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BRIEF REVIEW

NK cells in hepatitis B virus infection: a potent target for immunotherapy

Ziba Shabani · Masomeh Bagheri · Mohammad Zare-Bidaki · Gholamhossein Hassanshahi · Mohammad Kazemi Arababadi · Mozafar Mohammadi Nejad · Derek Kennedy

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Abstract Viruses, including hepatitis B virus (HBV), are the most prevalent and infectious agents that lead to liver disease in humans. Hepatocellular carcinoma (HCC) and cirrhosis of the liver are the most serious complications arising from prolonged forms of hepatitis B. Previous studies demonstrated that patients suffering from long-term HBV infections are unable to eradicate HBV from hepatocytes completely. The mechanisms responsible for progression of these forms of infection have not yet been clarified. However, it seems that there are differences in genetic and immunological parameters when comparing patients to subjects who successfully clear HBV infections, and these may represent the causes of long-term infection. Natural killer (NK) cells, the main innate immune cells that target viral infections, play important roles in the eradication of HBV from hepatocytes. NK cells carry several stimulatory and inhibitor receptors, and binding of receptors with their ligands results in activation and suppression of NK cells, respectively. The aim of this review is to address the recent information regarding NK cell phenotype, functions and modifications in hepatitis B. This review addresses the recent data regarding the roles of NK cells as novel targets for immunotherapies that target hepatitis B infection. It also discusses the potential to reduce the risk of HCC or cirrhosis of the liver by targeting NK cells.

Z. Shabani · M. Bagheri · M. Zare-Bidaki · M. K. Arababadi
Immunology of Infectious Diseases Research Center, Rafsanjan University of Medical Sciences, Rafsanjan, Iran

G. Hassanshahi
Molecular Medicine Research Center, Rafsanjan University of Medical Sciences, Rafsanjan, Iran

M. Mohammadi Nejad (✉)
Razi Herbal Medicine Research Center, Lorestan University of Medical Sciences, Khorramabad, Iran
e-mail: dr.kazemi@rums.ac.ir

D. Kennedy
School of Biomolecular and Physical Science, Eskitis Institute for Drug Discovery, Griffith University, Nathan, Queensland, Australia

Introduction

Hepatitis viruses, including hepatitis B virus (HBV), are the most dangerous and prevalent infectious agents that lead to liver disease in humans [66]. The major causes of the clinical presentations of hepatitis B are related to immune system responses, especially those mediated by natural killer (NK) cells and cytotoxic T lymphocytes, against infected hepatocytes [26]. A complete and specific immune response from the innate and adaptive immune systems against HBV can lead to the eradication of the virus from infected hepatocytes [2], but previous studies demonstrated that variations in the efficacy of immune responses against HBV led to five forms of the disease, including fulminant, acute, chronic, asymptomatic and occult HBV infection [26]. Moreover, prolonged infection with HBV, such as that seen in the chronic, asymptomatic and occult forms of the disease, can lead to hepatocellular carcinoma (HCC) and cirrhosis of the liver [28]. The mechanisms responsible for the progression of prolonged infections have yet to be fully clarified. Researchers have suggested that different genetic and immunological parameters in the patients may play critical roles in the etiology of prolonged hepatitis forms when compared to individuals who are able to clear the infection [10]. Recent studies demonstrated that NK cells play important roles in

the induction of appropriate immune responses to eradicate viral infection from the infected liver [10]. NK cells act against virus-infected cells by inducing cytolysis, apoptosis and the secretion of inflammatory cytokines. Previous studies have shown that immune responses to HBV infection play dual roles [10]: (1) destruction of hepatocytes and induction of hepatitis [43] and (2) HBV eradication [76]. Based on the fact that NK cells play important roles in immune responses against viral infections [76], this review summarizes recent information regarding NK cell phenotype, functions and modifications. It also discusses the potential for novel immunotherapeutic approaches against hepatitis B infection and its related disorders, including HCC and liver cirrhosis.

General features of NK cells

NK cells play key roles in immune responses against tumors and intracellular pathogens, including HBV [76]. NK cell functions include cytolysis and cytokine secretion, which induces the activation of antigen-presenting cells (APCs) [14, 56].

Cytolysis

The cytolysis pathway needs direct NK cell-target cell contact and the formation of immunological synapses. NK cells lyse target cells using two mechanisms. In the first mechanism, they use interactions between Fas-L (on the NK cell) and Fas (on the target cell) resulting in the activation of apoptosis via the extrinsic pathway [58]. In the second mechanism, NK cells release perforins and granzymes at immunological synapses [76]. Researchers believe that perforins permeabilize target-cell membranes to introduce granzymes to the cytoplasm [76]. Perforin makes a 20-nm pore in target-cell membranes, and granzyme granules are then transported into the target cell [45]. Granzymes are apoptotic effectors with serine protease activities that cleave target-cell proteins after lysine or arginine residues [69]. Granzymes also activate caspases such as caspase 1, 3 and 7 by cleaving the proenzyme forms of the enzyme [86]. To initiate the process, granzyme B cleaves pro-caspase 3 and converts it to its active form, which has several downstream activities, including the activation of DNase, which damages target cell DNA [86]. The overall result of these combined activities is apoptosis of the target cell. Interestingly, granzyme B is also able to kill target cells without the activation of caspases, but these mechanisms are yet to be fully defined [1]. NK cells also use these mechanisms to kill HBV-infected cells [48], hence, they can be considered important immune cells against HBV.

Cytokine production

Previous investigations revealed that pro-inflammatory cytokines play important roles in induction of appropriate immune responses against HBV [10]. NK cells are important for the fight against HBV and for the protection of the liver from hepatitis B complications, and they do this via the production of several pro- and anti-inflammatory cytokines such as interferon-gamma (IFN-γ), tumor necrosis factor-alpha (TNF-α), granulocyte monocyte-colony stimulatory factor (GM-CSF), interleukin-2 (IL-2), IL-22, IL-10 and IL-13 [12, 19, 54, 77]. Interestingly, previous studies demonstrated that the pattern of cytokine expression is disrupted in prolonged forms of hepatitis B infection, causing the serum levels of inflammatory cytokines to decrease and the secretion of anti-inflammatory cytokines to increase [8, 9, 44]. This alteration in cytokine levels may lead to suppression of immune responses against HBV. NK cells are important sources for both inflammatory and anti-inflammatory cytokines, and it appears that the function of NK cells is disrupted by HBV. Additionally, it has been documented that cytokines play important roles during the development of hepatitis-B-related complications including cirrhosis of the liver and HCC [4]; hence, it may be concluded that NK cells play crucial roles in the pathogenesis of liver cirrhosis and HCC via alterations in the secreted levels of cytokine patterns as a result of the initial viral infection.

NK cell/APC interaction

Previous studies demonstrated that NK cells physically interact with APCs, such as B cells, macrophages (MQs) and dendritic cells (DCs), to drive specific responses [64]. NK cell interactions with B cells occur through contact of CD40L or CD154 (on the NK cell surface) to CD40 (on the B cell surface), which leads to antibody secretion, isotype switching and B cell maturation as well as IFN-γ production by NK cells [71]. Antibody production derived from NK cell/B cell interactions only occurs in the spleen [35]. NK cells can also kill CD40-expressing cells, but this process is inhibited when CD40-expressing cells present major histocompatibility complex (MHC) class I on their surface [80]. The crosstalk between NK cells and MQs and DCs by CD40L/CD40 interactions results in IL-12 and IL-18 production by MQs and DCs, and these cytokines have a synergistic effect on the cytotoxic effects of NK cells. In addition, these interactions promote inflammatory cytokine production, including IFN-γ secretion [5]. Furthermore, IFN-γ activates DCs and macrophages to increase IL-12 production [5]. IFN-γ also increases expression levels of the IL-12 receptor on T and NK cells [5]. Additionally, MQs produce inflammatory cytokines, including IL-15 and

IL-27, in response to viral infection, leading to NK cell differentiation and activation [15]. Therefore, it appears that NK cells not only kill HBV-infected cells directly but also suppress HBV replication via crosstalk with other immune cells such as macrophages and DCs to induce them to produce pro-inflammatory cytokines such as IL-12. IL-12 is an important innate immune cytokine that plays crucial roles in induction of pivotal immune responses against HBV [9].

NK cell receptors

NK cells are the main arm of the innate immune system, which combats viral infections, including hepatitis B [21]. *In vivo* and *in vitro* studies have demonstrated that NK cells express several stimulatory and inhibitory receptors [20]. The four main families of molecules that are considered stimulatory receptors include (1) receptors associated with immunoreceptor tyrosine-based activation motif (ITAM)-coupled adaptor proteins, (2) receptors containing ITAM motifs in their cytoplasmic domains, (3) receptors containing no ITAM activation motif, and (4) adhesion molecules [20]. One family of receptors inhibits NK cell functions via immunoreceptor tyrosine-based inhibition motifs (ITIMs) within their cytoplasmic domains, which leads to phosphatase recruitment [20]. Table 1 lists all of the recently discovered NK cell stimulatory and inhibitory receptors. In addition to the mentioned receptors, NK cells carry several chemokine and cytokine receptors that modulate NK cell activation and recruit them to inflamed tissues. Table 2 lists NK cell chemokine and cytokine receptors. It is well established that the balance between signals from activating receptors and inhibitory receptors regulates NK cell activation [33]. Therefore, interactions between NK cell receptors and their corresponded ligands determine the fate of NK cells [33]. Interestingly, several viruses, including HBV, use various mechanisms to engage the inhibitory receptors of NK cells to prevent NK cell activation. The mechanisms that are used by HBV to suppress NK cells function are further discussed in section 3.

NK cell subtypes

Recent studies have demonstrated that there is heterogeneity in the responses of peripheral blood NK cells, suggesting that there are several subpopulations of NK cells in humans [39]. Characterisation of these cells has confirmed the hypothesis and revealed the existence of NK cell subpopulations, including $CD56^{dim}$ $CD16^{+}$, $CD56^{bright}$ $CD16^{+}$, thymic, NK22 and memory NK cells.

The $CD56^{dim}$ $CD16^{+}$ cell subpopulation is distinguished by its high expression of CD16 (FCγRIII) with respect to other NK cells, and these cells are the principal cytotoxic population [39]. This population is the main NK cell population that is active against virally infected cells and tumors [29]; hence, impaired $CD56^{dim}$ $CD16^{+}$ cells can lead to incomplete viral eradication. Accordingly, it has been documented that decreased numbers of $CD56^{dim}$ $CD16^{+}$ NK cells led to HBV persistence [81], which is discussed in the section 3. Therefore, it can be concluded that this subset of cells plays key roles in the fight against HBV. These cells constitute the majority of NK cells circulating in the peripheral blood and are considered developmentally mature [29]. It has been established that $CD56^{dim}$ $CD16^{+}$ cells predominantly mediate cytotoxicity functions, whereas $CD56^{bright}$ cells (see section 4.2.) principally secrete inflammatory cytokines, including IFN-γ [36].

$CD56^{bright}$ $CD16^{+}$ cells are the principal cytokine-producing subpopulation. More specifically, they are the main source of IFN-γ among the NK cell populations [36]. IFN-γ is an important cytokine that participates in stimulation of APCs to present HBV antigens and produce pro-inflammatory cytokines including IL-12, IL-6, and others [29, 42]. In contrast to $CD56^{dim}$ cells, $CD56^{bright}$ CD16 cells are a minority of the NK cells circulating in the peripheral blood and are thought to be at an earlier stage of maturation [36].

Cells of the thymic population expresses Gata3 and IL-7 receptor alpha (IL-7Rα) as their main markers and produce cytokines in high volumes while expressing low amounts of LY49, CD11b and granzyme B and hence are unable to lyse virus-infected cells as well as $CD56^{dim}$ $CD16^{+}$ cells [84]. Considering the important roles played by this subset of cells in cytokine production during viral infections, and based on the fact that prolonged-term HBV-infected patients suffer from altered cytokine patterns [8–10], there is potential to target thymic NK cells in the future as a target for immunotherapy against HBV.

NK22 subsets are the main IL-22-producing NK cells, and they express granzyme B and other cytokines at lower levels than other subpopulations [77]. NK22 cells show a $CD56^{+}$, $CD117^{high}$ and $CD94^{-}$ phenotype [77]. It has been documented that IL-22 is an important cytokine in the pathogenesis and outcome of HBV infection. To the best of our knowledge, there are no data regarding the status of NK22 in hepatitis B [27]. Therefore, more research is required before these cells can be considered a new candidate for study of the etiology of hepatitis B disease progression; however, they should not be ruled out as potential targets in treatments to eradicate HBV from the host.

Memory NK cells are killer cell lectin-like receptor subfamily G member 1 (KLRG1) positive and have a potent capacity for both cytolysis and cytokine production [68].

Table 1 Inhibitor, activator and cell adhesion molecules on NK cells

Function	Receptor	Ligand
Inhibitor		
ITIM-associated inhibitor	NKG2A (CD94/159a)	HLA-E
	LIR-1/ILT2 (CD85j)	Multiple HLA class1 alleles
	KLRG1	E/N/R-cadherin
	KLR2DL1 (CD158a)	HLA-C (C2 group)
	KLR2DL2/3 (CD158b)	HLA-C (C1-group)
	KIR2DL5 (CD158f)	?
	KIR3DL1 (CD158e1)	HLA-B alleles
	KIR3DL2 (CD158K)	HLA-A (A3,A11)
	KIR3DL3 (CD158z)	?
	LAIR-1 (CD305)	Collagen
	Siglec-7 (CD328)	Sialic acid
	Siglec-9 (CD329)	Sialic acid
	IRp60 (CD300a)	?
	NKR-P1 (CD161)	LLT1
	CEACAM-1 (CD66a)	CEACAM
	TIGIT	CD155,CD112
Activator		
ITAM-associated activator	CD16 (FCγRIIIA)	IgG
	NKP46 (CD335)	Viral haemagglutinin
	NKP30 (CD337)	B7-H3
	NKG2C (CD94/159c)	HLA-E (have low affinity)
	KIR2DS1 (CD158h)	HLA-C (C2, have low affinity)
	KIR2DS4 (CD158i)	HLA-A,C (have low affinity)
	KIR2DS2 (CD158j)	?
	KIR3DS1 (CD158e2)	HLA-B (BW4 have low affinity)
	KIR2DS5 (CD158g)	?
	KIR2DS3-6	?
Non-ITAM-associated activator	CD2	LFA-3 (CD58)
	CD7	Galectin, SECTM1
	CD44	Hyaloronan
	CD59	C8,C9
	DNAM-1 (CD266)	PVR (CD155), CD112
	NKp65	KACL
	NKp80 (KLRF-1)	AICL
	NKG2D (CD314)	ULBPs, MICA, MICB
	2B4 (CD244)	CD48
	KIR2DL4 (CD158d)	HLA-G (soluble)
	CRACC (CD319)	CRACC (CD319)
	NTB-A	NTB-A
	BY55 (CD160)	HLA-C
Cell adhesion		
Integrin	VLA-4 (α4β1,CD49d/29)	VCAM1, fibronectin
	VLA-5 (α5β1,CD49e/29)	Fibronectin
	VLA-6 (α6β1,CD49f/29)	Laminin
	CR3 (αMβ2,CD11b/18)	LPS,ICAM-1, fibrinogen, iC3b, β-glucan
	CR4 (αxβ2,CD11c/18)	ICAM-1, iC3b, CD23 LPS, fibrinogen
	LFA-1 (αLβ2,CD11a/18)	ICAM1-5

Table 2 Chemokine and cytokine receptors on NK cells

Chemokine receptors
CCR1
CCR2
CCR3
CCR4
CCR5
CCR6
CCR7
CCR8
CXCR1
CXCR2
CXCR3
CXCR4
CXCR5
CX3CR1
XCR1
Cytokine receptors
C-kit
IL-2R
IL-15R
IL-18R
IL-7R
IL-1R1
IL-10R
IL-21R
IL-12R
IL-4R
TNF-R
IL-23R

This population has a low threshold of activation and therefore responds to target cells expressing low activator ligand levels [68]. Like NK22 cells, the status and relationship between memory NK cells and hepatitis has not yet been clarified.

NK cells and hepatitis B

The actions of NK cells represent the main immune responses against hepatitis B infection, and typically there are elevated numbers of these cells in the peripheral blood and liver of HBV-infected patients [6]. Individuals that are capable of clearing HBV have good NK cell responses against HBV, which leads to the eradication of HBV from the infected liver [94]. Zhao et al. [94] reported that HBV-infected patients have abundant NK cells in their liver in comparison to healthy controls. Interestingly, their report revealed that NK cells of acutely infected patients have more activating receptors and fewer inhibitory receptors than those of chronic infected patients or healthy controls [94]. In addition, they showed that NK cell activation levels were correlated negatively with HBV DNA levels in acutely infected patients [94]. They also followed up the patients with acute hepatitis and found that NK cell activation was positively correlated with HBV clearance [94]. Yin et al. [90] also demonstrated that IL-15 administration led to upregulation of IFN-β in the liver of an animal model and subsequently suppressed HBV replication. This is not surprising, since IL-15 is the main cytokine for NK cell maturation and activation. Another study demonstrated that adoptive transfer of granzyme-H-overexpressing NK cells into HBV-infected mice led to HBV eradication [76]. Li and colleagues demonstrated that NK cells are involved in the early, nonspecific immune responses to HBV clearance [50] and reported that NK cell frequency was negatively correlated with the numbers of HBcAg-specific cytotoxic T lymphocytes [50]. Based on the studies mentioned above, the evidence suggests that NK cells play crucial roles in immune responses against HBV, and correctly orchestrated NK cell responses can lead to complete HBV eradication from the infected liver.

Interestingly, NK cells play important roles in eradication of HBV, but NK cell function is disrupted in patients with prolonged HBV infection; hence, it is plausible that HBV infection causes aberrations in NK function, leading to persistence and long-term infection of some patients. For instance, previous studies demonstrated that HBV can downregulate MHC class 1 expression on infected cells, which can lead to evasion of T cytotoxic immune responses [24]. Downregulation of MHC class 1, which is the inhibitory ligand for NK cells (Table 1), leads to NK cell activation and killing of infected cells (Fig. 1) [50]. Therefore, it appears that in some cases HBV is able to escape from host adaptive immunity through the downregulation of MHC molecules, which may in turn lead to persistent infection. However, downregulation of MHC class 1 in infected cells results in activation of NK cells. Evidence has demonstrated that the functions of NK cells and their related pro-inflammatory molecules are disrupted in long-term hepatitis B. For instance, our previous studies revealed that the number of NK cells expressing C-C chemokine receptor type 5 (CCR5) was significantly decreased in occult [7] and chronic (unpublished data) HBV infection. Li et al. [51] also demonstrated that NK cells from chronically HBV-infected patients with high levels of HBV DNA replication produced lower levels of IFN-γ than the normal control group. Ratnam and colleagues [65] showed that NK cells display impaired responses to Toll-like receptor 9 (TLR9) in chronically HBV-infected patients. TLR9 is one of the main innate immune cell receptors that recognize pathogen-associated molecular patterns (PAMPs), especially viral dsDNA [85].

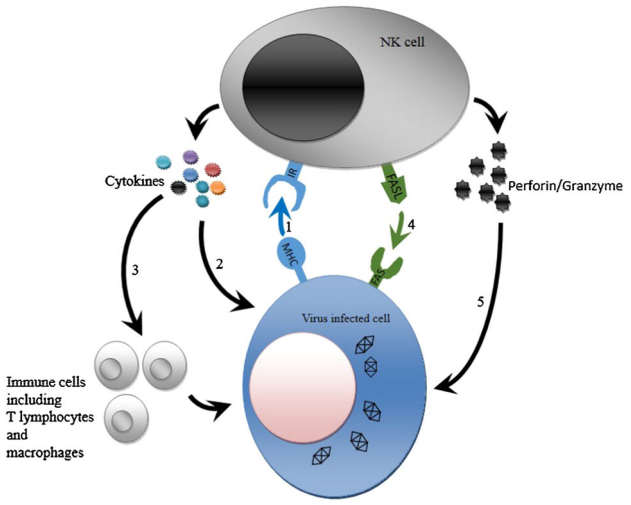

Fig. 1 The roles of NK cells against HBV infection. The figure illustrates the pivotal roles of NK cells in the killing of virus-infected cells. Virus-infected cells downregulate MHC class I (1); hence, the repression of NK cells by its inhibitory receptors (IR) is released and the NK cells are activated. Activated NK cells secrete proinflammatory cytokines, which directly (2) or indirectly (3) kill the infected cells. NK cells also express FASL molecules, which induce apoptosis of infected cells via interaction with FAS molecules (4). Finally, NK cells directly kill HBV-infected cells via production and secretion of perforin/granzyme complexes (5)

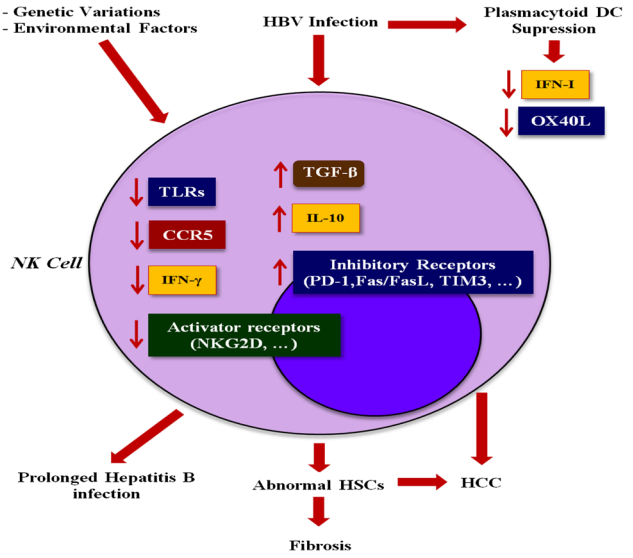

Fig. 2 Illustration of the processes of induction of tolerance to HBV. HBV infection can induce tolerance in NK cells via the downregulation of inflammatory cytokines (especially IFN-γ), TLRs, chemokine receptors (such as CCR5), and activation receptors while also causing the upregulation of anti-inflammatory cytokines (such as IL-10 and TGF-β) and inhibitory receptors. HBV infection also alters plasmacytoid DC functions, leading to NK cell inactivation. NK cell inactivation results in prolonged HBV infection, which can progress to fibrosis and HCC

Therefore, it seems that the activation of NK cells is defective in that patient cohort. Previous studies have also demonstrated that the serum levels of TGF-β, an immune inhibitory cytokine, were increased in chronically HBV-infected patients [44]. TGF-β downregulates natural killer group 2, member D (NKG2D)/DAP10 and 2B4/SAP expression on NK cells in chronically HBV-infected patients [74]. However, NKG2D is a non-ITAM-associated activator receptor for NK cells (Table 1); hence, it appears that there is a correlation between the high expression of TGF-β and impaired NK cell function, which may be another factor responsible for persistent HBV infection. Cao et al. found that expression levels of Programmed Death-1 (PD-1), an immune response inhibitory molecule, were increased on the hepatocytes of chronically HBV-infected patients [22]. Based on the fact that NK cell functions are regulated directly by PD-1 molecules via decreased NK cell cytotoxicity [13] and pro-inflammatory cytokine production [87] and also indirectly through increased numbers and functions of regulatory T lymphocytes [38], it seems reasonable to speculate that NK cell functions are disrupted in the liver of chronically HBV-infected patients. Bonorino et al. also showed that HBV can affect NK cell subsets according to the status of the disease [18]. They reported that levels of NKG2A-expressing NK cells were significantly decreased in HBV patients when compared to healthy controls [18]. These researchers also demonstrated that the percentage of NK $CD56^{bright}$ $CD16^{+}$ cells was increased, while NK $CD56^{dim}$ $CD16^{+}$ cell numbers in the liver of HBV-infected patients decreased [18]. Therefore, it appears that NK cell numbers and functions are disrupted in prolonged forms of HBV infection.

The main mechanisms that leads to impaired functions of NK cells in prolonged forms of hepatitis B has yet to been clarified, but it appears that HBV can affect NK cell functions both directly and indirectly. In addition, it seems that genetic variations within genes that regulate NK function suppress their activity, and these are discussed in the next paragraphs.

Studies have been performed to determine the direct and indirect inhibitory effects of HBV on NK cells. For example, a study showed that HBV infection can cause increased expression of immunoglobulin- and mucin-domain-containing molecule 3 (Tim-3) on NK cells in chronically HBV-infected patients [40]. Tim-3 is an inhibitory molecule that suppresses NK cell function; hence, HBV can suppress NK cell function by the upregulation of Tim-3 [40]. Previous studies also demonstrated that plasmacytoid DCs (pDCs) initiate antiviral immunity by the production of type 1 interferons and also by modifying NK cell function (Fig. 2) [75]. Interestingly, Shi et al. [72] reported that HBV significantly suppresses pDC-induced IFN-γ production by NK cells. They also revealed

that HBV does not directly affect IFN-γ production by NK cells cultured alone; hence, it seems that the pDC-NK cell interaction plays an important role in the suppressive effect of HBV on NK cell function [72]. These findings were supported by similar results reported by Martinet and colleagues [55]. They demonstrated that pDC of chronically HBV-infected patients express lower OX40L when compared to healthy controls [55]. Their results also revealed that incubating pDCs derived from healthy control subjects with pDC from chronically HBV-infected patients led to downregulation of OX40L on the pDCs of healthy controls [55]. OX40L is an NK cell activator molecule; hence, it seems that HBV can suppress NK cells by modifying pDCs. Therefore, it appears that HBV can suppress NK cell functions via upregulation of anti-inflammatory cytokines and downregulation of pro-inflammatory cytokines and co-stimulatory molecules. Thus, it seems that some aspects of attenuated immune responses observed in patients with prolonged HBV infection are related to impaired NK cell functions caused by the infection.

However, as mentioned above, the observed impaired functions may not all necessarily be confined to inhibitory affects initiated by HBV, and it would appear that genetic variations within the host also affect NK cell function. For example, Park et al. [61] reported that genetic variation of granulysin (a granule enzyme of NK cells) is associated with chronic HBV infection. Ma et al. [53] have analysed NKG2D polymorphisms in chronic hepatitis B patients of a Han Chinese population and found that the polymorphism rs2617160 in the NKG2D gene is associated with susceptibility to hepatitis B. Another study on the Chinese population demonstrated that in the killer cell immunoglobulin-like receptors, short cytoplasmic tail 2 (KIR2DS2) and KIR2DS3 gene polymorphisms are negatively associated with HBV clearance. Conversely, KIR2DS1, KIR3DS1 and KIR2DL5 are positively associated with HBV clearance [95]. In addition, Lu et al. [52] revealed that KIR haplotypes 4 and 5 are markers of HBV clearance susceptibility and protection, respectively. These studies demonstrate that NK-cell-related genes and molecules play important roles in inducing appropriate immune responses against HBV. Therefore, it appears that NK cells are a promising target for future immuno- and gene therapies to induce host responses against HBV to overcome prolonged HBV infections.

NK cell responses and liver damage

Apart from the important role of NK cell functions that are required for the eradication of HBV from infected hepatocytes, they also play a pivotal role in liver complications, including liver cirrhosis and HCC, especially following viral hepatitis [11]. Thirty to fifty percent of liver lymphocytes are NK cells that migrate from peripheral blood into the liver to produce liver-specific NK cells, and this specific NK cell population has higher levels of cytotoxicity function against tumors and virus-infected target cells in comparison to NK cells from other organs [89]. NK cells extracted from normal livers, but not from livers with cirrhosis, were able to kill the human HCC cell line HepG2 [93]. On the other hand, another study in animal models revealed that NK cells inhibit liver fibrosis [25]. Interestingly, studies in humans also demonstrated that NK cells may play a greater role in inhibiting liver fibrosis in humans than in mice [30]. Therefore, it seems that NK cells are important in the response against virus-infected hepatocytes and HCC as well as in the control of fibrosis. It appears that this balance changes during viral hepatitis, when infected hepatocytes produce inflammatory cytokines, including IL-12 and IFN-α/β, which activate cytotoxicity and infiltration of NK cells into the infected liver. However, the increased cytotoxicity is lower in patients with chronic HCV infection when compared to healthy controls [59]. This suggests that although intrahepatic NK cells exhibit higher activity than peripheral NK cells under normal conditions, their activity in HCV and HBV infection is decreased [59].

Glassner and colleagues [32] reported that human NK cells play crucial roles in controlling liver fibrosis in HCV patients by killing hepatic stellate cells (HSCs). Recent data demonstrated that HSCs play an important role in the development of liver fibrosis [63]. Although HSCs play a central role in the storage of retinol (a vitamin A compound) in normal liver, they change during injury, becoming activated and transdifferentiated into myofibroblasts (matrix-producing cells) [34]. The activation of HSCs is regulated by cytokines, growth factors, and immune cells, including NK cells [34]. Therefore it would appear that abnormal HSC killing is one of the mechanisms of NK cells to control fibrosis, and impaired NK cell functions may lead to an increased incidence of liver complications, including cirrhosis and HCC. In parallel with this conclusion, several studies have demonstrated that NK cell functions are impaired during cirrhosis and HCC, especially after viral infection. Ishimaru et al. [37] demonstrated that IL-2-positive NK cell levels were lower in HCC patients than in HBV ASC patients and healthy controls. IL-2 production is an activating marker of NK cells; therefore, it seems that the activity of NK cells decreases in patients with HBV-induced HCC.

Lee et al. [46, 47], in two different studies, demonstrated that there were no differences between HCC patients and healthy controls regarding NK cell numbers, while their results revealed that NK cell activity was significantly decreased in HCC patients in comparison to healthy

controls [46]. Nakamura and colleagues [57] also reported that NK cell activity was decreased in patients with liver cirrhosis. As mentioned in the previous section, NKG2D is an activation marker of NK cells [96]. However, Zheng et al. [92] showed that either NK cell activity or NKG2D expression were decreased in HCC patients when compared to healthy controls. Based on the data presented, it may be concluded that NK cells play crucial roles in controlling fibrosis, cirrhosis and HCC, especially in hepatitis patients, and further studies will be helpful to confirm these findings. Figure 2 illustrates the effect of HBV infection on NK cell function based on the reported data and shows the various interactions and stimuli that can influence NK function. Targeting these pathways could be considered candidate approaches for the treatment of persistent HBV infection. However, we must take into account genetic factors that can influence patient responses to viral infection or treatment if we are to consider immunological manipulation of NK cells as a therapy for viral hepatitis and liver complications. We discuss recent NK-cell-based therapies against viral hepatitis and liver complications in the next section.

NK cells as potential targets for hepatitis B immunotherapy

Currently, there are seven drugs that are used for the treatment of hepatitis B [83]. All of these drugs inhibit HBV replication and disease progression [83]. The seven drugs are categorized in two man classes, including nucleos(t)ide analogues (NAs) and interferons [70]. Lamivudine, adefovir dipivoxil, entecavir, telbivudine and tenofovir disoproxil fumarate are NAs, while, standard and pegylated interferons belong to the interferon class [83]. The advantages of these therapies are as follows: (1) administration of these drugs is easy, (2) tolerance and safety of NAs are excellent, and (3) HBV eradication is excellent in HBeAg-negative patients [70]. Although these drugs have several advantages, they are also associated with several limitations: (1) chronically HBV-infected patients need long-term therapies with NAs drugs [60], (2) use of interferons is associated with adverse events and affects the patient's quality of life, (3) the cost of the drugs is high, making them inaccessible to patients with low incomes, (4) none of these drugs can eliminate HBV covalently closed circular DNA (cccDNA) from liver cells, (5) long-term therapies with NAs drugs results in genetic drug resistance in HBV [83]. Therefore, it appears that novel therapeutic approaches are strongly needed to eliminate HBV particles from infected patients. It seems that immunotherapies using immune cells, including NK cells, against HBV in prolonged-term HBV-infected patients can be considered for future treatment of hepatitis B. Accordingly, Li et al. [49] showed that blocking the NK cell inhibitory receptor NKG2A increases the activity of NK cells and clears HBV from the infected liver. Another study demonstrated that blocking immunosuppressive cytokines, including IL-10 and TGF-β, increased the antiviral function of NK cells in chronic HBV infection [62]. Also, Tjwa et al. reported that restoration of myeloid dendritic cells, which are potent inducers of NK cells, leads to improved NK cell function in patients with chronic hepatitis B [82]. The same study also demonstrated that HBV viral load reduction led to activation of NK cells in chronic HBV infection [81]. Zhu et al. [97] reported that using a DNA-based immunization led to maturation and activation of NK cells and consequently HBV clearance in an animal model. Another study demonstrated that NK cell activation inhibits HBV replication *in vivo* [41]. It has also been established that the cross-talk between NK cells and DCs regulates the functions of adaptive immunity [91], which could significantly improve patient outcomes. Interestingly, several studies have used human recombinant IL-12 (rhIL-12) directly without HBV antigens [23] and with lamivudine [67] in chronically HBV-infected patients and shown that IL-12 reduces HBV copy numbers in those patients. It seems that activation of NK cells is one of the effector mechanisms of immunotherapy through the use of cytokines. Therefore, it may be concluded that immunotherapy using APCs may also trigger NK cells to respond against HBV. These data indicate that NK cells can be considered a potent target for use in immunotherapies directed against hepatitis B. Additionally, it has been demonstrated that other immune cells, including T cytotoxic and helper lymphocytes [3, 88], B lymphocytes, neutrophils [79] and APCs including DCs and macrophages [17], participate in the induction of appropriate immune responses against HBV. For instance, T cytotoxic lymphocytes are activated via recognition of HBV antigens on self MHC class 1 molecules and kill infected cells [78]. T helper lymphocytes produce pro-inflammatory molecules such as cytokines and co-stimulatory molecules following recognition of HBV antigens on self MHC class 2 molecules [31]. Also, APCs participate in immune responses against HBV via phagocytosis of the virus, and viral components are either presented as antigens on MHC class 2 or result in the production of pro-inflammatory cytokines as well as the ligands for co-stimulatory molecules (such as CD80 and CD86) for T helper cells [16]. Neutrophils also fight against HBV via phagocytosis and the production of pro-inflammatory cytokines such as IL-8 [73]. Therefore, it appears that immunotherapy directed against HBV using activators of NK cells as well as the aforementioned immune cells can be considered important candidates for future hepatitis B therapy.

Concluding remarks

The published data regarding the roles of NK cells in HBV infection overwhelmingly suggest that these cells not only play important roles in the eradication of HBV from the infected hepatocytes but are also important factors that inhibit the development of hepatitis-B-related liver diseases including fibrosis, cirrhosis and HCC. Based on the important roles of NK cells, future therapies could be directed towards restoring NK cell functions as an intervention to inhibit liver injuries via several mechanisms, which could include the suppression of HSC development. These therapies could also extend to the treatment of virus-induced liver cancer. Although some of the data are still controversial, several studies have indicated that NK cell functions, including cellular cytotoxicity and cytokine production, are disrupted in chronic HBV infections. Therefore, HBV infection may persist in the liver, potentially leading to fibrosis, cirrhosis and HCC. On the other hand, the mechanisms of immune and antiviral therapy to improve NK cell functions have not yet been clarified, and future research should be focused on using novel therapies to improve NK cell function during HBV infection for the treatment of long-term HBV disease and the control of HBV-related liver damage.

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