1

2 Antagonism of RNase L is required for murine coronavirus replication in Kupffer cells and liver

3 sinusoidal endothelial cells but not in hepatocytes

4 Yize Li and Susan R. Weiss

5 Department of Microbiology, Perelman School of Medicine, University of Pennsylvania, Philadelphia,
6 PA 19104

7

8

9 Running title: KC and LSEC limit MHV NS2 mutant liver replication

10 Key words: Murine coronavirus, MHV, phosphodiesterase, hepatocytes, Kupffer cells, liver
11 sinusoidal endothelial cell, hepatitis

*Corresponding Author

Susan R. Weiss
Department of Microbiology
University of Pennsylvania
Perelman School of Medicine
203A Johnson Pavilion
36th Street and Hamilton Walk
Philadelphia, PA 19104-6076
PHONE: 215-898-8013
FAX: 215-573-4858
Email: weisssr@upenn.edu

Abstract: 250 words

Text: 3544 words

12 **ABSTRACT**

13   Mouse hepatitis virus strain A59 infection of mice is a useful tool for studying virus-host interaction

14   during hepatitis development. The NS2^{H126R} mutant is attenuated in liver replication due to loss of

15   phosphodiesterase activity, which the wild-type virus uses to block the 2′,5′-oligoadenylate

16   synthetase (OAS)-ribonuclease L (RNase L) antiviral pathway. The activation of RNase L by

17   NS2^{H126R} is cell-type dependent and correlates with high basal expression levels of OAS, as found in

18   myeloid cells. We tested the hypothesis that resident liver macrophages, Kupffer cells (KC), are the

19   most likely cell type to restrict NS2^{H126R} and prevent hepatitis. As found previously, A59 and NS2^{H126R}

20   replicate similarly in hepatocytes and neither activates RNase L, as assessed by an rRNA

21   degradation assay. In contrast, in KCs, A59 exhibited a 100-fold higher titer than NS2^{H126R} and

22   NS2^{H126R} induced rRNA degradation. Interestingly, in liver sinusoidal endothelial cells (LSEC), the

23   cells that form a barrier between blood and liver parenchymal cells, NS2^{H126R} activates RNase L,

24   which limits viral replication. Similar growth kinetics were observed for both viruses in KC and LSEC

25   from RNase L$^{-/-}$ mice, demonstrating that both use RNase L to limit NS2^{H126R} replication. Depletion of

26   KC by gadolinium(III) chloride or LSEC by cyclophosphamide partially restores liver replication of

27   NS2^{H126R}, leading to hepatitis. Thus, during MHV infection, hepatitis, which damages the

28   parenchyma, is prevented by RNase L activity in both KC and LSEC but not in hepatocytes. This

29   may be explained by the undetectable levels of RNase L as well as OASs expressed in hepatocytes.

30

31

32

33

34

35

36

37

38   **IMPORTANCE**

39   Mouse hepatitis virus infection of mice provides a useful tool for studying virus-host interactions

40   during hepatitis development. The NS2^{H126R} mutant is attenuated in liver replication due to loss of

41   phosphodiesterase activity, with which the wild-type virus blocks the potent OAS-RNase L antiviral

42   pathway. RNase L activation by NS2^{H126R} is cell-type dependent and correlates with high basal

43   expression levels of OAS, as found in myeloid cells. We showed that hepatocytes that comprise the

44   liver parenchyma do not activate RNase L when infected with NS2^{H126R} nor do they restrict

45   replication. However, both Kupffer cells (KC), liver resident macrophages and liver sinusoidal

46   endothelial cells (LSEC) which line the sinusoids activate RNase L in response to NS2^{H126R}. These

47   data suggest that KC and LSEC prevent viral spread into the parenchyma, preventing hepatitis.

48   Furthermore, hepatocytes express undetectable levels of OASs and RNase L, which likely explains

49   the lack of RNase L activation during NS2^{H126R} infection.

50

51

52

53

54

55

56

57

58

59

60

61

62

63

64   **INTRODUCTION**

3

65   Mouse hepatitis virus (MHV) belongs to the order *Nidovirales*, family *coronaviridae*, and genus

66   *Betacoronavirus.* Coronaviruses are enveloped, nonsegmented positive-strand RNA viruses (1).

67   MHV strain A59 induces moderate to severe hepatitis in infection of mice (2); replication and the

68   consequent liver pathogenesis are dependent on the 2',5'-phosphodiesterase (PDE) activity of the

69   viral accessary protein NS2 (3).

70   The 2′,5′-oligoadenylate (2-5A) synthetase (OAS)-ribonuclease (RNase) L pathway is a potent IFN-

71   induced antiviral activity (4). Upon infection by diverse viruses, including the human liver pathogenic

72   hepatitis C virus (5, 6), dsRNA is detected by OAS proteins. Four mouse OAS species (OAS1a/g,

73   OAS2, OAS3 and OASL2) and 3 human OAS species (OAS1, OAS2, OAS3) can bind dsRNA *in*

74   *vitro* and be activated to generate 2-5A (7), the latter binding to and promoting activation and

75   dimerization of RNase L, which results in both cellular and viral RNA degradation and thus inhibition

76   of viral replication (7, 8).

77   The PDE (NS2) of MHV cleaves 2-5A and inhibits the activation of RNase L(3). An A59 mutant,

78   NS2^{H126R}, which encodes a catalytically inactive PDE replicates to a minimal extent in the mouse

79   liver and causes minor to no liver damage during infection (3). Previous studies have illuminated that

80   the activation of the OAS-RNase L antiviral pathway is cell type dependent. Bone marrow derived

81   macrophages (BMM) and bone marrow derived dendritic cells (BMDC) as well as microglia, brain

82   resident macrophages, limit NS2^{H126R} replication by activating the OAS-RNase L pathway. The basal

83   mRNA expression levels of OASs vary among the cell types and high levels of OAS correlate with

84   activation of the pathway (9, 10).

85   While the liver is known as a digestive organ, it also serves as an immunological organ (11-13). The

86   liver is composed of two populations of cells, including approximately 80% liver parenchymal cells

87   (hepatocytes) and 20% nonparenchymal cells (NPC). About 10% NPC are Kupffer cells (KC), or

88   liver macrophages, and 50% of NPC are liver sinusoidal endothelial cells (LSEC) (13). We found

89   previously that NS2^{H126R} infection failed to induce RNase L activation in hepatocytes and that

90   NS2^{H126R} replicated to similar extent as A59 in that cell type (9). However, little is known about the

91   activation of the OAS-RNase L pathway in the other liver cell types and specifically in which cell type

92   RNase L activation will limit NS2^{H126R} replication, although previous studies implicated a protective

93   role of KC(3). Here, we isolated the various primary liver cells and infected them with WT A59 and

94   NS2^{H126R} to assess the activation of RNase L as well as quantify viral replication from these cells.

95   We found KC and LSEC but not hepatocytes can limit NS2^{H126R} replication, indicating they have an

96   active OAS-RNase L pathway and this is likely due to differential levels of expression of RNase L as

97   well as OAS among these liver cell types. To further study the protective role of KC and LSEC, we

98   performed *in vivo* cell depletion in mice and showed depletion of KC or LSEC enhance NS2^{H126R} liver

99   replication and pathogenicity.

100   **MATERIAL AND METHODS**

101   **Cells lines, mice and viruses.** Mouse L2 fibroblasts were cultured as described previously (14).

102   Recombinant coronaviruses inf-MHV-A59 (A59) and inf-NS2^{H126R} (NS2^{H126R}) have been described

103   previously (15, 16). C57BL/6 (B6) mice were purchased from the National Cancer Institute

104   (Frederick, MD), and bred in the University of Pennsylvania animal facility. All procedures were

105   approved by the University of Pennsylvania Institutional Animal Care and Use Committee (IACUC).

106   **Isolation of primary liver cells.** Mice were euthanized with CO_2, and the livers were perfused.

107   Briefly, the animal was opened to expose the inferior vena cava (IVC) and portal vein, a catheter

108   was inserted into the IVC, and connected to a pump filled with dissociation buffer (NaCl, 8g/L, KCl,

109   0.4g/L, NaH$_2$PO$_4$ •H2O, 78mg/L, Na$_2$HPO$_4$ 120.45mg/ml, HEPES, 2380mg/L, Na$_2$CO$_3$, 350mg/L,

110   ethylene glycol tetraacetic acid (EGTA), 190mg/L, glucose 900mg/L, pH=7.4). The portal vein was

5

111   cut immediately to allow the buffer (20-30 ml) to flow through the liver.

112   For hepatocytes, the liver was digested with collagenase IV (0.05%) (Sigma-Aldrich) in liver

113   digestion buffer (NaCl, 8g/L, KCl, 0.4g/L, NaH$_2$PO$_4$ •H2O, 78mg/L, Na$_2$HPO$_4$ 120.45mg/ml, HEPES,

114   2380mg/L, Na$_2$CO$_3$, 350mg/L, phenol red, 6mg/L, CaCl$_2$ •2H2O, 560mg/L, pH=7.4). After digestion,

115   the liver was removed from the mouse and cells were dissociated from Glisson's capsule and were

116   filtered through a 100µm cell strainer (Becton Dickinson, BD). The cells were centrifuged at 40×g for

117   3min at 4°C, and the pellet was re-suspended in digestion buffer containing 1mg/ml DNase I

118   (Roche). The cells were centrifuged and washed with MEM (minimum essential medium Eagle,

119   Sigma-Aldrich) three times and were re-suspended in William's E media (Gibco) supplemented with

120   10% FBS, 100U/ml of penicillin and 100mg/ml streptomycin, 2mM L-Glutamine. The cells were

121   placed on plates coated with rat-tail collagen (Sigma-Aldrich). Four hours after plating, the cells were

122   washed with phosphate buffer saline (PBS) and used for experimentation.

123   Kupffer cells (KC) were isolated from mixed primary liver cell cultures using previously described

124   methods (17). Briefly, after perfusion with dissociation buffer, the liver was digested with collagenase

125   I (0.05%)(Sigma-Aldrich) in digestion buffer supplemented with 50µg/ml Trypsin inhibitor (Sigma-

126   Aldrich). Cells were harvested and washed three times with MEM, and cultured in DMEM (Gibco,

127   10566) supplemented with 10% FBS, 100U/ml of penicillin and 100mg/ml streptomycin, 100µM β-

128   mercaptoethanol, 10µg/ml insulin, 10mM HEPES and 50µg/ml gentamicin, in a flask coated with rat-

129   tail collagen, 1X10^7 cells per T175 flask. The next day, the flasks were shaken to remove the dead

130   cells, and on days 4 and day 7, fresh medium was added and the KC were harvested at days 9 to 12

131   post plating by shaking and then selected by binding to a petri dish (BD). KC were recovered by

132   TrpLE select enzyme (Invitrogen) digestion and plated for experiments.

133   The liver sinusoidal endothelial cell (LSEC) isolation protocol was adapted from previously described

134   methods (18). Briefly, after perfusion with dissociation buffer, the liver was digested with collagenase

135   I (0.025%) and collagenase II (0.025%)(Sigma-Aldrich) in digestion buffer supplemented with

136   50µg/ml Trypsin inhibitor (Sigma-Aldrich). After digestion, the liver was removed and cells were

137   dissociated from Glisson's capsule in preservation buffer [NaCl, 4.15g/L, 0.25g/L KCl, 1.12g/L

138   HEPES, 0.24g/L NaOH, bovine serum albumin (BSA),10g/L)], and filtered through a 100µm cell

139   strainer (BD). The supernatant was centrifuged at $60\times g$ for 2 min three times to remove

140   parenchymal cells. The supernatant was further centrifuged at $1350\times g$ 4°C for 10min to harvest the

141   non-parenchymal cells (NPC). The NPC were re-suspended in preservation buffer and were applied

142   on top of a 25/50% Percoll (GE Amersham) bilayer. The cells were centrifuged at $1350\times g$ 4°C for

143   10min. LSEC and KC were collected from the interface between the two density layers.. Cells were

144   washed with preservation buffer and resuspended in RPMI 1640 medium (Gibco) supplemented

145   with100U/ml of penicillin and 100mg/ml streptomycin. Cells then were plated onto a petri dish and

146   incubated for 8 min to remove KC which attach to the surface; the removal of KC was repeated once

147   more. The supernatant containing the LSEC was harvested and centrifuged at $1350\times g$ 4°C for

148   10min. The pellet was resuspended in RPMI 1640 medium (Gibco) supplemented with 100U/ml of

149   penicillin and 100mg/ml streptomycin and placed onto rat-tail collagen coated plates. Two hours

150   after plating, the cells were washed vigorously with ice cold PBS twice. The cells remaining on the

151   plates were used for further experiments.

152   **Antibodies**. Mouse anti human HNF-4α (cross reactive with mouse HNF-4α) monoclonal antibody

153   (mAb) (5µg/ml, R&D), and goat anti mouse IgG conjugated to Alexa Fluor-488 (1:400, Invitrogen)

154   were used to detect HNF-4α by immunofluorescence assay (IFA); rat anti mouse CD68 (1:100,

155   Serotech) mAb, conjugated to Alexa Fluor-488 were used to detect CD68 by IFA. OAS1A (clone E-

156   2, Santa Cruz; 1:200) mAb, OAS2 (clone G-9, Santa Cruz, 1:200), OAS3 (clone D-7, Santa Cruz,

157   1:200), goat anti RNase L (clone T-16, Santa Cruz, 1:200), as well as anti-GAPDH (Thermo-Fisher,

158   1:1000), goat anti-mouse IgG-HRP (Santa Cruz, 1:5000), and donkey anti-goat IgG-HRP (1:5000)

7

159 secondary antibodies were used to detect the primary antibodies of the appropriate species.

160 **Immunolabeling.** Cells were fixed in PBS containing 4% paraformaldehyde (Bio-Rad), blocked with

161 2% BSA, and immunolabeling with anti HNF-4α antibodies was used to detect hepatocytes;

162 immunolabeling with anti-CD68 antibodies conjugated with Alexa Fluor-488 was used to distinguish

163 KC (CD68 positive) and LSEC (CD68 negative). Primary anti-HNF-4α antibody was detected with

164 goat anti-mouse Alexa Fluor 488 (Invitrogen). DAPI (4',6-Diamidino-2-Phenylindole, Dihydrochloride)

165 (Invitrogen) was used to stain the nucleus. Fluorescence was visualized with a Nikon Eclipse 2000E-

166 U fluorescence microscope, and images were acquired using Nikon NIS-Element BR software

167 (Nikon)

168 Low density lipoprotein (LDL) uptake assay of LSEC. After isolation, cells were cultured in RPMI

169 1640 without FBS, medium containing 5µg/ml of LDL conjugated with Alexa Fluor-488 (Invitrogen)

170 was added, the cells were incubated at 37°C for 4 hours, and at 30 minutes before imaging, 1µg/ml

171 of Hoechst (Sigma-Aldrich) was add to the medium. Fluorescence was visualized with a Nikon

172 Eclipse 2000E-Ufluorescence microscope, and images were acquired using Nikon NIS-Element BR

173 software(Nikon)

174 **Virus growth kinetics.** Hepatocytes (1.5×10^5 cell per well in 24-well plates), KC (1×10^5 cell in 48-

175 well plates) or LSEC (5.0×10^5 cell per well in 48-well plates) were infected with A59 or NS2[H126R]

176 (MOI=1 PFU/cell) and at the indicated time points, cells and supernatants were harvested together,

177 and then frozen (-80°C) and thawed three times and finally viruses were titrated by L2 cell plaque

178 assay (19).

179 **KC and LSEC depletion and MHV infection.** Gadolinium(III) chloride was used deplete KC from

180 the livers of mice. B6 mice were intravenously injected with 20mg/kg of Gadolinium(III) chloride in

181 100µl of saline (20). Cyclophosphamide was used to deplete LSEC from the livers of mice. B6 mice

8

182 were intraperitoneally (i.p.) injected with 300mg/kg of cyclophosphamide in 200µl saline. Twenty-four

183 hours post injection, mice were infected with 200 pfu of virus intrahepatically (i.h.). On day 5 post

184 infection, mice were sacrificed and livers were harvested for virus titration (19) and histological

185 analysis (3).

186 **Western blotting**. Cells were treated or mock treated with 1000U of IFN-α (PBL) overnight,

187 harvested and washed in PBS and lysed with NP40 buffer [1% NP-40, 2 mM EDTA, 10% glycerol

188 150 mM NaCl, 50 mM Tris pH 8.0) with protease inhibitor cocktail (Roche)]. Cell lysates were mixed

189 with 4X Laemmli buffer and boiled at 95°C for 5 minutes and analyzed by electrophoresis on 4-15%

190 gradient SDS gels. Proteins were transferred to polyvinylidene difluoride (PVDF) membranes, which

191 were treated with 5% nonfat milk in TBST (Tris-HCl buffer saline with 0.5% Tween-20) blocking

192 buffer for one hour, followed by incubation overnight at 4°C with antibodies diluted into TBST.

193 Membranes were then washed three times with TBST and incubated with secondary antibodies for 1

194 hour at room temperature, washed three times with TBST and then incubated with SuperSignal

195 West Dura Extended Duration substrate (Thermo) and the signal was detected using an Amersham

196 Imager 600 (GE)**.**

197

198 **Histology**. Livers were isolated and fixed in 4% paraformaldehyde, embedded in paraffin and

199 sectioned. Sections were stained with hematoxylin and eosin as described previously (3).

200

201 **Statistical analysis.** Two-tailed student t-test was performed to determine statistically significant

202 differences for *in vitro* experiments. The Mann-Whitney test was used to analyze differences in virus

203 titer in different mouse tissues. Any undetectable titers from *in vitro* and *in vivo* infections were

204 entered as the limit of detection value for each experiment. All data were analyzed by using Prism

205 software (GraphPad Software, Inc., CA).

206 **RESULTS**

9

207 **Isolation and purification of Kupffer cells (KC), liver sinusoidal endothelial cells (LSEC) and**

208 **hepatocytes from mouse liver**

209 To study the role of individual liver cell types in limiting NS2^{H126R} replication, we purified each of the

210 three major cell types, representing >95% of liver cells, and cultured them *in vitro*. Specifically, we

211 focused on the parenchymal cells (the hepatocytes) and the non-parenchymal Kupffer cells (KC)

212 and liver sinusoidal endothelial cells (LSEC), using established isolation and culture methods, as

213 described elsewhere (17, 18). To assess the purity of the liver cell preparations, we used the

214 nonspecific stain DAPI in all the tests to identify the nuclei. To identify hepatocytes, we stained with

215 an antibody that recognizes hepatocyte nuclear factor 4α (HNF4α), a marker for hepatocytes (green)

216 (21). Over 99% of the cells in the hepatocyte preparation were HNF-4α positive, as seen in Fig 1A-

217 C, which show the merged blue and green stains. KC cells, the resident liver macrophages, were

218 detected by immunostaining for CD68 (Fig 1D), a KC specific marker (22). In the KC-purified

219 preparation, we found that >95% the cells were CD68-positive cells. LSEC were isolated from total

220 liver non-parenchymal cells. LSEC readily take up LDL (low density lipoprotein) (23). Therefore, we

221 incubated the LSEC with Alexa Fluor-488-conjugated-LDL for 2 hours, and then monitored the cells.

222 Over 95% of the cells were Alexa Fluor-488 positive (Fig 1F). To confirm there was little to no KC

223 cell contamination, the LSEC were stained with anti-CD68 antibodies; less than 1% of cells were

224 CD68 positive cells (Fig 1E). These results indicate that our isolation methods for hepatocytes, KC

225 and LSEC yielded highly purified products.

226

227 **Kupffer cells (KC) and liver sinusoidal endothelial cells (LSEC) but not hepatocytes limit**

228 **NS2^{H126R} replication *in vitro* through activation of RNase L.** To determine which cells limit

229 NS2^{H126R} replication, hepatocyte, KC, and LSEC cells derived from B6 (WT) and RNase L KO mice

230 were infected *in vitro* with WT A59 and mutant NS2^{H126R}. As observed previously in B6 hepatocytes,

231 A59 and NS2^{H126R} replicated similarly (9). However, in B6 KC and LSEC, A59 showed 10-100 fold

232 higher titer than NS2^{H126R} at 15 and 24 hours post-infection. When KC and LSEC from RNase L KO

233 mice were infected with NS2^{H126R}, the virus replicated to a similar titer as A59. These results suggest

234 that both KC and LSEC limit NS2^{H126R} virus replication by activation of the OAS-RNase L pathway,

235 as evidenced by the fact that replication in both these cell types was robust in cells from RNase L

236 KO mice and minimal in cells from B6 animals. To directly determine whether RNase L is activated

237 in KC and LSEC, cultures of each liver cell type as well as BMM, were infected with A59 and

238 NS2^{H126R} and at 12 hour (KC and BMM), 13 hour (LSEC), 15 hour (hepatocytes) post infection cells

239 were lysed total cellular RNA was harvested and analyzed for degradation by a Bioanalyzer, as

240 shown in Fig 3. Degradation of ribosomal RNA (rRNA) was observed in KC, LSEC and BMM but not

241 hepatocytes, consistent with the replication phenotype (Fig 2).

242

243 **Depletion of either KC or LSEC from mouse liver partially restores NS2^{H126R} replication and**

244 **liver pathogenicity.** To determine the role of KC and LSEC during MHV infection of intact animals,

245 KC or LSEC were depleted from the livers of B6 mice by intravenous injection of Gadolinium(III)

246 chloride (KC depletion) (20) or by intraperitoneal injection of cyclophosphamide (LSEC

247 depletion)(20, 24). The depletion of KC was monitored by India ink uptake which occurs only by KC

248 (25) and the disruption of the endothelial barrier was monitored by morphology as assessed by

249 electronic microscopy (24). We observed that >80% of KC or >50% of LSEC cells were depleted

250 from mouse liver (data not shown). Following depletion of KC from B6 mice, NS2^{H126R} replicated to

251 100-500 fold higher level than in the non-depleted livers. The depletion of LSEC showed a less

252 dramatic increase in virus titer than KC depletion but titers were still significantly higher than that in

253 the control mice (Fig 4A). Liver pathogenicity was monitored by H&E staining of liver sections (Fig

254 4B). Necrosis was readily apparent in livers from KC- and LSEC-depleted samples, but not in the

255 non-depleted animals. These results suggest that both the KC and LSEC protect the liver from

256 coronavirus infection *in vivo*.

257

258   **Hepatocytes express undetectable levels of RNase L and OAS.** Previous studies have indicated

259   that the activation of the OAS-RNase L pathway by NS2^{H126R} is cell type dependent and independent

260   of virus-induced IFN (3, 9). High basal levels of OAS mRNA and protein expression correlate with

261   activation of RNase L while RNase L expression was similar across the cell types examined,

262   including several primary CNS derived primary cells as well as BMM and BMDC (10) . Since we

263   found that among liver cells, the OAS-RNase L pathway can be activated in KC and LSEC but not in

264   hepatocytes, we hypothesized that KC and LSEC might have higher basal expression levels of OAS

265   genes than hepatocytes. Thus we assessed the basal and IFN induced expression levels of OAS1a,

266   OAS2, OAS3 and RNase L protein in hepatocytes, KC and LSEC by western blotting. OAS1a was

267   detected in KC and LSEC with or without IFN treatment (Fig 5) but not in hepatocytes. OAS2 was

268   not detected in any cell type without IFN treatment; however, OAS2 was induced to a detectable

269   level by IFN in KC. Basal expression of OAS3 was detected in KC but not in hepatocytes and LSEC;

270   however upon IFN treatment, OAS3 was further induced in KC and detected in LSEC but not

271   hepatocytes. The results are consistent with our previous data, in that myeloid cells express higher

272   basal levels of OAS genes and that murine OAS mRNA is induced by IFN treatment (9, 10, 26).

273   RNase L is constitutively expressed in many types of cells and is not generally induced by IFN

274   treatment (9, 10, 26). Surprisingly, while we detected RNase L in KC and LSEC, RNase L

275   expression was below the level of detection in hepatocytes. Consistent with the immunblot data,

276   RNase L mRNA as quantified by qRT-PCR was also 100-1000 fold lower in hepatocytes as

277   compared with KC and LSEC (data not shown), suggesting there is very tight control of this pathway

278   in this cell type.

279

280   **DISCUSSION**

12

281   Based on previous findings that the OAS-RNase L pathway can be activated in BMM and BMDC as

282   well as microglia during NS2 [H126R] infection (9, 10), it is not surprising that the KC also activate

283   RNase L in response to viral infection. KC can be divided into two populations, one is yolk sac

284   derived which develop in the liver before the formation of bone marrow and the other population is

285   myeloid derived (27). A previous study showed that the yolk sac derived KC play roles in

286   immunosuppression, while myeloid derived KC are more immune active(27). We would predict that

287   the myeloid derived cells will be more readily activated to degrade RNA during viral infection and

288   future studies will include separating these types of KC and determining whether the two populations

289   have differences in the activation of the OAS-RNase L pathway during infection.

290

291   LSEC and KC form a barrier between the parenchymal hepatocytes and the blood (12, 13). Previous

292   studies showed that depletion of KC from rat or mouse liver improves the efficacy of gene therapy

293   mediated by lentivirus and adenovirus vectors (25). We have shown here that depletion of KC or

294   LSEC leads to a partial restoration of NS2[H126R] liver replication, demonstrating that KC and LSEC

295   protect the liver from infection by limiting viral replication through activation of OAS-RNase L antiviral

296   pathways. These data are consistent with a previous reported that IFN signaling in myeloid cells was

297   crucial for protection from MHV induced hepatitis (28) and extend this finding to show that RNase L

298   activity specifically can restrict MHV NS2 mutant replication in the liver. Indeed, this is the first report

299   to find that KC and LSEC use the OAS-RNase L pathway to protect the mouse from viral liver

300   infection. It will be important to determine if this pathway limits other viral infections of the liver.

301

302   A key characteristic of LSEC is fenestration; these cells forms pores with diameters of 100-200nm

303   (29). While viruses need to cross the fenestration to infect hepatocytes, our results suggest that

304   MHV either cannot cross the fenestration or can cross inefficiently. Our results suggests that LSEC

305   limit MHV NS2[H126R] liver spread by activation of the OAS-RNase L antiviral pathway as well as by

306   formation of a physical barrier. We previously observed in the primary cells derived from the central

13

307   nervous system that while activation of the OAS-RNase L pathway occurs in microglia, cells of

308   myeloid lineage, non-myeloid cells such as neurons or astrocytes could not be activated (9). LSECs

309   are the first example of non-myeloid cells in which rRNA degradation and inhibition of viral

310   replication are observed in NS2^{H126R} infection. This may reflect their role in protecting the liver

311   parenchyma from viral invasion.

312

313   RNase L is constitutively expressed in many types of cells, including myeloid and non-myeloid and is

314   not induced by IFN treatment (10, 26). While OAS genes are induced by IFN, the activation of the

315   OAS-RNase L pathway in myeloid cells is not dependent on induction of IFN during viral infection

316   (10, 26). Rather, it is the high basal expression level of OAS proteins that play a key role in

317   activation of the pathway (10). We found that by reducing the levels of basal OAS gene expression

318   in BMM we were able to prevent RNase L activation (10). Thus we hypothesized that lack of RNase

319   L activation in hepatocytes would be due to low basal expression levels of OAS genes. We were

320   surprised to find undetectable levels of RNase L as well as OAS1a, OAS2 and OAS3 in

321   hepatocytes, which would provide an explanation for the lack of RNA degradation observed in virus-

322   infected cells. This was the first cell type in which we found a lack of activation of the OAS-RNase

323   pathway correlated with low expression of RNase L as well as low levels of basal expression of

324   OAS. We speculate that since the liver has many pathogens and debris flowing though the

325   sinusoids, this may induce type I IFN expression and subsequently activation of RNase L, which can

326   lead to apoptosis (30). Thus, parenchymal hepatocytes have tightly controlled the OAS-RNase L

327   pathway, which may protect them from pathogen induced cell death, which can result from RNase L

328   activation.

329

330

331   **ACKNOWLEDGEMENTS**

14

335

336

337

338

339

340

341

342

343

344

345

346

347

348

349

350

351

352

353

354

355

356

357   **REFERENCES**

15

358   1.   **Cavanagh D.** 1997. Nidovirales: a new order comprising Coronaviridae and Arteriviridae.
359   Arch Virol **142:**629-633.
360   2.   **Weiss SR, Leibowitz JL.** 2011. Coronavirus pathogenesis. Adv Virus Res **81:**85-164.
361   3.   **Zhao L, Jha BK, Wu A, Elliott R, Ziebuhr J, Gorbalenya AE, Silverman RH, Weiss SR.**
362   2012. Antagonism of the interferon-induced OAS-RNase L pathway by murine coronavirus
363   ns2 protein is required for virus replication and liver pathology. Cell Host Microbe **11:**607-
364   616.
365   4.   **Dong B, Silverman RH.** 1995. 2-5A-dependent RNase molecules dimerize during activation
366   by 2-5A. J Biol Chem **270:**4133-4137.
367   5.   **Han JQ, Barton DJ.** 2002. Activation and evasion of the antiviral 2 '-5 ' oligoadenylate
368   synthetase/ribonuclease L pathway by hepatitis C virus mRNA. Rna-a Publication of the Rna
369   Society **8:**512-525.
370   6.   **Washenberger CL, Han JQ, Kechris KJ, Jha BK, Silverman RH, Barton DJ.** 2007.
371   Hepatitis C virus RNA: dinucleotide frequencies and cleavage by RNase L. Virus Res
372   **130:**85-95.
373   7.   **Kakuta S, Shibata S, Iwakura Y.** 2002. Genomic structure of the mouse 2',5'-oligoadenylate
374   synthetase gene family. J Interferon Cytokine Res **22:**981-993.
375   8.   **Justesen J, Hartmann R, Kjeldgaard NO.** 2000. Gene structure and function of the 2'-5'-
376   oligoadenylate synthetase family. Cell Mol Life Sci **57:**1593-1612.
377   9.   **Zhao L, Birdwell LD, Wu A, Elliott R, Rose KM, Phillips JM, Li Y, Grinspan J, Silverman**
378   **RH, Weiss SR.** 2013. Cell-type-specific activation of the oligoadenylate synthetase-RNase L
379   pathway by a murine coronavirus. J Virol **87:**8408-8418.
380   10. **Birdwell LD, Zalinger ZB, Li Y, Wright PW, Elliott R, Rose KM, Silverman RH, Weiss SR.**
381   2016. Activation of RNase L by Murine Coronavirus in Myeloid Cells Is Dependent on Basal
382   Oas Gene Expression and Independent of Virus-Induced Interferon. J Virol **90:**3160-3172.
383   11. **Racanelli V, Rehermann B.** 2006. The liver as an immunological organ. Hepatology
384   **43:**S54-S62.
385   12. **Crispe IN.** 2009. The liver as a lymphoid organ. Annu Rev Immunol **27:**147-163.
386   13. **Racanelli V, Rehermann B.** 2006. The liver as an immunological organ. Hepatology
387   **43:**S54-62.
388   14. **Yount B, Denison MR, Weiss SR, Baric RS.** 2002. Systematic assembly of a full-length
389   infectious cDNA of mouse hepatitis virus strain A59. J Virol **76:**11065-11078.
390   15. **Roth-Cross JK, Stokes H, Chang G, Chua MM, Thiel V, Weiss SR, Gorbalenya AE,**
391   **Siddell SG.** 2009. Organ-specific attenuation of murine hepatitis virus strain A59 by
392   replacement of catalytic residues in the putative viral cyclic phosphodiesterase ns2. J Virol
393   **83:**3743-3753.
394   16. **Schwarz B, Routledge E, Siddell SG.** 1990. Murine coronavirus nonstructural protein ns2 is
395   not essential for virus replication in transformed cells. J Virol **64:**4784-4791.
396   17. **Kitani H, Takenouchi T, Sato M, Yoshioka M, Yamanaka N.** 2011. A Simple and Efficient
397   Method to Isolate Macrophages from Mixed Primary Cultures of Adult Liver. Jove-Journal of
398   Visualized Experiments.
399   18. **Oie CI, Appa RS, Hilden I, Petersen HH, Gruhler A, Smedsrod B, Hansen JB.** 2011. Rat
400   liver sinusoidal endothelial cells (LSECs) express functional low density lipoprotein receptor-
401   related protein-1 (LRP-1). J Hepatol **55:**1346-1352.
402   19. **Gombold JL, Hingley ST, Weiss SR.** 1993. Fusion-defective mutants of mouse hepatitis
403   virus A59 contain a mutation in the spike protein cleavage signal. J Virol **67:**4504-4512.
404   20. **Duffield JS, Forbes SJ, Constandinou CM, Clay S, Partolina M, Vuthoori S, Wu S, Lang**
405   **R, Iredale JP.** 2005. Selective depletion of macrophages reveals distinct, opposing roles
406   during liver injury and repair. J Clin Invest **115:**56-65.
407   21. **Li J, Ning G, Duncan SA.** 2000. Mammalian hepatocyte differentiation requires the
408   transcription factor HNF-4alpha. Genes Dev **14:**464-474.

409 22. **Klein I, Cornejo JC, Polakos NK, John B, Wuensch SA, Topham DJ, Pierce RH, Crispe
410 IN.** 2007. Kupffer cell heterogeneity: functional properties of bone marrow-derived and
411 sessile hepatic macrophages. Blood **110:**4077-4085.
412 23. **Ling W, Lougheed M, Suzuki H, Buchan A, Kodama T, Steinbrecher UP.** 1997. Oxidized
413 or acetylated low density lipoproteins are rapidly cleared by the liver in mice with disruption of
414 the scavenger receptor class A type I/II gene. J Clin Invest **100:**244-252.
415 24. **Malhi H, Annamaneni P, Slehria S, Joseph B, Bhargava KK, Palestro CJ, Novikoff PM,
416 Gupta S.** 2002. Cyclophosphamide disrupts hepatic sinusoidal endothelium and improves
417 transplanted cell engraftment in rat liver. Hepatology **36:**112-121.
418 25. **van Til NP, Markusic DM, van der Rijt R, Kunne C, Hiralall JK, Vreeling H, Frederiks
419 WM, Oude-Elferink RP, Seppen J.** 2005. Kupffer cells and not liver sinusoidal endothelial
420 cells prevent lentiviral transduction of hepatocytes. Mol Ther **11:**26-34.
421 26. **Li YZ, Banerjee S, Wang YY, Goldstein SA, Dong BH, Gaughan C, Silverman RH, Weiss
422 SR.** 2016. Activation of RNase L is dependent on OAS3 expression during infection with
423 diverse human viruses. Proc Natl Acad Sci U S A **113:**2241-2246.
424 27. **Schulz C, Gomez Perdiguero E, Chorro L, Szabo-Rogers H, Cagnard N, Kierdorf K,
425 Prinz M, Wu B, Jacobsen SE, Pollard JW, Frampton J, Liu KJ, Geissmann F.** 2012. A
426 lineage of myeloid cells independent of Myb and hematopoietic stem cells. Science **336:**86-
427 90.
428 28. **Cervantes-Barragan L, Kalinke U, Zust R, Konig M, Reizis B, Lopez-Macias C, Thiel V,
429 Ludewig B.** 2009. Type I IFN-mediated protection of macrophages and dendritic cells
430 secures control of murine coronavirus infection. J Immunol **182:**1099-1106.
431 29. **Elvevold K, Smedsrod B, Martinez I.** 2008. The liver sinusoidal endothelial cell: a cell type
432 of controversial and confusing identity. Am J Physiol Gastrointest Liver Physiol **294:**G391-
433 400.
434 30. **Castelli JC, Hassel BA, Maran A, Paranjape J, Hewitt JA, Li XL, Hsu YT, Silverman RH,
435 Youle RJ.** 1998. The role of 2'-5' oligoadenylate-activated ribonuclease L in apoptosis. Cell
436 Death Differ **5:**313-320.
437
438
439
440
441
442
443
444
445
446
447
448

449   **FIGURE LEGENDS**

450

451   Figure 1. Isolation and characterization of hepatocytes (Hep), KC and LSEC. Hep, KC and LSEC

452   were isolated as described in Materials and Methods, (A) Hep were stained with mouse anti human

453   HNF-4α and followed by secondary Alexa488-conjugated goat anti mouse antibody (Green), (B)

454   DAPI and   (C) both stains merged.   (D) KC and (E) LSEC were stained with Alexa Fluor -488

455   conjugated anti-CD68 antibodies (Green) and with DAPI to identify the nuclei. (F) LSEC were

456   incubated with Alexa Fluor-488 conjugated LDL (Green) in serum free media at 5µg/ml for 2 hours

457   and Hoechst (Blue) was added in the media the to stain the nucleus 30 minutes before imaging.

458

459   Figure 2. The replication of NS2^{H126R} is limited in KC and LSEC isolated from B6 mice but not from

460   RNase L-/- mice. (A) Hepatocytes (Hep) from B6 mice, (B) KC from B6 mice (C) KC from RNase L$^{-/-}$

461   mice; (D) LSEC from B6 mice, (E) LSEC from RNase L$^{-/-}$ mice were infected with A59 and NS2^{H126R}

462   (MOI=1, n=3). At the indicated time points, infectious virus was quantified by plaque assay on L2

463   cells from the combined cell lysates and supernatants. Data shown were analyzed by two-tailed

464   student t-test and expressed as means ± S.D. (***, P<0.001) and are from one representative of two

465   independent experiments.

466

467

468   Figure 3. RNase L is activated in BMM, KKC and LSEC but not hepatocytes (Hep) during infection

469   with NS2^{H126R}. BMM, hepatocytes, KC and LSEC were infected with MHV A59 and ns2^{H126R} (MOI=1).

470   At twelve hours (BMM and KC), 13 hours (LSEC) or 15 hours (Hep) post infection, RNA was

471   harvested and analyzed by a Bioanalyzer, for ribosomal RNA (rRNA) integrity. The positions of 28S

472   and 18S rRNA are indicated. Data shown are from one representative of two independent

473   experiments.

474

18

475   Figure 4. Depletion of KC or LSEC partially restores NS2^{H126R} replication and liver pathogenicity. B6

476   mice were intravenously injected with 20mg/kg of gadolinium(III) chloride (KC depletion) or

477   intraperitoneally injected with 300mg/kg of cyclophosphamide (LSEC depletion) or with saline as

478   control (n=5 for each group). Twenty-four hours post injection, mice were infected with 200 pfu of

479   virus intrahepatically and sacrificed 5 days pi. (A) Virus was quantified from liver lysates by plaque

480   assay on L2 cells. The data were analyzed by the Mann-Whitney test and expressed as mean ± SD

481   (**, $p<0.01$; ***, $p< 0.001$, titers in depleted mice compared to the saline control). B. Liver sections

482   from NS2^{H126R} infected mice were stained with hematoxylin and eosin for histological analysis.

483   Arrows indicate foci of pathology. Data shown as are from one representative of two independent

484   experiments.

485

486   Figure 5. Expression of OAS1a, OAS2, OAS3 and RNase L are below the level of detection in

487   hepatocytes. Hepatocytes (Hep), KC and LSEC were mock treated or treated with IFN-α (1000U/ml)

488   overnight. Cells were lysed, and proteins were analyzed by immunoblotting with antibodies against

489   OAS1a, OAS2, OAS3, RNase L and GAPDH. The positions and molecular weights of the indicated

490   proteins are indicated with arrows. Data shown as are from one representative of two independent

491   experiments. The additional bands detected with OAS2 antibody in all cell types and with OAS3

492   antibody in Hep and LSEC are not induced by IFN as expected for OAS proteins and are nonspecific
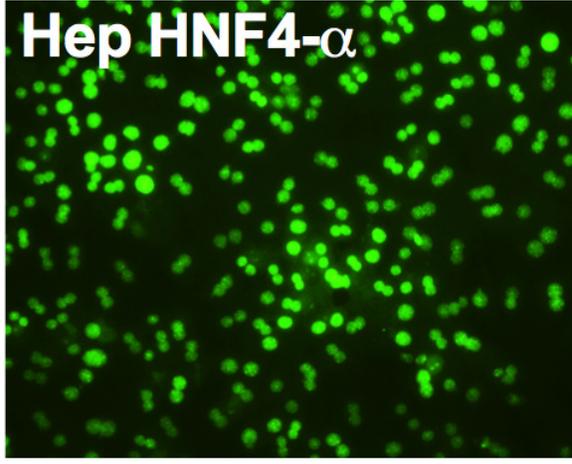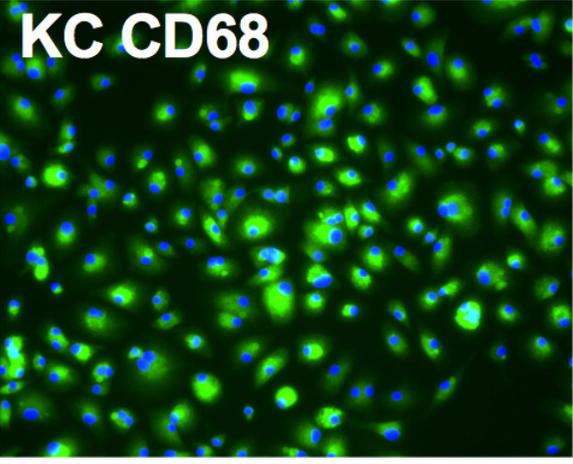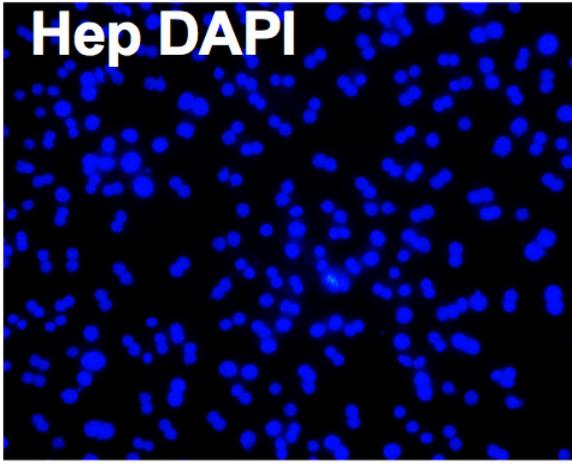
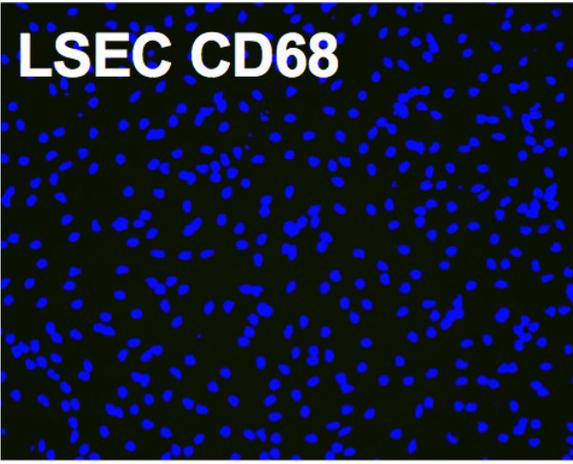493   (10).

494

495

496

497

498

499

19

A Hep HNF4-α

B Hep DAPI

C Hep Merge

D KC CD68

E LSEC CD68

F LSEC LDL-A488

A

B