IMMUNOPATHOLOGY AND INFECTIOUS DISEASES

Pulmonary Eosinophilic Granulomatosis with Polyangiitis Has IgG4 Plasma Cells and Immunoregulatory Features

Zachary M. Dong,^{*} Edwin Lin,^{*†} Michael E. Wechsler,[‡] Peter F. Weller,[§] Amy D. Klion,[¶] Bruce S. Bochner,^{||} Don A. Delker,^{*} Mark W. Hazel,^{*} Keke Fairfax,^{*} Paneez Khoury,[¶] Praveen Akuthota,^{**} Peter A. Merkel,^{††} Anne-Marie Dyer,^{‡‡} Carol Langford,^{§§} Ulrich Specks,^{¶¶} Gerald J. Gleich,^{*} Vernon M. Chinchilli,^{††} Benjamin Raby,^{‡‡|||} Mark Yandell,^{*†} and Frederic Clayton^{*}

From the University of Utah,^{*} Salt Lake City, Utah; the Department of Human Genetics,[†] USTAR Center for Genetic Discovery, Salt Lake City, Utah; the National Jewish Health,[‡] Denver, Colorado; the Beth Israel Deaconess Medical Center[§] and the Brigham and Woman's Hospital,^{||} Harvard Medical School, Boston, Massachusetts; the Laboratory of Parasitic Diseases,[¶] National Institute of Allergy and Infectious Diseases, NIH, Bethesda, Maryland; the Northwestern University Feinberg School of Medicine,^{||} Chicago, Illinois; the University of California, San Diego,^{**} San Diego, California; the University of Pennsylvania,^{††} Philadelphia, Pennsylvania; the Penn State College of Medicine,^{‡‡} Hershey, Pennsylvania; the Cleveland Clinic,^{§§} Cleveland, Ohio; and the Mayo Clinic,^{¶¶} Rochester, Minnesota

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Address correspondence to
Frederic Clayton, M.D., Hunts-
man Cancer Institute, 2000
Circle of Hope, Room 3100,
Salt Lake City, UT 84112. E-
mail: fred.clayton@path.utah.edu.

The immunologic mechanisms promoting eosinophilic granulomatosis with polyangiitis (EGPA) are unclear. To characterize the mechanisms underlying pulmonary EGPA, we examined and compared EGPA paraffin-embedded lung biopsies with normal lung biopsies, using immunostaining, RNA sequencing, and RT-PCR. The results revealed novel type 2 as well as immunoregulatory features. These features included basophils and increased mast cell contents; increased immunostaining for tumor necrosis factor ligand superfamily member 14; sparse mast cell degranulation; numerous forkhead box protein P3 (FoxP3)+ regulatory T cells and IgG4 plasma cells; and abundant arachidonate 15-lipoxygenase and 25-hydroxyvitamin D-1 α hydroxylase, mitochondrial. Significantly decreased 15-hydroxyprostaglandin dehydrogenase [NAD(⁺)], which degrades eicosanoids, was observed in EGPA samples. In addition, there was significantly increased mRNA for chemokine (C-C motif) ligands 18 and 13 and major collagen genes, IgG4-rich immune complexes coating alveolar macrophages, and increased immunostaining for phosphorylated mothers against decapentaplegic homolog 2/SMAD2, suggesting transforming growth factor- β activation. These findings suggest a novel self-promoting mechanism of activation of alveolar macrophages by arachidonate 15-lipoxygenase-derived eicosanoids to express chemokines that recruit a combined type 2/immunoregulatory immune response, which produces these eicosanoids. These results suggest that the pulmonary EGPA immune response resembles the immune response to a

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Eosinophilic granulomatosis with polyangiitis (EGPA) is a rare systemic disease characterized by eosinophilic asthma, sinus disease, pulmonary infiltrates, and vascular eosinophil infiltrates with vasculitis. Because lung biopsies are rarely done in EGPA and because there are no good EGPA animal models, the lung immunopathology of this rare disease has not been carefully studied.

Therapeutic trials, gene association studies, and flow cytometry of blood from EGPA patients all provide clues to its immune pathophysiology. Beneficial effects have been shown for treatments targeting eosinophils¹ (mepolizumab, anti-IL-5), B cells^{2,3} (rituximab, anti-CD20), and antibodies⁴ (intravenous immunoglobulins and plasmapheresis). Leukotriene modifier therapy is linked to an increased risk for developing EGPA.^{5,6} Patients with active EGPA have high serum IgG4.⁷ IgG4 is IL-10 induced.⁸ EGPA is associated with specific human leukocyte antigen-DR variants⁹ and an *IL10* promotor allele that increases IL-10 expression.¹⁰ These suggest mechanistic roles for eosinophils, B cells or antibodies, eicosanoids, T-helper cells, and IL-10.

Terrier et al¹¹ had predicted the pathogenic role for eosinophils and T cells on the basis of a possible self-promoting pathway involving eosinophils, T cells, and IL-25. Tsurikisawa and Saito and colleagues^{12–17} showed that peripheral blood regulatory T cell (Treg) content is decreased in active EGPA patients relative to healthy controls and to patients with inactive EGPA, asthma, and chronic eosinophilic pneumonia. They concluded that Tregs prevent or suppress EGPA and that Treg content was reduced in active disease. However, evidence against this included the *IL10* gene promoter association,¹⁰ that EGPA bronchoalveolar lavage specimens have increased *IL10* and *FOXP3* RNA,¹⁸ and the presence of abundant serum IgG4,⁷ presumably IL-10 induced,⁸ in active EGPA. Alternatively, Tregs might not be lost, but instead recruited from blood into tissue during active disease. In this study, we characterize the immune infiltrates in EGPA using both RNA sequencing and immunostaining. We show a combined type 2 and immunoregulatory infiltrate with abundant Tregs, IgG4 plasma cells, basophils, and immune complexes, as well as chemokines needed to recruit and sustain this infiltrate.

Materials and Methods

Criteria and Subjects

Lung biopsies were obtained from six EGPA subjects meeting American College of Rheumatology criteria,¹⁹ with active disease at the time of biopsy. All available cases were used without regard for statistical power. All studies were institutional review board approved. Informed consent was

obtained as required. Controls were histologically normal lung tissue removed in tumor resections.

Histopathology and Immunostaining

Routine hematoxylin and eosin sections and immunostains were examined in a blinded manner (F.C.). Immunostaining was done either manually or with a Ventana Benchmark Ultra (Santa Clara, CA) or Leica Bond (Richmond, IL) stainer. Manual immunoperoxidase staining was done with SignalStain reagents (Cell Signaling Technology, Danvers, MA). Manual immunofluorescence staining was done with goat anti-rabbit and goat anti-mouse antibodies conjugated with Alexa488, Alexa 647, or Cy3 (Jackson ImmunoResearch, West Grove, PA) and DAPI counterstained. Primary antibodies are listed in Table 1.

An Olympus BX60 fluorescent microscope (Waltham, MA) was used (0.54-mm high-power field diameter). Confocal microscopy was done with a Nikon A1 confocal microscope (Melville, NY). Manual immunostains were done after pressure cooker EDTA-based antigen retrieval (Trilogy; Cell Marque, Rocklin, CA), and incubated in 3.5% normal goat serum for 30 minutes. Antibody incubations were 9 to 12 hours at room temperature. Manual immunoperoxidase stains were done with SignalStain immunostaining kits from Cell Signaling Technology, using their methods. Automated immunostains were done on Ventana Benchmark Ultra or Leica Bond. Statistical significance was assessed by *U*-test (GraphPad Prism, version 8; GraphPad Software, San Diego, CA).

RNA Extraction and RNA Sequencing

RNA methods were as previously described.²⁰ Total RNA was extracted from formalin-fixed, paraffin-embedded tissue (10 μ m thick) using the High Pure FFPE RNA Isolation kit (Roche, Indianapolis, IN). DNA was removed from the RNA samples using the manufacturer's suggested DNase one-digestion step. RNA quantity was determined with a NanoDrop spectrophotometer (Thermo Fisher Scientific, Waltham, MA). RNA samples were prepared for RNA sequencing using the Illumina TruSeq Stranded Total RNA Sample Prep protocol (Illumina, San Diego, CA), which included removing ribosomal RNA. An Illumina HiSeq 2500 was used to sequence the PCR amplified libraries using 50-cycle single-read chemistry.

Bioinformatics Analysis

FASTQC version 0.11.6 (Babraham Institute, Cambridge, UK) was used to perform quality control checks on raw sequence data. Quantification of Ensembl human cDNA

(release 91) was performed using Kallisto and Taxonomer software.²¹ DESeq2 was used for differential expression analysis. Criteria for differentially expressed genes were as follows: a mean read count of at least 10, a log₂ fold change >2, and $P < 0.05$ after correction for false discovery rate. The mean number of reads mapped to human genes per case were 4,668,600 among the RNA-sequencing cases and 7,124,500 among controls. A total of 2309 genes were differentially expressed; 1493 genes were significantly up-regulated and 816 genes were significantly down-regulated in EGPA relative to controls. RNA-sequencing data generated in this study are available on National Center for Biotechnology Information Gene Expression Omnibus (<https://www.ncbi.nlm.nih.gov/geo>; accession number GSE144302).

RT-PCR

Selected genes crucial to the proposed mechanism were also examined by real-time quantitative RT-PCR. Total RNA was reverse transcribed to cDNA using Applied Biosystems (Foster City, CA) High-Capacity RNA-to-cDNA kit. Relative mRNA levels for *IL10*, *IL13*, *CCL18*, and the reference genes *GLYR1* and *WDFY2* were measured using intron-spanning TaqMan gene expression assays and TaqMan fast advanced master mix (Applied Biosystems). Reference genes were chosen on the basis of their moderate expression and lack of shift between sample categories by RNA sequencing. *GLYR1* was chosen as the reference gene for relative quantitation. Similar results were obtained using *WDFY2* as the reference gene. Quantitative PCRs were performed in 384-well plates and run in triplicate in a Life Technologies (Carlsbad, CA) QuantiStudio 12K Flex Real-Time PCR instrument. Fold change was determined by the $\Delta\Delta C_T$ method. Statistical significance was assessed by *t*-test in GraphPad Prism.

Results

Study Population

All six subjects had active EGPA at the time of lung biopsy and were previously diagnosed with asthma. Five biopsies were open lung; one was transbronchial. Two subjects were not on corticosteroid therapy at the time of biopsy, two were taking inhaled corticosteroids, and two were on oral prednisone. See [Supplemental Table S1](#) for details.

For RNA sequencing, three cases (mean age, 36 years) and four controls (mean age, 34 years) were studied. All three EGPA RNA-sequencing subjects were female; two of four controls were male. One subject from each category (no corticosteroids, inhaled corticosteroids, and oral corticosteroids) was studied by RNA sequencing.

For RT-PCR, four EGPA cases (mean age, 33 years) and 10 controls (mean age, 32 years) were studied. All four EGPA cases and eight of 10 controls were female.

For histology and immunohistology, six EGPA cases (mean age, 36 years) and seven controls (mean age, 31 years) were studied. Two of six EGPA cases and two of seven controls were male. The transbronchial biopsy was only used for some stains; the block was exhausted.

Histologic Findings

All five open lung biopsies from EGPA patients had alveolar and interstitial infiltrates. The alveolar infiltrates were abundant in four cases, and were composed of many eosinophils with focal degranulation, numerous alveolar macrophages, and occasional giant cells, all focally embedded in fibrin ([Figure 1](#), A and B, and [Supplemental Figure S1](#)). Both alveolar and, rarely, interstitial macrophages formed aggregates, some of which were cohesive enough to be considered granulomas.

Interstitial infiltrates were mostly pleural, septal, and around airways, and had lymphocytes, eosinophils, some macrophages, and plasma cells ([Figure 1](#), A and C). Interstitial edema was minimal to absent. Three EGPA open lung biopsies had eosinophil-rich infiltrates in small artery and vein walls without fibrinoid necrosis (necrotizing vasculitis) ([Supplemental Figure S2](#)). Sparse eosinophil and macrophage-rich areas of parenchymal necrosis were present in two open biopsies. Small areas with neutrophils were present in each case ([Figure 1D](#)). Trichrome stains (not shown) demonstrated only minimal, mostly subpleural fibrosis. The transbronchial biopsy, from a patient on oral corticosteroid therapy, had similar interstitial infiltrates, but lacked alveolar infiltrates. The open lung biopsy from a patient on oral corticosteroids had less alveolar infiltrate and fewer eosinophils than the other open biopsies; the interstitial findings were similar.

Main Cell Populations and Phosphorylated SMAD2

By immunostaining, many interstitial lymphocytes were CD19⁺ B cells or CD4⁺ T cells, with fewer CD8⁺ T cells ([Figure 2](#), A–E). Most CD68⁺ macrophages were in alveolar aggregates/granulomas ([Figure 2F](#)). Many alveolar macrophages were CD4⁺ ([Figure 2C](#)).

The five open lung biopsies from patients with EGPA had extensive nuclear plus cytoplasmic immunostaining in many cell types for phosphorylated mothers against decapentaplegic homolog 2 (phosphorylated SMAD2) ([Figure 3A](#)). The seven controls had sparse but definite phosphorylated SMAD2 nuclear staining, far less than in EGPA cases ($P = 0.0025$) ([Figure 3B](#) and [Supplemental Figure S3](#)). Tissue basophils were seen in four of five EGPA open biopsies ([Figure 3C](#)). No basophils were seen in seven control lung biopsies ($P = 0.0091$) ([Figure 3D](#) and [Supplemental Figure S3](#)). Mast cells, stained by tryptase δ (mast cell tryptase), were abundant in the EGPA patient open lung biopsies ([Figure 3E](#)), more than the control lungs

Table 1 Primary Antibodies Used

Antibody specificity	Clone	Manufacturer	Stainer used
<i>ALOX15</i>	EPR22138	Abcam, Cambridge, MA	Manual
<i>ALOX15</i>	7H6	Origene, Rockville, MD	Manual
CD68	KP-1	Dako, Santa Clara, CA	Ventana, manual
CD3	LN10	Biocare, Pacheco, CA	Leica, manual
CD3	SP7	Abcam	Manual
CD4	4B12	Leica, Buffalo Grove, CA	Leica
CD8	4B11	Leica	Leica
CD19	BT51E	Leica	Leica
<i>CYP27B1</i>	EPR20271	Abcam	Manual
FoxP3	236A/E7	Abcam	Manual
FoxP3	D2W8E	Cell Signaling Technology, Beverly, MA	Manual
15-PGDH	EPR14332	Abcam	Manual
15-PGDH	Rabbit polyclonal	Abcam	Manual
IgG4	EP4420	Abcam	Manual
IgG4	MRQ-44	Cell Marque, Rocklin, CA	Ventana
LIGHT (<i>TNFSF14</i>)	Rabbit polyclonal	Abcam	Manual
LIGHT (<i>TNFSF14</i>)	Rabbit polyclonal	Novus, Centennial, CO	Manual
Phosphorylated SMAD2	EPR2856	Abcam	Manual
Pro-MBP, for basophils	J175-7D4	Gift from Hirohita Kita (Mayo Clinic, Rochester, MN)	Manual
Rhodamine red X anti-human IgG	Goat polyclonal	Jackson ImmunoResearch, West Grove, PA	Manual

CD68, macrosialin; CD4, T-cell surface glycoprotein CD4; CD19, B-lymphocyte antigen CD19; FoxP3, forkhead box protein P3; 15-PGDH, 15-hydroxyprostaglandin dehydrogenase [NAD(+)]; LIGHT (*TNFSF14*), tumor necrosis factor ligand superfamily member 14; SMAD2, mothers against decapentaplegic homolog 2; Pro-MBP, bone marrow proteoglycan.

($P = 0.0025$) (Figure 3F and Supplemental Figure S3). Mast cell degranulation was sparse.

RNA Sequencing and RT-PCR

RNA sequencing was done on lung biopsy tissue of three patients with EGPA and four controls. Mean numbers of reads mapped to human genes per case were 4,668,600 among the RNA-sequencing cases and 7,124,500 among controls. Using stringent criteria, 2309 genes were differentially expressed; 1493 genes were significantly up-regulated and 816 genes were significantly down-regulated in EGPA relative to controls. Selected results are shown in Table 2 and in the volcano plot (Supplemental Figure S4).

Chemokine RNAs for *CCL18* and *CCL13* were markedly increased in EGPA, as was that for *ALOX15* (arachidonate 15-lipoxygenase), which induces alveolar macrophages *CCL18* and *CCL13*. *HPGD* RNA was decreased 24-fold in the EGPA cases.

Type 2–related RNAs for *SIGLEC8*, *TNFSF14*, and *IGHE* were increased in EGPA. *IL13*, *IL4*, and *IL5* had too few reads for RNA-sequencing evaluation, but RT-PCR indicated significantly increased *IL13* expression in EGPA (see below). *TNFSF14* (tumor necrosis factor ligand superfamily 14, protein is also called LIGHT, which has type 2 and immunoregulatory effects) was increased 17.5-fold in EGPA.

Immunoregulatory RNAs *FOXP3*, *IGHG4*, and *CYP27B1* were all significantly more abundant in EGPA. *IGHG4* was increased 1885-fold. RNA for the Treg-related gene *FOXP3* was increased eightfold in EGPA relative to

controls. *CYP27B1*/25-hydroxyvitamin D-1 α hydroxylase, mitochondrial, hereafter abbreviated as VD3 1A hydroxylase, which synthesizes calcitriol, was increased 11-fold in EGPA relative to controls. *CYP24A1* (which metabolizes calcitriol) was essentially unchanged in EGPA. *IL10* RNA was increased eightfold, but had insufficient reads for reliable interpretation.

RNAs characteristic of B cells, macrophages, and Ig heavy chains were increased in EGPA, but IgG4 was the most increased isotype (increased 1885-fold; $P = 1.7 \times 10^{-15}$). In addition to the increased *ALOX15*, there was decreased *HPGD* RNA content.

Compared with controls, lung tissue in EGPA had significantly increased *IL13* (20-fold; $P = 0.0085$) and *CCL18* RNA (21-fold; $P = 0.03$), but not *IL10* (3.4-fold increase; $P = 0.18$).

IgG4

IgG4 plasma cell infiltrates were extensive (Figures 2G and 4A). The five EGPA open lung biopsies had a median of 90 (range, 21 to 140) IgG4⁺ plasma cells per maximal high-power field, versus a median of 0 (range, 0 to 1) IgG4 plasma cells in seven control samples ($P = 0.0013$) (Supplemental Figure S3). By combined IgG and IgG4 immunofluorescence staining, most IgG⁺ plasma cells were IgG4⁺ (Supplemental Figure S5). The IgG4 plasma cell infiltrates were often subpleural, peribronchial, or septal, and were exclusively in the interstitium rather than alveolar spaces. There was interstitial and intra-alveolar IgG4

Figure 1 Eosinophilic granulomatosis with polyangiitis (EGPA) has distinctive alveolar and interstitial infiltrates. **A:** The alveolar infiltrate has abundant eosinophils, macrophages, and fibrin. The interstitium has abundant lymphocytes, eosinophils, and macrophages. **B:** Intra-alveolar giant cells are present but sparse. **C:** Focally, the interstitium has abundant plasma cells. **D:** In occasional fields of each EGPA open biopsy, neutrophils are also present. Original magnification: $\times 67$ (**A**); $\times 83$ (**B**); $\times 140$ (**C**); $\times 150$ (**D**).

staining, often coating alveolar macrophages and giant cells (Figure 4B). By confocal microscopy, the extracellular IgG and IgG4 was often granular, resembling immune complexes, focally coating CD68⁺ alveolar macrophages (Figure 4C). Both IgG4 antibodies stained similarly. The granular extracellular staining for IgG4 and for total IgG was generally similar (not shown).

Regulatory T Cells

Confirming the RNA-sequencing finding of increased *FOXP3* RNAs, FoxP3⁺ cells, presumed Tregs, were abundant in EGPA lung tissue but rare in controls ($P = 0.011$; *U*-test) (Figure 4D and Supplemental Figure S3). All FoxP3⁺ cells were CD3⁺ (Supplemental Figure S6). Many FoxP3⁺ cells were in subpleural or interstitial lymphoid aggregates; some were in alveolar macrophage infiltrates. Both FoxP3 antibodies stained similarly.

Eicosanoid-Related Immunostains

Eosinophils were the main site of arachidonate 15-lipoxygenase immunostaining (Figures 2H and 5A). All eosinophils stained strongly for arachidonate 15-lipoxygenase, as did some alveolar macrophages. Control lung cases had virtually no arachidonate 15-lipoxygenase

staining, substantially less than in the EGPA cases ($P = 0.0012$) (Figure 5B and Supplemental Figure S3). Respiratory epithelial cell and pleural mesothelial arachidonate 15-lipoxygenase staining was found in both EGPA cases and controls (not shown). Both arachidonate 15-lipoxygenase antibodies stained similarly.

In the EGPA open lung biopsies, immunostaining for 15-hydroxyprostaglandin dehydrogenase [NAD⁺], referred to as 15-PGDH, in the EGPA open biopsies was minimal to negative in both alveolar macrophages and alveolar epithelium (Figure 5C), but confluent present in control case pneumocytes (Figure 5D). In the EGPA cases, a minority of lymphocytes and sparse intermediate sized cells had 15-PGDH staining. The 15-PGDH⁺ intermediate-sized cells had abundant staining, whereas staining was far weaker in the positive-staining lymphocytes (Supplemental Figure S7). By RNA sequencing, HPGD expression was decreased 24-fold in the EGPA cases (see *RNA Sequencing and RT-PCR* above and Table 2).

Figure 2 Sections of the same eosinophilic granulomatosis with polyangiitis (EGPA) lung tissue, showing staining for various antigens. **A:** Hematoxylin and eosin (H&E) stain. **B:** CD3 (T cells). **C:** CD4 (T-helper cells and, more faintly, some macrophages). **D:** CD8 (cytotoxic T cells). **E:** CD19 (B cells). **F:** CD68 (macrophages). **G:** IgG4. **H:** Arachidonate 15-lipoxygenase (eosinophils, mesothelium, and, more faintly, a minority of macrophages). Original magnification, $\times 12$ (**A–H**).

Figure 3 Increased phosphorylated SMAD2, increased numbers of basophils, and mast cells are present in eosinophilic granulomatosis with polyangiitis (EGPA) lung biopsies. **A:** Phosphorylated SMAD2 immunostains in EGPA, showing abundant alveolar macrophage and epithelial cell staining. **B:** Phosphorylated SMAD2 immunostain in a control, showing definite nuclear staining, but far less than in the EGPA biopsies. **C:** Basophil immunostain (green) in EGPA. **Inset:** A basophil at high magnification with coarse cytoplasmic basophil granules. **D:** Basophil immunostain in control lung, showing no basophils. **E:** EGPA lung mast cell tryptase immunostain (green), showing abundant mast cells. Mast cell degranulation was sparse (not shown). **F:** Control lung, showing fewer mast cells. Original magnification: $\times 150$ (A–D); $\times 500$ (inset in C); $\times 55$ (E and F).

Other Immunoregulatory and Type 2–Related Immunostains

VD3 1A hydroxylase⁺ cells were increased in the EGPA open lung biopsies (Figure 6A) compared with the seven controls ($P = 0.0025$) (Figure 6B and Supplemental Figure S3). Alveolar macrophages had extensive granular cytoplasmic staining resembling mitochondria. Alveolar macrophages also stained for VD3 A1 hydrolase and pneumocytes, and other cells were extensively stained in the EGPA cases only. LIGHT immunostaining was present mainly in alveolar and interstitial macrophages. Macrophages were generally LIGHT⁺, and were substantially more abundant in the EGPA biopsies (Figure 6C) than in controls ($P = 0.0025$) (Figure 6D and Supplemental Figure S3).

Discussion

In summary, the data show that EGPA has both type 2 and immunoregulatory features. Type 2 features include infiltrates with eosinophils, basophils, and mast cells, and RNAs for *IL13*, *TNFSF14*, and *SIGLEC8*. Immunoregulatory features include the presence of Foxp3⁺ Tregs, IgG4 plasma cells, VD3 1A hydroxylase⁺ macrophages (synthesize calcitriol), and phosphorylated SMAD2 in most cells [suggesting active transforming growth factor (TGF)- β], each corroborated by RNA sequencing.

Chemokines *CCL18* and *CCL13* are significantly up-regulated, substantially explaining what cells are recruited to form the infiltrates. *ALOX15* contents are increased, explaining the presence of *CCL18* and *CCL13*. *HPGD* contents (15-PGDH, which metabolizes eicosanoids) are substantially decreased in EGPA, mainly in alveolar epithelium, although there was staining compatible with known basophil and Treg 15-PGDH expression.²² Decreased 15-PGDH suggests that eicosanoid signaling could be accentuated in EGPA.

Except for a study of CD83 and CD208,¹⁷ this is, to our knowledge, the first immunostaining study of lung tissue in EGPA. This is the first lung tissue transcriptome study in EGPA. Prior studies examined the routine histopathology of EGPA, showing abundant eosinophils, macrophages with giant cells, lymphocytes, plasma cells, and eosinophilic vasculitis in lung tissue.^{23–26} Bronchoalveolar lavage studies have shown significant EGPA-related increases in type 2–related cytokines, *FOXP3* and *IL10*,¹⁸ and *ALOX15*-related eicosanoids,²⁷ supporting our findings of Tregs and other immunoregulatory features. Although a prior study suggested that eosinophils have FoxP3,²⁸ all FoxP3⁺ were found to be CD3⁺ T cells. Although IgG4 plasma cells have not been previously reported in the lungs in EGPA and they are reportedly absent in the upper respiratory tract⁷ of EGPA patients, modest numbers of IgG4 plasma cells have been demonstrated in nerve biopsies from EGPA patients.²⁹

Table 2 RNA-Sequencing Results

Gene name	Base mean	Log2 fold change	RNA-sequencing adjusted <i>P</i> value	Comments
Genes significantly different				
Chemokines and eicosanoids				
<i>CCL18</i>	45.7	6.13	5.8×10^{-5}	Recruits Treg and Th2 via <i>CCR8</i> .
<i>CCL13</i>	10.9	4.25	0.0022	Recruits macrophages, eosinophils, and basophils.
<i>ALOX15</i>	203	3.55	0.024	Arachidonate 15-lipoxygenase. Its eicosanoid products induce macrophages <i>CCL18</i> , <i>CCL13</i> , and <i>CCL22</i> .
<i>HPGD</i>	397	-4.57	6.7×10^{-7}	15-PGDH. Metabolizes eicosanoids.
<i>LTC4S</i>	49	4.35	0.0022	Leukotriene C4 synthase. Makes leukotriene C4 and eoxin C4.
Type 2 related				
<i>IL13</i>	0.58	3.98		Type 2 cytokine. Significantly increased by RT-PCR.
<i>SIGLEC8</i>	34.7	4.11	8.5×10^{-5}	Receptor on mast cells and eosinophils.
<i>TNFSF14</i>	26.9	4.13	2.1×10^{-8}	LIGHT. Induces eosinophil IL-13, macrophage TGF- β , and T-cell activation.
<i>IGHE</i>	295	9.18	2.7×10^{-30}	IgE heavy chain.
Immunoregulatory and profibrotic				
<i>FOXP3</i>	17.3	3.04	0.0015	Tregs. Can be expressed in other cell types.
<i>IGHG4</i>	26,478	10.88	1.7×10^{-15}	IgG4 heavy chain. IgG4 is induced by IL-10.
<i>CYP27B1</i>	12.8	3.44	0.004	VD3 1A hydroxylase. Makes calcitriol (active vitamin D).
<i>INHBA</i>	40.0	-2.10	0.013	Inhibin β A chain (activin A). Causes SMAD2 phosphorylation.
<i>COL1A1</i>	20,163	4.32	2.0×10^{-7}	Major collagen gene.
<i>COL3A1</i>	20,529	3.03	0.0021	Major collagen gene.
Genes not significantly different				
<i>IL10</i>	9.3	3.02		Regulatory cytokine. Increased in EGPA BAL in a prior study.
<i>IL5</i>	2.26	-2.08		Induces eosinophils. Increased in EGPA BAL in a prior study.
<i>TGFB1</i>	338	1.14	0.062	TGF- β 1. Activated form is a crucial immunoregulatory signal.
<i>CCL22</i>	3.15	5.04		Recruits Th2 and Treg via <i>CCR4</i> .
<i>CCL26</i>	3.01	6.03		Eotaxin-3. Significantly increased in EGPA serum and BAL.
<i>CYP24A1</i>	8.21	0.08		Metabolizes calcitriol.

15-PGDH, 15-hydroxyprostaglandin dehydrogenase [NAD(+)]¹; BAL, bronchoalveolar lavage; LIGHT, tumor necrosis factor ligand superfamily member 14; Th2, type 2 helper T cell; TGF, transforming growth factor; Treg, regulatory T cell; VD3 1A hydroxylase, 25-hydroxyvitamin D-1 α hydroxylase, mitochondrial.

The *CCL18* and *CCL13* chemokine pattern is novel in EGPA and differs from the finding that *CCL17* and *CCL22*, made by respiratory epithelium, are dominant in asthma.³⁰ The presence of *CCL18* also explains the apparent discrepancy between the increase in Tregs found in the present study and earlier work by two different groups demonstrating decreased peripheral blood Tregs in EGPA.^{12–17,31} Although Saito and Tsurikisawa and colleagues^{12–17} concluded that Tregs have a protective effect in EGPA and are lost during active disease, these results suggest that, rather than being lost, Tregs are recruited to lung tissue, presumably by chemokine (C-C motif) ligand (CCL) 18, where they likely contribute to the immunoregulatory findings in EGPA. Other novel findings (to our knowledge for EGPA at any site) include expression of *CYP27B1*, *HPGD*, *TNFSF14*, *ALOX15*, *HPGD*, and their protein products, as well as TGF- β activation (indicated by

increased phosphorylated SMAD2), IgG4⁺ macrophage immune complexes, and increased numbers of Tregs and basophils.

Because EGPA is associated with an *IL10* promotor haplotype with increased IL-10 secretion,¹⁰ the immunoregulatory features could be mechanistically relevant. Tissue-invasive filarial parasites induce an analogous IL-10 and IgG4 immune response that modulates disease by blocking parasite-specific IgE responses, avoiding elephantiasis,³² as well as impairing type 1 and type 2 helper T-cell immune pathways.³³ We hypothesize that EGPA is a pathologic IL-10 and IgG4-related⁸ immune reaction analogous to an immune response to a tissue-invasive parasite, as previously proposed for eosinophilic esophagitis.³⁴

We propose a mechanism that integrates our findings with prior functional studies (Figure 7). Arachidonate 15-lipoxygenase produces eicosanoids that, in alveolar

Figure 4 Eosinophilic granulomatosis with polyangiitis (EGPA) patients have abundant IgG4 plasma cells, granular extracellular and macrophage IgG4, and FoxP3 staining, suggesting regulatory T cells. **A:** Lung biopsies from EGPA patients have, in some areas, abundant interstitial IgG4 plasma cells by IgG4 immunoperoxidase stain. Pleural surface is at bottom. **B:** Some alveolar macrophages and giant cells have predominantly cell membrane staining for IgG4. Eosinophils generally lack staining for IgG4. **C:** Confocal microscope image showing distinctly granular staining for IgG4 (green), resembling immune complexes. Focally, the IgG4 staining is at the surface of macrophages, with coarsely granular cytoplasmic CD68 immunostaining (red). **D:** EGPA biopsy. FoxP3 immunoperoxidase stain, with presumptive regulatory T cells in alveolar macrophage aggregates (at left) and pleural interstitium (at right). Original magnification: $\times 88$ (**A**); $\times 125$ (**B** and **D**); $\times 500$ (**C**).

macrophages *in vitro*, induce CCL18, CCL13, and CCL22.³⁵ This effect is potentiated by decreased 15-PGDH, which metabolizes eicosanoids, effectively prolonging eicosanoid signaling. CCL18 recruits C-C chemokine receptor type 8-containing cells, particularly Tregs, which

abundantly express this receptor, per the Human Protein Atlas.²² CCL18 also induces IL-10 and ALOX15.³⁶ Tregs can produce IL-10 and TGF- β , induce basophil IL-13,³⁷ and, in part via IL-10, induce macrophage CCL18.³⁸ CCL18 also induces collagen synthesis,³⁹ which induces further CCL18.⁴⁰ Although each of our lung biopsy cases are from early in the course of the disease before the EGPA diagnosis, the increased collagen mRNA is not surprising given the extensive fibrosis in advanced lesions.²³ CCL13 and CCL26 recruit eosinophils and basophils via CCR3.

IL-10 induces IgG4 plasma cells⁸ and macrophage TGF- β .⁴¹ IgG4 alveolar macrophage immune complexes induce macrophage IL-10.⁴² IgG4 blocking antibodies could suppress IgE-mediated macrophage activation and thus classic allergy. TGF- β is also induced by LIGHT.⁴³ TGF- β induces Tregs, CYP27B1, collagen synthesis, and other regulatory effects. Although the concept of M2 macrophages⁴¹ is an oversimplification, EGPA lung has four main M2 stimuli: IL-13 (M2a), immune complexes (M2b), IL-10, and TGF- β (M2c). Regulatory B cells, which are IgG4⁺, could also be present.^{44,45} Calcitriol, the product of CYP27B1/VD3 1A hydroxylase, has immunoregulatory features,⁴⁶ but suppresses collagen synthesis and TGF- β .⁴⁷ These findings are summarized in Figure 7.

Figure 5 Eosinophilic granulomatosis with polyangiitis (EGPA) lung tissue immunostaining suggests increased expression of arachidonate 15-lipoxygenase (ALOX15)-related eicosanoids. **A:** EGPA lung biopsy has extensive ALOX15 staining of all eosinophils and some alveolar macrophages. **B:** Control lungs have minimal ALOX15 staining. **C:** EGPA lung biopsy had 15-hydroxyprostaglandin dehydrogenase (15-PGDH) staining of a minority of lymphocytes and generally mononuclear cells with moderately abundant cytoplasm (presumed regulatory T cells and basophils, respectively). **D:** Control lungs have extensive type 1 pneumocyte and alveolar macrophage 15-PGDH immunostaining. Original magnification: $\times 150$ (**A** and **B**); $\times 120$ (**C** and **D**).

Figure 6 Eosinophilic granulomatosis with polyangiitis (EGPA) lung tissue immunostaining is increased relative to controls for 25-hydroxyvitamin D-1 α hydroxylase, mitochondrial (VD3 1A hydroxylase), and tumor necrosis factor superfamily member 14 (TNFSF14, also known as LIGHT), suggesting immunoregulatory and type 2 effects. **A:** EGPA biopsy showing extensive VD3 1A hydroxylase immunoperoxidase granular cytoplasmic staining, resembling mitochondria, in alveolar macrophages. **B:** Control lung showing similar VD3 1A hydroxylase staining of alveolar macrophages, but alveolar macrophages are sparse. **C:** EGPA lung biopsy showing cytoplasmic LIGHT immunostaining, mainly of alveolar macrophages. **D:** Control lung also has alveolar macrophage LIGHT immunostaining, but alveolar macrophages are sparse. Original magnification, $\times 135$ (A–D).

There remains a pressing need for improved EGPA treatment. To find appropriate treatment targets, we looked for self-promoting pathways (ie, positive feedback loops). A self-promoting EGPA pathway was previously found in a

blood leukocyte study of eosinophils, IL-25, and type 2 helper T cells, which correctly predicted that eosinophils are mechanistically relevant in EGPA.¹¹ Our data suggest that multiple potential self-promoting pathways could be activated in the lungs of EGPA patients, such as *CCL18/CCR8*/Tregs,^{35,36,39,40,48} *ALOX15*,^{35,36} IL-10 and IgG4,^{8,41,49,50} IL-13,^{35,37,50} TGF- β and Treg,^{41,43} *CCL18*, and collagen synthesis.^{39,40} These could be important in inducing or sustaining active inflammation and are thus potential treatment targets. We hypothesize that blocking *CCL18* with pirfenidone⁵¹ or blocking IL-13 plus IL-4 could be a useful treatment for EGPA. Other potential therapeutic targets include arachidonate 15-lipoxygenase–related eicosanoids, *CCR8* (the *CCL18* receptor), *SIGLEC8*, *TNFSF14*, and basophils.

Future studies could also explore the diagnostic value of abundant IgG4 plasma cells in distinguishing EGPA from eosinophilic pneumonia and eosinophilic pleuritis, which overlap substantially in both clinical manifestations and pathologic findings. Further studies are needed to examine the effects of IgG4 in EGPA, which could be pathogenic, protective, or both.

Limitations of this study are the number of biopsies available for study, all from patients with early EGPA. All lung biopsies for RNA studies were from females; sex-influenced RNAs could be misleading. This study did not compare EGPA with other eosinophil-rich or vasculitis-associated lung disorders; the EGPA specificity of this immunoregulatory immune response is unknown. Poorly preserved RNA from paraffin-embedded tissue precluded assessment of low abundance RNAs; IL-5, IL-10, and CCL26 are particularly likely to be increased in EGPA and mechanistically relevant. However, the main novel findings (abundant IgG4 plasma cells and immunoregulatory features) were validated by immunostaining. Finally, this is an observational study. Functional studies are needed to assess the effects of these treatments, which will likely be complex and difficult to predict.

Figure 7 Hypothesized mechanism for eosinophilic granulomatosis with polyangiitis (EGPA), showing a combined type 2 and immunoregulatory immune response. Findings novel to this study (in the context of lung EGPA) are underlined. Findings based solely on prior work (that were not studied or not proved significant in our data) are in parentheses. Numbers by the arrows or findings in parentheses refer to references on which they are based. Despite the complexity, this is simplified; some pathways are left out for clarity. See [Discussion](#) for details. CCL, chemokine (C-C motif) ligand; TGF- β , transforming growth factor- β ; Treg, regulatory T cell.

In summary, lung biopsies in EGPA have both type 2 and immunoregulatory features with Tregs and abundant IgG4. C-C motif chemokine 13 and C-C motif chemokine 18 are prominent chemokines. Eicosanoids are plausible intermediate signaling molecules. We propose a mechanism involving these to form a hybrid type 2/immunoregulatory infiltrate resembling the immune response to a tissue-invasive parasite infection.

Supplemental Data

Supplemental material for this article can be found at <http://doi.org/10.1016/j.ajpath.2020.03.005>.

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