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GSDMB induces an asthma phenotype characterized by increased airway responsiveness and remodeling without lung inflammation

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Gasdermin B (GSDMB) on chromosome 17q21 demonstrates a strong genetic linkage to asthma, but its function in asthma is unknown. Here we identified that GSDMB is highly expressed in lung bronchial epithelium in human asthma. Overexpression of GSDMB in primary human bronchial epithelium increased expression of genes important to both airway remodeling [TGF- β 1, 5-lipoxygenase (5-LO)] and airway-hyperresponsiveness (AHR) (5-LO). Interestingly, hGSDMB^{Zp3-Cre} mice expressing increased levels of the human GSDMB transgene showed a significant spontaneous increase in AHR and a significant spontaneous increase in airway remodeling, with increased smooth muscle mass and increased fibrosis in the absence of airway inflammation. In addition, hGSDMB^{Zp3-Cre} mice showed increases in the same remodeling and AHR mediators (TGF- β 1, 5-LO) observed in vitro in GSDMB-overexpressing epithelial cells. GSDMB induces TGF- β 1 expression via induction of 5-LO, because knockdown of 5-LO in epithelial cells overexpressing GSDMB inhibited TGF- β 1 expression. These studies demonstrate that GSDMB, a gene highly linked to asthma but whose function in asthma is previously unknown, regulates AHR and airway remodeling without airway inflammation through a previously unrecognized pathway in which GSDMB induces 5-LO to induce TGF- β 1 in bronchial epithelium.

GSDMB | asthma | airway-hyperresponsiveness | remodeling | inflammation

Chromosome 17q21 was initially linked to asthma in genome-wide association studies (GWAS) in 2007, with confirmation in multiple GWAS and non-GWAS studies in populations of diverse ethnic backgrounds (1, 2). Chromosome 17q21 contains a cluster of four genes [ORMDL3, gasdermin B (GSDMB), IKZF3, and ZPBP2] (2), which may, either individually or in combination, be responsible for its genetic association to asthma. A precedent for linkage of a cluster of genes in a single chromosomal region with asthma is evident from chromosome 5q31-33, where the genes IL-4, IL-5, IL-9, and IL-13 are located (3). Although there are many genetic epidemiologic studies linking chromosome 17q21 with asthma, there are limited functional studies of each of these four genes to understand how they contribute to the pathogenesis of asthma. We have previously demonstrated the importance of one of the 17q21 localized genes (i.e., ORMDL3) to asthma, airway responsiveness, and airway remodeling (4, 5). Mice overexpressing human ORMDL3 regulated downstream pathways (ATF6 α , SERCA2b, metalloproteases) important in asthma. Interestingly, mice overexpressing human ORMDL3 developed spontaneous increased airway responsiveness and airway remodeling in the absence of airway inflammation (5).

In this study, we are focusing on a second gene on chromosome 17q21, namely GSDMB, because little is known about its expression and function in the normal lung or asthmatic lung, and published studies of GSDMB in asthma are limited to

genetic association studies. The human GSDMB gene located on chromosome 17q21 belongs to the gasdermin (GSDM) protein family (6, 7). The human GSDM family consists of four members: GSDMA, GSDMB, GSDMC, and GSDMD. The GSDMA and GSDMB genes are located at 17q21, and the GSDMC and GSDMD genes are located at 8q24. Several genetic linkage studies have shown an association with GSDMB on chromosome 17q21 and asthma (2, 8), but none of the studies have linked asthma with chromosome 8q24 (where GSDMC and GSDMD are located). This finding suggests that GSDMB (and perhaps GSDMA) are the only GSDM family members linked to asthma. The function of GSDMB (a 416-amino acid protein) is largely unknown because GSDMB is a novel protein without characteristic domains related to known function (6, 7). The single-nucleotide polymorphism (SNP) linked to chromosome 17q21 and asthma is associated with increased expression of both GSDMB and ORMDL3 (genes located next to each other on chromosome 17q21) (2). Several studies indicate a strong linkage disequilibrium between the SNPs of *ORMDL3* and *GSDMB* (1, 2), suggesting that these genes could be acting in concert toward asthma pathophysiology. However, at present the function of GSDMB in the lung and in asthma is largely unknown.

Significance

Because the SNP linking chromosome 17q21 to asthma is associated with increased gasdermin B (GSDMB) expression, we generated transgenic mice expressing increased levels of the human GSDMB transgene (hGSDMB^{Zp3-Cre}), which develop an asthma phenotype characterized by a spontaneous increase in airway responsiveness and airway remodeling (increased peribronchial smooth muscle) in the absence of the development of airway inflammation. These results challenge the current paradigm in asthma that airway inflammation induces smooth muscle remodeling and airway responsiveness, as these hGSDMB^{Zp3-Cre} mice develop increased airway-hyperresponsiveness and smooth muscle in the absence of airway inflammation. Furthermore, this study adds to our understanding of gene networks in asthma that we have identified can act in sequential pathways (i.e., GSDMB induces 5-lipoxygenase to induce TGF- β 1).

Author contributions: S.D., M.M., H.M.H., and D.H.B. designed research; S.D., A.K.B., J.M., M.D.M., C.V., M.R.K., P.R., and F.C. performed research; R.C.K. and H.M.H. contributed new reagents/analytic tools; S.D., M.M., T.A.D., Q.H., and D.H.B. analyzed data; and S.D. and D.H.B. wrote the paper.

The authors declare no conflict of interest.

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Because the SNP linking chromosome 17q21 to asthma is associated with increased GSDMB expression (1, 2), we generated transgenic mice expressing increased levels of the human GSDMB transgene (hGSDMB^{Zp3-Cre}), which develop a significant spontaneous increase in airway-hyperresponsiveness (AHR) and a significant spontaneous increase in airway remodeling (increased smooth muscle, increased fibrosis) in the absence of development of airway inflammation. This study, combined with our previous studies on ORMDL3 (4, 5), suggest that at least two genes localized to chromosome 17q21 (GSDMB and ORMDL3) contribute to airway remodeling in the absence of airway inflammation, and challenges the current paradigm that inflammation precedes lung remodeling and airway responsiveness in asthma.

Results

GSDMB Is Highly Expressed in Human Bronchial Epithelial Cells in Asthma.

To determine which human lung cells express GSDMB, we examined human lung sections from asthmatics and nonasthmatic controls. Immunohistochemistry analyses showed that levels of GSDMB were significantly increased in the bronchial epithelial cells in the lungs of asthmatics compared with control lungs (Fig. 1A–C). We also examined the effect of asthma severity on GSDMB expression and found GSDMB⁺ cells to be significantly elevated in bronchial biopsy specimens from severe asthmatics compared with nonasthmatic controls (Fig. 1D). We further investigated which human lung structural cells express *GSDMB* or its isoforms *GSDMB-1*, *-2*, *-3*, and *-4* (Fig. S1A). We found that bronchial epithelial cells, but not alveolar epithelial cells, were a significant source of the *GSDMB-1* isoform as quantitated by quantitative PCR (qPCR) (Fig. 1E and F). In addition, we did not detect significant *GSDMB-1* expression in other human lung structural cells (i.e., smooth muscle cells, fibroblasts), or in human lung macrophages. Moreover, *GSDMB-1* was the most predominant isoform expressed in human bronchial epithelial cells (Fig. 1F).

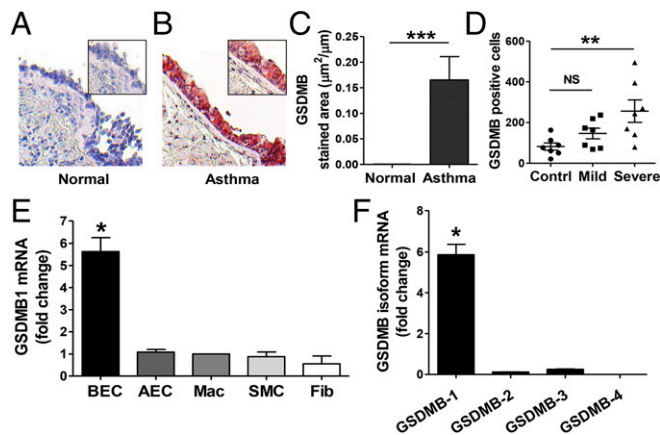


Fig. 1. GSDMB is highly expressed in human bronchial epithelial cells in asthma. Human lungs from (A) healthy controls and (B) asthmatic patients were examined by immunohistochemistry with an anti-GSDMB Ab ($n = 7$ subjects per group). (Magnification: A and B, 200 \times ; Insets, 400 \times .) (C) The number of peribronchial GSDMB⁺ cells were quantitated in each group by light microscopy (LM) and image analysis. (D) GSDMB⁺ cells in bronchial biopsies from healthy controls, mild asthmatics, and severe asthmatics were quantitated by LM ($n = 7$ subjects per group). (E) Relative quantification of GSDMB-1 mRNA in primary human bronchial epithelial cells (BEC), alveolar epithelial cells (AEC), macrophages (Mac), smooth muscle cells (SMC), and fibroblasts (Fib). (F) RNA from bronchial epithelial cells was examined using primers specific to GSDMB-1, -2, -3, and -4 isoforms (Table S1). GAPDH mRNA was used as normalization control. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; NS, not significant. Fold-change is expressed as mean \pm SEM and results are from three independent experiments.

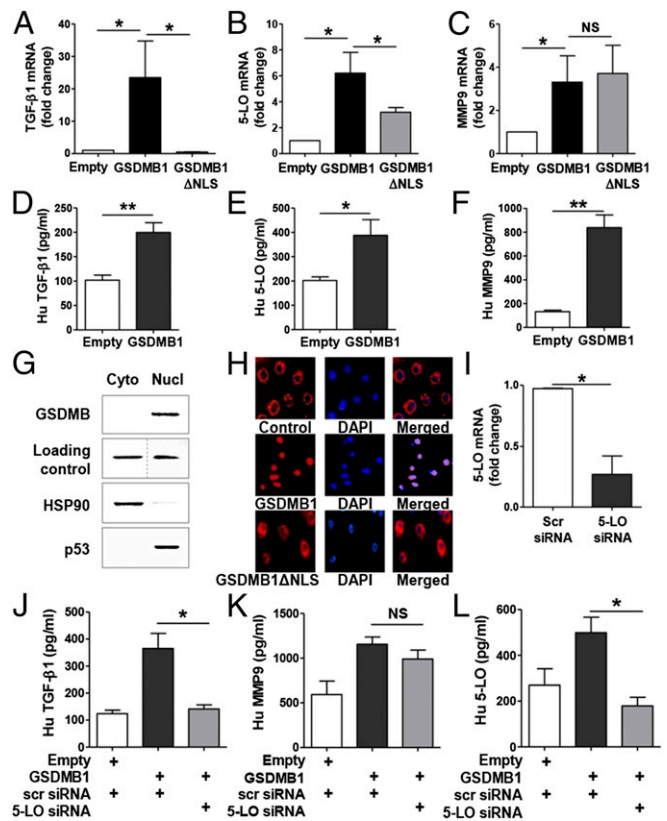


Fig. 2. Nuclear localization of GSDMB activates TGF- β 1, 5-LO, and MMP9 in human bronchial epithelial cells transfected with GSDMB. Primary bronchial epithelial cells were transfected with either empty, GSDMB-1, or GSDMB-1 vector lacking nuclear localization signal (GSDMB-1 Δ NLS) for 72 h and qPCR was performed to measure (A) TGF- β 1, (B) 5-LO, and (C) MMP9 mRNA. β -Actin mRNA was used as normalization control. Fold-change is expressed as mean \pm SEM from four independent experiments. ELISA was performed to measure (D) active TGF- β 1, (E) 5-LO, and (F) MMP9 levels in cells transfected with GSDMB-1 or empty vector. (G) Quantification of cellular localization of GSDMB in bronchial epithelial cells by Western blot. β -Tubulin and histone H3 were used as loading controls for cytoplasmic and nuclear extracts, respectively. The purity of these extracts was checked using HSP90, a cytoplasmic marker, and p53, a nuclear marker. Western blot image is a representative of three independent experiments. (H) Bronchial epithelial cells were transfected with RFP-tagged GSDMB-1, GSDMB-1 Δ NLS, or empty vector (red) and cells were mounted with DAPI (blue). (Magnification: 20 \times .) (I–L) Bronchial epithelial cells overexpressing GSDMB-1 or empty vector were transfected with scrambled (scr.) or 5-LO siRNA. (I) Efficiency of 5-LO mRNA knockdown was assessed by qPCR. β -Actin mRNA was used as normalization control. Protein levels of (J) active TGF- β 1, (K) MMP9, and (L) 5-LO were measured by ELISA. Results are expressed as mean \pm SEM from three independent experiments. * $P < 0.05$; ** $P < 0.01$; NS, not significant.

GSDMB-1 Up-Regulates In Vitro Expression of Remodeling Genes (TGF- β 1, 5-LO, MMP9), CC Chemokines (Eotaxin-3, CCL28), CXC Chemokines (CXCL6, CXCL17), and Heat-Shock Proteins (HSP60, HSP70) in Human Bronchial Epithelial Cells.

Because GSDMB-1 showed the highest level of expression among the four isoforms of GSDMB in bronchial epithelial cells, we focused our in vitro study on the GSDMB-1 isoform. We investigated the effect of GSDMB-1 overexpression in primary human bronchial epithelial cells on the levels of several genes important to airway remodeling and airway inflammation in asthma. There was a significant increase in levels of key remodeling genes, such as TGF- β 1, 5-lipoxygenase (5-LO), and matrix metalloproteinase 9 (MMP9) mRNA as assessed by qPCR (Fig. 2A–C), as well as a significant increase in TGF- β 1, 5-LO, and MMP9 protein levels in GSDMB-1-overexpressing cells compared with empty vector-overexpressing control cells (Fig. 2D–F).

Because GSDMB has been previously demonstrated to interact with heat-shock protein 90 (HSP90) in cancer cells (7), we investigated whether GSDMB-1 could regulate the levels of HSPs in epithelial cells. Because HSP60 and HSP70 have been previously associated with asthma (9), we examined the effect of GSDMB-1 overexpression on these genes and found a significant increase in HSP60 and HSP70 mRNA levels (Fig. S1B). In addition, because ORMDL3 regulates expression of CC and CXC chemokines in epithelial cells (4), we examined whether GSDMB also regulated the expression of chemokines. GSDMB-1 overexpression in human bronchial epithelial cells induced a significant increase in expression of CXC chemokines, CXCL6 (granulocyte chemotactic protein 2 or GCP-2) and CXCL17 (VEGF coregulated chemokine 1 or VCC-1) (Fig. S1C). We also found an increase in expression of CC chemokines, CCL26 (eotaxin-3) and CCL28 (mucosa-associated epithelial chemokine or MEC) mRNA but no change in the levels of CCL11 (eotaxin-1) (Fig. S1D).

GSDMB-1 Localizes to the Nucleus in Human Bronchial Epithelium.

Our studies of the cellular localization of ORMDL3 in the endoplasmic reticulum (ER) provided important insights into ORMDL3's function (4, 5), because we demonstrated that ORMDL3 activates only one of the three pathways of the ER unfolded response (i.e., the ATF-6 α pathway) and regulates Serca2b (a calcium pump localized in the sarcoplasmic ER). We have used a similar approach to examine the cellular expression of GSDMB in human bronchial epithelial cells and made the observation that GSDMB-1 is localized in the nucleus by using two approaches: (i) fractionating epithelial cells into cytoplasmic and nuclear components (Fig. 2G), and (ii) transfection of red fluorescent protein (RFP)-labeled GSDMB-1 into bronchial epithelial cells to see where it localizes compared with the RFP-labeled empty vector (Fig. 2H).

Western blots of the fractionated epithelial cell compartments demonstrated that GSDMB-1 was highly expressed in the nucleus, but not expressed in the cytoplasm. We validated the purity of our nuclear and cytoplasmic fractions by demonstrating that the nuclear extracts contained high levels of the nuclear histone protein p53 (not detected in the cytoplasmic fraction), whereas the cytoplasmic extracts contained high levels of the cytoplasmic HSP (HSP90), which was not detected in the nuclear fraction (Fig. 2G). In addition, RFP-labeled GSDMB-1 localized to the nucleus, whereas the empty RFP-labeled vector remained in the cytoplasm (Fig. 2H).

GSDMB-1 Requires Nuclear Localization to Up-Regulate Key Remodeling Gene TGF- β 1 in Human Bronchial Epithelial Cells.

Because previous studies have demonstrated the presence of a nuclear localization signal (NLS) in GSDMB-1 (10), we investigated whether the NLS is critical in regulating remodeling genes, chemokines, and HSPs. We observed that whereas overexpression of tagged GSDMB-1 in epithelial cells showed nuclear localization, the GSDMB-1 construct with the deleted NLS (GSDMB-1 Δ NLS) showed cytoplasmic localization similar to the control plasmid (Fig. 2H). Interestingly, overexpression of GSDMB-1 Δ NLS in bronchial epithelial cells failed to induce TGF- β 1 mRNA compared with full-length GSDMB-1, indicating that nuclear localization of GSDMB-1 is critical for GSDMB-1-mediated induction of TGF- β 1 (Fig. 2A). In contrast, nuclear localization of GSDMB-1 was only partially required for GSDMB-1 induction of 5-LO mRNA (Fig. 2B) and was not required for induction of MMP9 mRNA (Fig. 2C).

To understand the potential function of nuclear localized GSDMB-1, we performed GSDMB protein modeling and predictions using the GenBank GSDMB amino acid sequence, in conjunction with the Phyre2 bioinformatics tool (www.sbg.bio.ic.ac.uk/phyre2/html/page.cgi?id=index). The Phyre2 bioinformatics tools did not predict any DNA binding motifs in GSDMB, indicating that GSDMB may not function as a transcription factor. Protein sequence and secondary structure analysis using Phyre2 software predicted a plausible transcription initiation factor and chaperone domains in GSDMB with a confidence level of 42%, indicating that it might be involved in the regulation of expression

of genes, such as TGF- β 1, by acting as either a transcriptional coactivator or enhancer.

5-LO Is Critical for GSDMB-1 Mediated Up-Regulation of TGF- β 1 in Human Bronchial Epithelial Cells.

Because we demonstrated that GSDMB up-regulates remodeling genes, such as TGF- β , 5-LO, and MMP9, we performed small-interfering RNA (siRNA) knockdown of 5-LO to understand the relationship between TGF- β 1, 5-LO, and MMP9 during GSDMB-1 up-regulation. We observed that 5-LO knockdown after GSDMB-1 overexpression in bronchial epithelial cells significantly down-regulates TGF- β 1 protein levels (Fig. 2J). In contrast, 5-LO knockdown did not have a significant effect on MMP9 protein levels (Fig. 2K), indicating that 5-LO is critical for up-regulation of TGF- β 1 but not required for MMP9. We also observed a significant down-regulation in 5-LO mRNA (Fig. 2I) and protein levels (Fig. 2L), thus demonstrating that 5-LO siRNA knockdown was efficient in primary bronchial epithelial cells. Additionally, 5-LO overexpression alone was sufficient to induce TGF- β 1 mRNA and protein expression (Fig. S2).

Mice Expressing hGSDMB Transgene Exhibit Increased Airway Responsiveness Without Airway Inflammation.

To understand the potential role of GSDMB in relation to asthma in vivo, we generated hGSDMB^{Zp3-Cre} mice, as detailed in *SI Materials and Methods*. Briefly, conditional hGSDMB transgenic (Tg) floxed mice (RFP-Stop^{FL}-GSDMB-Tg) were crossed with zona pellucida 3 (Zp3) Cre mice, resulting in offspring expressing hGSDMB in all cells (hGSDMB^{Zp3-Cre} mice). The presence of loxP-flanked RFP and a transcriptional stop codon at the transcriptional initiation site of the hGSDMB transgene prevents transcription of hGSDMB until crossed with a Cre-mouse, which excises the transcriptional stop codon and histone H2B (H2B)-RFP (Fig. S3A and B). DNA from hGSDMB^{Zp3-Cre} mice and littermate controls was used to confirm the presence or absence of the transgene by PCR (Fig. S3C and D).

No developmental or morphologic defects were observed in hGSDMB^{Zp3-Cre} mice, and their lung size and weights were similar to those of WT mice (Fig. S3E and F). A significant increase in hGSDMB protein levels was detected in hGSDMB^{Zp3-Cre} mouse lungs (Fig. S3G) but no changes in mouse Gsdma, -c, -d (mouse do not have a GSDMB gene) was observed in hGSDMB^{Zp3-Cre} mice compared with WT mice (Fig. S4A). Interestingly, hGSDMB^{Zp3-Cre} mice exhibit a spontaneous increase in airway responsiveness to methacholine (MCh) compared with WT mice (Fig. 3A). However, we did not observe any significant changes in bronchoalveolar lavage (BAL) inflammatory cells associated with the increased AHR (Fig. 3B).

Mice Expressing hGSDMB Transgene Exhibit Increased Peribronchial Smooth Muscle and Collagen Deposition.

Because we did not observe any changes in BAL inflammatory cells in hGSDMB^{Zp3-Cre} mice, we examined whether increased peribronchial smooth muscle or other pathways could contribute to AHR. Interestingly, we detected several features of airway remodeling in hGSDMB^{Zp3-Cre} mice, including an increase in the area of peribronchial smooth muscle as assessed by immunostaining with an anti- α -smooth muscle actin Ab (Fig. 3C and D). In addition, there was an increase in peribronchial fibrosis as assessed by the area of peribronchial trichrome staining to detect lung collagen (Fig. 3E and F). There was no significant increase in mucin expression as detected by periodic acid Schiff (PAS) staining (Fig. 3G and H).

Mice Expressing hGSDMB Transgene Have Increased Expression of Pathways Associated with Airway Remodeling in Asthma: TGF- β 1, MMP9, 5-LO, and Cysteinyl Leukotrienes.

We quantitated levels of 5-LO, TGF- β 1, and MMP9 mRNA in hGSDMB^{Zp3-Cre} mouse lungs (Figs. S4B–D and S5A–J) because these genes were up-regulated in human bronchial epithelial cells upon in vitro GSDMB-1 overexpression. We found a significant increase in TGF- β 1 lung mRNA, lung protein, and serum levels in hGSDMB^{Zp3-Cre} mice

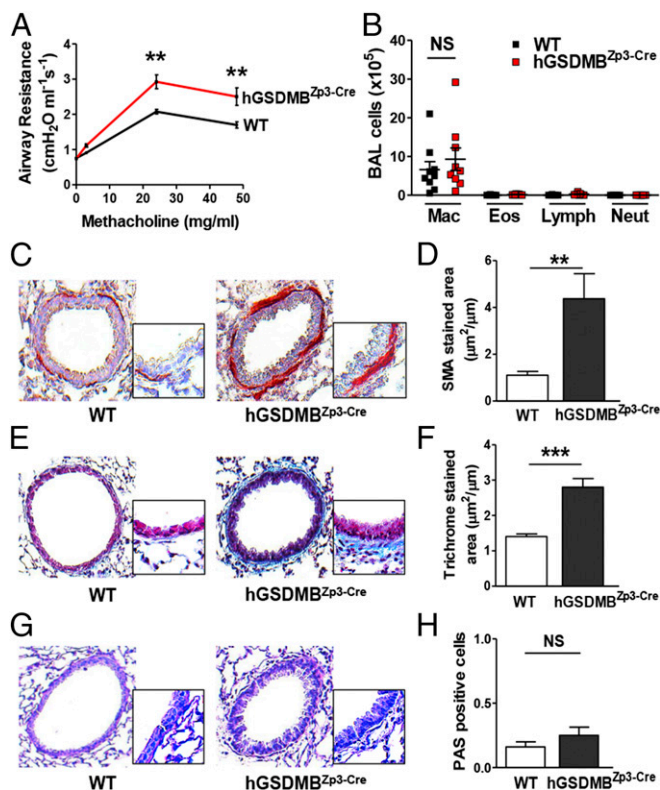


Fig. 3. hGSDMB^{Zp3-Cre} mice exhibit increased AHR, with an increase in peribronchial smooth muscle and collagen deposition without airway inflammation. (A) AHR to MCh and (B) BAL inflammatory cells were assessed in intubated and ventilated WT and hGSDMB^{Zp3-Cre} mice. (C) Levels of peribronchial smooth muscle were quantitated by immunohistochemistry using an anti- α -smooth muscle actin Ab and (D) image analysis. Results are expressed as the α -smooth muscle actin-stained area (μm^2) per circumference (μm) of basement membrane of bronchioles, 150- to 250- μm internal diameter in WT and hGSDMB^{Zp3-Cre} mice. (E) Levels of peribronchial trichrome staining were imaged using LM and (F) similarly quantitated by image analysis. (G) Levels of mucin were examined by PAS staining and (H) quantitated by image analysis. (Magnification: C, E, and G, 200 \times ; Insets, 400 \times .) * $P < 0.01$; *** $P < 0.001$; NS, not significant. $n = 9$ –12 mice per group. Results are expressed as mean \pm SEM from four independent experiments.

lungs compared with WT mice (Fig. S5A–C). hGSDMB^{Zp3-Cre} mice also exhibited a significant increase in MMP9 lung mRNA, lung protein, and serum levels (Fig. S5D–F). We also observed a significant increase in 5-LO lung mRNA and 5-LO protein levels, in conjunction with a significant increase in downstream mediators of 5-LO pathway [i.e., cysteinyl leukotrienes (LT) LTC₄, LTD₄, and LTE₄] (Fig. S5G–I) and a trend for increase in LTB₄ levels ($P = 0.09$) (Fig. S5J). Because previous studies demonstrated that the 5-LO pathway plays an important role in the pathogenesis of IL-13-induced remodeling (11), we examined the levels of IL-13 and found a significant increase in lung IL-13 mRNA and protein levels in hGSDMB^{Zp3-Cre} mice (Fig. S5K and L). hGSDMB^{Zp3-Cre} mice also showed an increase in total serum IgE levels (Fig. S6A).

We also examined hGSDMB^{Zp3-Cre} mouse lungs for other genes (i.e., chemokines and HSPs) that were up-regulated in human bronchial epithelial cells upon in vitro GSDMB-1 over-expression. Levels of Hsp1 (mouse ortholog of HSP60) but not Hspa2 (mouse ortholog of HSP70) were found to be up-regulated in mouse lungs (Fig. S6B). We also found a significant increase in mRNA expression of CXC chemokines Cxcl5 (mouse ortholog of CXCL6) and Cxcl17 (Fig. S6C). There was a significant increase in the mRNA levels of CC chemokine Ccl28 (also known as MEC), but no change in eotaxin-1 (Ccl11) and eotaxin-3 (Ccl26) mRNA levels (Fig. S6D). Although we

observed a significant increase in Cxcl5, Cxcl17, and Ccl28 mRNA, we did not observe any significant differences in Cxcl5, Cxcl17, and Ccl28 protein levels (Fig. S6E and F).

Dust Mite Allergen-Challenged Mice Expressing hGSDMB Transgene Exhibit Increased AHR, Airway Inflammation, Th2 Cytokines, and IgE.

To examine the effect of house dust mite (HDM) allergen on GSDMB mRNA expression in epithelial cells, we stimulated primary human bronchial epithelial cells with HDM in vitro and found a significant increase in GSDMB-1 mRNA expression (Fig. 4A). Although LPS is a component in dust mites, LPS alone did not induce GSDMB-1 expression.

We used a mouse model to examine the effect of HDM allergen challenge on AHR, airway inflammation, and airway remodeling in hGSDMB^{Zp3-Cre} mice (Fig. S7A). There was a significant increase in AHR in hGSDMB^{Zp3-Cre} mice challenged with HDM compared with challenged WT mice (Fig. 4B). This increase in AHR was associated with a significant increase in peribronchial major basic protein (MBP)⁺ eosinophils (Fig. 4C) and CD4⁺ cells (Fig. 4D) in HDM-challenged hGSDMB^{Zp3-Cre} mice, indicating that increased GSDMB expression in vivo exacerbates HDM allergen-mediated airway inflammation. There was no significant difference in neutrophil elastase (NE)⁺ lung neutrophils (Fig. 4E) in HDM-challenged hGSDMB^{Zp3-Cre} mice compared with WT mice. As observed in the lung tissue, there was a significant increase in BAL eosinophils and BAL lymphocytes, but not BAL neutrophils in HDM-challenged hGSDMB^{Zp3-Cre} compared with WT mice (Fig. S7B–D).

We measured lung Th2 cytokines, IL-4 and IL-13, and found a significant increase in HDM-challenged hGSDMB^{Zp3-Cre} mice

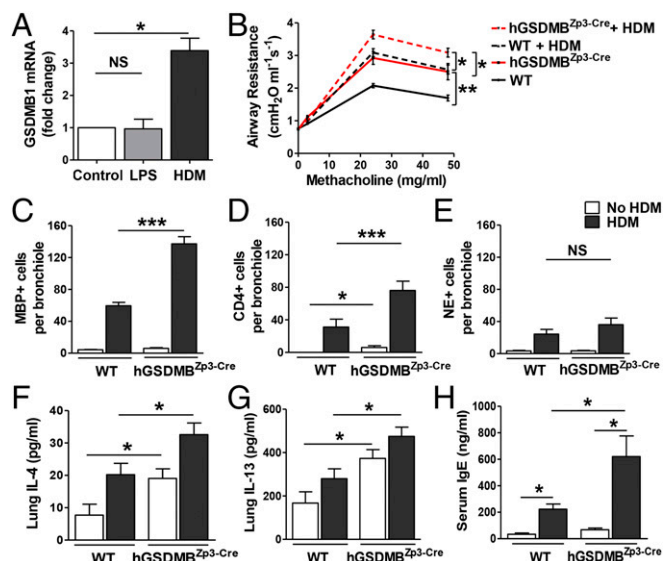


Fig. 4. Mice expressing human GSDMB transgene exhibit increased AHR, airway inflammation, Th2 cytokines, and IgE upon dust mite allergen challenge. (A) Primary human bronchial epithelial cells were incubated with either HDM (25 $\mu\text{g}/\text{mL}$) or LPS (100 ng/mL) for 48 h and GSDMB-1 mRNA levels were measured by qPCR. Cells treated with media alone served as experimental control. β -Actin mRNA was used as normalization control and fold-change is expressed as mean \pm SEM. (B) WT and hGSDMB^{Zp3-Cre} mice were administered either HDM extract (100 μg) or PBS via intranasal challenges on days 0, 7, 14, and 21. AHR to MCh was assessed on day 24 in intubated and ventilated mice. Lungs of WT and hGSDMB^{Zp3-Cre} mice challenged with or without HDM were examined for the number of lung (C) MBP⁺ peribronchial eosinophils, (D) CD4⁺ T cells, and (E) NE⁺ neutrophils per bronchiole of 150- to 250- μm internal diameter by immunohistochemistry and quantitated by image analysis. Levels of (F) lung IL-4, (G) lung IL-13, and (H) total serum IgE were quantitated by ELISA. * $P < 0.05$; *** $P < 0.01$; **** $P < 0.001$; NS, not significant. $n = 9$ –12 mice per group. Results are expressed as mean \pm SEM from four independent experiments.

compared with challenged WT mice (Fig. 4 *F* and *G*). However, we did not observe any significant difference in lung IL-5 levels (Fig. *S7E*). We also found a significant increase in total serum IgE levels in HDM-challenged hGSDMB^{Zp3-Cre} mice compared with challenged WT mice (Fig. 4*H*). There was no significant lung IFN- γ response in unchallenged or HDM-challenged hGSDMB^{Zp3-Cre} mice compared with WT mice (Fig. *S8*).

HDM Challenge in hGSDMB^{Zp3-Cre} Mice Induced Mucin and Exacerbates Peribronchial Smooth Muscle and Collagen Deposition. Interestingly, although unchallenged hGSDMB^{Zp3-Cre} mice did not exhibit increased mucin expression, HDM-challenged hGSDMB^{Zp3-Cre} mice showed an increase in mucin expression compared with WT mice, as detected by PAS staining (Fig. *S9 A* and *B*). HDM-challenged hGSDMB^{Zp3-Cre} mice had a significant increase in airway remodeling compared with hGSDMB^{Zp3-Cre} mice, as evident in the further increased thickness of the peribronchial smooth muscle layer (Fig. *S9 C* and *D*), and further increased level of peribronchial fibrosis (Fig. *S9 E* and *F*).

Overall, our results indicate that allergen challenge in hGSDMB^{Zp3-Cre} mice exacerbates airway inflammation, IgE levels, the thickness of the peribronchial smooth muscle layer, collagen deposition, mucin, and AHR.

Discussion

Because the SNP linking chromosome 17q21 is linked to increased expression of GSDMB (2, 8), we generated mice expressing increased levels of GSDMB and made the observation that these hGSDMB^{Zp3-Cre} mice had spontaneous increased AHR and increased peribronchial smooth muscle in the absence of airway inflammation. Although the classic paradigm for airway remodeling in asthma suggests that repeated episodes of inflammation of the airways results in airway remodeling, our studies with GSDMB suggest that this concept may need to be revised, not solely based on the evidence that GSDMB can induce airway smooth muscle remodeling and AHR in the absence of inflammation, but also based on several clinical studies (12, 13). For example, in preschool children with established severe asthma, increased airway smooth muscle mass and reticular basement thickening are present and is dissociated from inflammation (12, 13). The presence of increased airway smooth muscle mass in large and small airways in asthma is well described and is considered to be a major contributor to bronchoconstriction of airways and persistent airflow obstruction (14).

To understand the mechanism by which GSDMB induces increased airway remodeling and AHR, we performed studies examining which pathways were activated by GSDMB overexpression in vitro and in vivo. Our studies of epithelial cells transfected with GSDMB in vitro, as well as our studies of hGSDMB^{Zp3-Cre} mice in vivo, identified that GSDMB induced high levels of expression of 5-LO and TGF- β 1, mediators associated with AHR and smooth muscle remodeling. The 5-LO products can directly induce smooth muscle contraction with rapid onset kinetics, whereas TGF- β 1 can directly increase smooth muscle contractility with slow onset kinetics (15). Previous studies have demonstrated that TGF- β 1 and leukotrienes can produce synergistic effects to increase smooth muscle contraction, collagen synthesis, and epithelial cell proliferation (16). For example, LTC4 can stimulate airway epithelial cells to increase TGF- β 1 expression, which in turn can induce the expression of MMPs and tissue inhibitors of metalloproteinases, thus affecting airway remodeling (16). In addition to leukotrienes, there is considerable evidence that other GSDMB-regulated genes, such as TGF- β 1 and MMP9, can directly contribute to airway remodeling in asthma (17). Increased TGF- β 1 mRNA levels have been observed in bronchial biopsies of asthmatics compared with normal subjects, and levels of TGF- β 1 correlate with the depth of subepithelial fibrosis (17, 18). Levels of MMP9 are significantly increased in BAL fluid, blood, and sputum from allergic asthmatic patients (19).

To understand the relationship between GSDMB, 5-LO, and TGF- β 1, we performed 5-LO knockdown studies in GSDMB-1-overexpressing epithelial cells and established that 5-LO knockdown

led to a significant down-regulation in TGF- β 1 protein levels. These studies demonstrate that GSDMB-induced TGF- β 1 expression is critically dependent upon 5-LO, suggesting a remodeling pathway induced by GSDMB. Because nuclear localization of GSDMB is essential for induction of TGF- β 1, we used bioinformatics approaches to determine whether GSDMB could function as a transcription factor but did not identify any such DNA binding motifs in GSDMB. However, secondary structure analysis predicted a plausible transcription initiation factor and chaperone domains in GSDMB, indicating that it might be involved in the regulation of genes, such as TGF- β 1, by either acting as a transcriptional coactivator or enhancer. However, further studies are needed to define whether GSDMB's nuclear localization regulates TGF- β 1 gene expression by acting as either a transcription initiation factor, chaperone, coactivator, chromatin remodeler, histone acetylase, deacetylase, kinase, or methylase, which can all play crucial roles in gene regulation but lack DNA-binding domains, and therefore are not classified as transcription factors (20).

We demonstrate the importance of the GSDMB-1 isoform in asthma because it shows the highest expression among the four isoforms of GSDMB (GSDMB-1, -2, -3, -4). Interestingly, the GSDMB isoform expressed in epithelial cells from the lungs of asthmatics (GSDMB-1) differs from the isoform expressed in cancer-derived epithelial cells (GSDMB-2) (7). Alternative splicing can have diverse effects on protein folding and function (21) and can affect protein–ligand interactions. Recent studies have demonstrated an important role of alternative splicing in several human diseases (22), thus underscoring the need to further explore the role of GSDMB isoforms in asthma. The four GSDMB isoforms differ in the presence or absence of exons 6 and 7 of the *GSDMB* gene. In cancer, GSDMB-2 (lacking exons 6 and 7) has effects on tumor growth and development (7). In contrast we demonstrate that in asthma, GSDMB-1 (the only isoform that lacks only exon 6), is significantly expressed. We decided in our initial in vivo studies to overexpress the whole GSDMB gene rather than an individual isoform, as this would allow us to determine whether any GSDMB isoform influenced the development of an asthma phenotype. Further studies are needed to understand the roles of the individual GSDMB isoforms in asthma.

Although leukocytes, such as eosinophils, are a major source of 5-LO products in asthma, studies have demonstrated that human bronchial epithelial cells significantly express an active 5-LO and inducible biosynthetic pathway for LTC4, with levels similar to blood leukocytes (23). A higher number of bronchial epithelial cells relative to eosinophils in the lung may allow the bronchial epithelium to be the most significant source of leukotrienes in asthma. In our immunohistochemistry studies of GSDMB expression in human lungs from asthmatics, we observed that both epithelial cells and peribronchial inflammatory cells expressed GSDMB, indicating that epithelial cells might not be the sole source of GSDMB in human lungs. We also observed a mild increase in the number of CD4⁺ cells/bronchus in naïve hGSDMB^{Zp3-Cre} mice compared with WT mice, which is associated with an increase in IL-4 and IL-13 levels. GSDMB is known to be expressed in T cells (2) and may be expressed in other inflammatory cells in the lung. Because this study examines whether increased GSDMB expression results in an asthma phenotype, we elected to initially generate universal transgenic mice with increased expression of GSDMB in all cells, including epithelial cells and T cells known to express GSDMB. Future studies using cell specific-Cre mice (such as CC10-Cre, or CD4-Cre mice) might be helpful to delineate distinct functions for epithelial cell- and T-cell-expressed GSDMB.

GSDMB-1 overexpression led to a significant increase in HSP60 and HSP70 mRNA, which have been implicated in inflammation and asthma (9). Increased HSP60 is noted in alveolar macrophages of asthmatics, whereas HSP70 has demonstrated implications in inflammatory immune responses (9). Levels of the mouse orthologs of HSP60 (Hspd1) but not HSP70 (Hspa2) were up-regulated in the lungs of hGSDMB^{Zp3-Cre} mice. We also

investigated the effect of GSDMB-1 overexpression on selected chemokines involved in asthma and found a significant increase in mRNA levels of CCL26 (eotaxin-3), CCL28 (MEC), CXCL6 (GCP-2), and CXCL17 (VCC-1). Eotaxin-3 and CCL28 are up-regulated during an asthmatic response and is expressed on immune cells, such as eosinophils and T-cell subsets (24). CXC chemokines (such as CXCL6) can activate and attract neutrophils to the asthmatic airways (25). Elevated CXCL17 levels have been reported in lung fibrosis (26). Lung mRNA levels of mouse orthologs of CCL28 (Ccl28), CXCL6 (Cxc15), and CXCL17 (Cxc17) were up-regulated in hGSDMB^{Zp3-Cre} mice. Epithelial cells are known cellular sources of chemokines (27), which are the likely source of Ccl28, Cxc15, and Cxc17 in hGSDMB^{Zp3-Cre} mouse lungs. However, no significant changes in the protein levels of these chemokines were observed in hGSDMB^{Zp3-Cre} mice.

Because the human transgene in hGSDMB^{Zp3-Cre} mice is not regulated by the allergen challenge, we hypothesized that HDM-induced inflammatory and immune response would synergize with GSDMB-regulated genes (such as chemokines) expressed spontaneously in hGSDMB^{Zp3-Cre} mice. This process would simulate an asthmatic with the chromosome 17q21 SNP (which is associated with increased GSDMB expression) when exposed to an allergen. These studies demonstrated that HDM allergen challenge induced a significant increase in airway responsiveness, Th2 response (Th2 cytokines, MBP⁺ eosinophils, CD4⁺ cells, IgE), airway smooth muscle, subepithelial fibrosis, and mucin in HDM-challenged hGSDMB^{Zp3-Cre} mice compared with HDM-challenged WT mice. Epidemiologic studies of asthma link IgE levels to chromosome 17q21 variants in some (28) but not all studies (29).

In summary, we have made the observation that GSDMB is expressed at increased levels in the lungs of human subjects with severe asthma, and that hGSDMB^{Zp3-Cre} mice exhibits significant spontaneous increases in AHR and airway remodeling, including an increase in peribronchial smooth muscle and increased peribronchial fibrosis in the absence of airway inflammation. We

identified a pathway by which GSDMB regulates two genes previously implicated in asthma (i.e., TGF- β 1 and 5-LO) and established that nuclear localization of GSDMB-1 was essential for the induction of TGF- β 1. Overall, this study, in combination with previous data from our laboratory regarding ORM DL3 (4), suggests that at least two of the genes localized to chromosome 17q21 (GSDMB and ORM DL3) can contribute to increased AHR and increased airway remodeling in the absence of airway inflammation, which contrasts with the four chromosome 5q genes (IL-4, IL-5, IL-9, IL-13) that induce airway inflammation in asthma.

Materials and Methods

Methods and statistical analyses for qPCR, immunohistochemistry, immunofluorescence microscopy, Western blot, in vitro transfection, siRNA knockdown, generation of hGSDMB^{Zp3-Cre} mice, mouse model of HDM-induced asthma, AHR measurements, collection of mouse specimens including lungs, BAL and blood, histology, and ELISAs were performed as described in *SI Materials and Methods*. All of the mouse experimental protocols were approved by the University of California, San Diego Institutional Animal Care and Use Committee.

Postmortem human lungs from asthmatics and normal organ donors were procured by the Arkansas Regional Organ Recovery Agency and by the National Disease Research Interchange, and delivered to the Lung Cell Biology Laboratory at the Arkansas Children's Hospital Research Institute under conditions identical to those used for transplant. The acquisition of deceased donor tissue was reviewed by the University of Arkansas for Medical Sciences Institutional Review Board and determined not to be human subject research. After processing, tissue was shipped to San Diego under a protocol approved by the University of California, San Diego Human Research Protections program. The protocols using bronchoscopy to obtain bronchial biopsies from patients with mild or severe asthma and control nonasthmatics at McGill University and Université de Montréal were performed with the approval of the respective Institutional Review Boards, as previously described (30).

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- Moffatt MF, et al. (2007) Genetic variants regulating ORM DL3 expression contribute to the risk of childhood asthma. *Nature* 448(7152):470–473.
- Verlaan DJ, et al. (2009) Allele-specific chromatin remodeling in the ZPBP2/GSDMB/ORM DL3 locus associated with the risk of asthma and autoimmune disease. *Am J Hum Genet* 85(3):377–393.
- Marsh DG, et al. (1994) Linkage analysis of IL4 and other chromosome 5q31.1 markers and total serum immunoglobulin E concentrations. *Science* 264(5162):1152–1156.
- Miller M, et al. (2012) ORM DL3 is an inducible lung epithelial gene regulating metalloproteinases, chemokines, OAS, and ATF6. *Proc Natl Acad Sci USA* 109(41):16648–16653.
- Miller M, et al. (2014) ORM DL3 transgenic mice have increased airway remodeling and airway responsiveness characteristic of asthma. *J Immunol* 192(8):3475–3487.
- Saeki N, Kuwahara Y, Sasaki H, Satoh H, Shiroishi T (2000) Gasdermin (Gsdm) localizing to mouse chromosome 11 is predominantly expressed in upper gastrointestinal tract but significantly suppressed in human gastric cancer cells. *Mamm Genome* 11(9):718–724.
- Hergueta-Redondo M, et al. (2014) Gasdermin-B promotes invasion and metastasis in breast cancer cells. *PLoS One* 9(3):e90099.
- Wu H, et al. (2009) Genetic variation in ORM1-like 3 (ORM DL3) and gasdermin-like (GSDML) and childhood asthma. *Allergy* 64(4):629–635.
- Madore A-M, et al. (2010) Alveolar macrophages in allergic asthma: An expression signature characterized by heat shock protein pathways. *Hum Immunol* 71(2):144–150.
- Sun Q, et al. (2008) Expression of GSDML associates with tumor progression in uterine cervix cancer. *Transl Oncol* 1(2):73–83.
- Shim YM, et al. (2006) Role of 5-lipoxygenase in IL-13-induced pulmonary inflammation and remodeling. *J Immunol* 177(3):1918–1924.
- Bossley CJ, et al. (2012) Pediatric severe asthma is characterized by eosinophilia and remodeling without T(H)2 cytokines. *J Allergy Clin Immunol* 129(4):974–82.e13.
- O'Reilly R, et al. (2013) Increased airway smooth muscle in preschool wheezers who have asthma at school age. *J Allergy Clin Immunol* 131(4):1024–1032, 1032.e1–16.
- Noble PB, et al. (2014) Airway smooth muscle in asthma: Linking contraction and mechanotransduction to disease pathogenesis and remodeling. *Pulm Pharmacol Ther* 29(2):96–107.
- Beppu LY, et al. (2014) TGF- β 1-induced phospholamban expression alters esophageal smooth muscle cell contraction in patients with eosinophilic esophagitis. *J Allergy Clin Immunol* 134(5):1100–1107.e4.
- Perng DW, et al. (2006) Leukotriene C4 induces TGF- β 1 production in airway epithelium via p38 kinase pathway. *Am J Respir Cell Mol Biol* 34(1):101–107.
- Halwani R, Al-Muhsen S, Al-Jahdali H, Hamid Q (2011) Role of transforming growth factor- β in airway remodeling in asthma. *Am J Respir Cell Mol Biol* 44(2):127–133.
- Das S, et al. (2014) MicroRNA-326 regulates profibrotic functions of transforming growth factor- β in pulmonary fibrosis. *Am J Respir Cell Mol Biol* 50(5):882–892.
- Mautino G, Oliver N, Chanez P, Bousquet J, Capony F (1997) Increased release of matrix metalloproteinase-9 in bronchoalveolar lavage fluid and by alveolar macrophages of asthmatics. *Am J Respir Cell Mol Biol* 17(5):583–591.
- Briantlou AH, Darnell JE, Jr (2002) Signal transduction and the control of gene expression. *Science* 295(5556):813–818.
- Xiong HY, et al. (2015) RNA splicing. The human splicing code reveals new insights into the genetic determinants of disease. *Science* 347(6218):1254806.
- García-Blanco MA, Baraniak AP, Lasda EL (2004) Alternative splicing in disease and therapy. *Nat Biotechnol* 22(5):535–546.
- Jame AJ, et al. (2007) Human bronchial epithelial cells express an active and inducible biosynthetic pathway for leukotrienes B4 and C4. *Clin Exp Allergy* 37(6):880–892.
- Lukacs NW (2001) Role of chemokines in the pathogenesis of asthma. *Nat Rev Immunol* 1(2):108–116.
- Rohde G, et al. (2014) CXC chemokines and antimicrobial peptides in rhinovirus-induced experimental asthma exacerbations. *Clin Exp Allergy* 44(7):930–939.
- Burkhardt AM, et al. (2012) CXCL17 is a mucosal chemokine elevated in idiopathic pulmonary fibrosis that exhibits broad antimicrobial activity. *J Immunol* 188(12):6399–6406.
- Loxham M, Davies DE, Blume C (2014) Epithelial function and dysfunction in asthma. *Clin Exp Allergy* 44(1):1299–1313.
- Galanter J, et al. (2008) ORM DL3 gene is associated with asthma in three ethnically diverse populations. *Am J Respir Crit Care Med* 177(11):1194–1200.
- Moffatt MF, et al.; GABRIEL Consortium (2010) A large-scale, consortium-based genome-wide association study of asthma. *N Engl J Med* 363(13):1211–1221.
- Miller M, et al. (2015) Fst1 promotes asthmatic airway remodeling by inducing oncostatin M. *J Immunol* 195(8):3546–3556.
- Miller M, et al. (2016) Segmental allergen challenge increases levels of airway follistatin-like 1 in patients with asthma. *J Allergy Clin Immunol* 138(2):596–599.e4.
- Kelley LA, Mezulis S, Yates CM, Wass MN, Sternberg MJE (2015) The Phyre2 web portal for protein modeling, prediction and analysis. *Nat Protoc* 10(6):845–858.