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Identification and Characterization of Immunogenic RNA Species in HDM Allergens that Modulate Eosinophilic Lung Inflammation

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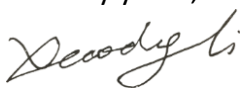
March 30, 2020

Dear Dr. Upponi,

Thank you so much for handling the review of our manuscript JoVE61183, entitled "***Identification and Characterization of Immunogenic RNA Species in HDM Allergens that Modulate Eosinophilic Lung Inflammation***". We really appreciate the constructive comments and valuable suggestions from you and three reviewers for improving the paper. We have revised the manuscript accordingly. The changes in text are highlighted in blue. Our point-by-point responses have been included with this submission.

Thank you for the kind consideration of publication in *JoVE*. We look forward to hearing from you.

Sincerely yours,



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TITLE:

Identification and Characterization of Immunogenic RNA Species in HDM Allergens that Modulate Eosinophilic Lung Inflammation

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KEYWORDS:

BAL, bronchoalveolar lavage, dsRNA, FACS, fluorescence-activated cell sorting, HDM, house dust mites, ISGs, interferon stimulated genes, PAMP, pathogen-associated molecular pattern, RT-qPCR

SUMMARY:

Environmental allergens such as house dust mites (HDM) often contain microbial substances that activate innate immune responses to regulate allergic inflammation. The protocol presented here demonstrates the identification of dsRNA species in HDM allergens and characterization of their immunogenic activities in modulating eosinophilic lung inflammation.

ABSTRACT:

Environmental allergens such as house dust mites (HDM) are often in complex forms containing both allergic proteins that drive aberrant type 2 responses and microbial substances that induce innate immune responses. These allergen-associated microbial components play an important role in regulating the development of type 2 inflammatory conditions such as allergic asthma. However, the underlying mechanisms remain largely undefined. The protocol presented here determines the structural characteristics and in vivo activity of allergen-associated immunostimulatory RNA. Specifically, common allergens are examined for the presence of double-stranded RNA (dsRNA) species that can stimulate IFN responses in lungs and can lead to the development of severe lung eosinophilia in a mouse model of HDM-induced allergic asthma. Here, we have included the following three assays: Dot blot to show the dsRNA structures in total RNA isolated from allergens including HDM species, RT-qPCR to measure the activities of HDM RNA in interferon stimulating genes (ISGs) expression in mouse lungs and FACS analysis to determine the effects of HDM RNA on the number of eosinophils in BAL and lung, respectively.

INTRODUCTION

Based on the hygiene hypothesis originally proposed by Strachan[1], early childhood exposure to environmental microbial factors such as endotoxin can protect against the development of allergic disorders[2,3]. During microbial infections, e.g., viral infections, the innate immune detection of foreign nucleic acids (RNA/DNA) triggers host defense responses[4-6]. However, the existence and prevalence of immunogenic nucleic acids such as long double-stranded RNA (dsRNA) species in house dust mites (HDM) or other insect allergens remain unknown. This protocol was designed to determine whether HDM or insect and non-insect allergens contain long dsRNA species that can activate a protective immune response to counteract the development of severe eosinophilic lung inflammation in a mouse model of allergic asthma. Here, we provide three simple and fast methods to evaluate the structural determinants in HDM total RNA that are required for regulating allergen-induced eosinophilic lung inflammation.

The mucosal immune system is the largest immune organ in the body and serves as the first line of host defense against both microbial infections and allergic insults[7,8]. The long dsRNA, the replication intermediate of many viruses, is known to function as a pathogen-associated molecular pattern (PAMP) to potently stimulate innate responses via Toll like receptor 3 (TLR3) to induce the expression of interferon stimulated genes (ISGs)[9-14]. We have recently shown that HDM total RNA contained dsRNA structures, which upregulated the expression of ISGs and reduced severe eosinophilic lung inflammation when administered via the intratracheal instillation in a murine model of allergic asthma induced by HDM extracts[15]. The severity of lung inflammations is determined by analyzing the immune cell types in bronchoalveolar lavage (BAL) and lung tissue via flow cytometry[16-20].

This protocol includes three assays: 1) rapid detection of dsRNA structures with RNA dot blot using a mouse monoclonal antibody J2 which specifically binds to the dsRNA (≥40bp) in a sequence-independent manner; 2) quick evaluation for in vivo effects of immunostimulatory RNA in mouse lungs by measuring the induction of ISGs using RT-qPCR; 3) accurate quantification of eosinophils in BAL and lung in the context of HDM-induced lung inflammation using flow cytometry analysis.

The above assays can be used to study not only allergic lung diseases, but also respiratory bacterial and viral infections. For example, the dsRNA specific J2 antibody can also be used in other applications such as immunoaffinity chromatography, immunohistochemistry, enzyme-linked immunosorbent assay (ELISA) and immunostaining[21-23]. In addition, several applications downstream of BAL fluid collection can be utilized for quantifying soluble contents such as cytokines and chemokines using ELISA, and transcriptional profiling of cells in the airways (e.g., alveolar macrophages). Although there are a variety of protocols available in the literature to evaluate lung conditions, most of these protocols often focus on the target validation. The procedures described here can be applied to identify components in environmental allergens that are important for regulating the development of allergic diseases.

PROTOCOL:

Experimental procedures described here were approved by the Institutional Animal Care and Use Committee of University of Texas Health San Antonio.

1. Dot blot to show the presence of dsRNA structures in HDM total RNA

1.1 Total RNA isolation from allergens, insects, and non-insect allergens

1.1.1 Put HDM, insects, or non-insect animals collected alive or obtained commercially into 50 mL tubes, and quickly freeze with liquid-N_2. Then store at -70 °C for subsequent total RNA isolation.

NOTE: In this experiment, HDM, insect, and non-insect animals were selected because they are known to be common sources of allergens. Further, an immunostimulatory function of their RNAs remain unclear.

1.1.2 Transfer a proper amount (equivalent to 100 µL in volume or less) of HDM, insects or non-insect animals stored at -70 °C into a 2 mL tube containing beads (1.4 mm ceramic spheres), then freeze tubes in a liquid-N_2 container for ~10 min.

1.1.3 For the total RNA isolation, add 1 mL of guanidinium thiocyanate-based RNA isolation reagent[24] to each tube, then break the insect and non-insect small animals with a high-energy cell disrupter at the maximum speed for 45 s and chill on ice. Repeat this step twice.

1.1.4 Transfer the solution from step 1.1.3 into a new 1.5 mL tube and add 200 µL of chloroform to each tube and vortex. Centrifuge tubes at 14,000 *x g* for 14 min at 4 °C.

1.1.5 Once centrifugation is completed, transfer the upper aqueous phase (200 µL) into a new 1.5 mL tube containing 500 µL of isopropanol to precipitate RNA pellet. Do not disturb the interphase. The recommended volume ratio of the upper phase versus isopropanol is 1:2.5 ratio.

1.1.6 Mix by gentle vortexing, then centrifuge tubes at 14,000 *x g* for 14 min at 4 °C.

1.1.7 Aspirate the supernatant with caution then wash the RNA pellet with 500 µL of 75% ethanol and centrifuge at 7,500 *x g* for 10 min at 4 °C. Remove all liquid with caution, air-dry the pellet and dissolve the RNA pellet with 20-50 µL of RNase-free H_2O.

1.1.8 Measure the RNA concentration with a spectrophotometer using the following parameters:

1.1.8.1 Open the associated software and select the type of nucleic acids to measure. Change the sample type to RNA.

1.1.8.2 Perform the blank measurement with 1-2 µL of RNase-free H_2O. Wipe off the RNase-free H_2O. Now, the instrument is ready for the measurement.

1.1.8.3 Load 1-2 µL of the RNA sample and measure the RNA concentration (µg/µL).

NOTE: The ratio of the absorbance at 260 and 280 nm (A260/280) at ~2.0 (1.9-2.2) is generally accepted as "pure" for RNA. If not processed immediately, store RNA samples at -70 ˚C and avoid the freeze-thaw cycles to keep the RNA intact.

1.2 Detection of dsRNA structure in the total RNA using dsRNA specific J2 antibody

1.2.1 Prepare two 20 μL of RNA samples (200 ng/μL). One with RNase-III treatment (1 μL for 1 μg RNA, incubate at 37 °C for 60 min), and the other without RNase-III treatment.

NOTE: RNase III is used here to specifically degrade dsRNA, but not single-stranded RNA[25].

1.2.2 Use a pencil to draw grids where RNA samples will be blotted on the membrane.

1.2.3 Spot 2 μL of the 200 ng/μL of the RNA sample onto the positively charged nylon membrane.

1.2.4 Crosslink the samples to the membrane at 1,200 microjoules x 100 in a UV crosslinker. Repeat steps 1.2.3 and 1.2.4 two more times in the sample spot place. This will result in total 1 μg per blot.

NOTE: Do not spot more than 2 μL of RNA sample on the membrane at a time.

1.2.5 Block non-specific binding with 5% milk in TBS-T for 1 h with shaking at room temperature. Remove the blocking solution from step 1.2.5 and add the anti-dsRNA J2 antibody at the 1:1,000 dilution in 1% milk in TBS-T and incubate overnight with shaking at 4 °C.

1.2.6 Wash the membrane with TBS-T for 5 min and repeat this step for 3 times. Add the secondary antibody (Alkaline phosphatase-conjugated Anti-Mouse IgG diluted in 1% milk 1:5,000) and incubate for 1 h at room temperature. Wash the membrane with TBS-T for 5 min and repeat this step for 3x.

1.2.7 Add the substrate (BCIP/NBT) and incubate for 5-15 min until a desired signal is visible.

1.2.8 Stop the reaction by rinsing the membrane with ddH_2O.

1.2.9 Dry the membrane on tissue papers and take a photograph using a smartphone (a representative result is shown in **Figure 1**).

2. RT-qPCR to measure the ability of HDM total RNA in stimulating lung ISGs expression

2.1 RNA isolation from mice lung tissues

NOTE: Mice (female, 8-12 weeks old, C57BL/6J) were maintained under specific pathogen-free conditions.

2.1.1 Briefly anesthetize the animal with isoflurane and administer via the intratracheal instillation with 5 μg (diluted in 80 μL PBS) of HDM RNAs treated with or without RNase III.

2.1.2 After 16-18 h post HDM RNA treatment, sacrifice the mouse by CO_2 inhalation for a few minutes. Then, place the mouse on a platform and pin limbs with needles.

2.1.3 Disinfect the mouse with 70% ethanol then cut the skin starting from abdomen to the neck with a sterilized scissor.

2.1.4 Fix the skin with needles and cut the ribs to expose the lungs. Remove the whole lungs and wash them with cold PBS. Place the lungs on tissue papers and excise one small piece of each lung-lobe into a 2 mL tube containing beads (200-300 μL in volume, 1.4 mm ceramic spheres).

NOTE: The purpose of using ceramic beads is to grind whole lung tissues

2.1.5 Freeze the lung samples by placing tubes into a liquid-N_2 container for ~10 min.

2.1.6 Add 500 μL of guanidinium thiocyanate-based RNA isolation reagent to each tube and break the lung tissues with a homogenizer for 45 s. Chill on ice between each step. Repeat this step twice.

2.1.7 Follow the steps 1.1.4- 1.1.7 for lung RNA isolation.

2.1.8 Air-dry the pellet and dissolve the RNA pellet with proper amount of RNase-free H_2O (~20-30 μL).

2.1.9 Measure the RNA concentration as described in step 1.1.10.

2.2 RT-qPCR to determine the ability of HDM RNA in stimulating lung gene expression.

2.2.1 Using 100 ng/μL of RNA extracted from lung tissues as the template, perform the cDNA synthesis according to the referenced protocol[26].

2.2.2 Set up an RT-qPCR reaction at 10 μL/well for a 384-well plate using cDNA generated above and the gene-specific primer pairs (**Table 1** and **Table 2**).

2.2.3 Seal the wells tightly with a transparent adhesive film and vortex the plate for 30 s. Spin the plate at 1,000 *x g* for 30 s to collect samples at the bottom of the wells.

2.2.4 Load the plate onto a RT-qPCR machine and start to run the RT-qPCR reaction using the thermal cycler protocol (**Table 3**).

2.2.5 Export the results into a spreadsheet file or analyze the data using the software provided by the manufacture after the program is completed (a representative result is shown in **Figure 2**).

3. FACS analysis to determine the effects of HDM RNA on the infiltration of eosinophils in BAL and lung

3.1 BAL fluid collection for FACS analysis

3.1.1 Euthanize mice (female, 8-12 weeks old, C57BL/6J) that were treated with HDM allergen extracts (according to the experimental design shown in **Figure 3B**) by CO_2 inhalation.

3.1.2 Place the mouse on a platform and pin limbs with needles.

3.1.3 Disinfect the mouse with 70% ethanol. Use scissors to cut the skin from the upper area of the abdomen to the neck.

3.1.4 Gently, pull the salivary glands and the sternohyoid muscle carefully apart using the forceps to expose the trachea. Place a nylon string (~10 cm) under the trachea using forceps.

3.1.5 Make an incision in the trachea (~2 mm under the larynx) just enough to insert a cannula. Do not cut through the trachea. Knot the string around trachea and cannula.

3.1.6 Load the syringe with 1 mL of PBS+EDTA and attach it to the end of the cannula. Inject 1 mL of PBS+EDTA into the lung and completely aspirate the solution. Detach the syringe from the cannula carefully and remove the solution into a 15 mL tube on ice.

3.1.7 Reload the syringe with the fresh PBS+EDTA and repeat this step 2x.

3.1.8 Centrifuge the tube containing the pooled BAL obtained in step 3.1.7 to pellet the cells at 500 *x g* for 7 min at 4 °C. Record the volume of BAL fluid then transfer the supernatant to two 1.5 mL tubes without disturbing the pellet.

NOTE: The supernatant of BAL can be stored at -70 °C for future analysis e.g., ELISA.

3.1.9 In case there are RBCs present in the pellet due to severe lung inflammation, after removing the supernatant, add 500 µL of RBC lysis buffer and mix well by resuspension. Transfer the solution into a new 1.5 mL tube and centrifuge for 7 min at the speed of 500 *x g* at 4 °C.

3.1.10 Remove the supernatant and resuspend the pellet in 150 µL of FACS buffer.

3.1.11 Transfer the 150 µL of the resuspended sample into 96-well plate and centrifuge the plate for 7 min at the speed of 500 *x g* at 4 °C.

3.1.12 Quickly, invert the plate on tissue papers to collect the cells residing at the bottom of the wells.

3.1.13 Stain the cells with antibodies in FACS buffer in the presence of 2.4G2 blocking antibody (2.5 µg / 100 µL). Incubate the plate at room temperature for 30 min in a dark place.

3.1.14 After staining, centrifuge the plate to pellet the cells at 500 *x g* for 7 min at 4 °C.

3.1.15 Remove the staining solution by inverting the plate on tissue paper then wash by resuspending with 100 µL of FACS buffer. Next, centrifuge the plate again at 500 *x g* for 7 min at 4 °C and remove the FACS buffer by inverting the plate on tissue paper.

3.1.16 Resuspend samples into 150 µL of FACS buffer and transfer samples to the FACS tubes containing 350 µL of FACS buffer. Add 25 µL of counting beads to each sample. Samples are now ready for flow cytometry analysis.

NOTE: Various cell types in BAL fluid were labeled with antibodies as indicated. Counting beads were added before the FACS run. Flow cytometry data were analyzed using a commercially available software. Refer to **Figure 3** and **Table 4** for gating strategy.

3.2. Lung tissue digestion for the FACS analysis

3.2.1. Follow steps 2.1.1 - 2.1.3.

3.2.2. Sacrifice mice by CO_2 inhalation.

3.2.3. Place and pin the mouse on a platform and disinfect the mouse with 70% ethanol.

3.2.4. Cut the skin starting from abdomen to the neck with a sterilized scissor. Fix the skin with needles and cut the ribs to expose the lungs.

3.2.5. Remove the whole lungs and wash them with cold PBS. Place samples in the 1.5 mL tube containing 50 µL of lung digestion solution.

3.2.6. Mince the lung tissues into small pieces with a curved scissor. Transfer the lung tissues into a 6-well plate, then add 8 mL of lung digestion solution. Place the plate on a shaker in 37 °C incubator for 45 min.

3.2.7. After incubation, use the top of 1.5 mL tube to grind the lung tissues. Place a 70 µm strainers on a new 6-well plate and apply the sample through 0.22 µm filter.

3.2.8. Transfer the filtered solution into a 15 mL tube, then centrifuge the tubes at 500 *x g* for 7 min at 4 °C. Aspirate the supernatant and resuspend the pellet in 1 mL of RBC lysis buffer and leave it on ice for 3 min.

3.2.9. Transfer the sample into 1.5 mL tube and centrifuge at 500 *x g* for 7 min at 4 °C. Repeat 2x

3.2.10. Wash the lung cells 2x with 1 mL FACS buffer. Aspirate the supernatant and resuspend the pellet in 1 mL of FACS buffer, and then transfer 100 µL of the sample into 96 well plate.

3.2.11. Centrifuge the plate for 7 min at the speed of 500 *x g* at 4 °C. Follow the steps described in BAL fluid collection for FACS analysis (3.1.13 to 3.1.16) to stain the cells indigested lung tissue samples.

NOTE: Eosinophils in the lungs were labeled with antibodies as indicated, then mixed with counting beads for further FACS analysis. Flow cytometry data were analyzed using associated software. Refer to **Figure 3** for evaluating HDM RNA-induced immune responses.

4. Statistical analysis

4.1 Perform statistical analysis using a commercially available software.

4.2 Determine the *p* values by unpaired two-tailed Student t test for the comparison of two groups.

4.3 Calculate the absolute numbers of eosinophils based on reference beads (top panel) using the formula

$$(Number\ of\ cells\ per\ ml) = (Total\ Beads/\#\ events\ of\ Beads)\ x\ (\#\ event\ of\ Sample)\ /Volume\ of\ Tested\ Sample.$$

4.4 Determine the *p* values by two-way ANOVA and Sidak's multiple comparisons test for the comparison of more than two groups.

4.5 Consider a *p* value smaller than 0.05 as statistically significant. The *p* values are indicated on plots as *p <0.05, **p<0.01, ***p<0.001, and ****p<0.0001.

NOTE: All buffer recipes are provided in **Table 5**.

REPRESENTATIVE RESULT:

The presence of long dsRNA structures in HDM, insects and non-insect small animals was examined by dot blot using a dsRNA-specific mouse monoclonal antibody J2 (≥ 40bp). RNase III was used to digest dsRNA into 12–15 bp dsRNA fragments, which were undetectable by J2 (**Figure 1**).

The ability of HDM total RNA in a dose-dependent manner to stimulate an innate immune response in mouse lungs was analyzed by RT-qPCR (**Figure 2, upper)**. The RNase III treatment abolished the immunostimulatory activity of HDM total RNA, indicating that dsRNA structures in HDM total RNA is essential for innate immune activity in the lungs (**Figure 2, lower**).

The inhibitory effects of HDM total RNA on the development of a severe type 2 lung inflammation were evaluated with the FACS analysis (**Figure 3A**). In this study, the eosinophilic lung inflammation was induced by HDM extracts, which were treated with or without RNase III as depicted in the experimental design (**Figure 3B**). RNase III treatment was used to remove long dsRNA species from

HDM extracts. As expected, the degradation of long dsRNA species resulted in severe type 2 lung inflammation reflected by the increased eosinophils numbers in BAL and lungs. Notably, the number of eosinophils in the HDM total RNA-treated group is comparable to the group treated with the original HDM extract that endogenously contains the long dsRNA species (**Figure 3B**).

FIGURE AND TABLE LEGENDS:

Figure 1: Detection of the dsRNA structures in HDM RNAs by dsRNA specific J2 Ab using dot blot. Total RNA from different aeroallergens including *Dermatophagoides farinae* (D.f.) and *Dermatophagoides pteronyssinus* (D.p.) was blotted on a nylon membrane for the detection of dsRNA (**left panel**). HDM (D.f. and D.p.) RNA was left untreated (-), treated with RNase III (dsRNA-specific nuclease) and RNase T1 (ssRNA-specific nuclease) (**right panel**). This figure is reprinted from She et al.[15].

Figure 2: Induction of ISG mRNA expression by total RNA from HDM (D.f.) was measured by RT-PCR. When delivered into mouse lungs, HDM RNA was able to stimulate the expression of ISGs in a dose-dependent manner (**upper panel**). RNase III treatment eliminated the immune stimulating activity of HDM RNA (**lower panel**). This figure is reprinted from She et al.[15].

Figure 3: Characterization of specific cell types in the airways and evaluation of BAL fluid and lung eosinophils. (**A**) Gating strategy used to identify cells recovered from BAL fluid were stained for cell surface markers as indicated. (**B**) Administration of HDM (D.f.) RNA at 5 µg/mouse (blue bar) versus control mouse lung RNA (red bar). HDM (D.f.) RNA but not dsRNA-depleted HDM extract decreased the number of eosinophils in both airways and lungs of animals treated with HDM extract. This figure is reprinted from She et al.[15].

Table 1: RT-qPCR Primers.

Table 2: Master mix setup for RT-qPCR.

Table 3: Program for running the RT-qPCR.

Table 4: FACS running

Table 5: Recipes for buffers and solution

DISCUSSION

The current protocol describes how to evaluate the immunostimulatory properties of allergen-associated microbial RNA and their impacts on the development of eosinophilic lung inflammation in a mouse model of allergic asthma. Although long dsRNAs are known as the replication intermediates of many viruses that can potently activate interferon responses in mammalian cells, their presences in HDM allergens have been unknown until our recent work[15]. The combination of RNA dot blot, RT-qPCR and FACS analysis presented in this manuscript may provide a good example

to dissect innate components such as the dsRNA species in environmental allergens that are critically involved in regulating allergen-induced eosinophilic inflammation.

In this protocol, the RNA dot blot has been employed to detect the presence of dsRNA structures in natural allergens using a mouse monoclonal antibody J2, which specifically binds to the dsRNA (≥40bp) independent of sequence. This method is highly reliable because J2 antibody can still recognize dsRNA samples pretreated with RNase T1 (single stranded RNA-specific endonuclease), but not samples pretreated with RNase III (a dsRNA-specific endonuclease). However, it is worth pointing out that a widely used synthetic analogue of dsRNA, Polyinosinic:polycytidylic acid [Poly(I:C)], has been reported to preferentially bind to another anti-dsRNA monoclonal antibody K1, instead of J2[21-23]. Therefore, the use of J2 antibody for the detection of Poly(I:C) is not recommended.

Cell type analysis on samples collected from BAL or lung tissues is useful for assessing the progression of allergic lung inflammation. Although BAL procedure is a common technique, the results may vary among research laboratories. Numerous factors may cause these variations such as the amount of bronchoalveolar lavage collected. The ideal volume of BAL recovered from an 8-12 weeks old mice is ~3 ml[19]. Another factor that may contribute to the lack of reproducibility is how deep the catheter should be inserted into the trachea (~0.5 cm is optimal) because deeper insertion of the catheters may cause damage to the trachea. In addition, researchers should also consider other factors such as the age, strain, and gender of the mice as these factors can greatly impact the experiment results[27-29].

Here, we provide a technical protocol to characterize immunomodulatory effects of HDM RNA in vitro and in vivo using RNA dot blot, RT-qPCR and FACS analysis of BAL and lung tissues. Proper practices can ensure successful reproducibility of results obtained when performing these techniques. For instance, try to avoid the contamination of RNases when performing RNA dot blot. Also, the centrifugation speed should be properly adjusted since the unnecessary higher centrifugation speed may compromise cell viability. Finally, cells used for the FACS analysis should be fixed if not analyzed on the same day.

Since innate immunity plays a pivotal role in host defense and inflammation[2,3], the techniques and methods described in this paper will be very useful for studying the immunomodulatory role of other innate immune components such as microbial DNA in natural allergens in the development of type 2 inflammation.

DISCLOSURES

We have nothing to disclose.

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24 TRIzol Reagent Literature. RNA Isolation with TRIzol (Invitrogen) and Qiagen RNAeasy. Invitrogen Life Technologies, http://www.ocg.cancer.gov/sites/default/files/RNA_Trizol_NBL.pdf.
25 Biolabs, New England. ShortCut RNase III Digestion Protocol (M0245). NEB, www.neb.com/protocols/0001/01/01/shortcut-rnase-iii-digestion-protocol-m0245.
26 “IScript cDNA Synthesis Kit.” IScript cDNA Synthesis Kit Technical Manual. www.bio-rad.com/webroot/web/pdf/lsr/literature/4106228.pdf.
27 Redente, E. F. et al. Age and sex dimorphisms contribute to the severity of bleomycin-induced lung injury and fibrosis. *American Journal of Physiology-Lung Cellular and Molecular Physiology.* **301**, L510-L518 (2011).
28 Card, J. W. et al. Gender differences in murine airway responsiveness and lipopolysaccharide-induced inflammation. *Journal of Immunology.* **177**, 621-630 (2006).
29 Gueders, M. M. et al. Mouse models of asthma: a comparison between C57BL/6 and BALB/c strains regarding bronchial responsiveness, inflammation, and cytokine production. *Inflammation Research.* **58,** 845-854 (2009).

Figure 1.

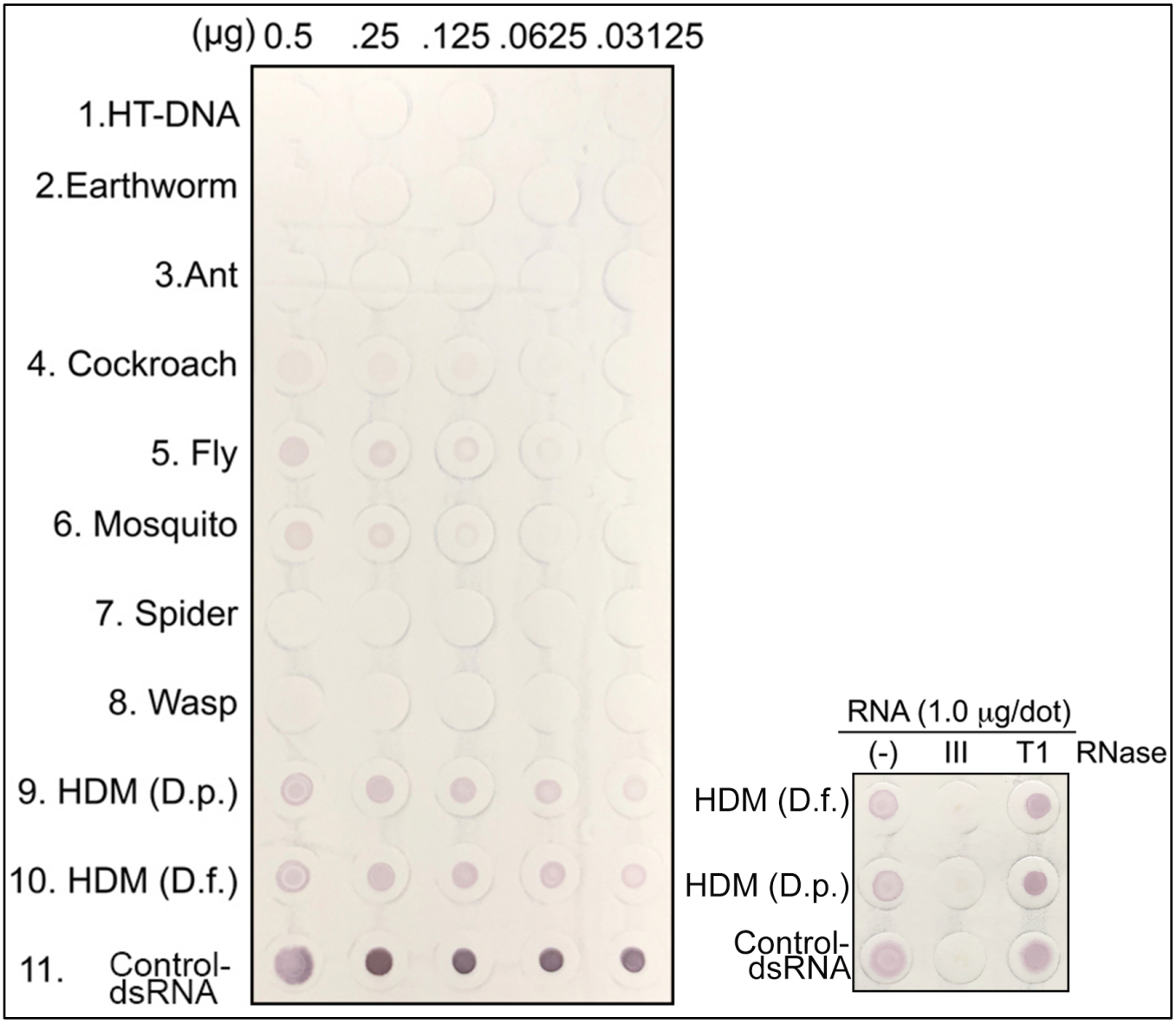

Figure 2.

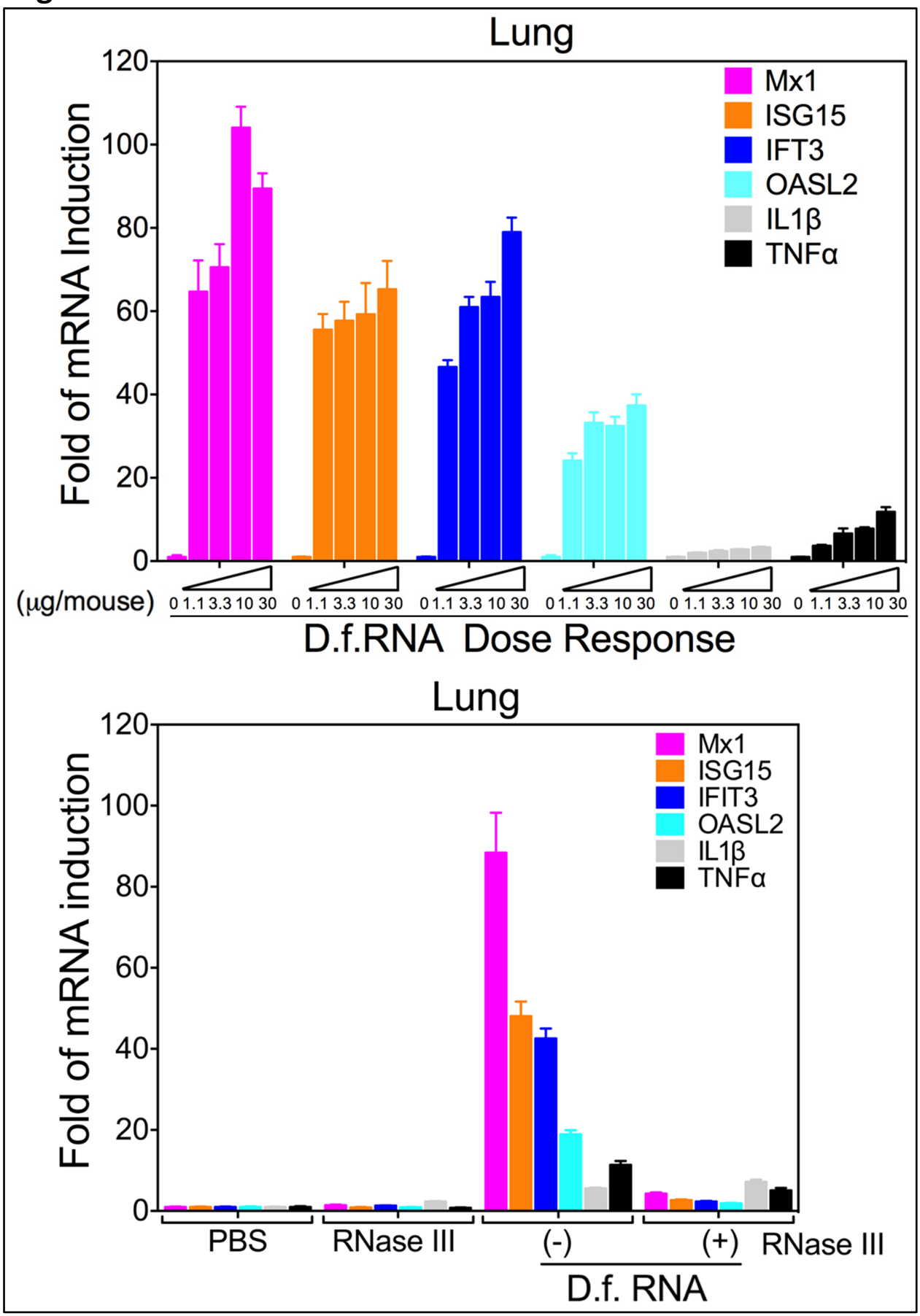

Figure 3.

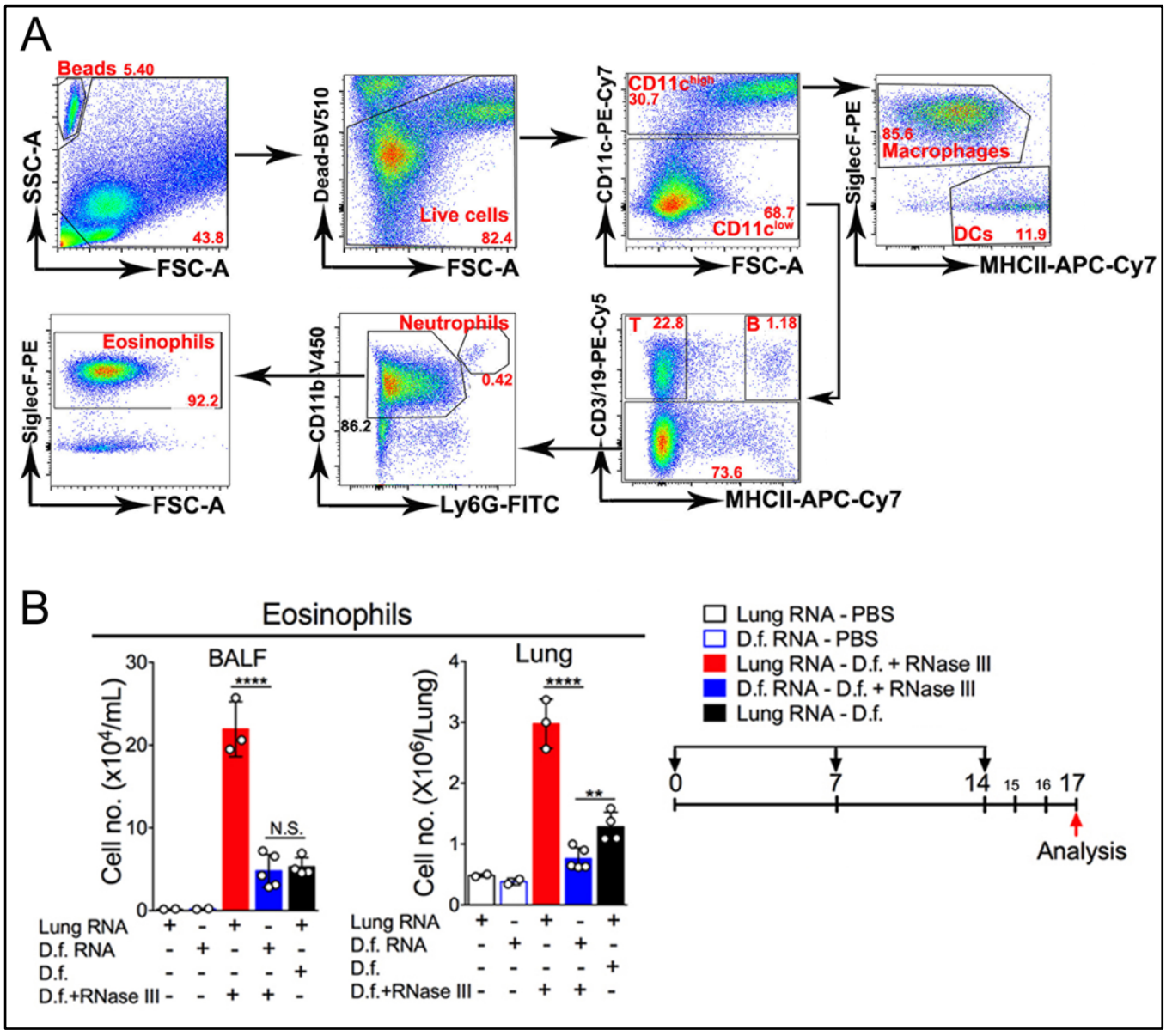
A
Beads 5.40
SSC-A
FSC-A
43.8
Dead-BV510
Live cells
82.4
CD11c-PE-Cy7
CD11c^high
30.7
CD11c^low
68.7
SiglecF-PE
85.6
Macrophages
DCs 11.9
MHCII-APC-Cy7
Eosinophils
92.2
CD11b-V450
Neutrophils
0.42
86.2
Ly6G-FITC
CD3/19-PE-Cy5
T 22.8
B 1.18
73.6
B
Eosinophils
BALF
Cell no. (x10^4/mL)
****
N.S.
Lung
Cell no. (X10^6/Lung)
**
Lung RNA
D.f. RNA
D.f.
D.f.+RNase III
Lung RNA - PBS
D.f. RNA - PBS
Lung RNA - D.f. + RNase III
D.f. RNA - D.f. + RNase III
Lung RNA - D.f.
0
7
14
15
16
17
Analysis

Table 1. RT-qPCR Primers.

Mouse Primers	Sequence (Forward-Reverse, 5'→3')
RPL19	AAATCGCCAATGCCAACTC; TCTTCCCTATGCCCATATGC
IL1β	TCTATACCTGTCCTGTGTAATG; GCTTGTGCTCTGCTTGTG
IFIT3	TGGCCTACATAAAGCACCTAGATGG; CGCAAACTTTTGGCAAACTTGTCT
ISG15	GAGCTAGAGCCTGCAGCAAT; TTCTGGGCAATCTGCTTCTT
Mx1	TCTGAGGAGAGCCAGACGAT; ACTCTGGTCCCCAATGACAG
OASL2	GGATGCCTGGGAGAGAATCG; TCGCCTGCTCTTCGAAACTG
TNFα	CCTCCCTCTCATCAGTTCTATGG; GGCTACAGGCTTGTCACTCG

Table 2. Set up the mastermix

Reagents	Volume (10 µl)
Universal SYBR Gree	5 µl
Forward and reverse	0.5 µl
cDNA template	0.2 µl
DNase- and RNase-fr	4.3 µl

3. The program for running the RT-qPCR.

Steps	Temperature
Step 1	95 ^{o}C
Step 2	95 ^{o}C
Step 3	55 ^{o}C
Step 4	**Go to step 2, (Repeat 2-3 for 39 cycles)**
Step 5 (Melt Curve)	55 ^{o}C to 95 ^{o}C

Time
3 minutes
10 seconds
30 seconds
0.5 ^{o}C, increments (hold time is 5 seconds)

Table 4: FACS running

1. Create a plot composed of forward (FSC) and side scatter (SSC).
2. Create a small plot for counting beads (FSC low, FITC high).
3. Create a plot to only gate for live cells while excluding dead cells using BV510 dye.
4. Live cells can then be separated into CD11chigh and CD11c low populations.
5. From CD11chighpopulation gate for macrophages (SiglecF high MHCII low) and DCs (Sig
6. From CD11clow gate for T cells (CD3/19 high, MHCII low), and B cells (CD3/19 high, MH
7. From CD11clow CD3/19null cell population, gate for neutrophils (CD11bhigh, Ly-6Ghigh
8. For gating strategy of Eosinophils in the lung tissues, use these markers aftere excludin

lecF low, MHCII high).
CII high).
) and Eosinophils (CD11b high, Ly-6G low, SiglecF high).
g dead cells using BV510 dye (CD45, SiglecF, CD11C).

Table 5. Recipes

TBS:
20 mm Tris-HCl
150 mm NaCl
pH 7.5
TBS-T:
0.05% Tween-20 in TBS
Blocking Buffer
5% non-fat milk diluted in TBS-T
Antibody dilution Buffer
1% non-fat milk diluted in TBS-T
PBS+EDTA
1x PBS + 0.1 mM EDTA
FACS buffer
2% Fetal Calf Serum (FCS) in 1x PBS
Total cell medium
RPMI 1640, 1X Glutamax, 10% FCS, 50 µM 2-mercaptoethanol and Penicillin-Streptomyc
Lung digestion solution
Total cell medium plus Liberase (50 µg/ml) and DNase I (1 µg/ml)

cin.

Name	Company
0.40 µm Falcon Cell Strainer	Thermo Fisher Scientific
1 mL syringes	Henke Sass Wolf
15 mL Tube	TH.Geyer
50 mL Tube	TH.Geyer
70% ethanol	Decon Labs
Absolute Counting Beads	Life Technologies Europe B.V.
ACK-RBC lysing buffer	Lonza
Amersham Hybond-N+ Membrane	GE Healthcare
Ant	San Antonio
Antibody dilution buffer	
Anti-Mouse CD11b V450 Rat (clone M1/70)	BD Bioscience
Anti-Mouse CD11c PE-Cy7 (clone N418)	BioLegend
Anti-Mouse CD19 Alexa Flour 647 (clone 1D3)	eBioscience
Anti-Mouse CD3e APC (clone 145-2C11)	Invitrogen
Anti-Mouse CD45 APC-Cy7 (clone: 30-F11)	BioLegend
Anti-Mouse Fixable Viabillity Dye eFluor 506	Invitrogen
Anti-Mouse IgG (H+L), AP Conjugate	Promega
Anti-Mouse Ly-6G FITC (clone RB6-8C5)	Invitrogen
Anti-Mouse MHC II APC-eFluor 780 (clone M5/114.15.2)	eBioscience
Anti-Mouse Siglec-F PE (clone E50-2440)	BD Pharmingen
BCIP/NBT substrate	Thermo Fisher Scientific

Blocking Buffer	
Cannual, 20G X 1.5”	CADENCE SCIENCE
Centrifuge	Thermo Fisher Scientific
CFX384 Touch Real-Time PCR Detection System	Bio-Rad Laboratories
Chloroform	Thermo Fisher Scientific
Cockroach	Greer Laboratories
Counting beads	Thermo Fisher Scientific
D. farinae	Greer Laboratories
D. pteronyssinus	Greer Laboratories
Denville Cell Culture Plates with lid, 96 well cell culture plate	Thomas Scientific
Digital Dry Bath - Four Blocks	Universal Medical, Inc.
Earthworm	San Antonio
Ethylenediaminetetraacetic acid (EDTA)	Sigma-Aldrich
FACS buffer	
Falcon Round-Bottom Polypropylene Tubes, 5 mL	STEMCELLTM TECHNOLOGIES
Flow cytometer (BD FACS Celesta)	BD Biosciences
Fly	Greer Laboratories
Forceps	Roboz Surgical Instrument
Hemocytometer	Hausser Scientific
HT-DNA	Sigma
In Vivo MAb anti-mouse CD16/CD32 (clone: 2.4G2)	Bio X Cell
iScript cDNA Synthesis Kit	Bio-Rad Laboratories
Isoflurane	Abbott Labs
Isopropanol	Thermo Fisher Scientific

J2 anti-dsRNA monoclonal antibody	SCICONS
Lung digestion solution	
Lysing Matrix D	MP Biomedicals
Lysing Matrix D, 2 mL tube	MP Biomedicals
Mice (female, 8-12 weeks old, C57BL/6J)	Jackson Laboratory
Microcentrifuge tube 1.5 mL	Sigma-Aldrich
Microscope	Olympus
Mini-BeadBeater	Homogenizers
Mini-Beadbeater-16	Biospec
Mosquito	Greer Laboratories
NanoDrop 2000C	Thermo Scientific Spectophotometer Medex Supply
Needle, 21 G x 1 1/2 in	BD Biosciences
Non-fat milk	Bio-Rad Laboratories
Nylon string	Dynarex
Phosphate-buffered Saline (PBS)	Lonza
RNase III	Thermo Fisher Scientific
RNase T1	Thermo Fisher Scientific
Scissors	Roboz Surgical Instrument
Shaker or Small laboratory mixer	Boekel Scientific
SPHERO AccuCount Fluorescent	Spherotech
Spider	San Antonio
TBS	
TBS-T	
Total cell medium	
TRIzol Reagent	Thermo Fisher Scientific
Tween 20	Sigma-Aldrich
UV Stratalinker 2400 UV	LabX
Wasp	San Antonio

Catalog number	Comments
08-771-1	
5010.200V0	
7696702	
7696705	
2701	
C36950	
10-548E	
RPN203B	
Note: Locally collected	
(see Table 5 for recipe)	
560456	1 to 200 dilution
117317	1 to 200 dilution
15-0193-81	1 to 200 dilution
15-0031-81	1 to 200 dilution
103130	1 to 200 dilution
65-0866-14	1 to 200 dilution
S3721	
11-5931-82	1 to 200 dilution
47-5321-80	1 to 200 dilution
552126	1 to 200 dilution
PI34042	

(see Table 5 for recipe)	
9920	
75004030	
1855485	
C298-500	
B26	
01-1234-42	
B81	
B82	
1156F03	
BSH1004	
Note: Locally collected	
E6511	
(see recipe in Table 5)	
38056	
B8	
RS-5135	
3110	
D6898	
BE0307	
1708891	
sc-363629Rx	
BP2618500	

10010200	
(see recipe in Table 5)	
116913050-CF	
SKU:116913100	
#000664	
30120.094	
CK30	
SKU:BS:607	
607	
B55	
TSCND2000C	
305167	
1706404	
3243	
BE17-516F	
AM2290	
AM2283	
RS-6802	
201100	
ACFP-70-5	1 to 10 dilution
Note: Locally collected	
(see recipe in Table 5)	
(see recipe in Table 5)	
(see recipe in Table 5)	
15596018	
P9416	
20447	
Note: Locally collected	

Alanazi et al., JoVE61183

"Identification and Characterization of Immunogenic RNA Species in HDM Allergens that Modulate Eosinophilic Lung Inflammation"

We thank the editor for fast handling our manuscript and three reviewers for their constructive critiques. Based on the comments from the editor and all three reviewers, we have expanded the introduction and discussion by clearly stating the overall goal and rationale for this manuscript and by providing more information on the advantages and limitations of the methods described in this manuscript. The detailed changes in text have been highlighted in blue. Our point-to-point responses are shown below.

Editorial comments:

Changes to be made by the Author(s):

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. The JoVE editor will not copy-edit your manuscript and any errors in the submitted revision may be present in the published version.

Re: we appreciate these instructions. We have carefully checked the manuscript and tried our best to correct any spelling and grammar errors.

2. Please format the manuscript as: paragraph Indentation: 0 for both left and right and special: none, Line spacings: single. Please include a single line space between each step, sub-step and note in the protocol section. Please use Calibri 12 points

Re: yes. we have now formatted the manuscript according to the above guidelines.

3. Please do not include the running title.

Re: yes. we have now removed the running title from the front page.

4. Please provide an email address for each author.

Re: yes. we have now added email addresses for all authors on the front page of this manuscript.

5. Please make the abbreviations and expanded term as two separate keywords.

Re: yes. we have expanded the abbreviation of each keyword into two separate words.

6. Please include a Summary to clearly describe the protocol and its applications in complete sentences between 10-50 words: “Present here is a protocol to …”

Re: yes. we have now provided a Summary on the front page of the manuscript.

7. Please do not include the video link details in the text. This is automatically generated later.

Re: yes. we have now removed the video link.

8. Please ensure that the Abstract is between 150-300 words.

Re: yes. we have revised the Abstract and confirmed the word number within the limit.

9. Please expand the Introduction to include all of the following:

a) A clear statement of the overall goal of this method

b) The rationale behind the development and/or use of this technique

c) The advantages over alternative techniques with applicable references to previous studies

d) A description of the context of the technique in the wider body of literature

e) Information to help readers to determine whether the method is appropriate for their application

Re: yes. we have added a new paragraph in the Introduction section to clearly state the overall goal and rationale

for this manuscript. Additionally, we have revised the text to provide more information on the advantages of the methods described in this manuscript.

10. JoVE cannot publish manuscripts containing commercial language. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials and Reagents. For example: Lysing Matrix D, Falcon, Trizol, Mini Beadbeater, Eppendorf, Styrofoam, etc.
Re: yes. all commercial languages have been removed from the text and placed into the corresponding tables.

11. Please include an ethics statement before your numbered protocol steps, indicating that the protocol follows the animal care guidelines of your institution.
Re: yes. we have now included an ethics statement before the protocol steps.

12. Please revise the protocol text to avoid the use of any personal pronouns in the protocol (e.g., "we", "you", "our" etc.).
Re: yes. we have verified that personal pronouns are not used in the protocol text.

13. The protocol steps should be numbered action steps. All the headings, subheadings and steps should be numbered. Please adjust the numbering of the Protocol to follow the JoVE Instructions for Authors. For example, 1 should be followed by 1.1 and then 1.1.1 and 1.1.2 if necessary. Please refrain from using bullets, alphabets, or dashes.
Re: yes. we have re-numbered the action steps by following above suggestions.

14. Please ensure that all text in the protocol section is written in the imperative tense as if telling someone how to do the technique (e.g., “Do this,” “Ensure that,” etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as “could be,” “should be,” and “would be” throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a “Note.”
Re: yes. we have used the imperative tense to describe the experimental procedures in the manuscript.

15. The Protocol should contain only action items that direct the reader to do something.
Re: yes. we confirm that this protocol contains only the sequential actions on how to perform the experiments.

16. Please ensure you answer the “how” question, i.e., how is the step performed?
Re: yes. the direction on how to perform the experiment at each step has been clearly spelled out in the text.

17. Please ensure that individual steps of the protocol should only contain 2-3 actions per step.
Re: yes. we confirm that each step contains no more than three actions.

18. There is a 10-page limit for the Protocol, but there is a 2.75-page limit for filmable content. Please highlight 2.75 pages or less of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol.
Re: yes. we thank the editor for the kind reminder and we have confirmed that content of this manuscript is within the page limit.

19. Please include at least one paragraph of text to explain the Representative Results in the context of the technique you have described, e.g., how do these results show the technique, suggestions about how to analyze the outcome, etc. The paragraph text should refer to all of the figures. Data from both successful and sub-optimal experiments can be included.
Re: yes. we have added one paragraph to explain the Representative Result associated with the described

technique.

20. Please obtain explicit copyright permission to reuse any figures from a previous publication. Explicit permission can be expressed in the form of a letter from the editor or a link to the editorial policy that allows re-prints. Please upload this information as a .doc or .docx file to your Editorial Manager account. The Figure must be cited appropriately in the Figure Legend, i.e. "This figure has been modified from [citation]."
Re: yes. we are currently in the process of obtaining explicit copyright permission to reuse the figures that was recently published in Journal of Immunology.

21. Please include all the Figure Legends together at the end of the Representative Results in the manuscript text. Please do not embed this in the figure.
Re: yes. we have moved the Figure Legends to the corresponding sections in the manuscript text.

22. As we are a methods journal, please revise the Discussion to explicitly cover the following in detail in 3-6 paragraphs with citations:
a) Critical steps within the protocol
b) Any modifications and troubleshooting of the technique
c) Any limitations of the technique
d) The significance with respect to existing methods
e) Any future applications of the technique
Re: yes. we have revised the Discussion by adding two new paragraphs and modified the relevant texts accordingly.

23. Please move all the recipes into a table and upload separately as .xlsx file to your editorial manager account.
Re: yes. we have moved all the recipes into a new **Table 9**.

24. All tables should be uploaded separately to your Editorial Manager account in the form of an .xls or .xlsx file. Each table must be accompanied by a title and a description after the Representative Results of the manuscript text and must be referenced in the text.
Re: yes. all tables have been uploaded separately and referenced in the manuscript text.

25. All figures should be uploaded separately to your Editorial Manager account. Each figure must be accompanied by a title and a description after the Representative Results of the manuscript text.
Re: yes. all figures have been uploaded separately. All figure legends have been placed at the relevant position of the manuscript text.

26. Please ensure that the references appear as the following: [Lastname, F.I., LastName, F.I., LastName, F.I. Article Title. Source. Volume (Issue), FirstPage – LastPage, (YEAR).] For more than 6 authors, list only the first author then et al.
Re: yes. we have formatted each reference to according to the above guideline.

Reviewer #1:
Major Concerns:
In the introduction section:
I am suggesting to elaborately explain the purpose of these experiments connecting real world application.
Re: yes, we have revised "the Introduction section" by adding a new paragraph to explain the goal of this protocol, which is "Based on the hygiene hypothesis orginally propoposed by Strachan [1], early childhood exposure to environmental microbial factors such as endotoxin can protect against the development of allergic disorders [2,3]. However, the underlying mechanisms remain largely undefined. Natural allergens such as house

dust mites (HDM) are often in complex forms containing both allergic proteins that drive aberrant type 2 responses and microbial substances that induce innate immune responses. Innate immune detection of those allergen-associated microbial components plays an important role in regulating the development of type 2 inflammatory conditions such as allergic asthma [4-6]. The overall goal of this protocol is to determine whether allergen-associated microbial RNA can activate innate immune responses to counteract the development of eosinophilic lung inflammation in a mouse model of allergic asthma."

In the discussion section:
Please discuss the specificity of the J2 antibody. What is it actually detecting? Any dsRNA structure, any sequence specificity? Cross-reactivity of the antibody, its limitation etc.
Re: we have added a new paragraph in the section of "discussion" to explain the specificity and limitations of J2 Ab, which is "In this protocol, the RNA dot blot has been employed to detect the presence of dsRNA structures in natural allergens with a mouse monoclonal antibody J2, which specifically binds to the dsRNA (≥40bp) independent of sequence. This method is highly reliable because J2 is unable to recognize dsRNA-containing samples pretreated with RNase III (a dsRNA-specific endonuclease), but not RNase T1 (a ssRNA-specific endonuclease). However, it is worth pointing out that a widely used synthetic analogue of dsRNA, Polyinosinic:polycytidylic acid [Poly(I:C)], has been reported to bind to J2 at a very low affinity [22-26]."

FACS analysis: Please provide description in detail how the analysis was done, what machine was used, etc.
Re: we have added a new Table (**Table 8**) to describe how to set up the running of FACS samples with the instrument, BD FACSCelesta™, and the obtained flow data was analyzed by FlowJo software with the gating strategy as shown in **Figure 3**.

Minor Concerns:
Minor errors in the manuscript to be corrected:
Line 65: Frozen > change to freeze
Re: corrected.

Lines 73, 76,112,115: Do > change to lower case letter (do)
Re: corrected.

Lines 77,95: H20 > H20
Re: corrected.

Line 87: Ant> Anti
Re: corrected.

Line 118: please provide the amount of RNA used in cDNA synthesis
Re: we used 100ng of RNA for the cDNA synthesis.

124: ……"treated with HDM allergens as…": how was treated? What was used to treat? What do you specifically mean by HDM allergens? HDM lysate, live HDM…?
Re: we mean HDM extract (100μg) that was treated with RNase III (1μl) in the RNase III reaction buffer.

Figure 1 legend: left and right panel switch
Re: corrected.

What is HT_DNA? How did you extract RNA (referencing to line 63)?
Re: HT-DNA is herring testis DNA. We used Trizol in combination with lysin matrix to extract RNA from HDM and

insects.

Figure 3: Correct the name of the figure (figure1 > figure3)
Table 1: D. farina > D. farinae
Re: corrected.

Reviewer #2:
Major Concerns:
In general, the authors failed to provide a detailed protocol for the different techniques. Lack of information, lack of rational of why this method and not another one, and lack of validation, controls, quality assessments. In the figures, authors are providing a bit more details, use of controls, and details about antibodies… but none of those controls are detailed in the manuscript. Tables are incomplete, procedures are simplistically described. No details about analysis and quality assessment.
Re: we thank the reviewer's constructive critiques. We have modified our manuscripts extensively to provide more details to address the reviewer's concerns. The changes are highlighted in blue.

Those protocols are known for long time, the authors should provide evidence of efficacy. Why are the procedures, in the context of HDM mouse models inflammations, described are providing more consistency, robustness, efficacy.
Re: the primary goal of this manuscript is to address an unknown role for natural allergens-associated immunostimulatory RNAs, dsRNA species in particular, in the development of allergic inflammation. The RNA Dot Blot and the gene expression analysis using RT-qPCR were used to build up the confidence to move into a function in vivo experiment, in this case, HDM-induced allergic inflammation. Thus, the sequential application of all these protocols are required.

Minor Concerns:
Introduction
1. The introduction is not adequate for the content of the paper. The introduction should provide a rational to explain why those methods can be used and how have they been validated (reference other papers that describe the methods and alternatives).
Re: we have added a new paragraph in the discussion to explain the specificity and limitations of J2 Ab. "In this protocol, the RNA dot blot has been employed to detect the presence of dsRNA structures in natural allergens with a mouse monoclonal antibody J2, which specifically binds to the dsRNA (≥40bp) independent of sequence. This method is highly reliable because J2 is unable to recognize dsRNA-containing samples pretreated with RNase III (a dsRNA-specific endonuclease), but not RNase T1 (a ssRNA-specific endonuclease). However, it is worth pointing out that a widely used synthetic analogue of dsRNA, Polyinosinic:polycytidylic acid [Poly(I:C)], has been reported to bind to J2 at a very low affinity [22-26]."

2. Confusion about HDM RNA? can author clarify what would be the source of dsRNA in house dust mites? Do they refer to existence of commensal viruses or bacteria present in the HDM? A bit of background would help.
Re: HDM RNA was extracted and purified from either HDM extracts or whole bodies (Greer lab). The source of dsRNA in HDM RNA currently remain unknown.

3. dsRNA extraction is not easy, many methods are available. Author should mention in references the different methods to isolates dsRNA.
Re: Here we used the Trizol reagent in combination with lysing matrix D to isolate total RNA (not dsRNA) from HDM and insects.

4. How do author justify the use of Trizol method and how did they validate this method?

Re: we used Trizol to extract total RNA, not dsRNA, from HDM, insects and mouse lung tissues. The RNA purity is based on the measurement of NanoDrop 200c, which the ration of absorbance 260/280 nm is ~2.

5. How does authors validate their dsRNA extraction? Please provide paper or Qiagen protocol where it is mentioned that dsRNA can be isolated using Trizol? Authors can bring that method as a novelty but then they have to show that using positive controls like culture of Viruses that have a lot of dsRNA.
Re: Trizol was used here to extract total RNA, not dsRNA, from HDM, insects and mouse lung tissues. The presence of dsRNA structures in total RNA was evaluated by the dsRNA-specific J2 Ab in combination with the treatment of dsRNA-specific endonuclease, RNase III.

6. Please provide evidence that Trizol will perform properly when diluted by more than 25%. (Qiagen usually recommend use of Trizol LS that is more concentrated) > see Qiagen information about Tissue RNA extraction using Trizol LS. However I'm not sure this is appropriate for dsRNA from Bacteria and viruses.
Re: Trizol reagent should not be diluted. We used 1 mL of the stock solution was used to extract RNA from allergens. The instruction can be found at:
https://www.thermofisher.com/order/catalog/product/15596026#/15596026.

7. The ratio Isopropanol / Trizol aquous phase is critical to obtain High quality RNA specifically for small RNA species. Please provide information about dsRNA solulbility in isopropanol. Author should suggest a ratio of isopropanol like 0.65x aqueous volume.
Re: we used chloroform (0.2 ml) / Trizol (1 ml) phase separation to extract total RNA. 2.5X volume of Isopropanol was added into a new tube that contains the aqueous sample (upper phase) to precipitate the total RNA.

8. The method stop at line 78. Then what to do? What are the Nanodrop dynamic range? what parameter to use on the Nanodrop? dsRNA, sRNA, DNA?? What are the parameter to allow experimentalist to assess the quality of extraction/dsRNA?
Re: Total RNAs isolated from HDM or insects will be applied for the Dot blot analysis and mouse lung delivery. We used Nanodrop 2000c to measure RNA samples, which has dynamic range, 2-15,000 ng/μl. The ratio of absorbance at 260 and 280 nm is used to assess the purity of RNA. A ratio of ~2.0 is generally accepted as "pure" for RNA.

9. What/how authors recommend storage of RNA extracted?
Re: It is recommended that the extracted RNA samples should be dissolved in RNase-free water and stored at -70 ^{o}C.

Immunoblotting assay for dsRNA.
10. A quick introduction would be helpful.
Re: Thanks for the excellent suggestion. We have added more information on the immunoblotting assay for dsRNA in the sections of "introduction" by saying that rapid detecting the dsRNA structure with RNA Dot blot in combination with a dsRNA-specific monoclonal antibody J2 and "discussion".

11. What control do authors suggest using, this is particularly important in radio-immuno assays since intensity of bands are not linear.
Re: we agree with the reviewer that control RNA or DNA samples are important in radio-immuno assays. As shown in Figure 1, HT-DNA and earthworm RNA should be added as the negative controls.

12. How many replicate would authors suggest? Any dilutions?
Re: In general, two replicates are recommended.

13. Author stop the description of the method at point 10. Line 95. So what is next? The method is incomplete.
Re: The next is to use these RNA samples isolated from HDM or insects to compare their in vivo immunostimulatory and modulatory activities using the RT-qPCR and FACS analysis, respectively.

RT-qPCR HDM RNA

14. What is the role of RNAse III? A 2-3 sentences introduction would facilitate the understanding of the reason why the method.
Re: RNase III is a dsRNA-specific nuclease. Together with ssRNA-specific nuclease RNase T1, they were used for the structure-function (immunostimulatory) analysis of RNA samples. More details have now been provided in the section of discussion.

15. The points 1 to 4 refers to mouse experiments not to RT-qPCR (title) > those points summarize a lot of technical procedures that should require more details or should be referred to another paper. " as recommended by …"
Re: yes, we have modified this part by providing more details on how to perform the RT-qPCR.

16. Point 5 to 15 list all points to isolate RNA and are not linked to RT-qPCR
Re: this point has been well taken. We revised this part by including two sections: **2.1** RNA isolation from lung tissues from mice treated with HDM RNA; **2.2** RT-qPCR to determine the ability of HDM RNA in stimulating lung gene expression.

17. No description of steps to RT-qPCR is described. Authors report to a kit without any description.
Re: we now have added all steps on how to set up and run a RT-qPCR.

18. No details about how to perform RT-qPCR and how to analyze the data. What is the linear dynamic rage of primers …
Re: we have added more details on how to perform RT-qPCR and analyze the data. **Table 5** shows the program of RT-qPCR on a CFX384 Touch™ Real-Time PCR Detection System.

19. This method is not to identify HDM RNAs (for what is it), but to measure differential gene expression induced by HDM-RNA in mouse lung. Title should be rephrased.
Re: yes, we have changed the title to "RT-qPCR to measure the activities of HDM RNA in stimulating ISG expression in mouse lungs."

BAL fluid collection for FACS

19. One quick introduction could help.
Re: Thanks for the excellent suggestion. We have added more information about BAL fluid collection for FACS analysis in the sections of "introduction" by accurate quantifying the number of eosinophils in BAL and lung in the context of HDM-induced lung inflammation using flow cytometry analysis and "discussion".

20. What is the objective of the method? BAL collection, cell isolation, FACs staining, cell counting?
Re: The overall goal of this protocol is to determine whether allergen-associated microbial RNA can activate innate immune responses to counteract the development of eosinophilic lung inflammation in a mouse model of allergic asthma.

21. Why do author use PBS+ EDTA? is there a paper that refer to this? If not maybe author should provide a small experience showing the worth to use EDTA.
Re: EDTA at 0.1 mM (**Table 9**) is used here to prevent the formation of cell clumping and should not cause any harms to cells at such a low concentration.

22. The g force of 1700g is quite high! Majority of protocol don't exceed 800g for 5 minutes (vast majority centrifuge at 400g for 5 min). Authors could explain why 1700g. 1700G may affect cell viability.
Re: we have changed the centrifuge speed to 500 g.

23. RBC is an osmolytic reagents, did authors test the effect of osmolytic shock on immune cells isolated from BAL? usually RBC treatment is suggested if BAL are very bloody. In that case, the experimentalist failed to produce a good BAL collection, 2. If slight RBC contamination due to hemorrhagic in the lung due to HDM reaction, FACS settings can avoid RBC lysis. Please comment.
Re: we agree with the reviewer that RBC treatment may not be necessary for BAL under normal conditions. However, inflammatory conditions induced by HDM treatment, severe lung inflammation leads to massive infiltrations of immune cells (Eos) into BAL. In such conditions, we incubate all BAL samples with RBC for a very short time (< 1min). Sun et al has previously reported that BAL fluid can be treated with RBC lysis buffer (below).

Sun, F., G. Xiao, and Z. Qu, *Murine Bronchoalveolar Lavage.* Bio Protoc, 2017. **7**(10).

24. Point 17 - line148: what is the solution?
Re: I change the "solution' into "cell suspension".

25. The number of centrifugations at 1500g is excessive.
Re: we have changed the centrifugation speed to 500g.

26. Once again, nothing is detailed about the FACS experiment per se. Authors describe the collection of the BAL and the isolation of the cellular fraction but nothing about FACS (antibody staining, FCgR blocking, FACS strategy).
Re: more details on FACS experiment have been added into **Table 7** and **8**.

Lung dispersion for FACS
27. Same questions as above.
Re: we have revised the section based on the above comments.

Discussion
28. The comments on RNAse is important and should be addressed in the procedure description.
Re: RNase III is a dsRNA-specific nuclease. Together with ssRNA-specific nuclease RNase T1, they are used here for the structure-function (immunostimulatory) analysis of RNA samples. More details have been provided in the section of discussion.

29. Discussion is incomplete.
Re: we have added a new paragraph in the discussion to explain the specificity and limitations of J2 Ab. "In this protocol, the RNA dot blot has been employed to detect the presence of dsRNA structures in natural allergens with a mouse monoclonal antibody J2, which specifically binds to the dsRNA (≥40bp) independent of sequence. This method is highly reliable because J2 is unable to recognize dsRNA-containing samples pretreated with RNase III (a dsRNA-specific endonuclease), but not RNase T1 (a ssRNA-specific endonuclease). However, it is worth pointing out that a widely used synthetic analogue of dsRNA, Polyinosinic:polycytidylic acid [Poly(I:C)], has been reported to bind to J2 at a very low affinity [22-26]."

Table with reagents
Authors should provide information about how to purchase those reagents
Re: we have included the vendor name and catalogue numbers for each product. For details, please refer to the **Tables (2, 3, 6, 7).**

Table 4 PCR primers: no sequences provided.
Re: the sequences of PCR primers are shown in **Table 4**.

Table 5. RT-PCR steps???
Re: we had added more information on how to set the RT-qPCR reaction in this section.

Table 7. Authors provide a list of antibodies but don't describe how to stain the cells, which was supposed to an objective of this paper. Please edit.
Re: **Table 7** shows the details on how to stain cells for FACS analysis.

Reviewer #3:
Major Concerns:
There are missing procedures: Length of isolated RNA (e.g. RNA seq)
Re: when resolved in gel, total RNAs isolated from HDMs and insects were observed to span over a wide (10-10,000 bp).

Purity and quality control of isolated RNA (e.g. microarray system)
Re: corrected. RNA purity was determined by NanoDrop 2000c and the 260/280 ratio around 2 is considered "pure".

Endotoxin content determination of isolated RNA when diluted in solution.
Re: we did not directly measure the level of endotoxin in our RNA samples isolated from HDM and insects. It is possible that endotoxin may be present in those samples.

Experiment on animal (information on gender, age, ethic approval, housing condition, …)
Re: The animal information is now provided in Table 6 and the ethic statement, which "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."

For FACS analysis indicate the name of the Flow cytometer used, data acquisition and analysis softwares.
Re: we have added a new Table (**Table 8**) to describe how to set up the running of FACS samples with the instrument, BD FACSCelesta™, and the obtained flow data was analyzed by FlowJo software with the gating strategy as shown in **Figure 3**.

Statistical analysis used in this study should be described on each figure legend if necessary.
Re: A separate paragraph of Statistical analysis has now been added.

Minor Concerns:
Line 87; correct Ant-dsRNA by anti-dsRNA
Re: corrected.

Table 7: include the dilution factor for each antibody used for FACS.
Re: we have added the dilution factor into Table 7.