RIG-I-like Receptor Triggering by Dengue Virus Drives Dendritic Cell Immune Activation and TH1 Differentiation

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RIG-I like receptor triggering by dengue virus drives DC immune activation and T helper 1 differentiation

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ABSTRACT

Dengue virus (DENV) causes 400 million infections annually and is one of several viruses that can cause viral hemorrhagic fever. This is characterized by uncontrolled immune activation resulting in high fever and internal bleeding. Although the underlying mechanisms are unknown, massive cytokine secretion is thought to be involved. Dendritic cells (DCs) are the main target cells of DENV and here we investigated the role of DCs in DENV-induced cytokine production and adaptive immune responses. DENV infection induced DC maturation and secretion of IL-1β, IL-6 and TNF. Inhibition of DENV RNA replication abrogated these responses. Notably, silencing of RNA sensors RIG-I or MDA5 abrogated DC maturation as well as cytokine responses by DENV-infected DCs. DC maturation was induced by type I interferon (IFN) responses as inhibition of IFNα/β receptor signaling abrogated DENV induced DC maturation. Moreover, DENV infection of DCs resulted in CCL2, CCL3 and CCL4 expression, which was abrogated after RIG-I and MDA5 silencing. DCs play an essential role in T helper cell differentiation and we show that RIG-I and MDA5 triggering by DENV leads to T helper 1 polarization, characterized by high levels of IFNγ. Notably, cytokines IL-6, TNF and IFNγ and chemokines CCL2, CCL3 and CCL4 have been associated with disease severity, endothelial dysfunction and vasodilation. Therefore, we identified RIG-I and MDA5 as critical players in innate and adaptive immune responses against DENV and targeting these receptors has the potential to decrease hemorrhagic fever in patients.

INTRODUCTION

Dengue virus (DENV) is a blood-borne pathogen that infects almost 400 million people annually (1). Most patients experience mild disease, but dengue fever can progress to life-threatening dengue hemorrhagic fever (DHF) or dengue shock syndrome (DSS), which are characterized by thrombocytopenia and increased vascular permeability resulting in plasma leakage and hemorrhages (2). With no specific antivirals available treatment relies on fluid replacement and the use of analgesics. There is therefore an urgent need to elucidate the factors that are involved in DSS and DHF to improve treatment.

The underlying cause of DHF and DSS remains unclear, but it is believed that massive cytokine secretion is one of the major factors contributing to the pathogenesis of DENV (3). High serum levels of IFNγ, IL-1β, IL-6, TNF, CCL2, CCL4, CCL5, CXCL8 and CXCL10 have been detected in patients with DHF compared to dengue fever (4). In particular IL-6, IL-8 and TNF are associated with increased vascular permeability and plasma leakage (4). The cellular source of DENV-induced cytokines and chemokines is unknown, but dendritic cells (DCs) and macrophages are the main target cells of DENV (5–8). Especially DCs are highly susceptible to DENV infection due to the expression of DENV entry receptor DC-SIGN (5, 9, 10).

DENV-infected DCs show increased expression of maturation markers and produce cytokines IL-6, IL-10 and TNF, chemokines CCL2, CXCL8, CXCL10 and type I interferons (IFN) α and β (11, 12). DCs express numerous pattern recognition receptors (PRRs) that recognize conserved pathogen-associated molecular patterns to induce DC maturation and specific T helper cell (T_H) polarization. Several PRRs have been implicated in DENV sensing including Toll-like receptors (TLRs), RIG-I-like receptors (RLRs) and C-type lectin receptors (CLRs) (9, 13–16). TLRs and RLRs have in common that activation leads both to cytokine responses

via NFκB as well as induction of type I IFNs via IRFs. However, it is unknown which sensor in DCs is responsible for DC maturation and cytokine production.

Here we have identified that DENV RNA replication is essential for DENV-induced DC maturation and cytokine responses. Notably, the intracellular RLRs RIG-I and MDA5 sense DENV RNA replication products and induce innate signaling via adapter protein MAVS to upregulate CD86 expression via type I IFN. DENV induced IL-1β, IL-6 and TNF secretion by DCs and induction of CCL2, CCL3 and CCL4 and RIG-I and MDA5 were critical for these responses. RLR-activation by DENV was also essential for the induction of adaptive immune responses as inhibiting RLR signaling in DCs abolished T_H1 responses. These findings have the potential to enable novel therapeutic strategies that interfere with RIG-I and MDA5 activation to limit DENV pathogenesis.

METHODS

DC stimulation and RNA interference

Peripheral blood monocytes were isolated from buffy coats of healthy donors (Sanquin) by Lymphoprep (Axis-Shield) gradient followed by Percoll (Amersham Biosciences) gradient steps. Monocytes were differentiated into immature DCs in the presence of 500 U/ml IL-4 and 800 U/ml GM-SCF (both Invitrogen) for 6-7 days in RPMI supplemented with 10% fetal calf serum, 10 U/ml penicillin, 10 mg/ml streptomycin (all Invitrogen) and 2 mM L-glutamine (Lonza). DC purity was determined by flow cytometry based on DC-SIGN, CD1c, CD11c and HLA-DR expression and the absence CD14, CD19, CD3 and CD56 respectively and was routinely more than 95% pure. This study was done in accordance with the ethical guidelines of the Academic Medical Center.

DCs were stimulated with 10 ng/ml LPS (Sigma) or 2 µg/ml poly(I:C)Lyovec (Invivogen), which is poly(I:C) complexed with liposomes to transfect poly(I:C) into the cytoplasm and stimulate RIG-I and MDA5. DENV replication inhibitor SDM25N (10µM, Tocris Bioscience), IFNα/β capture protein B18R (eBioscience), N-acetyl-L-cysteine (1-3mM, Sigma) or Catalase (100-500U/ml, Sigma) were added simultaneous with DENV to DCs.

DCs were transfected with 500 nM short interfering RNAs (siRNAs) using the Neon® Transfection System (ThermoFisher) according to the manufacturer's instructions. In brief, DCs were washed with PBS, resuspended in Buffer R (ThermoFisher) and divided over different siRNAs. DCs were transfected with a single pulse of 1500V for 20 ms, mixed with complete RMPI and incubated for 48h before stimulation. SMARTpool siRNA used were MAVS (M-024237-02), RIG-I (M-012511-01), MDA5 (M-013041-00), and non-targeting siRNA (D-001206-13) as control (all Dharmacon). Silencing was confirmed by real-time PCR, flow cytometry and immunoblotting (Fig. S3). Antibodies used for flow cytometry were anti-RIG-I (1:50, 3743, Cell Signaling), anti-MDA5 (1:50, 5321, Cell Signaling) and anti-

MAVS (1:50, 3993, Cell Signaling) in combination with PE-conjugated anti-rabbit (1:200; 711-116-152, Jackson ImmunoResearch) and cells were analyzed on a Canto II (BD Biosciences). Antibodies used for Western Blot were similar as for flow cytometry but in a different dilution (all 1:1000) except for anti-β-actin (1:500, sc-81178, Santa Cruz).

T cell differentiation

Naive $CD4^+$ T cells were isolated from buffy coats of healthy blood donors (Sanquin) with human $CD4^+$ T-cell isolation kit II (Miltenyi) by negative selection and subsequent depletion of $CD45RO^+$ memory T cells using phycoerythrin (PE)-conjugated anti-CD45RO (80μg/ml; R0843; Dako) and anti-PE beads (Miltenyi). This study was approved by the Medical Ethics Review Committee of the AMC.

DCs were silenced for indicated proteins and stimulated for 48h as indicated. DCs were combined with allogeneic naïve $CD4^+$ T cells (5,000 DCs/20,000 T cells) in the presence of 10 pg/ml *Staphylococcus aureus* enterotoxin B (Sigma). SDM25N (1 μM, Tocris Bioscience) was added to cocultures of SDM25N-treated DCs to maintain inhibition of DENV replication. After 5 days, cells were further cultured in the presence of 10 U/ml IL-2 (Chiron). Resting T cells were restimulated with 100 ng/ml PMA and 1 μg/ml ionomycin (both Sigma) for 6h. For flow cytometry analysis of restimulated T cells, cells were fixed in 4% *para*-formaldehyde for 20 min, followed by permeabilization in 0.1% saponin in PBS for 10 min. Cells were stained with APC-conjugated anti-IL-4 and FITC-conjugated anti-IFNγ (both BD Bioscience) before cells were analyzed on a FACS Canto II (BD Biosciences).

Virus production and infection

80% confluent C6/36 cells were infected with DENV-2/16681 at an MOI of 0.01 in HMEM medium supplemented with 2% fetal calf serum, 10 U/ml penicillin, 10 mg/ml streptomycin

(all Invitrogen), 2 mM L-glutamine and 100 µM non-essential amino acids (both Lonza). After 5-7 days, supernatant was harvested and cleared from cellular debris by centrifugation and subsequent filtration using a 0.2 µM filter. Supernatant was aliquoted, snap-frozen in liquid nitrogen and stored at -80 °C. Viral titers were determined as described previously (17). Supernatant of uninfected C6/36 cultured for 5-7 days was used for mock-treatment of DCs.

DCs were infected with DENV at an MOI of 1 unless stated otherwise. Infection was determined after 36-48h by measuring the percentage of $NS3^+$ cells by flow cytometry. Cells were fixed in 4% *para*-formaldehyde for 15 min followed by permeabilization in PBS supplemented with 0.1% saponin for 10 min. Cells were stained with anti-NS3 (1:800, SAB2700181, Sigma) followed by PE-conjugated anti-rabbit (1:200; 711-116-152, Jackson ImmunoResearch), AlexaFluor 488-conjugated anti-rabbit (1:400, A-21206, ThermoFisher) or AlexaFluor 647-conjugated anti-rabbit (1:400, A-21245, ThermoFisher) in combination with FITC-conjugated anti-HLA-A2 (1:25, 551285, BD Pharmingen), PE/Cy7-conjugated anti-HLA-DR (1:200, 560651, BD Pharmingen), PE-conjugated anti-CD80 (1:25, 557227, BD Pharmingen), APC-conjugated anti-CD83 (1:25, 551073, BD Pharmingen), FITC-conjugated anti-CD86 (1:25, 555657, BD Pharmingen), or PE-conjugated anti-CD86 (1:50, 555658, BD Pharmingen). Cells were analyzed on a FACS Canto II (BD Biosciences).

ELISA

Cell culture supernatant were harvested 24 or 48 hours after stimulation and IL-1β, IL-6, IL-12p70 and TNF (eBioscience) were measured by ELISA according to the manufacturer's instructions

Real-time quantitative PCR

mRNA was isolated using mRNA capture kit (Roche) and cDNA was synthesized with reverse transcriptase kit (Promega). PCR amplification was performed in the presence of SYBR Green in an ABI 7500 Fast PCR detection system (Applied Biosystems). Specific primers were designed using Primer Express 2.0 (Applied Biosystems; Table S1). Expression of target genes was normalized to GAPDH ($N_t=2^{Ct\ (GAPDH)\ -\ Ct\ (target)}$) and set at 1 in DENV-infected DCs for each donor within one experiment.

Statistical Analysis

Statistical analyses were performed using the Student's *t*-test for paired observations. Statistical significance was set at $P<0.05$.

RESULTS

DENV induces DC maturation and cytokine responses

To investigate DC maturation and cytokine induction by DENV, we infected DCs with a multiplicity of infection (MOI) of 1 and 3 for 24 and 48 hour and measured expression of DENV non-structural (NS) protein 3 (Fig. S1). NS viral proteins are only produced during replication and therefore indicate active viral infection (18). NS3 protein was detected 24 hour post infection (h.p.i) and DC infection increased over time for MOI 1, whereas MOI 3 reached maximal infection at 24 h.p.i. (Fig. 1A). A plateau of infection has been shown before in monocyte-derived DCs and $CD1c^+$ DCs from human skin (19, 20). Next we investigated DC maturation upon infection by measuring the expression of DC maturation markers CD80, CD83 and CD86 as well as expression of MHC class I (HLA-A2) and class II (HLA-DR). Interestingly, DC maturation was observed at 48 h.p.i but not at 24 h.p.i. even at MOI 3 despite active viral replication at this time point (Fig. 1B). DENV has been shown to induce apoptosis and interfere with DC activation and maturation (11, 21, 22). Therefore we analyzed the expression of DC maturation markers in infected ($DENV^+$) and bystander ($DENV^-$) DCs. Notably, maturation of the $DENV^-$ population is increased compared to the $DENV^+$ population, indicating that DENV limits DC maturation (Fig. 1C).

We next investigated the induction of different cytokines involved in fever (IL-1β, IL-6), T_H skewing (IL-1b, IL-6, IL-12p70) or activation of endothelial cells (IL-6, TNF). Similar to DC maturation, DENV infection induced IL-1β, IL-6 and TNF at 48 but not 24 h.p.i. (Fig. 1D). DENV infection did not induce IL-12p70 while DCs were capable to produce IL-12p70 in response to LPS (Fig. 1D). IL-12p70 is a heterodimeric protein consisting of subunits p40 and p35. Therefore we measured the induction of both subunits at mRNA level as well as mRNA expression of IL-1β, IL-6 and TNF 32 h.p.i. when transcription is maximal as determined by time course experiments (data not shown). Remarkable, DENV infection of DCs induced

mRNA expression of IL-1β, IL-6, IL-12p35 and TNF but not IL-12p40 (Fig. 1E). These results show that DENV induces DC maturation and cytokine responses 48 h.p.i. and that virus concentration affects the strength but not the time of the activation. These data suggest that sensing of DENV requires productive viral replication in DCs.

DENV RNA replication is essential for DC activation

DENV viral particles consist of different proteins and contain single stranded RNA that can activate TLR7 (15). During DENV RNA replication double-stranded RNA intermediates are formed that can be sensed by RLRs and TLR3 (16, 23). To investigate whether DENV replication is required for DC maturation and cytokine responses we used the DENV replication inhibitor SDM25N, which efficiently inhibits DENV RNA replication as well as infection (24). We have focused on CD86 expression for investigating the role of DENV replication as this maturation marker was highly induced by DENV. DENV-induced upregulation of CD86 was completely abrogated when DENV replication was inhibited by SDM25N (Fig. 2A). Also DENV-induced expression of IL-1β, IL-6, IL-12p35 and TNF was dependent on DENV RNA replication (Fig. 2B,C). Reactive oxygen species (ROS) have been implicated in DENV-induced maturation (21). However, treatment of DCs with ROS inhibitors N-acetyl-L-cysteine (NAC) and Catalase did not decrease DENV-induced DC maturation (Fig. S2). These data strongly suggest that DENV RNA replication is pivotal for DCs activation. These data also explain the delayed response of DCs to DENV infection as DENV RNA intermediates are formed during replication and are not present in viral particles that initiate infection.

DENV-induced cytokine responses depend on RIG-I and MDA5

DENV replication products are formed in the cytosol and can be sensed by RLRs RIG-I and MDA5 (13, 16, 23). We have recently shown that type I IFN responses induced by RLRs to DENV induce IL-27, which is crucial for T follicular helper cell polarization (Sprokholt *et al.*, submitted). To investigate if RIG-I and MDA5 are also involved in IL-1β, IL-6 and TNF responses, we used RNA interference to silence RIG-I and MDA5 to investigate if these RNA sensors are responsible for DENV-induced DC activation. We have confirmed effective silencing on mRNA as well as protein level. Furthermore, we have confirmed the specificity of the silencing using different RLR and TLR ligands and silencing did not affect cell viability (Fig. S3). Notably, silencing RIG-I and MDA5 alone or together abrogated IL-1β, IL-6 and TNF expression upon DENV infection (Fig. 3A,B). RIG-I and MDA5 triggering by DENV also resulted in IL-12p35 expression (Fig. 3A). RLRs signal via adapter molecule MAVS, which forms a scaffolding platform from which IRF and NFκB activation is orchestrated (25). Similar to RIG-I and MDA5, silencing MAVS abrogated DENV-induced cytokine expression (Fig. 3C,D). These data strongly suggest that RIG-I and MDA5 are critical sensors of DENV RNA replication and drive cytokine responses of DCs in response to DENV via adapter protein MAVS.

RIG-I and MDA5 activation leads to chemokine responses

In addition to elevated levels of cytokines that are associated with disease severity, several chemokines can impact vascular integrity, including CCL2, CCL3, CCL4 and CXCL8 (4, 26). Therefore we investigated whether DENV-infected DCs produced these chemokines in a RLR-dependent manner. DENV infection induced the expression of CCL2, CCL3 and CCL4, which was abrogated after silencing of either MAVS or both RIG-I and MDA5 (Fig. 4A,B). CXCL8 expression was not induced (data not shown). These data indicate the DENV-induced chemokine responses by DCs critically depend on RLR-MAVS activation.

225

226 **RLR-dependent type I IFN responses induce DC maturation**

227 DC maturation is essential for inducing adaptive immune responses by providing co-
228 stimulatory signals to T cells that strengthen T cell receptor signaling (27). CD86 expression
229 can be induced by NFκB as well as IRFs, which are both induced by RLRs (28, 29). We
230 therefore investigated whether DENV-induced DC maturation is dependent on RIG-I and
231 MDA5, and compared the level of CD86 expression induced by DENV with LPS. DENV
232 induced CD86 comparable to LPS, and silencing MAVS or both RIG-I and MDA5 strongly
233 decreased DENV-induced DC maturation (Fig. 5A), indicating that RLR activation is
234 essential for DC-T cell interaction during DENV infection. We next investigated how RLRs
235 drive DC maturation in response to infection. As type I IFNs are known to induce DC
236 maturation (30), we used recombinant IFNα/βR protein (B18R) to neutralize IFN-α/β secreted
237 by DCs and thereby prevent the induction of IFN-stimulated genes (ISGs) by IFNα/βR
238 signaling. Numerous ISGs have antiviral properties that limit viral replication and interfering
239 with IFNα/βR activation greatly enhanced DENV-infection of DCs (Fig. 5B). B18R
240 effectively inhibited the induction of ISGs MxA, IRF7, ISG15, OAS1 and ADAR1 by DENV
241 (Fig. 5C). Remarkably, inhibiting IFNα/βR signaling abrogated DC-maturation in response to
242 DENV despite enhanced infection (Fig. 5D). These data indicate that RLR activation by
243 DENV drives DC maturation and that this is mediated via type I IFN responses.

244

245 **DENV-induced T_H skewing requires RIG-I and MDA5 activation**

246 IFNγ is associated with severe disease in dengue patients. T_H1 cells produce high levels of
247 IFNγ and therefore we investigated DENV-induced T_H cell polarization. First, we evaluated
248 our assay by coculturing LPS-stimulated DCs, either alone or in combination with IFNγ or
249 prostaglandin E2 (PGE2), with naïve T_H cells for 10-12 days before measuring the percentage

of IFNγ or IL-4-positive cells, indicative for T_H1 or T_H2 cells, respectively (Fig. S4). LPS-stimulated DCs induced mixed skewing of T_H1 and T_H2 cells, whereas LPS+IFNγ-stimulated DCs induced more T_H1 polarization and less T_H2 differentiation compared to LPS (Fig. 6A,B). In contrast, coculturing naïve T_H cells with LPS+PGE2-stimulated DCs resulted in a greater percentage of T_H2 cells and fewer T_H1 cells compared to LPS (Fig. 6A,B). Next, we examined T_H polarization induced by DENV-infected DCs and the involvement of RIG-I and MDA5. DENV-infected DCs resulted in more T_H1 cells and less T_H2 cells compared to mock-treated DCs and this effect was dependent on viral replication as SDM25N decreased T_H1 polarization (Fig. 6C). Strikingly, silencing MAVS abrogated T_H1 skewing by DENV-infected DCs (Fig. 6D). Moreover, direct stimulation of RIG-I and MDA5 using the chemical RLR ligand poly (I:C)Lyovec also resulted in strong T_H1 polarization (Fig. 6E). These data indicate that DENV infection of DCs results in T_H1 skewing and that this effect is mediated by RIG-I and MDA5.

DISCUSSION

RNA viruses can induce viral hemorrhagic fever and this effect is thought to be mediated by immune activation, but little is known about the underlying mechanisms. Here we show that DENV infection of DCs results in DC maturation, high levels of cytokine secretion, chemokine expression and T_H1 polarization and that these responses depend on viral RNA replication. Notably, we identified RIG-I and MDA5 as key sensors that mediate immune activation of DCs by DENV. Inhibiting RLR activation or signaling abrogated DC maturation, cytokine secretion and chemokine expression in response to DENV. Therefore, we have identified RIG-I and MDA5 as key players in DENV-induced immune activation in DCs.

DENV viral particles contain multiple components that can directly activate several receptors. The viral envelope proteins can interact with CLR while the viral genome can activate endosomal TLRs that recognize single-stranded RNA (5, 10, 31). During viral replication double-stranded RNA intermediates are formed that can trigger cytoplasmic RNA sensors (16, 23). We did not observe DC maturation nor cytokine secretion during the first 24h of infection, indicating that DENV viral particles do not directly activate DCs via cell surface receptors or endosomal TLRs. Instead, DENV RNA replication was essential for DC immune activation and these responses were mediated by cytoplasmic RLRs RIG-I and MDA5.

RIG-I and MDA5 activation leads to signaling cascades that involve adapter molecule MAVS. RIG-I and MDA5 association to MAVS leads to the recruitment of IkB kinase (IKK)-related kinase IKKε and Tank-binding protein 1 (TBK1), which activate IRF3 for nuclear translocation and IFN-β transcription (32). Our data indicate that RLR-induced IFN-β limits DENV infection in DCs and is crucial for DC maturation by inducing IFNα/βR signaling. We have previously shown that DENV infection of DCs leads to type I IFN responses that are initiated by IRF3 and followed by IRF7, which is induced by IFNα/βR signaling.

Interestingly, IRF7 is a transcription factor of CD86 (33), indicating that RLRs might induce DC maturation via IFNα/βR-depended IRF7 expression during DENV infection. As DC maturation is crucial for inducing adaptive immune responses, type I IFN is not only involved in innate suppression of DENV replication but could also play a role in adaptive T cell responses.

The importance of IFNα/βR in DC maturation can explain the difference in maturation between infected and bystander DCs (11, 22). DENV is known to inhibit IFNα/βR signaling by targeting STAT2 for degradation by DENV protein NS5 (34, 35). As NS5 is only expressed in infected cells and not bystander cells, this could explain why bystander cells show increased maturation compared to infected cells.

Oxidative stress is another factor important for DCs maturation by DENV. DENV infection of DCs induces oxidative stress resulting in ROS and enhanced DC maturation (21). Interestingly, ROS is known to enhance RIG-I and MDA5 activation (36). The production of ROS by infected DCs might therefore be an antiviral strategy to limit viral replication via enhanced RIG-I and MDA5 activation, although our results did not show that neutralizing ROS affects DC maturation.

In parallel to IRF3-mediated responses, RLR activation leads to NFκB activation via CARD9 and Bcl-10 (37). NFκB activation is essential for the expression of most cytokines including IL-1β, which potently induces fever (38, 39). Persistent high fever is one of the clinical symptoms of DENV, but the underlying mechanisms that lead to IL-1β secretion are unknown. IL-1β expression is tightly regulated and requires a multi-stage process to be functionally active. Priming signals induce pro-IL-1β transcription and translation, whereas secondary signals induce inflammasome activation to process pro-IL-1β into mature IL-1β by caspases. Normally, pro-IL-1β synthesis and inflammasome activation are induced by separate signals and only few receptors have the capacity to provide both signals (40). In

313 human macrophages, CLR CLEC5A is required for NLPR3 expression and subsequent
314 processing of pro-IL-1β by NLPR3-inflammasomes in response to DENV, but requires
315 priming by LPS for efficient IL-1β secretion (41). In contrast, RIG-I can induce pro-IL-1β
316 expression and associate with inflammasome adapter molecule ASC to induce pro-IL-1β
317 processing by caspase 1 (37). We observed that DENV infection of DCs resulted in pro-IL-1β
318 expression and that DCs did not require priming signals for efficient IL-1β secretion. Notably,
319 both the induction of pro-IL-1β as well as the secretion of mature IL-1β depended on RIG-I
320 and MDA5. As IL-1β is also a potent inducer of vasodilation (42), inhibiting RLR-dependent
321 inflammasome activation has the potential to lower DHF.
322 In addition to cytokines, DENV infection of DCs resulted in robust chemokine expression of
323 CCL2, CCL3, CCL4 and CCL5. CCL2 and CCL4 are strong chemo-attractants for
324 monocytes, while CCL3 potently attracts neutrophils to sites of inflammation. Early during
325 DENV infection, monocytes are recruited to the skin were they differentiate into DCs and
326 thereby increase the pool of susceptible cells for DENV (43). This could lead to a self-
327 sustained loop were DENV-infected DCs attract monocytes that differentiate into DCs,
328 become infected and recruit more DCs. We show that DENV-induced CCL2 and CCL4
329 expression in DCs is under the control of RIG-I and MDA5. Interfering with RIG-I and
330 MDA5 function could therefore prevent the recruitment of susceptible cells to the site of
331 infection.
332 Notably, our data strongly suggest that RIG-I and MDA-5 have non-redundant roles as
333 silencing of either RIG-I or MDA-5 alone already decreased cytokine responses, DC
334 maturation and chemokine production.
335 Studies investigating RLR function in adaptive immune responses are scarce, but RLR
336 activation can inhibit T_H1 polarization in responses to TLR ligands in mice (44). IL-12p70
337 plays a vital role in T_H1 responses and RLR-dependent IRF3 activation blocks IL-12p40

mRNA expression and thereby prevents IL-12p70 formation (44). We also did not observe IL-12p40 induction by DENV in contrast to IL-12p35, which resulted in undetectable levels of mature IL-12p70. However, our data show that DENV infection of DCs did result in T_H1 formation and this was dependent on RLR-adapted protein MAVS. Also direct stimulation of RIG-I and MDA5 with chemical ligands resulted in T_H1 polarization. Other factors besides IL-12p70 have the potential to induce T_H1 polarization, including IFN-β (45). Our data show that DENV induces functional type I IFN responses in DCs and RLR-dependent IFN-β could be driving T_H1 differentiation by DENV-infected DCs. Interestingly, Chase *et al.* (2011) suggest that infected DCs are less efficient in inducing T_H1 responses (46). However, our data are supported by upregulation of co-stimulatory molecules and IFN responses that promote T_H differentiation. Moreover, Chase *et al.* (2011) observed T cell polarization, although to a lesser extent. Possible differences in assays might explain the apparent disparity between our studies.

Our data show that RLR activation by DENV leads to strong cytokine and chemokine responses that are associated with disease severity (4). We have recently shown that type I IFN responses induced by RLRs is crucial for T follicular helper cell polarization and antibody production (Sprokholt *et al.*, submitted). This indicates that RLRs induce a dual response against DENV: (i) an inflammatory response that is based on NFκB and involves cytokines and chemokines and (ii) a protective response that depends on IRF3 and involves type I IFN and antibody production.

Interestingly, only RNA viruses can induce viral hemorrhagic fever which could indicate that RNA sensors are key players in viral hemorrhagic fever. TNF directly activates endothelial cells and is critical for DENV induced vascular leakage in animal models, probably in concert with other factors (47, 48). We show that DENV potently stimulates TNF production by DCs several magnitudes larger than LPS stimulation. Notably, DENV-induced TNF responses

depended critically on RIG-I and MDA5. Numerous other RNA viruses that cause viral hemorrhagic fever activate RIG-I and/or MDA5 and RLR triggering could be a general mechanism underlying viral hemorrhagic fever (49, 50).

In conclusion, we have identified RIG-I and MDA5 as key sensors of DENV infection in DCs and as critical players in DC-depended innate and adaptive immune responses against DENV. Many of the cytokines and chemokines produced by DCs in response to DENV are associated with disease severity and endothelial dysfunction. Hence, molecules targeting RIG-I and MDA5 signaling components have the potential to be used as therapeutic agents for DHF, DSS and possibly other viruses.

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AUTHORS CONTRIBUTION

J.K.S. designed, performed and interpreted most experiments and prepared the manuscript. T.M.K. performed DENV infections, T cell differentiation, FACS analyses and cDNA synthesis. J.L.H. performed DENV infections, T cell assays and cDNA synthesis. R.J.O.

performed DENV infections and cDNA synthesis. S.I.G. helped prepare the manuscript. T.B.H.G. supervised all aspects of this study and helped prepare the manuscript.

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544 12.
545

FIGURE LEGENDS

Figure 1. DENV induces DC maturation and cytokine responses

(A-C) DCs were mock-treated or infected with an MOI of 1 or 3 and the percentage of $NS3^+$ cells (A) or the expression of CD80, CD83, CD86, HLA-A2 and HLA-DR (B,C) was measured 24 or 48 h.p.i. by flow cytometry. The $DENV^+$ and $DENV^-$ population in (C) was gated as shown in Fig. S1. (D) DCs were similarly infected as in (A) and the production of IL-1β, IL-6, IL-12p70 and TNF was measured in the cell culture supernatant by ELISA. (E). DCs were infected with an MOI of 1 and mRNA expression of indicated genes was measured 32 h.p.i. by real-time PCR, normalized to GAPDH and set as 1 in DENV infected samples. **P<0.01 (student's t-test). Data represent three (B,C) or four (A,D) donors of different experiments or are collated data (mean ± s.d.) of at least four donors (E) from different experiments (mean ± s.d. of duplicates in A,D).

Figure 2. DENV RNA replication is essential for DC maturation and cytokines responses

(A) DCs were infected with DENV at an MOI of 1 in the presence or absence of 10 μM SDM25N and the expression of CD86 was measured 48 h.p.i. by flow cytometry. (B-C) DC were infected similarly as in (A) and the mRNA expression of indicated genes was measured 32 h.p.i. by real-time PCR, normalized to GAPDH and set as 1 in DENV infected samples without SDM25N (B) or the secretion of indicated proteins in the cell culture supernatant was measured by ELISA (C). **P<0.01 (student's t-test). Data are representative of at least four (C) or two (A) donors of different experiments or are collated data (mean ± s.d) of four (B) donors from different experiments (mean ± s.d. of duplicates in C).

Figure 3. DENV induces DC immune activation via RIG-I and MDA5

(A,C) DCs were mock-treated or infected with DENV at an MOI of 1 after RIG-I, MDA5 or MAVS silencing and the mRNA expression of indicated genes was measured 32 h.p.i. by real-time PCR, normalized to GAPDH and set as 1 in DENV infected samples treated with control siRNA. (B,D) DCs were treated similarly as in (A) and the production of indicated proteins in the cell culture supernatant was measured by ELISA. **P<0.01 (student's t-test). Data are representative of at least two donors (B,D) of different experiments or collated data (mean ± s.d.) of four (C) or three (A) donors of different experiments (mean ± s.d. of duplicates in B,D).

Figure 4. RIG-I and MDA5 activation by DENV induces chemokine expression

(A,B) DCs were infected with DENV after RIG-I, MDA5 or MAVS silencing and the mRNA expression of indicated genes was measured 32 h.p.i. by real-time PCR, normalized to GAPDH and set as 1 in DENV infected samples treated with control siRNA. *P<0.05, **P<0.01 (student's t-test). Data are collated (mean ± s.d.) of at least five donors from different experiments.

Figure 5. RIG-I and MDA5 induce DC maturation via type I IFN

(A,D) DCs were stimulated with LPS (A), mock-treated or infected with DENV at an MOI of 1 after RIG-I, MDA5 or MAVS silencing (A) or in the presence or absence of B18R (D) and the expression of CD86 was measured 48 h.p.i. by flow cytometry. (B,C) Similar as in (D) but MxA, IRF7, ISG15, OAS1 and ADAR1 expression was measured by real-time PCR, normalized to GAPDH and set as 1 in DENV infected samples without B18R (C) or the percentage of $NS3^+$ cells was measured by flow cytometry (B). **P<0.01 (student's t-test). Data are representative of at least four (A,B,D) donors of different experiments or are collated data (mean ± s.d.) of four (C) donors of different experiments (mean ± s.d. of duplicates in B).

Figure 6. RIG-I and MDA5 induce T_H1 polarization

(A-B) Flow cytometry analysis of intracellular IFNγ or IL-4 expression in differentiated T cells after coculture of naïve $CD4^+$ T cells with DCs that were unstimulated (iDCs) or stimulated with LPS alone or in combination with IFNγ or PGE2 (A,B). Numbers in quadrants in (A) indicate percentage of gated cells. (C,D) DCs were mock-treated or infected with DENV at an MOI of 1 in the presence or absence of SDM25N (C) or after MAVS silencing (D) and cocultured with naïve $CD4^+$ T cells similar as in (A) and the percentage of IFNγ or IL-4 positive T cells was measured by flow cytometry. (E) Similar as in (A) but DCs were left untreated or stimulated with poly (I:C)Lyovec before coculture with naïve $CD4^+$ T cells. Data are representative of at least three (A,B,E) or two (C,D) independent experiments (mean ± s.d. of duplicates B-E). iDCs, immature DCs; p (I:C)LV, poly (I:C)Lyovec.

Fig. 1

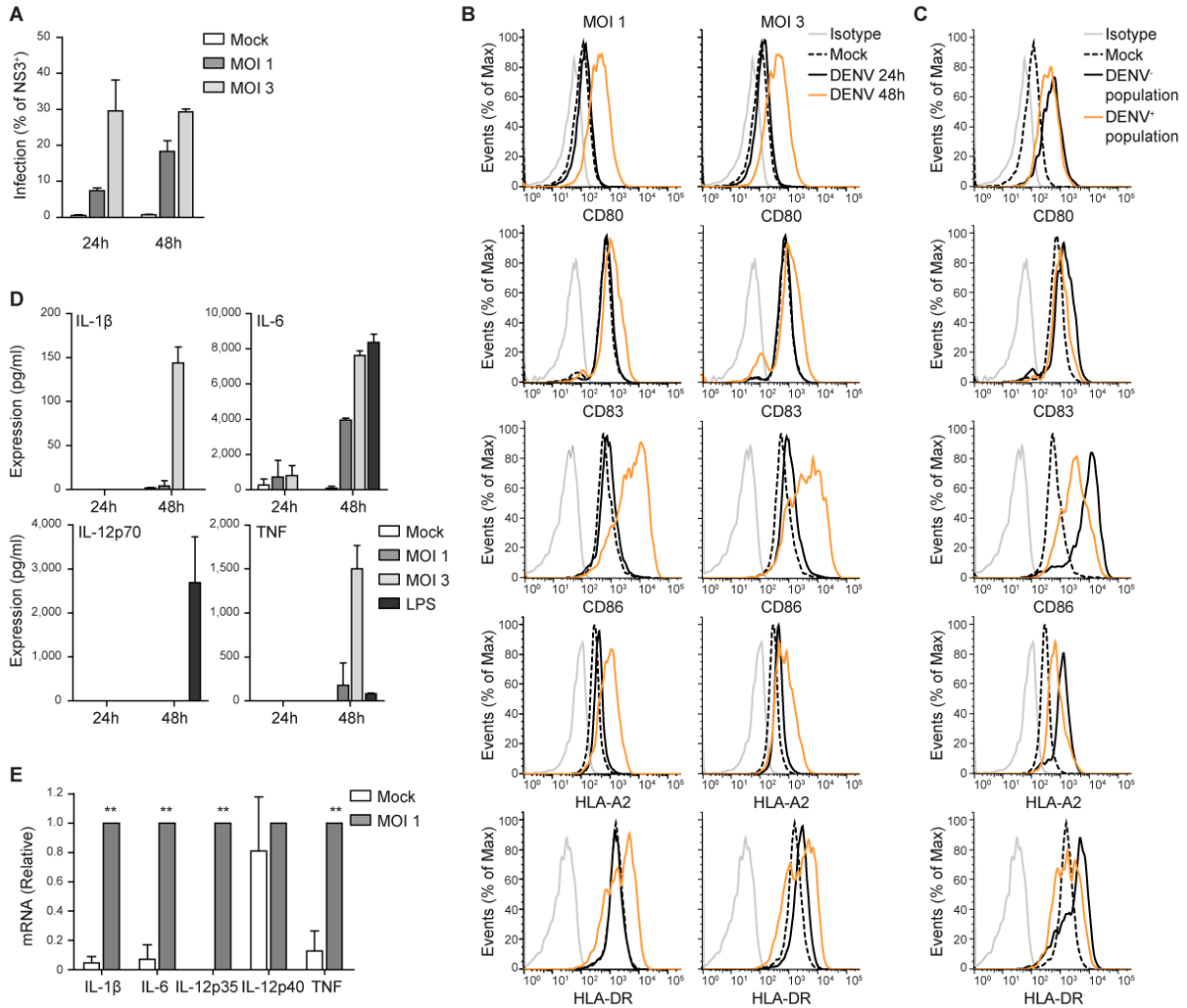

A
Infection (% of NS3+)
Mock
MOI 1
MOI 3
24h
48h
B
MOI 1
MOI 3
Isotype
Mock
DENV 24h
DENV 48h
Events (% of Max)
CD80
CD83
CD86
HLA-A2
HLA-DR
C
Isotype
Mock
DENV- population
DENV+ population
D
Expression (pg/ml)
IL-1β
IL-6
IL-12p70
TNF
Mock
MOI 1
MOI 3
LPS
E
mRNA (Relative)
IL-1β
IL-6
IL-12p35
IL-12p40
TNF

Fig. 2

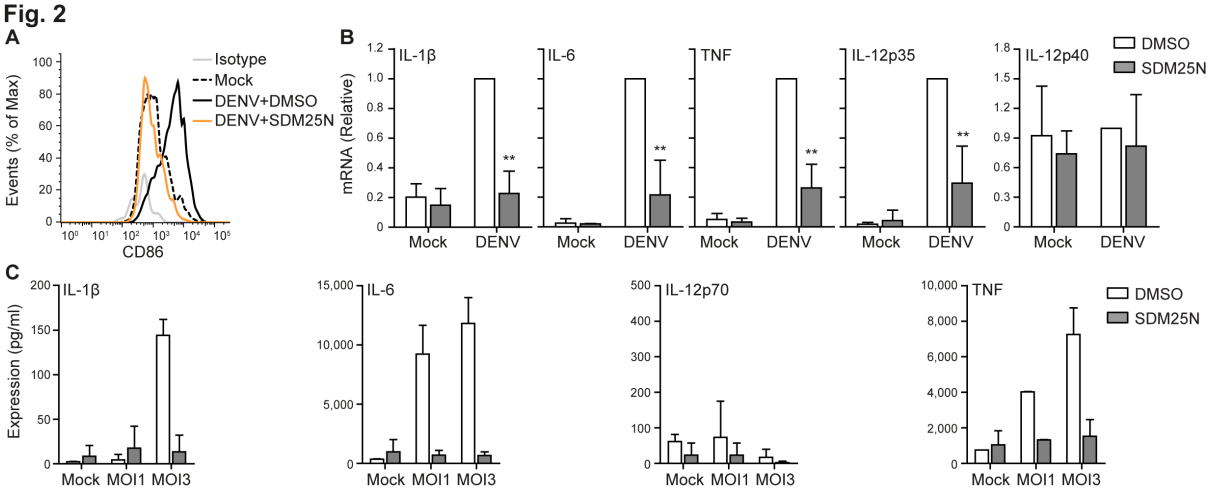

A
Isotype
Mock
DENV+DMSO
DENV+SDM25N
Events (% of Max)
CD86
B
mRNA (Relative)
IL-1β
IL-6
TNF
IL-12p35
IL-12p40
DMSO
SDM25N
Mock
DENV
C
Expression (pg/ml)
IL-1β
IL-6
IL-12p70
TNF
Mock
MOI1
MOI3

Fig. 3

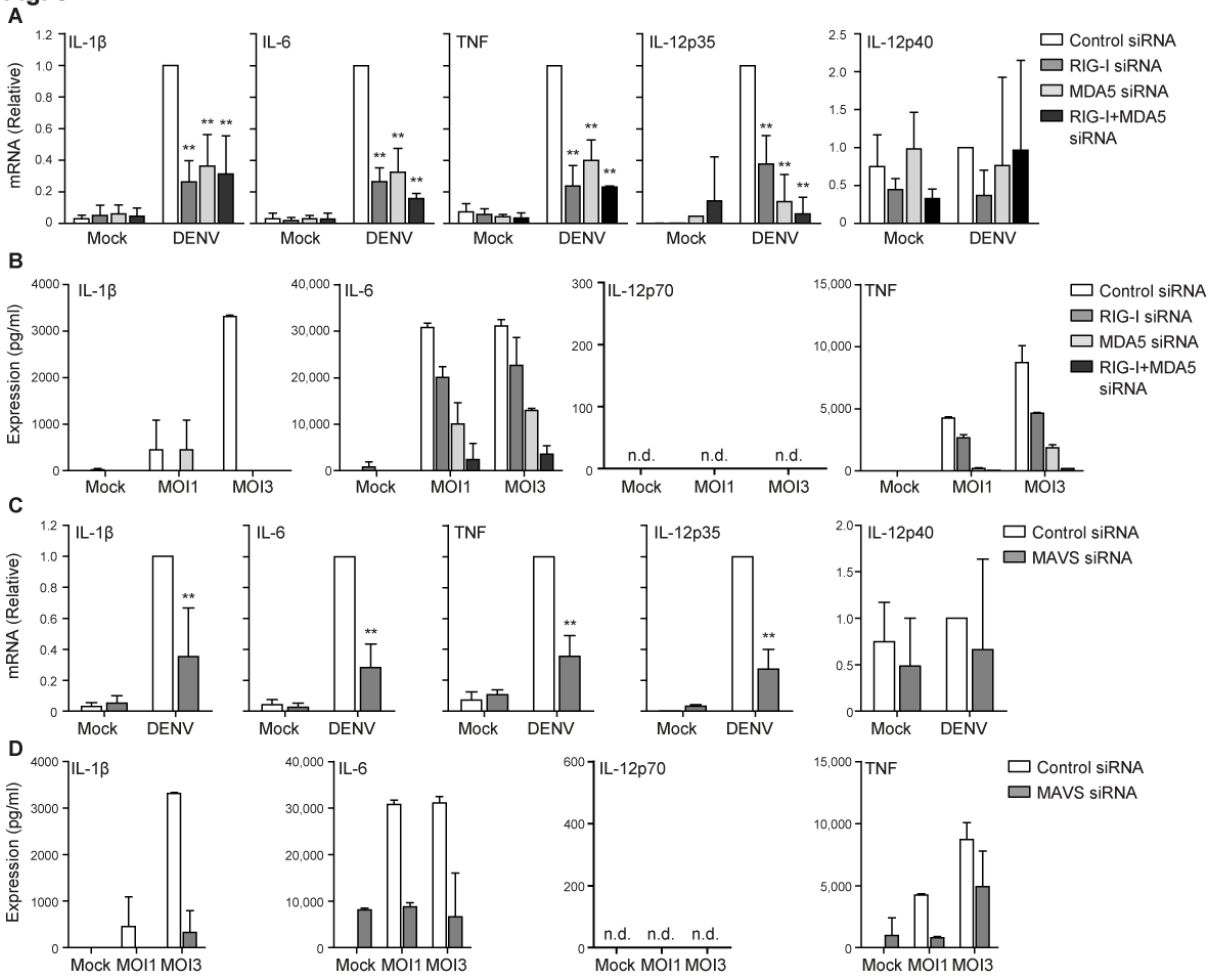

A
IL-1β
IL-6
TNF
IL-12p35
IL-12p40
mRNA (Relative)
Mock
DENV
Control siRNA
RIG-I siRNA
MDA5 siRNA
RIG-I+MDA5 siRNA
B
IL-1β
IL-6
IL-12p70
TNF
Expression (pg/ml)
n.d.
Mock
MOI1
MOI3
C
IL-1β
IL-6
TNF
IL-12p35
IL-12p40
mRNA (Relative)
Control siRNA
MAVS siRNA
D
IL-1β
IL-6
IL-12p70
TNF
Expression (pg/ml)
n.d.
Mock MOI1 MOI3
Control siRNA
MAVS siRNA

Fig. 4

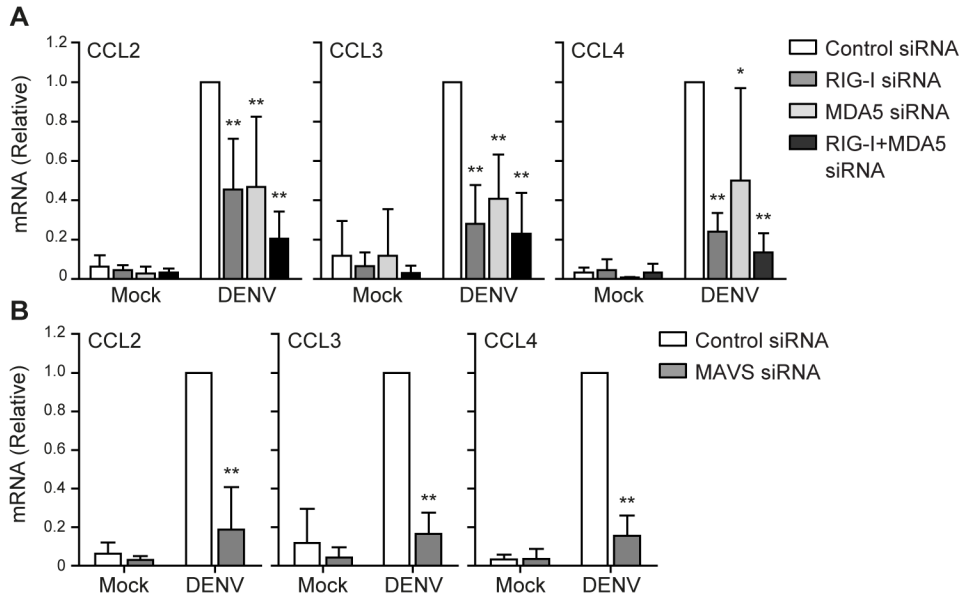

A
CCL2
CCL3
CCL4
mRNA (Relative)
1.2
1.0
0.8
0.6
0.4
0.2
0
Mock
DENV
Control siRNA
RIG-I siRNA
MDA5 siRNA
RIG-I+MDA5 siRNA
B
CCL2
CCL3
CCL4
mRNA (Relative)
Mock
DENV
Control siRNA
MAVS siRNA

Figure 5

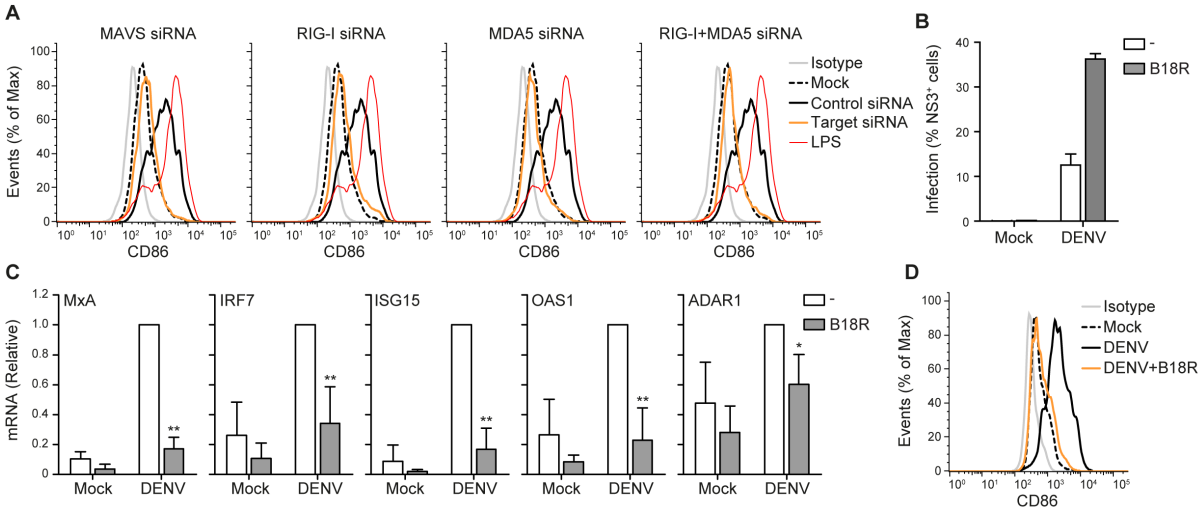
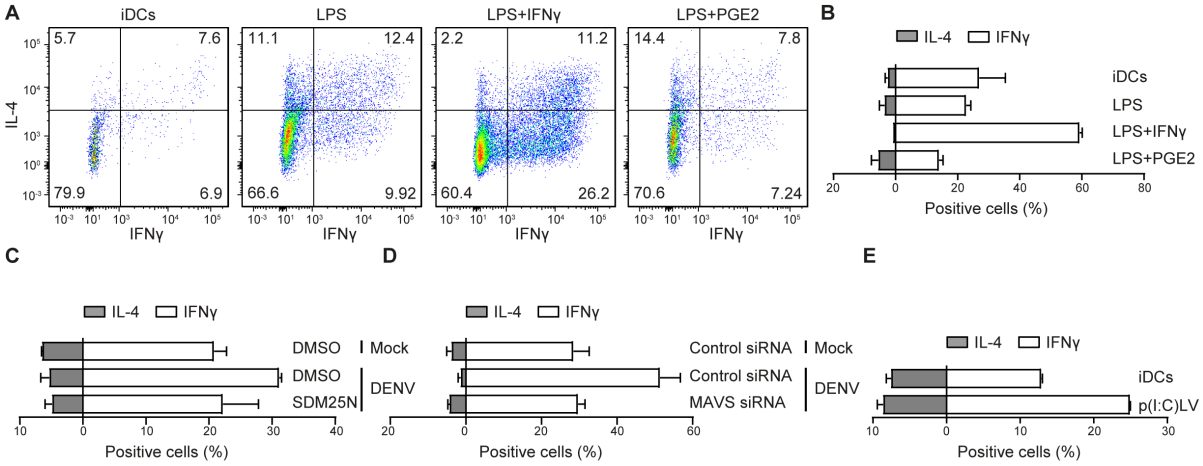

A
MAVS siRNA
RIG-I siRNA
MDA5 siRNA
RIG-I+MDA5 siRNA
Events (% of Max)
CD86
Isotype
Mock
Control siRNA
Target siRNA
LPS
B
Infection (% NS3+ cells)
-
B18R
Mock
DENV
C
mRNA (Relative)
MxA
IRF7
ISG15
OAS1
ADAR1
D
Events (% of Max)
Isotype
Mock
DENV
DENV+B18R
CD86

Figure 6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iDCs
LPS
LPS+IFNγ
LPS+PGE2
5.7
7.6
79.9
6.9
11.1
12.4
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9.92
2.2
11.2
60.4
26.2
14.4
7.8
70.6
7.24
IL-4
IFNγ
B
IL-4
IFNγ
iDCs
LPS
LPS+IFNγ
LPS+PGE2
Positive cells (%)

C
IL-4
IFNγ
DMSO
DMSO
SDM25N
Mock
DENV
Positive cells (%)
D
IL-4
IFNγ
Control siRNA
Control siRNA
MAVS siRNA
Mock
DENV
Positive cells (%)
E
IL-4
IFNγ
iDCs
p(I:C)LV
Positive cells (%)