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The work was supported by grants from PTR (18-16) from Institut Pasteur (to AT, MC and IGB), by ANR 18-CE14-0020 (to AT, MC and IGB), and by CNRS of Orléans (France) and European

funding in Region Centre-Val de Loire (FEDER N° 2016-00110366 BIO-TARGET and EX005756 BIO-TARGET II). AR was supported by Investissement d'Avenir program, Laboratoire d'Excellence "Integrative Biology of Emerging Infectious Diseases" (ANR-10-LABX-62-IBEID).

Disclosure of potential conflict of interest: The authors declare that they have no relevant conflict of interest.

Total word count: 4555

ABSTRACT

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

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

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

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

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

Key messages:

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

Capsule summary

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

Keywords

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

Abbreviations

AAI: Allergic airway inflammation

AHR: Airway hyperresponsiveness

I.n.: Intranasal

NOD1: Nucleotide-binding oligomerization domain protein 1

NOD2: Nucleotide-binding oligomerization domain protein 2

RIPK2: Receptor-interacting protein kinase 2

MDP: Muramyl di peptide

BAL: Bronchoalveolar lavage

PG: Peptidoglycan

NHBE: Normal human bronchial epithelial

HEK: Human embryonic kidney

INTRODUCTION

An increasing body of evidence from clinical and experimental studies suggests that microbiota encompass a wide range of dynamic microbial communities that play a significant role in host immunity including protecting against asthma. Besides the putative effect of the respiratory microbiota in asthma[1-3], intestinal dysbiosis appears to also play an important role[4, 5]. Accordingly, host immune responses are Th2 biased under germ-free (GF) conditions[6] and antibiotic-treated or GF animal models of allergen-induced asthma exhibit an exacerbated phenotype in either neonates[7, 8] or adults[9]. However, much less attention has been paid to the microbiota derived from house dust mite (HDM), which is the most frequent cause of allergic asthma, although analysis of HDM extracts has revealed the presence of Gram-negative bacteria that may likely arise from their intestine[10, 11]. Although it is well established that viral infections are involved in 40 to 85% of asthma exacerbations[12], a link has been established between adult asthma severity and exposure to indoor Gram-negative bacteria[13]. Unique muropeptides from the peptidoglycan (PG) of such bacteria are sensed by the pattern recognition receptors Nucleotide binding oligomerization domain protein 1 (NOD1)[14, 15] and NOD2, each receptor recognizing distinct PG structures. While NOD1 recognizes meso-diaminopimelic containing muramyl tripeptide (MTP), NOD2 recognizes muramyl dipeptide (MDP)[16]. Both receptors signal via the downstream adaptor RIPK2, which then activates MAPK and the NF κ B pathway. Of note, NOD1-mediated sensing of the gut microbiota participates in the development of lymphoid tissues, while its absence results in dysbiosis[17]. Additionally, genome-wide association studies have revealed a molecular link between polymorphisms of NOD1, asthma and high levels of IgE[18]. In agreement, we have previously shown that a NOD1 agonist used as a systemic adjuvant exacerbates ovalbumin (OVA)-induced Th2-mediated allergic asthma through dendritic cell

activation[19], and a recent paper has shown that RIPK2 promotes HDM-induced allergic airway inflammation by favoring type 2 immunity[20]. The aim of this study was to evaluate if either host or HDM-derived microbiota may contribute to NOD1-dependent disease severity. Here we provide evidence that NOD1-dependent sensing of muropeptides from Gram-negative bacteria present in HDM extracts exacerbate the severity of allergic airway inflammation, independently of the control by NOD1 of the composition of the gut microbiota from its host. These data identify a new mechanism of asthma aggravation linking HDM-derived microbiota and NOD1, and suggest that interfering with NOD1 signaling pathway may provide a therapeutic approach in this cumbersome disease, as well as for exacerbations of asthma driven by Gram-negative bacteria.

METHODS

Reagents

Key reagents used are listed in Table E1.

Mice

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

HDM-induced allergic airway inflammation and treatment of extracts

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

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

Airway responsiveness measurement

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

BAL analysis

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

Serum collection and analysis

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

Pulmonary histology

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

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

Lung protein extracts

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

Human cell lines and primary human epithelial cells

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

HEK293 luciferase reporter cell assay

The luciferase NFκB reporter assays, were performed by seeding HEK293 cells in 96-well plates at $5x10^4$ cells per well and transfecting them with expression plasmids producing either human NOD1, mouse Nod1 or mouse Nod2 (0,5ng/well) in combination with the reporter plasmids pBxIV-luc (1 ng/well) and pEF-BOS-β-gal (25ng/well) using Lipofectamine LTX & PLUS Reagent (Invitrogen) following manufacturer's recommendations. The reporter plasmids and the expression plasmids for hNOD1, hNOD2, mNod1 and mNod2 were kindly provided by Gabriel Nuñez and Naohiro Inohara (Ann Arbor, MI, USA). Details are provided in the online repository. The results were normalized by the expression of β-Galactosidase to reflect the efficiency of transfection and expressed as fold expression of the plasmid-transfected cells.

Epithelial cell line stimulation and transfection with siRNA

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

Generation of chimeric mice by bone marrow transplantation

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

Quantitative Real-Time PCR

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

ELISA Measurement of Cytokine Chemokine

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

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

16sRNA analysis

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

LC-MS/MS Method

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

Statistical analysis

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

RESULTS

Nod1 signaling aggravates HDM-induced allergic airway disease through Ripk2

The *in vivo* effect of Nod1 signaling was investigated in a model of 5 IR (i.e. 15µg protein/mL) HDM-induced asthma[21] (Fig. 1A) in C57BL6/J mice deficient or not in *Nod1*. HDM-challenged WT mice exhibited all the cardinal features of allergic airway inflammation, including increase in total cell, eosinophil, neutrophil and macrophage numbers in BAL (Fig. 1B), total IgE and HDM-specific IgG1 antibodies (Fig. 1C), and levels of lung Th2/Th17 cytokines and some pro-Th2 chemokines (Figs 1D, E1A). All these features were strongly diminished in *Nod1*$^{-/-}$ mice, except for the humoral response and Th17-type cytokine levels (Figs 1B, 1C, 1D and E1A). Lung sections from HDM-challenged mice showed that the increases in PAS-stained mucus and in Hematoxylin Eosin-stained cellular infiltrates in WT mice were decreased in the absence of Nod1 (Figs 1E and E1B respectively). Functionally, airway resistance in response to methacholine challenge was totally inhibited in HDM-challenged *Nod1*$^{-/-}$ versus WT mice (Fig. 1F). Likewise, *Nod2* deficiency led to a decrease in BAL cell recruitment after HDM challenge (Fig. 1G) and not in the humoral response (Fig. 1H). By contrast, there was no statistically significant decrease in Th2 and pro-Th2 lung cytokines and chemokines except for CCL2 and KC (Figs 1I and E1C), and no decrease in airway resistance (Fig. 1J). Given that Nod1 and Nod2 signal via the downstream adaptor Ripk2, we next evaluated whether a similar pattern of response was observed in *Ripk2*$^{-/-}$ mice. Miller et al. have recently reported that HDM-induced allergic airway inflammation is reduced in *Ripk2*$^{-/-}$ mice in a model similar to our 5IR model[20]. Therefore, we assessed *Ripk2*$^{-/-}$ mice in a 1IR (3µg protein/mL) model of HDM-induced allergic inflammation. There was a total inhibition of BAL cell recruitment, in particular of eosinophils (Fig. 2A), no changes in the humoral response (Fig. 2B), a decrease of Th2 type cytokines and chemokines (Fig. 2C), of mucus production (Fig. 2D) and of airway resistance (Fig. 2E). In contrast to the

Miller paper[20], it is of note that we did not observe changes in the humoral response, but we additionally observed abolition of a cardinal feature of asthma, i.e. airway resistance in *Ripk2*$^{-/-}$ and *Nod1*$^{-/-}$ but not *Nod2*$^{-/-}$ mice. Taken together, these data demonstrate that Nod1, but not Nod2 signaling, is involved in the severity of HDM-induced airway disease through Ripk2 activation.

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

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

HDM–associated muropeptides are sensed by NOD1 in epithelial cells via RIPK2.

Quantification of bacterial diversity in HDM extracts by 16S rRNA sequencing revealed that 87.7% of the assembled reads associated with members of the Gram-negative Bartonellaceae family (Fig. 4A). To identify specific PG moieties, HDM extracts were analyzed by mass spectrometry through a targeted approach specifically looking at well-characterized muropeptides. Both MTriDap and MDP, sensed by NOD1 and NOD2 respectively, were detected and their presence confirmed by fragmentation (Figs 4B and 4C, and table E3). Next, the effect of HDM was evaluated on human embryonic kidney 293 (HEK293) cells harboring a NF-kB-dependent luciferase reporter and expressing either h*NOD1*, m*Nod1*, or m*Nod2* and normalized for transfection efficiency using a β-galactosidase control vector. All positive controls induced NF-κB activation (Fig. 4D). HDM extracts elicited a dose dependent increase in both hNOD1- and mNod1- dependent luciferase activity, but only an effect at the highest dose of 4IR (i.e. 60µg protein/mL) for mNod2 activity (Fig. 4D). As the allergen first encounters epithelial cells, human bronchial epithelial BEAS-2B cells were primed with IFN-β[22], to increase the baseline expression of *NOD1*. The role of Nod1 signaling pathway was subsequently assessed by quantifying their cytokine production in response to HDM. HDM stimulation elicited a dose dependent increase in the production of IL-6 and IL-8 by BEAS-2B cells with an effect starting at 0.2IR (6µg protein/mL) and maximal at 4IR (120µg protein/mL) (Fig. E2A). BEAS-2B stimulation by HDM induced a clear increase in *NOD1* and *NOD2* mRNA (Fig. 4E). Although IFN-β increased *NOD1/2* expression and FK565-induced cytokine production (Fig. E2B), no effect was noticed on the response to HDM (Fig E2C). As expected the addition of a specific RIPK2 inhibitor belonging to the 4-amino-quinolines inhibited both IL-6 and IL-8 increases in response to FK565 positive control (Fig. 4F), but did not modulate the response to LPS (Fig. E2D). HDM

stimulation induced a stronger production of IL-6 and IL-8 (Fig 4F) with a partial inhibitory non-cytotoxic effect (Fig E2E) of RIPK2 antagonist (Fig. 4F). The same results were observed for the human alveolar epithelial cell A549 (Fig. E2F). The respective role of NOD1 and NOD2 was next assessed by transfecting BEAS-2B cells with specific siRNA. As compared to control and *NOD2* siRNAs, *NOD1* siRNA strongly inhibited FK565- and HDM- induced IL-6 and IL-8 production (Figs 4G and E2G). Similar observation was noticed on HDM-induced IL-33 mRNA expression (Fig. E2H). Results were further confirmed using NHBE cells. Stimulation with HDM elicited a dose dependent increase in IL-8 production as well as in *NOD1/2* mRNA expression in primary epithelial cells (Fig. 4H). Treatment with the RIPK2 antagonist strongly inhibited HDM-induced IL-8 production (Fig. 4H). The lack of effect of NOD2 led us to evaluate the mRNA expression of *PGLYRP2*, an enzyme which displays amidase activity known to degrade some NOD2-activating muropeptides. HDM was able to induce this enzyme both *in vitro* in BEAS-2B cells as well as *in vivo* in the lungs of HDM-challenged mice (Fig. 4I). In contrast, we failed to detect any change in the expression of PGLYRP1, another member of the peptidoglycan recognition protein family, that was shown to be involved in HDM-induced allergic airway inflammation in the allergy-prone Balb/c mice[23-25] (Fig. E2I). Furthermore, the transporter PEPT2, allowing uptake of NOD1 ligands, was highly expressed in both BEAS-2B cells and in the lungs of HDM-challenged mice as compared with PEPT1 transporter, specialized in the transport of NOD2 ligand MDP (Fig. 4J). These data show that HDM triggers cytokine production by epithelial cells through a NOD1 dependent pathway involving RIPK2 signaling.

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

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

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

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

Among bacterial moieties, LPS has been described to either favor or inhibit OVA-induced experimental asthma, according to its abundance[26]. To evaluate if LPS present in HDM was playing a role in the observed results, we depleted it by purification on a resin column. *In vitro,* in NF-κB reporter cells, LPS-low HDM was still able to dose dependently activate Nod1 (Fig. E3A). As the concentration of LPS was not biologically relevant for exacerbating the immunopathology in response to 1IR (2ng/IR) we only used the 5IR HDM-induced protocol. *In vivo*, LPS-low HDM challenges led to an increase in most of the parameters of experimental

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

DISCUSSION

In this study, HDM-induced allergic airway disease was strongly decreased in *Nod1* but not *Nod2*-deficient mice, including BAL cell recruitment, airway resistance, and Th2-type cytokine expression. Only BAL showed reduced cell recruitment in *Nod2*$^{-/-}$ compared to WT mice. We presume that this is possibly a consequence of lowered CCL2 levels, as neutralization of this chemokine pathway attenuates macrophage and eosinophil accumulation in the BAL of asthmatic monkeys[27]. HDM challenged *Nod2*$^{-/-}$ mice were still able to induce lung IL-33 in contrast to *Nod1*$^{-/-}$ mice, in agreement with the known direct effect of Nod1 on induction of mucosal IL-33 responses[28]. Likewise, similar findings were observed in BEAS-2B cells. One explanation may be that Nod2 ligands are degraded by an amidase activity that is either present in or induced by HDM extracts. Indeed, the presence of a γ-D-glutamyl-L-diamino acid endopeptidase has been described in such extracts[29]. Furthermore, PGLYRP2, an N-acetylmuramoyl-L-alanine amidase, was induced *in vitro* by HDM stimulation in BEAS-2B cells and *in vivo* in the lungs of the HDM model. Alternatively, PEPT2, a transporter mediating the uptake of Nod1 ligands but not MDP[30], is thoroughly expressed by the epithelial cells from the respiratory tract[31], in contrast to PEPT1, a MDP transporter mainly expressed by the gastro intestinal tract[32]. Indeed, PEPT2 mRNA was highly expressed in BEAS-2B cells as well as in the lungs of HDM-challenged mice as compared with PEPT1, likely also explaining the differential activation of Nod1/2 receptors in the lung by HDM. Upon recognition of PG, both NOD1 and NOD2 undergo self-oligomerization, leading to NF-κB activation and transcription of multiple inflammatory genes upon the recruitment of the scaffolding kinase protein RIPK2. In agreement with a recent report[20] and with our data using *Nod1*$^{-/-}$ mice, *Ripk2*$^{-/-}$ mice exhibited decreased asthma features in response to HDM, including a decrease in airway resistance. Previous studies have reported conflicting data about the role of Ripk2 in allergic airway inflammation, some finding a promoting effect[20], and others none[33, 34].

In the light of our own data, these results can be reconciled by the presence or not of unique muropeptides in the allergen extracts that are used to induce airway inflammation. Consistently, we have previously shown that Nod1$^{-/-}$ mice exhibited similar ovalbumin (OVA)-induced Th2-mediated allergic asthma than WT mice, although a NOD1 agonist used as a systemic adjuvant exacerbated experimental asthma features[19]. It is now well established that alterations in gut microbiota are involved in the susceptibility to asthma[35]. Along this line, Nod1-deficiency has been implicated in intestinal dysbiosis[17] and inhibits the priming of neutrophils by gut-derived PG resulting in increased susceptibility to pneumococcal lung infection[36]. Even if fecal transplantation experiments excluded the possibility of a pro-allergenic role of the gut microbiota, there is still a possibility in theory that airway microbiota from *Nod1*$^{-/-}$ mice might contribute to the allergic response in cooperation with the one derived from HDM.

Mass spectrometry analysis of HDM showed the presence of the specific NOD1 ligand M-TriDap, and of the NOD2 ligand MDP. However, in contrast to HDM-induced mNod1 reporter activity, mNod2 reporter activity was only observed in response to four times more HDM, suggesting again a putative Nod2 ligand degradation. Although LC-MS analysis confirmed the complete PG depletion, a residual mNod1 reporter activity was observed *in vitro*. This suggests a potential endocytosis of trace PG contaminants that are contained in the animal serum used in the cultures[37]. BEAS-2B cells exhibited a HDM-induced cytokine production that was only partly inhibited by RIPK2 antagonist and by NOD1 siRNA. This partial inhibition may reflect other mechanisms able to induce cytokine production in bronchial epithelial cells in response to other HDM components[38] such as proteases through PAR2 activation[39] or LPS through TLR4 stimulation[40]. Bone marrow chimeric mice showed that *Nod1*-expressing structural cells were the major cells involved in the aggravated features of HDM-induced airway inflammation which is consistent with the in vitro effect observed in epithelial cells. However, the expression of some

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

It has been proposed that low dose endotoxin promotes Th2 responses, whereas high dose promotes Th1 responses. However, the degree of endotoxin contamination in our extracts was 10 times lower (e.g. 10ng) than the dose used to promote Th2 immune responses to OVA (e.g. 100ng)[26]. Furthermore, LPS-depleted HDM extracts were still able to elicit all the parameters of allergic airway inflammation except for humoral responses, suggesting that LPS, as a major direct stimulus for B cells[41], is essential in the induction of antibody responses to HDM. This may relate to the predominance of *Bartonella* in HDM extracts, as its LPS has sharply reduced interactions with TLR4[42]. PG depletion from HDM resulted in a similar inhibition than observed in $Nod1^{-/-}$ mice, confirming its role in the exacerbation of HDM-induced allergic airway inflammation. It is of interest that neutralizing antibodies targeting the muropeptide MDP have been recently developed and shown to inhibit PG-dependent auto-immune arthritis[43]. Future development of such PG neutralizing antibodies would provide further information on the specific role of NOD1 in HDM-induced allergic airway inflammation.

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

ACKNOWLEDGMENT

We thank Stallergenes/Greer for providing Dermatophagoïdes farinae extract, and Gabriel Nuñez and Naohiro Inohara (Ann Arbor, MI, USA) for providing the reporter and expression plasmids for hNOD1, mNod1 and mNod2.

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568

FIGURE LEGENDS

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

(A) Protocol of HDM-induced experimental asthma

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

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

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

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

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

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

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

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

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

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

Fig2. Nod1 signaling aggravates HDM-induced allergic airway disease through Ripk2

(A) BAL cell counts in WT and *Ripk2*$^{-/-}$ mice challenged with PBS or low dose of HDM

(B) ELISA detection of total IgE and HDM-specific IgG1 antibody in WT and *Ripk2*$^{-/-}$ mice challenged with PBS or low dose of HDM

(C) Protein and mRNA relative expression (RE) of cytokines and chemokines assessed by ELISA and Q-RT-PCR in lung extracts from WT and *Ripk2*$^{-/-}$ mice challenged with PBS or low dose of HDM

(D) Representative microphotographs of PAS- stained lung sections in WT and *Ripk2*$^{-/-}$ mice. Scale bar: 100 µm

(E) Airway resistances of WT and *Ripk2*$^{-/-}$ mice challenged with PBS or low dose of HDM

Data are presented as mean ± SEM of n=6-12 for Ripk2. *p<0.05 **p<0.01 ***p<0.001 versus PBS, $^{\neq}$p<0.05, $^{\#\#}$p<0.01, $^{\#\#\#}$p<0.001 one-way ANOVA for all except airway resistance: two-way ANOVA.

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

(A) Protocol of fecal transplantation

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

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

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

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

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

(F) PCoA plots using unweighted UniFrac distances of feces from WT FM →GF (WT) and *Nod1*$^{-/-}$ FM →GF mice (KO). $p\leq0.001$ between the two groups, ANOSIM analysis

(G) Alpha diversity assessed by the Shannon index of feces from WT FM →GF (WT) and *Nod1*$^{-/-}$ FM →GF mice (KO). $p\leq0.01$ between the two groups, Mann Whitney test

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

Fig 4. HDM–associated muropeptides are sensed by NOD1 in epithelial cells via RIPK2.

(A) High throughput sequencing analysis of HDM

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

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

(D) Dose dependent effect of HDM on hNOD*1*, mNod1 and mNod2 reporter activity of HEK-293 cells normalized with β-galactosidase. Positive controls: FK565 for hNOD1, FK156 for mNod1, MDP for mNod2. IR: index of reactivity

(E) Relative expression (RE) of h*NOD1* and h*NOD2* mRNA in BEAS-2B cells stimulated with HDM

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

(G) Effect of control (C) h*NOD1* and h*NOD2* siRNA (KD) on HDM-, FK565-, and MDP-induced production of IL-6 by BEAS-2B cells

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

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

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

646 Data are presented as mean ± 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, $^{\neq}$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.

Fig 5. Nod1 expressed by structural cells mediate HDM-induced allergic airway inflammation

Chimeric mice were generated by bone marrow transplantation

(A) BAL cell counts in WT→WT, *Nod1*$^{-/-}$→WT (hematopoietic cells deficient in *Nod1*), *Nod1*$^{-/-}$ →*Nod1*$^{-/-}$, and *Nod1*$^{-/-}$→WT (structural cells deficient in *Nod1*) mice challenged with PBS or high dose of HDM

(B) ELISA detection of Th2 humoral response in chimeric mice challenged with PBS or high dose of HDM

(C) mRNA relative expression (RE) of cytokines and chemokines assessed by Q-RT-PCR in lung extracts from chimeric mice challenged with PBS or high dose of HDM. Data are presented as mean ± SEM of n=3-6 animals per group. *$p<0.05$ **$p<0.01$ versus PBS, $^{\#\#}p<0.01$. One-way ANOVA.

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

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

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

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

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

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

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

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

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