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1 **NOD1 sensing of house dust mite-derived microbiota promotes allergic**
2 **experimental asthma**

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32 **ABSTRACT**

33 • **Background:** Asthma severity has been linked to exposure to Gram-negative bacteria from the
34 environment, that are recognized by NOD1 receptor and are present in HDM extracts. NOD1
35 polymorphism has been associated with asthma.

36 • **Objective:** To evaluate if either host or HDM-derived microbiota may contribute to NOD1-
37 dependent disease severity.

38 • **Methods:** A model of HDM-induced experimental asthma was used and the effect of NOD1
39 deficiency evaluated. Contribution of host microbiota was evaluated by fecal transplantation.
40 Contribution of HDM-derived microbiota was assessed by 16S ribosomal RNA (rRNA)
41 sequencing, mass spectrometry analysis, and peptidoglycan depletion of the extracts.

42 • **Results:** In this model, loss of the bacterial sensor NOD1 and its adaptor receptor-interacting
43 protein kinase 2 improved asthma features. Such inhibitory effect was not related to dysbiosis
44 caused by NOD1 deficiency, as shown by fecal transplantation of Nod1-deficient microbiota to
45 wild-type germ-free mice. 16S rRNA gene sequencing and mass spectrometry analysis of HDM
46 allergen, revealed the presence of some muropeptides from Gram-negative bacteria that belong to
47 the Bartonellaceae family. While such HDM-associated muropeptides were found to activate
48 NOD1 signaling in epithelial cells, peptidoglycan-depleted HDM had a decreased ability to
49 instigate asthma *in vivo*.

50 • **Conclusion:** These data show that NOD1-dependent sensing of HDM-associated Gram-
51 negative bacteria aggravates the severity of experimental asthma, suggesting that inhibiting
52 NOD1 signaling pathway may be a therapeutic approach to treating asthma.

53 **Key messages:**

- 54 • NOD1-dependent sensing of HDM-associated Gram-negative bacteria aggravates
55 experimental asthma
- 56 • Inhibition of NOD1 signaling pathway may provide a new therapeutic approach in asthma

57

58 **Capsule summary**

59 The sensing by NOD1 of HDM-derived Gram-negative bacteria aggravates experimental asthma
60 suggesting that inhibiting this pathway may provide a therapeutic approach in a near future.

61

62 **Keywords**

63 Asthma, allergy; house dust mite; Th2; NOD1; peptidoglycan; epithelial cells, microbiota

64

65 **Abbreviations**

66 AAI: Allergic airway inflammation

67 AHR: Airway hyperresponsiveness

68 I.n.: Intranasal

69 NOD1: Nucleotide-binding oligomerization domain protein 1

70 NOD2: Nucleotide-binding oligomerization domain protein 2

71 RIPK2: Receptor-interacting protein kinase 2

72 MDP: Muramyl di peptide

73 BAL: Bronchoalveolar lavage

74 PG: Peptidoglycan

75 NHBE: Normal human bronchial epithelial

76 HEK: Human embryonic kidney

77 **INTRODUCTION**

78 An increasing body of evidence from clinical and experimental studies suggests that microbiota
79 encompass a wide range of dynamic microbial communities that play a significant role in host
80 immunity including protecting against asthma. Besides the putative effect of the respiratory
81 microbiota in asthma¹⁻³, intestinal dysbiosis appears to also play an important role^{4, 5}.
82 Accordingly, host immune responses are Th2 biased under germ-free (GF) conditions⁶ and
83 antibiotic-treated or GF animal models of allergen-induced asthma exhibit an exacerbated
84 phenotype in either neonates^{7, 8} or adults⁹. However, much less attention has been paid to the
85 microbiota derived from house dust mite (HDM), which is the most frequent cause of allergic
86 asthma, although analysis of HDM extracts has revealed the presence of Gram-negative bacteria
87 that may likely arise from their intestine^{10, 11}. Although it is well established that viral infections
88 are involved in 40 to 85% of asthma exacerbations¹², a link has been established between adult
89 asthma severity and exposure to indoor Gram-negative bacteria¹³. Unique muropeptides from the
90 peptidoglycan (PG) of such bacteria are sensed by the pattern recognition receptors Nucleotide
91 binding oligomerization domain protein 1 (NOD1)^{14, 15} and NOD2, each receptor recognizing
92 distinct PG structures. While NOD1 recognizes meso-diaminopimelic containing muramyl
93 tripeptide (MTP), NOD2 recognizes muramyl dipeptide (MDP)¹⁶. Both receptors signal via the
94 downstream adaptor RIPK2, which then activates MAPK and the NF κ B pathway. Of note,
95 NOD1-mediated sensing of the gut microbiota participates in the development of lymphoid
96 tissues, while its absence results in dysbiosis¹⁷. Additionally, genome-wide association studies
97 have revealed a molecular link between polymorphisms of NOD1, asthma and high levels of
98 IgE¹⁸. In agreement, we have previously shown that a NOD1 agonist used as a systemic adjuvant
99 exacerbates ovalbumin (OVA)-induced Th2-mediated allergic asthma through dendritic cell

100 activation¹⁹, and a recent paper has shown that RIPK2 promotes HDM-induced allergic airway
101 inflammation by favoring type 2 immunity²⁰. The aim of this study was to evaluate if either host
102 or HDM-derived microbiota may contribute to NOD1-dependent disease severity. Here we
103 provide evidence that NOD1-dependent sensing of muropeptides from Gram-negative bacteria
104 present in HDM extracts exacerbate the severity of allergic airway inflammation, independently
105 of the control by NOD1 of the composition of the gut microbiota from its host. These data
106 identify a new mechanism of asthma aggravation linking HDM-derived microbiota and NOD1,
107 and suggest that interfering with NOD1 signaling pathway may provide a therapeutic approach in
108 this cumbersome disease, as well as for exacerbations of asthma driven by Gram-negative
109 bacteria.

110 **METHODS**

111 **Reagents**

112 Key reagents used are listed in Table E1.

113 **Mice**

114 WT Female C57BL/6 mice (6 weeks of age) were purchased from Charles River. *Nod1*^{-/-}, *Nod2*^{-/-}
115 and *Ripk2*^{-/-} mice were backcrossed on the C57BL/6 background at least 8 times. All animals
116 were housed under specific pathogen-free (SPF) conditions, in ventilated cages with absorbent
117 bedding material, maintained on a 12-hour daylight cycle and with free access to commercial
118 pelleted food and water *ad libitum*. GF mice were housed in flexible isolators. All animal
119 experiments were approved by the regional ethical committee and authorized by the ministry of
120 research and innovation (APAFIS#7874-2016070417344442 v3), and the animals' care was in
121 accordance with institutional guidelines. For fecal transplantation, fresh fecal pellets from
122 untreated WT or *Nod1*^{-/-} female mice were resuspended in 1 mL of sterile PBS, and GF mice
123 were reconstituted by oral gavage with 200 µl of the suspension. The remaining homogenate was
124 kept frozen and analyzed for the status of fecal colonization.

125 **HDM-induced allergic airway inflammation and treatment of extracts**

126 HDM mice were sensitized i.n. with Dermatophagoides farinae extract kindly provided by
127 Stallergenes/Greer (lot 9702026) at a high dose of 5 index of reactivity (IR) (i.e. 15 µg protein/
128 40 µL of PBS) or a lower dose of 1IR (i.e. 3 µg protein/ 40 µL) in order to have a strong and a
129 moderate model of allergic inflammation respectively. Control mice received 40 µL of PBS.
130 Seven days after sensitization, mice were challenged i.n. with 40 µL of HDM or PBS daily for 5
131 consecutive days. Forty-eight hours later mice were anesthetized, assessed for airway
132 hyperresponsiveness and sacrificed (Fig 1A). For some experiments HDM extracts were depleted
133 in LPS or in PG (see online repository). For all conditions, airway hyperresponsiveness and lung

134 inflammation were assessed. Broncho alveolar lavage (BAL) fluids were recovered. Lung
135 samples were collected for protein extraction, RNA isolation and histology analysis.

136 **Airway responsiveness measurement**

137 Mice were anesthetized with 0.5 mg/kg medetomidine (Domitor®; Pfizer) and ketamine
138 (Imalgene® 1000; Merial), and immediately intubated with a 18-gauge catheter, followed by
139 mechanical ventilation using the FlexiVent (SCIREQ ®). Mice were exposed to nebulized PBS
140 followed by increasing concentrations of nebulized methacholine (0-100 mg/mL) (Sigma-Aldrich)
141 using an ultrasonic nebulizer (Aeroneb, Aerogen). Return to baseline resistance was ensured prior
142 to the administration of the next doses of methacholine. The mean value of measured resistances
143 was calculated for each dose.

144 **BAL analysis**

145 A total volume of 1mL of ice-cold PBS was used to gently wash the lungs. Cells from the lavage
146 fluid were recovered by centrifugation at 135 g for 5 min at 4°C. Cells were then resuspended in
147 PBS and counted. Samples of this resuspended BAL were spun onto slides (Shandon cytospin 4;
148 Thermo Fisher Scientific) and stained with May-Grünwald Giemsa (Diapath) for differential cell
149 count.

150 **Serum collection and analysis**

151 Blood was drawn from the abdominal vein. Serum was collected by centrifugation (5000 g for 5
152 min) and stored at -20°C. Levels of total IgE and Der f-specific IgG1 were measured in collected
153 sera by ELISA as indicated in the online repository.

154 **Pulmonary histology**

155 The left lobe of the lung from each mouse was fixed in Antigenfix (Diapath) and embedded in
156 paraffin (Histowax, HistoLab) according to the manufacturer's indications. Lung sections of 5µm
157 were stained with a standard Hematoxylin-Eosin stain and Periodic acid-Schiff (PAS) staining kit

158 (Diapath) to evaluate the peribronchial inflammation and mucopolysaccharide staining for mucus
159 respectively.

160 **Lung protein extracts**

161 Lung lobe was homogenized in 1 mL of T-PER (Thermo Scientific) buffer containing protease
162 inhibitors (Roche diagnostics). After 10 min on ice, the lysates were centrifuged at 13,000g for 5
163 minutes at 4°C and supernatants were collected for further cytokine and chemokine
164 measurements. Total protein concentrations of lung extracts were measured using the Pierce BCA
165 protein Assay kit (Thermo Scientific).

166 **Human cell lines and primary human epithelial cells**

167 Human embryonic kidney HEK293, human bronchial epithelium BEAS-2B and human alveolar
168 epithelial A549 cell lines, as well as normal human bronchial epithelial (NHBE) cells were
169 cultured as indicated in the online repository.

170 **HEK293 luciferase reporter cell assay**

171 The luciferase NFκB reporter assays, were performed by seeding HEK293 cells in 96-well plates
172 at 5×10^4 cells per well and transfecting them with expression plasmids producing either human
173 NOD1, mouse Nod1 or mouse Nod2 (0,5ng/well) in combination with the reporter plasmids
174 pBxIV-luc (1 ng/well) and pEF-BOS-β-gal (25ng/well) using Lipofectamine LTX & PLUS
175 Reagent (Invitrogen) following manufacturer's recommendations. The reporter plasmids and the
176 expression plasmids for hNOD1, hNOD2, mNod1 and mNod2 were kindly provided by Gabriel
177 Nuñez and Naohiro Inohara (Ann Arbor, MI, USA). Details are provided in the online repository.
178 The results were normalized by the expression of β-Galactosidase to reflect the efficiency of
179 transfection and expressed as fold expression of the plasmid-transfected cells.

180 **Epithelial cell line stimulation and transfection with siRNA**

181 BEAS-2B cells were stimulated with HDM at a final dose of 0.2 IR (i.e. 6µg protein/mL) unless
182 otherwise stated in the figures, the synthetic NOD1 agonist FK565 (Fujisawa Inc) (50 µg/mL) or
183 MDP (Invivogen) (10 µg/mL), after a 4-hour human recombinant IFN-β (PeproTech) (20 ng/mL)
184 priming. Culture supernatants and cell pellets were collected for cytokine quantification and RNA
185 isolation. In some experiments the stimuli were combined with a specific RIPK2 kinase inhibitor
186 (AGV discovery) at a final concentration of 5 µM. BEAS-2B cells were also stimulated with LPS
187 (Invivogen) in combination with the RIPK2 inhibitor as a specificity control of the inhibitor.
188 Except for mRNA analysis, HDM, FK565 and MDP stimulations were performed in presence of
189 Lipofectamine LTX to enhance intracellular transport. Knockdowns of either *NOD1* or *NOD2* in
190 BEAS-2B were obtained by a 48 hours siRNA transfection after a 4-hour human recombinant
191 IFN-β (PeproTech) (20 ng/ml) priming. NHBE cells were cultured as described in the online
192 repository.

193 **Generation of chimeric mice by bone marrow transplantation**

194 Total body irradiated WT or *Nod1*^{-/-} mice were transplanted with either WT or *Nod1*^{-/-} bone
195 marrow cells. Two months later, mice were sensitized and challenged with HDM as described
196 above. Details are provided in the method online repository.

197 **Quantitative Real-Time PCR**

198 Q-RT-PCR was performed using standard procedures described in the online repository. Primers
199 used are listed in Table E2.

200 **ELISA Measurement of Cytokine Chemokine**

201 Murine cytokines (TSLP, IL-33) and chemokines (CCL17, CCL22) levels in lung protein extracts
202 were assessed using commercial ELISA according to the instructions provided by the
203 manufacturers (R&D Systems and e-Biosciences). Human IL-6 and IL-8 (CXCL8)
204 concentrations in BEAS-2B, A549 and NHBE cell culture supernatants were measured by ELISA

205 using the ELISA Duoset kits (R&D systems). The reasons of the choice of the different cytokines
206 and chemokines are indicated in the online repository.

207 **16sRNA analysis**

208 Fresh fecal samples were collected at the end of the experiment and immediately snap frozen in
209 liquid nitrogen. Fecal DNA was isolated and sequenced at Genoscreen
210 (<https://www.genoscreen.fr>). Quantification of bacterial diversity in HDM was assessed by 16S
211 rRNA sequencing on V3-V4 region. Details are provided in the online repository.

212 **LC-MS/MS Method**

213 HDM extracts were analyzed by LC-MS/MS method as described in the online repository.

214 **Statistical analysis**

215 For bacterial taxonomy analysis we used the microbiome analyst software to determine
216 community properties. PCoA plots using unweighted UniFrac distances were used to analyze the
217 segregation between WT fecal microbiota (FM)→GF and Nod1^{-/-}FM→GF mice using analysis of
218 group similarities (ANOSIM statistical analysis) . Alpha diversity, calculated as the Channon
219 index, was analyzed using Mann Whitney U test. The *p* values below 0.05 were considered to be
220 statistically significant. For animal and cell analyses, data were analyzed using Prism 8.0
221 (GraphPad Software). For normally distributed data, significance of differences between groups
222 was evaluated by one-way analysis of variance (ANOVA) with Bonferroni's *post hoc* test for
223 multiple comparisons. For pairwise comparisons the two-tailed Student's t-test was used. For
224 airway resistance, the two-way analysis of variance test was used. Non normally distributed data
225 were analyzed using the Kruskal-Wallis *H* with Dunn's *post hoc* test. For *in vitro* cell
226 experiments, 3 to 4 biological replicates were performed, and the experiment was repeated two to
227 three times. For *in vivo* experiments, the number of mice per group is indicated in the figure
228 legends. *p* values below 0.05 were considered to be statistically significant.

229 **RESULTS**

230 **Nod1 signaling aggravates HDM-induced allergic airway disease through Ripk2**

231 The *in vivo* effect of Nod1 signaling was investigated in a model of 5 IR (i.e. 15µg protein/mL)
232 HDM-induced asthma²¹ (Fig. 1A) in C57BL6/J mice deficient or not in *Nod1*. HDM-challenged
233 WT mice exhibited all the cardinal features of allergic airway inflammation, including increase in
234 total cell, eosinophil, neutrophil and macrophage numbers in BAL (Fig. 1B), total IgE and HDM-
235 specific IgG1 antibodies (Fig. 1C), and levels of lung Th2/Th17 cytokines and some pro-Th2
236 chemokines (Figs 1D, E1A). All these features were strongly diminished in *Nod1*^{-/-} mice, except
237 for the humoral response and Th17-type cytokine levels (Figs 1B, 1C, 1D and E1A). Lung
238 sections from HDM-challenged mice showed that the increases in PAS-stained mucus and in
239 Hematoxylin Eosin-stained cellular infiltrates in WT mice were decreased in the absence of Nod1
240 (Figs 1E and E1B respectively). Functionally, airway resistance in response to methacholine
241 challenge was totally inhibited in HDM-challenged *Nod1*^{-/-} versus WT mice (Fig. 1F). Likewise,
242 *Nod2* deficiency led to a decrease in BAL cell recruitment after HDM challenge (Fig. 1G) and
243 not in the humoral response (Fig. 1H). By contrast, there was no statistically significant decrease
244 in Th2 and pro-Th2 lung cytokines and chemokines except for CCL2 and KC (Figs 1I and E1C),
245 and no decrease in airway resistance (Fig. 1J). Given that Nod1 and Nod2 signal via the
246 downstream adaptor Ripk2, we next evaluated whether a similar pattern of response was
247 observed in *Ripk2*^{-/-} mice. Miller et al. have recently reported that HDM-induced allergic airway
248 inflammation is reduced in *Ripk2*^{-/-} mice in a model similar to our 5IR model²⁰. Therefore, we
249 assessed *Ripk2*^{-/-} mice in a 1IR (3µg protein/mL) model of HDM-induced allergic inflammation.
250 There was a total inhibition of BAL cell recruitment, in particular of eosinophils (Fig. 2A), no
251 changes in the humoral response (Fig. 2B), a decrease of Th2 type cytokines and chemokines
252 (Fig. 2C), of mucus production (Fig. 2D) and of airway resistance (Fig. 2E). In contrast to the

253 Miller paper²⁰, it is of note that we did not observe changes in the humoral response, but we
254 additionally observed abolition of a cardinal feature of asthma, i.e. airway resistance in *Ripk2*^{-/-}
255 and *Nod1*^{-/-} but not *Nod2*^{-/-} mice. Taken together, these data demonstrate that Nod1, but not Nod2
256 signaling, is involved in the severity of HDM-induced airway disease through Ripk2 activation.

257 **Dysbiotic gut microbiota caused by Nod1 deficiency does not impact HDM-induced allergic**
258 **airway disease risk**

259 Because *Nod1*^{-/-} mice harbor a dysbiotic gut flora, we next assessed if such dysbiosis may
260 influence the development of experimental asthma. Transplantation of fresh fecal homogenates
261 from WT or *Nod1*^{-/-} donor mice was performed in control GF recipients (referred to as WT or
262 *Nod1*^{-/-} fecal microbiota (FM)→GF respectively). Five weeks later recolonized mice were
263 subjected to the HDM protocol (Fig. 3A). Both groups of mice challenged with HDM exhibited
264 similar increased BAL cell recruitment (Fig. 3B), humoral response (Fig. 3C), Th2 type response
265 (Fig. 3D) and airway resistance (Fig. 3E) compared to the PBS groups. To evaluate the gut
266 bacterial ecosystem of recolonized mice, fecal pellets were collected at the end of the
267 experiments and analyzed by 16S ribosomal RNA (rRNA) phylogenetic profiling. PCoA plots
268 using unweighted UniFrac distances highlighted the segregation between ex-GF mice ($p \leq 0.001$,
269 ANOSIM statistical analysis) (Fig. 3F). Alpha diversity, calculated as the Shannon index, showed
270 significant differences between the groups of ex-GF mice (Fig. 3G, $p \leq 0.01$, Mann Whitney test).
271 Heatmap of the top most abundant genera illustrates the major differences in the composition of
272 both groups of mice and the similarities with the microbiota composition from donors' feces (Fig.
273 3H). No difference was observed between PBS- and HDM- treated mice whatever the genotype
274 of the donor mice. Altogether these data underline that the compositional changes in the gut
275 microbiota of *Nod1*^{-/-} mice were not responsible for their protection against HDM-induced
276 disease.

277 **HDM-associated muropeptides are sensed by NOD1 in epithelial cells via RIPK2.**

278 Quantification of bacterial diversity in HDM extracts by 16S rRNA sequencing revealed that
279 87.7% of the assembled reads associated with members of the Gram-negative Bartonellaceae
280 family (Fig. 4A). To identify specific PG moieties, HDM extracts were analyzed by mass
281 spectrometry through a targeted approach specifically looking at well-characterized
282 muropeptides. Both MTriDap and MDP, sensed by NOD1 and NOD2 respectively, were detected
283 and their presence confirmed by fragmentation (Figs 4B and 4C, and table E3). Next, the effect of
284 HDM was evaluated on human embryonic kidney 293 (HEK293) cells harboring a NF- κ B-
285 dependent luciferase reporter and expressing either *hNOD1*, *mNod1*, or *mNod2* and normalized
286 for transfection efficiency using a β -galactosidase control vector. All positive controls induced
287 NF- κ B activation (Fig. 4D). HDM extracts elicited a dose dependent increase in both *hNOD1*-
288 and *mNod1*- dependent luciferase activity, but only an effect at the highest dose of 4IR (i.e. 60 μ g
289 protein/mL) for *mNod2* activity (Fig. 4D). As the allergen first encounters epithelial cells, human
290 bronchial epithelial BEAS-2B cells were primed with IFN- β ²², to increase the baseline expression
291 of *NOD1*. The role of Nod1 signaling pathway was subsequently assessed by quantifying their
292 cytokine production in response to HDM. HDM stimulation elicited a dose dependent increase in
293 the production of IL-6 and IL-8 by BEAS-2B cells with an effect starting at 0.2IR (6 μ g
294 protein/mL) and maximal at 4IR (120 μ g protein/mL) (Fig. E2A). BEAS-2B stimulation by HDM
295 induced a clear increase in *NOD1* and *NOD2* mRNA (Fig. 4E). Although IFN- β increased
296 *NOD1/2* expression and FK565-induced cytokine production (Fig. E2B), no effect was noticed
297 on the response to HDM (Fig E2C). As expected the addition of a specific RIPK2 inhibitor
298 belonging to the 4-amino-quinolines inhibited both IL-6 and IL-8 increases in response to FK565
299 positive control (Fig. 4F), but did not modulate the response to LPS (Fig. E2D). HDM

300 stimulation induced a stronger production of IL-6 and IL-8 (Fig 4F) with a partial inhibitory non-
301 cytotoxic effect (Fig E2E) of RIPK2 antagonist (Fig. 4F). The same results were observed for the
302 human alveolar epithelial cell A549 (Fig. E2F). The respective role of NOD1 and NOD2 was
303 next assessed by transfecting BEAS-2B cells with specific siRNA. As compared to control and
304 *NOD2* siRNAs, *NOD1* siRNA strongly inhibited FK565- and HDM- induced IL-6 and IL-8
305 production (Figs 4G and E2G). Similar observation was noticed on HDM-induced IL-33 mRNA
306 expression (Fig. E2H). Results were further confirmed using NHBE cells. Stimulation with HDM
307 elicited a dose dependent increase in IL-8 production as well as in *NOD1/2* mRNA expression in
308 primary epithelial cells (Fig. 4H). Treatment with the RIPK2 antagonist strongly inhibited HDM-
309 induced IL-8 production (Fig. 4H). The lack of effect of NOD2 led us to evaluate the mRNA
310 expression of *PGLYRP2*, an enzyme which displays amidase activity known to degrade some
311 NOD2-activating muropeptides. HDM was able to induce this enzyme both *in vitro* in BEAS-2B
312 cells as well as *in vivo* in the lungs of HDM-challenged mice (Fig. 4I). In contrast, we failed to
313 detect any change in the expression of PGLYRP1, another member of the peptidoglycan
314 recognition protein family, that was shown to be involved in HDM-induced allergic airway
315 inflammation in the allergy-prone Balb/c mice²³⁻²⁵ (Fig. E2I). Furthermore, the transporter
316 PEPT2, allowing uptake of NOD1 ligands, was highly expressed in both BEAS-2B cells and in
317 the lungs of HDM-challenged mice as compared with PEPT1 transporter, specialized in the
318 transport of NOD2 ligand MDP (Fig. 4J). These data show that HDM triggers cytokine
319 production by epithelial cells through a NOD1 dependent pathway involving RIPK2 signaling.

320 **Nod1 expressed by radio-resistant cells mediate HDM-induced allergic airway**
321 **inflammation**

322 To evaluate the specific contribution of non-hematopoietic structural cells, in particular epithelial
323 cells, versus the role of hematopoietic cells on Nod1-dependent HDM-induced airway

324 inflammation, chimeric mice were generated expressing either Nod1^{-/-} hematopoietic cells and
325 structural WT cells (Nod1^{-/-}→ WT) or Nod1^{-/-} structural cells together with WT hematopoietic
326 cells (WT→ Nod1^{-/-}). WT→ WT and Nod1^{-/-}→ Nod1^{-/-} groups served as controls. Analysis of
327 BAL showed that the total cell, as well as eosinophil and neutrophil numbers, were increased
328 after HDM challenge in the Nod1^{-/-}→ WT and the WT→ WT mice, whereas they were
329 diminished in the WT→ Nod1^{-/-} and Nod1^{-/-}→ Nod1^{-/-} mice (Fig. 5A). The humoral response was
330 not significantly modified after HDM challenge, whatever the group (Fig. 5B). While most tested
331 cytokines were increased after HDM challenge in the WT→ WT and Nod1^{-/-}→ WT mice, these
332 were either decreased or abolished in WT→ Nod1^{-/-} and Nod1^{-/-}→ Nod1^{-/-} mice (Fig. 5C). In
333 agreement with our *in vitro* data, chimera experiments demonstrated that Nod1 expressed by
334 structural cells mediates HDM-induced airway inflammation. However, CCL2, IL-13 and IL-33
335 were significantly decreased after HDM challenge in the Nod1^{-/-}→ WT group compared to the
336 WT→ WT mice (Fig.5C). This suggests that such induction of CCL2, IL-13 and IL-33 by HDM
337 in WT mice reconstituted with Nod1-expressing hematopoietic cells is not sufficient for
338 exacerbating HDM-induced airway inflammation.

339 **Severity of allergic airway disease is decreased in response to PG- but not LPS- depleted**
340 **HDM**

341 Among bacterial moieties, LPS has been described to either favor or inhibit OVA-induced
342 experimental asthma, according to its abundance²⁶. To evaluate if LPS present in HDM was
343 playing a role in the observed results, we depleted it by purification on a resin column. *In vitro*, in
344 NF-κB reporter cells, LPS-low HDM was still able to dose dependently activate Nod1 (Fig.
345 E3A). As the concentration of LPS was not biologically relevant for exacerbating the
346 immunopathology in response to 1IR (2ng/IR) we only used the 5IR HDM-induced protocol. *In*
347 *vivo*, LPS-low HDM challenges led to an increase in most of the parameters of experimental

348 asthma including BAL total cell recruitment, Th2-type cytokine expression and airway
349 hyperreactivity (Figs E3B, E3D, E3E) similar to that observed with native HDM challenges. The
350 only parameter that was not induced by LPS-low HDM was the humoral response, which
351 remained at basal levels (Fig. E3C). To evaluate the role of PG in NOD1-dependent effect, HDM
352 extracts were digested with mutanolysin and proteins precipitated by organic solvents, and the
353 corresponding pellets and supernatants assessed on mNod1 activity. Presence or absence of PG
354 was confirmed by mass spectrometry in the supernatants and pellets respectively (Fig. 6A). The
355 PG-free pellets did not exhibit dose dependent activation of mNod1 reporter activity, whereas the
356 PG-containing supernatants activated the luciferase activity with significant effects at 4 to 6 IR
357 (i.e. 60 μ g to 90 μ g protein/mL) of initial HDM input (Fig. 6B). Next, the effect of PG depletion
358 after normalization for final allergen content, was evaluated in both HDM models to ascertain
359 the results. In the 5IR HDM model, PG-depleted HDM challenges led to a reduction in BAL total
360 cell recruitment, including mainly eosinophils (Fig. 6C), no changes in the humoral response
361 (Fig. 6D), but led to a strong decrease in Th2-type cytokines and an increase in IFN- γ (Fig. 6E).
362 Airway resistance was also reduced (Fig. 6F), as well as mucus production (Fig. 6G). In the 1IR
363 model, PG-depleted HDM led to attenuated recruitment of total cells in the BAL, which did not
364 reach significance (Fig. E3F) whereas the IgE humoral response was still induced (Fig. E3G).
365 Th2-type cytokines were nonetheless reduced (Fig. E3H), as was mucus production (Fig. E3I).
366 PG-depleted HDM was also not able to increase airway resistance, as compared to native HDM
367 (Fig. E3J). Collectively, these data demonstrate that HDM-induced allergic airway disease
368 depends to a large extent on Nod1 sensing of unique muropeptides derived from the microbiota
369 of the allergen per se.

370 **DISCUSSION**

371 In this study, HDM-induced allergic airway disease was strongly decreased in *Nod1* but not
372 *Nod2*-deficient mice, including BAL cell recruitment, airway resistance, and Th2-type cytokine
373 expression. Only BAL showed reduced cell recruitment in *Nod2*^{-/-} compared to WT mice. We
374 presume that this is possibly a consequence of lowered CCL2 levels, as neutralization of this
375 chemokine pathway attenuates macrophage and eosinophil accumulation in the BAL of asthmatic
376 monkeys²⁷. HDM challenged *Nod2*^{-/-} mice were still able to induce lung IL-33 in contrast to
377 *Nod1*^{-/-} mice, in agreement with the known direct effect of Nod1 on induction of mucosal IL-33
378 responses²⁸. Likewise, similar findings were observed in BEAS-2B cells. One explanation may
379 be that Nod2 ligands are degraded by an amidase activity that is either present in or induced by
380 HDM extracts. Indeed, the presence of a γ -D-glutamyl-L-diamino acid endopeptidase has been
381 described in such extracts²⁹. Furthermore, PGLYRP2, an N-acetylmuramoyl-L-alanine amidase,
382 was induced *in vitro* by HDM stimulation in BEAS-2B cells and *in vivo* in the lungs of the HDM
383 model. Alternatively, PEPT2, a transporter mediating the uptake of Nod1 ligands but not MDP³⁰,
384 is thoroughly expressed by the epithelial cells from the respiratory tract³¹, in contrast to PEPT1, a
385 MDP transporter mainly expressed by the gastro intestinal tract³². Indeed, PEPT2 mRNA was
386 highly expressed in BEAS-2B cells as well as in the lungs of HDM-challenged mice as compared
387 with PEPT1, likely also explaining the differential activation of Nod1/2 receptors in the lung by
388 HDM. Upon recognition of PG, both NOD1 and NOD2 undergo self-oligomerization, leading to
389 NF- κ B activation and transcription of multiple inflammatory genes upon the recruitment of the
390 scaffolding kinase protein RIPK2. In agreement with a recent report²⁰ and with our data using
391 *Nod1*^{-/-} mice, *Ripk2*^{-/-} mice exhibited decreased asthma features in response to HDM, including a
392 decrease in airway resistance. Previous studies have reported conflicting data about the role of
393 Ripk2 in allergic airway inflammation, some finding a promoting effect²⁰, and others none^{33,34}.

394 In the light of our own data, these results can be reconciled by the presence or not of unique
395 mucopeptides in the allergen extracts that are used to induce airway inflammation. Consistently,
396 we have previously shown that *Nod1*^{-/-} mice exhibited similar ovalbumin (OVA)-induced Th2-
397 mediated allergic asthma than WT mice, although a NOD1 agonist used as a systemic adjuvant
398 exacerbated experimental asthma features¹⁹. It is now well established that alterations in gut
399 microbiota are involved in the susceptibility to asthma³⁵. Along this line, *Nod1*-deficiency has
400 been implicated in intestinal dysbiosis¹⁷ and inhibits the priming of neutrophils by gut-derived
401 PG resulting in increased susceptibility to pneumococcal lung infection³⁶. Even if fecal
402 transplantation experiments excluded the possibility of a pro-allergenic role of the gut microbiota,
403 there is still a possibility in theory that airway microbiota from *Nod1*^{-/-} mice might contribute to
404 the allergic response in cooperation with the one derived from HDM.

405 Mass spectrometry analysis of HDM showed the presence of the specific NOD1 ligand M-
406 TriDap, and of the NOD2 ligand MDP. However, in contrast to HDM-induced mNod1 reporter
407 activity, mNod2 reporter activity was only observed in response to four times more HDM,
408 suggesting again a putative *Nod2* ligand degradation. Although LC-MS analysis confirmed the
409 complete PG depletion, a residual mNod1 reporter activity was observed *in vitro*. This suggests a
410 potential endocytosis of trace PG contaminants that are contained in the animal serum used in the
411 cultures³⁷. BEAS-2B cells exhibited a HDM-induced cytokine production that was only partly
412 inhibited by RIPK2 antagonist and by NOD1 siRNA. This partial inhibition may reflect other
413 mechanisms able to induce cytokine production in bronchial epithelial cells in response to other
414 HDM components³⁸ such as proteases through PAR2 activation³⁹ or LPS through TLR4
415 stimulation⁴⁰. Bone marrow chimeric mice showed that *Nod1*-expressing structural cells were the
416 major cells involved in the aggravated features of HDM-induced airway inflammation which is
417 consistent with the *in vitro* effect observed in epithelial cells. However, the expression of some

418 pro inflammatory cytokines was also dependent upon hematopoietic cells, and probably upon
419 dendritic cells that also express NOD1.

420 It has been proposed that low dose endotoxin promotes Th2 responses, whereas high dose
421 promotes Th1 responses. However, the degree of endotoxin contamination in our extracts was 10
422 times lower (e.g. 10ng) than the dose used to promote Th2 immune responses to OVA (e.g.
423 100ng)²⁶. Furthermore, LPS-depleted HDM extracts were still able to elicit all the parameters of
424 allergic airway inflammation except for humoral responses, suggesting that LPS, as a major
425 direct stimulus for B cells⁴¹, is essential in the induction of antibody responses to HDM. This
426 may relate to the predominance of *Bartonella* in HDM extracts, as its LPS has sharply reduced
427 interactions with TLR4⁴². PG depletion from HDM resulted in a similar inhibition than observed
428 in *Nod1*^{-/-} mice, confirming its role in the exacerbation of HDM-induced allergic airway
429 inflammation. It is of interest that neutralizing antibodies targeting the muropeptide MDP have
430 been recently developed and shown to inhibit PG-dependent auto-immune arthritis⁴³. Future
431 development of such PG neutralizing antibodies would provide further information on the
432 specific role of NOD1 in HDM-induced allergic airway inflammation.

433 Collectively, this study highlights an unprecedented interaction between NOD1 and HDM, one of
434 the most common allergen and unveils a new mechanism whereby HDM-derived microbiota is
435 sensed by *Nod1* and potentiates disease severity. It paves the way towards novel therapeutic
436 strategies targeting the NOD1 pathway for fighting against the epidemic of asthma.

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440 for hNOD1, mNod1 and mNod2.

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- 568

569 **FIGURE LEGENDS**

570 **Fig 1.** Nod1 signaling aggravates HDM-induced allergic airway disease

571 (A) Protocol of HDM-induced experimental asthma

572 (B) BAL cell counts in WT and *Nod1*^{-/-} mice challenged with PBS or HDM

573 (C) ELISA detection of Th2 humoral response in WT and *Nod1*^{-/-} mice challenged with PBS or
574 HDM

575 (D) Protein and mRNA relative expression (RE) of cytokines and chemokines assessed by ELISA
576 and Q-RT-PCR in lung extracts from WT and *Nod1*^{-/-} mice challenged with PBS or HDM

577 (E) Representative microphotographs of PAS- stained lung sections in WT and *Nod1*^{-/-} mice.
578 Scale bar: 100µm

579 (F) Airway resistance of WT and *Nod1*^{-/-} mice challenged with PBS or HDM

580 (G) BAL cell counts in WT and *Nod2*^{-/-} mice challenged with PBS or HDM

581 (H) ELISA detection of Th2 humoral response in WT and *Nod2*^{-/-} mice challenged with PBS or
582 HDM

583 (I) Protein and mRNA relative expression (RE) of cytokines and chemokines assessed by ELISA
584 and Q-RT-PCR in lung extracts from WT and *Nod2*^{-/-} mice challenged with PBS or HDM

585 (J) Airway resistance of WT and *Nod2*^{-/-} mice challenged with PBS or HDM

586 Data are presented as mean ± SEM of n=12-22 animals per group for Nod1 and n=5-9 animals
587 per group for Nod2 experiments. *p<0.05 **p<0.01 ***p<0.001 versus PBS, †p<0.05 ††p<0.01,
588 one-way ANOVA for all except airway resistance: two-way ANOVA. ns: not significant

589 **Fig2.** Nod1 signaling aggravates HDM-induced allergic airway disease through Ripk2
590 (A) BAL cell counts in WT and *Ripk2*^{-/-} mice challenged with PBS or low dose of HDM
591 (B) ELISA detection of total IgE and HDM-specific IgG1 antibody in WT and *Ripk2*^{-/-} mice
592 challenged with PBS or low dose of HDM
593 (C) Protein and mRNA relative expression (RE) of cytokines and chemokines assessed by ELISA
594 and Q-RT-PCR in lung extracts from WT and *Ripk2*^{-/-} mice challenged with PBS or low dose of
595 HDM
596 (D) Representative microphotographs of PAS- stained lung sections in WT and *Ripk2*^{-/-} mice.
597 Scale bar: 100 μ m
598 (E) Airway resistances of WT and *Ripk2*^{-/-} mice challenged with PBS or low dose of HDM
599 Data are presented as mean \pm SEM of n=6-12 for Ripk2. *p<0.05 **p<0.01 ***p<0.001 versus
600 PBS, #p<0.05, ##p<0.01, ###p<0.001 one-way ANOVA for all except airway resistance: two-way
601 ANOVA.

602 **Fig 3.** Dysbiotic gut microbiota caused by *Nod1* deficiency does not impact HDM-induced
603 allergic airway disease risk

604 (A) Protocol of fecal transplantation

605 (B) BAL cell counts in WT and *Nod1*^{-/-} fetal microbiota (FM)→germ free (GF) mice challenged
606 with PBS or HDM

607 (C) ELISA detection of Th2 humoral response in WT and *Nod1*^{-/-} FM→GF mice challenged with
608 PBS or HDM

609 (D) Protein and mRNA relative expression (RE) of cytokines and chemokines assessed by ELISA
610 and Q-RT-PCR in lung extracts from WT and *Nod1*^{-/-} FM →GF mice challenged with PBS or
611 HDM

612 (E) Airway resistance of WT and *Nod1*^{-/-} FM →GF mice challenged with PBS or HDM

613 Data are presented as mean ± SEM of n=4-7 animals per group. Data are representative of 2
614 independent experiments. *p<0.05 **p<0.01 versus PBS. One-way ANOVA for all except
615 airway resistance: two-way ANOVA

616 (F) PCoA plots using unweighted UniFrac distances of feces from WT FM →GF (WT) and
617 *Nod1*^{-/-} FM →GF mice (KO). p≤0.001 between the two groups, ANOSIM analysis

618 (G) Alpha diversity assessed by the Shannon index of feces from WT FM →GF (WT) and *Nod1*^{-/-}
619 FM →GF mice (KO). p≤0.01 between the two groups, Mann Whitney test

620 (H) Heatmap of the top most abundant genera of feces from receivers (R) of WT FM →GF (WT),
621 *Nod1*^{-/-} FM →GF mice (KO) challenged with PBS or HDM, and from initial donors (D)

622 **Fig 4.** HDM-associated mucopeptides are sensed by NOD1 in epithelial cells via RIPK2.

623 (A) High throughput sequencing analysis of HDM

624 (B) Extract Ion Chromatograms (XICs) of HDM corresponding to the protonated MTriDap (m/z
625 666.2843) and its MS/MS fragmentation with a normalized collision energy (NCE) equal to 25%
626 in the high energy collision dissociation (HCD) cell of QExactive Focus. The specific fragments
627 are recorded in table E3

628 (C) XICs of HDM corresponding to the protonated MDP (m/z 494.1990) and its MS/MS
629 fragmentation as described above. The specific fragments are recorded in table E3

630 (D) Dose dependent effect of HDM on hNOD1, mNod1 and mNod2 reporter activity of HEK-
631 293 cells normalized with β -galactosidase. Positive controls: FK565 for hNOD1, FK156 for
632 mNod1, MDP for mNod2. IR: index of reactivity

633 (E) Relative expression (RE) of hNOD1 and hNOD2 mRNA in BEAS-2B cells stimulated with
634 HDM

635 (F) Inhibitory effect of RIPK2 inhibitor on IL-6 and IL-8 production by HDM- and FK565-
636 stimulated BEAS-2B cells

637 (G) Effect of control (C) hNOD1 and hNOD2 siRNA (KD) on HDM-, FK565-, and MDP-
638 induced production of IL-6 by BEAS-2B cells

639 (H) HDM-stimulated NHBE cells were examined in terms of dose dependent cytokine response,
640 of NOD1 and NOD2 relative expression (RE) and of IL-8 production in the presence of RIPK2
641 inhibitor

642 (I) PGLYRP2 mRNA relative expression (RE) in HDM-stimulated BEAS-2B cells and in lungs
643 from HDM-challenged WT mice

644 (J) PEPT1 and PEPT2 mRNA relative expression (RE) in HDM-stimulated BEAS-2B cells and
645 in lungs from HDM-challenged WT mice

646 Data are presented as mean \pm SEM and are representative of 2 to 3 independent in vitro
647 experiments and of 10-12 mice per group. *p<0.05 **p<0.01 ***p<0.001 versus plasmids or
648 medium, [#]p<0.05 ^{##}p<0.01 ^{###}p<0.001, one-way ANOVA except for figure 4D: Kruskal-Wallis,
649 and t-tests for figures 4E, 4H (NOD1/NOD2) 4I and 4J.

650 **Fig 5.** Nod1 expressed by structural cells mediate HDM-induced allergic airway inflammation
651 Chimeric mice were generated by bone marrow transplantation
652 (A) BAL cell counts in WT→WT, *Nod1*^{-/-}→WT (hematopoietic cells deficient in *Nod1*), *Nod1*^{-/-}
653 →*Nod1*^{-/-}, and *Nod1*^{-/-}→WT (structural cells deficient in *Nod1*) mice challenged with PBS or
654 high dose of HDM
655 (B) ELISA detection of Th2 humoral response in chimeric mice challenged with PBS or high
656 dose of HDM
657 (C) mRNA relative expression (RE) of cytokines and chemokines assessed by Q-RT-PCR in lung
658 extracts from chimeric mice challenged with PBS or high dose of HDM. Data are presented as
659 mean ± SEM of n=3-6 animals per group. *p<0.05 **p<0.01 versus PBS, [#]p<0.01. One-way
660 ANOVA.

661 **Fig 6.** Severity of allergic airway disease is decreased in response to PG- depleted HDM

662 (A) Extract Ion Chromatograms (XICs) in the peptidoglycan (PG)-containing supernatants and
663 PG-free pellets of HDM extracts after digestion with mutanolysin and precipitation of proteins by
664 organic solvents, corresponding to the protonated MTriDap (m/z 666.2843) and the protonated
665 MDP (m/z 494.1990)

666 (B) HDM extracts were digested with mutanolysin and proteins precipitated by organic solvents
667 and the activity of the peptidoglycan (PG)-free lysates and the PG-containing supernatants
668 assessed on mNod1 reporter activity. Positive control: FK156. IR: index of reactivity

669 (C) BAL cell counts in WT mice challenged with PBS or high dose of HDM depleted or not in
670 peptidoglycan (PG)

671 (D) ELISA detection of Th2 humoral response in WT mice challenged with PBS or high dose of
672 HDM depleted or not in peptidoglycan (PG)

673 (E) Protein and mRNA expression of cytokines and chemokines assessed by ELISA and Q-RT-
674 PCR in lung extracts from WT mice challenged with PBS or high dose of HDM depleted or not
675 in peptidoglycan (PG)

676 (F) Airway resistance of WT mice challenged with PBS or high dose of HDM depleted or not in
677 peptidoglycan (PG)

678 (G) Representative microphotographs of PAS- stained lung sections in WT mice challenged with
679 PBS or high dose of HDM depleted or not in peptidoglycan (PG). Scale bar: 100 μ m

680 Data are presented as mean \pm SEM of at least 2 independent *in vitro* experiments, **p<0.01
681 ***p<0.001 versus plasmids, and as mean \pm SEM of n=5-10 animals per group *p<0.05
682 **p<0.01 ***p<0.001 versus PBS, #p<0.05 ##p<0.01, ###p<0.001. One-way ANOVA for all
683 except for airway resistance: two-way ANOVA