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Heterozygous *CARD9* mutation favors the development of allergic bronchopulmonary aspergillosis

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Abstract

Background: Previous research demonstrated that a homozygous mutation of g.136372044G>A (S12N) in caspase recruitment domain family member 9 (*CARD9*) is critical for producing *Aspergillus fumigatus*-induced (*Af*-induced) T helper 2 (T_H2)-mediated responses in allergic bronchopulmonary aspergillosis (ABPA). However, it remains unclear whether the $CARD9^{S12N}$ mutation, especially the heterozygous occurrence, predisposes the host to ABPA.

Methods: A total of 61 ABPA patients and 264 controls (including 156 healthy controls and 108 asthma patients) were recruited for sequencing the *CARD9* locus to clarify whether patients with this heterozygous single-nucleotide polymorphisms are predisposed to the development of ABPA. A series of *in vivo* and *in vitro* experiments, such as quantitative real-time polymerase chain reaction, flow cytometry, and RNA isolation and quantification, were used to illuminate the involved mechanism of the disease.

Results: The presence of the p.S12N mutation was associated with a significant risk of ABPA in ABPA patients when compared with healthy controls and asthma patients, regardless of *Aspergillus* sensitivity. Relative to healthy controls without relevant allergies, the mutation of p.S12N was associated with a significant risk of ABPA (OR: 2.69 and 4.17 for GA and AA genotypes, $P = 0.003$ and 0.029, respectively). Compared with patients with asthma, ABPA patients had a significantly higher heterozygous mutation (GA genotype), indicating that p.S12N might be a significant ABPA-susceptibility locus (*aspergillus* sensitized asthma: OR: 3.02, $P = 0.009$; *aspergillus* unsensitized asthma: OR: 2.94, $P = 0.005$). The mutant allele was preferentially expressed in ABPA patients with heterozygous $CARD9^{S12N}$, which contributes to its functional alterations to facilitate *Af*-induced T_H2-mediated ABPA development. In terms of mechanism, *Card9* wild-type ($Card9^{WT}$) expression levels decreased significantly due to *Af*-induced decay of its messenger RNA compared to the heterozygous $Card9^{S12N}$. In addition, ABPA patients with heterozygous $CARD9^{S12N}$ had increased *Af*-induced interleukin-5 production.

Conclusion: Our study provides the genetic evidence showing that the heterozygous mutation of $CARD9^{S12N}$, followed by allele expression imbalance of $CARD9^{S12N}$, facilitates the development of ABPA.

Keywords: Allergic bronchopulmonary aspergillosis; *CARD9* polymorphism; Allelic expression imbalance; Asthma

Introduction

Allergic bronchopulmonary aspergillosis (ABPA) is a hypersensitivity lung disease attributed to bronchial colonization by the mold *Aspergillus fumigatus* (*Af*) in susceptible patients with asthma or cystic fibrosis (CF). ABPA affects approximately 12% of patients with severe asthma and 7–9% of those with CF,[1,2] and has low but significant prevalence in European, Hispanic, African American, and Asian people. ABPA affects

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approximately 30,000 individuals in the United States and a further 70,000 people worldwide.[3] The inflammatory response in ABPA is characterized by T helper 2 (T_H2) responses to *Af* allergens that stimulate immunoglobulin E (IgE) synthesis, eosinophil activation, and production of the cytokines interleukin-4 (IL-4) and IL-5.[4,5]

Clinically, ABPA is characterized by wheezing, pulmonary infiltrates, bronchiectasis, and parenchymal fibrosis. Both genetic and environmental influences drive the development of ABPA. To date, several genetic risk factors for the development of ABPA have been identified, including human leukocyte antigen DR (HLA-DR) and human leukocyte antigen DQ (HLA-DQ) restriction, the Ile75Val polymorphism in the IL-4 receptor alpha chain (IL-4Rα), IL-10 promoter polymorphisms, the Ala91Pro variant of the surfactant protein A2 (SP-A2), and heterozygous mutations of the CF transmembrane conductance regulator (*CFTR*) gene.[6–11] ABPA patients are genetically at risk to develop heightened T_H2 responses to *Af* antigens, given the prevalence of *Af* colonization.

Caspase recruitment domain family member 9 (CARD9) is a key adaptor protein involved in orchestrating C-type lectin receptor (CLR)-mediated canonical nuclear factor kappa B (NF-κB [p65]) signaling to initiate IL-12, IL-6, and IL-1β production, which drives T_H1 and T_H17 responses.[12,13] As a critical adaptor protein, CARD9 is mainly expressed on myeloid cells, downstream of pattern recognition receptors (PRRs) involved in innate and adaptive immunity, especially for macrophages and dendritic cells (DCs).[14] Clinically, *CARD9* single-nucleotide polymorphisms (SNP) have been found to be involved in autoimmune diseases, such as Crohn's disease, ulcerative colitis (UC), ankylosing spondylitis, immune globulin A (IgA) nephropathy, and rheumatoid arthritis.[15]

CARD9 null or loss-of-function mutations are responsible for defective innate and adaptive immunity, leading to life-threatening fungal infections in patients.[16,17] Inheritance of the $CARD9^{S12N}$ mutation is reported to confer susceptibility to Crohn's disease and UC (Genome Wide Association Study [GWAS] analyses with an odds ratio of 1.2 for both diseases).[18] In addition, $CARD9^{S12N}$ is also associated with primary sclerosing cholangitis in patients with UC.[19] Our previous study demonstrated that the homozygous $Card9^{S12N}$ is critical for *Af*-induced T_H2 responses.[20] In terms of mechanism, $Card9^{S12N}$ facilitates *Af*-induced activation of non-canonical NF-κB (RelB) in alveolar macrophages, which regulates IL-5 expression to induce T_H2 responses. However, we did not clarify whether and how patients with this heterozygous SNP are predisposed to the development of ABPA.

Methods

Ethical approval

The study design, which conforms to the ethical guidelines of the 1975 *Declaration of Helsinki*, was approved by the Human Research Committee of Tongji University School of Medicine (No. 2015YXY95) and other hospitals where an individual committee review was required. Written informed consents were obtained from all participants. All animal experiments were performed in compliance with institutional guidelines and the protocol, which was approved by the Animal Ethics Committee of Tongji University School of Medicine (No. 2015YXY95).

Human subject enrollment, sample collection, and antigen detection

A case-control study was conducted involving 61 ABPA patients, 108 asthma patients, and 156 healthy controls (HCs) from Shanghai Pulmonary Hospital, Ruijin Hospital, and Shanghai Putuo District People's Hospital from 2015 to 2019. Diagnosis of ABPA was based on the 2013 International Society for Human and Animal Mycology (ISHAM) criteria.[21] All patients with asthma fulfilled the definition given by the Global Initiative for Asthma (update 2015).[22] All asthma patients were proved to be sensitive to special allergens and divided by the presence of a positive specific immunoglobulin E (sIgE) in their serum into asthma patients with *Aspergillus* sensitivity, as well as those with other sensitivities. Healthy controls without clinical symptoms and a clinical history of asthma or allergic diseases were recruited from medical examination population. The level of total serum IgE and the levels of sIgE specific to allergens such as *Aspergillus*, house dust, mixed pollens, and other fungi were determined for ABPA patients, asthma patients, and healthy controls using the Phadia ImmunoCAP 1000 instrument system (Pharmacia Diagnostic, Uppsala, Sweden). Peripheral blood mononuclear cells (PBMCs) were obtained from patients and controls with informed consent.

Af exposure and sensitization animal model

Wild-type (WT), homozygous $Card9^{S12N}$ knock-in mice, and heterozygous $Card9^{S12N}$ knock-in mice (Het), on a C57BL/6J background, described previously,[20] were housed in the specific pathogen-free animal facilities at Tongji University. We established chronic asthma model (five animals per group) in homozygous and heterozygous $Card9^{S12N}$ knock-in mice and $Card9^{WT}$, and high dose exposure model (six animals per group) in heterozygous $Card9^{S12N}$ knock-in mice and $Card^{WT}$ mice. The chronic asthma model was established as described in the previous study.[25] Briefly, eight-week-old female mice were first administered *Af* (5×10^6) intratracheally in 50 μL of phosphate buffer saline (PBS). Seven days after the initial exposure, mice were intratracheally re-administered *Af* conidia (1×10^6) daily for 5 days; after resting for 2 days, all mice were re-challenged with *Af* conidia (1×10^6) for another 3 days, and were sacrificed 24 h after the final challenge (day 18). For high dose exposure model, mice were sacrificed at day 8 after a single exposure to *Af* conidia (5×10^7).

Cell culture

Primary cultures of bone marrow-derived macrophages (BMDMs) from $Card^{WT}$ mice, and heterozygous and homozygous $Card9^{S12N}$ knock-in mice were prepared as previously described.[24] After 6–7 days, flow cytometry

analysis indicated that the cell population contained >97% $CD11b^+F4/80^+$ cells, which were subsequently prepared for use. PBMCs from both patients with ABPA and healthy donors were isolated from heparinized blood using Lymphoprep (MP Biomedicals, Irvine, CA, USA), and then cultured for use. To detect messenger RNA (mRNA) decay of CARD9, the cells were treated with actinomycin D (5 μg/mL), and then challenged with *Af* conidia for indicated time.

RNA isolation and quantification of CARD9 expression

Complete RNA was extracted from PBMCs using the RNeasy Minikit (Qiagen, Duesseldorf, Germany) and subsequently reversely transcribed into complementary deoxyribonucleic acid (cDNA) using a PrimeScript™ II 1st Strand cDNA Synthesis Kit (TaKaRa, Tokyo, Japan), according to the manufacturer's instructions. Then, the cDNA samples were analyzed by pyrosequencing at Sangon Biotech in Shanghai to determine allelic expression levels according to the $Card9^{S12N}$ polymorphism. The fundamental basis of pyrosequencing is that pyrophosphate is released when a deoxyribonucleotide triphosphate is added to the end of a nascent strand of DNA. As the deoxyribonucleotide triphosphates are sequentially added to the reaction and the pyrophosphate concentration is continuously monitored, the DNA sequence can be determined.[23]

Flow cytometry

Antibodies to mouse integrin αX subunit (CD11c), integrin αM (CD11b), lymphocyte antigen 6 complex locus G6D (Ly6G), SiglecF, and antibodies to human IL-5, CD14, and CD11b were used in flow cytometry [Supplementary Table 1, http://links.lww.com/CM9/B644]. For IL-5 intracellular staining, PBMCs were cultured for 2 h, and non-adherent cells were gently removed. After stimulation with heat-killed swollen conidia (SC) for 2 h, cells were cultured and collected for CD14 and CD11b staining, followed by intracellular IL-5 staining as previously described.[20] Cells were examined using a BD LSRFortessa cell analyzer (BD Immunocytometry Systems, San Jose, CA, USA), and data were analyzed with FlowJo (Tree Star, Ashland, OR, USA).

Quantitative real-time polymerase chain reaction (qRT-PCR)

Total RNA was isolated using TRIzol (Invitrogen, Carlsbad, CA, USA) and then RNA was reverse-transcribed using PrimeScript RT Master Mix (Takara, Tokyo, Japan). qRT-PCR was performed using Power SYBR Green PCR Master Mix (TaKaRa, Tokyo, Japan). The amounts of transcript were normalized to glyceraldehyde-phosphate dehydrogenase (GAPDH). CARD9 mRNA expression was tested by qRT-PCR. The primers used in this study are shown in Supplementary Table 2, http://links.lww.com/CM9/B644.

Cytokine measurement

BMDM culture supernatants and mouse serum were collected. IL-4, IL-5 and IgE concentrations were measured by SET-Ready-GO ELISA kits (eBioscience, San Diego, CA, USA) according to the manufacturer's protocol.

Statistical analysis

For clinical data, values are presented as mean ± standard deviation, median (Q_1, Q_3), or number (percentage). For animal experimental data, all values in the paper are presented as the mean ± standard error of the mean (SEM), unless stated otherwise. To analyze the *in vitro* cell stimulation and *in vivo* measurements, the experimental data were analyzed using Student's *t* test, Mann-Whitney *U* test, or one-way analysis of variance with GraphPad Prism 6.0 (La Jolla, CA, USA). For the analysis of single nucleotide polymorphisms (SNPs), Pearson's chi-squared test was used for exploring the differences in categorical variables among groups, and Hardy-Weinberg equilibrium was evaluated by testing for deviation from the expected values. Logistic regression analysis was used to evaluate the relationship between *CARD9* genotype and ABPA. Statistical analyses were performed using SAS software (version 9.4, SAS Institute Inc., Raleigh, NC, USA). All tests were two-sided, and a $P < 0.05$ was considered statistically significant.

Results

Detailed information on recruited ABPA patients, asthma patients, and healthy controls

All recruited Han Chinese ABPA patients ($n = 61$) in this study met the ISHAM criteria for ABPA diagnosis [Table 1 and Supplementary Table 3, http://links.lww.com/CM9/B644]. The detailed information on enrolled asthma patients ($n = 108$) and healthy controls ($n = 156$) is listed in Supplementary Tables 4 and 5, http://links.lww.com/CM9/B644, respectively. The serum aspergillus-specific IgE, total serum IgE levels, and median eosinophil count of ABPA patients were 1.09 (0.57–3.78) kUA/L, 2435 (1694–2500) IU/mL, and 1.06 (0.81–1.65) × 10^9 cells/L, respectively, and were significantly higher than those in asthma patients and healthy controls (all

Table 1: Diagnostic data of ABPA patients ($n = 61$) .

Diagnostic items	Number (%)
Predisposing conditions	
Only asthma	1 (2)
Only bronchiectasis	0 (0)
Both asthma and bronchiectasis	60 (98)
Obligatory criteria	
Total IgE >1000 IU/mL	61 (100)
Af specific IgE >0.35 kUA/L	60 (98)
Type I *Af* skin test (+)	52 (85)
Other criteria	
Peripheral EOS >0.5 cells/μL	61 (100)
ABPA chest CT features exist	61 (100)

Diagnosis of ABPA was based on the 2013 ISHAM criteria. ABPA: Allergic bronchopulmonary aspergillosis; *Af*: *Aspergillus fumigatus*; CT: Computed tomography; EOS: Eosinophils; IgE: Immunoglobulin E; ISHAM: International Society for Human and Animal Mycology.

P <0.001) [Table 2]. The classic radiological features in chest CT scans of ABPA patients are showed in Supplementary Table 3, http://links.lww.com/CM9/B644.

The p.S12N mutation in CARD9 was associated with ABPA development

We examined whether *CARD9*^{S12N} mutations occur in ABPA patients by sequencing the *CARD9* locus in the genomic DNA from 61 confirmed ABPA patients, 108 asthma patients, and 156 healthy controls. Notably, 55.7% (34/61) of ABPA patients had the GA genotype of p.S12N in *CARD9* and 9.8% (6/61) had AA genotype [Table 2]. As the frequency of the A allele of p.S12N was higher in ABPA patients than that in asthmatic or healthy individuals, we analyzed their associations with ABPA [Table 3]. Relative to healthy controls without relevant allergies, ABPA patients had a significantly higher frequency of mutation, which means that *CARD9*^{S12N} mutations are associated with the risk of ABPA (OR: 2.69 and 4.17 for GA and AA genotypes, $P = 0.003$ and 0.029, respectively), and relative to healthy controls with allergies, the association of ABPA and *CARD9*^{S12N} mutations was significant (OR: 3.09 for GA genotypes, $P = 0.015$, respectively). It is worth noting that when comparing with patients with asthma, regardless of *Aspergillus* sensitivity, the association of ABPA risk and the heterozygote (GA genotype) was both significant, indicating that p.S12N is a significant ABPA-susceptibility locus (*Aspergillus* sensitized asthma: OR: 3.02, $P = 0.009$; *Aspergillus* unsensitized asthma: OR: 2.94, $P = 0.005$).

The heterozygous S12N mutation can facilitate Af-induced type-2 responses

As both GA and AA genotypes of the non-synonymous SNP of the *CARD9* gene were significantly associated with ABPA, it seems likely, therefore, that carrying the A allele has a dominant effect in conferring susceptibility to ABPA. To explore this, we determined whether the heterozygous *CARD9* GA genotype of this SNP affected *CARD9* function in patients with ABPA and healthy controls by intracellular IL-5 cytokine staining in PBMCs [Figure 1A]. There were few *Af*-induced IL-5-producing PBMCs (defined as live CD11b$^+$CD14$^+$) in samples from healthy controls (GG or GA genotypes) [Figure 1B and Supplementary Figure 1A, http://links.lww.com/CM9/B644]. By contrast, numerous *Af*-induced IL-5-producing PBMCs were observed in samples from ABPA patients with GA genotypes but not GG genotypes [Figure 1C and Supplementary Figure 1B, http://links.lww.com/CM9/B644]. These data imply that in ABPA patients, the A allele of S12N in *CARD9* dominantly confers host susceptibility to ABPA.

Heterozygous p.S12N mutation with allelic expression imbalance (AEI) facilitates host susceptibility to ABPA

We further sequenced cDNA in PBMCs randomly selected from 10 ABPA patients and 22 healthy controls to examine the AEI of heterozygous S12N of *CARD9* [Figure 2A and Supplementary Figure 2A, http://links.lww.com/CM9/B644]. Pyrosequencing showed that healthy control subjects expressed the S12N allele and the WT allele, whereas ABPA patients showed allelic imbalance, prominently expressing the *CARD9* allele with S12N mutation but not the WT allele [Figures 2B, 2C and Supplementary Figure 2B, http://links.lww.com/CM9/B644]. Next, we determined whether AEI of *CARD9* was dependent on *Af* stimulation. Our data showed that *Af* stimulation significantly increased the relative percentage of allele A expression from an average of 60% to 80% in four healthy controls with the GA genotype [Figure 2D and Supplementary Figure 2C, http://links.lww.com/CM9/B644], indicating that AEI of *CARD9* is promoted by *Af* stimulation.

The p.S12N mutation decreased mRNA decay of CARD9 induced by Af conidia

To further assess whether the A allele of p.S12N mutation is associated with *CARD9* gene expression levels, patients with ABPA were classified according to their genotypes, GG, GA, and AA, and *CARD9* expression levels in their PBMCs were determined by qRT-PCR [Figure 3A]. ABPA patients with GA or AA genotypes exhibited significantly higher levels of *CARD9* expression than those with the GG genotype. To further explore this phenomenon, we stimulated PBMCs from

Table 2: Demographic information of the ABPA patients, asthma patients and healthy controls.

		Asthma with positive allergens (n = 108)				Healthy control (n = 156)			
Variables	ABPA (n = 61)	*Aspergillus* sensitized (n = 43)	P values (*vs.* ABPA)	*Aspergillus* unsensitized (n = 65)	P values (*vs.* ABPA)	With allergy (n = 34)	P values (*vs.* ABPA)	Without allergy (n = 122)	P values (*vs.* ABPA)
Age (years)	48 (38, 59)	56 (43, 63)	0.033	54 (34, 62)	0.209	35 (28, 40)	<0.001	33 (27, 39)	<0.001
Gender			0.144		0.445		0.060		0.007
Male	24 (39.3)	24 (55.8)		30 (46.2)		21 (61.8)		75 (61.5)	
Female	37 (60.7)	19 (44.2)		35 (53.8)		13 (38.2)		47 (38.5)	
Af-specific IgE (kUA/L)	1.09 (0.57, 3.78)	0.26 (0.18, 0.37)	<0.001	0.01 (0, 0.02)	<0.001	0.02 (0, 0.06)	<0.001	0 (0, 0)	<0.001
Total IgE (IU/mL)	2435 (1694, 2500)	154 (125, 306)	<0.001	183 (82, 392)	<0.001	179 (41, 327)	<0.001	33 (15, 80)	<0.001
EOS counts (×10^9/L)	1.06 (0.81, 1.65)	0.24 (0.10, 0.38)	<0.001	0.29 (0.13, 0.56)	<0.001	0.09 (0.06, 0.18)	<0.001	0.08 (0.05, 0.14)	<0.001
Genotype			0.002		0.009		0.036		0.003
G/G	21 (34.4)	28 (65.1)		40 (61.5)		21 (61.8)		73 (59.8)	
G/A	34 (55.7)	15 (34.9)		22 (33.8)		11 (32.4)		44 (36.1)	
A/A	6 (9.8)	0 (0)		3 (4.6)		2 (5.9)		5 (4.1)	

Data are expressed as number (percentage) or median (Q_1, Q_3). ABPA: Allergic bronchopulmonary aspergillosis; *Af*: *Aspergillus fumigatus*; EOS: Eosinophils; IgE: Immune globulin E.

Table 3: Association of p.S12N polymorphism of *CARD9* and ABPA risk.

Items	Odds ratio	95% Confidence interval	*P* values
ABPA *vs.* healthy control without allergy			
GG (reference)	1.00		
GA	2.69	(1.39–5.20)	0.003
AA	4.17	(1.16–15.04)	0.029
ABPA *vs.* healthy control with allergy			
GG (reference)	1.00		
GA	3.09	(1.24–7.68)	0.015
AA	3.00	(0.54–16.60)	0.208
ABPA *vs. Aspergillus* sensitized asthma			
GG (reference)	1.00		
GA	3.02	(1.32–6.93)	0.009
AA*	–	–	–
ABPA *vs. Aspergillus* unsensitized asthma			
GG (reference)	1.00		
GA	2.94	(1.39–6.25)	0.005
AA	3.81	(0.86–16.79)	0.077

*Participants with aspergillus sensitized asthma had no AA genotype. Logistic models were used to estimate the odds ratios (ORs) and 95% confidence intervals (95% CIs) for ABPA compared with controls respectively. All statistical tests were 2-sided, and probabilities of less than 0.05 were considered to be statistically significant. ABPA: Allergic bronchopulmonary aspergillosis; –: Not avaiable.

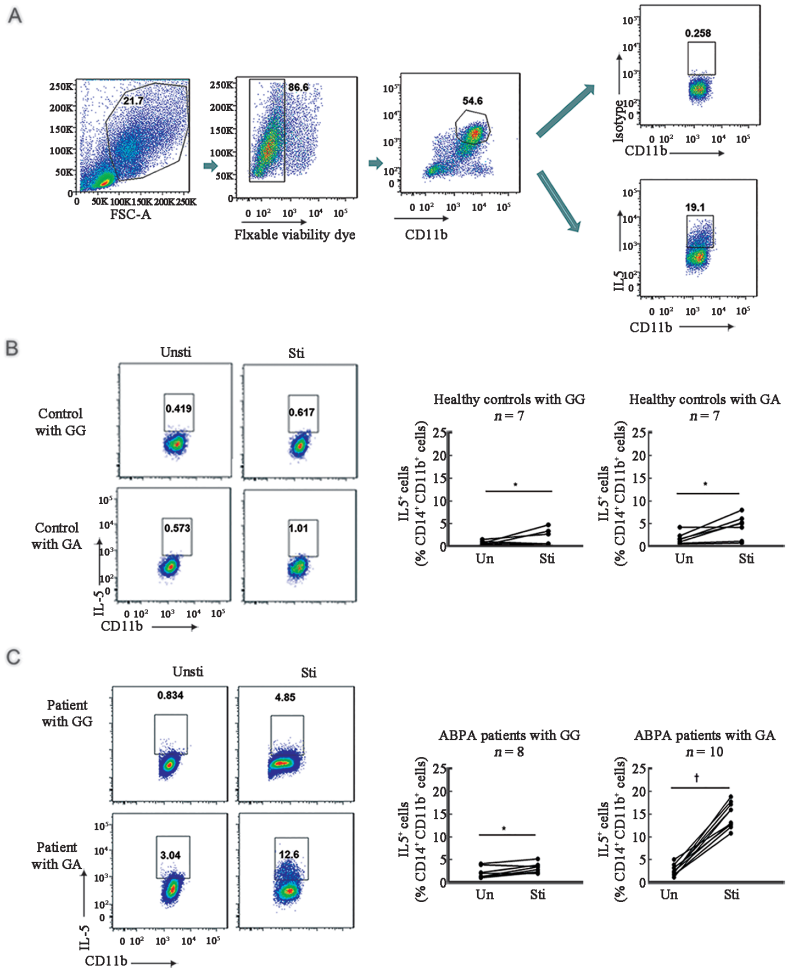

Figure 1: Heterozygous *CARD9*^{S12N} facilitated *Af*-induced Type-2 responses. (A) Gating strategy of immunocytochemical staining of IL-5 in PBMCs by flow cytometry. (B) Intracellular levels of IL-5 in PBMCs from healthy controls with WT or heterozygous *CARD9*^{S12N} mutation were determined before and after stimulation with *Af* by flow cytometry. (C) Intracellular levels of IL-5 in PBMCs from ABPA patients with WT or heterozygous *CARD9*^{S12N} mutation were determined before and after stimulation with *Af* by flow cytometry. $^{*}P > 0.05$, $^{\dagger}P < 0.001$; two-tailed paired *t* test was used for comparison. ABPA: Allergic bronchopulmonary aspergillosis; *Af: Aspergillus fumigatus*; CARD9: Caspase recruitment domain family member 9; CD: Cluster of differentiation. GA: Heterozygous *CARD9*^{S12N}; GG: Wild type *CARD9*^{S12N}; IL-5: Interleukin 5; n.s: No statistical significance; PBMCs: Peripheral blood mononuclear cells; Sti: Stimulation; Un/Unsti: Unstimulation; WT: Wild type.

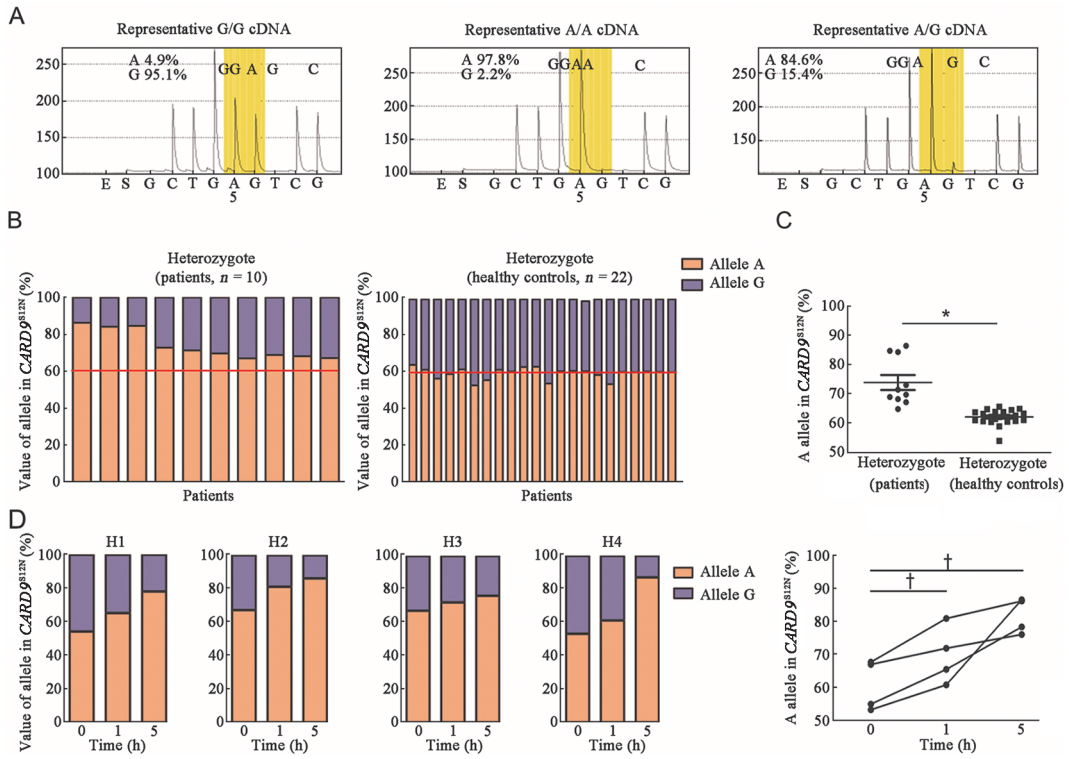

Figure 2: Allelic expression imbalance of *CARD9*^{S12N} confers host susceptibility to ABPA. (A) Allelic expression imbalance of *CARD9*^{S12N} polymorphism was determined by pyrosequencing analysis. (B and C) Quantifications of allelle of *CARD9*^{S12N} polymorphism by pyrosequencing in PBMCs from 10 ABPA patient and 22 healthy control samples. (D) PBMCs from 4 healthy control samples were isolated and challenged with *Af* swollen conidia. Allelic expression imbalance of *CARD9*^{S12N} polymorphism was determined. $^{*}P$ <0.001,$^{\dagger}P$ <0.05. Mann–Whitney *U*-test (C) or one-way ANOVA (D) was used for comparison. ABPA: Allergic bronchopulmonary aspergillosis; *Af*: *Aspergillus fumigatus*; cDNA: Complementary deoxyribonucleic acid; H: Healthy control; PBMCs: Peripheral blood mononuclear cells.

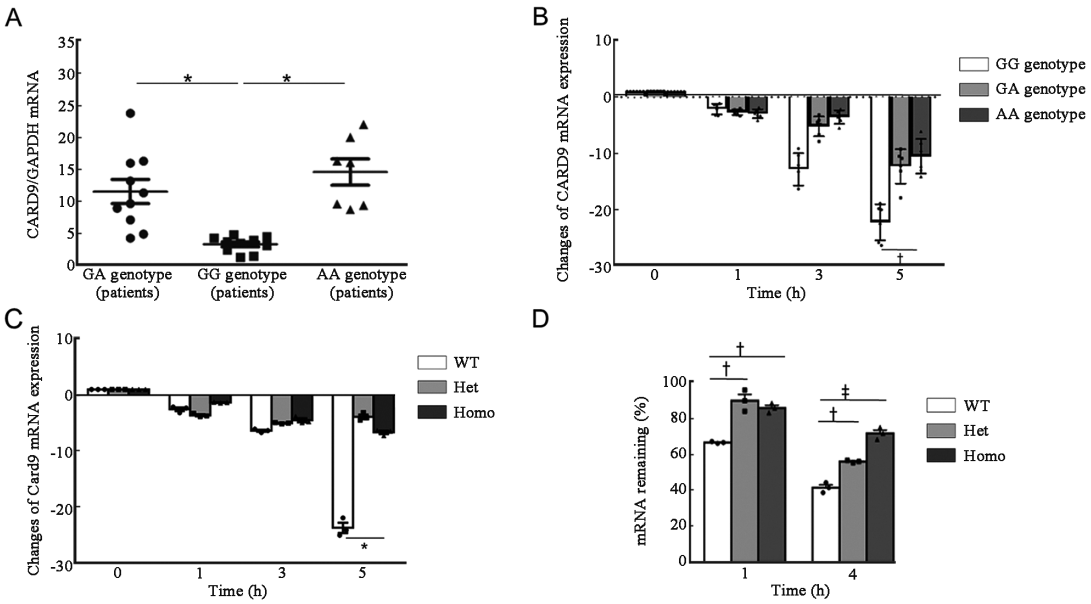

Figure 3: *CARD9*^{S12N} decreased the mRNA decay of *CARD9* induced by *Af*. (A) qRT-PCR results for *CARD9* expression, normalized to that of GAPDH, in PBMCs from ABPA patients expressing wild-type, or heterozygous or homozygous S12N variant of *CARD9*. (B) *CARD9* expression in *Af*-challenged PBMCs from patients with ABPA who were wild-type, heterozygous, or homozygous for the S12N variant was determined by qRT-PCR. (C) qRT-PCR results for *Af*-induced *Card9* expression in BMDMs isolated from wild-type, heterozygous and homozygous mice. (D) Remaining mRNA was determined by qRT-PCR 1 h and 4 h after treatment with actinomycin D (5 μg/mL) in BMDMs isolated from WT, heterozygous, and homozygous *Card9*^{S12N} knock-in mice. Data are mean ± s.e.m.$^{*}P$ <0.001, $^{\dagger}P$ <0.05, $^{\ddagger}P$ <0.01; Mann–Whitney *U* test (A) or two-tailed unpaired *t*-test (B–D) was used for comparison. ABPA: Allergic bronchopulmonary aspergillosis; *Af*: *Aspergillus fumigatus*; BMDMs: Bone marrow-derived macrophages; CARD9: Caspase recruitment domain family member 9; GAPDH: Glyceraldehyde-phosphate dehydrogenase; Het: Heterozygous; Homo: Homozygous; mRNA: Messenger RNA; PBMCs: Peripheral blood mononuclear cells; qRT-PCR: Quantitative reverse transcription-polymerase chain reaction (PCR); s.e.m: Standard error of mean; WT: Wild type.

ABPA patients with GG, GA, or AA genotypes with *Af* conidia, and then detected *CARD9* expression levels. The results showed that *Af* conidia stimulation dramatically decreased the mRNA abundance of *CARD9* in PBMCs with the GG genotype, compared with GA or AA genotypes [Figure 3B]. We further stimulated BMDMs isolated from wild-type, heterozygous, and homozygous mice with *Af* conidia, and found that *Card*^{WT} mRNA levels decreased more rapidly than heterozygous *Card9*^{S12N} and homozygous *Card9*^{S12N} [Figure 3C].

Next, we performed *Card9* mRNA stability assays in BMDMs from mice with different genotypes and found that *Af* stimulation significantly induced *Card9* mRNA decay in BMDMs from wild-type mice relative to those from either heterozygous or homozygous *Card9*^{S12N} knock-in animals [Figure 3D]. Together, these data confirm that *Af* stimulation can induce more mRNA decay in *Card*^{WT} mice than that in heterozygous and homozygous *Card9*^{S12N} mice.

The p.S12N mutation aggravated Af-induced type-2 responses in heterozygous Card9^{S12N} mice

The IL-4 and IL-5 levels are below the detection limit and there was no difference in IgE level in serum between naive WT and heterozygous mice [Supplementary Figure 3A, http://links.lww.com/CM9/B644]. These results showed that the S12N mutation had no substantial influence on physiological responses before fungal exposure. To determine the function of *Card9*^{S12N} in the development of ABPA, we proposed repeated challenges with low doses of *Af* conidia to mimic persistent local antigenic stimulation [Figure 4A]. We found that no statistically significant differences in fungal burden were observed between wild-type and heterozygous *Card9*^{S12N} knock-in mice [Supplementary Figure 3B, http://links.lww.com/CM9/B644]. The results also showed that *Af* conidia stimulation significantly induced lung eosinophilia in wild-type and heterozygous *Card9*^{S12N} knock-in mice [Figure 4B]. However, protein levels of

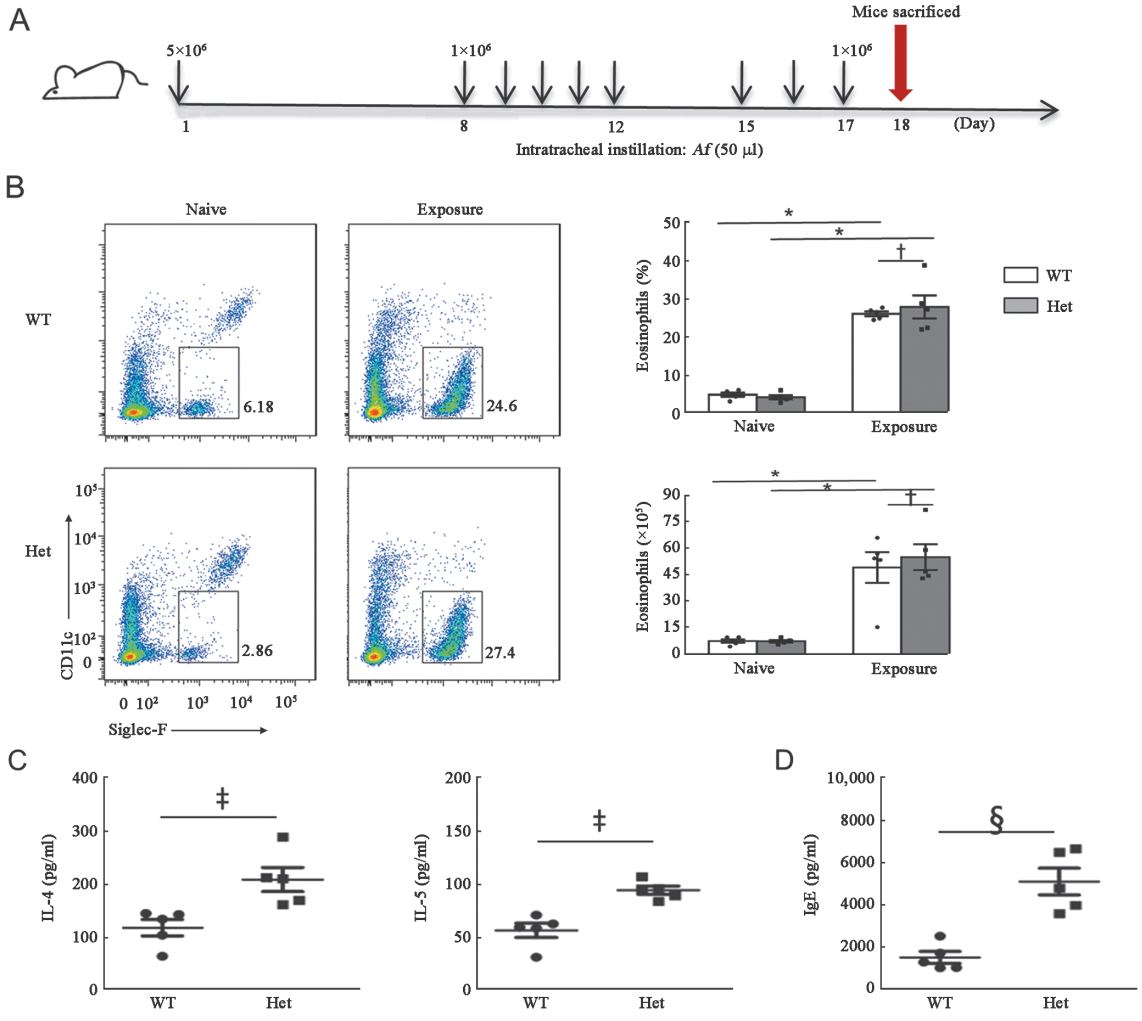

Figure 4: Heterozygous *Card9*^{S12N} can facilitate *Af*-induced T_H2-mediated responses. (A) Strategy for development of the chronic murine asthma model, in which WT and heterozygous *Card9*^{S12N} knock-in mice were sensitized with 5 × 10^6 *Af* conidia, challenged with 1 × 10^6 *Af* conidia for eight times, and sacrificed for subsequent assay at day 18. (B) Eosinophil (Siglec-F+) counts in lungs of the mice after multiple exposures to *Af* conidia. (C and D) ELISA results for lung IL-4 and IL-5 (C), and serum IgE (D), in the mice described above. Data are shown as mean ± s.e.m. $^*P$ <0.001, $^\dagger P$ >0.05, $^\ddagger P$ <0.05, $^\S P$ <0.01; two-tailed unpaired *t*-test was used for comparison (B–D). In B–D, n = 5 mice per group. *Af*: *Aspergillus fumigatus*; *CARD9*: Ccaspase recruitment domain family member 9; CD: Cluster of differentiation; ELISA: Enzyme linked immunosorbent assay; Het: Heterozygote; IgE: Immunoglobulin E; IL: Interleukin; s.e.m.: Standard error of mean; T_H2: T helper 2: WT: Wild type.

pulmonary IL-4, IL-5, and serum IgE were higher in heterozygous *Card9*^{S12N} knock-in mice than those in WT mice [Figures 4C and 4D]. There was no significant difference in neutrophil accumulation between *Card9*^{WT} and heterozygous *Card9*^{S12N} knock-in mice [Supplementary Figure 3C, http://links. lww.com/CM9/B644]. Our data confirmed that heterozygous *Card9*^{S12N} could facilitate *Af*-induced T_H2-mediated development of ABPA symptoms.

High dose exposure of Af induced T_H2-mediated responses in heterozygous Card9^{S12N} mice

Furthermore, our recent study demonstrated that a challenge with a low dose of *Af* conidia failed to induce T_H2-mediated responses in wild-type and heterozygous *Card9*^{S12N} knock-in mice, whereas a high dose of *Aspergillus* infection always caused an acute lung inflammatory response.[20,26] Therefore, in this study, we administered intratracheal injections with high doses of *Af* conidia to determine whether heterozygous *Card9*^{S12N} is critical for directing *Af*-induced T_H2 polarization [Figure 5A]. We observed that the levels of pulmonary IL-5 and serum IgE were significantly higher in the heterozygous *Card9*^{S12N} knock-in mice compared with those in wild-type controls [Figures 5B and 5C]; the number and percentage of eosinophils were increased more in the heterozygous *Card9*^{S12N} knock-in mice than in *Card9*^{WT} [Figure 5D]. However, there was no significant difference

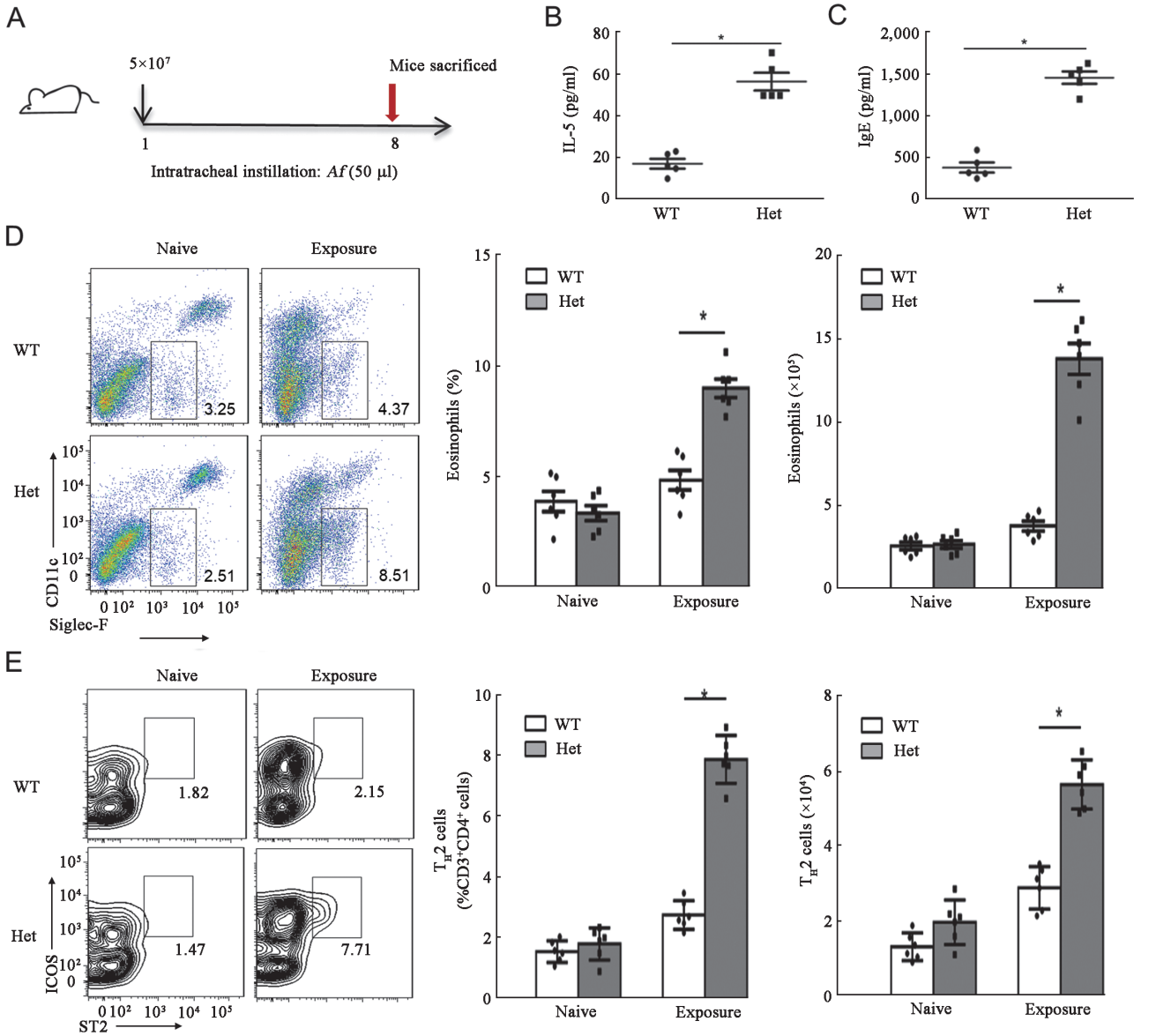

Figure 5: T_H2-mediated responses can be induced by high-dose *aspergillus* conidia in heterozygous *Card9*^{S12N} mice. (A) Eosinophil (SiglecF$^+$) counts in lungs from *Card9*^{WT} and heterozygous *Card9*^{S12N} knock-in mice after a single exposure to *Af* conidia (5×10^7) assayed at day 8 by flow cytometry. (B and C) ELISA results for IL-5 (B) in extracts of lung homogenates and serum IgE (C) levels from *Card9*^{WT} and heterozygous *Card9*^{S12N} knock-in mice. (D and E): Eosinophils (D) and T_H2 (E) cell counts (CD3$^+$CD4$^+$ICOS$^+$ST2$^+$) in the lungs of *Card9*^{WT} and heterozygous *Card9*^{S12N} knock-in mice. Data are shown as means ± SEM. Two-tailed unpaired *t*-test was used for comparison, $^*P$ <0.001 (B–E). In B–C, $n = 5$ (one sacrificed during infection); In D–E, $n = 6$ mice per group. *Af*: *Aspergillus fumigatus*; *CARD9*: Caspase recruitment domain family member 9; CD: Cluster of differentiation; ELISA: Enzyme linked immunosorbent assay; Het: Heterozygote; ICOS: Inducible costimulator; IgE: Immunoglobulin E; IL: Interleukin; SEM: Standard error of mean; ST2: Growth stimulation expressed gene 2; WT: Wild type.

in *Af*-induced neutrophil accumulation in the comparison between *Card9*^{WT} and heterozygous *Card9*^{S12N} mice [Supplementary Figure 4A, http://links.lww.com/CM9/B644]. Quantification of lung T_H2 lymphocytes, which are defined here as live $SSC^{low}CD3^+CD4^+ICOS^+ST2^+$ cells[27] [Supplementary Figure 4B, http://links.lww.com/CM9/B644], revealed significantly higher levels of these immune cells in the lungs of heterozygous *Card9*^{S12N} knock-in mice than those of wild-type mice, after challenged with *Af* conidia [Figure 5E]. These results indicate that heterozygous *Card9*^{S12N} can facilitate *Af*-induced T_H2 differentiation.

Discussion

The inflammatory response of ABPA is characterized by T_H2 responses to *Aspergillus* allergens that stimulate immunoglobulin E (IgE) synthesis, eosinophil activation and the production of IL-4, IL-5, and IL-13 cytokines rather than T_H1 molecules such as IL-12 and interferon-γ (IFN-γ). Recent studies indicate that ABPA patients carry genetic risk factors that contribute to skewed and heightened T_H2 responses to *Af* antigens.[28] Our results demonstrate that the non-synonymous SNP in *CARD9*, rs4077515, encoding the S12N amino acid substitution, favors ABPA development in patients with heterozygous and homozygous genotypes by orchestrating *CARD9*^{S12N}-mediated signaling to induce T_H2 responses. Our studies provide the genetic and functional evidence that indicates the role of S12N polymorphism in *CARD9* in regulating immune responses to the development of ABPA. As the frequency of the rs4077515 AA genotype in *CARD9* was significantly lower in the Chinese Han population with *Aspergillus* unsensitized asthma, the prediction of this polymorphism for ABPA did not have enough power, and the differences between ABPA patients and *Aspergillus* unsensitized asthma patients need a larger number of samples to identify.

To date, more than 24 mutations in *CARD9* have been reported to be associated with severe fungal infections.[29] Among these, two loss-of-function homozygous mutations, Q289X (c.865C >T) and Q295X (c.883C >T), in *CARD9* are associated with an elevated risk of candidiasis and dermatophytosis, respectively.[16,17] Besides deleterious mutations predisposing to fungal infection, our previous and present studies show that a genetic variant in *CARD9* is critical for the development of *Af*-induced T_H2 responses.[20] Our data show that ABPA patients with heterozygous or homozygous genetype of *CARD9*^{S12N} showed significantly higher *CARD9* expression levels than those with wild-type *CARD9*, hinting that the S12N variant induces the occurrence of AEI in *CARD9*.

AEI is an indicator of the effects of regulatory polymorphisms residing within a gene locus and is defined by the percentage levels of RNA alleles expressed, and determined by the measurement of cDNA. Epigenetic mechanisms are fundamental players that determine the allele expression. DNA methylation and histone modifications are two mediators of the epigenetic phenomena. Noncoding RNAs (ncRNAs) are also players in regulating gene expression.[30,31] For example, allelic imbalance of variant rs1047643 and B-cell-specific super-enhancer was associated with systemic lupus erythematosus mediated by signal transducer and activator of transcription 3 (STAT3).[32] Besides epigenetic events, AEI exists in autosomal non-imprinted genes and about 4.6% of heterozygous SNP-sample pairs show AEI.[33] However, in most cases, functional significance resulting from AEI and the causes of AEI phenomenon are still unknown. We observed that 10 samples from ABPA patients with heterozygous *CARD9*^{S12N} showed AEI, with dominant expression of the minor A allele. Furthermore, we showed that AEI of *CARD9* was dependent on *Af* stimulation. Heterozygous Y91H (c.439T >C) mutation of *CARD9* has also been reported to be associated with AEI, resulting in *CARD9* deficiency in two patients with spontaneous central nervous system candidiasis.[34] Taken together, our results identify the S12N SNP as a major regulatory variant in *CARD9*, accounting for the AEI observed in this study.

Our study has some limitations. First, we did not collect bone marrow-derived dendritic cells (BMDC) from asthma patients but this doesn't affect our conclusion. Then, the samples of ABPA patients are limited because of the low morbidity. Third, in most cases, functional significance resulting from AEI and the causes of AEI phenomenon are still unknown.

In summary, we reported that a SNP of *CARD9* gene encoding S12N is a genetic risk factor for human ABPA disease. Further, we demonstrated that *Af*-induced AEI of the minor *CARD9*^{S12N} allele can contribute to autosomal dominant functional alterations, favoring T_H2-mediated ABPA development. This study provides genetic evidence to show that the heterozygous mutation of *CARD9*^{S12N}, followed by AEI of *CARD9*^{S12N}, favors the development of ABPA. It could be a good screening factor in determining patient susceptibility to ABPA.

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Conflicts of interest

None.

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