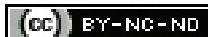


# Journey of Natural Killer Cells and their Role in Liver Diseases

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## ABSTRACT

Natural Killer (NK) cells are the key lymphocyte subset of the natural immune system that arbitrates antiviral and anticancer responses. In the human body NK cells inhabit in the bone marrow, lymph nodes, tonsils, skin, liver, gut, and lungs. This bibliographic study covers the origins and evolution of these cells. This review of NK cells includes synopsis of their well-known and evolving themes including their development, functions of cytokine production, anticancer cytotoxicity, clearing of viral infections and exhaustion. Within the liver, NK cells are enhanced in lymphocytes and possess distinctive phenotypic characters and useful properties, which contain tumour cytotoxicity and explicit cytokine profiles. NK cells while providing innate immunity in the liver, play important roles in providing protection versus pathogens and tumours utilising their cytotoxicity and cytokine production. Accruing substantiation from the last few decades proposes that NK cells perform a vital role in regulating viral hepatitis and liver tumours. In addition, they contribute to the pathogenesis of liver damage including its inflammation. Understanding the description of hepatic NK cell functions has aided us in better understanding the pathogenesis of diseases of the liver and consequently divulging novel therapeutic goals for treating these illnesses.

**Keywords:** Alcoholic liver disease, Hepatitis, Hepatocellular carcinoma, Liver transplant

## INTRODUCTION

The NK cells belong to the innate lymphocyte population and are involved in the first line of immune defense system against virally infected and transformed cells [1]. NK cells were initially discerned for their aptitude to kill tumour cells without preparing or previous initiation in divergence to cytotoxic T-cells. Hence, they were named as NK cells. NK cells are extensively dispersed throughout the body in lymphoid and non lymphoid tissues. The highest incidence of NK cells is seen in the lung, then the liver, outlying blood, spleen, bone marrow, lymph nodes, and thymus [2]. Successive discoveries showed the presence of NK cells in other tissues like skin, uterus, salivary gland and adipose tissue [3]. During various physiological and pathological conditions, NK cells secrete an array of cytokines, among which Interferon- $\gamma$  (IFN- $\gamma$ ) is predominant [4]. Morphologically, they are bone marrow derived large, granular lymphocytes and phenotypically they are Cluster of Differentiation 56+ (CD56+) and CD3- in humans. They are the third largest population of lymphocytes following B and T-cell and represent 10% of total peripheral blood mononuclear cells [5].

Circulating NK cells can be differentiated from liver NK cells by CD16+, presence of chemokine receptors like Chemokine Receptor 1 (CXCR1), CX3CR1 (CD56 dim) and low Natural Killer Cell Receptor (NKG2A), CD27, TNF-Related Apoptosis-Inducing Ligand (TRAIL), perforin, granzyme B, cytokine production and high Antibody Dependent Cell-Mediated Cytotoxicity (ADCC) [2]. In contrast to peripheral NK cells, NK cells inside a vigorous liver display an advanced level of cytotoxicity versus cancer cells and express advanced levels of cytotoxicity mediators [6]. The liver also harbours another set of cells in a large percentage called the invariant Natural Killer T (iNKT) cells as compared to any other organ [7].

Glycolipid  $\alpha$ -galatosylceramide activates the invariant NKT cells, presented by CD1d which is expressed by various lymphoid and non lymphoid cell types [8]. NK cells in liver are five times higher than in peripheral blood. They play a very vital role in avoidance of Hepatocellular Carcinoma (HCC). They are also considered as potential cell therapy resource for the treatment of HCC [9].

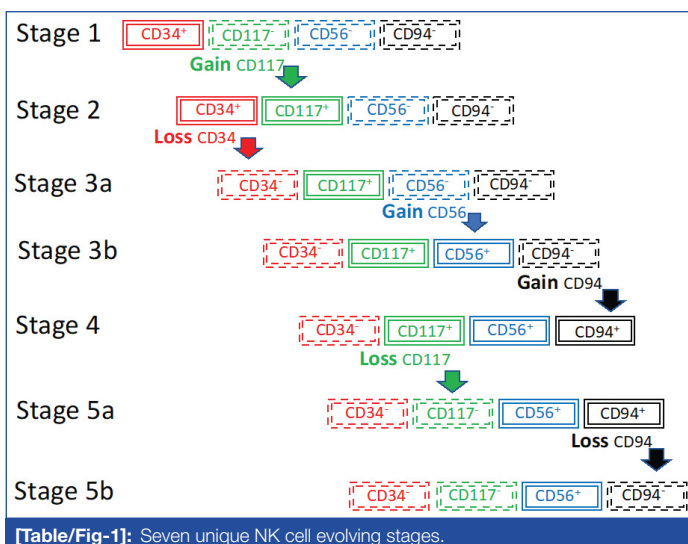
This review highlights the recent advances in understanding the journey and functions of NK cells and their role in various liver diseases.

## NK CELL DEVELOPMENT

Human NK cells are present as early as 6 and 15 weeks of gestation in foetal liver and spleen, respectively. Foetal NK cells are functionally immature and they are hyporesponsive compared to adult NK cells. NK cells originate from self-renewing Haematopoietic Stem Cells (HSCs) that reside in the bone marrow. According to recent studies, NK cells can also develop in lymph nodes and liver. NK cell Precursors (NKP) segregates into NK cells however not to other lineages, followed by phenotypic and working NK cell development. Later, NK cells undergo homeostasis. Transcription factors like Erythroblast Transformation Specific-1 (ETS-1), Inhibitor of Deoxyribonucleic Acid (DNA) binding-2 (ID2), Ikaros and PU.1 regulate NK cell development and maturation. GATA-3 and Interferon Regulatory Factor-2 (IRF-2) are involved in maturation of immature NK cells. The cytokine Interleukin-15 (IL-15) has been disclosed to be necessary for NK cell growth, homeostasis and subsistence [5].

Findings by Eissens D et al., have linked the role of 10-colour flow cytometry and recognised seven unique NK cell evolving stages in bone marrow and discovered that NK cell growth is complemented by initial appearance of stimulatory co-receptor CD244. They proposed the seven stages (depicted in [Table/Fig-1]) [10].

- **Stage 1:** Begins with CD34+, CD117-, CD56-, CD94- cells.
- **Stage 2:** The 'Gain' of CD117 to Stage 1 attains Stage 2 of CD34+, CD117+, CD56-, CD94-.
- **Stage 3:** A 'Loss' of CD34 expression from Stage 2 is Stage 3a (CD34-, CD117+, CD56-, CD94-). Stage Stage 3b (CD34-CD117+CD56+CD94-) is attained with a 'Gain' of CD56 from Stage 3a.
- **Stage 4:** In Stage 4, subsequent to NK cell lineage pledge, cells 'Gain' CD94 expression from Stage 3b to grow into undeveloped CD56 bright NK cells or CD34-, CD117+, CD56+, CD94+.
- **Stage 5:** Stage 5a is attained by a 'Loss' of CD117 expression from Stage 4, CD56 dim cells begin to grow, which results into CD34-, CD117-, CD56+, CD94+. Stage 5b (CD34-, CD117-, CD56+, CD94-) results with a 'Loss' of CD94 from Stage 5a.



More importantly, both Stage 1 and Stage 2 cells still acquire multi-lineage prospective and consequently comprises of NKPs but they have the ability to provide additional cell lineages. Additional investigation of cord blood, peripheral blood, inguinal lymph node, liver, lymph node, and spleen samples indicated that differential NK cell variation may happen at diverse anatomical sites due to unique appearance profiles of early progress marker CD33 and receptor NKG2A [10].

## NK CELL FUNCTIONS

Human NK cells in peripheral blood have diverse biological functions which can be classified into two subsets:

- CD56 dim (90%): High cytotoxic activity, and
- CD56 bright (10%): Production of cytokines.

NK cell functioning is controlled by wide range of receptors which are either inhibitory or activating in nature. NK cell receptors identify self Major Histocompatibility Complex (MHC) class 1 molecule and this averts NK cell triggering thereby explaining self-tolerance. The cells which lack self-MHC class 1 molecule activate the NK cells known as 'missing-self' hypothesis. Virally infested cells and cancer cells downregulate MHC class 1 expression which helps to avoid killing by cytotoxic T lymphocyte. However, it induces potent stimulatory signals tripping the balance in favour of NK cell activation, which is referred as 'induced-self recognition'.

## NK Cells in Uterus

NK cells are also detected in the uterus known as uterine Natural Killer (uNK) cells. uNK cells lack CD16 but express CD94 and secrete cytokines like Macrophage Inflammatory Protein 1 $\alpha$  (MIP1 $\alpha$ ), Granulocyte Macrophage-Colony Stimulating Factor (GM-CSF), CSF1 and IFN- $\gamma$ . The uNK cells are also seen in the implantation site and regulate pathogenic T helper 17 (Th17) cells at maternal-foetal border and encourage immune acceptance during pregnancy. Once the target is recognised by NK cells, the two major functions of NK cells are cytotoxicity and cytokine production. NK cells perform ADCC through CD16, as they express a low-affinity Fc $\gamma$  receptor IIIA. Through apoptosis NK cells kill tumours and virally infected cells. Perforin is a membrane-disrupting protein that is released on NK cell activation and its expression is enhanced by stimulation of IL-2. Granzymes belongs to a line of intrinsically associated serine proteases which are discharged by exocytosis, which along with perforin prompt apoptosis of the target cell. NK cells also express TRAIL and Fas Ligand (FasL) which results in caspase-dependent apoptosis which is associated with death receptors (e.g., FasL/CD95) [5].

## NK Cells in Liver Disease

Liver diseases are broadly categorised into viral hepatitis, Alcoholic Liver Disease (ALD), chronic hepatitis like autoimmune liver disease,

drug induced liver disease, extrahepatic biliary obstruction, cirrhosis, and Hepatocellular Carcinomas (HCC). [Table/Fig-2] shows these liver disease categories in conjunction with the various roles NK cells play in fighting them.

| Disease                | NK Cells' Roles   |
|------------------------|---|
| Cancer                 | 1) NK Cells STOP cancer growth<br>2) Cancer Environment STOPS NK Cell Functions   |
| Fibrosis               | 1) NK Cells KILL HSCs $\rightarrow$ STOP fibrosis growth<br>2) Late stage fibrosis STOPS NK Cells' 'anti-fibrotic' effects  |
| HBV                    |   |
| - Acute                | 1) NK cells NOT activated   |
| - Chronic              | 1) Peripheral/Intrahepatic NK cells ACTIVATED<br>$\rightarrow$ Hepatocellular Injury  |
| HCV                    |   |
| - Acute                | 1) # of NK cells/Cytokine Production INCREASED<br>2) NK cells STOP viral replication  |
| - Chronic              | 1) Peripheral NK cells ACTIVATED<br>$\rightarrow$ ENHANCED cytotoxicity<br>$\rightarrow$ REDUCED IFN- $\gamma$ production<br>2) Intrahepatic NK cells:<br>Not clear if ACTIVATED or NOT |
| Autoimmune Disease     | 1) NK cells KILL epithelial cells $\rightarrow$ PROMOTE the disease<br>2) NK cells STOP adaptive immunity $\rightarrow$ STOP the disease  |
| Alcoholic Disease      | 1) NK cells SUPPRESSED  |
| Biliary Atresia        | 1) NK cells KILL cholangiocytes $\rightarrow$ PROMOTE the disease   |
| HBV: Hepatitis B Virus | HCV: Hepatitis C Virus    HSCs: Hepatic Stellate Cells  |

**[Table/Fig-2]:** NK Cells' roles in liver diseases.

## NK Cells in Viral infection

NK cell has a role as immune-protective agent in viral infection by reducing its number and activity. In various virus infections like Flavi viruses, Human Immunodeficiency Virus (HIV), respiratory syncytial viruses and influenza there is shift from CD56 dim to CD56 bright which causes increased cytokine production [11]. In viral associated asthma, NK cells contribute to Immunoglobulin E (IgE) mediated immune response and cause resolution of acute allergic airway inflammation. They migrate from circulation towards tissues like lung and lymphoid organs due to antigenic stimulation [12].

**NK cells in viral hepatitis:** The scientists have suggested that NK cells play a critical role in defense against viral hepatitis. Globally, viral hepatitis has been the leading source of end-stage liver disease and hepatocellular carcinoma. Since the mid-1960s, five hepatitis viruses, types A, B, C, D and E, have been identified. More than half a billion people are persistent carriers of the Hepatitis B Virus (HBV), the Hepatitis C Virus (HCV), or the Hepatitis Delta Virus (HDV).

NK cells are known to play an important role in viral infection. Initial findings claimed that HCV obstructs NK cell tasks and getaways from immune reconnaissance of NK cells resulting in lingering infection. During acute infection, NK cells are activated by INF- $\alpha/\beta$ , IL-12, IL-15, IL-18 which in turn cause killing of HCV infected hepatocytes [13]. It has been observed that peripheral NK cells are also triggered during continuing HCV infection which shows an upsurge cytotoxicity with raised expression of NKG2D, NK p46, TRAIL and STAT1 which contributes to liver injury. Decrease IFN- $\gamma$  production may facilitate inability to clear HCV resulting in chronic stage [14].

**Hepatitis B virus (HBV):** Acute HBV infection in early stage shows upregulation of IL-10 in absence of IFN- $\alpha/\beta$  and IL-15 induction which contributes to inhibition of peripheral NK cell functions. Chronic HBV infection causes liver inflammation by activation of peripheral and intrahepatic NK cells induced by IFN- $\alpha$ , IL-12, 15, 18. Activated NK cells cause hepatocellular damage. However its role in control of HBV replication is questionable [15,16].

Ghosh S et al., study showed that NK cells cause hepatic harm and aid in viral resolve during advancement of hepatitis B. This is mediated by insufficient IFN- $\gamma$  production and NK mediated, perforin dependent depletion of CD4+T cells which causes liver damage and HBV persistence contributing to advance liver damage. This finding demonstrated that immunotherapy that combines INF- $\alpha$  and therapeutic weakening of NK cells or obstruction of NK triggering receptors can be effective in HBV suppression [17].

### NK Cells in COVID-19

NK cells play an important role in moderating the immune response in Coronavirus Disease-2019 (COVID-19) patients. There is decrease in NK cell number and its function, resulting in reduced clearance of activated and infected cells, and unhindered increase of tissue-damaging irritation markers. COVID-19 infection tilts the immune reaction towards a tremendously inflammatory phenotype. Re-establishment of NK cell effector functions has the prospective to appropriate the delicate immune balance necessary to successfully overcome COVID-19 infection [12].

### NK Cell in Autoimmune Disease

In autoimmune diseases like immune encephalomyelitis and multiple sclerosis, there is a reduction of number of NK cell with decrease cytotoxic function. It is also observed that cytotoxicity of NK cells can augment an autoimmune disease by destruction of cells in a target organ [18].

In type 1 diabetes, NK p46, the activating NK cell receptor binds to a known ligand on  $\beta$ -cell of pancreas and effectively killing them [19]. This is essential for development of type I diabetes. Various other studies revealed low expression of NK p30, NK p46 receptors in patients with long standing type I diabetes. They also displayed reduced perforin messenger Ribonucleic Acid (mRNA) expression along with decreased lysis activity by NK cells [20]. This was thought to be a consequence of diabetes rather than a cause.

In other autoimmune diseases role of NK cells is either disease promoting or disease controlling. Like in rheumatoid arthritis, there is NK cell accumulation in synovial fluid by secretion of IFN- $\gamma$  via CD56 bright [21].

In systemic lupus erythematosus, function of NK cells is reduced and there is shift from CD56 dim to CD56 bright subset [22]. Abnormalities in number and function in this inflammatory condition play protective or disease controlling role. NK cells also have a crosstalk with other immune cells like macrophages and Treg cells [23]. Treg cells have been seen to subdue NK cells via IL-21 arbitration in autoimmune illnesses. Exact mechanism and role of NK cell in autoimmune disease as a friend or foe is debatable. This also suggests the possibility of role of NK cell in causing remission and exacerbation of these autoimmune conditions [24].

## ROLE OF NK CELLS IN LIVER

NK cells in liver were first studied in mouse. Liver resident NK cells were DX5+ and DX5-. Hepatic NK cells were approximately half enriched in DX5- NK cells. CD49a is considered to be specific marker for liver DX5- NK cells, rarely expressed by liver DX5+ NK cells [25]. The CD49a+DX5- NK cells were present in liver sinusoid blood, neither in the afferent nor efferent blood vessels of the liver [26]. But CD49a-DX5+ is present in all blood sources. In human liver CD56 bright NK cells expressed CD49a and these CD49a+ NK cells express transcription factors like -bet+ Eomes- (T-box expressed in T-cells and Eomesodermin) [27]. In human liver-resident NK cells surface markers are CD56 bright CD16-, CXCR6+, CCR5+, CD69+, CD49a+/-, CX3CR1-, CD57-, CD49e-. Effector molecules include TRAIL, granzyme K, IFN- $\gamma$ , TNF $\alpha$ , GM-CSF. Human liver NK cell lacks T-bet expression and it highly expresses Enomes [28].

NK cell is a key component of the innate immune system in the liver. They act by fabrication of cytokines, killing cancer cells,

pathogens, strained hepatocytes and HSCs. NK cells acts as a governing cell which influences other cells in the vicinity like Dendritic Cells (DCs), T-cells, B-cells, Kupffer cells and endothelial cells by producing various cytokines like INF- $\gamma$ , TNF- $\alpha$ , IL-10 and TLR ligands, chemokines and growth factors. T regulatory cells and activated HSCs can inhibit NK cell formation via inhibitory cytokines predominantly TGF- $\beta$ , IL-10. Triggered NK cells mark HSCs, hepatocytes, and cholangiocytes and achieve range of vital functions in the pathogenesis of liver illness [13].

### NK Cell in Alcoholic Liver Disease (ALD)

Alcohol consumption contributes to an increase in NK cell cytotoxicity in normal individuals which may contribute to development of ALD. However, it is observed that patients with ALD show decrease NK cell numbers and reduce cytotoxic activity [29]. Chronic exposure of alcohol causes reduced expression of NKG2D, TRAIL and IFN- $\gamma$  on NK cells. This results in decreased antiviral, antifibrotic and antitumour effects of NK cells which contributes to susceptibility to infection, accelerated liver fibrosis and HCC in patients with liver alcoholic disease.

Obesity adds to decrease circulating NK cells with lower level of cytotoxicity [30]. However, Kahraman A et al., study reported NK cell associated cytotoxic mediators (such as TRAIL, NKG2D and MIA/B mRNAs), and number of hepatic NK cells were strikingly amplified in overweight patients with Non Alcoholic Steatohepatitis (NASH) and to smaller degree with non alcoholic fatty liver when rivalled to normal individuals [31]. In NASH patients, the expression of MICA/B mRNAs correlates with the non alcoholic fatty liver disease (NAFLD) activity score and hepatocyte apoptosis thus, NK cells are triggered due to the raised levels of numerous cytokines (e.g., IL-12, IL-18, and IFN- $\gamma$ ) and ligands (e.g., MICA/B) and contribute to the pathogenesis of NASH [31,32].

### Fibrosis and Cirrhosis

NK cells plays important role in controlling liver fibrosis which were recently seen in HCV patients by several clinical studies [33]. The first study demonstrated, human NK cells contributed to production of TRAIL and FasL which resulted in killing of the activated primary human HSCs in vitro. Second, activation of NK cell mediated killing of human HSCs through NKG2D and NK p46 activating receptors. Third, contribution of INF- $\alpha$  in patient with HCV increased the ability of NK cells to kill primary human HSCs. Fourth, cytotoxicity of NK cells were isolated from HCV patients against primary human HSCs demonstrated inverse correlation with stage of liver fibrosis. Fifth, lymphocytes of HCV patients were transfected with the help of KIR small interfering RNAs (siRNAs) inhibited the activation of human HSCs [34]. Lastly, buildup of NK p46 high NK cells in liver was contrariwise linked with the fibrosis phase of HCV patients. All these discoveries propose that NK cells perform a vital role in reducing liver fibrogenesis. Though, chronic alcohol consumption can reduce the antifibrotic function of NK cells and raised levels of TGF- $\beta$  are associated with final stage liver fibrosis, which leads to advancement of liver fibrogenesis [35].

### Autoimmune Liver Illness

Various human autoimmune diseases of liver like autoimmune hepatitis, Primary Biliary Cirrhosis (PBC), primary sclerosing cholangitis reduces the NK cell functions. In the pathogenesis of these disorders NK cells play dual roles [36]. NK cells cause destruction of biliary epithelial cells via a TRAIL-dependent mechanism and by cytokine production which promotes adaptive immune response and progression of disease. In contrast, NK cells may also cause regression of PBC by production of IL-10 and killing of T-cells and DCs [37].

### NK Cells in Biliary Disease

Biliary atresia, an advanced fibro-obliterative cholangiopathy with unidentified aetiology, disturbs the biliary tree in infants and upsets



bile flow from liver to the intestine. Investigational simulations implied that NK cells destroy cholangiocytes via NKG2D [38]. Postnatal absence of T regulatory cells in these patient allow hepatic DCs to act unopposed in NK cell activation and cholangiocyte destruction [35,39].

## Liver Cancer

NK cells are enriched in healthy liver and they play an important role in the immune surveillance for tumours which is mediated via the production of perforin, granzyme, TRAIL, IFN- $\gamma$  [40]. In HCC patients, the number of peripheral CD56dim CD16+ NK cells decreases with impaired cytotoxic activity and IFN- $\gamma$  production [41]. Several mechanisms involved in pathogenesis of HCC are NK cell malfunction, decrease in its number resulting in escape of tumour cells from immune surveillance.

Fibroblasts present within HCC produces Indoleamine 2,3-dioxygenase (IDO) and Prostaglandin E2 (PGE2) which trigger NK cells dysfunction and downregulates activating NK receptors [42]. In HCC patients, Myeloid-Derived Suppressor Cells (MDSCs) interact with NK cells and contribute to decreased cytotoxicity and cytokine production of NK cells [43].

## NK CELLS AS THERAPEUTIC TARGETS FOR THE TREATMENT OF LIVER DISEASE

In view of the important role of NK cell in pathogenesis of liver disorders, there is strong possibility of using hepatic NK cells as potential therapeutic target. Several approaches include cytokine treatment to increase the cytotoxicity of NK cell, antibodies to modulate NK cytotoxic function and use of agonist of NK cell activating receptors and adoptive transfer of NK cells. IL-12 and IL-18 have been displayed to successfully obstruct liver carcinogenesis by enhancing NK cell anticancer task. Recombinant IL-2 and IL-15 triggers both NK cell and CD8+ T-cell without exciting Tregs [44]. These are presently being verified for haematological malignancies. MiR-182 has been shown to rise NK cell cytotoxicity by controlling expression of NKG2D and NKG2A in HCC [45].

In this new era of immunotherapy for cancer, several inhibitory check points are targeted on NK cell through blocking monoclonal antibodies (mAbs) [46]. Killer cell immunoglobulin like receptors KIR are expressed on NK cells and is also present on T-cell in minor amounts. Antibodies against anti-KIR or inhibitory KIR showed promising effect in haematological cancer. NKG2A is currently been tested in solid tumours [47]. NK cells also express PD1. mAbs blocking PD-1/PD-L1 can be the future therapeutic target for liver cancer [48]. More recently genetic engineering with different technological approach like bi- and tri-specific killer engagers (BiKEs and TriKEs) or Chimeric Antigen Receptors (CARs) showed improved ability of NK cells to infiltrate tumour tissue [49]. This can be used as NK cell transfer therapies in liver cancer in future.

## NK CELLS ROLE IN LIVER TRANSPLANT

Liver transplantation or LT can save lives for patients with acute and persistent liver malfunction. It is the last treatment option for patients with:

- a) Major complications caused by final stage chronic liver disease;
- b) Sudden failure of a previously healthy liver.

Transplant receivers need lifelong immuno-suppression to avert immune responsiveness directed versus the donor organ, causing in graft refusal. NK cells role in organ transplantation has been inadequately defined due to contradictory clinical and experimental statistics. However, the liver contains main resident populations of immune cells, particularly enriched with NK cells,  $\gamma$ - $\delta$  T-cells, and NKT cells. Transplantation of liver thus ends in a rare assembly of recipient and donor immune systems. This potentially inflammatory meeting results in attenuated immune response leading to less requirement of immunosuppression. Thus, liver NK cell play an

important role in tolerance induction and post liver transplant outcome [50].

## CONCLUSION(S)

Recent studies have facilitated us to increase an understanding of NK cell in relations of its development, role, distinct phenotypical features and receptor interactions. Their assignment in confronting cancer cells is well recognised. The identification of liver resident NK cells have opened various dimensions in understanding the pathogenesis of liver diseases like hepatitis, ALD, autoimmune liver disease, non alcoholic steatohepatitis, biliary atresia and HCC. Interestingly, they play an important role in induction of tolerance post liver transplant. Over the last two decades, research in NK cells has gained some traction in therapeutics for the diseases of liver and other organs. However, how to deploy NK cells for the therapy of diseases remains in infancy as most of this work is still in the preclinical stage. A nationwide or global NK strategy is therefore a must to improve ailment therapies further.

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