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

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

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 of gut virus communities)<sup>1</sup>. This is of particular importance for unravelling the pathogenesis 83 of viruses that are establishing persistent infections<sup>2,3</sup>. Amongst enteric viruses yielding pro-84 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 of noroviruses from genogroups I, II, IV, VIII and IX<sup>4-6</sup> that infect humans (HuNoVs), 88 mechanistic details were mostly obtained from studies using the mouse norovirus (MNoV)<sup>7</sup>. 89 Indeed, MNoVs can be easily produced *in vitro* in macrophages or dendritic cells<sup>8</sup>. Several 90 91 MNoV strains have been described based on their shedding time in the stool and classified as persistent or acute (non-persistent) strains<sup>9</sup>. 92

93 Multiple intracellular pattern recognition receptors (PRR) have been associated with indirect 94 noroviral detection, especially during NoV genome replication, when double stranded RNA intermediates are generated<sup>10</sup>. The Toll like receptor 3 (TLR3) that recognizes any dsRNA in 95 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 MNoV CW3<sup>11</sup>. Amongst the Nod like receptor (NLR) family, Nlrp6 was shown to bind with 99 the encephalomyocarditis virus (EMCV), a single strand RNA (+) enteric virus, via the RNA 100 101 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 (MAVS) protein. Interestingly, this study also showed that, Nlrp6<sup>-/-</sup> mice infected with 103 MNoV\_1 had higher viremia, suggesting similar mechanisms of NoV sensing by  $Nlrp6^{12}$ . 104 105 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>.

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

Interestingly, Cadwell and colleagues have demonstrated how infection with the MNoV persistent strain CR6 rendered  $Atg16l1^{HM}$  mice more susceptible to dextran sodium sulphate (DSS)-induced colitis<sup>21</sup>. Functional autophagy is critical for microbial elimination. And NOD2's interaction with ATG16L1 was shown to regulate the autophagy clearance of pathogenic bacteria<sup>22</sup>. Improper detection of microbial content in the digestif tract leads to intestinal inflammation<sup>23</sup> and loss-of-function point mutations of either *NOD2* or *ATG16L1* 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 uncontrolled132 proinflammatory signalling are still missing.

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

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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 establish persistent infections<sup>26</sup>, we decided to assess MNoV\_S99's implication in the 145 146 vulnerability to inflammatory responses of the intestinal mucosa. Hence, we examined MNoV\_S99's impact in a chemically-induced colitis model. C57BL/6J mice were first 147 infected with a single dose of 10<sup>7</sup> TCID<sub>50</sub>PFU/mL of MNoV S99 or mock-treated with PBS. 148 149 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 significant weight loss (purple and orange curves Fig. 1B and C). After 7 days of DSS 152 153 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 measured in mice treated with the virus in presence of both 3 and 5% DSS in comparison with 157 158 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 with MNoV S99 and treated with 5% DSS in comparison with non-infected control animals 162 163 (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 of over-night in vitro cultured colon explants from MNoV\_S99 infected + 5% DSS treated 165 166 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.

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#### 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 chemically-induced epithelial injury<sup>27</sup>. To determine whether NOD2 is also implicated in the 173 174 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 virus on the daily weight-loss was absent upon MNoV\_S99 infection of Nod2<sup>-/-</sup> mice (Fig. 177 178 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 after mice were autopsied. Colon tissue sections staining confirmed the decreased levels of 182 colonic inflammation from MNoV\_S99 infected Nod2<sup>-/-</sup> vs WT mice (Fig. 2C). In agreement, 183 muscle thickness quantification from these colon sections showed significantly increased 184 185 thickness in MNoV\_S99-infected WT mice under 3% DSS treatment vs 3% DSS alone (Fig. 2D). While in  $Nod2^{-/-}$  mice subjected to similar conditions, the excess of inflammation caused 186 by the viral infection was lost. Consequently, the variations of colon length measurements 187 that were induced upon MNoV\_S99 infection were absent in Nod2<sup>-/-</sup> mice when compared to 188 189 WT infected mice (Fig. S1A). Similarly, differences in histological scores measured from MNoV S99-infected WT mice vs DSS alone were also lost in Nod2<sup>-/-</sup> infected mice (Fig. 190 191 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 mice infected with MNoV S99 and treated with 3% DSS vs those with DSS only. This effect 193 was absent in the supernatants from  $Nod2^{-/-}$  mice infected and treated similarly (Fig. S1C). 194 195 Overall, these data show the importance of bacterial sensing by NOD2 and the excessive

inflammatory response measured in the presence of MNoV\_S99 in our DSS-induced colitismouse model.

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

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

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

Interferon production being one of the most common response to the intracellular presence of RNA genomes of foreign origin, we examined if the STAT1 signalling pathway that lays upstream of ATG16l1-mediated interferon production was activated in response to murine 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 detectable as early as 15 minutes post-infection and its expression further increased at 30 min 224 225 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 NFkB activation, another pro-228 inflammatory signalling pathway. For this purpose, pIkBa levels were quantified from 229 infected cell lysates. Despite basal low-level activation in mock-infected cells, pIkBa 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 time frame, MNoV S99 failed to induce any activation of these signalling pathways (Fig. 232 S2). Next, we analysed the response of BMDM derived from WT or  $Nod2^{-/-}$  mice that were 233 infected with increasing doses of MNoV\_S99 for 30 or 60 minutes (Fig. 3C). STAT1 234 235 activation increased over the course of the acute infection in cells derived from WT mice. In contrast, the levels of pSTAT1 were barely detectable in  $Nod2^{-/-}$  cells at Moi 1. However, at a 236 237 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.

Next, we examined if Stat1 activation was detectable after prolonged times of infection. Indeed, we measured significant differences in pStat1 expression levels between WT and  $Nod2^{-/-}$  MNoV S99-infected cells 24h post-infection at Moi 0.1 (Fig. 3D).

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

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

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

To investigate the mechanisms behind the excessive inflammatory response induced by MNoV\_S99 infection in our *in vivo* experiments, we further explored how NOD2 expression levels were affected by the viral infections. NOD2 expression was determined by RT-qPCR in Raw264.7 cells or BMDM derived from
WT mice treated with MNoV\_S99. Interestingly, NOD2 expression levels are significantly
increased in response to the viral infection in both cellular models (Fig. 4A and 4B).

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

Accordingly, when we analyzed the viral replication in BMDM derived from WT or *Nod2*<sup>-/-</sup> mice infected *in vitro* with MNoV\_S99 (Moi 0.1 for 24h), the quantity of viral genomes was 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 infection especially for the MNoV\_CR6 persistent strain<sup>19</sup>. WT and Nod2<sup>-/-</sup> mice were 268 269 cohoused 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 any viral genome in stools from non-cohoused WT mice (Fig. 4D). 5.49  $10^2 \pm 1.78 \ 10^2$  mean 271  $(\pm \text{ sem})$  genome copies numbers were measured in Nod2<sup>-/-</sup> mice co-housed with WT mice and 272 4.12  $10^2 \pm 2.63 \ 10^2$  in WT mice co-housed with Nod2<sup>-/-</sup> animals. These preliminary results 273 274 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.

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

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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, IkBa 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 IkBa activation by single MNoV S99 infection was not achievable (Fig. 4E, MNoV\_S99+, MDP- condition), MNoV\_S99 infection followed by 286 287 secondary MDP treatment was associated with both pathways' moderate but significant 288 activation (Fig. 4F, MNoV\_S99+, MDP+ vs mock treated cells).

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

Finally, we compared WT and  $Nod2^{-/-}$  derived BMDM cultures' TNF $\alpha$  response to MNoV\_S99 infection followed by MDP treatment. TNF $\alpha$  levels were increased with increasing doses of virus in WT cells, while they remained undetectable (Moi 0.1) or much lower (Moi 1) in  $Nod2^{-/-}$  cells (Fig. 4H).

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

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#### 301 Discussion

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

thoroughly under investigation<sup>2</sup>. The cytosolic sensor NOD2 is very well known for its 305 pathogen associated molecular pattern recognition activity against bacteria<sup>13</sup>, and recently it 306 appears it can also participate in detection of certain viruses<sup>14</sup>. Hence, the combination of 307 genetic susceptibility factors and opportunistic viral infections could be one of the triggers of 308 CD<sup>31</sup>. Using pro-inflammatory signalling activation downstream of NOD2 as a read-out, we 309 310 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 responses were abrogated in *Nod2<sup>-/-</sup>* and *Mavs<sup>-/-</sup>* mice derived BMDM. 314

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  $Atg16l1^{HM}$  mice presenting defective autophagy in a DSS colitis model<sup>21</sup>.

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

Our *in vivo* data show significantly exacerbated inflammation when applying either 3% or 5% DSS colitis protocol to WT mice. The excessive MNoV-S99 associated inflammatory response was abrogated in *Nod2<sup>-/-</sup>* mice or in BMDM derived from these mice showing NOD2's implication in the pathogenesis and the viral sensing respectively. The genetic ablation of NOD2 also improved the disease risk conferred by MNoV\_CR6 in Atg16l1<sup>HM</sup> mice (data not shown).

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

knocking-down NOD2 prevents gut inflammation, it has the opposite effect with Influenza A virus (IAV) infection in the lung of  $Nod2^{-/-}$  or  $Ripk2^{-/-}$  mice<sup>32</sup>. Indeed, the NOD2-RIPK2 signalling axe has been shown to downmodulate the NLRP3 dependent mitophagy activation 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 JAK-STAT antiviral activity<sup>33</sup>. Interestingly, in our cellular models, in addition to the 337 338 phosphorylation of STAT1 that we observed when cells were infected with MNoV\_S99 339 alone, the subsequent treatment with MDP induced an increased NfkB signalling pathway via 340 IκBα 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 observed in cultures derived from WT but not in  $Nod2^{-/-}$  mice, at least at a lower dose (Moi 346 347 0.1) of infection. At higher doses, NOD2-independent alternative mechanisms seem to be 348 active to promote Stat1 activation and TNF production, but still at a lesser level than in WT 349 cells.

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

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#### 357 Methods:

358 Unless otherwise indicated, all media and reagents were purchased from ThermoFischer359 Scientific.

360

#### 361 Colitis mouse model and viral infection

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

For cohousing experiments, WT or  $Nod2^{-/-}$  (n = 5 each) C57BL/6J mice (CDTA, Orléans, France) coming from non-specific pathogen free area were cohoused for 4 weeks. A group of non-cohoused WT mice (n = 5) were kept separated. Stools were collected and kept frozen (-80°C) until processed for RNA extraction and RT-qPCR measurement of viral genomes (see 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. Bone marrow derived macrophages (BMDM) were isolated from femurs of wild-type,  $Nod2^{-/-}$ or  $Mavs^{-/-}$  mice of C57BL/6J background <sup>34</sup>. Briefly, bone marrow cells were flushed out of the bones, red blood cells were lysed using a 160mM NH4CL and 170mM Tris solution for 5 minutes at RT. 3 to  $6.10^6$  viable cells were plated in non-cell-culture-treated petri dishes and grown for 5-7 days in Iscove's modified Dulbecco's medium (IMDM), supplemented with 10% foetal bovine serum, 1% penicillin/streptomycin, 1% non-essential amino acid, 1% sodium pyruvate, 1% glutamine, and 20% (v/v) conditioned-media form L929 cells.

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

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

The MNoV-S99 strain (GenBank accession no. DQ911368) was provided by Prof. P. Maris from ANSES Fougères Laboratory (France) and was propagated in RAW 264.7 cells. Concentration of stock was determined by plaque assay. Mice were infected orally by pipette with  $3x10^7$  plaque forming units (PFU) resuspended in  $25\mu$ L PBS.

For in vitro infection experiments, 3-5.10<sup>5</sup> viable cells were seeded in 6 wells plates. The next day, just before viral infection, cells were counted from 1 well to determine precisely the multiplicity of infection (Moi). In the remaining wells medium was removed and replaced with variable quantities of MNoV-S99 from a common viral stock in a final volume of 1 mL fresh medium per well. Mock treated cells were incubated in parallel with matching quantities 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>.

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

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

421 For TNF $\alpha$  ELISA from BMDM, 2.10<sup>5</sup> 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 TNF $\alpha$  levels.

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

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

For *Nod2* quantification from MNoV\_S99 infected BMDM or RAW264.7 cells, total RNA
extraction was performed using the RNeasy Mini Kit (Qiagen). One µg of RNA was reversed
transcribed in the presence of 2.5mM of oligo-dT using the Reverse Transcriptor kit (Roche)
following the manufacturer's instructions. 75ng of cDNAs were amplified using Q5 HighFidelity 2X Master Mix (New England BioLabs). Primers used in PCR amplification are
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 analysis as previously described<sup>36</sup>. Briefly, total RNA was extracted from BMDM cell 444 cultures using the RNeasy Mini Kit (Qiagen) and from stool using QIAamp Viral RNA Kit 445 446 (Qiagen). 5µL of RNA were used per reaction of RT-qPCR. Viral genome levels were 447 determined using the Takyon<sup>™</sup>Dry No Rox One-Step RT Probe Mastermix (Eurogentec). 448 Viral specifically amplified genomes were 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 of453 MNoV1 (Dr Christiane Wobus, University of Michigan, USA).

454

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

NOD2<sup>GFP</sup> mice<sup>37</sup> in the C57BL/6 background were maintained at the Institut Pasteur Central 456 Animal facility. We used heterozygous  $NOD2^{GFP}$  mice since the presence of one functional 457 458 copy of NOD2 ensures that physiological responses dependent on functional NOD2 are not impaired. Heterozygous NOD2<sup>GFP</sup> female mice (8-12 weeks of age) were sacrificed and 459 several centimetres of the distal ileum were removed, opened longitudinally and washed in 460 461 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 ( $1\mu g/mL$ , 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  $2.4.2.0^{38}$ .

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#### 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  $TCID_{50}PFU/mL$ ), 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  $\pm$  MNoV S99 (n = 6). C)- 5% 584 DSS-induced colitis  $\pm$  MNoV\_S99 (n = 6). In B) and C), statistical differences were determined by 2 ways ANOVA test, \* p < 0.05, \*\* p < 0.01, \*\*\* p < 0.001. D)- Representative images from H&E 585 586 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  $\pm$  MNoV\_S99 591 treated mice (n = 6). G)- Colon length comparison between Control and 5% DSS  $\pm$  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

Weight loss comparison in A)- 3% DSS-induced colitis  $\pm$  MNoV\_S99 in WT mice (n=6) and B)- in Nod2<sup>-/-</sup> mice (n=6), statistical differences were determined by 2 ways ANOVA test, \* p < 0,05, \*\* p < 0,01, \*\*\* p < 0,001. C)- Representative images of H&E staining of colon sections from mice in A). Insets showing higher magnifications are presented below each image. D)- Percentages of increase of muscle wall thickness from colon section images are plotted after baseline subtraction relatively to the values obtained from WT mice treated only with DSS (n = 6), statistical differences were determined 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

A)- Representative image showing an intestinal villus from a  $Nod2^{GFP}$  mouse (GFP is shown in green, 610 611 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  $\pm$  SEM), statistical differences were determined by 1 way ANOVA test \* p < 0,05, \*\* p < 0,005. C)- STAT1 activation in response to MNoV\_S99 (Moi 1 615 or 5) in BMDM from WT vs Nod2<sup>-/-</sup> infected for 30 or 60 minutes. The lower panel shows 616 617 quantification of pSTAT1, bands intensities normalized to β-actin and relative to mock-infected cells 618  $(n = 3, mean \pm SEM)$ . 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  $\pm$  SEM) is shown in the lower panel, statistical differences were determined by 2 ways ANOVA test \*\*\* p < 0,0005. E)- STAT1 activation in 621 622 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  $\pm$  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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## Figure 4: NOD2 dependent pro-inflammatory signalling associated with MNoV\_S99 infectionand 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 IkBa signalling pathways modulation in cell lysates from monocytes that were either infected with MNoV S99 (Moi 5, 2h), treated with MDP (10ng/mL, 2h), 637 638 or a combination of both. E)- Quantification and comparison of pSTAT1 and pIkBa 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  $\beta$ -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 (CH-WT<sub>Nod2-/-</sub>) or WT mice cohoused with Nod2<sup>-/-</sup> (CH-Nod2-/-wT) (n=5 per group). G) TNF $\alpha$ 642 production by BMDM cells infected with MNoV\_S99 (Moi 0.1 or 1) for 6h, prior to being treated 643 644 with MDP (10µg/mL) overnight. H) TNFa 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

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

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

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

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

Table1: Antibodies used for Western Blotting						
Primary antibodies	Dilution	Supplier				
anti-phosphoStat1 (Tyr 701) (58D6) (85kDa)	1/1000					
anti-Stat1(D1K9Y)(85kDa)	1/1000					
anti-phosphol $\kappa$ B $lpha$ (Ser32/36) (5A5) (40kDa)	1/1000					
anti-ΙκΒα (44D4) (39kDa)	1/1000	Cell				
anti-phosphoAKT (Ser473) (D9E) (60kDa)	1/1000	Signaling				
anti-AKT (60kDa)	1/1000	Technology				
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	Supplier				
anti-Rabbit_HRP (#711-035-152)	1/5000	Jackson				
anti-Mouse_HRP (#115-005-003)	1/5000	Laboratories				