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Running Head: Gleich’s syndrome is a multilineage cell cycling disorder

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Abstract

Episodic Angioedema with Eosinophilia (Gleich’s Syndrome) is a rare disorder characterized by episodes of angioedema and eosinophilia that occur at monthly intervals and resolve spontaneously without therapy. Despite the striking periodicity of this disorder, its similarity to other cyclic hematopoietic disorders with multilineage involvement has not been assessed. To characterize the involvement of cell lineages in the etiology and pathogenesis of Episodic Angioedema with Eosinophilia, 4 subjects were evaluated with blood counts over the course of 1-2 months. Surface marker expression was assessed on T cells by flow cytometry and clonality by polymerase chain reaction. Intracellular cytokine evaluation, bone marrow and skin biopsies were performed during different parts of the cycle. Cycling of multiple lineages, including neutrophils, lymphocytes and eosinophils, was observed in 4 subjects with the disorder with a periodicity of 25-35 days. An aberrant CD3-CD4+ T cell population was detected in all 4 subjects, and T cell receptor rearrangement studies showed a clonal pattern in 3 subjects. A peak of type II cytokines was detected in the serum of subjects prior to onset of symptoms and eosinophil cycling and corresponded to ex-vivo type II cytokines detected intracellularly in CD3+CD4+CD154+ T cells. Although the etiology of episodic angioedema with eosinophilia is not yet known, multiple lineages, including lymphocytes, neutrophils and mast cells, are involved and may be related to disease pathogenesis. Whether these cells act directly or promote eosinophilia and eosinophil
activation remains to be elucidated. All subjects gave informed consent and were evaluated under an IRB-approved protocol (NCT00001406).

Key words: episodic angioedema, hypereosinophilic syndrome, eosinophilia, angioedema, cyclic hematopoiesis.

Introduction

Episodic Angioedema with Eosinophilia (EAE), also known as Gleich’s Syndrome, is a rare disorder characterized by recurrent episodes of urticaria, fever, angioedema, weight gain and dramatic eosinophilia that occur at 3-4 week intervals and resolve with spontaneous diuresis in the absence of therapy (1). Although the syndrome is often classified in the broad category of idiopathic HES (2), EAE is a distinct eosinophilic syndrome that is remarkably homogenous in clinical presentation, suggesting a common etiology in affected subjects. Early studies described cyclic elevations of serum IL-5 preceding the rise in eosinophilia, increased numbers of activated T cells and eosinophilic degranulation in the dermis during symptomatic episodes (1, 3), supporting the hypothesis that activation of blood and tissue eosinophils by T lymphocytes drives EAE. More recently, cycling of additional serum cytokines, including IL-3, IL-6, IL-1 and sIL-2R, has been described in isolated case reports (4, 5), although the sources of these cytokines have not been determined, and some cytokines, such as IL-6, reach maximum levels only after the peak of eosinophilia (5). The presence of a clonal T cell population with an aberrant CD3-CD4+ surface phenotype, commonly present in the lymphocytic variant of HES (L-HES), has been reported in 3 patients with EAE (6-8).
Whereas patients with L-HES can present with intermittent angioedema and/or urticaria (9), the regular periodicity of eosinophilia and symptoms in EAE is a distinguishing feature that is reminiscent of other cyclic hematologic disorders, such as cyclic neutropenia (10, 11) and cyclic thrombocytopenia (12, 13). Of note, cycling of multiple additional cell lineages, including eosinophils, platelets, monocytes and lymphocytes, has been reported in patients with these disorders (10, 13). Furthermore, populations of abnormal lymphocytes, specifically large granular lymphocytes, are often present in patients with the adult onset form of cyclic neutropenia (11) and have been described in cyclic thrombocytopenia (12). Although mutations in neutrophil elastase (ELANE) (14) have been identified in many patients with cyclic neutropenia, the role of these mutations in neutrophil cycling remains controversial.

Unlike patients with cyclic neutropenia, who require therapy once the diagnosis is made to prevent life-threatening complications of neutropenia, patients with EAE can often be followed without therapy providing a unique opportunity to investigate the etiology and pathogenesis of this disorder. In the present study, we identified 4 patients with definite EAE and monitored their clinical and laboratory characteristics, including absolute cell counts and cytokine responses, over the course of an entire cycle. Our data confirm that eosinophils are likely the primary cell responsible for the pathogenesis of clinical symptoms in EAE; however, cyclic variation in the numbers of peripheral blood cells other than eosinophils, including neutrophils and lymphocytes, was observed in all 4 patients, suggesting that EAE is a multilineage cell cycling disorder.
Methods

Baseline clinical assessment of study subjects.
All subjects signed informed consent, and research was performed under an IRB-approved protocol (NCT00001406) to evaluate subjects with eosinophilia. Four subjects with suspected EAE underwent a thorough history and physical examination with attention paid to timing of cycling and exclusion of iatrogenic causes of episodic eosinophilia, including intermittent administration of corticosteroids. Baseline diagnostic testing included complete blood count with differential, routine chemistries, CK, LDH, CRP, ESR, total serum immunoglobulin, vitamin B12 and tryptase levels, assessment for the *FIP1L1/PDGFRα* mutation by reverse-transcriptase polymerase chain reaction (RT-PCR), EKG, echocardiography, spirometry with lung volumes and CT scans of the chest, abdomen and pelvis. T cell clonality was determined in peripheral blood by PCR of T cell receptor (TCR)-γ rearrangement patterns (15). Additional studies to assess end organ involvement were performed as clinically indicated.

Longitudinal assessment of clinical and laboratory parameters.
Complete blood counts with differential and serum collection was performed every 2-3 days over the course of 1-2 months in 4 subjects. During this time, subjects maintained a symptom diary. Serum was stored at -80°C.
Serologic assessments.

Serum IL-5, IL-8, IL-9, IL-13, sIL-2Rα, eotaxin-1, GM-CSF, G-CSF, IFN-γ, MIP-1-β, TNFα and IL-1-β levels (limits of detection listed in the online supplement) were assessed by suspension array technology in multiplex (Millipore). Serum CCL17/TARC (R&D) and total serum tryptase was quantified as previously described (15, 16). Stem cell factor (SCF) (R&D) was assessed by ELISA according to the manufacturer’s instructions, limit of detection 9 pg/mL. Total serum IgE levels were determined by ImmunoCAP (Phadia).

Whole blood flow cytometry.

Whole blood was collected in EDTA tubes, diluted with PBS and spun at 1000 rpm at room temperature (RT). Following aspiration of the supernatant, 100µL of the well-mixed pellet was added to polystyrene tubes, pre-wetted with 0.5mL of 5% fetal calf serum, and stained with Blue-O LSA-1 and Blue-O LSA-2 (Blue Ocean Medical, FL) at 4° for 30 minutes. Red blood cells were lysed with Facs lysing solution (BD Biosciences). Cells were washed and fixed with 1.0% formaldehyde prior to analysis within 2 hours on a Blue Ocean CR 300 automated instrument.

Intracellular cytokine analysis.

Peripheral blood mononuclear cells (PBMCs) were isolated using density gradient separation (Ficoll-Paque Plus, GE Healthcare) and cryopreserved in liquid nitrogen. Thawed PBMCs were stimulated with PMA/ionomycin and stained with a panel of
antibodies to detect intracellular cytokines (IL-4, IL-13, IL-5, and IFN-γ) in CD4+ cells. Detailed methods are provided in the online supplement.

**Immunohistochemistry.**

Skin and bone marrow core biopsies were stained with hematoxylin & eosin (H&E) for morphologic examination. Immunohistochemical staining for eosinophil peroxidase and tryptase, CD3 and CD4 were performed and are further described in the online supplement.

**Sequencing of the gene for neutrophil elastase (ELANE).**

DNA was purified from frozen PBMCs using the Gentra Puregene kit (Qiagen) and 3 fragments of DNA spanning all 5 exons of *ELANE* were amplified using published primers (17). PCR products were eluted from bands cut out of 2% agarose gels and sequenced using standard capillary electrophoresis (Macrogen, Inc).

**Results**

**Clinical and laboratory characteristics of study subjects.**

Four subjects (subjects 1, 3 and 4 off therapy, subject 2 on stable low dose prednisone) were determined to have definite EAE based on differential blood counts over a period of 42-70 days (Figure 1). A fifth subject with probable EAE had clearly documented episodic cycling in the past during a serial collection, but took a burst of corticosteroids to treat a flare during evaluation at our institution precluding analysis of his data.
Baseline demographic, clinical and laboratory characteristics of the 4 subjects with definite EAE are given in Table 1. Symptoms at the time of cycling were similar in all subjects and included angioedema, urticaria, fatigue, weight gain, and fever. The location and extent of angioedema varied with symptom severity, but most subjects complained of worse symptoms in the face and upper body. Inter-individual cycling time and magnitude of eosinophilia varied between the subjects, but was consistent for each subject (Table 1). All subjects, with the exception of subject 3, had persistent eosinophilia above the normal range even at the nadir of cycles. One subject experienced complete resolution of symptoms on low dose (<5mg/day) corticosteroid therapy, and one subject reported moderate improvement in symptoms on chronic moderate dose corticosteroids (10-20mg/day). None of the subjects reported a family history of EAE. The reported onset of symptoms and eosinophilia was between 10 and 34 years of age, and despite disease durations ranging from 9-16 years, none of the subjects had evidence of chronic eosinophil-related end organ pathology at the time of presentation.

As has been previously reported in EAE (3, 18), all subjects had elevated serum IgM levels on initial assessment. Three subjects also had elevated serum IgE levels. Aberrant CD3-CD4+ lymphocyte populations were detected in all 4 EAE subjects and clonal lymphocyte populations were detected by PCR in 3 of 4 subjects (Table 1). Myeloproliferative features, including anemia, thrombocytopenia, elevated serum B12 and tryptase levels, were absent, and *FIP1L1/PDGFRA* testing was negative in all 4 subjects. Bone marrow aspirates from all 4 subjects demonstrated increased eosinophils, but no increase in blasts or cytogenetic abnormalities.
Multilineage cell cycling in EAE

Serial CBCs demonstrated not only cyclic eosinophilia, but also cycling of other cell populations (Figure 1). The absolute neutrophil count demonstrated a cyclic pattern in all 4 subjects with the peak neutrophil count preceding the peak eosinophil count in 2 subjects (Figure 1). Neutropenia was not observed with the exception of a single low absolute neutrophil count of 580/µL in subject 3 during one cycle. Absolute lymphocyte counts showed a cyclic pattern peaking either with the AEC (subjects 2 and 3) or slightly after the cycle (subjects 1 and 4) with an approximate two-fold increase in absolute lymphocyte count around the time of the peak in all 4 subjects. Subject 4 had a milder flare during the second peak of the sample collection, which is reflected in the less impressive changes noted in cellular and cytokine changes during the second peak. The absolute monocyte and platelet counts and hemoglobin levels did not cycle, and there was no apparent relationship between AEC and absolute counts of other cell lineages in 4 hypereosinophilic subjects without EAE or in one normal control subject tested serially (2-3 times/week) over a one month period (data not shown).

Flow cytometric analysis was performed on whole blood from subject 1 when he was asymptomatic and at three time points surrounding the peak of eosinophilia during a cycle and from subject 3 at 4 separate times over the course of two cycles (Supplemental figure 1). The absolute numbers of CD3+CD4+ and CD3+CD8+ T cells, CD19+ B cells and NK cells increased during the flare in subject 1, peaking simultaneously with the absolute eosinophil count. Although less dramatic, increased numbers of CD3+CD4+ and
CD3+CD8+ T cells and CD19+ B cells were also associated with peak eosinophilia in subject 3. The absolute number of CD3-CD4+ cells was assessed in subject 1 and demonstrated a similar pattern, with the number of aberrant cells increasing from 83/µL to 196/µL at the peak of the flare (data not shown).

Bone marrow biopsies were performed at the peak and nadir of symptoms in subject 1. The aspirate smear cell counts paralleled the findings in peripheral blood with 63% eosinophils (53% of them immature) at the peak and only 12% eosinophils (48% of them immature) at the nadir (Figure 2a, b). The core biopsy was hypercellular (70%) with prominent eosinophilia at the peak, and hypocellular (35%) with mild eosinophilia at the nadir (Figure 2c, d). Immunostaining showed mild increases in CD117 and tryptase-positive mast cells (Figure 2e, f), but no increases in CD34-positive blasts, CD20 or CD5 positive cells, or reticulin fibrosis at either time point.

Mutations in the gene encoding neutrophil elastase (ELANE) are common in patients with cyclic neutropenia and have been implicated in the pathogenesis of the disorder (19). Neutrophil elastase is also a potent activator of eosinophils (20). Since neutrophil cycling was observed in all 4 subjects with definite EAE and preceded the eosinophilia in 2 subjects, sequencing of the coding regions of ELANE was performed using DNA from 3 subjects and 2 normal controls. No polymorphisms were identified in the DNA from the EAE subjects compared to normal controls or the published reference sequence (NCBI Accession AC_Y00477).
Longitudinal analysis of serum and intracellular cytokine levels

Serum levels of the type II cytokines, IL-5, IL-13, IL-9 and IL-10, showed cyclic variation and peaked prior to the peak of eosinophilia in all 4 subjects (Figure 3). A similar pattern was also seen with the eosinophil and CD4+ recruiting chemokines, eotaxin-1 in all 4 subjects and TARC/CCL17 in subjects 1-3 but not subject 4 (Figure 3). Despite occasional variation in levels of serum GM-CSF, G-CSF, IFN-γ, MIP-1-β, TNFα and IL-1-β and IL-6, there was no clear relationship between serum levels of any of these cytokines or chemokines and the onset of symptoms or absolute AEC (data not shown).

In order to explore the role of lymphocytes as a possible source of the type II cytokine variation, PBMCs were obtained from subject 1 near the peak (8 days after onset of symptoms), and nadir (7 days prior to onset of symptoms, and 14 days prior to peak of next cycle) of eosinophilia, stimulated with PMA/ionomycin and assessed for cytokine production using intracellular flow cytometry. Elevated levels of IL-4, IL-13, and IL-5 were detected in the CD3+CD4+CD154+ (stimulated (21)) population at the nadir of eosinophilia with a decline in intracellular Th2 cytokines at the peak of eosinophilia, consistent with exhaustion of intracellular cytokine stores (Figure 4). The numbers of CD3-CD4+CD154+ cells were inadequate in the frozen samples to perform intracellular staining. IL-5 mRNA expression was detected in purified CD4+ cells from each of the 3 definite EAE subjects tested \( \left( \frac{1}{2^{ΔCT}} \right) \times 10^3 \) from 80 to 30,000, but not in 2 normal controls. Although mRNA levels varied dramatically (>100-fold) in the 2 patients for whom samples were available from two time points during the cycle, the pattern of change was inconsistent (data not shown).
Assessment of cellular involvement in skin manifestations of EAE

To assess the role of different cell lineages in the pathophysiology of EAE, two skin biopsies of the upper arm were performed in subject 1, the first at the site of angioedema during symptoms, and the second, near the same site as the first biopsy, after symptom resolution. Although neither skin biopsy showed an eosinophilic cellular infiltrate on routine staining (figure 2g,h), immunohistochemical staining with antibody to eosinophil peroxidase (EPX) revealed a marked increase in eosinophil granule protein deposition at the time of symptoms (figure 2k,l) compared to the asymptomatic period. Anti-CD4 antibodies revealed an increased infiltrate during the symptomatic period (figure 2p) with less dramatic increases in anti-CD3 (figure 2n). In addition, tryptase immunostaining demonstrated a mild increase in mast cells (39-44/hpf) in both biopsies (figure 2i,j). Of note, tryptase immunostaining of bone marrow examinations performed at the same two time points also revealed a mild, but comparable, increase in mast cell numbers (above the normal range of <1 mast cell/hpf at our institution) with an average of 5.6 mast cells/hpf and 8.6 mast cells/hpf at the peak and nadir respectively (figure 2e,f). Too few mast cells were present in the bone marrow aspirate to quantify CD2 and CD25 expression by flow cytometry at either time point.

Total serum tryptase and SCF levels were measured at all time points in two subjects (1 and 2). Despite the relative stability of mast cell numbers in the skin (Figure 2 i,j) and bone marrow (Figure 2 e, f) observed in subject 1, total serum tryptase levels exhibited a cyclic pattern in all 3 subjects tested, varying two-fold over the course of the cycle in all
three subjects tested, albeit remaining in the normal range (1-11.4 ng/ml). Serum SCF levels showed a similar pattern (Figure 5). SCF peaked before the first cycle in Subject 4 and was not detected during the second eosinophil peak during a milder flare of symptoms. SCF was detectable in serum at only one time point in the normal subject who had blood drawn 3 times per week for 4 weeks. Mature tryptase was not detectable in any of the three subjects tested.

Discussion

EAE is an exceedingly rare disorder with less than 50 cases reported in the literature to date. Consistent with prior case reports, the 4 subjects reported in the present series had cyclic eosinophilia and symptoms, including angioedema, with a defined periodicity that was preceded by a rise in serum IL-5. The age of onset (10-32 years of age) was typical and despite prolonged and dramatic eosinophilia, none of the subjects experienced chronic end organ manifestations typical of HES. Finally, as has been reported previously (1), all 4 subjects with definite EAE had elevated serum IgM levels, an uncommon laboratory abnormality in patients with HES. In fact, on review of laboratory data from 224 subjects with HES, elevated serum IgM was documented on more than one occasion in only 12 (5%) percent of subjects in whom ≥2 serum IgM levels were available for analysis (unpublished data).

A novel finding in the present study was cycling of hematopoietic cell lineages other than eosinophils in all 4 subjects with definite EAE. This finding is similar to what is seen in other cyclic hematopoietic disorders, including cyclic neutropenia (10) and cyclic
thrombocytopenia (13), and could be consistent with isolated involvement of a single
lineage with secretion of cytokines or chemokines leading to secondary cycling of other
cell types or with primary involvement of a hematopoietic precursor. In order to explore
the etiology of the cycling in EAE, serum and intracellular cytokine and chemokine
levels were examined every 2-3 days over the course of an entire cycle in 4 subjects on
no or stable low dose prednisone therapy. Serum levels of type II cytokines, including the
primary eosinophilopoietic cytokine, IL-5, were consistently elevated prior to the
eosinophilia in all subjects and intracellular staining of mitogen-stimulated lymphocytes
at the peak and nadir of the cycle demonstrated an increased capacity for production of
these cytokines by CD3+CD4+ lymphocytes prior to the development of eosinophilia in
two subjects, consistent with a primary role for lymphocytes in driving the eosinophilia in
EAE. Since activated eosinophils are known to secrete a wide variety of mediators and
cytokines (22), including the neutrophil chemoattractant IL-8 (23), cyclic changes in
eosinophil activation could, in turn, explain the variation in neutrophil counts. Although a
primary stem cell disorder could also explain multilineage involvement in EAE, the bone
marrow did not show changes in CD34+ cell numbers. Moreover, lymphocytes, but not
monocytes, appear to be involved. In addition, human androgen receptor (HUMARA)
assay analysis performed using purified eosinophils, neutrophils and lymphocytes from
the one female subject failed to demonstrate clonality in any of the lineages tested
(Supplemental Figure 2).

Lymphocyte-driven eosinophilia and dermatologic manifestations, including urticaria and
angioedema, are characteristic of the lymphocytic variant of HES (L-HES). In these
patients, aberrant and/or clonal lymphocytes, most commonly CD3-CD4+, have been
shown to produce increased amounts of type II cytokines driving the eosinophilia in these patients. All 4 of the subjects with EAE in the present study had detectable CD3-CD4+ aberrant T cells that were increased in number at the peak of eosinophilia in those patients seen at the time of symptoms. In three subjects, clonality could be demonstrated by TCR rearrangement studies. Unfortunately, there were insufficient numbers of aberrant CD3-CD4+ cells to assess intracellular cytokine production in frozen samples.

Angioedema and urticaria are typically associated with mast cell and basophil release. Despite the presence of multilineage cycling in the peripheral blood, the skin biopsy findings in the current study are most consistent with a primary role for eosinophils in the angioedema of EAE. The presence of eosinophil granules in the skin of patients with EAE has been previously reported (1). Not only can intact eosinophil granules release pre-formed cytokines, including IL-4 (24), which could lead to mast cell activation, but eosinophil granule proteins have been shown to directly activate mast cells in vitro (25-26), to increase vasopermeability (27-28), and to produce erythematous, indurated palpable lesions when injected intradermally in rabbits and guinea pigs (27). Although eosinophil granule proteins can cause vasopermeability independent of histamine (28), the association with cycling serum tryptase and SCF, as well as mild non-cyclic increases in skin and bone marrow mast cell numbers in these subjects, suggests that mast cells play a role in the clinical manifestations of EAE.

In summary, the present study provides evidence that EAE is a multilineage cell cycling disorder associated with the presence of aberrant and/or clonal T cell populations and eosinophil-driven pathology. Although the intracellular cytokine profile in T lymphocytes suggests that the dramatic eosinophilia and eosinophil activation may be lymphocyte-
driven, the etiology of the cycling in EAE, as well as in the other cyclic hematologic disorders, remains to be elucidated.

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Authorship and Disclosures

PK designed the research, performed and analyzed research and wrote the manuscript, JH, AA ED, JS, SG, IM, OS, LS, JF, LX, ZW and MR performed research and analyzed data, NHT collected data and recruited subjects for the protocol, CRL analyzed data and created figures, and ADK designed research, analyzed data and wrote the manuscript. LBS discloses that VCU receives royalties from tryptase assay sales, and shares those with the inventor (LBS). No other authors disclose any conflicts of interest.
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Table 1. Demographic, clinical and laboratory characteristics of subjects with EAE.

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Usual Symptoms: A-angioedema, U-urticaria, T-fevers, F-fatigue, W-weight gain. TCRγ results: C-Clonal population detected, R- Restricted pattern (two peaks), AEC-Absolute eosinophil count, ANC-absolute neutrophil count, NA-not applicable (prednisone not taken), TCR-γ-T cell receptor gamma. *Normal ranges are 34-342 mg/dL for IgM, 642-1730 mg/dL for IgG, 91-499 mg/dL for IgA and 0-99 IU/mL for IgE, 359-1565 for CD3+CD4+ cell and <1 for CD3-CD4+ cells.

**Figure 1.** Cyclic variation in eosinophils, neutrophils and lymphocytes in subjects with EAE. Absolute numbers of eosinophils, neutrophils and lymphocytes are shown as a function of time during an entire cycle for 4 subjects.

**Figure 2.** Cyclic variation in bone marrow and skin eosinophils and skin lymphocytes, but not mast cells in the skin of subject 1. Bone marrow aspirates (panels a and b; 500X), bone marrow biopsies (panels c-f; 500x magnification) and skin biopsies (panels g-h, k-l; 100x original magnification, insets 600x magnification, i-j, m-n, o-p; 200x original magnification) during the asymptomatic (top) and symptomatic (bottom) phases of the cycle. Panels a-d, g and h are stained with H&E, panels e, f, i and j with tryptase, and panels k and l with anti-EPX antibody, m and n with anti-CD3, and o and p with anti-CD4.
**Figure 3.** Cyclic variation in serum cytokine levels in subjects with EAE. Serum levels of IL-5 and IL-13 (panel 1), CCL17/TARC and eotaxin (panel 2) and IL-9 and IL-10 (panel 3) are shown as a function of time during an entire cycle for 4 subjects. Symbols represent individual measurements, and an arrow indicates the peak eosinophil count during a cycle.

**Figure 4.** Variation in intracellular production of IL-4, IL-5 IL-13 and IFN-γ by activated CD4⁺ lymphocytes in a subject with EAE. After gating on viable CD3⁺CD4⁺CD154⁺ cells, side scatter vs. intracellular cytokine (% cells positive) dot plots were generated. Intracellular IL-4, IL-5, IL-13 and IFN-γ levels are shown for subject 1 before and during symptoms (nadir and cycle, respectively) and for a normal control.

**Figure 5.** Cyclic variation of serum tryptase and SCF (limit of detection 9 pg/mL) levels in EAE. Serum levels of tryptase and SCF are shown as a function of time during an entire cycle for 2 subjects. Symbols represent individual measurements, and an arrow indicates the peak eosinophil count during a cycle.
Figure 3
Panel 1
Panel 2
Panel 3
Subject 1
IL-5 (pg/mL)
(μg/mL) IL-1
Eotaxin (ng/mL)
Subject 2
TARC/CCL17 (ng/mL)
Subject 3
Eotaxin (pg/mL)
Subject 4
TARC/CCL17 (ng/mL)

IL-10
IL-9
peak eosinophil count
days elapsed
Figure 4

IL-4 | IL-13 | IL-5 | IFN-γ

Cycle (±sx)

Nadir (±sx)

Normal control
Supplemental Methods:

Serologic Assessments

Serum IL-5, IL-8, IL-9, IL-13, sIL-2Rα, eotaxin-1, GM-CSF, G-CSF, IFN-γ, MIP-1-β, TNFα and IL-1-β levels were assessed by suspension array technology in multiplex (Millipore). The limits of detection were 0.1, 0.3, 1.1, 0.3, 7.5, 2.1, 2.3, 3.9, 0.4, 3.2, 0.2, and 0.7 pg/mL, respectively.

Immunohistochemistry methods

Immunohistochemical staining for lymphocytes was performed on a DAKO autostainer (Agilent Technologies, Carpinteria, CA) using a CD3 monoclonal antibody (1:200 dilation) and CD4 monoclonal antibody (1:80 dilation) and detected using diaminobenzine as the chromogen.

Immunohistochemical staining for eosinophil peroxidase was performed with a mouse anti-mouse monoclonal antibody (EPX; 1:500, provided by Dr. J.J. Lee). Heat-induced epitope retrieval was accomplished with Leica retrieval solution (Citric buffer) for 25 min and antibody was detected using the BondMax biotin-avidin free polymer-based detection system with diaminobenzine as the chromogen. Samples were analyzed on a Leica BondMax autostainer (Leica Microsystems, Bannockburn, IL).

Mast cells were identified in tissue by tryptase immunostaining performed on an automated immunostainer (Benchmark XT, Ventana Medical Systems, Inc., Tucson, AZ), according to the manufacturer’s protocol. Antigen retrieval was accomplished using
a Protease 1 solution and detected with a Ventana UltraView DAB Detection Kit. Mast Cell Tryptase (clone AA1, 1:400; Dako Corp, CA) was used as the primary antibody. Mast cells were quantified in the bone marrow and skin by averaging counts over 10 high-powered fields.

**Intracellular flow cytometry methods**

Peripheral blood mononuclear cells (PBMCs) were isolated using density gradient separation (Ficoll-Paque Plus, GE Healthcare) and cryopreserved in liquid nitrogen. PBMCs were thawed, washed twice, resuspended in AIM V medium (Invitrogen) with 20 ng/ml phorbol myristate acetate, 1 µM ionomycin calcium salt and 10 µg/ml brefeldin A (all EMD Biosciences, San Diego, CA) and cultured at 4x10^6 cells/well in 24 well plates in a 5% CO_2 incubator. After 6 h, 60 mg DNase (EMD Biosciences) were added to each well. Cells were harvested after 5 minutes, washed twice in cold PBS, labeled with LIVE/DEAD Fixable Violet Dead Cell Stain (Invitrogen), washed twice in cold PBS, and fixed in 4% paraformaldehyde (Sigma, St Louis, Mo). For staining, cells were blocked in PBS with 0.1% saponin (Sigma) and 5% nonfat dried milk for 30 minutes and then stained with the following panel: CD3-Pacific Blue (UCHT1-BD Pharmingen), CD4-Qdot 605 (S3.5 Invitrogen), CD8- PE-Cy5.5 (3B5 Invitrogen), CD154-APC-Cy7 (24-31 Biolegend), IL-4-PE (MP4-25D2, BD Pharmingen), IL-5-APC (TRFK5 BD Pharmingen), IL-13-PE (JES105A2 BD Pharmingen), IFN-γ-AF700 (B27 BD Pharmingen). Samples were acquired on an LSRII cytometer (BD Biosciences) and analyzed using FlowJo (v9.4.8 Stanford California).
Human Androgen Receptor (HUMARA) assay methods

DNA was extracted using Purgene Tissue and Blood kit (Qiagen). DNA concentration and purity was calculated and 1.25 µg of DNA was incubated with 1:10 proteinase K at 37°C for 75 minutes. The total volume was split in half and was digested with or without 0.5µL of 10U HpaII for 10 minutes at 65°C. The HUMARA locus was amplified using the primers (1pmol/ µL): FW 5’-/56-FAM/TCC AGA ATC TGT TCC AGA GCG TGC – 3’; RV 5’ – GCT GTG AAG GTT GCT GTT CCT CAT – 3’, and the PCR protocol: initial denaturation at 95°C for 5 minutes, 40 cycles of denaturation at 95°C for 45 seconds, annealing at 61°C for 45 seconds, and elongation at 72°C for 1 minute. Products were then separated by capillary electrophoresis and the relative quantity of gene product peaks was assessed to determine skewing.
**Supplemental Data Figure Legend:**

Supplement 1. Cycling of T cell subset (CD3+CD4+, CD3+CD8+, B cells (CD19+) and NK cell (CD16+CD56+) numbers parallel the eosinophil count in episodic angioedema with eosinophilia in Subject 1 during a flare and subject 3 during one full cycle.

Supplement 2. HUMARA analysis showing polyclonal populations of cells in a female subject with episodic angioedema with eosinophilia (subject 4). E-eosinophils, CD4+-CD4+ lymphocytes, N-Neutrophils, B-B lymphocytes, PC-Positive control HES subject with a lymphocyte clone.