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# **NOD2 dependent signalling exacerbates the intestinal epithelium injury and limits murine Norovirus propagation**

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1 **NOD2 dependent signalling exacerbates the intestinal epithelium injury and limits**  
2 **murine Norovirus propagation**

3

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48 **Abstract.** Over 90% of epidemic nonbacterial gastroenteritis are caused by human  
49 Noroviruses (NoV) which are persisting in a substantial subset of people allowing their spread  
50 worldwide. It leads to a significant number of endemic cases and up to 70,000 children deaths  
51 in developing countries. NoVs are primarily transmitted through the fecal-oral route. To date  
52 studies have focused on the influence of the gut microbiota on viral clearance by enteric  
53 immunity. In this study, the use of the persistent mouse Norovirus S99 strain (MNoV\_S99)  
54 allowed us to provide evidence that bacterial sensing by nucleotide-binding oligomerization  
55 domain 2 (Nod2) promotes the noroviral associated pro-inflammatory pathology in a dextran  
56 sodium sulphate induced colitis mouse model. By contrast, the establishment of the pathology  
57 in the colon is improved in the absence of Nod2. We also show in our cellular models a  
58 greater phosphorylation of Signal Transducer and Activator of Transcription1 (STAT1) in  
59 macrophages infected by MNoV\_S99 when compared with mock-infected cells. This  
60 increased STAT1 phosphorylation is associated with an induction of NOD2 expression in  
61 macrophages. This in turn enhances myeloid cells response to muramyl dipeptide (MDP)  
62 resulting in downstream pro-inflammatory cytokine secretion. Hence, our results uncover a  
63 previously unidentified virus-host-bacterial interplay that is particularly important for  
64 protecting against several common illnesses, such as Crohn's disease.

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81 **Introduction.** With the development of high throughput sequencing techniques, we are  
82 progressively gaining a better knowledge of the composition of the enteric virome (ensemble  
83 of gut virus communities)<sup>1</sup>. This is of particular importance for unravelling the pathogenesis  
84 of viruses that are establishing persistent infections<sup>2,3</sup>. Amongst enteric viruses yielding pro-  
85 inflammatory phenotypes, noroviruses (NoV) are responsible for the majority of non-bacterial  
86 gastroenteritis worldwide. **These** single stranded RNA (+) viruses, belonging to the family of  
87 *Caliciviridae* have been studied for several years. Until recent progress in cultivation methods  
88 of **noroviruses from** genogroups I, II, IV, VIII and IX<sup>4-6</sup> **that infect humans** (HuNoVs),  
89 mechanistic details were mostly obtained from studies using the mouse norovirus (MNoV)<sup>7</sup>.  
90 **Indeed, MNoVs** can be easily produced *in vitro* in macrophages or dendritic cells<sup>8</sup>. Several  
91 MNoV strains have been described based on their shedding time in the stool and classified as  
92 persistent or acute (non-persistent) strains<sup>9</sup>.

93 **Multiple intracellular pattern recognition receptors (PRR) have been associated with indirect**  
94 **noroviral detection, especially during NoV genome replication, when double stranded RNA**  
95 **intermediates are generated**<sup>10</sup>. The Toll like receptor 3 (TLR3) that recognizes any dsRNA in  
96 the endosomal compartment and the melanoma differentiation associated protein-5 (MDA-5),  
97 a member of the RIG-I like receptor (RLR) family that recognizes long dsRNA, have been  
98 shown to participate in the detection of the MNoV\_1 strain and its plaque isolate the  
99 MNoV\_CW3<sup>11</sup>. Amongst the Nod like receptor (NLR) family, Nlrp6 was shown to bind with  
100 the encephalomyocarditis virus (EMCV), a single strand RNA (+) enteric virus, via the RNA  
101 helicase Dhx15. Following the formation of these complexes, interferon (IFN) type I and III  
102 signalling responses are triggered via the recruitment of the mitochondrial antiviral signalling  
103 (MAVS) protein. Interestingly, this study also showed that, Nlrp6<sup>-/-</sup> mice infected with  
104 MNoV\_1 had higher viremia, suggesting similar mechanisms of NoV sensing by Nlrp6<sup>12</sup>.  
105 Another member of the NLR family, the nucleotide-binding oligomerization domain 2

106 (NOD2) mostly known as a sensor of muropeptides from Gram positive and negative  
107 bacteria<sup>13</sup>, was also shown to directly bind with the genome (ssRNA-) of the respiratory  
108 syncytial virus (RSV) to induce antiviral responses<sup>14</sup>. The recruitment of MAVS by NOD2  
109 was needed to activate the interferon-regulatory factor 3 (IRF3) dependent signalling and  
110 provoke IFN- $\beta$  production. So far, no PRR has been identified as a direct sensor of  
111 noroviruses. However, NOD2 dependent signalling was shown to potentiate gut inflammation  
112 and increase lethality in an *E. Coli* and MNoV\_1 co-infection mouse model<sup>15</sup>.

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

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

124 Interestingly, Cadwell and colleagues have demonstrated how infection with the MNoV  
125 persistent strain CR6 rendered *Atg16l1*<sup>HM</sup> mice more susceptible to dextran sodium sulphate  
126 (DSS)-induced colitis<sup>21</sup>. Functional autophagy is critical for microbial elimination. And  
127 NOD2's interaction with ATG16L1 was shown to regulate the autophagy clearance of  
128 pathogenic bacteria<sup>22</sup>. Improper detection of microbial content in the digestive tract leads to  
129 intestinal inflammation<sup>23</sup> and loss-of-function point mutations of either *NOD2* or *ATG16L1*  
130 predispose humans to Crohn's disease<sup>24,25</sup>. Insights into the mechanisms of how the loss of

131 tolerance of NOD2 against gut microbial communities can lead to uncontrolled  
132 proinflammatory signalling are still missing.

133 In this study, we have investigated the underlying mechanism of the pathogenesis of the  
134 persistent MNoV strain S99 (*Berlin*)<sup>26</sup>. MNoV\_S99 dependent inflammatory response to DSS  
135 was compared in WT vs *Nod2*<sup>-/-</sup> mice. NOD2-dependent signalling pathways activation was  
136 further examined in response to MNoV\_S99 during early or prolonged times of infection in  
137 Raw264.7 cell line, bone marrow derived macrophages, monocytes and dendritic cells.  
138 Finally, we have evaluated whether the priming effect of MNoV\_S99 can fine-tune *Nod2*  
139 dependent pro-inflammatory signalling.

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141

## 142 **Results.**

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

144 Since MNoV\_S99 is a natural strain commonly found in animal facilities that is able to  
145 establish persistent infections<sup>26</sup>, we decided to assess MNoV\_S99's implication in the  
146 vulnerability to inflammatory responses of the intestinal mucosa. Hence, we examined  
147 MNoV\_S99's impact in a chemically-induced colitis model. C57BL/6J mice were first  
148 infected with a single dose of 10<sup>7</sup> TCID<sub>50</sub>PFU/mL of MNoV\_S99 or mock-treated with PBS.  
149 One week later, drinking water was replaced with either a 3% DSS or 5% DSS solution for a  
150 period of seven consecutive days (Fig. 1A). Weight-loss was monitored on a daily basis. **With**  
151 **both concentrations of DSS, the pre-treatment with MNoV\_S99 had induced a more**  
152 **significant weight loss (purple and orange curves Fig. 1B and C).** After 7 days of DSS  
153 treatment, mice were sacrificed. Histological analysis of colon sections revealed an increased  
154 infiltration of inflammatory cells and epithelial damage in infected mice treated with DSS  
155 when compared to similarly challenged animals that were not infected (Fig. 1D). Next, we

156 quantified the muscle thickness from these colon sections. An increased thickness was  
157 measured in mice treated with the virus in presence of both 3 and 5% DSS in comparison with  
158 Mock-treated control mice (Fig. 1E). Similarly, histological scoring of the haematoxylin and  
159 eosin (H&E) stained colonic sections expectedly showed significantly more severity in mice  
160 treated with MNoV\_S99 + 3% or 5% DSS than DSS alone (Fig. 1F). Upon mice sacrifice,  
161 resected colons were measured. A significantly reduced length was obtained in mice infected  
162 with MNoV\_S99 and treated with 5% DSS in comparison with non-infected control animals  
163 (PBS) (Fig. 1G). The increased level of colitis was also evidenced by a significantly  
164 augmented secretion of the pro-inflammatory cytokine interleukin-6 (IL-6) in the supernatants  
165 of over-night *in vitro* cultured colon explants from MNoV\_S99 infected + 5% DSS treated  
166 mice versus those from DSS only mice (Fig. 1H). Taken together, these data clearly show a  
167 much worse inflammation state in mice infected with MNoV\_S99 in our chemically induced  
168 colitis model.

169

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

171 In cells from the myeloid lineage, NOD2 was shown to participate in the sensing of  
172 *Citrobacter rodentium* and of commensal bacteria that are translocating in response to  
173 chemically-induced epithelial injury<sup>27</sup>. To determine whether NOD2 is also implicated in the  
174 immunopathology of MNoV in our preclinical model of colitis, we compared the severity of  
175 DSS-induced colitis in the presence of MNoV\_S99 or not between WT and *Nod2*-deficient  
176 mice. In contrast to what was observed in WT mice (Fig. 2A), the sensitizing effect of the  
177 virus on the daily weight-loss was absent upon MNoV\_S99 infection of *Nod2*<sup>-/-</sup> mice (Fig.  
178 2B). These data suggest that the pro-inflammatory effect of MNoV\_S99 observed in WT  
179 mice is a consequence of a loss of tolerance to bacterial MDP. Indeed, DSS induces an  
180 abrasion of the epithelial barrier allowing commensal bacteria to cross the mucosa and



181 unleash a pro-inflammatory response from *Nod2* expressing cells. This was further confirmed  
182 after mice were autopsied. Colon tissue sections staining confirmed the decreased levels of  
183 colonic inflammation from MNoV\_S99 infected *Nod2*<sup>-/-</sup> vs WT mice (Fig. 2C). In agreement,  
184 muscle thickness quantification from these colon sections showed significantly increased  
185 thickness in MNoV\_S99-infected WT mice under 3% DSS treatment vs 3% DSS alone (Fig.  
186 2D). While in *Nod2*<sup>-/-</sup> mice subjected to similar conditions, the excess of inflammation caused  
187 by the viral infection was lost. Consequently, the variations of colon length measurements  
188 that were induced upon MNoV\_S99 infection were absent in *Nod2*<sup>-/-</sup> mice when compared to  
189 WT infected mice (Fig. S1A). Similarly, differences in histological scores measured from  
190 MNoV\_S99-infected WT mice vs DSS alone were also lost in *Nod2*<sup>-/-</sup> infected mice (Fig.  
191 S1B). And we could observe a clear trend in the increased levels of secretion of Tumour  
192 necrosis factor  $\alpha$  (TNF $\alpha$ ) in the supernatants of *in vitro* cultured colon explants from WT  
193 mice infected with MNoV\_S99 and treated with 3% DSS vs those with DSS only. This effect  
194 was absent in the supernatants from *Nod2*<sup>-/-</sup> mice infected and treated similarly (Fig. S1C).  
195 Overall, these data show the importance of bacterial sensing by NOD2 and the excessive  
196 inflammatory response measured in the presence of MNoV\_S99 in our DSS-induced colitis  
197 mouse model.

#### 198 **MNoV\_S99 associated inflammation relies on NOD2 associated signalling**

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

203 Making use of the GFP-Nod2 reporter mice line, we first examined in which cellular lineages  
204 *Nod2* is expressed. We failed to observe GFP-Nod2 expression in epithelial cells, while  
205 plenty of signal was detectable in the bulk of the cells bellow the lamina propria where

206 myeloid cells are located (Fig. 3A). Hence, our investigations regarding NOD2 expression  
207 variations in response to MNoV\_S99 infection focused on macrophages and monocytes in our  
208 *in vitro* cellular models.

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

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

222 Using the macrophage cell-line Raw264.7, we confirmed that MNoV\_S99 is able to induce  
223 phosphorylation of STAT1 (Tyrosine 701) at different times at Moi 1 (Fig. S3). pSTAT1 was  
224 detectable as early as 15 minutes post-infection and its expression further increased at 30 min  
225 and 1h, while total STAT1 was detectable in control non-infected cells and its expression only  
226 slightly increased at later time-points. In addition to the STAT1 activation through the Janus  
227 kinases JAK1 and Tyk2, we also examined the status of NF $\kappa$ B activation, another pro-  
228 inflammatory signalling pathway. For this purpose, pI $\kappa$ B $\alpha$  levels were quantified from  
229 infected cell lysates. Despite basal low-level activation in mock-infected cells, pI $\kappa$ B $\alpha$   
230 intensity increased slightly at 30 min and 1h post-infection. However, no significant increase

231 was measured. When checking PI3K-AKT or the MAPK-ERK signalling axes **in the same**  
232 **time frame**, MNoV\_S99 failed to induce any activation of these signalling pathways (**Fig.**  
233 **S2**). Next, we analysed the response of BMDM derived from WT or *Nod2*<sup>-/-</sup> mice that were  
234 infected with increasing doses of MNoV\_S99 for 30 or 60 minutes (Fig. 3C). STAT1  
235 activation increased over the course of the acute infection in cells derived from WT mice. In  
236 contrast, the levels of pSTAT1 were barely detectable in *Nod2*<sup>-/-</sup> cells at Moi 1. However, at a  
237 higher dose (Moi 5), Nod2-dependent STAT1 activation was only observable at the 30  
238 minutes time point. At 60 minutes post-infection, the decrease in pSTAT1 levels in response  
239 to MNoV\_S99 infection was not significant anymore when compared with WT cells.

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

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

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

250

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

252 To investigate the mechanisms behind the excessive inflammatory response induced by  
253 MNoV\_S99 infection in our *in vivo* experiments, we further explored how NOD2 expression  
254 levels were affected by the viral infections.

255 NOD2 expression was determined by RT-qPCR in Raw264.7 cells or BMDM derived from  
256 WT mice treated with MNoV\_S99. Interestingly, NOD2 expression levels are significantly  
257 increased in response to the viral infection in both cellular models (Fig. 4A and 4B).

258 Thus, augmented NOD2 expression plus STAT1 activation following MNoV\_S99 infection  
259 suggests a proinflammatory signalling loop, that could **ultimately** limit viral propagation on  
260 one hand and induce increased levels of inflammation on the other hand. **The norovirus**  
261 **induced NOD2 up-regulation** could explain the viral-induced pathogenesis that we observed  
262 in our colitis model.

263 **Accordingly, when we analyzed the viral replication in BMDM derived from WT or *Nod2*<sup>-/-</sup>**  
264 **mice infected *in vitro* with MNoV\_S99 (Moi 0.1 for 24h), the quantity of viral genomes was**  
265 **significantly lower in the WT cultures (Fig. 4C).**

266 **Several reports in the literature have shown an interplay between gut microbiota composition**  
267 **and the intestinal epithelium that modulates the interferon response that in turn favors MNoV**  
268 **infection especially for the MNoV\_CR6 persistent strain<sup>19</sup>. WT and *Nod2*<sup>-/-</sup> mice were**  
269 **co-housed for 4 weeks before we measured the viral load in stools by RT-qPCR after RNA**  
270 **extraction. In line with our *in vitro* data on viral replication in BMDM, we could not detect**  
271 **any viral genome in stools from non-cohoused WT mice (Fig. 4D).  $5.49 \cdot 10^2 \pm 1.78 \cdot 10^2$  mean**  
272 **( $\pm$  sem) genome copies numbers were measured in *Nod2*<sup>-/-</sup> mice co-housed with WT mice and**  
273  **$4.12 \cdot 10^2 \pm 2.63 \cdot 10^2$  in WT mice co-housed with *Nod2*<sup>-/-</sup> animals. These preliminary results**  
274 **need to be further explored but they hint towards the hypothesis that the knock-out of NOD2**  
275 **favors the multiplication of one or more phylum of the gut microbiota that in turn allows a**  
276 **prolonged persistence of MNoV\_S99.**

277 In order to have a better comprehension of how these dual stimuli are articulated together, we  
278 examined Stat1 and I $\kappa$ B $\alpha$  signalling pathways activation in response to MNoV\_S99 infection  
279 followed by secondary MDP (commonly expressed by all bacteria) treatment in monocytes

280 isolated from WT mice. As previously shown with BMDM or Raw264.7 cells, MNoV\_S99  
281 infection did also induce Stat1 activation (Fig. 4E and 4F) in monocytes. Similarly, IκBα was  
282 not activated by the viral infection alone. Though, relatively high basal levels were already  
283 detectable in mock-infected cells. As expected the treatment with the peptidoglycan MDP  
284 alone triggered potent IκBα activation without activating Stat1 (Fig. 4E, MNoV\_S99-, MDP+  
285 condition). Interestingly, while IκBα activation by single MNoV\_S99 infection was not  
286 achievable (Fig. 4E, MNoV\_S99+, MDP- condition), MNoV\_S99 infection followed by  
287 secondary MDP treatment was associated with both pathways' moderate but significant  
288 activation (Fig. 4F, MNoV\_S99+, MDP+ vs mock treated cells).

289 **Similarly, we noticed a significantly augmented pro-inflammatory cytokine TNFα response to**  
290 **MDP in dendritic cells that were first primed with Noroviral RNA (Fig. 4G).**

291 Finally, we compared WT and *Nod2*<sup>-/-</sup> derived BMDM cultures' TNFα response to  
292 MNoV\_S99 infection followed by MDP treatment. TNFα levels were increased with  
293 increasing doses of virus in WT cells, while they remained undetectable (Moi 0.1) or much  
294 lower (Moi 1) in *Nod2*<sup>-/-</sup> cells (Fig. 4H).

295 Overall, these *in vitro* data fit nicely with our *in vivo* models where excessive bacterial-  
296 induced inflammatory responses were observed when mice were infected with MNoV\_S99  
297 and treated with DSS. The increase in *Nod2* transcription levels in response to noroviral  
298 infection favours in turn excessive inflammatory signalling pathways activation. This suggest  
299 that MNoV\_S99 induces a loss of tolerance to bacterial MDP **in WT mice.**

300

## 301 **Discussion**

302 The breach in gut flora homeostasis termed dysbiosis is becoming central in the onset of  
303 chronic inflammatory bowel disease, such as Crohn's disease<sup>29,30</sup>. With the development of  
304 molecular biology techniques, alterations in the virome relevant to disease pathogenesis are

305 thoroughly under investigation<sup>2</sup>. The cytosolic sensor NOD2 is very well known for its  
306 pathogen associated molecular pattern recognition activity against bacteria<sup>13</sup>, and recently it  
307 appears it can also participate in detection of certain viruses<sup>14</sup>. Hence, the combination of  
308 genetic susceptibility factors and opportunistic viral infections could be one of the triggers of  
309 CD<sup>31</sup>. Using pro-inflammatory signalling activation downstream of NOD2 as a read-out, we  
310 have analysed if NOD2 can also participate in MNoV detection. All our *in vitro* experiments  
311 with monocytic lineage derived primary cells (e.g. BMDM, BMDC and BM) and cell line  
312 (e.g. Raw264.7) infected with different MNoV strains showed activation of the STAT1  
313 signalling pathway implicated in the pro-inflammatory IFN response. By contrast, these  
314 responses were abrogated in *Nod2*<sup>-/-</sup> and *Mavs*<sup>-/-</sup> mice derived BMDM.

315 Amongst enteric viruses commonly found in the microbiota, the Norovirus is considered as a  
316 good candidate in the hypothesis of an environmental-driver in genetically susceptible hosts  
317 at the origin of Crohn's disease onset. MNoV\_CR6 strain was shown to induce colitis in  
318 *Atg1611*<sup>HM</sup> mice presenting defective autophagy in a DSS colitis model<sup>21</sup>.

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

322 Our *in vivo* data show significantly exacerbated inflammation when applying either 3% or 5%  
323 DSS colitis protocol to WT mice. The excessive MNoV-S99 associated inflammatory  
324 response was abrogated in *Nod2*<sup>-/-</sup> mice or in BMDM derived from these mice showing  
325 NOD2's implication in the pathogenesis and the viral sensing respectively. The genetic  
326 ablation of NOD2 also improved the disease risk conferred by MNoV\_CR6 in *Atg1611*<sup>HM</sup>  
327 mice (data not shown).

328 Hence, NOD2 dependent signalling adds a layer of regulation in viral sensing and finetunes  
329 the immunopathology levels caused by the infection. While in our Norovirus + DSS model

330 knocking-down NOD2 prevents gut inflammation, it has the opposite effect with Influenza A  
331 virus (IAV) infection in the lung of *Nod2*<sup>-/-</sup> or *Ripk2*<sup>-/-</sup> mice<sup>32</sup>. Indeed, the NOD2-RIPK2  
332 signalling axis has been shown to downmodulate the NLRP3 dependent mitophagy activation  
333 and the associated inflammatory response in the lung tissue of infected mice.

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

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

354

355

356

357 **Methods:**

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

360

361 **Colitis mouse model and viral infection**

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

369 For cohousing experiments, WT or *Nod2*<sup>-/-</sup> (n = 5 each) C57BL/6J mice (CDTA, Orléans,  
370 France) coming from non-specific pathogen free area were cohoused for 4 weeks. A group of  
371 non-cohoused WT mice (n = 5) were kept separated. Stools were collected and kept frozen (-  
372 80°C) until processed for RNA extraction and RT-qPCR measurement of viral genomes (see  
373 below).

374 **Cells and virus**

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



379 Bone marrow derived macrophages (BMDM) were isolated from femurs of wild-type, *Nod2*<sup>-/-</sup>  
380 or *Mavs*<sup>-/-</sup> mice of C57BL/6J background<sup>34</sup>. Briefly, bone marrow cells were flushed out of  
381 the bones, red blood cells were lysed using a 160mM NH<sub>4</sub>CL and 170mM Tris solution for 5  
382 minutes at RT. 3 to 6.10<sup>6</sup> viable cells were plated in non-cell-culture-treated petri dishes and  
383 grown for 5-7 days in Iscove's modified Dulbecco's medium (IMDM), supplemented with  
384 10% foetal bovine serum, 1% penicillin/streptomycin, 1% non-essential amino acid, 1%  
385 sodium pyruvate, 1% glutamine, and 20% (*v/v*) conditioned-media from L929 cells.

386 Bone marrow derived dendritic cells were isolated similarly as BMDM cells. Then, 2.10<sup>6</sup>  
387 viable cells were plated in non-cell-culture-treated petri dishes and grown for 7 days in RPMI  
388 1640, supplemented with 10% FBS, 1% P/S, 1% L-glutamine, 1% HEPES and 20% (*v/v*)  
389 conditioned-media from J558 cell line producing murine granulocyte monocyte colony  
390 stimulating factor (GM-CSF).

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

393 The MNoV-S99 strain (GenBank accession no. DQ911368) was provided by Prof. P. Maris  
394 from ANSES Fougères Laboratory (France) and was propagated in RAW 264.7 cells.  
395 Concentration of stock was determined by plaque assay. Mice were infected orally by pipette  
396 with 3x10<sup>7</sup> plaque forming units (PFU) resuspended in 25μL PBS.

397 For in vitro infection experiments, 3-5.10<sup>5</sup> viable cells were seeded in 6 wells plates. The next  
398 day, just before viral infection, cells were counted from 1 well to determine precisely the  
399 multiplicity of infection (Moi). In the remaining wells medium was removed and replaced  
400 with variable quantities of MNoV-S99 from a common viral stock in a final volume of 1 mL  
401 fresh medium per well. Mock treated cells were incubated in parallel with matching quantities  
402 of culture media from non-infected cells that was generated at the same time as viral stocks.

403 At various time-points after infection, supernatant was removed and cells were washed once  
404 with PBS, before lysis in RIPA buffer for WB analysis or RLT buffer for total RNA  
405 extraction (RNAeasy kit, Quiagen) for RT-qPCR experiments.

406

#### 407 **Immunohistochemistry, ELISA, western blot and RT-qPCR analysis**

408 For immunohistochemistry analysis, formalin-fixed colon samples were embedded in  
409 paraffin. 5µm sections were stained with H&E. Slides were imaged with an AxioPlan 2  
410 (Zeiss) microscope and blindly scored for inflammation severity by two investigators as  
411 previously described<sup>35</sup>.

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

418 For TNFα ELISA from BMDC,  $5.10^5$  cells were plated in 24 wells plate and treated with  
419 MNoV\_S99's ssRNA (extracted with RNeasy Quiagen kit) ± MDP or MDP-DD (Invivogen)  
420 over-night and supernatants were measured for TNFα levels.

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

424 For western blot analysis, total proteins from cell lysates were quantified by BCA protein  
425 assay kit (Pierce), equalized amounts of proteins (10-20µg) were resolved by 4-15% gradient  
426 SDS-PAGE gels and transferred to nitrocellulose membranes. Membranes were blocked in  
427 0,05% PBS-Tween, 5% skimmed milk solution, before incubation subsequently with primary

428 and secondary antibodies diluted (see Table 1) in blocking solution. Following secondary  
429 antibodies incubations, signals of interest were detected using a chemiluminescence reader  
430 (ImageQuant<sup>TM</sup> Las 4000, GE Healthcare Life Sciences), images were processed and band  
431 ROI were quantified with Image J software (NCBI). Mean band intensities  $\pm$  SD of protein of  
432 interest were normalized to  $\beta$ -actin. Relative values to control mock-infected conditions were  
433 compared between different experiments.

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

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

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

452 standard curve with serial dilutions of the plasmid pMNoV1 corresponding to the full genome of  
453 MNoV1 (Dr Christiane Wobus, University of Michigan, USA).

454

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

456 *NOD2*<sup>GFP</sup> mice<sup>37</sup> in the C57BL/6 background were maintained at the Institut Pasteur Central  
457 Animal facility. We used heterozygous *NOD2*<sup>GFP</sup> mice since the presence of one functional  
458 copy of *NOD2* ensures that physiological responses dependent on functional NOD2 are not  
459 impaired. Heterozygous *NOD2*<sup>GFP</sup> female mice (8-12 weeks of age) were sacrificed and  
460 several centimetres of the distal ileum were removed, opened longitudinally and washed in  
461 PBS. The tissue was spread flat over a 4% w/v LMP agarose (Merck) pad and fixed with 4%  
462 v/v paraformaldehyde (VWR). The ileal tissue was then embedded in a 4% LMP agarose  
463 block and cut into 200µm-thick sections using a Microm HM 650 V Vibration microtome.

464 For staining, tissues were blocked, permeabilized and marked with an anti-GFP (rabbit  
465 polyclonal antibody A-11122) primary antibody and secondary Alexa Fluor 488 goat anti-  
466 rabbit antibody. Tissues sections were counter stained with DAPI (1µg/mL, BD Biosciences)  
467 and Phalloidin- iFluor 647 (1:200 dilution, Abcam and mounted using ProLong Gold  
468 Antifade reagent. Confocal acquisitions were performed using a Leica HyD SP5 confocal  
469 microscope. Image analysis was performed using Icy version 2.4.2.0<sup>38</sup>.

470

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- 556

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570

571 **Authors' contribution.** G.M. and M.T. performed the majority of the experimental  
572 procedures. T.G. contributed to the preclinical studies in mice. M.D., T.G. and O.B.  
573 performed RT-qPCR and ELISA analysis. M.D. contributed to histological analysis of colon  
574 sections. E.W. performed viral genome quantification by RT-qPCR. R.W. and I.G.B.  
575 performed histological analysis of tissue sections from NOD2- $\beta$ -Gal reporter mice. All  
576 authors contributed to interpretation of raw data and critically reviewed and/or modified the  
577 manuscript. G.M. and M.C. conceived, designed and wrote the paper.

578

579 **Figures legends:**

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

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

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

598 **Weight loss comparison in A)- 3% DSS-induced colitis ± MNoV\_S99 in WT mice (n=6) and B)- in**  
599 ***Nod2*<sup>-/-</sup> mice (n=6), statistical differences were determined by 2 ways ANOVA test, \* p < 0,05, \*\* p <**  
600 **0,01, \*\*\* p < 0,001. C)- Representative images of H&E staining of colon sections from mice in A).**  
601 **Insets showing higher magnifications are presented below each image. D)- Percentages of increase of**  
602 **muscle wall thickness from colon section images are plotted after baseline subtraction relatively to the**  
603 **values obtained from WT mice treated only with DSS (n = 6), statistical differences were determined**  
604 **with Student's t-Test.**

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608 **Figure 3: MNoV\_S99 associated pro-inflammatory signalling is Nod2 and MAVS-dependent in**  
609 **myeloid lineage cells**

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

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630 **Figure 4: NOD2 dependent pro-inflammatory signalling associated with MNoV\_S99 infection**  
 631 **and bacterial MDP**

632 Quantification of *Nod2* mRNA levels measured by RT-qPCR in **A)**- in Raw264.7 cells infected with  
 633 MNoV\_S99 (Moi 0.1 or 1, **for 24h**) and in **B)**- in BMDM from WT mice (Moi 0.1, **for 24h**)  
 634 normalized to *ActB* and relative to mock-infected cells (n = 3). **C)**- MNoV genome quantification from  
 635 WT and *Nod2*<sup>-/-</sup> BMDM cells infected with MNoV\_S99 (Moi 0.1 for 24h) (n = 3). **D)**- Representative  
 636 western blot showing STAT1 and IκBα signalling pathways modulation in cell lysates from  
 637 monocytes that were either infected with MNoV\_S99 (Moi 5, 2h), treated with MDP (10ng/mL, 2h),  
 638 or a combination of both. **E)**- Quantification and comparison of pSTAT1 and pIκBα signals between  
 639 MNoV\_S99 infected alone cells or in combination with MDP (10ng/mL, 2h), (n = 3), with the band  
 640 intensity being normalized to β-actin and relative to mock-treated cells. **F)** **Mouse norovirus genome**  
 641 **quantification from stool of non-cohoused WT mice (WT), *Nod2*<sup>-/-</sup> mice cohoused with WT mice**  
 642 **(CH-WT<sub>Nod2</sub><sup>-/-</sup>) or WT mice cohoused with *Nod2*<sup>-/-</sup> (CH-*Nod2*<sup>-/-</sup>-WT) (n=5 per group). **G)** TNFα  
 643 production by BMDM cells infected with MNoV\_S99 (Moi 0.1 or 1) for 6h, prior to being treated  
 644 with MDP (10μg/mL) overnight. **H)** TNFα production by BMDC subjected to MNoV\_S99's ssRNA  
 645 (10μg/mL) alone, or supplemented with either MDP (1μg/mL) or MDP-DD (1μg/mL) overnight. In all  
 646 experiments statistical differences were analyzed with Student's t Test.**

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650 **Supplementary Figures**

651

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

654 **A)** Colon length comparison from WT and *Nod2*<sup>-/-</sup> 3% DSS ± MNoV\_S99 treated mice, statistical  
 655 differences were determined with a t-Test, \* p <0,02. **B)**- Histological scores comparison between WT  
 656 and *Nod2*<sup>-/-</sup> ± MNoV\_S99 treated mice, statistical differences were determined with a t-Test, \* p  
 657 <0,05. **C)**- **TNFα production of explants (ON) from WT and *Nod2*<sup>-/-</sup> 3% DSS ± MNoV\_S99 treated**  
 658 **mice was measured by ELISA.**

659

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

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

665

666 **Tables:**

<b>Table1: Antibodies used for Western Blotting</b>		
Primary antibodies	Dilution	Supplier
anti-phosphoStat1 (Tyr 701) (58D6) (85kDa)	1/1000	Cell Signaling 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

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