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Natural Killer cells: Multi-faceted players with key roles in Hepatitis C immunity

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Summary

Natural killer cells (NKs) are involved in every stage of hepatitis C viral (HCV) infection, from protection against HCV acquisition and resolution in the acute phase to treatment-induced clearance. In addition to their direct antiviral actions, NKs are involved in the induction and priming of appropriate downstream T-cell responses. In the setting of chronic HCV, overall NK cell levels are decreased, altered subset distribution is altered, and changes in NK receptor (NKR) expression have been demonstrated, although the contribution of individual NKRs to viral clearance or persistence remains to be clarified. Enhanced NK cell cytotoxicity accompanied by insufficient interferon-γ production may promote liver damage in the setting of chronic infection. Treatment-induced clearance is associated with activation of NK cells, and it will be of interest to monitor NK cell responses to triple therapy. Activated NK cells also have antifibrotic properties, and the same hepatic NK cell populations that are actively involved in control of HCV may also be involved in control of HCV-associated liver damage. We still have much to learn, in particular: how do liver-derived NKs influence the outcome of HCV infection? Do NK receptors recognize HCV-specific components? And, are HCV-specific memory NK populations generated?

Keywords

innate immunity; liver; human; interferon

Hepatitis C viral infection

The hepatitis C virus (HCV) is a positive-stranded RNA enveloped virus, a member of the flavivirus family (1). The HCV genome is approximately 9600 nucleotides and encodes a single polyprotein precursor of approximately 3000 amino acids. The polyprotein is cleaved by both host and viral proteases into structural and nonstructural (NS) proteins (2) (Fig. 1). Replication is mediated by NS5B, the viral RNA-dependent RNA polymerase that is devoid of proof-reading capacity, resulting in a high mutation rate. The inherent sequence diversity of HCV represents one of the most substantial challenges to the development of an effective HCV vaccine. To date, seven major HCV genotypes demonstrating >30% nucleotide sequence divergence from each other and numerous subtypes have been identified.

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Approximately 200 million people (an estimated 3% of the world's population) throughout the world have chronic HCV infection (3); HCV persists in up to 80% of people infected, whereas only a minority (~20%) of individuals exposed to HCV are able to spontaneously clear the infection. HCV, primarily transmitted via contaminated blood, is a leading cause of liver cancer and indication for liver transplantation (3). In the US alone, the burden over the next 10–20 years is expected to reach over $10 billion in direct medical costs and double this in overall societal costs (4).

Pegylated interferon-based regimens, with ribavirin, had been the standard of treatment over the past decade. When results are stratified according to genotype, sustained virologic response (SVR) was about 40% to 50% in patients with genotype 1 and 75% to 85% in patients with genotype 2/3 (5–7). Although recent advances in treatment, including the addition of NS3/4A protease inhibitors, have significantly enhanced SVR, drug toxicities and costs remain significant hurdles for many patients, and triple therapy may not be available for the majority of HCV-infected patients (8). The immune response to HCV is complex involving multi-cellular division of labor and includes components of innate and adaptive immunity (3). Enhanced understanding of HCV-host interactions and the mechanisms that regulate immunity within the liver is required to combat this virus and to develop improved therapies.

Natural killer cells

Natural killer (NK) cells are considered the principal innate effectors representing the first line of defense in the control of viral infections (9–11). They provide antiviral protection through surveillance of danger signals, downregulation of major histocompatibility complex (MHC) class I molecules 'missing-self' (12), and upregulation of MHC class I homologues 'induced-self' ligands (13), in addition to direct recognition of pathogen-associated molecules (14, 15). Their role may be direct, as NK cells can kill without prior sensitization via the release of granzyme and perforin containing cytotoxic granules (16). In addition, they produce cytokines such as interferon-γ (IFN-γ), which can limit viral replication (17). NKs also act indirectly by influencing the activation and/or trafficking of other key immune cell populations (18, 19) including dendritic cells (DCs) (20) and T cells (21, 22). An appreciation of this immune-regulatory function of NKs has provided insight into the critical role played by NKs in the crosstalk between innate and adaptive immunity and has highlighted their continuing role in chronic viral infections) (Fig. 2). The suggestion that NK memory responses develop and persist, at least in mice (23–25) and that human NK cells have functional memory-like properties after cytokine activation (26) adds a new level of complexity to this population opening up exciting possibilities for NK cell-based immunotherapy or vaccination in cases where more traditional approaches have been unsuccessful (27).

Until recently, mainly because of the paucity of well described acute HCV cohorts and the perception that as part of the innate immune response NK cell function would impact only in the early stages of infection, the role of NK cells in HCV infection remained relatively unexplored. We hypothesized that NK cells were likely important at all stages of HCV infection, not just in the acute setting (28), and since then several studies have supported this premise. In this article, we review recent data from our own laboratory as well as others that have contributed significantly to our understanding of the role played by these complex and versatile immune effector cells in the setting of infection with HCV.

Natural killer cell activation

NK cell activity is stringently controlled by inhibitory NK receptors (NKRs), which in steady-state conditions override signals provided by engagement of activating receptors.

Negative signaling induced by inhibitory receptors opposes NK cell activation and provides an important safeguard from NK cell reactivity toward normal, healthy cells (29). In the setting of viral infection, the balance favoring inhibition seen under normal conditions is shifted towards activation (30). The main classes of NKRs include the predominantly inhibitory killer immunoglobulin-like receptors (KIR), C-type lectin-like receptors of the CD94/ natural killer group 2 (NKG2) family comprising inhibitory (NKG2A) and activatory (NKG2C/D) isoforms, as well as the natural cytotoxicity receptors (NCRs) NKp30 (NCR3/ CD337), NKp44 (NCR2/CD336), and NKp46 (NCR1/CD335) that deliver activation signals (15, 31–35). Additional surface receptors are involved in the activation of NK cells. Some of these are not exclusively expressed on NK cells and are mainly involved in NK cell adhesion to target cells. These include DNAX accessory molecule-1 (DNAM-1) and NKp80, also known as killer cell lectin-like receptor subfamily F, member 1 (KLRF1), involved in epithelial and myeloid cell interactions (36). Tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL/Apo2L), responsible for extrinsic induction of cell death, can also be expressed on NK cells. TRAIL has been implicated in immunosuppressive, immunoregulatory, and immune-effector functions. With respect to pathological challenges, TRAIL and its receptors have been shown to play important roles in the immune response to viral infections (37). The majority of activating NKRs function as co-receptors requiring a second signal provided by loss of inhibition, cytokine stimulation, or a second activating receptor (38, 39).

As mentioned above, one of the main classes of NK inhibitory receptors is the KIR family. Diverse and polymorphic, they interact with highly polymorphic MHC class I ligands. Their main function lies in constitutive inhibition and in the generation of diversity in immune responses to pathogens and as such have received considerable attention as potential disease association markers (40). The other dominant NK inhibitory receptor is the evolutionary conserved NKG2A (38). This receptor forms dimers with CD94 and binds human leukocyte antigen-E (HLA-E), which presents leader peptides derived from classical MHC class I molecules (41). CD94/NGK2A serves to monitor appropriate expression of MHC class I and senses changes in overall MHC class I expression that may arise from viral infection (36). Another member of the NKG2 family, NKG2D, is a potent activating receptor on NK cells (42). Multiple inducible ligands, all of which are homologues of MHC class I molecules are recognized by NKG2D. Human ligands include MHC class I chain-related A and B (MICA, MICB) and UL16-binding proteins (ULBPs) (13, 43). Through recognition of ligands induced by stress or infection, NKG2D plays an important role in the control of viral infections. The importance of this receptor in host antiviral defense is emphasized by the multiple redundant mechanisms viruses, including human cytomegalovirus (HCMV) (44), human immunodeficiency virus (HIV) (45), and hepatitis B virus (HBV) (46), have evolved to counteract the NKG2D-dependent immune response. Another important group of activating receptors is the NCRs, which include NKp30, NKp44, and NKp46. All three NCRs are involved in the clearance of both tumor and virus-infected cells (35). Several tumor-specific and viral ligands of the NCRs have been described (35). B7-H6 expressed on transformed cells has been identified as a tumor-specific ligand for NKp30 (47, 48). BCL-2-associated athanogene 6 (BAG6), also known as HLA-B-associated transcript 3 (BAT3), released by tumor cells in exosomes binding to NKp30 can activate NK cells (49), and BAG6 expressed on the membrane of immature DCs (iDCs) is involved in the elimination of iDCs by NK cells (50). NKp30 also binds several viral ligands leading to inhibition including HCMV pp65 (51) and vaccinia virus haemagglutinin (HA) (52). Unlike NKp30 and P46 which are expressed on both resting and activated NK cells, NKp44 is detected only on activated NKs (35). Viral HA and HA-neuraminidase (HN) are activating ligands (53, 54), and tumor-derived recently proliferating cell nuclear antigen (PCNA) has been reported as an inhibitory ligand (55). Among the NCRs, NKp46 is the only receptor that has an orthologue in other species. This specific evolutionary conservation suggests that NKp46 is

the primary NCR involved in tumor and pathogen recognition (35). NKp46 recognizes unknown ligands on pancreatic β-cells leading to the development of type I diabetes (56). Engagement of NKp46 by an as yet unidentified ligand on hepatic stellate cells protects from liver fibrosis (57). NKp46 is important for the recognition of HA from several viruses including influenza and sendai viruses (58).

The current model of NK cell activation includes loss of constitutive inhibition through downregulation of MHC class I (59), upregulation of activating receptors and/or their ligands (30), cell adhesion and, response to inflammatory cytokines including IFNs induced by viral infection, interleukin-2 (IL-2), IL-15, and IL-12 (38, 60–64)(Fig. 3).

The role of natural killer cells early in HCV infection

Recent studies have implicated NK cells as important players in host defense in all stages of HCV infection and suggest that NKs may even protect from HCV acquisition. Genetic studies have linked KIR and MHC Class I polymorphisms to resistance to HCV (65, 66). These data suggest that heightened NK cell activity may prevent HCV infection in the setting of low-dose exposure. However, as KIR can also be expressed by T-cell subsets, the direct relevance of some of these data to NK cell biology remains to be fully established (67). We characterized NK cells in a unique cohort of prospectively collected peripheral blood samples from HCV-exposed injection drug users (IDUs). NK cell profiles in exposed individuals who remained uninfected despite being repeatedly exposed to HCV (n=11) were compared with pre-infection samples (median 90 days prior to HCV seroconversion) collected from 14 IDUs who were exposed and subsequently became infected. We demonstrated, in patients who remain protected from HCV infection, enrichment for $CD56^{low}$ effector NKs displaying enhanced IL-2 induced cytolytic activity against the NK-sensitive cell line K562 and higher levels of the NKp30 activating NCR (68). A role for NKs in preventing HCV infection is further supported by a recent study from Barbara Rehermann's group at the National Institutes of Health (NIH) (69). Eleven healthcare workers with accidental percutaneous exposure to HCV-infected blood who remained negative for HCV RNA and HCV-antibodies were studied. All but one of these cases displayed increased multifunctional NK cell responses with enhanced NCR (NKp44, NKp46) and NKG2A expression, cytotoxicity (as determined by TRAIL and CD107a expression), and IFN-γ production (69). We have also demonstrated clear race- and gender-related differences in expression of NKp46, which correlates with differential HCV natural history, supporting the biological relevance of NKp46 in innate protection (70). NKp46 is considered the major human NCR involved in NK cell-mediated killing (58, 71). Taken together, these data support the hypothesis that NK cell activity contributes to anti-HCV defense in the earliest stages of infection, providing innate protection from HCV acquisition and point to a significant role for NCRs and enhanced cytotoxicity in this process.

Natural killers in the acute phase

Little is known of the role of NK cells in determining the outcome of acute HCV infection. It is thought that NK cells are activated early in acute HCV infection, although the precise role they play is unclear (72–74). Functionally, NK cell IFN-γ production (72) and cytotoxicity or degranulation were higher in individuals with acute HCV infection than in healthy controls (72, 73). In another study, the absolute percentage of circulating NK cells was significantly elevated in the acute phase of HCV infection compared to HCV-negative controls. In addition, NK cells from acutely infected patients showed increased degranulation in response K562 target cells (74). In the studies above, the activity of NK cells did not correlate with subsequent outcome. However, an indirect role for NKs through induction and priming of T-cell responses was suggested by the finding that peak NK cell

activation and degranulation preceded peak T-cell responses and, of note, NK cell degranulation correlated with the magnitude of HCV-specific T-cell responses (73). Phenotypic alterations of NK cells in acute HCV infection have been reported but are difficult to interpret. Amadei *et al.* (72) observed an increased expression of NKG2D on NK cells, irrespective of the outcome, as compared with healthy controls which is consistent with activation. Alter *et al.* (74) showed that NK cells from acute infected patients demonstrated lower frequencies of NKp46- and NKp30-expressing NK cells, and these lower levels correlated with HCV clearance. This finding is somewhat counterintuitive, as high levels of NKp30 (68) and NKp46 (69) expression have recently been associated with protection against HCV infection in exposed uninfected individuals and as NKp46 expression correlates with anti-HCV activity *in vitro* (70, 75, 76). The authors suggest that activation-induced downregulation of NCRs may account for the diminished percentage of NK cells expressing NKp46 and NKp30 in patients who resolve acute infection and may reflect that early NK cell activation results in the onset of an effective innate immune response that participates in viral clearance (74). Further studies using well defined cohorts of patients with acute HCV infection are needed to define the contributions of individual NKRs to resolution.

Studies to date suggest direct involvement of NK cells in the acute phase of HCV infection; NK cell activation and phenotypic alterations have clearly been demonstrated. A direct role for NK cells in resolution of acute HCV infection has yet to be demonstrated. Activation of NK cells early in HCV infection likely favors induction and priming of downstream T-cell responses and HCV clearance (77).

Natural killer cell levels and phenotype in chronic HCV infection

Significantly more is known of the role played by NK cells in the outcome of chronic HCV infection. NK cell frequency is reduced in chronic HCV compared to healthy controls (78–81). The reason for this decrease is currently unknown but is probably not due to NK cell recruitment to and compartmentalization in the liver as hepatic NK cell levels are also decreased (79, 82, 83). In humans, NKs can be identified by the expression of N-CAM (CD56) and relative expression of this antigen identifies functionally distinct immature/regulatory ($CD56^{bright}$) and effector ($CD56^{dim}$) NK subsets. The $CD56^{dim}$ subset, which are strongly cytolytic mature effector cells characterized by high perforin expression, account for the majority of circulating NK cells. In contrast, $CD56^{bright}$ NK cells are focused on production of cytokines such as IFN-γ (84). This subset is considered less mature and can give rise to the $CD56^{dim}$ NK cells (85). In addition to these conventional NK cell subsets, a highly dysfunctional subset of $CD56^{neg}CD16^{pos}$ NKs has been described that appears to be terminally differentiated, has impaired cytolytic function, and poor cytokine production (86). Altered subset distribution (decreased $CD56^{dim}$ and/or increased $CD56^{bright}$) is a consistent finding in several chronic HCV cohorts (79, 87). Increased circulating levels of dysfunctional $CD56^{neg}CD16^{pos}$ have also been reported (88, 89) (Fig. 4). While changes in phenotype are clearly demonstrated in chronic HCV, conflicting data exist with respect to the expression of NKRs. These variances may arise from differences in methodologies, control groups used, the use of fresh or frozen blood samples, and small sample sizes (90). Increased NKG2A expression (79, 91–93) is a consistent findings in chronic HCV, which suggests inhibition of NK function, although this may simply reflect altered subset distribution as $CD56^{bright}$ NKs express high levels of this receptor. The evidence with respect to NCR expression in chronic HCV is conflicting as both decreased expression (94) and increased expression (91, 95, 96) have been reported. A significant role for the NKG2D pathway in the defense against HCV infection is suggested by several studies, although the overall contribution of the NKG2D pathway in the control of HCV infection is not fully elucidated (81, 91). The HCV-NS5A protein downregulates expression of NKG2D on NK

cells via theTLR4 pathway, thus impairing their function. The suggested mechanism is that NS5A triggers IL-10 secretion from monocytes, which in turn promotes TGFβ production, which leads to downmodulation of NKG2D expression and impaired effector functions both IFN-γ and CD107a degranulation (97). In a direct infection system (HL7702 cells infected with HCV-positive serum), the HCV protease NS3/4A was shown to reduce the expression of NKG2D ligands MICA and MICB (98). Direct contact with HCV-infected cells impaired NK cell degranulation, lysis activity, and IFN-γ production, and this inhibition was associated with downregulation of NKG2D and NKp30 on NK cells. These observations suggest that direct cell-to-cell interaction between NK cells and HCV-infected hepatocytes may impair NK cell function *in vivo* and thereby contribute to the establishment of chronic infection (99). Augmentation of NKG2D activity may enhance immunity to some cancers or infections. For this to be possible, more research is needed to further understand mechanisms that regulate NKG2D function, expression, and signaling (43). NKG2D expression has been reported to be upregulated or downregulated or unchanged in HVC infection (91, 92, 95).

The activity of natural killer cells in chronic HCV infection

In chronic HCV infection, overall levels of NK cells are decreased, the NK cell subset distribution is perturbed, and NKR expression is altered possibly reflecting activation in response to chronic virus-induced stimulation. The question remains, how do these changes impact on activity, and, does the functionality of NKs influence the outcome of infection. Several *in vitro* and *ex vivo* studies suggested that NK cell activity was inhibited in chronic HCV infection (100–104). However, the reported depressed activity of NK cells in chronic HCV may be a consequence of decreased levels of $CD56^{dim}$ effector cells, as more recent studies suggest that activity on a per cell basis is intact (78, 87). Several lines of evidence suggest that skewing or polarization of NK cell function away from IFN-γ production towards cytotoxicity may promote viral persistence and liver damage (81, 95, 105–106). Polarization of NK cell function towards cytokine production or cytotoxicity is clearly demonstrated *in vitro*. Cytokine stimulation (IL-12/IL-15) of isolated human NKs induces production of IFN-γ but not degranulation, whereas, phorbol myristate acetate (PMA) and calcium ionophore ionomycin stimulation induces degranulation (70). An NK cell polarization model as a mechanism of HCV evasion of effective NK cell responses and promotion of liver injury is supported by the available data. This phenomenon may be linked to the requirement for caspase activation for IFN-γ and TNF production, which is dispensable for cytotoxicity (107). Insufficient IFN-γ responses may result in increased viral replication, as IFN-γ has direct anti-viral properties and can control viral replication *in vitro* in a dose-dependent manner (108, 109). In addition to antiviral activity, IFN-γ is important for the differentiation and trafficking of appropriate helper T-cell responses (110, 111). Enhanced NK cell cytotoxicity accompanied by insufficient IFN-γ production may promote liver damage (95).

The above studies suggest that in addition to a decrease in overall levels of NK cells that NKR expression is altered reflecting activation in response to chronic virus-induced stimulation. The contribution of individual NKRs to viral clearance or persistence remains to be clarified. Data with respect to the functionality of NK cells in the setting of chronic HCV infection favors a polarization model. More research is needed to further understand mechanisms that regulate NK cells in the setting of HCV infection.

Natural killer cells in treatment

As antiviral therapy for chronic HCV infection continues to evolve, IFN-α remains as an integral component of current therapies. NK cells are one of the primary cell populations

responding to IFN-α; therefore, it is logical to assume that NKs will be intimately involved in the response to antiviral therapy for chronic HCV. Human NK cells recognize HCV-infected hepatoma cells after IFN-α stimulation in a DNAM-1-dependent manner. Furthermore, interaction of IFN-α-stimulated NK cells with HCV-infected hepatoma cells efficiently reduces HCV replication (112). IFN-α also induces TRAIL expression on NK cells and increased expression of TRAIL on NK cells has been associated with control of HCV infection; these observations might account for the second-phase decline in HCV-RNA levels during pegylated-IFN-α therapy (113).

Different NK cell levels and phenotypic and functional features in patients with chronic hepatitis C treated with standard therapy (pegylated IFN-α) and ribavirin observed between non-responder versus SVR patients supports a role for NK cells in the response to treatment (114). Baseline frequencies of $CD56^{dim}$ NK cells and perforin content were significantly higher in SVR vs. non-responder subjects. NK cells were more activated, as evidenced by increased expression of CD69, in rapid virological responders (RVR). Moreover, higher natural and antibody-dependent NK cytolyticity were associated with SVR (114). We have demonstrated higher expression levels of inhibitory NKG2A in patients who failed to achieve SVR (89). Levels of NK cells and the IFN-γ expression upon stimulation with K562 were reversed after successful treatment with pegylated IFN-α and ribavirin; however, these skewed functions were not recovered in treatment-resistant patients (105). Circulating $CD56^{neg}$ functionally impaired NK cells are increased in chronic HCV-infected patients compared to uninfected controls with the highest levels seen in those who fail to respond to standard pegylated IFN-α and ribavirin therapy. Higher levels of these dysfunctional NK cells also correlate with poor early viral kinetics (viral decline < 1.4 log10 in the first 28 days of treatment) (89). Successful antiviral therapy restores NK cell levels in the liver. Analysis of paired liver biopsy samples has shown that SVR is associated with an increase in the total number of intrahepatic NK cells following treatment with IFN-α alone or combined with ribavirin (115, 116).

Taken together, the above studies suggest that activation of NK cells by IFN-α is important to achieve treatment-induced viral clearance. It will be of interest to monitor NK cell responses to triple therapy.

Hepatic NK cells and HCV

The human liver is relatively enriched in NK cells (117–119). The role of liver-derived NKs has been studied extensively in animal models, but their functions in human liver disease are largely unexplored (118, 119). Hepatic NKs comprise 30–50% of lymphocytes in the liver (117, 119). The relative enrichment and constitutive activation of NKs in normal liver reflects their role in immune surveillance and elimination of pathogens encountered in the liver (119). Phenotypic studies are limited but have shown that hepatic NK cells differ from peripheral NK cells in that the majority do not express CD16 (120). Increased CD94:NKG2A and decreased KIR expression is also characteristic of hepatic NK cells (121). Two recent comprehensive reviews highlight the paucity of data on human liver-derived NK cells (90, 122).

Intrahepatic NK cells may behave differently to NK cells in other areas due to the ‘tolerogenic’ environment in the liver (117). Murine intrahepatic NK cells express high levels of NKG2A and are hyporesponsive. They are less cytotoxic and have an altered cytokine profile producing lower levels of IFN-γ and greater levels of immunoregulatory cytokines, such as IL-10, compared to peripheral blood and splenic NK cells (123). This hyporesponsive state has been described in the early stages of hepatitis B virus infection and may contribute to the establishment of chronic viral infection (124). Similar studies on

human liver-derived NK cells in chronic HCV infection have yet to be carried out. Much of what we know about hepatic NK cells in chronic HCV is inferred from our knowledge of the altered expression of important NK cell ligands in infected liver and cell culture systems. Direct contact with HCV-infected cells results in impaired NK cell degranulation and IFN-γ production. The observed inhibition was associated with a decrease in NK-activating receptor (NKG2D and NKp30) surface expression on NK cells. These observations suggest that direct interaction between NKs and HCV-infected hepatocytes may impair NK cell function *in vivo* contributing to the establishment of chronic infection (99). In HCV infection, there is an impairment of MIC-A/B expression which may result in lower levels of NK cell activation *via* the NKG2D ligand (97, 98, 125). The increased expression of NKG2A on hepatic NK cells (79) may be important, as HCV can upregulate HLA-E, the ligand for NKG2A, *in vivo* and *in vitro* thus representing a mechanism by which HCV may modulate the hepatic NK cell response (125, 126). HCV core protein can upregulate MHC class I expression on hepatocytes (127), which acts as a ligand for inhibitory KIR, another potential NK inhibitory strategy at play in the liver.

Hepatic NK cell numbers are decreased in chronic HCV and further decreased in cirrhosis (79, 82, 83). NK cells comprise 38% of lymphocytes in HCV-infected liver compared to 55% in non-HCV liver and have similar activation status, as evidenced by the expression of CD69. In chronic HCV infection, NK cell populations do not correlate with histological parameters (128) and decrease with histological progression (82, 129), suggesting they may not be directly involved in liver damage, making them an attractive population for immune intervention. NK cells are localized to necrotic areas in liver biopsy specimens in chronic HCV, but not chronic HBV (79). Increased proportions of $CD56^{bright}$ NK cells, increased expression of NKG2A in the liver of chronic HCV-infected patients (compared to chronic HBV infection) has been demonstrated which inversely correlated with viral load (79). Successful antiviral therapy restores the NK cell levels in the liver. Analysis of paired liver biopsy samples has shown that SVR is associated with an increase in the total number of intrahepatic NK cells following treatment with IFN-α alone or combined with ribavirin (115, 116).Activated NKs kill hepatocytes by releasing TRAIL in mice (130), and an HBV study suggests that this is also true for humans (131). IFN-α induces TRAIL expression on NK cells and increased expression of TRAIL on NK cells has been associated with control of HCV infection (113). Therefore TRAIL expression by hepatic NK cells may be important for HCV clearance (132). A recent study from Jacob Nattermann's group (75) found an intrahepatic accumulation of highly cytolytic NK cells expressing high levels of NKp46. Of note, the frequency of intrahepatic $NKp46^{High}$ NK cells was inversely correlated with HCV-RNA levels. This observation suggests that hepatic NK cell populations are actively involved in control of HCV.

Natural killer cells and fibrosis

In the face of heightened immunity, control of immune responses to limit collateral damage is as important as sustaining anti-viral defense, and NK cells may be central to maintaining this balance (27, 133). Chronic liver injury leads to liver fibrosis that is associated with accumulation of collagen in the liver (134). Immune cells play important and opposing roles in the regulation liver fibrosis; $CD8^+$ T cells promote (135) and NK cells inhibit fibrosis. In addition to their role in protection against pathogens and tumor transformation, intrahepatic NK cells have been demonstrated to have anti-fibrotic functions via inhibition of hepatic stellate cells (HSCs). They are capable of directly inducing HSC apoptosis in a TRAIL and NKG2D-dependent manner (136). Early activated stellate cells (HSCs) express increased levels of the NKG2D ligand MICA (136) (137) and upregulate TRAIL receptors upon activation (138). IFN-α treatment enhances while other factors (e.g. alcohol, TGF-β) attenuate the cytotoxicity of NK cells against HSCs, thereby differentially regulating liver

fibrogenesis (118). Production of IFN-γ by NK cells directly inhibits HSC activation (139, 140). Inhibitory KIR knockdown stimulates NK cells and promotes their anti-fibrogenic activity in mice and in human cell co-cultures. These findings have implications for possible immune therapeutic strategies in patients with advanced liver disease (141). These studies have revealed that human NK cells can kill primary human HSCs. The ability of NK cells from HCV patients to kill HSCs is enhanced and correlates inversely with the stages of liver fibrosis (78). As mentioned above, an intrahepatic accumulation of $NKp46^{high}$ was demonstrated in chronic HCV. The frequency of this population not only inversely correlated with HCV-RNA levels but also with fibrosis stage (75). This finding suggests that the same hepatic NK cell populations that are actively involved in control of HCV may also be involved in control of HCV-associated liver damage.

Regulatory natural killer cells

The immune-regulatory role of NK cells appears to play a critical role in shaping subsequent T-cell responses. IFN-γ, produced by NK cells, can promote the development of appropriate inflammatory T-helper 1 (Th1) responses (142). NK cells through interaction with DCs may also be required for the initiation of T-cell responses (143). DCs can be broadly classified into two major subsets myeloid and plasmacytoid (mDC and pDCs, respectively), which play distinct roles in the immune system. As major antigen-presenting cells (APCs), mDCs are critical for the priming of virus-specific $CD4^+$ and $CD8^+$ T cells (144). Upon activation, they produce IL-12 and IL-15, which can activate NK cells (145, 146) in addition to promoting the differentiation of pathogen-specific $CD4^+$ Th1 cells and cytotoxic $CD8^+$ T cells (CTLS) (147). Upon pathogen sensing, pDCs produce type I and type III IFNs (148), which also play a role in the activation and expansion of NK cells (149). Crosstalk between NKs and DCs is bidirectional: DCs activate NK cells (150, 151), and NK cells induce the maturation of DCs (152). Maturation of immature DCs (iDCs) into efficient APCs capable of initiating effective T-cell responses is dependent on NK cells through cell surface contact and cytokine secretion (153, 154). Moreover, DCs might also activate NK cells indirectly by promoting the expansion of antigen-specific T cells, which secrete IL-2, which in turn activates NK cells. IL-2-activated NK cells eliminate iDCs and thus may also play a role in downregulation of immune responses, as IL-2 is primarily produced by activated inflammatory T cells (155, 156). Bidirectional crosstalk between DCs and NK cells is important for the priming, activation, and expansion of T-cell responses (157), and disruption of this pathway is emerging as an important immune evasion mechanism employed by several viruses and direct infection of DCs appears to be an important factor (158–161). It is unlikely that DCs support replication of HCV, although HCV RNA has been detected in both mDCs and pDCs (162, 163) while other studies have not been able to detect viral replication or protein synthesis in DCs after co-culture with infectious recombinant HCV (164). A recent study suggests that exosomal transfer of HCV RNA occurs between hepatocytes and DCs that would explain the presence of HCV RNA in cells that do not support replication (165). DCs have been shown to be defective in chronic HCV (166–170), although the mechanism has not been fully elucidated. Circumstantial evidence, such as downregulation of MICA/B and defective IL-15 production (125, 171) by DCs, suggests that dysregulation of NK:DC crosstalk may be involved further studies are required to provide direct evidence for dysregulation of this pathway in HCV infection. The numerous mechanisms evolved by viruses to inhibit NK cell activity may not be directed at the innate immune response but may represent a strategy to prevent effective induction of adaptive immune responses (172). Defective T-cell or DC activity observed in viral infection may represent a bystander effect of viral NK cell inhibition (28).

Memory NK cells

Evidence of NK cell memory populations involved in antiviral immunity is accumulating in murine studies (173–177). Adoptive transfer experiments demonstrate that hepatic NK cells are sufficient and required for anti-viral recall responses to vesicular stomatitis virus (VSV), influenza A, and HIV (174). NK cell memory of haptens and at least some viruses are dependent on CXCR6, a chemokine receptor on hepatic NK cells that was found to be required for the persistence of memory NK cells but not for antigen recognition. Thus, hepatic NK cells can develop adaptive immunity to structurally diverse antigens, an activity that may depend on NK cell-expressed CXCR6 (178). Murine cytomegalovirus (MCMV)-experienced NK cells confer 10-fold the level of protection from infection when transferred into newborn MCMV-susceptible mice. However, MCMV-specific NK cells are not confined to the liver (24). Identification of these long-lived memory NK cell populations in humans is more challenging and data is sparse (179). Human NK cells have functional memory-like properties after cytokine activation, which provides a novel rationale for integrating preactivation with combinations of cytokines into NK cell immunotherapy strategies (26). It has been suggested that in humans, that NKG2C^{pos} NK cells represent a CMV-specific memory NK cell population. NKG2C^{pos} NK cells transplanted from seropositive donors exhibit heightened function in response to a secondary CMV event compared with NKG2C^{pos} NK cells from seronegative donors (180).

NK cell vaccinations might provide new opportunities to immunize against pathogens that have proven difficult to control using conventional B- and T-cell vaccination strategies (181). Given the similarities with the NK cell response to virus in mice, it is not unreasonable to consider that antigen-specific stimulation of human NK cell receptors may lead to NK cell immunological memory. It has recently become appreciated that NK cells directly recognize pathogen-associated molecules (182–184). Direct pathogen recognition by NK cells adds a new dimension to a cell that is regulated not only by integration of a complex balance of inhibitory and activating receptor signals (15, 34, 185) but also by a wide array of cytokines (60–64). It is possible that NKRs may recognize HCV-specific components, as has been demonstrated for other flaviviruses, dengue virus (DV) and West Nile virus (WNV) (186). Human NK cell memory might pave the way for new vaccine approaches, not only against chronic virus infections but also cancer, through controlled exposure to NK cell-dependent antigens (27).

The observations that NK memory responses develop and persist at least in mice (23–25) and that inflammatory cytokines (25, 187) direct recognition of viral antigens and activating receptors are central to the generation and maintenance of NK memory suggests that NK cell vaccination may provide new opportunities to immunize against pathogens where conventional approaches have proved to be ineffective (181).

Concluding remarks

NK cells play important roles in every stage of HCV infection from protection against infection in IDUs to prediction of antiviral success or failure with IFN-based therapies (Fig. 5). They provide innate protection from HCV acquisition. Lack of constitutive inhibition and activation via NCRs are likely important in this process. NKs are activated early in HCV infection, and activation and phenotypic alterations have clearly been demonstrated. A direct role for NK cells in resolution of acute HCV infection has yet to be demonstrated. Activation of NK cells early in HCV infection likely favors induction and priming of downstream T-cell responses and HCV clearance. In the setting of chronic HCV, in addition to a decrease in overall NK cell levels and altered subset distribution, NKR expression is altered, reflecting activation in response to chronic virus-induced stimulation. The

contribution of individual NKRs to viral clearance or persistence remains to be clarified. Data with respect to the functionality of NK cells in the setting of chronic HCV infection favor a polarization model with overactive cytotoxic and inadequate IFN-γ responses. Treatment-induced clearance is associated with activation of NK cells, and these activated NK cells may perform dual roles. On one hand, they are antiviral, but they may also be antifibrotic. We still have much to learn, in particular how liver-derived NK cells influence the outcome of HCV infection. The demonstration of NK cell antiviral memory and the possibility that NKRs may recognize HCV-specific components open up challenging but exciting avenues of investigation for the future.

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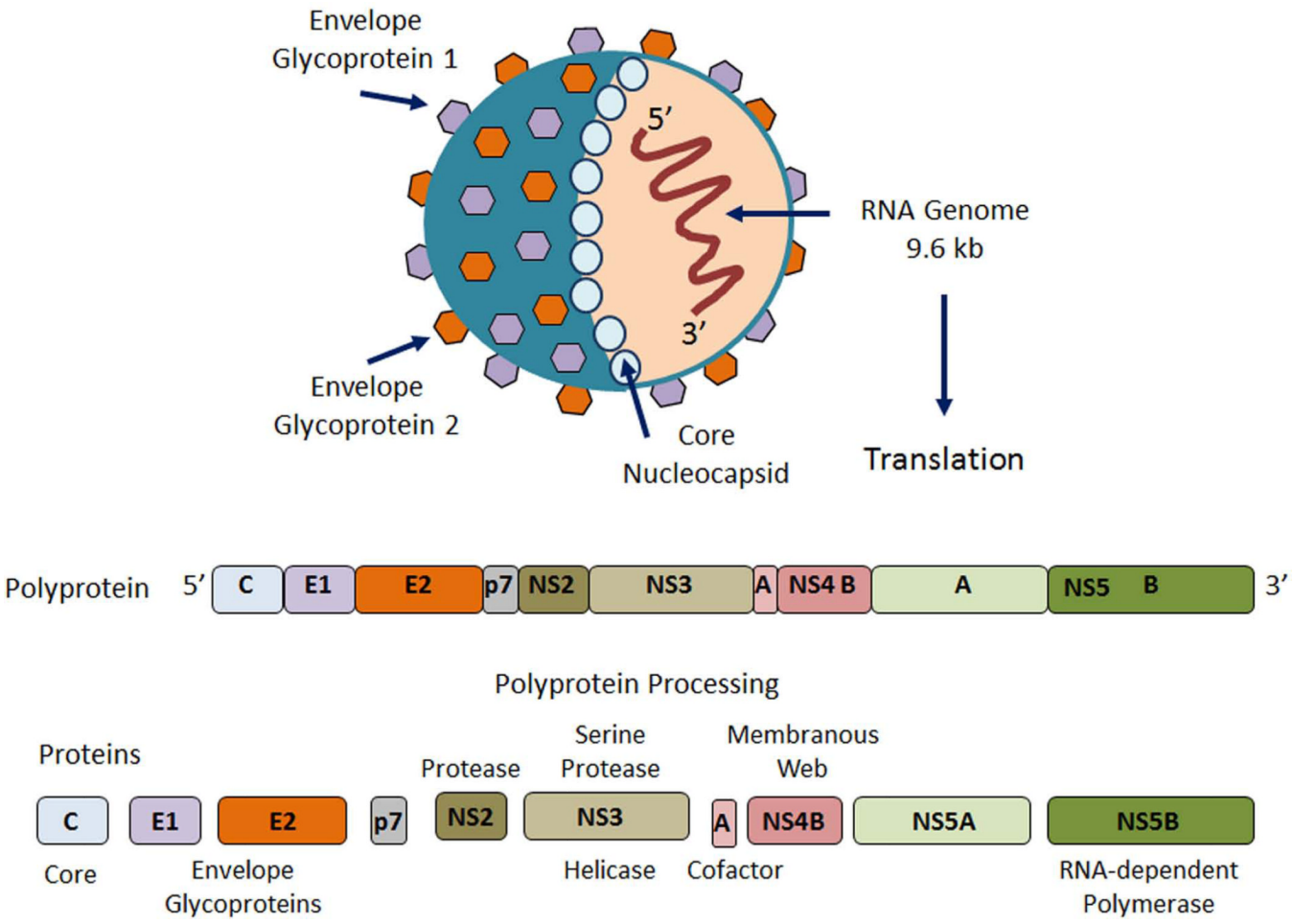

Fig. 1. Hepatitis C virus (HCV)

HCV is an enveloped, positive-stranded RNA virus. The 9.6 kilo base (kb) genome is translated into a single polyprotein precursor of approximately 3000 amino acids. Cleavage of the polyprotein by viral and host-cell proteases yields structural viral proteins (core protein and envelope proteins E1 and E2) and nonstructural viral proteins (NS2 through NS5B), with a number of putative activities and functions.

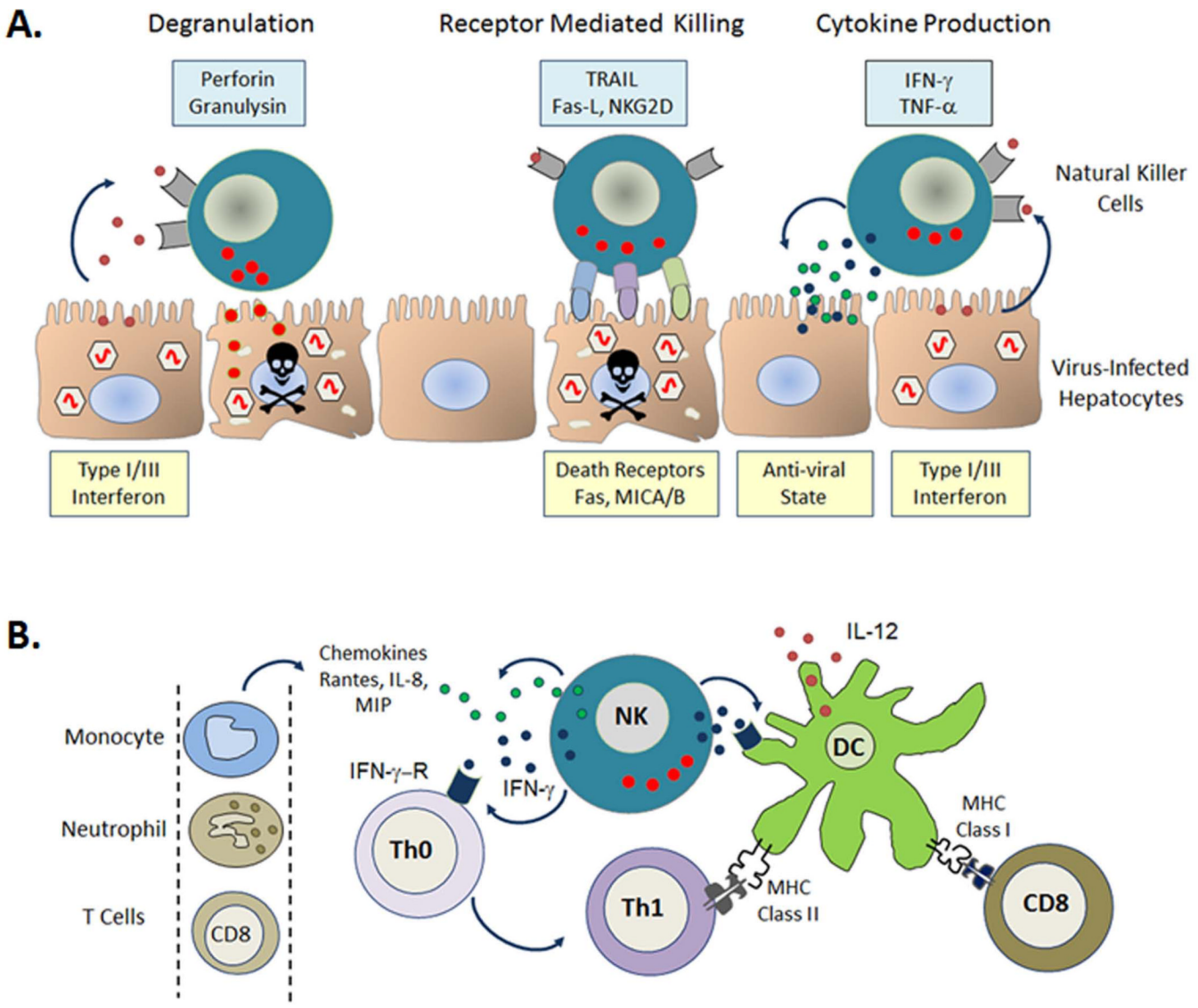

Fig. 2. Direct and indirect anti-viral effector mechanisms of natural killer (NK) cells

In the setting of viral infection, infected cells produce Type I/III interferon (IFN). NK cells respond through direct mechanisms; degranulation and receptor mediated lysis of infected cells as well as production of anti-viral cytokines such as IFN-γ (A). NK cells also act indirectly to prime the adaptive immune response promoting dendritic cell (DC) maturation and the differentiation of immature helper T cells (Th0) towards an inflammatory phenotype (Th1). Production of chemokines by NK cells attracts other immune cells to sites of inflammation (B). TRAIL, TNF-related apoptosis-inducing ligand; Fas-L, Fas ligand; TNF-α, tumor necrosis factor α; IFN-γ-R, interferon-γ receptor; IL-8/12, interleukin-8/12; MIP, macrophage inflammatory protein; MHC, major histocompatibility complex.

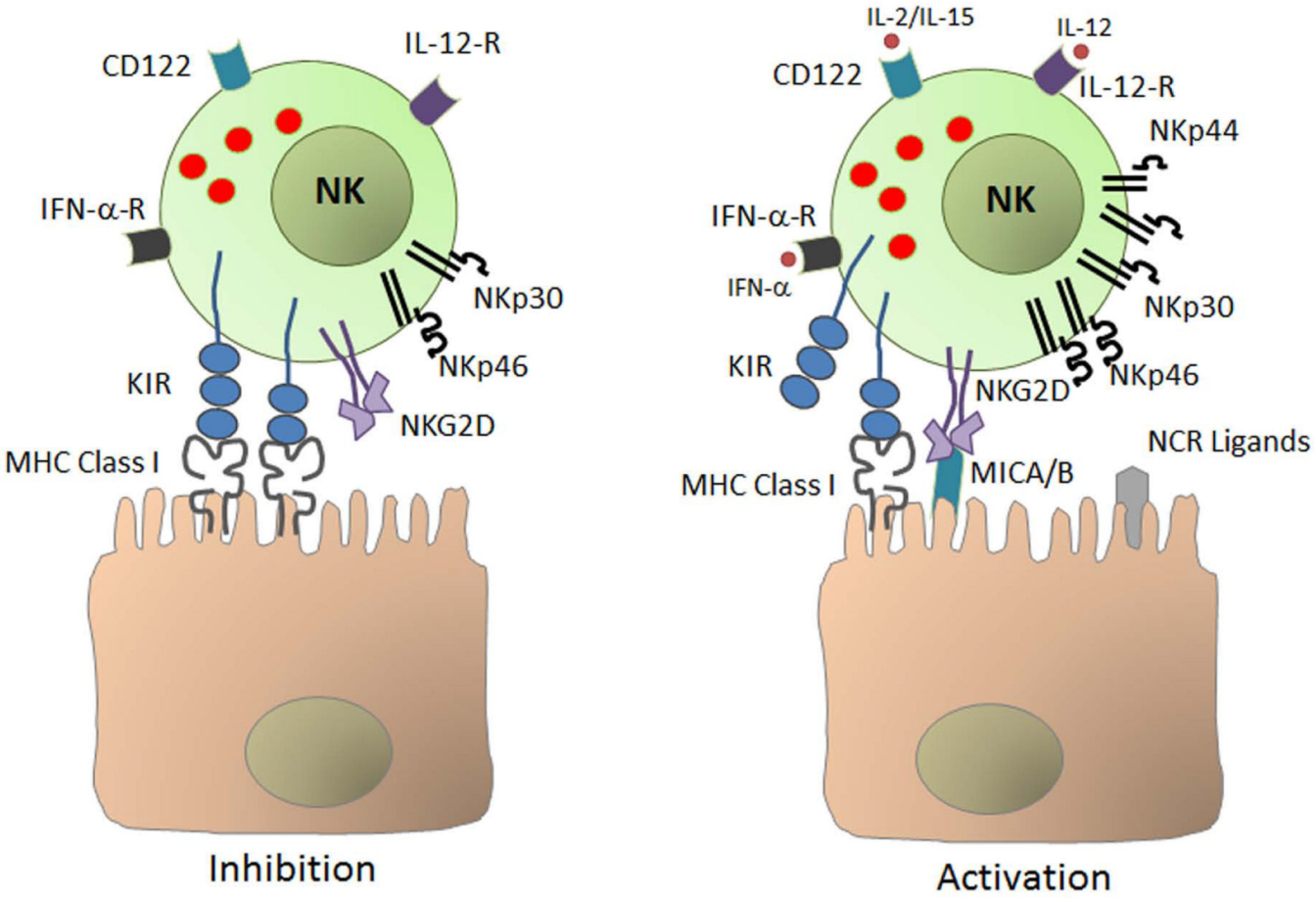

Fig. 3. Activation of natural killer (NK) cells

Under normal conditions, NK cells are constitutively inhibited mainly through engagement of major histocompatibility complex (MHC) class I molecules on normal cells by NK cell-expressed killer immunoglobulin-like receptor (KIR). Under conditions of stress such as viral infection, loss of constitutive inhibition through downregulation of MHC class I, upregulation of activating receptors and/or their ligands, cell adhesion, and response to inflammatory cytokines including interferon-α (IFN-α) and interleukin-2 (IL-2), IL-12, and IL-15 results in activation of NK cells. MICA/B, MHC class I polypeptide-related sequence A/B; NCR, natural cytotoxicity receptor; TNF-α, tumor necrosis factor α; IFN-α-R, IFN-α receptor.

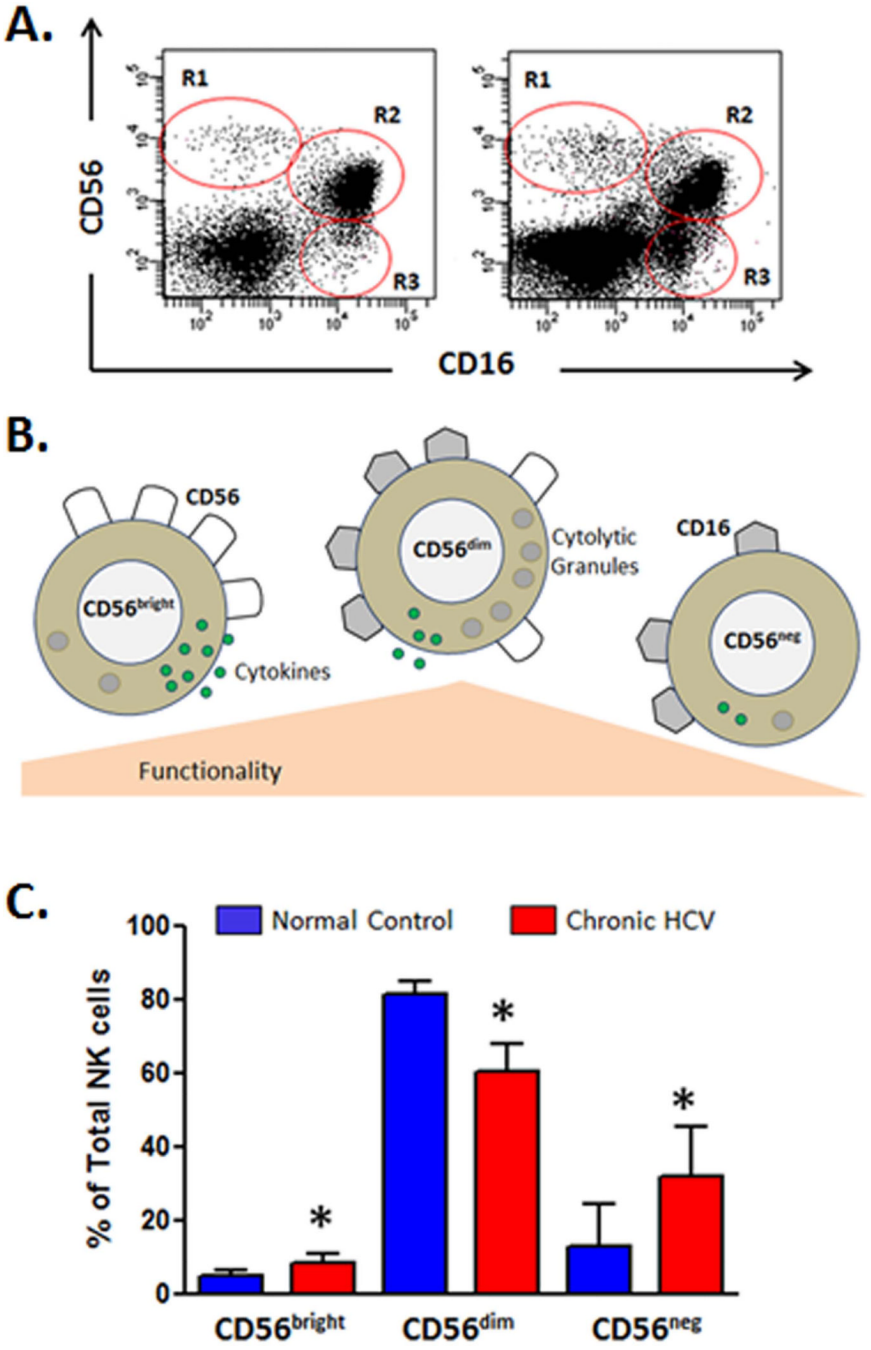

Fig. 4. Natural killer (NK) cell subset distribution is altered in HCV infection

The expression patterns of CD56 and CD16 can identify three distinct NK cell subsets $CD56^{bright}CD16^{neg}$ (R1), $CD56^{dim}CD16^{pos}$ (R2), and $CD56^{neg}CD16^{pos}$ (R3). Representative flow cytometric dot plots of $CD3^{neg}$ lymphocytes (low forward and side scatter) from one normal control subject and one chronic HCV patient are shown (A). $CD56^{bright}$ NK cells do not display significant natural cytotoxicity and are focused on production of cytokines such as IFN-γ. This subset is considered less mature and can give rise to the $CD56^{dim}$ mature effector cells which are strongly cytolytic and characterized by high perforin-containing granule content. Although $CD56^{dim}$ NKs produce less cytokine than their $CD56^{bright}$ counterparts, because they represent the predominant NK subset in

circulation, they are the main contributor to overall cytokine levels. The $CD56^{neg}CD16^{pos}$ NK subset is highly dysfunctional, has impaired cytotoxic function and poor cytokine production, and appears to be terminally differentiated (B). The bar chart shows the NK cell subset distribution which is altered in HCV infection. Median values and interquartile range are shown for normal control subjects (n = 5) compared to chronic HCV patients (n = 5). $CD56^{dim}$ mature effector NK cells account for the majority of circulating NK cells in both groups. Decreased $CD56^{dim}$ and increased $CD56^{bright}$ is a consistent finding in several chronic HCV cohorts (79, 87). Increased circulating levels of dysfunctional $CD56^{neg}CD16^{pos}$ are also evident. *$p<0.05$ calculated using a two sided Mann Whitney test.

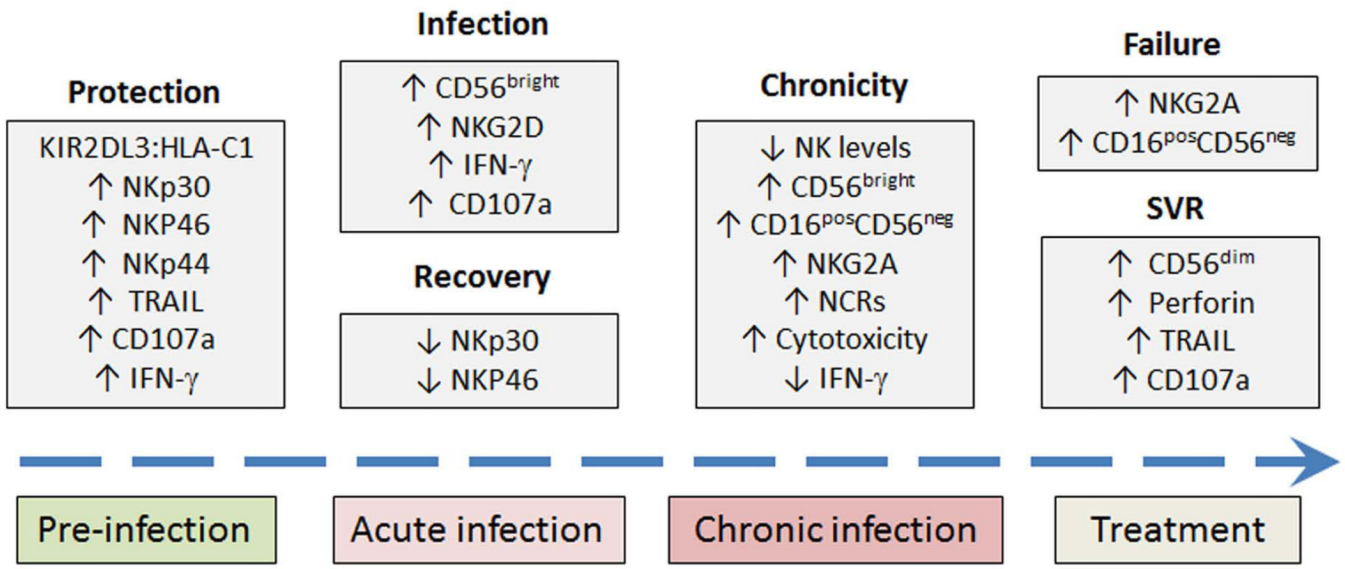

Fig. 5. Natural killer (NK) cells play important roles in every stage of HCV infection

NK cells play important roles in every stage of HCV infection from protection against infection in IDUs to prediction of antiviral success or failure with IFN-based therapies. Several NKRs and functional properties of NK cells have been implicated. Their association with natural history, stage of infection and treatment outcome are shown. KIR, killer immunoglobulin-like receptor; HLA, human leukocyte antigen; IFN-γ, interferon-γ; TRAIL, TNF-related apoptosis-inducing ligand; NCRs, natural cytotoxicity receptors; SVR, sustained virological response.