

TLR ligands up-regulate RIG-I expression in human plasmacytoid dendritic cells in a type I IFN-independent manner

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Running title: IFN-independent up-regulation of RIG-I in pDCs by TLRs

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ABSTRACT

Plasmacytoid dendritic cells (pDCs) are professional type I interferon (IFN) producing cells that play an essential role in antiviral immunity. In many cell types, detection of intracellular pathogens is mostly dependent on endosomal Toll-like receptors (TLRs) and cytosolic sensors, such as retinoic acid-inducible gene I (RIG-I). However, the possible interplay between these two systems has not yet been elucidated. Here we aimed to study the collaboration of endosomal TLRs and RIG-I in primary human pDCs. We found that under steady-state conditions pDCs express RIG-I at very low level, but the expression of this receptor is rapidly and dramatically up-regulated upon stimulation by the TLR7 ligand imiquimod or the TLR9 ligand type A CpG. We also demonstrated that pDCs are able to sense and respond to 5'-ppp-dsRNA only following activation by endosomal TLRs. Experiments on primary pDCs with functionally blocked IFN-alpha/beta receptor 1 (IFNAR1) and those on human pDC leukemia (pDC-L) cells defective in type I IFN secretion indicated that the up-regulation of RIG-I expression in pDCs upon stimulation by endosomal TLR occurs in a type I IFN-independent manner. Selective phosphorylation of STAT1 on tyrosine 701 could be identified as an early signaling event in this process. Our results show that in contrast to many other cell types, where RIG-I expression is induced by type I IFN, in pDCs a disparate mechanism is responsible for the up-regulation of RIG-I. Our findings also indicate that along with autophagy, an additional mechanism is operating in pDCs to promote the detection of replicating viruses.

Key words: cell activation, plasmacytoid dendritic cells, RIG-I-like receptors, STAT1, Toll-like receptors, viral infection

INTRODUCTION

Recognition of pathogen-derived nucleic acids and initiation of innate immune responses, including the production of type I IFNs and pro-inflammatory cytokines are crucial for the host's defense against infections in particular those caused by viruses. To detect viral nucleic acids, plasmacytoid dendritic cells (pDCs) are equipped with endosomal Toll-like receptors (TLRs) such as TLR7 and TLR9.¹ In contrast to the cell type-specific and limited expression of membrane-bound TLRs, a wide spectrum of immune and non-immune cells have been identified to express cytosolic pattern-recognition receptors (PRRs) with the capability to detect non-self RNA.² A group of these receptors is referred to as retinoic acid-inducible gene I (RIG-I)-like receptors (RLRs).³ The currently known family members are RIG-I, melanoma differentiation associated gene 5 (MDA5), and laboratory of genetics and physiology-2 (LGP2).⁴ RLRs recognize RNA structures highly specific for viral RNAs, but distinct from endogenous 5'-capped mRNA. RIG-I preferentially binds to 5'ppp-dsRNA and short dsRNA, while MDA5 recognizes long dsRNA.⁵ LGP2 was initially thought to negatively regulate RLR signaling⁶ however, a recent study on mice lacking LGP2 has demonstrated that this receptor may positively influence antiviral responses.⁷

Although TLR-mediated signaling pathways are relatively well characterized in human pDCs,⁸ the expression and the functional importance of RLRs in this cell type have remained poorly described. Initial data from murine models indicated that pDCs preferentially use the TLR system rather than RIG-I for the detection of viral infections⁹ and unlike monocytes, pDCs express only marginal levels of RIG-I under steady-state conditions.² However, a possible collaboration between TLRs and RLRs in human pDCs has not been investigated so far. Therefore, here we aimed to study the effects of stimulation by endosomal TLRs on RIG-I expression in human pDCs.

RESULTS

Activation of primary pDCs by TLR ligands up-regulates RIG-I expression

In order to investigate the effects of exposure to TLR ligands on the expression of RIG-I in pDCs, primary human pDCs isolated from peripheral blood were treated with increasing concentrations of the TLR7 ligand imiquimod and the TLR9 ligands type A or type B CpG. In accordance with previous studies, the expression of RIG-I was undetectable in untreated primary pDCs at both mRNA and protein levels (Figure 1a-d). However, both type A CpG (Figure 1a and c) and imiquimod (Figure 1b and d) treatments increased the expression of RIG-I in a dose-dependent manner, while stimulation of the cells with type B CpG did not trigger any changes in RIG-I expression levels (data not shown). Kinetic measurements showed that the expression of RIG-I mRNA could be detected as early as 2 h after activation by either CpG A or imiquimod (Figure 1e and f). Co-stimulation of primary pDCs with type A CpG and imiquimod resulted in a lower level of RIG-I expression than the activation of cells with either one of the TLR ligands applied individually (Figure 2a and b).

Sensing 5'ppp-dsRNA by RIG-I occurs in primary pDCs only when pre-activated by type A CpG or imiquimod

To assess the possible consequences of increased RIG-I expression in pDCs activated via endosomal TLRs, blood-derived primary pDCs were treated with type A CpG or imiquimod for 24 h. After removal of the culture supernatants the cells were re-stimulated in fresh medium with 5'ppp-dsRNA, a highly specific synthetic ligand of RIG-I.¹⁰ According to the low steady-state expression of RIG-I in freshly isolated primary pDCs and in unstimulated cultured cells (Figure 2a and b), exposure to 5'ppp-dsRNA without previous TLR-mediated activation did not result in IFN- α or IL-6 secretion (Figure 3a-d). However, pre-treatment of

primary resting pDCs with type A CpG (Figure 3a and c) or imiquimod (Figure 3b and d) allowed pDC stimulation by 5'ppp-dsRNA in a dose-dependent manner and was able to induce the production of IFN- α (Figure 3a and b) and IL-6 (Figure 3c and d). However, this occurred exclusively, when the cells had previously been activated with either TLR7 or TLR9 ligands. These findings suggest that ligation of endosomal TLRs brings about the ability of pDCs to sense of and respond to cytosolic viral RNA through the RIG-I receptor.

Type A CpG and imiquimod enhance RIG-I expression in pDCs in a type I IFN-independent manner

It has been previously reported that TLR7 stimulation results in the expression of early IFN-inducible genes even in the absence of type I IFNs.¹¹ Based on this previous observation we next investigated whether the cellular events triggered by TLR9 and TLR7 ligands would lead to the up-regulation of RIG-I expression in primary pDCs independent on type I IFN-mediated signals. To test this assumption, primary pDCs were treated with a blocking antibody of IFN-alpha/beta receptor 1 (IFNAR1), shared by IFN- α , - β and - ω , prior to stimulation by type A CpG or imiquimod. The efficacy of receptor-masking was controlled by measuring the expression of Mx1 and OAS1 genes recognized as early, type I IFN-induced factors.¹² Blocking of IFNAR1 receptors almost completely prevented Mx1 and OAS1 up-regulation in type I IFN-stimulated primary pDCs (Figure 4a and b) while it did not modify the ability of type A CpG or imiquimod to elevate RIG-I expression levels significantly (Figure 4c and d). Furthermore, treatment of primary pDCs for 6 h with various doses (10-100 ng) of IFN- α in control experiments did not lead to RIG-I up-regulation tested at both mRNA and protein levels (data not shown).

To further confirm the involvement of a type I IFN-independent mechanism of TLR

ligand-induced up-regulation of RIG-I expression in pDCs, we harnessed the defective type I IFN secreting capacity of previously characterized malignant pDC cells isolated from cryopreserved bone marrow samples of a patient diagnosed with pDC leukemia (pDC-L).¹³ Our previous control experiments revealed that non-malignant primary pDCs secrete high levels of IFN- α as a result of imiquimod or type A CpG stimulation, but pDC-L cells failed to do so (Figure 5a). These results clearly indicated that TLR ligands used at a broad concentration range are able to trigger type I IFN release by primary pDCs but not by pDC-L cells.

To confirm that TLR-mediated signaling is unimpaired in pDC-L cells, we measured the expression of IFNA genes after TLR stimulation at the mRNA level. Data from these experiments showed that the expression of IFNA gene transcripts was significantly increased as a result of imiquimod or type A CpG treatment for 6 h, while treatment with type B CpG did not induce remarkable changes in either IFNA-1 or IFNA-2 expression levels (Figure 5b and c). Furthermore, co-stimulation with imiquimod and type A CpG induced lower expression of IFNA genes than either of these TLR ligands individually.

To further verify the functionality of the IFN- α signaling pathway, we also assessed the expression of the IFN regulatory factor 7 (IRF-7), a master regulator of type I IFN production upon TLR-ligand stimulation at the mRNA level. As shown in Figure 5d, the expression pattern of IRF-7 was similar to that of IFNA-1 and IFNA-2 genes. While administration of imiquimod or type A CpG triggered a remarkable increase in IRF-7 levels, treatment with type B CpG rather had an inhibitory effect on IRF-7 expression. Combined treatment with imiquimod and type A CpG induced lower expression of IRF-7 than either of the ligands individually (Figure 5d). These results are in line with previous observations on IFN- α production by non-malignant primary pDCs tested after exposure to different TLR

ligands¹⁴ and suggest that the TLR induced signaling pathway is functional in pDC-L cells even though the secretion of type I IFN proteins is impaired.

Next, we investigated the expression of RIG-I in pDC-L cells before and after treatment with TLR ligands. Under steady-state conditions very low level of RIG-I mRNA was detected in pDC-L cells (Figure 2c). Activation of pDC-L cells by type A CpG or imiquimod up-regulated the expression of this cytoplasmic nucleic acid sensor; this effect was abrogated when the TLR ligands were administered in combination, whereas the treatment of pDC-L cells with type B CpG did not modify the expression of RIG-I (Figure 2c). These data demonstrated that TLR7 and TLR9 ligands are able to induce the expression of RIG-I in both non-malignant primary pDCs and pDC-L cells via a type I IFN-independent mechanism.

Activation of primary pDCs by endosomal TLRs leads to rapid phosphorylation of STAT1 on tyrosine 701

It has been previously demonstrated that in TLR7-activated GEN2.2 cells (a human pDC-derived cell line) the expression of several “IFN-inducible” genes is independent on the presence of type I IFN, but they remained dependent on p38 mitogen-activated protein kinase (MAPK)-mediated STAT1 phosphorylation on Tyr701.¹¹ Beside this phosphorylation site STAT1 can also be phosphorylated at Ser727, and type I IFNs are known to be able to induce STAT1 phosphorylation both on serine and tyrosine residues.¹⁵ To define the possible role of STAT1 in early signaling events leading to RIG-I expression in primary pDCs upon stimulation with endosomal TLR ligands, we examined the phosphorylation of STAT1 at both residues within a 90-min time period. Stimulation of blood-derived primary pDCs for 90 min by TLR7 or TLR9 ligands induced STAT1 phosphorylation on Tyr701 but not on Ser727

(Figure 6). This finding raised the possibility that a MAPK-dependent, but IFNAR1-independent STAT1 activation is involved in endosomal TLR-induced up-regulation of RIG-I in human pDCs.

DISCUSSION

The tightly regulated production of type I IFN provides a powerful defense mechanism against viruses.¹⁶ Previous studies have shown that primary pDCs, conventional DCs and alveolar macrophages secrete large amounts of type I IFNs and thus are considered as the primary source of type I IFNs during viral infections.¹⁷ The pDCs can detect RNA and DNA viruses by two endosomal receptors, TLR7 and TLR9.¹ Due to the vesicular localization of these TLRs, the recognition of viral nucleic acids occurs when viruses and/or their components are ingested by pDCs and transported into the lysosomes in a TLR transmembrane domain dependent manner¹⁸ or via the process of autophagy.¹⁹ In contrast, conventional DCs and alveolar macrophages use cytosolic RLRs to recognize replicating viral RNA intermediates.²⁰ In this study we report for the first time that the expression of the RIG-I receptor is dramatically up-regulated in a type I IFN-independent manner in pDCs upon stimulation via TLR7 or TLR9, challenging the current paradigm that RIG-I has no significant function in these cells. We also demonstrate an inhibitory rather than synergistic collaboration of the vesicular TLR7 and TLR9 receptors in the induction of this phenomenon suggesting that co-activation of TLR7 and TLR9 in pDCs does not support uncontrolled RIG-I expression but rather keeps the response under the control of TLR specificity.

Vesicular TLRs sense viruses in a replication-independent manner; consequently pDCs have the capacity to mount antiviral responses against viruses for which they are not serving as host cells. Furthermore, as material delivery from the endosomal compartment to the cytosol occurs in pDCs to facilitate cross-presentation,²¹ it could be presumed that viral replication intermediates can come into contact with cytosolic RLRs upon phagocytosis of the debris of infected cells. Our data support the existence of such mechanisms but also delineate a novel mechanism for TLR-RLR collaboration in pDCs. We have found that pDCs

are able to sense 5'ppp-dsRNA in case the cells had previously been activated by TLR7/9 ligands. These results suggested that TLR7/9-mediated signals are able to prepare pDCs for sensing cytosolic viral nucleic acids by up-regulating RIG-I expression and consequently for boosting antiviral responses. A previous study using respiratory syncytial virus demonstrated that pDCs are in fact capable of detecting ssRNA viruses that enter the cytosol directly.²² The recognition of replicating viruses leads to the production of vast amount of IFN- α independently on endosomal TLRs.²² In these experiments pDCs responded to cytosolic viral replication without previous TLR-mediated activation, which could be explained by the fact that pDCs produce only marginal level of RIG-I under steady-state conditions, but they are able to express considerable amount of MDA5 at the mRNA level.²³ Another study has demonstrated that MDA5 is indispensable for sustained expression of type I IFN in response to paramyxovirus infection in mice,²⁴ however, the involvement of MDA5 in sensing respiratory syncytial virus infection by human pDCs remains to be determined.

To test whether TLR7/9-triggered up-regulation of RIG-I expression in pDCs depends on type I IFN-mediated signals we blocked IFNAR1 and, in separate experiments, we used pDC-L cells isolated from bone marrow samples of a former patient with pDC leukemia defective in type I IFN secretion.¹³ To investigate whether pDC-L cells share functional properties with primary pDCs and thus provide a suitable model for studying TLR-mediated cellular events in pDCs, we analyzed their responses to stimulation by type A or B CpG acting through TLR9 and imiquimod targeting TLR7. It has already been shown that type A CpG induces type I IFN responses, whereas type B CpG predominantly activates pDCs in a manner that results in phenotypic changes and pro-inflammatory cytokine production.¹⁴ The functional activity of type B CpG is attributed to its single stranded monomeric form localized to lysosome-associated membrane protein 1-positive endosomes, while type A CpG

forms aggregates with long retention time in early transferrin receptor-positive endosomes,²⁵ thus, favoring the prolonged activation of the MyD88-TRAF6-IRAK1-IRF-7 signaling complex associated with robust production of IFN- α .²⁶ The TLR7 ligand imiquimod is able to trigger the production of both type I IFN and the activation/maturation program of pDCs.²⁷ Retention of the TLR signaling complex within early endosomes in pDCs has been shown to correlate with IRF-7 recruitment and the induction of type I IFN production.²⁸ In line with these observations, exposure of pDC-L cells to imiquimod or type A CpG induced significantly increased expression of IRF-7, IFNA-1 and IFNA-2 mRNA, respectively, indicating the functionality of the coupled signaling pathways. However, we could not detect the secretion of IFN- α in the culture supernatants of TLR-ligand-activated pDC-L cells. This observation is supported by previous data showing defective IFN- α secretion by malignant pDCs isolated from the bone marrow.²⁹ The finding that bone marrow-derived leukemic pDCs are not competent for type I interferon production even though their signaling machinery is intact suggests that pDC-L cells may acquire this potential in the periphery through additional signals that may contribute to the symptoms of leukemia patients.

In this study we have shown for the first time that TLR7- and TLR9-mediated signals are able to up-regulate RIG-I expression in both primary pDCs and pDC-L cells via a type I IFN-independent mechanism. This finding was unexpected, because RIG-I was considered as one of the IFN-inducible genes in several human cell types.³⁰ Interestingly, it has previously been demonstrated that MxA, CXCL10 and TRAIL, all involved in the inhibition of virus replication and described to be tightly regulated by IFNs, are rapidly expressed in TLR7-stimulated pDCs in the absence of type I IFNs.¹¹ The existence of a novel pathway downstream of TLR7 ligation and involving MAPK-mediated early STAT1 phosphorylation on Tyr701 has been identified behind this phenomenon.¹¹ In an earlier study, 2-h treatment of

human pDCs with CpG DNA induced MAPK-dependent phosphorylation of STAT1 on both Tyr701 and Ser727 in a type I IFN independent manner.¹⁵ In control experiments, stimulation of cells with type I IFN also led to STAT1 phosphorylation on both residues; however, it was not influenced by the MAPK pathway.¹⁵ In our experiments STAT1 phosphorylation could be detected exclusively on the tyrosine residue when tested 90 min after TLR7/9 ligation. This observation is in good agreement with a recent report¹¹ suggesting that the phosphorylation of this tyrosin by an intermediate MAPK-dependent tyrosine kinase precedes the direct serine phosphorylation in STAT1 by MAPK. However, the tyrosine kinase responsible for partial phosphorylation of STAT1 remains to be identified.

In all experiments where combined treatments were used, we observed antagonistic effects of TLR7- and TLR9-mediated signals. Similar inhibitory effects of simultaneous TLR7- and TLR9-mediated activation have been observed by Marshall and co-workers, who have found that the capacity of TLR9 ligands to induce potent IFN- α responses is markedly reduced by concurrent TLR7 stimulation.³¹ Another study has also demonstrated a strong inhibitory effect of TLR7 stimulation on IFN- α production induced by CpG-A- and CpG-C-oligodeoxynucleotides.³² One possible explanation for the molecular background of these antagonistic effects could be the competition of TLR7 and TLR9 receptors for the N-terminal domain of the membrane spanning protein Unc93B1^{33, 34} that is known to interact with the transmembrane domain of these structurally related TLR receptors in the acidified endo-lysosomal compartments.³⁵ It also cannot be excluded that imiquimod suppresses TLR9-mediated responses in a TLR7-independent molecular mechanism.³⁶

Our results show that upon recognition of viral replication intermediates, early type I IFN production depends on TLR-mediated signals, whereas the second wave of type I IFN responses is guided by RLR signaling. These results however, raise the question of which

biological situations would need pre-stimulation by strong type I IFN inducers (endosomal TLR ligand) to establish subsequent responsiveness to a late and weak IFN inducer (cytosolic dsRNA). In this context, the TLR-driven acute but transient activation of pDCs resulting in direct release of huge amount of IFNs into the lymph and to blood circulation³⁷ should be considered in contrast to pathological conditions, including viral infections going on in non-lymphoid tissues.³⁸ We suggest that at the site of infection moderate RIG-I-mediated production of IFNs by recruited pDCs may be sufficient for supporting potent antiviral responses, while unraveling the significance of this spatiotemporal regulation requires further studies.

METHODS

Isolation and culturing of non-malignant primary pDCs

Leukocyte-enriched buffy coats were obtained from healthy blood donors drawn at the Regional Blood Center of the Hungarian National Blood Transfusion Service (Debrecen, Hungary) with the written approval of the Director of the National Blood Transfusion Service and the Regional and Institutional Ethics Committee of the University of Debrecen, Medical and Health Science Center, Debrecen, Hungary. Written, informed consent was obtained from the donors prior to blood donation, and their data were processed and stored according to the directives of the European Union. Peripheral blood mononuclear cells were isolated by Ficoll density gradient centrifugation (GE Healthcare, Little Chalfont, UK). Primary pDCs were separated from peripheral blood mononuclear cells by negative selection using magnetic cell separation kit on QuadroMACS magnet (both from Miltenyi Biotec, Bergisch Gladbach, Germany). The homogeneity of the pDC fraction was 91 – 96%, as confirmed by flow cytometry. The purified cells were cultured in RPMI-1640 medium (Invitrogen, Carlsbad, CA, USA) supplemented with 2 mM L-glutamine, 110 mg/l Na-pyruvate (both from Sigma-Aldrich, St. Louis, MO, USA), penicillin (100 U/ml), streptomycin (100 µg/ml), 10% fetal calf serum (all from Invitrogen) and 10 ng/ml recombinant IL-3 cytokine (PeproTech, Rocky Hill, NJ, USA), which are essential for *in vitro* survival of primary pDCs.

Isolation and culturing of pDC-L cells from cryopreserved bone marrow samples

Leukemic pDCs were isolated from the bone marrow samples of a 71-year-old patient diagnosed with pDC leukemia.¹³ The study was approved by the Ethical Committee of the National Medical Center, Institute of Hematology and Immunology, Budapest, Hungary. For cell separation, $2.5 - 5 \times 10^7$ bone marrow cells were stained with 10 µl anti-CD123-PECy5

antibody (Clone No. 9F5, BD Biosciences, San Jose, CA, USA) for 30 min at room temperature, washed two times in PBS (PAA Laboratories, Pasching, Austria) and sorted with a FACSDiVa cell sorter (BD Biosciences). We identified pDC-L cells based on their CD123 positivity and light scatter properties,³⁹ and the viability of the sorted cells was evaluated on a FACSCalibur cytometer (BD Biosciences) based on 7-amino-actinomycin D (Sigma-Aldrich) staining. In the thawed samples, the percentage of pDC-L cells was 61.5±3% as determined in three independent experiments. After cell sorting, more than 95% of the cells displayed the pDC phenotype and the viability ranged from 87% to 93%. The separated cells were cultured at a final density of 10⁶ cells/ml in flat-bottom Nunclon 48-well cell culture plates (Thermo Fisher Scientific, Rochester, NY, USA) in RPMI-1640 medium (Invitrogen) supplemented with 2 mM L-glutamine, 110 mg/l Na-pyruvate (both from Sigma-Aldrich), penicillin (100 U/ml), streptomycin (100 µg/ml) and 10% fetal calf serum (all from Invitrogen).

Activation of non-malignant primary pDCs and pDC-L cells

Freshly isolated primary pDCs and pDC-L cells were treated with imiquimod (InvivoGen, San Diego, CA, USA) at a final concentration of 2.5 µg/ml and type A (CpG 2216) or type B (CpG 2006) CpG (both from Hycult Biotechnology, Uden, The Netherlands) at a final concentration of 5 µg/ml for 8 h in Q-PCR or for 24 h in Western blot and ELISA experiments. In separate experiments, cells were incubated with imiquimod or type CpG A for 24 h at concentrations indicated in Figure 3, and then washed two times with fresh medium. Thereafter, 5'ppp-dsRNA treatment of the cells was performed in freshly added medium. The introduction of 5'ppp-dsRNA was performed with the LyoVec transfection system (InvivoGen) according to the manufacturer's recommendations. The LyoVec+5'ppp-dsRNA complex containing 1 µg/ml working concentration of the RIG-I ligand was added to

the cells, and the supernatants of the cultures were collected for ELISA after 16 h (IL-6) or 24 h (IFN- α) of incubation. Control experiments were performed with “LyoVec-only” and LyoVec+control-oligo complexes (provided by InvivoGen).

RNA isolation, cDNA synthesis and Q-PCR

To analyze the relative changes in gene expression, Q-PCR was performed as described previously.⁴⁰ Briefly, the total RNA was isolated by TRIzol reagent (Invitrogen). 1.5-2 μ g of the total RNA were reverse transcribed using SuperScript II RNase H reverse transcriptase (Invitrogen) and Oligo(dT)15 primers (Promega, Madison, WI, USA). Gene-specific TaqMan assays (Applied Biosystems, Foster City, CA, USA) were used to perform Q-PCR in a final volume of 25 μ l in triplicates using AmpliTaq DNA polymerase and ABI Prism 7900HT real-time PCR instrument (Applied Biosystems). Amplification of 36B4 was used as normalizing control. Cycle threshold values were determined using the SDS 2.1 software (Applied Biosystems). Constant threshold values were set for each gene throughout the study. The sequence of the primers and probes are available upon request.

Evaluation of cytokine secretion from culture supernatants

Concentrations of secreted cytokines in the cell culture supernatants were measured by ELISA. The amount of IFN- α was measured using an ELISA kit from PBL InterferonSource (Piscataway, NJ, USA). Level of IL-6 secreted by primary pDCs was measured using OptEIA kit (BD Biosciences) after 24 h of stimulation.

Western blotting

Protein extraction was performed by lysing the cells in lysis/loading buffer (0.1% SDS, 100

mM Tris pH 6.8, bromophenol blue, 10% glycerol, 5 v/v% β -mercaptoethanol). Proteins were denatured by boiling for 5 min. Samples were separated by SDS-PAGE (10% gels), and transferred to nitrocellulose membranes. Nonspecific binding was blocked by TBS-Tween-5% non-fat dry milk for 1h at room temperature. Anti-RIG-I, anti-STAT1, anti-phospho-STAT1(Ser727), anti-phospho-STAT1(Tyr701) (Cell Signaling, Danvers, MA, USA) and anti- β -actin antibodies (Sigma-Aldrich) were used at a dilution of 1:1000. Membranes were washed three times in TBS-Tween, and then incubated with anti-rabbit secondary antibody conjugated to horseradish peroxidase (GE Healthcare) at a 1:5000 dilution at room temperature for 30 min. After three washes with TBS-Tween, protein samples were visualized by enhanced chemiluminescence (SuperSignal West Pico Chemiluminescent Substrate, Thermo Scientific, Rockford, IL, USA). After the membranes had been probed for RIG-I or phospho-STAT1, they were stripped and re-probed for β -actin or native STAT1. For densitometry analysis, the system was first calibrated by a gray optical wedge (Kodak, Rochester, NY, USA). Consistent optical and light conditions were maintained during the whole CCD camera capturing session, performed with a Kodak Image Station 2000mm device (Kodak).

IFNAR1 receptor neutralization in non-malignant primary pDCs

To block IFNAR1 receptors, cells were treated with 50 μ g/ml anti-IFNAR1 monoclonal antibody (Abcam, Cambridge, UK) for 1 h prior to activation with 10 ng/ml recombinant human IFN- α (R&D Systems, Minneapolis, MN, USA) or TLR7/9 ligands. To analyze RIG-I expression by Q-PCR, the total RNA from activated cells was extracted at 3 h after IFN- α or TLR7/9-specific stimulation.

Statistics

One-way ANOVA followed by Bonferroni *post hoc* test was used for multiple comparisons. All analyses were performed by using GraphPad PRISM software, version 5.04. Differences were considered to be statistically significant at $p < 0.05$. Significance is indicated by * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

DISCLOSURE

The authors declare that they have no conflict of interest.

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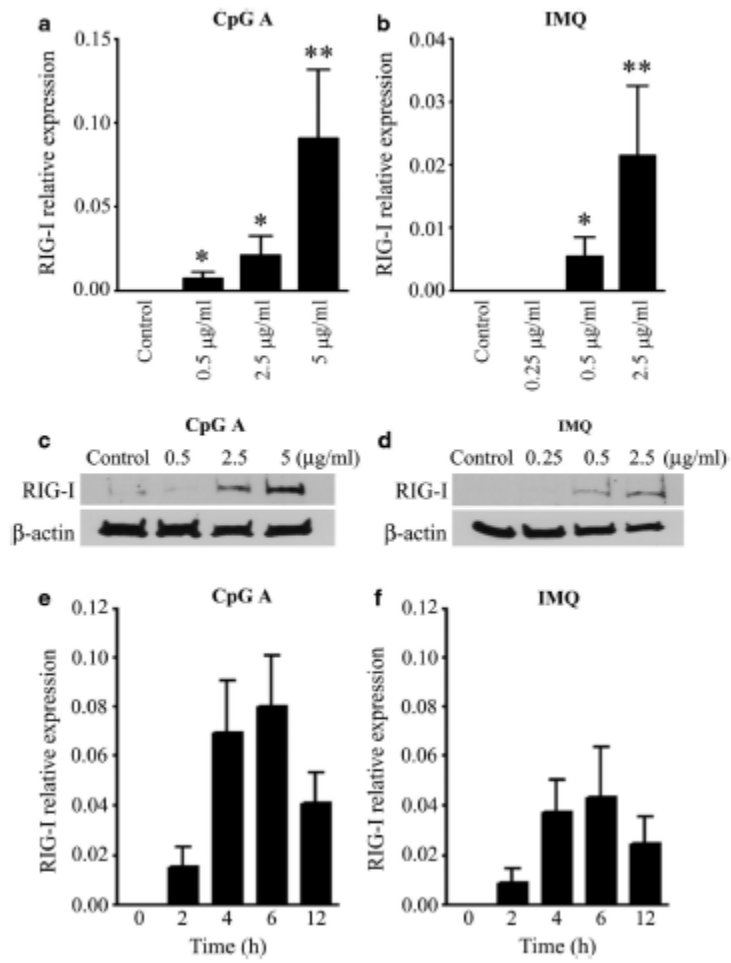


FIGURE LEGENDS

Figure 1. Expression of RIG-I in primary pDCs after stimulation by CpG A and imiquimod (IMQ).

Expression level of RIG-I was measured by Q-PCR (8 h) (a and b) and Western blotting (24 h) (c and d) after activation of pDCs at the indicated concentrations of type A CpG or IMQ.

* $P < 0.05$; ** $P < 0.01$ versus untreated control. Kinetics of RIG-I expression was analyzed

by Q-PCR after treatment of pDCs with 5 $\mu\text{g}/\text{mL}$ type A CpG and 2.5 $\mu\text{g}/\text{ml}$ IMQ (e and f).

Gene expression data are presented as means \pm SD of triplicates within one representative experiment out of the two independent ones.



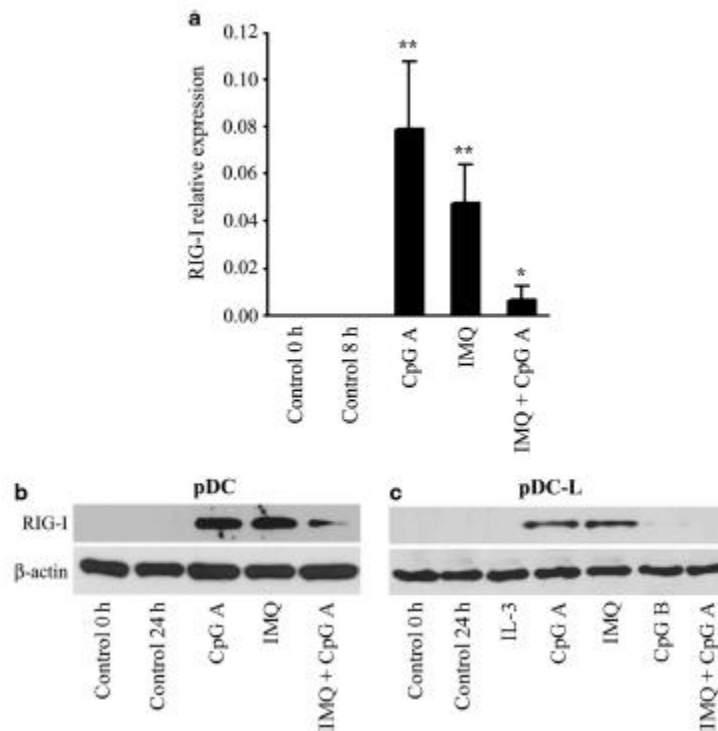


Figure 2. Expression of RIG-I in primary pDCs and in malignant pDC-L cells after co-stimulation with type A CpG and imiquimod (IMQ).

Expression of RIG-I followed by stimulation of primary pDCs with 5 μ g/mL type A CpG, 2.5 μ g/ml of IMQ or the combination of both TLR-ligands was determined by Q-PCR (8 h) (a) or Western blotting (24 h). RIG-I protein expression was also determined in pDC-L cells 24 h after stimulation (c). As primary pDCs were cultured in the presence of 10 ng/ml recombinant IL-3 cytokine, some experiments with pDC-L cells were also performed in this medium.

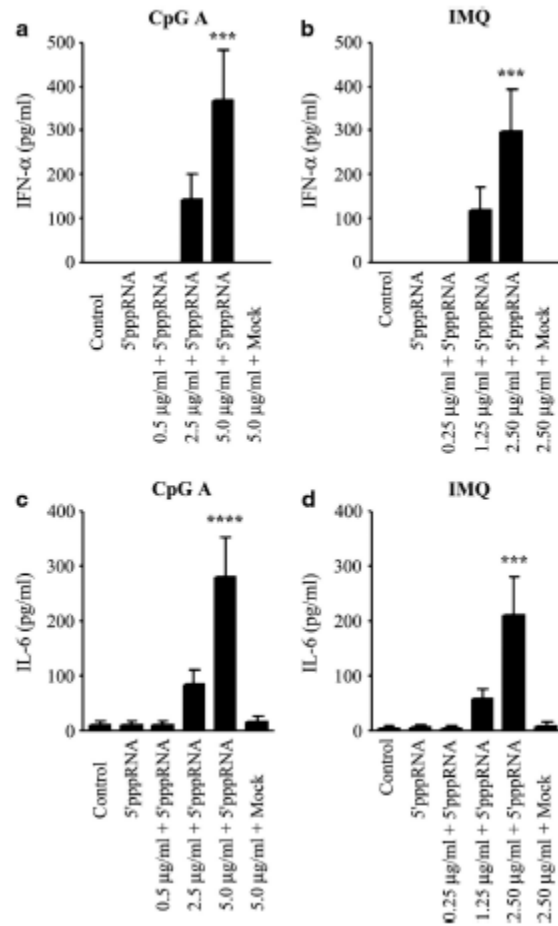


Figure 3. Cytokine secretion of primary pDCs in response to 5'ppp-dsRNA after induction of RIG-I expression with type A CpG or imiquimod (IMQ).

Primary pDCs were treated with the indicated concentrations of type A CpG (a, c) or IMQ (b, d) for 24 h and washed twice with fresh medium. Thereafter, the cells were treated by 5'ppp-dsRNA (1 μg/ml) using the LyoVec transfection system in fresh medium. Mock treatments were performed with “LyoVec-only” and LyoVec+control-oligo complexes. The concentration of the secreted IFN-α (a-b) and IL-6 (c-d) cytokines in the culture supernatants, collected after 16 h (IL-6) or 24 h (TNF-α) of incubation was measured by ELISA. Data are presented as means ± SD of triplicates within one representative experiment out of three independent ones. *** $P < 0.001$; **** $P < 0.0001$ versus untreated control.

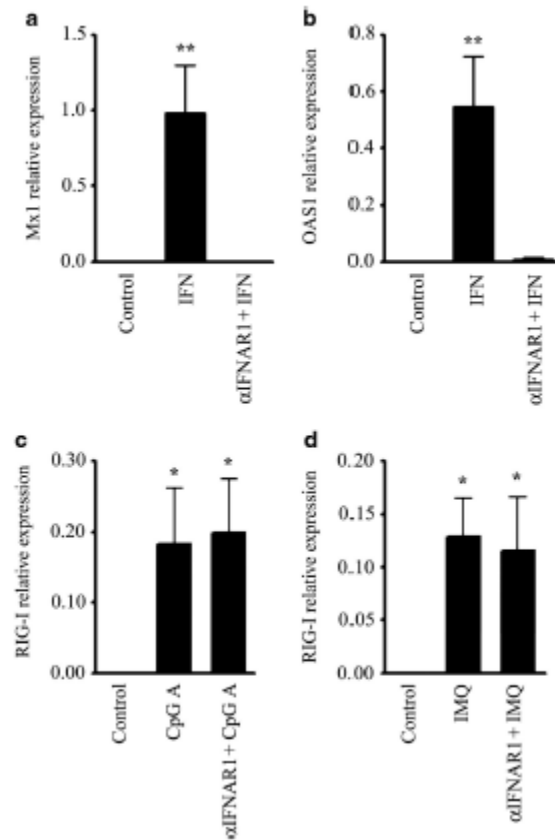


Figure 4. Induction of RIG-I expression in the primary pDCs after type A CpG and imiquimod (IMQ) treatment in the presence or absence of anti-IFNAR1 blocking antibodies.

To confirm the blocking potential of anti-IFNAR1 antibodies, primary pDCs were pre-incubated with or without 50 μ g/ml anti-IFNAR1 monoclonal antibodies for 1 h, washed, and stimulated with 10 ng/mL recombinant human IFN- α for 3 h. Relative expression levels of the early type I IFN-induced factors Mx1 (a) and OAS1 (b) were measured by Q-PCR. To control the dependence of RIG-I up-regulation in primary pDCs induced by type I IFN-mediated signals, the cells were treated with or without 50 μ g/ml anti-IFNAR1 for 1 h prior to activation by 5 μ g/ml type A CpG (c) or 2.5 μ g/ml imiquimod (d) for 3 h. Gene expression data are presented as means \pm SD of triplicates within one representative experiment out of the two independent ones. * P < 0.05, ** P < 0.01 versus untreated control.

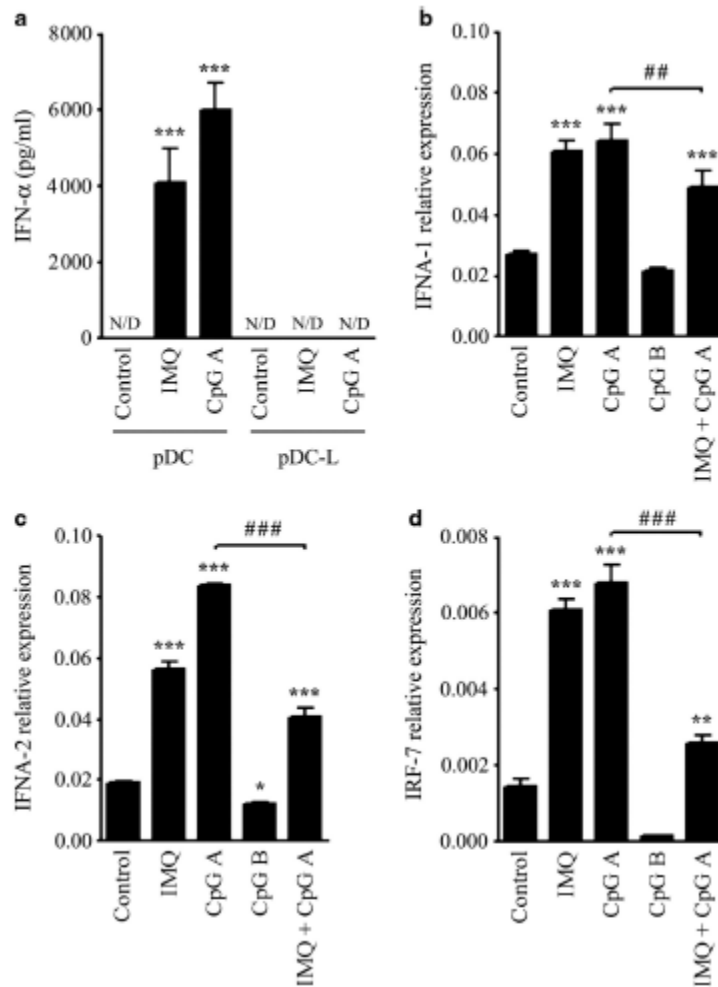


Figure 5. Type I interferon production and RIG-I expression of the activated primary pDCs and malignant pDC-L cells.

The amount of secreted IFN- α (a) in the culture supernatants of primary pDCs and malignant pDC-L cells was measured 24 h after activation with 2.5 μ g/ml imiquimod (IMQ) by cytometric bead array. Relative expressions of IFNA-1 (b), IFNA-2 (c) and IRF-7 (d) genes in pDC-L cells were measured 6 h after stimulation with the indicated TLR agonists by Q-PCR. TLR agonists were applied at the following concentrations: type A CpG and type B CpG, 5 μ g/ml; IMQ, 2.5 μ g/ml. Data are presented as means \pm SD of triplicates within one representative experiment out of the two independent ones. * P < 0.05; ** P < 0.01; *** P < 0.001 versus non-treated control, ## P < 0.01; ### P < 0.001 for imiquimod plus type A CpG

treatment vs. type A CpG treatment alone (panels b-d).

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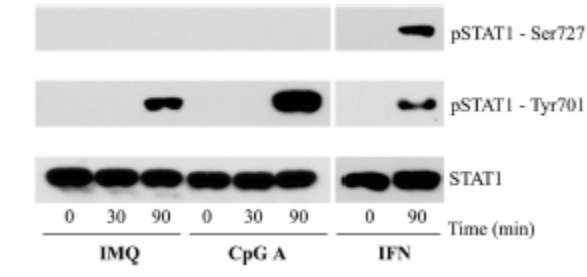


Figure 6. Induction of STAT1-phosphorylation in primary pDCs by CpG A or imiquimod (IMQ) treatment.

Time course of STAT1 phosphorylation was detected in primary pDC after treatment with type A CpG (5 $\mu\text{g/ml}$), IMQ (2.5 $\mu\text{g/ml}$) or recombinant human IFN- α (10 ng/ml). Whole cell lysates were prepared and 20 μg of the cell extract per assay were used to determine STAT1 phosphorylation at the Tyr701 and Ser727 residues and the total STAT1 levels by Western blotting. Results from a representative experiment out of the three independent ones are shown.