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Progressive increase of FcεRI expression across several PBMC subsets is associated with atopy and atopic asthma within school-aged children

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CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

AUTHOR CONTRIBUTIONS

JL, JR, ACJ, DM, EMH performed the experiments and analysed data. JL, JR and NHK performed statistical analysis. JL and JR drafted the manuscript. IAL, PNLS, PDS, MMHK, AB, PGH, DHS assisted in conceiving the project, data interpretation and manuscript editing. All authors read and approved the final version of the manuscript.

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ABSTRACT

Background Antigen specific IgE binds the Fcε receptor I (FcεRI) expressed on several types of immune cells, including dendritic cells (DC). Activation of FcεRI on DCs in atopics has been shown to modulate immune responses that potentially contribute to asthma development. However, the extent to which DC subsets differ in

FcεRI expression between atopic children with or without asthma is currently not clear.

This study aimed to analyse the expression of FcεRI on peripheral blood mononuclear cells (PBMC) from atopic children with and without asthma, and non-atopic/non-asthmatic age-matched healthy controls.

Methods We performed multiparameter flow cytometry on PBMC from 391 children across three community cohorts and one clinical cohort based in Western Australia.

Results We confirmed expression of FcεRI on basophils, monocytes, plasmacytoid and conventional DCs, with higher proportions of all cell populations expressing FcεRI in atopic compared to non-atopic children. Further, we observed that levels of FcεRI expression was elevated across plasmacytoid and conventional DC as well as basophils in atopic asthmatic compared to atopic non-asthmatic children also after adjusting for serum IgE levels.

Conclusion Our data suggest that the expression pattern of FcεRI on DC and basophils differentiates asthmatic and non-asthmatic atopic children. Given the significant immune modulatory effects observed as a consequence of FcεRI expression, this altered expression pattern is likely to contribute to asthma pathology in children.

Key words FcεRI expression, dendritic cells, basophils, monocytes, atopic asthma, IgE, childhood asthma, PBMC, flow cytometry

BACKGROUND

Allergic IgE sensitisation and early life respiratory infections are major risk factors for development of atopic asthma during childhood[1]. Asthma affects 300 million

people, consists of several phenotypes and atopic asthma is the most common in children. The disease manifests initially as wheeze which is triggered at the time of respiratory viral infection, but in the majority of children the wheezy phenotype is only transient and resolves during the preschool years, whereas in a smaller subset dominated by sensitized children it persists and intensifies and eventually develops into full-blown atopic asthma. At the time of puberty, asthma transitions from being most common in boys to becoming more common in females[2].

IgE sensitization is characterised by induction of allergen-specific T helper 2 (Th2) immune memory and the production of corresponding specific IgE. Allergen specific IgE binds Fcε receptors that are expressed on several cell types within the airways and circulation including mast cells, basophils, monocytes and dendritic cells (DC)[3]. Fcε receptor I (FcεRI) binds IgE with high affinity and consists of three subunits; the IgE binding α-subunit and the signalling/regulatory β and γ subunits. Mast cells and basophils express a $\alpha\beta\gamma_2$ tetrameric complex, whereas DC expresses a $\alpha\gamma_2$ trimeric complex[4]. Crosslinking of IgE-bound FcεRI complexes by allergen leads to release of inflammatory mediators by mast cells/basophils and this is largely dependent on signal amplification by the β-subunit[5]. Thus, the function of trimeric FcεRI on DCs and monocytes is more complex. On the one hand conventional DC (cDC) utilize surface FcεRI expression to enhance their allergen capture functions[6], which can result in multi-logfold increased capacity to re-activate Th2-memory cells[7] but have also been reported to be involved in IgE clearance through internalisation of serum IgE[8] and induction of immune modulatory mechanisms such as tolerance[9]. Conversely, cross-linking of FcεRI on plasmacytoid DC (pDC) results in reduced type I-interferon signalling and impaired anti-viral responses[10,11]. Expression of FcεRI on

pDCs and cDC has been reported to be dependent on serum IgE levels[3], and in sensitised children with elevated levels of specific IgE (above 0.35 IU/mL), respiratory viral infections are often more prevalent/severe compared to non-atopic children as reviewed[12]. The proportion of FcεRI^{+} DCs, and FcεRI expression intensity, is generally higher in atopic and/or asthmatic individuals compared to healthy controls but variation exists between studies[8,11], The association of FcεRI expression and total serum IgE levels appears strongest for basophils and pDCs[8,13].

Although FcεRI expression has been reported to be elevated in atopic and/or asthmatic individuals compared to healthy controls[13-15], there are currently few studies that have investigated whether FcεRI expression across cell populations within the PBMC fraction can differentiate atopic asthmatics from atopic but non-asthmatic individuals. In the current study we aimed to identify and characterise FcεRI expressing cell populations in atopic children with or without asthma and healthy controls across four different allergy/asthma cohorts based in Western Australia. Our findings suggest that FcεRI expression may have clinical relevance to the pathology of childhood asthma, and has potential to be used as a biomarker related to disease susceptibility and phenotype.

METHODS

Study cohorts

A subset of school-age individuals (age range 7-15 years) from four independent cohorts based in Perth, Western Australia, were analysed. The participant numbers and clinical parameters are summarised in Table I. The Childhood Asthma Study (CAS) cohort is a birth cohort which investigated respiratory infections in children

130 from families with a history of allergy or asthma and thus with a high risk for
131 developing atopic disease at the age of 10 years[16]. The Western Australian Twin study
132 of Child Health (WATCH) was established as a population-based twin register to
133 examine the genetics of asthma and attention deficit hyperactivity disorder. This was
134 extended to include a follow-up subsample of participant families enriched for (but
135 not exclusively) physician-diagnosed asthmatics[17] at the median age of 10 years. The
136 Western Australian Pregnancy Cohort (Raine) is a large (>2000 children) unselected
137 general birth cohort where approximately 10% of participants developed asthma for a
138 period during their first 14 years of life[18,19]. From this cohort, 79 atopic individuals
139 were included in the analysis and selected so that 40 individuals also had an asthma
140 diagnosis at the age of 14 years. Peripheral blood samples from the CAS, WATCH,
141 and Raine cohorts were collected when the children did not display any clinical
142 symptoms of airway disease. The Mechanism of Acute Viral Respiratory Infection in
143 Children (MAVRIC) cohort enrols children with median age of 9 years, who seek
144 emergency medical attention with an acute respiratory wheeze at Princess Margaret
145 Hospital in Perth, Australia[20]. MAVRIC cohort samples were collected during an
146 acute wheezy respiratory episode requiring hospitalization, with follow-up samples
147 collected ≥ 4 weeks later at convalescence. All work on human samples was carried
148 out after informed consent was given in accordance with the declaration of Helsinki.
149
150 **Clinical parameters and measurements**
151 Atopy was defined as a positive skin prick test (SPT, wheal size ≥3mm) or levels of
152 specific IgE above 0.35 IU/mL as measured using ImmunoCap (Phadia, Sweden) for
153 any of the most common aeroallergens in the Perth region including house dust mite
154 (HDM, Dermatophagoides pteronyssinus), cat dander, dog dander, Alternaria

alternata, Aspergillus spp., cockroach, Rye grass (Lolium perenne), mixed grass and mixed mould. SPT, total IgE and specific IgE were measured in the CAS, Raine and MAVRIC cohorts; SPT and total IgE were measured in the WATCH study. Current asthma was defined as (i) ever having physician diagnosed asthma, (ii) a wheezy episode within the last 12 months and (iii) current use of asthma medication, such as short acting adrenic β2 agonists. This definition of asthma excluded children who only wheeze at a very young age.

PBMC collection, storage and thawing

Peripheral blood was collected and processed within 2 hours (CAS and Raine), or 18 hours (WATCH). For the acute cohort (MAVRIC), samples were collected within 72 hours of presentation to Princess Margaret Hospital (Perth, Western Australia) and processed within 18 hours[20]. PBMCs were obtained using Lymphoprep™ (Axis-Shield, Norway) and cryopreserved in liquid nitrogen. Frozen PBMC were thawed as previously[21]. Identical PBMC isolation, cryopreservation, storage and thaw protocols were applied to all cohorts. Viability was assessed using trypan blue exclusion and was above 80%.

Flow cytometry analysis

Approximately $1x10^6$ thawed PBMCs were stained as previously described[21]. The same FACS staining panel and protocol was used for all cohorts. Antibodies were titrated and a single batch was used across all samples within a cohort. Additionally, unstained and FcεR1 isotype stained samples (pooled per run) were included as controls for each staining and acquisition run. Samples were acquired using the LSR-Fortessa platform with FACSDiva software (BD Bioscience, Franklin Lakes, NJ).

Quality control was preformed prior to each cytometry run for all cohorts; CS&T beads (BD) to ensure standardised setup and technical performance, and Rainbow calibration particles (BD) to determine alignment and track performance across detection channels and lasers. Following acquisition, data were analysed using FlowJo 10.3 (FlowJo, Ashland, Ore) and FlowSOM in R (v3.2.4)[22].

Statistical Analysis

The statistical significance of difference between two groups was estimated using Student's t-test or the Mann-Whitney U test depending on the data distribution; for comparison of three groups the Kruskal-Wallis test followed by Dunn's post test was used; for comparison of paired samples Wilcoxon's Signed rank test was used and for comparison of categorical data between groups, Pearson's χ^2 test was used. Adjustment for IgE levels was calculated using Nominal logistic regression for current asthma within atopic individuals using log(serum IgE levels) and proportion of FcεRIα$^+$ subset or log(FcεRIα MFI). Experimental and technical variation was minimised within and between cohorts, however the presence of latent variables between cohorts is a common consideration inherent to cross-cohort studies. To account for this when combining cohorts to compare FcεRIα expression, each cohort was normalised with respect to the 10% of subjects with the highest MFI for each cohort and cell type. The 10% threshold was considered reasonable as it captures the characteristic plateau observed in all cell types for the highest FcεRIα expressing individuals (Fig S3). Additionally, complete randomisation of subjects with respect to asthmatic status within the Raine cohort was not possible due to the experimental design. For analysis of FcεRIα MFI in the Raine cohort, expression was normalised to the run-specific isotype control. This consideration was not required for the CAS,

WATCH and MAVRIC cohorts determined by the consistency of the isotype controls (data not shown). All statistical analysis was performed using JMP 13 (SAS, NSW, Australia) and data was visualised using Prism 7.0a (GraphPad, La Jolla, Calif).

RESULTS

To identify FcεRI expressing cell types in the PBMC fraction, we used a 12-colour flow cytometry panel[21] (Fig S1) and an unsupervised learning approach (FlowSOM)[22] overlayed with manual gating to determine key cell populations (Fig 1A-B) and expression of FcεRIα across these populations (Fig 1C). Using this approach, we identified FcεRIα expression on pDCs, monocytes, cDCs and basophils (Fig 1D), as previously demonstrated[23].

To investigate the association between atopy and asthma with FcεRIα expression, we compared proportion and level of FcεRIα expression in above identified cell populations from (i) non-atopic/non-asthmatic, (ii) atopic/non-asthmatic or (iii) atopic/asthmatic individuals across the CAS, WATCH and Raine cohorts. Compared to non-atopic/non-asthmatic individuals, there was a significantly higher proportion of FcεRIα$^+$ pDCs in both atopic groups from the WATCH and CAS cohorts, as well as increased proportion of FcεRIα$^+$ monocytes in atopic/non-asthmatic compared to non-atopic in the WATCH or compared to atopic/asthmatic in the Raine cohort (Fig 2A). FcεRIα expression amongst FcεRIα$^+$ pDC, cDC, monocyte and basophil populations displayed a progressive gradation of FcεRIα expression from non-atopics to atopic/non-asthmatics, and ultimately to atopic/asthmatics across all cohorts (Fig 2B). As the cohorts consisted of different proportions of individuals with the clinical phenotypes of interest, the power of each individual cohort to detect differences

between some groups differed, and therefore we additionally combined the cohorts as part of the analyses performed as a way of interrogating trends observed within each individual cohort. Once combined, we observed an increased proportion of FcεRIα^{+} pDCs in atopic/asthmatics compared to atopic/non-asthmatics (Fig 2C) and we also observed significantly greater FcεRIα expression levels on FcεRIα^{+} pDCs, cDCs and basophils in atopic individuals (non-asthmatic and asthmatic) compared to non-atopic/non-asthmatics (Fig 2D). Amongst the cohorts the atopic/asthmatics stand out most prominently by this criterion in Raine cohort (Fig 2A-B), and this may be a reflection of relative atopic disease chronicity given that these subjects represent the oldest group tested. We further investigated whether FcεRIα expression was influenced by biological sex (Fig S2A-C) or use of corticosteroid medication within asthmatic individuals (Fig S3A-D) observing that asthmatic individuals on corticosteroids expressed higher levels of FcεRIα compared to individuals on other asthma medication.

Given the well established correlations of FcεRIα expression and total IgE serum levels, which we also observed (Fig S4A-D), we compared total serum IgE between our groups after combining cohorts (Fig 3A). Atopics (non-asthmatic and asthmatic) displayed significantly higher serum IgE levels compared to non-atopic/non-asthmatics. In addition, atopic/asthmatics also displayed higher levels compared to atopic/non-asthmatics. Therefore, to determine if the observed differences in proportion of FcεRIα^{+} pDCs between atopic/non-asthmatics and atopic/asthmatics as well as the increased levels of FcεRIα expression were dependent of total IgE levels, we adjusted for IgE levels using logistic regression on atopic individuals with asthma as the outcome. We observed that both the proportion of FcεRIα^{+} pDCs (Fig 3B) as

well as increased level of expression on FcεRIα^{+} pDCs, cDCs and basophils (Fig 3C) were associated with asthma independently of serum IgE levels, suggesting the operation of a mechanism by which FcεRIα expression is associated with asthma which goes beyond the control of IgE production.

We finally sought to assess the impact of asthma exacerbations on circulating FcεRIα^{+} cell subsets in atopic children. Through the MAVRIC cohort, we recruited subjects presenting at hospital during an acute wheezing illness. For each subject, an acute sample was assessed against a paired convalescent sample obtained once symptoms had resolved several weeks later (Fig 4). Plasmacytoid DC, cDC, and basophil cell numbers were significantly decreased during respiratory exacerbation compared to convalescence (Fig 4A-B), consistent with their likely exit from circulation and migration to sites of inflammation in the airways and their subsequent participation in the local host immunoinflammatory response. No correlation between systemic steroid administration and cell numbers were observed (Fig S5A-D). Together this suggests that these subsets are systemically influenced by wheezing airway disease in atopic individuals.

DISCUSSION

In the present study, we have systematically characterised the expression of FcεRI on cells in the PBMC fraction in non-atopic and atopic children with and without asthma. Our findings are broadly consistent with upregulated expression of FcεRI on both effector (basophil) and regulatory (myeloid, especially pDC/cDC) populations and this upregulation is associated with current atopic asthma. Interstingly, the correlation of FcεRI expression and total IgE levels is not as strong as previously reported[24],

potentially due to the young age of the participants. There are also indications that these subsets migrate to the airways during an acute exacerbation[25]. However given the small sample size and potential interference from administration of systemic steroids, this requires further investigation.

Regarding underlying mechanism(s), enhanced FcεRI expression on basophils equates to reducing the allergen-exposure threshold required for triggering IgE-mediated degranulation and attendant acute inflammation, but the implications of comparable upregulation on myeloid cells are more complex. Circulating cDC are the immediate source of precursors required to renew the airway mucosal DC network which turns over rapidly during infections/exacerbations[26], and their migration to this tissue pre-armed with FcεRI would increase the intensity of acute aeroallergen-specific Th2-effector responses and promote expansion of corresponding Th2-memory populations. A similar mechanism applies to circulating monocytes which traffic to airways following allergen challenge[27] and further give rise to monocyte derived DC[28]. However, expression of FcεRI on monocytes does not appear to separate atopic/non-asthmatic and asthmatic individuals in this data set.

Plasmacytoid DCs are recognised as the major producers of type I interferons, the most important mediators of host viral defence[29]. Consequently, pDCs are a likely candidate to bridge the gap between IgE sensitisation and respiratory viral infection as major asthmatic risk factors and may have a central role in regulating airway inflammation during asthma exacerbations triggered by viruses[29]. In a subsample from the COAST cohort, an increased proportion of pDCs expressing FcεRI in atopic asthmatic children was observed compared to non-atopic non-asthmatics children[11].

However, no significant difference was observed when compared to allergic non-asthmatics, possibly a result of sample size. Notably within the COAST study, allergic asthmatic children demonstrated significantly impaired ability to produce type I interferons following FcεRI cross-linking with IgE in the presence of human rhinovirus, indicating that FcεRI expressing pDCs may have a reduced ability to initiate viral defence[11]. Omalizumab treatment decreases circulating IgE levels and associated FcεRI cell surface expression. Importantly, omalizumab treatment reduces asthma exacerbation frequency and results in restored type I interferon response to rhinovirus[30].

In conclusion, our findings support the previous reports outlining an important role for FcεRI in atopic disease. Our study also suggest that FcεRI expression may be used to identify children with maximal susceptibility to allergic asthma and thus identify kids that may benefit the most from intervention therapies targeting atopy or allergic/Th2 mediated inflammation.

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TABLES

Table 1 – Clinical and demographic features for the respective cohorts.

	CAS	WATCH	Raine	MAVRIC	p†
Number of individuals, n (% of total	119 (32.0%)	174 (46.8%)	79 (21.2%)	19 n/a	n/a

individuals)					
Males, n (%)	71 (59.7%)	100 (57.5%)	44 (55.7%)	12 (63.2%)	0.91
Age, median years (range)	10.1 (10.0-10.7)	10.6 (7.2-13.0)	14.1 (13.5-14.9)	8.8 (6.9-13.2)	<0.001
Total IgE, median IU/mL (range)	166.8 (0-9892.1)	61.5 (1.0-747.0)	378.9 (38.3-5206.6)	585.3 (123.7-2342.1)	<0.001
SPT^{+}, n (%)	74 (62.2%)	132/172 (76.7%)‡	65/75 (86.7%)‡	11/13 (84.6%)‡	<0.001
Atopy (SPT^{+} or sIgE > 0.35 IU/mL), n (%)	98 (82.4%)	132 (75.9%)	79 (100%)	19 (100%)	<0.001
Current Asthma, n (%)	7 (5.9%)	28 (16.1%)	40 (50.6%)	19 (100%)	<0.001
Additional atopic disease§ within atopic individuals, n (%)	63 (64.3%)	73/90 (81.1%)‡	51 (64.6%)	n/a	0.02
Admitted to hospital with acute wheeze, n (%)	n/a	n/a	n/a	15/17 (88.2%)‡	n/a
Hours from last systemic corticosteroid administration (1 mg/kg) to blood collection, median (range)	n/a	n/a	n/a	4.1 (0.4-14.8)‡, n = 13	n/a
Respiratory viral infection positive, n (%)	n/a	n/a	n/a	11/14 (78.6%)‡	n/a
Total PBMC fraction cell	1.9 (0.8-4.2)	1.1 (0.6-5.1)	1.5 (0.8-4.1)	0.5 (0.3-2.3)	<0.001

count, x 10^6/mL median (range)						

[†]p-values of statistical difference between cohorts using either Kruskal-Wallis or Pearson's χ^2 test depending on characteristics of the data.

[‡]data missing from some individuals.

[§]additional atopic diseases were allergic rhinitis or eczema within last 12 months.

FIGURE LEGENDS

Figure 1

A Algorithm (FlowSOM) based clustering of cells found within the PBMC fraction followed by manual classification using surface marker expression. **B** Cell numbers per mL of blood of the major cell populations of individuals included in the WATCH study. **C-D** Mapping of FcεRIα expression across the cell populations identified by FlowSOM (**C**) and representative gates of FcεRIα$^+$ cel subsets (**D**) Representative figures from one individual are shown in A, C and D. In B, data is displayed as median, 25-75% percentile and range, n=174.

Figure 2

A-D Proportion (A,C) and expression of FcεRIα (B,D) on FcεRIα$^+$ cells within each cell type found in PBMC fraction in non-atopic/non-asthmatic, atopic/non-asthmatic or atopic/asthmatic individuals from each cohort (**A-B**) or combined (**C-D**). Significance of difference was calculated using Kruskal-Wallis test followed by Dunn's post test or Mann-Whitney's for comparisons of two groups in the Raine cohort and indicated as; *** $p<0.001$, ** $p<0.01$, * $p<0.05$. Data are displayed as median, 25-75% percentile and range, CAS $n_{(non\text{-}atopic/non\text{-}asthmatic)}=21$, CAS $n_{(atopic/non\text{-}}$

446 asthmatic)=91 and CAS $n_{(atopic/asthmatic)}$=7. WATCH $n_{(non-atopic/non-asthmatic)}$=42, WATCH
447 $n_{(atopic/non-asthmatic)}$=104 and WATCH $n_{(atopic/asthmatic)}$=28. Raine $n_{(atopic/non-asthmatic)}$=39 and
448 Raine $n_{(atopic/asthmatic)}$=40. combined $n_{(non-atopic/non-asthmatic)}$=63, combined $n_{(atopic/non-}$
449 $_{asthmatic)}$=235 and combined $n_{(atopic/asthmatic)}$=75.
450
451 **Figure 3**
452 **A** Total IgE serum levels in non-atopic/non-asthmatic, atopic/non-asthmatic or
453 atopic/asthmatic individuals in the combined cohorts. **B-C** Association of FcεRIα$^+$
454 cells proportion in PBMC fraction (log odds ratio ±95% CI; **B**) or normalised
455 expression levels of FcεRIα on FcεRIα$^+$ subsets (log expression level ±95% CI; **C**) to
456 current asthma within atopics of the combined cohorts, adjusted for log(serum IgE).
457 Data in A are displayed as median, 25-75% percentile and range, combined $n_{(non-}$
458 $_{atopic/non-asthmatic)}$=63, combined $n_{(atopic/non-asthmatic)}$=235 and combined $n_{(atopic/asthmatic)}$=75.
459 In B-C the estimated effect size (log odds ratio) for asthma among atopics ± 95% CI
460 per unit increase in % positive cells (B) or in log(expression level) (C) is displayed,
461 $n_{(atopic)}$=309.
462
463 **Figure 4**
464 **A-B** Concentrations of cells in the PBMC fraction at exacerbation and convalescence
465 (**A**) or fold change of cell concentration in the same individual during exacerbation
466 compared to convalescence (**B**). Significance of difference on paired samples was
467 calculated using Wilcoxon's Signed ranks test and indicated as; *** $p<0.001$. Data
468 are displayed as individual values, median, 25-75% percentile and range, n=19.

Figure 2

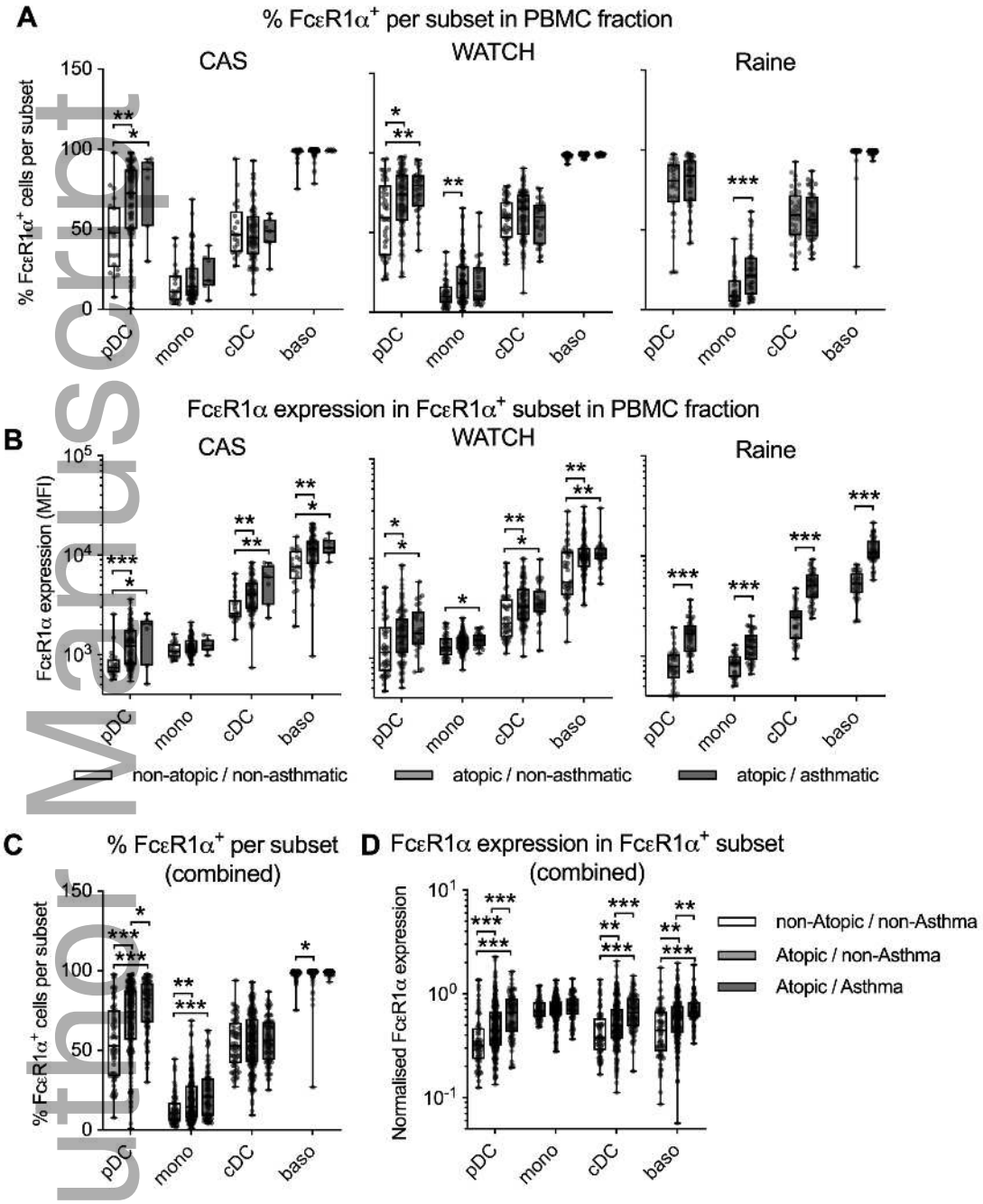

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Figure 3

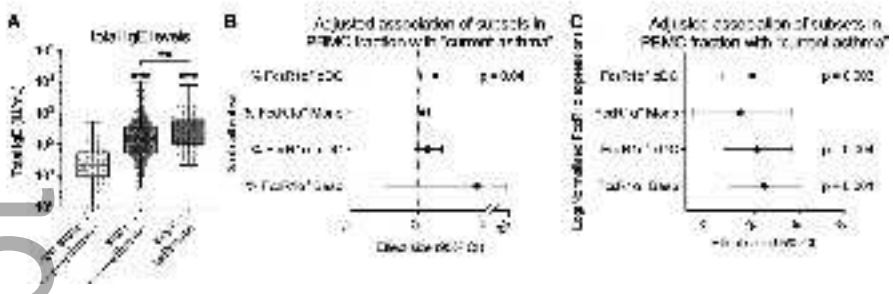

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Figure 4

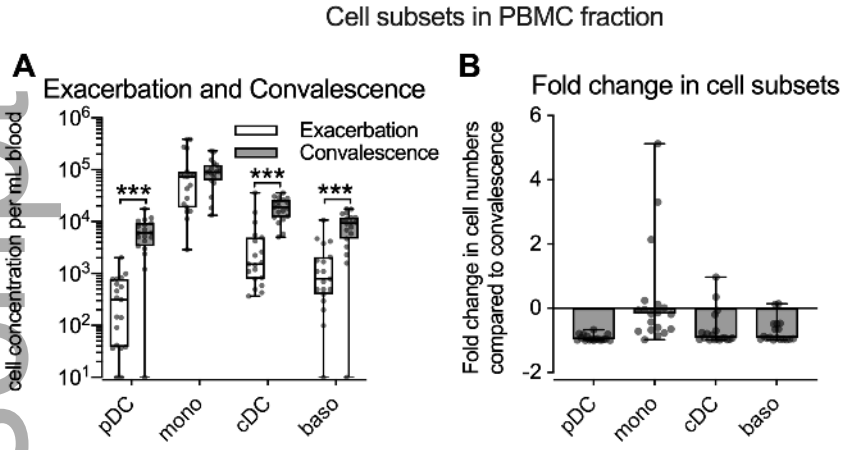

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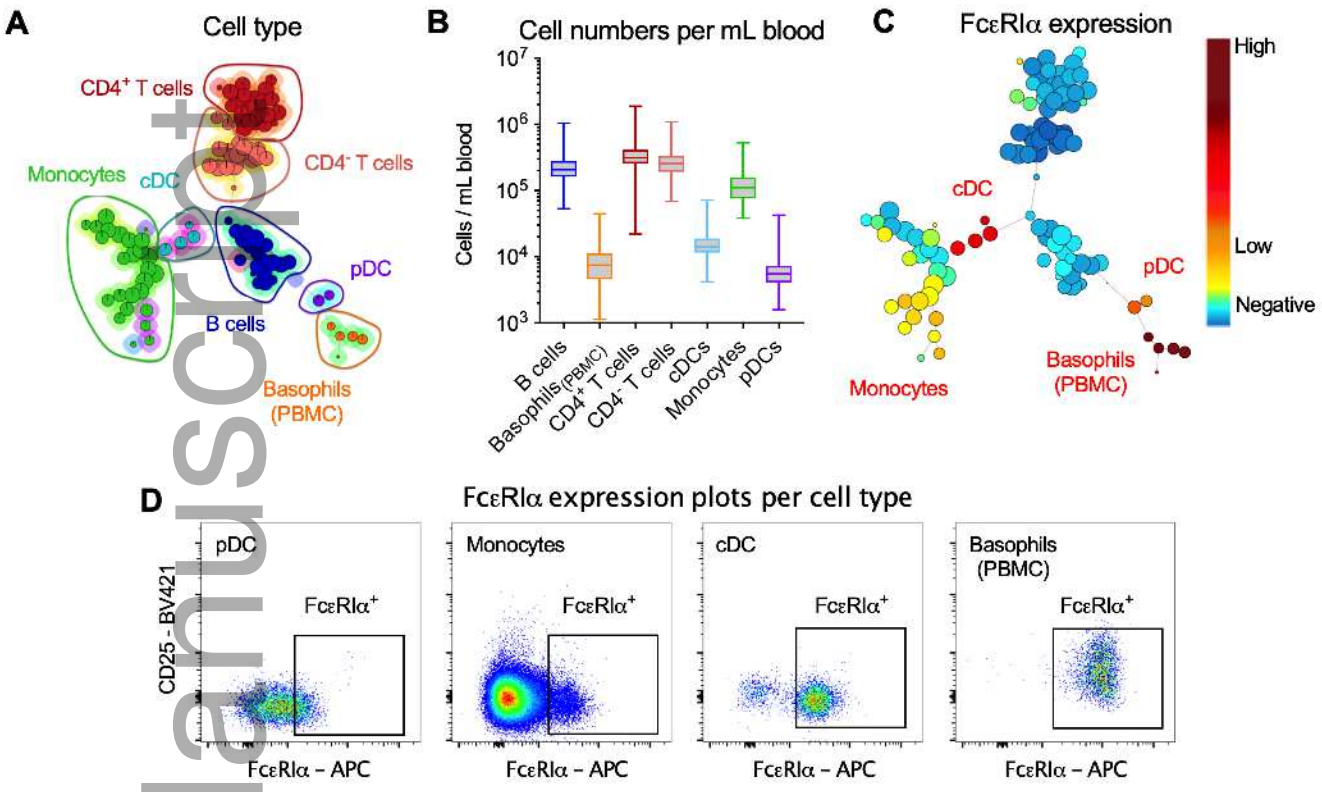
Figure 1



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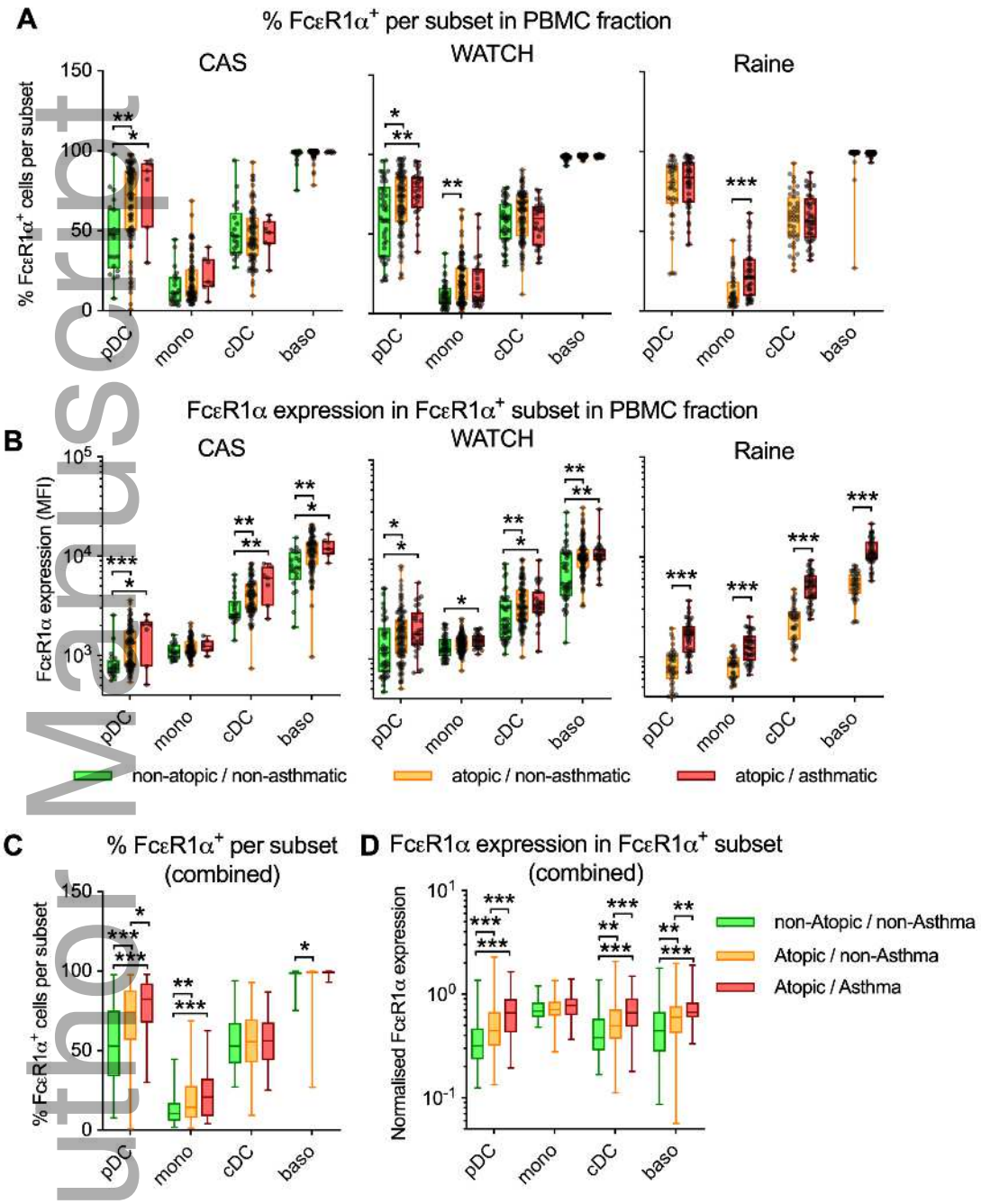

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Figure 3

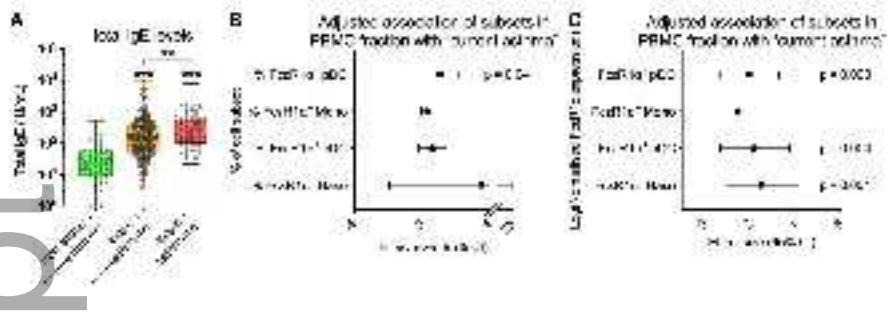

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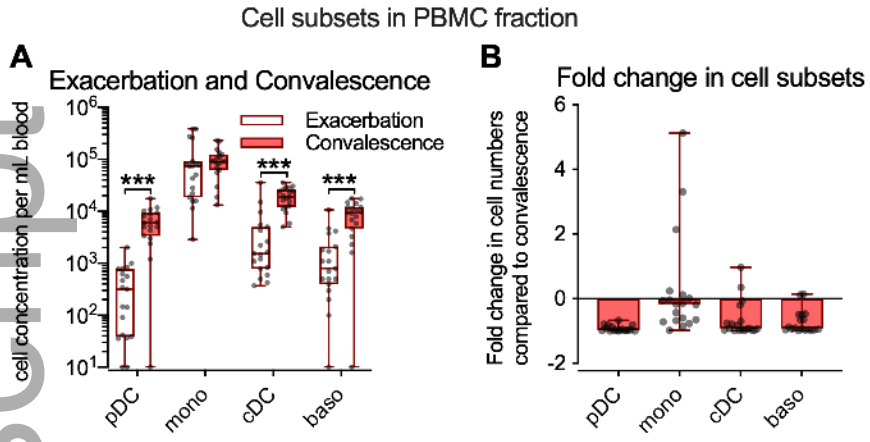
Figure 4



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