1 **Suppression of MDA5-mediated antiviral immune responses by NSP8 of SARS-CoV-2**

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4 Running title: SARS-CoV-2 NSP8 suppresses MDA5-mediated antiviral responses

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Abstract

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27   Melanoma differentiation-associated gene-5 (MDA5) acts as a cytoplasmic RNA sensor to detect viral

28   dsRNA and mediates type I interferon (IFN) signaling and antiviral innate immune responses to infection by

29   RNA viruses. Upon recognition of viral dsRNA, MDA5 is activated with K63-linked polyubiquitination and then

30   triggers the recruitment of MAVS and activation of TBK1 and IKK, subsequently leading to IRF3 and NF-κB

31   phosphorylation. Great numbers of symptomatic and severe infections of SARS-CoV-2 are spreading

32   worldwide, and the poor efficacy of treatment with type I interferon and antiviral agents indicates that SARS-

33   CoV-2 escapes from antiviral immune responses via an unknown mechanism. Here, we report that SARS-

34   CoV-2 nonstructural protein 8 (NSP8) acts as an innate immune suppressor and inhibits type I IFN signaling

35   to promote infection of RNA viruses. It downregulates the expression of type I IFNs, IFN-stimulated genes and

36   proinflammatory cytokines by binding to MDA5 and impairing its K63-linked polyubiquitination. Our findings

37   reveal that NSP8 mediates innate immune evasion during SARS-CoV-2 infection and may serve as a potential

38   target for future therapeutics for SARS-CoV-2 infectious diseases.

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Importance

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42   The large-scale spread of COVID-19 is causing mass casualties worldwide, and the failure of antiviral

43   immune treatment suggests immune evasion. It has been reported that several nonstructural proteins of

44   severe coronaviruses suppress antiviral immune responses; however, the immune suppression mechanism

45   of SARS-CoV-2 remains unknown. Here, we revealed that NSP8 protein of SARS-CoV-2 directly blocks the

46   activation of the cytosolic viral dsRNA sensor MDA5 and significantly downregulates antiviral immune

47   responses. Our study contributes to our understanding of the direct immune evasion mechanism of SARS-

48   CoV-2 by showing that NSP8 suppresses the most upstream sensor of innate immune responses involved in

49   the recognition of viral dsRNA.

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51   **Key words:**

52   SARS-CoV-2, NSP8, MDA5, interferon, immune evasion

Introduction

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) is an emerging severe coronavirus that is currently causing a global outbreak of Coronavirus Disease 2019 (COVID-19). It has infected more than twenty million patients and caused more than two-thirds of a million deaths. The number of patients and deaths are still rapidly increasing; however, no effective therapy, vaccine or cure are available. Knowledge of SARS-CoV-2 infection, pathogenesis, diseases and treatments remains limited, and novel targets of therapeutics and drug development are quite urgently needed.

Analysis of clinical data from different SARS-CoV-2 patients has shown that excessive cytokine release, known as a "cytokine storm", is closely related to disease severity [1-3]. To characterize the host immune and inflammatory responses in COVID-19 patients, genome-wide RNA-sequencing analysis was performed, which indicated that the proportion of immune cells in the blood was reduced in patients who required non-ICU admission, with lower levels of *GCSF*, *CXCL10*/*IP-10*, *CCL2*/*MCP-1* and *CCL3*/*MIP-1A* detected. In addition, some anti-inflammatory cytokines, such as IL-10 and TGF-β, were found to be induced during SARS-CoV-2 infection [4]. Accordingly, compared with ICU care patients, non-ICU care patients had lower plasma levels of cytokines, including IL-2, IL-7, IL-10, GSCF, IP10, MCP1, MIP1A, and TNFα. Furthermore, mild cases of COVID-19 exhibited decreased plasma levels of IL2R and IL6 compared with severe cases, while an excessive inflammatory response was observed in dead cases [5,6]. These studies reported a correlation between the release of inflammatory cytokines and pathogenesis of SARS-CoV-2.

SARS-CoV-2 and other coronaviruses generate massive amounts of RNA products during their infection that are then recognized by host cytosolic RNA sensors, including retinoic acid-inducible gene I (RIG-I) and melanoma differentiation-associated gene-5 (MDA5) [7]. Activation of RIG-I and MDA5 triggers the formation of signalosomes that induce the expression of type I IFN and ISGs and the subsequent execution of an antiviral state within the cell [8]. To escape immune elimination and to survive and then replicate, coronaviruses, including SARS-CoV and MERS-CoV, have evolved strategies to inhibit or delay IFN production and responses. SARS-CoV encodes a set of accessory proteins, several of which target the innate immune response. Its nonstructural protein 1 (NSP1) binds to the 40S ribosome to inactivate translation and induces host mRNA degradation [9,10]; NSP15 was identified as an IFN antagonist to enhance immune evasion [11,12]; NSP16, a 2′O-

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80 methyltransferase (2′O-MTase), provides a cap structure at the 5′-end of viral mRNAs to evade MDA5

81 detection [13-15]; furthermore, open reading frame 6 (ORF6) also reduces IFN production [16]. In addition, ORF-

82 9b localizes to mitochondrial membranes to induce the degradation of MAVS, TRAF3 and TRAF6, severely

83 limiting host cell IFN production [17]. In addition, MERS ORF4b is involved in evasion of the innate immune

84 response by binding α-karyopherin proteins, leading to the inhibition of NF-κB nuclear translocation [18].

85 Considering the numerous existing asymptomatic infected populations, it is reasonable to suggest that

86 SARS-CoV-2 has evolved effective strategies to inhibit the host immune response, which is also a challenge

87 for preventing and treating COVID-19. Consistent with these observations, a recent study showed that NSP1

88 of SARS-CoV-2 shuts down host mRNA translation by binding to 40S and 80S ribosomes, effectively blocking

89 RIG-I-dependent innate immune responses [19]. However, the potential immune inhibitory mechanism mediated

90 by viral components of SARS-CoV-2 is not well known.

91 We performed a systematic screening and determined that SARS-CoV-2 NSP8 is a suppressor of the type

92 I immune response upon RNA viral infection. It decreases type I IFN production and ISG expression, by which

93 it interacts with the MDA5 CARD domain and then inhibits MDA5 K63-linked polyubiquitination and thus

94 terminates MDA5-mediated immune responses.

95

96 **Results**

97 **NSP8 inhibits viral RNA-related type I interferon and antiviral responses.**

98 To investigate a factor of SARS-CoV-2 that suppresses the type I IFN signaling pathway, we screened a

99 panel of viral nonstructural proteins (NSPs) that regulate the expression of interferon and inflammatory factors

100 using RT-PCR arrays. 293T cells were transfected with empty vector or NSP-expressing plasmids for 24 h.

101 The cells were left untreated or challenged with dsRNA analog poly(I:C) for 18 h or 24 h separately and then

102 harvested for analysis of gene expression. Several NSPs exhibited the inhibitory effects on immune and

103 inflammatory gene expression (Supplementary Fig.1). We found that NSP8 significantly downregulated the

104 expression of *TNF-α*, *IFN-β*, interferon-stimulated genes (ISGs) *IFIT1* and *IFIT2* and proinflammatory

105 cytokines *IL-6* and *CCL-20* (Fig.1a). Consistent with our results, NSP1 was previously reported to inhibit the

106 translation of type I interferon to evade the immune response, which validated our screening approach [9,19].

107   To confirm that the attenuation of the type I IFN response by NSP8 is correlated with a decrease in the

108   antiviral response, *NSP8*-overexpressing 293T cells were subsequently infected with vesicular stomatitis virus

109   tagged with enhanced GFP (VSV-eGFP). As analyzed by fluorescence microscopy and flow cytometry, NSP8

110   overexpression increased the percentage of GFP-positive cells with the extended response time compared

111   with empty vector-transfected cells (Fig.1b,c), suggesting that NSP8 promoted the efficiency of viral infection

112   and gene expression, hence favoring VSV-eGFP replication. These data demonstrated that NSP8 is an

113   inhibitory protein in type I IFN signaling and antiviral responses during SARS-CoV-2 infection.

114

115   **NSP8 decreases MAVS-dependent antiviral responses**.

116   To further understand whether the inhibitory effects of NSP8 are exerted on both the IRF3 and NF-κB

117   pathways, 293T cells were cotransfected with empty vector or IRF3 or p65 expressing plasmid and with NSP8-

118   expressing plasmid, followed by VSV infection for the indicated time points. By immunoblotting analysis to

119   detect the effect of NSP8 on the phosphorylation of TBK1 (pTBK1), the phosphorylation of IRF3 (pIRF3)

120   (Fig.2a), the phosphorylation of IKKα/β (pIKKα/β), the phosphorylation of p65 (p-p65) and IκBα (Fig.2b), we

121   found that in NSP8-overexpressing cells, pTBK1 level was four- to nine-fold lower than that in control cells

122   after VSV infection. As a downstream transcription factor that is phosphorylated and activated by TBK1, IRF3

123   phosphorylation was entirely blocked in NSP8-overexpressing cells (Fig.2a). Similarly, inhibition of IKKα/β

124   phosphorylation by NSP8 led to the stabilization of IκBα, and NF-κB signaling was strongly inhibited by NSP8

125   overexpression, as indicated by the decrease in p65 phosphorylation (Fig.2b). These results confirm that

126   NSP8 suppresses the activation of both IRF3 and NFκB and the upstream cascade of the type I IFN signaling

127   pathway.

128   To investigate whether NSP8 plays a role in the early step of the type I IFN signaling cascade, we detected

129   the inhibitory effect of NSP8 in MAVS-deficient vs. complemented cells. *MAVS* knockout HEK293T cells

130   (HEK293T[MAVS-/-]) were cotransfected with *NSP8*-expressing plasmid or empty vector and with *MAVS*-

131   expressing plasmid or empty vector and then stimulated with poly(I:C) for different times. As expected, the

132   expression of *TNF-α*, *IFN-β*, *IFIT1*, *IFIT2*, *IL-6* and *CCL-20* was not downregulated by NSP8 upon stimulation

133   (Fig.2c, black vs. white columns), while MAVS overexpression in HEK293T[MAVS-/-] cells significantly restored

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134   their induction upon stimulation, and NSP8 once again had suppressive effects on the expression of these

135   cytokines (Fig.2c, red vs. blue columns). Taken together, these results suggest that NSP8 suppresses MAVS-

136   dependent innate immune responses, probably by acting on either MAVS or upstream RNA sensors.

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138   **NSP8 interacts with MDA5 and suppresses MDA5 K63-linked polyubiquitination**

139   Since viral RNA of coronaviruses contains a methylated 5'-cap and 3'-polyA tail that is similar to cellular

140   mRNA, we assumed that NSP8 may preferentially regulate MDA5-mediated responses rather than RIG-I-

141   mediated responses that recognize 5'-pppRNA. Hence, we performed coimmunoprecipitation (co-IP) analysis

142   and found that NSP8 interacts with MDA5 (Fig.3a). Furthermore, we mapped the binding domain and

143   determined that its CARD domain is responsible for the NSP8 interaction (Fig.3b). As a result, MDA5-mediated

144   ISRE-luc activity was inhibited by NSP8 in a dose-dependent manner (Fig.3c). In addition, confocal

145   microscopy demonstrated that NSP8 tightly colocalized with MDA5 inside cells (Fig.3d). These results suggest

146   that NSP8 interacts with MDA5 and directly suppresses MDA5-mediated immune responses.

147   To further understand the molecular mechanism by which NSP8 interacts and interferes with MDA5, we

148   predicted the MDA5 CARD, NSP8 and K63-Ub tertiary structures with SWISS-MODEL, and then the predicted

149   structures were input into ZDOCK SERVER for simulation. Predicted docking models were processed in

150   PyMOL for visualization. Surprisingly, we found that NSP8 possesses a long α-helix (Supplementary Fig.2a),

151   which is tightly packed in the ravines formed by the two α-helixes of MDA5 CARDs. The random coil and a

152   short α-helix in the N terminus of NSP8 occupy the area or space that interacts with K63-Ub (Fig.3e and

153   Supplementary Fig.2b). Further calculation of vacuum electrostatics for this binding model demonstrated that

154   the contact area in the chain of MDA5 CARDs is positively charged, while the corresponding area in the chain

155   of NSP8 is negatively charged (Fig.3f and Supplementary Fig.2c), implying that there is a likely interaction of

156   these two structures. We further searched the polar contacts in the interface of the binding model with PyMOL

157   and found that 7 paired residues anchor with each other, one locates in the N-terminal coil of NSP8 while the

158   others are in the long α-helix (Fig.3g).Thus, computer-based molecular structural prediction and modeling

159   implies that NSP8 interacts with the MDA5 CARD domain probably through ionic interactions and dipolar

160   surfaces between the NSP8 and MDA5 CARD binding pockets.

161   Next, we sought to determine how NSP8 inhibits MDA5 activation. It is well documented that upon virus

162   infection, the MDA5 CARD domain undergoes K63-linked polyubiquitination and recruits MAVS to form a

163   signalosome [20]. The structural prediction of the NSP8-MDA5 CARD interaction showed that NSP8 may

164   interrupt this process since it interacts with MDA5 at its CARD domain and shields the binding area or space

165   for K63-ubiquitin linkage (Fig.3e, Supplementary Fig.2, and pdb file). The polyubiquitination of MDA5 was

166   analyzed in the presence or absence of NSP8 expression. The *MDA5*-expressing plasmid was cotransfected

167   into HEK293T cells with a WT-, K48-, or K63-linked ubiquitin-expressing plasmid, and an in vivo ubiquitination

168   assay showed that MDA5 WT- and K63-linked polyubiquitination were strongly inhibited (Fig.4a,c), while K48-

169   linked polyubiquitination was barely affected (Fig.4b). Thus, these results reveal that NSP8 interferes with the

170   MDA5-MAVS signalosome by inhibiting the K63-linked polyubiquitination of MDA5.

171

172   **NSP8 decreases the expression of antiviral immune and inflammatory genes**.

173   Next, we investigated whether NSP8 affects the downstream expression of immune and inflammatory genes

174   and cytokines. NSP8-expressing A549 cells were collected, and total RNA was subjected to RT-PCR array

175   analysis for immune and inflammatory gene expression (Primer listed in Supplementary Table.1). The

176   expression of the majority of cytokines, including *IL-1β*, *IL-2*, *IL-5*, *IL-6*, *IL-26*, *IL-33*, *IFN-β*, *IFIT1* and *IFIT2,*

177   was downregulated by NSP8 expression. The transcription of some pleiotropic chemoattractant cytokines,

178   such as *IL-16*, *IL-17A*, *IL-17F*, and *IL17C*, was also downregulated. Furthermore, a decreasing transcription

179   tendency was also observed for the inflammatory receptors *IL-1RI*, *IL-1RII*, *IL-2Rα*, and *IL18RII*; NK cell-

180   associated activation receptors, such as *NKp44*, *NKp46*, and *NKG2B*; and the trans-acting T-cell-specific

181   transcription factor *GATA3* (Fig.5a), indicating that the activation of T cells and NK cells was attenuated by

182   NSP8 through the suppression of these key factors. Although these decreased cytokines and receptors may

183   not be directly activated by IRF3 or NFκB, they could be regulated by downstream cytokines or other factors

184   derived from these two pathways. In contrast, the cytokine IL-2 and IFN-gamma suppression gene *FOXP3*

185   was significantly increased with NSP8 overexpression.

186   To further confirm that the downregulation of these immune and inflammatory cytokines and genes is

187   mediated by NSP8 under physiological conditions, A549 cells were transfected with an NSP8-expressing

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188 plasmid or empty vector and then stimulated with poly(I:C) mimicking viral RNA for the indicated times. The

189 inhibition of the expression of key cytokines and related genes was verified, and NSP8 negatively regulated

190 the expression of these immune and inflammatory genes (Fig.5b). Collectively, these results suggest that

191 NSP8 could strongly impair the expression of genes involved in antiviral immune and inflammatory responses.

192

193 **Discussion**

194 It has not been well investigated whether SARS-CoV-2 components have immunosuppressive functions, so

195 we are very interested in exploring the roles of SARS-CoV-2 proteins, especially the nonstructural protein

196 family (NSP), in viral immune evasion. In the present study, we screened a panel of NSPs and identified NSP8

197 as an immune suppressor of the type I IFN signaling pathway. We have shown that NSP8 overexpression

198 impaired type I IFN production and the gene expression of immune and inflammatory factors, including *TNF-*

199 *α*, *IFN-β*, *IFIT1*, *IFIT2*, *IL-6* and *CCL-20*. As a consequence, we observed that VSV-eGFP infection was

200 significantly increased in cells with NSP8 overexpression. The subsequent detection of phosphorylation of

201 kinases TBK1 and IKKα/β and substrates IRF3 and p65 provided evidence that both IRF3 and NFκB activity

202 were inhibited by NSP8. MAVS is an upstream regulator of the type I IFN signaling cascade and regulates

203 both pathways; hence, we overexpressed NSP8 in MAVS knockout HEK293T cells and observed that the

204 inhibitory function of NSP8 towards type I IFN and cytokines was completely abolished. Re-expressing MAVS

205 in MAVS KO HEK293T cells that also express NSP8 successfully restored the NSP8 inhibitory activity.

206 Consequently, a series of antiviral immune and inflammatory cytokines and related genes were further strongly

207 downregulated by NSP8 expression.

208 Herein, we speculated that NSP8 may act on RIG-I or MDA5, two upstream viral RNA sensors. Our results

209 showed that NSP8 directly interacted with MDA5 on its CARD domains, and MDA5-mediated type I IFN

210 signaling activities were strongly inhibited at the same time. Polyubiquitinated modification of MDA5 is crucial

211 for its antiviral responses, K48-linked polyubiquitination mediates MDA5 proteasomal degradation [21], and K63-

212 linked polyubiquitination mediates MDA5-induced type I IFN expression [20]. We speculated that NSP8 may

213 inhibit type I IFN signaling through the polyubiquitinated modification of MDA5. To test our hypothesis, we

214 determined the status of WT-, K48- and K63-linked polyubiquitination of MDA5 in the absence or presence of

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215 NSP8 and confirmed that WT- and K63-linked polyubiquitination were impaired by NSP8, while K48-linked

216 polyubiquitination was barely changed. We therefore conclude that under certain circumstances, NSP8

217 jeopardizes antiviral responses by impairing MDA5 K63-linked polyubiquitination.

218 Based on our existing experimental data, we propose a simple working model to illustrate how NSP8

219 negatively regulates innate immune responses by inhibiting MDA5 K63-linked polyubiquitination (Fig.5c).

220 Upon SARS-CoV-2 infection, cytosolic viral dsRNA is recognized by MDA5 and triggers type I IFN expression;

221 meanwhile, NSP8 is largely expressed and localized in the cytoplasm of host cells and then interacts with

222 MDA5 on its CARD domains to inhibit its K63-linked polyubiquitination, consequently negatively regulating

223 type I IFN signaling and antiviral immune responses, thus favoring viral infection and replication.

224 Interestingly, a recent study showed that in the carrier and intermediate host of coronaviruses—pangolin—

225 the MDA5 gene is mutated and dysfunctional [22], while RIG-I and TLR 3, 7 and 8 are conserved among three

226 different species. Considering the coexisting relationship between pangolins and coronaviruses and the

227 tolerance of pangolins for SARS-CoV-2 infection, this finding implied that coronaviruses may be inclined to

228 silence MDA5-mediated innate immunity because they could generate more drastic reactivity and injury upon

229 viral RNA recognition. Based on this observation and the findings of our present study, the suppressive

230 function of NSP8 in antiviral responses and its impairment of MDA5 activation have been identified,

231 representing a direct immune evasion mechanism of the recognition of viral dsRNA.

232 Multiple posttranslational modifications, including phosphorylation, acetylation, methylation and

233 polyubiquitination, are employed to regulate the antiviral signalosome [23]. Among these modifications,

234 polyubiquitination is commonly used for the degradation or activation of MDA5 and RIG-I. The E3 ubiquitin

235 ligase TRIM40 targets MDA5 and RIG-I to promote their K27- and K48-linked ubiquitination, thus leading to

236 their proteasomal degradation for immune silencing, but upon RNA virus infection, TRIM40 is downregulated

237 to allow the activation of a sufficient antiviral immune response [21]. Likewise, some viral proteins, such as

238 Epstein-Barr virus protein BPLF1, which has ubiquitin- and NEDD8-specific deconjugase activity, interact with

239 scaffold proteins 14-3-3 and TRIM25 to form a tri-molecular complex, consequently promoting the dimerization

240 and ubiquitination of TRIM25. Consequently, K63-linked polyubiquitination of RIG-I is downregulated, leading

241 to the attenuation of RIG-I-mediated type I IFN antiviral responses [24]. In addition, the NS3 protein of ZIKA virus

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242   interacts with scaffold proteins 14-3-3ε and η separately through its 14-3-3 binding motif, hence blocking the

243   translocation of RIG-I and MDA5 from the cytosol to mitochondria, impairing signalosome formation with MAVS,

244   and antagonizing innate immunity [25]. Our studies revealed that NSP8 of SARS-CoV-2 acts as a binding partner

245   of MDA5 to shield its K63-linked polyubiquitination and then impairs the formation or activation of the MDA5

246   signalosome.

247   In summary, our study provides insights into the potential mechanisms of SARS-CoV-2 NSP8 in the

248   inhibition of type I IFN signaling and antiviral responses. We provide compelling evidence that NSP8 plays a

249   critical negative role in MDA5-mediated antiviral responses and demonstrate specific orchestration of the viral

250   dsRNA-triggered signalosome and signal cascade by NSP8. Importantly, considering that MDA5 plays a key

251   pathological role in antiviral immunity towards severe coronaviruses, antagonists of NSP8 could serve as a

252   promising therapeutic target for COVID-19 therapies.

253

254   **Materials and Methods**

255   Cell culture and antibodies

256   HEK293T cells were cultured in DMEM (Gibco) supplemented with 10% fetal bovine serum (FBS). A549

257   cells (human adenocarcinoma lung tissue-derived epithelial cells) were cultured in RPMI 1640 (Gibco) medium

258   containing 10% FBS. The following antibodies were used in this study: goat anti-mouse IRDye680RD

259   (C90710-09) and goat anti-rabbit IRDye800CW (C80925-05), which were purchased from Li-COR; anti-HA

260   (M132-3), purchased from MBL; anti-Flag (AE005), anti-GFP (AE0122) and anti-IRF3 (A2172), purchased

261   from ABclonal; anti-β-actin (HC201-01), purchased from TransGen; and anti-pTBK1 (#5483P), anti-TBK1

262   (#3504), anti-pIRF3 (#4947), anti-phospho-NF-κB p65 (#3033), and anti-NF-κB p65 (#8242), purchased from

263   Cell Signaling Technology.

264

265   Plasmids

266   The following plasmids were used. Flag-NSP8 was kindly provided by the Peihui Wang lab (Shandong

267   University). HA-tagged Ub, K48-Ub (K48 only), and K63-Ub (K63 only) were kindly provided by Dr Yang Du

268   (Sun Yat-Sen University). To generate GFP- and mCherry-tagged NSP8 and GFP- and Flag-tagged MDA5,

269   the NSP8 and MDA5 fragments were subcloned into the pEGFP-C2, p-mCherry-C2 and pcDNA3.1 vectors.

270

271   Transfection and luciferase reporter assays

272   HEK293T cells were seeded in 24-well plates overnight and then transfected using Lipofectamine 2000

273   (Invitrogen) with 100 ng ISRE luciferase reporter (firefly luciferase), 20 ng pRL-TK plasmid (Renilla luciferase),

274   150 ng Flag-MDA5 expressing plasmid and increasing amounts (0, 100, or 200 ng) of NSP8-expressing

275   plasmid. Twenty-four hours post transfection, cells were collected, and luciferase activity was measured with

276   a Dual-Luciferase Assay kit (Promega) with a Synergy2 Reader (Bio-Tek) according to the manufacturer's

277   protocol. The relative level of gene expression was determined by normalization of firefly luciferase activity to

278   Renilla luciferase activity.

279

280   Virus infection

281   VSV-eGFP was kindly provided by Dr Meng Lin, School of Life Sciences, Sun Yat-Sen University. Cells were

282   infected at various MOIs, as previously described [26].

283

284   Real-time PCR

285   Total RNA was extracted using TRIzol reagent (Invitrogen) and subjected to reverse transcription using

286   HiScript® III RT SuperMix (Vazyme). Real-time PCR was performed with a LightCycler® 480 SYBR Green I

287   Master Mix kit (Roche). The primers used in the indicated gene array are listed in Table S1. The following

288   primers were used for real-time PCR:

289   *TNFα*:

290   Forward 5'-CTCTTCTGCCTGCTGCACTTTG-3'

291   Reverse 5'-ATGGGCTACAGGCTTGTCACTC-3'

292   *IFNβ*:

293   Forward 5'-CCTACAAAGAAGCAGCAA-30-3'

294   Reverse 5'-TCCTCAGGGATGTCAAAG-30-3'

295   *IFIT1*:

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296   Forward 5'-GCCTTGCTGAAGTGTGGAGGAA-3'

297   Reverse 5'-ATCCAGGCGATAGGCAGAGATC-3'

298   *IFIT2*:

299   Forward 5'-GGAGCAGATTCTGAGGCTTTGC-3'

300   Reverse 5'-GGATGAGGCTTCCAGACTCCAA-3'

301   *IL-6*:

302   Forward 5'-AGACAGCCACTCACCTCTTCAG-3'

303   Reverse 5'-TTCTGCCAGTGCCTCTTTGCTG-3'

304   *CCL20*:

305   Forward 5'-AAGTTGTCTGTGTGCGCAAATCC-3'

306   Reverse 5'-CCATTCCAGAAAAGCCACAGTTTT-3'

307

308   Immunoprecipitation and immunoblot analysis

309   For immunoprecipitation, whole cell extracts were prepared after transfection or stimulation with appropriate

310   ligands, followed by incubation for 1 h at 4°C with anti-GFP agarose beads (AlpaLife). Beads were washed 4

311   times with low-salt lysis buffer, and immunoprecipitants were eluted with 2x SDS loading buffer and then

312   resolved by SDS-PAGE. Proteins were transferred to PVDF membranes (Millipore) and further incubated with

313   the appropriate primary and secondary antibodies. The images were visualized using Odyssey Sa (LI-COR).

314

315   Computer-based prediction and structural modeling

316   NSP8.pdb, MDA5-CARDs.pdb and K63-Ub.pdb were generated in SWISS-MODEL [27]. MDA5-CARDs.pdb

317   was input into ZDOCK-SERVER [28] as a receptor, while NSP8 or K63-Ub was input as a ligand for docking

318   computation. MDA5-CARDs with NSP8.pdb and MDA5-CARDs with K63-Ub.pdb were the best fit prediction

319   models chosen from the results. All the pdb files were processed and visualized with PyMOL (Schrödinger.

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321

322   **Author contributions**

323 X.L. and E.K. initiated the concept. Z.Y., X.L. and E.K. designed the experiments and analyzed the data. P.W.

324 provided the reagents. Z.Y., X.Z., F.W. performed the experiments. Z.Y. and E.K. wrote the paper.

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333 **Conflict of interest statement**

334 The authors declare no competing financial interest.

335
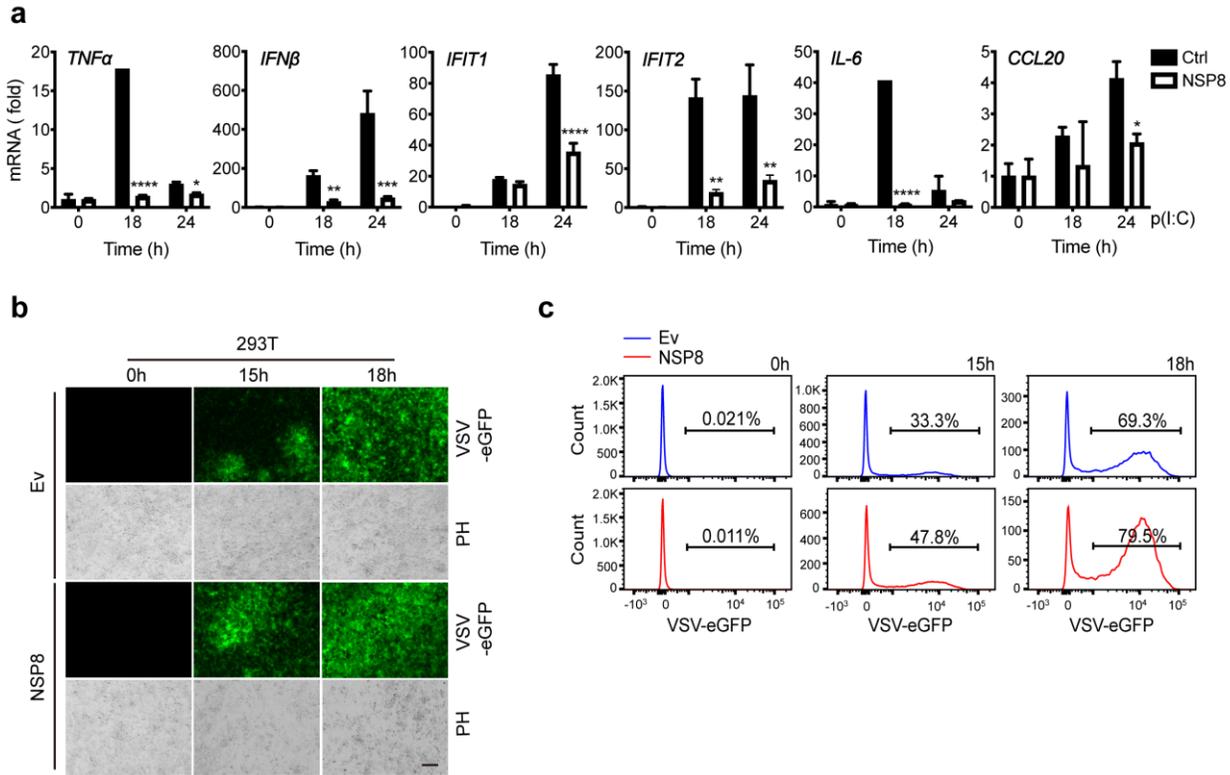
Figure 1. NSP8 inhibits the viral RNA-induced type I IFN signaling pathway.

a. HEK293T cells were transfected with a control vector (Ctrl) or *Flag-NSP* plasmids. Twenty-four hours post transfection, cells were treated with poly(I:C) (5 μM) for the indicated time points and then subjected to RT-PCR analysis for *TNF-α*, *IFN-β*, *IFIT1*, *IFIT2*, *IL-6* and *CCL20* expression. Data are shown as the mean values ± SD (n = 3). *, $p < 0.0332$; **, p < 0.0021; ***, p < 0.0002; ****, p < 0.0001; by Sidak's multiple comparisons test.

b. HEK293T cells were transfected with an empty vector or *Flag-NSP8*. Twenty-four hours post transfection, cells were infected with VSV-eGFP (MOI = 0.01) for the indicated time points and subjected to phase-contrast (PH) or fluorescence analyses. Scale bar, 15 μm.

c. eGFP-positive cells were detected by flow cytometry. Numbers indicate the representative percentage of eGFP-positive cells of three independent experiments.

14
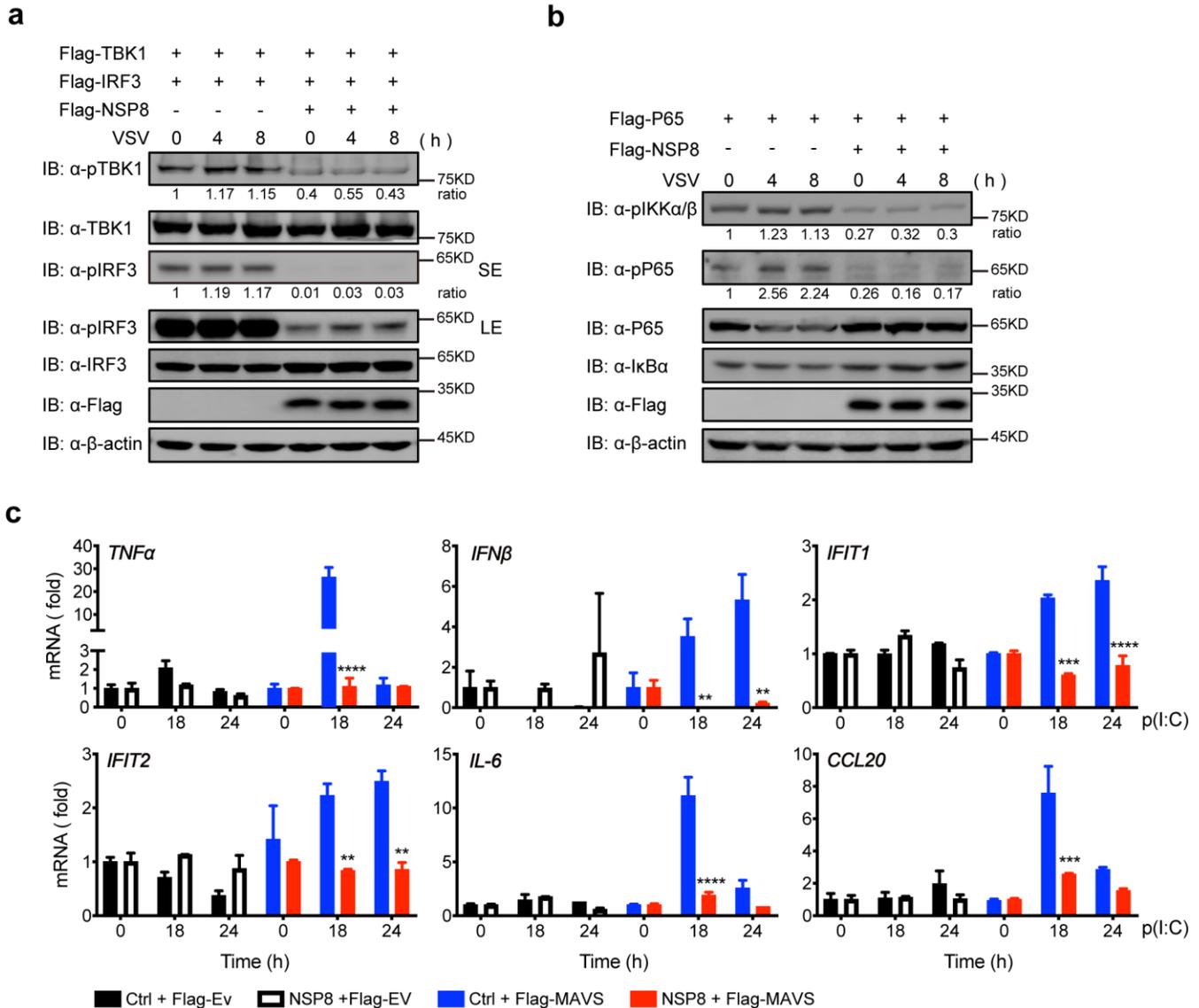
Figure 2. NSP8 inhibits the MAVS-dependent immune signaling pathway.

a-b. HEK293T cells were transfected with an empty vector or *Flag-NSP8* for 8 h and then transfected with *Flag-TBK1* and *Flag-IRF3* (a) or transfected with *Flag-p65* (b). Twenty-four hours post transfection, cells were infected with VSV-eGFP (MOI = 1) for the indicated time points and then harvested. Whole cell extracts were analyzed by immunoblotting as indicated. Representative images of three independent experiments are shown.

c. *MAVS* knockout HEK293T cells were cotransfected with *Flag-NSP8* and an empty vector or *Flag-MAVS*. Twenty-four hours post transfection, cells were treated with poly(I:C) (5 µg/ml) for the indicated time points, and then total RNA was extracted and subjected to RT-PCR analysis for *TNF-α*, *IFN-β*, *IFIT1*, *IFIT2*, *IL-6* and *CCL20* expression. The data are shown as the mean values ± SD (n = 3). *, $p < 0.0332$; **, $p < 0.0021$; ***, $p < 0.0002$; ****, $p < 0.0001$; by Sidak's multiple comparisons test.

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a

a-Flag-MDA5   -   +   +
GFP-NSP8   +   +   -

IP: α-GFP
- IB: α-Flag
- IB: α-GFP

WCL
- IB: α-Flag
- IB: α-GFP
- IB: α-β-actin

b

a-Flag-MDA5-CARDs   -   +   +
GFP-NSP8   +   +   -

IP: α-GFP
- IB: α-Flag
- IB: α-GFP

WCL
- IB: α-Flag
- IB: α-GFP
- IB: α-β-actin

c

Flag-MDA5   -   +   +   +

NSP8

d

mCherry-NSP8 + GFP-MDA5

mCherry   GFP   DAPI   Merge

- Red (NSP8)
- Green (MDA-5)
- Blue (Nuclear)

e Predicted binding model

MDA5-CARDs + NSP8   model Alpha   MDA5-CARDs + K63-Ub   schematic diagram of the competition binding area

f Vacuum Electrostatics

MDA5-CARDs + NSP8 (model Alpha)   MDA5-CARDs (A1)   NSP8 (A2)

A1 / A2

−60.057   60.057

g Polar Contacts

#2, 3, 4, 5, 6, 7   #1   B1 / B2

interface residues pair #1 (B1)   interface residues pairs #2, 3, 4, 5, 6, 7 (B2)

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366 **Figure 3. NSP8 interacts with MDA5 at the CARD domain.**

367 a-b. HEK293T cells were transfected with *GFP-NSP8* and full-length *Flag-MDA5* (a) or *Flag-MDA5-CARD* (b),

368 and after 36 h, the cells were collected and lysed. Then, cell lysates were subjected to coimmunoprecipitation

369 using anti-GFP beads, followed by immunoblotting with the indicated antibodies.

370 c. HEK293T cells were transfected with an empty vector or increasing amounts of *NSP8*-expressing plasmid

371 plus an *ISRE-luc* reporter and *MDA5*-expressing plasmids. Twenty-four hours post transfection, the cells were

372 collected, and then cell lysates were analyzed for ISRE-luc activi*ty*. The representative results of three

373 independent experiments are shown as the mean values ± SD (n = 3).

374 d. HEK293T cells were cotransfected with mCherry-NSP8 and GFP-MDA5. Twenty-four hours post

375 transfection, the images were visualized by confocal microcopy analysis. Scale bar, 10 μm. The colocalization

376 coefficient was determined by qualitative analysis of the fluorescence intensity of the selected area in Merge.

377 e-g. Computer-based prediction and structural modeling of the interaction of NSP8-MDA5 CARD domains.

378 e. Structural prediction of the MDA5-CARD-NSP8 interaction and MDA5-CARD linkage with K63-Ub. PDB

379 structures were input into ZDOCK Server for docking calculation separately. The predicted binding models of

380 MDA5 CARDs with NSP8 and MDA5 CARDs with K63-Ub were processed in PyMOL for demonstration. Model

381 alpha simulates the protein surface. Red chain, NSP8; violet chain, MDA5 CARDs; brown chain, K63-Ub.

382 f. Model alpha in (e) was subjected to vacuum electrostatics calculation in PyMOL. A1 and A2 indicate the

383 viewing angle in the green frame. The green frame indicates the contact area demonstrated in A1 and A2.

384 Scale bar indicates the range of vacuum electrostatics.

385 g. Polar contacts within interface of MDA5-CARD-USP8 were demonstrated with PyMOL. B1, B2 indicates

386 the viewing angle of dashed borders. #1, 2, 3, 4, 5, 6, 7 indicates paired residues. Paired residues were

387 highlighted with sticks model in yellow color. Green dashed lines indicate polar contacts between paired

388 residues, number besides dashed line indicates distance between two atoms connected (Å).

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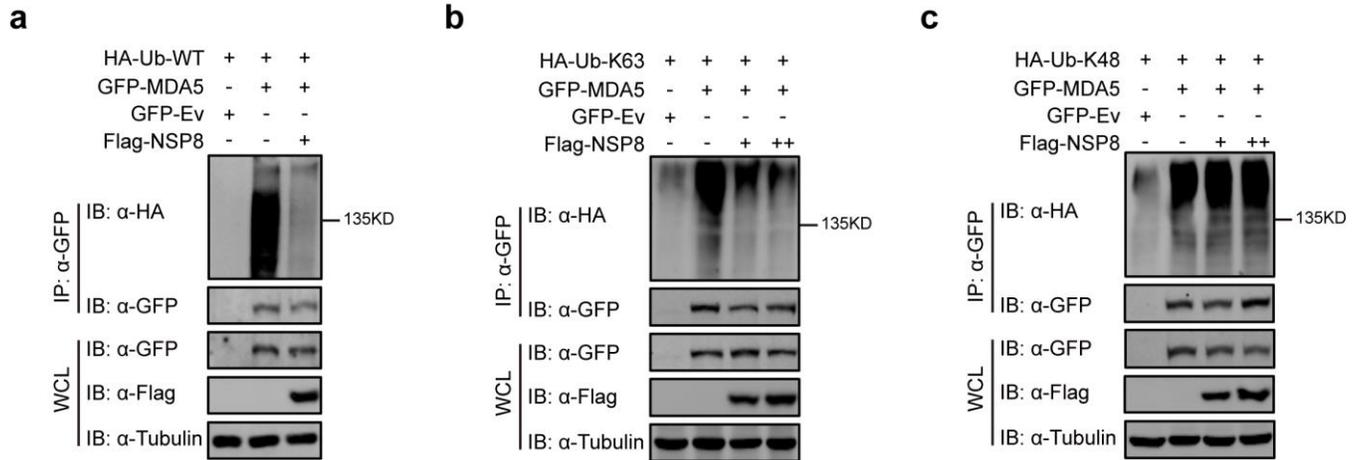
Figure 4. NSP8 suppresses K63-linked polyubiquitination of MDA5.

a-c. HEK293T cells were cotransfected with *HA-Ub-WT* (a), *HA-Ub-K63* (b) or *HA-Ub-K48* (c) and *GFP-MDA5* or *GFP*-tagged empty vector plus *Flag-NSP8.* Twenty hours post transfection, cells were treated with MG132 (5 µM) for 4 h, and then cells were collected and lysed in 0.1% SDS-containing lysis buffer. Cell lysates were subjected to coimmunoprecipitation using anti-GFP beads, followed by immunoblotting analysis with the indicated antibodies. Representative images of three independent experiments are shown.

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430   **Figure 5. NSP8 decreases the expression of antiviral immune and inflammatory factors.**

431   a. A549 cells were transfected with an empty vector or *Flag-NSP8*. Thirty-six hours post transfection, cells

432   were collected, and total RNA was extracted and subjected to RT-PCR analysis for the expression of the

433   indicated genes. "▼" indicates genes that were significantly changed in two independent *NSP8*-expressing

434   samples.

435   b. A549 cells were transfected with an empty vector or *Flag-NSP8*. Twenty-four hours post transfection, cells

436   were treated with poly(I:C) (5 µg/ml) for the indicated time points, and then total RNA was extracted and

437   subjected to RT-PCR analysis of the expression of selected genes.

438   The data are shown as the mean values ± SD (n = 3). *, $p < 0.0332$; **, $p < 0.0021$; ***, $p < 0.0002$; ****, $p <$

439   0.0001; by Sidak's multiple comparisons test.

440   c. Working model of SARS-CoV-2 NSP8 negatively regulating the MDA5-mediated type I IFN signaling

441   pathway. NSP8 interacts with MDA5 and impairs its K63-linked polyubiquitination, thus inhibiting the

442   phosphorylation of TBK1 and IRF3 and subsequently downregulating the production of type I IFNs, immune

443   cytokines and inflammatory factors.

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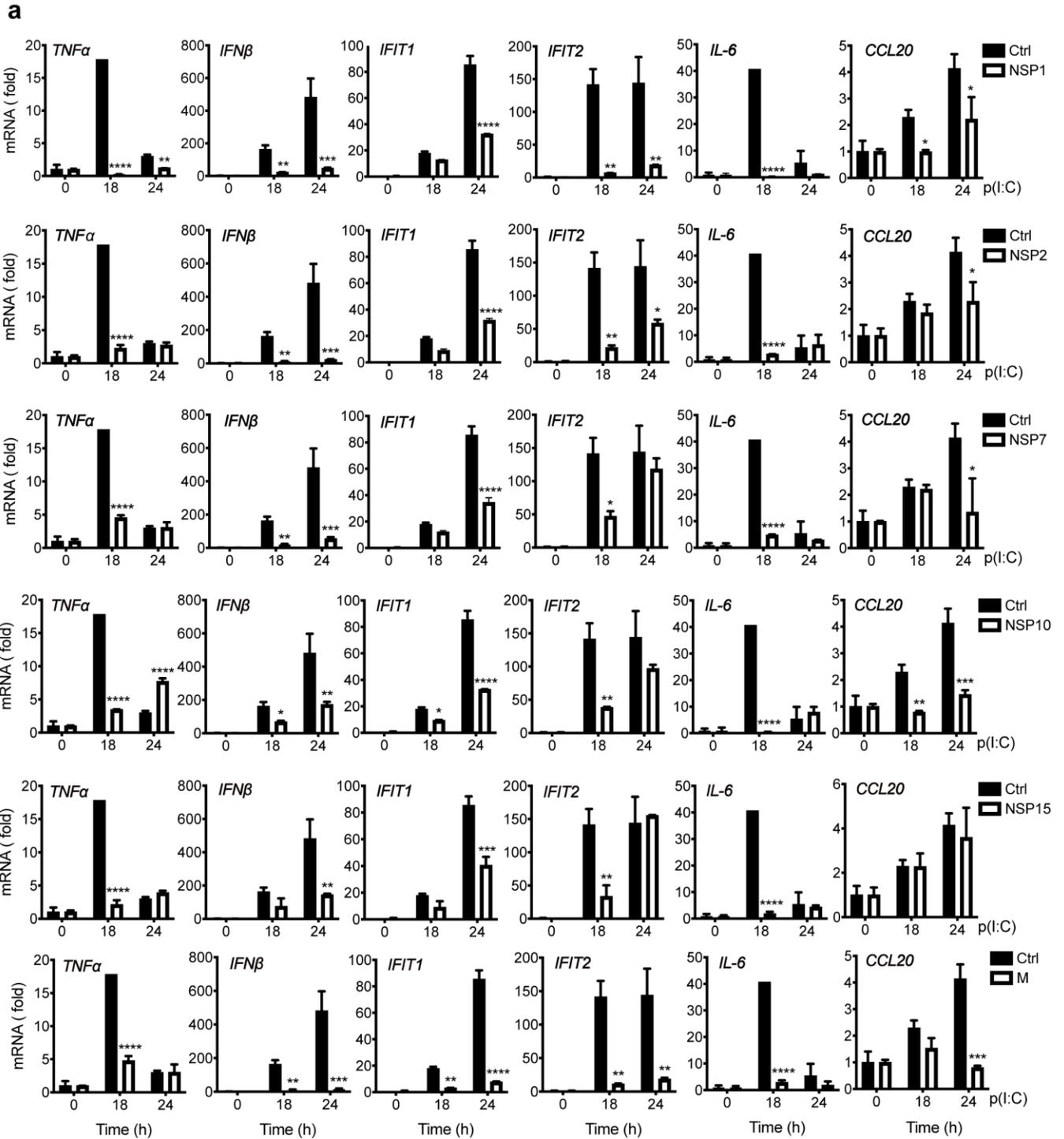
Supplementary Figure.1. Screening results of SARS-CoV-2 nonstructural proteins in the regulation of type I IFN signaling. HEK293T cells were transfected with empty vector (Ctrl) or Flag-NSP plasmids. Twenty-four hours post transfection, cells were treated with poly(I:C) (5 μM) for the indicated time, and total RNA was subjected to RT-PCR analysis of TNF-α, IFN-β, IFIT1, IFIT2, IL-6 and CCL20 expression.
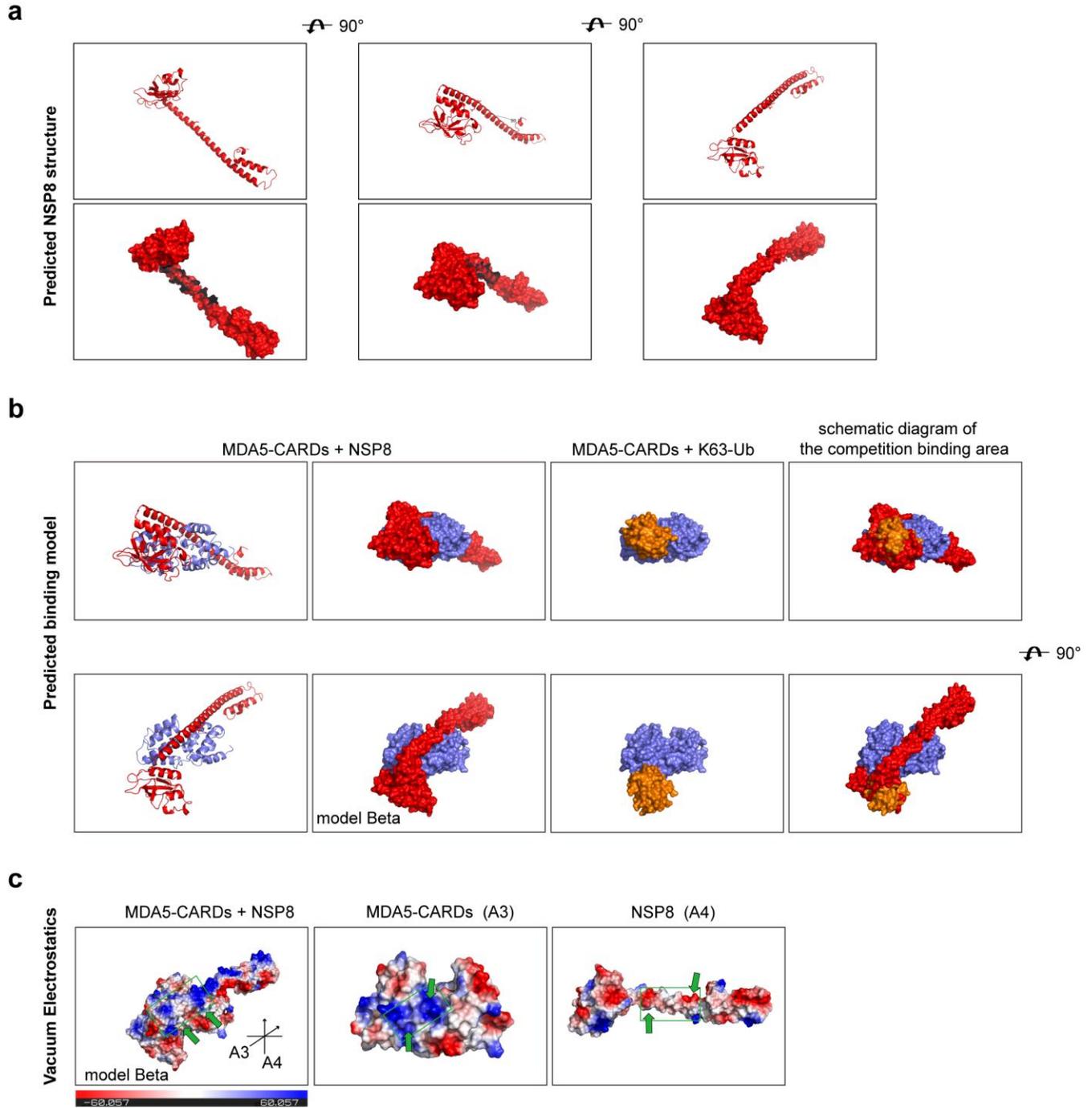
Supplementary Figure.2. Predicted structure of NSP8 and its binding with MDA5 CARDs

a. The PDB structure of NSP8 was processed in PyMOL. The cartoon structure and surface structure are demonstrated.

b. PDB structure of MDA5 CARD with NSP8 and MDA5 CARD with K63-Ub. PDB structures were input into the ZDOCK Server separately for docking calculations. The predicted binding models of MDA5 CARDs with

478   NSP8 and MDA5 CARDs with K63-Ub were processed in PyMOL for demonstration. Model alpha simulates

479   the protein surface. Red chain, NSP8; violet chain, MDA5 CARDs; brown chain, K63-Ub.

480   c. Model alpha in (b) was subjected to vacuum electrostatics calculation in PyMOL. A3 and A4 indicate the

481   viewing angle in the green frame. The green frame indicates the contact area demonstrated in A3 and A4. The

482   scale bar indicates the range of vacuum electrostatics.

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Gene		Sequence	Gene		Sequence
IL-1α	forward	5'-TGTATGTGACTGCCCAAGATGAAG-3'	TNFSF13	forward	5'-CGGAAAAGGAGAGCAGTGCTCA-3'
	reverse	5'-AGAGGAGGTTGGTCTCACTACC-3'		reverse	5'-GCCTAAGAGCTGGTTGCCACAT-3'
IL-1β	forward	5'-CCACAGACCTTCCAGGAGAATG-3'	TNFSF13B	forward	5'-ACCACGCGGAGAAGCTGCCAG-3'
	reverse	5'-GTGCAGTTCAGTGATCGTACAGG-3'		reverse	5'-CTGCTGTTCTGACTGGAGTTGC-3'
IL-2	forward	5'-AGAACTCAAACCTCTGGAGGAAG-3'	IFN-γ	forward	5'-GAGTGTGGAGACCATCAAGGAAG-3'
	reverse	5'-GCTGTCTCATCAGCATATTCACAC-3'		reverse	5'-TGCTTTGCGTTGGACATTCAAGTC-3'
IL-3	forward	5'-AAGCAGCCACCTTTGCCTTTGC-3'	IFN-β	forward	5'-CCTACAAAGAAGCAGCAA-30-3'
	reverse	5'-ACAGCCCTGTTGAATGCCTCCA-3'		reverse	5'-TCCTCAGGGATGTCAAAG-30-3'
IL-4	forward	5'-CCGTAACAGACATCTTTGCTGCC-3'	IFIT1	forward	5'-GCCTTGCTGAAGTGTGGAGGAA-3'
	reverse	5'-GAGTGTCCTTCTCATGGTGGCT-3'		reverse	5'-ATCCAGGCGATAGGCAGAGATC-3'
IL-5	forward	5'-GGAATAGGCACACTGGAGAGTC-3'	IFIT2	forward	5'-GGAGCAGATTCTGAGGCTTTGC-3'
	reverse	5'-CTCTCCGTCTTTCTTCTCCACAC-3'		reverse	5'-GGATGAGGCTTCCAGACTCCAA-3'
IL-6	forward	5'-AGACAGCCACTCACCTCTTCAG-3'	TGF-β	forward	5'-TACCTGAACCCGTGTTGCTCTC-3'
	reverse	5'-TTCTGCCAGTGCCTCTTTGCTG-3'		reverse	5'-GTTGCTGAGGTATCGCCAGGAA-3'
IL-7	forward	5'-GACAGCATGAAAGAAATTGGTAGC-3'	SPP1	forward	5'-CGAGGTGATAGTGTGGTTTATGG-3'
	reverse	5'-CAACTTGCGAGCAGCACGGAAT-3'		reverse	5'-GCACCATTCAACTCCTCGCTTTC-3'
IL-10	forward	5'-TCTCCGAGATGCCTTCAGCAGA-3'	BCL-6	forward	5'-CATGCAGAGATGTGCCTCCACA-3'
	reverse	5'-TCAGACAAGGCTTGGCAACCCA-3'		reverse	5'-TCAGAGAAGCGGCAGTCACACT-3'
IL-13	forward	5'-ACGGTCATTGCTCTCACTTGCC-3'	FOXP3	forward	5'-GGCACAATGTCTCCTCCAGAGA-3'
	reverse	5'-CTGTCAGGTTGATGCTCCATACC-3'		reverse	5'-CAGATGAAGCCTTGGTCAGTGC-3'
IL-16	forward	5'-TTGGACACAGGGTTCTCGCTCA-3'	MIF	forward	5'-AGAACCGCTCCTACAGCAAGCT-3'
	reverse	5'-AGCAGGGAGATAACGGACTGAC-3'		reverse	5'-GGAGTTGTTCCAGCCCACATTG-3'
IL-17A	forward	5'-CGGACTGTGATGGTCAACCTGA-3'	OSM	forward	5'-GAAAGAGTACCGCGTGCTCCTT-3'
	reverse	5'-GCACTTTGCCTCCCAGATCACA-3'		reverse	5'-CTCTCAGTTTAGGAACATCCAGG-3'
IL-17F	forward	5'-AACCAGCGCGTTTCCATGTCAC-3'	TBX21	forward	5'-ATTGCCGTGACTGCCTACCAGA-3'
	reverse	5'-GAGCATTGATGCAGCCCAAGTTC-3'		reverse	5'-GGAATTGACAGTTGGGTCCAGG-3'
IL-17C	forward	5'-GCCCTCAGCTACGACCCAGTG-3'	CXCR5	forward	5'-TGAAGTTCCGCAGTGACCTGTC-3'
	reverse	5'-AGCTTCTGTGGATAGCGGTCCT-3'		reverse	5'-GAGGTGGCATTCTCTGACTCAG-3'
IL-18	forward	5'-GATAGCCAGCCTAGAGGTATGG-3'	LTα	forward	5'-ACACCTTCAGCTGCCCAGACTG-3'
	reverse	5'-CCTTGATGTTATCAGGAGGATTCA-3'		reverse	5'-TCCGTGTTTGCTCTCCAGAGCA-3'
IL-22	forward	5'-GTTCCAGCCTTATATGCAGGAGG-3'	LTβ	forward	5'-GGTTTCAGAAGCTGCCAGAGGA-3'
	reverse	5'-GCACATTCCTCTGGATATGCAGG-3'		reverse	5'-CGTCAGAAACGCCTGTTCCTTC-3'
IL-26	forward	GGAAGACGTTTTTGGTCAACTGC-3'	NKP30	forward	5'-CCAGCATCTACGTGTGCAGAGT-3'
	reverse	5'-CTCTCTAGCTGATGAAGCACAGG-3'		reverse	5'-GCATAGAATCCAGCCCGAAGGA-3'
IL-33	forward	5'-GCCTGTCAACAGCAGTCTACTG-3'	NKP44	forward	5'-CTGAGTCTCCATCTACCATCCC-3'
	reverse	5'-TGTGCTTAGAGAAGCAAGATACTC-3'		reverse	5'-TCTTGGCTACGAGGAGTCCACA-3'
IL-1RII	forward	5'-GGCTATTACCGCTGTGTCCTGA-3'	NKP46	forward	5'-CAGCAACTTGCTGGATCTGGTG-3'
	reverse	5'-GAGAAGCTGATATGGTCTTGAGG-3'		reverse	5'-AGACGGCAGTAGAAGGTCACCT-3'
IL-1RAP	forward	5'-CTGAGGATCTCAAGCGCAGCTA-3'	NKG2A	forward	5'-GCCTCTGTGGTAACGATAGTTGT-3'
	reverse	5'-AGCAGGACTGTGGCTCCAAAAC-3'		reverse	5'-ATCCACTCCTCAGGACAATGGC-3'
IL-2Rα	forward	5'-GAGACTTCCTGCCTCGTCACAA-3'	NAMPT	forward	5'-AGGGTTACAAGTTGCTGCCACC-3'
	reverse	5'-GATCAGCAGGAAAACACAGCCG-3'		reverse	5'-CTCCACCAGAACCGAAGGCAAT-3'
IL-2Rβ	forward	5'-GGTGGAACCAAACCTGTGAGCT-3'	Peforin	forward	5'-ACTCACAGGCAGCCAACTTTGC-3'
	reverse	5'-GGTGACGATGTCAACTGTGGTC-3'		reverse	5'-CTCTTGAAGTCAGGGTGCAGCG-3'
IL-7R	forward	5'-ATCGCAGCACTCACTGACCTGT-3'	GATA3	forward	5'-ACCACAACCACACTCTGGAGGA-3'
	reverse	5'-TCAGGCACTTTACCTCCACGAG-3'		reverse	5'-TCGGTTTCTGGTCTGGATGCCT-3'
IL-18R1	forward	5'-GGAGGCACAGACACCAAAAGCT-3'	TNFα	forward	5'-CTCTTCTGCCTGCTGCACTTTG-3'
	reverse	5'-AGGCACACTACTGCCACCAAGA-3'		reverse	5'-ATGGGCTACAGGCTTGTCACTC-3'
IL-18BP	forward	5'-GTGTCCAGCATTGGAAGTGACC-3'	IL-6	forward	5'-AGACAGCCACTCACCTCTTCAG-3'
	reverse	5'-GGAGGTGCTCAATGAAGGAACC-3'		reverse	5'-TTCTGCCAGTGCCTCTTTGCTG-3'
TNFSF4	forward	5'-CCTACATCTGCCTGCACTTCTC-3'	CCL20	forward	5'-AAGTTGTCTGTGTGCGCAAATCC-3'
	reverse	5'-TGATGACTGAGTTGTTCTGCACC-3'		reverse	5'-CCATTCCAGAAAAGCCACAGTTTT-3'
TNFSF10	forward	5'-TGGCAACTCCGTCAGCTCGTTA-3'			
	reverse	5'-AGCTGCTACTCTCTGAGGACCT-3'			

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515 **Supplementary Table.1. The primer pairs of the RT-PCR array.**

516 The sequences of primer pairs used in the RT-PCR array (Fig.5a) are listed.

517

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