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1 **The endosomal Toll-like receptors 7 and 9 cooperate in detection of MHV68**

2 **infection**

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23 **ABSTRACT**

24 Murine gammaherpesvirus 68 (MHV68) is an amenable small animal model for study of the
25 human pathogens Epstein-Barr virus and Kaposi's sarcoma-associated herpesvirus. Here,
26 we have characterized the roles of the endosomal TLR escort protein UNC93B, endosomal
27 TLR7, 9, and 13, and cell surface TLR2 in MHV68 detection. We found that the interferon α
28 (IFN α) response of plasmacytoid dendritic cells (pDC) to MHV68 was reduced in *Tlr9*^{-/-}
29 cells compared to wildtype (WT), but not completely lost. *Tlr7*^{-/-} pDC responded similarly
30 to WT. However, we found that in *Unc93b*^{-/-} pDC, as well as in *Tlr7/Tlr9*^{-/-} double knockout
31 pDC, the IFN α response to MHV68 was completely abolished. Thus, the only pattern
32 recognition receptors contributing to the IFN α response to MHV68 in pDC are TLR7 and
33 TLR9, but the contribution of TLR7 is masked by the presence of TLR9. To address the role
34 of UNC93B and TLR for MHV68 infection *in vivo*, we infected mice with MHV68. Lytic
35 replication of MHV68 after intravenous infection was enhanced in the lungs, spleen, and
36 liver of UNC93B-deficient mice, in the spleen of TLR9-deficient mice, and in the liver and
37 spleen of *Tlr7/Tlr9*^{-/-} mice. The absence of TLR2 or TLR13 did not affect lytic viral titers.
38 We then compared reactivation of MHV68 from latently infected WT, *Unc93b*^{-/-}, *Tlr7/Tlr9*^{-/-},
39 *Tlr7*^{-/-}, and *Tlr9*^{-/-} splenocytes. We observed enhanced reactivation and latent viral loads,
40 particularly from *Tlr7/Tlr9*^{-/-} splenocytes, compared to WT. Our data show that UNC93B-
41 dependent TLR7 and TLR9 cooperate in and contribute to detection and control of MHV68
42 infection.

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52 **IMPORTANCE STATEMENT**

53 The two human gammaherpesviruses, Epstein-Barr virus (EBV) and Kaposi's sarcoma-
54 associated herpesvirus (KSHV), can cause aggressive forms of cancer. These herpesviruses
55 are strictly host specific and therefore their murine homolog murid gammaherpesvirus 4
56 strain 68 (MHV68) is a widely used model to obtain *in vivo* insights into the interaction
57 between these two gammaherpesviruses and their host. Like EBV and KSHV, MHV68
58 establishes lifelong latency in B cells. The innate immune system serves as one of the first
59 lines of host defense, with pattern recognition receptors such as the Toll-like receptors
60 playing a crucial role in mounting a potent antiviral immune response to various
61 pathogens. Here we shed light on a yet unanticipated role of Toll-like receptor 7 in the
62 recognition of MHV68 in a subset of immune cells called plasmacytoid dendritic cells, as
63 well as the control of this virus in its host.

64

65 INTRODUCTION

66 The double-stranded DNA virus murine gammaherpesvirus 4, also known as murine
67 gammaherpesvirus 68 (MHV68), was isolated in 1980 (1). Due to strict host species
68 specificity of the gammaherpesviruses, MHV68 is used as a small animal model for the
69 human gammaherpesviruses Kaposi's sarcoma-associated herpesvirus (KSHV) and
70 Epstein-Barr virus (2). The MHV68 life cycle is typical for herpesviruses and includes both
71 lytic and latent phases.

72 The pattern recognition receptors (PRR) are a crucial first line of host defense
73 against pathogens, including viruses. One class of PRR is the Toll-like receptors (TLR) that
74 are located on the cell surface and intracellularly in endosomes and detect a wide range of
75 non-self patterns. Cell surface TLR typically recognize exposed surface molecules on
76 bacteria or viruses. TLR2 recognizes bacterial lipoproteins and lipoteichoic acid, as well as
77 viral structural proteins (3). Recently, TLR2 was found to recognize repeating protein
78 subunit patterns, including those present in viral capsids (4).

79 The endosomal TLR sense nucleic acids. TLR3, TLR7, and TLR9 recognize dsRNA,
80 ssRNA, and CpG-rich dsDNA, respectively (5). TLR13 detects a specific sequence of
81 bacterial 23S ribosomal RNA and also responds to vesicular stomatitis virus (VSV) (6, 7).
82 Proper trafficking and signaling of endosomal TLR are dependent on the protein UNC93B;
83 mutation or deletion of UNC93B eliminates endosomal TLR signaling (8-12).

84 To date, twelve murine TLR have been described: TLR1-9 and TLR11-13. However,
85 very few have been characterized during MHV68 infection. So far, surface TLR2 and
86 endosomal TLR9 have been found to be important for detection of MHV68. TLR2
87 contributed to IL-6 and IFN α production in murine embryonic fibroblasts and elevated

88 titers were observed in the lungs of *Tlr2*^{-/-} mice after intranasal infection (13). TLR9 was
89 important for control of viral replication after intraperitoneal infection, as titers were
90 elevated in the spleens of *Tlr9*^{-/-} mice compared to WT controls (14). Reactivation and
91 latent viral load were correspondingly increased in *Tlr9*^{-/-} splenocytes (14). TLR9 also
92 partially contributes to the IFN α response to MHV68 in primary Fms-related tyrosine
93 kinase 3 ligand (Flt-3L)-induced dendritic cells (FLDC) (14). The role of TLR in detection of
94 human gammaherpesviruses has been analyzed in more detail. In humans, 10 TLRs, TLR1-
95 10, are described. To date, TLR2, TLR3, TLR7, and TLR9 have been shown to recognize at
96 least one human gammaherpesvirus (15). Whether TLR13 can detect herpesviral infection
97 is not yet known.

98 With the exception of TLR3, TLR recruit the adapter protein MyD88 to activate a
99 signaling cascade resulting in production of type I interferon (IFN) and proinflammatory
100 cytokines (16). The role of MyD88 during MHV68 infection is somewhat unclear; one study
101 found that viral titers were elevated in the lungs of *Myd88*^{-/-} mice 3 and 5 days post
102 infection (13), while another study found that on days 4 and 9 post infection, replication in
103 the lungs of *Myd88*^{-/-} mice was unaffected (17). The latent viral load was markedly
104 decreased in *Myd88*^{-/-} spleens after intranasal infection, with a concomitant decrease in
105 reactivation. However, upon intraperitoneal infection, the latent viral load in peritoneal
106 exudate cells (PEC) and splenocytes was similar in *Myd88*^{-/-} and WT mice, and reactivation
107 was increased from PEC in the absence of MyD88, but decreased from *Myd88*^{-/-}-deficient
108 splenocytes (17).

109 To clarify the role of additional individual and multiple TLR in detection of MHV68
110 *in vitro*, we analyzed the type I IFN response to MHV68 of primary plasmacytoid dendritic

111 cells derived from the bone marrow of WT, *Unc93b*^{-/-}, *Tlr2*^{-/-}, *Tlr7*^{-/-}, *Tlr9*^{-/-}, *Tlr13*^{-/-}, and
112 *Tlr7/9*^{-/-} mice. To understand the role of TLR during the acute phase of infection, we
113 analyzed lytic viral titers in WT and all six knockout genotypes mentioned. We observed
114 that lytic replication was enhanced in the lung, spleen, and liver of *Unc93b*^{-/-} mice, in the
115 spleen of *Tlr9*^{-/-} mice, and in the liver and spleen of *Tlr7/9*^{-/-} mice. Lastly, we analyzed latent
116 genome copy numbers and *ex-vivo* reactivation of MHV68 in WT and *Unc93b*^{-/-}, *Tlr7*^{-/-}, *Tlr9*^{-/-},
117 and *Tlr7/9*^{-/-} mice, and found that they were increased, particularly in the absence of
118 both TLR7 and TLR9. Our data have identified a novel role for TLR7 in detection of MHV68
119 *in vitro* and clarify the cooperative contribution of TLR7 and TLR9 to detection and control
120 of MHV68 replication *in vivo*.

121

122 RESULTS

123 MHV68 is detected by both TLR7 and TLR9 in pDC

124 A previous study has shown that the production of type I interferon in response to MHV68
125 stimulation is partially dependent on TLR9; in primary murine FLDC derived from *Tlr9*^{-/-}
126 mice, the IFN α response to MHV68 is abrogated, but not completely lost (14). When murine
127 bone marrow cells are cultured with FMS-like tyrosine kinase 3 ligand (Flt-3L), cells
128 differentiate into conventional and plasmacytoid dendritic cells (cDC and pDC,
129 respectively). Of these, the pDC are the major type I IFN producing cells (18) and detection
130 of infection in pDC is almost exclusively dependent on TLR, suggesting that another TLR
131 besides TLR9 may be responsible for the remaining IFN α response to MHV68 in *Tlr9*^{-/-}
132 FLDC. To identify the TLR that also contribute(s) to detection of MHV68 in pDC, we
133 analyzed the response to MHV68 infection of pDC purified from primary FLDC cultures
134 derived from WT and TLR-deficient mice.

135

136 First, we infected WT, *Tlr7*^{-/-}, or *Tlr9*^{-/-} pDC with MHV68 and measured IFN α secretion by
137 ELISA. We found that compared to WT pDC, *Tlr9*^{-/-} pDC produced significantly less IFN α in
138 response to MHV68 treatment (**Fig 1A**) as previously shown for FLDC (14). In contrast,
139 *Tlr7*^{-/-} pDC responded to MHV68 similarly to WT cells (**Fig 1A**). A similar pattern was
140 observed in response to MCMV infection (**Fig 1A**). The response to Newcastle disease virus
141 (NDV), an RNA virus, was almost entirely dependent on the presence of the RNA sensor
142 TLR7, as expected (**Fig 1A**). We next analyzed the IFN α response to MHV68 in pDC
143 deficient in multiple endosomal TLR. We used either *Unc93b*^{-/-} pDC, deficient in all
144 endosomal TLR signaling, or *Tlr7/9*^{-/-} pDC, lacking only TLR7 and TLR9. To our surprise,

145 *Unc93b*^{-/-} and *Tlr7/9*^{-/-} pDC did not mount an IFN α response upon infection with MHV68.
146 The same result was observed upon MCMV and NDV infection (**Fig 1B**). We successfully
147 verified the cell phenotypes using the synthetic TLR7 ligand polyU and the TLR9 ligand CpG
148 DNA (**Fig 1C and 1D**). Finally, we analyzed the IFN α response to MHV68 in pDC lacking
149 TLR2 or TLR13, with WT and *Unc93b*^{-/-} pDC as controls (**Fig 1E**). We found that *Tlr2*^{-/-} pDC
150 and *Tlr13*^{-/-} pDC were not impaired in their IFN α response compared to WT pDC (**Fig 1E**).
151

152 FLDC cultures include pDC as well as conventional dendritic cells (cDC). To clarify whether
153 pDC alone are responsible for the observed phenotype, or understand if cDC also
154 contribute, we next compared the response to MHV68 in total FLDC cultures derived from
155 WT, *Tlr7*^{-/-}, *Tlr9*^{-/-}, and *Tlr7/9*^{-/-} bone marrow. The results were similar to what was
156 observed for purified pDC; the response was reduced in *Tlr9*^{-/-} FLDC and completely lost in
157 *Tlr7/9*^{-/-} FLDC (**Fig 1F**). When we compared the IFN α response from WT FLDC to FACS-
158 purified CD11c^{mid}B220⁺ pDC and CD11c⁺B220⁻ cDC, we found that the pDC were primarily
159 responsible for the IFN α response upon MHV68 infection (**Fig 1G**). These data show that
160 both TLR7 and TLR9, but not TLR2 or TLR13, are required for IFN α production after
161 MHV68 infection of pDC. Interestingly, the contribution of TLR7 was only observed in the
162 absence of TLR9.

163

164

165 **The absence of endosomal TLR does not affect weight loss or lytic replication in**
166 **organs after intranasal infection with MHV68**

167 Next, we wanted to determine the role of TLR during *in vivo* infection with MHV68. The
168 natural route of infection in wild rodents is still unclear. Many routes of infection, including
169 intranasal and intravenous, are used in the literature (19). Intranasal infection is often
170 described as a more natural route than intravenous infection and it is anticipated that
171 murine noses play an important role in animal interactions (19, 20). However, horizontal
172 transmission among female laboratory mice has not been observed (21, 22). In contrast,
173 sexual transmission has been reported (22). MHV68 DNA has been identified in wild ticks
174 (23) and there is experimental evidence for tick-borne transmission of MHV68 (24). Both
175 sexual and tick-borne transmission are more similar to intravenous or blood-borne
176 infection than they are to intranasal infection. In humans, transmission of EBV and KSHV
177 can be salivary or sexual (reviewed in (25)). To comprehensively address the role of TLR
178 during MHV68 infection, we utilized both intranasal and intravenous infection.

179
180 First, we infected WT, *Unc93b*^{-/-}, *Tlr7*^{-/-}, and *Tlr9*^{-/-} mice intranasally and monitored body
181 weight. We observed minimal differences between *Unc93b*^{-/-} mice and their respective B6N
182 controls (**Fig 2A**). There were no differences observed in body weight change of *Tlr7*^{-/-} or
183 *Tlr9*^{-/-} mice compared to their respective B6J controls (**Fig 2B and 2C**). Mice of all
184 genotypes lost weight but recovered.

185
186 Next, we determined acute lytic viral titers in the lungs and spleens 3 days after intranasal
187 infection. We included endosomal TLR-deficient *Unc93b*^{-/-} mice, *Tlr13*^{-/-}, *Tlr2*^{-/-}, *Tlr7*^{-/-}, *Tlr9*^{-/-},
188 and *Tlr7/9*^{-/-} mice, as well as B6N and B6J WT mice as controls. There were no significant
189 differences in viral titers in the lungs among the genotypes examined (**Fig 2D**). Very little

190 lytic virus was detected in spleen samples, as expected based on the kinetics of viral
191 spread; many samples were around the approximate detection limit (horizontal gray line).
192 Many were negative, and those samples are displayed as points on the X-axis where $\log_{10}=0$
193 (Fig 2E).

194

195 To summarize, we did not observe a role of TLR in control of lytic replication upon
196 intranasal infection, but this finding was not surprising since endosomal TLR are not the
197 predominant pattern recognition receptors expressed in the lung. Previously, TLR9 was not
198 found to play a role during intranasal infection, but it was important in control of
199 replication upon intraperitoneal infection (14). Like intraperitoneal infection, intravenous
200 infection allows direct infection of the spleen without initial viral replication in the lung.

201

202 We thus utilized intravenous infection for further experiments. We found that *Unc93b*^{-/-}
203 mice lost significantly more weight and recovered more slowly from weight loss compared
204 to their WT B6N counterparts upon MHV68 infection (Fig 3A). In general, *Tlr7*^{-/-}, *Tlr9*^{-/-},
205 and *Tlr7/9*^{-/-} mice lost weight similarly to WT B6J mice (Fig 3B-3D).

206

207 We next examined replication of MHV68 in organs at day 3 post intravenous infection. We
208 compared lytic titers in the lungs (Fig 4A), spleen (Fig 4B), and liver (Fig 4C) of WT,
209 *Unc93b*^{-/-}, *Tlr2*^{-/-}, *Tlr7*^{-/-}, *Tlr9*^{-/-}, *Tlr13*^{-/-}, and *Tlr7/9*^{-/-} mice. Titers in the lungs of *Unc93b*^{-/-}
210 mice were increased 4-fold on average, a statistically significant increase, compared to
211 their B6N controls (Fig 4A). Average titers from *Tlr7*^{-/-}, *Tlr9*^{-/-}, *Tlr7/9*^{-/-}, and *Tlr13*^{-/-} lungs
212 were increased 3-, 1.5-, 2.2-, and 1.3-fold, respectively, compared to B6J WT, but these

213 increases were not statistically significant (**Fig 4A**). As previously reported for TLR9 upon
214 intraperitoneal infection, we found that titers in the spleens from *Tlr9*^{-/-} mice were
215 significantly increased upon intravenous infection; we observed an approximately 3.3-fold
216 increase in titers (**Fig 4B**). Additionally, titers in spleens derived from *Unc93b*^{-/-} and *Tlr7/9*^{-/-}
217 ^{-/-} mice were significantly elevated compared to their B6N and B6J controls, approximately
218 6.3-fold in both cases (**Fig 4B**). Finally, we found that lytic titers were significantly elevated
219 in the livers of *Unc93b*^{-/-} and *Tlr7/9*^{-/-} mice, approximately 2.3- and 2.7-fold, respectively,
220 but not in *Tlr9*^{-/-} livers (**Fig 4C**). In summary, after intravenous infection, viral titers are
221 elevated in the lungs of *Unc93b*^{-/-} mice. TLR9 is important for control of MHV68 replication
222 in the spleen, and both TLR7 and TLR9 are important for control of MHV68 replication in
223 the liver.

224

225 Like all herpesviruses, after a period of lytic replication, MHV68 establishes latency *in vivo*.
226 During MHV68 infection, independently of the route of infection, latency is established in
227 the spleen, predominantly in activated germinal center B220⁺PNA^{high} B cells (26). The co-
228 culture of latently-infected B cells or total splenocytes with NIH/3T3 fibroblasts leads to
229 reactivation and spread of lytic virus to fibroblasts, which are then scored for cytopathic
230 effect (CPE) (27, 28). By comparing serial dilutions of splenocytes from latently infected
231 mice, the reactivation frequency can be determined.

232

233 We next wanted to analyze the role of TLR signaling in the reactivation of MHV68. We
234 infected mice intravenously with MHV68, then waited 22 days for the establishment of
235 latency. For an *ex vivo* reactivation assay, total splenocytes were isolated as a single cell

236 suspension, diluted in 3-fold serial dilutions, and 24 replicates were plated per dilution.
237 The frequency of reactivating wells at each dilution was then determined by analyzing CPE.
238 To compare reactivation frequencies, the regression line intersect was set to 63.2% based
239 on the Poisson distribution. Slightly more reactivation was observed for *Unc93b*^{-/-}
240 splenocytes compared to B6N controls. Approximately 1 in 22,719 *Unc93b*^{-/-} splenocytes
241 reactivated compared to 1 in 30,170 B6N splenocytes, for a slight 1.33-fold increase in
242 reactivation of *Unc93b*^{-/-} compared to B6N splenocytes (**Figure 5A**). For B6J, *Tlr7*^{-/-}, and
243 *Tlr9*^{-/-} splenocytes, we found that approximately 1 in 47,982 B6J, 1 in 33,859 *Tlr7*^{-/-}, and 1
244 in 22,145 *Tlr9*^{-/-} splenocytes reactivated, for a 1.42-fold increase in reactivation of *Tlr7*^{-/-}
245 splenocytes over B6J and a 2.17-fold increase in reactivation of *Tlr9*^{-/-} splenocytes
246 compared to B6J (**Fig 5B**). Next, we examined reactivation of *Tlr7/9*^{-/-} splenocytes. At
247 multiple dilutions, reactivation was significantly more frequent than that of WT
248 splenocytes. The frequency of reactivation was approximately 1 in 10,350, for a 4.64-fold
249 increase in reactivation of *Tlr7/9*^{-/-} splenocytes compared to WT (**Fig 5C**).
250
251 Enhanced reactivation could be due to an increased ability of individual cells to reactivate,
252 or due to an increase in the number of latent genome copies per spleen or cell. To
253 differentiate between an improved ability to reactivate or an increase in the number of
254 latent genome copies, we performed qPCR for the MHV68 glycoprotein B (gB) gene to
255 determine the latent viral load in splenocytes. We normalized to the murine beta-2-
256 microglobulin gene to control for the number of cells. For ease of comparison, we then
257 scaled the gB values to the average of the appropriate B6 control. We found that for an
258 equal number of splenocytes, *Unc93b*^{-/-} splenocytes contained about 1.57-fold more gB

259 copies than B6N WT splenocytes (**Fig 5D**). In contrast, the number of gB positive *Tlr7*^{-/-} and
260 *Tlr9*^{-/-} splenocytes did not significantly differ from the number of gB-positive B6J
261 splenocytes (0.91-fold and 1.04-fold, respectively) (**Fig 5D**). However, when both TLR7 and
262 TLR9 were absent, the number of gB positive splenocytes was significantly increased
263 compared to B6J splenocytes, with *Tlr7/9*^{-/-} splenocytes containing about 1.8-fold more gB
264 copies than B6J splenocytes (**Fig 5D**). Collectively, both the latent viral load and
265 reactivation frequency of *Unc93b*^{-/-} and *Tlr7/9*^{-/-} splenocytes were elevated compared to
266 WT controls. In the case of *Unc93b*^{-/-} splenocytes, the increase in reactivation and increase
267 in latent viral load were similar, suggesting a direct relationship between viral load and
268 reactivation in B6N-background mice. However, in the case of *Tlr7/9*^{-/-} splenocytes,
269 genome copies were increased 1.8-fold, while reactivation was increased 4.6-fold ,
270 suggesting increased reactivation is only partially due to increased latent viral load on the
271 B6J background.

272

273 TLR7 is an important pattern recognition receptor that senses multiple viruses. So far, our
274 data indicate that TLR7 is important for detection and control of MHV68, but the ligand is
275 unknown. Furthermore, the effect of TLR7 appears to be masked by the presence of TLR9,
276 particularly in primary dendritic cell cultures. Thus, to analyze various ligands for their
277 contribution to TLR7 signaling during MHV68 infection, we utilized stimulation of primary
278 *Tlr9*^{-/-} FLDC with various MHV68 mutants.

279

280 One potential TLR7 ligand during MHV68 infection is viral microRNAs (miRNA). In MHV68,
281 pre-miRNA stem-loops give rise to 28 mature miRNAs (29-31). The stem-loops, however,

282 also contain viral genome-encoded 5' tRNAs (vtRNAs) (32). Due to the tight linkage of
283 vtRNAs and miRNAs, an MHV68 virus mutant lacking small noncoding RNAs (Δ miR) lacks
284 both miRNAs and vtRNAs (33). We stimulated *Tlr9*^{-/-} FLDC with MHV68 Δ miR, the
285 corresponding revertant virus (REV), or MHV68-GFP (GFP) as a control, then determined
286 the IFN α response. All three viruses induced an IFN α response in *Tlr9*^{-/-} FLDC, strongly
287 indicating that miRNAs are not the sole TLR7 ligand during MHV68 infection (**Figure 6A**).
288

289 Another potential TLR7 ligand during MHV68 infection is cleaved cellular mRNA. During
290 lytic replication, the MHV68 protein muSOX induces host shutoff via global mRNA
291 degradation. A host-shutoff deficient muSOX mutant (Δ HS) lacks the ability to degrade
292 mRNA due to a single amino acid change, but is well-expressed and retains DNase activity
293 (34). We stimulated *Tlr9*^{-/-} FLDC with MHV68 Δ HS, the corresponding revertant virus
294 (REV), or MHV68-GFP for comparison (**Figure 6B**). At an MOI of 0.5, Δ HS and REV induced
295 a similar IFN α response. At an MOI of 2, the response was slightly reduced upon
296 stimulation with Δ HS compared to REV, but a response was still present (**Figure 6B**).
297

298 It is possible that the TLR7 ligand is packaged into MHV68 virions or transcribed before the
299 onset of viral replication, or expressed later during infection in pDC. To help clarify this
300 question, we treated FACS-sorted *Tlr9*^{-/-} pDC with the viral DNA polymerase inhibitor
301 acyclovir or DMSO as a control, then infected them with MHV68 (**Figure 6C**). The IFN α
302 response to MHV68 was similar in both the presence and absence of acyclovir (**Figure 6C**).
303 To confirm that acyclovir treatment did not affect the IFN α response in general, we also
304 determined the response to the TLR7 agonist polyU in the presence of DMSO and acyclovir.

305 No differences were observed (**Figure 6D**). To verify that acyclovir treatment inhibited
306 viral replication, we measured mRNA levels for immediate-early RTA, early ORF68, early-
307 late ORF4, and late ORF8 and ORF65/M9 (**Figure 6E**). In the presence of acyclovir, early,
308 early-late, and late mRNA levels were greatly reduced.

309

310 In summary, as both the Δ miR and Δ HS viruses induced an IFN α response in *Tlr9*^{-/-} FLDC,
311 our results suggest that neither muSOX nor cleaved cellular mRNAs are exclusively
312 responsible for the induction of IFN α in *Tlr9*^{-/-} FLDC. Furthermore, experiments with
313 acyclovir suggest that the TLR7 ligand during MHV68 infection is packaged into virions,
314 expressed before the onset of viral DNA replication, or is not of viral origin.. In summary,
315 we have identified TLR7 as an important TLR for detection of MHV68 *in vitro* and control of
316 MHV68 infection *in vivo*, and it works cooperatively with TLR9 in detection of MHV68.

317

318 **DISCUSSION**

319 Our data identify the RNA sensor TLR7 as an important pattern recognition receptor
320 during infection with the DNA virus MHV68. Interestingly, we have found that the role of
321 TLR7 is frequently masked by the presence of TLR9. In *Tlr9*^{-/-} pDC, however, we observe
322 IFN α production that is entirely dependent on TLR7, as the response is completely lost in
323 *Tlr7/9*^{-/-} pDC. pDC have long been known to be the major IFN α -producing cells in response
324 to viral infection. Additionally, pDC detection of infection is dependent on their expression
325 of TLR7 and TLR9 (reviewed in (35)). pDC have been shown to detect both MCMV (36) and
326 MHV68 (37). However, until now, the two pattern recognition receptors required for
327 MHV68 recognition in pDC were not fully known.

328

329 While the role of TLR9 has been described (14), TLR7 had not been studied in conjunction
330 with TLR9 until now. Similar to our *in vitro* results, *in vivo*, we also see that both TLR7 and
331 TLR9 contribute to MHV68 recognition, but the strongest phenotype is present when both
332 TLR7 and TLR9 are absent. Reactivation has been previously studied in splenocytes
333 derived from *Tlr9*^{-/-} (14, 38) and *Tlr7*^{-/-} mice (38). While these authors observed that
334 reactivation was enhanced in the absence of TLR9, as we also show, no effect was seen
335 when TLR7 was absent. This is likely due to the fact that the presence of TLR9 masks the
336 contribution of TLR7, which we now see in this report. A similar overlapping role for TLR7
337 and TLR9 in detection of MCMV has also been described (39). In *Tlr7/9*^{-/-} pDC, there is a
338 markedly reduced response to MCMV, as we also show, and *in vivo*, *Tlr7/9*^{-/-} mice are more
339 susceptible to MCMV infection (39). The interferon response we observed in pDC infected
340 with MHV68 was clearly lower than the interferon response we detected upon MCMV or
341 NDV infection. This has previously been shown for MCMV and MHV68 in wild-type FLDC,
342 due to deamination and thus suppression of stimulatory CpG motifs in the MHV68 genome,
343 but not in that of MCMV (37).

344

345 Here, we have identified a role for TLR7 and TLR9 in detection of MHV68. Endosomal TLR
346 have been studied during KSHV and EBV infection and are important for detection and
347 control of reactivation. For example, primary human pDC detect KSHV in a primarily TLR9-
348 dependent manner; the use of a TLR9 inhibitor reduced or eliminated the IFN α response
349 with donor-to-donor variability (40). Although the role of TLR7 could not be tested, in the
350 donors where a response was still present despite TLR9 inhibition, the remaining response

351 could be due to TLR7 signaling (40), as we have observed in the case of MHV68 infection of
352 pDC. A role for TLR7 has been shown during reactivation of KSHV; TLR7 ligands or VSV
353 infection induced lytic gene transcription and replication (41). Similarly, in the case of EBV
354 infection, both TLR7 and TLR9 contribute to pDC production of IFN α (42, 43). TLR9 also
355 recognizes EBV in monocytes (43), but whether TLR7 plays a role in these cells has not
356 been studied.

357

358 One question raised by this study is the source of the TLR7 ligand in MHV68. Infection
359 induces transcription of many different cellular and viral RNAs, including mRNA,
360 microRNAs (miRNAs), and long non-coding RNAs, among others. First, we considered
361 miRNAs, which are RNA molecules about 22 nucleotides long and important for post-
362 transcriptional regulation of mRNA. miRNA control many cellular processes, including
363 innate immunity (44) so it is not surprising that many viruses, like their hosts, encode
364 miRNAs (45). MHV68 is no exception; it encodes 14 pre-miRNA stem-loops that form 28
365 mature miRNAs (29, 31, 32). Viral miRNAs were thus a potential source of TLR7 activation
366 during infection. In *Tlr9*^{-/-} FLDC, however, the response to infection with MHV68 Δ miR or
367 its revertant led to similar IFN α responses, suggesting miRNAs are not responsible for the
368 TLR7 response to MHV68.

369

370 Next, we considered the MHV68 muSOX protein and its function as a possible source of
371 TLR7 ligands during infection. The human gammaherpesviruses KSHV and EBV encode a
372 conserved alkaline exonuclease gene that has an additional function in repression of host
373 gene expression, or host shutoff. This function is also conserved in the MHV68 homolog

374 muSOX. We hypothesized that cleaved cellular mRNAs could be a TLR7 ligand during
375 MHV68 infection (46, 47). While our data do not exclude that TLR7 activation during
376 MHV68 infection is partially due to muSOX-dependent cleavage of host cell mRNA, our data
377 verify that cleaved host cell mRNA is not the sole TLR7 ligand during MHV68 infection. If
378 cleaved cellular mRNA was the only TLR7 ligand present during MHV68 infection, no IFN α
379 response would be anticipated in *Tlr9*^{-/-} FLDC. In contrast, an IFN α response was still
380 observed in *Tlr9*^{-/-} FLDC infected with MHV68 Δ HS.

381

382 In general, it is inadvisable for a cell to detect its own nucleic acid sequences, as that could
383 potentially cause unnecessary inflammation. However, cleaved cellular mRNAs could be an
384 exception to this standard as they are not normally present in a healthy cell. One possible
385 explanation is that to prevent autoimmune responses, TLR7 cannot detect these cleaved
386 host-derived mRNAs, in which case, other potential ligands must be considered. A second
387 possibility is that these cleaved mRNAs are simply not produced in pDC. However, this
388 would not change the interpretation of our data. If cleaved mRNAs are not produced, they
389 cannot be the ligand. Whether muSOX functions in primary pDC is not currently known.

390 Studies comparing the function of Δ HS and revertant viruses primarily used murine
391 embryonic fibroblasts, NIH/3T3, and the dendritic cell line DC2.4 (48), but did not compare
392 them in primary pDC.

393

394 Another open question is whether the TLR7 ligand is MHV68 RNA that is packaged into
395 virions, viral RNA that is transcribed prior to viral DNA replication, or viral RNA that is
396 transcribed later during the life cycle. To help address this question, we treated *Tlr9*^{-/-} pDC

397 with the viral DNA polymerase inhibitor acyclovir and measured the IFN α response to
398 MHV68. We found that acyclovir treatment did not affect the IFN α response. We verified
399 DNA polymerase inhibition by quantitative reverse transcription polymerase chain
400 reaction (q-RT-PCR) and found that expression of multiple viral genes was inhibited by
401 acyclovir. The observed effect of acyclovir on gene expression was in agreement with a
402 study that used the DNA polymerase inhibitor phosphonoacetic acid (PAA) (49).

403

404 It is possible that even a limited amount of immediate-early gene expression is sufficient to
405 activate the TLR7-dependent IFN α response in pDC. It is also plausible that packaged RNA
406 induces the response; we cannot distinguish between these possibilities. Unfortunately,
407 well-characterized immediate-early inhibitors of MHV68 replication are lacking. As MHV68
408 uses host RNA polymerase II for mRNA synthesis, we cannot inhibit all viral mRNA
409 synthesis; since we would also inhibit cellular mRNA synthesis, we would not be able to
410 measure host IFN α . Further studies will need to use a global approach to conclusively
411 identify the TLR7 ligand during MHV68 infection, but this is beyond the scope of this study.

412

413 *In vitro*, we found that *Unc93b*^{-/-} and *Tlr7/9*^{-/-} pDC responded identically to MHV68
414 infection. *In vivo*, however, we observed differences between the two strains. One obvious
415 explanation is that the *Unc93b*^{-/-} and *Tlr7/9*^{-/-} mice are on different genetic backgrounds.
416 The *Unc93b*^{-/-} mice were created as part of the KOMP knockout mouse project, and like all
417 International Knockout Mouse Consortium mutant clones, they were generated in
418 C57BL/6N embryonic stem cells (50). Chimaeras were then bred to C57BL/6N mice. In
419 contrast, the *Tlr7*^{-/-} and *Tlr9*^{-/-} mice were made using genomic DNA from 129/Sv mice and

420 most likely 129/Ola ES cells that were then injected into C57BL/6 blastocysts (51, 52). The
421 *Tlr7*^{-/-} and *Tlr9*^{-/-} mice were then backcrossed for a number of generations onto C57BL/6J
422 mice.

423

424 While C57BL/6N and C57BL/6J are closely related, the two strains diverged around 220
425 generations ago (53). There are a number of single-nucleotide polymorphisms, including
426 one in the NLR Family Pyrin Domain Containing 12 (*Nlrp12*) gene that leads to an amino
427 acid change in NLRP12, as well as small indels and structural variants (53). C57BL/6J mice
428 exhibit impaired glucose tolerance and have increased lean mass compared with
429 C57BL/6N mice, and control of *Listeria monocytogenes* varied by strain. Furthermore, the
430 activity of splenic natural killer (NK) cells from C57BL/6N mice was reduced compared to
431 that of C57BL/6J cells (53). Taken together, these differences in NLRP12, metabolism, and
432 NK cell activity may help clarify differences we observed between *Unc93b*^{-/-} and *Tlr7/9*^{-/-}
433 mice.

434

435 We observed route-dependent differences in viral replication in our studies that can be
436 explained by the distribution of TLR and UNC93B expression in the mouse. Levels of full-
437 length TLR9 and UNC93B are greatly reduced in the lung compared to the spleen, and
438 cleaved TLR9, required for signaling upon detection of viral DNA, is not detectable in the
439 lung (11) (54). Likewise, TLR7 is strongly expressed in the spleen, but not the lung (51). In
440 contrast, mRNA for Mab-21 domain containing 1, which encodes the PRR cGAS, is
441 expressed similarly in the lung and spleen (55). MHV68 infection of cGAS-deficient mice
442 leads to a 2-fold increase of viral titers in the spleen and 3.5-fold increase of viral titers in

443 the lung compared to WT mice (56). During primary infection of the lung after intranasal
444 infection, MHV68 replication is thus controlled primarily in a non-endosomal TLR
445 dependent manner; cGAS is the most likely PRR involved in detection, although RIG-I and
446 other non-TLR PRR may also be involved. As all of the mice analyzed in our experiments
447 were TLR-deficient, we would not expect to see a difference in primary viral replication in
448 these mice upon intranasal infection. In contrast, upon intravenous infection, the spleen is
449 one of the first sites of viral replication (19). In the spleen, UNC93B and endosomal TLR
450 expression is high, leading to an effect of TLR on early viral replication.

451

452 At least three publications have examined TLR ligand stimulation of latently infected cell
453 lines and mice (38, 57, 58). In one of these studies, treatment of infected A20 B cell lines
454 with TLR3, 4, 5, or 9 ligands enhanced MHV68 reactivation (57). In a separate study, TLR7
455 and TLR9 ligand stimulation inhibited reactivation of S11 cells, which are derived from
456 naturally occurring MHV68-induced lymphoproliferation (38). These results likely differ
457 due to the different cell types or establishment of infection in them. *In vivo*, however,
458 repeated stimulation of MHV68-infected WT mice with the TLR7 ligand R848 increased the
459 number of latently infected splenocytes (38). Likewise, injection of latently-infected mice
460 with the TLR9 ligand CpG DNA resulted in increased MHV68 genome copies and enhanced
461 reactivation of MHV68 in an *ex-vivo* reactivation assay (58).

462

463 These results regarding TLR stimulation and latency may seem at odds with the increased
464 frequency of latent genome copies we have observed in *Tlr7/9*^{-/-} mice. However, these are
465 two separate mechanisms. When multiple endosomal TLR are nonfunctional, for example

466 in *Unc93b*^{-/-} mice, viral replication after intravenous infection is already increased
467 compared to WT mice. The presence of more virus increases latent genome copy numbers
468 of MHV68, which we observed. In WT mice however, viral replication is controlled at the
469 beginning. Establishment of latency occurs, but at a reduced frequency compared to *Tlr*^{-/-}
470 mice. However, when TLR signaling is activated in WT cells with synthetic ligands, latently
471 infected cells are reactivated, allowing for increased viral spread, leading to establishment
472 of latency in additional cells.
473
474 TLR signaling is initiated via the adaptor protein MyD88, but as mentioned, whether
475 MyD88 is important for MHV68 detection or control is unclear. In a study that reported no
476 effect on early replication, the authors observed a decreased anti-MHV68 B-cell response in
477 *Myd88*^{-/-} mice and a decreased ability of MHV68 to establish latency in the spleen of *Myd88*^{-/-}
478 mice after intranasal infection (17). The authors found that this effect was directly related
479 to the absence of MyD88 in B cells, and was independent of IL-1R signaling (17). However,
480 after intraperitoneal infection, latent viral loads were not affected. Interestingly, though,
481 reactivation was actually enhanced in *Myd88*^{-/-} peritoneal exudate cells (PEC), and the
482 authors suggested MyD88 may be important for suppressing reactivation from latently
483 infected PEC (17). Likewise, we found that two MyD88-dependent TLR, TLR7 and TLR9,
484 may be important for suppressing reactivation, but in a different cell type. Further study to
485 understand this apparent discrepancy is required, but differences in infection route and
486 infectious dose are possible explanations.
487

488 In summary, two TLR, TLR7 and TLR9, cooperate in recognition of MHV68. This is the first
489 report of the contribution of TLR7 to MHV68 detection, and demonstrates the complexity
490 of overlapping and redundant pattern recognition pathways. Further studies on TLR and
491 PRR will thus likely benefit from analyzing combinations of PRR, rather than individual
492 PRR alone.

494 **MATERIALS AND METHODS**

495 **Ethics statement**

496 All animal experiments were performed in compliance with the German animal protection
497 law (TierSchG BGBI S. 1105; 25.05.1998). The mice were handled in accordance with good
498 animal practice as defined by FELASA and GV-SOLAS. All animal experiments were
499 approved by the responsible state office (Lower Saxony State Office of Consumer
500 Protection and Food Safety) under permit numbers #33.9-42502-04-12/0930 and #33.19-
501 42502-04-17/2657.

503 **Mice**

504 WT and KO mice were bred and maintained under specific-pathogen-free conditions at the
505 animal facility of the Helmholtz Centre for Infection Research in Braunschweig, Germany.
506 WT C57BL/6J and C57BL/6NCrl were originally obtained from Charles River Laboratories.
507 For most experiments, WT controls were bred in-house. For some experiments, C57BL/6J
508 Rj were purchased from Janvier Labs and C57BL/6NCrl were purchased from Charles River
509 Laboratories.

510 *Tlr13*^{-/-} (C57BL/6N-*Tlr13*^{tm1(KOMP)Vlcg}) and *Unc93b*^{-/-} (B6.*Unc93b1*^{tm1(KOMP)Vlcg}) mice are on
511 the C57BL/6NTac background and were originally obtained as *Tlr13*^{-/-} ES cells, clone
512 *Tlr13*_BC7, or *Unc93b*^{-/-} sperm from the mouse strain derived from ES cell clone 10049A-
513 G9, from the NCRR-NIH supported mouse strain KOMP Repository www.komp.org (U42-
514 RR024244). Velocigene targeted alleles (50) were generated by Regeneron
515 Pharmaceuticals, Inc. for KOMP. Germline positive *Tlr13*^{-/-} mice were backcrossed for 2
516 generations onto C57BL/6NTac mice, then interbred and maintained as a homozygous
517 colony. For *Unc93b*^{-/-} mice, oocytes of C57BL/6NCrl were *in vitro* fertilized. Heterozygous
518 offspring were interbred and maintained as a homozygous colony.
519 *Tlr2*^{-/-} (B6.129-*Tlr2*^{tm1Kir/J}) mice (59) were backcrossed to C57BL/6J mice for 9 generations
520 and were originally obtained from the Jackson Laboratory (004650) (60). *Tlr7*^{-/-}
521 (B6.129P2-*Tlr7*^{tm1Aki}) mice (51) were kindly provided by Stefan Bauer (Marburg, Germany)
522 and were backcrossed to C57BL/6J for 10 generations (61). *Tlr9*^{-/-} (B6.129P2-*Tlr9*^{tm1Aki})
523 mice (52), backcrossed to C57BL/6J mice for 12 generations, were kindly provided by
524 Stefan Bauer (Marburg, Germany). *Tlr7/9*^{-/-} double knockout mice were bred for this study
525 by crossing *Tlr7*^{-/-} and *Tlr9*^{-/-} mice.

526

527 **Cell lines and cell culture**

528 Murine bone marrow stromal M210-B4 (ATCC CRL-1972) and murine embryonic fibroblast
529 NIH/3T3 (ATCC CRL-1658) cells were obtained from the American Type Culture Collection
530 (ATCC) and cultured in basal medium of high-glucose DMEM containing glutamine,
531 supplemented with 8% fetal calf serum (FCS), 1% pen/strep, and in the case of NIH3T3
532 cells, 1mM sodium pyruvate and 1% non-essential amino acids. B16 Flt-3L cells, expressing

533 FMS-like tyrosine kinase 3 ligand (Flt-3L), were made by Dr. H Chapman (62), and like
534 primary Flt-3L induced dendritic cells (FLDC), were cultured in RPMI containing glutamine
535 and supplemented with 10% FCS, 1% pen/strep, and 50 μ M β -mercaptoethanol. All cells
536 were cultured at 37°C in a humidified 7.5% CO₂ incubator.

537

538 **Virus stock preparation**

539 MHV68 WUMS is from the ATCC (VR-1465). Recombinant viruses MHV68-GFP (63), the
540 MHV68 sncRNA ko, lacking all known sncRNAs (Δ miR), and its revertant (REV) (33), the
541 MHV68 muSOX mutant deficient in host shutoff activity, Δ HS, and its revertant (REV) (34),
542 and MCMV-GFP (64) have all been previously described. Virus stocks were amplified in
543 M210-B4 cells, concentrated, purified over a 10% Nycodenz cushion in virus standard
544 buffer (VSB, 50mM Tris-HCl, 12mM KCl, 5mM Na-EDTA, pH 7.8), resuspended in VSB, and
545 flash frozen as previously described (65-68). Virus stock titers were determined on M210-
546 B4 cells using the median tissue culture infective dose (TCID₅₀ method) for MHV68 strains
547 and plaque assay for MCMV. Newcastle disease virus LaSota strain (NDV) was kindly
548 provided by Andrea Kröger (Helmholtz Centre for Infection Research, Braunschweig,
549 Germany).

550

551 **Preparation and sorting of primary FLDC**

552 To obtain sufficient pDC numbers after MACS or FACS sorting, bone marrow was combined
553 from multiple mice of the same genotype for all experiments. Briefly, the femur and tibia of
554 mice of the indicated genotypes were cleaned of tissue and bone marrow was either
555 extruded using a medium-filled syringe equipped with a 25G needle, or by centrifugation.

556 After resuspension in RPMI, red blood cells were lysed. Cells were cultured for 8 days at 1.5
557 $\times 10^6$ cells/ml in complete RPMI supplemented with 2.5% conditioned medium from B16
558 Flt-3L cells. The resulting FLDC were then either seeded for experiments directly, or sorted
559 using MACS or FACS into plasmacytoid or conventional dendritic cells (pDC or cDC,
560 respectively). For MACS sorting, pDC were enriched from FLDC using the discontinued
561 negative-selection plasmacytoid dendritic cell isolation kit II (Miltenyi Biotec 130-092-786)
562 according to the manufacturer's instructions. For FACS sorting, FLDC were labeled with
563 APC-coupled anti-mouse/human CD45R/B220, clone RA3-6B2 (Biolegend 103212), and
564 PE-coupled anti-mouse CD11c, clone N418 (Biolegend 117308), then sorted on a FACS Aria
565 into CD45R/B220⁺ CD11c^{mid} pDC and CD45R/B220⁻ CD11c^{high} cDC.

566

567 **Stimulation of primary DC**

568 Equal cell numbers were seeded in 100 μ l volume in 96-well plates in each experiment, then
569 mock treated, infected at an MOI of 0.5 or 2 with the indicated virus, or stimulated with
570 TLR agonists by addition of 100 μ l of the appropriate control or stimulus diluted in FLDC
571 medium. For TLR9 activation, cells were treated with 1 μ M final concentration CpG DNA
572 ODN 2336. For stimulation of TLR7 with polyU (Invivogen tlrl-sspu), polyU was first
573 complexed with DOTAP (Roche 11811177001). 2 μ g polyU was diluted to a final volume of
574 20 μ l with HBS. Separately, 12 μ l DOTAP was diluted to 40 μ l with HBS. Diluted DOTAP and
575 polyU were combined, incubated for 15 minutes, and diluted to 1ml total volume with
576 FLDC medium. 100 μ l were added per well, for a final concentration of 1 μ g/ml. Cells were
577 stimulated with TLR ligands for 18h or viruses for 24h, then supernatants were harvested,
578 clarified, and stored at -20°C for ELISA analysis.

579

580 **Antiviral Treatment of pDC**

581 FACS-sorted *Tlr9*^{-/-} pDC were seeded into 96-well plates for ELISA (3×10^5 cells in
582 150 μ l/well) or 6-well plates for q-RT-PCR and ELISA (3×10^6 cells in 1.5ml/well). Cells
583 were then pre-treated for approximately 3h with 20 μ M acyclovir in DMSO in 0.1% final
584 DMSO concentration or 0.1% DMSO vehicle control. 20 μ M acyclovir is approximately 3 \times
585 the EC₅₀ for MHV68 and about 10 \times less than the IC₅₀ (69) . Cells were then infected with
586 MHV68 or stimulated with DOTAP or polyU with the final concentrations described under
587 **Stimulation of primary DC**. Cells were incubated for 24h post infection or stimulation
588 with a final concentration of 15 μ M acyclovir in 0.75% DMSO or 0.75% DMSO vehicle
589 control. Supernatants were harvested, clarified, and stored at -20°C for ELISA analysis.
590 Cells from 6-well plates were collected for RNA extraction and q-RT-PCR.

591

592 **IFN α ELISA**

593 IFN α levels were measured by ELISA. For most experiments, a rat anti-mouse IFN α capture
594 antibody (PBL 22100-1), a rabbit anti-mouse IFN α detection antibody (PBL 32100-1), and
595 an HRP-coupled donkey anti-rabbit IgG antibody (Jackson ImmunoResearch 711-036-152)
596 were used with mouse interferon α standard (PBL 12100-1) concentrations between 78
597 and 5000pg/ml. Similar results were obtained with the more sensitive PBL Verikine mouse
598 IFN α ELISA kit with IFN α standard concentrations between 12.5 and 400pg/ml.

599

600

601 **Quantitative reverse transcription polymerase chain reaction analysis of viral gene**

602 **expression**

603 RNA was isolated using the innuPREP RNA mini kit (Analytik Jena). The kit includes a
604 genomic DNA removal column step. RNA was diluted to equal concentrations and cDNA
605 was synthesized using the iScript gDNA clear cDNA synthesis kit (Biorad). Transcript levels
606 were determined using GoTaq qPCR master mix (Promega) and oligonucleotide pairs
607 specific for ORF50/RTA, ORF68, ORF4, ORF8, ORF65/M9, and 18s rRNA (48, 65). The viral
608 mRNA expression levels were normalized to 18s rRNA and compared using the $2^{-\Delta\Delta Ct}$
609 method (70).

610

611 ***In vivo* infections and analysis of lytic titers in organs**

612 Mice were anesthetized with ketamine/xylazine and infected intranasally (IN) with 2×10^6
613 PFU MHV68 WUMS in 20 μ l PBS/mouse. Alternatively, mice were humanely restrained and
614 infected intravenously (IV) with 2×10^6 PFU MHV68 WUMS in 100 μ l PBS/mouse. 3 days
615 post infection, mice were euthanized with CO₂. Organs were harvested, weighed,
616 homogenized using the FastPrep24 (MP Biomedicals), and frozen at -70°C. To determine
617 lytic titers, homogenates were thawed, mixed, and clarified supernatants were titered by
618 TCID₅₀ on M210-B4 cells. The limit of detection was $10^{2.10}$ TCID₅₀/ml for spleen and lungs
619 and $10^{3.10}$ TCID₅₀/ml for liver, based on organ toxicity and the minimum number of
620 positive wells. Data are combined from multiple independent experiments; individual mice
621 are shown.

622

623 **Reactivation assay and determination of latent viral load**

IV-infected mice were maintained for 22 days to allow establishment of MHV68 latency and reactivation assays were performed essentially as described (14). On day 21, 10,000 NIH/3T3 cells were plated per well of 96-well plates in 100µl/well. On day 22, 2-4 spleens per genotype were harvested, disrupted over 70µm mesh filters, and pooled to produce single-cell suspensions. After red blood cell lysis, splenocytes were 3-fold serially diluted from 1.5×10^6 cells/ml to 690 cells/ml. Twenty-four 100µl replicates of each dilution were seeded onto NIH/3T3 cells and co-cultured for 14 days. Wells were scored for CPE and the percent of positive wells was determined. To control for preformed infectious virus, splenocyte dilutions were twice frozen and thawed and plated similarly onto NIH/3T3 cells; positive wells were subtracted from the relevant live splenocyte dilution. Reactivation assays were performed at least 3 independent times. To determine the frequency of reactivation, the variable slope sigmoidal dose-response equation was used with the regression line intersect set to 63.2% based on the Poisson distribution. To determine the latent viral load, DNA was isolated in duplicate from 2×10^7 splenocytes using a modified version of a published protocol (71). First, splenocytes were digested overnight at 56°C with proteinase K in lysis buffer (100mM Tris-HCl, pH 8.5, 200mM NaCl, 5mM EDTA, 0.2% SDS, supplemented with 5µl 20mg/ml proteinase K per ml buffer) followed by isopropanol precipitation. DNA was then wound around a pipette tip, transferred to a fresh tube, dried, and resuspended in 500µl TE Buffer (10mM Tris-HCl, 10mM EDTA, pH 8.0). 50ng DNA was then used to determine copy numbers, usually in duplicate, for MHV68 gB and murine *B2M* by qPCR using the Roche Universal Probe Library and specific primers (MHV68 gB: cgtatgtcagccaaccactct and agtctctgttgagcgtcct, Probe 46, *B2M*: GTGGTGCCAGCAGAGACTTA and GGACAGTGG GTAGGGAAGT, Probe 40). gB copies

647 were normalized to *B2M* for each sample, then scaled to the average of the appropriate
648 control. Per pooled splenocyte sample analyzed for reactivation, 3-4 qPCR reactions were
649 analyzed. Each data point represents one qPCR reaction.

650

651 **Statistical analysis**

652 Statistical analysis was performed using Graphpad Prism. Significance for ELISA data and
653 MHV68 genome copies was determined by two-tailed unpaired t test of log-transformed
654 data. For viral titers in organs, significance was determined by two-tailed Mann-Whitney
655 test.

656

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668

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896 **FIGURE LEGENDS**897 **Figure 1. Both TLR7 and TLR9 are required for the IFN α response to MHV68 in pDC.**

898 (A-D) Bone marrow cells were cultured in the presence of Flt3 ligand (Flt-3L) for 8 days to
899 produce FLDC and pDC were MACS purified. (A) WT, *Tlr7*^{-/-}, and *TLR9*^{-/-} or (B) WT, *Unc93b*^{-/-},
900 ^{-/-}, or *Tlr7/9*^{-/-} pDC were mock infected or infected with MHV68, MCMV, or NDV as indicated.
901 (C, D) pDC of the indicated genotype were treated with (C) DOTAP (control), the TLR7
902 agonist polyU complexed with DOTAP, (D) medium (-), or the TLR9 agonist CpG DNA as
903 indicated. (E) pDC from WT, *Unc93b*^{-/-}, *Tlr2*^{-/-}, and *Tlr13*^{-/-} FLDC were prepared by MACS
904 negative selection. (F) FLDC from WT, *Tlr7*^{-/-}, *Tlr9*^{-/-}, and *Tlr7/9*^{-/-} mice were prepared. (G)
905 WT FLDC were reserved or stained with APC-coupled anti-mouse/human CD45R/B220
906 and PE-coupled anti-mouse CD11c, then separated into CD45R/B220⁺ CD11c^{mid}
907 plasmacytoid dendritic cells (pDC) and CD45R/B220⁻ CD11c^{high} conventional dendritic cells
908 (cDC) by FACS. (E-G) Equal cell numbers were plated, mock infected or infected with
909 MHV68. (A-G) The IFN α response was measured by ELISA. (A-B) Combined duplicates from
910 two independent experiments are shown for MHV68 and log-transformed data for MHV68
911 were analyzed by two-tailed unpaired t test. **: P<0.01, ***: P<0.001. For all other graphs
912 (MCMV and NDV in A-B and C-G), duplicates from one representative experiment are
913 shown.

914

915 **Figure 2. Early acute replication of MHV68 after intranasal infection is TLR-**

916 **independent and limited to the lungs.** Mice of the indicated genotype were infected
917 intranasally with 2 \times 10⁶ PFU MHV68. (A-C) Body weight was measured daily. Data are
918 combined from at least two independent experiments, with 8 or more mice total per

919 genotype. Data were analyzed by two-tailed Mann-Whitney test. *: $P < 0.05$ (D) Lungs and
920 (E) spleen were harvested 3 days post infection and viral titers were determined by the
921 median tissue culture infectious dose method (TCID₅₀) on M2-10B4 cells. Splenic titers
922 were almost exclusively around the average detection limit (approximate line in gray) or
923 negative (indicated by \log_{10} TCID₅₀/g = 0). Each data point represents one mouse.

924
925 **Figure 3. The absence of endosomal TLR minimally affects weight loss after**
926 **intravenous infection with MHV68.** Mice of the indicated genotype were infected
927 intravenously with 2×10^6 PFU MHV68. Body weight was measured daily. Data are
928 combined from at least two independent experiments, with 8 or more mice total per
929 genotype. Statistical significance was determined by Mann-Whitney test for days 3, 7, 8, 9,
930 and 10. *: $P < 0.05$, **: $P < 0.01$, ***: $P < 0.001$

931
932 **Figure 4. Early acute replication of MHV68 after intravenous infection is enhanced in**
933 **the absence of endosomal TLR, especially TLR9.** Mice of the indicated genotypes were
934 infected intravenously with 2×10^6 PFU MHV68. 3 days post infection, (A) lungs, (B) spleen,
935 or (C) liver were harvested and viral titers were determined by TCID₅₀ on M2-10B4 cells.
936 Data were analyzed by two-tailed Mann-Whitney test. *: $P < 0.05$, **: $P < 0.01$, ***: $P < 0.001$

937
938 **Figure 5. Reactivation and latent viral load are increased in the absence of**
939 **endosomal TLR.** Mice of the indicated genotypes were infected with 2×10^6 PFU MHV68
940 intravenously. 21 days post infection, spleens were harvested and single-cell splenocyte
941 suspensions pooled from multiple mice were prepared. (A-C) Three-fold serial dilutions of

splenocytes were co-cultured with NIH3T3 fibroblasts for two weeks and reactivation was determined based on the presence of cytopathic effect in the NIH/3T3 monolayer. Curve fit lines were derived from nonlinear regression analysis based on the variable slope sigmoidal dose-response equation. The regression line intersect was set to 63.2% based on the Poisson distribution. (D) DNA was isolated from pooled splenocytes and the gB copy number was determined relative to B2M and scaled to the average of the B6N or B6J WT control as appropriate. For each set of pooled splenocytes, copy numbers were determined for 3-4 replicates. Data points represent individual replicates. Log-transformed data (data points for $>10^3$ splenocytes, A-C, and all samples in D) were analyzed by two-tailed unpaired t test. *: $P<0.05$, **: $P<0.01$, ***: $P<0.001$

952

Figure 6. MHV68 miRNA, MHV68-mediated host shutoff, and MHV68 DNA polymerase activity minimally affect TLR7 signaling. *Tlr9*^{-/-} bone marrow cells were cultured in the presence of Flt-3L for 8 days to produce FLDC. (A-B) Cells were mock infected or stimulated with (A) miRNA-deficient MHV68 (Δ miR), its revertant (REV), or MHV68-GFP (GFP), or (B) host shutoff-deficient MHV68 (Δ HS), its revertant (REV), or MHV68-GFP (GFP), (A, B) at the indicated MOI. The IFN α response was measured by ELISA. Combined quadruplicates from three independent experiments are shown. (C-E) *Tlr9*^{-/-} FLDC were sorted into B220⁺CD11c^{mid} pDC and pretreated with DMSO or acyclovir for three hours. Cells were then infected with MHV68 (C,E) or stimulated with the TLR7 ligand polyU (D) for 24 hours. (C-D) The IFN α response was measured by ELISA. Combined duplicates from three independent experiments are shown. (E) Viral mRNA expression of RTA, ORF68, ORF4, ORF8, or ORF65 was measured by q-RT-PCR, normalized to 18s rRNA, and compared

965 using the $2^{-\Delta\Delta C_t}$ method. Combined duplicates from two independent experiments are
966 shown. (A-D) Log-transformed data were analyzed by two-tailed unpaired t test. ns, not
967 significant; ***: $P < 0.001$
968











