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TLR7/8-Mediated Activation of Human NK Cells Results in Accessory Cell-Dependent IFN-γ Production

Orla M. Hart,* Veronica Athie-Morales,† Geraldine M. O’Connor,* and Clair M. Gardiner2*

NK cells express receptors that allow them to recognize pathogens and activate effector functions such as cytotoxicity and cytokine production. Among these receptors are the recently identified TLRs that recognize conserved pathogen structures and initiate innate immune responses. We demonstrate that human NK cells express TLR3, TLR7, and TLR8 and that these receptors are functional. TLR3 is expressed at the cell surface where it functions as a receptor for polynucleotidic acid:cytidylic acid (poly(I:C)) in a lysosomal-independent manner. TLR7/8 signaling is sensitive to chloroquine inhibition, indicating a requirement for lysosomal signaling as for other cell types. Both R848, an agonist of human TLR7 and TLR8, and poly(I:C) activate NK cell cytotoxicity against Daudi target cells. However, IFN-γ production is differentially regulated by these TLR agonists. In contrast to poly(I:C), R848 stimulates significant IFN-γ production by NK cells. This is accessory cell dependent and is inhibited by addition of a neutralizing anti-IL-12 Ab. Moreover, stimulation of purified monocyte populations with R848 results in IL-12 production, and reconstitution of purified NK cells with monocytes results in increased IFN-γ production in response to R848. In addition, we demonstrate that while resting NK cells do not transduce signals directly in response to R848, they can be primed to do so by prior exposure to either IL-2 or IFN-α. Therefore, although NK cells can be directly activated by TLRs, accessory cells play an important and sometimes essential role in the activation of effector functions such as IFN-γ production and cytotoxicity. The Journal of Immunology, 2005, 175: 1636–1642.

Natural killer cells are lymphocytes that are best known for their ability to kill virally infected and malignant cells (1). They preferentially lyse virally infected cells while sparing uninfected cells (2), and patients deficient in NK cells suffer from recurrent viral infections (3, 4). Several advances have been made in recent years regarding the mechanisms by which NK cells recognize virally infected target cells. Human NK cells have different receptor families, including killer cell Ig-like receptors, which recognize HLA class I Ag directly (5, 6). They also express NKP44 and NKP46 receptors, which directly recognize hemagglutinin from the influenza virus (6–8). In the mouse, Ly49H on NK cells directly binds murine cytomegalovirus (MCMV)3-encoded protein m157, and NK cells confer resistance to infection (9). Therefore, it is becoming apparent that NK cells can directly recognize and respond to viral pathogens.

TLRs are receptors of the innate immune system that directly recognize conserved pathogen structures (10). There are at least 10 mammalian TLRs identified to date, including TLR2, TLR3, TLR7, TLR8, and TLR9, all of which have been implicated in the recognition of virus (10). TLR3 recognizes dsRNA produced during viral replication (11). TLR7 and TLR8, in humans, recognize ssRNA (12–14), whereas TLR9 recognizes unmethylated CpG motifs (15, 16). Given the critical role that NK cells play in the early immune response to viral infections, it is attractive to speculate that TLRs will be present and functional on NK cells. Furthermore, the discovery that TLR3 is a receptor for polynucleotidic acid:cytidylic acid (poly(I:C)), a synthetic analog of dsRNA (11) and the observation that poly(I:C) activates NK cells in vivo (17, 18) clearly implicates it as a possible receptor through which NK cells are activated. Indeed, a role for TLR agonists in NK cell activation has been demonstrated recently with reports that TLR2 (19), TLR3 (20–22), and TLR9 (22) agonists stimulate NK cell functions. In TLR3 and TLR9 knockout mice, MCMV infections have more severe pathologies (23, 24). As it is known that NK cells are key effector cells in the immune response to MCMV (25), these data suggest that deficiencies in TLR signaling could result in diminished NK cell activation and function.

The role of accessory cell help in the NK cell response to viral infections has been well documented (26). IFN-γ is a key cytokine produced by NK cells in response to particular infections, including MCMV (27) and influenza virus (2). It has several antiviral effects, including direct inhibition of viral replication in infected cells (28), activation of inducible NO synthase-dependent antiviral pathways (29, 30), and activation of macrophages (31). During MCMV infection, IFN-γ production by NK cells is dependent on accessory cell-derived IL-12 as neutralizing Abs to IL-12 in vivo abrogate NK cell activation (32). The role of accessory cell involvement in TLR-mediated activities is unclear with both accessory cell-dependent (21) and -independent activation (20) of NK cells reported. It is likely that both direct and indirect mechanisms of NK cell activation are required for an optimal innate immune response, a finding supported by our study. We demonstrate direct activation of human NK cells by poly(I:C) and show that this occurs through TLR3. Accessory cells contribute to this response through the production of the proinflammatory cytokine, IL-12. In addition to expanding on the role of TLR3, this study defines a novel role for TLR-7/8 signaling in NK cells. These receptors are present in NK cells, and functions, including cytotoxicity and cytokine production, are potently stimulated by TLR7/8 engagement.

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Abbreviations used in this paper: MCMV, murine cytomegalovirus; poly (I:C), polynucleotidic acid:cytidylic acid; rhIL-2, recombinant human IL-2; NCR, natural cytotoxicity receptor.

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We demonstrate both direct and indirect activation of NK cells through TLR7/8 and report a central role for monocyte-derived IL-12 in the differential IFN-γ produced by NK cells in response to different TLR agonists.

Materials and Methods

Cell culture

The NK leukemia cell line, NKL, was maintained in RPMI 1640 medium (Invitrogen Life Technologies) supplemented with 10% FCS (PAA Laboratories) and 200 U/ml rhIL-2 (Biological Resources Branch, National Cancer Institute). The NK lymphoma cell line, NK92, was maintained in MEM-α (Invitrogen Life Technologies) supplemented with 10% FCS, 10% horse serum (Sigma-Aldrich), and 200 U/ml rhIL-2. The T/NK cell leukemia cell line, YT, was maintained in IMDM (Invitrogen Life Technologies) supplemented with 20% FCS. The Burkitt’s lymphoma cell line, Daudi, was maintained in RPMI 1640 medium supplemented with 10% FCS. The fibroblast cell line 293T and its derivatives 293TLR7 or 293TLR8, which have been stably transfected with TLR7 and TLR8, respectively, were maintained in DMEM (Sigma-Aldrich) supplemented with 10% FCS and 10 µg/ml blasticidin (InvivoGen). All media were supplemented with 100 U/ml penicillin and 100 µg/ml streptomycin (both from Invitrogen Life Technologies). For primary NK cell culture, blood samples were obtained from normal healthy donors from whom written consent had been obtained. PBMCs were isolated by Lymphoprep (Axis-Shield) gradient. Highly purified NK cells were obtained by magnetic bead isolation (Miltenyi Biotec), according to the manufacturer’s instructions. Monocytes were isolated by magnetic bead isolation using anti-CD14 microbeads (Miltenyi Biotec), according to the manufacturer’s instructions. Purity was assessed by flow cytometry. NK cells were routinely purified to 95% CO2 atmosphere.

NK1 cells were obtained from normal healthy donors from whom written consent had been obtained. PBMCs were isolated by Lymphocult (Pall Biodesign) and anti-CD14 microbeads (Miltenyi Biotec), according to the manufacturer’s instructions. Purity was assessed by flow cytometry. NK cells were routinely purified to >93% with an average of 0.5% contaminating CD14 accessory cells. Isolations with >1% contaminating CD14 cells were discarded. For cell sorting experiments, primary NK cells were isolated as described above using magnetic beads. Highly purified NK cells were then labeled with anti-CD56 and anti-CD3. CD56-positive, CD3-negative cells were sorted on a BD FACSaria cell sorter from the lymphocyte gate, and purity was routinely 99.7-99.9%, with no contaminating CD14-positive cells. Short-term culture of isolated NK cells and monocytes was conducted in IMDM supplemented with 20% FCS, 100 U/ml penicillin, and 100 µg/ml streptomycin. All cell culture was conducted at 37°C in humidified 5% CO2 atmosphere.

RT-PCR

Total RNA was extracted from NKL, NK92, YT, PBMCs, and primary NK cells with Tri Reagent (Molecular Research Center). cDNA was generated from total RNA using TaqDNA polymerase (Invitrogen). Total RNA was extracted from NKL, NK92, YT, PBMCs, and primary NK cell lines with Tri Reagent (Molecular Research Center). cDNA was generated from cDNA samples with the following primer sets: TLR3 (F: 5'-GGTACAGCTTCTGTGCTGCTC-3'; R: 5'-TAGTGAAGGCTCCAGGAGGATG-3'); TLR7 (F: 5'-AGGTTGCTGATGAGGATGTTGACTAC-3'; R: 5'-GGAAAGAGGACGCAACATC-3'); TLR8 (F: 5'-CAGAATAGAGGATGACCTCAACATCA-3'; R: 5'-AATGTCACAGGTGTGCTCAGGAGG-3'); (34) using TaqDNA polymerase (Invitrogen Life Technologies). KIR2DL4 gene was amplified to control for genomic DNA contamination. PCR was conducted in IMDM supplemented with 20% FCS, 100 U/ml penicillin, and 100 µg/ml streptomycin. All cell culture was conducted at 37°C in humidified 5% CO2 atmosphere.

SDS-PAGE and Western blotting for IkBα

After appropriate stimulation with poly(C) (Sigma-Aldrich) or R848 (Invivogen), 1 x 10^6 cells were washed with PBS and lysed in radioimmune precipitation buffer (1% (v/v) Nonidet P-40, 0.5% (v/v) sodium deoxycholate, 0.1% SDS in PBS, 10 µg/ml PMSF, 7 µg/ml aprotinin, and 1 mM sodium orthovanadate) for 45 min at 4°C. For inhibition of lysosomal acidification, cells were incubated with 20 µg/ml chloroquine (Sigma-Aldrich) for 1 h before stimulation. Where applicable, cells were cultured in 500 U/ml IFN-α (Strathmann Biotec) or 500 U/ml IL-2 before stimulation with R848. Bradford assays were conducted on samples, and protein concentrations were normalized. Sample buffer (62.5 mM Tris (pH 6.8), 2% (w/v) SDS, 50 mM DTT, 10% glycerol (v/v), and 0.1% bromophenol blue (w/v)) was added to the sample to a final concentration of 20%. Samples were boiled at 100°C for 5 min and placed on ice before electrophoresis on a 10% polyacrylamide gel. Samples were blotted using a wet transfer system onto polyvinylidene difluoride membranes and blocked in 5% nonfat milk in PBS-Tween 20. Membranes were incubated with anti-IkBa mAb (a gift from Prof. R. Hay, St. Andrews University, St. Andrews, Scotland), washed, and incubated with secondary anti-mouse HRP, followed by West Pico ECL (Pierce) development. Equal loading was verified by reprobing membranes with anti-β-actin Ab (Sigma-Aldrich).

SDS-PAGE and Western blotting for TLR7 and TLR8

A total of 1.25 x 10^5 (293T, 293TLR7, or 293TLR8) or 2 x 10^5 cells (NKL, NK92, or YT) was resuspended in PBS, and SDS was added to 1%. Complete cell lysis was achieved by immediate vortexing and boiling. Samples were centrifuged at 16,000 × g for 40 min at 4°C. Proteins were acetone precipitated at −20°C from cleared lysates. Following rehydration, proteins were resuspended in reducing sample buffer (30 mM Tris (pH 6.8), 10% (v/v) glycine, 1% (w/v) SDS, 0.05% (w/v) bromophenol blue, and 2.5% (v/v) β-mercaptoethanol), separated using 7.5% SDS-PAGE, electroblotted, and stained with either anti-TLR7 or anti-TLR8 mAb (both from Immgenex), followed by anti-mouse-HRP (Sigma-Aldrich) using West Pico ECL development. The TLR8 protein ran at a lower apparent m.w. than expected.

31Cr release cytotoxicity assay

A total of 1 x 10^5 Daudi target cells were labeled with 1 mCi of Na3CrO4 for 1 h at 37°C. Cells were then washed twice with complete medium and incubated with effector cells at an E:T ratio of 10:1. After incubation for 4 h at 37°C, a sample of supernatant was counted on a Microbeta Trilux scintillation counter (PerkinElmer). Elution efficiency was calculated using the formula: % release = [activity at E > s] / [activity at E > s] x 100. One-way ANOVA followed by Newman-Keuls multiple comparison test was used to test for statistical significance of differences between experimental groups; *p < 0.05, **p < 0.01, and ***p < 0.001.

Human IFN-γ and IL-12 ELISA

Production of IFN-γ and IL-12 by NK cells and monocytes was assessed by ELISA (eBioscience Ready-Set-Go kit and match pair Ab kit (BD Biosciences), respectively) on cell culture supernatants according to the manufacturer’s instructions.

Statistical analyses

One-way ANOVA followed by Newman-Keuls multiple comparison test was used to test for statistical significance of differences between experimental groups; *p < 0.05, **p < 0.01, and ***p < 0.001.

Results

Human NK cells express functional TLR3, TLR7, and TLR8

Based on the hypothesis that TLRs are important in the NK cell antiviral response, we demonstrated TLR7, TLR8, and TLR3 expression by NK cells. We found mRNA transcript for all three TLRs in all three NK cell lines (NKL, NK92, and YT) by West Pico ECL. Expression of TLR3, TLR7, and TLR8 was used to test for statistical significance of differences between experimental groups; *p < 0.05, **p < 0.01, and ***p < 0.001.

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Having demonstrated expression of the receptors, we wanted to investigate whether NK cells activated components of TLR-signal-
TLR7 and TLR8 in human cells (38). R848 was extremely potent at activating NKL cells with degradation of IκBα apparent after only 5 min stimulation (Fig. 2a). Increased degradation was observed at 15 and 30 min, and the signal started to recover by 60 min. In contrast, the YT cell line was resistant to R848 stimulation only 5 min stimulation (Fig. 2a). Therefore, as previously shown. Activation was supported by functional data in which TLR agonists increased primary NK cell cytotoxicity against the Daudi cell line. R848 was more potent than poly(I:C) at the concentrations tested and levels approached those induced by IL-2 (Fig. 3a). It is known that TLR3 is a receptor for poly(I:C) (11) and a role. Therefore, we performed experiments with highly purified NK cells in which we blocked IL-12 using a neutralizing Ab. If the effect of TLR stimulation was accessory cell independent, addition of the Ab would have no effect. However, we found that blocking IL-12 partially decreased the stimulatory effect on NK cells (Fig. 3c). These data strongly suggest that in addition to having a direct effect on NK cells, accessory cells also play a role in the NK cell response to poly(I:C) and R848. In contrast, neutralizing IL-12 had no effect on the IL-2-induced cytotoxicity as expected given that IL-2 is known to directly activate NK cells in the absence of accessory cell help (Fig. 3c).

Accessory cells contribute to NK cell activation

Although we were using highly purified NK cell populations (average < 0.5% contaminating monocytes), the possibility remained that accessory cells, through TLR3 stimulation, were still playing a role. Therefore, we performed experiments with highly purified NK cells in which we blocked IL-12 using a neutralizing Ab. If the effect of TLR stimulation was accessory cell independent, addition of the Ab would have no effect. However, we found that blocking IL-12 partially decreased the stimulatory effect on NK cells (Fig. 3c). These data strongly suggest that in addition to having a direct effect on NK cells, accessory cells also play a role in the NK cell response to poly(I:C) and R848. In contrast, neutralizing IL-12 had no effect on the IL-2-induced cytotoxicity as expected given that IL-2 is known to directly activate NK cells in the absence of accessory cell help (Fig. 3c).

TLR7 signaling in NK cells is independent of lysosomal acidification

Many TLRs, including TLR3, are reported to be expressed in subcellular compartments where increased acidification is required for their activation (32–34, 36). However, we have found TLR3 at the cell surface of NK cells where we propose it to be functionally active and independent of any lysosomal signaling. To test this hypothesis, we used chloroquine to block lysosomal acidification and stimulated NKL cells in the presence of TLR agonists. As a positive control, chloroquine blocked R848 signaling in NK cells (Fig. 2f). This supports localization of TLR7/8 to lysosomes in NK cells and, furthermore, the requirement of a low pH for signaling as seen for other cell types (39). In contrast, chloroquine failed to block poly(I:C)-induced signaling (Fig. 2f), demonstrating that TLR3 signaling is independent of lysosomal acidification in NK cells. This data is consistent with functional expression of TLR3 on the surface of NK cells and supports a possible role for NK cell sensing of extracellular viral dsRNA.
Ag as expected \((n = 5; \text{data not shown})\). The cytotoxicity data for individual donors is shown in Fig. 3d. Poly(I:C) increased cytotoxicity in four of five donors, and although it was not to the same level as IL-2, it supports direct activation of primary NK cells by poly(I:C). R848 increased cytotoxicity in only one donor, which in conjunction with our signaling data (Fig. 2d and cytokine blocking experiments (Fig. 3c) suggests that accessory cells are critical for R848, but not poly(I:C), mediated activation of functional responses in primary resting NK cells.

**R848 induces IFN-γ production by NK cells in an IL-12-dependent manner**

IFN-γ is an important cytokine produced by NK cells, which plays an essential role in the early innate immune response to infection (26). In agreement with a previous report (21), poly(I:C) failed to induce IFN-γ production from NK cells after 18 h of stimulation (Fig. 4c). In contrast, R848 (3 \(\mu g/ml\)) induced IFN-γ production from purified NK cells, although there was significant donor-to-donor variation observed in amounts of IFN-γ produced (average, 752 pg/10^6 cells; range, 222-1892 pg/10^6 cells, \(n = 4\)). The absence of TLR7/8 signal transduction in resting primary NK cells (Fig. 2d) and the role that IL-12 plays in R848-induced cytotoxicity (Fig. 3c) strongly suggested an important role for accessory cells in IFN-γ production by NK cells. Indeed, this was found to be the case as addition of a neutralizing Ab to IL-12 significantly inhibited IFN-γ produced in all donors (Fig. 4a). These data support a model in which R848 induces relatively high levels of IL-12 from monocytes. This in turn activates NK cell cytotoxicity and also induces them to produce significant quantities of IFN-γ. In the case of poly(I:C), it increases cytotoxicity, but the amount of IL-12 produced is not enough to stimulate IFN-γ production. To test our model, we purified monocytes from peripheral blood and measured their ability to produce IL-12 in response to either R848 or poly(I:C) stimulation. R848 was extremely potent, while as predicted, the amount of IL-12 produced by monocytes in response to poly(I:C) was below detection levels (Fig. 4b). We titrated monocytes back into highly purified autologous NK cells and measured IFN-γ production in response to the TLR agonists. In further support of our model, addition of monocytes in the presence of poly(I:C) resulted in limited IFN-γ production when compared with the effects of R848 in the presence of monocytes (Fig. 4c).

**TLR agonist-induced modulation of NK cell activatory receptors**

Freshly isolated NK cells have only basal levels of killing against Daudi target cells but are induced to kill after activation, presumably by induction of receptors on the NK cells, which allow target recognition. We investigated whether R848 or poly(I:C) caused up-regulation of some of the recently defined activatory receptors, including NKp30, NKp44, NKp46, and NKG2D. NKp30 and NKp44 were expressed on few NK cells, and their expression increased only slightly in response to poly(I:C) or R848 (see Table I). NKp46 was found on most NK cells, and its expression levels did not change significantly in response to either stimulation. Results with NKG2D were variable. This receptor was expressed on very few NK cells with some donors exhibiting up-regulation and others down-regulation of NKG2D in response to poly(I:C) or R848 stimulation. Thus, NKG2D is unlikely to be the receptor that mediates TLR-induced recognition of Daudi target cells by human NK cells. This was further supported by functional experiments in which blocking Abs against NKG2D had no effect on poly(I:C)-induced cytotoxicity (data not shown). Although there were only minor changes in the expression of natural cytotoxicity receptors (NCRs), we cannot rule out a functional role for these receptors in the induction of NK cell cytotoxicity in response to poly(I:C) or R848.
Discussion

NK cells respond to conserved microbial products through TLRs. In this study, we demonstrate that both direct and indirect mechanisms are used and that their relative contribution varies for specific TLRs. As previously reported, TLR3 is directly activated on accessory cells and on NK cells. This activation of accessory cells produces cytokines such as IL-12, which in turn helps control the initial infection until an adaptive immune response finally eliminates the pathogen (43, 44). In the case of the TLR7/8 agonist, we have similar results for human NK cells. The presence of extremely low levels of accessory cells is sufficient to result in potent R848 stimulation of NK cell effector functions. Importantly, although resting NK cells are unresponsive to R848, they become responsive if accessory cells are prescent. Accessory cells help potentiate this response through the production of cytokines such as IL-12. In contrast, activation of the NK cell effector functions of cytokine production and cytotoxicity in response to the TLR7/8 agonist R848 is mediated primarily through accessory cells. The role of IL-12 appears to be less dominant in the case of TLR7/8; however, we must consider the likely role that IFN-α plays in this system. A potent activator of NK cells, IFN-α is known to be produced in response to TLR7/8 signaling (40, 41). In the absence of accessory cells, activation of resting NK cells fails to occur. Thus, although different TLRs modulate accessory cells, they do so in significantly different ways and target different effector functions. Accessory cells have long been known to influence NK cell activity: in MCMV viral infections, NK cells are key effector cells through the early production of IFN-γ (42). This production is IL-12- and therefore accessory cell dependent (32). Similarly, in murine models of bacterial infection, IL-12 produced by accessory cells stimulates NK cell production of IFN-γ, which in turn helps control the infection until an adaptive immune response finally eliminates the pathogen (44, 45).
Cytokine production is an important effector function of NK cells (32, 42–44). In particular, IFN- is a key antiviral cytokine produced early in infection. Poly(I:C) failed to stimulate rapid production of IFN- with minimal amounts detected in the supernatants of NK cells after 18 h. In marked contrast, R848 was extremely potent at inducing IFN- from purified NK cells. This may reflect different signaling through TLR7/8 and TLR3 (10) and a more potent contribution of accessory cells in the R848 induced activation. Production of IFN- by NK cells in response to R848 was almost completely inhibited by a neutralizing Ab to IL-12. Our data in the human system parallel precedents set in the mouse where IL-12 was absolutely required for IFN- production in murine MCMV infections (32). Our results suggest a model in which poly(I:C) directly activates both NK cells and monocytes. Low levels of IL-12 are produced by activated monocytes. These levels of IL-12 are enough to stimulate increased cytoxicity, as we can partially inhibit cytoxicity in the presence of a neutralizing anti-Ab IL-12. In contrast to poly(I:C), R848 induces large amounts of IL-12 from monocytes (39), which in turn promotes high IFN- production and increased cytoxicity by NK cells. IL-12 on its own is inefficient for induction of IFN- production and optimally synergizes with cytokines such as IL-2 and IL-18 (45). We propose that accessory cell-derived cytokines provide a primary signal to the NK cell and that direct activation by R848 provides the second signal required for IFN- production.

R848 activates human cells through both TLR7 and TLR8 (38) in contrast to the mouse, where TLR8 is considered to be non-functional (38, 46). mRNA for both receptors was expressed by primary human NK cells and NK cell lines, and R848 was extremely potent at activating NK cell functions. The present data do not allow the assessment of the relative contribution of TLR7 and TLR8 in our system. We have established the presence of TLR7 protein in NK cell lines that respond to R848 stimulation and its absence in cells that do not signal in response to R848. In contrast, Western blotting suggested the presence of TLR8 protein even in cell lines that do not respond to R848. This anecdotal evidence might suggest that TLR7 is the functional receptor in human NK cells. Additional experiments such as the use of TLR7-specific agonists (imiquimod and lexoribine) and/or specific inhibition of TLR7 and TLR8 signaling will address this issue. In addition, some components of the TLR7/8 signaling pathways, such as IFN regulatory factor-5 and IFN regulatory factor-7, are not constitutively expressed in most cell types and require induction, e.g., by type I IFNs. We demonstrated that in contrast to DCs (12) primary NK cells require priming by cytokines such as IL-2 or IFN- inhibiting their cytotoxicity in the presence of a neutralizing Ab to IL-12. These levels of IL-12 are produced by activated monocytes. These dual activities lead to optimal NK cell activation, which is accompanied by both NK cell cytotoxicity and IFN- production.

Poly(I:C) has a number of receptors through which it can signal. These include TLR3 and the intracellular receptors, dsRNA-dependent protein kinase R (47, 48) and retinoic acid-inducible gene-I (49). However, we suggest that human NK cells are responding to poly(I:C) exclusively through cell surface TLR3 because a subclone of the NLK cell line negative for TLR3 at the cell surface does not respond to poly(I:C), as measured by $\text{I}_\text{IkB}$ degradation (data not shown). The cellular localization of TLRs is important for their function. It is known that several of the TLRs are found in lysosomes where they encounter pathogen-derived, nucleoside-based ligands (39). It has been hypothesized that encountering of a ligand in lysosomes restricts autoimmune responses against endogenous RNA/DNA molecules while facilitating appropriate responses against pathogens (50). We postulate here that the immune system has evolved versatile strategies to deal with similar pathogen-associated molecular pattern encountered in different ways. Although TLR3 has been found in endosomes of DCs/monocytes where acidification is essential for function (32–34, 36), we have demonstrated that TLR3 is found at the cell surface of NK cells where its function is independent of lysosomes. We suggest that this allows NK cells to circulate in the peripheral blood where cell surface TLR3 searches for its cognate ligand. NK cells respond to exogenously added poly(I:C) in a lysosomal-independent manner, unlike DCs where phagocytosis of dsRNA is necessary for stimulation of intracellular TLR3 (48, 51). This reflects the location of the TLR3 receptors in each cell type. Yet where is a NK cell likely to encounter ligand? Many viruses have a lytic stage in their life cycle during which newly synthesized virions are released to allow the infection to propagate in new target cells. Given an inherent asynchronicity in the generation of virions, it is highly probable that intermediate viral replication products will be released into the extracellular space. This provides the opportunity for patrolling effector NK cells to sense the infection through cell surface TLR3.

In summary, the present report proposes a model in which specific TLR agonists activate human NK cells both directly and indirectly through activation of monocytes. These dual activities lead to optimal NK cell activation, which is accompanied by both NK cell cytoxicity and IFN- secretion. In the case of TLR3, we have found that cytokines such as IL-12 provide costimulation to dsRNA-activated NK cells. In contrast, cytokines provide the primary signals for NK cells to allow recognition of and costimulation by TLR7/8 agonists. These findings highlight the important role of NK cells in the recognition of conserved pathogen structures, which is in agreement with their essential role in innate immune responses.

### Disclosures

The authors have no financial conflict of interest.
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