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

Running title: RIG-I like receptors drive dengue virus immune activation

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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.

31 **INTRODUCTION**

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

39 The underlying cause of DHF and DSS remains unclear, but it is believed that massive
40 cytokine secretion is one of the major factors contributing to the pathogenesis of DENV (3).
41 High serum levels of IFNγ, IL-1β, IL-6, TNF, CCL2, CCL4, CCL5, CXCL8 and CXCL10
42 have been detected in patients with DHF compared to dengue fever (4). In particular IL-6, IL-
43 8 and TNF are associated with increased vascular permeability and plasma leakage (4). The
44 cellular source of DENV-induced cytokines and chemokines is unknown, but dendritic cells
45 (DCs) and macrophages are the main target cells of DENV (5–8). Especially DCs are highly
46 susceptible to DENV infection due to the expression of DENV entry receptor DC-SIGN (5, 9,
47 10).

48 DENV-infected DCs show increased expression of maturation markers and produce cytokines
49 IL-6, IL-10 and TNF, chemokines CCL2, CXCL8, CXCL10 and type I interferons (IFN) α
50 and β (11, 12). DCs express numerous pattern recognition receptors (PRRs) that recognize
51 conserved pathogen-associated molecular patterns to induce DC maturation and specific T
52 helper cell (T_H) polarization. Several PRRs have been implicated in DENV sensing including
53 Toll-like receptors (TLRs), RIG-I-like receptors (RLRs) and C-type lectin receptors (CLRs)
54 (9, 13–16). TLRs and RLRs have in common that activation leads both to cytokine responses

55 via NFκB as well as induction of type I IFNs via IRFs. However, it is unknown which sensor
56 in DCs is responsible for DC maturation and cytokine production.
57 Here we have identified that DENV RNA replication is essential for DENV-induced DC
58 maturation and cytokine responses. Notably, the intracellular RLRs RIG-I and MDA5 sense
59 DENV RNA replication products and induce innate signaling via adapter protein MAVS to
60 upregulate CD86 expression via type I IFN. DENV induced IL-1β, IL-6 and TNF secretion by
61 DCs and induction of CCL2, CCL3 and CCL4 and RIG-I and MDA5 were critical for these
62 responses. RLR-activation by DENV was also essential for the induction of adaptive immune
63 responses as inhibiting RLR signaling in DCs abolished T_H1 responses. These findings have
64 the potential to enable novel therapeutic strategies that interfere with RIG-I and MDA5
65 activation to limit DENV pathogenesis.

66 **METHODS**

67 **DC stimulation and RNA interference**

68 Peripheral blood monocytes were isolated from buffy coats of healthy donors (Sanquin) by
69 Lymphoprep (Axis-Shield) gradient followed by Percoll (Amersham Biosciences) gradient
70 steps. Monocytes were differentiated into immature DCs in the presence of 500 U/ml IL-4 and
71 800 U/ml GM-SCF (both Invitrogen) for 6-7 days in RPMI supplemented with 10% fetal calf
72 serum, 10 U/ml penicillin, 10 mg/ml streptomycin (all Invitrogen) and 2 mM L-glutamine
73 (Lonza). DC purity was determined by flow cytometry based on DC-SIGN, CD1c, CD11c
74 and HLA-DR expression and the absence CD14, CD19, CD3 and CD56 respectively and was
75 routinely more than 95% pure. This study was done in accordance with the ethical guidelines
76 of the Academic Medical Center.

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

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

91 MAVS (1:50, 3993, Cell Signaling) in combination with PE-conjugated anti-rabbit (1:200;
92 711-116-152, Jackson ImmunoResearch) and cells were analyzed on a Canto II (BD
93 Biosciences). Antibodies used for Western Blot were similar as for flow cytometry but in a
94 different dilution (all 1:1000) except for anti-β-actin (1:500, sc-81178, Santa Cruz).
95
96 **T cell differentiation**
97 Naive $CD4^+$ T cells were isolated from buffy coats of healthy blood donors (Sanquin) with
98 human $CD4^+$ T-cell isolation kit II (Miltenyi) by negative selection and subsequent depletion
99 of $CD45RO^+$ memory T cells using phycoerythrin (PE)-conjugated anti-CD45RO (80μg/ml;
100 R0843; Dako) and anti-PE beads (Miltenyi). This study was approved by the Medical Ethics
101 Review Committee of the AMC.
102 DCs were silenced for indicated proteins and stimulated for 48h as indicated. DCs were
103 combined with allogeneic naïve $CD4^+$ T cells (5,000 DCs/20,000 T cells) in the presence of
104 10 pg/ml *Staphylococcus aureus* enterotoxin B (Sigma). SDM25N (1 μM, Tocris Bioscience)
105 was added to cocultures of SDM25N-treated DCs to maintain inhibition of DENV replication.
106 After 5 days, cells were further cultured in the presence of 10 U/ml IL-2 (Chiron). Resting T
107 cells were restimulated with 100 ng/ml PMA and 1 μg/ml ionomycin (both Sigma) for 6h. For
108 flow cytometry analysis of restimulated T cells, cells were fixed in 4% *para*-formaldehyde for
109 20 min, followed by permeabilization in 0.1% saponin in PBS for 10 min. Cells were stained
110 with APC-conjugated anti-IL-4 and FITC-conjugated anti-IFNγ (both BD Bioscience) before
111 cells were analyzed on a FACS Canto II (BD Biosciences).
112
113 **Virus production and infection**
114 80% confluent C6/36 cells were infected with DENV-2/16681 at an MOI of 0.01 in HMEM
115 medium supplemented with 2% fetal calf serum, 10 U/ml penicillin, 10 mg/ml streptomycin

116 (all Invitrogen), 2 mM L-glutamine and 100 µM non-essential amino acids (both Lonza).
117 After 5-7 days, supernatant was harvested and cleared from cellular debris by centrifugation
118 and subsequent filtration using a 0.2 µM filter. Supernatant was aliquoted, snap-frozen in
119 liquid nitrogen and stored at -80 °C. Viral titers were determined as described previously
120 (17). Supernatant of uninfected C6/36 cultured for 5-7 days was used for mock-treatment of
121 DCs.
122 DCs were infected with DENV at an MOI of 1 unless stated otherwise. Infection was
123 determined after 36-48h by measuring the percentage of $NS3^+$ cells by flow cytometry. Cells
124 were fixed in 4% *para*-formaldehyde for 15 min followed by permeabilization in PBS
125 supplemented with 0.1% saponin for 10 min. Cells were stained with anti-NS3 (1:800,
126 SAB2700181, Sigma) followed by PE-conjugated anti-rabbit (1:200; 711-116-152, Jackson
127 ImmunoResearch), AlexaFluor 488-conjugated anti-rabbit (1:400, A-21206, ThermoFisher)
128 or AlexaFluor 647-conjugated anti-rabbit (1:400, A-21245, ThermoFisher) in combination
129 with FITC-conjugated anti-HLA-A2 (1:25, 551285, BD Pharmingen), PE/Cy7-conjugated
130 anti-HLA-DR (1:200, 560651, BD Pharmingen), PE-conjugated anti-CD80 (1:25, 557227,
131 BD Pharmingen), APC-conjugated anti-CD83 (1:25, 551073, BD Pharmingen), FITC-
132 conjugated anti-CD86 (1:25, 555657, BD Pharmingen), or PE-conjugated anti-CD86 (1:50,
133 555658, BD Pharmingen). Cells were analyzed on a FACS Canto II (BD Biosciences).
134
135 **ELISA**
136 Cell culture supernatant were harvested 24 or 48 hours after stimulation and IL-1β, IL-6, IL-
137 12p70 and TNF (eBioscience) were measured by ELISA according to the manufacturer's
138 instructions
139
140 **Real-time quantitative PCR**

141 mRNA was isolated using mRNA capture kit (Roche) and cDNA was synthesized with
142 reverse transcriptase kit (Promega). PCR amplification was performed in the presence of
143 SYBR Green in an ABI 7500 Fast PCR detection system (Applied Biosystems). Specific
144 primers were designed using Primer Express 2.0 (Applied Biosystems; Table S1). Expression
145 of target genes was normalized to GAPDH ($N_t=2^{Ct\ (GAPDH)\ -\ Ct\ (target)}$) and set at 1 in DENV-
146 infected DCs for each donor within one experiment.
147

148 **Statistical Analysis**

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

151 **RESULTS**

152 **DENV induces DC maturation and cytokine responses**

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

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

176 mRNA expression of IL-1β, IL-6, IL-12p35 and TNF but not IL-12p40 (Fig. 1E). These
177 results show that DENV induces DC maturation and cytokine responses 48 h.p.i. and that
178 virus concentration affects the strength but not the time of the activation. These data suggest
179 that sensing of DENV requires productive viral replication in DCs.
180
181 **DENV RNA replication is essential for DC activation**
182 DENV viral particles consist of different proteins and contain single stranded RNA that can
183 activate TLR7 (15). During DENV RNA replication double-stranded RNA intermediates are
184 formed that can be sensed by RLRs and TLR3 (16, 23). To investigate whether DENV
185 replication is required for DC maturation and cytokine responses we used the DENV
186 replication inhibitor SDM25N, which efficiently inhibits DENV RNA replication as well as
187 infection (24). We have focused on CD86 expression for investigating the role of DENV
188 replication as this maturation marker was highly induced by DENV. DENV-induced
189 upregulation of CD86 was completely abrogated when DENV replication was inhibited by
190 SDM25N (Fig. 2A). Also DENV-induced expression of IL-1β, IL-6, IL-12p35 and TNF was
191 dependent on DENV RNA replication (Fig. 2B,C). Reactive oxygen species (ROS) have been
192 implicated in DENV-induced maturation (21). However, treatment of DCs with ROS
193 inhibitors N-acetyl-L-cysteine (NAC) and Catalase did not decrease DENV-induced DC
194 maturation (Fig. S2). These data strongly suggest that DENV RNA replication is pivotal for
195 DCs activation. These data also explain the delayed response of DCs to DENV infection as
196 DENV RNA intermediates are formed during replication and are not present in viral particles
197 that initiate infection.
198
199 **DENV-induced cytokine responses depend on RIG-I and MDA5**

200 DENV replication products are formed in the cytosol and can be sensed by RLRs RIG-I and
201 MDA5 (13, 16, 23). We have recently shown that type I IFN responses induced by RLRs to
202 DENV induce IL-27, which is crucial for T follicular helper cell polarization (Sprokholt *et al.*,
203 submitted). To investigate if RIG-I and MDA5 are also involved in IL-1β, IL-6 and TNF
204 responses, we used RNA interference to silence RIG-I and MDA5 to investigate if these RNA
205 sensors are responsible for DENV-induced DC activation. We have confirmed effective
206 silencing on mRNA as well as protein level. Furthermore, we have confirmed the specificity
207 of the silencing using different RLR and TLR ligands and silencing did not affect cell
208 viability (Fig. S3). Notably, silencing RIG-I and MDA5 alone or together abrogated IL-1β,
209 IL-6 and TNF expression upon DENV infection (Fig. 3A,B). RIG-I and MDA5 triggering by
210 DENV also resulted in IL-12p35 expression (Fig. 3A). RLRs signal via adapter molecule
211 MAVS, which forms a scaffolding platform from which IRF and NFκB activation is
212 orchestrated (25). Similar to RIG-I and MDA5, silencing MAVS abrogated DENV-induced
213 cytokine expression (Fig. 3C,D). These data strongly suggest that RIG-I and MDA5 are
214 critical sensors of DENV RNA replication and drive cytokine responses of DCs in response to
215 DENV via adapter protein MAVS.
216
217 **RIG-I and MDA5 activation leads to chemokine responses**
218 In addition to elevated levels of cytokines that are associated with disease severity, several
219 chemokines can impact vascular integrity, including CCL2, CCL3, CCL4 and CXCL8 (4, 26).
220 Therefore we investigated whether DENV-infected DCs produced these chemokines in a
221 RLR-dependent manner. DENV infection induced the expression of CCL2, CCL3 and CCL4,
222 which was abrogated after silencing of either MAVS or both RIG-I and MDA5 (Fig. 4A,B).
223 CXCL8 expression was not induced (data not shown). These data indicate the DENV-induced
224 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

250 of IFNγ or IL-4-positive cells, indicative for T_H1 or T_H2 cells, respectively (Fig. S4). LPS-
251 stimulated DCs induced mixed skewing of T_H1 and T_H2 cells, whereas LPS+IFNγ-stimulated
252 DCs induced more T_H1 polarization and less T_H2 differentiation compared to LPS (Fig.
253 6A,B). In contrast, coculturing naïve T_H cells with LPS+PGE2-stimulated DCs resulted in a
254 greater percentage of T_H2 cells and fewer T_H1 cells compared to LPS (Fig. 6A,B). Next, we
255 examined T_H polarization induced by DENV-infected DCs and the involvement of RIG-I and
256 MDA5. DENV-infected DCs resulted in more T_H1 cells and less T_H2 cells compared to mock-
257 treated DCs and this effect was dependent on viral replication as SDM25N decreased T_H1
258 polarization (Fig. 6C). Strikingly, silencing MAVS abrogated T_H1 skewing by DENV-
259 infected DCs (Fig. 6D). Moreover, direct stimulation of RIG-I and MDA5 using the chemical
260 RLR ligand poly (I:C)Lyovec also resulted in strong T_H1 polarization (Fig. 6E). These data
261 indicate that DENV infection of DCs results in T_H1 skewing and that this effect is mediated
262 by RIG-I and MDA5.

263 **DISCUSSION**

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

273 DENV viral particles contain multiple components that can directly activate several receptors.
274 The viral envelope proteins can interact with CLR while the viral genome can activate
275 endosomal TLRs that recognize single-stranded RNA (5, 10, 31). During viral replication
276 double-stranded RNA intermediates are formed that can trigger cytoplasmic RNA sensors
277 (16, 23). We did not observe DC maturation nor cytokine secretion during the first 24h of
278 infection, indicating that DENV viral particles do not directly activate DCs via cell surface
279 receptors or endosomal TLRs. Instead, DENV RNA replication was essential for DC immune
280 activation and these responses were mediated by cytoplasmic RLRs RIG-I and MDA5.

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

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

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

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

304 In parallel to IRF3-mediated responses, RLR activation leads to NFκB activation via CARD9
305 and Bcl-10 (37). NFκB activation is essential for the expression of most cytokines including
306 IL-1β, which potently induces fever (38, 39). Persistent high fever is one of the clinical
307 symptoms of DENV, but the underlying mechanisms that lead to IL-1β secretion are
308 unknown. IL-1β expression is tightly regulated and requires a multi-stage process to be
309 functionally active. Priming signals induce pro-IL-1β transcription and translation, whereas
310 secondary signals induce inflammasome activation to process pro-IL-1β into mature IL-1β by
311 caspases. Normally, pro-IL-1β synthesis and inflammasome activation are induced by
312 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

338 mRNA expression and thereby prevents IL-12p70 formation (44). We also did not observe
339 IL-12p40 induction by DENV in contrast to IL-12p35, which resulted in undetectable levels
340 of mature IL-12p70. However, our data show that DENV infection of DCs did result in T_H1
341 formation and this was dependent on RLR-adapted protein MAVS. Also direct stimulation of
342 RIG-I and MDA5 with chemical ligands resulted in T_H1 polarization. Other factors besides
343 IL-12p70 have the potential to induce T_H1 polarization, including IFN-β (45). Our data show
344 that DENV induces functional type I IFN responses in DCs and RLR-dependent IFN-β could
345 be driving T_H1 differentiation by DENV-infected DCs. Interestingly, Chase *et al.* (2011)
346 suggest that infected DCs are less efficient in inducing T_H1 responses (46). However, our data
347 are supported by upregulation of co-stimulatory molecules and IFN responses that promote
348 T_H differentiation. Moreover, Chase *et al.* (2011) observed T cell polarization, although to a
349 lesser extent. Possible differences in assays might explain the apparent disparity between our
350 studies.
351 Our data show that RLR activation by DENV leads to strong cytokine and chemokine
352 responses that are associated with disease severity (4). We have recently shown that type I
353 IFN responses induced by RLRs is crucial for T follicular helper cell polarization and
354 antibody production (Sprokholt *et al.*, submitted). This indicates that RLRs induce a dual
355 response against DENV: (i) an inflammatory response that is based on NFκB and involves
356 cytokines and chemokines and (ii) a protective response that depends on IRF3 and involves
357 type I IFN and antibody production.
358 Interestingly, only RNA viruses can induce viral hemorrhagic fever which could indicate that
359 RNA sensors are key players in viral hemorrhagic fever. TNF directly activates endothelial
360 cells and is critical for DENV induced vascular leakage in animal models, probably in concert
361 with other factors (47, 48). We show that DENV potently stimulates TNF production by DCs
362 several magnitudes larger than LPS stimulation. Notably, DENV-induced TNF responses

363 depended critically on RIG-I and MDA5. Numerous other RNA viruses that cause viral
364 hemorrhagic fever activate RIG-I and/or MDA5 and RLR triggering could be a general
365 mechanism underlying viral hemorrhagic fever (49, 50).
366 In conclusion, we have identified RIG-I and MDA5 as key sensors of DENV infection in DCs
367 and as critical players in DC-depended innate and adaptive immune responses against DENV.
368 Many of the cytokines and chemokines produced by DCs in response to DENV are associated
369 with disease severity and endothelial dysfunction. Hence, molecules targeting RIG-I and
370 MDA5 signaling components have the potential to be used as therapeutic agents for DHF,
371 DSS and possibly other viruses.
372

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378

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382

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

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

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544 12.
545

546 **FIGURE LEGENDS**

547 **Figure 1. DENV induces DC maturation and cytokine responses**

548 (A-C) DCs were mock-treated or infected with an MOI of 1 or 3 and the percentage of $NS3^+$
549 cells (A) or the expression of CD80, CD83, CD86, HLA-A2 and HLA-DR (B,C) was
550 measured 24 or 48 h.p.i. by flow cytometry. The $DENV^+$ and $DENV^-$ population in (C) was
551 gated as shown in Fig. S1. (D) DCs were similarly infected as in (A) and the production of IL-
552 1β, IL-6, IL-12p70 and TNF was measured in the cell culture supernatant by ELISA. (E). DCs
553 were infected with an MOI of 1 and mRNA expression of indicated genes was measured 32
554 h.p.i. by real-time PCR, normalized to GAPDH and set as 1 in DENV infected samples.
555 **P<0.01 (student's t-test). Data represent three (B,C) or four (A,D) donors of different
556 experiments or are collated data (mean ± s.d.) of at least four donors (E) from different
557 experiments (mean ± s.d. of duplicates in A,D).

558

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

560 (A) DCs were infected with DENV at an MOI of 1 in the presence or absence of 10 μM
561 SDM25N and the expression of CD86 was measured 48 h.p.i. by flow cytometry. (B-C) DC
562 were infected similarly as in (A) and the mRNA expression of indicated genes was measured
563 32 h.p.i. by real-time PCR, normalized to GAPDH and set as 1 in DENV infected samples
564 without SDM25N (B) or the secretion of indicated proteins in the cell culture supernatant was
565 measured by ELISA (C). **P<0.01 (student's t-test). Data are representative of at least four
566 (C) or two (A) donors of different experiments or are collated data (mean ± s.d) of four (B)
567 donors from different experiments (mean ± s.d. of duplicates in C).

568

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

570 (A,C) DCs were mock-treated or infected with DENV at an MOI of 1 after RIG-I, MDA5 or
571 MAVS silencing and the mRNA expression of indicated genes was measured 32 h.p.i. by
572 real-time PCR, normalized to GAPDH and set as 1 in DENV infected samples treated with
573 control siRNA. (B,D) DCs were treated similarly as in (A) and the production of indicated
574 proteins in the cell culture supernatant was measured by ELISA. **P<0.01 (student's t-test).
575 Data are representative of at least two donors (B,D) of different experiments or collated data
576 (mean ± s.d.) of four (C) or three (A) donors of different experiments (mean ± s.d. of
577 duplicates in B,D).
578
579 **Figure 4. RIG-I and MDA5 activation by DENV induces chemokine expression**
580 (A,B) DCs were infected with DENV after RIG-I, MDA5 or MAVS silencing and the mRNA
581 expression of indicated genes was measured 32 h.p.i. by real-time PCR, normalized to
582 GAPDH and set as 1 in DENV infected samples treated with control siRNA. *P<0.05,
583 **P<0.01 (student's t-test). Data are collated (mean ± s.d.) of at least five donors from
584 different experiments.
585
586 **Figure 5. RIG-I and MDA5 induce DC maturation via type I IFN**
587 (A,D) DCs were stimulated with LPS (A), mock-treated or infected with DENV at an MOI of
588 1 after RIG-I, MDA5 or MAVS silencing (A) or in the presence or absence of B18R (D) and
589 the expression of CD86 was measured 48 h.p.i. by flow cytometry. (B,C) Similar as in (D) but
590 MxA, IRF7, ISG15, OAS1 and ADAR1 expression was measured by real-time PCR,
591 normalized to GAPDH and set as 1 in DENV infected samples without B18R (C) or the
592 percentage of $NS3^+$ cells was measured by flow cytometry (B). **P<0.01 (student's t-test).
593 Data are representative of at least four (A,B,D) donors of different experiments or are collated
594 data (mean ± s.d.) of four (C) donors of different experiments (mean ± s.d. of duplicates in B).

595

596 **Figure 6. RIG-I and MDA5 induce T_H1 polarization**

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

607

608

609

610

Fig. 1

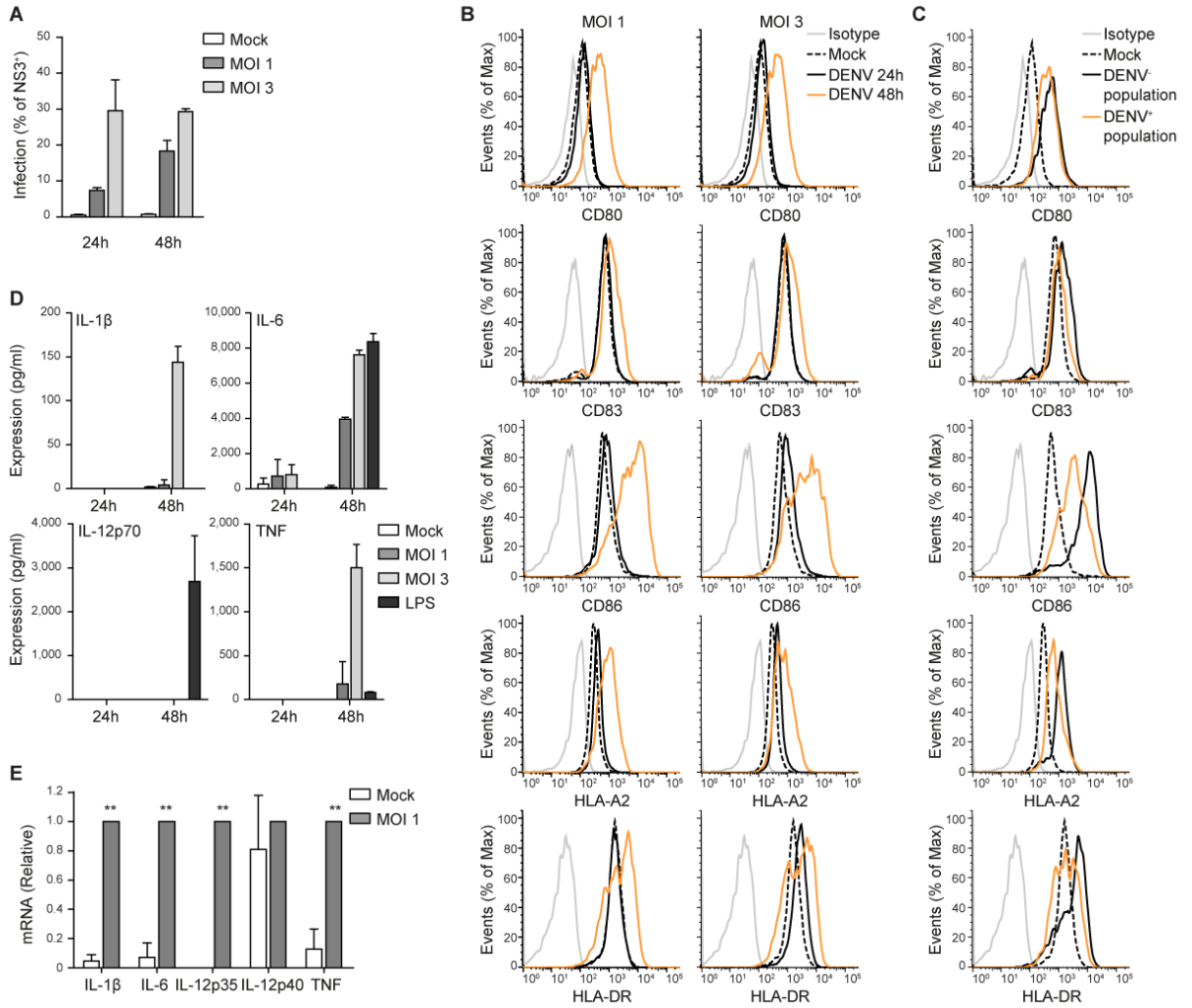

A
Infection (% of NS3+)
Mock
MOI 1
MOI 3
24h
48h
B
MOI 1
MOI 3
Isotype
Mock
DENV 24h
DENV 48h
Events (% of Max)
CD80
CD83
CD86
HLA-A2
HLA-DR
C
Isotype
Mock
DENV- population
DENV+ population
D
IL-1β
IL-6
IL-12p70
TNF
Expression (pg/ml)
Mock
MOI 1
MOI 3
LPS
E
mRNA (Relative)
Mock
MOI 1
IL-1β
IL-6
IL-12p35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

Fig. 2

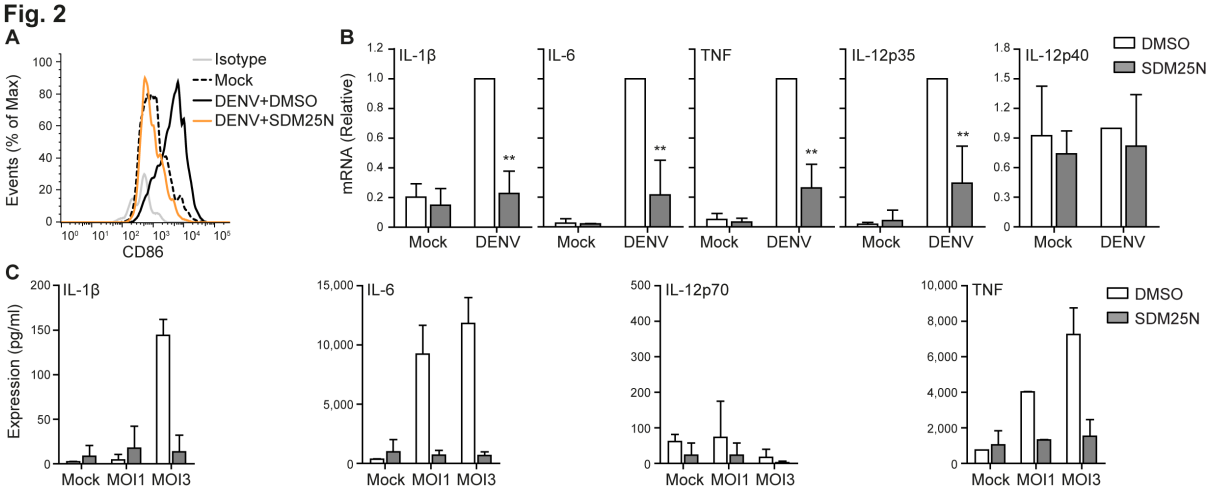

A
Isotype
Mock
DENV+DMSO
DENV+SDM25N
Events (% of Max)
CD86
B
IL-1β
IL-6
TNF
IL-12p35
IL-12p40
mRNA (Relative)
DMSO
SDM25N
Mock
DENV
C
IL-1β
IL-6
IL-12p70
TNF
Expression (pg/ml)
Mock
MOI1
MOI3

Fig. 3

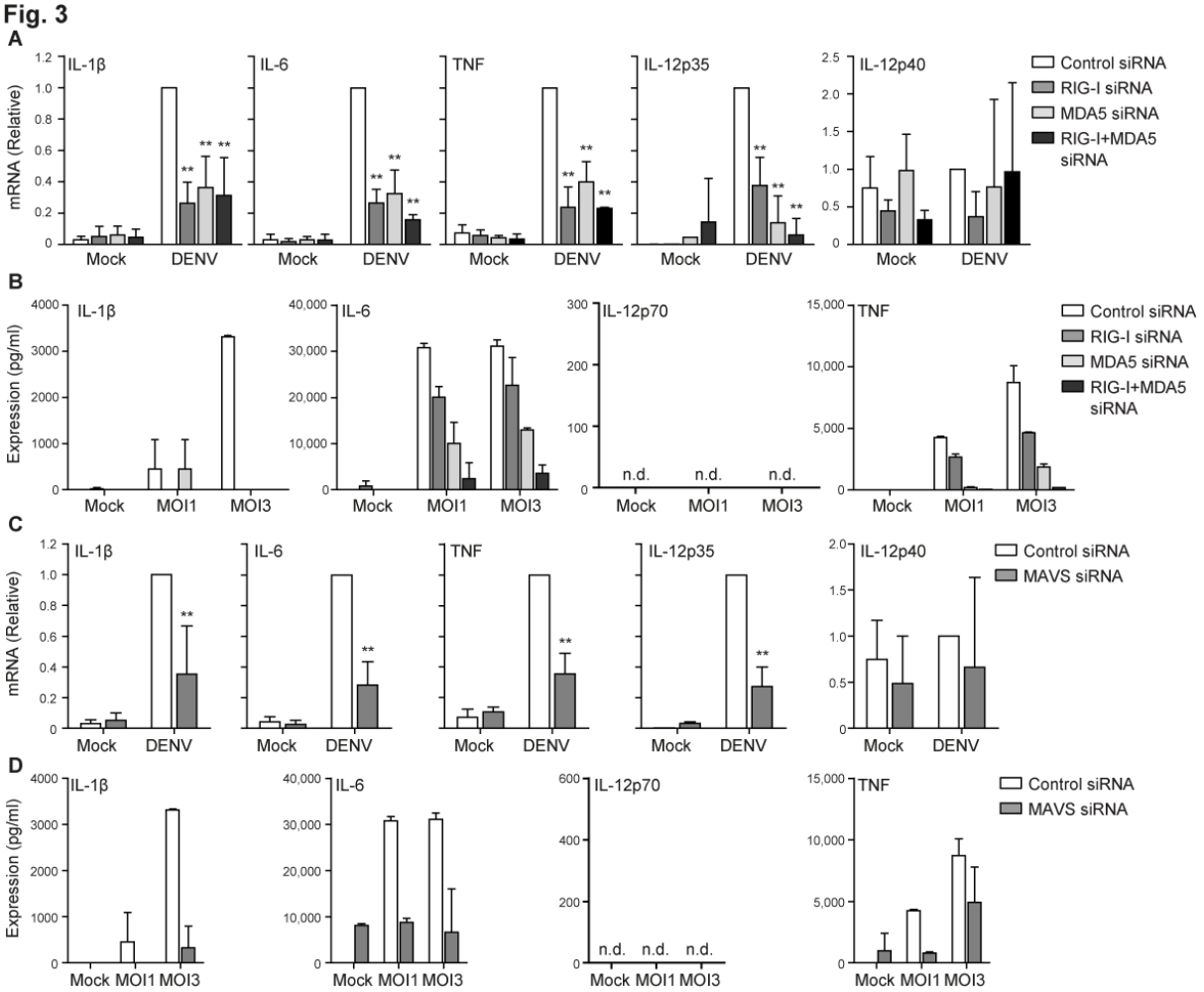

A
mRNA (Relative)
IL-1β
IL-6
TNF
IL-12p35
IL-12p40
Mock
DENV
Control siRNA
RIG-I siRNA
MDA5 siRNA
RIG-I+MDA5 siRNA
B
Expression (pg/ml)
IL-12p70
n.d.
MOI1
MOI3
C
MAVS siRNA
D

Fig. 4

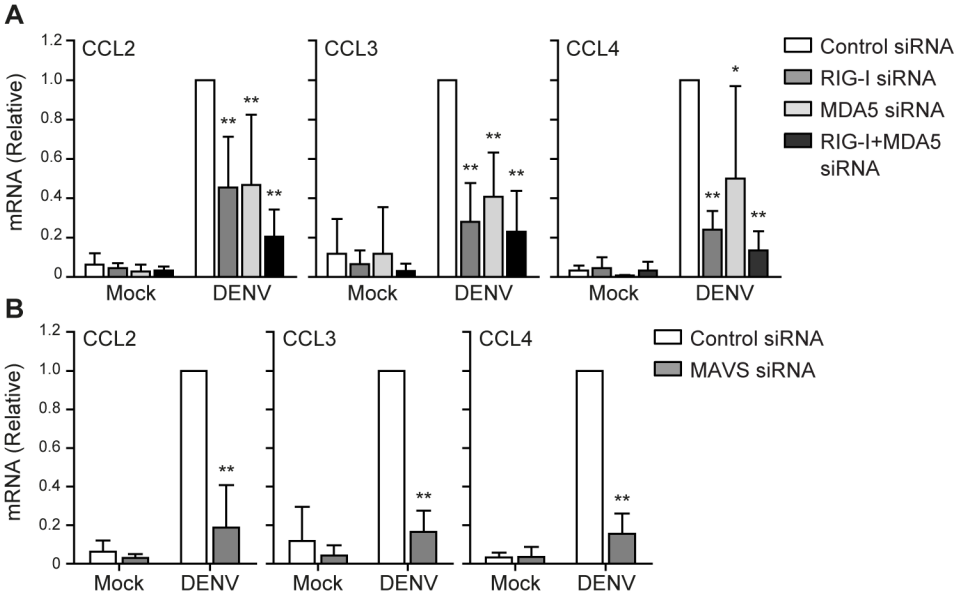

A
CCL2
CCL3
CCL4
mRNA (Relative)
1.2
1.0
0.8
0.6
0.4
0.2
0
Mock
DENV
Control siRNA
RIG-I siRNA
MDA5 siRNA
RIG-I+MDA5 siRNA
B
MAVS siRNA

Figure 5

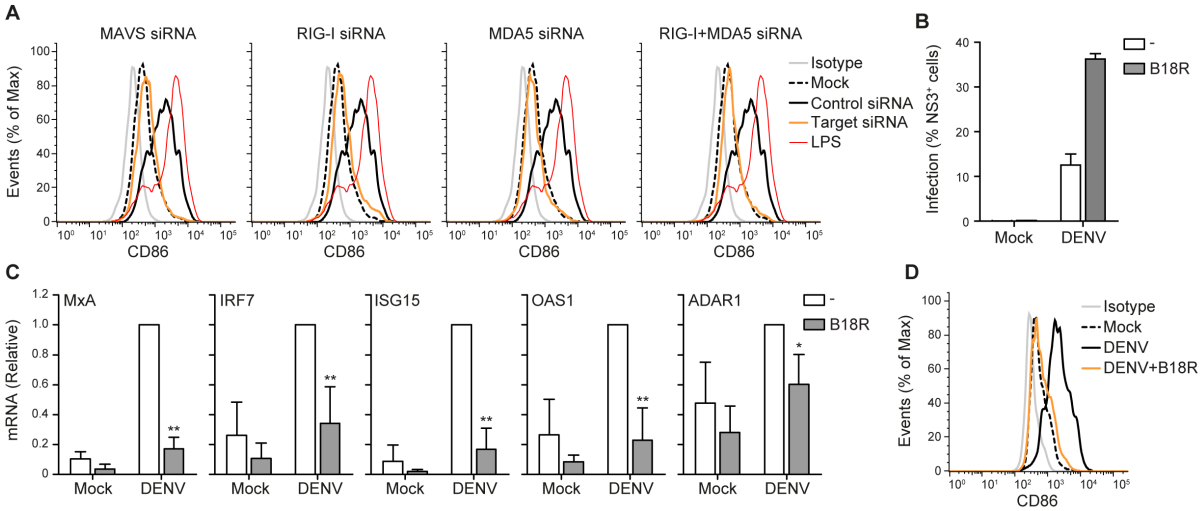
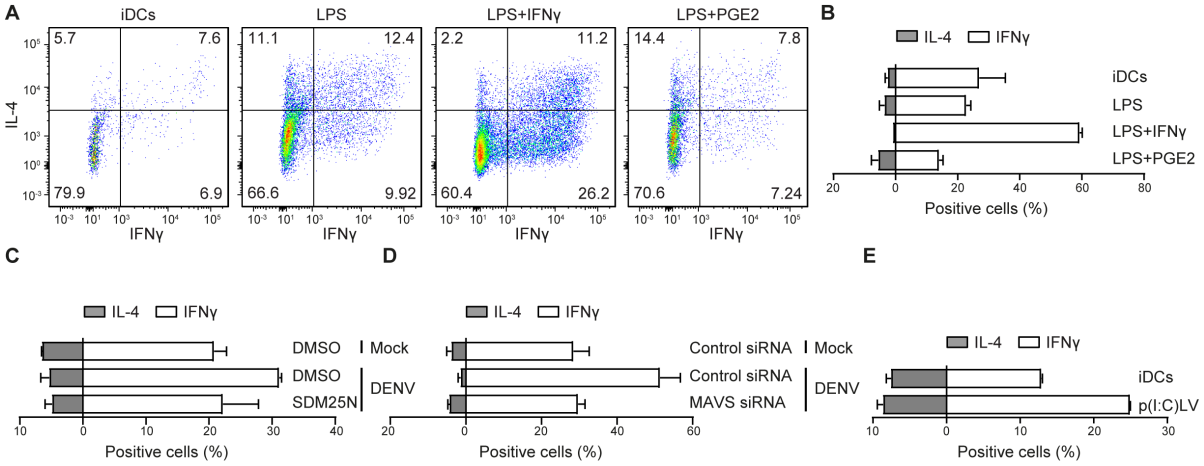

A
MAVS siRNA
RIG-I siRNA
MDA5 siRNA
RIG-I+MDA5 siRNA
Events (% of Max)
CD86
Isotype
Mock
Control siRNA
Target siRNA
LPS
B
Infection (% NS3+ cells)
-
B18R
Mock
DENV
C
mRNA (Relative)
MxA
IRF7
ISG15
OAS1
ADAR1
D
Isotype
Mock
DENV
DENV+B18R

Figure 6


A
iDCs
LPS
LPS+IFNγ
LPS+PGE2
IL-4
IFNγ
5.7
7.6
79.9
6.9
11.1
12.4
66.6
9.92
2.2
11.2
60.4
26.2
14.4
7.8
70.6
7.24
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 (%)