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Activation of cytokine-producing and antitumor activities of natural killer cells and macrophages by engagement of Toll-like and NOD-like receptors

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Abstract

Macrophages and natural killer (NK) cells are important antitumor effectors by virtue of their ability to produce cytokines, chemokines and interferons (IFNs) and to mediate tumor cytotoxicity. Little is known about the impact of Toll-like receptor (TLR) and nucleotide binding and oligomerization domain (NOD)-like receptor (NLR) pathways on NK cell functions, and the role of TLRs and NLRs in macrophage activation is incompletely understood. In this study, we examined the capacities of expressed TLRs and NLRs to elicit cytokine production in human NK cells and THP1 macrophages, and to activate NK cytotoxicity against the squamous cell carcinoma of head and neck cell line Tu167 and erythroleukemia K562 cells. We found that NK cells express high levels of NOD2, NLRP3, TLR3, TLR7, and TLR9, while NOD1 was expressed at low levels. All tested NLR and TLR agonists potentiated NK cytotoxicity against Tu167 cells, whereas only poly (I:C) increased NK cytotoxicity against K562 cells. Poly (I:C) and *Escherichia coli* RNA markedly up-regulated TNF-α and IFN-γ expression in the NK92 cell line and human $CD56^{+}CD3^{-}$ primary NK cells. High levels of NOD2, TLR7 and TLR9 proteins were observed in human THP1 cells, followed by TLR3, NOD1, and NLRP3. Stimulation of NLRP3 with *E. coli* RNA led to the highest induction of TNF-α, IL-6, IL-12p40, RANTES and IFN-β, whereas TLR7, TLR3, TLR9, NOD1 and NOD2 agonists had lower effects. Our data reveal involvement of TLRs and NLRs in potentiation of antitumor cytotoxicity and cytokine-producing activities of human NK cells and macrophages.

Keywords

Innate immunity, Toll-like receptors, signal transduction, cell activation, NK cells/macrophages

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Introduction

Natural killer (NK) cells and macrophages are innate cells that mediate first-line defense against microbial infections and play a key role in antitumor immune responses. Macrophages and NK cells secrete a number of cytokines, chemokines and interferons (IFNs), influencing activities of each other, regulating functions of dendritic cells (DCs), and promoting adaptive antitumor immune responses.[1–4] Macrophages, DCs and NK cells can recognize tumor cells and cause tumoristatic and tumoricidal effects by engagement of lectin receptors (*e.g.* macrophage galactose/*N*-acetyl-galactosamine-specific C-type lectin), Fas-ligand and a subset of activating receptors (*e.g.* NKG2D).[2,5–7] Elevated activity of macrophages and NK cells and/or their increased infiltration in the tumor micro-environment have been shown to correlate with prolonged survival of tumor-bearing mice and

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cancer patients.[8–12] Conversely, suppression of functional activities of NK cells and macrophages, or their depletion leads to enhanced tumor growth in experimental tumor animal models.[12–14] Furthermore, macrophage- and NK cell-deficient mice show deficiencies in antitumor immune responses, and cancer patients with impaired macrophage or NK cell functions exhibit increased mortality rates.[12] Many tumors, including squamous cell carcinoma of the head and neck (SCCHN), the sixth common cancer in the world,[15] develop multiple mechanisms of escape from immune surveillance. These include down-regulated expression of HLA class I molecules, antigenic adaptation with selection of low-immunogenic tumor-associated antigens, production of immunosuppressive molecules, and induction of immune tolerance and suppression.[15–22] These result in limited efficacy of currently available therapeutic regimens (such as surgery, radiation, and chemotherapy) and dictate an urgent need for the development of new therapeutic approaches aimed at activation of antitumor immunity of immune cells, including NK cells and macrophages.

Toll-like receptors (TLRs) and nucleotide binding and oligomerization domain (NOD)-like receptors (NLRs) sense pathogen-associated molecular patterns or endogenous 'danger' molecules released from host cells upon damage inflicted by infection, inflammation or stress.[23–25] The TLRs are membrane-associated sensors that are expressed on the cell surface (TLR1, TLR2, TLR4–6, TLR11) or in intracellular endosomes (TLR3, TLR7–9), whereas NLRs recognize their ligands in the cytoplasm.[26] Toll-like receptor2 in combination with TLR1 or TLR6 senses lipopeptides expressed by Gram-positive bacteria, mycobacteria and fungi, TLR3 recognizes viral dsRNA, while TLR4 is the main sensor of Gram-negative bacterial lipopolysaccharide (LPS). Bacterial flagellin is recognized by TLR5 expressed on the cell surface, endosomal TLR7/8 sense viral ssRNA, TLR9 reacts with hypomethylated CpG motifs present in bacterial, viral and fungal DNA, whereas TLR11 is the predominant sensor of uropathogenic *Escherichia coli* and *Toxoplasma* profilin-like molecules.[23,24,26] Amongst NLRs, NOD1 and NOD2 recognize products of bacterial peptidoglycan, *meso*-diaminopimelic (ie-DAP) and muramyl dipeptide (MDP), NLRC4 is the cytoplasmic sensor of bacterial flagellin, and NLRP3 senses *E. coli* RNA, alum adjuvants, silica and uric acid crystals.[25,27–31] Both TLRs and NLRs share the ability to activate the MAP kinase cascades and the transcription factor nuclear factor (NF)-κB, while having unique capacities to activate caspases via engagement of inflammasomes (a subset of NLRs, including cryopyrin/NLRP3) or induction of type I IFNs (TLR3, TLR4, TLR7-9).[23–25] The TLRs and NLRs are expressed in various cell types, including macrophages, neutrophils, epithelial and endothelial cells, dendritic cells,[23–25] and in malignant tumors, including melanoma and SCCHN.[32–34] Engagement of TLR3 and TLR4 has been shown to cause apoptosis of human melanoma and SCCHN cells,[33,35] poly (I:C) (TLR3) and R837 (TLR7) have been used for potentiation of antitumor responses,[36,37] and TLRs expressed on tumor cells have been proposed as targets for antitumor therapy.[38] Since engagement of TLRs/NLRs leads to activation of innate immune cells, it is plausible that stimulation of these receptors has the potential to reactivate tumor-infiltrating macrophages and NK cells that exhibit suppression of effector functions.

Previous reports demonstrated expression of various TLRs and NLRs in human macrophages;[24] however, controversy exists in the literature with regard to regulation of their relative expression levels and functional activities. For instance, two groups reported IFN-γ and GM-CSF mediated up-regulation of TLR2 and TLR4 expression in human monocytes,[39,40] whereas others observed no differences in expression levels of TLR4 before and after treatment with these cytokines.[41] Whereas NK cells have been shown to express functional TLR3 and TLR7,[42,43] no information is available with respect to expression levels and functions of NLRs in these cells. The purpose of the present study was to examine relative expression of TLRs and NLRs in human THP1 macrophages and primary $CD56^+$ NK cells, and to elucidate whether engagement of expressed pattern recognition receptors would stimulate their cytokine-producing and antitumor activities. We demonstrate that engagement of TLR/NLR with their defined agonists activates cytokine expression in human THP1 macrophages and NK cells, and potentiates NK cell cytotoxicity against SCCHN tumor cells.

Materials and methods

Reagents and cell culture

Antibodies against IκB-α, β-actin, donkey anti-goat IgG-Texas Red, and donkey anti-rabbit IgG-FITC were from Santa Cruz Biotechnology (Santa Cruz, CA, USA), anti-phospho (p)-p38 and total p38 were from Promega (Madison, WI, USA), and anti-HLA A, B, C Ab was from BD Biosciences Pharmigen (San Diego, CA, USA). Anti-caspase-1, anti-IL-1β and anti-NLRP3 Abs were obtained from Abcam (Cambridge, MA, USA), anti-NOD1 and anti-NOD2 were from Cell Signaling (Danvers, MA, USA) and Merk KGaA (Darmstadt, Germany), respectively. Antibodies against TLR3, TLR7 and TLR9 were from Imgenex (San Diego, CA, USA). Ultrapure LPS from *E. coli* O111:B4, ie-meso-DAP, MDP, loxoribin, poly (I:C), oligodeoxynucleotide (ODN) 2216 and ODN 2395 were obtained from Invivogen (San Diego, CA, USA). Phorbol myristate acetate was from Sigma, recombinant interleukin (IL)-2 was provided by the NIH Cytokine Repository (NCI, NIH), and *E. coli* RNA was purchased from Applied

BioSystems (Foster City, CA, USA). The human Tu167 head and neck tumor cell line was kindly provided by Dr G. Clayman (MD Anderson Cancer Center, Houston, TX, USA), the human K562 chronic myelogenous leukemia, the human NK 92 cell line and human monocytic cell line THP1 were obtained from the ATCC (Manassas, VA, USA). All cells were cultured in RPMI 1640 medium (Mediatech, Inc.) supplemented with 2 mM L-glutamine, 100 μg/ml penicillin/streptomycin (Invitrogen), 5×10^{-5} M β-mercaptoethanol and 5% fetal bovine serum (Sigma). Human monocytes were isolated from whole blood by counter flow centrifugal elutriation from peripheral blood mononuclear cells (PBMCs) that were obtained by leukapheresis of blood from healthy human volunteers at the Department of Transfusion Medicine, NIH, and were cultured as described above for THP1 cells. All studies were approved by the Institutional Review Board, University of Maryland, Baltimore.

Isolation of RNA and real-time PCR

Total RNA was isolated with RNeasy kits (Invitrogen), followed by DNase digestion and re-purification as recommended by the manufacturer. cDNA was prepared from 1 μg RNA using Reverse Transcription System (Promega), and subjected to quantitative real-time PCR analysis with gene-specific primers for human hypoxanthine-guanine phosphoribosyltransferase (HPRT), IL-8, IL-6, tumor necrosis factor (TNF)-α, IL-1β, IFN-β, IL-12p40 and IFN-γ on a MyIQ real-time PCR system (Bio-Rad). The following primers were used: HPRT, forward: 5′-ACCAGTC AACAGGGGACATAAAAG-3′, reverse: 5′-GTCT GCATTGTTTTGCCAGTGTC-3′; TNF-α, forward: 5′-CCCAGGCAGTCAGATCATCTTC-3′, reverse: 5′-GCTTGAGGGTTTGCTACAACATG-3′; IL-6, forward: 5′-GAAAGCAGCAAAGAGGCACTG-3′, reverse: 5′-GAAGCATCCATCTTTTTCAGCC-3′; IL-12p40, forward: 5′-TCATCAGGGACA TCATC AAACCT-3′, reverse: 5′-CCTCCACCTGCCGAG AATT-3′; IL-8, forward: 5′-CACCGGAAGGAACC ATCTCACT-3′, reverse: 5′-TGCACCTTCACACA GAGCTGC-3′; IFN-β, forward: 5′-ACTGCCT CAAGGACAGGATG-3′, reverse: 5′-AGCCAGGA GGTTCTCAACAA-3′; IL-1β, forward: 5′-AAAT GATGGCTTATTACAGTGGCA-3′, reverse: 5′-CTT CATCTGTTT AGGGCCATCAG-3′; IFN-γ, forward: 5′-ACGAGATGACTTCGAAAAGCTGA-3′, reverse: 5′-CATGTATTGCTTTGCGTTGGAC-3′; TLR3, forward: 5′-AGCCTTCAACGACTGATGCT-3′, reverse: 5′-GTTTCCAGAGCCGTGCTAAG; TLR7, forward: 5′-CCTTGAGGCCAACAACATCT, reverse: 5′-GTA GGGACGGCTGTGACATT-3′; TLR9, forward: 5′-AAGCTGGACCTCTACCACGA-3′, reverse, 5′-TTGGCTGTGGATGTTGTTGT-3′; NOD1, forward: 5′-TGACTACTTCTCGGCCGAAG-3′, reverse: 5′-GGTCAGTGTTGACCACGACT-3′; NOD2, forward: 5′-CAACATTGGCAGTGTGGGTG-3′, reverse: 5′-CAGACTTCCAGGATGGTGTC-3′; NLRP3, forward: 5′-CCCCGTAATCAACGGGACAA-3′, reverse: 5′-TCTCCTGTTGGCTCGATCCA-3′. The mRNA levels of the genes of interest were normalized to HPRT gene, and relative gene expression levels were determined using the $2^{-\Delta\Delta CT}$ method,[44] where CT is the cycle threshold. Melting curve analysis and sequencing of purified PCR products were employed to ensure the specificity of PCR amplification.

Preparation of cell extracts and immunoblotting

Cell extracts were prepared using a lysis buffer containing 20 mM HEPES (pH 7.4), 0.5% Triton X-100, 150 mM NaCl, 12.5 mM α-glycerophosphate, 50 mM NaF, 1 mM DTT, 1 mM sodium orthovanadate, 2 mM EDTA, 1 mM PMSF, and protease inhibitor cocktail (Roche). Protein concentration of cell extracts was determined by the Bradford protein reagent (Bio-Rad), using BSA as a standard. Equal amounts of cell extracts were resuspended in Laemmli loading buffer (BioRad), boiled and subjected to SDS-polyacrylamide gel electrophoresis to separate proteins on 4–20% polyacrylamide minigels (Invitrogen). Proteins were electrotransferred to Immobilon-P membranes (Millipore), membranes were blocked with Tris-buffered solution (TBS) with 0.1% Tween 20 (TBS-T), containing 5% non-fat dry milk (Bio-Rad), and probed for 20 h at 4°C with the corresponding primary Abs. After washing 5 times in TBS-T, membranes were incubated with the corresponding anti-rabbit, anti-mouse, or anti-goat secondary IgG-HRP conjugates diluted at 1:12,000 in TBS-T/5% milk, washed three times, and bands were revealed by incubation with enhanced chemiluminescence reagents (ECL+, Amersham) followed by exposure to X-ray films. Densitometric analysis of intensities of the p-p38, IκB-α, caspase-1 p20, IL-1β p17, and β-actin protein bands from three independent experiments was performed using the Quantity One software (Bio-Rad). Levels of p-p38, IκB-α, caspase-1 p20, IL-1β p17 and were normalized to those of β-actin, and data were expressed as arbitrary units.

Isolation of human NK cells

Human $CD56^+$ NK cells were isolated from donor buffy coats (Biospecialty Inc, Colmar, PA, USA) by MACS-based negative selection, using the human NK cell isolation kit (Miltenyibiotech) to deplete T- and B-cells, DCs, monocytes, granulocytes and erythrocytes, according to the manufacturer's instructions. In brief, blood was incubated with biotin-conjugate Abs against monocytes, DCs, B- and T-lymphocytes, granulocytes and erythrocytes and the NK cell Microbead cocktail. Non-NK cells were depleted by

magnetic separation on LS columns using MACS separator, and NK cells were analyzed by FACS with PERPC-CD3 (T-cells), APC-CD56 (NK cells) and PE-CD19 (B-cells) Abs. As assessed by FACS, this method consistently yielded isolation of $CD56^+CD3^-$ NK cells with the purity exceeding 97%.

FACS analysis

THP1 and $CD56^+$ NK cells were pre-incubated with 20% FBS to block Fc receptors, washed and permebialized, using the CytoFix-CytoPerm kit (BD Biosciences). Cells were stained for 30 min on ice with commercial Abs (Imgenex, Abcam) against TLRs and NLRs, isotype control Abs (negative control) or anti-HLA Ab (used as a positive control) at the concentrations recommended by the manufacturers. Negative controls included cells treated without Abs (to adjust for autofluorescence) or with secondary Abs only. After washing three times with ice-cold PBS, cells were incubated for 30 min on ice with the corresponding secondary IgG-FITC conjugates (anti-mouse or anti-rabbit), washed extensively with ice-cold PBS, and fixed in PBS containing 2% paraformaldehyde. FACS analyses were performed on a LSRII flow cytometer (BD Biosciences). Image acquisition was carried out using FACSDiva software (Beckton Dickinson, Franklin Lakes, NJ, USA), and image analyses was performed using FlowJo software (FlowJo Inc., Ashland, OR, USA).

Natural killer cell activation and cytotoxicity

Purified $CD56^+CD3^-$ NK cells were incubated for 24 h at 37°C in an atmosphere of 5% CO_2 with medium and TLR/NLR agonists, as described in the figure captions, washed three times and resuspended in complete medium. The cytotoxicity of NK cells were measured by standard ^{51}Cr-release assays, as described previously.[45,46] Briefly, target cells (2×10^6 in 0.3 ml of complete media) were incubated for 90 min at 37°C in 5% CO_2 with 150 μCi of ^{51}Cr (GE Healthcare, Piscataway, NJ, USA). The labeled cells were then washed and resuspended in complete medium at a concentration of 5×10^4 cells/ml. Serial dilutions of effector cells (100 μl/well) were added into each well of 96-well V-bottomed plates (Corning, NY, USA). Aliquots of ^{51}Cr-labeled target cells (100 μl/well) were dispensed into wells containing effector cells, cultures were incubated for 4 h at 37°C in an atmosphere of 5% CO_2, the plates were centrifuged again at 400 *g* for 5 min and 100 μl aliquots of the supernatants from each well were transferred to a new plate containing 100 μl/well of Optiphase Supermix scintillation fluid (Perkin Elmer, Boston, MA, USA). The radioactivity was measured using a 1450 Microbeta counter (Wallac, Turku, Finland). The percentage of specific cytotoxicity was calculated as (experimental release – spontaneous release)/(maximum release – spontaneous release) × 100. Spontaneous release was determined by incubating the targets with 100 μl of complete medium, maximum release was determined by incubating the target cells with 100 μl of 0.5% Triton-X100.

Determination of cytokine production by ELISA

Levels of cytokines and chemokines in cell-free supernatants were determined by ELISA in the University of Maryland, Baltimore Cytokine Core Laboratory using commercially paired antibodies and recombinant standards obtained from R&D (TNF-α, IFN-γ, RANTES and IL-12p40), and Pierce Biotech (IL-6). The lower detection limits for these assays (pg/ml) were 3.9, 6.25, 15.6, 3.9, and 31.25 for TNF-α, IL-6, IFN-γ, RANTES, and IL-12 p40, respectively.

Statistical analysis

Statistical analysis was performed using the GraphPad Prism v5 program for Windows (GraphPad Software, San Diego, CA, USA). Statistical differences among experimental groups were evaluated by a one-way ANOVA with repeated measures, followed by post hoc comparisons with Tukey's multiple paired comparison test. Values are expressed as mean ± SD.

Results

Expression of TLRs and NLRs in the human NK92 cell line and human $CD56^+CD3^-$ NK cells

First, we examined expression levels of NLRs and TLRs in human NK cells, using real-time PCR and FACS analyses. As sources of NK cells, we used the IL-2-dependent NK92 human cell line and primary human NK cells isolated from healthy donors by MACS-based negative selection to deplete T- and B-lymphocytes, dendritic cells, monocytes, granulocytes and erythrocytes. As determined by FACS, this NK cell purification yielded ~98% pure population of $CD56^+CD3^-$, $CD19^-CD56^+$ cells, corresponding to the reported NK phenotype2 (Fig. 1). Real-time PCR analysis revealed that NK cells express the highest amounts of mRNA encoding TLR3 (TLR3 mRNA expression normalized to HPRT mRNA levels as determined by $2^{-\Delta\Delta CT}$ method[44] is 0.58 ± 0.15), followed by NOD2 and TLR9 mRNA (Fig. 2A). NLRP3 and TLR7 mRNAs were expressed to a lower extent, while NOD1 gene was expressed at the minimal levels (0.0121 ± 0.004; Fig. 2A). We then used intracellular FACS to examine protein expression of pattern recognition receptors in permeabilized NK cells with Abs against NOD1, NOD2, NLRP3, TLR3, TLR7 and TLR9, isotype control Ig (negative control) or anti-HLA ABC Ab (positive control). Natural killer cells were found

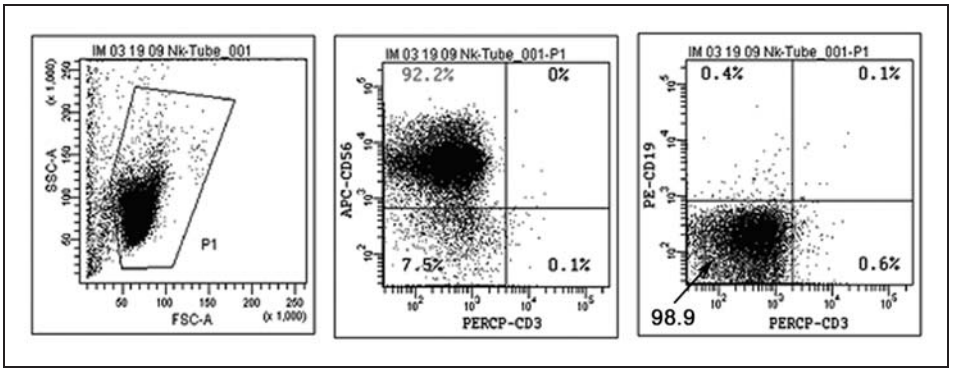

Figure 1. Isolation of human NK cells. Peripheral blood mononuclear cells were incubated with biotin-conjugate Abs against monocytes, DCs, B- and T-lymphocytes, granulocytes and erythrocytes and the NK cell Microbead cocktail. Non-NK cells were depleted by magnetic separation on LS columns, using MACS separator. Natural killer cells were analyzed by FACS with PerCP-CD3 (T cells), APC-CD56 (NK cells) and PE-CD19 (B cells) Abs. Shown are results of a typical NK cell isolation ($n = 8$).

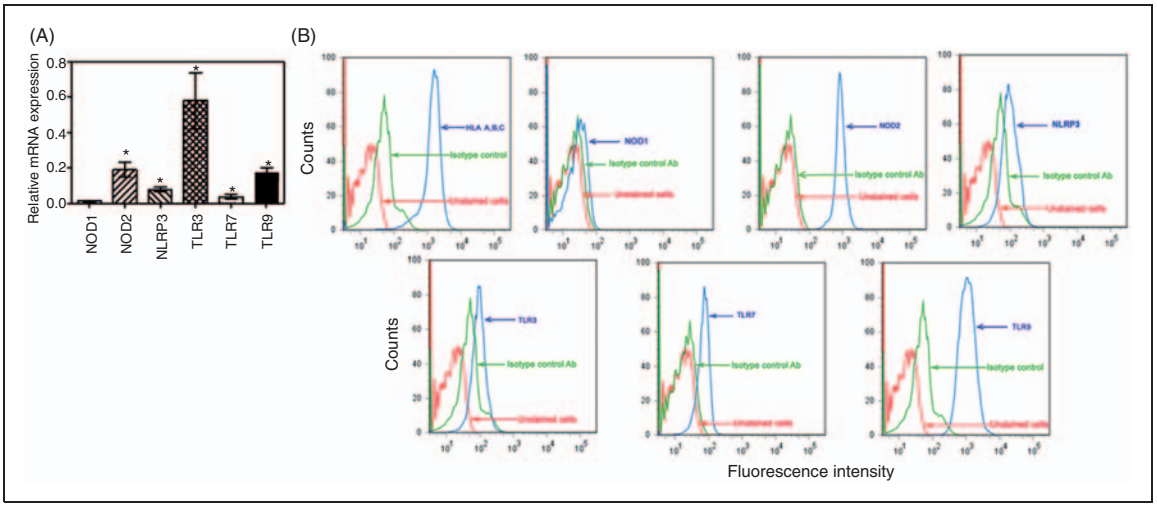

Figure 2. Expression of TLRs and NLRs in human primary NK cells. Human $CD56^{+}CD3^{-}$ NK cells were used either for isolation of RNA that was subjected to real-time PCR analyses of TLR/NLR mRNA expression (relative to HPRT levels) with the corresponding gene-specific primers (A) or were permeabilized using the CytoFix-CytoPerm™ kit and stained for 30 min on ice with either isotype control antibodies or antibodies against NOD1, NOD2, NALP3, TLR3, TLR7 or TLR9 (B). As a positive control, cells were incubated with anti-HLA A, B, C antibody. Cells were washed, stained with anti-rabbit IgG-FITC, and analyzed by FACS on a LSRII flow cytometer. To calculate statistical significance, relative gene expression values for pattern recognition receptors (normalized to HPRT) were compared to 'no template control' values, using a one-way ANOVA with post hoc Tukey's multiple paired comparison test. *$P < 0.05$. The data of a representative experiment ($n = 3$, total of three independent experiments (A); $n = 5$, total of five independent experiments (B) are depicted (mean ± SD, A).

to express high levels of NOD2 and TLR9, followed by TLR7, TLR3 and NLRP3, whereas NOD1 expression was the lowest (Fig. 2B). The human NK92 cell line showed expression profiles of NOD1, NOD2, NLRP3, TLR3, TLR7 and TLR9 mRNA and proteins similar to those observed in primary human NK cells (data not shown).

The effect of TLR and NLR agonists on cytokine expression in human NK cells

To study functionalities of NLRs and TLRs expressed in the human NK92 cell line and primary $CD56^{+}CD3^{-}$ NK cells, we first determined the ability of NLR and TLR agonists to activate TNF-α and IFN-γ mRNA expression and secretion, using real-time PCR and ELISA, respectively. In the NK92 cell line, poly (I:C) stimulation led to ~10-fold and ~25-fold increases in expression TNF-α and IFN-γ mRNA (Fig. 3A,B) and up-regulated levels of secreted TNF-α and IFN-γ by ~6.5-fold and 9-fold, respectively (Fig. 3C,D). Stimulation of NK92 cells with ODN2216 (a TLR9 agonist) and loxoribin (a TLR7 agonist) increased expression of TNF-α mRNA by 3.9-fold and 2.6-fold, respectively, while not appreciably inducing IFN-γ mRNA (Fig. 3A,B). Loxoribin failed to stimulate

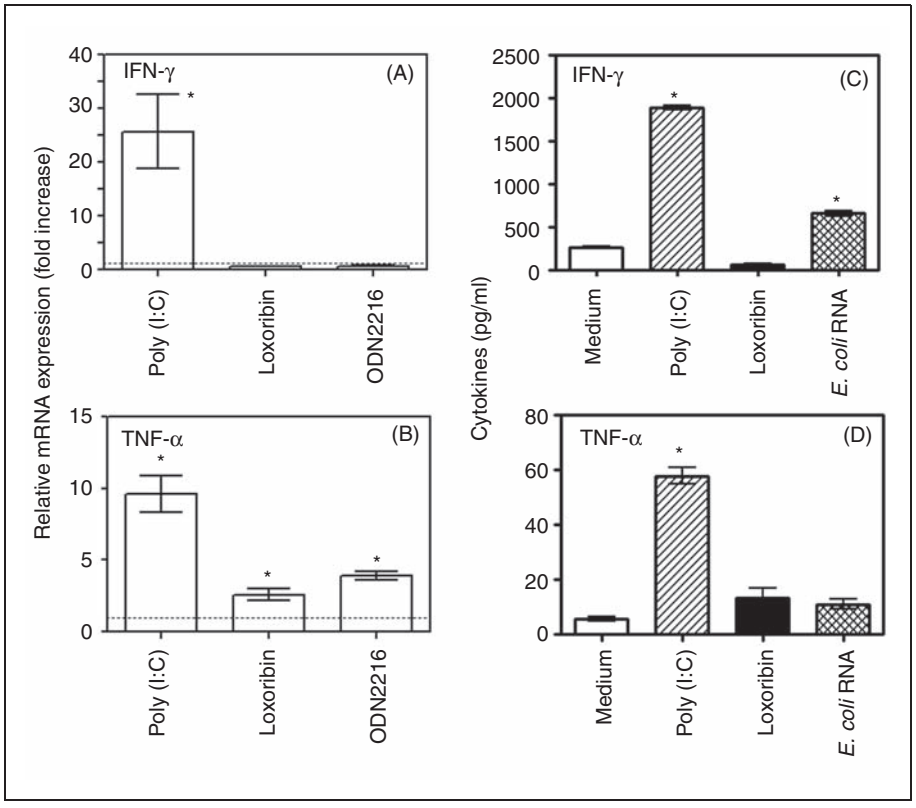

Figure 3. Cytokine-inducing capacities of TLR and NLR agonists in the human NK92 cell line. Natural killer92 cells were treated with medium or stimulated for 4 h (A and B) or for 24 h (C and D) with 1 μg/ml ie-DAP, 5 μg/ml MDP, 4 μg/ml *E. coli* RNA, 100 μg/ml poly (I:C), 5 μM loxoribin and ODN2216. (A and B): RNA samples were isolated, reverse-transcribed and subjected to real-time PCR using gene-specific primers to determine expression levels of TNF-α and IFN-γ mRNA relative to HPRT mRNA. (C and D): levels of secreted TNF-α and IFN-γ were determined in supernatants by ELISA. $^{*}P < 0.05$. The data of a representative experiment ($n = 3$) are depicted (mean ± SD).

IFN-γ secretion but showed a trend towards increasing secretion of TNF-α (that did not reach statistical significance), while *E. coli* RNA mediated a modest increase in IFN-γ secretion but did not cause statistically significant changes in TNF-α production (Fig. 3C,D).

In primary $CD56^{+}CD3^{-}$ NK cells, *E. coli* RNA and poly (I:C) caused 4.5-fold and 6.3-fold increases in TNF-α mRNA levels and up-regulated IFN-γ mRNA expression by 427-fold and 687-fold increases, respectively (Fig. 4A,B). The CpG oligonucleotide ODN2395 and ODN2216 increased IFN-γ gene expression by 423-fold (Fig. 4B) and 70-fold (data not shown), and up-regulated IFN-γ release by 6.4–9.2-fold (Fig. 4D and data not shown), while failing to exert statistically significant potentiation of TNF-α mRNA and protein expression (Fig. 4A,C). Stimulation of NK cells with ie-DAP, MDP and loxoribin led to 1.3–1.8-fold activation of TNF-α mRNA and 156–199-fold induction of IFN-γ mRNA (Fig. 4A,B). As determined by ELISA, MDP and loxoribin were the strongest activators of TNF-α release, *E. coli* RNA had ~1.5-fold lower activity, while poly (I:C) had a lower, albeit statistically significant, ability to elicit TNF-α secretion (Fig. 4C). With respect to IFN-γ production, poly (I:C) and *E. coli* RNA showed the strongest activity (9.3–16.4-fold increases), whereas MDP and loxoribin had a lower effect and caused 5.8-fold and 6.3-fold stimulation, respectively (Fig. 4D). Stimulation of NK cells with ie-DAP had the lowest effect on the IFN-γ gene expression but did not yield statistically significant up-regulation of TNF-α and IFN-γ release compared to the levels observed in medium-treated NK cells (Fig. 4C,D). These data suggest differences in mechanisms of induction of TNF-α and IFN-γ in NK cells, and show functional activities of expressed TLRs and NLRs in human NK cells.

TLR and NLR agonists enhance NK cytotoxicity against K562 and Tu167 tumor cells

Next, we analyzed whether stimulation of primary human NK cells with NLR and TLR agonists affects their cytotoxicity against K562 cells, classical NK cell targets, and the SCCHN cell line Tu167, using a standard ^{51}Cr release assay. As a positive control, we used IL-2 that mediated statistically significant increases in NK cytotoxicity against K562 and Tu167 cells (Fig. 5), consistent with published data.[45,47] Incubation with

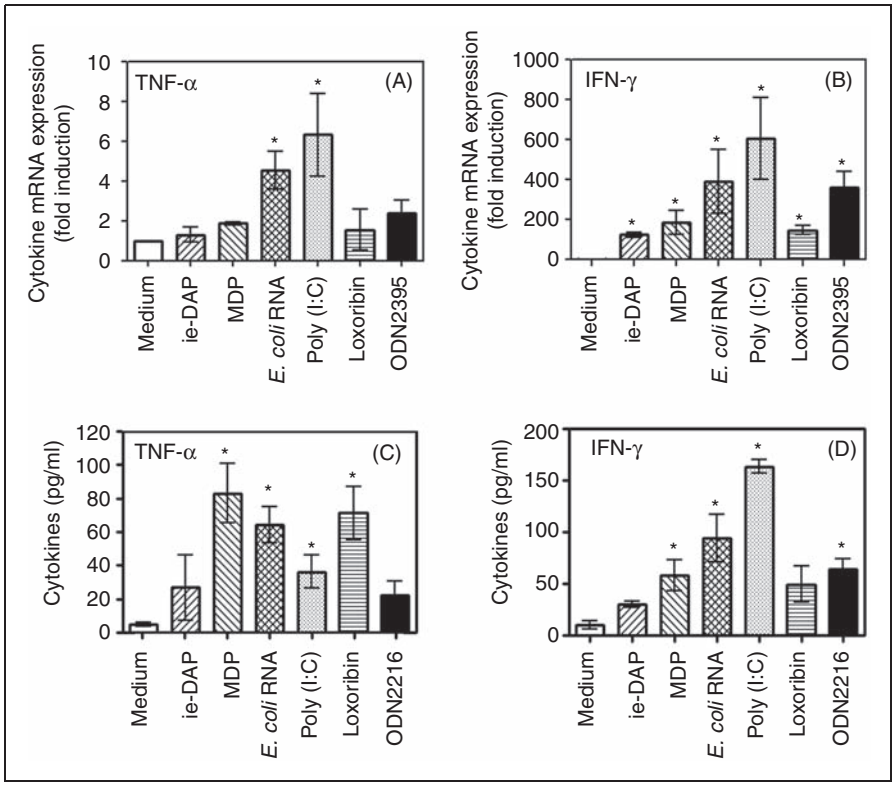

Figure 4. Cytokine-inducing capacities of TLR and NLR agonists in human $CD56^+CD3^-$ NK cells. $CD56^+CD3^-$ NK cells were treated with medium or stimulated for 4 h (mRNA studies) or 24 h (ELISA experiments) with 1 μg/ml ie-DAP, 5 μg/ml MDP, 4 μg/ml *E. coli* RNA, 100 ng/ml LPS, 100 μg/ml poly (I:C), 5 μM loxoribin and ODN2216. (A and B): RNA was isolated, reverse-transcribed and relative levels of TNF-α and IFN-γ mRNA (A) were determined by real-time PCR. (C and D): secretion levels of TNF-α and IFN-γ were measured in culture supernatants by ELISA. Shown are the data of a representative ($n = 2$) experiment. *$P < 0.05$.

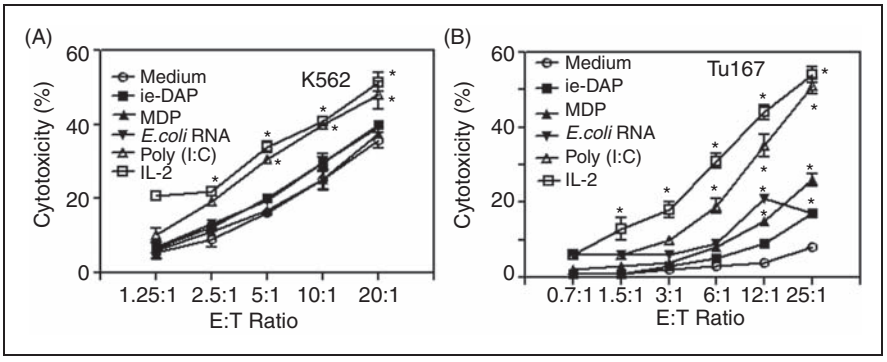

Figure 5. The effect of TLR and NLR agonists on cytotoxic activity of human $CD56^+CD3^-$ NK cells. $CD56^+CD3^-$ NK cells were treated for 24 h with medium or 1 μg/ml ie-DAP, 5 μg/ml MDP, 4 μg/ml *E. coli* RNA, 100 μg/ml poly (I:C), or 1000 u/ml IL-2. Cells were washed, incubated for 4 h with ^{51}Cr-labeled K562 (A) or Tu167 (B) target cells, and ^{51}Cr release from target cells was measured in supernatants. *$P < 0.05$. The results of a representative ($n = 5$) experiment are depicted (mean ± SD).

poly (I:C) (TLR3 agonist) also elicited statistically significant enhancement of NK cytotoxicity towards K562 and Tu167 cells compared to the response observed in medium-treated NK cells (Fig. 5). *Escherichia coli* RNA, MDP and ie-DAP did not exert statistically significant modulation of NK cytotoxicity against K562 (Fig. 5A). Interestingly, all tested NLR and TLR agonists exhibited a marked capacity to potentiate NK cell cytotoxicity against Tu167 cells, with poly (I:C) exerting a stronger effect than *E. coli* RNA and MDP that showed comparable activities, which were yet higher than those elicited by ie-DAP (Fig. 5B and data not shown). Collectively, our results demonstrate that engagement of TLRs and NLRs in NK cells increases their capacity to mediate lysis of tumor cell targets.

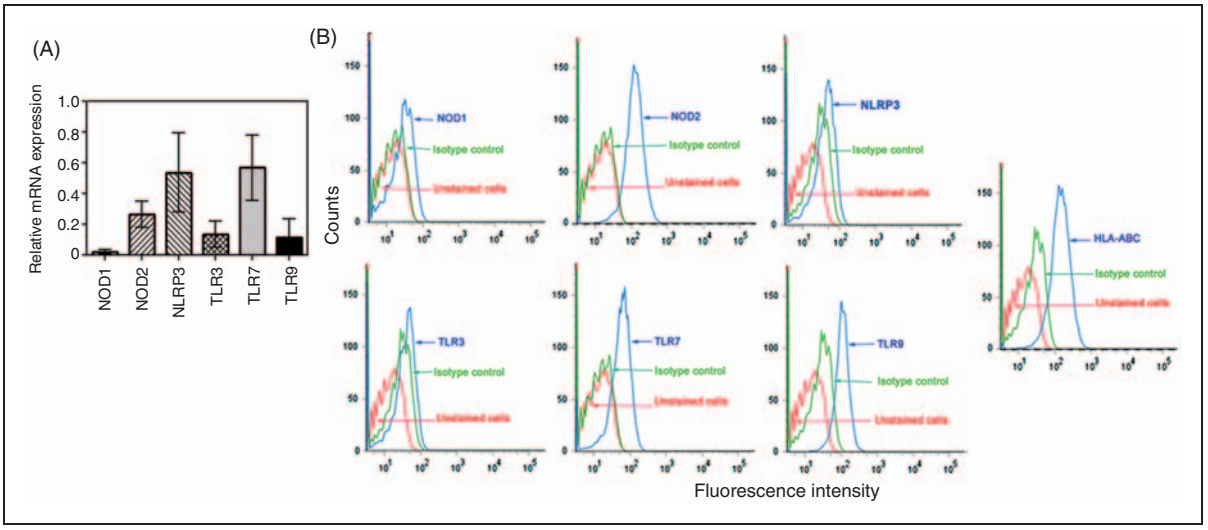

Figure 6. Expression of TLRs and NLRs in THP1 cells. THP1 cells were used for real-time PCR analyses of expression of the indicated TLR/NLR mRNA (A) or were permeabilized and stained for 30 min on ice with either isotype control antibodies or antibodies against NOD1, NOD2, NLRP3, TLR3, TLR7 or TLR9 (B). As a positive control, cells were incubated with anti-HLA Ab. Cells were washed, stained with anti-rabbit IgG-FITC, and analyzed by FACS on a LSRII flow cytometer. The data of a representative experiment ($n = 5$) are depicted.

Expression profiles of TLRs and NLRs in human THP1 macrophages

As our macrophage source, we used THP1 cells differentiated with PMA (20 ng/ml) for 72 h to attain macrophage characteristics such as increased CD14 expression, cytokine production and phagocytosis.[48] First, we employed real-time PCR analyses to determine mRNA expression levels of TLRs and NLRs. Figure 6A shows that THP1 cells express high levels of TLR7 and NLRP3, followed by NOD2, TLR3, and TLR9, whereas NOD1 mRNA was expressed at minimal levels. To confirm gene expression data, we then examined protein expression of intracellular TLRs (TLR3, TLR7 and TLR9) and NLRs (NOD1, NOD2 and NLRP3) by FACS analysis of permeabilized THP1 cells, using commercial Abs against TLRs and NLRs, isotype control Abs (negative control) or anti-HLA Ab (used as a positive control). Our results demonstrate that THP1 cells express high levels of NOD2, TLR7 and TLR9 proteins, followed by NOD1, TLR3 and NLRP3 (Fig. 6B).

Functional activities of expressed TLRs and NLRs in human THP1 macrophages

Differential utilization of TLR7 versus RIG-I for sensing of dsRNA has been reported in plasmacytoid DCs versus conventional DCs, mouse embryonic fibroblasts and other cells,[49] underscoring the importance of confirming functionality of expressed PRRs in different cell types. To analyze signaling capabilities of NOD1, NOD2 and NLRP3, THP1 cells were stimulated with the respective agonists, ie-DAP (NOD1), MDP (NOD2) and *E. coli* RNA (NLRP3), and activation of p38 MAP kinase, the transcription factor NF-κB, caspase-1 and generation of the processed IL-1β were determined. All three NLR agonists, ie-DAP, MDP and *E. coli* RNA, induced THP1 activation, as evidenced by IκB-α degradation and phosphorylation of p38, with *E. coli* RNA exhibiting the highest potency (Fig. 7A,C). In contrast, only *E. coli* RNA, a known NLRP3 agonist, mediated caspase-1 activation and processing, as judged by the appearance of p20 processed caspase-1 (Fig. 7B, top panel and Fig. 7D). Consistent with the requirement for active caspase-1 to mediate proteolytic cleavage of IL-1 precursor, stimulation of NLRP3 with *E. coli* RNA resulted in the appearance of processed IL-1β protein (mw ~17 kDa, Fig. 7B, middle panel and Fig. 7D). Neither NOD1 nor NOD2 engagement activated caspase-1 and both failed to yield mature IL-1β (Fig. 7B,D).

Next, we used real-time PCR and ELISA analyses of cytokine expression to examine functionalities of NLRs and TLRs in THP1 cells treated with the respective agonists. *E. coli* RNA had the highest activity to upregulate TNF-α, IL-1β, and IL-8 mRNA (Fig. 8A–C) and to stimulate IL-6 and IL-12p40 secretion (Fig. 9), followed by loxoribin, while ie-DAP and MDP exerted significantly lower and comparable effects. Lipopolysaccharide, a TLR4 agonist[50] used as a positive control for THP1 activation, was the most potent in activating gene expression and secretion of all cytokines, while ODN2216 (a TLR9 agonist) had the weakest effect (Figs 8 and 9). Only LPS, poly (I:C) and *E. coli* RNA increased IFN-β mRNA levels (18–36-fold stimulation) after treatment of THP1 cells, whereas all other tested TLR/NLR agonists failed to elicit the

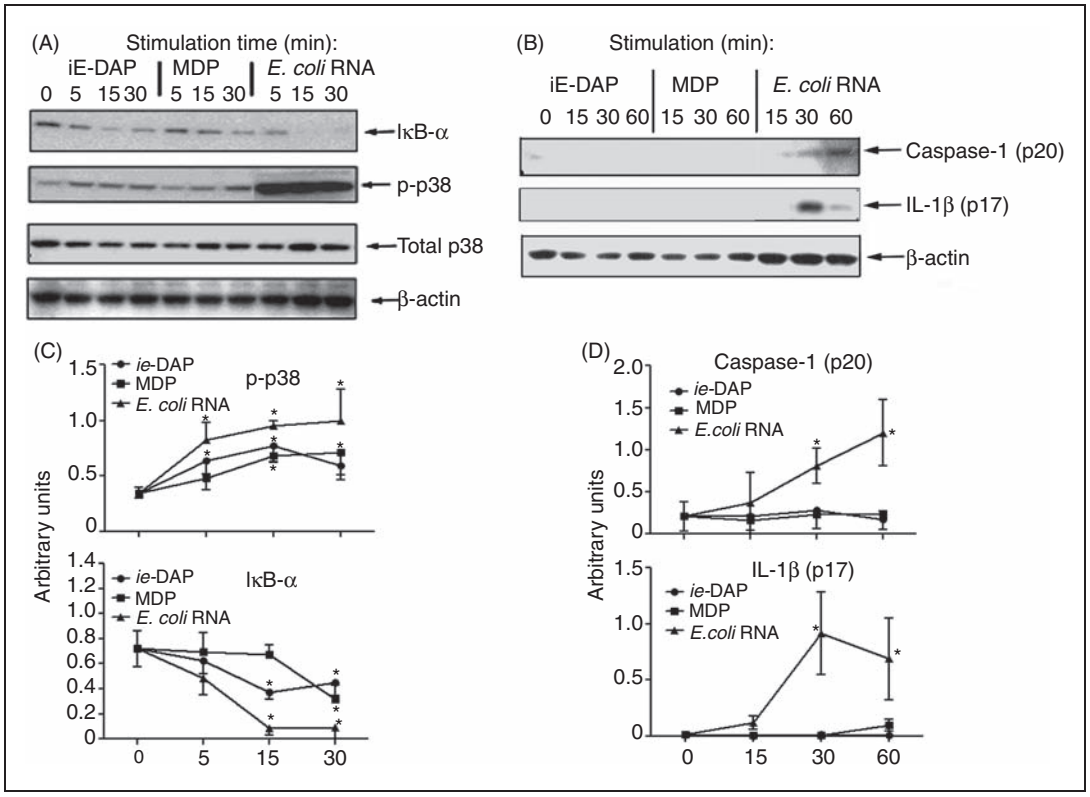

Figure 7. The NLR-mediated functional responses in THP1 cells. THP1 cells were treated with medium or stimulated with 1 μg/ml ie-DAP (NOD1 agonist), 4 μg/ml MDP (NOD2 agonist), 4 μg/ml *E. coli* RNA (a NALP3 agonist) for the indicated time periods. Cell extracts were prepared and analyzed by immunoblotting, using the indicated antibodies against IκB-α, p-p38, total p38 (A) or capspase-1, IL-1β (B) and β-actin (A and B). Shown are results of a representative ($n = 3$) experiments. (C and D): densitometric quantification of levels of p-p38, IκB-α, caspase-1 p20, IL-1β p17 and β-actin was performed for three independent experiments, and protein expression of the indicated proteins normalized to levels of β-actin was presented as arbitrary units (mean ± SD). $^*P < 0.05$.

response (Fig. 8D). These data indicate that THP1 cells express functional NLRs and TLRs.

Discussion

Macrophages and NK cells play a central role in tumor immunosurveillance, due to their abilities to exert tumoristatic and tumoritoxic effects, produce cytokines and chemokines that can facilitate recruitment and activation of other immune cell types, and to present tumor-associated antigens for initiation of adaptive immune responses.[3,5,9,10,12,14,18,21] The process of tumor immunoediting involves the generation of tumor cell clones with increased capacities to exert immune suppression,[18,20] decrease expression of MHC molecules,[17] and de-activate tumor-associated macrophages.[19,21] Therefore, the development of new approaches for re-activation of tumor-associated NK cells and macrophages is essential for enhancing anti-tumor immune responses and improving immunotherapeutic protocols for cancer patients.

This study was undertaken to examine the abilities of chemical TLR and NLR agonists to potentiate cytokine-secreting and antitumor activities of human macrophages, monocytes and NK cells. To the best of our knowledge, this paper is the first demonstration that human NK cells express functional NOD1, NOD2, and NLRP3 whose stimulation results in activation of their cytokine-producing and antitumor functions. Indeed, engagement of NOD2 with MDP and NLRP3 with *E. coli* RNA led to a marked increase in NK cytokine secretion and cytotoxicity against Tu167, whereas ie-DAP, a NOD1 agonist, exerted the weakest immunostimulating effects. We also support previously reported data on expression and functionality of TLR3 and TLR7 in isolated human NK cells,[42,43] showing that the TLR3 and NLRP3 agonists, poly (I:C) and *E. coli* RNA, were the most potent cytokine inducers and activators of NK cytotoxicity against Tu167 cells. Contradictory data have been reported regarding expression and functionality of TLR7, TLR8, and TLR9 in human NK cells.[42,43,51–55] Our FACS data demonstrated high levels of TLR9 in $CD56^+CD3^-$ human NK cells, whereas TLR7 was expressed at lower, albeit significant, levels. Furthermore, we showed that TLR9-activating ODN2216 and ODN2395, as well as TLR7-activating loxoribin induced expression of TNF-α and IFN-γ, supporting

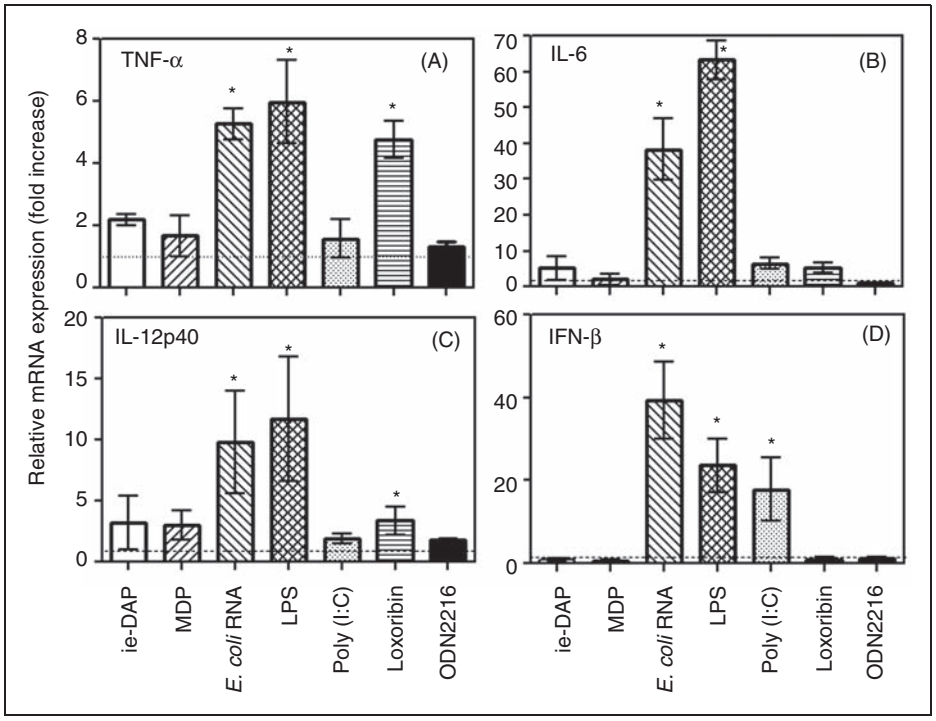

Figure 8. Effect of TLR and NLR agonists on cytokine gene expression in THP1 cells. Cells were treated with medium or stimulated for 4 h with 1 μg/ml ie-DAP, 5 μg/ml MDP, 4 μg/ml *E. coli* RNA, 100 ng/ml LPS, 100 μg/ml poly (I:C), 5 μM loxoribin and ODN2216. RNA samples were reverse-transcribed and analyzed by real-time PCR to determine expression of TNF-α (A), IL-6 (B), IL-12p40 (C) and IFN-γ (D) mRNA relative to HPRT mRNA. Data of four independent experiments (mean ± SD) are shown. *$P < 0.05$.

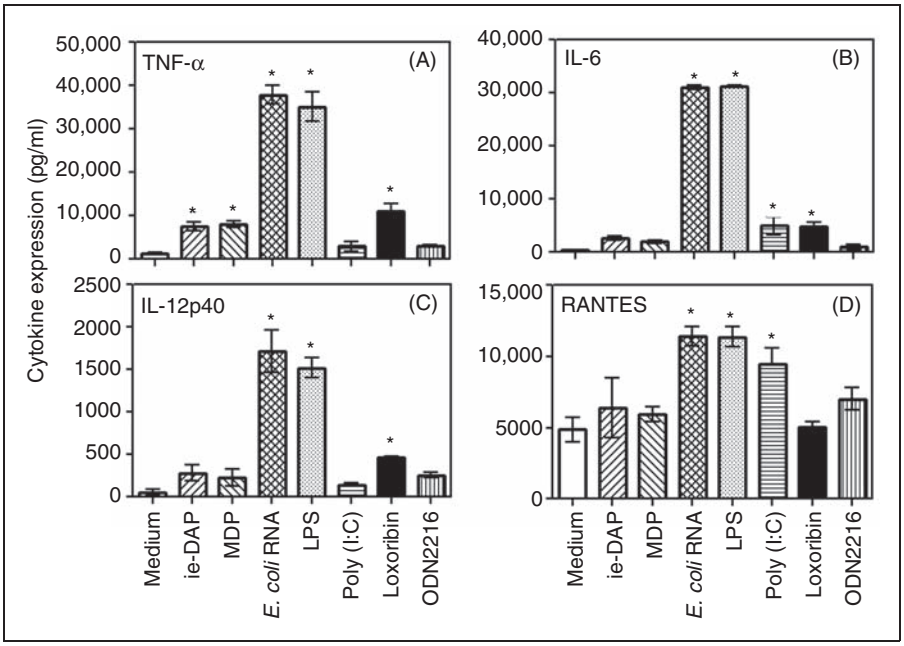

Figure 9. Effect of TLR and NLR agonists on IL-6 and IL-12p40 secretion by THP1 cells. Cells were treated for 24 h with medium or stimulated for 4 h with 1 μg/ml ie-DAP, 5 μg/ml MDP, 4 μg/ml *E. coli* RNA, 100 ng/ml LPS, 100 μg/ml poly (I:C), 5 μM loxoribin and ODN2216. Cell-free supernatants were collected and levels of TNF-α (A), IL-6 (B), IL-12p40 (C) and RANTES (D) were determined by ELISA. Data of a representative experiment (mean ± SD) out of three total experiments are shown. *$P < 0.05$.

the notion that NK cells express functional TLR7 and TLR9. Our findings also indicate that, despite the weakest effect on NK cytotoxicity, loxoribin, a TLR7 agonist, strongly up-regulated TNF-α expression. In this respect, it is noteworthy that loxoribin-mediated activation of TLR7 on NK cells cultured with suboptimal doses of IL-12 was similarly reported to increase IFN-γ secretion without modulating NK cytotoxicity against tumor targets.[42] Hence, these results suggest that different signaling pathways could be responsible for activation of cytokine expression versus up-regulation of cytotoxicity upon engagement of certain TLRs and NLRs.

Of interest, we report that while poly (I:C) significantly potentiate NK cytotoxicity against both 'classical' NK cell targets, K562 cells and the SCCHN cell line, Tu167, other tested TLR and NLR agonists selectively increased NK cytotoxic activity against Tu167 cells only. These results suggest that NK cells could engage different effector mechanisms (*e.g.* Fas-L vs perforin/granzyme) to mediate cell killing of SCCHN cells versus classical NK targets. It is also plausible that NK cell-mediated killing of different tumor targets may require distinct combinations of NK-activating receptors, such as NKG2D, that operates as an activating receptor on NK cells and co-stimulates the effector function of alphabeta $CD8^+$ T cells.[56] Ligands of NKG2D, the MHC class I chain-related and UL16 binding protein molecules, are expressed on many human tumors, and both NKG2D and its ligands are subjects to modulation in response to TLR engagement,[56] and might contribute to distinct NK cell killing of different tumor targets. Studies are in progress to address these possibilities and to define the molecular basis for preferential up-regulation of NK cytotoxicity against the SCCHN cell line Tu167 by TLR/NLR agonists.

This paper also shows expression profiles and functional activities of TLRs and NLRs in the human monocytic cell line, THP1 cells, differentiated with PMA to macrophages to enable increased CD14 expression and enhanced TLR responses, as reported.[48] Our findings indicate that the degree of expression of the studied PRRs does not always correspond to their functional activities. Indeed, we found the highest expression levels of NOD2, TLR7 and TLR9 in PMA-differentiated THP1 cells, while NLRP3, NOD1 and TLR3 were expressed to a lower extent. However, NLRP3–acting *E. coli* RNA mediated the highest extent of THP1 cell activation, as judged by IκB-α degradation and p38 phosphorylation. NLRP3 has been reported to activate caspase-1 maturation, cleavage and leading to caspase-1-mediated processing of pro-IL-1 that yields mature IL-1 capable of secretion.[57,58] In keeping with these reports, we observed that THP1 cell stimulation with NLRP3-activating *E. coli* RNA, but not with ie-DAP and MDP that do not activate inflammasomes,[58] led to the generation of active caspase-1 and mature IL-1β. NLRP3 and TLR7 exhibited the most potent capacity to up-regulate TNF-α and IL-8 mRNA expression, while NLRP3, TLR3 and TLR7 activated IL-6 and IL-12p40 release to the greatest extents. Poly (I:C) and LPS elicited potent induction of IFN-β mRNA, in line with a well-characterized capacity of TLR4 and TLR3 to activate the TRIF-dependent signaling pathway responsible for expression of type I IFN.[23,37]

Interestingly, *E. coli* RNA, a NLRP3 agonist, was also found to be a potent IFN-β inducer in THP1 cells. It should be noted that a subset of NLRs (*e.g.* NLRX1) has been implicated in the regulation of type I IFN expression elicited by other pattern recognition receptors, such as RIG-like helicases,[59] and bacterial RNA has recently been reported to stimulate expression of IFN-β in human PBMCs, supporting our results.[60] *Escerichia coli* RNA-mediated stimulation of IFN-β in THP1 cells could be indirectly mediated by IL-1β, due to a well-documented capacity of NLRP3 to induce mature, secreted IL-1β.[25,27,57] Notably, IL-1β was reported to induce IRF3 phosphorylation, nuclear translocation, and to activate expression of IFN-β and IFN-β-dependent genes in human fetal astrocytes cultured *in vitro*.[61] Studies are in progress to address whether *E. coli* RNA-mediated induction of IFN-β occurs directly via NLRP3 signaling or indirectly, via NLRP3-induced IL-1β.

Conclusions

This paper demonstrates for the first time that human NK cells express functional NOD2 and NLRP3 whose activation by the defined chemical agonists up-regulates NK cytotoxicity against the human head and neck tumor carcinoma cell line Tu167 and increases expression of TNF-α and IFN-γ. Our data support the conclusion that human NK cells express functional TLR3, TLR7 and TLR9 that respond to stimulation with poly(I:C), loxoribin and ODN2216 or ODN2395 by increasing NK cell cytokine expression and cytotoxicity. This manuscript reports an earlier unknown property of *E. coli* RNA to increase IFN-β expression in human macrophage-like THP1 cells, and shows expression of functional TLR3, TLR7, TLR9, NOD1, NOD2 and NLRP3 in these cells. Given NK-stimulating properties of IFN-β produced by THP1 cells, a plethora of other TLR/NLR-inducible cytokines observed in macrophages and NK cells, and the capacity of TLR/NLR agonists to potentiate NK cytotoxicity against SCCHN tumors, this study demonstrates a potential for the application of selected TLR and NLR agonists for further development of new immunotherapeutic strategies to treat patients with head and neck tumors.

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