Distinct indirect pathways govern human NK-cell activation by TLR-7 and TLR-8 agonists

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Abstract

NK cells limit the emergence of cancers and viral infections by surveillance of ‘missing-self’ and ‘induced-self’ ligands, and by direct recognition of pathogen-associated molecules. We examined individual roles for Toll-like receptors (TLRs)-7 and -8 in human NK-cell activation using synthetic, small molecule agonists of either TLR-7 (imiquimod and 3M-001), TLR-8 (3M-002) or both TLR-7/8 (3M-003 and R-848) for comparison with known ligands of TLR-2 to -9. Tracking cytokine production in PBMC initially revealed that a subset of TLR agonists including polyinosinic–polycytidylic acid (poly I:C), 3M-002, 3M-003, R-848 and single-stranded RNA trigger relatively high levels of IFN-γ expression by NK cells. Isolated NK cells did not express TLR-7 or TLR-8. Unlike MALP-2 and poly I:C, 3M-001-3 did not induce expression of either CD69 or IFN-γ by purified NK cells suggesting indirect activation. IL-18 and IL-12p70 were primarily required for induction of IFN-γ by both synthetic and natural TLR-8 ligands, while type I IFN was required for induction of CD69 on NK cells by the TLR-7 agonist 3M-001. In addition to expression of IFN-γ and CD69, relative induction of NK-cell cytotoxicity by TLR-7 and TLR-8 agonists was compared. Immune response modifiers (IRMs) with a TLR-8 agonist component (3M-002 and 3M-003) stimulated greater levels of K562 cytolysis than achieved with 3M-001 or IL-2 (1000 units ml⁻¹). In vivo NK-cell cytotoxicity was also enhanced by R-848, but not in type I IFNR-deficient mice. We conclude that IRMs can modulate NK-cell function both in vitro and in vivo and that distinct indirect pathways control human NK-cell activation by TLR-7 and TLR-8 agonists.

Introduction

NK cells are innate effector cells that assist in limiting the emergence of viral infections (1) and cancers (2), and cooperate with dendritic cells (DCs) to shape adaptive immune responses (3). It has recently become appreciated that NK cells directly recognize pathogen-associated molecules. Specifically, murine NK cells recognize m157 from mouse cytomegalovirus through the CMV1 locus-encoded Ly49h (4–6). NK cells are brought into the endoscopic world of NK cells by a variety of pathogen-derived ligands, in vertebrates (7, 8) but also by a wide array of cytokines including IFNs (9–13).

Toll-like receptors (TLRs) have been implicated in pathogen recognition by NK cells as well (14–17). TLRs are a conserved set of receptors that trigger innate immune activation in the presence of invading microbes (18, 19). TLRs-1 to -10 have been described in humans (20) and distinct immunosurveillance roles for individual TLRs are taking shape. Differences in TLRs have been identified across species. Notably, mouse TLR-8 is reported to be non-functional (21) and human TLR-11 is a pseudogene (22). TLR ligands are generally thought of as conserved pathogen structures and macromolecules [such as bacterial lipopeptides (TLR-1/6, 2/6), double-stranded RNA (TLR-3), LPS (TLR-4), flagellin (TLR-5) and pathogen DNA (TLR-9) (23)] and yet, they also include a family of synthetic, small molecule agonists of TLR-7 and TLR-8. These imidazoquinoline molecules include imiquimod (R-837) and resiquimod (R-848), and have been termed immune response modifiers (IRMs) (24). A link between IRMs and TLR-7 was first reported as a loss of imiquimod and resiquimod activity in mice lacking TLR-7 (25). Resiquimod is often reported as a TLR-7 agonist (26, 27) but is known to act through both human TLR-7 and TLR-8 (21). More recently, synthetic TLR-8...
selective IRMs have been characterized (28), and viral single-stranded RNA (ssRNA) has been identified as a natural agonist of TLR-7 (29, 30) and TLR-8 (31).

The diversity of microbes recognized by a fixed set of TLRs suggests that a division of labor exists for TLRs with regard to specific pathogen classes (23). Division of innate immune labors in mammals extends beyond receptors to include cell subsets specialized for control of distinct infections. Differential expression of TLRs in distinct innate immune cell subsets has been observed (14, 32) and correlates with induction of different types and quantities of cytokines by different TLR agonists. Human peripheral blood DCs have particularly well-defined roles in TLR-based immunosurveillance (33). Complementary sets of TLRs direct either plasmacytoid dendritic cell (pDC: TLRs-7 and -9) to secrete anti-viral IFN-α or myeloid dendritic cell (mDC: TLRs-2, -3, -4, -5, -6 and -8) to secrete IL-12 and promote expansion of antigen-specific T cells (27, 34, 35). Exceptions include IFN-α induction by polyinosinic–polycytidylic acid (poly I:C) in mDC (36) and IL-12 production by pDC activated with CpG oligodeoxynucleotides (ODNs) and CD40 ligand-expressing cells (33, 37). Synthetic TLR-7 (3M-001), TLR-8 (3M-002) and TLR-7/8 (3M-003) agonists were recently characterized for the ability to induce cytokines from isolated pDC and mDC (28). While 3M-001, a TLR-7 selective agonist similar to imiquimod, was shown to selectively activate pDC and 3M-002 was shown to preferentially activate mDC, 3M-003, a TLR-7/8 agonist closely related to R-848, induced cytokine production from both pDC and mDC correlating with TLR-7 and TLR-8 expression, respectively, by these cell subsets. Thus, TLR agonists including IRMs can act preferentially on specialized cell subsets leading to distinct immune responses.

While a critical interplay between DCs and NK cells has been identified in the context of immunotherapy (38), the degree to which NK cells utilize the TLR network for direct pathogen recognition is unclear. Like DC, there are at least two distinct types of NK cells in humans (10). In peripheral blood, the predominant NK-cell population (>90%, CD56dim, CD16+) is perforin rich and specialized for cytoxicity, while the minor peripheral population (<10%, CD56bright, CD16-) is also found in the T-cell areas of lymph nodes (39, 40). These human lymph node NK cells can produce a diverse set of cytokines in polarized fashion, suggesting a regulatory role in adaptive immunity (41) consistent with recent observations in vivo (3). Furthermore, both NK cells and DCs have been targeted for expansion in patients treated with cytokines. For example, CD56bright NK cells are dramatically expanded in patients treated with IL-2 (42), much like DC expansion with Flt3 ligand (43). Unlike DCs, however, little is known regarding activation of NK-cell subsets through TLRs.

NK cells are generally regulated by a balanced set of inhibitory and activating receptors that act together with IFNs to discriminate between healthy and infected cells (44). In addition to the loss of surface MHC, stress-induced ligands (MIC A/B and ULBPs) can mark altered cells for elimination (45, 46). A third pathway for NK-cell immunosurveillance is by direct recognition of pathogen-associated molecules. The purpose of this study was to determine the response of human NK cells to various TLR ligands. Herein, we take advantage of synthetic agonists of human TLR-7 and -8 for comparison with HIV-derived ssRNA to investigate activation of human DCs through different TLR pathways.

Methods

TLR agonists and antibodies

Synthetic agonists of TLR-7, imiquimod [molecular weight (MW) 240.3] and 3M-001 (MW 361.5), TLR-8, 3M-002 (MW 243.3) and TLR-7/8, R-848 (MW 314.4) and 3M-003 (MW 318.4), described previously (28, 47), were dissolved in dimethyl sulfoxide (Sigma–Aldrich, St Louis, MO, USA) at a concentration of 10 mM and stored at 4°C. Pam3CSK4, MALP-2, LPS (‘Ultrapure’ from Salmonella minnesota) and flagellin (from Bacillus subtilis) were obtained from InvivoGen (San Diego, CA, USA). Poly I:C was obtained from Sigma–Aldrich, reconstituted at 2.5 mg ml⁻¹ at 50°C and re-annealed before storage at −20°C. The CpG ODNs, ODN 2216, ODN 7909, immunostimulatory sequence (ISS) 1018 and ISS C274, described previously (48, 49), were reconstituted at 5 mg ml⁻¹ and stored at −20°C. ssRNA40 was synthesized as previously described (31) and complexed with DOTAP (liposomal transfection reagent; Roche, Indianapolis, IN, USA) before addition to cell cultures.

Fluorochrome-conjugated antibodies including anti-TCRαβ (PE), CD3 (PerCP), CD11a (PE), CD11b (PE) and CD54 (PE) were obtained from BD Biosciences (San Jose, CA, USA). Antibodies to CD56 (FITC, PE and allophycocyanin), IFN-γ (allophycocyanin), CD69 (FITC), CD3 (FITC), CD14 (FITC), CD16 (FITC) and CD19 (FITC) were obtained from eBioscience (San Diego, CA, USA). Fc-blocking reagent was obtained from Miltenyi Biotec (GmbH, Germany). All antibodies were titrated and used according to standard test methods (50). Neutralizing antibodies, anti-tumor necrosis factor (TNF)-α (mAb1, mlgG1) and anti-IL-12p40/70 (C8.6, mlgG1) were obtained from BD Biosciences; anti-IL-12p70 (24910, mlgG1) was obtained from R&D systems (Minneapolis, MN, USA); anti-IL-18 (125-2H, mIgG1) was obtained from Medical & Biological Laboratories Co., Ltd, (MBL; Naka-ku Nagoya, Japan) and anti-IL-10 (JES3-9D7, rtlgG1) was obtained from BD Biosciences. Anti-IFN-αβR chain 2 (MMHAR-2, mlgG2a) was obtained from PBL Biomedical (New Brunswick, NJ, USA). Neutralizing antibodies were used at optimal concentrations for blocking cognate cytokines according to manufacturer’s specifications. Functional grade antibodies (without azide or endotoxin) from BD Biosciences were used as controls including mlgG1 (107.3), mlgG2a (G155-178), rtlgG1 (R3-34) and rtlgG2a (R35-95).

Cell isolation and TLR agonist stimulation

Blood was acquired from healthy volunteers providing informed consent under protocols reviewed and approved by an institutional review committee. PBMCs were isolated by density gradient centrifugation as previously described (28) with minor modifications including use of Accuspin tubes (Sigma–Aldrich) according to the manufacturer’s specifications. Cells were washed and re-suspended in complete RPMI 1640 medium containing 10% heat-inactivated fetal bovine serum, 1 mM sodium pyruvate, 1 mM l-glutamine and 1% penicillin/streptomycin. PBMCs were cultured with various TLR
agonists as previously described (28). For neutralizing antibody experiments, pre-incubation of PBMC with cytokine blocking antibody was performed for 1 h prior to TLR agonist stimulation.

For analysis of NK cells in isolation, NK cells were first enriched from leukopherosed blood (Memorial Blood Centers, Minneapolis, MN, USA) using magnetic negative selection (Miltenyi NK-cell Isolation Kit II, AutoMACS). The enriched NK-cell fraction was stained with anti-CD16 (FITC) and anti-CD56 (PE) to identify NK cells, together with anti-CD3 (PerCP) and HLA-DR (PerCP) for exclusion of T cells and contaminating antigen-presenting cells (APC), respectively. CD56dimCD16* and CD56brightCD16+ NK-cell sub-populations were each separated to >99% purity using the BD FASCARia flow cytometer. Alternatively, >99% pure total NK cell fractions were obtained by sorting cells expressing CD56, but not CD3 or HLA-DR. Purified NK cells were cultured with TLR agonists for 48 h in complete RPMI 1640 medium supplemented with 50 units ml⁻¹ recombinant human IL-2 (Peprotech, Rocky Hill, NJ, USA) followed by analysis of secreted IFN-γ. CD69 expression by purified NK cells was assessed at 48 h after stimulation. Fold increases in the proportion of CD69 expression by NK cells expressing CD56dimCD16* or CD56brightCD16+ NK cells were determined.

Cytokine analysis
IFN-α was quantified in culture supernatants using the Multi-Species IFN-α ELISA (PBL Biomedical) as described (51). IFN-γ was monitored by the IGEN method (IGEN International, Gaithersburg, MD, USA) using IFN-γ antibody pairs (clone 67F12A8 and MD-1) obtained from BioSource and recombinant human IFN-γ standard obtained from R&D Systems. The minimum level of IFN-γ detection by this method is 0.02 ng ml⁻¹. ELISA was used to measure IL-18 (MBL). The minimum level of IL-18 detection by this method is 0.02 ng ml⁻¹.

Flow cytometry
For analysis of CD69 expression by NK cells, PBMCs were stained with antibodies for CD56 (PE), CD3 (PerCP) and CD69 (FITC). The proportion of CD69-positive NK cells and the geometric mean fluorescence intensity (MFI) of CD69 were determined. Similarly, for analysis of CD11a, CD11b and CD54 (ICAM-1) expression, PBMCs were stained with antibodies to CD3 (PerCP) and CD56 (allophycocyanin) together with anti-CD11a, -CD11b or -CD54 (PE) followed by analysis of geometric MFI values. For characterization of intracellular cytokine production, brefeldin A (Sigma–Aldrich) was added into PBMC cultures at 10 μg ml⁻¹ during the last 5 h of TLR agonist stimulation. Data were collected using a FACSCalibur flow cytometer (BD) and analyzed using FlowJo6.3.1 (Treestar, Bar Harbor, ME, USA) followed by analysis of secreted IFN-γ. CD69 expression by purified NK cells was assessed at 48 h after stimulation. Fold increases in the proportion of CD69 expression by NK cells expressing CD56dimCD16* or CD56brightCD16+ NK cells were determined.

Analysis of TLR mRNA expression
Expression of TLR-2 to -9 was determined by quantitative reverse transcription (RT)–PCR. RNA was isolated using Qiagen RNA Easy Midi kits followed by ethanol precipitation and quantification using the NanoDrop® and Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA, USA). Random-primed cDNAs were prepared (Superscript II, Invitrogen, San Diego, CA, USA) from 0.1 to 0.5 μg of total RNA. Quantitative PCR was performed using an ABI 7900 (Applied Biosystems, Foster City, CA, USA) and the 384-well Microfluidic Card with Taqman chemistry. TLR-2 to -9 mRNAs were monitored with ABI pre-developed assay reagents. Cycling conditions were 50°C for 10 min, 95°C for 2 min followed by 35 cycles of 95°C for 30 s and 60°C for 1 min. Cycle threshold (Ct) values were converted into copy number relative to glyceraldehyde-3-phosphate dehydrogenase.

Analysis of NK-cell cytotoxicity by chromium release
Cytotoxicity was evaluated on ⁵¹chromium-labeled K562 or YAC cells (American Type Tissue Collection) as previously described (52–54). Effector to target (E:T) ratios of 20:1, 6.6:1, 2.2:1, 0.7:1, 0.24:1 and 0.08:1 were evaluated for K562 target cells. E:T ratios of 30:1, 10:1, 3:1 and 1:1 were evaluated for YAC target cells. Release of ⁵¹chromium into cell-free supernatants was determined and percent lysis calculated. Paired, one-sided Student’s t-tests were performed to determine statistical significance of K562 lysis.

Assessment of in vivo NK cell-mediated cytotoxicity
All studies involving laboratory animals were reviewed and approved by Institutional Animal Care and Use Committee. Methods for analysis of in vivo NK cytotoxicity were adapted from Oberg et al. (55). Splenocytes from C57BL/6 and TAP1 (Tap1tm1Arp)-deficient mice (Jackson Laboratories, Bar Harbor, ME, USA) were differentially labeled with 5,6-carboxyfluorescein diacetate succinimidyl ester (CFSE) (Molecular Probes, Eugene, OR, USA). Briefly, the spleens were dissociated, washed 1× with PBS (containing 0.1% BSA) and re-suspended at a concentration of 2 × 10⁶ cells ml⁻¹ in PBS/0.1% BSA. CFSE was added to 5 μM for C57BL/6 splenocytes or to 0.5 μM for TAP1-deficient splenocytes for 10 minutes at 37°C. After washing 1× with PBS, the splenocytes were counted and re-suspended in PBS at 2.5 × 10⁷ cells ml⁻¹. C57BL/6 and TAP1-deficient splenocytes were then mixed at a 1:1 ratio and 5 × 10⁶ total cells were injected into C57BL/6 recipient animals by tail vein injection. Recipient animals were treated as follows: Half the animals received 50 μg of anti-NK1.1 antibody (PK136, eBioscience) intra-peritoneally 1 day prior to CFSE-labeled splenocyte transfer, and the other half received PBS. Mice were treated with poly I:C, R-848 or PBS alone. Fifteen hours after treatment, the spleens of the recipient mice were harvested, dissociated and placed on ice. Analysis of CFSEdim and CFSEbright cells was performed in triplicate using the BD FASCARia HTS Option according to the manufacturer’s specifications. A separate set of mice that did not receive transfer of CFSE-labeled splenocytes were treated similarly with either poly I:C, R-848 or PBS alone. Twenty hours after treatment splenocytes were used for analysis of cytotoxicity against YAC target cells by chromium release.
Results

IFN-γ is preferentially stimulated in human PBMC by agonists of TLR-3 and TLR-8

Initially, the TLR-7 agonist 3M-001 and the TLR-8 agonist 3M-002 were compared for the ability to trigger IFN-γ in human PBMC cultures within 24 h (Fig. 1A). The TLR-8 agonist 3M-002 induced a dose-dependent increase in IFN-γ in PBMC from all six donors tested. TLR-7 (3M-001) agonist did not induce IFN-γ above the minimum level of detection (0.02 ng ml⁻¹) at the concentrations tested. Next, PBMCs were stimulated with either 3M-003 or the natural TLR-8 ligand ssRNA40. Both 3M-003 and ssRNA40 triggered dose-dependent secretion of IFN-γ demonstrating that synthetic and natural TLR-8 agonists behave similarly (Fig. 1B). IFN-γ elicited from PBMC by R-848 in separate experiments (mean ± SEM of 22 donors) is plotted for comparison. Data highlight the similarity between the closely related TLR-7/8 agonists, 3M-003 and R-848, which are shown to induce IFN-γ at similar concentrations and to similar levels.

Next, PBMCs were stimulated with the TLR-3 ligand, poly I:C or a panel of TLR-9 ligands including so-called A-type (ODN 2216), B-type (ISS 1018 or ODN 7909) and C-type (ISS C274) CpG ODN. Treatment of PBMC with the mixed TLR-7/8 agonist 3M-003 was included for comparison. IFN-γ was induced by poly I:C in a dose-dependent fashion but not by CpG ODN (supplemental Fig. 1A, available at International Immunology Online). Both poly I:C and CpG ODNs, however, did induce IFN-α in a dose-dependent fashion demonstrating that TLR-9 agonists are active in these cultures (supplemental Fig. 1B, available at International Immunology Online). As expected, the A-type CpG, ODN 2216, induced the highest peak level of IFN-α (19 ng ml⁻¹). The B-type CpG ODN, ISS 1018, also induced dose-dependent induction of IFN-α but with a lower peak response (2.9 ng ml⁻¹). Results with the other B-type CpG, ODN 7909, were similar to published reports showing marginal IFN-α induction and finally, the C-Type CpG ODN, ISS C274 induced higher peak IFN-α relative to B-type ODN (data not shown). Agonists of TLR-2, -4 and -5 were tested in separate experiments. Like CpG ODN, Pam3CSK, MALP-2, LPS and flagellin did not induce detectable IFN-γ from stimulated PBMC (data not shown). These results further demonstrate that a subset of TLR ligands, including poly I:C, ssRNA, 3M-002, 3M-003 and R-848, trigger high levels of IFN-γ in human PBMC cultures.

Imiquimod and R-848 are comparable to 3M-001 and 3M-002, respectively, for activation human NK cells within PBMC

A direct comparison of the ability of imiquimod and R-848 to activate human NK cells has not been reported to date. We, therefore, analyzed NK cells within PBMC cultures stimulated with either imiquimod or R-848 for comparison with the TLR-7 and TLR-8 selective analogues 3M-001 and 3M-002 (Fig. 2). TLR-3 and -9 agonists (poly I:C and ODN 2216) were included as controls. Co-expression of CD69 and IFN-γ by NK cells was induced by poly I:C (50 and 27% for donor 1 and 2, respectively), R-848 (34 and 8.6%) and 3M-002 (33.4 and 11.2%). In contrast, CD69 single-positive NK cells were primarily triggered by TLR-7 (imiquimod: 17 and 15%; 3M-001: 39 and 36%) and TLR-9 agonists (ODN 2216: 24 and 16%). While 3M-001 induced CD69 on NK cells at concentrations of 0.03 μM and above the minimum effective concentration for imiquimod was 3 μM (data not shown). Data are consistent with the previous description of 3M-001, a selective TLR-7 agonist, more pharmacologically potent (induction of higher type I IFN levels at lower concentrations) than imiquimod (28). NK cells were observed to be the major population of IFN-γ-producing cells in human PBMC cultures stimulated with TLR-8 ligands (supplemental Fig. 2, available at International Immunology Online). The TLR-7/8 agonist 3M-003 also induced IFN-γ and CD69 expression and was shown to induce cell adhesion molecules (CAMs) on NK cells in a dose-dependent fashion, including CD11a, CD11b and ICAM-1 (supplemental Fig. 3, available at International Immunology Online). Expression of IFN-γ by NK cells was also induced by the TLR-8 agonist ssRNA40. These results clearly show that

![Fig. 1. IFN-γ is preferentially induced in human PBMC by agonists of TLR-7 and TLR-8. (A) IFN-γ induction by synthetic TLR-7 and -8 agonists in human PBMC. 3M-001 and 3M-002 were tested at concentrations of 0.1, 0.3 or 1 μM. Box-whisker plots represent median (-), minimum (lower whisker) and maximum (upper whisker) IFN-γ levels induced in PBMC from six donors. First and third quartiles are represented by the lower and upper boundaries of the box plots. (B) Comparison of IFN-γ induction in PBMC by synthetic and natural TLR-8 ligands. PBMCs were treated with 3M-003 at concentrations of 0.03, 0.1, 0.3, 1, 3 and 10 μM or with ssRNA40 at concentrations of 0.02, 0.05, 0.14, 0.43, 1.28 and 3.85 μM (corresponding to 0.1, 0.3, 0.9, 2.7, 8.3 and 25 μg ml⁻¹ ssRNA40, respectively). Mean IFN-γ levels ± SEMs of three donors are shown. R-848 was tested in separate experiments and the mean IFN-γ levels ± SEMs of 22 donors are shown.](image-url)
ligands of TLR-3 and -8, and ligands of TLR-7 and -9 favor distinct modes of NK-cell activation.

**CD56<sup>dim</sup> and CD56<sup>bright</sup> NK cells do not express TLR-7 or TLR-8, or respond directly to IRMs**

Recent reports suggest that TLR ligands can directly activate human NK cells (15, 17, 56–58). We examined the direct activation of NK cells individually through TLR-7 and/or TLR-8. We first sorted CD56<sup>dim</sup> and CD56<sup>bright</sup> NK cells from normal healthy donors (Fig. 3A) and determined expression of TLR-2 to -9 by quantitative PCR (Fig. 3B). We found that NK cells express relatively high levels of TLR-2, -3, -5 and -6 but very little, if any, TLR-4, -7, -8 or -9. Quantitative differences in TLR expression between CD56<sup>dim</sup> and CD56<sup>bright</sup> NK-cell populations were identified. While CD56<sup>dim</sup> NK cells were found to preferentially express TLR-3, CD56<sup>bright</sup> NK cells were found to preferentially express TLR-2.

We also measured the response of purified NK cells (in bulk or CD56<sup>dim</sup>) to relevant TLR ligands in the presence of IL-2. NK cells were isolated to >99% purity by flow cytometric sorting. We routinely tested for contamination by monocytes, mDCs or pDCs, B cells, T cells, T-regulatory cells, CD<sup>T</sup> cells and NKT cells but did not observe these cells in our purified NK-cell preparations. Poly I:C directly stimulated IFN-γ in NK-cell cultures from all four donors, while LPS, 3M-001, 3M-002, 3M-003 and ODN 2216 did not elicit IFN-γ (Fig. 3C). Sufficient numbers of rare CD56<sup>bright</sup> NK cells were not obtained for functional testing. Therefore, activation of the CD56<sup>dim</sup> and CD56<sup>bright</sup> subsets within isolated total NK-cell cultures was monitored by flow cytometry using the activation marker CD69 (Fig. 3D). LPS, 3M-001, 3M-002, 3M-003, ssRNA40 and CpG ODN 2216 did not induce increased levels of CD69 on isolated NK cells. In contrast, poly I:C induced a 3-fold increase in CD69 expression preferentially on CD56<sup>dim</sup> NK cells, while the TLR-2/6 agonist MALP-2 induced a 2.5-fold increase in CD69 preferentially on CD56<sup>bright</sup> NK cells. MALP-2 also induced CD69 expression on CD56<sup>dim</sup> cells (1.8-fold). These studies further suggested the importance of indirect pathways for human NK-cell activation by TLR-7 and TLR-8 agonists.

**TLR-8 agonists indirectly trigger IFN-γ in human PBMC via IL-18**

Because cytokines such as IL-12 are known to have a role in the induction of IFN-γ, we next considered the role of indirect mediators in IFN-γ production triggered by TLR-8 agonists. PBMCs were pre-treated with neutralizing antibodies including anti-TNF-α, anti-IL-12p40/70, anti-IFN-γR, anti-IL-18 and anti-IL-10 before stimulation with 3M-001, ssRNA40 or poly I:C.

**Fig. 2.** Imiquimod and R-848 trigger comparable NK-cell activation to 3M-001 and 3M-002, respectively. PBMCs were treated for 25 h with TLR agonists with brefeldin A added to 10 μg ml<sup>-1</sup> for the final 5 h of culture. Specifically, PBMCs were treated with poly I:C (25 μg ml<sup>-1</sup>), imiquimod (3 μM), 3M-001 (3 μM), R-848 (3 μM), 3M-002 (3 μM) or CpG ODN 2216 (2.5 μg ml<sup>-1</sup> (0.4 μM)) and stained with antibodies for CD69 (FITC), CD56 (PE), CD3 (PerCP) and intracellular IFN-γ (allophycocyanin). Contour plots display CD69 and IFN-γ expression by NK cells.
Induction of IFN-γ by 3M-003 (TLR-7/8) and ssRNA40 (TLR-8) was most dramatically reduced in cultures treated with anti-IL-18 (Fig. 4A). Anti-18 reduced IFN-γ levels >100-fold (to 0.9% of control) for 3M-003 and >10-fold (to 7.5% of control) for ssRNA40, while an isotype matched (mIgG1) control antibody did not reduce IFN-γ levels in these cultures. Similar IL-18 dependence was observed for R-848 and the TLR-8 selective IRM, 3M-002 (data not shown). In contrast, poly I:C-induced IFN-γ was not affected by anti-IL-18, but was reduced ~3-fold by anti-IL-12.

Demonstration that IL-18 is required for IFN-γ induction by 3M-003 but not poly I:C suggested that IL-18 is selectively induced by TLR ligands. Therefore, IL-18 was next measured in the same PBMC supernatants. IL-18 was induced by 3M-002 (~100 pg ml⁻¹), 3M-003 (~125 pg ml⁻¹) and ssRNA40 (~250 pg ml⁻¹) but not by 3M-001 or poly I:C (Fig. 4B). The amount of IL-18 stimulated by ssRNA40 was at least 2-fold more than that observed for either 3M-002 or 3M-003, consistent with the relatively higher levels of IFN-γ induced by ssRNA in these experiments.

To further confirm the predominant role of IL-18, we analyzed intracellular expression of IFN-γ by NK cells in PBMC cultures treated with control or IL-18-neutralizing antibodies (Fig. 4C). Again blocking IL-18 had a pronounced affect on NK cell IFN-γ production in response to 3M-003 (97% inhibition) and ssRNA40 (78% inhibition).

Although TLR-8 agonist stimulation in the presence of anti-IL-18 resulted in >10-fold reduced levels of IFN-γ, residual IFN-γ was observed (3M-003, <0.1 ng ml⁻¹; ssRNA40, 0.1–1 ng ml⁻¹). Additional neutralizing antibodies were paired with anti-IL-18 for induction of IFN-γ by TLR-8 agonists. Anti-IL-18 was used to determine if bioactive IL-12p70 is an important co-factor for IFN-γ induction by TLR-8 agonists. 3M-002 and ssRNA40 were used to stimulate PBMC. IFN-γ levels were reduced in the presence of anti-IL-18 to 57% of control (3M-002) and 52% of control (ssRNA40) and when anti-IL-12p70 was paired with anti-IL-18 induction of IFN-γ by TLR-8 agonists was not observed (Fig. 4D). Paired neutralization of IL-18 together with anti-IFN-γR or IFN-γR did not affect residual levels of IFN-γ (data not shown). These results indicate that the induction of IFN-γ by TLR-8 agonists results from indirect NK-cell activation by IL-18 and IL-12p70 with potential contributions from other cytokines as well.

We also investigated cytokine mediators responsible for induction of CD69 expression on NK cells. Pre-treatment with antibody against the IFN-γR reduced induction of CD69 by
the TLR-7 agonist 3M-001 by >80% (supplemental Fig. 4, available at International Immunology Online). For the TLR-8 agonist 3M-002 optimal induction of CD69 on NK cells was inhibited by both type I IFNR-blocking antibody (31% inhibition) and by anti-IL-18 (67% inhibition) (supplemental Fig. 5A, available at International Immunology Online). These results confirm that TLR-7 and TLR-8 agonists activate NK cells in PBMC through distinct indirect pathways.

TLR-7 and -8 agonists enhance cytotoxicity against MHC class I-negative cells

NK cells limit viral infections and outgrowth of tumors by direct cellular cytotoxicity. Therefore, TLR agonists were compared for their ability to prime enhanced lysis of the NK-cell sensitive leukemia cell line K562. Following overnight stimulation with IL-2, poly I:C, 3M-001, 3M-002 or 3M-003, relative percent lysis of K562 cells by PBMC effectors was measured in chromium release assays. Data from four donors are shown (Table 1). Significant increases in K562 cytosis (\(P < 0.05\)) were observed for IL-2, 3M-001, 3M-002 and 3M-003 at E:T ratios of 20:1, 6:1:1 and 2.2:1. Comparison of mean percent lysis values indicates that enhancement of cytotoxicity by 3M-002 and 3M-003 [25 and 28% over background (E:T = 20:1), respectively] matched even high-dose IL-2 [18% over background (E:T = 20:1)]. Poly I:C and the TLR-7 agonist 3M-001 also induced elevated K562 lysis [11 and
Percent lysis (K562) was monitored by 51chromium release as described previously. E:T ratios below 2.2:1 were tested as well but did not result in comparison. For each donor, PBMC effectors (E) were plated at the indicated ratio with a fixed number of NK-cell sensitive, K562 cells (T).

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<th>Donor number</th>
<th>E:T ratio</th>
<th>Percent lysis K562</th>
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<td>1</td>
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</tr>
<tr>
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<td>12 (±2)</td>
</tr>
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TLR-7 and -8 agonists differentially activate NK cells

Table 1. TLR-7 and -8 agonists elevate cytolyis of K562 cells.

14% over background (E:T = 20:1, respectively] but to a lesser degree than the TLR-8 agonist 3M-002 or the TLR-7/8 agonist 3M-003, both of which induced 2-fold increased K562 lysis values at E:T ratios of 20.1 and 6.6:1. These results suggest that TLR ligands differentially activate human NK cells leading to distinct patterns of CD69 and IFN-γ expression, and ultimately, different levels of tumor cytotoxicity.

**Discussion**

While the ‘missing-self hypothesis’ laid the groundwork for understanding NK-cell immunosurveillance, we now know that NK cells also recognize bacterial and viral pathogen-associated molecular patterns (PAMPs) through either NKRs or TLRs. Our data support the following model for activation of human NK cells by TLR agonists (supplemental Fig. 7, available at International Immunology Online). NK cells express TLR-2, -3, -5 and -6 but relatively little TLR-4, -7, -8 or -9. In addition, like DC subsets, NK-cell subsets are differentially activated based on expression of TLRs. CD56bright NK cells preferentially express TLR-2 and respond to the TLR-2 ligand MALP-2 by increasing the expression of CD69, while CD56dim NK cells preferentially express TLR-3 and respond to the TLR-3 ligand poly I:C increasing expression of CD69 and by producing IFN-γ. While TLR-2/6, -3 and -5 agonists directly activate NK cells (15, 17, 56, 59), TLR-4, -7, -8 and -9 agonists act indirectly on NK cells. Overall, the data suggest that NK cells have the potential to see a subset of bacterial and viral PAMPs directly, while APCs [either myeloid APC (mAPC) or pDC] generally respond to a larger set of TLR agonists and instruct distinct modes of indirect NK-cell activation accordingly.

A direct comparison of NK-cell activation by 3M-001-3, imiquimod and R-848 has not been reported previously; however, the ability of these molecules to act on TLR-7 and/or TLR-8 has been reported in separate publications (21, 25, 28, 51). Briefly, 3M-001 and imiquimod have selectivity for human TLR-7, while 3M-003 and R-848 act through both TLR-7 and TLR-8 and are therefore designated as mixed TLR-7/8 agonists. Finally, 3M-002 and ssRNA40 are both TLR-8 selective ligands (28, 31). We used these selective TLR-7 and TLR-8 ligands to identify the contributions of individual TLRs to activation of human NK cells.

In a recent publication, Hart et al. (57) suggest both direct and indirect NK-cell activation by the mixed TLR-7/8 agonist R-848. In general, our data are consistent with this publication;
however, we did not observe evidence of direct activation when pure NK cells cultured with IL-2 (Fig. 3C) or recombinant IFN-α (K. S. Gorski, unpublished results) were treated with TLR-7, TLR-8 or TLR-7/8 agonists. The ability of R-848 and 3M-002 to stimulate IL-12 production by mAPC including monocytes has been published previously (28, 47). We extend on these findings by identifying a role for type I IFN in NK-cell activation by TLR-7 agonists and by clarifying the individual roles of TLR-7 versus TLR-8 for induction of IL-18, IFN-γ and NK-cell cytolytic function.

We found that TLR-8 agonists including ssRNA trigger IFN-γ production in NK cells through an IL-18-dependent mechanism. We showed that IL-18 is induced by 3M-002, 3M-003 and ssRNA40 but not poly I:C or the TLR-7 agonist 3M-001 and that IL-18 cooperates with IL-12p70 for stimulation of IFN-γ production by NK cells. IL-18 was also required for IFN-γ induction by R-848 tested in separate experiments (K. S. Gorski, unpublished results). T cells lacking expression of TCRαβ were also found producing IFN-γ in response to TLR-8 agonists (supplemental Fig. 2, available at International Immunology Online). The effects of TLR-8 agonist stimulation on this population of cells which includes γδ T cells is currently under investigation. By depleting individual cell subsets within PBMC, monocytes were identified as a primary source of IL-18 stimulated by TLR-8 agonists (J. P. Vasilakos and K. S. Gorski unpublished results). The ability of recombinant IL-12 and IL-18 to induce IFN-γ has been well characterized (12, 60).

CD4 T-cell contributions to TLR-7/8 agonist stimulated IFN-γ in 96-h PBMC cultures have been described previously (47). The early IFN-γ provided by NK cells stimulated with IRMs such as 3M-002 and 3M-003 may facilitate generation of Th1 CD4 T cells as described for resiquimod when used as an adjuvant in vivo (3). TLR-3, -7 and -8 agonists were all found to enhance NK-cell cytolytic function. Similar enhancement of K562 cytosis has been reported for CpG ODN (48, 61, 62). TLR-8 agonists 3M-002 and 3M-003 stimulated the highest level of K562 cytotoxicity, surpassing the maximum response stimulated by 3M-001, poly I:C or treatment with recombinant IL-2. In general, IFN-α, IL-12 and IL-18 have all been shown to stimulate tumor killing by NK cells (9, 12, 60). One possible explanation for the potency of 3M-002 and 3M-003 for enhancement of K562 cytotoxicity is their ability to induce the broadest profile of NK-cell activation including induction of CD69, CAMs and IFN-γ.

NK-cell activation by TLR-7 and TLR-9 agonists correlates strongly with type I IFN production by pDC. Accordingly, 3M-001 was shown to induce type I IFN-dependent expression of CD69 on NK cells. Activation of NK cells by recombinant type I IFN (9, 63) or inducers of type I IFN such as CpG ODN have been described previously (14). While IL-18 was primarily responsible for CD69 induction by the TLR-8 agonist 3M-002, we were surprised to find that induction of CD69 was also partially type I IFN dependent (supplemental Fig. 5A, available at International Immunology Online). While pDC do not express
TLR-8, the TLR-8 selective agonists ssRNA40 (31) and 3M-002 (28) both require pDC for induction of type I IFN in PBMC. While IL-18 appears to be largely responsible for TLR-8 agonist-induced NK-cell cytotoxicity, blockade of type I IFN did reduce lysis of K562 cells (supplemental Fig. 5B, available at International Immunology Online). How TLR-8 agonists stimulate pDC-dependent IFN-α is currently under investigation.

The ability of IRMs to enhance NK-cell cytolytic function in vivo has not been previously reported. Differences in TLR-7 and -8 ligand recognition between mouse and man precluded extension of our comparison in vivo. Briefly, an inter-species comparison was performed demonstrating that human small molecule TLR-8 agonists trigger NFκB activation through mouse TLR-7 (supplemental Fig. 6, available at International Immunology Online). In mice, the TLR-7/8 agonist R-848 has been shown to act through TLR-7 (25). Studies with ssRNA (31) have also suggested the possibility that mouse TLR-7 can recognize selective ligands for human TLR-8. In tests with human TLRs, 3M-001 triggered NFκB activation through TLR-7, 3M-002 triggered activation through TLR-8 and 3M-003 triggered activation through both TLRs as reported previously (supplemental Fig. 6A, available at International Immunology Online). In contrast, 3M-001, 3M-002 and 3M-003 triggered NFκB activation through mouse TLR-7 but not mouse TLR-8 (supplemental Fig. 6B, available at International Immunology Online). Therefore, differences between TLR-7 and TLR-8 selective small molecule IRMs could not be addressed in vivo, and NK-cell activation by R-848 is representative of ligands acting through mouse TLR-7. Recently, R-848 was found to stimulate a DC–NK cell interaction in vivo leading to type I IFN dependent, IFN-γ production (64). We found that R-848 failed to prime cytotoxicity in IFN-α/βR-deficient mice, but impaired natural cytotoxicity was also observed suggesting a more general NK defect.

While our model of human NK-cell activation by distinct TLR ligands is generally consistent with published findings, our data differ from several published reports. The profile of TLRs that we observed in NK cells is consistent with the data from investigators that also used quantitative RT-PCR (14, 15). In contrast, semi-quantitative RT-PCR data from Chalifour et al. (17) suggest that NK cells express TLR-1 to -8 and that TLRs are not differentially expressed between human NK-cell subsets and similar data from Sivori et al. (16) suggest that NK cells express TLR-9. In contrast, we did not observe expression of TLR-4, -7, -8 or -9 in either CD56dim or CD56bright NK cells and a direct response to IRMs or CpG ODNs was not observed. Previously, CpG sequences have been characterized as inducers of IFN-γ from human PBMC (48, 49). In contrast, the studies described herein do not show induction of IFN-γ regardless of the type of CpG used. These ODNs were clearly active, as they all induced IFN-α in the same cultures lacking IFN-γ (supplemental Fig. 1A and B, available at International Immunology Online). Differences in culture conditions used may also help to explain the differences between our studies and previous reports.

Topical treatment with 5% imiquimod cream (Aldara™) has been shown to be an effective treatment for external genital warts, basal cell carcinoma (BCC) and actinic keratosis. Because NK cells are important for control of viral infections and cancers, it is intriguing to consider the possibility that NK cells play a role in the mechanism of action for TLR-7 agonists in these diseases. Indeed, results demonstrate an increase in NK-cell infiltration into BCC treated with Aldara™ (65). Furthermore, increased IFN-γ mRNA was seen in warts treated with Aldara™ and these increases correlated with clearance (66). Finally, actinic keratosis lesions treated with Aldara™ analyzed by genomic array strongly suggest an increase in NK-cell activity in these lesions (W. Birmachu, in preparation).

Given the ability of TLR-7 and TLR-8 agonists to activate human NK cells through distinct pathways, different IRMs may be better suited for particular disease applications. Clinical evaluation of the ability of IRMs to activate NK cells in cancer patients is currently under investigation. Patients with refractory solid organ tumors have received systemic administration of an IRM, TLR-7 agonist in a phase I study. Results from the study demonstrate heightened NK-cell activity ex vivo (Dudek, A. Z., Yunis, C., Kumar, S., Harrison, L. I., Hawkinson, R. W. and Miller, J. S. 2005. Immune response activation by a Toll-like receptor 7 agonist: results of a phase 1 study. American Society of Clinical Oncology (ASCO) Annual Meeting, Orlando, FL.). The success of imiquimod (Aldara™) in the treatment of genital warts, basal cell carcinoma and actinic keratosis highlights the potential of IRMs as TLR-based therapeutics for cancer and chronic viral infections where NK cells may have an impact.

In summary, TLR-7 and TLR-8 ligands promote distinct pathways leading to activation of human NK cells. While TLR-2 and TLR-3 agonists appear to directly activate NK cells, TLR-8 agonists utilize mAPC-derived IL-18 and IL-12 to indirectly drive NK-cell activation, including induction of IFN-γ, high levels of CD69 and tumor killing. In contrast, TLR-7 and TLR-9 agonists do not induce high levels of IFN-γ production by NK cells, but do induce both CD69 expression and tumor lysis. Thus, TLR agonists can access NK-cell pathways either directly (TLR-2 and -3) or indirectly (TLR-7, -8 and -9). The consequences of these NK-cell activation pathways on viral infections and cancer in vivo remain to be determined.

Supplementary data
Supplemental Figures 1–7 are available at International Immunology Online.

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Abbreviations
APC antigen-presenting cell
CAM cell adhesion molecule
CFSE 5,6-carboxyfluorescein diacetate succinimidyl ester
DC dendritic cell
E:T effector to target
IRM immune response modifier
ISS immunostimulatory sequence
mAPC myeloid antigen-presenting cell
mDC myeloid dendritic cell
References


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