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Original Article



# Serum Amyloid A1: A Biomarker for Neutrophilic Airway Inflammation in Adult Asthmatic Patients

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## ABSTRACT

**Purpose:** We evaluated the role of serum amyloid A1 (SAA1) in the pathogenesis of airway inflammation according to the phenotype of asthma.
**Methods:** One hundred twenty-two asthmatic patients and 60 healthy control subjects (HCs) were enrolled to measure SAA1 levels. The production of SAA1 from airway epithelial cells (AECs) and its effects on macrophages and neutrophils were investigated *in vitro* and *in vivo*.
**Results:** The SAA1 levels were significantly higher in sera of asthmatic patients than in those of HCs ($P = 0.014$); among asthmatics, patients with neutrophilic asthma (NA) showed significantly higher SAA1 levels than those with non-NA ($P < 0.001$). *In vitro*, polyinosinic:polycytidylic acid (Poly I-C) treatment markedly enhanced the production of SAA1 from AECs, which was further augmented by neutrophils; SAA1 could induce the production of interleukin (IL)-6, IL-8, and S100 calcium-binding protein A9 from AECs. Additionally, SAA1 activated neutrophils and macrophages isolated from peripheral blood of asthmatics, releasing neutrophil extracellular traps (NETs) and secreting proinflammatory cytokines presenting M1 phenotype, respectively. In ovalbumin-induced asthma mice, Poly I-C treatment significantly increased SAA1 levels as well as IL-17A/interferon-gamma/IL-33 levels in bronchoalveolar lavage fluid (BALF), leading to airway hyperresponsiveness and inflammation. The highest levels of SAA1 and neutrophilia were noted in the BALF and sera of the NA mouse model, followed by the mixed granulocytic asthma (MA) model. Especially, SAA1 induced IL-17/retinoic acid receptor-related orphan receptor γt expression from activated CD4+ T lymphocytes in asthmatic mice.
**Conclusions:** The results show that SAA1 could induce neutrophilic airway inflammation by activating neutrophils along with NET formation, M1 macrophages, and Th2/Th17 predominant cells, contributing to the phenotype of NA or MA.

**Keywords:** Serum amyloid A protein; asthma; phenotype; neutrophils; macrophages; epithelial cells; Poly I-C; inflammation; biomarkers

## INTRODUCTION

Asthma is traditionally characterized by eosinophilic inflammation in the airways; however, adult asthmatic patients are classified into various subtypes based on cellular profiles in the

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sputum: eosinophilic asthma (EA), neutrophilic asthma (NA), mixed granulocytic asthma (MA), and pauci-granulocytic asthma.[1-3] Especially, NA is associated with airway microbiology that may influence the response to steroid resistance with more severe asthma (SA) symptoms.[4,5] Moreover, tumor necrosis factor-α (TNF-α) and interleukin (IL)-8 (released from macrophages), as well as IL-17 (released from $CD4^+$ T lymphocytes), have been known to be involved in neutrophilic airway inflammation.[6-8] Nevertheless, persistent airway inflammation and frequent asthma exacerbations in NA representing SA have not been completely understood.[9,10]

Acute-phase serum amyloid A (SAA) proteins, including SAA1 and SAA2, are released from airway epithelial cells (AECs), macrophages, monocytes, and dendritic cells.[11,12] The association between higher SAA1 levels and asthma prevalence has been reported in several studies.[13,14] To date, SAA has been proposed as a biomarker for NA, which acts via N-formyl peptide receptor 2 signaling, and relates to lower forced expiratory volume in the first second (FEV1) (%) among asthmatic patients.[15-17] Moreover, SAA1 could bind to house dust mite (HDM) allergens, driving type 2 responses via inducing the production of IL-33 from AECs *in vitro*.[18] In addition to asthma, SAA levels were elevated in acute exacerbation of chronic obstructive pulmonary disease with induced neutrophilic airway inflammation.[15,17,19] Therefore, there is a need to clarify the contribution of SAA1 to neutrophilic airway inflammation according to the asthma phenotypes and the degree of airway inflammation.

AECs act as the key upstream in asthma pathogenesis by producing not only alarmins (*e.g.*, thymic stromal lymphopoietin [TSLP], IL-33 and IL-25) but also predominant source cells of proinflammatory cytokines and chemokines, which characterize infiltrating cells and asthma phenotypes.[20] In addition, AECs produce chemokines, such as C-C motif chemokine ligand 5 and IL-8, following respiratory syncytial virus infection, which drives eosinophil and neutrophil/monocyte recruitment.[21] Currently, pulmonary macrophages have been supposed to be central cells for regulating airway inflammation in asthma pathogenesis as they are in the host defense and produce abundant pro-inflammatory cytokines in response to surrounding environments; overproduction of these cytokines loses their protective properties and enhances innate and adaptive immune responses in the asthmatic airway.[22,23]

Here, we evaluated serum SAA1 levels according to various asthma phenotypes with clinical relevance. Moreover, the effects of SAA1 on neutrophils and macrophages in induction of airway inflammation were investigated *in vitro* and *in vivo*.

## MATERIALS AND METHODS

### Study subjects

Human studies were approved by the Institutional Review Board of Ajou University Hospital (AJIRB-GEN-SMP-13-108). All patients provided written informed consent at the time of recruitment. In this study, adult asthmatic patients (n = 122) and healthy control subjects (HCs, n = 60) were enrolled. A diagnosis of asthma was based on the Global Initiative for Asthma (GINA) 2020 guideline.[24] According to sputum neutrophil count, adult asthmatic patients were classified into NA (sputum neutrophils ≥ 65% among the leukocytes counted) and non-NA (sputum neutrophils < 65%).[25] Severe asthma was determined on the basis of the American Thoracic Society/European Respiratory Society guideline.[26] Atopy status was defined when a positive skin prick test to at least one of the following common inhalant allergens: 2 HDMs (*Dermatophagoides pteronyssinus, Dermatophagoides farinae*), cat,


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dog, cockroach, aspergillus, and alternaria, mugwort and ragweed pollens, and tree and grass pollen mixtures (Bencard, Bradford, UK). Spirometry was performed to evaluate the degree of airway obstruction, and methacholine bronchial challenge test was performed to examine the degree of airway hyperresponsiveness (AHR) as previously described.[27] Serum samples from all subjects were collected, stored at −70°C, and thawed before use. Total Immunoglobulin E (IgE) levels in serum were measured by the ImmunoCAP system (ThermoFisher Scientific, Waltham, MA, USA) in the detection range of 2–5,000 kU/L.

## Epithelial cell culture and stimulation

Two kinds of AECs were used in this study, primary small airway epithelial cells (SAECs) and human AEC line (A549), both of which were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). SAECs were cultured in basal medium (ATCC) plus bronchial epithelial cell growth kit (ATCC), 10 UI/mL penicillin G sodium, 10 μg/mL streptomycin sulfate (Gibco, Grand Island, NY, USA), and 25 ng/mL amphotericin B (Sigma Aldrich, St. Louis, MO, USA) at 37°C in humidified air with 5% $CO_2$. A549 cells were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum, penicillin G sodium (100 UI/mL), and streptomycin sulfate (100 μg/mL). SAECs ($1 \times 10^5$ cells) or A549 ($2 \times 10^5$ cells) were seeded onto a 12-well plate and stimulated with 50 ng/mL IL-5/IL-13/IL-6/IL-1β (Sigma Aldrich), or 50 μg/mL Poly I-C (Sigma Aldrich) for 24 hours. In some experiments, AECs were stimulated with 50 μg/mL Poly I-C with/without 50 ng/mL human recombinant SAA1/SAA (LifeSpan Biosciences, Franklin Lakes, NJ, USA).

## Human immune cell isolation and culture

Blood from asthmatic patients was layered on a lymphoprep™ solution (Axis-Shield, Oslo, Norway), followed by centrifugation at 2,500 rpm, at 20°C for 25 minutes without braking. The red layer was sedimented in Hank's balanced salt solution (HBSS from Gibco) buffer containing 2 mM ethylenediaminetetraacetic acid and 2% dextran at room temperature for 45 minutes. Then, peripheral blood eosinophils (PBEs), neutrophils (PBNs) and monocytes were isolated using Eosinophil or Monocyte Isolation Kit (Miltenyi Biotec Inc., Auburn, CA, USA) according to the manufacturer's instructions. The cells were maintained in RPMI-1640 medium supplemented with 10% heat-inactivated fetal bovine serum, 100 IU/mL penicillin, and 100 μg/mL streptomycin.

## Induction of neutrophil extracellular traps (NETs)

To investigate formation of NETs, PBNs were treated with 100 nM phorbol myristate acetate (PMA; Sigma Aldrich) or 50 ng/mL SAA1 with/without 1 μM dexamethasone (dex) for 5 hours as previously described.[28] Supernatants were harvested for ELISA. The images of NETs were taken by using a Zeiss LSM710 confocal microscope (Cal Zeiss Microscopy GmbH, Jena, Germany) and the images were analyzed by microscope software ZEN lite. To detect NET formation by using immunofluorescence, cells were incubated overnight with anti-myeloperoxidase (MPO) from Abcam (Cambridge, UK), and anti-neutrophil elastase (NE) from Santa Cruz (Biotechnology, Inc, Dallas, TX, USA), followed by Alexa fluor 488 donkey anti-rabbit and 594 donkey anti-goat antibodies (Thermo-Life Technology, Waltham, MA, USA) for 1 hour. The nuclear DNA was stained with 4′,6-diamidino-2-phenylindole (DAPI with 1.000-fold dilution) for 5 minutes. PicoGreen assay was performed to measure cell-free DNAs in the NETs using Quanti-iTTM PioGreen® double stranded DNA (dsDNA) kits (Invitrogen, Paisley, UK) according to the manufacturer's instructions. In some experiments, PBNs were stimulated with 50 ng/mL SAA1 for 30 minutes, followed by being pretreated 30 minutes with/without 1 μM dex to detect SAA1-induced signaling expression in PBNs.


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## Quantification for reactive oxygen species (ROS) production

The production of ROS from isolated PBNs were stained with 2'7′ dichlorofluorescein diacetate (H2DCFDA) (Life Technologies, Eugene, OR, USA) within 30 minutes at 37°C, followed by one wash with 1× HBSS, and then treated with 50 ng/mL SAA1 in phenol red-free RPMI medium for 30 minutes. After that 100 μL medium in each well was transferred to a black-plate (Thermo Fisher Scientific). The signal was read at 480 nm of excitation, and 520 nm of emission wavelengths under a fluorescence microplate reader (BioTek Instrument, Inc., Winooski, VT, USA)

## Neutrophil migration assay

The transwell system with a pore size of 3.0 μm (Neuro Probe, Gaithersburg, MD, USA) was used to evaluate PBN migration rate, according to the manufacturer's instructions. Isolated PBNs were stained with 2 μmol calcium aceto-methyl ester (Life Technologies) and seeded on the upper chamber of the transwell system in phenol red-free RPMI medium, while the lower chamber contained 50 ng/mL SAA1 in phenol red-free RPMI medium. The transwell plate was incubated at 37°C for 2 hours. Then, 100 μL medium of the lower chamber was transferred to a black-wall plate, and the signal was read at 485-nm excitation and 528-nm emission wavelengths under a fluorescence microplate reader (BioTek Instrument).

## Macrophage polarization and activation protocol

For macrophage polarization, monocytes were maintained in RPMI medium for 7 days without any stimulation to differentiate into M0 macrophages. After that, M0 macrophages were treated with 50 μg/mL Poly I-C, 100 ng/mL lipopolysaccharides (LPS; Sigma Aldrich) and 50 ng/mL SAA1 alone for 48 hours. To detect M1 macrophage polarization by using immunofluorescence, cells were stimulated overnight with anti-inducible nitric oxide synthase (iNOS) and anti-CD68 from Abcam, followed by Alexa fluor 488 donkey anti-rabbit and 594 donkey anti-mouse antibodies (Thermo-Life Technology) for 1 hour. For western blotting, M0 macrophages were treated with SAA1 in dose-dependent assays (0.5, 5 and 50 ng/mL).

## Asthma mouse models

Female BALB/c mice at 6 weeks old (weight 20 ± 2 g) were purchased from the Jackson Laboratory (Bar Harbor, ME, USA) and maintained under specific pathogen-free conditions. All animal experimental protocols were approved by the Institutional Animal Care and Use Committee of Ajou University (IACUC2018-0041). To evaluate the effect of viral infection on airway inflammation, asthma mouse model was established as a previous protocol.[29] Briefly, on days 0 and 14, 6-week-old female BALB/c mice were intraperitoneally sensitized with 10 μg/mL ovalbumin (OVA; Sigma Aldrich) with aluminum hydroxide solution (Sigma Aldrich). On days 28–30, the mice were challenged with 2% OVA for 30 minutes using an ultrasonic nebulizer (KTMED Inc., Seoul, South Korea). Prior to sensitization/challenge, mice were intranasally treated with Poly I-C (100 μg/mouse). To investigate the significance of SAA1 according to inflammatory phenotypes of asthma including EA, NA, and MA, mice were treated with/without LPS as described in **Supplementary Fig. S1**.[30] Briefly, mice were intraperitoneally sensitized with 10 μg of OVA in aluminum hydroxide solution on days 0 and 7. From days 14 to 17, the mice were challenged with 6% OVA for 30 minutes using an ultrasonic nebulizer for the EA group. Mice were intranasally administered 10 μg of LPS from days 15 to 17 for NA groups, while 0.1 μg of LPS on day 15 for MA groups. The mice from NA and MA were challenged with 6% OVA for 30 minutes using an ultrasonic nebulizer 30 minutes after LPS administration. Mice were assayed at 24 hours after the last challenge. Hematoxylin and eosin (H&E) staining was conducted for lung tissues to investigate immune


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cell infiltration. To determine the expression of SAA1 in neutrophils in lung tissues by using immunofluorescence, slides were stained with anti-SAA1 (LifeSpan BioSciences) and anti-MPO antibodies.

### Measurement of AHR

Airway resistance to inhaled methacholine (Sigma Aldrich) was measured using the FlexiVent System (SCIREQ, Montreal, Canada) as previously described.[29] Each mouse was anesthetized with pentobarbital sodium, followed by intubating with a cannula, and then aerosol methacholine at increasing concentrations (0, 1.56, 3.12, 6.25, 12.5 and 25 mg/mL), and the peak airway responses to the inhaled methacholine were noted.

### Differential cell count

To collect cell-free bronchoalveolar lavage fluid (BALF), BALF was centrifuged at 1,200 rpm within 5 minutes at 4°C. The cell pellets were used to stain with H&E. These cells were counted and classified as macrophages, eosinophils, neutrophils, or lymphocytes.

### T-cell stimulation

The spleen was ground completely in RPMI-1640 media plus 1% antibiotic and 10% of fetal bovine serum, followed by expelling several times with a 19-G needle and filtering with a mesh nylon screen to collect single cells. Then, $CD4^+$ T cells were isolated from mononuclear cells by using a T-cell isolation kit (Miltenyi Biotec). These cells ($2 \times 10^5$) were treated with or without 50 ng/mL mouse recombinant SAA/SAA1 (R&D Systems, Minneapolis, MN, USA) for 48 hours in the presence of anti-CD3/anti-CD28 antibodies from eBioScience (San Diego, CA, USA). In addition, CD4+ T cells were stimulated with 50 ng/mL SAA1 for 24 hours in the presence of anti-CD3/anti-CD28 antibodies, followed by flow cytometry.

### Flow cytometry

To analyze activated CD4+ T-cell cytokine patterns by intracellular cytokine staining and flow cytometry, antibodies were used as follows: fluorochrome-conjugated anti-IL-5 (PE signal), anti-IL-17 (PerCP-Cy 5.5 signal) or anti-interferon gamma (IFN-γ) (APC signal). All monoclonal antibodies were purchased from BioLegend (San Diego, CA, USA). Stained cells were analyzed with BD FACSCanto™ II (BD Bioscience), and graphs were produced using FlowJo software.

### Enzyme-linked immunosorbent assay

To measure concentration of the inflammatory cytokines, ELISA kits were used as follows: human SAA1, S100 calcium-binding protein A9 (S100A9), MPO, IL-6, IL-1β, IL-8, transforming growth factor-beta 1 (TGF-β1), and TNF-α (R&D Systems), mouse SAA1/SAA (LifeSpan BioSciences), and human/mouse IL-5, IFN-γ, IL-17A and IL-33 (R&D Systems).

### Western blot analysis

For the detection of several proteins, the antibodies were used as follows: SAA1 (LifeSpan BioSciences), iNOS, MPO, CD68, CD163, CD86, eosinophil-derived neurotoxin (EDN), IL-17, GATA binding protein 3 (GATA-3) from Abcam, retinoic acid receptor-related orphan receptor γt (RORγt) (Invitrogen), phosphor-extracellular-signal-regulated kinase (ERK), total ERK, phosphor-p38 mitogen-activated protein kinase (MAPK), total p38 MAPK from Cell Signaling (Minneapolis, MN, USA), NE, and β-actin from Santa Cruz.


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### Statistical analysis

All statistical analyses were performed using IBM SPSS, version 22 for Windows (IBM SPSS Statistics, IBM Corporation, Chicago, IL, USA) and GraphPad Prism 8 software. Categorical variables were analyzed by using Pearson's chi-square test. In clinical data, continuous variables showing normal distribution are presented as means ± standard deviations and were analyzed by Student t-test; those not normally distributed were analyzed by the Mann-Whitney *U* test. One-way ANOVA with *post hoc* Tukey HSD test was used for multiple comparisons of data from multiple groups. In *in vitro* and *in vivo* experiments, all values are represented as mean values ± SEM. *P* values of less than 0.05 were considered significant.

## RESULTS

### Clinical characteristics of the study subjects

The demographic data of the study subjects are depicted in **Table 1**. In this study, a total of 122 adult asthmatic patients and 60 HCs were enrolled. When compared between the NA and non-NA groups, no statistical significance was detected in age, sex, atopic status, serum total IgE, or total eosinophil counts. FEV1(%) values were significantly lower in the NA group than in the non-NA group ($P = 0.044$); PC20 methacholine values tended to be lower in the NA group ($P = 0.072$). In addition, the serum SAA1 levels were significantly higher in the NA group than in the non-NA group ($P = 0.001$). The asthmatic patients were divided into high- and low-SAA1 groups based on serum cutoff value at 2.6 ng/mL (the mean plus standard deviation of log-transformed SAA1 in HCs), and their clinical characteristics are summarized in **Table 2**. High-SAA1 group showed significantly lower FEV1 (%) ($P = 0.018$), but had a higher prevalence of SA ($P = 0.008$), a larger number of sputum neutrophils ($P = 0.031$), and higher levels of serum IL-6 ($P = 0.001$) and S100A9 ($P = 0.013$) than low-SAA1 group.

### Factors for inducing SAA1 production from AECs

Significant release of SAA1 was noted from both AECs treated with Poly I-C, IL-1β or IL-6 (**Fig. 1A and B**). Moreover, PBNs, but not PBEs, significantly increased SAA1 production (**Fig. 1C and D**). Sequentially, SAA1 stimulated AECs to induce IL-6, IL-8, and S100A9 production, which was further elevated by Poly I-C treatment. However, dex did not effectively suppress these responses (**Fig. 1E-K**). In addition, SAA1 increased the phosphorylation of ERK and p38 MAPK in AECs (**Fig. 1L**). These results suggest that SAA1 levels are increased in

**Table 1.** Characteristics of the study subjects

| Characteristics | Asthma (n = 122) | HCs (n = 60) | *P* value | NA (n = 78) | Non-NA (n = 44) | *P* value |
|---|---|---|---|---|---|---|
| Age (year) | 45.1 ± 15.8 | 48.2 ± 9.5 | 0.191 | 45.8 ± 15.6 | 43.1 ± 16.1 | 0.357 |
| Sex (male) | 41 (34) | 25 (42) | 0.303 | 25 (31) | 16 (39) | 0.367 |
| Atopy (positive) | 53 (43) | 20 (33) | 0.567 | 35 (43) | 18 (44) | 0.363 |
| FEV1 (% predicted) | 86.7 ± 17.0 | - | - | 84.5 ± 17.4 | 91.9 ± 15.1 | 0.044 |
| PC20 (mg/mL) | 7.3 ± 8.3 | - | - | 6.4 ± 7.8 | 9.9 ± 9.4 | 0.072 |
| Severe asthma | 40 (33) | - | - | 28 (36) | 12 (27) | 0.319 |
| Sputum neutrophil counts (%)* | 66.9 ± 33.3 | - | - | 82.9 ± 16.1 | 30.3 ± 32.5 | 0.001 |
| Sputum eosinophil counts (%)* | 22.1 ± 24.3 | - | - | 12.5 ± 26.1 | 30.1 ± 39.8 | 0.472 |
| TEC (cells/μL)* | 439.5 ± 593.1 | - | - | 369.9 ± 442.0 | 427.7 ± 304.1 | 0.307 |
| Serum total IgE (kU/L)* | 457.8 ± 825.7 | - | - | 444.7 ± 650.5 | 488.9 ± 17.8 | 0.122 |
| Log† (SAA1, ng/mL)* | 2.6 ± 0.3 | 2.4 ± 0.2 | 0.014 | 2.7 ± 0.3 | 2.3 ± 0.5 | 0.001 |

Values are given as number (%) for categorical variables and as mean ± standard deviation for continuous variables.
FEV1, forced exhaled volume at 1 second; HCs, healthy controls; IgE, immunoglobulin E; NA, neutrophilic asthma; PC20, concentration of methacholine to induce a 20% decline in FEV1; SAA1, serum amyloid A1; TEC, total eosinophil count.
*P* values were applied by Pearson chi-square test for categorical variables and Student's *t* test or *Mann-Whitney *U* test for continuous variables. Neutrophilic asthma was classified in asthma patients with sputum neutrophil count ≥ 65%; †Log: skewed data were log-transformed.


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**Table 2.** Comparison of demographic characteristics between high- and low-SAA1 groups

| Characteristics | High-SAA1 (n = 40) | Low-SAA1 (n = 82) | *P* value |
|---|---|---|---|
| Age (year) | 47.6 ± 15.2 | 43.6 ± 16.0 | 0.162 |
| Sex (male) | 13 (33) | 28 (34) | 0.857 |
| Atopy (positive) | 36 (55) | 70 (47) | 0.392 |
| FEV1 (% predicted) | 81.4 ± 20.5 | 89.4 ± 14.3 | 0.018 |
| PC20 (mg/mL) | 5.8 ± 7.5 | 8.4 ± 8.7 | 0.254 |
| Severe asthma | 18 (45) | 22 (27) | 0.008 |
| Sputum neutrophil counts (%)* | 77.3 ± 28.0 | 61.8 ± 34.7 | 0.031 |
| Sputum eosinophil counts (%)* | 32.8 ± 38.7 | 15.3 ± 30.1 | 0.060 |
| TEC (cells/μL)* | 361.8 ± 324.0 | 380.0 ± 418.8 | 0.866 |
| Serum total IgE (kU/L)* | 409.1 ± 705.0 | 471.3 ± 847.5 | 0.141 |
| Log† (S100A9, pg/mL)* | 1.4 ± 0.8 | 0.9 ± 1.0 | 0.013 |
| Log† (IL-6, pg/mL)* | 0.8 ± 1.0 | 0.3 ± 0.5 | 0.001 |

Identification of high and low SAA1 responders based on cutoff point at 2.6 that calculated by sum of mean and standard deviation of log (SAA1, ng/mL) in healthy controls. Values are given as number (%) for categorical variables and as mean ± SD for continuous variables.
SAA1, serum amyloid A1; FEV1, forced exhaled volume at 1 second; PC20, concentration of methacholine to induce a 20% decline in FEV1; TEC, total eosinophil count; IgE, immunoglobulin E; S100A9, S100 calcium-binding protein A9; IL, interleukin.
*P* values were applied by Pearson chi-square test for categorical variables and Student's *t* test or *Mann-Whitney *U* test for continuous variables; †Log: skewed data were log-transformed.

neutrophilic airway inflammation and they may promote Poly I-C-induced inflammation in AECs through activating ERK/p38MAPK signaling pathway.

## Effect of SAA1 on the induction of immune responses in neutrophils and macrophages

SAA1 significantly induced the release of MPO and dsDNA from PBNs (isolated from asthmatics), which were not suppressed by dex (**Fig. 2A and B**). In addition, SAA1 significantly enhanced ROS production and migration of PBNs, which were not suppressed by dex (**Fig. 2C and D**). The NET-positive cell numbers were remarkably larger in the SAA1-treated group than in the control group, which were not suppressed by dex (**Fig. 2E and F**). SAA1 remarkably enhanced the phosphorylation of ERK and p38 MAPK in PBNs (**Fig. 2G**). Here, we also found that SAA1 activated macrophages to produce pro-inflammatory cytokines such as IL-1β, IL-6, TNF-α, and TGF-β1 (**Fig. 3A-D**) as well as IL-8 (**Fig. 3E**). Especially, levels of IL-1β and IL-6 induced by 100 ng/mL SAA1 were similar with those by 50 μg/mL Poly I-C stimulation, but they were significantly lower than those by 100 ng/mL LPS stimulation, while SAA1-induced levels of TNF-α, TGF-β1 and IL-8 were significantly higher than Poly I-C-induced ones without any differences from LPS-stimulated ones (**Fig. 3A-E**). Moreover, the shape of SAA1-activated macrophages tended to be similar to that of LPS-activated macrophages detected by immunofluorescent (**Fig. 3F**). SAA1 remarkably enhanced the expression of iNOS (M1 marker) and CD68 (maturation marker) in M0 macrophages (**Fig. 3F**) and the expression of CD86 (M1 marker) in a dose-dependent manner (**Fig. 3G**). These findings indicate that SAA1 may play a role in neutrophilic airway inflammation via activating neutrophils (NET formation), M1 macrophages and AECs.

## Effect of virus infection on airway inflammation in allergic asthmatic mice

In allergic asthma mouse model, treatment of Poly I-C further increased AHR in allergic asthmatic mice (**Fig. 4A**). Moreover, more markedly enhanced total cell, eosinophil, and neutrophil counts in BALF were noted in the OVA/IC group than in the OVA group (**Fig. 4B**). Regarding cytokine patterns, elevated levels of IFN-γ, IL-17A, and IL-33, but not IL-5 were observed in the BALF of the OVA/IC group compared to those of the OVA group (**Fig. 4C-F**). In addition, the SAA1 levels in both BALF and sera were significantly higher in the OVA/IC group compared to the OVA group (**Fig. 4G and H**). Western blot analysis showed that expression


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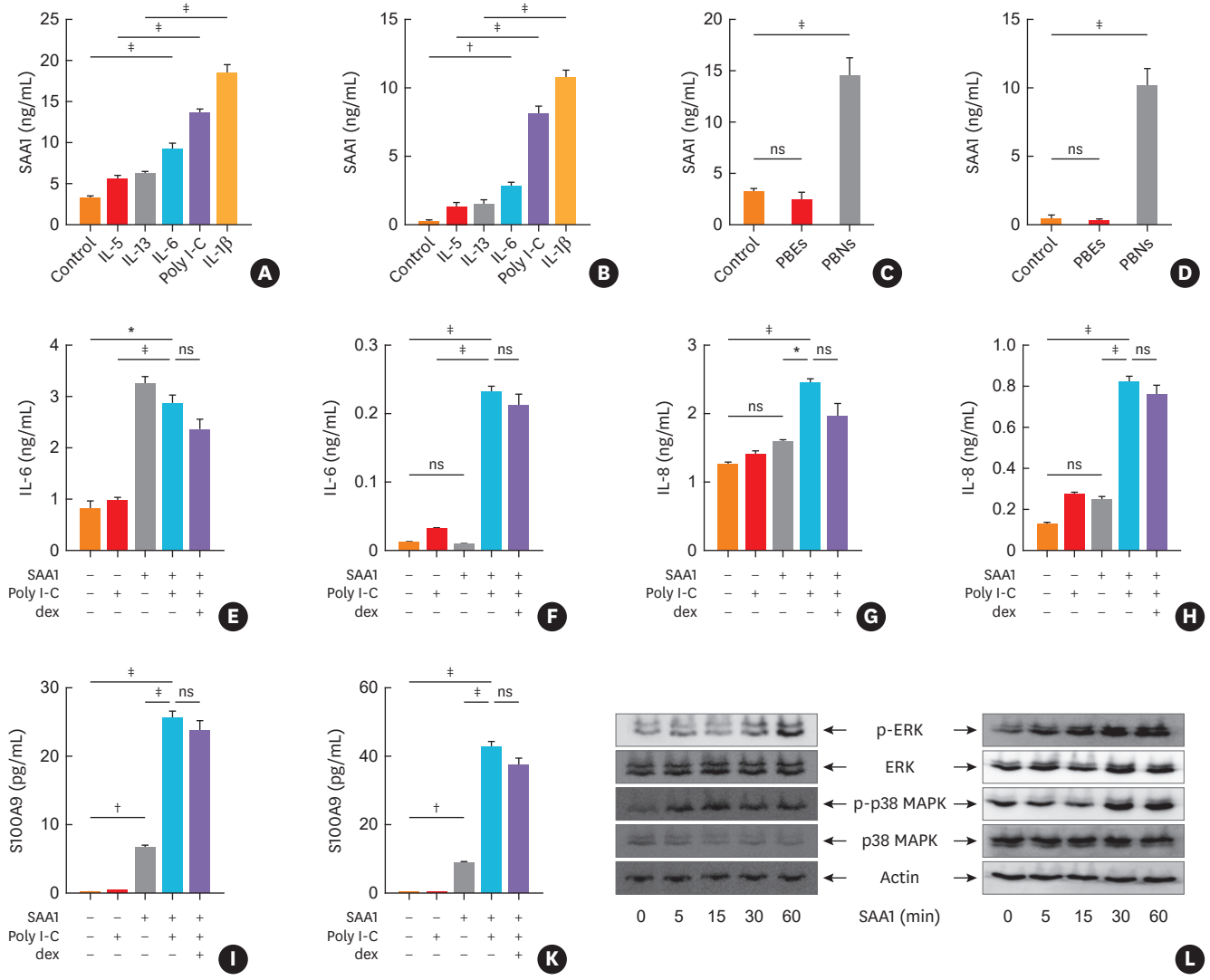


**Fig. 1.** The production of SAA1 from AECs and the effects of SAA1 on proinflammatory cytokine production. SAA1 proteins were produced from AECs (SAECs and A549, respectively) in response to various stimuli (A, B), and in the coculture with PBNs, PBEs (C, D). SAA1-induced release of IL-6, IL-8 and S100A9 from AECs (SAEC and A549, respectively) (E-K). Data are presented as means ± standard error of mean (n = 6). *P* values were calculated by using one-way analysis of variance with Bonferroni's *post hoc* test. SAA1 enhanced the expression of p-ERK and p-p38 MAPK (L) in AECs (SAECs and A549, respectively).
IL, interleukin; PBEs, peripheral blood eosinophils; PBNs, peripheral blood neutrophils; dex, dexamethasone; S100A9, S100 calcium-binding protein A9; p-ERK, phospho-extracellular signal-regulated kinase; ERK, extracellular signal-regulated kinase; p-p38 MAPK, phospho-p38 mitogen-activated protein kinase; MAPK, mitogen-activated protein kinase; AECs, airway epithelial cells; SAEC, small airway epithelial cells.
*$P < 0.050$, †$P < 0.010$, ‡$P < 0.001$, ns = not significant.

of SAA1 was more remarkably enhanced in the lung tissues of the OVA and OVA/IC groups compared to those of the control group. Furthermore, the expressions of EDN, NE and MPO were increased in the OVA/IC group, while only EDN expression was increased in the OVA group (**Fig. 4I-K**).

## Comparison between EA, NA, and MA mouse models

The SAA1 levels were compared among 3 mouse models. AHR and immune cell counts in the BALF were higher in the 3 mouse models than in the control group (**Fig. 5A, B, and G**); significantly higher eosinophil counts were noted in the BALF of the EA and MA models, whereas the highest neutrophil counts were noted in the BALF of the NA model ($P < 0.001$; **Fig. 5B**). The SAA1 levels in the BALF and sera were highest in the NA group; moreover, those


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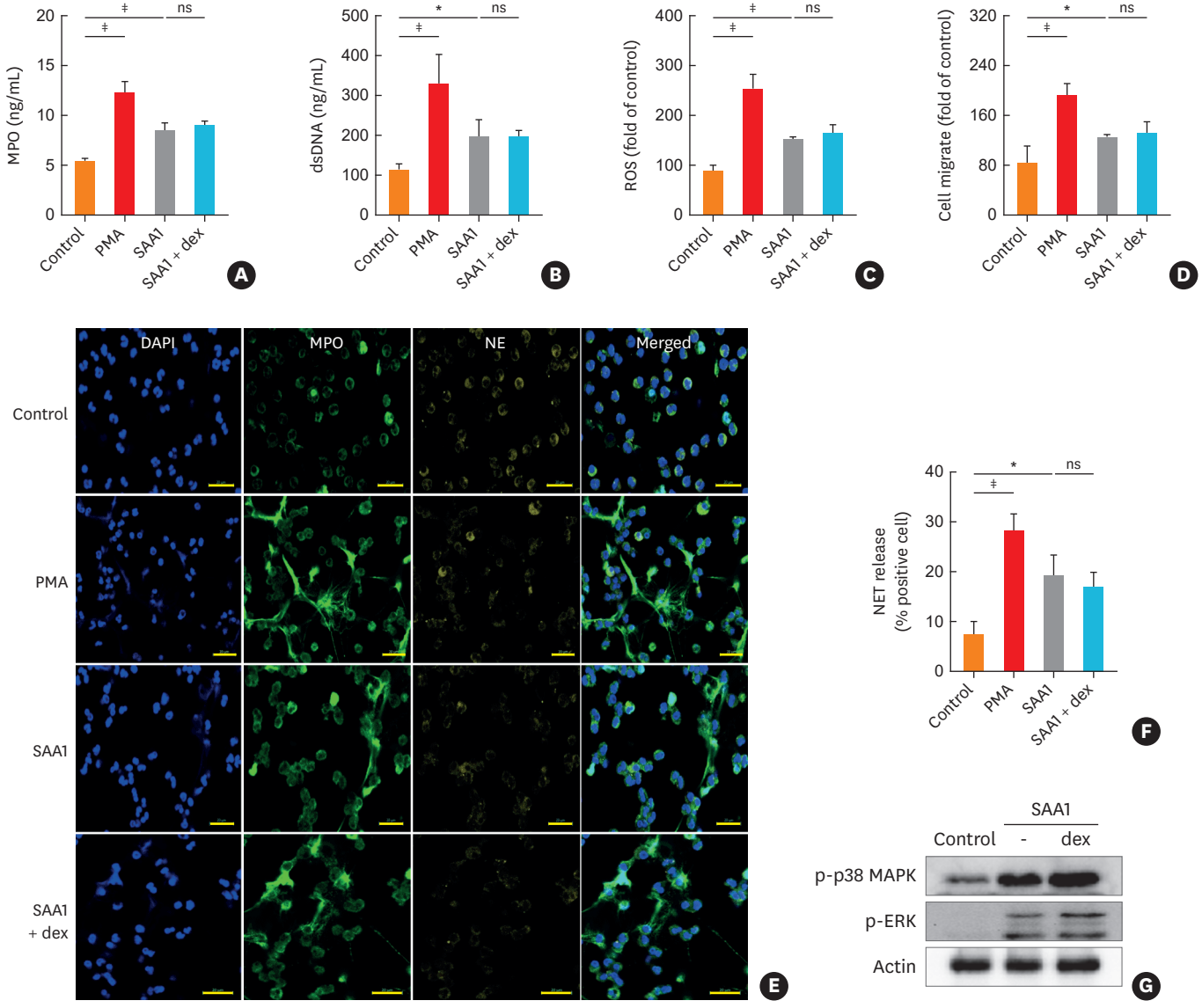


**Fig. 2.** Effects of SAA1 on neutrophil activation and the formation of neutrophil extracellular traps from PBNs in patients with asthma. SAA1-induced MPO production (A). SAA1-induced extracellular dsDNA from PBNs were evaluated by PicoGreen assay (B), and ROS formation from PBNs and neutrophil migration (C, D). PBNs were stained with MPO, NE, and DAPI. NET levels were measured by confocal microscopy (E, scale bar = 20 μm). NET levels were evaluated by % of positive cells using confocal microscopy (F). Data are shown as the mean ± standard error of mean (n = 8) for each group. *P* values were calculated by using one-way analysis of variance with Bonferroni's *post hoc* test. SAA1-induced expression of p-ERK and p-p38 MAPK in PBNs for 30 minutes, which were further enhanced by dex (pretreated 30 minutes) as detected via western blotting (G).
MPO, myeloperoxidase; PMA, phorbol myristate acetate; SAA1, serum amyloid A1; dex, dexamethasone; dsDNA, double stranded DNA; ROS, reactive oxygen species; DAPI, 4′,6-diamidino-2-phenylindole; NE, neutrophil elastase; NETs, neutrophil extracellular traps; p-p38 MAPK, phospho-p38 mitogen-activated protein kinase; p-ERK, phospho-extracellular signal-regulated kinase; PBNs, peripheral blood neutrophils.
$^{*}P < 0.050$, $^{\dagger}P < 0.010$, $^{\ddagger}P < 0.001$, ns = not significant.

of the MA and EA models were significantly higher compared to the control group ($P < 0.050$; **Fig. 5C and D**). In addition, the expression of SAA1 was more markedly enhanced in lung tissues of the NA, and MA groups compared to those of the control group. The IL-17 family (IL-17A, B, C, D, E and F) exerts their functions with a molecular weight of the monomer ranging from 17 to 21 kDa; however, IL-17A and IL-17F can form heterodimers that migrate between 25 and 37 kDa in which the upper band is IL-17F and the lower band is IL-17A.[31,32] When anti-IL-17 antibody was used for western blotting, 2 bands were detected (migrated between 30 and 37


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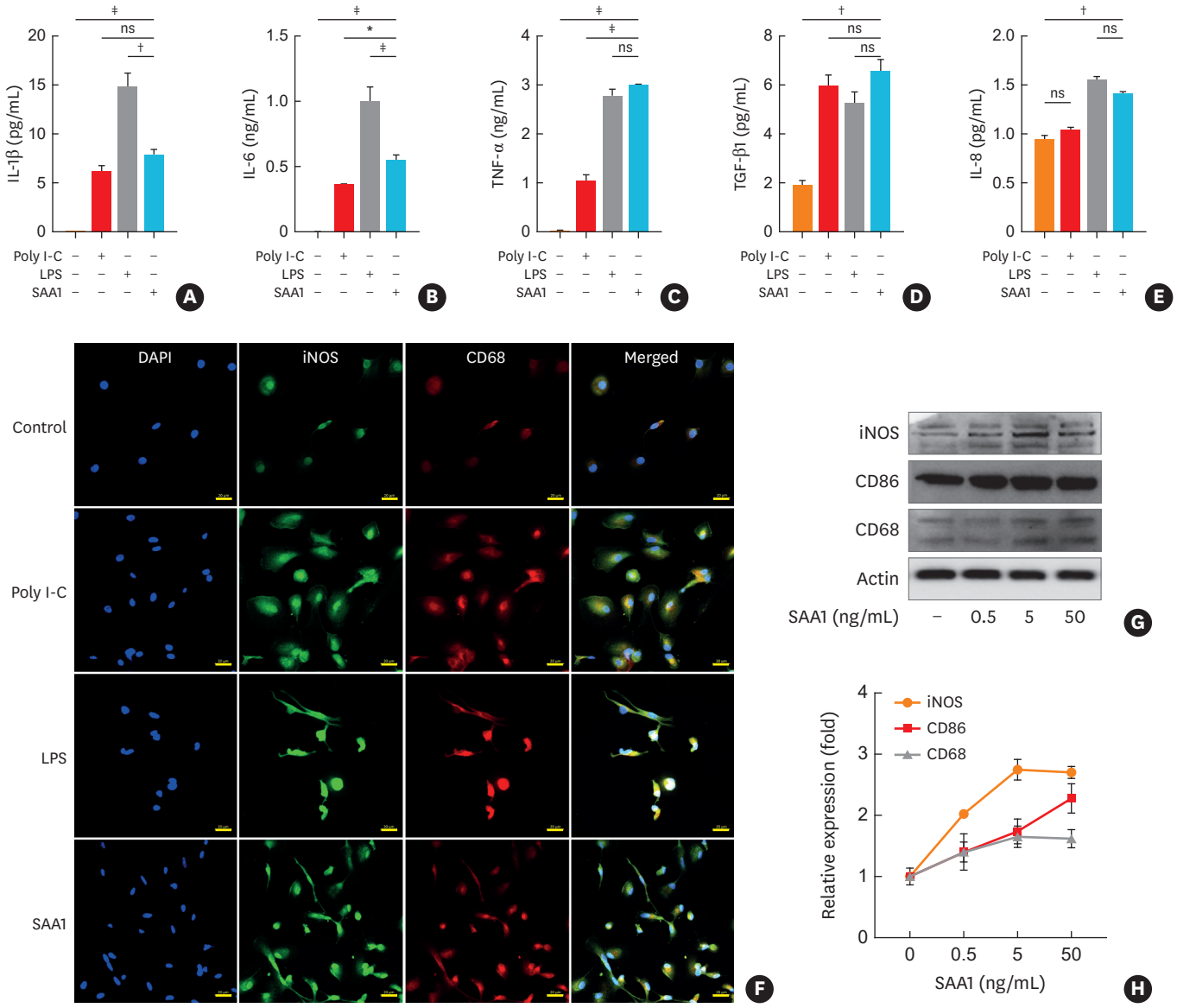


**Fig. 3.** SAA1-induced proinflammatory cytokine and chemokine release from M0 macrophages. SAA1-induced release of IL-1β (A), IL-6 (B), TNF-α (C), TGF-β1 (D) and IL-8 (E) from M0 macrophages in patients with asthma. Data are presented as means ± standard error of mean (n = 6). *P* values were calculated by using one-way analysis of variance with Bonferroni's *post hoc* test. SAA1-enhanced expression of M1 macrophages (iNOS, CD86) and a mature macrophage marker (CD68) from M0 macrophages, detected via immunofluorescence staining for iNOS (green) and CD68 (red) in M0 (F, scale bar = 20 μm) and western blot in a dose-dependent manner of SAA1 (G), the relative expression of protein/actin (H, fold change).
IL, interleukin; LPS, lipopolysaccharides; SAA1, serum amyloid A1; TNF-α, tumor necrosis factor alpha; TGF-β1, transforming growth factor beta; iNOS, inducible nitric oxide synthase.
*$P < 0.050$, †$P < 0.010$, ‡$P < 0.001$, ns = not significant.

kDa) in the control group. Meanwhile, lower band (#30 kDa) was detected in asthma mouse models and markedly increased in the NA and MA groups, suggesting a higher expression of IL-17A in the NA and MA models compared to the control group (**Fig. 5E and F**). Furthermore, more significantly increased expression of MPO was noted in the NA group (not MA group) compared to the control group (**Fig. 5F**). SAA1 expression was significantly higher in the NA and MA groups than in the EA and control groups and was localized with MPO expression in the lung tissues (**Fig. 5H**). These results suggest the associations of neutrophilic airway inflammation with SAA1 production and expression of IL-17A/MPO in the airway.


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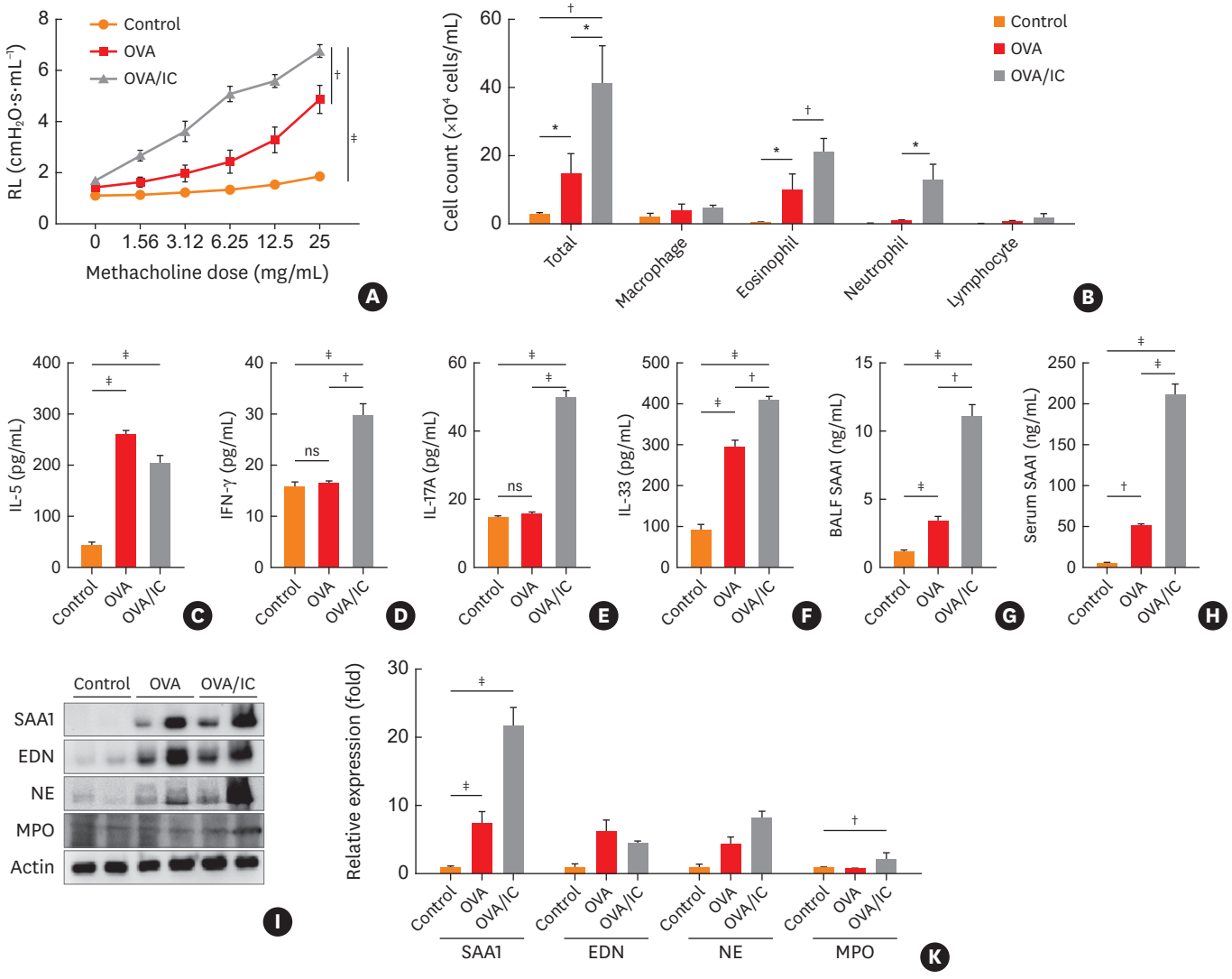


**Fig. 4.** Characteristics of the virus-infected allergic asthma mouse model. Changes in airway hyperresponsiveness to methacholine in the Poly I-C-infected asthma mouse model (OVA/IC), compared to healthy mice (control) and the allergic asthma mouse model (OVA) (A). Increased numbers of total cells, neutrophil and eosinophil cell counts in BALF of asthma mouse models (B). Increased levels of IL-5 (C), INF-γ (D), IL-17A (E) and IL-33 (F) in BALF of the asthma mouse models. Increased levels of SAA1 in BALF (G) and sera (H) in the asthma mouse models. Data are presented as means ± standard error of mean (n = 6). *P* values were calculated by using one-way analysis of variance with Bonferroni's *post hoc* test. The expression of SAA1, EDN, NE and MPO in lung tissue of the asthma mouse models as detected via western blotting (I) and presented as the relative expression of protein/actin (K, fold change).
OVA, ovalbumin; IC, Poly I-C; IL, interleukin; INF-γ, interferon gamma; BALF, bronchoalveolar lavage fluid; SAA1, serum amyloid A1; EDN, eosinophil-derived neurotoxin; NE, neutrophil elastase; MPO, neutrophil myeloperoxidase; RL, resistance to airflow across the lung.
*$P < 0.050$, †$P < 0.010$, ‡$P < 0.001$, ns = not significant.

## Effect of SAA1 on Th2/Th17 cytokine releases

T helper (Th) cytokine patterns in response to SAA1 were evaluated using the activated CD4+ T cells in the asthma mouse models. As a result, SAA1 strongly enhanced intracellular expression of IL-5/IL-17 (not IFN-γ) in the control group, whereas it increased intracellular IL-17 expression (not IL-5 or IFN-γ) in the OVA group (**Fig. 6A**). When evaluating released cytokines from activated CD4+ T cells, SAA1 significantly induced IL-13 and IL-17A (not INF-γ) production in the OVA group ($P < 0.010$ for all, **Fig. 6B-E**). In addition, SAA1 markedly increased expression of IL-17 and RORγt (Th17 differentiation) in activated CD4+ T cells from the OVA group, but not GATA-3 (Th2 differentiation) (**Fig. 6F and G**).


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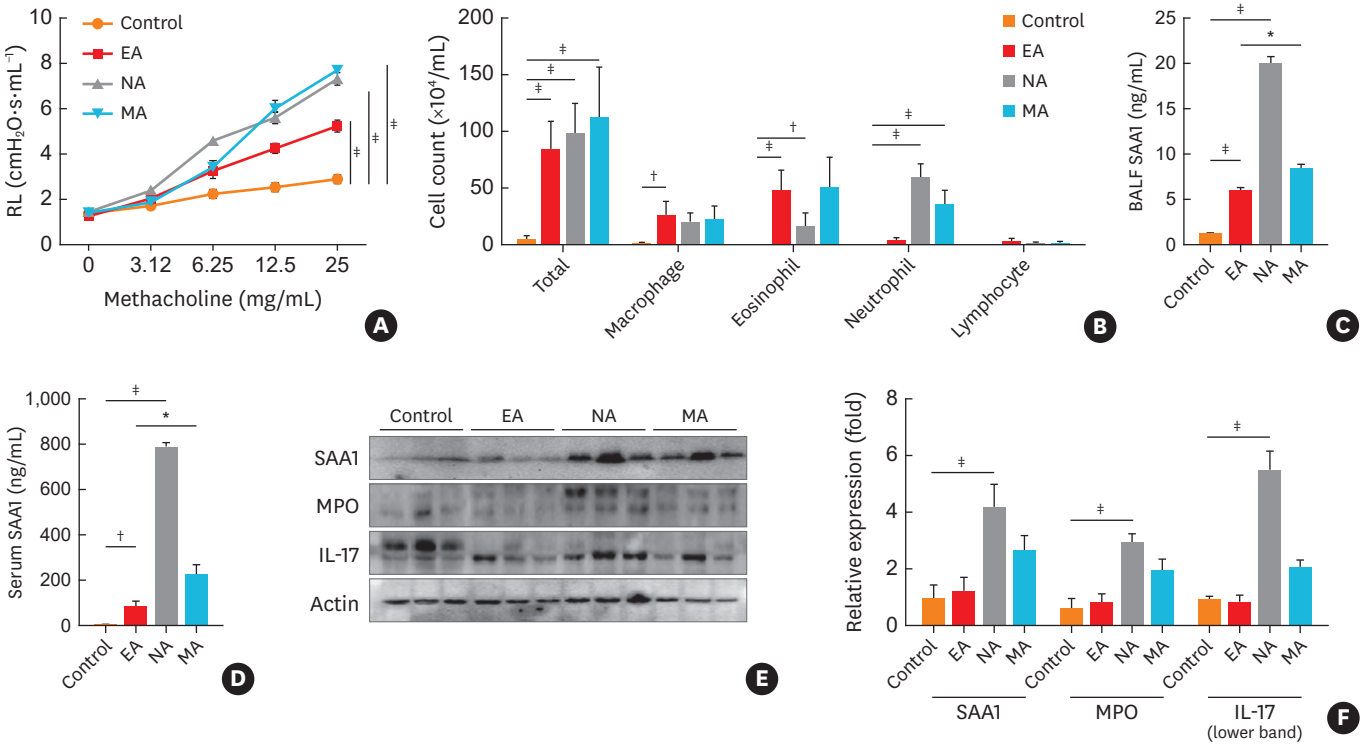


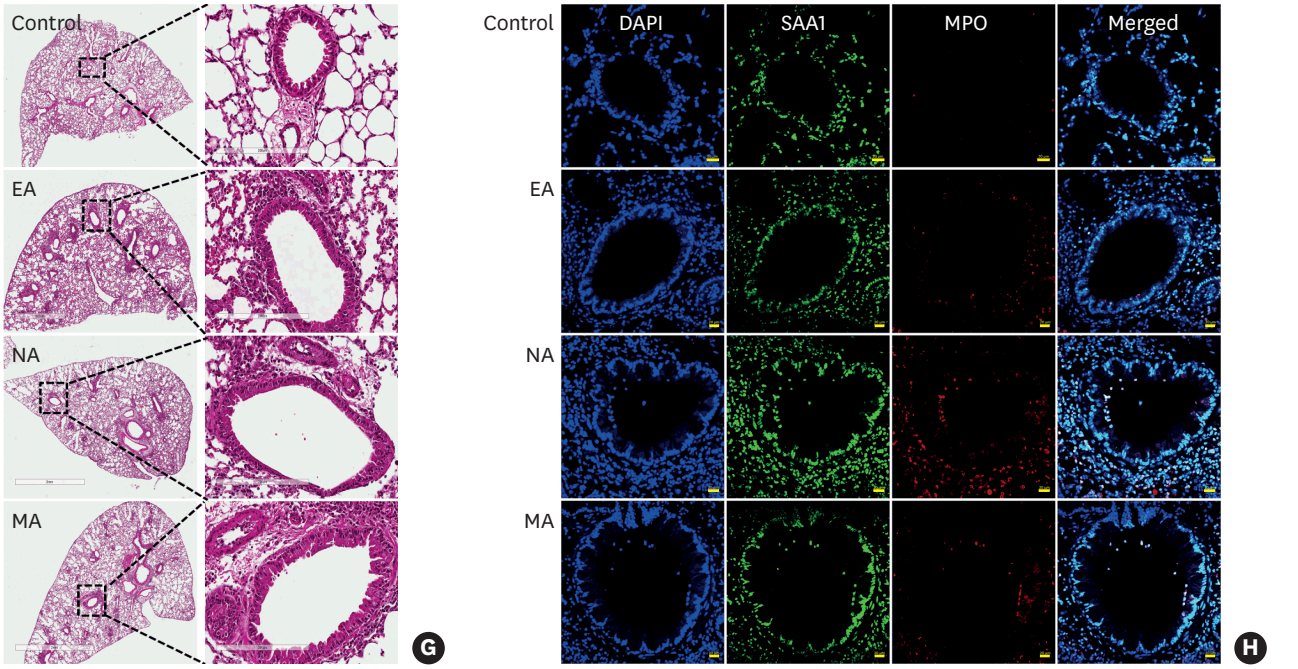


**Fig. 5.** Investigation of SAA1 in the mouse models of EA, NA and MA. Changes in airway hyperresponsiveness to methacholine in each asthma model (A). Total cell, neutrophil and eosinophil cell counts in the BALF of each asthma model (B). Increased levels of SAA1 in BALF (C) and sera (D) of asthma mouse phenotypes. Data are presented as means ± standard error of mean (n = 6). *P* values were calculated by using one-way analysis of variance with Bonferroni's *post hoc* test. The expressions of SAA1, MPO and IL-17 in lung tissues were detected via western blot (E) and presented as the relative expression of protein/actin (F, fold change). Lung histology stained with hematoxylin and eosin (G). Immunofluorescence staining for SAA1 (green) and MPO (red) in the lung tissues (H, scale bar = 20 μm).
EA, eosinophilic asthma; NA, neutrophilic asthma; MA, mixed granulocyte asthma; BALF, bronchoalveolar lavage fluid; SAA1, Serum amyloid A1; MPO, neutrophil myeloperoxidase; IL, interleukin; RL, resistance to airflow across the lung.
$^{*}P < 0.050$, $^{\dagger}P < 0.010$, $^{\ddagger}P < 0.001$, ns = not significant.


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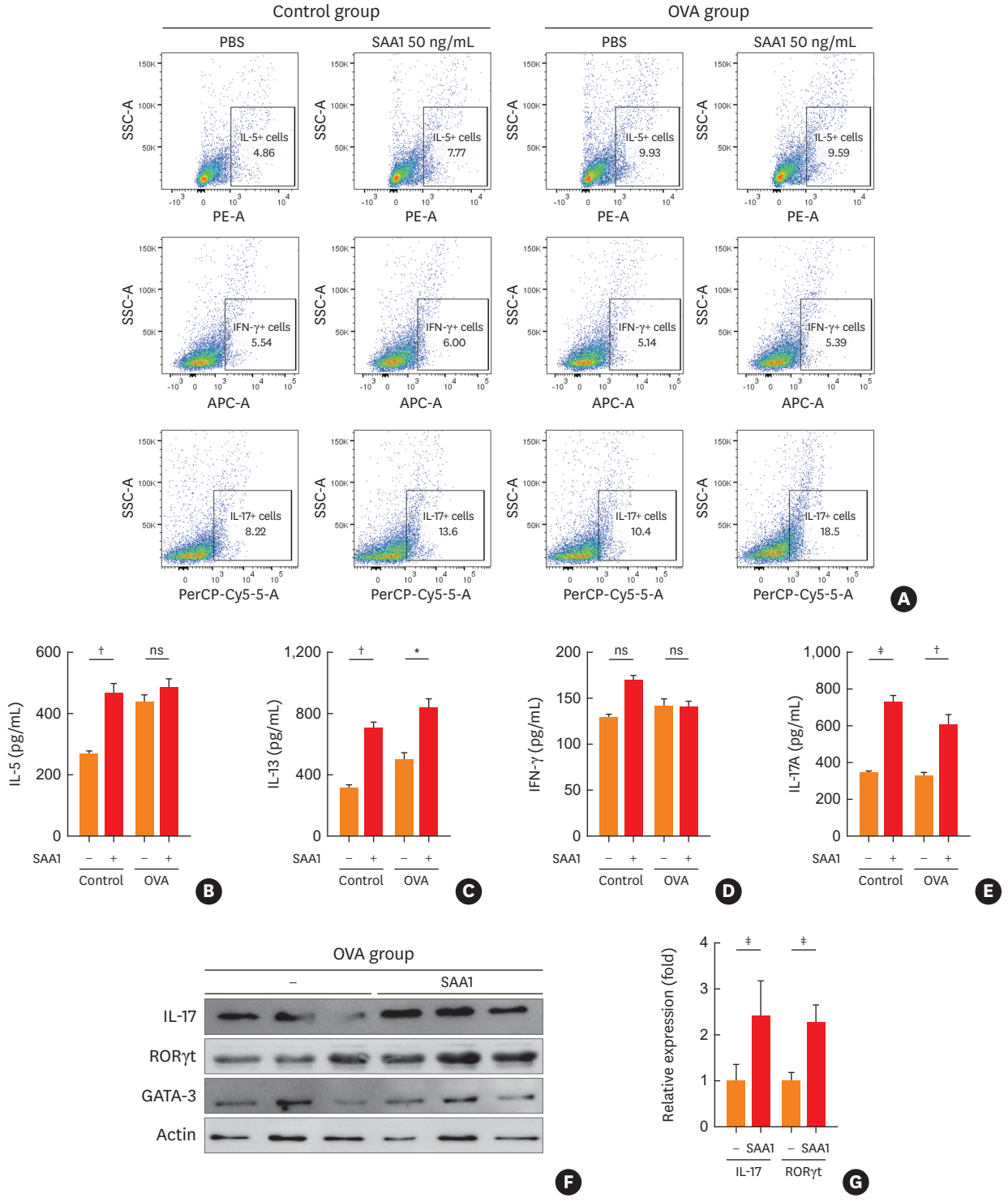


**Fig. 6.** SAA1-enhanced Th2/Th17 cytokine release from splenic CD4+ T cells of allergic asthma model. To examine the effect of SAA1 on T cell-derived cytokines in the OVA group compared to the control group, their CD4+ T cells were stimulated with or without mouse recombinant SAA1 in the presence of anti-CD3/anti-CD28 antibodies. Flow cytometry analysis of intracellular IL-5, INF-γ and IL-17 staining in activated CD4+ T cells (A). The releases of IL-5 (B), IL-13 (C), INF-γ (D) and IL-17A (E) from CD4+ T cells in response to SAA1 were noted. Data are presented as means ± standard error of mean (n = 6) per group. *P* values were calculated by using one-way analysis of variance with Bonferroni's *post hoc* test. The expression of IL-17, RORγt and GATA-3 in the lung tissues of asthma mouse models as detected via western blotting (F), the relative expression of protein/actin (G, fold change).
IL, interleukin; SAA1, serum amyloid A1; INF-γ, interferon gamma; OVA, ovalbumin; RORγt, retinoic acid receptor-related orphan receptor γt; GATA-3, GATA binding protein 3.
$^{*}P < 0.050$, $^{\dagger}P < 0.010$, $^{\ddagger}P < 0.001$, ns = not significant.


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## DISCUSSION

The study demonstrates a significant association of high SAA1 levels with neutrophilic airway inflammation and lower lung function in adult asthmatics. The serum SAA1 levels were higher in patients with NA compared to those with non-NA; high-SAA1 group had significantly higher levels of serum S100A9/IL-6/sputum neutrophil counts and prevalence of SA as well as lower FEV1% than their counterparts, indicating that adult asthmatics having higher SAA1 levels could present neutrophilic airway inflammation, lower lung function, and poor clinical outcomes.

AECs are the first-line affected cells in response to external stimuli (allergens, pollution, viruses, *etc*.) and endogenous cytokines (IL-1β, IL-6), driving airway inflammation.[33] Respiratory viruses are the most common cause of acute exacerbation in patients with asthma.[34] Virus-induced airway inflammation is characterized by numerous alarmin production and neutrophil inflammation as well as activation of Th2/group 2 innate lymphoid cells.[35] Regarding the source of SAA1, the present study showed that Poly I-C, representing a viral infection (neither bacteria nor HDM), is a major triggering factor for inducing SAA1 production from AECs. Moreover, the production of S100A9 and IL-6/IL-8 were induced by SAA1 stimulation in AECs, which was further increased after Poly I-C exposure, while these responses could not be suppressed by corticosteroid treatment. S100A9 released from AECs is known as a key mediator for neutrophilic airway inflammation in asthma via inducing IL-1β, IL-17 and INF-γ productions.[36] Higher serum levels of S100A9 are noted in wheat flour-exposed workers with significant correlations with serum MPO and IL-8 levels.[37,38] Moreover, IL-6 and IL-8 (major epithelial cytokines) could promote neutrophil activation and recruitment to the lungs, subsequently inducing epithelial dysfunction.[39,40] Taken together, respiratory viral infection plays a role in SAA1 production from AECs, leading to neutrophilic airway inflammation via producing S100A9, IL-6, and IL-8 in asthmatic airway, which may not be suppressed by steroid administration.

Increased sputum/blood neutrophil counts were higher in patients with SA compared to those with non-SA, and those with neutrophilic airway inflammation did not respond to conventional anti-inflammatory medications including inhaled corticosteroids.[41] The present study demonstrated that SAA1 could activate PBNs via increasing ROS production and PBN migration in asthmatics. In addition, SAA1 induced the phosphorylation of ERK and p38 MAPK in PBNs (derived from asthmatics). ROS-induced activation of ERK and p38 MAPK (protein arginine deiminase 4 dependent mechanism) has been reported to mediate NET release from human neutrophils.[42] SAA1 is known as a pro-inflammatory ligand of formyl peptide receptor 2 (FPR2) and Toll-like receptors (TLR-2, -4),[12] while ROS production through FPR2 (decreased nuclear factor erythroid 2–related factor 2 [Nrf2] expression) and TLR-2/-4 (MAPK/ERK activation) has been reported.[42,43] Moreover, attenuated Nrf2 expression relates to steroid resistance.[44,45] Regarding the role of NETs in asthma, NET-forming neutrophil counts were significantly higher in patients with SA than with non-SA.[28] In addition, NET induced epithelial dysfunction, eosinophil degranulation, and neutrophil activation, suggesting that NET formation may be a driving mechanism of type 2/eosinophilic airway inflammation and neutrophilic inflammation, contributing to steroid-resistance in patients with NA.[28] Taken together, SAA1 could enhance neutrophilic airway inflammation via neutrophil activation along with NET formation through the ROS-dependent manner, contributing to steroid resistance and progression to SA.


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Along with neutrophil activation, the effect of SAA1 on macrophage-mediated airway inflammation was evaluated in the present study, suggesting that macrophages play an important role in chronic airway inflammation and remodeling in asthma pathogenesis.[23,46] In the present study, macrophage-derived proinflammatory cytokines and chemokine as well as M1 markers (iNOS, CD86), and macrophage maturation marker (CD68) were evaluated.[47] SAA1 served as an immune mediator to induce the production of pro-inflammatory cytokines and chemokine (IL-1β, IL-6, TNF-α, TGF-β1 and IL-8) from M0 macrophages, and could stimulate the differentiation process of M0 macrophages shifting to M1 (iNOS, CD86) phenotype. TGF-β1 has been reported to enhance goblet cell proliferation, to regulate AEC adhesion, and to induce fibroblast proliferation, promoting airway remodeling in asthma.[48] Moreover, human monocytes and monocytes-derived macrophages express SAA1 gene in an inflammatory milieu, especially after M1 differentiation.[49] These findings suggest that SAA1 may contribute to persistent neutrophilic airway inflammation via shifting toward M1 macrophages and TGF-β1- mediated airway modeling.

The present study investigated the mechanisms underlying SAA1-induced neutrophilic inflammation in *in vivo* asthma models. Increased SAA1 levels were noted in the allergic asthma model which were further increased by viral infection with increased production of IL-5, IL-17 and IL-33 (not IFN-γ). The highest SAA1 levels were noted in the NA model, followed by MA, although SAA1 levels were slightly increased in the EA model. Moreover, SAA1 up-regulated IL-13/IL-17 release and IL-17/RORγt expression from activated CD4+ T cells in the allergic asthma mice. A recent study showed that SAA1 induces IL-17 production from the splenocytes of naive mice (C57BL/6).[50] In addition, RORγt is well known in Th17 differentiation; however, recently, it is required for optimal Th2 cell differentiation through suppressing B cell lymphoma 6 (Bcl6) expression.[51] These findings suggest that SAA1 may serve as a factor for inducing Th2/Th17 subtype of asthma (MA type) following virus infections. Therefore, SAA1 may be a new therapeutic target for better control of airway inflammation in NA or MA.

The present study has 2 limitations. One is that SAA1 levels were measured in the sera of adult asthmatics, not confirmed in airway secretion. The other is that monocytes and neutrophils were isolated from the peripheral blood of asthmatics, not from sputum or airway mucosa. Therefore, the SAA1 level will not be able to represent each phenotype of airway inflammation. However, the effects of SAA1 were evaluated in *in vitro* settings, including AECs, neutrophils and macrophages, as well as *in vivo* settings, including various mouse models of asthma.

In conclusion, SAA1 triggered by viral infection is an endogenous danger to induce neutrophilic airway inflammation, leading to present NA or MA phenotype via stimulating pro-inflammatory cytokine production from AECs, neutrophil activation/NET formation and macrophages differentiating to M1.

## ACNOWLEDGEMENTS

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## SUPPLEMENTARY MATERIALS

### Supplementary Fig. S1

Experimental design. 100 μg/mouse Poly I-C was administered intranasally to mice either for 5 days or prior to OVA sensitization/challenge to induce the Poly I-C -infected asthma mouse model (A). Mouse models of each asthma phenotype (e.g., eosinophilic asthma [EA], neutrophilic asthma [NA] and mixed granulocytic asthma [MA]). On days 0 and 7, mice were intraperitoneally sensitized with 10 μg of OVA in aluminum hydroxide solution. From days 14 to 17, the mice were challenged with 6% OVA for 30 minutes using an ultrasonic nebulizer for the EA group (B). To establish the mouse models of the NA (C) and MA groups (D), mice were intranasally administered LPS. The MA group received 0.1 μg of LPS on day 15, while the NA group received 10 μg of LPS from days 15 to 17. The mice were challenged with 6% OVA for 30 minutes using an ultrasonic nebulizer 30 minutes after LPS administration.

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### Supplementary Fig. S2

Effect of SAA1 on Th2 cytokine release from the Jurkat T cell line. Jurkat cells ($2 \times 10^5$ cells) were seeded onto a 24-well plate and stimulated with 10 ng/mL PMA, followed by stimulation of 50 ng/mL SAA1 for 24 hours. Supernatants were harvested for T helper (Th) cytokine quantification by ELISA, including IL-2 (A), IL-5 (B), IL-13 (C) and IL-10 (D). Data are presented as means ± SEM, n = 6 per group. *P* values were calculated by using one-way ANOVA with Bonferroni's post hoc test.

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### Supplementary Fig. S3

SAA1-induced proinflammatory cytokine and chemokine release from Mo macrophages in asthmatics. SAA1-induced production of IL-1β (A), IL-6 (B), TNF-α (C), IL-8 (D) and TGF-β1 (E) from M0 macrophages in a time-dependent manner (24, 48 and 72 hours). Data are presented as means ± SEM, n = 6. *P* values were calculated by using one-way ANOVA with Bonferroni's post hoc test. Increased expression of iNOS from M0 macrophages in response to SAA1 in a time-dependent manner (F).

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