Role of S1P/S1PR3 axis in release of CCL20 from human bronchial epithelial cells

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Abstract

Background

Sphingosine kinase phosphorylates sphingosine to generate sphingosine 1 phosphate (S1P) following stimulation of the five plasma membrane G-protein-coupled receptors. The objective of this study is to clarify the role of S1P and its receptors (S1PRs), especially S1PR3 in airway epithelial cells.

Methods

The effects of S1P on asthma-related genes expression were examined with the human bronchial epithelial cells BEAS-2B and Calu-3 using a transcriptome analysis and siRNA of S1PRs. To clarify the role of *CCL20* in the airway inflammation, BALB/c mice were immunized with ovalbumin (OVA) and subsequently challenged with an OVA-containing aerosol to induce asthma with or without intraperitoneal administration of anti-CCL20. Finally, the anti-inflammatory effect of VPC 23019, S1PR1/3 antagonist, in the OVA-induced asthma was examined.

Results

S1P induced the expression of some asthma-related genes, such as *ADRB2*, *PTGER4*, and *CCL20*, in the bronchial epithelial cells. The knock-down of SIPR3 suppressed the expression of S1P-inducing *CCL20*. Anti-CCL20 antibody significantly attenuated the eosinophil numbers in the bronchoalveolar lavage fluid ($P<0.01$). Upon OVA challenge, VPC23019 exhibited substantially attenuated eosinophilic inflammation.

Conclusions

S1P/S1PR3 pathways have a role in release of proinflammatory cytokines from bronchial epithelial cells. Our results suggest that S1P/S1PR3 may be a possible candidate for the treatment of bronchial asthma.

Introduction

Sphingosine 1 phosphate (S1P) is a bioactive sphingolipid metabolite that regulates diverse
biological responses such as proliferation, migration, cytoskeletal organization, adherent junc-
tion assembly and morphogenesis [1]. S1P is exported outside of cells by ATP-binding cas-
sette-type or other transporters and extracellular S1P activates five G protein-coupled
receptors, known as S1P receptors (S1PRs) 1–5 via autocrine and paracrine signaling [2].

S1PRs have diverse functions depending on the contexts. For example, S1PR1 has a role in
lymphocyte trafficking, from the lymph nodes or thymus to the lymph or blood and in den-
dritic cell (DC) maturation [2]. In immune cells, the localizations of S1PRs are well-character-
ized, and the physiological role of the interaction between S1P and S1PRs in the immune cell
function has been clarified using FTY720, which is a prodrug that is phosphorylated *in vivo* by
sphingosine kinases (SPHK) 2 to biologically active FTY720-phosphate [3]. FTY720-phosphate
binds to S1PRs except for S1PR2. Although it acts as a super agonist of S1PR1, FTY720-pho-
phate leads to sustained S1PR1 internalization and lymphocyte sequestration [4]. The adminis-
tration of FTY720 to the lung abrogates experimental asthma by inhibiting the migration of
lung DCs to the regional lymph nodes [5]. In contrast, VPC23019 is an aryl amide-containing
S1P analog that acts as an unselective competitive antagonist at both S1PR1 and S1PR3 [6].
Our laboratory previously showed that the administration of SPHK inhibitors prevented
eosinophil inflammation [1].

A recent study showed that S1P elicits the gene expression of inflammatory cytokines, such
as cyclooxygenase (COX)-2 mediated by phospholipase Cε (PLCε) in astrocytes [7]. We
showed that PLCεis expressed in the bronchial epithelial cells (ECs) and has a role in asthma
through upregulating the inflammatory cytokine production by the bronchial ECs in the elici-
tation phase [8]. In addition, we found that S1PR1-3 expressed on mouse airway ECs and
S1PR2 had a role in nuclear factorκB (NF-κB) activation and CC chemokine ligand 3 (CCL) 3
production in the bronchial ECs [9]. However, JTE013, S1PR2 antagonist did not completely
attenuate the ovalbumin (OVA)-induced airway inflammation. We think S1PR2 as well as
S1PR1/3 regulate the chemokine production from lung structured cells.Therefore, this study
builds upon our previous work and we would like to fully characterize the role of remaining
S1P/S1PR3 axis in the bronchial ECs.

The aim of this study is to evaluate the role of S1P and S1PR3 in the airway ECs using
human bronchial EC lines and experimental asthma mouse models.

Materials and methods

Reagents

VPC23019 (also known as BMS-345541; 401480, Calbiochem, Darmstadt, Germany) and
JTE23019 (Cayman, Ann Arbor, MI) were commercially obtained. Sphingosine 1 phosphate
(62570; Cayman chemical, Ann Arbor, MI, USA) and monoclonal rat anti-CCL 20 antibody
(Clone # 114906, R & D Systems, Minneapolis, MN, USA) were used. S1P was supplied as a
crystalline solid and was prepared by directly dissolving in basic buffers and 0.1% bovine
serum albumin solutions according to the manufacturer's protocol.

Bronchial epithelial cell cultures

Human bronchial EC lines BEAS-2B (CRL-9609) [10] and Calu-3 (HTB-55) [11] were pur-
chased from ATCC (Manassas, VA, USA) and maintained in BEGM with BulletKit (#CC-

3170; Lonza, Walkersville, MD, USA) and EMEM, respectively, in a humidified atmosphere containing 5% CO_2 at 37°C. S1P induced interleukin-8 release via S1PR2 and nuclear factor κB in BEAS-2B cells [12]. In addition, the production of interleukin-8 was also observed in Calu-3 [13]. Therefore, these bronchial ECs were treated with or without 1 μM S1P for 2 h.

RNA isolation and microarray

Total cellular RNA preparation from BEAS-2B and Calu-3 before and after S1P stimulation was performed as described [8, 9]. Total RNA labeled with Cy3 or Cy5 was hybridized to a 3D-Gene Human Oligo chip 25 k (Toray Industries Inc., Tokyo, Japan). Genes with Cy3/Cy5 normalized ratios greater than 2.0 were identified.

siRNA and transfection

S1PR3#1 siRNA (s4455) and S1PR3#2 siRNA (s4454) were purchased from Life Technologies (Carlsbad, CA, USA). The negative control siRNA (sc37007) was purchased from Santa Cruz Biotechnology (Dallas, TX, USA). A total of $2×10^5$ cells were transfected with siRNA or control siRNA using the Lipofectamine® RNA-iMAX Reagent (Life Technologies) in serum-free Opti-MEM® Medium (Thermo Fisher Scientific, Waltham, MA, USA). After 24-h incubation, the cells were used for further experiments.

Quantitative reverse transcription-polymerase chain reaction (qRT-PCR)

QRT-PCR was performed as described [8]. Relative human mRNA levels were calculated with the ΔΔCt method using the glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) mRNA as an internal control. The primers used in this study are as followed: 5'-GCACCGTCAAGGCT GAGAAC-3' and 5'-ATGGTGGTGAAGACGCCAGT-3' for *GAPDH* [14], 5'-CAGGTCTCC ACTGCTGCC-3' and 5'- CACTCAGCTCCAGGTCACT-3' for *CCL3* [9], 5'- CCACCCAG AAGAAGAGCCTGAA-3' and 5'-TGGACCCATGGGATGACTGTT-3' for *TIMP2* [9], 5'-A TGACTTCCAAGCTGGCCGTG-3' and 5'-TTATGAATTCTCAGCCCTCTTCAAAAACTTCT C-3' for *IL-8* [15], 5'-TCAGGGAGGGCAGTATGTTC-3' and 5'-GAGTAGAGGGGCAGGAT GGT-3' for *S1PR3* [16], and 5'-GACATAGCCCAAGAACAGAAA-3' and 5'-GACAAGTCC AGTGAGGCACAA-3' for *CCL20* [17].

Western blot analysis

The detailed protocol for Western blotting has been described previously [14]. The indicating antibodies to the following proteins were used in this study: β-actin (#4967, Cell Signaling Technology, Danvers, MA, USA) and S1PR3 [EPR4540(2), Abcam, Cambridge, UK].

Animals

Female BALB/c mice were purchased from CLEA Japan (Tokyo, Japan). Our research was approved by the Institutional Animal Care and Use Committee of Kobe University (Institutional Animal Care and Use Committee At Kusunoki and Myodani Campus Kobe University, Permit Numbers: P130610-R1, and P171009) and carried out according to the Kobe University Animal Experimentation Regulations. All surgery was performed under general anesthesia as follows: Mice were given dexmedetomidine (0.3 mg/kg; Maruishi Pharmaceutical, Osaka, Japan), midazolam (4 mg/kg; Astellas Pharma, Tokyo, Japan) and butorphanol tartrate (5 mg/ kg; Meiji Seika Pharma, Tokyo, Japan) by intraperitoneal (i.p.) administration, and all efforts were made to minimize suffering.

Induction of bronchial asthma

Six- to eight-week-old female BALB/c mice were sensitized with an i.p. injection of 10 μg of ovalbumin (Sigma-Aldrich, St. Louis, MO, USA) mixed with 1 mg of aluminum hydroxide in 0.5 ml of sterile phosphate-buffered saline (PBS) on days 0 and 7. On days 21 and 22, mice were placed in an acrylic chamber and exposed to aerosolized 1.0% OVA in sterile PBS for 30 min. Negative controls were injected and exposed to PBS on the same schedule.

Experimental mouse models

To prepare the administered groups of mice, mice were given VPC23019 (1 μg/mouse), or anti-CCL20 antibody (20 μg/mouse) via i.p. injection 15 minutes before OVA inhalation on days 21 and 22.

Bronchoalveolar lavage fluid (BALF)

Twenty-four hours after the final challenge of OVA inhalation, BAL was performed 3 times with 800 μl of PBS. Fluid was regularly recovered at 80%-90%. The BALF was collected and centrifuged at $1,500 \times$ rpm for 5 min at 4˚C. The pellet was suspended in PBS, and the leukocyte number was determined using hemocytometer. The leukocytes were then subjected to cytospin preparation and stained with Diff-Quick (Sysmex, Kobe, Japan). At least 200 leukocytes on each slide were subjected to differential counting of macrophages, neutrophils, lymphocytes and eosinophiols according to the standard morphological criteria [18].

Statistical analysis

All data are expressed as the means ± standard error of the mean (SEM). We use an unpaired two-tailed Student's *t*-test to compare the two different subjects. To test statistical differences between more than two groups, we use an analysis of variance (ANOVA). If *P* value was smaller than 0.05, the difference was considered to be statistically significant.

Results

Role of S1P in asthma-related gene expression in ECs

To evaluate the effect of S1P on the gene expression in ECs, we carried out a microarray analysis in two lines of ECs (GSE111746. BEAS-2B with or without S1P and Calu-3 with or without S1P). The expression of asthma-related genes is summarized in Table 1 [19, 20]. In contrast to S1P-untreated ECs, S1P-treated ECs expressed *ADRB2* and *PTGER4*. A hierarchical clustering analysis was performed using Pearson's correlation coefficient based on the log2 ratio data (Fig 1A and S1 Fig). We confirmed the increase of mRNA levels of *CCL3* and *TIMP2* in BEAS-2B cells (Fig 1B) and Calu-3 cells (Fig 1C). Intriguingly, not only JTE013 but also VPC23019 suppressed the increase of mRNA level of *CCL3*. Out of 967 genes that were up-regulated to more than 2-fold or down-regulated to less than 2-fold in at least 1 pair, only 14 genes were up-regulated 4-fold in both pairs (Table 2). Among these genes, *CCL20* was the gene most up-regulated in BEAS-2B. No previously reported asthma-related genes except for *CCL20* showed more than 4-fold upregulation or down-regulation in both cell lines [21]. CCL20 was reported to be secreted from bronchial ECs and regulates the recruitments of DCs which is critical for type 2 helper (T$_H$2) responses in asthma [22]. The secretion of CCL20 was regulated by clusterin, an oxidative stress regulatory molecule [22]. In general, sphingolipids has nothing to do with clusterin, although sphingolipids also have a crucial role in the production of cytokine from epithelial cells [9]. Therefore, it is thought to be key question whether and how S1P regulates the production of CCL20 from bronchial ECs.

Table 1. The effects of S1P on the asthma-related gene expression in bronchial epithelial cells.

Gene	description	BEAS-2B	Calu-3
ADRB2	adrenoceptor beta 2	6.45	2.52
LIFR	leukemia inhibitory factor receptor alpha	0.89	1
SCGB1A1	secretoglobin family 1A member 1	1.13	1
CTLA4	cytotoxic T-lymphocyte associated protein 4	0.89	1.32
ETV3	ETS variant 3	1.52	1.37
HAVCR2	hepatitis A virus cellular receptor 2	0.71	1.8
TLR1	toll like receptor 1	N. D.	1.67
GPRA(NPSR1)	neuropeptide S receptor 1	N. D.	N. D.
CCL11	C-C motif chemokine ligand 11	0.92	1.04
CCL24	C-C motif chemokine ligand 24	0.96	1.01
SFSWAP(SFRS8)	splicing factor, suppressor of white-apricot family	0.71	1.08
TNF	tumor necrosis factor	N. D.	N. D.
TLR2	toll like receptor 2	1.01	0.98
ADAMTS12	ADAM metallopeptidase with thrombospondin type 1 motif 12	N. D.	N. D.
TLR8	toll like receptor 8	0.96	0.81
TLR10	toll like receptor 10	N. D.	N. D.
PTGER4	prostaglandin E receptor 4	8.53	4.64
CCL2	C-C motif chemokine ligand 2	1.37	N. D.
PHF11	PHD finger protein 11	1.1	1.34
PRLR	prolactin receptor	0.95	1.82
TLR3	toll like receptor 3	1.21	1.5
SETDB2	SET domain bifurcated 2	0.94	1.47
CCL5	C-C motif chemokine ligand 5	0.87	N. D.
LTA	lymphotoxin alpha	0.97	0.99
IFNG	interferon, gamma	2.42	N. D.
GSTM1	glutathione S-transferase mu 1	1.01	0.98
ADAM33	ADAM metallopeptidase domain 33	0.93	0.86
ZFR	zinc finger RNA binding protein	1.37	1.06
TLR9	toll like receptor 9	N. D.	N. D.
PCDH1	protocadherin 1	1.07	1.09
NPR3	natriuretic peptide receptor 3	0.83	N. D.
IL4	interleukin 4	0.46	0.83
TLR7	toll like receptor 7	1.13	0.65
DPP10	dipeptidyl peptidase like 10	0.95	1.05
TGFB1	transforming growth factor beta 1	1.2	0.87
ORMDL3	ORMDL sphingolipid biosynthesis regulator 3	1.03	1.03
GSTP1	glutathione S-transferase pi 1	0.98	1.04
TLR8	toll like receptor 8	N. D.	N. D.
TLR5	toll like receptor 5	0.8	2.88
CHRM1	cholinergic receptor, muscarinic 1	N. D.	N. D.
CYFIP2	cytoplasmic FMR1 interacting protein 2	1.19	1.09
HLA-G	major histocompatibility complex, class I, G	0.7	0.86
MS4A2	membrane spanning 4-domains A2	0.92	0.38
STAT6	signal transducer and activator of transcription 6, interleukin-4 induced	1.11	1.11
IL13	interleukin 13	0.92	0.92
IL5	interleukin 5	0.81	1.27
RCBTB1	RCC1 and BTB domain containing protein 1	0.87	0.9

(*Continued*)

Table 1. (Continued)

Gene	description	BEAS-2B	Calu-3
ETS2	ETS proto-oncogene 2, transcription factor	1.34	2.7
HAVCR1	hepatitis A virus cellular receptor 1	N. D.	N. D.
IL4R	interleukin 4 receptor	1.39	1.25
KITLG	KIT ligand	1.07	1.21
IL9	interleukin 9	N. D.	N. D.
MUC8	mucin 8	0.97	1.21
IL7R	interleukin 7 receptor	1.31	2.12
PTGDR	prostaglandin D2 receptor (DP)	N. D.	N. D.
LTC4S	leukotriene C4 synthase	0.7	0.91
CD14	CD14 molecule	1.07	0.84
TLR6	toll like receptor 6	1.5	1.4
NOS1	nitric oxide synthase 1	N. D.	N. D.

The fold difference was obtained by dividing the mRNA level in S1P-treated ECs with that in S1P-untreated ECs.

https://doi.org/10.1371/journal.pone.0203211.t001

Role of S1P/S1PR3 axis in release of CCL20 from bronchial ECs

We analyzed the expression of *S1PRs* in the Calu-3 cells and confirmed the expression of *S1PR3* (Fig 2A). It could be possible that S1PR3 is more labile than S1PR2, and that is why the mRNA expression of *S1PR3* is higher than S1PR2. Therefore, we knocked down the *S1PR3* genes using siRNA. The efficacies of gene knockdown of *S1PR3* were about 80% (Fig 2B–2D). Ablation of *S1PR3* by siRNA significantly suppressed the gene expression of *CCL20* induced by S1P ($P<0.01$ to 0.05, Fig 2E). These results suggested that S1P induced *CCL20* expression via S1PR3.

Attenuation of eosinophilic inflammation in the lung of VPC23019-treated mice

To evaluate the role of CCL20 on bronchial asthma, we employed OVA-induced asthma mouse model. Twenty-four hours after the last OVA challenge, we performed BAL analyses and confirmed that the anti-CCL20 antibody reduced eosinophil percentage of total from 17% to 2% and significantly decreased eosinophil counts in the OVA-induced asthma mouse model ($P<0.01$, Fig 3A). This result suggested that CCL20 has a crucial role in the experimental asthma mouse model. To evaluate the effect of VPC23019, S1PR1 and S1PR3 antagonist, on experimental asthma, we carried out histologic analyses of the bronchus at 24 h after the last challenge. The accumulation of inflammatory cells around the bronchiole and mucin-producing epithelial cells positive for PAS staining was substantially reduced in VPC23019-treated mice (Fig 3B). Next, we performed a BAL examination for inflammatory cell counts using cytospin 24 h after the final OVA challenge using VPC23019). VPC23019 pretreatment significantly decreased the number of total cell and eosinophil cell compared vehicle pretreatment ($P<0.05$, Fig 3C). A similar trend was seen in the number of lymphocytes and neutrophils, but the difference was not statistically significant ($P>0.05$). Immunostaining demonstrated that S1P-induced CCL20 expression was suppressed by VPC23019 in lung structural cells (Fig 3D). On the oher hand, VPC23019 did not suppress the expression of mRNAs of *CCL3*, *TIMP2*, and *IL-8* (Fig 3E). These results suggested that VPC23019 attenuated the eosinophilic inflammation.
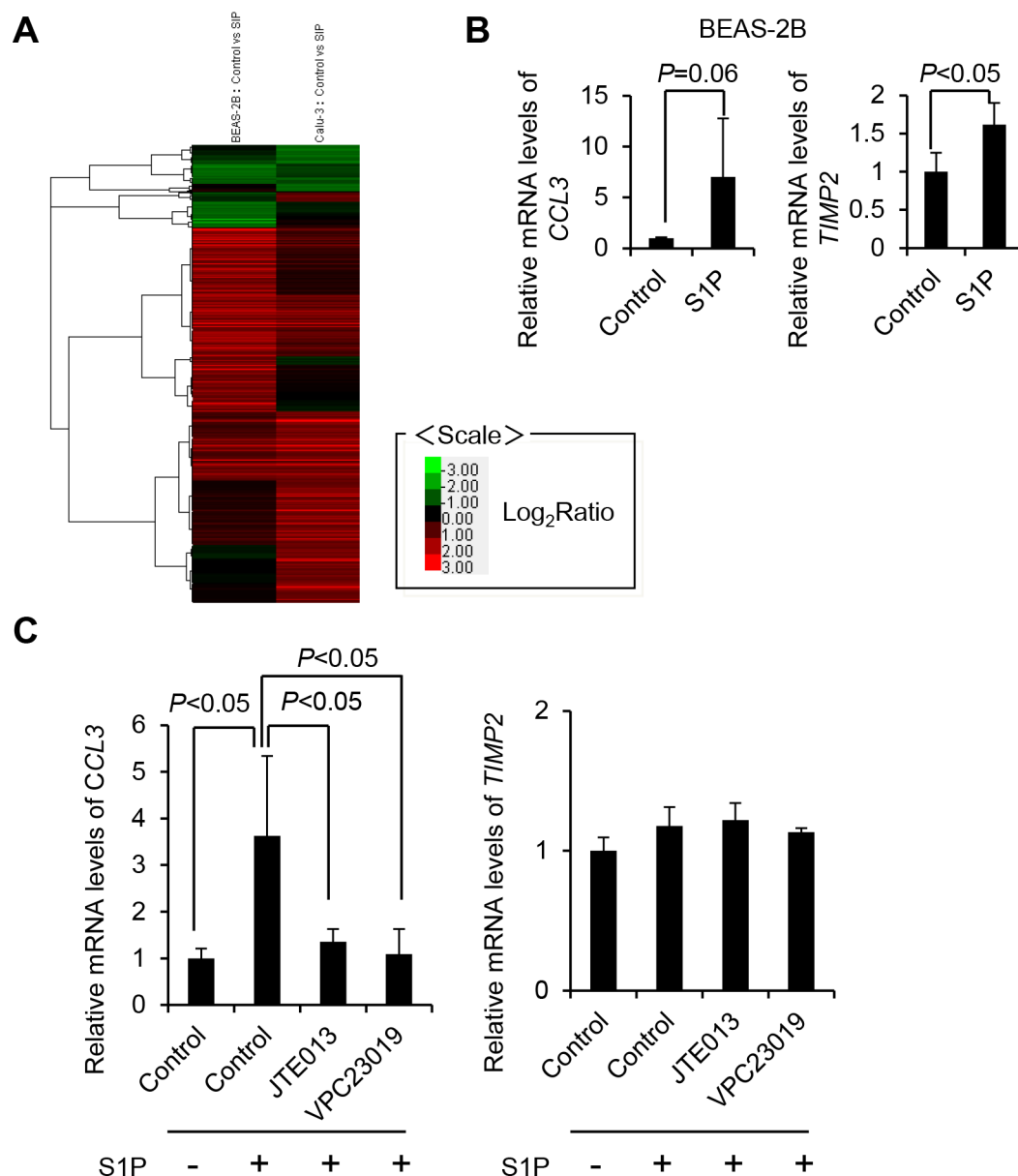
Fig 1. A hierarchical cluster analysis performed with Pearson's correlation coefficient using log2 ratio data and qRT-PCR analysis of *CCL3* and *TIMP2*. BEAS-2B and Calu-3 were stimulated with or without S1P (1 μM) for 2 h. Total RNA labeled with Cy3 or Cy5 was hybridized to a 3D-Gene Human Oligo chip 25 k (n = 1). Genes with Cy3/Cy5 normalized ratios greater than 2.0 were identified. A total of 967 genes that were upregulated to more than 2-fold or downregulated to less than 2-fold in at least 1 pair were analyzed (A). Relative mRNA levels of *CCL3* and *TIMP2* were analyzed by qRT-PCR in BEAS-2 (B) and Calu-3 (C).

Discussion

Sphingolipid metabolites have a role in the pathogenesis of asthma. S1P has been shown to promote airway hyper-reactivity through S1PR1 and S1PR3 and regulates immune cells, such as mast cells, eosinophils and DCs [23]. In the current study, we newly clarified the possible effects of S1P/S1PR3 axis on the expression of the CCL20 from the airway ECs and showed therapeutic effect of VPC23019 on airway eosinophilic inflammation.

Table 2. The S1P-inducing genes whose expression increased to four-fold or greater.

SYMBOL	description	BEAS-2B	Calu-3
KRT34	keratin 34, type I	8.84	5.56
CCL20	C-C motif chemokine ligand 20	53.65	7.33
ZFP57	ZFP57 zinc finger protein	11.27	6.13
RNF39	ring finger protein 39	5.73	6.95
TAGLN	transgelin	5.2	28.71
EREG	epiregulin	14.2	5.01
PPP1R3B	protein phosphatase 1 regulatory subunit 3B	6.43	10.33
FST	follistatin	5.47	9.47
PTGS2	prostaglandin-endoperoxide synthase 2	12.59	23.29
EDN1	endothelin 1	18.45	13.07
SERPINB2	serpin peptidase inhibitor, clade B (ovalbumin), member 2	25.01	59.43
IL1B	interleukin 1 beta	6.39	8.18
GBP1	guanylate binding protein 1	9	6.16
CTGF	connective tissue growth factor	20.12	12.83

Only 14 genes were up-regulated 4-fold in both pairs.

https://doi.org/10.1371/journal.pone.0203211.t002

The current study also showed that S1P induced *ADRB2* and *PTGER4* in the airway ECs. The β2-adrenoceptor gene (*ADRB2*) has genetic variability at amino acids 16 and 27 and correlates with some clinical features of bronchial asthma, including bronchial hyper-responsiveness [24, 25]. The Arg16 allele of *ADRB2* also has an important role in differential responses to β2-agonist therapy in bronchial asthma [26]. It is important to ensure consistency between humans and mice, which are one of the most commonly used species for modeling bronchial asthma. Similar to that of humans, the distribution of murine β2 adrenergic receptor (β2AR) subtypes is heterogeneous in lung [27]. B2AR is reportedly expressed in mouse tracheal epithelium (71%) and airway smooth muscle (ASM) (31%) [28, 29], and β2AR in bronchial smooth muscle plays an important role in bronchodilation in mice [30]. In addition, it was recently reported that β2AR signaling in airway ECs promotes eosinophilic inflammation, mucous metaplasia, and airway contractility [31]. In contrast, single nucleotide polymorphisms (SNPs) of prostaglandin E4 receptor (*PTGER4*) are reported to be associated with asthma symptoms [32]. The prostaglandin E (PGE) receptor 4 is expressed in a variety of tissues including lung, and has important roles in inflammation and immediate hypersensitivity. Recently, it was reported that S1P represses β2-adrenergic activity in human ASM cells by increasing cyclo-oxygenase (COX)-2-mediated PGE2 production [33]. Therefore, *ADRB2* and *PTGER4* may contribute to the S1P-induced experimental asthma mouse model. Table 1 show there is significant difference in the fold change of gene expression between BEAS-2B and Calu-3 cells. The kinetic difference among these cells in the $[Ca^{2+}]_i$ response which is induced by protease activated receptor 2 agonist peptide and Alternaria extract may be the cause of difference of cellular response [13].

In the current study, we focused on *CCL20*. A range of chemokines, including CCL20, CCL19, and CCL27, are primarily released by the bronchial ECs in response to stimulants, such as microbes or irritants [34]. These chemokines direct DC migration toward the epithelium and underlying mucosa through the ligands for CCR6, CCR7 and CCR10 [35]. Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) is abundantly expressed in the airway ECs of allergic mice and is related to the production of the chemokine CCL20. Inhibition of TRAIL is reported to impair the homing of myeloid DCs and T cells expressing CCR6 and CD4 to the airways, thereby suppressing several phenotypes of bronchial asthma, including
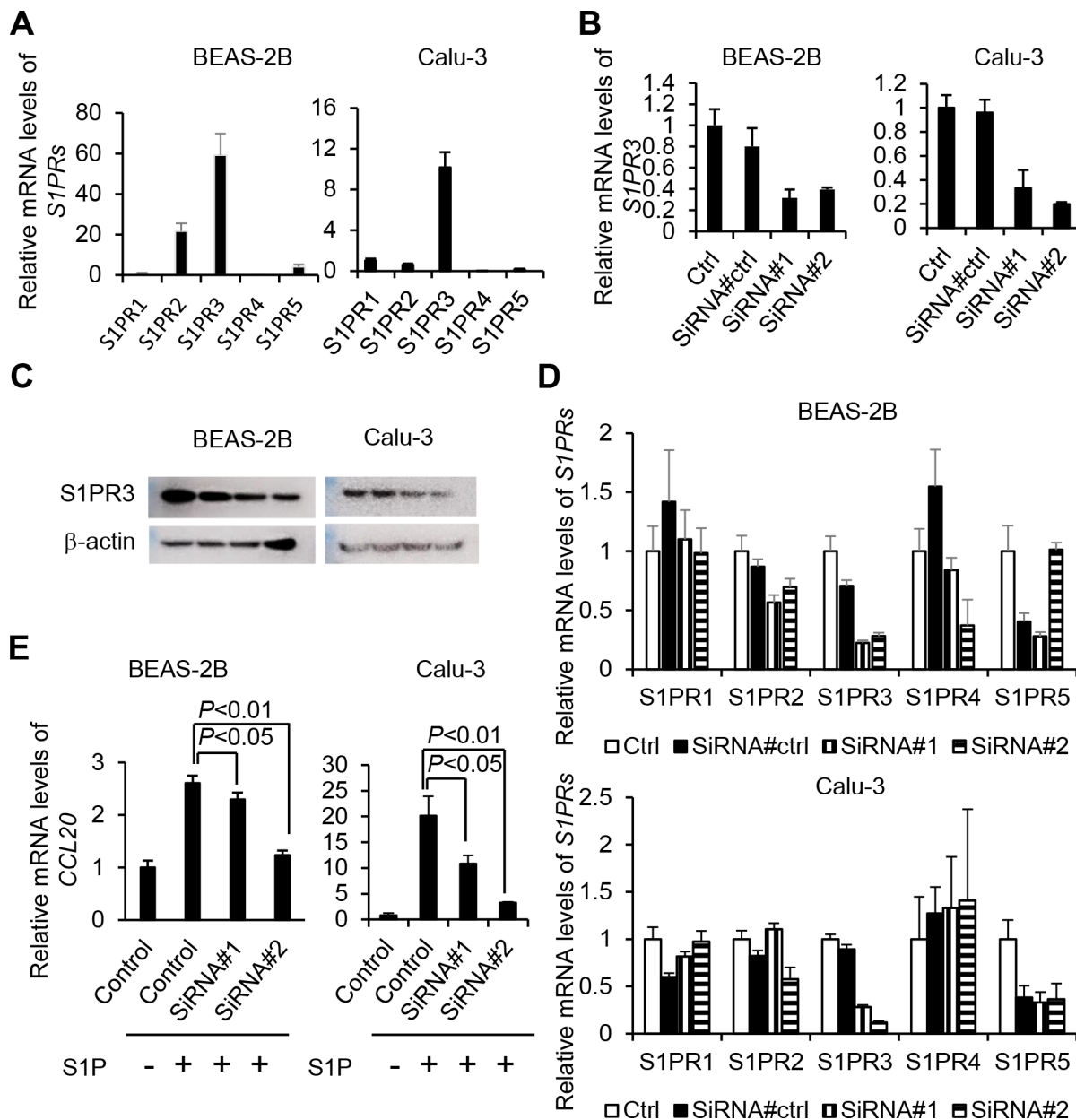
Fig 2. The assessment of role of S1PR3 in the bronchial epithelial cells. Relative mRNA levels of *S1PRs* in BEAS-2B and Calu-3 were analyzed by qRT-PCR (A). Gene expression knockdown of *S1PR3* was performed by the two pairs of siRNA, as described in the Materials and Methods. Data are expressed as the mean ± SEM (B). Protein levels of S1PR3 after the treatment of siRNA in BEAS-2B and Calu-3 were analyzed by western blot analyses (C). Relative mRNA levels of S1PRs after the treatment of siRNA in BEAS-2B and Calu-3 were analyzed by qRT-PCR (D). Relative mRNA levels of *CCL20* were analyzed by qRT-PCR in BEAS-2 and Calu-3 that had been treated with two pairs of siRNA of *S1PR3* and stimulated by PBS or 1 μM S1P for 2 h. Data are expressed as the mean ± SEM (E).

T$_H$2 cell cytokine release, inflammation, airway hyper-reactivity and the expression of the transcriptional activator STAT6 [36]. The current findings suggest that CCL20 has a crucial role in our experimental asthma model.

In conclusion, S1P/S1PR3 pathway has a role in release of CCL20, which has a role in experimental asthma mouse model, from bronchial ECs. Our results suggest that S1P/S1PR3 axis may be a possible candidate for the treatment of bronchial asthma.
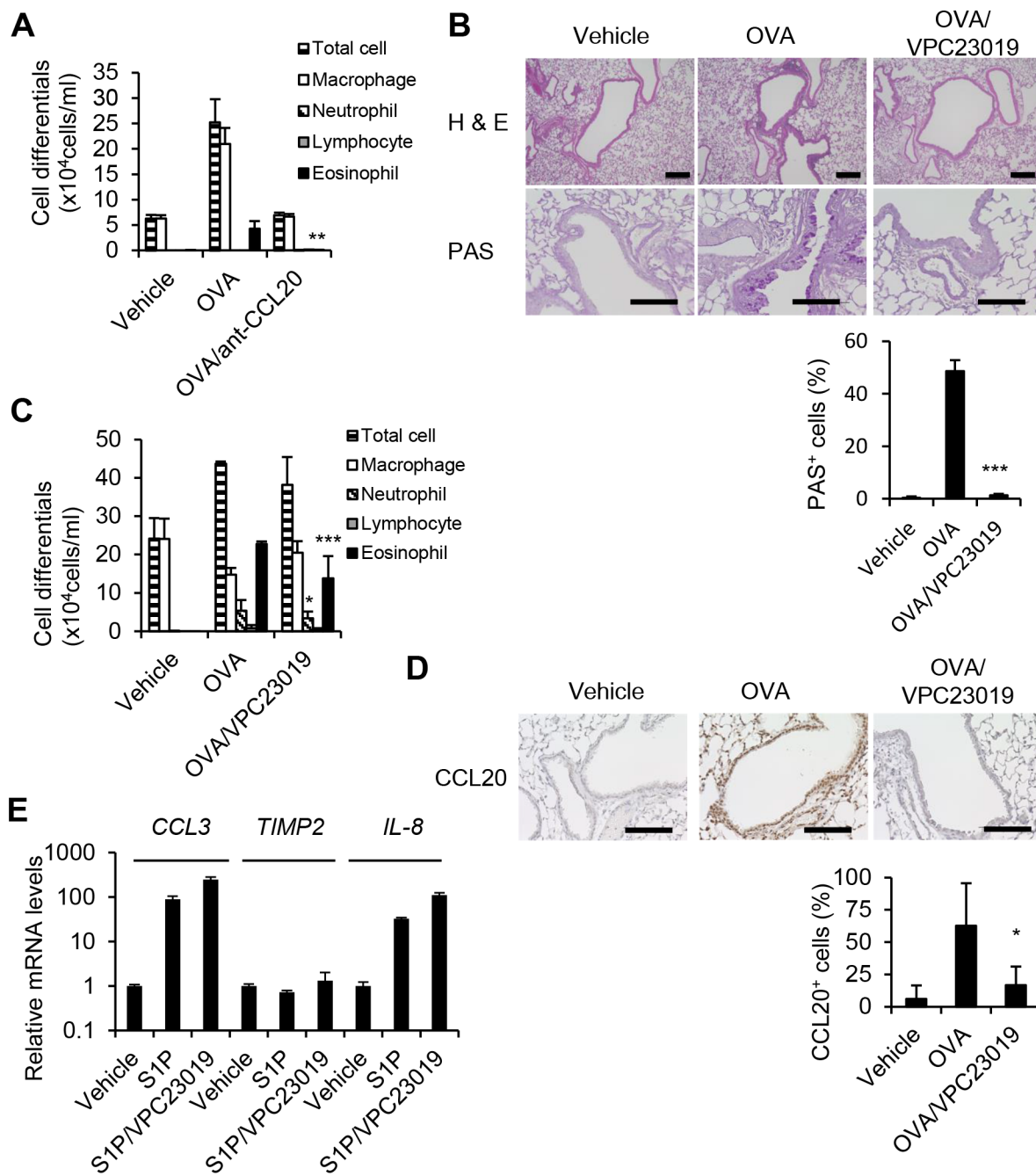
Fig 3. The analysis of the effect of VPC23019 in the experimental asthma mouse model. OVA-sensitized mice were administered PBS or anti-CCL20 antibody (20 µg/mouse) via i.p. injection before OVA inhalation on days 21 and 22. At day 23, BALF was collected, and inflammatory cells were analyzed. Data are expressed as the mean ± SEM obtained from three mice per group (A). Lungs and BALF were collected one day after the last challenge of the OVA-sensitized mice with the aerosol containing OVA or with PBS alone. Some mice received an intraperitoneal injection of 1 µg VPC23019 in a volume of 500 µl sterile PBS 30 minutes before each OVA exposure. Lung sections were subjected to H&E staining or PAS staining. *Bars*, 100 µm. PAS+ bronchial epithelial cells and total epithelial cells were counted on the specimens and the percentage of PAS+ epithelial cells was determined as 100 × (PAS+ cell number)/(total epithelial cell number) (%). Data are expressed as the mean ± SEM (n = 3) (B). The total inflammatory cells in the BALF were counted. For differential inflammatory cells counting, inflammatory cells were spun down and stained with Diff-Quick. Data are expressed as the mean ± SEM obtained for three mice in each group (C). Paraffin-embedded sections of the lung were stained with the antibody against CCL20 (*brown*) CCL20+ bronchial epithelial cells and total epithelial cells were counted on the specimens and the percentage of CCL20+ epithelial cells was determined as 100 × (CCL20+ cell number)/(total epithelial cell number) (%). Data are expressed as the mean ± SEM (n = 3) (D). BEAS-2B cells were treated with S1P (1 µM) and VPC23019 (10 µM) (B) for 3 h, and CCL3, TIMP2, and IL-8 gene expressions were analyzed by quantitative real-time RT-PCR. Data are expressed as the mean ± SEM (n = 3) (E). *, $P<0.05$ between three groups; **, $P<0.01$ between three groups; ***, $P<0.001$ between three groups.

Supporting information

S1 Fig. A hierarchical cluster analysis was performed with Pearson's correlation coefficient using log2 ratio data.
(XLSX)

Acknowledgments

Author Contributions

Conceptualization: Tatsuya Nagano, Hiroshi Kamiryo, Kazuyuki Kobayashi, Yoshihiro Nishimura.

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Software: Masatsugu Yamamoto.

Supervision: Kazuyuki Kobayashi, Yoshihiro Nishimura.

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Writing – review & editing: Tatsuya Nagano.

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