

# Human basophils express amphiregulin in response to T cell-derived IL-3

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**Background:** Amphiregulin, a member of the epidermal growth factor family, is expressed by activated mouse T<sub>H</sub>2 cells. Amphiregulin produced by mouse hematopoietic cells contributes to the elimination of a nematode infection by a type 2 effector response.

**Objective:** To identify the human peripheral blood cell population expressing amphiregulin.

**Methods:** Amphiregulin-expressing cells were identified by flow cytometry of cell surface markers and histologic staining.

**Histamine and amphiregulin in supernatants were measured by enzyme immunoassay. Quantitative real-time PCR was used to measure mRNA expression.**

**Results:** Stimulation of human PBMCs by anti-CD3 + anti-CD28 antibodies induced expression of amphiregulin mRNA and protein by a non-T-cell population. The amphiregulin-producing cells were basophils, as judged by morphology and expression of CD203c and CD123 (IL-3 receptor  $\alpha$  chain). Activated mouse basophils also produced amphiregulin. Amphiregulin expression by basophils in response to anti-TCR stimulation required IL-3 produced by T cells, and IL-3 alone induced high levels of amphiregulin expression by purified basophils. Amphiregulin was expressed at much higher levels when human basophils were stimulated by IL-3 than by IgE cross-linking, whereas the opposite was true for IL-4 expression and histamine release. Heparin-binding epidermal growth factor–like growth factor was also expressed by IL-3–stimulated human basophils. PBMCs from human subjects with asthma contained significantly higher numbers of basophils able to produce amphiregulin compared with controls with or without allergy.

**Conclusion:** IL-3 can induce basophils to express high levels of amphiregulin, which may contribute to tissue remodeling during type 2 immune responses such as asthma. (J Allergy Clin Immunol 2010;■■■:■■■-■■■.)

**Key words:** Basophils, amphiregulin, IL-3

## Abbreviations used

ECP: Eosinophil cationic protein

EGF: Epidermal growth factor

Gr-1: Granulocyte-differentiation antigen-1

HB-EGF: Heparin-binding epidermal growth factor–like growth factor

MFI: Mean fluorescent intensity

rh: Recombinant human

qPCR: Quantitative real-time PCR

TCR: T-cell receptor

Amphiregulin is a member of the epidermal growth factor (EGF) family, which includes EGF, amphiregulin, TGF- $\alpha$ , heparin-binding EGF-like growth factor (HB-EGF), betacellulin, and epiregulin.<sup>1,2</sup> These ligands share a conserved EGF-like motif (3-disulfide loop structure), and all are initially expressed as transmembrane precursor proteins that are released from the cell surface by proteolytic cleavage.<sup>2,3</sup> EGF receptors also make up a multigene family of integral membrane tyrosine kinases that are activated on binding of the ligands. Amphiregulin (and EGF) bind to the homodimer EGF receptor (ErbB1/ErbB1) or heterodimer ErbB1/ErbB2.<sup>4,5</sup>

Amphiregulin is widely expressed in human tissues.<sup>6</sup> EGF family members, including amphiregulin, induce proliferation and differentiation of normal and malignant epithelial cells, fibroblasts, and keratinocytes.<sup>1,7</sup> This is potentially important for embryogenesis, tissue remodeling, and repair.<sup>2</sup> Although amphiregulin-deficient mice show a defect in ductal elongation during mammary gland development in puberty,<sup>8</sup> these mice can still nurse young effectively. Other tissue remodeling functions appear to be normal in amphiregulin<sup>-/-</sup> mice, possibly because these functions are mostly redundant with other EGF family members.<sup>2,8</sup>

We previously reported that amphiregulin is expressed by T-cell receptor (TCR)–activated mouse CD4 T cells,<sup>9</sup> particularly the T<sub>H</sub>2 cells that are involved in allergic responses. Amphiregulin-deficient mice<sup>8</sup> showed slower kinetics of clearance of the helminth parasite, *Trichuris muris*, that is cleared most effectively by T<sub>H</sub>2-biased responses. Lack of amphiregulin was associated with reduction of the hyperproliferation of gut villus epithelium cells<sup>9</sup> that has been implicated in the removal of intestinal worms.<sup>10</sup> Hemopoietic cells produced the amphiregulin needed for this response, because reconstitution of irradiated amphiregulin<sup>-/-</sup> mice with wild-type bone marrow cells restored normal worm elimination kinetics.<sup>9</sup>

Human mast cells also produce amphiregulin on stimulation by IgE cross-linking, or constitutively in tissue-resident mast cells in patients with asthma.<sup>11,12</sup> Human eosinophils express amphiregulin

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in response to GM-CSF and IL-5 stimulation.<sup>13</sup> Therefore, amphiregulin is produced in the immune system in at least 3 cell types (mouse T<sub>H</sub>2 cells, human mast cells, and human eosinophils) that are strongly involved in allergic responses.

In allergic diseases including asthma and atopic dermatitis, EGF family members such as amphiregulin have been implicated in tissue remodeling.<sup>14</sup> Amphiregulin can promote the proliferation of primary human lung fibroblasts,<sup>12</sup> increase mucin gene expression by airway epithelial cells,<sup>11</sup> and enhance migration of T<sub>H</sub>2 cells into the inflamed tissue by increasing thymus and activation-regulated chemokine expression.<sup>15</sup> Amphiregulin levels in sputum were significantly higher in subjects with asthma during acute attacks and correlated with the severity of asthma symptoms and with tryptase or eosinophil cationic protein (ECP) in the sputum.<sup>16,17</sup> Thus, amphiregulin might significantly contribute to human allergic diseases.

We therefore tested whether human PBMCs produced amphiregulin in response to T-cell activation. Although we found that amphiregulin expression was indeed increased after anti-TCR stimulation of PBMCs, unexpectedly we found that very little of this amphiregulin could be attributed to T-cell production. Instead, a substantial proportion of basophils strongly upregulated amphiregulin mRNA and protein in response to TCR ligation in the overall PBMC population. The link between T-cell activation and basophil production of amphiregulin was found to be IL-3, which was both necessary and sufficient to stimulate amphiregulin production by basophils.

## METHODS

### Antibodies and reagents

Biotinylated goat antihuman and antimouse amphiregulin antibodies and recombinant human (rh) IL-3 were obtained from R&D Systems (Minneapolis, Minn). Antibodies specific for human CD3 $\epsilon$  (OKT3), IL-3 (BVD8-3G11), CD69 (FN50, Pacific Blue), CD123 (6H6, phycoerythrin-Cy7), and CD203c (NP4D6, phycoerythrin) were purchased from BioLegend (San Diego, Calif). Antibodies specific for human CD28 (CD28.2), CD4 (RPA-T4, APC-AF750, or phycoerythrin-Cy7), CD19 (HIB19, phycoerythrin-Cy5), and mouse granulocyte-differentiation antigen-1 ([Gr-1], RB6-8C5, AlexaFluor700), IL-4 (BVD6-24G2, phycoerythrin-Cy7), and Fc $\epsilon$ RI $\alpha$  (MAR-1, phycoerythrin) were obtained from eBioscience (San Diego, Calif). Antibodies specific for human CD8 $\alpha$  (3B5, phycoerythrin-Texas Red), CD14 (T $\mu$ K4, phycoerythrin-Cy5), mouse CD4 (RM4-5, AF405), and mouse CD19 (6D5, phycoerythrin-Texas Red) were obtained from CALTAG (Carlsbad, Calif). Allophycocyanin-conjugated antihuman CD303 was a generous gift from Dr Jyh-Chiang Wang (University of Rochester). Polyclonal goat antihuman IgE ( $\epsilon$ -chain specific) was obtained from Sigma (St Louis, Mo). 7AAD was obtained from Calbiochem (Gibbstown, NJ). The Basophil Isolation Kit II was obtained from Miltenyi Biotec (Auburn, Calif).

### Human PBMC activation and cell surface staining

Heparinized blood was obtained from healthy donors under a protocol approved by the University of Rochester Medical Center Research Subjects Review Board. PBMCs were isolated by Ficoll-Hypaque (Cellgro, Herndon, Va) density gradient centrifugation. Cells were suspended in RPMI-1640 medium containing 100 U penicillin/streptomycin (Invitrogen, Carlsbad, Calif) supplemented with 8% heat-inactivated FCS (HyClone, Logan, Utah). A total of 10<sup>6</sup> PBMCs per well were stimulated with medium alone or 5  $\mu$ g/mL anti-CD3 $\epsilon$  + 1  $\mu$ g/mL anti-CD28 in round-bottom 96-well plates (Costar; Corning Inc, Corning, NY) for 6 hours at 37°C. After stimulation, the cells were stained for cell surface markers amphiregulin, CD4, CD8, CD14, CD19, CD69, CD123, CD203c, CD303, and live/dead 7AAD staining, then with APC-conjugated streptavidin (BD Bioscience, San Jose, Calif) at 4°C for 15 minutes, and fixed with 2% paraformaldehyde. Samples were analyzed

by using an LSR II flow cytometer (BD Bioscience) and FlowJo software (Tree Star Inc, Ashland, Ore).

### Human basophils preparation and activation

Peripheral blood basophils were purified by cell sorting or enriched by negative selection. For studying the direct induction of amphiregulin in human basophils by IL-3, basophils were sorted from fresh PBMCs by surface markers CD4<sup>+</sup>CD8<sup>+</sup>CD14<sup>+</sup>CD19<sup>+</sup>7AAD<sup>+</sup>CD123<sup>+</sup> on a FACSaria (BD Bioscience). Purified basophils were treated with medium alone, 5  $\mu$ g/mL anti-CD3 $\epsilon$  + 1  $\mu$ g/mL anti-CD28 (as a negative control), and 5 ng/mL rhIL-3 for 16 hours. Cells were stained with 7AAD and antibodies against amphiregulin, CD123, CD203c, and CD69.

For the remaining experiments, basophils were enriched from peripheral blood by depletion of magnetically labeled cells according to the manufacturer's instructions (basophil isolation kit II; Miltenyi Biotec). The purity of live basophils was about 20% to 80%. Enriched basophils were suspended in Iscove's modified Dulbecco's medium supplemented with 5% FCS and 1x minimum essential medium nonessential amino acids (Invitrogen), ~10<sup>5</sup> cells/well in 96-well plates, and treated for 0.5, 1, 4, or 16 hours with medium alone, 10 ng/mL rhIL-3, or 10 ng/mL anti-IgE. Supernatants (4 hours) were collected to measure histamine release. Half of the cells were collected for extraction of total RNA, and the remaining cells were stained with 7AAD and antibodies against amphiregulin, CD123, CD203c, Fc $\epsilon$ RI $\alpha$ , and CD69 to measure surface amphiregulin expression.

### Quantitative real-time PCR for gene expression

Total RNA was extracted by using TRIzol (Invitrogen) according to the manufacturer's instructions. cDNA was prepared by reverse transcription from total RNA by using SuperScript III Reverse Transcriptase (Invitrogen) and Oligo(dT)<sub>12-18</sub> (Invitrogen) as primer. Real-time PCR was performed by using the Applied Biosystems 7900HT Sequence Detection System (Foster City, Calif). Primers and probes specific for amphiregulin, IL-3, IL-4, IL-13, HB-EGF, and glyceraldehyde-3-phosphate dehydrogenase were obtained from the TaqMan Gene Expression Assays (Applied Biosystems). mRNA amounts were normalized to endogenous glyceraldehyde-3-phosphate dehydrogenase gene expression. Samples were run in triplicate, and data were analyzed by using SDS software (Applied Biosystems).

### Histamine release assay

Histamine in enriched basophil supernatants was measured in duplicate by the Histamine Enzyme Immunoassay Kit (Cayman, Ann Arbor, Mich) according to the manufacturer's protocol.

### Measurement of amphiregulin release

Enriched basophils were treated with medium alone, 10 ng/mL rhIL-3, or 10 ng/mL anti-IgE, with or without 5  $\mu$ g/mL anti-IL3R $\alpha$  (anti-IL-3 receptor  $\alpha$ , R&D Systems) or 50  $\mu$ mol/L TNF- $\alpha$  protease inhibitor-1, an a disintegrin and metalloproteinase domain 17 protease inhibitor<sup>18</sup> (Calbiochem). After 24 hours, supernatant amphiregulin was measured by using the human Amphiregulin DuoSet ELISA Development kit (R&D Systems; detection limit, 7.8 pg/mL). This kit may underestimate the absolute levels of amphiregulin, because the ELISA standard and the immunogen for the polyclonal antibody reagent were unglycosylated bacterial recombinant human amphiregulin, whereas natural amphiregulin is heavily glycosylated. However, studies on amphiregulin produced by eosinophils and mast cells used the same ELISA kit,<sup>12,13</sup> so our results can be directly compared with these other studies.

### Intracellular staining of mouse cells

Amphiregulin-deficient (amphiregulin<sup>-/-</sup>) mice,<sup>8</sup> originally obtained from Dr David Lee (University of North Carolina School of Medicine), were backcrossed to C57BL/6.PL mice for 10 generations and then maintained by homozygous breeding.<sup>9</sup> C57BL/6.PL mice were bred in our facility.

Peripheral blood from mice was obtained by cardiac puncture. Red cells were lysed by ammonium chloride-potassium lysis buffer (0.8% NH<sub>4</sub>Cl, 10 mmol/L

KHCO<sub>2</sub>, and 0.1 mmol/L EDTA), and the remaining cells were stimulated with medium alone, 20 ng/mL recombinant mouse IL-3 (R&D Systems), or 20 ng/mL rat antimouse IgE (R35-92; BD Bioscience) in a 96-well plate at 10<sup>6</sup> cells/well. After 16 hours stimulation (with 2  $\mu$ mol/L monensin present for the last 6 hours), the cells were stained with antibodies specific for CD4, CD19, Gr-1, Fc $\epsilon$ RI $\alpha$ , and 7AAD, then fixed and permeabilized using Fix-Perm (BD Bioscience) and stained with antiampfiregulin and anti-IL-4 intracellularly. Cells were analyzed by using an LSR II flow cytometer and FlowJo software.

## RESULTS

### Basophils from anti-CD3/CD28-stimulated human PBMCs express amphiregulin

Mouse T<sub>H</sub>2 cells produced amphiregulin in response to TCR-mediated activation.<sup>9</sup> To determine whether human T<sub>H</sub>2 CD4 T cells produce amphiregulin, we stimulated human PBMCs with soluble anti-CD3 and anti-CD28 for 6 hours *ex vivo*. Because IL-4-producing and IL-5-producing cells can be detected in polyclonally stimulated PBMCs, this approach should have revealed T<sub>H</sub>2 production of amphiregulin, if it occurred.

Because amphiregulin is initially expressed as a surface protein, stimulated and unstimulated PBMCs were stained for cell-surface amphiregulin as well as other surface markers to identify CD4 T cells and other major PBMC populations. Analysis by flow cytometry (Fig 1, A) showed that anti-CD3 + CD28 stimulation induced a substantial population of amphiregulin-expressing cells, but these cells did not appear to be T cells. The amphiregulin-positive cells induced by T-cell activation (about 0.1% of total PBMCs) were mainly CD4<sup>+</sup>CD8<sup>+</sup>CD123<sup>+</sup>. Within the CD4<sup>+</sup>CD8<sup>+</sup>CD123<sup>+</sup> population, 26% expressed amphiregulin after anti-CD3/CD28 stimulation, whereas less than 1% of the cells expressed amphiregulin in the corresponding unstimulated population (Fig 1, B). The induction of amphiregulin expression by CD123<sup>+</sup> PBMCs is highly reproducible, and we have observed this in more than 20 experiments (data not shown).

CD123, the ligand-specific  $\alpha$ -chain of the heterodimeric IL-3 receptor (CD123/CD131) is expressed at high levels on blood basophils and plasmacytoid dendritic cells.<sup>19,20</sup> These 2 populations were distinguished by expression of 2 additional surface markers. Among hemopoietic cells, CD203c (E-NPP3, ectonucleotide pyrophosphatase/phosphodiesterase 3) is expressed on human basophils and mast cells but not on plasmacytoid dendritic cells. Expression of CD203c is increased on activated basophils.<sup>21</sup> Conversely, CD303 (plasmacytoid dendritic cell-specific antigen, a type II transmembrane C-type lectin) is expressed on blood plasmacytoid dendritic cells but not basophils.<sup>20</sup> The major population expressing amphiregulin after anti-TCR stimulation of PBMCs expressed the basophil pattern (CD123<sup>+</sup>CD203c<sup>+</sup>CD303<sup>+</sup>; Fig 1, A). Forward and side scatter were also consistent with the properties of small granulocytes, as expected for basophils.

Final identification of these amphiregulin-positive cells as basophils was made by sorting CD4<sup>+</sup>CD8<sup>+</sup>CD14<sup>+</sup>CD19<sup>+</sup>7AAD<sup>+</sup> amphiregulin<sup>+</sup> cells from anti-CD3 + CD28-stimulated human PBMCs and analyzing these by cytosin and histologic staining. More than 90% of these cells contained the lobulated nuclei and basophilic granules characteristic of basophils (Fig 1, C).

To confirm the expression of amphiregulin protein on basophils revealed by the polyclonal goat antibodies, RNA was extracted from CD4<sup>+</sup> and CD123<sup>+</sup> cells sorted from nontreated and anti-CD3 + CD28-stimulated PBMCs. As measured by quantitative real-time PCR (qPCR), amphiregulin transcription in stimulated

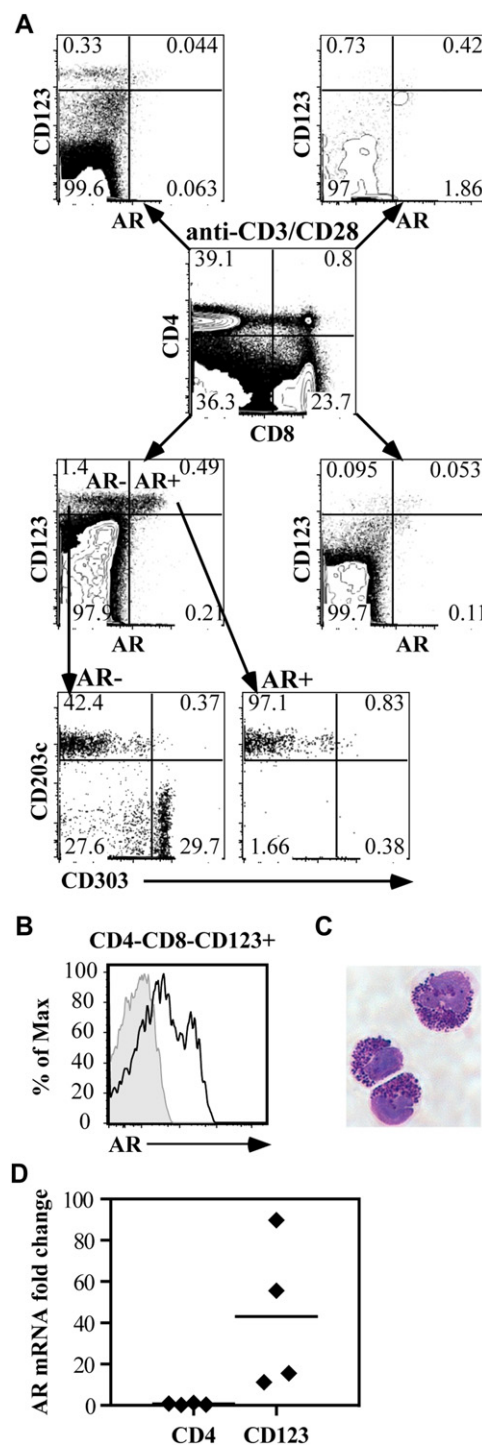


**FIG 1.** Human basophils express amphiregulin (AR). **A**, PBMCs were stimulated with anti-CD3/CD28, stained, and gated as shown. **B**, AR expression on unstimulated (shaded) and stimulated (line) CD4<sup>+</sup>CD8<sup>+</sup>CD123<sup>+</sup> cells. **C**, Sorted CD4<sup>+</sup>CD8<sup>+</sup>CD14<sup>+</sup>CD19<sup>+</sup>7AAD<sup>+</sup>AR<sup>+</sup> cells were stained with Wright-Giemsa. **D**, Stimulation-induced AR mRNA changes were measured in CD4<sup>+</sup>CD8<sup>+</sup>CD14<sup>+</sup>CD19<sup>+</sup>7AAD<sup>+</sup>CD123<sup>+</sup> ("CD4") and CD4<sup>+</sup>CD8<sup>+</sup>CD14<sup>+</sup>CD19<sup>+</sup>7AAD<sup>+</sup>CD123<sup>+</sup> ("CD123") cells sorted from anti-CD3/CD28-stimulated PBMCs (4 subjects; 16 hours). Results are representative of at least 3 experiments. Max, Maximum.

CD123<sup>+</sup> cells increased greatly, whereas CD4 cells expressed much lower amphiregulin mRNA levels in either untreated or stimulated PBMCs (Fig 1, D). The mRNA analysis suggests that

amphiregulin on basophils after stimulation represented new expression rather than release of preformed protein. This was supported by staining of permeabilized cells, showing increased intracellular amphiregulin after stimulation (data not shown).

### Basophils are induced to express amphiregulin by IL-3, which is expressed by activated human T cells

Basophils do not express the TCR, so why do human basophils upregulate amphiregulin expression after anti-CD3 stimulation of PBMCs? We proposed that amphiregulin expression by basophils might be indirectly induced by a mediator released by activated T cells. IL-3 primes and activates basophils,<sup>22</sup> enhancing expression of CD203c and CD69.<sup>23,24</sup> The IL-3 receptor chain CD123 is highly expressed on the amphiregulin-expressing basophils (eg, Fig 1, A). By qPCR, IL-3 mRNA levels were significantly increased in CD4 T cells after PBMC activation by anti-CD3 + CD28 (15-fold, 34-fold, 41-fold, 49-fold, and 53-fold in 5 individuals), and also in human T<sub>H</sub>1 and T<sub>H</sub>2 cell lines (data not shown).

Anti-IL-3 almost completely blocked the amphiregulin expression on basophils when added during stimulation of PBMCs by anti-CD3 + CD28. rhIL-3 (in the absence of anti-CD3 + CD28 stimulation) strongly induced basophil expression of amphiregulin and 2 other activation markers, CD203c and CD69 (Fig 2, A). IL-3-induced amphiregulin expression was rapid, peaked at 12 to 24 hours (see this article's Fig E1 in the Online Repository at [www.jacionline.org](http://www.jacionline.org)), and was induced by low IL-3 concentrations (Fig 2, B).

To test whether IL-3 (but not anti-CD3 + CD28) directly induced amphiregulin expression on basophils, basophils (CD4<sup>+</sup>CD8<sup>+</sup>CD14<sup>+</sup>CD19<sup>+</sup>7AAD<sup>+</sup>CD123<sup>+</sup>) were separated from PBMCs by cell sorting. IL-3 induced strong amphiregulin expression on the purified basophils, whereas anti-CD3 + CD28 had no effect, as expected (Fig 2, C). Thus, IL-3 is necessary for amphiregulin expression on basophils in anti-TCR-activated PBMCs and is sufficient potentially to induce amphiregulin expression on purified basophils.

### Amphiregulin expression is induced more strongly by IL-3 than by IgE cross-linking

Human basophils are strongly activated by cross-linking of high-affinity IgE Fc receptors (FcεRI), which leads to secretion of various mediators including histamine, leukotrienes, IL-4, and IL-13.<sup>25</sup> IL-3 alone is less effective than FcεRI cross-linking, but IL-3 can prime basophils for enhanced responses to subsequent FcεRI cross-linking.<sup>22</sup> IL-3 also directly enhances expression of CD69 and CD203c on basophils.<sup>23,24</sup> We therefore tested whether FcεRI cross-linking enhanced amphiregulin expression.

IgE cross-linking was more effective than IL-3 for inducing histamine release by basophils (Fig 3, A). In contrast, IL-3 was more effective than cross-linking IgE for inducing amphiregulin protein expression on the surface of basophils (Fig 3, A). The ability of anti-IgE to stimulate histamine release but not amphiregulin expression was confirmed with additional anti-IgE concentrations (from 1 ng/mL to 1 μg/mL; see this article's Fig E2 in the Online Repository at [www.jacionline.org](http://www.jacionline.org)) and incubation times (data not shown). IL-3 consistently induced higher levels of amphiregulin expression than any of the anti-IgE treatments tested. Fig 3, A, shows mean fluorescent intensity (MFI) values for amphiregulin. Similar conclusions were obtained if the percentages of cells

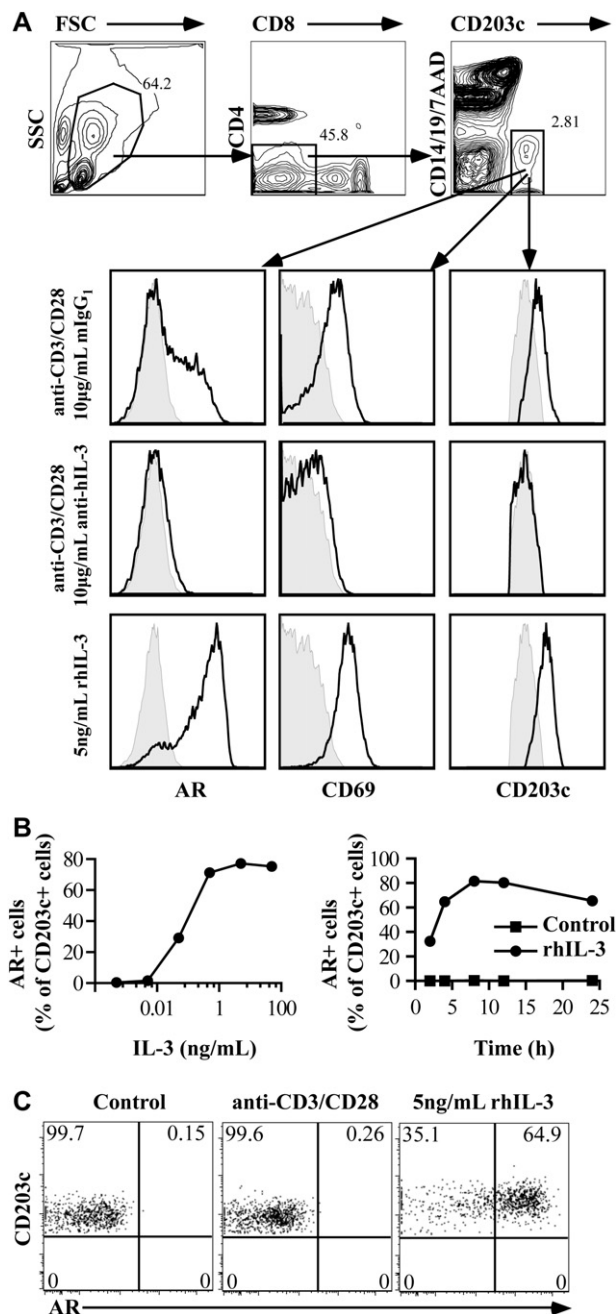


**FIG 2.** IL-3 is necessary and sufficient for induction of amphiregulin (AR) expression on basophils by activated T cells. **A**, Surface antigens on basophils (CD4<sup>+</sup>CD8<sup>+</sup>CD14<sup>+</sup>CD19<sup>+</sup>7AAD<sup>+</sup>CD203c<sup>+</sup>) were analyzed by flow cytometry in unstimulated (shaded) or stimulated (line) PBMCs. **B**, PBMCs were treated with rhIL-3 (left, 6 hours; right, 5 ng/mL). Means of triplicate cultures are shown. **C**, Sorted basophils treated for 16 hours as indicated were analyzed by flow cytometry. Results are representative of at least 3 experiments. FSC, Forward scatter; hIL, human interleukin; mlgG1, normal mouse IgG1; rhIL-3, recombinant human IL-3; SSC, side scatter.

positive for amphiregulin were plotted. Although IL-3 primes synergizes with IgE cross-linking to induce IL-4 production and histamine release,<sup>26,27</sup> there was no significant difference in the induction of surface amphiregulin expression during treatment with IL-3 with or without anti-IgE (Fig 3, B).

Because amphiregulin can exist as an initial membrane-bound form or a soluble cleaved molecule, we tested the possibility that

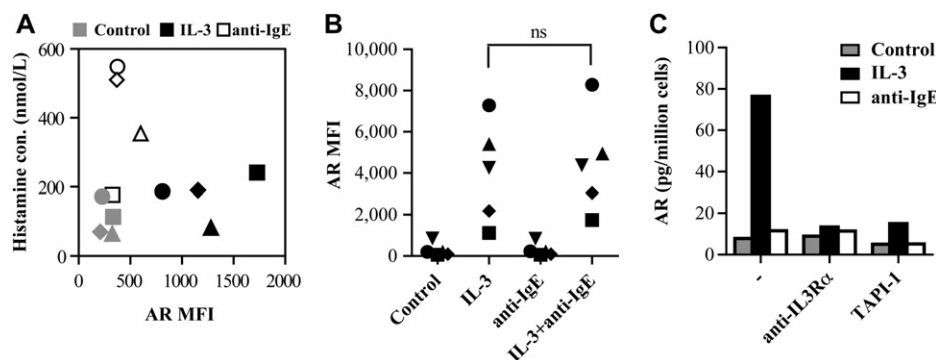


**FIG 3.** Amphiregulin (AR) expression in human basophils is induced more strongly by IL-3 than IgE cross-linking. Enriched basophils were stimulated as indicated. **A**, Histamine release and surface AR expression (4 hours). Symbols represent different individuals. **B**, Surface AR on basophils from 5 individuals (16 hours). **C**, Supernatant AR after stimulation with or without anti-IL-3R $\alpha$  or TAPI-1 (24 hours). con., Concentration; ns, not significant.

IgE cross-linking induced amphiregulin production, but this amphiregulin was cleaved off the basophil surface. IL-3 increased the levels of soluble amphiregulin in the supernatant more effectively than IgE cross-linking (Fig 3, C), and this could be inhibited by anti-IL-3 receptor antibodies or by the cleavage inhibitor TAPI-1. The supernatant levels of amphiregulin induced by IL-3 treatment for 24 hours were  $71 \pm 28$  pg/million basophils in 6 different experiments, comparable to the amphiregulin levels produced by eosinophils (estimated 18 pg/million cells from reference<sup>13</sup>), and mast cells (360 pg/million cells).<sup>12</sup> Cross-linking of IgE did not enhance soluble amphiregulin levels (Fig 3, C). However, in some experiments, anti-IgE further enhanced (up to 2-fold) the levels of amphiregulin released by IL-3-stimulated basophils (see this article's Fig E3 in the Online Repository at [www.jacionline.org](http://www.jacionline.org)).

Analysis of mRNA expression led to similar conclusions. Although anti-IgE induced rapid expression of IL-4 and IL-13 mRNA, within 1 hour (Fig 4), only IL-3 induced high levels of amphiregulin mRNA expression, with somewhat slower kinetics. As in previous studies,<sup>28</sup> IL-13 mRNA expression was induced by IL-3 at longer times (Fig 4).

Basophils can secrete IL-3 after IgE cross-linking.<sup>29</sup> Low levels of IL-3 expression by basophils were also detected by qPCR during our anti-IgE stimulation (data not shown). However, this IL-3 was not sufficient to induce substantial levels of amphiregulin expression (Figs 3 and E3). The possibility that anti-IgE stimulation induced both IL-3 and an inhibitor of amphiregulin expression was ruled out by the strong amphiregulin response of anti-IgE-treated basophils to exogenous IL-3 (Fig 3, B).

Overall, amphiregulin mRNA and protein expression as well as protein shedding by basophils was induced consistently and strongly via an IL-3-dependent pathway, whereas anti-IgE stimulation, even though more effective for inducing expression of other mediators, induced lower levels of amphiregulin expression.

Because IL-3 induces the synthesis of several mediators by basophils, we tested whether these conditions activated the synthesis of other EGF family members by measuring mRNA levels using qPCR. Similar to amphiregulin, HB-EGF was expressed by basophils in response to IL-3, but at lower, more transient levels by anti-IgE. Fig 4 shows the extent of induction of HB-EGF mRNA relative to unstimulated cells. When normalized to glyceraldehyde-3-phosphate dehydrogenase mRNA levels, HB-EGF

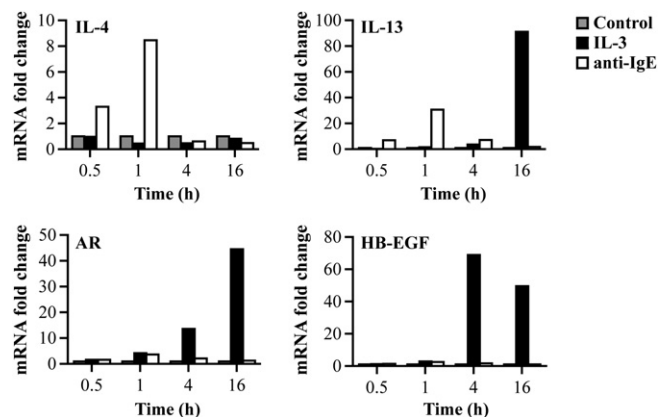


**FIG 4.** IL-3 and IgE cross-linking induce different activation profiles in human basophils. Enriched basophils were stimulated as indicated, and mRNA was measured by qPCR. Kinetics of amphiregulin (AR), IL-4, IL-13, and HB-EGF mRNA expression in response to IL-3 or anti-IgE stimulation (expressed as the fold change relative to unstimulated cells) are shown. Similar results were obtained in at least 4 experiments.

mRNA levels were lower than those of amphiregulin (data not shown). Other EGF family members were expressed at lower or undetectable levels (data not shown).

### Activated mouse basophils express amphiregulin

Because human basophils expressed amphiregulin, we tested whether mouse blood basophils could also express amphiregulin. After red blood cell lysis, mouse blood cells were stimulated with IL-3 or anti-IgE and stained for expression of surface markers, intracellular IL-4, and amphiregulin. Basophils were identified as CD4<sup>+</sup>CD19<sup>+</sup>Gr-1<sup>+</sup>Fc $\epsilon$ RI<sup>+</sup> cells.<sup>30</sup> IL-3 induced substantial numbers of wild-type C57BL/6.PL cells in this population to express IL-4, and about 29% of these cells also expressed amphiregulin. The specificity of amphiregulin staining in basophils was confirmed by showing that the equivalent population from amphiregulin<sup>-/-</sup> mice expressed similar levels of IL-4 but undetectable amphiregulin (Fig 5). Thus, IL-3-activated mouse basophils also expressed amphiregulin. Interestingly, anti-IgE stimulation was more effective on mouse than human basophils, stimulating low levels of amphiregulin production. Similar to

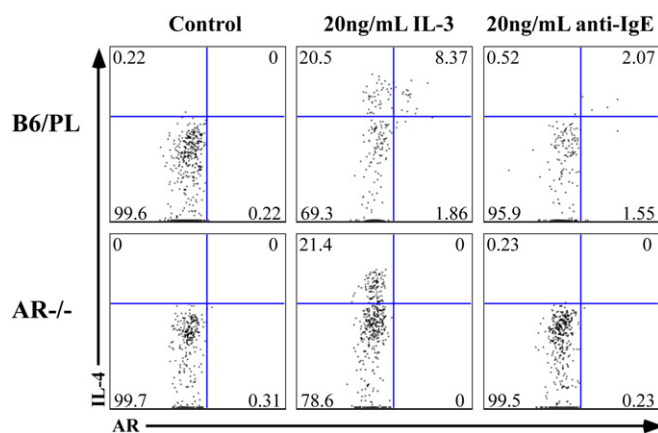


**FIG 5.** Mouse basophils express amphiregulin (AR) in response to IL-3 activation. Mouse blood leukocytes from C57BL/6.PL (B6/PL) or AR<sup>-/-</sup> mice were treated as indicated and stained for surface markers and intracellular IL-4 and AR expression. Basophils (CD4<sup>-</sup>CD19<sup>-</sup>Gr-1<sup>-</sup>FcεR1<sup>+</sup>) were gated and then analyzed for IL-4 versus AR. Results are representative of 3 experiments.

human basophils, mouse basophils produced cytokines more rapidly in response to anti-IgE and produced amphiregulin more slowly in response to IL-3 (results not shown).

### Increased numbers of basophils from subjects with asthma can produce amphiregulin

To investigate potential links between asthma and the ability of basophils to produce amphiregulin, we measured the levels of amphiregulin-producing basophils in PBMCs from 3 groups of human subjects: those (1) with allergic asthma, (2) with allergy but not asthma; and (2) without allergy. This article's Fig E4 in the Online Repository at [www.jacionline.org](http://www.jacionline.org) describes this study and the results. The numbers of basophils able to produce amphiregulin were increased in subjects with asthma compared with either of the other groups. This difference was significant ( $P < .05$ ) in response to stimulation with IL-3 or *Staphylococcal* enterotoxin B but did not reach significance with anti-CD3 + anti-CD28. Consistent with our previous data, anti-IL-3 antibodies substantially reduced the numbers of amphiregulin<sup>+</sup> cells induced by anti-CD3 + anti-CD28 or SEB.

## DISCUSSION

These results clearly show that human basophils can produce the EGF ligands amphiregulin and HB-EGF, particularly in response to stimulation by IL-3. Thus basophils would be expected to produce these cytokines at inflammatory sites where T cells were activated to produce IL-3. Amphiregulin is also produced on stimulation of human mast cells by IgE cross-linking,<sup>11</sup> constitutively in tissue-resident mast cells in patients with asthma,<sup>12</sup> and on activation of eosinophils by IL-5 or GM-CSF.<sup>13</sup>

Immune cells known to produce amphiregulin now include T<sub>H</sub>2 cells (mouse), basophils (mouse and human), mast cells (human), and eosinophils (human).<sup>9,11-13</sup> Mouse or human neutrophils (data not shown) do not express significant levels of amphiregulin.<sup>13</sup> Recent studies on mouse basophils suggest that basophils also provide a positive feedback loop enhancing type 2 responses, acting as one of the major sources of local IL-4 secretion, and

directly priming T cells to induce T<sub>H</sub>2 responses.<sup>30-33</sup> Thus, the hemopoietic cell types expressing amphiregulin are all crucial components of the allergic inflammatory response,<sup>34</sup> suggesting that the effects of immune amphiregulin are more prominent in allergic than type 1 inflammation.

IL-3 also enhances mouse basophil migration into sites of inflammation.<sup>35</sup> Expression of specific chemotactic receptors, such as the prostaglandin D<sub>2</sub> receptor chemoattractant receptor-homologous molecule expressed on T<sub>H</sub>2 cells,<sup>36</sup> on human basophils also contributes to the selective recruitment of basophils. Although IL-3 is produced by both T<sub>H</sub>1 and T<sub>H</sub>2 cells after activation,<sup>37</sup> selective migration of basophils into allergic sites because of other receptors may result in association between type 2 responses and basophils, resulting in IL-3-dependent amphiregulin expression on basophils infiltrating the sites of allergic inflammation.

Human CD4 T cells stimulated through the TCR did not express amphiregulin in this study, which was unexpected on the basis of mouse data showing that amphiregulin is produced by T<sub>H</sub>2-cell populations.<sup>9</sup> However, in other studies we found that TCR stimulation and intracellular cyclic adenosine monophosphate upregulation synergize to induce human CD4 T cells to express amphiregulin (Y. Qi, D. J. Operario, and T. R. Mosmann, manuscript in preparation). Partially-purified human CD4 T cells have also been reported to produce amphiregulin in response to cyclic adenosine monophosphate agonists in another study,<sup>38</sup> but the purification method used (depletion of CD14<sup>+</sup> and CD19<sup>+</sup> cells) would have resulted in a population containing both basophils and T cells; thus, the amphiregulin detected in that study was probably derived mainly from basophils.

Amphiregulin can contribute to effector functions by enhancing parasite expulsion during worm infection,<sup>9,10</sup> and can also cause tissue remodeling, which may be positive for repairing immunopathology, but could play a negative role in the development of asthma.<sup>14</sup> A characteristic feature of asthma is airway remodeling, including epithelial proliferation, subepithelial fibrosis, increased airway smooth muscle mass, goblet cell hyperplasia, and mucus gland hyperplasia. Amphiregulin not only promoted the proliferation of primary human lung fibroblasts<sup>12</sup> but also enhanced mucin gene expression in the airway epithelium.<sup>11</sup> IL-13 can also contribute to tissue remodeling,<sup>39</sup> and both IL-13 and amphiregulin are produced by basophils in response to IL-3.

Consistent with a role for amphiregulin in asthma, mast cells expressing amphiregulin have been described in patients with asthma,<sup>12</sup> and amphiregulin levels in sputum were associated with the severity of asthma and with tryptase and ECP levels in sputum.<sup>16,17</sup> Although not directly addressing the role of basophils in the lung, our studies demonstrated that subjects with asthma had increased numbers of peripheral blood basophils capable of producing amphiregulin. IL-3-dependent production of amphiregulin by human basophils could occur as part of a coordinated type 2 response in which mast cells, eosinophils, and basophils produce amphiregulin, consistent with the correlation of amphiregulin and the mast cell and eosinophil products tryptase and ECP. The discovery that amphiregulin is produced by basophils as well as mast cells, eosinophils, and potentially T<sub>H</sub>2 cells strongly suggests that amphiregulin is selectively produced at the site of allergic reactions and may participate in allergic reactions involving tissue remodeling, such as asthma and allergic dermatitis.

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### Key messages

- **Activation of T cells in human peripheral blood mononuclear cells leads to high expression of amphiregulin in basophils.**
- **IL-3 induces amphiregulin expression by human basophils.**
- **IL-3 is more effective than IgE cross-linking for inducing amphiregulin expression.**

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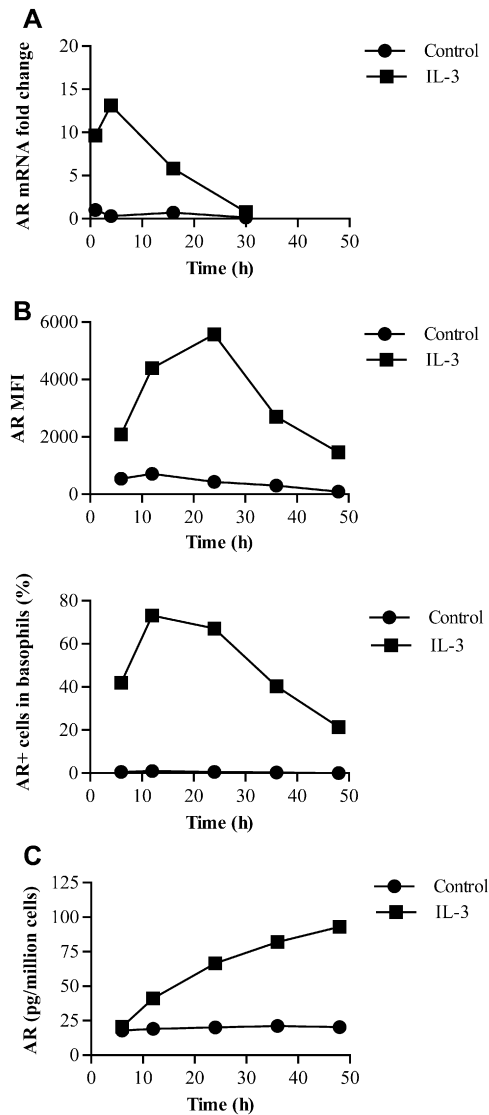


**FIG E1.** Kinetics of amphiregulin (AR) mRNA, cell surface, and soluble form expression in response to IL-3. Enriched basophils were treated with medium alone or rhIL-3 (10 ng/mL) for indicated times. **A**, AR mRNA was measured by RT-PCR. Data are shown as the fold change relative to 1 hour unstimulated cells. **B**, Surface AR expression on basophils was analyzed by flow cytometry. AR MFI of basophils and the percentage of AR<sup>+</sup> cells are shown. **C**, Soluble AR expression was measured by ELISA. Results are representative of at least 3 experiments.

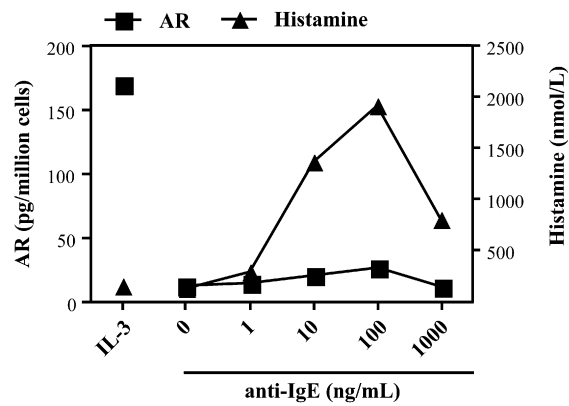


**FIG E2.** Soluble amphiregulin (AR) expression in response to IL-3 or IgE cross-linking. Enriched basophils were treated with medium alone, rhIL-3 (5 ng/mL), or anti-IgE (1 ng/mL to 1  $\mu$ g/mL). Histamine release (at 4 hours) and soluble AR (at 24 hours) were measured by ELISA. Results are representative of 3 experiments.

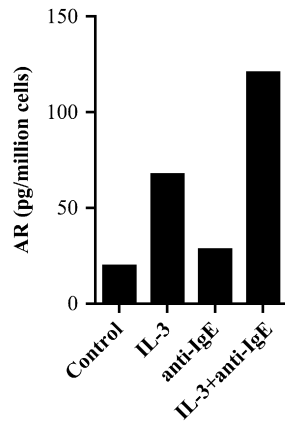


**FIG E3.** Synergy between IL-3 and anti-IgE for soluble amphiregulin (AR) expression. Enriched basophils were treated with medium alone, IL-3 (5 ng/mL), anti-IgE (10 ng/mL), or IL-3 (10 ng/mL) + anti-IgE (10 ng/mL) for 24 hours. Soluble AR was measured by ELISA. Representative of 3 experiments.

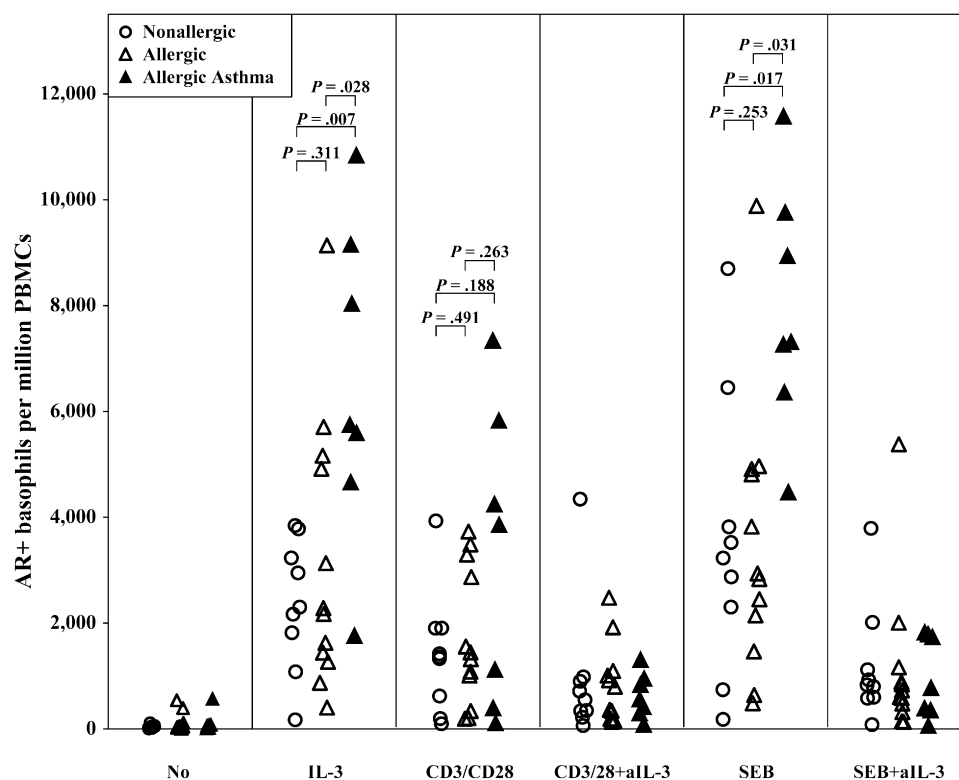


**FIG E4.** Increased amphiregulin (AR)-expressing basophils in subjects with asthma. This study was approved by the institutional review board of the University of Rochester Medical Center, and written informed consent was obtained from all participating subjects. Eleven subjects with allergic asthma (age, 19-50 years; 5 women, 6 men), 17 subjects with allergic rhinitis but no asthma (age, 23-55 years; 9 women, 8 men), and 11 normal subjects without allergy (age, 19-50 years; 5 women, 6 men) were enrolled for the study. The screening and classification of subjects into 3 groups were based on the results from skin tests, RAST, and lung function tests. Exclusion criteria included a history of cigarette smoking or drug or alcohol abuse, significant occupational exposure to respiratory irritants or toxins, chronic use of medications (other than medications for allergies/asthma and oral contraceptives), cardiac or severe lung disease, history of allergen immunotherapy or underlying illnesses that may alter lung function, and pregnancy. Peripheral blood was drawn from participating subjects with no recent exacerbations. PBMCs were isolated as described in Methods. Cells were stimulated with medium alone (No), 5 ng/mL rhIL-3, 5  $\mu$ g/mL anti-CD3 + 1  $\mu$ g/mL anti-CD28, or 1  $\mu$ g/mL SEB. Anti-CD3/CD28 and SEB stimulations were also performed in the presence of anti-IL-3 (aIL-3, 10  $\mu$ g/mL). After 16 hours, the cells were stained with antibodies specific for CD4, CD8, CD14, Fc $\epsilon$ R1 $\alpha$ , CD123, CD203c, CD69, AR, and 7AAD and analyzed by flow cytometry. All analysis of flow cytometry was performed by a blind investigator. Basophils expressing AR (Fc $\epsilon$ R1 $\alpha$ <sup>+</sup>CD123<sup>+</sup>AR<sup>+</sup>) are expressed as the numbers per million PBMCs. Statistical comparisons were made by 1-sided Mann-Whitney test. No, No treatment; aIL, anti-IL-3.