among the patients with early recurrence of HCV infection [44].

HCV core protein has been reported to inhibit IL-12p40 mRNA synthesis and the subsequent IL-12p70 production through its interaction with the receptor for the complement component C1q expressed on activated macrophages [67]. This interaction suppresses IFN-γ secretion by stimulated peripheral blood mononuclear cells (PBMC). In addition,

serum IL-12 levels were increased in HCV patients responders to the treatment with IFN-α plus Ribavirin, whereas non-responders returned to normal values [68], indicating the necessary effective production of Th1 cytokines.

A characteristic of chronic HCV infection is the development of lymphoid follicles in portal areas. This feature has led to the suggestion that naïve T cells are recruited to the liver. Compared with blood, liver has a 30-fold higher percentage of HCV-specific T cells. However, the vast majority of intrahepatic $CD8^+$ T cells are not virus-specific. These bystander cells secrete IFN-γ and modulate immune response to allow chronic inflammation to become established [69,70] implying that bystander-primed but not HCV-specific $CD8^+$ T cells contribute to liver damage [69,71]. *In situ* hibridation studies confirmed that T cells, but not macrophages, Kupffer cells or hepatocytes, expressed both IFN-γ and the IFN-γ-inducible isoform of nitric oxide synthase in chronic HCV patients [72]. In contrast, the hepatic source of IL-2 is mainly from hepatocytes [73].

A pivotal role has been attributed to IL-10 on the outcome of HCV infection. An increased frequency of the -1082GG high producer genotype was recently reported by us in chronic non cirrhotic HCV patients [74]. In spite of ethnic differences these results are in line with similar findings reported by the Mayo Clinic [39], an extensive study performed in the United Kingdom [75] and in the Japanese population [76]. Interestingly, in our study, we observed a gender effect, because the presence of a high IL-10 producer genotype, was observed only in female HCV patients, in particular those $RNA^+$ patients with elevated levels of transaminases (ALT) and low score of fibrosis [74]. In contrast, low IL-10 producers were reported associated with a self-limited HCV infection [42,74,75]. However, contradictory results exist regarding the association of the IL-10 promoter with HCV infection. Many of those studies were performed either in a small number of patients, or failed to take into account the putative gender effect described by us [40,42,43,47,77]. Additional experiments support the role of IL-10 in liver infected with hepatotrophic viruses. For instance, the antibody-induced blockage of IL-10R generates a favourable balance of $CD4^+$ T cell response to HCV. Also, this anti-IL-10R reverses the inhibitory effect of IL-10 on HCV-specific T cell proliferation, demonstrating the major role of IL-10 in the suppression of antiviral T cell responses [78].

Genetic IL-10 results are supported by elevated serum levels of IL-10 found in chronic untreated HCV-infected patients [79,80] and by a higher *in vitro* production of IL-10 by PBMC from those patients [81,82]. Similarly, high serum levels of IL-10 were reported in a prospective study of patients with acute infection who developed a chronic disease [38].

Conversely, several reports indicate that Th1 cytokines mediate the tissue injury and the chronic evolution of HCV infection. In this sense, it has been reported that the IL-18 and IFN-γ mRNA expression in the liver were significantly correlated with each other and both upregulated in chronic HCV patient [83]. Interferon-γ secretion by intrahepatic lymphocytes was almost absent in patients with persistently normal ALT levels compared with patients with abnormal ALT values [84]. These evidences might suggest that an initial inefficient Th1 response would generate the inflammatory mechanism responsible for the tissue injury observed in chronic patients.

#### 4.2.2. Role of cytokines in HBV infection

It is generally accepted that the presence of neutralizing anti-HBV antibodies is a marker of resolution of infection. These antibodies contain the spread of infection, facilitate the removal of viral particles and prevent the HBV reinfection but together with a Th2 antibody-mediated immune response, a Th1 cell-mediated immunity is very important in the HBV clearance (review in [85]).

During HBV infection, intrahepatic production of Th1 inflammatory cytokines and type-I IFNs activate two functionally independent pathways: an early elimination of HBV nucleocapsid particles from the hepatocytes; and a later post-transcriptional downregulation of viral RNA. Most of these effects are mediated direct or indirectly by IFN-α, β and γ [86,87]. Additionally, chronic HBV patients who clear the virus have higher levels of IL-12 than patients who remain HBV positive [88].

The role of IFN-γ in the antiviral T cell response to HBV was well documented in the transgenic mouse model. These experiments demonstrated that IL-12 can inhibit the replication of HBV through the induction of IFN-γ [89]. Similarly, reports regarding pediatric patients demonstrated the synergistic action of IL-12 and IL-18 which results in strong activation of HBcAg-specific Th1 lymphocytes, followed by a high IFN-γ production [90]. Analysis of IFN-γ gene polymorphisms suggested that diminished production levels of this cytokine was associated with susceptibility to HBV [91]. Supporting a role of Th1 cytokines as being also responsible for liver injury, serum levels of IFN-γ, TNF-α, IL-2, IL-6 and IL-18 were found increased during the chronic phase of the liver disease [92].

Regulatory T cells also seem to play a role in the outcome of HBV infection in chronic patients. The frequency of $CD4^+CD25^+$ Treg producing IL-10 was found increased in liver and PBMC of these patients [93]. Interestingly, experimental depletion of Treg led to an increase of IFN-γ production by Ag-stimulated PBMC [94]. Finally, modulation of function and expansion of HBV-specific $CD8^+$ cells by $CD4^+CD25^+$ has also been reported [95]. Thus, the effect of Treg cells over Th1-type cytokine production could contribute to viral persistence in chronic infection by interfering with the critical role that IFN-γ plays in protection against viral infections.

As described for HCV, IL-10 also seems to affect HBV disease progression. Self-limited HBV infection has been found associated with the low producer IL-10 allele [96].

## 5. Link between infection and autoimmunity

The breakdown of tolerance to self-antigens results in a chronic inflammatory state that may cause loss of organ function. The presence of a preexisting hepatic inflammation seems to be a crucial element in providing an environment for an effective activation of autoreactive T cells [10].

Although triggers leading to this pathway are unknown, it was suggested that infectious agents may induce autoimmune responses through the mechanism of molecular mimicry. For instance, HCV-specific $CD8^+$ cytotoxic T lymphocytes that recognize infected cells and contribute to viral clearance and tissue injury during HCV infection could be involved in the induction of autoimmune hepatitis (AH). A recent report [97] demonstrated that synthetic peptides derived from HCV core 178–187 (having a sequence homology with human cytochrome P450) are able to induce a naïve CTL repertoire which cross-reacts with self peptides derived from cytochrome P450 in PBMC from healthy or HCV-infected donors. These results indicate that HCV may induce autoreactive $CD8^+$ T cells via the mechanism of molecular mimicry.

### *5.1. Is there an association between type-I autoimmune hepatitis (AH) and hepatitis A virus infection?*

The AH is a progressive liver disease, with female prevalence, characterized by the presence of piecemeal necrosis, hypergammaglobulinemia and response to corticosteroid treatment. The presence of antinuclear (ANA) and/or smooth muscle (SMA) autoantibodies are the serologic hallmarks of the so-called type 1 AH [98]. The pathogenesis of AH may involve an environmental agent that triggers a cascade of T cell-mediated events directed to liver antigens in a host genetically predisposed to this disease. The so triggered immune response leads to a progressive necroinflammatory and fibrotic process in the liver. In addition of environmental factors, multiple genes are probably involved in the pathogenesis. Human leukocyte antigen (HLA) genes appear to play the dominant role in a predisposition to AH. Several reports from our laboratory have identified genetic and clinical differences between pediatric (PAH) and adult (AAH) forms of the type 1 AH which suggest that they may represent different clinical entities [99–101]. The comparison between PAH and AAH demonstrated that the HLA-DRB1*1301 DQB1*0603 haplotype is strongly associated with PAH [99,102,103], while HLA-DR3 and HLA-DR4 represent the HLA susceptibility genes associated with AAH [100,104].

Within environmental factors, infection by the hepatitis A virus (HAV) was postulated as a possible trigger factor of AH [105]. Argentina and Brazil are regions with a high prevalence of HAV infection [106] and also share the very strong association of PAH with the HLA allele DRB1*1301 [101,103]. We also investigated whether HLA may influence the outcome of the HAV infection. We found that children carrying the HLA DRB1*1301 haplotype, were associated with a higher susceptibility to develop a prolonged HAV infection. This susceptibility does not seem to be related to a higher predisposition to become infected by HAV, because this allele is not increased in children with self-limited HAV infection. Although these protracted forms of HAV infection are accompanied by the presence of anti-SMA antibodies, a long-term follow up of those patients (in some cases for as long as 5 years) failed to detect the development of AH [107]. These findings are in line with the current view that most autoimmune diseases are polygenic.

Besides molecular mimicry, other putative mechanisms of immunopathogenesis are involved. It has been recently demonstrated that a defective suppressor cell activity might contribute to the development of AH. In addition of a decreased number of $CD4^+CD25^+$ T cells, Tregs obtained from AH patients at diagnosis failed to inhibit $CD8^+$ T cell proliferation and cytokine production. Of note, the number of Treg negatively correlated with the levels of autoantibodies [108].

## 6. Role of cytokines in liver autoimmunity

### *6.1. Type I autoimmune hepatitis*

The constitutive production of cytokines is absent or minimal in most tissues including the liver [109]. Limited data are available for the participation of cytokines in the development of AH. In a previous study, we analyzed the expression of cytokines in liver biopsies from PAH patients in comparison with liver control samples obtained from cadaveric liver donors [110]. While the expression of IFN-$\gamma$ and IL-12p40 was not detectable in control livers, it was clearly upregulated in PAH patients. In addition, these samples showed an increased expression of IL-18, IL-4 and the IL-12 $\beta_2$ chain receptor. The unexpected increase of mRNA for IL-4, a typical Th2 cytokine, was found in conjunction with a severe histological inflammation. A similar association was reported in HCV [111] and primary biliary cirrhosis (PBC) [112]. Although IL-4 induces the differentiation of the Th2 subset [113], the presence of this cytokine in PAH livers did not prevent the expression of mediators of a type 1 immune response. In contrast to the finding in pediatric patients, IL-4 mRNA was amplified inconsistently at a very low level in hepatic tissue from adults with AH [114]. Therefore, the upregulation of IL-4 in PAH but not in another disease control group clearly suggests a more complex immunopathologic mechanism. The similar levels of IL-10 found in PAH and controls could reflect its constitutive expression by liver sinusoidal cells.

Th2 cytokines activate B cells and induce their differentiation into antibody-producing cells. Liver-infiltrating autoreactive B cells, in addition to their role in producing autoantibodies, also play a critical role in the development of fibrosis [115]. The mechanism of suppressing fibrosis by B-cell depletion is independent of antibodies or T cells, raising

the possibility that cytokines, produced or induced by autoimmune B cells, are responsible for fibrosis in autoimmune diseases targeting the liver.

Hypergammaglobulinemia is another characteristic feature of AH. The receptor-ligand pair CD40–CD154 provides key communication signals between cells of the adaptive immune system during inflammation and autoimmunity. CD154 is primarily expressed by activated T cells and CD40 is constitutively expressed on B lymphocytes and provides important signals that regulate clonal expansion and antibody production. Increased levels of CD154 mRNA were found to correlate positively with secretory Ig mRNA levels in the liver of patients with AH or PBC. This finding suggests its involvement in the hypergammaglobulinemia associated with these diseases.

As mentioned above, human liver contains an uncommonly high number of NKT cells. These cells participate in the early regulation of Th1/Th2 cell differentiation through the release of IFN-γ and IL-4. We found an increased number of Vα24 positive cells and transcripts coding for this invariant Vα24 chain in the liver of PAH patients, pointing to a probable involvement of these regulatory cells as mediators of the hepatocellular injury in PAH.

### 6.2. Primary biliary cirrhosis (PBC)

This is an organ-specific chronic cholestatic liver disease that usually develops in the adult life, predominantly in females often in perimenopausal age. The serologic hallmark of PBC is the presence of antimitochondrial antibodies (AMA) specific to the E2 component of the pyruvate dehydrogenase complex. These autoantibodies are unique among autoimmune serologic reactants because of their extremely high association with the disease index.

Primary biliary cirrhosis is characterized by a chronic and progressive destruction of small intrahepatic bile duct with portal inflammation and ultimately fibrosis, leading to liver failure in the absence of treatment. The current theory on the pathogenesis of PBC indicates that environmental factors might trigger autoimmunity in genetically susceptible individuals. In spite of the lack of a strong HLA-association, the recently reported concordance rate among monozygotic twins strengthens the importance of genetic factors in disease onset and progression. Additional factors, possibly infectious agents may intervene to trigger disease and the concept of induction via molecular (epitope) mimicry has been suggested [116].

The clinical features are heterogeneous, ranging from an asymptomatic presentation to end-stage liver disease. Fibrosis, cirrhosis and hyper IgM-globulinemia are associated with the progression of PBC [116]. Eosinophilia is also a characteristic finding in PBC patients. The deposit of major basic protein activated by IL-5 and released from eosinophils, is observed within the portal inflammatory infiltrate, and cause epithelial cell lysis [117]. Whereas 90% of stage 3 or 4 PBC patients express IL-5 mRNA in the liver, AH patients and healthy controls express none. Increased serum levels of TNF-α and TGF-β were found associated with an increased severity of PBC. Thus, a role for these two cytokines was proposed in the progression of the disease.

### 6.3. Primary sclerosing cholangitis (PSC)

This is a chronic cholestatic liver disease characterized by stricture of the biliary tree caused by a progressive inflammatory destruction of intrahepatic and extrahepatic bile ducts, and ultimately cirrhosis. It is often associated with inflammatory bowel disease [118]. Although the precise etiopathogenesis of PSC remains unknown, it was proposed the existence of an enterohepatic circulation of lymphocytes, whereby some mucosal lymphocytes produced in the gut during active inflammation persist as memory cells capable of recirculation throughout the liver [119]. The survival of these cells might initially require the presentation of peptide antigens delivered to the liver via the portal vein, as a result of the increased permeability of the inflamed gut, but the process could become independent of the gut if the recruited lymphocytes include T cells which recognize cross-reacting self-antigens. Under certain circumstances these gut-derived lymphocytes might become activated resulting in hepatic inflammation.

#### 6.3.1. Cytokines and PSC

Human β-defensins (hBDs) 1 and 2 are antimicrobial peptides that contribute to innate immunity at mucosal surfaces. hBD-1 is nonspecifically expressed in intrahepatic biliary epithelium and hepatocytes. By contrast, hBD-2 is only expressed in response to local infection and/or active inflammation induced in the large intrahepatic bile ducts by the effect of *Escherichia coli*, lipopolysaccharide (LPS), IL-1β and TNF-α, which induce Toll like receptor (TLR) signaling [120]. In conclusion, these observations suggest that in the intrahepatic biliary tree, hBD-2 is expressed in response to a local trigger, whereas hBD-1 may constitute a preexisting component of the biliary antimicrobial defense system.

Liver is always exposed to bacterial components derived from the intestine, thus expression of hBD-2 may be particularly relevant to PSC. Repeated stimulation with LPS and other bacterial products may result in the enhancement of the production of pro-inflammatory cytokines such as TNF-α. In fact, liver T cells from PSC individuals produce higher levels of TNF-α than T cells from PBC or AH patients, which might be associated to the periductular fibrosis present in these patients.

### 6.4. Nonalcoholic fatty liver disease (NAFLD)

NAFLD is a clinicopathological term that encompasses a disease spectrum ranging from simple triglyceride accumulation in hepatocytes (hepatic steatosis) to hepatic steatosis with inflammation (steatohepatitis), fibrosis and cirrhosis (the most severe outcome of steatohepatitis).

The importance of cytokines as effector molecules in liver damage has been particularly well demonstrated in patients and animals with alcoholic or nonalcoholic liver diseases ranging from steatosis to cirrhosis. TNF-α is involved in the progression from steatohepatitis to cirrhosis, since it promotes activation of stellate cells, matrix-gene expression, and matrix remodeling [121]. However, additional factors must also be present. Even after cirrhosis is well established, the ongoing production of TNF-α and related inflammatory cytokines modulates the expression of enzymes such as inducible nitric oxide synthase (iNOS), that regulates the production of vasoactive molecules mediating the hemodynamic abnormalities of cirrhosis, such as portosystemic shunting and the hepatorenal syndrome [122].

Although excess hepatic triglyceride accumulation is associated with various drugs, nutritional factors, and multiple genetic defects, the most common disorder associated with hepatic steatosis is insulin resistance [123], and as such, it has been proposed that NAFLD might be included as a component of the metabolic syndrome [124].

A ''two-hit'' model was initially proposed to explain the progression of NAFLD. The ''first hit'' constitutes the deposition of triglycerides in the cytoplasm of the hepatocyte. The disease does not progress unless additional cellular events that promote inflammation, cell death and fibrosis occurs, inducing a ''second hit''. The conventional explanation for the ''first hit'' is that obesity and insulin resistance result in increased release of fatty free acids (FFAs) from adipocytes. Increased adipocyte mass and increased hydrolysis of triglycerides through increased hormone-sensitive lipase activity contribute to elevated plasma levels of FFAs.

Fatty free acids taken up by the liver are metabolized by one of two pathways: oxidation to generate ATP or esterification to produce triglycerides, which are either incorporated into very low density lipoprotein particles for export, or stored within the hepatocyte. Defects in one or both of these pathways can lead to hepatic steatosis.

Within NAFLD, nonalcoholic esteatohepatitis (NASH) is histologically similar to alcohol-induced steatohepatitis. Arbitrarily, NASH is sub-categorized into ''primary'' and ''secondary'' [125,126]. Primary NASH, is the predominant form and refers to steatohepatitis that is associated with the dysmetabolic syndrome (obesity, type-2 diabetes, dyslipidaemia). Secondary NASH includes the steatohepatitis that accompanies other syndromes (for example, lipodystrophy) or caused by certain drugs (for example, amiodarone). Although unproven, it is likely that primary and secondary NASH share common pathogenic mechanisms.

There are two broad categories of factors implicated both in the development of NASH and alcoholic steatohepatitis: factors causing an increase in oxidative stress and those promoting expression of proinflammatory cytokines. The potential role of lipid-induced cellular injury and oxidative stress in the development of NASH escapes from the scope of this review and can be revised elsewhere [127]. Long-term ingestion of alcohol increases intestinal permeability [128], translocation of bacterial products from the intestinal lumen to the mesenteric circulation and its lymphatic vessels, and induces regional and systemic production of TNF-α and other proinflammatory cytokines. Furthermore, serum concentrations of both TNF-α and soluble TNF receptors are correlated with the degree of endotoxemia and the stage of liver disease in patients with alcoholic liver disease [129].

The possibility that increased production of TNF-α is the consequence, rather than the cause of alcohol-related liver injury cannot be ruled out. Indeed, the former possibility is suggested by evidence that serum concentrations of various cytokines are increased in patients with acute or chronic liver diseases of diverse origin [130,131]. Either being the cause or consequence of liver injury, the recent finding that exposure to alcohol does not induce steatohepatitis in mice in which the gene for type-I TNF receptor is disrupted constitutes the best evidence that TNF-α is a key pathogenic factor in alcohol-related liver injury [132]. Even though TNF-α appears to be necessary for the development of alcohol-related steatohepatitis, increased production of this cytokine is not sufficient to cause liver injury.

Obesity, especially visceral adiposity, is a major risk factor for NASH in humans [133]. Adipose tissue is a source of free fatty acids that are delivered to the liver and a depot for triglycerides that are synthesised by hepatocytes and released into the blood. As producers of TNF-α and IL-6, adipocytes are considered a component of the immune system [134]. Visceral fat, which appears to be less ''mature'' than subcutaneous fat, produces more TNF-α and free fatty acids but less adiponectin than subcutaneous fat. Adiponectin antagonizes both the production and activity of TNF-α; thus the effect of this cytokine is potentiated when adiponectin is scarce. In addition, TNF-α inhibits adiponectin [135]. Adiponectin also inhibits synthesis and uptake of FFA by hepatocytes, while stimulating FA oxidation enhancing their sensitivity to insulin. The combination of low adiponectin and high TNF-α levels in the context of increased hepatic exposure to FFA results in hepatic steatosis and severe hepatic insulin resistance.

### 6.5. *Cytokines in fibrosis and cirrhosis*

Chronic inflammation induced by viruses, toxins, and conditions like alcoholic and nonalcoholic steatohepatitis upset the balance between the synthesis and degradation of the liver matrix. This alteration in the composition of the matrix allows fibrosis to occur, compromising portal venous blood flow, which in turn compromises hepatic regeneration and promotes the portosystemic shunting of blood. All these changes lead to some of the clinical and more severe manifestations of advanced liver disease.

TGF-β, in addition to a key role in controlling T cells in the periphery [136], has been implicated in hepatic fibrogenesis. In chronic HCV, TGF-β stimulates the

production of extracellular matrix proteins and their receptors and inhibits the synthesis of matrix-degrading proteolytic enzymes [44,137,138]. Untreated HCV patients demonstrated a correlation between the fibrosis score and serum and liver levels of TGF-$\beta_1$. Similarly, those patients who respond to treatment with IFN-α showed a decrease in TGF-$\beta_1$ levels [138]. We analyzed the single-nucleotide polymorphisms (SNPs) of TGF-$\beta_1$ gene (G/C codon 25) in chronic HCV patients. By taking into account the clinical outcome, we found a significantly decreased frequency of the codon 25 C allele (low TGF-$\beta_1$ producer), in patients with fibrosis. Conversely, patients who did not develop fibrosis showed a higher frequency of this low producer allele (Fainboim et al., unpublished results). In patients with recurrent HCV infection after orthotopic liver transplantation, development of fibrosis was also associated with the presence of TGF-$\beta_1$ high producer alleles [44].

Experimental and clinical data suggest a protective role of IL-10 in hepatic fibrogenesis [78,139]. Chronically HCV-infected patients who received a short treatment with recombinant IL-10 showed a decreased hepatic inflammation and reduced liver fibrosis [139]. Similarly, a 12-month therapy with recombinant IL-10, in chronically HCV-infected patients with advanced fibrosis, led to increased levels of serum HCV RNA and a reduction in fibrosis score [140]. Our own study on the polymorphism of the promoter region of the IL-10 gene, demonstrated that those HCV patients carrying the polymorphism of the IL-10 which encodes for high IL-10 production, are associated with a low fibrosis score [74].

In addition to chronic viral infections, most liver diseases like AH, PBC, PSC and NASH, liver-associated morbidity and mortality are stronger in the subpopulation that progresses to cirrhosis. Excessive production of extracellular matrix by periductal and periportal fibroblasts causes these pathological changes. Cytokines such as TNF-α and TGF-β, as well as matrix metalloproteinases and tissue inhibitors of metalloproteinases are involved in fibrogenesis. Also, impediment to bile drainage results in progressive intrahepatic fibrosis.

## 7. Wound healing in the biliary tract significantly contributes to the development of liver disease

Bile duct epithelial cells (BEC) are normally protected from apoptosis by bile containing salts [141]. Bile acids activate EGF receptors via a TGF-α dependent mechanism and induce cyclooxygenase-2 (COX-2) expression [142], or trigger BEC IL-6 production [143].

Bile duct epithelial cells lining the smallest intrahepatic ducts are thought to contain a population of liver stem cells that can differentiate into either hepatocytes or BEC [144]. Virtually any bile duct insult, such as obstruction, infection, or immunologic damage triggers a sharp increase in IL-6 mRNA and protein production by BEC and peribiliary hematolymphoid cells [145]. This, in essence, alerts BEC to environmental stimuli and leads to subsequent autocrine, paracrine, and juxtacrine glycoprotein (gp) 130. The gp130 is one of the most promiscuous cytokine receptors [146], which binds to many different ligands including, IL-6, IL-11, leukemia inhibitory factor, oncostatin M, ciliary neurotrophic factor, cardiotrophin-1 and cardiotrophin-like cytokine.

A primitive or innate mucosal defense system in the liver protects against injury and stimulates repair. Trefoil family factor (TFF) proteins are comprised of one or more trefoil motifs, which consist of 6 cysteine residues. TFF proteins increase mucous viscosity and thereby contribute to optimal protection of the intestinal mucosa from injury [147]. There are three known TFF proteins which are differentially regulated in the gastrointestinal tract, of which TFF3 predominates in the biliary tree of mouse and human livers [148].

Wound healing responses can be triggered in BEC lining, the smallest intrahepatic bile ducts, as occurs in chronic necroinflammatory liver disease regardless of the underlying cause. In PSC, an ineffective wound healing can induce mural scarring and alterations in the large extra-hepatic bile ducts. Conversely, an exuberant wound healing response or ductular reaction contributes to the development of cirrhosis. During chronic necroinflammatory liver diseases a variety of insults, such as cholestasis, HCV replication, steatosis, copper deposition, and alcohol can cause oxidative stress, mainly in hepatocytes. The stress upregulates hepatocyte nuclear p21 expression which in turn, inhibits hepatocytes proliferation and accentuates ductular reactions [149]. A close relationship between BEC and periductal myofibroblasts in the smallest bile ductules usually results in co-activation of these populations, which often precede the development of cirrhosis. Cytokines and growth factors like hepatocyte growth factor (HGF) and TGF-β can down-regulate BEC TFF3 expression [150] and lead to an impaired biliary barrier function.

## 8. Cytokines and cachexia

Cachexia is a wasting syndrome, characterized by loss of lean body mass, weight loss, anorexia, and metabolic breakdown, frequently associated with chronic diseases including liver failure. Cachexia is believed to be a significant factor contributing to morbidity and mortality in these chronic diseases. Prolonged production of cytokines associated with chronic infections and other long-term immune reactions is increasingly recognized as a main causal factor. TNF was initially held responsible for causing these changes. Hypoalbuminemia, a frequent feature of cachectic patients afflicted with chronic diseases, is also a major contributor to their morbidity. Strong evidences suggest that TNF-α [151–153], in concert with IL-6 and other proinflammatory cytokines [154,155] are critical mediators of cachexia.

The progressive loss of fat and muscle mass observed in liver cirrhosis is likely to progress, on one hand due to an hypermetabolism mediated by factors such as frequent episodes of endotoxinemia, activation by inflammatory cytokines and/or increase in the β-adrenergic system and on the other hand reduced volitional food intake and malabsorption. Some of these factors may also be responsible for reduced appetite. Obviously, these mechanisms may also be operative in other disease entities [156].

## 9. Conclusions and future directions

In Fig. 2 we provide a summary of the classical and current view of the immunological behavior of the liver in health and disease.

The phenomenon of tolerance to self-antigens within a healthy liver may result from a steady-state migration of immature DCs to the RLN that occurs in the absence of activating stimuli and during normal tissue turnover. Additionally KC and resident liver DC are known to secrete IL-10 that contributes to the tolerance state. In response to a virus infection, a classic model for primary T cell activation can be established by the infection or cross-priming by resident DC. After migration to the RLN, DC differentiate and can activate naïve T cells. Activated $CD4^+$ are expanded and become effector T cells which once in the presence of infected hepatocytes secrete Th1 cytokines. Activated NK cells also contribute to the clearence of virus from infected hepatocytes. An alternative view was recently postulated [29]. Exogenous Ag expressed in hepatocytes can be presented to naïve $CD8^+$ T cells which after clonal expansion become efficient CTLs and also secrete Th1 cytokines. This study not only demonstrates that infected hepatocytes can act as an antigen presenting cell for naive $CD8^+$ T cells, but is also a provocative view of the "current" concept that $CD8^+$ T cells activated by Ag expressed on hepatocytes undergo abortive activation leading to premature apoptosis. These observations lead to the introduction of the concept that the liver is a "graveyard" or a killing field [30]. Additionally, the demonstration that Ag expression can be restricted to some hepatocytes, might explain

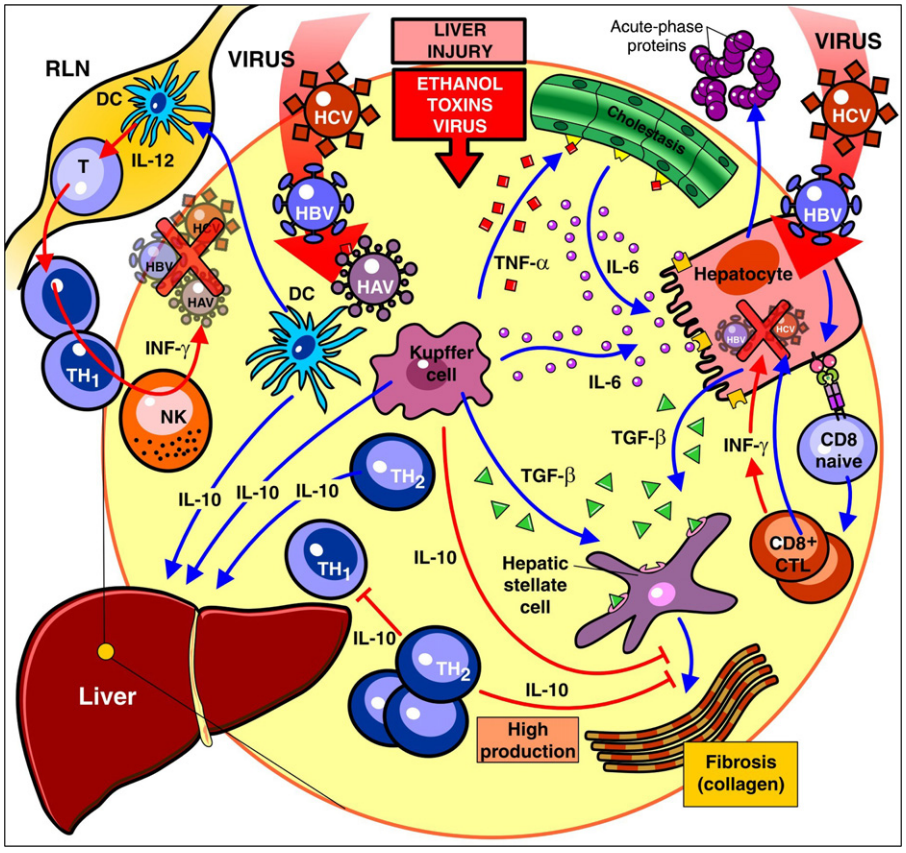


Fig. 2. Overview of immune and parenchymal cells during liver injury. A steady-state migration of immature dendritic cells (DC) to the regional lymph node (RLN) and the production of IL-10 by Kupffer cells and resident DC are involved in the phenomenon of tolerance to self-antigens within a healthy liver. After a virus infection, viral particles are incorporated into DC either because they become infected or through cross-priming and then migrate to the RLN, where they differentiate and activate naïve T cells. Effector $CD4^+$T cells return to the liver and through secretion of Th1 cytokines and collaboration with activated NK cells, might contribute to the virus clearance. In an alternative view, exogenous Ag expressed in hepatocytes can be presented to naïve $CD8^+$ T cells which after clonal expansion become efficient CTLs and secrete Th1 cytokines Under conditions of liver injury, KC play a critical role through secretion of TNF-α, TGF-β and IL-6. The latter acting on hepatocytes induces the production of the acute phase proteins. TGF-β activates the induction of fibrosis through the action of stellate cells and TNF-α seems to play a critical role in the induction of cholestasis. A high production of IL-10 is able to modulate the development of fibrosis.

that some liver pathogens such as hepatitis C might use this mechanism of $CD8^+$ T cell activation. In addition to the role of proinflammatory cytokines in the viral clearance, they also play a critical role in the liver injury under conditions of viral persistence. It is of note the role of high levels of IL-10, both by increasing viral load through inhibition of Th1 responses and for their role of controlling fibrosis development.

We have also discussed here the contribution of KC to different types of liver injury. During the early phase of chronic liver disease, agents like virus, ethanol or toxins induce secretion of TNF-α, TGF-β and IL-6. The latter, acting on hepatocytes, induces the production of the acute phase proteins. TGF-β activates the induction of fibrosis through the action of stellate cells and TNF-α seems to play a critical role in the induction of cholestasis. Clinical features of chronic liver disease that are mediated by cytokines include in addition to fibrosis and cholestasis, the hypergammaglobulinemia associated to autoimmune diseases and cachexia present at late stages of chronic liver diseases.

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