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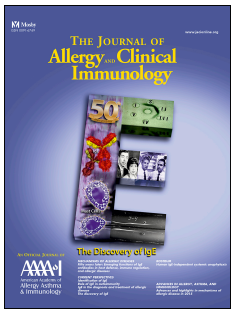

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Jonatan Leffler, PhD, Anya C. Jones, MSc, Elysia M. Hollams, PhD, Franciska Prastanti, MD, Peter N. Le Souëf, MD, Patrick G. Holt, PhD, Anthony Bosco, PhD, Ingrid A. Laing, PhD, Deborah H. Strickland, PhD

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Basophil levels in PBMC population during childhood acute wheeze/asthma are associated with future exacerbations

Jonatan Leffler PhD[1, #], Anya C. Jones MSc[1,2], Elysia M. Hollams PhD[1], Franciska Prastanti MD[3], Peter N. Le Souëf MD[1,2], Patrick G. Holt PhD[1,4], Anthony Bosco PhD[1], Ingrid A. Laing PhD[1,3,*], Deborah H. Strickland PhD[1,*]

[1]*Telethon Kids Institute, University of Western Australia, WA, Australia*
[2]*Division of Paediatrics, University of Western Australia, WA, Australia*
[3]*School of Biomedical Sciences, University of Western Australia, WA, Australia*
[4]*Child Health Research Centre, University of Queensland, QLD, Australia*

[*]Shared senior author
[#]Corresponding author
Dr. Jonatan Leffler, Telethon Kids Institute, 100 Roberts Road, Subiaco, Western Australia 6008, Australia
Email: Jonatan.Leffler@telethonkids.org.au
Phone: +61 8 9489 7897
Fax +61 8 9489 7700

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Conflicts of interest
The authors declare no conflicts of interest.

Key words acute asthma; basophils; PBMC; children; recurrent wheeze

Encapsulating summary Our data suggest that a basophil level above 0.18% of the PBMC population during an acute respiratory exacerbation is associated with an increased risk for future exacerbations in children with asthma and/or wheeze.

To the Editor

During early childhood, respiratory infections may result in wheezing episodes, which together with atopy commonly precede asthma development (1). A subset of wheezing children experience frequent exacerbations and are overrepresented among respiratory hospitalisations, and are estimated to be responsible for ~80% of health care costs associated with asthma and related diseases (2). Patients with recurrent exacerbations exist throughout the full range of the asthma exacerbation severity spectra but are at high risk of irreversible airflow limitations and loss of responsiveness to available therapeutics (3). Hence, early identification of these children is crucial for tailored treatment regimens and may also help to better understand the mechanisms of the development of recurrent exacerbations. There are currently no appropriate biomarkers that reliably identify children at risk of recurrent exacerbations.

Asthma-associated airway inflammation results in the recruitment of immune cells from the circulation and promotes an altered immune cell output from the bone marrow. Thus, the circulating peripheral blood mononuclear cell (PBMC) population reflects disease activity and phenotype (4, 5). Since these cells are *en route* to respiratory tissues and are more easily accessible in children than lung tissue, we have focused on the PBMC population, as a source of immune cells, to identify cellular response profiles associated with recurrent exacerbations. The cellular PBMC profile was characterised during an exacerbation using multi-parameter flow cytometry, to identify how proportions and activation status of dendritic cells, basophils, B cells, monocytes and T cells within the PBMC preparation (Fig E1A-B), differ between patients with few versus multiple previous exacerbations.

To define an exacerbation-prone phenotype, we divided the patients into two groups comprising those with high versus low exacerbation recurrence. By using a cut-off of 4 prevous wheezing or acute asthma exacerbations, we separated approximately the top quarter of recurrent patients (Fig 1A-B). Between the two patient groups, there was no difference in sex, age at recruitment, atopic status, virus prevalence or exacerbation severity (Table E1). Given that the age of the patients ranged from 0.6-12.3 years and the frequencies of some cell subsets (such as B cells and $CD4^+$ T effector cells) change with increasing age (6), we used multiple regression to adjust

for age in our analyses. To account for varying effect size for common and rare cell subsets, we also normalised each subset to the median. We observed that elevated proportions of $CD123^{+}/CD11c^{lo}/HLA^{-}$ basophils (as per Figure E1A) were positively associated with a history of >4 exacerbations, whereas high proportions of $CD3^{+}/CD4^{+}/CD25^{+}/FoxP3^{-}$ T effector cells were negatively associated with >4 exacerbations (Fig 1C). Within the basophil subset, we further observed that CD25 expression was decreased in patients with >4 previous exacerbations (data not shown). The identified associations were also confirmed using the number of previous exacerbations as a continuous variable, basophils (p=0.02) and $CD4^{+}$ T effector cells (p=0.04).

After establishing that a history of >4 exacerbations was associated with altered proportions of basophils and $CD4^{+}$ T effector cells during an acute exacerbation, we stratified the patients into two groups based on a median split of these cell types and assessed the risk of a future exacerbation within the follow-up period. By plotting exacerbation free time, we observed that patients with high basophil levels were more likely to experience a subsequent exacerbation (Fig 2A), whereas no difference was observed for patients with $CD4^{+}$ T effector cell levels above or below the median (p = 0.13), (data not shown) suggesting that not all changes associated with a history of exacerbations contribute to a recurrent disease phenotype. To confirm our findings, we studied an independent set of patients, and stratified them into two groups based on a median split of basophil levels during an asthma exacerbation. Of note, this group of patients were slightly older, had a higher prevalence of atopy, but had less severe exacerbations (Table E2). We again observed that patients with high basophil levels were more likely to experience an additional exacerbation within the follow-up period, compared to patients with low basophil levels (Fig 2B). Since patients with a history of >4 exacerbations were more likely to experience an additional exacerbation (p<0.001, data not shown), we investigated patients from our study population who had been sampled at their first ever exacerbation (n = 19). Of the patients with basophil levels above the median level for the study population (0.18% of PBMC), 7 (70%) patients experienced a subsequent exacerbation within the follow-up period, whereas only 1 (11%) with basophils levels below the median had an additional exacerbation (χ^2 p < 0.01) and Fig 2C.

These results suggest that patients with a high proportion of basophils within the PBMC preparation (i.e. patients with many basophils with a density that is below 1.077 g/mL), were more likely to experience another exacerbation during the follow-up period. These findings were not mirrored/reflected in total PBMC or whole blood basophil numbers, with the latter determined from clinical measurements of differential cell counts (data not shown), indicating that the predictive effect is specific to the proportion of basophils with lower density. This is likely a direct consequence of recent degranulation or activation, and elevated levels of these cells in PBMC may be indicative of enhanced basophil activity in the exacerbation event in the exacerbation-prone subset. In healthy subjects, basophils in the PBMC fraction indeed display elevated expression levels of selected activation markers (Figure E2A-B), however no difference in granularity / side scatter profile between activated and resting basophils were observed in whole blood (Figure E2C). Of note, in subjects with both whole blood and PBMC basophil data available (n = 16), the ratio of low density PBMC basophils:total blood basophils was higher in the exacerbation prone group, 12.2% ± 7.2% compared to 4.4 ± 5.7% ($p = 0.04$). Basophils are known to be a significant producer of Th2 cytokines and are involved in defence against parasites (7). Moreover, they traffic to the airways during allergen challenge (8) and contribute to disease pathology in eosinophilic asthma (9). Our findings suggest that the proportion of de-granulated basophils may also be associated with recurrent exacerbations. These results will need to be confirmed using a larger cohort and particularly in patients experiencing their first ever exacerbation. Given that early identification of patients with recurrent asthma exacerbations is difficult, the use of basophil proportion and density as a potential marker warrants further exploration.

Jonatan Leffler, PhD[1]
Anya C. Jones, MSc[1,2]
Elysia M. Hollams, PhD[1]
Franciska Prastanti, MD[3]
Peter N. Le Souef, MD[1,2]
Patrick G. Holt, PhD[1,4]
Anthony Bosco, PhD[1]
Ingrid A. Laing, PhD[1,3,*]

Deborah H. Strickland, PhD[1,*]

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CONFLICTS OF INTEREST

The authors declare no conflicts of interest

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FIGURE LEGENDS

Figure 1

A-**B** Patient exacerbation history at the time of sampling plotted against age (**A**) or per patient distribution (**B**). Shaded area indicates patients with >4 exacerbations. **C** Normalised proportions of cellular subsets comparing children with a history of >4 or ≤4 exacerbations, adjusted for age. * $p<0.05$, $n = 40$.

Figure 2

A-**B** Exacerbation free time after recruitment and sampling is plotted for all patients comparing the proportion of basophils either above or below median levels in the original cohort (**A**) as well as an independent patient set (**B**). **C** Exacerbation free time in the combined patient cohort following recruitment and sampling, comparing patient basophil levels either above or below combined median levels. Significance of difference was calculated using log-rank test.

Figure 1
Leffler et al. (2 columns)

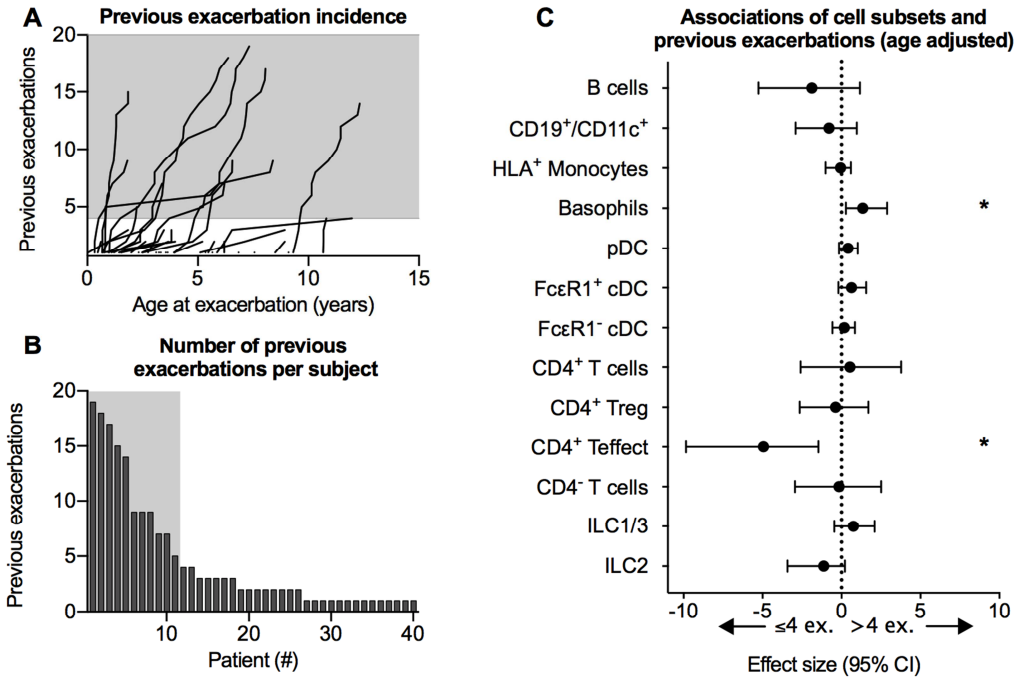

Figure 2
Leffler et al.
(1.5 column)

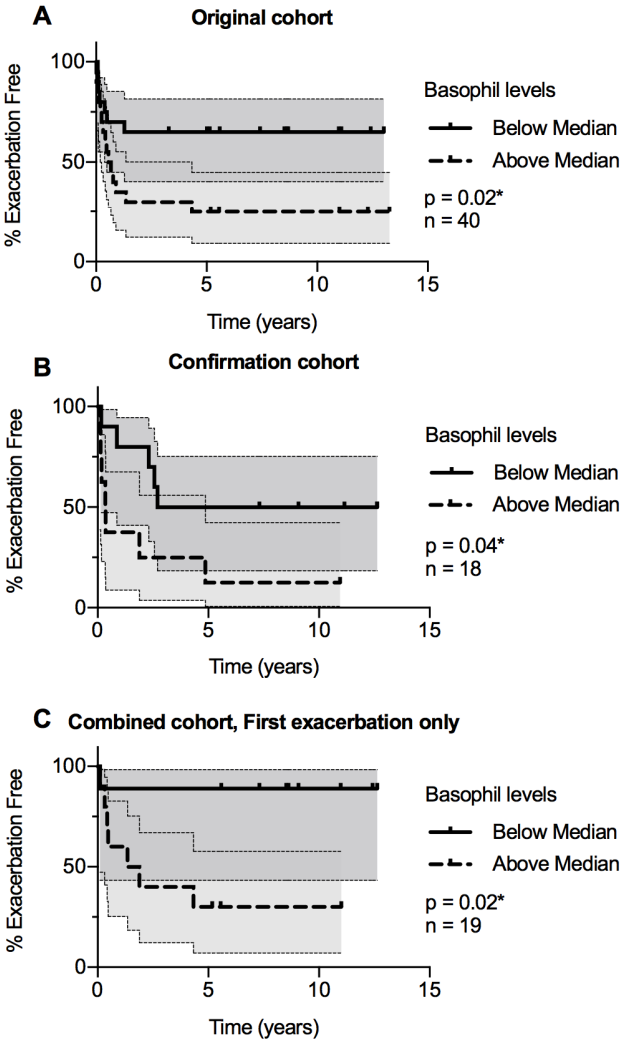

Table E1 – Patient demographics at an acute exacerbation in patients with more than 4 and 4 or less previous exacerbations. For assessments of statistical significance between groups, Pearson's χ^2 or one-way ANOVA were used for categorical or continuous variables, respectively, as appropriate.

	≤4 previous exacerbations	>4 previous exacerbations	Significance of difference (p)
Number of patients, n (%)	29 (73%)	11 (28%)	n/a
Age, mean years (range)	4.9 (0.6-12)	5.9 (1.8-12.3)	0.35
Male gender, n (%)	17 (58.6%)	7 (63.6%)	0.77
Family history of asthma (parents or siblings), n (%)	20 (83.3%)	11 (100%)	0.15
Atopy to aeroallergen, n (%)	20 (69.0%)	6 (54.5%)	0.39
Total IgE, mean IU/mL (range)	334.3 (21.1-1270.4)	328.6 (24.4-1938.0)	0.97
Season recruited			0.27
Spring (Sept – Nov)	7 (24.1%)	3 (27.3%)	
Summer (Dec – Feb)	4 (13.8%)	0 (0%)	
Autumn (Mar – May)	10 (34.5%)	2 (18.2%)	
Winter (Jun – Aug)	8 (27.6%)	6 (54.6%)	
Admitted to hospital, n (%)	27 (93.1%)	10 (90.9%)	0.82
Hours from last oral corticosteroid administration to blood collection, mean (range)	7.6 (0.8-49.5)	11.8 (2-25.2)	0.23
Exacerbation severity Z-score, mean (range)	-.07 ((-2.2)-1.6)	-0.2 ((-1.4)-1.2)	0.68
Total PBMC cell count, x 10^5/mL mean (range)	11.0 (4.4-21)	13.1 (5.4-28.2)	0.22
Respiratory viral infection positive, n (%)	21 (91.3%)	10 (90.1%)	0.97
Diagnosis at sample			0.35

Acute asthma, n (%)	23 (79.3%)	8 (72.73%)	
Virus induced wheeze, n (%)	5 (17.2%)	2 (18.2%)	
Other, n (%)	1 (3.5%)	1 (9.1%)	

Table E2 – Patient demographics of the two cohorts. For assessments of statistical significance between groups, Pearson's χ^2 or one-way ANOVA were used for categorical or continuous variables, respectively, as appropriate.

	Original cohort	Confirmation cohort	Significance of difference (p)
Number of patients, n	40	18	n/a
Age, mean years (range)	5.1 (0.6-12.3)	9.3 (6.9-13.2)	<0.001
Male gender, n (%)	24 (60.0%)	11 (61.1%)	0.94
Family history of asthma (parents or siblings), n (%)	31 (88.6%)	13 (81.3%)	0.48
Atopy to aeroallergen, n (%)	26 (65.0%)	18 (100%)	0.004
Total IgE, mean IU/mL (range)	333.0 (21.1-1938.0)	618.1 (123.7-1765.2)	0.02
Season recruited			0.94
Spring (Sept – Nov)	10 (25%)	4 (22.2%)	
Summer (Dec – Feb)	4 (10%)	1 (5.6%)	
Autumn (Mar – May)	12 (30%)	6 (33.3%)	
Winter (Jun – Aug)	14 (35%)	7 (38.9%)	
Admitted, n (%)	37 (92.5%)	14 (87.5%)	0.55
Hours from last oral corticosteroid administration to blood collection, mean (range)	8.7 (.8-49.5)	5.3 (0.4-14.8)	0.21
Exacerbation severity Z-score, mean (range)	-0.1 ((-2.2)-1.6)	-0.8 ((-2.2)-1.2	0.003
Total PBMC cell count x 10^5/mL, mean (range)	11.6 (4.4-28.2)	10.6 (3.4-20.1)	0.46
Respiratory viral infection positive, n (%)	31 (91.2%)	10 (71.4%)	0.08
Diagnosis at sample			0.19
Acute asthma, n (%)	31 (77.5%)	18 (100%)	

Virus induced wheeze, n (%)	7 (17.5%)	0	
Other, n (%)	2 (5%)	0	

Figure E1
Leffler et al.

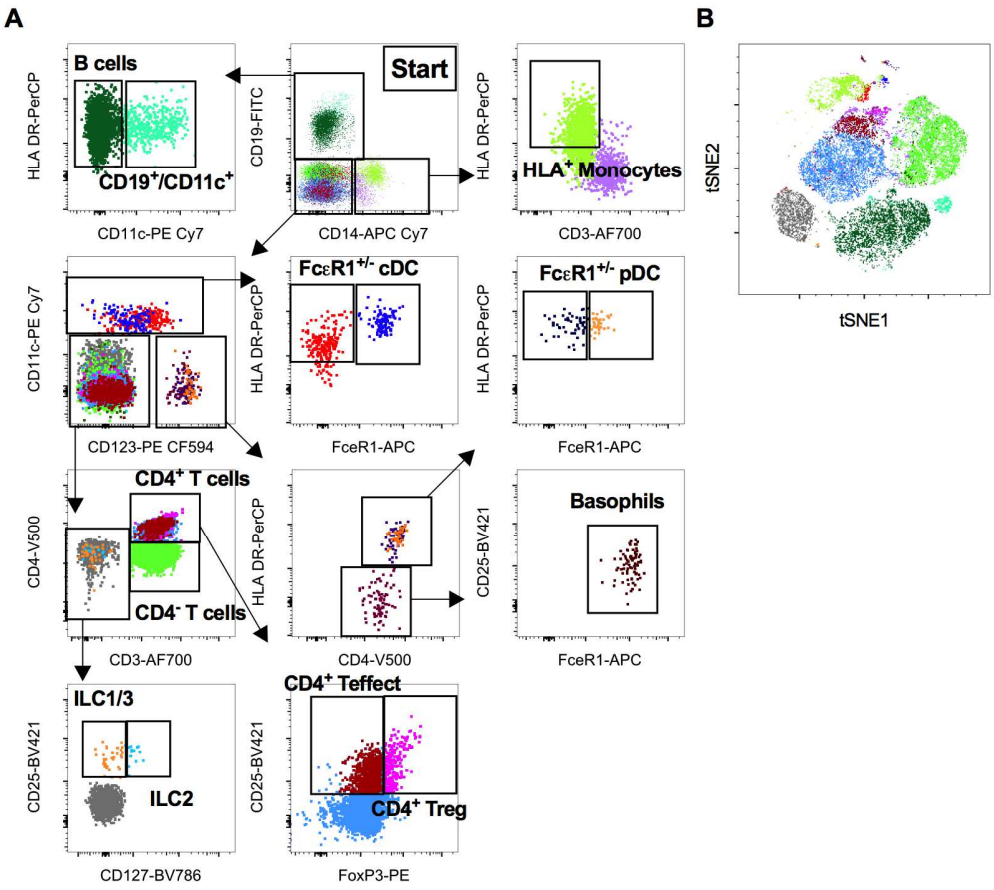

Figure E2
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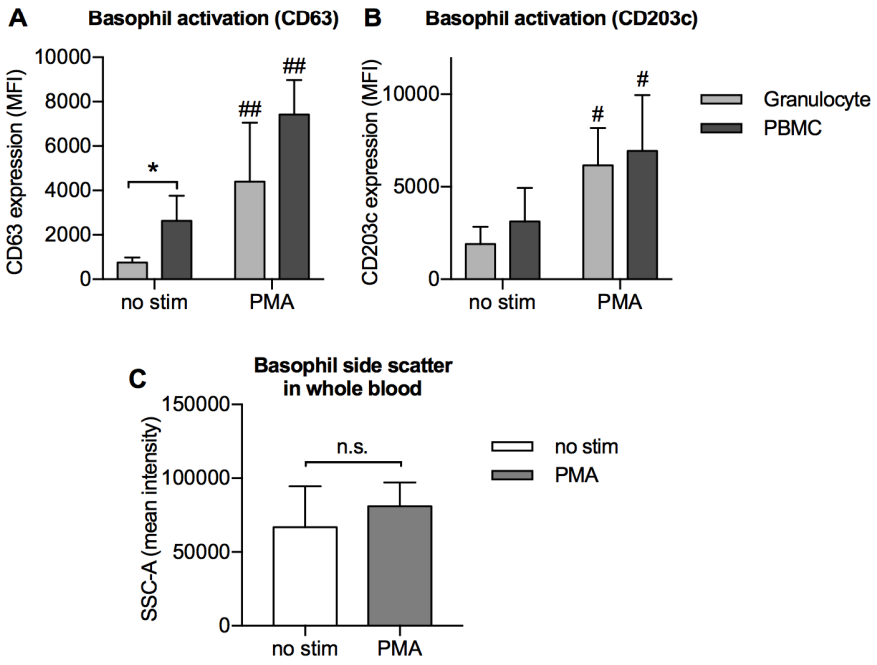

SUPPLEMENTARY DATA – Leffler et al.

METHODS

Patients and cell samples

Two cohorts of patients (n=40 and n=18) with asthma exacerbations, were selected from the Perth based MAVRIC cohort, which was approved by the local ethics committee (Ethics approval# 1761EP). Patients were recruited when they presented to a tertiary children's hospital with a lower respiratory illness in Perth, Western Australia. All respiratory visits to hospital from birth were determined for each child using the Western Australia public hospital database, which records all visits to a public hospital. Blood was collected within 24 hours of presentation to hospital and PBMCs were obtained using lymphoprep (Axis-Shield) and stored in liquid nitrogen. Atopy to an aeroallergen was defined as returning at least one of the following (i) positive skin-prick test (≥3 mm wheal diameter), (ii) specific-IgE against HDM, cat or mixed grasses ≥0.35 kU/L or (iii) history of allergic / anaphylactic reactions following exposure to an aeroallergen. Acute asthma severity scores were assigned to each child at recruitment using a modified National Institute of Health score with separate scales for children over or under 2 years of age as previously (1, 2). Separate severity Z scores were calculated for each child within each of the two age groups to provide standardized scores across the whole cohort. This standardised score was then used across all the subjects analysed. Viral prevalence was determine using a panel of common respiratory pathogens as well as genetic characterisation of respiratory viral infections as previously described (3). Patient characteristics are summarised in Table E2. Additionally, fresh blood from healthy volunteers was stimulated with 10 ng/ml PMA for 20 min at 37 °C prior to separation on lymphoprep as above and/or red blood cell lysis in 0.144 M NH_4Cl and 1 mM $NaHCO_3$ for 15 min. Granulocyte and PBMC fraction was analysed using flow cytometry as below.

Flow cytometry

Frozen PBMC were thawed at 37 °C, suspended in ice-cold RPMI supplemented with 10% fetal calf serum, spun down and resuspended in RPMI. Viability, which in general was above 80% was assessed using trypan blue exclusion. One million cells were stained with CD19-FITC (#555412, BD), CD123-PE CF594 (#562391, BD),

HLA-DR PerCP (#347364, BD), CD11c PE-Cy7 (#561356, BD), FcεR1α APC (#17-5899-42, eBioscience), CD14 APC-Cy7 (#641394, BD), CD4 V500 (#560768, BD), CD11b BV605 (#562723, BD), CD127 BV786 (#563324, BD), CD25 BV421 (#562442, BD), CD3 AF700 (#561027, BD) for 20 min on ice followed by fixation and permeabilisation using FoxP3 staining kit (eBioscience) and stained using FoxP3 PE (#560852, BD). For basophil specific analysis the following markers were used: CD19-FITC, CD123-PE CF594, HLA-DR PerCP, CD11c PE-Cy7, FcεR1α APC, CD14 APC-Cy7, CD63 V450 (#561984, BD) and CD203c BV510 (#563297, BD). Samples were acquired using a LSR-Fortessa (BD) and analysed using FlowJo 10.3 (FlowJo LLC) and viSNE at Cytobank.org (Cytobank).

Statistics

The statistical difference between two patient groups was calculated using One-way ANOVA or Pearsons χ^2 test depending on data characteristics. Age-adjusted associations were calculated using nominal logistic-regression analysis. Time to next exacerbation was plotted using Kaplan-Meier and statistical significance of difference between patient groups was calculated using the Log-Rank test. All statistical analysis was performed using JMP 13 (SAS, NSW, Australia) and data was visualised using Prism 7.0a (GraphPad, CA, USA).

FIGURE LEGENDS

Figure E1 A-B Gating strategy to identify relevant PBMC cell subset. Cell population data used in this study was derived using manual gating (**A**). Gating was confirmed using t-distributed stochastic neighbour embedding (t-SNE)-based algorithm which is used to reduce multi-dimensional flow cytometry data into two dimensions (**B**).

Figure E2 A-B Surface expression of CD63 (**A**) and CD203c (**B**) on $CD123^{+}/CD11c^{lo}/HLA^{-}$ basophils in granulocyte and PBMC fractions following control or PMA stimulated fresh blood. **C** Mean side scatter (SSC) of total basophils in whole blood following control or PMA stimulated samples. Statistically significant difference between cell fractions is indicates as *: $p<0.05$ or between control or PMA-stimulated samples within cell fraction as #: $p<0.05$ and ##: $p<0.01$, n.s: not significant.

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