

DR. SEUNG-HYUN KIM (Orcid ID : 0000-0003-1789-8964)

DR. GA YOUNG BAN (Orcid ID : 0000-0002-7961-742X)

PROF. HAE-SIM PARK (Orcid ID : 0000-0003-2614-0303)

Article type : Original Article: Asthma and Lower Airway Disease

Ceramide/sphingosine-1-phosphate imbalance is associated with distinct inflammatory phenotypes of uncontrolled asthma

Seung-Hyun Kim^{1,2 *}, Hae-Won Jung², Minji Kim^{1,2}, Ji-Young Moon², Ga-Young Ban^{2,3}, Su Jung Kim⁴, Hyun-Ju Yoo⁴, Hae-Sim Park^{2*}

¹Translational Research Laboratory for Inflammatory Disease, Clinical Trial Center, Ajou University Medical Center, Suwon; ²Department of Allergy and Clinical Immunology, Ajou University School of Medicine, Suwon; ³Department of Pulmonary, Allergy, and Critical Care Medicine, Kangdong Sacred Heart Hospital, Hallym University College of Medicine Institute for Life Sciences; ⁴Asan Institute for Life Sciences, Asan Medical Center, University of Ulsan College of Medicine, Seoul, South Korea.

Running title: Ceramide/S1P imbalance in uncontrolled asthma

Funding sources: This research was supported by the Basic Science Research Program of the National Research Foundation (NRF) grant funded by the Korea government (NRF-2018R1A2B6004905) and the Korea Health Technology R&D Project through the Korea Health Industry Development Institute (KHIDI) grant funded by the Ministry of Health and Welfare, Republic of Korea (Grant no. HI16C0992).

Key words: Asthma; ceramide; neutrophil; platelet-adherent eosinophil; sphingosine-1-phosphate

This article has been accepted for publication and undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process, which may lead to differences between this version and the [Version of Record](#). Please cite this article as [doi: 10.1111/ALL.14236](https://doi.org/10.1111/ALL.14236)

This article is protected by copyright. All rights reserved

***Corresponding author:**

Seung-Hyun Kim, PhD

Translational Research Laboratory for Inflammatory Disease, Clinical Trial Center, Ajou University
Medical Center, Suwon, South Korea.

Tel: +82-31-219-4264; Fax: 82-31-219-4265

E-mail: kimsh@ajou.ac.kr

Hae-Sim Park, MD, PhD

Department of Allergy & Clinical Immunology, Ajou University

School of Medicine, Suwon, South Korea.

Tel: +82-31-219-5150; Fax: +82-31-219-5154

E-mail: hspark@ajou.ac.kr

Abstract

Background: Asthma is associated with inflammatory dysregulation, but the underlying metabolic signatures are unclear. This study aimed to classify asthma inflammatory phenotypes based on cellular and metabolic features.

Methods: To determine cellular and metabolic profiles, we assessed inflammatory cell markers using flow cytometry, sphingolipid (SL) metabolites using LC-MS/MS, and serum cytokines using ELISA. Targeted gene polymorphisms were determined to identify genetic predispositions related to the asthma inflammatory phenotype.

Results: In total, 137 asthmatics and 20 healthy controls (HCs) were enrolled. Distinct cellular and metabolic profiles were found between them; asthmatic patients showed increased expressions of inflammatory cell markers and higher levels of SL metabolites compared to HCs ($P<0.05$ for all). Cellular markers (CD66⁺ neutrophils, platelet-adherent eosinophils) and SL metabolic markers (C16:0 and C24:0 ceramides) for uncontrolled asthma were also identified; higher levels were observed in uncontrolled asthma compared to controlled asthma ($P<0.05$ for all). Asthmatics with higher levels of CD66⁺ neutrophils had lower FEV1(%), higher ACQ (but lower AQLO) scores, and higher sphingosine and C16:0 ceramide levels compared to those with low levels of CD66⁺ neutrophils. Asthmatics with higher levels of platelet-adherent eosinophils had higher S1P levels compared to those with lower levels of platelet-adherent eosinophils. Patients carrying TT genotype of *ORMDL3* had more CD66⁺ neutrophils; those with AG/ GG genotypes of *SGMS1* exhibited higher platelet-adherent eosinophils.

Conclusion: Uncontrolled asthmatics possess distinct inflammatory phenotypes including increased CD66⁺ neutrophils and platelet-adherent eosinophils, with an imbalanced ceramide/S1P rheostat, potentially involving *ORMDL3* and *SGMS1* gene polymorphisms. Ceramide/S1P synthesis could be targeted to control airway inflammation.

Introduction

Asthma is a common and chronic inflammatory disease with reversible airway obstruction and hyper-responsiveness with a considerable financial burden ¹. Given the heterogeneity of asthma phenotypes with distinct pathological and inflammatory mechanisms/endotypes, patients exhibit differences in severity, disease control, and response to treatment ².

Heterogeneous airway inflammation involving complex pathological mechanisms has been composed in the severe type of asthma. T helper (Th) 2-dominant eosinophilic inflammation has been considered as the main target in severe asthma ³, neutrophilic inflammation and/or mixed airway granulocytic inflammation has also been noted in severe and poorly controlled asthma ^{4,5}. Neutrophils contain cytotoxic granules and release various cytokines, chemokines, and lipid mediators, that contribute to persistent airway inflammation, even with high-dose glucocorticoid treatment ⁶. Furthermore, aberrant neutrophil processes such as autophagy and the neutrophil extracellular DNA trap could enhance the inflammatory responses of human airway epithelial cells in severe asthma ⁷. Recent work reported that activated platelets could induce the release of numerous inflammatory mediators ⁸, recruit eosinophils, form platelet–eosinophil complex, and finally exacerbate eosinophilic airway inflammation ^{9,10}. A multifactorial adaptive immune response (Th1/Th2/Th17) also contributes to the heterogeneous inflammatory phenotype of asthma, which allows asthmatics to be classified into Th2-predominant, Th2/Th17-predominant, and Th2/Th17-low groups with different inflammatory mechanisms ¹¹. In addition, an epithelial derived cytokine-mediated innate immune response could exacerbate airway inflammation ¹².

Persistent airway inflammation caused by immune dysfunction of various types of inflammatory cells is suspected to be the main contributing factor to poor disease control, however, the underlying cellular and molecular features have not yet been fully examined. Precise definition of pathophysiologic endotypes across asthma phenotypes is necessary to better understand the heterogeneity and to facilitate targeted therapies for the most effective therapy ¹³. Therefore, examination of inflammatory immune cells in blood would be valuable in defining the inflammatory phenotypes of asthma.

In recent decades, the importance of sphingolipids (SLs) in the pathophysiology of asthma has been highlighted. The orosomucoid-like 3 (*ORMDL3*) gene has been strongly linked to the genetic susceptibility of childhood ¹⁴ and adult-onset asthma in a number of ethnically diverse populations ¹⁵. Due to its inhibitory action on serine palmitoyl transferase (SPT)¹⁶, which is the rate-limiting enzyme in SL biosynthesis, impaired SL synthesis has been shown to be associated with methacholine-induced airway hyper-reactivity ¹⁷. Bioactive SLs are regulated by a set of enzymes through which *de novo* synthesis, catabolism, and recycling of the lipids and their combined action allows complex SL metabolites, which is further complicated by the action of additional elongase, hydroxylase and desaturase ¹⁸. Several

metabolomic studies on asthma have addressed SL metabolic changes according to asthma phenotype¹⁹⁻²², in which genetic variants were closely associated with metabolism¹⁹. Thus, it is of great clinical relevance to search for metabolic signatures discriminating a certain type of asthma and the underlying immunological inflammation and clinical outcomes.

In this study, we hypothesized that immune dysfunction of various types of inflammatory cells could contribute to persistent airway inflammation, leading to asthma exacerbation, and that metabolic changes may be involved. To examine any immune dysfunction at the cellular level, we performed flow cytometry analyses on peripheral blood inflammatory cells such as basophils, eosinophils, neutrophils, and platelets. Metabolic changes at the molecular level was also determined by assessing SL metabolite levels. Finally, we analyzed the cellular and metabolic profiles by integrating the clinical features, disease severity, symptom control status, and genetic predisposing factors in asthmatics.

Materials and methods

Study subjects and collection of clinical data

This study enrolled 137 asthmatic patients and 20 healthy controls (HCs) from Ajou University Hospital (Suwon, Korea). Written informed consent was obtained from each subject and the study was approved by the Institutional Review Board of Ajou University Hospital (AJIRB-GEN-SMP-13-108, AJIRB-BMR-SUR-15-498).

Comprehensive phenotypic characterization of asthmatic patients was performed as follows; complete blood count with cell differential, spontaneous sputum cell counts, skin prick test, serum total IgE measurements, fractional exhaled nitric oxide (FeNO) measurements, and spirometry for measuring forced expiratory volume in 1 second (FEV1) percent, forced vital capacity (FVC) percent, FEV1/FVC ratio, and maximal mid-expiratory flow (MMEF) percent predicted values.

Atopy was defined as one or more positive reactions on the skin prick test²³. Serum total IgE levels were measured using the ImmunoCAP system (ThermoFisher Scientific, Waltham, MA, USA). FeNO was measured with a NIOXTM analyzer (Aerocrine AB, Solna, Sweden) in accordance with international guidelines²⁴.

Asthma was diagnosed based on recurrent episodes of wheezing, dyspnea, cough, and evidence of airway hyper-responsiveness to methacholine (a decrease in FEV1 percent of 20% on methacholine challenge test [PC20])²⁵. All the study subjects had maintained the same dose of anti-asthmatic medications including medium dose of inhaled corticosteroid-long-acting beta agonist (ICS-LABA) such as budesonide-formoterol or fluticasone-salmeterol with/without leukotriene receptor antagonist (LTRA) such

as montelukast or pranlukast according to the control status for more than 1 month prior to the enrolment date; the patients who had used high dose of ICS-LABA and systemic oral corticosteroid (OCS) were excluded. Asthma control status was defined according to the Global Initiative for Asthma (GINA) guidelines²⁶ using a composite measure comprising daytime symptoms, the restriction of daily activities, night-time symptoms, and the need for reliever/rescue treatment; controlled, none of the all features, partly controlled, one or two features present; uncontrolled, three or more features present in any week. In addition, as objective measures, patients completed extensive questionnaires such as Asthma Control Test (ACT)²⁷, Asthma Control Questionnaire (ACQ)²⁸ and Asthma Quality of Life Questionnaire (AQLQ) to measure their control status. Lower ACT and AQLQ scores indicate uncontrolled symptom scores, while ACQ indicate well-controlled. Clinical and demographic characteristics of the study subjects are presented in Table 1.

Flow cytometric analysis for immunophenotyping

Immunophenotyping was performed within 1 hour after blood sampling by identifying circulating immune cells using cell type-specific selection markers and activation markers. The following antibody panels were used for whole blood flow cytometry analysis (Table S1): a) CD63⁺ activated basophils, b) CD63⁺ and P-selectin⁺ activated platelets, c) Siglec8⁺ eosinophils and platelet-adherent eosinophils (CD61⁺ platelet on Siglec8⁺ eosinophils), d) CD66⁺ activated neutrophils. Whole blood was incubated with cell type specific antibodies for 20 minutes at room temperature in the dark, lysed with RBC Lysis/Fixation solution (Biolegend, San Diego, CA, USA) for 10 min, washed with 1× phosphate-buffered saline, and then analyzed immediately by flow cytometry. All monoclonal antibodies were purchased from BD Biosciences (San Jose, CA, USA) and Biolegend. Details of antibody panels that used for immunophenotyping are shown in Table S1 and S2. Gating strategies for flow cytometry analysis are shown in Figure S1. Samples were processed on a BD FACS Canto II and data were analyzed with FlowJo software (Tree Star, Ashland, OR, USA).

Measurement of serum SL metabolites using liquid chromatography-tandem mass spectrometry (LC-MS/MS)

Sera were separated using a BD Vacutainer® SST tube according to the manufacturer's instructions and stored at -20°C until analysis. Human serum was well mixed and internal standard solutions (1 μM of C17 sphingosine-1-phosphate [S1P] for S1P, 500 nM of C17 ceramide for other SLs) were added to the samples before extraction. SLs were extracted using the Bligh and Dayer method²⁹. After lipid extraction, organic solutions containing lipids were dried using a vacuum centrifuge and stored at -20°C until LC-MS/MS analysis. Dried matter was reconstituted with methanol and injected into the LC-MS/MS system.

All lipid standards including internal standards were purchased from Avanti-Polar Lipids (Alabaster, AL, USA). Lipid levels were determined using an LC-MS/MS system equipped with a 1290 HPLC (Agilent, Waldbronn, Germany) and QTRAP 5500 (AB Sciex, Toronto, Canada). Multiple reaction monitoring (MRM) was performed in the positive ion mode and the extracted ion chromatogram corresponding to the specific transition for each lipid was used for quantification. Data analysis was performed using Analyst 1.5.2 software.

Serum cytokine analysis

The levels of tissue inhibitor of metalloproteinase (TIMP1), matrix metalloproteinase 9 (MMP9), myeloperoxidase (MPO), and transforming growth factor beta 1 (TGFβ1) were measured from the sera of asthmatic patients using enzyme-linked immunosorbent assay (ELISA) according to the manufacturer's instructions (R&D systems, Minneapolis, MN, USA). Serum samples were collected, frozen at -70°C and thawed before use.

Transcriptome gene expression profiling

Transcriptome expression profiling was performed using whole blood samples from 18 randomly selected asthmatic patients using the Affymetrix Human Gene ST 2.0 chip (Affymetrix, Santa Clara, CA, USA). Details are shown in the supplementary methods section.

Single nucleotide polymorphism (SNP) genotyping

Four SNPs of the genes (*ORMDL3*, *SPTLC2*, *SPTLC3*, *SGMS1*, and *CERS2*) encoding proteins that are responsible for *de novo* ceramide synthesis or ceramide metabolism were genotyped. Genotyping was determined by a primer extension method, which was performed with the SNaPshot ddNTP primer extension kit from Applied Biosystems (Foster City, CA, USA). The sequences of the amplifying and extension primers are shown in Table S3. Each SNP did not deviate from the Hardy-Weinberg equilibrium, as tested by a chi-square goodness-of-fit test.

Statistical analysis

Independent *t*-test, Fisher's exact test, one-way analysis of variance (ANOVA), *post-hoc* test, and Pearson's correlation test were applied. A *P*-value ≤ 0.05 was regarded as significant. Statistical analyses were performed using SPSS version 20.0 (IBM SPSS Statistics, IBM Corporation, Chicago, IL, USA). GraphPad Prism 5.0 software (GraphPad Inc., San Diego, CA, USA) was used to produce graphs.

Results

Immunological and metabolic changes in asthma

Overall, 137 asthmatic patients and 20 HCs were recruited for this study. The demographics of the study subjects are presented in Table 1. There were no significant differences in female sex and age between the asthma and HC groups. Asthmatic patients had a significantly higher atopy rate, serum total IgE levels, peripheral eosinophil count, and FeNO levels than HCs ($P<0.05$ for all).

Among inflammatory cell markers, CD63⁺ basophils, Siglec8⁺ eosinophils, CD66⁺ neutrophils, activated platelets (P-selectin⁺ platelets, CD63⁺ platelets), and platelet-adherent eosinophils (CD61⁺platelets/ Siglec8⁺eosinophils) were significantly higher in asthmatics than in HCs ($P<0.05$ for all, Figure 1).

Targeted metabolomic analysis of SLs showed that levels of sphingosine and total ceramides were increased significantly in asthmatics compared to HCs ($P<0.001$ and $P=0.008$, respectively), whereas there were no significant differences in the levels of S1P or total sphingomyelins (Table 1). For individual SL metabolites, the levels of ceramide (C16:0, C18:0, C18:1, C24:0, and C24:1 ceramides) were significantly higher in asthmatics compared to HCs ($P<0.05$ for all), however, there were no significant differences in sphingomyelins between groups (Table S1). C16:0 and C24:0 ceramides were the most abundant serum ceramide species in this study cohort. Furthermore, separate classifications designed to examine ceramide metabolism (5 ratios), desaturation (2 ratios), and elongation (4 ratios) were also analyzed. Significant metabolic changes in ceramide, the sphingomyelin/ceramide ratio, was noted in asthmatics compared to HCs (SM18:0/Cer18:0, SM24:0/Cer24:0, and SM24:1/Cer24:1, $P<0.05$ for all). However, there were no significant changes in ceramide elongation and desaturation between the 2 study groups (Table S4).

Immunological and metabolic changes according to asthma control status

Patients were classified according to their symptom control status using the Global Initiative for Asthma (GINA) guidelines: controlled, partly controlled, or uncontrolled asthma. When clinical characteristics are compared according to their control status, significant differences were noted in lung function parameters (FEV1%, FEV1/FVC, MMEF%) and ACT/ACQ/AQLQ scores. In addition, for inflammatory cell markers, the percentage of CD66⁺ neutrophils and platelet-adherent eosinophils was significantly different according to asthma control status; the percentage of CD66⁺ neutrophils was significantly higher in patients with uncontrolled asthma, while the percentage of platelet-adherent eosinophils was significantly higher in patients with partly controlled than in those with controlled asthma ($P<0.05$ for all, Figure 2). Among SL metabolites, the levels of C16:0 and C24:0 ceramides were significantly different according to asthma control status; C16:0 ceramide was significantly higher in

patients with partly controlled asthma than in those with controlled asthma ($P<0.05$). However, there was no difference in the sphingomyelin/ceramide ratio according to asthma control status (Table 2).

Association of CD66⁺ neutrophils with lung function parameters and ceramide levels

When asthmatic patients were stratified according to the level of CD66⁺ neutrophils or platelet-adherent eosinophils, distinct clinical, metabolic, and serologic features were found (Table 3). Asthmatics with higher levels of CD66⁺ neutrophils (greater than 90% of HCs) were older, less atopic, had lower baseline FEV1 % predicted and FEV1/FVC ratio, and higher sphingosine and C16:0 ceramide levels compared to those with lower levels of CD66⁺ neutrophils. For inflammatory cell markers, asthmatics with higher levels of CD66⁺ neutrophils had lower percentages of CD63⁺ basophils and activated platelets (CD63⁺CD61⁺ and P-selectin⁺CD61⁺ platelets) than those with lower levels of CD66⁺ neutrophils. Serum levels of MMP9, TIMP1, MPO, and TGFβ1 were significantly higher in asthmatics with higher levels of CD66⁺ neutrophils than those with low levels ($P<0.05$ for all). The SM16:0/Cer16:0 and SM24:0/Cer24:0 ratios were significantly different according to CD66⁺ neutrophil levels ($P<0.05$ for all).

The percentage of CD66⁺ neutrophils was significantly associated with lung function parameters (baseline FEV1 % predicted, FVC % predicted, FEV1/FVC ratio, MMEF % predicted values) and asthma symptom and quality of life scores (ACQ, and AQLQ), was also correlated with serum inflammatory cytokine levels such as MMP9, TIMP1, MPO, and TGFβ1 (Table S5). In addition, the percentage of CD66⁺ neutrophils were positively correlated with reactive oxygen species (ROS) production from peripheral blood neutrophils both at baseline and after formyl methionyl-leucyl-phenylalanine (fMLP) stimulation. In addition, significant correlations were noted between the percentage of CD66⁺ neutrophils and the levels of sphingosine and C16:0 ceramide as well as between the percentage of CD66⁺ neutrophils and the SM16:0/Cer16:0 and SM24:0/Cer24:0 ratios ($P<0.05$ for all).

Association of platelet-adherent eosinophils with S1P levels and asthma control symptom scores

Asthmatics with higher levels of platelet-adherent eosinophils (greater than 90% of HCs) had more activated platelets (CD63⁺CD61⁺ platelets) and higher S1P levels compared to those with lower levels of platelet-adherent eosinophils. There were no significant differences in ceramide levels according to platelet-adherent eosinophils (Table 3).

The percentage of platelet-adherent eosinophils was associated with asthma symptom and quality of life scores (ACT, ACQ, and AQLQ) in positive correlations with the levels of TIMP1 and TGFβ1 (Table S5). It was also positively correlated with the serum levels of S1P and sphingosine.

Ceramide-mediated neutrophilic inflammation in patients with uncontrolled asthma

To assess the role of ceramide-mediated signaling in neutrophil-dominant asthma, the expression

profiles of genes involved in the SL signaling pathway were examined using whole blood transcriptome data (Supplementary Method). Patients with high levels of CD66⁺ neutrophils exhibited increased expression of genes (*SIPR1*, *SIPR2*, *SIPR4*) encoding S1P receptors and *CERS5* encoding ceramide synthase 5 (Figure S2). The expression of *BID* and *JNK* genes encoding the Bcl-2 family protein, BH3 interacting domain death agonist and c-Jun N-terminal kinase, respectively, was also increased.

Association between gene polymorphisms involved in SL metabolism and inflammatory phenotypes in uncontrolled asthma

To examine dysfunction in inflammatory cells and metabolic changes according to genetic predisposition, 4 SNPs involved in *de novo* SL synthesis and metabolism were genotyped (Table S3). Among the target SNPs investigated, distinct inflammatory phenotypes were found according to *ORMDL3* and *SGMS1* gene polymorphisms (Table 4). Asthmatic patients carrying the TT genotype of *ORMDL3* rs7216389 had higher percentages of CD66⁺ neutrophils, higher levels of inflammatory cytokines (MMP9, MPO, and TGFβ1), higher sphingosine levels, and lower SM16:0/Cer16:0 ratio compared to those with CT or CC genotypes ($P < 0.05$ for all). Asthmatic patients carrying the AG or GG genotype of *SGMS1* rs10740762 exhibited higher percentages of peripheral eosinophils and platelet-adherent eosinophils compared to those with AA genotypes ($P < 0.05$ for all).

Discussion

As asthma shows heterogeneous and complicated endotypes with different immunologic and metabolic dysfunctions, carefully defining the endotype of asthma is necessary to understand the complex pathophysiology and to achieve better clinical outcomes in the management of asthmatics^{2,3}. In the present study, we examined cell surface activation markers of circulating inflammatory cells and SL metabolic changes to define immunologic and metabolic dysfunction across different asthma phenotype. Moreover, we used a combinatorial approach by correlating clinical data with disease severity, cellular and molecular profiles, and genetic predisposing factors in asthmatics.

Regarding inflammatory cell markers, asthmatics exhibited increased CD63⁺ basophils, Siglec8⁺ eosinophils, CD66⁺ neutrophils, activated platelets (CD63⁺CD61⁺ and P-selectin⁺ CD61⁺ platelets), and platelet-adherent eosinophils (CD61⁺ platelets/Siglec8⁺ eosinophils) compared to HCs. These results demonstrate that there are continuous immune activations in asthmatics, therefore careful examination on inflammatory cell profiles would be valuable for monitoring control status.

Of interest, among these inflammatory cell markers, the percentage of activated neutrophils and platelet-adherent eosinophils were increased significantly in uncontrolled asthma. However, there were no significant differences in sputum eosinophil or neutrophil counts according to asthma control status. Moreover, sputum cell counts did not correlate with measures of lung function and airway inflammation

markers in asthmatics, which raise concern that these markers may not allow for the evaluation of activated neutrophils or eosinophils in peripheral blood migrating to the lungs and exacerbating airway inflammation. Because the expression of CD66 on neutrophils is required for promoting neutrophil adhesion, phagocytosis, and activation of superoxide production in response to chemotactic stimuli^{30,31}, examination of CD66⁺ neutrophils could identify the activation status of neutrophils, which is supported by the strong correlation of this with lower FEV1(%) and higher FeNO levels in the present study. In addition, the percentage of CD66⁺ neutrophils was associated with asthma control symptom scores and the levels of serum inflammatory cytokine such as MMP9, MPO, TIMP1, and TGFβ1, which are correlated with asthma severity^{32,33}.

Neutrophilic asthma shows persistent airflow limitations and poor disease control in adult asthmatics^{34,35}, despite treatment with high-dose glucocorticoids⁶. A significantly higher percentage of CD66⁺ neutrophils in uncontrolled asthma suggests that activated neutrophils play a functional role in contributing to severity and poor disease control status. Previously, we reported that the *in vitro* release of ROS from activated neutrophils in asthmatics was associated with poor lung function and increased bronchial hyper-reactivity to methacholine³⁶. In the present study, the expression of CD66⁺ on peripheral neutrophils was correlated with ROS production from neutrophils, while peripheral and sputum neutrophil counts were not associated with ROS production from neutrophils. Therefore, these findings suggest that activation markers of neutrophils may efficiently represent the biological function of neutrophils migrating to the lungs and exacerbating airway inflammation. Moreover, the percentage of CD66⁺ neutrophils was positively correlated with circulating ceramide levels, particularly C16:0 ceramide, which raises concern about the role of ceramide in airway inflammation. The gene expression profiles of the SL signaling pathway using whole blood transcriptome data from asthmatics indicated increased *CERS5* expression, which encodes ceramide synthase 5 and mostly synthesizes C16:0 ceramide³⁷, and ceramide mediated pro-apoptotic signals via the activation of pro-apoptotic transcription factors BID and JNK in patients with higher levels of CD66⁺ neutrophils. These findings are predicted to ceramide accumulation and consequent ceramide mediated pro-apoptotic signals in airways. This result is consistent with that of a previous report of ROS-mediated acid sphingomyelinase (aSMase) activation and consequent ceramide accumulation and death receptor clustering in ceramide-rich lipid rafts on neutrophils³⁸.

ROS secreted from activated neutrophils may contribute to the pathogenesis of asthma exacerbation by transferring apoptotic signals to structural epithelial cells, thereby affecting the integrity and inflammation of airway epithelial cells. In a preliminary study, we cultured A549 epithelial cells with cell supernatant from activated peripheral blood neutrophils via fMLP stimulation and observed increased expression of *SMPD1*, which encodes aSMase on A549 cells (data not shown). Since aSMase was implicated as an additional site for ceramide formation³⁹, increased *SMPD1* expression on epithelial cells may contribute to ceramide accumulation and subsequent ceramide-mediated epithelial cell damage.

However, this should be further examined to obtain consistent findings using an *ex vivo* functional study.

There is also a link between neutrophil elastase secreted by neutrophils and bronchial hyper-reactivity ⁴⁰. Neutrophil elastase can upregulate serine palmitoyltransferase long chain base subunit 2 (SPTLC2) via a posttranscriptional mechanism, leading to increased airway ceramide levels, particularly very long-chain ceramides including C22:0, C24:0, and C24:1 ceramides, which ultimately contributes to neutrophil-dominant airway inflammation ⁴¹. Taken together, these results suggest that activated neutrophils may be prone to ceramide signal- favoring persistent inflammation and epithelial damage in asthmatic airways.

Ceramides are generated through the *de novo* synthesis pathway or recycling, referred to as the salvage pathway. Under normal conditions of *de novo* SL synthesis, ceramides are the central intermediate of the SL metabolic pathway that converts ceramides into sphingomyelins via sphingomyelin synthase or into sphingosine via ceramidases. However, in response to various stress stimuli including tumor necrosis factor- α and oxidative stress, abnormal ceramide accumulation has frequently been reported in lung diseases such as acute lung injury, cystic fibrosis, chronic obstructive pulmonary disease ⁴², and allergic asthma ⁴³. C16:0 and C24:0 ceramides were the most abundant ceramide species in the current study cohort, which are the main ceramides found in human lung cells and mouse lung ⁴⁴. Several studies have shown that ceramides with different acyl-chain lengths may play roles in different cellular processes ⁴⁵; however, cellular functions related to specific ceramide species seem to be more complicated. It is often unclear how changes in ceramide composition depend on substrate availability and metabolizing enzymes with posttranscriptional actions ⁴⁶, which is further complicated by the fact that ceramides can be metabolized into complex SLs or sphingosine.

In the present study, the percentage of CD61⁺platelets/Siglec8⁺ eosinophils indicating platelet-adherent eosinophils was significantly increased in partly controlled asthma. Furthermore, it was positively correlated with serum levels of S1P and sphingosine along with associations with the serum levels of TIMP1 and TGF β 1. Patients with high levels of platelet-adherent eosinophils exhibited significantly higher S1P levels than those with low levels of platelet-adherent eosinophils. This result is consistent with those of previous reports that activated platelets, the major source of S1P in plasma, play an important role in the development of asthma by releasing various inflammatory mediators, facilitating granulocyte recruitment into airways in a manner correlated with the severity of airflow obstruction ^{10,47}. Local administration of S1P causes inflammation via eosinophils, which is caused by increased expression of S1P receptors and the chemokine receptor CCR3, which are crucial for the recruitment of eosinophils ⁴⁸. Platelet activation has been linked to not only the exacerbation of asthma but also the pathophysiology of aspirin-exacerbated respiratory disease (AERD), a severe type of eosinophilic asthma with a hypersensitivity to non-steroidal anti-inflammatory drugs ⁴⁹. Patients with AERD have increased numbers of platelet-adherent eosinophils in a manner correlated with urinary LTE4 levels ^{50,51}. Furthermore, S1P and sphingosine have been reported

as metabolic biomarkers for AERD ²¹. This may be supported by the current finding demonstrating a positive correlation between platelet-adherent eosinophils and circulating S1P levels in asthmatics. However, it remains unclear whether the interaction between platelets and eosinophils could lead to the over-production of S1P and severe eosinophilic inflammation. Besides of platelets and eosinophils, mast cells can release S1P upon agonist stimulation. However, there was no any correlation between serum S1P and CD63⁺ activated basophils, even though increased CD63⁺ activated basophils were noted in asthmatics in this study. That might be from relatively low expression levels of CD63 on CD123⁺HLA-DR⁻ basophils in basal status, so further examination on CD63⁺ activated basophils upon agonist stimulation is needed to clarify the association between them.

Since the balance between individual SL species could be influenced by genetic variants of factors involved in SL metabolism, it is important to assess genetic components and predisposing factors that influence inflammatory phenotypes in asthmatics. Among 4 SNPs in the genes (*ORMDL3*, *SPTLC3*, *SGMS1*, and *CERS2*), *ORMDL3* rs716389 and *SGMS1* rs10740762 were associated with distinct inflammatory phenotypes in asthmatics. Patients carrying the TT genotype in the *ORMDL3* gene exhibited many features of the neutrophilic inflammatory phenotype, whereas patients with AG or GG genotypes in the *SGMS1* gene had features of the activated platelet-eosinophil inflammatory phenotype. *ORMDL3* rs716389 has been functionally associated with *ORMDL3* expression that could affect SPT enzyme activity and SL metabolite levels ¹⁴. *SGMS1* rs10740762 has been reported to be associated with the sphingomyelin/ceramide ratio ⁵². Considering its function as a regulator involved in ceramide and sphingomyelin homeostasis, the functional contribution to circulating SL metabolite levels should be further examined.

Taken together, neutrophil activation and platelet-adherent eosinophils closely linked with SL metabolic changes, which could contribute to SL metabolite accumulation (S1P or ceramide) into airway, persist ongoing airway inflammation through further recruitment of inflammatory cells (neutrophils and eosinophils) into airways. Therefore, targeting the SL metabolic pathway could break a link of SL metabolism with airway inflammation. Pharmacological inhibition of SL metabolism has exhibited a promise as a novel therapeutic target for controlling chronic airway inflammation in asthmatics ⁵³. FTY-720, a structural analog of sphingosine, and myriocin, an SPT inhibitor, could attenuate airway hyper-responsiveness and inflammation by antagonizing S1P signaling ^{54,55} and inhibiting ceramide synthesis ^{41,56}. Considering their pleotropic actions on various types of immune cells, careful examination on which inflammatory phenotype could be efficiently controlled by these pharmacological candidates should be beneficial.

The current study suggested that inflammatory phenotypes of uncontrolled asthma were correlated with differential SL metabolic signatures; however, the study had some limitations. First, the number of asthmatic patients was relatively small to dissect asthmatics according to the asthma phenotype,

particularly asthma control status. This is a real-world clinical trial to compare inflammatory profiles and related serologic biomarkers in adult asthmatics who had maintained anti-asthmatic medications according to their control status. Therefore, it is not possible to allocate the even number of patients into 3 study groups. Secondly, although we excluded patients who had used high-dose ICS-LABA and systemic OCS and all asthmatics had maintained the same dose of anti-asthmatic medications, including medium-dose ICS-LABA with/without LTRA, according to the control status more than 1 month prior to enrolment, any effect of current medications on SL metabolite levels could not completely excluded. However, examination on metabolic changes along with inflammatory cell profiles in asthmatics would be valuable in a real-world practice setting. Thirdly, body fluids from local tissues are more valuable in representing inflammatory status in asthmatics. However, it is very difficult to recruit adult asthmatic patients who can voluntarily participate in studies including nasal or bronchoalveolar lavage. Therefore, we could not measure cytokine levels in nasal nor bronchoalveolar lavage fluids. Lastly, we did not show direct evidence of the association between SL metabolic changes and certain types of inflammation using *in vitro/ex vivo* functional studies. Further studies are necessary to define distinct inflammatory mechanisms according to different SL species in asthmatics.

In conclusion, this study demonstrated differential immunologic and metabolic features according to asthma control status. Imbalance of the ceramide-S1P rheostat may contribute to asthma exacerbation and poor disease control by worsening airway inflammation, which may facilitate development of targeted therapeutic strategies for SL metabolism.

Conflicts of interest: The authors declare no competing financial interests.

Author Contributions

Seung-Hyun Kim designed the study, analyzed the data, wrote the manuscript, and supervised all the processes; Hae-won Jung, Minji Kim, and Ji-Young Moon performed the experiments; Ga-Young Ban collected samples; Su Jung Kim and Hyun-Ju Yoo analyzed sphingolipid metabolites using LC-MS/MS; and Hae-Sim Park collected samples and supervised all the processes.

Supporting information

Supplementary tables (Tables S1- S5), figures (Figures S1 – S2), and methods are available in the online version of this manuscript.

REFERENCES

1. Croisant S. Epidemiology of asthma: prevalence and burden of disease. *Advances in experimental medicine and biology*. 2014;795:17-29.
2. Chung KF, Adcock IM. How variability in clinical phenotypes should guide research into disease mechanisms in asthma. *Ann Am Thorac Soc*. 2013;10 Suppl:S109-117.
3. Fajt ML, Wenzel SE. Development of New Therapies for Severe Asthma. *Allergy Asthma Immunol Res*. 2017;9(1):3-14.
4. Louis R, Lau LC, Bron AO, Roldaan AC, Radermecker M, Djukanovic R. The relationship between airways inflammation and asthma severity. *Am J Respir Crit Care Med*. 2000;161(1):9-16.
5. Green RH, Brightling CE, Woltmann G, Parker D, Wardlaw AJ, Pavord ID. Analysis of induced sputum in adults with asthma: identification of subgroup with isolated sputum neutrophilia and poor response to inhaled corticosteroids. *Thorax*. 2002;57(10):875-879.
6. Wenzel SE, Szeffler SJ, Leung DY, Sloan SI, Rex MD, Martin RJ. Bronchoscopic evaluation of severe asthma. Persistent inflammation associated with high dose glucocorticoids. *Am J Respir Crit Care Med*. 1997;156(3 Pt 1):737-743.
7. Pham DL, Ban GY, Kim SH, et al. Neutrophil autophagy and extracellular DNA traps contribute to airway inflammation in severe asthma. *Clin Exp Allergy*. 2017;47(1):57-70.
8. Steinhubl SR. Platelets as mediators of inflammation. *Hematol Oncol Clin North Am*. 2007;21(1):115-121.
9. Takeda T, Unno H, Morita H, et al. Platelets constitutively express IL-33 protein and modulate eosinophilic airway inflammation. *The Journal of allergy and clinical immunology*. 2016;138(5):1395-1403. e1396.
10. Johansson MW, Han ST, Gunderson KA, Busse WW, Jarjour NN, Mosher DF. Platelet activation, P-selectin, and eosinophil beta1-integrin activation in asthma. *Am J Respir Crit Care Med*. 2012;185(5):498-507.

11. Liu W, Liu S, Verma M, et al. Mechanism of TH2/TH17-predominant and neutrophilic TH2/TH17-low subtypes of asthma. *The Journal of allergy and clinical immunology*. 2017;139(5):1548-1558 e1544.
12. Robinson D, Humbert M, Buhl R, et al. Revisiting Type 2-high and Type 2-low airway inflammation in asthma: current knowledge and therapeutic implications. *Clin Exp Allergy*. 2017;47(2):161-175.
13. Lötval J, Akdis CA, Bacharier LB, et al. Asthma endotypes: a new approach to classification of disease entities within the asthma syndrome. *Journal of Allergy and Clinical Immunology*. 2011;127(2):355-360.
14. Moffatt MF, Kabesch M, Liang L, et al. Genetic variants regulating ORMDL3 expression contribute to the risk of childhood asthma. *Nature*. 2007;448(7152):470-473.
15. Zhao CN, Fan Y, Huang JJ, et al. The Association of GSDMB and ORMDL3 Gene Polymorphisms With Asthma: A Meta-Analysis. *Allergy Asthma Immunol Res*. 2015;7(2):175-185.
16. Siow DL, Wattenberg BW. Mammalian ORMDL proteins mediate the feedback response in ceramide biosynthesis. *J Biol Chem*. 2012;287(48):40198-40204.
17. Worgall TS, Veerappan A, Sung B, et al. Impaired sphingolipid synthesis in the respiratory tract induces airway hyperreactivity. *Sci Transl Med*. 2013;5(186):186ra167-186ra167.
18. Hannun YA, Obeid LM. Sphingolipids and their metabolism in physiology and disease. *Nat Rev Mol Cell Biol*. 2018;19(3):175-191.
19. McGeachie MJ, Dahlin A, Qiu W, et al. The metabolomics of asthma control: a promising link between genetics and disease. *Immunity, inflammation and disease*. 2015;3(3):224-238.
20. Reinke SN, Gallart-Ayala H, Gomez C, et al. Metabolomics analysis identifies different metabotypes of asthma severity. *Eur Respir J*. 2017;49(3).
21. Trinh HK, Kim SC, Cho K, et al. Exploration of the Sphingolipid Metabolite, Sphingosine-1-phosphate and Sphingosine, as Novel Biomarkers for Aspirin-exacerbated Respiratory Disease.

- Sci Rep.* 2016;6:36599.
22. Kowal K, Zebrowska E, Chabowski A. Altered Sphingolipid Metabolism Is Associated With Asthma Phenotype in House Dust Mite-Allergic Patients. *Allergy Asthma Immunol Res.* 2019;11(3):330-342.
23. Pham LD, Kim M-A, Yoon MG, Lee SI, Shin YS, Park H-S. Serum specific IgG response to toluene diisocyanate-tissue transglutaminase conjugate in toluene diisocyanate-induced occupational asthmatics. *Annals of Allergy, Asthma & Immunology.* 2014;113(1):48-54.
24. American Thoracic S, European Respiratory S. ATS/ERS recommendations for standardized procedures for the online and offline measurement of exhaled lower respiratory nitric oxide and nasal nitric oxide, 2005. *Am J Respir Crit Care Med.* 2005;171(8):912-930.
25. Ulambayar B, Lee SH, Yang EM, Ye YM, Park HS Association Between Epithelial Cytokines and Clinical Phenotypes of Elderly Asthma . *Allergy Asthma Immunol Res.* 2019 Jan;11(1):79-89.
26. 2019 GINA Report, Global Strategy for Asthma Management and Prevention. Global Initiative for Asthma; 2019. <http://ginasthma.org/>. Accessed April 10th, 2019.
27. Nathan RA, Sorkness CA, Kosinski M, et al. Development of the asthma control test: a survey for assessing asthma control. *The Journal of allergy and clinical immunology.* 2004;113(1):59-65.
28. Barnes PJ, Casale TB, Dahl R, Pavord ID, Wechsler ME. The Asthma Control Questionnaire as a clinical trial endpoint: past experience and recommendations for future use. *Allergy.* 2014;69(9):1119-1140.
29. Bligh EG, Dyer WJ. A rapid method of total lipid extraction and purification. *Can J Biochem Physiol.* 1959;37(8):911-917.
30. Stocks SC, Ruchaud-Sparagano MH, Kerr MA, Grunert F, Haslett C, Dransfield I. CD66: role in the regulation of neutrophil effector function. *Eur J Immunol.* 1996;26(12):2924-2932.
31. Ruchaud-Sparagano MH, Stocks SC, Turley H, Dransfield I. Activation of neutrophil function via CD66: differential effects upon beta 2 integrin mediated adhesion. *Br J Haematol.*

- 1997;98(3):612-620.
32. Cundall M, Sun Y, Miranda C, Trudeau JB, Barnes S, Wenzel SE. Neutrophil-derived matrix metalloproteinase-9 is increased in severe asthma and poorly inhibited by glucocorticoids. *The Journal of allergy and clinical immunology*. 2003;112(6):1064-1071.
33. Al-Alawi M, Hassan T, Chotirmall SH. Transforming growth factor beta and severe asthma: a perfect storm. *Respiratory medicine*. 2014;108(10):1409-1423.
34. Shaw DE, Berry MA, Hargadon B, et al. Association between neutrophilic airway inflammation and airflow limitation in adults with asthma. *Chest*. 2007;132(6):1871-1875.
35. Uddin M, Nong G, Ward J, et al. Prosurvival activity for airway neutrophils in severe asthma. *Thorax*. 2010;65(8):684-689.
36. Kim SH, Uuganbayar U, Trinh HKT, et al. Evaluation of Neutrophil Activation Status According to the Phenotypes of Adult Asthma. *Allergy Asthma Immunol Res*. 2019;11(3):381-393.
37. Levy M, Futerman AH. Mammalian ceramide synthases. *IUBMB Life*. 2010;62(5):347-356.
38. Scheel-Toellner D, Wang K, Craddock R, et al. Reactive oxygen species limit neutrophil life span by activating death receptor signaling. *Blood*. 2004;104(8):2557-2564.
39. Marchesini N, Hannun YA. Acid and neutral sphingomyelinases: roles and mechanisms of regulation. *Biochem Cell Biol*. 2004;82(1):27-44.
40. Stockley RA, Cole PJ. Effects of human neutrophil elastase and *Pseudomonas aeruginosa* proteinases on human respiratory epithelium. *Am J Respir Cell Mol Biol*. 1991;4:26-32.
41. Karandashova S, Kummarapurugu AB, Zheng S, Chalfant CE, Voynow JA. Neutrophil elastase increases airway ceramide levels via upregulation of serine palmitoyltransferase. *Am J Physiol Lung Cell Mol Physiol*. 2018;314(1):L206-L214.
42. Ghidoni R, Caretti A, Signorelli P. Role of Sphingolipids in the Pathobiology of Lung Inflammation. *Mediators Inflamm*. 2015;2015:487508.
43. Oyeniran C, Sturgill JL, Hait NC, et al. Aberrant ORM (yeast)-like protein isoform 3 (ORMDL3)

- expression dysregulates ceramide homeostasis in cells and ceramide exacerbates allergic asthma in mice. *The Journal of allergy and clinical immunology*. 2015;136(4):1035-1046 e1036.
44. Petrache I, Kamocki K, Poirier C, et al. Ceramide synthases expression and role of ceramide synthase-2 in the lung: insight from human lung cells and mouse models. *PLoS One*. 2013;8(5):e62968.
45. Grosch S, Schiffmann S, Geisslinger G. Chain length-specific properties of ceramides. *Prog Lipid Res*. 2012;51(1):50-62.
46. Laviad EL, Kelly S, Merrill AH, Jr., Futerman AH. Modulation of ceramide synthase activity via dimerization. *J Biol Chem*. 2012;287(25):21025-21033.
47. Pitchford SC, Riffo-Vasquez Y, Sousa A, et al. Platelets are necessary for airway wall remodeling in a murine model of chronic allergic inflammation. *Blood*. 2004;103(2):639-647.
48. Roviezzo F, Del Galdo F, Abbate G, et al. Human eosinophil chemotaxis and selective in vivo recruitment by sphingosine 1-phosphate. *Proc Natl Acad Sci U S A*. 2004;101(30):11170-11175.
49. Laidlaw TM, Boyce JA. Aspirin-Exacerbated Respiratory Disease--New Prime Suspects. *The New England journal of medicine*. 2016;374(5):484-488.
50. Laidlaw TM, Kidder MS, Bhattacharyya N, et al. Cysteinyl leukotriene overproduction in aspirin-exacerbated respiratory disease is driven by platelet-adherent leukocytes. *Blood*. 2012;119(16):3790-3798.
51. Mitsui C, Kajiwarra K, Hayashi H, et al. Platelet activation markers overexpressed specifically in patients with aspirin-exacerbated respiratory disease. *The Journal of allergy and clinical immunology*. 2016;137(2):400-411.
52. Hicks AA, Pramstaller PP, Johansson A, et al. Genetic determinants of circulating sphingolipid concentrations in European populations. *PLoS Genet*. 2009;5(10):e1000672.
53. Sun WY, Bonder CS. Sphingolipids: a potential molecular approach to treat allergic inflammation. *Journal of allergy*. 2012;2012:154174.

54. Graler MH, Goetzl EJ. The immunosuppressant FTY720 down-regulates sphingosine 1-phosphate G-protein-coupled receptors. *FASEB journal : official publication of the Federation of American Societies for Experimental Biology*. 2004;18(3):551-553.
55. Sawicka E, Zuany-Amorim C, Manlius C, et al. Inhibition of Th1- and Th2-mediated airway inflammation by the sphingosine 1-phosphate receptor agonist FTY720. *J Immunol*. 2003;171(11):6206-6214.
56. Berdyshev EV, Gorshkova I, Skobeleva A, et al. FTY720 inhibits ceramide synthases and up-regulates dihydrosphingosine 1-phosphate formation in human lung endothelial cells. *J Biol Chem*. 2009;284(9):5467-5477.

Table 1. Characteristics of the study subjects

	HC [†]	Asthma	<i>P</i> -value*
	(N=20)	(N=137)	HC vs. Asthma
Demographics			
Sex (female), no. (%)	13 / 20 (65%)	89 / 137 (64.96%)	1.000
Age (year)	44.55 ± 10.45	50.19 ± 14.8	0.102
Atopy (presence, %)	0/20 (0.00%)	79 / 134 (58.96%)	<0.001
Total serum IgE (KU/L)	27.42 ± 34.3	346.34 ± 440.07	<0.001
Inflammatory subtype			
WBC (x10 ³ /μL)	5.79 ± 1.56	6.8 ± 1.99	0.030
Peripheral eosinophil count (/μL)	155 ± 105.01	312.77 ± 243.61	<0.001
Peripheral eosinophil (%)	2.63 ± 1.68	5.02 ± 4.17	<0.001
Peripheral neutrophil (%)	53.49 ± 10.38	54.28 ± 12.42	0.786
FeNO (ppb)	22.4 ± 11.56	34.35 ± 31.89	0.002
Sputum eosinophils (%)	ND	19.74 ± 30.17	NA
Sputum neutrophils (%)	ND	65.96 ± 34	NA
Pulmonary function			
Baseline FEV1 (% pred.)	106.75 ± 11.75	90.48 ± 17.74	<0.001
Baseline FVC (% pred.)	103.46 ± 9.78	90.86 ± 14.96	<0.001
Baseline FEV1/FVC	87.12 ± 3.75	83.04 ± 8.66	<0.001
Baseline MMEF (% pred.)	102.27 ± 27.1	71.83 ± 28.26	<0.001
Inflammatory cell markers [‡]			
CD63 ⁺ Basophils	0.2 ± 0.15	9.37 ± 19.55	<0.001
Siglec8 ⁺ Eosinophils	5.19 ± 3.05	9.47 ± 10.32	0.028
CD66 ⁺ Neutrophils	40 ± 24.82	49.78 ± 29.35	0.014
CD63 ⁺ Platelets	5.92 ± 2.11	10.66 ± 9.39	<0.001
P-selectin ⁺ Platelets	17.37 ± 5.98	25.66 ± 16.72	<0.001
CD61 ⁺ Platelets/Siglec8 ⁺ Eosinophils	15.43 ± 7.35	23.37 ± 11.22	0.003
SL metabolic markers (ng/ml) [‡]			
S1P	914.25 ± 467.42	1001.21 ± 603.71	0.463
Sphingosine	1.38 ± 0.47	2.34 ± 1.12	<0.001
Ceramides (total)	3290.87 ± 816.28	3870.58 ± 1000.61	0.008
Sphingomyelins (total)	156497.6 ± 26123.72	165060.89 ± 29243.45	0.218

[†] HC, healthy control; N, number; WBC, whole blood cells; ND, not done; NA, not applicable; SL, sphingolipid; S1P, sphingosine-1-phosphate; Cer, ceramide; SM, sphingomyelin.

The continuous data are presented as the means $\pm$ SD. The dichotomous data are presented as numbers (%).

* For continuous variables, independent t-tests were used to test for differences when variances were normal distribution, otherwise nonparametric Mann–Whitney U test. For categorical variables, Fisher’s exact test were used. Bold values indicate significant *P* value. [†] Inflammatory cell markers on peripheral blood cells. [‡] Metabolites were from human serum samples.

Table 2. Immunological and metabolic changes according to asthma control status

	Controlled (C)	Partly controlled(P)	Uncontrolled(U)	<i>P</i> - value *	<i>Post-hoc</i> test significant pairs (<i>P</i> <0.05)
Demographics					
	43 / 70 (61.4%)	37 / 51 (72.5%)	9 / 16 (56.2%)	0.332	
Sex (female), no. (%)					
Age (year)	46.86 $\pm$ 14.8	52.22 $\pm$ 14.29	58.13 $\pm$ 12.88	0.010	(C, U)
	44 / 70 (62.9%)	29 / 50 (58.0%)	9 / 14 (42.9%)	0.376	
Atopy (presence, %)					
	397.04 $\pm$ 524.85	265.22 $\pm$ 287.97	381.47 $\pm$ 415.52	0.266	
Total serum IgE (KU/L)					
Inflammatory cell markers ¶					
CD63 ⁺ Basophils	9.08 $\pm$ 19.46	9.98 $\pm$ 20.02	8.15 $\pm$ 19.32	0.952	
Siglec8 ⁺ Eosinophils	8.2 $\pm$ 5.94	11.5 $\pm$ 15.28	9.58 $\pm$ 8.58	0.266	
CD66 ⁺ Neutrophils	45.46 $\pm$ 27.35	49.61 $\pm$ 29.24	71.53 $\pm$ 31.66	0.009	(C, U)
CD63 ⁺ Platelets	11.09 $\pm$ 10.13	10.39 $\pm$ 8.3	9.36 $\pm$ 9.1	0.800	
P-selectin ⁺ Platelets	26.83 $\pm$ 18.75	25.67 $\pm$ 13.91	19.83 $\pm$ 13.18	0.363	
CD61 ⁺ Platelets/Siglec8 ⁺ Eosinophils	21.12 $\pm$ 6.72	26.09 $\pm$ 11.65	26.35 $\pm$ 21.83	0.043	(C, P)
SL metabolic markers (ng/ml) ‡					
<i>SL concentration</i>					
S1P	923.1 $\pm$ 634.92	1003.26 $\pm$ 760.48	1017.96 $\pm$ 472.66	0.853	
Sphingosine	2.27 $\pm$ 0.92	2.26 $\pm$ 1.21	2.87 $\pm$ 1.49	0.127	
<i>Ceramide (ng/ml)</i>					
Cer14:0	14.73 $\pm$ 3.86	14.58 $\pm$ 3.89	13.85 $\pm$ 2.2	0.696	
Cer16:0	277.4 $\pm$ 53.45	302.63 $\pm$ 49.23	309.01 $\pm$ 48.05	0.012	(C, P)
Cer18:0	142.73 $\pm$ 46.57	143.8 $\pm$ 41.95	137.83 $\pm$ 37.42	0.895	

Cer18:1	23.78 ± 7.53	23.65 ± 6.65	21.31 ± 4.26	0.424
Cer20:0	111.18 ± 45.33	111.26 ± 35.88	100.43 ± 27.54	0.607
Cer24:0	2310.26 ± 700.6	2569.04 ± 614.07	2714.28 ± 860.77	0.042
Cer24:1	807.64 ± 278.06	856.47 ± 258.88	928.82 ± 389.4	0.282
Ceramide metabolism (ratio)				
SM16:0/Cer16:0	238.57 ± 64.4	247.81 ± 69.37	241.13 ± 50.33	0.754
SM18:0/Cer18:0	127.39 ± 32.15	131.21 ± 34.38	138.04 ± 34.35	0.493
SM18:1/Cer18:1	446.34 ± 110.87	471.9 ± 109.83	473.66 ± 91.65	0.393
SM24:0/Cer24:0	9.72 ± 2.55	9.61 ± 2.81	9.54 ± 3.38	0.960
SM24:1/Cer24:1	59.59 ± 17.8	58.6 ± 18.99	61.9 ± 20.5	0.829

† SL, sphingolipid; S1P, sphingosine-1-phosphate; Cer, ceramide; SM, sphingomyelin. The continuous data are presented as the means ± SD.

Asthma control status is defined according to the Global Initiative for Asthma (GINA) guidelines.

* The data among 3 groups were analyzed by ANOVA test with *post-hoc* Dunnett T3 test.

Bold values indicate significant *P* value. Bold characters indicates significant pair within the group comparison.

‡ Inflammatory cell markers on peripheral blood cells. † Metabolites were from human serum samples.

Table 3. Characteristics of asthmatics according to the level of inflammatory cell markers (CD66⁺ neutrophils and platelet-adherent eosinophils).

		CD66 ⁺ Neutrophil		P-value*	CD61 ⁺ Platelets/Siglec8 ⁺ Eosinophils		P-value
		Low	High †		Low	High †	
		(N=91)	(N=34)		(N=81)	(N=44)	
Demographics							
	Sex (female), no. (%)	62 / 91 (68.1%)	21 / 34 (61.8%)	0.528	52 / 81 (64.2%)	31 / 44 (70.5%)	0.554
	Age (year)	48.36 ± 14.96	54.82 ± 13.32	0.029	49.07 ± 14.07	49.96 ± 15.05	0.862
	Atopy (presence, %)	58 / 91 (63.74%)	14 / 34 (41.18%)	0.027	46 / 81 (56.8%)	26 / 44 (59.1%)	0.851
	Total serum IgE (KU/L)	330.64 ± 451.67	324.79 ± 413.94	0.948	274.94 ± 365.43	316.11 ± 523.13	0.867
Inflammatory subtype							
	Peripheral eosinophil (%)	4.52 ± 3	5.99 ± 6.38	0.205	4.7 ± 4.37	4.41 ± 3.27	0.976

	Peripheral neutrophil (%)	54.1 ± 11.99	55.33 ± 14.5	0.633	54.25 ± 12.99	54.42 ± 10.98	0.697
	FeNO (ppb)	30.49 ± 32.66	40.65 ± 26.03	0.106	30.77 ± 26.28	33.89 ± 35.63	0.693
	Sputum eosinophils (%)	23.79 ± 31.49	19.74 ± 30.17	0.599	23.83 ± 31.63	20.26 ± 30.11	0.617
	Sputum neutrophils (%)	66.16 ± 31.12	65.96 ± 34	0.98	66.79 ± 31.33	64.74 ± 33.12	0.725
Pulmonary function							
	Baseline FEV1 (% predicted)	93.68 ± 13.33	83.72 ± 23.74	0.026	94.27 ± 16.95	90.74 ± 18.59	0.397
	Baseline FVC (% predicted)	93.41 ± 11.63	85.57 ± 19.99	0.070	94.25 ± 13.69	90.19 ± 16.63	0.195
	Baseline FEV1/FVC	84.45 ± 7.79	80.27 ± 10.04	0.033	83.64 ± 8.36	84.24 ± 7.99	0.761
	Baseline MMEF (% predicted)	75.38 ± 23.95	64.56 ± 33.46	0.091	78.44 ± 30.55	72.57 ± 25.22	0.367
	PC20 (mg/ml, methacholine)	6.8 ± 8.04	5.83 ± 8.44	0.616	6.19 ± 8.19	7.42 ± 7.96	0.181
Symptom control							
	ACT score	20.8 ± 3.73	19.85 ± 3.99	0.217	20.67 ± 3.33	20.32 ± 4.6	0.768
	ACQ score	4.97 ± 4.44	7.27 ± 6.13	0.024	5.46 ± 4.55	5.82 ± 5.84	0.803
	AQLQ score	171.59 ± 34.47	156.56 ± 45.11	0.049	168.48 ± 34.3	165.7 ± 44.56	0.719
Inflammatory cell markers							
	CD63 ⁺ Basophils	10.11 ± 20.4	1.12 ± 3.66	<0.001	6.91 ± 17.81	10.47 ± 19.5	0.285
	Siglec8 ⁺ Eosinophils	7.52 ± 5.14	12.99 ± 16.94	0.083	9.73 ± 10.28	7.04 ± 8.34	0.013
	CD66 ⁺ Neutrophils	33.38 ± 13.7	93.98 ± 3.93	<0.001	48.83 ± 28.8	47.56 ± 29.38	0.776
	CD63 ⁺ Platelets	11.74 ± 9.11	4.77 ± 5.65	<0.001	9.37 ± 9.17	11.38 ± 8.22	0.049
	P-selectin ⁺ Platelets	26.2 ± 15.82	19.4 ± 15.35	0.081	24.09 ± 17.71	25.44 ± 11.31	0.101
	CD61 ⁺ Platelets/Siglec8 ⁺ Eosinophils	21.9 ± 10.0	23.5 ± 14.0	0.694	17.22 ± 5.42	33.17 ± 12.35	<0.001
Serum biomarkers							
	MMP9	273.56 ± 105.19	330.84 ± 123.01	0.010	279.51 ± 114.12	303.39 ± 106.03	0.304
	TIMP1	134.46 ± 43.02	153.15 ± 52.14	0.040	135.26 ± 44.75	146.72 ± 47.65	0.158
	TGFβ1	37.78 ± 22.17	51.67 ± 17.17	<0.001	38.35 ± 22.16	46.63 ± 20.32	0.059
	MPO	402.87 ± 223.21	599.94 ± 282.77	0.001	434.89 ± 250.51	476.34 ± 253.13	0.366
SL metabolic pathway							
SL concentration (ng/ml)							
	S1P	1007.28 ± 604.78	944.68 ± 592.35	0.472	920.62 ± 521.92	1144.28 ± 723.36	0.047
	Sphingosine	1.98 ± 0.9	2.98 ± 1.37	<0.001	2.11 ± 1.02	2.47 ± 1.29	0.132
Ceramide (ng/ml)							
	Cer16:0	283.28 ± 52.91	303.02 ± 45.19	0.025	288.82 ± 53.49	286.49 ± 48.17	0.743
	Cer24:0	2368.85 ± 671.98	2603.94 ± 769.1	0.087	2362.76 ± 720.33	2560.7 ± 646.14	0.062
Ceramide metabolism (ratio)							
	SM16:0/Cer16:0	251.76 ± 70.01	212.38 ± 40.1	<0.001	238.49 ± 64.3	248.82 ± 63.87	0.304
	SM24:0/Cer24:0	9.83 ± 2.54	8.49 ± 2.63	0.011	9.86 ± 2.87	9.48 ± 2.39	0.644

*Definition of high-CD66⁺Neutrophil (or CD61⁺ platelets/Siglec8⁺ eosinophils) asthma is greater than 90% of HC.

† ACT, Asthma Control Test; ACQ, Asthma Control Questionnaire; AQLQ, Asthma Quality of Life Questionnaire; SL, sphingolipid; Cer, ceramide; SM, sphingomyelin; MMP9, matrix metalloproteinase 9; TIMP1, tissue inhibitor of metalloproteinase; MPO, myeloperoxidase; TGFβ1, transforming growth factor beta 1.

* Independent t-test and Fisher's exact test were used to assess differences between groups. Bold values indicate significant *P* value.

Table 4. Characteristics of asthmatics according to the gene polymorphism (*ORMDL3* rs7216389 and *SGMS1* rs10740762).

		ORMDL3, rs7216389 (T>C)		P-value*	SGMS1, rs10740762 (A>G)		P-value*
		TT	CT + CC		AA	AG + GG	
		(N=68)	(N=61)		(N=64)	(N=64)	
Demographics							
	Sex (female), no. (%)	47 / 68 (69.1%)	39 / 61 (63.9%)	0.578	44/ 64 (68.8%)	41 / 64 (64.1%)	0.708
	Age (year)	51.45 ± 14.53	48.51 ± 15	0.310	50.52 ± 14.47	49.91 ± 15.04	0.971
	Atopy (presence, %)	31 / 68 (45.6%)	42 / 59 (71.2%)	0.003	35 / 62 (56.5%)	37 / 64 (57.8%)	1.000
	Total serum IgE (KU/L)	295.59 ± 445.45	349.28 ± 357.46	0.244	299.05 ± 299.08	336.37 ± 495.11	0.656
Inflammatory subtype, no. (%)							
	Peripheral eosinophil (%)	5.1 ± 4.57	4.96 ± 3.79	0.817	4.1 ± 2.83	6.03 ± 5.08	0.021
	Peripheral neutrophil (%)	55.86 ± 10.51	53.55 ± 11.89	0.487	56.58 ± 10	52.59 ± 11.76	0.064
	FeNO (ppb)	33.9 ± 32.66	34.54 ± 31.84	0.950	32.13 ± 31.56	35.89 ± 32.97	0.357
	Sputum eosinophils (%)	22.67 ± 31.22	27.62 ± 33.88	0.416	21.24 ± 32.23	29.02 ± 32.58	0.294
	Sputum neutrophils (%)	65.98 ± 32.63	62 ± 33.72	0.611	68.15 ± 33.36	59.8 ± 32.61	0.279
Pulmonary function							
	Baseline FEV1 (% predicted)	89.29 ± 20	95.75 ± 15.06	0.063	95.73 ± 17.18	89.93 ± 18.09	0.050
	Baseline FVC (% predicted)	90.57 ± 16.59	90.95 ± 13.04	0.947	90.57 ± 15.2	90.57 ± 14.71	0.840
	Baseline FEV1/FVC	83.65 ± 8.76	82.74 ± 8.71	0.481	85.19 ± 7.52	81.23 ± 9.49	0.008
	Baseline MMEF (% predicted)	74.93 ± 29.75	69.79 ± 25.84	0.317	75.8 ± 26.51	68.94 ± 29.38	0.108
	PC20 (mg/ml, methacholine)	6.17 ± 7.53	6.66 ± 8.52	0.820	6.07 ± 7.67	6.36 ± 7.96	0.708
Symptom control							
	ACT score	20.35 ± 4.23	20.59 ± 3.26	0.725	20.67 ± 3.88	20.31 ± 3.72	0.398
	ACQ score	5.9 ± 5.13	5.72 ± 5.24	0.760	5.11 ± 4.76	6.44 ± 5.5	0.144
	AQLQ score	165.44 ± 38.87	168.02 ± 38.9	0.673	168.55 ± 38.17	165.23 ± 39.68	0.600
Inflammatory cell markers							
	CD63+ Basophils	9.4 ± 20.26	9.41 ± 19.07	0.998	9.03 ± 17.68	9.94 ± 21.66	0.803
	Siglec8+ Eosinophils	9.95 ± 12.88	8.97 ± 6.33	0.583	7.67 ± 5.47	11.29 ± 13.6	0.195
	CD66+ Neutrophils	54.72 ± 31.13	43.54 ± 25.85	0.048	47.41 ± 27.78	51.17 ± 30.5	0.507
	CD63+CD61+Platelets	9.92 ± 8.21	11.21 ± 10.59	0.524	10.24 ± 7.44	10.95 ± 11.04	0.645
	P-selectin+CD61+ Platelets	26.61 ± 18.71	23.8 ± 13.72	0.578	26.48 ± 16.25	24.41 ± 17.08	0.303
	CD61+ Platelets/Siglec8+ Eosinophils	20.12 ± 10.06	21.69 ± 12.71	0.467	18.51 ± 8.88	23.58 ± 13.42	0.010
Serum biomarkers							
	MMP9	328.95 ± 98.42	275.82 ± 100.14	0.002	305.71 ± 101.25	301.12 ± 104.87	0.921
	TIMP1	142.08 ± 32.77	137.11 ± 60.99	0.087	141.67 ± 45.88	137.86 ± 50.91	0.572
	TGFβ1	47.78 ± 19.02	39.86 ± 22.45	0.049	44.46 ± 21.52	43.45 ± 20.76	0.702
	MPO	526.3 ± 263.25	434.32 ± 229.65	0.048	484.06 ± 268.78	480.41 ± 237.09	0.853
SL metabolic pathway							

*SL concentration
(ng/ml)*

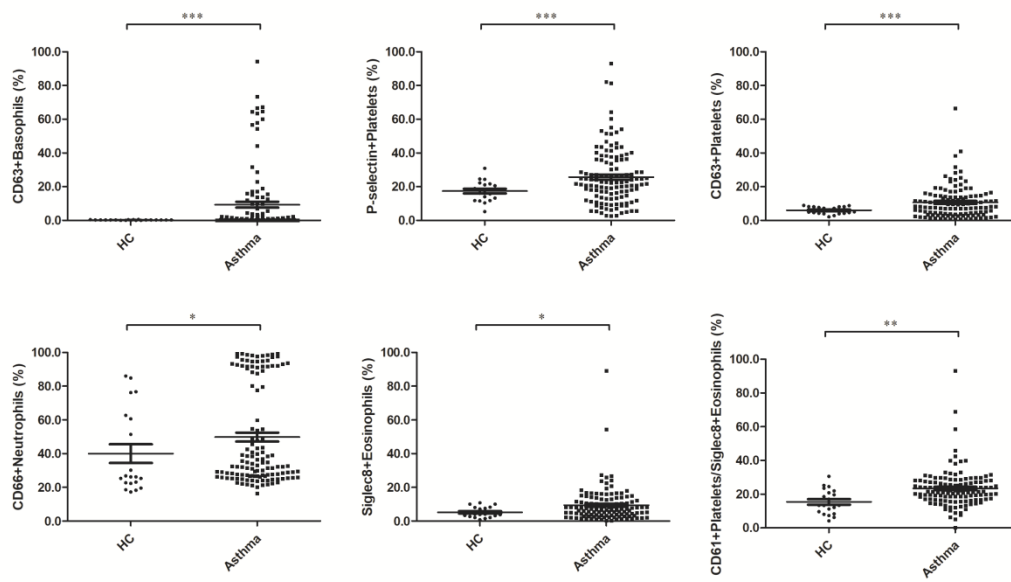
S1P	1057.98 ± 638.15	946.91 ± 569.39	0.232	987.41 ± 562.94	1026.9 ± 655.53	0.886
Sphingosine	2.57 ± 1.22	2.08 ± 0.95	0.042	2.25 ± 1.18	2.43 ± 1.07	0.268
<i>Ceramide (ng/ml)</i>						
Cer16:0	295.42 ± 56.58	283.22 ± 48.28	0.224	286.82 ± 52.39	292.49 ± 54.22	0.569
Cer24:0	2431.74 ± 719.1	2485.24 ± 700.87	0.702	2486.49 ± 755.11	2444.36 ± 655.52	0.883
<i>Ceramide metabolism (ratio)</i>						
SM16/Cer16	234.97 ± 69.14	251.43 ± 58.48	0.035	249.12 ± 72	236.77 ± 56.64	0.463
SM24/Cer24	9.78 ± 2.68	9.5 ± 2.82	0.549	10.02 ± 2.99	9.26 ± 2.45	0.207

† ACT, Asthma Control Test; ACQ, Asthma Control Questionnaire; AQLQ, Asthma Quality of Life Questionnaire; SL, sphingolipid; Cer, ceramide; SM, sphingomyelin; MMP9, matrix metalloproteinase 9; TIMP1, tissue inhibitor of metalloproteinase; MPO, myeloperoxidase; TGFβ1, transforming growth factor beta 1.

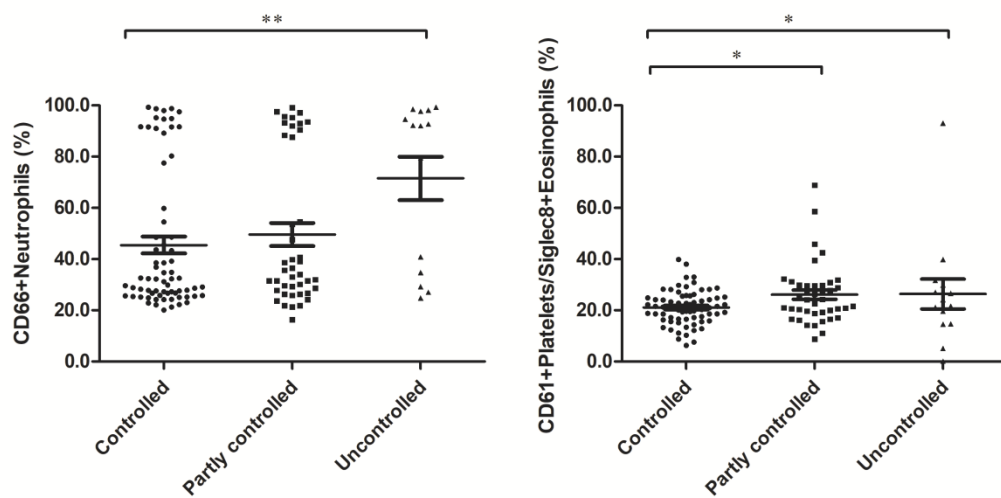
FIGURE LEGENDS

Figure 1. Inflammatory cell markers of study subjects (125 asthmatics and 20 HCs). Whole blood flow cytometry analysis was performed for immunophenotyping. * means P -value ≤ 0.05 ; ** means P -value ≤ 0.01 ; *** means P -value ≤ 0.001 .

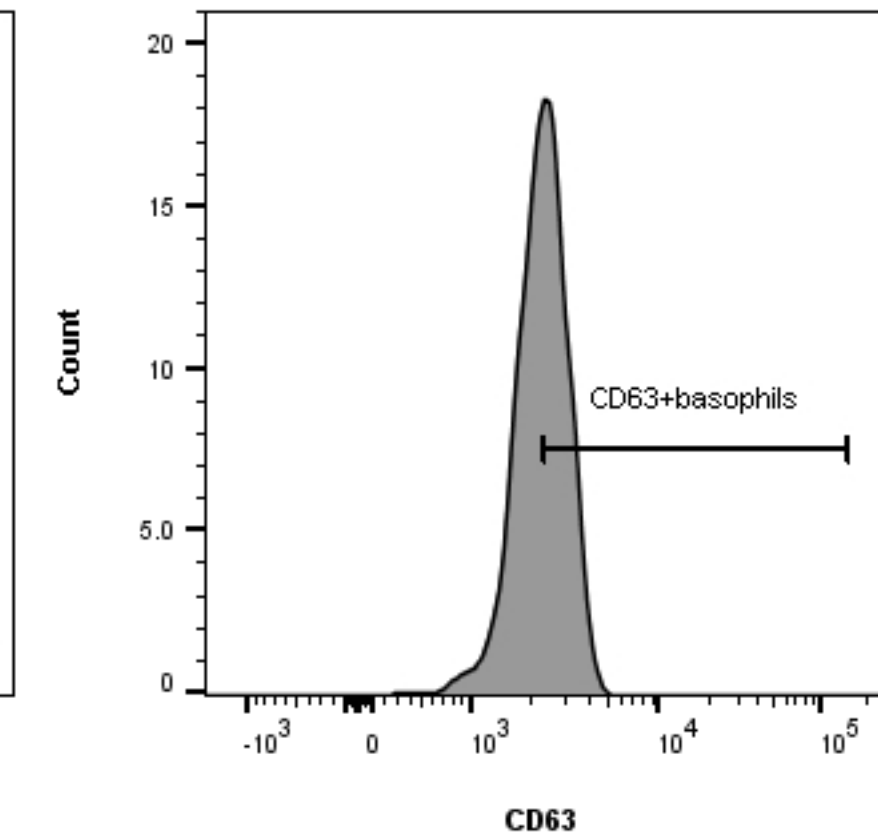
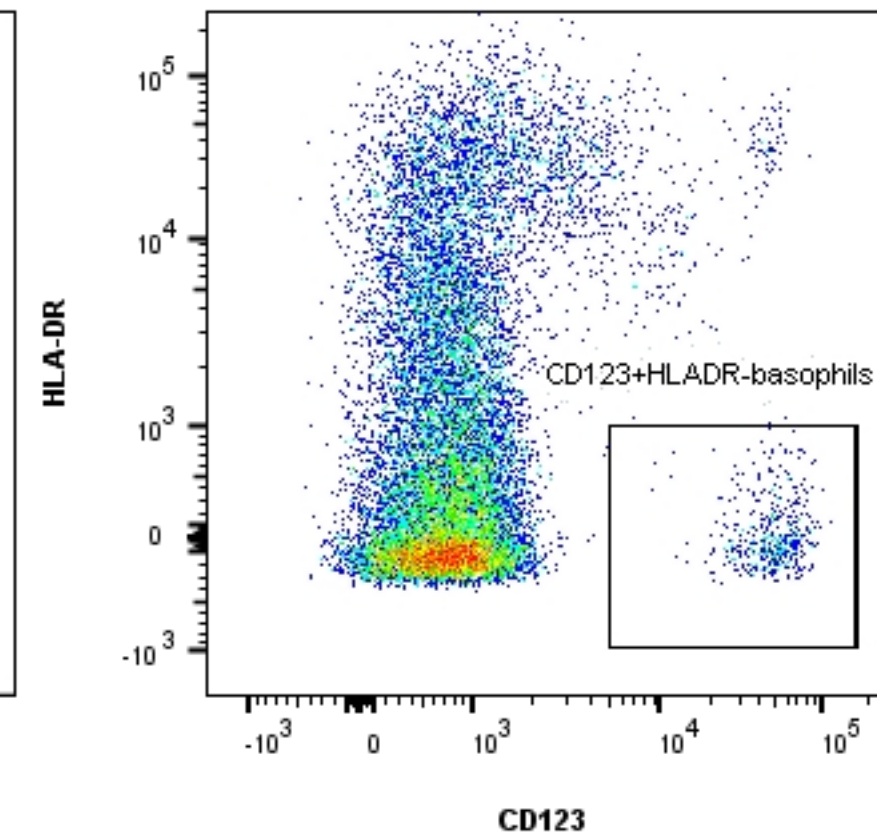
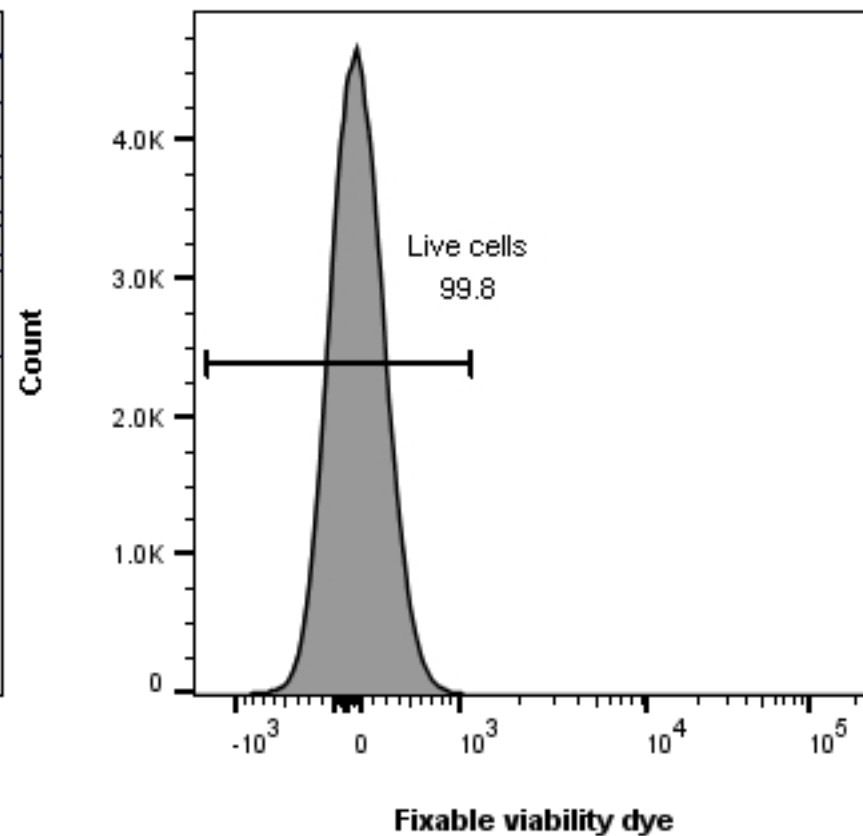
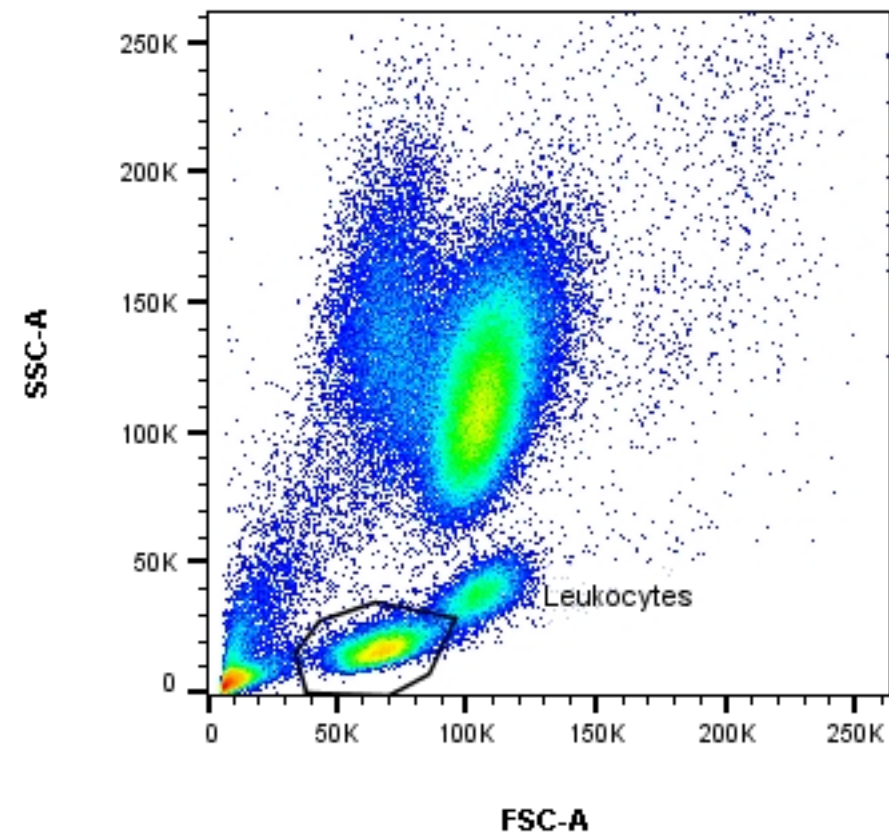
Figure 2. The percentage of CD66⁺ neutrophils and CD61⁺platelets/Siglec8⁺ eosinophils according to asthma control status (69 controlled, 42 partly controlled, and 14 uncontrolled asthmatics). * means P -value ≤ 0.05 ; ** means P -value ≤ 0.01 .

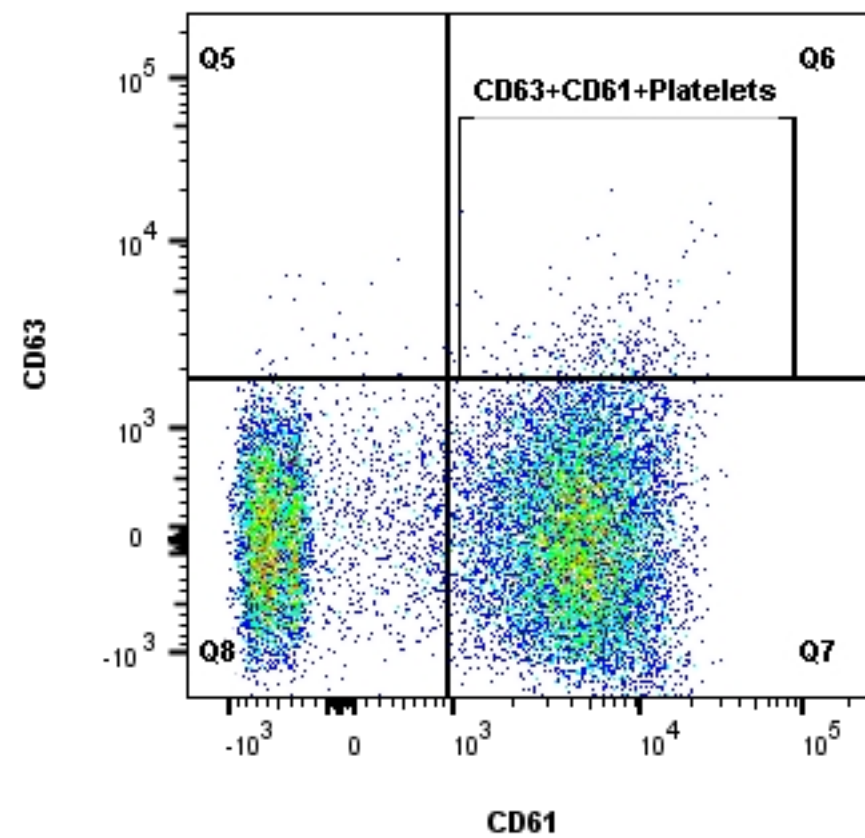
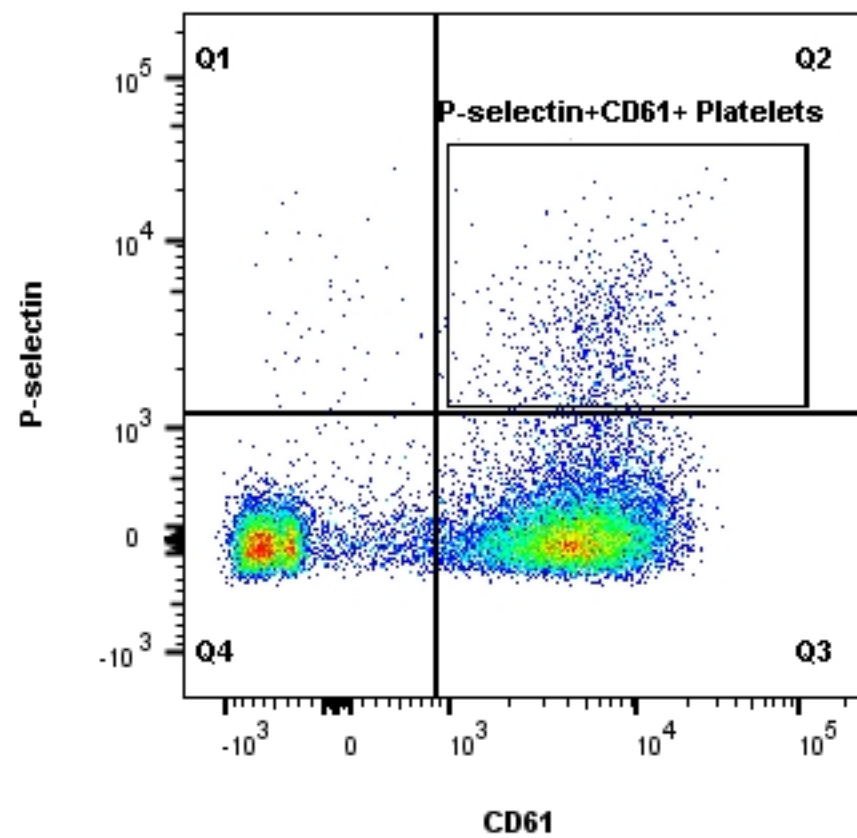
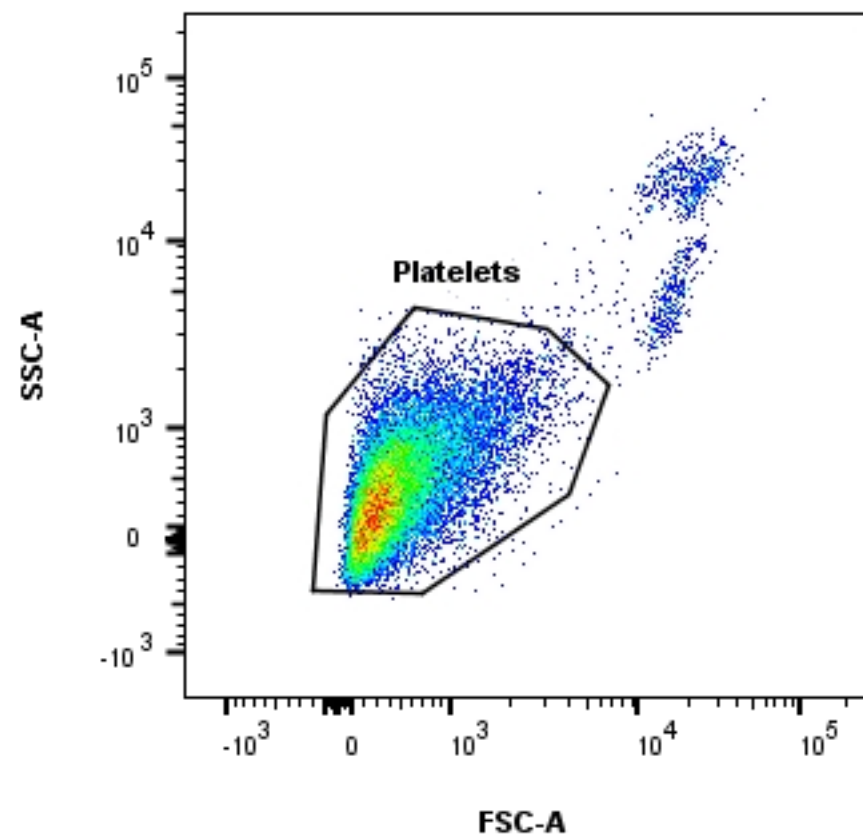


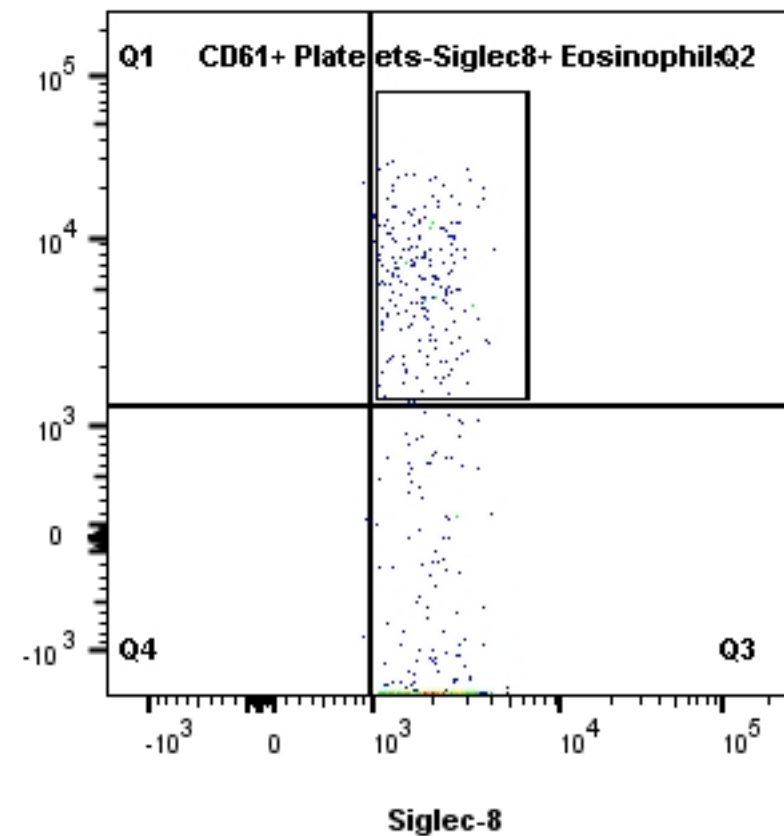
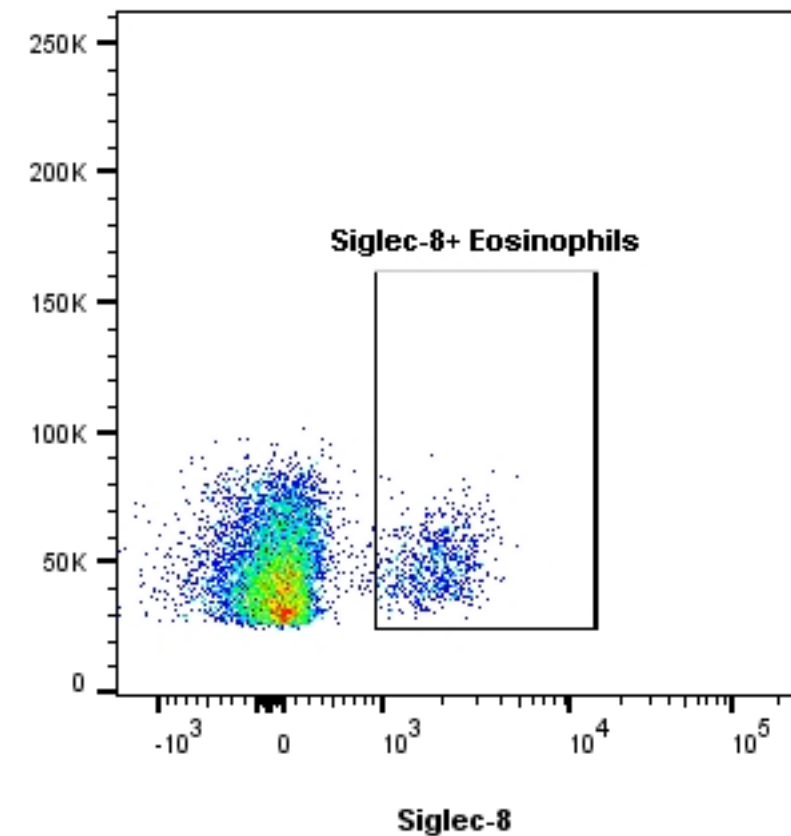
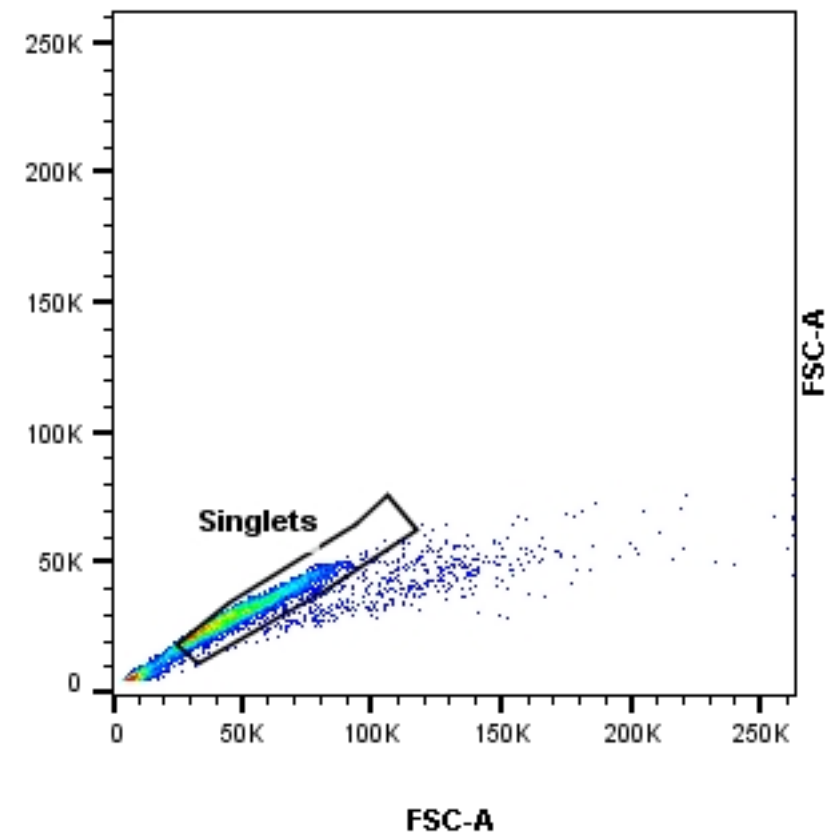
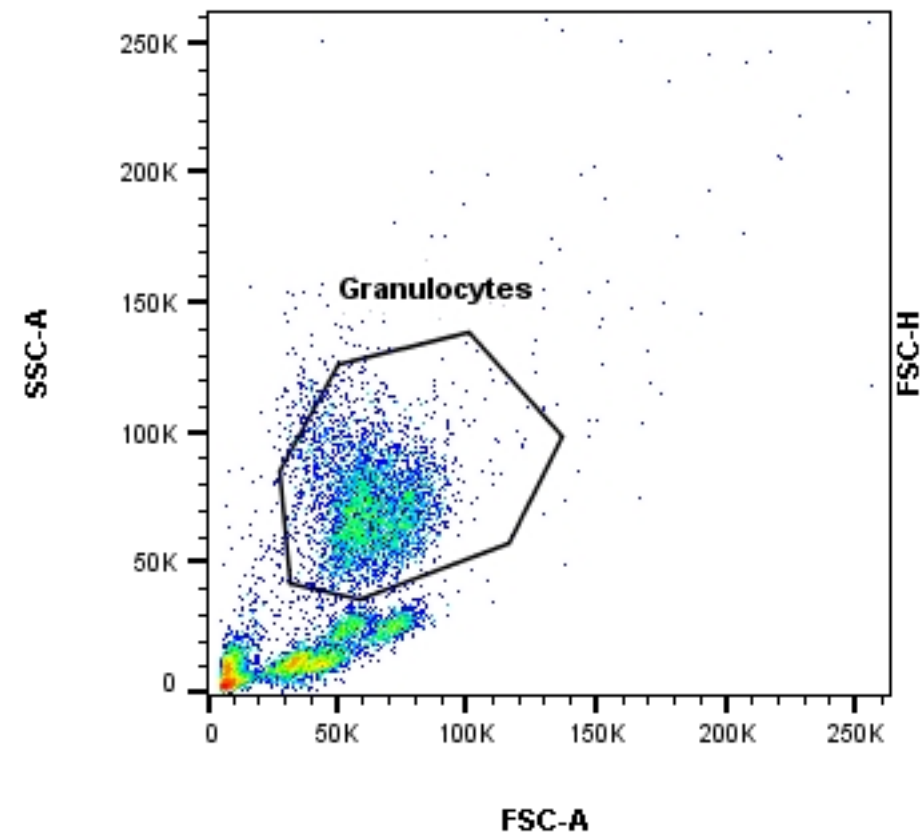
all_14236_f1.tif

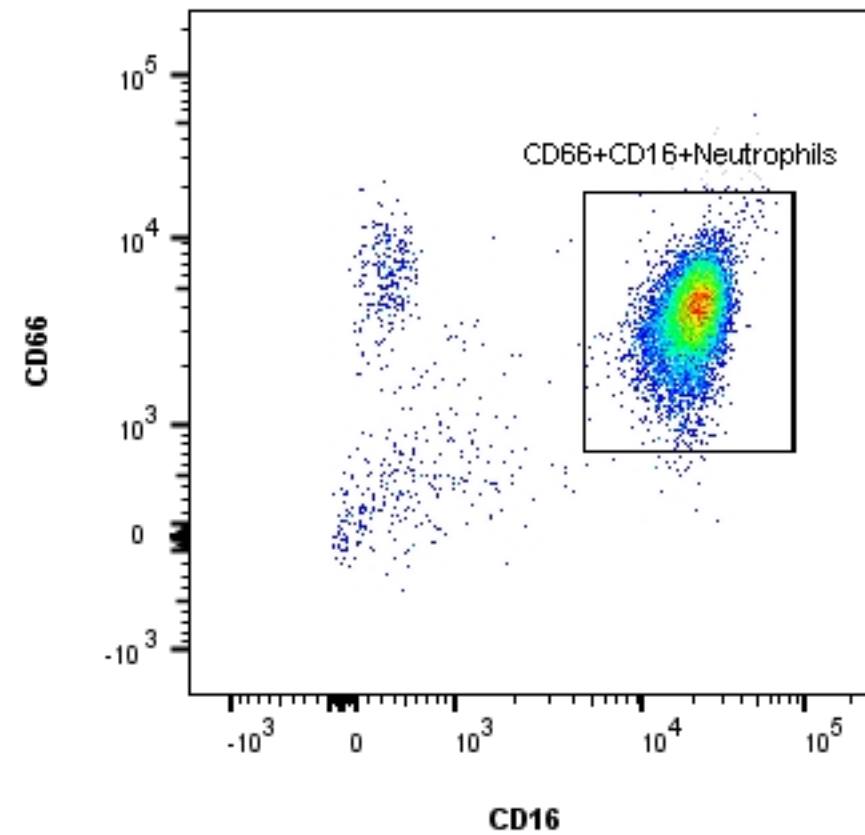
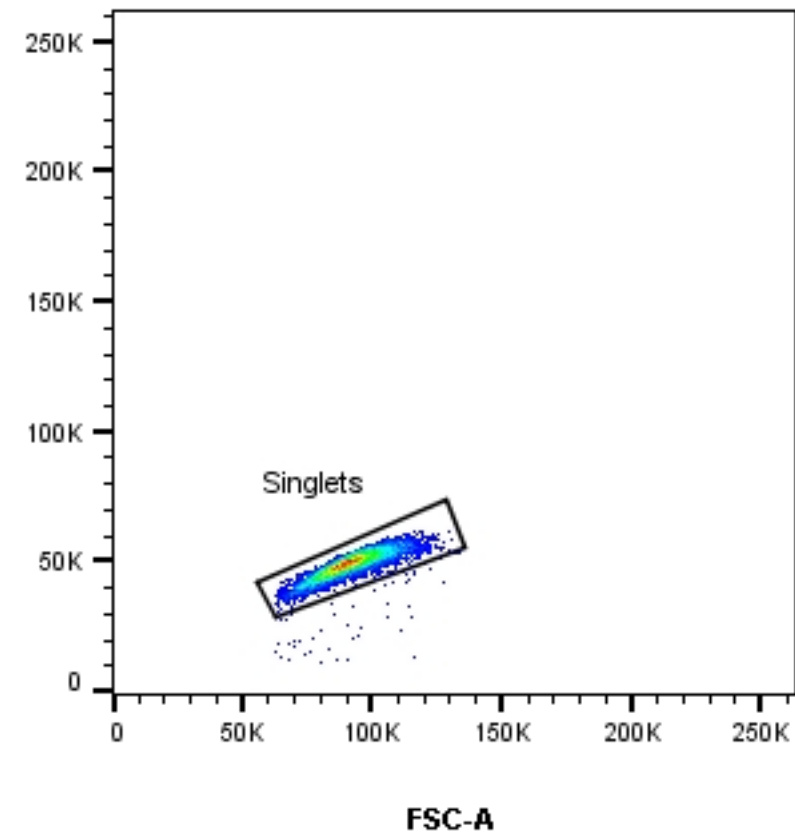
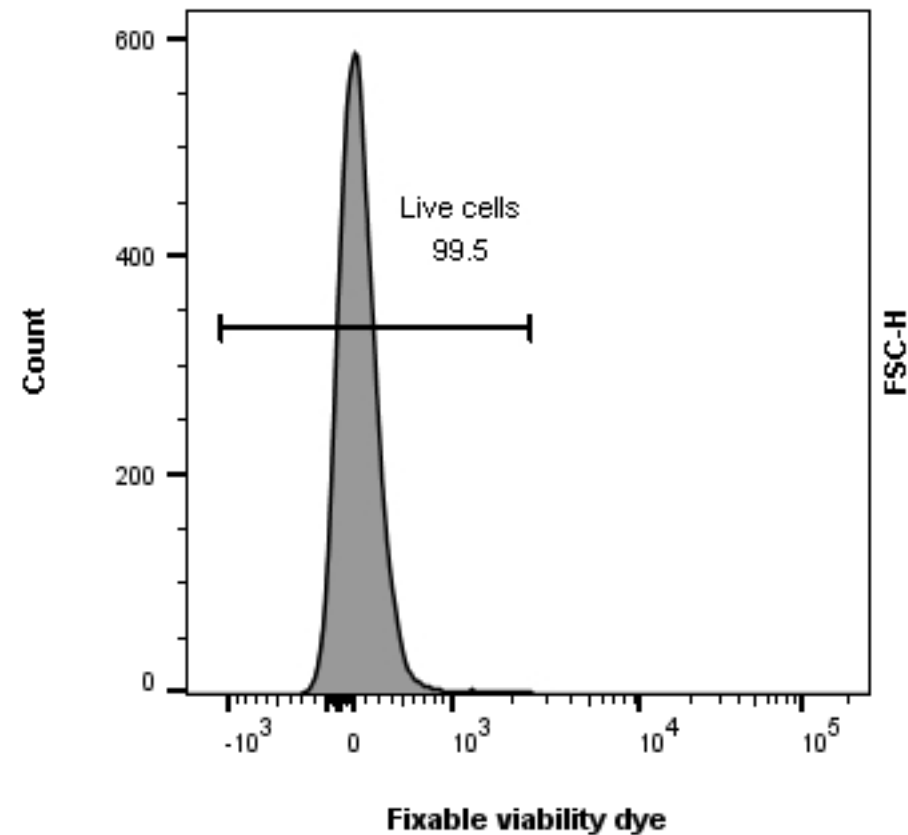
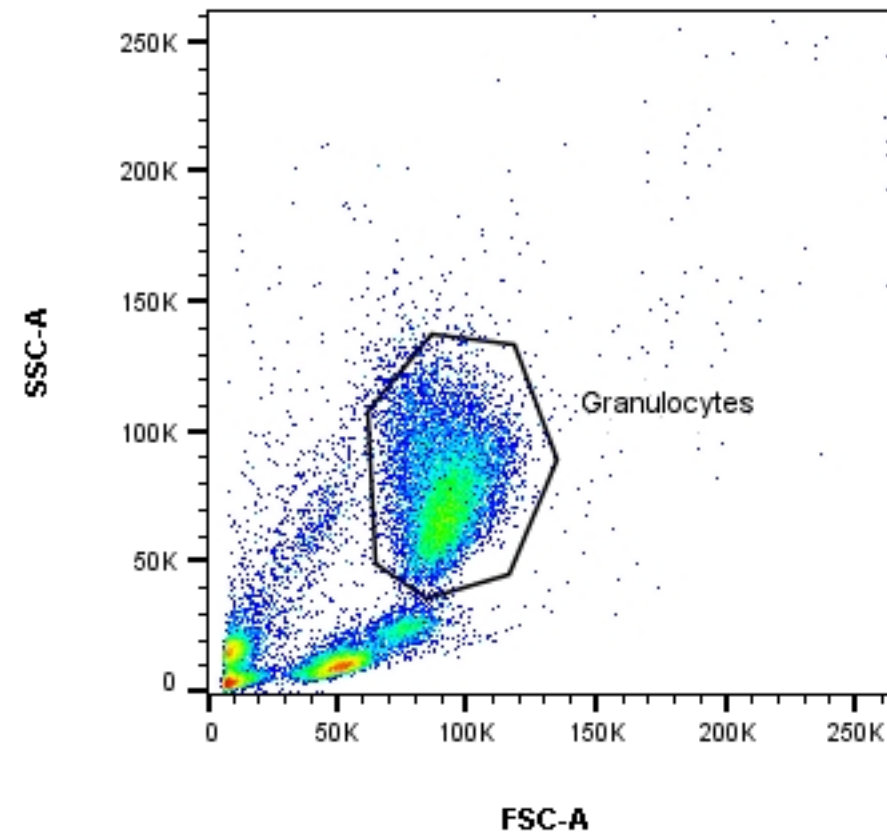


all_14236_f2.tif

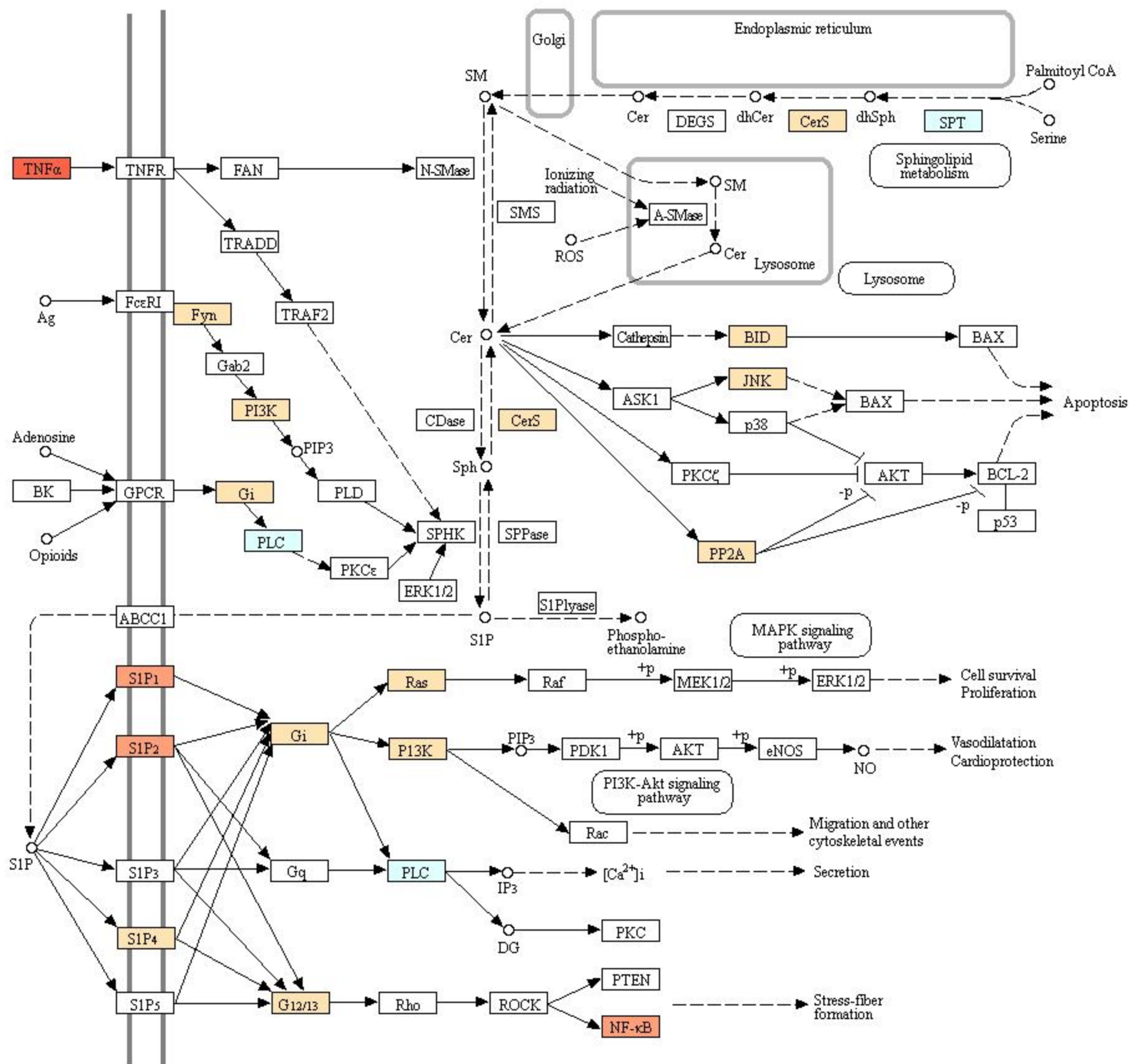








SPHINGOLIPID SIGNALING PATHWAY



Supporting Information

Ceramide/sphingosine-1-phosphate imbalance is associated with distinct inflammatory phenotypes of uncontrolled asthma

Seung-Hyun Kim^{1,2 †}, Hae-won Jung², Minji Kim^{1,2}, Ji-Young Moon², Ga-Young Ban^{2,3}, Su Jung Kim⁴, Hyun-Ju Yoo⁴, Hae-Sim Park^{2†}

¹Translational Research Laboratory for Inflammatory Disease, Clinical Trial Center, Ajou University Medical Center, Suwon; ²Department of Allergy and Clinical Immunology, Ajou University School of Medicine, Suwon; ³Department of Pulmonary, Allergy, and Critical Care Medicine, Kangdong Sacred Heart Hospital, Hallym University College of Medicine Institute for Life Sciences; ⁴Asan Institute for Life Sciences, Asan Medical Center, University of Ulsan College of Medicine, Seoul, South Korea.

[†] Corresponding authors:

Seung-Hyun Kim, PhD

Translational Research Laboratory for Inflammatory Disease, Clinical Trial Center, Ajou University Medical Center, Suwon, South Korea.

Tel: +82-31-219-4264; Fax: 82-31-219-4265

E-mail: kimsh@ajou.ac.kr

Hae-Sim Park, MD, PhD

Department of Allergy & Clinical Immunology, Ajou University

School of Medicine, Worldcup-ro 164, Youngtong-gu, Suwon-si, Korea, 443-380

Tel: +82-31-219-5150; Fax: +82-31-219-5154

E-mail: hspark@ajou.ac.kr

Supplementary methods

Flow cytometry analysis for immunophenotyping

Flow cytometry for immunophenotyping was performed within 1 hour of blood sampling using the following antibody panels as shown in Table S1 and S2. All monoclonal antibodies were purchased from BD Biosciences (San Jose, CA, USA) and Biolegend (San Diego, CA, USA). The whole blood was incubated with fluorochrome-conjugated monoclonal antibodies of cell type-specific selection and activation markers for 20 minutes at room temperature in the dark. Next, red blood cells (RBCs) were lysed with the RBC Lysis/Fixation solution (Biolegend, San Diego, CA, USA) for 10 minutes and washed once with 1× phosphate-buffered saline (PBS). The cells were analyzed immediately by flow cytometry with the BD FACSCanto II (BD Bioscience) and data were analyzed with FlowJo software (Tree Star, Ashland, OR, USA). Cell viability was achieved above 99.8% in the procedure when we checked that by using the fixable viability dye eFlourTM 780 purchased from eBioscience (Carlsbad, CA, USA). We also used individual isotype controls to exclude nonspecific binding. At least 10000 events were used in a gate to define the sample as positive except for basophils (1000 events).

Transcriptome gene expression profiling and data analysis

To examine target gene expression profile in SL metabolism, we stored whole blood stored in RNA stabilization solution (RNAlater) from 30 randomly selected patients with asthma in this study cohort. Total RNA was extracted from whole blood cells using RiboPureTM-Blood extraction kit (Applied Biosystems/Ambion, Austin, TX, USA) according to the manufacturer's instructions. RNA quality and quantity were assessed using ND-1000 Spectrophotometer (NanoDrop, Thermo Scientific, Wilmington, DE, USA) and Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). Among them, 18 samples succeeded to meet the chip quality control (QC) criteria assessed by RNA Integrity Number (RIN) ≥ 8.0 and 260/280 ratio > 1.8 .

For transcriptome profiling of 18 asthmatics, the Affymetrix Human Gene ST 2.0 chip was used (Affymetrix, Santa Clara, CA, USA). The Affymetrix Whole Transcript Expression array process was executed according to the manufacturer's protocol (GeneChip Whole Transcript PLUS reagent Kit). cDNA synthesis and hybridisation were performed according to the manufacturer's recommendations. The data were summarized and normalized with robust multi-average (RMA) method implemented in Affymetrix® Power Tools. We exported the result with gene level RMA analysis and performed the differentially expressed gene analysis. Statistical significance of the expression data was determined using independent t-test and fold change in which the null hypothesis was that no difference exists among groups. All data analysis was conducted using R 3.3.2 (www.r-project.org).

Visualization of gene expression levels on sphingolipid signaling pathway was performed using KEGG mapper (https://www.genome.jp/kegg/tool/map_pathway2.html).

SNP genotyping and data analysis

Four SNPs of the genes such as *ORMDL3*, *SPTLC3*, *SGMS1*, *CERS2* involved in ceramide synthesis and metabolism were selected by previously reported functional SNPs ¹⁻⁴.

SNP genotyping was performed using the SNaPshot ddNTP primer extension kit from Applied Biosystems (Foster city, CA, USA). Sequences of amplifying and extension primers are shown in Table S3. A significant departure of genotype frequency from the Hardy-Weinberg equilibrium (HWE) at each SNP was tested by a chi-square goodness-of-fit test. All SNPs were not deviated from the Hardy-Weinberg equilibrium.

Measurement of ROS in peripheral blood neutrophils under fMLP stimulation

In vitro neutrophil stimulation assay was performed according to the previous report ⁵. Peripheral blood neutrophils were isolated, stimulated with 1 μ M fMLP for 1 hour, and then analyzed ROS production as previously described.

SUPPLEMENTARY TABLES

Table S1. Antibody panels for immunophenotyping of whole blood samples.

Panel	Fluorochromes and markers			Target cells	References
	Gating (SSC/FSC)	Selection marker	Activation marker		
a	Leukocytes	FITC-CD123 ⁺ /APC-HLADR ⁺	PE-CD63 ⁺	Activated basophils	6
b	Platelets	PerCP-CD61 ⁺	FITC-P-selectin ⁺	Activated platelets	7
b	Platelets	PerCP-CD61 ⁺	PE-CD63 ⁺	Activated platelets	8
c	Granulocytes	APC-Siglec8 ⁺		Eosinophils	9
c	Granulocytes	APC-Siglec8 ⁺	PerCP-CD61 ⁺	Platelet-adherent eosinophils	10
d	Granulocytes	FITC-CD16 ⁺	PE-CD66 ⁺	Activated neutrophils	11

APC, Allophycocyanine; FITC, Fluorescein isothiocyanate; PE, Phycoerythrin; PerCP, Peridinin Chlorophyll Protein Complex

Table S2. Antibodies and staining reagents.

Marker	Isotype	Fluorochrome conjugate	Company	Catalog #
P-selectin	Mouse IgG2a	FITC	Biolegend	304904
CD63	Mouse IgG1, κ	PE	BD	556020
CD16	Mouse IgG1, κ	FITC	Biolegend	302006
CD61	Mouse IgG1, κ	PerCP	Biolegend	336410
Siglec8	Mouse IgG1, κ	APC	Biolegend	347106
CD66	Mouse IgG2a, κ	PE	BD	555779
CD123	Mouse IgG2a, κ	FITC	BD	558663
HLADR	Mouse IgG2a, κ	APC	BD	559866

APC, Allophycocyanine; FITC, Fluorescein isothiocyanate; PE, Phycoerythrin; PerCP, Peridinin Chlorophyll Protein Complex

Table S3. Sequences of amplifying and extension primers of 4 single nucleotide polymorphisms involved in SL metabolism.

Gene name	rs number	Location	Allele (M/m)*	q **	Primer sequence	Tm(°C)	References	
ORMDL3	rs7216389	intron	T/C	0.271	Forward Primer	CAGTTCTGTCGCTGTGTGTT	60	1
					Reverse Primer	TCTGTCACATTTCACCAG		
					Genotyping Primer	AGGCAACCCTGGAAAGTCA CAAACA		
SPTLC3	rs6078905	5'UTR	T/C	0.264	Forward Primer	CTTCTGGGTAAAAGAGAAA GTT	60	2
					Reverse Primer	TGCCTGAAGTCCTCAATA		
					Genotyping Primer	GAGAAAGTTAAATTGGGAT CTAGA		
CERS2	rs267738	exon	A/C	0.091	Forward Primer	TGTTAGCTCTGGCAGGTGAA	60	3
					Reverse Primer	CCAAATCCATGGCCTCA		
					Genotyping Primer	GGCTCTCTGGCCRCCAGGTR G		
SGMS1	rs10740762	intron	A/G	0.293	Forward Primer	TTTATGTCTCCTATCCACCT TC	60	4
					Reverse Primer	GTGGCTGGTTATTGGAAGT		
					Genotyping Primer	GATTTGGGATGCTCAACCA ATACCC		

ORMDL3, ORMDL Sphingolipid Biosynthesis Regulator 3; *SPTLC3*, Serine palmitoyltransferase, long chain base subunit 3; *CERS2*, Ceramide synthase 2; *SGMS1*, Sphingomyelin Synthase 1.

* M, major allele ; m, minor allele ** q, Minor allele frequency in the study subjects. Tm, melting temperature.

Table S4. Levels of individual ceramide and sphingomyelin species in study subjects.

No.		HC [†]	Asthma	P-value [*]
		(N=20)	(N=137)	HC vs. Asthma
Ceramide (ng/ml)				
	Cer14:0	12.9 ± 2.92	14.57 ± 3.69	0.055
	Cer16:0	264.72 ± 39.98	290.12 ± 52.77	0.041
	Cer18:0	112.06 ± 40.8	142.51 ± 43.68	0.004
	Cer18:1	19.3 ± 5.96	23.43 ± 6.9	0.012
	Cer20:0	99.15 ± 45.89	109.89 ± 40.25	0.277
	Cer24:0	2117.82 ± 575.25	2450.47 ± 704.8	0.046
	Cer24:1	664.94 ± 230.19	839.58 ± 287.68	0.011
Sphingomyelin (ng/ml)				
	SM(d18:0/16:0)	64421.97 ± 16310.84	69215.29 ± 18891.13	0.284
	SM(d18:0/18:0)	16036.65 ± 3882.54	17495.34 ± 3598.15	0.097
	SM(d18:0/18:1)	9220.59 ± 2338.73	10395.6 ± 2757.92	0.073
	SM(d18:0/24:0)	22994.62 ± 4690.32	22270.89 ± 4531.8	0.509
	SM(d18:0/24:1)	43823.77 ± 8447.88	45683.78 ± 8332.04	0.355
Ceramide metabolism (ratio)				
	SM16:0/Cer16:0	244.95 ± 55.01	242.13 ± 64.39	0.853
	SM18:0/Cer18:0	151.83 ± 39.44	130.03 ± 33.14	0.008
	SM18:1/Cer18:1	492.11 ± 98.87	458.65 ± 108.38	0.196
	SM24:0/Cer24:0	11.36 ± 2.86	9.66 ± 2.73	0.011
	SM24:1/Cer24:1	72.45 ± 24.44	59.52 ± 18.44	0.006
Ceramide elongation (ratio)				
	Cer16:0/Cer24:0	0.13 ± 0.03	0.12 ± 0.03	0.382
	Cer18:0/Cer24:0	0.05 ± 0.02	0.06 ± 0.02	0.182
	Cer18:1/Cer24:1	0.03 ± 0.01	0.03 ± 0.01	0.704
Ceramide desaturation (ratio)				
	Cer18:0/Cer18:1	5.79 ± 0.93	6.14 ± 1.1	0.178
	Cer24:0/Cer24:1	3.34 ± 0.9	3.07 ± 0.86	0.187

† HC, healthy control; N, number; SL, sphingolipid; Cer, ceramide; SM, sphingomyelin.

The continuous data are presented as the means ± SD. The dichotomous data are presented as numbers (%).

* The data between asthma and HC were analyzed with independent t-test and Fisher's exact test. Bold values indicate significant P value.

Table S5. Correlations of inflammatory cell markers (CD66⁺Neutrophils and CD61⁺ Platelets/Siglec8⁺Eosinophils) with clinical features and biomarkers in asthmatics

	CD66 ⁺ Neutrophils	CD61 ⁺ Platelets/Siglec8 ⁺ Eosinophils
Clinical features (N=137)		
Total serum IgE (KU/L)	.000	.042
Sputum eosinophils (%)	-.052	-.201
Sputum neutrophils (%)	.004	.072
FeNO (ppb)	.238**	.131
PC20 (mg/ml, methacholine)	-.048	.135
Baseline FEV1 (% predicted)	-.266**	-.030
Baseline FVC (% predicted)	-.218**	-.124
Baseline FEV1/FVC	-.265**	.132
Baseline MMEF (% predicted)	-.235**	.001
ACT score	-.139	-.276**
ACQ score	.215*	.200*
AQLQ score	-.202*	-.236**
Sphingolipid metabolites (N=137)		
S1P	-.045	.282**
Sphingosine	.395**	.220**
Cer16:0	.188*	-.007
Cer24:0	.150	.103
SM16:0/Cer16:0	-.261**	-.053
SM24:0/Cer24:0	-.183*	-.011
Serum cytokines (N=137)		
MMP9	.222**	.112
TIMP1	.167*	.219**
TGFβ1	.251**	.198*
MPO	.344**	.079
ROS production from neutrophils (N=50)		
Control	.404**	-.334*
fMLP stimulation	.356*	-.541**

† ROS, reactive oxygen species; fMLP, formyl methionyl-leucyl-phenylalanine; SL, sphingolipid; Cer, ceramide; SM, sphingomyelin; MMP9, matrix metalloproteinase 9; TIMP1, tissue inhibitor of metalloproteinase; MPO, myeloperoxidase; TGFβ1, transforming growth factor beta 1. Data are presented as correlation coefficient ^{P value}. * $P < 0.05$, ** $P < 0.001$

Supplementary Figure

Figure S1. Whole blood flow cytometry for immunophenotyping. Flow cytometric plots of (A) CD123⁺/HLADR⁻ basophils, (B) activated platelets, CD63⁺ or P-selectin⁺ on CD61⁺ platelets, (C) Siglec8⁺ eosinophils and CD61⁺ platelets on Siglec8⁺ eosinophils (platelet-adherent eosinophils), and (D) CD66⁺ neutrophils.

Figure S2. Expression profiles of the genes encoding enzymes in the sphingolipid signaling pathway in patients with higher levels of CD66⁺ neutrophils. Boxes in red represent strongly up-regulated genes encoding that enzyme. Boxes in orange represent up-regulated genes encoding the enzymes. Boxes in blue represent down-regulated genes. TNF- α , tumor necrosis factor- α ; S1P1, sphingosine-1-phosphate receptor 1, CerS; ceramide synthase, SPT, serine palmitoyl transferase, BID, BH3 interacting domain death agonist; JNK, c-Jun N-terminal kinase; NF- κ B, Nuclear Factor-kappaB.

References

1. Moffatt MF, Kabesch M, Liang L, et al. Genetic variants regulating ORMDL3 expression contribute to the risk of childhood asthma. *Nature*. 2007;448(7152):470-473.
2. Shahin MH, Gong Y, Frye RF, et al. Sphingolipid Metabolic Pathway Impacts Thiazide Diuretics Blood Pressure Response: Insights From Genomics, Metabolomics, and Lipidomics. *J Am Heart Assoc*. 2017;7(1).
3. Shiffman D, Pare G, Oberbauer R, et al. A gene variant in CERS2 is associated with rate of increase in albuminuria in patients with diabetes from ONTARGET and TRANSCEND. *PLoS One*. 2014;9(9):e106631.
4. Hicks AA, Pramstaller PP, Johansson A, et al. Genetic determinants of circulating sphingolipid concentrations in European populations. *PLoS Genet*. 2009;5(10):e1000672.
5. Kim SH, Uuganbayar U, Trinh HKT, et al. Evaluation of Neutrophil Activation Status According to the Phenotypes of Adult Asthma. *Allergy Asthma Immunol Res*. 2019;11(3):381-393.
6. Chirumbolo S, Vella A, Ortolani R, et al. Differential response of human basophil activation markers: a multi-parameter flow cytometry approach. *Clin Mol Allergy*. 2008;6:12.
7. van der Zee PM, Biro E, Ko Y, et al. P-selectin- and CD63-exposing platelet microparticles reflect platelet activation in peripheral arterial disease and myocardial infarction. *Clin Chem*. 2006;52(4):657-664.
8. van Velzen JF, Laros-van Gorkom BA, Pop GA, van Heerde WL. Multicolor flow cytometry for evaluation of platelet surface antigens and activation markers. *Thromb Res*. 2012;130(1):92-98.
9. Bochner BS. Siglec-8 on human eosinophils and mast cells, and Siglec-F on murine eosinophils, are functionally related inhibitory receptors. *Clin Exp Allergy*. 2009;39(3):317-324.
10. Trinh HKT, Nguyen TVT, Choi Y, Park HS, Shin YS. The synergistic effects of clopidogrel with montelukast may be beneficial for asthma treatment. *J Cell Mol Med*. 2019;23(5):3441-3450.
11. Stocks SC, Ruchaud-Sparagano MH, Kerr MA, Grunert F, Haslett C, Dransfield I. CD66: role in the regulation of neutrophil effector function. *Eur J Immunol*. 1996;26(12):2924-2932.