Identification of Genes Expressed by Human Airway Eosinophils after an *In Vivo* Allergen Challenge

Stephane Esnault[1]*, Elizabeth A. Kelly[1], Elizabeth A. Schwantes[1], Lin Ying Liu[1], Larissa P. DeLain[1], Jami A. Hauer[1], Yury A. Bochkov[2], Loren C. Denlinger[1], James S. Malter[3], Sameer K. Mathur[1], Nizar N. Jarjour[1]

1 Department of Medicine, Allergy, Pulmonary, and Critical Care Medicine Division, University of Wisconsin School of Medicine and Public Health, Madison, Wisconsin, United States of America, **2** Department of Pediatrics, University of Wisconsin School of Medicine and Public Health, Madison, Wisconsin, United States of America, **3** Department of Pathology, University of Texas Southwestern Medical Center, Dallas, Texas, United States of America

Abstract

Background: The mechanism for the contribution of eosinophils (EOS) to asthma pathophysiology is not fully understood. Genome-wide expression analysis of airway EOS by microarrays has been limited by the ability to generate high quality RNA from sufficient numbers of airway EOS.

Objective: To identify, by genome-wide expression analyses, a compendium of expressed genes characteristic of airway EOS following an *in vivo* allergen challenge.

Methods: Atopic, mild asthmatic subjects were recruited for these studies. Induced sputum was obtained before and 48h after a whole lung allergen challenge (WLAC). Individuals also received a segmental bronchoprovocation with allergen (SBP-Ag) 1 month before and after administering a single dose of mepolizumab (anti-IL-5 monoclonal antibody) to reduce airway EOS. Bronchoalveolar lavage (BAL) was performed before and 48 h after SBP-Ag. Gene expression of sputum and BAL cells was analyzed by microarrays. The results were validated by qPCR in BAL cells and purified BAL EOS.

Results: A total of 299 transcripts were up-regulated by more than 2-fold in total BAL cells following SBP-Ag. Mepolizumab treatment resulted in a reduction of airway EOS by 54.5% and decreased expression of 99 of the 299 transcripts. 3 of 6 post-WLAC sputum samples showed increased expression of EOS-specific genes, along with the expression of 361 other genes. Finally, the intersection of the 3 groups of transcripts (increased in BAL post SBP-Ag (299), decreased after mepolizumab (99), and increased in sputum after WLAC (365)) was composed of 57 genes characterizing airway EOS gene expression.

Conclusion: We identified 57 genes that were highly expressed by BAL EOS compared to unseparated BAL cells after *in vivo* allergen challenge. 41 of these genes had not been previously described in EOS and are thus potential new candidates to elucidate EOS contribution to airway biology.

Citation: Esnault S, Kelly EA, Schwantes EA, Liu LY, DeLain LP, et al. (2013) Identification of Genes Expressed by Human Airway Eosinophils after an *In Vivo* Allergen Challenge. PLoS ONE 8(7): e67560. doi:10.1371/journal.pone.0067560

Editor: Phillip A. Stumbles, Murdoch University, Australia

Received March 21, 2013; **Accepted** May 20, 2013; **Published** July 2, 2013

Copyright: © 2013 Esnault et al. This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

Funding: This work was supported in part by a Program Project Grant (NIH HL088594), the University of Wisconsin General Clinical Research Center grant (NIH M01RR03186), and the University of Wisconsin Institute for Clinical and Translational Research (NCRR/NIH 1UL1RR025011). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: N.N. Jarjour received honoraria for lectures sponsored by GSK that did not exceed $5000. The other authors have no financial conflict of interest. The other authors have declared that no competing interests exist. This does not alter the authors' adherence to all the PLOS ONE policies on sharing data and materials.

* E-mail: sesnault@medicine.wisc.edu

Introduction

Recruitment of EOS to the lung has been reproducibly reported in allergic asthma [1]. While airway eosinophilia is commonly associated with increased risk for asthma exacerbation, severity, and poor prognosis [2–4], the precise correlation of EOS to the pathophysiology of asthma remains controversial. Reduction of airway EOS is associated with decline of submucosal matrix protein deposition and airway smooth muscle hyperplasia [5,6] suggesting that EOS contribute to airway remodeling. Through the production and release of pro-inflammatory mediators, EOS can amplify the expression of Th1, Th2, and Th17 cytokines and chemokines [7–9] indicating they play a role in the adaptive immune response. Recent trials of anti-IL-5 antibodies (mepolizumab and reslizumab) have shown benefits in asthma, particularly in reducing rates of exacerbations [10–12].

One approach to understanding the biology of EOS in asthma is gene expression analysis by microarrays. Initial GeneChip analysis, which was performed using *in vitro* IL-5-activated circulating EOS, identified 66 genes that were up-regulated by IL-5 and predicted to have functions in adhesion, recruitment, activation and survival [13]. A subsequent study performed by our group showed that the expression of more than 200 genes was increased *in vitro* in IL-5- and GM-CSF-activated EOS, including

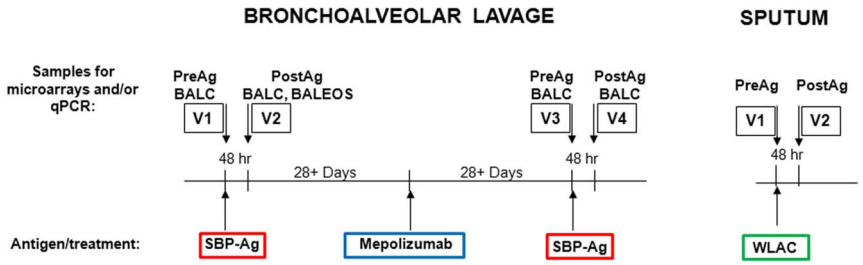

Figure 1. Bronchoalveolar lavage and sputum timelines. Atopic mild asthmatics underwent bronchoalveolar lavage (BAL) followed by segmental challenge with an allergen (SBP-Ag) on visit 1 (BAL V1). 48 h later on visit 2 (BAL V2), BAL was performed at the site of the challenged segment. One month later, a single dose of mepolizumab was administered. One month after dosing, BALs were repeated both before and after SBP-Ag. BAL cells (BALC) were prepared on visits 1, 2, 3, and 4 and airway EOS (BALEOS) were purified on visit 2. For the induced sputum study, sputum collection was performed followed by a whole lung allergen challenge (WLAC) on visit 1 (sputum V1). Collection of a second induced sputum was done 48 h after WLAC on visit 2 (sputum V2).
doi:10.1371/journal.pone.0067560.g001

the anti-apoptotic serine/threonine protein kinase Pim-1 [14,15]. During their egress to the airway, in response to allergen, the phenotype of peripheral blood EOS changes dramatically [16–18]; however, gene analysis with microarrays of airway EOS has not been explored.

We performed gene expression array analysis on sputum samples obtained following whole lung allergen challenge (WLAC), and on bronchoalveolar lavage (BAL) cells obtained following segmental bronchoprovocation with an allergen (SBP-Ag). These two *in vivo* allergen challenge models are well-established asthma models that lead to eosinophilic airway inflammation [19–21]. Typically SBP-Ag causes EOS to increase in the BAL from ~0.5% at baseline to ~70% after allergen challenge [9] while WLAC induces an increase of EOS in sputum from ~3% to ~10% [21–24]. Therefore, we anticipated that analysis of total BAL and sputum cells by microarrays after SBP-Ag and WLAC and purified BAL EOS would facilitate identification of genes specifically expressed by airway EOS.

Materials and Methods

Subjects

The study was approved by the University of Wisconsin-Madison Health Sciences Institutional Review Board (IRB). Informed written consent was obtained from subjects prior to participation. Subjects had a history of mild atopic asthma as defined by at least one positive skin prick test and a history of allergen-induced asthma exacerbation with reversibility to albuterol >12% and/or a provocative concentration of methacholine producing a 20% fall in FEV_1 (PC_{20}) of <8 mg/ml. None of the subjects were using inhaled or oral corticosteroids. 10 subjects finished the SBP-Ag protocol and 2 were excluded for lower percentage of EOS in BAL after SBP-Ag (<67%) or low purity of BAL EOS (<99%). 18 subjects completed the sputum protocol and 12 were excluded due to having <500,000 cells, greater than 60% epithelial cells in the sputum, a greater than 2-fold difference in the percentage of epithelial cells pre- and post- WLAC, lack of RNA after extraction, or RNA integrity that was insufficient for microarrays.

Bronchoscopy, SBP-Ag, and Anti-IL-5 Administration

Detailed methods for bronchoscopy, SBP-Ag, and BAL cell preparation have previously been described [25]. A graded inhaled Ag challenge with *Dermatophagoides farinae* (Der p 1, dust mite), *Felis catus domesticus* (Fel d 1, cat) or *Ambrosia artemisiifolia* (Amb a 1, ragweed), obtained from Greer Laboratories (Lenoir, NC, USA) was performed to determine the participant's $AgPD_{20}$ (the provocative dose of Ag leading to a 20% fall in FEV_1). One month later, a baseline bronchoscopy with BAL (4 × 40 ml aliquots of sterile 0.9% NaCl) was performed followed by administration of Ag at a dose of 20% of the $AgPD_{20}$ into the lavaged segment (BAL V1, Figure 1). Bronchoscopy with BAL was repeated 48 h later (BAL V2, Figure 1). One month after the first SBP-Ag, a single dose of anti-IL-5 (750 mg, mepolizumab generously donated by GlaxoSmithKline) was administered intravenously to reduce the number of airway EOS. One month after mepolizumab treatment, the second SBP-Ag was performed with BAL immediately before (BAL V3) and 48 h later (BAL V4). Circulating EOS number was <100 per µl at the time of the second SBP-Ag (BAL V3). BAL cells were assessed by hemocytometer using Turk's counting solution containing acetic acid and methylene blue and cell differentials were determined on cytospin preparations stained with the Wright-Giemsa-based Hema-3 (ThermoFisher/Thermo Scientific, Rockford, IL, USA). On BAL V2 (Figure 1), airway EOS were purified from BAL cells using a two-step Percoll gradient as previously described [8]. EOS were collected from the 1.085/1.100 g/ml interface.

Induced Sputum

Induced sputum was obtained as previously described [22] on visit 1 (sputum V1, Figure 1) and 48 h after a graded WLAC leading to a 20% fall in FEV_1 (sputum V2). Briefly, patients inhaled nebulized 3% saline for 5 minutes, rinsed out their mouth and then coughed with collection of the sputum produced. This was repeated a total of three times. The sputum was diluted 1:1 with PBS. After centrifugation, cytospins were prepared and stained with Giemsa to determine cell distributions. Cells were stored in Trizol solution for RNA purification.

RNA Preparation and Microarray Hybridization

Total RNA was extracted from BAL cells or purified BAL EOS using the RNeasy Mini Kit (Qiagen, Valencia, CA, USA), treated with DNase (RNase-free DNase kit, Qiagen), and RNA from BAL cells were submitted to the University of Wisconsin-Madison Gene Expression Center (Madison, WI) for labeling and hybridization. RNA quality and integrity was evaluated via Agilent 2100 Bioanalyzer platform (Agilent Technologies). Sense-strand cDNA was generated from total RNA using the Ambion Whole Transcript (WT) Target Expression Kit (Life Technologies, Grand Island, NY, USA). Fragmentation and labeling of the single-stranded cDNA was performed using the Affymetrix GeneChip WT Terminal Labeling Kit (Affymetrix, Santa Clara, CA, USA).

The labeled cDNAs were hybridized to Human Gene ST 1.0 GeneChips (Affymetrix) according to the manufacturer's protocols.

Induced sputum samples were shipped to Miltenyi Biotech for RNA extraction using standard RNA Trizol extraction protocols. Sample integrity was evaluated via Agilent 2100 Bioanalyzer platform (Agilent Technologies). 50 ng of RNA was amplified and labeled using the Aglient Low Input Quick Amp Labeling Kit (AgilentTechnologies). Hybridization was performed using the Agilent Gene Expression Hybridization Kit to Agilent Whole Human Genome Oligo Microarrays 8X60K for microarray analyses. The Agilent Feature Extraction Software was used to read out and process the microarray image files.

Microarray Data Analysis

For BAL cells, raw data (CEL files) were uploaded into ArrayStar software version 4.0 (DNASTAR Inc., Madison, WI, USA) for normalization and statistical analysis. The robust multichip analysis (RMA) algorithm was used for background correction, quantile normalization and median polish summarization. Microarrays are deposited in the Gene Expression Omnibus repository (accession number: GSE46159). Four pairs of data comparisons that might reveal genes up-regulated in BAL cells after allergen challenge with or without mepolizumab were performed. The transcripts were filtered on the basis of ≥2-fold difference. The 99 genes up-regulated by SBP-Ag and decreased 1.5-fold by mepolizumab (Table S5) were determined using both the direct fold change between BAL V4 and V2 and the fold change between BAL V2 and V1 divided by the fold change between BAL V4 and V3 (see Figure 1).

For induced sputum samples, raw data files were analyzed using the Agilent Feature Extraction Software (FES) (Agilent Technologies). The software was used to determine feature intensities, perform background subtraction, reject outliers, and calculate statistical confidences. Microarrays are deposited in the Gene Expression Omnibus repository (accession number: GSE46238).To determine differential gene expression, the data outputted from FES was then analyzed using the Rosetta Resolver gene expression data analysis system (Rosetta Biosoftware). Ratios were calculated as control vs. sample, or before and after allergen challenge, by division of sample intensity through control signal intensity.

Real-time qPCR

The reverse transcription reaction was performed using the Superscript III system (Invitrogen/Life Technologies, Grand Island, NY, USA). mRNA expression was determined by qPCR using SYBR Green Master Mix (SABiosciences, Frederick, MD, USA) and human IL5RA, LTC4S, FFAR2, CNR2, ARAP3, DAPK2, PRSS33, TNFSF14, IL1R1 and FCER2 (CD23ß) forward and reverse specific primers (see Table S1 for primer sequences) were designed using Primer Express 3.0 (Applied Biosystems, Carlsbad, CA, USA) and blasted against the human genome to determine specificity using http://www.ncbi.nlm.nih.gov/tools/primer-blast. The reference gene, ß-glucuronidase ((GUSB), forward: caggacctgcgcacaagag, reverse: tcgcacagctggggtaag), was used to normalize the samples. Standard curves were performed and efficiencies were determined for each set of primers. Efficiencies ranged between 91 and 96%. Data are expressed as fold change using the comparative cycle threshold (ΔΔCT) method as described previously [9]. The values presented in Figure 4 are fold change = ($2^{-\Delta\Delta C_t}$) compared to expression in BAL cells at V1 (before SBP-Ag).

Statistical Analyses

To compare expression of genes in total BAL cells and purified BAL EOS by qPCR, data were log transformed and analyzed using the Student's paired *t*-test. Statistical analyses were performed using SigmaPlot 11.0 software package.

Results

Gene Expression Analysis in BAL Cells 48 h after SBP-Ag by Microarrays

While less than 1% of BAL cells were EOS before SBP-Ag, they increased to 73.9% ±4.2 (n = 8) of the total BAL cell population after SBP-Ag. After mepolizumab, SBP-Ag elicited a significant but much attenuated increase in EOS counts compared to before mepolizumab (34% ±9 versus 73.9% ±4.2; $p<0.001$, paired *t* test, n = 8) [26]. 2 of 8 subjects (Figure 2) were randomly chosen for gene expression analysis using a whole human genome transcript microarray. For these 2 subjects, EOS constituted 72 and 74% of the BAL cells after SBP-Ag and were reduced to 24 and 39% of the BAL when SBP-Ag was performed after mepolizumab.

In total BAL cells, expression of 299 genes was up-regulated by more than 2-fold 48 h after SBP-Ag (Figure 2 and Table S2). In both subjects, 22 transcripts were increased by more than 5-fold (Table S3) and 4 transcripts by more than 10-fold (ALOX15, CD1A, CD24 and FAM101B). The 299 genes up-regulated after SBP-Ag included marker genes for EOS (IL5RA, RNASE2, RNASE3, and SIGLEC8), and other genes well-known to be expressed by EOS (ADAM8, CCR3, CD69, CDA, GATA1, HRH4, ICAM3, IL1RL1, LTC4S, OXER1, P2RY2, PADI4, and SIGLEC10). Genes associated with lung remodeling were also found such as metalloproteinase genes (MMP12, MMP9), as well as some IL-13-induced chemokines (CCL13, CCL2) and Th2-associated genes (CCL22, FCER2, and IL1R2) [27–29]. While expression of genes coding for soluble mediators (e.g. cytokines) were unchanged, receptors for several mediators known to participate in lung fibrosis were up-regulated (CCR2, FGFR2, FLT1, IGF1R, IL1R1 and IL6R) [27,29,30]. 19 transcripts mostly expressed in dendritic cells, lymphocytes or mast cells (Table S4), Swissprot research at http://www.uniprot.org/) were also included in the 299 transcripts. Further analysis using DAVID Bioinformatics Resources indicated that 31 genes have been previously associated with asthma, allergy or a pulmonary disease (DAVID Bioinformatics Resources 6.7; National Institute of Allergy and Infectious Diseases (NIAID), NIH) (Table S5).

99 (33.1%) of the 299 transcripts elevated by more than 2-fold in BAL cells after SBP-Ag were decreased by more than 1.5-fold after reduction of EOS by mepolizumab (Table S6). Of note, 1.5- versus 2-fold reduction was chosen because mepolizumab decreased EOS percentage in BAL cells by ~50%. Among the 99 genes whose expression was down-regulated after SBP-Ag are the well-known EOS-specific genes (IL5RA, RNASE2, RNASE3 and SIGLEC8) and all the other transcripts mentioned above as previously known to be expressed by EOS (ADAM8, CD69, GATA1, HRH4, ICAM3, IL1RL1, LTC4S, OXER1, P2RY2, PADI4 and SIGLEC10) except for CCR3. In addition, other genes that are not known to be expressed by EOS were down-regulated by mepolizumab. Whether these genes are expressed by other cell types (lymphocytes, dendritic cells, macrophages, or mast cells) that are affected directly or indirectly by the lack of IL-5 or the reduction of EOS or directly expressed by EOS is not known.

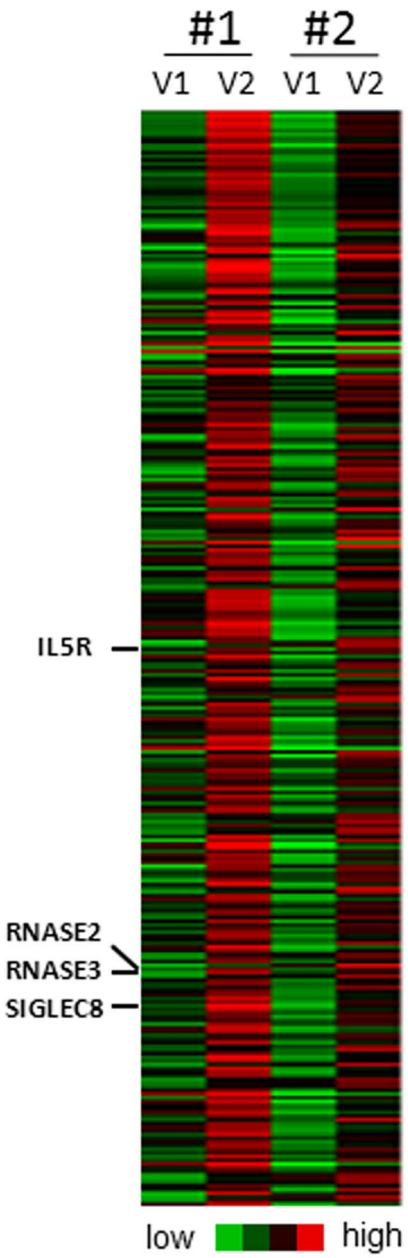

Figure 2. Heat map generated using Cluster 3.0 and presenting the 299 genes up-regulated by more than 2-fold in BAL cells from both subjects #1 and #2, 48 h after SBP-Ag (BAL V2 as shown in Figure 1) compared to before SBP-Ag (V1). Eosinophil-markers (IL5RA, RNASE2, RNASE3 and SIGLEC8) are highlighted. The full list of gene names is presented on Table S2.
doi:10.1371/journal.pone.0067560.g002

Identification of EOS-associated Genes by Microarray Using Induced Sputum after WLAC

Sputum cells from 6 subjects were analyzed by a whole genome transcript microarray. WLAC increased the percentage of EOS in sputum from 2.0 to 8.2% ($p<0.001$). Marker genes for EOS (RNASE2, RNASE3, IL5RA and SIGLEC8) were increased by more than 2-fold after WLAC in 3 of the 6 subjects involved in this analysis. Sputum samples from these 3 individuals allowed us to establish a list of 365 EOS-associated genes whose expression was increased by more than 1.5-fold after WLAC (Table 1). In addition to the original EOS markers (RNASE2, RNASE3, IL5RA and SIGLEC8), the 365 transcripts (Table 1) included some other well-known genes expressed by EOS. In fact, 11 of the 13 genes (ADAM8, CCR3, CD69, CDA, HRH4, ICAM3, IL1RL1, LTC4S, P2RY2, PADI4 and SIGLEC10) known to be expressed by EOS and up-regulated in BAL cells after SBP-Ag (Table S2) were also seen in sputum after WLAC.

Identification of Airway EOS Gene Expression

We have utilized 3 airway models that either augmented or reduced the EOS population, and defined genes concomitantly expressed with markers of EOS genes in the context of allergen challenges *in vivo*. Figure 3 shows that 168 (111+57) (46%) of the 365 EOS-associated genes in sputum were also identified in the 299 genes up-regulated in BAL cells. This suggests that despite distinct anatomical sites and provocative protocols, infiltrating EOS express a consistent group of transcripts. Furthermore, the intersection of these 2 sets with the genes decreased by mepolizumab identified a group of 57 genes (Figure 3 and Table 2). We propose that this intersection represents a stringent selection for gene expression by airway EOS. A literature search in PubMed (http://www.ncbi.nlm.nih.gov/pubmed/) indicates that only 16 of the 57 genes (28%) have been previously linked to EOS. Therefore, 41 genes (>70%) in this subset have not previously been associated with EOS.

Validation of the Airway EOS-associated Genes

To validate the expression of these genes by airway EOS, we performed qPCR for 8 of the 57 genes (Table 2). The samples from the 6 subjects not used in the microarray analyses were analyzed by qPCR. Gene expression in highly purified BAL EOS (BAL V2, see Figure 1) and total BAL cells after SBP-Ag before (BAL V2) and after mepolizumab infusion (BAL V4) are presented as fold change ($2^{-\Delta\Delta Ct}$ method) compared to gene expression in BAL cells before SBP-Ag (BAL V1). Initially, 2 established genes expressed by EOS were chosen as controls (IL5RA and LTCS4). As shown in Figure 4A, expression of both genes was highly up-regulated by SBP-Ag, and strongly decreased (>80%) by mepolizumab. Both genes displayed very high levels in pure BAL EOS compared to total BAL cells, suggesting that EOS are likely the main source of IL5RA and LTCS4 in BAL cells obtained after SBP-Ag. In Figure 4B, FFAR2 and CNR2 were selected as genes that have been connected with asthma and/or allergy and have been reported to be expressed by EOS [31,32]. Both genes exhibited the same profile as IL5RA and LTCS4, indicating that EOS are also an important source of FFAR2 and CNR2 in BAL cells. In addition, 4 of the 41 genes for which expression in EOS has not previously been described were randomly selected. As shown in Figure 4C, all 4 genes (ARAP3, DAPK2, PRSS33 and TNFSF14) presented the same profile of expression as IL5RA, LTCS4, FFAR2 and CNR2 demonstrating that these transcripts are highly expressed in airway EOS. IL1R1 and FCER2 (CD23β) were chosen as negative controls as they were up-regulated in unseparated BAL cells after SBP-Ag (Table S2) and part of the EOS-associated genes as established in sputum (Table 2), but were not affected by mepolizumab (Table S6). Conversely to IL5RA, LTCS4, FFAR2, CNR2, ARAP3, DAPK2, PRSS33 and TNFSF14, IL1R1 and FCER2 displayed very low expression in BAL EOS compared to total BAL cells (Figure 4D). Figure 4 validates the data obtained by microarrays and demonstrates that BAL EOS are likely the source of the 57 genes that increased post SBP-Ag in BAL cells. Using DAVID Bioinformatics Resources, genes were clustered according to

Table 1. EOS-associated genes in sputum after WLAC.

AATK	CD48	DNAJB5	GPR56	KCNK5	NKX6-2	RAB37	ST6GAL1
ABCB1	CD69	DNASE1L3	GPR84	KCNMB4	NOV	RAB3D	ST8SIA4
ABLIM2	CD93	DUSP2	GPR97	KCNN4	NPDC1	RASAL1	STAB1
ACAP1	CDA	DYSF	GPT	KCTD15	NRARP	RASSF2	SULF2
ACOX2	CDKN1C	ECE1	GRASP	KIF21B	NRG1	RASSF5	SYNE1
ACPP	CDRT8	EGR3	GRB10	KLF10	NTRK1	RD3	SYNE2
ADAM19	CECR6	EGR4	GSDMA	KRTAP19-1	ODF4	RGAG4	TAC4
ADAM28	CHI3L1	EIF2C2	HAS1	LBH	OLIG1	RGL4	TAGAP
ADAM8	CHST15	EMB	HBA2	LGALS12	OLIG2	RGS16	TAS2R50
ADAMTS10	CHST2	EMR4P	HBB	LGALS2	OSBPL3	RGS2	TBC1D3B
ADORA2A	CHSY1	ENHO	HBD	LIMK2	OSM	RHOH	TESC
AGER	CLC	ENPP1	HCG27	LPPR2	P2RY14	RNASE2	TET2
AGPAT9	CLEC10A	ENPP2	HDAC4	LRG1	P2RY2	RNASE3	TGM2
ALPL	CLEC4F	EPHB3	HDC	LTC4S	P2RY6	RNASE6	THBS4
AMPD2	CLEC4G	ETV3	HGF	LTF	PADI2	RNF19B	TIAM2
ANKH	CLEC5A	F13A1	HHIP	LY9	PADI4	RUFY4	TIMP1
APOBEC3B	CMTM2	FAM101B	HIC1	MARCKSL1	PAK1	RUNX2	TMEM154
ARAP3	CNR2	FAM198B	HIF1A	MAST4	PALLD	RYBP	TMEM156
AREG	COL18A1	FAM20A	HIP1R	MATK	PARM1	S100B	TMEM71
ASB2	COMP	FAM46B	HIST1H1D	MBOAT7	PDCD1	S1PR1	TNFRSF10C
ASGR2	CORO1A	FAM65B	HIST1H1E	MCTP2	PDE2A	SATB1	TNFSF14
ATHL1	CREB3L3	FAM83A	HIST1H2AC	MECOM	PGLYRP1	SCHIP1	TPCN1
ATL2	CREB5	FCAR	HIST1H2AE	MGAM	PHF19	SCNN1D	TREML2
ATP2A3	CSGALNACT2	FCER1A	HIST1H2BO	MGC24103	PHLDB1	SDS	TRERF1
ATP8B4	CST7	FCER2	HIST1H4A	MMP1	PIK3R5	SELL	TRPM6
B3GNT8	CTTNBP2	FCGR2B	HIST2H2BE	MMP10	PIK3R6	SEMA4B	TSPAN18
B4GALNT3	CX3CR1	FCN1	HSPA6	MMP12	PIP5K1B	SEMA7A	TTYH2
BIRC3	CXCR1	FFAR2	ICAM3	MMP25	PLAUR	SIGLEC10	VDR
CACNA1E	CXCR2	FFAR3	IFITM1	MMP7	PLD4	SIGLEC8	VEGFA
CACNA2D3	CXCR2P1	FGF11	IGFL1	MPZL3	PLEKHG2	SLC16A10	VENTX
CALCRL	CYP1B1	FHL3	IL10	MRC2	PLUNC	SLC24A3	VNN2
CAMK1	CYP4F12	FLJ11710	IL18R1	MRVI1	PPP1R14A	SLC26A8	VSTM1
CASS4	CYSLTR2	FNDC1	IL1R1	MSRB3	PPP2R2C	SLC2A13	WNT5A
CBFA2T3	CYTIP	FRY	IL1R2	MST4	PRDM1	SLC3A1	WTAP
CCL17	DAB2IP	FZD2	IL1RAP	MT1G	PRKCB	SLC40A1	XYLT1
CCL26	DACH1	GADD45A	IL1RL1	MTVR2	PRSS33	SLC6A10P	YPEL3
CCR3	DAPK2	GAGE7	IL21R	MXD1	PSTPIP1	SLC6A8	ZBTB46
CD177	DCHS1	GAS6	IL4	NCAM2	PTGIR	SLC7A5	ZBTB46
CD1A	DEFB122	GATA2	IL5RA	NCRNA00085	PTGS2	SNAI1	ZDHHC18
CD1B	DEFB130	GCOM1	IL6R	NCRNA00152	PTP4A3	SNHG3	ZDHHC8
CD1C	DENND3	GFRA2	INSIG1	NCRNA00287	PTPN7	SOCS2	ZNF395
CD1E	DGKA	GGT5	IRX6	NDE1	PVRL2	SORBS1	ZNF469
CD207	DGKD	GLYCTK	ITGB1BP2	NDEL1	PXN	SORL1	ZNF507
CD209	DIRAS1	GPR160	JHDM1D	NECAB2	PYROXD2	SPATA9	
CD226	DNAH17	GPR183	KCNH2	NHSL2	QPCT	SPNS3	
CD300LB	DNAJB1	GPR35	KCNJ15	NKD1	RAB33A	SPOCK1	

365 genes upregulated by more than 1.5 in each of the 3 subjects with high levels of EOS-characterizing genes (IL5RA, RNASE2, RNASE3 and SIGLEC8) after WLAC.
doi:10.1371/journal.pone.0067560.t001

their known functions. Six genes have been associated with a respiratory disease, 7 genes produce secreted proteins and 27 genes code for plasma membrane protein. Some other important clusters included cell adhesion (7 genes), immune

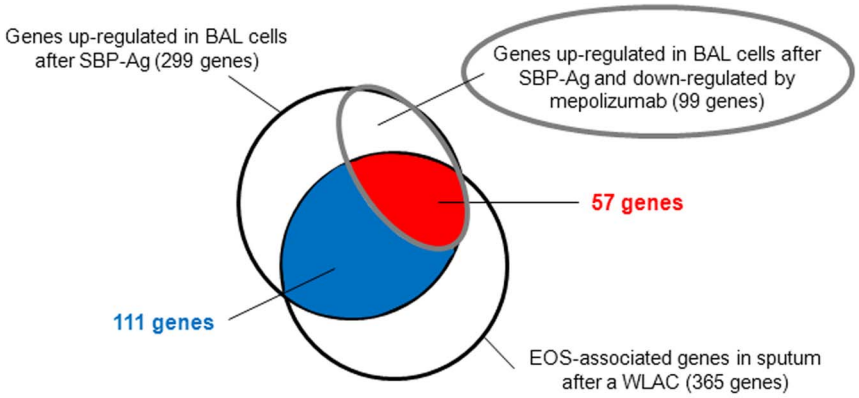

Figure 3. Venn diagram identifying 57 genes highly associated with airway EOS after an allergen challenge. 57 genes defined at the intersection of genes up-regulated in BAL cells after SPB-Ag, down-regulated when SBP-Ag was performed following mepolizumab, and part of the EOS-associated genes in the sputum following WLAC.
doi:10.1371/journal.pone.0067560.g003

and defense response (6 genes), and receptors for a variety of stimuli such as carbohydrates and nucleotides (15 genes) (Table S7).

Discussion

We have used a unique approach combining 3 whole genome expression microarrays (sputum cells after WLAC, BAL cells after SBP-Ag, and BAL cells after mepolizumab and SBP-Ag) to identify 57 genes highly expressed by airway EOS after an allergen challenge. We demonstrated that EOS are likely the main source of these 57 genes expressed by total BAL cells. The 57 genes included genes such as CD69, IL5RA, LTC4S, RNASE2, RNASE3, SIGLEC8, ADAM8, CDA, ICAM3, IL1RL1 (ST2), and P2RY2 that are known to be expressed in EOS, and other genes not previously linked to EOS. Cluster analysis indicated these genes might have an impact on EOS migration and activation, as well as the innate and adaptive immune responses.

Of the 57 transcripts associated with airway EOS, 16 have been previously detected in circulating EOS, including IL1RL1, CNR2, and FFAR2. IL1RL1 (also known as ST2) is the receptor for IL-33, a cytokine that is released following epithelial damage during an allergic reaction. IL-33 can activate $CD4^+$ T cells, type-2 innate lymphoid cells, and mast cells, as well as EOS [33]. The function of IL-33 on circulating EOS includes induction of degranulation, cytokine release, and increased survival. *In vivo*, blockade of the IL-33/IL1RL1 pathway reduces allergic inflammation and airway responsiveness [34]. Our study suggests that EOS might be the main source of IL-33 receptor among the airway inflammatory cells present after SBP-Ag, and EOS could contribute to IL-33-mediated effects in allergic asthma. CNR2 (also called CB2 receptor) is the cannabinoid receptor expressed in lymphoid organs [35]. CNR2 is known to be expressed by circulating EOS, and an endogenous ligand (2-arachidonoylglycerol) induces EOS migration [36]. In mice, genetic deletion of Cnr2 exacerbated contact allergic inflammation and influenza-induced excessive airway injury possibly via induction of pro-inflammatory and pro-remodeling mediators (IL-17, IL-13, TNF-α, GM-CSF) [37,38]. CNR2 agonists reduced bronchoconstriction, mast cell degranulation and pulmonary inflammation in guinea pigs [39]. Another endogenous CNR2 ligand, anandamide is increased in BAL fluids after SBP-Ag, and correlated with the number of EOS in the airway [40]. Finally, FFAR2 expression by EOS has only been reported once [32]. FFAR2 (also known as GPR43) is a lipid G-protein coupled receptor for short-chain fatty acids that is produced by bacteria after fermentation of dietary fibers. Interestingly, the lack of FFAR2 led to unresolved inflammation in several animal models of inflammation, including allergen-induced airway inflammation [32], suggesting FFAR2 activation by short-chain fatty acids would be beneficial to reduce asthma.

One example of potentially novel EOS-derived mediators is TNFSF14 (also called LIGHT), a membrane-expressed or secreted protein that interacts with TNFRSF14 membrane receptor (HVEM), and was characterized as a co-stimulatory ligand for lymphoid cells [41]. As most of the co-stimulatory members of the TNF/TNFR superfamily, TNFSF14 has been studied for its impact on autoimmune disease and transplant rejection [41]. More recently, LIGHT has been implicated in induction of airway fibrosis and smooth muscle hyperplasia via TGF-ß and IL-13 production in an allergic animal model [42]. Interestingly, in human sputum, LIGHT protein level has been associated with lower lung function ($FEV_{1\%}$ predicted) [43].

Another example of novel mediators in human EOS is PGLYRP1 or PGRP-S (also called Tag7), which recognizes bacterial peptidoglycan and has an antibacterial function [44]. PGLYRP1 is a 21Kd protein with a signal peptide for secretion.

Table 2. 57 genes upregulated in BAL cells after SBP-AG, down-regulated when SBP-Ag was performed following mepolizumab, and part of the EOS-associated genes in sputum after WLAC.

ADAM28	**CNR2***	FFAR3	LGALS12	**PRSS33***	SPNS3
ADAM8	CORO1A	FHL3	**LTC4S***	RAB37	TESC
ARAP3*	CYP4F12	GGT5	MCTP2	RAB3D	**TNFSF14***
ASB2	DACH1	GPR56	MMP25	RD3	TREML2
ATP2A3	**DAPK2***	GPR97	NHSL2	RNASE2	TRPM6
CASS4	DGKD	ICAM3	P2RY2	RNASE3	TSPAN18
CD300LB	EMR4P	IFITM1	PADI2	SIGLEC10	VSTM1
CD69	FAM101B	IL1RL1	PADI4	SIGLEC8	
CDA	FAM65B	**IL5RA***	PGLYRP1	SLC24A3	
CHST15	**FFAR2***	KIF21B	PIK3R6	SORL1	

*genes chosen for validation of the list by qPCR.
IL5RA, RNASE2, RNASE3 and SIGLEC8 are known markers of EOS.
doi:10.1371/journal.pone.0067560.t002

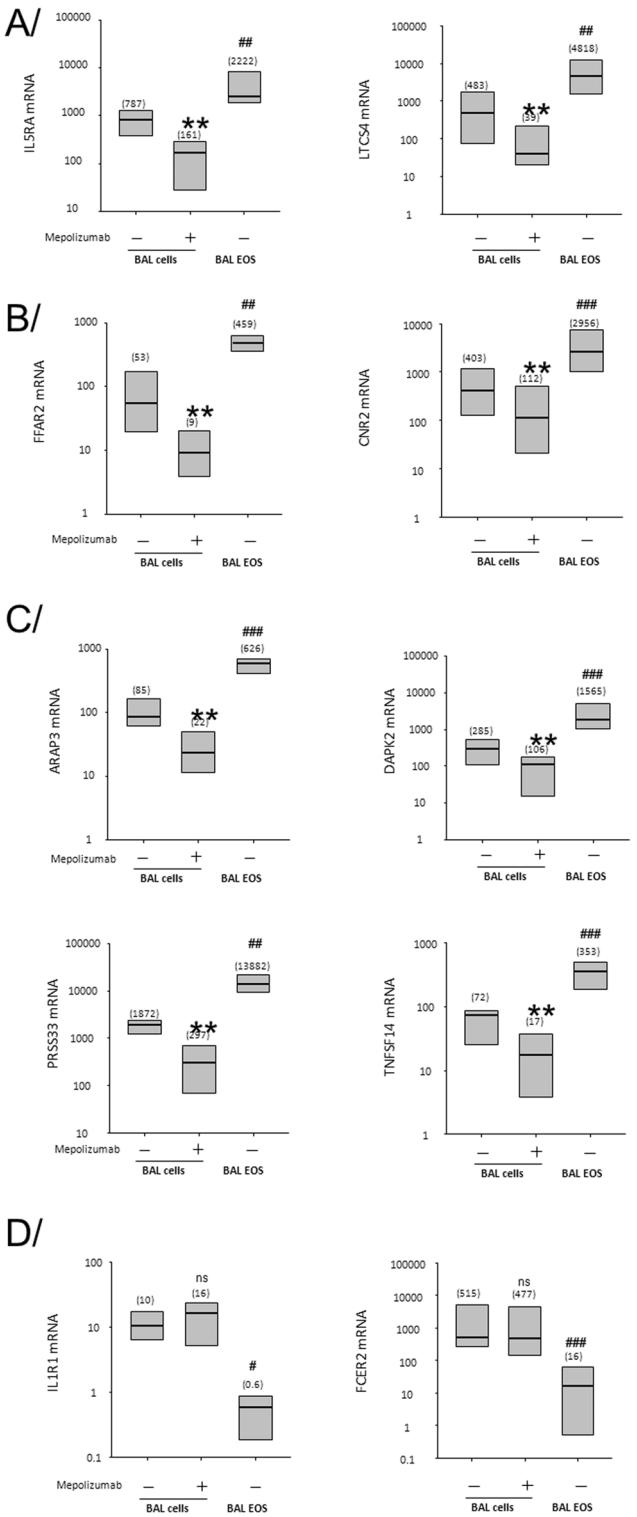

Figure 4. Validation by qPCR of the 57 genes associated with airway EOS after airway allergen challenge. qPCR was performed on BAL cells before SBP-Ag (BAL V1 as shown on Figure 1), 48 h after allergen with (V4) or without mepolizumab treatment (V2), and on purified BAL EOS (BALEOS) after allergen challenge (no mepolizumab, V2). Fold changes compared to expression in BAL cells before allergen challenge and mepolizumab (V1) are presented. Box plots depict the median and the interquartile range between the 25th and the 75th percentiles. 6 subjects are included in each group and means are presented in parentheses. ** indicates that mRNA level in BAL cells is significantly decreased by mepolizumab compared to BAL cells on V2 ($p<0.01$). # indicates that mRNA level in BAL EOS at V2 is significantly higher or lower compared to total BAL cells on V2 ($^{\#}$, $p<0.05$; $^{\#\#}$, $P<0.01$: $^{\#\#\#}$, $P<0.001$).
doi:10.1371/journal.pone.0067560.g004

While PGLYRP1 has not been described in human EOS, bovine and mouse EOS are known to express and store the bovine and murine ortholog of PGLYRP1 [45,46]. In addition, to its antimicrobial function, several mouse models using PGLYRP1 knock-out, have demonstrated both an anti-inflammatory role for PGLYRP1 that down-regulated the production of type-1 cytokines and chemokines [47], and a pro-inflammatory role in atopic dermatitis, contact dermatitis, and pulmonary inflammation models promoting the Th1, Th2 and Th17 response [48,49]. Also, through its interaction with Hsp70 on cytotoxic lymphocytes, PGLYRP1 possesses anti-tumor cell activity [50].

Another intriguing gene is PRSS33 (also called Protease EOS). This member of the trypsin-like serine proteases has been described to be predominantly produced by macrophages [51]. However, an international patent has described PRSS33 as expressed by EOS as well (Pub. No.: WO/2001/016290). Proteases have crucial roles in maintaining homeostasis and regulating inflammation and immune reactions, thus their expression in EOS is under active investigation. For instance, PRSS21 (esp-1) has been found in EOS but not in neutrophils [52], and we have shown very recently that EOS could generate high amounts of another protease, MMP-9 [53]. Yet, the production of PRSS33 by human EOS remains uncertain.

There is little overlap between airway EOS genes described in the present study and previous microarrays using *in vitro* activated blood EOS. *In vitro*, IL-5 induced the up-regulation of CD69 by blood EOS [13,14]. Surprisingly, CD69 was the only common gene between our 57 EOS-associated genes and the genes up-regulated by IL-5 or GM-CSF in these 2 previous studies. This suggests that a short-term *in vitro* activation using IL-5 or GM-CSF might not be an adequate model to reflect *in vivo* EOS activation after an allergen challenge. In addition, the present study analyzed the presence of genes associated with eosinophilia while the 2 previous studies analyzed genes that are cytokine-induced. Thus, in addition to their exposure to cytokines, airway EOS likely become activated as a result of their transmigration out of the blood and through the tissues. Future studies will be required to determine if the 57 genes are inducible or constitutively expressed by airway EOS. Comparison of gene expression in airway EOS after allergen challenge with blood EOS obtained from individuals who do not receive challenge would provide information on the inducible versus constitutive expression of these genes.

In a study by Nakajima et al. [54], microarray analysis examined EOS-specific transcripts by comparison to other nucleated peripheral blood cells. Among the 30 EOS-specific genes, 3 were present in our list of 57 genes (RNASE2, IL5RA and CNR2). 11 of the 30 genes were also up-regulated in our BAL cells after SBP-Ag including GPR44, ALOX15, P2RY14, IDO1, ADORA3, CAMK1, CCR3 and P2RY10; while 10 of the 30 genes were part of the EOS-associated genes in induced sputum (CLC, PLAUR, MARCKSL1, P2RY14, CAMK1, CCR3, and OLIG2).

Gene expression analysis by microarrays has been previously performed on bronchial biopsies from asthmatic patients and on airway epithelial cells [55,56] but microarray analysis using BAL cells after *in vivo* challenge has not been reported. In our whole genome expression analysis, 299 genes were up-regulated after SBP-Ag in BAL cells. Cluster analysis by DAVID Bioinformatics Resources identified 36 genes involved in defense, immune, or

wound-healing responses, 32 genes implicated in cell adhesion or locomotion, 23 genes that regulate transcription, 21 genes associated with apoptosis and 12 genes that are part of the second-messenger-mediated signaling. Importantly, the EOS markers (RNASE2 and RNASE3, also called EDN and ECP) as well as 15 other well-known EOS-associated genes were up-regulated concomitantly with EOS accumulation in the airway following SBP-Ag. Of these 17 genes, all but CCR3 were decreased by the mepolizumab infusion. Among the 13 genes up-regulated by SBP-Ag and related to lung remodeling (CCL2, CCL13, CCL22, CCR2, FCER2, FGFR2, FLT1, IGF1R, IL1R1, IL1R2, IL6R, MMP9 and MMP12), 11 of them were unchanged following mepolizumab. This suggests that in this model mepolizumab did not impact expression of remodeling-associated genes.

Potential limitations of this study include the inability to assess gene expression in non-hematopoietic airway cells (including epithelial cells) or to detect by microarray transcripts, such as cytokines, that are in low abundance. Unlike microarray analysis, qPCR allowed detection of IL-13 and IL-17A in BAL after SBP-Ag [9]. This suggests that some genes might have been excluded from the final eosinophilic genes and that the list of 57 genes is not an exhaustive inventory. Finally, it is important to note that a portion of the genes that are not affected by mepolizumab may in fact represent genes associated with a distinct phenotype of eosinophils that were recruited to or differentiated in the airway following administration of mepolizumab.

In conclusion, we have identified 57 genes highly expressed *in vivo* by airway EOS (BAL and sputum). Most of the 57 genes have not been previously studied in EOS and are important candidates to understand tissue EOS biology and to find new targets for therapies associated with an excessive eosinophilic response.

Supporting Information

Table S1 Primer sequences used for real-time PCR.
(DOCX)

Table S2 299 Genes upregulated in BAL cells 48 h after segmental allergen challenge.
(DOCX)

Table S3 22 Genes upregulated by more than 5 fold in both subjects in BAL cells 48 h after segmental allergen challenge.
(DOCX)

Table S4 Main cellular sources (non-eosinophilic) of the transcripts up-regulated in BAL cells after SBP-Ag.
(DOCX)

Table S5 Among the 299 genes upregulated in BAL cells after SBP-Ag, 31 have been previously genetically associated with asthma, allergy or a pulmonary disease.
DAVID Bioinformatics Resources 6.7; National Institute of Allergy and Infectious Diseases (NIAID), NIH; http://david.abcc.ncifcrf.gov/.
(DOCX)

Table S6 99 genes up-regulated in BAL cells by SBP-Ag and down-regulated after mepolizumab.
(DOCX)

Table S7 Genes up-regulated in BAL cells after allergen challenge, down-regulated by mepolizumab and part of the EOS-associated genes in the sputum: functional annotation clustering (DAVID Bioinformatics Resources 6.7, National Institute of Allergy and Infectious Diseases, NIH).
(DOCX)

Acknowledgments

The authors thank Mary Jo Jackson, RN, BSN, Holly Eversoll, RN, BSN, Michele Wolff, RN, MSN, and Evelyn Falibene, BS, for patient recruitment and screening; the Laboratory core that recruited and screened subjects and purified blood eosinophils; Paul Fichtinger, BS, for eosinophil purification; and the UW-Asthma Program Project Grant group for helpful suggestions.

Author Contributions

Conceived and designed the experiments: SE EAK EAS LYL LCD JSM SKM NNJ. Performed the experiments: SE EAK EAS LYL LPD. Analyzed the data: SE YAB JAH SKM. Wrote the paper: SE EAK YAB LCD JSM SKM NNJ.

References

1. Bousquet J, Chanez P, Lacoste JY, Barnéon G, Ghavanian N, et al. (1990) Eosinophilic inflammation in asthma. New Engl J Med 323: 1033–1039.
2. Broekema M, Timens W, Vonk JM, Volbeda F, Lodewijk ME, et al. (2010) Persisting Remodeling and Less Airway Wall Eosinophil Activation in Complete Remission of Asthma. Am J Respir Crit Care Med.
3. Broekema M, Volbeda F, Timens W, Dijkstra A, Lee NA, et al. (2010) Airway eosinophilia in remission and progression of asthma: accumulation with a fast decline of FEV(1). Respir Med 104: 1254–1262.
4. Busse WW, Ring J, Huss-Marp J, Kahn JE (2010) A review of treatment with mepolizumab, an anti-IL-5 mAb, in hypereosinophilic syndromes and asthma. J Allergy Clin Immunol 125: 803–813.
5. Flood-Page P, Menzies-Gow A, Phipps S, Ying S, Wangoo A, et al. (2003) Anti-IL-5 treatment reduces deposition of ECM proteins in the bronchial subepithelial basement membrane of mild atopic asthmatics. J Clin Invest 112: 1029–1036.
6. Humbles AA, Lloyd CM, McMillan SJ, Friend DS, Xanthou G, et al. (2004) A critical role for eosinophils in allergic airways remodeling. Science 305: 1776–1779.
7. Spencer LA, Szela CT, Perez SA, Kirchhoffer CL, Neves JS, et al. (2009) Human eosinophils constitutively express multiple Th1, Th2, and immunoregulatory cytokines that are secreted rapidly and differentially. J Leukoc Biol 85: 117–123.
8. Liu LY, Mathur SK, Sedgwick JB, Jarjour NN, Busse WW, et al. (2006) Human airway and peripheral blood eosinophils enhance Th1 and Th2 cytokine secretion. Allergy 61: 589–598.
9. Esnault S, Kelly EA, Nettenstrom LM, Cook EB, Seroogy CM, et al. (2012) Human eosinophils release IL-1ß and increase expression of IL-17A in activated CD4(+) T lymphocytes. Clin Exp Allergy 42: 1756–1764.
10. Castro M, Mathur S, Hargreave F, Boulet LP, Xie F, et al. (2011) Reslizumab for Poorly Controlled, Eosinophilic Asthma: A Randomized, Placebo-Controlled Study. Am J Respir Crit Care Med.
11. Haldar P, Brightling CE, Hargadon B, Gupta S, Monteiro W, et al. (2009) Mepolizumab and exacerbations of refractory eosinophilic asthma. New Engl J Med 360: 973–984.
12. Nair P, Pizzichini MM, Kjarsgaard M, Inman MD, Efthimiadis A, et al. (2009) Mepolizumab for prednisone-dependent asthma with sputum eosinophilia. N Engl J Med 360: 985–993.
13. Temple R, Allen E, Fordham J, Phipps S, Schneider HC, et al. (2001) Microarray analysis of eosinophils reveals a number of candidate survival and apoptosis genes. Am J Respir Cell Mol Biol 25: 425–433.
14. Bates ME, Liu LY, Esnault S, Stout BA, Fonkem E, et al. (2004) Expression of IL-5- and GM-CSF-responsive genes in blood and airway eosinophils. Am J Respir Cell Mol Biol 30: 736–743.
15. Stout BA, Bates ME, Liu LY, Farrington NN, Bertics PJ (2004) IL-5 and granulocyte-macrophage colony-stimulating factor activate STAT3 and STAT5 and promote Pim-1 and cyclin D3 protein expression in human eosinophils. J Immunol 173: 6409–6417.
16. Bates ME, Sedgwick JB, Zhu Y, Liu LY, Heuser RG, et al. (2010) Human airway eosinophils respond to chemoattractants with greater eosinophil-derived neurotoxin release, adherence to fibronectin, and activation of the Ras-ERK pathway when compared with blood eosinophils. J Immunol 184: 7125–7133.

17. Liu LY, Sedgwick JB, Bates ME, Vrtis RF, Gern JE, et al. (2002) Decreased expression of membrane IL-5R alpha on human eosinophils: I. Loss of membrane IL-5 alpha on eosinophils and increased soluble IL-5R alpha in the airway after antigen challenge. J Immunol 169: 6452–6458.
18. Johansson MW, Kelly EA, Busse WW, Jarjour NN, Mosher DF (2008) Up-regulation and activation of eosinophil integrins in blood and airway after segmental lung antigen challenge. J Immunol 180: 7622–7635.
19. Calhoun WJ, Jarjour NN, Gleich GJ, Stevens CA, Busse WW (1993) Increased airway inflammation with segmental versus aerosol antigen challenge. Am Rev Respir Dis 147: 1465–1471.
20. Jarjour NN, Calhoun WJ, Kelly EA, Gleich GJ, Schwartz LB, et al. (1997) The immediate and late allergic response to segmental bronchopulmonary provocation in asthma. Am J Respir Crit Care Med 155: 1515–1521.
21. Boulay ME, Boulet LP (2002) Lower airway inflammatory responses to repeated very-low-dose allergen challenge in allergic rhinitis and asthma. Clin Exp Allergy 32: 1441–1447.
22. Liu LY, Swenson CA, Kita H, Kelly EAB, Busse WW (2000) The relationship of airway eosinophilia and sputum cell generation of IL-5 following antigen challenge. J Allergy Clin Immunol 106: 1063–1069.
23. Lopuhaa CE, Out TA, Jansen HM, Aalberse RC, van der Zee JS (2002) Allergen-induced bronchial inflammation in house dust mite-allergic patients with or without asthma. Clin Exp Allergy 32: 1720–1727.
24. Pin I, Freitag AP, O'Byrne PM, Girgis-Gabardo A, Watson RM, et al. (1992) Changes in the cellular profile of induced sputum after allergen-induced asthmatic responses. Am Rev Respir Dis 145: 1265–1269.
25. Liu LY, Bates ME, Jarjour NN, Busse WW, Bertics PJ, et al. (2007) Generation of Th1 and Th2 chemokines by human eosinophils: evidence for a critical role of TNF-alpha. J Immunol 179: 4840–4848.
26. Johansson MW, Gunderson KA, Kelly EA, Denlinger LC, Jarjour NN, et al. (2012) Anti-IL-5 attenuates activation and surface density of ß2-integrins on circulating eosinophils after segmental antigen challenge. Clin Exp Allergy 43: 292–303.
27. Royce SG, Cheng V, Samuel CS, Tang ML (2012) The regulation of fibrosis in airway remodeling in asthma. Mol Cell Endocrinol 351: 167–175.
28. Homer RJ, Elias JA, Lee CG, Herzog E (2011) Modern concepts on the role of inflammation in pulmonary fibrosis. Arch Pathol Lab Med 135: 780–788.
29. Al Muhsen S, Johnson JR, Hamid Q (2011) Remodeling in asthma. J Allergy Clin Immunol 128: 451–462.
30. Elias JA, Zhu Z, Chupp G, Homer RJ (1999) Airway remodeling in asthma. J Clin Invest 104: 1001–1006.
31. Kishimoto S, Oka S, Gokoh M, Sugiura T (2006) Chemotaxis of human peripheral blood eosinophils to 2-arachidonoylglycerol: comparison with other eosinophil chemoattractants. Int Arch Allergy Immunol 140 Suppl 1: 3–7.
32. Maslowski KM, Vieira AT, Ng A, Kranich J, Sierro F, et al. (2009) Regulation of inflammatory responses by gut microbiota and chemoattractant receptor GPR43. Nature 461: 1282–1286.
33. Cherry WB, Yoon J, Bartemes KR, Iijima K, Kita H (2008) A novel IL-1 family cytokine, IL-33, potently activates human eosinophils. J Allergy Clin Immunol 121: 1484–1490.
34. Kearley J, Buckland KF, Mathie SA, Lloyd CM (2009) Resolution of allergic inflammation and airway hyperreactivity is dependent upon disruption of the T1/ST2-IL-33 pathway. Am J Respir Crit Care Med 179: 772–781.
35. Munro S, Thomas KL, Abu-Shaar M (1993) Molecular characterization of a peripheral receptor for cannabinoids. Nature 365: 61–65.
36. Oka S, Ikeda S, Kishimoto S, Gokoh M, Yanagimoto S, et al. (2004) 2-arachidonoylglycerol, an endogenous cannabinoid receptor ligand, induces the migration of EoL-1 human eosinophilic leukemia cells and human peripheral blood eosinophils. J Leukoc Biol 76: 1002–1009.
37. Karsak M, Gaffal E, Date R, Wang-Eckhardt L, Rehnelt J, et al. (2007) Attenuation of allergic contact dermatitis through the endocannabinoid system. Science 316: 1494–1497.
38. Karmaus PW, Chen W, Crawford RB, Harkema JR, Kaplan BL, et al. (2011) Deletion of cannabinoid receptors 1 and 2 exacerbates APC function to increase inflammation and cellular immunity during influenza infection. J Leukoc Biol 90: 983–995.
39. Giannini L, Nistri S, Mastroianni R, Cinci L, Vannacci A, et al. (2008) Activation of cannabinoid receptors prevents antigen-induced asthma-like reaction in guinea pigs. J Cell Mol Med 12: 2381–2394.
40. Zoerner AA, Stichtenoth DO, Engeli S, Batkai S, Winkler C, et al. (2011) Allergen challenge increases anandamide in bronchoalveolar fluid of patients with allergic asthma. Clin Pharmacol Ther 90: 388–391.
41. del Rio ML, Lucas CL, Buhler L, Rayat G, Rodriguez-Barbosa JI (2010) HVEM/LIGHT/BTLA/CD160 cosignaling pathways as targets for immune regulation. J Leukoc Biol 87: 223–235.
42. Doherty TA, Soroosh P, Khorram N, Fukuyama S, Rosenthal P, et al. (2011) The tumor necrosis factor family member LIGHT is a target for asthmatic airway remodeling. Nat Med 17: 596–603.
43. Hastie AT, Moore WC, Meyers DA, Vestal PL, Li H, et al. (2010) Analyses of asthma severity phenotypes and inflammatory proteins in subjects stratified by sputum granulocytes. J Allergy Clin Immunol 125: 1028–1036.
44. Osanai A, Sashinami H, Asano K, Li SJ, Hu DL, et al. (2011) Mouse peptidoglycan recognition protein PGLYRP-1 plays a role in the host innate immune response against Listeria monocytogenes infection. Infect Immun 79: 858–866.
45. Tydell CC, Yount N, Tran D, Yuan J, Selsted ME (2002) Isolation, characterization, and antimicrobial properties of bovine oligosaccharide-binding protein. A microbicidal granule protein of eosinophils and neutrophils. J Biol Chem 277: 19658–19664.
46. Park SY, Jing X, Gupta D, Dziarski R (2013) Peptidoglycan Recognition Protein 1 Enhances Experimental Asthma by Promoting Th2 and Th17 and Limiting Regulatory T Cell and Plasmacytoid Dendritic Cell Responses. J Immunol.
47. Saha S, Jing X, Park SY, Wang S, Li X, et al. (2010) Peptidoglycan recognition proteins protect mice from experimental colitis by promoting normal gut flora and preventing induction of interferon-gamma. Cell Host Microbe 8: 147–162.
48. Park SY, Gupta D, Kim CH, Dziarski R (2011) Differential effects of peptidoglycan recognition proteins on experimental atopic and contact dermatitis mediated by Treg and Th17 cells. PLoS ONE 6: e24961.
49. Park SY, Jing X, Gupta D, Dziarski R (2013) Peptidoglycan Recognition Protein 1 Enhances Experimental Asthma by Promoting Th2 and Th17 and Limiting Regulatory T Cell and Plasmacytoid Dendritic Cell Responses. J Immunol.
50. Sashchenko LP, Dukhanina EA, Shatalov YV, Yashin DV, Lukyanova TI, et al. (2007) Cytotoxic T lymphocytes carrying a pattern recognition protein Tag7 can detect evasive, HLA-negative but Hsp70-exposing tumor cells, thereby ensuring FasL/Fas-mediated contact killing. Blood 110: 1997–2004.
51. Chen C, Darrow AL, Qi JS, D'Andrea MR, Andrade-Gordon P (2003) A novel serine protease predominately expressed in macrophages. Biochem J 374: 97–107.
52. Inoue M, Kanbe N, Kurosawa M, Kido H (1998) Cloning and tissue distribution of a novel serine protease esp-1 from human eosinophils. Biochem Biophys Res Commun 252: 307–312.
53. Kelly EA, Liu LY, Esnault S, Quinchia-Rios BH, Jarjour NN (2012) Potent synergistic effect of IL-3 and TNF on matrix metalloproteinase 9 generation by human eosinophils. Cytokine 58: 199–206.
54. Nakajima T, Matsumoto K, Suto H, Tanaka K, Ebisawa M, et al. (2001) Gene expression screening of human mast cells and eosinophils using high-density oligonucleotide probe arrays: abundant expression of major basic protein in mast cells. Blood 98: 1127–1134.
55. Laprise C, Sladek R, Ponton A, Bernier MC, Hudson TJ, et al. (2004) Functional classes of bronchial mucosa genes that are differentially expressed in asthma. BMC Genomics 5: 21.
56. Lilly CM, Tateno H, Oguma T, Israel E, Sonna LA (2005) Effects of allergen challenge on airway epithelial cell gene expression. Am J Respir Crit Care Med 171: 579–586.

Table S1: Primer sequences used for real-time PCR

GENE	Forward primer	Reverse primer
IL5R	aagtgtgcggaccatcctgca	ccaggagaccctggtggggc
LTC4S	gcccaggtgaactgcagcga	gccgccgccccttcatgaaa
FFAR2	ggctgcgtcgaacttccgct	gccaaaactcgtgagggcgc
CNR2	cccacaacacaacccaaagccttct	tggggtgggcccttcagattcc
IL1R1	tgtgagcccagctaatgagaca	ccggtgacattacagatcaattgt
ARAP3	gcccccgacagaagagagagca	tagccagcatcctcccgtgcc
DAPK2	agagacccggaaacggctcaca	ccacagactccctgcgcacc
PRSS33	aggtcctgctccttctggtgctg	tccgactggacatgcggggct
TNFSF14	ctaggagagatggtcacccgcctg	ggttgacctcgtgagaccttcgc
CD23b	tggaacaagcagaatttagca	ctgggaagctcctcgatctc

Table S2. 299 Genes upregulated in BAL cells 48 h after segmental allergen challenge

Probe Set ID	Gene_assignment	Probe Set ID	Gene_assignment	Probe Set ID	Gene_assignment	Probe Set ID	Gene_assignment	Probe Set ID	Gene_assignment	Probe Set ID	Gene_assignment
8017964	ABCA6	7921667	CD48	7970763	FLT1	7963614	ITGB7	8166408	PHEX	8003298	SLC7A5
8082673	ACPP	7961075	CD69	8056408	GALNT3	8048995	ITM2C	8016540	PHOSPHO1	8113369	SLCO4C1
8115490	ADAM19	7898655	CDA	8105481	GAPT	8068583	KCNJ15	7897482	PIK3CD	8014248	SLFN13
8145293	ADAM28	7977928	CEBPE	8167360	GATA1	7963851	KIAA0748	8012539	PIK3R6	8002249	SMPD3
7937150	ADAM8	7936856	CHST15	8127130	GCM1	7923233	KIF21B	8119161	PIM1	7999423	SOCS1
8069178	ADARB1	8092726	CLDN1	8158939	GFI1B	8092348	LAMP3	8172471	PIM2	7944667	SORL1
7918533	ADORA3	8012013	CLEC10A	8149629	GFRA2	8025382	LASS4	8126784	PLA2G7	8114964	SPINK1
8170364	AFF2	7960832	CLEC4C	8074991	GGT5	8131143	LFNG	7977319	PLD4	8003861	SPNS3
8096116	AGPAT9	7966089	CMKLR1	8029728	GIPR	7940762	LGALS12	7957570	PLXNC1	7934215	SPOCK2
8011680	ALOX15	7913705	CNR2	7974341	GNG2	8075956	LGALS2	8062557	PPP1R16B	8041995	SPTBN1
7938396	AMPD3	7994769	CORO1A	7996064	GPR114	8031213	LILRA1	7994131	PRKCB	8112940	SSBP2
8111101	ANKH	8083260	CPA3	7981530	GPR132	8017393	LIMD2	7939383	PRR5L	8084717	ST6GAL1
8101260	ANTXR2	8151092	CRH	8131067	GPR146	8025877	LPPR2	7998861	PRSS33	8113358	ST8SIA4
8115234	ANXA6	8171105	CRLF2	7972557	GPR183	7954436	LRMP	8157650	PTGS1	7929012	STAMBPL1
8114733	ARAP3	8061416	CST7	7948470	GPR44	8110562	LTC4S	7923406	PTPN7	8122600	STXBP5
8059854	ARL4C	8163063	CTNNAL1	7996081	GPR56	8166925	MAOA	7949792	PTPRCAP	8066822	SULF2
7981020	ASB2	8055465	CXCR4	8166919	GPR82	8113790	MARCH3	7931353	PTPRE	7947147	SVIP
8011516	ATP2A3	8109528	CYFIP2	7996100	GPR97	8050160	MBOAT2	8041508	QPCT	8130211	SYNE1
7905754	ATP8B2	8051583	CYP1B1	8013319	GRAP	7986293	MCTP2	8169898	RAB33A	8108050	TCF7
8096617	BANK1	8028991	CYP2S1	7978366	GZMB	8044391	MERTK	8009666	RAB37	7966749	TESC
7943413	BIRC3	8026468	CYP4F12	7988672	HDC	8136662	MGAM	8034202	RAB3D	8103226	TMEM154
8062444	BPI	7971950	DACH1	7948973	HRASLS5	8172266	MIR221	8119102	RAB44	8099850	TMEM156
7934185	C10orf54	7989596	DAPK2	8110106	HRH2	8167971	MIR223	8035050	RASAL3	8143790	TMEM176B
7945734	C11orf21	8049317	DGKD	8020684	HRH4	7951297	MMP12	8064790	RASSF2	8152976	TMEM71
7923875	C1orf186	7913357	ECE1	7909446	HSD11B1	7992811	MMP25	7924085	RD3	8033248	TNFSF14
8060940	C20orf103	8132305	EEPD1	8026533	HSH2D	8063115	MMP9	7908125	RGL1	8062782	TOX2
8063785	C20orf197	7949264	EHD1	8033987	ICAM3	8058824	MREG	8110327	RGS14	7958960	TPCN1
8080511	CACNA1D	8088803	EIF4E3	8146092	IDO1	7940216	MS4A3	7967202	RHOF	8133155	TPST1
8085206	CAMK1	8112007	EMB	8124848	IER3	8169949	MST4	8094743	RHOH	7909455	TRAF3IP3
8063497	CASS4	7954090	EMP1	7937335	IFITM1	8079462	NBEAL2	7977615	RNASE1	8136727	TRBV27
8079117	CCBP2	8034873	EMR2	7986383	IGF1R	8133314	NCF1	7973110	RNASE2	8126288	TREML2
8006459	CCL13	8033332	EMR4P	7981740	IGHA1	8043363	NCRNA00152	7973105	RNASE3	8126428	TRERF1
8006433	CCL2	8152522	ENPP2	7981724	IGHD	7969544	NDFIP2	7903393	S1PR1	8161774	TRPM6
7996022	CCL22	7929511	ENTPD1	8100827	IGJ	7977621	NDRG2	7922219	SELL	8034199	TSPAN16
8093294	CCR2	7952601	ETS1	8043476	IGKC	8067040	NFATC2	7906107	SEMA4A	7939559	TSPAN18
8079383	CCR3	8092578	ETV5	7981730	IGLJ3	8168359	NHSL2	7990345	SEMA7A	8131179	TTYH3
8078442	CCR4	8123744	F13A1	8014891	IKZF3	8162455	NINJ1	8000869	SEPT1	8024900	UHRF1
8015031	CCR7	7922174	F5	7942300	IL18BP	8087935	NT5DC2	8021635	SERPINB2	7928444	VCL
7906339	CD1A	8010915	FAM101B	8044035	IL18R1	8158890	NTNG2	8123609	SERPINB9	8169263	VSIG1
7921346	CD1B	8164343	FAM102A	8044049	IL18RAP	8033818	OLFM2	8038824	SIGLEC10	8039109	VSTM1
7906348	CD1C	7901466	FAM159A	8043995	IL1R1	8051793	OXER1	8038839	SIGLEC8	7899417	XKR8
7906355	CD1E	8103415	FAM198B	8043981	IL1R2	8011415	P2RX5	8066038	SLA2	7999754	XYLT1
8089568	CD200R1	7904361	FAM46C	8044021	IL1RL1	8168524	P2RY10	8059642	SLC16A14	8000791	YPEL3
8052916	CD207	8124280	FAM65B	7931914	IL2RA	8091511	P2RY14	7930837	SLC18A2	7926916	ZEB1
8033445	CD209	7906443	FCER1A	8075886	IL2RB	7942400	P2RY2	8061227	SLC24A3		
8177222	CD24	8033420	FCER2	8085062	IL5RA	8177026	P2RY8	7915472	SLC2A1		
7921677	CD244	7906777	FCGR2B	7905789	IL6R	7912937	PADI2	8109350	SLC36A1		
7922040	CD247	8027862	FFAR2	7901804	INADL	7898448	PADI4	8143367	SLC37A3		
7953333	CD27	8027860	FFAR3	7931479	INPP5A	8095751	PARM1	7962516	SLC38A1		
8047677	CD28	7936734	FGFR2	8116559	IRF4	8112220	PDE4D	8057677	SLC40A1		
8018169	CD300LB	7915147	FHL3	7995096	ITGAM	8037742	PGLYRP1	8102800	SLC7A11		

Eosinophil markers

Table S3. 22 Genes upregulated by more than 5 fold in both subjects in BAL cells 48 h after segmental allergen challenge

Probe Set ID	gene_assignment	Fold change, subject 1	Fold Change, subject 2
7906339	CD1A	31.5	10.0
8006433	CCL2	31.1	9.5
8082673	ACPP	20.9	9.8
8011680	ALOX15	15.5	15.2
7912937	PADI2	17.0	8.5
8010915	FAM101B	14.0	11.2
8177222	CD24	10.7	12.1
8027860	FFAR3	15.6	5.9
7918533	ADORA3	15.1	6.3
8038839	SIGLEC8	15.2	5.8
8011516	ATP2A3	12.6	7.5
7906355	CD1E	5.3	13.3
7921346	CD1B	7.2	11.1
7996022	CCL22	11.9	6.4
7937150	ADAM8	11.3	6.9
8006459	CCL13	10.5	7.7
8085206	CAMK1	11.2	5.4
8033987	ICAM3	8.0	6.5
7921677	CD244	8.9	5.2
8012539	PIK3R6	5.5	7.1
7898448	PADI4	5.7	5.7
8027862	FFAR2	5.2	6.0

Table S4. Main cellular sources (non-eosinophilic) of the transcripts up-regulated in BAL cells after SBP-Ag

Cell type	GENES
Dendritic cells	CCL2, CCL22, CD1A, CD1E, CD207, CD209, CLEC4C, LAMP3, NDRG2
Lymphocytes	CCR7, CRH, GPR183, IK2F3, PHEX, SELL, BIRC3
Mast cells	CPA3, FCER1, HDC

Swissprot research at http://www.uniprot.org/

Table S5. Among the 299 genes upregulated in BAL cells after SBP-Ag, 31 have been previously genetically associated with asthma, allergy or a pulmonary disease

PROTEIN	DISEASES	GENE
CD1a molecule	pulmonary disease	CD1A
CD1b molecule	pulmonary disease	CD1B
CD1c molecule	pulmonary disease	CD1C
CD1e molecule	pulmonary disease	CD1E
CD28 molecule	asthma, atopy, specific IgE, total serum IgE	CD28
Fc fragment of IgE, high affinity I, receptor for; alpha polypeptide	airway hyperresponsiveness atopy, allergic diseases, asthma, atopy, IgE	FCER1A
Fc fragment of IgE, low affinity II, receptor for (CD23)	atopy	FCER2
G protein-coupled receptor 44	asthma, asthma, atopy, IgE,	GPR44
chemokine (C-C motif) ligand 2	asthma, asthma severity, atopic asthma	CCL2
chemokine (C-C motif) receptor 3	asthma, rhinitis	CCR3
chemokine (C-C motif) receptor 4	atopic dermatitis, dermatitis and eczema	CCR4
chemokine (C-X-C motif) receptor 4	asthma	CXCR4
coagulation factor V (proaccelerin, labile factor)	bronchopulmonary dysplasia, pulmonary thromboembolism	F5
coagulation factor XIII, A1 polypeptide	bronchopulmonary dysplasia, pulmonary thromboembolism	F13A1
histamine receptor H2	asthma, urticaria/angioedema	HRH2
integrin, beta 7	asthma, inflammatory bowel disease	ITGB7
intercellular adhesion molecule 3	severe acute respiratory syndrome	ICAM3
interleukin 1 receptor, type I	asthma, pulmonary fibrosis	IL1R1
interleukin 18 receptor 1	pulmonary fibrosis	IL18R1
interleukin 2 receptor, alpha	IgE levels	IL2RA
interleukin 5 receptor, alpha	asthma, asthma atopy, atopic asthma, dermatitis and eczema	IL5RA
leukotriene C4 synthase	allergic disease, allergic rhinitis, aspirin-induced asthma, aspirin-intolerant asthma, asthma, asthma, IgE, atopy	LTC4S
matrix metallopeptidase 12 (macrophage elastase)	chronic obstructive pulmonary disease/COPD	MMP12
matrix metallopeptidase 9 (gelatinase B, 92kDa gelatinase, 92kDa type IV collagenase)	asthma, chronic obstructive pulmonary disease/COPD, emphysema	MMP9
membrane-spanning 4-domains, subfamily A, member 3 (hematopoietic cell-specific)	asthma	MS4A3
phosphodiesterase 4D, cAMP-specific (phosphodiesterase E3 dunce homolog, Drosophila)	chronic obstructive pulmonary disease/COPD	PDE4D
prostaglandin-endoperoxide synthase 1 (prostaglandin G/H synthase and cyclooxygenase)	asthma	PTGS1
purinergic receptor P2Y, G-protein coupled, 2	cystic fibrosis	P2RY2
ribonuclease, RNase A family, 2 (liver, eosinophil-derived neurotoxin)	tropical pulmonary eosinophilia,	RNASE2
ribonuclease, RNase A family, 3 (eosinophil cationic protein)	asthma, tropical pulmonary eosinophilia,	RNASE3
suppressor of cytokine signaling 1	asthma	SOCS1

DAVID Bioinformatics Resources 6.7; National Institute of Allergy and Infectious Diseases (NIAID), NIH ; http://david.abcc.ncifcrf.gov/

Table S6.

99 genes up-regulated in BAL cells by SBP-Ag and down-regulated after mepolizumab

ADAM28	EHD1	KIF21B	RAB37
ADAM8	EMR4P	LASS4	RAB3D
ADARB1	FAM101B	LFNG	RAB44
ARAP3	FAM65B	LGALS12	RD3
ASB2	FFAR2	LTC4S	RNASE2
ATP2A3	FFAR3	MARCH3	RNASE3
BPI	FGFR2	MCTP2	SIGLEC10
C10orf54	FHL3	MIR223	SIGLEC8
C11orf21	GATA1	MMP25	SLC16A14
C20orf197	GCM1	NCF1	SLC18A2
CASS4	GFI1B	NDFIP2	SLC24A3
CCBP2	GGT5	NHSL2	SOCS1
CD244	GIPR	NINJ1	SORL1
CD300LB	GPR114	NTNG2	SPNS3
CD69	GPR146	OLFM2	TCF7
CDA	GPR44	OXER1	TESC
CEBPE	GPR56	P2RY2	TNFSF14
CHST15	GPR97	PADI2	TOX2
CNR2	HRASLS5	PADI4	TREML2
CORO1A	HRH2	PGLYRP1	TRPM6
CYP4F12	ICAM3	PHEX	TSPAN16
DACH1	IFITM1	PHOSPHO1	TSPAN18
DAPK2	IL1RL1	PIK3R6	VSTM1
DGKD	IL5RA	PIM1	XKR8
EEPD1	INPP5A	PRSS33	

EOS markers

Table S7.

Genes up-regulated in BAL cells after allergen challenge, down-regulated by mepolizumab and part of the EOS-associated genes in the sputum: functional annotation clustering (DAVID Bioinformatics Resources 6.7, National Institute of Allergy and Infectious Diseases, NIH)

Secreted		Gene name
	ADAM metallopeptidase domain 28	ADAM28
	interleukin 1 receptor-like 1	IL1RL1
	matrix metallopeptidase 25	MMP25
	peptidoglycan recognition protein 1	PGLYRP1
	protease, serine, 33	PRSS33
	sialic acid binding Ig-like lectin 10	SIGLEC10
	tumor necrosis factor (ligand) superfamily, member 14	TNFSF14
Plasma membrane		
	ADAM metallopeptidase domain 28	ADAM28
	ADAM metallopeptidase domain 8	ADAM8
	ArfGAP with RhoGAP domain, ankyrin repeat and PH domain 3	ARAP3
	CD300 molecule-like family member b	CD300LB
	CD69 molecule	CD69
	Cas scaffolding protein family member 4	CASS4
	G protein-coupled receptor 56	GPR56
	G protein-coupled receptor 97	GPR97
	RAB3D, member RAS oncogene family	RAB3D
	cannabinoid receptor 2 (macrophage)	CNR2
	coronin, actin binding protein, 1A	CORO1A
	diacylglycerol kinase, delta 130kDa	DGKD
	free fatty acid receptor 2	FFAR2
	free fatty acid receptor 3	FFAR3
	gamma-glutamyltransferase 5	GGT5
	intercellular adhesion molecule 3	ICAM3
	interferon induced transmembrane protein 1 (9-27)	IFITM1
	interleukin 1 receptor-like 1	IL1RL1
	interleukin 5 receptor, alpha	IL5RA
	matrix metallopeptidase 25	MMP25
	purinergic receptor P2Y, G-protein coupled, 2	P2RY2
	sialic acid binding Ig-like lectin 10	SIGLEC10
	sortilin-related receptor, L(DLR class) A repeats-containing	SORL1
	tescalcin	TESC
	transient receptor potential cation channel, subfamily M, member 6	TRPM6
	triggering receptor expressed on myeloid cells-like 2 pseudogene	TREML2
	tumor necrosis factor (ligand) superfamily, member 14	TNFSF14

Table S7…

… Table S7

Cell adhesion	Gene name
ADAM metallopeptidase domain 8	ADAM8
Cas scaffolding protein family member 4	CASS4
G protein-coupled receptor 56	GPR56
coronin, actin binding protein, 1A	CORO1A
intercellular adhesion molecule 3	ICAM3
sialic acid binding Ig-like lectin 10	SIGLEC10
sialic acid binding Ig-like lectin 8	SIGLEC8
Immune response	
CD300 molecule-like family member b	CD300LB
cannabinoid receptor 2 (macrophage)	CNR2
coronin, actin binding protein, 1A	CORO1A
interleukin 1 receptor-like 1	IL1RL1
peptidoglycan recognition protein 1	PGLYRP1
tumor necrosis factor (ligand) superfamily, member 14	TNFSF14
Defense response	
cannabinoid receptor 2 (macrophage)	CNR2
coronin, actin binding protein, 1A	CORO1A
interleukin 1 receptor-like 1	IL1RL1
matrix metallopeptidase 25	MMP25
peptidoglycan recognition protein 1	PGLYRP1
ribonuclease, RNase A family, 3 (eosinophil cationic protein)	RNASE3
Carbohydrate binding	
CD69 molecule	CD69
lectin, galactoside-binding, soluble, 12	LGALS12
peptidoglycan recognition protein 1	PGLYRP1
sialic acid binding Ig-like lectin 10	SIGLEC10
sialic acid binding Ig-like lectin 8	SIGLEC8
Nucleotide binding	**Gene name**
ATPase, Ca++ transporting, ubiquitous	ATP2A3
RAB37, member RAS oncogene family	RAB37
RAB3D, member RAS oncogene family	RAB3D
carbohydrate (N-acetylgalactosamine 4-sulfate 6-O) sulfotransferase 15	CHST15
dachshund homolog 1 (Drosophila)	DACH1
death-associated protein kinase 2	DAPK2
diacylglycerol kinase, delta 130kDa	DGKD
kinesin family member 21B	KIF21B
purinergic receptor P2Y, G-protein coupled, 2	P2RY2
transient receptor potential cation channel, subfamily M, member 6	TRPM6
Cytoskeleton	
ArfGAP with RhoGAP domain, ankyrin repeat and PH domain 3	ARAP3
Cas scaffolding protein family member 4	CASS4
coronin, actin binding protein, 1A	CORO1A
family with sequence similarity 65, member B	FAM65B
four and a half LIM domains 3	FHL3
interleukin 1 receptor-like 1	IL1RL1
kinesin family member 21B	KIF21B

…Table S7

Other genes		Gene name
	ankyrin repeat and SOCS box containing 2	ASB2
	cytidine deaminase	CDA
	cytochrome P450, family 4, subfamily F, polypeptide 12	CYP4F12
	EGF-like module containing, mucin-like, hormone receptor-like 4	EMR4P
	family with sequence similarity 101, member B	FAM101B
	leukotriene C4 synthase	LTC4S
	multiple C2 domains, transmembrane 2	MCTP2
	NHS-like 2	NHSL2
	peptidyl arginine deiminase, type II	PADI2
	peptidyl arginine deiminase, type IV	PADI4
	phosphoinositide-3-kinase, regulatory subunit 6	PIK3R6
	retinal degeneration 3	RD3
	ribonuclease, RNase A family, 2 (eosinophil-derived neurotoxin)	RNASE2
	solute carrier family 24 (sodium/potassium/calcium exchanger), member 3	SLC24A3
	spinster homolog 3 (Drosophila)	SPNS3
	tetraspanin 18	TSPAN18
	V-set and transmembrane domain containing 1	VSTM1

Genetic association with a respiratory disease		
DISEASES		
severe acute respiratory syndrome	intercellular adhesion molecule 3	ICAM3
atopic asthma, dermatitis and eczema	interleukin 5 receptor, alpha	IL5RA
allergic disease, rhinitis, asthma, atopy	leukotriene C4 synthase	LTC4S
cystic fibrosis	purinergic receptor P2Y, G-protein coupled, 2	P2RY2
pulmonary eosinophilia	ribonuclease, RNase A family, 2 (eosinophil-derived neurotoxin)	RNASE2
asthma, pulmonary eosinophilia	ribonuclease, RNase A family, 3 (eosinophil cationic protein)	RNASE3