Prominent role of IFN-γ in patients with aspirin-exacerbated respiratory disease

John W. Steinke, PhD,[a,b] Lixia Liu, MD,[a] Phillip Huyett, MD,[a] Julie Negri, BS, MBA,[a] Spencer C. Payne, MD,[a,c] and Larry Borish, MD[a,b] *Charlottesville, Va*

Background: Aspirin-exacerbated respiratory disease (AERD) is distinguished from aspirin-tolerant asthma/chronic sinusitis in large part by an exuberant infiltration of eosinophils that are characterized by their overexpression of metabolic pathways that drive the constitutive and aspirin-induced secretion of cysteinyl leukotrienes (CysLTs).

Objective: We defined the inflammatory milieu that in part drives CysLT overproduction and, in particular, the role of IFN-γ in the differentiation of eosinophils.

Methods: Quantitative real-time PCR was performed for T_H1 and T_H2 signature cytokines on tissue from control subjects, patients with chronic hyperplastic eosinophilic sinusitis, and patients with AERD, and their cellular source was determined. The influence of IFN-γ on maturation, differentiation, and functionality of eosinophils derived from hematopoietic stem cells was determined.

Results: Gene expression analysis revealed that tissue from both aspirin-tolerant subjects and patients with AERD display a T_H2 cytokine signature; however, AERD was distinguished from chronic hyperplastic eosinophilic sinusitis by the prominent expression of IFN-γ. Intracellular and immunohistochemical cytokine staining revealed that the major sources of these cytokines were the eosinophils themselves. IFN-γ promoted the maturation of eosinophil progenitors, as measured by increased mRNA and surface expression of CCR3 and sialic acid–binding immunoglobulin-like lectin 8 (Siglec-8). Additionally, IFN-γ increased the expression of genes involved in leukotriene synthesis that led to increased secretion of CysLTs. IFN-γ–matured eosinophil progenitors were also primed, as demonstrated by their enhanced degranulation.

Conclusions: High IFN-γ levels distinguish AERD from aspirin-tolerant asthma and underlie the robust constitutive and aspirin-induced secretion of CysLTs that characterize this disorder. (J Allergy Clin Immunol 2013;132:856-65.)

Key words: *Aspirin-exacerbated respiratory disease, aspirin-tolerant asthma, chronic sinusitis, cytokines, eosinophils, IFN-γ, nasal polyps*

Aspirin-exacerbated respiratory disease (AERD) is a disease of the upper (chronic sinusitis/nasal polyposis) and, usually but not always, the lower (asthma) respiratory tract that is distinguished from aspirin-tolerant asthma/chronic sinusitis by the pathognomonic sensitivity to aspirin and other nonselective inhibitors of COX-1. AERD comprises as many as 20% of cases of adult-onset asthma and up to 30% of cases of adult asthma with nasal polyps (NPs).[1,2] There are several features of AERD that make it clear that this is a unique disorder (endotype) and not just a variant of aspirin-tolerant asthma/chronic sinusitis. Patients with AERD are distinguished from those who are aspirin tolerant through a distinctive natural history, including the almost universal tendency to develop *de novo* in adulthood in patients without a previous history of asthma or allergies.[1,3] The usual absence of sensitization to inhalant allergens in patients with AERD suggests that, when present, allergies are just the coincidental presence of this common disorder. AERD can further be distinguished from aspirin-tolerant disease through the presence of markedly increased circulating and tissue eosinophilia. For example, in published studies from our group[4,5] and others,[6,7] NPs from patients with AERD expressed an approximately 3-fold greater numbers of eosinophils or eosinophil cationic protein (ECP) compared with those from patients with aspirin-tolerant eosinophilic sinus disease, the condition termed chronic hyperplastic eosinophilic sinusitis (CHES). Similarly, when asthma is present, endobronchial biopsy tissue expresses approximately 5-fold greater numbers of eosinophils in patients with AERD.[8] However, the most impressive feature that distinguishes these conditions is that patients with AERD display particularly robust aspirin-induced but also constitutive overproduction of cysteinyl leukotrienes (CysLTs) secondary to the overexpression of the CysLT synthesis pathway, particularly the rate-limiting enzyme leukotriene C_4 synthase.[5,7,9] Reflecting the increased expression of leukotriene receptors, patients with AERD display hyperreactivity to these lipid mediators.[10-12] As a result, AERD is uniquely therapeutically responsive to leukotriene modifiers.[13]

Elucidation of the unique histologic and inflammatory mediators for each asthma/sinusitis endotype is required to further understand and ultimately to better treat these diseases. However, little is understood regarding the immunologic basis for AERD. Eosinophilia categorically reflects a cytokine-dependent process. We speculated that aspirin-tolerant asthma/CHES and AERD could be distinguished based on patterns of cytokine expression. Asthma and nasal polyposis are, in general, characterized by a T_H2 cytokine signature, with prominent expression of IL-4,

From [a]the Asthma and Allergic Disease Center, [b]the Carter Immunology Center, and [c]the Department of Otolaryngology–Head and Neck Surgery, University of Virginia Health System.

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Corresponding author: John W. Steinke, PhD, Asthma and Allergic Disease Center, Box 801355, Charlottesville, VA 22903. E-mail: js3ch@virginia.edu.

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Abbreviations used
AERD: Aspirin-exacerbated respiratory disease
APC: Allophycocyanin
CHES: Chronic hyperplastic eosinophilic sinusitis
CysLT: Cysteinyl leukotriene
ECP: Eosinophil cationic protein
EDN: Eosinophil-derived neurotoxin
EF1α: Elongation factor 1α
Flt3L: Fms-like tyrosine kinase 3 ligand
H&E: Hematoxylin and eosin
LTC_4S: Leukotriene C_4 synthase
NP: Nasal polyp
PE: Phycoerythrin
qPCR: Quantitative real-time PCR
Siglec-8: Sialic acid–binding immunoglobulin-like lectin 8

IL-13, GM-CSF, and especially IL-5 and other features conducive to an eosinophilic infiltrate, including expression of eosinophil-targeting chemokines.[6,14-19] However, these studies have not fully delineated unique patterns of expression of these mediators in patients with aspirin-tolerant asthma/chronic sinusitis and patients with AERD to address the basis for their differentiation. Therefore the current studies were performed to assess the cytokine profile and cellular source of these cytokines in NP tissue from patients with CHES or AERD. In particular, we were able to demonstrate the ability of IFN-γ, which is overexpressed in patients with AERD, to drive the distinctive feature that characterizes that condition, specifically the robust increased expression of metabolic pathways driving CysLT production.

METHODS

Subjects

NP tissue was obtained from patients referred to the University of Virginia Health System for sinus surgery under a protocol approved by the University of Virginia Institutional Review Board. Exclusion criteria included the presence of cystic fibrosis, allergic fungal sinusitis, sinonasal tumor or carcinoma, or immunodeficiency. Control tissue was harvested from the sinus cavities of patients undergoing surgery that required access to their paranasal sinuses for reasons other than chronic sinusitis (eg, orbital decompression, cerebrospinal fluid leak repair, or transsphenoidal pituitary surgery). Immediately after removal, tissue specimens were transported to the research laboratory for processing and analysis. Depending on the quantity of tissue available, specimens were divided and used for subsets of the various experimental procedures outlined below. NPs were obtained from 64 patients, including 43 with CHES and 21 with AERD, and additionally, control tissue was obtained from 12 subjects. Eosinophilic sinusitis was histologically defined, as described below. AERD was diagnosed based on clinical criteria and was defined by the presence of a compelling history involving a hypersensitivity reaction within 2 to 3 hours of ingestion of either aspirin or another nonsteroidal anti-inflammatory drug. Diagnosis of AERD was confirmed in 12 of 21 patients who underwent a subsequent aspirin challenge/desensitization procedure after sinus surgery.

Histologic evaluation for diagnosis of CHES

CHES was distinguished from idiopathic noneosinophilic nasal polyposis by means of histologic evaluation, as we have previously described.[4] Briefly, polyp tissue was fixed in 4% paraformaldehyde, paraffin embedded, sectioned, and hematoxylin and eosin (H&E) stained by the Histology Core Laboratory of the University of Virginia. Sections were examined at ×400 magnification in a blinded fashion, and eosinophils were counted in 10 random sections for each sample, with the final number being the average number of cells per 10 high-power fields. CHES was distinguished by the presence of 5 or more eosinophils/high-power field.

Quantitative real-time PCR

Quantitative real-time PCR (qPCR) was performed on NP tissue for IL-4, IL-5, IL-13, and IFN-γ and on newly differentiated eosinophils for ECP, CysLT1, CysLT2, and $P2Y_{12}$ receptors; sialic acid–binding immunoglobulin-like lectin 8 (Siglec-8); and leukotriene C_4 synthase (LTC_4S). For the NP studies, polyps were minced and digested with Accutase (Innovative Cell Technologies, San Diego, Calif) for 1 hour at 37°C, and the leukocyte-containing fraction was collected by passing the cell suspension through a 70-μm nylon mesh strainer (BD Falcon, Bedford, Mass). Total RNA was extracted with TRI reagent (Sigma, St Louis, Mo). Conversion of mRNA to cDNA was performed with a TaqMan Reverse Transcription kit (Roche, Branchburg, NJ). Briefly, 200 ng of RNA was added to each reaction along with oligo dT primers, 5.5 mmol/L $MgCl_2$, 2 mmol/L dNTPs, RNasin, and reverse transcriptase. Reactions underwent 10 minutes at 25°C, 30 minutes at 48°C, and 5 minutes at 95°C in a Bio-Rad iCycler thermocycler (Bio-Rad, San Jose, Calif). The PCR mix consisted of iQ SYBR Green Supermix (Bio-Rad), cDNA, and 200 μmol/L of each primer. Data were analyzed as the change in the cycle threshold of each cytokine transcript in comparison with either elongation factor 1α (EF1α) or β-actin (as appropriate based on concordance of their cycle threshold values with those of the gene of interest). Primers for β-actin, CysLT1 receptor, CysLT2 receptor, IL-4, IL-5, IL-13, and IFN-γ were as we have previously described,[20,21] and those for EF1α, $P2Y_{12}$, ECP, LTC_4S, and Siglec-8 were purchased from SABiosciences (Frederick, Md).

Flow cytometry of NP-derived leukocytes and intracellular cytokine staining

Flow cytometry with intracellular cytokine staining was performed to identify the cellular source for cytokine expression. Polyps were processed as described above, after which red blood cells were lysed by resuspending the cellular pellet in lysis buffer. Cells were subsequently incubated with 50 μL of mouse IgG (1 mg/mL; Lampire Biologicals, Pipersville, Pa) to block nonspecific binding. Cells were incubated with the panleukocyte marker CD45 (phycoerythrin [PE]–Cy5 conjugate; BD Biosciences, San Jose, Calif) along with either anti-CD4, anti-CD8, or anti-CCR3 antibodies (PE conjugate, BD Biosciences). Intracellular staining was performed by addition of 100 μL of Solution A from the Invitrogen Fix & Perm kit for 10 minutes at room temperature (Invitrogen, Grand Island, NY). The cells were washed, and 100 μL of Solution B was added along with 50 μL of blocking solution and 5 μL of appropriate antibody (allophycocyanin [APC] conjugate; anti–IL-4, anti–IL-5, or anti–IFN-γ; BD Biosciences). Flow cytometry was performed on a Becton Dickinson FACSCalibur machine equipped with CellQuest software, version 5.2 (BD Biosciences). The flow cytometer was restricted to analyses of the live gate by using forward and side scatter characteristics, and data were subsequently analyzed for the $CD45^+$ population. Data were analyzed with FlowJo software, version 6.4.1 (Tree Star, Ashland, Ore).

ImageStream cytometry

Flow cytometry with the ImageStream (Amnis, Seattle, Wash) cytometer combines elements of flow cytometry with confocal microscopy. As with conventional flow cytometry, cells are stained with different fluorophores and passed through the machine in a single-cell suspension. Unlike conventional flow cytometry, a picture of each cell is taken as it passes by the detector. In addition to staining for cell-surface markers, a nuclear dye is added, which allowed us, through observer visualization, to confirm the cellular identification suggested by surface marker staining. This is of particular use for surface markers that are expressed on multiple cell types. Two-color surface staining for CD45 and CCR3 was performed as described for standard flow cytometry, after which the cells were permeabilized and the nuclei were stained with 4′,6-diamidino-2-phenylindole (Sigma).

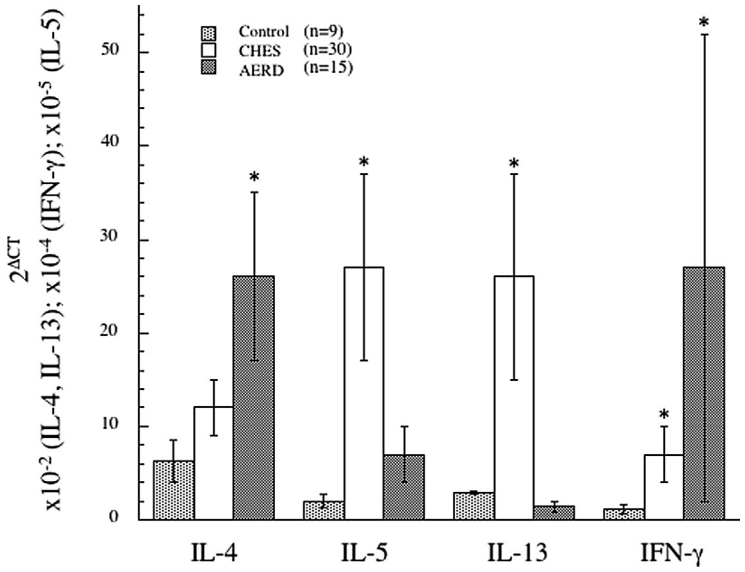

FIG 1. T_H1/T_H2 cytokine signature in sinus tissue from control subjects, patients with CHES, and patients with AERD, as determined by using qPCR. Tissue samples were homogenized after surgical removal and RNA isolated. Transcript levels for IL-4, IL-5, IL-13, and IFN-γ were quantified by using PCR with SYBR Green detection. Data (mean ± SEM) reflect relative expression of each gene in comparison with β-actin ($2^{\Delta CT}$). Control samples (n = 9) are depicted in *black bars*, CHES samples (n = 30) are depicted in *gray bars*, and AERD samples (n = 15) are depicted in *white bars*. **P* < .05 compared with control samples.

Immunohistochemical staining

Samples were deparaffinized and rehydrated. Antigen retrieval was performed by heating sections for 15 minutes at 37°C with proteinase K (0.6 U/mL) in TE buffer with 0.5% triton X-100. Slides were washed in Tris-buffered saline and blocked with 1% bovine albumin and 10% mouse serum in Tris-buffered saline for 2 hours at room temperature. Specific staining for IFN-γ was performed with an APC-conjugated mouse anti-human IFN-γ antibody (1:250; BD PharMingen, San Jose, Calif) for 2 hours and compared with results obtained with an APC-labeled isotype control rabbit IgG antibody (BD PharMingen). Nuclei were stained with 100 ng/mL 4′,6-diamidino-2-phenylindole (Sigma) for 30 minutes. Samples were aqueous mounted with VectaMount AQ (Vector Laboratories, Burlington, Calif) and analyzed with a Zeiss AxioImager Z2 equipped with Apotome for optical sectioning (Zeiss, Thornwood, NY).

Eosinophil differentiation

We investigated the ability of cytokines associated with aspirin-tolerant disease and AERD to modulate expression of LTC_4S by eosinophils. Eosinophils were enriched from peripheral blood by means of Ficoll-Hypaque (Sigma) density centrifugation, followed by dextran sedimentation and hypotonic lysis. Eosinophils were enriched from granulocytes by using negative magnetic affinity column purification ($CD16^-$ cells; Miltenyi Biotec, Auburn, Calif) and were greater than 99% pure, as measured by using flow cytometry.[20] Eosinophils were incubated with IL-3, IL-4, IL-5, GM-CSF, and IFN-γ alone or in various combinations (all cytokines used at 10 ng/mL, BD Biosciences). At the end of varying time periods (2 hours to 7 days), mRNA was extracted, and LTC_4S transcripts were quantified, as previously described.

Eosinophil progenitor activation

PBMCs were isolated through Ficoll-Hypaque (Sigma) density centrifugation from blood obtained from 24 healthy volunteers; again, not all studies could be performed on all subjects. $CD34^+$ cells were enriched from PBMCs by using positive magnetic affinity column purification ($CD34^+$ cells, Miltenyi Biotec), and eosinophil progenitors were derived with the technique of Hudson et al[22] by culturing purified $CD34^+$ cells in complete medium (RPMI 1640 and 10% FBS) supplemented with stem cell factor (25 ng/mL, BD Biosciences), thymopoietin (25 ng/mL; R&D Systems, Minneapolis, Minn), Fms-like tyrosine kinase 3 ligand (Flt3L; 25 ng/mL, BD Biosciences), IL-3 (25 ng/mL, BD Biosciences), and IL-5 (25 ng/mL, BD Biosciences) with or without IFN-γ (20 ng/mL, BD Bioscience) for 3 days and then cultured for an additional 3 weeks with just IL-3 and IL-5 (again with or without IFN-γ). Cells were washed, and fresh media and cytokines were applied weekly.

Influence of IFN-γ on generation of mature eosinophils

Eosinophils newly differentiated from progenitors with or without IFN-γ were evaluated for eosinophil maturation by means of flow cytometry and histochemistry. Samples were cytospun and stained (H&E). Flow cytometry was performed for expression of the markers of mature eosinophils PE-conjugated anti-CCR3 and Siglec-8 and for mature basophils by using APC-conjugated CD203c (Miltenyi Biotec) and PE-conjugated FcεRI (eBioscience, San Diego, Calif) along with relevant control antibodies. Anti–Siglec-8 was a gift from Dr Bruce Bochner and was conjugated with Alexa Fluor 488 (Invitrogen). qPCR was performed as described above for ECP, Siglec-8, CysLT1, CysLT2, and $P2Y_{12}$ receptors and LTC_4S.

Functional behavior of newly differentiated eosinophils

Eosinophils differentiated with or without IFN-γ were either left in a resting state or stimulated with the combination of calcium ionophore A23187 (2 μg/mL, Sigma) and phorbol 12-myristate 13-acetate (100 ng/mL; Thermo Fisher Scientific) for 15 minutes. Supernatants were collected and assayed by means of an EIA for eosinophil-derived neurotoxin (EDN; MBL International, Woburn, Mass) and CysLTs (Assay Designs, Ann Arbor, Mich), according to the manufacturer's directions.

Statistical analyses

Data were contrasted between paired samples by using independent *t* tests with or without equal variance, where appropriate, with SPSS 17.0 software (SPSS, Cary, NC).

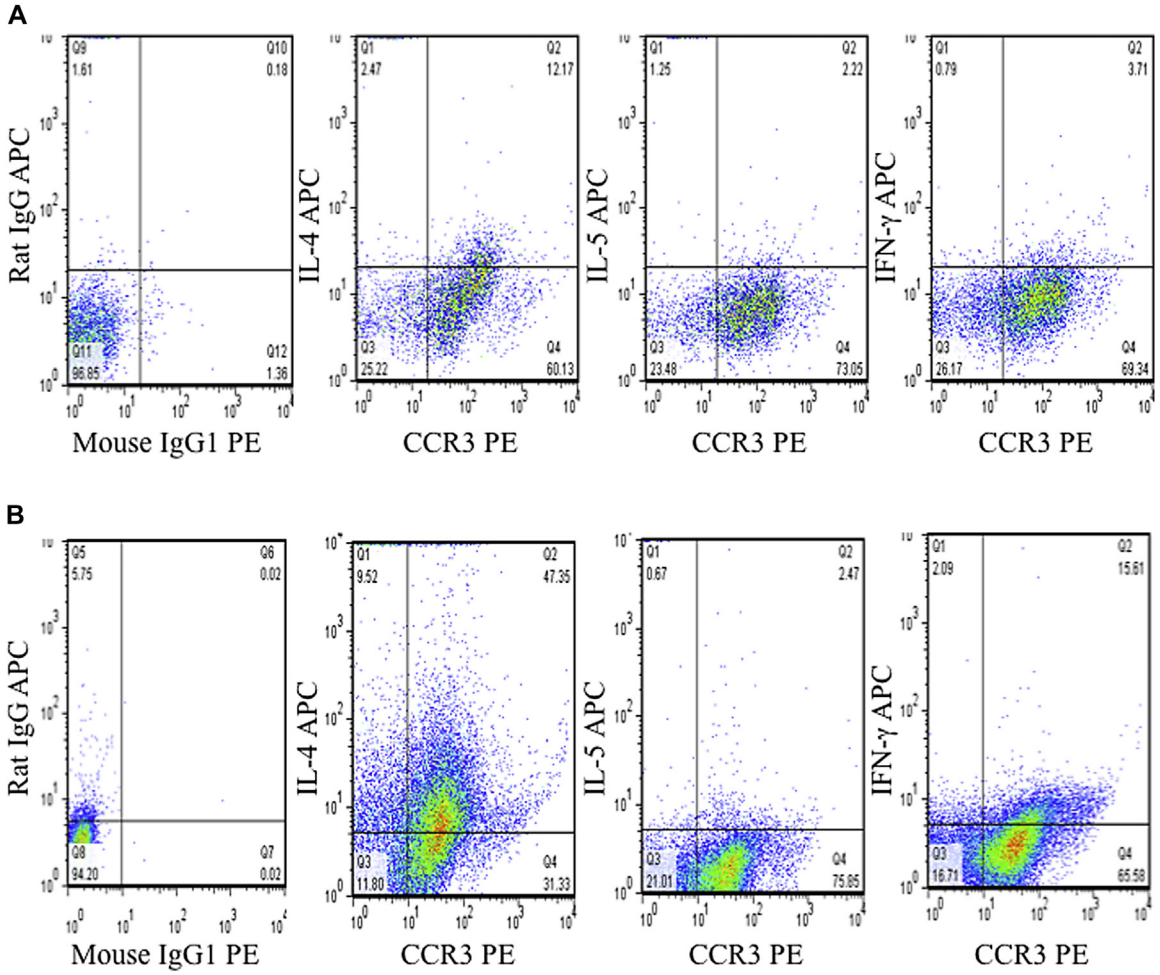

FIG 2. Intracellular cytokine staining of CCR3$^+$ eosinophils in patients with CHES and those with AERD. NPs were digested, and cells were separated from collagenous support material. Cells were stained with appropriate markers and analyzed by means of flow cytometry. Initial gating was performed to include only CD45$^+$ cells. This was followed by extracellular staining for CCR3, CD4, CD8, or CD19 and intracellular staining for cytokines. **A,** Representative staining of CCR3$^+$ NP cells from patients with CHES, showing intracellular levels of IL-4, IL-5, and IFN-γ. **B,** Representative staining of CCR3$^+$ NP cells from patients with AERD, showing intracellular levels of IL-4, IL-5, and IFN-γ.

RESULTS

qPCR cytokine profile of CHES and AERD

Our initial interest was to identify unique features of the cytokine profile that might contribute to the distinct phenotypes of aspirin-tolerant chronic sinusitis (CHES) and AERD. NPs from patients with CHES (n = 30) or AERD (n = 15) and control sinus tissue (n = 9) were used for the qPCR component of these studies. Quantitative PCR indicated that CHES, as previously reported,[6,16,23] was characterized as having a prominent T_H2 cytokine profile, which is indicated by increased expression of IL-4, IL-5, and IL-13 compared with control tissue (Fig 1), with the increase in IL-5 and IL-13 expression achieving statistical significance. In contrast, polyps from patients with AERD had significantly increased levels of IL-4 mRNA ($P < .05$). However, the more striking feature was their robust expression of IFN-γ ($P < .05$), a cytokine traditionally more ascribed to a T_H1 signature (Fig 1). IL-5 levels were also increased in patients with AERD when compared with those seen in control tissue ($P < .05$), although they were not as high as in patients with CHES, and surprisingly, IL-13 levels were actually decreased in patients with AERD compared with those seen in control subjects.

Intracellular cytokine staining of NP leukocytes

To confirm the posttranslational expression of these cytokines and to define their cellular source, we performed intracellular cytokine staining for IL-4, IL-5, and IFN-γ. As a percentage, very few T cells (CD4$^+$ or CD8$^+$) were observed within the NP samples (data not shown), and, consistent with numerous reports regarding the histologic appearance of polyps,[4,7,8] most of the CD45$^+$ leukocytes within polyps from both patients with CHES and those with AERD were eosinophils (CCR3$^+$ cells, Fig 2). Within the constraints imposed by the sensitivity of intracellular cytokine staining, only modest cytokine staining was observed among the CD45$^+$CCR3$^-$ cells. In contrast, the majority of IL-4 and IFN-γ staining was observed in eosinophils, reflecting, in contrast to lymphocytes, the constitutive intracellular accumulation of these cytokines in eosinophil granules (Fig 2).[24] Also

TABLE I. Percentage of eosinophil intracellular cytokine expression

	Patients with CHES (n = 17)	Patients with AERD (n = 5)
IL-4	30.6 ± 5.9	33.1 ± 10.4
IFN-γ	18.1 ± 3.4	27.5 ± 10.4

consistent with the modest production of IL-5 by eosinophils, intracellular cytokine staining for this cytokine was not observed. In patients with CHES and those with AERD, IL-4 expression was similar and was detected in concentrations significantly greater than background staining in 30.6% ± 5.9% and 33.1% ± 10.4% of the eosinophils, respectively (Table I). In contrast, in patients with AERD, the percentage of eosinophils expressing IFN-γ was nearly twice that seen in patients with CHES (27.5% ± 10.4% vs 18.1% ± 3.4%).

ImageStream flow cytometry and immunofluorescent staining of NPs

Because CCR3 can be expressed on cells other than eosinophils, including lymphocytes and basophils, we performed ImageStream flow cytometry of the $CD45^+CCR3^+$ cells to examine their appearance. Consistent with the recognition that eosinophils comprise the overwhelmingly predominant leukocyte in both patients with CHES and those with AERD, greater than 99% of the $CD45^+CCR3^+$ population were eosinophils, as determined by their bilobed nucleus and robust granularity (see Fig E1 in this article's Online Repository at www.jacionline.org). Immunofluorescence staining on paraffin-embedded sections from patients with AERD and those with CHES was performed to verify that the IFN-γ staining observed during flow cytometry was specific to the eosinophils. Greater than 90% of eosinophils from the patients with AERD had positive results for IFN-γ, with intense staining observed in most cells (Fig 3, *A*). In contrast, in the CHES samples less than 50% of eosinophils did not stain positively for IFN-γ or were weakly positive (Fig 3, *B*). Additionally, in the CHES samples mononuclear cells could also be detected that were positive for IFN-γ, which was not seen in the patients with AERD.

Influence of IFN-γ on eosinophil differentiation

We next began to investigate the mechanism of how IFN-γ could contribute to the unique phenotypic features of AERD. As noted, AERD is characterized by strikingly increased eosinophil numbers in the NPs, and these eosinophils are characterized by their overexpression of LTC_4S.[4,7,8] Initially, we investigated the ability of IL-1, TNF-α, IL-3, IL-4, IL-5, GM-CSF, and IFN-γ to induce LTC_4S transcripts in eosinophils. Perhaps reflecting their terminal differentiation status, no influence of any of these cytokines, either alone or in various combinations, was observed (data not shown).

Influence of IFN-γ on eosinophilopoiesis

We therefore hypothesized that IFN-γ would primarily act by contributing to the development and differentiation of eosinophil progenitors. Eosinophils were derived by culturing $CD34^+$-enriched cells with Flt3L, thymopoietin, stem cell factor, IL-3, and IL-5 with or without the additional presence of IFN-γ for 3 days, followed by just IL-3 and IL-5 (with or without IFN-γ) for an additional 3 weeks. Similar to previous reports,[25] we observed a decrease in total cell numbers when the $CD34^+$ cells were cultured in the presence of IFN-γ ($1.33 \pm 0.21 \times 10^6$ without IFN-γ compared with $0.56 \pm 0.17 \times 10^6$ with IFN-γ, mean ± SEM of 10 experiments; $P < .01$). Although overall cell numbers were decreased, this reflected the enhanced terminal differentiation of eosinophils in the additional presence of IFN-γ. A portion of the cells were removed and examined by using H&E staining. Along with a preponderance of apoptotic bodies, all of the viable cells had the appearance of granulocytes at varying stages of maturation, including fully mature cells with readily apparent bilobed nuclei (see Fig E2 in this article's Online Repository at www.jacionline.org). We next examined these $CD34^+$-derived cells using flow cytometry for markers of mature eosinophils, specifically for their surface expression of CCR3 and Siglec-8, with the flow cytometer gated exclusively on the viable population (representative data are displayed in Fig 4, *A* and *B*, and summarized in Fig 4, *C*). Dual $CCR3^+$Siglec-8^+ expression was observed on a significantly greater percentage of viable cells grown in the presence of IFN-γ (17.73% ± 3.94%) compared with those without IFN-γ (3.06% ± 1.31%, mean ± SEM of 5 experiments; $P < .03$). Flow cytometry was used to examine surface expression of CD203c and FcεRI on $CD34^+$ cells differentiated in the presence or absence of IFN-γ to exclude the possibility that the dual $CCR3^+$Siglec-8^+ cells were basophils. Less than 3% of the cells stained dual positive for both markers, indicating an absence of basophils (see Fig E3 in this article's Online Repository at www.jacionline.org).

Influence of IFN-γ on eosinophil-associated gene expression

Expression of mRNA for the eosinophil maturation marker Siglec-8 was significantly increased (2.88-fold, $P < .05$) when progenitors were coincubated with IFN-γ (Table II). In contrast, no further increase in ECP gene expression was observed in cells exposed to IFN-γ, reflecting that more mature cells decrease their expression of transcripts for these preformed mediators.[22,26] As noted, the distinctive feature of AERD in comparison with aspirin-tolerant disease is the marked overexpression of CysLTs and their receptors.[5,7,9-12] Our previous work indicated that IFN-γ increases CysLT receptor expression on circulating eosinophils.[20] On the basis of those results, we measured gene expression for leukotriene receptors (CysLT1, CysLT2, and $P2Y_{12}$) and for LTC_4S in eosinophil progenitors stimulated with IFN-γ. As with mature eosinophils, IFN-γ resulted in a modest 1.46-fold increase in CysLT1 receptor expression ($P < .05$). We also observed a slight increase in the newly described CysLT receptor $P2Y_{12}$ (2.11-fold). Under these conditions, no further increase was observed for the CysLT2 receptor. Most importantly, however, LTC_4S expression was significantly increased after IFN-γ stimulation (2.38-fold, $P < .05$). In contrast, no further increase in LTC_4S levels were observed in the additional presence of IL-4 (data not shown).

Influence of IFN-γ on eosinophil activation

Finally, we investigated the influence of IFN-γ on the differentiation and priming of viable eosinophils because of their

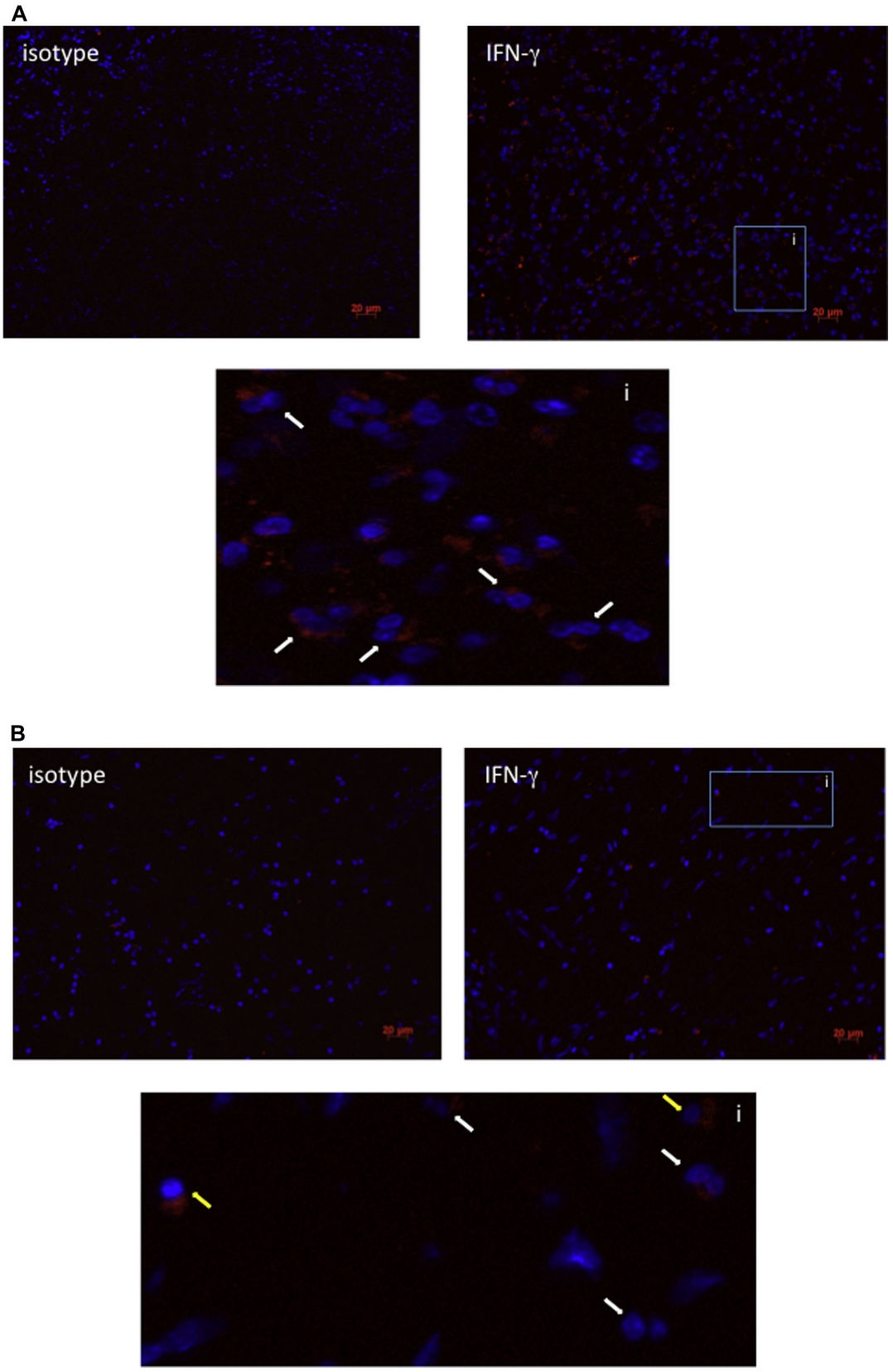

FIG 3. Immunofluorescence for IFN-γ in polyps from patients with AERD and those with CHES. Sections from paraffin-embedded NPs from patients with AERD **(A)** and patients with CHES **(B)** were stained with an APC-labeled antibody directed against IFN-γ and counterstained with 4′,6-diamidino-2-phenylindole to display nuclei. Each grouping displays an isotype control, specific IFN-γ staining, and an enlarged region to show specific cells. In these figures *red* represents IFN-γ staining, and *blue* represents nuclear staining. *White arrows* show eosinophils, and *yellow arrows* show mononuclear cells. *i*, Insert.

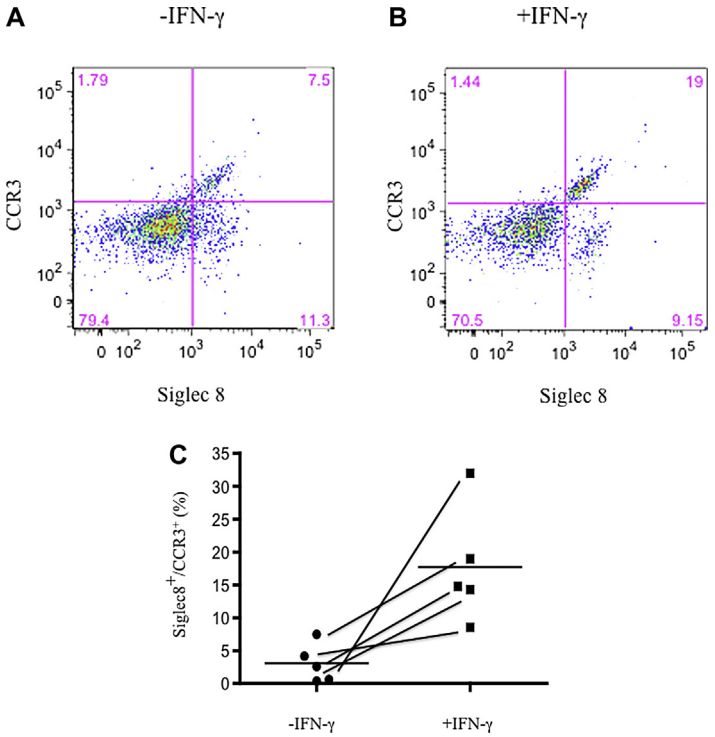

FIG 4. IFN-γ potentiation of expression of markers for mature eosinophils. $CD34^+$-enriched hematopoietic stem cells were cultured for 3 days with stem cell factor, thymopoietin, Flt3L, IL-3, and IL-5, after which they were cultured for 3 additional weeks with just IL-3 and IL-5 with or without the additional presence of IFN-γ. Media were changed weekly. Maturation status was assessed by means of flow cytometry for CCR3 and Siglec-8. **A,** Cells matured with IL-5 and IL-3 only. **B,** Cells matured in the additional presence of IFN-γ. **C,** Summary data for all 5 samples.

TABLE II. Influence of IFN-γ on gene expression in newly differentiated eosinophils

	Siglec-8	ECP	CysLT1R	CysLT2R	$P2Y_{12}$	LTC_4S
Without IFN-γ	0.0063 ± 0.0017	0.109 ± 0.030	0.052 ± 0.024	0.00059 ± 0.00031	0.0068 ± 0.0021	0.033 ± 0.014
With IFN-γ	0.018 ± 0.006	0.093 ± 0.018	0.076 ± 0.027	0.00053 ± 0.00012	0.0144 ± 0.0042	0.080 ± 0.021
Fold increase	2.88*	0.85	1.46*	0.90	2.11*	2.38*

qPCR data reflect relative expression in comparison with the housekeeping gene EF1α, with data reflecting the $2^{\Delta\Delta CT}$ value (n = 12). Fold increase represents that induced by IFN-γ in comparison with IL-5 and IL-3 alone.
*$P < .05$ compared with without IFN-γ.

capacity to degranulate and secrete CysLTs (the means ± SEMs of 10 and 7 results, respectively, are displayed in Fig 5). The presence of IFN-γ induced a significantly enhanced capacity to degranulate (EDN; $P < .001$) and to secrete CysLTs ($P = .02$).

DISCUSSION

Many features of aspirin-tolerant asthma and AERD lead to the conclusion that these are distinct disorders and not different presentations of a spectrum of the same underlying process. In addition to the eponymous feature of aspirin intolerance, this includes their distinct clinical presentations and natural histories. Aspirin-tolerant asthma usually presents in childhood and adolescence in allergic subjects. As noted, AERD most commonly presents in the third or fourth decades of life, and the frequency with which it develops in nonallergic subjects suggests that, when present, allergies are merely the coincidental presence of this common disorder.[2,3] Their natural histories are distinguished by the refractoriness of the nasal polyposis in patients with AERD to treatment[27] and the greater tendency of AERD to lead to irreversible loss of lung function.[28,29] These disorders are especially distinguished by their inflammatory component. One of the striking features of AERD is the virtually diagnostic upregulation of LTC_4S in both the lungs and NPs,[5,7,8] largely within eosinophils, and this upregulation leads to a constitutive overproduction of CysLTs and promotes the surge in CysLT production that occurs after ingestion of aspirin.[9] Patients with AERD also display overresponsiveness to CysLTs,[10] reflecting the overexpression of CysLT receptors,[11,12] including perhaps specific leukotriene E_4 receptors.[30-32] Previously, we found that even though both CHES and AERD are characterized by eosinophilic infiltration, as with lung tissue,[8] the numbers of eosinophils infiltrating the NPs of patients with AERD were dramatically higher.[4] Eosinophilia is a cytokine-dependent process, and many facets of eosinophil function are further regulated by cytokines. This prompted us to query whether differences in cytokine expression might underlie these distinct presentations.

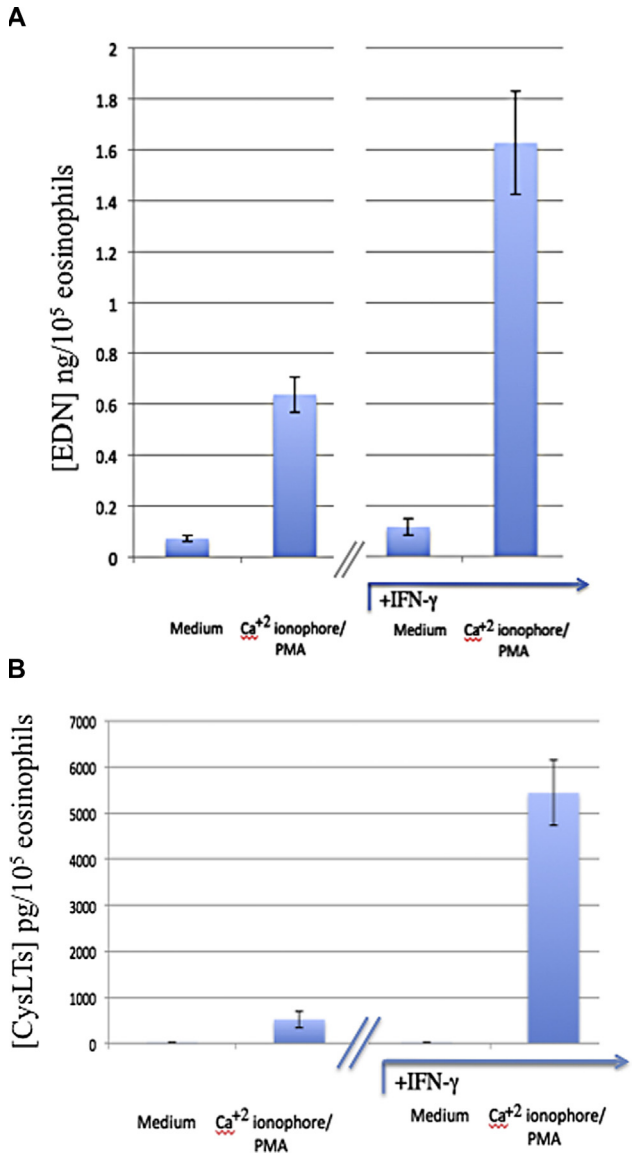

FIG 5. Enhanced secretion by eosinophils differentiated in the additional presence of IFN-γ. Eosinophils matured from $CD34^+$ progenitors, as described, were activated with or without calcium ionophore and phorbol 12-myristate 13-acetate for 20 minutes. Supernatants were collected and EDN **(A)** and CysLT **(B)** levels were quantified.

Not surprisingly, given the prominent eosinophilia, the presence of a T_H2 cytokine signature in NPs has previously been well established.[6,14-19] However, these studies have not fully addressed the distinct signature of AERD compared with CHES. Initially, we screened for cytokine expression using qPCR of NP (or control sinus) tissue. We confirmed the variable expression of T_H2 cytokines in patients with both diseases (Fig 1) and also demonstrated that eosinophils themselves were an important source for these cytokines (Figs 2 and 3 and Table I), although other cells, such as innate lymphoid type 2 cells, likely contribute to the T_H2 cytokine expression. Eosinophils can secrete more than 30 cytokines and chemokines when stimulated,[33] and unlike other lymphocytes, in which these factors are synthesized and immediately secreted, eosinophils store preformed cytokines that can be rapidly released from granules.[24,34] In interpreting these qPCR results, it is important to note the limitations posed by performing these studies on heterogeneous tissue and that these results might thereby be skewed by the distinct cellular makeup of CHES and AERD polyps. For example, most of the AERD mRNA would have been derived from eosinophils, whereas CHES represents a more diffuse distribution of leukocytes, and as a percentage, a greater contribution of the mRNA would have come from stromal tissue. Within these constraints, the more robust expression of IL-4 in patients with AERD, in comparison especially with IL-5 expression, likely represents the physiology of eosinophil cytokine transcript expression and specifically that IL-5 is not an important eosinophil-derived cytokine. It is also worth considering that the high IL-5/IL-13 and low IL-4 signature in patients with CHES is consistent with the pattern of cytokines observed in innate lymphoid type 2 cells, an increasingly recognized source of these cytokines in patients with allergic disorders and a cell type that is specifically expressed in NPs.[35,36]

However, the most striking feature of these studies was the increased expression of IFN-γ mRNA and protein levels in patients with AERD (Figs 1 and 3), which is consistent with recognition that this cytokine can be expressed by eosinophils in substantial amounts.[24,33,37] One previous study investigated cytokine expression in patients with "nonallergic" sinusitis, a cohort within which patients with AERD are likely to be overrepresented, and reported similarly high levels of IFN-γ expression.[23] Van Zele et al[38] previously described high IFN-γ levels in tissue from patients with chronic sinusitis but not NPs, although their study was not designed to investigate patients with AERD. We did not identify T lymphocytes as an important source for these cytokines. This does not rule out a role for T cell–derived cytokines in patients with these disorders because it remains quite plausible that the relevant T cells orchestrating the inflammation within NPs might reside in sinonasal-associated lymphatic tissue or other lymphoid structures. Interestingly, the T_H1/T_H2 balance we observed in NP eosinophils in patients with AERD is consistent with a prior report that detected higher levels of IFN-γ in circulating $CD8^+$ cells compared with those seen in aspirin-tolerant control subjects,[39] a pattern that decreased after aspirin desensitization.

IFN-γ, despite its primary association with the T_H1 cytokine signature, is present and contributes to the severity of asthma and other allergic inflammatory disorders.[37] Given the capacity of IFN-γ to block IgE class-switch recombination, it is possible that this coexpression of IFN-γ might contribute to the absence of allergy (despite the shared expression of IL-4).

We speculated that the primary function of IFN-γ would be to contribute to the distinct inflammatory features of AERD, specifically the hypereosinophilia and overexpression of CysLTs and their receptors.[10-12,40] It is known that an IFN-γ–induced transcription factor can be critical for regulating the development of eosinophils.[41] Eosinophil and basophil progenitors or colony-forming units are bone marrow–derived mononuclear cells that express CD34 and IL-5 receptors that are capable of responding to appropriate cytokine signals to differentiate into mature eosinophils and basophils.[42] Given that eosinophil and basophil progenitors or colony-forming units are increased in the blood and bone marrow of asthmatic patients and have also been identified in NP tissue,[43,44] we hypothesized that IFN-γ would synergize with IL-5 to enhance eosinophil maturation in patients with AERD. As previously reported,[25] we observed a decrease in the overall cell numbers when the $CD34^+$ cells were cultured in the

presence of IFN-γ. Despite the lower cell numbers, our studies demonstrate that the addition of IFN-γ significantly accelerated the terminal maturation of eosinophils, as evinced by their surface expression of CCR3 and Siglec-8 (Figs 4 and 5) and increased their mRNA expression of Siglec-8 (Table II). Consistent with a role in driving the CysLT overresponsiveness observed in patients with AERD[45] and consistent with our previous studies performed with mature eosinophils,[20] we also demonstrated modest increases in expression of transcripts for CysLT1 and a putative third CysLT receptor, $P2Y_{12}$ (Table II).[30,31]

Overexpression of LTC_4S underlies the generation of CysLTs in patients with AERD, and this has been ascribed to the eosinophil.[8] To date, only 1 study has shown modulation of LTC_4S gene expression and that was by IL-4 in mast cells.[46] No basis for the increase in eosinophil LTC_4S expression in patients with AERD has been demonstrated. Our studies did not demonstrate an ability of numerous innate or adaptive cytokines, including IL-3, IL-4, IL-5, GM-CSF, IL-1, TNF-α, or even IFN-γ, to modulate LTC_4S expression on circulating eosinophils, which is perhaps consistent with their terminal differentiation status (data not shown). In contrast, our data do demonstrate a statistically significant increase in IFN-γ–driven LTC_4S expression in eosinophil progenitors (Table II). Most importantly, this translated into increased capacity of these newly differentiated eosinophils to secrete CysLTs and release EDN (Fig 5). In contrast to its effect on mast cells,[46] this capacity did not extend to IL-4 (data not shown). Thus, at present, IFN-γ uniquely carries the capacity to drive this disease-defining characteristic of AERD.

In summary, our studies demonstrate that although both aspirin-tolerant disease (CHES) and AERD have components of T_H2 disorders, AERD is distinguished by the prominent expression of IFN-γ. Interestingly, much of the cytokine expression, including IFN-γ, was derived from the eosinophils themselves. Eosinophil-derived IFN-γ synergizes with IL-3 and IL-5 to drive maturation of eosinophils, and these eosinophils display an upregulated capacity to not only respond to CysLTs but also, comprising the most important diagnostic feature of AERD, to secrete CysLTs.

We thank Dr Bruce Bochner for kindly providing us with the Siglec-8 antibody we used for flow cytometry and Dr Steven Ackerman for helpful conversations on differentiating eosinophils from $CD34^+$ progenitor cells.

Clinical implications: High IFN-γ levels in patients with AERD might explain the absence of IgE-mediated allergies associated with the disease, and increased CysLT synthesis driven by IFN-γ underlies why leukotriene modifiers are partially effective in the treatment of this disease.

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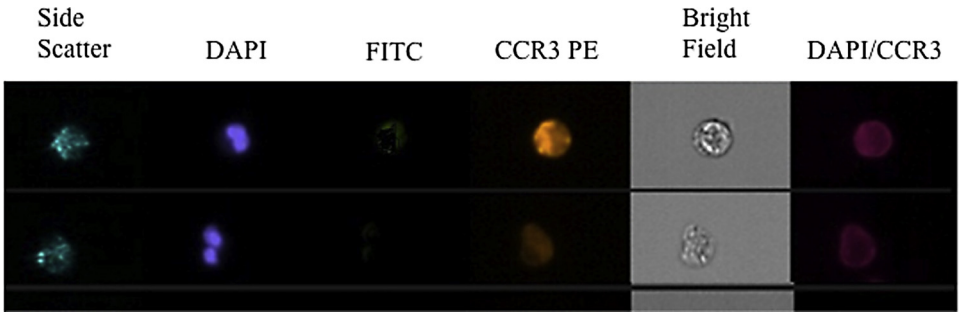

FIG E1. ImageStream flow cytometry of $CD45^{+}CCR3^{+}$ leukocytes in patients with CHES, patients with AERD, or both. We used ImageStream flow cytometry, as described in the text, to confirm that the $CCR3^{+}$ cells were eosinophils. *DAPI*, 4′,6-Diamidino-2-phenylindole; *FITC*, fluorescence isothiocyanate.

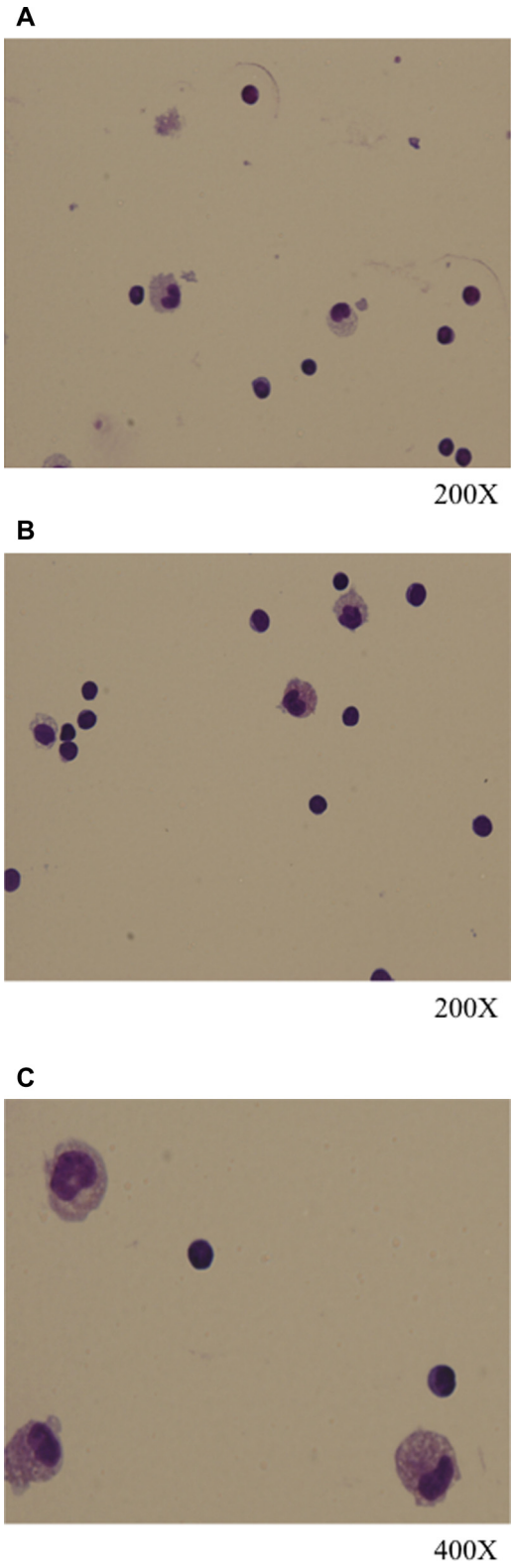

FIG E2. IFN-γ potentiation of eosinophilopoiesis. $CD34^{+}$-enriched hematopoietic stem cells were cultured for 3 days with stem cell factor, thymopoietin, Flt3L, IL-3, and IL-5, after which they were cultured for 3 additional weeks with just IL-3 and IL-5 with or without the additional presence of IFN-γ. Media were changed weekly. After 3 weeks, aliquots were cytospun and stained (H&E). **A,** Cells matured with only IL-5 and IL-3 (×200 magnification). **B,** Cells matured in the additional presence of IFN-γ (×200 magnification). **C,** Cells matured in the additional presence of IFN-γ (×400 magnification). Viable cells with bilobed nuclei and granules are demonstrated along with numerous apoptotic bodies. All viable cells have the appearance of granulocytes.

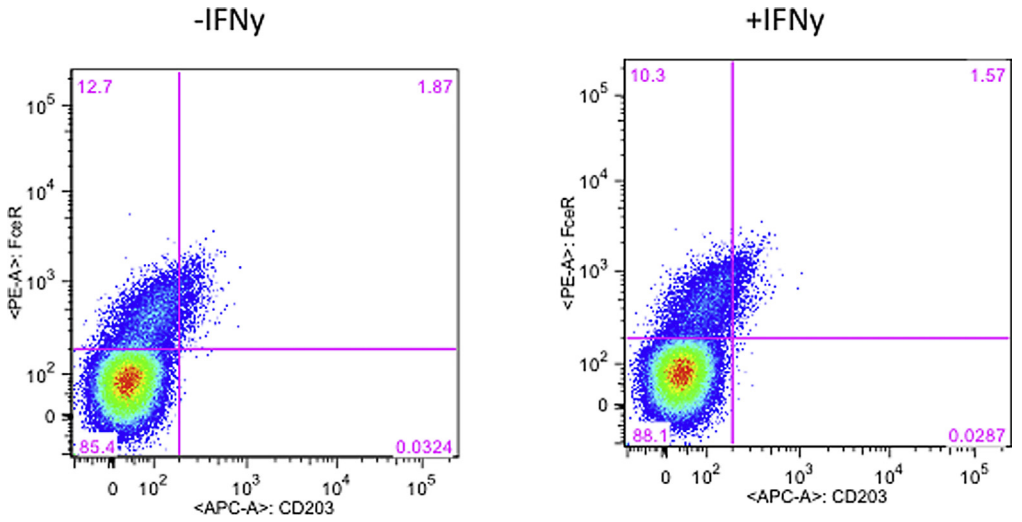

FIG E3. Absence of basophil markers on $CD34^+$-derived eosinophils. $CD34^+$-enriched hematopoietic stem cells were cultured for 3 days with stem cell factor, thymopoietin, Flt3L, IL-3, and IL-5, after which they were cultured for 3 additional weeks with just IL-3 and IL-5 with or without the additional presence of IFN-γ. Media were changed weekly. After 3 weeks, aliquots were collected and analyzed by means of flow cytometry for expression of CD203c and FcεRI.