

Natural Killer Cell Responses to Viral Infection

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Key Words

Natural killer cells · Viral infection · Innate immunity

Abstract

Natural killer (NK) cells, as part of the innate immune system, play a key role in host defense against viral infections. Recent advances have indicated that NK cell activation and function are regulated by the interplay between inhibitory and activating signals. Thus, a better understanding of mechanisms responsible for NK cell activation and function in the control of viral infections will help develop NK cell-based therapies. In this review, we will first discuss how NK cells are activated in response to viral infections. We will then focus on the recruitment of activated NK cells to the site of infection as well as on NK cell effector mechanisms against virally infected cells.

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Introduction

Natural killer (NK) cells are unique lymphocytes without a clonally specific receptor [1]. As lymphocytes of the innate immune system, NK cells lack antigen-specific receptors and do not resemble T and B cells. This identity contributed to speculation that NK cells, origi-

nally discovered through their ability to destroy tumor cells without prior sensitization, mediate cytolysis in a nonspecific fashion [2, 3]. Subsequent observations that NK cells can kill certain tumor cells without major histocompatibility complex (MHC) class I [4] has led to the ‘missing self’ hypothesis, which proposes MHC class I functions as a ligand for inhibitory receptors in NK cell-mediated cytolysis. It is now better understood that the activation of NK cells and their functions are regulated by both activating and inhibitory signals.

While initial work demonstrated their antitumor activities, NK cells are also critical for the control of certain infections, particularly viral infections. In humans, NK cells are important to the innate immune response against members of the herpesvirus, poxvirus and papillomavirus families [5, 6]. Patients with identified NK cell deficiencies are predisposed to particularly severe, recurrent viral infections. Mouse models provide additional evidence that NK cells give critical help to control several viral infections, most notably murine cytomegalovirus (MCMV), poxviruses and influenza [7, 8].

In this review, we will detail how NK cells are activated in response to viral infections. We will then proceed to describe the recruitment of activated NK cells to the site of infection and NK cell effector mechanisms against virally infected cells.

NK Cell Activation in Response to Viral Infection

NK Cell-Activating Receptors

The 'missing self' hypothesis predicted the mechanism whereby NK cells destroy virus-infected cells that have a downregulated expression of MHC class I. However, in many circumstances, NK cells can efficiently eliminate virus-infected cells that maintain expression of the inhibitory MHC class I [9, 10]. Recent advances have indicated that NK cell activation and function are regulated by the interplay between the inhibitory and activating receptors [11, 12]. Indeed, accumulating evidence has revealed the importance of NK cell-activating receptors in antiviral defense.

The first NK cell-activating receptor identified to be critical for viral control in vivo was Ly49H, which is necessary to clear MCMV infection [13]. Ly49H, a C-type lectin-like receptor, specifically recognizes the m157 open reading frame of MCMV. This ligand was identified by two independent groups using heterologous reporter cells exposed to MCMV-infected cells [14, 15]. Activation of Ly49H by m157 is required for NK cell-mediated clearance in MCMV-resistant mice. Deletion of the genetic locus of Ly49H, *Cmv1'*, or use of Ly49H-blocking antibodies confers susceptibility to the virus [16]. Deletion of m157 also facilitates viral escape and persistence later in the infection. The Ly49 lectin-like receptors do not exist in humans. However, structurally different killer immunoglobulin-like receptors (KIRs) function similarly and recognize peptide-loaded MHC class I molecules. Patients homozygous for KIR2DL3 or KIR3DS1 and particular human leukocyte antigen haplotypes are much more likely to clear acute hepatitis C virus rather than progress to chronic infection [10]. KIR2DS1 can activate NK cells by recognizing MHC class I molecules loaded with peptide during Epstein-Barr virus infection [9].

The natural cytotoxicity receptors represent another class of activating receptors that recognize viral-derived products [17]. In humans, there are three members of this receptor family: NKp30, NKp44 and NKp46. Of these, NKp46 is the most prominent and is found on all NK cells. NKp46 recognizes hemagglutinin of influenza virus and hemagglutinin-neuraminidase of parainfluenza virus, suggesting that it may be involved in resistance to these viruses [17]. Indeed, mice deficient in NCR1, a murine homolog of NKp46, fail to protect against lethal influenza infection [18]. NKp46 and another activating receptor, DNAM-1, are critical for activation in response to human CMV-infected myeloid dendritic cells; however, a cellular ligand for NKp46 remains to be identified [19].

Besides direct recognition of viral-derived products or viral peptide-loaded MHC molecules, NK cells can also recognize stress-induced ligands through the NKG2 family, most notably the NKG2D receptor [20]. NKG2D, a C-type lectin-like homodimer, promotes NK cell activation by recognizing host stress proteins induced upon viral infections. The stress-induced NKG2D ligands are Rae-1, Mult-1 and H60 classes in mice and the ULBP and MIC classes in humans. Stress ligands have been shown to play an important role in the control of human CMV and MCMV infections [21, 22]. NKG2D is also critical in NK cell-mediated control of infection with vaccinia virus [23] and adenovirus [24]. The importance of NKG2D in viral defense is highlighted by the viral evasion mechanisms that seem to escape NKG2D recognition [25]. It has been shown that MCMV can downregulate H60 to evade NK cell-mediated clearance and that human CMV can produce antagonists to directly block NKG2D recognition of ULBP. The expression of NKG2D ligands on accessory cells such as dendritic cells and macrophages also mediates 'crosstalk' with NK cells. One recent study described a pathway in which lipopolysaccharide treatment of macrophages upregulated MICA expression by increasing its transcription, promoting its translational or posttranslational processing, and downregulating micro-RNAs that target MICA [26].

Downstream of these activating receptors, ligand recognition triggers an intracellular kinase cascade to transmit the activation signal [11]. This cascade begins with Src tyrosine kinase phosphorylation of the immunoreceptor tyrosine-based activation motifs (ITAMs), which are found on adaptor subunits KARAP/DAP-12, Fc ϵ RI γ and CD3 ζ . The phosphorylated ITAMs then recruit tandem SH2 tyrosine kinases Syk and ZAP70, which propagate a phosphorylation signaling cascade through transmembrane and cytosolic adaptors similar to T/B-cell receptor signaling in fellow lymphocytes. Ultimate downstream activation pathways include PI3K/ERK, Ras/ERK and PLC γ /DAG/IP $_3$. Importantly, NKG2D signaling differs from the traditional ITAM activation pathway. In humans, NKG2D relies on DAP-10 to transmit its activation signal, unlike other activating receptors that signal through DAP-12. DAP-10 requires the PI3K signaling pathway, which it activates via phosphorylation cascades involving either the p85 subunit of PI3K, Grb2 directly or the Sos1-Vav1-Grb2 complex [27]. Recent studies have shown that NKG2D signaling alone is not sufficient to activate NK cells and that efficient NK cell activation requires cooperation of other activating receptors acting synergistically [28]. In mice, NKG2D can signal through

both DAP-10 and DAP-12, leading to NK cell activation [29, 30].

In addition to NK cell-activating receptors, direct Toll-like receptor (TLR) stimulation on NK cells has emerged to play an important role in NK cell activation. It has been shown that TLR3, TLR7, TLR8 and TLR9 are expressed on human NK cells and that ligands for these TLRs can activate human NK cells in vitro [31–33]. Intranasally administered CpG can in fact activate and recruit NK cells to the lung [34]. Recent studies have demonstrated that direct TLR2 stimulation on murine NK cells is critical for their activation and function in the control of vaccinia viral infection in vivo [23]. This is achieved by activating the downstream MyD88-PI3K-ERK pathway. Similarly, direct activation of TLR4 by a bacterial component fimbrial protein, FimH, appears to be important for the activation of both mouse and human NK cells [35].

NK Cell Inhibitory Receptors

To control inappropriate activating signals, there is a repertoire of inhibitory receptors that repress NK cell activation [36]. These include lectin-like heterodimers such as CD94-NKG2A [37], KIRs found in humans [38] or lectin-like Ly49 homodimers found in mice [39]. These inhibitory receptors survey MHC class I molecules and seem to protect healthy cells from inappropriate NK cell-mediated killing. The ability of KIRs and Ly49 homodimers to be either activating or inhibitory while recognizing class I MHC molecules is dependent on the specific receptors' downstream signaling and the different human leukocyte antigen subtypes they recognize. Some inhibitory receptors also recognize other ligands such as cadherins and collagen. Despite the structural differences between the lectin and immunoglobulin receptors, these classes of receptors signal downstream similarly.

The balance between activating and inhibitory receptors is achieved within the cell, downstream of receptor-ligand binding [36]. Contradictory signaling between the activating and inhibitory receptors depends on the ITAMs and immunoreceptor tyrosine-based inhibition motifs (ITIMs), respectively. Upon stimulation of inhibitory receptors, ITIMs on the cytosolic domains of inhibitory receptors become phosphorylated by Src kinases. Phosphorylated ITIMs activate phosphatases to counter the kinase cascade of the activating receptors. They can recruit SHP-1 and SHP-2 protein phosphatases and SHIP-1, a lipid phosphatase, which dephosphorylate many downstream molecules in the activation signaling pathway to dampen Ca²⁺ influx and effector function. Only Vav1, a guanine nucleotide exchange factor for Rac1, has

emerged as a direct SHP-1 substrate from a 'functional substrate trapping' screen that employed a catalytically inactive KIR2DL1-SHP-1 chimera [40]. Dephosphorylation of Vav1 may prevent its promotion of Rac1 remodeling of the actin cytoskeleton for cell cytotoxicity.

The model for integration of inhibitory and activating signaling has progressed further with the identification of subsets of 'unlicensed' NK cells in mice [41]. These 'unlicensed' cells lack any known inhibitory receptor, but are apparently self-tolerant and hyporesponsive upon ligation of activating receptors in vitro. This important finding followed work characterizing the hyporesponsiveness of NK cells from MHC class-I-deficient mice. The hyporesponsiveness could be reversed only in NK cells expressing inhibitory receptors capable of binding the particular class I MHC allele reintroduced [42]. However, a more recent study indicates that only 'unlicensed' NK cells are capable of mediating MCMV control in vivo [43], suggesting that NK cells lacking inhibitory receptors may be critical for NK cell control of certain infections.

Cytokines Involved in NK Cell Activation

In addition to direct stimulation through activating receptors, NK cells can be activated by cytokines during the initial stages of viral infection [44]. Cytokines can also enhance activating receptor-mediated NK cell activation. The four principal cytokines involved in NK cell activation are type I interferons (IFNs), interleukin (IL)-12, IL-15 and IL-18. These cytokines can be produced directly by infected cells, or by activated dendritic cells or macrophages [45].

Type I IFNs, which include over a dozen different subtypes of IFN- α and one IFN- β , signal through IFN- α/β R1 and IFN- α/β R2 [46]. They directly activate NK cells to enhance cell-mediated cytotoxicity. This role of type I IFNs has been well established in the setting of infection with MCMV, lymphocytic choriomeningitis virus (LCMV) and several other viruses. They can also induce secretion of IL-15, a cytokine capable of inducing NK cell proliferation.

Unlike type I IFNs, IL-12 activates NK cells to augment the production of IFN- γ [47]. IL-12 is produced in response to MCMV infection and is capable of activating NK cells alone. In LCMV infection, the absence of high levels of IL-12 likely account for the weak NK cell response. Herpes simplex virus (HSV) provokes robust IL-12 and type I IFN responses in both mice and humans, whereas patients with HIV show little IL-12 response, which correlates to the degree of NK cell activation, respectively [8]. Another cytokine, IL-18, can also induce

IFN- γ production by NK cells [48]. IL-18 has been shown in at least one setting to act synergistically with IL-12 in its ability to induce IFN- γ expression in NK cells. Both IL-12 and IL-18 appear necessary to prime NK cells in response to viral infection. The mechanism of this priming is not entirely clear, but involves increasing translation of IFN- γ mRNA.

Cytokine receptors signal through the JAK-STAT pathway to induce NK cell activation [46]. Seven members of the STAT family facilitate the differential effects of various cytokines. Type I IFNs, for example, induce phosphorylation of STAT1 and STAT2 for STAT1-STAT2 heterodimers and STAT1 homodimers to drive gene expression that enhances cell cytotoxicity [49]. IL-15 also uses STAT1 to trigger NK cell proliferation. STAT1, on the other hand, impairs IL-12 sensitivity and IFN- γ production. Instead, STAT4 drives IFN- γ production in response to IL-12. The regulation of STAT molecules has clear consequences for cytokine signaling, and loss of STAT molecules can increase viral sensitivity at early times after infection.

NK Cell Recruitment to the Site of Infection

Viral control necessitates not just appropriate NK cell activation, but also effective recruitment of activated NK cells to the site of infection [50]. In the steady state, NK cells can be found in the spleen, lung, bone marrow, lymph nodes, peripheral blood mononuclear cells, and liver. Sphingosine-1-phosphate plays an important role in lymphocyte migration, and S1P5R, a G-protein-coupled receptor (GPCR), is differentially expressed on NK cells to control their movement in development and the steady state [51].

During infection, NK cells migrate towards and accumulate at the sites of infection for a wide range of viruses, including LCMV, MCMV, mouse hepatitis virus and vaccinia virus [52]. Broadly, four chemokine receptors have been implicated in NK cell recruitment to sites of inflammation: CCR2, CCR5, CXCR3 and CX₃CR1 [50]. All except CX₃CR1 have been shown to play a role in chemotaxis to sites of viral infection. Specifically, CCR2 and CCR5 are critical for trafficking to the liver in MCMV infection [53, 54]. CCR5 also induces NK cell recruitment to the central nervous system during HSV-2 infection [55]. CXCR3 aids NK cell movement to the liver in the setting of Dengue virus infection and, along with CD62L, generally facilitates migration to the lymph nodes in inflammation [50, 56].

However, the mechanisms of NK cell migration are only beginning to become understood. NK cell migration to chemokines *in vitro* or to the peritoneum of mice intraperitoneally infected with vaccinia virus has been shown to be pertussin-toxin sensitive, suggesting a role for GPCRs [57, 58]. The PI3K pathway, specifically the p110 γ and p110 δ isoforms, plays a role in the motility of leukocytes in general. NK cell migration is similarly controlled by these two PI3K isoforms where p110 γ signals downstream of GPCRs in response to chemokines and p110 δ functions downstream of tyrosine kinase-linked receptors in response to other steady-state or inflammatory signals [57]. However, the role of these PI3K isoforms in NK cell trafficking in response to viral infection has not yet been investigated.

NK Cell Effector Responses to Virally Infected Cells

Upon activation and recruitment to the site of infection, NK cells employ three main strategies to kill virally infected cells: the production of cytokines, the secretion of cytolytic granules, and the use of death receptor-mediated cytotoxicity [8]. Production of IFN- γ is an important effector function of activated NK cells. IFN- γ exerts direct effects in making host cells less hospitable to the virus and can act distally to prevent infection in other cells. It also recruits and activates other effector leukocytes, including cytotoxic T lymphocytes and CD4⁺ T helper type 1 cells [59]. NK cell production of IFN- γ helps to control MCMV. Mice with deficiencies in IFN- γ or IFN- γ R are increasingly susceptible to vaccinia virus, HSV and other viral infections. Similar susceptibilities are seen in humans with identified IFN- γ or IFN- γ R deficiencies [60].

NK cells, called 'large granular lymphocytes' in early work, can also directly kill infected cells by mediating cytotoxicity through preformed granules [8, 52]. These granules, notably perforin and granzymes, are similar to those used by cytotoxic T lymphocytes and function after direct interaction between the NK and infected cells. Perforin, a membrane pore-forming molecule, can permeabilize the cell [61]. On the other hand, granzymes, a family of serine proteases, disrupt cell cycle progression, inflict DNA damage and dissolve the nucleus upon entrance into the cell. Humans with identified deficiencies in perforin are susceptible to herpesvirus infections, and perforin knockout mice have impaired clearance of MCMV, influenza and several other viruses [5, 8].

FasL and tumor-necrosis factor-related apoptosis-inducing ligand can also be used in NK cell-mediated cy-

tolysis of infected cells [62]. In these pathways, NK cells express ligands capable of activating death receptors on the target cell that can trigger the extrinsic pathway of apoptosis. Tumor-necrosis factor-related apoptosis-inducing ligand-mediated cytotoxicity is particularly important for immature NK cells, which cannot use perforin-dependent mechanisms [62].

Concluding Remarks

In this review, we have sought to illustrate the role of NK cells in viral control. We have described the mechanisms of their activation, recruitment and effector responses against virally infected cells. Research on their response to viruses has demonstrated that NK cells are

not the unbridled, non-MHC-restricted killers they were originally depicted to be. Instead, their activation and function are highly regulated. How NK cells respond to particular viral infections is of continuing interest in the development of new treatment options. Specifically, delineating the key signaling pathways in the activation and migration of NK cells in response to particular infections remains an essential avenue of future research necessary for the development of future NK cell-based therapies.

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