

# MAP kinase p38 $\alpha$ regulates type III interferon (*IFN- $\lambda$ 1*) gene expression in human monocyte-derived dendritic cells in response to RNA stimulation

Miao Jiang,<sup>\*,†,‡,1</sup> Pamela Österlund,<sup>\*</sup> Riku Fagerlund,<sup>§</sup> Diana N. Rios,<sup>§</sup> Alexander Hoffmann,<sup>§,¶</sup> Minna M. Poranen,<sup>‡</sup> Dennis H. Bamford,<sup>†,‡</sup> and Ilkka Julkunen<sup>\*,||,1</sup>

<sup>\*</sup>Virology Unit, Department of Infectious Disease Surveillance and Control, National Institute for Health and Welfare, Helsinki, Finland; <sup>§</sup>Department of Chemistry and Biochemistry, University of California, San Diego, La Jolla, California, USA; <sup>¶</sup>Department of Microbiology, Immunology, and Molecular Genetics, Institute for Quantitative and Computational Biosciences, University of California, Los Angeles, California, USA; <sup>†</sup>Institute of Biotechnology and <sup>‡</sup>Department of Biosciences, University of Helsinki, Finland; and <sup>||</sup>Department of Virology, University of Turku, Finland

RECEIVED JANUARY 29, 2014; REVISED OCTOBER 22, 2014; ACCEPTED OCTOBER 23, 2014. DOI: 10.1189/jlb.2A0114-059RR

## ABSTRACT

Recognition of viral nucleic acids leads to type I and type III *IFN* gene expression and activation of host antiviral responses. At present, type III *IFN* genes are the least well-characterized *IFN* types. Here, we demonstrate that the p38 MAPK signaling pathway is involved in regulating *IFN- $\lambda$ 1* gene expression in response to various types of RNA molecules in human moDCs. Inhibition of p38 MAPK strongly reduced *IFN* gene expression, and overexpression of p38 $\alpha$  MAPK enhanced *IFN- $\lambda$ 1* gene expression in RNA-stimulated moDCs. The regulation of *IFN* gene expression by p38 MAPK signaling was independent of protein synthesis and thus, a direct result of RNA stimulation. Moreover, the RIG-I/MDA5-MAVS-IRF3 pathway was required for p38 $\alpha$  MAPK to up-regulate *IFN- $\lambda$ 1* promoter activation, whereas the MyD88-IRF7 pathway was not needed, and the regulation was not involved directly in IRF7-dependent *IFN- $\alpha$ 1* gene expression. The stimulatory effect of p38 $\alpha$  MAPK on *IFN- $\lambda$ 1* mRNA expression in human moDCs did not take place directly via the activating TBK1/IKK $\epsilon$  complex, but rather, it occurred through some other parallel pathways. Furthermore, mutations in ISRE and NF- $\kappa$ B binding sites in the promoter region of the *IFN- $\lambda$ 1* gene led to a significant reduction in p38 $\alpha$  MAPK-mediated *IFN* responses after RNA stimulation. Altogether, our data suggest that the p38 $\alpha$  MAPK pathway is linked with RLR signaling pathways and regulates the expression of early *IFN*

genes after RNA stimulation cooperatively with IRF3 and NF- $\kappa$ B to induce antiviral responses further. *J. Leukoc. Biol.* **97**: 307–320; 2015.

## Introduction

Innate immune responses constitute the first-line defense system against invading microbial pathogens, and they also play an essential role in regulating antigen-specific adaptive-immune responses. The first step in the activation of innate immune responses is the recognition of pathogen-associated molecular patterns by PRRs [1]. The *IFN* system, including type I and type III *IFN*, is activated as a host response to viral infection, and it forms an important part of innate antiviral responses by interfering directly with the replication of viruses and activating downstream *IFN*-stimulated genes to elicit subsequent antiviral responses [2–5]. As sentinel cells of the host, DCs are the key mediators linking innate and adaptive immunity. They are professional APCs, playing a pivotal role in initiation, regulation, and maintenance of innate and antigen-specific adaptive-immune responses against viruses [6]. Human DCs express multiple RNA-sensing PRRs that recognize viral genomic RNA or RNA replication intermediates during infection [7]. RLRs, such as RIG-I and MDA5, reside in the cytoplasm, where they preferentially recognize uncapped RNA molecules containing 5'-triphosphate moieties and base-paired regions or long dsRNAs, respectively [8–12]. In contrast to RLRs, TLR3 senses viral dsRNA on the plasma membrane or in endosomes, depending on the cell type, whereas TLR7 and TLR8 sense ssRNA in endosomes [13, 14]. Upon binding with RNAs, RLRs and TLRs activate their downstream signaling pathways via different adaptors.

Abbreviations:  $\Delta$  = change/difference, ATF = activating transcription factor, CHX = cycloheximide, Ct = threshold cycle, DC = dendritic cell, dnp38 = dominant-negative form of p38 $\alpha$ , HEK293 = human embryonic kidney cell 293, IAV = influenza A virus, IKK = I $\kappa$ B kinase, IRF = *IFN*-regulatory factor, ISRE = *IFN*-stimulated response element, KO = knockout, MAPKAPK = MAPK-activated protein kinase, MAVS = mitochondrial antiviral-signaling protein,

(continued on next page)

The online version of this paper, found at [www.jleukbio.org](http://www.jleukbio.org), includes supplemental information.

1. Correspondence: M.J., Mannerheimintie 166, 00300, Helsinki, Finland. E-mail: miao.jiang@helsinki.fi or miao.jiang@thl.fi; I.J., University of Turku, FIN-20520 Turku, Finland. E-mail: ilkka.julkunen@thl.fi or ilkka.julkunen@utu.fi

Mitochondria-associated adaptor molecule IFN- $\beta$  promoter stimulator 1 (also called MAVS, Cardif, or virus-induced signaling adaptor) is recruited by activated RIG-I or MDA5 [15–18], whereas TRIF is recruited by TLR3 activation. In addition, the adaptor protein MyD88 is mediating TLR7- and TLR8-dependent signaling [19]. The stimulation of both RLR and TLR signaling pathways results in the activation of different transcription factors, such as NF- $\kappa$ B and IRF3 and -7, leading to the expression of *IFN* genes and activation of subsequent antiviral responses [14, 20–23].

MAPKs are ubiquitously expressed serine/threonine kinases that are engaged in regulating multiple cellular functions, including gene expression, cell proliferation, differentiation, and apoptosis [24]. The MAPK family includes 3 major groups: 1) ERKs, which are stimulated by growth factors and mainly regulate cell proliferation, 2) JNKs, and 3) p38 MAPKs, which are activated by cytokines and cell stress and mediate cellular stress responses and apoptosis [25–28]. The p38 MAPK family consists of 4 isoforms of p38 kinases: 1) p38 $\alpha$  [29], 2) p38 $\beta$  [30], 3) p38 $\delta$  [31] and 4) p38 $\gamma$  [32], among which p38 $\alpha$  MAPK is ubiquitously expressed at high levels in almost all cell types [33]. Moreover, previous studies have shown that the p38 MAPK pathway is activated by type I IFNs, and it is also involved in regulating type I IFN-dependent antiviral responses [34, 35].

Activation of IFN responses is regulated by at least 3 groups of transcription factors: 1) ATF/c-jun, 2) IRFs, and 3) NF- $\kappa$ B. They bind to specific sites on promoters upstream of *IFN* genes and cooperatively activate the transcription [36]. The promoter regions of all type I and type III *IFN* genes contain PRDI/III-like motifs, which function as IRF-specific binding sites, indicating that IRFs play an important role in transcriptional regulation of type I and III *IFN* genes. Among 9 IRF family members, IRF3 and IRF7 are essential for regulating *IFN* gene expression [37–39]. Besides PRDI/III-like motifs, the promoter regions of *IFN*- $\beta$ , *IFN*- $\lambda$ 1, and *IFN*- $\lambda$ 3 genes also contain a NF- $\kappa$ B-binding motif, suggesting that these genes are regulated by IRFs and NF- $\kappa$ B [40].

The mechanism of induction and regulation of type I IFN (IFN- $\alpha$ / $\beta$ ) signaling have been investigated extensively during the last decades. Our previous study also showed that RNAs of different sizes and forms induce differential *IFN* gene expression via different signaling pathways in human moDCs [41]. The divergent cellular signaling pathways activated by various types of RNAs may lead to differential activation of transcription factors and *IFN* promoters. Relatively little is known about the mechanisms by which signaling pathways, apart from those leading to the activation of IRFs and NF- $\kappa$ B, are involved in regulating *IFN* gene expression. Moreover, less attention has been paid to the structure of promoter regions of type III *IFN* genes, such as *IFN*- $\lambda$ 1. In the current study, by use of a pharmacological approach to inhibit different signaling

pathways, we show that several signaling pathways are involved in regulating *IFN*- $\lambda$ 1 gene expression after stimulation with different types of RNAs in human moDCs. Furthermore, with the experimental approach of nucleofection of the p38 $\alpha$  MAPK expression plasmid in human moDCs and overexpression of p38 $\alpha$  MAPK or the dnp38 MAPK in HEK293 cells, our study strongly suggests that p38 $\alpha$  MAPK positively regulates the induction of the *IFN*- $\lambda$ 1 gene. We also obtained evidence that the RIG-I/MDA5-MAVS-IRF3 signaling pathway but not the MyD88-IRF7 pathway is essential for *IFN* gene regulation by p38 $\alpha$  MAPK. Moreover, p38 $\alpha$  MAPK appears to regulate *IFN* gene expression via a pathway apart from the activation of TBK1/IKK $\epsilon$ . With the use of mouse 3T3 IRF3/IRF7 and NF- $\kappa$ B KO cells, we observed that these transcription factors are essential in regulating *IFN*- $\beta$ 1 gene expression, and overexpression of p38 $\alpha$  MAPK could not compensate for the lack of IRFs or NF- $\kappa$ B transcription factors. The DNA affinity-binding assay in human moDCs and the promoter mutation assay in HEK cells further confirmed that ISRE and NF- $\kappa$ B1 binding sites of the *IFN*- $\lambda$ 1 promoter are essential for p38 MAPK to regulate *IFN*- $\lambda$ 1 gene expression. Altogether, our data suggest that p38 $\alpha$  MAPK positively regulates the induction of the *IFN*- $\lambda$ 1 gene cooperatively with IRF3 or NF- $\kappa$ B transcription factors in RNA-stimulated human moDCs.

## MATERIALS AND METHODS

### Cell cultures

The HEK293 cell line (ATCC CLR 1573; American Type Culture Collection, Manassas, VA, USA) was maintained with continuous growth in Eagle's MEM (Sigma-Aldrich, St. Louis, MO, USA).

The primary WT and *Ir3 $\beta$ <sup>-/-</sup>Ir7 $\gamma$ <sup>-/-</sup>* MEFs and the *RelA<sup>-/-</sup>c-Rel<sup>-/-</sup>Nfkb1<sup>-/-</sup>* (Nfkb<sup>-/-</sup>) 3T3 cell lines were generated from E12.5–14.5 embryos and maintained as described previously [42]. Primary MEFs were immortalized by a rigorous passaging protocol to obtain WT and IRF3/7 KO 3T3 cell lines. The MEFs and 3T3 cell lines were maintained with continuous growth in DMEM (Sigma-Aldrich).

Human primary 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 [43]. Monocytes were differentiated into immature DCs for 6 d in the presence of 10 ng/ml human rGM-CSF (BioSource International, Camarillo, CA, USA), and 20 ng/ml human rIL-4 (R&D Systems, Minneapolis, MN, USA) in RPMI-1640 medium.

All media were supplemented with 0.6  $\mu$ g/ml penicillin, 60  $\mu$ g/ml streptomycin, 2 mM L-glutamine, 20 mM HEPES, and 10% FCS (Integro, Englewood, CO, USA). All cells were maintained at 37°C in a humidified atmosphere in the presence of 5% CO<sub>2</sub>.

### RNA preparations

**Enzymatic synthesis of ssRNA and dsRNA molecules.** ssRNAs and dsRNAs were produced by in vitro transcription and replication with bacteriophage T7 DNA-dependent RNA polymerase and bacteriophage  $\phi$ 6 RNA-dependent RNA polymerase, as described previously [41]. Enzymatically synthesized ssRNA and dsRNA molecules were purified with TRIzol (Invitrogen, Carlsbad, CA, USA)/chloroform (5:1) extraction, followed by preparative agarose gel electrophoresis and precipitation with 3 M sodium acetate (88 nt RNAs) or stepwise lithium chloride precipitation (1800 nt RNAs). All RNAs were washed with 70% ethanol, dissolved in sterile water, and precipitated with 0.75 M NH<sub>4</sub>Ac–70% ethanol. The pellets were suspended in sterile water and desalted with NAP-5 columns (GE Healthcare, Pittsburgh, PA, USA) before HPLC purification (Gen-Pak FAX; Waters, Milford, MA, USA).

(continued from previous page)

MDA5 = melanoma differentiation-associated gene 5, MEF = mouse embryonic fibroblast, moDC = monocyte-derived dendritic cell, pcDNA = plasmid construct DNA, PRD = positive regulatory domain, PRR = pattern recognition receptor, PVDF = polyvinylidene difluoride, RIG-I = retinoic acid-inducible gene I, RLR = retinoic acid-inducible gene-like receptor, RSV = Rous sarcoma virus, TBK = TNFR-associated factor family member-associated NF- $\kappa$ B activator-binding kinase, TRIF = Toll/IL-1R domain-containing adaptor-inducing IFN- $\beta$ , WT = wild-type

**H3N2 IAV viral RNAs.** IAV/Beijing/353/89 virus was concentrated by sedimentation through a 30% sucrose cushion by ultracentrifugation at 26,000 rpm in an SW32 rotor for 90 min (Beckman Coulter, Brea, CA, USA). Viral RNA was isolated from virions by use of the QIAmp viral RNA extraction kit (Qiagen, Valencia, CA, USA).

**RNAs isolated from H3N2 IAV-infected moDCs.** moDCs from 4 different blood donors were infected separately with H3N2 IAV. The infected moDCs from different blood donors were harvested and pooled, and total cellular RNA was isolated by use of the RNeasy Mini Kit (Qiagen), including DNase digestion (RNase-free DNase kit; Qiagen).

## Expression plasmid of p38 $\alpha$ MAPK, dnp38 MAPK, and IFN- $\lambda$ 1 promoter mutants

The cDNA encoding for p38 $\alpha$  MAPK was amplified from total cellular RNA isolated from human moDCs and cloned into the *Bam*HI site of the pcDNA3.1(+) expression vector by use of primers (upstream) 5' TCGGGATCCATGTCTCAGGAGAGGCCACGT 3' and (downstream) 5' TCGGGATCCCTTATCAGGACTCATCTCTTCTTGGTC 3' (*Bam*HI site underlined; initiation codon in bold). This construct served as the template to generate further the plasmid of dnp38 MAPK by replacing threonine 180 and tyrosine 182 with alanine and phenylalanine, respectively. The oligonucleotides used to create the dnp38 MAPK construct are 5'-CAGATGATGAAATGGCAGGCTTCGTGGCCACTAGG-3' and 5'-CCTAGTGGCCACGAAGCCTGCCATTTTCATCATCTG-3'. Mutations were incorporated by use of the QuikChange Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA, USA).

Plasmid pGL3-IFN- $\lambda$ 1-luc [40] was applied as a template in the generation of the single-site mutations at the ISRE, NF- $\kappa$ B1, or NF- $\kappa$ B2 sites of the IFN- $\lambda$ 1 promoter region. The construct harboring the mutation at the ISRE site of the IFN- $\lambda$ 1 promoter region was used further as a template to generate an ISRE/NF- $\kappa$ B1 double mutant, in which the ISRE and NF- $\kappa$ B1 sites were mutated. The oligonucleotides used to generate the promoter mutants are described elsewhere [44]. Mutations were incorporated by use of the QuikChange Site-Directed Mutagenesis Kit. All plasmids were maintained and propagated in *Escherichia coli* strain DH5 $\alpha$ .

## Stimulation of cells with RNA molecules

**moDC stimulations.** All experiments were performed with moDCs obtained from 4 blood donors, and the cells were stimulated separately. Cells were first treated with different pharmacological signaling inhibitors (10  $\mu$ M) or CHX (10  $\mu$ g/ml), 0.5 h before RNA transfection. Enzymatically synthesized RNAs, viral RNAs isolated from H3N2 IAV virions, or RNAs isolated from H3N2 IAV-infected moDCs (50 ng/ml) were transfected with Lipofectamine 2000 (Invitrogen) into moDCs, according to the manufacturer's instructions. Four hours after transfection, cells from different donors were collected and pooled. As there is a considerable individual variation in the responses in different blood donors (see Supplemental Fig. 2C), we used pooled cellular RNA specimens to obtain a more global view of RNA-stimulated responses in moDCs.

**HEK293 cell stimulations.** HEK293 cells were plated on 12-well culture plates ( $5 \times 10^5$  cells/well), 1 d before transfection, after which, the expression plasmids of p38 $\alpha$  MAPK, dnp38, RIG-I, or empty vectors (pcDNA) were transfected into cells by use of TransIT-LT1 transfection reagent (Mirus Bio, Madison, WI, USA). Sixteen hours after the plasmid transfection, 88 bp dsRNA (200 ng/ml) molecules was transfected into the cells with Lipofectamine 2000 (Invitrogen). Three hours after RNA transfection, cells were used for isolation of total cellular RNA or lysed for protein samples with Passive Lysis buffer (Dual Glo kit; Promega, Madison, WI, USA).

**Mouse 3T3 cell stimulations.** 3T3 WT, IRF3/IRF7 KO 3T3, or NF- $\kappa$ B KO 3T3 cells were plated on 12-well culture plates ( $5 \times 10^5$  cells/well), 1 d before transfection, after which, the expression plasmids of p38 $\alpha$  MAPK, RIG-I, or empty vectors (pcDNA) were transfected into cells by use of TransIT-LT1 transfection reagent (Mirus Bio). Five hours after the plasmid transfection, 88 bp dsRNA (100 ng/ml) molecules were transfected into the cells with Lipofectamine 2000 (Invitrogen). Three hours after RNA transfection, cells were used for isolation of total cellular RNA or lysed for protein samples with Passive Lysis buffer (Dual Glo kit; Promega).

## Nucleofection in moDCs

Monocytes were differentiated into immature DCs for 6 d in the presence of 10 ng/ml human rGM-CSF (BioSource International) and 20 ng/ml human rIL-4 (R&D Systems) in RPMI-1640 medium, as described above. Cells ( $2 \times 10^6$ ) were then collected and resuspended in 100  $\mu$ l nucleofector solution and combined with 5  $\mu$ g plasmid, followed by the nucleofection, according to the manual of the nucleofector kit (Amaxa Human Dendritic Cell Nucleofector kit; Lonza, Cologne, Germany). Transfected cells were then plated onto a 12-well plate and incubated in a humidified 37°C/5% CO $_2$  incubator for a further 20 h, followed by RNA stimulation.

## Luciferase reporter assay

HEK293 cells were plated in 96-well culture plates ( $3 \times 10^4$  cells/well), 1 d before transfection. Cells were first transfected with IFN- $\lambda$ 1/ $\beta$ / $\alpha$ 1 promoter or IFN- $\lambda$ 1 mutant promoter-reporter constructs that harbor the firefly luciferase gene under the IFN gene promoter [40, 45] together with expression plasmids (see above) by use of TransIT-LT1 transfection reagent (Mirus Bio). Renilla luciferase plasmid (Promega) was used to control transfection efficiency. RNAs (20 ng/well) were transfected with Lipofectamine 2000 (Invitrogen), 4 h after initial transfection with reporter and expression plasmids, according to the manufacturer's instructions. Luciferase assays were performed 20 h after RNA stimulation with a Dual Glo kit (Promega), according to the manufacturer's instructions, and the results were normalized to the intensity of Renilla luciferase (Promega).

## Quantitative RT-PCR

Total cellular RNA was isolated from moDCs derived from 4 pooled donors or from HEK293 cells by use of the RNeasy Mini Kit. DNase-treated total cellular RNA (1  $\mu$ g) was reverse transcribed into cDNA by use of the TaqMan RT kit (Applied Biosystems, Carlsbad, CA, USA). cDNA samples were then amplified by use of a TaqMan Universal PCR Master Mix buffer (Applied Biosystems) and the commercial gene expression system assay (Applied Biosystems) with primers and probes for human IFN- $\alpha$ 1 (Hs00256882\_s1), IFN- $\beta$  (Hs00277188\_s1), IFN- $\lambda$ 1 (Hs00601677\_g1), and  $\beta$ -ACTIN (Hs99999903\_ml) or mouse *Irf- $\beta$* 1 (Mm00439552\_s1) and *Gapd* (4352339E-1207040). Each cDNA sample was amplified in duplicate with an Mx3005P QPCR System (Stratagene). The relative amount of cytokine mRNAs was calculated with the  $\Delta\Delta C_t$  method by use of human  $\beta$ -ACTIN or mouse *Gapd* mRNA in standardization.

## Western blot analysis

Whole-cell lysates were prepared from moDCs, derived from 4 pooled donors or from HEK293 cells in Passive Lysis buffer of the Dual Luciferase assay kit (Promega) containing 1 mM Na $_3$ VO $_4$ . Protein aliquots of whole-cell lysates (30  $\mu$ g) were separated on 10% SDS polyacrylamide gels by use of the Laemmli buffer system [46]. Proteins were transferred onto Immobilon-P membranes, followed by blocking with 5% milk in PBS (or in TBS for cell-signaling anti-phospho-protein antibodies). Rabbit anti-IRF3 antibody, guinea pig anti-RIG-I, and anti-IRF7 were used, as described previously [41, 47]. Antibodies against the phosphorylated forms of IRF3 (Ser396), the phosphorylated forms of p38 MAPK (T180/Y182), TRIF, and p38 MAPK were from Cell Signaling Technology (Beverly, MA, USA). Rabbit anti-MyD88 and anti-MAVS antibodies were prepared by immunizing rabbits with baculovirus-expressed, preparative SDS-PAGE (Bio-Rad Laboratories, Hercules, CA, USA)-purified proteins (4 immunizations, 20  $\mu$ g/immunization). HRP-conjugated goat anti-rabbit or rabbit anti-guinea pig antibodies (DakoCytomation, Carpinteria, CA, USA) were used in secondary staining. Antibody binding was visualized by the ECL system on HyperMax films (GE Healthcare). Software ImageJ was used for densitometry analysis of Western blots.

## DNA affinity-binding assay

dsRNA-induced activation of transcription factors was studied by use of the DNA affinity-binding method, as described previously [47]. The used, putative ISRE and NF- $\kappa$ B1 binding-site oligonucleotide sequences from the IFN- $\lambda$ 1



promoters were described elsewhere [40, 47]. The bound proteins were separated on SDS-PAGE and visualized by use of specific antibodies against the phosphorylated form of IRF3 (Ser396) and against p65 and p50 proteins (Santa Cruz Biotechnology, Santa Cruz, CA, USA).

### Statistical analysis

All experiments are presented as means  $\pm$  sd. Statistical analyses were performed by use of unpaired, two-tailed Student's *t*-test. The differences were considered to be statistically significant when  $P < 0.05$ .

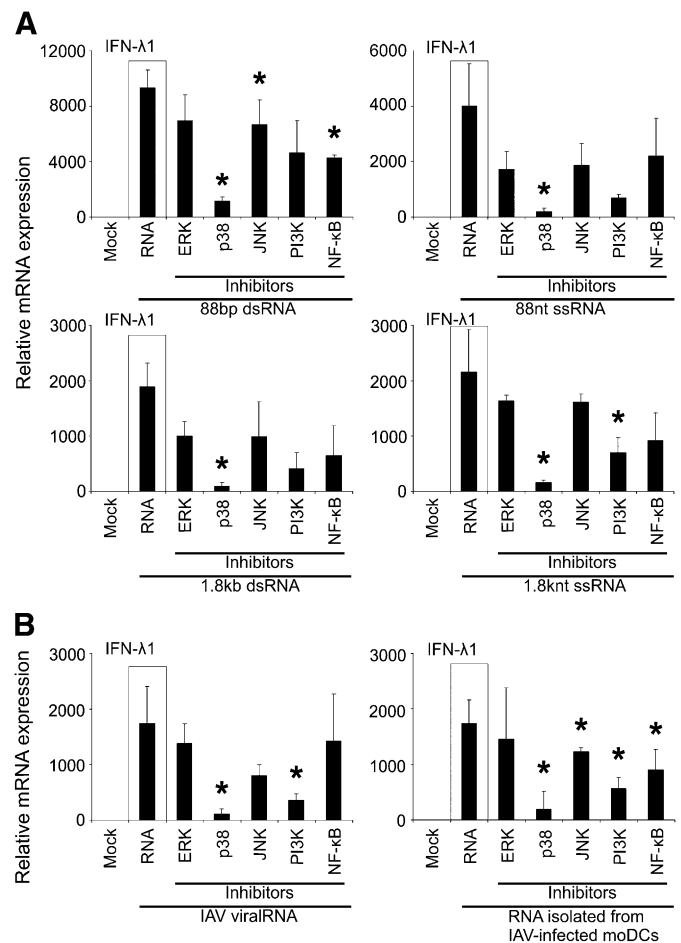
## RESULTS

### Effects of MAPK, PI3K, and NF- $\kappa$ B inhibitors on RNA-induced *IFN- $\lambda$ 1* gene expression in human moDCs

In human moDCs, different types of RNAs can induce type III *IFN* gene expression, such as *IFN- $\lambda$ 1*, through multiple PRR signaling pathways with different efficiency [41]. To investigate further the role of multiple signaling pathways in regulating *IFN- $\lambda$ 1* gene expression after RNA stimulation, we analyzed the role of several pharmacological inhibitors on the key component of selected signaling pathways. moDCs were transfected with various types of enzymatically synthesized ds/ssRNAs in the presence or absence of inhibitors PD98059 (ERK inhibitor), SB202190 (p38 MAPK inhibitor), SP600125 (JNK inhibitor), Ly294002 (PI3K inhibitor), and pyrrolidine dithiocarbamate (NF- $\kappa$ B inhibitor). The expression of *IFN- $\lambda$ 1*, *IFN- $\beta$* , and *IFN- $\alpha$ 1* was studied at the mRNA level at 4 h after RNA stimulation (Fig. 1A and Supplemental Fig. 1A and B). Consistent with our previous study [41], short dsRNA and ssRNA molecules induced higher levels of *IFN- $\lambda$ 1* mRNA expression compared with long RNA molecules. We observed that all studied inhibitors suppressed the up-regulation of *IFN- $\lambda$ 1* mRNA after RNA stimulation to a varying degree. Notably, the production of *IFN- $\lambda$ 1* mRNA was most dramatically and significantly inhibited by the p38 MAPK inhibitor, SB202190 (Fig. 1A and Supplemental Fig. 2B), and this inhibition was clearly dose dependent (Supplemental Fig. 2A). Similar results were seen with the *IFN- $\beta$*  and *IFN- $\alpha$ 1* genes (Supplemental Fig. 1A and B). The explicit inhibition of *IFN- $\lambda$ 1* gene expression by the p38 MAPK inhibitor, SB202190 was also evident in cells from all different blood donors that were analyzed separately (Supplemental Fig. 2C). To study the ability of real viral RNA to induce *IFN- $\lambda$ 1* mRNA expression, viral RNA isolated from purified IAV virions or RNA isolated from IAV-infected moDCs was used to stimulate moDCs, in the presence or absence of signaling inhibitors (Fig. 1B). Both RNA preparations induced the expression of *IFN- $\lambda$ 1* (Fig. 1B) and *IFN- $\alpha$ 1* (Supplemental Fig. 1C) mRNAs. Furthermore, the p38 MAPK inhibitor suppressed the expression of *IFN- $\lambda$ 1* (Fig. 1B) and *IFN- $\alpha$ 1* (Supplemental Fig. 1C) mRNAs after stimulation with viral RNA or cellular RNAs from IAV-infected moDCs.

### p38 MAPK is regulating *IFN- $\lambda$ 1* gene expression in RNA-stimulated human moDCs

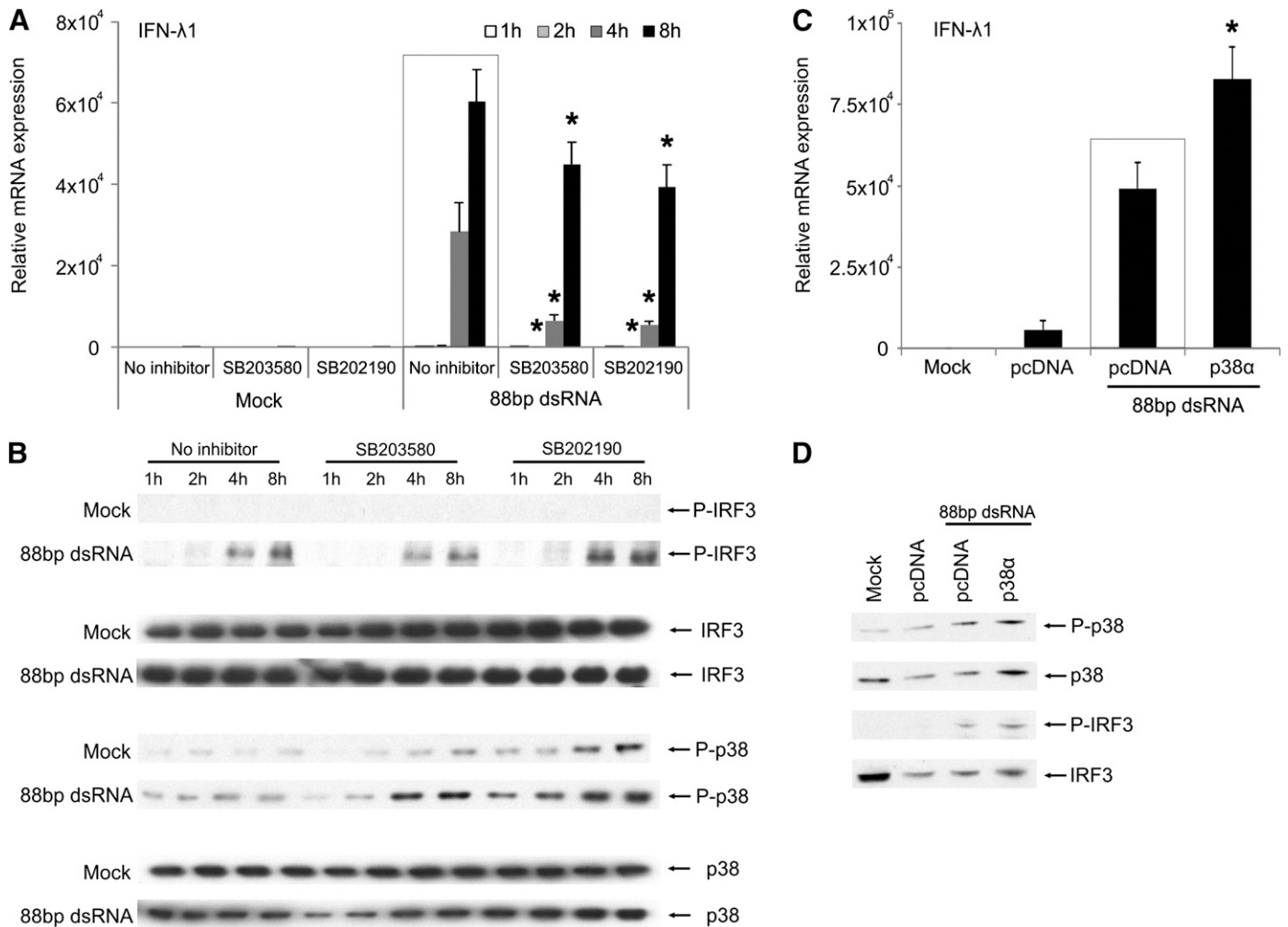
To investigate further the role of p38 MAPK in regulating *IFN- $\lambda$ 1* gene expression in RNA-stimulated human moDCs, we analyzed the time-dependent kinetics of 2 p38 MAPK inhibitors SB203580 and SB202190 on regulating *IFN- $\lambda$ 1* mRNA expression. moDCs were transfected with 88 bp dsRNAs in the presence or absence



**Figure 1. Analysis of host cell-signaling pathways regulating *IFN- $\lambda$ 1* mRNA expression in RNA-stimulated human moDCs.** moDCs from 4 different blood donors were grown separately on 24-well plates. Various pharmacological signaling inhibitors (10  $\mu$ M) were added into the growth media, 0.5 h before RNA transfection with different in vitro-synthesized RNAs (50 ng/ml; A) or with viral RNA preparations originating from H3N2 IAV virions or RNA extracted from H3N2 IAV-infected moDCs (B). Cells were collected at 4 h after RNA transfection. Total cellular RNA was isolated, and relative expression of *IFN- $\lambda$ 1* mRNA was measured by quantitative RT-PCR. The values were normalized against human  $\beta$ -ACTIN mRNA, and relative expression levels were calculated with the  $\Delta\Delta$ Ct method by use of untreated cells as a calibrator. The means ( $\pm$ sd) of 3 parallel determinations are shown. The data are representative of 3 individual experiments. Results were considered statistically significant when  $*P < 0.05$  compared with the uninhibited samples (boxed bars).

of inhibitors, and *IFN- $\lambda$ 1* mRNA expression was studied at 1, 2, 4, and 8 h after RNA stimulation. Both inhibitors suppressed the up-regulation of *IFN- $\lambda$ 1* mRNA expression in a similar fashion, and inhibition was already observed at 2 h after RNA stimulation, reaching the highest level at 4 h and reducing within 8 h after stimulation (Fig. 2A).

It is well known that multiple *IFN* types are regulated by IRF3 [5, 40]. Next, we investigated whether the p38 MAPK was involved in dsRNA-induced phosphorylation of IRF3 in human moDCs. The expression of phosphorylated IRF3, phosphorylated p38, total cellular IRF3, and p38 was analyzed by Western blotting



**Figure 2. The role of p38 MAPK in regulating *IFN- $\lambda$ 1* mRNA expression in short dsRNA-stimulated human moDCs.** moDCs from 4 different blood donors were grown separately on 24-well plates. (A) Cells remained untreated or were pretreated with p38 MAPK inhibitor SB203580 or SB202190 (10  $\mu$ M), 0.5 h before transfection with 88 bp dsRNA (50 ng/ml). Cells were collected at indicated time-points after RNA transfection, total cellular RNA was isolated, and *IFN- $\lambda$ 1* mRNA expression was determined by quantitative RT-PCR. (C) *IFN- $\lambda$ 1* mRNA expression in short dsRNA-stimulated human moDCs after nucleofection of p38 $\alpha$  MAPK. Cells ( $2 \times 10^5$  cells/well) were un-nucleofected or nucleofected with an empty pcDNA or a p38 $\alpha$  MAPK-expressing plasmid (5  $\mu$ g/well), 20 h before transfection of 88 bp dsRNA (50 ng/ml); cells were collected 4 h after RNA transfection; total cellular RNA was isolated; and relative expression of *IFN- $\lambda$ 1* mRNA was measured by quantitative RT-PCR. The values were normalized against  $\beta$ -ACTIN, and relative *IFN- $\lambda$ 1* mRNA levels were calculated with the  $\Delta\Delta$ Ct method by use of untreated cells as a calibrator. The means ( $\pm$ SD) of 3 parallel determinations are shown. Data are representative of 3 individual experiments. Results were considered statistically significant when  $*P < 0.05$  compared with uninhibited and pcDNA-transfected cellular samples, respectively (boxed bars). (B and D) Western blot analysis for the expression of phospho (P)-IRF3 and total IRF3 protein and phospho (P)-p38 MAPK and total p38 protein in short dsRNA-stimulated human moDCs. Cells were collected at 1, 2, 4, and 8 h after RNA transfection, with or without the presence of p38 MAPK inhibitors (B) or 4 h after RNA transfection (D), with or without the nucleofection of the p38 MAPK expression plasmid, and whole-cell lysates were prepared. Cellular proteins (30  $\mu$ g/lane) were separated on 10% SDS-PAGE, followed by electrophoretic transfer of the proteins onto PVDF membranes and detection of the transferred proteins by antibodies that are specific for phospho-IRF3, IRF3, phospho-p38, and p38, as indicated. The data of 1 representative experiment of 3 independent experiments are shown.

at different time-points after 88 bp dsRNA stimulation in the presence or absence of SB203580 and SB202190 (Fig. 2B). Consistent with the kinetics of *IFN- $\lambda$ 1* mRNA expression (Fig. 2A), the amount of phosphorylated IRF3 increased up to the 8 h time-point in the absence of p38 MAPK inhibitors (Fig. 2B). Neither one of the inhibitors decreased the phosphorylation of IRF3, whereas the presence of p38 inhibitors led to increased phosphorylation level of p38 at 4 h and 8 h in RNA-stimulated and unstimulated moDCs (Fig. 2B). The total amount of IRF3 and p38 remained relatively constant.

As off-target effects of p38 MAPK inhibitors have been described in some studies [48, 49], we investigated whether p38 MAPK is involved in regulating *IFN- $\lambda$ 1* mRNA expression in human moDCs by nucleofection expression of cDNA for the human p38 $\alpha$  isoform in human moDCs. Among the 4 isoforms of p38 MAPK, p38 $\alpha$  MAPK is the one that is ubiquitously expressed in different cell types [33] and is involved in regulating IFN signaling [35]. The overexpression of p38 $\alpha$  MAPK increased *IFN- $\lambda$ 1* mRNA expression in RNA-stimulated moDCs (Fig. 2C). Western blot analysis showed that the protein amount of

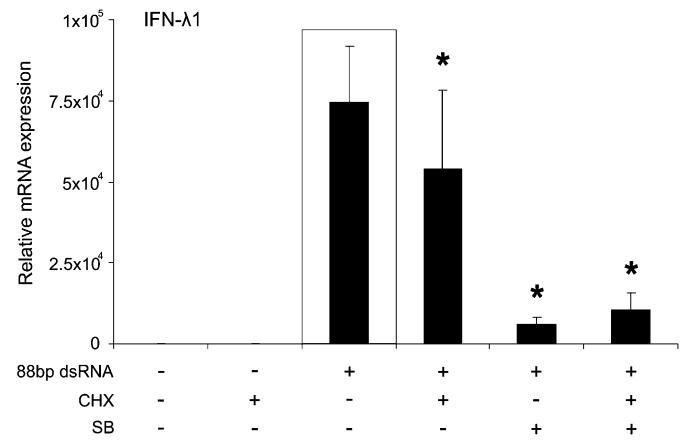
phosphorylated and unphosphorylated p38 MAPK slightly increased after nucleofection of p38 $\alpha$  MAPK in moDCs, and the phosphorylation level of IRF3 was increased after 88 bp dsRNA stimulation, whereas it remained approximately the same after p38 $\alpha$  MAPK overexpression (Fig. 2D).

### dsRNA-induced *IFN- $\lambda$ 1* gene expression is regulated by p38 MAPK in the absence of protein synthesis

To analyze whether ongoing protein synthesis was needed for RNA-induced *IFN- $\lambda$ 1* gene expression via the p38 MAPK pathway, we used CHX to block protein synthesis during the stimulation. As CHX is toxic to the cells, we selected a relatively early time-point of 3 h for our stimulation experiment. Human moDCs were stimulated with 88 bp dsRNA, and *IFN- $\lambda$ 1* mRNA expression was analyzed by quantitative PCR (Fig. 3). We found that 88 bp dsRNA was capable of inducing *IFN- $\lambda$ 1* mRNA expression in the presence and absence of CHX, and CHX did not affect SB202190-mediated inhibition of *IFN- $\lambda$ 1* expression, indicating that the p38 MAPK pathway is involved directly in the regulation of *IFN- $\lambda$ 1* gene expression. Similar results were also obtained for the *IFN- $\alpha$ 1* gene (Supplemental Fig. 3). However, CHX treatment led to superinduction of *IFN- $\alpha$ 1* mRNA expression (Supplemental Fig. 3), which is a previously identified phenomenon related to the accumulation of mRNAs as a result of blocking protein synthesis or of removal of a negative-feedback mechanism of cytokine mRNA degradation.

### The p38 MAPK signaling pathway regulates *IFN- $\lambda$ 1* gene expression through RLR/TLR3 signaling pathways in dsRNA-stimulated HEK293 cells

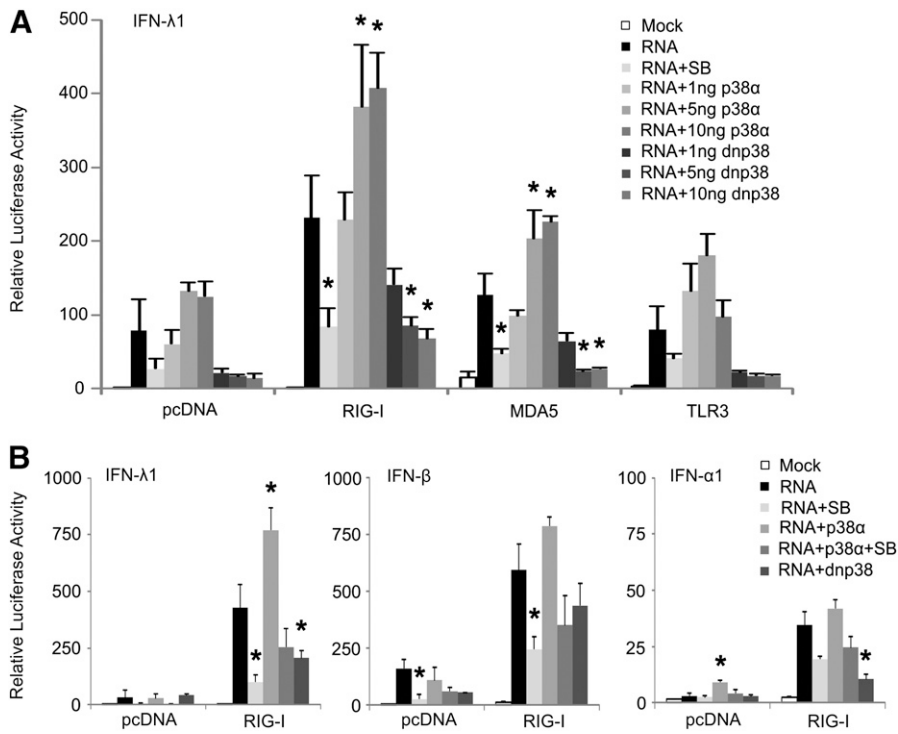
It is well known that RLRs and TLR3 are the main cellular dsRNA-recognizing receptors that activate *IFN* gene expression and antiviral responses [14, 50]. Having seen that the p38 MAPK signaling pathway is involved in regulating *IFN- $\lambda$ 1* gene expression in human moDCs after RNA stimulation (Figs. 1 and 2 and Supplemental Figs. 1 and 2), we transiently expressed different RLR/TLR receptors in HEK293 cells to identify which signaling pathways are positively regulated by p38 MAPK. For these stimulation experiments, the expression construct of p38 $\alpha$  MAPK and dnp38 together with 88 bp dsRNA was used to study the role of p38 $\alpha$  MAPK in dsRNA-regulated *IFN* gene expression. In RIG-I-, MDA5-, and TLR3-overexpressing HEK293 cells, SB202190 inhibited the activation of the *IFN- $\lambda$ 1* gene promoter triggered by 88 bp dsRNA (Fig. 4A), indicating a positive role for p38 $\alpha$  MAPK in RLR/TLR-stimulated gene expression. Overexpression of human p38 $\alpha$  MAPK enhanced the activation of the *IFN- $\lambda$ 1* promoter in a dose-dependent fashion, whereas overexpression of dnp38 decreased the promoter activation (Fig. 4A), further demonstrating the positive role of p38 $\alpha$  MAPK in RLR/TLR signaling. Especially, the inhibition of *IFN- $\lambda$ 1* promoter activation by SB202190 and overexpression of dnp38 and the enhancement of promoter activation by overexpression of p38 $\alpha$  MAPK were more significant in RIG-I- or MDA5-expressing HEK293 cells (Fig. 4A), indicating that the regulation of *IFN- $\lambda$ 1* promoter activation by p38 $\alpha$  MAPK is more likely through RLR signaling. SB202190 was also able to inhibit dsRNA-induced activation of *IFN- $\beta$*  and *IFN- $\alpha$ 1* promoters (Fig. 4B). The positive



**Figure 3. Inhibition of *IFN- $\lambda$ 1* mRNA expression by the p38 MAPK inhibitor in human moDCs after stimulation with short dsRNA molecules, with or without inhibition of protein biosynthesis by CHX.** moDCs from 4 different blood donors were treated separately with CHX (10  $\mu$ g/ml) and/or  $\pm$  SB202190 (SB; 10  $\mu$ M), 1 or 0.5 h before RNA transfection, respectively, as indicated in the figure. The cells were subsequently transfected with 88 bp dsRNA molecules (50 ng/ml) for 3 h. Cells were collected, total cellular RNA was isolated, and relative expression of *IFN- $\lambda$ 1* mRNA was measured by quantitative RT-PCR. The values were normalized against  $\beta$ -ACTIN mRNA, and the relative expression levels were calculated with the  $\Delta\Delta$ Ct method by use of untreated cells as a calibrator. The means ( $\pm$ SD) of 3 parallel determinations are shown. The data are representative of 3 individual experiments. Results were considered statistically significant when  $*P < 0.05$  compared with the dsRNA-stimulated and uninhibited sample (boxed bar).

regulatory role of overexpressed p38 $\alpha$  MAPK was more modest on *IFN- $\beta$*  and *IFN- $\alpha$ 1* promoters compared with that of the *IFN- $\lambda$ 1* promoter, but *IFN- $\beta$*  promoter activation was still sensitive to the inhibitory effect by SB202190, and *IFN- $\alpha$ 1* promoter activation was reduced by overexpression of dnp38 (Fig. 4B).

We also analyzed dsRNA-induced expression of *IFN- $\lambda$ 1* in HEK293 cells in the presence of SB202190 or during overexpression of p38 $\alpha$  MAPK and dnp38 (Fig. 5A). Stimulation of HEK293 cells with 88 bp dsRNA led to the induction of endogenous *IFN- $\lambda$ 1* mRNA expression, and this induction was enhanced with ectopic expression of RIG-I, whereas overexpression of p38 $\alpha$  MAPK or dnp38 could not enhance the induction of *IFN- $\lambda$ 1* mRNA expression (Fig. 5A). Moreover, co-overexpression of RIG-I and p38 $\alpha$  MAPK significantly enhanced the induction of *IFN- $\lambda$ 1* mRNA expression, whereas co-overexpression of RIG-I and dnp38 decreased the induction of the *IFN- $\lambda$ 1* mRNA compared with cells that were transfected only with the RIG-I-expression plasmid (Fig. 5A). Again, in RIG-I-overexpressing cells, the induction of *IFN- $\lambda$ 1* mRNA expression by 88 bp dsRNA was inhibited by SB202190 (Fig. 5A). Western blot analysis showed that the amount of phosphorylated p38 was increased after transfection with 88 bp dsRNA and decreased in dnp38-overexpressing cells (Fig. 5B, 1st row). Moreover, after 88 bp dsRNA stimulation, the phosphorylation level of IRF3 was increased in RIG-I overexpressing HEK293 cells compared with cells that were not overexpressing RIG-I, and co-overexpression of RIG-I together with p38 $\alpha$  MAPK or dnp38 did not affect the phosphorylation of IRF3 compared with RIG-I-overexpressing



**Figure 4. Regulation of *IFN* gene promoter activity by the p38 MAPK signaling pathway in HEK293 cells overexpressing dsRNA receptors after stimulation with short dsRNA molecules.** HEK293 cells (in 96-well plates;  $3 \times 10^4$  cells/well) remained untreated or were pretreated with the p38 MAPK inhibitor SB202190 (10  $\mu$ M), 0.5 h before transfection with pcDNA (empty vector) or RIG-I, MDA5, or TLR3 (20 ng/well) expression plasmids, a construct containing the firefly luciferase gene under the *IFN $\lambda$ 1* promoter (20 ng/well) and RSV-Renilla luciferase plasmids (5 ng/well). Selected transfections were supplemented further with the p38 $\alpha$  MAPK or dnp38 expression plasmid as indicated (A). Alternatively, cells were transfected with the pcDNA or RIG-I (20 ng/well) expression plasmid together with a construct-containing firefly luciferase gene under the *IFN $\lambda$ 1*, *IFN $\beta$* , or *IFN $\alpha$ 1* promoter (20 ng/well) and RSV-Renilla luciferase plasmids (5 ng/well), with or without the p38 $\alpha$  MAPK or dnp38 expression plasmid (10 ng/well; B). After 4 h of incubation, cells were stimulated by transfection with 88 bp dsRNAs (20 ng/well) for an additional 20 h. Cells were collected, and luciferase assays were carried out to determine the relative promoter activity. The means ( $\pm$ SD) of 3 parallel determinations are shown. The data are representative of 3 individual experiments. Results were considered statistically significant when  $*P < 0.05$  compared with the *IFN* promoter activity after RNA stimulation alone (black bars).

nations are shown. The data are representative of 3 individual experiments. Results were considered statistically significant when  $*P < 0.05$  compared with the *IFN* promoter activity after RNA stimulation alone (black bars).

cells (Fig. 5B, 3rd row), even though the *IFN $\lambda$ 1* mRNA expression level was greatly changed after co-overexpression (Fig. 5A). It is of interest to note that the amount of total p38 MAPK was increased slightly in HEK293 cells co-overexpressing RIG-I and p38 $\alpha$  MAPK or RIG-I and dnp38 (Fig. 5B, 2nd row), whereas the expression level of IRF3 remained approximately the same in all conditions (Fig. 5B, bottom row).

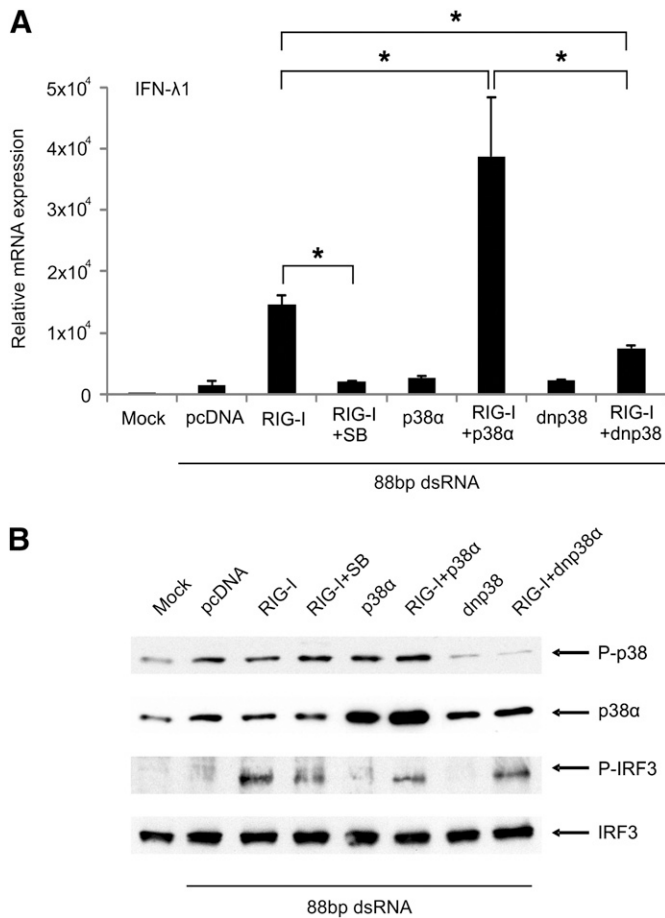
### ISRE and NF- $\kappa$ B1 sites are essential for p38 $\alpha$ MAPK-dependent activation of *IFN $\lambda$ 1* gene expression

To investigate further the mechanism how the *IFN $\lambda$ 1* gene is activated and regulated by p38 $\alpha$  MAPK, we analyzed the role of ISRE and NF- $\kappa$ B promoter elements in the *IFN $\lambda$ 1* promoter activation after stimulation by different RNA molecules. To characterize the role of these transcription factor binding sites on *IFN $\lambda$ 1* gene activation, different single and double mutations were introduced into the promoter region of *IFN $\lambda$ 1* (Fig. 6A). In RIG-I-overexpressing cells, the WT *IFN $\lambda$ 1* promoter was well activated by 88 bp dsRNA, but the activation level was reduced if ISRE and NF- $\kappa$ B1 sites of the promoters were mutated (Fig. 6B). However, mutations at the NF- $\kappa$ B2 site did not change the level of promoter activation in response to RNA stimulation, suggesting that the NF- $\kappa$ B2 site is not as important in the activation of the *IFN $\lambda$ 1* promoter as the NF- $\kappa$ B1 site (Fig. 6B). Moreover, if both the ISRE and NF- $\kappa$ B1 sites were mutated, the *IFN $\lambda$ 1* promoter did not respond to RNA stimulation in RIG-I-overexpressing cells (Fig. 6B), suggesting that both of these sites are required for maximal activation of the promoter. Similar results were also found in MDA5-overexpressing HEK cells (Supplemental Fig. 4).

To study further the role of the p38 $\alpha$  MAPK signaling pathway on ISRE- and NF- $\kappa$ B1-dependent *IFN $\lambda$ 1* gene activation, the effect of SB202190 and overexpression of p38 $\alpha$  MAPK were analyzed on WT, ISRE, or NF- $\kappa$ B1 mutant *IFN $\lambda$ 1* promoter activation. Overexpression of an active form of RIG-I,  $\Delta$ RIG-I, as well as MAVS and TRIF activated the WT *IFN $\lambda$ 1* promoter, whereas treatment with SB202190 decreased this activation (Fig. 7A, 1st bar chart). In addition, overexpression of p38 $\alpha$  MAPK together with other signaling components ( $\Delta$ RIG-I, MAVS, or TRIF) led to clearly enhanced activation of the *IFN $\lambda$ 1* promoter. Moreover, mutations at ISRE and NF- $\kappa$ B1 sites on the *IFN $\lambda$ 1* promoter resulted in reduced promoter activation and high sensitivity to SB202190 (Fig. 7A, 2nd and 3rd bar charts). Interestingly, SB202190 treatment and overexpression of p38 $\alpha$  MAPK did not affect the activation of the WT *IFN $\lambda$ 1* promoter or the promoter containing mutated ISRE or NF- $\kappa$ B1 sites if the cells were stimulated by cotransfection with MyD88 and IRF7 expression constructs (Fig. 7A).

It has been shown that besides being regulated by IRF3, the human *IFN $\alpha$ 1* gene is mainly regulated by IRF7 [39, 51, 52]. To demonstrate further that the IRF7 signaling pathway is not essential for p38 $\alpha$  MAPK to regulate the *IFN $\alpha$ 1* gene, the activation of the *IFN $\alpha$ 1* promoter was measured after stimulation by IRF7 expression or coexpression of IRF7 and MyD88 in the presence or absence of SB202190 or overexpression of p38 $\alpha$  MAPK. Consistent with the data above (Fig. 7A), SB202190 did not inhibit the activation of the *IFN $\alpha$ 1* promoter stimulated by expression of IRF7 alone or by coexpression of IRF7 and MyD88 (Fig. 7B), whereas the protein expression level of MyD88 or IRF7 remained the same (Fig. 7C). The overexpression of p38 $\alpha$  MAPK



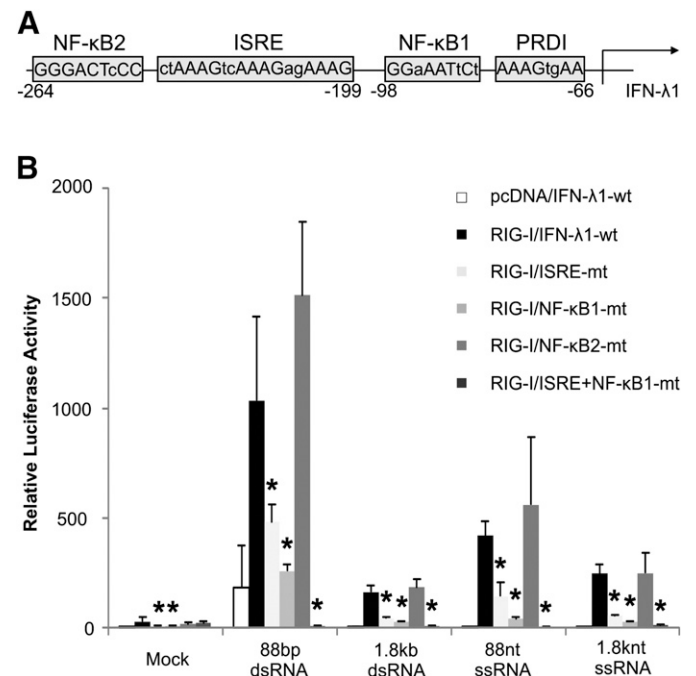


**Figure 5. Regulation of *IFN-λ1* mRNA expression and phospho-IRF3 expression by p38α MAPK in RIG-I-expressing HEK293 cells after stimulation with short dsRNA molecules.** (A) HEK293 cells (in 12-well plates;  $5 \times 10^5$  cells/well) remained untreated, or they were pretreated with the p38 MAPK inhibitor SB202190 (10  $\mu$ M), 0.5 h before transfection with indicated expression plasmids (100 ng/ml). After 16 h of incubation, cells were stimulated by transfection with 88 bp dsRNAs (200 ng/ml) for an additional 3 h, followed by cell collection, RNA isolation, and quantitative RT-PCR analysis. The values were normalized against  $\beta$ -ACTIN mRNA, and the relative *IFN-λ1* mRNA level was calculated with the  $\Delta\Delta C_t$  method by use of untreated cells as a calibrator. The means ( $\pm$ SD) of 3 parallel determinations are shown. Data are representative of 3 individual experiments. *IFN-λ1* gene expression results were considered statistically significant when  $*P < 0.05$  compared with the experimentally relevant stimulatory conditions. (B) Western blot analysis for the expression of phospho-IRF3, total IRF3, phospho-p38 MAPK, and total p38α MAPK proteins in RNA-stimulated HEK293 cells, which were collected at 3 h after 88 bp dsRNA transfection, and whole-cell lysates were prepared. Cellular proteins (30  $\mu$ g/lane) were separated on 10% SDS-PAGE, followed by electrophoretic transfer of the proteins onto PVDF membranes and visualization of the transferred proteins by protein-specific antibodies, as indicated. The data of 1 representative experiment of 3 independent experiments are shown.

also failed to enhance the promoter activation effectively under these conditions (Fig. 7B), whereas overexpression of p38α MAPK reduced the expression of MyD88 or IRF7 proteins to some extent (Fig. 7C). Altogether, our data suggest that the MyD88-IRF7 pathway is not essential for p38α MAPK to regulate *IFN* promoter activity.

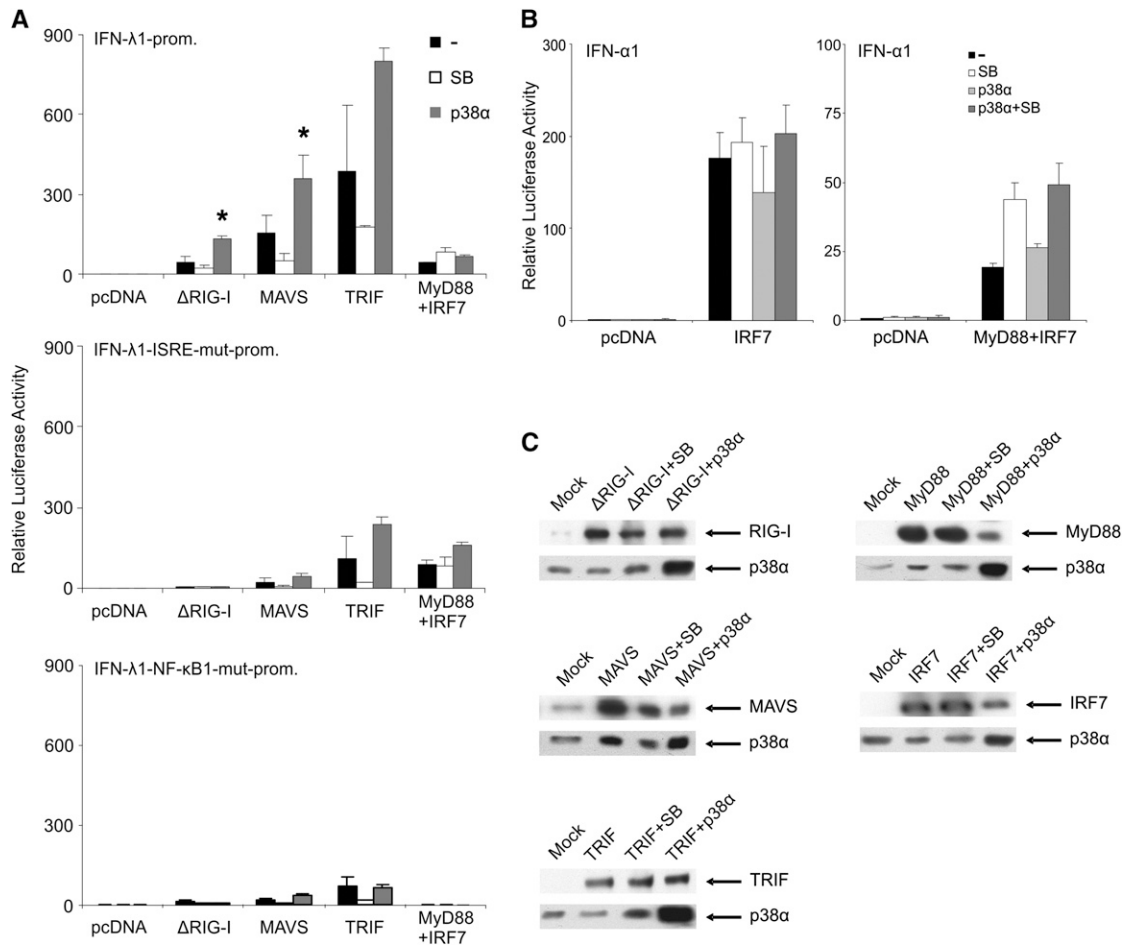
## IRF3/IRF7 and NF-κB are essential for p38α MAPK-dependent regulation of *IFN-β1* gene expression in mouse 3T3 cells

As described previously, IRFs regulate *IFN-λ1* gene expression, mainly through binding to the ISRE site, whereas the regulation by NF-κB takes place through its binding to cognate NF-κB sites. Having observed that ISRE and NF-κB1 sites at the *IFN-λ1* promoter are essential for p38α MAPK-dependent activation of *IFN-λ1* transcription, we further studied the role of IRF3, IRF7, and NF-κB in the regulation of *IFN-β1* gene expression by p38α MAPK in mouse 3T3 cells. As mice lack the human counterpart of *IFN-λ1*, we concentrated on analyzing mouse *IFN-β1* gene expression in IRF3/IRF7 KO and NF-κB KO mouse 3T3 cells. Consistent with the data on *IFN-λ1* gene expression in HEK293 cells (Fig. 5A), mouse *IFN-β1* mRNA expression was activated by stimulation with 88 bp dsRNA in WT mouse 3T3 cells, and the



**Figure 6. Promoter structure of the *IFN-λ1* gene and the differences between *IFN-λ1* mutated and natural promoter activation in RIG-I-expressing HEK cells after different RNA stimulations.** (A) The promoter structure of the human *IFN-λ1* gene. Computer analysis was performed to elucidate 4 putative transcription factor binding sites on the promoter region of *IFN-λ1* near the start codon. The putative binding sites for IRFs (ISRE and PRDI) and NF-κB (gray boxes), their sequences, and relative positions with respect to the start codon (arrow) are shown. (B) HEK293 cells (in 96-well plates;  $3 \times 10^4$  cells/well) were transfected with pcDNA or RIG-I (20 ng/well) expression plasmid and together with RSV-Renilla luciferase plasmids (5 ng/well) and constructs containing the firefly luciferase gene under the WT *IFN-λ1* promoter or *IFN-λ1* ISRE or/and NF-κB mutant (mt) promoters, as indicated (20 ng/well). After 4 h of incubation, cells were stimulated by transfection with 88 bp dsRNAs (20 ng/well) for an additional 20 h. Cells were collected, and luciferase assays were carried out to determine the relative promoter activity. The means ( $\pm$ SD) of 3 parallel determinations are shown. The data are representative of 3 individual experiments. Results were considered statistically significant when  $*P < 0.05$  compared with the WT *IFN-λ1* promoter activity (black bars).





**Figure 7. Effects of the p38 MAPK signaling pathway on *IFN- $\lambda$ 1* promoter activity in HEK293 cells expressing different components of the RLR/TLR signaling pathways.** HEK293 cells (in 96-well plates;  $3 \times 10^4$  cells/well) were left untreated or were pretreated with p38 MAPK inhibitor SB202190 (10  $\mu$ M), 0.5 h before transfection with pcDNA or indicated expression plasmids for different signaling components (20 ng/well), RSV-Renilla luciferase plasmids (5 ng/well) and constructs containing the firefly luciferase gene under the WT *IFN- $\lambda$ 1* promoter (prom.) or mutated *IFN- $\lambda$ 1* promoters (mut-prom.; A) or under the *IFN- $\alpha$ 1* promoter (B; 20 ng/well). After 24 h incubation, cells were collected, and luciferase assays were carried out to determine the relative promoter activity. The means ( $\pm$ sd) of 3 parallel determinations are shown. The data are representative of 3 individual experiments. Results were considered statistically significant when  $*P < 0.05$  compared with the black bar of the control *IFN- $\lambda$ 1* promoter activity. (C) Western blot analysis for the expression of RIG-I, MAVS, TRIF, IRF7, MyD88, and p38 $\alpha$  MAPK in HEK cells. HEK293 cells (in 12-well plates,  $3 \times 10^5$  cells/well) were left untreated or were pretreated with the p38 MAPK inhibitor SB202190 (10  $\mu$ M), 0.5 h before transfection with pcDNA or indicated expression plasmids for different signaling components (200 ng/well). The whole-cell lysates were prepared, and proteins (20  $\mu$ g/lane) were separated on 10% SDS-PAGE, followed by electrophoretic transfer of the proteins onto PVDF membranes and detection of the transferred proteins by antibodies that are specific for RIG-I, MAVS, TRIF, IRF7, MyD88, and p38 $\alpha$  MAPK, as indicated. The data of 1 representative experiment of 3 independent experiments are shown.

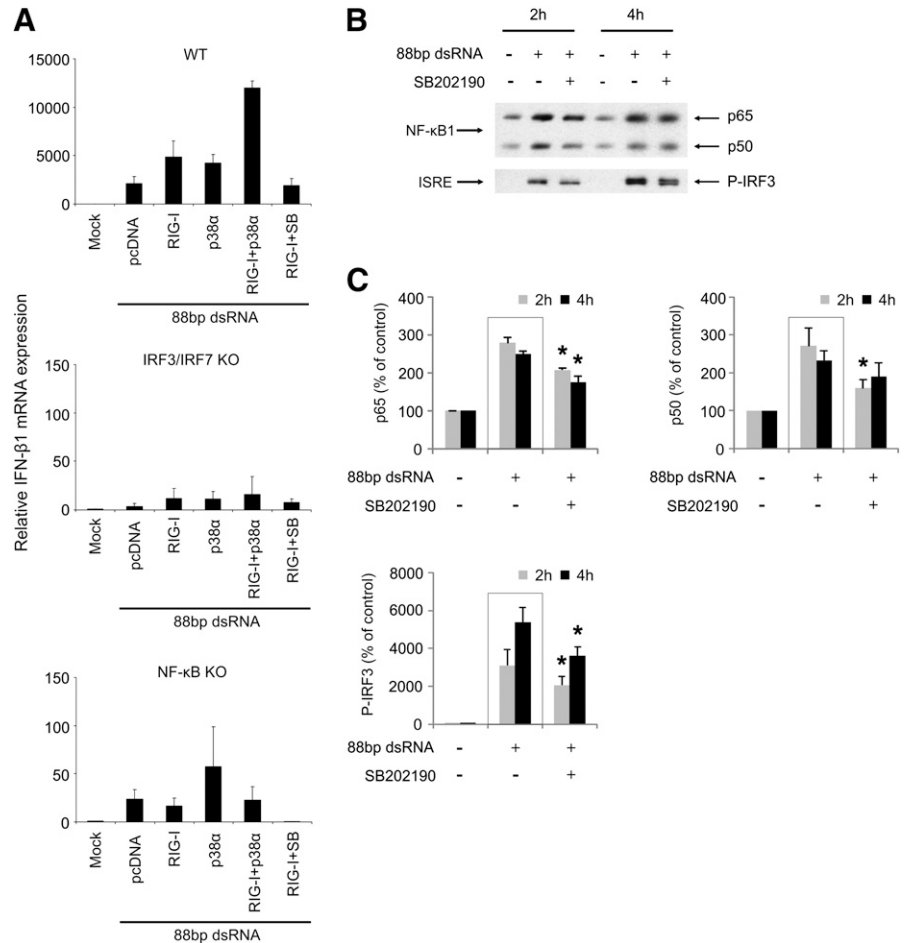
activation was weakly enhanced by overexpression of RIG-I or p38 $\alpha$  MAPK (Fig. 8A, 1st bar chart). However, coexpression of RIG-I and p38 $\alpha$  MAPK led to a clearly detectable increase in *Ifn- $\beta$ 1* mRNA expression. SB202190 efficiently inhibited *Ifn- $\beta$ 1* mRNA expression in RIG-I-overexpressing WT 3T3 cells. However, in IRF3/IRF7 KO and NF- $\kappa$ B KO cells, 88 bp dsRNA-induced expression of *Ifn- $\beta$ 1* mRNA was decreased significantly compared with the WT mouse 3T3 cells, and coexpression of RIG-I and p38 $\alpha$  MAPK failed to enhance the *Ifn- $\beta$ 1* mRNA expression (Fig. 8A, 2nd and 3rd bar charts). This data suggest that IRF3/IRF7 and NF- $\kappa$ B are the key transcription factors that work in concert with p38 $\alpha$  MAPK to regulate early *IFN* gene expression, such as those of mouse *Ifn- $\beta$*  and human *IFN- $\lambda$ 1* genes.

### p38 $\alpha$ MAPK is involved in the binding of IRF3 and NF- $\kappa$ B to the *IFN- $\lambda$ 1* promoter

To characterize further the role of the p38 $\alpha$  MAPK signaling pathway on regulating the binding of IRF3 and NF- $\kappa$ B to the *IFN- $\lambda$ 1* promoter, DNA affinity-binding assays were carried out. moDCs were transfected with 88 bp dsRNA in the presence or absence of SB202190, cells were collected at 2 h and 4 h after RNA stimulation, and nuclear proteins were extracted and precipitated by use of the *IFN- $\lambda$ 1* gene promoter ISRE- and NF- $\kappa$ B1-site oligonucleotides. The binding of phosphorylated IRF3 to the ISRE site and p65 and p50 to the NF- $\kappa$ B1 site was clearly enhanced already at 2 h after RNA stimulation, whereas the presence of SB202190 reduced the binding of both transcription factors, especially at the early stage—2 h after RNA stimulation (Fig. 8B and C).

**Figure 8. Effect of the p38 MAPK signaling pathway on regulating *IFN- $\lambda$ 1* mRNA expression in mouse 3T3 WT cells, IRF3/IRF7 KO cells, and NF- $\kappa$ B KO cells after stimulation with short dsRNA molecules and the role of the p38 MAPK signaling pathway on regulating the binding of activated IRF3 and NF- $\kappa$ B to the ISRE and NF- $\kappa$ B1 sites of the *IFN- $\lambda$ 1* promoter in human moDCs. (A)**

Indicated cells (in 12-well plates;  $5 \times 10^5$  cells/well) were left untreated or were pretreated with p38 MAPK inhibitor SB202190 (10  $\mu$ M), 0.5 h before transfection with the indicated expression plasmids (100 ng/ml). After 5 h of incubation, cells were stimulated by transfection with 88 bp dsRNAs (200 ng/ml) for an additional 3 h, followed by collection of cells, RNA isolation, and quantitative RT-PCR analysis. The values were normalized against mouse *Gapd* mRNA, and relative *IFN- $\lambda$ 1* mRNA levels were calculated with the  $\Delta\Delta$ Ct method by use of untreated cells as a calibrator. The means ( $\pm$ SD) of 3 parallel determinations are shown. The data are representative of 2 individual experiments. (B) moDCs from 4 different blood donors were left untreated or were pretreated with p38 MAPK inhibitor SB202190 (10  $\mu$ M), 0.5 h before RNA transfection. The cells were subsequently transfected with 88 bp dsRNA molecules (50 ng/ml) for 2 h and 4 h. Cells were pooled and collected, and nuclear proteins were extracted and precipitated with agarose-bound oligonucleotides representing ISRE and NF- $\kappa$ B1 elements of the *IFN- $\lambda$ 1* promoter. Oligonucleotide-bound proteins were boiled, separated on SDS-PAGE, transferred to PVDF membranes, and analyzed by Western blotting with antibodies that are specific for the phosphorylated form of IRF3 and p65 and p50 proteins. Antibodies and promoter elements are indicated in the figure. This experiment was performed 2 times with similar results. (C) Densitometry analysis of Western blots in a DNA affinity binding assay. Error bars show means  $\pm$  SD. Results were considered statistically significant when  $*P < 0.05$  compared with dsRNA-stimulated, uninhibited samples (boxed bars).



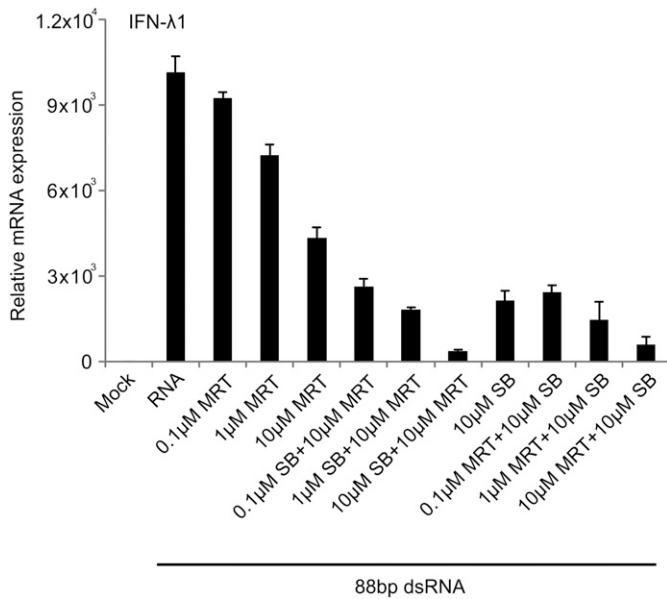
### The regulation of *IFN- $\lambda$ 1* mRNA expression by p38 $\alpha$ MAPK likely occurs via a pathway apart from the TBK1 signaling pathway in human moDCs

It is well known that the activation of the TBK1/IKK $\epsilon$  complex is essential for phosphorylation and activation of IRF3 [14, 21, 22]. As we clearly show that the activation of IRF3 through RLR signaling and the binding of the phosphorylated IRF3 to ISRE site on the promoter of the *IFN- $\lambda$ 1* gene are required for the regulation of the *IFN- $\lambda$ 1* gene by p38 $\alpha$  MAPK (Figs. 6, 7A, and 8A and B), we wanted to analyze whether the positive regulatory effect of p38 MAPK takes place via the TBK1/IKK $\epsilon$  or through another pathway. moDCs were pretreated with increasing doses of the TBK1 inhibitor MRT67307 or with the combination of MRT67307 and SB202190, followed by transfection with 88 bp dsRNA and analysis of the expression of *IFN- $\lambda$ 1* mRNA at the 4 h time-point after RNA stimulation. The induction of *IFN- $\lambda$ 1* mRNA expression was inhibited by MRT67307 in a dose-dependent fashion (Fig. 9). However, in cells pretreated with the combination of MRT67307 and SB202190, *IFN- $\lambda$ 1* expression was reduced even further when the dose of SB202190 increased, and the dose of MRT67307 remained the same (Fig. 9). Similar results were observed when the dose of MRT67307 increased, and the SB202190 dose remained

constant (Fig. 9). These data suggest that p38 MAPK regulates *IFN- $\lambda$ 1* gene expression, not directly through the activation of the TBK1/IKK $\epsilon$  complex but through another parallel pathway instead.

## DISCUSSION

It is well established that different types of RNAs bind and activate specific members of the TLR and RLR families. This, in turn, leads, via certain adaptor proteins, to the activation of downstream transcription factors, especially those belonging to the IRF and NF- $\kappa$ B families, which play an essential role in *IFN* gene expression. However, despite the rapid advances in the studies of IFN induction pathways by PRRs, the precise mechanisms by which additional signaling cascades participate in the regulation of *IFN* gene expression have remained uncharacterized, especially for the regulation of type III *IFNs*. In the present study, we used several pharmacological signaling inhibitors to identify previously unrecognized signaling pathways that are involved in the regulation of *IFN* gene expression in human DCs. We found that depending on the size and type of RNAs, PI3K and several MAPK pathways contribute to RNA-induced *IFN* gene expression (Fig. 1 and Supplemental Fig. 1).



**Figure 9. The role of the p38 MAPK inhibitor and TBK1 inhibitor on regulating *IFN- $\lambda$ 1* mRNA expression in short dsRNA-stimulated human moDCs.** moDCs from 4 different blood donors were grown separately on 24-well plates. Cells remained untreated or were pretreated with an indicated amount of p38 MAPK inhibitor SB202190 or TBK1 inhibitor MRT67307 (MRT), 0.5 h before transfection with 88 bp dsRNA (50 ng/ml). Cells were collected 4 h after RNA transfection, and total cellular RNA was isolated, and *IFN- $\lambda$ 1* mRNA expression was determined by quantitative RT-PCR. The values were normalized against  $\beta$ -*ACTIN*, and relative *IFN- $\lambda$ 1* mRNA levels were calculated with the  $\Delta\Delta C_t$  method by use of untreated cells as a calibrator. The means ( $\pm$ SD) of 3 parallel determinations are shown. Data are representative of 3 individual experiments.

Interestingly, the p38 MAPK inhibitors were very efficient in inhibiting *IFN* gene expression (Figs. 1 and 2A), and this inhibition took place also in the presence of protein synthesis inhibitor (Fig. 3 and Supplemental Fig. 3), indicating that p38 MAPK is contributing to the very early transcriptional events regulating type I and type III *IFN* gene expression. Phosphorylation of p38 MAPK increased after 88 bp dsRNA stimulation (Figs. 2B and D and 5B), suggesting that short dsRNA molecules can also activate the p38 MAPK signaling pathway in addition to the classic PRR signaling pathways. Nucleofection of p38 $\alpha$  MAPK in dsRNA-stimulated moDCs enhanced *IFN- $\lambda$ 1* mRNA expression (Fig. 2C), further demonstrating the positive role of p38 MAPK in *IFN- $\lambda$ 1* gene regulation. The expression of phosphorylated forms of IRF3 and p38 MAPK was increased after 88 bp dsRNA stimulation (Fig. 2B and D), suggesting that these 2 signaling pathways cooperatively regulate the induction of the *IFN- $\lambda$ 1* gene. In addition, overexpression studies with p38 $\alpha$  MAPK, dnp38, and some TLR/RLR family members suggest that the RIG-I and IRF3 pathway, but not the MyD88 and IRF7 pathway, is required for the regulation of *IFN* gene expression by p38 $\alpha$  MAPK (Figs. 4, 5, and 7). Moreover, the effects of mutations in the *IFN- $\lambda$ 1* promoter (Figs. 6 and 7), DNA affinity-binding experiments in human moDCs (Fig. 8B), and experiments in mouse IRF3/IRF7 and NF- $\kappa$ B KO cells (Fig. 8A) suggest that the IRF and NF- $\kappa$ B transcription factors and their respective binding sites ISRE and

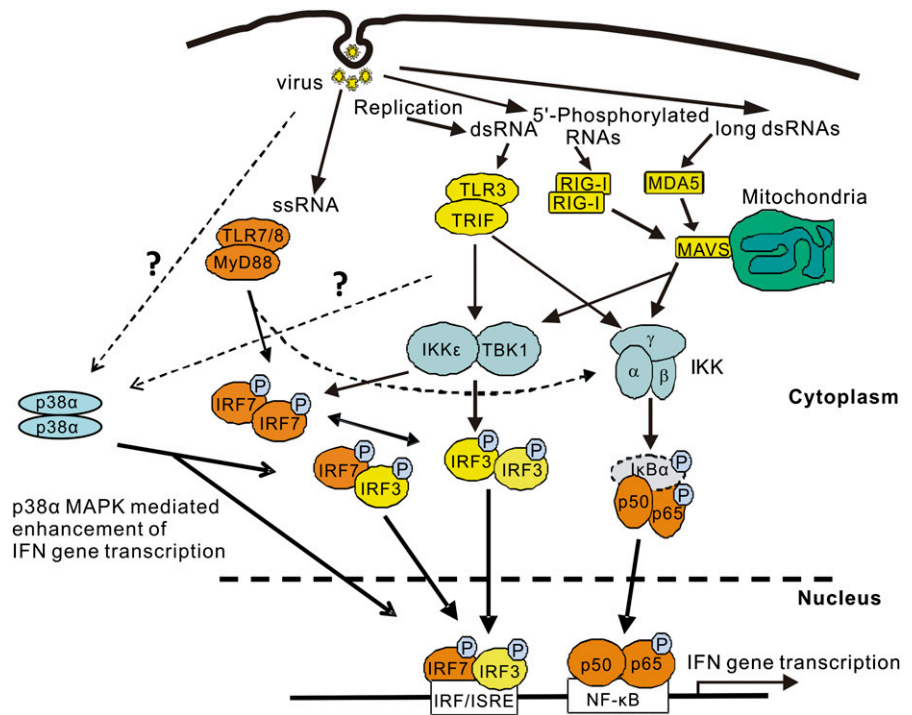
NF- $\kappa$ B1 in the *IFN- $\lambda$ 1* promoter are essential in RNA-regulated *IFN* gene expression. Furthermore, in these experiments, p38 $\alpha$  MAPK had a positive regulatory role (Figs. 7 and 8). In addition, the inhibitor-combination experiments in human moDCs suggest that the regulation of *IFN- $\lambda$ 1* mRNA expression by p38 $\alpha$  MAPK does not take place directly through activation of the TBK1/IKK $\epsilon$  complex (Fig. 9), and Western blot analysis also showed that the addition of inhibitors of p38 MAPK and overexpression of p38 $\alpha$  MAPK or dnp38 could not significantly affect the phosphorylation of IRF3 (Figs. 2B and D and 5B), further suggesting that p38 MAPK regulates *IFN- $\lambda$ 1* gene expression, not directly through activation of IRF3. Therefore, our data strongly indicate that p38 $\alpha$  MAPK positively regulates early *IFN- $\lambda$ 1* gene expression by cooperating with the transcription factors IRF3 and NF- $\kappa$ B through some parallel signaling pathway after RNA stimulation (Fig. 10).

It has been shown previously that p38 MAPK plays a central role in inflammatory responses induced by environmental stress, growth factors, and inflammatory factors [53–55]. Furthermore, the p38 MAPK signaling pathway was shown to be involved in IFN signaling and regulation of antiviral responses [34]. However, less is known about the role of p38 MAPK in regulating *IFN* gene expression. It has been reported that the activation of RIG-I during Sendai virus infection leads to the activation of p38 MAPK, which contributes to type I IFN-mediated antiviral responses and the activation of conventional DCs [56]. It has also been found that 2 p38 $\alpha$  MAPKAPKs—MAPKAPK2 and MAPKAPK3—regulate the expression of IFN-inducible genes and antiviral responses [35]. The present study demonstrates that besides the regulation of IFN signaling and antiviral response [34, 35, 56], p38 MAPK is activated by short dsRNA stimulation (Figs. 2B and D and 5B) and is taking part in the regulation of RNA-induced early *IFN* gene expression in human moDCs, especially those of *IFN- $\lambda$ 1* and *IFN- $\beta$*  genes (Figs. 1 and 2 and Supplemental Figs. 1 and 2). Our data are also consistent with a previous study, which showed that in human macrophages, IFN induction by the infection with an RNA virus—IAV—is predominantly regulated by IRF3 and p38 MAPK [57]. The PI3K inhibitor also decreased the expression of the *IFN- $\lambda$ 1* gene, especially in response to stimulation with short 88 nt ssRNA and long 1.8 kb dsRNA molecules (Fig. 1), suggesting some selectivity of the PI3K pathway in RNA-induced innate immune response. However, further studies are needed to elucidate the potential role of these pathways in regulating *IFN* gene expression.

To investigate further the mechanism by which p38 MAPK regulates the expression of the *IFN* gene in response to RNA stimulation, the relationship of different RNA receptors was studied. Overexpression of p38 $\alpha$  or dnp38 MAPK regulated the activation of the *IFN- $\lambda$ 1* promoter positively or negatively, respectively, through RLR (RIG-I and MDA5) and TLR (TLR3) pathways (Fig. 4A), indicating a broader role for p38 $\alpha$  MAPK in intracellular signaling. Moreover, co-overexpression of RIG-I and p38 $\alpha$  MAPK enhanced the *IFN- $\lambda$ 1* promoter activity at higher levels compared with *IFN- $\beta$*  and *IFN- $\alpha$ 1* promoters (Fig. 4B), suggesting that the promoter of type III *IFN* genes can be regulated by a broader scale of transcription factors than type I *IFN* genes. Even though the precise site of action of p38 $\alpha$  MAPK



**Figure 10. Schematic illustration of the role of p38 $\alpha$  MAPK in regulating *IFN* gene expression at early stages of RNA virus infection or RNA stimulation in human moDCs.** Infection of DCs with RNA viruses led to expression of different types of RNA molecules that activate cellular PRRs. Transfected RNA mimics RNA virus infection. The figure outlines the most important signaling pathways activated by TLRs and RLRs (RIG-I and MDA5). RNA binding to TLRs and/or RLRs leads to activation of adaptor molecules and downstream kinases (IKK, IKK $\epsilon$ /TBK1), followed by phosphorylation of IRFs and I $\kappa$ B $\alpha$ . This triggers the nuclear translocation and binding of activated IRFs and NF- $\kappa$ B to the promoter elements of *IFN* genes, including *IFN*- $\lambda$ 1, leading to recruitment of the enhanceosome complex and RNA polymerase II (not shown) to regulate gene transcription. As shown in the present study, RNA stimulation in moDCs triggers the activation of p38 $\alpha$  MAPK, leading to enhancement of *IFN* gene transcription. The mechanisms responsible for the activation of p38 $\alpha$ , its downstream transcription factors, and transcriptional enhancement of *IFN* genes remain elusive.



remains partially unresolved, we were able to show that p38 $\alpha$  MAPK is activated by RNA stimulation, and it works in concert with RIG-I/MDA5-MAVS-IRF3 signaling without directly activating the TBK1/IKK $\epsilon$  complex. This suggests that p38 $\alpha$  MAPK enhances *IFN* gene transcription via a pathway apart from but in cooperation with IRF3 and NF- $\kappa$ B, the key transcription factors regulated by all RNA sensors (Fig. 10).

As our results indicated that p38 $\alpha$  MAPK was regulating *IFN*- $\lambda$ 1 gene expression downstream of the RNA sensors, we studied the functionality of the *IFN*- $\lambda$ 1 promoter by creating IRF and NF- $\kappa$ B binding-site mutations to the promoter (Fig. 6A). We demonstrated that ISRE and NF- $\kappa$ B1 sites in the *IFN*- $\lambda$ 1 promoter are indispensable for the activation of the promoter (Fig. 6B). Although there are also other important transcription-factor binding sites on the *IFN*- $\lambda$ 1 promoter, such as the PRDI site, and also 4 distal NF- $\kappa$ B binding sites far away from the start coding site [58], mutations targeting ISRE and NF- $\kappa$ B1 sites were able to abolish completely the activation of the *IFN*- $\lambda$ 1 promoter (Fig. 6B), suggesting that these 2 sites play an essential role in regulating *IFN*- $\lambda$ 1 promoter activity. Furthermore, these 2 promoter sites were required for the p38 $\alpha$  MAPK-mediated positive regulatory effect on *IFN*- $\lambda$ 1 promoter activity (Fig. 7A). Moreover, the DNA affinity-binding experiments showed that SB202190 can reduce the transcriptional activation of IRF3 and the active subunits of NF- $\kappa$ B (p65 and p50) on the *IFN*- $\lambda$ 1 promoter (Fig. 8). This suggests that p38 $\alpha$  MAPK together with IRF3 and NF- $\kappa$ B transcription factors contributed to RNA-stimulated induction of the *IFN*- $\lambda$ 1 gene. However, Western blot analysis did not show any specific phospho-p38, total p38, phospho-ATF2, or total ATF2 bands after precipitation with ISRE and NF- $\kappa$ B oligonucleotides in the DNA affinity-binding assay, and mutations on putative binding sites of p38-regulated

transcription factors (ATF2, CREB, and p53) on the *IFN*- $\lambda$ 1 promoter failed to reduce the promoter activity (data not shown), suggesting that p38 MAPK regulates *IFN*- $\lambda$ 1 gene expression via another mechanism rather than enhancing the binding of these p38-regulated transcription factors on the *IFN*- $\lambda$ 1 promoter. Further experiments in mouse IRF3/IRF7 or NF- $\kappa$ B KO 3T3 cells demonstrated the importance of IRFs and NF- $\kappa$ B in the regulation of *Ifn* gene expression by p38 $\alpha$  MAPK (Fig. 8A). Our data are consistent with the study of Dr. Peiris and coworkers [57], who reported that both IRF3 and p38 MAPK function in enhancing cytokine production in H5N1 influenza virus-infected cells. Moreover, the same authors showed that the p38 MAPK inhibitor (SB203580) did not affect the nuclear translocation of IRF3. Accordingly, our data also showed that p38 MAPK inhibitors (SB203580 and SB202190) failed to decrease the phosphorylation of IRF3 after 88 bp dsRNA stimulation (Fig. 2B). This may suggest that p38 MAPK cooperatively regulates *IFN* gene expression only after the phosphorylation and translocation of IRF3. Therefore, in our experimental approaches, we monitored *IFN* mRNA expression and used the *IFN*- $\lambda$ 1 promoter-reporter construct, as well as 2 specific p38 MAPK inhibitors, to demonstrate the cooperability of p38 $\alpha$  MAPK with IRF3 and NF- $\kappa$ B.

It was of interest to note that p38 $\alpha$  MAPK did not regulate *IFN*- $\lambda$ 1 gene expression via the MyD88-IRF7 pathway (Fig. 7A). In addition, p38 $\alpha$  MAPK failed to enhance IRF7 or MyD88-IRF7-dependent activation of the *IFN*- $\lambda$ 1 promoter in transfected HEK293 cells (Fig. 7B). As IRF7 is the key transcription factor regulating late *IFN* genes (almost all *IFN*- $\alpha$  subtypes), this data suggest that p38 $\alpha$  MAPK mainly regulates the expression of early *IFN* genes (*IFN*- $\lambda$ 1 and *IFN*- $\beta$ ) in cooperation with IRF3 or NF- $\kappa$ B transcription factors. Indeed, early RIG-I-mediated

promoter activities of human *IFN- $\lambda$ 1*, *IFN- $\beta$* , and *IFN- $\alpha$ 1* were inhibited by SB202190 and enhanced by p38 $\alpha$  MAPK over-expression (Fig. 4B). Furthermore, RNA-induced *IFN- $\lambda$ 1* or *IFN- $\alpha$ 1* expression was inhibited by SB202190, also in the presence of protein synthesis inhibitor CHX, indicating that the regulation of *IFN* mRNA expression by p38 MAPK takes place directly after RNA stimulation and does not require de novo protein synthesis. Our data are also consistent with the study of Dr. Ludwig and coworkers [59], who reported that inhibition of p38 MAPK by the p38-specific inhibitor SB202190 led to reduced expression of IFN- $\beta$  and other cytokines in H5N1 and H7N7 virus-infected cells. The studies by us and others indicate a clear role of p38 MAPK in regulating *IFN* gene expression at early stages of RNA stimulation or viral infection.

Our data, as concluded from multiple experimental approaches, strongly suggest that p38 $\alpha$  MAPK, together with IRF3 and NF- $\kappa$ B, regulates the expression of early *IFN* genes, especially that of the type III *IFN- $\lambda$ 1* gene. This suggests that p38 MAPK is not only involved in IFN signaling and induction of an antiviral response, but it also contributes to transcriptional regulation of *IFN* genes in RNA-stimulated cells. Our study thus provides new mechanistic evidence for the function of p38 $\alpha$  MAPK in the early antiviral signaling pathways and provides further evidence for the complexity of signal transduction pathways and the fine-tuning of IFN responses.

## AUTHORSHIP

M.J. designed the study, performed laboratory experiments, analyzed the results, and wrote the paper. P.Ö. and I.J. designed the study and wrote the paper. R.F. performed laboratory experiments. D.N.R. performed laboratory experiments and wrote the paper. A.H., M.M.P., and D.H.B. wrote the paper.

## ACKNOWLEDGMENTS

This work was supported by the Sigrid Juselius Foundation (to I.J., M.M.P., and D.H.B.), Finnish Foundation for Research on Viral Diseases (to M.J.), Research Council for Health of the Academy of Finland (Grants 252252 and 256159 to I.J. and 256197 to D.H.B.), and Research Council for Biosciences and Environment of the Academy of Finland (Grants 250113, 256069, and 272507 to M.M.P. and 255342 and 256518 to D.H.B.). The authors thank Hanna Valtonen and Teija Aalto for their excellent technical assistance in cell experiments and protein expression assays.

## DISCLOSURES

The authors declare no conflict of interest.

## REFERENCES

- Janeway, Jr., C. A. (1989) Approaching the asymptote? Evolution and revolution in immunology. *Cold Spring Harb. Symp. Quant. Biol.* **54**, 1–13.
- Dupuis, S., Jouanguy, E., Al-Hajjar, S., Fieschi, C., Al-Mohsen, I. Z., Al-Jumaah, S., Yang, K., Chapgier, A., Eidenschenk, C., Eid, P., Al-Ghoniaim, A., Tufenkeji, H., Frayha, H., Al-Gazlan, S., Al-Rayes, H., Schreiber, R. D., Gresser, I., Casanova, J. L. (2003) Impaired response to interferon-alpha/beta and lethal viral disease in human STAT1 deficiency. *Nat. Genet.* **33**, 388–391.
- Takeuchi, O., Akira, S. (2009) Innate immunity to virus infection. *Immunol. Rev.* **227**, 75–86.
- Ank, N., Iversen, M. B., Bartholdy, C., Staeheli, P., Hartmann, R., Jensen, U. B., Dagnaes-Hansen, F., Thomsen, A. R., Chen, Z., Haugen, H., Klucher, K., Paludan, S. R. (2008) An important role for type III interferon (IFN-lambda/IL-28) in TLR-induced antiviral activity. *J. Immunol.* **180**, 2474–2485.
- Ank, N., West, H., Paludan, S. R. (2006) IFN-lambda: novel antiviral cytokines. *J. Interferon Cytokine Res.* **26**, 373–379.
- Palucka, K., Banchereau, J. (2002) How dendritic cells and microbes interact to elicit or subvert protective immune responses. *Curr. Opin. Immunol.* **14**, 420–431.
- Iwasaki, A., Medzhitov, R. (2010) Regulation of adaptive immunity by the innate immune system. *Science* **327**, 291–295.
- Bruns, A. M., Horvath, C. M. (2012) Activation of RIG-I-like receptor signal transduction. *Crit. Rev. Biochem. Mol. Biol.* **47**, 194–206.
- Yoneyama, M., Kikuchi, M., Natsukawa, T., Shinobu, N., Imaizumi, T., Miyagishi, M., Taira, K., Akira, S., Fujita, T. (2004) The RNA helicase RIG-I has an essential function in double-stranded RNA-induced innate antiviral responses. *Nat. Immunol.* **5**, 730–737.
- Kawai, T., Akira, S. (2006) Innate immune recognition of viral infection. *Nat. Immunol.* **7**, 131–137.
- Takeuchi, O., Akira, S. (2008) MDA5/RIG-I and virus recognition. *Curr. Opin. Immunol.* **20**, 17–22.
- McCartney, S. A., Colonna, M. (2009) Viral sensors: diversity in pathogen recognition. *Immunol. Rev.* **227**, 87–94.
- Mogensen, T. H., Paludan, S. R. (2005) Reading the viral signature by Toll-like receptors and other pattern recognition receptors. *J. Mol. Med. (Berl)* **83**, 180–192.
- Akira, S., Uematsu, S., Takeuchi, O. (2006) Pathogen recognition and innate immunity. *Cell* **124**, 783–801.
- Kawai, T., Takahashi, K., Sato, S., Coban, C., Kumar, H., Kato, H., Ishii, K. J., Takeuchi, O., Akira, S. (2005) IPS-1, an adaptor triggering RIG-I and Mda5-mediated type I interferon induction. *Nat. Immunol.* **6**, 981–988.
- Seth, R. B., Sun, L., Ea, C. K., Chen, Z. J. (2005) Identification and characterization of MAVS, a mitochondrial antiviral signaling protein that activates NF-kappaB and IRF 3. *Cell* **122**, 669–682.
- Meylan, E., Curran, J., Hofmann, K., Moradpour, D., Binder, M., Bartschlager, R., Tschopp, J. (2005) Cardif is an adaptor protein in the RIG-I antiviral pathway and is targeted by hepatitis C virus. *Nature* **437**, 1167–1172.
- Xu, L. G., Wang, Y. Y., Han, K. J., Li, L. Y., Zhai, Z., Shu, H. B. (2005) VISA is an adapter protein required for virus-triggered IFN-beta signaling. *Mol. Cell* **19**, 727–740.
- O'Neill, L. A., Bowie, A. G. (2007) The family of five: TIR-domain-containing adaptors in Toll-like receptor signalling. *Nat. Rev. Immunol.* **7**, 353–364.
- Ghosh, S., May, M. J., Kopp, E. B. (1998) NF-kappa B and Rel proteins: evolutionarily conserved mediators of immune responses. *Annu. Rev. Immunol.* **16**, 225–260.
- Mogensen, T. H. (2009) Pathogen recognition and inflammatory signaling in innate immune defenses. *Clin. Microbiol. Rev.* **22**, 240–273 (Table of Contents).
- Hiscott, J. (2007) Triggering the innate antiviral response through IRF-3 activation. *J. Biol. Chem.* **282**, 15325–15329.
- Hiscott, J. (2007) Convergence of the NF-kappaB and IRF pathways in the regulation of the innate antiviral response. *Cytokine Growth Factor Rev.* **18**, 483–490.
- Kyriakis, J. M., Avruch, J. (1996) Sounding the alarm: protein kinase cascades activated by stress and inflammation. *J. Biol. Chem.* **271**, 24313–24316.
- Schaeffer, H. J., Weber, M. J. (1999) Mitogen-activated protein kinases: specific messages from ubiquitous messengers. *Mol. Cell. Biol.* **19**, 2435–2444.
- Chang, L., Karin, M. (2001) Mammalian MAP kinase signalling cascades. *Nature* **410**, 37–40.
- Platanias, L. C. (2003) Map kinase signaling pathways and hematologic malignancies. *Blood* **101**, 4667–4679.
- Davis, R. J. (2000) Signal transduction by the JNK group of MAP kinases. *Cell* **103**, 239–252.
- Lee, J. C., Laydon, J. T., McDonnell, P. C., Gallagher, T. F., Kumar, S., Green, D., McNulty, D., Blumenthal, M. J., Heys, J. R., Landvatter, S. W., Strickler, J. E., McLaughlin, M. M., Siemens, I. R., Fisher, S. M., Livi, G. P., White, J. R., Adams, J. L., Young, P. R. (1994) A protein kinase involved in the regulation of inflammatory cytokine biosynthesis. *Nature* **372**, 739–746.
- Jiang, Y., Chen, C., Li, Z., Guo, W., Gegner, J. A., Lin, S., Han, J. (1996) Characterization of the structure and function of a new mitogen-activated protein kinase (p38beta). *J. Biol. Chem.* **271**, 17920–17926.

31. Jiang, Y., Gram, H., Zhao, M., New, L., Gu, J., Feng, L., Di Padova, F., Ulevitch, R. J., Han, J. (1997) Characterization of the structure and function of the fourth member of p38 group mitogen-activated protein kinases, p38delta. *J. Biol. Chem.* **272**, 30122–30128.
32. Li, Z., Jiang, Y., Ulevitch, R. J., Han, J. (1996) The primary structure of p38 gamma: a new member of p38 group of MAP kinases. *Biochem. Biophys. Res. Commun.* **228**, 334–340.
33. Schieven, G. L. (2009) The p38alpha kinase plays a central role in inflammation. *Curr. Top. Med. Chem.* **9**, 1038–1048.
34. Platanias, L. C. (2003) The p38 mitogen-activated protein kinase pathway and its role in interferon signaling. *Pharmacol. Ther.* **98**, 129–142.
35. Li, Y., Sassano, A., Majchrzak, B., Deb, D. K., Levy, D. E., Gaestel, M., Nebreda, A. R., Fish, E. N., Platanias, L. C. (2004) Role of p38alpha Map kinase in type I interferon signaling. *J. Biol. Chem.* **279**, 970–979.
36. Carey, M. (1998) The enhanceosome and transcriptional synergy. *Cell* **92**, 5–8.
37. Yoneyama, M., Suhara, W., Fukuhara, Y., Fukuda, M., Nishida, E., Fujita, T. (1998) Direct triggering of the type I interferon system by virus infection: activation of a transcription factor complex containing IRF-3 and CBP/p300. *EMBO J.* **17**, 1087–1095.
38. Sato, M., Hata, N., Asagiri, M., Nakaya, T., Taniguchi, T., Tanaka, N. (1998) Positive feedback regulation of type I IFN genes by the IFN-inducible transcription factor IRF-7. *FEBS Lett.* **441**, 106–110.
39. Honda, K., Yanai, H., Negishi, H., Asagiri, M., Sato, M., Mizutani, T., Shimada, N., Ohba, Y., Takaoka, A., Yoshida, N., Taniguchi, T. (2005) IRF-7 is the master regulator of type-I interferon-dependent immune responses. *Nature* **434**, 772–777.
40. Osterlund, P. I., Pietilä, T. E., Veckman, V., Kotenko, S. V., Julkunen, I. (2007) IFN regulatory factor family members differentially regulate the expression of type III IFN (IFN-lambda) genes. *J. Immunol.* **179**, 3434–3442.
41. Jiang, M., Osterlund, P., Sarin, L. P., Poranen, M. M., Bamford, D. H., Guo, D., Julkunen, I. (2011) Innate immune responses in human monocyte-derived dendritic cells are highly dependent on the size and the 5' phosphorylation of RNA molecules. *J. Immunol.* **187**, 1713–1721.
42. Hoffmann, A., Levchenko, A., Scott, M. L., Baltimore, D. (2002) The IkkappaB-NF-kappaB signaling module: temporal control and selective gene activation. *Science* **298**, 1241–1245.
43. Veckman, V., Miettinen, M., Pirhonen, J., Sirén, J., Matikainen, S., Julkunen, I. (2004) *Streptococcus pyogenes* and *Lactobacillus rhamnosus* differentially induce maturation and production of Th1-type cytokines and chemokines in human monocyte-derived dendritic cells. *J. Leukoc. Biol.* **75**, 764–771.
44. Nousiainen, L., Sillanpää, M., Jiang, M., Thompson, J., Taipale, J., Julkunen, I. (2013) Human kinome analysis reveals novel kinases contributing to virus infection and retinoic-acid inducible gene I-induced type I and type III IFN gene expression. *Innate Immun.* **19**, 516–530.
45. Lin, R., Génin, P., Mamane, Y., Hiscott, J. (2000) Selective DNA binding and association with the CREB binding protein coactivator contribute to differential activation of alpha/beta interferon genes by interferon regulatory factors 3 and 7. *Mol. Cell. Biol.* **20**, 6342–6353.
46. Laemmli, U. K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680–685.
47. Osterlund, P., Veckman, V., Sirén, J., Klucher, K. M., Hiscott, J., Matikainen, S., Julkunen, I. (2005) Gene expression and antiviral activity of alpha/beta interferons and interleukin-29 in virus-infected human myeloid dendritic cells. *J. Virol.* **79**, 9608–9617.
48. Menon, M. B., Kotlyarov, A., Gaestel, M. (2011) SB202190-induced cell type-specific vacuole formation and defective autophagy do not depend on p38 MAP kinase inhibition. *PLoS ONE* **6**, e23054.
49. Lavoie, H., Thevakumaran, N., Gavory, G., Li, J. J., Padeganeh, A., Guiral, S., Duchaine, J., Mao, D. Y., Bouvier, M., Sicheri, F., Therrien, M. (2013) Inhibitors that stabilize a closed RAF kinase domain conformation induce dimerization. *Nat. Chem. Biol.* **9**, 428–436.
50. Kawai, T., Akira, S. (2009) The roles of TLRs, RLRs and NLRs in pathogen recognition. *Int. Immunol.* **21**, 317–337.
51. Levy, D. E., Marié, I., Smith, E., Prakash, A. (2002) Enhancement and diversification of IFN induction by IRF-7-mediated positive feedback. *J. Interferon Cytokine Res.* **22**, 87–93.
52. Génin, P., Lin, R., Hiscott, J., Civas, A. (2009) Differential regulation of human interferon A gene expression by interferon regulatory factors 3 and 7. *Mol. Cell. Biol.* **29**, 3435–3450.
53. Kumar, S., Boehm, J., Lee, J. C. (2003) p38 MAP kinases: key signalling molecules as therapeutic targets for inflammatory diseases. *Nat. Rev. Drug Discov.* **2**, 717–726.
54. Cuadrado, A., Nebreda, A. R. (2010) Mechanisms and functions of p38 MAPK signalling. *Biochem. J.* **429**, 403–417.
55. Rincón, M., Davis, R. J. (2009) Regulation of the immune response by stress-activated protein kinases. *Immunol. Rev.* **228**, 212–224.
56. Mikkelsen, S. S., Jensen, S. B., Chiliveru, S., Melchjorsen, J., Julkunen, I., Gaestel, M., Arthur, J. S., Flavell, R. A., Ghosh, S., Paludan, S. R. (2009) RIG-I-mediated activation of p38 MAPK is essential for viral induction of interferon and activation of dendritic cells: dependence on TRAF2 and TAK1. *J. Biol. Chem.* **284**, 10774–10782.
57. Hui, K. P., Lee, S. M., Cheung, C. Y., Ng, I. H., Poon, L. L., Guan, Y., Ip, N. Y., Lau, A. S., Peiris, J. S. (2009) Induction of proinflammatory cytokines in primary human macrophages by influenza A virus (H5N1) is selectively regulated by IFN regulatory factor 3 and p38 MAPK. *J. Immunol.* **182**, 1088–1098.
58. Thomson, S. J., Goh, F. G., Banks, H., Krausgruber, T., Kotenko, S. V., Foxwell, B. M., Udalova, I. A. (2009) The role of transposable elements in the regulation of IFN-lambda1 gene expression. *Proc. Natl. Acad. Sci. USA* **106**, 11564–11569.
59. Börgeling, Y., Schmolke, M., Viemann, D., Nordhoff, C., Roth, J., Ludwig, S. (2014) Inhibition of p38 mitogen-activated protein kinase impairs influenza virus-induced primary and secondary host gene responses and protects mice from lethal H5N1 infection. *J. Biol. Chem.* **289**, 13–27.

## KEY WORDS:

RIG-I-like receptors · signaling · transcription · IRF3 · NF-κB