

## Natural Killer Cell Dysfunction in Hepatocellular Carcinoma: pathogenesis and clinical implications

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## Abstract

Hepatocellular carcinoma (HCC) is currently the third leading cause of malignancy-related mortalities worldwide. Natural killer (NK) cells are involved in the critical role of first line immunological defense against cancer development. Defects in NK cell functions are recognized as important mechanisms for immune evasion of tumor cells. NK cell function appears to be attenuated in HCC, and many previous reports suggested that NK cells play a critical role in controlling HCC, suggesting that boosting the activity of dysfunctional NK cells can enhance tumor cell killing. However, the detailed mechanisms of NK cell dysfunction in tumor microenvironment of HCC remain largely unknown. A better understanding of the mechanisms of NK cell dysfunction in HCC will help in the NK cell-mediated eradication of cancer cells and prolong patient survival. In this review, we describe the various mechanisms underlying NK cell dysfunction in HCC. Further, we summarize current advances in the approaches to enhance endogenous NK cell function and in adoptive NK cell therapies, to cure this difficult-to-treat cancer.

## 1. Introduction

Worldwide, hepatocellular carcinoma (HCC) is the third leading cause of cancer-related mortalities and the sixth most predominant type of malignancy (1). The majority of patients with HCC are diagnosed at an advanced stage, contributing to the very low rate of five-year survival (2). HCC often develops in patients with a history of chronic hepatitis B virus (HBV) and hepatitis C virus (HCV) infections (1). In addition, obesity and diabetes, which cause non-alcoholic steatohepatitis (NASH), have become significant risk factors for the development of HCC in patients from developed countries, in recent years (1, 3). Antiviral therapies for HCV and HBV assist in reducing HCC occurrence (2), but the limitations of accessibility to these antivirals in developing countries and a rising incidence of NASH, guarantee that HCC is going to remain an impending perplexing disease (2).

It is challenging to treat patients with HCC in the advanced stage. The two drugs that FDA has approved for treating advanced HCC, as a first line therapy, are sorafenib and lenvatinib (4-6). Nevertheless, only a modest survival improvement, as well as the substantial adverse effects, underscore the need for new therapeutics (5). After decades of failure of immune therapies, immune checkpoint inhibitors have been proven as effective treatments for patients with advanced HCC (7, 8). Monoclonal antibodies that are able to block immune checkpoint molecules displayed antitumor activity against HCC (8). Clinical trials were performed with two checkpoint inhibitors in HCC: the anti-cytotoxic T lymphocyte-associated antigen 4 (CTLA-4) agent tremelimumab (9) as well as the anti-programmed death 1 (PD-1) agent nivolumab (10). The latter has shown unprecedented antitumor activity in both sorafenib-naïve and sorafenib-experienced patients with advanced HCC (10). However, the response rate of nivolumab was still below 20 percent (10). Novel approaches that can harness antitumor immune response are therefore urgently required for HCC.

The liver possesses a uniquely tolerogenic immune environment. The liver contains a diverse population of innate lymphocytes, such as NK cells, natural killer T (NKT) cells, as well as adaptive lymphocytes like B cells and T cells (11). Immune cells that reside in the liver are exposed to numerous antigens through the portal circulation and therefore, unfavorable immune responses are easy to be triggered (12, 13). It appears that the liver has attained specialized immune tolerance mechanisms to evade the undesirable immune over-activation (14). The development of chronic infectious disease by HCV or HBV, also appears to be related to the liver's tolerogenic properties (14). Furthermore, immune dysfunction is also associated with liver cirrhosis, which is the final outcome of unresolved hepatic inflammation and fibrosis (7).

HCC represents a classic paradigm of inflammation-associated malignancy, since most of the tumors are arising in the context of hepatic inflammation and the resultant fibrosis (15). The risk factors of HCC usually provoke a non-resolving inflammatory response characterized by infiltration of lymphocytes, macrophages, and immature myeloid cells. These immune cells produce various kinds of inflammatory cytokines, resulting in the perpetuation of the wound-healing response that ends up with cirrhosis and HCC (15). In the early hepatocarcinogenesis, the natural killer (NK) cells have critical functions. NK cells are innate lymphocytes that are capable of killing virus-infected or cancer cells (16). Deregulation of many NK cell-related genes was identified in the early stages of hepatocarcinogenesis in a genetically engineered mouse model of HCC (17), suggesting that the disruption of immune surveillance by NK cells denotes the onset of HCC development (17).

In this review, we will discuss the mechanisms of NK cell dysfunction in patients with HCC. Then, we will summarize the recent developments and future directions in HCC

management by enhancing the functions of endogenous NK cells and by infusing patients with cultured NK cells.

## 2. Biology of human NK cells

NK cells originate from the bone marrow, where they develop in a stepwise manner, leading to the progressive maturation in their functions and phenotypes (18). NK cells mediate cytotoxicity and the cytokine-producing effector functions (19). NK cells are defined as CD3<sup>-</sup>CD56<sup>+</sup> cells in humans and CD3<sup>-</sup>NK1.1<sup>+</sup> or CD3<sup>-</sup>NKp46<sup>+</sup> cells in mice (20). In humans, two main subsets of NK cells, the CD56<sup>bright</sup> and the CD56<sup>dim</sup> NK cells are distinguished by their cell surface CD56 levels. Amongst them, the CD56<sup>dim</sup> NK cells constitute approximately 90% of the total NK cells, show mature phenotype, and mediate cytolytic reactions. In contrast, the immature CD56<sup>bright</sup> NK cells constitute 5% to 15% of the total NK cells and have been regarded as cytokine producers (21, 22). The most important cytolytic targets of NK cells are the abnormal cells having the downregulated expression of the major histocompatibility complex (MHC) class I, which is highly expressed on the surface of most of the healthy, nucleated cells (23). NK cells can be quickly activated when they encounter virus-infected cells or tumor cells, since the loss of MHC class I is a common mechanism in these cells to avoid recognition by the cytotoxic T cells (24).

Activated NK cells rapidly establish dynamic contacts with the target cells via integrins, such as the leukocyte functional antigen-1, followed by the exocytosis of perforins and granzymes which perforate the plasma membrane and elicit apoptosis (25). Death-receptor pathways involving Fas ligand and TNF-related apoptosis-inducing ligand (TRAIL) are also used to kill the target cells. Mature NK cells are not activated when they contact normal cells that express MHC class I molecule, which can bind to inhibitory receptors on the surface of

NK cells. The inhibitory receptors recruit tyrosine phosphatase to remove the phosphates from several signaling proteins or lipids that were generated by the activating receptors (25). When a NK cell comes across abnormal cells lacking the MHC class I, inhibitory receptors are not engaged, and the killing of the target cells is triggered by the unsuppressed activating signals (26). In this manner, NK cell function is regulated by a dynamic balance between signals produced from the activating and inhibitory receptors (26).

Among the activating receptors of NK cells, Fc $\gamma$ RIIIA (CD16) is the only receptor that can individually evoke the signal that is sufficient enough to stimulate degranulation after crosslinking by monoclonal antibody (27). CD16 binds to the Fc portion of immunoglobulins and transmit activating signals in NK cells (28). Once activated by Fc receptors through monoclonal antibodies that are bound to target cells, NK cells kill target cells with cytotoxic molecules, and secrete cytokines to recruit the adaptive lymphocytes. This is known as the antibody-dependent cell cytotoxicity (ADCC) function, which is the primary mechanism of action for some monoclonal antibodies (28). All other activating receptors such as the NK group 2D (NKG2D), natural cytotoxicity receptors (NCRs), DNAX Accessory Molecule-1 (DNAM-1), 2B4, and CD2 can only work in combination with each other (26).

NK cells have not only cytotoxic function but also immunoregulatory roles that can have positive or negative influences on the overall immune responses by modulating the function of T cells and dendritic cells (DCs) (20). NK cells secrete proinflammatory cytokines and chemokines such as TNF- $\alpha$ , IFN- $\gamma$ , IL-6, and CCL5 that may contribute to innate and adaptive immune responses (20). Furthermore, NK cells respond to a variety of cytokines and chemokines from the external environment. Cytokines such as the interleukin (IL)-2, IL-12, IL-15, and IL-18 are strong activators of NK cells. These cytokines have been applied for NK cell-based immunotherapy against various types of cancers (29). Responding

to these cytokines, NK cells secrete cytotoxic molecules such as perforin, granzyme, IFN- $\gamma$ , TNF- $\alpha$ , and other chemokines and cytokines.

### 3. Intrahepatic NK cells: liver-resident or liver-infiltrating

Human liver has a large population of NK cells which form between 30% to 50% of the intrahepatic lymphocytes (30). This percentage is two to five times the number of peripheral NK cells. In humans, NK cells normally exist in the liver sinusoids (31). Previous reports have demonstrated that up to 80 percent of intrahepatic NK cells are liver-resident (30, 32-36). Human liver-resident NK cells are characterized by expression of CXCR6 and CD69, have a distinct transcriptional and functional signature, and display memory-like features (37). This subset expresses CD49a, and has a narrow killer-cell immunoglobulin-related receptor (KIR) profile that indicates a clonal-like expansion (37). These human liver-resident NK cells survive for a relatively long duration in the liver and are incapable of recirculation, whereas the liver-infiltrating NK cells have the transcriptional characteristics of the peripheral NK cells (34). Furthermore, the liver-resident NK cells have been found to have some attributes related to the tolerogenic characteristics of the liver (36, 38). Compared to the NK cells found in peripheral blood, liver-resident NK cells express the inhibitory receptor natural killer group 2 member A (NKG2A), which binds to the human leukocyte antigen (HLA)-E in humans, and MHC class I-associated protein Qa-1 in mice. Tolerogenic immune profile of the liver may partly be influenced by the expression of NKG2A on the surface of NK cells (39).

In the liver, NK cells actively interact with other immune cell subsets, hepatocytes, and stellate cells. NKT cells, DCs and Kupffer cells can stimulate the activation of NK cell by producing various cytokines, such as type I interferon (IFN), IFN- $\gamma$ , IL-2, IL-12, IL-15, and IL-18 (30, 39). On the other hand, certain cells can inhibit the functions of NK cell by

producing IL-10 and transforming growth factor- $\beta$  (TGF- $\beta$ ) (30). Activated NK cells attack the cholangiocytes, hepatic stellate cells, and hepatocytes, and carry out a range of essential roles in the pathogenesis of liver diseases (30, 39).

#### 4. NK cells in chronic viral hepatitis

The tolerogenic properties of the liver make it vulnerable to pathogens and sustained chronic infection. In fact, several widespread pathogens, including HCV and HBV, preferentially attack the liver and cause persistent infections. Co-culture experiments demonstrated that NK cells suppress HCV replications by the production of IFN- $\gamma$  (40). Earlier genetic studies on KIRs and HLA in HCV-exposed individuals demonstrated the critical function of NK cells in HCV infection (41). This study was the first to show that the spontaneous HCV clearance is linked to the KIR2DL3/HLA-C1 genotype (41). In a study performed in Korea, a lower frequency of KIR2DS2 was reported among patients with chronic HCV infection compared to healthy controls, suggesting that KIR2DS2 might facilitate HCV clearance by enhancing the innate immune response (42).

During chronic HCV infection, NK cells are functionally deviated toward increased cytotoxicity and decreased IFN- $\gamma$  production, by chronic exposure to type I IFNs (43). This phenomenon is caused by the increased level of signal transducer and activator of transcription 1 (STAT1), and the preferential phosphorylation of STAT1 over STAT4 in NK cells by type I IFN (44, 45). As a consequence, NK cells display accentuated cytotoxicity and TRAIL upregulation, rather than non-cytolytic IFN- $\gamma$  production (46, 47).

Activated NK cells might suppress HBV and contribute to HBV clearance during acute HBV infection (48). However, in chronic HBV infection, NK cells are functionally altered similar to that in chronic HCV infection. In particular, their capacity for IFN- $\gamma$  and TNF- $\alpha$

production is reduced, while their cytotoxic activity is maintained and the TRAIL, CD38, and Ki-67 expressions are increased (49-51). This deviated NK cell function in chronic HBV infection, suppresses the HBV-specific T-cell function (52). This “inflammatory” phenotype of the NK cells significantly disappears when the viral DNA titer is reduced after successful antiviral therapy (52). The functional alteration of NK cells in HBV infection is caused by the immunosuppressive cytokines such as IL-10 and TGF- $\beta$  (50, 53).

Chronic liver diseases finally lead to the deposition of extracellular matrix and fibrosis (31, 54). At this stage, the human intrahepatic NK cells take an anti-fibrotic role by targeting the activated stellate cells, which is the main cell type that deposits the extracellular matrix (54). However, it is still not clear whether these anti-fibrotic functions are performed by the conventional NK cells or the liver-resident NK cells.

## 5. NK cell dysfunction in HCC

NK cells play critical roles in the surveillance and control of HCC. However, NK cells are dysfunctional in the microenvironment of HCC, and various mechanisms seem to be involved in their malfunction.

### 5.1. NK cell dysfunction in HCC: Decreased frequency and defective cytokine secretion

As mentioned earlier, NK cells play a critical role in controlling HCC. High frequency of NK cells with functional activity, expressing a wide spectrum of activating receptors and low amounts of inhibitory receptors, take part in controlling the HCC (55). In addition, the density of the infiltrating intratumoral NK cells is positively correlated with the overall survival in patients with HCC (55, 56). These results imply that NK cells play an important role in the immune-mediated defense against HCC. However, the frequency of NK cells in

the tumor regions is lower than that in the non-tumor regions, which would increase the chances of the tumor's evasion of immune surveillance. Previous report studied the immune profile of 110 patients with HCC and found that NK cells were abundant in the liver tissues of HCC individuals, with higher number of NK cells being detected in non-tumor liver tissues, than in the intratumoral regions (57). Consistent with this finding, a recent report demonstrated that NK cells were infiltrated in the intratumoral regions of HCC at a lower frequency than in the non-tumor liver tissues (34). Most intratumoral NK cells exhibited CXCR6<sup>+</sup> CD69<sup>+</sup> liver-resident phenotype (34). In addition, the intratumoral NK cell subgroups (CD56<sup>dim or bright</sup>) displayed a distorted population ratio, with a considerable drop in the number of CD56<sup>dim</sup> NK cells (57). The functional capacity of intratumoral NK cells is also attenuated in the HCC. Figure 1 describes various mechanisms of NK cell dysfunction in the tumor microenvironment of HCC. A previous research revealed that the intratumoral NK cells have more defective IFN- $\gamma$  and TNF- $\alpha$  secretion than non-tumor NK cells (56). The attenuated cytokine secretion in intratumoral NK cells is mainly caused by the defective recognition of tumor cells, or by inhibitory cells surrounding the NK cells, which will be discussed in the following sections. In summary, intratumoral NK cells in HCC are functionally defective, with relatively low level of cytotoxic potential and cytokine secretion ability, as compared to the liver-resident NK cells in non-tumor regions.

## 5.2. NK cell dysfunction in HCC: Defective recognition of tumor

The activating receptors of NK cells are important in tumor immunosurveillance (58). NKG2D detects several molecules, such as the cytomegalovirus UL-16 protein (ULBP1-6) and polymorphic MHC class I chain-associated molecules (MIC) A/B, which are not expressed on normal cells, but are upregulated on stressed cells (58). The role of NKG2D in

HCC has also been emphasized by Chu et al. (59). These authors found that, at the end of the antiviral therapy, there was a fast down-regulation of NKG2D on peripheral NK cells, in individuals who developed HCC immediately after HCV eradication. One recent study explained that the intratumoral NK cells have NKG2D downregulation in comparison to NK cells in non-tumor liver, leading to the defective recognition of tumor (34).

Furthermore, some tumor cells also downregulate NKG2D ligands, or downmodulate NKG2D function on effector cells. Among the NKG2D ligands, MICA was highlighted because the unidentified locus in the 5' flanking region of *MICA* was strongly associated with HCC occurrence in HCV-infected patients (60). Membrane-bound MICA triggers NK cell-mediated cytotoxicity. However, some proteases in the tumor microenvironment are known to shed the membrane-bound MICA, releasing soluble MICA into the bloodstream. According to Jinushi et al., the amount of soluble MICA was high in several patients with HCC, and their respective peripheral NK cells had reduced amounts of NKG2D expression and showed impaired activation (61). This detrimental function of soluble MICA, enables cancer cells to evade the NK cell-mediated immune surveillance (62). Among the MICA-shedding proteases, the roles of a disintegrin and metalloproteases 17 (ADAM17), were emphasized recently (62). ADAM17 knockdown reduced the soluble MICA levels and increased the membrane-bound MICA expression in HCC cells, thereby allowing for the cells to be killed by NK cells (Figure 1) (62).

The NCR family is type I transmembrane glycoproteins including NKp30 (NCR3), NKp44 (NCR2) and NKp46 (NCR1) (26). The *NCR3* gene is transcribed and undergoes alternative splicing, resulting in production of three major isoforms of the NKp30 protein (63). Recent study demonstrated that NKp30-expressing NK cells have a reduced expression of NCR3 immunostimulatory splice variants and an increased expression of the inhibitory

variant in patients with advanced HCC, leading to defective NKp30-mediated functionality (63).

### 5.3. NK cell dysfunction in HCC: Role of the KIR-HLA complex

KIRs are known to inhibit NK cell activation by interacting with diverse MHC class I molecules (64). The KIR genes, as well as their cognate HLA genes, illustrate extensive polymorphism and produce varied NK cell responses to cancer cells. There are two different points that should be considered in the role of KIR-HLA complex in patients with HCC: the ‘licensing’ of NK cells during maturation, and the control of NK cell activity when stimulated by the activating signal (Figure 1).

The binding of high-affinity inhibitory KIRs to their HLA cognates exerts a strong inhibitory effect on NK cell activation. However, during the maturation of competent NK cells, the ‘licensing’ of NK cells occurs, to avoid attacking the self (65). The ‘licensing’ refers to a process by which NK cells expressing the inhibitory KIRs for self-HLA, finally obtain a higher resting response capacity. Five inhibitory KIRs and their cognate HLA ligands that cause the NK cell licensing have been defined till now: KIR3DL1 for the HLA-Bw4 group alleles, KIR3DL2 for the HLA-A3/11 alleles, KIR2DL2 and KIR2DL3 for the HLA-C1 group, and KIR2DL1 for the HLA-C2 group of alleles (66, 67). In line with the ‘licensing’ model, an immunogenetic study discovered that the presence of KIR2DL2 and homozygosity for HLA-C1 that conferred NK cell licensing, correlated with the prolonged recurrence-free survival in patients with HCV-related HCC, after radiofrequency ablation (68). This means that the concurrent presence of KIR2DL2 and HLA-C1 is associated with longer time-to-recurrence, due to the protective effect of NK cells that had attained full functional competence after licensing (68). However, for patients with HBV infection, the KIR-HLA

types with the high functional maturation of NK cells, which had been ‘licensed’, were associated with HCC progression (69). This can be partly explained by the assumption, that an enhanced phenotype of NK function might lead to tissue damage as well as constant inflammation, which promoted HCC development in patients with chronic HBV infection (70). Although not fully defined yet, the progression of HCC is somewhat attributed to the dysfunction of NK cells, and the immunogenetic profile of KIR/HLA is linked to the activity of NK cells, which affects the prognosis for patients with HCC.

#### **5.4. NK cell dysfunction in HCC: Inhibitory roles of the immunoregulatory cells and the immunosuppressive cytokines**

One mechanism that modulates the function of NK cells is the crosstalk between immune cells in the tumor microenvironment (Figure 1). In the liver, multiple cellular constituents such as MDSCs, Tregs, macrophages polarized to the immunoregulatory phenotype, and immature DCs facilitate the development of cancer by promoting local immune tolerance (71). Normally, Tregs infiltrate the HCC, and the HCC stages have correlated with their frequencies (72, 73). They impair NK cell responses via membrane-bound and secreted TGF- $\beta$  (74, 75). MDSCs are myeloid cells with a potent immunosuppressive activity. They accumulate in the intratumoral and stromal lesions of various types of cancer, including HCC (76). MDSC-mediated inhibition of NK cells is contact dependent via membrane-bound TGF- $\beta$  on MDSC (77). Furthermore, recent data has shown that MDSCs from patients with HCC hamper cytokine production and cytotoxicity by autologous NK cells, and the suppression is cell contact dependent and primarily relies on NKp30 on the surface of NK cells (78). Tumor-associated macrophages (TAM) are located in the stroma of HCC and are polarized toward M2 phenotype (79). TAMs foster tumor cell

proliferation and spread in the HCC (79). TAMs are therefore related with the increased recurrence of tumor after the surgical resection of HCC (80). The primary role of TAM is their cytokine-dependent inhibition of NK cells and other lymphocytes with IL-10 and TGF- $\beta$  (81). The immunosuppressive activity of TGF- $\beta$  in the inhibition of NK cell cytotoxicity was also reported when NK cells are co-cultured with DCs (77). Secretion of IL-6 and IL-10 is also involved in DC-mediated NK cell inhibition (82). HCC-associated fibroblasts also induce the NK cell dysfunction by producing prostaglandin E2 (PGE2) and indoleamine 2,3-dioxygenase (IDO) (83).

## 6. Strategies to boost NK cell function in the HCC microenvironment

To reverse the malfunction of NK cells in HCC, various strategies were developed. These approaches include either endogenous stimulation of the NK cells in patients, or adoptive NK cell therapy to the patients. This review will cover both approaches in the following sections and table 1.

### 6.1. Strategies to boost NK cell function in the HCC microenvironment: Current treatment options

Currently, there are some effective therapies accessible to patients with HCC. The curative therapeutic options for HCC include the liver transplantation, radiofrequency ablation and surgical resection (1, 84). Radiofrequency ablation and surgical resection may have different impacts on the NK cell function. Ohira et al. discovered that the NK cells had a lower expression of CD69 and TRAIL after partial hepatectomy in mice. In addition, after injecting the Hepa 1-6 cell line, these mice were vulnerable to liver metastases in the remaining portion of the liver (85). This observation needs to be validated in humans. In

contrast, the radiofrequency ablation was linked to an evident increase in the IFN- $\gamma$  secretion and NK cell cytotoxicity (86). Very recently, transcriptomic analysis and deep immunophenotyping were performed with HCC tissues from patients who underwent Y<sup>90</sup>-radioembolization (87). The data showed strong immune activation in the tumor microenvironment and in the peripheral blood of patients with HCC with sustained response to Y<sup>90</sup>-radioembolization (87). Among the immune cells that were recruited and activated, NK cells were the strongest producer of granzyme B, suggesting the critical role of NK cells in the control of tumor, after Y<sup>90</sup>-radioembolization (87). Sorafenib also enhanced the NK cell-mediated cytotoxicity by increasing the expression of membrane MICA and reducing the levels of soluble MICA from HCC cells (88). Furthermore, through the modulation of the crosstalk between NK cells and TAMs, sorafenib strengthened the cellular anticancer effector functions (79). In contrast, another group claimed that sorafenib reduces the number and cytotoxicity of NK cells against tumor cells in tumor-bearing mice through downregulation of CD69 on the surface of NK cells (89), which needs further validation. Cisplatin has recently been shown to upregulate an NKG2D ligand, ULBP2, which might consequently enhance the NK cell-induced cytotoxicity against HCC cells. This supports a new immunomodulatory role for cisplatin in HCC therapy (90).

## **6.2. Strategies to boost NK cell function in the microenvironment of HCC: Modulating ADCC and the function of activating receptors**

Clinical trials have shown that patients with HCC having a high affinity Fc $\gamma$ RIII polymorphic variant have better outcome after monoclonal antibody treatment (28). This supports the potential advantageous function of ADCC and CD16 in HCC treatment. The ADCC of tumor cells by NK cells has been applied in the treatment of various cancers

overexpressing unique antigens (28). To directly target HCC cells, antibodies targeting the glypican-3 (GPC-3), which is expressed on up to 70% of tumors but not on normal hepatocytes, were prepared and tested (91). Humanized anti- GPC-3 antibody, codrituzumab, was proven to induce the ADCC. Although the phase I trial for codrituzumab was promising (92), the phase II trial for patients with advanced HCC for whom the standard treatment had failed, did not show an overall survival benefit (NCT01507168) (93). The efficacy of such antibody-based therapies might have been restricted by lower levels of NK cell activation due to the shedding of cell surface CD16, or by CD16 polymorphism (28).

For activating receptors of NK cells other than CD16, the results are slightly complicated. In a recent report, Sheppard et al. showed an unexpected tumor-promoting role of NKG2D in a model of inflammation-driven HCC (94). They showed that NKG2D-sufficient mice displayed an increased tumor growth associated with an increased infiltration of cytotoxic CD8<sup>+</sup> T cells to the liver and an intensified pro-inflammatory environment, which finally caused HCC (94). NKp30, another activating receptor of NK cells, mediated the interaction of NK cells with the expanded pool of MDSCs in HCC leading to the inhibition of NK cell activity (78). Similarly, another activating receptor, NKp46, was found to be upregulated on circulating NK cells in patients with HCC, with a poor prognosis (95). Collectively, these data suggest that enhancing the function of activating receptors on NK cells might not be beneficial in the control of HCC.

In regard to other aspects of NKG2D signaling, the modulation of NKG2D ligands on the tumor cells can boost the NK cell activity against HCC cells. Soluble MICA, as mentioned earlier, was demonstrated to work as a decoy to prevent anticancer surveillance by NK cells, while the upregulation of membrane MICA expression enhances the NK-mediated cytotoxicity. Histone deacetylase (HDAC) inhibitors are a new class of anticancer agents for

the treatment of various types of cancers including HCC (96). Several groups have reported that HDAC inhibitors promote MICA or MICB expression on HCC cells and increase the vulnerability of HCC cells to NK cell-mediated lysis (97-99). This suggests that the HDAC inhibitors promote the recognition of tumor cells by immune cells, in addition to their direct role in proliferation inhibition and apoptosis induction in tumor cells (96). Furthermore, it was recently reported that lomofungin, an antifungal drug, drastically decreases the enzymatic activity of ADAM17 and enhances the membrane MICA expression in a dose-dependent manner (100). Another recent report demonstrated that the enhancer of zeste homolog 2 (EZH2) functions as a transcriptional suppressor of NKG2D ligands in HCC cells, and targeting of EZH2 by small-molecule inhibitors causes killing of HCC cells by NK cells in an NKG2D ligand-dependent manner (101).

### **6.3. Strategies to boost NK cell function in the microenvironment of HCC: Modulating the function of inhibitory receptors on NK cells**

Blocking inhibitory receptors can also provide a robust method to enhance NK cell function. A recent clinical trial using the human HLA-haplotype mismatched hematopoietic stem cells to treat leukemia, showed efficient NK cell-mediated elimination of the leukemic cells (102). This indicates that the unmatched KIR-HLA haplotypes are potent NK cell activators, suggesting a rationale of blocking inhibitory KIR of NK cells to kill tumor cells. The two IgG4 monoclonal antibodies in clinical advancement that target inhibitory NKG2A and KIR2D (such as KIR2DL1-3/S1-2) receptor functions are monalizumab (IPH2201) and lirilumab (IPH2101), respectively (103, 104). Lirilumab infusion resulted in rapid decrease in the surface KIR2D expression and the inhibition of KIR2D<sup>+</sup> NK cell function, although it did not show clinical efficacy in multiple myeloma (103, 104). The CD94-NKG2A complex

provides another inhibitory stimulus, when it interacts with the non-classical MHC-I, HLA-E, on target cells (26). Monalizumab is an antibody which targets the CD94/NKG2A receptor and improves the cytolytic action of NK cells in pre-clinical investigation. Earlier researches have proposed that HLA-E is differently expressed in HCCs, highlighting the possible use of this monoclonal antibody for HCC therapy (105).

PD-1, which is a well-known exhaustion marker in T cells, was shown to be expressed on the surface of CD56<sup>dim</sup> NK cells (104). PD-1-expressing NK cells exhibited defective antitumor action and cytokine-stimulated proliferation, and such characteristics were reversed by blocking the PD-1/PD-L1 activities (106). Nevertheless, NK-cell contribution to the antitumor action targeting PD-1 axis in HCC remains to be investigated in future research. Another checkpoint molecule, T cell immunoglobulin and immunoreceptor tyrosine-based inhibitory motif domain (TIGIT) was recently found to be highly expressed on the exhausted tumor-infiltrating NK cells (107). Monoclonal antibody-mediated blockade of TIGIT or combined blockade of TIGIT and PD-L1 boosted the antitumor activity of NK cells in mouse models of colorectal cancer and efficiently delayed tumor growth (107). TIGIT-mediated NK cell exhaustion needs to be confirmed in human and mouse models of HCC.

#### **6.4. Strategies to boost NK cell function in the microenvironment of HCC: Stimulation of NK cells with interferons and cytokines**

After binding of IFNs to their receptors, cellular or extracellular effects are mediated through various interferon-stimulated genes (ISGs) with immunoregulatory and antiviral effects (108, 109). IFNs are grouped into three main categories, namely type I, type II, and type III. Each category of IFNs signals to the host cell by binding to the respective receptor complexes (109-111). An experimental treatment for HCC involving the type I and III IFNs

in a BNL hepatoma model showed an important role for NK cells in the antitumor action of IFNs (112). A phase I clinical trial (NCT01628640) has been initiated for patients with advanced HCC, with a recombinant vesicular stomatitis virus expressing the IFN- $\beta$ , which may perform an anticancer activity by stimulating the NK cells (113, 114).

IL-15 supports the survival, proliferation, and cytolytic activity of NK cells (34, 115). IL-15 is capable of recovering the antitumor function of NK cells hampered by *in vitro* contact with HCC cell lines or cells taken out directly from HCC tissues (34). K562 cells transfected with membrane-bound IL-15 and 4-1BB ligand encoding plasmid, can proliferate and activate the NK cells for application in HCC immunotherapy (116). Continuous infusions of IL-15 in cancer patients is accompanied by a preferential expansion of CD56<sup>bright</sup> NK cells with increased abilities to recognize tumor cells and with enhanced cytokine production and cytotoxicity (117). Nevertheless, systemic administration of IL-15 can result in major toxicity, which is associated with IFN- $\gamma$  secretion by NK cells (118). Novel approaches to specifically enhance IL-15 signaling in NK cells and reduce the systemic adverse effects will be promising immunotherapy areas to focus on.

## 7. Current status and future direction of adoptive NK cell therapy in HCC

Adoptive therapy of NK cells requires *ex vivo* expansion, and *in vivo* specificity to tumor cells, and *in vivo* maximal activity and persistence (20). The source of NK cells for adoptive therapy can be autologous or allogeneic NK cells, stem cell-derived NK cells, and NK cell lines such as NK-92 cell line (Figure 2) (7). Transfer of autologous NK cells failed to show improved survival outcomes in several types of cancers, because these cells had low efficacy and tended to remain in circulation rather than in tumor microenvironment (Figure 2) (119, 120). Therefore, allogeneic NK cell transfer is recently being widely used in various clinical

trials. Most cases of allogeneic NK cell trials have been performed using HLA-haploididentical NK cells with or without allogeneic hematopoietic stem cell transplantation (HSCT) (16). Adoptive transfer of allogeneic NK cells from KIR-mismatched donors has shown promising results in patients with acute myeloid leukemia (AML) (20). KIR-ligand incompatibility seems to be critical in efficacy of allogeneic NK cell therapy because the mismatch prevents the generation of negative signal and guarantees adequate NK cell activation (Figure 2). Allogeneic NK cells are expanded with cytokine stimulation, and T cells should be removed before adoptive transfer to avoid graft-versus-host disease (16).

Recently, the clinical efficacy of the allogeneic NK cell immunotherapy to treat various types of hematologic and solid tumors has also been evaluated by many groups. In HCC, there are on-going clinical trials using adoptive NK cell therapies (Table 1). A Chinese group recently demonstrated that irreversible electroporation combined with allogeneic NK cell transfer significantly increased the median overall survival of patients with stage IV HCC (NCT03008343) (121). Allogeneic NK cell transfer trial for preventing HCC recurrence after curative resection had been performed in Korea (NCT02008929), although the results are not available. Recently, a Korean multicenter group started a new phase 2 clinical trial to evaluate the safety and efficacy of allogeneic NK cells therapy after transarterial chemoembolization (NCT02854839). In another clinical trial, allogeneic, IL-2-stimulated NK cells from liver allografts were intravenously injected to transplant recipients. The safety and feasibility of this therapy was demonstrated in the phase I trial (NCT01147380) (122).

Genetic modification is another option for redirecting the function of NK cells. Genetic manipulation of NK cells has proven to be difficult until recently. Viral transduction was reported to be associated with very low expression levels of inserted genes and harmful effects on cell viability in NK cells. The recent optimization of viral transduction for efficient

gene transfection, have been shown to boost the interaction and activities of tumors and NK cells. First, it can be performed to express cytokine transgenes. NK cell function is enhanced by forced expression of cytokine transgenes such as IL-15, IL-2, or IL-12 (16). Gene therapy with an adenovirus expressing *IL-12* against orthotopic HCC in rat model, showed a considerable control of tumor growth. Activation of NK cells was the primary antitumor mechanism involved (123). Intratumoral gene transfer of *IL-12* in the mouse HCC model inhibited the spontaneous lung metastasis, neovascularization, and tumor growth (124). Here again, the tumor growth inhibition was almost completely dependent on NK cells, and this result was confirmed by the depletion of NK cells (124).

Chimeric antigen receptor (CAR)-expressing NK cells represent another promising modality for genetic modification. Currently, 9 clinical trials are being performed to evaluate the safety and efficacy of CAR-NK cells (119). CAR-NK cells are short-lived, and known to cause cytokine storms or graft-versus host diseases less frequently than do the CAR-T cells (125). NK-92 cell line has been used as a source for adoptive CAR-NK cell therapy due to its efficiency in expansion and transduction (126). Very recently, their potential efficacy was demonstrated in a preclinical model of HCC by a Chinese group (127). GPC3-specific CAR-NK-92 cells showed potent antitumor activities only to HCC cells expressing GPC3 molecule, reflecting the safety and specificity of CAR-NK cells (127).

## Conclusions

The strong antigen-independent cytotoxicity of NK cells can be applied to various types of cancer immunotherapy. The signals required to elicit robust antitumor responses by NK cells have not yet been entirely elucidated. The role of NK cells in controlling HCC has been underrated. NK cells have been demonstrated to play critical roles in the immune responses against HCC, providing a rationale for developing novel treatment strategies that enhance the NK cell response for treating HCC. A more comprehensive understanding of the detailed receptor interactions with tumor cells, intracellular signaling, and interactions with other immune cells should be reached. This would be critical for evaluating the current efforts to establish novel ways to enhance the activity of NK cells against HCC.

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**Table 1. Clinical approaches for targeting NK cells to treat HCC**

| Treatment method          | Development stage  | Features   | Country       | ClinicalTrials.gov identifier | Reference |
|---------------------------|--|--|---------------|-------------------------------|-----------|
| Current treatment options | Radiofrequency ablation  | May enhance the NK cell-mediated cytotoxicity  |               |                               | 86        |
|                           | $Y^{90}$ -radioembolization  | May enhance the NK cell-mediated cytotoxicity  |               |                               | 87        |
|                           | Sorafenib  | May enhance the NK cell-mediated cytotoxicity  |               |                               | 88        |
|                           | Cisplatin  | Upregulates an NKG2D ligand in HCC cells   |               |                               | 90        |
| Monoclonal antibodies     | Codrituzumab (anti-GPC-3-antibody)                                 | Advanced HCC who failed prior systemic therapy<br>No survival benefit                                      | Multinational | NCT01507168                   | 93        |
| Cytokines                 | Recombinant vesicular stomatitis virus expressing the IFN- $\beta$ | Phase I<br>Sorafenib-refractory or -intolerant HCC   | USA           | NCT01628640                   |           |
| Adoptive cell therapy     | Allogeneic NK cells  | Phase II<br>Performed after transarterial chemoembolization  | Korea         | NCT02854839                   |           |
|                           | Allogeneic NK cells  | Phase I, II<br>Stage IV HCC<br>Combined with irreversible electroporation<br>Survival benefit demonstrated | China         | NCT03008343                   | 126       |
|                           | Allogeneic NK cells  | Phase I<br>NK cells from liver allografts  | USA           | NCT01147380                   | 127       |

NK, natural killer cell; HCC, hepatocellular carcinoma; GPC-3, glypican-3; IFN, interferon

## Figure legend

### Figure 1. Mechanisms of NK cell dysfunction in tumor microenvironment of HCC

HCC cells inhibit NK cell activity via 1) downregulation of NKG2D ligands and 2) shedding of membrane-bound MICA. 3) Soluble MICA works as a decoy to prevent anticaner surveillance by NK cells. 4) Inhibitory receptors such as KIRs and NKG2A on the surface of NK cells recognize their ligands and suppress NK cell activity. 5) MDSCs inhibit NK cell cytotoxicity, via membrane bound TGF- $\beta$  and the NKp30 receptor on NK cells. 6) TAMs, 7) Tregs, 8) DCs, and 9) tumor-associated fibroblasts inhibit NK cells via immunosuppressive cytokines. 10) Defective ADCC also occur between tumor cells and NK cells in HCC.

NK, natural killer cell; HCC, hepatocellular carcinoma; MDSC, myeloid-derived suppressor cell; TAM, tumor-associated macrophage; Treg, regulatory T cell; MHC-I, major histocompatibility complex class 1; TGF- $\beta$ , transforming growth factor- $\beta$ ; MICA, MHC-I polypeptide-related sequence A; KIR, killer cell immunoglobulin-like receptor; ADCC, antibody-dependent cell cytotoxicity; PGE2, prostaglandin E2; IDO, indoleamine 2,3-dioxygenase; NKG2A, natural killer group 2 member A; NKG2D, natural killer group 2 member D; HLA-E, human leukocyte antigen E; PD-1, programmed death 1; NCR, natural cytotoxicity receptor.

### Figure 2. Adoptive transfer of NK cells: autologous and allogeneic NK cell transfer

(A) In autologous NK cell transfer, anti-tumor activity of NK cells might be limited by the inhibitory signal transmitted by the complex of matched KIR and self MHC class I molecule.

(B) In allogeneic NK cell transfer, high cytotoxic responses can be obtained when donor NK cells do not express KIRs matching the MHC class I molecules of the tumor cells. KIR-

ligand incompatibility is critical in efficacy of allogeneic NK cell therapy because the mismatch prevents the generation of negative signal and guarantees adequate NK cell activation.

NK, natural killer cell; HCC, hepatocellular carcinoma; KIR, killer cell immunoglobulin-like receptor; NKG2D, natural killer group 2 member D; MHC-I, major histocompatibility complex class 1

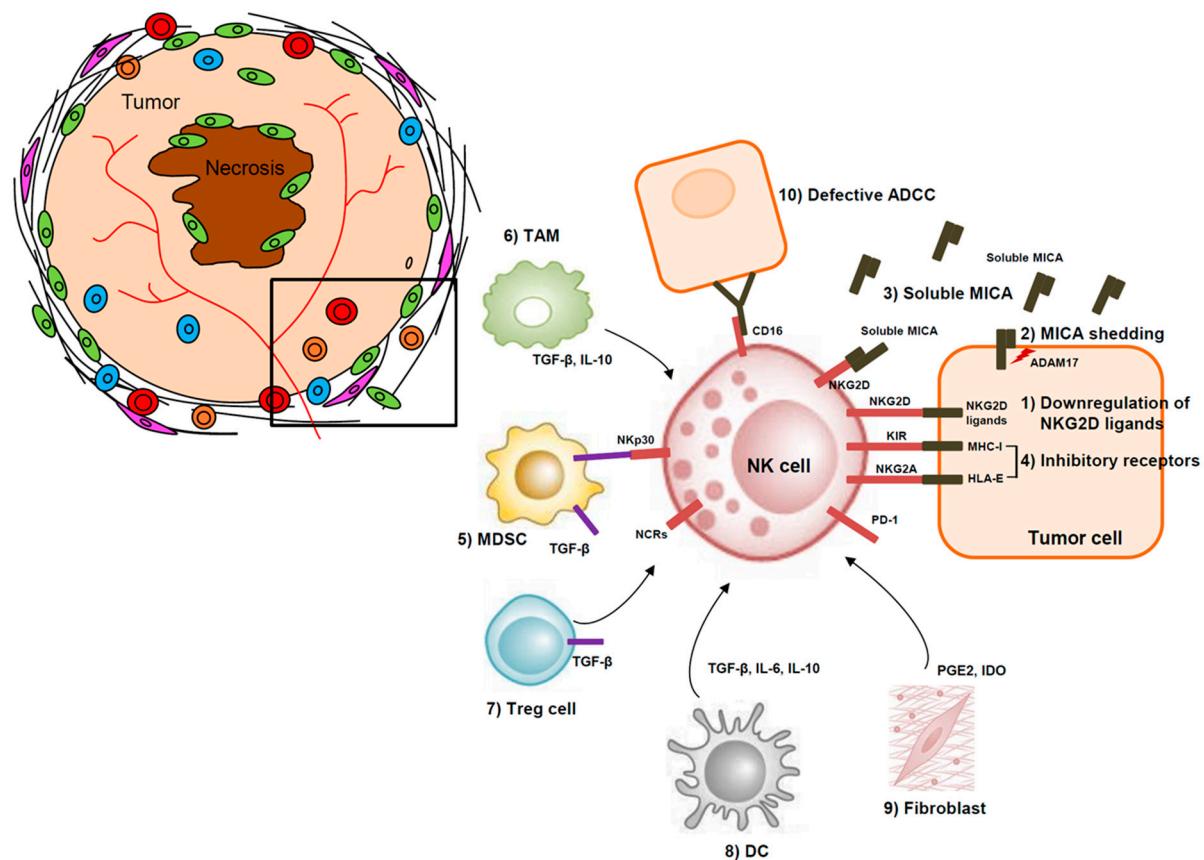


Figure 1

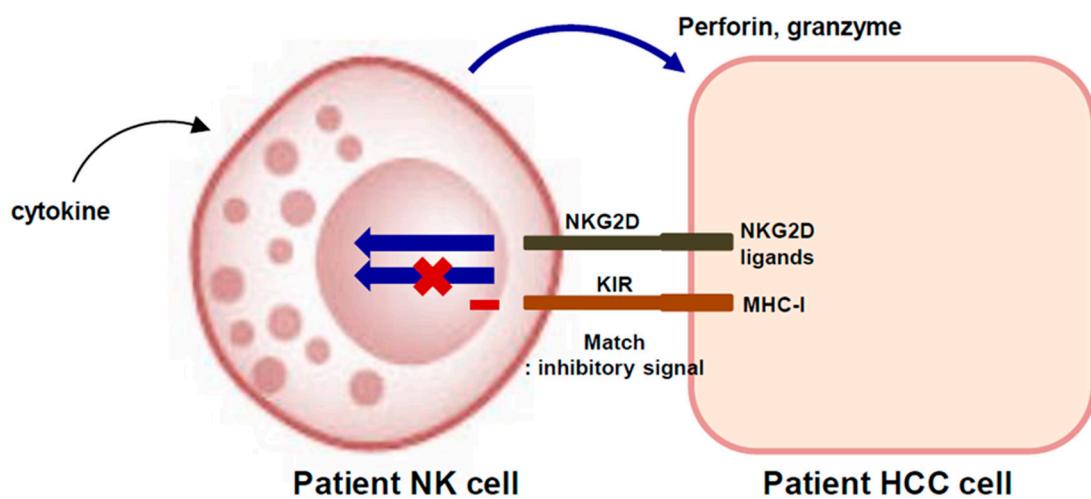
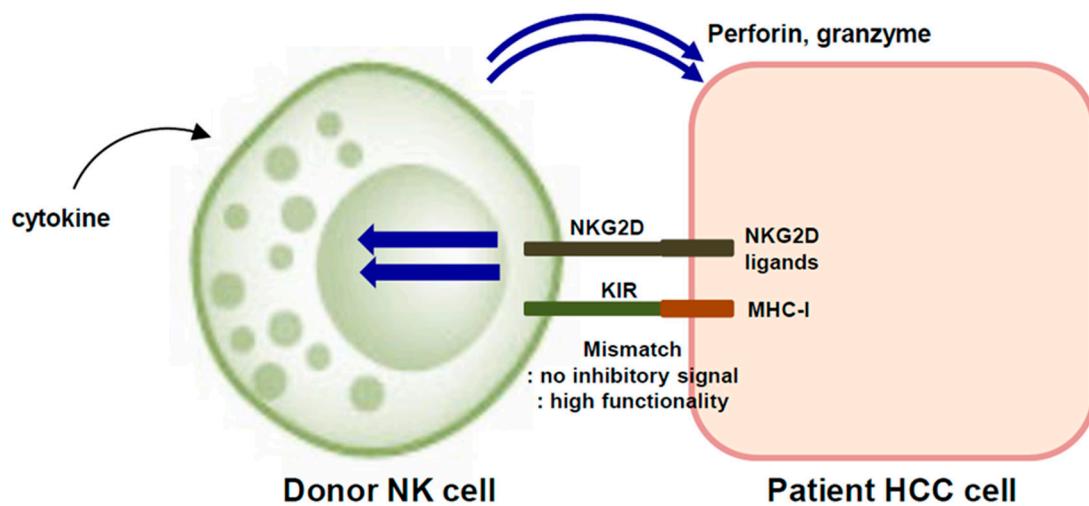
**A. Autologous NK cell therapy: low efficacy****B. Allogeneic NK cell therapy: high efficacy**

Figure 2

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