NK cells in liver disease

Victoria Male, Kerstin A. Stegmann, Nicholas J. Easom and Mala K. Maini

Dr Victoria Male (PhD)
Affiliation: Division of Infection and Immunity, University College London
Postal address: Institute of Immunity and Transplantation, UCL Medical School, Rowland Hill Street, London NW3 2PF
Telephone: 0207 794 0500 x22463

Dr Kerstin Stegmann (PhD), Dr Nicholas Easom (MBBChir), Prof Mala Maini (F Med Sci)
Postal address: Division of Infection and Immunity, Rayne Building, 5 University Street, WC1E 6JF
Telephone: 020 3108 2170

Abstract

NK cells comprise one of the most abundant immune cell populations in human liver and the nature and functions of these cells have been a focus of recent interest. Here, we consider the possible roles of NK cells in diverse liver diseases, concentrating on data from patient studies. NK cells can be protective, killing virally infected and cancerous cells in the liver and limiting fibrosis by eliminating hepatic stellate cells. However, they can also be deleterious, contributing to pathology in viral hepatitis by killing hepatocytes and downregulating virus-specific T cell responses. It has recently emerged that a large fraction of hepatic NK cells constitute a distinct liver-resident subset and we highlight the need to distinguish between circulating and liver-resident NK cells in future studies. There is also a need for further investigation into how NK cells are influenced by the liver microenvironment and what scope there is to harness their immunotherapeutic potential.

Natural killer cells

Natural killer (NK) cells are immune cells of the lymphoid lineage that recognise and kill virally infected and malignant cells. NK cells also modulate the immune response by their production of cytokines, particularly IFNγ, and by their interactions with other immune cells, notably dendritic cells and T cells. Unlike T and B cells, NK cells do not undergo antigen receptor rearrangement but instead recognise their targets using a range of germ-line encoded receptors. Therefore, in contrast to T and B cells, NK cells make a rapid and robust response without the requirement for prior sensitisation [1-3].

NK cells were first described as recognising targets that lack MHC class I: the "missing self" response. They do this by expressing a variety of inhibitory receptors with a broad specificity for MHC class I, notably the inhibitory killer immunoglobulin-like receptors (KIRs) and CD94-NKG2A. When these are not engaged, the inhibitory signal is lifted leading to target cell killing. However, some cell types constitutively express low levels of MHC class I, and some virally infected cells evade recognition by NK cells by encoding molecules that mimic MHC class I. These phenomena limit the utility of target cell recognition based entirely on "missing self". More recently, it has become appreciated that NK cells also express a number of activating receptors whose engagement promotes killing. One such receptor is NKG2D, whose ligands, MICA, MICB and the ULPBs, are upregulated on stressed and transformed cells, providing another way for NK cells to recognise their targets. NK cells also express a group of activating receptors known as "natural cytotoxicity receptors" (NCR) which include NKp30, NKp44 and NKp46. The ligands of these receptors are not yet well-defined, but they are known to recognise virally infected and cancerous cells. Binding to antibody-coated cells via the Fc receptor CD16 allows a subset of NK cells to mediate antibody-dependent cell-mediated cytotoxicity (ADCC). The balance between ligation of activating and inhibitory receptors determines the outcome of the interaction between an NK cell and a potential target, although the precise threshold for NK cell activation can be modulated by the cytokine milieu. Selected NK cell receptors and their ligands in humans are summarised in Table I.

Having recognised a target, an NK cell may kill it either by releasing cytotoxic granules or by ligating target cell death receptors. Cytotoxic granules contain perforin and granzymes. Perforin forms a pore in the target cell membrane allowing granzymes to enter the cytosol and induce apoptosis. Therefore, the ability of NK cells to degranulate and their expression of perforin and granzymes are often used as proxy measures for their cytotoxicity. NK cells may also promote target cell death via tumour necrosis factor (TNF) receptor superfamily molecules expressed by the target cell. Some NK cells express TNF superfamily molecules, such as TRAIL or Fas ligand, which bind to these receptors, triggering apoptosis in the target cell.

The functions of circulating NK cells, then, are well-established, and the mechanisms by which they achieve target cell recognition, killing and cytokine production are well-understood. However, many non-lymphoid organs are enriched in NK cells compared to the blood. The NK
75 populations in these organs differ phenotypically from blood NK cells, and in some cases, notably in the uterus, these organ-specific NK cells are thought to have specialist physiological functions in their home tissue [4,5]. The liver contains a large number of NK cells, and these have recently been a focus of intense research interest.

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NK cells in the liver
NK cells have long been recognised to be extremely abundant in human liver, ordinarily representing between 30% and 50% of liver lymphocytes [6-8]. Their abundance has fueled interest in their potential role in liver disease. The specialised hepatic circulation and
85 surrounding parenchymal and non-parenchymal cells are important local influences on the features and functions of hepatic NK cells. The extensive narrow-lumen sinusoidal vasculature is lined by a fenestrated endothelium, allowing direct contact of intravascular lymphocytes with hepatocytes and stellate cells within the space of Disse [9]. Liver NK cells were originally described as "pit cells" that were noted to be in close contact with sinusoidal endothelial cells
90 and Kupffer cells [10]; the latter have subsequently been shown to shape NK cells by differential cytokine production following TLR signalling [11].

Human and mouse livers both contain large NK cell populations with an "immature" phenotype [7,8,12]. In mice, these "immature" NK cells are now known to represent a separate, liver-
95 resident population that is identified by its expression of CD49a and is less cytotoxic but has a greater capacity for TNFα and GM-CSF production than circulating NK cells [13,14]. In the light of these findings, recent attempts have been made to define an equivalent NK cell population in human liver. Some human livers contain a small CD49a^{+} NK cell population [15], but these differ from CD49a^{+} NK cells in mouse liver in a number of respects and may not be equivalent [16].

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More promisingly, all human livers seem to contain a large NK cell population that is phenotypically distinct from NK cells found in the blood [17-20] (Table II). Experiments carried out in a transplant setting have shown that these cells are unable to exit the liver and are long-lived, and are therefore considered liver-resident [19]. The liver-resident NK cells appear less
105 mature than circulating NK cells, on the basis of their expression of the maturation marker CD56, the terminal differentiation marker CD57 and by their expression of various NK cell receptors [17-20]. They express the C-type lectin CD69, which is associated with tissue-residence [5], as well as the chemokine receptors CCR5 and CXCR6, which can mediate liver homing and residency by recognition of their ligands CCL3, CCL5 and CXCL16, which are
110 present in hepatic sinusoids [17-19].

Liver-resident NK cells express lower levels of perforin and granzyme B than their circulating counterparts, are less cytotoxic and less able to produce pro-inflammatory cytokines [17-19]. This is perhaps not unexpected, since it is in line with the immunotolerant nature of the liver, but
115 it does suggest that liver-resident NK cells do not have the same function as circulating NK cells. The large number and tissue residence of these cells perhaps point to a tissue-specific function, although it is not yet clear what this might be. There is some evidence from mice that liver-resident NK cells are able to mediate immune memory responses to haptens and viral vaccinations [21,22]. This suggests the intriguing possibility that liver-resident NK cells in
120 humans may be "memory" cells, a function that would be consistent with their longevity [19].

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Regardless of their function, the discovery of these liver-resident NK cells has a number of implications for the interpretation of older studies into human NK cells in liver disease. Firstly, it should be noted that they express CD69. In the past, this was considered a marker of immune cell activation, but is now known to also be associated with tissue-resident cells. Therefore, studies which report an increase in the proportion of CD69$^+$ intrahepatic NK cells could be interpreted either as demonstrating activation of circulating NK cells, an expansion of the resident compartment, or even a combination of the two. Furthermore, the existence of liver-resident cells means that the behavior of NK cells isolated from blood will not necessarily represent the responses of all the NK cells present in the liver. Indeed, circulating and liver-resident NK cells may later be shown to have different, even opposing, roles in liver disease.

NK cells in liver fibrosis

The liver is exceptional in its regenerative capacity, but repeated destruction and regeneration results in fibrosis, which can progress to cirrhosis. Fibrosis occurs largely as a result of extracellular matrix production by activated hepatic stellate cells. It is a process common to all chronic inflammatory diseases of the liver and, as a result, any role for NK cells in fibrosis is likely to be relevant to a broad range of liver diseases.

A number of studies in mice have shown that chemically-induced liver fibrosis is exacerbated in the absence of NK cells, suggesting that NK cells control fibrosis. This is a result of the greater susceptibility of activated hepatic stellate cells to NK cell killing, an effect that may be mediated by their reduced expression of MHC class I [23], increased expression of ligands for NKG2D [24,25], NKp46 [26] or receptors for TRAIL [27], or some combination of these.

Consistent with the findings in mice, NK cells in humans also have a role in controlling liver fibrosis. Human peripheral blood NK cells are able to kill hepatic stellate cells in culture and this is dependent on NKp46 [26], NKG2A [28], NKG2D, TRAIL and FasL [29]. NK cells that are cytotoxic [30], and specifically more effective at killing hepatic stellate cells [29], are associated with a lower degree of fibrosis in chronic hepatitis C. Furthermore, increased NK cell infiltration into the liver is associated with reduced fibrosis in primary sclerosing cholangitis [31].

NK cells in viral hepatitis

One of the major causes of liver disease is viral hepatitis brought about by the unrelated Hepatitis viruses A, B, C and E. Each of these is considered below, although the majority of NK cell studies have focused on Hepatitis B and C [32].

Hepatitis B

In acute Hepatitis B virus (HBV) infection, there is an early expansion of NK cells [33,34] but their antiviral potential is temporarily impaired [35], coinciding with peak viraemia and a surge in IL-10, which has suppressive effects on NK cells. Only when viral load, and therefore IL-10 levels, decrease, do NK cells upregulate TRAIL and regain the ability to produce pro-inflammatory cytokines.

Studies of NK cell frequency and function in chronic hepatitis B (CHB) have been complicated by the heterogeneity of patients cohorts, comprised of varying proportions in the different clinical phases. Even the nomenclature and diagnostic criteria of these four phases (currently usually known as immune-tolerant, immune-active, immune-control and reactivation), based on markers such as eAg, viral load and ALT, are controversial [36-38]. NK cell frequency in the periphery and the liver is highest in immune-tolerant patients, while immune-active patients have a

reduction in NK cells [39,40]. There is some disagreement on the precise way in which activating and inhibitory NK cell receptors are altered in the immune-tolerant phase, but the consensus seems to be that there is a general shift towards inhibition. In immune-active patients, the consensus points to peripheral and intrahepatic NK cells expressing more activating markers such as NCRs, TRAIL, CD69 and HLA-DR, and fewer inhibitory receptors [41-44], although one study reported increased expression of the inhibitory receptor NKG2A by peripheral NK cells in immune-active patients [45]. Consistent with a generalised activation, NK cells from immune-active patients have an increased ability to degranulate and produce cytokines compared to immune-tolerant patients and healthy controls. These changes might be attributable to increased proinflammatory cytokine production in the liver of immune-active patients [40].

A broad consensus from all studies is that NK cells in CHB have a functional dichotomy, with selective impairment in cytokine production but relative preservation of cytotoxicity [42-44,46]. IFNγ is key to the non-cytolytic immune control of HBV replication without excessive liver damage, so this reduction in IFNγ production is particularly significant [47]. The selective defects in NK cell function could be recapitulated by IL-10 treatment *in vitro*, whilst blockade of IL-10 and TGF-β led to restoration of IFNγ production in both circulating and intrahepatic NK cells from patients with CHB. A role for these immunosuppressive cytokines in shaping the NK cell functional profile is in line with their preponderance in the liver and increased expression in CHB [46]. Additional mechanisms proposed to contribute to the impaired NK cell effector function in CHB are their reduced levels of activating receptors [48] and impaired activation by plasmacytoid dendritic cells [49].

Patients in the eAg-negative reactivation phase experience temporary increases in viraemia and liver damage, known as "hepatic flares", associated with increased serum IFNα and IL-8 levels, together with enhanced NK cell activation and TRAIL expression. This cytokine combination promotes TRAIL-mediated killing by upregulating TRAIL on NK cells, and altering the balance of death-inducing versus decoy TRAIL receptors on hepatocytes. Human NK cells are able to kill both hepatoma cells and primary human hepatocytes in a partially TRAIL-dependent manner. These findings provided a paradigm in which TRAIL+ NK cells, which are predominantly liver-resident [18], eliminate virally-infected hepatocytes and propagate immune-mediated liver damage [50].

While NK cells can play a protective role by eliminating virally infected hepatocytes or activated hepatic stellate cells, their potential to regulate immunopathology is complicated by their interaction with antiviral T cells [51] (Figure 1). NK cells selectively kill HBV-specific CD8+ T cells rather than T cells of other specificities and this susceptibility is partly determined by upregulation of the death-inducing receptor TRAIL-R2 by CD8+ T cells during HBV-specific responses. Expression of TRAIL-R2 is greatly enriched on intrahepatic CD8+ T cells, to a degree determined by viral load. The *in vivo* relevance of this is underscored by the finding that antiviral T cell responses from HBV-infected livers can be rescued following overnight TRAIL blockade [52]. This finding was supported by several studies in murine models showing a vital rheostat function for NK cells in regulating T cell responses in persistent viral infections [53,54]. Another example of NK cell/T cell crosstalk in viral hepatitis is the finding that MICA is expressed on HBV-specific and intrahepatic CD4+ T cells. This first demonstration of uninfected human T cells expressing an NKG2D ligand was supported by *in vitro* and *ex vivo* data suggesting that this could drive NK cell cytotoxicity [55]. Further results pointing to a role for NK cells in negatively regulating HBV-specific T cells came from a study of patients treated with nucleoside/nucleotide analogs (NUCs). NUC treatment reduces NK cell activation and TRAIL

expression [46]; reversion to a quiescent NK cell phenotype is associated with restoration of HBV-specific CD4^{+} T cells [56].

Interferon-alpha (IFNα) is another mainstay of treatment for CHB, providing an opportunity to study the *in vivo* impact of this cytokine on NK cells. Administration of therapeutic pegylated-IFNα to cohorts with either eAg-positive or eAg-negative CHB led to an increase in serum levels of the pro-proliferative cytokine IL-15, accompanied by a cumulative expansion of CD56^{bright} NK cells expressing TRAIL, NKp46 and HLA-DR [57,58]. Whereas NUCs were unable to reverse the defective NK cell IFNγ production characteristic of CHB [44,46,56], this could be restored by pegylated IFNα [57,58]. Surprisingly, pegylated-IFNα was also able to induce sustained changes in NK cell responsiveness to sequential treatment with NUCs [58]. These prolonged *in vivo* changes in NK cells are in line with emerging data on adaptive features of NK cells [3].

Hepatitis C
An early indication of the importance of NK cells in the outcome of Hepatitis C virus (HCV) infection came from genetic studies, revealing that patients expressing the inhibitory receptor KIR2DL3 together with its ligand HLA-C1 had an increased chance of spontaneous or treatment-induced HCV clearance [59,60]. Similarly, HCV-exposed uninfected individuals were more likely to have KIR2DL3-expressing NK cells with enhanced activation and effector function [61-63]. A number of studies have reported associations between NK cell receptor expression and function with outcomes in HCV infection, including disease activity as measured by ALT and AST [43,64], viral load [43,65-67] and outcome of treatment [66,68]. Broadly speaking, higher NK cell cytotoxicity and expression of activating receptors are associated with viral clearance and good responses to treatment, while higher expression of inhibitory receptors is linked to poor responses. Therefore, activated NK cells seem to be beneficial in the control of HCV.

In chronic HCV, the total frequency of NK cells and the proportion of the more cytotoxic CD56^{dim} subset of cells within the NK cell compartment are reduced [30,69,70]. There is no consensus on the expression of activating and inhibitory receptors by peripheral NK cells from chronic HCV patients, although the slim majority of studies report an overall increase in activating receptors [41,43,67,69,70]. This lack of consensus could potentially be attributed to differences in patient cohorts. Hepatic NK cells in chronic HCV also seem to be broadly activated, with one study reporting that intrahepatic NK cells express increased levels of activating receptors compared to peripheral NK cells [64] while another found increased expression of activating receptors by NK cells from HCV infected liver, compared to control liver [71].

NK cells in chronic HCV infection display reduced cytokine production, but sustained cytotoxicity, similar to the situation in CHB. Cytotoxic function of NK cells has been reported either to be increased [43] or unaltered in HCV patients compared to healthy controls [30,70]. The HCV-infected liver is enriched in NK cells expressing high levels of NKp46, which are highly cytotoxic, produce IFNγ and block HCV replication more efficiently than NKp46^{lo} cells. The frequency of NKp46^{hi} NK cells in the liver correlates inversely with viral load and fibrosis [72], in line with NK cells having an anti-fibrotic [29] and anti-viral activity [65]. On the other hand, NK cells from HCV infected patients have an impaired ability to produce IFNγ [43,64,73].

IFNα is a major regulator of NK cell activity during HCV infection, and a key determinant of outcome. *In vitro,* treatment with IFNα leads to a shift towards activation with increased TRAIL expression on both peripheral and intrahepatic NK cells [64,65,68] and increased degranulation in peripheral NK cells [64]. IFNα stimulation is necessary for NK cells to effectively recognise

HCV-replicating hepatoma cells and IFNα-stimulated NK cells challenged with these targets degranulate, produce cytokines and mediate cytotoxicity via TRAIL [65,74].

Until recently, administration of pegylated IFNα was the standard treatment for HCV and examination of NK cell responses in patients undergoing therapy sheds light on the importance of IFNα in NK cell responses to HCV *in vivo*, with TRAIL emerging as a critical effector of virus control. NK cells in acute HCV patients have elevated TRAIL expression, which increases during IFNα therapy. Patients who cleared chronic HCV infection during IFNα treatment showed a greater upregulation of TRAIL compared to patients with ongoing chronic HCV infection. Furthermore, in patients with chronic HCV infection, TRAIL upregulation during the first 48h of IFNα treatment correlated with decreases in viral load [65]. Others studies have shown an upregulation of CD69, NKp30, NKp46 and NKG2A and downregulation of CD16, perforin and NKG2D during IFNα treatment [66]. There is also a decreased frequency of NK cells expressing CXCR3 and CCR7, suggesting recruitment to the liver [68]. More recently, studies of blood and liver samples from HCV patients treated with the new interferon-free regimes (direct-acting antivirals) have demonstrated normalisation of the disease-associated NK cell phenotype and function.

Hepatitis A and E
The role of NK cells in Hepatitis A virus (HAV) has not been investigated in detail. One study suggested NK cells have the potential to control HAV infection, although this was carried out in HAV-infected fibroblasts rather than hepatocytes [75,76]. There is controversy over changes in NK cell frequency during Hepatitis A [77,78] but elevated NK cell NKG2A expression correlates with ALT, suggesting a possible role for NK cells in acute liver damage [78].

In patients with acute Hepatitis E virus (HEV) infection, NK cells produce proinflammatory cytokines in response to stimulation faster than in patients with resolved infection [79]. NCR, NKG2D and CD69 expression increase during acute and resolved HEV infection compared to healthy controls [79]. These findings suggest that NK cells are activated in HEV infection. However, NK cell degranulation is impaired [79] and NK cell numbers decrease and remain low in resolved patients [80].

NK cells in alcoholic and non-alcoholic fatty liver disease
Alcoholic liver disease (ALD) and non-alcoholic fatty liver disease (NAFLD) are histologically similar disorders, both of which encompass a spectrum from simple steatosis to steatohepatitis to cirrhosis and finally hepatocellular carcinoma (HCC). Hepatocyte ballooning and immune cell infiltration are features of both ALD and NAFLD and the two disorders are likely to have a number of immunological characteristics in common, including the role of NK cells.

Alcoholic liver disease
The role of NK cells in ALD has not yet been extensively investigated. In mice, chronic ethanol consumption reduces NK cell number and function by inhibiting their maturation. This in turn accelerates the development of liver fibrosis in the presence of other injurious stimuli [81,82]. In human peripheral blood, a similar reduction in NK cell frequency and cytotoxic function is observed in ALD patients, compared to controls [83].

Non-alcoholic fatty liver disease
The role of NK cells in NAFLD has been considered in more depth. To the extent that NAFLD is regarded as the hepatic manifestation of the metabolic syndrome, NK cells in adipose tissue are likely to be implicated in NAFLD through their involvement in altering glucose homeostasis [84-

86]. Similar to ALD patients, NK cell frequency and cytotoxic function is decreased in the peripheral blood of obese patients, compared to lean controls [87]. Non-alcoholic steatohepatitis (NASH) patients have increased NK cell numbers in their liver, compared to those with simple steatosis or healthy controls, suggesting that the decrease in peripheral blood NK cell frequency may be a result of their recruitment to the liver [88]. Notably, this study identified NK cells using CD57, demonstrating that the increase is specifically in the CD57$^+$ liver-infiltrating, and not the CD57$^-$ liver-resident, population, and supporting the idea that these NK cells have been recruited from the blood. The study also reported increased expression of NKG2D ligands and apoptotic hepatocytes in NASH patients, suggesting increased NKG2D-dependent activation of these NK cells [88].

An increase in NK cells in the liver during NASH was also observed in a mouse model of the disease, and again this was attributable to an increase in liver-infiltrating, rather than liver-resident NK cells [89]. Intriguingly, in this study depletion of NK cells aggravated liver disease by allowing macrophages to become polarised to an M2 phenotype. Therefore, although a number of associations between NK cells and NAFLD have been reported, it is not yet clear if NK cells are helpful or deleterious in the disease.

NK cells in autoimmune diseases of the liver
NK cells are implicated to some extent in the pathology of all three main autoimmune diseases of the liver: autoimmune hepatitis (AIH), primary biliary cirrhosis (PBC, formerly primary biliary cirrhosis), and primary sclerosing cholangitis (PSC).

Autoimmune hepatitis
AIH is characterised by hepatocyte necrosis associated with T cell and plasma cell infiltrates. NK cells have been observed to interact with hepatocytes in AIH [90]. Different HLA alleles have been associated with AIH in different ethnic groups [91,92], and recently in a Sardinian cohort the activating receptor KIR2DS1 has been shown to be a risk factor for AIH, while presence of the inhibitory receptor-ligand pairs KIR2DL3/HLA-C1 and KIR3DL1/HLA-Bw4 were protective [93], implicating NK cell activation in disease.

Primary biliary cirrhosis
In PBC there is destruction of the small intrahepatic bile ducts, with contributions from both adaptive and innate cells. NK cells are more numerous in both liver and blood in PBC, with increased perforin expression and cytotoxicity [94]. This is likely to reflect recruitment of peripheral blood NK cells to the liver via CXCR3 and CX3CR1 [95,96]. *In vitro*, NK cells can be induced to kill autologous biliary epithelial cells via TRAIL, by cross-talk with TLR-3 and -4 stimulated macrophages [97]. Further, IFNγ produced by NK cells after co-culture with biliary epithelial cells can induce cytotoxicity in autoreactive CD4$^+$ T cells [98].

Primary sclerosing cholangitis
PSC is an autoimmune disorder with inflammation of all parts of the biliary tree and associated T cell infiltrates. It is associated with increased NK cell numbers in the peripheral blood but not the liver [99] and these NK cells are functionally impaired due to the action of TNFα [100]. A number of lines of immunogenetic evidence point to the involvement of NK cell receptors and ligands, and by extension NK cells themselves, in the progression of PSC. The development of the disease is associated with certain alleles of the NKG2D ligands MICA and MICB [101,102], and also with the absence of HLA-Bw4 and HLA-C2, which are ligands for inhibitory KIR, an effect that seems to require homozygosity for MICA5.1 [103]. Furthermore, two SNPs in the

NKG2D gene are associated with development of cholangiocarcinoma in the context of PSC [104].

NK cells in liver cancer

Primary liver cancer is the third commonest cause of cancer death worldwide and the liver is also a major site of metastatic spread for many other malignancies. One major role of NK cells throughout the body is tumour surveillance and NK cells have the potential to play crucial roles in the immune response to both primary and metastatic liver cancers.

Hepatocellular carcinoma

HCC arises from hepatocytes in the context of pre-existing liver disease, most commonly viral hepatitis, ALD or NAFLD in the developed world, and is strongly associated with aflatoxin exposure in the developing world. Apart from HBV, which can be directly carcinogenic, HCC arises only in cirrhotic livers and is associated with inflammation in all settings, with systemic inflammation carrying a poor prognosis [105].

As in other malignancies, NK cells are functionally impaired in HCC. Tumour-infiltrating NK cells have reduced expression of granzymes and perforin and reduced cytotoxic potential compared to healthy controls, and are also reduced as a proportion of infiltrating lymphocytes compared with non-tumour liver tissue [106]. Tumour environments can subvert NK cell immunity through a variety of mechanisms, including increased numbers of immunosuppressive cells such as regulatory T cells and myeloid-derived suppressor cells and increased expression of immunoregulatory cytokines such as TGFβ [107]. Changes in NK receptors or their ligands within the tumour milieu can also downregulate immunity. For example, loss of expression of the NKG2D ligands ULBP1 and MICA by HCC is associated with a poor prognosis [108,109]. The SNP at rs2596542 is associated with MICA shedding in HCC. Among hepatitis C patients, those with the A/A genotype, which is associated with low soluble MICA, are at higher risk of HCC, whereas among hepatitis B patients, those with the GG genotype are at higher risk [110-112]. The differential effects of this SNP may be due to differences in induction and processing of MICA between the two viruses. The high-affinity allele MICA-129Met is associated with an increased risk of HCC in the context of HBV [112], providing further evidence to suggest that NKG2D may play a role in both HCC surveillance and control.

Conversely, there is some immunogenetic evidence to suggest that high basal NK cell activity may be deleterious. KIR-HLA interactions which allow the development of highly responsive ("licensed") NK cells and activating KIR in the presence of appropriate HLA ligands are associated with increased progression to HCC in the context of HBV [113]. Similarly, different murine models have pointed to tumorigenic and well as protective roles for the NKG2D pathway in HCC [114]. Therefore it remains to be seen whether increasing NK cell activation and/or promoting NKG2D will be helpful or harmful in HCC.

Attempts to overcome tumour evasion strategies and harness NK cells for immunotherapy of HCC are now being tested in the clinic. One strategy is enhancing the function of endogenous NK cells; an example is the use of an antibody that blocks engagement of the inhibitory receptors KIR2DL-1/2/3, which is currently being tried, in combination with PD-1 blockade, in patients with advanced solid tumours (NCT01714739). Alternatively, adoptive cell therapy can be carried out, using infusions of autologous or allogeneic NK cells, expanded, activated or genetically modified to optimise their anti-tumour capacity [107]. Since NK cells may function better to prevent metastases than to penetrate established solid tumours, several ongoing trials are using allogeneic NK adoptive immunotherapy once the HCC has been resected or

425   explanted (NCT01147380, NCT02008929). Previous *in vitro* studies suggested NK cells extracted from donor liver perfusates may be a promising source for such adoptive therapy because of an enhanced capacity to kill HCC following IL-2 activation [7]. However, peripheral NK cells can also be rendered effective by expansion using the NK cell-stimulating cell line K562-mb15-41BBL or by transduction with the chimeric receptor NKG2D-CD3z-DAP10 [115].

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Cholangiocarcinoma
Cholangiocarcinoma is a primary malignancy of the biliary tree, and is closely associated with inflammation of the biliary system, primarily due to PBC, PSC or infection with liver flukes. In mice, cytokine induced killer cells and IFNγ are associated with control of subcutaneously
435   injected cholangiocarcinoma cells [116,117]. In humans, two SNPs in the NKG2D gene are associated with the development of cholangiocarcinoma in the context of PSC [104] but it remains to be seen whether this effect is NK cell-mediated.

Liver metastases
440   In most major malignancies including those of the lung, breast and GI tract, spread to the liver is common. In mice, NK cells are well-known to have a role in the control of tumour metastases and intrahepatic NK cells control metastases via TRAIL [118,119]. The role of NK cells in metastases to the liver in humans is best studied in colorectal cancer, where tumours resected from the liver are available for investigation. In one study, infiltrating T cells, but not NK cells,
445   were associated with a good prognosis [120], and in other studies tumour infiltrating CD8^{+} T cells have been shown to contribute to good prognosis [121,122]. In many solid tumours, NK cells become functionally impaired and unable to lyse tumour cells. These autologous NK cells may represent an effector population that could be recruited therapeutically to target metastases *in vivo*, although cytokine infusion approaches have so far been limited by clinical toxicity [123].
450   Combining cytokine activation of NK cells with ADCC using monoclonal antibodies that engage CD16, such as trastuzumab for HER2+ metastatic breast cancer, may be a more promising approach [124]. Lenalidomide, which decreases the activation threshold of NK cells via an effect on actin remodelling [125], did not induce clinical response in metastatic renal carcinoma including in patients with liver metastases as monotherapy, but could be combined with ADCC-
455   inducing antibodies, as in various haematological malignancies [126].

NK cells in liver transplantation
Liver transplantation is the only definitive treatment for patients suffering from end-stage liver
460   disease, but its success is heavily dependent on a number of immune-mediated factors, which may involve the action of NK cells. One early complication of transplantation is ischaemia-reperfusion (IR) injury, in which hypoxic organ damage is exacerbated following the re-establishment of blood supply and oxygen delivery. Acute rejection occurs days to weeks after a transplant and is primarily mediated by alloreactive T cells, whereas chronic rejection can occur
465   up to years later, is characterised by occlusion of the blood vessels leading to ischaemia, and is a result of mixed mechanisms, including low-grade T cell responses and anti-graft antibody.

The role of NK cells in IR injury during liver transplant has not yet been investigated in humans, but there is evidence from mouse models that NK cells are involved in exacerbating this
470   disease. Treatment of mice with the NK cell depleting antibody NK1.1 reduces hepatic IR injury [127] and CD39-deficient mice, in which NK cells are sub-functional, are less susceptible to hepatic IR injury than wild-type controls [128].

NK cell-mediated skin graft rejection occurs in mice [129] so it is possible that some component
475   of liver transplant rejection may be NK cell-mediated. In rats, recipient NK cells are rapidly

recruited to the allogeneic transplanted liver. These cells are the main producers of IFNγ in the transplanted liver, and antibody depletion of NK cells can prolong the survival of the allograft [130]. During human liver transplantation, recipient NK cells are also rapidly recruited to the graft [19]. A number of immunogenetic studies have attempted to determine whether recipient NK cells may be involved in rejection of the transplanted liver, with mixed results. One study of 100 transplants showed that matching of the main HLA ligand recognised by NK cells, HLA-C, between donor and recipient, reduced the likelihood of acute rejection, with recipients who expressed no HLA-C group 2 alleles further protected from acute rejection [131]. However, later, larger studies were unable to replicate these results [132,133]. A study of HLA-C effect on chronic rejection of 595 liver transplants showed that the presence of at least one HLA-C group 2 allele in the donor was protective [132], but again this finding could not be replicated in a later study [133]. It is unclear, then, if NK cells are important mediators of either acute or chronic rejection in liver transplantation.

Future directions
NK cells are one of the most prevalent immune cell types in the human liver and are implicated as players in many liver diseases (Figure 2). However, in the healthy liver and most disease settings, their dominant roles remain to be clarified. Murine models are an important adjunct, but not a substitute, for studies in humans, especially given some notable differences in NK cells between mouse and man [16]. Researchers in this field are fortunate to have access to human liver tissue from diagnostic biopsies, along with transplant perfusates, metastatic resections and cirrhotic explants, giving more access to NK cells at the site of disease than is possible for most human organs. Nevertheless, the demanding logistics of obtaining such samples has meant that the majority of studies are conducted on peripheral blood; this is becoming more of an issue as non-invasive diagnostic methods start to replace liver biopsies. Recent studies revealing large liver-resident NK cell populations that do not recirculate [19] underscore the importance of continued access to liver sampling, exemplified by a recent study tracking the kinetics of NK cell responses by an alternative approach of repeated fine needle aspiration [134].

Future studies will need to analyse liver-resident and non-resident NK subsets separately, since prior analyses of the whole intrahepatic fraction may have masked important disease-associated changes. Another factor that may have confounded interpretation of results in previous studies is CMV serostatus; it is now clear that prior CMV infection is a major driver of NK cell adaptive differentiation [135] that needs to be controlled for in studies of diverse infections and liver diseases. In mice, the liver is a site where "memory" NK cells accumulate [21,22]. Whether this is the case in humans remains to be established but elegant work in HCV has already revealed another "adaptive" feature of NK cells: their capacity for mediating fine specificity through altered KIR binding to subtle variants of peptide/MHC class I complexes, impairing NK cell inhibition [136,137]. It will be interesting to consider precise NK cell target selectivity in other settings.

In addition to analysing freshly isolated hepatic NK cells, enhanced technology for multiparameter *in situ* immunostaining will facilitate improved studies of their anatomical localisation and interactions in liver sections. More in-depth mechanistic studies of how NK cells kill their targets in the liver, and how other liver cell types influence hepatic NK cells, will take advantage of emerging 3D models. Such scaffold systems constitute exciting progress in the attempt to recapitulate the complexity of the liver microanatomy and to allow analysis of NK cell cross-talk with, for example, primary hepatocytes and hepatic stellate cells, rather than immortalised cell lines [138]. Ongoing studies are starting to address the profound influence of local factors such as hypoxia and nutrient metabolism on NK cells in the unique liver niche.

Initial work suggests that exposure of NK cells to hypoxic conditions mimicking those present in the liver (which receives more than 70% of its blood supply from the portal vein) has divergent effects on function: while low oxygen tensions prevent activating receptors driving target cell killing, they maintain ADCC by NK cells [139]. As such, it may be necessary to revisit ideas about NK cell function in the liver that are based on experiments carried out at atmospheric oxygen tensions. Hypoxia may also interact with the unique cytokine milieu of the liver, since TGFβ is able to inhibit the mTOR-dependent metabolic reprogramming central to IL-15-induced NK cell responses [140-142]. It will also be necessary to examine interactions between hypoxia and infection. Co-culturing NK cells from HCV patients with HCV-replicating hepatocytes or with stellate cells revealed that hypoxia constrained their antiviral capacity but not their anti-fibrotic activity [143] and one of the metabolic adaptations of NK cells exposed to hypoxia, HIF-1α-induced glycolytic gene transcription [144], appears to be defective in HCV [143].

Studies addressing these novel aspects of NK cell biology in state-of-the art *in vitro* and *in vivo* models will enhance our understanding of their diverse roles in liver disease and the scope for immunotherapeutic modification. Meanwhile NK cells are already reaching the clinic for primary and secondary liver cancers and these trials will provide powerful insights into their potential as therapeutic tools.

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Tables

Receptor	Ligand	Activating/inhibitory
Killer Immunoglobulin-like Receptors (KIRs)	MHC class I (primarily HLA-C)	Either, depending on variant
CD94-NKG2A	MHC class I (HLA-E)	Inhibitory
CD94-NKG2C	MHC class I (HLA-E)	Activating
NKG2D	ULBPs, MICA, MICB	Activating
NCR (NKp30, NKp44, NKp46)	Not well defined	Activating
CD16	IgG-coated cells	Activating

Table I: Selected NK cell receptors and their ligands

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	Circulating NK cells	Liver-resident NK cells
Adhesion, recruitment and residence		
CD56	Mostly low ("dim")	Mostly high ("bright")
CD57	Mostly positive	Negative
CD69	Negative	Positive
CCR5	Negative	Positive
CCR7	Positive	Negative
CXCR6	Negative	Positive
L-selectin	Positive	Negative
NK cell receptors		
CD16	Mostly positive	Negative
KIR	Mostly positive	Mostly negative
NKG2A/CD94	~50% positive	Mostly positive
NKG2D	Positive	Positive
Transcription factors		
Eomes	Low	High
T-bet	High	Low

Table II: Key phenotypic differences between circulating and liver-resident NK cells

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Figure Legends

Figure 1: NK cell interference with the antiviral T cell response in CHB

During CHB infection, NK cells target HBV-specific T cells, dampening the antiviral T cell response. HBV-specific CD8^{+} T cells upregulate TRAILR2, becoming susceptible to NK cell killing via the TRAIL pathway. Meanwhile HBV-specific CD4^{+} T cells upregulate the NKG2D ligand MICA, which activates NK cells leading to degranulation and cytotoxicity.
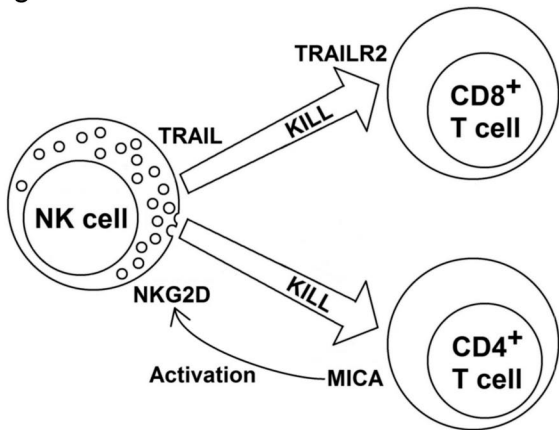
Figure 2: Roles of NK cells in liver diseases

Figure 1

Figure 2