1   **Activation of the chicken type I IFN response by infectious bronchitis coronavirus.**

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14

15   **Abstract**

16   Coronaviruses from both the *Alpha* and *Betacoronavirus* genera, interfere with the type I interferon

17   (IFN) response in various ways, ensuring limited activation of the IFN response in most cell types.

18   Of *Gammacoronaviruses* that mainly infect birds, little is known about activation of the host

19   immune response. We show that the prototypical *Gammacoronavirus*, infectious bronchitis virus

20   (IBV), induces a delayed activation of the IFN response in primary renal cells, tracheal epithelial

21   cells and in a chicken cell line. *Ifnβ* expression in fact, is delayed with respect to the peak of viral

22   replication and accompanying accumulation of dsRNA. In addition, we demonstrate that MDA5 is

23   the primary sensor for *Gammacoronavirus* infections in chicken cells. Furthermore, we provide

24   evidence that accessory proteins 3a and 3b of IBV modulate the IFN response at the transcriptional

25   and translational level. Finally, we show that, despite the lack of activation of the IFN response

26   during the early phase of IBV infection, signalling of non-self dsRNA through both MDA5 and TLR3

27   remains intact in IBV-infected cells. Taken together, this study provides the first comprehensive

28   analysis of host-virus interactions of a *Gammacoronavirus* with avian innate immune responses.

29

30   **Importance:** Our results demonstrate that IBV has evolved multiple strategies to avoid activation

31   of the type I interferon response. Taken together, the present study closes a gap in the

32   understanding of host-IBV interaction*,* and paves the way for further characterization of the

33   mechanisms underlying immune evasion strategies as well as pathogenesis of

34   *Gammacoronaviruses*.

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Introduction

46 Coronaviruses constitute a large family of positive-stranded RNA viruses and cause a range of

47 human and veterinary diseases. Infectious bronchitis virus (IBV) is the prototype avian coronavirus

48 from the *Gammacoronavirus* genus and the causative agent of a highly contagious respiratory

49 disease of major economic importance to the poultry industry (1). IBV enters the avian host

50 through the respiratory tract, where it causes destruction of the epithelium leading to respiratory

51 distress and initiation of secondary bacterial infections. Depending on the strain, IBV can also

52 spread to other epithelial surfaces such as the gastrointestinal tract, the kidneys and the oviduct,

53 the latter causing problems in egg production and quality (1-6). Contrary to coronaviruses from the

54 *Alpha* and *Beta* genera, including human coronavirus HCoV-229E, Severe Acute Respiratory

55 Syndrome (SARS-CoV), Middle East Respiratory Syndrome (MERS-CoV) and mouse hepatitis virus

56 (MHV), very little is known about how *Gammacoronaviruses* including IBV evade or interfere with

57 innate immune responses of their host.

58 Innate immune responses consist of a network of antimicrobial mechanisms, of which the type I

59 interferon (IFN) response is an essential defence mechanism against viruses. Typically, the type I

60 IFN response, from hereafter referred to as IFN response, is initiated upon activation of host

61 pattern recognition receptors (PRRs), present in all animal cells. Two families of PRRs have been

62 shown to be involved in the recognition of RNA viruses namely the membrane-bound Toll-like

63 receptors (TLRs) and the cytosolic RIG-I-like receptors (RLRs) (7). The primary ligands for

64 activation of these PRRs are double-stranded RNA (dsRNA) and 5' triphosphate-containing RNA,

65 normally absent from uninfected host-cells. Activation of RLRs leads to the transcription of genes

66 encoding type I interferons (IFNα and IFNβ). These interferons are secreted from the infected cell

67 providing a signal for the infected as well as the neighbouring cells that induce the transcription of

68 anti-viral effector genes collectively called interferon stimulated genes (ISGs).

69   The ability of a virus to replicate and produce infectious progeny depends for a large part on its

70   ability to avoid induction or counteract the IFN response of its host. Indeed, a common feature of

71   *Alpha-* and *Betacoronaviruses*, including HCoV-229E, SARS-CoV, and MHV, is their limited

72   activation of the IFN response (8-13). This limited activation can be partially explained by

73   intracellular membrane rearrangements that might shield dsRNA and other viral components from

74   recognition by host PRRs (14, 15). In addition, coronavirus nsp16 displays 2'-O-methylase activity,

75   which results in 2'-O-methylation of a ribose moiety on the 5' cap of coronavirus mRNAs, making

76   them indistinguishable from host mRNAs (16). Furthermore, many other coronavirus proteins, such

77   as nsp1, nsp3, the nucleocapsid and many of the accessory proteins have been shown to interfere

78   with the IFN response in various ways (reviewed in (17, 18)).

79

80   Interaction between G*ammacoronaviruses* and innate immune responses of their avian hosts is

81   poorly understood. Early studies on G*ammacoronaviruses* in chicken suggest that IBV-induced IFN

82   production is variable and dependent on both virus strain and cell type. (19-22). Further, two

83   transcriptional studies on tissues collected after *in vivo* and *in ovo* IBV infections, found only limited

84   upregulation of ISGs at 1 - 3 days post-infection (23-25). Functional studies using IBV Beaudette

85   showed that it induced cell-cycle arrest and apoptosis (26, 27), that IBV interacts with eIF3f (28)

86   and that IBV inhibits protein kinase R activation, thereby maintaining protein synthesis (29).

87   Although these studies did provide a number of details on the interactions between IBV and the

88   host cell, most experiments were carried out in Vero cells. This non-avian cell line is one of the

89   very few cell lines in which the IBV-Beaudette strain has been adapted to grow, facilitating *in vitro*

90   experiments. Vero cells, however, lack the *Ifnβ* gene, preventing them from mounting a type I IFN

91   response (30, 31), reducing the value of Vero cells for research on innate immune responses to

92   IBV. In addition, the Beaudette strain is non-pathogenic *in vivo* with limited replication in host

93   tissues (32), reducing the value of these *in vitro* studies for translation to *in vivo* situations. For

94   these reasons, we used pathogenic isolates of IBV to infect primary chicken cells, and a chicken cell

95   line, as these isolates are known to infect, spread and cause clinical disease *in vivo*.

96

97   In the current study, we show that IBV infection leads to a significant induction of *Ifnβ* transcription

98   through an MDA5-dependent activation of the IFN response, albeit delayed with respect to both

99   virus replication and accumulation of dsRNA. This delayed induction of *Ifnβ* was further confirmed

100   through RNA FISH analysis showing that accumulation of *Ifnβ* mRNA is restricted to IBV-infected

101 and not neighbouring uninfected cells. Although the time lag between accumulation of dsRNA and

102 induction of *Ifnβ* transcription might suggest that IBV interferes with recognition of dsRNA, we

103 observed that sensing of exogenous (non-self) dsRNA remained functional in IBV-infected cells.

104 Using mutant IBV viruses we demonstrate that both accessory proteins 3a and 3b are involved in

105 limiting *Ifnβ* expression, as both 3a and 3b null viruses induced increased *Ifnβ* expression.

106 Nevertheless, 3a and 3b seem to have a differential effect on IFN protein production, infection with

107 3a null virus induced lower IFN levels whereas a 3b null virus increased IFN production compared

108 to the parental virus. Altogether, our data suggest that IBV delays but does not prevent detection

109 by MDA5, and that accessory proteins 3a and 3b modulate the IFN response in avian cells. This is

110 the first study addressing immune evasion and interference strategies of IBV in chicken and not in

111 mammalian cells, providing information essential to further understanding of the pathogenesis of

112 *Gammacoronaviruses*.

113

114 **MATERIALS AND METHODS**

115 **Cells**

116 Chicken embryonic kidneys were aseptically removed from 17- to 19-day-old chicken embryo's

117 (Charles River, SPAFAS). A cell suspension was obtained by trypsinisation for 30 min at 37°C and

118 filtered through a 100 μm mesh. The resulting chicken embryo kidney (CEK) cells were seeded at 4

119 x 10^5 cells/cm^2 in 199 medium (Invitrogen) supplemented with 0.5% fetal bovine serum (FBS,

120 FASC) and 1% PenStrep (Gibco®, Invitrogen). Chicken trachea cells were isolated from 8- to 10-

121 week-old chickens (white leghorn). Tracheas were collected in ice-cold PBS, washed and stripped

122 from adipose tissue. Trachea were filled with a solution of 3.5 U/ml protease type XIV (Sigma), 4

123 U/ml DNase I (Qiagen) and 1% PenStrep in EMEM, sealed with clamps, and incubated overnight at

124 4°C. The next day, cells lining the luminal side of the trachea were flushed out with cold EMEM,

125 filtered through a cell strainer and seeded at 4 x 10^5 cells/cm^2 in DMEM supplemented with 10%

126 FBS and 1% PenStrep. The RIG-I^{wt}, RIG-I^{KO}, MDA5^{wt} and MDA5^{KO} MEFs were provided by Prof. S.

127 Akira (33). The MAVS^{wt} and MAVS^{KO} MEFs were provided by Z.J. Chen (34). DF-1, CEC-32 and

128 MEFs cells were cultured in DMEM (Gibco®, Invitrogen) supplemented with 10% FBS and 1%

129 PenStrep. All cells were incubated in a humidified incubator at 37°C and 5% CO_2.

130

131

132 **DF-1 Ifnβ-luc reporter cell line**

133 DF-1 cells were transfected using Fugene (Promega) according to the manufacturer's instructions

134 with a construct expressing firefly luciferase under the control of the -110bp proximal region of the

135 human *IFNβ* promotor (35). Stably expressing cells were selected over a period of 3 weeks using

136 geneticin (500 μg/ml). DF-1 Ifnβ-luc stable cells were cultured in DMEM supplemented with 10%

137 FBS and 1% PenStrep and were not further subcloned.

138

139 **Viruses**

140 IBV-M41, IBV-QX and IBV-Italy-O2, Rift Valley Fever Virus clone 13 (RVFV Cl13) and Infectious

141 Pancreatic Necrosis Virus (IPNV) were obtained from Merck Animal Health, Boxmeer, The

142 Netherlands. Sindbis-GFP was a kind gift from J. Fros, (Laboratory of Virology, Wageningen

143 University). IBV Beaudette, strain Beau-R, as well as the generation of the ScAUG3a, ScAUG3b,

144 ScAUG3ab and ScAUG5ab Beau-R null viruses has been published previously (36-38). In these

145 mutant IBV viruses, the start codons of the indicated accessory genes were mutated to stop

146 codons. All IBV strains were amplified and titrated on CEK cells. Sindbis-GFP was amplified on baby

147 hamster kidney (BHK) cells and titrated on CEK cells. RVFV Cl13 was amplified and titrated on Vero

148 cells, an African green monkey cell line. IPNV was amplified and titrated on the CHSE-214,

149 Chinook-salmon cell line. IPNV was inactivated by 20 min UV exposure on a 48W BXT-26-M

150 (Uvitec).

151

152 **Poly I:C stimulation and RNase treatment**

153 Polyinosinic-poly(C) [p(I:C)] sodium was purchased from Sigma, dissolved in nuclease-free water

154 and stored at -80°C. p(I:C) was either directly added to the medium or transfected using

155 Lipofectamine 2000 (Invitrogen) as per manufacturer's instructions. DF-1 cells (3 x 10^5/well) were

156 cultured in 24 well plates and transfected with 500 ng p(I:C). RNase treatment of CEK cell culture

157 supernatant was performed by addition of 10 μg/ml RNase A (Invitrogen) before IBV infection or

158 before stimulation with 2 μg/ml p(I:C).

159

160 **RNA isolation and cDNA synthesis**

161 Approximately 8 x 10^5 CEK cells or 3 x 10^5 DF-1 cells were lysed in RLT buffer (Qiagen) at various

162 time points after treatment or infection. RLT cell lysis buffer was spiked with 1 ng/sample of

163 luciferase mRNA (Promega) immediately prior to RNA isolation. Luciferase expression will later be

164   used as external reference gene for normalization during the gene expression analysis. Total RNA

165   was isolated using the RNeasy Mini Kit (Qiagen) according to the manufacturer's instructions,

166   including an on-column DNase treatment with RNase-free DNase (Qiagen). Before cDNA synthesis

167   of 0.5–1 µg total RNA, a second DNase treatment was performed using DNase I, amplification

168   grade (Invitrogen). Synthesis of cDNA was performed using SuperScript III (Invitrogen) using

169   random primers. cDNA samples were further diluted 1:50 in nuclease-free water before real-time

170   quantitative PCR analysis.

171

172   **Gene expression analysis**

173   Real-time quantitative PCR was performed on a Rotor-Gene 6000 (Corbett Research), using

174   Brilliant SYBR Green quantitative PCR (Stratagene) and primers (39-42) as listed in Table 1. Cycle

175   thresholds and amplification efficiencies were calculated by the Rotor-Gene software (version 1.7).

176   The relative expression ratio of the target gene was calculated using the average reaction efficiency

177   for each primer set and the cycle threshold (C_t) deviation of sample vs. control at time point 0h, as

178   described in (43). For calculation of the fold change of IBV total RNA, C_t deviation was calculated

179   versus C_t 30, as no IBV was present in the non-infected cells that were used as control in all the

180   experiments. Because expression of various housekeeping genes was unstable during virus

181   infections at time points later than 24 hours (data not shown), gene-expression ratios were

182   normalised using an external reference gene (luciferase).

183

184   **Immunohistochemistry**

185   CEK cells were seeded on fibronectin-coated glass Biocoat coverslips (BD Biosciences) at a density

186   of 1×10^5 cells/cm^2. After incubation at 37°C for 48 hours, cells were infected with IBV strain M41

187   at an MOI of 1, and fixed at different time points with 3.7% paraformaldehyde and permeabilized

188   using 0.1% Triton X-100. Infected cells were probed with anti-dsRNA antibody (English & Scientific

189   Consulting) and polyclonal chicken serum raised against IBV M41 was obtained from Merck AH.

190   Detection was done performed using Alexa 488 goat anti-mouse antibody (Invitrogen) and FITC

191   labelled goat anti-chicken antibody (Kirkegaard and Perry laboratories). Nuclei were stained with

192   4',6-diamidino-2-phenylindole (DAPI). Cells were imaged using a Zeiss Primo Vert microscope and

193   Axiovision software. Image overlays were made in ImageJ.

194

195 **RNA fluorescence *in situ* hybridization**

196 RNA fluorescence *in situ* hybridization (FISH) was performed according to previously described

197 protocols (44-46). A set of forty RNA FISH probes (20 bp), each labelled with one CAL Fluor® Red

198 610 fluorophore and targeting chicken *Ifnβ* (ENSGALT39477), was designed using the Stellaris®

199 probe designer (Biosearch Technologies; https://www.biosearchtech.com/stellarisdesigner/). The

200 coding sequence of chicken Ifnβ is 601 bp, therefore to accommodate the optimum number of

201 fluorescent probes (48, as explained in reference (44)), the 3'UTR was included in the probe design

202 tool. CEK cells were grown on fibronectin-coated coverslips (BD Biosciences) at a density of 2 x 10^5

203 cells/cm^2. After incubation at 37°C for 48 hours, cells were infected with IBV M41, and at the

204 indicated time points fixed in 70% ethanol at 4 °C. Hybridisation of the probes was performed

205 using the manufacturer's protocol for adherent cells. Imaging was performed using a Roper (Evry,

206 France) Spinning Disc Confocal System on a Nikon Eclipse Ti microscope using a 100 × Plan apo oil

207 immersion objective (NA 1.4) and a 491 nm laser line. Z-stacks were collected with 0.25 μm Z-

208 intervals. For each channel, maximum Z-stack projections were made and processed with ImageJ.

209

210 **Chicken type I IFN bioassay.**

211 Bioactive chicken type I interferon (chIFN) was measured using a bioassay based on the CEC-32

212 quail reporter cell line expressing luciferase under the control of the chicken *Mx* promotor (47)

213 (kindly provided by Prof. Peter Staeheli). Briefly, CEC-32 were incubated with serial dilutions of

214 chIFN-containing samples for 6 hours, after which luciferase activity was quantified and IFN

215 concentrations calculated using a chIFN standard. To avoid influence of IBV on the assay, samples

216 were heat inactivated at 56°C for 30 min, which did not influence type I chIFN bioactivity.

217

218 **Gene silencing**

219 siRNAs targeting chicken *Tlr3* and *Mda5* were designed by and purchased from Microsynth,

220 Switzerland (sequences in Table 1). Transfections were performed using siLentFect (Biorad) at a

221 final siRNA concentration of 20 nM. For one well, 160 ng siRNA was combined with 1 μL siLentFect

222 in 100 μL OptiMEM (Gibco) and incubated for 20 min. The siRNA complexes were added to 2 x 10^5

223 DF-1 cells grown in 500 μL medium per well in a 24 well plate. siRNA complexes were left on the

224 cells for 48 hours before further experiments were performed.

225

226 **Statistics**

227 All statistical analyses were performed in GraphPad Prism 5.0. RT-qPCR fold changes were first log

228 transformed and then used for statistical analysis. For all tests, equality of variance was assessed

229 using Bartlett's test. Significant differences ($P<0.01$) were determined by a one way or two-way

230 ANOVA (indicated in the figure legend) followed by a Bonferroni post-hoc test.

231

232

233 **Results**

234

235 **IBV delays the onset of an IFN response during infection of primary chicken cells**

236 To investigate the kinetics of viral replication and IFN induction upon infection with the avian

237 G*ammacoronavirus* IBV, we infected primary CEK cells (24) with the IBV M41 strain. To monitor

238 the kinetics of the IFN response in relation to IBV replication, we quantified transcription of *Ifnβ,* a

239 set of genes involved in innate immunity, extracellular IFN protein production, virus titres and IBV

240 RNA in M41-infected CEK cells. In line with previous observations (48), progeny virus was produced

241 after 6 hpi and virus titres reached a maximum around 24 hpi (Fig. 1A). Total intracellular IBV RNA

242 levels reflected the kinetics of infectious IBV virus in the supernatant (Fig. 1A), reaching maximum

243 levels around 24 hpi. *Ifnβ* expression was delayed with respect to the peak of viral replication and

244 remained low until 18 hpi, after which it was strongly upregulated, peaking around 36 hpi (Fig.

245 1B). IFN protein activity levels were quantified using a chicken IFN-specific *Mx*-luc cell-based

246 bioassay showing accumulation of IFN from 36 hpi onwards (Fig. 1B). Concomitant with *Ifnβ,* a

247 subset of genes involved in innate immunity, including *Mx, Oas* and *Il8,* were upregulated whereas

248 others, such as *Tlr7, Adar*, *Isg12, MHC-I* and *Ifnar2* appeared not, or only marginally affected by

249 IBV infection (Fig. 1C). Pattern recognition receptors *Mda5* and *Tlr3* and the transcription factor

250 *Irf3* were also upregulated (Fig. 1C), which is of interest given the role of these PRRs in virus

251 recognition.

252

253 **The delayed IFN response is independent from the cell type or virus strain**

254 *Ifnβ* transcription during infection with coronaviruses such as MHV and SARS-CoV is generally low

255 (9, 10, 12, 13, 49), and was shown to be dependent on cell type and virus strain (50). The delayed

256 induction of *Ifnβ* transcription observed in IBV M41-infected CEK cells prompted us to investigate

257 whether induction of *Ifnβ* would be dependent on the cell type or IBV strain. Epithelial cells isolated

258 from trachea of 10-week-old SPF chickens and DF-1 chicken fibroblast cells were infected with IBV

259 M41 or IBV Beaudette (Beau-R, (38)). At several time points after infection, *Ifnβ* levels were

260 monitored by RT-qPCR (Fig. 2A and 2B). In both cell types *Ifnβ* transcription followed the same

261 kinetics observed in CEK cells (Fig. 1B), indicating that induction of *Ifnβ* by IBV is independent of

262 cell type. To study whether induction of *Ifnβ* transcription differs between different strains of IBV,

263 we also infected CEK cells with the QX and ItO2 strains of IBV (Fig. 2C). Although we observed

264 some differences in absolute levels of *Ifnβ* upregulation induced by QX, ItO2 and M41, kinetics of

265 *Ifnβ* transcription were similar, suggesting that delayed induction of *Ifnβ* transcription could be

266 considered a general feature of IBV infection in chicken cells.

267 To assess whether CEK and DF-1 cells do have the intrinsic ability to express *Ifnβ* earlier than 18h,

268 we stimulated these cells with extracellular polyI:C (pI:C), transfected pI:C (t[pI:C]) or with the

269 dsRNA virus Infectious Pancreatic Necrosis Virus (IPNV). We found that stimulation of CEK cells

270 with pI:C could induce *Ifnβ* transcription as early as one hour after stimulation (Fig. 3A). In DF-1

271 cells, stimulation with IPNV, and t[pI:C], but not pI:C, induced *Ifnβ* already at 4h (Fig. 3B). The

272 observation that DF-1 cells do not respond to stimulation with extracellular dsRNA, is in accordance

273 with previous findings and is most likely due to the lack of surface expression of TLR3 (51). In

274 addition, a 12h infection of CEK cells with Sindbis, IPNV or Rift Valley Fever Virus clone 13 (RVFV

275 Cl13) induced a clear transcription of *Ifnβ* (Fig. 3C). These results suggest that delayed expression

276 of *Ifnβ* is a specific feature of IBV infection and not an intrinsic characteristic of chicken cells.

277

278 **The intracellular pattern recognition receptor MDA5 is the primary sensor of IBV**

279 In general, dsRNA has been shown to be the canonical inducer of *Ifnβ* during infection with *Alpha-*

280 and *Betacoronaviruses* (16, 50). To determine which pattern recognition receptor (PRR) would be

281 involved in sensing (ds)RNA of the *Gammacoronavirus* IBV, leading to subsequent *Ifnβ*

282 transcription, we first examined the possibility that IBV-(ds)RNA could be sensed extracellularly by,

283 for example, cell-surface receptors. To investigate this, CEK cells were infected with IBV M41 in the

284 presence of RNase A and *Ifnβ* expression was analysed. As a positive control, CEK cells were

285 stimulated with pI:C in the presence or absence of RNase A. The IFN response to pI:C was greatly

286 inhibited by addition of RNase A, which had no effect on *Ifnβ* levels induced by infection with IBV

287 M41 (Fig. 4A). These data suggest that *Ifnβ* upregulation during the late stage (>18 hpi) of IBV

288 infection could be the result of sensing of IBV-(ds)RNA by an intracellular rather than an

289 extracellular pattern recognition receptor. This is consistent with our observation that IBV infection

290 can be detected by DF-1 cells, which show only a marginal upregulation of *Ifnβ* transcription in

291 response to extracellular dsRNA (see Fig. 3B). In general, dsRNA can be recognised by membrane-

292 bound TLR3 and cytosolic RLRs such as MDA5 and RIG-I. Genome mining strongly indicates that

293 chickens do not express a *RIG-I* homologue (52), leaving TLR3 and MDA5 as the two PRRs

294 potentially involved in dsRNA sensing. Silencing of *Mda5*, but not *Tlr3*, in DF-1 cells resulted in a

295 70% decrease in *Ifnβ* transcription (Fig. 4B). Similar results were obtained with an *Ifnβ*-luc DF-1

296 reporter cell line in which silencing of *Mda5*, but not *Tlr3*, resulted in a 70% decrease in luciferase

297 activity by the reporter cells (Fig. 4C). Because no antibody against chicken MDA5 is currently

298 available for protein detection, successful knockdown was evaluated using RT-qPCR demonstrating

299 a silencing efficiency for both *Tlr3* and *Mda5* of approximately 60% (data not shown). Replication of

300 IBV at the investigated time point was not affected by knockdown of neither *Tlr3* nor *Mda5*, as

301 measured by both virus titre and intracellular IBV total RNA (Fig. 4D). These results indicate that

302 MDA5 is the primary PRR responsible for sensing *Gammacoronavirus* IBV-(ds)RNA in chicken cells.

303

304 **Early accumulation of dsRNA in IBV-infected cells does not result in early induction of**

305 ***Ifnβ***

306 Having assessed that chicken cells can indeed promptly respond to stimulation with dsRNA (Fig. 3)

307 and having identified MDA5 as the primary sensor involved in the detection of IBV (Fig. 4), we

308 investigated whether there would be a temporal difference between IBV-induced accumulation of

309 dsRNA and the upregulation of *Ifnβ* transcription in CEK cells. Indeed, dsRNA could clearly be

310 detected, even at low MOI of 0.01, by 12 hpi (Fig. 5A). In contrast, *Ifnβ* levels at this time point

311 remained low (Fig. 5B) even in cell cultures infected at higher MOIs of 1 or 10 and despite the

312 increased abundance of dsRNA. To further investigate the time lag between early accumulation of

313 dsRNA and late *Ifnβ* expression, we performed a time course analysis. Foci of dsRNA could be

314 detected as early as 3 hours post-infection only in IBV-infected cells (Fig. 5C, inset 3hpi) indicating

315 that dsRNA starts accumulating very early in IBV-infected cells but apparently only leads to late

316 (>18 hpi) *Ifnβ* transcription.

317 Primary CEK cells consist of a heterogeneous mix of cell types. Even at high MOI, IBV M41 infects

318 only ~70% of the cells, indicating that not all cells are permissive to IBV M41 infection. In order to

319 assess whether the time lag between accumulation of dsRNA and *Ifnβ* expression could be due to

320 the induction of *Ifnβ* in bystander rather than IBV-infected cells, we used RNA fluorescent *in situ*

321 hybridisation to visualise *Ifnβ* mRNA in IBV-infected CEK cell cultures (Fig. 5D). At 12 hpi and low

322 MOI (0.1), with most cells showing clear foci of dsRNA, none of the IBV-infected cells displayed an

323 accumulation of *Ifnβ* mRNA. At 12 hpi and a higher MOI, a few cells stained positive for *Ifnβ* mRNA

324 and only later, at 24 hpi, did most IBV-infected cells also stain positive for *Ifnβ* mRNA, the kinetics

325 of which closely following that observed in Figure 5A. In all cases, detection of *Ifnβ* mRNA was

326 restricted to cells that contained dsRNA. Altogether our data shows that IBV-infected, but not

327 adjacent uninfected cells, upregulate *Ifnβ* transcription in response to IBV infection. The significant

328 time lag between accumulation of dsRNA and *Ifnβ* transcription further suggests the presence of a

329 mechanism adopted by IBV to circumvent the onset of an IFN response.

330

331 **Accessory proteins 3a and 3b regulate IFN transcription and protein production**

332 To investigate whether the accessory proteins of IBV might play a role in the observed delay in *Ifnβ*

333 transcription, we infected CEK cells with IBV scAUG3ab and scAUG5ab null viruses and the parental

334 Beau-R virus (scAUG viruses possess a scrambled AUG start codon resulting in transcription but not

335 translation of either ORFs 3a and 3b or 5a and 5b (36, 37)). Infection with the scAUG3ab, but not a

336 scAUG5ab null virus resulted by 24 hpi in increased upregulation of *Ifnβ* expression (Fig. 6A).

337 Indicating that either one, or a combination of, accessory proteins 3a and 3b play a role in down

338 regulating *Ifnβ* transcription. The difference in *Ifnβ* transcription between the scAUG3ab and the

339 parental (Beau-R) virus could not be ascribed to differences in kinetics of virus replication, as all

340 viruses displayed similar growth kinetics until 24 hpi (Fig. 6B). To determine whether 3a, 3b or

341 both accessory proteins are involved in the observed down regulation of the IFN response, we

342 quantified *Ifnβ* transcription and IFN protein production in CEK cells infected with scAUG3a,

343 scAUG3b and scAUG3ab mutant viruses, and compared the values observed in cells infected with

344 Beau-R (Fig. 6C and 6D). Infection with all mutant viruses led to an increased transcription of *Ifnβ*

345 when compared to the Beau-R (Fig. 6C), indicating that the presence of either one of the two

346 accessory proteins is sufficient to limit *Ifnβ* transcription. The kinetics of *Ifnβ* transcription in

347 response to AUG3a/b differs between Fig. 6A and Fig. 6C. In Fig 6C there is a significant difference

348 in *Ifnβ* transcription between AUG3a/b and Beau-R, which is absent in Fig 6A. This difference can

349 probably be attributed to variation in the kinetics of *Ifnβ* transcription between primary CEK cells

350 isolated from embryos originating from different flocks. Nonetheless, this difference does not affect

351 the conclusion that knockout of 3a and 3b leads to an increase in transcription of *Ifnβ*.

352 No significant differences in IFN protein production were observed between cells infected with the

353 Beau-R and the scAUG3ab double null virus, except at 36 hpi. However, infection with scAUG3b

354 virus led to an increase in IFN protein levels, whereas infection with the scAUG3a virus led to a

355 decrease in IFN when compared to both Beau-R and scAUG3ab double null virus (Fig. 6D). Taken

356 together, these results indicate that accessory proteins 3a and 3b both play a role in the inhibition

357 of *Ifnβ* transcription but have distinct and opposing effects on protein production. Accessory protein

358 3b seems to be involved in limiting IFN protein activity whereas 3a is involved in promoting it.

359

360 **Signalling of non-self dsRNA remains intact in IBV-infected cells**

361 Since IBV showed the intrinsic ability to delay *Ifnβ* transcription in several cell types (Fig. 1 and

362 Fig. 2) even in the presence of high levels of intracellular dsRNA (Fig. 5), we investigated the

363 ability of IBV to interfere with sensing of non-self dsRNA by TLR3 or MDA5. We infected CEK cells

364 with IBV M41 and subsequently used extracellular poly I:C to trigger TLR3 signalling (Fig. 7A).

365 Stimulation with pI:C alone led to a significant increase in *Ifnβ* transcription whereas stimulation

366 with pI:C following an infection with IBV led to an enhanced increase in *Ifnβ* transcription in an

367 MOI-dependent manner. These results indicated that IBV infection does not interfere with TLR3-

368 mediated *Ifnβ* transcription, on the contrary IBV infection appears to result in a synergistic

369 activation of the TLR3 pathway triggered by pI:C. Next, we investigated whether IBV infection

370 could interfere with MDA5-mediated transcription of *Ifnβ*. Although transfection of pI:C into the

371 intracellular compartment is a commonly used ligand of MDA5, this method induced very little

372 transcription of *Ifnβ* in primary CEK cells, because of low transfection efficiency (data not shown).

373 As an alternative route to stimulate MDA5 in primary chicken cells, we investigated the use of

374 either RVFV Cl13 or IPNV, that induce *Ifnβ* transcription in CEK cells (Fig 3C). RVFV Cl13 is a (-)

375 ssRNA virus with a truncated IFN antagonist (53), for which RIG-I, but not by MDA5 or TLR3, was

376 previously shown to be the most likely PRR in mammalian cells (54, 55). Since chickens, as

377 opposed to most mammals, do not have a *RIG-I* homologue, the most likely PRR for RVFV in CEK

378 cells would be MDA5. IPNV is a birnavirus with a dsRNA genome that naturally infects salmonids

379 but has been shown to enter but not replicate in cells of warm-blooded animals (56). To date, the

380 PRR responsible for sensing IPNV dsRNA has not been described. Knockdown experiments in DF-1

381 *Ifnβ*-luc reporter cells, using siRNAs against chicken MDA5 or TLR3, revealed that MDA5, but not

382 TLR3, is the prime PRR for IPNV (Fig. 7B). These findings were confirmed using MEFs (mouse

383 embryo fibroblasts) from knockout mice deficient in expression of either MDA5, RIG-I or the

384 downstream adaptor protein MAVS. Here, knockout of either MDA5 or MAVS abrogated sensing of

385 IPNV as shown by a strong reduction of *Ifnβ* transcription, whereas knockout of RIG-I did not (Fig.

386 7C). Both IPNV and RVFV Cl13 were subsequently used to investigate whether IBV infection could

387 interfere with MDA5-mediated transcription of *Ifnβ* in CEK cells.

388 Using quantification of *Ifnβ* transcription by RT-qPCR as read out, we could show that IBV infection

389 does not interfere with MDA5-mediated signalling of IPNV (Fig. 7D) or RVFV Cl13 (Fig. 7E), in fact

390 it had a synergistic effect on *Ifnβ* transcription as previously observed for TLR3-mediated signalling

391 (Fig. 7A). Similar results were obtained when stimulating IBV-infected DF-1 cells with IPNV or

392   t[pI:C] (Fig. 7F), indicating that the observed synergistic effect is not specific to CEK cells. Taken

393   together, IBV infection very efficiently prevents sensing of IBV (ds)RNA, but our results indicate

394   that it does not interfere with sensing and downstream signalling of other non-self (ds)RNA ligands.

395

396   **DISCUSSION**

397   In this study we performed a comprehensive analysis of the kinetics of IBV infection in avian cells

398   and studied the mechanisms by which IBV interferes with the onset of the type I IFN response. We

399   show that infection with the *Gammacoronavirus* IBV leads to a considerable activation of the type I

400   IFN response, albeit delayed with respect to the peak of viral replication and accumulation of viral

401   dsRNA. Using an siRNA knockdown approach we show that MDA5 is the main receptor involved in

402   the induction of *Ifnβ* expression during IBV infection. We present evidence that IBV accessory

403   proteins 3a and 3b play a role in the modulation of the delayed IFN response, by regulating

404   interferon production both at the transcriptional as well as translational level. In addition, we show

405   that although IBV alone effectively prevents *Ifnβ* induction in IBV-infected cells, it does not block

406   *Ifnβ* induction upon stimulation of IBV-infected cells with other RIG-I, MDA5 or TLR3 ligands. To

407   our knowledge, this study provides the most comprehensive analysis of the interplay between a

408   *Gammacoronavirus* and the avian type I IFN response.

409

410   Much of our current knowledge about the interaction of coronaviruses with the innate immune

411   response (reviewed in (57)) comes from studies in mice and mouse cells using mouse hepatitis

412   virus (MHV). MHV activated IFN production only in specific cell types and an efficient IFN response

413   was only mounted by plasmacytoid dendritic cells (58), bone marrow derived macrophages (10,

414   59) and oligodendrocytes (10). In a recent study on SARS-CoV and MERS-CoV in an epithelial lung

415   cell line, ISGs started to be upregulated at 12 hpi (60), when virus titres were already reaching

416   their maximum. The kinetics of IFN response observed in our study are in line with aforementioned

417   studies, however it must be noted that in most cell types, infection with *Alpha* or *Betacoronaviruses*

418   induced very little, if any, *Ifnβ* transcription (8-13, 49). This suggests that all coronaviruses are

419   able to modulate the activation of the type I IFN response.

420

421   We found that IBV infection is detected by various chicken cell types, but until now it was unknown

422   which PRR was involved. MHV has been shown to be detected by MDA5 and not RIG-I or TLR3 in

423   brain macrophages (50), by both MDA5 and RIG-I in an oligodendrocyte derived cell line (10) and

424   by TLR7 in plasmacytoid dendritic cells (58). Analysis of the chicken genome suggests that chicken

425   lack a *RIG-I* homologue (52), and basal expression of *Tlr7* was found to be very low in CEK cells

426   (data not shown). We therefore silenced the remaining candidate RNA sensors MDA5 and TLR3,

427   and were able to show that MDA5, but not TLR3, is involved in the sensing of IBV. Silencing of

428   *Mda5* did not lead to an increase in replication of IBV, suggesting that IBV might have developed

429   strategies to counteract the activated IFN response.

430

431   We recently reported membrane rearrangements in chicken cells infected with IBV (48), similar to

432   those found in cells infected with *Betacoronaviruses*. In theory, the formation of intracellular

433   membrane rearrangements might partly explain the discrepancy observed in the kinetics of dsRNA

434   accumulation and *Ifnβ* upregulation. Indeed, for SARS-CoV it has been shown that virus-induced

435   double membrane vesicles (DMVs) contain dsRNA (14), suggesting that coronaviruses might

436   exploit membrane structures to shield dsRNA from recognition by host PRRs (61). However, the

437   kinetics of *Ifnβ* transcription were not investigated in these studies. The presence of coronavirus-

438   induced DMVs has been demonstrated as early as 2 hpi in SARS-CoV-infected cells (14). Although

439   we did not demonstrate the presence of DMVs in IBV-infected chicken cells at time points earlier

440   than 7 hpi (48), it is likely that DMVs could also be present at earlier time points. As such, the

441   timing of DMV formation in coronavirus-infected cells could suggest that membrane

442   rearrangements play a role in the delayed activation of the IFN response by shielding dsRNA from

443   cellular PRRs.

444

445   In addition to membrane rearrangements, coronavirus-encoded proteins, including numerous

446   accessory genes, have been shown to interfere with the type I IFN response pathway (reviewed in

447   (17, 18). To investigate the possible role of IBV accessory proteins in the regulation of the IFN

448   response we made use of our previously constructed mutant IBV Beau-R viruses that do not

449   express either one or more of the four accessory proteins 3a, 3b, 5a and 5b. Previously we have

450   demonstrated the accessory genes of IBV are not essential for replication (36, 37). In the present

451   study we show that infection of CEK cells with 3a or 3b null viruses as well as a 3a/3b double null

452   virus led to increased *Ifnβ* transcription compared to Beau-R. Because the kinetics of *Ifnβ*

453   transcription of 3a, 3b and 3a/3b null viruses are comparable to the parental virus, we conclude

454   that, 3a and 3b are probably not responsible for the delay in *Ifnβ* transcription, suggesting that IBV

455   utilises additional strategies to delay transcription of *Ifnβ*. Apart from their effect on *Ifnβ*

456   transcription, 3a and 3b seem to have opposing effects on IFN protein production by IBV infected

457   cells. Infection with the 3b null virus resulted in increased IFN production whereas infection with

458   the 3a null virus resulted in reduced IFN levels compared to the Beau-R virus. Together with the

459   observation that IFN production induced by the 3a/3b double null virus is comparable to that

460   induced by Beau-R virus, our data suggests that accessory proteins 3a and 3b antagonise each

461   other to tightly regulate IFN production (Fig. 6B).

462

463   Using the eukaryotic linear motif server (62), we identified a Protein phosphatase 1 (PP1)-binding

464   [17]KISF[20] domain in the IBV 3b protein sequence. The canonical PP1-binding motif is

465   [R/K][V/I/L]X[F/W], in which x can be any amino acid except proline (63). Interestingly,

466   *Alphacoronavirus* TGEV accessory protein 7 (TGEV-7) has been shown to bind PP1 via a binding

467   motif similar to that found in IBV 3b (64). Similar to IBV scAUG3b, infection with TGEV-Δ7 led to

468   increased mRNA and protein levels of IFNβ (65). The fact that both TGEV-7 and IBV 3b contain a

469   PP1 binding domain indicates that interaction with PP1 could be a common strategy of

470   coronaviruses to inhibit the host innate immune response. The mechanism by which interaction of

471   coronavirus accessory proteins with PP1 counteracts the innate immune response still needs to be

472   determined. One clue might come from the PP1-binding domain of Measles virus V, which was

473   recently shown to be essential for inhibition of MDA5 signalling (66, 67). Measles V protein binds

474   PP1 and inhibits dephosphorylation of MDA5, which is required for activation and subsequent

475   signalling by MDA5. Motif analysis for IBV 3a protein did not reveal the presence of relevant

476   motives that might explain the observed activity of 3a on IFN regulation. We conclude that both

477   accessory proteins 3a and 3b limit *Ifnβ* transcription but have distinct and opposing effects on

478   protein production. Whilst 3a seems to promote IFN production, 3b seems to be involved in limiting

479   IFN protein production, possibly through a similar mechanism as described for protein 7 of TGEV.

480   The fact that IBV 3a and 3b have opposing roles in regulating IFN production indicates that CoV's

481   tightly regulate IFN production to balance their own survival with that of the host. This hypothesis

482   is supported by the observation that field isolates lacking 3a and 3b display reduced virulence *in*

483   *vitro* as well as *in vivo* (68). Elucidation of the exact mechanisms of action of 3a and 3b will be the

484   subject of further investigation.

485

486

487   To investigate whether IBV interferes with a general sensing of (ds)RNA ligands or downstream

488   signalling that leads to *Ifnβ* transcription, we stimulated IBV-infected cells with TLR3, RIG-I, and

489   MDA5 ligands. Surprisingly, we found that infection with IBV did not reduce *Ifnβ* transcription but

490   rather increased *Ifnβ* levels upon stimulation with these PRR ligands. Similar to IBV, MHV has been

491   shown unable to inhibit expression of *Ifnβ* induced by either t[pI:C] or Sendai virus (69, 70), but in

492   these studies no synergistic effect was observed. Currently, we can only speculate about the cause

493   of this synergistic effect. It appears that IBV infection 'arms' the *Ifnβ* induction pathway, without

494   actually triggering it, possibly by enhancing the activity of one or more components of the pathway

495   leading to Ifnβ upregulation. One possibility is that IBV-proteins interact with host-proteins that

496   regulate this pathway through ubiquitination and phosphorylation (reviewed in (71)). The fact that

497   stimulation with either TLR3 or MDA5 ligands resulted in exacerbated transcription of *Ifnβ* indicates

498   that IBV influences a component which is downstream of both MDA5 and TLR3.

499

500   Taken together, our study provides the first comprehensive analysis of host-virus interactions of a

501   *Gammacoronavirus* with the avian innate immune response. We show that the *Gammacoronavirus*

502   IBV, induces activation of the type I IFN response in primary chicken renal cells, tracheal epithelial

503   cells and in a chicken cell line. We show that activation of the IFN response is dependent on MDA5

504   but is delayed with respect to the peak of virus replication. We demonstrate that *Ifnβ* transcription

505   is restricted to IBV-infected, dsRNA-containing cells and provide evidence that accessory proteins

506   3a and 3b of IBV are involved in regulating transcription as well as protein production of type I IFN.

507

518

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699

700 **FIGURE LEGENDS**

701

702 **FIG 1. IBV infection delays *Ifnβ* upregulation.**

703 Chicken embryo kidney (CEK) cells were infected with IBV M41 at an MOI of 0.1. (A) Replication of

704 IBV was quantified by titration in cell culture supernatants of infected cells; in a parallel

705 experiment, intracellular IBV RNA was quantified using RT-qPCR. (B) *Ifnβ* mRNA levels were

706 determined using RT-qPCR and IFN protein levels using a chicken IFN-specific *mx*-luc cell-based

707 bioassay, respectively. (C) Expression of genes involved in the antiviral response. All gene

708 expressions were calculated as fold changes relative to uninfected control cells and normalised

709 against an external reference gene (luciferase). For IBV total RNA, fold changes were calculated

710 relative to C_t 30. Depicted are the results of a representative experiment out of three independent

711 experiments.

712

713 **FIG 2. Delayed induction of *Ifnβ* transcription is independent of cell type or IBV strain.**

714 (A) Epithelial cells from adult chicken trachea were infected with IBV M41 at MOI 0.1. (B)

715 Fibroblast DF-1 cells were infected with IBV Beau-R at MOI 0.1. (C) CEK cells were infected with

716 IBV strains M41, QX and It02, at an MOI 0.1. Intracellular IBV total RNA (open diamonds) and *Ifnβ*

717 mRNA (bars) are depicted as fold changes as assessed by RT-qPCR. Gene expression of *Ifnβ* was

718 calculated as fold changes relative to uninfected control cells and normalised against an external

719 reference gene (luciferase).. For IBV total RNA, fold changes were calculated relative to C_t 30. Error

720 bars indicate standard deviation.

721

722 **FIG 3. Chicken cells have the intrinsic ability to respond rapidly to dsRNA.**

723 (A) CEK cells were seeded in 24 well plates and 48 hours later stimulated with extracellular poly

724 I:C for the indicated times. (B) DF-1 cells were infected with IPNV, a non-replicating dsRNA virus,

725 or stimulated with extracellular pI:C or transfected pI:C (t[pI:C]). Four hours later, *Ifnβ* fold

726 changes were determined by RT-qPCR. Bars represent the mean (plus standard deviation) of

727 triplicate wells from a representative experiment. Asterisks indicate significant differences (P<0.01)

728 with respect to the non-stimulated control as assessed by one-way ANOVA followed by a Bonferroni

729 post-hoc test. (C) CEK cells were infected with IBV M41 (MOI 1), IBV Beau-R, (MOI 1), Sindbis-GFP

730 (MOI 1), IPNV (MOI 50) and RVFV Cl13 (MOI 5). Depicted are *Ifnβ* fold changes at 12 hpi relative

731 to uninfected control cells as assessed by RT-qPCR.

732

FIG 4. MDA5, and not TLR3, is the prime sensor of IBV.

(A) CEK cells were infected with IBV M41 for 24 hours, in the presence or absence of RNase A. *Ifnβ* expression was analysed by RT-qPCR. Stimulation with pI:C in the presence or absence of RNase A was included as a positive control. (B,D) DF-1 cells and (C) DF-1 Ifnβ-luc reporter cells were transfected with siRNAs against *Tlr3*, *Mda5* or a control siRNA and 48 hours later infected with IBV M41 (MOI 0.1). (B) *Ifnβ* mRNA, (C) Ifnβ-luciferase activity, and (D) IBV titres and intracellular RNA were analysed 18 hpi. Bars represent the mean (plus standard deviation) of triplicate wells from a representative experiment. Asterisks indicate significant differences (P<0.01) with respect to the non-RNaseA-treated control (A) or to the siRNA control (B-C), as assessed by one-way ANOVA followed by a Bonferroni post-hoc test.

743

FIG 5. Early accumulation of dsRNA in IBV-infected cells does not result in early induction of *Ifnβ*

CEK cells were infected with IBV M41 or IBV Beau-R at the indicated MOIs. At time point 12 hpi (A) dsRNA was visualised in M41-infected cells using an antibody against dsRNA. (B) Expression of *Ifnβ* mRNA was analysed by RT-qPCR. (C) CEK cells were infected with IBV M41 and accumulation of dsRNA was visualised at the indicated time post infection. (D) RNA fluorescent *in situ* hybridisation of *Ifnβ* mRNA in IBV M41-infected CEK cells. Open arrowheads indicate cells that contain dsRNA and no Ifnβ mRNA. Solid white arrowheads indicate cells that contain both dsRNA and *Ifnβ* mRNA.

752

FIG 6. Accessory proteins 3a and 3b are involved in regulation of IFN transcription and protein production.

(A) CEK cells were infected with IBV Beau-R 3a/3b (scAUG3ab) or 5a/5b (scAUG5ab) null viruses (MOI 0.1). *Ifnβ* levels were determined using RT-qPCR. (B-D) CEK cells were infected with scAUG3a, scAUG3b or scAUG3ab null IBV viruses (MOI 0.1). In the same cultures (B) *Ifnβ* mRNA, (C) virus titres and (D) type I IFN protein were quantified. Bars represent the mean (plus standard deviation) of triplicate wells from a representative experiment. Significant differences (P<0.01) relative to the Beau-R virus at the same timepoint (*) or between the indicated bars (#) as assessed by a two-way ANOVA followed by a Bonferroni post-hoc test.

762

763 **FIG 7. Signalling of non-self RNA remains intact in IBV-infected cells**

764 (A) CEK cells were infected with IBV M41 for 3 hours and stimulated with extracellular poly I:C (50

765 μg/ml) for an additional 3 hours after which *Ifnβ* transcription was analysed by RT-qPCR. (B) DF-1

766 Ifnβ-luc reporter cells were transfected with siRNAs against *Tlr3*, *Mda5* or a control siRNA and 48

767 hours later infected with IPNV (MOI 50); at 6 hpi luciferase activity was quantified. (C) Knockout

768 (KO) and wild-type (wt) MEFs were infected with IPNV (MOI 50) for 8 hours. (D) CEK cells were

769 infected with IBV M41 (MOI 10) for 6h and super-infected with IPNV or UV-inactivated IPNV (MOI

770 50) for an additional 6 h. (E) CEK cells were co-infected with IBV M41 (MOI 5) and RVFV clone 13

771 (MOI 5) and sampled at 6 hpi. (F) DF-1 cells were infected with IBV Beau-R (MOI 1) for 3 h and

772 super-infected with IPNV (MOI 50) or transfected with pI:C (t[pI:C], 500 ng/well) for an additional

773 4 h. (C-F) *Ifnβ* levels were quantified by RT-qPCR. Bars represent the mean (plus standard

774 deviation) of triplicate wells. Significant differences (P<0.01) are indicated by (*) as assessed by

775 one-way ANOVA followed by a Bonferroni post-hoc test.

gene	kind	sense	sequence (5'-3')	Accession nr.	reference
TLR3	siRNA	S	UCGAAUACUUGGCUUUAAA	NM_001011691	
		AS	UUUAAAGCCAAGUAUUCGA		
ctrl	siRNA	S	AGGUAGUGUAAUCGCCUUG	--	
		AS	CAAGGCGAUUACACUACCU		
MDA5	siRNA	S	ACACUGGUAUCAAGUUAUU	GU570144	
		AS	AAUAACUUGAUACCAGUGU		
IFNβ	RQ primer	FW	GCTCTCACCACCACCTTCTC	ENSGALT00000039477	
		RV	GCTTGCTTCTTGTCCTTGCT		
IFNα	RQ primer	FW	ATCCTGCTGCTCACGCTCCTTCT	XM_004937096	40
		RV	GGTGTTGCTGGTGTCCAGGATG		
IRF3	RQ primer	FW	CAGTGCTTCTCCAGCACAAA	NM_205372	
		RV	TGCATGTGGTATTGCTCGAT		
IRF1	RQ primer	FW	CAGGAAGTGGAGGTGGAGAA	ENSGALG00000006785	
		RV	TGGTAGATGTCGTTGGTGCT		
TLR7	RQ primer	FW	TTCTGGCCACAGATGTGACC	NM_001011688	40
		RV	CCTTCAACTTGGCAGTGCAG		
TLR3	RQ primer	FW	TCAGTACATTTGTAACACCCCGCC	NM_001011691	40
		RV	GGCGTCATAATCAAACACTCC		
MDA5	RQ primer	FW	TGGAGCTGGGCATCTTTCAG	GU570144	
		RV	GTTCCCACGACTCTCAATAACAGT		
Mx	RQ primer	FW	TTGTCTGGTGTTGCTCTTCCT	ENSGALT00000025999	
		RV	GCTGTATTTCTGTGTTGCGGTA		
OAS	RQ primer	FW	CACGGCCTCTTCTACGACA	NM_205041	41
		RV	TGGGCCATACGGTGTAGACT		
IL8	RQ primer	FW	TTGGAAGCCACTTCAGTCAGAC	NM_205498	41
		RV	GGAGCAGGAGGAATTACCAGTT		
PKR	RQ primer	FW	CCTCTGCTGGCCTTACTGTCA	NM_204487	42
		RV	AAGAGAGGCAGAAGGAATAATTTGCC		
ADAR	RQ primer	FW	TGTTTGTGATGGCTGTTGAG	AF403114	
		RV	AGATGTGAAGTCCGTGTTG		
ISG12	RQ primer	FW	TAAGGGATGGATGGCGAAG	NM_001002856	
		RV	GCAGTATCTTTATTGTTCTCAC		
MHC-I	RQ primer	FW	CTTCATTGCCTTCGACAAAG	NM_001031338	41
		RV	GCCACTCCACGCAGGT		
IFNAR2	RQ primer	FW	GCTTGTGTTCGTCAGCATT	ENSGALT00000036778	41
		RV	TTCGCAATCTTCCAGTTGT		
IBV-N	RQ primer	FW	GAAGAAAACCAGTCCCAGA	AY851295	
		RV	TTACCAGCAACCCACAC		
Luciferase	RQ primer	FW	TGTTGGGCGCGTTATTTATC	X65316	
		RV	AGGCTGCGAAATGTTCATACT		

Fig.1

A

B

C

Fig.2

A trachea cells

B DF-1 cells

C CEK cells

Fig.3

A

CEK

pI:C (µg/ml)
☐ 1
■ 10
■ 100

Ifnβ mRNA

400
300
200
100
0

1   3   6

hours post treatment

B

DF-1

Ifnβ mRNA

150
100
50
0

IPNV   pI:C   t[pI:C]

*   *

C

CEK

Ifnβ mRNA

600
400
200
0

M41   Beau-R   Sindbis   IPNV   RVFV Cl13

Fig.4

Fig. 5

A

B

C

Inset 3 hpi

D

Fig.6

Fig.7

A CEK

$Ifn\beta$ mRNA

800, 600, 400, 200, 0

IBV, pI:C, IBV + pI:C (MOI 1), IBV + pI:C (MOI 10)

*

*

B DF-1

Ifnβ -luc activity (×10⁴ RLU)

5, 4, 3, 2, 1, 0

siCtrl, siTLR3, siMDA5

*

*

C MEFs

$Ifn\beta$ mRNA

10^4, 10^3, 10^2, 10^1, 10^0

wt, KO

RIG-I, MDA5, MAVS

*

*

D CEK

$Ifn\beta$ mRNA

4000, 3000, 2000, 1000, 0

IPNV, IBV, IPNV (UV)

+, +, −, −
−, +, −, +
−, −, +, +

*

*

E CEK

$Ifn\beta$ mRNA

500, 400, 300, 200, 100, 0

IBV, RVFV, IBV + RVFV

F DF-1

$Ifn\beta$ mRNA

30, 20, 10, 0

IBV, IPNV, IBV + IPNV, t[pI:C], IBV + t[pI:C]