

Granule protein changes and membrane receptor phenotype in maturing human eosinophils cultured from CD34⁺ progenitors

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Summary

Background Eosinophils are now recognized as major effector cells in allergic and asthmatic disease with a potent armoury of mediators whose release makes a major contribution to the inflammation underlying these conditions.

Objectives The purpose of this study was to compare cultured eosinophils (CE) with normal-density peripheral blood eosinophils (PBE) in terms of their membrane receptor expression and to analyse the expression and storage of the eosinophil granule proteins major basic protein (MBP) and eosinophil cationic protein (ECP) during eosinophil maturation *in vitro*.

Methods Purified human peripheral blood CD34⁺ cells were cultured in the presence of recombinant human IL-3, IL-5, rhGM-CSF, SCF, and FLT-3 ligand. PBE were isolated by density gradient centrifugation and negative immunomagnetic selection. Expression of CD11b, CD18, CD45, CD45RA, CD45RB, CD45RO, CD69, CD95, IL-5R α , IL-9R α , CCR1, CCR3, and CXCR4 by CE as they matured in culture were assessed by immunostaining and flow cytometry and expression of these receptors compared with freshly isolated PBE. Immunohistochemical staining and labophot-2TM light microscopy determined expression of MBP, ECP, and CD69 during eosinophil maturation.

Results Positive immunostaining for MBP and ECP was detectable in a proportion (15–20%) of CE as early as 3 days of culture even though these cells were mononuclear in appearance. The numbers of CE positive for both granule proteins increased in rhIL-3 and rhIL-5 treated cells to a maximum of ~80% by day 28. Maturing eosinophils exhibited positive immunostaining for CD69 after 14, 21 and 28 days of culture. Compared with PBE, CE had lower expression of pan-CD45 and CD45 isoforms, CD95 and CD11b. In contrast, the specific mean fluorescence for CD69, CD18, IL-5R α , and IL-9R α was significantly elevated for CE compared with PBE. CCR3 expression by CE and PBE was similar with no expression of CXCR4 detected by either CE or PBE. No significant difference in expression of CCR1 was found between CE and PBE.

Conclusion These data suggest that CE and PBE share many phenotypic properties and both MBP and ECP appear early in eosinophil development *in vitro*. However, there are quantitative differences that may be a consequence of their immaturity and/or the influence of the cytokines used in their culture.

Keywords CD34⁺ progenitors, ECP, eosinophilopoiesis, eosinophils, MBP, membrane receptor phenotype

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Introduction

Eosinophils are thought to play a beneficial role in immunity to parasitic worms [1] and have also been proposed to play a role in host defence against respiratory pathogens [2]. However, they are also recognized as major effector cells in the inflammatory process underlying much of the pathogenesis of asthma and other allergic diseases [3]. Large numbers of activated tissue eosinophils are typically observed in airway biopsies, sputum

and bronchoalveolar lavage fluid obtained from asthmatic patients [1, 3]. Eosinophils store and release, on appropriate activation, a wide spectrum of pro-inflammatory mediators including cationic granule proteins, major basic protein (MBP), eosinophil cationic protein (ECP), and eosinophil-derived neurotoxin (EDN) [4]. They have also been shown to synthesize up to 28 cytokines, chemokines, and growth factors, many of which are stored in their crystalloid granules [5]. Indeed, the morphologic characteristics of eosinophilopoiesis and the ontogeny of the formation of crystalloid granule have been partially investigated by electron microscopy and *in vitro* culture systems. This included the description of early commitment to the eosinophil phenotype by the appearance of numerous large immature granules in the cytoplasm [6]. The

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observation that immature granules appear early in eosinophil development was confirmed in later studies showing pro-MBP messenger RNA and protein expression in developing eosinophils *in vitro*, which localized to large immature granules containing 'hollow' electron-lucent cores [7].

The peripheral blood of normal individuals contains small numbers of cells with primitive CD34⁺ progenitor phenotype [8, 9]. These CD34⁺ peripheral blood progenitors are easily accessible and may serve as a helpful resource for studying the maturation process of haematopoietic cells. Several groups have demonstrated that IL-3, IL-5, and granulocyte-macrophage colony-stimulating factor (GM-CSF) are haematopoietic cytokines that induce the proliferation and differentiation of eosinophils from progenitor cells [10–13]. While we have previously examined the phenotype and function of eosinophils cultured from cord blood mononuclear cells [14], relatively little is known regarding the phenotypic and granule content changes in eosinophils as they mature from CD34⁺ peripheral blood progenitors in the presence of IL-3, IL-5, rhGM-CSF, SCF, and FLT-3 ligand. The aim of this study therefore was to assess the basic protein content of their crystalloid granules and the changes in eosinophil cell surface receptor expression as they mature in culture compared with peripheral blood normal-density eosinophils.

Material and methods

Cytokines and antibodies

Recombinant human IL-5 (rhIL-5) (rhIL-3) (rhSCF) (rhGM-CSF), rat IgG_{2A} mAb isotype-matched control, antihuman CCR-3, and anti-human CCR-1 mAb were all purchased from R&D systems (Abingdon, UK). Human fms-like tyrosine kinase-3 ligand (rhFLT-3 ligand) was obtained from Pepro Tech. Ltd (Rocky Hill, NJ, USA). CD45 mAb (Bra-55) was supplied by Sigma (Poole, UK). CD45RA mAb (MB1) and CD45RB mAb (MT4) were obtained from Immuno Quality Products (Groningen, the Netherlands). CD11b mAb (2LPM19C), CD45RO mAb (UCHL-1), CD18 mAb (MHM23), mouse IgG1 mAb isotype-matched control (DAK-GO1), mouse IgG2a mAb isotype-matched control (DAK-GO5), rabbit IgG polyclonal antibody (pAb) isotype-matched control, rabbit anti-mouse immunoglobulin, and alkaline phosphatase anti-alkaline phosphatase (APAAP) mouse mAb were all from Dako Ltd (Cambridgeshire, UK). FAS (B-10) mAb, IL-5R α (C-20) pAb, IL-9R α (C-19) pAb, and CXCR4 (12G5L) mAb were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-human ECP mAb (EG2) was supplied by Pharmacia® AB, Uppsala, Sweden. Mouse monoclonal anti-human MBP (BMK-13) was kindly provided by Professor Redwan Moqbel, University of Alberta, Canada and MLR3 anti-human CD69 mAb was a gift from Dr M. E. Cosulich, Instituto Scientifico Tumori, Genova, Italy.

Cell culture and eosinophil purification

Blood (80 mL) was obtained from normal donors or individuals with a history of mild allergic disease and an eosinophilia not greater than 0.5×10^6 eosinophils/mL, who gave informed consent. Eosinophils were purified using dextran sedimentation and centrifugation on Percoll gradients, followed by CD16-dependent negative immunomagnetic selection, as previously

described [15]. Isolated eosinophils were >99% pure with >98% viability as judged by trypan blue exclusion. For demonstration of CD69 expression by immunostaining, freshly purified PBE were washed with RPMI-1640 (supplemented with 10% FCS, 100 IU/mL penicillin, 100 mg/mL streptomycin, and L-glutamine), adjusted to a concentration of 2×10^6 /mL in the same medium and 1 mL of cells cultured overnight (20 h) in the presence of GM-CSF at a final concentration of 1.7 ng/mL in 24-well plates (Life Technologies, Paisley, UK) at 37 °C with 5% CO₂ in a humidified atmosphere.

Isolation of human CD34⁺ stem cells by magnetic cell sorting (miniMACS)

We used a slightly modified protocol from those previously described [13, 16, 17].

Briefly, human peripheral blood CD34⁺ cells were obtained from leukapheresis collection of $2\text{--}4 \times 10^8$ light density leucocytes from healthy adult donors after informed consent. After removal of erythrocytes by dextran sedimentation, mononuclear cells were centrifuged on isotonic Percoll cushions (1.129 g/mL, Pharmacia) at 400 *g* for 20 min. These cells were collected and washed twice in phosphate buffered saline (PBS) containing 0.5% bovine serum albumin (BSA) and 2 mM EDTA. The isolated mononuclear layer was then labelled with micro-magnetic beads coupled to anti-CD34-mAb (QBEND-10; Miltenyi Biotec GmbH, Bergisch Gladbach, Germany), followed by positive selection during elution through a MiniMACS separation column (Miltenyi Biotec). Enriched CD34⁺ cells were recovered by further elution after removing the column from the magnetic field. CD34⁺ cells were subjected to adherence in a plastic tissue culture flask for 2 h to deplete the few contaminating cells before determination of cell number and viability.

Culturing and differentiation of CD34⁺ cells

Between 5×10^4 and 1×10^5 CD34⁺ cells/mL were cultured in 24-well plates in Iscove's modified Dulbecco's medium (IMDM) (Ivotrogen, Gibco, UK) containing 10%FCS, L-glutamine, 100 IU/mL Penicillin, 100 mg/mL streptomycin, and 4 μ M mercaptoethanol (Sigma). Differentiation of progenitors into eosinophilic cells was induced by adding rhIL-5 (10 ng/mL), rhIL-3 (25 ng/mL), rhGM-CSF (10 ng/mL), SCF (100 ng/mL), and FLT-3 ligand (100 ng/mL). After 3 days, cells were counted and resuspended in IMDM containing IL-3, IL-5, and GM-CSF. After 7 days, GMCSF was omitted from the medium. From day 21, IL-3 was also omitted from the media. Growth and viability were determined by light microscopic evaluation with trypan blue exclusion. Viability remained >95% throughout the 28-day experimental period.

Expression of surface antigens on eosinophil precursor cells

Immunostaining and flow cytometry were performed as previously described [18]. Freshly isolated eosinophils and cultured eosinophils harvested at timed intervals during the course of development (2×10^5) were immunostained with the relevant antibodies or controls at saturating concentrations for 30 min on ice. After a wash and staining with FITC-conjugated anti-mouse Ig mAb or anti-rabbit Ig pAb, the cells were analysed on a flow cytometer (FACScan, Becton Dickinson, Oxon, UK). Flow cytometry was performed on eosinophils gated on the basis of their forward and side scatter profile with any cell debris

excluded from analysis. Non-specific fluorescence was controlled for by incubation with isotype-specific control antibodies.

Immunocytochemical labelling

Cytospin slides were prepared from freshly purified CD34⁺ and CE harvested at days 3, 7, 14, 21 and 28. Cells (1×10^5) were suspended in 100 μ L of PBS and cytopins prepared. The slides were fixed for 10 min in 99% methanol and stored at -20°C until required. Slides of cultured cells (days 3, 7, 14, 21 and 28) were washed two times in TRIS-buffered saline solution (TBS, pH 7.4) before incubation with primary mouse mAb against MBP (BMK13) (diluted 1:5), ECP (EG2) (diluted 1:4) and MLR3 anti-human CD69 (diluted 1:4). Negative controls consisted of slides incubated with an isotype-matched (IgG1) mouse monoclonal antibody instead of the primary antibody. Immunoreactivity to MBP, ECP, CD69 and IgG1 was detected by incubating slides with rabbit anti-mouse Ig (diluted 1:20) for 30 min. After another washing step, slides were incubated with APAAP (diluted 1:40) for 30 min. Positive cells stained red after development with Fast Red (Sigma). At least 200 cells were counted per slide and the proportion with positive staining expressed as a percentage.

Statistical analysis

All data are presented as mean $\pm$ SEM and where n is given this represents the number of experiments. Statistical analysis was performed with the unpaired 2-tailed Student t -test, and a P -value of < 0.05 was considered significant.

Results

The number of cells in culture and the time course of morphological features of cells belonging to the eosinophil lineage were monitored from day 0 to day 28. Haematoxylin-eosin (haema-'gurr' solution) staining of cytopsin preparations of freshly purified CD34⁺ cells demonstrated that they lacked eosinophilic features (Fig. 1a). By day 7 a proportion of the cultured cells began to exhibit evidence of granularity and the distinctive eosinophil bi-lobed nucleus was apparent by day 14. Both granules and a bi-lobed nucleus were present at day 21 with further culture to day 28 resulting in cells resembling mature eosinophils (Fig. 1a). Cell viability (as determined by trypan blue staining) was always $> 95\%$ throughout the culture period. As shown in Fig. 1(b) the total number of cells in culture at day 0 increased 9.75 ± 0.85 -fold by day 3, 14.01 ± 0.92 -fold by day 7, 57 ± 6.31 -fold by day 14, and 207 ± 19.53 -fold by day 28 of culture. The percentage of cells displaying eosinophil-like features rose to a maximum of 85% by day 28 of culture as judged by microscopic analysis (Fig. 1c).

We next examined the expression and storage of the granule cationic proteins MBP and ECP together with the eosinophil activation marker CD69 in cultured eosinophils collected at the indicated time-points. Cytopsin slides were prepared from CE after 3, 7, 14, 21 and 28 days of culture and single-labelled with BMK-13 (MBP), EG2 (ECP) or MLR3 (CD69) together with suitable isotype-matched control antibodies. Positive cells were identified by presence of a red substrate. A representative experiment of immunostaining for MBP and ECP is shown in Fig. 2. Positive staining for MBP and ECP could be detected in

a portion of cells at 3 and 7 days of culture (red regions in Figs 2g, h, k and l), respectively, when most of the cells were mononuclear in appearance with a large nucleus. After 14 days of culture large numbers of eosinophils exhibited a bi-lobed nucleus and also exhibited positive immunostaining for MBP (Fig. 2o) and ECP (Fig. 2p). After 21 days of culture both MBP and ECP were well expressed with little difference in the appearance of the cells when culture was continued to day 28. Control CE incubated with isotype negative control mAb were counterstained with Kimura. CE exhibited positive green staining from day 14 and this was associated with the developing granules. At 7 days, CE were weakly positive for CD69 (Fig. 2m) with strong expression of CD69 (Fig. 2q) observed after 14 days of culture and this remained stable up to day 28. CD69 expression remained intracellular with no evidence of membrane expression. CE expression of MBP, ECP and CD69 were also compared with normal density eosinophils. Overnight incubation of normal density eosinophils in GM-CSF (10^{-11} M) resulted in the expression of CD69 (Fig. 2e). No staining was apparent with appropriate isotype negative control mAb at any culture time-point (Fig. 2(a for PBE and f, j, n, r and v for CE at days 3, 7, 14, 21 and 28, respectively). Figure 3 demonstrates that developing eosinophils expressed MBP and ECP as early as after 3 days of culture and this increased to a maximum by day 28 of culture.

We next performed immunostaining and flow cytometry on developing eosinophils to assess membrane phenotypic changes in these cells as they mature in culture. Developing phenotype was compared with freshly isolated resting PBE. To enable a valid comparison using the full panel of mAb for membrane receptors, day 14 was chosen as the first time-point to ensure sufficient numbers of cells were available for these experiments. PBE and CE were compared for expression of CD45, CD45RA, CD45RB, CD45RO, CD11b, CD18, FAS, and CD69 by immunostaining and flow cytometric analysis. Table 1 shows the mean expression levels of these surface antigens in terms of specific mean fluorescence from three to four independent preparations of CE (28 days in culture) and PBE. In CE, these receptors were expressed after 14 days of culture and the mean fluorescence was increased with further culture to days 21 and 28 (Fig. 4). For the majority of receptors examined expression by CE increased over the culture period and was comparable to PBE. However, compared with PBE at 28 days CE expressed significantly higher amounts of CD69 ($P < 0.05$), the common β_2 -chain (CD18) ($P < 0.04$) and somewhat lower levels of CD11b ($P < 0.04$). There were no significant differences observed in expression of different CD45 clones or CD95 between the CE and PBE.

CE and PBE were also compared for expression of cytokine receptors (IL-5R α , IL-9R α) and chemokine receptors (CCR1, CXCR4, CCR3) over the same time-points (Table 2). Figure 5 is a representative flow cytometry experiment demonstrating that CE express IL-5R α , IL-9R α , CCR1, CCR3 after 14, 21 and 28 days of culture. Both IL-5R α and IL-9R α expression increased from day 14 through to day 28 of culture and we observed a greater variation in the range of specific mean fluorescence for IL-5R α (57.03 ± 2.24 , $P < 0.05$) and IL-9R α (34.47 ± 0.91 , $P < 0.05$) at 28 days compared with PBE. Interestingly, surface expression of both IL-5R α and IL-9R α were observed after 7 days of culture (data not shown) and by day 28 expression levels of both receptors were far greater on CE

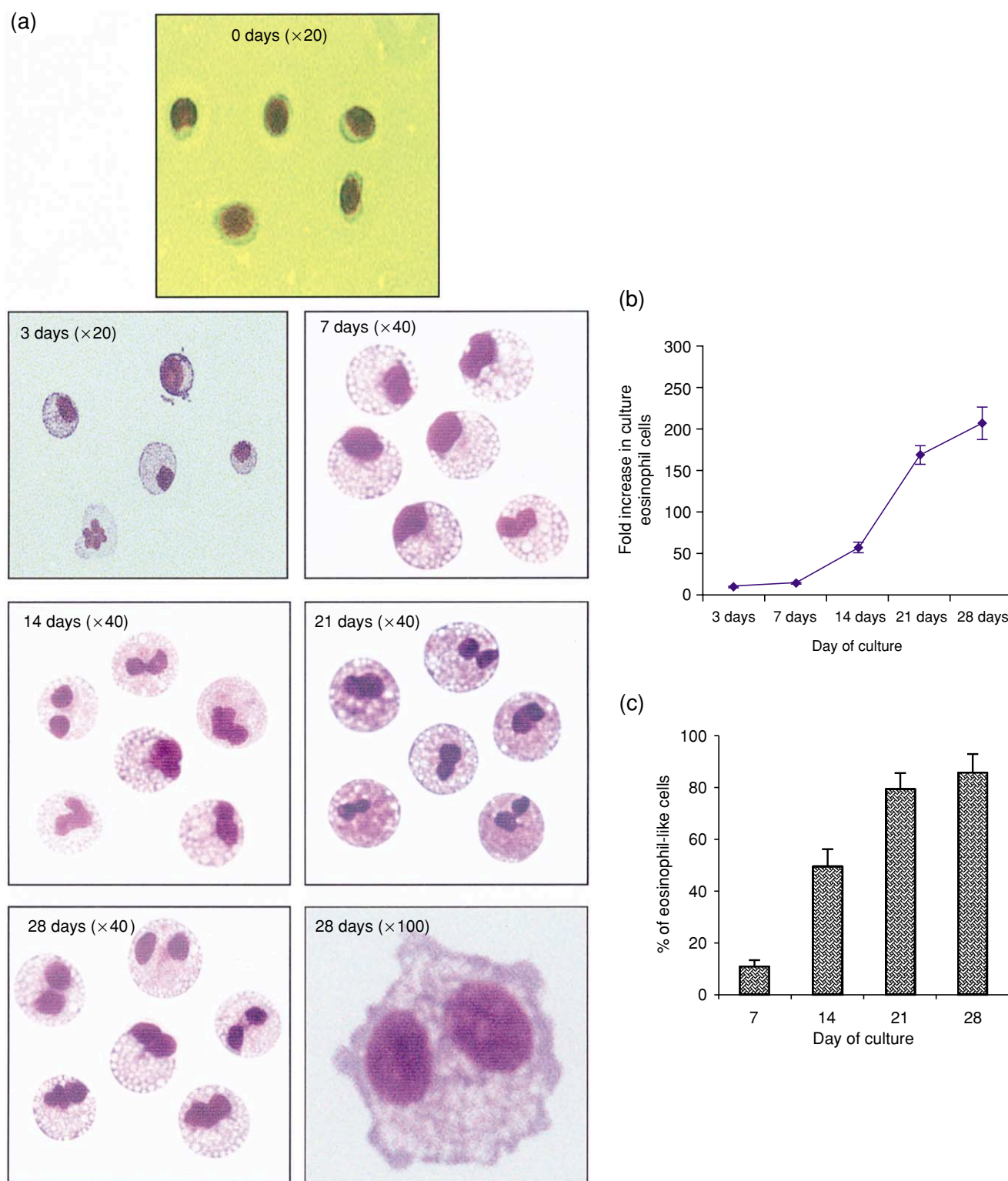


Fig. 1. Morphological characterization and expansion of $CD34^+$ cells undergoing eosinophilic differentiation. (a) Isolated $CD34^+$ stem cells from peripheral blood were cultured in the presence of FLT3-ligand (100 ng/mL), SCF (100 ng/mL), IL-3 (25 ng/mL), GM-CSF (10 ng/mL), and IL-5 (10 ng/mL) for eosinophil differentiation (see Materials and methods for details). At the indicated times, cytospin preparations were stained with haema-gurr[®] solution to identify eosinophil precursors (original magnification $\times 20$, $\times 40$, and $\times 100$). Representative photographs have been enhanced with Adobe PhotoShop. On indicated days, cell numbers (b) were counted and percentage of eosinophil-like cells identified (c). Results in (b) and (c) are from the mean $\pm$ SEM of three experiments.

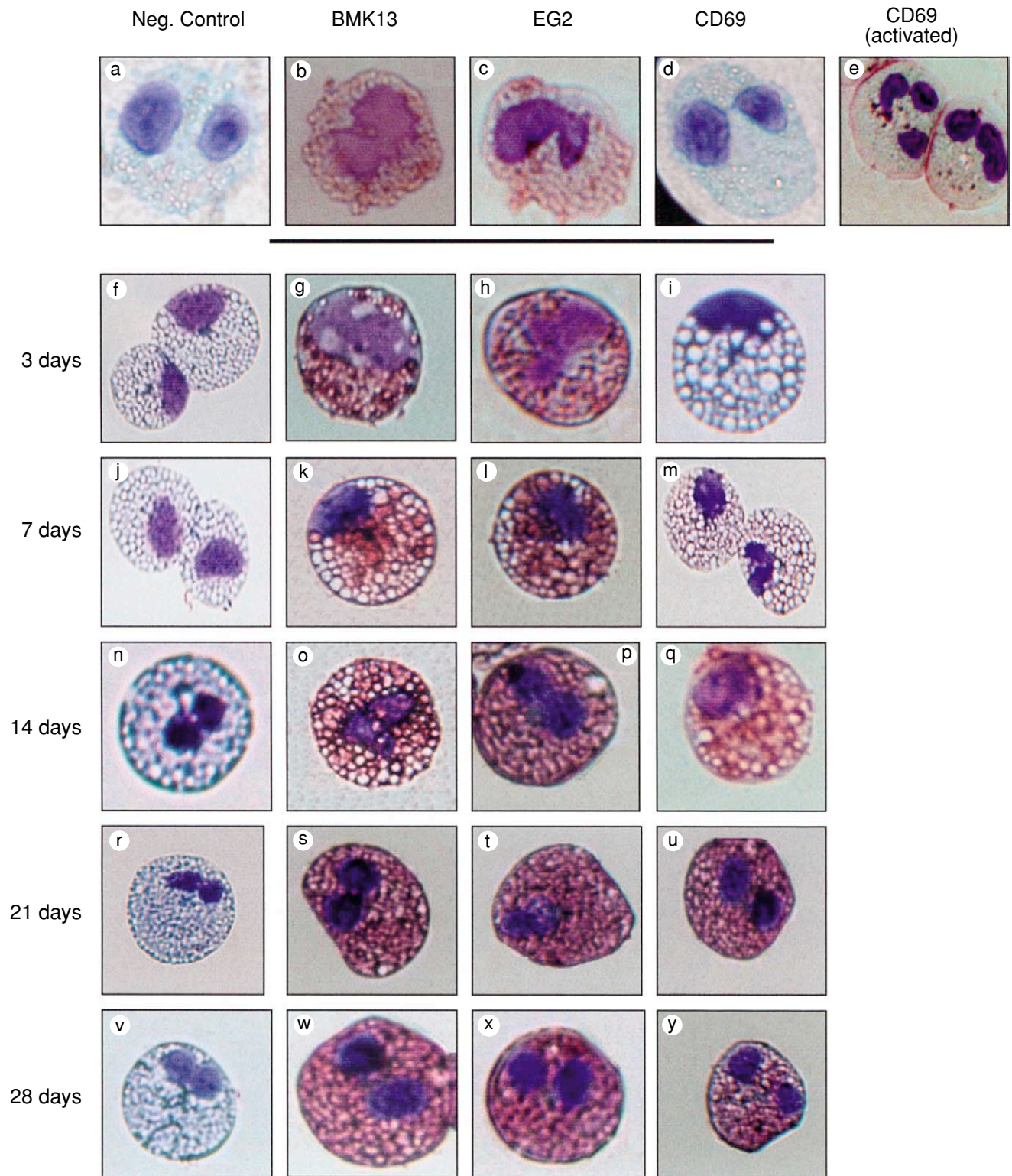


Fig. 2. Representative light photomicrographs (original magnification $\times 100$) showing alkaline phosphatase anti-alkaline phosphatase (APAAP) immunostaining of normal density eosinophils and developing eosinophils with antibodies for MBP, ECP, CD69⁺, and isotype negative control (IgG1). Positive immunostaining (red) of developing eosinophils corresponded to MBP, EG2, and CD69⁺ at days 3 (g, h, i), 7 (k, l, m), 14 (o, p, q), 21 (s, t, u) and 28 (w, x, y) of culture, respectively. Normal density eosinophils were labelled with APAAP (red) demonstrating MBP, EG2, and CD69⁺ (b, c, d), respectively. APAAP staining (red) corresponded to CD69⁺ immunoreactivity of activated normal density eosinophils (e). Isotype negative control of normal density eosinophils and developing eosinophils (days 3, 7, 14, 21 and 28 of culture) for MBP, EG2, and CD69⁺ are shown in (a), (f), (j), (n), (r) and (v), respectively. All cell nuclei stain dark blue with Kimura.

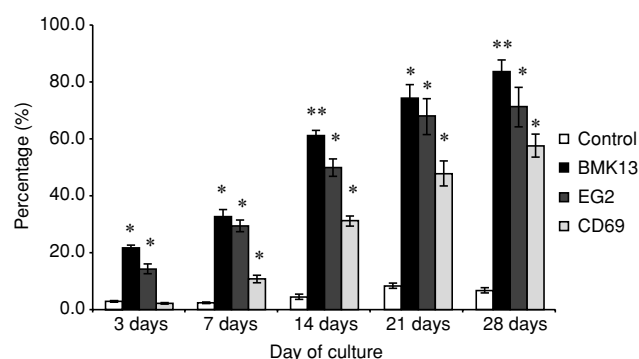


Fig. 3. The proportion of cultured eosinophils with positive immunostaining for MBP, ECP, CD69 after various periods of time in culture. Each bar represents the mean ($\pm$ SEM) of three independent experiments: * $P < 0.001$, ** $P < 0.0001$ compared with control.

Table 1. Membrane receptor expression by CE and PBE

Normal density eosinophils (PBE)		Cultured eosinophils (CE)		
		14 days	21 days	28 days
IgG1	7.6 $\pm$ 0.4	7.7 $\pm$ 0.4	5.9 $\pm$ 0.3	6.9 $\pm$ 0.5
IgG2a	6.2 $\pm$ 0.3	8.5 $\pm$ 0.2	7.1 $\pm$ 0.4	7.6 $\pm$ 0.3
CD45 (Bra55)	155.6 $\pm$ 11.5	31.7 $\pm$ 2.2	98.4 $\pm$ 1.5	130.7 $\pm$ 3.2
CD45RA	12.7 $\pm$ 1.1	10.2 $\pm$ 1.1	11.7 $\pm$ 1.3	11.4 $\pm$ 0.8
CD45RB	17.4 $\pm$ 1.5	11.6 $\pm$ 0.8	16.5 $\pm$ 0.9	17.0 $\pm$ 0.3
CD45RO	170.4 $\pm$ 9.3	40.2 $\pm$ 2.9	125.8 $\pm$ 5.3	149.0 $\pm$ 3.2
FAS (CD95)	14.2 $\pm$ 1.6	9.1 $\pm$ 0.4	11.3 $\pm$ 1.1	12.6 $\pm$ 1.3
CD69	8.5 $\pm$ 0.8	12.5 $\pm$ 1.0	17.6 $\pm$ 1.9	19.1 $\pm$ 1.6†
CD18	33.7 $\pm$ 7.4	31.0 $\pm$ 1.8	76.3 $\pm$ 4.2	96.3 $\pm$ 3.1*
CD11b	143.1 $\pm$ 9.2	26.5 $\pm$ 2.1	58.9 $\pm$ 3.4	89.4 $\pm$ 4.2*

*mAbs receptor expression (specific mean fluorescence) presented as the mean $\pm$ SEM ($n = 3$ for normal-density eosinophils, $n = 4$ for cultured eosinophils) compared with PBE: * $P < 0.04$; † $P < 0.05$.

Table 2. Chemokine and cytokine receptor expression by CE and PBE

Normal density eosinophils (PBE)		Cultured eosinophils (CE)		
		14 days	21 days	28 days
IgG1	5.0 $\pm$ 0.4	7.7 $\pm$ 0.4	5.9 $\pm$ 0.3	6.9 $\pm$ 0.5
IgG2a	5.2 $\pm$ 0.1	8.5 $\pm$ 0.4	7.1 $\pm$ 0.4	7.6 $\pm$ 0.3
Rat IgG2A	8.8 $\pm$ 0.6	7.7 $\pm$ 0.9	8.9 $\pm$ 0.4	10.0 $\pm$ 0.7
IgG(R)	5.5 $\pm$ 0.6	7.9 $\pm$ 0.2	7.5 $\pm$ 1.1	5.7 $\pm$ 0.4
IL-5R α	9.1 $\pm$ 0.4	22.0 $\pm$ 0.6	45.8 $\pm$ 1.0	57.0 $\pm$ 2.2†
IL-9R α	7.9 $\pm$ 0.1	15.2 $\pm$ 0.4	32.5 $\pm$ 1.1	34.5 $\pm$ 0.9†
CCR1	17.7 $\pm$ 0.8	10.4 $\pm$ 1.0	17.8 $\pm$ 2.2	19.4 $\pm$ 1.7*
CCR3	25.6 $\pm$ 1.5	21.0 $\pm$ 0.6	24.7 $\pm$ 1.7	24.8 $\pm$ 3.3*
CXCR4	6.2 $\pm$ 0.2	7.2 $\pm$ 0.4	8.9 $\pm$ 1.0	7.2 $\pm$ 0.1

*Chemokines and cytokines receptor expression (specific mean fluorescence) presented as the mean $\pm$ SEM ($n = 3$ for normal-density eosinophils, $n = 4$ for cultured eosinophils) compared with PBE: † $P < 0.05$.

compared with PBE. Like PBE, CE did not express measurable levels of CXCR4, and the reactivity of CE and PBE with mAb to CCR3 was virtually identical. No significant difference was demonstrated in expression of CCR1 between the CE and PBE.

Discussion

We used a combination of SCF, FLT3-ligand, IL-3, GM-CSF and IL-5 to induce maturation and differentiation of peripheral blood-derived CD34⁺ cells into eosinophils in long-term liquid culture in order to study the kinetics of expression and storage of granule cationic proteins together with examining a number of membrane receptors important in the function of human eosinophils. Under our culture conditions peripheral blood progenitors proliferated 14-fold by day 7 of liquid culture, with increased cell numbers seen by days 14–28 (77- to 207-fold). During culture the morphology and size of the cells changed dramatically as their maturation towards eosinophils progressed. Although at 7 and 14 days the cultures were heterogeneous, eosinophilic cells were clearly observed in increasing numbers.

In the present study we investigated the expression and storage pattern of MBP and ECP in maturing eosinophils. Other studies [19] have shown that both proteins are present in 16 day cultures of CE derived from cord blood mononuclear cells. Here we demonstrate that immunoreactivity for ECP and MBP can be seen as early as day 3 of culture in a minority of CE with increasing numbers becoming positive for BMK-13 and EG2 immunostaining from day 7 to day 28 of culture. Interestingly, an earlier study by Shalit et al. [20], demonstrated that peripheral blood CD34⁺ cells express messenger RNA for MBP and ECP after 3 days of culture and this stabilized between days 7 and 28. This finding is in agreement with our observation that the translated proteins for MBP and ECP were detectable early in CE development. After 3 and 7 days of culture, granule cationic proteins examined in this study appeared to be associated with round granule-like structures, few of which showed immunoreactivity for MBP and ECP, a finding which may be due to differential mediator storage pattern between immature granules. At later stages of culture (days 14, 21 and 28) eosinophils showed strong immunoreactivity, with more condensed and intense localization sites for both granule cationic proteins. We also examined the expression of CD69, a membrane receptor expressed by activated PBE [21]. To our knowledge, this is the first study to comprehensively examine CD69 expression by CE as they mature in culture. Weak CD69 expression was detected at day 7 with strong expression evident at days 14, 21 and 28 of culture. Overnight incubation of PBE in GM-CSF resulted in high levels of expression of CD69 on the periphery of PBE in contrast to CD69 expression in CE that was observed throughout the cell. Although we did not examine CD69 expression by CE maintained in the absence of the cytokines used in their differentiation, Ochiai et al. [22] demonstrated that at day 14 of culture, CE constitutively expressed CD69, expression of which was significantly enhanced by IFN- γ after 24 h of incubation.

This study also compared CE with normal-density human PBE in terms of surface receptor expression. While CE possessed many phenotypic features described for human peripheral blood eosinophils, a number of important differences were apparent. We have previously shown that CD45RO was the most predominant isoform of CD45 expressed by PBE followed by modest to weak expression of CD45RB and relatively low levels of CD45RA [17]. Comparison of CE membrane receptor expression with PBE showed somewhat lower but non-significant differences in the expression of pan-CD45 and the

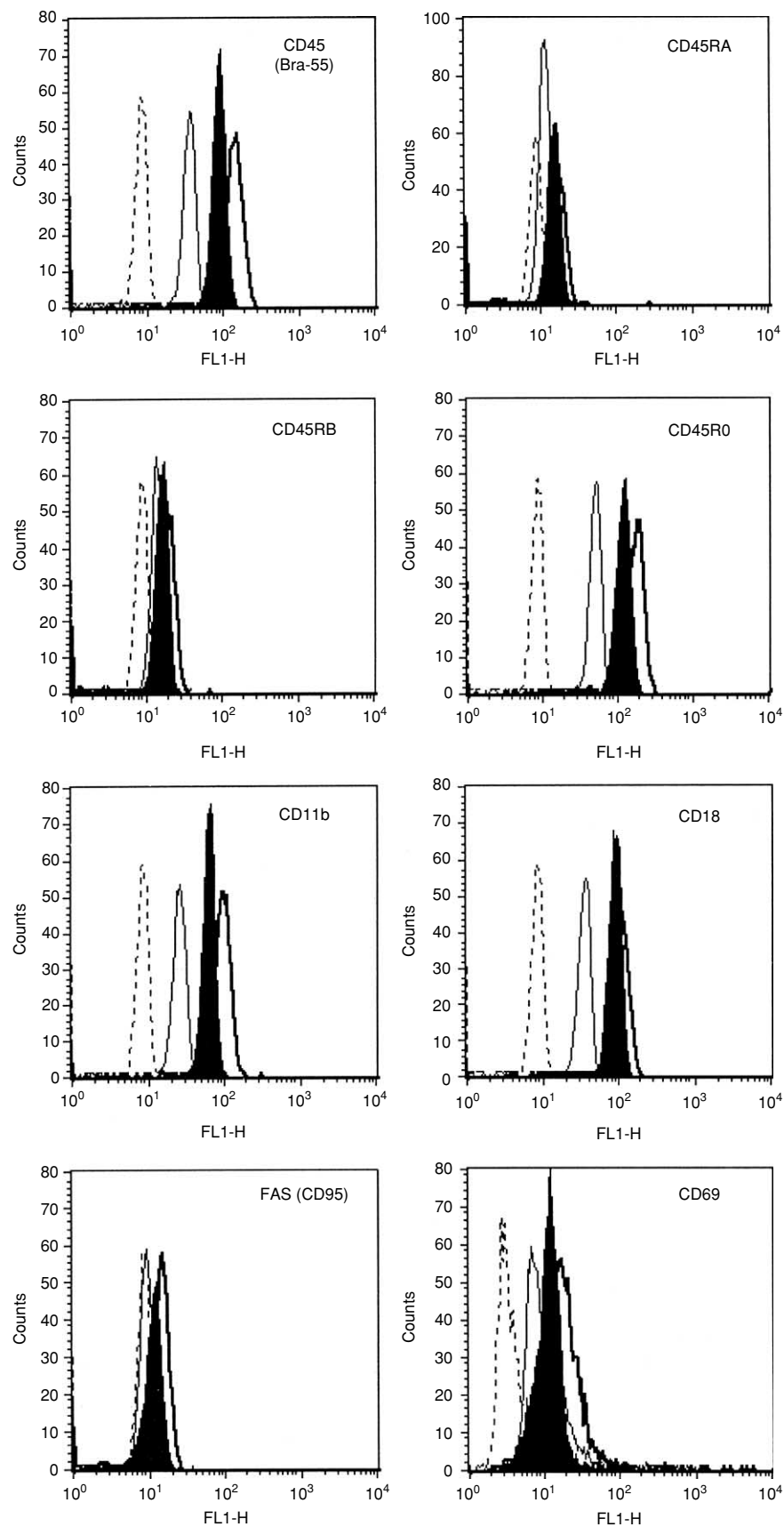


Fig. 4. Representative experiment of expression data from flow cytometric analysis of cultured eosinophils immunostained with mAb specific for pan-CD45, CD45RA, CD45RB, CD45RO, CD11b, CD18, CD95 and CD69. In each histogram the dotted line represents the isotype-matched negative control. The solid line, filled histogram, and the heavy solid line denote immunostaining with the indicated primary antibody of CE after 14, 21 and 28 days in culture, respectively.

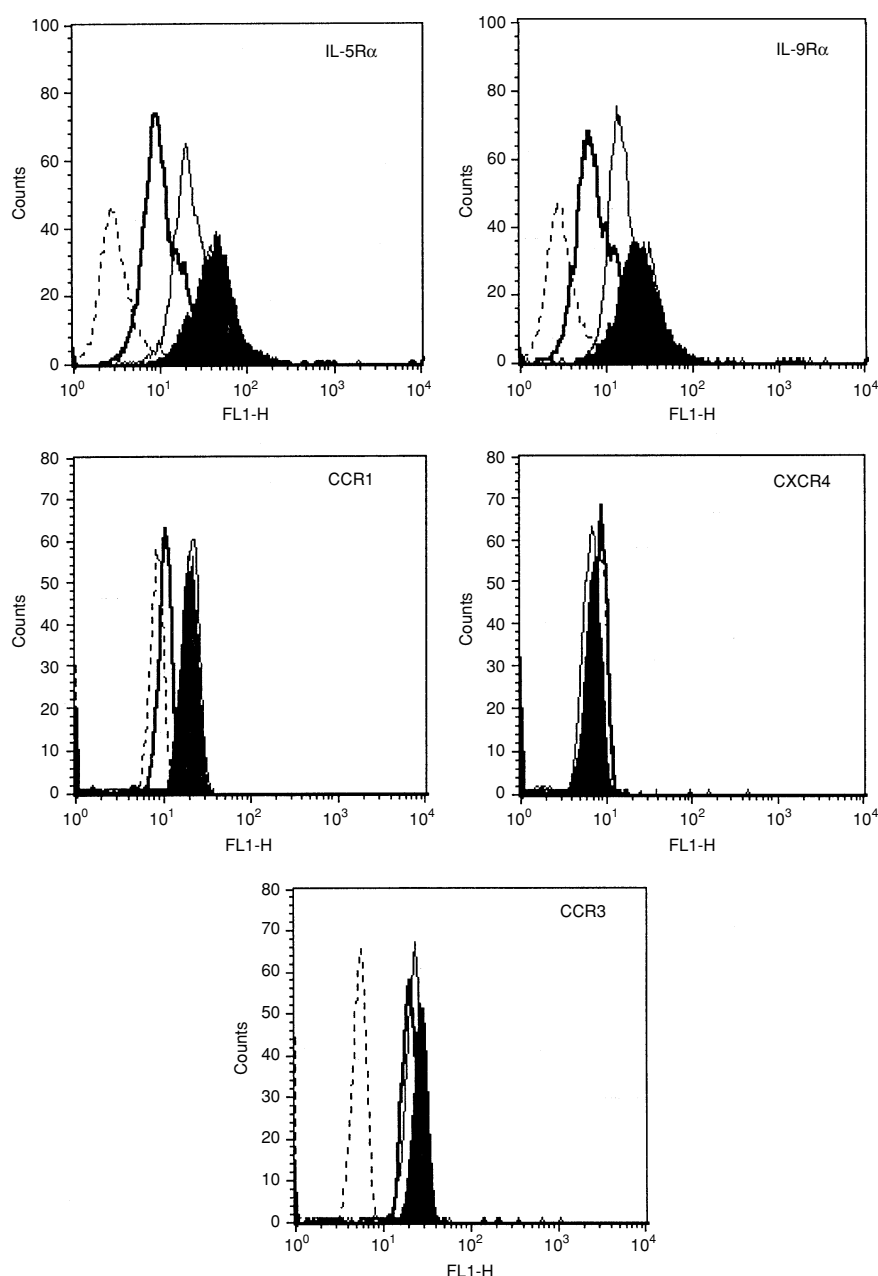


Fig. 5. Representative flow cytometric experiment illustrating expression of cytokines receptors for IL-5R α and IL-9R α and the chemokine receptors CCR1, CCR3 and CXCR4 by cultured eosinophils. In each histogram, the dotted line represents the isotype-matched negative control, the heavy solid line, solid line and the filled histogram denote immunostaining of CE with the indicated primary antibody after 14, 21 and 28 days in culture, respectively.

isoforms CD45RA, CD45RB and CD45RO. Higher expression CD18 by CE was observed while that of CD11b appeared to be lower than that of PBE. To date, most studies of the expression of eosinophil chemokine receptors have used PBE. Our observations suggest that CE also express CCR3 and CCR1 but not CXCR4 after selected culture time-points and this was comparable to that of PBE. One study has demonstrated that CCR1 and CCR3 are markers of IL-5-dependent late eosinophil differentiation from HL-60 cells [23]. IL-5 plays a pivotal role in promoting eosinophil accumulation with selective effects on eosinophil production, maturation and activation [1]. These actions are mediated via a membrane-bound IL-5R α that is composed of a ligand-specific α -subunit chain and a common α -chain. Our results demonstrate that both IL-5R α and IL-9R α are expressed by CE early in their development (days 7, 14),

findings that in most respects are in agreement with those of Gounni et al. [24]. Furthermore, the expression of IL-5R α and IL-9R α showed significantly greater variation in the range of mean fluorescence compared with PBE. Although IL-5R expression is often thought to be restricted to mature eosinophils and basophils and to late lineage-committed CD34⁻ CD33⁺ cells [25], the more recent demonstration that IL-5R α is also present on primitive CD34⁺ progenitor cells [26] suggests that expression of IL-5R may be a critical and early haemopoietic event that can determine commitment to the eosinophil-basophil lineage. Furthermore, IL-5 (but not IL-3 or GM-CSF) is able to regulate differential splicing of membrane and soluble isoforms of IL-5R α during *in vitro* differentiation of normal human progenitor cells [27] and IL-9 can enhance IL-5R expression [23]. Thus, there is a complex relationship

between the expression of IL-5R α and IL-9R α in developing eosinophils that may in turn have functional consequences for recruited eosinophils during an inflammatory reaction.

In conclusion, while CE share a number of membrane markers with PBE there are quantitative differences that may be a consequence of their immaturity and/or the influence of the cytokines used in their culture. Moreover, we cannot exclude the possibility that our results may have been different had we used CD34⁺ cells derived from bone marrow samples rather than peripheral blood. Our observations suggest that maturing eosinophils generated from peripheral blood-derived CD34⁺ cells express both MBP and ECP at early stages of their development. They may therefore provide important tools for further investigation of the role of the eosinophil in asthmatic and allergic inflammation.

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