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REVIEW ARTICLE

The Natural Killer Cell Response to HCV Infection

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In the last few years major progress has been made in better understanding the role of natural killer (NK) cells in hepatitis C virus (HCV) infection. This includes multiple pathways by which HCV impairs or limits NK cells activation. Based on current genetic and functional data, a picture is emerging where only a rapid and strong NK cell response early on during infection which results in strong T cell responses and possible subsequent clearance, whereas chronic HCV infection is associated with dysfunctional or biased NK cells phenotypes. The hallmark of this NK cell dysfunction is persistent activation promoting ongoing hepatitis and hepatocyte damage, while being unable to clear HCV due to impaired IFN-γ responses. Furthermore, some data suggests certain chronically activated subsets that are NKp46^{high} may be particularly active against hepatic stellate cells, a key player in hepatic fibrogenesis. Finally, the role of NK cells during HCV therapy, HCV recurrence after liver transplant and hepatocellular carcinoma are discussed.
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INTRODUCTION

Hepatitis C virus (HCV) infection presents a global health problem with ~3% of the world population currently infected (1). Of these, 70~80% develop chronic disease with a risk for progressive liver fibrosis and cancer (2). Accordingly, HCV-related liver disease is the most common cause for liver transplantation in the Western world with a high risk of HCV recurrence and rapidly progressive liver fibrosis post transplant (3). Therapy for chronic hepatitis C (CHC) comprises a backbone of IFN-α and ribavirin (RBV) (4). Sustained virological response (SVR), i.e. cure, is achieved in only 40~50% of cases of genotype 1 infection, although the addition of direct acting antivirals (DAAs), such as telaprevir (TPV) and boceprevir (BOC), improves response to ~70% (5-7).

NATURAL KILLER CELLS

Natural Killer (NK) cells are innate immune effector cells that are highly efficient in recognizing and killing virally infected cells and produce antiviral cytokines, such as IFN-γ and tumor necrosis factor (TNF)-α (8). Unlike T and B cells, however, they do not require priming and lack T cell receptor and immunoglobulins.

NK cells can be roughly divided into CD56^{bright} cells that produce IFN-γ and contribute to T helper cell type 1 priming, and CD56^{dim} that represent a fully mature, highly cytotoxic subset also capable of antibody dependent cell-mediated cytotoxicity via the Fc receptor γ III (CD16) (9). A third, dysfunctional subsets has been described as CD56-CD16+, which is rather rare and has been mainly studied in the context of HIV infection (10,11).

As NK cells can kill without prior sensitization, their activation needs to be very tightly regulated to prevent uncontrolled killing. This "friend or foe" detection system is provided by a plethora of receptors, most prominent amongst these are the killer immunoglobulin-like receptors (KIRs) and the lectin

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Abbreviations: HCV, hepatitis C virus; TRAIL, TNF related apoptosis inducing ligand

like receptors. The KIR system in particular operates on the basis of the "missing self" dogma (12) meaning NK cells are inhibited by major histocompatibility complex (MHC) class I molecules, preventing them from killing a targeted cells. If MHC class I is removed from the targeted cell, however, it is killed by the NK cell via direct cytotoxicity. Importantly, both the KIR and MHC or HLA gene cluster show significant genetic diversity, causing individual KIRs to recognize only specific subgroups of HLA class I alleles and for type and number of KIRs to differ between different KIR haplotypes. This combined with the extraordinary polymorphism of the HLA system results in high inter-individual variability. Adding to the complexity is that KIRs may be inhibitory or activating.

Lectin-like receptors, i.e. NKG2A to F, hetero-dimerize with CD94 with the exception of NKG2D. Whereas NKG2A and NKG2B exert inhibition upon recognition of HLA-E, NKG2C-F activates NK cells upon recognition of HLA-E. NKG2D presents an exception as it recognizes the stress ligands MICA and MICB. Finally, the natural cytotoxicity receptors NKp30/44/46 can activate NK cells upon recognition of their ligand, even in the absence co-stimulation (13). Overall, the number of different receptors expressed on NK cells results in a broad heterogeneity of different NK cell subpopulations.

NK cells are enriched among liver resident lymphocytes (30%) as compared to blood (5～20%) and this percentage increases further in the context of hepatitis (14,15). Their natural enrichment in the liver and the ability to eliminate virally infected hepatocytes places NK cells in a key position among effector lymphocytes in acute and chronic HCV infection.

DIRECT INTERACTION BETWEEN NK CELLS AND HCV

There has been long-standing controversy as to whether HCV directly inhibits NK cells. Two studies have previously shown that a plate-bound, recombinant form of the HCV envelope 2 (E2) glycoprotein may impair IFN-γ release by NK cells, as it binds to the tetraspanin CD81 expressed on the cell surface of NK cells (16,17). A later study suggested the same for plate bound HCVcc (cell culture) particles, though IL-8 release was actually increased (18). There is no convincing evidence, however, that the viral particle or soluble HCV-E2 protein inhibits NK cell function (18,19). There is, however, some suggestion that certain HCV-derived peptides may bind to HLA-E, stabilizing its expression and thus inhibiting NK cell cytotoxicity via an interaction with NKG2A (20).

Although activated NK cells can recognize and lyse HCV replicon containing hepatoma cell lines in a perforin-/granzyme-dependent manner (21), there is some suggestion that cellular contact with hepatoma cells may impair killing capabilities and IFN-γ response of NK cells (22); the reason for this observation is not entirely clear and indeed may be partially related to use of hepatoma cells to begin with. An alternative explanation was recently offered by Wang et al., where HCV seems to up-regulate "Killer cell lectin-like receptor subfamily G member 1" (KLRG1), which impairs IFN-γ response and proliferation of NK cells suggesting that KLRG1 negatively regulates NK cell function via the Akt pathway (23). Similarly, Holder et al. suggested that HCV infected hepatoma cells inhibit NK cell function in a contact-dependent manner proportional to HCV infection levels (24). This was the result of reduced NKp30 expression on NK cells. A similar decrease in NKp30 expression, but also NKG2D expression, was recently reported by Yoon et al. (22). Of note, there is significant controversy, on whether the expression of NK cells markers such as NKG2A/C/E and NKp30/44/46 is increased, decreased or unchanged in chronic HCV infection (25-29). The underlying cause for such controversy is not immediately clear, though they are likely related to small numbers and differences in patient selection.

Interestingly, once NK cells have been pre-activated by IFN-α, they can efficiently recognize and kill HCV-infected hepatoma cells in a DNAM-1-dependent manner, complementary to the well established role of NKG2D for cytotoxicity (30). Importantly, the IFN-α that so efficiently turns NK cells into activated killers, also promotes other NK cells functions, such as IFN-γ release in this context and is primarily derived from accessory cells such as plasmacytoid dendritic cells (pDCs) as shown in co-culture experiments with HCV infected hepatoma cells, NK cells and pDCs (31). Another study investigated the role of bystander monocytes in this context: Interestingly, the HCV-NS5b protein can bind to TLR-4 on monocytes and induce IL-10 production while inhibiting IL-12 induction. This subsequently induces release of TGF-β that results in down-modulation of NGK2D expression on NK cells, a receptor widely expressed by NK cells and playing an important role in direct cytotoxicity (32).

In summary, there are multiple possible avenues, by which HCV may impair NK cell activation or function, though the role of these mechanisms in the clinical context is not well understood.

ACUTE HEPATITIS C AND SPONTANEOUS RECOVERY FROM HCV INFECTION

NK activation is regulated by interactions of KIRs (among others) on NK cells with their specific HLA ligand on the target cell. Distinct KIR/HLA-C haplotypes, i.e. KIR2DL3/HLA-C1 vs. others, influence spontaneous and treatment induced HCV clearance (33-35). This effect was independent of *IFNL3* polymorphism (33,36), a well-established marker for spontaneous and treatment-induced clearance of HCV infection (33,37-41). Of note, HLA class I molecule expression may be up-regulated by HCV core protein, thus increasing recognition and therefore inhibition through HLA-C recognizing KIRs (42). The underlying reason for the KIR/HLA-C association seems to be *differential* NK cell activation predetermined by the KIR/HLA-C interaction allowing for faster, more profound activation, particularly in terms of IFN-γ release in the context of KIR2DL3/HLA-C1 interaction (43). Importantly, IFN-γ is considered essential for HCV clearance (44,45) and a more efficient mechanism than cytotoxicity, as the latter requires a 1:1 interaction between NK cells and infected cells, whereas the IFN-γ molecules secreted by an individual NK cell may reach more than a 100 hepatocytes (46).

IFNL3 polymorphisms have been associated with changes in expression levels of KIRs and other NK receptors such as NKp30, SIGLEC6 and NKG2A, as well as TRAIL, although this data was not analyzed with respect to HLA-C genotypes and the underlying mechanism leading to altered expression are poorly understood (47,48).

Alter et al. decribed lower frequencies of NKp30, NKp46, CD161 and NKG2D NK cells in patients with spontaneous recovery from HCV infection as compared to patients who developed chronic infection (26). Amadei et al. studied consecutive blood samples of patients with acute HCV infection and described an increase of NKG2D+ NK cells irrespective of outcome, i.e. spontaneous resolution or chronic infection (49). While IFN-γ production was increased in general, cytotoxicity was increased only in a KIR/HLA-C dependent manner with increased degranulation noted only in NK cells expressing the HLA-C1-specific KIR, which was maximal in self-limited infection (49). Pelletier et al. studying patients with acute HCV infection and Werner et al. studying healthcare workers with low level HCV exposure extended these results by linking increased NK cell cytotoxicity during early infection to a stronger T cell response, though not necessarily clearance (50,51). Particularly the second study is notable, as it suggests that even very low-level viral exposure will induce a strong NK cell response within weeks of infection (51), in keeping with the association between KIR and HLA-C being particularly strong in iv-drug users with likely low level viral exposure as compared to patients receiving contaminated blood products (35).

NK CELLS IN CHRONIC HCV INFECTION

Once chronic HCV infection is established, patients suffer from mild chronic hepatitis and are at risk of progressing to fibrosis and cirrhosis (2). NK cells seem to contribute to this phenomenon by remaining in a state of chronic activation: Particularly, enhanced cytotoxicity of $CD56^{dim}$ NK cells correlates with ALT levels, a marker for hepatocyte damage, in these patients (52). Interestingly, the NK cell phenotype in chronic HCV infection is not one of overall activation, but rather biased towards increased cytotoxicity and impaired ability to produce IFN-γ (29,52). This bias seems to be mediated by endogenous IFN-α that promotes STAT1 expression (53-55). This increased STAT1 expression is than preferentially phosphorylated as compared to STAT4 and promotes cytotoxicity, whereas the lack of STAT4 phosphorylation results in impaired IFN-γ responses.

Some controversy exists in the expression and role of TRAIL, as TRAIL was reported to be up-regulated on intrahepatic NK cells in one study and down-regulated with an associated of decreased degranulation in another study (52,56). NKp46, however, was up-regulated on intrahepatic NK cells in both studies. Importantly, $NKp46^{High}$ NK cells show stronger cytotoxicity and IFN-γ secretion than $NKp46^{Dim}$ NK cells and are capable of blocking HCV replication *in vitro* (57). $NKp46^{High}$ NK cells are enriched in livers of patients with chronic hepatitis C and maintain enhanced cytotoxicity particularly against hepatic stellate cells (57). Intrahepatic frequency of $NKp46^{High}$ NK cells was inversely correlated with HCV-RNA levels and fibrosis stage (57). This suggests a potential role of NK cells in eliminating hepatic stellate cells, one of the main drivers of fibrosis. Another study described pathological activation of intrahepatic $NKp46^{High}$ NK cells as one of the drivers of ongoing hepatitis and hepatocyte death in chronic HCV infection (58). Interestingly, the frequency of $NKp46^{High}$ cells seems to be related by ethnicity and gender (59). Further to this, CXCR3 seems to be an important chemokine receptor in this context, as CXCR3+CD56bright cells show impaired degranulation

and impaired IFN-γ secretion in response to hepatic stellate cells and accumulate in the liver with their frequency correlating to the degree of fibrosis (60).

In summary there is sufficient data to suggest an active role of NK cells in ongoing hepatic inflammation in chronic HCV infection, with the NKp46^{High} subset showing direct activity against hepatic stellate cells.

NK CELLS IN TREATMENT OF CHRONIC HCV INFECTION

Standard therapy for HCV infection consists of pegylated interferon-α (IFN-α) and ribavirin (RBV), with the recent addition of boceprevir or telaprevir for triple therapy in HCV genotype 1 infection (5-7). KIR/HLA-C genotypes are clearly associated with response to standard peg-IFN-α/RBV therapy of chronic HCV infection in keeping with an important role of NK cells in antiviral therapy of HCV infection (33,34).

IFN-α has strong immuno-modulatory properties and directly activates NK cells (61), particularly with respect to cytotoxicity (62). Thus, it is to be expected that NK cells play a role in antiviral therapy.

Lower pre-treatment levels of activating NK cells receptors NKp30 and NKp46 have been associated with treatment response (63) and higher pre-treatment levels of IFN-α receptor (IFNAR) on NK cells have been associated with a better response to therapy in an *IFNL3* genotype dependent manner (64). Two studies have addressed treatment changes of NK cells in chronic HCV infection: NK cells become activated and express CD69, NKG2D and NKp30 within hours of the first dose of IFN-α, particularly in patients with an early virological response (65). This seems to herald an increase in cytotoxicity and TRAIL within 24 hours of first IFN-α dose. Interestingly, this coincides with a decrease of NK cells expressing CXCR3, the receptor for IP-10 (CXCL10) whose up-regulation is strongly associated with response to IFN-α therapy (66), suggesting active recruitment of NK cells to the liver. NK cell cytotoxicity peaked after 24 h of therapy and correlated with a rise in ALT, implying that activated NK cells kill infected hepatocytes and support phase 1 viral clearance (65). Of note, the above-mentioned biased NK cell phenotype in chronic HCV (52), is further enhanced *in vivo* by IFN-α therapy and does not recover for more than 4 weeks on treatment (65). Importantly, within 72 h of therapy NK cells become refractory to IFN-α stimulation *in vivo* and *in vitro* supporting the concept of early, NK cell activation to be important for outcome (53). Another study reported a rapid increase in CD69+ NK cells during therapy which was associated with rapid virological response (67). Furthermore, SVR in this study was associated with higher NK cell perforin content and a sustained increase in NK cell degranulation in the presence of hepatoma cells, suggesting that a sustained NK cell response is important for SVR (67). Importantly, both studies did not find any association of IFNL3 genotype with NK cell response, suggesting an indirect effect of IFNL3 via accessory cells (65,67). Indeed there is controversy as to whether IFNL3 has a direct action on NK cell function (68-70). Finally, TRAIL seems to be more strongly up-regulated in response to IFN-α in patients who have achieved SVR and seems to control HCV replication by killing HCV replicating hepatoma cells in a TRAIL dependent manner (71).

In summary, there is strong data suggesting a role of NK cells in IFN-α/RBV therapy, especially early on during treatment. Future studies will have to establish the role of NK cells in triple or quadruple therapy and especially IFN-free regimens.

NK CELLS AND HCV RECURRENCE AFTER LIVER TRANSPLANTATION

Recurrence of HCV post transplant is common and associated with rapid progression to graft fibrosis and cirrhosis in up to 20% of these cases (72,73). A small study from Italy described an association of KIR/HLA-C mismatch between donor and recipient with recurrence of HCV hepatitis post-transplant and the presence of KIR2DL3 with rapid progression to fibrosis once HCV re-infection occurred (74). Considering that KIR/HLA-C interactions are important for NK cell regulation and that KIR2DL3 is the KIR associated with spontaneous resolution of HCV through rapid and vigorous NK cell activation (35,43), this suggests a prominent role of KIR and thus NK cells in the immuno-suppressed post-transplant setting. Other studies further reported a role of KIR/HLA-C mismatches also for liver transplant in general in terms of short-term allograft injury and survival (75,76).

Varchetta et al. investigated this further by studying NK cells in consecutive blood samples post liver transplant in patients with recurrent HCV (77): NKG2D+ NK cells declined post-transplant, but then increased accompanied by a rise in CD69+ NK cells at day 7 post-transplant suggesting early activation of NK cells despite immuno-suppression. The progressive increase in the frequency of CD94/NKG2C+ NK

cells over time was likely related to HCV recurrence. A significant correlation between NKp30 and NKp46 expression on NK cells with ALT levels was in keeping with a role of NK cell related cytotoxicity in determining the severity of hepatitis. This data clearly suggests a role of NK cells in HCV recurrence and graft fibrosis post-transplant. Future studies are required to better understand how this process may be modulated.

NK CELLS AND HCV-RELATED HEPATOCELLULAR CARCINOMA

A role of NK cells in tumor surveillance through direct activity against neoplastic cells is well established (78). KIR-HLA interaction is thought to contribute to this by NK cells killing off malignant cells that are down-regulating HLA expression in order to avoid T cell recognition and expressing stress ligands. Data regarding NK cells and hepatocellular carcinoma (HCC), however, remains scarce. A small Spanish study including 54 patients with HCC suggested an association of the activating KIR3DS1 and its ligand HLA-Bw4I80, with protection from HCC in patients with HCV related liver cirrhosis (79). A lower frequency of KIR expressing NK cells has been described among tumor-infiltrating NK cells in HCC, suggesting an adaptation of NK cell phenotype in this context (80).

CONCLUDING REMARKS

In summary there is growing evidence that NK cells play an important role in all aspects of HCV infection and that HCV has derived multiple ways of impairing the NK cell response. Future studies will need to address the important questions of 1) how to enhance the NK cell response to eliminate HCV in acute, chronic infection and post-transplant re-infection, 2) how to best modulate NK cell function in order to promote a T cell response to clear HCV infection during initial infection and 3) whether modulation NK cell function may assist vaccine development.

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CONFLICTS OF INTEREST

The author has no financial conflict of interest.

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