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Immune Sensing of Aeroallergen-Associated Double-Stranded RNA Triggers an IFN Response and Modulates Type 2 Lung Inflammation

Li She,^{*,†} Hamad H. Alanazi,^{*} Liping Yan,^{*} Yi Zou,[‡] Yilun Sun,^{*} Peter H. Dube,^{*} Edward G. Brooks,[§] Gema D. Barrera,^{*} Zhao Lai,[‡] Yidong Chen,[‡] Yong Liu,[†] Xin Zhang,[†] and Xiao-Dong Li^{*}

The innate immune sensing of allergens or allergen-associated components regulate the development of type 2 inflammatory responses. However, the underlying molecular basis by which allergens or allergen-associated components are detected by innate immune receptors remains elusive. In this study, we report that the most common aeroallergen, house dust mite (HDM), harbors a dsRNA species (HDM-dsRNA) that can activate TLR3-mediated IFN responses and counteract the development of an uncontrolled type 2 immune response. We demonstrate that the mouse strains defective in the dsRNA-sensing pathways show aggravated type 2 inflammation defined by severe eosinophilia, elevated level of type 2 cytokines, and mucus overproduction in a model of allergic lung inflammation. The inability to sense HDM-dsRNA resulted in significant increases in airway hyperreactivity. We further show that the administration of the purified HDM-dsRNA at a low dose is sufficient to induce an immune response to prevent the onset of a severe type 2 lung inflammation. Collectively, these results unveil a new role for the HDM-dsRNA/TLR3–signaling axis in the modulation of a type 2 lung inflammation in mice. *The Journal of Immunology*, 2019, 203: 000–000.

Although allergic diseases are known to be primarily mediated by a Th2-biased, allergen-specific immune response, accumulating evidence in recent years has suggested that innate immunity also plays a critical role in pathogenesis of allergic diseases (1, 2). Innate immunity, the first line of host defense, has evolved to sense the environment and activate signaling events that instruct the development of a proper adaptive immune response (3). The mammalian innate immune system mainly comprises four types of PRRs: TLRs, the nucleotide-binding oligomerization domain receptors (NOD-like receptors [NLRs]), the nucleic acid sensing receptors, including RNA sensors (retinoic acid-inducible gene I [RIG-I]–like receptors [RLRs]), including RIG-I, and melanoma differentiation-associated protein 5 [MDA5]), and DNA sensors like cyclic GMP-AMP synthase

(cGAS) and absent in melanoma 2 (AIM2) (4–7). TLRs mainly detect bacteria-derived PAMPs such as LPS, rRNA, unmethylated CpG DNA, etc. Except for TLR3 and TLR4, most TLRs activate the MyD88-mediated signaling cascades that primarily turn on the transcription factor NF- κ B. TLR3 recognizes dsRNA usually derived from viruses to activate the Trif-mediated pathway (8, 9). Upon the engagement of microbial ligand, innate immune receptors turn on signaling cascades to activate NF- κ B and IRF3, which then enter the nucleus and function together to elicit the production of type I and III IFNs (IFN- α/β and IFN- λ s) and a myriad of inflammatory cytokines, including TNF- α and IL-1 β (10–13).

Because the prevalence of allergic diseases has continued to rise globally in recent decades, it is imperative to further understand the

^{*}Department of Microbiology, Immunology and Molecular Genetics, University of Texas Health San Antonio, San Antonio, TX 78229; [†]Department of Otolaryngology, Head and Neck Surgery, Xiangya Hospital, Central South University, Changsha, Hunan 410008, China; [‡]Greehey Children's Cancer Research Institute, University of Texas Health San Antonio, San Antonio, TX 78229; and [§]Division of Immunology and Infectious Disease, Long School of Medicine, University of Texas Health San Antonio, San Antonio, TX 78229

ORCID: 0000-0002-5512-8349 (L.S.); 0000-0002-5248-1489 (H.H.A.); 0000-0001-5559-7842 (L.Y.); 0000-0001-6806-2185 (Y.C.); 0000-0002-9273-6212 (X.Z.); 0000-0002-4349-6996 (X.-D.L.).

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X.-D.L. planned and designed research, supervised the overall work, made the original findings, and wrote the manuscript; L.S., L.Y., Y.S., H.H.A., G.D.B., and X.-D.L.

performed most experiments; Y.Z., Z.L., and Y.C. did the RNA sequencing and bioinformatics analysis. L.S., L.Y., Y.S., P.H.D., Y.Z., Z.L., Y.C., and X.-D.L. analyzed data; P.H.D. performed the lung pathology assessment. All authors discussed the results and participated in writing and commenting on the manuscript.

The sequences presented in this article have been submitted to the National Center for Biotechnology Information Gene Expression Omnibus (<https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE102211>) under accession number GSE102211.

Address correspondence and reprint requests to Dr. Xiao-Dong Li, Department of Microbiology, Immunology and Molecular Genetics, University of Texas Health San Antonio, 7703 Floyd Curl Drive, MC 7758, San Antonio, TX 78229. E-mail address: lix8@uthscsa.edu

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Abbreviations used in this article: AHR, airway hyperreactivity; BALF, bronchoalveolar lavage fluid; BMDM, bone marrow–derived macrophage; HDM, house dust mite; HDM-dsRNA, HDM harboring dsRNA species; HRV, human rhinovirus virus; ISG, IFN-stimulated gene 15; PAS, periodic acid–Schiff; qPCR, quantitative PCR; RIG-I, retinoic acid-inducible gene I; RNA-Seq, RNA sequencing.

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mechanisms of pathogenesis in order to find better preventive and therapeutic strategies (14). It has been previously reported that allergen-associated components can engage their corresponding PRRs to activate the production of either pro- or anti-inflammatory cytokines, which direct the subsequent adaptive immune reactions. A high level of allergen-associated microbial endotoxin (LPS) triggers the TLR4-mediated signaling pathway, which culminates in the production of Th1-polarizing cytokines and, in turn, dampen the development of Th2 immune responses (15–17). Similarly, bacterial DNA, unmethylated CpG DNA in particular (18), in house and farm barn dust negatively regulate the development of allergic inflammation through the activation of a Th1 immune response (19). House dust mite (HDM), one of the most common indoor allergens worldwide, causes inflammatory diseases in atopic people, including allergic asthma, allergic rhinitis, and atopic dermatitis. HDM has recently emerged as a valuable model allergen for investigating the molecular mechanisms of the pathogenesis of allergic diseases (20–23). HDM includes two main species: *Dermatophagoides farinae* (American HDM) and *D. pteronyssinus* (European HDM) (24–26). Using clinical-grade HDM extracts that have been employed in many experimental settings (20–23), in this study, to our knowledge, we demonstrate for the first time that the HDM contains endogenous dsRNA species that can induce an IFN response and reduce the severity of HDM-dependent type 2 inflammatory responses.

Materials and Methods

Mice

MAVS^{-/-} (27), TLR3^{-/-} (8), and Trif^{cl-ps2} (28) mice have been described previously. Wild-type C57BL/6J mice were purchased from The Jackson Laboratory. The double knockouts TLR3^{-/-}MAVS^{-/-} mice were generated by intercrossing on campus. Mice were bred and maintained under specific pathogen-free conditions in the animal facility of University of Texas Health San Antonio according to the experimental protocols approved by the Institutional Animal Care and Use Committee.

Cells and reagents

Human cell lines A549 and HT29 were cultured in DMEM supplemented with 10% FCS plus antibiotics. BEAS-2B cell was cultured in LHC-9 medium supplemented with 10% FBS plus antibiotics. Bone marrow cells were collected from femurs and tibiae of mice. To obtain bone marrow–derived macrophages (BMDMs), ~10 million bone marrow cells were cultured in DMEM containing 10% FCS, antibiotics, and conditioned media from L929 cell culture. After 7 d, mature macrophages were harvested and cultured on six-well plates for experiments. Media was changed every other day. Most allergen source materials were purchased from Greer Laboratories, including *D. farinae* (no. B81, lyophilized extract was reconstituted in PBS with the protein concentration at 1.0 mg/ml and the endotoxin at 11,155 endotoxin units/ml, respectively), *D. pteronyssinus* (no. B82, lyophilized extract was reconstituted in PBS with the protein concentration at 1.0 mg/ml and the endotoxin at 11,280 endotoxin units/ml, respectively), flour mite (*Acarus siro*, no. B71), storage mite (*Lepidoglyphus destructor*, no. B72), cheese mite (*Tyrophagus putrescentiae*, no. B73), dog dander (no. E64), dog epithelia (no. E7), cockroach (no. B26), flea (no. B22), and mosquito (no. B55). For RNA isolation from allergens, we directly added the proper amount of TRIzol (Invitrogen) into the lyophilized extracts. Usually ~0.1–0.2 mg of RNA was obtained from 20 to 30 mg (protein) lyophilized HDM extract per bottle. Except for the frozen total bodies of *D. pteronyssinus* and *D. farinae* (Greer Laboratories), ant, wasp, spider, and earthworm were collected alive into 50-ml Falcon tubes and immediately frozen with liquid nitrogen for further RNA isolation.

ELISA to detect cytokines

For measuring cytokines after HDM restimulation, mediastinal lymph node cells (3×10^5 cells in a 96-well plate) were cultured with or without HDM (30 µg/ml). After 4 d, culture supernatants were assayed for IL-5 and IL-13 (Invitrogen). For measuring IFN-α in mouse lungs after HDM or HDM RNA stimulation, the harvested lungs were washed once with cold

PBS, transferred into 2 ml tubes, rapidly frozen into liquid N₂, and stored at -80°C. Later, to prepare lung homogenates, 1 ml tissue protein extraction reagent (catalog no. 78510; Thermo Fisher Scientific) containing protease inhibitors (catalog no. 11836153001; Roche) was added and homogenized by a BeadBeater (BioSpec). The lysates were transferred to a 1.5-ml tube and spun at $14,000 \times g$ for 30 min at 4°C. Supernatant was collected for the ELISA measurement of IFN-α. All final reactions were developed with tetramethylbenzidine substrate (Thermo Fisher Scientific) and stopped by sulfuric acid (0.16 M), and the OD at 450 nm was measured.

Detection of dsRNA with dot blot

Spotted RNAs as indicated from HDM or other organisms were cross-linked to Amersham Hybond-N+ Membrane (GE Healthcare) in a Stratalinker 2400 UV crosslinker. The membrane was washed with washing buffer (TBST) and blocked in blocking buffer (5% BSA in TBST) with gentle shaking. Incubate the membrane with J2 anti-dsRNA mAb (SCICONS) overnight at 4°C with gentle shaking. For detection, AP-conjugated anti-mouse was added at a dilution of 1:5000.

Real-time quantitative PCR

Reverse transcription and real-time quantitative PCR (qPCR) reactions were carried out using iScript cDNA Synthesis Kit and iQ SYBR Green Supermix (Bio-Rad Laboratories). qPCR was performed on a Bio-Rad Laboratories CFX384 Touch Real-Time PCR Detection System using the following primers (5'→3'): mouse primers Rpl19 (5'-AAATCGC-CAATGCCAACTC-3'; 5'-TCTTCCCTATGCCCATATGC-3'), IFN-β (5'-TCCGAGCAGAGATCTTCAGGAA-3'; 5'-TGCAACCACCACTCATTCTGAG-3'), IL-1β (5'-TCTATACCTGTCTGTGTAATG-3'; 5'-GCTTGTGCTCTGTGTG-3'), CXCL10 (5'-GCCGTCATTTCTGCCTCA-3'; 5'-CGTCCTTGCGAGAGGGATC-3'), IFIT3 (5'-TGGCCTACATAAGCACCTAGATGG-3'; 5'-CGCAAACCTTTGGCAAACCTTGTCT-3'), IFN-stimulated gene 15 (ISG15) (5'-GAGCTAGAGCCTGCAGCAAT-3'; 5'-TTCTGGGCAATCTGCTTCTT-3'), Mx1 (5'-TCTGAGGAGAGCCAGACGAT-3'; 5'-ACTCTGGTCCCCAATGACAG-3'), OASL2 (5'-GGA-TGCCTGGGAGAGAATCG-3'; 5'-TCGCCTGCTCTTCGAAACTG-3'), and TNF-α (5'-CCTCCCTCTCATCAGTTCTATGG-3'; 5'-GGCTACAG-GCTTGCTACTCG-3'). Human primers human GAPDH (5'-ATGACA-TCAAGAAGGTGGTG-3'; 5'-CATACCAGGAAATGAGCTTG-3'), human IFN-β (5'-AGGACAGGATGAACCTTTGAC-3'; 5'-TGATAGACATTAGC-CAGGAG-3'), and human IFN-λ1 (5'-CGCCTTGAAGAGTCACTCA-3'; 5'-GAAGCCTCAGGTCCCAATTC-3').

Transfection, allergen, and nucleic acid treatment

Transfection of RNA (0.5–1.0 µg/ml) or allergen extracts (2.5–5.0 µg/ml) as indicated into cultured epithelial cells (A549, BEAS-2B, and HT29) was carried out using Lipofectamine 2000 or 3000 (Invitrogen) or FuGENE 6 (Promega). Without transfection, BMDMs were treated with RNA at 25 µg/ml. For enzyme treatments of nucleic acids, allergen extracts or RNA were treated with various nucleases (Invitrogen), as indicated at 37°C for 1 h.

HDM administration and FACS analysis of bronchoalveolar lavage fluid and lung

Each mouse was administered via the intratracheal route with 100 µg dust mite extracts or PBS (Greer Laboratories) for three times on days 0, 7, and 14. Bronchoalveolar lavage fluids (BALFs) were collected on day 17. Briefly, after CO₂ euthanasia, the trachea was catheterized and flushed with 1 ml of ice-cold PBS-EDTA three times. Cell number in BALF was first evaluated using a hemocytometer. Differential cells in BALF were labeled with Abs as indicated, then mixed with counting beads (Spherotech) for further FACS analysis on a BD LSR II cell analyzer. Flow cytometry data were analyzed using FlowJo software. The Abs and reagents for FACS analysis are the following: SPHERO AccuCount Fluorescent (catalog no. ACFP-70-5; Spherotech), Anti-Mouse Siglec-F PE (clone E50-2440) (catalog no. 552126; BD Pharmingen), Anti-Mouse CD3e PE/Cy5 (clone 145-2C11) (catalog no. 15-0031-81; Invitrogen), Anti-Mouse CD19 PE/Cy5 (clone eBioD13) (catalog no. 15-0193-81; eBioscience), Anti-Mouse CD11c PE/Cy7 (clone N418) (catalog no. 1173170; BioLegend), Anti-Mouse CD11b V450 Rat (clone M1/70) (catalog no. 560456; BD Bioscience), Anti-Mouse Ly-6G FITC (clone RB6-8C5) (catalog no. 11-5931-82; Invitrogen), Anti-Mouse MHC Class II APC-eFluor 780 (clone M5/114.15.2) (catalog no. 47-5321-80; eBioscience), Anti-Mouse Fixable Viability Dye eFluor 506 (catalog no. 65-0866-14; Invitrogen), Anti-Mouse CD45 APC/Cy7 (clone: 30-F11) (catalog no. 103130; BioLegend), Anti-Mouse CD86

PE (clone PO3) (catalog no. 105106; BioLegend), and Anti-Mouse Isotype Control PE (clone RTK2071) (catalog no. 400408; BioLegend).

Lung inflammation and pathology

Each mouse was administered via the intratracheal route with 100 μ g dust mite extract treated with or without RNase III once a week for 3 wk. Two days after the last challenge, lung tissues were taken and fixed in 4% paraformaldehyde, paraffin embedded, cut into 4- μ m sections, and stained with H&E and periodic acid-Schiff (PAS). Complete images of control and treated lungs were obtained digitally using the Aperio ScanScope XT (Aperio, Vista, CA). Printed images of lungs from study groups were graded for disease severity using a panel of standards as previously described (29).

Measurement of pulmonary function

In the HDM-induced lung inflammation model, changes in mouse pulmonary function after allergen exposure were determined by invasive measurements using the flexiVent system (SCIREQ, Montreal, QC, Canada). On day 17, the trachea was intubated after anesthetization. The lungs were mechanically ventilated. Indicators of airway hyperreactivity (AHR), including airway resistance, elastance, and compliance, were measured after increasing dosages (6.25–50 mg/ml) of aerosolized methacholine.

Mouse lung RNA sequencing

Sequencing libraries were constructed following the manufacturer's protocol, and the libraries were sequenced by the Illumina HiSeq 3000 using single-read 50 bp sequencing protocol in duplicate. The reads were aligned to the mouse reference genome (build mm9; University of California Santa Cruz) by TopHat2. HTSeq-Count was used to count the gene expression reads, and R/DESeq was used to identify differentially expressed genes. Differentially expressed genes were identified with a filter of RPKM >1, Benjamini and Hochberg (30) adjusted $p \leq 0.05$, and absolute (log2-FoldChange) >2 or 4.

Statistical analysis

The statistical analysis was done using software GraphPad Prism 6. For comparison of two groups, p values were determined by unpaired two-tailed Student t test, unless otherwise indicated. A p value <0.05 was considered statistically significant. The p values are indicated on plots and in figure legends: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, and **** $p < 0.0001$.

Raw data availability

The accession number for the raw data files of RNA sequencing (RNA-Seq) reported in this paper is National Center for Biotechnology Information Gene Expression Omnibus: GSE102211 (<https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE102211>). The sequencing data analysis and bioinformatics methods are described in details in the related methods.

Results

HDM allergens contain immune stimulating dsRNA species

We postulated that environmental allergens might contain unidentified innate immune stimulatory factors capable of inducing an IFN response. To address this possibility, we examined various allergens in cultured cells. Epithelial cell lines were initially used because they are nonresponsive to LPS, which is a common contaminant of environmental allergens that may activate the TLR4/Trif-mediated pathway leading to the IFN production. Our initial efforts to detect IFN-inducing activities by directly adding allergens to cells were unsuccessful (data not shown). We then successfully delivered the allergens with transfection reagents. We first examined the immune stimulating activities of common allergen source materials from various organisms including dust mites, dog skin, and insects like cockroach, flea, and mosquito. Interestingly, among all tested allergens, only mites, including *D. pteronyssinus* and *D. farinae* in particular, induced significantly higher levels of a type III IFN IFN- λ 1 transcript (Fig. 1A). To further define the chemical nature of the immunogenic elements, we treated HDM extracts (*D. pteronyssinus* or *D. farinae*) with proteinase K or heat at 95°C for 2 h (Fig. 2A). Both treatments did not significantly reduce the IFN-inducing activity of HDM,

suggesting that the protein constituents may not be a contributing factor to the activity of HDM in these assays. To explore the potential role of nucleic acids RNA/DNA as the active factors, HDM was treated with various RNases or DNases. The removal of DNA by DNase I had little to no effect on the activity, whereas the treatment with either RNases (RNase If and RNase III) or Benzonase (digests both RNA and DNA) completely abolished the IFN-stimulatory property of HDM, suggesting that RNA is likely to be one of the active factors in HDM in this assay (Fig. 1B). To determine the structural features of HDM RNA, we treated the HDM RNA samples with either single- or double-strand-specific RNases, RNase T1 and III, respectively. RNase III, but not RNase T1, abolished the IFN-stimulating activity of HDM RNA, indicating the presence of dsRNA species (Fig. 1C). These data strongly suggest that the HDM-associated RNA, but not DNA or protein, has the ability to trigger the IFN production in the cultured epithelial cells.

We went on to purify total RNA from multiple organisms and examine their direct IFN-stimulating activities. Remarkably, the purified RNAs from both *D. pteronyssinus* and *D. farinae*, but not from other insects, were able to stimulate production of both IFNs (β and λ 1) transcript in BEAS-2B cells (Fig. 1D). HDM purchased from a non-United States vendor also activated the production of type I and III IFNs (Fig. 2B). In addition to *D. pteronyssinus* and *D. farinae*, RNA isolated from two other mite species *B. tropicalis* and *G. domesticus*, crude mattress dust, and various indoor dusts can also trigger the production of type I or III IFNs, albeit with less potency (Fig. 2C, 2D). More interestingly, the detection of long dsRNA structures in HDM was achieved in dot blot using a dsRNA-specific mAb J2, which was abrogated by the RNase III treatment (Fig. 1E, upper and lower panels). Further, when treated with the same amount of RNA, HDM RNA is more potent than other tested insects' RNAs to stimulate the expression of the costimulatory molecule CD86 in wild-type BMDMs (Fig. 1F). To determine the immune-sensing pathway that is responsible for recognizing HDM RNA, we turned to using primary BMDMs derived from wild-type, TLR3^{-/-}, MAVS^{-/-}, and TLR3^{-/-}MAVS^{-/-} mice. In contrast to epithelial cell lines, BMDMs are professional phagocytes being capable of taking up foreign materials like nucleic acids without help of transfection. We found that the expression of CD86 and IFN- β were dependent more on TLR3 than MAVS upon HDM RNA treatment (Fig. 1G, 1H). In summary, these results suggest that dsRNA, but not DNA or protein, is likely to be one of the major IFN-stimulating factors in HDM and indoor dusts when examined at the cellular level.

HDM harboring dsRNA species activates RNA-sensing pathways to trigger an IFN response in vivo

Because the type I IFN response is known to be a hallmark of dsRNA/TLR3 signaling, we wanted to determine whether HDM harboring dsRNA species (HDM-dsRNA) is able to induce an IFN response in vivo. We analyzed the production of IFN- α protein in mouse lungs by ELISA. *D. farinae* extracts (treated with or without RNase III) and purified *D. farinae* RNA were delivered into either wild-type or TLR3^{-/-}MAVS^{-/-} mice. As expected, HDM-dsRNA was observed to be necessary and sufficient to strongly induce the production of IFN- α protein in mouse lungs 24 h posttreatment (Fig. 3A, 3B). Next, we evaluated lung gene expression profiles using RNA-Seq. In the scatter-plot analysis, compared with the PBS-treated mice, HDM strongly induced up to 983 genes, whereas the RNase III treatment led to a substantial reduction in the induced genes to 44 genes (Fig. 3C). To identify the biologic relationships within each cluster of upregulated genes, we

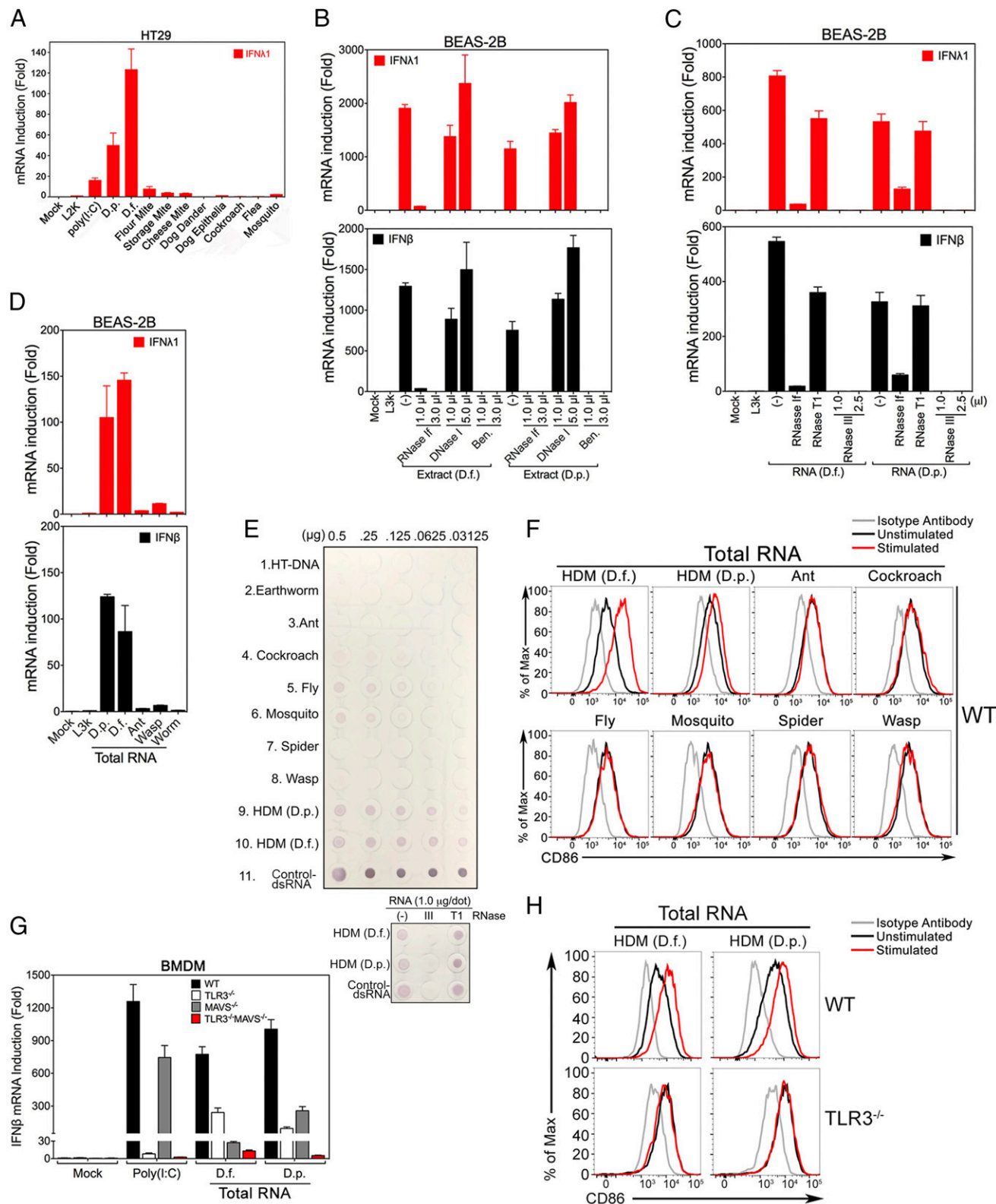


FIGURE 1. HDM allergens contain immunostimulatory dsRNA species. HT29 or BEAS-2B cells (**A–D**) were treated with extract (2.5 μ g/ml per 1×10^6 cells) or RNA (0.5 μ g/ml per 1×10^6 cells) from various allergens in the presence of the transfection reagent Lipofectamine 2000 or 3000 (L2K or L3K) for 16–18 h. (**A**) Induction of IFN- α 1 by extracts of *D. pteronyssinus* and *D. farinae*, but not by other allergens, including dog dander or epithelia, cockroach, flea, or mosquito. poly(I:C) was used as a positive control. (**B**) RNase If and Benzonase (Ben.), but not DNase I, completely destroyed the immune stimulating activity of HDM (*D. pteronyssinus*). (**C**) Total RNA isolated from HDM (*D. pteronyssinus*) contained double-stranded structural elements that are essential for its activity. RNA was left untreated (–) or treated with various RNases (RNase If, RNase T1, and RNase III). (**D**) Induction of IFNs (β or α 1) by the purified RNA isolated from either HDM (*D. pteronyssinus* or *D. farinae*), but not from ant, wasp, or earthworm. (**E**) Detection of the double-stranded structure in HDM RNAs by a dsRNA-specific Ab J2 with dot blot. (**F**) Upregulation of the costimulatory molecule CD86 by HDM RNA in BMDMs. The surface expression of CD86 was assessed by FACS analysis. Each RNA isolated from HDM (*D. pteronyssinus* or *D. farinae*), ant, cockroach, fly, mosquito, spider, and wasp was directly added into culture media (the final concentration at 25 μ g/ml) without transfection reagents and incubated for 16–18 h before analysis. (**G**) (Figure legend continues)

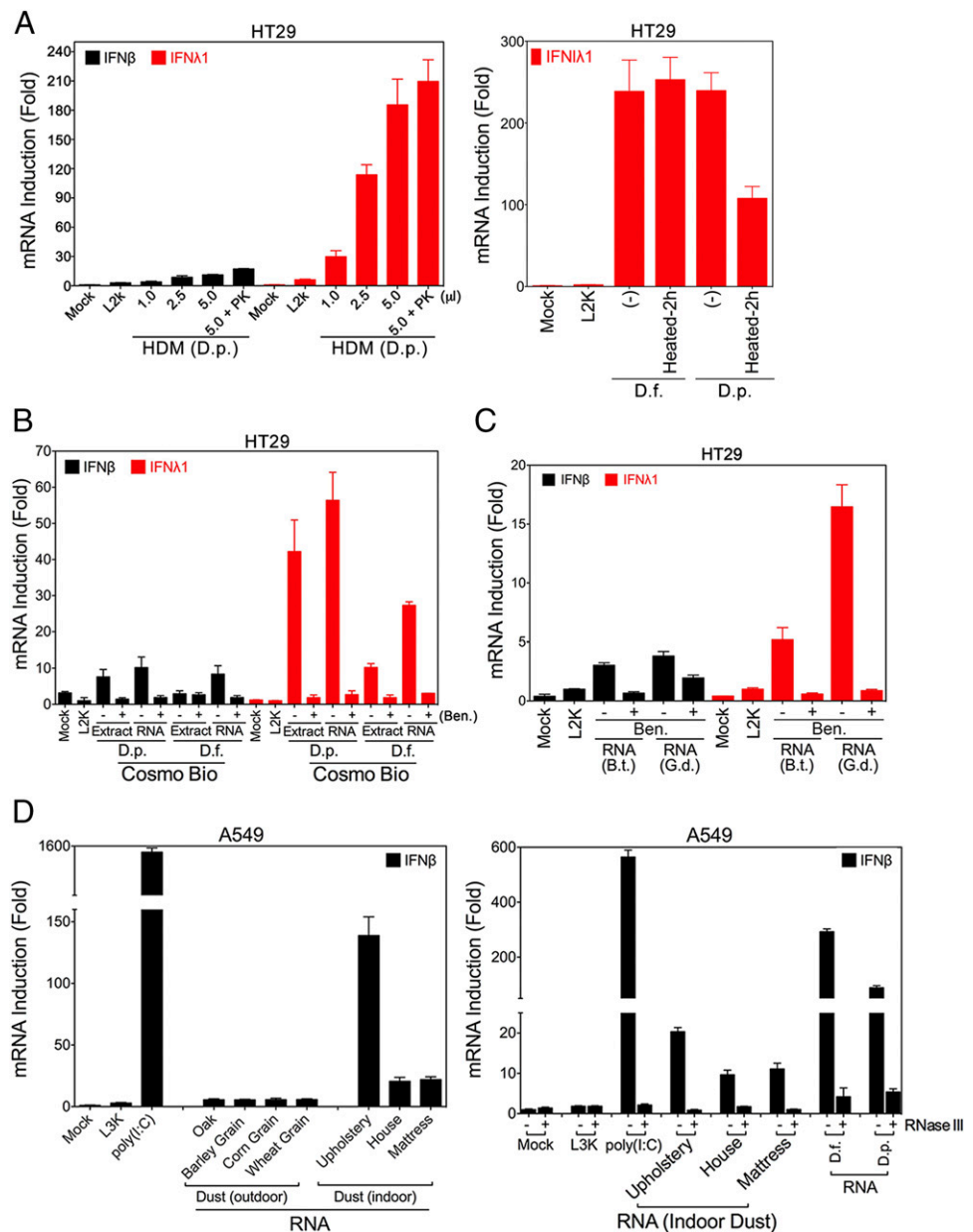


FIGURE 2. The immunostimulatory activities of HDM and indoor dusts depend on RNA, but not proteins. **(A)** Proteinase K (PK) digestion or heat at 95°C for 2 h did not affect the ability of *D. pteronyssinus* or *D. farinae* extract to activate the production of IFN-β or IFN-λ1 in HT29 cells. **(B)** Induction of IFN-β and IFN-λ1 by the extract or RNA of *D. pteronyssinus* and *D. farinae* purchased from Cosmo Bio. **(C)** RNAs from two other dust mites (*B. tropicalis* and *G. domesticus*) were also immunostimulatory when transfected into HT29 cells. **(D)** RNAs isolated from various sources of indoor dusts, but not outdoor dusts, were potent immune stimulators when transfected into A549 cells. B.t., *B. tropicalis*; D.f., *D. farinae*; D.p., *D. pteronyssinus*; G.d., *G. domesticus*.

used the Ingenuity Pathway Analysis (QIAGEN) to define functional connections. We found that genes of the HDM-treated group were significantly enriched in 10 biological processes, most related to innate immunity (Fig. 3D). Notably, ISGs (the fold-change > 4) are comprised mostly of the top 50 genes shown in the heat map analysis (Fig. 3E). We went on to examine the relative contribution of TLR3 and MAVS using various genetically deficient strains of mice exposed to HDM. In comparison, whereas 1189 genes and 1460 genes were significantly upregulated in wild-type and MAVS^{-/-} mice, the number of upregulated genes was reduced to 341 and 141 in TLR3^{-/-} and TLR3^{-/-}MAVS^{-/-} mice, respectively (Fig. 3F). Further, the top 50 induced genes from the heat map analysis, comprised of the same gene list as Fig. 3E, was abolished in TLR3^{-/-}MAVS^{-/-} mice (Fig. 3G). We verified these results using real-time-qPCR to analyze ISGs expression (Mx1,

ISG15, OASL2, and IFIT3) and chemokine and cytokine expression (CXCL10, TNF-α, IL-1β, and IFN-β) (Supplemental Fig. 1A, 1B). These results indicate that the TLR3- and MAVS-mediated pathways work together in vivo to recognize HDM-associated immunogenic RNAs and trigger the expression of ISGs in mouse lungs after inhalation of HDM.

RNA-sensing deficiency aggravates type 2 lung inflammation

As shown above, although HDM-dsRNA seems to be required for HDM allergens to induce an IFN response in vivo, it remains unclear whether HDM-dsRNA can modulate the allergen-driven type 2 inflammation. To address this issue, we have employed a widely used mouse model of lung inflammation as illustrated in Fig. 4A (20, 21, 23, 26). Upon completion of the experimental protocol, mouse lungs were processed for the H&E and PAS

Without transfection, the TLR3- and MAVS-mediated pathways are both required to induce the expression of IFN-β mRNA in BMDMs stimulated by either *D. farinae* or *D. pteronyssinus* RNA. **(H)** TLR3-dependent induction of the costimulatory molecule CD86 by HDM RNA in BMDMs. Representative data from one experiment are shown here. Similar results were obtained from at least three experiments. D.f., *D. farinae*; D.p., *D. pteronyssinus*.

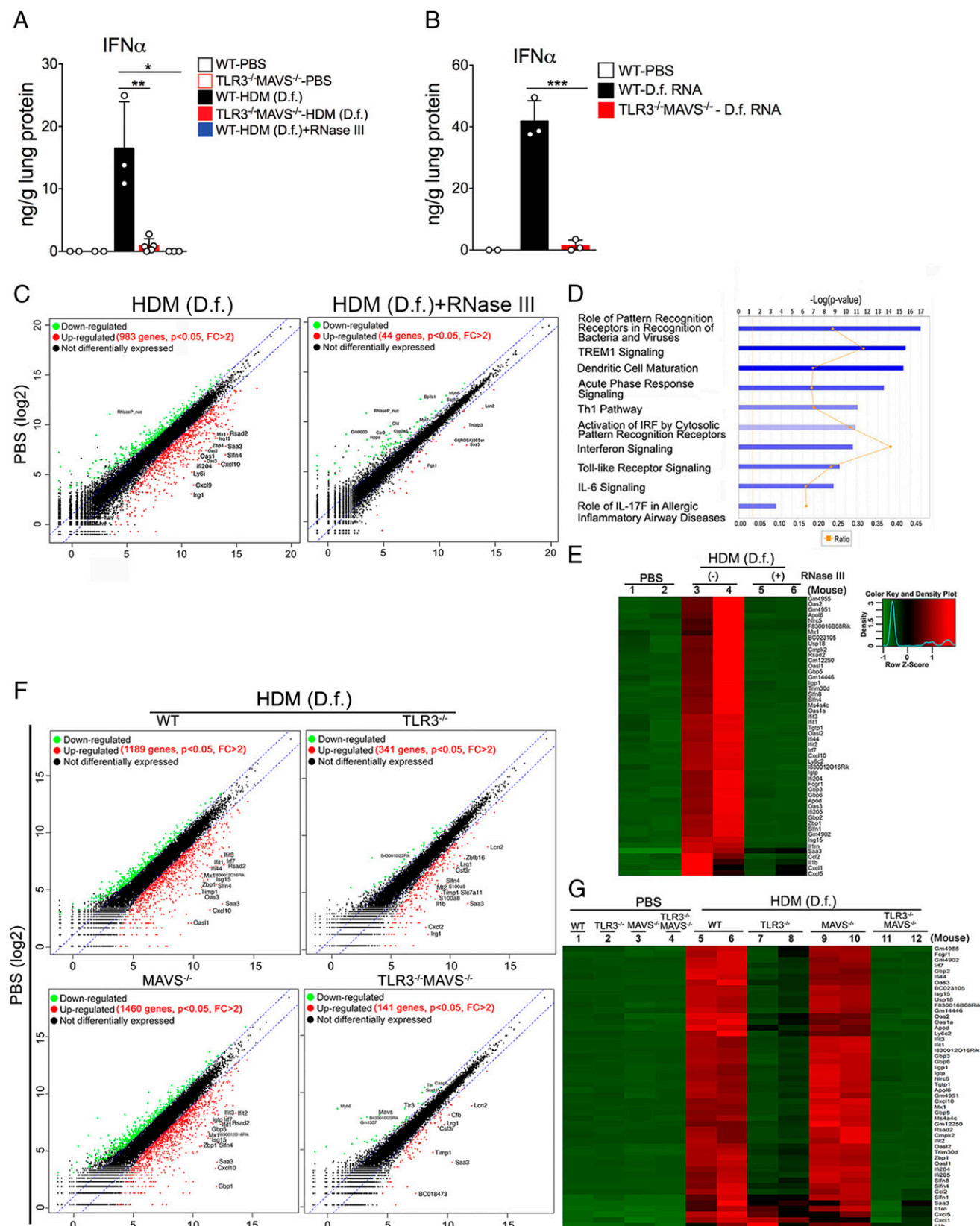


FIGURE 3. The HDM-dsRNA is required for HDM allergens to induce an IFN response in mouse lungs. **(A)** The induction of IFN- α protein after administration of 100 μ l of PBS or HDM (*D. fariniae*) (100 μ g/mouse) treated with or without RNase III as indicated for 24 h. The mouse lungs from wild-type or TLR3^{-/-}MAVS^{-/-} mice were processed for the ELISA measurement. **(B)** Similarly, instead of *D. fariniae* extracts, the purified *D. fariniae* RNA at 10 μ g/mouse was administered into mice as indicated for 24 h. The mouse lungs were processed for the IFN- α ELISA measurement. For both (A) and (B) a *p* value < 0.05 was considered statistically significant, unpaired *t* test. **p* < 0.05, ***p* < 0.01, ****p* < 0.001. **(C)** Each animal (wild-type, TLR3^{-/-}MAVS^{-/-}, TLR3^{-/-}, or MAVS^{-/-}) was exposed via the intratracheal route to 100 μ l of PBS or HDM (*D. fariniae*) (100 μ g/mouse) treated with or without RNase III as indicated. After 16–18 h, total RNA isolated from mouse lungs was subjected to RNA-Seq analysis. Differential gene expression analysis was performed by DESeq, and significant genes with a 4-fold change or above were chosen for analysis. The scatter-plot graph from the RNA-Seq analysis shows that the induction of global gene expression in mouse lungs by HDM exposure is largely dependent on the presence of HDM-associated immunogenic RNA. Wild-type mice were exposed to HDM (*D. fariniae*) that was (Figure legend continues)

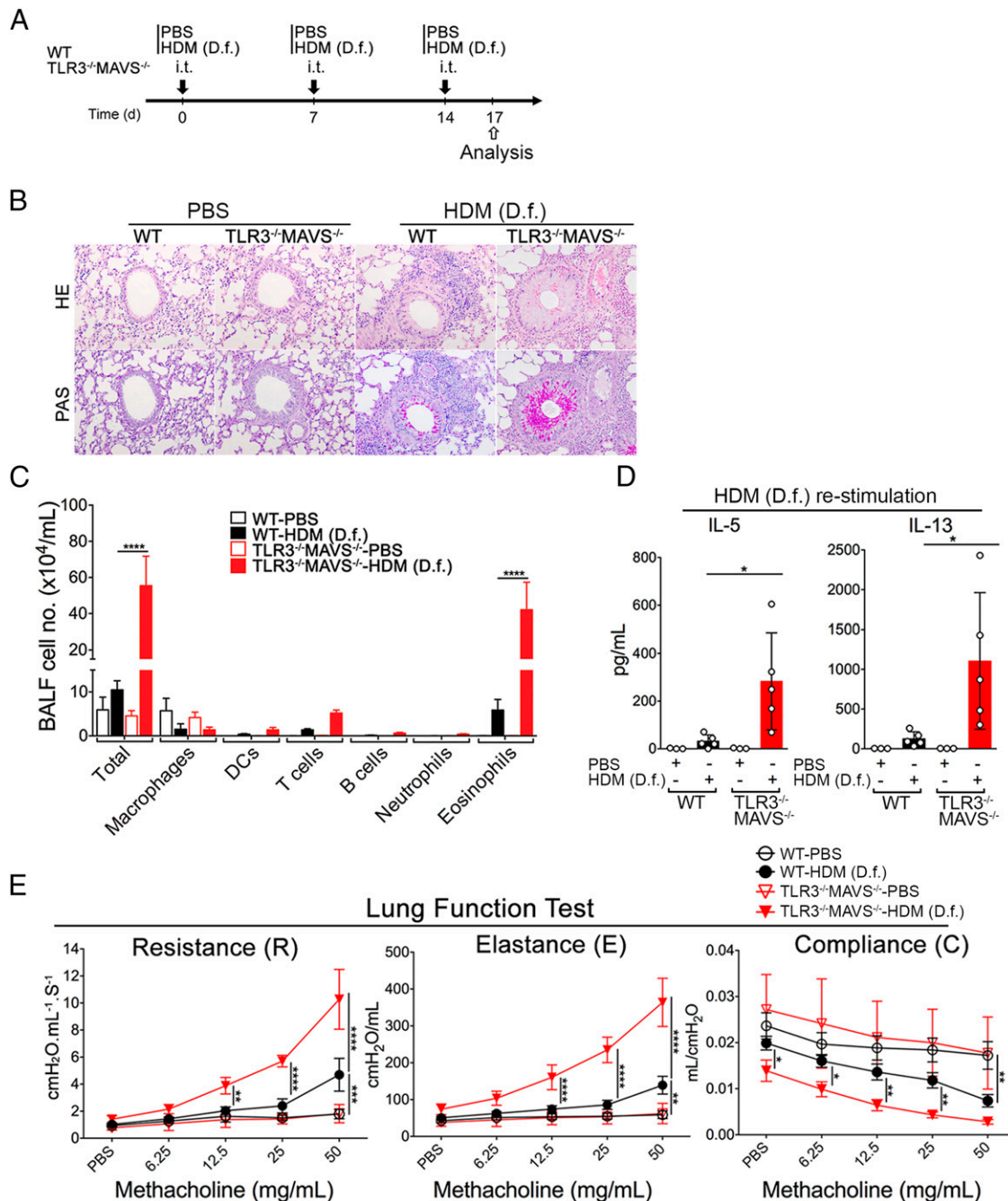


FIGURE 4. TLR3^{-/-}MAVS^{-/-} mice shows an exacerbated type 2 inflammation. **(A)** Experimental setup illustrating the animal groups, the corresponding treatment regimen, and timeline. **(B)** Representative lung pathologies. Four groups of mice (wild type and TLR3^{-/-}MAVS^{-/-}) were administered via the intratracheal route with PBS or HDM extracts once a week for 3 wk. Lung pathology was assessed with either H&E or PAS staining. Original magnification $\times 100$. **(C)** Groups of mice as indicated were treated with either PBS or HDM (*D. farinae*). BALF was collected and analyzed for total and differential immune cell types (a related FACS gating strategy is shown in Supplemental Fig. 3). The result is a representative of all three independent experiments ($n = 3$ –5). A p value < 0.05 was considered statistically significant, two way ANOVA, Sidak multiple comparisons test. **(D)** The production of IL-5 and IL-13 cytokines by cells of the mediastinal lymph node were measured by ELISA after Ag re-stimulation for 4 d. **(E)** AHR was examined by flexiVent (SCIREQ). Airway resistance (R) was measured after exposure to increasing doses (6.25–50 mg/mL) of aerosolized methacholine. The result is a representative of two independent experiments ($n = 5$ –10). A p value < 0.05 was considered statistically significant, two way ANOVA, Tukey multiple comparisons test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$. D.f., *D. farinae*.

staining. The representative histopathological images show that PBS-treated mice had little changes, whereas the groups treated with HDM developed the typical lung inflammatory cell infiltration

and mucus hyperplasia associated with allergic lung inflammation. Notably, TLR3^{-/-}MAVS^{-/-} mice appeared to have a much worse pathology illustrated by the bronchial epithelial hyperplasia and

treated with (+) or without (–) RNase III. **(D)** Top 10 biological processes were identified in the HDM (*D. farinae*)–treated group by the Ingenuity Pathway Analysis (IPA). **(E)** Heat map of the differentially expressed top 50 genes with an ISG signature. **(F)** The scatter-plot graph from the RNA-Seq analysis showed that the induction of global gene expression in mouse lungs by HDM exposure is largely dependent on the TLR3 and to a lesser extent MAVS. **(G)** Heat map of the differentially expressed top 50 genes. The induction of ISGs was abolished in TLR3^{-/-}MAVS^{-/-} mice. D.f., *D. farinae*.

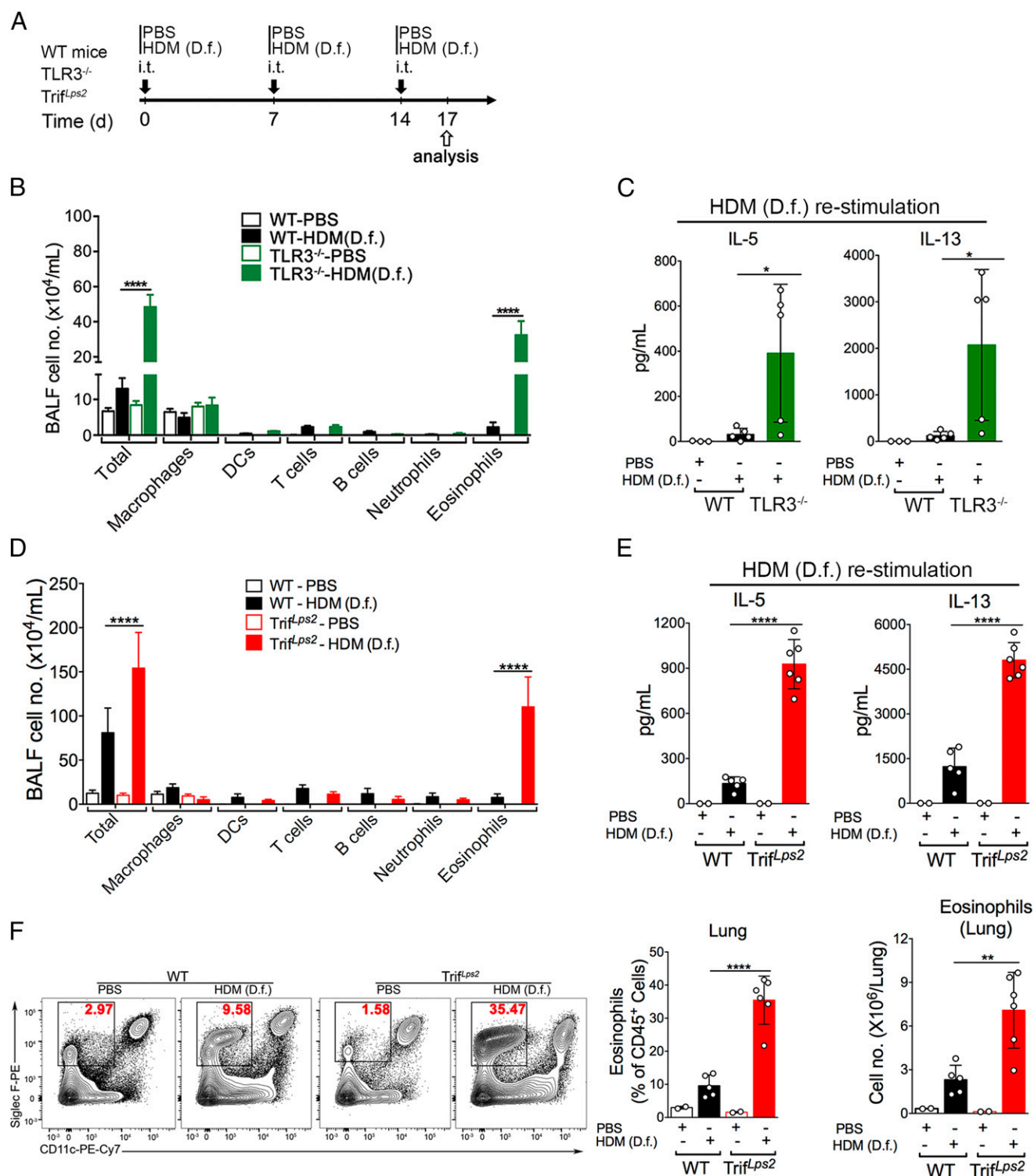
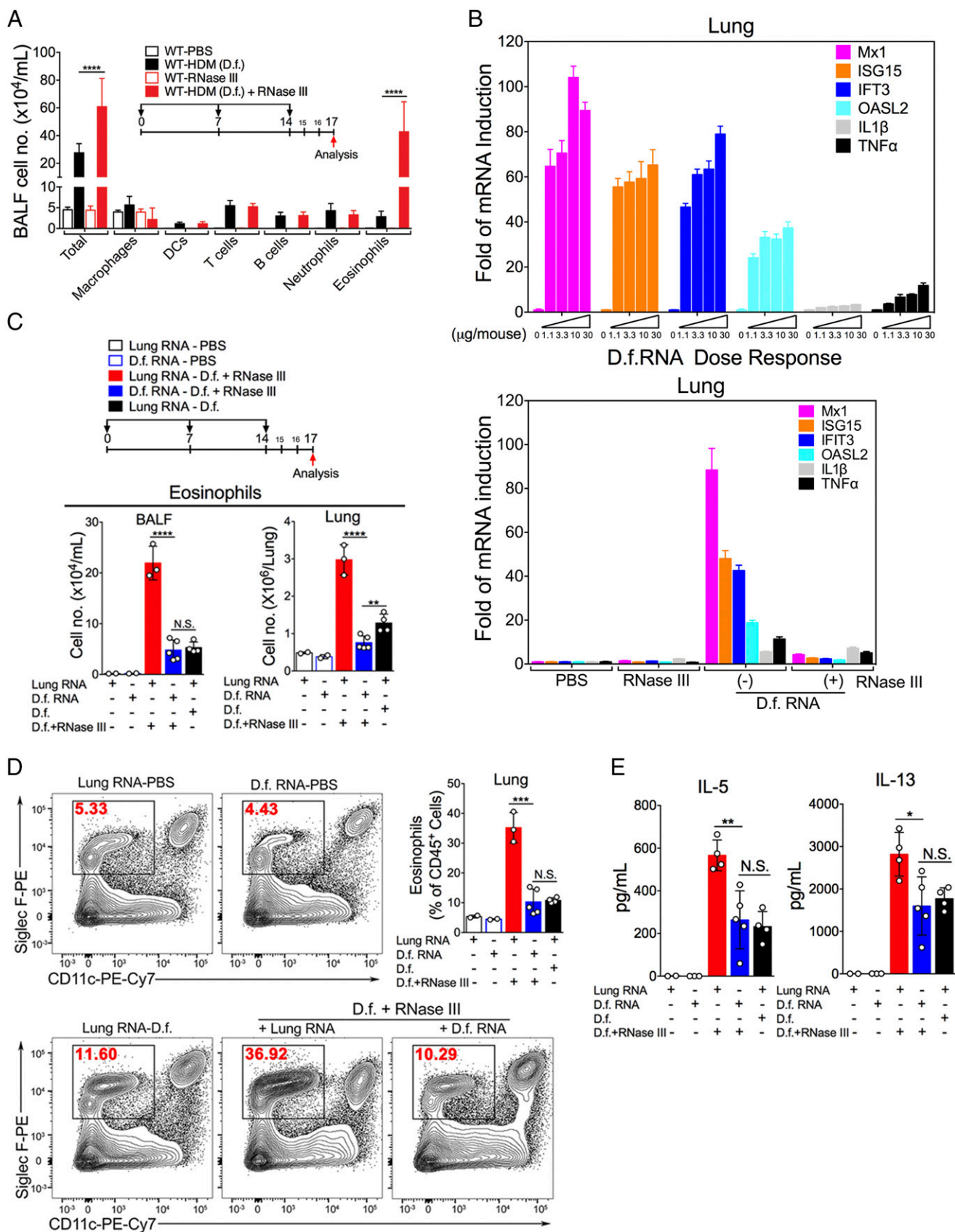


FIGURE 5. HDM allergen-induced type 2 lung inflammation in TLR3- and Trif- deficient mice. **(A)** Experimental setup illustrating the animal groups, the corresponding treatment regimen, and timeline. **(B)** Airway eosinophilic infiltration in TLR3^{-/-} mice. Four groups of mice (wild type and TLR3^{-/-}) were treated with either PBS or HDM (*D. farinae*). BALF was collected and analyzed for total and differential immune cell counts as described in the *Materials and Methods* ($n = 3-5$). A p value < 0.05 was considered statistically significant, two-way ANOVA, Sidak multiple comparisons test. **(C)** The elevated levels of type 2 cytokines IL-5 and IL-13 in TLR3^{-/-} mice. Cells of the mediastinal lymph node were collected from in wild-type and TLR3^{-/-} mice after HDM (*D. farinae*) exposure. A p value < 0.05 was considered statistically significant, unpaired t test. **(D)** Airway eosinophilic infiltration in wild-type and Trif^{Lps2} mice. Four groups of mice (wild type and Trif^{Lps2}) were treated with either PBS or HDM (*D. farinae*). BALF was collected and analyzed for total and differential immune cell counts as described in the *Materials and Methods* ($n = 3-5$). A p value < 0.05 was considered statistically significant, two-way ANOVA, Sidak multiple comparisons test. **(E)** The production of type 2 cytokines measured by ELISA. Cells of the mediastinal lymph node were collected from in wild-type and Trif^{Lps2} mice after HDM (*D. farinae*) exposure. **(F)** The percentage and number of eosinophils in mouse lungs. A p value < 0.05 was considered statistically significant, unpaired t test. * $p < 0.05$, ** $p < 0.01$, **** $p < 0.0001$. D.f., *D. farinae*.



mucus overproduction (Fig. 4B). Consistently, strikingly, we found that the number of eosinophils in the BALF of TLR3^{-/-}MAVS^{-/-} mice were significantly higher than the wild-type control group after HDM (*D. farinae*) extract challenge (Fig. 4C, the FACS gating strategy shown in Supplemental Fig. 2). After Ag restimulation, cells from lung draining lymph nodes (mediastinal lymph node) of these two mutant strains secreted more type 2 cytokines, including IL-5 and IL-13 (Fig. 4D). Functionally, TLR3^{-/-}MAVS^{-/-} mice exhibited a higher AHR, elastance, and decreased compliance in response to methacholine (Fig. 4E). Consistently, TLR3^{-/-} or Trif^{Lps2} (28) single mutant mice also had more eosinophils in both BALFs and lungs, along with higher levels of type 2 cytokines (IL-5 and IL-13) (Fig. 5A–F). Taken together, these results suggest that HDM-dsRNA may activate a distinct immune response via the TLR3/Trif-signaling pathway, which significantly tempers the development of allergen-induced type 2 lung inflammation and AHR in response to HDM exposure.

Administration of a low-dose HDM-dsRNA prevents the development of a severe type 2 lung inflammation

We hypothesized that eliminating dsRNA from HDM allergen would aggravate lung inflammation, phenocopying the inflammatory responses observed in mice deficient in RNA sensing. To test this hypothesis, we treated the HDM extract with RNase III, a dsRNA-specific nuclease. We found that mice treated with HDM (*D. farinae*) plus RNase III had more eosinophils in the airway compared with mice treated with HDM (*D. farinae*) extract alone (Fig. 6A). Next, we wanted to test whether pretreatment of mice with purified HDM RNA would inhibit the development of an exaggerated lung inflammation. First, we determined the dose response of HDM RNA in mouse lungs by measuring the expression of a few ISGs and two proinflammatory cytokines (IL-1 β and TNF- α) (Fig. 6B). Because a higher dose of HDM RNA might result in toxic effects and proinflammatory responses, we chose a lower dose of HDM RNA at 5 μ g/mouse for subsequent in vivo experiments. As expected, the administration of *D. farinae* RNA, but not the equal amount of control lung RNA (total RNA from mouse lungs), greatly reduced the number and percentage of eosinophils in both BALFs and lungs induced by exposure to dsRNA-depleted HDM extracts (*D. farinae* plus RNase III). Notably, the number of eosinophils in the *D. farinae* RNA-treated group is comparable to the group treated with the original *D. farinae* extract that endogenously contains the dsRNA species (Fig. 6C, 6D). Consistently, the production of IL-5 and IL-13 by cells of lung draining lymph nodes was significantly decreased in the *D. farinae* RNA-treated animals following Ag restimulation (Fig. 6E). These data indicate that the purified HDM RNA at a low dose is able to activate an immune response that attenuates the development of a severe type 2 lung inflammation. Collectively, our data indicate that HDM-dsRNA negatively regulates allergen-induced type 2 immune responses. Based on these findings, we propose that HDM-dsRNA is detected primarily by the TLR3-mediated pathway to induce an IFN response in mouse lungs, which may lead to a more balanced immune response through

counteracting the development of exaggerated type 2 inflammatory responses.

Discussion

In this study, we provide the biochemical, immunological, and genetic evidence that the common indoor allergen HDM contains endogenous dsRNA that specifically activates the TLR3-mediated IFN responses in mouse lungs. The HDM-dsRNA/TLR3-mediated signaling axis seems to be important in controlling the severity of HDM-induced type 2 lung inflammation. This is highlighted by the increases in eosinophils and type 2 cytokines in mice treated with HDM lacking dsRNA or in genetically modified mice unable to sense dsRNA because of deficiencies in TLR3 and/or MAVS. Additionally, the presence of HDM-dsRNA modulates physiological responses to HDM exposure by reducing mucus metaplasia and hyperplasia as well as AHR. Pretreating mice with HDM-derived dsRNA is sufficient to reduce the exaggerated inflammation induced by dsRNA-depleted HDM, suggesting a new role for nucleic acids in controlling the severity of allergic inflammation.

In the past decade, it has become increasingly appreciated that innate immunity may play a key role in modulating a Th2-biased immune responses in allergic diseases (16, 31, 32). Natural allergens are almost always contaminated with microbial products; however, there are no published results describing the association of an immunogenic RNA with any indoor allergens. Notably, it has been recently reported that HDM can induce an IFN response, which resembles that induced by viral infections or poly(I:C) treatment (21, 33–35), but the underlying molecular mechanisms remain unknown. LPS and several factors such as Der p2, ATP and uric acid have been suggested to serve as ligands that can directly engage the innate immune signaling pathways and cooperate with Ags to regulate an allergic immune response (15, 36–38). Der p2, ATP, and uric acid have not been previously associated with an IFN response. With regards to LPS, it can activate the TLR4/Trif/IRF3 pathway culminating in the production of type I and III IFNs (4). However, when we transfected the HDM extracts into TLR2, TLR4, and TLR13 triple-knockout BMDMs or delivered HDM extracts into mouse lungs, we observed a normal production of IFN- β and ISGs (data not shown), indicating that LPS is unlikely to be responsible for HDM-induced IFN responses.

Early childhood viral bronchiolitis caused by human respiratory syncytial virus is known to be associated with an increased risk of allergic asthma later in life (39). Likewise, infection with human rhinovirus virus (HRV) is the most common cause of acute asthma exacerbation. In humans, loss-of-function mutations have been identified in viral RNA sensors such as TLR3 (40) and RIG-I (41). In experimental settings using the synthetic dsRNA analogue poly(I:C) in conjunction with a model Ag OVA, TLR3 signaling has been reported to play an important role in the regulation of lung inflammation (42–44). TLR3 is known to recognize a dsRNA ligand with a minimum length of 30 bp (45, 46). Now our data show that HDM extract is able to elicit a robust IFN response in mouse lungs. Because it is known that the type I IFNs have an important function in promoting a Th1-polarized immune response, it will be important in the future to conduct more mechanistic studies on a regulatory role of the HDM-dsRNA/TLR3

the RNase III treatment) was included in the experiment (black bar). The experimental setup is shown on the top. (D) The percentage of eosinophils in CD45⁺ immune cells of mouse lungs was also decreased after pretreatment of *D. farinae* RNA. (E) The production of type 2 cytokines, IL-5 and IL-13, by cells of the mediastinal lymph node were measured by ELISA. A *p* value <0.05 was considered statistically significant, unpaired *t* test. **p* < 0.05, ***p* < 0.01, ****p* < 0.001, *****p* < 0.0001. D.f., *D. farinae*.

signaling in the context of allergic models induced by HDM or other environmental allergens.

In contrast to a protective role for HDM-associated dsRNA proposed in the current report, it has been previously reported that activation of the dsRNA/TLR3/IRF3 pathway by the synthetic dsRNA poly(I:C) and HRV infection might promote an exacerbated inflammation in experimental models of allergic asthma (44, 47–53). Poly(I:C) treatment at high doses and HRV infection could trigger the production of inflammatory cytokines and chemokines in cultured epithelial cells such as BEAS-2B or A549. We speculate the discrepancy could be ascribed in part to the difference in the experimental protocol. As many previous studies tried to use poly(I:C) to mimic HRV infection, which is known to exacerbate allergic asthma. Therefore, poly(I:C) was only included at the allergen challenge phase, but not at the sensitization phase in animal model of allergic asthma. In addition, sites for allergen sensitization and challenge were often separated. For example, allergens (HDM or OVA) were commonly mixed with adjuvants such as alum to sensitize mice via the i.p. route, then intranasally challenged with the same allergen. In our case, we performed the sensitization and challenge of HDM extracts without any formulations via the same route. Thus, HDM-associated dsRNA should concomitantly exist with HDM allergen at both experimental stages. However, much more works need to be done to completely resolve this controversy on the opposite role for the dsRNA/TLR3-signaling pathway in the context of allergic asthma.

We and others have recently reported that bacterial RNAs and viral RNAs are highly immunogenic when delivered into the cytosol of mammalian cells (54–58). In particular, the dsRNA byproduct of many RNA viruses is chemically very stable and has been known as one of the most potent triggers for induction of type I IFNs in mammalian cells (57). The identities of the HDM-dsRNA remain unknown. Further, why HDM allergens contain so much immunostimulatory dsRNAs is unclear. In addition, more work will be needed to establish a mechanistic link between the innate RNA-sensing pathway and the development of Th2-biased adaptive immune responses in the context of indoor allergen exposure. Nonetheless, the work reported in this study reveals a previously unrecognized role for an allergen-associated dsRNA that modulates the severity of allergen-associated lung inflammation.

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Disclosures

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Figure S1

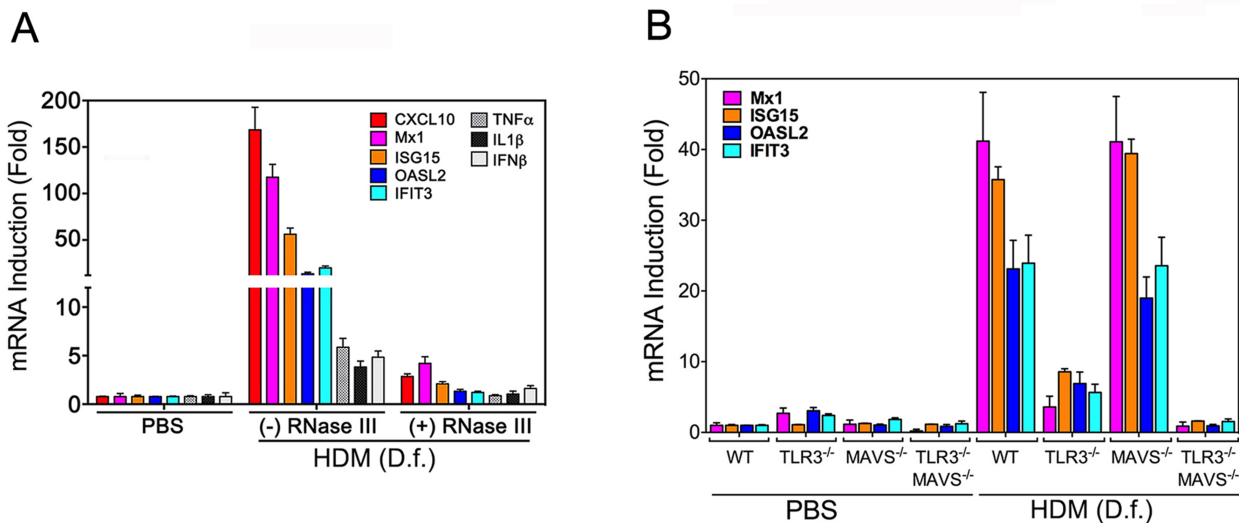


Figure S1. Transcriptional induction of multiple cytokines in mouse lung by the HDM treatment. **A.** In response to the treatment of HDM (D.f.) and HDM (D.f.) + RNase III, lung samples from wild type mice were further validated for a few selected genes including CXCL10, Mx1, ISG15, OASL2, IFIT3, TNF α and IL1 β by RT-qPCR. **B.** In response to the treatment of HDM (D.f.), lung samples from wild type, TLR3^{-/-}, MAVS^{-/-} and TLR3^{-/-}MAVS^{-/-} mice were further validated for a few selected differentially expressed ISGs by RT-qPCR.

Figure S2

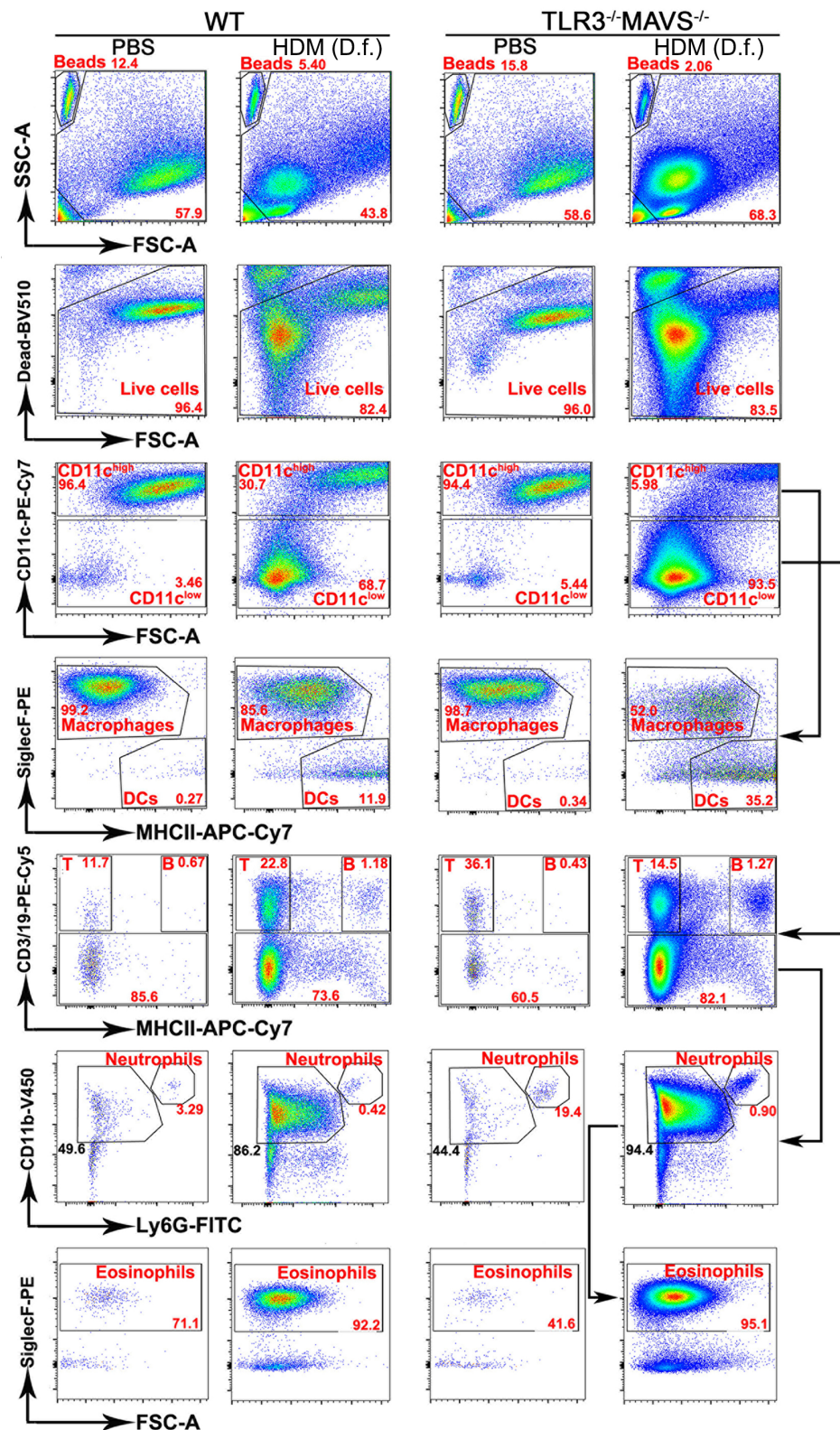


Figure S2. FACS Gating strategy for the identification of specific cell types including eosinophils. Cells recovered from the BAL fluid were stained for cell surface markers as indicated. The absolute numbers were calculated based on reference beads (top panel). Formula (Number of cells per mL): (Total Beads/# events of Beads) x (# event of Sample)/Volume of Tested Sample. (Related to **Figure 4**).