



COVID-19 Research Tools

Defeat the SARS-CoV-2 Variants

InVivoGen

The Journal of
Immunology

RESEARCH ARTICLE | AUGUST 01 2005

TLR7/8-Mediated Activation of Human NK Cells Results in Accessory Cell-Dependent IFN- γ Production¹ **FREE**

Orla M. Hart; ... et. al

J Immunol (2005) 175 (3): 1636–1642.

<https://doi.org/10.4049/jimmunol.175.3.1636>

TLR7/8-Mediated Activation of Human NK Cells Results in Accessory Cell-Dependent IFN- γ Production¹

Orla M. Hart,* Veronica Athie-Morales,[†] Geraldine M. O'Connor,* and Clair M. Gardiner^{2*}

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 polyinosinic 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 polyinosinic 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 potentially stimulated by TLR7/8 engagement.

*Department of Biochemistry, and [†]Xoma Ireland Limited, Department of Biochemistry, Trinity College, Dublin, Ireland

Received for publication December 22, 2004. Accepted for publication May 20, 2005.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

¹ This study was partially supported by a Cancer Research Ireland grant (to C.M.G.).

² Address correspondence and reprint requests to Dr. Clair M. Gardiner, Department of Biochemistry, Trinity College, Dublin 2, Ireland. E-mail address: clair.gardiner@ted.ie

³ Abbreviations used in this paper: MCMV, murine cytomegalovirus; poly(I:C), polyinosinic acid:cytidylic acid; rhIL-2, recombinant human IL-2; NCR, natural cytotoxicity receptor.

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 293T-TLR7 or 293T-TLR8, which have been stably transfected with TLR7 and TLR8, respectively, were maintained in DMEM (Sigma-Aldrich) supplemented with 10% FCS and 10 μ g/ml blasticidin (InvivoGen). All media were supplemented with 100 U/ml penicillin and 100 μ g/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 using NK isolation kit II (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 >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 μ g/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 with random hexamers using ImProm-II Reverse Transcription System (Promega). PCR was conducted on cDNA samples with the following primers: *TLR3* (F: 5'-GGTAACGATTCTTTGCTTGGCTTC-3', R: 5'-TAGTGGCTTGACAGCTCAGGGATG-3') (33); *TLR7* (F: 5'-AGTGTCTAAAGAACCCTGG-3', R: 5'-CTTGGCCTTACAGAAATG-3'); and *TLR8* (F: 5'-CAGAATAGCAGGCCTAACACATCA-3', R: 5'-AATGTCACAGGTGCATTCAAAGGG-3') (34) using TaqDNA polymerase (Invitrogen Life Technologies). *KIR2DL4* gene was amplified to control for genomic DNA contamination (F: 5'-AGGACAAGCCCTTCTGC-3', R: 5'-GGAAAGAGCCGAAGCATC-3').

Flow cytometry

NKL, NK92, and primary NK cells were stained with anti-TLR3 (eBioscience) and anti-CD56 (BD Biosciences) Abs. Cells were acquired on a FACSCalibur flow cytometer and analyzed using CellQuest software (BD Biosciences). Anti-CD3, anti-CD14, and anti-NKG2D were obtained from BD Biosciences, and Abs against NKp30, NKp44, and NKp46 were obtained from Immunotech. Isotype-matched control Abs were purchased from BD Biosciences.

SDS-PAGE and Western blotting for I κ B α

After appropriate stimulation with poly(I:C) (Sigma-Aldrich) or R848 (InvivoGen), 1×10^6 cells were washed with PBS and lysed in radioimmune precipitation buffer (1% (v/v) Nonidet P-40, 0.5% (w/v) sodium deoxycholate, 0.1% SDS in PBS, 10 μ g/ml PMSF, 7 μ g/ml aprotinin, and 1 mM sodium orthovanadate) for 45 min at 4°C. For inhibition of lysosomal acidification, cells were incubated with 20 μ g/ml chloroquine (Sigma-Aldrich) for 1 h before stimulation. Where applicable, cells were cultured with 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-I κ B α 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 1.25×10^5 (293T, 293T-TLR7, or 293T-TLR8) or 2×10^6 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 $16,000 \times g$ 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) glycerol, 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 Imgenex), followed by anti-mouse-HRP (Sigma-Aldrich) using West Pico ECL development. The TLR8 protein ran at a lower apparent m.w. than expected.

⁵¹Cr release cytotoxicity assay

A total of 1×10^6 Daudi target cells were labeled with 1 mCi of Na⁵¹CrO₄ 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). Percentage cytotoxicity was calculated using the formula (experimental - spon)/(maximum - spon) \times 100%, where spon = release from targets incubated with medium alone and maximum = release from targets induced by 5% Triton X-100 (Sigma-Aldrich). Blocking with anti-IL-12 Ab (R&D Systems), anti-TLR3 Ab (HyCult Biotechnology), or control IgG (Sigma-Aldrich) was conducted at 10 μ g/ml.

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 tested using NK cell lines and highly purified NK cells from peripheral blood (Fig. 1a). We next examined NK cells for protein expression of TLRs. TLR3 was present on the surface of virtually all primary NK cells in addition to all three NK cell lines tested (Fig. 1b). This result is unexpected because in most cell types TLR3 expression is restricted to intracellular vesicles (35–37). As expected, neither TLR7 nor TLR8 are expressed at the cell surface on NK cells (data not shown), but protein expression of TLR7 protein is found in the NKL cell line, whereas TLR8 protein can be detected in all three NK cell lines (NKL, NK92, and YT) by Western blotting (Fig. 1c).

Having demonstrated expression of the receptors, we wanted to investigate whether NK cells activated components of TLR-signaling cascades in response to TLR agonists. NF- κ B is a common component of all TLR-signaling pathways (10), and we demonstrated its activation using I κ B α degradation by Western blotting as a readout. R848 is an imidazoquinoline, which signals through

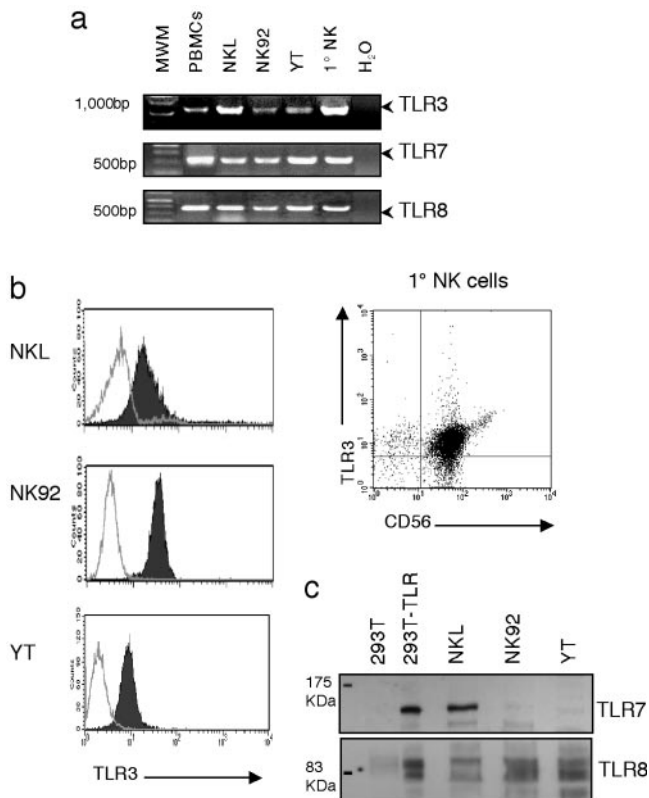


FIGURE 1. Expression of TLRs by NK cells. *a*, RT-PCR was conducted on RNA isolated from NKL, NK92, YT, and primary NK cells ($n = 3$) using primers specific for *TLR3*, *TLR7*, and *TLR8*. RNA from PBMCs was used as a positive control. *b*, Cell surface expression of TLR3 on NKL, NK92, YT, and primary NK cells ($n = 5$) was assessed by flow cytometry using a PE-conjugated anti-TLR3 mAb (filled histograms) and compared with isotype matched controls (empty histograms). *c*, Western blot analysis of protein extracts from the 293T, 293T-TLR7 (upper panel), 293T-TLR8 (lower panel), NKL, NK92, and YT cell lines. Blots were stained with anti-TLR7 (upper panel) or anti-TLR8 (lower panel) specific Abs.

TLR7 and TLR8 in human cells (38). R848 was extremely potent at activating NKL cells with degradation of $\text{I}\kappa\text{B}\alpha$ 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 with no $\text{I}\kappa\text{B}\alpha$ degradation observed over a range of concentrations and times tested (0.5, 1, 3, and 10 $\mu\text{g}/\text{ml}$; 15, 30, 60, and 90 min; data not shown). Poly(I:C) also induced $\text{I}\kappa\text{B}\alpha$ degradation, albeit with slower kinetics. Partial and total degradation were observed at 60 and 90 min, respectively, in the cell lines (Fig. 2b). Differences between the TLR agonists poly(I:C) and R848 were observed when experiments were performed on primary NK cells. Although poly(I:C) induced degradation of $\text{I}\kappa\text{B}\alpha$ between 60 and 90 min (Fig. 2c), R848 failed to do so (Fig. 2d). Therefore, as previously reported (21), NK cells directly respond to poly(I:C). However, the data with regard to TLR7/8 are less clear. R848 appeared not to signal directly on resting primary NK cells. Given that the IL-2-dependent NKL cell line signals potently in response to R848 (Fig. 2a), we hypothesized that activation or priming of NK cells may be required to facilitate TLR7/8 signaling. Preincubation of primary NK cells with IL-2 and, to a lesser extent, IFN- α consistently results in $\text{I}\kappa\text{B}\alpha$ degradation upon subsequent R848 stimulation, as measured by Western blotting (Fig. 2e). Therefore, in contrast to resting primary NK cells, which do not respond directly to R848, priming of NK cells with cytokine allows their direct activation.

TLR3 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.

TLR3 and TLR7/8 agonists activate NK cells

Using highly purified primary NK cells, we observed significant CD69 up-regulation in response to both R848 (11.6 \pm 6.0% baseline to 47.6 \pm 28.7% at 1 $\mu\text{g}/\text{ml}$ R848, $p < 0.01$, and 54.54 \pm 21.0% at 10 $\mu\text{g}/\text{ml}$ R848, $p < 0.01$; $n = 5$) and poly(I:C) stimulations (12.3 \pm 7.4% baseline to 45.9 \pm 25.5% at 10 $\mu\text{g}/\text{ml}$ poly(I:C), $p < 0.01$; $n = 7$), indicating that the cells were activated. It has been reported that levels of TLR3 RNA transcript increase in NK cells in response to poly(I:C) stimulation (21). However, we found no corresponding increase in cell surface expression of TLR3, although CD69, as positive control, increased as expected (data not 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 that NK cells respond to poly(I:C) (17, 18). To prove that poly(I:C) was signaling through TLR3 on NK cells, we blocked TLR3 function using a blocking Ab. Preincubation of highly purified NK cells with a blocking Ab against TLR3 completely abrogated the ability of poly(I:C) to induce cytotoxic activity of the NK cells (Fig. 3b). This is the first formal demonstration that poly(I:C) is mediating its effects on NK cells through TLR3.

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).

To further address the direct activation of primary NK cells, we purified NK cells using a cell sorter and measured CD69 activation and cytotoxicity. Poly(I:C) increased CD69 expression in two of five donors. R848 failed to change CD69 levels in any donor in contrast to IL-2, which significantly enhanced expression of this

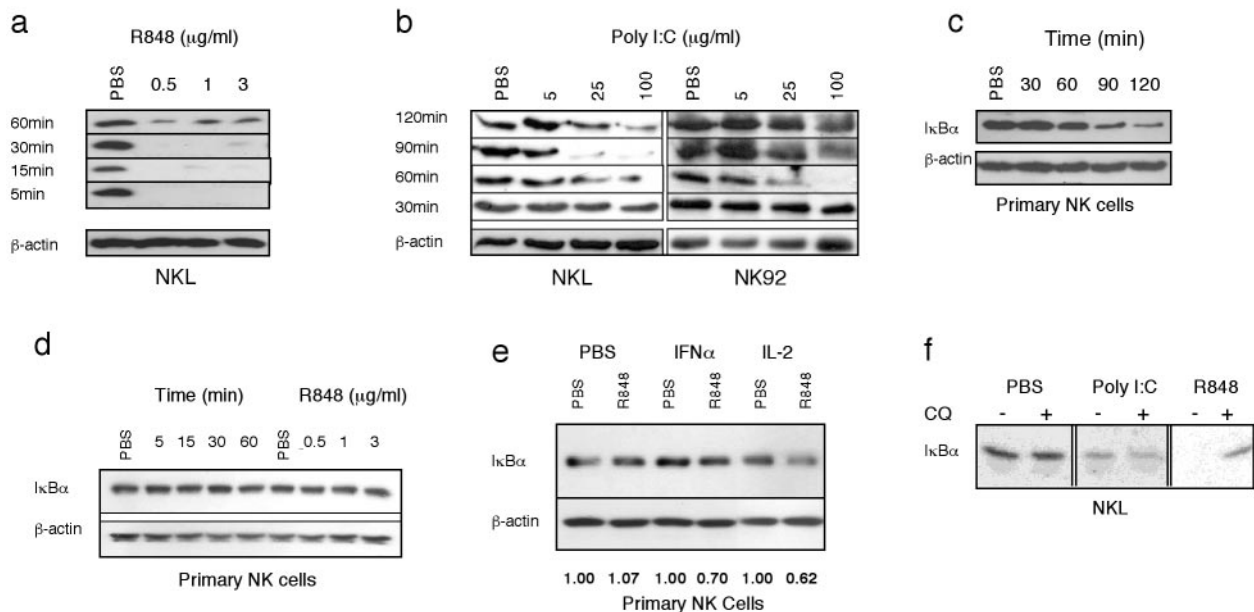


FIGURE 2. Signaling by TLR agonists and lysosomal-independent signaling of TLR3 in human NK cells. Blots show I κ B α degradation as a readout of NF- κ B activation after TLR agonist stimulation over a range of concentrations and time points. Blots were stripped and reprobed with an anti- β -actin Ab to control for protein loading. *a*, NK cells were stimulated with R848. SDS-PAGE and Western blotting were conducted on whole cell lysates. *b*, NK and NK92 cells were stimulated with poly(I:C). *c*, Primary NK cells were stimulated with 100 μ g/ml poly(I:C). *d*, Primary NK cells were stimulated with R848 at 1 μ g/ml for the times indicated or 15 min at the concentration indicated. *e*, Highly purified NK cells were cultured with 500 U/ml IFN- α or 200 U/ml IL-2 for 18 h as indicated and stimulated with 1 μ g/ml R848. Numbers represent values normalized to corresponding β -actin densitometry figures and then to the relevant PBS control. *f*, NK cells were stimulated with 100 μ g/ml poly(I:C) for 90 min or 0.75 μ g/ml R848 for 20 min. Degradation of I κ B α was assessed in the presence or absence of 20 μ g/ml chloroquine (CQ). Results shown are representative of at least three independent experiments.

Ag as expected ($n = 5$; data not shown). The cytotoxicity data for individual donors is shown in Fig. 3*d*. 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. 2*d*) and cytokine blocking experiments (Fig. 3*c*) 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. 4*c*). In contrast, R848 (3 μ 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/10⁶ cells; range, 222–1892 pg/10⁶ cells, $n = 4$). The absence of TLR7/8 signal transduction in resting primary NK cells (Fig. 2*d*) and the role that IL-12 plays in R848-induced cytotoxicity (Fig. 3*c*) 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. 4*a*). 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. 4*b*). 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. 4*c*).

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 either 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.

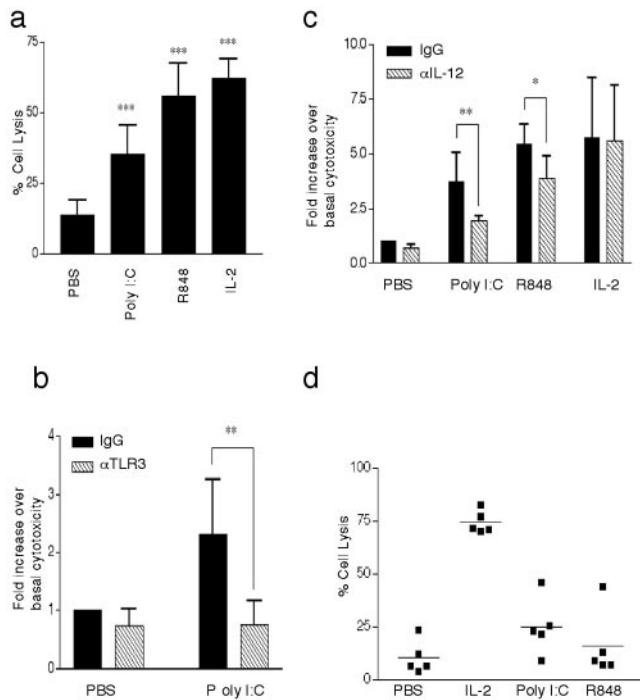


FIGURE 3. TLR agonists activate NK cell cytotoxicity. *a*, Highly purified primary NK cells were stimulated with 10 $\mu\text{g}/\text{ml}$ poly(I:C) or 1 $\mu\text{g}/\text{ml}$ R848 for 18 h, and cytotoxicity was assessed using the ^{51}Cr release assay. As a positive control, cells were stimulated with 200 U/ml rIL-2. *b*, Highly purified primary NK cells were preincubated with blocking anti-TLR3 mAb and stimulated with 10 $\mu\text{g}/\text{ml}$ poly(I:C) for 18 h. Cytotoxicity was assessed as for *a*. Data are expressed as fold increase in cytotoxicity over levels observed in resting cells. *c*, Highly purified primary NK cells were preincubated with blocking anti-IL-12 mAb and stimulated with 10 $\mu\text{g}/\text{ml}$ poly(I:C) or 3 $\mu\text{g}/\text{ml}$ R848 for 18 h. Cytotoxicity was assessed as for *a*. Data are expressed as fold increase in cytotoxicity over levels observed by resting cells. *d*, FACS-sorted NK cells were stimulated with 10 $\mu\text{g}/\text{ml}$ poly(I:C) or 1 $\mu\text{g}/\text{ml}$ R848 for 18 h, and cytotoxicity was assessed as for *a*. The data represent the average values obtained for six donors for *a* (three for IL-2 experiment) and four donors for each of *b* and *c*. Error bars show SEM. *, $p < 0.05$, **, $p < 0.01$, and ***, $p < 0.001$

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 resting NK cells by poly(I:C) to induce effector functions such as cytotoxicity (but not IFN- γ production). Accessory cell help potentiates 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 are 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 monocytes, activation of resting NK cells fails to occur. Thus, although different TLRs modulate NK 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 stim-

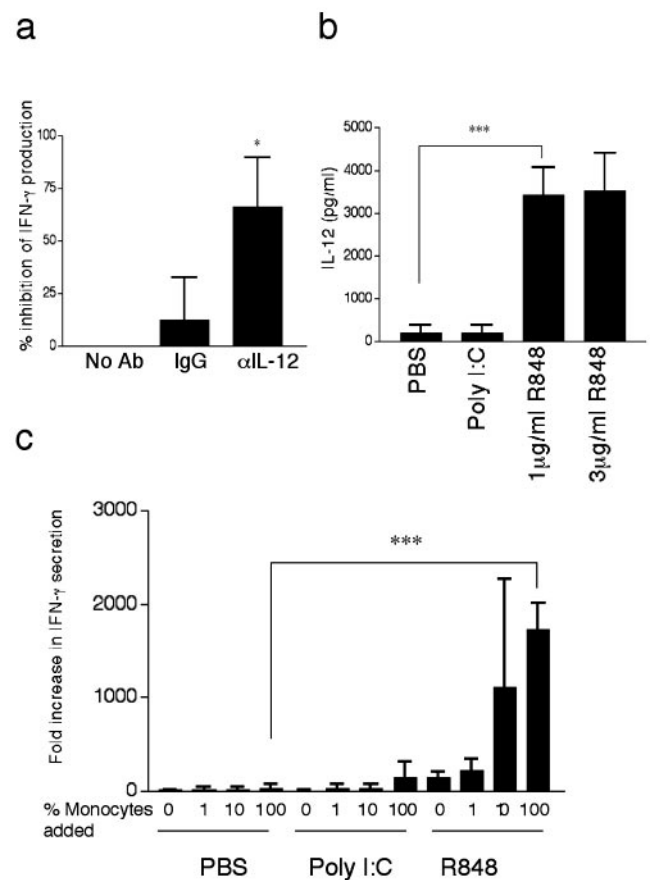


FIGURE 4. Production of IFN- γ by NK cells is dependent on IL-12 produced by accessory cells. Production of cytokines was measured by ELISA of cell culture supernatants. *a*, Purified NK cells were stimulated with 3 $\mu\text{g}/\text{ml}$ R848 for 18 h, and IFN- γ was measured. Either control IgG or anti-IL-12 Abs were added at the start of the cultures. Data are expressed as percentage inhibition compared with the PBS (no Ab) control ($n = 4$). *b*, Purified monocytes were stimulated for 18 h with 10 $\mu\text{g}/\text{ml}$ poly(I:C) or 1 and 3 $\mu\text{g}/\text{ml}$ R848. IL-12 secretion was measured. *c*, Highly purified NK cells were stimulated with 10 $\mu\text{g}/\text{ml}$ poly(I:C) or 1 $\mu\text{g}/\text{ml}$ R848 for 18 h. Purified monocytes were titrated back into the purified primary NK cell populations (0% = 0.1–0.25%, CD14 $^{+}$). One hundred percent refers to 1:1 NK cell-to-monocyte ratio. Data are expressed as fold increase in IFN- γ secretion over levels secreted by resting NK cells alone. Data in *b* and *c* represent the averages of at least $n = 3$ donors. Error bars show SEM. *, $p < 0.05$, and ***, $p < 0.001$

ulates NK cell production of IFN- γ , which in turn helps control the 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 activated by cytokines such as IL-2 or IFN- α . Our data presented here support a model in which TLR agonists directly target both NK cells and accessory cells to generate an optimal innate immune response. Depending on the TLR, NK cells will be directly activated or primed for direct TLR activation by accessory cell-derived cytokines. Activation of NK cell cytotoxicity will help control the initial infection. In addition, IFN- γ produced by the NK cell will help to initiate a Th1-type adaptive immune response, which is required for successful elimination of most viral pathogens.

Table I. NCR expression in response to stimulation with TLR3 or TLR7/8 agonists*

	NKp30 (%)	NKp44 (%)	NKp46 (%)	NKG2D (%)
PBS	11.9 ± 4.5	5.6 ± 6.5	88.3 ± 18.2	4.0 ± 6.5
Poly(I:C) (10 µg/ml)	15.9 ± 8.5	8.0 ± 6.0	91.1 ± 14.2	6.4 ± 4.9
R848 (3 µg/ml)	13.2 ± 9.4	7.6 ± 2.9	85.9 ± 17.7	5.1 ± 2.7
R848 (10 µg/ml)	12.6 ± 7.2	7.1 ± 2.8	85.8 ± 18.6	3.5 ± 1.5

* NK cells were purified and stimulated with poly(I:C) (10 µg/ml) or R848 (either 3 or 10 µg/ml) for 18 h at 37°C. Cells were stained for cell surface expression of NCRs and analyzed by flow cytometry. Results are expressed as the percentage of CD56⁺ cells that coexpress the indicated activatory receptor. A minimum of three donors were used (NKG2D experiment) and ranged from five to seven donors for the other activation antigens.

Cytokine production is an important effector function of NK cells (32, 42–44). In particular, IFN- γ is a key antiviral cytokine produced early in infection. Poly(I:C) failed to stimulate rapid production of IFN- γ with minimal amounts detected in the supernatants of NK cells after 18 h. In marked contrast, R848 was extremely potent at inducing IFN- γ 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- γ 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- γ 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 cytotoxicity, as we can partially inhibit cytotoxicity in the presence of a neutralizing anti-IL-12 Ab, but are insufficient to induce IFN- γ production. In support of this model, in the absence of costimulation, nanogram quantities of IL-12 are required to stimulate IFN- γ production by highly purified human NK cells (data not shown), whereas picogram amounts can stimulate increased NK cell cytotoxicity (45). In contrast to poly(I:C), R848 induces large amounts of IL-12 from monocytes (39), which in turn promotes high IFN- γ production and increased cytotoxicity by NK cells. IL-12 on its own is inefficient for induction of IFN- γ 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- γ 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 loxoribine) 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- α , indicating that some of the signaling components may not be constitutively present in NK cells.

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 NKL cell line negative for TLR3 at the cell surface does not respond to poly(I:C), as measured by $\text{I}\kappa\text{B}\alpha$ 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 cytotoxicity and IFN- γ 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.

References

- Cerwenka, A., and L. L. Lanier. 2001. Natural killer cells, viruses and cancer. *Nat. Rev. Immunol.* 1: 41–49.
- Fitzgerald, P. a. L., C. 1986. Natural killer cells active against viral, bacterial, protozoan and fungal infections. In *Immunobiology of Natural Killer Cells*, Vol. 2. H. Lotzora and R. B. Herberman, eds. CRC, Boca Raton, p. 107.
- Biron, C. A., K. S. Byron, and J. L. Sullivan. 1989. Severe herpesvirus infections in an adolescent without natural killer cells. *N. Engl. J. Med.* 320: 1731–1735.
- Orange, J. S. 2002. Human natural killer cell deficiencies and susceptibility to infection. *Microbes Infect.* 4: 1545–1558.
- Vilches, C., and P. Parham. 2002. KIR: diverse, rapidly evolving receptors of innate and adaptive immunity. *Annu. Rev. Immunol.* 20: 217–251.
- Moretta, L., and A. Moretta. 2004. Unravelling natural killer cell function: triggering and inhibitory human NK receptors. *EMBO J.* 23: 255–259.
- Mandelboim, O., N. Lieberman, M. Lev, L. Paul, T. I. Arnon, Y. Bushkin, D. M. Davis, J. L. Strominger, J. W. Yewdell, and A. Porgador. 2001. Recognition of haemagglutinins on virus-infected cells by NKp46 activates lysis by human NK cells. *Nature* 409: 1055–1060.
- Arnon, T. I., M. Lev, G. Katz, Y. Chernobrov, A. Porgador, and O. Mandelboim. 2001. Recognition of viral hemagglutinins by NKp44 but not by NKp30. *Eur. J. Immunol.* 31: 2680–2689.
- Brown, M. G., A. O. Dokun, J. W. Heusel, H. R. Smith, D. L. Beckman, E. A. Blattenberger, C. E. Dubbelde, L. R. Stone, A. A. Scalzo, and W. M. Yokoyama. 2001. Vital involvement of a natural killer cell activation receptor in resistance to viral infection. *Science* 292: 934–937.
- Akira, S., and S. Sato. 2003. Toll-like receptors and their signaling mechanisms. *Scand. J. Infect. Dis.* 35: 555–562.
- Alexopoulou, L., A. C. Holt, R. Medzhitov, and R. A. Flavell. 2001. Recognition of double-stranded RNA and activation of NF- κ B by Toll-like receptor 3. *Nature* 413: 732–738.
- Heil, F., H. Hemmi, H. Hochrein, F. Ampenberger, C. Kirschning, S. Akira, G. Lipford, H. Wagner, and S. Bauer. 2004. Species-specific recognition of single-stranded RNA via Toll-like receptor 7 and 8. *Science* 303: 1526–1529.
- Lund, J. M., L. Alexopoulou, A. Sato, M. Karow, N. C. Adams, N. W. Gale, A. Iwasaki, and R. A. Flavell. 2004. Recognition of single-stranded RNA viruses by Toll-like receptor 7. *Proc. Natl. Acad. Sci. USA* 101: 5598–5603.
- Diebold, S. S., T. Kaisho, H. Hemmi, S. Akira, and C. Reis e Sousa. 2004. Innate antiviral responses by means of TLR7-mediated recognition of single-stranded RNA. *Science* 303: 1529–1531.
- Hemmi, H., O. Takeuchi, T. Kawai, T. Kaisho, S. Sato, H. Sanjo, M. Matsumoto, K. Hoshino, H. Wagner, K. Takeda, and S. Akira. 2000. A Toll-like receptor recognizes bacterial DNA. [Published erratum appears in 2001 *Nature* 409: 646.] *Nature* 408: 740–745.
- Bauer, S., C. J. Kirschning, H. Hacker, V. Redecke, S. Hausmann, S. Akira, H. Wagner, and G. B. Lipford. 2001. Human TLR9 confers responsiveness to bacterial DNA via species-specific CpG motif recognition. *Proc. Natl. Acad. Sci. USA* 98: 9237–9242.
- Gidlund, M., A. Orn, H. Wigzell, A. Senik, and I. Gresser. 1978. Enhanced NK cell activity in mice injected with interferon and interferon inducers. *Nature* 273: 759–761.
- Biron, C. A. 1997. Activation and function of natural killer cell responses during viral infections. *Curr. Opin. Immunol.* 9: 24–34.
- Becker, I., N. Salaiza, M. Aguirre, J. Delgado, N. Carrillo-Carrasco, L. G. Kobeh, A. Ruiz, R. Cervantes, A. P. Torres, N. Cabrera, et al. 2003. *Leishmania* lipophosphoglycan (LPG) activates NK cells through Toll-like receptor-2. *Mol. Biochem. Parasitol.* 130: 65–74.
- Pisegna, S., G. Pirozzi, M. Piccoli, L. Frati, A. Santoni, and G. Palmieri. 2004. p38 MAPK activation controls the TLR3-mediated up-regulation of cytotoxicity and cytokine production in human NK cells. *Blood* 104: 4157–4164.
- Schmidt, K. N., B. Leung, M. Kwong, K. A. Zarembler, S. Satyal, T. A. Navas, F. Wang, and P. J. Godowski. 2004. APC-independent activation of NK cells by the Toll-like receptor 3 agonist double-stranded RNA. *J. Immunol.* 172: 138–143.
- Sivori, S., M. Falco, M. Della Chiesa, S. Carlomagno, M. Vitale, L. Moretta, and A. Moretta. 2004. CpG and double-stranded RNA trigger human NK cells by Toll-like receptors: induction of cytokine release and cytotoxicity against tumors and dendritic cells. *Proc. Natl. Acad. Sci. USA* 101: 10116–10121.
- Krug, A., A. R. French, W. Barchet, J. A. Fischer, A. Dzionek, J. T. Pingel, M. M. Orihuela, S. Akira, W. M. Yokoyama, and M. Colonna. 2004. TLR9-dependent recognition of MCMV by IPC and DC generates coordinated cytokine responses that activate antiviral NK cell function. *Immunity* 21: 107–119.
- Tabeta, K., P. Georgel, E. Janssen, X. Du, K. Hoebe, K. Crozat, S. Mudd, L. Shamel, S. Sovath, J. Goode, et al. 2004. Toll-like receptors 9 and 3 as essential components of innate immune defense against mouse cytomegalovirus infection. *Proc. Natl. Acad. Sci. USA* 101: 3516–3521.
- Biron, C. A., H. C. Su, and J. S. Orange. 1996. Function and regulation of natural killer (NK) cells during viral infections: characterization of responses in vivo. *Methods* 9: 379–393.
- Biron, C. A., K. B. Nguyen, G. C. Pien, L. P. Cousens, and T. P. Salazar-Mather. 1999. Natural killer cells in antiviral defense: function and regulation by innate cytokines. *Annu. Rev. Immunol.* 17: 189–220.
- Orange, J. S., B. Wang, C. Terhorst, and C. A. Biron. 1995. Requirement for natural killer cell-produced interferon γ in defense against murine cytomegalovirus infection and enhancement of this defense pathway by interleukin 12 administration. *J. Exp. Med.* 182: 1045–1056.
- Lucin, P., S. Jonjic, M. Messerle, B. Polic, H. Hengel, and U. H. Koszinowski. 1994. Late phase inhibition of murine cytomegalovirus replication by synergistic action of interferon γ and tumour necrosis factor. *J. Gen. Virol.* 75(Pt. 1): 101–110.
- MacMicking, J., Q. W. Xie, and C. Nathan. 1997. Nitric oxide and macrophage function. *Annu. Rev. Immunol.* 15: 323–350.
- Tay, C. H., and R. M. Welsh. 1997. Distinct organ-dependent mechanisms for the control of murine cytomegalovirus infection by natural killer cells. *J. Virol.* 71: 267–275.
- Heise, M. T., and H. W. T. Virgin. 1995. The T cell-independent role of γ interferon and tumor necrosis factor α in macrophage activation during murine cytomegalovirus and herpes simplex virus infections. *J. Virol.* 69: 904–909.
- Orange, J. S., and C. A. Biron. 1996. An absolute and restricted requirement for IL-12 in natural killer cell IFN- γ production and antiviral defense: studies of natural killer and T cell responses in contrasting viral infections. *J. Immunol.* 156: 1138–1142.
- Visintin, A., A. Mazzoni, J. H. Spitzer, D. H. Wylie, S. K. Dower, and D. M. Segal. 2001. Regulation of Toll-like receptors in human monocytes and dendritic cells. *J. Immunol.* 166: 249–255.
- Kadowaki, N., S. Ho, S. Antonenko, R. W. Malefyt, R. A. Kastelein, F. Bazan, and Y. J. Liu. 2001. Subsets of human dendritic cell precursors express different Toll-like receptors and respond to different microbial antigens. *J. Exp. Med.* 194: 863–869.
- Takeda, K., and S. Akira. 2005. Toll-like receptors in innate immunity. *Int. Immunol.* 17: 1–14.
- Nishiya, T., and A. L. DeFranco. 2004. Ligand-regulated chimeric receptor approach reveals distinctive subcellular localization and signaling properties of the Toll-like receptors. *J. Biol. Chem.* 279: 19008–19017.
- Matsumoto, M., S. Kikkawa, M. Kohase, K. Miyake, and T. Seya. 2002. Establishment of a monoclonal antibody against human Toll-like receptor 3 that blocks double-stranded RNA-mediated signaling. *Biochem. Biophys. Res. Commun.* 293: 1364–1369.
- Jurk, M., F. Heil, J. Vollmer, C. Schetter, A. M. Krieg, H. Wagner, G. Lipford, and S. Bauer. 2002. Human TLR7 or TLR8 independently confer responsiveness to the antiviral compound R-848. *Nat. Immunol.* 3: 499.
- Lee, J., T. H. Chuang, V. Redecke, L. She, P. M. Pitha, D. A. Carson, E. Raz, and H. B. Cottam. 2003. Molecular basis for the immunostimulatory activity of guanine nucleoside analogs: activation of Toll-like receptor 7. *Proc. Natl. Acad. Sci. USA* 100: 6646–6651.
- Barnes, B. J., P. A. Moore, and P. M. Pitha. 2001. Virus-specific activation of a novel interferon regulatory factor, IRF-5, results in the induction of distinct interferon α genes. *J. Biol. Chem.* 276: 23382–23390.
- Izaguirre, A., B. J. Barnes, S. Amrute, W. S. Yeow, N. Megjugorac, J. Dai, D. Feng, E. Chung, P. M. Pitha, and P. Fitzgerald-Bocarsly. 2003. Comparative analysis of IRF and IFN- α expression in human plasmacytoid and monocyte-derived dendritic cells. *J. Leukocyte Biol.* 74: 1125–1138.
- Biron, C. A., K. B. Nguyen, and G. C. Pien. 2002. Innate immune responses to LCMV infections: natural killer cells and cytokines. *Curr. Top. Microbiol. Immunol.* 263: 7–27.
- Byrne, P., P. McGuirk, S. Todyrk, and K. H. Mills. 2004. Depletion of NK cells results in disseminating lethal infection with *Bordetella pertussis* associated with a reduction of antigen-specific Th1 and enhancement of Th2, but not Tr1 cells. *Eur. J. Immunol.* 34: 2579–2588.
- Bancroft, G. J. 1993. The role of natural killer cells in innate resistance to infection. *Curr. Opin. Immunol.* 5: 503–510.
- Aste-Amezaga, M., A. D'Andrea, M. Kubin, and G. Trinchieri. 1994. Cooperation of natural killer cell stimulatory factor/interleukin-12 with other stimuli in the induction of cytokines and cytotoxic cell-associated molecules in human T and NK cells. *Cell. Immunol.* 156: 480–492.
- Hemmi, H., T. Kaisho, O. Takeuchi, S. Sato, H. Sanjo, K. Hoshino, T. Horiuchi, H. Tomizawa, K. Takeda, and S. Akira. 2002. Small anti-viral compounds activate immune cells via the TLR7 MyD88-dependent signaling pathway. *Nat. Immunol.* 3: 196–200.
- Williams, B. R. 2001. Signal integration via PKR. *Sci. STKE.* 2001: RE2.
- Diebold, S. S., M. Montoya, H. Unger, L. Alexopoulou, P. Roy, L. E. Haswell, A. Al-Shamkhani, R. Flavell, P. Borrow, and C. Reis e Sousa. 2003. Viral infection switches non-plasmacytoid dendritic cells into high interferon producers. *Nature* 424: 324–328.
- Yoneyama, M., M. Kikuchi, T. Natsukawa, N. Shinobu, T. Imaizumi, M. Miyagishi, K. Taira, S. Akira, and T. Fujita. 2004. The RNA helicase RIG-I has an essential function in double-stranded RNA-induced innate antiviral responses. *Nat. Immunol.* 5: 730–737.
- Crozat, K., and B. Beutler. 2004. TLR7: a new sensor of viral infection. *Proc. Natl. Acad. Sci. USA* 101: 6835–6836.
- Schulz, O., S. S. Diebold, M. Chen, T. I. Naslund, M. A. Nolte, L. Alexopoulou, Y. T. Azuma, R. A. Flavell, P. Liljestrom, and C. Reis e Sousa. 2005. Toll-like receptor 3 promotes cross-priming to virus-infected cells. *Nature* 433: 887–892.