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## **Activation of Nod2 signalling upon Norovirus infection enhances antiviral immunity and susceptibility to colitis**

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1 **Activation of Nod2 signalling upon Norovirus infection enhances antiviral immunity and**  
2 **susceptibility to colitis**

3

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48 **Abstract.** Over 90% of epidemic nonbacterial gastroenteritis are caused by human Noroviruses  
49 (NoV) which are persisting in a substantial subset of people allowing their spread worldwide.  
50 It leads to a significant number of endemic cases and up to 70,000 children deaths in developing  
51 countries. NoVs are primarily transmitted through the fecal-oral route. To date studies have  
52 focused on the influence of the gut microbiota on viral clearance by enteric immunity. **In this**  
53 **study, the use of the persistent mouse Norovirus S99 strain (MNoV\_S99) allowed us to provide**  
54 **evidence that the norovirus-driven exacerbation of colitis severity relied on bacterial sensing**  
55 **by nucleotide-binding oligomerization domain 2 (Nod2). Similarly, another persistent**  
56 **MNoV\_CR6 strain failed to exacerbate colitis severity in Nod2-deficient mice. In parallel, the**  
57 **viremia was heightened in these mice in comparison with control animals. Accordingly, a**  
58 **reduced level of phosphorylation of Signal Transducer and Activator of Transcription1**  
59 **(STAT1) in Nod2-deficient macrophages infected by MNoV\_S99 was measured. By contrast,**  
60 **STAT1 phosphorylation was increased in wild-type macrophages and associated with an**  
61 **induction of NOD2 expression. This in turn enhances myeloid cells response to muramyl**  
62 **dipeptide (MDP) resulting in downstream pro-inflammatory cytokine secretion and reduced**  
63 **noroviral production. Hence, our results uncover a previously unidentified virus-host-bacterial**  
64 **interplay that may represent a novel therapeutic target for treating noroviral origin**  
65 **gastroenteritis that may be linked with susceptibility to several common illnesses such as**  
66 **Crohn's disease.**

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79 **Introduction.** With the development of high throughput sequencing techniques, we are  
80 progressively gaining a better knowledge of the composition of the enteric virome (ensemble  
81 of gut virus communities)<sup>1</sup>. This is of particular importance for unravelling how some enteric  
82 viruses can establish persistent infections and the subsequent consequences on health<sup>2,3</sup>.  
83 Amongst enteric viruses yielding pro-inflammatory phenotypes, noroviruses (NoV) are  
84 responsible for the majority of non-bacterial gastroenteritis worldwide. **These** single stranded  
85 RNA (+) viruses, belonging to the family of *Caliciviridae* have been studied for several years.  
86 Until recent progress in cultivation methods of **noroviruses from** genogroups I, II, IV, VIII and  
87 IX<sup>4-6</sup> **that infect humans** (HuNoVs), mechanistic details were mostly obtained from studies  
88 using the mouse norovirus (MNoV)<sup>7</sup>. **Indeed, MNoVs** can be easily produced *in vitro* in  
89 macrophages or dendritic cells<sup>8</sup>. Several MNoV strains have been described based on their  
90 shedding time in the stool and classified as persistent or acute (non-persistent) strains<sup>9</sup>.  
91 **Multiple intracellular pattern recognition receptors (PRR) have been associated with indirect**  
92 **noroviral detection, especially during NoV genome replication, when double stranded RNA**  
93 **intermediates are generated<sup>10</sup>. The Toll like receptor 3 (TLR3) that recognizes any dsRNA in**  
94 **the endosomal compartment and the melanoma differentiation associated protein-5 (MDA-5),**  
95 **a member of the RIG-I like receptor (RLR) family that recognizes long dsRNA, have been**  
96 **shown to participate in the detection of the MNoV\_1 strain and its plaque isolate the**  
97 **MNoV\_CW3<sup>11</sup>. Amongst the Nod like receptor (NLR) family, Nlrp6 was shown to bind with**  
98 **the encephalomyocarditis virus (EMCV), a single strand RNA (+) enteric virus, via the RNA**  
99 **helicase Dhx15. Following the formation of these complexes, interferon (IFN) type I and III**  
100 **signalling responses are triggered via the recruitment of the mitochondrial antiviral signalling**  
101 **(MAVS) protein. Interestingly, this study also showed that, Nlrp6<sup>-/-</sup> mice infected with**  
102 **MNoV\_1 had higher viremia, suggesting similar mechanisms of NoV sensing by Nlrp6<sup>12</sup>.**  
103 **Another member of the NLR family, the nucleotide-binding oligomerization domain 2 (NOD2)**

104 mostly known as a sensor of muropeptides from Gram positive and negative bacteria<sup>13</sup>, was  
105 also shown to directly bind with the genome (ssRNA-) of the respiratory syncytial virus (RSV)  
106 to induce antiviral responses<sup>14</sup>. The recruitment of MAVS by NOD2 was needed to activate the  
107 interferon-regulatory factor 3 (IRF3) dependent signalling and provoke IFN- $\beta$  production. In  
108 the same line, NOD2 dependent signalling was shown to potentiate gut inflammation and  
109 increase lethality in an *E. Coli* and MNoV\_1 co-infection mouse model<sup>15</sup>.

110 Another molecular player that is essential to activate antiviral signalling is the cytosolic protein  
111 signal transducer and activator of transcription 1 (STAT1). MNoV\_1 infections were shown to  
112 be lethal in STAT1 knock-out mice<sup>16</sup>. Furthermore, STAT1 dependent interferon responses  
113 were shown to limit MNoV\_1 replication and dissemination *in vivo*<sup>17</sup>.

114 Owing to the variety of circulating NoV strains, strain-dependent effects are expected to occur  
115 and should be studied more in details. Even a single amino acid change in the NS1/2 viral  
116 protein is sufficient to switch the acute MNoV\_CW3 strain to a persistent state<sup>18</sup>, showing the  
117 high degree of adaptability of NoV genomes and hence their versatility to counteract host  
118 defence mechanisms. Type III interferon (IFN- $\lambda$ ) response has been shown to control the *in*  
119 *vivo* propagation of the persistent MNoV\_CR6 strain<sup>19</sup>, while type I and II interferons mediate  
120 innate immune responses against acute MNoV infections<sup>16,20</sup>.

121 Interestingly, Cadwell and colleagues have demonstrated how infection with the MNoV  
122 persistent strain CR6 rendered *Atg16l1<sup>HM</sup>* mice more susceptible to dextran sodium sulphate  
123 (DSS)-induced colitis<sup>21</sup>. Interestingly, NOD2's interaction with ATG16L1 was shown to  
124 regulate the autophagy clearance of pathogenic bacteria<sup>22</sup>. Indeed, improper interaction with  
125 muropeptides and other metabolites from the gut microbiota leads to intestinal inflammation<sup>23</sup>  
126 and loss-of-function point mutations of either *NOD2* or *ATG16L1* predispose humans to  
127 Crohn's disease<sup>24,25</sup>. Insights into the mechanisms of how NOD2 signaling is regulated upon

128 noroviral infection and whether it may subsequently modulate colitis susceptibility remained to  
129 be assessed.

130 In this study, we have investigated the underlying mechanism of the pathogenesis of two  
131 persistent MNoV strains, namely S99 (*Berlin*)<sup>26</sup> and CR6. MNoV\_S99 dependent inflammatory  
132 response to DSS was compared in WT and *Nod2*<sup>-/-</sup> mice. Similarly, the consequences of  
133 infections with MNoV\_CR6 on disease risk were assessed in WT, *Nod2*<sup>-/-</sup> and *Atg1611*<sup>HM</sup> mice.  
134 Nod2-dependent pro-inflammatory signalling pathways activation were further examined in  
135 response to MNoV\_S99 infection in myeloid cells to determine how this in turn may affect  
136 viral production.

137

138

## 139 **Results.**

### 140 **MNoV\_S99 exacerbates the DSS-induced inflammation**

141 Since MNoV\_S99 is a natural strain commonly found in animal facilities that is able to establish  
142 persistent infections<sup>26</sup>, we assessed MNoV\_S99's implication in the vulnerability to  
143 exacerbated inflammatory responses of the intestinal mucosa. Hence, we examined  
144 MNoV\_S99's impact in a widely used chemically-induced colitis model that is induced upon  
145 administration of Dextran Sodium Sulfate (DSS). Specifically, C57BL/6J mice were first  
146 infected with a single dose of 10<sup>7</sup> TCID<sub>50</sub>PFU of MNoV\_S99 or mock-treated with PBS. One  
147 week later, drinking water was replaced with either a 3% DSS or 5% DSS solution for a period  
148 of seven consecutive days (Fig. 1A). Body weight-loss was monitored on a daily basis together  
149 with signs of diarrhea and rectal bleeding. With both concentrations of DSS, the pre-treatment  
150 with MNoV\_S99 had induced a more significant body weight loss (purple and orange curves  
151 Fig. 1B and C). After 7 days of DSS treatment, mice were euthanized. Histological analysis of  
152 colon sections revealed an increased infiltration of inflammatory cells and epithelial damage in

153 infected mice treated with DSS when compared to similarly challenged animals that were not  
154 infected (Fig. 1D). Next, we quantified the muscle thickness from these colon sections. An  
155 increased thickness was measured in mice treated with the virus in presence of both 3 and 5%  
156 DSS in comparison with Mock-treated control mice (Fig. 1E). Similarly, histological scoring  
157 of the haematoxylin and eosin (H&E) stained colonic sections expectedly showed significantly  
158 more severity in mice treated with MNoV\_S99 + 3% or 5% DSS than DSS alone (Fig. 1F).  
159 Upon mice sacrifice, resected colons were measured. A significantly reduced length was  
160 obtained in mice infected with MNoV\_S99 and treated with 5% DSS in comparison with non-  
161 infected control animals (PBS) (Fig. 1G). The increased level of colitis was also evidenced by  
162 a significantly augmented secretion of the pro-inflammatory cytokine interleukin-6 (IL-6) in  
163 the supernatants of over-night *in vitro* cultured colon explants from MNoV\_S99 infected + 5%  
164 DSS treated mice versus those from DSS only mice (Fig. 1H). Taken together, these data clearly  
165 show a much worse inflammation state in mice infected with MNoV\_S99 in our chemically  
166 induced colitis model.

167

#### 168 **MNoV\_S99 associated inflammation is abrogated in *Nod2*<sup>-/-</sup> mice**

169 In cells from the myeloid lineage, NOD2 was shown to participate in the sensing of *Citrobacter*  
170 *rodentium* and of commensal bacteria that are translocating in response to chemically-induced  
171 epithelial injury<sup>27</sup>. To determine whether NOD2 is also implicated in the immunopathology of  
172 MNoV in our preclinical model of colitis, we compared the severity of DSS-induced colitis in  
173 the presence of MNoV\_S99 or not between WT and *Nod2*-deficient mice. In contrast to what  
174 was observed in WT mice (Fig. 2A), the sensitizing effect of the virus on the daily weight-loss  
175 was absent upon MNoV\_S99 infection of *Nod2*<sup>-/-</sup> mice (Fig. 2B). These data suggest that the  
176 pro-inflammatory effect of MNoV\_S99 observed in WT mice is a consequence of a loss of  
177 tolerance to bacterial MDP. Indeed, DSS induces an abrasion of the epithelial barrier allowing



178 commensal bacteria to cross the mucosa and unleash a pro-inflammatory response from *Nod2*  
179 expressing cells. This was further confirmed after mice were autopsied. Colon tissue sections  
180 staining confirmed the decreased levels of colonic inflammation from MNoV\_S99 infected  
181 *Nod2*<sup>-/-</sup> vs WT mice (Fig. 2C). In agreement, muscle thickness quantification from these colon  
182 sections showed significantly increased thickness in MNoV\_S99-infected WT mice under 3%  
183 DSS treatment vs 3% DSS alone (Fig. 2D). While in *Nod2*<sup>-/-</sup> mice subjected to similar  
184 conditions, the excess of inflammation caused by the viral infection was lost. Consequently, the  
185 variations of colon length measurements that were induced upon MNoV\_S99 infection were  
186 absent in *Nod2*<sup>-/-</sup> mice when compared to WT infected mice (Fig. S1A). Similarly, differences  
187 in histological scores measured from MNoV\_S99-infected WT mice vs DSS alone were also  
188 lost in *Nod2*<sup>-/-</sup> infected mice (Fig. S1B). **And a clear trend was observed regarding the increased**  
189 **levels of secretion of Tumour necrosis factor  $\alpha$  (TNF $\alpha$ ) in the supernatants of *in vitro* cultured**  
190 **colon explants from WT mice infected with MNoV\_S99 and treated with 3% DSS vs those**  
191 **with DSS only. This effect was absent in the supernatants from *Nod2*<sup>-/-</sup> mice infected and treated**  
192 **similarly (Fig. S1C).**

193 Overall, these data show the importance of bacterial sensing by NOD2 and the excessive  
194 inflammatory response measured in the presence of MNoV\_S99 in our DSS-induced colitis  
195 mouse model.

196

### 197 **Mouse noroviruses infections triggers NOD2-dependent pro-inflammatory signalling**

198 Mouse noroviruses are known to have a tropism for the myeloid cell lineages, namely  
199 macrophages and dendritic cells. Recently it was shown that Tuft cells belonging to the gut  
200 epithelial cellular lineages, that possess the CD300lf MNoV receptor can also be targeted by  
201 the MNoV\_CR6 strain<sup>28</sup>.

202 Making use of the GFP-Nod2 reporter mice line, we first examined in which cellular lineages  
203 Nod2 is expressed. We failed to observe GFP-Nod2 expression in epithelial cells, while plenty  
204 of signal was detectable in the bulk of the cells **below** the lamina propria where myeloid cells  
205 are located (Fig. 3A). Hence, our investigations regarding NOD2 expression variations in  
206 response to MNoV\_S99 infection focused on macrophages, monocytes and dendritic cells in  
207 our *in vitro* cellular models.

208 Interferon production being one of the most common response to the intracellular presence of  
209 RNA genomes of foreign origin, we examined if the STAT1 signalling pathway that lays  
210 upstream of ATG1611-mediated interferon production was activated in response to murine  
211 norovirus infection.

212 To this end, bone marrow derived macrophages (BMDM) from WT mice were infected with  
213 several MNoV strains at a multiplicity of infection (Moi) of 1, for 1h and pSTAT1 levels of  
214 expression from cell lysates were measured by western blot (Fig. 3B). In contrast to the non-  
215 persistent strain MNoV\_CW3 that caused a striking increase in pSTAT1 level, the persistent  
216 strains S99 and CR6 infections induced a much milder STAT1 signalling pathway activation.  
217 The CW1 strain was associated with the lowest level of activation. Since MNoV\_S99 is a  
218 natural strain commonly found in animal facilities that is able to establish persistent infections,  
219 we further analysed the mechanistic details behind its infection in our cellular models.

220 Using the macrophage cell-line Raw264.7, we confirmed that MNoV\_S99 is able to induce  
221 phosphorylation of STAT1 (Tyrosine 701) at different times at Moi 1 (Fig. S3). pSTAT1 was  
222 detectable as early as 15 minutes post-infection and its expression further increased at 30 min  
223 and 1h, while total STAT1 was detectable in control non-infected cells and its expression only  
224 slightly increased at later time-points. In addition to the STAT1 activation through the Janus  
225 kinases JAK1 and Tyk2, we also examined the status of NFκB activation, another pro-  
226 inflammatory signalling pathway. For this purpose, pIκBα levels were quantified from infected

227 cell lysates. Despite basal low-level activation in mock-infected cells, pI $\kappa$ B $\alpha$  intensity increased  
228 slightly at 30 min and 1h post-infection. However, no significant increase was measured. When  
229 checking PI3K-AKT or the MAPK-ERK signalling axes **in the same time frame**, MNoV\_S99  
230 failed to induce any activation of these signalling pathways (**Fig. S2**). Next, we analyzed the  
231 response of BMDM derived from WT or *Nod2*<sup>-/-</sup> mice that were infected with increasing doses  
232 of MNoV\_S99 for 30 or 60 minutes (Fig. 3C). STAT1 activation increased over the course of  
233 the acute infection in cells derived from WT mice. In contrast, the levels of pSTAT1 were  
234 barely detectable in *Nod2*<sup>-/-</sup> cells at Moi 1. However, at a higher dose (Moi 5), Nod2-dependent  
235 STAT1 activation was only observable at the 30 minutes time point. At 60 minutes post-  
236 infection, the decrease in pSTAT1 levels in response to MNoV\_S99 infection was not  
237 significant anymore when compared with WT cells.

238 Next, we examined if Stat1 activation was detectable after prolonged times of infection. Indeed,  
239 we measured significant differences in pStat1 expression levels between WT and *Nod2*<sup>-/-</sup>  
240 MNoV\_S99-infected cells 24h post-infection at Moi 0.1 (Fig. 3D).

241 To examine whether the Mitochondrial antiviral signalling (MAVS) adaptor protein is required  
242 for sensing MNoV\_S99, BMDM derived from *Mavs*-deficient mice were compared to WT  
243 cells. Interestingly, the higher pStat1 levels measured in WT cells (Moi 0.1, 1h) were  
244 significantly decreased in cells derived from *Mavs*<sup>-/-</sup> cells (Fig. 3E).

245 Altogether, these data show a mild but significant activation of pro-inflammatory signalling  
246 pathways following MNoV\_S99 infection in our *in vitro* cellular models. These signalling  
247 responses can be at least partially abrogated in the absence of MAVS and NOD2.

248

249 **Lower levels of inflammation in *Nod2*<sup>-/-</sup> mice benefit noroviral propagation**

250 **MNoV\_CR6 infection in DSS-induced colitis was previously shown to promote inflammation**  
251 **in a functional Atg1611 dependent manner<sup>21</sup>. Using this viral strain, we checked how loss of**

252 *Nod2* influenced resolution of inflammation in response to 3% DSS treatment. As shown with  
253 the S99 strain, the significantly increased inflammation levels measured by colon wall muscle  
254 thickening were abrogated in *Nod2*<sup>-/-</sup> mice (Fig. 4A). However, a CR6 strain-specific effect can  
255 be observed with these experiments since no significantly increased inflammation was  
256 measured in WT mice as opposed to *Atg16l1* hypomorph mice.

257 Next, we examined the impact of the pro-inflammatory signalling activation on viral  
258 propagation in these mice. In the absence of DSS induced inflammation no significant  
259 differences could be found when comparing the viral load in the stool of WT, *Atg16l1*<sup>HM</sup> or  
260 *Nod2*<sup>-/-</sup> mice (Fig. 4B). But, when gut epithelium tissue sections were assessed from mice  
261 treated with DSS, the measured viral load was significantly increased in ileum and colon of  
262 *Nod2*<sup>-/-</sup> mice in comparison with inflamed tissues of *Atg16l1*<sup>HM</sup> mice (Fig. 4C).

263 We have also analyzed the viral replication in BMDM derived from WT or *Nod2*<sup>-/-</sup> mice  
264 infected *in vitro* with MNoV\_S99 (Moi 0.1 for 24h). In that case a slight but significant increase  
265 in the number of viral genomes was measured from *Nod2*<sup>-/-</sup> derived cultures (Fig. 4D).

266 Hence, albeit some strain effect differences between CR6 and S99, the lower levels of  
267 inflammation found in *Nod2*<sup>-/-</sup> mice seems to favour a higher propagation of these two persistent  
268 strains.

269

#### 270 **MNoV\_S99 persistence is decreased with loss of tolerance to Bacterial MDP**

271 To further explore the mechanisms behind the excessive inflammatory response associated with  
272 noroviral infections we examined *Nod2* expression levels by RT-qPCR. Interestingly, they were  
273 significantly increased in response to MNoV\_S99 infection in Raw264.7 cells or BMDM  
274 derived from WT mice (Fig. 5A and 5B).

275 This increased *Nod2* expression following norovirus infection could explain the viral-induced  
276 pathogenesis that we observed in our colitis models. Indeed, with augmented NOD2 levels the

277 inflammatory response of myeloid cells from the lamina propria should spike up when suddenly  
278 interacting with intestinal commensal bacteria following DSS-induced epithelial abrasion.  
279 To gain a better understanding of how these two stimuli work in tandem, we examined in our  
280 *in vitro* cellular models, Stat1 and I $\kappa$ B $\alpha$  signalling pathways activation in response to  
281 MNoV\_S99 infection followed by MDP treatment. As previously shown with BMDM or  
282 Raw264.7 cells, MNoV\_S99 infection did also induce Stat1 activation in monocytes isolated  
283 from WT mice (Fig. 5C and 5D, MNoV\_S99+, MDP- condition). Similarly, I $\kappa$ B $\alpha$  was not  
284 activated by the viral infection alone. Though, relatively high basal levels were already  
285 detectable in mock-infected cells. As expected the treatment with the peptidoglycan MDP alone  
286 triggered potent I $\kappa$ B $\alpha$  activation without activating Stat1 (Fig. 5C, MNoV\_S99-, MDP+  
287 condition). Interestingly, while I $\kappa$ B $\alpha$  activation by single MNoV\_S99 infection was not  
288 achievable, MNoV\_S99 infection followed by secondary MDP treatment was associated with  
289 both pathways' moderate but significant activation (Fig. 5D, MNoV\_S99+, MDP+ vs mock  
290 treated cells). In the same line of investigation, we noticed a significantly augmented pro-  
291 inflammatory cytokine TNF $\alpha$  response to MDP in dendritic cells that were first primed with  
292 Noroviral RNA (Fig. 5E). Next, we compared WT and *Nod2*<sup>-/-</sup> derived BMDM cultures' TNF $\alpha$   
293 response to MNoV\_S99 infection followed by MDP treatment. TNF $\alpha$  levels were increased  
294 with increasing doses of virus in WT cells, while they remained undetectable (Moi 0.1) or much  
295 lower (Moi 1) in *Nod2*<sup>-/-</sup> cells (Fig. 5F). Consequently, when noroviral replication levels were  
296 compared between Raw264.7 cells only infected with MNoV\_S99 and those infected + MDP  
297 treated, viral titers measured in supernatants showed significantly decreased yields in the latter  
298 case (Fig. 5G).

299 Overall, these *in vitro* data fit nicely with our *in vivo* models. The increased *Nod2* transcription  
300 levels following noroviral infection in turn favours excessive inflammatory signalling pathways  
301 activation in the presence of bacterial MDP, leading ultimately to a limitation in the viral

302 propagation. Hence the lower inflammation in *Nod2*<sup>-/-</sup> mice is associated with increased  
303 propagation of MNoV\_S99 and MNoV\_CR6 strains.

#### 304 **Discussion**

305 The breach in gut flora homeostasis termed dysbiosis is becoming central in the onset of chronic  
306 inflammatory bowel disease, such as Crohn's disease<sup>29,30</sup>. With the development of molecular  
307 biology techniques, alterations in the virome relevant to disease pathogenesis are thoroughly  
308 under investigation<sup>2</sup>. The cytosolic sensor NOD2 is very well known for its pathogen associated  
309 molecular pattern recognition activity against bacteria<sup>13</sup>, and recently it appears it can also  
310 participate in detection of certain viruses<sup>14</sup>. Hence, the combination of genetic susceptibility  
311 factors and opportunistic viral infections could be one of the triggers of CD<sup>31</sup>. Using pro-  
312 inflammatory signalling activation downstream of NOD2 as a read-out, we have analysed if  
313 NOD2 can also participate in MNoV detection. All our *in vitro* experiments with monocytic  
314 lineage derived primary cells (e.g. BMDM, BMDC and BM) and cell line (e.g. Raw264.7)  
315 infected with different MNoV strains showed activation of the STAT1 signalling pathway  
316 implicated in the pro-inflammatory IFN response. By contrast, these responses were abrogated  
317 in *Nod2*<sup>-/-</sup> and *Mavs*<sup>-/-</sup> mice derived BMDM.

318 Amongst enteric viruses commonly found in the microbiota, the Norovirus is considered as a  
319 good candidate in the hypothesis of an environmental-driver in genetically susceptible hosts at  
320 the origin of Crohn's disease onset. MNoV\_CR6 strain was shown to induce colitis in  
321 *Atg16l1*<sup>HM</sup> mice presenting defective autophagy in a DSS colitis model<sup>21</sup>.

322 Several PRR are implicated in viral sensing. MDA-5 and TLR3 have been shown to participate  
323 in detection of either MNoV\_1 or CW3<sup>11</sup>. These are both strains which do not persist *in vivo*,  
324 contrary to the S99 (*Berlin*)<sup>26</sup> and CR6 that were used in our study.

325 Our *in vivo* data show significantly exacerbated inflammation when applying either 3% or 5%  
326 DSS colitis protocol to WT or *Atg16l1*<sup>HM</sup> mice. The excessive MNoV-S99 or MNoV\_CR6

327 associated inflammatory response was abrogated in *Nod2*<sup>-/-</sup> mice or in BMDM derived from  
328 these mice showing NOD2's implication in the pathogenesis and the viral sensing respectively.  
329 Hence, NOD2 dependent signalling adds a layer of regulation in viral sensing and finetunes the  
330 immunopathology levels caused by the infection. While in our Norovirus + DSS model  
331 knocking-down NOD2 prevents gut inflammation, it has the opposite effect with Influenza A  
332 virus (IAV) infection in the lung of *Nod2*<sup>-/-</sup> or *Ripk2*<sup>-/-</sup> mice<sup>32</sup>. Indeed, the NOD2-RIPK2  
333 signalling axe has been shown to downmodulate the NLRP3 dependent mitophagy activation  
334 and the associated inflammatory response in the lung tissue of infected mice.

335 Noroviruses propagation or prolonged persistence is tightly regulated by the interplay between  
336 several signalling pathways. MNoV\_1 replication was shown to be inhibited in macrophages  
337 following LPS mediated NF- $\kappa$ B activation that in turn triggers IFN- $\beta$  dependent JAK-STAT  
338 antiviral activity<sup>33</sup>. Interestingly, in our cellular models, in addition to the phosphorylation of  
339 STAT1 that we observed when cells were infected with MNoV\_S99, *Nod2* expression levels  
340 were also increased. Consequently, the subsequent treatment with MDP induced an increased  
341 Nf $\kappa$ B signalling pathway via I $\kappa$ B $\alpha$  activation. The sequential activation of these two pro-  
342 inflammatory signalling pathways resulted in the inhibition of the MNoV\_S99 propagation on  
343 one hand and the augmented production of the pro-inflammatory cytokine TNF $\alpha$  on the other  
344 hand. Hence, the aggravated inflammatory pathology we observed in our DSS colitis model  
345 can be explained by this NOD2-dependent signalling loop in MNoV\_S99 or MNoV\_CR6  
346 infected mice. Similarly, *in vitro* the signalling and cytokine responses were NOD2 dependent  
347 since they were only observed in cultures derived from WT but not in *Nod2*<sup>-/-</sup> mice, at least at  
348 a lower dose (Moi 0.1) of infection. At higher doses, NOD2-independent alternative  
349 mechanisms seem to be active to promote Stat1 activation and TNF $\alpha$  production, but still at a  
350 lesser level than in WT cells.

351 In conclusion, our study shows a NOD2 dependent mechanism that prevents low level  
352 propagation of the persistent MNoV\_S99 and MNoV\_CR6 strains. When the noroviral  
353 infection is coupled with digestive tract injury a positive signalling loop generates a potent pro-  
354 inflammatory response that limits viral propagation.

355

356

357

### 358 **Methods:**

359 Unless otherwise indicated, all media and reagents were purchased from ThermoFischer  
360 Scientific.

361

### 362 **Colitis mouse model and viral infection**

363 All animal experiments were approved by the local ethical committee n° CEEA-  
364 2016030717519903. Age and gender related *Nod2*<sup>-/-</sup> and wild type littermates C57BL/6J mice  
365 (CDTA, Orléans, France) were housed 4-6 per cage, under strict pathogens-free environment.  
366 Mice were gavaged with 10<sup>7</sup> TCID<sub>50</sub>PFU/mL of MNoV\_S99 diluted in 200µl PBS or mock  
367 treated with PBS only. 7 days post-infection, drinking water was substituted with a 3-5%  
368 dextran sodium sulphate (DSS) (35,000-40,000 MW; TdB Consultancy) solution replaced  
369 every 2 days for a total duration of 7 days. **3% DSS colitis experiments with MNoV\_CR6 were**  
370 **conducted as previously described in<sup>21</sup>.**

### 371 **Cells and virus**

372 Mouse leukemic monocyte macrophage cell line RAW264.7 were purchased from ECCAC  
373 (Sigma-Aldrich) and maintained in Dulbecco's modified Eagle's medium (DMEM),  
374 supplemented with 10% (v/v) foetal bovine serum, 1% penicillin/streptomycin and 1% sodium  
375 pyruvate.



376 Bone marrow derived macrophages (BMDM) were isolated from femurs of wild-type, *Nod2*<sup>-/-</sup>  
377 or *Mavs*<sup>-/-</sup> mice of C57BL/6J background<sup>34</sup>. Briefly, bone marrow cells were flushed out of the  
378 bones, red blood cells were lysed using a 160mM NH<sub>4</sub>CL and 170mM Tris solution for 5  
379 minutes at RT. 3 to 6.10<sup>6</sup> viable cells were plated in non-cell-culture-treated petri dishes and  
380 grown for 5-7 days in Iscove's modified Dulbecco's medium (IMDM), supplemented with 10%  
381 foetal bovine serum, 1% penicillin/streptomycin, 1% non-essential amino acid, 1% sodium  
382 pyruvate, 1% glutamine, and 20% (v/v) conditioned-media from L929 cells.

383 Bone marrow derived dendritic cells were isolated similarly as BMDM cells. Then, 2.10<sup>6</sup> viable  
384 cells were plated in non-cell-culture-treated petri dishes and grown for 7 days in RPMI 1640,  
385 supplemented with 10% FBS, 1% P/S, 1% L-glutamine, 1% HEPES and 20% (v/v) conditioned-  
386 media from J558 cell line producing murine granulocyte monocyte colony stimulating factor  
387 (GM-CSF).

388 Monocytes were isolated from bone marrow cells by using the mouse Monocyte Isolation Kit  
389 (Miltenyi Biotec) and a QuadroMACS separator.

390 The MNoV-S99 strain (GenBank accession no. DQ911368) was provided by Prof. P. Maris  
391 from ANSES Fougères Laboratory (France) and was propagated in RAW 264.7 cells. **MNoV-**  
392 **CR6 concentrated stocks were prepared as described in<sup>35</sup>. Briefly, supernatant from 293T cells**  
393 **transfected with a plasmid containing the viral genome was applied to RAW264.7 cells to**  
394 **amplify virus production, and virions were concentrated by ultracentrifugation and**  
395 **resuspension in endotoxin-free PBS. Concentration of stocks was determined by plaque assay.**

396 For in vitro infection experiments, 3-5.10<sup>5</sup> viable cells were seeded in 6 wells plates. The next  
397 day, just before viral infection, cells were counted from 1 well to determine precisely the  
398 multiplicity of infection (Moi). In the remaining wells medium was removed and replaced with  
399 variable quantities of MNoV\_S99 from a common viral stock in a final volume of 1 mL fresh

400 medium per well. Mock treated cells were incubated in parallel with matching quantities of  
401 culture media from non-infected cells that was generated at the same time as viral stocks.  
402 At various time-points after infection, supernatant was removed and cells were washed once  
403 with PBS, before lysis in RIPA buffer for WB analysis or RLT buffer for total RNA extraction  
404 (RNAeasy kit, Quiagen) for RT-qPCR experiments.

405

#### 406 **Immunohistochemistry, ELISA, Western Blot, RT-qPCR and Titration**

407 For immunohistochemistry analysis, formalin-fixed colon samples were embedded in paraffin.  
408 5µm sections were stained with H&E. Slides were imaged with an AxioPlan 2 (Zeiss)  
409 microscope and blindly scored for inflammation severity by two investigators as previously  
410 described<sup>37</sup>.

411 For ELISA analysis, briefly 1cm colon samples were recovered from mice, luminal content was  
412 flushed with PBS, samples were cut opened and resuspended over-night in 250µl DMEM  
413 supplemented with 1% P/S in 24 wells plate at 37°C. The following day, supernatants were  
414 collected and stored at -80°C before cytokine measurement. TNFα and Interleukin-6 proteins  
415 levels were measured specifically using pre-coated 96 wells ELISA plates (R&D System)  
416 following manufacturers indications.

417 For TNFα ELISA from BMDC, 5.10<sup>5</sup> cells were plated in 24 wells plate and treated with  
418 MNoV\_S99's ssRNA (extracted with RNeasy Quiagen kit) **with Lipofectamine 2000**<sup>14</sup> ± MDP  
419 or MDP-DD (Invivogen) over-night and supernatants were measured for TNFα levels.

420 For TNFα ELISA from BMDM, 2.10<sup>5</sup> cells were plated in 96 wells plate infected with  
421 MNoV\_S99 (Moi 0.1 or 1) for 6h prior to MDP treatment (10ug/mL) over-night and  
422 supernatants were measured for TNFα levels.

423 For western blot analysis, total proteins from cell lysates were quantified by BCA protein assay  
424 kit (Pierce), equalized amounts of proteins (10-20µg) were resolved by 4-15% gradient SDS-

425 PAGE gels and transferred to nitrocellulose membranes. Membranes were blocked in 0,05%  
426 PBS-Tween, 5% skimmed milk solution, before incubation subsequently with primary and  
427 secondary antibodies diluted (see Table 1) in blocking solution. Following secondary antibodies  
428 incubations, signals of interest were detected using a chemiluminescence reader  
429 (ImageQuant™ Las 4000, GE Healthcare Life Sciences), images were processed and band ROI  
430 were quantified with Image J software (NCBI). Mean band intensities  $\pm$  SD of protein of  
431 interest were normalized to  $\beta$ -actin. Relative values to control mock-infected conditions were  
432 compared between different experiments.

433 For *Nod2* quantification from MNoV\_S99 infected BMDM or RAW264.7 cells, total RNA  
434 extraction was performed using the RNeasy Mini Kit (Qiagen). One  $\mu$ g of RNA was reversed  
435 transcribed in the presence of 2.5mM of oligo-dT using the Reverse Transcriptor kit (Roche)  
436 following the manufacturer's instructions. 75ng of cDNAs were amplified using Q5 High-  
437 Fidelity 2X Master Mix (New England BioLabs). Primers used in PCR amplification are  
438 available upon request.

439 *Actb* was used as an internal reference gene in order to normalize the transcript levels. Relative  
440 mRNA levels ( $2^{-\Delta\Delta Ct}$ ) were determined by comparing (a) the PCR cycle thresholds (Ct) for  
441 *Nod2* and *Actb* ( $\Delta Ct$ ) and (b)  $\Delta Ct$  values for treated and control groups ( $\Delta\Delta Ct$ ).

442 MNoV\_S99 load was determined by measuring MNoV genome copy numbers by RT-qPCR  
443 analysis as previously described<sup>36</sup>. Briefly, total RNA was extracted from BMDM cell cultures  
444 using the RNeasy Mini Kit (Qiagen). 5 $\mu$ L of RNA were used per reaction of RT-qPCR. Viral  
445 genome levels were determined using the Takyon™Dry No Rox One-Step RT Probe  
446 Mastermix (Eurogentec). Viral genomes were specifically amplified using the  
447 Fwd\_5'GTGCGCAACACAGAGAAAACG3' and Rev\_5'CGGGCTGAGCTTCCTGC3'  
448 primers that target MNoV ORF1 region combined to a TaqMan probe using the Stratagene  
449 Mx3005P (Agilent Technologies). The quantification of viral genome copies was obtained

450 using a standard curve with serial dilutions of the plasmid pMNoV1 corresponding to the full  
451 genome of MNoV1 (Dr Christiane Wobus, University of Michigan, USA). **Viral load for**  
452 **MNoV\_CR6 strain was determined by measuring genome copy numbers by RT-qPCR analysis**  
453 **as previously described in<sup>21</sup>.**

454 **The net production of MNoV\_S99 was determined using the TCID<sub>50</sub> method as described in<sup>36</sup>.**  
455 **Briefly, Raw264.7 cells were infected at Moi 1 with MNoV\_S99 for 2h, before adding MDP**  
456 **(100ng/mL) or not in the supernatant for 24h. Titers (n = 3) were obtained from each**  
457 **supernatant. The net production is calculated by normalizing the amount of viruses produced**  
458 **with the amount of viruses used for the infection.**

459

#### 460 ***NOD2<sup>GFP</sup>* immunohistochemistry imaging**

461 *NOD2<sup>GFP</sup>* mice<sup>38</sup> in the C57BL/6 background were maintained at the Institut Pasteur Central  
462 Animal facility. We used heterozygous *NOD2<sup>GFP</sup>* mice since the presence of one functional  
463 copy of *NOD2* ensures that physiological responses dependent on functional *NOD2* are not  
464 impaired. Heterozygous *NOD2<sup>GFP</sup>* female mice (8-12 weeks of age) were sacrificed and several  
465 centimeters of the distal ileum were removed, opened longitudinally and washed in PBS. The  
466 tissue was spread flat over a 4% w/v LMP agarose (Merck) pad and fixed with 4% v/v  
467 paraformaldehyde (VWR). The ileal tissue was then embedded in a 4% LMP agarose block and  
468 cut into 200µm-thick sections using a Microm HM 650 V Vibration microtome.

469 For staining, tissues were blocked, permeabilized and marked with an anti-GFP (rabbit  
470 polyclonal antibody A-11122) primary antibody and secondary Alexa Fluor 488 goat anti-  
471 rabbit antibody. Tissues sections were counter stained with DAPI (1µg/mL, BD Biosciences)  
472 and Phalloidin- iFluor 647 (1:200 dilution, Abcam and mounted using ProLong Gold Antifade  
473 reagent. Confocal acquisitions were performed using a Leica HyD SP5 confocal microscope.  
474 Image analysis was performed using Icy version 2.4.2.0<sup>39</sup>.

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- 563

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577

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579 T.G. contributed to the preclinical studies in mice. M.D., T.G. and O.B. performed RT-qPCR  
580 and ELISA analysis. M.D. contributed to histological analysis of colon sections. E.W.  
581 performed viral genome quantification by RT-qPCR. R.W. and I.G.B. performed histological  
582 analysis of tissue sections from NOD2- $\beta$ -Gal reporter mice. All authors contributed to  
583 interpretation of raw data and critically reviewed and/or modified the manuscript. G.M. and  
584 M.C. conceived, designed and wrote the paper.

585

586 **Figures legends:**

587 **Figure 1: MNoV\_S99 aggravates inflammation in DSS-induced colitis**

588 **A)**- *In vivo* experimental design, C57BL/6J mice were orally gavaged with PBS or MNoV\_S99 ( $10^7$   
589 TCID<sub>50</sub>PFU/mL), 7 days later, all the mice were given DSS in drinking water for a week. Mice were  
590 weighed daily for weight loss comparison. **B)**- 3% DSS-induced colitis ± MNoV\_S99 (n = 6). **C)**- 5%  
591 DSS-induced colitis ± MNoV\_S99 (n = 6). In B) and C), statistical differences were determined by 2  
592 ways ANOVA test, \* p < 0.05, \*\* p < 0.01, \*\*\* p < 0.001. **D)**- Representative images from H&E  
593 staining of colon sections from mock-treated (Control) or 3% DSS ± MNoV\_S99 treated mice. Insets  
594 showing higher magnifications from each image are presented next to each image. **E)**- Muscle wall  
595 thickness was determined with Image J from colon section images. Percentages of increase are plotted  
596 after baseline subtraction relatively to the values obtained from Control mice (n = 6). **F)**- Histological  
597 intestinal epithelial inflammation scores comparison between Control, 3% or 5% DSS ± MNoV\_S99  
598 treated mice (n = 6). **G)**- Colon length comparison between Control and 5% DSS ± MNoV\_S99 treated  
599 mice **H)**- IL6 secretion levels from colon explants from mock or 5% DSS ± MNoV\_S99 treated mice (n  
600 = 6). Statistical differences were determined with Student's t-Test in E, F, G and H.

601  
602  
603

604 **Figure 2: MNoV\_S99 associated inflammation is NOD2 dependent**

605 Weight loss comparison in **A)**- 3% DSS-induced colitis ± MNoV\_S99 in WT mice (n=6) and **B)**- in  
606 *Nod2*<sup>-/-</sup> mice (n=6), statistical differences were determined by 2 ways ANOVA test, \* p < 0,05, \*\* p <  
607 0,01, \*\*\* p < 0,001. **C)**- Representative images of H&E staining of colon sections from mice in A) and  
608 B). Insets showing higher magnifications are presented below each image. **D)**- Percentages of increase  
609 of muscle wall thickness from colon section images are plotted after baseline subtraction relatively to  
610 the values obtained from WT mice treated only with DSS (n = 6), statistical differences were determined  
611 with Student's t-Test.

612  
613  
614

615 **Figure 3: MNoV\_S99 associated pro-inflammatory signalling is NOD2 and MAVS-dependent in  
616 myeloid lineage cells**

617 **A)**- Representative image showing an intestinal villus from a *Nod2*<sup>GFP</sup> mouse (GFP is shown in green,  
618 Phalloidin in grey and DAPI in blue); scale bare represents 50µm. **B)**- Representative western blot  
619 showing STAT1 signalling pathway activation after 1h infection (Moi 1) with the mentioned MNoV  
620 strains in BMDM. Below are shown quantification of pSTAT1 bands intensities normalized to β-actin  
621 and relative to mock-infected cells (n = 3, mean ± SEM), statistical differences were determined by 1  
622 way ANOVA test \* p < 0,05, \*\* p < 0,005. **C)**- **STAT1 activation** in response to MNoV\_S99 (Moi 1 or  
623 5) in BMDM from WT vs *Nod2*<sup>-/-</sup> infected for 30 or 60 minutes. The lower panel shows quantification  
624 of pSTAT1, bands intensities normalized to β-actin and relative to mock-infected cells (n = 3, mean ±  
625 SEM). Statistical differences were determined by 2 ways ANOVA test \* p < 0,05. **D)**- STAT1 activation  
626 in response to MNoV\_S99 (Moi 0.1) in WT or *Nod2*<sup>-/-</sup> BMDM infected for 24h. Relative quantification  
627 of pSTAT1 (n = 3, mean ± SEM) is shown in the lower panel, statistical differences were determined  
628 by 2 ways ANOVA test \*\*\* p < 0,0005. **E)**- STAT1 activation in response to MNoV\_S99 (Moi 0.1) in  
629 BMDM from WT vs *Mavs*<sup>-/-</sup> infected for 60 minutes. Relative quantification of pSTAT1 (n = 4, mean ±  
630 SEM) are shown below. The lower panel shows the mock vs Moi 0.1 infected cells quantification of  
631 pSTAT1. Statistical differences were determined by 2 ways ANOVA test \* p < 0,05.

632

633 **Figure 4: NOD2 dependent noroviral load**

634 **A)**- WT, *Nod2*<sup>-/-</sup> and *Atg1611*<sup>HM</sup> mice (n = 5 each) were treated with 3% DSS and infected with  
635 MNoV\_CR6 ( $3.10^7$  PFU). Inflammation was measured as percentage of increase in muscle thickness at  
636 the anal-rectal junction in colonic section images plotted after baseline subtraction relatively to the  
637 values obtained from WT mice. **B)**- The viral load measured in stools from WT, *Nod2*<sup>-/-</sup> and *Atg1611*<sup>HM</sup>  
638 mice at day 3, 5 and 7 post-infection with MNoV\_CR6 ( $3.10^7$  PFU) (n = 8). **C)**- The viral load measured



639 from indicated tissue samples from MNoV\_CR6 infected ( $3.10^7$  PFU) WT, *Nod2*<sup>-/-</sup> and *Atg16l1*<sup>HM</sup> mice  
 640 that were treated for 1 week with D55 3% (n = 5). **D)**- MNoV\_S99 genome quantification from WT  
 641 and *Nod2*<sup>-/-</sup> BMDM cells infected with MNoV\_S99 (Moi 0.1 for 24h) (n = 3). All the statistical  
 642 differences were measured with Student's t-Test.

643  
 644 **Figure 5: NOD2 dependent pro-inflammatory signalling associated with MNoV\_S99 infection and**  
 645 **bacterial MDP**

646 Quantification of *Nod2* mRNA levels measured by RT-qPCR in **A)**- in Raw264.7 cells infected with  
 647 MNoV\_S99 (Moi 0.1 or 1, for 24h) and in **B)**- in BMDM from WT mice (Moi 0.1, for 24h) normalized  
 648 to *ActB* and relative to mock-infected cells (n = 3). **C)**- Representative western blot showing STAT1  
 649 and IκBα signalling pathways modulation in cell lysates from monocytes that were either infected with  
 650 MNoV\_S99 (Moi 5, 2h), treated with MDP (10ng/mL, 2h), or a combination of both. **D)**- Quantification  
 651 and comparison of pSTAT1 and pIκBα signals between MNoV\_S99 infected alone cells or in  
 652 combination with MDP (10ng/mL, 2h), (n = 3), with the band intensity being normalized to β-actin and  
 653 relative to mock-treated cells. **E)** TNFα production by BMDC subjected to MNoV\_S99's ssRNA  
 654 (10μg/mL) alone, or supplemented with either MDP (1μg/mL) or MDP-DD (1μg/mL) overnight. **F)**  
 655 TNFα production by BMDM cells infected with MNoV\_S99 (Moi 0.1 or 1) for 6h, prior to being treated  
 656 with MDP (10μg/mL) overnight. **G)** Net production of MNoV\_S99 in Raw264.7 cells infected with  
 657 MNoV\_S99 (Moi 1 for 2h before MDP treatment (100ng/mL) for 24h). Statistical differences were  
 658 analyzed with Student's t Test.

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 660

## 661 Supplementary Figures

662

663 **Supplementary Figure 1: Analysis of MNoV\_S99 associated NOD2 dependent inflammation**  
 664 **measurements from mice showed in Figure 2**

665 **A)** Colon length comparison between WT and *Nod2*<sup>-/-</sup> from 3% DSS ± MNoV\_S99 treated mice,  
 666 statistical differences were determined with Student's t-Test, \* p <0,02. **B)**- Histological scores  
 667 comparison between WT and *Nod2*<sup>-/-</sup> ± MNoV\_S99 treated mice, statistical differences were determined  
 668 with Student's t-Test, \* p <0,05. **C)**- TNFα production of explants (ON) from WT and *Nod2*<sup>-/-</sup> 3% DSS  
 669 ± MNoV\_S99 treated mice was measured by ELISA.

670

671 **Supplementary Figure 2: MNoV\_S99 associated pro-inflammatory signalling pathways activation**

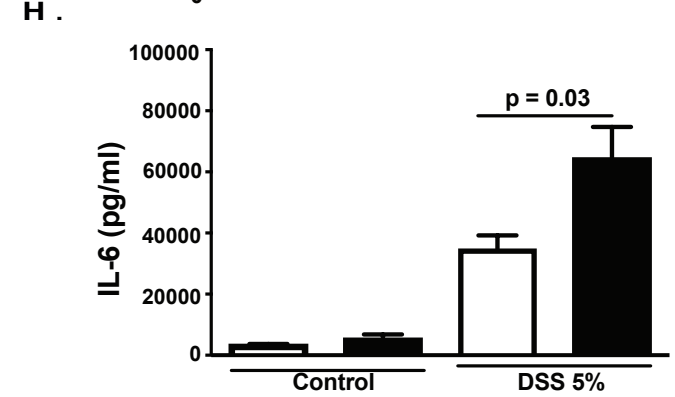
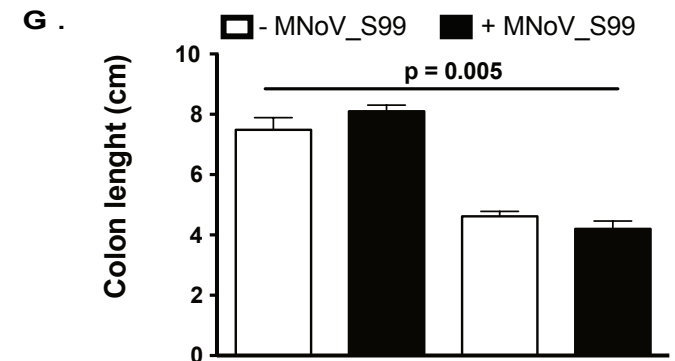
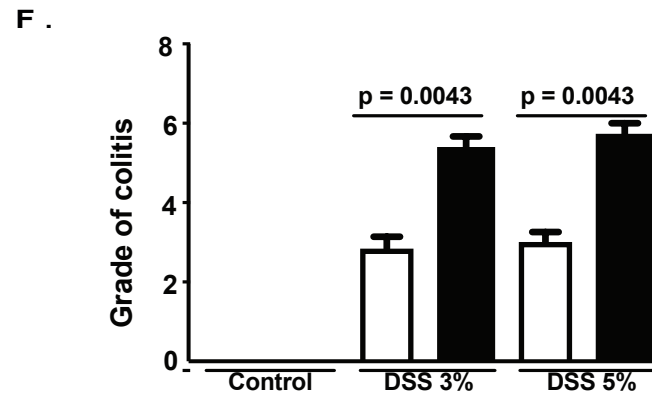
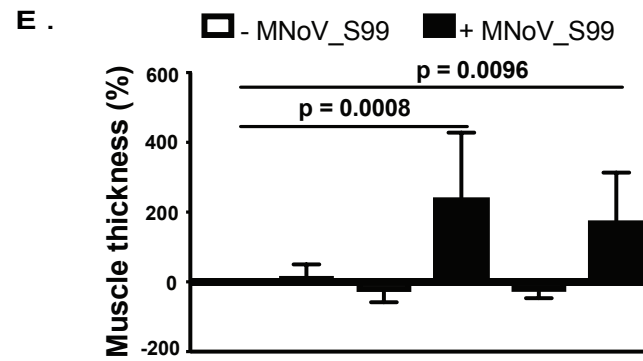
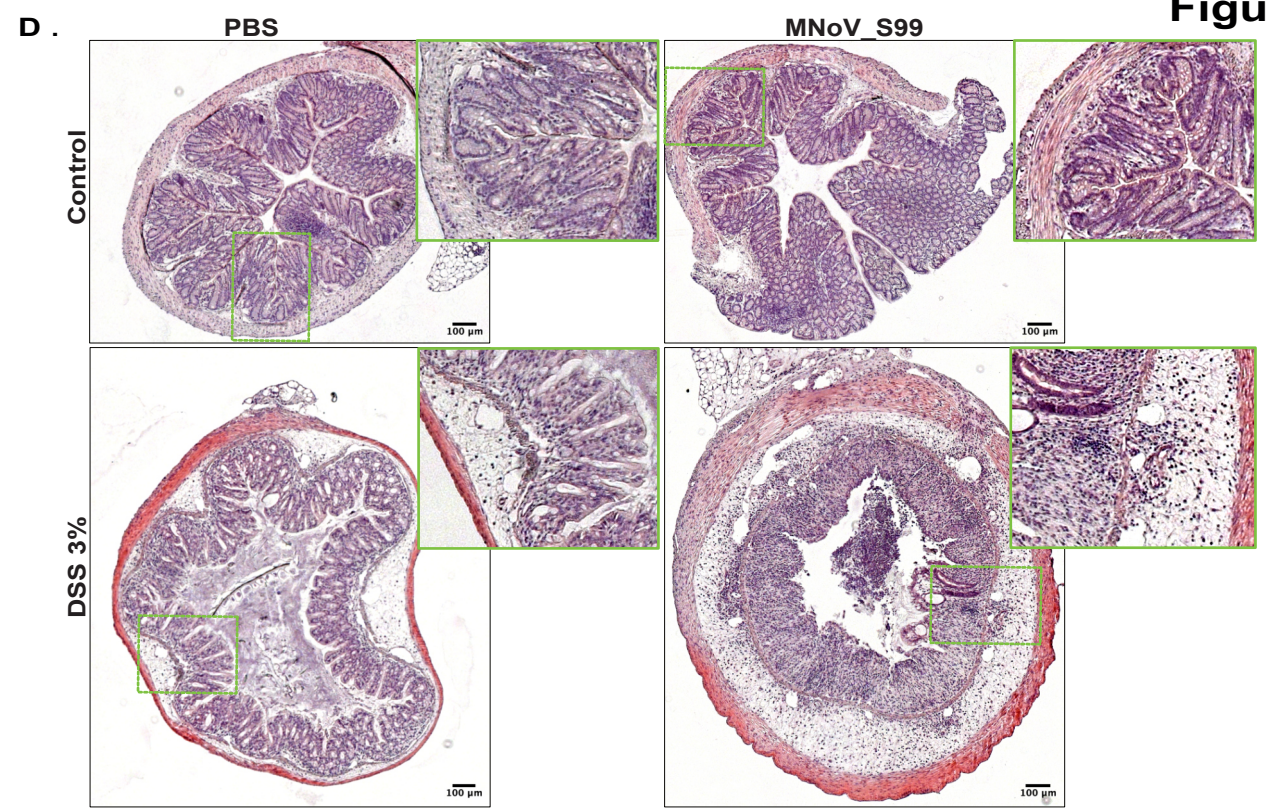
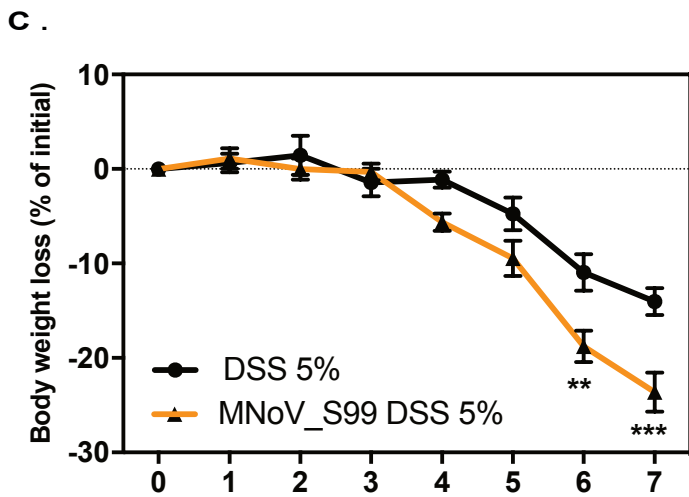
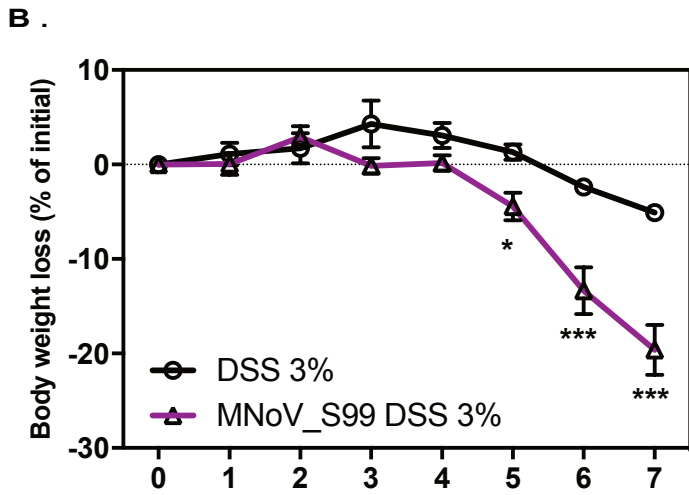
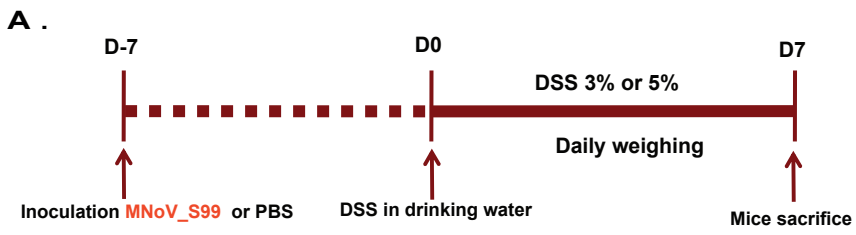
672 Representative western blot showing STAT1, IκBα, AKT and ERK signalling pathways' modulation in  
 673 cell lysates from Raw264.7 that were either infected with MNoV\_S99 (Moi 1) for various times or mock  
 674 treated for 60 min. β-actin was used as loading control.

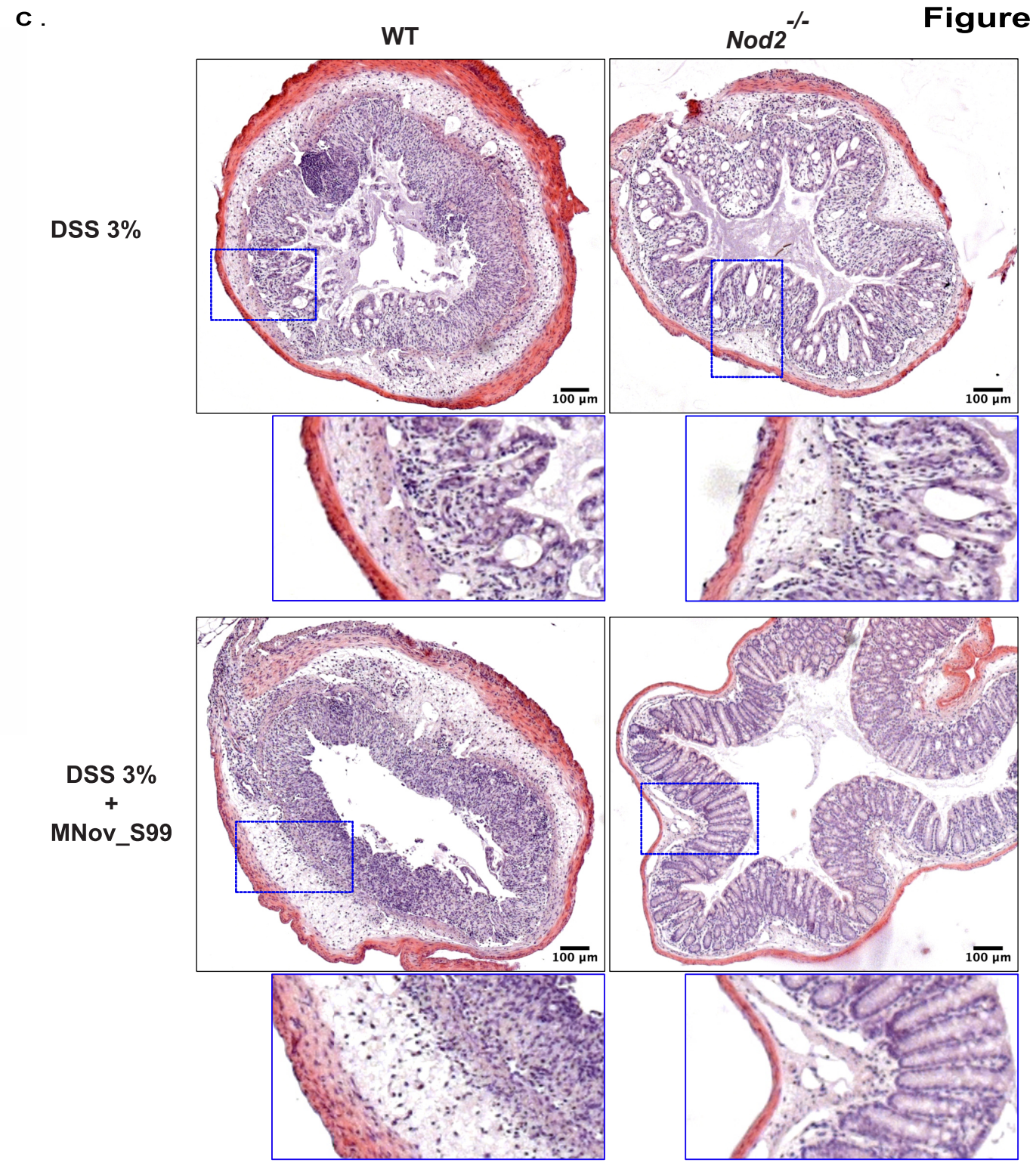
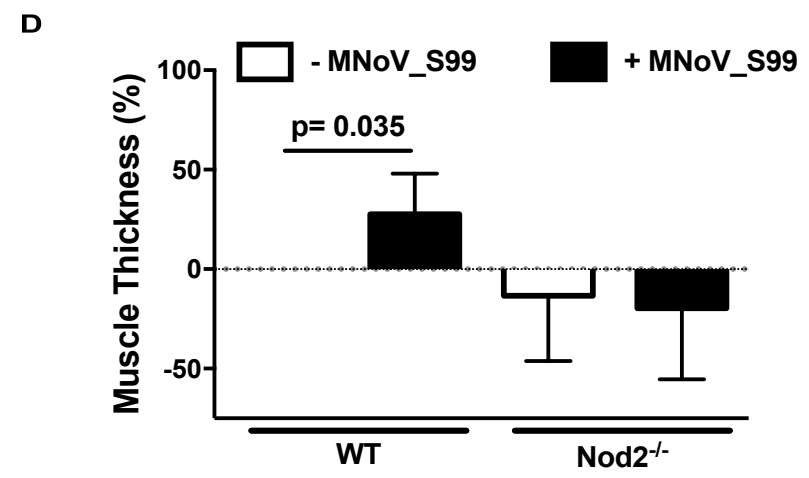
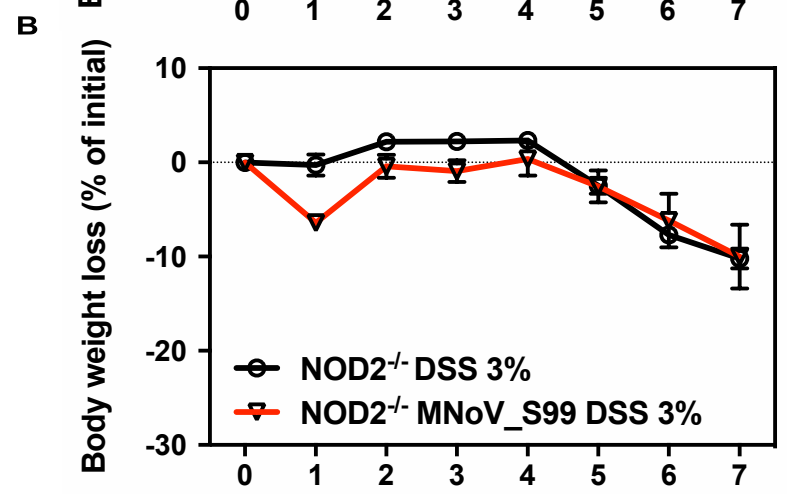
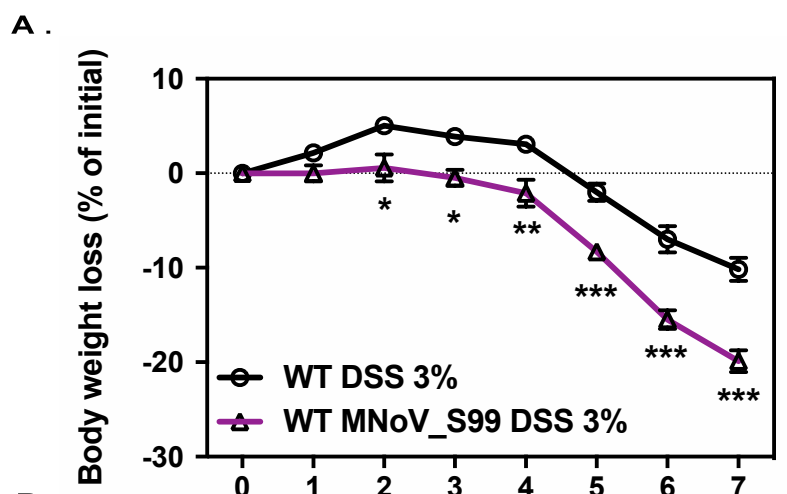
675

## 676 **Tables:**

| Table1: Antibodies used for Western Blotting                         |          |                                 |
|--|----------|---------------------------------|
| Primary antibodies   | Dilution | Supplier                        |
| anti-phosphoStat1 (Tyr 701) (58D6) (85kDa)                           | 1/1000   | Cell<br>Signaling<br>Technology |
| anti-Stat1(D1K9Y)(85kDa)   | 1/1000   |                                 |
| anti-phosphoIκBα (Ser32/36) (5A5) (40kDa)                            | 1/1000   |                                 |
| anti-IκBα (44D4) (39kDa)   | 1/1000   |                                 |
| anti-phosphoAKT (Ser473) (D9E) (60kDa)                               | 1/1000   |                                 |
| anti-AKT (60kDa)   | 1/1000   |                                 |
| anti-phospho44/42 MAPK (Erk1/2) (Trh202/Tyr204) (197G2) (42, 44 kDa) | 1/1000   |                                 |
| anti-p44/42 (Erk1/2) (42, 44 kDa)                                    | 1/1000   |                                 |
| anti-βactin (45kDa)  | 1/500    |                                 |
| Secondary antibodies   | Dilution |                                 |
| anti-Rabbit_HRP (#711-035-152)                                       | 1/5000   | Jackson                         |
| anti-Mouse_HRP (#115-005-003)  | 1/5000   | Laboratories                    |

677





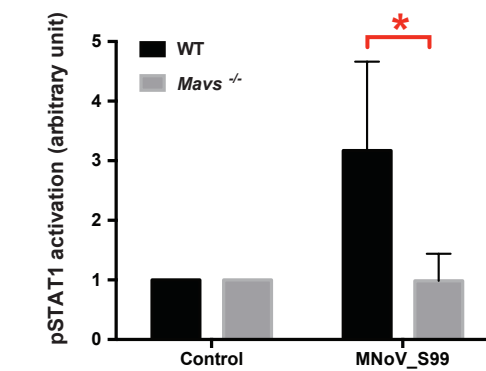
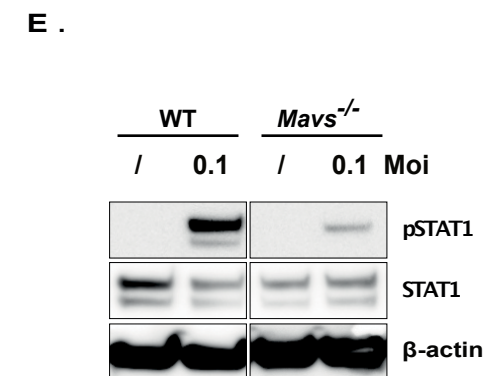
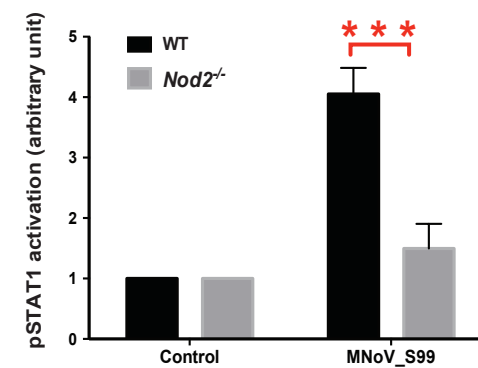
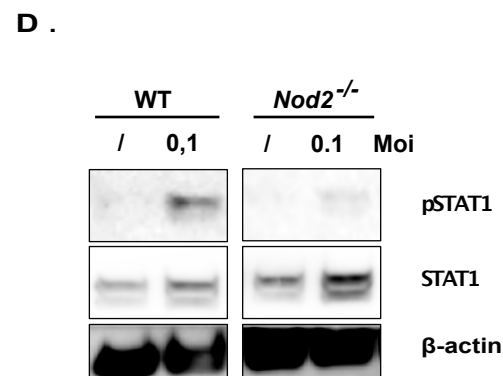
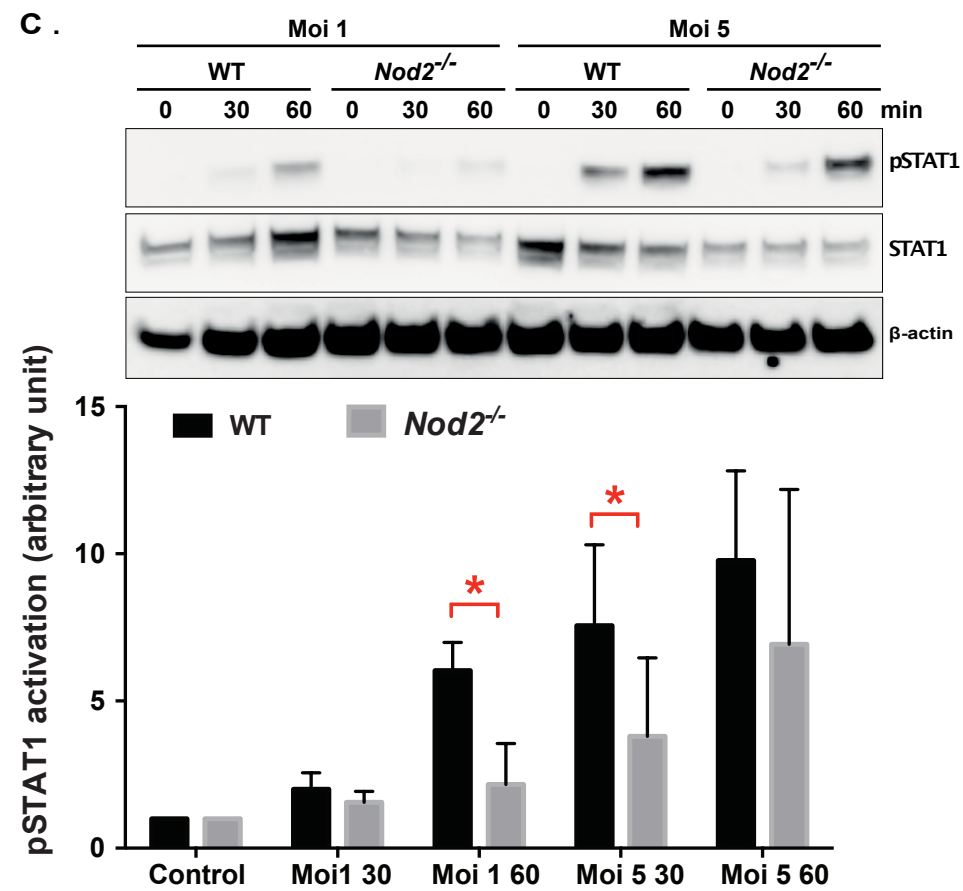
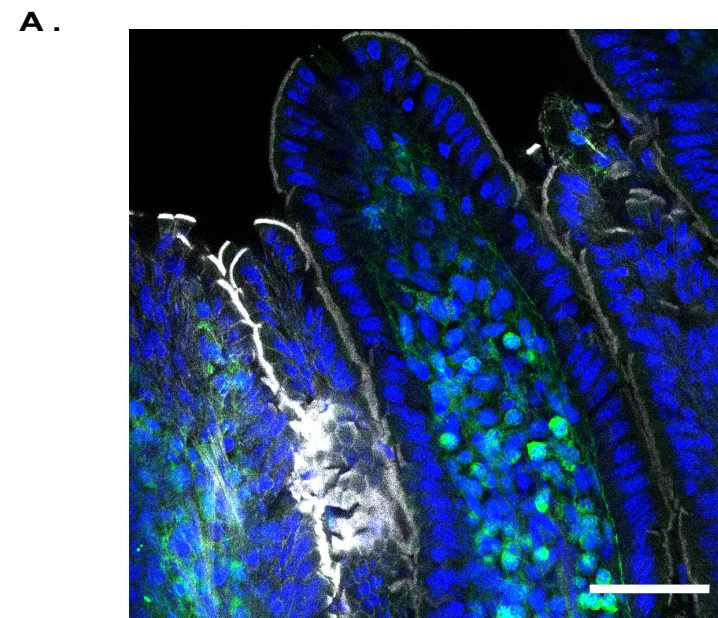
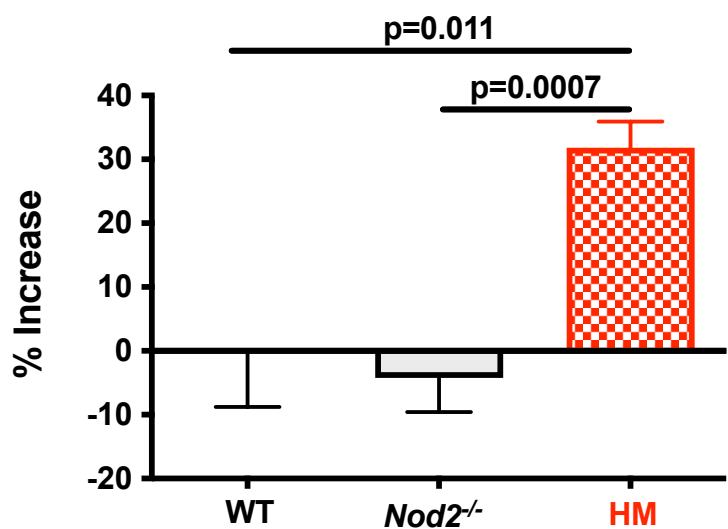
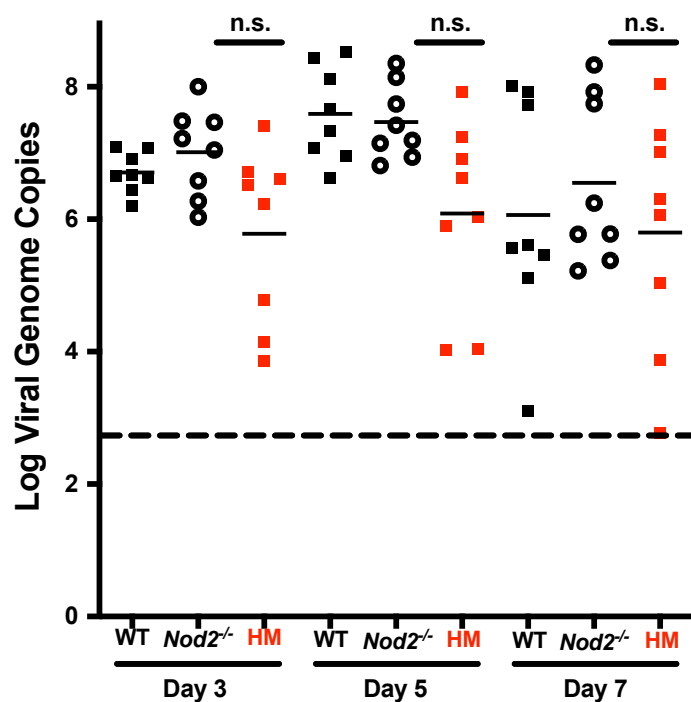


Figure 4

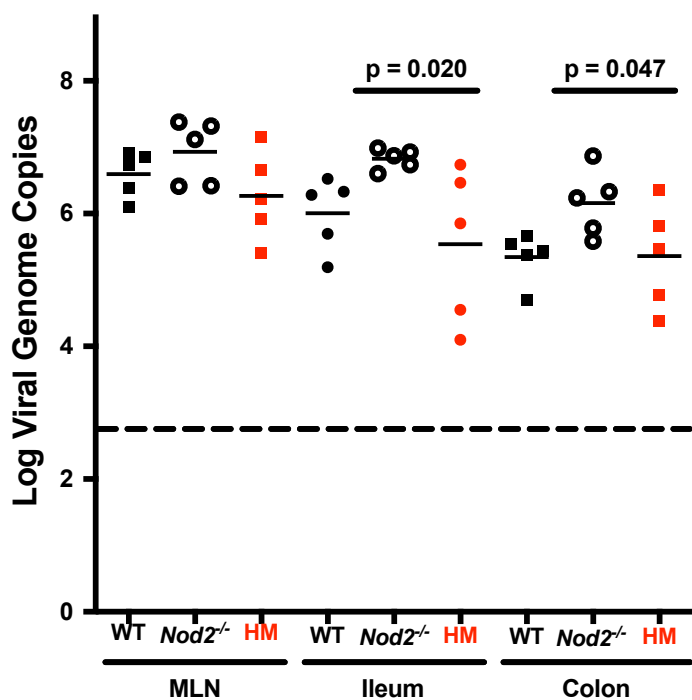
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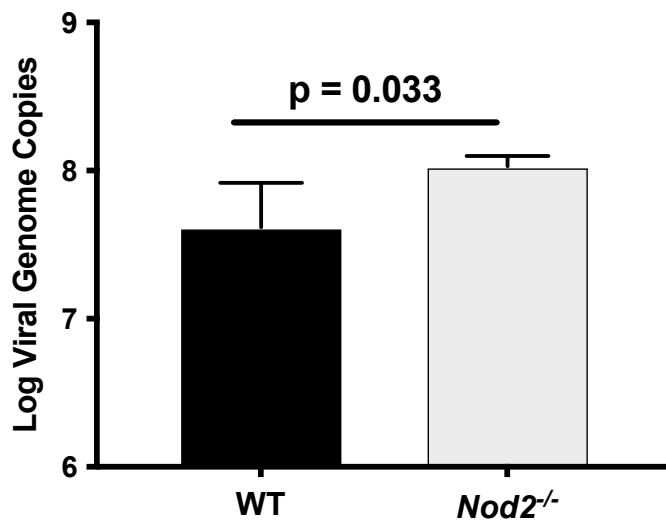
B.



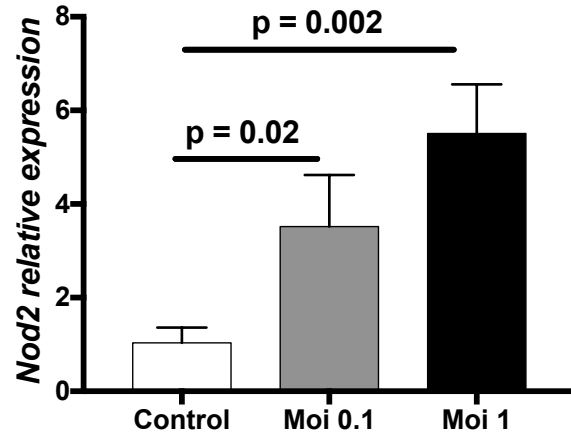
C.



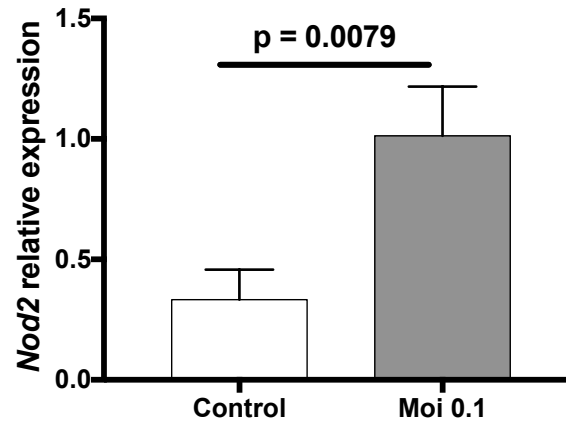
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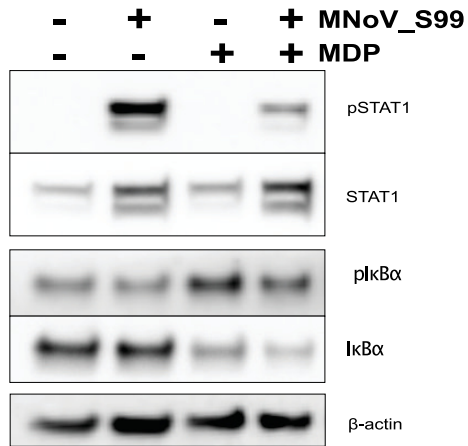
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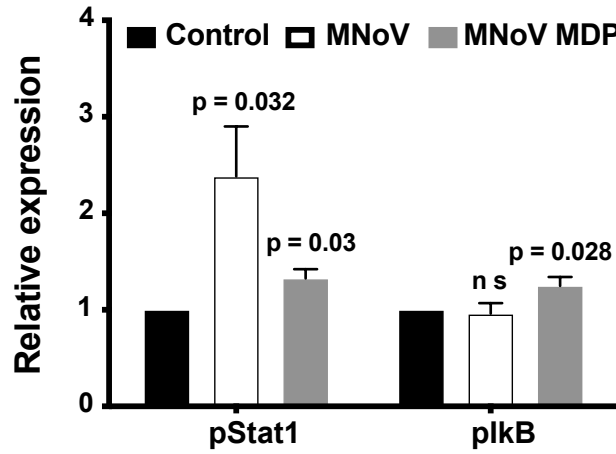
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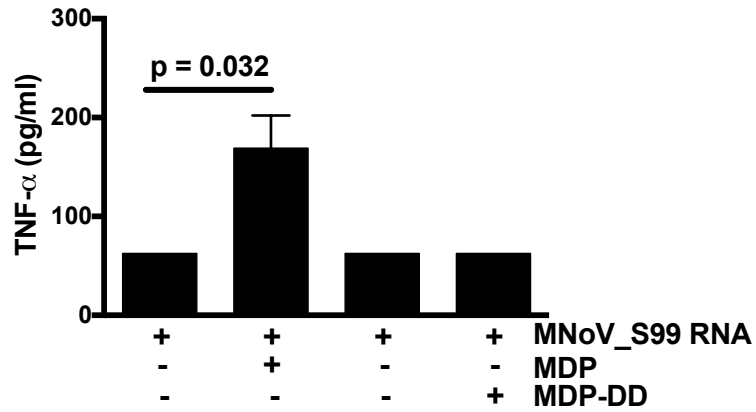
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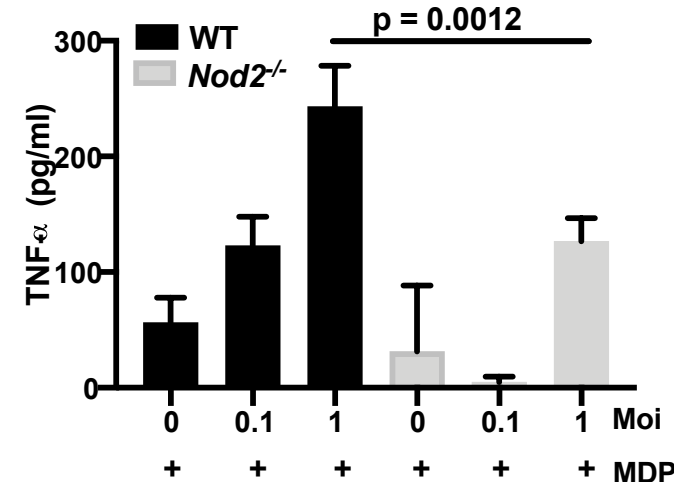
D.



E.



F.



G.

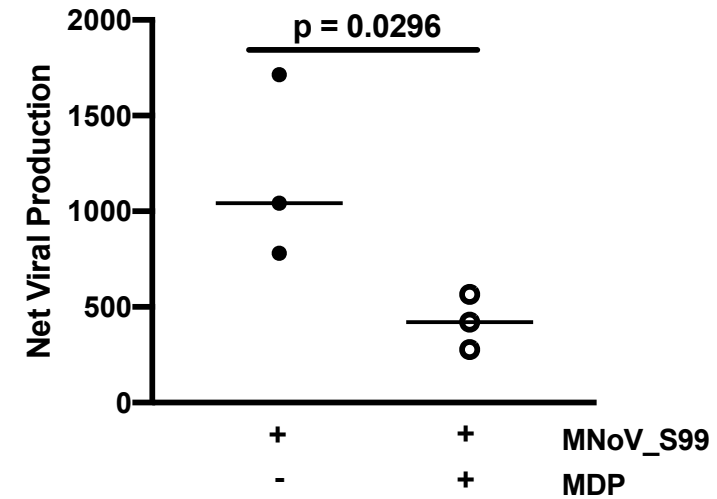
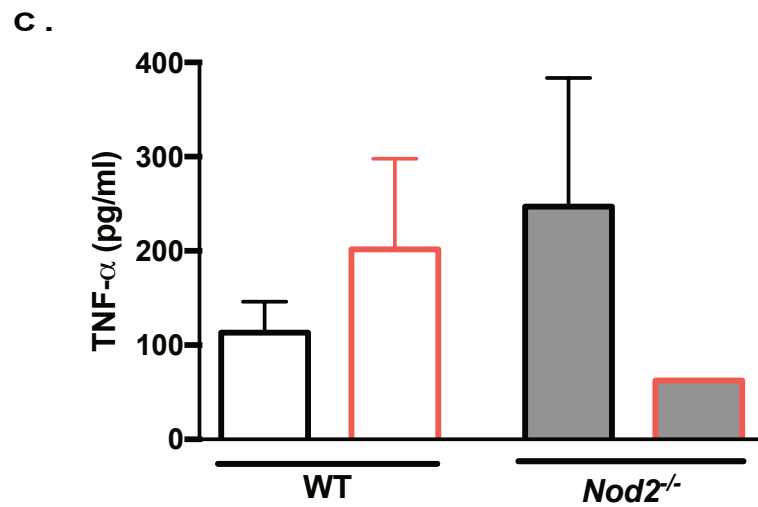
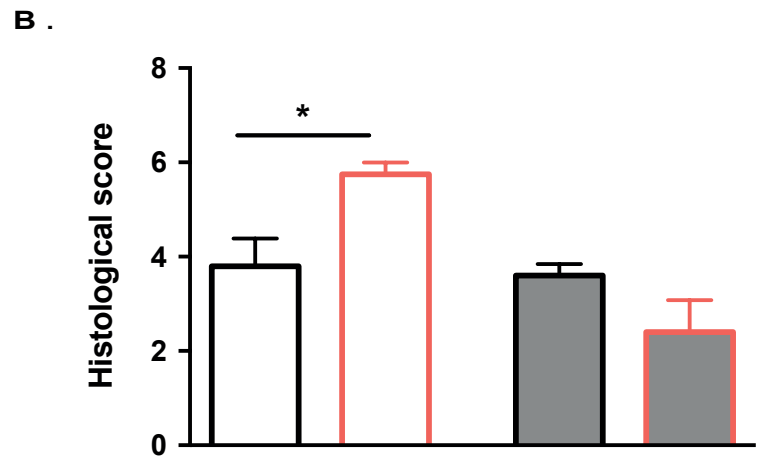
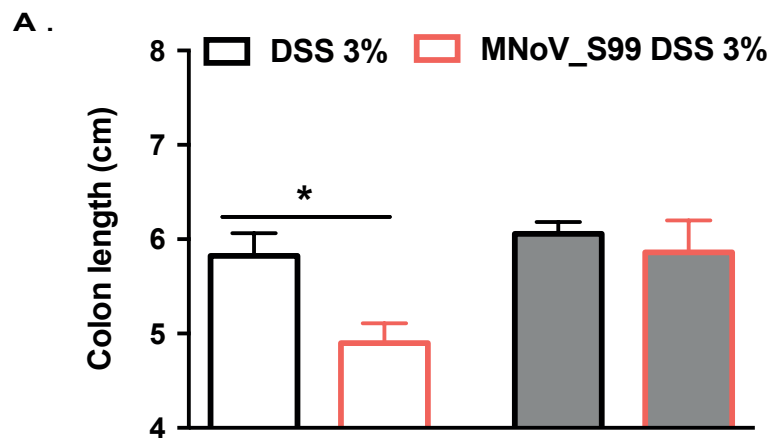


Figure S1



**Figure S2**

