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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 signaling upon norovirus infection enhances antiviral immunity and**  
2 **susceptibility to colitis**

3

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49 **Abstract.** Over 90% of epidemic nonbacterial gastroenteritis are caused by human Noroviruses  
50 (NoV) which are persisting in a substantial subset of people allowing their spread worldwide.  
51 It leads to a significant number of endemic cases and up to 70,000 children deaths in developing  
52 countries. NoVs are primarily transmitted through the fecal-oral route. To date studies have  
53 focused on the influence of the gut microbiota **on enteric viral clearance by mucosal immunity.**  
54 In this study, the use of mouse norovirus S99 (MNoV\_S99) and CR6 (MNoV\_CR6), two  
55 persistent strains, allowed us to provide evidence that the norovirus-**induced** exacerbation of  
56 colitis severity relied on bacterial sensing by nucleotide-binding oligomerization domain 2  
57 (Nod2). **Consequently, *Nod2*-deficient mice showed reduced levels of gravity of Dextran**  
58 **sodium sulphate (DSS)-induced colitis with both viral strains. And MNoV\_CR6 viremia was**  
59 **heightened in *Nod2*<sup>-/-</sup> mice in comparison with animals hypomorphic for *Atg16ll1*, that are prone**  
60 **to aggravated inflammation under DSS. Accordingly, the infection of macrophages derived**  
61 **from WT mice promoted the phosphorylation of Signal Transducer and Activator of**  
62 **Transcription1 (STAT1) and NOD2's expression levels. Higher secretion of Tumor Necrosis**  
63 **Factor alpha (TNF $\alpha$ ) following NOD2 activation and better viral clearance were measured in**  
64 **these cells. By contrast, reduced levels of pSTAT1 and blunted downstream secretion of TNF $\alpha$**   
65 **were found in *Nod2*-deficient macrophages infected by MNoV\_S99. Hence, our results uncover**  
66 a previously unidentified virus-host-bacterial interplay that may represent a novel therapeutic  
67 target for treating noroviral origin gastroenteritis that may be linked with susceptibility to  
68 several common illnesses such as Crohn's disease.

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79 **Introduction.** The development of high throughput sequencing techniques progressively  
80 helped us gain a better comprehension of the composition of the enteric virome<sup>1</sup>. This is of  
81 particular importance for unravelling how some enteric viruses can establish persistent  
82 infections and subsequently influence our health<sup>2,3</sup>. Amongst those, noroviruses (NoV) have  
83 been studied for several years. These single stranded RNA (+) viruses, belonging to the family  
84 of *Caliciviridae* are responsible for the majority of non-bacterial gastroenteritis worldwide.  
85 Until recent progress in cultivation methods of noroviruses from genogroups I, II, IV, VIII and  
86 IX<sup>4-6</sup> that infect humans (HuNoVs), mechanistic details were mostly obtained from studies  
87 using the mouse norovirus (MNoV)<sup>7</sup>. Indeed, MNoVs can be easily produced *in vitro* in  
88 macrophages or dendritic cells<sup>8</sup>. Several MNoV strains have been described based on their  
89 shedding time in the stool and classified as either persistent or acute (non-persistent) strains<sup>9</sup>.  
90 Owing to the variety of circulating NoV strains, strain-dependent effects are expected to occur  
91 and should be studied more in details. Even a single amino acid change in the NS1/2 viral  
92 protein is sufficient to switch the acute MNoV\_CW3 strain to a persistent state<sup>10</sup>, showing the  
93 high degree of adaptability of NoV genomes and hence their versatility to counteract host  
94 defence mechanisms. Type III interferon (IFN- $\lambda$ ) response has been shown to control the *in*  
95 *vivo* propagation of the persistent MNoV\_CR6 strain<sup>11</sup>, while type I and II interferons mediate  
96 innate immune responses against acute MNoV infections<sup>12,13</sup>.  
97 Multiple intracellular pattern recognition receptors (PRR) have been associated with indirect  
98 noroviral detection, especially during NoV genome replication, when double stranded RNA  
99 intermediates are generated<sup>14</sup>. As such, the Toll like receptor 3 (TLR3) that recognizes any  
100 dsRNA in the endosomal compartment and the melanoma differentiation associated protein-5  
101 (MDA-5), a member of the RIG-I like receptor (RLR) family that recognizes long dsRNA, have  
102 been shown to participate in the detection of the MNoV\_1 strain and its plaque isolate the  
103 MNoV\_CW3<sup>15</sup>. Amongst the Nod like receptor (NLR) family, it was shown that the loss of

104 *Nlrp6* impairs antiviral immunity to MNoV\_1<sup>16</sup>. Besides its ability to sense muropeptides from  
105 Gram positive and negative bacteria<sup>17</sup> the nucleotide-binding oligomerization domain 2  
106 (NOD2) was also shown to directly bind with the genome (ssRNA-) of the respiratory syncytial  
107 virus (RSV) to induce antiviral responses<sup>18</sup>. The recruitment of MAVS by NOD2 was needed  
108 to activate the interferon-regulatory factor 3 (IRF3) dependent signaling and promote IFN- $\beta$   
109 production. In the same line, NOD2-dependent signaling was shown to disrupt intestinal  
110 homeostasis and increase lethality in an *E. Coli* and MNoV\_1 co-infection mouse model<sup>19</sup>.  
111 Another molecular player that is essential to activate antiviral signaling is the cytosolic protein  
112 signal transducer and activator of transcription 1 (STAT1). MNoV\_1 infection was shown to  
113 be lethal in STAT1-deficient mice<sup>12</sup>. Furthermore, STAT1 dependent interferon responses were  
114 shown to limit MNoV\_1 replication and dissemination *in vivo*<sup>20</sup>.  
115 Interestingly, Cadwell and colleagues have demonstrated how infection with the MNoV  
116 persistent strain CR6 rendered *Atg16l1<sup>HM</sup>* mice more susceptible to DSS-induced colitis<sup>21</sup>.  
117 Interestingly, the interaction of NOD2 with ATG16L1 was shown to regulate the autophagy  
118 clearance of pathogenic bacteria<sup>22</sup>. Furthermore, it has been established that loss-of-function  
119 point mutations of either *NOD2* or *ATG16L1* predispose humans to Crohn's disease<sup>23,24</sup>.  
120 Insights into the mechanisms of how NOD2 signaling is regulated upon noroviral infection and  
121 whether it may subsequently modulate colitis susceptibility remained to be assessed.  
122 In this study, we have investigated how NOD2 influences the pathogenesis of the two persistent  
123 murine norovirus strains MNoV\_S99 (*Berlin*)<sup>25</sup> and MNoV\_CR6 *in vivo* in DSS-induced colitis  
124 models. At the cellular level, the *Nod2*-dependent pro-inflammatory signaling pathways  
125 activation were further examined in response to MNoV\_S99 and MNoV\_CR6 infection in  
126 myeloid cells to determine how this in turn subsequently affect viral production.  
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129 **Results.**

130 **MNoV\_S99 exacerbates the severity of DSS-induced inflammation**

131 Since MNoV\_S99 is a natural strain commonly found in animal facilities that is able to establish  
132 persistent infections<sup>25</sup>, we assessed the implication of MNoV\_S99 in the exacerbation of colitis.  
133 Hence, we examined MNoV\_S99's impact in a widely used chemically-induced colitis model  
134 that is induced upon administration of Dextran Sodium Sulfate (DSS). Specifically, C57BL/6J  
135 mice were first infected with a single dose of  $5 \times 10^7$  TCID<sub>50</sub>/mL of MNoV\_S99 or mock-treated  
136 with PBS (200 $\mu$ L). One week later, drinking water was replaced with either a 3% DSS or 5%  
137 DSS solution for a period of seven consecutive days (Fig. 1A). Body weight-loss was monitored  
138 on a daily basis together with signs of diarrhea and rectal bleeding. With both concentrations  
139 of DSS, the pre-treatment with MNoV\_S99 had induced a more significant body weight loss  
140 (purple and orange curves Fig. 1B and C). After 7 days of DSS treatment, mice were euthanized  
141 and the disease severity was evaluated. Histological analysis of colon sections revealed an  
142 increased infiltration of inflammatory cells and more pronounced epithelial damages in infected  
143 mice treated with DSS when compared to similarly challenged animals that were not infected  
144 (Fig. 1D). Accordingly, increased muscle thickness was measured in colon sections from mice  
145 treated with the virus in presence of both 3 and 5% DSS in comparison with Mock-treated  
146 control mice (Fig. 1E). Similarly, histological scoring of the hematoxylin and eosin (H&E)  
147 stained colonic sections expectedly showed significantly more severity in MNoV\_S99-infected  
148 mice treated with either 3% or 5% DSS than DSS alone (Fig. 1F). Upon mice sacrifice, resected  
149 colons were measured. A significantly reduced length was obtained in mice infected with  
150 MNoV\_S99 and treated with 5% DSS in comparison with non-infected control animals (PBS)  
151 (Fig. 1G). The increased level of colitis was also evidenced by a significantly augmented  
152 secretion of the pro-inflammatory cytokine interleukin-6 (IL-6) in the supernatants of over-  
153 night *in vitro* cultured colon explants from MNoV\_S99 infected + 5% DSS treated mice versus

154 those from DSS only mice (Fig. 1H). Taken together, these data clearly show that MNoV\_S99  
155 infection **is able to lower the resistance of mice to chemically induced colitis.**

156

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

158 In cells from the myeloid lineage, NOD2 was shown to participate in the sensing of *Citrobacter*  
159 *rodentium* and of commensal bacteria that are translocating in response to DSS-induced  
160 epithelial injury<sup>26</sup>. To determine whether NOD2 is also implicated in the immunopathology of  
161 MNoV in our preclinical model of colitis, we compared the severity of DSS-induced colitis in  
162 the presence **or absence** of MNoV\_S99 between WT and *Nod2*-deficient mice. In contrast to  
163 what was observed in WT mice (Fig. 2A), the sensitizing effect of the virus on the daily weight-  
164 loss was absent upon MNoV\_S99 infection of *Nod2*<sup>-/-</sup> mice (Fig. 2B). These data suggest that  
165 the pro-inflammatory effect of MNoV\_S99 observed in WT mice **may depend on the degree of**  
166 **bacterial sensing through NOD2.** Indeed, DSS induces an abrasion of the epithelial barrier  
167 allowing commensal bacteria to cross the mucosa and unleash a **NOD2-dependent** pro-  
168 inflammatory response. This was further confirmed after mice were autopsied. Colon tissue  
169 sections staining showed decreased levels of colonic inflammation from MNoV\_S99 infected  
170 *Nod2*<sup>-/-</sup> vs WT mice (Fig. 2C). In agreement, muscle thickness quantification from these colon  
171 sections showed significantly increased thickness in MNoV\_S99-infected WT mice under 3%  
172 DSS treatment vs 3% DSS alone (Fig. 2D). The variations of colon length measurements that  
173 were induced upon MNoV\_S99 infection in WT mice were absent **by contrast** in *Nod2*<sup>-/-</sup> mice  
174 (Fig. S1A). Similarly, differences in histological scores **and mucosal wall thickness** measured  
175 from MNoV\_S99-infected WT mice vs DSS alone were also **blunted** in *Nod2*<sup>-/-</sup> infected mice  
176 (Fig. S1B). And a clear trend was observed regarding the increased levels of secretion of  
177 Tumour necrosis factor  $\alpha$  (TNF $\alpha$ ) in the supernatants of *in vitro* cultured colon explants from  
178 WT mice infected with MNoV\_S99 and treated with 3% DSS vs those with DSS only. This



179 effect was absent in the supernatants from *Nod2*<sup>-/-</sup> mice infected and treated similarly (Fig.  
180 S1C).

181 Overall, these data **demonstrate that** the excessive inflammatory response measured in the  
182 presence of MNoV\_S99 **depends on NOD2-dependent bacterial sensing** in our DSS-induced  
183 colitis mouse model.

184

### 185 **Mouse noroviruses infections triggers NOD2-dependent pro-inflammatory signaling**

186 Mouse noroviruses are known to have a tropism for the myeloid cell lineages, namely  
187 macrophages and dendritic cells. Recently it was shown that Tuft cells belonging to the gut  
188 epithelial cellular lineages, that possess the CD300lf MNoV receptor can also be targeted by  
189 the MNoV\_CR6 strain<sup>27</sup>.

190 Making use of the GFP-Nod2 reporter mice line, we first examined in which cellular lineages  
191 Nod2 is expressed **within the intestinal mucosa**. We failed to observe **any** GFP-Nod2 expression  
192 in epithelial cells. **By contrast, GFP** signal was detectable in the bulk of the cells below the  
193 lamina propria where **intestinal mononuclear phagocytes** are **primarily** located (Fig. 3A).  
194 Hence, **the NOD2-dependent response to MNoV\_S99 infection was further** studied in  
195 macrophages, monocytes and dendritic cells in our *in vitro* cellular models.

196 Interferon production being one of the most common response to the intracellular presence of  
197 RNA genomes of foreign origin, we **next** examined if the STAT1 signaling pathway that lays  
198 upstream of ATG1611-mediated interferon production was activated in response to murine  
199 norovirus infection. To this end, bone marrow derived macrophages (BMDM) from WT mice  
200 were infected with several **persistent and non-persistent** MNoV strains at a multiplicity of  
201 infection (Moi) of 1, for 1h and pSTAT1 levels of expression from cell lysates were measured  
202 by western blot (Fig. 3B). In contrast to the non-persistent strain MNoV\_CW3 that caused a  
203 striking increase in pSTAT1 levels, **infections with** the persistent strains S99 and CR6 induced

204 a much milder STAT1 signaling pathway activation. The CW1 strain was associated with the  
205 lowest level of STAT1 activation.

206 Since MNoV\_S99 is a natural strain commonly found in animal facilities that is able to establish  
207 persistent infections, we used it to further analyze whether the response to viral infections may  
208 rely on NOD2-associated signaling. Using the macrophage cell-line Raw264.7, we confirmed  
209 that MNoV\_S99 is able to induce phosphorylation of STAT1 (Tyrosine 701) at different times  
210 at Moi 1 (Fig. S2). pSTAT1 was detectable as early as 15 minutes post-infection and its  
211 expression further increased at 30 min and 1h, while only a slight increase of STAT1 level was  
212 detectable at later time-points. In addition to the STAT1 activation through the Janus kinases  
213 JAK1 and Tyk2, we also examined the status of NF $\kappa$ B activation. For this purpose, pI $\kappa$ B $\alpha$  levels  
214 were quantified from infected cell lysates. Despite basal low-level activation in mock-infected  
215 cells, pI $\kappa$ B $\alpha$  intensity increased slightly at 30 min and 1h post-infection even if it failed to reach  
216 significance. When checking AKT or the MAPK-ERK signaling axes in the same time frame,  
217 MNoV\_S99 failed to induce any activation of these signaling pathways (Fig. S2). Next, we  
218 analyzed the response of BMDM derived from WT or *Nod2*<sup>-/-</sup> mice that were infected with  
219 increasing doses of MNoV\_S99 for 30 or 60 minutes (Fig. 3C). STAT1 activation increased  
220 over the course of the acute infection in cells derived from WT mice. In contrast, the levels of  
221 pSTAT1 were barely detectable in *Nod2*<sup>-/-</sup> cells at Moi 1. Similarly, with prolonged times of  
222 infection (24h, Moi 0.1) we measured significantly lowered pStat1 levels in MNoV\_S99-  
223 infected *Nod2*<sup>-/-</sup> cells in comparison with WT cells (Fig. 3D). However, with a higher dose (Moi  
224 5) this lowered phosphorylation of STAT1 in response to MNoV\_S99 infection progressively  
225 vanished (Fig. 3C), suggesting NOD2-independent alternative mechanisms of action operate  
226 when the viral load is too important.

227 To examine whether the Mitochondrial antiviral signaling (MAVS) adaptor protein is required  
228 for sensing MNoV\_S99, BMDM derived from Mavs-deficient mice were generated.

229 Interestingly, the higher pStat1 levels measured in WT cells (Moi 0.1, 1h) were significantly  
230 decreased in **BMDM** from *Mavs*<sup>-/-</sup> cells (Fig. 3E).

231 Altogether, these data show a mild but significant activation of **STAT1** following MNoV\_S99  
232 infection in our *in vitro* cellular models. These signaling responses can be at least partially  
233 abrogated in the absence of MAVS and NOD2.

234

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

236 MNoV\_CR6 infection in DSS-induced colitis was previously shown to promote inflammation  
237 in a functional *Atg16l1* dependent manner<sup>21</sup>. Using this viral strain **that promote STAT1**  
238 **phosphorylation in similar degree than MNoV\_S99 (Fig. 3B)**, we checked how loss of *Nod2*  
239 influenced resolution of inflammation in response to 3% DSS treatment **in mice. As shown with**  
240 **the MNoV\_S99 strain, loss of NOD2 improved the muscle thickening of the colon wall in mice**  
241 **infected with MNoV\_CR6 (Fig. 4A). However, a CR6 strain-specific effect can be observed**  
242 **with these experiments since significantly increased inflammation was only measured in**  
243 ***Atg16l1* hypomorph mice but not in WT mice as opposed to MNoV\_S99.**

244 Next, we examined the impact of the pro-inflammatory signaling activation on viral  
245 propagation in these mice. In the absence of DSS-induced inflammation no significant  
246 differences could be found when comparing the viral load in the stool of WT, *Atg16l1*<sup>HM</sup> or  
247 *Nod2*<sup>-/-</sup> mice (Fig. 4B). **By contrast, the measured viral load was significantly increased in ileum**  
248 **and colon of DSS-treated *Nod2*<sup>-/-</sup> mice in comparison with tissues of *Atg16l1*<sup>HM</sup> mice (Fig. 4C).**  
249 We have also analyzed the viral replication in BMDM derived from WT or *Nod2*<sup>-/-</sup> mice  
250 infected *in vitro* with MNoV\_S99 (Moi 0.1 for 24h). In that case a slight but significant increase  
251 in the number of viral genomes was measured from *Nod2*<sup>-/-</sup> derived cultures (Fig. 4D).

252 **Hence, the lower levels of inflammation found in *Nod2*<sup>-/-</sup> mice seems to favor a higher**  
253 **propagation of these two persistent strains.**

## 254 **MNoV\_S99 infection promotes bacterial sensing by NOD2**

255 To further explore the mechanisms behind the excessive inflammatory response associated with  
256 noroviral infections, we examined **whether noroviral infection may enhance** *Nod2* expression  
257 levels by RT-qPCR. **As what was previously observed with MNoV-1<sup>15</sup>, the transcription level**  
258 **of NOD2 was significantly enhanced** in response to MNoV\_S99 infection in **either** Raw264.7  
259 cells or BMDM derived from WT mice (Fig. 5A and 5B).

260 This increased *Nod2* expression following norovirus infection could explain **at least partially**  
261 the viral-induced pathogenesis that we observed in our colitis models. **Indeed, the greater**  
262 **expression of NOD2 should spike up when suddenly intestinal mononuclear phagocytes**  
263 interact with intestinal commensal bacteria following DSS-induced epithelial abrasion. **We next**  
264 **evaluated whether this heightened expression of NOD2 in response to MNoV\_S99 infection**  
265 **may enhance bacterial sensing. While MNoV\_S99 infection did induce Stat1 (but not I $\kappa$ B $\alpha$ )**  
266 **activation** in monocytes isolated from WT mice (Fig. 5C and 5D, MNoV\_S99+, MDP-  
267 condition), MDP alone triggered potent I $\kappa$ B $\alpha$  activation without activating Stat1 (Fig. 5C,  
268 MNoV\_S99-, MDP+ condition). Interestingly, MNoV\_S99 infection followed by secondary  
269 MDP treatment was associated with significant **phosphorylation of both Stat1 and I $\kappa$ B $\alpha$**  (Fig.  
270 5D, MNoV\_S99+, MDP+ vs mock treated cells). In the same line of investigation, we noticed  
271 a significantly augmented pro-inflammatory cytokine TNF $\alpha$  response to MDP in dendritic cells  
272 that were first primed with noroviral RNA (Fig. 5E). **Next, the secretion of TNF $\alpha$  was measured**  
273 **in the supernatants from WT and *Nod2*<sup>-/-</sup> BMDM cultures** infected with MNoV\_S99 followed  
274 by MDP treatment. TNF $\alpha$  levels were increased with increasing doses of virus in WT cells,  
275 while they remained undetectable (Moi 0.1) or much lower (Moi 1) in *Nod2*<sup>-/-</sup> cells (Fig. 5F).  
276 **Equally of importance, viral titers measured in supernatants showed significantly decreased**  
277 **yields when infected cells were treated with MDP** (Fig. 5G).

278 Overall, these data show that the increased *Nod2* transcription levels following noroviral  
279 infections may at least partially promote an inflammatory response to bacterial MDP, leading  
280 ultimately to a limitation in the viral propagation. Hence the lowered inflammation in *Nod2*<sup>-/-</sup>  
281 mice is associated with increased dissemination of MNoV\_S99 and MNoV\_CR6 strains,  
282 suggesting that bacterial sensing through NOD2 may regulate their persistence.

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303 **Discussion**

304 Intestinal homeostasis is tightly regulated by the interplay between the microorganisms found  
305 in the gut microbiota, intestinal epithelial cells and mononuclear phagocytes. The breach in gut  
306 flora homeostasis termed dysbiosis is becoming central in the onset of chronic inflammatory  
307 bowel disease, such as Crohn's disease<sup>28,29</sup>. With the development of molecular biology  
308 techniques, alterations in the virome relevant to disease pathogenesis are thoroughly under  
309 investigation<sup>2</sup>. As such, the role played by noroviruses was studied in several gastrointestinal  
310 inflammatory models. An endemically circulating mouse norovirus strain was shown to  
311 promote intestinal barrier dysfunctions and inflammatory lesions caused by loss of tolerance to  
312 commensal bacteria in *III0*<sup>-/-</sup> mice<sup>30</sup>. The secondary challenge with a pathogenic *E. coli* strain  
313 following MNoV-1's infection can even cause lethal lesions due to the induction of an  
314 unresolved proinflammatory cytokine secretion in a *Nod1/2* dependent manner<sup>19</sup>. The  
315 hypothesis of an environmental-driver in genetically susceptible hosts that could be at the origin  
316 of Crohn's like disease was also explored with the MNoV\_CR6 strain in the *Atg16l1*<sup>HM</sup> mice  
317 presenting defective autophagy in a DSS colitis model<sup>21</sup>.

318 NOD2 is very well known for its pathogen associated molecular pattern recognition activity  
319 against bacteria<sup>17</sup>, and recently it appears it can also participate in detection of certain viruses<sup>18</sup>.  
320 Using pro-inflammatory signaling activation downstream of NOD2 as a read-out, we have  
321 analyzed if NOD2 can also participate in MNoV detection and elimination. All our *in vitro*  
322 experiments with monocytic lineage derived primary cells (e.g. BMDM, BMDC and BM) and  
323 cell line (e.g. Raw264.7) infected with different MNoV strains showed activation of the STAT1  
324 signaling pathway implicated in the pro-inflammatory IFN and TNF $\alpha$  responses. By contrast,  
325 these responses were abrogated in *Nod2*<sup>-/-</sup> and *Mavs*<sup>-/-</sup> mice derived BMDM.

326 Amongst enteric viruses commonly found in the microbiota, noroviruses have been proposed  
327 to be good candidates in the hypothesis of an environmental-driver in genetically susceptible

328 hosts at the origin of Crohn's disease onset. MNoV\_CR6 strain was shown to induce colitis in  
329 *Atg16l1<sup>HM</sup>* mice presenting defective autophagy in a DSS colitis model<sup>21</sup>.

330 Several PRR are implicated in viral sensing. MDA-5 and TLR3 have been shown to participate  
331 in detection of either MNoV\_1 or CW3<sup>15</sup>. These are both strains which do not persist *in vivo*,  
332 contrary to the S99 (*Berlin*)<sup>25</sup> and CR6 that were used in our study.

333 Our *in vivo* data show significantly exacerbated inflammation when applying either 3% or 5%  
334 DSS colitis protocol to WT or *Atg16l1<sup>HM</sup>* mice. The excessive MNoV\_S99 or MNoV\_CR6  
335 associated inflammatory response was abrogated in *Nod2<sup>-/-</sup>* mice or in BMDM derived from  
336 these mice.

337 Hence, NOD2 dependent signaling adds a layer of regulation in **the viral sensing and**  
338 **propagation**. While in our norovirus + DSS model knocking-down NOD2 prevents gut  
339 inflammation, it has the opposite effect with influenza A virus (IAV) infection in the lung of  
340 *Nod2<sup>-/-</sup>* or *Ripk2<sup>-/-</sup>* mice<sup>31</sup>. Indeed, the NOD2-RIPK2 signaling axis has been shown to  
341 downmodulate the NLRP3 dependent mitophagy activation and the associated inflammatory  
342 response in the lung tissue of infected mice.

343 Noroviruses propagation or prolonged persistence is tightly regulated by the interplay between  
344 several signaling pathways. MNoV\_1 replication was shown to be inhibited in macrophages  
345 following LPS mediated NF- $\kappa$ B activation that in turn triggers IFN- $\beta$  dependent JAK-STAT  
346 antiviral activity<sup>32</sup>. Interestingly, in our cellular models, in addition to the phosphorylation of  
347 STAT1 that we observed when cells were infected with MNoV\_S99, **the heightened *Nod2***  
348 **expression levels preceded a greater ability of cells to respond to bacterial MDP**. Consequently,  
349 the subsequent treatment with MDP induced an increased Nf $\kappa$ B signaling pathway via I $\kappa$ B $\alpha$   
350 activation. The sequential activation of these two pro-inflammatory signaling pathways resulted  
351 in the inhibition of the MNoV\_S99 propagation on one hand and the augmented production of  
352 the pro-inflammatory cytokine TNF $\alpha$  **in response to MDP** on the other hand. Hence, the

353 aggravated inflammatory pathology we observed in our DSS colitis model can be explained by  
354 this NOD2-dependent signaling loop in MNoV\_S99 or MNoV\_CR6 infected mice.

355 In conclusion, our study shows a **NOD2-dependent bacterial sensing that prevents**  
356 **dissemination of the persistent MNoV\_S99 and MNoV\_CR6 strains.**

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378 **Methods:**

379 Unless otherwise indicated, all media and reagents were purchased from ThermoFischer  
380 Scientific.

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382 **Colitis mouse model and viral infection**

383 All animal experiments were approved by the local ethical committee n° CEEA-  
384 2016030717519903. Age and gender related *Nod2*<sup>-/-</sup> and wild type C57BL/6J mice (CDTA,  
385 Orléans, France) were housed 4-6 per cage, under strict pathogens-free environment. Mice were  
386 gavaged with  $5 \times 10^7$  TCID<sub>50</sub>/mL of MNoV\_S99 diluted in 200µl PBS or mock treated with PBS  
387 only. 7 days post-infection, drinking water was substituted with a 3-5% dextran sodium sulphate  
388 (DSS) (35,000-40,000 MW; TdB Consultancy) solution replaced every 2 days for a total  
389 duration of 7 days. 3% DSS colitis experiments with MNoV\_CR6 were conducted as previously  
390 described in<sup>21</sup>.

391 **Cells and virus**

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

396 Bone marrow derived macrophages (BMDM) were isolated from femurs of wild-type, *Nod2*<sup>-/-</sup>  
397 or *Mavs*<sup>-/-</sup> mice of C57BL/6J background<sup>33</sup>. Using a 26G ½" needle, bone marrow cells were  
398 flushed out of the bones with Iscove's modified Dulbecco's medium (IMDM), supplemented  
399 with 10% foetal bovine serum, 1% penicillin/streptomycin, 1% non-essential amino acid, 1%  
400 sodium pyruvate, 1% glutamine, and 20% (v/v) conditioned-media from L929 cells. Red blood  
401 cells were lysed using a 160mM NH<sub>4</sub>CL and 170mM Tris solution for 5 minutes at RT. 3 to

402  $6 \times 10^6$  viable cells were plated in non-cell-culture-treated petri dishes and grown for 5-7 days  
403 in the above mentioned fully supplemented IMDM medium.

404 Bone marrow derived dendritic cells were isolated similarly as BMDM cells. Then,  $2 \times 10^6$   
405 viable cells were plated in non-cell-culture-treated petri dishes and grown for 7 days in RPMI  
406 1640, supplemented with 10% foetal bovine serum, 1% penicillin/streptomycin, 1% L-  
407 glutamine, 1% HEPES and 20% (v/v) conditioned-media from J558 cell line producing murine  
408 granulocyte monocyte colony stimulating factor (GM-CSF).

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

411 The MNoV-S99 strain (GenBank accession no. DQ911368) was provided by Prof. P. Maris  
412 from ANSES Fougères Laboratory (France) and was propagated in RAW 264.7 cells. MNoV-  
413 CR6 concentrated stocks were prepared as described in<sup>34</sup>. Briefly, supernatant from 293T cells  
414 transfected with a plasmid containing the viral genome was applied to RAW264.7 cells to  
415 amplify virus production, and virions were concentrated by ultracentrifugation and  
416 resuspension in endotoxin-free PBS. Concentration of stocks was determined by plaque assay.

417 For in vitro infection experiments,  $3-5 \times 10^5$  viable cells were seeded in 6 wells plates. The next  
418 day, just before viral infection, cells were counted from 1 well to determine precisely the  
419 multiplicity of infection (Moi). In the remaining wells medium was removed and replaced with  
420 variable quantities of MNoV\_S99 from a common viral stock in a final volume of 1 mL fresh  
421 medium per well. Mock treated cells were incubated in parallel with matching quantities of  
422 culture media from non-infected cells that was generated at the same time as viral stocks.

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

426

427 **Immunohistochemistry, ELISA, Western Blot, RT-qPCR and Titration**

428 For immunohistochemistry analysis, formalin-fixed colon samples were embedded in paraffin.  
429 5µm sections were stained with H&E. Slides were imaged with an AxioPlan 2 (Zeiss)  
430 microscope and blindly scored for inflammation severity by two investigators as previously  
431 described<sup>35</sup>. Briefly, tissue lesions were scored from 0 to 3, so was the infiltration of  
432 inflammatory cells in the lamina propria. A combined score was calculated, ranging from 0 (no  
433 changes) to 6 (wide-spread cellular infiltrations and extensive tissue damage).

434 The muscularis propria thickness was measured from light microscopy images of H&E stained  
435 colon sections using Image J (NCBI). 5 measurements from different locations were performed  
436 for each animal and compared with colon sections from mock treated animals.

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

443 For TNFα ELISA from BMDC, 5x10<sup>5</sup> cells were plated in 24 wells plate and treated with  
444 MNoV\_S99's ssRNA (extracted with RNeasy Quiagen kit) with Lipofectamine 2000<sup>18</sup> ± MDP  
445 or MDP-DD (Invivogen) over-night and supernatants were measured for TNFα levels.

446 For TNFα ELISA from BMDM, 2x10<sup>5</sup> cells were plated in 96 wells plate infected with  
447 MNoV\_S99 (Moi 0.1 or 1) for 6h prior to MDP treatment (10ug/mL) over-night and  
448 supernatants were measured for TNFα levels.

449 For western blot analysis, total proteins from cell lysates were quantified by BCA protein assay  
450 kit (Pierce), equalized amounts of proteins (10-20µg) were resolved by 4-15% gradient SDS-  
451 PAGE gels and transferred to nitrocellulose membranes. Membranes were blocked in 0,05%

452 PBS-Tween, 5% skimmed milk solution, before incubation subsequently with primary and  
453 secondary antibodies diluted (see Table 1) in blocking solution. Following secondary antibodies  
454 incubations, signals of interest were detected using a chemiluminescence reader  
455 (ImageQuant™ Las 4000, GE Healthcare Life Sciences), images were processed and band ROI  
456 were quantified with Image J software (NCBI). Mean band intensities  $\pm$  SD of protein of  
457 interest were normalized to  $\beta$ -ACTIN. Relative values to control mock-infected conditions were  
458 compared between different experiments.

459 For *Nod2* quantification from MNoV\_S99 infected BMDM or RAW264.7 cells, total RNA  
460 extraction was performed using the RNeasy Mini Kit (Qiagen). One  $\mu$ g of RNA was reversed  
461 transcribed in the presence of 2.5mM of oligo-dT using the Reverse Transcriptor kit (Roche)  
462 following the manufacturer's instructions. 75ng of cDNAs were used for the qPCR analyses  
463 using Q5 High-Fidelity 2X Master Mix (New England BioLabs). *Nod2* and *Actb* were  
464 specifically amplified using the Fwd\_5'GGAAACAACATTGGCAGCAT3' and  
465 Rev\_5'TCTTGAGTCCTTCTGCGAGA3' Fwd\_5'TTCTTTGCAGCTCCTTCGTT3' and  
466 Rev\_5'ATGGAGGGGAATACAGCCC3' respectively. *Actb* was used as an internal reference  
467 gene in order to normalize the transcript levels. Relative mRNA levels ( $2^{-\Delta\Delta Ct}$ ) were  
468 determined by comparing (a) the PCR cycle thresholds (Ct) for *Nod2* and *Actb* ( $\Delta Ct$ ) and (b)  
469  $\Delta Ct$  values for treated and control groups ( $\Delta\Delta Ct$ ).

470 MNoV\_S99 load was determined by measuring MNoV genome copy numbers by RT-qPCR  
471 analysis as previously described<sup>36</sup>. Briefly, total RNA was extracted from BMDM cell cultures  
472 using the RNeasy Mini Kit (Qiagen). 5 $\mu$ L of RNA were used per reaction of RT-qPCR. Viral  
473 genome levels were determined using the Takyon™Dry No Rox One-Step RT Probe  
474 Mastermix (Eurogentec). Viral genomes were specifically amplified using the  
475 Fwd\_5'GTGCGCAACACAGAGAAAACG3' and Rev\_5'CGGGCTGAGCTTCCTGC3'  
476 primers that target MNoV ORF1 region combined with the TaqMan probe 5'[6-FAM]-

477 **CTAGTGTCTCCTTTGGAGCACCTA-[BHQ1]3'** using the Stratagene Mx3005P (Agilent  
478 Technologies) **with 1 RT cycle and 40 cDNA amplification cycles**. The quantification of viral  
479 genome copies was obtained using a standard curve (**ranging from  $2 \times 10^1$  to  $2 \times 10^6$  genome**  
480 **copies**) with serial dilutions of the plasmid pMNoV1 corresponding to the full genome of  
481 MNoV\_1 (Dr Christiane Wobus, University of Michigan, USA). Viral load for MNoV\_CR6  
482 strain was determined by measuring genome copy numbers by RT-qPCR analysis as previously  
483 described in<sup>21</sup>.

484 The net production of MNoV\_S99 was determined using the TCID<sub>50</sub> method as described in<sup>35</sup>.  
485 Briefly, Raw264.7 cells were infected at Moi 1 with MNoV\_S99 for 2h, before adding MDP  
486 (100ng/mL) or not in the supernatant for 24h. Titers (n = 3) were obtained from each  
487 supernatant. The net production is calculated by normalizing the amount of viruses produced  
488 with the amount of viruses used for the infection.

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

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

499 For staining, tissues were blocked and **permeabilized overnight in solution containing 0.4%**  
500 **Triton X-100 (Merck)** and marked with an anti-GFP (rabbit polyclonal antibody A-11122,  
501 Invitrogen) primary antibody and secondary Alexa Fluor 488 goat anti-rabbit antibody

502 (Invitrogen). Tissues sections were counter stained with DAPI (1µg/mL, BD Biosciences) and  
503 Phalloidin- iFluor 647 (1:200 dilution, Abcam) and mounted using ProLong Gold Antifade  
504 reagent. Confocal acquisitions were performed using a Leica HyD SP5 confocal microscope.  
505 Image analysis was performed using Icy version 2.4.2.0<sup>38</sup>.

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632

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

640

641 **Figures legends:**

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

643 **A)**- In vivo experimental design, C57BL/6J mice were orally gavaged with PBS (200 $\mu$ L) or MNoV\_S99  
644 ( $5 \times 10^7$  TCID<sub>50</sub>/mL), 7 days later, all the mice were given DSS in drinking water for a week. Mice were  
645 weighed daily for weight loss comparison. **B)**- 3% DSS-induced colitis  $\pm$  MNoV\_S99 (n = 6). **C)**- 5%  
646 DSS-induced colitis  $\pm$  MNoV\_S99 (n = 6). In B) and C), statistical differences were determined by 2  
647 ways ANOVA test, \* p < 0.05, \*\* p < 0.01, \*\*\* p < 0.001. **D)**- Representative images from H&E  
648 staining of colon sections from mock-treated (Control) or 3% DSS  $\pm$  MNoV\_S99 treated mice. Insets  
649 showing higher magnifications from each image are presented next to each image. **E)**- Muscle wall  
650 thickness was determined with Image J from colon section images. Percentages of increase are plotted  
651 after baseline subtraction relatively to the values obtained from Control mice (n = 6). **F)**- Histological  
652 intestinal epithelial inflammation scores comparison between Control, 3% or 5% DSS  $\pm$  MNoV\_S99  
653 treated mice (n = 6). **G)**- Colon length comparison between Control and 5% DSS  $\pm$  MNoV\_S99 treated  
654 mice **H)**- IL6 secretion levels from colon explants from mock or 5% DSS  $\pm$  MNoV\_S99 treated mice (n  
655 = 6). Statistical differences were determined with Student's t-Test in E, F, G and H.

656

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

658 Weight loss comparison in **A)**- 3% DSS-induced colitis  $\pm$  MNoV\_S99 in WT mice (n = 6) and **B)**- in  
659 *Nod2*<sup>-/-</sup> mice (n = 6), statistical differences were determined by 2 ways ANOVA test, \* p < 0,05, \*\* p <  
660 0,01, \*\*\* p < 0,001. **C)**- Representative images of H&E staining of colon sections from mice in A) and  
661 B). Insets showing higher magnifications are presented below each image. **D)**- Percentages of increase  
662 of muscle wall thickness from colon section images are plotted after baseline subtraction relatively to  
663 the values obtained from WT mice treated only with DSS (n = 6), statistical differences were determined  
664 with Student's t-Test.

665

666 **Figure 3: MNoV\_S99 associated pro-inflammatory signaling is NOD2 and MAVS-dependent in**  
667 **myeloid lineage cells**

668 **A)**- Representative image showing an **intestinal villus** from a *Nod2*<sup>GFP</sup> mouse. NOD2-GFP is shown in  
669 green, Actin is stained with Phalloidin in grey and nuclei were stained with DAPI in blue; scale bare  
670 represents 50 $\mu$ m. **B)**- Representative western blot showing STAT1 signaling pathway activation after  
671 1h infection (Moi 1) with the mentioned MNoV strains in BMDM. Below are shown quantification of  
672 pSTAT1 bands intensities normalized to  $\beta$ -actin and relative to mock-infected cells (n = 3, mean  $\pm$   
673 SEM), statistical differences were determined by 1 way ANOVA test \* p < 0,05, \*\* p < 0,005. **C)**-  
674 STAT1 activation in response to MNoV\_S99 (Moi 1 or 5) in BMDM from WT vs *Nod2*<sup>-/-</sup> infected for  
675 30 or 60 minutes. The lower panel shows quantification of pSTAT1, bands intensities normalized to  $\beta$ -  
676 actin and relative to mock-infected cells (n = 3, mean  $\pm$  SEM). Statistical differences were determined  
677 by 2 ways ANOVA test \* p < 0,05. **D)**- STAT1 activation in response to MNoV\_S99 (Moi 0.1) in WT  
678 or *Nod2*<sup>-/-</sup> BMDM infected for 24h. Relative quantification of pSTAT1 (n = 3, mean  $\pm$  SEM) is shown  
679 in the lower panel, statistical differences were determined by 2 ways ANOVA test \*\*\* p < 0,0005. **E)**-  
680 STAT1 activation in response to MNoV\_S99 (Moi 0.1) in BMDM from WT vs *Mavs*<sup>-/-</sup> infected for 60  
681 minutes. Relative quantification of pSTAT1 (n = 4, mean  $\pm$  SEM) are shown below. The lower panel  
682 shows the mock vs Moi 0.1 infected cells quantification of pSTAT1. Statistical differences were  
683 determined by 2 ways ANOVA test \* p < 0,05.

684

685 **Figure 4: NOD2-dependent noroviral load**

686 **A)**- WT, *Nod2*<sup>-/-</sup> and *Atg16l1*<sup>HM</sup> mice (n = 5 each) were treated with 3% DSS and infected with  
687 MNoV\_CR6 ( $3 \times 10^7$  PFU). Inflammation was measured as percentage of increase in muscle thickness  
688 at the anal-rectal junction in colonic section images plotted after baseline subtraction relatively to the  
689 values obtained from WT mice. **B)**- The viral load measured in stools from WT, *Nod2*<sup>-/-</sup> and *Atg16l1*<sup>HM</sup>  
690 mice at day 3, 5 and 7 post-infection with MNoV\_CR6 ( $3 \times 10^7$  PFU) (n = 8). **C)**- The viral load measured  
691 from indicated tissue samples from MNoV\_CR6 infected ( $3 \times 10^7$  PFU) WT, *Nod2*<sup>-/-</sup> and *Atg16l1*<sup>HM</sup> mice  
692 that were treated for 1 week with D55 3% (n = 5). **D)**- MNoV\_S99 genome quantification from WT and  
693 *Nod2*<sup>-/-</sup> BMDM cells infected with MNoV\_S99 (Moi 0.1 for 24h) (n = 3). All the statistical differences  
694 were measured with Student's t-Test.

695 **Figure 5: NOD2-dependent pro-inflammatory signaling associated with MNoV\_S99 infection and**  
 696 **bacterial MDP**

697 Quantification of *Nod2* mRNA levels measured by RT-qPCR in **A)**- in Raw264.7 cells infected with  
 698 MNoV\_S99 (Moi 0.1 or 1, for 24h) and in **B)**- in BMDM from WT mice (Moi 0.1, for 24h) normalized  
 699 to *ActB* and relative to mock-infected cells (n = 3). **C)**- Representative western blot showing STAT1  
 700 and IκBα signaling pathways modulation in cell lysates from monocytes that were either infected with  
 701 MNoV\_S99 (Moi 5, 2h), treated with MDP (10ng/mL, 2h), or a combination of both. **D)**- Quantification  
 702 and comparison of pSTAT1 and pIκBα signals between MNoV\_S99 infected alone cells or in  
 703 combination with MDP (10ng/mL, 2h), (n = 3), with the band intensity being normalized to β-ACTIN  
 704 and relative to mock-treated cells. **E)** TNFα production by BMDC subjected to MNoV\_S99's ssRNA  
 705 (10μg/mL) alone, or supplemented with either MDP (1μg/mL) or MDP-DD (1μg/mL) overnight. **F)**  
 706 TNFα production by BMDM cells infected with MNoV\_S99 (Moi 0.1 or 1) for 6h, prior to being treated  
 707 with MDP (10μg/mL) overnight. **G)** Net production of MNoV\_S99 in Raw264.7 cells infected with  
 708 MNoV\_S99 (Moi 1 for 2h before MDP treatment (100ng/mL) for 24h). Statistical differences were  
 709 analyzed with Student's t Test.

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712 **Supplementary Figures legends:**

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

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

721

722 **Supplementary Figure 2: MNoV\_S99 associated pro-inflammatory signaling pathways activation**

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

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727 **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-β-ACTIN (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

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