Identification of the human eosinophil lineage-committed progenitor: revision of phenotypic definition of the human common myeloid progenitor

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To establish effective therapeutic strategies for eosinophil-related disorders, it is critical to understand the developmental pathway of human eosinophils. In mouse hematopoiesis, eosinophils originate from the eosinophil lineage-committed progenitor (EoP) that has been purified downstream of the granulocyte/macrophage progenitor (GMP). We show that the EoP is also isolatable in human adult bone marrow. The previously defined human common myeloid progenitor (hCMP) population (Manz, M.G., T. Miyamoto, K. Akashi, and I.L. Weissman. 2002. *Proc. Natl. Acad. Sci. USA.* 99:11872–11877) was composed of the interleukin 5 receptor α chain$^+$ (IL-5Rα$^+$) and IL-5Rα$^-$ fractions, and the former was the hEoP. The IL-5Rα$^+$CD34$^+$CD38$^+$IL-3Rα$^+$CD45RA$^-$ hEoPs gave rise exclusively to pure eosinophil colonies but never differentiated into basophils or neutrophils. The IL-5Rα$^-$ hCMP generated the hEoP together with the hGMP or the human megakaryocyte/erythrocyte progenitor (hMEP), whereas hGMPs or hMEPs never differentiated into eosinophils. Importantly, the number of hEoPs increased up to 20% of the conventional hCMP population in the bone marrow of patients with eosinophilia, suggesting that the hEoP stage is involved in eosinophil differentiation and expansion in vivo. Accordingly, the phenotypic definition of hCMP should be revised to exclude the hEoP; an "IL-5Rα–negative" criterion should be added to define more homogenous hCMP. The newly identified hEoP is a powerful tool in studying pathogenesis of eosinophilia and could be a therapeutic target for a variety of eosinophil-related disorders.

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Abbreviations used: BaP, basophil lineage-committed progenitor; CCR3, CC chemokine receptor 3; CMP, common myeloid progenitor; EoP, eosinophil lineage-committed progenitor; Epo, erythropoietin; EPX, eosinophil peroxidase; FOG-1, friend of GATA-1; GMP, granulocyte/macrophage progenitor; HDC, histidine decarboxylase; HES, hypereosinophilic syndrome; HSC, hematopoietic stem cell; IL-5Rα, IL-5R α chain; MegE, megakaryocyte/erythroid; MEP, megakaryocyte/erythrocyte progenitor; MNC, mononuclear cell; MPO, myeloperoxidase; MPP, multipotent progenitor; PMN, polymorphonuclear cell; SCF, stem cell factor; Tpo, thrombopoietin.

Eosinophils mainly reside in the gastrointestinal mucosa and normally constitute only 1–5% of blood nucleated cells. Eosinophils play an important role in host defense against parasitic infections and are major effectors in a variety of allergic reactions (1, 2). Upon diverse stimuli, infiltrating eosinophils cause chronic inflammatory tissue damage by releasing a wide spectrum of proinflammatory mediators, including major basic protein, eosinophil cationic protein, and eosinophil-derived neurotoxin (2, 3).

Like other hematopoietic lineages, eosinophils originate from the hematopoietic stem cell (HSC). In mouse hematopoiesis, the eosinophil lineage-committed progenitor (EoP) (4) exists as a distinct population downstream of the granulocyte/macrophage progenitor (GMP) (5). The mEoP expressed the receptor for IL-5 that plays an important role in controlling eosinophil numbers (6–8). The mouse bipotent basophil/mast cell progenitor and the basophil

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lineage-committed progenitor (BaP) were also identified downstream of the mGMP (9), suggesting that the commitment of eosinophil and basophil/mast cell lineages independently occurs after the multipotent progenitor (MPP) has lost a megakaryocyte/erythroid (MegE) lineage potential. In contrast, in human hematopoiesis, cells possessing both basophil and eosinophil granules have been found in leukemia patients (10, 11), suggesting the close relationship between the basophil and the eosinophil lineages. The early works using methylcellulose colony assays of human bone marrow cells demonstrated that eosinophils were scattered preferentially within erythroid (12) or myeloid (13) colonies. These data raise the possibility that the eosinophil developmental pathway is considerably different between the human and mouse. However, such retrospective assessments of progenitor functions based on lineage readouts in colony assays does not necessarily reflect their full lineage potential because MPPs could decide lineage fates in a random manner, at least in vitro (14).

In this paper, we identified the EoP in human bone marrow. The previously defined human common myeloid progenitor (hCMP) (15) was divided into the IL-5R α chain$^+$ (IL-5Rα$^+$) and IL-5Rα$^-$ fractions, and the former was fully committed to the eosinophil lineage lacking the MegE, the neutrophil/monocyte, and the basophil/mast cell potentials. The hEoP was generated from the IL-5Rα$^-$ hCMP but not from the hGMP or the human megakaryocyte/erythrocyte progenitor (hMEP). Thus, the prospectively purified hEoP represents the initial stage of eosinophil development independent of the hGMP, the hMEP, the putative human basophil/mast cell progenitor, or the hBaP. Interestingly, the hEoP population expanded significantly in the bone marrow of patients with eosinophilia, suggesting that the hEoP might be a promising therapeutic target for eosinophil-related allergic and inflammatory disorders. These data led us to revise our original definition of the hCMP population by using "IL-5Rα-negative" as an additional phenotypic criterion.

RESULTS

Three granulocyte subclasses such as neutrophils, eosinophils, and basophils can be purified from human blood by FACS

Isolation of a lineage-committed progenitor population by FACS is highly dependent on lineage-specific surface markers. In mouse hematopoiesis, eosinophils but not basophils or neutrophils possessed a high level of IL-5Rα, and the expression of IL-5Rα was a key to isolate the mEoP (4). We first tested the expression of IL-5Rα on human blood leukocytes and found that both eosinophils and basophils expressed IL-5Rα at high levels. We further found that eosinophils and basophils independently resided within the polymorphonuclear cell (PMN) gate and the mononuclear cell (MNC) gate, respectively (Fig. 1 A). Thus, by combinatory analysis of the expression pattern of IL-5Rα and of the forward scatter versus the side scatter cytogram, three classes of human blood granulocytes became clearly visible: IL-5Rα$^-$ PMNs were neutrophils, IL-5Rα PMNs were eosinophils, and IL-5Rα MNCs were basophils (Fig. 1 A). The purity of sorted populations was >99% in all of these fractions.

Fig. 1 B shows the expression patterns of cell-surface molecules related to the eosinophil and/or the basophil/mast cell lineages. CC chemokine receptor 3 (CCR3), a high-affinity receptor for eotaxin that promotes the migration of eosinophils and basophils into the inflammatory tissue (16, 17), was expressed on both purified eosinophils and basophils. β7 integrin is an essential molecule for tissue-specific homing of precursors for mouse intestinal mast cells (18), and is expressed on mouse mast cells and basophils but not on eosinophils (9, 19). In human blood, however, not only basophils but also eosinophils expressed β7 integrin. CD203c, the ectonucleotide pyrophosphatase/phosphodiesterase 3, is reported to be a marker for human basophils, mast cells, and their precursors (20). In our hands, however, CD203c was expressed on not only basophils but also eosinophils, indicating that this molecule is not specific for the basophil/mast cell lineage. Thus, none of these markers appeared to be useful to differentiate human eosinophils from basophils on FACS, except for a high-affinity receptor for IgE α chain (FcεRIα) that was expressed only on human basophils (Fig. 1 B).

We further tested the gene expression of lineage-specific markers in each FACS-purified granulocyte subclass. The expression of myeloperoxidase (MPO), eosinophil peroxidase (EPX), and histidine decarboxylase (HDC) transcripts was evaluated. As shown in Fig. 1 C, human neutrophils exclusively possessed MPO but lacked the expression of EPX or HDC. Human eosinophils and basophils did not express MPO but possessed only EPX and HDC, respectively. Thus, in addition to the morphological analysis, the evaluation of these functional molecules should be useful to differentiate eosinophils from basophils and neutrophils.

Eosinophils develop from hHSCs and hCMPs but not from hGMPs or hMEPs

To delineate the developmental origin of human eosinophils, we tested the eosinophil lineage readout of myeloid progenitor populations in steady-state bone marrow. As shown in Fig. 2 A, the lineage-affiliated antigen (Lin$^-$) and CD34$^+$ human bone marrow MNC fraction was subdivided into the CD38$^-$ hHSC and the CD38$^+$ progenitor populations (21). The Lin$^-$CD34$^+$CD38$^+$ human progenitors were further fractionated into hCMP, hGMP, and hMEP populations according to the expression patterns of IL-3Rα and CD45RA, as we previously reported (15): hCMPs, hGMPs, and hMEPs were visualized as the IL-3Rα$^+$CD45RA$^-$, IL-3Rα$^+$CD45RA$^+$, and IL-3Rα$^-$CD45RA$^-$ populations, respectively (Fig. 2 A). In liquid cultures supplemented with a cytokine cocktail containing stem cell factor (SCF), IL-3, IL-5, GM-CSF, erythropoietin (Epo), and thrombopoietin (Tpo), hHSCs and hCMPs generated CCR3-expressing eosinophils/basophils as well as other myelomonocytic and MegE cells, whereas hGMPs and hMEPs gave rise mainly to neutrophils/monocytes/macrophages and MegE cells, respectively, without developing into the eosinophil lineage (Fig. 2 B). Basophils were also scattered

in the progeny of hHSCs, hCMPs, and hGMPs but not of hMEPs (unpublished data). In agreement with FACS and morphological analyses, progeny of hHSCs and hCMPs expressed both the eosinophil-affiliated EPX and the basophil-affiliated HDC transcripts in addition to the MPO transcript, whereas progeny of hGMPs or hMEPs did not express EPX

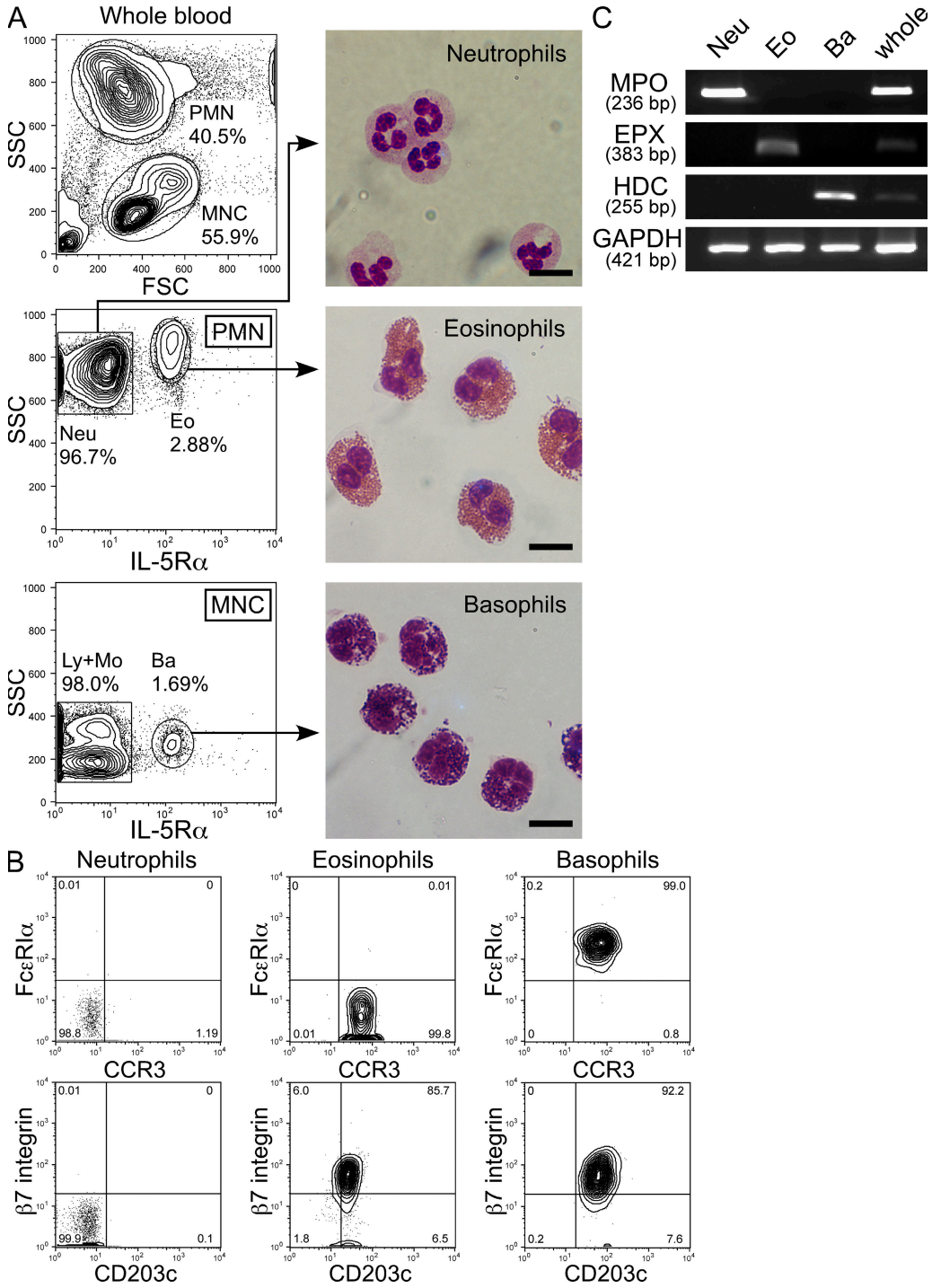

Figure 1. Purification of neutrophils, eosinophils, and basophils. (A) Sorting gates for three granulocyte subclasses. The representative FACS plots of normal blood leukocytes are shown. IL-5Rα$^-$ and IL-5Rα$^+$ fractions within the PMN gate were neutrophils (Neu) and eosinophils (Eo), respectively. Basophils (Ba) resided within the MNC gate and expressed IL-5Rα at a high level. The purity of each sorted population was >99%, as determined morphologically by cytospin preparations (May-Giemsa staining). Bars, 10 µm. (B) FACS analyses of cell-surface molecules that relate to the eosinophil and/or the basophil lineages on each granulocyte subclass (percentages are shown). (C) RT-PCR analyses of lineage-affiliated gene expression in FACS-purified granulocyte subclasses (whole, whole blood leukocytes). Data were reproducible in two independent analyses using different blood samples. FSC, forward scatter; SSC, side scatter.

(Fig. 2 C). The HDC transcript was also detected in progeny of hGMPs but not of hMEPs (Fig. 2 C). We further performed methylcellulose assays, where we picked up all single colonies and made cytospin preparations to morphologically define cell components. As shown in Fig. 2 D, ∼3% of hHSC- and ∼5% of hCMP-derived colonies contained eosinophils. hGMPs and hMEPs never generated eosinophil-containing colonies. More interestingly, ∼4% of hCMP colonies were composed only of eosinophils (Fig. 2 D), suggesting that a fraction of cells within the hCMP gate may be progenitors already restricted to the eosinophil lineage.

The IL-5Rα$^+$ fraction within the original hCMP is the hEoP

To separate the putative hEoP, we tested the expression of IL-5Rα in stem and progenitor populations. The expression of IL-5Rα was detectable only in the hCMP fraction: ∼10% of hCMPs expressed IL-5Rα (Fig. 3 A). The IL-5Rα$^+$ fraction of hCMPs also expressed very low levels of CCR3 and β7 integrin (not depicted) that were detected on blood mature eosinophils and basophils at high levels (Fig. 1 B). FcεRIα was specifically expressed on blood basophils (Fig. 1 B) but not detected in the hCMP population (not depicted). Strikingly, in methylcellulose assays, purified IL-5Rα$^+$ hCMPs gave rise only to pure eosinophil colonies (Fig. 3, B and C). In contrast, the IL-5Rα$^-$ fraction of hCMPs generated a variety of myeloid colonies including rare (∼2%) eosinophil-containing colonies (Fig. 3 B). In liquid cultures, the day 14 progeny of IL-5Rα$^+$ hCMPs significantly up-regulated CCR3 on their surface (Fig. 3 D). These cells possessed EPX but not MPO or HDC transcripts, whereas progeny of IL-5Rα$^-$ hCMPs

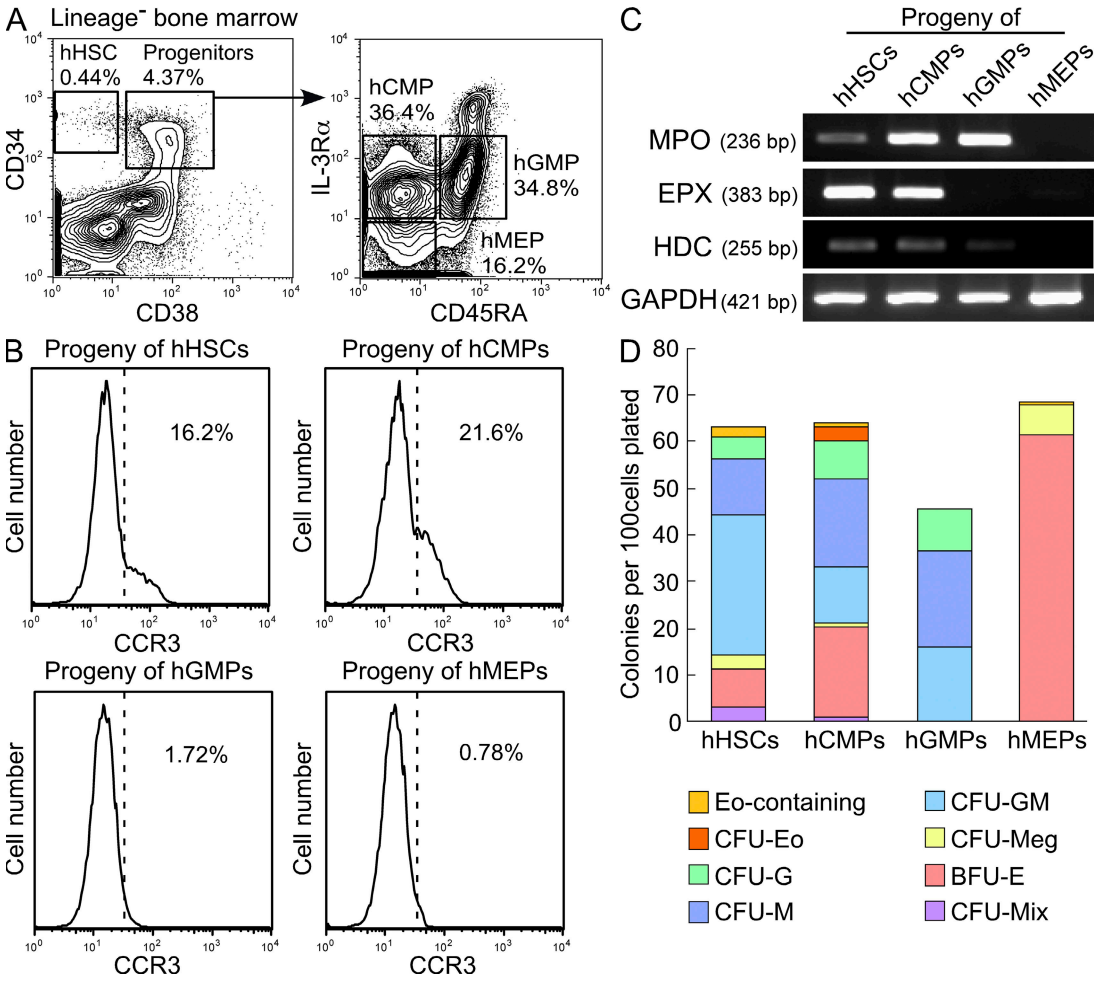

Figure 2. Eosinophils develop from hHSCs and hCMPs but not from hGMPs or hMEPs. (A) Sorting gates for hHSC and downstream myeloid progenitor populations. (B) The emergence of CCR3$^+$ eosinophils in cultures of FACS-purified progenitor populations. A cytokine cocktail used in this experiment contained SCF, IL-3, IL-5, GM-CSF, Epo, and Tpo. CCR3$^+$ eosinophils were detected in hHSC and hCMP cultures but not in hGMP or hMEP cultures. Dashed lines indicate cut-off lines of negative controls. (C) RT-PCR analyses of lineage-affiliated gene expression in the progeny of each progenitor population. The expression of eosinophil-specific EPX was detected in progeny of hHSCs and hCMPs but not of hGMPs or hMEPs. (D) The methylcellulose colony assays of FACS-purified progenitor populations. The eosinophil-containing (Eo-containing) colonies developed from hHSCs and hCMPs but not from hGMPs or hMEPs. Approximately 4% of hCMP-derived colonies were composed only of eosinophils (CFU-Eo). Data were reproducible in two independent experiments using different bone marrow samples.

expressed all of these molecules (Fig. 3 E), supporting the eosinophil lineage-restricted potential of the IL-5Rα$^+$ hCMP population. These observations collectively suggest that the IL-5Rα$^+$ fraction of original hCMP has committed to the eosinophil lineage. Thus, the hEoP was as isolatable as the IL-5Rα$^+$hCD34$^+$hCD38$^+$IL-3Rα$^+$hCD45RA$^-$ population in steady-state human bone marrow. These data also in turn show that the original hCMP (15) includes the hEoP contaminant, and that its phenotypic definition should be revised as IL-5Rα$^-$hCD34$^+$hCD38$^+$IL-3Rα$^+$hCD45RA$^-$.

hEoPs develop from hCMPs independent of hGMPs and hMEPs

The question was of the lineage relationship between hEoPs and other myeloid progenitors. We tried to track the emergence of hEoPs within the culture of IL-5Rα$^-$ hCMPs and other myeloid progenitors. The hCMPs gave rise to a minor fraction of progeny expressing IL-5Rα on day 5 (Fig. 4 A), together with hGMPs and hMEPs (not depicted). The IL-5Rα$^+$ progeny gave rise exclusively to pure eosinophil colonies (Fig. 4 B), and they possessed the surface phenotype identical to that of hEoPs (Fig. 4 C). Neither hGMPs nor hMEPs generated hEoPs at any time point during the culture (unpublished data). These data strongly suggest that the hEoPs develop from IL-5Rα$^-$ hCMPs but not from hGMPs.

Consistent with their capability of functional readouts, IL-5Rα and EPX transcripts were exclusively expressed in hEoPs, whereas other eosinophil-specific genes such as Charcot-Leyden crystal protein and major basic protein were

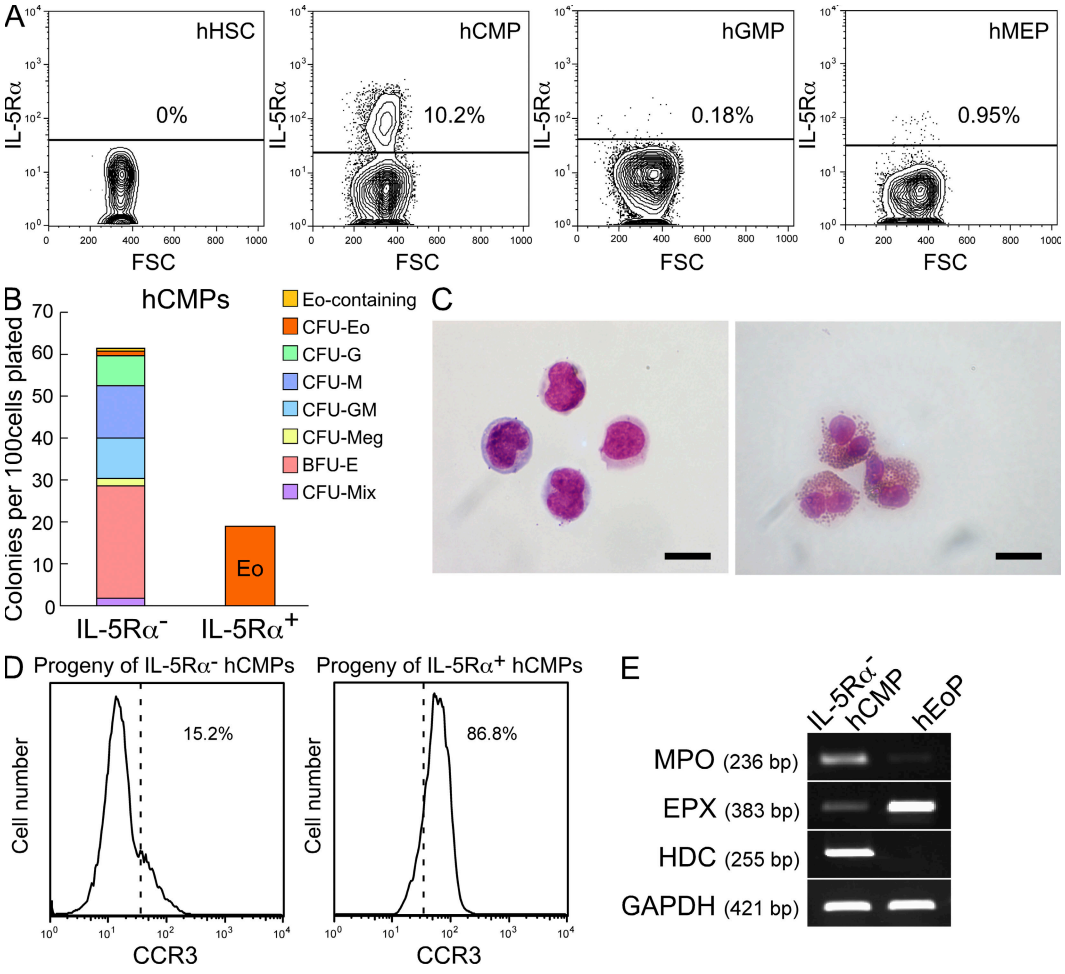

Figure 3. The IL-5Rα$^+$ fraction within the original hCMP represents the hEoP. (A) FACS analysis of IL-5Rα expression on myeloid progenitor populations. The representative FACS plots of normal bone marrow progenitors are shown. (B) The methylcellulose colony assays of IL-5Rα$^-$ and IL-5Rα$^+$ fractions within the hCMP. The cytokine cocktail used in this experiment was the same as in Fig. 2 B. All of the single colonies were picked up and mounted on cytospin preparations to define cell components. The IL-5Rα$^+$ fraction of hCMP only generated pure eosinophil colonies (CFU-Eo). (C) The morphologies of purified hEoPs and their progeny. hEoPs represented a blastic morphology with fine cytoplasmic granules (left), and their progeny were composed only of eosinophils (right; May-Giemsa staining). Bars, 10 µm. (D) FACS analysis of CCR3 expression on the progeny of IL-5Rα$^-$ hCMPs and hEoPs. Dashed lines indicate cut-off lines of negative controls. (E) RT-PCR analyses of lineage-affiliated gene expression in progeny of IL-5Rα$^-$ hCMPs and hEoPs. The progeny of hEoPs expressed a high level of EPX but never possessed MPO or HDC transcripts. Data were reproducible in two independent experiments using different bone marrow samples.

expressed at low levels in hCMPs but were up-regulated at the hEoP stage (Fig. 5 A). hMEPs and hGMPs never expressed any of these eosinophil-related molecules (Fig. 5 A).

Expression profiles of lineage-instructive transcription factors in hEoPs

In mouse hematopoiesis, GATA-1 and GATA-2 play critical roles in the eosinophil lineage development. GATA-1–deficient mice lack eosinophils (22, 23), and the enforced expression of GATA-1 or GATA-2 instructed both mouse and human myeloid progenitors to develop into the eosinophil lineage (23–25). As shown in Fig. 5 B, hEoPs possessed approximately two- to threefold increased levels of GATA-1 and GATA-2 as compared with those in hCMPs, whereas hGMPs completely shut down these GATA factors. The expression level of GATA-1 in hEoPs was significantly lower than that of hMEPs, whereas the GATA-2 level in hEoPs was higher than that in hMEPs (Fig. 5 B). Friend of GATA-1 (FOG-1) is a critical cofactor of GATA family transcription factors and plays an indispensable role in the mouse MegE lineage development (26, 27), whereas it antagonizes the eosinophil development presumably interfering with eosinophil lineage-related functions of GATA factors (28). Consistent with these mouse data, hEoPs significantly down-regulated the FOG-1 expression, whereas hMEPs expressed FOG-1 at the highest level (Fig. 5 B). The distribution of these GATA family transcription factors in human myeloid progenitors was identical to that in mouse counterparts (29).

C/EBPα is required for the development of mGMPs as well as their progeny, including eosinophils (30). In our hands, in mouse hematopoiesis, the eosinophil lineage commitment can be activated by up-regulation of GATA-2 in the presence of C/EBPα (29). As shown in Fig. 5 B, the expression level of C/EBPα was highest in hGMPs but was diminished in hMEPs. Importantly, hCMPs, which are immediate precursors of hEoPs, possessed a significant amount of C/EBPα, and hEoPs maintained its expression with up-regulation of GATA-1 and GATA-2. PU.1 is indispensable for mouse granulocyte and lymphoid cell development (31, 32). The expression of PU.1 was detected in both hGMPs and hEoPs but was suppressed at the hMEP stage.

These data show that changes in expression patterns of lineage-instructive transcription factors in these myeloid progenitors are similar between human and mouse hematopoiesis (29). Therefore, the developmental machinery of eosinophil lineage might be well preserved between these species.

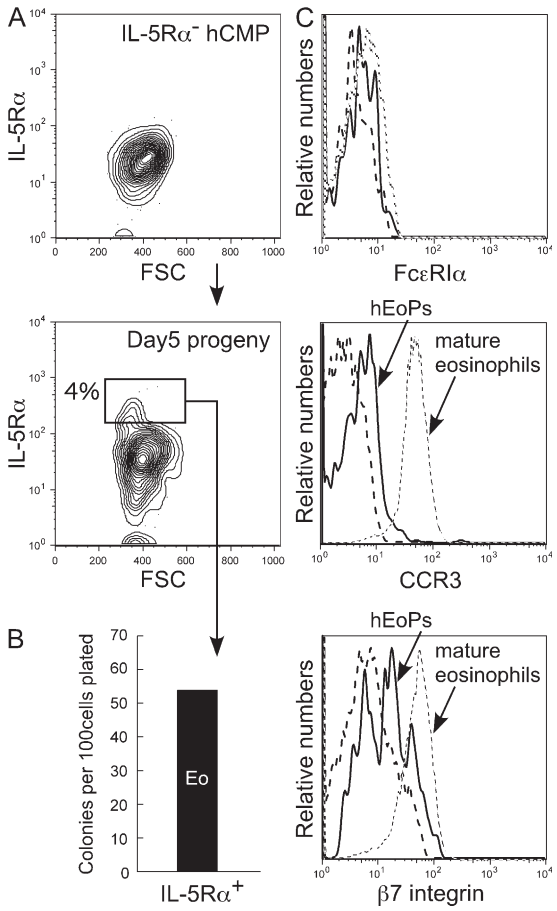

Figure 4. hEoPs develop from hCMPs independent of hGMPs and hMEPs. (A). Development of IL-5Rα⁺ hEoPs from IL-5Rα⁻ hCMPs in liquid culture. (B) The IL-5Rα–expressing cells purified from the primary hCMP culture formed only eosinophil colonies in the presence of the multiple cytokines used in Fig. 2 B. All single colonies were picked up and were mounted on cytospin preparations to define cell components. (C) FACS analyses of cell-surface molecules on the hEoP (continuous line, hEoPs; dashed line, blood eosinophils; bold dashed line, control antibodies). Data were reproducible in two independent experiments using different bone marrow samples. FSC, forward scatter.

The hEoP population significantly expanded in patients with eosinophilia

To evaluate whether the hEoP contributes toward eosinophil production in vivo, we enumerated the number of hEoPs in the bone marrow of eosinophilia patients. Patients' characteristics are summarized in Table I. In normal bone marrow samples, the hEoP population accounted for only 0.033% of bone marrow MNCs or 2.38% of Lin^-CD34^+ cells, respectively (Table I and Fig. 6 A). We analyzed 15 patients with eosinophilia: 5 patients with hypereosinophilic syndrome (HES), 2 patients with T cell malignancies, 1 chronic myelogenous leukemia patient in accelerated phase, 2 patients with Churg-Strauss syndrome, and 5 eosinophilia patients with unknown etiology. The *FIP1L1/PDGFRα* fusion gene was not detected in any of these patients. The blood eosinophil count was 13,799 μl, and eosinophil lineage cells accounted for 38.4% of whole bone marrow cells on average (Table I). The bone marrow hEoP in eosinophilia patients consisted of 0.125% of MNCs or 7.44% of Lin^-CD34^+ cells, respectively (Table I and Fig. 6 A). Thus, eosinophilia patients possessed three- to fourfold higher numbers of hEoPs compared with normal controls ($P < 0.01$; Fig. 6 A). These data suggest that the hEoP stage is actively involved in generation of eosinophils in patients with eosinophilia.

DISCUSSION

In the present study, we identified the hEoP in steady-state human bone marrow. This population gave rise to eosinophils but not neutrophils or basophils in vitro. It possessed the eosinophil-specific EPX transcript but did not have the basophil-specific HDC or the neutrophil-specific MPO transcripts. Furthermore, the hEoP significantly expanded in number in the bone marrow of patients with eosinophilia. Thus, like mouse hematopoiesis, the developmental pathway initiating from the distinct hEoP stage exists in human bone marrow, and the hEoP should actively contribute toward generation of eosinophils in vivo.

We have also developed a method to ultimately purify mature neutrophils, eosinophils, and basophils from human

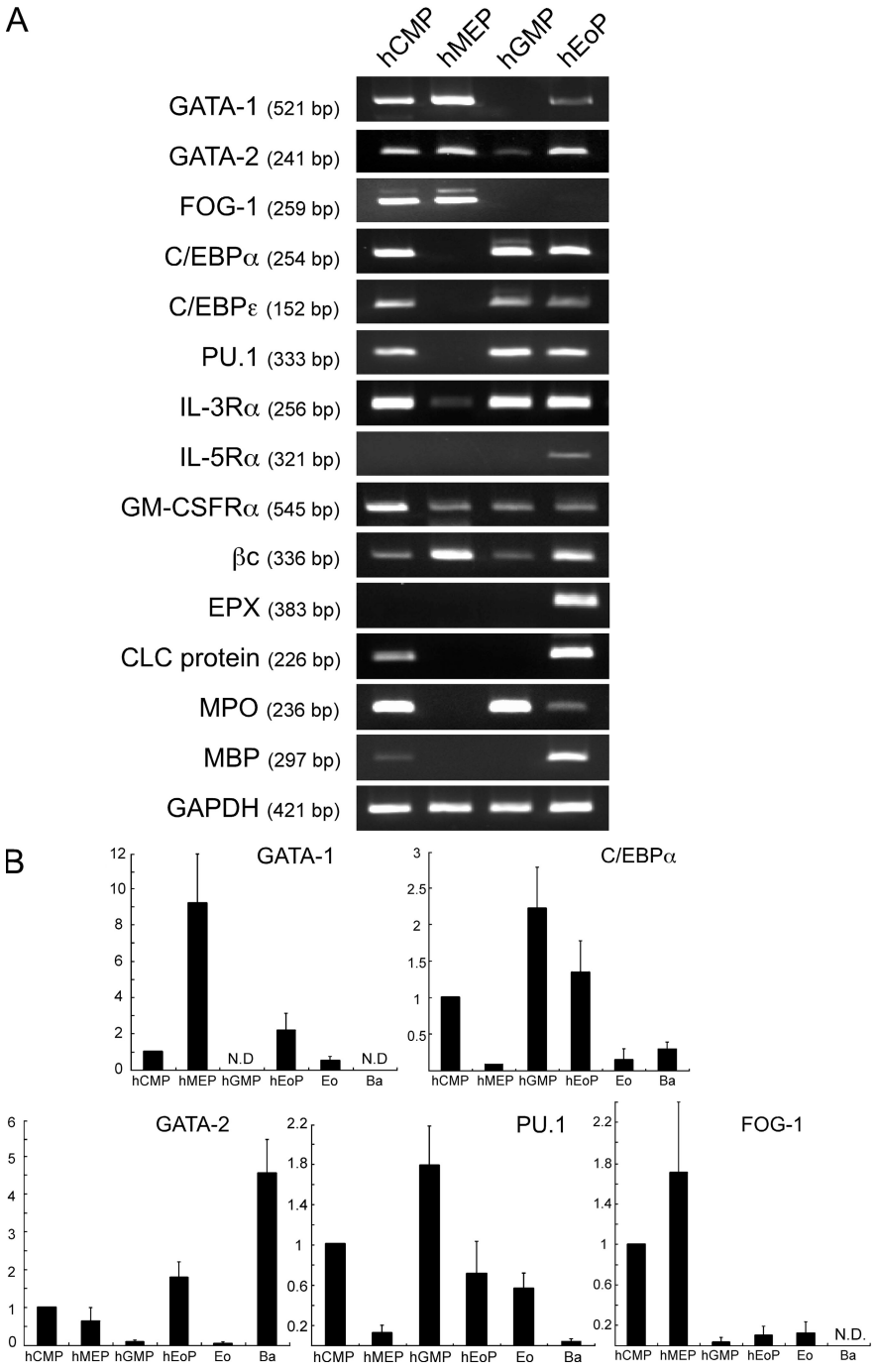

Figure 5. The comparison of lineage-affiliated gene expression between hEoPs and other myeloid progenitors. (A) RT-PCR analyses of lineage-affiliated genes. (B) Quantitative real-time PCR analyses of lineage-instructive transcription factors (Ba, blood basophils; Eo, blood eosinophils). Data were reproducible in three independent analyses using different RNA samples. Data represent means ± SEM.

blood (Fig. 1 A). In contrast to mouse hematopoiesis, where IL-5Rα is expressed in the eosinophil but not the basophil lineage, mature human basophils expressed IL-5Rα at a level comparable to that of eosinophils (Fig. 1 A). Interestingly, the IL-5Rα$^+$CD34$^+$CD38$^+$ population (i.e., hEoP) gave rise only to eosinophils and activated the EPX, whereas they did not differentiate into basophils or neutrophils nor did they activate the HDC or the MPO after culture (Fig. 3). We found that the hEoP having the identical IL-5Rα$^+$CD34$^+$CD38$^+$ phenotype resided also in the umbilical cord blood and in the adult peripheral blood. They were extremely rare populations (~0.005% of cord blood and 0.001% of blood MNCs, respectively) but differentiated only into eosinophils at a similar efficiency (unpublished data). Thus, the IL-5Rα$^+$CD34$^+$CD38$^+$ phenotype is common to the hEoP in multiple locations, including the bone marrow, the peripheral blood, and the cord blood. hEoPs may emigrate from the bone marrow into circulation and may be recruited into sites of allergic inflammation.

The expression of IL-5Rα in hEoPs might occur as a result of eosinophil lineage commitment without exerting instructive functions for eosinophil lineage specification. We have previously shown that the enforced IL-5 signaling at the mGMP stage by transducing mouse IL-5Rα induced mainly neutrophil/macrophage- but not eosinophil-specific differentiation (4). Consistent with this data, hEoPs generated only eosinophils even when we removed IL-5 from the cytokine cocktail, suggesting that IL-3 and/or GM-CSF signaling is sufficient to support the survival/proliferation and the terminal maturation of hEoPs. In addition, G-CSF, a cytokine that promotes neutrophil differentiation, did not affect the fate of hEoPs. It corresponds with our finding that hEoPs lack the expression of G-CSF receptor transcript (unpublished data). These observations collectively suggest that the hEoP is absolutely committed to the eosinophil lineage irrespective of a cytokine milieu.

Basophils were produced from populations within the IL-5Rα$^-$CD34$^+$CD38$^+$ fraction, such as hCMPs and hGMPs, as well as the CD34$^+$CD38$^-$ hHSCs (Fig. 2 C). Therefore, in normal human hematopoiesis, the IL-5Rα$^+$CD34$^+$CD38$^+$ hEoP does not contain progenitors common to eosinophils and basophils. It is still unclear at what stage basophils up-regulate IL-5Rα. Because all IL-5Rα$^+$CD34$^+$CD38$^+$ cells have committed to the eosinophil lineage, and because hGMPs were capable of producing basophils (Fig. 2 C), putative hBaP may exist within or downstream of the hGMP population. Basophils may up-regulate IL-5Rα at a later stage of their maturation after cells shut off CD34 expression. To isolate hBaPs, the identification of new surface antigens that mark early basophil lineage commitment is necessary.

This study also indicates that the conventional hCMP population defined by our earlier work (15) was heterogeneous because it contained at least the hEoP. Therefore, we should revise our original definition of hCMP: the true hCMP (i.e., revised hCMP) resides in the IL-5Rα$^-$ fraction of conventional hCMP (Fig. 6 B). The IL-5Rα$^-$ hCMP gave rise to the IL-5Rα$^+$ hEoP (Fig. 4), suggesting that the eosinophil

Table I. Percentages of hEoP in normal and eosinophilia bone marrow

Patient no.	Diagnosis	Age/sex	WBC (/μl)	Eosinophils in PB (%)	Eosinophils in BM (%)	hEoP in BMMNCs (%)	hEoP in Lin$^-$CD34$^+$ (%)
1	HES	17/M	18,510	12,957 (70)	NA	0.227	7.72
2	HES	50/M	29,530	14,647 (49.6)	NA	0.253	6.87
3	HES	51/M	13,220	4,680 (35.4)	21.2	0.186	3.85
4	HES	62/F	16,800	9,593 (57.1)	36.4	0.047	12.7
5	HES	68/M	12,800	3,430 (26.8)	32.8	0.048	4.73
6	T cell lymphoma	75/F	30,180	27,011 (89.5)	66.8	0.039	11.8
7	ATL	60/M	57,510	24,442 (42.5)	38	0.056	5.66
8	CML	48/M	436,000	34,008 (7.8)	9.6	0.33	8.46
9	AGA	34/F	23,210	14,669 (63.2)	49.6	0.074	5.82
10	AGA	66/F	40,000	33,240 (83.1)	76.4	0.072	9.91
11	unknown	81/F	9,300	2,632 (28.3)	36.4	0.043	7.56
12	unknown	26/F	10,970	5,331 (48.6)	22.4	0.052	3.84
13	unknown	50/F	13,610	3,062 (22.5)	28.8	0.076	8.67
14	unknown	57/M	21,020	13,747 (65.4)	37.6	0.207	6.92
15	unknown	76/M	10,380	3,529 (34)	43.2	0.161	7.09
Average		57	49,536	13,799 (48.3)	38.4	0.125	7.44
SD		(17–81)	107,711	11,026 (23.2)	18.1	0.094	2.61
Normal BM (n = 7)							
Average		46	5,164	176 (3.26)	3.09	0.033	2.38
SD		(28–72)	1,652	110 (1.21)	1.17	0.013	0.46

AGA, allergic granulomatous angitis; ATL, adult T cell leukemia; BMMNC, bone marrow MNC; CML, chronic myelogenous leukemia; NA, not applicable; PB, peripheral blood.

lineage commitment occurs at least within the IL-5Rα⁻ hCMP stage. However, we cannot exclude the possibility that a portion of hEoPs develop directly from the earlier progenitors such as hHSCs and hMPPs, bypassing the hCMP stage. The hGMP does not faithfully correspond to the mGMP because the hGMP lacked the eosinophil potential (Fig. 2), whereas the mGMP was capable of producing all granulocyte subclasses, including eosinophils and basophils via their progenitor populations such as the mEoP and the mBaP. Therefore, it is also possible that the true hGMP with all granulocyte subclass potentials may reside within the IL-5Rα⁻ hCMP fraction (Fig. 6 B). Another possibility is that a putative erythroid/eosinophil progenitor that preferentially differentiates into the erythroid and the eosinophil lineages (12) may exist upstream of the hEoP within the IL-5Rα⁻ hCMP fraction independent of the hEoP, hMEP, and hGMP. To fully understand the myeloerythroid lineage relationship, these possibilities should be tested in future studies.

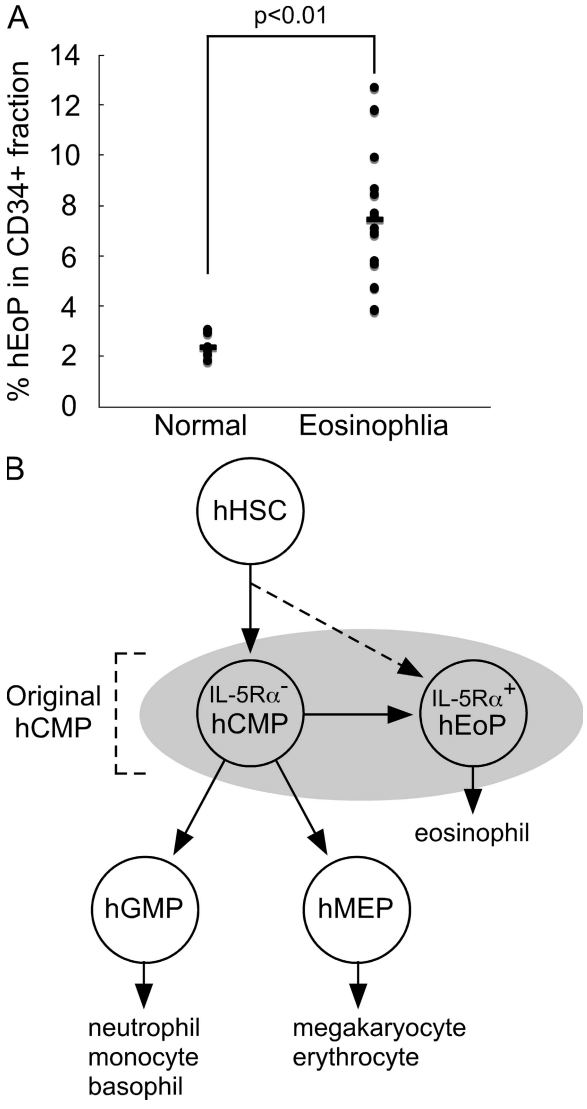

Figure 6. The in vivo expansion of hEoP in the bone marrow of eosinophilia patients. (A) The percentage of the hEoP population in the bone marrow CD34⁺ fraction. Eosinophila patients possessed approximately fourfold increased numbers of hEoPs (horizontal lines indicate means). Patients' characteristics are summarized in Table I. (B) The lineal relationship between the hEoP and other myeloid progenitor populations. The original hCMP (shaded) contained the hEoP. The revised hCMP is defined as the IL-5Rα⁻ fraction of the original hCMP. The hEoP develops from the hCMP or its upstream MPP independent of the hGMP and the hMEP.

Although the eosinophil developmental pathway could be somewhat different between the human and mouse, the use of key transcription factors appeared to be well preserved between these species (Fig. 5) (29). The hCMP expressed only low levels of GATA-1 and GATA-2 but both were upregulated in the hEoP. The expression of C/EBPα was maintained, whereas FOG-1 was suppressed in the hEoP. In mouse hematopoiesis, we have shown that the order of expression of C/EBPα and GATA-2 plays a pivotal role in the eosinophil versus the basophil lineage commitment (29). It is of interest whether such interplay of transcription factors is also critical in human hematopoiesis.

In summary, we have identified the hEoP as an IL-5Rα⁺ fraction of conventional hCMP. Thus, we propose to revise our original definition of hCMP by excluding an IL-5Rα⁺ fraction from the previous one: the revised phenotype of hCMP is IL-5Rα⁻Lin⁻CD34⁺CD38⁺IL-3Rα⁺CD45RA⁻. The hEoP significantly expands in patients suffering from diseases with eosinophilia, presumably reflecting their contribution toward eosinophil production in vivo. Therefore, this population should be useful to study the normal and abnormal eosinophil development, and could be a therapeutic target of diseases with eosinophila such as HES and allergic disorders.

MATERIALS AND METHODS

Bone marrow and blood samples. Bone marrow samples were obtained from healthy volunteers ($n = 7$) and eosinophilia patients ($n = 15$) by a conventional bone marrow aspiration procedure. All donors gave informed consent, and the study was approved by the Institutional Review Board of Kyushu University Hospital. Patients' characteristics are summarized in Table I. The diagnosis of HES was made according to the World Health Organization criteria. The *FIP1L1/PDGFRα* fusion gene, which relates to the pathogenesis of chronic eosinophilic leukemia, was analyzed by a nested RT-PCR method, as previously described (33). The EoL-1 cell line was used as a positive control for the PCR assay. All patients' samples analyzed in this study were negative for the *FIP1L1/PDGFRα* fusion gene.

Antibodies, cell staining, and sorting. Anti–human IL-5Rα monoclonal antibodies (KM1266) (34) were biotinylated with an EZ-Link NHS-PEO_4 Solid Phase Biotinylation Kit (Thermo Fisher Scientific) according to the manufacturer's protocol. To separate mature granulocyte subclasses, heparinized whole blood was stained with biotinylated anti–IL-5Rα antibodies followed by streptavidin-PE (eBioscience) in combination with anti-CCR3 (R&D Systems), anti-FcεRIα (eBioscience), anti-CD203c (Beckman Coulter), or anti–β7 integrin (BD) antibodies. The sorting procedures for hHSCs and myeloid progenitor populations that we previously reported (15) were slightly modified. In brief, bone marrow MNCs obtained by a density-gradient centrifugation method were first stained with PE-Cy5–conjugated lineage antibodies, including anti-CD3, -CD4, -CD8, -CD10, -CD19, -CD20, -CD14, -CD56, and –glycophorin A. Subsequently, cells were stained with allophycocyanin-conjugated anti-CD34 (BD), PE-conjugated anti-CD38 (Invitrogen),

PE-Cy7–conjugated anti–IL-3Rα (eBioscience), and FITC-conjugated anti-CD45RA (eBioscience) antibodies. hHSCs, hCMPs, hGMPs, and hMEPs were isolated as $Lin^-CD34^+CD38^-$, $Lin^-CD34^+CD38^+IL\text{-}3R\alpha^+CD45RA^-$, $Lin^-CD34^+CD38^+IL\text{-}3R\alpha^+CD45RA^+$, and $Lin^-CD34^+CD38^+IL\text{-}3R\alpha^-CD45RA^-$ populations, respectively. To sort hEoPs and redefined hCMPs, Pacific blue–conjugated anti-CD38 antibodies were used, and biotinylated anti–IL-5Rα antibodies followed by streptavidin-PE were added. hEoPs and hCMPs were purified as $Lin^-CD34^+CD38^+IL\text{-}3R\alpha^+CD45RA^-IL\text{-}5R\alpha^+$ and $Lin^-CD34^+CD38^+IL\text{-}3R\alpha^+CD45RA^-IL\text{-}5R\alpha^-$ populations, respectively. Dead cells were excluded by propidium iodide staining. Appropriate isotype-matched control monoclonal antibodies were used to determine the background staining level in each channel. All sorting and analyses were performed on three laser-equipped FACSAria machines (BD). To minimize contamination, the second round of sorting was performed routinely with the same sorting gates as the first round. The automatic cell-deposition system was used for single-cell assays. FACS data were analyzed with FlowJo software (Tree Star, Inc.).

Cell culture. For liquid cultures, purified progenitor populations were suspended in 12-well plates with the following medium: IMDM (Invitrogen) supplemented with 20% FCS (StemCell Technologies Inc.), antibiotics, 20 ng/ml of human recombinant IL-3, 20 ng/ml IL-5, 20 ng/ml SCF, 50 ng/ml GM-CSF, 4 U/ml Epo, and 20 ng/ml Tpo (R&D Systems). For clonogenic analyses of hHSCs and myeloid progenitors, including hEoPs, cells were cultured 14 d in IMDM-based methylcellulose medium (Methocult H4100; StemCell Technologies Inc.) with 20% FCS, 1% BSA, 2 mM L-glutamine, and 50 μM 2-mercaptoethanol (StemCell Technologies Inc.). The same cytokines described were added at the initiation of cultures. All cultures were incubated at 37°C in a humidified chamber under 5% CO_2. All single colonies were picked up and were mounted on cytospin preparations to define cell components.

Gene expression analysis. Total RNA was extracted from purified progenitor populations, cultured cells, or mature blood granulocytes using ISOGEN reagent (Nippon Gene) according to the manufacturer's protocol. All RNA samples were reverse transcribed with Oligo dT primers using the SuperScript III First-Strand Synthesis System (Invitrogen). The conventional RT-PCR and the quantitative real-time PCR assays were performed with the GeneAmp 9700 PCR System and the PRISM 7500 Fast Real-Time PCR System, respectively (Applied Biosystems). The specific primer and probe sequences for PCR analyses are provided in Table S1 (available at http://www.jem.org/cgi/content/full/jem.20081756/DC1). Human β2 microglobulin transcript was simultaneously amplified as an internal standard for quantification.

Online supplemental material. The specific primer and probe sequences for PCR analyses are listed in Table S1. Online supplemental material is available at available at http://www.jem.org/cgi/content/full/jem.20081756/DC1.

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