



Research paper

Efficient purification of eosinophils from human tissues: A comparative study

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ABSTRACT

Background: Eosinophils are key effector cells in allergy and in other inflammatory diseases. Although they carry out their function in the tissues, no efficient method exists allowing for consistent purification of tissue eosinophils for culture. Rather, studies rely mainly on peripheral blood eosinophils. This study aimed to determine the most efficient protocol for purifying eosinophils from nasal polyp tissue.

Methods: Nasal polyps were obtained from patients undergoing surgical polypectomy. The polyps were minced and enzymatically digested. Surface receptor analysis was performed by flow cytometry. In order to obtain optimal purification, the nasal polyp cell suspension was subjected to two methods of purification: 1) positive magnetic selection of CCR3+ cells, or 2) negative selection using CD3/CD14/CD16 magnetic beads. Enriched tissue eosinophils were cultured with or without IL-3, IL-5 or GM-CSF, and their survival was evaluated by flow cytometry.

Results: Tissue-derived eosinophils exhibited surface expression of NEC2, DNAM-1, NTBa, 2B4, and CD300a comparable to similarly prepared eosinophils obtained from the peripheral blood of the same patients. Positive selection consistently yielded eosinophils of high purity (>90%) with 63% viability. In contrast, negative selection yielded better viability (88%), reduced purity (66%), and could be utilized for in vitro activation experiments.

Conclusion: Eosinophils can be purified from nasal polyps. Negative selection appears to be advantageous due to improved viability of the eosinophils, which may be cultured and activated in vitro. This methodology is an important advance in studying tissue eosinophils for further investigations on inflammatory tissue responses.

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1. Introduction

Eosinophils are bone marrow-derived granulocytes normally low in number in peripheral blood or tissues. Blood and tissue eosinophilia are present in conjunction with specific conditions such as allergic, parasitic, malignant, and idiopathic disorders, and diseases such as allergic rhinitis and nasal polyposis (Rothenberg and Hogan, 2006; Munitz and Levi-Schaffer, 2004).

In these conditions, eosinophils infiltrate inflamed tissues, where they become activated and degranulate, thus releasing preformed major basic protein, phospholipid metabolites, and an array of cytokines, chemokines, and growth factors (Gleich, 2000).

Although eosinophils carry out their function once they infiltrate into tissues, most of the data concerning their biology has been accumulated from peripheral blood, as this is a readily available source of eosinophils. Currently, the most common and efficient way to purify these cells from peripheral blood is by negative selection with immunomagnetic separation (Hansel, 1991; Munitz et al., 2005,

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2006). With this technique a mixed granulocyte population is incubated with immuno-magnetic beads coated with CD16 mAb. Neutrophils, bound to the beads through FcγRIII, are removed with a magnet and a highly pure eosinophil population is obtained.

Tissue eosinophils differ from peripheral blood eosinophils in their biologic properties (Malm-Erfjefält, 2005). It is therefore essential for researchers to be able to study isolated tissue eosinophils in order to obtain unequivocal data about their functions.

Nasal polyposis is a chronic inflammatory disease of the upper airways affecting up to 4% of the general population. Nasal polyps (NP), benign growths of the nasal and sinus mucosa, are often associated with asthma and allergy. NP are characterized by chronic inflammation, fibrosis and angiogenesis, and are usually associated with a large eosinophilic infiltrate. Since NP are often surgically removed, they may become a readily available source of tissue eosinophils (Eliashar and Levi-Schaffer, 2005; Pawankar, 2003).

In the present study we initially compared peripheral blood and tissue eosinophils for surface molecules. We also established a method for the purification of eosinophils from NP tissue. This method preserves their viability and functional activity, and may therefore serve as an important tool for *ex vivo* studies of tissue eosinophils.

2. Materials and methods

2.1. Human nasal polyp digestion

Cells were isolated from NP of patients who underwent endoscopic nasal polypectomy according to the guidelines established by the Hadassah–Hebrew University Internal Review Board. NP (2–9 g wet weight), were kept for a maximum of 1 h at room temperature in sterile saline. Thereafter, NP were washed twice in RPMI-1640 with Penicillin/Streptomycin (100 U/ml) and L-glutamine (300 mg/l) enriched with 2% heat-inactivated fetal calf serum (FCS) [Biological Industries, Beit Haemek, Israel] (wash medium). The tissue was finely minced with surgical scissors to fragments of approximately 1 mm³. The fragments were subsequently digested by incubation with an enzymatic cocktail containing collagenase type-I (6 mg/g tissue), hyaluronidase (3 mg/g tissue), and DNase (100 µg/g tissue) (Sigma Chemicals, St Louis, MO, USA), in wash medium with shaking for 90 min at 37 °C. The digested tissue was filtered through a 150-micron nylon mesh (BD Falcon™ BD Bioscience, Transduction Laboratories, Erembodegem, Belgium), and cells were collected in a 50 ml tube. Samples were centrifuged (150 g, 4 °C) and re-suspended in 10 ml medium (RPMI, 10% FCS). Cell viability was assessed by trypan blue exclusion (Sigma). Eosinophils in the cell suspension were identified with Kimura's staining and as SSC^{high} cells using FACS analysis (as described below). Eosinophils in the cell suspension were stained for surface receptors in some experiments, while in others they were purified (see below).

2.2. Enrichment of leukocytes from peripheral blood

Blood was collected from patients undergoing polypectomy and white blood cells were enriched by hypotonic lysis

[Samoszuk 2006]. Briefly, 10 ml blood was added to 40 ml of cold distilled water and shaken for 2 min. PBS was added to a final 1X concentration to stop the lysis effect. The samples were centrifuged (150 g, 5 min, 4 °C). Supernatant was removed and the cell pellet was re-suspended in cold distilled water for another cycle of lysis and centrifugation. The samples were subjected to the same enzymatic digestion as the tissue samples to equalize for cleavage of surface receptors. Cells were re-suspended in 5 ml medium and eosinophils were evaluated by Kimura's staining. The obtained cells were fixed for subsequent Flow Cytometry analysis (see below).

2.3. Flow cytometric analysis of the expression of cell surface receptors

Flow cytometric analysis was performed using a BD Biosciences FACSCalibur and CellQuest software. Eosinophils [10⁶ cells/ml] were fixed in 2% formaldehyde for 15 min at 4 °C. All experiments were performed in u-shaped 96-well culture plates (Nunc) in a volume of 100 µl. Cells (10⁵/sample) were washed with ice-cold HBA buffer (Hank's solution containing BSA (0.1% w/v) and NaN₃ (0.01% w/v)), and incubated (4 °C, 30 min) with either anti-CD300a, -DNAM-1, -NTBa, -2B4, or -Nec2 antibodies (1:10), kindly provided by Prof. A. Moretta (Department of Experimental Medicine, University of Genoa, Genoa, Italy), or the appropriate isotype control, followed by two washes with cold PBS. The cells were then incubated with anti-mouse FITC-conjugated IgG antibodies at the recommended dilutions (RT, 30 min) followed by two additional washes, and analyzed immediately by FACS.

2.4. Purification of eosinophils from nasal polyps

Cell samples obtained after NP digestion and filtration were used for subsequent purification of eosinophils. The average cell yield from the polyps was 0.5–1 × 10⁶, of which approximately 30% were eosinophils.

2.4.1. Negative selection

The recovered cells were re-suspended in 500 µl medium-2% FCS with anti-CD3 (70 µl), -CD14 (150 µl), and -CD16 (50 µl) conjugated magnetic beads (MACS, Miltenyi Biotec, Bergisch Gladbach, Germany) and incubated for 45 min on ice with gentle tilting. The mixture was layered onto a steel wire column (MACS CS column), and application of a magnetic field caused retention of CD16⁺ neutrophils, CD3⁺ T lymphocytes, and CD14⁺ monocytes. The column was then washed with 30 ml wash medium and the eluted CD16⁻CD3⁻CD14⁻ eosinophils were collected in a 50 ml polypropylene Falcon tube. Purity was assessed with Kimura's staining and viability was evaluated by Trypan blue exclusion.

2.4.2. Positive selection

The cells were re-suspended in medium-1% FCS [1 × 10⁷ cells/ml] with rat anti-human CCR3 antibody (20 µg/ml. R&D systems) and incubated for 20 min on ice with gentle tilting. Samples were washed three times with PBS containing 0.1% BSA and 2 mM EDTA (pH 7.4) (buffer), re-suspended in the buffer at a concentration of 1 × 10⁷ cells/ml with sheep anti-rat IgG covalently linked to magnetic beads (4 × 10⁶ beads/

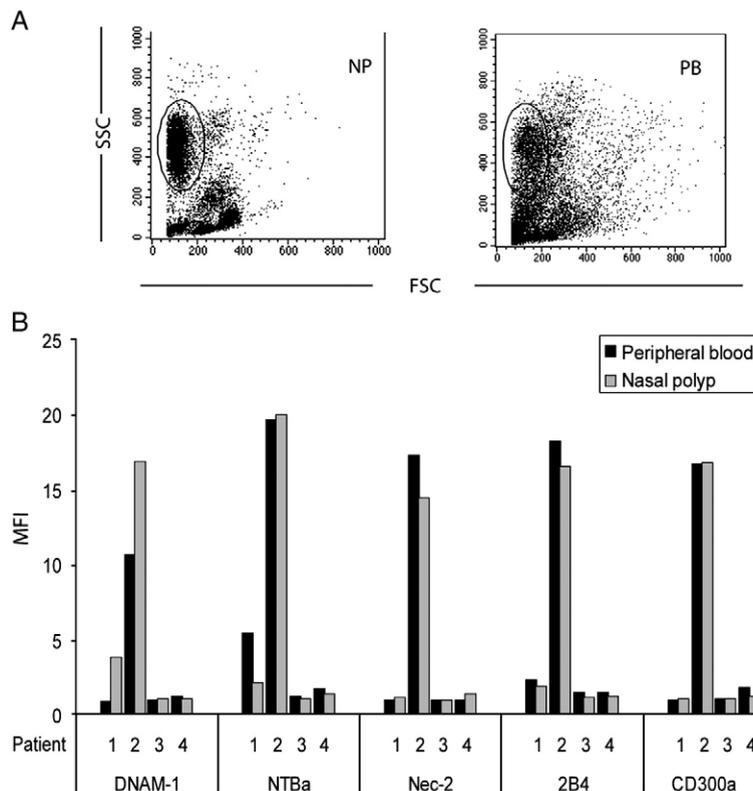


Fig. 1. Comparison of NP-derived and peripheral blood eosinophils. A: Physical parameters of cells from NP-digested tissue and from enriched peripheral blood obtained from the same patient. B: FACS analysis of NP-derived and peripheral blood eosinophils with anti-DNAM-1, NTBa, Nec-2, 2B4, and CD300a antibodies.

ml) (Dynabeads, Dynal Biotech Invitrogen, Carlsbad, CA, USA), and incubated on ice with gentle tilting for 30 min. The volume was doubled with buffer and the tube was placed in a magnetic field for 2 min in order to allow bead-bound cell retention. Supernatant was discarded and the tube was removed from the magnet. The remaining bead-bound cells were washed 4 times by re-suspending the cells in 1 ml buffer, placing the tube in the magnet for 1 min and discarding supernatant. Purity was assessed by Kimura's staining and viability was evaluated by Trypan blue exclusion.

Eosinophils obtained both from negative and positive selection, were re-suspended in medium-10% FCS for further experiments.

2.5. Culture of eosinophils purified from nasal polyps

Eosinophils purified from NP by negative selection were re-suspended in culture medium-10% FCS. Cells [10^6 cells/ml] were cultured in u-shaped 96-well culture plates [2×10^5 eosinophil/well] in 200 μ l of medium with or without 20 ng/ml of either IL-3, IL-5, or GM-CSF (PeproTech Inc., Rocky Hill, NJ, USA). Cell viability was evaluated after 18, 24 and 48 h by Trypan blue exclusion and by flow cytometric analysis after annexinV/ PI staining.

2.6. Assessment of eosinophil viability by trypan blue exclusion

Eosinophil samples (20 μ l) were mixed with 20 μ l Trypan Blue solution (Sigma). Cells were immediately placed in a

hemacytometer and evaluated under a light microscope (Olympus, MH-2, X40). Non stained cells were considered viable and the percentage of viable cells was determined in relation to total cells.

2.7. Assessment of eosinophil viability by Annexin V and propidium iodide staining

Viable vs. necrotic or apoptotic eosinophils were assessed by Annexin V-FITC (IQ Products, Groningen, Netherlands) -propidium iodide (PI) (Sigma) double staining. After culture, eosinophils [2×10^6 cells] were washed and re-suspended in 0.2 ml Annexin V buffer (218 mM HEPES, 1.4 mM sodium chloride, 38.1 mM calcium chloride), and incubated for 20 min on ice with Annexin V-FITC [10μ l/ 1×10^6 cells]. The cells were washed, PI [5μ g/ml] was added, and apoptosis and viability were subsequently analyzed by flow cytometry.

2.8. Eosinophil activation and measurement of eosinophil peroxidase (EPO) release

After purification by negative selection, eosinophils [10^7 cells/ml] were incubated overnight in medium with 10 ng/ml GM-CSF and for 30 min in medium-10% FCS alone, or with phorbol myristate acetate (PMA) (2.5 or 25 μ g/ml). EPO release was determined by a colorimetric assay as previously described (Munitz et al., 2005). Briefly, eosinophil culture supernatants (50 μ l) were incubated in flat 96 well plate (10–15 min, 37 $^{\circ}$ C, 5% CO₂) with 50 μ l of substrate

solution consisting 0.1 mM *O*-phenylenediamine dihydrochloride (Sigma) in 0.05 M Tris buffer [pH 8.0] containing 0.1% Triton X-100 and 1 mM hydrogen peroxide (Merck). The reaction was stopped by adding 100 μ l of 4 M sulfuric acid to each well, and absorbance was determined at 492 nm in a spectrophotometer (PowerWave XS; Bio-Tek Instruments, Winooski, VT).

2.9. Statistical analysis

Statistical significance was calculated using parametric analysis (ANOVA, followed by paired Student's *t* test assuming equal variance). Data is expressed as mean \pm SD. *p* values < 0.05 were considered significant.

3. Results

3.1. Peripheral blood and NP eosinophils have a similar surface receptor profile

Peripheral blood and NP eosinophils obtained from the same patients were compared for the expression pattern of different surface receptors. Peripheral blood samples were subjected to the same enzymatic digestion as the tissue samples, in order to rule out any cleavage of surface receptors that could have resulted in differences due to the preparation. Blood and tissue granulocytes were identified by their physical parameters and gated (Fig. 1A). Blood and NP granulocytes, which are mostly eosinophils, (Munitz, 2006)

expressed similar levels of NEC2, DNAM-1, NTB4, 2B4, and CD300a as seen in Fig. 1B, although intra-patient variability could be detected in the intensity of expression of these receptors.

3.2. Comparison of two methods for the purification of tissue eosinophils

Starting from a typical enzymatically digested NP containing 30–40% eosinophils, we compared two methods for purifying the eosinophils: negative and positive selection. In the negative selection method, cell suspension from enzymatically digested NP tissue was incubated with mAbs anti-CD3, -CD14, and -CD16 surface receptors, which are expressed on T lymphocytes, monocytes, and neutrophils respectively, but not on eosinophils. With this method eosinophils were obtained with $66.31 \pm 23.8\%$ purity and at a viability of $88.53 \pm 3.4\%$. The yield in this case was $50.73 \pm 32.6\%$ (Fig. 2; *n* = 6).

In the positive-selection method the cell suspension was initially incubated with rat anti-human CCR3 antibodies for selective and specific tagging of the eosinophils. Subsequently, the samples were incubated with magnetic beads of sheep anti-rat antibodies, and the mixture was placed in a magnetic particle concentrator. The eosinophils remained trapped, thus allowing elimination of the other cells. With this method a high purity ($90.66 \pm 3.2\%$) of eosinophils was obtained. However, they were only about 63% viable and the yield was rather low ($20.51 \pm 10\%$) (Fig. 3; *n* = 7). Moreover, incubation of the purified eosinophils for more than 1 h with

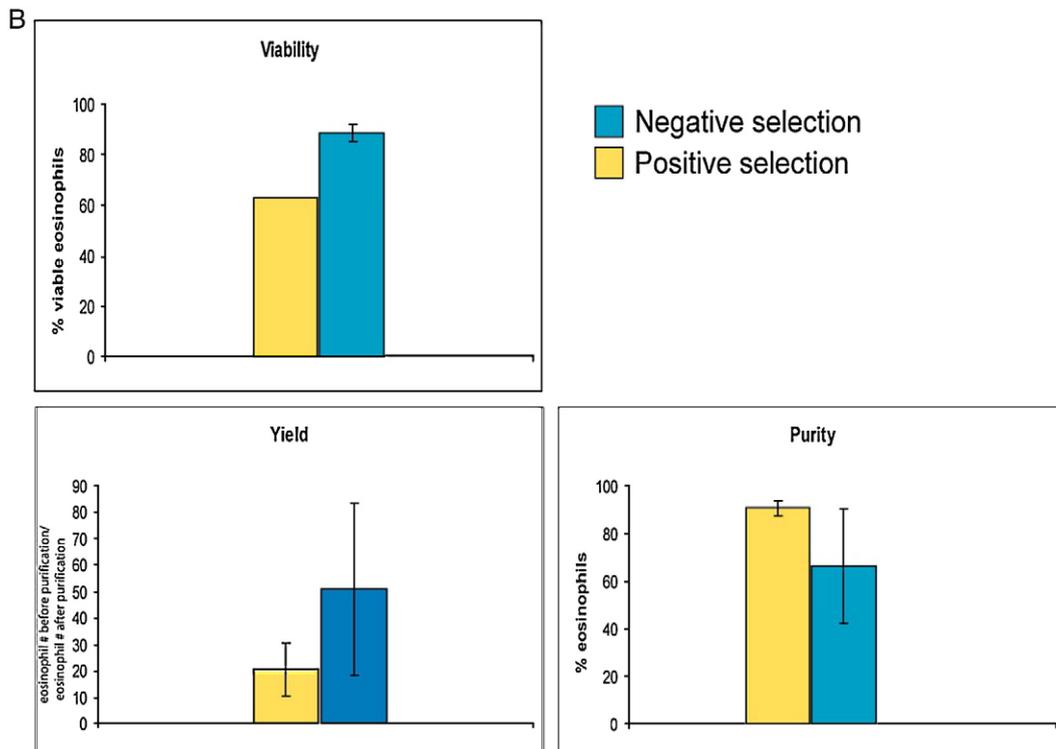


Fig. 2. Characteristics of eosinophils purified from human NP by negative and positive selection. Eosinophils were obtained from NP by enzymatic digestion and subsequently purified by either positive (*n* = 6) or negative (*n* = 7) selection (Colors correspond to Fig. 1). Purity was assessed by Kimura's staining; viability was evaluated by Trypan blue exclusion test. The yield was calculated as the ratio between the number of eosinophils before and after purification. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

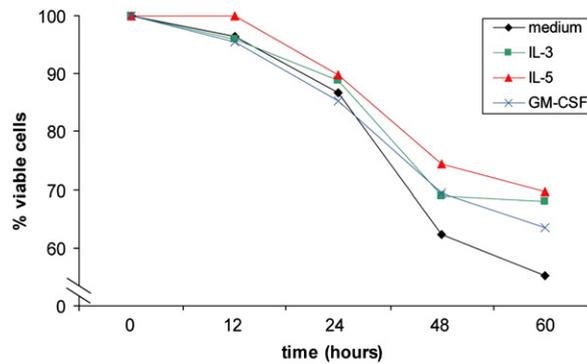


Fig. 3. Eosinophils purified from NP tissue can survive in culture. Eosinophils purified by negative selection [10^6 eos/ml] were incubated either in medium alone or with GM-CSF, IL-3, or IL-5 [20 ng/ml]. Cell viability was assessed by PI staining. The percentage of eosinophil survival was calculated as: (the number of viable cells at the end of the culture/the number of viable cells at the start of culture) \times 100. The result is the mean of four different experiments performed in duplicates.

pepsin was not successful in separating the antibody–bead complexes from the cells (data not shown).

3.3. NP-derived eosinophils maintain their viability under tissue culture conditions

After isolation of the eosinophils from the tissue, it was essential to evaluate how long these cells remained viable and under what conditions. Eosinophils purified by negative selection were cultured in medium alone or with an optimal concentration of IL-3, IL-5, or GM-CSF [20 ng/ml]. As shown in Fig. 3, ~80% of the eosinophils were viable after 24 h of incubation in all culture conditions (medium: 86.62 ± 8.8 ; IL-3: 88.88 ± 7.1 %; IL-5: 89.72 ± 7.1 ; GM-CSF: 85.4 ± 9.3 , $n = 4$). After 60 hours, the viability of eosinophils cultured in medium was 55.14 ± 14.32 %, while cells cultured in the presence of survival cytokines was improved, although not significantly (IL-3: 68 ± 21.5 %; IL-5: 69.6 ± 11.1 %; GM-CSF: 63.51 ± 3.8 %).

3.4. Release of eosinophil peroxidase from NP-derived eosinophils

Eosinophils purified by negative selection were evaluated for their functional activity by degranulation and consequent release of eosinophil peroxidase (EPO). After overnight incubation with 10 ng/ml GM-CSF, eosinophils were incubated with PMA (2.5 μ g/ml and 25 μ g/ml) for 30 min. Incubation with PMA increased EPO release (2.5 μ g/ml: 146%; 25 μ g/ml: 178.5%. Fig. 4).

4. Discussion

In this study we attempted to develop a technique for purifying eosinophils from NP, due to the importance of studying tissue eosinophils, and the need to find a way to analyze these cells *in vitro*. To the best of our knowledge, only one study has been previously published on this topic (Ramis et al., 1995). In this study, the authors used NP and performed enzymatic digestion followed by density centrifugation of the cells on a discontinuous Percoll gradient. They reported good purification of eosinophils with prolonged life span. However, a comparison between gradient and immuno-magnetic separation of peripheral blood eosinophils showed, that with Percoll gradients, only a specific hypodense population

of granulocytes was obtained, whereas the primed eosinophil subpopulation was lost. In contrast, with immuno-magnetic separation it is possible to obtain a heterogeneous (i.e. comprising resting and activated, mature and immature), highly pure population of eosinophils (Blom et al., 1995). To date, immuno-magnetic separation is the conventionally used method for eosinophil purification.

In the present study, we initially obtained a cell suspension containing ~30% eosinophils from enzymatically digested NP, and compared them with peripheral blood eosinophils from the same patients. This was done by studying the expression of a series of inhibitory and activating cell surface receptors, which were previously found by us to be expressed on peripheral blood eosinophils (i.e. CD300a, 2B4, NTB-A, Nec2, and DNAM-1) (Munitz et al., 2005, 2006; Bachelet et al., 2006). CD300a is an inhibitory cell surface molecule, while 2B4, Nec2, and DNAM-1 are co-activating molecules, and NTB-A is a CD2 subfamily receptor (Munitz et al., 2005). We found that tissue eosinophils expressed a comparable receptor profile to peripheral blood eosinophils similarly treated.

Next, we aimed to obtain an enriched and maximally purified, highly viable, tissue-derived eosinophil population, in order to study their properties *in vitro*. The NP digestion technique was combined with positive or negative magnetic

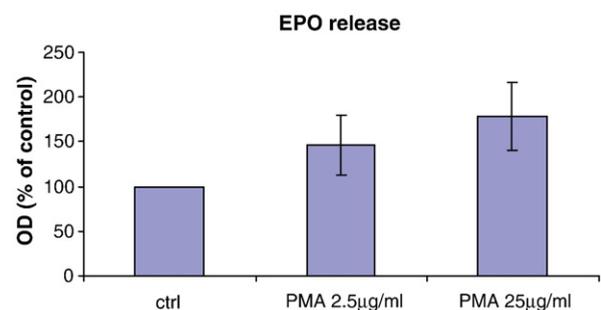


Fig. 4. EPO release from NP-derived eosinophils. After overnight priming with GM-CSF, eosinophils were incubated for 30 min in medium alone or with different concentrations of PMA. EPO release was evaluated by a colorimetric assay. The results are presented as percentage relative to control. Data are the mean \pm SEM of two different experiments performed in triplicates.

cell sorting. CCR3 was chosen for its relatively high specificity for eosinophils. Although this receptor is expressed on other cell populations such as Th2, basophils and mast cells, the percentage of these cells was low (<3%) and most of the contaminating cells were neutrophils and macrophages. With positive selection, high eosinophil purity was achieved (~90%), but the yield was low (~20%). This is a characteristic of positive selection procedures, in which relatively few cells are trapped in the magnetic field. Additionally, with this technique, the viability of the purified eosinophils was lower (~60%). We assume that the attached beads associated with the highly magnetic field contributed to cell death. Additionally it was difficult to separate the antibody–bead complexes from the cells, which made analysis by flow cytometry or by microscopy virtually impossible. We concluded that positive selection may be useful for assays in which there is a need for high sample purity, but in which the viability of the cells is not the highest priority (such as PCR or immunoblot).

In contrast, negative selection, in which lymphocytes, monocytes and neutrophils were tagged with magnetic beads, achieved ~85% viability leading to a relatively high eosinophil yield (~50%). These eosinophils were assessed for their survival under standard culture conditions with one of three survival cytokines (IL-3, IL-5 and GM-CSF). Tissue eosinophils cultured with cytokines (particularly with GM-CSF and IL-5), were still viable after two days of incubation at a percentage comparable to that reported for peripheral blood eosinophils (Tai et al., 1991). Therefore, in this regard, eosinophils isolated from NP behave similarly to peripheral blood cells. Priming and activation of these eosinophils revealed that these cells maintained their ability to become activated and release EPO, thus confirming that these eosinophils are suitable for further investigations.

Our finding of high viability, comparable surface expression and activation parameters in NP purified eosinophils, makes our methodology a potentially important advance in studying this cell population. Although we observed similarities in both cell survival and expression of cell-surface molecules, purification of tissue eosinophils is essential for further investigations of eosinophil function during inflammatory tissue responses.

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