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## Pathogenesis of Chronic Viral Hepatitis: Differential Roles of T cells and NK cells

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### Abstract

Chronic hepatitis B (HBV) and hepatitis C virus (HCV) infections account for 57% of cases of liver cirrhosis and 78% of cases of primary liver cancer worldwide and cause a million deaths per year. Although HBV and HCV differ in their genome structures, replication strategies and life cycles, they have common features, including their noncytopathic nature and the capacity to induce chronic liver disease, which is thought to be immune-mediated. However, the rate of disease progression from chronic hepatitis to cirrhosis varies greatly among infected individuals, and the factors that regulate it are largely unknown. This review summarizes our current understanding of the roles of antigen-specific and nonspecific immune cells in the pathogenesis of chronic hepatitis B and C, and discusses recent findings that identify natural killer cells as regulators of T cell function and liver inflammation.

### Introduction

HBV and HCV are both parenterally transmitted, enveloped viruses that exist as multiple genotypes. Despite different replication strategies and life cycles, both viruses have developed highly successful ways to establish chronic hepatitis (reviewed in <sup>1–3</sup>), resulting in 350 million chronic HBV infections and in 130–170 million chronic HCV infections worldwide. About one million people die from the sequelae of chronic HBV and HCV infections each year, mostly due to end-stage liver disease and liver cancer.

Disease progression to cirrhosis is nonlinear, and large fluctuations in viral titer and disease activity are particularly observed in HBV-infected patients over time (Box 1). Whereas the viral titer is generally more consistent and the disease activity lower in chronic HCV than HBV infection, there is still significant variation in both parameters among HCV-infected patients. Liver injury and disease progression are thought to be driven by host immune responses in both infections <sup>1–3</sup>, and previous research has mostly focused on the role of virus-specific T cells in this process. Virus-specific CD8 T cells are recognized as the main effector cells and their experimental depletion delays the clearance of acute HBV <sup>4</sup> and HCV <sup>5</sup> infection in chimpanzees. Work over the past 5 years revealed multiple, nonredundant mechanisms that drive attenuation and exhaustion of HBV- and HCV-specific

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T cells in chronically infected patients<sup>6–11</sup>. The few virus-specific T cells that remain tend to target sequences in which the virus has mutated and, thus, cannot eliminate infected target cells<sup>12–16</sup>. Based on these observations, it is plausible that most of the immune-mediated liver injury in chronic HBV and HCV infection is mediated by immune cells other than virus-specific T cells.

Here, we review virus and host mechanisms that downregulate effector functions of antigen-specific T cells in the liver and examine the role of antigen-nonspecific inflammatory cells, in particular natural killer (NK) cells in the pathogenesis of chronic viral hepatitis and regulation of inflammation.

## Downregulation of virus-specific T cell responses in chronic hepatitis

Strong CD4 helper and CD8 effector T cell responses have been observed in patients<sup>17–19</sup> and experimentally infected chimpanzees<sup>4,5,20</sup> that clear HBV and HCV infection. In HCV infection, protective immunity is more likely in the presence of HLA-B27, HLA-B57 and HLA-A3 alleles. Protective T cell responses tend to target epitopes that do not allow escape mutations because of high costs to viral replicative fitness<sup>21–23</sup>

Whereas neutralizing antibodies are an important component of protective immunity against HBV<sup>19</sup>, neutralizing antibodies are not required to clear HCV as demonstrated in hypogammaglobulinemic patients<sup>24</sup> and their titer decreases to undetectable levels long-term after HCV clearance<sup>25</sup>. Broad neutralizing antibody responses only appear after chronic HCV infection is established - they are unable to clear the infection at this stage and rather select viral escape mutants<sup>26</sup>.

While the immunological mechanisms for viral persistence differ for HBV and HCV (Box 2) a common feature is the downregulation of virus-specific T cell responses in the chronic phase, which is marked by progressive functional exhaustion and ultimately deletion of virus-specific CD4 and CD8 T cells<sup>8,9</sup>. In addition, an inflammation-induced increase in regulatory T cells<sup>27–29</sup>, a decrease in intrahepatic arginine levels<sup>30</sup> and a shift in the ratio between T cell-sustaining cytokines such as IL-2<sup>31,32</sup> and suppressive cytokines such as IL-10 and TGF- $\beta$ <sup>33–35</sup> dampen the virus-specific T cell response of infected patients. Lastly, viral mutations in dominant T cell epitopes that have been selected as a means to establish chronic infection render the remaining T cell responses irrelevant (Box 2). These viral escape mutations have been described in both HBV-<sup>12</sup> and HCV-infected patients<sup>14–16,36</sup> but are more frequent in HCV infection as a result of the high replicative fitness of HCV paired with the high error rate of its polymerase.

Prolonged exposure to viral antigens is the main cause for the reduced frequency and impaired effector function of virus-specific CD8 T cells<sup>37,38</sup> as first revealed in mice infected with lymphocytic choriomeningitis virus (LCMV). LCMV is a noncytopathic arenavirus that is widely used as a model to study the pathogenesis of viral hepatitis given that HBV and HCV do not infect rodents. Consistent with findings in the LCMV model, CD8 T cells that are not stimulated anymore due to viral escape mutations do not display an exhausted phenotype in HCV-infected patients<sup>39</sup>. In HBV infection, chronic T cell stimulation may be caused by both viral peptides presented on the MHC of infected cells

and by viral antigens such as soluble HBsAg and HBeAg in HBV infection that can access the MHC class I pathway for crosspresentation<sup>40</sup>. This is supported by a recent translational study that showed a smaller gain in HBV-specific T cell function in patients who turned HBV DNA-negative but remained HBsAg-positive upon treatment than in patients who turned negative for both HBV DNA and HBsAg<sup>41</sup>.

The downregulation of T cell responses follows a similar pattern in patients with HBV and HCV infection and in LCMV-infected mice. In all three infections, virus-specific T cells display increased levels of inhibitory molecules such as programmed death-1 (PD-1)<sup>6,8,10,42-44</sup>, the cytotoxic T-lymphocyte antigen 4 (CTLA-4)<sup>7,10,43</sup>, the T cell immunoglobulin and mucin domain-containing molecule 3 (Tim-3)<sup>1145</sup> and 2B4 (CD244)<sup>7,43,46-48</sup>, and the corresponding ligands are upregulated in the inflamed liver. The PD-1 ligand PD-L1, for example, is upregulated on human hepatocytes in an IFN- $\alpha$ - and IFN- $\gamma$ -dependent manner<sup>49</sup>, in addition to its constitutive expression on intrahepatic liver sinusoidal endothelial cells (LSEC), Kupffer cells and stellate cells as demonstrated in mouse models<sup>50,51</sup>. Thus, when fully functional HBV-specific CD8<sup>+</sup> T cells are transferred into transgenic mice that express HBsAg or replicate HBV in hepatocytes, they upregulate PD-1 upon recognition of their cognate antigen and rapidly lose their capacity to produce IFN- $\gamma$ <sup>52</sup>. Consistent with these findings, HBV and HCV-infected patients have a more severely impaired CD8 T cell phenotype in the liver than in the blood<sup>10,53</sup>.

Overall, T cell exhaustion follows a predictable pattern as first shown in mice with LCMV hepatitis (reviewed in<sup>54</sup>). T cells that undergo exhaustion in this model first lose their capacity to produce IL-2, a cytokine that supports proliferation. IL-2 is predominantly produced by CD4 T cells whereas CD8 T cells produce little IL-2 themselves and depend on CD4 T cell help. This is followed by sequential loss of cytotoxicity, TNF- $\alpha$  and IFN- $\gamma$  production. In addition, the intracellular expression of pro-apoptotic genes such as Bcl2-interacting mediator (Bim) increases in virus-specific CD8 T cells of LCMV-infected mice<sup>55</sup> as well as in those of HBV- and HCV-infected patients<sup>56,57</sup>.

From a therapeutic angle, the blockade of these inhibitory pathways along with selective stimulation of costimulatory pathways is being pursued both *in vitro* and *in vivo* as a means to rescue exhausted T cells and to restore their function. The most effective strategy depends on the etiology of viral hepatitis. For example, PD-L1 blockade combined with CD137 costimulation has been shown to reverse the exhaustion of virus-specific CD8 T cells from patients with chronic HBV infection *in vitro*<sup>58</sup>. CD137 costimulation results in downregulation of Bim-expression and upregulation of Bcl-2 family members, thus supporting survival of activated virus-specific T cells<sup>58</sup>. However, the same strategy does not reverse the dysfunction of virus-specific T cells in chronic HCV infection even though they appear to be less impaired in their capacity to produce IFN- $\gamma$  and IL-2 than HBV-specific T cells<sup>58</sup>. Rather, functional recovery of HCV-specific T cells requires a combined CTLA-4 and PD-1/PD-L1 blockade<sup>43,59</sup>. Finally, PD-1/PD-L1 blockade alone is sufficient to rescue virus-specific T cells in LCMV-infected mice<sup>44</sup>. Thus, the therapeutic blockade of the inhibitory pathways needs to be tailored to the specific viral infection.

In addition, there appears to be a general antigen-nonspecific suppression of the effector phase of the intrahepatic immune response. Decreased arginine levels may contribute to this dysfunction, because arginine deprivation triggers TCR-CD3 $\zeta$  downregulation, impaired T cell proliferation and reduced IL-2 production as shown in HBV-infected patients<sup>30</sup>. Accordingly, addition of arginine rescues these T cell responses *in vitro*<sup>30</sup>. Second, an increased frequency of CD4 T cells with regulatory function (Foxp3<sup>+</sup> Tregs) has been observed in the blood and in the liver of HBV-<sup>27,28</sup> and HCV-infected patients<sup>29</sup>. Tregs appear to proliferate in viral hepatitis because they display fewer T cell receptor excision circles in chimpanzees with chronic HCV infection than in HCV-naïve chimpanzees<sup>60</sup>.

Finally, an increased level of the immunosuppressive cytokine IL-10 has been observed in both blood and liver of HBV-<sup>61</sup> and HCV-infected patients<sup>34</sup>. IL-10 is produced by regulatory T and B cells<sup>33</sup>, monocytes<sup>62</sup> and intrahepatic T cells<sup>34</sup> as shown in HBV- and HCV-infected patients and by human Kupffer cells<sup>63</sup>. IL-10 inhibits IFN- $\alpha$  production<sup>62</sup> and promotes apoptosis of human plasmacytoid dendritic cells<sup>64</sup>. It also inhibits antigen-presenting cells and attenuates the induction of antigen-specific CD8 T cells in humans<sup>65</sup>. While the frequency of dendritic cells has generally been reported to be reduced in the blood and increased in the liver in patients with chronic HBV and HCV infection, there is no consensus on their functionality (reviewed in<sup>66</sup>). The lack of generalized immune suppression in patients with established chronic HCV infection argues for an antigen-specific immune suppression. Consistent with this notion, T cell responses against new HCV epitopes but not against unrelated immunogens are rarely detected in patients with chronic hepatitis C<sup>67</sup>, and attempts to induce hepatitis virus-specific immune responses via vaccination have failed not only for chronic hepatitis C but also for chronic hepatitis B.

Collectively, these findings demonstrate that virus-specific T cell responses that are qualitatively or quantitatively insufficient to clear the virus in the acute phase of hepatitis B and C are downregulated in frequency and function in the chronic phase. Indeed, once chronic hepatitis is established, the antiviral effect of these remaining virus-specific CD8 T cells is negligible and they rarely select HCV escape mutations in the chimpanzee model<sup>68</sup>. Accordingly, experimental *in vivo* depletion of CD8 T cells affects neither ALT levels nor viremia in chronically HCV-infected chimpanzees (C. Walker, personal communication) whereas the absence of CD8 T cells in acute HCV infection reduces liver injury and prevents viral clearance<sup>5</sup>. Thus, the benefit of downregulating virus-specific CD8 T cell responses in the chronic phase of hepatitis may lie in the prevention of immunopathology, which is amplified by secondarily recruited mononuclear cells (Box 3).

In contrast to the detailed knowledge on CD8 T cell responses in chronic hepatitis, much less is known about the downregulation of CD4 T cell responses. Although antigen persistence has been implicated in causing CD4 T cell dysfunction, CD4 T cell function is only partially reversible by PD-1/PD-L1 blockade or by antigen removal in mouse models<sup>69,70</sup>. Furthermore, CD4 T cell-driven immune escape is not a significant factor in the chimpanzee model of HCV infection<sup>71</sup>. Recent studies have now identified a role of NK cells in the direct regulation of CD4 and indirect regulation of CD8 T cell responses in viral hepatitis as described below. This regulatory activity of NK cells against CD4 T cells in particular may account for the antigen-specific nature of defective T cell immunity in viral

hepatitis – which to date, has been difficult to explain mechanistically because hepatitis viruses such as HBV, HCV and LCMV do not infect T cells.

## NK cell function in chronic viral hepatitis

NK cells account for the majority of innate immune cells in the healthy human liver<sup>72</sup> and their frequency increases further in the liver and decreases in the blood in chronic HBV and HCV infection<sup>73–75</sup>. NK cells depend on chemokines from Kupffer cells for recruitment and on cytokines from Kupffer cells, LSEC and T cells for survival (reviewed in<sup>76</sup>). In particular, LSEC-derived CXCL16 is of interest because CXCL16 is recognized by NK cells with antigen-specific memory function<sup>77,78</sup>. Mechanisms of NK cell activation include cytokines such as type I IFN, IL-8, IL-12, IL-15 and IL-18, a relative reduction of signals from inhibitory receptors, such as reduced MHC expression on virus-infected cells, or an increase in signals from activating receptors, such as recognition of antibody-coated viral antigens and/or stress-induced ligands on infected cells (reviewed in<sup>76</sup>).

Peripheral blood NK cells from HBV-infected individuals express higher levels of the activating receptors NKp30, NKp46 and NKG2C<sup>79</sup> and lower levels of the inhibitory marker NKG2A<sup>73,79</sup> than those from HCV-infected patients, but NK cells from HCV-infected patients also express higher levels of several activating receptors, such as NKp30<sup>80</sup>, NKp44<sup>81</sup>, NKp46<sup>79,81,82</sup>, NKG2C<sup>81</sup>, NKG2D<sup>74</sup> and CD122<sup>81</sup>, and one inhibitory receptor, NKG2A<sup>81</sup> than those from healthy controls. The integration of signals from these and other receptors results in NK cell activation in both HBV and HCV infection, as evidenced by the expression of CD69<sup>74,81</sup>, an inducible cell surface glycoprotein acquired during lymphoid activation. Intrahepatic NK cells are more activated than their counterparts in the peripheral blood<sup>74,80,81</sup>.

Unexpectedly, NK cell activation does not induce all effector functions to an equal degree. Whereas correlates of cytotoxicity such as degranulation and TRAIL expression are increased in chronic HBV and HCV infection<sup>74,81</sup>, IFN- $\gamma$  and TNF- $\alpha$  production is suppressed<sup>61,74,75,81,83</sup>. This divergent functional phenotype has been attributed to chronic exposure to cytokines. In HBV infection, the selective defect in NK cell function appears to be induced by IL-10 and TGF- $\beta$  and can be restored upon *in vitro* blockade of these cytokines<sup>61</sup>. While IL-10 is also present in the HCV-infected liver<sup>34</sup>, an additional causative factor appears to be IFN- $\alpha$ <sup>81</sup>. IFN- $\alpha$  can be produced by human plasmacytoid dendritic cells<sup>84</sup> and Kupffer cells<sup>85</sup> that sense HCV RNA, whereas both IFN- $\alpha/\beta$  induction and IFN- $\alpha/\beta$  receptor signaling are attenuated by HCV in infected hepatocytes (reviewed in<sup>2</sup>). Chronic exposure of NK cells to endogenous IFN- $\alpha$  results in increased STAT1 levels and preferential STAT1 over STAT4 phosphorylation, as first described for NK cells from LCMV-infected mice<sup>86,87</sup>, and confirmed for NK cells from HCV-infected patients<sup>88,89,90</sup>. This concomitant increase in pSTAT1-dependent cytotoxicity and decrease in pSTAT4-dependent IFN- $\gamma$  production of NK cells<sup>81,88</sup> (Fig. 3) can be reproduced *in vitro* when primary NK cells of healthy uninfected blood donors are stimulated with IFN- $\alpha$ <sup>81</sup> and is further enhanced when patients with chronic HCV infection undergo IFN- $\alpha$ -based therapy<sup>88,90</sup>. Within hours after therapy initiation, NK cell degranulation and TRAIL production peak, concomitant with a slight rise in serum ALT levels, whereas IFN- $\gamma$

production decreases<sup>88,90</sup>. These events correlate with induction of pSTAT1 and reduction in HCV titer during the first 48 hours of therapy, which mark the first phase virological response<sup>88</sup>. Thus, NK cells from individuals with rapid first phase HCV RNA decline display maximal pSTAT1 induction *in vivo* and are refractory to further IFN- $\alpha$  stimulation *in vitro*. In contrast, NK cells from individuals with slow first phase HCV RNA decline exhibit significantly lower pSTAT1 levels and retain their *in vitro* responsiveness to IFN- $\alpha$ <sup>88</sup>. This is consistent with differential NK cell phenotype and function in treatment responders and nonresponders at later time points<sup>90,91</sup>. Specifically, treatment responders exhibit greater levels of NK cell degranulation than nonresponders for at least the first 12 weeks of IFN-based therapy<sup>88</sup>, the period that defines an early virological response. Patients with a sustained virological response also display enhanced NK cytotoxicity<sup>92</sup>. Thus, NK cell responses can be used as an indicator of a patient's IFN responsiveness, which along with the expression level of ISGs<sup>93-95</sup> and *IFNL* SNPs<sup>96-99</sup> predict the treatment response. Specifically, high pretreatment ISG levels combined with low inducibility during treatment predict a poor virological response, whereas low pretreatment ISG levels combined with strong inducibility during treatment predict treatment success<sup>93-95</sup>. Likewise, high pretreatment levels of the inhibitory NKG2A and KIR3DL1 receptors on NK cells predict treatment failure<sup>100</sup>. Such NKG2A+ NK cells co-express higher levels of the activation marker NKp44 pretreatment than NKG2A- NK cells and produce less CXCL10 and TRAIL upon *in vitro* IFN- $\alpha$  stimulation<sup>100</sup>. Thus, the responsiveness of NK cells to interferon-based therapy parallels the virological response and recapitulates what has been reported for ISGs<sup>93,95</sup>.

### Antiviral versus regulatory role of NK cells in viral hepatitis

The decreased ability to produce IFN- $\gamma$  likely reduces the antiviral function of NK cells because both HBV and HCV are sensitive to this cytokine. For HBV, important information on the antiviral role of IFN- $\gamma$  has been forthcoming from mouse models where IFN- $\gamma$  from adoptively transferred HBV-specific CD8 T cells<sup>101</sup> and from IL-12-activated NK cells downregulates HBV replication<sup>102</sup>. The molecular mechanisms of this antiviral effect have been identified in the same model<sup>103,104</sup>. For HCV, the antiviral effect of IFN- $\gamma$  has been demonstrated in hepatoma cell lines that harbor subgenomic HCV replicons<sup>105</sup>. This concept is supported by the observation that the initial decrease in HBV and HCV titer in infected patients and nonhuman primates coincides with the increase of IFN- $\gamma$  and CD8 mRNA in the liver<sup>106,107</sup> and with the appearance of IFN- $\gamma$  secreting virus-specific T cells in both blood and liver<sup>108-110</sup>. HBV titers typically decline prior to maximal ALT elevation in acute hepatitis<sup>106,108,109</sup>, which supports the notion of cytokine-mediated antiviral effects by CD8 T cells. However, HBV titers do not decrease at earlier time points when the frequency of NK cells increases in the blood<sup>111</sup>. In fact, NK cell activation and IFN- $\gamma$  production are reduced during peak viremia in prospectively studied patients with acute hepatitis B<sup>112</sup>. Thus, NK cells, although activated, appear to be unable to clear the infection on their own.

Whether this is a general feature of human NK cells or the result of viral escape is not clear at this time. The effect of HBV on NK cells has not been examined, and the effect of HCV has only been analyzed *in vitro* with tissue-culture derived virus. In these studies human NK

cells maintain their function if exposed to infectious HCV <sup>113</sup>, unless the HCV virions are coated on tissue culture plates, which increases their ability to crosslink CD81 <sup>114</sup>. Further *in vitro* studies demonstrated that HCV NS5A-induced IL-10 and TGF- $\beta$  <sup>115</sup> and direct interaction between human NK cells and HCV-infected hepatoma cells <sup>116</sup> downregulate NK cell NKG2D expression and effector function. If these mechanisms extend to the interaction between NK cells and infected hepatocytes in the liver NK cell-mediated elimination of virus-infected hepatocytes may be impaired. However, the sole study comparing NK cells in livers of HCV-infected patients and healthy controls reported that a potential inhibitory effect of the virus on intrahepatic NK cells must be transient rather than pervasive because these intrahepatic NK cells maintained their cytotoxic response to *in vitro* stimulation with inflammatory cytokines such as IFN- $\alpha$  <sup>80</sup>.

If NK cells fail to clear the virus why is there no overall downregulation of NK cell cytotoxicity in both blood and liver as reported for T cells? Recent studies in the LCMV model suggest that NK cell cytotoxicity fulfills an important regulatory role, namely the control of the inflammatory cascade that virus-specific CD4 and CD8 T cells initiate as well as the fibrogenesis that stellate cells drive. As in HBV and HCV infection, NK cells do not appear to have a direct antiviral effect in LCMV infection <sup>117</sup>, yet maintain their cytotoxic effector function (reviewed in <sup>118</sup>). In a seminal report, NK cell cytotoxicity was shown to be beneficial to LCMV and the host after high dose LCMV infection, because NK cell-mediated killing of activated CD4 T cells eliminates help for CD8 T cells (Fig. 3)<sup>119</sup>, which results in CD8 T cell exhaustion, allowing LCMV to persist and the host to survive with minimal immunopathology (Fig. 4A). CD4 T cells are predominantly targeted, because CD8 T cells express higher levels of CD48, which engages the inhibitory receptor CD244 [2B4] on NK cells (Fig. 3) <sup>120</sup>. There are also conditions such as medium-dose LCMV infection, in which NK cell cytotoxicity is not beneficial because this viral dose does not drive CD8 T cell exhaustion when NK cells eliminate CD4 T cell help, resulting in viral persistence and extensive immunopathology (Fig. 4A). In this condition, the virus-specific CD8 T cell response is stronger in the absence than presence of NK cells, and clears LCMV prior to the development of enhanced immunopathology by secondarily recruited mononuclear cells (Fig. 4A). Finally, at low dose LCMV infection, NK cells do not affect viral persistence and immunopathology because the virus is cleared in the presence or absence of NK cells. Thus it has been suggested that NK cells function as “rheostats” for virus-specific T cell responses <sup>119</sup> and that therapeutic targeting of NK cells may have differential effects based on the degree of virus-driven T cell exhaustion (Fig. 4B).

NK cells have also been shown to indirectly regulate CD8 T cell function in the LCMV model through effects on antigen presenting cells <sup>121</sup>. While the specific mechanism of this pathway is still unclear it does not appear to be an antiviral effect because the initial level of viremia, the number of antigen-presenting cells and their expression of costimulatory or inhibitory molecules are not affected. Rather, via an unknown mechanism, NK cells limit the ability of antigen presenting cells to stimulate CD8 T cell proliferation <sup>121</sup>. In addition to the indirect regulation of CD8 T cell function, NK cells can directly lyse in a perforin-mediated manner CD8 T cells that upregulate NKG2D ligands in LCMV-infected

mice <sup>122,123</sup> (Fig. 3). Consequently, NK cell depletion or NKG2D blockade increases the frequency of functional antigen-specific CD8 T cells in this model <sup>122</sup>.

The NK cell-mediated regulation of virus-specific T cell responses is supported by recent translational studies in HBV-infected patients, where *in vitro* depletion of NK cells from PBMC of HBV-infected patients increased HBV- but not CMV-specific CD8 T cell responses <sup>124</sup>. The specificity of the NK cell response was attributed to a higher proportion of HBV-specific than CMV-specific CD8 T cells expressing TRAIL death receptor 2 (TRAIL-R2), which rendered them susceptible to apoptosis by TRAIL-expressing NK cells. Increased TRAIL-R2 expression was specifically observed on intrahepatic HBV-specific CD8 T cells in correlation to HBV titer and in close contact to TRAIL-expressing NK cells <sup>124</sup>. This regulatory role of NK cells may be relevant during HBV flares. HBV-infected patients who develop flares typically develop increased HBV viremia and HBV antigen levels, followed by increased serum IFN- $\alpha$ , IL-8 <sup>31</sup> and ALT levels. IFN- $\alpha$  can contribute to the observed increased TRAIL expression by NK cells during HBV flares <sup>31</sup>, and the concomitant increase in IL-8 levels can promote the recruitment of activated NK cells to the liver and the induction of TRAIL-R2 on intrahepatic T cells (Fig. 3). Thus, NK cells may contribute the control of enhanced intrahepatic CD8 T cell responses during the flare because flare-associated increases in inflammatory cytokines, such as IL-12 by activated Kupffer cells and IL-15 by LSEC render human T cells more susceptible to TCR-mediated signals, and enhances their effector functions <sup>125</sup>.

Finally, NK cell-mediated cytotoxicity may also contribute to the control of stellate cells, which drive liver fibrosis. Stellate cells are located in the space of Dissé in close contact with NK cells (Fig. 2), store most of the body's vitamin A and are the primary source of type I and III collagen (reviewed in <sup>126</sup>). Collagen production requires stellate cell activation, which is triggered by cytokines, reactive oxygen species, phagocytosis and TLR-9-mediated recognition of DNA from apoptotic hepatocytes (reviewed in <sup>127</sup>). Upon activation, stellate cells differentiate into myelofibroblastic cells and convert vitamin A into retinoic acid, which induces the activating NK cell ligand RAE1 in mouse models of liver fibrosis (Fig. 3)<sup>128</sup>. Activated mouse stellate cells downregulate the inhibitory NK cell ligand MHC-class I <sup>129</sup> and upregulate TRAIL receptors <sup>130</sup>, which converts them into targets for NKG2D-, TRAIL- and granzyme-mediated killing by NK cells <sup>128,129</sup>. NKG2D and TRAIL-mediated killing of stellate cells has also been shown for NK cells from HCV-infected patients <sup>131</sup> and NKp46+ NK cells have been shown to accumulate in the liver of HCV-infected patients and to lyse human stellate cells *in vitro* (Fig. 3)<sup>132</sup>. Accordingly, the absence of either NK cells <sup>128,129</sup> or the murine orthologue of the NKp46 receptor <sup>133</sup> enhances liver fibrosis in mouse models. Collectively, these studies suggest that preservation of NK cell cytotoxicity serves a nonclassical regulatory rather than a classical antiviral role in viral hepatitis.

## Conclusion

CD4 and CD8 T cell responses play a unique part in HBV and HCV infection because they contribute not only to viral clearance and protective immunity in those who recover from infection but also to significant liver injury. If the viruses cannot be cleared and chronic



infection ensues, responses by virus-specific T cells and by secondarily recruited mononuclear cells need to be tightly controlled. The induction of inhibitory molecules such as PD-1, CTLA-4, TIM-3 on T cells and their ligands in the liver, the production of immunosuppressive cytokines such as IL-10 and TGF- $\beta$  and the recently discovered regulatory role of NK cells allow the host to counteract liver inflammation and disease progression. Importantly, these mechanisms target virus-specific immune responses and thus, do not result in global immune suppression. These findings have therapeutic implications because all treatment strategies to date are aimed at elimination of the virus, either via antiviral drugs, which is still problematic for chronic HBV infection, or enhancement of antiviral immune responses, which has the potential of inducing significant immunopathology. A better understanding of the endogenous mechanisms that regulate liver inflammation and disease pathogenesis may help us to determine why liver disease progresses more rapidly in some patients than others. At the same time it may result in new therapeutic treatment options to decrease the rate of disease progression in viral infections that cannot be cleared with antiviral agents.

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### Box 1 Natural history of chronic hepatitis

#### Chronic HBV infection

Chronic HBV infection results mostly from vertical transmission from mother to neonate. HBV-infected neonates and children typically experience an immunotolerant phase with normal alanine aminotransferase levels despite high levels of circulating HBV DNA and HBe antigen, the secreted form of the HBV core antigen. This phase may last for decades and eventually transitions into an immunoactive phase with more severe liver disease and fluctuations in HBV titer (Fig. 1A). Whereas an extended immunoactive phase is associated with rapid disease progression and liver cirrhosis, it may also convert into a low replicative phase with reduced inflammation, low HBV titers less than 2,000 IU/ml, and conversion to HBeAg- / anti-HBe+ status. A subset of patients later develops recurrent necroinflammatory liver disease with high level replication of either wildtype HBeAg+ HBV or mutant HBeAg- HBV (reviewed in <sup>3,140</sup>). Increased age, male gender, alcohol consumption and HIV coinfection increase the risk for an adverse outcome of HBV infection.

Even though HBV infection can be prevented by a vaccine there is still no curative treatment for most who are already chronically infected. Cure is defined as clearance of HBV surface antigen (HBsAg) and seroconversion to anti-HBs, which occurs spontaneously in about 1% of chronically infected patients per year. Interferon (IFN)-based therapies achieve this goal in less than 10% of treated patients (reviewed in <sup>141</sup>). Therapy with nucleos(t)ide analogues decreases the HBV titer but results in viral resistance <sup>141</sup> because it does not eliminate covalently closed circular HBV DNA, the transcriptional template of HBV.

In contrast to vertical HBV transmission during the neonatal period, horizontal transmission during adulthood causes acute hepatitis, which is resolved by more than 95% of infected adults and results in lifelong T cell- and antibody-mediated immunity. The small percentage of individuals who progress to chronic infection typically do not experience a long immunotolerant phase and enter the immunoactive phase of chronic hepatitis sooner than after vertical transmission.

#### Chronic HCV infection

Chronic HCV infection is typically acquired during adulthood and about 70–85% of infected persons develop viral persistence. Chronic hepatitis is characterized by relatively mild liver inflammation without significant fluctuations in HCV RNA titers and disease progression is accelerated with increased age, obesity, alcohol consumption and HIV coinfection (Fig. 1B). IFN/ribavirin-based therapy is less effective in the chronic phase than in the acute phase of hepatitis, but the recently FDA-approved directly acting antivirals have significantly improved treatment responses <sup>142</sup>.

Both hepatitis B and C are also associated with extrahepatic disease manifestations, which can be mediated by virus-specific immune complex injury and include arthritis, vasculitis and glomerulonephritis. In addition, mono- and polyclonal B cell expansions

have been observed in chronic hepatitis C and can evolve into mixed cryoglobulinemia and B cell malignancies such as non-Hodgkin's lymphoma (reviewed in <sup>143</sup>).

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### Box 2 Virus and host factors that result in HBV and HCV persistence

As outlined in Box 1 most chronic HBV infections are caused by vertical transmission from mother to child either in utero or during early infancy. Based on studies in rodent models, immaturity of the neonatal immune system<sup>144</sup>, a tolerogenic effect of HBeAg in utero<sup>145</sup>, and more recently, decreased IL-21 production by CD4 follicular helper T cells<sup>146</sup> have been proposed to contribute to the high rate of HBV persistence but not yet confirmed in humans. In contrast to HBV most chronic HCV infections are caused by horizontal transmission between immunocompetent adults. A key factor in the development of chronic HCV infection are viral escape mutations, which are selected not just in individual patients<sup>14,16</sup> but in entire populations resulting in patterns (“footprints”) of mutations within or near virus-encoded T cell epitopes depending on the prevalent HLA haplotypes<sup>15,36</sup>. If HCV is transmitted to individuals that share the respective HLA alleles it does not stimulate T cell responses against the mutated epitopes, thereby increasing the likelihood of chronic infection. A second factor appears to be decreased help by virus-specific CD4 T cells, which has been reported not only for HCV<sup>147</sup> but also for HBV-infected patients<sup>148</sup> and is associated with reduced function<sup>18,149,150</sup> and breadth<sup>148</sup> of the virus-specific CD8 T cell response. Consistent with this finding, in vivo depletion of CD4 T cells from HCV-recovered chimpanzees abrogates protective CD8 T cell-mediated immunity upon HCV re-challenge<sup>20</sup>. Likewise, CD4 T cell-depleted chimpanzees develop persistent HBV infection when inoculated with a dose of HBV that is typically cleared<sup>151</sup>.

The role of innate immune responses in the outcome of acute HBV and HCV infection is less clear. HBV remains at low to undetectable levels in the circulation for weeks after infection and does not stimulate proinflammatory cytokines or interferon-induced genes during this period in patients<sup>152</sup> and experimentally infected chimpanzees<sup>153</sup>. Recent studies in mouse models where HBV replication is launched from a recombinant adenovirus demonstrated that NKT cell activation by HBV-induced self lipids contribute to adaptive immune responses<sup>154</sup> but it remains unknown whether the same occurs in patients.

For HCV, the outcome of infection has been linked to several SNPs within or near *IFNL* genes<sup>99,155</sup>, but the underlying immunological mechanisms are largely unknown. While NKT and NK cells are activated in the early phase of infection along with the induction of ISGs, HCV has developed sophisticated means to escape from innate immune responses (reviewed in<sup>2</sup>) and a notable decrease in HCV titer occurs only weeks later when IFN- $\gamma$  producing CD8 T cells appear in the liver<sup>110,156</sup>.

### Box 3 Contribution of secondarily recruited mononuclear cells to disease pathogenesis

Viral escape mutations and downregulation of T cell responses against conserved viral epitopes during chronic infection suggest that virus-specific T cells are not the major mediators of liver disease. Indeed, most of the necroinflammatory liver disease is due to secondarily recruited mononuclear cells (Fig. 2). This is supported by the observation that chronically HBV-infected patients with and without liver inflammation do not differ in the frequency of intrahepatic HBV-specific CD8 T cells, but in the size of the nonspecific mononuclear cell infiltrate<sup>157</sup>. Much has been learned from transgenic mice that express HBsAg or replicate the complete HBV genome in their hepatocytes and are intravenously injected with HBs-specific CD8 T cells (reviewed in<sup>1</sup>). The injected T cells travel via the portal vein into the liver sinusoids, where the slow blood flow and trapping by liver-resident macrophages (Kupffer cells) exposes them to liver sinusoidal endothelial cells (LSECs), and via fenestrae in the LSEC layer to hepatocytes. Recognition of their cognate antigen triggers cytotoxicity as well as IFN- $\gamma$  release<sup>101</sup>. IFN- $\gamma$  blocks the assembly of HBV nucleocapsids and destabilizes HBV RNA<sup>103,104</sup>. IFN- $\gamma$  also activates macrophages and Kupffer cells to produce TNF- $\alpha$ , more IFN- $\gamma$ <sup>101</sup> and, along with hepatocytes, stellate cells and LSEC the chemokines CXCL9, CXCL10 and CXCL11. Hepatocyte-derived chemokines are then transported via transcytosis from the basolateral to the luminal surface of the endothelium and expressed on LSEC (reviewed in<sup>158</sup>). Combined with CCL3, CCL5, CXCL1-3 and CXCL5, which are produced by the portal tract vascular endothelium, and IL-1 $\beta$ , IL-6, IL-8, TNF- $\alpha$  and other cytokines from hepatocytes and IFN- $\gamma$ -activated monocytes and macrophages, they recruit mononuclear cells (reviewed in<sup>159</sup>). Their entry into the liver parenchyma is facilitated by neutrophil-derived matrix metalloproteinases, which remodel the extracellular matrix<sup>137</sup>. Activated platelets, which are detectable in intrahepatic necroinflammatory foci as well as hepatic sinusoids, also contribute to chronic liver injury by facilitating lymphocyte entry<sup>135</sup>.

In the HBV mouse model, the recruitment of mononuclear cells can be prevented by depletion of neutrophils<sup>138</sup>, blocking of MMPs<sup>137</sup> or neutralization of chemokines<sup>136</sup>. All three measures reduce liver disease while maintaining the antiviral activity of HBV-specific CD8 T cells. The secondarily recruited mononuclear cells therefore contribute to the liver injury but not to viral clearance. Inflammatory liver injury is perpetuated into chronic disease and ultimately, hepatocellular carcinoma if a continued supply of HBs-specific CD8 T cells is provided<sup>139</sup>.

The results from this animal model are relevant for viral hepatitis in humans. For example, serum IFN- $\alpha$ , IL-8, CXCL9 and CXCL10 levels<sup>31,160,161</sup> rather than the frequency or effector functions of HBV-specific CD8 T cells<sup>160,162</sup> increase during flares of chronic hepatitis B suggesting that the increase in disease activity is due to an amplification of the intrahepatic lymphocyte infiltrate. Correlations between the serum levels of several chemokines and liver inflammation have also been observed in HCV-infected patients<sup>163,164</sup> and are consistent with chemokine-mediated recruitment of T

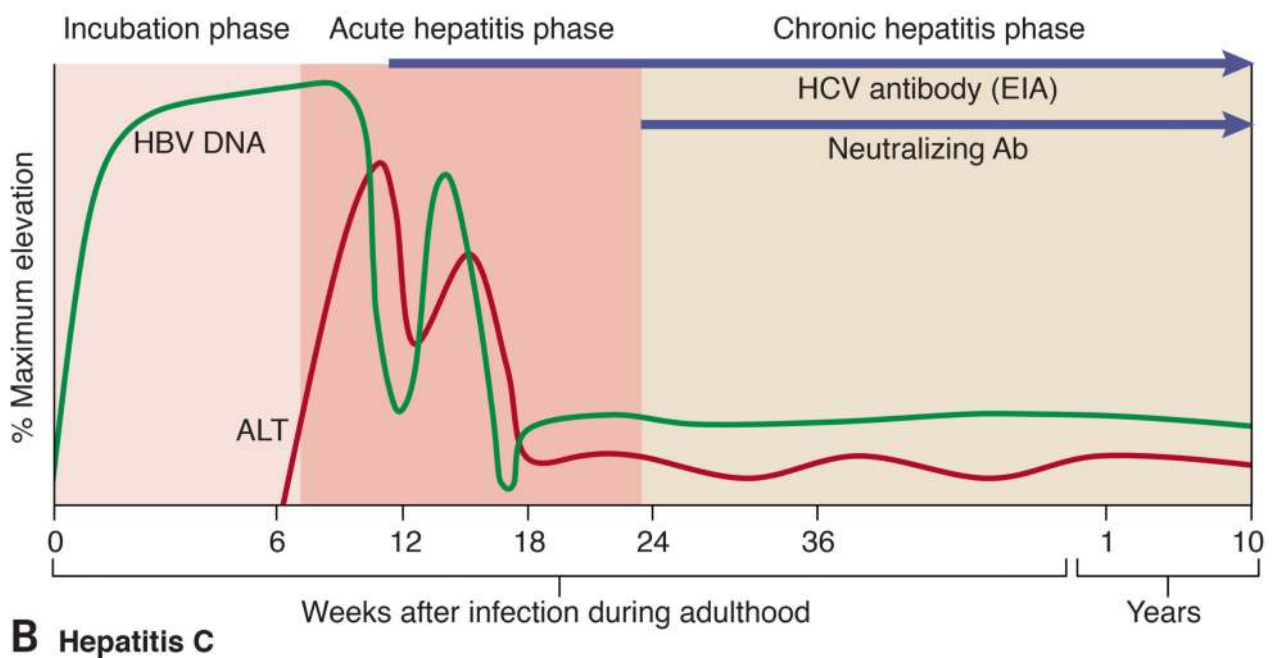
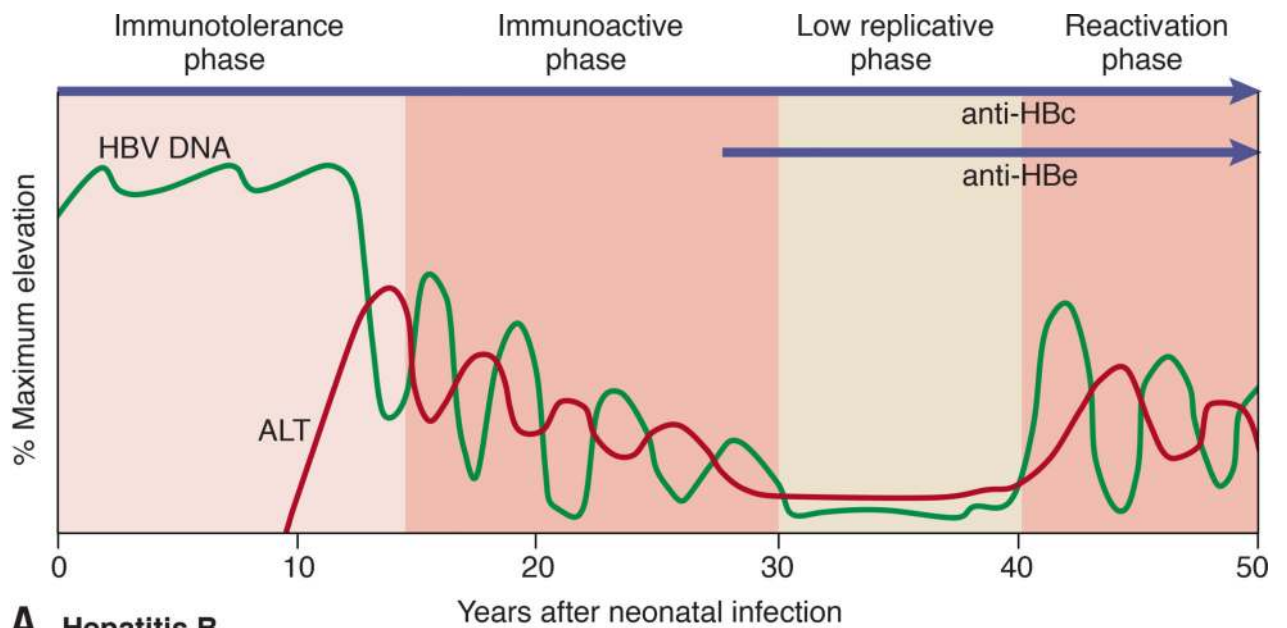
cells to the liver even though one chemokine (CXCL10) has been shown to exist in a nonfunctional form <sup>165</sup>.

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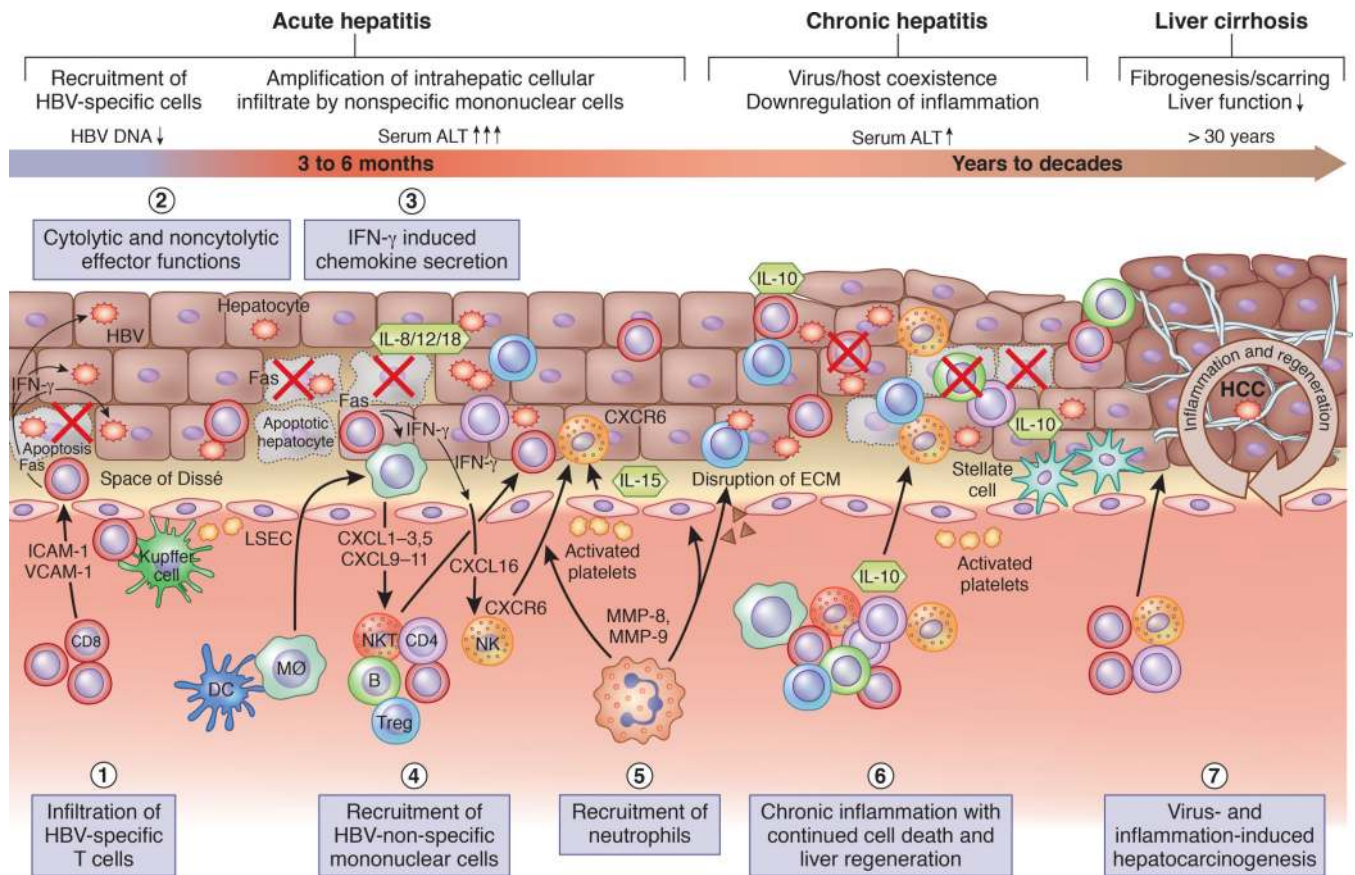
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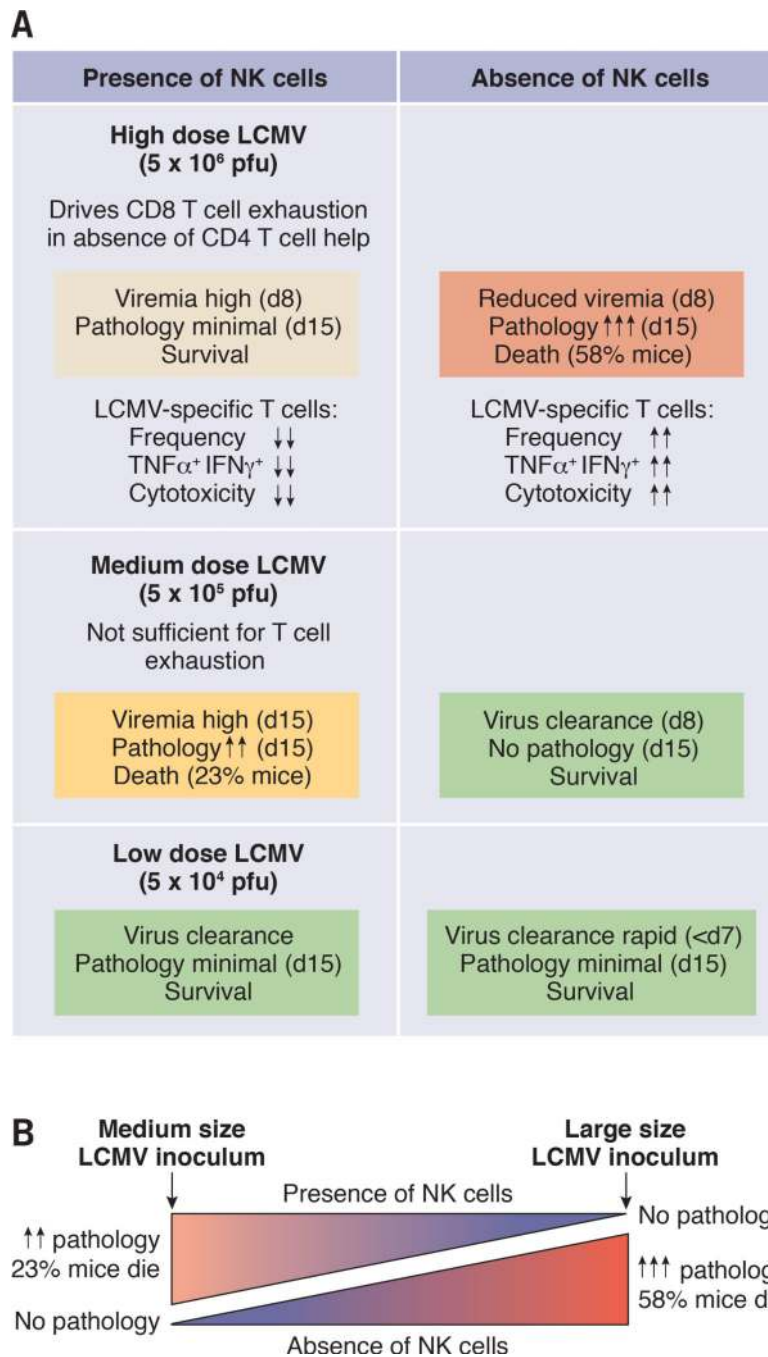
**Fig. 1. Natural history of chronic HBV and HCV infections**

A. Most chronic HBV infections result from vertical transmission from infected mother to neonate or young infant.

B. Most chronic HCV infections result from horizontal transmission during adulthood. Whereas patients with chronic HBV infection experience multiple phases with distinct viremia and pathology patterns, these parameters tend to be less pronounced and more stable in chronic HCV infection (see text for details).



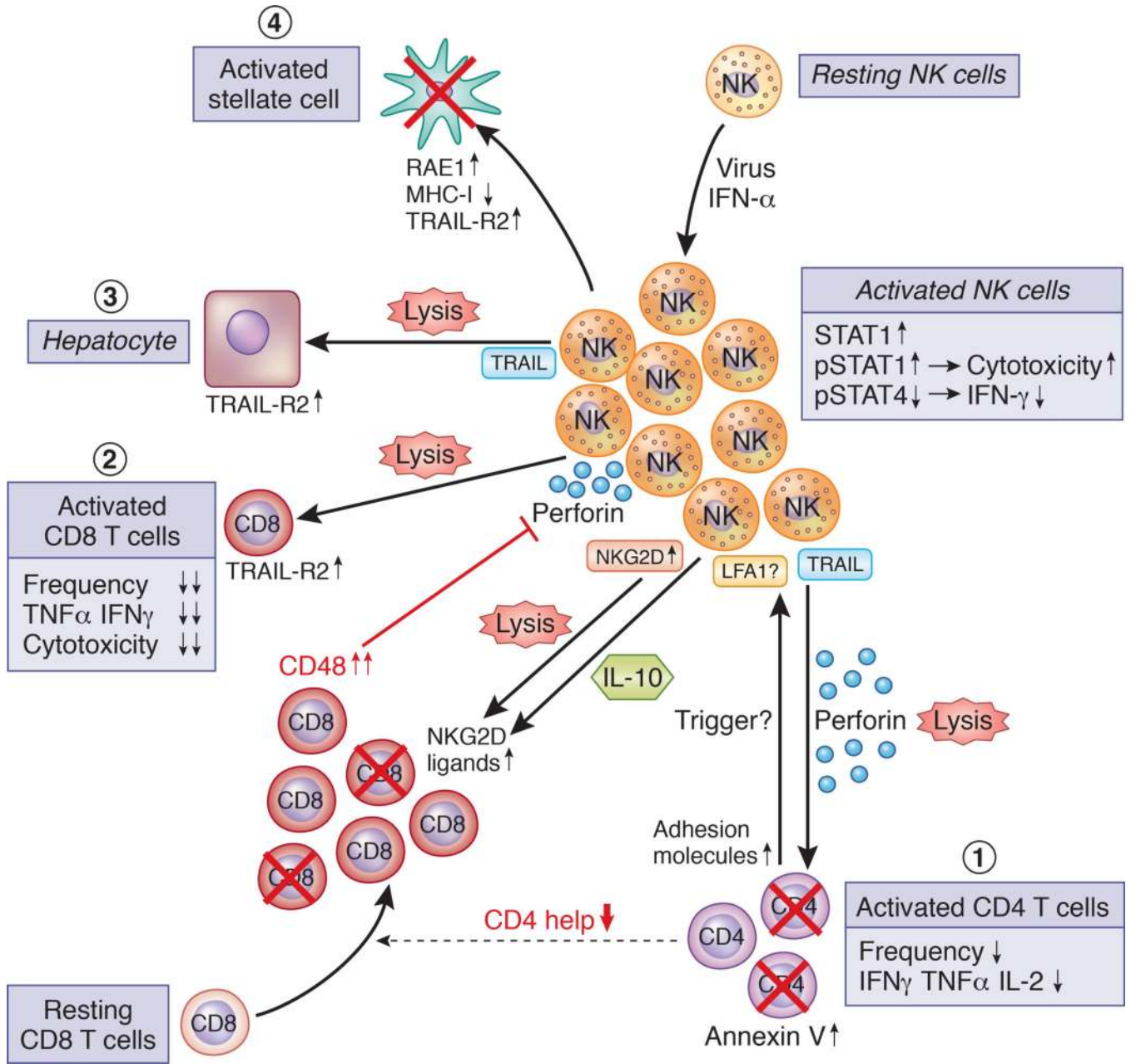
**Fig. 2.** Pathogenesis of HBV-related liver disease. Simplified schematic presentation of key factors that contribute to the pathogenesis of acute and chronic liver disease. As described in box 3 these findings are mostly based on observations made in transgenic mice that express HBsAg or replicate the complete HBV genome in their hepatocytes and were reconstituted with HBs-specific CD8 T cells <sup>101,103,134–139</sup>.



**Fig. 3.** NK cell-mediated lysis of intrahepatic cells involved in the pathogenesis of viral hepatitis. As described in detail in the main text, the drawing integrates results from the LCMV-model of viral hepatitis and from translational studies with biospecimens from HBV and HCV infected patients. Chronic exposure to IFN- $\alpha$  induces increased STAT1 levels and preferential STAT1 over STAT4 phosphorylation<sup>88,89</sup> resulting in increased cytotoxicity rather than IFN- $\gamma$  production in HCV-infected patients<sup>74,81</sup> and LCMV-infected mice<sup>86,87</sup>. In HBV infection, increased NK cell cytotoxicity has been attributed to IL-10 and TGF- $\beta$



exposure<sup>61</sup>. (1) NK cells kill activated CD4 T cells in a perforin-dependent manner, which results in reduced CD4 help to CD8 T cells and exhaustion of the LCMV-specific T cell response after high dose LCMV infection<sup>120</sup>. (2) Additional mechanisms of CD8 T cell regulation include perforin-mediated lysis of activated CD8 T cells that upregulate NKG2D in response to IL-10 in the LCMV model<sup>122,123</sup>, and TRAIL-mediated lysis of activated CD8 T cells that express TRAIL-R2 in HBV infection<sup>124</sup>. CD8 T cells can upregulate CD48 to engage the inhibitory NK cell receptor CD244 (2B4) as a negative feedback mechanism. (3) NK cells also lyse human hepatocytes in HBV infection<sup>31</sup> as well as (4) activated human and mouse stellate cells with altered expression of activatory and inhibitory NK cell ligands.



**Fig. 4. NK cell-mediated regulation of antiviral T cell responses as observed in the LCMV model of viral hepatitis**

A. The differential effect of NK cells on viremia and pathology depends on the size of the LCMV inoculum and is associated with differential T cell response patterns <sup>119</sup>.

B. NK cells serve as rheostats of disease activity <sup>119</sup> (see text for details).