

María Bravo García-Morato, BSc^{a,b}
 Ane Calvo Apalategi, BSc^{c*}
 Luz Yadira Bravo-Gallego, MD^{a,b*}
 Alfonso Blázquez Moreno, PhD^c
 Miriam Simón-Fuentes, BSc^d
 Jenny V. Garmendia, MD^e
 Ana Méndez Echevarría, MD, PhD^f
 Teresa del Rosal Rabes, MD, PhD^f
 Ángeles Domínguez-Soto, PhD^d
 Eduardo López-Granados, MD, PhD^{a,b}
 Hugh T. Reyburn, PhD^{c*}
 Rebeca Rodríguez Pena, MD, PhD^{a,b}

From ^athe Department of Immunology, La Paz University Hospital, Madrid, Spain; ^bthe Lymphocyte Pathophysiology Group, La Paz Institute of Biomedical Research, IdiPAZ, Madrid, Spain; ^cthe Department of Immunology and Oncology, CNB-CSIC, Madrid, Spain; ^dCentro de Investigaciones Biológicas/Consejo Superior de Investigaciones Científicas, Madrid, Spain; ^eInstituto de Immunología, Facultad de Medicina, Universidad Central de Venezuela; and ^fthe Paediatric Infectious Diseases Department, La Paz University Hospital, Madrid, Spain. E-mail: hreyburn@cnb.csic.es.

*These authors contributed equally to this work.

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Galectin-3 is essential for IgE-dependent activation of human basophils by A549 lung epithelial cells



To the Editor:

Recently, we reported that human basophils are activated to release robust levels of IL-4/IL-13 when cocultured with A549 epithelial cells (ECs), a lung adenocarcinoma.¹ These responses proved dependent on cell-to-cell contact and basophil-associated IgE. They were also inhibited by compounds targeting

FcεRI-dependent signaling and by n-acetyllactosamine. Involvement of galectin-3 (Gal-3) was hypothesized, given its initial description as an IgE-binding lectin.² Moreover, Gal-3 expression is common to ECs, especially those of cancerous origin, and is unique among galectins in possessing multivalent binding capacity.³ Therefore we theorized that A549 cell-associated Gal-3 nonspecifically binds IgE on basophils, triggering a unique activation similar to standard IgE-dependent stimulation but in a manner independent of allergen.

Here we address the direct involvement of Gal-3 in basophil activation (see the [Methods](#) section in this article's Online Repository at www.jacionline.org). First, we used short hairpin RNA (shRNA) to suppress Gal-3 expression in A549 cells, creating stable clones whereby Gal-3 protein is nearly ablated, as is their capacity to activate basophils for IL-4/IL-13. Second, we coupled rhGal-3 to microspheres (MSs), showing that Gal-3, when immobilized on this matrix, activates basophils much like A549 ECs.

As shown in [Fig 1, A](#), 2 clones (1E2 and 1B10) demonstrated greater than 95% suppression of total Gal-3 protein, as determined by using Western blotting. In contrast, Gal-3 was only partially knocked down in a third clone, 3G8, showing approximately 60% reduction compared with wild-type (WT) A549 cells (A549-WT cells). Flow cytometry validated these findings, showing that Gal-3 expression by the clones 1E2, 1B10, and 3G8 was similarly reduced by 90%, 88%, and 49%, respectively, when compared with A549-WT cells ([Fig 1, C](#)). No phenotypic features distinguished the clones from WT cells ([Fig 1, D](#)).

As shown in [Fig 1, E](#), basophils alone secreted low levels of IL-4 (8 ± 2 pg/10⁶, n = 6) to IL-3. This response was augmented approximately 10-fold when basophils were cocultured with A549-WT cells, confirming our previous findings.¹ However, these augmented responses were ablated (to basophil-alone levels) when cocultured with the Gal-3-deficient clones 1E2 and 1B10. In contrast, the Gal-3⁺ clone 3G8 retained the capacity to activate basophils for IL-4, with levels nearly equal to those induced by A549-WT cells.

Basophils cocultured with A549-WT cells also produced approximately 30% to 50% more IL-4 in response to stimulation with an activating anti-IgE antibody ([Fig 1, E](#)). In contrast, these enhanced responses were not evident when basophils were cocultured with Gal-3-deficient clones ($P = .100$ and $P = .190$, respectively; n = 6-7). In contrast, clone 3G8 retained near full capacity to augment IgE-dependent IL-4 production, much like A549-WT ECs.

Comparable patterns were observed for IL-13 secretion ([Fig 1, F](#)) and were substantially greater than those for IL-4, an observation consistent with our initial findings.¹ For example, IL-13 averaged 69 ± 14 pg/10⁶ (n = 11) when basophils were cultured with IL-3 alone. These increased approximately 30-fold to 2001 ± 268 pg/10⁶ when basophils were cocultured with A549-WT cells. In contrast, IL-13 levels were markedly suppressed (82% and 79%) when basophils were cocultured with the clones 1E2 and 1B10, respectively ($P = .00001$, n = 11). This contrasts to just 14% suppression when basophils were cocultured with the clone 3G8 ($P = .541$, n = 11).

Basophils also secreted approximately 9-fold greater levels of IL-13 in response to anti-IgE when additionally cocultured with A549-WT cells. In contrast, the Gal-3-deficient clones 1E2 and 1B10 only slightly augmented this response ($P \leq .006$, n = 11).

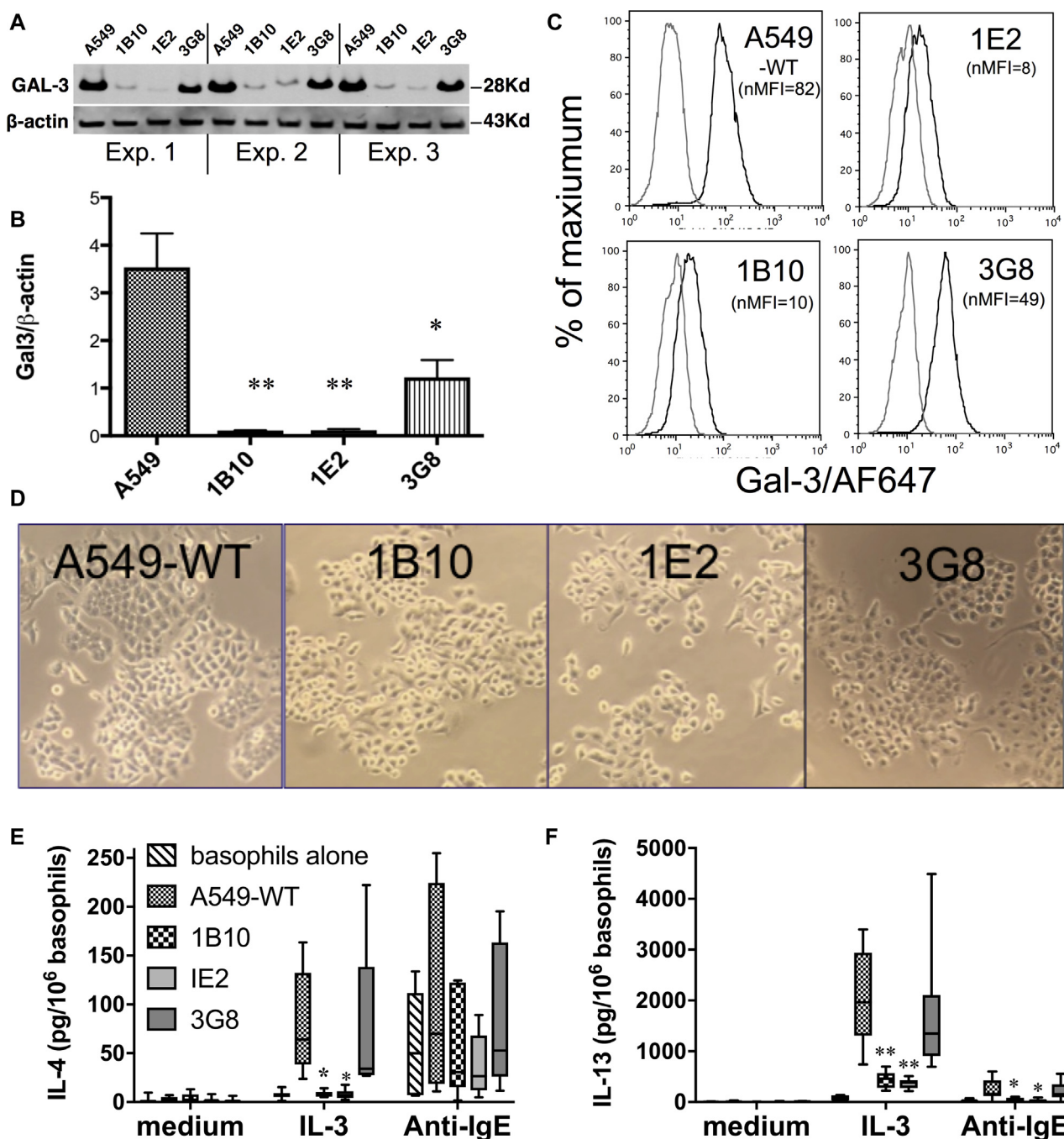


FIG 1. **A** and **B**, Western blot analysis of lysates from A549-WT cells and clones after using shRNA to repress Gal-3 protein (3 experiments conducted at weekly intervals; Fig 1, **A**), with densitometric analyses (Fig 1, **B**). **P* = .047 and ***P* < .009 versus A549-WT cells. **C**, Gal-3 on A549-WT cells and clones, as indicated by using flow cytometry. **D**, Light-field images as indicated (original magnification ×400). **E** and **F**, Box-and-whisker plots of IL-4 (*n* = 6-7; Fig 1, **E**) and IL-13 (*n* = 11; Fig 1, **F**) secreted in basophil/EC cocultures containing IL-3 (10 ng/mL), anti-IgE (20 ng/mL), or medium alone. **P* ≤ .006 and ***P* = .00001 versus corresponding conditions with basophils cocultured with A549-WT cells.

Again, clone 3G8 retained near capacity to costimulate basophils for IL-13, inducing responses (220 ± 57 pg/10⁶ basophils, *n* = 11) nearly equal to those seen in cocultures with A549-WT cells.

To further support the concept that Gal-3 mediates basophil activation, we conducted experiments using recombinant protein. Importantly, there was no indication that A549-WT cells secreted

Gal-3 into the culture medium, as determined by means of ELISA (data not shown). Therefore we added rhGal-3 to basophils alone and to A549-WT cell/basophil cocultures. As shown in Fig 2, **A**, rhGal-3 mediated no effect on basophil IL-4 production until used at 10 μg/mL, which only marginally increased IL-3-dependent secretion approximately 2-fold (from 20 to 50 pg/10⁶ basophils). Once again, basophils cocultured with A549-WT cells produced

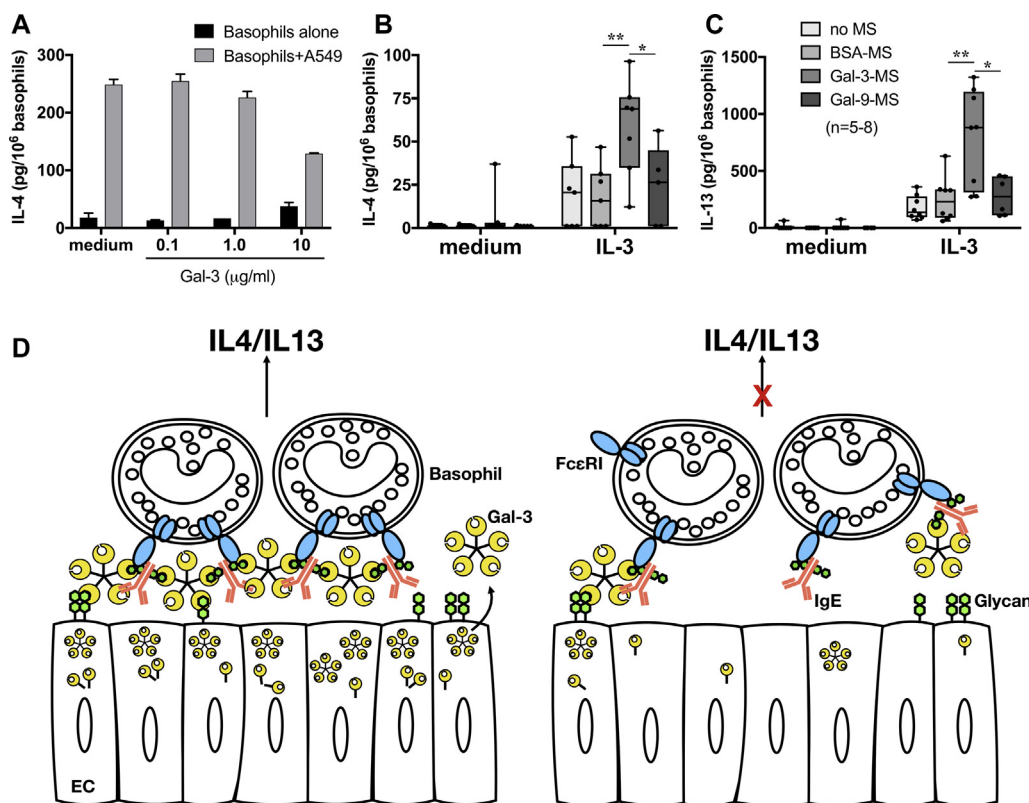


FIG 2. A, Effects of Gal-3 protein on IL-4 secreted by basophils alone and in coculture with A549-WT cells in the presence of IL-3 (10 ng/mL [mean $\pm$ SEM], $n = 3$). B and C, IL-4 (Fig 2, B) and IL-13 (Fig 2, C) secreted by basophils cocultured with MSs (5 μ m in size) precoated with BSA, Gal-3, or Gal-9 in the absence and presence of IL-3 (10 ng/mL). * $P < .05$ and ** $P < .01$. D, Current conception of how EC-associated Gal-3 might crosslink IgE on basophils, resulting in activation for IL-4/IL-13.

approximately 10-fold greater levels of IL-4, yet the addition of rhGal-3 did not augment this response. In fact, 10 μ g/mL rhGal-3 actually suppressed IL-4 (approximately 50%), hinting that this high concentration competes with A549 cell-associated Gal-3, hindering its capacity to activate basophils.

Observing that rhGal-3 only marginally activated basophils and at concentrations approximately 1000-fold greater than even the serologic levels associated with disease,⁴ we considered that immobilized Gal-3 might better mimic A549 cell-dependent basophil activation. Therefore we coupled rhGal-3 to polycarbonate MSs, testing whether these activate basophils.

MSs coupled with BSA (MS-BSA) and MSs coupled with Gal-9 (MS-Gal-9) were also tested. When cocultured with basophils at a 5:1 ratio (MSs/basophils) and in the presence of IL-3, only MS-Gal-3 significantly activated basophils, both for IL-4 (Fig 2, B) and IL-13 (Fig 2, C). Cytokine levels from basophils costimulated with MS-BSA and MS-Gal-9 were not significantly different from those induced by using IL-3 alone.

Overall, these data support our hypothesis that Gal-3 is essential for A549 cell-dependent basophil activation. Whether this mode of activation will translate to other cell lines and/or primary ECs (normal and/or diseased) requires further investigation, as do the mechanisms controlling Gal-3 expression. Instead, the significance of these data currently lies with the fact that Gal-3 is now implicated as a biomarker and/or factor contributing to the fibrosis associated with and/or pathogenesis of a wide range of diseases/conditions, including asthma and atopic dermatitis but also in patients with cancer, autoimmunity (lupus), cardiovascular

disease, and wound healing. Indeed, publications now link Gal-3 to disorders beyond the realm of allergic disease, with the overall number having grown exponentially in recent years.⁵ Ironically, most of this work seldom focuses on Gal-3's interaction with IgE, despite its initial description as an IgE-binding lectin.² We predict that the data herein will spark renewed interest in Gal-3/IgE interactions by providing novel insight into how this lectin, when expressed on matrix cells (eg, ECs), activates basophils (Fig 2, D) and possibly other IgE-bearing cells. Such findings could provide foresight regarding how these basophils participate in diseases not typically driven by specific IgE/allergen. For example, IL-4-producing basophils have been identified in patients with lupus nephritis⁶ and recently in those with cancer.⁷ It is intriguing to contemplate that the increased levels of Gal-3 also seen in the affected tissues of patients with these diseases might play a role in activating infiltrating basophils and the consequences thereof.

John T. Schroeder, PhD
Abiodun A. Adeosun, MS*
Danh Do, PhD
Anja P. Bieneman, BS

From the Department of Medicine, Division of Allergy and Clinical Immunology, Johns Hopkins Asthma and Allergy Center, Johns Hopkins University, Baltimore, Md. E-mail: schray@jhmi.edu.

*Abiodun A. Adeosun, MS, is currently a doctoral candidate within the Department of Pharmacology at Baylor College School of Medicine, Houston, Texas.

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Dust mite induces leukocyte exosomes with proinflammatory effects toward airway epithelial cells



To the Editor:

House dust mite (HDM), a major indoor perennial aeroallergen, is a prevalent cause of atopic sensitization worldwide. Exposure to mite allergens is in fact a common cause and trigger for asthma, a disease that affects 300 million persons worldwide.¹ Crosstalk between multiple cell types is known to maintain chronic inflammation in patients with allergic airway disease, although the exact mechanisms are complex and still uncertain.² To date, intercellular communication between leukocytes and airway epithelial cells has been largely attributed to cytokines and chemokines. However, the inflammatory milieu also contains other soluble mediators that are yet unappreciated and potentially pathogenic in airway inflammation. Growing evidence suggests

that exosomes, nano-sized membrane-bound vesicles, are contributors to cell-to-cell signaling and important conveyors of inflammation in patients with airway disease.

Initial studies have implicated exosomes in the regulation of immune responses and inflammation in patients with allergic disease.³⁻⁵ Exosomes containing nucleic acid and protein cargo are released by virtually all cell types and act as a shuttle to transfer material to recipient cells, where they exert functional and regulatory effects. In our study we aimed to determine whether dust mite extract (DME) regulates production of exosomes from human mononuclear cells and whether they have immunoregulatory effects on airway epithelial cells.

Across disciplines, exosome release appears to be cell dependent and regulated by both internal conditions and exogenous factors, although the external stimuli required for exosome secretion are still largely unknown.⁶ Additionally, the majority of studies on exosome release have been completed with cancer cell lines. By using HDM extract as a relevant inflammatory model, our study fills this gap by examining the effects of DME exposure on exosome secretion in human PBMCs from patients with dust mite allergy.

We analyzed exosomes derived from PBMCs of donors with dust mite allergy (for subjects' characteristics, see Table E1 in this article's Online Repository at www.jacionline.org) cultured with DME or PBS vehicle control for 48 hours. Exosomes were isolated from media by using a well-established, volume-crowding, polymer precipitation method reported in the Methods section in this article's Online Repository at www.jacionline.org and then analyzed by using electron microscopy and nanoparticle tracking analysis. Exosomes from both unstimulated and DME-stimulated leukocytes had similar characteristics, including size (108.68 ± 3.00 and 112.88 ± 1.93 nm, respectively) and morphology (see Fig E1 in this article's Online Repository at www.jacionline.org).

PBMCs constitutively produced exosomes without stimulation. PBS vehicle-treated cells produced an average of 512 ± 51 million particles/mL in a 48-hour period. Exposure to DME increased PBMC exosome levels to 1.081 ± 0.886 billion particles/mL, nearly doubling exosome release in the same

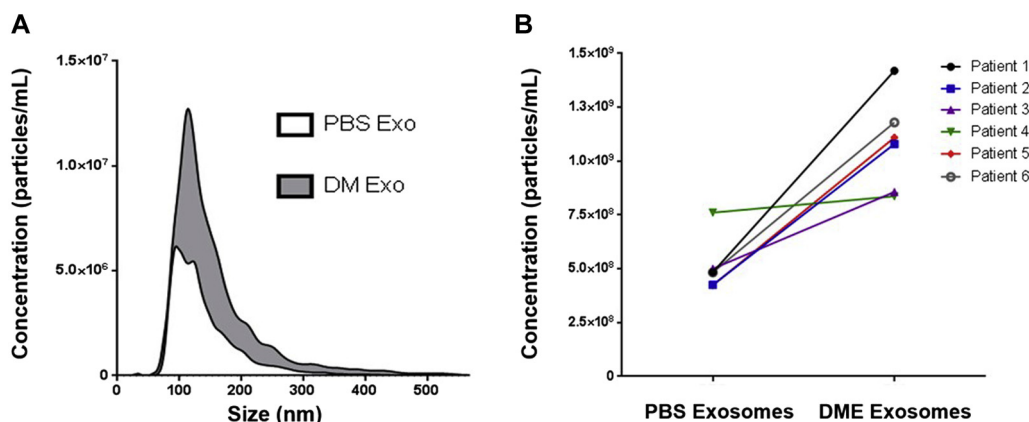


FIG 1. DME stimulates PBMC production of exosomes. PBMCs from donors with dust mite allergy ($n = 6$) were stimulated *in vitro* with $50 \mu\text{g}$ of DME or PBS vehicle for 48 hours. Exosomes were isolated from cultured media and measured by using nanoparticle tracking analysis. **A**, Histogram of mean particle concentration for each size. **B**, Plot of total particle concentration from unstimulated versus stimulated patient-matched cells. Dust mite allergen significantly increases leukocyte production of exosomes, $P = .0313$ determined by the Wilcoxon signed pairs rank test. *DM Exo*, DME exosome; *PBS Exo*, PBS-exosome.

METHODS

Special reagents, buffers, and media

The following reagents were purchased: crystallized human serum albumin (Calbiochem-Behring, La Jolla, Calif); PIPES, FCS, and crystallized BSA (Sigma-Aldrich, Allentown, Pa); gentamicin, Iscove modified Dulbecco medium, and nonessential amino acids (Life Technologies, Grand Island, NY); Percoll (Pharmacia Biotech, Piscataway, NJ); rHL-3 (Biosource, Camarillo, Calif); monoclonal anti-Gal-3 (clone B2C10) and IgG₁ isotype (clone MOPC-21) control (Santa Cruz Biotechnology, Dallas, Tex); Gal-3 ELISAs (eBioscience, San Diego, Calif); goat anti-IgG₁-Alexa Fluor 647 (Invitrogen, Eugene, Ore); 10× RIPA buffer and rabbit polyclonal anti-Gal-3 (Abcam, Cambridge, Mass); anti-β-actin (clone 2F1-1; BioLegend, San Diego, Calif); protease inhibitor cocktail (catalog no. 5871; Cell Signaling Technology, Danvers, Mass); and goat polyclonal anti-human IgE (provided by Dr Robert Hamilton, Johns Hopkins University).

A549 cell culture and Gal-3 knockdown by using shRNA

A549 ECs were from the American Type Culture Collection (Manassas, Va). A549 cells (of bronchial origin) were maintained in medium consisting of F-12K Nutrient Mixture-Kaigh Modification (Sigma-Aldrich), with 10% heat-inactivated FBS and 1% penicillin-streptomycin. Cultures were split twice weekly during the duration of these experiments, with no detectable changes in their capacity to activate basophils. Seeding of ECs for coculture with basophils has been previously described.^{E1} Briefly, ECs were plated into wells of 96-well plates in 0.100 mL of medium. These were incubated for up to 48 hours in a humidified incubator (37°C in a 5% CO₂ atmosphere) to allow cells to adhere and achieve 50% to 80% confluency before basophil coculture.

Knockdown of Gal-3 was achieved by using a Mission Lentiviral Transduction Kit (Sigma-Aldrich, St Louis, MO). This kit was supplied with shRNA constructs cloned and sequence verified to ensure a match to the human Gal-3 gene (LGALS3, NM_002306). Constructs were further cloned into the TRC2-pLKO.1-Puro-CMV-tGFP vector, and transduction of A549-WT cells was done according to the company protocol. After overnight transduction, medium was removed and replaced with fresh medium containing 2 μg/mL puromycin to select for transduced cells. Medium was replaced every 3 days for a total of 12 days to ensure selection of cells resistant to the antibiotic.

Because of random integration of lentivirus into the genome, which often causes varying levels of target gene knockdown, we performed limiting dilution with the goal of identifying clones showing marked reduction of Gal-3 expression, as well as transduced clones whereby Gal-3 expression remained intact. This involved seeding wells of microtiter plates at an average density of 0.75 cells per well, selecting only those cultures showing a single colony after 3 to 5 days of growth. These were then transferred to 6-well plates for expansion. At the time of passage, 2.5×10^5 cells from each clone were transferred to microcentrifuge tubes, resuspended to 1×10^6 /ml in 0.250 mL medium, and snap-frozen 3 times to induce cell lysis. Lysates were collected after centrifugation and assayed for Gal-3 protein by means of ELISA. This screening approach (data not shown) identified 2 clones (1E2 and 1B10) showing marked reduction of Gal-3 protein compared with lysates of WT cells (A549-WT). In contrast, lysate from clone 3G8 retained relatively high levels of Gal-3 protein similar to those in A549-WT cells. Cells from all 3 clones were expanded for further analysis of Gal-3 expression and assessed for their capacity to activate basophils in coculture.

Coupling of Gal-3, Gal-9, and BSA to MSs and/or wells of microtiter plates

Polystyrene MSs 5 μm in size were purchased from Phosphorex (Hopkinton, Mass) and washed twice with sterile ddH₂O, according to the

manufacturer's specifications. MSs were then coated with BSA, Gal-3, or Gal-9 by using the same conditions commonly used for coating microtiter wells for ELISA. In particular, a saturating solution (10 μg/mL in PBS buffer, pH 7.2) was used for each protein. MSs were incubated with solutions (0.5 mL per approximately 3×10^7 MSs) for 1 hour at room temperature (approximately 23°C), with mixing every 15 minutes before incubation overnight at 4°C. After coating, MSs were centrifuged, and supernatants were removed and then washed 2 times with PBS and 0.1% BSA. MSs were counted, adjusted to 1×10^7 /mL, and stored at 4°C. MSs proved functional for several months when prepared/kept under sterile conditions.

Basophil purification

Basophils were prepared from residual TRIMA cassettes from subjects undergoing platelet pheresis. In some instances venipuncture was performed on consenting adults (age range, 21-65 years) by using a protocol approved by the Johns Hopkins University Institutional Review Board. Subjects were selected regardless of allergic status. Buffy coats from both specimen sources were subjected to double-Percoll density centrifugation, which produced both basophil-depleted cell and basophil-enriched cell suspensions, as previously described.^{E2} Basophils were purified from the basophil-enriched cell suspensions by using negative selection reagents (STEMCELL Technologies, Vancouver, British Columbia, Canada) and immunomagnetic LS columns (Miltenyi Biotec, Gaithersburg, Md). Cell suspensions were routinely 99% or more basophils, as assessed by using Alcian blue staining.^{E2}

Flow cytometry

A549 ECs and shRNA clones were investigated for Gal-3 expression by using flow cytometry, as previously described.^{E1} In both instances blocking was done with 1% human IgG. The anti-Gal-3 mAb and IgG₁ isotype control were each used at 10 μg/mL in 20-minute incubations at room temperature. Cells were then washed before staining for 30 minutes on ice with goat anti-IgG₁-Alexa Fluor 647 (2.5 μg/mL). Flow cytometry was performed with a FACSCalibur machine (BS Biosciences, San Jose, Calif).

Western blot

Lysates of A549-WT ECs and shRNA clones were made by using RIPA buffer (made from 10× stock, Cell Signaling) containing protease inhibitor cocktail and shearing by aspirating up and down 10 times with a tuberculin syringe/needle (25 gauge). After clearing particulate matter by means of centrifugation, total protein content was determined by using a BCA protein assay kit (Thermo Scientific, Rockford, Ill) before adding β₂-mercaptoethanol and boiling for 5 minutes. Novex WedgeWell 4-12% Tris-glycine gels (Thermo Fisher Scientific, Waltham, Mass) were loaded with 10 to 15 μg of total protein per sample for electrophoresis. Protein was then transferred to 0.2-μm nitrocellulose blots (Bio-Rad Laboratories, Hercules, Calif). Blocking was with 3% wt/vol BSA in TBS-Tween before probing for Gal-3 and β-actin with polyclonal rabbit antibodies. Blots were visualized by using a LI-COR Odyssey Imaging System after incubation with IRDye 800CW conjugated secondary antibodies (LI-COR, Lincoln, Neb). Image quantification was carried out with Image Studio Lite software (version 5.2.5; LI-COR).

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