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A rapid two-step procedure for the purification of human peripheral blood basophils to near homogeneity

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Summary

Background Basophils are increasingly utilized as indicators of allergic inflammation and as primary allergic effector cells to study signalling pathways. However, until the present, their enrichment has been time consuming, costly and limited to relatively few specialized laboratories.

Objective We have therefore devised a reproducible and rapid method for the purification of human basophils from small quantities of peripheral blood within 1.5 h, which does not require the use of specialized equipment such as elutriators.

Methods Human basophils were obtained from healthy volunteers undergoing venipuncture. Heparinized or K3-ethylenediaminetetraacetic acid blood samples were first subjected to centrifugation in Hetasep, directly followed by negative selection using immunomagnetic beads. Basophil morphology and purity were assessed by May–Grünwald staining of cytopins. IgE-mediated histamine release was analysed spectrofluorometrically and IL-4 and IL-13 production by quantitative RT-PCR. CD203c and CD63 surface expression was measured using flow cytometry before and after activation with anti-IgE.

Results Using this protocol, basophils were enriched close to homogeneity in most cases with a mean purity of $99.34 \pm 0.88\%$ (range 97–100%, $n = 18$) and a mean recovery of 75.6 (range 39–100%, $n = 8$). Basophil viability following purification was $99.6 \pm 0.89\%$ using Trypan blue exclusion. The purification procedure gave rise to basophils with normal functional responses to anti-IgE regarding histamine release as well as IL-4 and IL-13 mRNA expression. Moreover, constitutive cell-surface CD203c/CD63 expressions were not elevated before anti-IgE stimulation.

Conclusion The rapidity, simplicity and reproducibility of this method will facilitate the employment of basophils in high-output *ex vivo* studies.

Keywords basophils, CD203c, CD63, histamine, IgE, IL-4, purification techniques

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Introduction

Human basophils generate key inflammatory and immunomodulatory mediators (e.g. histamine, LTC₄, IL-4 and IL-13) during Type I hypersensitivity reactions (reviewed in [1]). These mediators are rapidly released upon high-affinity IgE receptor (FcεRI) stimulation by allergens and non-specific triggers such as certain parasitic antigens [2], lectins [3] and superantigens [4]. Basophils are also stimulated by various IgE-independent stimuli such as toll-like receptor 2 (TLR2) ligands [5], suggesting that they may contribute to innate immune responses as well as support underlying T-helper type 2 (Th2)-type immunity [1].

Because of the above, basophils are increasingly employed as vital tools for studying immune/inflammatory responses. This is complemented by the emergence of basophil-specific staining antibodies (such as BB1) showing, for example, that they invade the lungs of patients with fatal allergic asthma [6]. Analysis of basophil degradation (e.g. using CD63 and CD203c antibodies) is increasingly employed in clinical studies and for routine diagnosis in allergy clinics [7, 8]. However, when considering more-detailed basophil studies, where homogeneous populations are necessary, their relatively low numbers (<1% of peripheral blood leucocytes) still constitute a major problem. This is compounded by the lack

of suitable animal models or human cell lines for studying the biochemistry of basophils.

Methodologies for the purification of basophils have evolved considerably in the past decade, owing largely to the possibility of negatively selecting partially enriched basophil preparations using immunomagnetic particles [9–11]. However, in order to obtain pure basophil preparations, as well as reduce costs and eliminate residual erythrocyte contamination, magnetic cell selection required prior enrichment steps. These included Ficoll or Percoll density centrifugation [9–11] and countercurrent centrifugal elutriation [9], techniques that require considerable patience and time to perform (>3 h) and, in the case of elutriation, expensive apparatus. Our aims were therefore to optimize a reliable protocol for the purification of functionally viable basophils in <1.5 h, circumventing the use of density gradients and elutriation. This simplified technique should enable high output, simultaneous purifications of multiple donors, thereby increasing the feasibility of examining *ex vivo* basophil function as part of larger clinical studies/trials.

Methods

Basophil purification

Thirty millilitres of K₃-ethylenediaminetetraacetic acid (EDTA) or lithium-heparin blood obtained from healthy, unselected blood donors with permission of the local ethics committee were mixed with 6 mL Hetasep (StemCell Technologies, Vancouver, Canada), split in two 15 mL tubes and centrifuged at 110 g for 6 min at room temperature (RT). This usually resulted in a neat separation of red blood cells from the leucocyte-containing upper phase. However, in a small subset of experiments, it was necessary to let the tubes stand for another 15–20 min until a clear separation was obtained.

The upper phases were carefully collected and pooled, topped up to 50 mL using chilled purification buffer [Dulbecco's phosphate-buffered saline (DPBS) without Ca²⁺/Mg²⁺ with 1 mM EDTA and 2% fetal bovine serum (FBS) v/v], and subjected to a low-acceleration centrifugation (450 g, 7 min) to deplete non-adherent platelets. The pellet was resuspended in 1 mL purification buffer, transferred to a standard fluorescence-activated cell sorter (FACS) tube and mixed, at RT, with 100 µL Human Basophil Enrichment Cocktail (EasySep Human Basophil Enrichment Kit, StemCell Technologies) containing monoclonal antibodies to the following cell-surface antigens: CD2, CD3, CD14, CD15, CD16, CD19, CD24, CD34, CD36, CD45RA, CD56 and Glycophorin A. After 10 min, the suspension was topped up to 2.5 mL with purification buffer and 70 µL of EasySep Magnetic Nanoparticles were added to the antibody–cell suspension followed by 10 min incubation at RT. Finally, cells were transferred to an

EasySep Magnet and incubated for another 10 min at RT, following which unbound cells were decanted into a fresh FACS tube. After washing, cells were resuspended in HEPES-buffered Tyrode's solution (without calcium). Aliquots were taken for Trypan blue viability determination and cyto-spin staining using May–Grünwald's process as described elsewhere [9]. In addition, cell recovery was determined using Alcian blue staining (which permits for rapid basophil-specific staining) in a haemocytometer, as described by Gilbert and Ornstein [10].

Cell stimulation and mediator assay

Purified basophils (0.5–1 × 10⁵ mL) were resuspended in HEPES-buffered Tyrode's solution, containing 1 mM CaCl₂, warmed for 15 min at 37 °C and incubated for 30 min with various titrations of anti-IgE (polyclonal goat anti-human IgE, Sigma, Poole, UK) or buffer alone. Samples were processed and assessed for histamine contents using spectrofluorometric autoanalysis as described elsewhere [9]. Percentage histamine releases were determined from the total histamine content in the sum of pellet and supernatant tubes.

RT-PCR and quantitative RT-PCR (RT-qPCR)

Two hours after stimulation with anti-IgE, 0.2 × 10⁶ cells were harvested, washed with 1 mL DPBS and processed for mRNA extraction followed by reverse transcription using the µMACS One-step cDNA Kit (Miltényi Biotec, Bergisch Gladbach, Germany). All primers were chosen from regions spanning an intron in order to discriminate genomic DNA from cDNA. The following sequences were used: IL-4-FOR, 5'-CTCATTTTCCTCGGTTTCA-3'; IL-4-REV 5'-GTCCTTCTCATGGT GGCTGT-3' (expected sizes: 383 bp cDNA, 5863 bp gDNA); IL-13-FOR 5'-TACTGTG-CAGCCCTGGAATC-3', IL-13-REV 5'-CAAGGGGAAGGC TGAGGT-3' (401 bp cDNA, 747 bp gDNA); GAPDH-FOR 5'-TGATGACATCAAGAAGGTGGTGAAG-3', GAPDH-REV 5'-TCCTTGGAGGCCATGTGGGCCAT-3' (240 bp cDNA, 344 bp gDNA). 1 µL of cDNA was amplified in 20 µL total reaction volume containing 10 µL of ReddyMix PCR mixture (ABgene Ltd, Epsom, UK), 7 µL of PCR-grade water and 2 µL of primers (5 µM each). Cycling conditions were as follows: 94 °C for 5 min, followed by 35 cycles of 94 °C for 30 s, 58 °C for 45 s and 72 °C for 90 s followed by 72 °C for 5 min. qPCR primers were designed using Primer3 software [12] and following the guidelines for qPCR with SYBR-green I fluorescence [13]. For each primer pair, one primer was designed across an exon boundary to prevent amplification of genomic DNA. To ensure the absence of near and exact matches, primer sequences were checked for regions of similarity using nucleotide blast ('search for short, nearly exact matches') on the NCBI blast server. Primer validity was determined using the

melt curve functionality with the Icyler IQ multicolour real-time PCR detection system (Optical system software 3.1, Bio-Rad, Hemel Hempstead, UK) and annealing temperatures were raised to 62 °C to prevent the formation of unspecific products. Primer sequences were HsIL-4 SYBR FOR: 5'-TGCCTCCAAGAACACAACACTG-3', HsIL-4 SYBR REV: 5'-GTCCTTCTCATGGTGGCTGT-3', HsIL-13 SYBR FOR: 5'-ACCAGAAGGCTCCGCTCT-3', HsIL-13 SYBR REV: 5'-CTGACACGTTGATCAGGGATT-3', HsGAPDH SYBR FOR: 5'-CGTGGAAGGACTCATGACC-3', HsGAPDH SYBR REV: 5'-GAGGCAGGGATGATGTTCTG-3'. Forty-five amplification cycles were run with an annealing temperature of 62 °C. Each qPCR reaction contained 1 × iQ SYBRGreen Supermix (Bio-Rad), 200 nM forward and 200 nM reverse primers (Sigma-Genosys, Haverhill, UK), 2.5 µL cDNA and DEPC-treated water to a final volume of 25 µL. Data were normalized using the Pfaffl equation [14] using GAPDH as the internal reference gene.

Fluorescence-activated cell sorter

A total of 1×10^5 purified basophils in DPBS with Ca^{2+} / Mg^{2+} were stimulated with goat anti-human anti-IgE ($1:10^5$, Sigma) for 10 min at 37 °C and cooled on ice for 10 min to stop activation. The cells were then incubated with 10 µL of fluorescein isothiocyanate (FITC)-conjugated anti-CD63 and 10 µL phycoerythrin (PE)-conjugated anti-CD203c (Immunotech, Marseille, France) or isotype controls (PE-mouse IgG1, BD Pharmingen, Oxford, UK FITC-mouse IgG1, Immunotech) for 30 min at 4 °C in the dark. Purified basophils were washed twice with HBSS buffer and fixed in DPBS/0.5% (v/v) formaldehyde. Flow cytometric analysis was performed on an EPICS Altra

sorter (Coulter, High Wycombe, UK), and data were analysed with WinMDI v2.8.

Statistics

Statistical significance was assessed using Student's *t*-test or Mann-Whitney's *U*-test (SPSS v15.0) as indicated in the figure legends.

Results

The first purification step resulted in a rapid and efficient separation of erythrocytes from leucocytes. We found this step to be critical in achieving the highest possible purity with only one round of immunomagnetic selection. Although the antibody cocktail contains an anti-Glycophorin A antibody, which efficiently removes residual erythrocytes, we observed that excessive carryover of red blood cells compromised final purity, making a second round of immunomagnetic selection necessary.

The purification protocol resulted in highly purified basophils (97–100%, $n = 18$) independent of the donor (Fig. 1a). The average purity obtained was $99.34 \pm 0.88\%$, higher than previously reported for purification protocols based on negative selection [9, 11, 15], and viability was $99.6 \pm 0.89\%$. The total number of basophils obtained from 20 mL of peripheral blood with this method was $320\,000 \pm 158\,000$ (comparable to previously reported values of 318 000/20 mL [9]). The mean recovery of 75.6% (range 39–100%) also compared favourably with previous reports of 49.7% [9]. Morphologically, basophils appeared fully granulated on May–Grünwald-stained

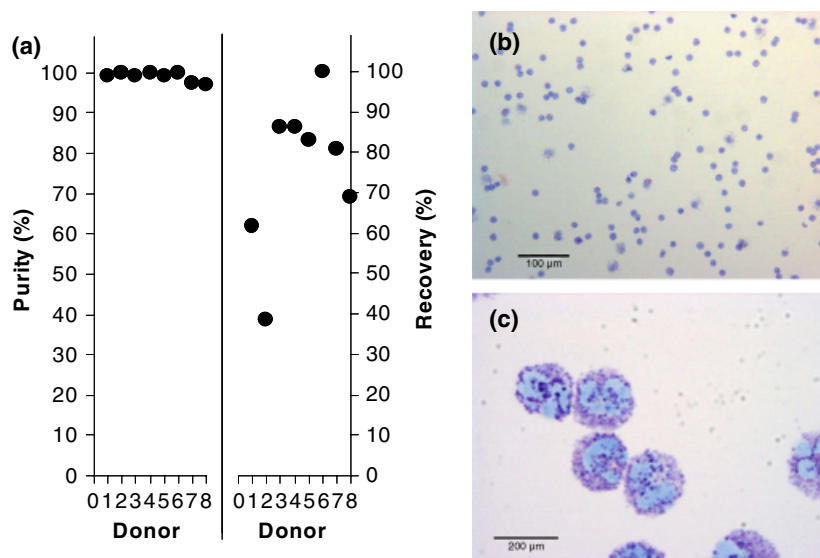


Fig. 1. (a) Percentage purity and recovery of basophils purified from fresh peripheral blood with the new two-step procedure ($n = 8$). Recovery was determined by Alcian blue staining of cells both before and after magnetic cell sorting. Cytospin preparations stained with May–Grünwald stain showing 100% pure basophils at $\times 100$ magnification (b) and at $\times 1000$ magnification (c), showing that basophils are fully granulated.

cytospins (Figs 1b and c). The whole procedure was usually performed within 75–90 min.

To assess whether the negative selection procedure leads to unwanted basophil activation, we monitored the levels of the basophil-specific surface activation marker CD203c [16] and the degranulation marker CD63 [17] before and after degranulation (Figs 2a–e). Figure 2b shows that no CD63 is detectable on unstimulated basophils, confirming the lack of spontaneous activation, while CD63 intensity rises strongly after anti-IgE stimulation. CD203c is present at intermediate levels on resting basophils in peripheral blood, and is further increased upon IgE-dependent activation [16]. This can be clearly seen in Fig. 2c. Based on the mean fluorescence intensity of the gated basophil population, stimulation with an optimal dilution of anti-IgE resulted in a 4.58-fold increase after 10 min of incubation, which fits well with the study by Ocmant *et al.* [18], who found a CD203c stimulatory index ranging from 3.05 to 4.55 using a 15 min incubation. We did not observe any significant difference in CD203c levels between freshly purified basophils before and after 10 min incubation at 37 °C (data not shown). Overall, the FACS study suggests that the purification protocol does not lead to unwanted activation.

Next, we examined whether basophils purified with this protocol displayed normal responses to IgE-mediated stimulation in terms of cytokine (IL-4, IL-13) induction and histamine release. IL-4 and IL-13 mRNA, both present at lower levels in resting basophils, was increased 2 h after anti-IgE stimulation (Fig. 3a). RT-qPCR showed the increase of IL-4 mRNA expression upon activation to be 4.5-fold for IL-4 and 75-fold for IL-13. IL-4 levels were easily measurable in resting basophils and were much higher than the unstimulated IL-13 mRNA levels. This is consistent with our previous finding that small, but detectable levels of pre-formed IL-4 levels are present in resting basophils, and transiently increased after stimulation, while IL-13 is not measurable in resting basophils, but strongly induced at the protein level after activation [19].

Furthermore, dose-dependent stimulation with dilutions of anti-IgE (Fig. 4) resulted in the expected bell-shaped histamine release curve [8]. There were no significant differences between purified and unpurified basophil preparations from the same donor (results not shown). Together, these findings indicate that the basophils are not affected by the purification procedure and display a normal functional response to anti-IgE stimulation.

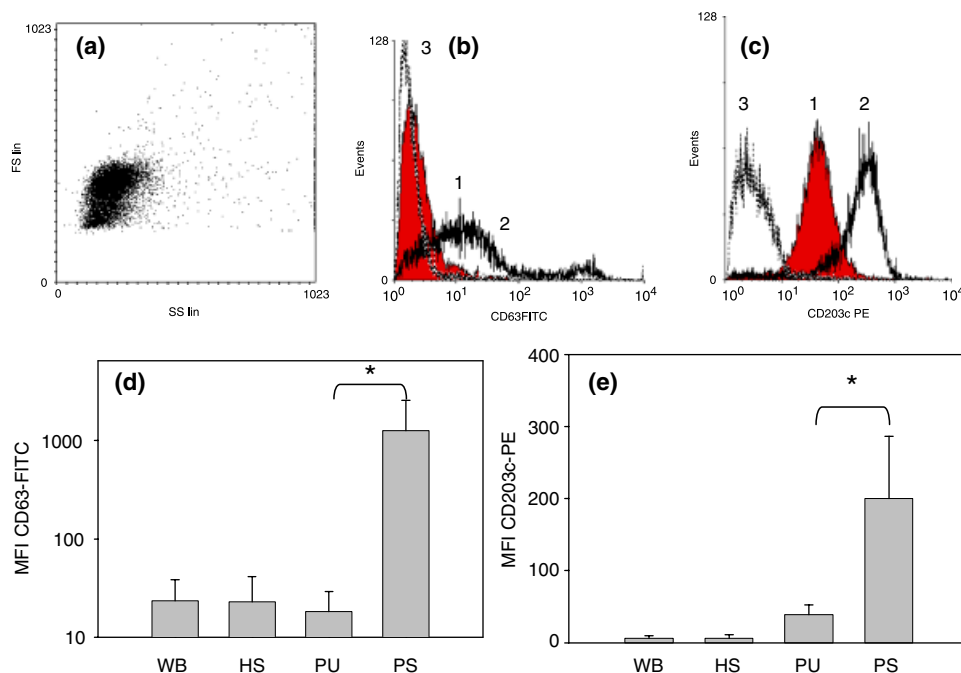


Fig. 2. (a) Side scatter (SS lin) vs. forward scatter (FS lin) dot plot of purified basophils showing a homogeneous basophil population. (b) Flow cytometric staining of purified basophils labelled with a fluorescein isothiocyanate (FITC)-conjugated antibody to CD63. CD63 is not present on the membrane of resting basophils (1, full histogram), but appears on the surface during basophil degranulation (2). (3) Is the isotype control. (c) Flow cytometric staining of purified basophils labelled with a phycoerythrin (PE)-conjugated antibody to basophil surface activation marker CD203c. CD203c is only found on basophils in peripheral blood and is expressed at low to intermediate levels on resting basophils, but increased upon activation. (3) Is the isotype control, (1) is resting basophils and (2) is 10 min after stimulation with anti-IgE. (d, e) Mean fluorescence intensity (MFI) $\pm$ SD for CD-63-FITC- (d) and CD203c-PE (e) -stained whole blood (WB), post-HetaSep (HS) leucocytes, purified unstimulated (PU) and purified stimulated (PS) basophils, gated based on size and granularity according to (a) ($n = 4$ independent experiments, $*P < 0.05$, Mann–Whitney *U*-test).

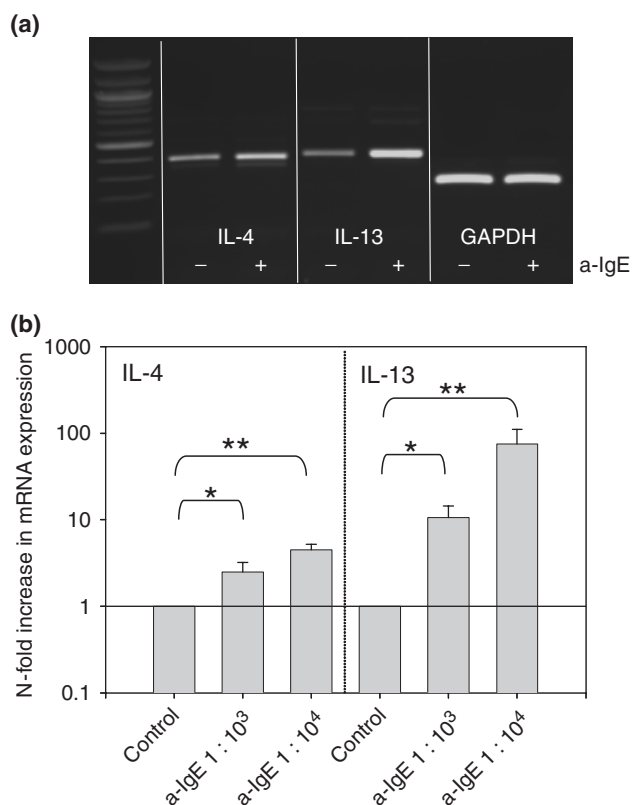


Fig. 3. (a) RT-PCR demonstrating expression of IL-4 mRNA (lanes 1,2); IL-13 (lanes 3,4) and GAPDH (lanes 5,6) before (–) and 2 h after (+) stimulation with anti-IgE (a-IgE) in highly purified basophils. The results suggest that transcription is increased for both cytokines after IgE crosslinking. (b) Quantitative RT-PCR demonstrating increased expression (N-fold increase over unstimulated control $\pm$ SD) of IL-4 and IL-13 mRNA 1 h after stimulation with a-IgE ($n = 3$ independent experiments). Statistical significance was assessed using Student's *t*-test (* $P < 0.05$; ** $P < 0.01$).

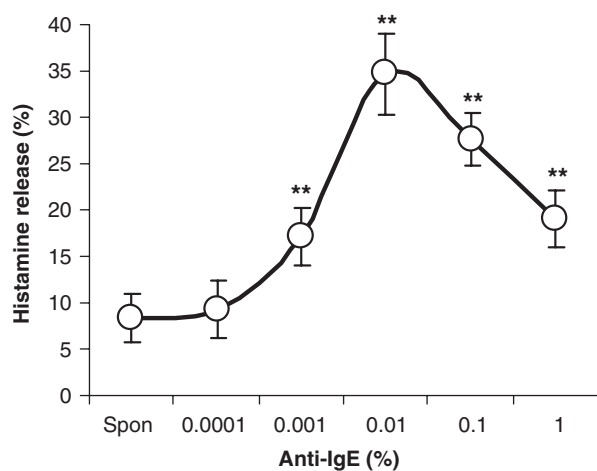


Fig. 4. Histamine release from purified basophils stimulated with increasing amounts of anti-human IgE. The curve shows the typical expected bell-shaped curve demonstrating basophil functionality unaffected by the purification. IgE-mediated histamine release was analysed spectrofluorometrically ($n = 8$). **Significant releases ($P < 0.01$) compared with spontaneous secretions.

Discussion

This protocol allows basophils to be purified to near homogeneity within 75–90 min with excellent recovery. Time-consuming steps such as Percoll gradients are avoided, and the use of specialist or expensive equipment such as elutriators is not necessary. We have found the protocol to result in high final purities independently of the donor. The technique could easily be upgraded to an automated system allowing for simultaneous purification of several donors, or a larger blood volume of the same donor. One important advantage of such a shortened purification protocol is that it is less likely to influence gene expression patterns, which is of relevance, e.g. to gene array studies. The main limitation of this method is where larger numbers of basophils are needed; in this case, it is advisable to reduce the number of contaminating cells before immunomagnetic selection to reduce the costs, by introducing a middle purification step such as elutriation [20].

Overall, the procedure presented here is fast, efficient and straightforward to perform, making basophil purification accessible to any interested laboratory without the need for specialist or expensive equipment.

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