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Human Airway Eosinophils Exhibit Preferential Reduction in STAT Signaling Capacity and Increased CISH Expression

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Allergic asthma, a chronic respiratory disorder marked by inflammation and recurrent airflow obstruction, is associated with elevated levels of IL-5 family cytokines and elevated numbers of eosinophils (EOS). IL-5 family cytokines elongate peripheral blood EOS (EOS_{PB}) viability, recruit EOS_{PB} to the airways, and, at higher concentrations, induce degranulation and reactive oxygen species generation. Although airway EOS (EOS_A) remain signal ready in that GM-CSF treatment induces degranulation, treatment of EOS_A with IL-5 family cytokines no longer confers a survival advantage. Because the IL-5 family receptors have common signaling capacity, but are uncoupled from EOS_A survival, whereas other IL-5 family induced endpoints remain functional, we tested the hypothesis that EOS_A possess a JAK/STAT-specific regulatory mechanism (because JAK/STAT signaling is critical to EOS survival). We found that IL-5 family-induced STAT3 and STAT5 phosphorylation is attenuated in EOS_A relative to blood EOS from airway allergen-challenged donors. However, IL-5 family-induced ERK1/2 phosphorylation is not altered between EOS_A and EOS from airway allergen-challenged donors. These observations suggest EOS_A possess a regulatory mechanism for suppressing STAT signaling distinct from ERK1/2 activation. Furthermore, we found, in EOS_{PB}, IL-5 family cytokines induce members of the suppressors of cytokine signaling (SOCS) genes, *CISH* and *SOCS1*. Additionally, following allergen challenge, EOS_A express significantly more *CISH* and *SOCS1* mRNA and CISH protein than EOS_{PB} counterparts. In EOS_{PB}, long-term pretreatment with IL-5 family cytokines, to varying degrees, attenuates IL-5 family-induced STAT5 phosphorylation. These data support a model in which IL-5 family cytokines trigger a selective downregulation mechanism in EOS_A for JAK/STAT pathways. *The Journal of Immunology*, 2013, 191: 000–000.

Eosinophils (EOS) contribute to the pathophysiology of allergic diseases, including asthma, through activation and subsequent release of inflammatory mediators and profibrotic factors (1–5). Our laboratory and others (2, 6–9) have observed that allergic airway inflammation leads to an elevated number of airway EOS (EOS_A) and that this increased number of EOS_A is associated with higher concentrations of proinflammatory IL-5 family cytokines (i.e., IL-5, IL-3, and GM-CSF) found in asthmatic bronchoalveolar lavage (BAL) fluid 48 h after segmental bronchoprovocation with allergen (SBP-Ag) (6, 8,

10–12). Incidentally, these IL-5 family cytokines are critical for normal EOS biology and function and signal through heterodimeric receptors comprised of a shared common β -chain (IL-5R β_c) and a ligand-specific α -chain (i.e., IL-5R α) (13, 14). EOS activation via these cytokines leads to a number of signaling pathways (including the JAK/STAT and Ras–Raf–ERK/ERK pathways (13, 15–19) and physiologically relevant end points, such as differentiation and recruitment, enhanced survival, and release of cytotoxic proteins and reactive oxygen species (4, 12, 20–23).

Important to the pathophysiology of allergic diseases, such as allergic asthma, there are distinct physiologic differences between EOS_A and circulating blood EOS (peripheral blood EOS [EOS_{PB}]). Such differences include upregulated surface expression of certain integrins in EOS_A relative to EOS_{PB} (6), allowing for greater adherence and motility and elongated viability in EOS_A (12, 20). Interestingly, upon exposure to IL-5 family cytokines, EOS_{PB} downregulate surface expression of the IL-5-specific subunit of the IL-5R (IL-5R α) as well as the IL-5R β_c (24–26) and increase IL-3R α surface expression (23, 24, 26). This receptor expression profile of decreased IL-5R α and IL-5R β_c as well as increased IL-3R α is reflected in EOS_A (27–29), which furthermore exhibit elevated GM-CSFR α surface expression (27). Once in the airway, and upon conclusion of the immunological response to challenge, it would be crucial that there exist some regulatory mechanism to inhibit continuous IL-5 family cytokine-induced survival signals and to guard against unremitting EOS activation. Given the evidence that IL-5 family cytokines continue signal in EOS_A [e.g., GM-CSF continues to induce degranulation (27), which is dependent on MEK/ERK pathway signaling (21)] but that EOS_A survival is no longer enhanced by IL-5 family cytokines (12, 20, 30), we tested the hypothesis that, in addition to changes observed in

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Abbreviations used in this article: BAL, bronchoalveolar lavage; Ct, cycle threshold; EOS, eosinophil; EOS_A, airway eosinophil; EOS_{PB}, peripheral blood eosinophil from segmental bronchoprovocation with allergen-challenged individual; EOS_{PB}, peripheral blood eosinophil; GPCR, G-protein-coupled receptor; SBP-Ag, segmental bronchoprovocation with allergen; SOCS, suppressor of cytokine signaling.

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IL-5 family receptor expression, there is/are (a) pathway-specific mechanism(s) regulating EOS_A responsiveness to IL-5 family cytokines.

One possible selective attenuation pathway involves the suppressor of cytokine signaling (SOCS) family proteins. JAK/STAT signaling upregulates SOCS family proteins, which serve as negative-feedback regulators in several immune systems (31, 32). SOCS family proteins block phosphorylation of membrane receptors and JAK family members and, subsequently, inhibit STAT binding and phosphorylation (31, 33, 34), all necessary to induce downstream JAK/STAT signaling, which has been linked to IL-5 family-induced survival in EOS (22, 23). Interestingly, in a murine model of allergic inflammation, Lee and colleagues (35) found two members of the SOCS family of genes, *SOCS1* and *CISH*, also referred to as *CIS1*, were, respectively, modestly and highly expressed in hematopoietic cells trafficking to the lungs after OVA challenge. These findings, combined with the observation that *CISH* is inducible in human EOS_{PB} (17), led us to *CISH* and *SOCS1* upregulation as a possible IL-5 family signaling regulatory mechanism in human EOS.

The data presented in this study represent a unique opportunity to test these questions with highly purified EOS_{PB} and EOS_A from patients with asthma undergoing an allergic airway challenge, of which the literature is extremely sparse, as well as with control EOS_{PB} from unchallenged participants. We found EOS_A exhibit decreased IL-5 family-stimulated p-STAT5 and p-STAT3 compared to IL-5 family stimulation of same-day isolation of blood EOS from the SBP-Ag-challenged individuals (EOS_{CPB}), whereas IL-5 family-stimulated p-ERK1/2 remained unaltered between EOS_A and EOS_{CPB}. We also found that EOS_A express significantly more *CISH* and *SOCS1* mRNA and CISH protein than EOS_{CPB}, whereas EOS_{CPB} in turn express more *CISH* and *SOCS1* mRNA and CISH protein than EOS_{PB} from independent, nonchallenged donors. Additionally, we observed 24-h pretreatment of EOS_{PB} with IL-5 family cytokines, to varying degrees, attenuates the ability of subsequent IL-5 family stimulation to induce p-STAT5. Taken together, these data suggest distinct regulation mechanisms by which STAT3 and STAT5 signal transduction may be altered in EOS_A, as compared with regulation of other pathways stimulated by IL-5 family cytokines.

Materials and Methods

Subjects

These studies were approved by the University of Wisconsin-Madison Center for Health Sciences Human Subjects Committee. Each subject provided informed, written consent prior to participation. We recruited atopic and nonatopic volunteer donors who had EOS comprising 2–10% of their peripheral blood leukocytes.

Isolation and treatment of peripheral EOS_{CPB} and EOS_A

Human EOS_{PB} were purified from heparinized peripheral blood, as described previously (23). Briefly, a granulocyte pellet was obtained by centrifugation of the blood through a Percoll monolayer (1.090 g/ml) and subsequent hypotonic lysis of erythrocytes. The resulting granulocytes were resuspended in HBSS supplemented with 2% newborn calf serum and incubated with anti-CD16-conjugated paramagnetic microbeads (MACS system; Miltenyi Biotec, San Jose, CA) to deplete contaminating neutrophils. Eosinophil populations were at least 98% pure and 97% viable.

Fluid from BAL was recovered, and EOS_A were isolated 48 h after SBP-Ag, in which Ag dose for SBP defined and BAL performed as described (27, 36). In brief, EOS_A were obtained after centrifuging BAL fluid through a Percoll bilayer (1.085/1.100 g/ml), with the recovered EOS population at the interface between the two layers. As above, purification continued with hypotonic lysis of erythrocytes, resuspension in HBSS plus 2% newborn calf serum, and incubation with anti-CD16-conjugated paramagnetic microbeads. Eosinophil populations were at least 98% pure and 97% viable.

EOS_{PB} were isolated and purified from the same donors who underwent SBP-Ag (challenged peripheral blood, EOS_{CPB}), obtained from phlebotomy that occurred immediately after recovery from the bronchoscopy procedure. Control experiments were performed in all cases on the same day

with peripheral blood from a different, unchallenged donor to validate that the purification process did not result in EOS activation.

For all experiments, freshly isolated EOS (2–4 million per recovery source [i.e., EOS_A, EOS_{CPB}, or EOS_{PB}]) were divided evenly between treatments and incubated at 37°C in 25 mM HEPES-buffered RPMI 1640 containing 0.1% human serum albumin for 30 min and then stimulated with IL-5, IL-3, or GM-CSF at the concentrations and times indicated in the figure legends. Loading controls were used to appropriately compare for differences in cell number between groups and donors.

Immunoblotting

Primary human EOS cultures were diluted with ice-cold STOP buffer (20 mM Tris [pH 7.4], 137 mM NaCl, 1 mM EDTA, 1 mM Na₃VO₄, 20 mM β-glycerophosphate, 10 mM NaF, and 1% mammalian protease inhibitor mixture), pelleted by centrifugation, and lysed in RIPA buffer (STOP buffer containing 0.1% v/v glycerol, 0.01% Triton X-100, 0.1% SDS, and 0.25% deoxycholic acid). Lysates were sonicated and centrifuged at 15,800 × g for 10 min to remove the insoluble fraction. Supernatants were assayed for total protein content using the Pierce Micro BCA protein assay (Pierce, Rockford, IL), electrophoresed on SDS-PAGE gels, and immunoblotted as described in the figure legends using Abs against the following: phospho-Tyr⁶⁹⁵ STAT5 and phospho-Tyr⁷⁰⁵ STAT3 (Cell Signaling Technology, Danvers, MA), p-Thr¹⁸⁵, p-Tyr¹⁸⁷ ERK1/2 (Invitrogen, Carlsbad, CA), CISH (R&D Systems, Minneapolis, MN), STAT5 and STAT3 (Santa Cruz Biotechnology, Santa Cruz, CA), and actin Ab-5 (BD Biosciences, San Jose, CA). Membranes were visualized using the Epichemi II darkroom (UVP, Upland, CA) equipped with a 12-bit cooled CCD camera. Relative immunoblot band densities were obtained via analysis with National Institutes of Health ImageJ software version 1.38X. Alterations in expression or phosphorylation of protein were normalized to appropriate loading control levels.

Isolation of mRNA and subsequent quantitative PCR

Total RNA was extracted using the RNeasy Mini Kit (Qiagen, Valencia, CA) from EOS_{PB}, media-treated or activated in vitro with GM-CSF, IL-3, or IL-5, and from EOS_{PB} (*n* = 3), EOS_A (*n* = 4), and EOS_{CPB} (*n* = 6) isolated 48 h after an in vivo allergen challenge. The reverse-transcription reaction was performed using the Superscript III system (Invitrogen/Life Technologies, Grand Island, NY). Expression of mRNA was determined by quantitative PCR using SYBR Green Master Mix (SABiosciences, Frederick, MD). Human *CISH*- (forward 5'-GTCCAGCCGAGTCCC-CCTCC-3' and reverse 5'-TGCTCACCCTGAACGCAGAG-3') and *SOCS1*-specific (forward 5'-GCTGGCCCCCTCTGTAGGAT-3' and reverse 5'-TGCTGTGGAGACTGCATTGTC-3') primers were designed using Primer Express 3.0 (Applied Biosystems, Carlsbad, CA) and blasted against the human genome to determine specificity using <http://www.ncbi.nlm.nih.gov/tools/primer-blast>. The reference gene, β-glucuronidase (*GUSB*) (forward 5'-CAGGACCTGCGCACAAGAG-3' and reverse 5'-TCGCA-CAGCTGGGGTAAG-3'), was used to normalize the samples. Standard curves were performed, and efficiencies were determined for each set of primers. Efficiencies ranged between 94 and 96%. Data are expressed as fold change using the comparative cycle threshold (ΔΔCt) method as described previously (37). The values presented in Fig. 4A and 4B are fold change (2^{-ΔΔCt}) compared with the level in untreated EOS_{PB} that was fixed at 1 (*n* = 5). The values presented in Fig. 5A and 5B are fold change (2^{-ΔΔCt}) compared with the level in EOS_{PB} that average level was fixed at 1 (± 0.168 for *CISH* and ± 0.287 for *SOCS1*; *n* = 3).

Statistical analyses

Phosphorylated signaling markers and mRNA expression measures were compared among cell sources (EOS_A, EOS_{CPB}, and EOS_{PB}) and stimulants (media, IL-3, IL-5, and GM-CSF) using either one-way ANOVA or linear mixed-effect ANOVA models with fixed-effect terms for cell source and stimulant and random-effect terms for donor and experiment to account for within-donor and within-experiment correlation. Measures were log-transformed as appropriate to achieve normal distributions and homogeneity of variance. A two-sided *p* value <0.05 was regarded as statistically significant.

Results

IL-5 family cytokine-induced ERK1/2 phosphorylation is unaltered between EOS_A and EOS_{CPB}, whereas STAT5 and STAT3 phosphorylation is reduced in EOS_A

Compared to EOS_{CPB}, EOS_A exhibit phenotypic differences, including enhanced inflammatory capacity and prolonged survival uncoupled from IL-5 family cytokines (20, 30), while remaining

responsive to IL-5 family cytokines regarding certain physiological end points, such as degranulation (27). Accordingly, we tested the idea that downregulation in specific intracellular signaling pathways is associated with these phenotypic differences observed between EOS_A and EOS_{PB}. In this regard, IL-5 family cytokines have been shown by us and others (4, 15–18, 21) to act via signaling cascades JAK/STAT (critical for IL-5 family–induced survival) and MAPK (crucial in IL-5 family–induced degranulation), thus we chose to examine these signaling cascades (at respective peak-activation time points) as potential markers of differences in EOS_A and EOS_{PB}/EOS_{CPB} activation. As shown in Fig. 1, we found IL-5, IL-3, and GM-CSF elevates p-ERK1/2 in EOS_A compared with media control ($p < 0.05$, $p < 0.05$, and $p < 0.001$ respectively) (Fig. 1A, 1B) to statistically equivalent levels relative to the respective cytokine stimulation of p-ERK1/2 in EOS_{CPB} (Fig. 1B). Control experiments using EOS_{PB} from unchallenged donors purified on the same day showed that the purification process does not alter baseline or IL-5 family cytokine–stimulated p-ERK1/2 levels (Supplemental Fig. 1A). These data are consistent with the previously observed reduction of IL-5R α and elevation of IL-3R α and GM-CSFR α on EOS_A cell surface relative to circulating EOS (27, 28). Collectively, EOS_A retain the capacity to activate ERK1/2 after stimulation ex vivo with IL-5 family cytokines.

In contrast to the IL-5 family activation of p-ERK1/2 in EOS_A, little to no p-STAT5 was detected in EOS_A despite the fact that IL-5, IL-3, and GM-CSF elevated p-STAT5 in EOS_{CPB}, whereas immunoblotting for total STAT5 indicates no change in total STAT expression (Fig. 2A). IL-5–, IL-3–, and GM-CSF–stimulated p-STAT5 is significantly decreased in EOS_A compared with the EOS_{CPB} from that same challenged donor ($p < 0.0001$, $p = 0.02$, and $p = 0.02$, respectively) (Fig. 2B). The purification process did not alter

basal or IL-5 family cytokine–stimulated p-STAT5 levels in EOS_{PB} from unchallenged donors (Supplemental Fig. 1B).

As shown in Fig. 3, the p-STAT3 observations are similar to p-STAT5 following stimulation with IL-5, IL-3, and GM-CSF. These treatments failed to induce p-STAT3 in EOS_A ($p = 0.001$, $p = 0.009$, and $p = 0.001$, respectively) relative to EOS_{CPB} but did not alter total STAT3 levels (Fig. 3A, 3B). Again, the purification process did not alter basal or IL-5 family cytokine–stimulated p-STAT3 levels in EOS_{PB} from unchallenged donors (Supplemental Fig. 1C). Combined with observations that IL-3R α and GM-CSFR α are present and even elevated on EOS_A surfaces relative to EOS_{PB} (27, 28), these data suggest there is potentially a JAK/STAT-specific inhibition of IL-5 family cytokine signaling in EOS_A.

IL-5 family stimulation of EOS_{PB} upregulates mRNA transcripts and proteins for the SOCS genes *CISH* and *SOCS1*

Because phosphorylation of STAT3 and STAT5 is selectively attenuated following IL-5 family cytokine stimulation in EOS_A, we sought to elucidate possible mechanisms by which this could occur. Others have observed that stimulation of immune cells with numerous cytokines activates both JAK/STAT pathways and up-regulates SOCS family proteins (31, 32, 34). SOCS proteins serve as a negative-feedback mechanism to attenuate signaling through JAK/STAT cascades. To assess the relative expression of transcripts for this gene family in EOS, we stimulated EOS_{PB} with IL-5 family cytokines and determined mRNA expression for the SOCS family members *CISH* and *SOCS1*. We found that stimulation with any of the IL-5 family cytokines results in a significant increase in expression of *CISH* and *SOCS1* transcripts, with increases varying between ~2.5- and 10-fold compared with control treated EOS_{PB} (Fig. 4A, 4B). Immunoblotting confirmed that

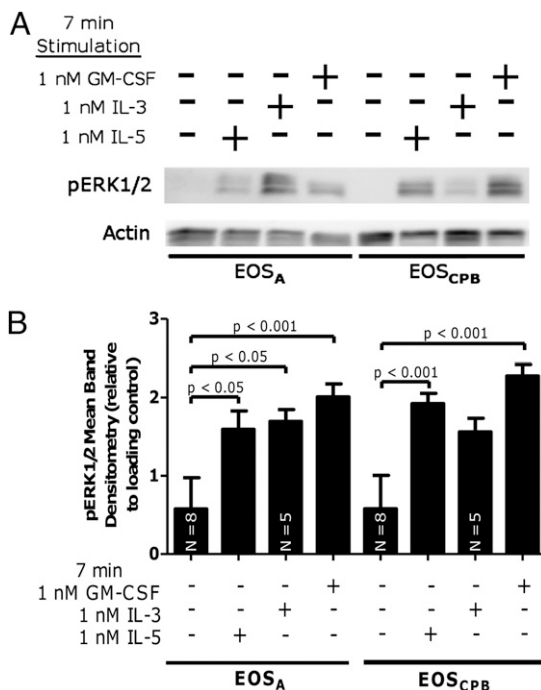


FIGURE 1. IL-5 family cytokine–induced ERK1/2 activation is unaltered in EOS_A. **(A)** Representative blot of EOS_A (lanes 1–4) and EOS_{CPB} (lanes 5–8) stimulated for 7 min with media $\pm$ 1 nM GM-CSF, 1 nM IL-3, or 1 nM IL-5. **(B)** Pooled data, $n = 7$ unless indicated on bar, corrected with loading control and log-transformed. Error bars indicate SEM. p values from one-way ANOVA.

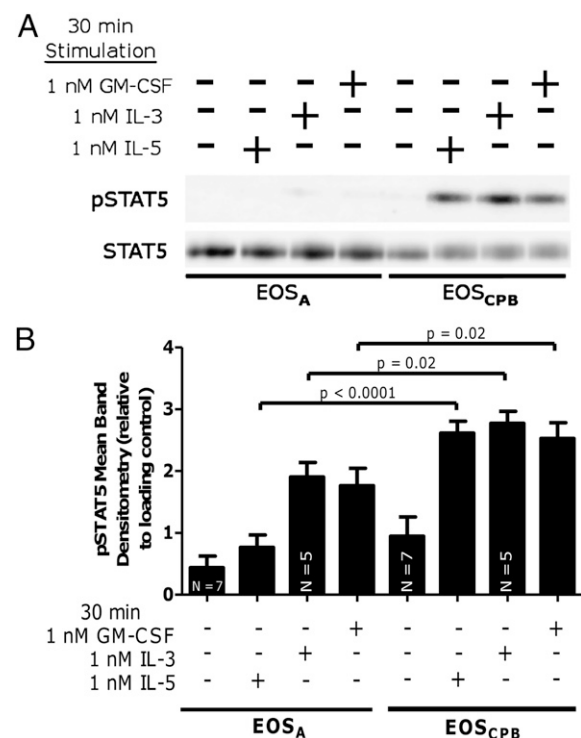


FIGURE 2. IL-5 family cytokine–induced STAT5 activation is attenuated in EOS_A. **(A)** Representative blot of EOS_A (lanes 1–4) and EOS_{CPB} (lanes 5–8) stimulated for 30 min with media $\pm$ 1 nM GM-CSF, 1 nM IL-3, or 1 nM IL-5. **(B)** Pooled data, $n = 6$ unless indicated, corrected with loading control and log-transformed. Error bars indicate SEM. p values from mixed-effect ANOVA.

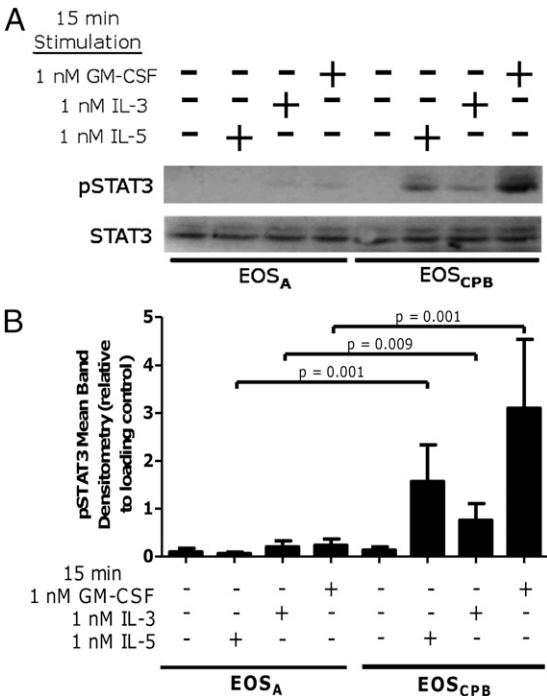


FIGURE 3. IL-5 family cytokine-induced STAT3 activation is attenuated in EOS_A. **(A)** Representative blot of EOS_A (lanes 1–4) and EOS_{CPB} (lanes 5–8) stimulated for 15 min with media ± 1 nM GM-CSF, 1 nM IL-3, or 1 nM IL-5. **(B)** Pooled data, $n = 5$, corrected with loading control. Error bars indicate SEM. p values from mixed-effect ANOVA.

each IL-5 family cytokine upregulates CISH protein expression at statistically significant levels ($n = 9$; IL-5, $p < 0.05$; IL-3, $p < 0.001$; and GM-CSF, $p < 0.05$) (Fig. 4C, 4D).

EOS isolated after SBP-Ag express more CISH/SOCS1 compared with unchallenged EOS

Given our observations that IL-5 family members induce SOCS family gene expression and that EOS_A are refractory to IL-5 family

cytokine-mediated STAT3/5 phosphorylation, we examined basal mRNA expression of *CISH* and *SOCS1* and protein levels of CISH from untreated EOS_A compared with untreated EOS_{CPB} and EOS_{PB}. At baseline, EOS_A exhibit statistically elevated *CISH* and *SOCS1* mRNA compared with EOS_{CPB} isolated from the same individual ($p = 0.005$ and $p = 0.03$, respectively) (Fig. 5A, 5B). Interestingly, mRNA transcripts from EOS_{CPB} are also statistically elevated compared with expression levels from EOS_{PB} at baseline (*CISH*, $p = 0.004$; *SOCS1*, $p = 0.001$) (Fig. 5A, 5B). This could suggest that the modest systemic elevation of these cytokines in acute inflammation causes a low-level activation of EOS. Immunoblotting for CISH protein confirmed the mRNA expression results, showing elevated CISH protein in EOS_A compared with undetectable-to-low protein levels in both EOS_{CPB} and EOS_{PB} ($n = 3$; $p < 0.05$, $p < 0.001$, respectively) (Fig. 5C).

EOS_{PB} exposed to GM-CSF recapitulate characteristic insensitivity of EOS_A to IL-5 family cytokine-induced STAT5 phosphorylation

In vitro priming, achieved by exposing purified EOS_{PB} to low concentrations of IL-5 family cytokines, recapitulates many behaviors/properties observed in EOS_A, which are considered to be primed in vivo (1, 5, 8, 30, 38). Among other changes to EOS biology, as mentioned previously, priming EOS_{PB} imparts decreased responsiveness to IL-5 family cytokines (24) through a relatively poorly understood mechanism that warrants additional research. To further our understanding of this decreased IL-5 family sensitivity (and therefore the process by which EOS_{PB} progress to an EOS_A state), EOS_{PB} were pretreated for 24 h with media, IL-5, GM-CSF, or IL-3, then restimulated for 30 min with media, IL-5, GM-CSF, or IL-3, and p-STAT5 was assessed. As hypothesized, 24-h media pretreatment permits each IL-5 family cytokine to induce significant levels of p-STAT5 after 30-min treatment relative to media-alone restimulation ($p < 0.0001$ for each cytokine; Fig. 6). Interestingly, 24 h GM-CSF pretreatment blocks any significant elevation of p-STAT5 relative to media-stimulated, regardless of which IL-5 family cytokine was used to restimulate. Furthermore, 24-h pretreatment with IL-5, IL-3, or

FIGURE 4. IL-5 family cytokines induce *CISH* and *SOCS1* gene mRNA and protein in EOS_{PB}. EOS_{PB} were purified and treated for 2 h with 1 nM GM-CSF, IL-3, or IL-5, then mRNA was extracted and purified, or lysates were prepared for immunoblots. Real-time PCR was used to quantify *CISH* **(A)** and *SOCS1* **(B)** mRNA levels (relative to housekeeping gene *GUSB*) in five sample sets. Whiskers indicate range, box indicates the inner quartiles, and central bar indicates the mean; p values from mixed-effect ANOVA. **(C)** Lysates were immunoblotted for CISH with pooled data $n = 9$ and normalized to actin. **(D)** Data displayed fold change from IL-5 treated. Error bars indicate SEM. p values from one-way ANOVA.

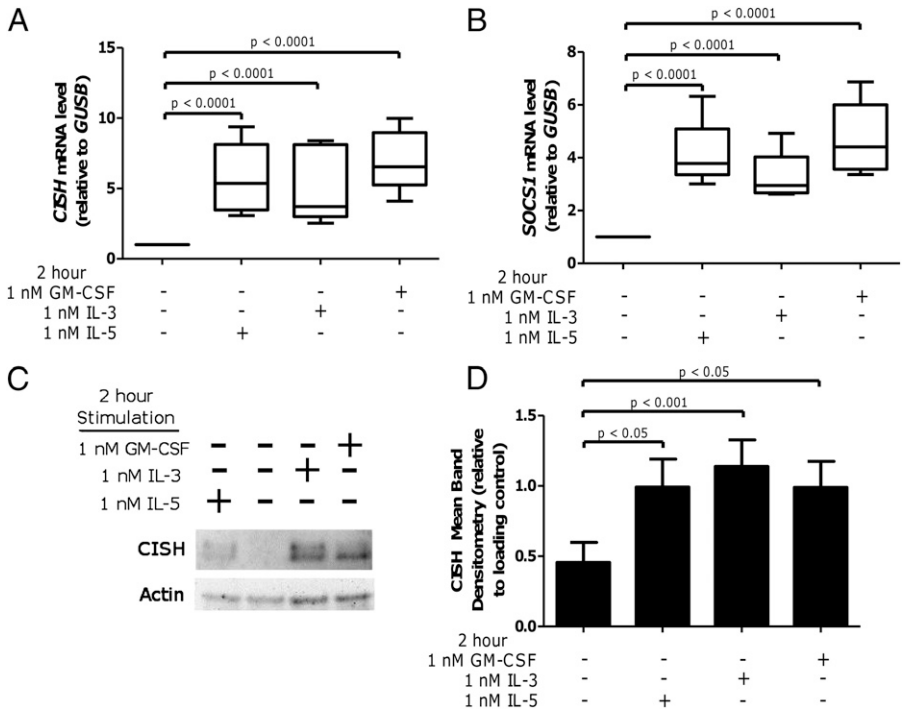
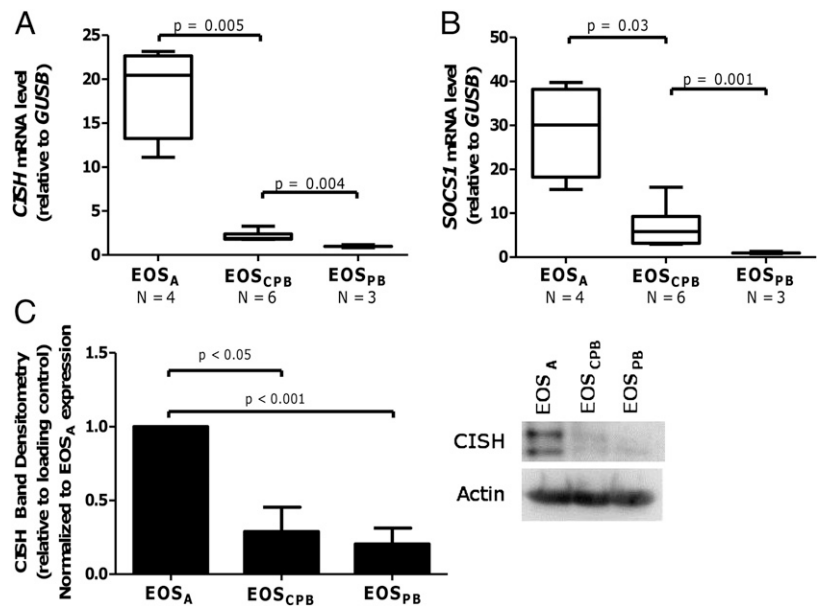


FIGURE 5. Compared to peripheral blood, EOS_A exhibit elevated SOCS family members *SOCS1* and *CISH*. Real-time PCR was used to quantify *CISH* (A) and *SOCS1* (B) mRNA levels (relative to *GUSB*) in indicated number EOS_A and peripheral blood EOS (EOS_{CPB} and EOS_{PB}). Whiskers indicate range, box indicates the inner quartiles, and central bar indicates the mean; *p* values from mixed-effect ANOVA. (C) Pooled data (*n* = 3) of *CISH* protein expression in purified nonstimulated EOS_A, EOS_{CPB}, and EOS_{PB}, normalized to actin. Data display fold change from EOS_A expression. Error bars indicate SEM. *p* values from one-way ANOVA. Right panel: representative immunoblot.



GM-CSF inhibits IL-5 restimulation from significantly elevating p-STAT5 significantly above the respective media-alone stimulation in agreement with previously discussed decreases in IL-5R α surface expression. In either IL-5 or IL-3 pretreatment conditions, both IL-3 and GM-CSF restimulation significantly elevates p-STAT5 (IL-5 priming: *p* < 0.001 both GM-CSF and IL-3; IL-3 priming: *p* < 0.001 both GM-CSF and IL-3) (Fig. 6).

Discussion

IL-5 family cytokines modulate many EOS functions, including enhancing the inflammatory capacity and potentiating survival of the cell. To better manage certain inflammatory diseases, including

allergic asthma, it is critical to better understand the phenotypic differences between EOS_A and EOS_{PB}. Elucidating mechanisms underlying the phenotypic differences would potentially provide insight into the efficacy of a range of therapeutics currently used as well as provide molecular targets for manipulation to alleviate EOS-associated symptoms present in allergic disorders like asthma. The goal of the current study was to determine the effect of IL-5 family cytokines on modulation of intracellular signaling in both EOS_A and EOS_{CPB}/EOS_{PB} in terms of p-ERK1/2, p-STAT5, and p-STAT3 and expression of SOCS family members *CISH* and *SOCS1*.

Accordingly, we find that EOS_A are refractory to IL-5 family-induced p-STAT3 and p-STAT5 while inducing p-ERK1/2 at levels with no significant difference to that induced by IL-5 family cytokines in EOS_{CPB}. Furthermore, expression levels of SOCS family members *CISH* and *SOCS1* and *CISH* protein are elevated by IL-5 family cytokine stimulation. Interestingly, we observed an elevation of both *CISH* and *SOCS1* mRNA and *CISH* protein in EOS_A relative to both EOS_{CPB}/EOS_{PB}, with EOS_{CPB} expressing significantly more *CISH* and *SOCS1* mRNA compared with EOS_{PB}. Also, 24-h IL-5 family cytokine pretreatment of EOS_{PB} attenuated the ability of IL-5 restimulation to induce p-STAT5. Furthermore, GM-CSF pretreatment additionally inhibited p-STAT5 induction by both IL-3 and GM-CSF. It is intriguing that only GM-CSF pretreatment attenuated IL-5 family restimulation of STAT5, regardless of the fact that all three IL-5 family cytokines induced *CIS1* and *SOCS1* genes. These collective data, gathered entirely from human donor samples from blood draws and/or BAL post-SBP-Ag, collectively point to a complex and specific system/mechanism of regulation that may influence EOS physiology and therefore inflammatory capacity and enhanced survival.

Given the differences our laboratory and others (23, 27, 28) have observed between EOS_A and EOS_{CPB}/EOS_{PB}, alterations in receptor subunit expression for the IL-5, GM-CSF, and IL-3R α -chains and the common β -chain following SBP-Ag cannot completely explain the general refractory nature of EOS_A toward IL-5 family cytokines. For example, although the expression levels of the common β -chain are decreased on EOS_A, GM-CSF still induces release of eosinophil-derived neurotoxin, whereas IL-5 cannot (27). If attenuation of downstream signaling was largely based on receptor common β -chain expression levels, we would hypothesize that phosphorylation of ERK1/2, STAT3, and STAT5 would

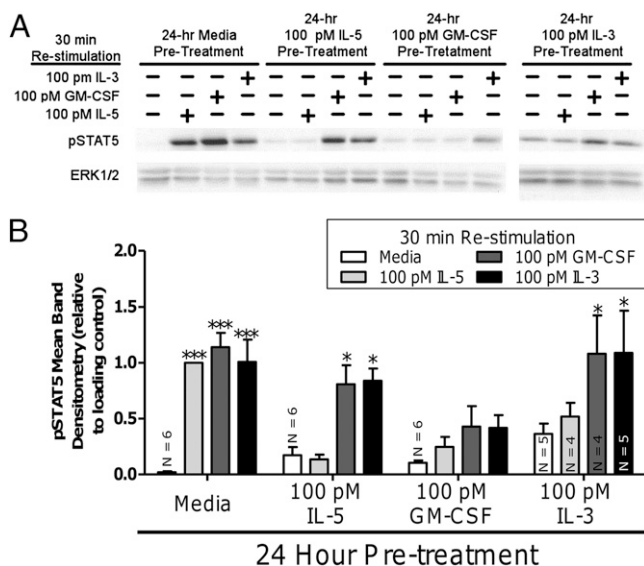


FIGURE 6. Pretreating human EOS_{PB} with GM-CSF recapitulates down-regulation of IL-5 family-activated p-STAT5 in EOS_A. (A) Human EOS_{PB} were treated 24 h with media (lanes 1–4), 100 pM IL-5 (lanes 5–8), 100 pM GM-CSF (lanes 9–12), or 100 pM IL-3 (lanes 13–16), then restimulated with 100 pM IL-5, 100 pM GM-CSF, or 100 pM IL-3, and probed for active STAT5. (B) Pooled data from five experiments, unless indicated on bar, was analyzed via two-way ANOVA using post hoc Bonferroni analysis *p* values: **p* < 0.05, ****p* < 0.0001 compared with respective media, 24-h pretreatment.

all be decreased in response to all IL-5 family cytokines in EOS_A. If the downstream signaling attenuation was due to alterations in the ligand-specific α -chains, it would follow that only IL-5-induced signaling only would be attenuated, with both IL-3- and GM-CSF-induced signaling potentially increasing due to the elevated surface expression. However, neither of these models fit with the observations. Another plausible explanation would be that the decreased signaling may be a function of a threshold response needed for activation of one pathway versus another or potentially represent a specific signaling pathway regulatory event. We know that IL-3 and GM-CSF have functional receptors on EOS_A (27), which led us to test the hypothesis of altered signaling instead of pursuing the idea of an activation threshold. Also, the concentration of IL-5, IL-3, and GM-CSF used in the STAT3/5 and ERK1/2 phosphorylation experiments is in excess of the K_D values of each receptor/ligand pairing (13, 22, 39–41). Our data indicate that phosphorylation of STAT5 and STAT3 is selectively attenuated in EOS_A relative to ERK1/2 phosphorylation. These observations are in line with previous findings that indicate even after long-term exposure to IL-5, additional stimulation with IL-5 can phosphorylate MEK (24), indicating MEK/ERK signaling remains intact. We therefore hypothesized there is/are selective mechanism(s) that differentially affect(s) responses to these cytokines in the signaling pathways under consideration.

Our laboratory and others (1, 5, 20, 30, 38, 42, 43) have found that in vitro stimulation of EOS_{PB} with IL-5 family cytokines recapitulates certain signaling and physiological behaviors of EOS_A regarding the enhanced responsiveness to proinflammatory chemokines, which EOS_{PB} are normally minimally responsive to. In this study, our data indicate that in vitro IL-5 family pretreatment of EOS_{PB} is able to also recapitulate to different degrees the decreased responsiveness to IL-5 family-induced p-STAT5 observed in EOS_A, proving a valuable model for EOS_A, which are far less readily available than EOS_{PB}. Although the data in this study regarding the IL-5 family pretreatment indeed only represent a small subset of EOS_A physiology, given the extremely limited literature surrounding the refractory nature of EOS_A relative to IL-5 family stimulation (24, 27), this in vitro EOS_{PB} system provides an exciting opportunity to research the EOS behavior upon IL-5 family exposure.

Reports from other immune cell systems have revealed that the SOCS family genes are upregulated following STAT-induced transcriptional activation and selectively downregulate signaling through JAK/STAT pathways (31, 34). Our data indicate that relative to EOS_{CPB}/EOS_{PB}, EOS_A express elevated levels of *CISH* and *SOCS1* mRNA and CISH protein. These data are in line with previous studies that indicate IL-5 and GM-CSF can induce *CISH* mRNA in human EOS_{PB} (18) as well as systems in which SOCS family members downregulate JAK/STAT signaling (31, 33, 34). For example, CISH has been found to block STAT5b activity by binding to activating/dockings sites on signaling receptors (44, 45), whereas other members of the SOCS family proteins (i.e., SOCS1, SOCS2, and SOCS3) additionally block STAT5b activity (44). Interestingly, SOCS1 is capable of binding directly to the activating loop of JAK2 (46), thereby inhibiting several JAK/STAT-regulated signaling systems (reviewed in Ref. 47). These previous data, combined with our findings, therefore present a possible mechanism for the JAK/STAT selective attenuation of signaling in response to IL-5 family cytokines in human EOS. Furthermore, based on our findings that all three IL-5 family cytokines induce *CIS1* and *SOCS1* mRNA products as well as CISH protein, we theorize that the systemic elevation of IL-5 family cytokines in disorders like allergic asthma may alter the responsiveness and inflammatory capacity of EOS_A in distinct ways, which is further supported by the results that EOS_{CPB} ex-

press significantly more *CISH* and *SOCS1* mRNA than the non-challenged EOS_{PB}. Additional support for the idea that expression of SOCS family members modulates EOS activation comes from a murine model of allergic airway inflammation. Deletion of *Socs1* in an *Ifng* knockout mouse showed an overall intensification of asthma-like symptoms, including elevated EOS recruitment to the lungs, enhanced IgE serum levels, and increased cytokine and gene transcript production compared with the *Ifng* knockout alone (35), suggesting that in humans, *SOCS1* could play a crucial role in normal regulation of airway inflammation and EOS_{PB} recruitment.

In terms of MEK/ERK signaling, IL-5 family cytokines are known to enhance EOS responsiveness to secondary stimuli that signal via G-protein-coupled receptors (GPCRs) (4, 38, 43, 48). Because GPCRs often exhibit extensive cross-talk with MEK/ERK signaling pathways (49), including chemokine-induced GPCR signaling in EOS_A (30), it would be crucial that many MEK/ERK signaling molecules remain signal-ready, which our observations support.

Given these data, future studies are necessary to further characterize the functional alterations that are observed between EOS_A and EOS_{PB}, including investigation of physiological end points and expression of any additional SOCS family members. One promising SOCS family member is SOCS3, which López and colleagues (32) found is inducible at the mRNA level in EOS_{PB} and elevated in EOS from asthmatics and patients with eosinophilic bronchitis when compared with EOS from healthy control donors. Furthermore, SOCS3 has been observed to perform similar functions to both CISH and SOCS1, blocking JAK2 tyrosine kinase activity, and has been linked to suppression of STAT3 activity (reviewed in Ref. 47). The overlapping nature of these SOCS family members and these *SOCS3* data further support the idea that there is a very finely tuned regulation system in these cells in which the smallest variations may lead to disease states and that further research is warranted.

In summary, this study supports the idea that phenotypic differences observed between EOS_{PB} and EOS_A may be explained as functions of alterations in the signaling pathways that are modulated by IL-5 family cytokines. These cytokines serve to both recruit and activate EOS_{PB} while providing negative-feedback mechanisms to regulate the inflammatory response of recruited EOS. This negative-feedback mechanism perhaps protects against inappropriate activation once the EOS_{PB} have migrated to the site of allergic inflammation and are thus inundated with proinflammatory cytokines, including those of the IL-5 family. Manipulation of these regulatory pathways may provide new and novel therapeutics to alleviate symptoms in eosinophil-driven disorders such as allergic asthma.

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Disclosures

The authors have no financial conflicts of interest.

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Supplemental Figure 1: **Eosinophil purification process does not alter baseline or IL-5 family cytokine stimulated pERK1/2, pSTAT5, or pSTAT3 levels.** (A) Representative blot of EOS_{PB} stimulated for 7 minutes with media +/- 1 nM IL-5, 1 nM IL-3, or 1 nM GM-CSF. (B) Representative blot of EOS_{PB} stimulated for 30 minutes with media +/- 1 nM IL-5, 1 nM IL-3, or 1 nM GM-CSF. (C) Representative blot of EOS_{PB} stimulated for 15 minutes with media +/- 1 nM IL-5, 1 nM IL-3, or 1 nM GM-CSF.

Supplemental Figure 1

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