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Research paper

Improved recovery of functionally active eosinophils and neutrophils using novel immunomagnetic technology☆,☆☆

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ABSTRACT

Clinically relevant and reliable reports derived from in vitro research are dependent on the choice of cell isolation protocols adopted between different laboratories. Peripheral blood eosinophils are conventionally isolated using density-gradient centrifugation followed by immunomagnetic selection (positive/negative) while neutrophils follow a more simplified dextran-sedimentation methodology. With the increasing sophistication of molecular techniques, methods are now available that promise protocols with reduced user-manipulations, improved efficiency, and better yield without compromising the purity of enriched cell populations. These recent techniques utilize immunomagnetic particles with multiple specificities against differential cell surface markers to negatively select non-target cells from whole blood, greatly reducing the cost/time taken to isolate granulocytes. Herein, we compare the yield efficiencies, purity and baseline activation states of eosinophils/neutrophils isolated using one of these newer protocols that use immunomagnetic beads (MACSxpress isolation) vs. the standard isolation procedures. The study shows that the MACSxpress method consistently allowed higher yields per mL of peripheral blood compared to conventional methods ($P < 0.001$, $n = 8$, Wilcoxon paired test), with high isolation purities for both eosinophils (95.0 ± 1.7%) and neutrophils (94.2 ± 10.1%) assessed by two methods: Wright's staining and flow cytometry. In addition, enumeration of $CD63^+$ (marker for eosinophil activation) and $CD66b^+$ (marker for neutrophil activation) cells within freshly isolated granulocytes, respectively, confirmed that conventional protocols using density-gradient centrifugation caused cellular activation of the granulocytes at baseline compared to the MACSxpress method. In conclusion, MACSxpress isolation kits were found to be superior to conventional techniques for consistent purifications of eosinophils and neutrophils that were suitable for activation assays involving degranulation markers.

1. Introduction

Eosinophils and neutrophils belong to the granulocyte family of leukocytes which are characterized by the presence of granules in the cytoplasm (Geering et al., 2013). The granules contain a diverse array of cytotoxic molecules that are secreted under specific conditions to combat invading extraneous agents like pathogens and allergens. In disease conditions, these molecules also collaterally damage host cell tissues and often contribute to the inflammatory state (Jacobsen et al., 2007; Mantovani et al., 2011). Indeed, both eosinophils and neutrophils are important effector cells with established pathogenicity in chronic airway diseases, including asthma, chronic obstructive pulmonary disease (COPD), and bronchitis (D'silva et al., 2011). Intact sputum eosinophil counts, and in particular measures of airway eosinophil activity (free granules and eosinophil peroxidase levels in sputum), are associated with disease severity in asthma (Nair, Ochkur et al. 2013). Again, a

Abbreviations: 7-AAD, 7-aminoactinomycin; ANOVA, analysis of variance; APC, allophycocyanin; CD, cluster of differentiation; COPD, chronic obstructive pulmonary disease; DNA, deoxyribonucleic acid; EDTA, ethylenediaminetetraacetic acid; EPX, eosinophil peroxidase; FACS, fluorescence-activated cell sorting; fMLP, N-formyl-methionyl-leucyl-phenylalanine; FITC, fluorescein isothiocyanate; HBSS, Hanks' Balanced Salt Solution; ICAM-1, intercellular adhesion molecule 1; Ig, immunoglobulin; LTC4, leukotriene C4; NH_4Cl, ammonium chloride; PAF, platelet-activating factor; PB, peripheral blood; PBS, phosphate-buffered saline; PMD, piecemeal degranulation; PE, phycoerythrin; SEM, standard error of mean.

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significantly higher percentage of neutrophils were found in the sputum of patients diagnosed with moderate to severe COPD relative to mild airflow obstruction (Kay, 2005; D'silva et al., 2011).

Both clinical studies and in vitro/ex vivo experimentation suggest several distinct mechanisms of degranulation in eosinophils including piecemeal degranulation (PMD) and cytolytic release of intact granules (ECL) (Lee et al., 2012). PMD and ECL degranulation in human eosinophils have been well studied and documented, degranulation mechanisms in mouse models of human disease, in particular to the airways, have been subjected to limited reproducibility and subjective interpretation (Stelts et al., 1998; Lacy and Moqbel, 2001; Ochkur et al., 2007; Ochkur et al., 2012). The inconsistencies of mouse eosinophils in replicating human eosinophil functions essentially highlight the importance of continued in vitro research using human peripheral blood eosinophils to investigate underlying mechanisms of recruitment, survival, migration, degranulation and other effector functions that contribute to disease progression and steroid insensitivity.

The accurate assessment and interpretation of cellular functions inferred from in vitro studies rely heavily on the isolation protocol used, as well as the population of target cells studied. In early studies, the standard isolation technique for individual granulocyte populations commonly involved sedimentation in high molecular weight polysaccharide solutions, such as dextran, followed by discontinuous density gradients which were greatly limited by low granulocyte recovery and purity, particularly for eosinophils. In fact, isolations using this technique generated extremely poor yields for eosinophils from healthy donors, and made comparative studies between healthy and diseased subjects a logistical impossibility.

In the 1990s, target-cell selection using antibody-conjugated microbeads post density-gradient centrifugation of whole blood was introduced. Negative immunomagnetic selection of eosinophils was made possible by using anti-CD16 antibody-coated magnetic beads, since neutrophils express high levels of this cell surface marker compared to eosinophils in granulocyte preparations (Pillay et al., 2013). This method was deemed to be preferable over discontinuous density (Percoll) gradient methods (Sedgwick et al., 1996) and, to this date, remains the most commonly used protocol. In some cases, isolation techniques were expanded to improve negative selection of eosinophils using biotinylated antibodies of multiple specificities (for example, Eosinophil Isolation Kit, Miltenyi Biotec, 130-092-010, uses anti-CD2, CD14, CD16, CD19, CD56, CD123, CD235a).

Conflicting data remains regarding the purity and activation states of eosinophils isolated by these methods (Schefzyk et al., 2009; Percopo et al., 2010). Nevertheless, the use of immunomagnetic beads of multiple specificities that exploit differential expression of surface cellular markers between target and non-target cells are commercially available, including the newly introduced MACSxpress kit (Miltenyi Biotec Inc., Bergisch Gladbach, Germany), along with the EasySep kit (STEMCELL Technologies Inc., BC, Canada), which are gaining popularity. However, eosinophils and neutrophils isolated using various approaches show variability in their activation profiles, with a tendency towards cells becoming refractory to stimulation if dextran, Lymphoprep, or Histopaque sedimentation of whole blood is carried out for too long a period (>30 min), if red blood cell lysis is damaging to cells, or if cells are subjected to excess mechanical handling because of repeated centrifugation steps (personal communication). In spite of this, neutrophil isolation involving dextran sedimentation of whole blood followed by Ficoll separation remains the method of choice (Oh et al., 2008), although similar immunomagnetic bead-based kits are available.

Here we report a comparative analysis of granulocyte isolation from healthy individuals using immunomagnetic microbead cocktail-based methods and standard protocols with respect to granulocyte recovery (yield), purity and baseline activation states of the freshly isolated cells.

2. Materials and methods

2.1. Subjects and blood sampling

Healthy donors (n = 8) with no known medical conditions were recruited with written consent (Hospital Research Ethics Board, St. Joseph's Healthcare, Hamilton, ON, Canada). The complete blood count was assessed clinically for determining the circulating absolute values of eosinophils and neutrophils (St. Joseph's Healthcare Core Lab). The neutrophil counts ranged from 3.3–4.6 $\times 10^9$ cells/mL whereas the eosinophil counts were 0.2 $\times 10^9$ cells/mL for all volunteers. A total of 60 mL of venous blood was drawn for eosinophil isolations and 40 mL for neutrophil isolations on separate days. The comparative isolation protocols were initiated in parallel, immediately after sample draw. A schematic of the work flow is summarized in Fig. 1.

2.2. Isolation protocols

2.2.1. Column-based CD16 negative selection isolation

A volume of 25 mL of peripheral blood collected into heparin anticoagulant blood collection tubes (BD Biosciences, ON, Canada) from venipuncture was mixed at a 1:1 ratio with RPMI media (Invitrogen, CA, USA) and layered on top of Lymphoprep (STEMCELL Technologies Inc.) at a 2:1 volume ratio for density-gradient sedimentation. Subsequent to centrifugation (20 min, 1200 *g*, 20 °C, no brakes), the bottom-most granulocyte layer was harvested. Erythrocytes were lysed with an ice-cold ammonium chloride solution (NH_4Cl, 155 mM; $KHCO_3$, 10 mM; EDTA, 0.1 mM; pH 7.4) and further centrifuged (10 min, 300 *g*, 4 °C) for removal from the granulocyte layer. The remaining granulocyte population was treated with immunomagnetic anti-CD16 microbeads (#130-45-701, Miltenyi Biotec Inc.) to negatively select for eosinophils. The remainder of the protocol was carried out as per manufacturer's protocol, and an aliquot of the final population was removed and stained with trypan blue for cell counts via hemocytometer.

2.2.2. EasySep (STEMCELL Technologies)

Briefly, peripheral blood samples were diluted with PBS and layered on top of Ficoll (GE Healthcare, Little Chalfont, UK) and centrifuged (30 min, 300 *g*, room temperature, no brakes) for density separation according to the manufacturer's protocol. The granulocyte layer was removed and residual erythrocytes were lysed with 1 mL of endotoxin-free water. Cells were divided into aliquots of 10^7 cells/mL, and were then treated with a human eosinophil enrichment cocktail (catalogue#: 19,256, STEMCELL Technologies Inc.) containing magnetic nanoparticles as per manufacturer's instruction. An aliquot of the final population was removed for cell counting as mentioned previously.

2.2.3. MACSxpress isolation kit (Miltenyi Biotec Inc.)

Samples of 30 mL of venous blood were collected into EDTA anticoagulant blood collection tubes for eosinophil isolation, while only 8 mL was procured for neutrophil isolation. The isolation procedure was performed according to the manufacturer's protocol (catalogue #: 130-104-446, for eosinophils; 130-104-434, for neutrophils). In brief, peripheral blood was mixed with cocktail containing magnetically-labelled antibodies targeting cell surface markers of cells of non-interest. Enriched cells remained in supernatants while other cell populations were segregated magnetically using the MACSxpress Separator (#130-098-308). Residual erythrocytes were lysed with 9 mL of cold sterile endotoxin-free water for 20 s (cold water/hypotonic lysis), then 1 mL of 10× HBSS media without calcium and magnesium (Invitrogen) was added. An aliquot of the final population was removed for cell counting as mentioned previously.