

Activation of mast cells by double-stranded RNA: Evidence for activation through Toll-like receptor 3

Marianna Kulka, PhD,^a Lena Alexopoulou, PhD,^b Richard A. Flavell, PhD,^c and Dean D. Metcalfe, MD^a Bethesda, Md, Marseille, France, and New Haven, Conn

Background: Although mast cells (MCs) have been clearly implicated in innate immune responses involving bacteria, their ability to respond to viral infection is less clear.

Objective: Given that MCs increase at sites of inflammation and are located at surfaces where exposure to invading viruses may occur, we explored the ability of MCs to produce cytokines including type I IFNs after exposure to viruses and to polyinosine-polycytidylic acid (polyI:C), a synthetic mimic of viral double-stranded RNA, and characterized the receptors involved, if any.

Methods: Human peripheral blood-derived cultured MCs and 2 MC lines, Laboratory of Allergic Disease MC line and human MC line 1, were stimulated with viruses and polyI:C, and cytokine production, degranulation, and signaling pathway activation were examined. Because polyI:C is a ligand for Toll-like receptor (TLR)-3, human MCs were also analyzed for TLR expression.

Results: Viruses and polyI:C induced IFN- α and IFN- β production. PolyI:C did not induce TNF, IL-1 β , IL-5, or GM-CSF production, in contrast with other TLR ligands (LPS, peptidoglycan, CpG-A, or flagellin). IFN- α production involved nuclear factor- κ B, p38, and C-Jun NH2-terminal kinase and mitogen-activated protein kinase. RT-PCR and Western blot analysis confirmed expression of TLR-3 by all MCs. Human cultured MCs also expressed TLR-1, TLR-2, TLR-4, TLR-5, TLR-6, TLR-7 and TLR-9. Antibodies to TLR-3 significantly decreased IFN- α production. Bone marrow-derived MCs from TLR-3 knockout mice showed an ablated response to polyI:C. **Conclusions:** Murine and human MCs produce type I IFNs after exposure to double-stranded RNA and/or virus, the former via specific interactions with TLR-3. These data suggest that MCs contribute to innate immune responses to viral infection via the production of type I IFNs. (*J Allergy Clin Immunol* 2004;114:174-82.)

Key words: Mast cells, human, Toll-like receptors, innate immunity, type I interferons, double-stranded RNA, viruses

From ^athe Laboratory of Allergic Diseases, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda; ^bthe Centre d'Immunologie de Marseille-Luminy, INSERM-CNRS, Marseille, and ^cthe Section of Immunobiology, Howard Hughes Medical Institute, Yale University School of Medicine, New Haven.

Supported by the intramural program at the National Institutes for Health Research. Dr Alexopoulou was supported in part by a National Institutes of Health grant (P01AI36529).

Received for publication November 12, 2003; revised March 11, 2004; accepted for publication March 12, 2004.

Reprint requests: Dean D. Metcalfe, MD, Laboratory of Allergic Diseases, NIAID/NIH, 10 Center Drive, MSC 1881, Building 10, Room 11C209, Bethesda, MD 20892-1881. E-mail: dmetcalfe@niaid.nih.gov.

0091-6749/\$30.00

© 2004 American Academy of Allergy, Asthma and Immunology

doi:10.1016/j.jaci.2004.03.049

Abbreviations used

BMMC: Bone marrow-derived mast cell
CysLT: Cysteinyl leukotriene
dsRNA: Double-stranded RNA
HCMC: Human cultured mast cell
HMC-1: Human mast cell line 1
LAD: Laboratory of Allergic Disease mast cell line
MC: Mast cell
NF: Nuclear factor
PKR: Protein kinase R
polyI:C: Polyinosine-polycytidylic acid
PR8: UV-inactivated influenza virus
RSV: Respiratory syncytial virus
ssRNA: Single-stranded RNA
TLR: Toll-like receptor

Mast cells (MCs) are long-lived CD34⁺-derived cells that migrate to sites of inflammation and regulate innate immune responses via production of cytokines, leukotrienes, and other inflammatory mediators.¹ Indeed, MCs of human and rodent origin have been shown to express Toll-like receptor (TLR)-1, TLR-2, TLR-4, and TLR-6 mRNA and to respond to lipopolysaccharide and peptidoglycan by producing TNF- α , GM-CSF, IL-1 β , IL-5, IL-13, and leukotriene C₄.^{2,3} Although these studies suggest that MCs contribute to innate immune responses against invading bacteria, little is known regarding the interaction of MCs with viruses or their products, and whether this interaction results in the production of innate cytokines like type I IFNs, known to play critical roles in host defense against viral infection via both virocidal and immunoregulatory properties.

To address this possibility, we examined the ability of human MC lines, as well as primary cultures of human CD34⁺ cell-derived MCs, to respond to both viruses and polyinosine-polycytidylic acid (polyI:C), a synthetic mimic of viral double-stranded RNA (dsRNA) that is thought to be an important viral pathogen-associated molecular pattern. We show that MCs produced type I IFNs after exposure to polyI:C, respiratory syncytial virus, influenza virus, and type 1 reovirus, and expressed TLR-3 as well as all other known TLRs except TLR-10. In addition, TLR-3 signaling with polyI:C was uniquely capable of inducing the production of type I IFNs and did not result in the production of other proinflammatory cytokines. Together with the fact that MCs are positioned at surfaces that interface the external environment, these

TABLE I. Primers for the human TLRs, signaling molecules, and IFN- α/β used for RT-PCR analysis*

Gene	Sequence	Size	Tm	Cycles
TLR-1	tgaatcagcaaggtcttgc catctgtgtagctattcagct	432	54	30
TLR-2	gagcatctgataatgacagagtta gttcagtaagtatttgaaga	773	60	40
TLR-3	gtttgagacccttaacatggaa tgcttagatccagaatgtgcaag	454	60	30
TLR-4	gcatacttagactactacctgat aataacaccattgaagctcagatc	342	60	35
TLR-5	acaccaatgtcactatagctg tgtacaaagcctctgatggat	645	50	30
TLR-6	cttggaatgcctgtcagagct atctgaaaacagagctcagtaagc	544	60	35
TLR-7	gacctaaagtgaaattgccct ctcttgaatcctctgaaggtg	538	60	35
TLR-8	aacagaatatcaccgttgtaaa ttcagttccacttaacactgtgag	293	60	35
TLR-9	ggacctctgttactactctcca aagctcgtttgacaccagctct	150	54	45
TLR-10	tgctcatctgcatctaaatctgt agctccagtttattgccattcaa	671	60	35
IFN- α (all types)	agaatctctcttctctctg catctgtgtagctattcagct	914	50	35
IFN- β	tgctctcccaaatgtctctcc gcatctgctggttgaagaatgc	635	60	35
β -actin	atctggcaccacaccttctacaat- gagctgcg cgtcatactctgctgtgatcca- catctgc	838	60	25

*All sequences are in the 5' to 3' orientation.

data suggest that MCs may contribute significantly to innate immunity to viral infection and contribute to the induction of adaptive immunity via the production of type I IFNs.

METHODS

Human MC culture

Human peripheral blood CD34⁺ progenitor cells were cultured as described.⁴ At 6 to 8 weeks of culture, aliquots of 2×10^4 cultured cells were spun onto glass slides (Cytospin 2; Thermo Electron Corporation, Houston, Tex) and stained with toluidine blue.⁴ More than 99% of the nonadherent cells contained metachromatic granules, and flow cytometry showed them to be positive for Kit and Fc ϵ RI receptors.

Human MC line 1 (HMC-1) cells were cultured in Iscove medium containing 10% FBS, 100 U/mL penicillin, and 100 μ g/mL streptomycin (Biosource International, Rockville, Md) in a humidified atmosphere of 5% CO₂ in air at 37°C. Laboratory of Allergic Disease mast cell line (LAD)-2 MCs⁵ were cultured in serum free media (StemPro-34 SFM; Life Technologies, Carlsbad, Calif) supplemented with 2 mmol/L L-glutamine, 100 U/mL penicillin, 50 μ g/mL streptomycin, and 100 ng/mL stem cell factor. The cell suspensions were seeded at a density of 10^5 cells/mL and maintained at 37°C and 5% CO₂.

Generation of bone marrow–derived MCs

Bone marrow–derived MCs (BMMCs) were generated from femoral bone marrow cells of TLR-3 knockout mice and wild-type

littermates backcrossed on a C57BL/6 background.⁶ Cells were maintained in RPMI medium (Biosource International) supplemented with 4 mmol/L L-glutamine, 1 mmol/L sodium pyruvate, 100 U/mL penicillin, 100 μ g/mL streptomycin, 50 μ mol/L β -mercaptoethanol, 0.1 mmol/L nonessential amino acids, and 30 ng/mL IL-3 (PeproTech, Rocky Hill, NJ). After 4 weeks of culture, >99% of the cells were Fc ϵ RI⁺/Kit⁺ by FACS analysis and toluidine blue–positive.

Bone marrow aspirate collection and flow cytometry

The bone marrow aspirate mononuclear cell fraction containing MCs was obtained after informed consent and was separated by using Histopaque (Sigma-Aldrich, St Louis, Mo) gradient centrifugation. Contaminating red cells were lysed in 0.8% ammonium chloride solution (Stem Cell Technologies, Vancouver, British Columbia, Canada) for 10 minutes. Bone marrow aspirate mononuclear cell fraction cells were stained with anti-TLR3-PE (eBioscience, San Diego, Calif) or anti-Kit allophycocyanin (Caltag, Burlingame, Calif) for 30 minutes at 4°C, then analyzed on a FACSCalibur (BD Biosciences, San Jose, Calif). For intracellular detection of IFN- α , cells were fixed with 4% paraformaldehyde for 5 minutes, permeabilized with 0.1% saponin/PBS, blocked with 5% milk/PBS, and stained with anti-IFN- α (R&D Systems, Minneapolis, Minn).

RT-PCR

Total RNA was isolated from MCs by using the SNAP Total RNA Isolation kit (Life Technologies, Carlsbad, Calif). Genomic DNA was digested by incubating 10 μ g total RNA with 2 U DNase (Life Technologies) in DNase buffer (200 mmol/L Tris-HCl, 20 mmol/L MgCl₂, 500 mmol/L KCl, pH 8.4; Life Technologies) and RNase-free H₂O for 10 minutes at room temp. RNA was precipitated with 3 mol/L C₂H₂O₂Na (pH 5.2; Sigma-Aldrich). Treated RNA (1 μ g) was reverse-transcribed by using 0.5 μ g oligo(dT), reverse transcriptase (RT) buffer, 10 mmol/L dithiothreitol, 10 mmol/L of each dNTP (dATP, dCTP, dGTP, and dTTP), 2'-deoxynucleoside 5'-triphosphate-treated H₂O, and 200 U Moloney murine leukemia virus RT enzyme (all from Life Technologies) at 37°C for 1 hour.

PCR was performed by using 1X PCR buffer, 0.8 mmol/L dNTP mix, 20 μ mol/L antisense primer, 20 μ mol/L sense primer, 1.5 mmol/L MgCl₂, RNase-free H₂O, 2 μ L cDNA, and 2.5 U Taq DNA Polymerase (all from Life Technologies). The sequences of each primer set are shown in Table I. The PCR mixture was amplified at the indicated annealing temperature for 20 to 35 cycles (Table I). The PCR product was then analyzed by 2% agarose gel electrophoresis and visualized by ethidium bromide staining. The optimal PCR conditions (amplification within the linear phase) for all primers were determined by amplifying human spleen total RNA (BD Bioscience, San Jose, Calif) over a range of cycle numbers, annealing temperatures, primer concentrations, and MgCl₂ concentrations.

Quantitative real-time PCR

Quantitative real-time PCR assay of transcripts was performed by using gene-specific fluorescently labeled primers and a 7700 Sequence Detector (Applied Biosystems, Foster City, Calif) as described.⁷ Primers and reagents were obtained from Invitrogen (Chantilly, Va). Each primer set consisted of 1 labeled (6-carboxy fluorescein [FAM] fluorescent reporter at the 5' end) and 1 unlabeled primer. Sequences of the primers were as follows: IFN- α forward primer, 5'-FAM-GACCTTTTGTGCTGAAGAGATTGAAGG5C-3'; IFN- α reverse primer, 5'-TGATGGCAACCAGTTCAG-3'; human β -actin forward primer, 5'-FAM-CAACTGTCTCCATGTCGTCAG5TG-3'; and human β -actin reverse primer, 5'-GACGAGGCCAGAGCAAGA-3'. Data were collected during

the annealing/extension phase of PCR and analyzed by using the comparative C_t method.⁷

β -Hexosaminidase release assay

A total of 2×10^5 cells were washed and resuspended in buffer (10 mmol/L HEPES, 137 mmol/L NaCl, 2.7 mmol/L KCl, 0.38 mmol/L $\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$, 5.6 mmol/L glucose, 1.8 mmol/L $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 1.3 mmol/L $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.04% BSA; pH 7.4) and stimulated with various concentrations of LPS (from *Escherichia coli* serotype 055:B5; Sigma-Aldrich), peptidoglycans (from *Staphylococcus aureus*; Sigma-Aldrich), polyI:C (Amersham Biosciences, Piscataway, NJ), CpG-A DNA (Coley Pharmaceuticals, Kanata, Ontario, Canada), and flagellin (Axxora LLC, San Diego, Calif). In the case of Fc ϵ RI-mediated stimulation, human cultured MCs (HCMCs) were sensitized with human IgE anti-4-hydroxy-3-nitrophenylacetyl (NP) (1 $\mu\text{g}/\text{mL}$; Serotec, Raleigh, NC) overnight and stimulated with NP-BSA (Sigma-Aldrich) at various concentrations. The β -hexosaminidase in supernatants and cell lysates was quantified by hydrolysis of *p*-nitrophenyl N-acetyl- β -D-glucosamide (Sigma-Aldrich) in 0.1 mol/L sodium citrate buffer (pH 4.5) for 90 minutes at 37°C. The percentage of β -hexosaminidase release was calculated as percent of total content.

Cytokine ELISAs

Human cultured MCs were washed with media and suspended at 1×10^6 cells per well, then stimulated with LPS, peptidoglycan, polyI:C, CpG, or antigen for indicated times. In some cases, cells were preincubated with SP600125 (0.1 $\mu\text{mol}/\text{L}$; a JNK inhibitor; Biosource), SB202190 (30 $\mu\text{g}/\text{mL}$, an inhibitor of p38), SN50 (50 $\mu\text{g}/\text{mL}$; an inhibitor of nuclear factor [NF]- κB ; Biosource), actinomycin D (5 $\mu\text{g}/\text{mL}$; transcription inhibitor; Biosource), anti-TLR-3 polyclonal antibody (5 $\mu\text{g}/\text{mL}$; clone Q-18 or L-13; Santa Cruz Biotechnology, Santa Cruz, Calif), anti-TLR-2 (5 $\mu\text{g}/\text{mL}$; clone N-17; Santa Cruz Biotechnology) or anti-TLR-4 (5 $\mu\text{g}/\text{mL}$; clone C-18; Santa Cruz Biotechnology) for 1 hour, then stimulated with polyI:C. Cell free supernatants were isolated and analyzed for cysteinyl leukotriene (CysLT), human cytokines (IFN- α , TNF, IL-5, IL-1 β , and GM-CSF), and murine cytokines (IFN- α and TNF) by using commercial ELISA kits (R&D Systems).

Western immunoblot

Human cultured MCs were washed with PBS and 1×10^6 cells lysed in buffer containing lithium dodecyl sulfate sample buffer (Life Technologies), 10% β -mercaptoethanol (Sigma-Aldrich), 0.1 mol/L DTT (Sigma-Aldrich), and protease inhibitor cocktail (Roche, Indianapolis, Ind). Whole cell lysates (30 μg) were separated on 4% to 12% Bis-Tris SDS-PAGE gels (Life Technologies) and transferred onto nitrocellulose membranes. The membranes were blocked with 3% milk in TRIS-buffered saline-0.05% Tween for 1 hour and then stained with primary antibodies, anti-TLR1 to TLR9 (Santa Cruz Biotechnology), anti-phospho-stress-activated protein kinase/JNK (Thr183/Tyr185; Cell Signaling Technology, Beverly, Mass), anti-phospho-p38 MAP kinase (Thr180/Tyr182; Cell Signaling Technology), anti-phospho-NF- κB p65 (Ser536; Cell Signaling Technology), or antiactin (Sigma-Aldrich) for 1 hour at room temperature. The membranes were washed with TRIS-buffered saline-Tween 3 times and stained with the secondary antibody sheep antirabbit horseradish peroxidase (Jackson ImmunoResearch Laboratories, West Grove, Pa), donkey antigoat HRP (Santa Cruz Biotechnology), or goat antimouse HRP (Santa Cruz Biotechnology) for 1 hour. The nitrocellulose membranes were developed with chemiluminescence reagent (Life Technologies) for 1 minute and exposed to high-performance chemiluminescence film for 30 minutes.

Viral infection

Human cultured MCs were washed with media and resuspended at 1×10^6 cells/mL in a 24-well plate. Cells were then treated with 8 plaque-forming units/cell of respiratory syncytial virus (RSV), reovirus type 1, Lang (10 PFU/cell; a gift from Terence Dermody, Vanderbilt University School of Medicine, Nashville, Tenn), or UV-inactivated influenza virus (40 hemagglutination units/mL; a gift from John Yewdell, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, Md) and incubated for 3 days at 37°C. Cell-free supernatants were assayed for IFN- α production by ELISA (R & D Systems).

Statistical analysis

Experiments were conducted in triplicate or with MCs obtained from 3 separate donors, and values represent means of $n = 3 \pm$ SEMs. *P* values were determined by 1-way ANOVA (between groups) or Student *t* test.

RESULTS

dsRNA induces IFN- α production by human MCs

Double-stranded RNA, a synthetic mimic of viral RNA, has been shown to induce type I IFNs in several cell types, including dendritic cells.^{8,9} For these studies, we stimulated LAD, HMC-1, and HCMCs with polyI:C for 0 to 24 hours, and total RNA samples were analyzed for the presence of IFN- α and IFN- β mRNA by using RT-PCR. PolyI:C induced IFN- α and IFN- β mRNA expression in LAD and HMC-1 cells, with gene induction occurring as early as 30 minutes (Fig 1, A). HCMCs constitutively expressed low levels of IFN- α mRNA, and expression was increased after polyI:C exposure. PolyI:C did not induce message for IFN- β in HCMCs. The increase in IFN- α mRNA after polyI:C treatment of HCMCs was confirmed by real-time PCR analysis. As shown in Fig 1, B, IFN- α mRNA expression increased after polyI:C treatment and reached a maximum at 3 to 5 hours.

To determine whether the increase in IFN- α mRNA was accompanied by secretion of IFN- α protein, HCMCs were treated with polyI:C (10 $\mu\text{g}/\text{mL}$) for 0 to 24 hours, and cell-free supernatants were analyzed by ELISA (Fig 1, C). Consistent with RT-PCR analysis, polyI:C induced IFN- α production, which continued over a period of 8 hours. Stimulation of HCMCs with polyI:C, polyA:U, polyG:C, poly C, and polydI:dC showed that polyI:C was the most potent inducer of IFN- α production (Fig 1, D) results similar to those with other cell types.⁶

We next verified that human MCs produce type I IFNs in response to viral exposure. HCMCs were treated with UV-inactivated influenza virus (PR8), type 1 reovirus, RSV, and polyI:C dsRNA at 37°C for 3 days. All 3 viruses induced substantial production of IFN- α (PR8, 407 ± 31.9 pg/mL; type 1 reovirus, 281.4 ± 17.6 pg/mL; and RSV, 503.4 ± 21.8 pg/mL).

Human MCs express TLR-3 as well as other TLRs

Because dsRNA is reported to signal via TLR-3, we examined HMC-1, LAD, HCMCs cultured for 4 weeks,

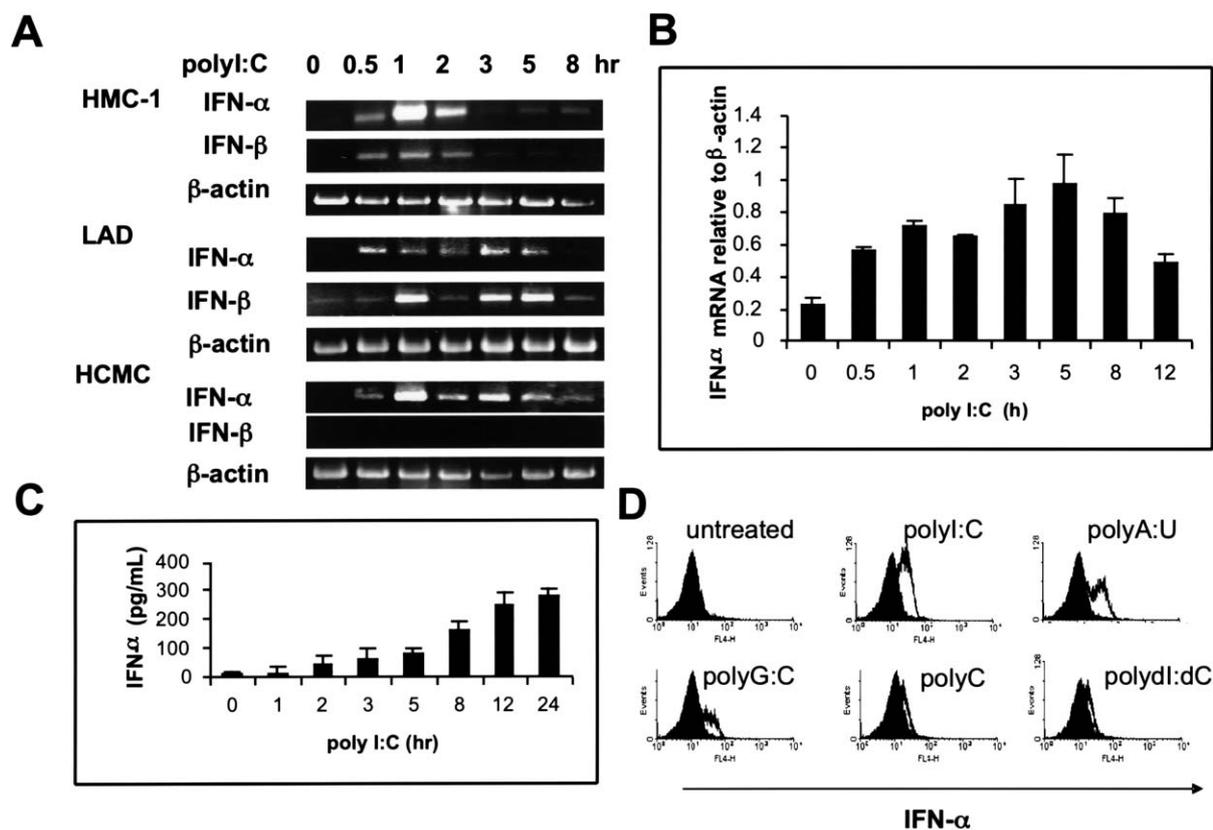


FIG 1. **A**, RT-PCR analysis of IFN- α/β mRNA production after dsRNA (polyI:C, 10 μ g/mL) treatment for 0 to 8 hours. **B**, Real-time PCR analysis of IFN- α gene expression in HCMCs treated with dsRNA (polyI:C, 10 μ g/mL). **C**, ELISA analysis of IFN- α production from HMCs stimulated with dsRNA (polyI:C, 10 μ g/mL). **D**, Flow cytometry analysis of IFN- α production after HMC stimulation with polyI:C, polyA:U, polyG:C, polyC, and polydI:dC (all at 10 μ g/mL) for 24 hours (n = 3).

and HCMCs cultured for 8 weeks for message and protein expression of TLRs. Previous reports had shown that human cord blood–derived MCs express mRNA for TLR-1, TLR-2, TLR-4, and TLR-6.^{10,11} Analysis revealed that all human MC types expressed TLR-3, TLR-5, TLR-7, and TLR-9 mRNA (Fig 2, A). HCMCs also expressed TLR-1, TLR-2, TLR-4, TLR-6, and TLR-8 (Fig 2, A). However, mature HCMCs (at 8 weeks of culture) did not express TLR-8. These observations were confirmed by Western blot analysis by using antibodies specific for TLR1 to TLR9. HCMCs (mature, cultured for 8 weeks) similarly expressed protein for all TLRs except TLR-8, and all MC types expressed TLR-3, TLR-5, TLR-6, TLR-7, and TLR-9 (Fig 2, B). The bone marrow aspirate mononuclear cell fraction containing MCs was also tested for TLR-3 expression by flow cytometry. MCs are readily identified as a population highly expressing CD117 (Kit¹²), and these cells also expressed TLR-3 (Fig 3).

TLR-3 antibodies inhibit dsRNA activation of human MCs, and MCs from TLR-3 knockout mice show impaired responses

To explore whether polyI:C-induced IFN- α production involved TLR-3, HCMCs were preincubated with 2

polyclonal anti-TLR-3 antibodies (clone Q-18 and L-13), then stimulated with polyI:C (10 μ g/mL) for 24 hours. IFN- α release was then measured by ELISA (Fig 3, A). Preincubation with either anti-TLR-3 antibody inhibited polyI:C-induced IFN- α production by more than 40%. Isotype, anti-TLR-2, and anti-TLR-4 antibodies did not have a significant effect on polyI:C-induced IFN- α production.

Bone marrow–derived MCs from TLR-3 knockout mice have a significantly ($P < .01$) impaired response to polyI:C (Fig 3, B) but respond normally to LPS (Fig 3, C) and antigen (Fig 3, D). This impaired MC response is similar to the impaired polyI:C response of macrophages and splenocytes from TLR-3^{-/-} mice.⁶ TLR-3^{-/-} BMMCs expressed the same level of Kit and Fc ϵ RI receptors as wild-type BMMCs and degranulated normally in response to antigen (data not shown).

Human cultured MCs do not secrete IFN- α in response to LPS, peptidoglycan, flagellin, or antigen

Because initial studies had shown that MCs express several TLRs (Fig 2), IFN- α production in response to diverse TLR ligands was next determined. HCMCs did

not produce IFN- α in response to LPS, peptidoglycan, or antigen (Fig. 4). TLR-3^{-/-} BMDCs and wild-type BMDCs also did not produce IFN- α in response to LPS, peptidoglycan, CpG, or antigen (data not shown). Thus, polyI:C and CpG are unique among TLR ligands in inducing IFN- α production by HCMCs.

Because LPS, peptidoglycan, and antigen induce production of TNF from MCs, we determined whether polyI:C would similarly induce TNF release. PolyI:C stimulation did not induce significant production of TNF from HCMCs (Fig 5, B). However, LPS, peptidoglycan, CpG, flagellin, and antigen induced TNF release from HCMCs and LAD cells (Fig 5, A and B). Furthermore, polyI:C augmented antigen-dependent induction of TNF (Fig 5, B). We similarly examined IL-1 β , GM-CSF, IL-5, and CysLT production. Peptidoglycan stimulated IL-5, IL-1 β , GM-CSF, and CysLT release (Fig 5, C-F), whereas LPS stimulated only TNF production (Fig 5, B). CpG stimulated production of TNF (Fig 5, B), IL-1 β (Fig 5, D) and CysLT (Fig 5, E), whereas flagellin stimulated production of TNF and IL-1 β but not CysLT. PolyI:C induced a small amount of CysLT production (220 ± 23 pg/mL). PolyI:C did not stimulate production of IL-5, IL-1 β , or GM-CSF but slightly augmented antigen-mediated induction of TNF, IL-5, IL-1 β , and CysLT (Fig 5, C-F). Thus, in terms of these additional cytokines, polyI:C generated little to no response, demonstrating selectivity to production of IFN- α , an antiviral cytokine.

Although IL-12 is also known to stimulate IFN- α production, we found that none of the TLR ligands induced IL-12 production. Further, the addition of exogenous IL-12 (as much as 1000 U/mL) did not stimulate IFN- α production from HCMCs (data not shown). Therefore, dsRNA induction of IFN- α is not dependent on IL-12.

dsRNA has no effect on Fc ϵ RI-mediated degranulation

Because polyI:C uniquely induced IFN- α production from human MCs and type I IFNs have been implicated in MC granule restructuring,¹² we examined the ability of polyI:C to modulate MC degranulation. PolyI:C (0.1-200 μ g/mL) treatment of HCMCs and LAD at 37°C for as long as 1 hour did not induce MC degranulation as assessed by β -hexosaminidase release (data not shown). We next examined whether polyI:C modified Fc ϵ RI-activated degranulation. PolyI:C (5-12 hours) pretreatment had no effect on antigen-driven release of β -hexosaminidase from HCMCs or LAD (data not shown). Thus, dsRNA does not lead to modulation of IgE-mediated MC degranulation.

dsRNA-induced IFN- α production is JNK-dependent and NF- κ B dependent

Because dsRNA activates a JNK/NF- κ B-dependent and p38-dependent signaling pathway in splenocytes, macrophages, and epithelial cells,^{6,13-15} we determined whether the JNK/NF- κ B pathway was involved in polyI:C-induced human MC IFN- α release. HCMCs were pretreated with inhibitors of JNK (SP600125), p38 (SB202190), or NF- κ B (SN50) for 1 hour and then

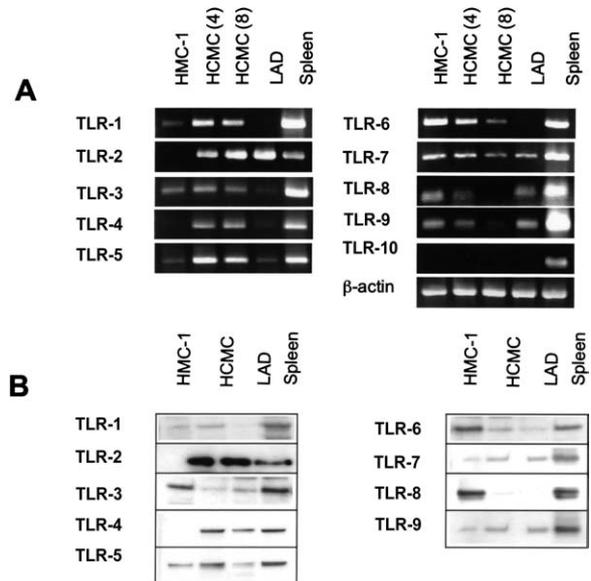


FIG 2. TLR expression in HMC-1, HCMC (at 4 weeks and 8 weeks of culture), and LAD cells determined by RT-PCR (A) and Western blot analysis (B). Human spleen RNA and protein lysate were used as a positive control.

stimulated with polyI:C. These inhibitors effectively block JNK, p38, and NF- κ B phosphorylation after IgE/antigen cross-linking of Fc ϵ RI (data not shown). ELISA analysis of cell-free supernatants showed that IFN- α production was partially inhibited by SP600125, SB202190, and SN50, suggesting that p38, JNK, and NF- κ B were at least partially involved in IFN- α release (Fig 6, A). Moreover, actinomycin D completely inhibited polyI:C-induced IFN- α production, suggesting that this process required de novo gene transcription. Vehicle alone (0.01% dimethyl sulfoxide in PBS) had no effect on IFN- α production (data not shown).

To examine further the signaling pathways activated by dsRNA, HCMCs were treated with polyI:C for 0 to 60 minutes, and cell lysates were examined for the presence of phosphorylated JNK, I κ B α , and p38 (Fig 6, B). PolyI:C exposure, as seen in Fig 6, B, leads to JNK, p38, and I κ B phosphorylation. Thus, dsRNA induces activation signaling pathways in human MCs consistent with TLR ligation.

DISCUSSION

This is the first report that human MCs express TLR-3 and are capable of responding to the TLR-3 ligand, polyI:C dsRNA, by producing IFN- α , a molecule heretofore not known to be synthesized and released by MCs of any species. This observation may be important for understanding innate host responses to viral infections in peripheral tissues. IFN- α effects are mediated by both direct inhibition of viral replication and activation of nearby immune cells such as NK cells, macrophages, and

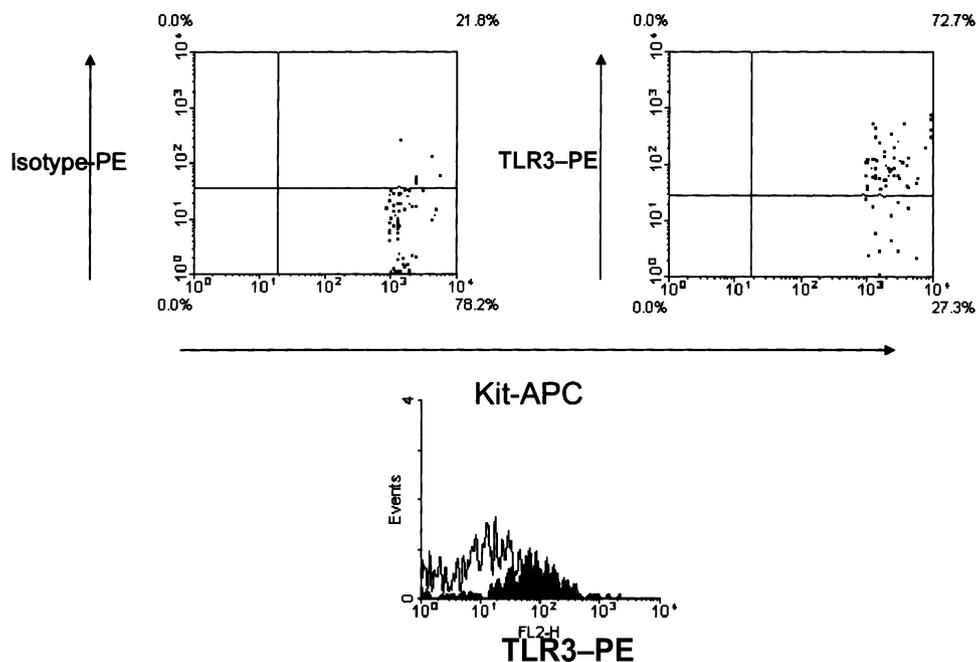


FIG 3. TLR-3 expression on BMMCs. Bone marrow aspirate mononuclear cell fraction was incubated with anti-TLR3-PE (5 μ g/mL) and anti-Kit-antigen-presenting cells (2 μ g/mL) for 30 minutes at 4°C and analyzed on a FACSCalibur.

T cells,^{16,17} and IFN- α is widely used to treat viral hepatitis.¹⁸

We further show polyI:C-induced IFN- α production to be TLR-3-mediated. Preincubation with anti-TLR-3 antibodies inhibited polyI:C-induced IFN- α production from HCMCs, whereas anti-TLR-2 or anti-TLR-4 antibodies did not have an effect (Fig 3, A). This finding suggests that TLR-3 is expressed by human MCs, and binding TLR-3 with these antibodies is able to disrupt dsRNA activation. Consistent with this finding, BMMCs from TLR-3^{-/-} mice show impaired polyI:C-induced IFN- α production. The 45% decrease in IFN- α production from TLR-3^{-/-} BMMCs is consistent with results obtained with bone marrow-derived macrophages and splenocytes from these mice.⁶

A recent study has shown that dendritic cells, which readily phagocytose extracellular particles, express TLR-3 in endocytic vesicles, requiring internalization of dsRNA for TLR-3 activation.¹⁹ However, this same group has shown that fibroblasts express TLR-3 on their surface, and blocking surface TLR-3 with antibodies can abrogate polyI:C-induced IFN- β in these cells.²⁰

Although all MC types expressed TLR-3, only HCMCs released significant amounts of IFN- α . LAD and HMC-1 produced very little IFN- α protein but also produced IFN- β mRNA. Because currently available IFN- β ELISAs are not sensitive, it was difficult to measure IFN- β in these supernatants, but it is possible that LAD-2 and HMC-1 also produce IFN- β protein.

dsRNA can activate various kinase pathways leading to the activation of transcription factors such as NF- κ B.²¹ In our study, polyI:C-mediated activation of IFN- α was

partially blocked by inhibitors of p38, JNK, and NF- κ B, and immunoblot analysis showed that polyI:C activated p38, JNK, and I κ B phosphorylation (Fig 6). This finding suggests that other cytokines whose promoters are activated by these pathways may also be produced. However, we found that polyI:C did not induce TNF, IL-1 β , or IL-5 production by human MCs. The relative absence of TNF production, despite evidence of NF- κ B activation, may reflect limited TNF release by these cells in these culture conditions.

We have also shown that infection of human MCs with live virus (RSV, reovirus) and PR8 induced IFN- α production. Evidence suggests that IFN- α production during viral infection can be either TLR-3-dependent or TLR-3-independent. Plasmacytoid dendritic cells that do not express TLR-3²² are able to produce large amounts of IFN- α in response to polyI:C if the dsRNA crosses the plasma membrane by electroporation or lipofectamine administration.⁹ This TLR-independent pathway likely involves protein kinase R (PKR), a cytosolic serine/threonine kinase activated by autophosphorylation on binding to dsRNA.²³ It is possible that TLR-3^{-/-} BMMC production of IFN- α may involve internalization of dsRNA and activation of the PKR pathway.⁶

It is likely that viral infection stimulates a variety of intracellular signaling pathways in human MCs. Previous reports have shown that dengue virus infection of human MCs stimulates RANTES, macrophage inflammatory protein 1 α , and macrophage inflammatory protein 1 β ,²⁴ which may recruit inflammatory cells to sites of infection. Still others have shown that rhinovirus infection alone does not alter cytokine production by human basophilic or

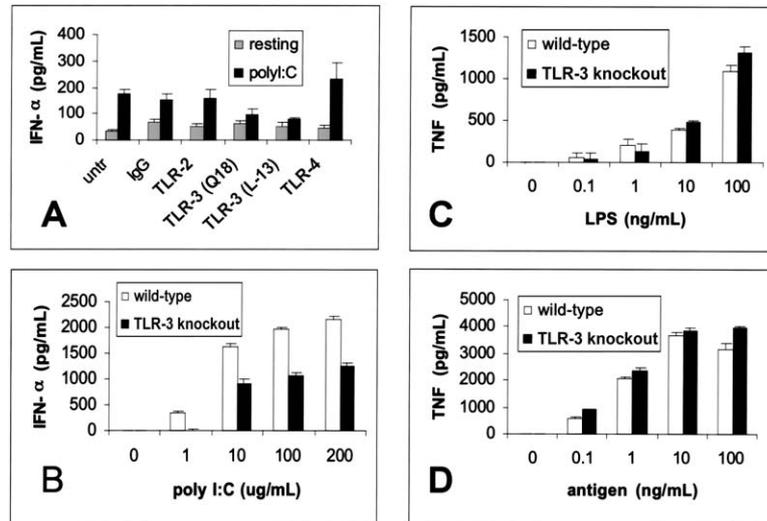


FIG 4. A, HCMCs were incubated with 5 μ g/mL IgG isotype control, anti-TLR-2, anti-TLR-3 (clone Q-18), anti-TLR-3 (clone L-13), and anti-TLR-4 for 1 hour, then stimulated with poly:I:C (10 μ g/mL) for 24 hours. BMMCs from wild-type and TLR-3 knockout mice were stimulated with poly:I:C (B), LPS (C), and antigen (D) for 24 hours, and IFN- α or TNF was measured in supernatants with ELISA ($n = 3$ mice). *untr*, Untreated.

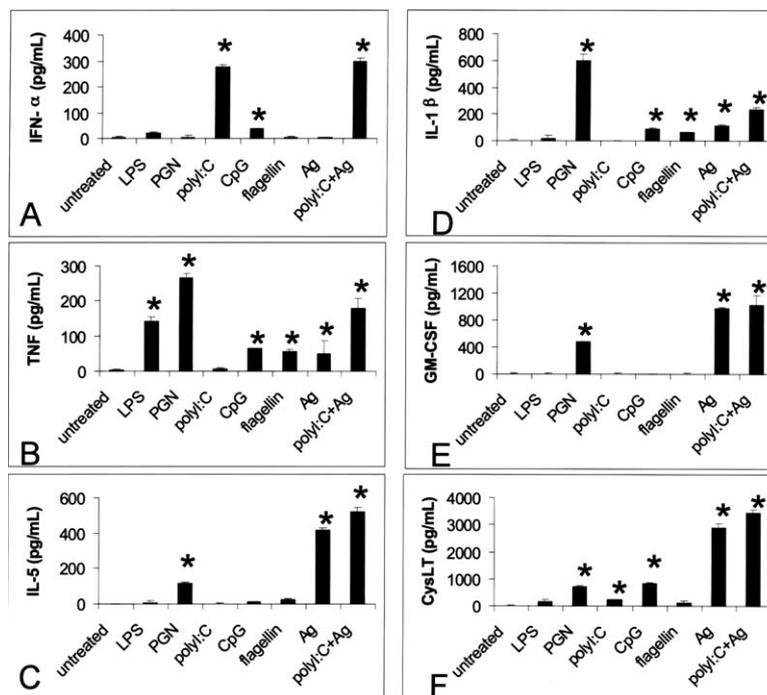


FIG 5. HCMCs were stimulated with poly:I:C (10 μ g/mL), LPS (100 ng/mL), peptidoglycan (10 μ g/mL), CpG-A oligonucleotides (1 μ g/mL), flagellin (100 ng/mL), antigen (Ag; 100 ng/mL), or poly:I:C + antigen for 24 hours. IFN- α (A), TNF (B), IL-5 (C), IL-1 β (D), GM-CSF (E), and CysLT (F) were measured in cell-free supernatants by using ELISA. *Statistical significance between untreated and treated ($P < .05$ compared with untreated, $n = 3$).

MC lines²⁵ but can potentiate IgE-activated IL-4, IL-6, and IL-8 release.²⁵ These studies suggest that MCs recruit nearby inflammatory cells during a viral infection. Our study suggests that MCs may also activate nearby cells to inhibit viral infection, because IFN- α activates various

antiviral pathways²⁶ and greatly reduces virus replication.^{27,28} Reovirus, influenza, and RSV have dsRNA, single-stranded RNA (ssRNA), and ssRNA genomes, respectively, and may activate IFN- α production by their dsRNA (via TLR-3 or PKR), ssRNA (via TLR7 or

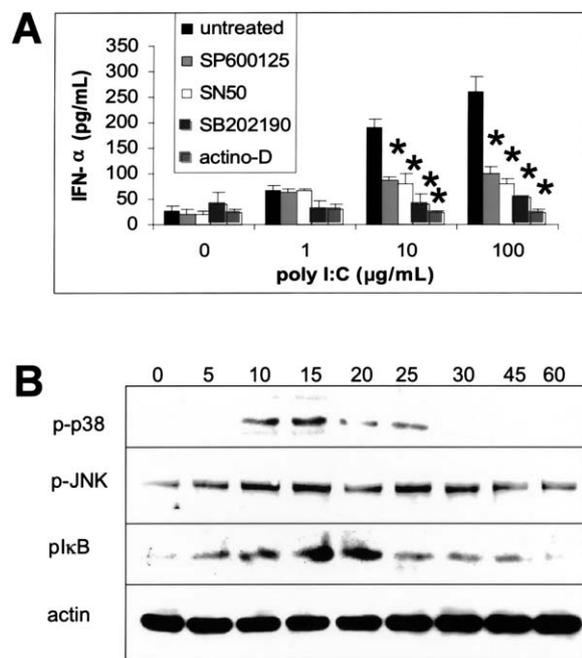


FIG 6. A, IFN- α production is mediated by JNK and NF- κ B activation. HCMCs were stimulated with polyI:C (10 μ g/mL) in the presence of p38 JNK inhibitor (SP600125; 0.1 mmol/L), NF- κ B inhibitor (SN50; 50 μ g/mL), p38 inhibitor (SB202190; 30 μ g/mL), or actinomycin-D (5 μ g/mL) for 24 hours. IFN- α production was measured by ELISA in cell-free supernatants ($n = 3$). **B**, HCMCs were treated with polyI:C (10 μ g/mL) for 0 to 60 minutes, and cell lysates were examined for the presence of phosphorylated JNK, I κ B, and p38 by using phospho-specific mAb. Blots were stripped and reprobed with antiactin mAb to monitor loading ($n = 3$).

TLR8^{29,30}), or as yet unknown pathways involving viral proteins.

Human MC responses to 5 TLR ligands (LPS, peptidoglycan, polyI:C, CpG-A, and flagellin) suggest that MC responses to polyI:C are unique because polyI:C stimulates IFN- α production but not TNF, IL-1 β , IL-5, or GM-CSF. Conversely, bacterial products LPS and peptidoglycan induce TNF, IL-1 β , IL-5, GM-CSF, and CysLT but not IFN- α . Furthermore, human MC activation with dsRNA via TLR-3 does not induce β -hexosaminidase release (data not shown). Previous reports have shown that TLR-2-mediated activation induces significant β -hexosaminidase release from mouse BMMCs¹⁰ and a low β -hexosaminidase release from human cord blood-derived mast cells.³ This observation supports the hypothesis that MCs differentially respond to external signals and preferentially release specific mediators.

For the first time, we also show that human MCs express TLR-5 and TLR-7. TLR-5 recognizes flagellin,³¹ and TLR-7 is activated by ssRNA²⁹ and the synthetic imiquimod drugs, which are used in the treatment of genital warts caused by human papillomavirus.³² We have also confirmed previous findings showing that human MCs express TLR-1, TLR-2, TLR-4, and TLR-6, all of which are involved in LPS-mediated and peptidoglycan-mediated signaling.^{2,3} Therefore, mature human MCs

express all but 2 TLRs, suggesting that they are able to participate in innate immune response to a wide variety of pathogens. Because MCs are resident in all peripheral tissues and because they are long-lived, it is likely that their response to viral infection may contribute significantly to localized innate immune response to viral infection.

We thank Brian Kelsall for reovirus and influenza virus and for critically revising this article. We also thank Helene Rosenberg for RSV and technical advice. Dr Flavell is an investigator of the Howard Hughes Medical Institute.

REFERENCES

- Kawakami T, Galli SJ. Regulation of mast-cell and basophil function and survival by IgE. *Nat Rev Immunol* 2002;2:773-86.
- Varadaradjalou S, Feger F, Thieblemont N, Hamouda NB, Pleau JM, Dy M, et al. Toll-like receptor 2 (TLR2) and TLR4 differentially activate human mast cells. *Eur J Immunol* 2003;33:899-906.
- McCurdy JD, Olynych TJ, Maher LH, Marshall JS. Cutting edge: distinct toll-like receptor 2 activators selectively induce different classes of mediator production from human mast cells. *J Immunol* 2003;170:1625-9.
- Kirshenbaum AS, Goff JP, Semere T, Foster B, Scott LM, Metcalfe DD. Demonstration that human mast cells arise from a progenitor cell population that is CD34(+), c-kit(+), and expresses aminopeptidase N (CD13). *Blood* 1999;94:2333-42.
- Kirshenbaum AS, Akin C, Wu Y, Rottem M, Goff JP, Beaven MA, et al. Characterization of novel stem cell factor responsive human mast cell lines LAD 1 and 2 established from a patient with mast cell sarcoma/leukemia; activation following aggregation of Fc ϵ RI or Fc γ RI. *Leuk Res* 2003;27:677-82.
- Alexopoulou L, Holt AC, Medzhitov R, Flavell RA. Recognition of double-stranded RNA and activation of NF- κ B by Toll-like receptor 3. *Nature* 2001;413:732-8.
- Nazarenko I, Lowe B, Darfler M, Ikononi P, Schuster D, Rashtchian A. Multiplex quantitative PCR using self-quenched primers labeled with a single fluorophore. *Nucleic Acids Res* 2002;30:e37.
- Kadowaki N, Antonenko S, Liu YJ. Distinct CpG DNA and polyinosinic-polycytidylic acid double-stranded RNA, respectively, stimulate CD11c-type 2 dendritic cell precursors and CD11c+ dendritic cells to produce type 1 IFN. *J Immunol* 2001;166:2291-5.
- Diebold SS, Montoya M, Unger H, Alexopoulou L, Roy P, Haswell LE, et al. Viral infection switches non-plasmacytoid dendritic cells into high interferon producers. *Nature* 2003;424:324-8.
- Supajatura V, Ushio H, Nakao A, Akira S, Okumura K, Ra C, et al. Differential responses of mast cell Toll-like receptors 2 and 4 in allergy and innate immunity. *J Clin Invest* 2002;109:1351-9.
- McCurdy JD, Lin TJ, Marshall JS. Toll-like receptor 4-mediated activation of murine mast cells. *J Leukoc Biol* 2001;70:977-84.
- Crivellato E, Candussio L, Mallardi F, Ribatti D. Recombinant human alpha-2a interferon promotes an atypical process of mast cell secretion with ultrastructural features suggestive for piecemeal degranulation. *J Anat* 2002;201:507-12.
- Tadlock L, Yamagiwa Y, Marienfeld C, Patel T. Double-stranded RNA activates a p38 MAPK-dependent cell survival program in biliary epithelia. *Am J Physiol Gastrointest Liver Physiol* 2003;284:G924-32.
- Chu WM, Ostertag D, Li ZW, Chang L, Chen Y, Hu Y, et al. JNK2 and IKKbeta are required for activating the innate response to viral infection. *Immunity* 1999;11:721-31.
- Maggi LB Jr, Heitmeier MR, Scheuner D, Kaufman RJ, Buller RM, Corbett JA. Potential role of PKR in double-stranded RNA-induced macrophage activation. *EMBO J* 2000;19:3630-8.
- Pfeffer LM, Dinarello CA, Herberman RB, Williams BR, Borden EC, Borden R, et al. Biological properties of recombinant alpha-interferons: 40th anniversary of the discovery of interferons. *Cancer Res* 1998;58:2489-99.
- Marrack P, Kappler J, Mitchell T. Type I interferons keep activated T cells alive. *J Exp Med* 1999;189:521-30.

18. Hadziyannis SJ, Papatheodoridis GV. Peginterferon-alpha2a (40 kDa) for chronic hepatitis C. *Expert Opin Pharmacother* 2003;4:541-51.
19. Matsumoto M, Funami K, Tanabe M, Oshiumi H, Shingai M, Seto Y, et al. Subcellular localization of toll-like receptor 3 in human dendritic cells. *J Immunol* 2003;171:3154-62.
20. Matsumoto M, Kikkawa S, Kohase M, Miyake K, Seya T. Establishment of a monoclonal antibody against human Toll-like receptor 3 that blocks double-stranded RNA-mediated signaling. *Biochem Biophys Res Commun* 2002;293:1364-99.
21. Fitzgerald KA, Palsson-McDermott EM, Bowie AG, Jefferies CA, Mansell AS, Brady G, et al. Mal (MyD88-adaptor-like) is required for Toll-like receptor-4 signal transduction. *Nature* 2001;413:78-83.
22. Kadowaki N, Ho S, Antonenko S, Malefyt RW, Kastelein RA, Bazan F, et al. Subsets of human dendritic cell precursors express different toll-like receptors and respond to different microbial antigens. *J Exp Med* 2001;194:863-9.
23. Williams BR. PKR; a sentinel kinase for cellular stress. *Oncogene* 1999;18:6112-20.
24. King CA, Anderson R, Marshall JS. Dengue virus selectively induces human mast cell chemokine production. *J Virol* 2002;76:8408-19.
25. Hosoda M, Yamaya M, Suzuki T, Yamada N, Kamanaka M, Sekizawa K, et al. Effects of rhinovirus infection on histamine and cytokine production by cell lines from human mast cells and basophils. *J Immunol* 2002;169:1482-91.
26. Goren T, Kapitkovsky A, Kimchi A, Rubinstein M. High and low potency interferon-alpha subtypes induce (2'-5') oligoadenylate synthetase with similar efficiency. *Virology* 1983;130:273-80.
27. Su B, Ceponis PJ, Lebel S, Huynh H, Sherman PM. Helicobacter pylori activates Toll-like receptor 4 expression in gastrointestinal epithelial cells. *Infect Immun* 2003;71:3496-502.
28. Choudhary S, Gao J, Leaman DW, De BP. Interferon action against human parainfluenza virus type 3: involvement of a novel antiviral pathway in the inhibition of transcription. *J Virol* 2001;75:4823-31.
29. Heil F, Hemmi H, Hochrein H, Ampenberger F, Kirschning C, Akira S, et al. Species-specific recognition of single-stranded RNA via Toll-like receptor 7 and 8. *Science* 2004;303:1526-9.
30. Diebold SS, Kaisho T, Hemmi H, Akira S, Reis E, Sousa C. Innate antiviral responses by means of TLR7-mediated recognition of single-stranded RNA. *Science* 2004;303:1529-31.
31. Hayashi F, Smith KD, Ozinsky A, Hawn TR, Yi EC, Goodlett DR, et al. The innate immune response to bacterial flagellin is mediated by Toll-like receptor 5. *Nature* 2001;410:1099-103.
32. Dockrell DH, Kinghorn GR. Imiquimod and resiquimod as novel immunomodulators. *J Antimicrob Chemother* 2001;48:751-5.