Human group 2 innate lymphoid cells do not express the IL-5 receptor

To the Editor:

Eosinophils, cardinal effector cells of type 2 inflammation, contribute to the clinical and immunopathologic manifestations of asthma[1] and chronic obstructive pulmonary disease[2] inflammatory endotypes. Eosinophil biology is governed by IL-5, a cytokine that binds with high affinity to a specific IL-5 receptor α subunit (IL-5Rα) before forming a heterodimeric receptor complex with the β subunit.[3] IL-5 signaling promotes differentiation, maturation, and survival of eosinophil-committed progenitors while acting on mature eosinophils to enhance their migratory potential and effector responses.[4] IL-5 signaling also promotes alternative splicing of the IL-5Rα gene to generate transmembrane forms of IL-5Rα.[5] Because the IL-5-IL-5R axis appears to be restricted to eosinophils and basophils (and their progenitors),[6] therapeutic regulation of these cells through the neutralization of circulating IL-5 (eg, mepolizumab and reslizumab)[1] or IL-5Rα ligation (eg, benralizumab),[1,2] have emerged as effective strategies to deplete blood, tissue, and airway eosinophils and consequently reduce exacerbation rates and improve lung function.[1]

Basophils and group 2 innate lymphoid cells (ILC2s) are important innate sources of type 2 cytokines, including IL-5, in response to epithelial-derived cytokines such as IL-33.[7,8] ILC2s, however, have emerged as central and critical innate coordinators of steady-state eosinophilopoiesis and epithelial cell–driven, type 2 immunopathology in asthma.[8] As an important upstream regulator of eosinophil function we asked whether ILC2s, like their basophil counterparts, express the IL-5Rα subunit because this may have important implications for our understanding of the role of the IL-5-IL-5R axis in disease and therapeutic targeting of these rare but important innate immune cells. Detailed methods are provided in this article's Online Repository at www.jacionline.org.

ILC2s are rare innate lymphocytes that lack the T-cell receptor complex and all known lineage markers but, similar to other type 2 cytokine-producing cells such as T_H2 cells, eosinophils, and basophils, express the type 2 prostaglandin D_2 receptor, DP2/CRT_H2 (CD294).[9] We defined blood ILC2s as cells with singlet, lymphocyte light scatter properties (Fig 1, *A* and *B*), lineage (CD2, 3, 14, 16, 19, 56, and 235a)$^-$ but CD294$^+$ (Fig 1, *C*). A large proportion of cells within this gate were basophils, (CD123$^+$ cells in Fig 1, *D*); however, CD294$^+$, CD123$^-$ ILC2s were present (Fig 1, *D*, and light scatter in *E*). Basophils displayed a higher level of CD125-phycoerythrin (PE) staining than did the ILC2s (Fig 1, *D*) that could be blocked in the presence of rhIL-5 (Fig 1, *F*), confirming the specificity of the antibody and basophil IL-5Rα expression. In contrast to basophils, there was no change in CD125-PE signal intensity when ILC2s were incubated with rhIL-5, indicating that there was no IL-5Rα expression on these cells (Fig 1, *F*). To confirm the reproducibility of these findings, we recruited 6 control and 13 volunteers with asthma (see clinical details in Table E1 in this article's Online Repository at www.jacionline.org) and measured IL-5Rα expression. The total mean (SD) % of ILC2s within the lymphocyte gate for both control and asthma groups were 0.1% ± 0.1% and 0.03% ± 0.03%, respectively ($P = .06$, Mann-Whitney). In total, combining asthma and control data sets, the basophil CD125 geometric mean fluorescence intensity (GMFI) (mean ± SD) in the absence or presence of rhIL-5 was 1285 ± 614 and 401 ± 315 respectively, representing a significant reduction in GMFI (Fig 1, *G* and *I*). In contrast, the ILC2 CD125 GMFI (mean ± SD) in the absence or presence of rhIL-5 was 45 ± 45 and 42 ± 33, respectively (Fig 1, *H* and *I*). In a subset of samples (n = 3), where basophils and ILC2s could be detected in whole blood, data were qualitatively similar. There were no significant differences between the control and asthma subjects for any of the flow cytometric measurements obtained.

We next sought to confirm whether the IL-5Rα data were reflected at the RNA level using real-time PCR probes spanning the exon boundaries present within IL-5Rα subunit variants (see Fig E1, *A*, in this article's Online Repository at www.jacionline.org). For this, ILC2s were isolated from 3 additional donors and cultured in the absence or presence of cytokines known to enhance survival (IL-2 and IL-7) and activate ILC2s (IL-25 and IL-33). RNA was isolated at 6 time points (day 0, 1, 2, 4, 7, and 14) and converted into cDNA. Eosinophil cDNA was included as a positive control. The IL-5Rα transcripts were not detected in ILC2s at any time point; however, an increase in IL-5 mRNA was observed, indicating that the culture conditions were sufficient to activate the cells (Fig E1, *B* and *C*).

Finally, using a similar approach to the blood analyses (Fig 1) and using surgically removed lung tissue from 7 subjects (see Table E2 in this article's Online Repository at www.jacionline.org), we asked whether tissue-derived ILC2s and basophils expressed IL-5Rα. Tissue-derived basophils were defined as viable, CD45$^+$Lin$^-$CD294$^+$CD123$^+$CD127$^-$ cells and ILC2s as viable, CD45$^+$Lin$^-$CD294$^+$CD123$^-$CD127$^+$ cells present within the singlet lymphocyte gate (Fig 2, *A-E*). Basophil CD125-PE GMFI (mean ± SD, 612 ± 327) could be reversed in the presence of rhIL-5 to a mean of 317 ± 91 (example in Fig 2, *F*, and cumulative data in Fig 2, *H*). Using the super enhanced Dmax (SED) algorithm to compare basophil populations stained in the absence or presence of IL-5 for each of the 7 donors revealed that 71.1% ± 4.2% basophils were CD125 positive (Fig 2, *I*). In contrast, ILC2 CD125 GMFI (mean ± SD) was significantly lower than that of basophils and showed little change in the presence of rhIL-5 (190 ± 42 and 145 ± 49, respectively) (example in Fig 2, *G*, and cumulative data in Fig 2, *H*). SED analysis of ILC2s stained in the absence or presence of rhIL-5 for each of the 7 donors (Fig 2, *I*) did not reveal a CD125-positive subset (mean ± SD, 3.3% ± 0.89%). Data from these 7 subjects consistently show that tissue-derived basophils express the IL-5Rα whereas tissue-derived ILC2s do not (Fig 2, *H*).

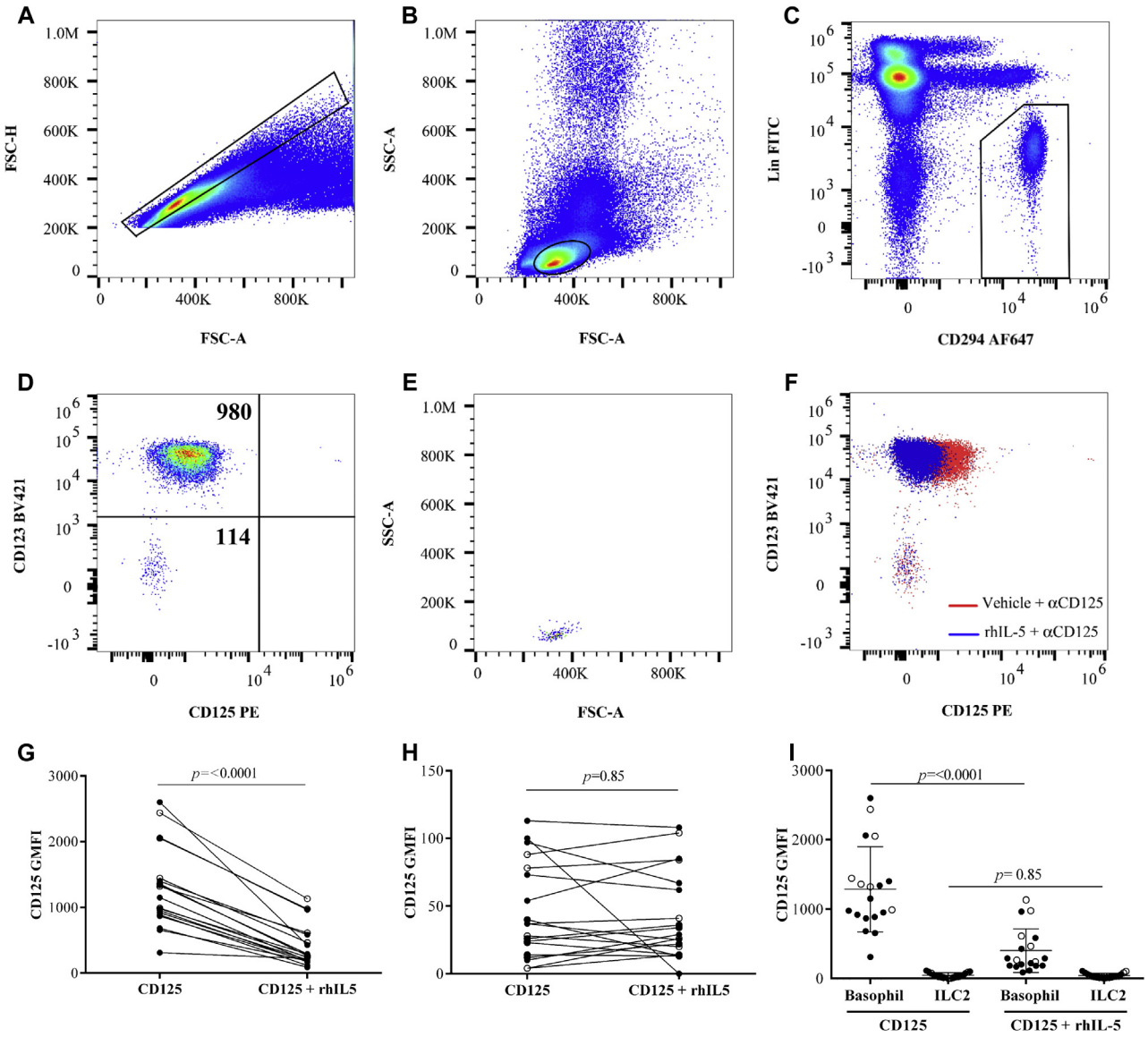

FIG 1. CD125 protein is absent on blood ILC2s. **A,** Total PBMCs, singlets within boxed region. **B,** Singlet light scatter with lymphocyte gate overlaid. **C,** Lineage$^-$, CD294$^+$ cells were identified (boxed region) encompassing **(D)** CD123$^+$ basophils and CD123$^-$ ILC2s, in the upper and lower left quadrant, respectively, with CD125 GMFI values. **E,** ILC2 light scatter properties. **F,** Overlay of basophil and ILC2 CD125-stained cells in the absence (red) or presence (blue) of rhIL-5. **(G)** Basophil and **(H)** ILC2 CD125 GMFI paired data (mean ± SD) from 6 healthy volunteers (open) and 13 (filled) volunteers with asthma, also shown in **(I)** unpaired on the same axes. Statistical comparisons were made using a Wilcoxon matched-pairs signed rank test. *APC*, Allophycocyanin; *FITC*, fluorescein isothiocyanate; *FSC-A*, forward scatter-area; *FSC-H*, forward scatter-height; *SSC-A*, side scatter-area.

The strength(s) of this study lies in the fact that we have measured protein IL-5Rα subunit expression in a rare critical regulator of type 2 inflammation, ILC2s, and in the context of rhIL-5–mediated receptor blockade/downregulation, rather than an isotype control, which is less robust. The robustness of our data using human peripheral blood (from donors with asthma), *ex vivo* activated cells, and lung tissue cells diminishes the likelihood that IL-5Rα–expressing ILC2s are present in asthmatic tissue. Although it would be desirable to extend our blood and tissue observations to investigate whether ILC2s in bronchial biopsies from patients with asthma express IL-5Rα, the paucity of tissue ILCs and the need for multiple immunological markers to positively identify them severely limits this approach. These results extend the list of cells[6] that are known not to express the IL-5Rα subunit, specific for the biological activities of IL-5. Moreover, we show that rare tissue-derived basophils, like their blood counterparts, express the IL-5Rα. Our data suggest that the success of therapeutic interventions targeting IL-5/R is unlikely to be mediated directly on ILC2s but may function via both eosinophils and basophils.

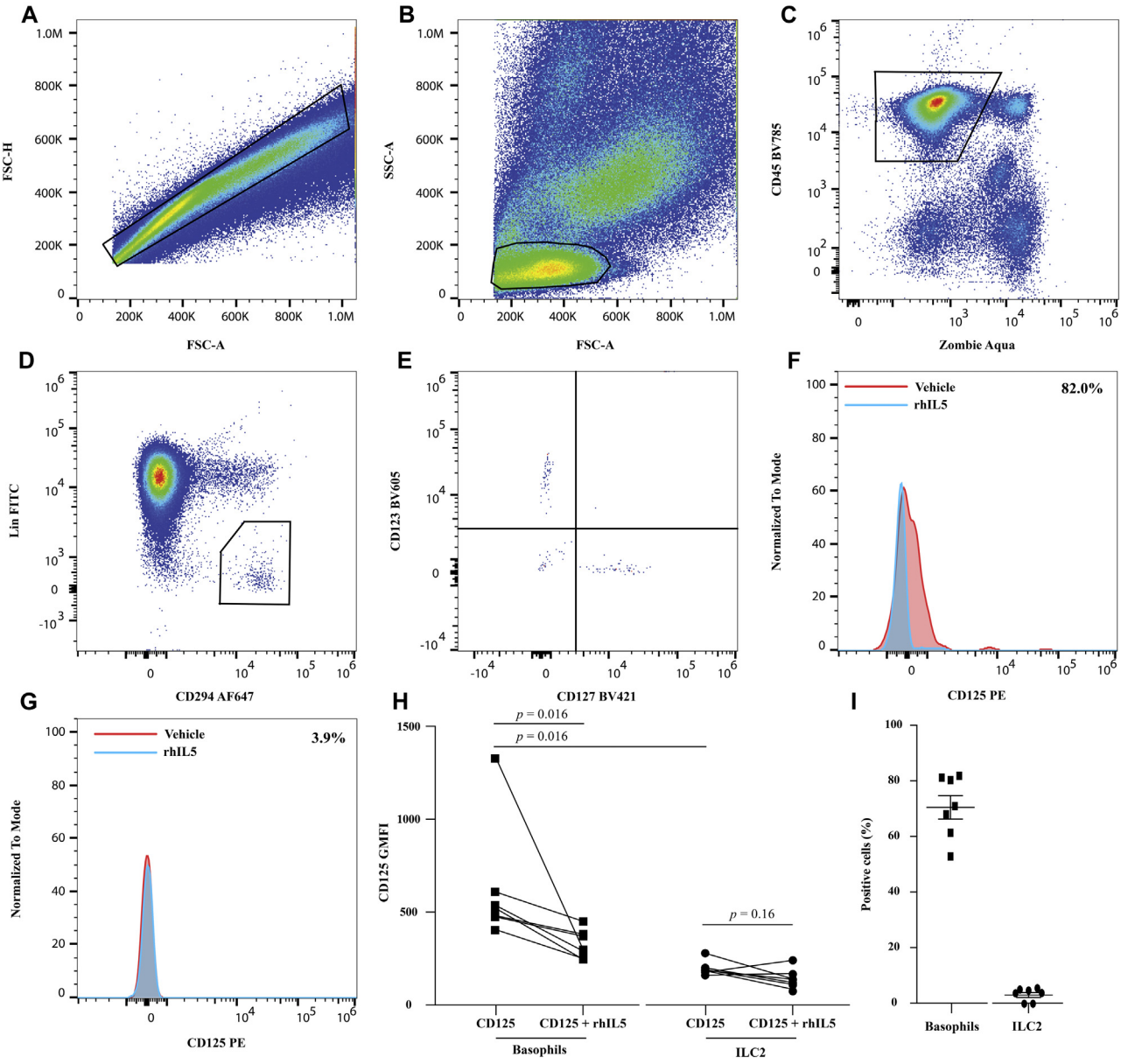

FIG 2. CD125 protein is absent on lung-derived ILC2s. **A,** Total lung tissue cells, singlets within boxed region. **B,** Singlet light scatter properties with lymphocyte gate overlaid. **C,** Viable CD45$^+$ cells (boxed region). **D,** Lineage$^-$ CD294$^+$ cells (boxed region). **E,** ILC2s (CD123$^-$CD127$^+$) and basophils (CD123$^+$CD127$^-$) were identified. **(F)** Basophil CD125 fluorescence and **(G)** ILC2 CD125 fluorescence in the absence (red) or presence of rhIL-5 (blue). **H,** Collated basophil and ILC2 CD125 GMFI (mean ± SD) data (n = 7). **I,** Proportion of CD125$^+$ basophils and ILC2s (calculated using SED); exemplar values given in Fig 2, *F* and *G*. Statistical comparisons were made using a Wilcoxon matched-pairs signed rank test. *FITC,* Fluorescein isothiocyanate; *FSC-A,* forward scatter-area; *FSC-H,* forward scatter-height; *SSC-A,* side scatter-area.

We thank all research participants for taking part in this study and Tracy Thornton and Marcia Soares for their assistance with volunteer recruitment and data collection. We also thank Prof Andy Wardlaw, Paige Tongue, Malgorzata Rekas, Will Monteiro, Dr Amanda Sutcliffe, Beverley Hargadon, Sarah Parker, and Hilary Marshall for their assistance in the procurement and processing of lung tissue.

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This work was part funded by (for C.E.B.) Airway Disease Predicting Outcomes through Patient Specific Computational Modelling (AirPROM) project (funded through FP7 European Union grant), Wellcome Senior Fellowship (for D.J.C.), Medical Research Council (MRC), and Asthma UK (Centre grant no. G1000758), from the National

Institute for Health Research (NIHR) Biomedical Research Centre based at Guy's and St Thomas' NHS Foundation Trust and King's College London, an equipment grant from the Midlands Asthma and Allergy Research Association and (for C.E.B. and D.J.C.) the NIHR Leicester Respiratory Biomedical Unit. B.M.J.R. was funded by an MRC and Asthma UK PhD studentship. This article presents independent research funded by the NIHR. The views expressed are those of the authors and not necessarily those of the National Health Service, the NIHR, or the Department of Health.

Disclosure of potential conflict of interest: C. E. Brightling has consultant arrangements with AstraZeneca/MedImmune, GlaxoSmithKline, Roche/Genentech, Pfizer, Chiesi, Novartis, Vectura, Theravance, PreP, and Boehringer Ingelheim and has received grants from AstraZeneca/MedImmune, GlaxoSmithKline, Pfizer, Boehringer Ingelheim, Chiesi, Novartis, and Roche/Genentech. D. J. Cousins has received grants from the Medical Research Council, Asthma UK, the National Institute for Health Research, GlaxoSmithKline, MedImmune, and the Biotechnology and Biological Sciences Research Council. The rest of the authors declare that they have no relevant conflicts of interest.

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Available online May 10, 2017.
http://dx.doi.org/10.1016/j.jaci.2017.04.025

Comparison of the immunogenicity of BM32, a recombinant hypoallergenic B cell epitope–based grass pollen allergy vaccine with allergen extract–based vaccines

To the Editor:

Allergen-specific immunotherapy (AIT) is a clinically and cost-effective allergy treatment that modifies the course of the disease and has long-lasting effects.[1,E7,E8] However, allergen extract–based forms of AIT require administration of multiple doses, which makes treatment cumbersome and leads to poor compliance in patients.[2] A number of approaches were proposed to address this issue, including use of AIT materials with higher safety, such as allergoids, recombinant allergen derivatives, and allergen-derived peptides, which allow shortening of the build-up phase.[E9]

In this study we have investigated the ability of the recombinant B cell epitope–based allergy vaccine BM32 to induce allergen-specific IgG antibodies and the ability of these antibodies to inhibit allergic patients' IgE binding to grass pollen allergens, as well as allergen-induced T-cell proliferation. BM32 is a recombinant grass pollen *(Phleum pratense)* allergy vaccine based on 4 fusion proteins consisting of peptides from the 4 major timothy grass pollen allergens (Phl p 1, Phl p 2, Phl p 5, and Phl p 6) fused to the PreS carrier protein from hepatitis B, which lacks relevant allergenic activity[E10] and therefore can be injected into allergic patients without need for updosing.[3,4] For our immunogenicity studies, we have used a dose (ie, 20 μg of each of the 4BM fusion proteins) that has been safely administered to allergic patients (ClinicalTrials.gov identifiers NCT01445002, NCT01538979, and NCT02643641).[4]

The main finding of our current study was that 3 monthly subcutaneous injections of aluminum hydroxide–adsorbed BM32 induced IgG antibody levels to the major grass pollen allergens Phl p 1, Phl p 5, and Phl p 6 in rabbits, which were comparable with natural allergen extract–based registered grass pollen allergy vaccines requiring more than 8 injections (Allergovit grass; Allergopharma, Reinbek, Germany; Alutard SQ grass mix; ALK-Abelló, Hørsholm, Denmark; and Phostal grasses + rye; Stallergenes, Antony, France; see the Methods section in this article's Online Repository at www.jacionline.org; Fig 1), whereas almost no response was observed with Pollinex (Pollinex Quattro Plus grasses + rye; Bencard Allergie GmbH, Munich, Germany), a vaccine based on 4 injections. Importantly, BM32 induced higher levels of Phl p 2–specific IgG antibodies than any of the registered allergen extract–based vaccines (Fig 1). We consider this an important finding because it has been shown that group 2 allergens are major grass pollen allergens recognized by more than 60% of patients with grass pollen allergy and, when compared with the other grass pollen allergens by using skin testing, were found to show high allergenic potency.[5] Thus our results indicate that it should be possible to build up sufficient levels of grass pollen allergen–specific IgG responses with only few injections (ie, 3-5) of BM32, whereas traditional allergy vaccines require more than double the number of updosing injections. Sublingual treatment even requires daily administration. Therefore we think that the treatment schedules based on BM32 will be more convenient for patients and should increase their compliance.

The kinetics of allergen-specific IgG antibodies induced with BM32 were similar to those induced by natural allergen extract–based vaccines in terms of increases and decreases (Fig 1). According to the hapten carrier principle described by the Nobel laureate B. Benacerraf,[E11] a robust antibody response can be obtained against a haptenic structure lacking T-cell epitopes if this structure is covalently bound to an unrelated carrier molecule, such as the PreS that contains T-cell epitopes. Carrier-specific T cells support antibody production against the carrier and the hapten. Therefore the question was whether an allergen-specific IgG immune response initiated with BM32 can be boosted by repeated immunization with BM32. To address this question, we kept rabbits that had received a first course of 3 monthly injections with BM32 for 5.5 months without immunization and then applied 1 booster injection; after an additional 4.5 months, we administered another booster injection (see Fig E1 in this article's Online Repository at www.jacionline.org). BM32-induced grass pollen allergen–specific IgG production

METHODS

Subjects

Control subjects (without a history of respiratory disease) and subjects with an asthma diagnosis were recruited at University Hospitals of Leicester NHS Trust, Leicester, United Kingdom (UK), and provided informed written consent, under ethics (REC reference no. 08/H0406/189) approved by the Leicestershire, Northamptonshire, and Rutland ethics committee, to take part in this study. The recruited subjects with asthma were mostly diagnosed with late-onset asthma. Lung resection material was ethically obtained at University Hospitals of Leicester NHS Trust, under the auspices of the Midlands lung tissue consortium (REC reference no. 07/MRE08/42), from a separate cohort of patients undergoing lung surgery. Clinical characteristics can be found in Table E1 (blood) and Table E2 (lung tissue). Additional control subjects (without a history of respiratory disease) were also recruited under ethical approval from the research ethics committee of Guy's Hospital, London, UK (REC reference no. 09/H804/077) as described previously[E1] to isolate ILC2 cDNA for downstream experiments.

Cells

Human eosinophils were purified from whole blood using CD16 microbead depletion of neutrophils over CS columns (both Miltenyi Biotec, Surrey, UK) to a purity of 97% as described previously.[E2] Purified eosinophils were immediately placed into Qiazol (Qiagen, Manchester, UK) for downstream RNA isolation and used as a positive control for the detection of transmembrane and soluble mRNA variants of the IL-5Rα gene. ILC2s were isolated from peripheral blood by flow cytometric cell sorting and cultured *in vitro* as described in detail previously.[E1] Briefly, ILC2s were grown at 37°C, 5% CO_2, in a humidified incubator at a density of 10,000 cells/mL in RPMI 1640 supplemented with 10% FBS, glutamine, penicillin, and streptomycin (all Thermofisher, Paisley, UK). Recombinant cytokines were added as described in the figure legend (rhIL-2 [50 U/mL; Eurocetus, Amsterdam, The Netherlands], rhIL-7 [50 ng/mL; R&D Systems, Abingdon, UK], rhIL-25 [125 ng/mL; R&D Systems], and rhIL-33 (50 ng/mL; R&D Systems]). Lung tissue cells were obtained from enzymatically digested lung obtained within 1 hour of resection (n = 7). Lung tissue bathed in media (Dulbecco modified Eagle medium, with 2% FBS) was cut into small pieces and enzymatically digested using hyaluronidase (0.75 mg/mL final, H3506, Sigma, Gillingham, Dorset, UK) and collagenase (0.75 mg/mL final, C2674, Sigma) for 75 minutes at 37°C. Digested tissue was passed through a syringe, filtered through a 100- and 50-μm gauze, then washed twice with media (centrifuging at 230*g* for 8 minutes, 4°C). Cell counts were performed using hemocytometer and Kimura stain, washed again, and the cell suspension was prepared for flow cytometry, as described below.

Flow cytometry

PBMCs were isolated following centrifugation of whole blood over Lymphoprep (Stem Cell Technologies, Cambridge, UK). PBMCs were washed in PBS containing 2% FBS (Thermofisher) and 2 mM EDTA (Thermofisher) and the cell density was adjusted to 1×10^6/100 μL before cell surface staining. For lung tissue cells, at least 5×10^5 cells were resuspended in PBS and stained with Zombie Aqua fixable viability kit (as described by the manufacturer [Biolegend, London, UK]) for 20 minutes at room temperature. Following incubation, lung tissue cells were washed and resuspended in 50 μL brilliant violet stain buffer (Becton Dickinson, Oxford, UK) before cell surface staining. A total of 1×10^6 PBMCs or lung tissue cells were then incubated at room temperature for 15 minutes with Human Trustain (Biolegend, London, UK) to block Fc receptors and either a vehicle control (PBS/2%FBS) or rhIL-5 (50 or 100 ng/mL final for PBMCs and lung tissue cells, respectively [Miltenyi Biotec]), to block cell surface staining of IL-5Rα. Cells were then incubated at room temperature with the following anti-human mAbs in a fluorescence minus one[E3] set-up: BV_{421}-conjugated CD123 (clone 6H6, Biolegend), fluorescein isothiocyanate–conjugated lineage (α-CD2, α-CD3, α-CD14, α-CD16, α-CD19, α-CD56, α-CD235a) cocktail (Affymetrix, Cheshire, UK), phycoerythrin-conjugated α-CD125 (clone A14, Becton Dickinson), and AF647-conjugated α-CD294 (clone BM16, Biolegend). In some experiments, whole blood (100 μL) was stained as described above. For single-cell suspensions derived from lung tissue, the lineage cocktail, CD125, and CD294 antibodies (described above) were supplemented with BV_{421}-conjugated CD127 (clone A019D5), BV_{605}-conjugated CD123 (clone 6H6), and BV_{785}-conjugated CD45 (clone HI30) (all Biolegend) to enable identification of tissue-derived ILC2s and basophils. PBMCs were washed with PBS/2%FBS before acquisition, whereas whole blood or lung tissue cells were fixed and lysed (1-step fix and lyse, Affymetrix) to remove erythrocytes according to the manufacturer's protocol. Cell acquisition was performed on an Attune flow cytometer operating NxT software v2.2 (Life Technologies). CD125-PE and rhIL-5 (for blocking purposes) were both titrated in pilot experiments. Compensation beads (Becton Dickinson, High Wycombe, UK) and unstained cells were used to determine compensation settings. Analysis was performed using FlowJo v10 (Tree star, Ashland, Ore).

RT-PCR

RNA was isolated from purified cell populations using the miRNeasy mini kit (Qiagen), followed by DNAse digestion (Thermofisher) and RNeasy minElute cleanup (Qiagen) according to the respective manufacturer's instructions. RNA integrity was assessed using a Bioanalyser (Agilent, Edinburgh, UK), according to the manufacturer's instructions. For eosinophil samples, cDNA synthesis was performed using superscript II reverse transcriptase (Thermofisher), random hexamers (Roche, West Sussex, UK), and dNTPs (Thermofisher) according to the manufacturer's instructions. Because of low cell numbers, ILC2 cDNA samples were amplified using the Ovation PicoSL WTA system V2 kit (NuGEN, Leek, Netherlands) as per the manufacturer's instructions. Real-time RT-PCR was performed as described previously[E4] using TaqMan Master mix II (Thermofisher) and TaqMan probe sets Hs00602482_m1 (transmembrane and soluble variants of the IL-5Rα), Hs00236871_m1 (transmembrane only variant of IL-5Rα), or Hs00174200_m1 (IL-5) (Thermofisher) and compared with an 18S rRNA endogenous control (Thermofisher), using FAM-MGB (IL-5Rα probe sets) and VIC-MGB (18S rRNA) dyes, respectively. Cycling conditions were as follows: 50°C for 2 minutes, 95°C for 10 minutes, 95°C for 15 seconds then 60°C for 1 minute for 50 cycles on ViiA7 real-time PCR system (Thermofisher). Because IL-5Rα transcripts were undetectable, to confirm that a sufficient amount of ILC2 cDNA from each condition was present in the reaction, the mean (±SEM) 18S CT values were as follows: D0 (18.4 ± 0.18), D1 (18.5 ± 0.23), D2 (18.0 ± 0.17), D4 (17.4 ± 0.01), D7 (17.4 ± 0.03), D14 (16.8 ± 0.08), eosinophils (16.2 ± 0.4) (representative results from 1 donor). Furthermore, to confirm that ILC2s were activated in the presence of rhIL-2/7/25 and 33, we have presented IL-5 transcript data alongside the IL-5Rα data in Fig E1.

Statistics

Descriptive and comparative (super enhanced Dmax SED) subtraction % positive[E5] analyses of flow cytometric population data were performed using FlowJo version 10 (Tree Star). Descriptive and comparative analyses were performed using Graph Pad PRISM v6 (GraphPad software, Inc, La Jolla, Calif), and Shapiro-Wilk normality test was used to determine data distribution. Statistical comparisons were made using paired or unpaired tests where relevant, as indicated, with statistical significance determined if the *P* value was less than .05.

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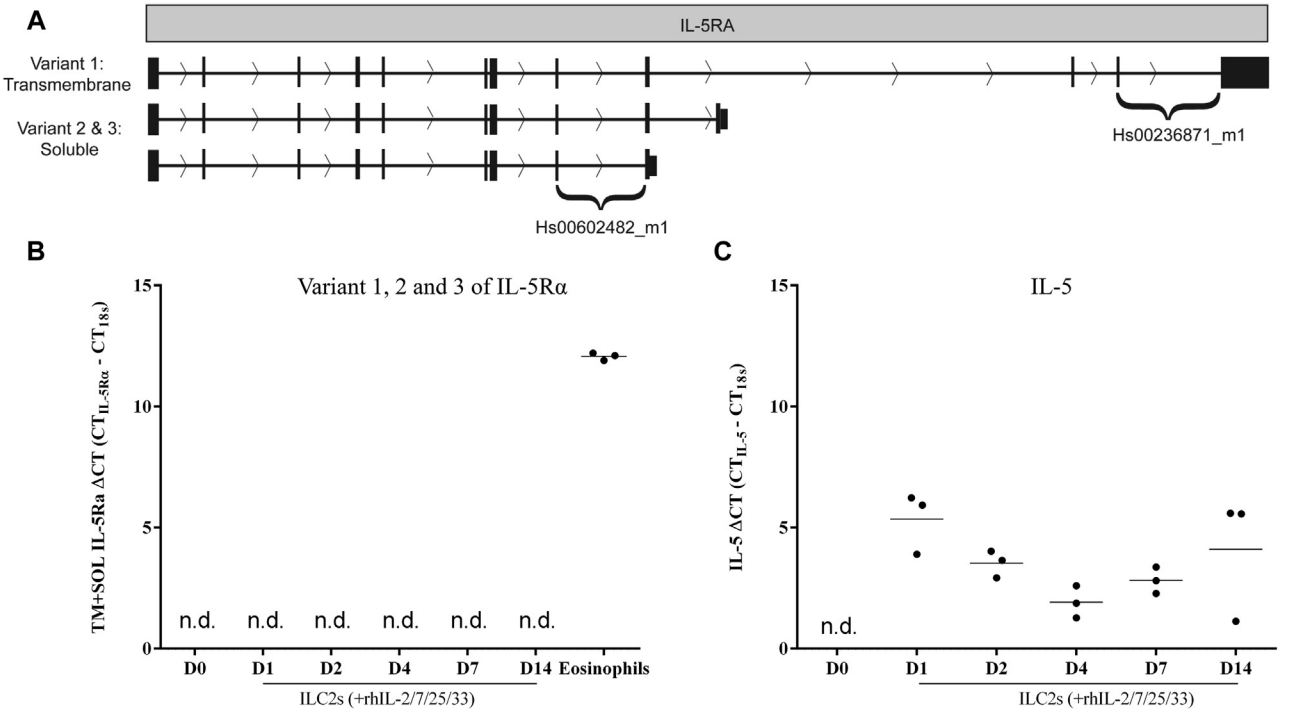

FIG E1. ILC2s do not express IL-5Rα transcripts. **A,** Schematic representation of IL-5Rα mRNA showing the splice variants and probe sets Hs00602482_m1 (detecting soluble and transmembrane variants) and Hs00236871_m1 (transmembrane only) used in this study. ΔCT values were calculated relative to 18S mRNA for **(B)** all IL-5Rα transcript variants using the Hs00602482_m1 probe and **(C)** IL-5 transcripts using Hs00174200_m1. In Fig E1, *B* and *C*, data are derived from isolated ILC2s (n = 3) that were grown for up to 14 days in the presence of rhIL-2, rhIL-7, rhIL-25, and rhIL-33. In Fig E1, *B*, cDNA generated from freshly isolated eosinophils was also included as a positive control in each IL-5Rα assay. In Fig E1, *C*, IL-5 transcript data are shown to confirm activation in the presence of cytokines; low ΔCT values reflect high abundance of the IL-5 transcript. *n.d.*, Not detected.

TABLE E1. Clinical characteristics of the study participants recruited for blood collection

Characteristic	Control	Asthma	*P* value*
n	6	13	
Age (y)†	50 ± 15	59 ± 13	.18
Sex: M:F	4:2	6:7	.63
Smoking history (never, ex, current)‡	5, 1, 0	9, 4, 0	1.0
BMI (kg/m^2)†	27 ± 4	29 ± 6	.47
Post-BD FEV_1 (L)†	3.6 ± 0.8	2.5 ± 0.9	.02
Post-BD FEV_1 (% predicted)†	109 ± 16	92 ± 24	.17
FEV_1/FVC (ratio)†	0.82 ± 0.06	0.71 ± 0.09	.01
WBC ($\times 10^9$/L)†	6.4 ± 0.9§	7.1 ± 2.8‖	.63
Eosinophils ($\times 10^9$/L)†	0.16 ± 0.04§	0.28 ± 0.16‖	.12
Eosinophil (%)	2.5 ± 0.6§	4.3 ± 2.4‖	.13
Basophils ($\times 10^9$/L)†	0.04 ± 0.02§	0.04 ± 0.02‖	.97
GINA 1, 2, 3, 4, 5	NA	1, 1, 0, 7, 4	NA
Beclomethasone dipropionate equivalents (μg)¶	NA	1000 (650-1600)	NA

BD, Bronchodilator; *BMI*, body mass index; *F*, female; *FVC*, forced vital capacity; *GINA*, Global Initiative for Asthma; *M*, male; *NA*, not applicable; *WBC*, white blood cell.
*Unpaired *t* test.
†Mean ± SD.
‡All ex-smokers have quit smoking for 3+ years.
§n = 5.
‖n = 12.
¶Median (interquartile range).

TABLE E2. Clinical characteristics of the study participants recruited for lung tissue collection

Characteristic	Control
n	7
Age (y)*	66 ± 9
Sex: M:F	4:3
Smoking history (never, ex, current)†	0, 4, 3
WBC ($\times 10^9$/L)*	9.5 ± 3.7
Eosinophils ($\times 10^9$/L)*	0.24 ± 0.18
Eosinophil (%)*	2.4 ± 1.1
Basophils ($\times 10^9$/L)*	0.04 ± 0.03
Diagnosis	Adenocarcinoma (n = 4) Squamous cell carcinoma (n = 2) Lung volume reduction surgery (n = 1)

F, Female; *M*, male; *WBC*, white blood cell.
*Mean ± SD.
†All ex-smokers have quit smoking for 2+ years.