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Does reduced ZPBP2 expression on chromosome 17q21 protect against asthma ?

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Capsule Summary:

ZPBP2 is located on chromosome 17q21, a gene region highly linked to asthma. House dust mite (HDM) challenge in wild type mice induced expression of ZPBP2 in the lung epithelium. ZPBP2 deficient mice have reduced AHR to HDM challenge. Lack of ZPBP2 expression may thus protect against AHR and asthma.

Keywords:

ZPBP2

Asthma

AHR

Abbreviations:

AHR	Airway hyperreactivity
BAL	Bronchoalveolar lavage
HDM	House dust mite
Mch	Methacholine
WT	Wild type
ZPBP2	Zona Pellucida Binding Protein 2
GSDMB	Gasdermin B
GSDMA	Gasdermin A
IKZF3	IKAROS family zinc finger 3
LRRC3C	Leucine rich repeat containing 3C

To the Editor:

Chromosome 17q21, is highly linked to asthma in genetic association studies¹, and contains a cluster of six genes (including Zona Pellucida Binding Protein or ZPBP2 the focus of this study, as well as ORMDL3, GSDMB, GSDMA, IKZF3, LRRC3C)(**Figure 1A**). Functional studies of the biology of these six chromosome 17q21 genes in relation to asthma have predominantly focused on ORMDL3 and to a lesser degree on GSDMB in lung structural, immune, and inflammatory cells²⁻⁸. These studies have identified that either increased ORMDL3 expression³ or increased GSDMB⁸ expression can result in increased AHR characteristic of asthma. In contrast, there is limited information on whether ZPBP2, located next to GSDMB on chromosome 17q21 (**Figure 1A**), is expressed in lung structural, immune, or inflammatory cells following allergen challenge and whether it plays a role in the pathogenesis of asthma. In this study we have used a mouse model of house dust mite (HDM) induced asthma to determine whether ZPBP2 is expressed in the lung following HDM challenge and whether airway hyperresponsiveness (AHR) is influenced in mice deficient in ZPBP2 (all methods described in the **Supplementary On-Line Repository Methods**).

ZPBP2 is highly expressed in sperm where it is known to be required for the formation of mature spermatozoa⁹. During fertilization ZPBP2 binds to the zona pellucida which surrounds the oocyte to allow sperm penetration⁹. As ZPBP2 is highly expressed in sperm, whether it could also be linked to expression in structural, immune, or inflammatory cells pertinent to the pathogenesis of asthma in males and females is at present uncertain. We therefore initially examined whether ZPBP2 was expressed in the lungs of wild type (WT) mice challenged with HDM. WT mice challenged with HDM expressed significantly increased ZPBP2 mRNA as

quantitated by copy number assessed by digital PCR (**Figure 1B**). The specificity of the ZPBP2 mRNA we detected in WT mouse lung challenged with HDM was validated by the lack of ZPBP2 mRNA detection in ZPBP2 KO mice challenged with HDM.

To determine which lung cells pertinent to the pathogenesis of asthma might express ZPBP2 we quantitated levels of ZPBP2 mRNA in purified populations of mouse structural as well as immune and inflammatory cells pertinent to the pathogenesis of asthma (**Fig 1C**). These studies demonstrated that macrophages, T cells, B cells, and eosinophils expressed detectable copy numbers of ZPBP2 mRNA. In addition, lower copy numbers of ZPBP2 mRNA were detected in structural cells (epithelium, smooth muscle, fibroblast). The specificity of the ZPBP2 copy number assay was validated in WT mouse testis which had very high ZPBP2 mRNA copy number, while lungs from ZPBP2 KO mice had no detectable levels of ZPBP2.

We also examined whether HDM challenge in WT mice induced increases in ZPBP2 mRNA copy number in selected lung cells (epithelium, eosinophils, T cells, macrophages) isolated by bronchial brushing or FACS sorting. These studies demonstrated that in WT mice there was a significant increase in ZPBP2 mRNA copy number expression in airway epithelium ($P < 0.05$; HDM WT vs no HDM WT)(**Fig 1D**), with no significant ZPBP2 copy number detected in eosinophils, T cells, or macrophages. Interestingly, HDM challenge increased lung protein levels of TSLP (**Fig 1E**) and IL-13 (**Fig 1F**) in WT but not in ZPBP2 KO mice (**Fig 1 E-F**). Neither WT nor ZPBP2 KO mice had an increase in lung IFN- γ following HDM challenge (**Fig 1G**).

Studies of AHR demonstrated that WT mice challenged with HDM had a significant increase in AHR compared to WT mice not challenged with HDM in both male mice ($p < 0.05$) (**Fig 2A**), and female mice ($p < 0.05$) (**Fig 2B**). In contrast, both male ZPBP2 KO mice (**Fig 2A**) ($p < 0.05$) and female ZPBP2 KO mice (**Fig 2B**) ($p < 0.05$) challenged with HDM had significantly reduced AHR compared to WT mice challenged with HDM.

To determine whether a deficiency of ZPBP2 influenced levels of HDM induced lung inflammation, T cells, or IgE levels, we quantitated levels of peribronchial MBP⁺ eosinophils and CD4⁺ cells by immunohistochemistry and image analysis, and IgE by Elisa as previously described³. These studies demonstrated that both male and female ZPBP2 KO mice challenged with HDM had reduced peribronchial eosinophils (**Fig 2C, 2D**) and reduced peribronchial CD4⁺ cells (**Fig 2E, 2F**) compared to WT mice challenged with HDM. Similarly, both male and female ZPBP2 KO mice challenged with HDM had reduced BAL eosinophils (**On-line Supplementary Fig E1 A-B**) and reduced BAL lymphocytes (**On-line Supplementary Fig E1 C-D**) compared to WT mice challenged with HDM. There was no difference in levels of IgE in HDM challenged WT compared to HDM challenged ZPBP2 KO mice in males or females (**Fig 2G, 2H**).

The similar level of reduction in AHR and airway inflammation in male and female HDM challenged ZPBP2 KO mice, suggests that cells other than sperm expressing ZPBP2 were responsible for the protection against AHR. Indeed, our studies demonstrate that several cell types pertinent to the pathogenesis of asthma can express low levels of ZPBP2 mRNA, and that HDM challenge induced increased ZPBP2 levels in airway epithelium. Interestingly, ORMDL3 is also expressed in airway epithelium², and ZPBP2 KO mice have reduced levels of ORMDL3

mRNA, but not *ORMDL1* or *ORMDL2* mRNA (**On-line Supplementary Fig E2**) suggesting that *ZPBP2* may be regulating *ORMDL3*. The specific cell type (s) expressing *ZPBP2* that are responsible for mediating the protection against AHR and airway inflammation in the mouse model of asthma are at present unknown, as is the mechanism and importance of reduced *ORMDL3* expression in the protection which will require further study.

Overall, these studies suggest that *ZPBP2* is expressed at low levels following HDM challenge in mouse lung cells pertinent to the pathogenesis of asthma, and that based on *in vivo* studies in *ZPBP2* KO mice a lack of *ZPBP2* protects against the development of HDM induced airway inflammation and AHR. In contrast to reductions in *ZPBP2* protecting against AHR, increased *ORMDL3* or increased *GSDMB* expression *in vivo* in mice induces increased AHR^{3,8}. Interestingly, a *cis*-regulatory haplotype regulating the expression of chromosome 17q21 genes (*ORMDL3*, *GSDMB*, *ZPBP2*) and linked to asthma has been reported^{E1} and is associated with higher expression of *ORMDL3* and *GSDMB* and lower expression of *ZPBP2*. Thus, our studies of the biology of these three genes would suggest that if an asthmatic had this haplotype, the increased *ORMDL3* and increased *GSDMB* associated with the haplotype would contribute to the pathogenesis of asthma, whereas reduced expression of *ZPBP2* associated with the haplotype would protect against the development of asthma. However, further studies are needed to determine whether human lung cells pertinent to the pathogenesis of asthma express *ZPBP2* and whether modulating levels of *ZPBP2* expression in these cells influences cellular functions pertinent to asthma to reduce the development of increased AHR and airway inflammation associated with asthma.

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FIGURE LEGENDS

Figure 1. ZPBP2 expression in lung and lung cells

A. Six chromosome 17q21 localized genes (IKZF3, ZPBP2, GSDMB, ORMDL3, LRRC3C, GSDMA) are depicted with arrows indicating the direction of transcription. **B.** WT mice were challenged with HDM intranasally on day 0, 7, 14, 21. On day 24 lung tissues (WT no HDM; WT HDM) (n = 8 mice/group) were processed for quantitation of ZPBP2 mRNA copy number. **C.** Levels of ZPBP2 mRNA copy number were quantitated in mouse testes and purified populations of mouse eosinophils (bone marrow), T cells (spleen), B cells (spleen), macrophages (mac)(bone marrow), smooth muscle cell (smc)(tracheal), fibroblast (fibr)(lung), epithelial cell line MLE12 (epi), as well as from lungs of ZPBP2 KO mouse (ZPBP2 KO). **D.** Levels of ZPBP2 mRNA copy number were quantitated in mouse airway epithelial cells obtained by bronchial brushing, and FACS sorted lung eosinophils, T cells, and macrophages before and after HDM challenge of WT mice (n=3 mice/group). **E-G.** Levels of lung TSLP (**E**), lung IL-13 (**F**), and lung IFN- γ (**G**) were quantitated by ELISA (n=8 mice/group).

Figure 2. Reduced AHR and inflammation in HDM challenged ZPBP2 KO mice

WT or ZPBP2 KO mice (**A**, male group; **B**, female group) were challenged with HDM intranasally on day 0, 7, 14, 21. On day 24 airway resistance in response to methacholine was measured by flexiVent. WT + HDM vs WT no HDM, $^{**}P < 0.01$, (Mch 48 mg/ml); ZPBP2 KO +HDM vs ZPBP2 KO no HDM, p= NS (not significant)(Mch 48 mg/ml). **C-F.** Lung tissue from WT or ZPBP2 KO mice (**C**, **E**, male groups; **D**, **F** female groups) was processed for immunohistochemistry to detect MBP⁺ eosinophils (**C**, **D**), and CD4⁺ lymphocytes (**E**, **F**). **G-H.**

230 Serum from WT or ZPBP2 KO mice (**G**, male group; **H**, female group) was processed for
231 ELISA to detect IgE.

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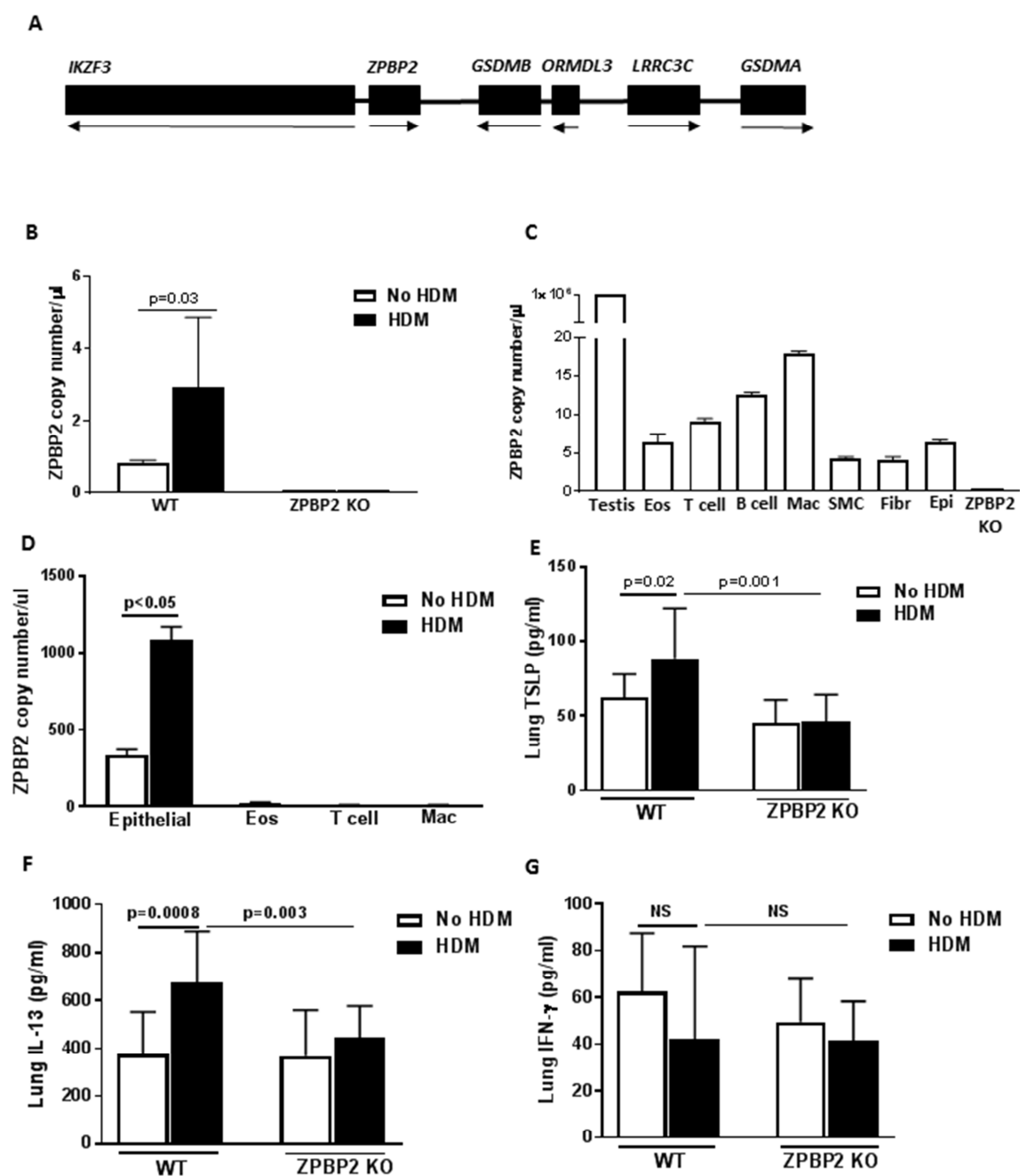


Figure 1

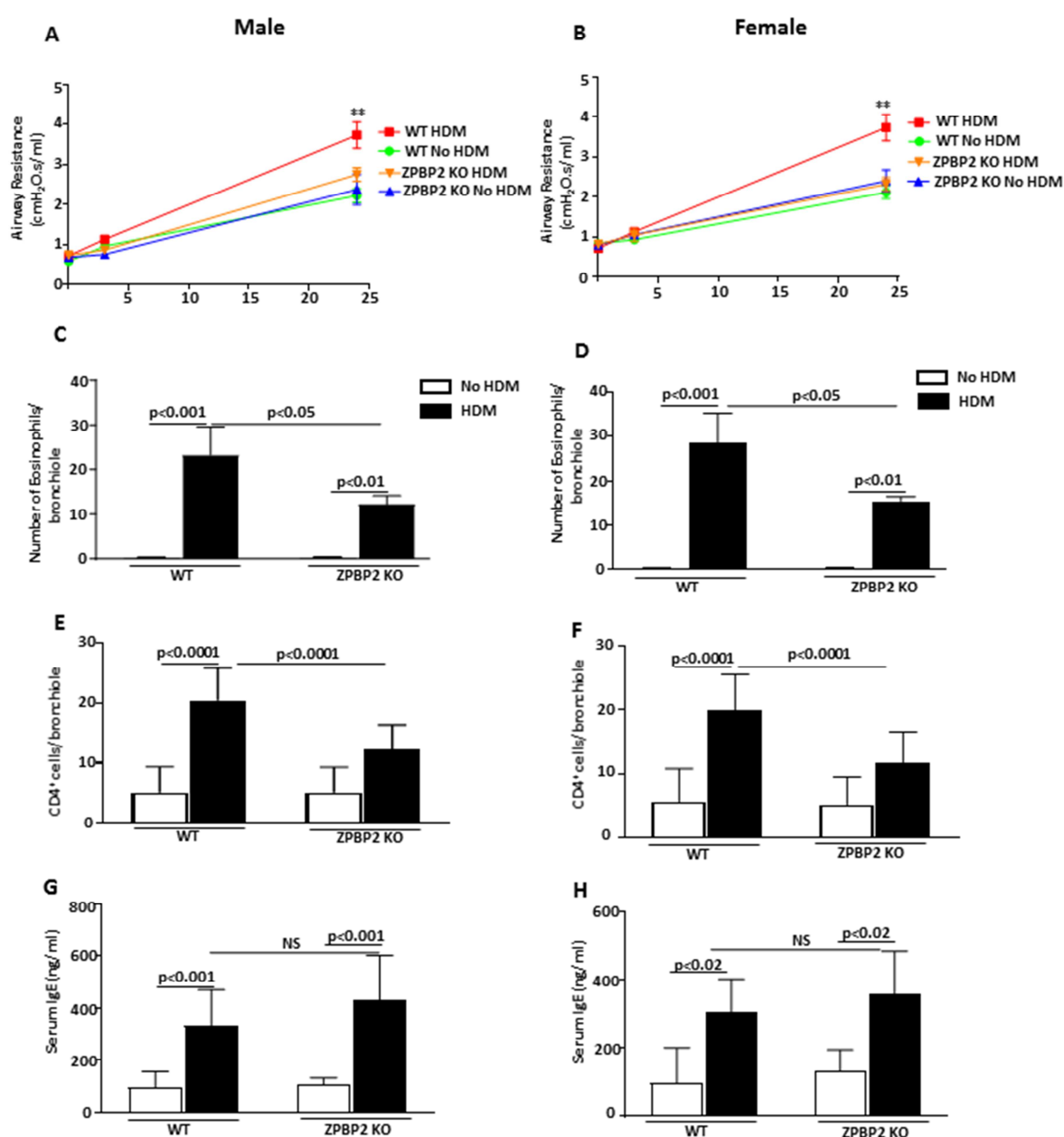


Figure 2

SUPPLEMENTARY ONLINE REPOSITORY**Does reduced ZPBP2 expression on chromosome 17q21 protect against asthma ?**

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SUPPLEMENTARY METHODS

Mouse model of house dust mite (HDM) induced asthma

ZPBP2 deficient mice (Jackson labs)^{E1,E2} on a C57Bl6 background and littermate control C57Bl6 mice (here after referred to as WT) aged approximately 12 weeks were either challenged with HDM (Greer)(HDM group)(n=8 mice/ group), or not challenged with HDM (no HDM group) (n=8 mice/ group) as previously described^{E3}. In brief in the HDM group, ZPBP2 deficient or WT mice were intranasally administered 100 µg HDM in 50 µl PBS on day 0, 7, 14, and 21. On day 24, AHR was measured, mice were sacrificed and lungs collected to quantitate levels of airway inflammation and ZPBP2 mRNA copy number as described below. All experimental mouse protocols were approved by the UCSD Institutional Animal Care and Use Committee.

Measurement of airway hyperresponsiveness

Airway responsiveness to methacholine was assessed in intubated and ventilated mice aged 12 wk ($n = 8$ mice/group) (flexiVent ventilator; Scireq) using Scireq software on day 24 of the HDM mouse protocol. Mice were anesthetized with ketamine (100 mg/kg) and xylazine (10 mg/kg) i.p. and exposed to nebulized PBS and methacholine (0, 3, and 24 mg/ml) as previously described^{E3}. The following ventilator settings were used: tidal volume (10 ml/kg), frequency (150/min), and positive end-expiratory pressure (3 cmH₂O).

Lung processing

Lungs were processed for immunohistology (paraffin-embedded lung sections) as previously described in this laboratory^{E3}. In brief, lungs were equivalently inflated with an intratracheal

injection of the same volume of 4% paraformaldehyde solution (Sigma Chemicals, St. Louis, MO) to preserve the pulmonary architecture.

Mouse Bronchial Epithelial Cells

Purified populations of mouse bronchial epithelial cells were obtained by bronchial brushing as previously described in detail in this laboratory^{E3}. Cytospun cells from bronchial brushings have the ultrastructural appearance of ciliated airway epithelial cells and immunostained positive for the epithelial marker E-cadherin^{E4}. Compared with whole-lung cell suspensions that contained both CD45⁺ and CD45⁻ cells, the cells from the airway brushing were nearly all CD45⁻ by FACS^{E4}. ZPBP2 mRNA copy number was quantitated in bronchial epithelial cells using a digital PCR method as described below.

Mouse lung T cells, eosinophils and macrophages

Whole lungs from no HDM and HDM challenged mice were processed into single cell suspensions using a lung dissociation kit and a tissue dissociator (Miltenyi Biotec, Auburn, CA). Lung single cell suspensions were pre-incubated with anti-mouse CD16/CD32 antibodies for 10 minutes on ice. To identify lung macrophages, T cells, and eosinophils, cells were stained with CD45.2, CD11c, SiglecF, CD3, Gr-1. Lungs cells were then sorted on a BD FACS Aria II (BD Biosciences) cell sorter. T-cells were identified as CD45.2+CD3+, eosinophils as CD45.2+SiglecF+CD11c-, and macrophages as CD45.2+CD11c+Gr1-. All antibodies used for the FACS sorter studies were from BioLegend (San Diego, CA) except for anti-SiglecF which was from BD Bioscience (San Jose, CA). ZPBP2 mRNA copy number was quantitated in lung macrophages, T cells, and eosinophils using a digital PCR method as described below.

Peribronchial lung eosinophils and lymphocytes.

Levels of lung eosinophils and CD4⁺ lymphocytes were quantitated in the peribronchial space in lung sections processed for immunohistochemistry using an anti-mouse MBP Ab (rabbit polyclonal Ab kindly provided by James Lee PhD, Mayo Clinic, Scottsdale, Arizona), and an anti-mouse CD4 Ab (rat monoclonal Ab, GTX85525, GeneTex) as previously described^{E3}. The number of individual cells staining positive in the peribronchial space was counted using a light microscope. Results are expressed as the number of peribronchial cells staining positive per bronchiole with 150–200 μ m of internal diameter. At least five bronchioles were counted in each slide.

Assessment of BAL inflammation

BAL fluid was collected by lavaging the lung with 1 ml PBS via a tracheal catheter as previously described^{E3}. BAL eosinophil and lymphocyte cell counts were quantified in Wright Giemsa stained slides.

ZPBP2 mRNA copy number

In brief, total RNA was extracted from WT and ZPBP2 deficient lung or testes with RNA-STAT-60 (Tel-Test) and reverse transcribed with Oligo-dT and SuperScript II kit (Life Technologies). In addition RNA was obtained from WT mouse bone marrow eosinophils (3H Biomedical), spleen T cells (3H Biomedical), spleen B cells (3H Biomedical), bone marrow macrophages (ATCC), primary tracheal smooth muscle cells (Cell Biologics), lung fibroblasts (ScienCell), and a mouse lung epithelial cell line MLE12 (ScienCell). ZPBP2 mRNA copy number was quantitated using a digital PCR method, in which the reaction mixture contained

500 ng of target cDNA, Supermix (Bio-Rad), as well as ZBPB2 specific primers and probe (Applied Biosystems). The reaction mixture was loaded into the sample well of the disposable droplet generator cartridge (Bio-Rad). After the droplet generation, the mixture was transferred into a 96-well PVR plate and then amplified on the Bio-Rad T100 Thermal Cycler. The plate was analysed with a QX200 droplet Reader and the dPCR data were processed using the QuantaSoft software (Bio-Rad)^{E5}. Results are expressed as ZBPB2 mRNA copy number/ μ l.

IgE

Levels of serum IgE were quantitated by ELISA (BD Biosciences, San Diego, CA) as previously described^{E3}.

ORMDL3 levels

Levels of ORMDL3 mRNA (as well as ORMDL1 and ORMDL2 mRNA) was quantitated by qPCR in the lungs of ZBPB2 Deficient and WT mice. Primers were obtained from Life Technology (Carlsbad, CA) for ORMDL3 (Cat # Mm00787910), ORMDL1 (Cat # Mm00523629, and ORMDL2 (Cat # MM01160074).

Statistical analysis

All results are presented as mean $\pm$ SEM. A statistical software package (Graph Pad Prism, San Diego, CA) was used for the analysis. A t test was used for analysis of two groups. ANOVA analysis was use when more than two groups were compared. P values of < 0.05 were considered statistically significant.

SUPPLEMENTARY FIGURE LEGENDS:**Supplementary Figure E1. ZPBP2 Deficient vs WT mice BAL eosinophils and T cells**

ZPBP2 deficient or WT mice (n = 8 mice/group) were challenged with HDM intranasally on day 0, 7, 14, 21. On day 24, levels of BAL eosinophil and T cell inflammation was quantitated. The number of Wright-Giemsa stained **A**, BAL eosinophils, and **B**, BAL lymphocytes were quantitated by light microscopy. * $P < 0.05$, *** $P < 0.001$, and NS (not significant).

Supplementary Figure E2. ORMDL3 mRNA levels in ZPBP2 Deficient vs WT mice

Levels of ORMDL3 mRNA (as well as ORMDL1 and ORMDL2 mRNA) was quantitated by qPCR in the lungs of ZPBP2 Deficient and WT mice (n=8 mice/group).

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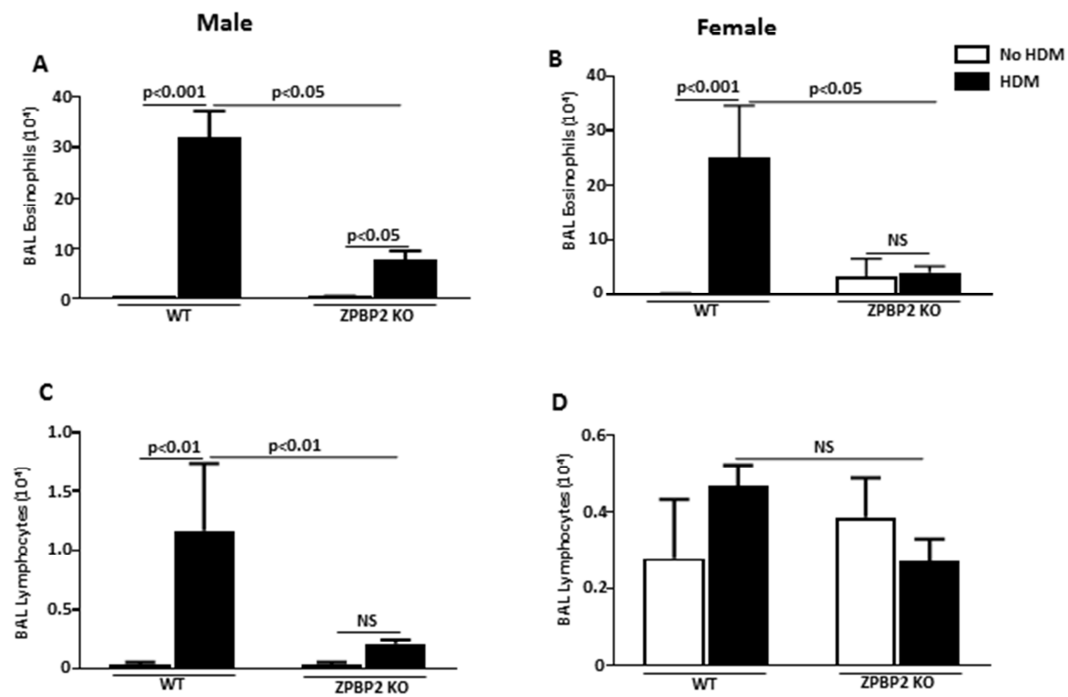


Figure E1

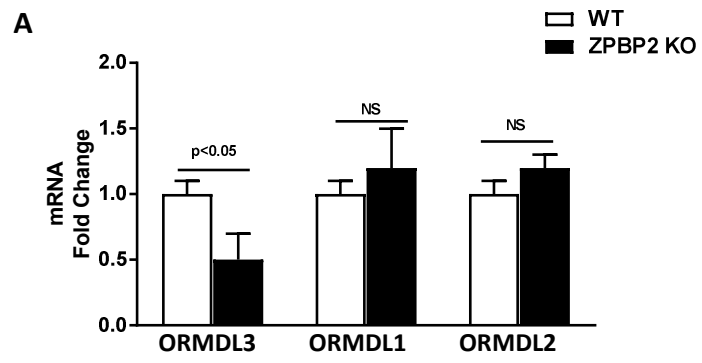


Figure E2