

ORIGINAL ARTICLE

Rhinitis, Sinusitis, and Upper Airway Disease

Dysregulated fatty acid metabolism in nasal polyp-derived eosinophils from patients with chronic rhinosinusitis

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Abstract

Background: Eosinophils are multifunctional granulocytes capable of releasing various cytokines, chemokines, and lipid mediators. We previously reported dysregulated fatty acid metabolism in peripheral blood-derived eosinophils from patients with severe asthma. However, functional characteristics of eosinophils present in allergic inflammatory tissues remain largely uncharacterized.

Methods: We established a method for isolating CD69^{hi} CCR3^{low} CXCR4⁻ siglec-8^{int} eosinophils from nasal polyps of patients with eosinophilic rhinosinusitis (NP-EOS). Multi-omics analysis including lipidomics, proteomics, and transcriptomics was performed to analyze NP-EOS as compared to peripheral blood-derived eosinophils from healthy subjects (PB-EOS).

Results: Lipidomic analysis revealed impaired synthesis of prostaglandins and 15-lipoxygenase (15-LOX)-derived mediators, and selective upregulation of leukotriene D₄ production. Furthermore, proteomics and transcriptomics revealed changes in the expression of specific enzymes (GGT5, DPEP2, and 15-LOX) responsible for dysregulated lipid metabolism. Ingenuity pathway analysis indicated the importance of type 2 cytokines and pattern recognition receptor pathways. Stimulation of PB-EOS with eosinophil activators IL-5, GM-CSF, and agonists of TLR2 and NOD2 mimicked the observed changes in lipid metabolism.

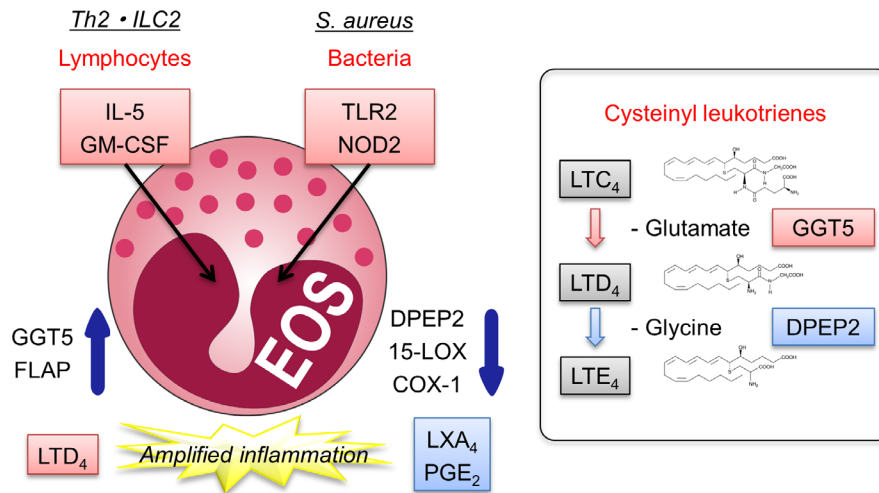
Conclusion: Inflammatory tissue-derived eosinophils possess a specific phenotype with dysregulated fatty acid metabolism that may be targeted therapeutically to control eosinophilic inflammatory diseases.

KEYWORDS

chronic rhinosinusitis, GGT5, human eosinophil, lipid mediator, multi-omics

Abbreviations: ALOX15, 15-lipoxygenase; ALOX5AP, 5-LOX-associated protein; CysLT, cysteinyl leukotriene; DPEP2, dipeptidase-2; GGT5, gamma-glutamyltransferase 5; GM-CSF, granulocyte-macrophage colony-stimulating factor; HETE, hydroxyeicosatetraenoic acid; IL, interleukin; IPA, ingenuity pathway analysis; LC, liquid chromatography; LOX, lipoxygenase; LTC₄S, leukotriene C₄ synthase; LTD₄, leukotriene D₄; LTE₄, leukotriene E₄; MS/MS, tandem mass spectrometry; NOD2, nucleotide-binding oligomerization domain-containing protein 2; NP-EOS, nasal polyp-derived eosinophils; PGE₂, prostaglandin E₂; PB-EOS, peripheral blood-derived eosinophils; PRR, pattern recognition receptor; RT-PCR, reverse transcription-PCR; TLR, Toll-like receptor.

Fatty acid metabolism in nasal polyp eosinophils in CRS



GRAPHICAL ABSTRACT

Eosinophils isolated from nasal polyps of patients with eosinophilic rhinosinusitis possess dysregulated fatty acid metabolism. Of the CysLT (cysteinyl leukotriene) biosynthetic pathway metabolites, LTD₄ production is selectively enhanced with increased expression of GGT5 (gamma-glutamyl transferase-5) and decreased expression of DPEP2 (dipeptidase-2). Type 2 cytokines (IL-5 and GM-CSF) and agonists of TLR2 and NOD2 induce similar changes of fatty acid metabolism.

COX: Cyclooxygenase; CRS: Chronic rhinosinusitis; DPEP2: Dipeptidase-2; GGT5: Gamma-glutamyl transferase-5; GM-CSF: Granulocyte-macrophage colony-stimulating factor; ILC2: Type 2 innate lymphoid cells; LTC₄: Leukotriene C₄; LTD₄: Leukotriene D₄; LTE₄: Leukotriene E₄; LXA₄: Lipoxin A₄; NOD2: Nucleotide binding oligomerization domain containing 2; PGE₂: Prostaglandin E₂; Th2: Type 2 helper T cells; TLR2: Toll-like receptor 2

1 | INTRODUCTION

Eosinophils are multifunctional cells involved in the pathogenesis of helminthic parasite infections and allergic disorders. Eosinophils contain abundant cytotoxic granules and can release several types of cytokines, chemokines, and lipid mediators. They also contribute to the amplification of inflammation and pathogen clearance. Importantly, eosinophils also play beneficial roles in the pathogenesis of obesity, diabetes, muscle regeneration, and neutrophilic inflammation.^{1–5} At present, it remains unclear whether eosinophils with specific phenotypes are responsible for these different immunological responses.

Eosinophils synthesize various types of lipid mediators, such as cysteinyl leukotrienes (CysLTs), prostaglandins, thromboxanes, and 15-lipoxygenase (15-LOX)-derived mediators.⁶ Increasing evidence suggests that 15-LOX mediates the local production of anti-inflammatory and/or pro-resolving lipid mediators (lipoxins, resolvins, maresins, and protectins) that contribute to the proper resolution of inflammation and tissue homeostasis.⁷ In contrast, CysLTs contribute to the amplification of allergic inflammatory responses. In particular, leukotriene D₄ (LTD₄) has a strong affinity for CysLT1 and is responsible for bronchial constriction, vascular leakage, and mucus production with accumulation of T-helper 2 (Th2)-related inflammatory cells. Collectively, different balance of lipid mediators directs immunological responses to specific inflammatory environments.

Severe asthma, one of the unresolved allergic diseases, is characterized by overproduction of CysLTs and reduced synthesis of prostaglandin E₂ (PGE₂) and 15-LOX-derived anti-inflammatory lipid mediators.^{8–11} We previously reported that synthesis of 15-LOX-derived mediators in eosinophils isolated from peripheral blood of patients with severe asthma was selectively impaired.⁶ These phenomena suggest that the pathophysiology of allergic disorders might result from impaired fatty acid metabolism in eosinophils and raise the questions of how the corresponding enzymatic production of lipid mediators in eosinophils could be changed.

In this study, we investigated the specific phenotype of eosinophils isolated from nasal polyps (NP-EOS) of patients with chronic eosinophilic rhinosinusitis. Chronic eosinophilic rhinosinusitis is characterized by bilateral ethmoidal-dominant inflammation and abnormal tissue accumulation of eosinophils in the nasal mucus, mucosa, and polyp.^{12–14} We performed comprehensive transcriptomic, proteomic, and lipidomic analyses of NP-EOS, and compared them to eosinophils isolated from the peripheral blood of healthy subjects (PB-EOS). These results demonstrated a selective upregulation of LTD₄ biosynthesis and a significant downregulation of 15-LOX and COX pathway products in NP-EOS as compared to PB-EOS, and a local inflammatory milieu is responsible for the development of the specific inflammatory phenotype of eosinophils.

2 | METHODS

2.1 | Isolation of eosinophils from human peripheral blood and nasal polyps

Cells positive for CD45 and negative for other cell surface markers (CD2, CD14, CD16, CD19, CD56, CD123, CD235a, and c-kit) were sort-purified from the peripheral blood of healthy volunteers and from single-cell suspensions of nasal polyps from patients with eosinophilic rhinosinusitis using a FACS Aria cell sorter (BD Biosciences, Franklin Lakes, NJ). Antibodies used were purchased from Miltenyi Biotec (anti-CD2, CD14, CD16, CD19, CD56, CD123, and CD235a; Bergisch Gladbach, Germany) and BioLegend (anti-CD45 and c-kit; San Diego, CA, USA). For patient classification, we used the diagnostic criteria for chronic rhinosinusitis based on the EPOS guidelines¹⁵ and eosinophilic rhinosinusitis defined in the JESREC Study, in which disease scores are based on bilateral lesions, presence of nasal polyps, dominant lesions of the ethmoid sinus detected by computed tomographic scanning, and blood eosinophilia.¹⁶ This study was approved by the Institutional Review Boards of the RIKEN Center for Integrative Medical Sciences, the Institutional Review Boards of Keio University School of Medicine, Ono Pharmaceutical Co., Ltd., and Nose Clinic Tokyo. All patients granted their written informed consent to participate. Demographic data of the subjects (six healthy subjects and six patients) included in the proteomic and transcriptomic analysis are presented in Table S1.

Single-cell suspensions from nasal polyps were prepared as follows. Nasal polyps resected during surgery were mechanically minced into small fragments. Samples transferred to a gentleMACS C Tube (Miltenyi Biotec, Bergisch Gladbach, Germany) were dissociated with the gentleMACS™ Dissociator (Miltenyi Biotec, Bergisch Gladbach, Germany) using the installed software program "h_tumor_01-03" and digested with collagenase and DNase. The suspension was centrifuged at $300 \times g$ for 10 minutes, and then, after hemolysis, the pellet was washed with PBS. The resulting single-cell suspension was diluted in PBS/0.5% BSA containing 2 mmol/L EDTA and filtered through a 100- μ m filter. Cells were stained with Diff-Quick (International Reagents Corp., Kobe, Japan) to determine morphology and measure cell size by BZ-9000 (Keyence Corp., Osaka, Japan).

2.2 | Flow cytometric analysis of cell surface markers

Eosinophils were labeled with the following monoclonal antibodies (mAbs): APC-conjugated CD44, CD52, CD69, C-C motif chemokine receptor 3 (CCR3), C-X-C motif chemokine receptor 4 (CXCR4), and siglec-8 (BioLegend, San Diego, CA, USA) for 30 minutes at 4°C. The expression of these markers was analyzed using a FACSCanto II flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA). All measurements were normalized to the value derived from the corresponding control isotype antibody.

2.3 | Targeted liquid chromatography (LC)-tandem mass spectrometry (MS/MS)-based lipidomics

The LC-MS/MS analysis was performed as previously described¹⁷ with technical modification in extraction protocol for fatty acid metabolites. Details were described in Methods section in this article's Supporting Information.

2.4 | RNA and protein extraction

NP-EOS and PB-EOS were lysed with 0.5 mL of TRIzol Reagent (Thermo Fisher Scientific, Waltham, MA, USA) and stored at -80°C until use. Total RNAs and proteins were isolated from the lysate according to the manufacturer's protocols. In particular, the final protein pellet was washed twice with 0.8 mL of acetone and then dissolved in a phase-transfer surfactant (PTS) buffer (12 mmol/L sodium deoxycholate, 12 mmol/L sodium N-lauroyl sarcosinate in 100 mmol/L Tris-HCl, pH 9.0) using a water bath-type sonicator (Bioruptor UCD-200; Cosmo Bio Co., Ltd., Tokyo, Japan) followed by centrifugation at $15\,000 \times g$ for 15 minutes at 4°C to remove insoluble materials.

2.5 | Proteome analysis

The extracted proteins were digested into peptides and analyzed as described in Methods section in this article's Supporting Information.

2.6 | RNA-seq analysis

RNA-seq libraries were prepared using an NEBNext Ultra RNA Library Prep Kit for Illumina (New England Biolabs, Ipswich, MA, USA) according to the manufacturer's protocols and were sequenced on a HiSeq 1500 DNA Sequencer (Illumina, San Diego, CA, USA) in a 50 base single-end read mode. The sequence reads were mapped to the human genome (NCBI version 19) using TopHat2 (version 2.0.8) and bowtie2 (version 2.1.0) with default parameters, and gene annotation was provided based on the NCBI RefSeq. The transcript abundances were estimated using Cufflinks (version 2.1.1), which was run with the same reference annotation with TopHat2 to generate FPKM (fragments per kilobase per million mapped reads) values for known gene models.

2.7 | Ingenuity pathway analysis of gene expression arrays

Molecular interactions among differentially regulated genes between NP-EOS and PB-EOS were explored using ingenuity pathway analysis (IPA) (Qiagen, Hilden, Germany). Each human gene identifier was mapped to its corresponding gene in the Ingenuity Pathway Knowledge Base. Families of genes that were up- or downregulated in NP-EOS and PB-EOS were integrated into predictive network models on the basis of gene interactions within a biologic pathway defined in the literature as contained in the Ingenuity Pathway Knowledge Base.

2.8 | Quantitative reverse transcription (RT)-PCR

Total RNA was extracted from human eosinophils isolated as specified above using the RNeasy® Mini Kit (Qiagen, Hilden, Germany). *GGT5*, *DPEP2*, and *ALOX5AP* transcripts were quantified by RT-PCR, using the High Capacity RNA-to-cDNA Master Mix (Applied Biosystems, Foster City, CA, USA), followed by quantitative PCR amplification with the Cyber Green method on an ABI PRISM® 7500 (Applied Biosystems, Foster City, CA, USA). The comparative threshold cycle method was validated and used to interpret the results.

2.9 | In vitro treatment of PB-EOS

We used a CD16-negative immunomagnetic selection method to isolate eosinophils from the peripheral blood of healthy volunteers, as previously reported.⁶ This study was approved by the Institutional Review Boards of the RIKEN Center for Integrative Medical Sciences. All patients granted their written informed consent to participate.

Purified eosinophils at 1.0×10^6 cells/mL in RPMI supplemented with 10% FBS, 100 IU/mL of penicillin, and 100 mg/mL of streptomycin were cultured with 10 ng/mL of type 2 cytokines (IL-4, IL-5, IL-33, GM-CSF) and TNF- α (R&D systems, Minneapolis, MN, USA) or 10 μ g/mL of TLR2 ligand (Pam3CSK4) and/or NOD2 ligand MDP (Invivogen, San Diego, CA, USA) for 6 hours or 3 days following analysis of mRNA expressions or lipid metabolism, respectively.

2.10 | Statistical analysis

Data are presented as mean $\pm$ standard error of the mean (SEM). Differences in lipid mediator biosynthesis, protein expression, and mRNA expression between two groups were examined using Student's *t* test. mRNA expression in eosinophils challenged with various stimulators or vehicle was evaluated by repeated analysis of variance measurements, followed by the Bonferroni/Dunn procedure as a *post hoc* test. Data were analyzed using GraphPad Prism version 4.0c (GraphPad Software, San Diego, CA, USA). A *P* value of 0.05 was considered significant.

3 | RESULTS

3.1 | Isolation of human eosinophils from nasal polyps of patients with eosinophilic sinusitis

To assess the functional characteristics of eosinophils in allergic inflammatory loci, we isolated eosinophils from nasal polyps of patients with eosinophilic sinusitis. Nasal polyps were collected by surgery during a therapeutic procedure. Given the many types of structural and hematopoietic cells in nasal polyps, it was difficult to isolate eosinophils with high purity. Therefore, we established an isolation method using negative selection with an antibody cocktail that excluded other cell types. To

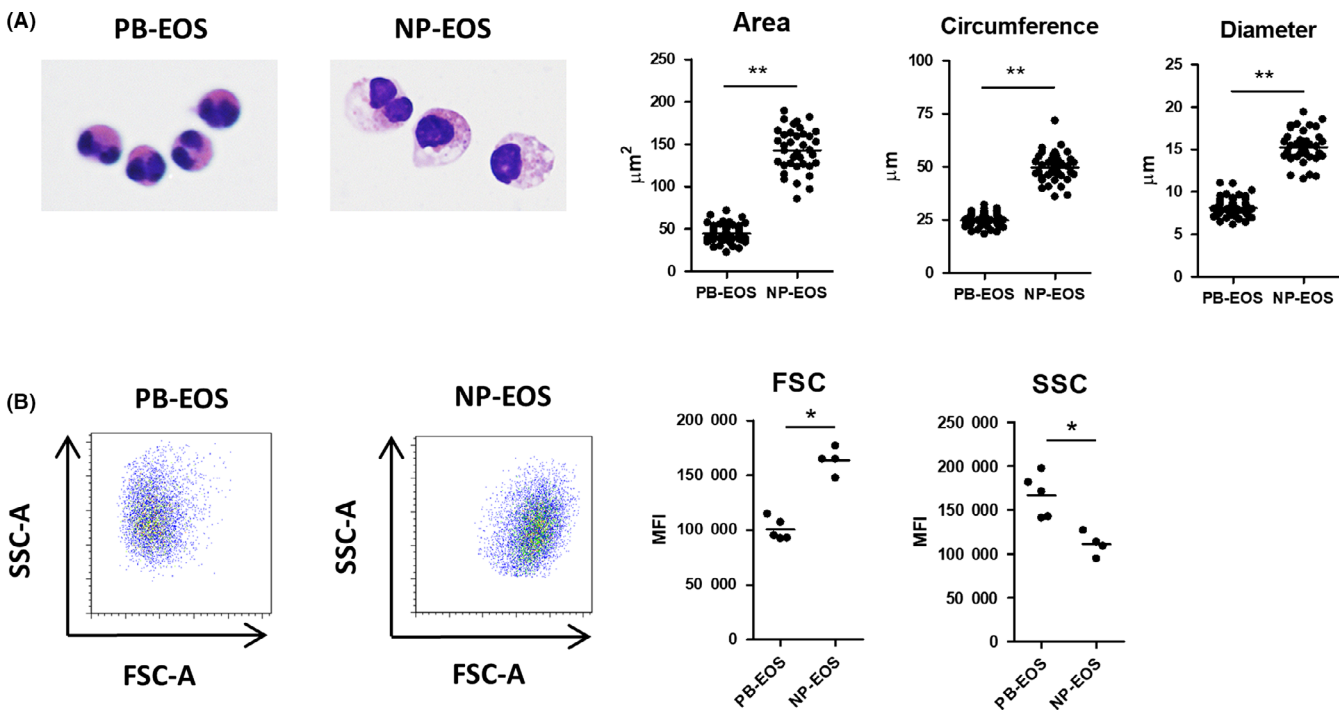


FIGURE 1 Morphological and structural character of tissue eosinophils from nasal polyps of patients with eosinophilic rhinosinusitis. The identity and purity of isolated cells using a cell sorter were confirmed by microscopy. Peripheral blood eosinophils from healthy subjects (PB-EOS) were used as a control. Morphological and structural differences between both groups were evaluated by an auto-detection system for microscopic analysis ($n = 37$ – 69 per group; data are obtained from three independent experiments) (A) and by flow cytometry ($n = 4$ – 6) (B). * $P < 0.05$, ** $P < 0.01$ compared between both groups. FSC, forward scatter; SSC, side scatter

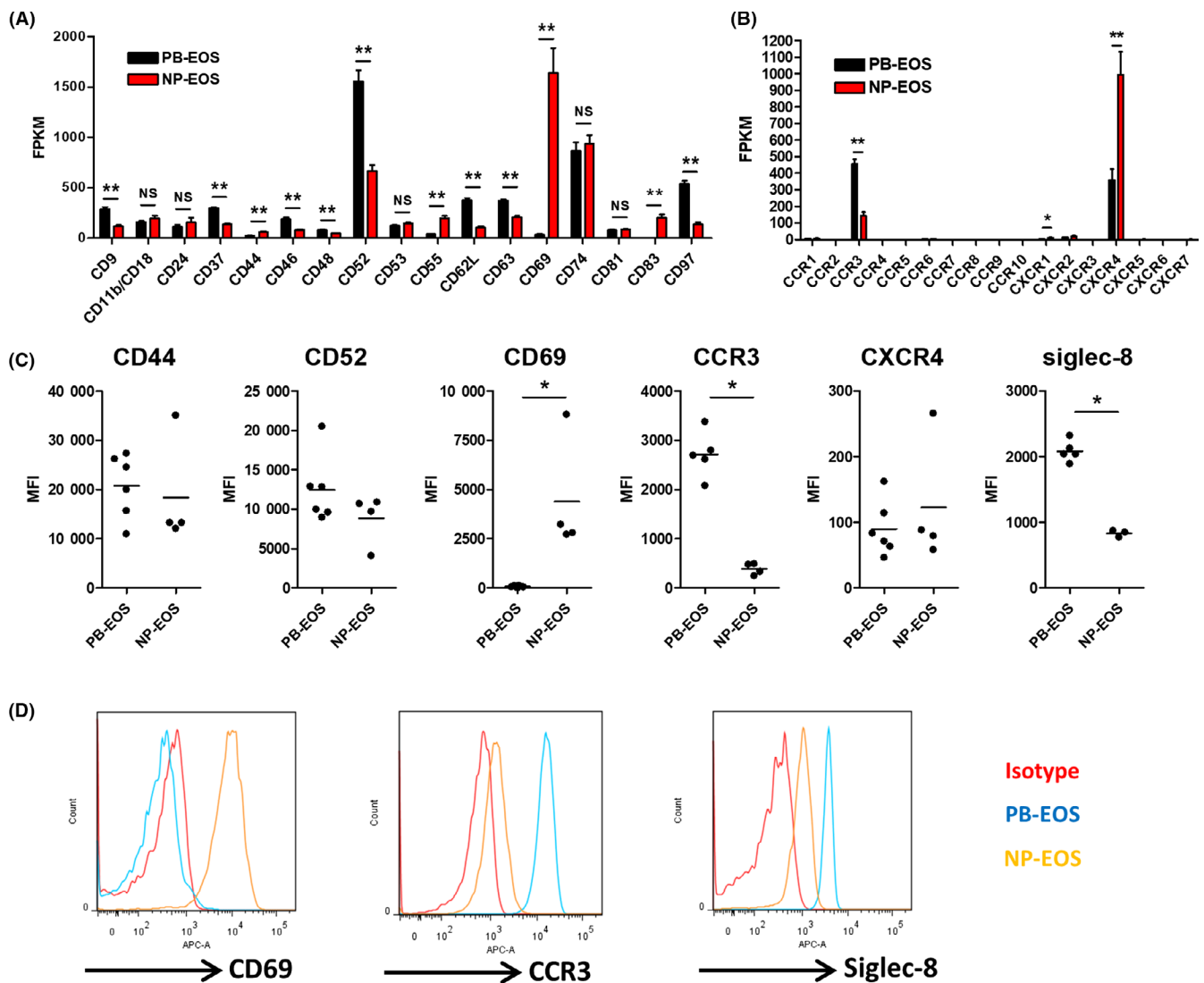


FIGURE 2 Flow cytometric analysis demonstrates that nasal polyp-derived eosinophils are CD69^{hi} CCR3^{low} CXCR4^{neg} siglec-8^{int}.

Transcriptomic and flow cytometric analyses of human eosinophils isolated from the peripheral blood of healthy subjects or nasal polyps of patients with eosinophilic rhinosinusitis. Gene expression of CD molecules (A) and chemokine receptors (B). Cell surface levels of CD44, CD52, CD69, C-C motif chemokine receptor 3 (CCR3), C-X-C motif chemokine receptor 4 (CXCR4), and siglec-8 on eosinophils were measured by flow cytometry (C). Histogram demonstrating the cell surface expression of CD69, CCR3, and siglec-8 on eosinophils (D). Histogram represent staining with control Ig (red) and antibodies specific for the indicated markers by PB-EOS (blue) and NP-EOS (yellow). Mean $\pm$ SEM of six samples from each group. * $P < 0.05$ compared between both groups [Colour figure can be viewed at wileyonlinelibrary.com]

further improve eosinophil identification and purity, we used CD45, a hematopoietic cell marker, and the cells' intrinsic auto-fluorescence (Figure S1). Isolated NP-EOS were >99% pure and larger in size than PB-EOS from healthy subjects. Morphological and structural features were observed by microscopic analysis using an auto-detection system. These analyses revealed significant differences between NP-EOS and PB-EOS, with NP-EOS having significant increases in cellular area, circumference, and diameter (Figure 1A). Furthermore, flow cytometric analysis revealed that NP-EOS had higher forward scatter and lower side scatter, indicating lower cell granularity as compared to PB-EOS (Figure 1B).

3.2 | Flow cytometric analysis of NP-EOS and PB-EOS

Next, we conducted flow cytometry to determine different levels of cell surface markers between PB-EOS and NP-EOS. Transcriptomic analysis identified several candidates with statistically significant differences, namely CD44, CD52, CD69, C-C motif chemokine receptor 3 (CCR3), and C-X-C motif chemokine receptor 4 (CXCR4) (Figure 2A and B). FACS analysis revealed a significant upregulation of CD69 and downregulation of CCR3 and siglec-8 in NP-EOS as compared to PB-EOS (Figure 2C and D). No difference was observed in CD44, CD52, or CXCR4 levels (Figure 2C), which might be related to post-transcriptional changes in these proteins. These results

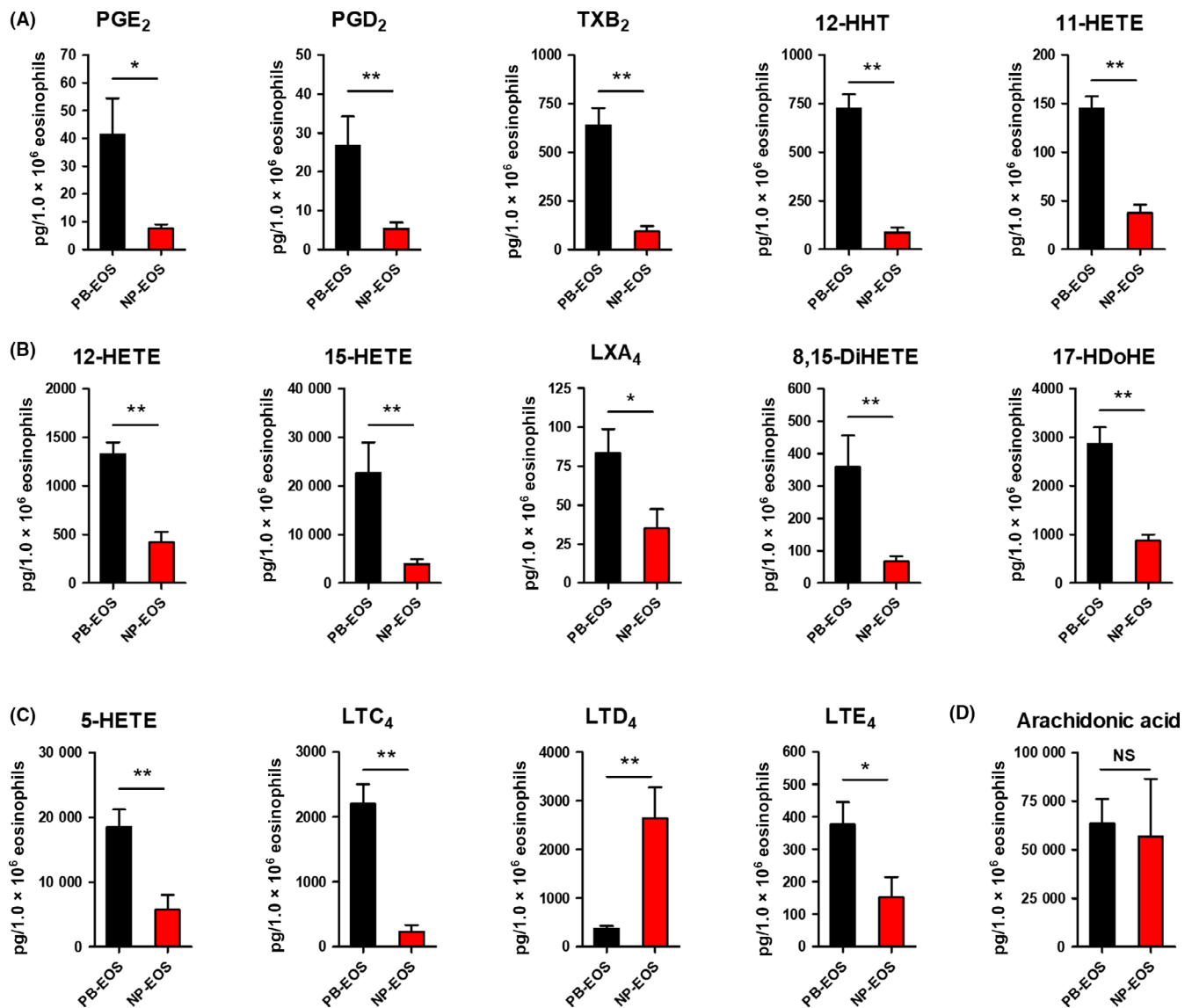


FIGURE 3 Dynamic changes in fatty acid metabolism between nasal polyp- and peripheral blood-derived eosinophils. Eosinophils isolated from peripheral blood of healthy subjects or nasal polyps of patients with eosinophilic rhinosinusitis were stimulated with 2 μ mol/L of A23187 for 30 minutes. Lipidomic analysis of eicosanoids biosynthesized by activated eosinophils was performed using the cell supernatant. Released amounts of arachidonic acid and arachidonic acid-derived lipid mediators from eosinophils: cyclooxygenase-derived mediators (prostaglandin E₂ (PGE₂), PGD₂, thromboxane B₂ (TXB₂), 12-hydroxyheptadecatrienoic acid (12-HHT), and 11-hydroxyeicosatetraenoic acid (HETE)) (A), 12/15-lipoxygenase-derived mediators (12-HETE, 15-HETE, lipoxin A₄, 8,15-diHETE, and 17-hydroxydocosahexaenoic acid (HDoHE)) (B), 5-lipoxygenase-derived mediators and cysteinyl leukotrienes (5-HETE, leukotriene C₄ (LTC₄), LTD₄, and LTE₄) (C), and arachidonic acid (D) were measured. Mean $\pm$ SEM of 4–7 samples from each group. * P < 0.05, ** P < 0.01 compared between both groups [Colour figure can be viewed at wileyonlinelibrary.com]

demonstrated that cell surface markers including CD69, CCR3, and siglec-8 could be used to distinguish NP-EOS from PB-EOS.

3.3 | Differences in fatty acid metabolism between NP-EOS and PB-EOS

To examine lipid metabolism, we measured fatty acid-derived metabolites released from both types of eosinophils stimulated with the calcium ionophore A23187. LC-MS/MS-based lipidomics demonstrated that NP-EOS produced significantly lower amounts of COX products (prostaglandin E₂ (PGE₂), PGD₂, thromboxane B₂ (TXB₂),

12-hydroxyheptadecatrienoic acid (12-HHT), and 11-hydroxyeicosatetraenoic acid (HETE)) and 12/15-LOX products (12-HETE, 15-HETE, lipoxin A₄, 8,15-diHETE, and 17-hydroxydocosahexaenoic acid (HDoHE)) as compared to PB-EOS (Figure 3A and B). Of the CysLT biosynthetic pathway products, LTD₄ was much enhanced and LTC₄ and E₄ were significantly decreased (Figure 3C). Despite dramatic changes in fatty acid metabolism, levels of arachidonic acid, a precursor of these mediators, were similar in both cell types (Figure 3D). These results demonstrated that NP-EOS display a specific inflammatory phenotype, with unique lipid mediator profiles as compared to PB-EOS.

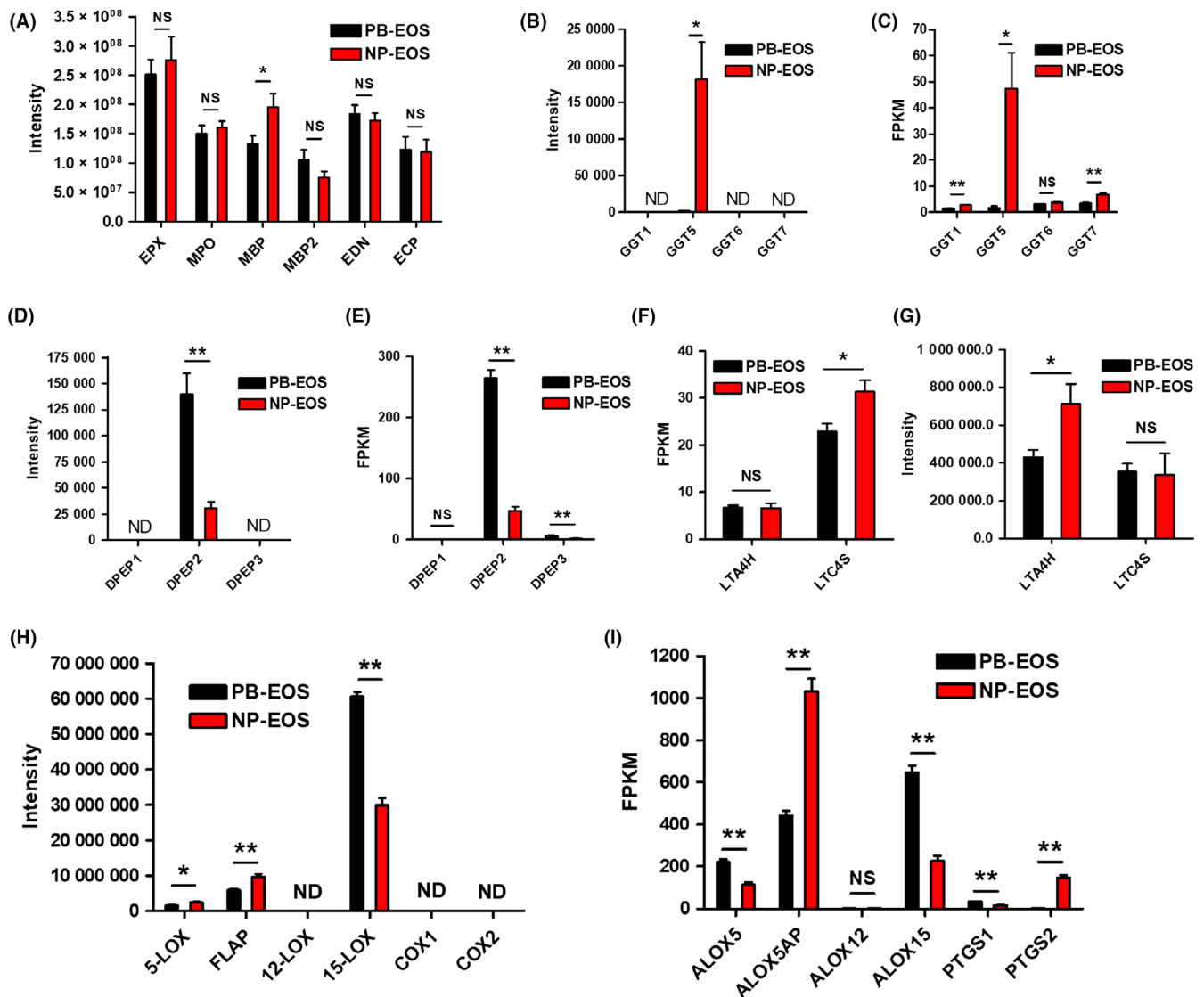


FIGURE 4 Proteomics and transcriptomics reveal that GGT5 and DPEP2 are key to enhancing LTD₄ synthesis. Proteomic and transcriptomic analyses of human eosinophils isolated from the peripheral blood of healthy subjects or nasal polyps of patients with eosinophilic rhinosinusitis were performed. Protein levels of eosinophilic granules (eosinophil peroxidase (EPX), myeloperoxidase (MPO), major basic protein (MBP), MBP2, eosinophil-derived neurotoxin (EDN), and eosinophil cationic protein (ECP)) (A), fatty acid metabolism-related enzymes: gamma-glutamyltransferases (GGT1, 5, 6, and 7) (B), dipeptidases (DPEP1, 2, and 3) (D), and other enzymes including lipoygenases and cyclooxygenases (leukotriene A4 hydrolase (LTA4H), leukotriene C4 synthase (LTC4S), 5-lipoxygenase (5-LOX), 5-lipoxygenase-activating protein (FLAP), 12-LOX, 15-LOX-1, cyclooxygenase 1 (COX1), and COX2) (F, H) were analyzed by proteomics. mRNA levels of GGT (C), DPEP (E), and other enzymes including lipoygenases and cyclooxygenases (LTA4H, LTC4S, arachidonate 5-lipoxygenase (ALOX5), arachidonate 5-lipoxygenase-activating protein (ALOX5AP), arachidonate 12-lipoxygenase (ALOX12), arachidonate 15-lipoxygenase (ALOX15), prostaglandin G/H synthase 1 (PGHS1), and prostaglandin G/H synthase 2 (PGHS2)) (G, I) were measured by transcriptomics. All data are Mean $\pm$ SEM of six samples from each group. * $P < 0.05$ compared between both groups [Colour figure can be viewed at wileyonlinelibrary.com]

3.4 | Proteomic and transcriptomic analyses of NP-EOS and PB-EOS

To understand the cellular characteristics of NP-EOS versus PB-EOS, we performed proteomic and transcriptomic analysis. In total, 2298 proteins and 19 529 transcripts were quantitatively evaluated for characterizing specific differences in protein and gene expression in the two cell types. Although upregulation of major basic protein (MBP) was observed in NP-EOS, protein

levels of other eosinophil-specific granules were similar in both groups (Figure 4A). Of note, changes in the CysLT-related enzymes in NP-EOS were characterized by upregulation of gamma-glutamyltransferase 5 (GGT5/GGT5) and arachidonate 5-lipoxygenase-activating protein (ALOX5AP/FLAP), and downregulation of dipeptidase-2 (DPEP2/DPEP2) at both mRNA and protein levels as compared to PB-EOS (Figure 4B-I). These changes should lead to enhanced LTD₄ levels and suppressed conversion into LTE₄, as was observed in the lipidomic analysis. The mRNA

level of GGT5 was also elevated in PB-EOS from patients with eosinophilic sinusitis, although the difference was not statistically significant (data not shown). The discrepancies between the protein and mRNA levels of some molecules might reflect the dynamic expression changes occurring during the maturation or activation period of eosinophils.¹⁸ Downregulation of arachidonate 15-LOX (ALOX15/15-LOX-1) in NP-EOS was observed at both mRNA and protein levels, concomitant with reduced levels of 15-LOX-derived mediators (Figure 4H and I). Protein levels of COX-1 (prostaglandin G/H synthase 1 (PGHS1)) and COX-2 (prostaglandin G/H synthase 2 (PGHS2)) were below the detection limit (Figure 4H). In addition, the protein level of hematopoietic prostaglandin D synthase (HPGDS/hPGDS) was below the detection limit, although the mRNA level of HPGDS was higher in NO-EOS, albeit without statistical significance (data not shown). There were slight differences in treatment prior to enrollment or surgery (Table S1); however, this had no significant effects on the results of omics analyses. Tables S2-S4 summarize the results of proteomic analysis.

3.5 | Role of type 2 cytokines and pattern recognition receptor signals in regulating NP-EOS-like phenotypes

To identify potential environmental factors and intracellular signals responsible for transforming conventional eosinophils into inflammatory phenotype, we performed ingenuity pathway analysis (IPA). This analysis revealed that NP-EOS had a characteristic gene expression pattern of type 2 cytokine responses such as GM-CSF, IL-4, IL-5, IL-13, and IL-33 (Table S5). IPA also identified activated signal transduction through pattern recognition receptors including TLRs and NODs (Tables S6 and S7). Previous reports indicated that *Staphylococcus aureus* is a major pathogen of the nasal mucosa and augments nasal inflammation by releasing exotoxins. In addition, components of *Staphylococcus aureus* functioned as TLR2 and NOD2 agonists.^{19,20} Indeed, TLR2 and NOD2 are functional receptors for eosinophil activation.^{21,22} Based on the above information, together with our IPA analysis, we hypothesized that both type 2 cytokines and pathogen-derived signals would be important to locally induce the NP-EOS-like inflammatory phenotype.

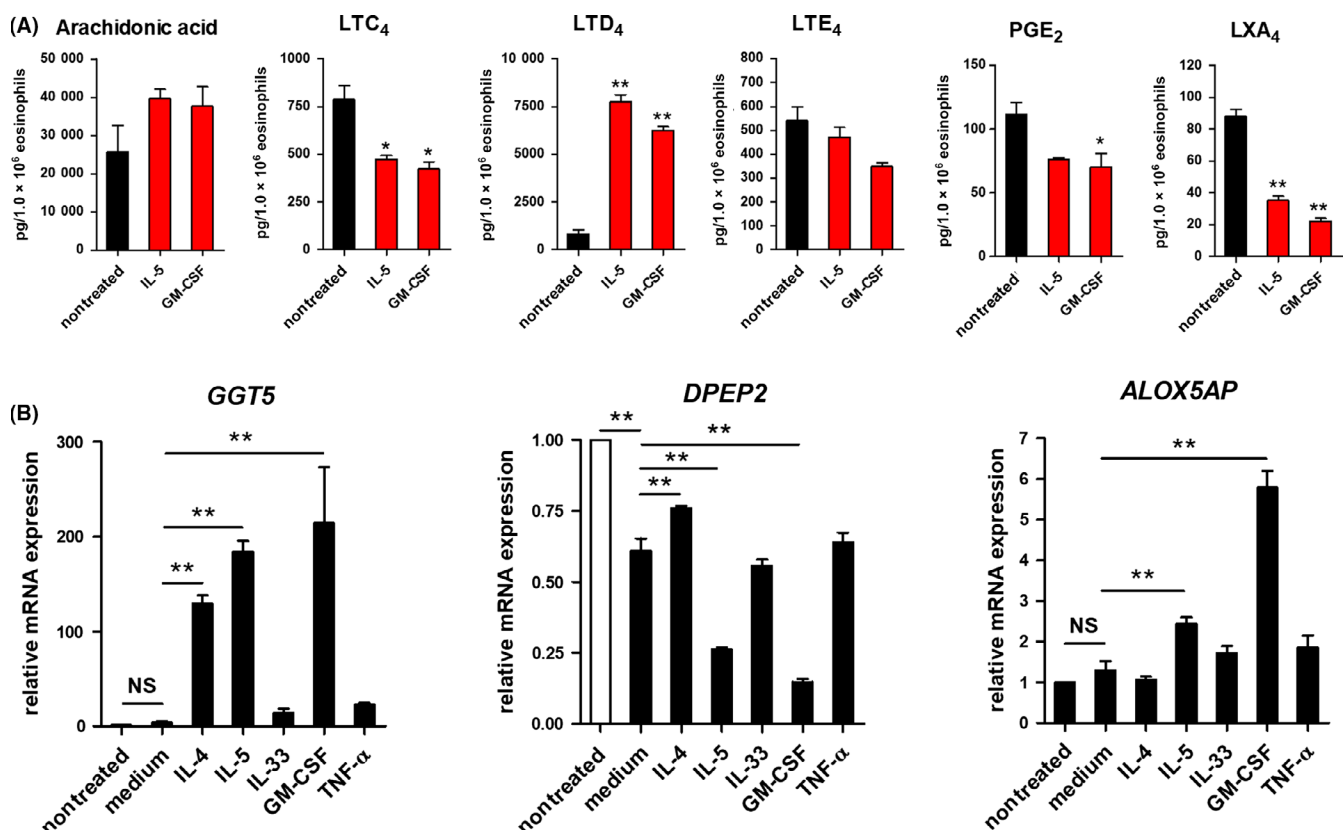


FIGURE 5 IL-5 and granulocyte-macrophage colony-stimulating factor (GM-CSF) induce high expression of gamma-glutamyltransferase 5 (GGT5) with LTD₄-synthesizing capacity. Human eosinophils from peripheral blood of healthy subjects were cultured with 10 ng/mL of type 2 cytokines (IL-4, IL-5, IL-33, and GM-CSF) and tumor necrosis factor-α (TNF-α) for 6 h or 3 d following quantitative RT-PCR and lipidomics, respectively. Effect of IL-5 and GM-CSF on the amounts of arachidonic acid and arachidonic acid-derived lipid mediators (leukotriene C₄ (LTC₄), LTD₄, LTE₄, lipoxin A₄ (LXA₄), and prostaglandin E₂ (PGE₂)) released from eosinophils stimulated with 2 μmol/L of A23187 for 30 min (A) and the mRNA levels of specific enzymes (GGT5, dipeptidase-2 (DPEP2), and arachidonate 5-lipoxygenase-activating protein (ALOX5AP)) required for modulation of cysteinyl leukotriene synthesis (B) were analyzed. Mean ± SEM of 3-15 samples from each group. *P < 0.05, **P < 0.01 compared with nontreated or medium control group [Colour figure can be viewed at wileyonlinelibrary.com]

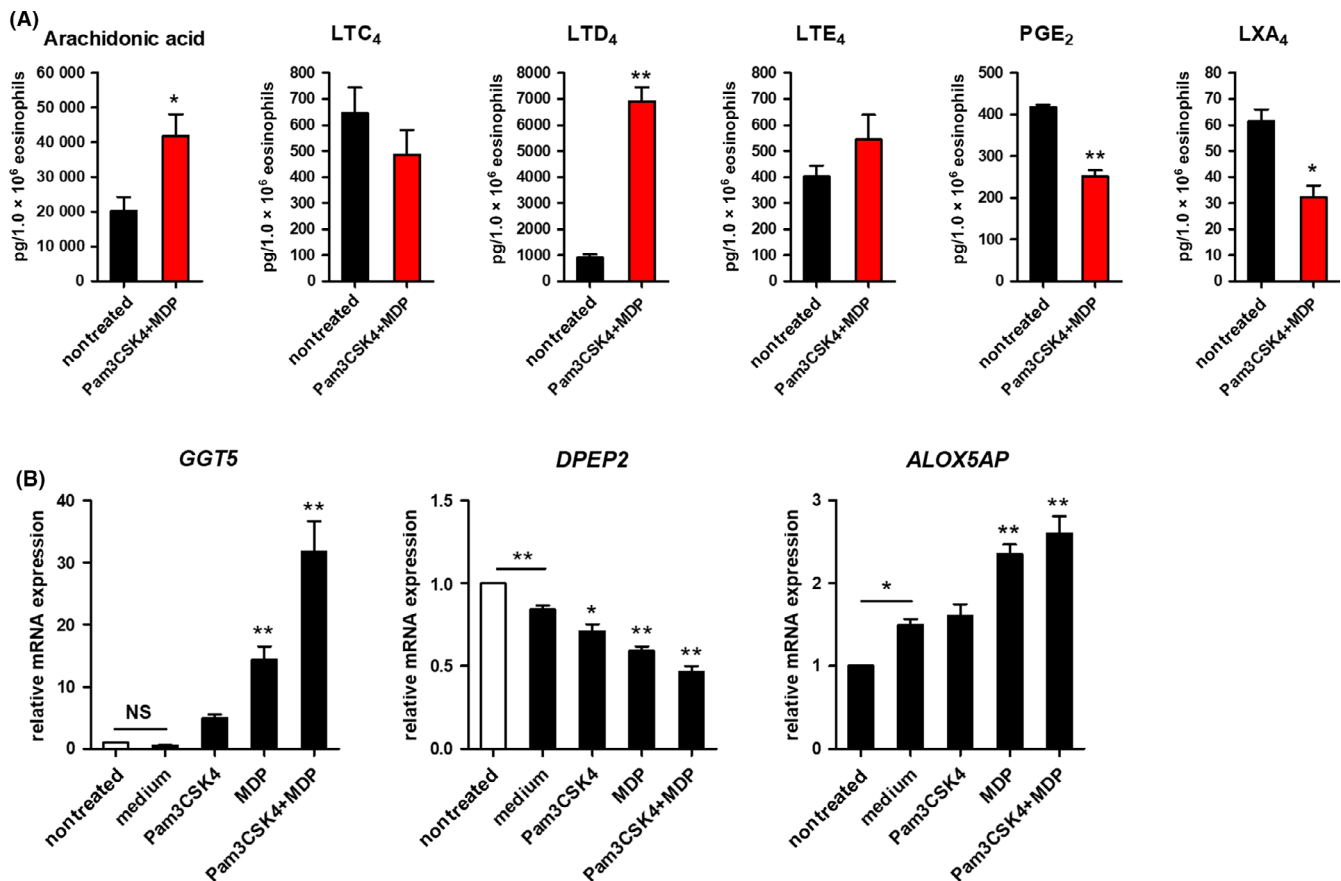


FIGURE 6 Combined stimulation with Toll-like receptor 2 (TLR2) and nucleotide-binding oligomerization domain-containing protein 2 (NOD2) agonists synergistically increases LTD₄-synthesizing capacity. Human eosinophils from peripheral blood of healthy subjects were cultured with 10 ng/mL of type 2 cytokines (IL-4, IL-5, IL-33, and granulocyte-macrophage colony-stimulating factor (GM-CSF)) and tumor necrosis factor- α (TNF- α) for 6 h or 3 d following quantitative RT-PCR and lipidomics, respectively. Effect of TLR2 and NOD2 agonists (Pam3CSK4 and MDP, respectively) on the amount of arachidonic acid and arachidonic acid-derived lipid mediators (leukotriene C₄ (LTC₄), LTD₄, LTE₄, lipoxin A₄ (LXA₄), and prostaglandin E₂ (PGE₂)) released from eosinophils stimulated with 2 μ mol/L of A23187 for 30 min (A) and the levels of mRNA encoding specific enzymes ((gamma-glutamyltransferase 5 (GGT5), dipeptidase-2 (DPEP2), and arachidonate 5-lipoxygenase-activating protein (ALOX5AP)) necessary for modulation of cysteinyl leukotriene synthesis (B) were analyzed. All data are Mean $\pm$ SEM of 3–8 samples from each group. * P < 0.05, ** P < 0.01 compared with nontreated or medium control group [Colour figure can be viewed at wileyonlinelibrary.com]

To test this hypothesis, we first treated PB-EOS with type 2 cytokines (IL-4, IL-5, IL-33, and GM-CSF) and other eosinophil activator (tumor necrosis factor- α (TNF- α)). Among those, IL-5 and GM-CSF induced morphological changes in PB-EOS including an enlarged cell size, CD69 upregulation, and prolonged survival (data not shown). Next, we tested the effect of IL-5 and GM-CSF on the eosinophil fatty acid metabolism. After incubation with these cytokines for 72 hours, PB-EOS produced higher amounts of LTD₄ and lower amounts of COX- and 15-LOX-derived mediators (Figure 5A). This lipid mediator profile was similar to that of NP-EOS. At the level of mRNA, IL-5 and GM-CSF upregulated GGT5 and ALOX5AP, and downregulated DPEP2 (Figure 5B). Similar to the IL-5 and GM-CSF treatment, TLR2 and NOD2 agonists induced metabolic changes in PB-EOS leading to increased synthesis of LTD₄ and decreased amounts of COX- and 15-LOX-derived mediators (Figure 6A). Moreover, the amount of arachidonic acid released increased, which was in contrast to the metabolic profile of NP-EOS, suggesting that NP-EOS might have enhanced capacity to convert arachidonic acid to

downstream mediators. At the level of mRNA, TLR2 and NOD2 agonists upregulated GGT5 and ALOX5AP, and downregulated DPEP2 (Figure 6B). Incubation with type 2 cytokines (IL-4, IL-5, and IL-33) and ligands of pattern recognition receptors (TLR2 and NOD2) for 24 hours also induced the LTD₄-synthesizing and/or LTC₄-converting capacity in eosinophils (Figure S2). These results indicate that stimulation with local activators (IL-5, GM-CSF, agonists for TLR2 or NOD2) is important to induce the NP-EOS-like inflammatory phenotype with dysregulated fatty acid metabolism in nasal polyps.

4 | DISCUSSION

In the present study, we characterized the phenotype of NP-EOS isolated from patients with eosinophilic rhinosinusitis. Multi-omics analyses revealed a unique inflammatory phenotype of NP-EOS with dysregulated fatty acid metabolism, that is, LTD₄ production was largely increased, while 15-LOX- and COX-derived mediators were

significantly reduced in NP-EOS as compared to PB-EOS. Treatment with eosinophil activators such as IL-5, GM-CSF, and agonists for TLR2 and NOD2 induced the dysregulated fatty acid metabolism in PB-EOS, suggesting that the local inflammatory milieu associated with nasal polyp is responsible for the development of the specific inflammatory phenotype of NP-EOS. Thus, control of type 2 cytokines using antibody-based drugs and targeting microorganisms with antibiotics or probiotics might be a novel therapeutic strategy for chronic eosinophilic rhinosinusitis.

For this study, we developed a method to isolate eosinophils from nasal polyps with >99% purity and high viability. A rapid procedure, including surgical resection of nasal polyps, shipment to the laboratory, and tissue disruption, is required to maintain viability of immune cells in the nasal polyp. Development of this procedure for clinical samples was critical, and it enabled us to conduct the phenotypic and multi-omics analyses to comprehensively characterize the unique properties of NP-EOS as compared to PB-EOS.

Human eosinophils are major producers of CysLTs such as LTC₄, D₄, E₄, and 15-LOX-derived mediators such as lipoxin A₄ and protectin D1.⁶ Eosinophils are also capable of producing substantial amounts of COX-derived prostaglandins. NP-EOS acquired the capacity to produce a large amount of LTD₄ concomitantly with a reduced release of lipoxins and prostaglandins. CysLT1, a G protein-coupled receptor with high affinity for LTD₄, is expressed by Th2 cells and type 2 innate lymphoid cells, and local production of LTD₄ would lead to accumulation of these cell types and promote a inflammatory milieu. Therefore, the molecular mechanisms underlying the enhanced production of LTD₄ by NP-EOS are of great interest.

A series of enzymes are required for LTD₄ biosynthesis in eosinophils. LTC₄ is synthesized from arachidonic acid by the sequential reaction of 5-LOX, FLAP, and LTC₄ synthase and is subsequently metabolized into LTD₄ and E₄ by GGT5 and DPEP2, respectively.^{23–25} Transcriptome and proteome analyses revealed that GGT5 expression was highly upregulated, while DPEP2 was significantly downregulated in NP-EOS. Changes in these enzymes appeared to define the inflammatory phenotype of NP-EOS with enhanced LTD₄ production, making them potential therapeutic targets to control eosinophilic inflammatory diseases.

It has been reported that dysregulated synthesis of 15-LOX- and COX-derived mediators is observed in aspirin-intolerant asthma and severe asthma.^{6,9–11,26,27} Also 15-LOX-derived products such as lipoxins, resolvins, and protectins are known to dampen allergic airway inflammation in mice.^{7,28–30} PGE₂, a major COX-derived product, also possesses anti-inflammatory properties in allergy and functions as an airway relaxant.^{31,32} NP-EOS exhibit a reduced capacity to synthesize these mediators, which might result in the loss of counter-regulatory signals locally in the nasal polyp. In addition, although we found reduced production of PGD₂, an important mediator to augment allergic airway inflammation, a previous study showed that PB-EOS from patients with aspirin-exacerbated respiratory disease acquired enhanced capacity for this mediator.³³ These findings might also lead to the novel therapeutic strategies by promoting these metabolic pathways and their products together with the local administration of stable analogs to enhance signals through their corresponding receptors.

Activated eosinophils identified in peripheral blood of individuals with eosinophilia are hypodense, have an altered capacity to produce CysLTs, and show prolonged survival.^{34,35} Eosinophils are capable of responding to several mediators, such as type 2 cytokines (IL-3,³⁶ IL-5,³⁷ IL-33,³⁸ and GM-CSF³⁹) and TNF- α .⁴⁰ Microbe-derived molecules (peptidoglycans, flagellin, ssRNA, and β -D glucan) also activate eosinophils to exert effector functions.^{41,42} Stimulation of PB-EOS with these eosinophil activators (IL-5, GM-CSF, and agonists of TLR2 and NOD2) induced dysregulated fatty acid metabolism as observed in NP-EOS. Indeed, eosinophils are activated by bacteria such as *S. aureus* and *Haemophilus influenza*, as well as by common environmental fungi, *Alternaria alternata* and *Aspergillus fumigatus*. These microbes induce inflammatory responses through TLR2 and NOD2,^{19,20,43–45} and should be considered as eosinophil activators together with the cytokines at local environment, such as in the nasal polyp.

Together, these findings have identified a unique phenotype with dysregulated fatty acid metabolism in inflammatory tissue-derived eosinophils such as NP-EOS. Development of methods to control key enzymes or metabolic pathways may be a novel therapeutic approach for diseases in which inflammatory eosinophils are suspected to be a key component of the pathogenesis.

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CONFLICTS OF INTEREST

The authors declare that they have no conflicts of interest.

AUTHOR CONTRIBUTIONS

JM, KF, OO, and MA designed the experiments. KF, OO, TB, and MA supervised the project. JM performed most of the experiments and compiled the data. YK and TW involved in experiments of proteomics and transcriptomics. AS and TH performed sample preparation and helped with multi-omics analysis. YA and TK provided the fresh nasal polyps. JM, KF, and MA wrote the manuscript. All authors provided feedback on the manuscript.

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REFERENCES

1. Qiu Y, Nguyen KD, Odegaard JI, et al. Eosinophils and type 2 cytokine signaling in macrophages orchestrate development of functional beige fat. *Cell*. 2014;157:1292–1308.

2. Wu D, Molofsky AB, Liang HE, et al. Eosinophils sustain adipose alternatively activated macrophages associated with glucose homeostasis. *Science (New York, NY)*. 2011;332(6026):243-247.
3. Heredia JE, Mukundan L, Chen FM, et al. Type 2 innate signals stimulate fibro/adipogenic progenitors to facilitate muscle regeneration. *Cell*. 2013;153:376-388.
4. Tani Y, Isobe Y, Imoto Y, et al. Eosinophils control the resolution of inflammation and draining lymph node hypertrophy through the proresolving mediators and CXCL13 pathway in mice. *FASEB J*. 2014;28:4036-4043.
5. Yamada T, Tani Y, Nakanishi H, Taguchi R, Arita M, Arai H. Eosinophils promote resolution of acute peritonitis by producing proresolving mediators in mice. *FASEB J*. 2011;25:561-568.
6. Miyata J, Fukunaga K, Iwamoto R, et al. Dysregulated synthesis of protectin D1 in eosinophils from patients with severe asthma. *J Allergy Clin Immunol*. 2013;131:353-360.
7. Serhan CN. Pro-resolving lipid mediators are leads for resolution physiology. *Nature*. 2014;510(7503):92-101.
8. Taniguchi N, Mita H, Saito H, Yui Y, Kajita T, Shida T. Increased generation of leukotriene C4 from eosinophils in asthmatic patients. *Allergy*. 1985;40:571-573.
9. Huynh ML, Malcolm KC, Kotaru C, et al. Defective apoptotic cell phagocytosis attenuates prostaglandin E2 and 15-hydroxyeicosatetraenoic acid in severe asthma alveolar macrophages. *Am J Respir Crit Care Med*. 2005;172:972-979.
10. Levy BD, Bonnans C, Silverman ES, Palmer LJ, Marigowda G, Israel E. Diminished lipoxin biosynthesis in severe asthma. *Am J Respir Crit Care Med*. 2005;172:824-830.
11. Planaguma A, Kazani S, Marigowda G, et al. Airway lipoxin A4 generation and lipoxin A4 receptor expression are decreased in severe asthma. *Am J Respir Crit Care Med*. 2008;178:574-582.
12. Bachert C, Zhang L, Gevaert P. Current and future treatment options for adult chronic rhinosinusitis: focus on nasal polyposis. *J Allergy Clin Immunol*. 2015;136:1431-1440.
13. Hamilos DL. Drivers of chronic rhinosinusitis: inflammation versus infection. *J Allergy Clin Immunol*. 2015;136:1454-1459.
14. Stevens WW, Lee RJ, Schleimer RP, Cohen NA. Chronic rhinosinusitis pathogenesis. *J Allergy Clin Immunol*. 2015;136:1442-1453.
15. Fokkens WJ, Lund VJ, Mullol J, et al. European position paper on rhinosinusitis and nasal polyps 2012. *Rhinol Suppl*. 2012;23:3. Preceding table of contents, 1-298
16. Tokunaga T, Sakashita M, Haruna T, et al. Novel scoring system and algorithm for classifying chronic rhinosinusitis: the JESREC Study. *Allergy*. 2015;70:995-1003.
17. Arita M. Mediator lipidomics in acute inflammation and resolution. *J Biochem*. 2012;152:313-319.
18. Kawashima Y, Miyata J, Watanabe T, Shioya J, Arita M, Ohara O. Proteogenomic analyses of cellular Lysates using a Phenol-Guanidinium Thiocyanate reagent. *J Proteome Res*. 2018. <https://doi.org/10.1021/acs.jproteome.8b00609>. [Epub ahead of print].
19. Schroder NW, Morath S, Alexander C, et al. Lipoteichoic acid (LTA) of *Streptococcus pneumoniae* and *Staphylococcus aureus* activates immune cells via Toll-like receptor (TLR)-2, lipopolysaccharide-binding protein (LBP), and CD14, whereas TLR-4 and MD-2 are not involved. *J Biol Chem*. 2003;278:15587-15594.
20. Hruz P, Zinkernagel AS, Jenikova G, et al. NOD2 contributes to cutaneous defense against *Staphylococcus aureus* through alpha-toxin-dependent innate immune activation. *Proc Natl Acad Sci USA*. 2009;106:12873-12878.
21. Wong CK, Cheung PF, Ip WK, Lam CW. Intracellular signaling mechanisms regulating toll-like receptor-mediated activation of eosinophils. *Am J Respir Cell Mol Biol*. 2007;37:85-96.
22. Kvarnhammar AM, Petterson T, Cardell LO. NOD-like receptors and RIG-I-like receptors in human eosinophils: activation by NOD1 and NOD2 agonists. *Immunology*. 2011;134:314-325.
23. Habib GM, Shi ZZ, Cuevas AA, Lieberman MW. Identification of two additional members of the membrane-bound dipeptidase family. *FASEB J*. 2003;17:1313-1315.
24. Carter BZ, Shi ZZ, Barrios R, Lieberman MW. gamma-glutamyl leukotrienase, a gamma-glutamyl transpeptidase gene family member, is expressed primarily in spleen. *J Biol Chem*. 1998;273:28277-28285.
25. Shi ZZ, Han B, Habib GM, Matzuk MM, Lieberman MW. Disruption of gamma-glutamyl leukotrienase results in disruption of leukotriene D(4) synthesis in vivo and attenuation of the acute inflammatory response. *Mol Cell Biol*. 2001;21:5389-5395.
26. Sanak M, Levy BD, Clish CB, et al. Aspirin-tolerant asthmatics generate more lipoxins than aspirin-intolerant asthmatics. *Eur Respir J*. 2000;16:44-49.
27. Roca-Ferrer J, Garcia-Garcia FJ, Pereda J, et al. Reduced expression of COXs and production of prostaglandin E(2) in patients with nasal polyps with or without aspirin-intolerant asthma. *J Allergy Clin Immunol*. 2011;128:66-72.
28. Levy BD, Kohli P, Gotlinger K, et al. Protectin D1 is generated in asthma and dampens airway inflammation and hyperresponsiveness. *J Immunol*. 2007;178:496-502.
29. Rogerio AP, Haworth O, Croze R, et al. Resolvin D1 and aspirin-triggered resolvin D1 promote resolution of allergic airways responses. *J Immunol*. 2012;189:1983-1991.
30. Levy BD, De Sanctis GT, Devchand PR, et al. Multi-pronged inhibition of airway hyper-responsiveness and inflammation by lipoxin A (4). *Nat Med*. 2002;8:1018-1023.
31. Tilley SL, Hartney JM, Erikson CJ, et al. Receptors and pathways mediating the effects of prostaglandin E2 on airway tone. *Am J Physiol Lung Cell Mol Physiol*. 2003;284:L599-L606.
32. Gavett SH, Madison SL, Chulada PC, et al. Allergic lung responses are increased in prostaglandin H synthase-deficient mice. *J Clin Invest*. 1999;104:721-732.
33. Feng X, Ramsden MK, Negri J, et al. Eosinophil production of prostaglandin D2 in patients with aspirin-exacerbated respiratory disease. *J Allergy Clin Immunol*. 2016;138:1089-1097.
34. Owen WF, Rothenberg ME, Petersen J, et al. Interleukin 5 and phenotypically altered eosinophils in the blood of patients with the idiopathic hypereosinophilic syndrome. *J Exp Med*. 1989;170:343-348.
35. Kajita T, Yui Y, Mita H, et al. Release of leukotriene C4 from human eosinophils and its relation to the cell density. *Int Arch Allergy Immunol*. 1985;78:406-410.
36. Rothenberg ME, Owen WF Jr, Silberstein DS, et al. Human eosinophils have prolonged survival, enhanced functional properties, and become hypodense when exposed to human interleukin 3. *J Clin Invest*. 1988;81:1986-1992.
37. Rothenberg ME, Petersen J, Stevens RL, et al. IL-5-dependent conversion of normodense human eosinophils to the hypodense phenotype uses 3T3 fibroblasts for enhanced viability, accelerated hypodensity, and sustained antibody-dependent cytotoxicity. *J Immunol*. 1989;143:2311-2316.
38. Cherry WB, Yoon J, Bartemes KR, Iijima K, Kita H. A novel IL-1 family cytokine, IL-33, potently activates human eosinophils. *J Allergy Clin Immunol*. 2008;121:1484-1490.
39. Owen WF Jr, Rothenberg ME, Silberstein DS, et al. Regulation of human eosinophil viability, density, and function by granulocyte/macrophage colony-stimulating factor in the presence of 3T3 fibroblasts. *J Exp Med*. 1987;166:129-141.
40. Roubin R, Elsas PP, Fiers W, Dessein AJ. Recombinant human tumour necrosis factor (rTNF)2 enhances leukotriene biosynthesis in neutrophils and eosinophils stimulated with the Ca2+ ionophore A23187. *Clin Exp Immunol*. 1987;70:484-490.
41. Nagase H, Okugawa S, Ota Y, et al. Expression and function of Toll-like receptors in eosinophils: activation by Toll-like receptor 7 ligand. *J Immunol*. 2003;171:3977-3982.

42. Yoon J, Ponikau JU, Lawrence CB, Kita H. Innate antifungal immunity of human eosinophils mediated by a beta 2 integrin, CD11b. *J Immunol*. 2008;181:2907-2915.
43. Shin SH, Kim YH, Jin HS, Kang SH. Alternaria induces production of thymic stromal lymphopoietin in nasal fibroblasts through toll-like receptor 2. *Allergy Asthma Immunol Res*. 2016;8:63-68.
44. Wu J, Zhang Y, Xin Z, Wu X. The crosstalk between TLR2 and NOD2 in *Aspergillus fumigatus* keratitis. *Mol Immunol*. 2015;64: 235-243.
45. Shuto T, Xu H, Wang B, et al. Activation of NF-kappa B by nontypeable *Hemophilus influenzae* is mediated by toll-like receptor 2-TAK1-dependent NIK-IKK alpha /beta-I kappa B alpha and MKK3/6-p38 MAP kinase signaling pathways in epithelial cells. *Proc Natl Acad Sci USA*. 2001;98:8774-8779.

SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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Online Supporting Information

Dysregulated fatty acid metabolism in nasal polyp-derived eosinophils from patients with chronic rhinosinusitis

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Methods

Targeted liquid chromatography (LC)-tandem mass spectrometry (MS/MS)-based lipidomics

Human eosinophils, isolated as detailed above, were suspended in Hank's buffered salt solution at 0.2×10^6 cells/mL and stimulated at 37°C for 30 min with 2 μ M A23187. The incubation was stopped by centrifugation at 4°C for 5 min. The stimulated cell supernatant was then diluted in same volume of methanol and applied to MonoSpin C18-AX columns (GL Sciences, Inc., Tokyo, Japan), an anion-exchange cartridge with a hydrophobic retention character, for solid-phase extraction. A deuterated internal standard consisting of 1 ng of LTB₄-d₄, LTD₄-d₅, PGE₂-d₄, and 15-HETE-d₈ (Cayman Chemical, Ann Arbor, MI) was added to the cell supernatants before extraction. We used a triple quadrupole linear ion trap mass spectrometer (QTRAP 5500; AB Sciex, Foster City, CA) equipped with a 1.7- μ m, 1.0 $\times$ 150 mm Acquity UPLC™ BEH C18 column (Waters Corp., Milford, MA). MS/MS analyses were performed in negative ion mode, and the eicosanoids and docosanoids were identified and quantified by multiple reaction monitoring. Calibration curves were obtained over a 1–1000-pg range. LC retention times for each compound were determined with corresponding synthetic standards.

Proteome analysis

The concentration of the extracted protein was measured using a BCA Protein Assay Kit (Thermo Fisher Scientific, Waltham, MA, USA) and adjusted to 1 mg/mL with PTS buffer. The extracted proteins were subjected to alkylation with 35 mM iodoacetamide in the dark at room temperature for 30 min after treatment with 10 mM dithiothreitol at 50 °C for 30 min. The reaction mixture was 4-fold diluted with 50 mM ammonium bicarbonate and then digested with Lys-C and trypsin for 18 h at 37 °C. After addition of an equal volume of ethyl acetate, the digestion mixture was acidified with 0.5% (final concentration) trifluoroacetic acid according to the PTS protocols^{1,2}. The mixture was shaken for 1 min, centrifuged at 15,000× g for 2 min for phase separation, and then the aqueous phase was retrieved. The volume of the aqueous phase thus recovered was reduced to half or less of the original volume by a centrifugal evaporator for complete removal of ethyl acetate. After desalting with C18-StageTips³, the recovered peptides were dried using a centrifugal evaporator and then finally dissolved in 3% acetonitrile and 0.1% formic acid.

Peptides were directly injected onto a 75 µm × 15 cm, PicoFrit emitter (New Objective, Woburn, MA, USA) packed in house with 2.7 µm core shell C18 particles (Capcell Core MP; Shiseido, Tokyo, Japan) and then separated with a 180-min gradient at a flow

rate of 300 nl/min using an Eksigent ekspert nanoLC 400 HPLC system (Sciex, Framingham, MA, USA). Peptides eluting from the column were analyzed on a TripleTOF 5600+ mass spectrometer (Sciex) for both shotgun-MS and sequential window acquisition of all theoretical mass spectra (SWATH)-MS analyses^{1,2,4,5}. For shotgun-MS based experiments, MS1 spectra were collected in the range of 400-1000 m/z for 250 ms. The top 25 precursor ions with charge states of 2+ to 5+ that exceeded 150 counts/s were selected for fragmentation with a rolling collision energy, and MS2 spectra were collected in the range of 100-1500 m/z for 100 ms. Dynamic exclusion time was set to 24 s. For SWATH-MS based experiments, the mass spectrometer was operated in a consecutive data-independent acquisition mode with 12 m/z increments in precursor isolation window. Using an isolation width of 13 m/z (1 m/z for the window overlap), a set of 50 overlapping windows was constructed covering the precursor mass range of 400–1000 m/z . SWATH MS2 spectra were in the range of 100–1500 m/z for 60 ms per MS2 experiment. Precursor ions were fragmented for each MS2 experiment using rolling collision energy.

All shotgun-MS files were searched against the human UniProt Swiss-Prot database using ProteinPilot software v. 4.5 with the Paragon algorithm (Sciex) for protein identification. The protein confidence threshold was a ProteinPilot unused score of 1.3

with at least one peptide with 95% confidence. Global false discovery rate for both peptides and proteins was lower than 1% in this study. The identified proteins were quantified from SWATH-MS data using PeakView v.2.2 (Sciex).

REFERENCES

1. Masuda T, Tomita M, Ishihama Y. Phase transfer surfactant-aided trypsin digestion for membrane proteome analysis. *Journal of proteome research* 2008; **7**(2): 731-740.
2. Masuda T, Saito N, Tomita M, Ishihama Y. Unbiased quantitation of Escherichia coli membrane proteome using phase transfer surfactants. *Molecular & cellular proteomics : MCP* 2009; **8**(12): 2770-2777.
3. Rappsilber J, Ishihama Y, Mann M. Stop and go extraction tips for matrix-assisted laser desorption/ionization, nanoelectrospray, and LC/MS sample pretreatment in proteomics. *Analytical chemistry* 2003; **75**(3): 663-670.
4. Gillet LC, Navarro P, Tate S, Rost H, Selevsek N, Reiter L *et al.* Targeted data extraction of the MS/MS spectra generated by data-independent acquisition: a new concept for consistent and accurate proteome analysis. *Molecular & cellular proteomics : MCP* 2012; **11**(6): O111.016717.
5. Collins BC, Gillet LC, Rosenberger G, Rost HL, Vichalkovski A, Gstaiger M *et al.* Quantifying protein interaction dynamics by SWATH mass spectrometry: application to the 14-3-3 system. *Nature methods* 2013; **10**(12): 1246-1253.

Figure legends

Supplementary Figure 1. Isolation of tissue eosinophils from nasal polyps of patients with eosinophilic rhinosinusitis.

Protocol for the isolation of nasal polyp-derived eosinophils (PB-EOS) using a cell sorter. Nasal polyps were dissected and lysed with collagenase to obtain a single-cell suspension. Single cells were incubated with antibodies specific for cell surface markers. Eosinophils were isolated by gating on CD45⁺ cells and selecting a negative population of specific cell surface markers for other hematopoietic cell types. Negative markers represent CD2, CD14, CD16, CD19, CD56, CD123, and CD235a. Increased autofluorescence in human eosinophils was detected in the panel to identify c-kit⁻ cells.

Supplementary Figure 2. Stimulation with type 2 cytokines (IL-4, IL-5, and IL-33) or agonists of pattern recognition receptors [nucleotide binding oligomerization domain containing 2 (NOD2) and/or Toll-like receptor 2 (TLR2)] affect cysteinyl leukotriene metabolism.

Human eosinophils from the peripheral blood of healthy subjects were cultured with 10 ng/ml of type 2 cytokines (IL-4, IL-5, and IL-33) and tumor necrosis factor- α (TNF- α) for 24 h followed by lipidomics analysis. Effect of type 2 cytokines (IL-4, IL-5, and

IL-33) (a) or TLR2 and NOD2 agonists (Pam3CSK4 and MDP, respectively) (b) on the amount of arachidonic acid and arachidonic acid-derived lipid mediators [leukotriene C4 (LTC₄), LTD₄, LTE₄, lipoxin A4 (LXA₄), and prostaglandin E2 (PGE₂)] released from eosinophils stimulated with 2 μM of A23187 for 30 min. All data are mean ± SEM of 4–8 samples per group. *p < 0.05, **p < 0.01 compared with non-treated or the medium control group.

Supplementary Table 1. Demographic data of the studied subjects

Demographic data of the studied subjects for proteomics and transcriptomics analysis are presented.

Supplementary Table 2. Upregulated proteins in nasal polyp-derived eosinophils

Eosinophils isolated from nasal polyps of patients with chronic eosinophilic rhinosinusitis (NP-EOS) and those from peripheral blood of healthy subjects (PB-EOS) were lysed with 0.5 mL of TRIzol Reagent and total proteins were isolated. The extracted proteins were digested into peptides and analyzed. Proteins that highly expressed in nasal polyp-derived eosinophils compared with peripheral blood-derived eosinophils are listed in this table. Protein expression levels presented in this table are

different between both groups with statistical significance ($p < 0.01$).

Supplementary Table 3. Downregulated proteins in nasal polyp-derived eosinophils

Eosinophils isolated from nasal polyps of patients with chronic eosinophilic rhinosinusitis (NP-EOS) and those from peripheral blood of healthy subjects (PB-EOS) were lysed with 0.5 mL of TRIzol Reagent and total proteins were isolated. The extracted proteins were digested into peptides and analyzed. Proteins that had lower expressions in nasal polyp-derived eosinophils compared with peripheral blood-derived eosinophils are listed in this table. Protein expression levels presented in this table are different between both groups with statistical significance ($p < 0.01$).

Supplementary Table 4. Specific proteins that determine the differences between eosinophil types

Eosinophils isolated from nasal polyps of patients with chronic eosinophilic rhinosinusitis (NP-EOS) and those from peripheral blood of healthy subjects (PB-EOS) were lysed with 0.5 mL of TRIzol Reagent and total proteins were isolated. The extracted proteins were digested into peptides and analyzed. Specific proteins that

determine the differences between eosinophil types are listed in this table. Protein expression levels presented in this table are different between both groups with statistical significance ($p < 0.01$).

Supplementary Table 5. Type 2 cytokines and related molecules identified as activators by ingenuity pathway analysis

Type 2 cytokines and related molecules in this table were identified as activators by ingenuity pathway analysis of human eosinophils isolated from either peripheral blood of healthy subjects (PB-EOS) or nasal polyps of patients with eosinophilic rhinosinusitis (NP-EOS). Families of genes that were up- or down-regulated in PB-EOS and NP-EOS were integrated into predictive network models on the basis of gene interactions within a biologic pathway defined in the literature as contained in the Ingenuity Pathway Knowledge Base.

Supplementary Table 6. Activated signal pathways predicted by ingenuity pathway analysis

Activated signal pathways predicted by ingenuity pathway analysis of human eosinophils isolated from either peripheral blood of healthy subjects (PB-EOS) or nasal

polyps of patients with eosinophilic rhinosinusitis (NP-EOS) are listed in this table. Families of genes that were up- or down-regulated in PB-EOS and NP-EOS were integrated into predictive network models on the basis of gene interactions within a biologic pathway defined in the literature as contained in the Ingenuity Pathway Knowledge Base.

Supplementary Table 7. Pattern recognition receptors in ingenuity pathway analysis

Analysis of Pattern recognition receptors by ingenuity pathway analysis of human eosinophils isolated from either peripheral blood of healthy subjects (PB-EOS) or nasal polyps of patients with eosinophilic rhinosinusitis (NP-EOS) are presented in this table. Families of genes that were up- or down-regulated in PB-EOS and NP-EOS were integrated into predictive network models on the basis of gene interactions within a biologic pathway defined in the literature as contained in the Ingenuity Pathway Knowledge Base.

Supplementary Table 1. Demographic data of the studied subjects

	Healthy Subjects	Patients with NP
n	6	6
Age (yrs old)	33 ± 2	45 ± 5
Sex (woman:man)	1:5	1:5
Peripheral blood eosinophils (%)	ND	9.1 ± 1.2
Peripheral blood eosinophils (cells/ μ l)	ND	509 ± 85
Total serum IgE levels (IU/L)	ND	360 ± 109
Nasal polyp	-	+
No. of surgeries	-	6
Hyposmia	-	6/6
Asthma	-	2/6
Aspirin intolerance	-	1/6
Treatment		
# of patients taking intranasal corticosteroids	-	4/6
# of patients taking inhaled corticosteroids	-	2/6
# of patients taking oral corticosteroids prior to surgery	-	1/6
# of patients taking leukotriene receptor antagonists	-	3/6
# of patients taking inhaled long-acting β -agonists	-	2/6
# of patients receiving anti-IgE antibody	-	0/6
# of patients receiving anti-IL5 antibody	-	0/6

IgE, immunoglobulin E; ND not determined; NP, nasal polyp

Supplementary Table 2. Upregulated proteins in nasal polyp-derived eosinophils

Protein name	Gene name	HS Ave	NP Ave	NP/HS
Gamma-glutamyltransferase 5	GGT5	1694	181934	107.42
Antileukoproteinase	SLPI	1967	196705	99.98
BPI fold-containing family A member 1	BPIFA1	48968	1987811	40.59
Collagen alpha-1(VI) chain	COL6A1	591	22879	38.70
Anterior gradient protein 2 homolog	AGR2	23384	564591	24.14
Immunoglobulin J chain	IGJ	7500	142160	18.95
Neutrophil defensin 1	DEFA1	2198624	36699588	16.69
Ig kappa chain V-III region GOL	LOC106504484	10065	142095	14.12
Lactotransferrin	LTF	852208	10267961	12.05
Protein-glutamine gamma-glutamyltransferase 2	TGM2	66294	796384	12.01

Supplementary Table 3. Downregulated proteins in nasal polyp-derived eosinophils

Protein name	Gene name	HS Ave	NP Ave	NP/HS
Semaphorin-4A	SEMA4A	64156	9919	0.15
Ena/VASP-like protein	EVL	94348	17265	0.18
Protein unc-45 homolog A	UNC45A	19978	3850	0.19
Condensin complex subunit 1	NCAPD2	8155	1712	0.21
Dipeptidase 2	DPEP2	139470	30361	0.22
Actin-related protein 10	ACTR10	45784	10288	0.22
Sorting nexin-2	SNX2	1933999	450566	0.23
Putative RNA-binding protein 15	RBM15	15550	3680	0.24
Nuclear autoantigenic sperm protein	NASP	207486	53990	0.26
Microtubule-associated serine/threonine-protein kinase 3	MAST3	17352	4786	0.28

Supplementary Table 4. Specific proteins that determine the differences between eosinophil types

Protein name	Gene name	HS Ave	NP Ave	NP/HS	p-value
Arachidonate 15-lipoxygenase	ALOX15	60649916	29890598	0.49	1.57E-06
CapZ-interacting protein	RCSD1	1467553	973570	0.66	4.53E-06
SH3 domain-containing kinase-binding protein 1	SH3KBP1	1162969	816145	0.70	5.37E-06
Semaphorin-4A	SEMA4A	64156	9919	0.15	1.34E-05
Pyruvate kinase PKM	PKM	37560789	29506340	0.79	2.40E-05
Histidine triad nucleotide-binding protein 1	HINT1	2162437	1657112	0.77	2.72E-05
Calponin-2	CNN2	3493901	1897552	0.54	3.01E-05
Programmed cell death protein 4	PDCD4	471323	215293	0.46	3.60E-05
Stathmin	STMN1	2242928	728188	0.32	5.14E-05
Protein-methionine sulfoxide oxidase MICAL1	MICAL1	864417	359990	0.42	7.99E-05

Supplementary Table 5. Type 2 cytokines and related molecules identified as activators by ingenuity pathway analysis

Upstream Regulator	Exp Fold Change	Predicted Activation State	Activation z-score	p-value of overlap
TNF	1	Activated	8.6	4.00E-79
CSF2	279	Activated	8.0	5.19E-20
IL4	1	Activated	7.3	4.48E-26
IL5	5	Activated	6.4	6.63E-11
leukotriene D4		Activated	5.3	2.02E-12
IL13	702	Activated	4.7	1.41E-28
CCL11	106	Activated	4.3	1.93E-07
Gm-csf		Activated	3.6	1.93E-07
leukotriene C4		Activated	3.3	1.38E-04
IL33	2491	Activated	3.0	6.83E-07

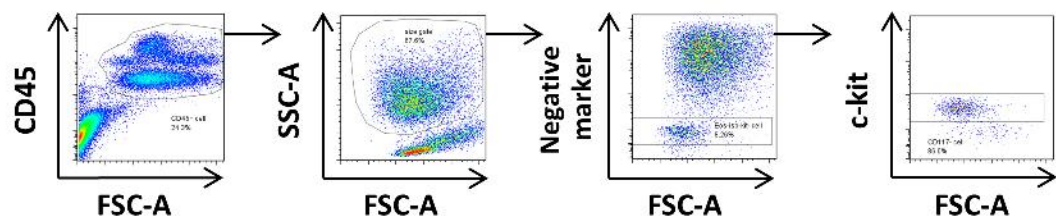
Supplementary Table 6. Activated signal pathways predicted by ingenuity pathway analysis

Ingenuity Canonical Pathways	-log(p-value)	zScore	Ratio
IL-6 Signaling	2.44E+00	4.52	3.88E-01
TREM1 Signaling	1.93E+00	3.65	3.95E-01
p38 MAPK Signaling	1.89E+00	3.57	3.68E-01
Toll-like Receptor Signaling	1.57E+00	3.54	3.78E-01
Chemokine Signaling	1.50E+00	3.40	3.73E-01
cAMP-mediated signaling	6.51E+00	2.95	4.26E-01
STAT3 Pathway	1.34E+00	2.89	3.65E-01
Role of IL-17F in Allergic Inflammatory Airway Diseases	1.83E+00	2.50	4.32E-01
Role of PRR in Recognition of Bacteria and Viruses	1.99E+00	2.35	3.68E-01

Supplementary Table 7. Pattern recognition receptors in ingenuity pathway analysis

Upstream Regulator	Exp Fold Change	Predicted Activation State	Activation z-score	p-value of overlap
TLR4	1.01	Activated	6.46	4.69E-18
TLR2	44.47	Activated	5.953	1.48E-08
TLR3	110.07	Activated	5.297	3.28E-12
NOD2	1.18	Activated	4.676	2.43E-09
TLR5	3.27	Activated	4.234	5.12E-05
TLR9	-1.20	Activated	3.983	4.61E-08
TLR7	-1.12	Activated	3.847	5.10E-12
TLR6	1.43	Activated	3.072	3.34E-02
CLEC7A	8.74	Activated	3.068	3.34E-02
TLR1	-1.57	Activated	2.944	1.02E-02
NOD1	-2.74	Activated	2.177	2.68E-02
TLR8	3.96	Activated	2.034	1.28E-04

Supplementary Figure 1



Supplementary Figure 2

