Innate Immune Responses in Human Monocyte-Derived Dendritic Cells Are Highly Dependent on the Size and the 5′ Phosphorylation of RNA Molecules

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Recognition of viral genetic material takes place via several different receptor systems, such as retinoic acid-inducible gene I-like receptors and TLRs 3, 7, 8, and 9. At present, systematic comparison of the ability of different types of RNAs to induce innate immune responses in human immune cells has been limited. In this study, we generated bacteriophage $\phi 6$ and influenza A virus-specific ssRNA and dsRNA molecules ranging from 58 to 2956 nt. In human monocyte-derived dendritic cells (moDCs), short dsRNAs efficiently upregulated the expression of IFN (IFN-α, IFN-β, and IFN–λ1) and proinflammatory (TNF-α, IL-6, IL-12, and CXCL10) cytokine genes. These genes were also induced by ssRNA molecules, but size-specific differences were not as pronounced as with dsRNA molecules. Dephosphorylation of short ssRNA and dsRNA molecules led to a dramatic reduction in their ability to stimulate innate immune responses. Such a difference was not detected for long ssRNAs. RNA-induced cytokine responses correlated well with IFN regulatory factor 3 phosphorylation, suggesting that IFN regulatory factor 3 plays a major role in both ssRNA- and dsRNA-activated responses in human moDCs. We also found that IFN gene expression was efficiently stimulated following recognition of short dsRNAs by retinoic acid-inducible gene I and TLR3 in human embryonic kidney 293 cells, whereas ssRNA-induced responses were less dependent on the size of the RNA molecule. Our data suggest that human moDCs are extremely sensitive in recognizing foreign RNA, and the responses depend on RNA size, form (ssRNA versus dsRNA), and the level of $5'$ phosphorylation. The Journal of Immunology, 2011, 187: 1713–1721.

RIG-I and melanoma differentiation-associated gene 5 (MDA5), can recognize foreign RNA in the cytoplasm and activate intracellular signaling cascades by interacting with the adaptor molecule IFN-β promoter stimulator-1 (4–6). In contrast, TLR3, TLR7, and TLR8 are transmembrane receptors expressed on the plasma membrane (TLR3) or endosomes and the endoplasmic reticulum (TLR7 and TLR8) (7). TLR7 and TLR8 sense viral ssRNA and use MyD88 as the adaptor molecule to transmit the signal, whereas TLR3 detects dsRNA triggering the innate immunity response via Toll/IL-1R domain-containing adaptor-inducing IFN-β (2, 8). Activation of RLR and TLR pathways leads to activation of NF-κB and phosphorylation of IFN regulatory factor (IRF) 3 and IRF7, leading to the expression of IFN genes (2, 5, 6, 9).

RIG-I is essential for eliciting IFN responses by many ssRNA viruses, including both negative-stranded (−)RNA (e.g., paramyxov-, rhadbo-, and orthomyxoviruses) and positive-stranded (+)RNA viruses (e.g., flaviviruses). Nonetheless, MDA5 plays an important role in recognizing other types of viruses, such as (+)RNA containing picornaviruses (10). In contrast, some viruses can be detected by both RIG-I and MDA5, such as West Nile and Dengue viruses that belong to the Flaviviridae family, or reovirus belonging to Reoviridae, a family of segmented dsRNA viruses (11, 12).

The ligands for RIG-I have extensively been studied during the last few years, revealing three major groups, as follows: 1) 5’ triphosphate containing RNAs, such as viral genomic RNAs, in vitro transcribed RNAs, Pol III-transcribed RNAs, and blunt end 5’-triphosphate double-stranded oligonucleotides; 2) RNAs lacking 5’ triphosphates, such as short polyinosinic:polycytidylic acid (poly I:C) molecules, 5’-monophosphorylated double-stranded oligoribonucleotides, and RNase L-cleaved cellular RNAs; and 3) RNAs with varying 5’-end characteristics, for example, viral transcription or replication intermediates and total cellular RNA.
extracted from virus-infected cells (13). However, the characteristics of RNA agonists for MDA5 are less clear. Poly I:C is known to be a ligand for MDA5 and, in mouse embryonic fibroblasts, RIG-I and MDA5 preferentially recognize short and long dsRNAs, respectively (14). TLR3, 7, and 8 are mainly responsible for detecting viral RNAs. The ligands for TLR3 involve synthetic dsRNA, poly I:C, viral genomic dsRNA, or transcription or replication intermediates of ssRNA or DNA viruses. The recognition of RNA is most likely not sequence specific, as also suggested by recent structural analysis on TLR3–RNA complexes (15). Dendritic cells (DCs) express several classes of PRRs, and they are the key cell types regulating both innate and adaptive immunity (16). PRRs recognize PAMPs by two ways, as follows: extracellularly via TLRs, and intracellularly via NOD-like receptors, RLRs, and certain TLR subtypes (17). Microbial (PAMP) stimulation or virus infection of DCs leads to the maturation of DCs, induction of costimulatory molecules, and production of cytokines, which together with MHC-dependent Ag presentation leads to the activation of adaptive immune responses.

The effects of small interfering RNAs, ranging in size from 17 to 31 nt on antiviral responses, have been extensively studied (18–20). However, at present systematic analysis on the role of RNA length, form, specificity, or the extent of phosphorylation in activating antiviral responses, especially in the human system, is lacking. In the current study, we have applied DNA-dependent T7 polymerase and RNA-dependent RNA polymerase (RdRP) of bacteriophage φ6 (21–24) to produce φ6 (25) and influenza virus (IAV)-specific ssRNA and perfectly duplexed dsRNA molecules in vitro. These RNA molecules and their 5′-dephosphorylated derivatives were used to stimulate human primary monocyte-derived DCs (moDCs) and human embryonic kidney (HEK) 293 cells expressing various RLRS or TLRs and the expression of IFN and other proinflammatory cytokine genes were analyzed. The data reveal that in human moDCs the RNA length, form, and the extent of 5′-triphosphate greatly affect cytokine gene expression. However, the origin of viral sequence (φ6 versus IAV) or differential secondary structure of ssRNA (based on prediction) is unlikely playing a role in RNA-induced stimulatory responses.

Materials and Methods

Cell lines and plasmids

The HEK293 cell line (American Type Culture Collection; ATCC CLR 1573) was maintained in continuous growth in Eagle MEM (Sigma-Aldrich) supplemented with antibiotics, t-glutamine, and 10% FCS (Integro).

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 (26). 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 supplemented with 0.6 μg/ml penicillin, 60 μg/ml streptomycin, 2 mM t-glutamate, 20 mM HEPES, and 10% FCS.

Plasmid pLM659 was the template for the production of full-length and truncated mRNA sense ssRNA (s)′ and dsRNA molecules of the small (S) genome segment of bacteriophage φ6 (27). Plasmid pLD18 was constructed by removing the egfp gene from multiple cloning site of plasmid pPS9 (24) and introducing a T7 terminator downstream of the 3′ φ6 replication signal. A chimera of IAV sequences from various conserved regions of the viral genome encoding viral internal proteins (Supplemental Fig. 1a, Supplemental Table I) was reverse transcribed into cDNA using Moloney murine leukemia virus reverse transcriptase (Fermentas) and amplified by PCR (28). The individual segments were ligated and cloned into the multiple cloning site of pLD18, thereby creating plasmid pLD19. All plasmids were maintained and propagated in Escherichia coli strain DH5α.

Synthesis of ssRNA and dsRNA molecules

ssRNAs were produced by in vitro transcription with T7 RNA polymerase. Templates for T7 transcription were prepared by PCR amplification using Phusion HF DNA polymerase (Finnzymes). Full-length and truncated cDNAs of bacteriophage φ6 S segment and chimeric IAV genes, covering a range from 58 to 2956 nt from the 3′ end (Fig. 1a, Supplemental Fig. 1a), were obtained by PCR amplification from plasmids pLM659 (27) and pLD19, respectively. The oligonucleotides used in PCR reactions are listed in Supplemental Table II. dsRNAs were generated by one-step in vitro transcription and replication using the Replicator RNAi kit (Finnzymes).

Enzymatically synthesized ssRNA and dsRNA molecules were isolated with TRizol (Invitrogen)/chloroform (5:1) extraction, followed by analytical agarose gel electrophoresis and 3 M sodium acetate precipitation (58–108 nt RNAs) (28), or stepwise lithium chloride precipitation (208–2956 nt RNAs) (24). All RNAs were washed with 70% ethanol, dissolved in sterile water, and precipitated with 0.75 M NaHAc–70% ethanol (24, 28). The pellets were suspended in sterile water and desalted with NAP5 columns (GE Healthcare) prior to HPLC purification (GenFax PAC, Waters).

RNA secondary structure calculations

The simulated two-dimensional RNA structure predictions (minimum free energy and energy parameter 37°C) were performed using the RNAfold WebServer program from the Vienna RNA Package.

Dephosphorylation of RNA

To remove 5′-triphosphates, enzymatically synthesized ssRNAs and dsRNAs were treated with FastAP thermosensitive alkaline phosphatase (Fermentas). The reactions were carried out at standard reaction conditions (10 mM Tris-HCl [pH 8.0], 5 mM MgCl2, 0.1 M KCl, 0.02% Triton X-100, 1 mM 2-ME, and 0.1 mg/ml BSA) for 30 min at 37°C, followed by inactivation of the enzyme at 75°C for 5 min. Dephosphorylated RNAs were subsequently purified, as described above.

Stimulation of cells with ssRNA and dsRNA molecules

moDC stimulations. To minimize interindividual variation, all experiments were performed with moDCs obtained from four blood donors, and the cells were stimulated separately. In initial experiments, optimal concentrations for synthetic ssRNAs, dsRNAs, and poly I:C were determined. Enzymatically synthesized RNAs (50 ng/ml) or poly I:C (10 μg/ml) were transfected with Lipofectamine 2000 (Invitrogen), according to manufacturer’s instructions. After transfection, cells from different donors were collected and pooled. Cells were used for isolation of total cellular RNA or lysed for protein samples with Passive Lysis buffer (Promega, Dual Glo kit). Because there is considerable individual variation in the responses in different blood donors (Supplemental Fig. 2), 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 in 96-well culture plates 1 d before transfection, after which the reporter and expression plasmids of RNA sensors (RIG-I, MDA5, or TLR3) or empty vectors were transfected into cells using TransIT-LT1 transfection reagent (Mirus Bio). Renilla luciferase plasmid (Promega) was used to control transfection efficiency. RNAs (20 ng/well) or poly I:C (1.5 μg/well) were transfected with Lipofectamine 2000 (Invitrogen) 4 h after transfection of reporter and expression plasmids, according to manufacturer’s instructions. Luciferase assays were performed 20 h after RNA stimulation with a Dual Glo kit (Promega), according to manufacturer’s instructions.

Quantitative RT-PCR

Total cellular RNA was isolated from moDCs derived from four pooled donors using the RNasy Mini RNA Isolation kit (Qiagen). DNase-treated total cellular RNA (1 μg) was reverse transcribed into cDNA by using the TaqMan Reverse Transcription kit (Applied Biosystems). cDNA samples were then amplified using a TaqMan Universal PCR Mastermix buffer (Applied Biosystems) and the commercial gene expression system assay (Applied Biosystems) with primers and probes for IFN-α (Hs00258882_s1), IFN-β (Hs00271888_s1), IFN-γ (Hs00601677_g1), CXCL10 (Hs00171042_m1), IL-1β (Hs01074979_m1), IL-6 (Hs00171431_m1), IL-10 (Hs00174096_m1), IL-12 p40 (Hs00233688_ml), TNF-α (Hs00171428_ml), and β-actin (Hs99999903_ml). Each cDNA sample was amplified in duplicate with a Mx3005P QPCR System (Stratagene). The relative amount of cytokine mRNA to β-actin was calculated with the ΔΔ comparative threshold cycle (Ct) method.

Western blot analysis

Whole-cell lysates were prepared in Passive Lysis buffer of Dual Luciferase Activity Kit (Promega) containing 1 mM NaVO₄. Protein aliquots of whole-
cell lysates (30 μg) were separated on 10% SDS polyacrylamide gels using the Laemmili buffer system (29). Proteins were transferred onto Immobilon-P membranes, followed by blocking with 5% milk in PBS (or in TBS for cell-signaling Abs). Guinea pig (anti-RIG-I, anti-MDA5, and anti-IRF3) and rabbit (anti-IRF3) Abs were used, as previously described (30–33). Abs against β-actin (actin H-300; SC-10731) were obtained from Santa Cruz Biotechnology. Abs against the phosphorylated forms of IRF3 (Ser396) were from Cell Signaling Technology. HRP-conjugated goat anti-rabbit or rabbit anti-guinea pig Abs (DakoCytomation) were used in secondary staining. Ab signals were visualized on HyperMax films using the ECL system (GE Healthcare).

ELISA

Cytokine levels of TNF-α and CXCL10 from moDC culture supernatants were analyzed using Ab pairs and standards from BD Pharmingen (San Diego, CA). IFN-λ1 was measured with a DuoSet kit ELISA (R&D Systems). Secreted levels of IFN-α (multisubtype) and IFN-β were measured with VeriKine ELISA kit supplied by PBL Biomedical Laboratories (Piscataway, NJ).

Results

Establishing a tool kit by enzymatic RNA synthesis and modification

Cellular RNA recognizing receptors (TLRs and RLRs) have different specificities for different types and sizes of ssRNA and dsRNA molecules (7, 15, 34). To systematically study the effects of RNA size, type (ssRNA versus dsRNA), specificity (Φ6 or IAV), or level of 5′ phosphorylation, we enzymatically synthesized different type and size RNA molecules using bacteriophage Φ6 S segment (Fig. 1A) or chimeric IAV-specific (Supplemental Fig. 1) cDNAs as templates. Using well-established T7 polymerase-based in vitro transcription system, we were able to generate a collection of 5′-phosphorylated ssRNA molecules of different molecular sizes (Fig. 1B). As a novelty, we used the ability of bacteriophage Φ6 RdRP to produce dsRNA molecules from the ssRNA templates. The dsRNA molecules were produced in a combined one-step T7 polymerase RNA transcription and bacteriophage Φ6 RdRP dsRNA synthesis reaction (Fig. 1C).

The dsRNA products generated by the Φ6 RdRP are blunt ended and completely base-paired dsRNA molecules (21; see also Fig. 1). According to the secondary structure calculations, the produced Φ6 S segment-specific ssRNA molecules contain various types of hairpin structures (Fig. 1D). Such structures may favor the recognition of these molecules by different types of RLRs.

Short dsRNA molecules are strong activators of IFN and proinflammatory cytokine genes

Although recognition of dsRNA by RNA sensors has been previously studied (14, 35), systematic analysis of dsRNA length dependence in triggering antiviral responses in the human system, such as in moDCs, has remained uncharacterized. Thus, we studied the ability of dsRNA molecules of three different size classes, short (58, 88, and 108 nt), medium (208, 308, and 508 nt), and long (708, 1808, and 2956 nt), to induce the expression of IFN-α1, IFN-β, IFN-λ1, proinflammatory (TNF-α, IL-1β, IL-6), Th1-type (IL-12, CXCL10), and anti-inflammatory Th2-type (IL-10) genes. A set of bacteriophage Φ6-specific dsRNAs (Fig. 1) was transfected into moDCs at equal concentrations. Cytokine mRNA expression was analyzed at three different time points. IFN family genes (IFN-α1, IFN-β, IFN-λ1) and TNF-α were very efficiently induced by short dsRNAs (58, 88, and 108 bp) as compared with induction by medium-size (308 bp) or long dsRNAs (708 and 1808 bp; Fig. 2A). Interestingly, IL-1β mRNA expression was induced only by long dsRNA molecules at early time points (peaking at 3 h). The kinetics and expression patterns of IL-6, IL-12, p40, and CXCL10 genes resembled those of IFNs.

However, the difference in the responses induced by the short and long dsRNAs was not as pronounced. The expression of the IL-10 gene was not induced with any of the dsRNAs studied.

We also measured the cytokine levels in the supernatants collected at three different time points (Fig. 2B). The data indicate that short dsRNAs (58, 88, and 108 bp) efficiently induced IFN-α, IFN-λ1, TNF-α, and CXCL10 protein production. Although medium-size and large dsRNAs induced IFN and TNF-α relatively weakly, CXCL10 production was almost equally well induced by short and longer RNAs (Fig. 2B). As a whole, there appeared to be a very good correlation between cytokine mRNA and protein production levels.

Cytokine gene expression is more sensitive to the size variation of dsRNA than ssRNA molecules

The ability of ssRNA and dsRNA molecules to induce innate antiviral responses in moDCs was compared. Commercial low m.w.
and high m.w. (HMW; 1.5–8 kb) poly I:C and selected in vitro produced short (88 nt) and long (708 and 1808 nt) dsRNA and ssRNA molecules were used to stimulate moDCs for different periods of time (Fig. 3A,3B, Supplemental Fig. 2). Consistent with the data above (Fig. 2), short dsRNA molecules were able to elicit higher IFN and CXCL10 mRNA expression and protein production levels as compared with long dsRNAs. In the case of ssRNAs, the differences were not as pronounced, because also long ssRNA molecules induced IFN-α and IFN-β. Cell culture supernatants were collected at 3, 8, and 24 h after transfection, and protein levels of IFN-α1, IFN-α, TNF-α, and CXCL10 were determined by ELISA. The mean (±SD) cytokine levels produced by the cells of four blood donors are shown. The data are representative of two individual experiments. Cytokines, time points, and the sizes of dsRNAs are indicated in the figure.

RNA stimulation activates IRF3 phosphorylation and enhances the expression of RLRs and IFRF7

To investigate which signaling pathways would contribute to triggering of innate immune responses after dsRNA or ssRNA stimulation in moDCs, analysis of phospho-IRF3, total IRF3, IRF7, RIG-I, and MDA5 expression was analyzed by Western blotting. Short (88-bp) dsRNA elicited efficient IFRF3 phosphorylation in moDCs, whereas ssRNA induced IFRF3 phosphorylation relatively weakly (Fig. 3C). The phosphorylation of IRF3 was clearly detected at 3 h after dsRNA or ssRNA stimulation, reaching its peak at 8 h poststimulation and thereafter declining to basal unphosphorylated levels within 24 h (Fig. 3C). The kinetics of LMW and HMW poly I:C-induced phospho-IRF3 expression was faster and more efficient as compared with that induced by in vitro produced short and long dsRNA and ssRNA molecules (Fig. 3C). The basal expression of IFRF7 was low, but it was strongly elevated at 8 and 24 h after RNA stimulation. The type or length of the RNA did not significantly affect IFRF7 expression levels. Initially,
The role of the 5'-triphosphate group of the in vitro produced dsRNA and ssRNA molecules in stimulating cytokine mRNA expression in moDCs was investigated and compared with the responses induced by commercial poly I:C. Equal amounts of phosphorylated and dephosphorylated short (88 nt long) dsRNA and ssRNA were transfected into human moDCs, and the expression of cytokine mRNAs (IFN-β, IFN-λ1, IFN-α1, and CXCL10) was analyzed at three different time points after transfection. Interestingly, the removal of the 5'-triphosphate group from the short dsRNAs significantly suppressed the induction of cytokine gene expression (Fig. 4A, 4B, left panel). Moreover, dephosphorylated short ssRNAs were practically unable to induce cytokine mRNA expression (Fig. 4A, 4B, right panels).

Phosphorylation of IRF3 was hardly detectable by Western blot analysis when moDCs were stimulated with dephosphorylated short dsRNA or ssRNA molecules, indicating that 5’-phosphate groups in short RNAs contribute to the activation of cytokine gene expression (Fig. 4C). In the case of long RNA molecules, the level of RNA 5’ phosphorylation did not significantly affect the efficiency of IRF3 phosphorylation (Fig. 4C).

Multiple RNA sensors recognize dsRNA and ssRNA in HEK293 cells

A previous study conducted in RIG-I−/− and MDA5−/− mouse embryonic fibroblasts demonstrated that RIG-I selectively recognizes medium-size poly I:C molecules (∼300 bp long), whereas long poly I:C (∼800 bp or longer) are ligands for MDA5 (14). To further elucidate whether the recognition of dsRNA by multiple RNA sensors is length dependent also in the human system, various size dsRNA molecules were transfected together with RNA sensor-expression constructs and IFN promoter-reporter constructs into HEK293 cells, with LMW and HMW poly I:C functioning as positive controls (Fig. 5).

Both in RIG-I−, MDA5−, and TLR3-overexpressing HEK293 cells, short (58–108 bp) dsRNAs triggered better activation of IFN-β and IFN-α1 promoters as compared with medium-size (208–508 bp) or long (708–2948 bp) dsRNAs (Fig. 5A). The IFN promoter activation in MDA5-expressing cells was somewhat stronger than in RIG-I-expressing cells, especially when cells were challenged with LMW or HMW poly I:C. In RIG-I gene-transfected cells, the dsRNA size dependency was clear, whereas in MDA5-expressing cells there appeared to be a trend that long dsRNAs induced slightly better responses as compared with medium-size dsRNA molecules (Fig. 5A). It is noteworthy that mere overexpression of MDA5 led to significant IFN promoter activation, rendering the dynamic range of the assay relatively insensitive.

In TLR3-overexpressing cells, the control poly I:C molecules and the short 88-bp dsRNA were the best activators of both IFN-β and IFN-α1 promoters (Fig. 5A, right panel). It is of interest that all tested dsRNA molecules, although with slightly different efficiency, were able to activate IFN promoters via TLR3 (Fig. 5A).

To study the length dependency of ssRNA recognition by RNA sensors, we stimulated RIG-I− or MDA5-overexpressing HEK293 cells by transfecting them with equal mass amount of 66 S
segment-specific ssRNAs of different lengths (Fig. 5B). In contrast to dsRNA, the length of the ssRNA did not have a major effect on the level of IFN-β or IFN-λ1 promoter activation in HEK293 cells (Fig. 5B). Apparently, RIG-I– or MDA5-mediated ssRNA recognition was less length dependent as compared with that of dsRNA.

To further investigate the recognition of the 5’-triphosphorylated and dephosphorylated dsRNA- and ssRNA-stimulated human moDCs. Cells were collected at 3, 8, or 24 h after RNA transfection, and lysed in passive lysis buffer, followed by separation of 30 μg samples of cellular proteins on 10% SDS-PAGE and Western blot analysis with the indicated Abs. One representative experiment of three independent experiments is shown.

Similar IFN expression patterns were also observed when equal mass (Fig. 5A) or molar amounts (Fig. 6A) of dsRNAs were used to transfect HEK293 cells. Furthermore, the activation patterns were not significantly changed when the 5’-triphosphorylated and dephosphorylated ssRNA molecules led to completely abolished IFN-β and IFN-λ1 promoter activation via the TLR3 signal pathway.

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a wide repertoire of PRRs and their flexibility in recognizing different types of RNAs.

It has previously been suggested that RIG-I preferentially recognizes 5'-triphosphorylated RNAs (14, 37, 38), including blunt-ended dsRNAs with 5'-triphosphate groups (42, 43). Because short 5'-triphosphorylated dsRNAs and ssRNAs induced strong immune responses in human moDCs, it is likely that RIG-I plays a dominant role in recognizing foreign dsRNA and ssRNA molecules triggering the expression of IFN and other proinflammatory cytokine genes. In the case of long dsRNA molecules, dimerization of RIG-I could be prevented (44), which would lead to inefficient RIG-I signaling by large dsRNA molecules. Consistent with this hypothesis, medium or long dsRNA molecules were thus quite poor inducers of IFN responses in human moDCs (Fig. 2) and in RIG-I-overexpressing HEK293 cells (Fig. 5A). However, long dsRNAs were able to induce inflammatory
responses, possibly through other signal pathways than RIG-I, because they efficiently enhanced IL-1β, IL-6, and CXCL10 gene expression (Fig. 2).

We also observed that short dsRNAs induced more efficient phosphorylation of IRF3 than long dsRNAs, especially at early stages of stimulation. Although it has been reported that in mouse fibroblasts long dsRNA molecules trigger antiviral responses in an IRF3-independent fashion (35), our result suggests that stimulation by short dsRNAs and to a lesser extent by long dsRNAs is mediated by IRF3 (Fig. 3C). This is also consistent with short dsRNAs being able to elicit strong IFN responses in transfected HEK293 cells through RIG-I–, MDA5–, and TLR3-mediated pathways (Fig. 5A). We also showed that stimulation of moDCs with short dsRNAs led to increased expression of RIG-I, MDA5, and IRF7 (Fig. 3C), which may provide a positive feedback to cellular responses. The increased expression of RLRs and IFR7 is most likely due to early IFN production, because these genes are under the regulation of type I and type III IFNs (30, 45). It was also of interest that none of the tested dsRNAs was able to induce IL-10 gene expression, indicating that moDCs preferentially respond to these danger signals by inducing IFN and proinflammatory cytokine-mediated responses.

MoDC responses to stimulation by ssRNAs were somewhat different from those induced by dsRNAs. In moDCs (Figs. 3A, 4B) and in RLR- or TLR3-expressing HEK293 cells (Fig. 5B), IFN induction was less dependent on the length of ssRNAs as compared with dsRNAs. This may be due to a more complex secondary structure of ssRNAs, which, in contrast to linear dsRNA, can form various hairpin structures that are recognized by RNA sensors, rendering the actual length of the ssRNA of secondary importance. Surprisingly, at early time points of stimulation, long ssRNAs were able to elicit more efficient IRF3 phosphorylation than short ssRNAs when equal molar amounts of ssRNAs were used (Fig. 4C). Interestingly, the induction of IFN mRNA expression remained at a similar level regardless of the ssRNA applied (Fig. 4B). It is therefore possible that in human moDCs, short ssRNAs induce antiviral responses simultaneously via multiple signaling pathways, including TLR3, TLR7/8, RIG-I, and MDA5, instead of using only the RIG-I/IRF3 signaling pathway (46, 47). Recent evidence (48, 49) suggests that in human moDCs, TLR3 and TLR7/8 ligands induce the expression of proinflammatory cytokines synergistically. However, for enhanced IFN gene expression, positive feedback via early IFN production and upregulation of TLR and IRF7 genes is required (48, 49). It is likely that certain TLR/RLR ligands, such as short ssRNAs, may activate multiple transcription factor systems, whereas long ssRNAs mainly stimulate the IRF3 signaling pathway to trigger antiviral responses.

It has been observed that the 5′-triphosphate group is essential for ssRNA recognition by RIG-I (36). In this study, we demonstrated that short 5′-triphosphorylated dsRNA and ssRNA molecules could elicit high antiviral responses in human moDCs (Figs. 3, 4). Removing the 5′-triphosphate group strongly reduced both dsRNA- and ssRNA-induced antiviral responses (Fig. 4A). This indicates that RIG-I–dependent recognition of small RNAs, especially that of ssRNA molecules, takes place mainly in a 5′-triphosphate–dependent fashion. These data are consistent with previous publications, which demonstrated that partially duplexed RNAs (e.g., RNase L digestion products) and longer siRNAs (27–31 bp), which both lack 5′-triphosphate groups, can activate RIG-I and IFN responses, respectively (19, 50, 51). Because IFN gene expression and phosphorylation of IRF3 were inefficiently induced by dephosphorylated dsRNA and ssRNA molecules, it can be expected that RNA sensing by RIG-I followed by IRF3 phosphorylation are the dominant signaling pathways in human moDCs for the detection of foreign or short RNAs.

Dephosphorylation of RNA molecules reduced the activation of the IFN-β and IFN-α promoters following dsRNA induction through the RIG-I and MDA5 signal pathway in HEK293 cells (Fig. 5). This is consistent with the data obtained in human moDCs. However, dephosphorylation completely abolished IFN activation through the TLR3 signal pathway as well (Fig. 5C). This strongly suggests that the 5′-triphosphate group is essential also for TLR3-dependent recognition of dsRNA.

Based on previous studies (2, 6, 7, 13, 14) and the data presented in this work, it appears that dsRNA recognition in human moDCs is clearly length dependent with short 5′-triphosphorylated dsRNA molecules eliciting the strongest antiviral responses. Moreover, the 5′-triphosphate group is essential for the recognition of short ssRNAs. Long ssRNA molecules, instead, are capable of activating cytokine gene expression also in the absence of 5′-phosphate groups. Furthermore, we found no evidence for viral sequence-specific recognition of RNAs in human moDCs, because no major differences were found between φ6 versus IAV RNA-induced responses. The findings presented in the present work expand our knowledge on how human moDCs recognize RNA molecular
patterns and viral pathogens, thereby extending our understanding of innate immune responses during viral infection and facilitating the design and development of RNA-based drugs.

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Disclosures

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