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Review Article

Natural Killer Cells in Hepatitis C Virus Infection

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Abstract

Natural Killer (NK) cells account for the majority of innate immune cells in the human liver (the primary site of HCV replication). $CD3^-CD56^+$ NK cells are significantly increased in the liver compared to the peripheral blood although this becomes especially evident in chronic HCV infection. NK cells have been implicated in all stages of HCV infection in both genetic and functional studies. This role may be either direct, by targeting infected hepatocytes, or indirect by influencing other key immunocytes such as dendritic cells (DCs) or T cells. Hepatotropic viruses such as HCV induce production of type I Interferon (IFN) by hepatocytes and other cells in the liver, which in turn promotes infiltration of NK cells in virus infected livers. Production of type I IFN and other cytokines (including Interleukin (IL)-12, IL-15, and IL-18) by hepatocytes activates NK cells and induces IFN-γ production by them, which recruits activated T cells to the liver. It has been shown in animal models that depletion of NK cells before hepatotropic viral infection leads to inhibition of a virus-specific T cell response, as well as inhibition of liver injury. It is possible that an adequate NK cell response may control HCV infection, even in the absence of virus-specific immune responses. NK cells have direct antiviral effects which are mediated by direct cytolytic (e.g., TRAIL or perforin mediated) or non-cytolytic (e.g., IFN-γ mediated) effector functions. Understanding the mechanisms by which HCV is successfully eradicated is especially important for therapeutic and vaccination strategies.

Keywords: apoptosis, Interferon, interleukins

NK Cell Subtypes

NK cells are large granular lymphocytes (LGL) constitute about 5–20% of peripheral blood mononuclear cells (PBMCs). Their immunophenotype is usually described as $CD56^+$, and $CD3^-$[1]. According to the expression of CD56, CD16 differentiation antigens and functional features, NK cells are further subdivided to several subsets[2]**.** NK cells express CD56, the 140-kD isoform of the neural cell adhesion molecule (NCAM). Thee major subset of NK cells (≥90%) in peripheral blood dimly expresses CD56 and shows co-expression of CD16 with a low affinity Fc receptor for the IgG ($CD56^{+dim}$ $CD16^+$ cells). It represents about 7% (2-14%) of all PBMCs. They have homing markers for inflamed peripheral sites. They exhibit strong antibody-dependent cell-mediated cytotoxicity (ADCC) and natural cell-mediated cytotoxicity but have low cytokine production capacity[2]. Another subset of NK cells is characterized by high expres-

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sion of CD56 and is negative for CD16 ($CD56^{+bright}$ $CD16^{-}$ cells). These are a minor subset of NK cells in peripheral blood (10%) and represent about 1-2% of all PBMCs[3]. It is regarded as a less mature subset. They express homing markers for secondary lymphoid tissues where they accumulate and are found predominantly in the liver. They have an immunoregulatory role as they have high cytokine production capacity. They secrete interferon (IFN)-γ as well as Tumor Necrosis Factor (TNF)-α, Granulocyte Monocyte Colony Stimulating Factor (GM-CSF), Interleukin (IL)-10 and IL-13. It has been shown that $CD56^{+bright}$ cells may transform into $CD56^{+dim}$ ones. Moreover, $CD56^{+dim}$ cells can also produce the mentioned cytokine[4]. In addition, $CD56^{-}CD16^{+}$ subset of NK cells may also be found, but their function remains unclear[5]. Recent data suggest that $CD56^{-}$ NK cells exhibit functional skewing manifested by low capacity to produce IFN-γ, and to degranulate, but may release significant amounts of chemokines such as MIP-1 α/CCL3 (Macrophage Inflammatory Protein-1), MIP-1β/CCL4 and RANTES/CCL5 (Regulated on Activation, Normal T Expressed and Secreted)[6].

NK Cell Receptors

Three groups of NK cell receptors have been distinguished: killer immunoglobulin-like receptors (KIRs), lectin-like receptors (e.g. NKG2D and CD94-NKG2A-F), natural cytotoxicity receptors (NCRs) and LIR (leukocyte inhibitory receptors)[7]. The main activating NK cell receptors are NKG2D and the natural cytotoxicity receptors (NCRs) while the inhibitory receptors are CD94/NKG2A and KIRs[8]. Ligands of KIRs are predominantly alleles of MHC class I molecules (classical MHC class I) such as HLA (human leucocyte antigen), mainly HLA-C antigens. Ligands of lectin-like CD94-NKG2A-F receptors are HLA-E antigens connected with HLA-A, -B, -C or -G leader peptide (non-classical MHC class I) While MIC-A, MIC-B (MHC-class I-related chain) are ligands of lectin-like NKG2D. Natural cytotoxicity (NKp30, NKp44, NKp46, and NKp80) receptors have various ligands, some unknown, but the most important are apparently viral hemagglutinins[9]. The $CD56^{dim}$ NK subset express higher levels of KIRs, CD16 and perforin. The $CD56^{bright}$ subset express a high level of the inhibitory receptor CD94:NKG2A and generally do not express KIRs or CD16[10]. In molecular terms, the difference between activating and inhibitory receptors is expressed in the cell cytoplasmic tails—the long immunoreceptor tyrosine-based inhibition motifs (ITIMs) mediate inhibitory signals, while the short immunoreceptor tyrosine-based activation motifs (ITAMs) are responsible for activating signals[11]. NK cell function is critically regulated by combinations of stimulatory, co-stimulatory and inhibitory receptors. The net balance of signals derived from these receptors determines whether the NK cell becomes activated[12].

Physiological Function of NK cells

1. Cytolytic granule mediated cell apoptosis: NK cells are cytotoxic; small granules in their cytoplasm contain proteins such as perforin, granzymes, and proteases. Upon release in close proximity to a cell slated for killing, perforin forms pores in the cell membrane of the target cell, creating an aqueous channel through which the granzymes and associated molecules can enter, inducing either apoptosis or osmotic cell lysis. The distinction between apoptosis and cell lysis is that: lysing a virus-infected cell could potentially only release

the virions, whereas apoptosis leads to destruction of the virus inside. Apoptosis can be mediated by degranulation of cytotoxic granules, and by surface expression of ligands such as Fas ligand (FasL) and TRAIL (TNF-related apoptosis-inducing ligand) that activate death receptors on target cells. Antimicrobial molecules, α-defensins, are also secreted by NK cells, and directly kill bacteria by disrupting their cell walls[13].

2. *Antibody-dependent cell-mediated cytotoxicity:*

Infected cells are routinely opsonized with antibodies for detection by immune cells. Antibodies that bind to antigens can be recognised by FcγRIII (CD16) receptors expressed on NK cells, resulting in NK activation, release of cytolytic granules and consequent cell apoptosis[14].

3. *Cytokine-induced NK and cytotoxic T lymphocytes (CTL) activation*

Cytokines play a crucial role in NK cell activation. As they released by cells upon viral infection, they serve to signal to the NK cell the presence of viral pathogens in the affected area. Cytokines involved in NK activation include IL-12, IL-15, IL-18, IL-2, and RANTES/CCL5[15]. NK cells are activated in response to interferons or macrophage-derived cytokines. They serve to contain viral infections while the adaptive immune response generates antigen-specific CTL that can clear the infection. NK cells work to control viral infections by secreting T-helper 1 (Th1) cytokines; IFN-γ and TNF-α. Th1 type cytokines can prime the adaptive immune response and IFN-γ, in particular, can have a direct antiviral effect. IFN-γ also activates macrophages for phagocytosis and lysis, and TNF-α acts to promote direct NK tumor cell killing[16]. NK cells play a major role in viral infections, predominantly in the early phase. Following recognition of infected cells as non-self via activating receptors, the response of NK cells is further enhanced by at least two cytokines—IL-12 released by dendritic cells (DCs) and monocytes, and IFN-γ secreted by T cells and activated NK cells themselves. Furthermore, type I IFNs secreted by virus-infected cells augment NK cell cytotoxicity[17].

4. *Missing 'self' hypothesis*

NK cells function to sense pathogen, infected and/or transformed cells, and to eliminate them from the body following their activation. In contrast to T and B cells, the NK cells do not require prior sensitization. NK cells are equipped into various receptors, allowing them to recognize self from non-self and unchanged healthy cells known as "missing-self hypothesis"[18]. The inhibitory receptors of NK cells recognize MHC (Major Histocompatibility Complex) class I alleles. MHC class I molecules are the main mechanism by which cells display viral or tumor antigens to cytotoxic T cells. A common evolutionary adaptation to this is seen in both intracellular microbes and tumors: the chronic down-regulation of MHC I molecules, which makes affected cells invisible to T cells, allowing them to evade T cell-mediated immunity. NK cells evolved as an evolutionary response to this adaptation (the loss of the MHC eliminates CD4/CD8 action), so NK cells evolved to fulfill the function[18].

5. *Tumor cell surveillance:*

NK cells play a role in tumor immuno-surveillance by directly inducing the death of tumor cells (NK cells act as cytolytic effector lymphocytes), even in the absence of surface adhesion molecules and antigenic peptides. This role of NK cells is critical to immune success particularly because T cells are unable to recognize pathogens in

the absence of surface antigens. Tumor cell detection results in activation of NK cells and consequent cytokine production and release[1]. If tumor cells do not cause inflammation, they will also be regarded as self and will not induce a T cell response. A number of cytokines are produced by NKs, including TNF-α, IFN-γ, and IL-10. TNF-α and IL-10 act as proinflammatory and immunosuppressors, respectively. The activation of NK cells and subsequent production of cytolytic effector cells impacts macrophages, dendritic cells, and neutrophils, which subsequently enables antigen-specific T and B cell responses. Instead of acting via antigen-specific receptors, lysis of tumor cells by NK cells is mediated by alternative receptors, including NKG2D, NKp44, NKp46, NKp30, and DNAM (DNAX Accessory Molecule). NKG2D is a disulfide-linked homodimer which recognizes a number of ligands, including ULBP and MICA, which are typically expressed on tumor cells[7]. NK cells express the Fc receptor (FcR), an activating biochemical receptor that binds the Fc portion of antibodies. This allows NK cells to target cells against which a humoral response has been mobilized and to lyse cells through ADCC. This response depends on the affinity of the Fc receptor expressed on NK cells, which can have high, intermediate, and low affinity for the Fc portion of the antibody or IgG[7].

6. *Adaptive features of NK cells - "memory-like" and memory NK cells*

The ability to generate memory cells following a primary infection and the consequent rapid immune activation and response to succeeding infections by the same antigen is fundamental to the role T and B cells play in the adaptive immune response[19]. For many years, NK cells have been considered to be a part of the innate immune system. However, recently increasing evidence suggests that NK cells can display several features that are usually attributed to adaptive immune cells (e.g. T cell responses) such as expansion and contraction of subsets, increased longevity and a form of immunological memory, characterized by a more potent response upon secondary challenge with the same antigen. In addition to, antigen-specific recall responses to some haptens[20]. The antigen specificity appeared to be supported by some RAG-1 and RAG-2 (recombination-activating genes)-indpendent mechanisms and consequently mice were able to express NK cells memory following viral infection[21]. Moreover, NK cells appear to be involved in a close bi-directional cross-talk with DCs. Activated NK cells enhance DC maturation and production of IL-12. Reciprocally, DCs augment cytotoxicity of NK cells. These interactions are mainly cell-contact dependent. Mature myeloid DCs (MDCs) are a major source of IL-12 which enhances NK cell-mediated cytotoxicity and IFN-γ production[22]. While Plasmacytoid DCs (PDCs) secrete Type I IFN which is crucial for DC-induced NK-cell activation and have an important role in the induction of NK cell cytotoxicity by modulating the expression of ligands for the NK cell receptor NKG2D. Also PDCs when activated by virus express the ligand for glucocorticoid-induced tumor necrosis factor receptor (GITR). The latter, in synergy with IL-2, IFN-α and some KIRs triggers NK cell cytotoxicity and IFN-γ secretion due to GITR expression on NK cells. DCs may also activate NK cells indirectly by promoting the expansion of antigen-specific T cells, which secrete IL-2, which in turn activates NK cells[23].

NK Cells and HCV Infection

Human NK cells co-cultured with HCV replicon inhibit the replicon expression at protein and RNA levels by secreting antiviral factors, including IFN-γ. Thus, NK cells could contribute towards control of HCV replication[24]. NK cells have direct antiviral effects which are mediated by direct cytolytic (e.g., TRAIL or perforin mediated) or non-cytolytic (e.g., IFN-γ mediated) effector functions[25]. NK cells were originally implicated in determining the outcome of HCV infection in an immunogenetic study of the KIR genes and their HLA-C ligands[26]. KIR2DL3, an inhibitory receptor, binds to MHC molecules encoded by HLA-C group 1 alleles (HLA-C1), triggering a comparatively weak inhibitory signal which directly influence resolution of HCV infection in patients homozygous for these genes because the KIR2DL3 binds HLA-C with a lower avidity than other inhibitory KIR, and thus NK cells expressing this specific inhibitory receptor have a lower threshold for activation[27]. Furthermore, $KIR2DL3^{+}$-$NKG2A^{-}$ NK cells are not inhibited in the presence of the NKG2A ligand HLA-E, which is upregulated in the liver during HCV infection. These have been suggested to control early HCV infection prior to seroconversion and may thus result in an apparent state of "natural resistance" to HCV in persons who inject drugs[28]. Similarly, a number of cytokines involved in NK activation or function have been implicated in the outcome of HCV infection. These include the NK cells associated cytokines IL-12, IL-18 and IFN-γ[29]. The type III interferon IL-28B (IFN-λ3) has received much attention as a mediator of clearance of HCV. It is not known whether IL-28B has a similar or complementary functions to the type I interferon, IFN-α, which is involved in NK cell activation. However, as these molecules share common signaling pathways there is likely to be at least some overlap in function[30].

Role of NK Cells in the Early Phase of HCV infection

NK cells are activated in the acute phase of HCV infection. Downregulation of MHC I on virus-infected hepatocytes may reduce the inhibitory signal to NK cells, shifting the balance towards NK cell activation. DCs engage with NK cells via the NKp30 receptor, and produce cytokines which boost NK cell proliferation towards an 'NK1' phenotype. Activated NK1 cells produce cytokines such as IFN-γ and TNF-α which suppress HCV replication, reciprocally activate DCs, and prime naive CD4 T cells inducing a Th-1 response[12]. A possible role of NK cells in HCV is further supported by the finding that they are activated in acutely infected subjects, as determined by an increased expression of the activating receptor NKG2D on both $CD56^{bright}$ and $CD56^{dim}$ subsets of NK cells that is accompanied by an increased production of IFN-γ and cytotoxicity. NK cell responses are also linked with T cell responses, e.g., increased degranulation of NK cells during acute HCV has been shown to correlate with the magnitude of virus-specific T cell responses. Also, an activated multifunctional NK cell response, i.e., cytotoxicity and IFN-γ production, has been reported early after HCV exposure in healthcare workers who do not develop acute infection, suggesting an important contribution to the prevention of high level viremia[31]. Amadei et al[31] also reported an increase in $CD56^{bright}$ NK cells (with an associated reduction in the $CD56^{dim}$ subset) in acute HCV patients compared to healthy individuals. Individuals who spontaneously cleared the virus showed a decline in the $CD56^{bright}$ population, with levels compara

ble to healthy control individuals after 1-3 months, indicating a return to baseline which was not observed in those that went on to have a chronic infection. Expression of the activating receptor NKG2D was also increased in the acute phase of infection. Functional experiments showed augmented IFN-γ production and cytotoxicity in these patients and a trend for more NK cell degranulation in individuals expressing HLA-C1 specific KIR receptors, which was maximal in those with self-limiting infection. Thus in the acute phase of HCV infection there is activation of NK cells indicating their role in the immune response at this stage. Pelletier et al (2010)[32] have also studied individuals in the acute phase of HCV infection. They also found increased activity of NK cells as determined by a degranulation assay, but found that the NK cells from intravenous drug users had generally lower levels of IFN-γ secretion as compared to healthy controls, and suggest that this may be related to opioid use. They found that the levels of the inhibitory receptor NKG2A, declined on $CD56^{bright}$ NK cells during the follow-up phase in those spontaneously resolving infection only. Furthermore they were able to correlate NK cell activity with T cell activity, implying a coordinated innate and adaptive immune response to acute HCV infection. Thus, there is activation of NK cells in the acute phase of HCV infection, which declines in those clearing HCV and persists in those remaining chronically infected. However, HCV has the ability to interfere with the action of NK cells and compromise their functions. A report suggests that NS5A-containing apoptotic bodies can trigger monocytes to produce increased amounts of IL-10 and decreased levels of IL-12. In consequence, this leads to a significant down-regulation of NKG2D on NK cells via Transforming Growth Factor-β (TGF-β)[33]. Another proposed mechanism for HCV-induced NK cell inhibition is cross-linking of CD81 by the HCV envelope protein E2. It has been shown that engagement of this tetraspanin on the surface of NK cells exerted an inhibitory effect, leading to inhibition of NK cytotoxicity as well as CD16- or IL-2-induced IFN-γ secretion by NK cells. The findings suggested a direct impairment of NK cell functions through direct contact with HCV virions or HCV-infected cells. It was observed that cell-to-cell contact with HCV-infected cells reduces functional capacity of NK cells although NK cell function remains intact after exposure to infectious virus. Also HCV mediated inhibition of NK cell mediated augmentation of complement synthesis has also been reported[34].

NK cell Responses in Chronic HCV Infection

In chronic HCV infection, NK cells are activated but may display alterations in phenotype and function. For example, NK cells from chronically HCV infected patients express higher levels of several activating receptors, such as NKp30, NKp44 and NKp46[35]. Chronic exposure of NK cells to endogenous IFN-α can result in increased STAT expression, and preferentially STAT1 over STAT4 phosphorylation[36]. NK cells in chronically infected patients, are impaired in their antiviral effector function due to impaired ability to secrete IFN-γ. Importantly, IFN-γ production by NK cells in response to HCV-infection is dependent on accessory cells, such as monocytes and plasmacytoid dendritic cells[37]. Peripheral blood NK cell frequencies are reduced in chronic HCV compared to healthy individuals. This reduction may be a consequence of HCV infection, or a predisposing factor to chronic HCV infection. In individuals

with chronic HCV infection, NK cell frequency increases following successful antiviral therapy while a reduction in peripheral blood NK cell frequency in individuals with chronic HCV as compared to spontaneous resolvers has also been noted[38]. IL-15, a pivotal cytokine for NK cell development, proliferation and function, may be relevant to this observation. Meier et al (2005)[39] showed a significant reduction in IL-15 levels in HCV patients as compared to healthy controls and demonstrated that exogenous IL-15 rescued HCV-NK cells from apoptosis, increasing ex vivo proliferation and function. Furthermore, DCs are an important source of IL-15 and have been shown to cross-talk with NK cells. In chronic HCV infection IL-15 production by IFN-α-stimulated DCs is deficient. Thus a downstream consequence of this DC dysfunction could be inadequate production or proliferation of NK cells[40].

• *Skewing of subset distribution*

A relative increase in circulating $CD56^{bright}$, but not $CD56^{dim}$ NK cells, in chronic HCV compared to healthy individuals and spontaneous resolvers[41, 42]. $CD56^{-}CD16^{+}$ NK cells appear to be more terminally differentiated NK cells and there is an expansion of this subset in chronic HCV infection. These cells have reduced perforin expression as compared with $CD56^{dim}$ NK cells and have been shown to be hypofunctional, particularly in their interactions with dendritic cells[43]. In HCV, chemokine production by $CD56^{-}CD16^{+}$ NK cells was skewed towards MIP-1b, and there was also a reduction in IFN-γ and TNF-α secretion compared to the $CD56^{+}$ NK population. Thus, overall, there is a skewing of NK cells away from the $CD56^{dim}$ $CD16^{+}$ subset, which is thought to be the main cytotoxic subset of NK cells. This may be an effect of IFN-α, as there is a strong IFN-α response to HCV infection, and therapy with pegylated IFN-α and ribavirin leads to an increase in $CD56^{bright}$ and a decline in $CD56^{dim}$ NK cells[38].

• *Alterations in phenotype*

Changes in phenotype may reflect changes in subset distribution and also the effect of cytokines on specific subsets of NK cells. $CD56^{bright}$ NK cells are KIR-negative and NKG2A-positive, and the most consistent finding has been an increase in NKG2A expression in chronic HCV infection. This occurs on both intrahepatic and peripheral blood NK populations[44, 38].

• *Altered function*

There is diminished natural cytotoxicity in chronic HCV which is restored by successful HCV clearance with IFN-α and ribavirin therapy[45]. However, the number of cytotoxic $CD56^{dim}$ NK cells in the peripheral blood is depressed. There is greater expression of activation markers such as CD122 (a subunit of IL-2 receptor which is crucial for IL-2 and IL-15 signaling), CD69, and NKp44. NK cell TRAIL expression is increased in chronic HCV, and these cells have a phenotype consistent with IFN-α stimulation. Upregulation of TRAIL on NK cells may also be an important mechanism underlying the anti-HCV effect of NK cells[46]. A change in the cytokine profile of NK cells in chronic HCV may be relevant to the persistence of HCV infection. Failure of NK cell production of IFN-γ in chronic HCV has been reported[38]. IFN-γ has potent direct anti-HCV properties, blocking HCV replication in a dose-dependent manner. IFN-γ also indirectly suppresses HCV activity by polarizing T cell differentiation towards a virus specific Th1 phenotype[47]. Additionally, increased HCV-NK cell production of Th2 cytokines such as IL-10 and TGF-B, and the chemokine IL-8, may skew the cytokine profile towards an environment which is

more permissive for HCV. This phenotype, is similar to the NK2 phenotype in which NK cells secrete the Th2 cytokines IL-5 andIL-13. In vitro NK cells can be polarized towards this phenotype under the influence of IL-4. Thus the Th2 environment found in chronic HCV infection may affect the differentiation and maturation of NK cells, towards this NK2 phenotype which further contributes to the Th2 environment in a positive feedback loop. This polarization may occur either in the periphery, or within the liver microenvironment. Thus the cytokine microenvironment may affect NK cell phenotype and also function[47]. In chronic HCV infection the dominant effect on NK cells appears to be of IFN-α. The profile of NK cells in chronic HCV is consistent with a reduced maturation of $CD56^{bright}$ NK cells, and also enhanced differentiation of $CD56^{dim}$ NK cells towards a $CD56^{-}CD16^{+}$ phenotype[12]. NK cells may also be modulated by direct cellular interactions, especially with DCs. All mature NK cells express the activating receptor NKG2D, the ligands for which are MIC proteins. In HCV infection there is an impairment of MIC-A/B expression which results in lower levels of NK cell activation. NK cells enhance maturation and activation of DCs to promote a Th1-polarised CD4 T-cell response. NK cells from HCV-infected individuals have a reduced capacity to activate DCs, due to NK cell inhibition by the CD94:NKG2A receptor and a consequent increase in NK expression of the immunoregulatory cytokines IL-10 and TGF-B, which promote Th2 type differentiation[48]. Interestingly, inhibition of NKG2A restored the ability of HCV-NK cells to activate DCs, and also the production of the Th1 cytokines IFN-γ and TNF-α. This may be important as HCV can upregulate HLA-E, the ligand for NKG2A, in vivo and so represents a mechanism by which HCV may modulate the NK cell response. The activation status of NK cells correlates with liver inflammation. Increased expression of NKG2A, CD69 and CD107a (a marker of NK cell degranulation) on peripheral blood NK cells have all been linked with disease activity[49].

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