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Dysregulation of interleukin 5 expression in familial eosinophilia

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Abstract

Background—Familial eosinophilia (FE) is a rare autosomal dominant inherited disorder characterized by the presence of lifelong peripheral eosinophilia (>1500/μL). Mapped to chromosome 5q31-q33, the genetic cause of FE is unknown, and prior studies have failed to demonstrate a primary abnormality in the eosinophil lineage.

Objective—The aim of the present study was to identify the cells driving the eosinophilia in FE.

Methods—Microarray analysis and real-time PCR were used to examine transcriptional differences in peripheral blood mononuclear cells (PBMC), and in purified cell subsets from affected and unaffected family members belonging to a single large kindred. Cytokine levels in serum and PBMC culture supernatants were assessed by suspension array multiplexed immunoassays.

Results—Whereas *IL5* mRNA expression was significantly increased in freshly isolated PBMC from affected family members, this was not accompanied by increased mRNA expression of other Th2 cytokines (*IL4* or *IL13*). Serum levels of IL-5 and IL-5 receptor α, but not IgE, were similarly increased in affected family members. Of note, *IL5* mRNA expression was significantly increased

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in purified CD3+ CD4+, CD14+, CD19+ and ILC2 cells from affected family members, as were IL-5 protein levels in supernatants from both stimulated PBMC and ILC2 cultures.

Conclusions—These data are consistent with the hypothesis that the eosinophilia in FE is secondary to dysregulation of IL-5 production in PBMC (and their component subsets).

Keywords

Eosinophil; hypereosinophilic syndrome; autosomal dominant; cytokine; interleukin 5

Introduction

Familial eosinophilia (FE) is a disorder characterized by the presence of sustained peripheral eosinophilia >1500/μL beginning at birth that has an autosomal dominant inheritance pattern (1,2). Despite prolonged eosinophilia in affected family members, clinical manifestations related to the eosinophilia are uncommon (2,3). This “benign” phenotype is likely related to a relative lack of eosinophil activation as evidenced by cellular morphology, surface activation markers and release of eosinophil granule proteins (3).

Although the gene responsible for FE has been mapped by genome wide linkage analysis in one large kindred to a region on chromosome 5q containing the cytokine gene cluster (multipoint LOD score of 6.49), targeted sequencing of candidate genes, including *IL5*, *IL3*, and *GMCSF*, and their promoters has failed to identify the causative mutation (4). Attempts to identify the causative mutation by whole genome sequencing are ongoing, but have been unsuccessful to date.

The increased levels of morphologically normal peripheral blood eosinophils observed in subjects with FE could be due to a primary abnormality in the eosinophil lineage or to mediators produced by cell types other than the eosinophil. Prior studies, including morphologic examination, ex vivo differentiation of eosinophils from CD34+ cells, and eosinophil survival assays, have provided little evidence for a primary eosinophil defect (3). Although serum cytokine levels measured previously were similar between affected and unaffected family members, the assay used to measure IL-5 was relatively insensitive, detecting IL-5 in sera from only 3/14 affected and 3/22 unaffected family members (1). The aim of the present study was to identify the cell population(s) responsible for driving the eosinophilia in FE.

Patient population and methods

Study subjects

Affected and unaffected family members from two kindreds with autosomal dominant hypereosinophilia (HE; absolute eosinophil count >1500/μL) documented over at least three generations were evaluated on institutional review board-approved clinical protocols to study familial eosinophilia (NCT00091871 and a prior protocol that preceded clinicaltrials.gov) (see pedigrees in Supplemental Figure 1). Healthy controls (HC) without eosinophilia were recruited on a separate institutional review board-approved clinical protocol to obtain normal blood samples for *in vitro* research (NCT00001846). All participants gave written informed

consent. As described previously (3), subjects underwent an extensive clinical evaluation to exclude secondary causes of eosinophilia and assess end organ manifestations of eosinophilia. Routine laboratory testing, including complete blood counts, serum immunoglobulin levels and assessment of T, B and NK cell subsets in whole blood by flow cytometry, was performed in the Department of Laboratory Medicine, NIH Clinical Center. Mast cells were quantified in bone marrow core biopsies by a single hematopathologist following tryptase immunostaining. Results are reported as the average count over 10 high-powered fields.

PBMC purification

PBMC were isolated from peripheral blood by density gradient centrifugation (Ficoll-Paque PLUS, GE Healthcare, Uppsala, Sweden). Red cells were lysed for 10 minutes at room temperature with ammonium-chloride-potassium (ACK) lysing buffer (Invitrogen, Carlsbad, CA). Cell number and viability were determined by staining with trypan blue. Aliquots of PBMC were cryopreserved in liquid nitrogen in freezing medium with C–RPMI containing 10% fetal bovine serum (FBS; Invitrogen) and 7.5 % dimethyl sulfoxide (DMSO; Thermo Fisher Scientific) or stored in Trizol (Invitrogen) at -80°C for future use.

Magnetic enrichment of CD3+ and CD3- PBMC subsets

PBMC (either fresh or from previously cryopreserved samples) from affected and unaffected family members were magnetically labeled with CD3 Microbeads (Miltenyi Biotec). Using positive selection in an autoMACS Pro separator, the magnetically labeled CD3+ T cells and the unlabeled CD3- T cells were isolated.

Flow sorting of PBMC subsets

PBMC from affected family members and healthy controls were stained with antibodies to CD3, CD4, CD8, CD19 and CD14 for isolation of CD3+CD4+ and CD3+CD8+ T cells, CD19+ B cells and CD14+ monocytes. Fresh PBMC were stained with a cocktail of lineage markers to deplete lineage positive cells and with subset markers to sort innate lymphoid cells (ILC) subsets. ILCs were defined as LIN-CD45+CD127+ and ILC subsets as ILC1 (CD117- CRTH2-), ILC2 (CRTH2+) and ILC3 (CRTH2- CD117+) as previously described (5). A complete listing of antibodies used can be found in Supplemental Table 1.

All cells were sorted using a FACSAriaTM II cell sorter (BD Biosciences) according to the manufacturer's instructions. The gating strategies are provided in Supplementary Figure 2.

Microarray analysis

Total RNA was isolated from freshly purified PBMC stored in Trizol according to the manufacturer's instructions (Invitrogen) prior to reverse-transcription using a T7 oligo dT (24) primer and Superscript II (Invitrogen). Second-strand cDNA synthesis was obtained with RNase H, E. coli DNA polymerase I, and DNA ligase (Invitrogen). cDNA was blunt-ended with T4 DNA polymerase (Invitrogen) and purified using the QIAQuick PCR purification Kit™ (Qiagen, Germantown, MD). Labeling was performed using the ENZO BioArray™ HighYield™ RNA Transcript Kit (Affymetrix) according to the manufacturer's instructions. After purification, labeled cRNA was quantified by OD, and the quality was

assessed on a Bioanalyzer (Agilent, Santa Clara, CA). Fragmented cRNA with hybridization controls and Oligo B2 from Affymetrix were hybridized on Affymetrix U133 Plus 2.0 Genechips for 18 hours followed by washing and staining on Affymetrix Fluidic 400. The arrays were scanned using the Affymetrix GeneChip Scanner 3000. Array images were analyzed using Affymetrix Gene Chip Operating System (GCOS) software using the MAS5 algorithm. The MAS5 algorithm (6) computes an adjusted signal level for each probe set to estimate expression of the target mRNA of that probe set. Values were normalized for each probe set across all arrays in the dataset.

Real-Time qRT-PCR analysis of PBMC and of PBMC subsets

Total RNA was extracted from PBMC or from flow sorted PBMC subsets in Trizol according to the manufacturer's instructions (Invitrogen). Total RNA (1 μg) was reverse-transcribed using the High Capacity cDNA Reverse Transcription kit (Applied Biosystems). qRT-PCR was carried out using Taqman^R Fast Universal PCR Master Mix using commercially available FAM-MGB primer probes (Taqman^R Gene Expression Assay, Applied Biosystems) for *IL5* (Hs00174200_m1), *IL13* (Hs00174379_m1), *IL4* (Hs00174122_m1) and *IFN-γ* (Hs00174143_m1). All reactions were performed in triplicate, and the relative expression was normalized to the corresponding 18S rRNA cycle threshold (C_t). Lack of expression was defined as a C_t of more than 35 cycles (for *IL5*) despite the presence of sufficient cDNA (18S C_t <12). Data are expressed as $1/\Delta C_t$.

Measurement of soluble mediators in serum and PBMC supernatants

Serum was collected from whole blood by centrifugation and stored immediately at −80°C for future use. Isolated PBMC were thawed, washed and resuspended in RPMI 1640 medium, supplemented with gentamicin, L-glutamine (2mM), HEPES (10mM), sodium pyruvate (all from Invitrogen, SanDiego, CA) in 24-well tissue culture plates (Costar, Corning, NY). After resting overnight, the cells were cultured with and without PMA and ionomycin (final concentrations: 15 ng/mL and 150 ng/ml, respectively) for 18 hours at 37°C. The culture supernatants were harvested and stored at -80 °C.

Flow-sorted ILC2 cells were cultured in 96-well round bottom plates at a concentration of 2×10^3/150 μL/well in X-Vivo™ 15 medium (Lonza) supplemented with 1% heat-inactivated human AB serum, 10 U/mL of IL-2 (PeproTech) and 50 ng/mL of rIL-7 (PeproTech) and activated with PMA and ionomycin for 18 hours. Supernatants were collected at day 2 and stored at -80°C.

Levels of IL-4, IL-5, IL-13, GM-CSF, and eotaxin1/CCL11 were measured in both serum and supernatants by suspension array multiplex immunoassays (Millipore). All assays were run in duplicate. The lower limits of detection were: IL-5 (0.1 pg/ml), IL-13 (0.4 pg/ml), IL-4 (0.6 pg/ml), IFN-γ (0.1pg/mL), GM-CSF (9.5 pg/ml) and eotaxin1/CCL11 (1.2 pg/ml). Serum TARC levels were measured by ELISA (Human CCL17/TARC DuoSet ELISA, R&D Systems) according to the manufacturer's instructions. Soluble IL-5 receptor alpha (sIL-5Rα) levels were quantified in serum using an in-house chemiluminescence capture ELISA as previously described (7).

Statistical analysis

Geometric means (GM) were used for measurements of central tendency. Group means were compared using the Mann-Whitney test, and paired samples were analyzed using the Wilcoxon signed rank test. A p-value of <0.05 was considered statistically significant for all tests. For the microarray data, the table of gene probe set expression estimates (using the MAS5 algorithm) for 17 arrays (9 affected subjects, 8 unaffected) was transformed by quantile normalization before calculating expression differences between the two groups using the SAS mixed-effects ANOVA procedure in JMP/Genomics version 8.0 (SAS Institute, Cary NC). P-values were adjusted for multiple testing using the False Discovery Rate (FDR) method (8).

Results

Increased IL5 mRNA expression in PBMC from affected family members

To investigate the role of PBMC in driving the eosinophilia in FE, gene expression microarray analysis was performed using PBMC RNA from 9 affected and 8 unaffected members of the previously described family with autosomal dominant FE mapped to chromosome 5q31-33 (Klion Blood 2004). Restricting the analysis to the probe sets for the 313 genes in the previously mapped region, the highest differential expression (after a p=0.05 positive or negative fold-change of 2 cutoff) between affected and unaffected family members was seen for the following genes: *IL5, ARHGEF37, CSF2, ECSCR, FGF1, GRIA1, IL12B, IL4, MYOZ3, MZB1, NRG2, PCDHGC4, PDGFRB, PSD2, SPARC* (Figure 1).

Given the dramatic increase in eosinophilia in FE and the known role of IL-5 in eosinophilia, *IL5* mRNA expression in unstimulated PBMC was assessed next by qRT-PCR. As can be seen in Figure 2, constitutive expression of *IL5* mRNA was detected in all 10 affected family members tested, in 8 of 9 unaffected family members and in 5 of 7 healthy controls (Figure 2A). Geometric mean *IL5* mRNA expression was significantly increased in affected family members ($1/\Delta C_t$ of 0.07 in affected family members vs. 0.03 in unaffected family members, and 0.04 in healthy controls, $p<0.0001$ and $p<0.005$, respectively). In contrast, mRNA from two other Th2 cytokines, *IL13* and *IL4* (also increased in affected family members based on the microarray analysis), was not detectable in any of the 8 affected or 8 unaffected family members tested using qRT-PCR. mRNA expression of the Th1 cytokine, *IFNγ*, was detectable in 10 affected and 8 unaffected family members, but was comparable between the two groups ($1/\Delta C_t$ of 0.05 in affected and unaffected family members) (Figure 2B). mRNA expression of *RAD50*, a gene in close proximity to *IL5*, was also comparable between the two groups (Supplemental Figure 3).

Increased serum levels of IL-5 and sIL5-Rα in affected family members

Similar to the mRNA expression data, serum IL-5 levels were low, but detectable, in all 13 of the affected family members tested and in 10/14 of the unaffected family members (p=NS, Fisher's exact test). However, geometric mean serum IL-5 levels were significantly increased in affected family members (GM 4.45 pg/mL vs. 1.60 pg/mL in unaffected family members; $p<0.05$). Serum levels of sIL-5Rα, a surrogate marker of cytokine-driven

eosinophilia (7), were also significantly increased in affected family members (GM 450.9 ng/mL vs. 174.9 ng/mL in unaffected family members; $p<0.01$). Serum levels of GM-CSF, another cytokine that plays a role in the induction of eosinophilia and has been shown to increase serum levels of sIL-5Rα, was significantly decreased in affected family members (GM 0.37 pg/mL vs. 1.33 pg/mL in unaffected family members; $p=0.02$). Surprisingly, serum levels of the eosinophil-attracting chemokine, eotaxin1/CCL11, were also decreased in affected family members (GM 128.6 pg/mL vs. 241.2 pg/mL in unaffected family members, $p=0.02$).

To explore whether the increased IL-5 was part of a more generalized Type 2 response, serum levels of the type 2 cytokine, IL-13 and IL-4, as well as serum IgE levels were assessed. Serum levels of IL-13 and IgE were significantly decreased in affected family members (GM serum IL-13 level of 1.52 pg/mL in affected vs. 5.02 pg/mL in unaffected family members and GM serum IgE level of 19.41 IU/mL in affected vs. 57.23 IU/mL in unaffected family members; $p=0.01$ and $p=0.03$, respectively; Figure 3). Serum IL-4 levels were not measurable in serum from any of the subjects tested. Serum levels of TARC/CCL17, a marker of T cell activation, and the Th1 cytokine, IFN-γ, were similar between the two groups (Figure 3).

Increased release of IL-5 following PMA/ionomycin stimulation of PBMC from affected family members

Although IL-5 levels tended to be higher in the supernatants from PMA/ionomycin-stimulated PBMC from affected family members, the difference did not reach statistical significance (GM 221.4 pg/mL vs. 26.49 pg/mL in unaffected family members; $p=0.059$). Stimulated PBMC supernatant levels of IL-4 (GM 73.46 pg/mL in affected vs. 191.4 pg/mL in unaffected family members) and IL-13 (GM 186.5 pg/mL in affected and 302.5 pg/mL in unaffected) were comparable between the two groups (Figure 4). No IL-5, IL-4 or IL-13 was detectable in supernatants from unstimulated PBMC.

Increased expression of IL5 mRNA is not restricted to CD4+ T cells in affected family members

Since CD4+ T cells are known to be a major source of IL-5, the percentages and absolute numbers of CD3+, CD4+, CD8+, CD4+CD3+, CD8+CD3+, CD3-CD4- T cells, CD3+HLADR+, CD3+CD25+, CD20+, CD3-CD16+CD56+ NK cells and CD3+CD16+CD56+ NKT cells were assessed by whole blood flow cytometry to determine whether alterations of particular PBMC components/subsets might be the reason for the observed increased *IL5* expression. No significant differences were detected between the affected and unaffected family members (Supplementary Figure 4).

As a next step, cDNA was prepared from magnetic bead-enriched CD3+ and CD3- cells and from flow-sorted CD3+CD4+, CD3+CD8+, CD3-CD14+ and CD3-CD19+ subsets from affected and unaffected family members. *IL5* mRNA was significantly upregulated in both CD3+ and CD3- subsets from affected family members (Figure 5A, $p<0.01$). Furthermore *IL5* mRNA was detectable, albeit at low levels, in flow-sorted CD4+, CD8+, CD14+ and

CD19+ from all 6 affected family members and none of the 3 unaffected family members tested (Figure 5B, $p<0.05$ for all subsets).

Since ILCs are a known source of IL-5 and could account for the increased *IL5* seen in the CD3- PBMC fraction, ILC subsets were assessed in affected family members (n=3) and compared to those from normal controls (n=6). Whereas the ILC2 subset accounted for a similar proportion of the total ILC population in the two groups (GM 7% (range 2-10%) in healthy controls compared to 4% (range 2-10%) in the affected family members; Figure 6A), flow-sorted ILC2s from the affected family members produced increased amounts of IL-5 following stimulation with PMA/ionomycin (GM net IL-5 of 185.9 vs. 6.194 in healthy controls; Figure 6B).

Discussion

Familial eosinophilia (FE) is a rare genetic disorder in which seemingly normal eosinophils are increased in the peripheral blood without clinical consequences. Although the gene has been mapped to chromosome 5q31-q33 (4), the causative mutation has not been identified. Whereas absolute eosinophil counts (AEC) have been linked to chromosome 5q31-q33 in other settings, including asthma in children (9,10), schistosomiasis (11) and, more recently, in adults with a high risk of cardiovascular disease (12), little is known about the specific mechanisms underlying these associations. Furthermore, the complex genetics of these conditions and the presence of elevated IgE and other findings suggestive of a more generalized Type 2 response, complicate the interpretation of functional studies. In this context, FE provides a unique opportunity to study the effect of a single genetic abnormality in chromosome 5q31-q33 on the regulation of eosinophilia. The major limitation of the present study is the fact that the studies to date have been restricted to one large kindred. Although a second family with asymptomatic eosinophilia over 3 generations and increased production of IL-5 has recently been identified, the small number of affected family members available for study and logistical constraints have precluded additional analyses.

Given the lack of evidence pointing to an abnormality in the eosinophil lineage in FE, the current study focused on the role of circulating mononuclear cells in driving the eosinophilia. Although microarray analysis revealed few differences in PBMC gene expression between affected and unaffected family members, a relative increase in *IL5* expression was observed and confirmed by qRT-PCR. Of note, mild but significant increases in basophils and mast cells, the only other lineages that express IL-5Rα in humans, were also seen in affected family members. Other lineages were similar between the two groups (Supplemental Figure 5). Increased *IL5* expression was also significant at the protein level in serum and in supernatants from stimulated PBMCs. In contrast to the generalized Th2 responses seen in allergen or helminth-driven eosinophilia, however, there was no concomitant increase in PBMC expression of *IL4* or *IL13* mRNA, and serum IL-13 and IgE levels were significantly decreased in affected family members.

The genes for the three major Th2 cytokines, *IL5*, *IL4* and *IL13*, are clustered in a 160 kb region of chromosome 5 and are most often expressed together (13,14). Nevertheless, selective regulators of expression of *IL4* and *IL13* have been described (15). Moreover, Th2

cells producing IL-4 and IL-13, but not IL-5, have been demonstrated by flow cytometry in normal and atopic individuals (16) as well as in children with measles (17) and adult patients with filarial infection (18). In contrast, prior to the present study, selective production of IL-5 had only been convincingly demonstrated in murine T cell clones stimulated with IL-2 (19) and in clonal T cell populations from some patients with lymphocytic variant HES (20,21).

Whereas coordinated expression of *IL4*, *IL5* and *IL13* has been shown to be regulated by a locus control region (LCR) containing the 3′ portion of the RAD50 gene (14,22), the genes encoding *IL4* and *IL13* are adjacent to each other and to the LCR, but separated from the *IL5* gene by the single large gene encoding *RAD50*. Furthermore, *IL5* transcription occurs in a direction opposite from that of *IL4* and *IL13*. Consequently, it is not difficult to imagine a scenario in which specific regulatory elements might lead to isolated expression of *IL5* (23). In fact, isolated upregulated *IL5* transcription has been shown to occur in response to mIR-1248 (24). In the present study, the lack of concomitant upregulation of RAD50 is consistent with the hypothesis that *IL5* is not being expressed under control of the previously described LCR and suggests a novel regulatory mechanism for *IL5* mRNA expression. The fact that all cell types examined expressed increased levels of *IL5* mRNA implies that this dysregulation of expression is lineage-independent.

In summary, FE is a rare genetic disorder of unknown etiology characterized by persistent eosinophilia and increased IL-5 production by peripheral blood mononuclear cells. The central role of IL-5 in the production, activation and survival of eosinophils has made it a prime target for therapeutic intervention in asthma and other eosinophilic disorders. FE provides a unique opportunity to explore a novel mechanism of IL-5 regulation in humans. Ultimately, this could lead to the development of new therapeutic strategies for the treatment of eosinophilic disorders.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

A	Affected family members
Ct	Cycle threshold
FE	Familial eosinophilia
FHES	Familial hypereosinophilic syndrome
GM	Geometric mean
HD	Healthy controls
HE	Hypereosinophilia
LOD	Logarithm of Odds
PBMC	Peripheral blood mononuclear cells
qRT-PCR	Quantitative reverse transcriptase polymerase chain reaction
UA	Unaffected family members

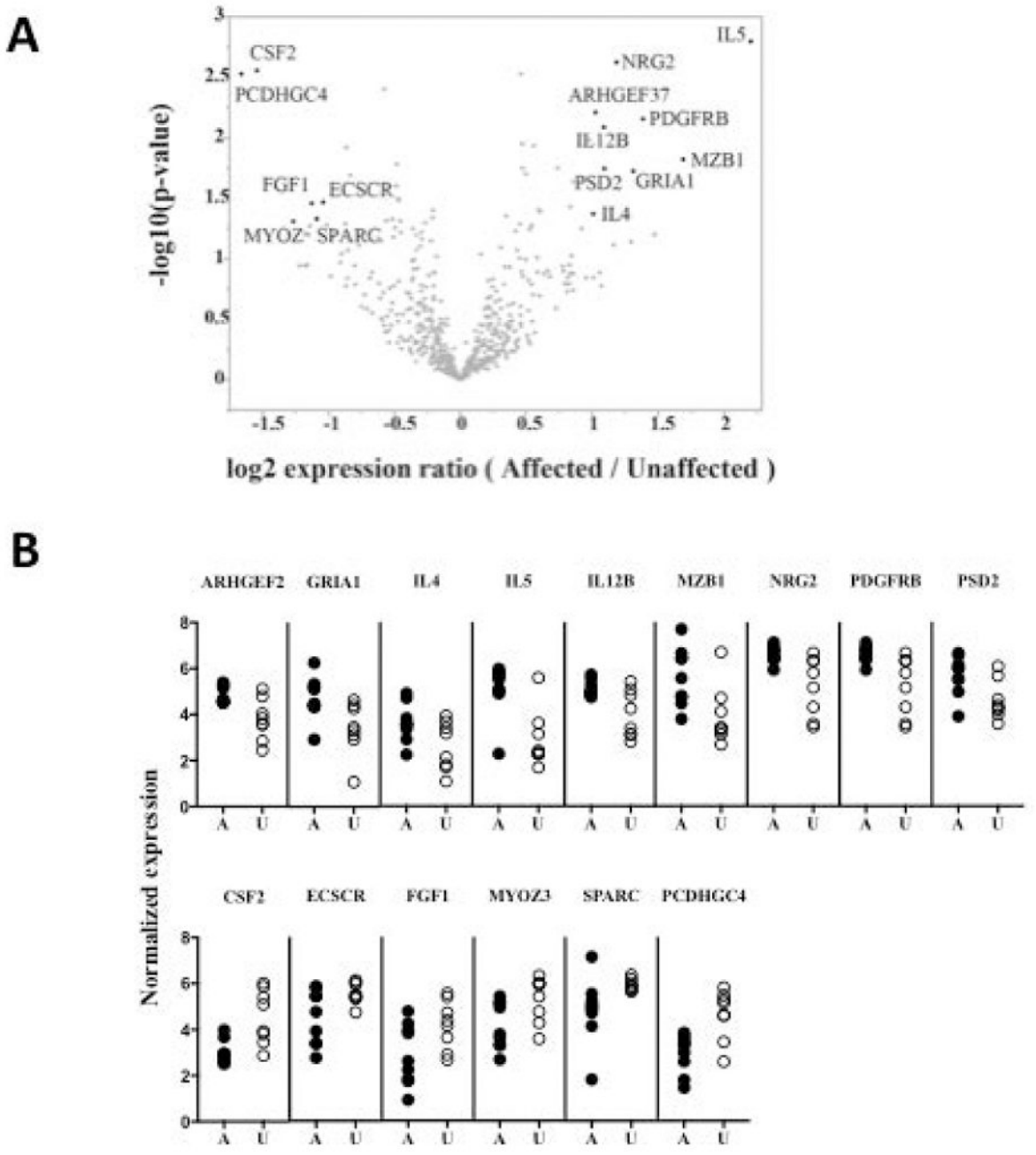

Figure 1.

Gene expression analysis for chromosomal region [5q31.1 - 5q33.1]. (A) Results of ANOVA test for difference in geometric average expression. Probe sets with expression differences greater than 2–fold and (unadjusted) p-value < 0.05 are labeled with their corresponding gene symbol. The False Discovery Rate values for these selected genes ranged from 0.32 to 0.70. Thus, none of the expression differences in the region were judged be statistically significant once this correction for multiple testing was applied. (B) Individual subject expression levels for the selected, most differentially expressed, genes.

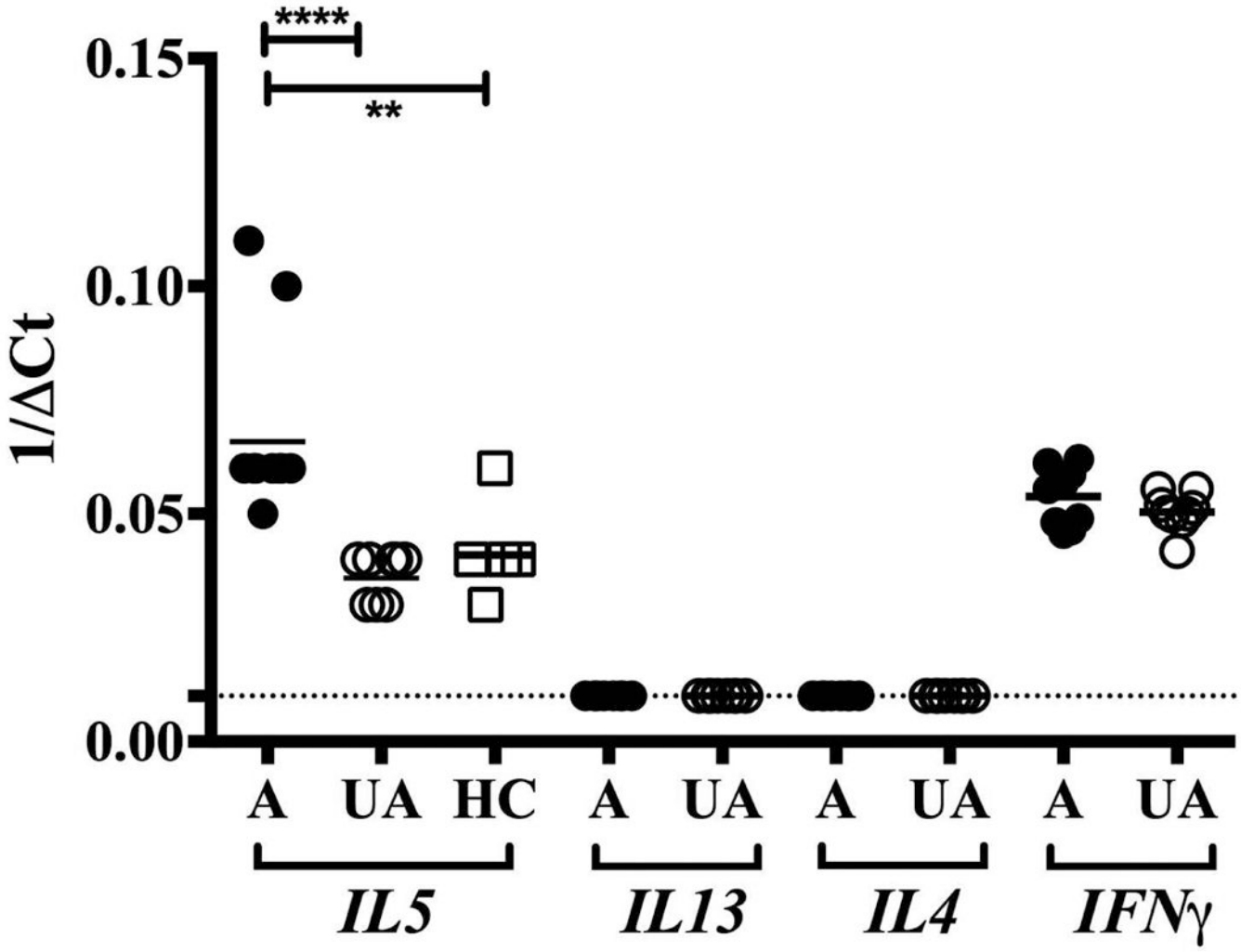

Figure 2.

Cytokine mRNA expression by PBMC in FE. *IL5*, *IL4*, *IL13* and *IFNγ* mRNA expression by unstimulated PBMC as assessed by qRT-PCR is shown as $1/\Delta C_t$ for affected (A) and unaffected (UA) family members and healthy controls (HC). Horizontal lines represent the geometric means for each group. **p<0.005 and ****p<0. 0001 as compared to affected family members.

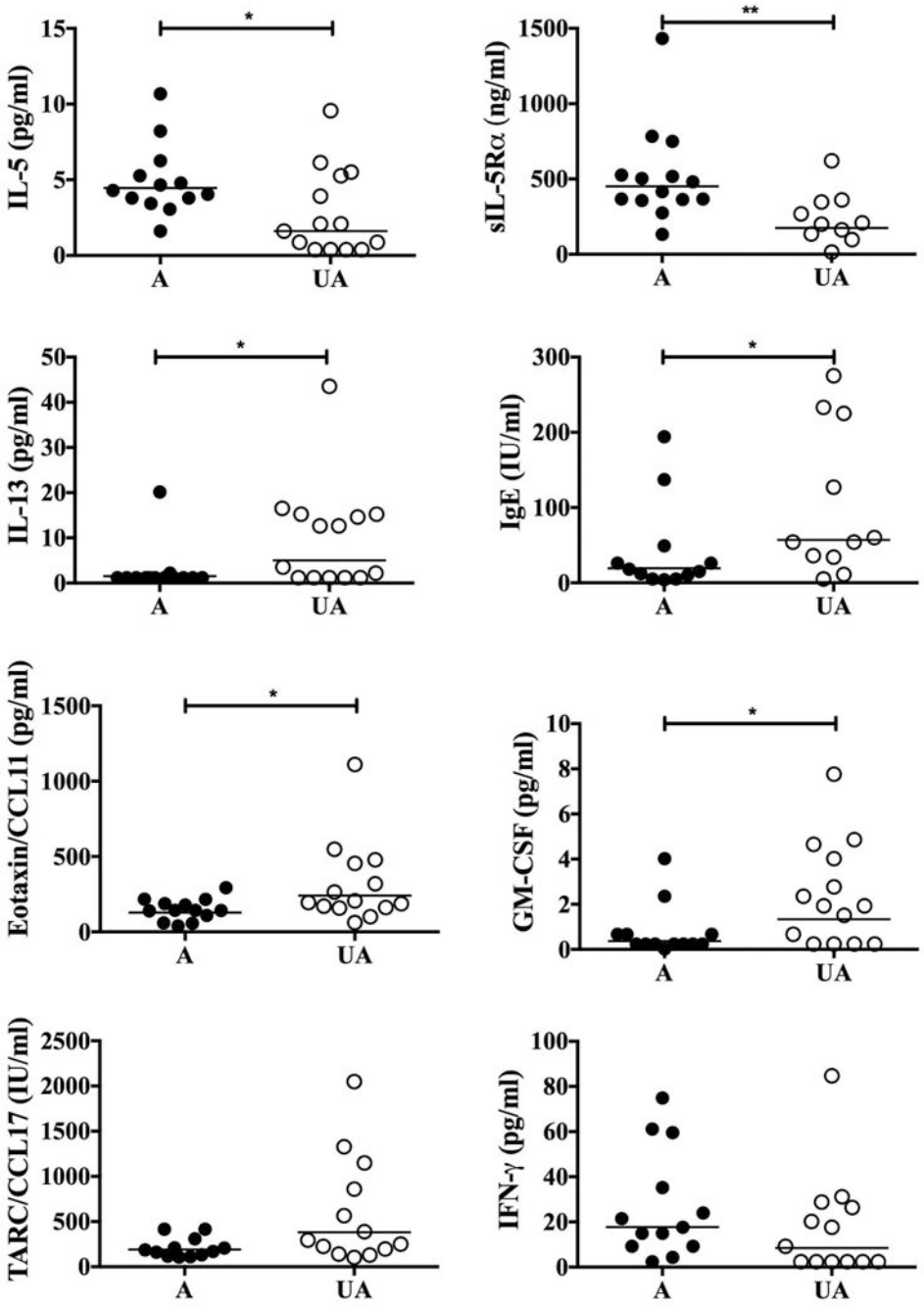

Figure 3.

Serum mediator levels in FE. Serum levels of IL-5, sIL-5Rα, IL-13, IgE, eotaxin1/CCL11, GM-CSF, TARC/CCL17 and IFN-γ are shown for individual affected (A) and unaffected (UA) family members. The horizontal lines represent the geometric means for each group. *p<0.05, **p<0.005

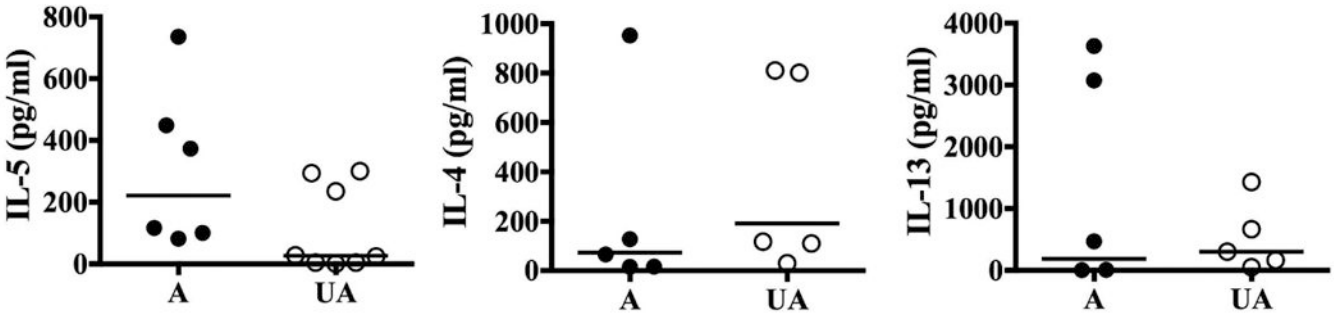

Figure 4.

Cytokine levels in supernatants from mitogen-stimulated PBMC in FE. Levels of IL-4, IL-5, and IL-13 in PMA/ionomycin stimulated PBMC supernatants are shown for individual affected (A) and unaffected (UA) family members. The horizontal lines represent the geometric means for each group.

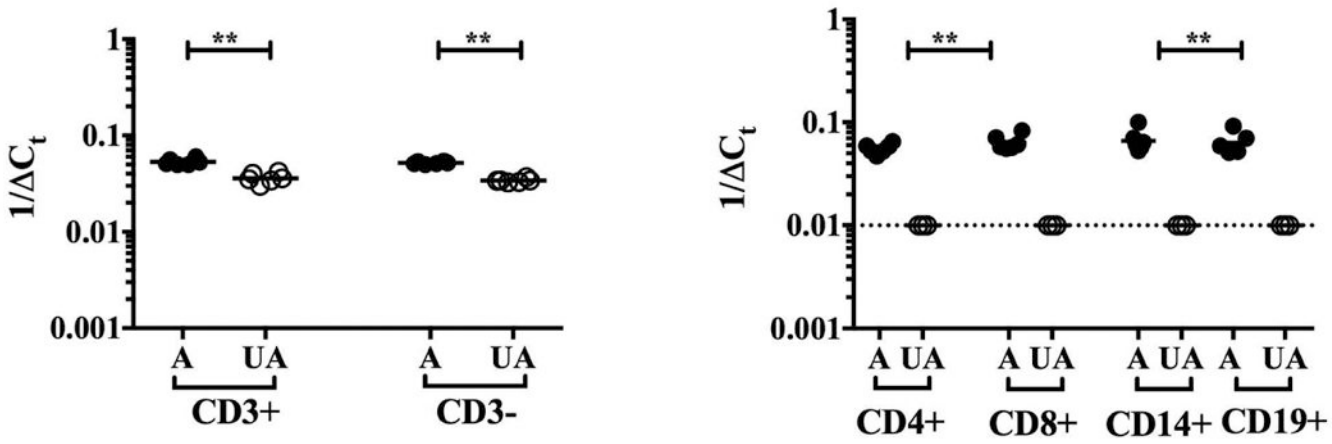

Figure 5.

IL5 mRNA expression by PBMC subsets in FE. *IL5* mRNA expression by unstimulated PBMC subsets as assessed by qRT-PCR is shown as $1/\Delta C_t$ for individual affected (A) and unaffected (UA) family members. The horizontal lines represent the geometric means for each group. *$p<0.05$ as compared to affected family members.

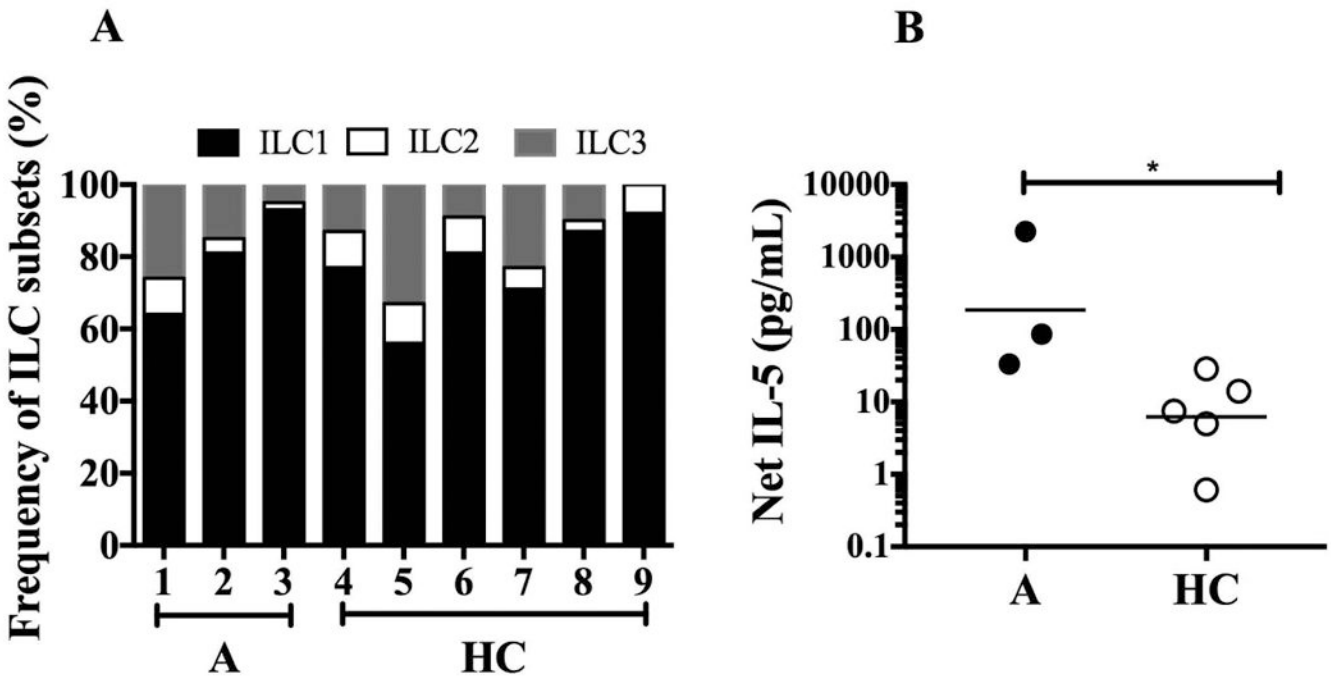

Figure 6.

ILC subset frequency and net IL-5 production in FE. A) Percentage distribution of ILC1, ILC2 and ILC3 subsets are shown for individual healthy controls (HC) and affected (A) family members. B) Net IL-5 release from unstimulated and PMA/Ionomycin stimulated, flow-sorted ILC2 subsets. Horizontal lines represent the geometric means for each group. *$p<0.05$ as compared to affected family members.