HHS Public Access

Author manuscript

J Allergy Clin Immunol. Author manuscript; available in PMC 2018 November 09.

Published in final edited form as:

J Allergy Clin Immunol. 2018 November ; 142(5): 1469–1478.e2. doi:10.1016/j.jaci.2017.11.040.

A functional splicing variant associated with decreased asthma risk abolishes the ability of gasdermin B (*GSMDB*) to induce epithelial cell pyroptosis

Ronald A. Panganiban, PhD[a,+], **Maoyun Sun, PhD**[a,+], **Amber Dahlin, PhD**[b,+], **Hae-Ryung Park, PhD**[a], **Mengyuan Kan, PhD**[c], **Blanca E. Himes, PhD**[c], **Jennifer A. Mitchel, PhD**[a], **Carlos Iribarren, MD, PhD**[d], **Eric Jorgenson, PhD**[d], **Scott H. Randell, PhD**[e], **Elliot Israel, MD**[f], **Kelan Tantisira, MD**[b], **Stephanie Shore, PhD**[a], **Jin-Ah Park, PhD**[a], **Scott T. Weiss, MD**[b], **Ann Chen Wu, MD**[b,g], and **Quan Lu, PhD**[a,*]

[a]Program in Molecular and Integrative Physiological Sciences, Departments of Environmental Health and Genetics & Complex Diseases, Harvard T.H. Chan School of Public Health, Boston, Massachusetts 02115

[b]Channing Division of Network Medicine, Brigham and Women's Hospital and Harvard Medical School, Boston, Massachusetts 02115

[c]Department of Biostatistics, Epidemiology and Informatics, University of Pennsylvania, Philadelphia, Pennsylvania 19104

[d]Division of Research, Kaiser Permanente Northern California, Oakland, California 94612

[e]Marsico Lung Institute/Cystic Fibrosis Center, University of North Carolina, 125 Mason Farm Road, Chapel Hill, North Carolina 27599

[f]Asthma Research Center, Brigham and Women's Hospital and Harvard Medical School, Boston, Massachusetts 02115

[g]Precision Medicine Translational Research Center, Department of Population Medicine, Harvard Medical School, Boston, Massachusetts 02115

Abstract

Background—Genetic variants in the chromosomal region 17q21 are consistently associated with asthma. However, mechanistic studies have not yet linked any of the associated variants to a function that could influence asthma, and as a result, the identity of the asthma gene(s) remains elusive.

Objectives—We sought to identify and characterize functional variants in the 17q21 locus.

Methods—We used the Exome Aggregation Consortium (ExAC) browser to identify coding (amino acid-changing) variants in the 17q21 locus. We obtained asthma association measures for these variants in both the GERA cohort (16,274 cases and 38,269 matched controls) and the EVE

*Correspondence author: Quan Lu, PhD, Program in Molecular and Integrative Physiological Sciences, Harvard T.H. Chan School of Public Health, 665 Huntington Avenue, Boston, Massachusetts 02115, USA. Phone number: 617-432-7145, FAX: 617-432-3468; qlu@hsph.harvard.edu.

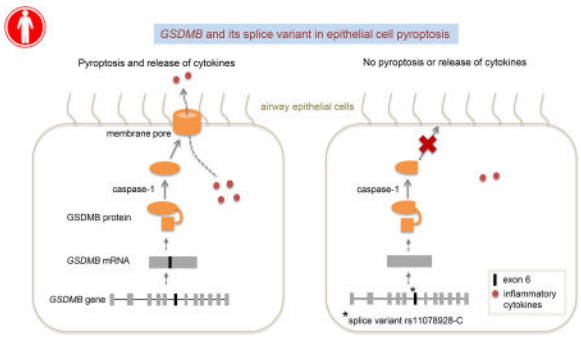
+These authors contribute equally to this work

Consortium study (5,303 asthma cases and 12,560 individuals). Gene expression and protein localization were determined by quantitative RT-PCR and fluorescence immunostaining, respectively. Molecular and cellular studies were performed to determine the functional effects of coding variants.

Results—Two coding variants (rs2305480 and rs11078928) of the gasdermin B (*GSDMB*) gene in the 17q21 locus were associated with lower asthma risk in both GERA (OR = 0.92; P= 1.01×10^{-6}) and EVE (OR= 0.85; Joint $P_{EVE} = 1.31 \times 10^{-13}$). In GERA, rs11078928 had a minor allele frequency (MAF) of 0.45 in unaffected (non-asthmatic) controls and 0.43 in asthma cases. For European Americans in EVE the MAF of rs2305480 was 0.45 for controls and 0.39 for cases; for all EVE subjects the MAF was 0.32 for controls and 0.27 for cases. *GSDMB* is highly expressed in differentiated airway epithelial cells, including the ciliated cells. We found that when the GSDMB protein is cleaved by inflammatory caspase-1 to release its N-terminal fragment, potent pyroptotic cell death was induced. The splicing variant rs11078928 deletes the entire exon 6, which encodes 13 amino acids in the critical N-terminus, and abolishes the pyroptotic activity of the GSDMB protein.

Conclusions—Our study identified a functional asthma variant in the *GSDMB* gene of the 17q21 locus and implicates GSDMB-mediated epithelial cell pyroptosis in pathogenesis.

Graphical Abstract



Keywords

Asthma; gasdermin B; GSDMB; 17q21 locus; genetics; airway epithelium; pyroptosis

INTRODUCTION

Asthma is a common chronic lung disease affecting over 300 million people worldwide[1] and 8% of the U.S. population[2]. The annual cost of asthma in the US alone is over 50 billion dollars, placing a significant burden on the healthcare system[2, 3]. Despite the increasing prevalence, morbidity and economic burden of the disease, the molecular basis of asthma remains poorly understood.

While asthma is heterogeneous clinically[4] and its prevalence and severity vary among racial/ethnic groups[5], genetic studies including genome-wide association studies (GWAS) have identified many genomic loci that are significantly associated with asthma [6–10]. Many asthma-associated genes identified by GWAS are known to regulate cellular phenotypes

directly relevant to asthma pathogenesis. For example *IL33* and its receptor *IL1RL1* regulate the Th2 (type 2 helper T-cell) immune response, which directly contributes to airway inflammation and asthma pathogenesis [11, 12].

GWAS have also identified genetic loci with less clear mechanistic links to asthma, most notably, the 17q21 locus, which is the strongest and most reproducible asthma GWAS signal [9, 13]. Many single nucleotide polymorphisms (SNPs) in this locus that are in linkage disequilibrium (LD) with each other have been associated with asthma in diverse and independent populations, and appear to be specific to childhood-onset asthma [9, 10, 14–16]. The 17q21 locus spans a region containing at least 6 genes (*IKZF3*, *ZPBP2*, *GSDMB*, *ORMDL3*, *LRRC3C* and *GSDMA*), and because most asthma-associated SNPs are in introns or intergenic regions, it is not clear which gene(s) is functionally related to asthma Expression quantitative trait loci (eQTL) studies have found that 17q21 SNPs are associated with mRNA expression levels of *ORMDL3* [13, 17–19], *GSDMA* [17] , and *GSDMB* [19], which prioritizes these genes for functional studies, but still leaves unclear which one modifies asthma risk and how.

In this study, we sought to identify potential functional variants in the 17q21 region. Rather than begin with SNPs as ranked by GWAS, we focused on SNPs that could alter protein coding. Subsequently, we prioritized which of these SNPs would most likely contribute to observed 17q21 association signals by obtaining GWAS results from two large asthma cohorts. We found that a splicing variant in *GSDMB* that was nominally associated with lower risk of asthma causes the deletion of an important exon from the *GSDMB* transcript and consequently abolishes the ability of the GSDMB protein to induce pyroptotic death of airway epithelial cells. Thus, this *GSDMB* splicing variant may reduce asthma risk by protecting airway epithelial cells from pyroptotic cell death.

METHODS

Primary study population

The Genetic Epidemiology Research in Adult Health and Aging (GERA) cohort, which comprises 110,266 adult men and women members of the Kaiser Permanente Medical Care Plan, Northern California Region (KPNC), has been described in detail in dbGAP (http://ncbi.nlm.nih.gov/gap; Study Accession: phs000674.v1.p1). In this study, we focused on subjects (16,274 cases and 38,269 matched controls) who were at least 21 years of age at time of the survey, of non-Hispanic white race/ethnicity, and who had self-reported or physician-diagnosed asthma. All study procedures were approved by the Institutional Review Board of the Kaiser Foundation Research Institute and Brigham and Women's Hospital.

Genotyping and imputation

DNA samples were extracted using Oragene kits (DNA Genotek Inc., Ottawa, ON, Canada) at KPNC and genotyped at the Genomics Core Facility of the University of California, San Francisco (UCSF). Design details and genome-wide coverage of those arrays have been previously described.[20, 21] High genotype quality control (QC) procedures for the GERA

cohort were performed on an array-wise basis as described in detail elsewhere.[22] Using strict QC criteria, including initial genotyping call rate ≥ 97%, allele frequency difference (≤ 0.15) between males and females for autosomal markers, and genotype concordance rate (> 0.75) across duplicate genetic markers, around 94% of samples and more than 98% of genetic markers assayed passed the QC procedures.[22] Prior to imputation, we additionally excluded genetic markers with a minor allele frequency (MAF) < 1%, or a genotype call rate < 90%.

Imputation was conducted on an array-wise basis. Following the pre-phase of the genotypes with Shape-IT v2.5,[23] genetic markers were imputed from the cosmopolitan reference panel 1000 Genomes Project (phase I integrated release) using IMPUTE2 v2.3.1[24–26]. As a QC metric, we used the info r^2 from IMPUTE2, which estimates the correlation between the true and imputed genotype[27]. We reported imputed markers with info-metric $r^2 \geq 0.9$ and MAF ≥ 1%; all reported genotyped markers exceeded a genotype call rate ≥ 98%, and a P-value ≥ 0.001 for Hardy-Weinberg equilibrium deviation.

Genetic association analyses

We obtained results from a recently-completed GWAS of asthma in the GERA cohort [28]. Briefly, a case-control GWAS was conducted to investigate the association of 7,230,512 common (MAF > 5%) genotypes (genotyped and imputed) with asthma status in 54,543 adult non-Hispanic white subjects from GERA. Covariate-adjusted logistic regression of case-control asthma status was performed with an additive genetic model using PLINK v.1.9 and additive genetic model. SNP identifiers, gene and allele annotations, summary statistics and relevant association parameters (MAF, Odds ratios, and P-values) were obtained for the coding SNPs investigated in this study. Population attributable risk (PAR) was computed as follows: $\mathrm{PAR} = Pe \times \frac{(\mathrm{OR} - 1)}{\mathrm{OR}}$ where Pe is the proportion of risk allele carriers among cases.

Variant analyses in EVE

Allele frequencies for European American, African American/Caribbean, and Latino American asthma cases and controls were obtained from the previously published EVE consortium asthma GWAS, along with p-values and allelic odds ratios (ORs) corresponding to each of these racial/ethnic groups and the overall meta-analysis[10]. To obtain PAR measures with the same reference allele as GERA, we computed genotype frequencies for EVE cases and controls according to Hardy-Weinberg equilibrium distributions [29]. Population attributable risk (PAR) was computed similarly as in GERA. Analyses were performed with R software [29].

Cell culture and transfection

Human embryonic kidney (HEK) 293T and BEAS2B cells were maintained in DMEM (Life Technologies) supplemented with 10% FBS (Life Technologies) and 100 U/ml penicillin and 100 μg/ml streptomycin (Life Technologies). Primary human airway smooth muscle (ASM) cells and normal human lung fibroblasts were obtained from Lonza. Transfections were performed on subconfluent cells using Turbofect transfection reagent (Life Technologies) for HEK293T and Lipofectamine 3000 (Life Technologies) for BEAS2B cells.

Expression constructs

Full length human GSDMB cDNA was generated by RT-PCR from RNAs extracted from normal HBE cells in ALI. All GSDMB constructs were made by cloning the cDNAs into a pEF6 vector. The pEF6 vector was digested by BamH1 and EcoR1. The inserts were generated using specific primers that allowed generation of cutting sites for BamH1 and Mfe1 as the GSDMB cDNA harbors a restriction site for EcoR1. EcoR1 and Mfe1 generate similar cohesive ends. The ligated products were transformed into TOP10 competent cells (Invitrogen). All restriction enzymes were from New England Biolabs. Mutations were introduced by site-directed mutagenesis using the QuikChange II Kit (Agilent Technologies) and were confirmed by DNA sequencing. Caspase-1 expression construct in pCDNA3.1(+) was from Dr. Tiffany Horng (Harvard School of Public Health). pCDNA3.1(+) (Thermo Fisher Scientific) was used as an empty vector control.

Air-liquid interface (ALI) culture of HBE cells

Normal human bronchial epithelial (NHBE) cells were obtained at passage 1 from the Marsico Lung Institute/Cystic Fibrosis Center at the University of North Carolina, Chapel Hill and expanded in bronchial epithelial basal media (BEBM, Lonza) supplemented with bovine pituitary extract (BPE, 52 μg/ml), hydrocortisone (0.5 μg/ml), human epidermal growth factor (hEGF, 25 ng/ml), epinephrine (0.5 μg/ml), insulin (5 μg/ml), triiodothyronine (6.5 ng/ml), transferrin (10 μg/ml), gentamicin (50 μg/ml), amphotericin-B (50 ng/ml), bovine serum albumin (1.5 μg/ml), and retinoic acid (50 nM). For the establishment of ALI culture, passage 2 HBE cells were fed with a 1:1 mixture of BEBM and Dulbecco's Modification of Eagle's Media (DMEM, Mediatech) supplemented with the same components detailed above, except hEGF (0.5 ng/ml). Briefly, cells were cultured on collagen-coated 12-Transwell plate (Corning) at a density of 6×10^4 cells/cm^2 under submerged conditions until confluence. Medium was then removed from the apical surface, and ALI culture was maintained up to 21 days until needed.

Western blotting

Whole cell lysates were prepared in RIPA buffer supplemented with Protease and Phosphatase Inhibitor Cocktails (Roche). Cleared lysates were heated in LDS buffer at 70°C for 10 minutes and run in SDS-PAGE gel under reducing conditions. Primary antibodies included anti-GSDMB (Proteintech), anti-FLAG (Sigma), anti-β-actin (Santa Cruz Biotechnology), and anti-GAPDH (Santa Cruz Biotechnology).

Quantitative real-time PCR (qRT-PCR)

RNA from NHBE cells was extracted using the RNeasy kit according to the manufacturer's instructions (Qiagen). RNA was then reverse-transcribed using using the First-Strand Synthesis Kit (Life Technologies). Quantitative PCR was then performed using SYBR green master mix (Qiagen) with the following primers: *GSDMB* forward: 5'-AAAGCGACCGGCAATATAAA, *GSDMB* reverse: 5'-ATAGCTCAGGACCCGATTTG, *ACTB* (β-actin) forward: 5'-CCAACCGCGAGAAGATGA, and *ACTB* reverse: 5'-CCAGAGGCGTACAGGGATAG, *FOXJ1* forward: 5'-CTTGCCTGGTTCGTCCTTCTC-3', *FOXJ1* reverse: 5'-ATCCGCCACAACCTGTCTCT-3', *MUC5AC* forward: 5'-

GTCACATTCCTCAGCGAGGTG-3' and *MUC5AC* reverse: 5'-GGAACTGTGGGGACAGCTCTT-3'. The delta delta CT method was used to determine relative expression. Values were normalized against β-actin expression.

Fluorescence activated cell sorting (FACS)

Intracellular sorting followed by RNA extraction was adapted from a previously described protocol [30]. NHBE cells in ALI culture (day 21) were trypsinized and fixed with 4% paraformaldehyde in PBS with RNASEout (1:100, Life Technologies) for 30 minutes at room temperature. Following 2 washes with wash buffer (PBS supplemented with 0.5% BSA, 0.05% Tween-20 and 1:100 RNASEout), cells were incubated with either anti-β-tubulin IV (Sigma) or anti-MUC5AC (Thermo Scientific) antibodies for 1 hour at 4°C in PBS with 1% BSA, 0.05% Tween-20 and RNASEout (1:25). Following 3 washes with wash buffer, cells were incubated with secondary antibody (1:100), washed again for another 3 washes and sorted by FACS (BD FACS-Aria Sorter). RNA was then extracted using the PFFE Kit (Qiagen) and transcribed using random hexamers in the First-strand Synthesis Kit (Life Technologies). Quantitative PCR was performed for GSDMB using primers above and with the following additional primers (*FOXJ1* and *MUC5AC*) to determine whether efficient separation of ciliated or goblet cells was achieved.

Immunofluorescence staining

Immunofluorescence staining on ALI-cultured NHBE cells and paraffin-embedded lung tissue sections was performed as described previously with some modifications.[31] For ALI-cultured HNBE cells, fixation was performed *in situ* on inserts using 4% paraformaldehyde. For lung tissue sections, which were received from the Marsico Lung Institute/Cystic Fibrosis Center at the University of North Carolina, Chapel Hill, deparaffinization was performed by processing through a series of ClearRite and ethanol solutions. Antigen retrieval was performed using 10mM citrate buffer, pH 6.0. Both ALI-cultured NHBE cells and lung tissue sections were blocked with PBS supplemented with 5% normal donkey serum and 0.2% Triton X-100 for 1 hour at room temperature. Primary antibody incubation was performed overnight at 4°C in PBS supplemented with 1% BSA and 0.2% Triton X-100 using anti-GSDMB (Abgent) and anti-β-tubulin IV (Sigma) at 1:250 dilution. Secondary antibodies conjugated with Alexa-fluor 488 or 594 (Life Technologies) were used at 1:100 dilution. DAPI was used to label the nuclear DNA and samples were mounted with Vectashield anti-fade mounting medium (Vector Labs). Confocal images were taken using Leica SPE Confocal Microscope and the images were processed using ImageJ.

Pyroptosis assay

CytoTox Non-Radioactive Cytotoxicity Assay (Promega) was used to measure lactate dehydrogenase (LDH) release. Conditioned media from BEAS-2B or HBE cell cultures were mixed with an equal volume of CytoTox96 reagent (tetrazolium salt) and incubated for 30 minutes at room temperature. Stop solution was then added and the absorbance signal was measured at 490 nm using a plate reader (SpectraMAX 190). The percent of cytotoxicity was calculated by dividing the absorbance value of experimental LDH release by the maximum LDH release obtained by complete lysis of cells using the kit's lysis solution.

Statistical analyses

Statistical analysis for functional experiments was performed using Student's t-test. Results presented were mean with standard error of the mean (SEM). The differences between groups were considered statistically significant if P value < 0.01.

RESULTS

Association of exon coding variants in *GSDMB* with reduced asthma risk

The asthma-associated 17q21 locus contains six protein-coding genes: *IKZF3*, *ZPBP2*, *GSDMB*, *ORMDL3*, *LRRC3C* and *GSDMA*. We searched for all common (i.e., minor allele frequency (MAF) > 5%) as well as lower frequency (MAF > 1%) coding variants in these six genes using the Exome Aggregation Consortium (ExAC) browser, which contains exome sequencing results of more than 60,000 individuals[32]. We identified eight common and six low-frequency coding variants (Table 1 **and** Supplementary Table 1). Six SNPs (rs11078928, rs2305480, rs2308479, rs16965388, rs35104165, rs12450091) were in *GSDMB*, four (rs3894194, rs7212938, rs7212944, rs56030650) in *GSDMA*, three (rs11557467, rs35829084, rs35302660) in *ZPBP2*, and one (rs112301322) in *IKZF3*. No common coding variant were found in *ORMDL3* or *LRRC3C*. Seven of the eight coding SNPs were genotyped in GERA. Each of these seven SNPs (rs2305480, rs11078928, rs11557467, rs7212944, rs56030650, rs7212938, rs3894194) had MAF > 30% and was nominally associated with asthma, although none reached traditional significance threshold for genome-wide significance ($P<5 \times 10^{-08}$) (Table 1). The strongest associations with asthma were for rs2305480 and rs11078928 in *GSDMB*, SNPs whose minor alleles were associated with decreased asthma risk (OR = 0.92; P-values 1.01×10^{-6} and 1.32×10^{-6} for rs2305480 and rs11078928, respectively). Specifically, the MAF for rs11078928 in GERA was 45% in non-asthmatic controls and 43% in asthma cases with a population attributable risk of 3.8% (Table 1), suggesting that the minor allele variant (C) may protect against asthma.

We next sought to replicate these *GSDMB* SNP associations in the EVE consortium, which consisted mostly of cases with childhood onset asthma (mean age of asthma onset for cohorts ranged between 1.4 and 13.1 years; median age of asthma onset for cohorts ranged between 1.0 and 8.0 years) [10]. Although results for the *GSDMB* splicing variant rs11078928 were not available in EVE, results for rs2305480, a SNP in perfect LD ($r^2=1$) with rs11078928 among European reference populations (Supplementary Fig. 1), were available. Furthermore, among European reference populations, these two SNPs of interest (i.e., rs2305480 and rs11078928) were in perfect LD ($r^2=1$) with the non-coding variant rs11078927, which was previously identified as the SNP most significantly associated with asthma in EVE[10]. Table 2 contains results for these two SNPs of interest, along with those of one other SNP (rs2305479) with available EVE results, each of which was associated with reduced asthma at genome-wide significant levels in the full EVE cohort. Association results for rs2305480, the only SNP available in both EVE and GERA, were consistent across cohorts.. The minor (T) allele of rs2305480 was associated with decreased asthma prevalence with suggestive significance in both European Americans (OR = 0.80; $P = 8.30 \times 10^{-7}$) and Latino Americans (OR = 0.78; $P = 2.27 \times 10^{-7}$) (Table 2). For EVE European

Americans, rs2305480 MAF was 0.45 for non-asthmatic controls and 0.39 for asthmatic cases; for all EVE cohorts, of rs2305480 MAF was 0.32 for controls and 0.27 for cases. The population attributable risks for these SNPs ranged from approximately 12% to 16% in each of the race/ethnicity-specific groups of EVE (Table 2). Altogether, GERA and EVE GWAS results identified two tightly linked common coding variants (rs2305480 and rs11078928) in *GSDMB*, whose minor alleles were nominally associated with decreased asthma risk in multiple diverse populations.

High expression of *GSDMB* in differentiated airway epithelial cells

GSDMB has been shown to be expressed in primary bronchial epithelium in asthmatic lung [33], and epithelium in the gastrointestinal tract [34]. We examined the expression of *GSDMB* in primary human lung cells that are relevant to asthma pathogenesis. Quantitative RT-PCR showed that *GSDMB* mRNA is moderately expressed in normal human bronchial epithelial (NHBE) cells and lung fibroblasts, but was barely detectable in human airway smooth muscle cells (Fig. 1A). NHBE cells cultured in the submerged condition are mostly basal cells, which can be differentiated into goblet cells and ciliated cells in the air-liquid interface (ALI) culture. Remarkably, *GSDMB* mRNA expression is significantly higher (~20 fold) in well-differentiated NHBE cell culture on ALI day 21 than in undifferentiated NHBE cells (ALI day 0) (Fig. 1A). To further distinguish which mature airway epithelial cell types (goblet or ciliated cells) express highest levels of *GSDMB*, we performed fluorescence activated cell sorting (FACS) to isolate goblet or ciliated cells. We sorted β-tubulin IV-positive cells as ciliated cells[35] and MUC5AC-positive cells as goblet cells[36]. QRT-PCR using selected markers (*FOXJ1* for ciliated cells[37] and *MUC5AC* for goblet cells[36]) confirmed that the FACS sorting successfully isolated specific cell populations (Fig. 1B). In β-tubulin-IV-positive ciliated cells, the expression of *GSDMB* is significantly higher (> 2 fold) than in β-tubulin IV-negative cells. The expression of *GSDMB* is not significantly different between MUC5AC-postive goblet cells and MUC5AC-negative cells.

Consistent with the qRT-PCR results, immunostaining showed that GSDMB protein is highly expressed in ciliated (β-tubulin-IV-positive) cells (Fig. 1C). GSDMB is also expressed in some β-tubulin-IV-negative (presumably goblet) cells (Fig. 1C). In addition, we also detected robust expression of GSDMB protein in the epithelium, but not other types of cells, of the human airways (Fig. 1D). Together, these data showed that GSDMB is highly expressed in the airway epithelium, including the fully differentiated ciliated cells.

Cleavage of GSDMB Protein by Caspase-1 Induces Pyroptotic Cell Death

GSDMB belongs to the family of gasdermin proteins[38]. While little was known about the biochemical function of GSDMB, recent studies have demonstrated that another family member gasdermin D (GSDMD) induces a particular type of cell death known as pyroptosis, in which pore formation at the plasma membrane causes cell bursting and release of inflammatory molecules[39, 40]. GSDMD, upon inflammasome activation, is cleaved by inflammatory caspases to remove the inhibitory C-terminus, resulting in the release of a functional N-terminal domain, which induces pore formation at the plasma membrane to cause pyroptosis[39–42]. We observed that, when co-expressed with inflammatory caspase-1, GSDMB protein is cleaved into at least two short forms (Fig. 2A). One of the cleaved forms

is about the predicted size (~20 kD) of the N-terminus of the GSDMB protein. To confirm the specificity of the cleavage, we mutated two potential caspase-1 cleavage sites (aspartate 236 [D236] and aspartate 250 [D250]) in the middle of the GSDMB protein. As shown in Fig. 2A, while mutation of D250 has little effect on the cleavage of GSDMB into the shorter form, mutation of the aspartate at residue 236 to alanine (D236A) completely abolished the cleavage as indicated by the disappearance of the shorter fragment. This result strongly supports specific cleavage of GSDMB by caspase-1 at the D236 position.

We next determined whether the GSDMB N-terminal fragment released from caspase-1-induced cleavage induces pyroptosis of cells. Expression of the N-terminal fragment induced potent pyroptotic cell death, as indicated by both the release of LDH into the medium (Fig. 2B) and by microscopic examination of cell morphology (Fig. 2C). In contrast, expression of the full length or the C-terminal fragment did not induce any increase in pyroptosis (Fig. 2B,C). Consistent with the cleavage of the GSDMB protein by caspase-1, co-expression of wild type GSDMB, but not the D236A mutant, which cannot be cleaved by caspase-1, resulted in pyroptotic cell death (Fig. 2D). These results show that the GSDMB N-terminus released by the inflammatory caspase-1 cleavage induces potent pyroptosis.

The Splicing Variant rs11078928 Abolishes Pyroptosis-inducing Activity of GSDMB

While genotypes of rs2305480 result in a change of proline at amino acid residue 311 of the GSDMB protein to serine (P311S), the splicing variant rs11078928 (T→C) destroys a splicing acceptor site and thus prevents the splicing of exon 6[43], resulting in the deletion of 13 amino acids from the N-terminus of GSDMB protein (Fig. 3A). We first examined the effect of rs11078928 genotypes on GSDMB splicing and protein expression in primary NHBE cells. We genotyped over 30 primary NHBE lines and identified 2 donors with homozygous CC alleles of rs11078928. We chose two age- and gender-matched donors with homozygous TT alleles. As shown in Fig. 3B, the *GSDMB* mRNA in NHBE cells with the CC genotype is smaller than that in NHBE cells with the homozygous TT genotype. Direct sequencing confirmed the deletion of exon 6 from the transcript (data not shown). Consistent with this, NHBE cells of TT genotype express the long (416 amino acids) and short (403 amino acids) forms of GSDMB protein, whereas the long form is absent in NHBE cells of CC genotype (Fig. 3C). Interestingly, although the long form *GSDMB* mRNA is more abundant in cells of TT genotype, the cells express more of the 403-aa-long GSDMB protein, suggesting that the longer transcript may be less efficiently translated than the shorter transcript. Nevertheless, the TT genotype allows, whereas the CC genotype abolishes, the expression of the longer GSMB form. Together, these results confirmed the effect of rs11078928 on GSDMB splicing and protein expression.

We next examined the effects of the variants (rs2305480 and rs11078928) on GSDMB protein function: induction of pyroptosis. We transfected GSDMB-416, GSDMB-416P311S (for rs2305480), and GSDMB-403 (for the splicing rs11078928) into HEK293T cells, either in the presence or absence of caspase-1. As shown in Fig. 3D, expression of GSDMB proteins alone (in the absence of caspase-1) did not induce pyroptosis. However, in the presence of caspase 1, GSDMB-416 induced pyroptosis (Fig. 3D). GSDMB-416 P311S (proline to serine change at residue 311), which was suggested to affect GSDMB protein

conformation[44], induced similar cytotoxicity as the wild type protein (Fig. 3D), indicating that rs2305480 has little effect on the pyroptotic activity of GSDMB. In contrast, GSDMB-403 did not induce pyroptosis even in the presence of caspase-1 (Fig. 3D). Consistent with this, the expression of the shorter N-terminus of GSDMB-403 (N220), unlike the normal GSDMB N-terminus (N232), failed to induce pyroptotic cell death (Fig. 3E). Moreover, in the bronchial epithelial BEAS-2B and 16HBE cells, GSDMB-403 was unable to increase pyroptosis in response to inflammasome induction (Fig. 3F). Together, these results indicate that the splicing variant rs11078928 abolished the ability of GSDMB to induce pyroptotic cell death.

DISCUSSION

In this study, we identified a *GSDMB* splicing variant (rs11078928*)* that abolishes the biochemical activity of GSDMB and that is nominally significantly associated with decreased asthma risk in a large asthma GWAS. Additionally, a SNP (rs2305480) in strong LD with it among Europeans is also associated with asthma in a second large asthma GWAS. Previous study by Igartua et al. reported an association between rs11078928 and asthma [45]. Another study showed that the asthma risk allele (T) of rs11078928 was associated with increased expression of *GSDMB* transcripts [43]. More recently, Chao et al reported that several apoptotic executioner caspases can cleave the GSDMB protein [44]. In addition, Das et al showed that *GSDMB* is expressed in human airway epithelial cells and that *GSDMB* expression increases with severity of asthma [33]. We extended these findings by showing that *GSDMB* is highly expressed in ciliated airway epithelial cells and, when activated, induces inflammatory caspase-mediated pyroptotic cell death. Importantly, we demonstrated that the loss of functional splicing by a genetic variant abolishes the biochemical activity of GSDMB. Together, our work identified the function of *GSDMB* coding variant rs11078928, an asthma-associated 17q21 locus SNP, thus supporting *GSDMB* as an asthma-related gene by linking one of its variants to a mechanism that plausibly contributes to asthma pathogenesis.

Many non-coding SNPs in the 17q21 locus have been associated with asthma, especially childhood-onset asthma [9, 10, 13]. A recent study that examined rare to low-frequency functional variants in this region, including miss-sense variants in the 17q21 region [45], measured associations but did not include functional follow-up studies. As a result, an asthma-related functional role for variants in the 17q21 locus has not been established. The rs11078928 splicing variant, by influencing pyroptotic cell death, may causally contribute to the previously observed 17q21 locus asthma associations, as we found that (1) the C allele of this SNP is nominally associated with decreased risk of asthma in non-Hispanic white adults from the GERA biobank-based cohort , and (2) the A allele of rs2305480, which is in perfect LD with rs11078928 among European populations (rs2305480), is associated with decreased asthma prevalence in diverse subjects from EVE, a cohort consisting mostly of cases with childhood-onset asthma. Further, rs2305480 is in perfect LD with the top-ranked EVE asthma-associated SNP rs11078927. Our findings do not preclude other genes and/or variants in the 17q21 locus from contributing to the well-known asthma-association signal. For example, the *GSDMA* gene present in this region is also expressed in the lung [46] and could have a functional role in asthma pathogenesis as several *GSDMA* coding variants are

associated with asthma, albeit at a less significant level than the *GSDMB* splicing variant. Whether these variants functionally perturb *GSDMA* gene function and contribute to asthma-relevant cell phenotypes awaits further investigation. Future studies that identify additional functional variants in *GSDMB* and in other 17q21 genes are warranted.

GSDMB is highly expressed in well-differentiated airway epithelial cells, including ciliated cells. The dominant cell type in the airway epithelium, ciliated cells are important for mucociliary clearance, transdifferentiation, and repair following injury to the airway epithelium [47, 48]. Asthmatic airways have repeated epithelial shedding that results in impaired mucociliary clearance. Epithelial biopsies from both asthmatic children and adults showed damage to ciliated cells with cytoplasmic blebbing and mitochondrial vacuolization, as well as loss of cilia and abnormal cilia structure [49]. Future studies investigating exactly how GSDMB-mediated pyroptosis of ciliated airway cells contributes to asthma pathogenesis may offer a new avenue for therapeutic intervention that protects the integrity and function of the airway epithelium. Based on our studies, we envision a model in which GSDMB in differentiated airway epithelial cells is activated by certain pro-inflammatory stimuli such as viruses and allergens. Such activation results in caspase-mediated cleavage of GSDMB, whose N-terminal fragment targets the membrane and induces the formation of plasma membrane pores. This GSDMB-mediated lysis of airway epithelial cells triggers the release of cytoplasmic contents, including, inflammatory molecules (e.g. IL-1β and IL-33) that alter the behavior of other cell types involved in hallmark asthma features. The GSDMB splicing variant abolishes the biochemical activity of the cleaved GSDMB fragment, and may thus confer decreased asthma susceptibility.

The rs11078928 SNP represents a “loss-of-function” mutation: the major allele (T) confers higher incidence of asthma, whereas the C allele, which causes the loss of pyroptosis-inducing activity of GSDMB is associated with lower asthma risk. This finding has important implications for potential asthma therapies that target GSDMB. For example, suppression of the active GSDMB fragment in individuals with rs11078928-TT could reduce asthma susceptibility by reducing airway epithelial pyroptosis and subsequent release of inflammatory factors. However, this GSDMB-targeting therapy is unlikely to have therapeutic benefit for asthmatics with rs11078928-CC, who lack the functional GSDMB protein. Thus, in the context of precision medicine, an effective therapeutic strategy targeting GSDMB would require the screening and selection of asthmatics with the major allele of rs11078928, who are more likely to respond to therapies targeting functional GSDMB.

Our study has some limitations. First, GERA consisted largely of adults with asthma, and we are unable to distinguish those with childhood- vs. adult-onset asthma. Because the 17q21 locus is most pronounced in childhood-onset asthma[9, 13], the associations we measured in GERA may not reflect those of a cohort with childhood-onset asthma only. Future fine-mapping studies that compare childhood asthma-specific cohorts to adult-onset cohorts may yield insights into functional 17q21 associations that vary by time of asthma onset. Second, none of the *GSDMB* SNPs investigated in the GERA cohort achieved a traditional significance threshold for genome wide significance ($P < 5 \times 10^{-8}$) with the highest significance being 1.3×10^{-6}. Third, due to the inherent difficulty of genome editing in primary cells, our study did not demonstrate directly the functional role of rs11078928 in

differentiated airway epithelial cells. Future development of more efficient methods of genome editing methods will help interrogate the role of *GSDMB* and its variants in primary airway epithelium, including the ciliated cells. Finally, *GSDMB* is human specific (there is no *GSDMB* gene encoded in the mouse genome), and thus, we were unable to investigate the function of the *GSDMB* splicing variant *in vivo*. However, whole body transgenic mice overexpressing the *GSDMB* minor (C) allele (403-aa form) do exhibit airway hyperresponsiveness without inflammation [33]. Future experiments using transgenic mice expressing the *GSDMB* splicing variant in airway epithelium may directly test the function of the variant *in vivo*.

In summary, by combining genetic association analyses and mechanistic studies, we identified and characterized a functional asthma variant in the *GSDMB* gene. Future studies that further investigate the physiological role of the variant and the gene may provide novel mechanistic insights into asthma pathogenesis that could ultimately lead to the development of potentially curative asthma therapies targeting GSDMB.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank Ian Stancil and Rebecca Hirsch for help with NHBE ALI cell culture and immunostaining, and the Harvard NeuroDiscovery Imaging Core for technical support with confocal imaging. We are grateful to the EVE Consortium, funded by RC2 HL101651, and Dr. Carole Ober, for generously providing us with their primary study results. This work was supported by an NIH grant (R01HL114769) and an American Asthma Foundation Scholar award to QL. AD was supported by K01 HL130629-01. BEH was partially supported by NIH R00 HL105663. JP was supported by American Heart Association (13SDG14320004) and by an NIH grant P01HL120839. SHR was supported in part by an NIH grant DK065988.

Abbreviations used

ALI	air-liquid interface
BEBM	bronchial epithelial basal media
DMEM	Dulbecco's Modification of Eagle's Media
ExAC	Exome Aggregation Consortium
FACS	fluorescence activated cell sorting
GERA	Genetic Epidemiology Research in Adult Health and Aging
GSDMA	Gasdermin A
GSDMB	Gasdermin B
GSDMD	Gasdermin D
GWAS	genome-wide association study
HBE	human bronchial epithelial

HEK293T	human embryonic kidney 293T
KPNC	Kaiser Permanente Medical Care Plan, Northern California Region
LD	Linkage disequilibrium
LDH	lactate dehydrogenase
MAF	minor allele frequency
NHBE	normal human bronchial epithelial
OR	odds ratio
PAR	population attributable risk
QC	quality control
qRT-PCR	quantitative real-time polymerase chain reaction
SNP	Single nucleotide polymorphism
Th2	type-2 helper T-cell

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Key Messages

- Multiple coding variants in the gasdermin B (*GSDMB*) gene in the 17q21 locus are associated with reduced asthma risk.
- *GSDMB* gene is highly expressed in the ciliated airway epithelial cells and encodes a protein with the ability to induce cell pyroptosis.
- A splicing variant (rs11078928) in *GSDMB* skips an essential exon from the transcript and abolishes the pyroptotic activity of the GSDMB protein.

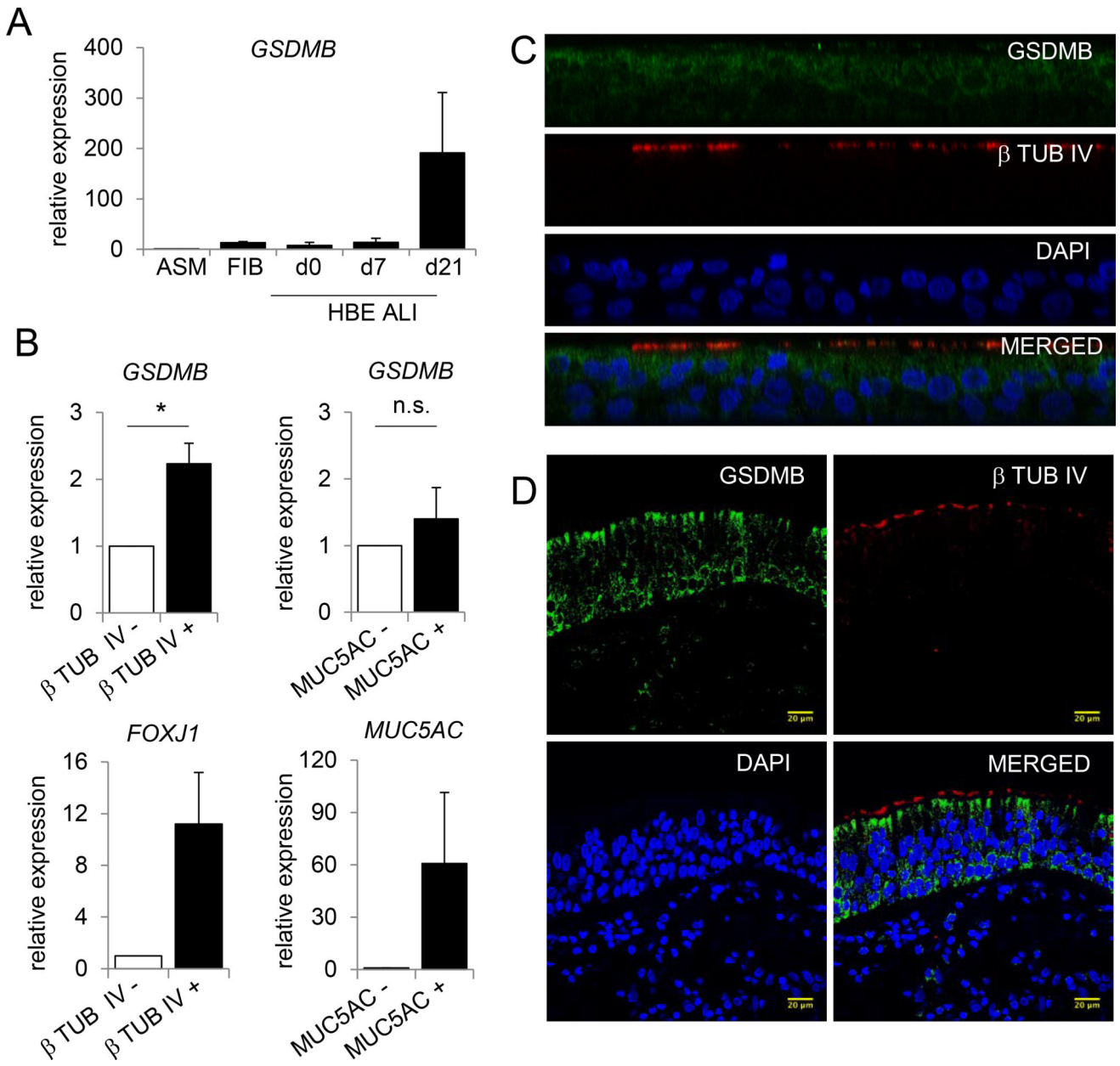

Figure 1. *GSDMB* expression in airway epithelium

A) Relative *GSDMB* mRNA expression in human primary airway smooth muscle (ASM), lung fibroblasts (FIB), and normal human bronchial epithelial (NHBE) cells over the course of air-liquid interface (ALI) culture. *ACTB* (β-actin) was used as an internal control. B) Relative *GSDMB* mRNA expression in sorted β−tubulin IV-positive ciliated NHBE and MUC5AC-postive goblet NHBE cells. Graphs show mean of fold-change from three different donors; n=3, +/− SEM. C and D) Immunostaining of GSDMB protein in NHBE (normal human bronchial epithelial) cells at day 21 of ALI culture (C) and lung tissue (D) shows GSDMB expression in ciliated cells. Ciliated cells were stained for β−tubulin IV and nuclei were visualized by DAPI staining.

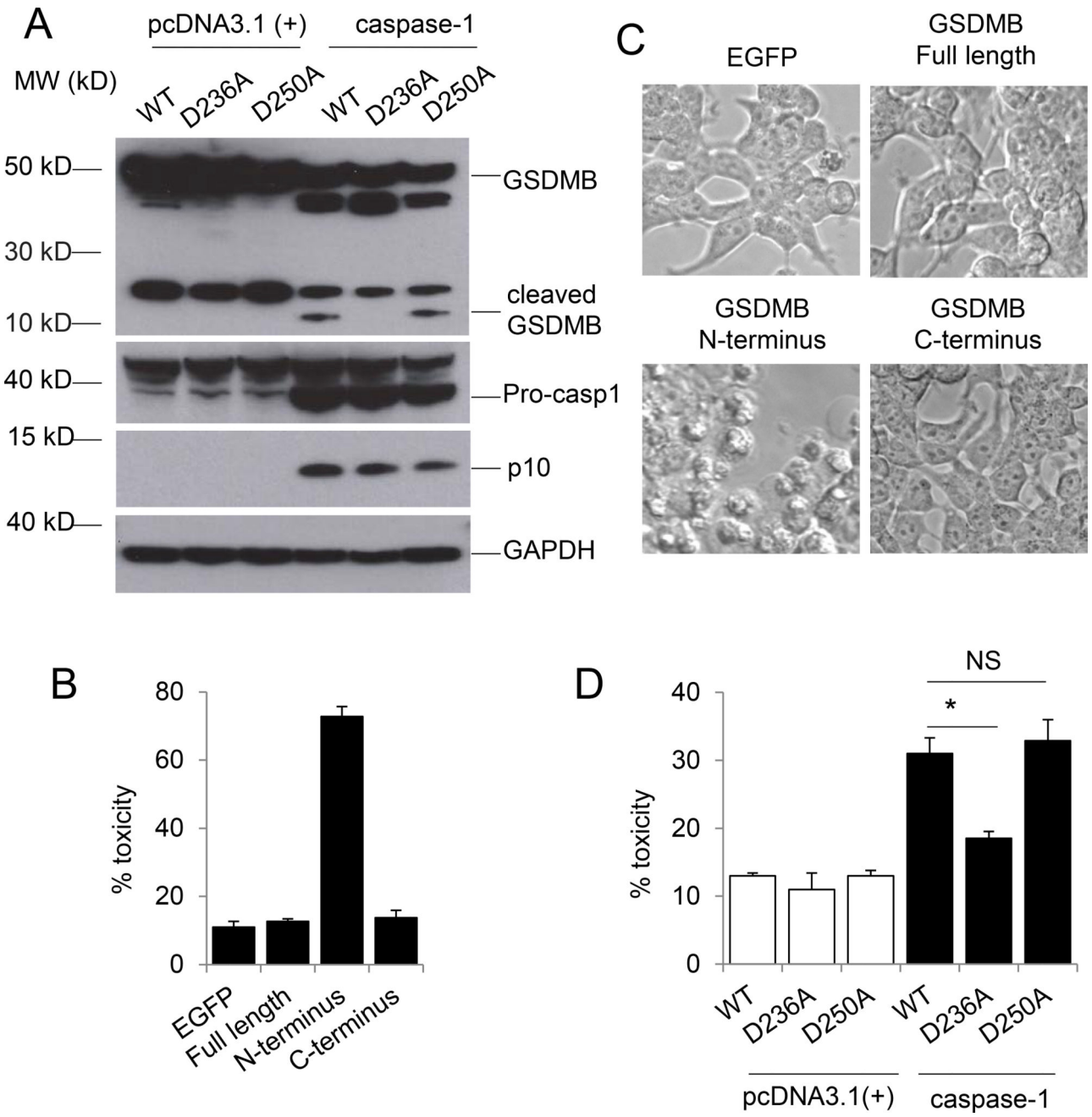

Figure 2. GSDMB is cleaved by caspase 1 to induce pyroptosis

A) Western blot showing GSDMB cleavage by caspase-1. Wild type or mutant forms (D236A and D250A) were co-transfected with either caspase-1 or vector plasmid. Twenty-four hours post-transfection, protein lysates were prepared for Western blotting..B and C) The N-terminal fragment of GSDMB induces cell death. HEK293T cells were transfected with EGFP, full length GSDMB, N-terminal, or C-terminal fragment of GSDMB protein. Twenty-four hours after transfection, cytotoxicity was assessed using the LDH assay (B) and changes in cell morphology were captured by light microscopy (C). D) LDH assay showing GSDMB-induced pyroptosis in the presence of caspase-1. HEK293T cells were transfected

with wild type, D236A, or D250A GSDMB (all full length) in the presence or absence of caspase-1. Twenty-four hours after transfection, cytotoxicity was assessed using the LDH assay. Graph shows mean of n=3, +/− SEM from a representative experiment; * $p<0.05$.

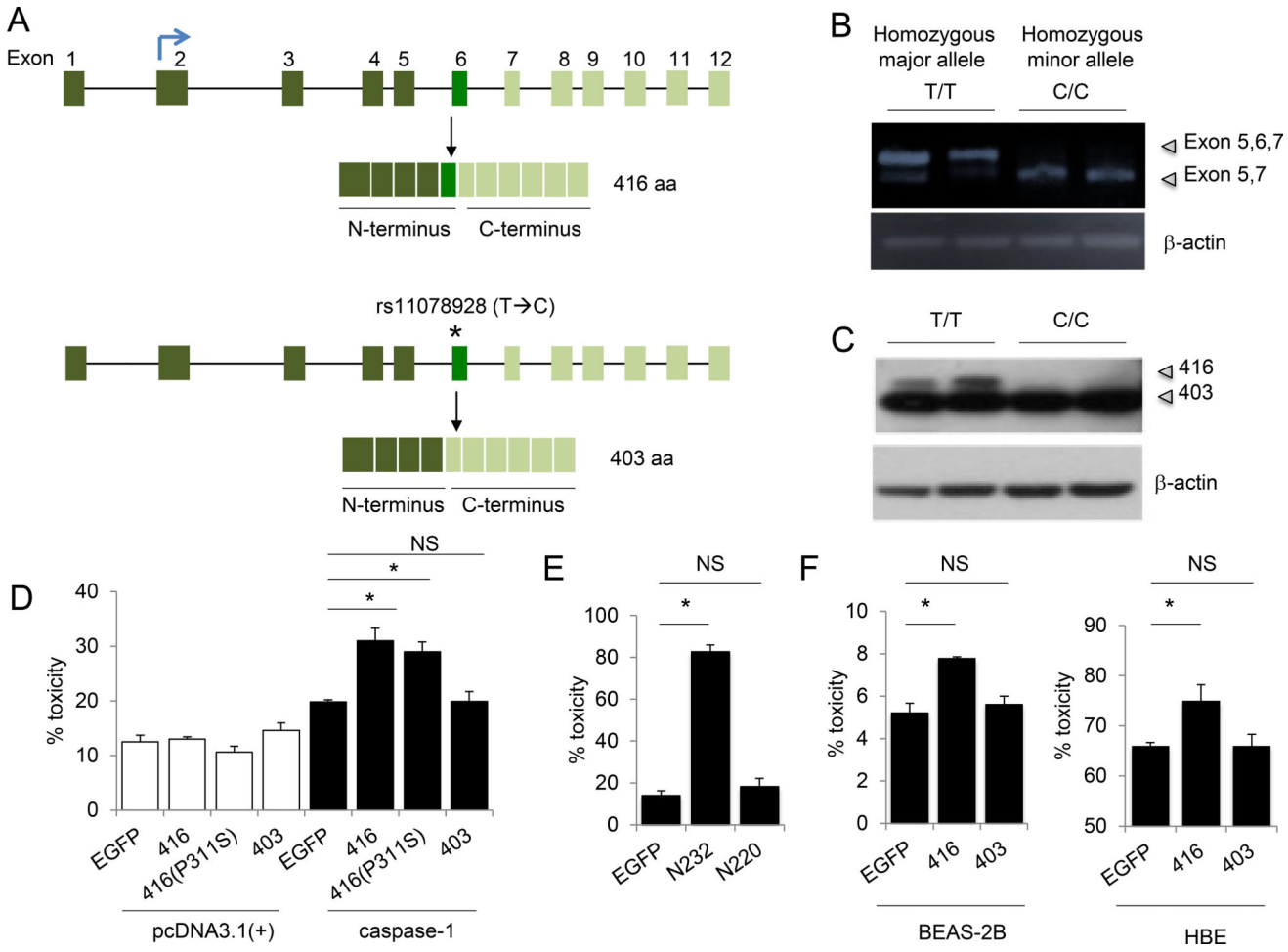

Figure 3. Splicing variant rs11078928 abolishes pyroptosis activity of GSDMB

A) Schematic representation of the GSDMB gene showing the exons and the two translational products. Boxes: exons; *: rs11078928. B) RT-PCR and C) Western blot showing the effect of rs11078928 on GSDMB splicing and expression in NHBE cells harboring the TT or CC allele and cultured in ALI. D) The 403 variant does not induce caspase-1-mediated pyroptosis in HEK293T cells. LDH assay was performed twenty-four hours post transfection with the indicated constructs. E) Cytotoxic effects of N-terminal fragments (N232: residues 1-232; N220: residues 1-220) transfected in HEK293T cells . LDH assay was performed twenty-four hours post-transfection. F) Effect of the expected product of rs11078928 on inflammasome-induced pyroptosis. BEAS2B or 16HBE cells transfected with either EGFP, 416 or 403 and caspase-1 were treated with 10 μM nigericin. Graph shows mean of n=3, +/− SEM from a representative experiment; * p<0.05.

TABLE 1

Association of 17q21 Coding Variants with Asthma in GERA.

SNP	Position_hg19	Gene	Minor_Allele	Major_Allele	Coding change	MAF, all	MAF, cases	MAF, controls	OR (95% CI)	P-value	PAR
rs2305480	38062196	*GSDMB*	A	G	P311S	0.45	0.43	0.45	0.92 (0.89–0.95)	1.01E-06	0.038
rs11078928	38064469	*GSDMB*	C	T	splicing	0.45	0.43	0.45	0.92 (0.89–0.95)	1.32E-06	0.038
rs2305479	38062217	*GSDMB*	T	C	G304R	-	-	-	-	-	-
rs11557467	38028634	*ZPBP2*	T	G	S173I	0.49	0.48	0.5	0.95 (0.90–0.96)	1.67E-05	0.025
rs7212944	38122686	*GSDMA*	A	G	E130K	0.34	0.33	0.34	0.95 (0.91–0.98)	2.75E-03	0.017
rs56030650	38131187	*GSDMA*	A	C	T314N	0.46	0.47	0.46	1.05 (1.02–1.08)	2.98E-03	0.023
rs7212938	38122680	*GSDMA*	G	T	V128L	0.49	0.5	0.49	1.05 (1.02–1.08)	5.38E-03	0.024
rs3894194	38121993	*GSDMA*	A	G	R18Q	0.46	0.47	0.45	1.05 (1.02–1.08)	8.11E-03	0.022

MAF: minor allele frequency; OR: Odds Ratio; PAR: Population Attributable Risks.

All P values are nominally significant based on P<0.05; however, none achieved genome-wide significance ($P< 5\times10^{-08}$).

TABLE 2

Association of *GSDMB* Coding Variants with Reduced Asthma Risk in EVE.

		SNP		
		rs2305480	**rs2305479**	**rs11078927**
EVE EA	Case_MAF	0.39	0.43	0.39
	Control_MAF	0.45	0.49	0.45
	OR (95% CI)	0.80 (0.71–0.9)	0.78 (0.69–0.89)	0.80 (0.71–0.91)
	PAR	0.123	0.123	0.120
	P-value	8.30E-07	2.79E-08 *	8.62E-07
EVE LA	Case_MAF	0.27	0.31	0.27
	Control_MAF	0.32	0.36	0.32
	OR (95% CI)	0.78 (0.67–0.90)	0.8 (0.69–0.92)	0.79 (0.68–0.92)
	PAR	0.163	0.14	0.152
	P-value	2.27E-07	4.90E-07	3.12E-07
EVE AA	Case_MAF	0.12	0.16	0.11
	Control_MAF	0.14	0.17	0.14
	OR (95% CI)	0.86 (0.69–1.06)	0.94 (0.78–1.15)	0.81 (0.65–1.01)
	PAR	0.125	0.047	0.164
	P-value	9.45E-03	1.44E-01	1.19E-03
Joint	Case_MAF	0.27	0.31	0.27
	Control_MAF	0.32	0.36	0.32
	OR (95% CI)	0.80 (0.73–0.86)	0.81 (0.75–0.88)	0.80 (0.73–0.87)
	PAR	0.148	0.129	0.148
	P-value	1.31E-13 *	1.21E-12 *	1.22E-14 *

EA: European American; LA: Latino American; AA: African American; PAR: Population Attributable Risks; OR: odds ratio.

P-values that reach genome-wide significance (i.e., are $< 5 \times 10^{-8}$) are designated with an asterisk (*)