

Identification of granulocyte subtype-selective receptors and ion channels by using a high-density oligonucleotide probe array

Toshiharu Nakajima, PhD,^{a,b} Motoyasu Ikura, MD, PhD,^c
Yoshimichi Okayama, MD, PhD,^d Kenji Matsumoto, MD, PhD,^{a,c}
Chitose Uchiyama, MD,^c Taro Shirakawa, MD,^e Xing Yang, PhD,^f
Chaker N. Adra, PhD,^{f,g} Koichi Hirai, MD, PhD,^c and Hirohisa Saito, MD, PhD^{a,d}
Tokyo, Kyoto, and Yokohama, Japan, and Boston, Mass

Background: During inflammation, neutrophils, basophils, and eosinophils release cell type-specific mediators and proteases through signaling molecules, such as G protein-coupled receptors and ion channels. As such, ion channels and receptors, including G protein-coupled receptors, are common drug targets.

Objective: We sought to identify, for the first time, ion channels and receptors preferentially expressed by each granulocyte subtype.

Methods: Using GeneChip, we compared approximately 20,000 transcripts present in 7 leukocyte types, platelets, mast cells, and fibroblasts to identify granulocyte subtype-selective transcripts for receptors and ion channels. Granulocyte subtype-selective transcripts were chosen on the basis of several conditions, such as the transcript having a 5-fold or greater expression level compared with the maximum level of other leukocytes.

Results: Fifty-one transcripts were chosen to be preferentially expressed by each granulocyte subtype. Seventeen of the 51 transcripts have not been previously reported as granulocyte subtype selective. Among the 17 receptors and ion channels, 6 were basophil selective, eosinophil selective, or both and were not highly expressed by other organs, indicating that they might be potential targets for antiallergy drugs.

Conclusion: Use of this database of potential cell type-selective

drug targets should minimize the efforts required for pharmaceutical development. (*J Allergy Clin Immunol* 2004;113:528-35.)

Key words: Basophils, eosinophils, granulocytes, G protein-coupled receptors, ion channels

Three types of human blood granulocytes, eosinophils, basophils, and neutrophils, play roles in protecting against microbial infection by releasing cell type-specific mediators and proteases. Specifically, eosinophils and basophils evoke allergic reactions and damage nematodes.^{1,2} As well as killing bacteria, neutrophils sometimes induce systemic vasculitis or multiple organ damage under certain conditions.^{3,4} Thus targeting granulocyte type-selective functions is considered an important strategy for drug discovery.

Activation of blood granulocytes and tissue mast cells is generally characterized by an influx of extracellular calcium (Ca^{2+}), which is essential for subsequent release of granule-derived mediators, newly generated lipid mediators, and cytokines.⁵ The mechanism by which granulocyte mediator secretion is sustained is therefore likely to include modulation of various types of ion channels. Flow of ions, including K^+ and Cl^- , might play an important role during granulocyte responses because they regulate cell membrane potential and thus influence Ca^{2+} influx.⁶ Treatment of mast cells and basophils with pertussis toxin inactivates the G_i type of G proteins and abolishes degranulation induced by nonimmunologic ligands, such as thrombin and N-formylpeptide; however, it fails to inhibit the influx of Ca^{2+} .⁷ Thus Ca^{2+} -independent stimulation of G_i is also involved in granulocyte degranulation. The thrombin (protease)-activated receptors and formylpeptide receptors are classified as G protein-coupled receptors (GPRs), having an extracellular N-terminal segment, a 7-transmembrane region that forms the transmembrane core, 3 exoloops, 3 cytoloops, and a C-terminal segment.⁸ Thus ion channels and GPRs both play essential roles in degranulation, as well as other cellular functions important for granulocytes. As a result, both ion channels and GPRs are targets of drug development.⁹

As the human genome project nears completion, the identification of potential drug targets by using gene expression profiles from specific cell types is becoming practical and important for drug discovery.^{10,11} The

From ^athe Department of Allergy and Immunology, National Research Institute for Child Health and Development, Tokyo; ^bThe Organization for Pharmaceutical Safety and Research, Tokyo; ^cthe Department of Bioregulatory Function, University of Tokyo, Graduate School of Medicine, Tokyo; ^dthe Laboratory for Allergy Transcriptome, RIKEN Research Center for Allergy and Immunology, Yokohama; ^ethe Department of Health Promotion and Human Behavior, Kyoto University Graduate School of Public Health, Kyoto; ^fthe Department of Medicine and Pathology, Beth Israel Deaconess Medical Center, Boston; and ^gthe Department of Medicine, Children's Hospital, Harvard Medical School, Boston.

Supported in part by a grant from the Organization for Pharmaceutical Safety and Research and the Ministry of Health, Labour and Welfare (the Millennium Genome Project, MPJ-5), a grant from RIKEN Research Center for Allergy and Immunology (to H.S.), National Institutes of Health grant AI 43663 from the National Institute of Allergy and Infectious Diseases, grant RSG-01-241-01-LIB from the American Cancer Society (to C.A.), and by the Adra family.

Received for publication September 30, 2003; revised December 6, 2003; accepted for publication December 12, 2003.

Reprint requests: Hirohisa Saito, MD, PhD, Department of Allergy and Immunology, National Research Institute for Child Health and Development, 3-35-31 Taishido, Setagaya-ku 154-8567 Tokyo, Japan, or Chaker N. Adra, PhD, Department of Medicine, Beth Israel Deaconess Medical Center, 99 Brookline Ave., Boston, MA 02215.

0091-6749/\$30.00

© 2004 American Academy of Allergy, Asthma and Immunology
doi:10.1016/j.jaci.2003.12.036

Abbreviations used

AD:	Average difference (of mRNA expression level)
Ca ²⁺ :	Extracellular calcium
EMR:	Epidermal growth factor–like module containing mucin-like receptor
GAPDH:	Glyceraldehyde–phosphate dehydrogenase
GPR:	G protein–coupled receptor
PAR:	Proteinase-activated receptor
Siglec:	Sialic acid–binding Ig-like lectin

sequencing of the human genome is offering an unprecedented opportunity for the pharmaceutical development of drugs. Receptor genes and ion channel genes are found only in 5% and 1.3% of all genes present in the human genome, respectively.¹⁰ However, receptors and ion channels are found in 45% and 5% of the molecular targets of all known drugs, respectively.^{9,12,13} Thus receptors, including GPRs, and ion channels are now considered the most important drug targets.

Until recently, it has been impractical to analyze genome-wide expression of leukocytes. A newly developed technology, the microarray or high-density oligonucleotide probe array (GeneChip), is one of the latest breakthroughs in experimental molecular biology, which allows approximately 39,000 transcripts derived from a cell's transcriptome to be simultaneously monitored. Using this technology, we previously reported the transcriptome profiling of various types of mast cells and eosinophils.^{14–16} In this study we used GeneChip (version U133A, containing approximately 22,000 gene probes) to examine the cell type–selective transcriptome expression of 7 types of leukocytes (basophils, eosinophils, neutrophils, CD4⁺ cells, CD8⁺ cells, CD14⁺ cells, and CD19⁺ cells), platelets, mast cells, and fibroblasts. Then we focused on the expression of granulocyte-selective genes for ion channels, GPRs, and other receptors. We identified 17 novel granulocyte subtype–selective transcripts that might prove useful for drug development.

METHODS

Purification of leukocytes

All human subjects in this study provided written informed consent, and the ethical review boards at the relevant hospitals (National Center for Child Health and Development and Jikei University School of Medicine) approved the study. The subjects used in this study were all healthy volunteers, specifically chosen for having no allergic diseases.

Granulocytes and mononuclear cells were separated from the venous blood of normal volunteers. Human basophils were semipurified by means of Percoll (Pharmacia, Uppsala, Sweden) density gradient centrifugation, and the cells were further purified by means of negative selection through use of a MACS Basophil Isolation Kit (Miltenyi Biotec, Bergisch-Gladbach, Germany), as described previously.¹⁷ Eosinophils were isolated by using Percoll (1.090 g/mL) density centrifugation. The eosinophils were further purified by means of negative selection with anti-CD16–bound micromagnetic beads, as described previously.¹⁸ Neutrophils were isolated by using Percoll (1.085 g/mL) density centrifugation and further purified by

means of negative selection with anti-CD81 antibody and anti-mouse IgG-bound micromagnetic beads to eliminate contaminating eosinophils. These granulocytes purified from human peripheral blood were spun down onto glass slides by using the Cytospin II (Shandon Southern Instruments Inc, Sewickley, Pa). The purity of these cells was evaluated on the basis of 500 cells stained with May-Grünwald and Giemsa solutions.

For preparation of lymphocytes and monocytes, PBMCs were isolated by means of centrifugation on lymphocyte separation medium (Organon Teknica Corp, Durham, NC). Monocytes (CD14⁺ cells) were prepared with magnetic bead–conjugated CD14⁺ antibody (CD14 MicroBeads; Miltenyi Biotec) from PBMCs. CD4⁺ and CD8⁺ cells were also sorted by using magnetic bead–conjugated CD4⁺ (CD4 MicroBeads; Miltenyi Biotec) and CD8⁺ antibodies (CD8 MicroBeads; Miltenyi Biotec), respectively, from PBMCs after depletion of CD14⁺ cells with MACS CD14 MicroBeads (Miltenyi Biotec). The purity of CD4⁺, CD8⁺, and CD14⁺ cells was evaluated by staining the magnetic bead–conjugated cells compared with feasible control cell preparations, such as unpurified cells, with FITC-labeled goat anti-mouse Ig (BD Pharmingen, Tokyo, Japan). Peripheral B cells were purified by using a combination of negative (MicroBead–conjugated antibodies to CD3, CD7, CD14, CD42b, and CD56; Miltenyi Biotec) and positive (CD19 MicroBeads; Miltenyi Biotec) selection with MicroBeads (Miltenyi Biotec). Blood samples were mixed with 3.8% (wt/vol) sodium citrate solution (9:1) and centrifuged at 260g for 15 minutes at 20°C to obtain platelet-rich plasma. The plasma was centrifuged again at 260g for 15 minutes to remove any contaminating erythrocytes and leukocytes.

Human mast cells were derived from cord blood CD34⁺ progenitor cells, as described previously.^{11–14} Briefly, progenitor cells purified from peripheral blood by using CD34⁺ isolation kits (Miltenyi Biotec) were cultured in Iscove modified Dulbecco medium supplemented with 1% insulin-transferrin-selenium supplements (Invitrogen, Tokyo, Japan), 50 μmol/L 2-mercaptoethanol, antibiotics, and 2% FCS in the presence of 100 ng/mL stem cell factor and 50 ng/mL IL-6. After 11 to 14 weeks of culture, tryptase-positive cells represented more than 99% of the cells.

Purification of human lung mast cells and nasal polyp–derived fibroblasts

Normal human lung tissue dissected during surgery was obtained macroscopically after obtaining informed consent. Human lung mast cells were dispersed from chopped lung specimens by means of an enzymatic procedure and were purified by using magnetic bead affinity selection with the mAb anti-kit YB5.B8 (BD Pharmingen, San Diego, CA), as described previously.¹⁹ The cells were further cultured in the presence of stem cell factor and IL-6 for several weeks. Human nasal polyp–derived fibroblasts were obtained as previously reported.²⁰

GeneChip expression analysis

Human genome–wide gene expression was examined by using the Human Genome U133A probe array (GeneChip; Affymetrix, Santa Clara, Calif), which contains the oligonucleotide probe set for 22,000 full-length genes. Experiments were performed in accordance with the manufacturer's protocol (Expression Analysis Technical Manual) and previous reports.^{11–14} Total RNA (1–5 μg) was extracted from 10⁷ cells. Double-stranded cDNA was synthesized by using a SuperScript Choice system (Invitrogen) and a T7-(dT)24 primer (Amersham Pharmacia Biotech, Buckinghamshire, United Kingdom). The cDNA was subjected to in vitro transcription in the presence of biotinylated nucleoside triphosphates by using a BioArray high-yield RNA transcript labeling kit (Enzo Diagnostics, Farmingdale, NY). The biotinylated cRNA was hybridized with a

probe array for 16 hours at 45°C. In some experiments, as indicated in the supplementary table, biotinylated cRNA was prepared by using 2 cycles of cDNA synthesis, and in vitro transcription for target amplification was performed according to the manufacturer's directions (Affymetrix, Inc). For the latter protocol, we used 100 ng of total RNA. After washing, the hybridized biotinylated cRNA was stained with streptavidin-phycoerythrin (Molecular Probes, Eugene, Ore) and then scanned with an HP gene array scanner. The fluorescence intensity of each probe was quantified by using a computer program, GeneChip Analysis Suite 5.0 (Affymetrix). The expression level of single mRNA was determined as the average fluorescence intensity among the intensities obtained with 11 paired (perfect-matched and single nucleotide-mismatched) probes. If the intensities of mismatched probes were very high, gene expression was judged to be absent, even if a high average fluorescence was obtained with the GeneChip Analysis Suite 5.0 program. The level of gene expression was determined as the average difference (AD) by using the GeneChip software. Each AD level was then normalized by dividing it with the median value of 22,283 AD levels obtained in an experiment (ie, the normalized AD level).

Real-time RT-PCR

Total RNA was isolated with Isogen (Nippon gene, Tokyo, Japan), according to the manufacturer's instructions, and quantified by measuring the absorbance at 260 nm. RNA was subsequently treated with DNase I (Invitrogen) reverse transcribed by using Superscript II reverse transcriptase (Invitrogen). Real-time RT-PCR was performed with 10 ng of cDNA in 25 μ L of final volume by using the primers and probes supplied by the Assays-on-Demand Gene Expression System (Applied Biosystem, Tokyo, Japan), according to the manufacturer's instructions. Measurement of gene expression was performed with the ABI PRISM 7700 Sequence Detector. The expression level of each gene was normalized to a reduced glyceraldehyde-phosphate dehydrogenase (GAPDH).

Staining of basophils with anti-HTm4

Basophils purified from human peripheral blood with the Basophil Isolation Kit (Miltenyi Biotec) were spun down onto glass slides by using the Cytospin II (Shandon). Cells were fixed with acetone for 1 minute and then blocked in 2% goat serum in 50 mmol/L Tris-Cl, pH 7.4, for 1 hour. Cells were further incubated for 2 hours with 2 μ g/mL of the polyclonal antibody rabbit anti-hHTm4. Cells were then washed 3 times with PBS and incubated with a secondary antibody, highly cross-adsorbed Alexa Fluor 546-conjugated goat anti-rabbit IgG (H+L; Red; Molecular Probes), for 1 hour. After 3 PBS washes, air-dried cells were further mounted by using the Prolong Anti-Fade Kit (Molecular Probes). Slides were scanned with a Zeiss Laser Scanning Microscope 5 Pascal (Carl Zeiss Microimaging Inc, Thornwood, NY).

Purity and viability of the leukocytes and RNA quality

We used leukocyte samples in this study only if the purity of each cell type was at least 98%, but there were greater than 0.5% contaminated cells in any of the samples. We could not evaluate the purity of CD19⁺ cells and platelets because of the lack of feasible controls or methods. However, specific transcript markers for non-granulocytes (eg, CD4, CD8, CD14, CD19, and IgG), as well as granulocyte subtype-specific transcripts, were reasonably expressed by each leukocyte type, as shown in Online Repository Table E1. Regarding the viability, we qualified the RNA before GeneChip assay by using Array Quality Metrics Comparisons Software (Affymetrix), as well as trypan blue staining (they were always >95% viable), because RNase-rich granules derived from degenerating cells rapidly destroy RNA transcripts. Briefly, to eval-

uate the quality of RNA, the ratio of the 3'-probe set and 5'-probe set of housekeeping genes were compared as shown in Online Repository Table E1. According to the above software's guidance, the ratio of greater than 2:1 at a standard sample (5 μ g total RNA) protocol and that of greater than 10:1 at a small sample (50 ng of total RNA) protocol were recommended. As shown in Online Repository Table E1, the cells used in the present study had the appropriate ratios of the 3'-probe set and 5'-probe set of housekeeping genes, suggesting that these cells were highly viable.

Statistical analysis

Because logarithmic normalized AD levels were normally distributed within each group, the unpaired, parametric, 2-tailed Student *t* test was used to analyze the data on a logarithmic scale.

RESULTS

In this study we have used a high-density oligonucleotide probe array (GeneChip) to measure the expression levels of approximately 20,000 different transcripts in highly purified cells. These cells were basophils, eosinophils, neutrophils, monocytes (CD14⁺), T lymphocytes (CD4⁺ and CD8⁺ cells), B lymphocytes (CD19⁺), lung-derived mast cells, cord blood-derived cultured mast cells, and nasal polyp-derived fibroblasts. The GeneChip assay allows the simultaneous measurement of large numbers of transcripts by using relatively small numbers of cells. Using this technology, we could even measure triplicate transcriptome levels of basophils, the most rare granulocytes in peripheral blood.

Cell type-selective transcripts were selected on the basis of the following criteria. First, the average normalized AD expression level of each gene in a certain cell type must be 3-fold or greater than the maximal level in other cell types, and second, it must be significantly ($P < .01$) greater than that in other cell types. Third, the AD expression level provided with "absence" or "marginal" call by GeneChip Suite Software should be observed only once or not at all in the 3 or 4 independent experiments (3 experiments for basophils and 4 experiments for eosinophils and neutrophils) by using different cell populations. Finally, for the transcripts preferentially expressed for the 2 different cell types, such as basophils and eosinophils, the average normalized AD expression levels in the 2 cell types should be within 3-fold of each other. By using these standards, we found 83 basophil-selective, 37 eosinophil-selective, 257 neutrophil-selective, 34 basophil/eosinophil-selective, 19 eosinophil/neutrophil-selective, and 17 basophil/neutrophil-selective transcripts. Because of the functional similarity with basophils, mast cell-selective transcripts were also examined, and 63 mast cell-selective and 11 mast cell/basophil-selective transcripts were also detected (Table I and Online Repository Table E1). Because mast cells, basophils, and eosinophils play similar roles in allergic inflammation, the transcripts preferentially expressed for the 3 granulocytes by comparing their average normalized AD levels with those of other leukocytes. Thirty-four transcripts were then selected; however, most of them were overlapped with the transcripts listed in

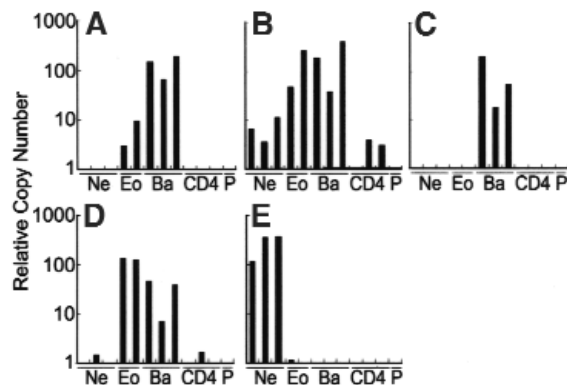


FIG 1. Real-time quantitative PCR analysis. The relative mRNA expression level by each cell type against PBMCs was shown after normalization of mRNA levels for HTm4 (0.42 per 100 GAPDH; **A**), Ca²⁺ receptor α 1D (0.003 per 100 GAPDH; **B**), prostaglandin E receptor 3a2 (0.12 per 100 GAPDH; **C**), EMR-1 (0.62 per 100 GAPDH; **D**), and aquaporin 9 (0.92 per 100 GAPDH; **E**), expressed by PBMCs. Ne, Neutrophils; Eo, eosinophils; Ba, basophils; CD4, CD4⁺ cells; P, PBMCs.

Online Repository Table E1. Only 4 transcripts (*MYB*, *SAMSN1*, *BACE2*, and *CASP3*) were found not to be overlapped, and they were not receptors or ion channels.

Among the 491 granulocyte-selective transcripts listed in Online Repository Table E1, 4 ion channels, 19 GPRs, and 28 other receptors were further selected (Table II). When plural transcripts obtained by different probe sets had identical Genbank or Unigene accession numbers (<http://www.ncbi.nlm.nih.gov/>), the transcript showing the highest expression level was selected.

Ion channels and receptors preferentially expressed by granulocytes other than neutrophils

Eosinophils, basophils, and mast cells play an important role in the pathogenesis of allergic diseases but do not play an essential role in killing microbes, except for nematodes. On the other hand, neutrophils play a crucial role in killing microbes, such as bacteria. Caution should be taken in regulating neutrophil function, even in the case of neutrophil-induced inflammation. Therefore the molecules present only in granulocytes, except for neutrophils, would be important pharmaceutical targets for allergic disorders.^{1,2}

Among the 51 granulocyte-selective transcripts for ion channels and receptors, we identified 17 granulocyte-selective transcripts that have not been reported for their selective expression (shown in bold letters in Table II). Of these 17 transcripts, 8 were preferentially expressed by granulocytes other than neutrophils. Among these 8 transcripts, the 2 transcripts for fibroblast growth factor receptor 2 and low-density lipoprotein receptor were found to be expressed by multiple tissue cell types (shown at http://www.lsbm.org/index_e.html, which displays genomic expression of 55 different human tissue cells, such as brain, heart, and lung cells, using the same experimental system [Affymetrix U133A] as ours). Thus they might not be suitable as a drug target because important organs that

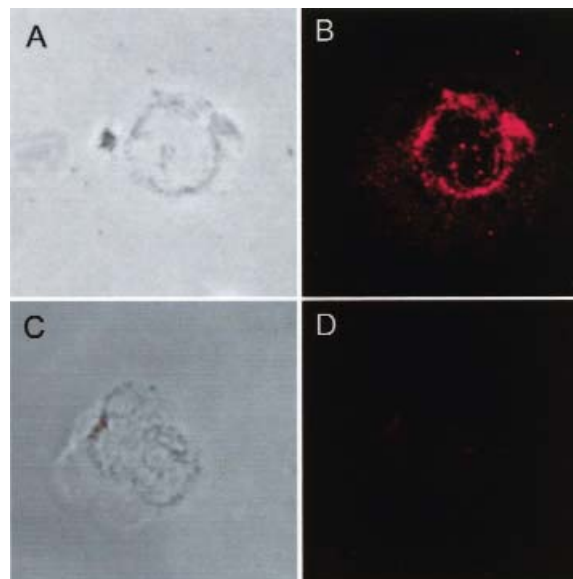


FIG 2. Demonstration of HTm4 protein on human basophils. Cells were incubated with 2 μ g/mL polyclonal rabbit anti-hHTm4 antibody (**A** = phase contrast, **B**) or 2 μ g/mL rabbit IgG (H+L) as a control (**C** = phase contrast, **D**), followed by incubation with a secondary antibody, highly cross-adsorbed Alexa Fluor 546-conjugated goat anti-rabbit IgG (H+L; Fig 2, **B** and **D**).

are unrelated to allergic inflammation (eg, the brain) express it. Among the 6 novel transcripts found to be preferentially expressed by granulocytes except for neutrophils, we focused on the following 4 transcripts expressed by granulocytes, including basophils. They were Ca²⁺ channel (*CACNA1D*), prostaglandin E receptor (*EP3A2*), epidermal growth factor-like module-containing mucin-like receptor (EMR) 1 (*EMR1*), and HTm4 (*MS4A3*).

Basophils are the rarest granulocytes present in human peripheral blood, and as such, their complete transcriptional profiles remain unclear, and no basophil-selective transcripts have previously been reported.²¹ Although eosinophils and mast cells have been considered important therapeutic targets for allergic diseases for a long time, recent studies suggest the importance of basophils in the pathogenesis of severe allergic diseases, such as fatal asthma.^{2,22} Therefore we further examined the selective expression of these 4 basophil- or basophil/eosinophil-selective transcripts by using real-time RT-PCR. As shown in Fig 1, including a known molecule preferentially expressed by neutrophils, aquaporin 9,²³ the results obtained with the GeneChip assay were confirmed by using this highly accurate and reproducible method.²⁴

Among these 4 molecules, we could obtain a suitable antibody against HTm4, which is a member of a family of 4-transmembrane proteins that includes CD20 and the Fc ϵ RI β -chain.²⁵ Genetics provided evidence for the existence of multiple loci relevant to atopic asthma on chromosome 11q13, including HTm4.²⁶ Most recently, we have published data identifying HTm4 as a hematopoietic cell-cycle regulator.²⁷ Using specific antibody against HTm4, we could detect the expression of HTm4 at the protein level in basophils (Fig 2). The

TABLE I. Representative cell type-selective transcripts in granulocytes

Accession no.*	Transcript	SI†	Normalized AD level
Basophil selective			
NM_000589.1	IL-4	73.3	13.3
L35848.1	HTm4	38.2	132.1
BC005912.1	FcεRIα	12.7	219.0
Eosinophil selective			
NM_001140.1	Arachidonate 15-lipoxygenase	74.1	18.3
NM_024703.1	FLJ22593	19.1	29.1
NM_014442.1	Siglec-8	9.8	16.9
Neutrophil selective			
NM_004633.1	IL-1R, type II	127.9	51.5
U73191.1	Inward rectifier K ⁺ channel Kir1.3	107.5	98
NM_001557.1	CXCR2 (IL-8Rβ)	39.3	105.2
Mast cell selective			
AF206667.1	Tryptase β	84.3	159.4
NM_001911.1	Cathepsin G	51.5	72.1
BC005929.1	Major basic protein	31.6	72.5
Basophil/eosinophil selective			
M75914.1	IL-5Rα	42.8	19.4 (B), 29.3 (E)
NM_004778.1	CRTH2	16.6	23.9 (B), 38.1 (E)
NM_001828.3	Charcot-Leyden crystal protein	15.2	229.2 (B), 198.6 (E)
Eosinophil/neutrophil selective			
NM_005306.1	GPR 43 (PAR1-like)	21.7	11.7 (E), 32.9 (N)
NM_004668.1	DHHC domain containing 18	6.6	16.2 (E), 44.5 (N)
Basophil/neutrophil selective			
NM_016006.1	CGI-58 protein	5.8	12.6 (B), 21.2 (N)
Basophil/mast cell selective			
NM_001870.1	Carboxypeptidase A3	59.2	111.7 (M), 137.3 (B)
NM_002529.2	TRK neurotrophin receptor	34.7	3.1 (M), 7 (B)
NM_000139.1	FcεRI β	21.2	22.2 (M), 43.8 (B)

*GenBank accession number (<http://www.ncbi.nlm.nih.gov>). †The selectivity index (SI) was calculated by comparing the normalized AD level in a cell type or the normalized AD level of 2 cell types with the maximal gene expression level of the other 8 or 9 cell types. The complete list of the genes having an SI of greater than 3 is shown in Online Repository Table E1.

amount of molecules expressed by various cell types should be quantified, and the effect of any identified antagonists should be tested on the cell types found to express these molecules to confirm whether these ion channels and receptors could be potential drug targets for diseases involving basophil activation.

As has been well documented and expected, IL-3 receptors, IL-5 receptors, chemokine receptor CCR3,^{1,2} sialic acid-binding Ig-like lectin (Siglec)-8,²⁸ Siglec-6,¹⁵ histamine H4 receptor,²⁹ and chemoattractant receptor-homologous molecule expressed on T_H2 cells (CRTH2)³⁰ were preferentially expressed by basophils, eosinophils, or both or mast cells. These consistent observations strengthened the reliability of the present methods and research strategy. Indeed, the antibody against Siglec-8 can induce selective apoptosis of eosinophils and is expected to be useful therapeutically.²⁸

Ion channels and receptors preferentially expressed by granulocytes, including neutrophils

Of the 17 transcripts that have not been reported as granulocyte selective, 9 were preferentially expressed by granulocytes, including neutrophils. Pharmaceutical targets of selective granulocyte transcripts should treat

inflammatory diseases without affecting the function of important organs that are unrelated to inflammation, as well as the function of lymphocytes. However, 4 of the 9 neutrophil-selective transcripts were expressed by multiple organ tissues. One of the 4 neutrophil-selective transcripts encoded proteinase-activated receptor (PAR)-2, a receptor for mast cell tryptase that is linked to the pathogenesis of allergic diseases, such as asthma.^{31,32} PAR-2 transcripts are also abundantly expressed by tissue types, including skin and intestine, which are often the target organs for allergic diseases. But the development of PAR-2 antagonists for use as antiallergic drugs might be unsuitable because it might downregulate neutrophil function and thereby induce bacterial infection.

DISCUSSION

We identified 51 granulocyte-selective genes for ion channels and receptors by examining approximately 20,000 kinds of transcripts derived from 16,000 genes from 10 different types of cells with the U133A GeneChip, which covers approximately half of the genes present in the human genome. The majority of these transcripts encoded molecules known or expected to be granulocyte subtype-selective, such as the IL-3 receptor and Fcε receptors.

TABLE II. Granulocyte subtype-selective transcripts for ion channels and receptors

Transcript (GenBank accession no.)	Cell type*	MC	Ba	Eo	Ne	Pl	CD4	CD8	CD14	CD19	Fb	TC†
Ion channels												
Ca²⁺ channel type A1 D (BE550599)	Ba, Eo	0.1	1.7	1.5	0.4	0.0	0.3	0.1	0.0	0.3	0.0	0
Aquaporin 9 (NM_020980.2)	Ne	0.7	0.1	0.4	137.4	0.4	0.9	0.1	8.6	0.1	0.2	7
K⁺ channel Kir1.3 (U73191.1)	Ne	0.9	0.2	0.4	99.5	0.7	0.2	0.0	0.8	0.0	0.0	5
K⁺ channel Kir2.1 (AF153820.1)	Ne	0.7	3.8	5.3	40.7	0.4	0.2	0.5	1.5	0.7	1.1	6
GPR												
Histamine H ₄ R (AF312230.1)	Ba	0.7	34.2	9.4	0.7	0.4	0.8	0.5	0.6	0.0	0.1	0
PGE R type 3a2 (X83858.1)	Ba	0.8	10.3	0.1	0.7	0.6	0.3	0.2	0.2	0.5	1.7	0
C3a R (U62027.1)	Ba, Eo	11.8	55.7	39.4	2.0	1.6	1.6	1.5	3.0	0.6	0.5	3
CCR3 (NM_001837.1)	Ba, Eo	0.6	117.4	90.9	24.9	0.2	0.5	0.4	0.2	0.2	0.4	0
CRTH2 (NM_004778.1)	Ba, Eo	1.1	26.0	38.2	2.0	0.8	1.4	1.0	1.2	0.9	0.5	0
EMR-1 (NM_001974.1)	Ba, Eo	0.8	33.5	90.9	4.2	3.4	1.7	0.8	7.1	1.6	0.5	1
Adenosine A ₃ R (NM_000677.2)	Eo	2.6	2.9	15.4	2.3	1.2	1.6	0.5	2.0	0.5	0.5	0
P2Y ₂ purinergic R (NM_002564.1)	Eo	0.1	0.1	5.5	0.1	0.2	0.1	0.3	1.2	0.2	0.1	0
GPR105 purinergic R (NM_014879.1)	Eo	2.6	2.9	15.4	2.3	1.2	1.6	0.5	2.0	0.5	0.5	2
GPR, Edg-4 (AF011466.1)	Eo, Ne	1.3	2.8	15.9	24.2	0.1	3.6	5.0	3.8	1.9	0.9	2
PAR1-like GPR43 (NM_005306.1)	Eo, Ne	0.3	0.7	12.4	35.2	0.8	0.1	0.1	0.7	0.4	0.1	0
C5a R (NM_001736.1)	Ne	2.3	21.6	13.6	92.6	2.3	1.2	0.5	25.6	1.0	0.4	5
CXCR1 IL-8R (NM_000634.1)	Ne	0.2	4.3	0.3	83.4	0.4	0.3	0.2	0.2	0.1	0.1	0
CXCR2 IL-8R (NM_001557.1)	Ne	0.2	1.1	1.5	112.1	2.7	0.3	0.8	0.7	0.6	0.0	1
Formyl peptide R 1 (NM_002029.1)	Ne	2.8	23.5	8.8	282.9	3.6	1.3	0.7	62.6	1.1	0.5	0
Formyl peptide R 2 (U81501.1)	Ne	0.4	0.5	0.6	75.5	0.1	1.0	0.6	6.0	0.8	0.1	5
GPR77 (NM_018485.1)	Ne	0.1	0.9	0.8	3.4	0.0	0.3	0.2	1.0	0.1	0.3	2
GPR86 purinergic R (NM_023914.1)	Ne	0.3	0.2	17.9	88.2	1.9	0.1	0.2	12.3	0.6	0.0	1
PAR2 (BE965369)	Ne	0.1	0.3	2.2	36.2	0.1	0.7	0.1	1.6	0.4	1.2	15
Other receptors (R)												
FcεRIα (BC005912.1)	Ba	19.4	219.0	4.6	5.5	1.4	5.1	0.8	1.4	0.4	0.2	0
HTm4 (L35848.1)	Ba	0.6	133.1	6.9	3.5	0.2	0.5	0.4	0.1	0.2	0.0	1
IL-3 R (NM_002183.1)	Ba	0.6	52.6	2.0	0.7	0.2	0.3	0.2	0.7	0.1	0.1	0
CD244 NK cell R (NM_016382.1)	Ba, Eo	0.3	56.0	16.5	1.2	0.1	0.4	3.5	5.2	1.1	0.1	0
Fibroblast growth factor R 2 (NM_022969.1)	Ba, Eo	0.1	27.9	12.1	0.2	0.1	0.1	0.2	0.1	0.1	1.0	17
IL-5R α (M75914.1)	Ba, Eo	0.9	20.5	30.4	0.6	0.1	0.1	0.1	0.1	0.2	0.1	0
Siglec-8 (NM_014442.1)	Eo	1.8	0.3	17.4	0.4	0.3	0.2	0.1	0.2	0.5	0.2	0
CD117 c-KIT (NM_000222.1)	MC	89.0	7.2	4.2	1.2	0.8	0.2	0.5	0.1	0.1	0.5	15
Siglec-6 (D86358.1)	MC	5.6	0.2	0.0	0.2	0.5	0.0	0.0	0.0	0.4	0.0	0
FcεRIβ (NM_000139.1)	MC, Ba	22.3	44.3	0.4	0.6	1.6	0.5	0.2	0.0	0.3	0.1	0
Low-density lipoprotein R (NM_000527.2)	MC, Ba	20.7	20.4	1.3	1.8	2.3	2.1	3.4	3.5	0.8	6.3	34
Tropomyosin-related kinase neurotrophin R (NM_002529.2)	MC, Ba	4.6	7.3	0.1	0.1	0.1	0.1	0.1	0.1	0.0	0.0	0
Butyrophilin-like R (AK025267.1)	Ne	1.1	1.7	1.5	6.9	1.5	1.7	1.6	0.9	1.5	1.3	1
CD120a, TNF-R-I (NM_001065.1)	Ne	1.7	1.2	7.8	74.7	1.3	2.2	5.9	17.4	0.6	16	35
CD95, Fas, APO-1 (AA164751)	Ne	1.4	10.9	7.3	50.4	1.3	5.6	4.8	3.6	2.6	9.7	7
Decoy R1, TRAILR3 (AF012536.1)	Ne	0.1	1.5	7.7	78.7	0.5	0.2	0.1	0.7	0.1	0.6	0
FcγRIIc2 (U90939.1)	Ne	1.5	2.4	7.8	59.9	0.3	0.2	0.1	7.2	2.3	0.2	2
FcγRIIc3 (U90940.1)	Ne	2.4	10.7	10.0	84.3	3.0	1.4	0.6	14.0	7.2	1.0	5
FcγRIII (J04162.1)	Ne	0.7	1.6	1.9	199.6	6.6	1.3	1.3	2.2	2.6	0.1	4
G-CSF R (NM_000760.1)	Ne	0.1	0.4	1.6	163.6	0.2	0.8	0.2	25.5	0.1	0.2	3
IL-13R (U81379.3)	Ne	0.3	0.2	2.0	14.1	0.4	0.4	0.3	2.9	1.3	1.3	0
IL-1R, type II (NM_004633.1)	Ne	0.1	0.1	0.1	53.5	0.2	0.4	0.1	0.2	0.0	0.0	3
Insulin-like growth factor R1 (NM_000875.2)	Ne	0.3	3.5	5.0	17.4	1.6	0.1	2.8	2.4	2.0	3.5	3
Insulin-like growth factor R2 (NM_000876.1)	Ne	4.6	0.9	5.4	85.3	1.7	2.9	8.7	8.4	4.8	15	32
Leukocyte Ig-like R A2 (NM_006866.1)	Ne	0.5	5.8	4.3	41.2	1.8	0.0	0.1	11.6	0.5	0.1	2
Toll-like R1 (AL050262.1)	Ne	0.6	0.3	1.2	31.5	1.6	0.8	0.7	3.0	1.5	0.3	0
Toll-like R2 (NM_003264.1)	Ne	0.9	6.0	1.3	83.8	1.6	1.3	0.1	26.3	0.9	0.4	0
Toll-like R6 (NM_006068.1)	Ne	0.5	1.0	0.9	8.8	0.1	0.9	0.9	2.1	1.2	0.6	0

MC, Mast cells; Ba, basophils; Eo, eosinophils; Ne, neutrophils; Pl, platelets; Fb, fibroblasts; TC, tissue cells; R, receptor.

*Cell-type selectivity was obtained by comparing the normalized AD levels of each gene in mast cells, basophils, eosinophils, neutrophils, platelets, CD4⁺ cells, CD8⁺ cells, CD14⁺ cells, CD19⁺ cells, and fibroblasts.

†The expression of each gene by various tissue cells was determined by searching http://www.lsbm.org/index_e.html. The italicized numeral indicates the number of tissues that have a raw AD level of greater than 200.

Mast cells expressed low levels of FcεRIα compared with basophils, and even neutrophils expressed a substantial level of the receptor (Table II). This raises the possibility that the GeneChip assay might not be suitable for detecting selective molecules. In the present study, however, only the GeneChip data obtained by using cord blood–derived mast cells and lung mast cells could be used because of the strict data selection based on the RNA quality (see the “Methods” section). As has been reported,^{14,33} peripheral blood–derived cultured mast cells or skin–derived mast cells expressed approximately 10-fold FcεRIα mRNA compared with that produced by cord blood–derived mast cells (data not shown). Also, as shown in Online Repository Table E1, only 2 of 4 neutrophil samples expressed FcεRIα mRNA. This might be explained by the observation that only neutrophils obtained from some allergic donors express the molecule.³⁴

We unexpectedly found 17 granulocyte-selective transcripts, including HTm4. Basophil-selective transcripts, eosinophil-selective transcripts, or both identified in our study could be potential therapeutic targets for allergic diseases because these granulocytes play a crucial role in allergic inflammation.^{1,2} Granulocyte-selective transcripts could also be drug targets for other inflammatory diseases, such as systemic vasculitis.^{3,4} Analysis of cell type–selective transcripts from database searches is expected to minimize the efforts required for drug discovery. The public database (http://www.lsbm.org/index_e.html) shows that some granulocyte-selective transcripts (18 of 51) detected in our study are abundantly expressed by multiple (>3) organ tissue cell types by using the same GeneChip U133A probe array. Thus the safety of any candidate drug must be evaluated by comparing its efficacy (on granulocytes) with its toxicity (to organs). Six of the 17 novel granulocyte-selective molecules could be excluded from drug development because of their expression in multiple organs unrelated to the diseases. Thus our approach has identified 11 receptors and ion channels with therapeutic potential. Among the 11 receptors and ion channels, 7 were basophil selective, eosinophil selective, or both and were not expressed by other organs, indicating that they might be potential targets for antiallergic drugs.

Finally, it should be stressed that basophils, the rarest leukocytes, have recently been found to play a more crucial role than we ever proposed in the pathogenesis of intractable allergic diseases, such as fatal asthma.^{35,36} Thus targeting basophil receptors and ion channels, such as HTm4 and the Ca²⁺ channel *CACNA1D*, is particularly expected for future drug discovery. The importance of molecules known to be expressed by basophils might be reevaluated regarding its selectivity. Freshly isolated resting basophils expressed the highest level of IL-4 compared with other cell types. Because the basophil purification procedure requires more isolation steps, ex vivo manipulation might activate the cells. However, it should also be noted that basophils have been recently found to be the major source of IL-4, at least in asthma models.^{37,38}

We thank Ms Noriko Hashimoto, Mr Keisuke Yuki, Mr Hisashi Tomita, Mr Nobuyuki Baba, and Ms Nao Aida at The National Research Institute for Child Health and Development for discussion and skillful technical assistance. We also thank Mr Igor A. Bagayev at The Confocal Microscope Facility, Massachusetts General Hospital, Harvard Medical School.

REFERENCES

- Bochner BS. Systemic activation of basophils and eosinophils: markers and consequences. *J Allergy Clin Immunol* 2000;106(suppl):S292-302.
- Hirai K, Miyamasu M, Takaishi T, Morita Y. Regulation of the function of eosinophils and basophils. *Crit Rev Immunol* 1997;17:325-52.
- Savage CO. The evolving pathogenesis of systemic vasculitis. *Clin Med* 2002;2:458-64.
- Oehler R, Weingartmann G, Manhart N, Salzer U, Meissner M, Schlegel W, et al. Polytrauma induces increased expression of pyruvate kinase in neutrophils. *Blood* 2000;95:1086-92.
- Church MK, Pao GJ, Holgate ST. Characterization of histamine secretion from mechanically dispersed human lung mast cells: effects of anti-IgE, calcium ionophore A23187, compound 48/80, and basic polypeptides. *J Immunol* 1982;129:2116-21.
- Lin CS, Boltz RC, Blake JT, Nguyen M, Talento A, Fischer PA, et al. Voltage-gated potassium channels regulate calcium-dependent pathways involved in human T lymphocyte activation. *J Exp Med* 1993;177:637-45.
- Saito H, Okajima F, Molski TF, Sha'afi RI, Ui M, Ishizaka T. Effects of ADP-ribosylation of GTP-binding protein by pertussis toxin on immunoglobulin E-dependent and -independent histamine release from mast cells and basophils. *J Immunol* 1987;138:3927-34.
- Ji TH, Grossmann M, Ji I. G protein-coupled receptors. I. Diversity of receptor-ligand interactions. *J Biol Chem* 1998;273:17299-302.
- Zambrowicz BP, Sands AT. Knockouts model the 100 best-selling drugs. Will they model the next 100? *Nat Rev Drug Discov* 2003;2:38-51.
- Venter JC, Adams MD, Myers EW, Li PW, Mural RJ, Sutton GG, et al. The sequence of the human genome. *Science* 2001;291:1304-51.
- Waterston RH, Lindblad-Toh K, Birney E, Rogers J, Abril JF, Agarwal P, et al. Initial sequencing and analysis of the human genome. *Nature* 2001;409:860-921.
- Drews J. Drug discovery: a historical perspective. *Science* 2000;287:1960-4.
- Hopkins AL, Groom CR. The druggable genome. *Nat Rev Drug Discov* 2002;1:727-30.
- Iida M, Matsumoto K, Tomita H, Nakajima T, Akasawa A, Ohtani NY, et al. Selective down-regulation of high-affinity IgE receptor (FcεRI) α-chain messenger RNA among transcriptome in cord blood-derived versus adult peripheral blood-derived cultured human mast cells. *Blood* 2001;97:1016-22.
- Nakajima T, Matsumoto K, Suto H, Tanaka K, Ebisawa M, Tomita H, et al. 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* 2001;98:1127-34.
- Nakajima T, Inagaki N, Tanaka H, Tanaka A, Yoshikawa M, Tamari M, et al. Marked increase in CC chemokine gene expression in both human and mouse mast cell transcriptomes following Fcε receptor I cross-linking: an interspecies comparison. *Blood* 2002;100:3861-8.
- Yoshimura C, Miyamasu M, Nagase H, Iikura M, Yamaguchi M, Kawanami O, et al. Glucocorticoids induce basophil apoptosis. *J Allergy Clin Immunol* 2001;108:215-20.
- Matsumoto K, Schleimer RP, Saito H, Iikura Y, Bochner BS. Induction of apoptosis in human eosinophils by anti-Fas antibody treatment in vitro. *Blood* 1995;86:1437-43.
- Okayama Y, Hunt TC, Kassel O, Ashman LK, Church MK. Assessment of the anti-c-kit monoclonal antibody YB5.B8 in affinity magnetic enrichment of human lung mast cells. *J Immunol Methods* 1994;169:153-61.
- Fukagawa K, Okada N, Fujishima H, Nakajima T, Tsubota K, Takano Y, et al. CC-chemokine receptor 3: a possible target in treatment of allergy-related corneal ulcer. *Invest Ophthalmol Vis Sci* 2002;43:58-62.
- Arock M, Schneider E, Boissan M, Tricottet V, Dy M. Differentiation of human basophils: an overview of recent advances and pending questions. *J Leukoc Biol* 2002;71:557-64.
- Kepley CL, McFeeley PJ, Oliver JM, Lipscomb MF. Immunohistochemical detection of human basophils in postmortem cases of fatal asthma. *Am J Respir Crit Care Med* 2001;164:1053-8.

23. Loitto V-M, Forslund T, Sundqvist T, Magnusson K-E, Gustafsson M. Neutrophil leukocyte motility requires directed water influx. *J Leukoc Biol* 2002;71:212-22.
24. Pfaffl MW. A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Res* 2001;29:e45.
25. Adra CN, Lelias JM, Kobayashi H, Kaghad M, Morrison P, Rowley JD, et al. Cloning of the cDNA for a hematopoietic cell-specific protein related to CD20 and the beta subunit of the high-affinity IgE receptor: evidence for a family of proteins with four membrane-spanning regions. *Proc Natl Acad Sci U S A* 1994;91:10178-82.
26. Adra CN, Mao XQ, Kawada H, Gao PS, Korzycka B, Donato JL, et al. Chromosome 11q13 and atopic asthma. *Clin Genet* 1999;55:431-7.
27. Donato JL, Ko J, Kutok JL, Cheng T, Shirakawa T, Mao XQ, et al. Human HTm4 is a hematopoietic cell cycle regulator. *J Clin Invest* 2002;109:51-8.
28. Nutku E, Aizawa H, Hudson SA, Bochner BS. Ligation of Siglec-8: a selective mechanism for induction of human eosinophil apoptosis. *Blood* 2003;101:5014-20.
29. O'Reilly M, Alpert R, Jenkinson S, Gladue RP, Foo S, Trim S, et al. Identification of a histamine H4 receptor on human eosinophils—role in eosinophil chemotaxis. *J Recept Signal Transduct Res* 2002;22:431-48.
30. Hirai H, Tanaka K, Yoshie O, Ogawa K, Kenmotsu K, Takamori Y, et al. Prostaglandin D2 selectively induces chemotaxis in T helper type 2 cells, eosinophils, and basophils via seven-transmembrane receptor CRTH2. *J Exp Med* 2001;193:255-61.
31. Moffatt JD, Jeffrey KL, Cocks TM. Protease-activated receptor-2 activating peptide SLIGRL inhibits bacterial lipopolysaccharide-induced recruitment of polymorphonuclear leukocytes into the airways of mice. *Am J Respir Cell Mol Biol* 2002;26:680-4.
32. Schmidlin F, Amadesi S, Vidil R, Trevisani M, Martinet N, Caughey G, et al. Expression and function of proteinase-activated receptor 2 in human bronchial smooth muscle. *Am J Respir Crit Care Med* 2001;164:1276-81.
33. Bradding P, Okayama Y, Kambe N, Saito H. Ion channel gene expression in human lung, skin and cord blood-derived mast cells. *J Leukoc Biol* 2003;73:614-20.
34. Gounni AS, Lamkhioued B, Koussih L, Ra C, Renzi PM, Hamid Q. Human neutrophils express the high-affinity receptor for immunoglobulin E (Fc epsilon RI): role in asthma. *FASEB J* 2001;15:940-9.
35. Koshino T, Teshima S, Fukushima N, Takaishi T, Hirai K, Miyamoto Y, et al. Identification of basophils by immunohistochemistry in the airways of post-mortem cases of fatal asthma. *Clin Exp Allergy* 1993;23:919-25.
36. Kepley CL, McFeeley PJ, Oliver JM, Lipscomb MF. Immunohistochemical detection of human basophils in postmortem cases of fatal asthma. *Am J Respir Crit Care Med* 2001;164:1053-8.
37. Schroeder JT, Lichtenstein LM, Roche EM, Xiao H, Liu MC. IL-4 production by human basophils found in the lung following segmental allergen challenge. *J Allergy Clin Immunol* 2001;107:265-71.
38. Luccioli S, Brody DT, Hasan S, Keane-Myers A, Prussin C, Metcalfe DD. IgE(+), Kit(-), I-A/I-E(-) myeloid cells are the initial source of IL-4 after antigen challenge in a mouse model of allergic pulmonary inflammation. *J Allergy Clin Immunol* 2002;110:117-24.