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Purification of functional eosinophils from human bone marrow

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Abstract

Eosinophils are granulocytic leukocytes that are best known for their involvement in host immune defense and pathologic states. More recently, they have also been shown to play a role in regulation of murine plasma cell homeostasis in the bone marrow, which prompted our investigation of human bone marrow eosinophils. However, effective methods to isolate eosinophils from human bone marrow thereby allowing comparisons with circulating eosinophils have not yet been described. Herein we describe the development of a novel, cost effective protocol for the purification of eosinophils from human bone marrow that allows us to obtain bone marrow eosinophils of near 100% purity after an 8-day culture system. Furthermore, we demonstrate that bone marrow eosinophils have characteristics similar to blood eosinophils, including the expression of IL-5R α , the presence of eosinophil-specific granules, and similar activation kinetics upon phorbol myristate acetate and high-dose IL-5 stimulation. While migratory responses toward the chemokine CXCL12 differed between purified bone marrow and freshly isolated blood eosinophils, migratory responses were similar upon comparison of bone marrow eosinophils with blood eosinophils cultured *ex vivo* for 8 days prior to assay. Interestingly, a concurrent upregulation of CXCR4 expression was not observed in these cultured blood eosinophils. Taken together, we have overcome the existing challenges to the study of bone marrow eosinophils through our novel strategy for cell purification and have thus enabled future investigations of these cells and their role(s) in human health and disease.

Keywords

eosinophils; bone marrow; eosinophil purification; activation; chemotaxis; CXCR4

1. Introduction

Eosinophils are innate immune cells that play critical roles in host defense against helminthes and in allergic diseases such as asthma. Within the bone marrow, these cells differentiate from hematopoietic stem cells (HSCs) into the granulocytic lineage. They eventually mature and exit the bone marrow through the circulation to reside in tissues such as the gut, mammary gland, uterus, and thymus (Kita, 2011). Recently, through work done in the mouse, eosinophils were shown to influence the humoral response of the adaptive immune system by promoting bone marrow plasma cell survival (Chu et al., 2011). Eosinophils involved in this mechanism may be from either the retention of a subpopulation

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of the eosinophils within the bone marrow or a homing of mature circulating eosinophils back into the bone marrow. It remains unknown whether peripheral blood eosinophils and bone marrow eosinophils represent distinct subsets of cells with intrinsic functional differences or if they are indeed biologically similar.

Human eosinophils comprise approximately 2–5% of the total peripheral blood leukocyte population. The isolation of these cells from peripheral blood has not always been a trivial task. In the 1980s, the study of these cells depended on the use of discontinuous multiple density Percoll gradients for cell purification, but the difficulty of the technique in conjunction with the rarity of these cells in non-allergic individuals limited the usefulness of this method in terms of percent recovery and purity (Gartner, 1980; Gleich et al., 1983). Subsequently, an approach was developed by Hansel et al. utilizing anti-CD16-conjugated magnetic beads to negatively select for eosinophils within the mixed granulocytic population (Hansel et al., 1989, 1991). Alternatively, CD16-negative depletion of neutrophils can also be achieved by the use of fluorescence-activated cell sorter (FACS) or complement reaction techniques (Chihara et al., 1995). Studies have provided comparisons between these various strategies for eosinophil isolation from peripheral blood to highlight the pros and cons of each method as well as the functionality of the eosinophils post-isolation (Chihara et al., 1995; Sedgwick et al., 1996; Percopo et al., 2010). Currently the standard protocol used for the isolation of these cells in the study of asthma and allergic diseases utilizes the anti-CD16-conjugated magnetic bead separation technique.

In the human bone marrow, eosinophils make up 1–4% of the cellular compartment (Terstappen and Levin, 1992; Fauci et al., 2009). Unlike the case for peripheral blood eosinophils, methodologies for isolation of bone marrow eosinophils are less refined. Due to the abundance of hematopoietic cells at different developmental stages in the bone marrow with variable size and morphology, compounded by the low levels or complete lack of CD16 expression on immature neutrophils (i.e. band neutrophils), protocols effective at isolating peripheral blood eosinophils, including anti-CD16 negative selection, are likely to be ineffective at purifying bone marrow eosinophils.

Thus far, studies of bone marrow eosinophils in both humans and mice have mostly relied on FACS sorting of these cells after antibody-labeling of surface markers. For example, in the mouse bone marrow, eosinophils can be gated using a combination of markers that include F4/80, Gr-1, CD11b, and Siglec-F (Chu et al., 2011, 2012). In human bone marrow, eosinophils can be identified based on FSC vs SSC and IL-5R α expression (Mori et al., 2009). While these sorting strategies yield relatively pure eosinophils, the labeling of these surface molecules with antibodies often results in cell activation (Kato et al., 1998). Eosinophils as terminal effector cells have a limited lifespan post-activation, sometimes on the order of hours. Consequently, while the FACS sorting method permits molecular studies of these cells on the nucleic acid and protein level, it has limited usefulness in studying the biology of eosinophils. Dyer et al. (2008), alternatively, described a series of culture conditions in which the addition of stem cell factor (SCF), FLT3-ligand, and IL-5 induced differentiation of mouse bone marrow progenitor cells into eosinophils. Human progenitor cells from the umbilical cord (Ten et al., 1991; Ohashi et al., 1999) and bone marrow (Clutterbuck et al., 1989) have also been successfully differentiated into eosinophils *in vitro* in the presence of IL-3 and IL-5. While these are viable options to obtain human bone marrow-derived eosinophils, it may not represent the best option as the differentiation of these cells from progenitor populations can be labor and/or time intensive (2–4 weeks) as well as costly.

Here we describe an optimized protocol for the purification of human bone marrow eosinophils through a minimal-cost, 8-day *in vitro* culture system. Unlike the differentiation

studies mentioned previously, our culture system allows for the direct purification of mature bone marrow eosinophils. We show that by day 8 of our bone marrow cultures, we can achieve near 100% purity for eosinophils and that these cells have a number of characteristics in common with freshly isolated peripheral blood eosinophils.

2. Materials and methods

2.1. Patients and healthy donors

Mayo Clinic Institutional Review Board (IRB) approval was obtained for collection of peripheral blood and bone marrow aspirates from multiple myeloma patients and bone marrow aspirates from otherwise healthy individuals undergoing hip replacement or spine surgery. Tissue was only collected after the patients provided written informed consent in accordance with the Declaration of Helsinki.

2.2. Isolation of granulocytes from peripheral blood and bone marrow

Granulocytes were obtained through Percoll density gradient centrifugation. Briefly, diluted whole peripheral blood or bone marrow aspirates (1:1 in PBS) were underlain with 56% Percoll (GE Healthcare, Piscataway, NJ, USA) and centrifuged at 700xg for 30 minutes. The plasma and interphase layers containing mononuclear cells (MNCs) were removed, leaving behind the pellet containing red blood cells (RBCs) and granulocytes. RBCs were subsequently lysed in dH₂O followed by the addition of equi-volume 2X PBS (hypotonic red cell lysis; HRCL).

2.3. Purification of peripheral blood eosinophils

Peripheral blood eosinophils were purified using anti-CD16-conjugated magnetic beads (Miltenyi Biotec, Auburn, CA, USA) following the manufacturer's protocol. Briefly, granulocytes were isolated using a Percoll density centrifugation technique as described above followed by incubation with anti-CD16-conjugated magnetic beads for 30 minutes on ice. The cell suspension was then allowed to run through a MACS column (Miltenyi Biotec) to allow for the retention of CD16⁺ neutrophils in the column.

2.4. Purification of bone marrow eosinophils

Granulocytes were first isolated as described above. Cells were then resuspended in RPMI 1640 (Gibco Life Technologies, Grand Island, NY, USA) supplemented with 100 U/ml penicillin G, 10 µg/ml streptomycin, 3 µg/ml L-glutamine (Invitrogen, Carlsbad, CA, USA), 50 µg/ml gentamycin (Gibco Life Technologies), 50 µM β-mercaptoethanol (Sigma-Aldrich, St. Louis, MO, USA), 20% heat-inactivated fetal calf serum (PAA Laboratories, Etobicoke, Ontario, Canada), and 1 ng/ml human recombinant IL-5 (Peprotech, Rocky Hill, NJ, USA), which we term RPMI_{eos}, to approximately 2×10⁶ cells/ml and plated in 75 cm² culture flasks (BD Biosciences, San Jose, CA, USA). Cells were incubated undisturbed for 4 days at 37 °C in a humidified 5% CO₂ incubator. On day 4 of the culture, cells were centrifuged at low speed (185xg) for 10 minutes to remove dead cell debris. Pelleted viable cells were resuspended in RPMI_{eos} to 1–2×10⁶ cells/ml, plated in new 75 cm² flasks, and incubated for another 2 days. On day 6, cells were harvested from the flasks and centrifuged at 185xg for 10 minutes before resuspending in RPMI_{eos} to 0.5–1×10⁶ cells/ml and plating in 10 cm tissue culture dishes (BD Biosciences). The change from culture flasks into dishes allows for removal of any macrophages that might have grown out from our cultures as a result of GM-CSF secretion by eosinophils. On day 7 of the culture, we performed density gradient centrifugation using 56% Percoll and centrifuging at 700xg for 30 minutes as described above to remove any remaining macrophages. Cell pellets were washed once, resuspended in RPMI_{eos}, and plated into new tissue culture dishes. On day 8, the purity of

the cultures was evaluated by cytopsin preparations stained with modified Wrights stain (see detail below). See Figure 1 for a schematic of the isolation protocol.

2.5. Viability assessment and cell counting

Cell viability was assessed at various time points by the trypan blue exclusion method. Cells were counted on a hemocytometer (Hausser Scientific, Horsham, PA, USA). Specific apoptosis analyses of the cultures were performed with flow cytometric staining for annexin and 7AAD (see Section 2.8).

2.6. Cytopsin and staining

Cytospins were prepared from the cell cultures by centrifugation at 350 rpm for 4 minutes (CytoSpin 4 Cyto centrifuge, Thermo Fisher Scientific, Waltham, MA, USA). Slides were air-dried prior to staining. For morphologic analysis, slides were stained with Hema 3 modified Wright-Giemsa stain (Thermo Fisher Scientific) according to manufacturer's protocol.

2.7. Polymerase chain reaction

Total RNA was extracted from cells at various time points throughout the 8-day incubation using Trizol reagent (Invitrogen) according to manufacturer's protocol. cDNA was generated using an iScript cDNA synthesis kit (Bio-Rad, Hercules, CA, USA). Conventional 25- μ l PCR reaction assays were performed using the Qiagen HotStarTaq MasterMix kit (Qiagen, Valencia, CA, USA). The following primers were used (Mori et al., 2009): MPO, 5'-GTATGACGGAGGCTTCTCTC (forward) and 5'-CAGTTGACGCCAGTGACGAA (reverse); IL-5R α , 5'-ATGAAGGAAGCTGCCTGAGA (forward) and 5'-GTAATACATGCGCCACGATG (reverse); EPX, 5'-CACGGTTTCAAGGGACATC (forward) and 5'-CTTTTCTTGCTGGGGTG (reverse); MBP, 5'-CGTACCTCCTGGTGAGAAG (forward) and 5'-GTAGCCTCCTCGGGTACACA (reverse); beta actin, 5'-GGATCCGACTTCGAGCAAGAGATGGCCAC (forward) and 5'-CAATGCCAGGGTACATGGTGGTG (reverse). The sizes of the PCR products were: MPO, 236 bp; IL-5R α , 321 bp; EPX, 383 bp; MBP, 297 bp; beta actin, 271 bp. The amplification was carried out in a PerkinElmer 9600 thermocycler with the following PCR reaction profile: 30 seconds at 94 °C, 1 minute at 58 °C, 1 minute at 72 °C, followed by a final extension of 7 minutes at 72 °C. PCR products were separated by size on a 1.5% TAE agarose gel containing 200 ng/ml ethidium bromide.

2.8. Flow cytometry and apoptosis analysis

For apoptosis analyses, day 8 bone marrow cell cultures were stained with annexin-APC and 7-AAD (BD Biosciences) following manufacturer's protocol and analyzed on a FACSCanto flow cytometer (BD Biosciences). For analyses of cell surface expression of Siglec-8 and CXCR4, the following antibodies were used: phycoerythrin-conjugated anti-human Siglec-8 (BioLegend, San Diego, CA, USA), phycoerythrin-conjugated mouse IgG1 κ isotype control (BD Biosciences), unconjugated anti-CXCR4 (Abcam, Cambridge, MA, USA), unconjugated rabbit IgG isotype control (Peprotech), and Alexa Fluor 488-conjugated goat anti-rabbit IgG (Molecular Probes, Eugene, Oregon, USA). Samples were analyzed on FACScan or FACSCanto flow cytometers (BD Biosciences).

2.9. Chemotaxis assay

Chemotaxis assays in response to various cytokines were performed using 3 μ m polycarbonate membrane Transwell inserts in 24-well tissue culture polystyrene plates (Corning, Corning, NY, USA). RPMI₁₆₄₀ was used as the chemotaxis assay medium. Chemokines used in the study included CCL5 (BioLegend) and CXCL12 (BioLegend).

1×10^6 eosinophils (in a total volume of 200 μ l) were seeded in the upper chamber and 500 μ l of media alone or media plus the indicated concentrations of chemokine was plated in the lower chamber. The plates were incubated at 37 °C and 5% CO₂ for 90 minutes. The number of cells that migrated to the bottom chamber in each condition was counted on a Vi-Cell XR (Beckman Coulter, Brea, CA, USA). The migration index was calculated as the ratio of the number of eosinophils migrated toward specific chemokines relative to the number of eosinophils migrated in response to media alone.

2.10. Superoxide assay

The release of superoxide by eosinophils upon activation was measured as the reduction of cytochrome *c*. Briefly, 5×10^4 cells/well were plated in 96-well plates in HBSS/10 mM HEPES/0.01% gelatin. Cells were stimulated with 15 ng/ml IL-5 (Peprotech), 0.5 ng/ml or 1 ng/ml phorbol myristate acetate (PMA) (Invitrogen) in the presence of 100 μ M cytochrome *c* (Sigma-Aldrich). Absorbance was read immediately after plating and every 15 minutes for 1 hour and every 30 minutes for another 2 hours at 550 nm using a SpectraMax Pro microplate reader (Molecular Devices, Sunnyvale, CA, USA). Plates were kept at 37 °C in between readings. Generation of superoxide was calculated by the following formula: nmol superoxide released per 1×10^6 cells = nmol cytochrome *c* reduced per 1×10^6 cells = $19.1 \times [(OD_{550}(\text{time} = x) - OD_{550}(\text{time} = 0))] / 0.05$.

3. Results and discussion

Anti-CD16 negative selection is a well established method for the purification of eosinophils from peripheral blood. The method relies on an initial density gradient centrifugation step to fractionate granulocytes and RBCs out from whole blood followed by lysis of the RBCs. Finally the eosinophils are separated from the neutrophils within the granulocyte fraction by anti-CD16-conjugated magnetic beads, which bind to the CD16 that is expressed by neutrophils but not eosinophils. This protocol consistently yields peripheral blood eosinophils at >95% purity in the CD16⁻ fraction (Figure 2A, **middle panel**).

We first attempted to purify eosinophils from human bone marrow aspirates using this identical protocol which proved to be unsuccessful. Although cytopsin preparations of the CD16⁺ fraction showed highly purified neutrophils (Figure 2B, **right panel**), the CD16⁻ fraction contained both eosinophils and neutrophils (Figure 2B, **middle panel**). Closer morphologic analysis of the cells revealed that the CD16⁺ fraction was comprised mostly of mature neutrophils as indicated by their nuclear segmentation whereas the neutrophils found in the CD16⁻ fraction were mostly immature cells with elongated and curved nuclei that lacked segmentation. This result was not surprising given that neutrophils only upregulate their expression of CD16 during the final stages of maturation in the bone marrow (Elghetany, 2002). These initial studies prompted us to then devise a methodology that would permit isolation of purified bone marrow derived eosinophils. Our optimized protocol (described fully in the Materials and Methods section; Figure 1) utilizes culture conditions that are optimal for eosinophil survival while lacking survival factors for neutrophils in order to recover healthy bone marrow eosinophils within 8 days of *in vitro* culture. During the optimization process, numerous culture conditions were tested using e.g., various cell densities, FCS concentrations, and time points for media change (results not shown). The optimized protocol allows for a recovery of eosinophils that varies between 25.6–60.3% (mean, 41.0%; standard deviation, 10.6).

To assess the purity of our eosinophil cultures, we first evaluated these cells based on morphology (Figure 3A). Eosinophils, as suggested by their name, are easily stained by acidic dye as a consequence of their basic granules. On cytopsin preparations stained with modified Wright-Giemsa stain, these cells appear pink and granular. Initially at day 0 after

Percoll density centrifugation, we recover anywhere between $46\text{--}95 \times 10^6$ granulocytes, depending on the starting volume of the bone marrow aspirates as well as the percent of granulocytes in the bone marrow cellular compartment for each sample. Eosinophils represented 3–13% (mean, 6.7%; standard deviation, 3.0) of the granulocytes. Remaining cells were predominantly neutrophils. During the first four days of culture, the majority of neutrophils underwent apoptosis due to the lack of pro-survival cytokines, whereas eosinophils remained alive as a result of the supplemental IL-5 in the culture medium (Figure 3A). Apoptotic neutrophils could be identified in the culture by their condensed nuclei. By day 4, the number of viable cells in the cultures was significantly diminished compared to numbers at day 0 (Figure 3B). Furthermore, in addition to the apoptotic neutrophils, bone marrow derived macrophages begin to appear, likely as a result of secretion of GM-CSF by eosinophils in the culture (Levi-Schaffer et al., 1995). Consistent with a recent description of embryonic stem cell-derived macrophages (Zhuang et al., 2012), we observed bone marrow derived macrophages that were proficient at phagocytosis of apoptotic cells (Figure 3A). On day 6 of the culture, the proportion of eosinophils in the culture outweighs the proportion of neutrophils. Macrophages were often found adherent to the bottom of the culture flask. The combination of low-speed centrifugation and replacement of the culture flasks at this point aids to remove apoptotic cellular debris and adherent macrophages, respectively. By day 7, cell cultures were comprised mostly of eosinophils. Eosinophil precursors could be detected morphologically by their blast-like appearance, kidney-shaped rather than bi-lobed nucleus, and sometimes the coexistence of acidophilic and basophilic granules (Figure 3A). The occasional loosely adherent macrophages persist in culture even after sequential culture flask/dish change; therefore, an additional Percoll step at day 7 was implemented to further enhance culture purity. The purity of the eosinophil culture at day 8 across 10 different experiments as assessed by morphology was calculated to be 99.6% (range, 98.8–100%; standard deviation, 0.43; Figure 3A, **right panels**, and 3C) with an overall viable cell yield of 2.21×10^6 (range, $0.83\text{--}3.79 \times 10^6$; standard error, 0.4×10^6 ; Figure 3B). Interestingly, at day 8 the vast majority of the eosinophils appear to be fully matured. This is likely the result of both the removal of less mature, blastic-eosinophils during the Percoll step at day 7 and the continual maturation of the immature eosinophils into fully mature cells between day 7 and 8. Formal apoptosis analysis was performed at day 8 of the culture (Figure 4). Our results indicate that approximately 90% of the purified eosinophils are viable and non-apoptotic. These 8-day-purified bone marrow eosinophils are observed to be viable in culture for at least 6 more days (unpublished observations). Furthermore, we have evidence to suggest that these cells remain functional during this time (Wong et al., manuscript in preparation).

We further characterized the cells throughout our 8-day culture using PCR to measure mRNA expression for various genes. Specifically, we assessed mRNA expression levels of several lineage-specific genes, including myeloperoxidase (MPO), IL-5R α , eosinophil peroxidase (EPX), and major basic protein (MBP) at various time points. Human neutrophils uniquely express MPO but not IL-5R α , EPX, or MBP. Conversely, human eosinophils while lacking MPO expression do express IL-5R α , EPX, and MBP (Mori et al., 2009). Figure 5A shows the expression patterns of these four genes at various time points throughout the eosinophil purification culture system. As expected, MPO expression was the highest at day 0 of the cultures when neutrophils predominate in numbers and MPO mRNA levels were observed to decrease with time. By day 8 of the culture, when neutrophils were almost never observed, MPO expression could no longer be detected. On the other hand, IL-5R α expression at day 0 was relatively low due to the low proportion of eosinophils in the mixed culture, but as eosinophil purity increased over the course of 8 days, IL-5R α expression became more prominent. A similar pattern was seen with EPX and MBP expression, with the exception of day 8 when expression levels were low or absent. This finding is consistent with the fact that at day 8 the majority of the eosinophils in the cultures

were of the mature type, which are known to contain preformed effector granules and are less active at transcribing mRNA for these particular genes.

While our mRNA analysis of lineage-specific genes are reflective of a decrease in neutrophil and increase in eosinophil percentages over the course of the 8-day culture, these data alone do not provide quantitative information regarding these percentages. Consequently, we utilized flow cytometry to analyze the percent of eosinophils at each time point by staining for surface expression of Siglec-8, an eosinophil-specific molecule. As shown in Figure 5B, the proportion of Siglec-8⁺ eosinophils increased from 5.7% at day 0 to 94.0% at day 8. Of note, although Siglec-8 analysis shows only 94.0% eosinophils at day 8, cyto-spin preparations from this sample revealed 100% pure eosinophils (data not shown). We suspect that the discrepancy is the result of the flow cytometer's mis-identification of cellular debris as intact cells, supported by the fact that the Siglec-8⁺ "cells" are found to have low forward and side light scatter properties.

We next aimed to assess the functionality of these purified bone marrow eosinophils. The measurement of superoxide production in response to various activatory stimuli is a standard methodology to evaluate activation of peripheral blood eosinophils (Kita, 2011). We therefore utilized this assay to compare the kinetics of activation of peripheral blood and bone marrow eosinophils. As shown in Figure 6A, the release of superoxide by freshly isolated peripheral blood eosinophils could be detected as early as 15 minutes following the addition of PMA. The maximal release of superoxide was reached within 30–45 minutes. Similarly, bone marrow eosinophils purified using our protocol showed comparable activation kinetics, with detectable superoxide release at 15 minutes of PMA stimulation and a peak response at 45 minutes. We further investigated the activation kinetics of peripheral blood eosinophils isolated by anti-CD16 negative selection and maintained in culture for 8 days in RPMI_{eos}. The activation kinetics of cultured and freshly isolated peripheral blood eosinophils were similar. Given that PMA stimulation is an unnatural way to activate these cells, we also examined the ability of bone marrow eosinophils to be activated upon high-dose IL-5 stimulation. Using the superoxide assay, we demonstrate that bone marrow eosinophils, as previously described with human peripheral blood eosinophils (Horie et al., 1996; Kita, 2011), can be activated by high-dose IL-5 (Figure 6B). Together, these results suggest that bone marrow eosinophils purified through this 8-day *in vitro* culture protocol still preserve their functionality. Furthermore, the comparable activation kinetics between bone marrow and peripheral blood eosinophils based on the superoxide assay indicate that these cells may represent cells with similar intrinsic properties that reside in different compartments within the human body.

Finally, we attempted to elucidate the mechanism by which bone marrow and peripheral blood eosinophils home to their respective locations by comparing the migratory abilities of these cells toward various chemokines. CCL5, a.k.a. RANTES, is a known chemoattractant for eosinophils. In a normal immune response, CCL5 is produced by activated T cells to recruit leukocytes, including eosinophils, to the site of inflammation by its binding to the chemokine receptors CCR3, CCR5, and CCR1. On the other hand, CXCL12 is a chemokine produced in abundance by bone marrow stromal cells. Its cognate receptor is CXCR4. CXCL12 is the chemokine toward which long-lived plasma cells migrate in order to take up residence in the bone marrow (Hargreaves et al., 2001). Using transwell assays, our initial experiments showed that while bone marrow eosinophils migrate similarly or better toward CXCL12 as compared to CCL5, freshly isolated peripheral blood eosinophils preferentially migrate toward CCL5 and exhibit little response to CXCL12 (Figure 7). We questioned the possibility that our 8-day *in vitro* culture of the bone marrow eosinophils may have influenced their migrational response toward CXCL12, therefore we further extended our analysis to test chemotaxis of peripheral blood eosinophils toward these chemokines after

being similarly maintained in culture for 8 days in RPMI_{eos}. Our data revealed that while freshly isolated blood eosinophils showed minimal migration toward CXCL12 (Figure 8A), cultured blood eosinophils responded to CXCL12 in a way that mirrors that which was seen with bone marrow eosinophils (Figure 8B–D). Of note, as the isolation of fresh, untouched eosinophils from the bone marrow is not yet possible, we are unable to assess whether the observed CXCL12-mediated migrational response of the bone marrow eosinophils purified using our protocol is intrinsic to these cells or if the response is upregulated only after culturing with IL-5 as in the case of blood eosinophils. We also note that our findings here regarding the effects of IL-5 on blood eosinophils are in contrast to reports from prior studies which showed a decreased responsiveness of these cells toward CXCL12 upon *in vitro* culture with IL-5 (Nagase et al., 2000; Dulkys et al., 2004). Although we cannot fully explain these differences, we hypothesize that differences in culture duration (24 hours vs. 8 days) and IL-5 concentration used (10 ng/ml vs. 1 ng/ml) between the studies may contribute to the discrepancy.

As mentioned previously, CXCR4 is the cognate receptor for the chemokine CXCL12. Prior studies that showed an attenuated response of blood eosinophils to CXCL12 upon culture in IL-5 also reported a simultaneous downregulation of CXCR4 expression (Nagase et al., 2000; Dulkys et al., 2004). We similarly investigated the surface expression of CXCR4 by peripheral blood and bone marrow eosinophils on day 0 and day 8 after culturing in RPMI_{eos}. In freshly collected peripheral blood and bone marrow samples, we found that eosinophils in the bone marrow expressed significantly higher levels of CXCR4 than those in the peripheral blood (Figure 9A). Given our chemotaxis data (Figure 8), we suspected that CXCR4 expression by blood eosinophils would increase upon 8-day culture in IL-5-containing media. Surprisingly, our data showed that while bone marrow eosinophils increase surface expression of CXCR4 over time in culture, peripheral blood eosinophils do not upregulate CXCR4 expression under the same culture conditions (Figure 9B and C). Our finding raises an interesting question regarding eosinophil biology with respect to how long-term culture of blood eosinophils in IL-5 might sensitize these cells' response to CXCL12 without changing the levels of the chemokine's known cognate receptor. Of note, as our protocol requiring an 8-day *in vitro* culture system is the first to isolate "untouched" eosinophils from bone marrow, similar studies investigating potential changes in chemotactic response of bone marrow eosinophils as a consequence of the *in vitro* culture is not yet possible.

4. Conclusions

In summary, we have developed a novel, low-cost method for the purification of eosinophils from human bone marrow. Notably, our culture system allows for isolation of bone marrow eosinophils of near 100% purity. These cells can be activated by various stimuli and show similar activation kinetics to that of peripheral blood eosinophils. Although our data do not clearly provide a mechanistic explanation for the *in vivo* localization of bone marrow and peripheral blood eosinophils, our chemotaxis results and CXCR4 expression data raise several interesting questions pertaining to eosinophil biology that warrant further study. We recognize the inherent limitations introduced by the 8-day *ex vivo* culture to the isolation of bona fide bone marrow eosinophils, nevertheless this method represents the only method to date for the purification of untouched eosinophils from human bone marrow. Our work provides a useful tool for laboratories that have easy access to bone marrow samples as an alternative source of eosinophils for the study these cells and their involvement in human health and disease.

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Highlights

- Novel methodology for the purification of eosinophils from human bone marrow
- Marrow- and blood-eosinophils exhibit similar activation kinetics
- Ex vivo cultured blood and marrow-, but not freshly isolated blood-, eosinophils show chemotaxis toward CXCL12
- Marrow-eosinophils, unlike blood-eosinophils, express surface CXCR4 and upregulate its expression further upon ex vivo culture with IL-5

Figure 1.
Schematic of the optimized protocol for the purification of human bone marrow eosinophils.

Figure 2. Anti-CD16 negative selection protocol effectively isolates eosinophils from human peripheral blood but not bone marrow. Anti-CD16-conjugated magnetic beads were used to separate CD16⁺ (right panel) and CD16⁻ (middle panel) cell populations from the peripheral blood (**A**) and bone marrow (**B**). Left panels represent granulocytes recovered after Percoll density centrifugation and red blood cell lysis.

Figure 3.

8-day *in vitro* culture system yields highly pure eosinophils from human bone marrow. (A) Cytopsin preparations were made at various time points throughout the 8-day culture system from 2 different patient samples. (B) The number of viable cells from 10 separate patient samples was counted during the 8-day culture. Differences in the initial (day 0) cell counts reflect differences in the bone marrow aspirate volume collected from the patients combined with variability in percentages of granulocytes amongst different bone marrow samples. Cell viability was assessed by trypan blue staining. (C) The percent eosinophils in the cultures on day 0, 4, 6, 7, and 8 for these 10 samples were calculated based on modified Wright-stained cytopsin preparations.

Figure 4. Apoptosis analysis of day 8 purified bone marrow eosinophils. Using annexin-7AAD, day 8 purified bone marrow eosinophils were analyzed by flow cytometry for the percentages of apoptotic cells.

Figure 5.

Characterization of cells during the 8-day culture by assessing mRNA expression of lineage-specific genes and surface expression of the eosinophil-specific protein, Siglec-8. **(A)** mRNA expression of MPO, IL-5R α , EPX, and MBP were evaluated at day 0, 4, 6, 7, and 8 of culture from 2 separate patients. Beta actin mRNA expression was used as a loading control. **(B)** The percentage of eosinophils in bone marrow cultures throughout the 8-day culture was assessed using flow cytometric analysis of surface Siglec-8 expression.

Figure 6.

Marrow- and blood-derived eosinophils show similar activation kinetics upon PMA and high dose IL-5 stimulation. Superoxide release as a consequence of eosinophil activation was assessed by cytochrome *c* reduction over a 3-hour period. **(A)** Bone marrow eosinophils (triangles; $n=3$; D8) purified using the 8-day culture system and peripheral blood eosinophils (circles; $n=2$; D0 and D8) isolated using anti-CD16 negative selection were stimulated with 1 ng/ml PMA (open). Closed symbols represent unstimulated controls. Results are shown as the mean levels of superoxide release ($\pm$ SEM) for the separate experiments. **(B)** Purified bone marrow eosinophils were stimulated with 15 ng/ml IL-5 (square), 0.5 ng/ml PMA (circle), or 1 ng/ml PMA (diamond). Closed triangles represent the unstimulated control.

Figure 7. Purified bone marrow, but not freshly isolated peripheral blood, eosinophils migrate toward CXCL12. Chemotaxis of peripheral blood (open; n=3) and bone marrow (closed; n=5) eosinophils in response to 100 ng/ml CCL5 and CXCL12 was assessed (mean ± SEM). * indicates $p < 0.05$.

Figure 8.

Peripheral blood eosinophils show enhanced migration toward CXCL12 upon 8-day *in vitro* culture with IL-5. Freshly isolated blood eosinophils (A), blood eosinophils after 8-day *in vitro* culture in RPMI_{eos} (B), and 8-day culture-purified bone marrow eosinophils (C) were evaluated for chemotaxis response toward various concentrations of CCL5 and CXCL12. Data shown are representative of 4 (peripheral blood) and 3 (bone marrow) separate experiments. (D) Mean migrational response toward 100 ng/ml CCL5 and CXCL12 was calculated ($\pm$ SEM; $n=3$) for peripheral blood eosinophils either immediately after anti-CD16-magnetic bead purification or after 8 days *in vitro* culture with IL-5 (1 ng/ml). * $p < 0.05$; n.s., not significant.

Figure 9.

Surface expression of CXCR4 increases in bone marrow, but not peripheral blood, eosinophils upon 8-day *in vitro* culture with IL-5. **(A)** Siglec-8⁺ eosinophils from freshly obtained human peripheral blood and bone marrow samples were analyzed for surface expression of CXCR4 by flow cytometry. ΔMFI were calculated relative to isotype control. Mean ± SEM are shown for each group. * indicates $p < 0.05$. **(B)** Surface expression of CXCR4 was analyzed for blood and bone marrow eosinophils at day 0 (solid line) and day 8 (dotted line) of the *in vitro* culture in RPMI_{eos} relative to isotype control (gray filled). **(C)** A total of 3 peripheral blood and 6 bone marrow samples were analyzed for CXCR4 as described in **(B)**. ΔMFI values were calculated relative to isotype control. Mean ± SEM are shown for each group. * indicates $p < 0.05$, ** indicates $p < 0.001$.