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TLR ligands induce synergistic interferon-β and interferon-λ1 gene expression in human monocyte-derived dendritic cells

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ABSTRACT

Toll-like receptors (TLRs) are pattern-recognition receptors of the innate immune system that recognize various pathogen-associated molecules. TLR ligands are potent activators of immune cells and certain TLR ligands have a synergistic ability to induce the production of pro-inflammatory cytokines. In the present study we have analyzed the potential synergy between TLR3, TLR4 and TLR7/8 ligands in type I and type III interferon (IFN) gene expression in human monocyte-derived dendritic cells (moDCs). We show that stimulation of moDCs with TLR7/8 ligand R848 together with TLR3 or TLR4 ligands, polyI:C or LPS, respectively, leads to a synergistic expression of IFN-β and IFN-λ1 mRNAs. Neutralization of type I IFNs as well as IFN priming prior to stimulation suggest that IFN-dependent positive feedback loop is at least partly responsible for the mechanism of synergy. Enhanced expression of TLR3 and especially TLR7, which are both under the regulation of type I IFNs, correlated to synergistic TLR ligand-dependent induction of IFN-β and IFN-λ1 genes. NF-κB, PI3 kinase and MAP kinase pathways were involved in TLR ligand-induced IFN gene expression as evidenced by pharmacological signaling inhibitors. The data indicates that IFNs contribute to TLR-dependent gene activation in human DCs stimulated with multiple TLR ligands.

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1. Introduction

Interferons (IFN) are cytokines that are crucial for the innate immune protection of the host against viral infections. Dendritic cells (DC) are one of the major producers of IFNs although many other cell types are also able to synthesize IFNs in response to viral infections (Coccia, 2008). Besides having direct antiviral effects, IFNs regulate the expression of many innate immune receptors, stimulate cytokine and chemokine production, regulate cell differentiation and polarization, inhibit cell proliferation and regulate apoptosis (Noppert et al., 2007). IFNs are classified into three types; type I IFNs include IFN-α (13 functional subtypes), IFN-β, IFN-ω, IFN-ε and IFN-κ, the sole member of type II IFN is IFN-γ and type III IFNs consist of IFN-λ1, IFN-λ2 and IFN-λ3. Type I IFNs signal via a receptor composed of two receptor chains, IFNAR1 and IFNAR2, while IFN-λs use their separate specific receptor that consists of IFNLR1 and IL10R2 chains. Type I and type III IFNs use the Jak-Stat pathway to activate cytoplasmic ISGF3 complex that is transported into the nucleus where it binds to the promoter regions of IFN stimulated genes (ISG) and induces their expression (Takaoka and Yanai, 2006).

The production of IFNs is initiated by the recognition of viral or bacterial structural components or genetic material by cellular pattern recognition receptors (PRR). An important class of PRRs is Toll-like receptors (TLR) of which TLRs 3, 4, 7, 8 and 9 are involved in the induction of type I and type III IFN gene expression (Coccia et al., 2004; Honda et al., 2005; Yang et al., 2005). TLR4 is located at the cell membrane and it recognizes many different ligands of which the bacterial lipopolysaccharide (LPS) is the most studied one. TLRs 3, 7, 8 and 9 are located at the endosomal membranes and they recognize various types of nucleic acids. TLR3 is responsive to dsRNA, TLR7 and 8, which are structurally homologous, recognize ssRNA and TLR9 is a specific receptor for CpG DNA. Recently, it was shown that also bacterial mRNA can induce IFN-β production via TLR7 in mice (Mancuso et al., 2009). TLR3 and TLR4 use the Toll/interleukin-1 receptor homology domain-containing adaptor protein inducing IFN (TRIF) adaptor molecule to activate IFN regulatory factor (IRF) 3 by phosphorylation which regulates type I and type III IFN gene expression. TLR7/8 and TLR9, instead,

Abbreviations: ERK, extracellular signal-regulated kinase; IFN, interferon; IL, interleukin; IRF, IFN regulatory factor; ISRE, interferon-stimulated response element; JNK, c-Jun N-terminal kinase; LPS, lipopolysaccharide; MDA5, melanoma differentiation associated gene 5, MAPK, mitogen-activated protein kinase; moDC, monocyte-derived dendritic cell; MyD88, myeloid differentiation factor 88; NF-κB, nuclear factor κB; pDC, plasmacytoid dendritic cell; PI3K, phosphatidylinositol 3-kinase; PRR, pattern recognition receptor; RIG-I, retinoic acid inducible gene 1; TLR, Toll-like receptor; TNF, tumor necrosis factor; TRAM, TRIF-related adaptor molecule; TRIF, Toll/interleukin-1 receptor homology domain-containing adaptor protein-inducing IFN.

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employ the Myeloid differentiation factor 88 (MyD88) adaptor to activate a pathway leading to the activation of IRF7 (Kawai and Akira, 2006). TLR stimulation also leads to the activation of nuclear factor (NF)-κB and mitogen-activated protein kinase (MAPK) pathways, which are known to contribute to TRIF-dependent IFN gene expression (Kawai and Akira, 2006). In addition to endosomal TLRs, cytoplasmic retinoic acid inducible gene-I (RIG-I) -like receptors (RLRs) recognize foreign nucleic acids in the cell (Yoneyama et al., 2004). RIG-I is activated by 5′p-ssRNA and short, transfected dsRNA molecules whereas melanoma differentiation associated gene 5 (MDA5) is a sensor for longer dsRNA molecules (Hornung et al., 2006; Kato et al., 2008).

We and others have recently shown that stimulation of monocyte-derived DCs (moDC) via TLR3 or TLR4 together with TLR7/8 leads to synergistic induction of pro-inflammatory cytokines such as TNF-α, IL-6 and IL-12 (Gautier et al., 2005; Makela et al., 2009; Napolitani et al., 2005). It has, however, not been analyzed whether type I or type III IFNs are expressed in a synergistic manner in TLR ligand stimulated moDCs. Recognition of microbes via different PRRs enables the host to react appropriately to various danger signals and regulate the immune response accordingly. It is thus important to study the mechanisms of synergy between different TLR ligands. In the present study we describe a TLR-mediated synergistic IFN-β and IFN-λ1 gene expression in human moDCs. We propose that early TLR3 or TLR4 ligand-induced IFN augments the expression of TLRs, especially that of TLR7, leading to sustained IFN gene expression when cells are stimulated with TLR7/8 and TLR3 or TLR4 ligands simultaneously.

2. Material and methods

2.1. Cell culture

Monocytes were purified from freshly collected, leukocyte-rich buffy coats obtained from healthy blood donors (Finnish Red Cross Blood Transfusion Service, Helsinki, Finland) as described previously (Veckman et al., 2004). Briefly, human peripheral blood mononuclear cells were isolated by density gradient centrifugation over a Ficoll-Paque gradient (Amersham Biosciences, Uppsala, Sweden), followed by Percoll gradient (Amersham Biosciences) centrifugation to obtain monocytes. The remaining T and B cells were depleted using anti-CD3 and anti-CD19 magnetic beads (Dynal, Oslo, Norway). Monocytes (2.5×10^6 cells/six-well plate well, 1.25×10^6 cells/24-well plate well) were allowed to adhere to plates (Falcon, Becton Dickinson, Franklin Lakes, NJ, USA) for 1 h at +37 °C in RPMI-1640 (Sigma–Aldrich, Saint Louis, MO, USA) supplemented with 0.6 μg/ml penicillin, 60 μg/ml streptomycin, 2 mM L-glutamine, and 20 mM HEPES. Adhered monocytes were washed with PBS and immature moDCs were generated by cultivating cells in RPMI-1640 medium with supplements as above plus 10% FCS (Integro, BV, Dieren, The Netherlands), 10 ng/ml human rGM-CSF and 20 ng/ml human rIL-4 (R&D Systems, Abingdon, UK). The cells were cultivated for 6 days and fresh media was added every 2 days.

2.2. Reagents

TLR3 ligand, polyinosic-polycytidylic acid (polyI:C) which is a synthetic analogue of dsRNA and TLR4 ligand, ion exchange chromatography purified LPS from *Escherichia coli* serotype O111:B4 were purchased from Sigma–Aldrich. TLR7/8 ligand R848 was from Alexis Biochemicals (Lausen, Switzerland). TLR stimulations were performed in RPMI-1640 medium in the presence of 10% FCS with the following ligand concentrations; polyI:C 30 μg/ml, LPS 100 ng/ml, and R848 10 μM. Cycloheximide (CHX) in dimethylsulfoxide (DMSO) (Sigma–Aldrich) was used at 10 μg/ml. Normal sheep antiserum and antisera specific for human IFN-α and IFN-β (containing 450 000 neutralizing U/ml for IFN-α and 3000 neutralizing U/ml for IFN-β, respectively) (Mogensen et al., 1975) were used. Recombinant IFN-β from Schering-Plough (Kenilworth, NJ, USA) was used at a concentration of 100 IU/ml and recombinant IFN-λ1 (Osterlund et al., 2005) from ZymoGenetics (Seattle, WA, USA) was used at a concentration of 10 ng/ml. Pharmacological inhibitors used were PD98059 (MEK1 inhibitor used at 10 μM) and Ly294002 (PI3K inhibitor, 50 μM) from Calbiochem (Darmstadt, Germany), SB202190 (p38 inhibitor, 5 μM), SP600125 (SAPK/JNK inhibitor, 10 μM) and pyrrolidine dithiocarbamate PDTC (NF-κB inhibitor, 100 μM) from Alexis Biochemicals. CHX and inhibitors were added to cells 30 min prior to stimulation with TLR ligands. IFN antiserum was added to the cells 1 h after TLR stimulation.

2.3. Quantitative real time PCR (qPCR)

Total cellular RNA was isolated from moDCs derived from three to four pooled donors using TRIZOL and the Qiagen Rneasy Mini kit. One microgram of total cellular RNA was reverse transcribed into cDNA in TaqMan RT buffer with 5.5 mM $MgCl_2$, 500 μM dNTPs, 2.5 μM oligo $d(T)_{16}$, 0.4 U/μl RNase inhibitor and 1.25 U/μl MultiScribe reverse transcriptase (Applied Biosystems, Foster City, CA, USA). cDNA samples were then amplified in TaqMan universal PCR master mix buffer (Applied Biosystems) with Gene Expression system assay mix oligonucleotides (Applied Biosystems) to analyze mRNA levels for IFN-α1 (Hs00256882_s1), IFN-β1 (Hs00277188_s1), IFN-λ1 (Hs00601677_g1), IFN-λ2/3 (Hs00820125_g1), IL-6 (Hs00174131-m1), IL-12p40 (Hs00233688_m1), CXCL10 (Hs00171042_m1), Mx1 (Hs00182073_m1), IRF7 (Hs00242190_g1), TLR3 (Hs00152933), TLR4 (Hs01061963_m1), TLR7 (Hs00152971_m1), TLR8 (Hs00607866_mh) and β-actin (Hs99999903_m1). All cDNA samples were amplified in duplicates with Stratagene Mx3500P (La Jolla, CA, USA). The mRNA levels were normalized against β-actin mRNA and the amounts of cytokine mRNA relative to unstimulated cells were calculated with $\Delta\Delta$Ct-method.

2.4. ELISA

The secreted levels of IFN- β and IFN- λ1 were analyzed from cell culture supernatants with an enzyme-linked immunosorbent assay (ELISA) kit supplied by PBL Biomedical Laboratories (Piscataway, NJ) or with a Duoset ELISA kit (R&D Systems, Minneapolis, MN), respectively.

2.5. Oligonucleotide precipitation assay

TLR ligand-induced activation of transcription factors was studied by oligonucleotide precipitation method (Osterlund et al., 2005) using IFN stimulated response element (ISRE) binding site-specific oligonucleotide sequences from the IFN-λ1 promoter (Osterlund et al., 2007). Equal amounts of cells were harvested and nuclear extracts were prepared by lysing the cells in a buffer containing 10 mM HEPES–KOH, 10 mM KCl, 1.5 mM $MgCl_2$, 0.5 mM DTT, 1 mM Na_3VO_4, and protease inhibitor mixture (Complete, Roche Diagnostics, Mannheim, Germany). The remaining nuclei were lysed in 400 mM KCl with 10 mM HEPES, 10% glycerol, 2 mM EDTA, 1 mM EGTA, 0.1% Triton X-100, 0,5 mM DTT, 1 mM Na_3VO_4 and a protease inhibitor mixture. The forward oligonucleotide was 5′-biotinylated and a BamHI site was added as a spacer (DNA Technology A/S, Aarhus, Denmark). Oligonucleotides were incubated at +4 °C for 2 h with streptavidin-agarose beads (Pierce, Rockford, IL, USA). The unbound oligonucleotide was washed after which 130 μg of the protein lysates were incubated with the agarose-bound oligonu-

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