



Activation of Human Basophils by A549 Lung Epithelial Cells Reveals a Novel IgE-Dependent Response Independent of Allergen

John T. Schroeder and Anja P. Bieneman

J Immunol published online 26 June 2017

<http://www.jimmunol.org/content/early/2017/06/24/jimmunol.1700055>

This information is current as of July 2, 2017.

Supplementary Material	http://www.jimmunol.org/content/suppl/2017/06/24/jimmunol.1700055.DCSupplemental
Subscription	Information about subscribing to <i>The Journal of Immunology</i> is online at: http://jimmunol.org/subscription
Permissions	Submit copyright permission requests at: http://www.aai.org/About/Publications/JI/copyright.html
Email Alerts	Receive free email-alerts when new articles cite this article. Sign up at: http://jimmunol.org/alerts



Activation of Human Basophils by A549 Lung Epithelial Cells Reveals a Novel IgE-Dependent Response Independent of Allergen

John T. Schroeder and Anja P. Bieneman

Evidence for epithelial cell (EC)-derived cytokines (e.g., thymic stromal lymphopoietin [TSLP]) activating human basophils remains controversial. We therefore hypothesize that ECs can directly activate basophils via cell-to-cell interaction. Basophils in medium alone or with IL-3 $\pm$ anti-IgE were cocultured with TSLP, IL-33, or IL-25. Analogous experiments cocultured basophils (1–72 h) directly with EC lines. Supernatants were tested for mediators and cytokines. Abs targeting receptors were tested for neutralizing effects. Lactic acid (pH 3.9) treatment combined with passive sensitization tested the role of IgE. Overall, IL-33 augmented IL-13 secretion from basophils cotreated with IL-3, with minimal effects on histamine and IL-4. Conversely, basophils (but not mast cells) released histamine and marked levels of IL-4/IL-13 (10-fold) when cocultured with A549 EC and IL-3, without exogenous allergen or IgE cross-linking stimuli. The inability to detect IL-33 or TSLP, or to neutralize their activity, suggested a unique mode of basophil activation by A549 EC. Half-maximal rates for histamine (4 h) and IL-4 (5 h) secretion were slower than observed with standard IgE-dependent activation. Ig stripping combined with passive sensitization $\pm$ omalizumab showed a dependency for basophil-bound IgE, substantiated by a requirement for cell-to-cell contact, aggregation, and Fc ϵ RI-dependent signaling. A yet unidentified IgE-binding lectin associated with A549 EC is implicated after discovering that LacNAc suppressed basophil activation in cocultures. These findings point to a lectin-dependent activation of basophil requiring IgE but independent of allergen or secreted cytokine. Pending further investigation, we predict this unique mode of activation is linked to inflammatory conditions whereby IgE-dependent activation of basophils occurs despite the absence of any known allergen. *The Journal of Immunology*, 2017, 199: 000–000.

Human basophils secrete inflammatory mediators and cytokines central to the pathogenesis of allergic disease (1). In particular, preformed histamine and newly generated leukotriene C4 are released within minutes upon cross-linking of the high affinity IgE receptor, Fc ϵ RI. This mode of activation also results in IL-4 production de novo, but with secretion first evident after 1 h and peaking by $\sim$ 4 h. Basophils also secrete IL-13 in response to IgE-dependent activation, but are generally more proficient in making this cytokine in response to IL-3.

The notion of basophils being a significant source of IL-4 has been substantiated with development of IL-4 reporter mouse strains, which clearly demonstrates the capacity of these cells to secrete this cytokine in vivo (2, 3). In fact, several studies have shown that basophils, by producing IL-4, facilitate the pro-Th2

activities of other immune cells, including T cells (4), B cells (5), monocytes (6, 7), eosinophils (8), and, most recently, type 2 innate lymphoid cells (9).

Although studies clearly indicate that human and mouse basophils are prolific producers of IL-4, differences persist between the two species regarding the stimuli that induce this cytokine. In vitro production of IL-4 by human basophils is tightly coupled to IgE-mediated activation and such responses are greatly augmented by IL-3 (1). In contrast, studies in mice show that epithelial cell (EC)-derived cytokines, particularly thymic stromal lymphopoietin (TSLP), are essential components in eliciting IL-4 from basophils (10). Indeed, several studies support the existence of a so-called basophil-TSLP axis whereby the latter plays an important role in conditioning basophils for IL-4 production (10, 11). Although evidence for this axis in humans has been less forthcoming, one group has recently reported on TSLP's capacity to activate basophils from asthmatic subjects: a response dependent on IL-3 (12). These authors additionally report that other EC-derived cytokines (IL-25 and IL-33) activate basophils from asthmatics, particularly post allergen challenge (13).

Therefore, in this study we probed for additional evidence of whether EC-derived cytokines activate human basophils. Among those investigated (e.g., TSLP, IL-33, and IL-25), we found that only IL-33 mediates activity on human basophils, thus confirming earlier reports (14, 15). We also explored the hypothesis that basophils might be activated by EC via other mechanisms, either through direct interaction and/or by unique cytokines or factors. As a result, our investigations revealed an unexpected finding that basophils cocultured with the lung-derived EC line, A549, are activated to release histamine but in a delayed manner (2–5 h) unlike classic mediator release via IgE-dependent activation. Basophils also generated large quantities of IL-4 and IL-13 in

Division of Allergy and Clinical Immunology, Department of Medicine, Johns Hopkins Asthma and Allergy Center, Johns Hopkins University School of Medicine, Baltimore, MD 21224

Received for publication January 10, 2017. Accepted for publication May 25, 2017.

This work was supported in part by Public Health Services Research Grants AI115703 and R21AI121766 to J.T.S. from the National Institute of Allergy and Infectious Diseases, National Institutes of Health.

Address correspondence and reprint requests to Dr. John T. Schroeder, Unit Office 2, Johns Hopkins Asthma and Allergy Center, Johns Hopkins University School of Medicine, 5501 Hopkins Bayview Circle, Office 3A.12A, Baltimore, MD 21224. E-mail address: schray@jhmi.edu

The online version of this article contains supplemental material.

Abbreviations used in this article: BDC, basophil-depleted cell; BHR, basophil histamine release; CD4, culture-derived basophil; CIU, chronic idiopathic urticaria; DC, dendritic cell; EC, epithelial cell; Gal, galectin; MC, mast cell; PAG, PIPES, albumin, glucose; TSLP, thymic stromal lymphopoietin.

Copyright © 2017 by The American Association of Immunologists, Inc. 0022-1767/17/\$30.00

these cocultures. These responses were dependent on cell-to-cell interaction and aggregation, a requirement for basophil-bound IgE, and of signaling through FcεRI. Additional experiments revealed that LacNAc suppressed basophil activation in the cocultures, thus indicating the involvement of a yet unidentified IgE-binding lectin associated with A549 cells. Overall, these data provide evidence for a unique mechanism whereby basophils are potentially activated by IgE-binding lectins expressed on EC, but with secretion parameters very different from those seen with conventional IgE-dependent activation. Such a phenomenon could have mechanistic relevance to basophil participation in allergic conditions, including those not typically driven by allergen (e.g., urticaria and atopic dermatitis), but also in nonallergic conditions recently implicating basophil involvement (e.g., lupus and cancer).

Materials and Methods

Special reagents, buffers, and media

The following reagents were purchased: crystallized human serum albumin (Calbiochem-Behring, La Jolla, CA); PIPES, FCS, crystallized BSA, and LacNAc (Sigma-Aldrich, Allentown, PA); gentamicin, IMDM, and non-essential amino acids (Life Technologies, Grand Island, NY); Percoll (Pharmacia Biotech, Piscataway, NJ); rhIL-3 (Biosource, Camarillo, CA); rhTSLP, rhGM-CSF, rhIL-5, anti-galectin (Gal)-9 Ab and ELISAs (R&D Systems, Minneapolis, MN); anti-Gal-3 and IgG₁ isotype control (Santa Cruz, Dallas, TX); Gal-3 ELISAs (eBioscience, San Diego, CA). Polyclonal goat anti-human IgE (provided by Dr. R. Hamilton, Johns Hopkins University). The Btk inhibitor, Ibrutinib (PCI-32765), was purchased from Apexbio Technologies, Houston, TX. The selective syk inhibitor, 161y (16), was provided by Dr. D.W. MacGlashan, Jr., Johns Hopkins University. The specificity of these inhibitors for syk and Btk activity induced through FcεRI signaling is documented (17, 18).

ECs and culture conditions

The A549 and BEAS-2B epithelial cell lines were from the American Type Culture Collection, Manassas, VA. A549 (bronchial origin) were maintained in medium consisting of F-12K nutrient mixture: Kaigh's Modification, with 10% heat-inactivated FBS, and 1% penicillin streptomycin. BEAS-2Bs (lung origin) were maintained in DMEM or Ham's F-12 medium, with 5% heat-inactivated FBS and 1% penicillin streptomycin. Cultures were split twice weekly for the duration of these experiments, with no detectable changes in their capacity to activate basophils. To facilitate the coculture experiments described below, 5×10^3 ECs were plated into 96-well plates in 0.100 ml of the medium specific for each. These were incubated up to 48 h in a humidified incubator (37°C, 5% CO₂) to allow cells to adhere and achieve 50–80% confluency.

Basophil purification

Venipuncture was performed on consenting adults (age 21–65 y) using a protocol approved by the Johns Hopkins University Institutional Review Board. Subjects were selected regardless of allergic status. An additional source of basophils included residual Trima cassettes from subjects undergoing platelet pheresis within the Hemapheresis Unit at Johns Hopkins University. Buffy coats from both specimen sources were subjected to double-Percoll density centrifugation, which produced both basophil-depleted cell (BDC) and basophil-enriched cell suspensions, as described (19, 20). Basophils were purified from the basophil-enriched cell suspensions using negative selection reagents (Stemcell Technologies, Vancouver, Canada) and immunomagnetic LS columns (Miltenyi Biotec, Gaithersburg, MD). Cell suspensions were routinely $\geq 99\%$ basophils, as assessed by Alcian blue staining (21).

In some experiments, basophil surface IgE was depleted (i.e., stripped) using lactic acid buffer (pH 3.9) (22). In brief, basophils were washed with 0.9% NaCl before resuspending in cold lactic acid (0.5 ml) for 1 min on ice. Cell suspensions were immediately flooded with cold PIPES, albumin, glucose (PAG) (~10 vol), equally portioned between two tubes and centrifuged. After removing supernatant, both pellets were resuspended in PAG containing heparin (10 μg/ml) and EDTA (4 nM), both added from a 50× stock. Those prepared for passive sensitization additionally received polyclonal JK IgE (500 ng/ml, provided by Dr. R. Hamilton, Johns Hopkins University) with or without omalizumab (10 μg/ml). A third tube containing an equal number of basophils not treated with lactic acid was

prepared but did not receive JK IgE. All tubes were incubated at 37°C for 30 min. Cells were washed twice with PAG before counting and adjusting cell densities in conditioned IMDM.

Culture-derived basophils and mast cells

BDC suspensions produced during density centrifugation (see above) were used as a source of PBMC for isolating CD34⁺ cells by positive selection (Miltenyi Biotec). When starting with $2-4 \times 10^9$ BDC obtained from the Trima cassettes, $2-5 \times 10^6$ enriched CD34⁺ cells were typically prepared. These CD34⁺ cells were differentiated into basophil-like cells by culturing 2 wk in IL-3 (10 ng/ml) alone or into mast cells (MC) after 8–10 wk in SCF/IL-6 (IL-3 during the first week), as described elsewhere (23, 24).

Flow cytometry

In some instances, EC were investigated for Gal-3 expression using flow cytometry. In brief, adherent cells were removed from culture flasks using EDTA without trypsin and then washed with PBS. Cells were prepared for surface and intracellular staining using 4% paraformaldehyde or by the Fix & Perm kit (Invitrogen) (25). Blocking was done using 1% human IgG. Anti-Gal-3 Ab (clone B2C10; Santa Cruz Biotechnology) and IgG₁ isotype control (clone MOPC-21) were each used at 10 μg/ml in 20 min incubations at room temperature. Cells were then washed before staining 30 min on ice with anti-IgG₁-Alex647 (2.5 μg/ml). Flow cytometry was performed using a FACSCalibur machine.

Real-time RT-PCR

Total RNA was isolated using the RNA-Bee protocol (Tel-Test, Friendswood, TX). After isopropanol precipitation, RNA was washed with ethanol before resuspending in DNase-free water. It was then subjected to RNA cleanup (Qiagen, Germantown, MD) and quantified using Nanodrop. Quantitative RT-PCR was performed as described (26), using validated primer and probe combinations for Gal-1, -3, -9 (Applied Biosystems, Foster City, CA).

Co-culture conditions

Cultures were conducted in IMDM supplemented with 5% FCS, non-essential amino acids, L-glutamine, 10 μg/ml gentamicin (pH 7.2–7.4) (conditioned IMDM). Depending on the set of experiments, basophils or MC (1.0×10^5) were added in 0.100 ml volumes to 96-well plates, plated with EC or without, both at 0.100 ml volumes in the F-12K nutrient mixture. Immediately after adding basophils or MC, 0.050 ml of 5× stimulus (e.g., medium, IL-3/SCF, anti-IgE, or a combination) was added and the cultures incubated as indicated at 37°C, 5% CO₂. In some experiments, LacNAc or inhibitors were added to EC before the addition of basophils and stimuli. Several experiments were done using Corning polycarbonate 0.4 μm transwell plates (Sigma-Aldrich, St. Louis, MO). Combinations were set up whereby bottom or insert wells were seeded with EC. Basophils were then added the next day to the opposite well to prevent direct contact with EC, or added directly with EC. Stimuli were added to bottom wells, the inserts, or both before culturing at 37°C, 5% CO₂. Supernatants were harvested after 20 h unless otherwise indicated and tested for cytokine secretion and basophil histamine release (BHR).

Histamine and cytokine measurements

Supernatants were analyzed for IL-4/IL-13 protein by ELISA (eBioscience), and histamine by automated fluorimetry (19, 27).

Statistical analysis

Statistical analyses were performed with Prism 7.0 software (GraphPad Software, LaJolla, CA). Analyses were performed using Wilcoxon non-parametric and paired *t* test analyses unless specified otherwise. Differences were considered statistically significant at a *p* value <0.05.

Results

We first investigated several recombinant EC-derived cytokines to test their capacity to activate human basophils and to establish a pattern of responsiveness to compare in the subsequent EC-basophil cocultures. Included in our analyses were TSLP, IL-25, and IL-33. Both TSLP and IL-25 showed no capacity to induce IL-4/IL-13 secretion when incubated for 20 h, and IL-33 alone was only marginal in inducing detectable levels of these cytokines (Supplemental Fig. 1). IL-3 induces IL-13 (>IL-4) from human

basophils (28, 29), and this was once again demonstrated in this study (Supplemental Fig. 1). However, adding TSLP or IL-25, along with IL-3, did not further augment IL-13 or IL-4 secretion. In contrast, IL-33 markedly increased IL-13 secretion in response to IL-3, and did so over several doses, consistent with findings published elsewhere (14, 15). A similar pattern was seen for IL-4 secretion, although levels of this cytokine, as predicted, were ~8- to 10-fold less than those for IL-13. None of the cytokines directly induced histamine release, and only IL-33 showed a marginal capacity to enhance this mediator when combined with IgE-dependent activation (data not shown).

The inability of TSLP to stimulate human basophils was not from a lack of biological activity, because it proved capable of inducing OX40L on myeloid dendritic cells (DCs) (data not shown). In addition, TSLPR was inducible on basophils following IgE-dependent activation, as reported elsewhere (30), with similar levels induced following incubation with IL-3 (data not shown). Thus, despite induction of TSLPR by these stimuli, adding TSLP did not yield a detectable response.

A549 EC-dependent activation of human basophils

In vitro coculture approaches have demonstrated that EC can directly activate MC, in part, via TSLP (31, 32). In addition, the activity of some cytokines, such as IL-15, is mediated through a phenomenon known as transpresentation, whereby they are presented *in trans* to other cells expressing the required receptors (33). Because TSLP is classified in the same family of cytokines as IL-15, we considered the possibility that it too may function through transpresentation, in this instance, by EC to basophils. We therefore conducted experiments whereby basophils were cocultured with lung-derived EC lines to allow direct cell-to-cell contact. By focusing on IL-4 secretion, we made several surprising observations with A549 EC, as shown in Fig. 1. First, the amount of IL-4 secreted after 20 h incubation was significantly greater in the cocultures compared with those with basophils alone. Although this was true regardless of the mode of stimulation, IL-4 was unusually high in cocultures receiving IL-3 where levels exceeded those without EC nearly 10-fold (332 ± 74 versus 37 ± 7 pg per 10^6 basophils, $p = 0.0005$, $n = 12$). For comparison, relatively low levels of IL-4 were detected when basophils were cultured in medium alone, but these increased ~7-fold if cocultured with EC (4 ± 1 versus 27 ± 10 pg per 10^6 basophils, $p = 0.0078$). Even the high levels of IL-4 produced in response to anti-IgE stimulation were consistently increased ~35% when basophils were cocultured with EC (225 ± 42 versus 304 ± 51 , $p < 0.001$). In contrast, coculturing basophils with BEAS-2B EC did not show

any effects on cytokine secretion, compared with basophils stimulated alone (Supplemental Fig. 2). Experimental controls indicated that A549 EC did not contribute to the production of IL-4, even when treated with supernatants from activated basophils (data not shown).

IL-3 priming of basophils augments their activation by EC

The capacity of IL-3 to augment IL-4 (and IL-13) in the basophil/A549 cocultures was further investigated by titrating its effects and comparing it to other growth factors. As shown in Fig. 2A, IL-4 levels secreted by basophils after 20 h incubation in IL-3 (1–100 ng/ml) averaged less than ~30 pg per 10^6 cells. However, these increased 10-fold when conducting the same experiment in the presence of A549 EC. Even IL-3 concentrations (0.1 ng/ml) not inducing detectable IL-4 from basophils when used alone, synergized with EC to result in levels approaching 100 pg per 10^6 basophils. Marked levels of IL-13 were detected in the cocultures, with levels exceeding ~10-fold those produced by basophils stimulated with IL-3 alone (Fig. 2B). Other cytokines, including GM-CSF, IL-5, and TSLP did not synergize with A549 EC to activate basophils (data not shown).

With the possibility that IL-3 additionally acted on A549 ECs, experiments were carried out whereby basophils were first primed with IL-3 (10 ng/ml) overnight, washed extensively, and then cocultured for 20 h with EC. As shown in Fig. 2C, no detectable IL-4 was produced by the primed basophils cultured without EC. In contrast, IL-3-primed basophils cocultured with EC secreted IL-4 in excess of 100 pg per 10^6 basophils: a response not significantly increased by adding IL-3 (or IL-33). As expected, primed basophils readily secreted IL-4 when stimulated by anti-IgE, with a trend for greater production if the cells were cocultured with EC. A similar pattern occurred for IL-13 production, with overall levels two to three times greater than those for IL-4 (Fig. 2D). IL-13 secreted by IL-3-primed basophils was not augmented by IL-33, whether cocultured alone or with EC, despite the fact that IL-3 and IL-33 synergized to induce IL-13 from basophils when both were added together (Supplemental Fig. 1).

Delayed time course for BHR and cytokine secretion in basophil/A549 EC cocultures

Although human basophils secrete IL-4 in response to IL-3, the levels produced are generally far lower than those seen with IgE-dependent activation (28). This latter mode of stimulation is also typically preceded by histamine release, which occurs within 30 min of activation, whereas IL-4 is secreted over several hours. Thus, the relatively high levels of IL-4 produced in the cocultures prompted testing to show its time course and whether BHR becomes evident with IL-3, an IgE-independent stimulus. The time course for maximal BHR (~30 min) and IL-4 secretion (~4 h) was as expected in control cultures with basophils alone stimulated with an activating anti-IgE Ab (Fig. 3A, 3B). Likewise, little to no BHR and IL-4 (above the medium control) was released by basophils alone in response to IL-3. In sharp contrast, both mediators were secreted in basophil/A549 cocultures, especially in response to IL-3, the major difference being their time course, with nearly twice the time for IL-3-induced release relative to the kinetics seen with IgE-dependent activation. For comparison, basophils, which typically secrete IL-13 whether stimulated with anti-IgE or IL-3 (as shown in Fig. 3C), generated on average ~25-fold greater levels of this cytokine when cocultured with EC in the presence of IL-3 (Fig. 3F). As a result of these findings, all subsequent experiments continued to look at cytokine secretion after 20 h, because this time allowed for peak or near-peak secretion. Those experiments investigating histamine release focused on times of

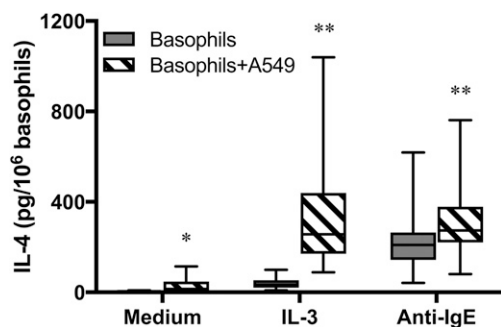


FIGURE 1. IL-4 production in basophil/EC cocultures. Basophils were cultured alone and with A549 EC in the presence of medium, IL-3 (10 ng/ml), or activating anti-IgE (10 ng/ml). After 20 h incubation, supernatants were harvested and assayed for IL-4 protein by ELISA. Box-whisker plots shown ($n = 12$). * $p < 0.008$, ** $p \leq 0.001$ versus corresponding conditions with basophils alone.

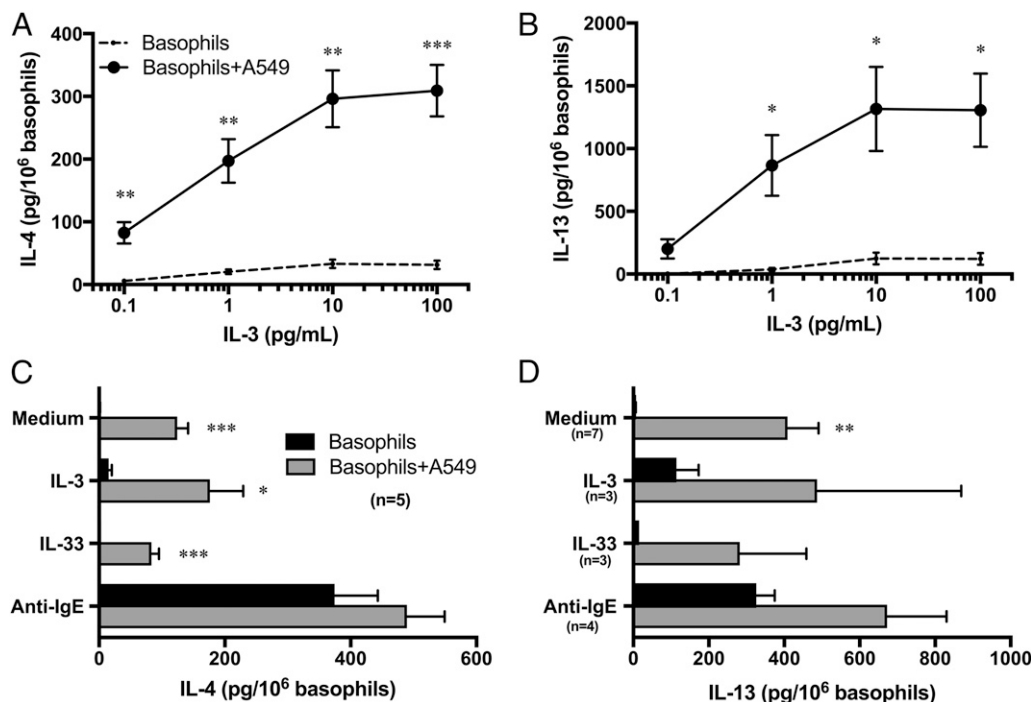


FIGURE 2. IL-3 parameters affecting IL-4/IL-13 production by basophil/A549 versus basophil alone. (A and B) The effect of IL-3 dosing on IL-4 and IL-13, respectively. (C and D) The effect of IL-3 (10 ng/ml) priming overnight followed by washing on IL-4 and IL-13 secretion, respectively, after rechallenging with IL-3, IL-33, anti-IgE (each at 10 ng/ml), or with medium alone. Supernatants were harvested (20 h) and assayed for cytokine by ELISA. Mean $\pm$ SEM, $n = 3-5$. * $p < 0.05$, ** $p < 0.009$, *** $p < 0.0008$.

up to 8 h, because peak responses for this preformed mediator were unlikely to extend beyond this time.

Cell-to-cell contact is required for A549 EC to activate basophils

Additional clues regarding the requirements for A549-dependent basophil activation were provided in experiments that produced negative results. First, supernatants from A549 EC did not support increased IL-4/IL-13 secretion from basophils, whether in the

absence or presence of IL-3 (data not shown). Second, attempts to detect TSLP and IL-33 in A549 supernatants were also negative, signifying that neither cytokine was secreted by A549 EC. Third, attempts to neutralize activity with Abs targeting TSLPR and IL-33R (ST2) did not suppress cytokine secretion in A549/basophil cocultures (data not shown). Collectively, these results argue against the likelihood that A549-derived cytokines (e.g., TSLP, IL-33) accounted for the marked induction of IL-4/IL-13 by basophils cocultured with the EC line. Of note, evidence of cell clumping in

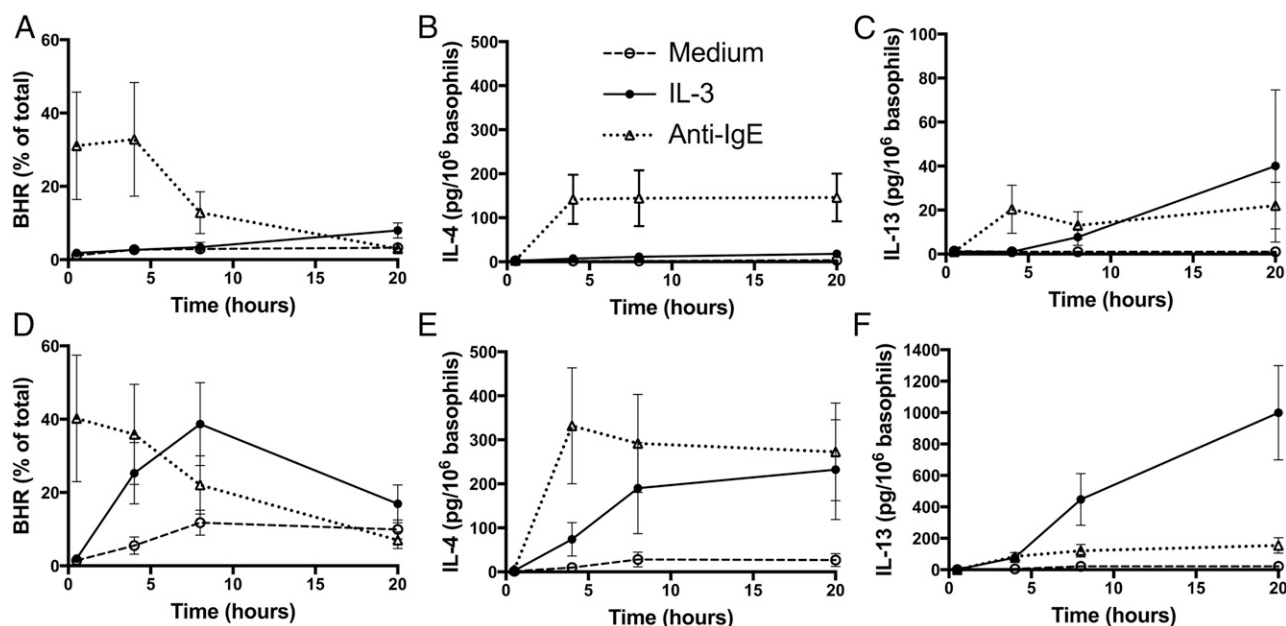


FIGURE 3. Delayed time course for BHR and cytokine secretion in basophil/A549 cocultures. Basophils cultured alone (upper panels) and with A549 EC (lower panels) were stimulated with medium, IL-3 (10 ng/ml), or anti-IgE (10 ng/ml). Supernatants were harvested at the indicated times and assayed for: (A and D) histamine, (B and E) IL-4, and (C and F) IL-13. Results are the mean $\pm$ SEM, $n = 3$.

the IL-3 treated cocultures was seen (Fig. 4A), prompting the hypothesis that cell-to-cell contact is necessary for A549 EC to activate basophils, a belief substantiated by conducting transwell experiments. As expected, coculturing basophils and A549 EC with IL-3 for 20 h resulted in high levels of IL-4 (Fig. 4B) and IL-13 (Fig. 4C). Under the same conditions, but separating the two cell types by transwells, the levels of both cytokines were no different from culturing basophils alone.

A549 EC-dependent activation of basophils is IgE dependent and requires FcεRI signaling

The combined observations of BHR and IL-4 secretion along with the need for cell-to-cell contact suggested that basophil-bound IgE is somehow involved. To address this hypothesis, we tested the effect of stripping IgE from basophils using lactic acid (pH 3.9) and restoring its expression via passive sensitization. Both IL-4 (Fig. 5C) and IL-13 (Fig. 5D) were significantly reduced in cocultures that included stripped basophils, compared with A549 cocultured with untreated basophils. Most remarkably, this was evident with IL-3 stimulation. Yet, the production of these cytokines was restored under the same conditions if stripped basophils were resensitized with IgE. The addition of omalizumab to neutralize IgE during passive sensitization prevented this from happening (Fig. 5E). For comparison, the results for these same parameters were as expected when tested in control cultures with basophils alone (Fig. 5A, 5B).

Given the requirement for basophil IgE expression, we next addressed whether FcεRI signaling is also involved by testing the effects of pharmacologically inhibiting syk and Btk tyrosine kinase activity. As shown in Fig. 5F, selective inhibitors of syk (161Y) and Btk (ibrutinib) used at IC₅₀s (50 and 5 nM, respectively) targeting standard IgE-dependent activation, likewise inhibited those induced by IL-3 in the cocultures. This latter response was inhibited nearly 90% by the Btk inhibitor, implying that this mode of activation is more sensitive to Btk inhibition than is standard IgE-dependent activation.

Basophils and MC derived from CD34⁺ precursors cultured in serum-free medium express FcεRI but are devoid of IgE (23). These cells therefore enabled further confirmation regarding the role of IgE in A549-dependent activation of basophils and to additionally test whether these EC activate MC ± IgE. As shown in Fig. 6, culture-derived basophils (CDBA) passively sensitized with IgE responded by releasing histamine, IL-4, and IL-13 when cocultured with A549 cells, especially in the presence of IL-3, and at peak times consistent with those by blood basophils. In contrast, the same CDBA, if not sensitized with IgE, failed to release these mediators when cocultured with A549. Interestingly, MC derived from progenitors cultured for 8–10 wk and passively sensitized with IgE released slightly more histamine to anti-IgE when cocultured with A549. However, this increased responsiveness was absent in cocultures stimulated with IL-3 or SCF (Fig. 7). Unlike basophils, MC (alone or cocultured for up to 20 h with A549) did not release detectable IL-4 and no or little amounts of IL-13 (data not shown).

Potential role for an IgE-binding lectin expressed by A549 EC

Because the capacity of A549 cells to promote mediator release and cytokine secretion was dependent on basophils expressing IgE, we considered the hypothesis that a protein expressed by these EC interacts with this Ig to promote these basophil responses. After finding no evidence for FcεRIα or CD23 using flow cytometry (data not shown), we considered the involvement of a lectin possibly binding and cross-linking IgE, thus triggering basophil activation. We therefore conducted a final set of experiments testing two sugars, lactose and LacNAc, for their capacity to act as competitive antagonists and inhibit cytokine secretion in basophil/A549 cocultures exposed to IL-3 for 20 h. In testing lactose, we found no significant effect of this sugar on basophil cytokine responses, even at concentrations of up to 25 mM (data not shown). At concentrations exceeding 25 mM, lactose began showing nonspecific inhibitory activity, as it began suppressing responses in control cultures of basophils alone (data not shown). In contrast,

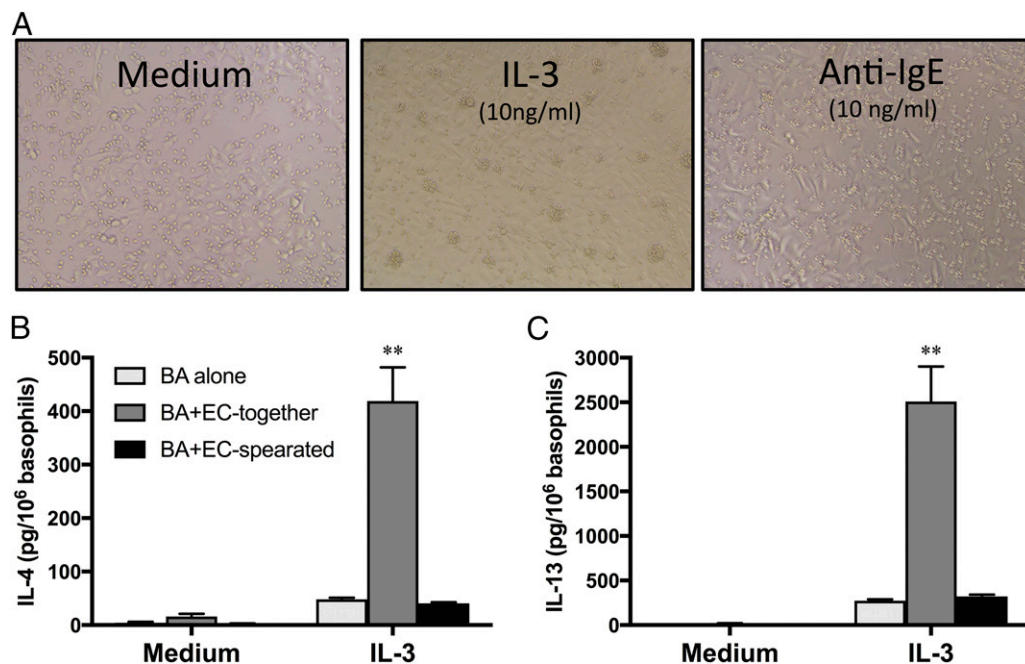


FIGURE 4. Cell-to-cell contact required for A549 EC-dependent activation of basophils. (A) Cell-clumping evident in basophil/A549 cocultures receiving IL-3 (middle panel). Original magnification $\times 40$. (B and C) IL-4 and IL-13, respectively, detected in culture supernatants (20 h) whereby basophils (BA) and A549 EC were cocultured together or separated in transwells. Cytokine levels compared with those from basophils cultured alone (mean $\pm$ SEM, $n = 3$). ** $p < 0.01$.

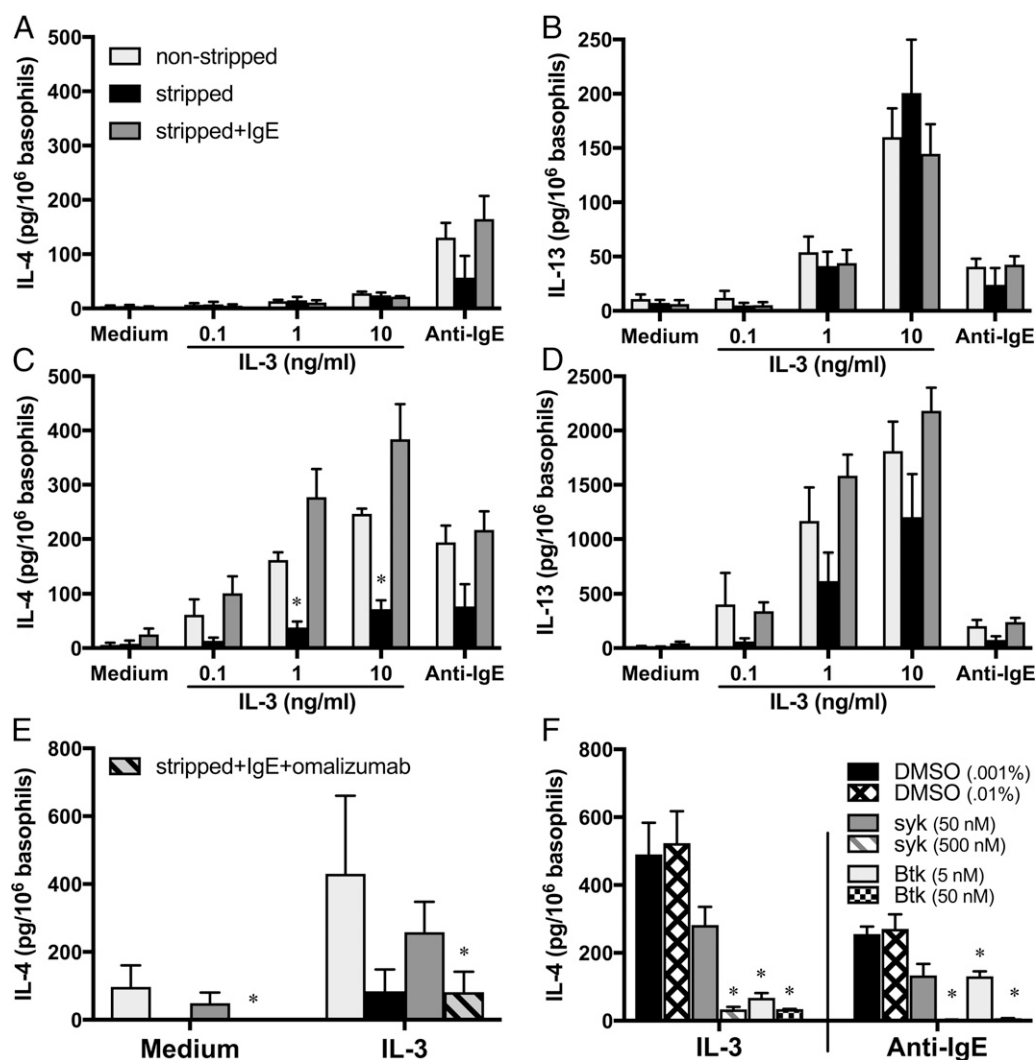


FIGURE 5. A549 EC activate basophils via IgE/Fc ϵ RI. (A and B) IL-4/IL-13, respectively, by basophils depleted of IgE (stripped), stripped, and sensitized with IgE (stripped+IgE), or left untreated (nonstripped), without A549 EC. (C and D) IL-4/IL-13 using basophils identically treated as in (A) and (B) but cocultured with EC. (E) IL-4 from cocultures that include stripped basophils sensitized with IgE in presence of omalizumab. (F) IL-4 secretion in basophil+A549 cocultures using inhibitors of syk and BTK tyrosine kinase. All cytokines assayed after 20 h incubation (mean $\pm$ SEM, $n = 3$), * $p < 0.05$.

LacNAc showed a dose-response suppression with ~20% inhibition of the IL-4 response mediated by this sugar at 1 mg/ml (~2.6 mM), which culminated in ~60% inhibition when used at 10 mg/ml (~26 mM) (Fig. 8). The same concentrations of LacNAc showed no suppressive activity on the IL-4 produced by basophils alone stimulated with IL-3, and actually mediated a 10–25% increase in this response. The IL-4 secreted in response to anti-IgE was also suppressed by the sugar in basophil/EC cocultures, but this became indistinct from the control IL-4 at the higher concentration.

Although results using LacNAc strongly indicated the involvement of a lectin, particularly a galectin, preliminary experiments proved inconclusive in linking A549-dependent activation of basophils to this family of lectins. First, there are some 15 family members of galectins, but only Gal-3 and Gal-9 reportedly bind IgE (34–36). We therefore assessed the probability of a Gal-3/Gal-9 involvement by investigating A549 EC for expression of these galectins. In doing so, we confirmed evidence that A549 EC expresses Gal-3 (Fig. 9A) (37). However, BEAS-2B cells, which lacked the capacity to activate basophils in coculture (Supplemental Fig. 2, online repository), also showed Gal-3 expression at levels equal to or greater than those detected on A549.

Both cell lines expressed Gal-1 equally (data not shown). BEAS-2B differed slightly from A549 cells in expressing nearly 50-fold greater levels of Gal-9 mRNA (Fig. 9B). Confirmation of this was also evident at the protein level in that BEAS-2B lysates showed detectable amounts of Gal-9 protein whereas A549 lysates did not (Fig. 9C). Overall, there was no clear pattern of Gal-3/Gal-9 found in or on A549 EC that readily linked the expression of these lectins to the activation of basophils, although further investigation is required.

Attempts were also made to test whether Abs specific for these galectins inhibit basophil activation mediated by A549 EC, even though none of the commercially available reagents (most containing azide) report neutralizing activity. At best, just ~20% inhibition was seen using the B2C10 Ab specific for Gal-3 (as compared with isotype control) and no effect was seen using anti-Gal-9 (data not shown). Both reagents do not appear to target the carbohydrate recognition domain of either lectin, which is presumably needed to neutralize activity.

Discussion

Our rationale for conducting this study initially sought translational evidence that human basophils respond to EC-derived cytokines

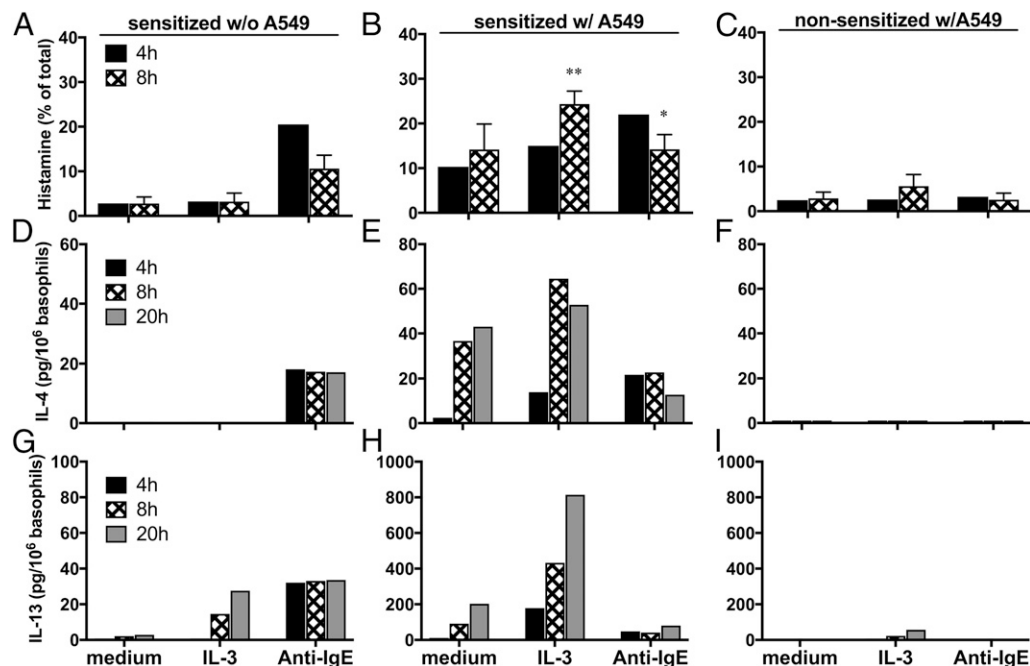


FIGURE 6. CD34⁺ precursors are activated by A549 EC only after sensitization with IgE. CD34⁺ precursors were grown from CD34⁺ precursors cultured 14 d in serum-free medium containing IL-3 (10 ng/ml). Cells were then sensitized for ~16 h with IgE (500 ng/ml) or left unsensitized, washed, and cultured with or without A549 EC. Histamine (**A–C**), IL-4 (**D–F**), and IL-13 (**G–I**) were then measured in culture supernatants at the times indicated (mean $\pm$ SEM, $n = 2–3$). * $p < 0.02$, ** $p = 0.05$.

(e.g., TSLP, IL-33, IL-25). When unable to detect any discernable *in vitro* effect on basophil function using two of these cytokines (TSLP and IL-25), we proceeded to coculture basophils with EC lines. This approach stems from the concept that some cytokines (e.g., TSLP) may exert their function, in part, through a phenomenon known as transpresentation (33), whereby a cytokine is physically presented to effector cells (i.e., basophils) by EC in a manner requiring cell-to-cell contact. Although our findings have not supported a role for transpresentation, they did expose a potentially unique mode of EC-dependent basophil activation, which may ultimately have significance in multiple diseases in addition to allergy. Indeed, the results in this study support a concept whereby a yet unidentified IgE-binding lectin expressed by A549 EC is capable of cross-linking IgE/Fc ϵ RI complexes on basophils to induce mediator release and cytokine secretion. Certainly, our findings do not exclude a role for TSLP (or any other EC cytokine) in activating human basophils, but every attempt thus far to test this hypothesis has proved negative. In this respect, it is important to note that Salter et al. (12) recently provided evidence of TSLP activating human basophils for mediator release and cytokine production. This group investigated allergic asthmatics whose basophils were somewhat hyper-responsive, spontaneously releasing ~40% of total histamine in buffer alone. These basophils were not only responsive to TSLP but were also shown to secrete histamine and cytokine in response to IL-3. In contrast, the basophils we investigated were predominately from normal subjects, which may account for why they did not respond to TSLP (or IL-25). They did respond to recombinant IL-33, as previously reported by others (14, 15), but in a manner quite different from that ultimately observed when cocultured with A549 EC.

Several experimental observations led to the hypothesis that an IgE-binding protein is a key player in the mechanism by which A549 EC activate basophils. The first came from the relatively high levels of IL-4 generated in the cocultures, which were comparable

(and generally higher) to those we typically measure following IgE-dependent activation. In fact, it is our experience that IL-4 secretion

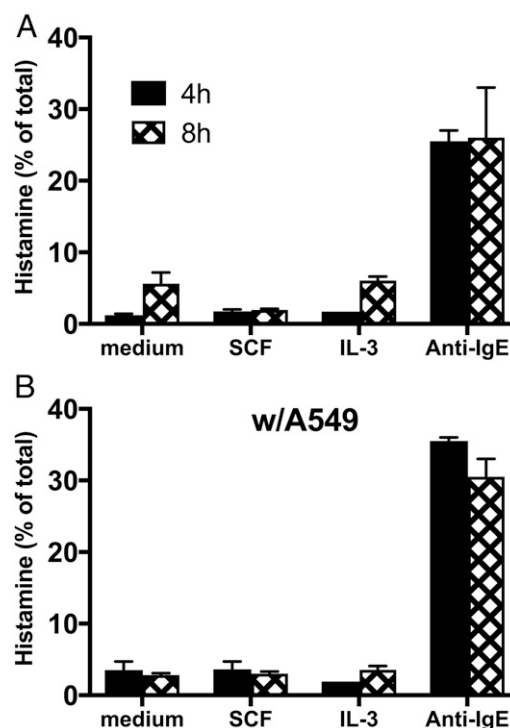


FIGURE 7. Mast cells sensitized with IgE are not activated by A549. Mast cells were grown from CD34⁺ precursors cultured 8 wk in serum-free medium containing IL-6 and SCF, as described in *Materials and Methods*. Cells were then sensitized for ~16 h with IgE (500 ng/ml), washed, and cultured without (**A**) and with (**B**) A549 EC as indicated. Histamine was measured at the times indicated (mean $\pm$ SEM, $n = 3$).

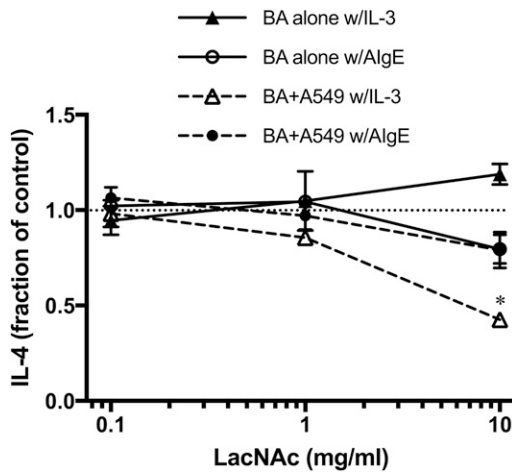


FIGURE 8. Putative role for an IgE-binding lectin. Effect of LacNAc on IL-4 secretion in basophil (BA)/A549 EC cocultures versus basophils alone after stimulating as indicated for 20 h. Shown are fractional responses (mean $\pm$ SEM, $n = 4-5$) of baseline IL-4 levels (dotted line) by basophils cultured alone with IL-3 (21 ± 6 pg per 10^6 basophils) or anti-IgE (115 ± 17 pg per 10^6 basophils) versus levels in cocultures with IL-3 (338 ± 82 pg per 10^6 basophils) or anti-IgE (190 ± 36 pg per 10^6 basophils). * $p < 0.02$.

does not typically exceed ~ 50 pg per 10^6 basophils unless the cells are activated via an IgE-dependent stimulus or by calcium ionophore. Certainly, IL-3 alone can induce IL-4 secretion but typically in the range of $\sim 10-50$ pg per 10^6 basophil, and only after prolonged (~ 20 h) incubation (28). Yet, the IL-4 levels generated in this study among the basophil-A549 EC cocultures stimulated with IL-3, often exceeded 500 pg per 10^6 basophils after 20 h.

Upon seeing these robust responses, we immediately predicted two additional parameters: 1) that basophils are likely secreting histamine under the same conditions, and 2) that the mode of activation somehow involves IgE/Fc ϵ RI cross-linking, despite no exogenous activator of this pathway (i.e., allergen or anti-IgE Ab). Again, the capacity of human basophils to secrete IL-4 has long been linked to IgE-dependent activation (38–40). In addition, this response is typically preceded by rapid degranulation (i.e., BHR) occurring within the first 30 min. In fact, the two mediators correlate quite well, despite having different kinetics and dose-response curves. As predicted, histamine was released in basophil/A549 EC cocultures stimulated with IL-3. Remarkably, the half-maximal time of 4 h for this response far exceeded the 30 min necessary for maximal BHR following standard IgE-dependent activation. IL-4 production in these cocultures was also slower (half maximal at 5 h), requiring nearly twice the time to be secreted compared with standard IgE-dependent activation. The exact cause(s) for these delayed time courses (from standard IgE-dependent activation) remains poorly understood but may point to the time necessary for optimal aggregation mediated by the proposed lectin. Nonetheless, the importance of IgE was clearly demonstrated 2-fold. First, by depleting this basophil-bound Ig (with pH 3.9 lactic acid buffer), which significantly diminished IL-4 production, and then restoring this response by resensitizing basophils with IgE. Second, by using CDDBA devoid of IgE, which were completely unresponsive when cocultured with A549. Only after sensitization with IgE did these cells become responsive to coculture with A549 EC. Then, the observation that inhibitors of syk and Btk kinases also blocked cytokine secretion in the cocultures, further linked the response to Fc ϵ RI-dependent activation. Most significantly, these findings were evident only in the cocultures and not with basophils alone.

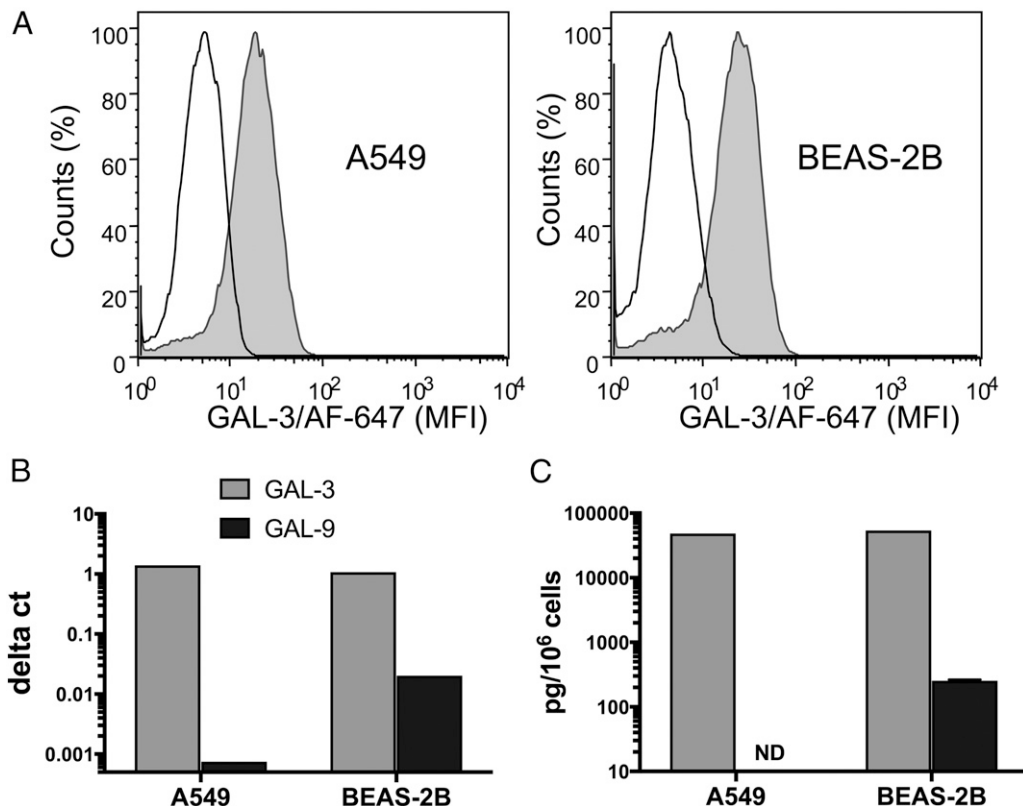


FIGURE 9. Gal-3, -9 expression by A549 and BEAS-2B cell lines. (A) Flow cytometry demonstrating Gal-3 expression by A549 and BEAS-2B cell lines. (B) Relative expression of Gal-3 and Gal-9 mRNA and (C) protein for these galectins in the two epithelial cell lines.

Collectively, they add to the notion that A549 ECs provide some sort of protein or factor that interacts with IgE on the surface of basophils, effectively cross-linking FcεRI, which results in activation.

The mechanism by which IL-3 (but not IL-5 or GM-CSF) facilitated the overall response also remains unclear, yet further points to multiple factors contributing to the capacity of A549 EC to activate basophils. For instance, basophils cocultured with A549 EC in medium alone or with anti-IgE, showed greater responsiveness than if cultured alone with these stimuli. However, the greatest magnitude in response was with those in which cocultures received IL-3. Even basophils primed overnight with IL-3 and then washed free of this cytokine, showed significantly greater responses, also indicating that the effect of IL-3 was on basophils rather than the A549 EC. Of course, IL-3 has long been known to amplify just about every biological response attributed to basophils, so the underlying mechanism(s) in this study are likely linked to the signaling mediated by this cytokine. It remains equally plausible that IL-3 augments the expression of a third component that facilitates interaction between the putative lectin (on EC) with IgE (on basophils), which then amplifies EC-dependent basophil activation. Obviously, this hypothesis requires further investigation and can only be tested upon identification of the lectin and/or cofactor augmenting basophil activation.

The observation that culture-derived MCs were unresponsive when cocultured with A549 further supports the importance of IL-3. Like basophils, MCs express FcεRI and bind IgE with high affinity, which should enable them to be responsive to A549 if IgE was the only requirement. However, human mast cells do not generally express IL-3 receptors and are unresponsive to this cytokine. We therefore considered that SCF might do for MCs what IL-3 does for basophils, but the addition of this factor (or IL-3) also failed to make MCs responsive to A549. Culture-derived mast cells are generally thought to possess a tryptase-positive phenotype. It therefore remains possible that skin mast cells bearing the tryptase/chymase-positive phenotype may respond to A549 cells, but this is still to be tested. Overall, the capacity of A549 EC to activate IgE-bearing cells in general might be unique to basophils, because they express IL-3 receptors (CD123) and bind IgE.

In observing that LacNAc significantly suppressed IL-4 production in the basophil-A549 cocultures but not in cultures with basophils alone, our attention quickly turned toward possible involvement of an IgE-binding lectin expressed on A549 cells. In particular, the galectin family consists of 15 members, but only Gal-3 and Gal-9 reportedly bind IgE (34, 35). Moreover, Gal-3 is widely expressed by EC and fibroblasts, especially those with a cancer origin (41, 42). In fact, it has been reported in mice that EC actually bind IgE via Gal-3 (43). Gal-3 is also the only chimera galectin, meaning it can exist as a monomer or form multivalent structures (e.g., pentamers) that facilitate lattice formation between glycoproteins on different cell types (44). This characteristic alone provided a good rationale as to how Gal-3 might facilitate IgE/FcεRI cross-linking to activate basophils. Gal-9 is more recently reported to bind IgE, and apparently does so with greater affinity than Gal-3. Interestingly, Gal-9 reportedly inhibits IgE-dependent activation, both *in vitro* and *in vivo* (36). Exactly how this galectin mediates this effect is currently unclear, but it seemingly does so by binding free IgE and preventing sensitization. Whether and how it might interact with cell-bound IgE has not been fully addressed, nor has whether it mediates FcεRI cross-linking. Nonetheless, LacNAc can target all galectins and thus lacks specificity for any given family member, although it has recently been shown to inhibit Gal-3 binding to IgE (45).

We therefore proceeded to investigate A549 cells for galectin expression, confirming what is well cited in the literature: A549 cells express Gal-3. However, BEAS-2B cells, which did not activate basophils for increased IL-4/IL-13 secretion, also expressed Gal-3 at nearly equal levels, thus confounding the hypothesis that this IgE-binding lectin mediates basophil activation. As it stands, these preliminary data challenge the hypothesis that Gal-3 is the sole component mediating A549-dependent basophil activation. Obviously, further investigation is required to fully address the role of Gal-3, Gal-9, and possibly other galectins; this work extends beyond the scope of the current study. Abs specific to these galectins did not reveal neutralizing activity, but are unlikely to do so as none target the carbohydrate recognition domain of the lectin. Instead, we are currently exploring the use of CRISPR, with the intent of systematically knocking-out galectins (e.g., Gal-3 and Gal-9) in A549 EC to directly address their importance. Of course, even this approach may prove negative, if a yet unidentified lectin is mediating A549-dependent activation of basophils. Our hypothesis regarding the role of galectins, particularly Gal-3 and Gal-9, rests solely on the fact that these bind IgE, are expressed by A549 EC, and are targeted by LacNAc. More direct approaches are required to fully address the role of these lectins.

The proposed mechanism in this study of how an A549 EC-associated lectin activates basophils raises several important questions requiring further investigation. First, it remains unknown whether other EC lines and/or normal or disease primary EC possess a similar capacity to activate basophils. And, with the putative IgE-binding lectin currently unidentified, such investigations will require the same rigorous testing as in this study to determine the dependency on IgE. Thus, it seems possible that some EC will activate basophils for mediator release and/or cytokine production, but the potential role of EC-derived cytokines (IL-33, TSLP, and IL-25) will have to be ruled out before concluding a mechanism identical to that seen using A549 EC. And, with BEAS2B cells not possessing the same capacity to activate basophils, then another important parameter requiring investigation pertains to how the expression of this putative lectin is regulated on EC. If it is not expressed on all EC, then what are the conditions regulating its expression? We are also hesitant to conclude that basophils are the only IgE-bearing cells activated in this manner, despite the augmentative effects seen using IL-3, a cytokine known to prime basophils. The conditions tested in this study for MC may not have been fully optimal. Likewise, IgE-DC could also be activated in a similar manner, even though the mediators and cytokines produced by these cells will differ from basophils (and MC). With MC already in organ tissue, they could be the primary targets responding to this mode of activation. In contrast, basophils and DC would respond only after infiltrating lesion sites expressing the lectin.

Finally, there is also the question of whether this mode of basophil activation is even relevant to human disease. Again, this issue may only be fully addressed upon identification of the supposed lectin. Nonetheless, there is emerging evidence that lectins, in particular IgE-binding galectins, are linked to a variety of conditions, including allergic disease. For example, Mauri et al. (46) recently identified Gal-3 as a predictive biomarker of airway remodeling in allergic asthmatics; this finding was unveiled in subjects responsive to anti-IgE therapy using omalizumab. Knowing that omalizumab administration rapidly decreases IgE/FcεRI expression on circulating basophils, DC, and eventually on MC, these cells could become unresponsive to tissue expressing Gal-3. Chronic idiopathic urticaria (CIU) and atopic dermatitis are other clinical conditions whereby lectin/IgE interactions could theoretically contribute to their pathogenesis. Again, at least one

report has shown that using a mouse model of atopic dermatitis, mice deficient for Gal-3 do not develop active disease (47). IL-4 levels in these mice were greatly reduced whereas IFN- γ remained high. Although there are currently no reports of whether galectins are expressed in CIU tissue, it is reasonable to hypothesize the possibility and these having a role in activating basophils, MC, and DC. Histamine, IL-4, and IL-13 are all inflammatory mediators identified in CIU (48) and basophils are known to infiltrate CIU lesions (49). As with allergic asthma, given the recent efficacy using omalizumab (50), it is apparent that an IgE component contributes to the pathogenesis of CIU, despite these patients not typically being sensitized to any known allergen. It is therefore intriguing to think that basophils infiltrating this skin disease become activated via a lectin/IgE pathway. Anti-IgE therapy potentially reduces the chance of this occurring by neutralizing IgE and depleting its expression on basophils. Finally, galectin-dependent activation of basophils is conceivably linked even to diseases for which basophils have only recently been implicated, such as lupus (51) and cancer (52). This is because other reports show that galectins are widely expressed in the same tissues (53, 54). Thus, a synthesis of these findings, along with our proposed mechanism, might further explain why IL-4-producing basophils are seen in both diseases: they are triggered through an IgE/galectin interaction. Obviously, this hypothesis and those above remain speculation, yet the findings in this study provide feasibility and thus warrant further investigation.

Acknowledgments

We thank Dr. Li Gao for instruction in maintaining the A549 and BEAS2B cell lines, Dr. Donald MacGlashan, Jr. for use of the 161y syk inhibitor, Abiodun Adefola Adeosun for RT-PCR work, and Dr. Danh Do for microscopy and helpful discussion.

Disclosures

The authors have no financial conflicts of interest.

References

- Schroeder, J. T. 2011. Basophils: emerging roles in the pathogenesis of allergic disease. *Immunol. Rev.* 242: 144–160.
- Min, B., M. Prout, J. Hu-Li, J. Zhu, D. Jankovic, E. S. Morgan, J. F. Urban, Jr., A. M. Dvorak, F. D. Finkelman, G. LeGros, and W. E. Paul. 2004. Basophils produce IL-4 and accumulate in tissues after infection with a Th2-inducing parasite. *J. Exp. Med.* 200: 507–517.
- Voehringer, D., K. Shinkai, and R. M. Locksley. 2004. Type 2 immunity reflects orchestrated recruitment of cells committed to IL-4 production. *Immunity* 20: 267–277.
- Sokol, C. L., G. M. Barton, A. G. Farr, and R. Medzhitov. 2008. A mechanism for the initiation of allergen-induced T helper type 2 responses. *Nat. Immunol.* 9: 310–318.
- Denzel, A., U. A. Maus, M. Rodriguez Gomez, C. Moll, M. Niedermeier, C. Winter, R. Maus, S. Hollingshead, D. E. Briles, L. A. Kunz-Schughart, et al. 2008. Basophils enhance immunological memory responses. *Nat. Immunol.* 9: 733–742.
- Egawa, M., K. Mukai, S. Yoshikawa, M. Iki, N. Mukaida, Y. Kawano, Y. Minegishi, and H. Karasuyama. 2013. Inflammatory monocytes recruited to allergic skin acquire an anti-inflammatory M2 phenotype via basophil-derived interleukin-4. *Immunity* 38: 570–580.
- Borriello, F., M. Longo, R. Spinelli, A. Pecoraro, F. Granata, R. I. Staiano, S. Loffredo, G. Spadaro, F. Beguinot, J. Schroeder, and G. Marone. 2015. IL-3 synergises with basophil-derived IL-4 and IL-13 to promote the alternative activation of human monocytes. *Eur. J. Immunol.* 45: 2042–2051.
- Cheng, L. E., B. M. Sullivan, L. E. Retana, C. D. Allen, H. E. Liang, and R. M. Locksley. 2015. IgE-activated basophils regulate eosinophil tissue entry by modulating endothelial function. *J. Exp. Med.* 212: 513–524.
- Motomura, Y., H. Morita, K. Moro, S. Nakae, D. Artis, T. A. Endo, Y. Kuroki, O. Ohara, S. Koyasu, and M. Kubo. 2014. Basophil-derived interleukin-4 controls the function of natural helper cells, a member of ILC2s, in lung inflammation. *Immunity* 40: 758–771.
- Siracusa, M. C., B. S. Kim, J. M. Spergel, and D. Artis. 2013. Basophils and allergic inflammation. *J. Allergy Clin. Immunol.* 132: 789–801, quiz 788.
- Noti, M., B. S. Kim, M. C. Siracusa, G. D. Rak, M. Kubo, A. E. Moghaddam, Q. A. Sattentau, M. R. Comeau, J. M. Spergel, and D. Artis. 2014. Exposure to food allergens through inflamed skin promotes intestinal food allergy through the thymic stromal lymphopoietin-basophil axis. *J. Allergy Clin. Immunol.* 133: 1390–1399, 1399.e1–1399.e6.
- Salter, B. M., J. P. Oliveria, G. Nusca, S. G. Smith, R. M. Watson, M. Comeau, R. Sehmi, and G. M. Gauvreau. 2015. Thymic stromal lymphopoietin activation of basophils in patients with allergic asthma is IL-3 dependent. *J. Allergy Clin. Immunol.* 136: 1636–1644.
- Salter, B. M., J. P. Oliveria, G. Nusca, S. G. Smith, D. Twarek, P. D. Mitchell, R. M. Watson, R. Sehmi, and G. M. Gauvreau. 2016. IL-25 and IL-33 induce Type 2 inflammation in basophils from subjects with allergic asthma. *Respir. Res.* 17: 5.
- Smithgall, M. D., M. R. Comeau, B. R. Yoon, D. Kaufman, R. Armitage, and D. E. Smith. 2008. IL-33 amplifies both Th1- and Th2-type responses through its activity on human basophils, allergen-reactive Th2 cells, iNKT and NK cells. *Int. Immunol.* 20: 1019–1030.
- Pecaric-Petkovic, T., S. A. Didichenko, S. Kaempfer, N. Spiegel, and C. A. Dahinden. 2009. Human basophils and eosinophils are the direct target leukocytes of the novel IL-1 family member IL-33. *Blood* 113: 1526–1534.
- Collingwood, S. P., J. Hayler, D. M. Le Grand, H. Mattes, K. A. Menear, C. V. Wilker, et al. 2001. Preparation of anilino-purine derivatives as inhibitors of tyrosine protein kinase syk. *PCT Int Appl.* Novartis, Canada.
- MacGlashan, D., Jr., L. A. Honigberg, A. Smith, J. Buggy, and J. T. Schroeder. 2011. Inhibition of IgE-mediated secretion from human basophils with a highly selective Bruton's tyrosine kinase, Btk, inhibitor. *Int. Immunopharmacol.* 11: 475–479.
- MacGlashan, D., Jr., and N. Vilariño. 2006. Nonspecific desensitization, functional memory, and the characteristics of SHIP phosphorylation following IgE-mediated stimulation of human basophils. *J. Immunol.* 177: 1040–1051.
- Frischmeyer-Guerrero, P. A., and J. T. Schroeder. 2012. Cellular immune response parameters that influence IgE sensitization. *J. Immunol. Methods* 383: 21–29.
- Schroeder, J. T., and A. P. Bieneman. 2016. Isolation of human basophils. *Curr. Protoc. Immunol.* 112: 7.24.1–7.24.8.
- Gilbert, H. S., and L. Ornstein. 1975. Basophil counting with a new staining method using alcian blue. *Blood* 46: 279–286.
- Pruzansky, J. J., L. C. Grammer, R. Patterson, and M. Roberts. 1983. Dissociation of IgE from receptors on human basophils. I. Enhanced passive sensitization for histamine release. *J. Immunol.* 131: 1949–1953.
- Langdon, J. M., J. T. Schroeder, B. M. Vonakis, A. P. Bieneman, K. Chichester, and S. M. Macdonald. 2008. Histamine-releasing factor/translationally controlled tumor protein (HRF/TCPTP)-induced histamine release is enhanced with SHIP-1 knockdown in cultured human mast cell and basophil models. *J. Leukoc. Biol.* 84: 1151–1158.
- Kirshenbaum, A. S., and D. D. Metcalfe. 2006. Growth of human mast cells from bone marrow and peripheral blood-derived CD34+ pluripotent progenitor cells. *Methods Mol. Biol.* 315: 105–112.
- Schroeder, J. T., A. P. Bieneman, K. L. Chichester, C. A. Keet, R. G. Hamilton, D. W. MacGlashan, Jr., R. Wood, and P. A. Frischmeyer-Guerrero. 2013. Spontaneous basophil responses in food-allergic children are transferable by plasma and are IgE-dependent. *J. Allergy Clin. Immunol.* 132: 1428–1431.
- Unverdorben, L., U. Jeschke, L. Santoso, S. Hofmann, C. Kuhn, P. Arck, and S. Hutter. 2016. Comparative analyses on expression of galectins 1–4, 7–10 and 12 in first trimester placenta, decidua and isolated trophoblast cells in vitro. *Histol. Histopathol.* 31: 1095–1111.
- Schroeder, J. T., and A. Kagey-Sobotka. 2001. Assay methods for the measurement of mediators and markers of allergic inflammation. In *Manual of Clinical Laboratory Immunology*, 6th Ed. N. R. Rose, and E. C. deMacario, and J. D. Folds, and H. C. Lane, and R. M. Nakamura. American Society for Microbiology Press, Washington, DC.
- Redrup, A. C., B. P. Howard, D. W. MacGlashan, Jr., A. Kagey-Sobotka, L. M. Lichtenstein, and J. T. Schroeder. 1998. Differential regulation of IL-4 and IL-13 secretion by human basophils: their relationship to histamine release in mixed leukocyte cultures. *J. Immunol.* 160: 1957–1964.
- Ochsenberger, B., G. C. Daepf, S. Rihs, and C. A. Dahinden. 1996. Human blood basophils produce interleukin-13 in response to IgE-receptor-dependent and -independent activation. *Blood* 88: 3028–3037.
- Agrawal, R., J. Wisniewski, M. D. Yu, J. L. Kennedy, T. Platts-Mills, P. W. Heymann, and J. A. Woodfolk. 2014. Infection with human rhinovirus 16 promotes enhanced IgE responsiveness in basophils of atopic asthmatics. *Clin. Exp. Allergy* 44: 1266–1273.
- Allakhverdi, Z., M. R. Comeau, H. K. Jessup, B. R. Yoon, A. Brewer, S. Chartier, N. Paquette, S. F. Ziegler, M. Sarfati, and G. Delespesse. 2007. Thymic stromal lymphopoietin is released by human epithelial cells in response to microbes, trauma, or inflammation and potently activates mast cells. *J. Exp. Med.* 204: 253–258.
- Nagarkar, D. R., J. A. Poposki, M. R. Comeau, A. Biyasheva, P. C. Avila, R. P. Schleimer, and A. Kato. 2012. Airway epithelial cells activate TH2 cytokine production in mast cells through IL-1 and thymic stromal lymphopoietin. *J. Allergy Clin. Immunol.* 130: 225–32.e4.
- Castillo, E. F., and K. S. Schluns. 2012. Regulating the immune system via IL-15 transpresentation. *Cytokine* 59: 479–490.
- Liu, F. T., K. Albrandt, E. Mendel, A. Kulczycki, Jr., and N. K. Orida. 1985. Identification of an IgE-binding protein by molecular cloning. *Proc. Natl. Acad. Sci. USA* 82: 4100–4104.

35. Albrandt, K., N. K. Orida, and F. T. Liu. 1987. An IgE-binding protein with a distinctive repetitive sequence and homology with an IgG receptor. *Proc. Natl. Acad. Sci. USA* 84: 6859–6863.
36. Niki, T., S. Tsutsui, S. Hirose, S. Aradono, Y. Sugimoto, K. Takeshita, N. Nishi, and M. Hirashima. 2009. Galectin-9 is a high affinity IgE-binding lectin with anti-allergic effect by blocking IgE-antigen complex formation. *J. Biol. Chem.* 284: 32344–32352.
37. Nita-Lazar, M., A. Banerjee, C. Feng, and G. R. Vasta. 2015. Galectins regulate the inflammatory response in airway epithelial cells exposed to microbial neuraminidase by modulating the expression of SOCS1 and RIG1. *Mol. Immunol.* 68(2 Pt A): 194–202.
38. Schroeder, J. T., D. W. MacGlashan, Jr., A. Kagey-Sobotka, J. M. White, and L. M. Lichtenstein. 1994. IgE-dependent IL-4 secretion by human basophils. The relationship between cytokine production and histamine release in mixed leukocyte cultures. *J. Immunol.* 153: 1808–1817.
39. MacGlashan, D., Jr., J. M. White, S. K. Huang, S. J. Ono, J. T. Schroeder, and L. M. Lichtenstein. 1994. Secretion of IL-4 from human basophils. The relationship between IL-4 mRNA and protein in resting and stimulated basophils. *J. Immunol.* 152: 3006–3016.
40. Brunner, T., C. H. Heusser, and C. A. Dahinden. 1993. Human peripheral blood basophils primed by interleukin 3 (IL-3) produce IL-4 in response to immunoglobulin E receptor stimulation. *J. Exp. Med.* 177: 605–611.
41. Sundblad, V., D. O. Croci, and G. A. Rabinovich. 2011. Regulated expression of galectin-3, a multifunctional glycan-binding protein, in haematopoietic and non-haematopoietic tissues. *Histol. Histopathol.* 26: 247–265.
42. Larsen, L., H. Y. Chen, J. Saegusa, and F. T. Liu. 2011. Galectin-3 and the skin. *J. Dermatol. Sci.* 64: 85–91.
43. Brassart, D., E. Kolodziejczyk, D. Granato, A. Woltz, M. Pavillard, F. Perotti, L. G. Frigeri, F. T. Liu, Y. Borel, and J. R. Neeser. 1992. An intestinal galactose-specific lectin mediates the binding of murine IgE to mouse intestinal epithelial cells. *Eur. J. Biochem.* 203: 393–399.
44. Nabi, I. R., J. Shankar, and J. W. Dennis. 2015. The galectin lattice at a glance. *J. Cell Sci.* 128: 2213–2219.
45. Chen, S. S., L. W. Sun, H. Brickner, and P. Q. Sun. 2015. Downregulating galectin-3 inhibits proinflammatory cytokine production by human monocyte-derived dendritic cells via RNA interference. *Cell. Immunol.* 294: 44–53.
46. Mauri, P., A. M. Riccio, R. Rossi, D. Di Silvestre, L. Benazzi, L. De Ferrari, R. W. Dal Negro, S. T. Holgate, and G. W. Canonica. 2014. Proteomics of bronchial biopsies: galectin-3 as a predictive biomarker of airway remodelling modulation in omalizumab-treated severe asthma patients. *Immunol. Lett.* 162(1 Pt A): 2–10.
47. Saegusa, J., D. K. Hsu, H. Y. Chen, L. Yu, A. Fermin, M. A. Fung, and F. T. Liu. 2009. Galectin-3 is critical for the development of the allergic inflammatory response in a mouse model of atopic dermatitis. *Am. J. Pathol.* 174: 922–931.
48. Ferrer, M., E. Luquin, A. Sanchez-Ibarrola, C. Moreno, M. L. Sanz, and A. P. Kaplan. 2002. Secretion of cytokines, histamine and leukotrienes in chronic urticaria. *Int. Arch. Allergy Immunol.* 129: 254–260.
49. Ying, S., Y. Kikuchi, Q. Meng, A. B. Kay, and A. P. Kaplan. 2002. TH1/TH2 cytokines and inflammatory cells in skin biopsy specimens from patients with chronic idiopathic urticaria: comparison with the allergen-induced late-phase cutaneous reaction. *J. Allergy Clin. Immunol.* 109: 694–700.
50. Maurer, M., K. Rosén, H. J. Hsieh, S. Saini, C. Grattan, A. Giménez-Arnau, S. Agarwal, R. Doyle, J. Canvin, A. Kaplan, and T. Casale. 2013. Omalizumab for the treatment of chronic idiopathic or spontaneous urticaria. *N. Engl. J. Med.* 368: 924–935.
51. Sanjuan, M. A., D. Sagar, and R. Kolbeck. 2016. Role of IgE in autoimmunity. *J. Allergy Clin. Immunol.* 137: 1651–1661.
52. De Monte, L., S. Wörmann, E. Brunetto, S. Heltai, G. Magliacane, M. Reni, A. M. Paganoni, H. Recalde, A. Mondino, M. Falconi, et al. 2016. Basophil recruitment into tumor-draining lymph nodes correlates with Th2 inflammation and reduced survival in pancreatic cancer patients. *Cancer Res.* 76: 1792–1803.
53. Kang, E. H., K. C. Moon, E. Y. Lee, Y. J. Lee, E. B. Lee, C. Ahn, and Y. W. Song. 2009. Renal expression of galectin-3 in systemic lupus erythematosus patients with nephritis. *Lupus* 18: 22–28.
54. Ruvolo, P. P. 2016. Galectin 3 as a guardian of the tumor microenvironment. *Biochim. Biophys. Acta* 1863: 427–437.

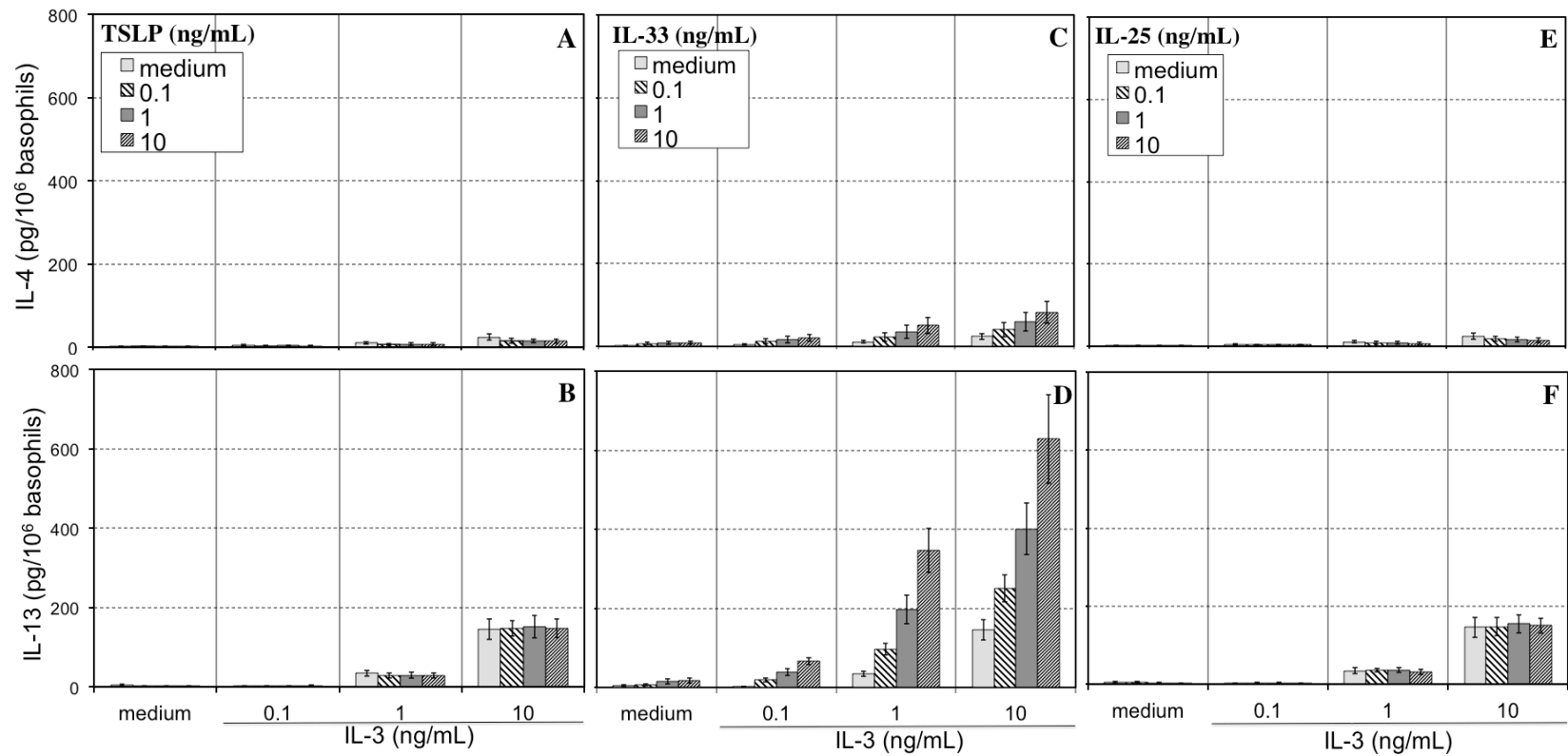


Fig. S1. Effect of recombinant EC-derived cytokines on IL-4/IL-13 secretion by human basophils. **A, C, E**, IL-4 protein secreted by basophils incubated for 20h with TSLP, IL-33, and IL-25, respectively, alone and combined with increasing concentrations of IL-3. **B, D, F**, IL-13 protein detected in the same supernatants. Results are the Mean $\pm$ SEM, n=4.

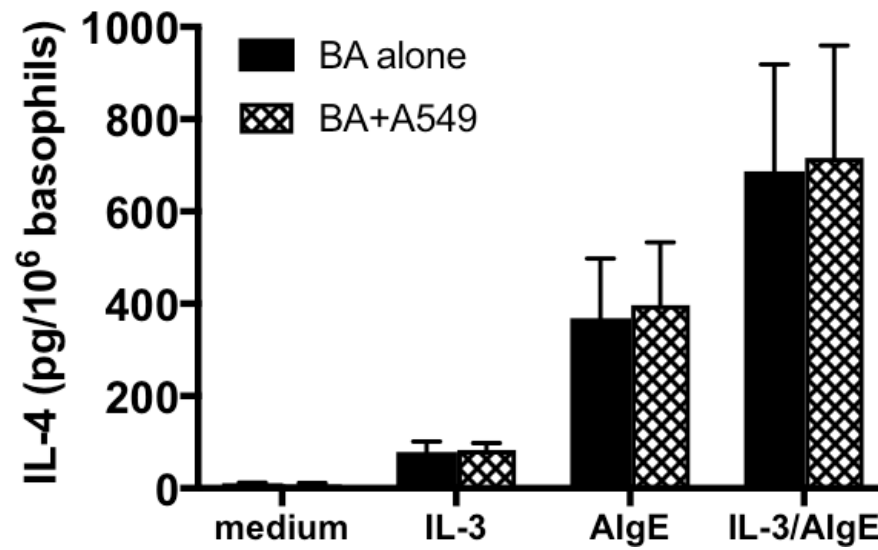


Fig. S2. *IL-4 production in basophil-BEAS2B co-cultures.* Basophils were cultured alone and with BEAS2B EC in the presence of medium, IL-3 (10 ng/mL), activating anti-IgE (10 ng/mL) or the combination. After 20h incubation, supernatants were harvested and assayed for IL-4 protein by ELISA. Results are the Mean $\pm$ SEM, n=3