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IMMUNOMODULATION IN ADENOVIRUS-INDUCED VIRAL HEPATITIS

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IMMUNOMODULATION IN ADENOVIRUS-INDUCED VIRAL HEPATITIS

by

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To my parents

Eppur si muove

Galileo Galilei (1564-1642)

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ABSTRACT

Hepatotropic viruses are one of the major etiologic causes of morbidity and mortality worldwide. Currently, there is a great interest in examining the mechanisms of viral clearance versus persistence. The liver produces and releases IL-33 and hepatocyte growth factor (HGF) upon viral infection, two molecules that favor hepatocyte survival. In this dissertation, we asked whether IL-33 and HGF could influence outcomes in viral hepatitis. We found that IL-33 and its receptor (ST-2) are expressed during Ad-induced hepatitis in mouse. Furthermore, treatment with exogenous IL-33 during the course of infection decreased hepatocyte apoptosis and ALT release by inhibiting TNF-α production. However, lymphocyte infiltration was not modified by IL-33. Interestingly, IL-33 enhanced both type 1 cytokines (IL-2 and IFN-γ and type 2 cytokines (IL-5 and IL-13). In addition, exogenous IL-33 promoted the expansion of innate lymphocytes class 2 (ILC2). When we co-cultured ILC2 with intrahepatic lymphocytes from Ad-infected mice, TNF-α production was decreased. Collectively, this study indicates that IL-33 could be explored as a promising therapeutic tool in viral hepatitis. Here, we provide evidence that the HGF axis is activated during viral hepatitis. We further examined HGF role during viral hepatitis by over-expressing it and found that HGF ameliorated liver damage by diminishing lymphocyte infiltration and the expression of type I interferon and IL-6. HGF also modulated dendritic cells activation *in vivo* and *in vitro* by decreasing IL-12p40 and CD40 expression. We also observed that HGF diminished TGF-β and IL-10 production in serum and liver tissues. In line with these results, the expression of CD8 T cell inhibitory markers (Tim-3 and PD-1) also decreased. Our findings suggest that HGF mediates organ protection by promoting hepatocyte survival, but like IL-33, it does not abolish viral clearance. In summary we have described the hepatoprotective and immune-regulatory role of IL-33 and HGF in Ad-induced hepatitis. Furthermore, these two naturally occurring molecules in the liver do not mediate liver protection by abolishing anti-viral responses. Therefore, immune regulation with both cytokines could be a promising therapeutic tool for treating viral hepatitis.

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LIST OF ABREVIATIONS

Ad	Adenovirus
AdLaZ	Adenovirus with recombinant β-galactosidase
APCs	Antigen presenting cells
Bcl-2	B-cell CLL/lymphoma 2
BMDCs	Bone marrow-derived dendritic cells
CCL17	Chemokine (C-C motif) ligand 17
CCL22	Chemokine (C-C motif) ligand 22
c-kit	Kit oncogene
c-Met	Mesenchymal epithelial transition
CVH	Chronic viral hepatitis
CXCL9	Chemokine (C-X-C motif) ligand 9
DAMP	Damage-associated molecular pattern
DCs	Dendritic cells
FLP2	Fibrinogen like protein 2
FVH	Fulminant viral hepatitis
HAV	Hepatitis A virus
HBsAg	Hepatitis B surface antigen
HBV	Hepatitis B virus
HCV	Hepatitis C virus
HDV	Hepatitis D virus
HEV	Hepatitis E virus

HGF	Hepatocyte growth factor
HIV	Human immunodeficiency virus
HSCs	Hepatic stellate cells
ICOS	inducible T cell co-stimulator
IFN	Interferon
IFN-γ	Interferon gamma
IHL	Intrahepatic lymphocytes
ILC2	Innate lymphocytes class 2
KCs	Kupffer cells
LCMV	Lymphocytic chroriomeningitis virus
LSECs	Liver sinusoidal endothelial cells
MAVS	mitochondrial antiviral signaling protein
mDCs	Myeloid dendritic cells
MHC-I	Major histocompatibility complex class I
MHC-II	Major histocompatibility complex class II
NF-kB	nuclear factor of kappa beta
NK	Natural killer
NKT	Natural killer T cells
TNFSF4	tumor necrosis factor (ligand) superfamily, member 4
PD1	Program death 1
pDCs	Plasmacytoid dendritic cells
PDL-1	Program death ligand 1
PRR	pathogen-recognition receptors

SENV	SEN virus
sST2	Soluble Suppressor of tumorgenesis
ST-2	Suppressor of tumorgenesis
TGF-β	Tumor growth factor beta
T regs	Regulatory T cells
Th2	T helper 2
Tim-3	T-cell Immunoglobulin and Mucin-domain 3
TLR	Toll like receptor
TLR2	Toll like receptor 2
TLR9	Toll like receptor 9
TNF-R1	Tumor necrosis factor receptor 1
TNF-α	Tumor necrosis factor alpha
TRIF	TIR domain containing adaptor inducing interferon-inhibitor
WHO	World health organization

CHAPTER 1: INTRODUCTION TO LIVER BIOLOGY

1.1 Liver microenvironment

The liver is a unique organ that sits in the interphase between the intestinal circulation and the systemic blood stream. It is exposed to numerous exogenous antigens from partially digested food, and also receives a considerable amount of lipopolysacharide from the gut commensal bacteria. Through its central role in vital metabolic and synthesis processes, the liver should restrain any possible infection coming from the gut [1]. The immune processes in the liver should be orchestrated to ensure tolerance to antigens from food, but also to mount a fast and effective immune response to potential invading pathogens. Hence, the liver microenvironment is particularly rich in TGF-β and IL-10, and both molecules can directly influence the immune response to infectious agents [2-4].

The liver is constituted mainly by hepatocytes (~80%), liver sinusoidal endothelial cells (LSECs) and immune cells (~20%). The percentage of immune cells is represented by resident macrophages (Kupffer cells), natural killer cells (NK), natural killer T cells (NKT), dendritic cells (DCs) and T cells [5]. All the liver antigen-presenting cells (APCs) can be potentially activated through pathogen-recognition receptors (PRR) [5].

Hepatic antigen-presenting cells comprise a heterogeneous population of cells: (1) the classic APCs represented by DCs and Kupffer cells (KCs), and (2) the non-classical populations such as LSECS and hepatic stellate cells (HSCs) [6]. Hepatocytes are also able to serve as antigen-presenting cells under pro-inflammatory conditions [7]. Despite being classified as non-immune cells, they can influence the immune response by synthesizing proteins of the complement system [8]. Under inflammatory conditions, hepatocytes can express co-stimulatory molecules [9] and present antigens to CD8 T cells [10]. *In vitro* studies showed that hepatocytes are not able to promote sustained CD8 T cell survival [11]. However, the relevance of hepatocytes as APCs in the liver is still to be defined.

Other non-classical immune cells such as HSCs and LSECs can also serve as APCS, but in contrast to hepatocytes, these cells are able to activate CD4 and CD8 T cells [7]. Between HSCs and LSECs, the latter are more efficient in priming naïve T cells [10]. LSECs may also exert some immunomodulatory roles by inhibiting CD8 T cell activation. This subset of cells display the co-inhibitory molecule programmed death ligand 1 (PD-1), which can halt CD8 T cell activation [12]. Studies *in vitro* have also demonstrated that virally- infected LSECs can promote efficient CD8 T cell priming as well [13]. LSECs may also impact the final clinical outcome of viral hepatitis because hepatitis B and HCV have particular tropism for this cell type [14, 15]. Therefore, it would be instrumental to elucidate the factors that can influence LSECs behavior.

HSCs are mesenchymal cells with the potential to differentiate into fibroblasts and synthesize the extracellular matrix. HSCs express MHC-I and MHC-II and, and they can present antigens to either CD8 or CD4 T cells. Furthermore, SC also display CD1b and CD1c molecules conferring on them the ability to present glycolipids to natural killer T cells (NKT), which can activate the antiviral response in a very acute manner. Like LSECS, the immune role of HSCs is far from being elucidated. Whereas some studies *in vitro* claim the ability of HSCs to prime effector T cells at comparable levels of DCs [16], other studies have shown that HSCS preferentially prime regulatory T (Tregs) cells [17] and also trigger apoptosis of effector T cells in a PD-L1 dependent manner [18]. The immune behavior of activated HSCs gains particular relevance in chronic viral infection because they are an important source of TGF-β which can also mediate liver fibrosis [19].

KCs are the most numerous resident APCs in the liver. Under homeostatic conditions, KCs trigger tolerogenic responses to food antigens and LPS that come from the portal circulation by producing prostaglandin E2 and IL-10. The role of KCs in the liver varies depending on the type of stimulus. KCs are able to protect the liver from ischemia-reperfusion injury by secreting IL-10. In viral hepatitis, depletion of KCs leads to an increase hepatocytes loss [20]. On the other hand, KCS can activate NK and NK T cells upon TLR3 activation [21, 22]. KCs are able to perform cross-presentation [10]. In the context of systemic viral infections, KCs mediate bystander activation of CD8 T cells in influenza virus infection. This can potentially mediate liver damage [23].

Hepatic DCs are composed of a heterogeneous population cells that are in continuous transit in the liver sinusoids and outside the liver [24]. The immune role of hepatic DCs, like KCs, cannot be defined in simple terms. For example, myeloid DCs (mDCs) in the liver produce more IL-10 in comparison with their counterparts in other organs [25]. However, hepatic DCs can be activated when the concentration of pro-inflammatory cytokines reaches high levels [26].

Plasmacytoid DCS (pDCs) are another subset of APCs that is present in considerable quantities in the liver [26]. Hepatic pDCs play an instrumental role in the primary response to viruses, they constitutively express toll-like receptor 7 (TLR7) and toll like receptor 9 (TLR9) and secrete large quantities of type I interferon when these receptors are engaged [27]. Hepatic pDCs are also able to prime CD8 T cells [28]. Like the mDCs, hepatic pDCs have a tolerogenic cytokine profile when compared to that of splenic counterparts, as they express more IL-10 and less IL-12p70. However, it is also true that these tolerogenic features in pDCs are in part mediated by their interactions with regulatory T cells [29], and pDCs have an instrumental role in containing chronic viral infections [30]. In patients with chronic hepatitis B infection, there is a severe abrogation in the production of IL-12p70 and expression of OX40L in pDCs. Moreover, it was showed that defective pDCs activation correlates with viral persistence [31].

1.2 Etiologic agents of acute and chronic viral hepatitis

Acute viral hepatitis (AVH) is a clinical entity defined as liver inflammation lasting less than 6 moths. Worldwide, acute viral hepatitis represents an important cause

of mortality and morbidity. Fulminant hepatitis (FH) is one serious complication of acute hepatitis of viral etiology. In the US, FH causes approximately 285 deaths a year [32]. Another clinical presentation of viral hepatitis is chronic viral hepatitis (CVH) is defined as the hepatocellular necrosis and inflammation caused by viruses that last more than 6 months. Hepatitis B virus (HBV) and C virus (HCV) are the major causes of CVH. Furthermore, CVH is directly related to 700,000 to one million of deaths annually [33, 34] and is ranked as one of the top five preventable causes of mortality worldwide [35].

A wide variety of viruses can cause a clinical presentation consistent with that of acute hepatitis: hepatitis A virus (HAV), hepatitis B virus (HBV), hepatitis C virus (HCV), hepatitis D virus (HDV) and HEV[32]. In contrast to acute viral hepatitis, CVH is typically caused by HCV and HBV or the co-infection of HCV and HBV. However, there have been reports on chronic damage due to HAV [34].

HAV is a member of the genus *Hepatovirus* and the *Picornavidirae* family [36]. It is a single-stranded RNA positive-sense virus, which encodes four structural and seven nonstructural proteins. HAV is not an envelope virus but it can resist changes of pH, conferring a lot of stability to the virions as they pass through the intestinal tract [37].

HAV is the most common cause of viral hepatitis worldwide. HAV is transmitted by direct person-to-person contact through the oral-fecal route. The seropositivity of any of the HAV envelope protein varies from 11% in children younger than 5 years to 70% in people older than 50 years. The incidence rates and prevalence of HAV are closely

related to socioeconomic development. Thus, areas with bad management of quality in water purification and poor hygiene conditions are by far the most endemic areas [34, 38].

HAV causes a self-limiting disease but on some occasions can lead to fulminant hepatitis. HAV infection has a window period of 15 to 50 days. HAV replicates exclusively and extensively in hepatocytes, before it is detected by the immune system [39]. After new virions are formed, they are secreted into the bile secretions. HAV is shed in the stool one week before the individuals show any clinical manifestation of viral hepatitis [39]. In some individuals HAV causes a silent infection without signs or symptoms. HAV-infected individuals have prodromal symptoms that are unspecific: fever, myalgia, malaise, weakness, vomiting and nausea. Thereafter, those infected have jaundice and dark urine, signs which last for several weeks, followed by a convalescence period [36]. Some extra-hepatic manifestations of HAV include Guillain-Barré syndrome, vasculitis, renal failure, pancreatitis, bradycardia and changes in electrocardiogram [prolongation of the P-R interval, T wave changes and left axis deviation [37]].

HEV is a non-enveloped virus part of the *Hepeviridae* family, which comprises four genotypes. HEV is a positive single-strand RNA genome, which encodes several structural and nonstructural proteins in over-lapping reading frames [40]. According to the World Health Organization (WHO) there are 20 million of HEV cases per year. Classically, HEV causes disease in areas of the world having limited resources and poor

hygiene. Southeast Asia has been particularly affected by HEV outbreaks [41]. However, there have been documented cases in European residents with no history of traveling to endemic areas, and the presence of sero-positive individuals indicates that HEV might be circulating in Europe [42-45].

After HEV infection, there is an incubation period of 2 to 8 weeks followed by a prodromal phase with unspecific symptoms. The classical symptoms last for around 2 to 8 weeks. Like HAV, HEV causes a self-limiting disease in immunocompetent individuals. However, in immunodeficient hosts the HEV can cause a chronic infection [46]. Furthermore, pregnant women are particularly susceptible to develop fulminant hepatitis when they are infected with HEV [47]. In Europe, there have been cases of fulminant hepatitis cause by HEV [48].

HBV is a circular double-stranded DNA member of the *Hepandaviridae* family. The HBV genome encodes four open reading frames. HBV is an enveloped virus [49] transmitted via perinatal, sexual intercourse, and direct contact with secretions from infected individuals. Importantly, HBV can be transmitted through unsafe injection practices. In 2012, it was estimated that this risk factor accounted for 20 million new cases of HBV [34].

HVB infection is a major health problem in certain regions of the world, e.g. like in sub-Saharan Africa, Southeast Asia, China and Alaska. In these regions, the prevalence can reach 20 percent of the population. In the industrialized countries,

vertical transmission is still a public health concern; around 20 to 50% of the infants who are infected via the perinatal route evolve to a chronic state. In the USA there are yearly 46,000 new cases of HBV infections, although the incidence may be an underestimation. African-Americans constitute the major risk group to acquire the HBV, followed by Hispanics and Whites [32].

HBV can cause signs and symptoms of acute viral hepatitis; the diagnosis is done using serological markers as a reference. HBV surface antigen (HBsAg) turns positive within 1 to 10 weeks post-exposure and this also matches with the appearance of jaundice in the patient. HBsAg is cleared in individuals who can eliminate the virus [32].

HBV has an error-prone reverse transcriptase, which confers it the ability to generate a diverse progeny. The diversity in the HBV new virions promotes immune evasion that may be involved in persistence [50]. In the individuals who cannot clear the virus, antibodies against HBV core protein start to increase along with the persistent levels of HBsAg [51].

HDV is a RNA virus part of the *Deltaviridae* family, considered a defective virus because it requires HBV surface antigen proteins for its assembly [52, 53]. Hence, HDV epidemiology is closely related to HBV infection [53]. In western countries, HDV incidence is particularly high in intravenous drug users. The prevalence of HDV and HBV co-infection is between 2 to 9 percent [52, 54].

The clinical relevance of HDV infection relies on the fact that HDV and HBV co-infection can modify the outcomes of the disease. When co-infection with HDV and HBV occurs at the same time only 2 % of the individuals develop a chronic infection. In contrast, when individuals chronically-infected with HBV are infected with HDV there is a rapid progression of liver damage to the establishment of cirrhosis or it may also lead to fulminant hepatitis [53, 54]. Some studies indicate that HDV infection in chronically-infected patients increases the risk of hepatocellular carcinoma by three fold [55].

HCV is a single-stranded positive sense RNA virus, member of the *Flaviviridae* family. The HCV genome encodes a core protein, envelope proteins, and nonstructural proteins with protease activity. Nonstructural proteins have protease activity and cleave virus and host proteins [56]. HCV is transmitted via parenteral routes and through the mucosal exposure to infected secretions. The normal course of the HCV infection is characterized by a silent primary infection followed by the establishment of a persistent infection, with the subsequent development of complications: cirrhosis or hepatocellular carcinoma.

HCV has an incubation period of 14 to 120 days. In a few cases, the primary HCV infection presents itself with any clinical manifestation. The pre-icteric phase of HCV acute infection is characterized by nonspecific symptoms: fatigue, anorexia, right upper quadrant pain, and febricula. The icteric phase lasts from 1 to 2 weeks and the liver enzyme activity is increased approximately 6 to 12 weeks after exposure [32]. Very few

patients with HCV infection develop acute symptoms of hepatitis. Importantly, the individuals who have clinical manifestations of acute HCV infection are less prone to develop the chronic complications of HCV infection.

The diagnosis of acute HCV infection relies on a high index of suspicion, occurring in individuals with a history of intravenous drug use, unsafe medical injections, recent blood transfusions (very low risk), or unprotected sexual intercourse in the last 12 weeks [33]. After HCV exposure is recognize, it is recommended that the individual be screened for anti-HCV antibodies in serum or the presence of HCV RNA. The current gold standard for HCV diagnosis is based on serology, which can allow detection of positive individuals from 4 to 10 weeks after infection [57].

HCV is perhaps the leading cause of chronic viral hepatitis. HCV replicates its genome by using an error-prone RNA-dependent RNA polymerase, and as a result, numerous groups of viruses emerge with wide antigenic variation or quasi-species [58]. This confers to HCV an enormous antigenic variation that helps it to escape from the immune system. Hence, in the vast majority of HCV-infected patients (around 75%), HCV primo-infection does not cause any signs or symptoms. Thus, it is recommended to screen individuals with risk factors.

Depending on the reference centers, up to 20% of the causes of viral hepatitis cannot be defined [59]. Hepatitis G virus (HGV) is a positive-sense RNA virus recently associated with human disease with concomitant markers of hepatitis [60]. A recently

described DNA virus, the SEN virus (SENV) has been also implicated in viral hepatitis in humans [61]. In a Greek center the HGV and SEV prevalence in patients with hepatitis of unknown etiology reaches up to 29% and 36%, respectively [62]. Other causes of viral hepatitis are represented by parvovirus B19 [63], herpes simples virus, and cytomegalovirus [64].

1.3 Pathogenesis of viral hepatitis

Most of the viruses that cause hepatitis are not directly cytolytic. Thus, it is generally accepted that immune recognition mediates liver damage during these infections. The immune players during acute versus chronic infections vary during the course of infection [65]. Immune recognition of hepatotropic viruses is primarily done through pattern recognition receptors or toll-like receptors (TLR). TLR3 activation in the liver mediates an overwhelming production of type I interferon, TNF-α, IFN-γ and chemokines that are able to recruit immune cells to the liver [66, 67]. There is also emerging and supporting evidence that stimulation of intracellular TLRs is essential for myeloid cell cluster formation and subsequent T cell priming [68]. Furthermore, locally primed T cells are shown to be more effective in clearing pathogens [69].

One of the effects downstream of the production of IFN-γ and TNF-α is the expression of co-stimulatory molecules and establishment of a chemokine gradient that leads to lymphocyte infiltration to the liver [11]. Many viruses evade innate immune recognition by subverting the TLRs pathway. HAV inhibits TLR3 signaling by cleaving the TIR domain containing adaptor inducing-interferon-β (TRIF) [70] to have ability to

subvert TLR3 signaling allows HAV to have robust replication before the immune system detects it [71]. HCV also inhibits TLR3 activation by cleaving the mitochondria antiviral signaling protein (MAVS) [72]. Similarly, HBV e antigen (HBeAg) suppresses TLR signaling pathway by physically interacting with TIR-containing proteins and toll like receptor 2 (TLR2) [73]. Furthermore, the direct association of HBV with TLR2 promotes the expansion of T regs.

The strength and the duration of the antiviral response also determine the clinical outcome and the pathologic changes seen in infected individuals. The immune response to viruses can trigger apoptosis in the liver by three different mechanisms: (1) the engagement of the Fas ligand on hepatocytes, (2) release of cytotoxins (granzyme and perforin), and (3) activation of TNF-α-TNF-R1 axis [74].

The mechanisms that mediate the establishment of ***fulminant viral hepatitis*** (FVH) are not well understood. The correlation of viral loads and FVH is not always strong. In studies of FVH caused by HAV the viral load directly correlated with the severity of the liver damage [75], whereas in some others this observation was inverse [37]. Conversely, in individuals with viral FVH caused by HBV, the viral copy numbers were not as high as that in individuals with the normal evolution of the infection [76]. The idea that hepatotropic viruses initiate pathology through immune-mediated mechanisms is supported by the fact that superinfection with HAV in individuals chronically infected with HCV may lead to FVH [77]. Therefore, it is possible that immune mechanisms mediate the loss of a large quantity of the hepatocyte pool [76].

Previous reports showed that in FVH, the deletion of infected hepatocytes was done in a Fas-Fas ligand-dependent manner. In mice, the use of anti-Fas antibody can emulate the liver destruction seen in fulminant hepatitis [78]. Later studies in human subjects undergoing fulminant hepatitis showed that the Fas/Fas ligand axis was up-regulated and that this phenomenon correlated with the severity of the disease [76, 79-82].

The mechanisms that lead to the excessive Fas-mediated apoptosis in viral fulminant hepatitis could be the deregulation of the immune response at different checkpoints. KCs over-activation may play an important role in the pathogenesis of FVH [83]. Additionally, disruption of inhibitory signals can exacerbate liver damage. PD1/PDL-1 deletion in mouse hepatitis virus strain 3 (MHV-3) infection leads to a severe form of fulminant hepatitis. Furthermore, in the same study it was shown that the absence of the PD-1/PD-L1 axis also promotes the synthesis of the fibrinogen-like protein 2 (FLP2), a pro-coagulant factor that can promote consumptive coagulopathy, one of the hallmarks of fulminant hepatitis. Furthermore, it was shown that exacerbation in liver pathology was done in an IFN-γ- and TNF-α-dependent manner [84]. Other studies using murine models of FVH showed that over-expression of CD40L and CD40 drastically increased liver necrosis and mortality in adenovirus (Ad)-induced hepatitis [9, 85]. On the other hand, the unbalance of cytoprotective cytokines IL-25 (IL-17E) has been also postulated to be in part responsible for the massive hepatocyte loss [86].

In ***acute viral hepatitis***, hepatotropic viruses trigger hepatic inflammation in an acute manner when they are sensed by components of the innate immune response. Of

note, the liver is densely populated by immune cells that do not need antigen-specific activation; NK and NKT cells together comprise 60% of the intrahepatic lymphocytes under steady conditions [87].

The early interactions of HBV with the innate immune system are not well understood. In non-human primates early HBV infection does not change the global gene profile [88]. There is evidence that HBV components interact with toll-like receptors and trigger the activation of KCs with the subsequent production of IL-6, IL-8 and TNF-α [89, 90]. Subsequently, KCs have been reported to trigger the activation of NK and NKT cells which produce pro-inflammatory cytokines [91]. Thus, it is possible that an early immune response to HBV is IL-6-dependent and type I IFN-independent.

APCs activation and subsequent T cell response during early stages of viral hepatitis may determine the clinical outcome to great extent [92, 93]. Chronic infection with HCV and HBV has been characterized by cycles of inflammation that promote liver fibrosis (cirrhosis) and loss of function [94]. Furthermore, the constant inflammation cycles without HCV and/or HBV clearance have been shown to be a risk factor for the development of hepatocellular carcinoma [95].

In ***chronic hepatitis*** apoptosis also plays a major role. However, the immune elements involved in this process differ [96]. As mentioned previously, the intrinsic ability of HCV and HBV to produce a diverse progeny plays an important role in the primary immune evasion. Subsequent liver intrinsic homeostatic mechanisms have been

reported to promote viral persistence and cycles of inflammation (discussed sections following) [65]. These two events combined generate waves of apoptotic hepatocytes which can modify the behavior of liver non-parenchymal cells, as the healing process is involved when the viral infection progresses. One of the hallmarks of wound healing is the expression of Th2 cytokines, major players in liver fibrosis [97]. Furthermore, the activating TLR9 in KCs and HSCs by apoptotic hepatocytes have been shown to promote the secretion of large TGF-β quantities which is also a central element in liver fibrosis [98, 99] and, unfortunately, in viral persistence [3].

In ***chronic viral hepatitis,*** a switch has been reported from a primary Th1 response to Th2, along with the expression of high concentrations of IL-10 and TGF-β were found to exert an inhibitory effect on T cells [2, 97]. IL-10 and TGF-β were found to be deleterious for viral clearance by promoting expression of T cell exhaustion markers on CD8 T cells [3, 100]. T cell exhaustion reportedly occurs at the point in which antigen-specific T cells play pivotal roles in the establishment of chronic infection [101]. CD8 T cells from chronically infected mice and individuals with HCV have displayed a combination of program death 1 (PD-1) and T-cell Immunoglobulin and Mucin-domain 3 (Tim-3) [102-104], and this combination was shown to correspond to a profound inhibition of T cell function [105]. On the other hand, monoclonal antibodies against PD-1 or Tim-3 molecules have helped to restore T cell activation *in vitro* [106]. Of note is that antibody neutralization against TGF-β does not appear to reverse the exhaustion phenotype in T cells [4]. Therefore, immuno-modulation that leads to a lower

production of TGF-β and IL-10 seems to be more efficient in restoring the anti-viral response.

In addition to the mechanisms described above, the liver has been found to have negative feedback loop that consists of the expression of PD-L1 in response to IFN-γ and TNF-α [107]. Subsequently, PD-L1 expression in the liver may lead to T cell inhibition or exhaustion, diminishing the anti-viral response [108]. In mice chronically infected with clone 13 of the lymphocytic choriomeningitis virus (LCMV), type I IFN antibody neutralization and the deletion of the type I IFN receptor has been found to accompany with fewer IL-10 production, diminished PD-1 expression and less viral titers [109, 110]. These data have helped to explain why higher expression of the interferon-inducible gene is correlated with failure to clear HCV in some individuals [111]. Altogether, these data indicate that viral persistence and the pathologic changes seen in chronic viral hepatitis are the sum of deregulated immune regulatory mechanisms.

No pathognomonic changes for the diagnosis of etiology and stage of viral hepatitis have been characterized in the histopathological examinations. Thus, the exact diagnosis must combine clinical and other serology markers. In fulminant hepatitis, massive necroinflammatory lymphocytic infiltration has been seen in the central vein or in portal to portal bridging necrosis [82, 112, 113]. In acute viral hepatitis there is early infiltration of mononuclear cells that mainly comprises the portal and periportal areas. Other findings are cholestasis with bile salts precipitation in the ducts. Another hallmark of acute viral hepatitis is the presence of atypical hepatocytes with nucleus condensation

and a prominent nucleolus, indicating apoptosis. Depending on the severity of the inflammation, there are areas of necrosis in the periportal regions. In chronic hepatitis the lymphocytic infiltration affects the biliary ducts preferentially rather than the portal vein as seen in acute hepatitis.

1.4 Prophylaxis and treatment modalities in hepatitis

It is suspected that an individual is undergoing fulminant hepatitis (FH) when there are clinical and paraclinical indicators: marked depletion of coagulation factors, prolongation of the prothrombin time, hypoglycemia, and cerebral edema manifested clinically with mental status changes [114]. In the case of FH, the clinical team should determine its etiology, and then, treat the life-threatening conditions. For example, hypoglycemia can be corrected by administering dextrose solution, while cerebral edema can be treated via hyperventilation, manitol administration and hypothermia. Consumption of coagulation factors should be corrected with fresh plasma [114]. However, should liver failure progress rapidly, a liver transplant is recommended. The long-term outcome varies with the etiologic agent of FH. For years FH of viral etiology has been a relative contraindication of liver transplantation. Currently, it is known that patients with FH caused by HBV have better prognosis and better graft survival than individuals infected with HCV [115]. Moreover, an improved understanding of liver regeneration has opened the possibility of treating FH without having to resort to a liver transplant. Hepatocyte growth factor (HGF) has been proven to be tolerated in patients with FH [116] (discussed in the following sections). A recent study showed that

manipulation of mitogen-activated kinases in hepatocytes can potentiate their capacity to replicate and engraft in a new recipient. Thus, cell therapy is also feasible [117].

In general, the treatment for uncomplicated acute hepatitis caused by either HAV or HEV infection relies on supportive therapy. In individuals without complications, good hydration and a well-balanced nutrition are recommended. For ***HAV*** treatment, an immunoglobulin is available that is effective in individuals who had recent contact with HAV-infected person within the previous 2 weeks. The immunoglobulin is given intramuscularly at 0.02 mL/kg [118]. In HAV infection, active immunization is also available for primary and secondary prevention. Currently, there are three different licensed vaccines composed of inactivated virus, and all of the options can be immunogenic in 95% of the recipients. HAV vaccination is recommended for individuals who travel to endemic areas, residents from those endemic areas, patients with hemophilia, and pre-existing hepatic disease, veterinarians or personnel that manage livestock, children in daycare facilities, and health workers [119]. In some endemic and non-endemic areas, the HAV vaccine has helped to decrease the incidence of new cases of HAV infection [36, 120]. Furthermore, the HAV vaccine, like passive immunization, is effective in recently exposed individuals [118].

For ***HEV*** infection, the treatment is only directed to decrease the burden of the symptoms. However, there is currently an HEV vaccine composed of recombinant antigens [121] that showed to confer protection rates of up to 95% following its use in a phase II clinical trial [122].

The most effective strategy for treating ***HBV*** infection is primary prevention by using a vaccine. In 1996, the WHO recommended universal immunization for the prevention of HBV [123]. In the USA and worldwide, the establishment of universal vaccination programs at birth resulted in a sustained decrease in cases of HBV infection. The HBV vaccine consists of virus recombinant proteins that are given in three doses and can elicit a humoral response in 95% of the vaccinated children. Moreover, the immune recall to HBV vaccine has been documented to be present two decades after intervention [124]. This universal immunization coverage at birth is important because neonates exposed to HBV are more prone to develop chronic complications as a result of the infection [125]. Additionally, the screening for HBV silent infection is also recommended during the first trimester of pregnancy (sustained positivity of HBsAg, anti-HBV and anti-HBs antibodies). Other groups at risk should also be examined for HBV presence, including: intravenous drug users, individuals with elevated enzyme activity with an unknown etiology, donors of fluids, and patients undergoing hemodialysis, individuals with HIV infection and health care workers [119].

Passive immunization is available for individuals recently exposed to HBV virus or persons who are not responsive to the HBV recombinant vaccine. The immunoglobulin is given via the intramuscular route and causes a sustained titer of anti-HB antibodies. HBV human immunoglobulin can be protective for 3 to 6 months [119]. In patients who were exposed to HBV, treatment with anti-HBV immunoglobulin dampens the signs and symptoms of the infection [125].

Interferon alpha (IFN-α)-based therapy has been used in individuals with proven acute HBV infection with promising results. IFN-α has dual functions; it works as an immuno-modulatory molecule and interferes with viral replication. Additionally, IFN-α treatment has serious side effects that include depression, leucopenia, and thrombocytopenia [118]. These serious adverse effects can influence the treatment scheme, tolerance of regimen, and the final therapeutic outcome. For chronic hepatitis, the treatment with pegylated IFN-α is effective and is associated with a sustained antiviral response. The success rate for pegylated IFN-α is around 30% [126]. Moreover, host factors are associated with this antiviral therapy success. However, individuals with a polymorphism in the IL-28B gene have been identified as non-responders to the IFN-α therapy [127].

Nucleosides or nucleotides analogs are viral-specific drugs that inhibit viral replication because they lack of the 3'-hydroxyl group. When nucleotide or nucleoside analogues are incorporated to the nascent DNA chain, the addition of another phosphodiester bond for a new nucleotide is blocked [49]. Lamivudine is a nucleoside prototype is administered by oral route. In the context of acute HBV infection, lamivudine has been effective and well tolerated [128]. In chronic HBV infection, lamivudine has shown effectiveness in reducing progression of fibrosis and restoring antiviral responses [118]. Lamivudine has uncommon adverse effects, among them anorexia, vomiting, diarrhea, rash, muscle pain and depression. Telbivudine, another

nucleoside analogue, is an HBV polymerase inhibitor that has the same antiviral spectrum as lamivudine. Like lamivudine, telbivudine is well tolerated by patients [129].

Adefovir is also another nucleotide analogue used in the treatment of HBV infection. This antiviral agent acts in a way similar to that of lamivudine, and the serologic response is dose dependent [118]. A higher incidence of renal damage has been documented with the use of higher doses of adefovir [130]. This drug is also used as a second line of treatment when resistance to lamivudine is detected [131].

Other nucleosides are available for the treatment of HBV infection. Etecavir is a guanosine analogue that has shown efficacy in reducing HBV DNA and sustained seroconversion [126]. Viral resistance to lamivudine overlaps with etecavir [132]. Tenofovir is another acyclic nucleoside that has an antiviral spectrum against HBV and HIV. Thus, it is especially useful in co-infection with both viruses. Tenofovir has been used with promising results in patients with chronic hepatitis because it was able to ameliorate disease progression in this group of patients [133].

Unfortunately, HDV does not encode any enzyme that could be a susceptible target of an antiviral agent [55]. Thus, there is not a current specific antiviral treatment for the HDV infection. IFN-α has shown some efficacy reducing viral numbers in HDV-infected patients. Another therapeutic strategy consists of the inclusion of a combination of pegylated interferon alpha and a nucleoside analogue to counteract HBV replication and, in that way, inhibit HDV replication [53].

HCV treatment is based on using pegylated interferon for a lengthy time period. The recommended scheme can last up to 24 weeks, and its duration is determined by the genotype of the HCV infecting the individuals. Usually genotype 1 requires a 24-week course of treatment, the genotypes 2, 3 and 4 generally require from 8 to12 weeks of treatment [134]. Usually the duration of the pegylated interferon treatment is not recommended to be longer than 12 weeks, since no clinical improvement was shown in patients who were responders during that period of time [135].

Combined therapy with ribavirin to the pegylated interferon-α has been recommended and shown to be effective. It is estimated that this scheme cures 50% of HCV-infected individuals [136]. Recently, two HCV-specific NS3/NS4 protease inhibitors have been approved in Europe and the USA: boceprevir and telaprevir. Boceprevir has been tested in a phase 3 clinical for safety and effectiveness in clearing the virus. Treatment with boceprevir was shown to be successful in getting a sustained antiviral response. Telaprevir has been also taken to phase 3 clinical trials and has been shown to be safe, and as effective as the established treatment with pegylated IFN-α and ribavirin [137].

Regarding primary prophylaxis, the use of universal precautions can help to diminish the rates of transmission in individuals who manage human fluids. Currently, there is no approved vaccine for human use. A new vaccine based on replication-deficient adenovirus that encodes the HCV NS3-5B proteins has been developed and

tested in humans. Interestingly, the vaccine was able to elicit the expansion of HCV-specific CD4 and CD8 T cells [138]. Other potential vaccines use the same principle of chimeric viruses with HCV antigens, however they consist of retrovirus or vaccinia Ankara strain [139].

1.5 Animal models to study viral hepatitis

As mentioned in previous sections, fulminant hepatitis of viral etiology still represents an important cause of mortality in developed countries such as France or Japan [140, 141]. Additionally, concomitant HEV infection during pregnancy is still associated with a high risk of developing fulminant hepatitis [142]. Several models of fulminant hepatitis have been proposed, some of which consist of introducing chemical injury to mice with D-galactosamine, tioacetamide, and amanita [143]. The mentioned models are useful to study fulminant hepatitis in which death of hepatocytes is a large component, but are not useful in examining the immune response in these situations. Other models consist of the use of surrogate viruses. For example, in rabbits infection with rabbit hemorrhagic disease virus produces a disease that emulates FVH [143], and in mice, infection with mouse hepatitis virus 3 (MHV-3) also emulates FVH [144]. In both models, almost of all the hallmarks of FH, such as extensive hepatic necrosis, coagulopathy, and increase in nitrogen products are seen. However, the use of small animals makes it difficult to measure intracranial pressure [144].

Hepatitis E has a wide spectrum of hosts; there have been HEV isolates from swine, rats, sheep, macaques, and rhesus monkeys [145]. This wide variety of hosts also opens the possibility of establishing different animal models of disease. Although, observations in nonhuman primates were instrumental for elucidating the course of the disease [146].

In addition to human beings, only nonhuman primates can harbor productive HCV infection. Research using nonhuman primates brings the following issues: (1) high cost to maintain a primate colony, (2) the need for special facilities and trained personnel to handle the animals and (3) genetic diversity [147]. Furthermore, the USA National Institutes of Health have recommended the cessation of biomedical research in nonhuman primates [148].Therefore, there have been numerous efforts to develop a small animal model for the study of HCV. The first basic model consisted of the development of a chimeric mouse transplanting human liver into an immune-compromised mouse (the SCID strain), and subsequently infecting animals with HCV or HBV. This model has the limitation of lacking a matching immune system that could react to infected human hepatocytes. Furthermore, the chimeric mouse cannot sustain HCV infection for longer periods of time. Thus, this model is only useful to study viral entry and to test antiviral compounds [136, 149].

Another approach for studying virus-host interactions is the expression of complete or sections of the HCV or HBV viral genome. Transgenic mice generated with this approach do not develop a complete productive infection [150]. However,

interactions between viral and host proteins can be studied in these models. Following this approach, it was shown that sustained expression of HBV genome [151] or HCV core protein can lead to the establishment of premalignant lesions in the liver [152]. Transgenic mouse models are also valuable to study the influence of viral proteins on cell signaling that potentially influence the global antiviral response. By using this approach it was elucidated that the NS3/4A protein of HCV cleaves MAVS protein [153].

After the identification of CD81, scavenger receptor type B class I (SCARB1), claudin 1 (CLDN1) and occluding (OCLN) as the co-receptors for HCV entry [154], the development of a genetically engineered mouse became possible. Following this rationale, a transgenic mouse bearing the human HCV co-receptors was developed and proved to harbor viral replication for at least 4 days [155]. This system allows the study *in vivo* of the mechanisms HCV infection and possibly immunity in immune-competent systems other than non-human primates.

1.6 The adenovirus model and its similarities with clinically relevant hepatotropic viruses

Throughout the studies discussed in this dissertation, we have used the Adenovirus (Ad)-induced hepatitis model to test our hypotheses. The (Ad)-induced hepatitis model unites some of the features seen in clinical hepatitis caused by human pathogens: high liver tropism, cross presentation and strong CD8 T cell activation with the subsequent loss of hepatocytes (discussed in section 1.3) [156, 157]. On the other hand, the Ad-induced hepatitis model has the limitation that the virus used is replication

deficient and does not cause chronic hepatitis. As discussed earlier, none of the clinically relevant hepatotropic viruses are cytopathic for hepatocytes. One of the hallmarks is hepatocytes killing through immune activation; this is also one characteristic of Ad-induced pathology [157].

The Ad-induced hepatitis model is an acute self-limiting model which emulates the clinical course of the human hepatotropic viruses that cause acute hepatitis: HAV, HEV and HBV. However, it is important to emphasize that among the mentioned viruses only HBV is a DNA virus and consequently has the same interaction with TLR9 like Ad [158]. Therefore, our results can be extrapolated to self-limiting, acute viral hepatitis with the mentioned limitations.

1.7 IL-33 and its immunologic functions

IL-33 is part of the IL1R super family; other members of this group are IL-18, IL-1α and IL-1β [159]. Like IL-1, IL-33 is released from the nucleus of necrotic cell and triggers the activation of the immune response in the absence of pathogens. This feature confers IL-33 with the denomination of *alarmin* or damage association pattern [160]. However, IL-33 can be also actively transcribed in epithelial cells, smooth muscle cells, keratinocytes, fibroblasts and DCs upon cell insults [160]. After its release, IL-33 is subject to proteolytic cleavage, possibly by caspases or calpain [161, 162]. It has been proposed that there are diverging roles between the non-cleaved form of IL-33 and its cleaved or mature forms [161].

The cleaved or mature IL-33 binds a dimeric receptor that is composed of ST2 and the interleukin 1 receptor (IL-1R) inhibitory molecule [163]. This dimeric receptor is present in cells from the innate immune system, lineage-negative cell in the bone marrow and Th2 cells predominantly [164]. IL-33 induces the expansion of innate lymphocytes class 2 (ILC2) or nuocytes, a subset of cells that are lineage negative with the capacity to secrete type-2 cytokines. ILC2 have been showed to be protective against parasitic infestations [165].

Mature IL-33 can act as a trans-element modeling gene transcription. IL-33 binds preferentially to heterochromatin [166, 167]. By binding the chromatin, IL-33 can modify the gene expression. IL-33 interacts and inhibits with p65 and p60 components of NF-κB transcription factor. When IL-1R signaling is activated, over-expression of IL-33 is able to diminish the expression of NF-κB dependent genes [168].

The immune effects of IL-33 are contradictory and vary among disease models. There is evidence of the protective immunologic role of IL-33 in colitis by inducing Th2 cytokines and regulatory T cells (T regs) [169]. In models of airway hypersensitivity, IL-33 has been shown to promote bronchial inflammation via Th2 cytokine production [170].

The exact role of IL-33 in the liver is not well understood. In a model of ischemia/reperfusion, IL-33 pre-treatment ameliorated liver damage [171]. In concavalin A-induced hepatitis, mice deficient in ST-2 receptor displayed more inflammation

compared to that seen wild type mice [172]. However, it is still not clear why IL-33 serum levels are increased in mice and humans with chronic liver damage [173].

IL-33 has an immune role in viral infections. It has been shown that IL-33 is produced in the lungs of influenza-infected mice [174, 175]. In the LCMV model of infection, IL-33 promotes the expansion of cytotoxic CD8 T cells [176]. Thus, it is possible that IL-33 may play an important role in the establishment of anti-viral responses in the liver.

1.8 Immune effects of HGF

The hepatocyte growth factor (HGF) is a dimeric molecule composed of a 69Kd (the α chain) 35 Kd chain (the β chain). HGF is a pleiotropic molecule that is primarily associated with liver regeneration agents [177]. HGF is produced by a wide variety of cells with a mesenchymal origin that are present in the entire organism: fibroblasts, liver endothelial cells, hepatic stellate cells, monocytes and neutrophils [178-180]. HGF can exert its biological function in a paracrine manner, and its classical targets are the epithelial cells or hepatocytes. Later observations showed that HGF is produced in anatomically distant organs and can act on damaged tissues as a hormone [181]. Recently, observations *in vivo* and *in vitro* showed that HGF can act also in an autocrine manner in immune cells and HSCs [182, 183].

Like IL-33, HGF is produced as a zymogen molecule (the pro-HGF). Under steady conditions, the newly formed pro-HGF interacts very closely with

glycosaminoglycans in the extracellular matrix [182, 184]. Subsequently, the pro-HGF is cleaved by matrix metalloproteinases when there is an insult. These physiological features assure the presence of a pre-formed pool of HGF when it is needed and also avoid continuous release to the microenvironment. Additionally, HGF can be produced *de novo* after stimulation with pro-inflammatory cytokines, such as IL-1, IL-6 and TNF-α [185], or with CD40 engagement [186], indicating that HGF production can be amplified by inflammation.

HGF exerts its biological effects through the receptor mesenchymal epithelial transition receptor or c-Met [187]. Activation of c-Met signaling activation promotes the transcription of the anti-apoptotic factor Bcl-2 [188]. This feature may exert some direct protective role in viral hepatitis (discussed previously). In the context of inflammation, the c-met/HGF axis also inhibits the activation of NF-κB by preventing the binding of p65 to its promoter [189]. The sustained presence of HGF promotes the differentiation of DCs efficiently in priming regulatory T cells, named tolerogenic DCs [186]. Of note, it is important that the classification of HGF as a merely anti-inflammatory molecule could be misleading. In addition to the inhibitory effects on NF-kB, HGF counteracts TGF-β by disrupting smad/mad signaling [190, 191]. HGF has been shown to promote lymphopoiesis and, by that, the establishment of cytotoxic activity [192]. Furthermore, in the absence of c-met, DCs from the skin are not able to migrate to the lymph nodes [193]. Hence, it is possible that HGF has an immune-modulatory role rather than a classical immunosuppressive or pro-inflammatory role.

HGF has been shown to preserve organ function in injury mediated by chemical agents in the heart [194], liver [195], and kidneys [195-198]. However, these studies did not elucidate whether HGF was mediating the organ protection through the pro-survival function or whether there was immune modulation. Recent studies showed that HGF can exert some inhibitory effects in DCs from OVA-sensitized animals [199]. In experimental autoimmune encephalitis, HGF also ameliorates disease and expands DCs that prime T regs [200]. However, the exact role of HGF in viral infections has never been examined.

In the clinical setting, HGF has been detected to be increased in patients having a wide diversity of diseases with infectious etiology [201-205]. HGF was purified for the first time in humans from serum collected from a patient suffering fulminant hepatitis caused by HBV [206]. Later studies demonstrated that HGF is produced in a wide spectrum of liver diseases. In patients with acute HAV infection, HGF serum levels are increased during the onset of the infection and then fall to undetectable levels in the convalescence stage of the disease. Similarly, chronic infection with HBV and HCV tend to increase HGF in blood in a cyclic manner. Furthermore, the HGF directly co-relates with higher scores in the child classification (categorical scale for liver damage) [207-209]. The mentioned studies have the limitation of being observational and raise the questions about the exact role of HGF in viral hepatitis: it is not well defined whether HGF is promoting liver damage or whether it is part of the counter regulatory mechanisms that protect the liver in chronic and acute infections. Thus, mechanistic

studies are needed to define the exact role of HGF in viral hepatitis and determine the putative therapeutic role of this molecule.

CHAPTER 2: ROLE OF IL-33 DURING ACUTE ADENOVIRUS-INDUCED HEPATITIS

2.1 Introduction

IL-33 is a member of the IL-1 family that is produced ubiquitously. Different cells in different organs are able to synthesize IL-33, although the major producers are vascular endothelial and lining epithelial cells of the respiratory and gastrointestinal tracts [210]. From the immunological aspect, IL-33 can initiate inflammation in the absence of pathogens, and thus this is molecule classified as an *alarmin* or a damage-associated pattern (DAMP) [160].

As mentioned before, IL-33 mediates its biological functions through a dimeric receptor composed of suppressor of tumorigenicity (ST-2) and the IL-1 receptor antagonist protein [163]. The ST-2 receptor is expressed in a wide variety of cells, including those from the innate immune responses such as macrophages, DCs, NK T and NK cells and other components from the adaptive immune response, e.g. Th2-polarized T cells [159, 162]. Thus, IL-33 can possibly modify the immune response at different levels. ST-2 can be present in a soluble form (sST2) or decoy receptor. This sST2 has been proposed to act as an inhibitor or regulator of the IL-33 in the context of inflammation, as its levels are undetectable under homeostatic conditions [162]. In the clinical setting, sST2 proposed a biomarker of disease activity in a wide variety of diseases, such as juvenile idiopathic arthritis [211], cardiac congestive failure [212], allergic-mediated diseases (rhinitis and asthma), and autoimmune-mediated diseases (dermatomyositis) [213].

Regarding the role of IL-33 during infections, this cytokine has been associated with protection against parasites as it is classically related to the expansion and establishment of Th2 responses. IL-33 *knockout* mice cannot clear *Nippostrongylus brasiliensis* infestations [214]. Conversely, the IL-33 supplementation is protective in a model of *Toxoplasma gondii* infection in the central nervous system and *Trichuris muris* infestation model [134, 215].

Despite the classical association of IL-33 to Th2-mediated immune responses, it has been reported that IL-33 also promotes the production of IFN-γ in NK T and NK when IL-12 is present [216]. Thus, IL-33 in addition to promoting Th2 responses also helps to mount a Th1-mediated response. However the IL-33 effects on Th1 responses *in vivo* are not well established. In the ST-2 *knockou*t mouse, the progression of mammary cancer was delayed due to significantly lower IL-10 production [217].

In the context of viral infection, it has been shown that IL-33 is induced in a murine model of viral influenza [174]. The amplifying effect on IFN-γ of IL-33 indicates that this cytokine may be instrumental in initiating the anti-viral response. Of note, a recent report showed that the ST-2 was necessary to initiate CD8 T cell expansion and priming with LCMV antigens. Furthermore, ST-2 deficient mice failed to contain viral replication [176].

In the context of liver disease, IL-33 gained recognition after it was detected at elevated levels in individuals with fulminant hepatitis [218]. The acute secretion of IL-33 indicates that this cytokine may influence ***hepatic immune responses***. As mentioned before, IL-33 is able to amplify the activation of NK and NKT cells two cell types that have been recognized as the early response to virus in the liver (discussed in previous sections). Previous studies in a concavalin A-induced hepatitis demonstrated that the IL-33 axis is important for the hepatocytes' survival. Furthermore, in this study it was shown that in the absence of the IL-33/ST-2 axis the production of pro-inflammatory cytokines is increased [172]. Thus, it is also possible that IL-33 may play an important role in preserving organ function during acute viral hepatitis. Hence, using an animal model of adenovirus-induced hepatitis, we explored the effects of exogenous IL-33 on the immune response and organ damage.

2.2 Materials and methods

Animal models and treatment

CBL57/6 mice were infected with 2 x10^9 of plaque forming units (*pfu*) of adenovirus via tail vein injection. One group received 0.8 μg of recombinant IL-33 via intra-peritoneal route, every day for 5 days. The control group was infected as previously described and received a daily dose of PBS as control. Both groups were sacrificed at day 6 of infection. Liver tissues and serum were collected for further analysis.

Evaluation of H&E stains

Histopathological changes in the liver were evaluated by using an *in house* standardized scale consisting of the following criteria: lymphocyte infiltration per acinar zones (1 point if they were present in zone 1, 2 points if they were present in zone 2, and 3 points if they were present in zone 3), inflammatory affection to the central vein (3 points); and number of Councilman bodies per view (0 to 1 bodies= 0 points, 2 to 3 = 1 point, 3 to 4 bodies = 2, and more than 5 bodies =3). Points were summed and a final grade was given by applying the following standards: 0 for the preparations with less than 1 point, 1 for the preparations with 2 to 4 points total, 2 for preparations with 5 to 6 points and 3 for those greater than 6 points. The slides were double blinded and read by three independent evaluators using the mentioned criteria. The mean of the average of the scores given by each reader was used for further statistical analysis.

Isolation of intrahepatic lymphocytes and splenocytes

Intrahepatic lymphocytes (IHL) were isolated from livers as follow: livers were perfused with ice cold PBS. Then, the organs were mechanically dispersed and digested in a solution or RPMI media with 0.05% collagenase. The cell suspension was passed through a cell strainer and separated in a discontinuous percoll gradient (Sigma Life Sciences, St. Louis MO) of 40%/70% phases. Mononuclear cells were collected in the

interphase between the mentioned concentrations. The mononuclear cells were washed twice, characterized and used for experiments downstream. Single cell suspensions were obtained from mechanically dispersed spleens. The suspensions were treated with red blood cell lysing buffer for 5 minutes and washed twice with ice cold PBS. The final suspension was passed through a cell strainer, and the cells were used for downstream experiments.

In vitro differentiation of ILC2

Splenocytes obtained from naïve mice were culture with 10 ng of each of recombinant IL-7, IL-33 and IL-2 (Peprotech, Rocky Hill, NY) for 5 days in 5% CO_2 at 37ºC. The culture supernatant was changed every 2 days. The lineage-negative cells were separated by using biotin-coated antibodies of the following specificities: CD3, CD4, CD8, CD11b, CD11c, B220, NK1.1 and Ter-119.Then, the cells had a second incubation with streptavidin magnetic beads and separated with LD column (Miltenyi, San Diego, CA); the eluted fraction was considered the nuocyte-enriched fraction. The ILC2 were characterized by using CD43, CD44, CD45, Cd69, CD25, CD127, c-kit and MHC-II as previously described [165].

For the detection of intracellular cytokines, splenocytes and IHL ($2x10^6$ cells/well) were cultured for 5 hours in complete RPMI media (10% FBS, penicillin, streptomycin, 2-ME) in the presence of Golgi stop (e-Bioscience, San Diego, CA). Cell cultures were incubated for 5 hours at 37 ºC with 5% CO_2. Following the incubation,

the cells were stained for surface markers and fixed and permeabilized by using cyto/fix kit (e-Bioscience) for intracellular cytokine detection as described in the following section.

Flow cytometry analysis

Isolated cells were incubated with FCγR blocker for 30 minutes at 4ºC. Then, they were treated with different combinations of primary antibodies conjugated with different cytochromes. Antibodies of the following specificities were used: CD3ε, CD4, CD8α, CD11b, CD25, CD43, CD44, CD45, NK1.1, Gr-1, IL-4, IL-13, TNF-α, IFN-γ and FoxP3. The cells were treated for 30 minutes at 4ºC and then washed with ice cold PBS. Following surface staining, the samples designated for intracellular cytokine staining were permeabilized and fixed by using a commercial kit (eBioscience). After the permeabilization and fixation, the samples were treated with combinations of antibodies with specificity for the mentioned cytokines. The samples were acquired in LSR II Fortessa (Becton Dickinson, San Jose, CA) and analyzed by using FlowJo 8.86 software (Tree Star, Ashland, OR).

Gene expression evaluation

Liver tissues were conserved in RNA later solution (Ambion) and stored at -90ºC until further processing. RNA was extracted done by using a commercial kit (Quiagen, Maryland, MA) and cDNA was obtained by using the super-script III first strand

synthesis system (Invitrogen, San Diego, CA). Real time PCR was done by using Sybr green as the molecular probe (BioRad), by following protocol: denaturing for 10 minutes at 95ºC, 40 cycles of 15 seconds at 95ºC and 60 seconds at 60ºC. Each molecule relative expression was calculated by using the melting curve as reference and normalized to GAPDH gene expression by using the $2^{-\Delta\Delta}$ CT method.

Evaluation of serum cytokines and ALT activity

ALT activity was measured in the Department of Clinical Chemistry at UTMB. IL-33 concentration was evaluated with a commercial ELISA kit (R&D systems, Minneapolis, MN). The levels of IL-2, IL-12(p40), IL-7, IL-10 and IL-17 were measured by using a multiplex magnetic beads kit (Millipore, Billerica, MA). Cytokines were detected in a Bioplex 200 system (Luminex XMap Technology, Bio-Rad, Hercules, CA).

2.3 Results

IL-33 is expressed during the course of Ad-induced hepatitis

We first examined whether IL-33 is secreted or expressed in the liver during the course of Ad-induced hepatitis. Thus, CBL57/6 mice were infected with Ad (2×10^9 *pfu* via IV). The expression of IL-33 and the ST-2 was followed by using RT-PCR. ST-2 expression increased at day 1 of infection reaching significant levels at day 3 and 6 of infection. Similarly, the expression of IL-33 increased at 24 hours of infection and continued to increase at day 6 (**Fig. 1.2 A**). Furthermore, the protein levels of IL-33

liver and in serum increased significantly at day 6 post-infection compared to levels in naïve mice (**Fig. 1.2 B**).

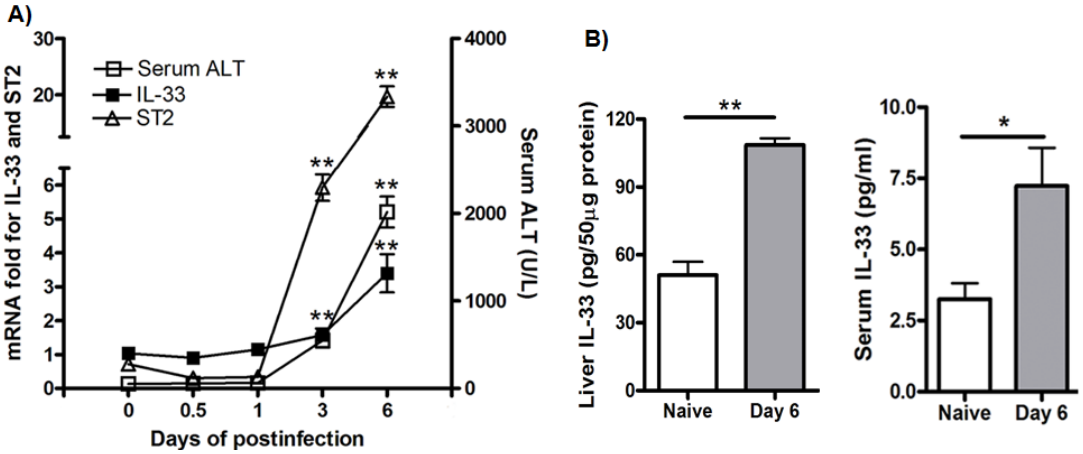

Figure 1.1 Expression of IL-33 during Ad-induced hepatitis. CBL57/6 mice were infected with 2×109 *pfu* via IV and sacrificed at different time points. Expression of IL-33 and ST-2 receptor was normalized by using a GAPDH housekeeping gene (A). IL-33 protein concentrations in liver tissue homogenates (left) and in serum (right). Error bars are standard deviation of the mean. *p<0.05.

IL-33 exerts protective effects in Ad-induced hepatitis

CBL57/6 mice were infected with 2×10^6 *pfu* of replication-deficient adenovirus carrying the α-galactosidase gene. Subsequently, mice were treated everyday with 0.8 μg of recombinant IL-33. At day 6 post-infection mice were sacrificed. Liver tissues were collected for further analysis. H&E stains of the livers from the experimental animals showed similar lymphocyte infiltration compared to that in control animals (**Fig. 1.2 A**). In line with this result, both groups had the same average of histological scores (**Fig. 1.2 B**).However, the animals treated with the IL-33 had lower numbers of apoptotic

hepatocytes (**Fig. 1.2C**). Consistent with lower numbers in hepatocytes loss, the ALT activity was decreased by IL-33 treatment (**Fig. 1.2 D**).

IL-33 does not change the total intra hepatic lymphocyte (IHL) infiltration

The totals of lymphocytes recovered from IL-33 and control groups were comparable (**Fig. 1.3 A**). The expression of CD44 activation marker in either CD4 or CD8 cells was not changed by IL-33 at day 6 post-infection (**Fig. 1.3 B**). However, the proportion of immune cell subsets in the liver and spleen was changed by IL-33. CD11b, CD11c and NK cells were significantly decreased in the liver by IL-33. A similar trend was seen in the spleen except for CD11b or CD11c, subsets that were not changed or increased, respectively. In contrast, regulatory T cells were significantly higher in the liver and spleen collected from IL-33 group (**Fig. 1.3 C and D**). Altogether, these data indicate that IL-33 exerts immune effects during Ad-induced hepatitis.

IL-33 does not interfere with viral clearance in Ad-induced hepatitis

Previous studies associated IL-33 with clearance of worms and intracellular parasites [134, 214]. However, to our knowledge there was no report showing the effects of IL-33 on viral clearance. To our surprise, treatment with IL-33 did not interfere with the viral clearance at week 2 post-infection (**Fig. 1.4**).

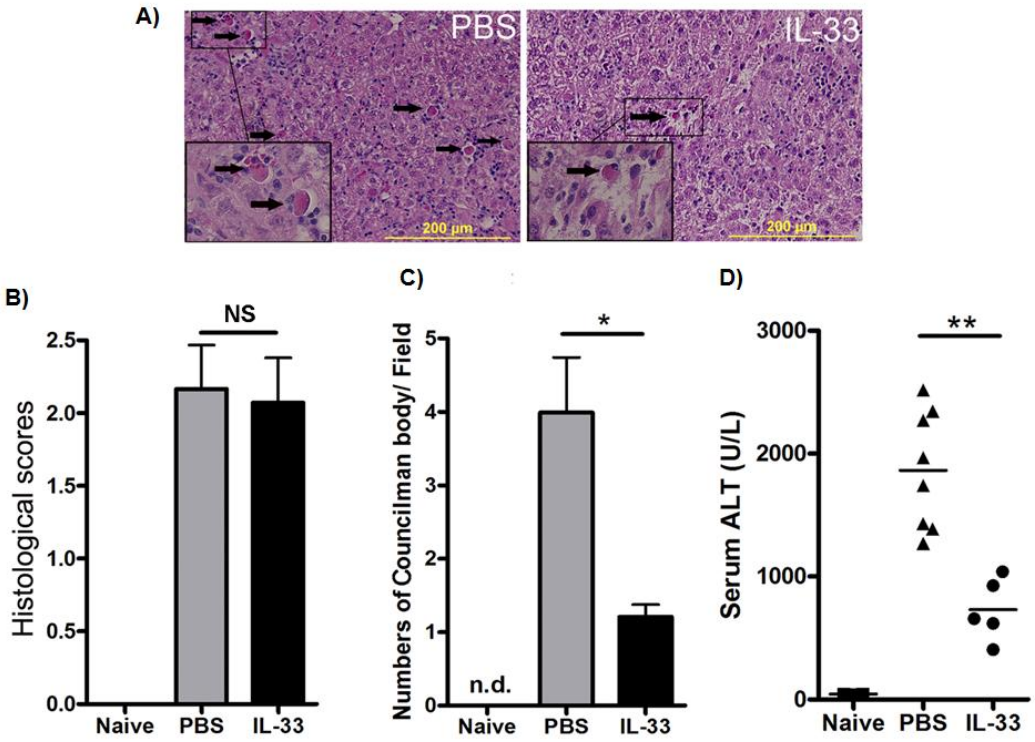

Figure 1.2 Exogenous IL-33 is protective in Ad-induced hepatitis. CBL57/6 mice were infected with Ad, as explained in figure 1, and then received exogenous IL-33 (0.8µg daily) or vehicle (PBS). Mice were sacrificed at day 6 of infection. H&E stains of the liver tissues from experimental animals (A). Histological scores of the H&E stains evaluated by three independent individuals (B). Average of apoptotic (councilman) bodies seen in five fields (C). ALT activity from serum of the experimental groups. Error bars represent standard deviation of the mean. *$p<0.05$.

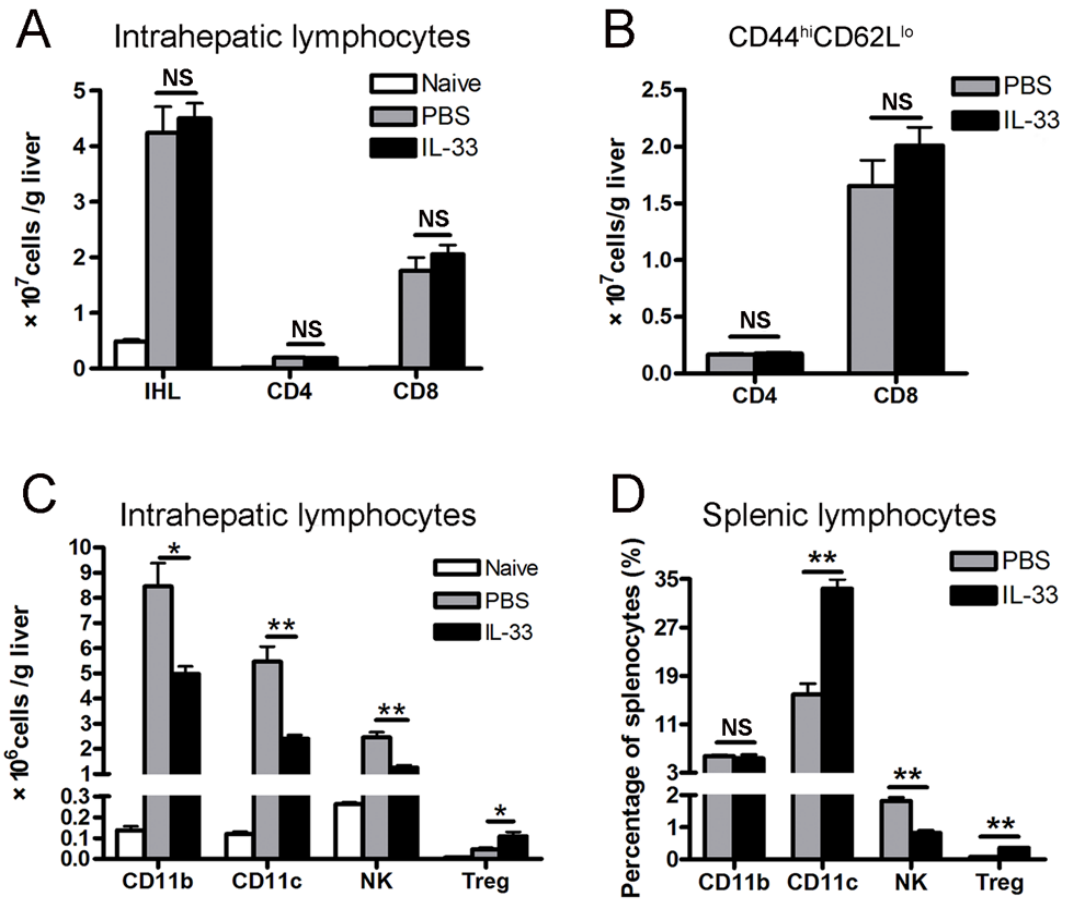

Figure 1.3 IL-33 favors the expansion of regulatory T cells in the liver during Ad-induced hepatitis. CBL57/6 mice were treated as explained in figure 2. Absolute numbers of CD4 and CD8 cells in experimental groups (A). Total numbers of activated T cells (B). Different subsets of immune cells in liver (C) and (D) spleen of the experimental animals. Error bars represent standard deviation of the mean. *$p<0.05$.

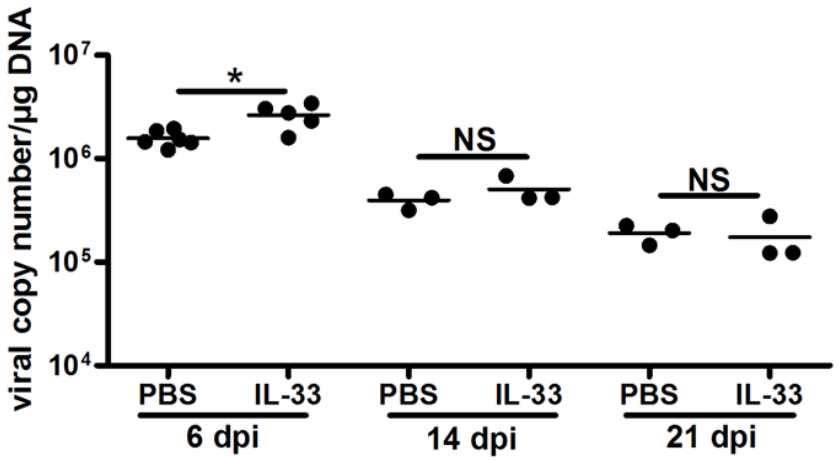

Figure 1.4 IL-33 does not interfere with viral clearance. Mice were treated as in figure 1 and sacrificed at different time points. DNA was isolated and an Ad-specific primer was used to determine the viral copy number by using a standard curve done with known Ad concentration. *$p<0.05$.

IL-33 promotes the expression of IFN-γ, granzyme B and perforin effector molecules in Ad-induced hepatitis

Further characterization of the lymphocyte infiltrates showed that IL-33 treatment increased the production of IFN-γ in CD4 and CD8 T cells (**Fig. 1.5 A**). Similarly, concentrations of IFN-γ and IL-2 in serum were increased in the IL-33-treated mice (**Fig. 1.5 B**). The mRNA expression in liver of IFN-γ, granzyme B and perforin were significantly increased by IL-33 (**Fig. 1.5 C**). In conclusion, these results indicate that the expression of effector molecules with antiviral effects is enhanced by alarmin IL-33. And altogether these data indicate that IL-33 exerts its protective effects in an IFN-γ-, IL-2, granzyme B- and perforin-independent manner. Hence, we aimed to examine the effects of IL-33 on the production of TNF-α, another important mediator of hepatocytes apoptosis during viral hepatitis (discussed in previous sections).

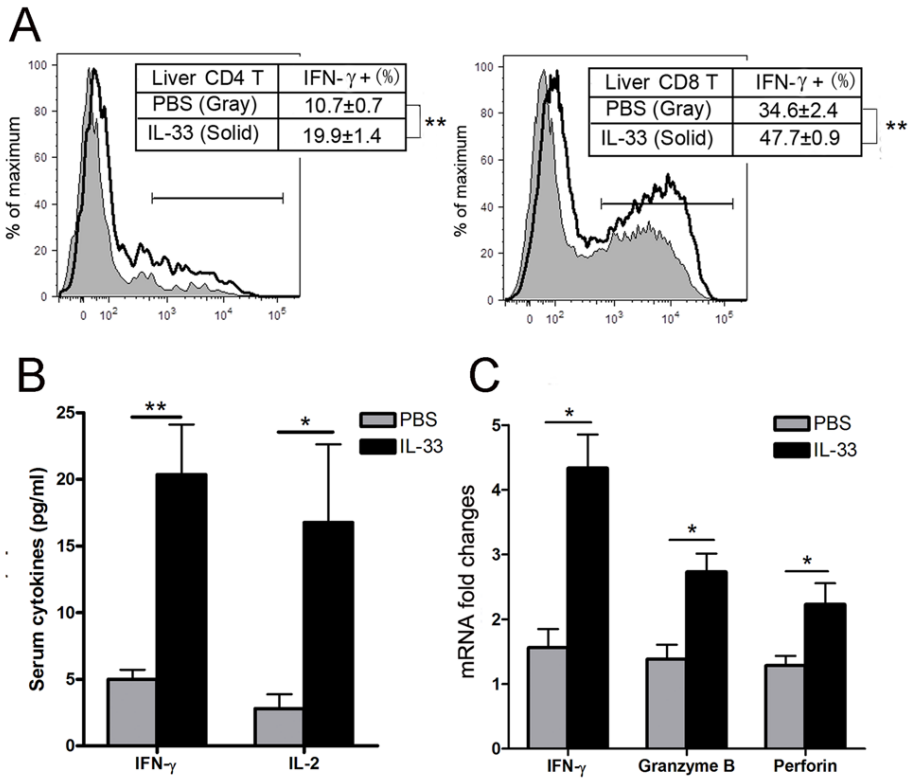

Figure 1.5 IL-33 promotes the production of IFN-γ and effector molecules. Mice were treated as in fig. 1 and sacrificed at day 6 of infection. Expression of IFN-γ evaluated by flow cytometry in CD4 and CD8 (A) T cells. Solid line represents the IL-33-treated group and gray area the control group. Serum concentration of IFN-γ and IL-2 from experimental animals measured with Bioplex assay (B). mRNA expression of IFN-γ, granzyme B and perforin normalized to GAPDH gene (C). Error bars represent standard deviation of the mean. *p<0.05.

IL-33 decreases the production of TNF-α in different immune cells subsets

It has been shown that TNF-α triggers hepatocyte death during viral hepatitis (discussed in previous sections)[156]. First, we evaluated the expression of TNF-α in liver tissues and found that IL-33 significantly decreased the TNF-α transcription (**Fig. 1.6 A**). Thus, we examined the effects of IL-33 on TNF-α production in different

immune subsets. In line with the previous result, the TNF-α-positive CD4, CD8 and CD11b cells were present in lower percentages in the IL-33-treated mice compared to those in control animals. Additionally, the mean fluorescence intensity in the examined gates was decreased in the IL-33 group (**Fig. 1.6 B**). To further determine whether this TNF-α inhibition was done in a direct manner, IHL from Ad-infected mice were isolated and treated with different concentrations of IL-33. IL-33 inhibited the expression of TNF-α in a dose dependent manner in CD11b cells, CD4 and CD8 T cells (**Fig. 1.6 C**). Thus, IL-33 mediates its hepatoprotective effects, in part, by decreasing TNF-α production.

IL-33 increases the expression of Th2 cytokines and chemokines but does not inhibit the expression of Th1-associated chemokines

Previous reports showed that IL-33 promotes the expression of type 2 cytokines. Thus, we examined the expression of Th2 cytokines with IL-33 treatment. As expected, the expression of IL-4, IL-5, IL-6 and IL-13 was significantly increased in the IL-33 group compared to that in control animals (**Fig. 1.7 A**). Similarly, there was an enhancement of production of CCL17 and CCL22 in the IL-33 group, two chemokines associated with the recruitment of Th2 cells. To our surprise, the transcription of chemokines that are involved in the recruitment of Th1 cells was not diminished by IL-33 treatment in the context of Ad-induced hepatitis (**Fig. 1.7 B**). Altogether, these results indicate that IL-33 does not interfere with the establishment of Th1 response, while at the same time it increases the Th2 responses.

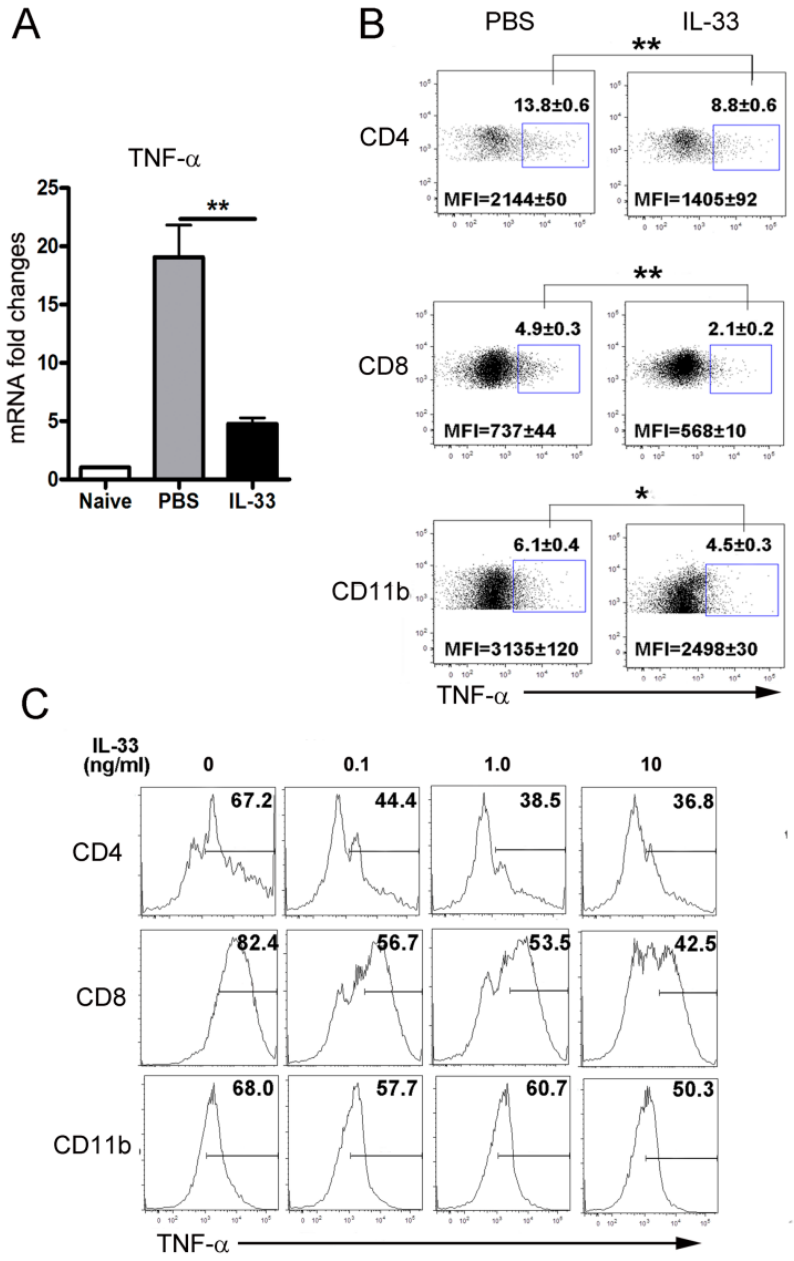

Figure 1.6 IL-33 diminishes the production of TNF-α in Ad-induced hepatitis. Mice were treated with IL-33 as in figure 1 and sacrificed at day 6 of infection. mRNA expression of TNF-α normalized to GAPDH (A). Intracellular stain of TNF-α detected by flow cytometry in different subsets of intrahepatic lymphocytes (IHL) (B). IHL from Ad-infected mice were collected and treated with different concentrations of IL-33. TNF-α expression was examined with flow cytometry (C). Error bars represent standard deviation of the mean. *$p<0.05$.

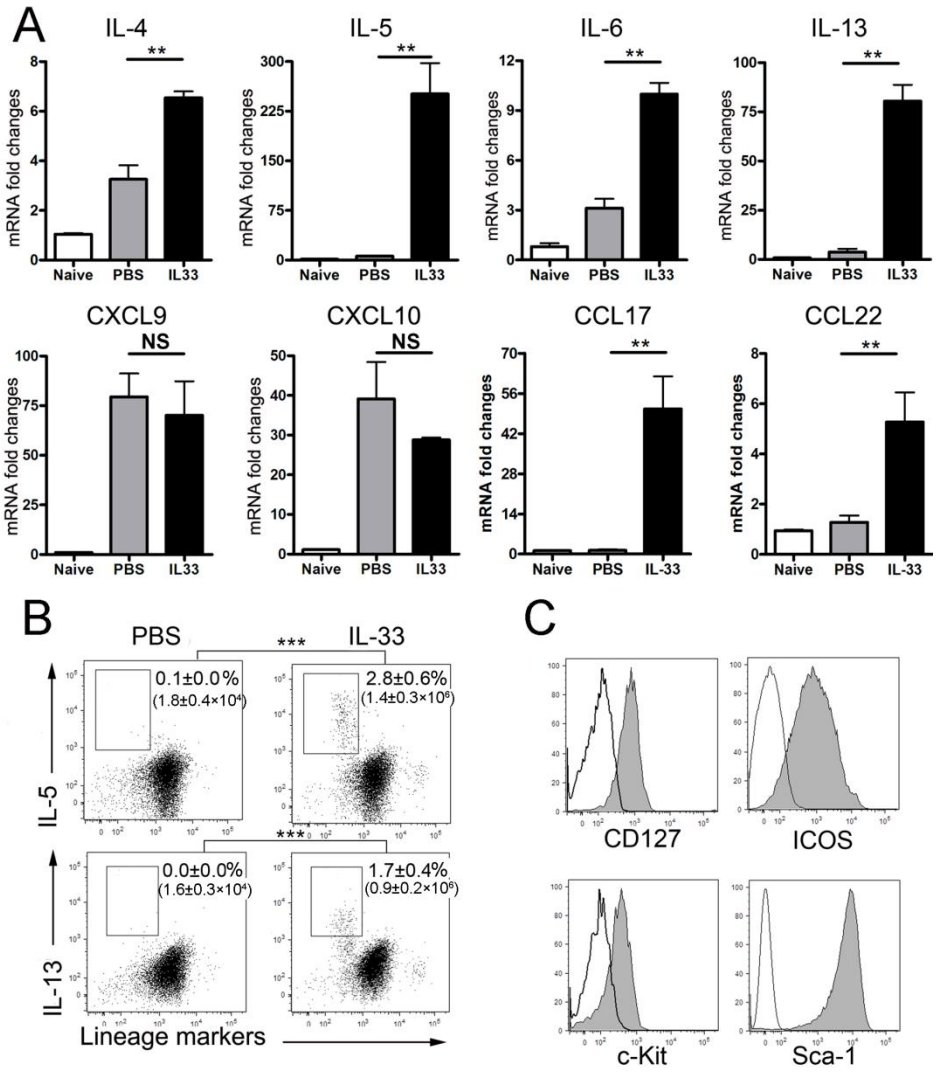

Figure 1.7 IL-33 induces the expression of Th2 cytokines and Th1 chemokines. Mice were treated and sacrificed as in Fig. 1. Expression of Th2 cytokines, Th1-related and Th2-related chemokines measured with RT-PCR and normalized to GAPDH expression (A). Percentage of ILC2 and their production of IL-5 and IL-13 detected with flow cytometry (B). Characterization ILC2 (gray shaded area), CD121, ICOS, c-Kit and Sca-1 positive cells (C). Error bars represent standard deviation of the mean. *$p<0.05$.

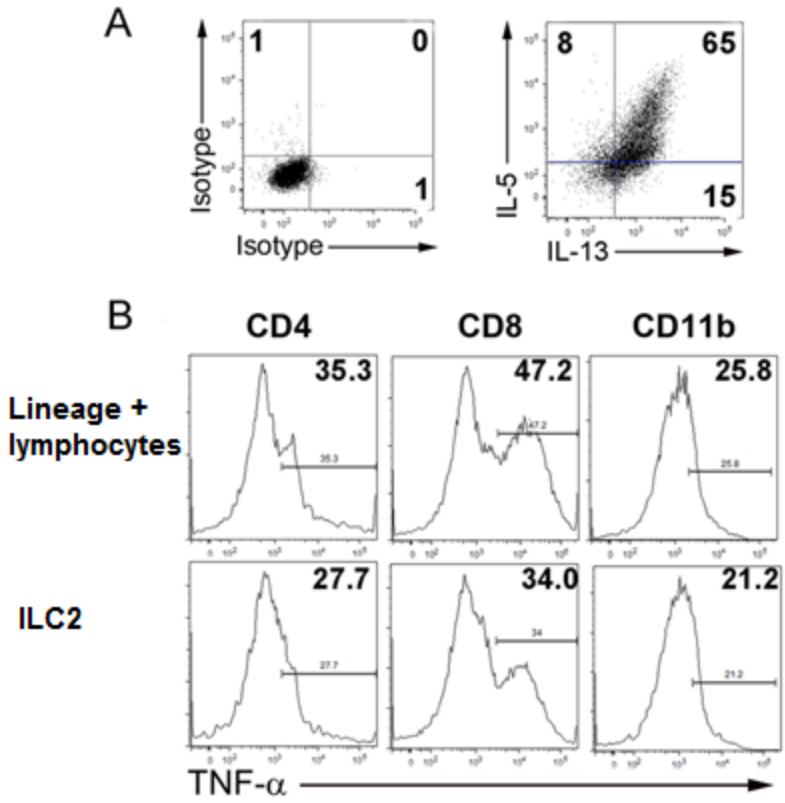

Figure 1.8 *In vitro* differentiated ILC2 diminish the production of TNF-α in IHL collected from Ad-infected mice. ILC2 were differentiated from splenocytes of naïve CBL57/6 mice by using IL-2, IL-7 and IL-3 recombinant cytokines for 5 days. Expression of IL-13 and IL-5 in the differentiated cell (A). *In vitro*-differentiated ILC2 (nuocytes) or a bulk of lineage positive lymphocytes were co-cultured with IHL from Ad-infected animals in 1:40 ratio. Expression of TNF-α after 48 hours of ILC2:IHL co-culture (B).

Exogenous IL-33 expands ILC2 during Ad-induced hepatitis

Recent studies showed that IL-33 was instrumental in expanding a newly identified of innate immune cells, the ILC2 [165]. This new subset is characterized by the absence of lineage markers and the production of Th2 cytokines (IL-5 and IL-13). In our experiments, we were able to identify the expansion of ILC2 when exogenous IL-33 was administered to Ad-infected mice (**Fig. 1.7 B**). Furthermore, these cells were able to

produce IL-5 and IL-13 and express CD127, ICOS, c-Kit and Sca-1, as it is reported in the literature (**Fig. 1.7 C**). Therefore, we wanted to elucidate the role of IL-33-induced ILC2 in Ad-induced hepatitis.

Adoptive transfer of ILC2 to Ad-infected mice ameliorates liver inflammation

To define whether IL-33 mediates the anti-inflammatory effects through ILC2, we expanded them with a cocktail of IL-2, IL-7 and IL-33 *in vitro.* As seen previously, they expressed IL-5 and IL-13 (**Fig 1.8 A**). Differentiated ILC2 were co-cultured with IHL lymphocytes isolated from Ad-infected mice in a 1:40 ratio (**Fig. 1.8 B**). As expected, the presence of ILC2 reduced the production of TNF-α in the CD4 and CD8 T cells as well as $CD11b^+$ cell components of the IHL.

To further determine the role of ILC2 in Ad-induced hepatitis, studies using *in vitro* differentiated ILC2 were conducted. Ad-infected mice received an adoptive transfer of ILC2 every day for six days. At the day of sacrifice ILC2 were present in the liver of the experimental animals. Furthermore, these cells retained their ability to produce IL-13 and IL-5 (**Fig. 1.9 A**). Higher IL-13 expression indicated that transferred ILC2 conserved their ability to produce Th2 cytokines (**Fig. 1.9 B**). Of note, adoptive transfer with ILC2 decreased the ALT activity in infected mice compared to that in controls, and these mice also had lower TNF-α production, although these changes were not significant (**Fig. 1.9 C and D**). Altogether these experiments indicate that IL-33 mediates its protective effect to the liver through the expansion of ILC2.

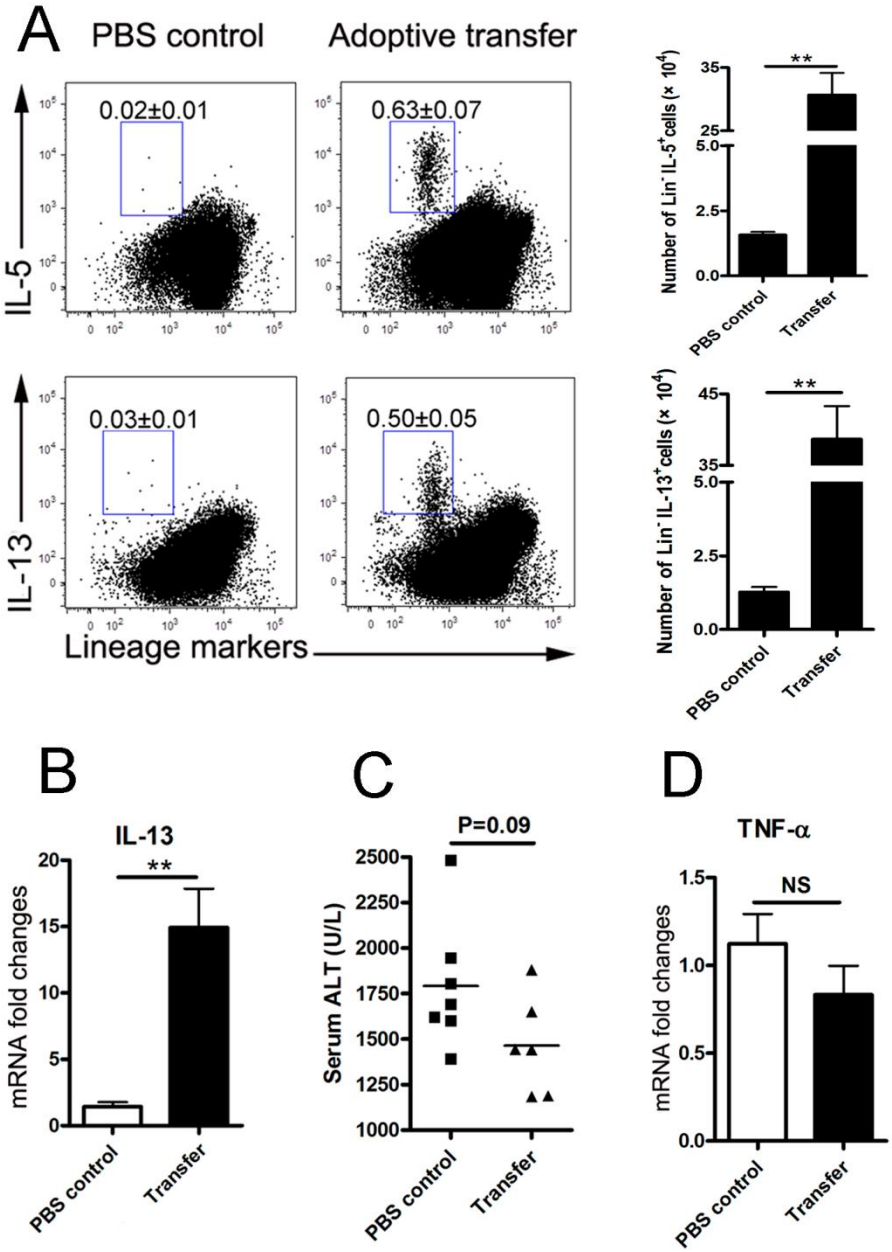

Figure 1.9 Adoptive transfer with ILC2 ameliorates Ad-induced hepatitis. ILC2 were in vitro differentiated as in figure 8. A total of 2×106 ILC2 were transferred to Ad-infected mice at 1, 3 and 5 days of infection. ILC2 transferred to infected animals produced IL-5 and IL-13 (A). Expression of IL-13 in the liver of experimental animals (B), ALT activity in the serum of the experimental animals (C). Expression of TNF-α in liver normalized to GAPDH (D).

2.4 Conclusions

In this study, we demonstrated for the first time that the alarmin IL-33 has an important role in the immune response to viruses in the liver. Classically, IL-33 was identified as an alarmin or damage-associated pattern, and this classification directed the IL-33 research towards its ability to initiate inflammation in the absence of pathogens. As mentioned in previous sections, IL-33 is recognized by its ability to polarizeTh2 cells [159].Thus, there is a body of data supporting IL-33's role in Th2-mediated diseases such as asthma [161], rheumatoid arthritis and Crohn's disease [166]. Our results indicate that IL-33 is produced in response to Ad-virus infection in the liver.

In this study we showed that IL-33 is produced and released in the liver during viral infections. Previous studies showed the prominent role of IL-33 in mounting a biased Th2 response that is instrumental for parasite clearance. Additionally, the early secretion of IL-33 during viral influenza infection indicates that IL-33 may play an important role in the anti-viral response [174]. Here, we showed that IL-33 along with its receptor was expressed during Ad-induced hepatitis (**Fig. 1.2**).

Studies in a concavalin A (con A)-induced hepatitis showed that IL-33/ST-2 axis exerts organ protection by increasing the expression of the *bcl-2* pro-survival gene [172]. Here we investigate whether IL-33 exerts the same protection in our model of Ad-induced hepatitis. Similar to the studies done with con A, IL-33 protected hepatocytes from apoptosis, giving as an outcome lower apoptotic bodies counts in the H&E slides and lower ALT activity in the experimental animals. However, the total lymphocyte

infiltration to the liver was not changed by IL-33 (**Fig. 1.2**). Thus, it is possible that IL-33 mediates its protective effects by promoting hepatocyte survival.

Regarding the lymphocyte subsets that infiltrated the liver under our experimental conditions, we observed that in general IL-33 did not affect the CD8/CD4 ratio or the expression of CD44 in T cells. However, IL-33 treatment decreased the CD11b+, CD11c+, and NK cell number and increased the number of Tregs. Despite this profile in the lymphocyte infiltration we did not find the viral clearance dramatically compromised (**Fig. 1.4**). Thus, these data indicate that IL-33 damps the immune response but does not completely abrogate it.

Regarding the immune effects of IL-33 in the liver, in the Con-A-induced hepatitis model IL-33/ST-2 axis was shown to be instrumental in containing the deleterious effects of IFN-γ and TNF-α during the course of disease. In our studies, we observed that IL-33 exogenous treatment promoted the expression of IFN-γ in different cell subsets (**Fig. 1.5**). In contrast, IL-33 affected the production of TNF-α in different immune cell subsets (**Fig. 1.6**). These immune effects indicate that IL-33 mediates immune modulation in viral infections compared to that due to chemically induced liver damage. Furthermore, our data indicate that IL-33 does not abrogate the establishment of the antiviral response. Indeed, recent studies showed that IL-33 is instrumental in the early activation of CD8 T cells [176].

As mentioned before, the IL-33 has been related to Th-2 response initiation [159]. In our experiments we found that IL-33 indeed promoted hepatic production of Th2 cytokines during the Ad infection. As expected, IL-33 induced the production of Th2-associated chemokines but, surprisingly, the production of Th1-associated chemokines was not affected. This indicates that IL-33 exerts a bystander activation in the liver. Further experiments will elucidate whether this bystander activation mediated by IL-33 treatment can be used in patients suffering acute viral hepatitis. However, the putative therapeutic role of IL-33 is jeopardized by the body of evidence supporting the concept that Th2 cytokines promote liver fibrosis which is deleterious for hepatic function [97]. Indeed, immunohistochemistry studies showed that IL-33 is over-expressed in cirrhotic human livers [173].

Recently, IL-33 has been associated with the expansion of ILC2, a cell subset that promotes tissue repair, early defense against helminthes and mucosal integrity in the lungs [175]. ILC2 have been associated with the Th2 response [165]. In our experiments, IL-33 treatment led to the expansion of ILC2 characterized by the production of IL-5 and IL-13. Subsequent characterization showed that *in vitro* expanded ILC2 with IL-33 are able to decrease TNF-α production *in vitro* (**Fig. 1.8**). Furthermore, ILC2 derived from IL-33 stimulation was able to ameliorate liver damage parameters during Ad infection (**Fig. 1.9**). Altogether these data indicate that IL-33 mediates its protective effects through ILC2.

Altogether, our data indicate that IL-33 protects the liver in viral hepatitis by reducing the expression of TNF-α, but at the same time Th1 and Th2 responses are conserved. Further studies will help to determine the putative therapeutic role of IL-33 in viral hepatitis.

CHAPTER 3: EFFECTS OF HGF IN VIRAL HEPATITIS

3.1 Introduction

Fulminant hepatitis is a rare, but fatal disease that is characterized by massive hepatocyte death that leads to a deteriorated liver function and encephalopathy [219, 220]. In addition to drug-related hepatotoxicity, especially from acetaminophen, patients with hepatitis A, hepatitis B, autoimmune hepatitis, and Wilson's disease may also develop acute liver failure. Adenovirus (Ad) are a promising tool in the field of gene and cancer therapy and as experimental vaccine vector [138, 221]. However, Ad can elicit strong and pathogenic immune activation in the liver in humans, non-human primates, and mice [222]. The failure to halt these immune responses can lead to necroinflammatory hepatitis, treatment failure, and even patient death [223]. The only effective treatment for ALF is liver transplantation. Other therapies such as lamivudine for acute hepatitis B and plasmapheresis, including corticosteroids, have no demonstrable benefit [220].

Hepatocyte growth factor (HGF) is a pleiotropic molecule that has pro-mitogenic and pro-survival functions [182]. HGF binds a receptor known as mesenchymal-epithelial transition factor (c-Met) inducing survival and proliferation [181]. Because of its role in hepatocyte proliferation, HGF is a potential therapeutic agent for treatment of fatal liver disease, and indeed has proven to be safe and tolerated in cases of fulminant hepatitis during in phase II clinical trials [116]. HGF is also pro-angiogenic, and it has been used successfully in patients with severe peripheral arterial disease [224]. On the

other hand, HGF is involved in cancer metastasis [225]; hence c-Met inhibitors are emerging as therapeutic candidates for breast, colon, gastric and prostate cancer [225, 226].

HGF is produced in injured tissues by stromal-derived cells, monocytes/macrophages [227] and neutrophils [180]. HGF up-regulates the *bcl-2* via AKT/PKB signaling pathway, conferring cyto-protection during oxidative stress [228]. HGF is also an anti-inflammatory, since it can also inhibit NF-kB activation [189, 229, 230]. However, HGF cannot be considered as a merely immune-suppressive molecule for the following reasons; (1) HGF can inhibit the production and signaling pathway of TGF-β, [190, 231] (2) HGF has been found to be instrumental for DCs activation in the skin, [193] and (3) HGF has preserved graft-versus-leukemia and T cell reconstitution in a model of bone marrow transplantation [192]. Accordingly, these data indicate that HGF may affect immune responses in a multi-layered function.

Despite the widely recognized protective role of HGF in fulminant hepatitis and liver regeneration [116], the immune effects of HGF in acute hepatitis are far from being elucidated. In this report, we studied the immune effects of HGF on dendritic cells (DCs) and on T cell activation during viral hepatitis. By using an animal model of adenovirus (Ad)-induced hepatitis and *in vitro* cell culture systems, we examined the immune effects of HGF on DCS and its putative hepatoprotective role in acute hepatitis. HGF diminished the expression of IFN-γ, TGF–β and IL-10. At the cellular level, DCs expression of co-stimulatory molecules and IL-12p40 was affected, but surprisingly

HGF also affected T cell function. Our findings that HGF ameliorates liver damage in viral hepatitis may provide insight into the therapeutic strategies for fulminant hepatitis having a viral etiology.

3.2 Materials and methods

Mouse experiments

Female C57BL\6 mice (6-8 weeks, Jackson Laboratories, Bar Harbor, ME) were maintained in germ-free conditions in the animal research facility at the University of Texas Medical Branch. All animal experiments were done under an IACUC protocol in accordance with the guidelines of the Animal Care and Use Committee to assure humane animal handling.

Mice received 20 μg of hydrodynamically delivered, naked DNA plasmid encoding the human HGF gene in a pCDNA3.1 vector, as previously described [232]. As a control, a group of mice received 20 μg of empty vector. The expression of human HGF was examined in the livers of these animals as previously described [233]. Three days after gene delivery, both groups received 3×10^9 pfu of Aflac virus (Vector Development Laboratory, Houston, USA). Mice were sacrificed at day 1 or 6 or 14 post-infection. Liver tissues were fixed in 10% formaldehyde solutions and stain with hematoxylin and eosin (H&E). Blood, liver and spleen samples were collected for further analyses.

Intrahepatic lymphocytes and splenocyte collection and *ex vivo* T cell stimulation

Livers were mechanically disrupted and digested in a solution of 0.05% of collagenase D (Roche, Mannheim, Germany) in RPMI media (Corning, Mannassas, VA). The obtained cellular suspension was washed once with PBS and then separated in a Percoll (Sigma Life Sciences, St. Louis, MO) discontinuous gradient. A splenocyte suspension was obtained after mechanical dispersion and treatment with a red blood cell lysing buffer (Sigma, USA). IHL or splenocytes collected from the experimental animals were plated in 24-well plates (2×10^6 cells/ well) and stimulated with phosphor 12-mystrate 13-acetate (10 ng/mL) and ionomycin (500 ng/mL) in the presence of 1 μg/mL of Golgi Plug (BD Biosciences, San Diego, CA) in complete RPMI media (10% heat inactivated FBS, Penicillin, ampicillin, pyruvate and 2-ME), cells were incubated for 5 hours at 37ºC and 5% CO_2. Stimulated cells were stained for surface markers, fixed and, then permeabilized with a Cytofix/Cytoperm kit (eBioscience, San Diego, CA) for intracellular cytokine staining.

Evaluation of ALT, cytokines and growth factor in serum

ALT activity in serum was measured in the Department of Clinical Chemistry (UTMB, Hospital University Clinics). HGF concentration was evaluated with an ELISA (R&D systems, Minneapolis, MN). The levels of IL-2, IL-12(p40), IL-7, IL-10 and IL-

17 were measured by using a multiplex magnetic beads kit (Millipore). Cytokines were detected in a Bioplex 200 system (Luminex XMap Technology, Bio-Rad, Hercules, CA).

Histological Grading

A categorical nominal grading scale was defined by taking into consideration the following parameters: lymphocyte infiltration per acinar zones (1 point if they were present in zone 1, 2 points if they were present in zone 2, and 3 points if they were present in zone 3), inflammatory affection to the central vein (3 points), number of Councilman bodies per view (0 to 1 bodies= 0 points, 2 to 3 = 1 point, 3 to 4 bodies = 2, and more than 5 bodies =3). Points were summed and a final grade was given by applying the following standard: 0 for the preparations with less than 1 point, I for the preparations with 2 to 4 points total, II for preparations with 5 to 6 points and III for the ones that summed more than 6 points. Slides were double-blinded and evaluated by 3 independent individuals following the mentioned criteria. The final grading consisted of taking the consensus of more than 2 evaluators, as in a Delphi test.

Flow cytometry analysis

IHL and splenocytes isolated from experimental animals were stained with monoclonal antibodies after they were treated with FC blocking antibody (BD, Franklin Lakes, NJ).

Antibodies against mouse IFN-γ (XMG1.2), CD4 (GK1.5), CD8 (53-6.7), CD3 (17A2), c-Met receptor; MHC-II (M5/114.15.2), CD40 (1C10), CD86 (GL-1), CD44 (IM7), CD62L (MEL-14), PD-1 (243) were purchased from eBioscience (San Diego, CA). Anti-mouse Tim-3 antibody (RMT3-23) was purchased from BD Biosciences (Franklin Lakes, NJ). For intracellular cytokine detection, T cells were stimulated as previously described. Then, cells were fixed and permeabilized by using a Cytofix/Cytoperm kit (BD Biosciences). Data were acquired with the LSR II Fortessa system (BD Biosciences) and analyzed with FlowJo 8.5 software (TreeStar, Ashland, OR).

Gene expression analysis and viral quantification

Freshly isolated tissues were preserved in RNA-later solution (Ambion, Austin, TX). RNA was isolated by using an RNAeasy isolation kit (Quiagen, Maryland, MA) and then used as a template for cDNA synthesis with a commercial kit (Bio-Rad). Our real-time PCR protocol was as follows: denaturing 10 min at 95°C, 40 cycles of 15 s at 95°C, and 60 s at 60°C. Melt-curve analysis was also used to check the specificity of the amplification reaction. The relative quantity of mRNA expression was calculated by using the $2^{-\Delta\Delta}$CT method. Similarly, virus concentration was calculated by using a standard curve of AdLacZ DNA coming from a stock with known viral concentration.

Bone marrow-derived dendritic cell (BMDCs) cultures

BMDCs were differentiated in IMDM media (Gibco, Grand Island, NY) supplemented with 20 ng/mL of recombinant GM-CSF (PeproTech, Rocky Hill, NY). Fresh media was added every 3 days. Non-adherent cells were harvested at day 7 and plated in 24-well plates at 1×10^6 cells/well density. BMDCs were stimulated with 100 ng of LPS (Sigma, St. Louis, MO), Poly IC (Invivogen, San Diego, CA), CL096, or AdLacZ virus (MOI 1:200) for 24 hours in the presence or absence of 40 pg/mL of recombinant murine HGF (PeproTech). Stimulated cells were harvested and stained under sterile conditions.

Statistical analyses

Data were analyzed with Graph Pad Prism 4 software. For normally distributed and non-parametric data, a two-tailed Student T test was done. For non-normally distributed data, a pair-wise Mann-Whitney test was done. For nominal ordinal variable, an X^2 test was performed. Spearman correlations were done to explore the association between parametric and non-parametric variables. A p value less than 0.05 was considered significant.

3.3 Results

HGF/c-Met axis is up-regulated in Ad-induced hepatitis

HGF is secreted in response to partial hepatectomy or chemical damage to the liver [182]. Hence, we evaluated the kinetics of HGF/c-met axis during the course of Ad-induced hepatitis. Mice received a single dose of AdLacZ (3×10^9 pfu) via a tail vein. We found that HGF serum concentrations increased considerably during the first 12 h followed by a second peak at day 6 **(Fig. 2.1 A)**. The hepatic expression of the *hgf* gene started at12 h of infection and peaked at day 3 prior to the second peak in the serum **(Fig. 2.1 B)**. We speculate that the biphasic increase of HGF resulted from protease-mediated activation and release of pro-HGF to the serum, and that *de novo* gene expression led to the second peak at day 6 [182]. Similarly, *c-met* expression in liver tissues was increased within the first 24 h and fell on day 6 **(Fig. 2.1 C)**. When we analyzed the c-Met expression in intra-hepatic lymphocytes (IHL), we detected a steady increase in c-Met levels on their surface (data not shown). Since c-Met prevents apoptosis through the activation of Bcl-2, we evaluated *bcl-2* expression in the liver [188]. The expression of *bcl-2* rose considerably during the first 12 h followed by a second increase at day 6 **(Fig. 2.1D)**. This pattern was reminiscent of that of serum HGF (**Fig. 2.1 A**), indicating an activated HGF expression and its role in Ad-induced hepatitis.

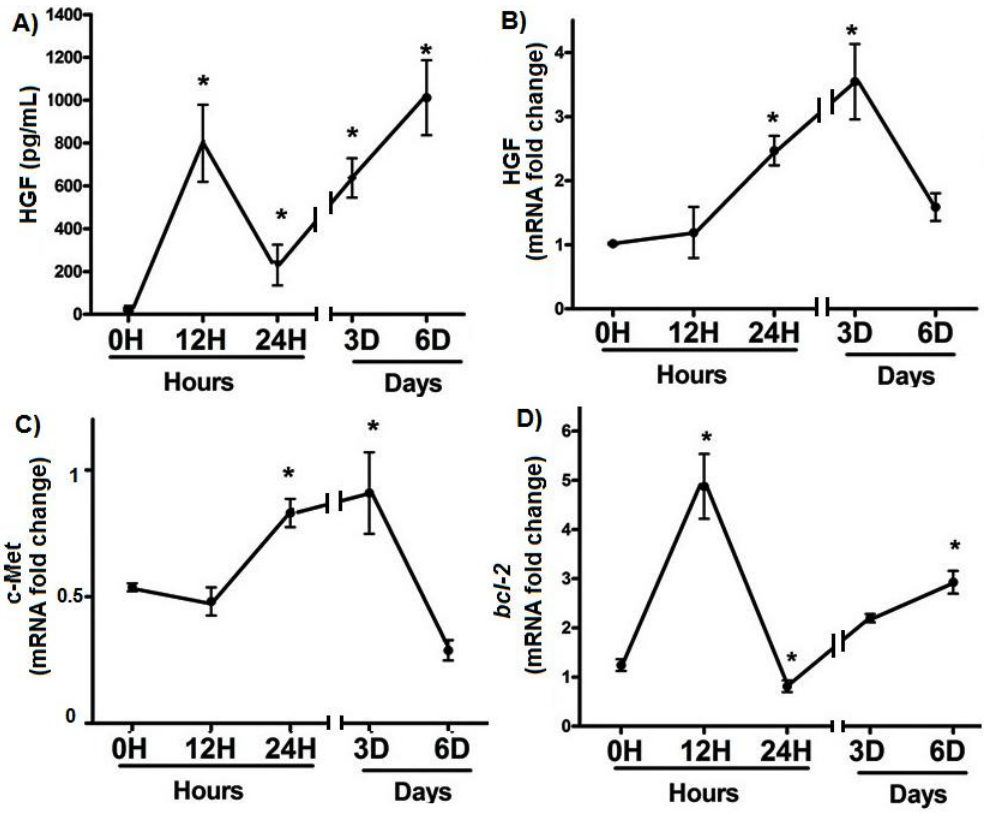

Figure 2.1 The HGF axis is activated in the course of viral hepatitis. C57BL/6 mice received 3×10^9 *pfu* of AdLacZ *i.v.* and sacrificed at each time point. Serum concentration of HGF (A). (B) Liver expression of the *hgf* gene was normalized to the GAPDH. (C and D) Liver expression of *c-met* (C) and *bcl-2* were evaluated with RT PCR and normalized to GAPDH. Data are representative of three independent experiments with three mice at each time-point (* $p<0.05$, Standard error of the mean).

HGF ameliorates immune-mediated pathological changes in the liver

HGF protects the organ in chemically induced injuries in the liver [234], kidneys [198] and heart [235]. To explore the biological role of HGF in viral hepatitis, we over-expressed HGF by using a hydrodynamically delivered plasmid or an empty vector [232]. Three days later, both groups were infected with AdLacZ virus (3×10^9 pfu). At day 6, the serum ALT levels in the HGF group were significantly decreased (**Fig. 2.2 A**). IHL and splenocyte counts were significantly lower in the HGF group than those in the

control group (**Fig. 2.2 B and C**), indicating that HGF can modify the inflammatory response locally and systemically. H&E-stained preparations showed a decreased lymphocytic infiltration and better-preserved hepatic architecture in the HGF-transfected group (**Fig. 2.2 D**). Consistently, histopathological scores were significantly lower in the HGF group (**Fig. 2.2 E**) and correlated with the ALT levels (Spearman's rho=0.92). Altogether, these data indicate that HGF exerts cyto-protection in viral hepatitis possibly by inhibiting the early lymphocytic recruitment to the liver and modulating antigen presentation by DCs.

HGF reduces DCs activation during Ad-induced hepatitis

HGF is known to modulate DCs activation in experimental allergic encephalomyelitis [200] and lung inflammation triggered by soluble antigens [199]. To further examine the role of HGF in DCs function, we examined the expression of HGF receptor c-Met. We analyzed hepatic DCs ($CD11c^+$ $CD11b^+$ $F4/80^-$) at different time points during the course of viral infection. Hepatic DCs steadily increased in the liver-derived DCs (**Fig. 1.3 A**). When we examined the effect of HGF over-expression on DCs activation, we observed a lower expression of serum IL-12 p40 in the HGF group at day 6 (**Fig. 1.3 B**). Thus, we further examined hepatic DCs activation in the IHL coming from transfected animals at 24 h post-infection. There was a significant decrease in the expression of CD40 and MHC-II in the DCs collected from the HGF group (**Fig. 1.3 C**, **D** and not shown). There were no such differences in splenic DCs (not shown).

To determine whether HGF exerts a direct effect on DCs, we performed *in vitro* studies in which bone marrow-derived DCS (BMDCs) were stimulated with 100 ng/mL of TLR4 (LPS), TLR7/8(CL097), TLR3 (poly I:C) agonists, or with AdLacZ (MOI 1:200). We found that BMDCs displayed c-Met upon TLRs stimulation and viral infection (**Fig. 1.4 A**). Then, BMDCs were stimulated for 24 h with different stimuli with or without recombinant HGF (40 ng/mL). Similarly, BMDCs stimulated in the presence of recombinant HGF did not up-regulate the expression of CD40 (**Fig. 1.4 B and C**) and CD86 (not shown). Furthermore, IL-12p40 production was significantly decreased by HGF in BMDCs stimulated with TLR3, TLR7/8 and Ad. However, the HGF did not abrogate IL-12p40 production in LPS-stimulated BMDCs (**Fig. 1.4 D**). Our *in vivo* and *in vitro* studies indicated that activated DCs expresses HGF receptor c-Met, and that HGF directly modulates DCs maturation and activation upon viral infection and TLR stimulation, which could down-modulate T cell activation and cytokine production.

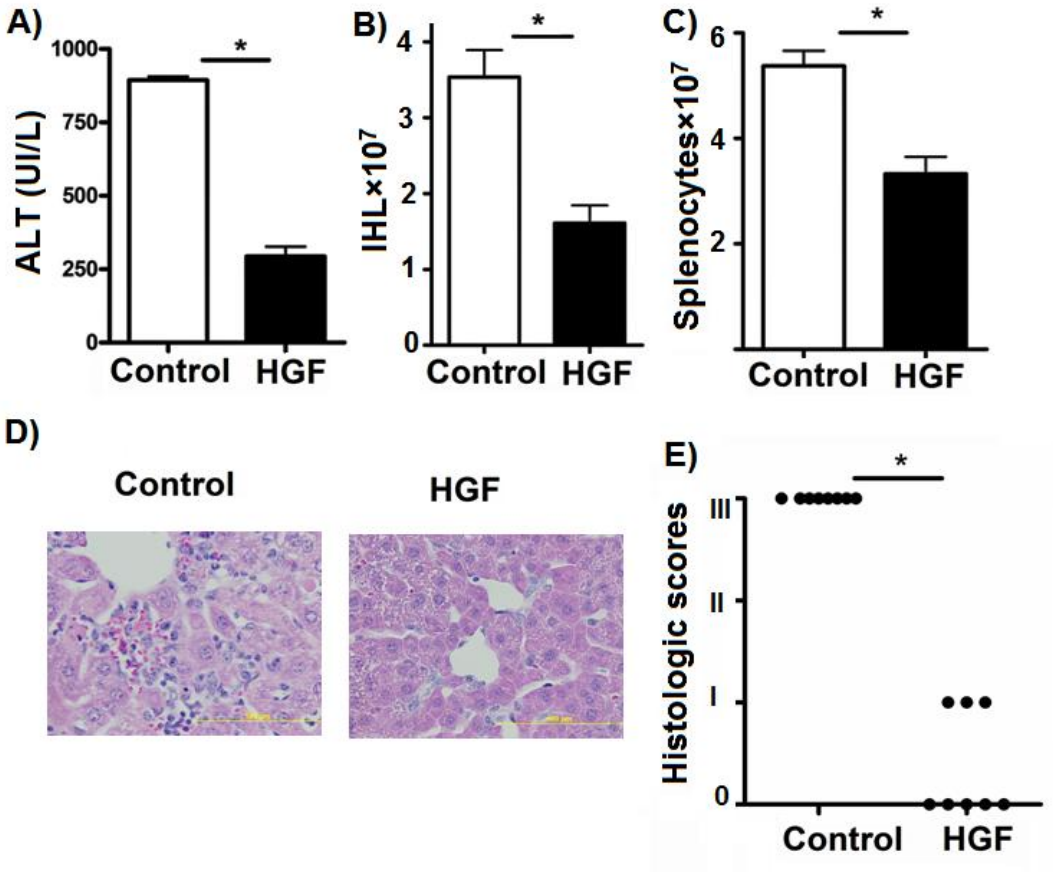

Figure 2..2 HGF over-expression ameliorated Ad-induced immune-pathology. Mice received 20 μg of the HGF plasmid or an empty plasmid i.v. Three days later each group received 3×10^9 *pfu* of AdLacZ virus *I.V.* Mice were sacrificed at 6 days post-infection. (A) ALT activity measured in serum. (B and C) Cell counts of intra-hepatic lymphocytes (IHL) and splenocytes recovered from each mouse. (D) H&E stains of liver from experimental animals. (E) Distribution of the histological scores from animals of two independent experiments. Data are representative of five independent experiments, four mice per group. *$p<0.05$.

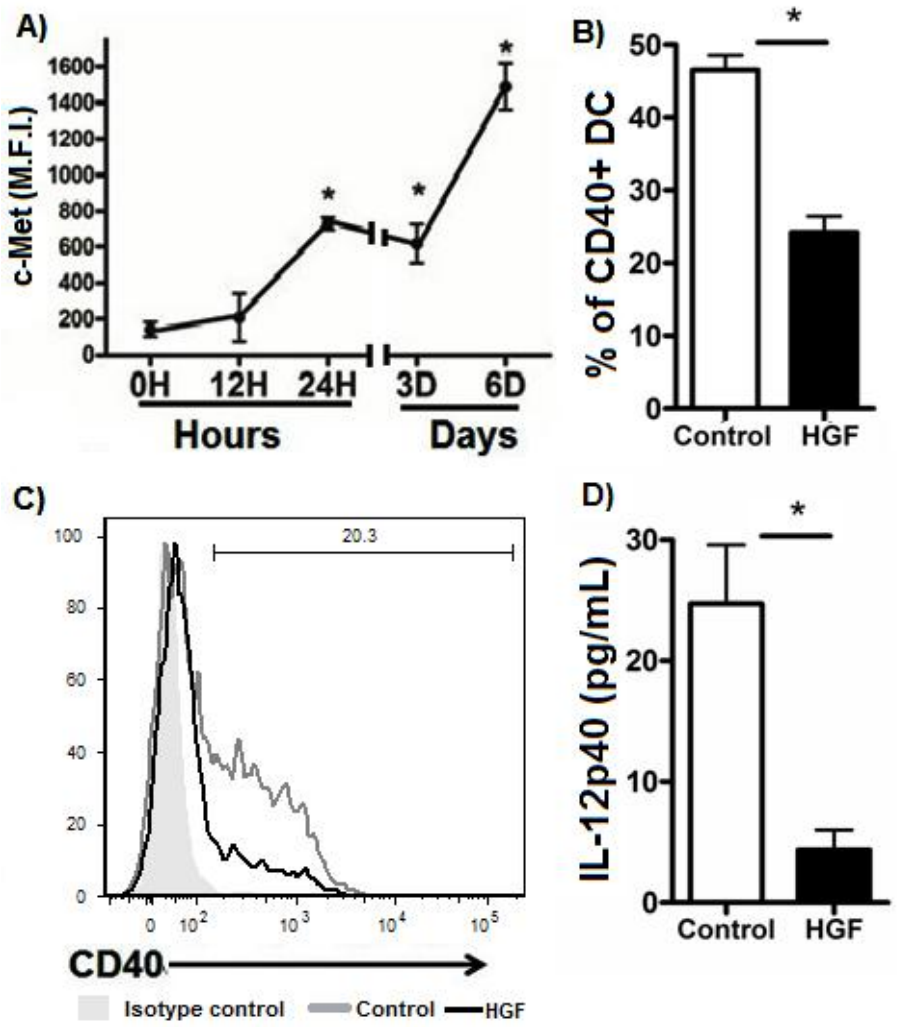

Figure 2.3 HGF diminishes DCs activation *in vivo*. Intra-hepatic lymphocytes were isolated from Ad-infected mice and stained with CD11c and c-Met (A). (B) The percentage of CD40+ DCS (C) and expression of CD40 in DCS collected at day 1 of infection. (D) Concentration of IL-12 (p40) in the HGF and control plasmid transfected mice at day 6 post-infection. Data are representative of three independent experiments, three mice per group. Error bars represent the standard error of the mean, *$p<0.05$.

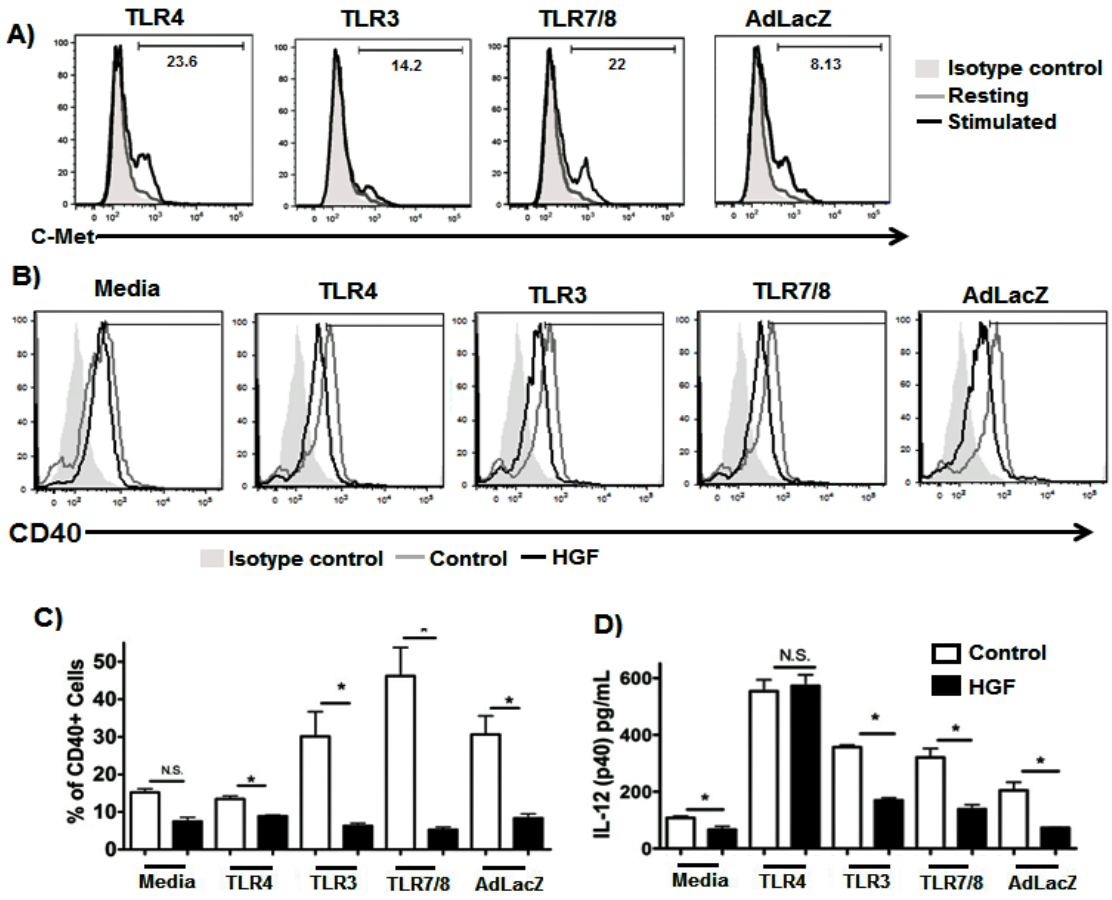

Figure 2.4 HGF modulates bone marrow-derived DCs. DCs were generated from bone marrow culture in the presence of recombinant GM-CSF. DCSs were stimulated for 24 h with LPS (TLR4), Poly I:C (TLR3), CL097 (TLR7/8), and AdLacZ (MOI 1:200), respectively. **(A)** Expression of c-Met in activated DCS. Expression of CD40 in DCs, histogram (B) and the pooled data from five technical replicates (C). (D) Concentration of IL-12p40 in the supernatant of the stimulated DCS. Data are representative of three independent experiments, five technical replicates per treatment,*$p<0.05$. Open bars represent control groups and solid bars the HGF groups.

HGF diminishes IFN-γ and acute phase protein production

IFN-γ is elicited by viral infections in the liver [107]. We examined IFN-γ expression in the liver and serum in Ad-induced hepatitis with or without HGF over-expression. In the HGF group, there was a significant decrease in the serum concentration and liver expression of IFN-γ (**Fig. 2.5 A and B**). Furthermore, the percentage of IFN-γ-producing T cells was significantly decreased in the IHLs from the HGF group when compared to those in the control group (**Fig. 2.5 C and D**). In contrast, no differences were found in the splenic T cells from both groups. These data demonstrated that IHLs, but not splenocytes, can produce large amounts of IFN-γ and that HGF limited IFN-γ production in hepatic T cells.

HGF diminishes type I interferon production

Type I interferon is secreted upon TLR activation in response to viral infection [153]. In addition, we showed that in the presence of HGF TLR-activated DCs had lower expression of co-stimulatory molecules and IL-12p40 (**Fig. 2.4**).Thus, we examine the effect of HGF type I interferon production after Ad infection. Type I interferon is also decreased in the presence of HGF transfection (**Fig. 2.6**). These data indicate that the inhibitory effect of HGF on T cell recruitment is at least, in part, attributable to a lower production of IL-6 and IFN-γ.

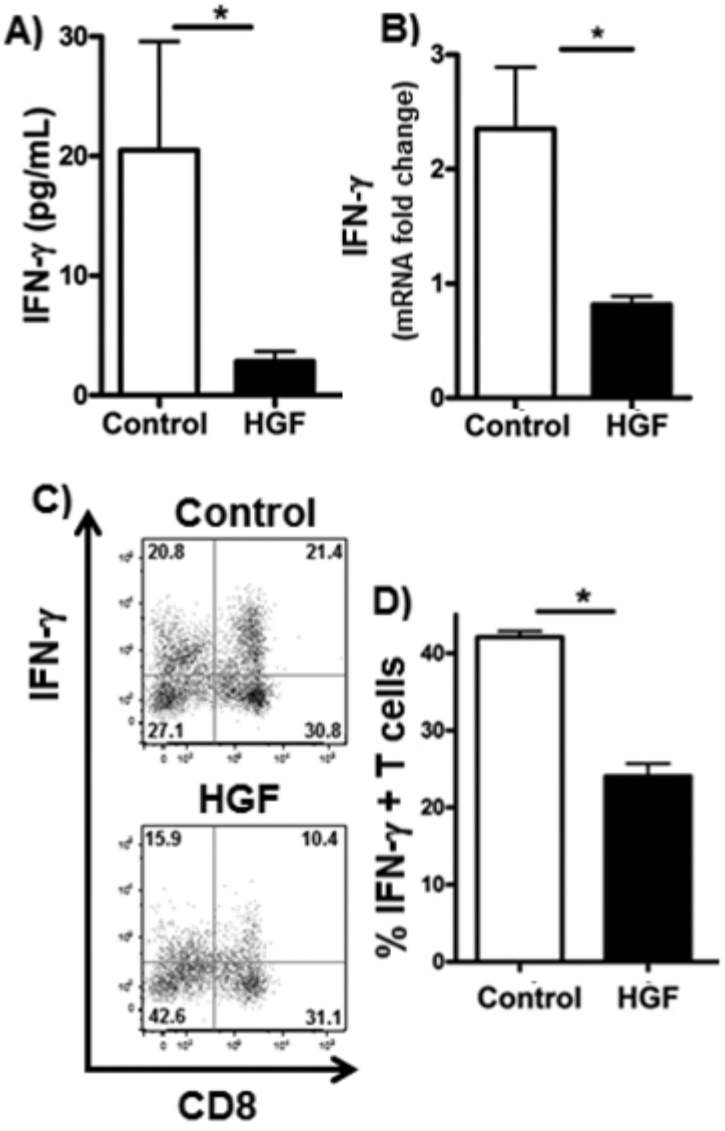

Figure 2.5 HGF abrogates the production of IFN-γ during Ad-induced hepatitis. Mice were treated and sacrificed at day 6 of infection as described in figure 2. (A and B) Serum concentration and liver expression of IFN-γ at day 6 post-infection. (C and D) Percentage of IFN-γ production in T cells. Results are representative of three independent experiments, four mice per group. Standard error represented in the error bars *$p<0.05$.

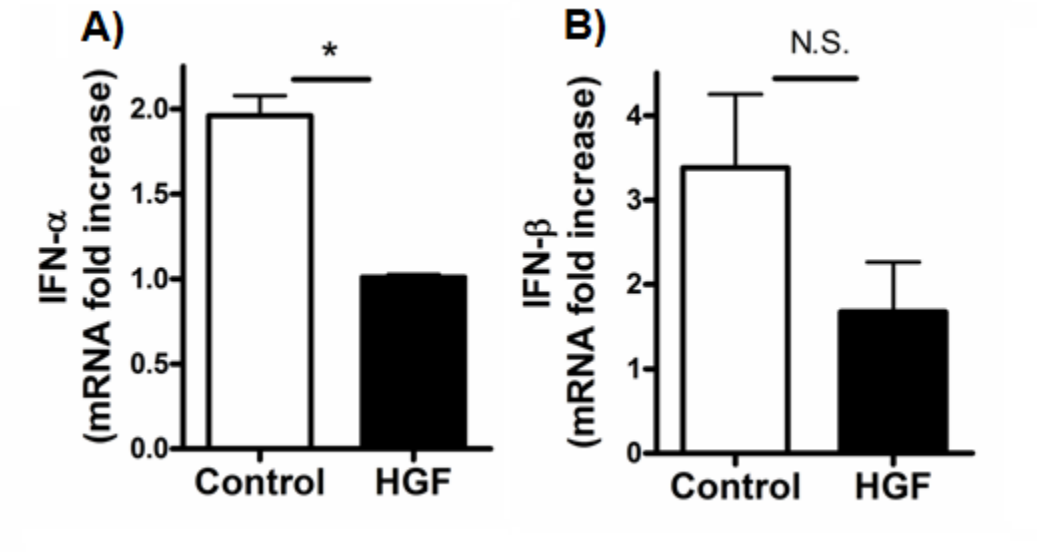

Figure 2.6 HGF over-expression diminishes the production of type I interferon in Ad-induced hepatitis. Mice were treated and infected as in figure 2,3 at day six of infection mice were sacrificed. Expression of IFN-α and IFN-β normalized to GAPDH gene expression (A and B). *p <0.05.

CXCL9 and IL-6 are decreased by HGF over-expression

CXCL9 and IL-6 are critical for T cell recruitment to the liver following stimulation with TLRs agonist and IFN-γ [66, 73]. We observed diminished T cell recruitment (**Fig. 2.3 B**) and less TLR-mediated DCs activation in the presence of HGF (**Fig. 2.4 B**). We also examined the effect of HGF on CXCL9 and IL-6 production. The expression of CXCL9 in the liver was significantly decreased by HGF over-expression (**Fig. 2.7 A**). Additionally, IL-6 serum concentration and liver expression were significantly diminished in the same group (**Fig. 2.7 B and C**).

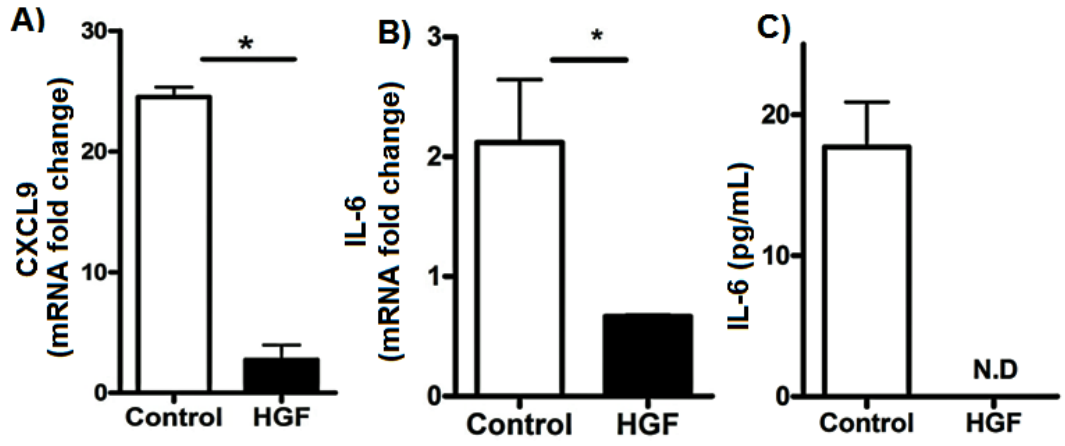

Figure 2.7 CXCL9 and IL-6 production is decreased by HGF. Mice were treated with HGF plasmid or control, infected and sacrificed as described in figure 2. (A)Liver expression of CXCL9 normalized with GAPDH gene. (B) Expression of IL-6 normalized with GAPDH. (C) Serum concentration of IL-6. Data are representative of three independent experiments, four mice per group. Standard error of mean, *$p<0.05$.

HGF decreases IL-10 and TGF-β production and modulates T cell activation

Having demonstrated that IFN-γ, IL-6 and CXCL9 were down-regulated by HGF (**Fig. 2.5 and 2.7**), we then asked whether this inhibition resulted from an augmented response from anti-inflammatory cytokines such as IL-10 and TGF-β, since previous reports showed contradictory effects of HGF on TGF-β and IL-10 in the liver, kidneys and central nervous system [200]. To our surprise, there was a significant reduction in the expression and production of IL-10 and TGF-β in the HGF group (**Fig. 2.8 A-C**). Since TGF-β and IL-10 are inhibitors of T cell activation and promote viral persistence [100, 236], we determined whether HGF exerted its immunomodulatory effect by measuring T cell activation marker CD44 and T cell homing molecule CD62L [237, 238]. There was a decrease in the percentage of $CD44^{hi}$ $CD62L^{lo}$ hepatic T cells from the HGF group (66.96%±5.16 vs. 35.9%±1.03, **Fig. 2.8 D and E**). However, $CD44^{hi}$ $CD62L^{hi}$ T cells were increased in the HGF group (**Fig. 2.8 D and F**). No major differences were seen in the splenic T cells in both groups. When taken together, these data indicated to us that HGF modulates the activation of hepatic T cells, but not of splenic T cells, in such a way that they do not display a phenotype of robust activation ($CD44^{hi}$).

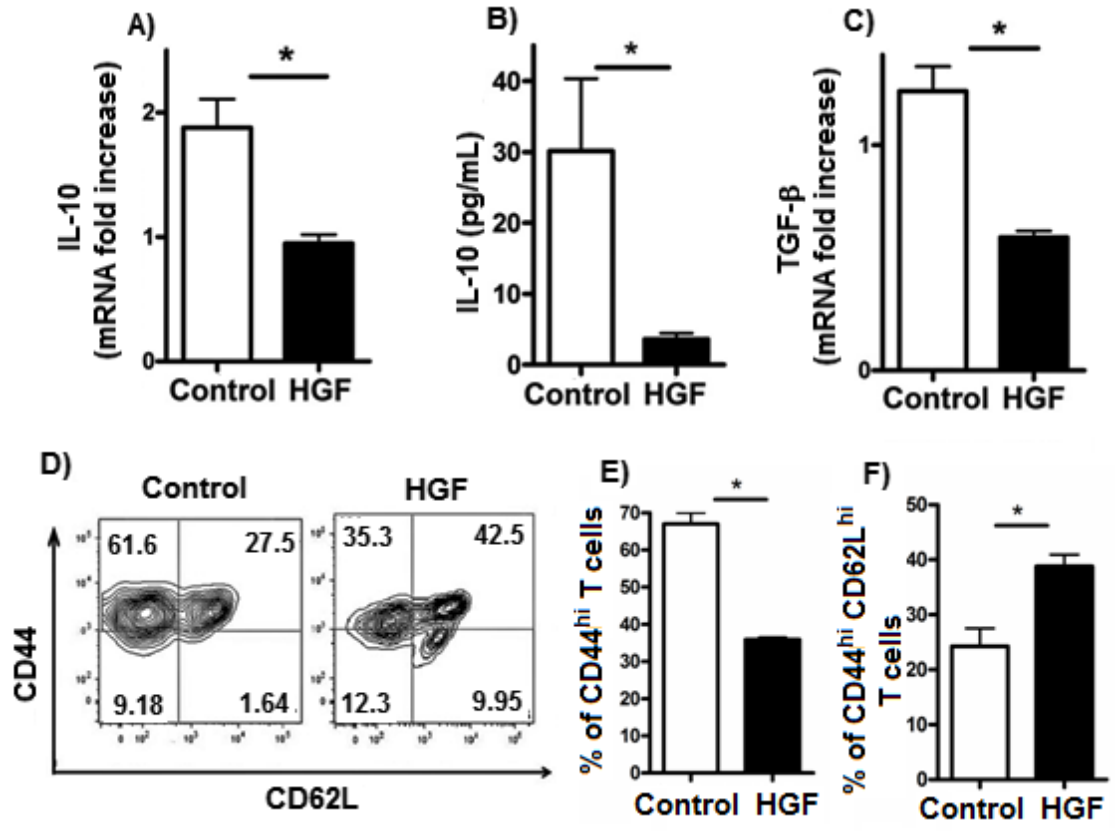

Figure 2.8 HGF modulates T cells by decreasing the expression of TGF-β and IL-10. Mice were treated as previously specified in figure 2.3 and sacrificed at day 6 of infection. (A) Liver expression of IL-10 normalized to GAPDH expression. (B) Serum concentration of IL-10. (C) Hepatic expression of TGF-β normalized to GAPDH expression. (D) Representative FACS plots of CD44 and CD62L in T cells collected from liver. (E) Percentage of $CD44^{hi}$ cells and (F) $CD44^{hi}CD62L^{hi}$ T cells from experimental animals in liver. Data representative of three independent experiments, three mice per group. Error bars are standard error of mean, *$p<0.05$.

HGF decreases the expansion of PD-1^{+}Tim-3^{+} CD8 T cells

Based on the inhibitory effect of HGF on TGF-β and IL-10 production during viral hepatitis, we explored whether it had an effect on the early expression of T cell inhibition markers and viral persistence. Previous reports showed that Tim-3 expression on CD8 induces tolerance [239] . Furthermore, in patients with hepatitis C, CD8 cells that display Tim-3 in conjunction with PD-1 have lower antiviral activity [240], and this CD8 population is refractory to antigen re-stimulation [105]. To our surprise, the frequency of PD-1^{+} Tim-3^{+} CD8 cells was significantly diminished in the livers of the HGF-transfected mice in comparison with those of control animals at day 14 post-infection (45%±3.6 vs. 25.25%±1.8, **Fig. 2.9 A and B**). PD-1^{+} Tim-3^{+} CD8 cells were more frequent in the liver than in the spleen of both groups. Regarding viral clearance, HGF inhibited early lymphocyte infiltration. However, viral clearance is restored 14 days post-infection (**Fig. 2.9 C**). Importantly, the expression of TGF-β, IL-10 and PD-1 was decreased by HGF at day 14 post-infection (not shown). Thus, HGF inhibited the production of IFN-γ, IL-6 and CXCL9 in the liver, but at the same time it decreased the expression of T cell inhibitors (PD-1 and Tim-3).

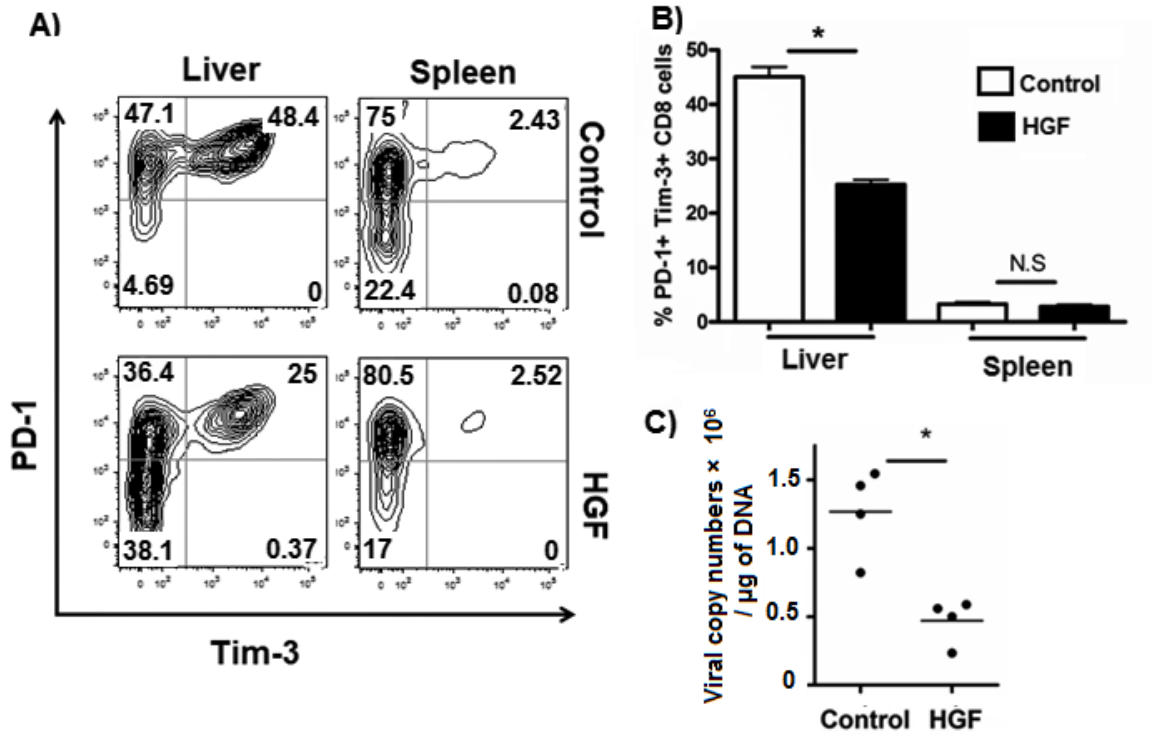

Figure 2.9 PD-1^{+}Tim-3^{+} CD8 T cells are decreased by HGF over-expression. Mice were treated, as described in figure 2. Mice were sacrificed at 6 and 14 days post infection. (A) Representative FACS plot of PD-1 and Tim-3 expression on CD8 T cells collected from liver and spleen at day 14 post-infection. (B) Percentage of PD1+Tim-3+ CD8 T cells collected from liver and Spleen of four animals. (C) Viral copy numbers of AdLacZ normalized with DNA concentration of the sample at day 14 post-infection. Data are representative of three independent experiments, four mice per group. Error bars are standard error of mean, *$p<0.05$.

3.4 Discussion

Fulminant hepatitis, caused by HAV, HBV and several other viruses (*e.g.* adenovirus), is a rare but fatal disease [140, 141, 223, 241]. Fortunately, HGF is a potential therapeutic agent for treatment of patients with this disease [116]. In addition, clinical observations have shown that serum HGF levels were elevated in patients infected with HCV compared to those who cleared it [181, 209, 242]. However, despite its importance, there is a paucity of mechanistic studies on the role of HGF in viral hepatitis. In this study, we found that HGF was drastically elevated during the first several hours of Ad infection **(Fig. 2.1)**. When HGF was over-expressed in mouse liver, it reduced the total numbers of IHLs and splenic lymphocytes, resulting in decreased serum ALT levels and liver damage in infected animals (**Fig. 2.2**). We concluded that HGF has immunomodulatory effects on DCs and T cell functions in a multi-layered fashion and that it protects the liver during acute hepatitis in mice infected with adenovirus.

In this study, we found that HGF over-expression in the liver and its presence in BMDCs cultures down-regulated DCs activation marker CD40 as well as IL-12p40 production (**Fig. 2.3 and 2.4**). Previous reports of studies in which soluble antigens and complete Freund's adjuvant were used, have indicated that HGF can inhibit DCs functions and significantly suppress collagen-induced arthritis [199, 243]. However, in another study, HGF receptor c-Met activation was found to be instrumental to DCs migration and skin hypersensitivity [193]. Also, the sustained expression of HGF was

found to induce tolerogenic DCs and Tregs and suppress autoimmunity in the central nervous system [200]. Therefore, HGF plays a complex immunomodulatory role, depending in part on the site of inflammation and the dose levels at which HGF is given. These results are in agreement with our observations, in that HGF suppressed DCs activation through intracellular TLR during Ad infection (**Figs. 2.3 and 2.4**). However, T cell activation was not fully abrogated, indicating that HGF did not fully suppress DCS activation in Ad-induced hepatitis (**Figs. 2.5 and 2.8**).

In addition, HGF has been reported to act directly on fully differentiated T cells or other immune cells [244, 245]. For instance, HGF has been found to induce tyrosine phosphorylation on immune cells via another receptor, *i.e.* mannose receptor [245]. In this study, we found that HGF over-expression resulted in decreased IFN-γ production and CXCL9 and IL-6 expression (**Figs. 2.5 and 2.7**). HGF can also influence T cell responses by enhancing T cell maturation in the bone marrow and thymus [246, 247]. Here, we found that higher percentages of T cells expressed $CD44^{hi}CD62L^{hi}$ markers in the HGF-transfected mice compared to those in the control group (38.8%±4.27% vs. 24.26%±5.6, **Fig. 2.8**). We speculate that these cells may represent T lymphocytes that were expanded by HGF. This conclusion is also supported by the fact that HGF can help expand CD8 T cells and maintain their cytotoxicity in a bone marrow transplant model [192, 248].

HGF can modulate the micro-environment of T cells. For example, it can suppress TGF-β production and reverse liver cirrhosis [231, 249]. In another case, HGF gene

therapy suppressed TGF-β expression in glomeruli and tubulointerstitium in the kidneys and ameliorated diabetic nephropathy in mice [230, 233]. In our study, HGF over-expression decreased TGF-β in Ad-induced hepatitis (**Fig. 2.8**), and TGF-β is directly related to PD-1 expression [236]. Suppression of TGF-β production, but not antibody neutralization [4, 250], can result in decreased levels of PD-1 expression on T cells and thus in sustained effector functions of antiviral T cells [3]. In an EAE murine model, the constitutive expression of HGF in transgenic mice led to increased production of IL-10 in the CNS, which promotes T cell tolerance [200]. In contrast with that study, hydrodynamic injection and protein expression of HGF plasmid did not induce IL-10 or TGF-β in our model of viral hepatitis. We speculate that sustained HGF expression causes up-regulation of IL-10, whereas transient HGF expression leads to down-regulation of IL-10. This HGF-induced IL-10 and TGF-β inhibition is relevant to other viral infections, since both cytokines are associated with viral persistence [100].

It has been reported that PD-1 expression on T cells depends on TGF-β production [3]. Both PD-1 and Tim-3 are associated with the contraction and exhaustion of the Cytotoxic T cell responses to viral infection [105, 239, 240]. In our study, hepatic CD8 T cells in the HGF-transfected mice had a lower percentage of PD-1^{+}Tim-3^{+} cells compared to those in the control group. Consistently, viral clearance was restored by day 14 (**Fig. 2.9**). In conclusion, we found that HGF was drastically elevated during the first several hours of Ad infection. HGF over-expression modulated DCs activation by down-regulating the expression of IL-12 and co-stimulatory molecules. In our studies, HGF was found to have immunomodulatory effects on T cell functions in a multi-layered

fashion, while limiting lymphocytic infiltration and immune-mediated pathological changes in the liver. In conclusion, HGF should be further explored in clinical studies as therapy for acute hepatitis and to possibly avert fulminant liver failure in patients.

CHAPTER 4: CONCLUDING REMARKS

As of today, there are five well-characterized human hepatitis viruses (from A-E). All of the mentioned viruses trigger the pathological changes seen in hepatitis indirectly after they are recognized by the immune system [32] (discussed in section 1.2). However, there are several differences between these pathogenic viruses that should be highlighted: HBV and HCV have an error system in transcribing their genomes [50, 58]. This feature confers both viruses the ability to evade the immune system. This ability is not present in HAV and HEV, two viruses that cause mainly acute viral hepatitis. It is nevertheless important to emphasize that among these viruses, HCV, HBV and HAV can delay innate immune recognition by subverting the TLR signaling [70, 153, 158]. In this regard, our model of Ad-induced hepatitis is similar to all the clinically relevant viruses in that their immune recognition triggers hepatocyte loss. On the other hand, our model does not consist of a chronic infection. Thus, our results are only extrapolated to the viruses that typically produce an acute presentation of hepatitis: HAV, HBV and HEV.

How do our findings contribute to the understanding of hepatitis?

In this dissertation work, we have studied the immune role of IL-33 and HGF in Ad-induced hepatitis. Before this dissertation, these two molecules were thought to be non-classical components of the antiviral response in the liver. By using systems that modify the course of Ad-induced hepatitis, we were able to examine the exact role of the pro-inflammatory circuits in the pathogenesis of viral hepatitis. Therefore, by affecting the production of TNF-α or IFN-γ by the use of IL-33 and HGF, we were provided with

a proof-of-the concept that immune recognition indeed triggered histopathological changes in viral hepatitis. Additionally, our findings also provided the concept that direct effects of these two hepatocyte pro-survival factors, such as IL-33 and HGF, could also change the paraclinical measurements, such as ALT levels. Thus, the number of Councilman bodies (apoptotic hepatocytes) was decreased by these afore pro-survival factors. However, in the case of IL-33, the numbers of Councilman bodies did not correlate with lower lymphocytic infiltration, thereby providing evidence that T cell cytotoxicity may be tempered by IL-33.

Our findings also helped to expand the concept that immune activation against viruses in the liver is the sum of effector molecules and inhibitory molecules. Previous observations showed the existence of a positive feedback loop between the expression of IFN-γ and TNF-α (effector molecules), with the program death ligand 1 (-PD-L1-inhibitory molecule on the T cell function) [107]. Thus, in our experiments with HGF, we were able to see that decreasing IFN-γ concentrations, along with type I interferon, were also correlated with lower PD-L1 expression, IL-10 and TGF-β expression.

IL-33 and HGF are secreted in early phases of tissue damage, and it is feasible that they have an important role in modulating the immune response. In our studies, we used Ad-induced hepatitis, a model of viral hepatitis that can be used to study early immune responses in the liver and emulate hepatotropic viruses that cause acute hepatitis.

4.1 Role of damage associated molecules in viral hepatitis

As previously described, IL-33 can initiate immune response activation in the absence of pathogens, and this feature allows its classification as a damage-associated molecular pattern (DAMP) or an alarmin [160]. In the liver, the high mobility group 1 (HMGB-1), another DAMP, has been directly associated with the activation of the immune system upon viral infection. Importantly, the activating signals initiated by HMGB-1could be deleterious in case that scavenger cells such as KCs fail to clear it from the microenvironment [251].

Our observations showed that IL-33 and its receptors are expressed during viral hepatitis. This up-regulation leads us to the conclusion that IL-33 may play a role in the pathogenesis of acute viral hepatitis (**Figure 1.2**). Clinical observations have shown IL-33 up-regulation in patients with fulminant liver failure stemming from a wide variety of etiologies [218]. Thus, IL-33 may correspond to an early signal of tissue damage that is independent of the origin of the insult.

When we examined the role of IL-33 in Ad-induced hepatitis, we found that ALT and apoptotic hepatocytes were diminished by IL-33 (**Fig. 1.2**). Similar results showed that IL-33 prevented hepatocytes apoptosis in the model of concavalin-A-induced hepatitis [172]. Conversely, antibody neutralization of IL-33 ameliorated disease in the same model [84]. Thus, it is possible that the role of IL-33 during viral infection

involves, on one hand, the orchestration of primary activation of immune cells as described in a LCVM model [176], and, on the other, the maintenance of hepatocyte survival. These two features may be beneficial in the context of FH having viral etiology. However, our results showed that IL-33 delays viral clearance at day 6 of infection (**Fig. 1.4**) and this effect should be taken into consideration in the event this molecule is considered for therapy in humans.

IL-33 causes numerous effects in the immune response to hepatotropic viruses. On one hand, exogenous IL-33 considerably increases the expression of IFN-γ and IL-2, two molecules involved in T cell effector functions [252, 253] (**Fig. 1.5**). At the same time, exogenous IL-33 keeps the TNF-α production low in viral hepatitis. We have also demonstrated that IL-33 inhibits TNF-α production in DCs and T cells in a dose-dependent manner (**Fig. 1.6**).This is relevant because TNF-α mediates hepatocyte apoptosis through the activation of caspase 8 [254]. Moreover, IL-33 treatment was shown to increase the expression of the anti-apoptotic gene *bcl-2* [162]. Thus, as indicated previously, IL-33 mediates two biological events apparently coordinated in the pathogenesis of viral hepatitis (Chapter 1): 1) immune cell activation and 2) hepatocyte apoptosis [255]. However, these data also indicate that the evolutionary role of IL-33 is to initiate early immune activation, but at the same time to confer organ protection.

IL-33 decreases $CD11b^+$, $CD11c^+$, NK cells in the liver. However, the population of regulatory T cells (Tregs) is expanded. Of note is that T regs are in part responsible for viral persistence in models of chronic infection [256]. Furthermore, in a murine

model of prostate cancer, the sole presence of T regs in the tissue interferes with immune activation against neoplasic cells [257]. Therefore, it would be important to take into consideration this possible deleterious effect of IL-33 on antiviral activity.

Our observations also showed that Th2 cytokines are increased by IL-33 (**Fig. 1.6**). This should be considered when proposing IL-33 as a therapeutic tool in viral hepatitis. Th2 cytokines, especially IL-4, have been involved in fibrosis progression in the liver [97]. IL-33 has been associated with fibrosis in mice and humans [173]. On the other hand, it was shown that the combined expression of IFN-γ, IL-2 and IL-4 in HCV patients is correlated with spontaneous viral clearance [258]. Thus, the possible deleterious effects of IL-33 on viral clearance may not happen in individuals chronically infected with hepatotropic viruses.

Our observations also helped in defining one of the mechanisms that IL-33 uses to confer organ protection in viral hepatitis: the expansion of the nuocytes or innate lymphoid class 2 (ILC2). ILC2 are, by definition, newly described cells that do not express lineage markers and produce IL-5 and IL-13 [259]. ILC2 are present in lymphoid tissue associated with mucosa and confer protection to parasites in the gut [165]. In our studies, we have shown that ILC2 are expanded in the liver when exogenous IL-33 is given (**Fig. 1.7**). Co-culturing *in vitro* generated ILC2 with IHL from Ad-infected animals diminishes the production of TNF-α (**Fig. 1.8**). Furthermore, when *in vitro* generated ILC2 are transferred to Ad-infected animals, there is a reduction in the markers of liver damage (**Fig. 1.9**). However, it is still not clear whether ILC2 are

expanded in the liver due to the treatment with recombinant IL-33 or whether they are recruited from mucosa-associated lymphoid tissue.

In sum, IL-33 exerts immune modulation in Ad-induced hepatitis by diminishing the production of TNF-α. This effect delays hepatocyte apoptosis. However, the production of effector cytokines (IFN-γ and IL-2) is conserved by IL-33 treatment (**Diagram 3.1**). The potential role of IL-33 as therapeutic in viral hepatitis should be examined after its exact role in viral persistence and fibrosis is well defined. Therefore, further studies in a model of chronic viral hepatitis will be instrumental in our understanding of IL-33 biology.

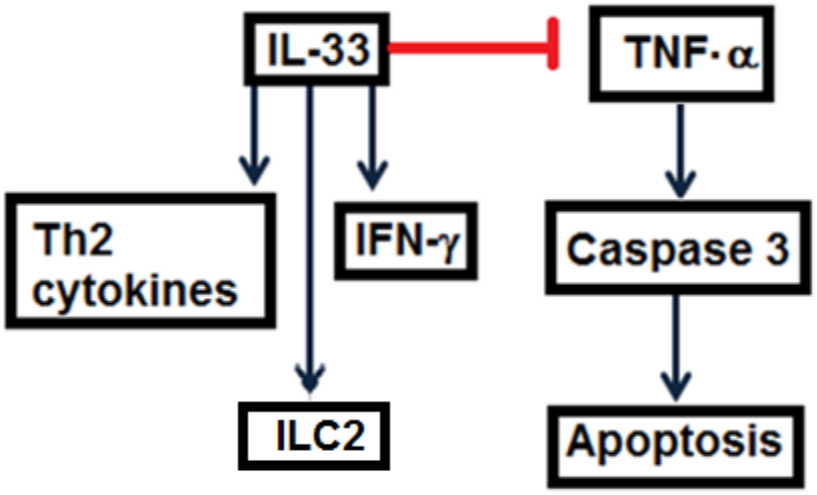

Diagram 3.1 Scheme of the IL-33 multilayered function during Ad-induced hepatitis. IL-33 inhibits the production of TNF-α in immune cells (red line) while inhibiting caspase activation and hepatocyte apoptosis. On the other hand, IL-33 promotes the expression of Th2-associated cytokines, IFN-γ and the expansion of innate lymphocytes class 2 (ILC2).

4.2 Hepatocyte growth factors and their role in modulation of immune response in the liver

Hepatocyte growth factor is a pleiotropic molecule that is secreted also when there is direct injury to the tissues [178]. In the context of damage driven by an infectious agent, HGF secretion has been observed in a wide variety of diseases [202, 205]. This feature is shared with IL-33. Furthermore, HGF associates with perlecan sulfate, a component of the extracellular matrix classified as an alarmin or DAMP [260]. Hence, HGF can be classified as a danger signal when there is injury.

Previous studies have examined the role of growth factors in liver diseases. The placenta growth factor has been associated with liver fibrosis in a model of portal hypertension with concomitant chemical injury [261]. On the other hand, HGF drives opposite effects in a similar model of liver fibrosis [231]. In patients with chronic HCV infection, HGF was found in high concentrations in serum [262]. Despite its association with HCV infection, there is a paucity of mechanistic studies that help us to elucidate the exact role of HGF in viral hepatitis.

In our studies, we showed that HGF and its associated genes were up-regulated by Ad-induced hepatitis (**Fig. 2.1**). Furthermore, when we modified HGF expression by using a hydrodynamically-delivered plasmid, we found a reduction in the markers of liver immune-mediated pathology (**Fig. 2.2**). Thus, our experimental design and results

allowed us to confirm that HGF modulates the immune response to hepatotropic viruses and protects hepatocytes from immune-mediated apoptosis.

In this study we did not examine KCs, because activation of was associated with the removal of DAMP and thus, KCs would dampen immune activation in the liver [251]. Furthermore, in the same model of Ad-induced hepatitis, KCs depletion did not change the immune response to Ad [263]. Thus DCs activation was investigated as the major initiator of immune response in viral hepatitis. Previous studies demonstrated that DCs activation was inhibited by HGF when they were exposed to soluble antigens and LPS [199]. However, the effect of HGF on virally-infected DCs was far from being elucidated. In our observations *in vivo*, we found that the expression of CD40, IL-12p40 and other co-stimulatory molecules was diminished by HGF. *In vitro* observations confirmed this effect as well (**Fig. 2.3 and 4**). The suppression of CD40 expression has particular relevance since the over-expression of this molecule is associated with exacerbation of liver damage but not with an increase of viral clearance [9]. These observations also led us to postulate that strong co-stimulatory signals in the liver do not necessarily translate into better viral clearance.

In our studies, the downstream effects of HGF inhibition on DCs were decreased in the production of type I interferon, IL-6 as well as CXCL9 giving as a result a lesser expansion of IFN-γ-producing T cells **(Fig. 2.5 to 2.7)**. On the other hand, there was also a decrease in the production of anti-inflammatory cytokines (IL-10 and TGF-β) (**Fig. 2.8**). These findings are consistent with clinical observations, in which the up-regulation

of interferon-inducible genes correlates with less viral clearance or therapy success in patients with HCV [111]. Furthermore, a recent study done during LCMV chronic infection demonstrated that ablation of type I interferon resulted in more effective viral clearance [109, 110]. Our studies showed that reduction of type I interferon and IFN-γ can be also beneficial in liver inflammation because this also brings down the concentration of anti-inflammatory and pro-fribrotic cytokines.

HGF shaped the microenvironment in a way that T cells were not fully activated. But at the same time, T cells were not fully inhibited. Hepatic T cells collected from the HGF-treated mice displayed an intermediate state of activation, $CD44^{hi}CD62L^{hi}$, rather than just $CD44^{hi}$ phenotype (**Fig. 2.8**). Moreover, in the presence of HGF, T cells did not display markers of T cell inhibition, giving as a result an enhancement of viral clearance in this group (**Fig. 2.9**). Furthermore, the downstream effect of this HGF-mediated immunomodulation was a delay in the activation of intrinsic inhibitory mechanisms in the liver. These inhibitory mechanisms consist of the expression of PD-L1 when IFN-γ and TNF-α were present in the environment [107] (**Diagram 3.2**).

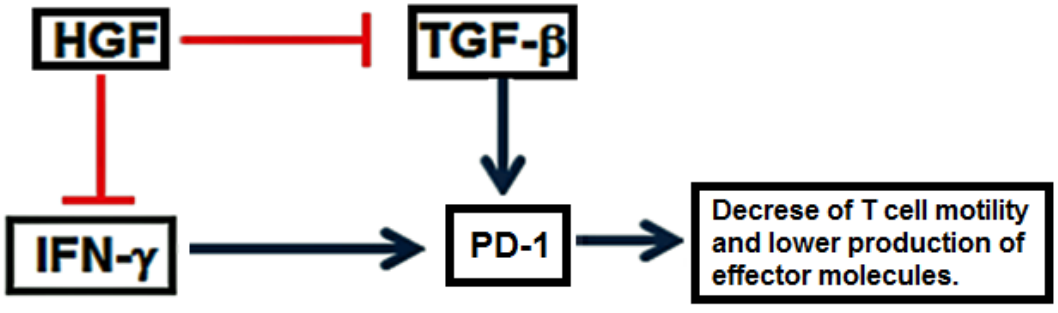

Diagram 3.2 Scheme of the HGF effects during Ad-induced hepatitis. HGF inhibits the production of TGF-β and IFN-γ (red lines). TGF-β and IFN-γ promote the expression of PD-1 (blue arrows), a major player in T cell inhibition.

Before proposing the use of HGF in the treatment of viral hepatitis, it is important to list the possible limitations this molecule may have when used in human subjects:

1) HGF is a mitogen and has been associated with cancer progression [264]; 2) HGF has a role in the expansion of lymphoid progenitors and its use could initiate leukemoid reactions in infected patients [192].

The possible carcinogenic role of HGF has been examined and found to be complex in the context of adenocarcinomas that have already developed into non-differentiated tumors. Thus, the pro-angiogenic properties of HGF are believed to be deleterious in this context [265]. However, an independent study did not find an increase in the tumor size and lung metastasis, when exogenous HGF was given to experimental animals [266]. Studies in the field of hepatocellular carcinoma (HCC) are also not definitive. Of note is that individuals chronically infected with HCV are at higher risk of developing HCC compared to the general population. Indeed, c-Met inhibitors proved to be helpful in HCC treatment [267]. On the other hand, treatment of an HCC cell line with HGF inhibited growth [268]. This is consistent with the fact that sustained growth signals in cancer cells activate apoptosis or cell senescence [269]. Hence, the future role of HGF as a therapeutic tool should be established after its exact effect on carcinogenesis is defined. Perhaps personalized treatment of individuals with a higher risk of HCC would be useful to avoid this possible deleterious effect of HGF.

To sum up, in this dissertation work, we evaluated the effect of IL-33 and HGF in the immune response to hepatotropic viruses. These two molecules are secreted and

synthesized in the liver after viral infection. A few previous studies examined their effects on the immune recognition of hepatotropic viruses. IL-33 and HGF conferred organ protection in viral hepatitis through immune-mediated mechanisms. However, their effects were not simply immunosuppressive and did not inhibit the immune response. On the other hand, our findings help us to understand that exacerbated immune activation can be deleterious to viral clearance and maintenance of liver function. Future studies using models of chronic infection would help to define the role and mechanisms of these two molecules in the immune response to viruses. Our current data suggest that IL-33 and HGF are promising candidates in the treatment of acute viral hepatitis.

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VITA

Renán Antonio Aguilar Valenzuela was born in Rosario, State of Sinaloa in Mexico on July 15th 1983. He graduated in 2008 from the medical school at the *Universidad de Monterrey* (University of Monterrey in Monterrey, NL, Mexico) with honors (*cum laude*). After graduating, the same year he joined the Microbiology and Immunology Ph.D. program at The University of Texas Medical Branch. During his early doctoral training Renán conducted clinical studies in the field of autoimmunity with the objective of finding biomarkers in patients with Lupus Erythematosus and Antiphospholipid Syndrome (APS). The results from these studies were presented at the 10th International Congress on Lupus in Vancouver, Canada and received an award as the best abstract presented in the mentioned meeting. Additionally, Renán also conducted experimental studies *in vivo* in which he explored the molecular bases of thrombosis in APS. At The International Congress on Antiphospholipid Antibodies 2010, Renán received the Young Investigator award for his contributions to the understanding of APS pathogenesis. In 2011, Renán conducted studies in the pathogenesis and immune regulation during viral hepatitis. His studies on the effects of HGF in adenovirus-induced hepatitis were recognized with a travel award to attend the American Society of Virology annual meeting 2012 in Madison, Wisconsin. After the completion of his Ph.D., Renán will pursue a medical residency in Internal Medicine and orient his research career toward development of translational medicine.

This dissertation was typed by Renán A. Aguilar Valenzuela.

EDUCATION

Degree	Institution	Field of Study	Years
M.D.	*Universidad de Monterrey*, School of Medicine, Monterrey, Nuevo León, México.	Academic curricula	2001-2008
Internship	Hospital General de Zona No. 33, IMSS, Monterrey, México.	Clinical training	2006-2007

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