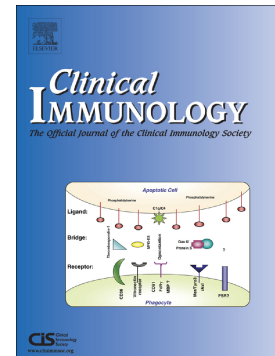


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Role of S100A9 in the development of neutrophilic inflammation in asthmatics and in a murine model

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Short title: S100A9 in neutrophilic asthma

Abbreviations

ELISA: Enzyme-linked immunosorbent assay

S100A9: S100 calcium binding protein A9

MMP: Matrix metalloproteinases

FEV1: Forced expiratory volume in one second

FVC: Forced vital capacity

TNF: Tumor necrosis factor

IFN: Interferon

IL: interleukin

LPS: Lipopolysaccharide

BAL: Bronchoalveolar lavage

CFA: Complete Freund's Adjuvant

OVA: Ovalbumin

PBS: Phosphate-Buffered Saline

TBS: Tris Buffered Saline

DAPI: 4',6-diamidino-2-phenylindole

PE: Phycoerythrin

FITC: Fluorescein Isothiocyanate

BMI: Body mass index

Abstract

S100A9 is an endogenous danger signal that promotes and exacerbates the neutrophilic inflammatory response. To investigate the role of S100A9 in neutrophilic asthma, S100A9 levels were measured in sputum from 101 steroid-naïve asthmatics using an ELISA kit and the levels were significantly correlated with percentages of neutrophils in sputum. Intranasal administration of recombinant S100A9 markedly increased neutrophil numbers at 8 h and 24 h later with concomitant elevation of IL-1 β , IL-17, and IFN- γ levels. Treatment with an anti-S100A9 antibody restored the increased numbers of neutrophils and the increased airway resistance in OVA/CFA mice toward the levels of sham-treated mice. Concomitantly, the S100A9 and neutrophil elastase double positive cells were markedly reduced with attenuation of IL-1 β , IL-17, and IFN- γ levels by the treatment with the anti-S100A9 antibody. Our data support a role of S100A9 to initiate and amplify the neutrophilic inflammation in asthma, possibly via inducing IL-1 β , IL-17 and IFN- γ .

Key Words: Calgranulin B, S100A9, asthma, IL-1 β , IL-17, and IFN- γ

Introduction

Asthma is a heterogeneous disease composed of various subtypes, which can be defined by etiology, pathology, severity, physiologic parameters, and response to treatment [1]. In recent years, cluster analysis of asthma cohorts has led to identification of distinct clinical sub-phenotypes, including early-onset atopic asthma with normal lung function, non-atopic older obese females with moderate reductions in FEV₁, and adult asthmatics with severe airflow obstruction [2-4]. Other parameters such as patterns of airway inflammation or cytokines have been used to define asthma, because clustering using clinical and demographic parameters is of limited utility for predicting clinical course. Airway inflammation is heterogeneous, with eosinophilic, neutrophilic, mixed cellular and pauci-granulocytic types, each of which has distinct physiologic and clinical characteristics [5, 6].

Early-onset allergic asthmatics have robust eosinophilic airway inflammation with Th2-type cytokine dominance [7], while 5–10% of late-onset asthmatics have severe manifestations with concomitant neutrophilic airway inflammation [5, 6, 8]. In addition, a cluster analysis of patients with asthma identified a severe asthma subgroup that showed high levels of sputum neutrophils and the sputum neutrophils was the second most influential variable [9]. Interleukin (IL)-4, IL-5, and IL-13 released from Th2-polarized lymphocytes contribute to the airway eosinophilia of asthma, while IL-8, TNF- α , interferon (IFN)- γ and IL-17 are associated with the neutrophilic inflammation [10]. However, the causative molecules of the airway neutrophilia in asthmatics have not been determined. Thus, we screened candidate proteins in neutrophil-dominant sputum, and found elevated S100A9 levels in uncontrolled severe asthmatics under treatment with high doses of inhaled steroid [11].

S100A9 is a small calcium-binding protein recognized as an alarmin released by stressed cells: an endogenous danger signal that promotes and exacerbates the inflammatory response. S100A9 induces activation of Toll-like receptor 4 [12], neutrophil chemotaxis [13], neutrophilic inflammation [14], and augmentation of IL-8 production [15]. Thus, S100A9 is speculated to induce airway neutrophilia in asthma. Thus, additional clinical evidence of the association of S100A9 with airway neutrophilia is needed in order to define the role of S100A9 in the neutrophilic subtype of asthma. Furthermore, the contribution of S100A9 to neutrophilic asthma should be clarified in animal models. In the present study, S100A9 protein levels were measured in sputum from steroid-naïve asthmatics to evaluate the association between S100A9 protein and neutrophilic inflammation. In addition, the role of S100A9 was elucidated in a murine model of asthma with neutrophilic inflammation.

Materials and Methods

Subjects and sputum collection

Asthma was diagnosed by physicians based on the Global Initiative for Asthma (GINA) guidelines [16]. At the baseline visit, demographic information, such as enrollment age, sex, BMI, asthma-onset age, asthma duration, and smoking amount was collected. At the same time, all patients underwent a standardized assessment, which included analyses of induced-sputum specimens, complete blood cell count with differential counts, total IgE, chest radiography, and allergy skin prick tests with 24 common inhalant allergens (Bencard Co, London, UK). Informed written consent forms were obtained from the participants, and the protocol was approved by the ethics committee of the hospital (SCHGM 2014-16). We enrolled the asthmatics, who did not use systemic or inhaled corticosteroids for at least 4 weeks or more before sputum examination. Sputum was induced using isotonic saline that contained a short-acting bronchodilator. Sputum was prepared as follows: all portions with visibly greater solidity were carefully selected and placed in a pre-weighed Eppendorf tube. The samples were treated by adding eight volumes of 0.05% dithiothreitol (Calbiochem, San Diego, USA) to Dulbecco's phosphate-buffered saline (PBS). One volume of protease inhibitor (0.1 M methylenediaminetetraacetic acid and 2 mg/mL phenylmethylsulfonylfluoride) was added to 100 volumes of homogenized sputum, and the total cell count was determined using a hemacytometer. The sputum cells were collected by cytocentrifugation and 500 cells were examined after staining with Diff-Quick (American Scientific Products, Chicago, USA). Sputum samples that contained >10% squamous epithelial cells were excluded from the study. The remainder of the homogenized sputum sample was centrifuged at 1,000 g for 5 min, and the supernatant was collected and stored at -70°C for subsequent protein analyses. Sputum samples were obtained from 34 normal controls (NC) and 101 asthmatics (BA) who had never smoked or quit smoking (<10 pack-years). The asthmatics were categorized into 4 inflammatory subtypes [17, 18]: neutrophil dominant ($\geq 60\%$, $n=42$), eosinophil dominant ($\geq 3\%$, $n=27$), co-dominant ($\geq 60\%$, $\geq 3\%$ $n=22$), and pauci-granulocytic ($<60\%$, $<3\%$ $n=10$) subtypes. The characteristics of the study subjects are summarized in Table 1.

Quantitative measurement using ELISA

S100A9 concentrations in sputum were measured using a quantitative sandwich enzyme-linked immunosorbent assay (ELISA) kit (Mybiosource, San Diego, USA) according to the manufacturer's protocol and normalized to the total protein concentration of the samples, which was determined using a BCA Kit (Pierce Biotechnology, Rockford, USA). The

detection limit for S100A9 was 0.1ng/mL. Levels below the detection limit were considered 0ng/mL. In addition, S100A8 was measured concentration using an ELISA kit (MyBioSource, San Diego, USA) in neutrophilic and eosinophilic sputum from uncontrolled, partially controlled and controlled asthmatics.

Production and delivery of recombinant S100A9 protein into the airways of mice

S100A9 protein was synthesized and purified using the the pCMV6-XL4/S100A9 (Origene, Rockville, USA) human cDNA clone was purchased and amplified with a forward primer (5'-GAC GGA TCC ATG ACT TGC AAC ATG TCG-3') that contained a BamHI restriction enzyme site and a reverse primer (5'-GAC CTC GAG TTA GGG GGT ACC CTC CCC -3') that contained an XhoI restriction enzyme site. The amplified S100A9 cDNA product was digested with BamHI and XhoI restriction enzymes (New England Biolabs, Ipswich, USA) and the digested products were ligated into a pPRO-EX HTb protein expression vector (Life Technologies, Grand Island, USA). At each step, the sequences were confirmed by direct sequencing (Macrogen, Seoul, Korea). As described previously [19], the recombinant a pPRO-EX HTb-S100A9 plasmid was transformed into *E. coli*, followed by culturing until the optical density at 600 nm (OD 600) approached 0.6. Next, 1 mM isopropyl-b-D-thiogalactopyranoside (IPTG) (Sigma-Aldrich) was added to induce S100A9 protein expression, and the culture was incubated at 25°C, 30°C, and 37°C for a further 3, 6, and 9 h to determine the most appropriate temperature for protein expression. The S100A9 fusion protein was analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (Fig. 1). S100A9 protein was expressed in *E. coli* and purified using a TALON affinity column (Life Technologies, Grand Island, USA) via a his⁶-tag at the N-terminus of the recombinant protein. The TALON affinity-purified proteins were subjected to size exclusion chromatography (Superdex 75, A⁺ KTAFFPLC) and then digested with AcTEV protease (Life Technologies) for 16 h at 4°C to remove the his⁶-tag fusion protein. The AcTEV-cleaved recombinant S100A9 protein was dialyzed in Tris-base buffer (50mM, pH 8), and the resulting materials were subjected to ion-exchange chromatography (HiTrapQFF, A⁺ KTAFFPLC). Endotoxin level of S100A9 synthetic protein was assayed using the ToxinSensor Chromogenic Limulus Amoebocyte Lysate Endotoxin Assay Kit (Genescript, Walkersville, USA) with a sensitivity range of 0.005–1.0 EU/mL. As the result, one µg/mL of the recombinant S100A9 protein had 0.034 EU/mL (equivalent to 3.34 pg of endotoxin).

The in vitro potency of S100A9 was analyzed using 293-hTLR4A-MD2-CD14 cells transfected with NF-κB-luc and pRL-Tk. In vitro potency of the synthetic S100A9 protein was measured as follows: Cells positive for 293-hTLR4A-MD2-CD14 (Invivogen, San Diego, USA) were co-transfected in 12-well plates with 2µg of NF-κB reporter vector (Panomics,

Santa Clara, USA) and 1 μ g of pRL-TK vector (Promega, Madison, USA) as an internal control. Subsequently, the cells were stimulated with LPS (1-100ng/mL), and S100A9 protein (100ng and 1 μ g/mL) in the absence or presence of anti-S100A9 antibody (1:100, 10 μ L/mL) for 8 hours. After then, the cells were lysed by cell lysis buffer (Promega) and the luciferase activities were measured by luminometer. The recombinant S100A9 and bovine serum albumin (BSA) were administered to 6-week-old C57BL/6 mice (Orientbio Inc, Seongnam, Korea) via the nasal route, and BAL and tissue morphologies were investigated at 2, 4, 8, 24, 48, and 72h as described previously[18].

Neutrophilic model of murine asthma

The neutrophil-dominant model was generated using OVA/complete Freund's adjuvant (CFA) as described in a prior study [20]. Briefly, 6-week-old female C57BL/6 mice were sensitized via intraperitoneal injection of grade V chicken egg OVA (20 μ g; Sigma-Aldrich, St. Louis, USA) with CFA (75 μ L; Sigma-Aldrich) emulsified in PBS on days 0, 7, and 14. On days 21 and 22, all mice received intra-nasal challenges with 0.1% of grade III OVA in 50 μ L PBS. Sham mice were sensitized and challenged with PBS. Temporal changes in S100A9 expression and neutrophil infiltration were analyzed on days 7, 14, and 23. To suppress the activity of S100A9 in the OVA/CFA model, mice were administered 20 μ L anti-S100A9 antibody (1.38 mg, Novus Biological, Littleton, USA), and the same amount of control antibody via the intranasal route 3 h before sensitization or challenge on days 7, 14, 17, and 21. A purified anti-yolk immunoglobulin polyclonal antibody (42094, Sabbiotech, Baltimore, USA) was used as a control in the neutralization experiment. The Institutional Animal Care and Use Committee of the hospital approved the animal study (SCHBC-animal-2014-012).

Measurement of airway resistance, bronchoalveolar lavage, and histology analysis including immunofluorescence staining

The mice were anesthetized with ketamine–xylazine, tracheostomized, and mechanically ventilated using the Flexi-Vent system (SCIREQ, Montreal, Canada) 24 h after the final OVA challenge as described previously [21]. Airway resistance was measured using PBS and increasing doses of methacholine (0, 20, and 100 mg/mL; Sigma-Aldrich). Immediately thereafter, BAL was performed, and lung tissues were processed for histological and protein analyses as described previously [18, 22]. BAL was performed in four instillations of 1 mL PBS and gentle retrieval. Cells were enumerated using a hemocytometer and differential cell counts were performed on slides prepared by cyto-centrifuge and Diff-Quick staining (Scientific Products, Gibbstown, USA). BAL fluid was centrifuged and the supernatants were stored at -80°C. A portion of the left lung was treated for Western blot in radio-

immunoprecipitation assay (RIPA) buffer (Scientific Products) with 0.5 mM EDTA and 100 mM PMSF, and stored at -80°C. The other portion of the lung was fixed in 4% buffered paraformaldehyde and embedded in paraffin. The tissues were cut into slices (4 µm thick) for hematoxylin eosin staining and for immunofluorescence (IF) staining of S100A9 and neutrophil elastase (NE). The tissue sections were de-paraffinized, re-hydrolyzed, and blocked with normal serum for 1 h at room temperature. The tissue slices were incubated at 4°C with rat anti-mouse S100A9 monoclonal antibody (1:200 dilution; Hycult Biotech, Uden, Netherlands), rabbit polyclonal anti-mouse neutrophil elastase (NE) antibody (1:200 dilution, Abcam, Cambridge, UK) and rabbit polyclonal anti-E-cadherin (1:500 dilution, R&D Systems, MN, USA). After washing three times in Tris-buffered saline (TBS), the slides were incubated for 1 h at room temperature with FITC-conjugated anti-rat IgG and phycoerythrin (FITC or PE)-conjugated anti-rabbit IgG (1:1000 dilution, Abcam) diluted in TBS of 1.5% normal rabbit and goat serum (Vector Laboratories, California, USA). After washing in Tris-buffered saline, the slides were incubated with DAPI (1:1000 dilution; Sigma-Aldrich). The slides washed in TBS, mounted, and observed under a confocal laser scanning microscope (LSM 510 META, Carl Zeiss, Oberkochen, Germany).

Western blot analysis

Western blot analysis was performed using mouse lung lysates as described previously [11]. The extracted lung tissues were homogenized in a protein lysis solution containing 50 mM Tris-HCl (pH 7.4), 50mM NaCl, 0.1% SDS, and 1% TritonX-100 in distilled water, centrifuged at 14000 rpm for 30 min at 4°C, and the soluble portions were collected. Protein (15–30µg) was loaded and electrophoresed with a discontinuous system consisting of a 15% polyacrylamide gel. The proteins were transferred to nitrocellulose membrane at 120 V for 1 h. The membranes were blocked for 1 h at room temperature in 5% skim milk in 0.1% Tween-20 in TBS and incubated overnight at 4°C with the primary antibodies.

The membranes were incubated overnight at 4°C with an anti-S100A9 polyclonal antibody (1:1000 dilution; Novus Biological), anti-caspase-1 polyclonal antibody (1:1000 dilution; Adipogen, San Diego, USA), anti-IL-1β monoclonal antibody (1:1000 dilution; Cell Signaling Technology, Danvers, USA), anti-IFN-γ monoclonal antibody (1:1000 dilution; Santa Cruz Biotechnology, Dallas, USA), anti-IL-17 monoclonal antibody (1:1000 dilution; R&D systems, Minneapolis, USA), and anti-β-actin monoclonal antibody (1:5000 dilution; Sigma-Aldrich). Then they were incubated for 1 h at room temperature with an HRP-conjugated secondary antibody (1:5000 dilution; Thermo, Fremont, USA). The target protein was detected using an enhanced chemiluminescence solution (GE Healthcare, Little Chalfont, UK) on X-ray film.

Statistical Analyses

The data were analyzed using SPSS ver. 20.0. The normality of the distribution was evaluated using the Shapiro-Wilk test. Comparisons of nonparametric variables and parametric variables were performed using Kruskal-Wallis tests and ANOVA respectively, and then post hoc analyses using tukey-HSD test or Mann-Whitney U-tests were performed. Correlations of the S100A9 levels with sputum cell counts were analyzed by Spearman's rank correlation coefficient analysis. The data are presented as median values with 25% and 75% quartiles for skewed variables, or as means $\pm$ SEM for those with a normal distribution. A value of $p < 0.05$ was considered significant.

Results

1. Clinical characteristics of the study subjects

The clinical and demographic characteristics of the study population (101 asthmatics and 34 normal controls) are shown in Table 1. FVC% predicted, FEV1% predicted, and FEV1/FVC% were significantly lower in the BA than the NC group ($P < 0.01$). The frequency of atopy was significantly higher in the BA group ($P < 0.05$). The BA group had significantly higher percentages of eosinophils, lymphocytes, columnar cells ($P < 0.01$), and neutrophils ($P < 0.05$) and a lower percentage of macrophages ($P < 0.05$) in sputum. Among the subtypes of asthma, enrollment age, onset age of asthma, smoking and atopy frequency were significantly different ($P = 0.034 - 0.002$).

2. Comparison of S100A9 protein levels in asthma subtypes and its correlation with inflammatory cells in sputum

S100A9 was detected in 12 of 34 NC (35.3%) and 79 of 101 BA (73%). The median S100A9 level normalized to total protein concentration in sputum was significantly higher in BA (10.37 [3.40–14.71] pg/ μ g) than in NC (0 [0–10.23] pg/ μ g, $P = 0.0002$, Fig. 2A). BA was divided into four groups according to the percentage of neutrophils and eosinophils in sputum: neutrophil dominant ($\geq 60\%$, $n = 42$), eosinophil dominant ($\geq 3\%$, $n = 27$), co-dominant ($\geq 60\%$, $\geq 3\%$, $n = 22$) and pauci-granulocytic ($< 60\%$, $< 3\%$, $n = 10$) subtypes. The median S100A9 level was increased in the neutrophil dominant group (13.06 [6.46–18.70] pg/ μ g) compared to the eosinophil dominant group (8.66 [0–12.84] pg/ μ g, $P = 0.038$) and the pauci-granulocytic group (5.48 [0–10.02] pg/ μ g, $P = 0.012$) (Fig. 2A). In BA, S100A9 levels were significantly correlated with the percentages of neutrophils in sputum ($n = 101$, $r = 0.289$, $P = 0.003$) (Fig. 2B) and not correlated with those of eosinophils ($r = 0.083$, $P = 0.409$) (Fig. 2C).

3. Effect of recombinant S100A9 protein on neutrophilic inflammation of the airway

Luciferase activities were measured at 8 h after treatment with the recombinant S100A9. The stimulating activity of 1 µg/mL was similar to that of 10 ng/mL LPS (Sigma-Aldrich) (Fig. 3). Following administration of S100A9 (0.2 µg) into the nose of C57BL/6 mice, neutrophil and macrophage numbers in BAL fluid were significantly increased at 4 h ($P < 0.05$) and peaked at 8 and 24 h ($P < 0.01$), and then decreased to basal levels at 72 h (Fig. 4A-1). On other hands, Treatment with bovine serum albumin (BSA, 0.2 µg) did not change neutrophil and macrophage numbers in BAL fluids (Fig. 4A-2). On histologic examinations, intense infiltration of inflammatory cells was observed in the peribronchial area treated S100A9. (Fig. 4B-1), but not in mice treated with BSA (Fig. 4B-2). On Western blot, S100A9 protein appeared at 2 h and peaked at 24 h. P20-activated caspase-1, IL-1 β , IL-17, and IFN- γ levels showed a similar pattern: a significant increase at 2 h, peak at 8 h, and return to the pre-challenge levels at 48 h after stimulation (Fig. 4C). In IF staining, S100A9 and neutrophil elastase (NE) double-positive cells were observed around the bronchus and bronchial epithelium at 24 h after S100A9 instillation (Fig. 4D). In addition, S100A9 was also expressed by the bronchial epithelial cells stained with E-cadherin. (Fig. 4E)

4. Temporal changes of S100A9 protein levels in murine asthma models and inhibitory effects of anti-S100A9 antibody

Inflammatory cell numbers and S100A9 protein levels were measured in the lungs of OVA/CFA mice on days 0, 7, 14, and 23 (Fig. 5A). Lung resistance showed a significant increase in OVA/CFA mice ($P < 0.05$) and treatment with an anti-S100A9 antibody significantly abolished the enhanced lung resistance ($P < 0.05$, Fig. 5B). Macrophage, neutrophil, eosinophil, and lymphocyte numbers were not significantly increased on days 7 and 14, but robustly increased on day 23 compared to those on day 0 and those of sham-treated mice ($P < 0.01$, Fig. 5C). Intranasal administration of 20 µL anti-S100A9 attenuated the increased numbers of neutrophils ($P < 0.01$), but did not affect the increased numbers of eosinophils ($P < 0.05$, Fig. 5C). In the IF staining, S100A9 and neutrophil elastase double-positive cells infiltrating the peribronchial area and bronchial epithelium were almost abolished by treatment with anti-S100A9 antibody on day 23 in OVA/CFA mice (Fig. 6A). In Western blots, the S100A9 protein level started to increase on day 7 and peaked on day 23 (Fig. 6B). Concomitantly, levels of P20-activated caspase-1 and the mature forms of IL-1 β , IL-17, and IFN- γ increased steadily up to day 23. The increments were markedly down-regulated by treatment with the anti-S100A9 antibody (Fig. 6B).

Discussion

We found that the S100A9 level was elevated in steroid-untreated asthmatics with mild to moderate airflow limitations and were positively correlated with the percentages of neutrophils in sputum. Considered together with our previous observation that S100A9 was elevated in neutrophilic sputum from patients with severe uncontrolled asthma on high-dose corticosteroids [11], S100A9 may be a biomarker for activated neutrophils in the asthmatic airways of mild to moderate as well as severe asthmatics. The active form of S100A9 protein is known to usually exert function in a heterodimeric form with S100A8 [23]. Thus, we measured S100A8 levels using ELISA in sputum, which were used for the measurement of S100A9 in our previous publication [24]. As seen in the supplement table and figure, S100A8 protein was detected in few patients with uncontrolled asthma, in none of partially controlled and controlled asthma as shown in the figure. This means that S100A9 may be responsible for the neutrophilic inflammation of asthma rather than S100A8. Recently, other investigators have found that S100A9 levels are elevated in BAL fluid of asthmatics as well [25]. In our *in vivo* animal studies, direct instillation of recombinant S100A9 protein induced robust neutrophilic inflammation in the airways, and blocking of S100A9 abolished the neutrophilic inflammation and the enhanced airway reactivity. To the best of our knowledge, this is the first report demonstrating that S100A9 is likely the main mediator responsible for the neutrophilic inflammation and enhanced reactivity of the asthmatic airway, and that sputum S100A9 levels may be used as a surrogate marker for activated neutrophils in the asthmatic airway.

Neutrophils as well as eosinophils are present in large numbers in the airways of asthmatics, in particular severe asthmatics [5, 8, 9]. Although prominent eosinophilic inflammation is related to frequent exacerbations in severe asthmatics, airway neutrophilia has been considered to be related to persistent airway obstruction [6, 26]. A study of 1197 asthmatics showed an association between sputum neutrophil counts with decrease in post-bronchodilator FEV1 [27]. In addition to the increased numbers, neutrophils are functionally activated in the airways. The levels of MMP-9, released mainly from neutrophils, are elevated in BAL fluids and biopsy specimens of asthmatics, and are inversely proportional to FEV1 [28]. Neutrophil elastase can cause morphologic changes reflecting airway remodeling such as metaplasia of mucus glands and mucus hypersecretion, epithelial damage [29], and disrupted elastic fibers [30]. Myeloperoxidase (MPO) released from neutrophils generates hypochlorous acid, which can injure the surrounding tissue. Neutrophilic elastase and MPO levels are elevated in BAL fluid of asthmatics [31]. These data support the fact that the mediators released by neutrophils are able to remodel the airway of asthmatics.

To reveal the direct role of S100A9 in the neutrophilic airway inflammation of asthma,

recombinant S100A9 protein was used to challenge the airways. Recombinant S100A9 protein induced a marked elevation of neutrophil numbers within 8 h, which peaked at 24 h. These results are consistent with studies of the *in vivo* chemotactic activities of S100A9 via shedding of L-selectin, and upregulation and activation of Mac-1 to fibrinogen [13]. Infiltration of S100A9 and NE double-positive cells around the bronchus peaked at 24 h. Interestingly, in Western blots, S100A9 appeared at 2 h after S100A9 administration, peaked at 24 h, and slowly decreased up to 72 h, similar to neutrophil numbers, suggesting that the newly recruited and activated neutrophils might produce endogenous S100A9 protein. Interestingly, P20-activated caspase-1, IL-1 β , IFN- γ , and IL-17 levels increased at 2 h after stimulation, and decreased at 24 h. These data suggest that S100A9 may directly induce activation of inflammasome and the production of IFN- γ and IL-17.

We also found a high level of S100A9 in the airways of neutrophilic asthma model of OVA/CFA [20]. To block the S100A9, an anti-S100A9 antibody was administered from day 7 to 21 because S100A9 began to appear at day 7 in Western blots. Firstly, we analyzed the inhibitory potential of the polyclonal anti-S100A9 antibody using 293-hTLR4A-MD2-CD14-positive cells. Luciferase activities were measured after treatment with 1 μ g/mL recombinant S100A9. The NF- κ B luciferase activity induced by 1 μ g/mL S100A9 was abolished by treatment with the anti-S100A9 neutralizing antibody (1:100 dilutions) and the treatment with 1:100 dilution of the antibody substantially restored the increases in neutrophil numbers and S100A9 levels to those of the sham-treated mice, with a concomitant decrease in airway reactivity. The treatment also attenuated the elevated IL-1 β , IFN- γ and IL-17 levels in lung lysates of OVA/CFA mice. These data, to the best of our knowledge, demonstrate for the first time that S100A9 may be able to function as a major modulator of neutrophilic inflammation of asthma by regulating IL-1 β , Th1, and Th17 levels in the airways.

The OVA/CFA model involves polarization of local CD4⁺ T lymphocytes toward Th1 and Th17 types [20], similar to severe neutrophilic asthma; i.e., elevation of IL-17, IL-8, and IFN- γ [10, 32]. In the present study, anti-S100A9 treatment significantly suppressed the number of lymphocytes in OVA/CFA mice. Although we did not assess Th17 and Th1 subsets, they may be suppressed by treatment with the anti-S100A9 antibody as the levels of these two cytokines in lung lysates were significantly decreased.

The expression levels of key inflammasome components, such as IL-1 β and NLRP3, are increased in the airways of patients with neutrophilic asthma [33]. Tissue injury or allergen exposure leads to NLRP3 activation, with formation of the inflammasome complex and caspase-1 activation, resulting in mature IL-1 β release [34]. S100A9 regulates the NLRP-3 inflammasome, resulting in IL-1 β processing and secretion by human monocytes [35]. We previously observed that S100A9 induces inflammasome activation and that IL-1 β stimulates

S100A9 induction in monocyte cell lines [36]. Thus, inflammasome may stimulate inflammatory cells and epithelial cells to synthesize S100A9, which in turn triggers inflammasome activation, leading to amplification of inflammation. IL-17 also rapidly recruits neutrophils into the lung [37]. IL-17 is produced not only by Th17 cells but also by innate immune cells, including iNKT [38] and gamma/delta T cells during the early stages of inflammation [39]. These cells can respond directly to microbial pathogens [40] such as CFA in the airway. Indeed, neutrophils also produce IL-17 [41]. These data suggest that IL-17 may participate in neutrophilic inflammation observed after stimulation with S100A9.

The main limitation of the present study was the absence of clear documentation on the contribution by macrophages and activated epithelial cells to S100A9 protein in human asthmatics, because macrophages and activated epithelial cells have been reported to produce S100A9 during chronic inflammation [42]. Another limitation was the use of recombinant human S100A9 protein in mice because murine and human S100A9 share 56% amino acid identity [43]. However, *in vivo* intranasal administration of 0.2 μ g human recombinant S100A9 protein induced robust neutrophilic inflammation, indicating that human and murine S100A9 may share a higher degree of functional homology than of sequence similarity [44]. In conclusion, S100A9 protein levels were markedly increased in the sputum of neutrophil dominant BA and were significantly correlated with the percentages of neutrophils. In animal models, recombinant S100A9 induced neutrophilic inflammation of the airways with concomitant elevation of IL-1 β , IL-17, and IFN- γ levels. Blocking of S100A9 using an anti-S100A9 antibody restored the neutrophilic inflammation and the increased airway resistance to those of sham-treated mice. These data indicate that S100A9 may initiate and amplify the neutrophilic inflammation of asthma directly or in collaboration with IL-1 β , IL-17, and IFN- γ . Clinically, S100A9 levels in sputum in terms of neutrophil activation would assist management of severe asthmatics, because neutrophilic inflammation is resistant to the corticosteroids recommended in conventional treatment guidelines: modulation of S100A9 protein levels in the airway may be a therapeutic strategy to control neutrophilic inflammation in asthma.

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Authors Contributions: TH Lee, HS Chang and CS Park designed research; TH Lee, DJ Bae, HJ Song and JA Jun performed research; JS Park, ST Uh and CS Park collected sputum samples; SY Lee, and SH Kim made recombinant S100A9; MS Kim analyzed the data; TH Lee, Hun Soo Chang, SH Kim and CS Park wrote the paper.

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Figure legends

Figure 1. Purification of recombinant S100A9 protein. A) SDS-gel electrophoresis and silver staining of recombinant S100A9 and B) Western blot of the S100A9 using an anti-S100A9 polyclonal antibody.

Figure 2. S100A9 protein levels in sputum between normal controls and subgroups of asthmatics. A) Comparison of S100A9 protein levels in sputum between the four subtypes of asthmatics according to the percentages of neutrophils and eosinophils: co-dominant ($\geq 60\%$, $\geq 3\%$ n=22), neutrophil dominant ($\geq 60\%$, n=42), eosinophil dominant ($\geq 3\%$, n=27), and pauci-granulocytic ($< 60\%$, $< 3\%$ n=10) subtypes. Open circle indicates the S100A9 levels below the detection limit ($< 0.1\text{ng/mL}$). S100A9 concentration was normalized with total protein. Box plots represent medians and interquartile ranges. P-values were obtained using Mann-Whitney U test. B) Correlation of the S100A9 levels with the percentages of neutrophils ($r=0.289$, $P=0.003$) and C) those of eosinophils ($r=0.083$, $P=0.409$) in sputum from the total asthmatics (n=101).

Figure 3. Effects of recombinant S100A9 protein and anti-S100A9 neutralizing antibody on NF- κ B luciferase activity. Cells positive for 293-hTLR4A-MD2-CD14 were co-transfected with NF- κ B-luciferase and pRL-Tk constructs, and luciferase activity was measured 8 h after treatment with LPS or recombinant S100A9 with/without anti-S100A9 antibody (Novus Biological, Littleton, USA, $10\mu\text{g/mL}$ of media). Data are means $\pm$ SEM of six experiments; P-values were calculated using t-tests. * $P<0.05$ vs. untreated control cells.

Figure 4. Effects of recombinant S100A9 protein on airway inflammation and cytokine levels. A-1,2) Temporal changes in total cells, macrophage, neutrophil, eosinophil, and lymphocyte numbers in bronchoalveolar lavage fluids of mice after single challenge of S100A9 and bovine serum albumin (BSA) ($20\mu\text{L}$ of $10\mu\text{g/mL}$). Data are means $\pm$ SEM of six experiments and p-values were determined by t-test. * $P<0.05$ compared to 0 h. † $P<0.01$ compared to 0 h. B-1,2) Representative images of H&E-stained lung tissues of 6 experimental mice after the challenge of S100A9 and BSA (400x). C) Representative Western blot of S100A9 and neutrophil-related cytokines in lung lysates pooled from sham (n=6) and S100A9-treated mice (n=6). D) Immunofluorescence staining for S100A9 (green, FITC) and neutrophil elastase (red, PE) in lung tissues at 0 and 24 h. Nuclei were stained with DAPI

(blue) (400x). E) Immunofluorescence staining for E-cadherin (green, FITC) and S100A9 (red, PE) in lung tissues at 0 and 24 h. Nuclei were stained with DAPI (blue) (400x).

Figure 5. Temporal changes in cellular profiles in the neutrophilic murine model of asthma and inhibitory effects of anti-S100A9 antibody. A) Protocol of the neutrophilic model of murine asthma using OVA/CFA and anti-S100A9 antibody treatment. IP, IN, and R_L indicate intra-peritoneal injection, intra-nasal instillation, and lung resistance, respectively. Anti-S100A9 antibody (Novus Biological, Littleton, CA, USA) (1.38mg/20 μ l) and control antibody (1.38mg/20 μ l Anti-Yolk Immunoglobulin polyclonal antibody, Sabbiotech, Baltimore, MD, USA) were administrated via intranasal route. B) Lung resistance (R_L : cmH₂O/mL/second) measured using the Flexivent with increasing concentrations of methacholine. *, † P<0.05 compared to sham mice. ‡ P<0.05 compared to OVA/CFA and OVA/CFA + control antibody. C) Inflammatory cell profiles in BAL fluids. Data are means $\pm$ SEM of six experiments. P-values were calculated by ANOVA and t-tests. *, † P<0.01 compared to days 0, 7, 14 and sham-treated mice. ‡ P<0.01 and § P<0.05 compared to day 23 and day 23 + control antibody.

Figure 6. Localization of S100A9 and temporal changes in S100A9, P20-activated caspase-1, IL-1 β , IL-17, and IFN- γ proteins in the neutrophilic murine asthma model and inhibitory effects of anti-S100A9 antibody. A) Representative image of H&E and immunofluorescence staining for S100A9 (green, FITC) and neutrophil elastase (red, PE) in lung tissues of sham and OVA/CFA mice on day 23. Nuclei were stained with DAPI (blue) (400 $\times$). B) Representative Western blot of S100A9 and neutrophil-related cytokine levels in lung lysates pooled from CFA/OVA-sensitized/challenged mice (n=6, days 0, 7, 14, and 23) and 20 μ l of anti-S100A9-treated mice on day 23 (n=6).

Supplement Figure legends

Supplement Figure 1. A) S100A8 protein was detected (>10 pg/mL) in 3 of 13 patients with COPD, 1 of 20 patients with uncontrolled asthma (UA), none of 20 patients with partially controlled asthma (PA), none of 20 patients with controlled asthma (CA) and 1 of 7 normal controls. There was no difference of S100A8 between the subgroups of asthma. B) S100A9 protein was detected (>1.17 ng/mL) in 10 of 20 patients with uncontrolled asthma(UA), 11 of 25 patients with partially controlled asthma (PA) and 5 of 21 patients with controlled

asthma(CA), 0 of 8 normal controls in reference [24]. C) Correlation between the levels of S100A8 and S100A9 in total study subjects (n=80).

ACCEPTED MANUSCRIPT

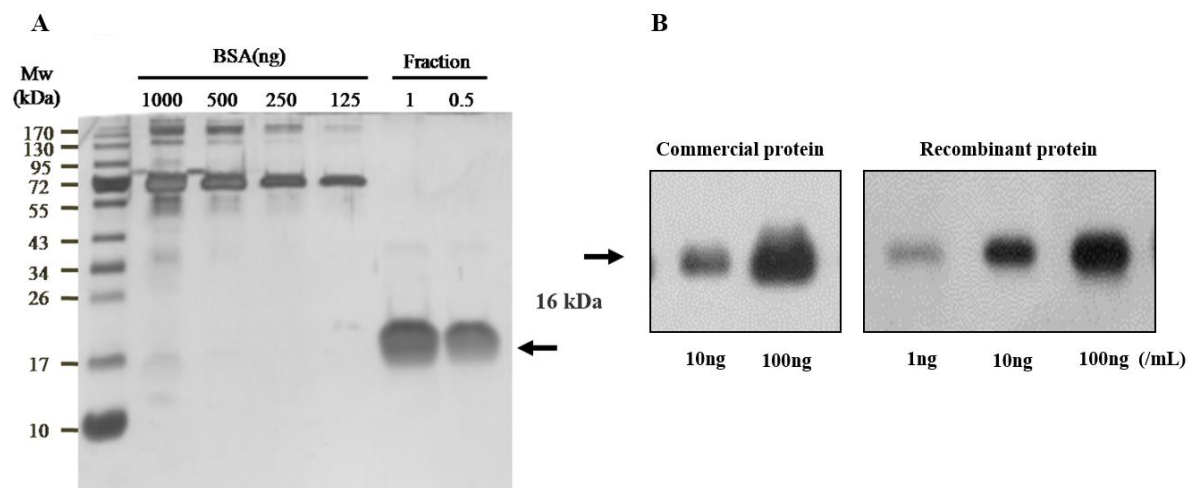


Figure 1

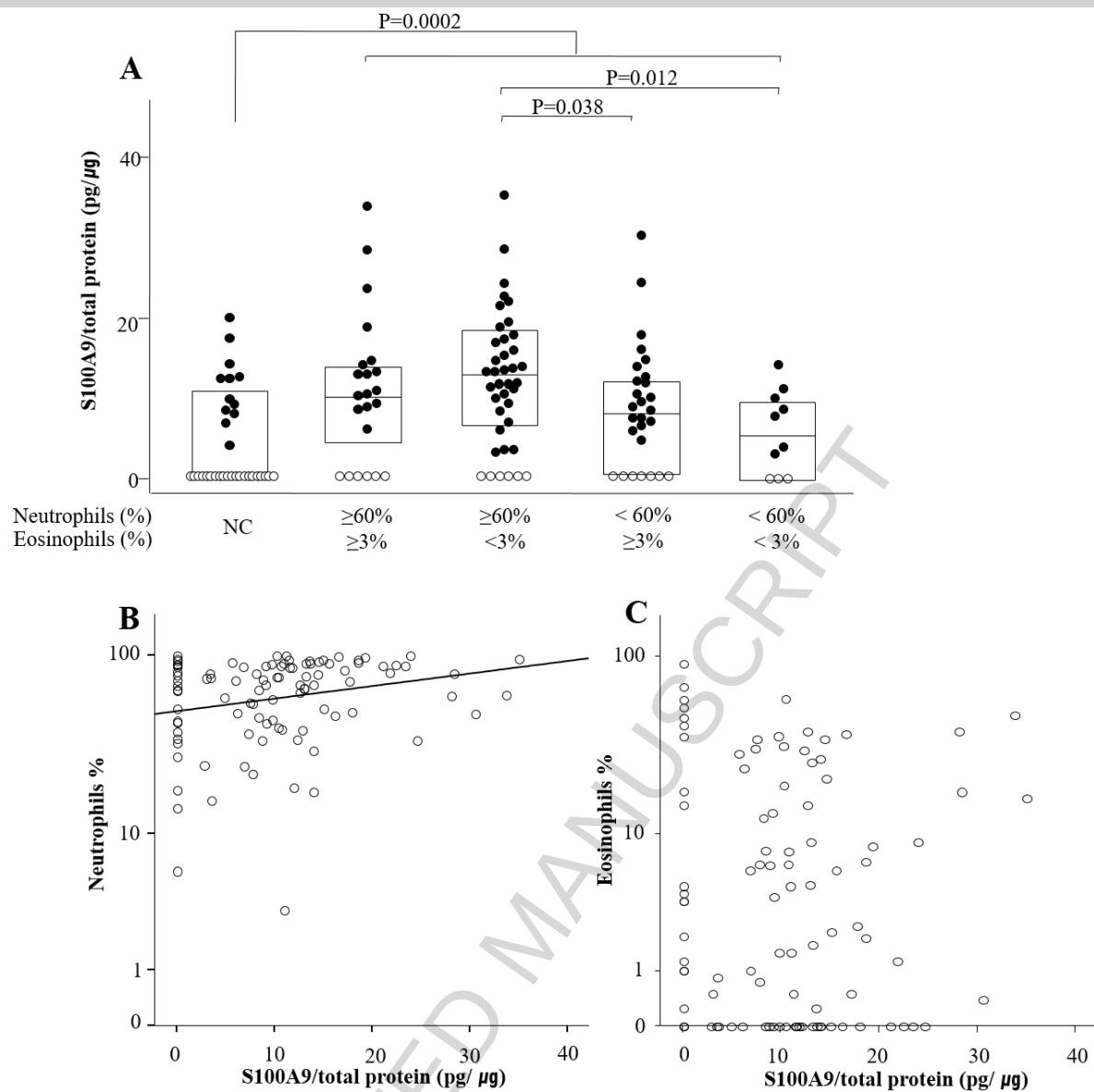


Figure 2

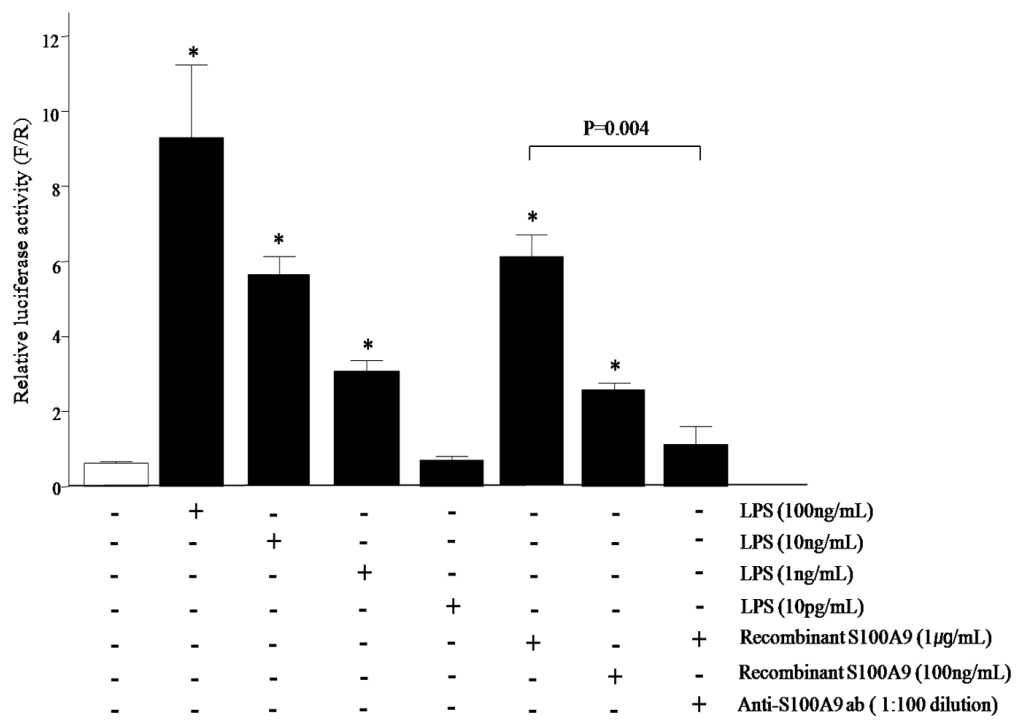


Figure 3

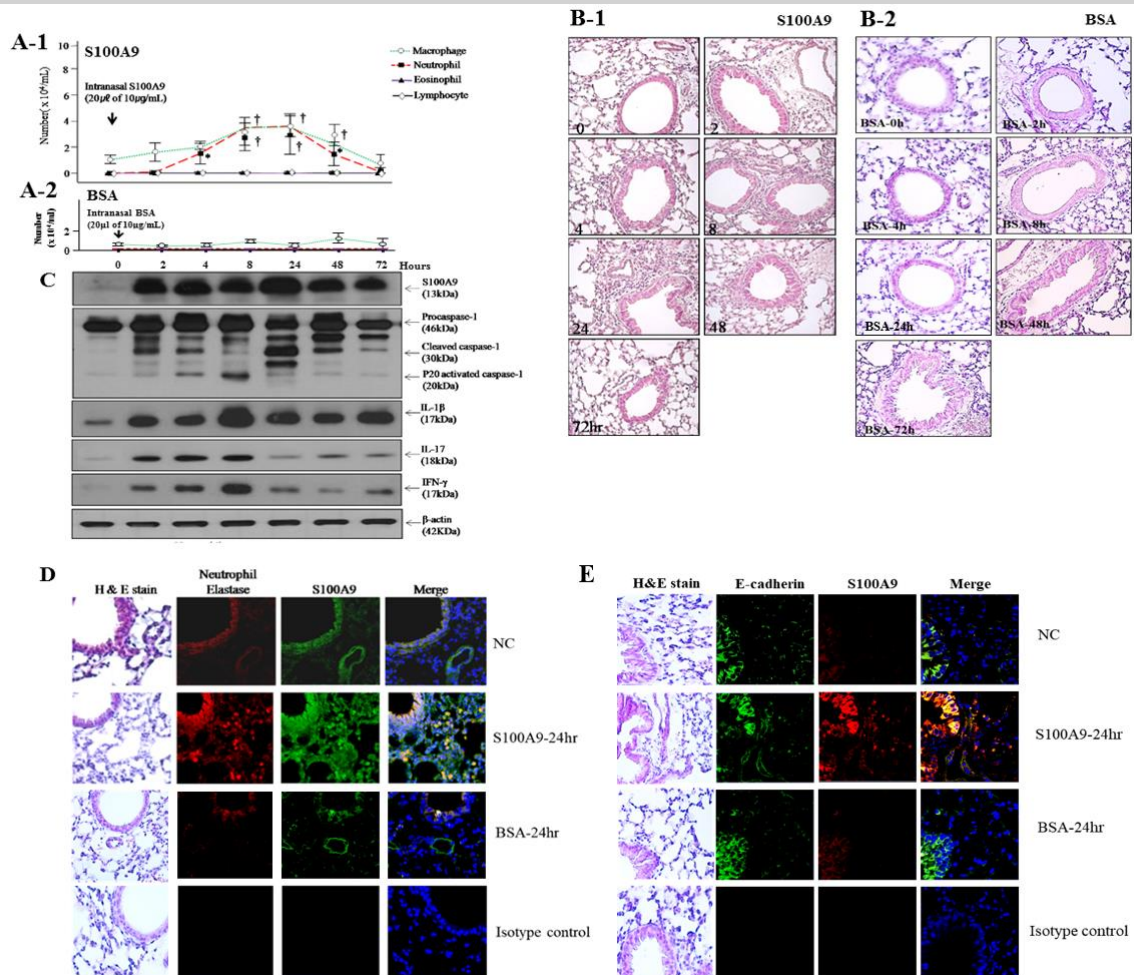
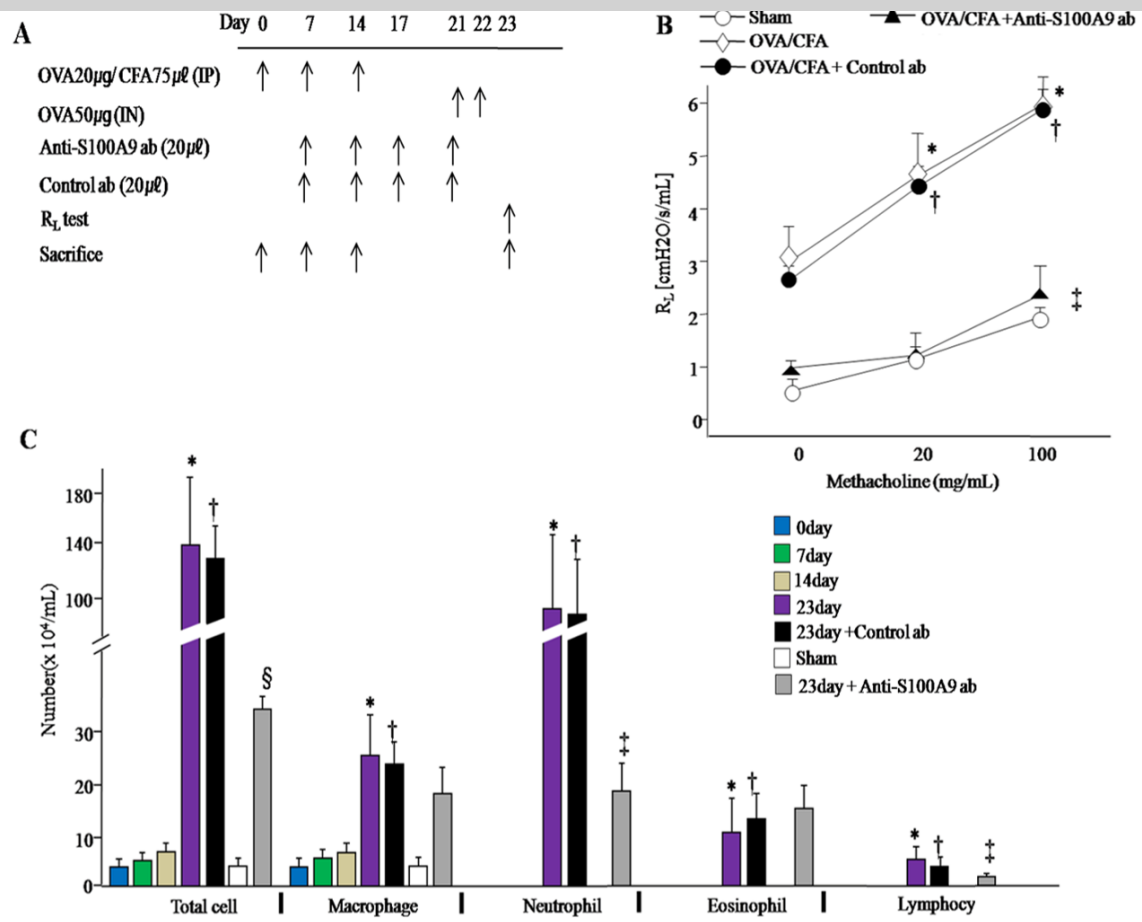


Figure 4



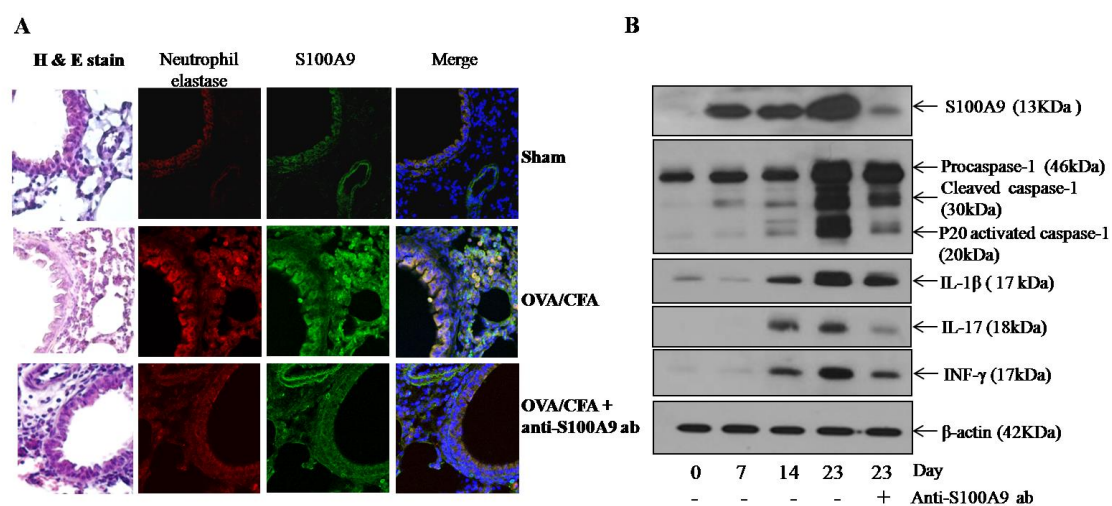


Figure 6

Table 1. Clinical and laboratory characteristics of the study subjects

	NC	BA	BA subtypes				P-value *
			Co-dominant	Neutrophilic	Eosinophilic	Pauci-granulocytic	
No.	34	101	22	42	27	10	
Enrollment age (year)	51.55 ± 3.2	50.68 ± 1.6	54.82 ± 3.6 §	54.97 ± 2.3 §	43.44 ± 3.1	43.7 ± 3.2	0.007
Onset of asthma (year)	NA	47.20 ± 1.6	50.18 ± 3.9 §	51.40 ± 2.6 §	40.26 ± 2.7	41.9 ± 3.5	0.002
Duration of asthma (year)	NA	5.65 ± 0.5	6.31 ± 1.3	5.93 ± 0.8	4.71 ± 0.7	5.56 ± 1.4	0.424
Smoke (NS/ES/CS)	22 / 12 / 0	87 / 14 / 0	18 / 4 / 0 †	38 / 4 / 0 §	22 / 5 / 0 §	9 / 1 / 0	0.012
FVC(% pred.)	90.15 ± 2.4	84.48 ± 1.7 †	84.73 ± 3.5	83.95 ± 3.2	82.81 ± 2.6	90.6 ± 3.4	0.615
FEV1(% pred.)	102.94 ± 2.6	85.19 ± 2.0 †	86.95 ± 4.3	84.88 ± 3.7	82.15 ± 3.0	90.8 ± 5.1	0.609
FEV1/FVC (%)	84.32 ± 1.2	76.03 ± 1.1 †	77.45 ± 1.8	75.14 ± 1.8	75.44 ± 2.1	78.2 ± 2.9	0.922
BMI(kg/m ²)	26.26 ± 1.4	24.37 ± 0.4	25.04 ± 0.8	24.11 ± 0.6	23.81 ± 0.7	25.37 ± 1.2	0.507
Atopy(N/Y/ND)	17 / 7 / 10	58 / 40 / 3 †	12 / 10 / 0	25 / 15 / 2	16 / 11 / 0	5 / 4 / 1	0.034
Sputum total cell count (x10 ⁵ /ml)	6.80 ± 1.4	8.96 ± 1.5	6.67 ± 1.5 §	14.98 ± 3.2 §	3.55 ± 0.8	3.34 ± 0.7	0.027
Neutrophils (%)	51.65 ± 4.0	65.83 ± 2.6 †	76.03 ± 2.3 §	86.87 ± 1.5 §	37.27 ± 2.6	32.15 ± 5.4	<0.001
Eosinophils (%)	1.31 ± 0.9	11.96 ± 1.8 †	12.59 ± 1.6 ¶	0.61 ± 0.1 §	33.44 ± 4.2	0.19 ± 0.1	<0.001
Macrophages (%)	45.31 ± 4.0	16.81 ± 2.0 †	7.40 ± 1.7 §	8.47 ± 1.3 §	21.28 ± 3.2	60.47 ± 5.4	<0.001
Lymphocytes (%)	0.68 ± 0.2	1.67 ± 0.3 †	1.10 ± 0.4	1.31 ± 0.3	2.63 ± 0.8	1.85 ± 0.7	0.179
Columnar cells (%)	1.04 ± 0.6	3.71 ± 0.7 †	2.87 ± 1.2	2.72 ± 0.9	5.35 ± 1.8	5.31 ± 1.7	0.082

NC: normal controls, BA: asthmatics, NS: non-smoker, ES: ex-smoker, CS: current smoker,

NA: not applied

Patients were categorized into four inflammatory subtypes based on the percentages of neutrophils and eosinophils in sputum; co-dominant (percentage of neutrophils and eosinophils: $\geq 60\%$ and $\geq 3\%$), neutrophil dominant ($\geq 60\%$), eosinophil dominant ($\geq 3\%$) and pauci-granulocytic subtypes. The normality of the distribution was evaluated using the

Shapiro-Wilk test. Comparisons of the variables among the subtypes were performed using ANOVA (* indicates p-value) and then post hoc analyses using tukey-HSD test was performed. The chi-squared test was used to compare qualitative variables. Data are presented as mean $\pm$ SEM.

† $P < 0.01$, compared with NC.

‡ $P < 0.05$, compared with NC.

§ $P < 0.05$, compared with eosinophilic BA.

¶ $P < 0.01$, compared with neutrophilic BA.

|| $P < 0.01$, compared with pauci-granulocytic BA.

Highlight

- S100A9 is an endogenous danger signal that promotes and exacerbates the neutrophilic inflammatory response.
- Our data support a role of S100A9 to initiate and amplify the neutrophilic inflammation in asthma, possibly via inducing IL-1 β , IL-17 and IFN- γ .
- modulation of S100A9 protein levels in the airway may be a therapeutic strategy to control neutrophilic inflammation in asthma.