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House dust mite impairs antiviral response in asthma exacerbation models through its effects on TLR3

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Abstract

Background: Impaired antiviral interferon expression may be involved in asthma exacerbations commonly caused by rhinovirus infections. Allergy is a known risk factor for viral-induced asthma exacerbation, but little is known whether allergens may affect interferon responses.

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Objective: Our hypothesis is that house dust mite (HDM) impairs viral stimulus-induced antiviral signalling.

Methods: Experimental asthma exacerbations were produced in vitro in human bronchial epithelial cells (HBECs) and in mice by using sequential challenges with HDM and a viral infection mimic, Poly(I:C). We examined rhinovirus pattern recognition receptors (PRRs) signalling pathways and potential mechanisms of impaired interferon response.

Results: HBECs and mice exposed to HDM prior to Poly(I:C) exhibited a reduced antiviral response compared to Poly(I:C) alone, including reduced IFN- β , IFN-lambda, TLR3, RIG-I, MDA5, IRF-3 and IRF-7. Heat-inactivation of HDM partially restored the TLR3-induced interferon response in vitro and in vivo. Our HBEC-data further showed that HDM directly affects TLR3 signalling by targeting the receptor glycosylation level.

Conclusions: Direct effects of allergens such as HDM on PRRs can present as potential mechanism for defective antiviral airway responses. Accordingly, therapeutic measures targeting inhibitory effects of allergens on antiviral PRRs may find use as a strategy to boost antiviral response and ameliorate exacerbations in asthmatic patients.

Introduction

Acute respiratory viral infections are the most common cause of asthma exacerbations in both children and adults (1, 2). Human rhinovirus (RV) is the most common pathogen identified during asthma exacerbations (3) and infected asthmatic patients are more prone to morbidities than the healthy population. This highlights the possibility of a defect in the antiviral response of asthmatics (4, 5).

Studies in children indicate that allergy plays a significant role as a risk factor for developing virus-induced asthma exacerbations (6, 7). Durrani et al. also observed that increased expression of high affinity IgE receptors on peripheral blood monocytes, obtained from asthmatic children, was associated with reduced secretion of interferons in vitro in response to RV infection (8). However, bronchial epithelial cells (BECs) emerge as main target cells both to inhaled allergens and respiratory viral infections. Yet, little is known whether exposure to appropriate allergens may affect the antiviral defence capacity of the bronchial epithelium. House dust mite (HDM) is a major allergen strongly associated with asthma.

HDM activates not only adaptive and Th2-derived immune response but also innate immunity (9-11). Allergenicity of HDM can be due to mites but also bacterial and fungal products, which are associated to mites (12). Protease activity of HDM can damage the airway epithelium and also activate immune responses (13). Allergenic peptides of HDM are able to activate intracellular signalling pathways in the airway epithelium through protease activity (14, 15). HDM components can also serve as ligands for pattern recognition receptors (PRRs) (TLRs and non-TLRs), which are present on airway mucosal surface cells and immune cells (16, 17). The cysteine protease of dust mite, Der p1, has also been shown to activate the innate immune response through cleavage of complement components into their active forms at the mucosal surface (18). Hence, HDM emerges as a multipotent pathogenic factor in asthma, and its effect on antiviral defence such as interferons is not known.

The innate immune system of airway mucosal lining cells is the first line of defence against RV. After entry into cells RV RNA is amplified. During replication, a double-stranded RNA (dsRNA) intermediate is formed which stimulates the host innate immune system involving PRRs (TLR3, RIG-I, MDA5). In response to RV infection, or to synthetic dsRNA, interferons (type I and III) are thus produced by the bronchial epithelium and play a critical role in the antiviral defence in the airways. A defective interferon response to RV has been proposed as an underlying mechanism for RV-induced asthma exacerbations (19-21). Interestingly, TLR3 stimulated BECs from asthmatic donors also expressed less interferon- β than cells from healthy donors (22).

We hypothesized that HDM-induced allergic bronchial inflammation may adversely affect the epithelial antiviral interferon response. We thus investigated the effects of HDM exposure in human bronchial epithelial cells as well as on antiviral immune response in a mouse model of asthma exacerbation in vivo (23). We demonstrate that pre-exposure to HDM impairs interferon responses to TLR3 stimulation both in vitro and in vivo. These effects were seen on the whole pathway from upstream PRRs (TLR3, MDA5 and RIG-I) to downstream type I IFN. Furthermore, we observed that the effects of HDM were mostly due to its heat-sensitive components since heat-inactivation of HDM partly restored interferon response. Our observations on silencing PRRs suggested that TLR3 is the main target involved in reducing the antiviral response by HDM. We suggest that allergic bronchial epithelial inflammation induced by HDM plays a role in impaired innate immunity in response to rhinovirus infection in asthmatic patients.

Material and Methods

Bronchial epithelial cell culture

Human bronchial epithelial cells (HBECs) from healthy and asthmatic donors were obtained by bronchoscopy using a fibre-optic bronchoscope (Olympus, IT160, Tokyo, Japan). Standard sterile-sheared nylon cytology brushes were used to sample epithelial cells from bronchi. The procedure was performed in accordance with standard published guidelines and processed as described previously (22). All subjects (8 healthy and 9 asthmatics) provided informed consent. Approval was obtained from the regional ethical review board at Lund University and patient characterizations can be found in Table S1. Bronchial epithelial cells were cultured in bronchial epithelial growth medium.

Human lung epithelial cell line (BEAS2B) was obtained from the American Type Culture Collection (Manassas, VA) and was cultured under recommended conditions in RPMI growth medium.

Animal study

In our model C57BL/6 mice with 8-10 weeks of age were used. All experiments were approved by the ethics committee at Lund University. Mice were challenged with 25 µg house dust mite or saline 3 days/week for 3 weeks and were subsequently exposed to 3 doses of 50 or 100 µg (in heat-inactivated HDM study) polyinosinic:polycytidylic acid (Poly(I:C); InvivoGen, San Diego, CA, USA) or saline. Lavage of the lungs was performed with phosphate buffered saline (PBS) 72h after final HDM or 24h after final Poly(I:C) exposure. Total cell count of the bronchoalveolar lavage fluid (BALF) was performed and total protein concentration was analysed by bicinchoninic acid (BCA) assay (Pierce, Thermo Scientific, Waltham, MA, USA).

Small interfering RNA transfection

BEAS2B cells were transfected with siRNA targeted against TLR3, MDA5 or RIG-I or with non-specific siRNA (Ambion, Thermo Scientific, Waltham, MA, USA) in a concentration of 10 µM using Lipofectamine RNAiMAX (Ambion, Thermo Scientific, Waltham, MA, USA) as a transfection agent.

ELISA

IL-4, IL-13 and IFN- β levels were measured in tissue homogenates and BALF of mice according to the manufacturer's instructions (R and D systems Abingdon, Oxon, UK).

Statistics

The data is presented as mean $\pm$ SEM except for the patients' data, which is depicted as median $\pm$ interquartile range. Statistical analyses were performed by Mann-Whitney *U* test using Prism software. A *P* value of <0.05 was considered statistically significant.

Description of reagents, RT-PCR and, western blotting, protease activity, and immunofluorescence microscopy are in the Methods section in this article's supplementary.

Results

HDM exposure impairs interferons I, III and PRR responses to Poly(I:C) in bronchial epithelial (BEAS2B) cells.

To determine effects of HDM exposure on bronchial epithelial antiviral defence, BEAS2B cells were incubated with HDM or control medium for 24 hours before being stimulated with Poly(I:C).

Poly (I:C) stimulation alone increased IFN- β and $-\lambda$ expression in BEAS-2B cells after 24h. However, their expression in response to Poly(I:C) was significantly lower after HDM exposure compared to non-exposed cells (Figure 1A and B).

We next analysed mRNA expression of relevant PRRs to see if they followed the same pattern as IFN- β and $-\lambda$. Twenty-four hours after stimulation with Poly(I:C), mRNA expression of TLR3, MDA5 and RIG-I was more reduced in cells pre-exposed to HDM compared to control (Figure 1C-E). Importantly, we observed the same pattern for protein expression of PRRs as the mRNA expression (Figure 1F-H). We also confirmed the western blot findings for MDA5 by staining BEAS-2B cells 24h after Poly(I:C) stimulation (Figure S1).

It has been shown that innate antiviral response to RV is impaired in several cohorts of asthmatic patients (19, 20). In accord, impaired production of IFN- β in response to TLR3 stimulation with its ligand Poly(I:C) has previously been recorded in HBECs obtained from asthmatic donors (22). However, any associated effects on PRRs were not determined. Hence, we assessed the expression of IFN- β , TLR3 and MDA5 mRNA following stimulation with 10 μ g/ml of Poly(I:C) at 3 and 24h in HBECs from both healthy and asthmatic subjects. At 3 and 24h, we observed significant induction of IFN- β expression in HBECs from healthy subjects as well as asthmatic patients (Figure S2A and D), though there was significantly less expression in HBECs from asthmatic patients compared to healthy subjects at 24h.

Then we measured mRNA expression of PRRs to further evaluate the impairment of IFN- β expression in asthmatic HBECs. There was no difference for basal expression of TLR3 and MDA5 between HBECs from healthy and asthmatic subjects (Figure S2B, C, E, and F). Poly(I:C) did not increase TLR3 mRNA expression in HBECs of neither healthy nor asthmatic subjects at 3h (Figure S2B), though at 24h there was higher expression of TLR3 in both groups (Figure S2E). At 3h post stimulation with Poly(I:C), MDA5 mRNA levels were induced in both asthmatic and healthy cells (Figure S2C). Interestingly, MDA5 gene expression in HBECs from asthmatic subjects after Poly(I:C) stimulation was reduced compared to healthy subjects at 24h ($P=0.0542$)(Figure S2F).

Heat-treatment of HDM opposes interferon-reducing effects of HDM in bronchial epithelial cells.

Many aeroallergens, including HDM, contain proteolytic activities that may affect PRR signalling. To examine if the effect of HDM on interferon signalling pathways reflects protease activity of HDM, we used heat treatment to inactivate protease activity (HI-HDM) (24) (Figure S3). BEAS2B cells were incubated with control medium, HDM or HI-HDM for 24h. Then the cells were stimulated with Poly(I:C) for an additional 24h. Epithelial cells that were pre-exposed to HDM had a lower expression of IFN- β and - λ compared to the cells that were not pre-exposed to HDM. Interestingly, epithelial cells which were pre-exposed to HI-HDM exhibited higher IFN- β and - λ in response to Poly(I:C) compared to HDM-Poly(I:C) – treated cells (Figure 2A and B).

We next investigated expression of PRRs in response to exposure to HDM and HI-HDM. Pre-exposure to HDM decreased the expression of TLR3, RIG-I and MDA5 compared to

medium-Poly(I:C) treated cells (Figure 2C-E). Protein expression of these receptors in cell lysates showed a similar pattern (Figure 2F-H). Heat-inactivation partly restored the mRNA expression of RIG-I like receptors but not TLR3 (Figure 2C-E), while at protein level there was an increased expression for all three receptors in cells pre-exposed to HI-HDM (Figure 2F-H).

We then studied the effects of HDM and HI-HDM on type I, III IFNs and PRRs in primary HBECs obtained from human subjects (Table-S1). Primary HBECs were incubated with control medium, HDM or HI-HDM for 24h. Then the cells were stimulated with Poly(I:C) for an additional 24h. We observed similar pattern to BEAS2B cells for the expression for type I, IFN- β and type III, IFN- λ . Their expressions were increased by Poly(I:C) compared to control. HDM exposure lowered their expression, which was partly restored by heat-inactivation of HDM extract (Figure S4A and B).

Pre-exposure to HDM did not have any effect on the gene expression of TLR3 in response to Poly(I:C) (Figure S4C) but the expression of RIG-I like receptors were lower compared to cells which only received Poly(I:C) (Figure S4D and E). The only PRR whose expression was partly restored by heat-inactivation of HDM extract was MDA5 (Figure S4D). This data suggests that effects of HDM on the IFN- β and- λ signalling pathway in both BEAS2B cells and primary HBECs reflect in part protease activity of HDM.

Exposure to TLR3 ligand induces a mixed neutrophilic and lymphocytic exacerbation on a background of HDM-induced allergic inflammation in vivo.

In order to test our in vitro findings in an in vivo setting, we used a model of experimental asthma in mice (as previously described) (23). In the present study, BALF and lungs were collected 72 hours after the last HDM or saline administration. Consistent with our previous observations (23) mice that received HDM for 3 weeks had higher cell count and percentage of eosinophils and neutrophils in their BALF compared to the saline group (Figure S5A- C). Both IL-4 and IL-13 concentration in the lung homogenates were higher in the HDM group compared to saline group (Figure S5G and H).

We next looked into effects of pre-established allergic inflammation on the response to Poly(I:C) administration. Three days after the last saline or HDM administration Poly(I:C) (exacerbation) or saline (exacerbation control) was administered intranasally for 3 consecutive days. Such dual allergic and viral challenge produces exaggerated inflammation

with features in common with viral induced exacerbations of human asthma (23). BALF cells were increased by Poly(I:C) on both saline and HDM background compared to the saline control group. Poly(I:C)-induced asthma exacerbation exhibited increased cell count in mice with pre-established allergic inflammation with a mixed neutrophilic and lymphocytic inflammatory profile in BALF (Figure 3A-C). Mice with an established allergic inflammatory background had a higher percentage of lymphocytes in response to Poly(I:C) compared to the saline background group (Figure 3C). We also analysed BALF total protein content as an indicator of the degree of inflammation. Poly(I:C) increased BALF protein content in both saline and HDM background and there was no difference between these groups (Figure 3E). In summary, Poly(I:C) induced a mixed neutrophilic and lymphocytic inflammatory profile which was exaggerated in mice with a pre-established HDM-induced allergic inflammation.

Established allergic airway inflammation modulates Poly(I:C)-induced expression of IFN- β , - λ and PRRs in vivo.

We investigated type I, IFN- β and type III, IFN- λ mRNA expression in response to Poly(I:C) in lung homogenates obtained from our mouse exacerbation model. The expression of IFN- β and - λ was increased by Poly(I:C) in lungs without prior exposure to HDM (Figure 4A-C). Interestingly, the interferon response to Poly(I:C) was lost in mice with a prior established pulmonary allergic inflammation to HDM; these mice also had lower IFN- β and - λ expression compared to the saline-Poly(I:C) group (Figure 4A and C). Poly(I:C) increased IFN- β protein in BALF compared to saline in both saline and HDM pre-exposed groups, though the HDM-Poly(I:C) group had significantly lower IFN- β concentration in the BALF (Figure 4B). Looking upstream of the interferon signalling pathways, we next examined IRF-3 and IRF-7 mRNA expression in lung homogenates. Gene expression of both IRF-3 and 7 was increased in the saline-Poly(I:C) group (Figure 4D and E). However, pulmonary IRF-7 mRNA expression was significantly lower in the HDM-Poly(I:C) group compared to the saline-Poly(I:C) group. IRF-3 expression had a similar trend to be attenuated though it did not reach statistical significance (Figure 4D). We then examined PRRs (TLR3 and RIG-I like receptors) gene and protein expression in lung tissue. At gene levels, all three PRRs showed a similar pattern; gene expression of TLR3, RIG-I and MDA5 in response to Poly(I:C) was thus increased in mice without a prior established pulmonary allergic inflammation compared to saline group, and notably, there was lower gene expression in the HDM-Poly(I:C) compared to the saline-Poly(I:C) group (Figure 4F,H and J). We also analysed the protein

expression of these three PRRs. TLR3 protein expression did not change in response to Poly(I:C) (Figure 4G). RIG-I expression was increased in response to Poly(I:C) but there was no difference between the saline-Poly(I:C) and the HDM-Poly(I:C) group (Figure 4I). The only PRR whose protein expression followed the pattern of IFN- β concentration in the BALF was MDA5. Its expression increased in lung homogenates from the saline-Poly(I:C) group compared to the saline-saline group. It also showed increased levels in the HDM-Poly(I:C) group compared to the HDM-saline group. However, there was a decreased expression of MDA5 in the lungs of the HDM-Poly(I:C) compared to the saline-Poly(I:C) group (Figure 4J and K).

To further explore the RIG-I like receptor signalling we investigated pulmonary gene and protein expression of LGP2. The role of LGP2 has been a matter of debate in RIG-I like receptors signalling (25). LGP2 mRNA expression had a similar pattern to our RIG-I like receptors gene expression, with increased levels in the saline-Poly(I:C) compared to the saline-saline group. LGP2 expression did not change in response to Poly(I:C) in mice with a pre-established allergic inflammation in their lungs. The HDM-Poly(I:C) group had a lower expression of LGP2 compared to the saline-Poly(I:C) group (Figure 4L). Yet LGP2 protein levels did not change in response to Poly(I:C) (Figure 4M).

Impaired IFN- β response to Poly(I:C) in mice pre-exposed to HDM is partially restored by heat-inactivation of HDM.

In vitro data showed that heat-inactivation of HDM opposes its IFN-lowering effects (Figure 3A and B). In order to test these results in vivo, we included HI-HDM in the same in-vivo model as mentioned above. Poly(I:C) increased cells in the BALF of mice which received 3 weeks of saline, HDM or HI-HDM compared to saline (Figure S6A). In summary, the data showed lower level of eosinophilia at exacerbation for the HI-HDM group compared to the HDM group. There was not much of difference for other BALF inflammatory indicators between these two groups (Figure S6B-F).

Next, we studied mRNA expression of IFN- β in lung homogenates. Intranasal administration of Poly(I:C) in mice, which were pre-exposed to saline, increased IFN- β gene expression. Similar to what we observed previously, this response was dampened in mice that received HDM. Heat-inactivation of HDM increased IFN- β response to Poly(I:C) compared to the HDM-Poly(I:C) group (Figure 5A). We then analysed BALF for IFN- β protein by ELISA. Poly(I:C) administration increased BALF IFN- β concentration in all three groups. Heat-

inactivation of HDM slightly increased BALF IFN- β compared to HDM ($P=0.0519$) (Figure 5B). We also analysed IFN- λ gene expression in lung homogenates. IFN- λ gene expression was increased by Poly(I:C) in the saline group (though not statistically significant) and was decreased in the HDM group. Heat-inactivation of HDM did not affect IFN- λ expression in response to Poly(I:C) (Figure 5C).

Moving up-stream in the interferon signalling pathway, we then looked into expression of IRF-3 and -7 in lung homogenates. Their expression showed a pattern quite similar to IFN- β expression. Poly(I:C) increased the expression of IRF-3 and IRF-7 in the saline group, which was reduced in the HDM group. Heat inactivation of HDM restored IRF-3 (partially) and IRF-7 (completely) expression (Figure 5D and E).

We then looked at mRNA and protein expression of TLR3 and RIG-I like receptors in lung homogenates. TLR3 gene expression was increased by Poly(I:C) in the saline group (though not statistically significant) and was decreased in the HDM group. Heat-inactivation of HDM did not affect TLR3 expression in response to Poly(I:C) (Figure 5F). This pattern of response was similar to our observation in vitro. TLR3 protein expression in the lungs also followed the same pattern (Figure S7A). RIG-I gene expression was increased by Poly(I:C) in the saline group and was decreased in HDM pre-exposed mice. There was no significant increase in RIG-I expression in response to Poly(I:C) by heat-inactivation (Figure 5G). RIG-I protein expression showed the same pattern (Figure S7B). In contrast, for the other RIG-I like receptor, MDA5, we observed an increased gene expression in the saline-Poly(I:C) group compared to the saline-saline group, which was dampened in the HDM-Poly(I:C) group, and was completely restored by heat-inactivation of HDM extract (Figure 5H). Protein expression followed the same pattern (Figure S7C). In summary we observed that impaired MDA5, IRF-3, IRF-7 and IFN- β response to Poly(I:C) in HDM pre-exposed lungs were all restored by heat-inactivation, though the extent of restoration varied among them.

HDM-induced IFN deficient response in BEAS2B cells is partly through affecting TLR3 glycosylation

It has been shown that RIG-I like receptors expression can be upregulated by IFNs. In order to investigate whether the up/down-regulation of MDA5 at gene and protein level by HDM-Poly(I:C) and Poly(I:C) is partly the result of this feedback, we stimulated BEAS2B cells with IFN- β . Incubation of BEAS2B cells with IFN- β for 24 hours increased both gene and protein expression of MDA5 (Figure 6A and B). In order to investigate the involvement of

MDA5 and TLR3 in IFN- β expression we knocked down MDA5 and TLR3 and stimulated the cells with Poly(I:C). MDA5 and TLR3 siRNA reduced the respective gene expression up to 90% (Figure S8). We also confirmed that the knock down was target specific and did not affect RIG-I expression (Figure S8). IFN- β expression was still significantly down-regulated after pre-exposure to HDM when MDA5 gene was knocked down (Figure 6C). These results indicated the possibility that HDM may affect IFN- β expression in response to Poly(I:C) through TLR3.

Glycosylation of TLR3 plays an important role in its function (26). Thus we investigated if HDM affects TLR3 glycosylation. Heavily-glycosylated TLR3 has a molecular weight of ~130kDa. In a TLR3 antibody-probed cell lysate of cells pre-exposed to HDM and treated with Poly(I:C) we detected a band at molecular weight ~70kDa, which had a higher intensity compared to non-HDM treated cells (Figure 6D), suggesting a higher amount of low-glycosylated TLR3. This band had weaker intensity in cells pre-exposed to heat-inactivated HDM. We then treated the cells with tunicamycin, which inhibits the addition of N-acetylglucosamine, to see the effect of glycosylation on IFN- β expression. Tunicamycin reduced the IFN- β expression in response to Poly(I:C) supporting the role of glycosylation (Figure 6E). The heavily-glycosylated bands at ~130kDa were reduced 24h after tunicamycin treatment (Figure 6F). These findings suggest that HDM-induced impaired interferon response to Poly(I:C) in bronchial epithelial cells can partly be due to its effects on TLR3 glycosylation.

Discussion

Both viral infections and allergic airway inflammation can contribute to early development of asthma as well as to acute asthma exacerbations. Exploring the interactions between innate antiviral responses and allergic inflammation would thus be of great help in order to identify underlying mechanisms of asthma onset and acute exacerbations (27, 28). In this study we investigated effects of a common aeroallergen implicated in asthma on interferon signalling in response to a synthetic viral infection surrogate. We showed that pre-exposure to HDM impairs the interferon response to Poly(I:C) both in vitro and in vivo. The effects of HDM on interferon signalling were partly due to its heat-sensitive components.

In several cohorts, BECs from asthmatic patients have shown impaired interferon response to rhinovirus and its surrogate (dsRNA) (19, 21, 22, 29). This was not restricted to bronchial epithelial cells and has been shown in peripheral blood mononuclear cells (30) and alveolar macrophages (20). Adding to previous observations, bronchial epithelial cells from our cohort of asthmatic patients had reduced expression of IFN- β in response to TLR3 stimulation compared to cells from healthy donors. The innate immune system senses rhinovirus by recognizing a variety of viral components, foremost dsRNA, and triggers antiviral responses (31). TLR3 recognizes viral dsRNA as well as the synthetic dsRNA analogue poly(I:C) (32). The cytoplasmic proteins RIG-I and MDA5 have also been identified as dsRNA detectors. RIG-I recruits a CARD-containing adaptor, MAVS (33), which relays the signal to the kinases that phosphorylate interferon-regulatory factor-3 (IRF-3) and IRF-7, transcription factors critical for the expression of interferons (34, 35). There is no evidence suggesting genetic defects in the interferon signalling pathway in asthmatic patients through array studies (36). We also did not observe any difference in expression of TLR3 and MDA5 between untreated healthy and asthmatic BECs. This is in contrast with previous findings in children that showed reduced expression of TLR3 in unstimulated, diseased cells (21). This may be explained by the fact that our cohort included mild to moderate asthmatic patients while the patients in the other study were children with severe, therapy-resistant asthma (21). However, at 24 hours after stimulation with Poly(I:C) the asthmatic cells showed a trend towards lower expression for MDA5 than cells from healthy donors.

We demonstrated that pre-exposure of BECs to HDM impaired IFN- β and IFN-lambda expression in response to Poly(I:C). This novel finding was accompanied by lower expression of both mRNA and protein of TLR3 and RIG-I like receptors. Hence, HDM allergens not only induce adaptive Th2-biased immune responses in susceptible subjects but also may affect innate immunity by reducing the antiviral response. HDMs are complex organisms. In addition to their well-characterized proteases they produce proteins and macromolecules that might have inflammatory effects (16). It has been shown that allergenic proteases of HDM can release a biologically active product by cleaving CX3CL1 from apical bronchial epithelial surface, which can be augmented by RV (37). Besides proteolytic allergens, HDMs contain Der p2, a non-proteolytic allergen. The extract, which we used, has been shown to contain the highest levels of Der p2 whose effects disappear upon heat inactivation (38). The present effects of HDM on IFN signalling on BECs in response to

Poly(I:C) were partially sensitive to heat-inactivation. Heat inactivation reduces the protease activity and the exochitinase levels in a HDM extract (39). HDMs have also other components, including chitin/chitinases, β -glucan and lipopolysaccharide (LPS) that may contribute to inflammatory responses (16, 17, 40). We have used serine and cysteine protease inhibitors in order to investigate if proteases are responsible for HDM effects on interferon signalling but we could not observe similar effects to heat inactivation (unpublished data). This can be due to much lower inhibition of protease activity by protease inhibitors compared to heat inactivation (39). Another explanation is that HDM effects on interferon signalling are independent of serine and cysteine proteases.

Importantly, we could extend our in vitro findings on HDM-induced effects on type I and III interferons expression to an in vivo model which we have characterized previously (23). In this model Poly(I:C) exacerbated neutrophilia on the background of HDM-induced allergic airway inflammation as also shown previously (23, 41). However, to our knowledge this is the first report that has compared the effects of Poly(I:C) between saline and HDM-exposed mice. Other reports have focused on the differences between saline and Poly(I:C) stimulation in allergen-exposed animals (23, 41, 42). The present design gave us the privilege of observing the difference in type I and III IFNs levels between HDM-exposed and saline-exposed mice in response to Poly(I:C), which indeed was much lower in the former group. HDM pre-exposure thus had grave effects on gene expression of IFN- β and - λ signalling pathway including TLR3 and RIG-I like receptors in vivo. However, this was not observed to the same degree at protein level. One explanation for these findings could be the time point of termination of the study in our model. We could not observe the same effects of HDM on PRRs' protein levels as we saw in vitro except for MDA5. Since we used whole tissue homogenates for analysis, we had other cell types present in our preparation, which may not respond in the same way as epithelial cells. There is also the possibility of a species difference and the multiple opportunities of differences between in vitro cells and cells in the complex and dynamic in vivo milieu.

LGP2 is the third member of the RIG-I like receptors and is less well characterized. LGP2 is a modulator that can modify RIG-I-dependent signalling via competition for dsRNA. It can also augment MDA5-dependent signalling (43). We analysed the expression of LGP2 in our mouse study to see if this RLR is also affected by HDM exposure. Gene expression of LGP2 followed the same pattern as the other two members of RLRs (RIG-I and MDA5), while

protein expression was reduced by HDM exposure for unknown reasons. Some reports suggest an inhibitory role for LGP2 on antiviral signalling (44, 45). However, our data demonstrated that LGP2 is not acting as a negative regulator of Poly(I:C) recognition in the HDM treated mice. Thus the inhibitory effect of HDM cannot be due to LGP2.

Inflammatory cells and protein content in BALF were not affected by heat inactivation of HDM in our model but percentage of eosinophils in BAL was reduced. De Alba et al. have reported that heat inactivation of HDM extract did not change the inflammatory profile in vivo but they used a different type of regimen for HDM administration compared to our model (10). Interestingly, we observed restoration of the interferon responses in vivo as well as in vitro by heat inactivation of HDM. MDA5, the only PRR affected at protein level by HDM, also exhibited restored expression in response to Poly(I:C) in the present experiments involving heat inactivation of HDM.

Lower expression of PRRs in HDM pre-exposed BECs suggested that the whole antiviral signalling cascade is affected. The role of TLR3 and RLRs in interferon signalling has not been fully understood. Apparently their role varies depending on which type of cells and viruses are involved. All these PRRs may be needed, perhaps in sequence, for a full interferon response at viral infection (46). In order to study which PRRs are affected directly by HDM exposure, we used gene silencing and observed that TLR3 signalling was reduced the most by HDM pre-exposure. Looking at TLR3 gene polymorphism it has been reported that L412F is present in 20% of the population and is higher in the asthmatic population (47). Mutant L412F may affect potential glycosylation of neighbouring residue Asn⁴¹³, which has been shown to have *N*-acetylglucosamines attached in the crystal structure of TLR3 ectodomain (47-49). Hence, we investigated the low-glycosylated and highly-glycosylated TLR3 expression in response to HDM. We showed lower expression of the highly-glycosylated TLR3 in BECs, which were pre-exposed to HDM. Interestingly, the ratio of highly-glycosylated TLR3 to low-glycosylated TLR3 was decreased by HDM exposure and restored by heat-inactivation of HDM. We also showed in BECs that tunicamycin, which is widely used as an inhibitor of glycosylation, reduced the IFN- β expression. This finding agrees with a previous report demonstrating that highly-glycosylated TLR3 is essential for its intact signalling (40). TLR3 biology is a complex process including different steps from synthesis, processing and trafficking to final recognition of dsRNA and start of the signalling cascade (50, 51). Our data demonstrates that regulation of receptor glycosylation is one

possible mechanism by which HDM can disturb signalling through TLR3. Hence, direct effects of allergens on PRRs involved in antiviral response can explain in part defective antiviral responses in patients. Another potential possibility is that the band with the lower molecular weight represents cleaved TLR3. TLR3 can be cleaved by proteases during endocytosis. HDM proteases may possibly contribute to the cleavage process. However, it has previously been shown that cleaved TLR3 has the ability to bind to Poly(I:C) and signal making it less likely that HDM-produced cleaved TLR3 present impaired interferon response (50).

Epidemiological data indicate increased susceptibility to effects of viral infection in atopic asthma (52). However, experimental HDM challenges in vivo in asthmatic individuals did not increase inflammatory and disease responses to subsequent rhinovirus inoculation (53). The failure to demonstrate the expected positive interaction in the challenge and inoculation study may be explained by antiviral components of acute responses to HDM challenge including nitrogen oxide (52) and exudation of potent plasma protein systems (54). Hence, it is important to ascertain that a reproducible pathogen burden is employed in experiments comparing responses with and without prior HDM exposure. This consideration was a factor supporting our choice of dsRNA challenges to mimic viral infection-evoked responses in the present exacerbation in vivo model.

In conclusion, HDM exposure impaired interferon response to TLR3 stimulation in BECs and in an in vivo model of asthma exacerbation. This impairment was mostly due to HDM effects on TLR3 signalling pathway. Our data showed that HDM can affect N-glycosylation of TLR3 receptor, which would impair the IFN- β and - λ response. Direct effects of allergens on PRRs can present as a potential mechanism for defective antiviral response in asthmatic patients who are atopic. Accordingly, therapeutic measures targeting inhibitory effects of allergens on antiviral PRRs may find use as a strategy to boost antiviral response in airway disease.

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Author contributions

HA and LU designed the studies. HA, MM, SR and IMP contributed to the data collection. HA, MM and LU analysed and interpreted the work. LB provided clinical samples. HA drafted the manuscript. All authors have read, revised and approved the final version of this manuscript.

Conflict of interest

The authors declare no competing interests.

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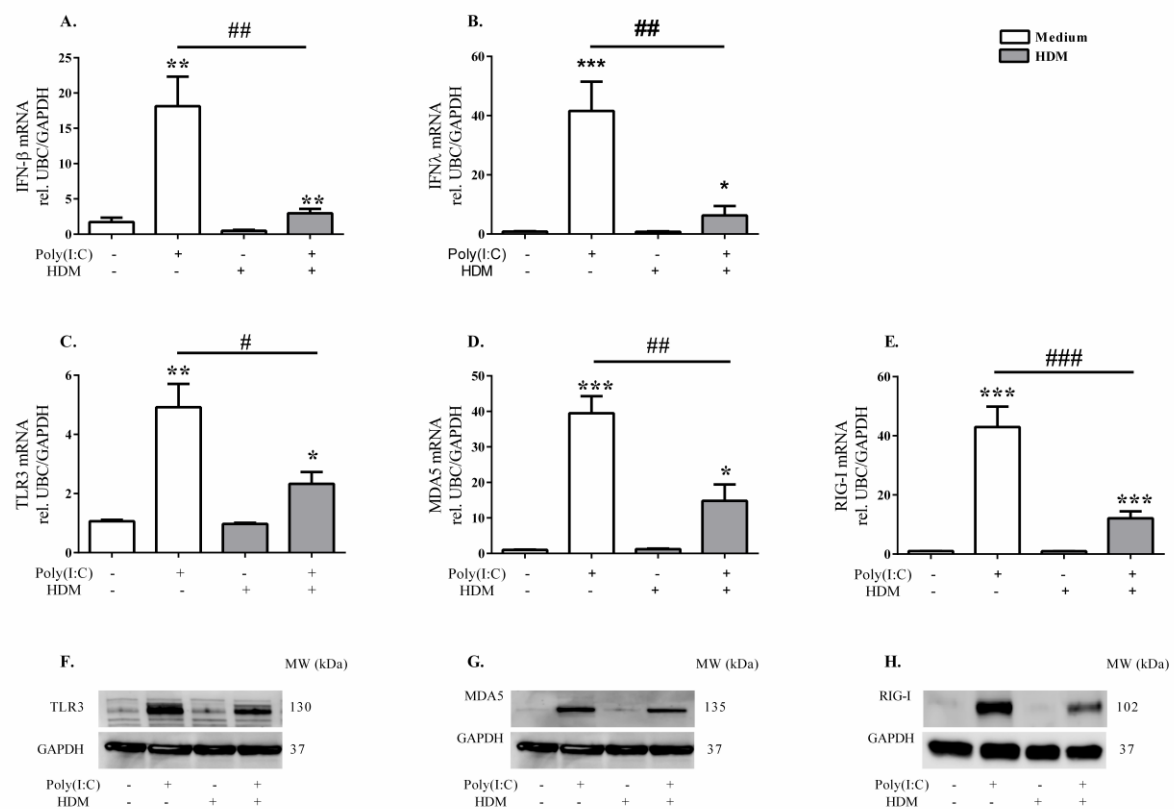


Fig-1 Pre-exposure of HDM impairs interferon I and III signalling in response to Poly(I:C) in bronchial epithelial cells. BEAS-2B cell monolayers were cultured for 24 h with medium or 20 µg/ml HDM, followed by stimulation with 10 µg/ml of Poly(I:C) or control medium for 24 h (A-E). The expression of IFN-β (A), IFN-λ (B), TLR3 (C), MDA5 (D) and RIG-I (E) was determined by qPCR. Representative immunoblots of cell lysates probed with anti-TLR3 (F), anti-MDA5 (G), or anti-RIG-I (H) are presented. Data is presented as mean ± SEM n= 8-10 from 6 experiments. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.005$ compared to control and HDM group, # $P < 0.05$, ## $P < 0.01$, ### $P < 0.001$ compared to HDM-Poly(I:C) group.

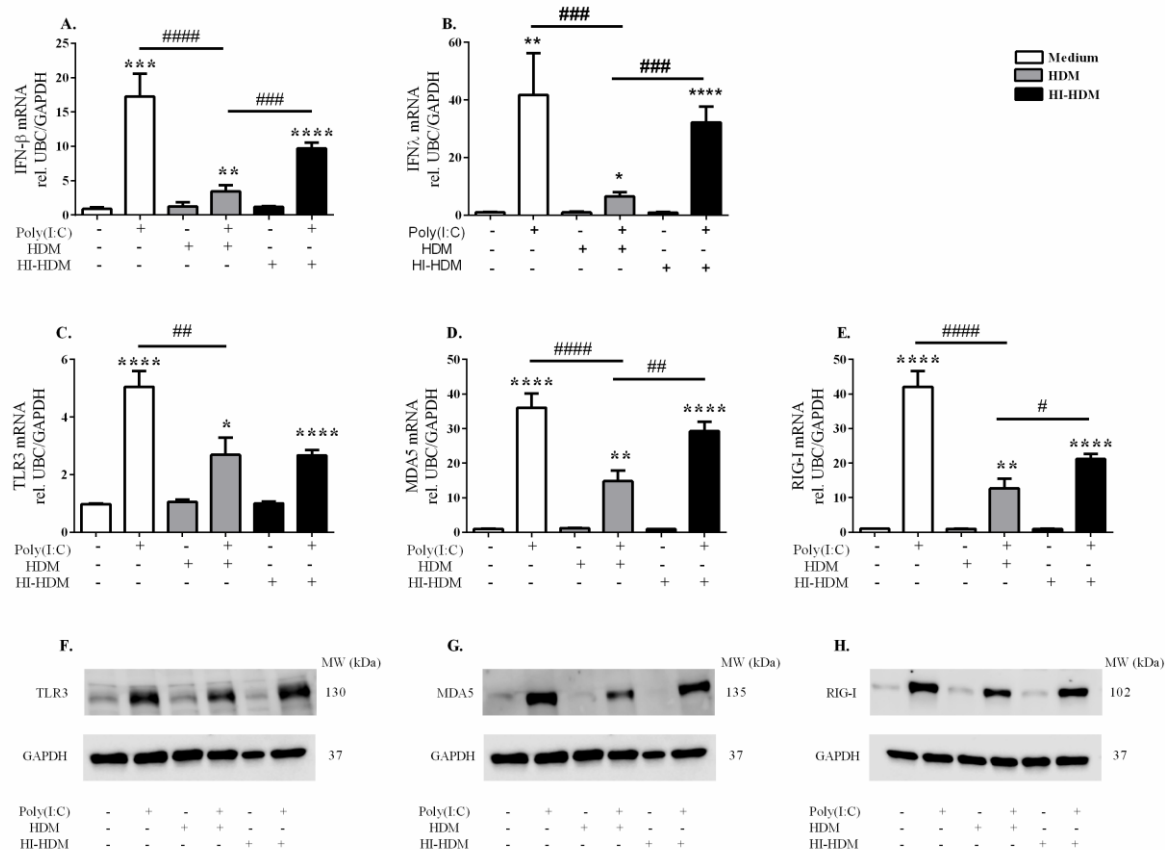


Fig-2 Heat-inactivation of HDM restores IFN- β , - λ and its downstream signalling pathways in response to Poly(I:C). The expression of IFN- β (A), IFN- λ (B), TLR3 (C), MDA5 (D), RIG-I (E) was determined by qPCR. Representative immunoblots of cell lysates which were probed with anti-TLR3 (F), anti-MDA5 (G), or anti-RIG-I (H) are presented. Data is presented as mean $\pm$ SEM $n = 5-10$ from up to 6 experiments. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$ compared to control and HDM group, # $P < 0.05$, ## $P < 0.01$, ### $P < 0.001$, #### $P < 0.0001$ compared to HDM-Poly(I:C) group.

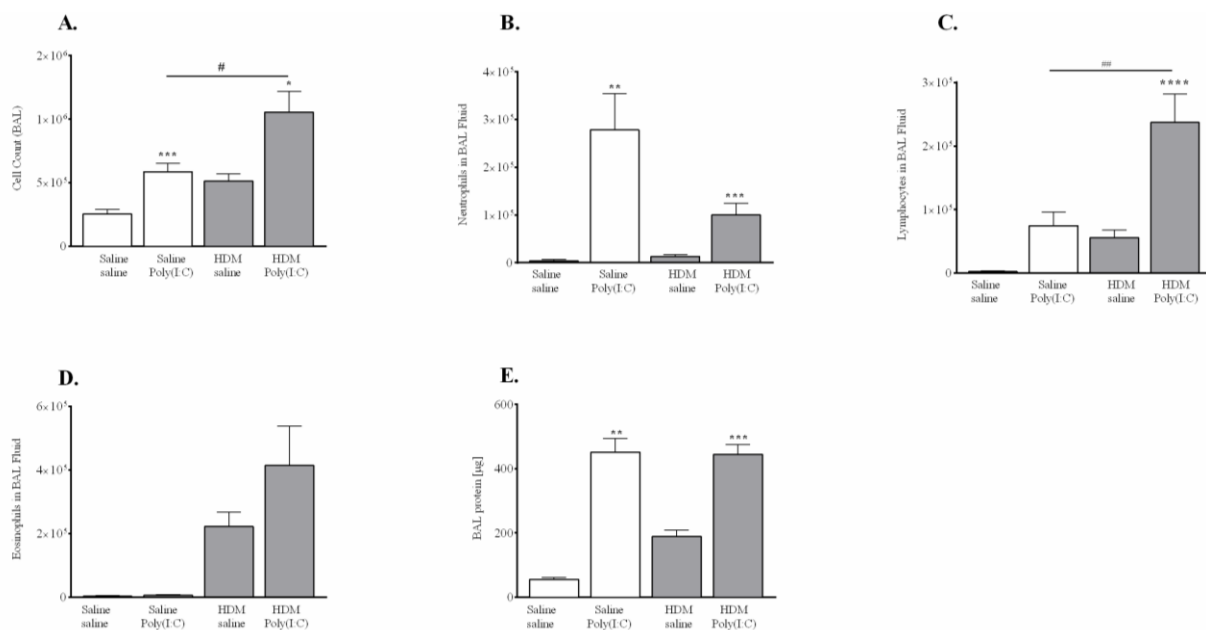


Fig-3 Poly(I:C) exacerbates the cellular inflammation in BALF in mice pre-exposed to HDM. BALF was collected and was analysed for cell count **(A)**, percentage of neutrophils **(B)**, lymphocytes **(C)**, eosinophils **(D)** and total protein content **(E)**. Data is presented as mean $\pm$ SEM $n = 6-8$ * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ compared to saline-saline or HDM-saline groups. # $P < 0.05$ compared to saline-Poly(I:C).

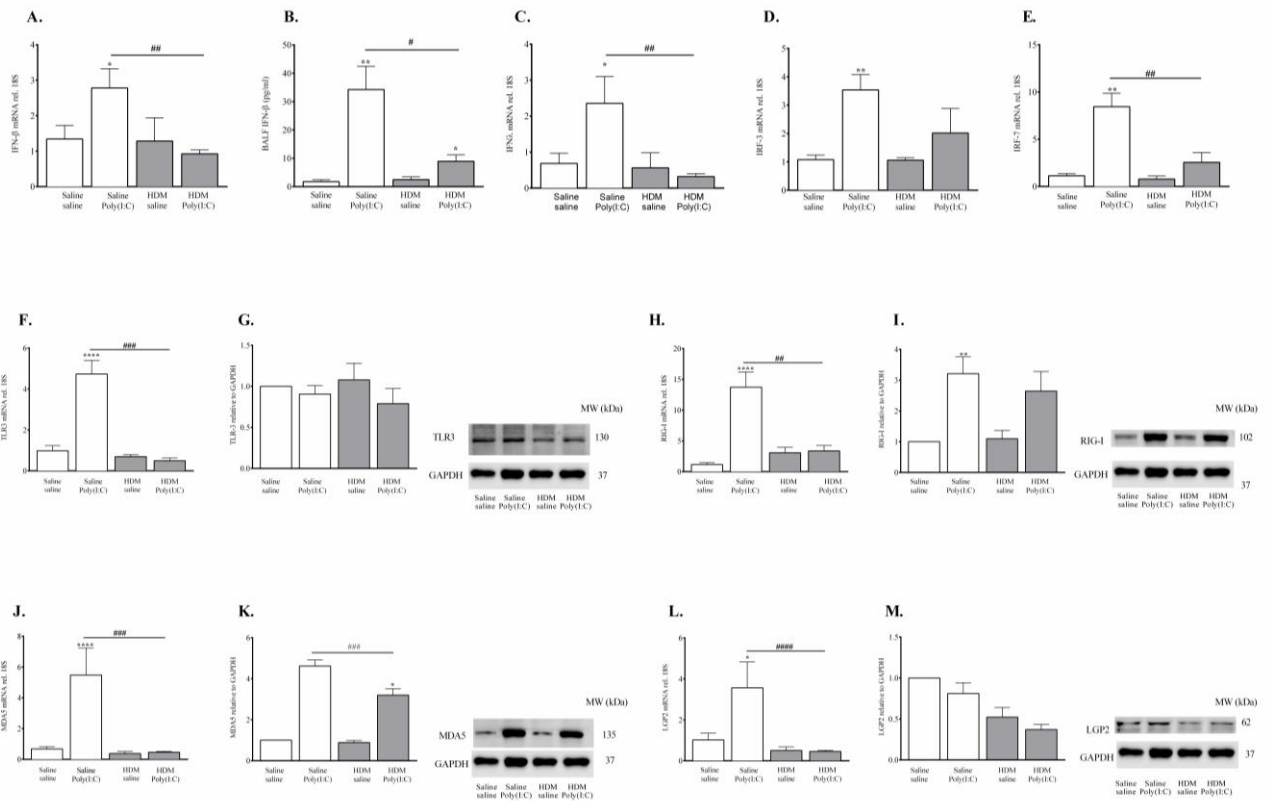


Fig-4 HDM-exposed mice showed impaired lung interferon signalling in response to Poly(I:C). Quantitative analysis of transcript levels of IFN-β (A) IFN-λ (C), IRF-3 (D), and IRF-7 (E) in the lung homogenates was done by qPCR. BALF samples were analysed for IFN-β (B) by ELISA. The expressions of TLR3 (F), RIG-I (H), MDA5 (J) and LGP2 (L) were determined by qPCR. Immunoblots of tissue lysates were probed with anti-TLR3 (G), anti-RIG-I (I), anti-MDA5 (K), or anti-LGP2 (M). Data is presented as mean ± SEM. n= 4-14 for gene expression and BALF IFN-β. n= 4-5 for immunoblots. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.0001$ compared to saline-saline or HDM-saline groups. # $P < 0.05$, ## $P < 0.01$, ### $P < 0.001$, #### $P < 0.0001$ compared to saline-Poly(I:C).

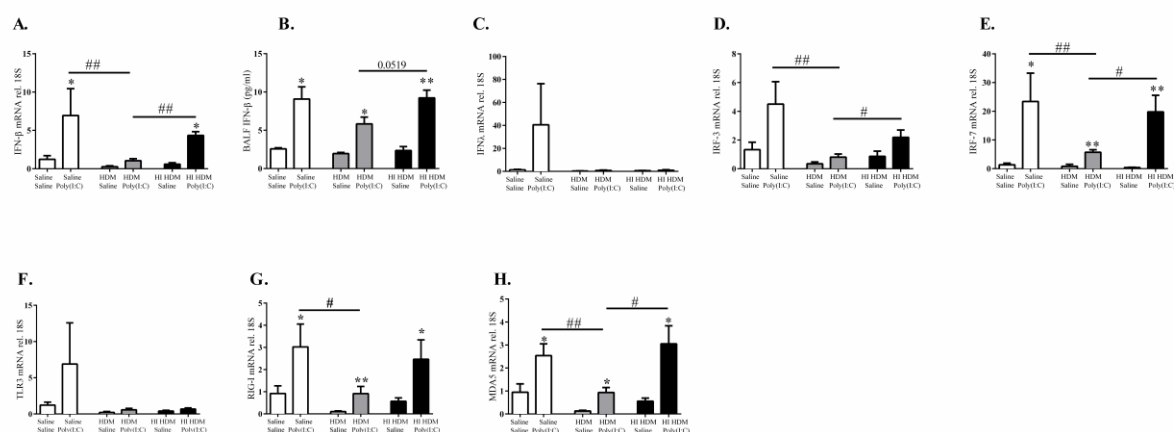


Fig-5 Heat-inactivation of HDM partially restores IFN- β signalling in response to Poly(I:C). Quantitative analysis of transcript levels of IFN- β (A), IFN- λ (C), IRF-3 (D), IRF-7 (E), TLR3 (F), RIG-I (G), and MDA5 (H) in the lung homogenates was done by qPCR. BAL fluid samples were analysed for IFN- β (B) by ELISA. Data is presented as mean $\pm$ SEM. $n = 4-7$ for gene expression and BALF IFN- β . * $P < 0.05$, ** $P < 0.01$ compared to saline-saline, HI-HDM-saline or HDM-saline groups. # $P < 0.05$, ## $P < 0.001$ compared to HDM-Poly(I:C).

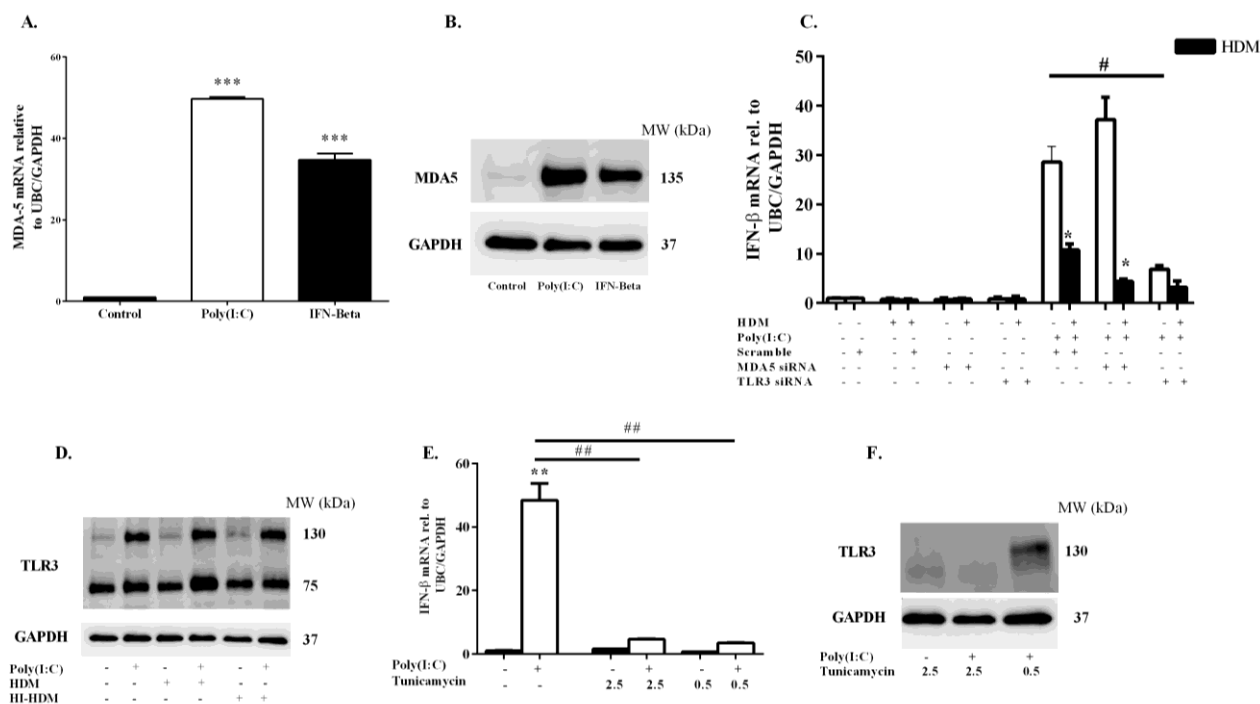


Fig-6 HDM impairs interferon signalling partly through its effects on TLR3. The expression of MDA5 (A) was determined by qPCR. Representative immunoblots of cell lysates which were probed with anti-MDA5 (B) are presented. The expression of IFN-β (C) was determined by qPCR. Representative immunoblots of cell lysates which were probed with anti-TLR3 (D). The expression of IFN β (E) was determined by qPCR. Cell lysates were probed with anti-TLR3 (F). Data is presented as mean ± SEM n= 3-7 from up to 5 experiments. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ compared to control or control-Poly(I:C) groups. # $P < 0.05$, ## $P < 0.01$.

**House dust mite impairs antiviral response in asthma exacerbation models
through its effects on TLR3**

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23 **Figure-S1** Poly(I:C)-induced MDA5 expression is reduced by HDM pre-exposure.

24 **Fig-S2** Lower expression of IFN- β and MDA5 in response to Poly(I:C) stimulation in
25 bronchial epithelial cells from asthmatic subjects.

26 **Figure-S3** Protease activity of HDM is reduced by heat exposure.

27 **Figure-S4** Heat-inactivation of HDM restores IFN- β , - λ and MDA5 in response to Poly(I:C)
28 in primary HBECs.

29 **Figure-S5** Intranasal HDM induced eosinophilia and neutrophilia in the BAL.

30 **Figure-S6** Heat inactivation of HDM reduced the percentage of eosinophils in the BAL in
31 response to Poly(I:C).

32 **Figure-S7** Heat inactivation of HDM restored MDA5 protein expression in the lungs in
33 response to Poly(I:C).

34 **Figure-S8** MDA5 and TLR3 siRNAs are specific for their intended targets.

35

Supplementary Material and Methods:

Reagents

House dust mite (HDM; Greer, Lenoir, NC, USA), Tunicamycin (Sigma-Aldrich, Stockholm, Sweden), and polyinosinic:polycytidylic acid (Poly(I:C)) High Molecular Weight, InvivoGen, San Diego, CA, USA, recombinant human IFN- β (R and D systems Abingdon, Oxon, UK) were used for in vitro and in vivo studies.

RNA extraction and quantification of gene expression by RT-qPCR

For in vivo experiments frozen lung tissue was mechanically homogenized (OmniPrep, Rotor Stator Generator, Omni International, Kennesaw, GA, USA) in lysis buffer (Nucleospin® RNA II, Macherey-Nagel, Düren, Germany). Total RNA was extracted from lung homogenates or HBECs using a RNA extraction kit (Nucleospin® RNA II, Macherey-Nagel, Düren, Germany). Then 2 μ g of RNA (from lung homogenates) or 1 μ g of RNA (from HBECs) was reverse transcribed to cDNA (Precision Nanoscript 2 Reverse Transcription Kit, PrimerDesign, Southampton, UK) and real-time quantitative PCR was performed on a Mx3005P qPCR system (Stratagene, La Jolla, CA, USA) using standard cycling parameters. Primers were obtained from PrimerDesign (Southampton, UK) and Qiagen (Sollentuna, Sweden). Samples were analysed by the $\Delta\Delta$ Ct method and related to UBC/GAPDH (in vitro samples) and 18S rRNA (in vivo samples) expression. Groups were normalized to control.

Protein expression by Western Blot

Protein expression of TLR3, RIG-I, MDA5, LGP2 and GAPDH was quantified by western blot. Lung tissue or cells were lysed in a lysis buffer containing 1% TritonX-100, 10 mM Tris-HCl, 50 mM NaCl, 5 mM EDTA, 30 mM Na₄P₂O₇, 50 mM NaF, 0.1 mM Na₃VO₄, 1% phosphatase and protease inhibitors (Sigma-Aldrich, Stockholm, Sweden). Protein

concentrations were determined by BCA protein assay (Pierce Thermo Scientific, Waltham, MA, USA) for each sample and equal amounts of protein were loaded and electrophoresed on a 10% TGX stain-free gel (Bio-Rad Laboratories AB, Solna, Sweden), before blotting on a Trans-Blot Turbo Transfer System (Bio-Rad Laboratories AB, Solna, Sweden). This was followed by blocking of the membrane in 5% milk in Tris-buffered saline Tween-20 and overnight incubation at 4°C with primary antibodies (anti-RIG-I Rabbit mAb, anti-MDA5 Rabbit mAb, anti-GAPDH Rabbit mAb (Cell Signaling Technology, Leiden, The Netherlands) or anti-LGP2 (Fisher Scientific, Waltham, MA, USA). Then the membrane was washed and incubated for 1 hour with secondary antibodies (anti-Rabbit IgG HRP-linked Ab; Fisher Scientific, Waltham, MA, USA). Restore PLUS Western Blot Stripping Buffer (Fisher Scientific, Waltham, MA, USA) was used to strip blots. Detection was performed by chemiluminescence using SuperSignal West FEMTO substrate (Pierce Thermo Scientific, Waltham, MA, USA) and immunoblots were visualized by LI-COR Odyssey Fc Imager (LI-COR Biosciences, Lincoln, NE, USA) and the density was analysed by Image Studio (v.3.1.4; LI-COR Biosciences, Lincoln, NE, USA).

Protease activity measurement

HDM extract was treated for 30 minutes at 65°C. Protease activity of both HDM and HI-HDM was measured by using azocasein (Sigma-Aldrich, Stockholm, Sweden) as substrate. In the azocasein assay, 10 µl of samples and 2% azocasein dissolved in Tris/HCl. After incubation at 37°C for 12 hours, the reaction was stopped by adding 100 µl of 10% trichloroacetic acid. After removing precipitates by centrifugation (8000 *g*, 5 min), 1 M NaOH was added and absorbance was quantified at 440 nm using a spectrophotometer. The protease activity of HI-HDM was expressed as percentage of absorbance of HDM.

Immunofluorescence Microscopy

A total of 20000 BEAS2B cells were placed into Lab-Tek four-chamber slides (Nalge Nunc International, Rochester, NY, USA). The next day they were incubated either with HDM or culture medium. After 24 hours they were stimulated with 10 µg/ml Poly(I:C) for 24 hours. Then cells were washed with PBS and fixed in 4% paraformaldehyde (Histolab Products AB, Gothenburg, Sweden) and permeabilised in 0.1% Triton X-100 (Sigma-Aldrich, Stockholm, Sweden) at room temperature for 15 min. The cells were incubated with anti-MDA5 Rabbit mAb (Cell Signaling Technology, Leiden, The Netherlands) overnight at 4°C. Cells were washed and incubated with anti-rabbit-Alexa Fluor 555 (1:200; Molecular Probes, Invitrogen, USA) for 1 hour at room temperature in the dark. Cells were washed and incubated with Nucblue (Molecular Probes, Invitrogen, USA) for 5 minutes. Cells were scanned by Olympus DP80 camera using a Nikon Eclipse 80i (Nikon, Japan) microscope with identical exposure times.

97 **Table-S1** Patient clinical characteristics

Age	Sex	FEV1	FEV % predicted	Atopy	Treatment
50	Male	2.94	68.1	NO	ICS
24	Female	3.74	107.3	YES	ICS/LABA
27	Male	4.06	85.4	YES	ICS/SABA
50	Female	2.14	74.7	YES	ICS/LABA
34	Male	5.15	108.8	YES	ICS/ LTRA
22	Female	3.74	92	NO	ICS/LABA
49	Male	4.39	116	YES	NO
25	Female	3.51	96.3	YES	NO
21	Male	3.87	89.2	YES	ICS/SABA

98

99 FEV₁, forced expiratory volume in 1s; ICS, inhaled corticosteroids; LABA, long-acting beta-

100 adrenoceptor agonist; SABA, short-acting beta-adrenoceptor agonist; LTRA, leukotriene

101 receptor antagonist

102

Supplementary Results

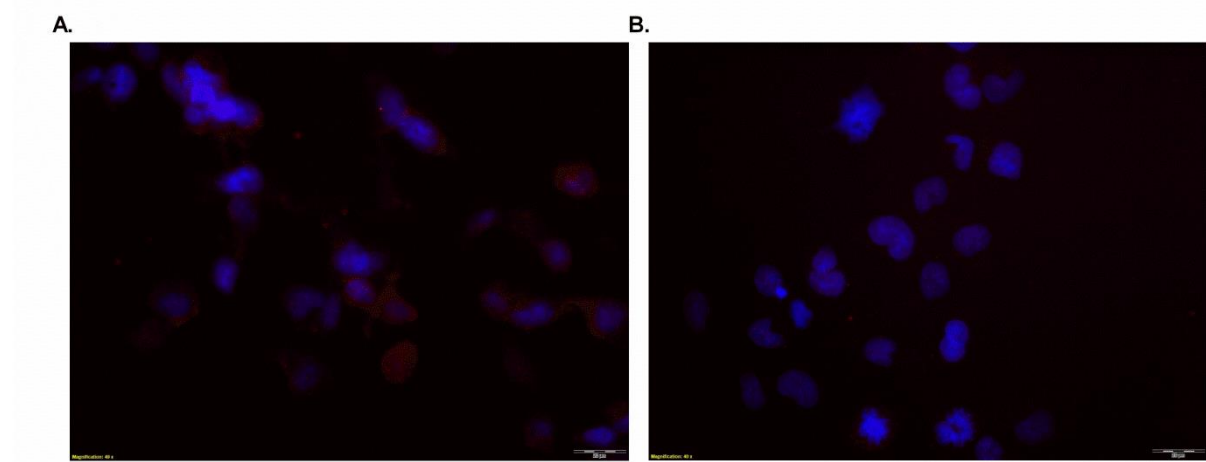


Figure-S1 Poly(I:C)-induced MDA5 expression is reduced by HDM pre-exposure.

MDA5 was detected 24 h after stimulation with Poly(I:C) on BEAS-2B cells pre-treated without (A) or with (B) HDM for 24 hours, by immunofluorescent staining. MDA5 is depicted in red.

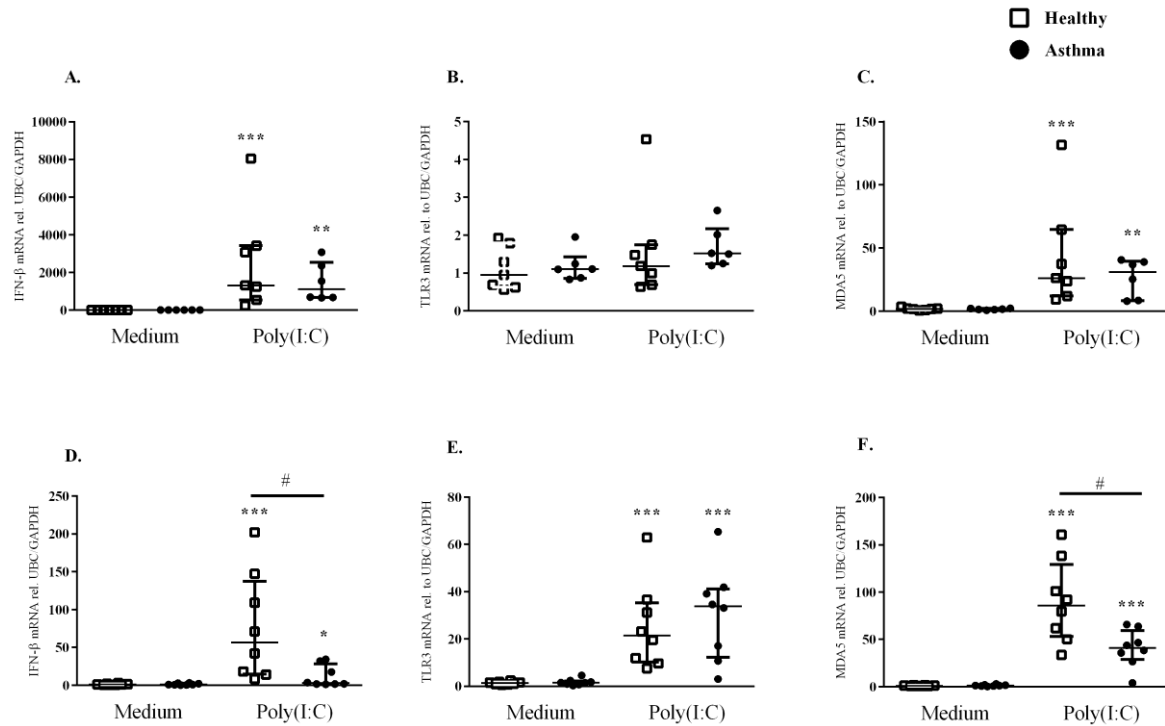
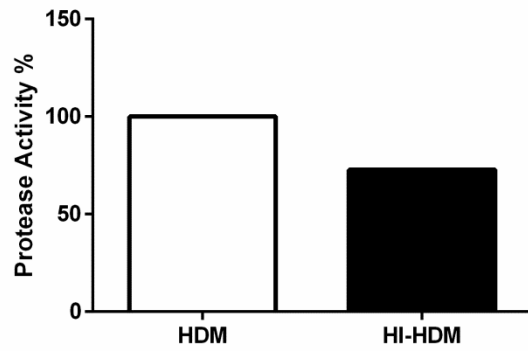


Fig-S2 Lower expression of IFN-β and MDA5 in response to Poly(I:C) stimulation in bronchial epithelial cells from asthmatic subjects. HBE cell monolayers from healthy and asthmatic subjects were either stimulated with 10μg/ml of Poly(I:C) or medium for 3 (A-C) and 24 hours (D-F). The expression of IFN-β (A, D), TLR3 (B, E), and MDA5 (C, F) was determined by qPCR at 3 and 24 hours. Data is presented as median (interquartile range) n= 6-7 per group for 3h and 8 per group for 24h. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ compared to medium group, # $P < 0.05$ compared to Healthy-Poly(I:C) group.



120

121 **Figure-S3 Protease activity of HDM is reduced by heat exposure.** HDM and HI-HDM

122 protease activity was measured by azocasein assay. HI-HDM protease activity in relation to

123 HDM is showed.

124

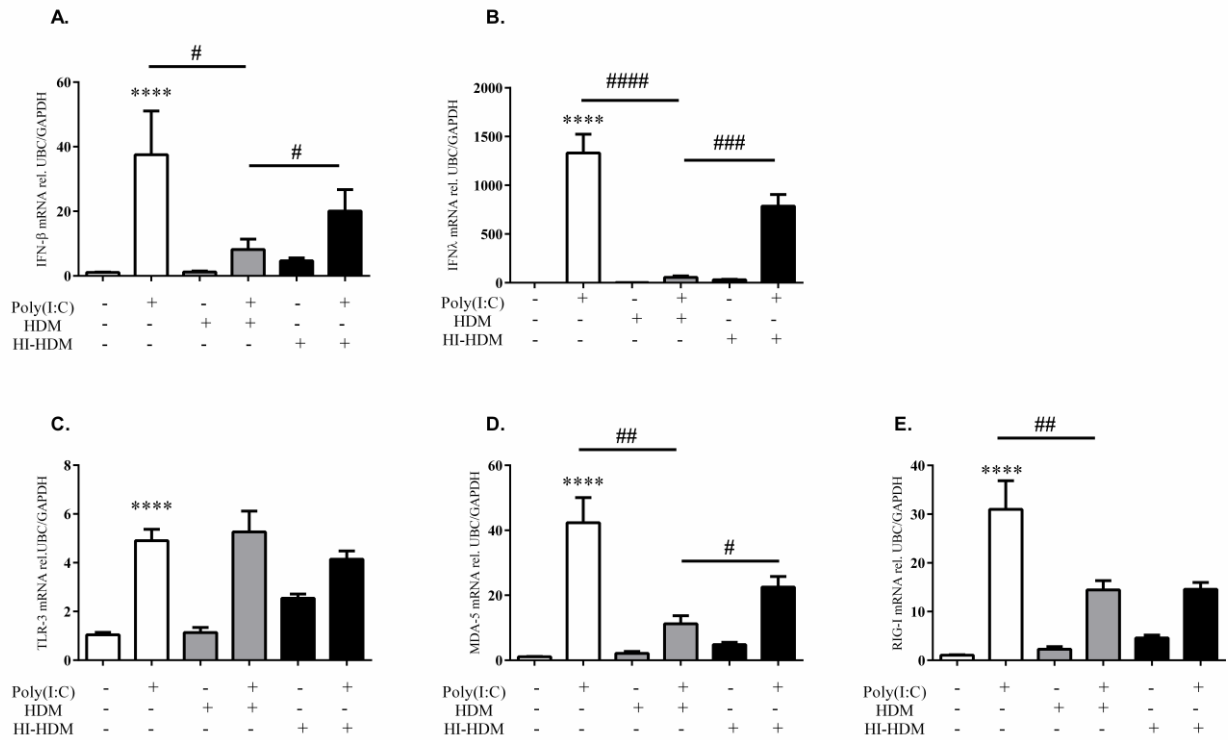


Figure-S4 Heat-inactivation of HDM restores IFN-β, -λ and MDA5 in response to Poly(I:C) in primary HBECs. The expression of IFN-β (A), IFN-λ (B), TLR3 (C), MDA5 (D), RIG-I (E) was determined by qPCR. Data is presented as mean ± SEM n= 4 * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$ compared to control and HDM group, # $P < 0.05$, ## $P < 0.01$, ### $P < 0.001$ #### $P < 0.0001$ compared to HDM-Poly(I:C) group.

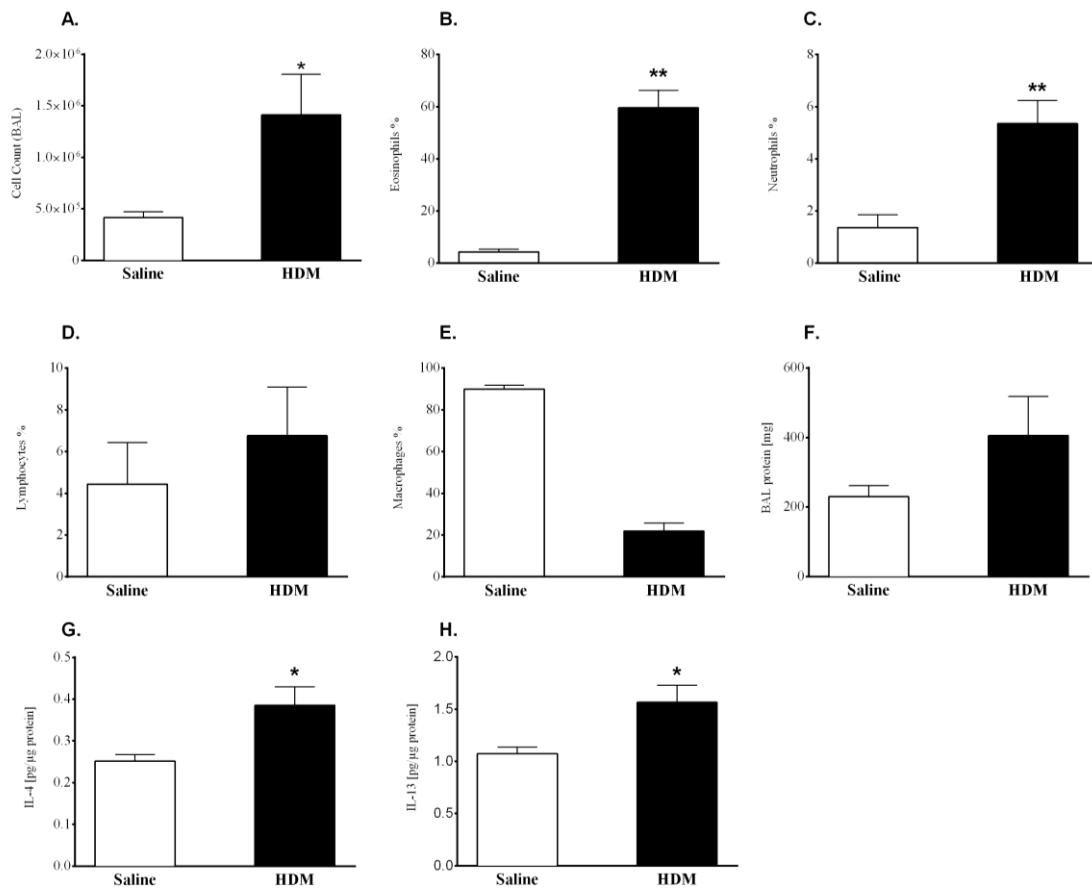
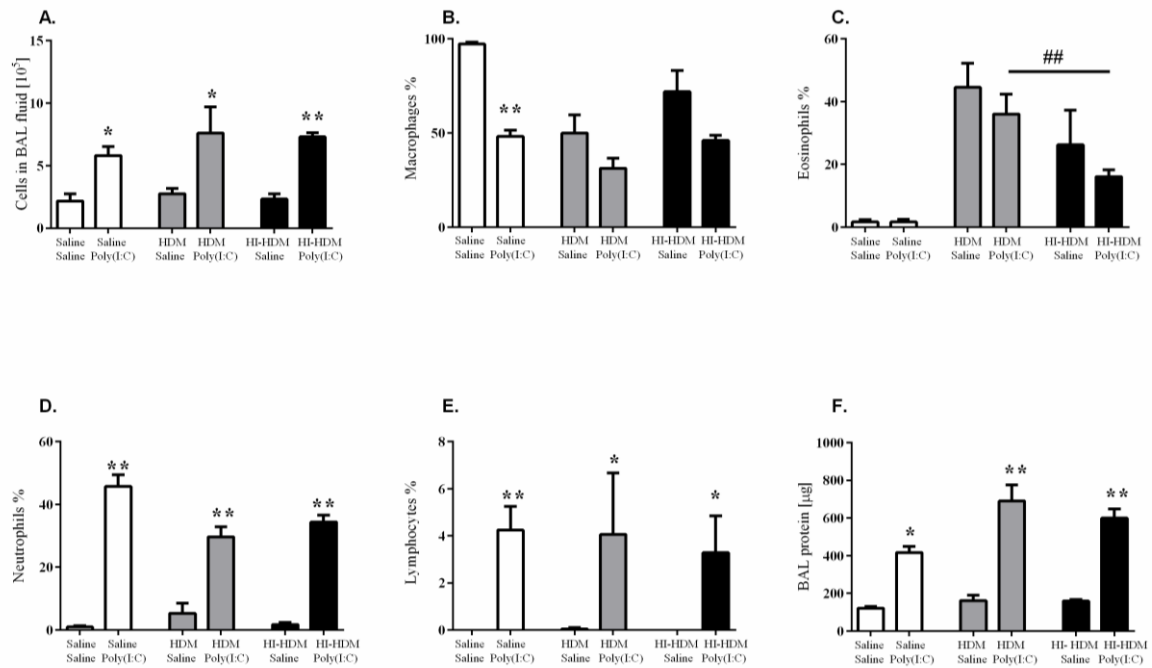


Figure-S5 Intranasal HDM induced eosinophilia and neutrophilia in the BAL. Mice were given intranasal HDM or saline 3 times per week for 3 weeks. BALF was collected and analysed for cell count (A), percentage of eosinophils (B), neutrophils (C), lymphocytes (D), macrophages (E) and total protein content (F). Lung homogenates were analysed for IL-4 (G) and IL-13 (H) by ELISA. Data is presented as mean $\pm$ SEM $n = 6-8$ * $P < 0.05$ ** $P < 0.01$



139

140 **Figure-S6 Heat inactivation of HDM reduced the percentage of eosinophils in the BAL**
 141 **in response to Poly(I:C).** Mice were given intranasal HDM, HI-HDM or saline 3 times per
 142 week for 3 weeks. They received saline or Poly(I:C) for 3 consecutive days the week after.
 143 BALF was collected 24 hours after and was analysed for cell count (A), percentage of
 144 macrophages (B), eosinophils (C), neutrophils (D), lymphocytes (E) and total protein content
 145 (F). Data is presented as mean $\pm$ SEM n = 4-7 * P < 0.05, ** P < 0.01, ### P < 0.01 compared
 146 to HDM-Poly(I:C) group.

147

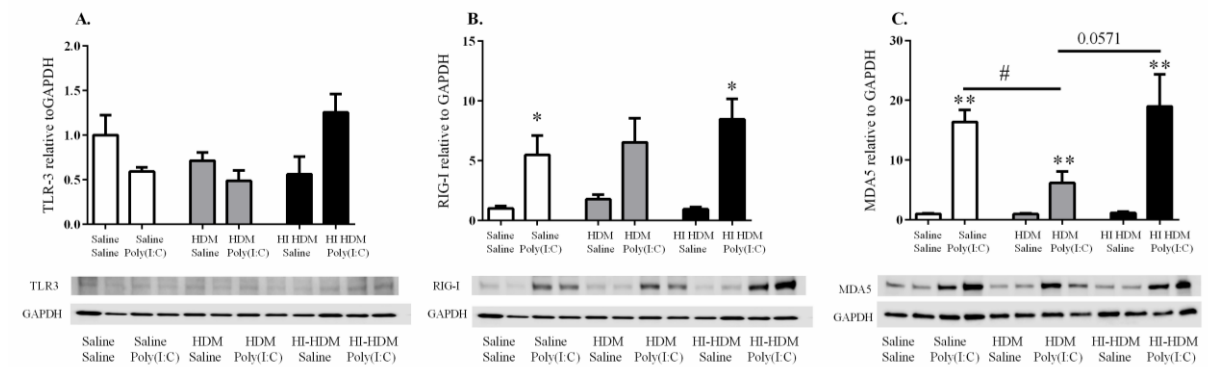


Figure-S7 Heat inactivation of HDM restored MDA5 protein expression in the lungs in response to Poly(I:C). Immunoblots of tissue lysates were probed with anti-TLR3 (A), -RIG-I (B) and -MDA5 (C). Representative immunoblots of tissue lysates are presented. Data is presented as mean ± SEM. $n = 4-6$. * $P < 0.05$, ** $P < 0.01$ compared to saline-saline, HI-HDM-saline or HDM-saline groups. # $P < 0.05$ compared to HDM-Poly(I:C).

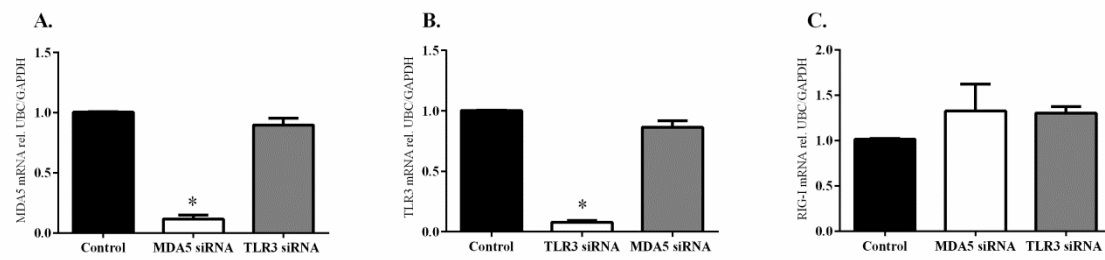


Figure-S8 MDA5 and TLR3 siRNAs are specific for their intended targets. Gene expression of MDA5 (A), TLR3 (B), and RIG-I (C) was determined by qPCR. * $P < 0.05$ compared to control (non-specific siRNA).