

Critical role of Toll-like receptor signaling in NK cell activation

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Toll-like receptors (TLRs) and NK cell receptors are the most important receptor superfamilies in innate immunity. TLRs act as the sensor of external pathogens, while NK cells detect alterations in endogenous protein expression on target cells through activating and inhibitory receptors. Accumulating data has demonstrated that TLRs and NK cell receptors can coordinate and regulate each other during immune responses, which contributes to the initiation of innate response and the priming of adaptive responses. TLRs can activate NK cell function directly or with the help of accessory cells in a cytokine or cell-to-cell contact dependent manner. More understanding of the recognition of innate receptors and interactions between them may provide important insights into the design of effective strategies to combat tumor and microbial infections. In this review, we summarize how TLRs and NK cells discriminate the self or non-self components respectively. And importantly, we pay more attention to the role of TLR signaling in induction of NK cell activation, responses and the crosstalk between them.

Toll-like receptor, natural killer (NK) cells, NK cell receptor, dendritic cells, crosstalk

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The innate immune system in mammalian has evolved a universal and conservative defense line against the invasion from exotic micro-organisms and autogenous transformed objects. The early concept about innate immunity believed that it can nonspecifically eliminate microbes because innate immune cells exert their function without pre-sensitization; however, the discovery of Toll-like receptors (TLRs) in the mid-1990s shows that pathogen recognition by the innate immune system actually relies on germline-encoded pattern-receptors (PRRs) that have evolved to detect components of foreign pathogens, also known as pathogen-associated molecular patterns (PAMPs) [1–3]. So far four classes of PRRs have been identified: TLRs, RIG-I-like receptors (RLRs), NOD-like receptors (NLRs) and C-type lectin receptors (CLRs) [4]. Among them, TLRs have the most greatly advanced understanding of how they recognize conserved structures in pathogens and trigger innate immune response to further prime antigen-specific adaptive immunity.

Natural killer (NK) cells are innate effector cells that play a critical role in immunosurveillance by eliminating virally infected and transformed cells via generating and secreting cytolytic granules or cytokines, as well as expressing several activating and inhibitory receptors on the surface [5]. Equally as the essential components of the innate immune system, NK cells and TLRs bear unique mechanism to sense dangerous signals and exert immune effect. On the other hand, they coordinate with each other to control the invasion of acataleptic challenges [6,7]. In this review, we survey how TLRs and NK cells discriminate the self and non-self components respectively. And importantly, we pay more attention to the present knowledge of how TLRs affect the function of NK cells and the crosstalk between them.

1 TLRs—The sensors of external stimulator

TLRs, acting as the sensors of external pathogens, are one of the most important PRRs. They are expressed on almost

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all immune cells, such as DCs, monocytes, macrophages, T cell, B cell, NK cells, and even on non-immune cells such as fibroblasts, epithelial cells and some tumor cells [8]. Although TLRs are evolutionarily conserved from the worm *Caenorhabditis elegans* to mammals, the expression of TLRs is not stable but constantly variable in response to environmental stress such as virus or bacteria infection [9]. TLRs generally detect microbes-related components which are usually called PAMPs to activate transcriptional programs that initiate innate immune responses and orchestrate acquired antigen-specific resistance to exogenous insults [10]. Stimulation of TLRs by these microbial products, such as lipids, lipoproteins, lipopolysaccharide (LPS) and nucleic acids, leads to the activation of signaling pathways that result in the up-regulation of antimicrobial genes and the secretion of some inflammatory cytokines or IFNs to start up the defense responses [11,12].

So far, over ten kinds of functional TLRs have been elucidated in mammals, including 10 in humans and 12 in mice respectively, with TLR1-TLR9 being conserved in both species [13]. These TLRs are roughly divided into two sub-families depending on their cellular localization and respective PAMP ligands. One group is composed of TLR1, TLR2, TLR4, TLR5, TLR6 and TLR11, which are expressed on cell surface and recognize mainly microbial lipids components such as LPS and lipoproteins; the other group mainly includes TLR3, TLR7, TLR8 and TLR9, which are expressed exclusively in intracellular vesicles such as endoplasmic reticulum (ER), endosomes, lysosomes and endolysosomes, where they recognize microbial nucleic acids components such as double-stranded RNA (dsRNA), single-stranded RNA (ssRNA), unmethylated-CpG DNA motifs respectively [14,15]. So it is essential for these "nucleic acid-sensing" TLRs to internalize to the endosome before signaling is possible. Study of mice deficient with each TLR has demonstrated that each TLR has a distinct deflection in terms of PAMP recognition to perform their duty to counteract the extraneous invasion.

For example, TLR4 stimulation with LPS is the main force to resist the Gram-negative bacterial infection. For recognition, TLR4 needs the presence of complex from CD14 and MD-2 in the assist of LPS-binding protein (LBP) [16]. For infection of Gram-positive bacteria, TLR2, rather than TLR4, plays a major role to clear away the pathogenic bacteria due to the deficiency of LPS. TLR2 forms heterodimers with TLR1 or TLR6 to recognize its ligands, the microbial components from Gram-positive bacterial, fungi, parasites and virus, including LTA, lipoproteins, and PG [17,18]. Moreover, some co-receptors on the cell surface can assist PAMP recognition of TLR2. These include CD36, which acts together with the TLR2-TLR6 heterodimer to mediate the sensing of some TLR2 agonists [19]; and dectin-1, a C-type lectin that binds to fungus β -glucan and induces its internalization [20]. In addition to recognizing compounds derived from Gram-positive bacteria, TLR2 also binds LPS

in the presence of LBP and CD14 and induces nuclear factor (NF)- κ B activation. LPS treatment has been demonstrated to enhance the oligomerization of TLR2. Concomitant with receptor oligomerization, the IL-1R-associated kinase is recruited to the TLR2 complex [21]. Viral structural components, including viral DNA, dsRNA, ssRNA, can activate "nucleic acid-sensing TLRs", including TLR3, TLR7, TLR8 and TLR9 to eliminate the viral-invasion cells via inducing type I IFN and antiviral factors production [22].

Structurally, TLRs are type I trans-membrane proteins characterized by the extracellular domains containing leucine-rich repeats that mediate the recognition of PAMPs, transmembrane domains, and the intracellular Toll-interleukin 1 (IL-1) receptor (TIR) domains [23]. After binding with corresponding ligands, TLRs dimerize and undergo conformational changes required for the recruitment of TIR-domain-containing adaptor molecules to the TIR domain of the TLRs. Although with structural similarity, accumulating evidence suggests that the signaling pathways associated with each TLR are not identical, and therefore, result in different biological responses. For example, activation of TLR3 and TLR4 signaling pathway generates both type I IFN and inflammatory cytokine responses whereas TLR1/TLR2, TLR2/TLR6 and TLR5 activation induces mainly inflammatory cytokines such as IL-6, TNF- α and IL-8 [24].

2 Recognition of NK cell receptors: Discrimination of "self" and "non-self"

NK cells participate in immunosurveillance by directly eliminating virally infected or transformed cells through killing target cells, and priming adaptive lymphocytes through secretion of cytokines, such as IL-12 and IFN- γ [25]. NK cells, identical to other lymphocytes, have the potential to attack autologous cells. However, there are unique mechanisms to ensure that NK cells can keep the "self-tolerance" to normal self cells but are standby to danger signals. The NK cell function relies upon signals from activation and inhibitory receptors whose ligands are largely endogenous, that is, encoded by host genes. During effector responses, NK cells may detect alterations in endogenous protein expression on target cells by integrating signals from the balance between inhibitory and activating receptors [26,27].

2.1 Missing-self recognition by NK cells or NK cell self-tolerance

NK cells are lymphocytes that derived from bone-marrow precursors. During the development of NK cells, NK-cell precursors (NKp) generated from multipotent haematopoietic precursors develop into immature NK cells, which express NK-cell-specific markers partially, including similar levels of NKp46, NKG2D, CD11b and CD16, and express perforin and granzyme B both in mice and humans [28,29].

With the further differentiation of immature NK cells, MHC-class-I-binding receptors, also known as the inhibitory receptors, are expressed in a multiplex and omnipresent manner. This process is also referred to as “NK-cell education” [28,30]. So far the MHC class I-specific inhibitory receptors include the killer cell immunoglobulin-like receptors (KIRs) in humans, the lectin-like Ly49 dimers in mouse, and the lectin-like CD94-NKG2A heterodimers in both species [30,31]. The education process generates NK cells that are tolerant to self and obtain the capacity to respond to stimulation with regard to their activating receptors. So the recognition and lysis of MHC-class-I^{low} or MHC-class-I defection target cells by NK cells is aptly referred to as “missing-self” recognition [32,33]. Self MHC recognition can activate an immunoreceptor tyrosine-based inhibitory motif (ITIM)-dependent phosphatase SHP-1 or -2 and thus initiates an inhibitory signaling cascade [34]. The suitable expression of MHC class I molecules in normal cells and engagement of inhibitory receptors can avoid the autoimmune activity of NK cells.

2.2 Induced-self recognition by NK cells

NK cells can be incompetent or in-activated via the recognition of inhibitory receptor while encountering “healthy-self” cells. On the other hand, NK cells are regarded as the major force to oppose the virus infection and tumor development. So the integration of activation signal from the stimulatory receptors should conquer the inhibition of NK cells to exert their lytic and cytokine producing function. Transformation or infection might induce expression of stimulatory ligands so that constitutive inhibition delivered by inhibitory receptors is overcome. The magnification of activation response due to the up-regulation of stimulatory ligands is known as “induced-self” recognition [35].

NKG2D is an important activating receptor of NK cells, and is the main force to initiate “induced-self” recognition by NK cells. NKG2D is expressed on all NK cells and a set of $\gamma\delta$ T cells and NKT cells in mouse, as well as on all activating CD8+ T cells and intraepithelial $\gamma\delta$ T cells in human [36]. NKG2D identifies MHC class I chain-related A chain (MICA) or B chain (MICB) in humans [37] and a diverse

family of ligands shared by human and mice called the retinoic acid early transcripts (RAET1) family, which includes RAE-1, H-60 and murine UL16-binding protein-like transcript 1 (MULT1) in mice and the UL16-binding protein (ULBP) in humans [38,39]. Interestingly, MICA and MICB are nearly not expressed on normal cells or tissues but can be induced in many epithelial tumor cells, bacterial or viral infected cells and in “stressed” cells [40]. Similarly, Rae-1 and H-60 are also barely located in tumor cells of diverse origin but not expressed on “non-stressed” cells. So NKG2D recognizes endogenous ligands that are upregulated on the surface of most tumor or infected cells. And all the ligands are “induced-self” relatives of MHC class I molecules and adopt a MHC class I-like structure. The activation of NK cells mediated by “induced-self” recognition is usually transmitted through ITAM-containing adaptor proteins, as known as KARAP/DAP12 [41]. But in CD8⁺T cells, NKG2D associates solely with the signaling molecule DAP10. Collectively, the crosslink of NKG2D and the self ligands which are induced in stress triggers responsive mechanisms including the production of cytokines and the enhancing natural cytotoxicity, which are vital for the elimination of infected or transformed cells.

3 TLR signaling on NK cell activation

It is well-known that NK cells act as first line in defense against tumor, viral and microbial infections. During microbial infections, NK cells can be activated to kill infected or transformed cells and secrete large amount of cytokines to prime innate and adaptive immune responses. However, for a long time, it is not known whether NK cells can directly recognize pathogens. Until recently, it has been pointed that NK cells can be activated directly via TLRs. In other words, TLRs on NK cells can induce NK cell activation via integrating with their respective ligands.

It has been reported that almost all TLRs can be differentially expressed on human NK cells (Table 1). TLR2, TLR3, TLR5 and TLR6 have the primary expression but relatively low expression of TLR4, TLR7, TLR8 or TLR9 on human NK cells [42]. The expression profile might differ in different

Table 1 Expression of TLRs in NK and their exogenous and endogenous ligands

TLR	Expression	Exogenous ligands	Endogenous ligands	Adaptor
TLR2	High	Peptidoglycans, glycolipids	Hsp60, HMGB1	TIRAP/MyD88
TLR3	High	dsRNA, polyI:C, siRNA	dsRNA	MDA5, TICAM-1
TLR5	High	Flagellin	Gp96, fibrinogen, α -defensin2	MyD88
TLR6	High	Di-acetylated lipopeptides		TIRAP/MyD88
TLR4	Low	LPS, lipid A		TIRAP/MyD88
TLR7	Low	ssRNA40	ssRNA	MyD88
TLR8	Low	ssRNA40	ssRNA	MyD88
TLR9	Low	CpG-DNA	DNA-containing immunocomplex	MyD88

NK-cell populations. For example, CD56^{bright} NK cells were found to preferentially express TLR2, while CD56^{dim} NK cells were found to preferentially express TLR3 [43].

Stimulation with various TLR3 ligands can directly promote NK function, leading to the secretion of cytokines, the enhancement of cell lysis against virally infected or tumor cells. Total NK cells and CD56^{dim} subset have been sorted from donors and were stimulated by poly I:C, an agonist of TLR3. As expected, poly I:C directly induced the secretion of IFN- γ , as well as pro-inflammatory cytokine IL-6 and IL-8 by NK cells in a dose-dependent manner without the help of other cytokines [44]. And NK cells showed a up-regulation of CD69 expression and enhanced cytotoxicity targeting K562 tumor cells [44,45]. Similarly, poly I:C also directly activated human NK cell lines, NK92, YTC12 and YTS, which all had a notable TLR3 expression [43]. Poly I:C stimulation up-regulated the cytotoxic activity of NK cells targeting K562 and 721.221 cell lines, promoted the secretion of IFN- γ and induced the CXCL10 mRNA without additional cytokines or cell assist [44,45]. Further study proved that the TLR3 stimulation can directly induce the activation of TRIF/TICAM-1-dependent transcription factor IRF-3 and p38 MAPK in these human NK cell lines, and activation of p38 MAPK was essential for the activation of NK cells [43]. The activation of NK cells mediated by poly I:C has been applied to the research of tumor treatment or anti-virus responses, looking forward to finding an effective method for tumor or infection control in clinic. In head and neck squamous cell carcinoma (HNSCC) patients, the HNSCC microenvironment made TLR3 on surface of NK cells internalized rapidly, but afresh stimulation of NK cells with poly I:C can impair the TLR3 internalization and lead to the activation of local NK cells, as shown as high levels of IFN- γ secretion and up-regulation of cytotoxicity targeting the tumor cells [46]. Similarly, TLR3 expressed on NK cells also plays a major role on other circumstances such as virus infection, especially for MCMV infection. Moreover, similar activation effect of NK cells stimulated by TLR9 and possibly TLR7 and TLR8 signaling also occurs during MCMV infection [47].

There are two disparate situations on the expression of TLR7/8 on NK cells. TLR7/8 proteins are relative low expressed in CD56^{dim} and CD56^{bright} NK cells from normal healthy donors but are distinctly expressed in NK cell lines [48]. TLR7 is solely located in the NK cell line, while TLR8 is highly expressed in all NK cell lines (NK1, NK92, and YT) [49]. Nevertheless, accumulating data show that TLR7/8 stimulation promotes NK cell activation and exerts powerful anti-tumor and anti-viral responses. NK cell activation through TLR7/8 agonists mainly requires the help of other cytokines, such as IL-12, IL-18, and IFN- α/β , which are secreted by DCs or monocytes [49,50]. ssRNA40 which contains a uridine-rich ssRNA sequence derived from the LTR region of HIV-1 can serve as a TLR7/8 ligand and activate DCs and even NK cells. The critical role of ssRNA40

on NK cell activation has been shown in patients with acute viremic HIV-1 infection, in which the CD14⁺ monocytes help the activation of NK cells by secretion of IFN- α and IL-12, and also with a cell-to-cell contact involvement [51]. During the past years, with the elicitation of ssRNA40, several other single-stranded immunostimulatory RNAs (ss-isRNAs), which are characterized as uridine-rich oligonucleotide, have been identified to be able to stimulate NK cells via TLR7 recognition [52]. And more important is that unlike other TLR7/8 agonists, these ssRNAs can mediate the activation of NK cells directly, represented as up-regulation of cytotoxicity against HNSCC cell lines BHY, PCI-1 and PCI-13, induction production of IFN- γ , granzyme B and perforin by NK cells [53]. VTX-2337, a novel small-molecular TLR8 agonist, is recently found to be able to activate NK cells directly and augment ADCC by rituximab in PBMCs [54]. Stimulation with imiquimod, also known as a TLR7 ligand, enhances the cytotoxic activity of expanded NK and $\gamma\delta$ T cells from cancer patients *in vitro* [55]. Our recent results showed that Imiquimod and another TLR7 agonist, Gardiquimod, promoted the proliferation and activation of splenic T, NK and NKT cells, and increased the cytolytic ability of NK cells against melanoma B16. When combined with DC vaccine, both Imiquimod and Gardiquimod exert more powerful inhibition of the growth of subcutaneous B16 melanoma and suppression of the pulmonary metastasis, demonstrating that TLR7-stimulated anti-tumor effect by NK cells needs the help of DCs [56]. Taken together, the activation of NK cells mediated by TLR7/8 agonists should be accompanied by the stimulation of other cytokines.

TLR9 regarded as another "nucleic acid-sensing" TLR also has a functional expression in NK cells. The classical ligand of TLR9 is oligodeoxynucleotides (ODN) containing unmethylated CpG dinucleotides (CpG ODNs) which is mimics of bacterial DNA. So far three CpG ODN classes, also known as ODN A, ODN B and ODN C, have been identified and proved to bear differential capability to stimulate human NK cells [57]. First of all, stimulation of freshly isolated NK cells with ODN C shows similar or higher levels of CD69 and CD25 expression as compared with ODN A, whereas treatment with ODN B is consistently less efficient [57,58]. However, in line with the TLR7/8, TLR9 ligands induce NK activation in virtue of suboptimal doses of pro-inflammatory cytokines, such as IL-12 or IL-18, due to low expression of TLR9 in human NK cells [59]. In addition, ODN C administration can up-regulate both cytolytic activity against tumor cell lines and cytokine production more significantly than ODN A and ODN B [60]. These results suggest that ODN C might represent a good volunteer to support anti-tumor therapy.

CpG ODN has gained much attention to the tumor treatment in mice model, because CpG ODN has been proved to be able to activate monocytes directly to secrete the Th1-like cytokine IL-12 and type I IFNs, promote NK cells re-

sponse with increased lytic activity and IFN- γ secretion, and even drive dendritic cell and B cell maturation and thus enhance their Ag presentation capability [61]. However, the spontaneous antitumor immunity primed by CpG ODN can be tampered when ODN is applied systemically *in vivo*, therefore developing some vector-based agents for selective delivery of CpG-ODN is important for the activation maintaining. In RIP1-Tag5-transgenic mouse model of pancreatic islet tumorigenesis, developing insulinomas are deeply embedded in the exocrine pancreas and thus out of reach for intra-tumoral injection. CpG-ODN has been packaged into the vascular targeting peptide (RGR peptide) which specifically gets to the tumor blood vessels. RGR peptide-coated stealth liposomes showed a long time stimulation of spontaneous antitumor immunity and anti-vascular effects. RGR-liposomes plus CpG-treated RIP1-Tag5 mice display long-term survival, and CD8⁺T and NK cells are major force for this effect, because CD8⁺T and NK cells but not the CD4⁺T cells depletion impaired the survival [62]. And more important is that sorted NK cells from tumor sites exhibit more vigorous ability of producing IL-12 and IFN- γ and natural cytotoxicity, even accompanied by distinct inhibition of angiogenesis target tumor cells [62,63].

In addition to controlling tumor growth, TLR9-dependent signaling in plasmacytoid dendritic cells (pDCs) is a key contributor to innate immune defense against virus or bacteria infection. For instance, TLR2 and TLR9 have been proved to act in concert to mount the first line of defense against HSV infection, in which the production of various proinflammatory cytokines, such as type I IFNs, IL-6, IL-12, RANTES, and TNF- α , plays the major role [64]. On the other hand, although expression of the early activation marker CD69 was comparable between splenic NK1.1⁺ cells from WT and TLR2/9 DKO, reduced NK cell recruitment to the spleen has been observed, resulting in more virus load [64]. Further study suggested that the secretion of chemokines such as CXCL9 and CXCL3 induced by TLR2 or TLR9 stimulation is important for the NK activation [64,65]. This is also the case for MCMV infection. Overall, during the virus infection, TLR2/9 stimulation can mediate NK recruitment directly via the production of chemokines, as well as enhance the CD69 expression and cytokine secretion. Just during the cutaneous leishmaniasis challenge, NK cells represent an important source for early IFN- γ , and TLR9 is essential for the induction of NK cell-related IFN- γ secretion and cytotoxicity during the innate phase of *Leishmania (L.) major* infection, because lymph node (LN) NK cells from TLR9^{-/-} mice acquired no cytolytic activity after stimulation with *L. major* [66]. Further study showed that activation of DCs via TLR9 was essential for the induction of hypertrophy in leishmaniasis, due to the crosstalk between NK cells and DCs [67].

Besides the TLRs mentioned above, TLR2 and TLR5 expressing on NK cells at a middle degree also can stimulate NK cells directly via combining with their ligands. NK

activation mediated by TLR2 and TLR5 mainly participates in the defense of bacteria and fungi challenge. *Klebsiella pneumoniae* (KpOmpA) and flagellin, which represent the ligand of TLR2 and TLR5 respectively, can activate CD56⁺CD3⁺ NK cells directly, demonstrated as induction of IFN- γ production or increment of proinflammatory cytokines such as TNF- α in the presence of IL-2 [68]. More importantly, KpOmpA and flagellin rapidly promote NK cells to constitutively produce α -defensins which disrupted the bacterial membrane, leading to pathogen death [68]. Similarly, TLR2 is involved to assist NK cells to recognize *H. pylori* to promote the production of IFN- γ which is the major inflammation factor in the human gastric mucosa [69]. In vaccinia viral infection, TLR2 was able to directly activate NK cell in a TLR2/MyD88-dependent manner and was critical for the control of vaccinia viral infection *in vivo* [70]. TLR2 agonist polysaccharide krestin (PSK) was also reported to activate human NK cells directly and indirectly with the help of IL-12. Notably, it can enhance the antitumor effect of HER2-targeted monoclonal antibody therapy [71]. In addition, a bacterial fimbrial protein FimH, a novel TLR4 ligand, was recently reported to directly activate human and murine natural killer cells via TLR4-MyD88 pathways [72].

Ligation of TLRs with respective agonists at the site of infection or cellular stress activates NK cells followed by the release of inflammatory mediators, including cytokines and chemokines, and initiation of target-cell lysis. However, the activation of NK cells mediated by TLRs is also involved in some pathologic process of autologous diseases. For example, poly I:C treatment can induce the accumulation and activation of NK cells in liver, leading to autoimmune hepatitis [73,74]. Pre-activation of T cells aggravates poly I:C-induced liver injury by up-regulating NK cell function [75]. HBs-transgenic mice are over-sensitive to poly I:C-induced liver injury, which is dependent on the presence of NK cells and IFN- γ produced by intrahepatic NK cells [76,77]. In primary biliary cirrhosis (PBC), stimulation with ligands for TLR3 and TLR4 enhanced NK cell-mediated cytotoxicity against biliary epithelial cells (BECs), during which IFN- α produced by TLR3 ligand-stimulated monocytes enhances the cytotoxicity of TLR4 ligand-activated NK cells [78]. In addition, the stimulation of TLR3 can also trigger murine uterus NK cell activation followed by amounts of TNF- α and IFN- γ secretion. These two factors can induce the reconstruction of artery and promote the abortion [79].

4 The crosstalk between TLR and NK cell receptors

Accumulating evidence has shown that NK cells can recognize pathogen-associated structures directly or indirectly.

However, it is not clear how TLR signaling results in NK cell activation. Increasing data has indicated that there exists crosstalk between TLR and NK cell receptors, and therefore regulates NK cell function.

KIRs are human NK receptors that recognize allotypic determinants of human leukocyte antigen (HLA) class I. The major function of inhibitory KIRs is discriminating normal cells from tumor or virus-infected cells via recognizing the HLA class I molecules (HLA-A, -B or -C allotypes). As one of the members of KIRs, KIR3DL2 has been reported to be capable of binding CpG-ODNs (such as ODN-C) and transiting themselves into the endosomal compartments of NK cells and therefore down-modulating surface KIR3DL2 expression [80]. The disappearance of KIR3DL2 mediated by TLR9 stimulation is beneficial for the activation of NK cells to counteract the pathogen challenge. The stimulation of CpG-ODNs did not impair the expression of other surface receptors, including NKG2D, NKG2A, CD16, CD56, DNAM-1, CD2, LFA-1, 2B4 and NTBA [81,82].

The expression of activating receptors and their ligands is also regulated by TLR pathway to influence NK cell function when suffering the microbe infection or cell stress. The NKG2D receptor which is the primary activating receptor can be upregulated when "stressed-induced" signals emerge. Accumulating data demonstrates that TLR signaling might tempt the NKG2D expression on NK cells or other NKG2D-expressing lymphocytes, and/or simultaneously induce expression of NKG2D ligands on NK-targeting cells to strengthen NK activation. For instance, during the infection of intracellular *Mycobacterium tuberculosis* (*M. tb*), lysis of infected mononuclear phagocytes mediated by NK cells is the main force to clear away microbial pathogens, in which natural cytotoxicity receptors on NK cells such as NKp30, NKp44 and NKp46 play a pivotal role [83,84]. The expression of NKp46, NKp30 and NKG2D has been identified to be markedly increased on NK cells upon exposure to *M. tb*-infected cells, and NKG2D and NKp46 are vital for NK cell lysis of *M.tb*-infected monocytes, which was confirmed by neutralization antibodies. More importantly, *M. tb* infection can up-regulate ULBP1 expression on monocytes through stimulating TLR2 signaling [84]. This induction of both receptor and ligand can enhance cytotoxicity of NK cells. So these results indicate that interactions between the TLR2 pathway and NK cells can contribute to the local immune response to *M. tb* infection.

Large amount of data has shown that TLR signaling enhances NK cell activation through strengthening the interactions between NKG2D and its ligands via up-regulating NKG2D ligands on macrophages, monocytes or tumor cells. Previously reports showed that LPS preferentially upregulated MICA but not MICB, while CL097 stimulation resulted in upregulation of both MICA and MICB. Multiple mechanisms downstream of TLR4 stimulation, such as ATM/ATR kinases and miRNAs, regulate expression of

NKG2D ligands, which were involved in the macrophage/NK cell crosstalk [85]. Our recent published data showed that poly I:C-stimulated murine macrophages can enhance NK cell-mediated anti-tumor effects, which is dependent on the interaction of NKG2D and its ligands RAE-1, H60, and MULT-1 [86]. Similar effects also occur in crosstalk between NK and monocytes. The up-regulation of MICA expression and human monocytes activation upon LPS stimulation in turn enhances the NK cells activity manifested by the induction of IFN- γ secretion via NKG2D-ligands interaction and augments NK cytotoxicity against tumor cells. More important was that activated NK cells did not display cytotoxicity against MICA-expressing monocytes to avoid autologous immune disorder [87].

In addition to inducing the activation of NK cells via up-regulating the NKG2D ligands on macrophages or monocytes to complete the mission of resisting microbe infection or tumor challenge, TLR signaling-induced NK cell activation may also mediate autologous disorder via affecting the reciprocity of NKG2D-ligands. In a polyI:C/D-GalN-induced fulminant hepatitis, in which TLR3 stimulation with polyI:C triggered the up-regulation expression of Rae-1 on the surface of Kupffer cells, the crosstalk between NK cells and Kupffer cells via NKG2D-Rae1 recognition resulted in IFN- γ and TNF- α secretion which were major factors involved in liver injury [88]. Poly I:C was also reported to activate hepatic NK cells and ameliorate liver fibrosis through enhancing NKG2D expression on NK cells and Rae-1 expression on hepatic stellate cells and then killing stellate cells in NKG2D- and TRAIL-dependent manner [89].

Besides mediating the liver injury via intensifying the interaction of NKG2D and respective ligand, TLR stimulation can induce other immune disorders through promoting NK activation. In human skeletal muscle cell lines such as myoblasts and human rhabdomyosarcoma cells (TE671), there are low mRNA expression of TLR1-7 and TLR9 but constitutively expression of TLR3 which can be up-regulated by poly(I:C) [90]. Moreover, TLR3 engagement with poly(I:C) or other dsRNA is capable of initiating the increase of NKG2D on NK cells, and also the up-regulation of respective ligands such as MICA, MICB, ULBP-2 and ULBP-3 in human muscle cells [91]. The interaction between NKG2D and ligands results in autologous cytotoxicity and the secretion of IL-8 and tissue inhibitor of metalloproteinase-2 (TIMP-2), which can aggravate the development of sarcolysis [90]. The activation of TLR signaling also breaks down epithelial homeostasis of small intestine in mice. Poly I:C stimulation could urge intestinal epithelial cells (IECs) to express RAE-1 and promote NKG2D expression on CD8⁺ intestinal intraepithelial lymphocytes in the presence of IL-15 derived from TLR3-activated IECs. The blockade of interaction between NKG2D and RAE-1 inhibited the cytotoxicity of intraepithelial lymphocytes

against IECs consequently to alleviate epithelial destruction and acute mucosal injury of small intestine mediated by poly I:C [92]. This is also the case for the full development of renal ischemia-reperfusion injury (IRI). Published results have proved that ischemic injury resulted in the generation or release of various endogenous ligands of TLRs, including HMGB1, biglycan, and heat shock proteins. The coalescent of TLR4 and its ligand HMGB1 has been found to be involved in the progress of IRI [93]. Recent finding showed that in ischemic kidney of BALB/c mouse, RAE-1 and MULT-1 expression on renal tubular epithelial cells and NKG2D expression on renal NK cells both could be up-regulated and TLR2/MyD88 signaling was responsible for the increase of NKG2D and respective ligands directly because further induction was restricted in both TLR2^{-/-} and MyD88^{-/-} mice [94].

Collectively, the crosstalk between TLR and NK cell receptors not only plays major roles in control of NK cell function and participates in the defense of NK cells against infection and transformation, but also participates in pathogenesis of some diseases.

5 TLRs—The hinge connecting DCs and NK cells

Customarily, DCs are known as the professional antigen presentation cells which present antigens to acquired immune cells such as T and B lymphocytes, followed by the robust activation of entire immune system. But more and more data have shown that DCs also play a very important role in control of the innate immune responses. It is undoubted that DCs can trigger NK cells directly via the secretion of cytokines, such as IL-12 and IL-15, which promote NK-cell survival and differentiation, NK cell-mediated cytotoxicity and IFN- γ secretion [95]. Herein we demonstrate that TLRs highly expressed on DCs act as a bridge between DCs and NK cells, that is, TLR ligands can stimulate DCs, and consequently activate NK cells through cytokine secretion and/or cell-to-cell contact.

pDCs are bone marrow-derived leukocytes that secrete large amounts of type I IFN in response to a variety of viruses. And two endosomal sensors, TLR7 and TLR9 both have a remarkable expression in pDCs. Moreover, pDCs are responsible for the early peak of IFN production in response to most viruses or TLR7 and TLR9 agonists [96]. In line with previous reports, the activation of NK cells mediated by TLR9 recognition depends on the presence of cytokines probably from other immune cells, but the exact role of pDCs played in the activation of NK cells induced by TLR has not been validated. It has been proved that TLR9-engagement with CpG-A in human pDCs induces NK cell activation, represented as the up-regulation of CD69 expression, as well as proliferation of CD56^{bright}CD16⁻ NK cells. Moreover, extensive research showed that the activation and proliferation of NK cells promoted by pDCs after TLR9

stimulation could be augmented by the addition of CD4⁺CD25⁻ Th cells in a IL-2-dependent manner, but was completely abrogated by the CD4⁺CD25^{hi} Treg cells [97]. It has been demonstrated that during MCMV infection, the production of type I IFN by pDCs is vital for NK cell activation and function. Recent findings show that during persistent MCMV infection, IFN- γ production and cytotoxic capacity of NK cells is inhibited dramatically due to the defect of type I IFN response from pDCs [98]. When wild-type (WT) BALB/c mice encounter the challenge of some Gram-negative bacteria, such as *Klebsiella pneumoniae*, pDCs expressing TLR9 accumulate rapidly, followed by the increase of total number of DX5⁺ cell. If CpG-stimulated CD11c^{dull} B220⁺ pDCs are injected into the infected mice, the recruitment of NK cells to the peritoneum can be induced drastically followed by the high expression of CXCR3 and CD62L, and moderate expression of CCR5 [99]. And these NK cells isolated from the peritoneum inoculated with TLR7/8 or TLR9-stimulated pDCs exhibit highlighted cytotoxicity against YAC-1 tumor cells. These findings suggest that although the TLRs stimulation on the NK cells can directly enhance their ability to produce cytokines and lyse infected or transformed cells, the response of DCs to TLR agonists is pivotal for the recruitment and proliferation of NK cells. About the recruitment of NK cells mediated by DCs, there are many related details. By using a mouse model for the ablation of DCs, the priming of NK cell responses to viral and bacterial pathogens was proved to require the help of CD11c^{high} DCs [98]. After peripheral TLR stimulation, NK cells were recruited to local lymph nodes, and their interaction with DCs resulted in the emergence of effector NK cells in the periphery. NK cell priming was dependent on the recognition of type I IFN signals by DCs and the subsequent production and trans-presentation of IL-15 by DCs to resting NK cells. CD11c^{high} DC-derived IL-15 was vital and sufficient for the priming of NK cells.

Conventional DCs (cDCs) in fact are veiled cells of myeloid origin which are capable of efficiently processing and presenting antigens to prime naive T cells [100]. Although pDCs are characterized as the type I IFN-production in response to virus or bacterium components, they cannot secrete IFN- α by poly I:C stimulation. In contrast, CD11c⁺-B220⁻ cDCs produce type I IFNs in response to poly I:C. Moreover, CD8 α ⁺ cDCs in response to poly I:C stimulation can enhance cytotoxicity of NK cells targeting YAC-1 tumor cells, and up-regulate the expression of granzyme B and IFN- γ due to the activation of IPS-1 and TRIF signaling pathway and consequently up-regulate the expression of type I IFNs, IL-6, and IL-12p40 in DCs [101]. Similar to pDCs, cDCs also promote the recruitment of NK cells after TLRs-related ligands stimulation via up-regulating the secretion of chemokines. Some investigators showed that in mice with depletion of CD11c⁺ MHC class II⁺ cDCs, the recruitment of CXCR5⁺DX5⁺ NK cells to draining lymph nodes is abated after treatment with TLR4 and TLR5 ago-

nists, although these TLR ligands also can activate NK cells in the draining lymph nodes [102]. DCs are now accepted as crucial regulators of innate and adaptive immunity, in particular NK cells function. It is undoubtful that the IFN- γ secretion, cytotoxicity, CD69 expression and proliferation of resting NK cells can be induced by mouse DC cell lines, mouse bone-marrow-derived DCs (BMDCs), human monocyte-derived DCs and human cord-blood-derived DCs [103]. So far the mechanisms involved in which DCs activate NK cells include the direct cell-cell contact and soluble factors, including IL-12, IL-18 and type I IFN. Moreover, some researchers have begun to reveal intrinsic relations between NK cells and DCs when stimulated by diversified TLR agonists or other stimulus. Myeloid DCs (mDCs), which comprise many subsets, including BMDCs and CD8+ DCs, possess subset-specific pattern-recognition systems. After stimulation with TLR agonists, adaptor MyD88 or the Toll/IL-1 receptor homology domain-containing adaptor molecule (TICAM)-1 (TRIF) is recruited and transduces signaling to engaged in type I IFN induction and NK activation via partly overlapped but distinct pathways in a cell type-specific manner [104]. MyD88 in pDC can be stimulated after administration of TLR7 or TLR9 ligand to promote IFN- γ secretion, and consequently, NK cells demonstrate further activation [95,96]. This is also the case for TLR2 agonist, because cell contact-mediated NK activation

is hampered when the cells are stimulated with Pam2 lipopeptide after coincubation with MyD88^{-/-} mDC [70]. In contrast, poly I:C-induced NK activation is tampered in double KO (IPS-1^{-/-} and TICAM-1^{-/-}) mice, suggesting that these two pathways are both required for poly I:C-mediated NK activation [105]. Moreover, regulatory molecule which is responsible for the crosstalk between NK and mDC has been reported. INAM (IRF-3-dependent NK cell activating molecule), a poly I:C-inducible membrane protein, has been identified to participate in mDC-mediated NK cell activation and contribute to mDC-NK reciprocal activation [104,106]. Notably, INAM does not act as an NK-activating ligand; it works only on mDC/macrophage for NK activation. In addition, MyD88 in NK cells also participates in direct NK activation.

6 Future prospect

TLRs and NK cell receptors are the most important receptor superfamilies in innate immunity. TLRs, acting as the sensor of external pathogens, lie in the first defense line to resist the invasion from xenogenic organisms by directly recognizing PAMPs. NK cells can eliminate pathogenic infection or transformed-cells via detecting alterations in endogenous protein expression on target cells through activating

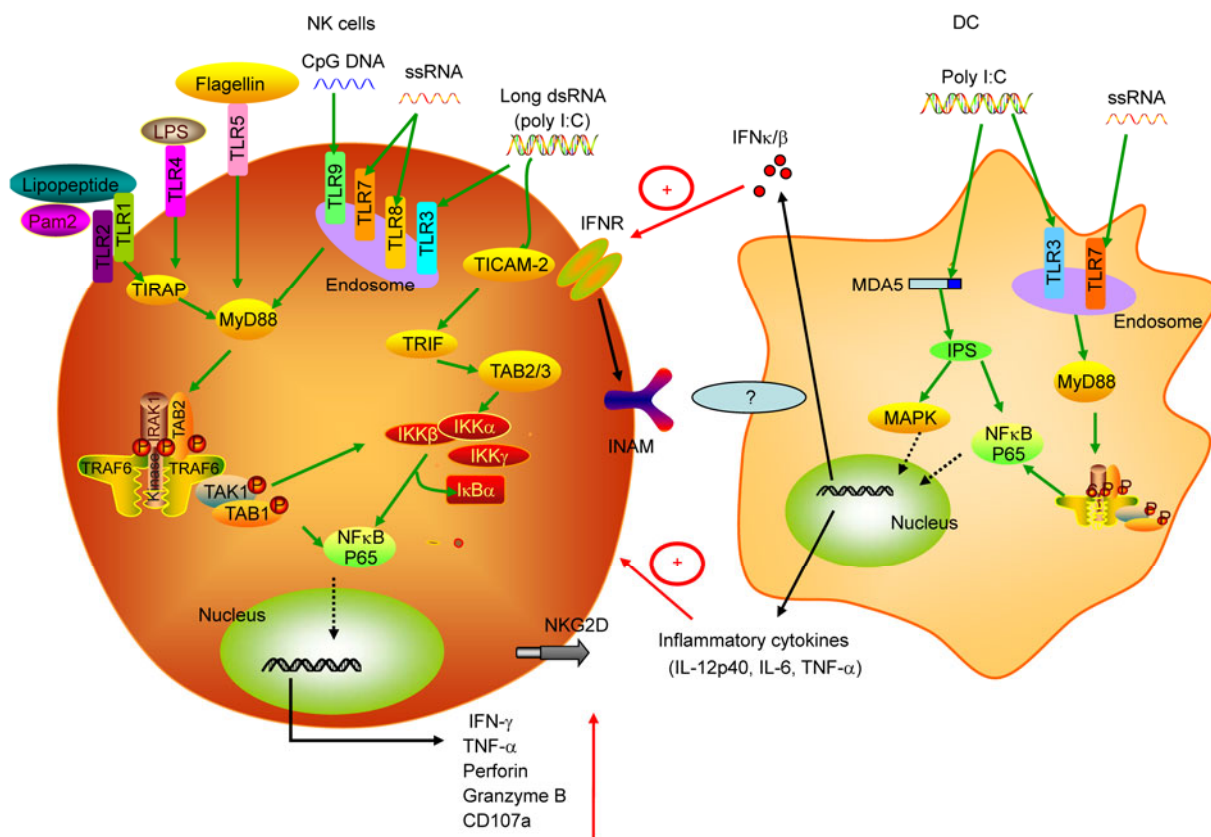


Figure 1 The TLR signaling in NK cell activation.

and inhibitory receptors. Accumulating data has demonstrated that TLRs and NK cell receptors can coordinate and regulate each other during immune responses, which contributes to the initiation of innate response and the priming of adaptive responses. TLRs can activate NK cell function directly or with the help of accessory cells in a cytokine or cell-to-cell contact dependent manner. In addition, TLR signaling can also induce the expression of NKG2D ligand and thus indirectly activate NK cells. As shown in Figure 1, NK cells express diversified TLRs, and they can be activated directly by TLR ligands, followed by the activation of TLR signaling pathway via the recruitment of TIR domain-containing adaptor molecules, including MyD88, TIRAP (Mal), TRIF and TRAM. In most cases, TLR-induced NK cell activation needs the help of accessory cells, such as DCs, macrophages and monocytes. For example, TLR agonists stimulate TLRs on the surface or in the endosome of DCs and finally promote the production of inflammatory cytokines such as IL-12, IL-15, and type I IFN, which are vital for NK activation. Moreover, cell-to-cell contact between DC and NK cells is crucial for NK cell activation. DC activation can induce the expression of INAM to enhance the function of NK, although the exact role of INAM players in the mDC-NK contact is still unidentified. Taken together, TLR signaling can directly or indirectly induce the activation of NK cells, thereby promoting IFN- γ secretion and enhancing cytotoxicity against tumor or infected cells.

In conclusion, the TLR signaling is critical for NK cell activation and function in the control of viral or bacterial infection and cancer. Other innate immune receptors, such as RLRs, NLRs, and CLRs, may also interact with NK cell receptors or intracellular signaling, and contribute to NK cell activity. Although the progress has been gained in recent years, the precise crosstalk between different immune cells and different receptors should be investigated more intensively. More understanding of the recognition of innate receptors and interactions between them may provide important insights into the design of effective strategies to combat tumor and microbial infections.

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