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DHX9 Pairs with IPS-1 To Sense Double-Stranded RNA in Myeloid Dendritic Cells

Zhiqiang Zhang, Bin Yuan, Ning Lu, Valeria Facchinetti, and Yong-Jun Liu

The innate immune system is equipped with many molecular sensors for microbial DNA/RNA to quickly mount antimicrobial host immune responses. In this paper, we identified DHX9, a DEAd helicase family member, as an important viral dsRNA sensor in myeloid dendritic cells (mDCs). Knockdown of DHX9 expression by small heteroduplex RNA dramatically blocked the ability of mDCs to produce IFN-α/β and proinflammatory cytokines in response to polyinosine-polyribidylic acid, influenza A, and reovirus. DHX9 could specifically bind polyinosine-polyribidylic acid via its double-strand RNA binding motifs. DHX9 interacted with IPS-1 via the HelicC-HA2-DUF and CARD domains of DHX9 and IPS-1, respectively. Knockdown of DHX9 expression in mDCs blocked the activation of NF-κB and IFN regulatory factor 3 by dsRNA. Collectively, these results suggest that DHX9 is an important RNA sensor that is dependent on IPS-1 to sense pathogenic RNA. The Journal of Immunology, 2011, 187: 4501–4508.

The innate immune system detects viral infection predominantly by sensing viral nucleic acids. Innate immune cells, such as macrophages, dendritic cells (DCs), and epithelial cells/fibroblasts, use at least three types of pattern recognition receptors, including the TLRs (TLR3, 7, 8, and 9) (1–3), retinoic acid-inducible gene I-like helicases (RLH, RIG-I, LGP2, and MDA5) (4–7), and dsRNA-activated protein kinase receptor (PKR) (8, 9), to sense different forms of viral nucleic acids for inducing IFN responses. Some RNA virus infections generate dsRNA, which are detected by different types of receptors within the innate immune system. DCs are in the frontline of the innate immune system—they can sense viral infection, process viral Ag, and mount both innate and adaptive immune responses to virus (10). Although previous studies using gene-targeting technology demonstrated that myeloid DCs (mDCs) or conventional DCs use TLR3, RIG-I, and MDA5 to sense viral RNA infection or synthetic dsRNA polyinosine-polycytidylic acid (poly I:C) that mimics viral dsRNA to produce type 1 IFN, whether there are additional dsRNA sensors in mDCs are unknown (7).

DHX9 is a member of the DExDc helicase family and is a homolog of Drosophila maleless protein, which is essential to regulate X-linked gene expression and the survival of male larvae (11). DHX9 recognizes a structured 5'–terminal posttranscriptional control element in retroviruses that regulates translation (12). The ATPase helicase function of DHX9 is necessary for efficient HIV-1 RNA translation (13). Several lines of evidence suggest that DHX9 plays important roles in the regulation of immune response. Results from luciferase reporter gene assays showed that DHX9 enhances the expression of p65 and TNF-α. Knockdown of DHX9 expression by RNA interference reduced levels of NF-κB–dependent transactivation by mediating the transcriptional activity of NF-κB (14). DHX9 is a microbial DNA sensor that is dependent on MyD88 and triggers NF-κB responses in plasmacytoid DCs (pDCs) (15). DHX9 was found as an autoantigen in the autoimmune disease systemic lupus erythematosus (16). DHX9 has two dsRNA-binding motifs. These motifs are conserved in the cis-acting transactivation response element (TAR)-binding protein and PKR, a dsRNA sensor. DHX9, TAR-binding protein, and PKR are all involved in the regulation of HIV-1 gene expression through their binding to TAR RNA (17). Further investigation indicated that DHX9 interacts with PKR and is identified as a substrate for PKR (18).

In this study, we show that DHX9 acts as an important viral sensor in myeloid DCs (mDCs). DHX9 could specifically bind to dsRNA, binding activity required the dsRNA binding motif (DSRM) of DHX9. DHX9 also interacted with IPS-1; this interaction was dependent upon the helicase-associated domain (HA2)-domain of unknown function (DUF) domains of DHX9 and the CARD domain of IPS-1. We demonstrate that knockdown of DHX9 expression by small heteroduplex RNA (shRNA) dramatically blocked the ability of mDCs to produce type I IFN and proinflammatory cytokines in response to dsRNA and RNA virus. Taken together, our results suggest that DHX9 is an important IPS-1–dependent RNA sensor.

Materials and Methods

Reagents

Poly I:C was purchased from InvivoGen. This poly I:C is composed of a strand of poly (I) annealed to a strand of poly (C). The size of the short poly I:C is from 0.2 to 1 kb. The size of the long poly I:C is from 1.5 to 8 kb. Poly deoxyadenylc-deoxythymidylic acid (poly DA-dT), poly deoxyguanylic-deoxyctydylid acid (poly dG-dC), and poly deoxyinosinic-deoxyctydylid acid (poly dI-dC) were purchased from Sigma-Aldrich. Lipofectamine 2000 was purchased from Invitrogen. The following Abs were used for immunoprecipitation and/or immunoblotting: anti-DHX9 and anti-IFN regulatory factor 3 (IRF3) (Santa Cruz Biotechnology), anti-MDA5, anti–RIG-I, anti–IPS-1, anti–p65, anti–phospho-IRF3, and anti–phospho-p65 (Cell Signaling Technology), anti–β-actin, anti–hemagglutinin (HA)-HRP, and anti–MyC-HRP (Sigma-Aldrich). Anti-β-HA and anti-MyC beads were purchased from Sigma-Aldrich. NeutAvidin beads were purchased from Pierce.
**D2SC mDCs cell culture**

The murine splenic DC cell line D2SC was maintained in IMDM containing 5% heat-inactivated FCS and 1% penicillin-streptomycin (Invitrogen Life Technologies). D2SC cells were infected with the lentiviral vector carrying a helicase-targeting shRNA or a scrambled shRNA. After 1–2 d of growth, cells were seeded at a density of 10^5 cells/ml in the medium. Knockdown efficiency and specificity was confirmed by immunoblot. Cells were stimulated with one of the following nucleic acids plus Lipofectamine 2000: long poly I:C, short poly I:C (both at 2.5 μg/ml concentration), 1 μg/ml poly dA-dT, and 2 mM CpG A or RNA virus, influenza A (Flu A), or reovirus, each virus at a multiplicity of infection of 10.

**GM-CSF–derived DCs preparation**

Single-cell suspensions of bone marrow cells were cultured in RPMI 1640 medium containing 10% FCS, 1 mM sodium pyruvate, HEPS, penicillin, streptomycin, and 2-ME (RPMI 1640 medium/FCS) supplemented with murine GM-CSF (50 ng/ml) (R&D Systems). Fresh GM-CSF was provided on days 3 and 5 of culture. Cells were recovered on day 6 for shRNA knockdown of a targeted helicase or scrambled control. The knockdown efficiency was detected with western blotting 36 h post-shRNA treatment.

**RNA interference**

shRNA lentiviral vectors were purchased from Open Biosystems: target for RIG-I, clone TRCN0000010388; target for MDA5, clone V2HS_202104; target for IPS-1, clone TRCN0000124770; target for MyD88, clone TRCN0000077236; and target for DHX9, clone TRCN and clone TRCN0000071116 (DHX9-a) and TRCN0000071117 (DHX9-b).

**Luciferase reporter gene assay**

HEK293T cells were seeded on 48-well plates (1 × 10^4 cells/well) and then transfected with 100 ng IFN-β luciferase and 2 ng Renilla luciferase reporter vectors plus 20, 100, or 200 ng DHX9, ΔDSRM, MyD88, or IPS-1 expression vector. Empty control vector was added so that a total of 500 ng vector DNA was transfected in each well of cells. After 30 h post-transfection, cells were transfected with 2.5 μg/ml poly I:C delivered by Lipofectamine 2000. Cells were harvested after 6 h of stimulation. The luciferase activity in the total cell lysate was detected with the Dual-Luciferase Reporter Assay.

**In vitro pulldown and immunoblotting assay**

Lysates from HEK293T cells transfected with HA-tagged DHX9 or DHX36 expression plasmids were incubated with anti-HA beads. Proteins were eluted from beads after washing six times with PBS. For nucleic acid pulldown assays, purified HA-DHX9 or HA-DHX36 proteins were incubated for 2 h with biotin-labeled poly I:C, poly dA-dT, poly dG-dC, poly dI-dC, CpG A, or CpG B. Following incubation with NA beads, bound complexes were pelleted by centrifugation and analyzed by immunoblot with anti–HA-HRP Abs. To ascertain DHX9 binding specificity, pulldown beads were repeated with HA-DHX9 protein plus biotin-labeled poly I:C in the absence or presence of increasing amounts (0.5, 5, and 50 μg/ml) of unlabelled CpG B, polyadenylic acid (poly A), polyadenylic-polyuridylic acid (poly A-U), poly I:C, or CpG A. For DHX9-IPS-1 interaction analysis, D2SC cells were unstimulated (−) or stimulated (+) with short poly I:C or long poly I:C for 4 h; whole-cell lysates were prepared from the cells and then immunoprecipitated with anti-DHX9 Ab; bound proteins were analyzed by immunoblotting with anti-DHX9, anti–IPS-1, or anti–RIG-I Abs. To map region(s) in DHX9 required for interaction with IPS-1, purified Myc-IPS-1 was incubated for 1 h with HA-DHX9 or DHX9 truncations proteins, individually, followed by one more hour of incubation after adding anti-Myc beads. Bound proteins were pelleted by centrifugation, and analyzed by immunoblotting with anti–Myc-HRP Abs.

**RNA extract and quantitative PCR**

Total RNA was isolated from cells using the RNeasy kit (Qiagen), and treated with DNase (Qiagen). cDNA was synthesized using SuperScript II (Invitrogen) and random hexamer primers. Quantitative PCR (qPCR) amplification was performed using TaqMan Universal Master Mix (Applied Biosystems) on the 7500 Fast Real-Time PCR system (Applied Biosystems). The following primers are used for qPCR: TNF-α, 5′-CTAGCCGAGTATGACGAGGAG-3′ (forward) and 5′-TCCACAGTCACCTTCTCCTC-3′ (reverse); reovirus, 5′-CAAGCCACATTTGGTTAGG-3′ (forward) and 5′-GCCCTAGCTGAGTGGTAC-3′ (reverse); and GAPDH, 5′-AAGCAGAGTACTGTTAGTTG-3′ (forward) and 5′-AAGCCGAGTTCCTCCGTAC-3′ (reverse).

**Results**

**DHX9 senses both short poly I:C and long poly I:C in D2SC mDCs**

The DExD/H helicase family members RIG-I, MDA5, LGP2, and DICER have been shown to play important roles in sensing dsRNA and viral infection (4–7, 19). In pDCs, DHX9 is able to sense CpG mediated by MyD88 (15). In addition, DHX9 regulates HIV-1 gene expression through its binding to TAR RNA, indicating that DHX9 is also an RNA binding protein (17). We decided to investigate the function of DHX9 in sensing dsRNA and RNA virus. We therefore established stable D2SC cell lines expressing shRNA to knockdown expression of DHX9, MyD88, IPS-1, RIG-I, or MDA5. D2SC cells expressing a scrambled shRNA targeting sequence served as the control. Knockdown efficiency of each cell line was assessed by western blot (Fig. 1A). IFN-α/β, TNF-α, and IL-6 production was measured after control and knockdown D2SC cells were stimulated with a short poly I:C (0.2–1 kb). As shown in Fig. 1B, control D2SC mDCs produced high levels of IFN-α/β, TNF-α, and IL-6 following stimulation with short poly I:C. This cytokine response was strongly attenuated in DHX9-ΔDSRM, MyD88-, and RIG-I-knockdown D2SC mDCs and was not or only slightly affected in MyD88− and MDA5−/−/−D2SC mDCs, confirming the previous report showing that MDA5 only plays an important role in sensing long poly I:C (4).

We next investigated whether DHX9 also plays a role in sensing long poly I:C (1.5–8 kb) in D2SC cells. We found that knocking down DHX9 expression resulted in 60% reduction in type I IFN, TNF-α, and IL-6 production by D2SC cells in response to long poly I:C (Fig. 1C). MDA5 and IPS-1 knockdown in D2SC cells led to 80–90% reduction in type I IFN, TNF-α, and IL-6 production, whereas RIG-I and MyD88 knockdown had little effect on IFN-α/β production by D2SC cells in response to long poly I:C. These data suggest that DHX9 plays important roles in sensing both short (0.2–1 kb) and long (1.5–8 kb) poly I:C in D2SC cells. Our data also confirms previous studies showing that MDA5/IPS-1 preferentially senses long poly I:C and RIG-I/IPS-1 preferentially senses short poly I:C in DCs (20).

To determine whether DHX9 senses other nucleic acids, cytokine production was measured after control and knockdown D2SC cells were stimulated with B form DNA (poly dA-dT) or CpG. As shown in Fig. 1D and 1E, DHX9 knockdown, as well as MyD88, IPS-1, RIG-I, and MDA5 knockdown in D2SC mDCs, had little effect on IFN-α/β production in response to poly dA-dT. Although DHX9 or MyD88 knockdown led to 90% reduction in IFN-α/β production in response to CpG, confirming the previous report showing that DHX9 senses CpG mediated by MyD88 (15).

**DHX9 senses RNA virus in D2SC mDCs**

To determine the function of DHX9 in sensing viral infection, cytokine production was measured after culturing control and knockdown D2SC cells with reovirus. DHX9− and IPS-1−/−knockdown D2SC mDCs had 80% reduction in IFN-α/β production in
response to reovirus (Fig. 1). Although both RIG-I- and MDA5-knockdown D2SC mDCs had 50% reduction in IFN-α/β levels, MyD88 knockdown in D2SC cells had little effect on IFN-α/β production in response to reovirus. To further determine whether DHX9 senses other RNA viruses, cytokine levels were measured after culturing control and knockdown D2SC cells with Flu A virus. As shown in Fig. 1G, DHX9 knockdown in D2SC mDCs led to 50% reduction in IFN-α/β production in response to Flu A. In contrast, RIG-I- and IPS-1-knockdown D2SC mDCs had almost 80% reduction in IFN-α/β production, whereas knockdown of MyD88 had little effect on IFN-α/β production by D2SC cells in response to Flu A. These data indicate that DHX9 plays an important role in mediating DC responses to RNA viruses. In addition, our study suggests that there are different and redundant sensors for detecting different RNA species during viral infection.

DHX9 knockdown in bone marrow-derived DCs abolishes their cytokine responses to poly I:C and RNA virus

To determine whether DHX9 senses dsRNA in primary cells, GM-CSF-derived mDCs were prepared and treated with shRNA to knockdown expression of DHX9, IPS-1, RIG-I, or MDA5. Fig. 2A shows the specificity and efficiency of shRNA knockdown at the protein level without affecting the expression of nontargeted proteins in the bone marrow-derived DCs (BMDCs). Control and knockdown BMDCs were examined for their IFN-α/β response following stimulation with nucleic acid or virus. As shown in Fig. 2B, DHX9- and IPS-1-knockdown BMDCs produced much reduced IFN-α/β levels in response to short poly I:C, long poly I:C or reovirus when compared with levels produced by the control cells. Although RIG-I- and MDA5-knockdown BMDCs had 50% reduction in IFN-α/β production in response to reovirus, the RIG-I-knockdown BMDCs produced reduced levels of IFNα/β in response to short poly I:C and normal levels of IFNα/β in response to long poly I:C. On the contrary, MDA5-knockdown BMDCs produced normal levels of IFNα/β in response to short poly I:C but much reduced levels of IFNα/β in response to long poly I:C. These data indicate that DHX9 and IPS-1 play a critical role in sensing both short and long poly I:C in BMDCs.

To determine the function of DHX9 in sensing viral infection in BMDCs, cytokine production was measured after culturing control and knockdown BMDCs with reovirus. DHX9- and IPS-1-knockdown BMDCs had 85% reduction, whereas both RIG-I- and
MDA5-knockdown BMDCs had 50% reduction in IFN-α/β production in response to reovirus (Fig. 2D). To determine whether DHX9 plays an important role in antiviral defense, reovirus RNA replication in BMDCs was measured by qPCR. The replication of reovirus substantially increased in DHX9- and IPS-1–knockdown BMDCs (Fig. 2E). These data indicate that DHX9 plays an important role in mediating BMDCs responses to reovirus.

DHX9 is also involved in basic cellular functions to selectively regulate mRNA through binding of 5′-UTRs. To further determine whether DHX9 directly affects the cytokine mRNA transcription or stability, the mRNA of TNF-α and the mRNA of IFN-β in BMDCs treated with scrambled shRNA or shRNA targeting DHX9 were measured. As shown in Fig. 2F, no change of TNF-α mRNA or IFN-β mRNA was observed, excluding the possibility of DHX9 directly regulating the cytokine production at the transcriptional or translational level.

**DHX9 is a poly I:C-binding protein**

To determine whether DHX9 directly binds nucleic acids, we conducted pulldown assays in which a purified HA-tagged DHX9 or DHX36 recombinant protein was incubated with biotin-labeled poly I:C, poly dA-dT, poly dG-dC, poly dI-dC, CpG A, or CpG B, followed by pulldown with NA-beads. We found recombinant DHX9 protein to associate with poly I:C RNA and CpG DNA, suggesting that DHX9 binds dsRNA directly (Fig. 3A). To map the dsRNA binding site of DHX9, we repeated pulldown assays using biotin-labeled poly I:C and truncated versions of DHX9. Results indicated that the DSRM of DHX9 was required for poly I:C binding (Fig. 3C). To confirm that DHX9 was specifically binding to poly I:C, we performed competition experiments by adding increasing amounts of unlabeled poly I:C, poly A:U, CpG, and poly A to HA-DHX9 protein plus biotin-labeled poly I:C, followed by pulldown with NA beads. We found that only unlabeled poly I:C could block the binding of biotin-labeled poly I:C to DHX9 (Fig. 3D), supporting the idea that DHX9 directly associates with poly I:C.

Overexpression of DHX9 leads to enhanced IFN-β promoter activity in response to poly I:C

To further confirm the role of DHX9 in sensing poly I:C, we conducted transactivation assays to determine whether DHX9 expression was associated with Ifnb promoter activity in response to poly I:C. MyD88, IPS-1, DHX9, and a DHX9 mutant lacking the DSRM were overexpressed in HEK293T cells containing a luciferase reporter controlled by the Ifnb promoter. As shown in Fig. 4, we found that overexpression of DHX9 and IPS-1 led to increased Ifnb promoter activation following stimulation with short or long poly I:C, whereas the DHX9 mutant with deletion of the DSRM failed to activate the Ifnb promoter. Overexpression of DHX9 and the DHX9 mutant with deletion of the DSRM had little

**FIGURE 2.** DHX9 knockdown in BMDCs abolishes their cytokine responses to poly I:C and reovirus. A, Immunoblot (IB) showing the knockdown efficiency of shRNA targeting the indicated genes in BMDCs. Nontargeting shRNA served as a control (first left lane). β-Actin blots are shown as loading controls (lower panel). ELISA of IFN-α/β production by BMDCs with the indicated shRNA after 16 h stimulation with 2.5 μg/ml short poly I:C (B), 2.5 μg/ml of long poly I:C (C), or reovirus (D) at a multiplicity of infection (MOI) = 10. Nucleic acids were delivered to the cells by Lipofectamine 2000. E, qPCR of reovirus copies from BMDCs with shRNA knockdown of indicated protein. BMDCs were incubated with reovirus at MOI of 10 for 2 h. Cells were collected, washed with PBS, and cultured for an additional 16 h, followed by RNA extract and RT-PCR. F, qPCR of TNF-α and IFN-β mRNA extract from BMDCs treated with scrambled shRNA (control) or shRNA targeting DHX9. N-STM, scrambled shRNA-treated D2SC cells without stimulation.
effect on the activation of the *Ifnb* promoter without poly I:C stimulation, indicating that this activation by DHX9 is poly I:C dependent. Overexpression of IPS-1 led to increased *Ifnb* promoter activation without poly I:C stimulation, confirming the previous report that the transfection of IPS-1 expression plasmid activates the *Ifnb* promoter (21). Overexpression of MyD88 had little effect on the activation of the *Ifnb* promoter with or without poly I:C stimulation. These data indicate that the dsRNA-binding activity of DHX9 is necessary for eliciting the IFN response to poly I:C.

**DHX9 interacts with IPS-1 in D2SC mDCs**

Because DCs with knockdown of DHX9 or IPS-1 displayed similar cytokine responses to short and long poly I:C, we hypothesize that DHX9 may use IPS-1 as an adaptor molecule for signal transduction. We next investigated the potential interaction between DHX9 and IPS-1 in mDCs. We performed anti-DHX9 immunoprecipitation experiments in resting or poly I:C-activated D2SC mDCs. As shown in Fig. 5A, endogenous DHX9 associates with the *Ifnb* promoter with or without poly I:C stimulation. These data indicate that the dsRNA-binding activity of DHX9 is necessary for eliciting the IFN response to poly I:C.

**FIGURE 3.** DHX9 requires DSRM domain to bind poly I:C. A, Immunoblot using anti-HA Ab of pulldown assays in which purified HA-DHX9 or HA-DHX36 recombinant proteins were incubated with biotinylated poly I:C, poly dA-dT, poly dG-dC, poly dl-dC, CpG A, or CpG B, followed by addition of NA beads. B, Schematic representations of full-length and serial truncations of DHX9. HA-DHX9, DHX9 full size; HA-9ΔDSRM, DHX9-deleted DSRM; HA-9ΔDEXDc, DHX9-deleted DSRM and DEXDc; HA-9ΔHelicC, DHX9-deleted DSRM, DEADc and HelicC; HA-9CADF, DHX9-deleted DUF; HA-9CASHD, DHX9-deleted HA2 and DUF. Numbers denote amino acid residues. C, Immunoblot using anti-HA Ab of pulldown assays in which purified HA-tagged serial truncations of DHX9 were individually incubated with biotinylated poly I:C, followed by addition of NA beads. D, Immunoblot using anti-HA Ab of pulldown competition assays in which 0.5, 5, or 50 μg/ml CpG B, poly A, poly A:U, poly I:C, or CpG A was added to a mixture of HA-DHX9 protein plus biotinylated poly I:C, followed by addition of NA beads. DEAD, Asp-Glu-Ala-Asp box motif.

As DHX9 or RIG-I knockdown displayed similar effect on the cytokine responses to short poly I:C, to further determine whether the DHX9 signaling is independent on RIG-I signaling, we performed experiments using RIG-I deficiency D2SC cell line to knockdown DHX9. The residual response to short poly I:C in RIG-I deficiency D2SC is existing. This residual response was decreased dramatically after knockdown DHX9 (Fig. 5E), suggesting that DHX9 is independent on RIG-I to sense poly I:C.

**DHX9 and IPS-1 are required for activating the NF-κB and IFN-response signal transduction pathways upon poly I:C stimulation**

It was previously shown that over-expression of IPS-1 in HEK293 cells resulted in robust activation of IRF3 and NF-κB (22). We investigated whether downstream signaling of DHX9 in

**FIGURE 4.** Overexpression of DHX9 leads to enhanced *Ifnb* promoter activity in response to poly I:C. Fold activation of the *Ifnb* reporter in HEK293T cells transfected with IFN-β luciferase reporter (100 ng) plus MyD88, IPS-1, DHX9, or DHX9-deleted DSRM expression vectors at 20, 100, or 200 ng concentrations. The Renilla-luciferase reporter gene (2 ng) was transfected simultaneously for the internal control. At 30 h posttransfection, cells were without stimulation or stimulated for 6 h with 2.5 μg/ml poly I:C delivered by Lipofectamine 2000 and then harvested.
D2SC mDCs induced by poly I:C involves IRF3 and NF-κB activation. Scrambled shRNA (control), DHX9- and IPS-1–knockdown D2SC cells were stimulated with poly I:C, total cell extracts were prepared and analyzed by SDS-PAGE, followed by Western blotting (Fig. 6). In scrambled shRNA D2SC cells, the phosphorylation of p65 and IRF3 was detected at 60–120 min after poly I:C stimulation. In contrast, the phosphorylation of p65 and IRF3 was barely detectable in DHX9- or IPS-1–knockdown D2SC cells at these time points. These data suggest that DHX9 and IPS-1 are required for activating the NF-κB and IFN-response signal transduction pathways upon poly I:C stimulation.

Discussion
Over the past decade, many molecular sensors for viral or self-nucleic acids that trigger type 1 IFN or inflammatory cytokine responses have been identified. PKR, 2′,5′-oligoadenylate synthetase, TLR3, DDX3, and the RLH family members RIG-I and MDA5 have been shown to recognize poly I:C or dsRNA with different subcellular localizations, lengths or structure (4–9, 23–26). Although MDA5 was found to preferentially sense long form of poly I:C (>1000 bp), RIG-I was found to preferentially sense short form of poly I:C (>300 and <1000 bp) or dsRNA with 5′-triphosphate (4). Interestingly, RIG-I was also shown to sense cytosolic B form DNA (poly dA-dT) through RNA polymerase III (Pol III) to trigger IRF-3–dependent type 1 IFN responses (27, 28). A number of cytosolic DNA sensors, such as Pol III, AIM2, and IFI16, have recently been identified. AIM2 was shown to sense cytosolic DNA by activating inflammasome responses via the adaptor molecule apoptosis-associated-speck-like protein containing a caspase recruitment domain (29–35). More recently, it was reported that IFI16, an AIM2-like molecule, uses STING for sensing cytosolic DNA (36). By direct biochemical approaches using biotin-labeled CpG-DNA or poly I:C, immunoprecipitation assays and mass spectrometry, we recently identified novel nucleic acid sensors within the (DExD/H)-box helicase superfamily in both pDC (15) and mDCs (37). We found that DHX9 and DHX36 were able to sense cytosolic CpG-DNA via MyD88 in pDCs and a DDX1/DDX21/DHX36 complex was able to sense both short and long poly I:C via TRIF in mDCs. Our bioinformatics analysis revealed the presence of at least 60 members of the (DExD/H)-box helicase superfamily, including the RLR subfamily (RIG-I, MDA5, and LGP-2). Our hypothesis is that members of the DExD/H-box helicase superfamily may play a more important role in sensing microbial nucleic acids than previously thought.

It was reported that DHX9 regulates translation by recognizing the posttranscriptional control element of a retrovirus RNA via its ATPase activity (12, 13). Overexpression of DHX9 leads to increasing expression of p65 and TNF-α genes. In addition, DHX9 is a CpG-DNA sensor in pDCs (15). DHX9 has two dsRNA-binding motifs in the N terminus. Those motifs bind the TAR element of HIV-1 to active PKR and regulate HIV-1 gene expression (17). Therefore, it is very interesting to investigate whether DHX9 is able to sense cellular dsRNA or DNA viruses.
In this study, we found that the ability of DHX9 in sensing both poly I:C and RNA virus was mediated by IPS-1 in mDCs. The DSRM domain of DHX9 was required for DHX9 binding to poly I:C, and the HelicC, HA2, and DUF domains of DHX9 and the CARD domain of IPS-1 were required for DHX9:IPS-1 interaction. We have demonstrated that the observed association between DHX9 and IPS-1 in overexpression studies. Our endogenously immunoprecipitation data indicate that the DHX9–IPS-1 complex exists in resting cells and the complex formation does not require stimulation. Additionally, DHX9–IPS-1 complex is independent on RIG-I to sense poly I:C. Previous study suggests that the TRIM25 regulated ubiquitination of RIG-I is essential for its antiviral activity in response to RNA virus infection (38). Furthermore, RIG-I is type I IFN-induced genes in responses to poly I:C (39). DHX9–IPS-1 complex may represent the early sensor of poly I:C or RNA virus to trigger the early IFN production. This early IFN production will up-regulate RIG-I to further amplify the IFN responses to virus infection. Targeting DHX9 with shRNA, and thus inhibiting poly I:C-induced NF-κB and IRF3 activation, resulted in reduced type I IFN and cytokine responses to dsRNA and RNA virus. Collectively, these results suggest that DHX9 is an important RNA sensor that is dependent on IPS-1 to sense pathogenic RNA. Interestingly, previous studies have shown that Flu A virus can activate multiple RNA sensors, including TLR7 and RIG-I (4, 40, 41). We found that DHX9 can sense Flu A infection resulted in reduced type I IFN and cytokine responses to dsRNA and thus inhibiting poly I:C-induced NF-κB activation. A role of RNA helicase A in cis-regulating transcription response element-mediated transcriptional regulation of human immunodeficiency virus type 1 (J. Biol. Chem. 276: 5445–5451).


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