LETTERS

The earliest thymic progenitors for T cells possess myeloid lineage potential

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There exists controversy over the nature of haematopoietic progenitors of T cells. Most T cells develop in the thymus, but the lineage potential of thymus-colonizing progenitors is unknown. One approach to resolving this question is to determine the lineage potentials of the earliest thymic progenitors (ETPs). Previous work has shown that ETPs possess T and natural killer lymphoid potentials, and rare subsets of ETPs also possess B lymphoid potential[1], suggesting an origin from lymphoid-restricted progenitor cells. However, whether ETPs also possess myeloid potential is unknown. Here we show that nearly all ETPs in adult mice possess both T and myeloid potential in clonal assays. The existence of progenitors possessing T and myeloid potential within the thymus is incompatible with the current dominant model of haematopoiesis, in which T cells are proposed to arise from lymphoid-restricted progenitors[2]. Our results indicate that alternative models for lineage commitment during haematopoiesis must be considered.

Haematopoiesis is a model system for tissue homeostasis and regeneration. Mature blood cell types originate from haematopoietic stem cells (HSCs) that are multipotent and self-renewing[3]. The first step in differentiation of HSCs involves the generation of multipotent progenitors (MPPs), which do not self-renew[4,5]. This is followed by an ordered series of irreversible lineage commitment events, resulting in the generation of increasingly lineage-restricted downstream intermediates. Subsequent to the generation of non-renewing MPPs, the standard classical model of haematopoiesis postulates a binary split between lymphoid and myelo-erythroid lineages[2,6,7]. In this model, MPPs give rise to either common lymphoid progenitors (CLPs) with lymphoid potential but devoid of myeloid and erythroid potentials, or to common myeloid progenitors possessing myeloid and erythroid potentials but lacking lymphoid potentials[6,7]. Further development along a given lineage is accompanied by continuing loss of alternative lineage potentials, until mature blood cell types are generated[2].

As lymphocytes, T cells are proposed to develop from CLPs[7]. However, the identity of thymus-settling progenitors is unknown[1]. The most immature intrathymic progenitors that have been identified are ETPs, defined phenotypically as expressing low levels of lineage markers (Lin) that are also $CD25^{-}Kit^{hi}$ cells[8,9]. In addition to efficient T lymphoid potential[9,10], ETPs possess natural killer and dendritic cell potentials[1]. These non-T-lineage potentials are maintained in downstream DN2 cells ($Lin^{-}CD25^{+}Kit^{hi}$), and are not lost until final commitment to the T-cell lineage, which occurs at the DN3 stage ($Lin^{-}CD25^{+}Kit^{-/lo}$)[1]. In addition to T, natural killer and dendritic cell potential, a rare subset of ETPs also possesses B lymphoid potential[11–13]. Whether ETPs possess potential for myeloid cells such as monocytes/macrophages and granulocytes, as distinct from their well-studied dendritic cell potential, is less clear. The ETP population possesses some degree of myeloid potential in bulk assays[9,14–16], leading to the suggestion that not all ETPs are derived from lymphoid-restricted CLPs[9]. It is unclear whether the same cells that have myeloid potential also have T lymphoid potential, or if the ETP population instead contains some myeloid-lineage-committed cells (Supplementary Fig. 1)[14]. Resolution of this question requires the development of clonal assays to measure the myeloid and T lineage potentials of single ETPs.

Culture of haematopoietic progenitors on bone-marrow-derived OP9 stromal cells has been extensively used to investigate lineage potentials of haematopoietic progenitors. OP9 stromal cells transduced with the Notch ligand delta-like 4 (OP9-DL4) support T-lineage development, whereas OP9 cells lacking delta-like Notch ligands (OP9) do not[17]. Using this system we found that ETPs placed in short-term cultures with Flt3 ligand and interleukin (IL)-7 underwent population expansion (Fig. 1a). Similar levels of expansion were seen when ETPs were cultured on OP9-DL4 or on OP9 stroma.

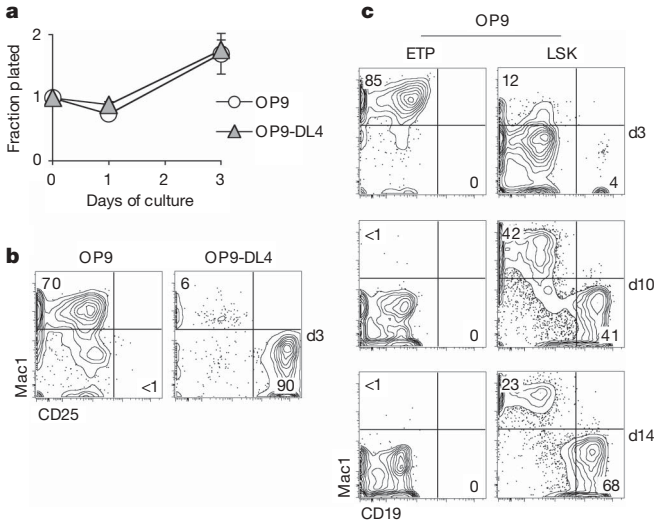

Figure 1 | ETPs have myeloid lineage potential. **a**, Sorted ETPs (500 per well) were cultured with either OP9 or OP9-DL4 stromal cells in medium supplemented with IL-7 and Flt3 ligand. Cultures were analysed at 1 and 3 days and assessed for the presence of live (4,6-diamidino-2-phenylindole (DAPI) negative) haematopoietic ($CD45^{+}$) cells. Recovery values are shown as the fraction of cells plated ± s.e.m. of triplicate wells. **b**, Cells cultured for 3 days were analysed for surface expression of Mac1 and CD25 by flow cytometry. Plots shown are gated on live haematopoietic cells. Numbers on plots represent percentages of cells in those regions. Most Mac1-negative cells lacked expression of lineage markers and so were undifferentiated cells. **c**, Mac1 and CD19 expression on cells derived from ETPs or bone marrow LSK cells (500 per well) cultured for 3, 10, or 14 days on OP9 stroma. Data in **a**–**c** are representative of at least three experiments.

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However, the identities of the resulting cell lineages were distinct. On OP9-DL4 stroma, ETPs gave rise to CD25$^+$ T-lineage cells (Fig. 1b), which co-expressed Thy-1 and other T-lineage-specific markers[17]. By day 3 on OP9 stroma, however, ETPs gave rise to cells that expressed the myeloid lineage marker Mac1 (Fig. 1b). Such Mac1$^+$ cells were also seen when DN2 cells were cultured, albeit in lower numbers, but not in cultures initiated with DN3 cells (Supplementary Fig. 2). B-lineage cells were not seen in these experiments initiated with 500 ETPs on OP9 stroma (Fig. 1c), even when cultures were extended until 2 weeks. This can be explained by the low frequency of ETPs with B potential (<1:500 in 6-week-old adult C57BL/6 mice)[12,13,18]. In contrast, primitive bone marrow Lin$^-$Sca1$^+$Kit^{hi} (LSK) progenitors efficiently generated B-lineage cells in these culture conditions, as evidenced by the presence of CD19$^+$ cells from day 3 until 2 weeks of culture (Fig. 1c).

We further characterized the Mac1$^+$ progeny of ETPs cultured on OP9 stroma (Fig. 2). Some Mac1$^+$CD11c$^+$ dendritic cells were evident in these cultures, as expected[11,19] (Fig. 2a). However, the majority of Mac1$^+$ cells lacked expression of CD11c. Some Mac1$^+$ cells co-expressed the monocyte/macrophage markers MCSF receptor (MCSFR) and F4/80. Other Mac1$^+$ cells co-expressed Gr1, expressed on granulocytes and some macrophages. To characterize better these populations, we made use of lysozyme M reporter mice, in which the gene for green fluorescent protein (GFP) is knocked into the

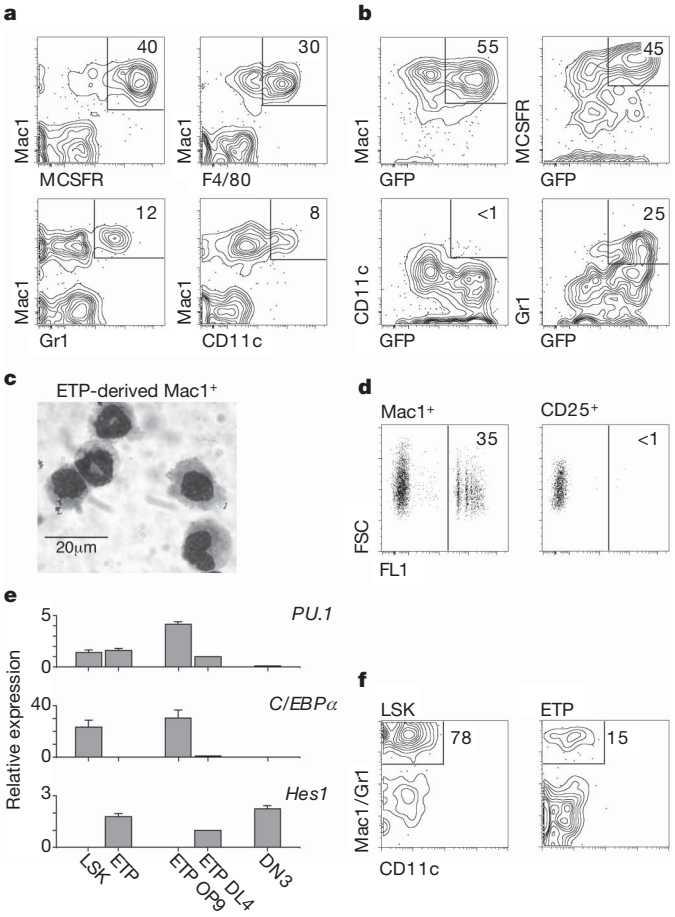

Figure 2 | Characterization of ETP-derived myeloid cells. **a**, ETPs were cultured for 3 days on OP9 stroma in medium supplemented with IL-7 and Flt3 ligand, and their progeny were assessed for myeloid lineage markers by flow cytometry. **b**, ETPs were sorted from lysozyme M reporter mice (where GFP is knocked into the lysozyme M locus) and cultured with OP9 stromal cells as before. After 3 days, cells were analysed for expression of myeloid markers and GFP. **c**, Mac1$^+$ cells resulting from ETPs cultured as in **a** were Wright-Giemsa stained. Scale bar, 20 μm. Data in **a**–**c** are representative of at least three experiments. **d**, Phagocytic ability of sorted Mac1$^+$ cells derived from ETPs cultured on OP9 was analysed by uptake of beads fluorescing in the FL1 channel (see Methods). CD25$^+$ cells from ETPs cultured on OP9-DL4 serve as a negative control. FSC, forward scatter. Data are representative of two experiments. **e**, ETP-derived cultures on OP9 stroma (ETP OP9) or OP9-DL4 stroma (ETP DL4) were harvested after 4 days, then analysed by real-time PCR for expression of *PU.1*, *C/EBPα* and *Hes1*. Freshly isolated bone marrow LSK progenitors, ETPs and DN3 cells served as controls. Data are averages of triplicate wells ± s.e.m., and are representative of two experiments. **f**, ETPs or control bone marrow LSK progenitors (1,000) from C57BL/6 mice (CD45.2) were transferred intravenously into irradiated B6.CD45.1 recipients. After 6 days the spleens were analysed for donor-derived cells using CD45 markers as shown in Supplementary Fig. 3. Mac1/Gr1 versus CD11c expression on gated donor-derived cells is shown. Data are representative of five experiments using 200–3,000 progenitors in which myeloid lineage progeny were always present.

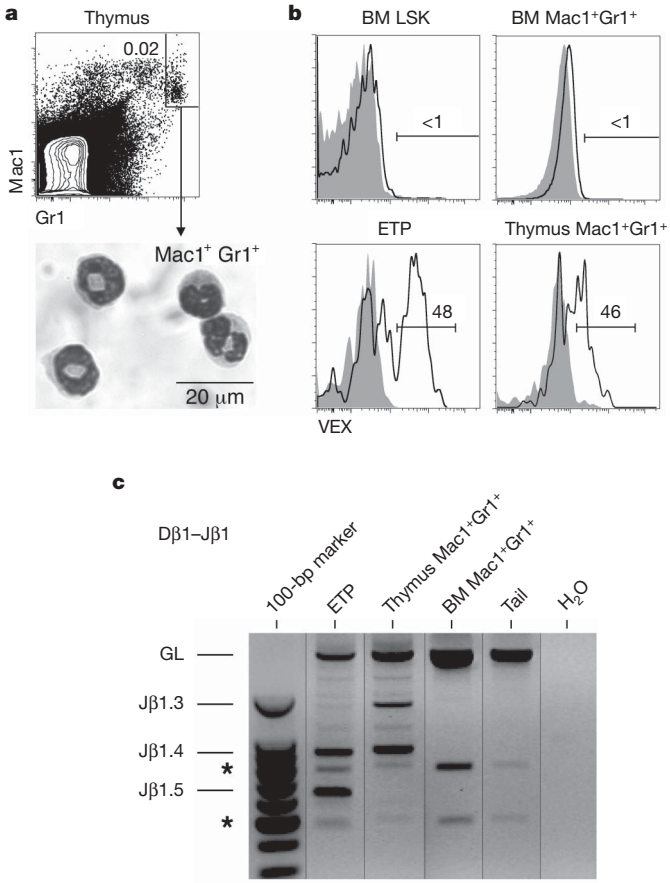

Figure 3 | Thymic granulocytes possess antigen receptor gene rearrangements. **a**, Thymic Mac1$^+$Gr1$^+$ cells were isolated by cell sorting and Wright-Giemsa stained. Band, ring and segmented granulocytes were present. Scale bar, 20 μm. Data are representative of two experiments. **b**, Bone marrow (BM) and thymic populations from H2-SVEX fluorescent transgenic recombination reporter mice were analysed for VEX fluorescence by flow cytometry. Plots are shown comparing H2-SVEX bone marrow and thymic progenitor and granulocyte populations (open histograms) with corresponding C57BL/6 control populations (shaded histograms). The gating strategies for analysis of these populations are shown in Supplementary Fig. 4. Data are representative of four experiments. **c**, Wild-type bone marrow and thymic granulocyte populations were analysed for Dβ1–Jβ1 rearrangements by PCR. ETPs, previously shown to possess such rearrangements, are also shown. The identities of marked Dβ1–Jβ1 rearrangements were confirmed by sequencing (Supplementary Fig. 5). The germline band (GL) is indicated. Background bands marked with an asterisk are present in all samples, including tail. DNA sequencing was used to confirm that these bands do not contain rearranged TCR or immunoglobulin genes (data not shown). No consistent difference was seen in TCR rearrangements within ETPs and thymic granulocytes. Data are representative of three experiments.

lysozyme M locus[20,21]. HSCs and ETPs express only low levels of GFP or are GFP-negative[21] (our own unpublished data). High-level GFP expression is seen in monocytes/macrophages and granulocytes, but not in dendritic cells or other non-myeloid lineages[20] (Fig. 2b). $Mac1^+$ cells developing from ETPs included GFP^{hi} cells. Indeed, the $MCSFR^+$ cells and $Gr1^+$ cells were mainly GFP^{hi}, whereas $CD11c^+$ dendritic cells lacked high-level expression of GFP (Fig. 2b). Microscopic examination of $Mac1^+$ cells derived from ETPs revealed some cells with monocytic morphology and other cells with granulocytic morphology (Fig. 2c). Some $Mac1^+$ cells phagocytosed fluorescent latex beads, indicating that they were functional macrophages (Fig. 2d). These results further establish that $Mac1^+$ progeny of ETPs include macrophages and granulocytes that are in the myeloid lineage.

We also determined that these cells possessed transcripts consistent with their myeloid lineage identity. Bone marrow LSK progenitors known to possess myeloid potential[5] and ETPs were compared for expression of *PU.1* (also called *Sfpi1*), *C/EBPα* (*Cebpa*) and the Notch gene target *Hes1*. Withdrawal of Notch signalling caused increased *PU.1* and *C/EBPα* expression in progeny of ETPs, consistent with the myeloid identity of cells in these cultures[22]. Maintenance of Notch signalling prevented these transcriptional changes (Fig. 2e). To determine whether the myeloid potential of ETPs was evident *in vivo*, we intravenously transferred 1,000 ETPs or bone marrow LSK cells into irradiated mice, and analysed spleens for donor-derived $Mac1/Gr1^+$ populations 6 days later. The overall level of donor chimaerism was similar between mice inoculated with ETPs and LSK cells at this early time point (Supplementary Fig. 3). Although the frequency of myeloid progeny was lower for mice receiving ETP donor cells compared with LSK donor cells, ETPs gave rise to myeloid cells in this experimental *in vivo* system (Fig. 2f).

We next wished to determine whether ETPs physiologically give rise to intrathymic myeloid progeny *in vivo*. We focused on $Mac1^+Gr1^+$ cells because we could easily distinguish this population even at the low frequency present within the thymus (Fig. 3a). Thymic $Mac1^+Gr1^+$ cells possessed a granulocytic morphology and were unambiguously myeloid lineage cells (Fig. 3a). To mark non-lymphoid progeny of ETPs permanently, we made use of a fluorescent transgenic V(D)J recombination reporter mouse[23]. This transgene is expressed in haematopoietic cells in the H2-SVEX mouse[23]. Rearrangement mediated by recombinase-activating genes (RAG) results in permanent expression of violet excited GFP (VEX), allowing detection by flow cytometry[23]. We found that bone marrow LSK progenitors and bone marrow $Mac1^+Gr1^+$ cells do not express VEX (Fig. 3b). In contrast, nearly half of ETPs express VEX, consistent with their possession of T-cell receptor (TCR) Dβ1–Jβ1 as well as additional rearrangements[9]. A fraction of thymic granulocytes also exhibited VEX fluorescence, demonstrating that they were derived from RAG-expressing progenitors. We confirmed that thymic granulocytes possessed Dβ1–Jβ1 rearrangements, similar to ETPs (Fig. 3c). The identity of Dβ1–Jβ1 rearrangements in thymic granulocytes was confirmed by DNA sequencing (Supplementary Fig. 5). A smaller fraction (5–10%) of the $Mac1^+MCSFR^+CD11c^-$ thymic macrophage population was also positive for VEX in H2-SVEX mice (data not shown), suggesting that other myeloid cell types might also be derived from ETPs. These results indicate that ETPs physiologically give rise to myeloid cells *in vivo*.

The prominent myeloid potential of ETPs is surprising, and suggests that this potential is not due to a rare myeloid committed progenitor. Instead, bipotent progenitors possessing T and myeloid potential may exist within the ETP population. To test this, we performed single cell assays. As ETPs developed predominantly into T-lineage cells on OP9-DL4 stroma, and developed into myeloid lineage cells on OP9 stroma, we cultured ETPs on a 50:50 mixture of OP9:OP9-DL4 for 10 days. Our rationale was that as lineage commitment is not instantaneous[11,24], uncommitted progeny of ETPs would segregate to different local environments in these cultures, and adopt different cell fates. The inclusion of cytokines promoting myeloid lineage and T-lineage development allowed expansion of myeloid lineage and T-lineage progeny of single ETPs to a degree that could be detected by flow cytometry. Unlike 3-day cultures where granulocytic progeny were abundant (Fig. 2c), most myeloid cells in these 10-day cultures were monocytes/macrophages (data not shown). This is consistent with the accompanying study[25] that examined longer-term cultures, in which macrophages also predominated. The *in vitro* system established here allowed analysis of the myeloid lineage and T-lineage potentials of single ETPs. Surprisingly, most ETPs contained both T and myeloid potential (Table 1 and Supplementary Fig. 6). Some progenitors with T and myeloid potential remained evident at the downstream DN2 stage; however, the majority of DN2 cells lacked myeloid potential (Table 1). Hence, myeloid potential is evident in most ETPs, is largely but not completely lost in DN2 cells, and is extinguished in DN3 cells (Supplementary Fig. 2).

Our results establish that most ETPs possess T and myeloid potential, and so establish that the majority of ETPs do not arise from lymphoid-restricted progenitors. Instead, we suggest that ETPs arise from multipotent bone marrow progenitors such as early lymphoid progenitors or lymphoid-primed multipotent progenitors, which possess T, B and myeloid potential[18,26–28]. We propose that such multipotent progenitor cells lose B potential, either prethymically or intrathymically, generating ETPs possessing T and myeloid potential (Supplementary Fig. 1). Our results further establish that whereas myeloid potential is lost in the bone marrow during B-cell development, it is lost in the thymus during T-cell development, suggesting that distinct mechanisms may be involved. The myeloid potential of ETPs was most clearly evident when ETPs were removed from the thymus and assessed in the absence of T-inductive Notch signalling, indicating a role for intrathymic Notch signals in suppression of the myeloid fate. However, we also found that granulocytes within the thymus can possess TCR rearrangements, suggesting that some ETPs produce myeloid progeny within the thymus. The development of such thymic myeloid cells may have commonalities with the previously described development of thymic dendritic cells from ETPs[19]. Notch signals antagonize the effects of PU.1 and C/EBPα, critical for development of dendritic cells and other myeloid lineages, suggesting that some thymic dendritic cells arise from ETPs in niches protected from Notch signalling[22,29]. Similarly, thymic myeloid cells may arise from ETPs that escape Notch signals. Thus, the development of dendritic cells and myeloid cells within the thymus may share some cellular requirements and molecular mechanisms.

The standard model of a binary split between lymphoid and myelo-erythroid lineages has been the prevailing idea of initial lineage restriction during haematopoiesis[6,7]. The present study, together with past work indicating that the majority of ETPs lack B potential[9,11–13,16,30], demonstrates that most ETPs possess T-lymphoid and myeloid potentials but lack B-lymphoid potential. The standard model cannot explain the lineage potentials of ETPs, because this model requires that myeloid potential be lost before the loss of any lymphoid potentials during T-cell development. Hence, the idea of

Table 1 | A high frequency of single ETPs have T and myeloid potential

Cell type	T (%)	T and myeloid (%)	Myeloid (%)
ETP	9	87	4
DN2	87	13	0

Single ETPs or DN2 cells from lysozyme M reporter mice were sorted into 96-well plates containing a 50:50 mix of OP9 and OP9-DL4, and medium supplemented with 1 ng ml^{-1} IL-7, 5 ng ml^{-1} Flt3 ligand and 10 ng ml^{-1} IL-3, IL-6, stem cell factor (SCF), macrophage colony-stimulating factor (M-CSF), granulocyte colony-stimulating factor (G-CSF), and granulocyte/macrophage colony-stimulating factor (GM-CSF). After 10 days the resulting colonies were analysed for expression of T (CD25) and myeloid (Mac1, MCSFR, Gr1 and GFP) markers as well as CD45 and CD11c. Shown is the percentage of T or myeloid unipotent and T and myeloid bipotent progenitors. The percentages are normalized based on the number of surviving colonies arising from single cells, excluding wells that did not support growth. Plating efficiencies were >70%. Colonies derived from 150 single ETPs (four experiments) and 55 single DN2 cells (two experiments) were analysed. All experiments gave similar results.

an obligatory initial split in haematopoiesis between lymphoid lineages and myelo-erythroid lineages[6,7] is incorrect.

METHODS SUMMARY

C57BL/6 (B6) mice and B6.CD45.1 mice were purchased from the National Cancer Institute. Lysozyme M reporter mice[21] were provided by T. Graf. H2-SVEX mice[23] were obtained from R. M. Gerstein. Progenitor populations were isolated as described[9]. OP9 and OP9-DL4 cells were gifts from J. C. Zuniga-Pflucker and were used as described[17]. IL-7 was added at a final concentration of 1 ng ml^{-1} and Flt3 ligand at 5 ng ml^{-1}.

Full Methods and any associated references are available in the online version of the paper at www.nature.com/nature.

Received 28 October 2007; accepted 15 February 2008.

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Supplementary Information is linked to the online version of the paper at www.nature.com/nature.

Acknowledgements We thank C. Bassing, M. Carroll, B. Schwarz and J. Thompson for advice. This work was supported by a grant from the NIH and a Scholar Award from the Leukemia and Lymphoma Society (A.B.) and an institutional training grant from NCI (J.J.B).

Author Contributions J.J.B. performed the majority of the experimental work. J.J.B. and A.B. together planned the project, analysed data and prepared the manuscript.

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METHODS

Mice. All mice were females used at 5–8 weeks of age. All animal experiments were done according to protocols approved by the Office of Regulatory Affairs of the University of Pennsylvania in accordance with guidelines set forth by the National Institutes of Health.

Flow cytometry. All antibodies were purchased from BD Biosciences or Ebiosciences. Antibodies used were fluorescein isothiocyanate (FITC)-conjugated CD45.1 (A20); phycoerythrin (PE)-conjugated CD25 (PC61), MCSFR (AFS98), F4/80 (BM8); PE-Cy7-conjugated Mac1 (CD11b) (M1/70); PE-Cy5.5-conjugated CD45.1, CD45.2 (104); peridinine-chlorophyll protein (PerCP)-conjugated CD19 (1D3); allophycocyanin (APC)-conjugated Kit (CD117) (2B8), CD25, CD11c (HL3); APC-Alexa-750-conjugated CD25; and biotin-conjugated CD45.2 (104), Gr1 (8C5). To exclude mature cells, a lineage cocktail of the following FITC-, PE-, or biotin-conjugated antibodies was used: B220 (RA3-6B2), CD19, CD11b, Gr1, CD11c, NK1.1 (PK136), Ter119, CD8α (53-6.7), CD8β (53-5.8), TCR-γδ (GL-3), TCR-β (H57) and CD3 (2C11). Biotin-conjugated antibodies were visualized with streptavidin-Pacific blue (Molecular Probes) or streptavidin-PerCP–Cy5.5 (BD Biosciences). Dead cells were excluded with 4,6-diamidino-2-phenylindole (DAPI). Cells were sorted on a BD FACSAria (Becton Dickinson) and sort purity was routinely checked. Cell analysis was done on a 4-laser LSR II (Becton Dickinson) and data were analysed using FlowJo version 4.6.2 (Tree Star).

Phagocytosis assay. ETP-derived $Mac1^+$ cells from OP9 cultures supplemented with M-CSF, GM-CSF, G-CSF, IL-3 and IL-6 to support maturation were sorted and incubated with 1 μm fluorescent beads (Fluoresbrite Yellow Green Microspheres, Polysciences, Inc.) for 5 h in medium supplemented with M-CSF. Control $CD25^+$ cells were obtained from OP9-DL4 cultures. Cell to bead ratio was 1:10. Cells were then treated with 0.05% trypsin-EDTA to free surface-bound beads and washed with PBS. Beads that had not been ingested were removed by centrifugation (100*g* for 10 min) on a cushion of PBS-3% BSA supplemented with 4.5% glucose. Fluorescence was then assessed by flow cytometry as above.

PCR. For real-time PCR, messenger RNA from sorted populations was isolated using the RNeasy kit (Qiagen) and reverse transcribed with Superscript II (Invitrogen). Resultant complementary DNAs were then amplified and detected using pre-made FAM-labelled primer/probes against *PU.1*, *C/EBPα* and *Hes1*, purchased from Applied Biosystems. Amplification and analysis were carried out on a 7300 Real-Time PCR System (Applied Biosystems). PCR analysis for TCR Dβ1–Jβ1 rearrangements using 1,000 cell equivalents was carried out as described[9].

Wright-Giemsa staining. Sorted populations were spun onto glass slides using a Shandon Cytospin 2 cytocentrifuge. Cells were then fixed in fresh methanol and stained in Wright-Giemsa reagent (Fisher Scientific) for 3 min, followed by Wright-Giemsa with Original Azure Blend (Harleco) for 10 min, then Wright-Giemsa with phosphate buffer pH 6.8 (Fisher Scientific) for 2 min. Stained slides were then washed with ddH_2O and allowed to dry, coverslipped, and examined under microscope. Microscopy pictures shown are at ×100 magnification.

OP9 and OP9-DL4 cell culture. OP9 and OP9-DL4 cells were used essentially as described[17]. For most cultures, IL-7 was added at a final concentration of 1 ng ml^{-1} and Flt3 ligand at 5 ng ml^{-1}. Myeloid progeny of ETPs cultured in these conditions included granulocytes and monocytes/macrophages that were evident at day 3 of culture. Myeloid progeny of ETPs did not survive to day 10 of culture in the absence of additional cytokine supplementation (shown in Fig. 1). For single cell assays, ETPs were cultured on a 50:50 mixture of OP9:OP9-DL4 for 10 days. IL-3, IL-6, stem cell factor, macrophage colony-stimulating factor, granulocyte colony-stimulating factor, and granulocyte/macrophage colony-stimulating factor were added at a final concentration of 10 ng ml^{-1}, in addition to IL-7 and Flt3 ligand as before. These myeloid-cytokine-supplemented 10-day cultures contained abundant monocytes/macrophages, but only rare granulocytes. The relative paucity of granulocytes compared to monocytes/macrophages after extended periods of *in vitro* culture is probably a result of the shorter lifespan of mature granulocytes. Stromal cells were plated 2 days before initiation of culture at a concentration of 20,000 cells ml^{-1} in 24-well plates for bulk cultures and 96-well plates for single cell assays. For bulk cultures, 500 progenitors were plated per well. For single cell assessment of ETPs, 150 colonies were analysed.