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MNP triggers nasal allergy by modulating dendritic cell properties**Running title:** MNP facilitates nasal allergy

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34 **Keywords:** Nasal allergy; Air pollution; 3-methyl-4-nitrophenol; Dendritic cell;

35 Antigen presentation.

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38 **Capsule Summary**

- 39 • Exposure to MNP generates Th2 response-inducing dendritic cells
- 40 • Transfer of OVA/MNP-primed dendritic cells induces nasal allergy
- 41 • Inhibition of Bcl2L12 prevents nasal allergy development

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To the Editor,

It is recognized that the air pollution is associated with the pathogenesis of airway allergy^{1,2}. Diesel exhaust particles are recognized as one of the major sources of air pollution³. The 3-methyl-4-nitrophenol (MNP) is a component of the diesel exhaust particles² as well as the primary breakdown product of the excessively used insecticide fenitrothion⁴. Although the association between air pollution and airway allergy has been recognized⁵, the underlying mechanism remains to be further investigated. This study aims to elucidate the role of 3-methyl-4-nitrophenol (MNP) in the regulation of dendritic cell property and facilitates the development of allergic rhinitis (AR).

MNP triggers DCs' Th2 response-inducing properties

Mouse bone marrow-derived dendritic cells (BMDC) were prepared with established procedures⁶. BMDCs were exposed to MNP in the culture at gradient concentrations for 48 h. The cell extracts were prepared and analyzed by RT-qPCR and Western blotting. The results showed that exposure to MNP markedly increased levels of TIM4, Bcl2L12, MHC II and CD80 in BMDCs in an MNP concentration-dependent manner (Fig. 1). The results indicate that exposure to MNP increases the expression of Bcl2L12 and other Th2 response-generation relevant co-stimulatory molecules in DCs.

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67 **Bcl2L12 facilitates the formation of MHC II/allergen complex in DCs**

68 BMDCs were cultured with OVA and MNP for 48 h. The cells were collected at the
69 end of culture. Cell extracts were prepared with the cells and analyzed by
70 co-immunoprecipitation (IP). The results showed that a triple complex of MHC
71 II/Bcl2L12/OVA was detected in DCs exposed to both OVA and MNP (Fig. 2A), which
72 was much less in those treated with OVA alone (Fig. 2B). The results suggest that
73 Bcl2L12 facilitates the formation of MHC II/OVA complex in DCs. To corroborate the
74 results, BMDCs were generated with the bone marrows of the mice with the Bcl2L12
75 gene knockout (KO, in short). The KO DCs were treated with MNP and OVA in the
76 culture for 48 h. Indeed, the amounts of complex of MHC II/OVA were much less in
77 KO BMDCs (Fig. 2C) as compared with the wild type (WT) DCs (Fig. 2A). The results
78 indicate that Bcl2L12 facilitates the formation of the MHC II/OVA complex in DCs. To
79 corroborate the results, His-MHC II-expressing and Flag-Bcl2L12-expressing plasmids
80 were constructed and transfected into BMDCs to produce recombinant (r) MHC II
81 and rBcl2L12. The BMDCs were exposed to OVA in the culture for 48 h. The cells were
82 then analyzed by co-IP with antibodies of His, Flag and OVA, or isotype IgG as IP
83 antibodies. The results showed that a complex in the extracts of the BMDCs was
84 precipitated by the antibodies of either His (for MHC II), or Flag (for Bcl2L12), or OVA,
85 respectively (Fig. 2D), indicating that the rMHC II, rBcl2L12 and OVA can be processed
86 by BMDCs to form a triple complex. The rMHC II did bind OVA to form a complex in

BMDCs (Fig. 2E), but the amounts were much less than that in the presence of Bcl2L12 (Fig. 2D). The results demonstrate that Bcl2L12 enhances the formation of MHC II/OVA complex, while inhibition of Bcl2L12 does not affect the baseline of MHC II/OVA complex formation in DCs.

Bcl2L12 facilitates antigen presentation by DCs

BMDCs were prepared and primed by exposure to MNP and OVA for 48 h to generate the complex of MHC II/OVA on the surface of DCs (Fig. 2A). The primed BMDCs were cultured with CD4⁺ T cells (Teff; isolated from the DO11.10 mouse spleen) for 3 days. As analyzed by flow cytometry, co-culture with primed BMDCs induced marked Teff proliferation (Fig. S2A-B). The levels of IL-4, IL-5 and IL-13, but not IFN- γ , were increased significantly in the culture supernatant (Fig. S2C-F). The results demonstrate that Bcl2L12 facilitates the antigen presentation of DC, while BMDCs generated from KO mice did not show such an ability to present antigens to T cells (Fig. S2).

MNP facilitates sensitization in the airway of mice

BMDCs were prepared and primed by incubating with MNP and OVA, or OVA alone, or saline, in the culture for 48 h. Grouped BALB/c mice were adoptively transferred with the primed BMDCs via tail vein injection, which was repeated one week later. The mice were sacrificed one week after the second transfer. Teffs were isolated from

lung mononuclear cells (LMC) and spleen cells and analyzed by CFSE-dilution assay.

The results showed that mice received the BMDCs primed with both MNP and OVA,

but not either one alone, were sensitized to OVA as the high frequency of

OVA-specific CD4⁺ T cells was detected in the lung tissue and the spleen (Fig. S3).

MNP facilitates the development of airway allergy

Next, the primed WT BMDCs or primed KO BMDCs were adoptively transferred into

naïve BALB/c mice. Parameters relevant to nasal allergy were analyzed. The results

showed that adoptive transfer of primed WT BMDCs induced nasal allergy-like

changes in the recipient mice, including rhinocnesmus, sneezing and rhinorrhea;

increases in the content of OVA-specific IgE, Th2 cytokines and mouse mast cell

protease-1 (mMCP1) in the nasal mucosa, which did not occur in mice received

primed KO BMDCs (Fig. S4). The data are corroborated by treating mice with a

mixture of MNP and house mite extracts (HME), in which WT mice were sensitized by

the nasal drops of MNP/HME while none of Bcl2L12 KO mice were sensitized (Fig. S5).

The results demonstrate that MNP facilitates the development of nasal allergy in

mice, which can be prevented by inhibiting the expression of Bcl2L12.

MNP is a small molecule (MW=306.27), which is somewhat similar to a hapten, such

as dinitrophenyl (DNP), in the induction of an immune response⁷. While a hapten

has to conjugate with a large molecular carrier to initiate allergic responses⁸, MNP

enhances allergic responses in a different way by facilitating the expression of Bcl2L12 as shown by the present data.

In summary, the present data show a previously unknown phenomenon that exposure to MNP, one of the components of diesel exhausts, enhances the DC's ability of antigen presentation and drives DCs to produce the allergy development-relevant factors. Inhibition of Bcl2L12 can diminish the DC's ability of antigen presentation and the development of nasal allergy in mice.

Declare of conflict of interest: None.

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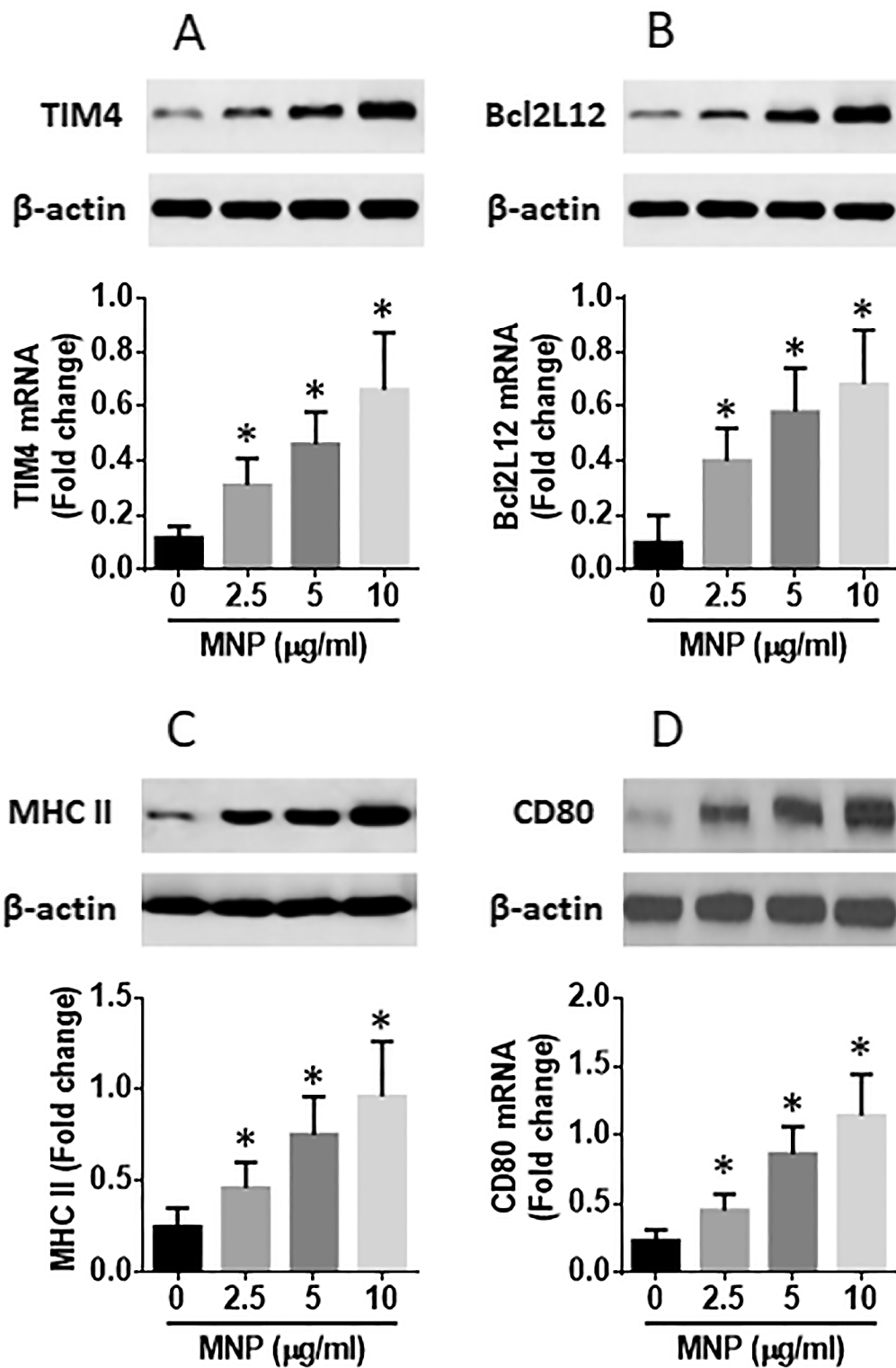
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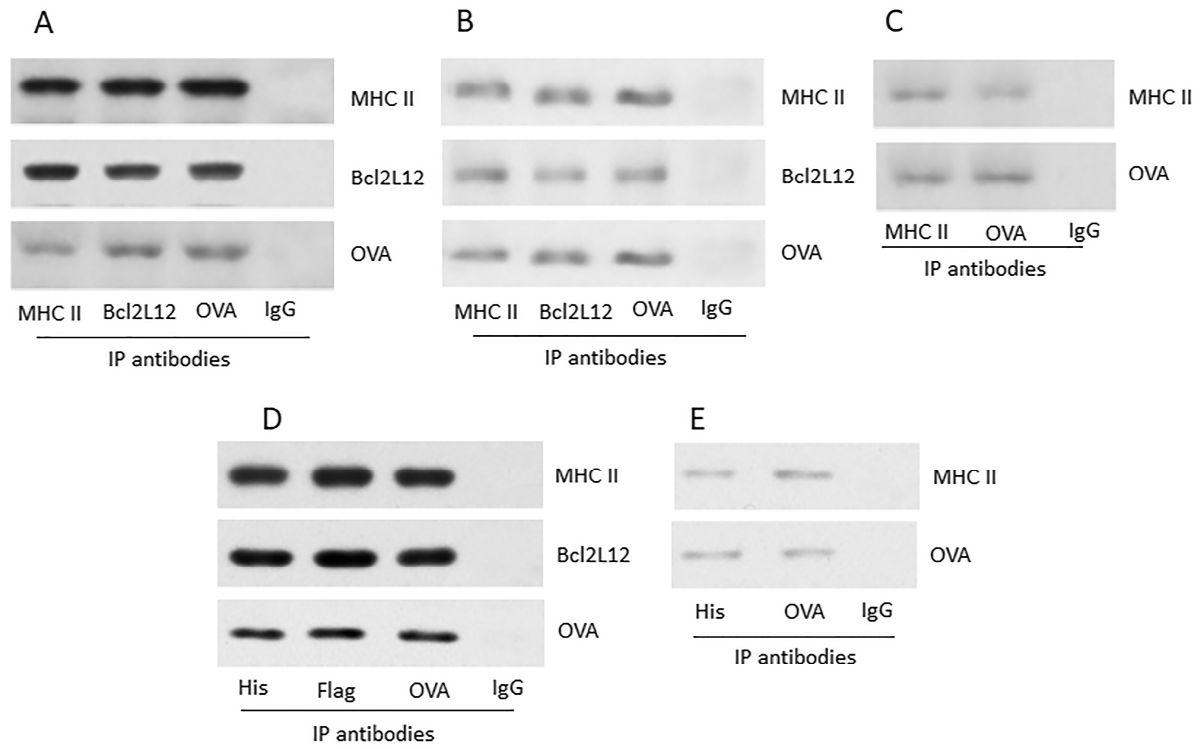
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Supplemental materials

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Running title: MNP facilitates nasal allergy

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Reagents

The antibodies of TIM4, Bcl2L12, MHC II, CD80, OVA, His and Flag were purchased from Santa Cruz Biotech (Santa Cruz, CA). The ELISA kits of OVA-specific IgE, mouse mast cell protease-1 (mMCP1), IL-4, IL-5 and IFN- γ were purchased from the BioMart (Beijing, China). The MNP, materials and reagents for immunoprecipitation were purchased from Sigma Aldrich (St. Louis., MO). The immune cell isolation kits were purchased from Miltenyi Biotech (San Diego, CA).

Mice

Male BALB/c mice (6-8 week old) were obtained from the Guangzhou Experimental Animal Center (Guangzhou, China). DO11.10 mice (BALB/c ground) were purchased from Jackson Laboratory (Bar Harbor, ME). The Bcl2L12 knockout (KO) mice were provided by the Animal Institute of Chinese Science Academy (Beijing, China. See the supplemental materials for detail). The mice were maintained in a specific pathogen-free facility with accessing water and food freely. The using mouse in the present study was approved by the Animal Ethics Committee at Shenzhen University.

Preparation of bone marrow-derived dendritic cells (BMDC)

BMDCs were prepared from the bone marrows of BALB/c mice following our established procedures {Feng, 2008 #20}. The cells were cultured in RPMI1640 medium supplemented with 10% fetal bovine serum, 0.1 mg/ml streptomycin, 100

U/ml penicillin and 2 mM glutamine. The medium was changed in 2-3 days. The cell viability was greater than 99% as checked by the Trypan blue exclusion assay.

Cell culture

Cells were cultured in RPMI1640 medium supplemented with 10% fetal bovine serum (BSA), 100 U/ml penicillin, 0.1 mg/ml streptomycin and 2 mM glutamine. The medium was changed in 2-3 days. The cell viability was greater than 99% as checked with Trypan blue exclusion assay. In some experiments, MNP was added to the culture to stimulate immune cells. The concentrations of MNP in the culture were referred to published data (1, Toxicology Letters 2009; 190: 61-65; 2, Biol. Pharm Bull 2003; 26: 394—395 and 3, Curr Opin Pulm Med 2012;18:144-50).

Enzyme-linked immunosorbent assay (ELISA)

Cytokine levels were determined by ELISA with commercial reagent kits following the manufacturer instructions.

Preparation of mice with the Bcl2L12 gene knockout (KO)

The BALB/c background Bcl2L12 KO mice were prepared by the Animal Institute of Chinese Science Academy (Beijing, China). The procedures of the Bcl2L12 KO mouse preparation are presented in the supplemental materials.

Preparation of protein extracts

Cells were collected from relevant experiments and incubated with a lysing buffer for 30 min. The supernatant was collected by centrifugation at 13,000 rpm for 10 min and used as the cytosolic extracts. The remained pellets were resuspended in a nuclear lysing buffer and incubated for 30 min. The supernatant was collected by centrifugation at 13,000 rpm for 10 min and used as the nuclear extracts. All the procedures were performed at 4 °C.

Western blotting

The proteins were fractioned by sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred onto a PVDF membrane. After blocking with 5% skim milk for 30 min, the membrane was incubated with the first antibodies of interest overnight at 4 °C, followed by incubating with the second antibodies (labeled with peroxidase) for 2 h at room temperature. The membrane was washed with Tris-buffered saline Tween 20 after each time of incubation. The immunoblots on the membrane were developed by the enhanced chemiluminescence and photographed with an imaging device.

Immunoprecipitation (IP)

The proteins were precleared by incubating with protein G agarose beads for 2 h to remove the non-specific immune complexes. The samples were then incubated with antibodies of interest or isotype IgG overnight. The immune complexes on the beads were collected by centrifugation (13,000 rpm; 10 min) and eluted with an eluting

buffer. The samples were then analyzed by Western blotting. All the procedures were performed at 4 °C.

Sensitization of mice with MNP/OVA-primed DCs

BMDCs were prepared and primed by incubating with MNP (10 µg/ml) and OVA (10 µg/ml) for 48 h. The primed BMDCs, or naive BMDCs, were adoptively transplanted into mice (10^6 cells per mouse) by tail vein injection for two times with one week apart. The mice were sacrificed one week after the second injection. $CD4^+ CD25^-$ T cells (Teff) were isolated from the lung and the spleen. The Teffs were labeled with CFSE, cultured with DCs in the presence of OVA (5 µg/ml) or bovine serum albumin (BSA; 5 µg/ml) for 3 days. The cells were analyzed by the CFSE-dilution assay. The levels of Th1 and Th2 cytokines in the culture supernatants were determined by ELISA. The rate of the Teff proliferation and Th2 cytokine levels in the culture were used as the indicators of mouse sensitization.

Challenging the airway mucosa with specific antigens

One week after the second transplantation of the primed DCs, the mice were treated with nasal drops containing OVA (50 µg/ml) daily for 7 days.

Assessment of nasal allergy in mice

The parameters of nasal allergy in the mice were assessed, including the following parameters: recording rhinocnesmus, sneezing and rhinorrhea within 1 h after the

last nasal challenge with the specific antigen (OVA). The levels of OVA-specific IgE, mouse mast cell protease-1 (mMCP1), Th2 cytokines and Th1 cytokine IFN- γ in the nasal mucosal protein extracts were determined by ELISA.

Preparation of the lung mononuclear cells (LMC) of mice.

The lungs were excised from mice upon sacrifice. The lung tissue was cut into small pieces and incubated with collagenase IV (1 mg/ml) at 37 °C for 1 h with mild agitation. The single cells were passed through a cell strainer (70 μ m) and collected by centrifugation.

Immune cell isolation

The effector T cells (Teff; CD3⁺ CD4⁺ CD25⁻) were isolated from the spleen cells or LMCs by the magnetic cell sorting (MACS) with purchased reagent kits following the manufacturer's instructions. If the purity of the cells did not reach 95%, the procedures of MACS were repeated with the cells.

T cell proliferation assay

T cell proliferation was assessed by the carboxyfluorescein succinimidyl ester (CFSE)-dilution assay. Teffs were isolated from the spleen and the lung, labeled with CFSE and cultured with DCs (Teff:DC = 10⁶:10⁵/ml) in the presence of OVA (5 μ g/ml) or BSA (5 μ g/ml) for 3 days. The cells were analyzed by flow cytometry.

Statistics

The data are presented as mean $\pm$ SD. The difference between two groups was determined by Student *t* test. ANOVA followed by followed by Dunnett's *t* test or SNK test was used for multiple comparisons. $P < 0.05$ was considered statistically significant.

Mouse Bcl2112 Knock out Project (CRISPR/Cas9)

The objective of this procedure is to create a Bcl2112 knockout mouse model (C57BL/6) by CRISPR/Cas-mediated genome engineering, which include:

The mouse Bcl2112 gene (GenBank accession number: NM_029410.3; Ensembl: ENSMUSG00000003190) was located on mouse chromosome 7. Seven exons were identified, with the ATG start codon in exon 2 and TGA stop codon in exon 7. Exon 2 to exon 3 and exon 3 were selected as target sites. Two pairs of gRNA targeting vectors were constructed and confirmed by sequencing. Cas9 mRNA, gRNA generated by in vitro transcription was co-injected into fertilized eggs for KO mouse production. The pups were genotyped by PCR followed by sequence analysis.

The targeting strategy includes:

A. Schematic depiction of targeting strategy

Genomic region of mouse Bcl2112 locus is diagrammed below (gene is oriented from left to right; total size is 6.36 kb). Solid bars represent ORF; open bars present UTRs.

B. gRNA target sequence

Pair 1

gRNA1 (matches forward strand of gene): AGCGTG TACGCCAGTGCACGGG

gRNA2 (matches forward strand of gene): TTCCGTGGTTGACC CGGGAGAGG

Pair 2

gRNA3 (matches reverse strand of gene): GGAGT TATCCCGAAGGGG TCTGG

gRNA4 (matches forward strand of gene): GTGGGACGTGTCCCG ATGGAGGGG

Fig. S1. Expression of Bcl2L12 in DCs of WT mice and Bcl2L12 KO mice. DCs were isolated from WT mice and Bcl2L12 KO mice. The total RNA was extracted from the organs and analyzed by RT-qPCR. The bars indicate the mRNA levels (A), the immunoblots (B) indicate the protein levels of Bcl2L12 in the main organs of WT mice and Bcl2L12 KO mice. The data are presented as mean $\pm$ SD. * $p < 0.01$, compared with the WT mice. The data represent 3 independent experiments.

Fig. S2. Bcl2L12 facilitates antigen presentation of DC. BMDCs were generated from the bone marrow of WT mice and Bcl2L12 KO mice. The cells were primed with MNP and OVA for 48 h. Naïve CD4⁺ T cells (Teff) were isolated from the spleen of DO11.10 mice. Teffs were labeled with CFSE and cultured with the primed BMDCs at gradient ratios for 3 days. The cells were analyzed by flow cytometry. A, gated histograms

indicate the proliferating Teffs. B, bars indicate the summarized data of proliferating Teffs in panel A. C-F, bars indicate the cytokine levels of CD4⁺ T cells in the culture supernatant. Data of bars are presented as mean $\pm$ SD. * $p < 0.01$, compared with the “10:0” group. The data represent 3 independent experiments.

Fig. S3. MNP/OVA-primed DCs sensitize mice. BMDCs were prepared and primed by incubating with MNP (10 $\mu\text{g/ml}$) and OVA ($\mu\text{g/ml}$), or OVA alone, or saline, in the culture for 48 h. BALB/c mice (6 mice per group) were adoptively transferred with the primed BMDCs (10^6 cells/mouse) via tail vein injection, which was repeated one week after. The mice were sacrificed one week after the second transfer. Teffs were isolated from the lung mononuclear cells (LMC) and spleen cells and analyzed by CFSE-dilution assay. A, gated histograms indicate the proliferating Teffs after treating with reagents in the culture as denoted above each panel in the presence of DCs and OVA (5 $\mu\text{g/ml}$) or BSA (#; 5 $\mu\text{g/ml}$). B, bars indicate the summarized data of proliferating Teffs in panel A. Data of bars are presented as mean $\pm$ SD. * $p < 0.01$, compared with the saline group.

Fig. S4. Adoptive transfer with the MNP/OVA-primed BMDCs induces AR in mice.

BMDCs were prepared with the bone marrows of WT and KO mice. The cells were primed by exposure to MNP/OVA in the culture for 48 h. The BMDCs (10^6 cells/mouse) or saline (control) were injected into BALB/c mice via tail vein puncture; the injection was repeated one week after. One week after the second injection, the

mice were challenged by nasal drops containing OVA (5 mg/ml). A-C, the nasal symptoms (rhinocnesmus, sneezing and rhinorrhea) were recorded within 1 h after the challenge with nasal drops. D-H, the mice were sacrificed 1 h after the nasal challenge. The nasal mucosa was collected from each mouse and processed for protein extracts. The levels of specific IgE, mMCP1, IL-4, IL-5 and IFN- γ in the protein extracts were determined by ELISA as shown by the bars. Data of bars are presented as mean $\pm$ SD. * $p < 0.01$, compared with the saline group. Each group consists of 6 mice.

Figure S5. Inhibition of Bcl2L12 abolishes MNP/HME-induced AR in mice. BALB/c (WT) mice (n=6) and Bcl2L12 knockout (KO) mice (n=6) were treated with a mixture of MNP (10 μ g/ml) and house mite extracts (HME; 10 μ g/ml) by nasal drops (50 μ l/nostril/time) daily for 21 consecutive days. Control mice were treated with saline nasal drops. Mice were sacrificed on day 21, 1 h after treating with the nasal drops. A-C, AR clinical symptoms, rhinocnesmus (A), sneezing (B) and rhinorrhea (C), were recorded on day 21, 0-60 min after treating with nasal drops. D-E, blood samples were collected from each mouse. The sera were isolated from the samples and analyzed by ELISA. Bars indicate serum levels of sIgE (D) and mMCP1 (E). F-H, nasal mucosa was collected from each mouse immediately after the sacrifice. Protein extracts were prepared with the nasal mucosal samples and analyzed by ELISA. The bars indicate levels of IL-4 (F), IL-5 (G) and IFN- γ (H) in nasal mucosal extracts. Data of

bars are presented as mean $\pm$ SD. Samples from individual mice were analyzed separately in triplicate.

