The earliest thymic T cell progenitors sustain B cell and myeloid lineage potential

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The stepwise commitment from hematopoietic stem cells in the bone marrow to T lymphocyte–restricted progenitors in the thymus represents a paradigm for understanding the requirement for distinct extrinsic cues during different stages of lineage restriction from multipotent to lineage-restricted progenitors. However, the commitment stage at which progenitors migrate from the bone marrow to the thymus remains unclear. Here we provide functional and molecular evidence at the single-cell level that the earliest progenitors in the neonatal thymus had combined granulocyte-monocyte, T lymphocyte and B lymphocyte lineage potential but not megakaryocyte-erythroid lineage potential. These potentials were identical to those of candidate thymus-seeding progenitors in the bone marrow, which were closely related at the molecular level. Our findings establish the distinct lineage-restriction stage at which the T cell lineage–commitment process transits from the bone marrow to the remote thymus.

At the heart of developmental and stem-cell biology, as well as regenerative medicine, is the fundamental process of lineage commitment from self-renewing multipotent stem cells to lineage-restricted progenitors. In all species and organ systems, this process occurs first during embryonic development but is recapitulated postnatally and in adult life by adult multipotent stem cells that replenish cell lineages with a limited lifespan. Hematopoiesis represents the mammalian paradigm of how multilineage diversity can be achieved through the commitment of multipotent stem cells to lineage-committed progenitors and the establishment of distinct blood cell lineages. However, the exact cellular commitment pathways remain unclear.

Whereas lineage-restricted progenitors for all other blood cell lineages can be generated from self-renewing hematopoietic stem cells (HSCs) in the postnatal bone marrow, the final steps of restriction to the T lymphocyte lineage take place in the thymus. Because the thymus cannot sustain HSCs, continuous thymopoiesis can be secured only through regular replenishment by bone marrow–resident thymus-seeding progenitors (TSPs). However, the commitment stage(s) at which these progenitors migrate from the bone marrow to the thymus is (are) unknown. The thymus contains multiple blood cell lineages, as does the bone marrow; however, the identification of multipotent progenitors in the thymus that match the lineage potential of candidate TSPs in the bone marrow has not been possible so far. Early thymic progenitors (ETPs) have been extensively studied in the adult thymus, but their exact lineage potentials and relationship to candidate TSPs in the bone marrow have remained unclear.

Studies evaluating the lineage potential of ETPs at the single-cell level have shown that a large fraction of ETPs from adult mice have combined T cell and myeloid (granulocyte-monocyte (GM)) potential. B cell lineage potential, however, was not detected for single, highly purified ETPs from adult mice, which suggests that the most primitive progenitor in the thymus might have potential restricted to T cells and granulocytes-monocytes. Similar studies of fetal thymus have supported the proposal that the potential of ETPs is restricted to T cells and granulocytes-monocytes and have failed to show any B cell potential. Other studies have reported even rarer ETPs from adult mice with combined T cell and B cell potential. Candidate TSPs identified in the bone marrow, such as common lymphoid progenitors (CLPs) and lymphoid-primed multipotent progenitors (LMPPs), all have B cell potential. The megakaryocyte-erythroid (MkE) potential of ETPs is of particular relevance to the ongoing debate on whether...
the first lineage-commitment step in hematopoiesis results in strict separation into common pathways for commitment to the myeloid and lymphoid lineage, as presented in the still-prevaling textbook hierarchical model of hematopoiesis\(^1,14\), or whether early lymphoid progenitors sustain GM potential but not MkE potential\(^2,13,15\), as reported in human studies as well\(^16,17\). The MkE potential of purified ETPs has yet to be investigated\(^5,6,8,9\).

The fact that no multipotent thymic progenitors with the same lineage potentials as those of candidate multipotent TSPs in the bone marrow have been identified yet contributes to the considerable gap in understanding of the distinct roles of the local bone marrow and thymus environments in promoting distinct prethymic and thymic stages of commitment to the T cell lineage. Here we demonstrate at the single-cell level the existence of postnatal ETPs with combined T cell, GM and B cell potential but no MkE potential, establishing the exact lineage commitment step at which the multipotent T lymphocyte progenitors must migrate to the thymus to allow the final steps of restriction to the T cell lineage to be completed. The data reported here provide further support for a lymphoid-based model of commitment of hematopoietic-lineage cells to the T cell lineage.

**RESULTS**

**ETPs have combined T cell, B cell and GM potential**

The present knowledge about candidate TSPs and ETPs can be reconciled (Supplementary Fig. 1) only if a progenitor restricted to the T cell–GM lineage can be identified in the bone marrow, a T cell–GM...
progenitor can be generated in the passage from the bone marrow to the thymus and/or a thymic cell population with combined T cell, GM and B cell lineage potential can be identified among or beyond the ETPs. In the last scenario, the ETP could either be a lymphoid-GM–restricted multipotent progenitor or a pluripotent hematopoietic stem cell or progenitor cell that also has MKE potential. ETPs have been studied mostly in adult mice6,8,9. However, thymic involution (the physiological shrinking of the thymus with age that occurs in all vertebrates) indicates that thymopoiesis, and therefore thymus seeding, is much more active in the early postnatal thymus1,8. The B cell potential of early erythroblasts, at the population level, is much higher (although still low) in the neonatal thymus than in the adult thymus1,8. In agreement with published studies, lineage-negative (Lin−) CD4−CD8− ETPs represented only 0.01% of adult thymocytes8, but as many as 40% of ETPs had cell-surface expression of the cytokine tyrosine kinase receptor Flt3, a greater frequency than reported before20 (Fig. 1a). Also in agreement with published findings6,8,9, a low but highly reproducible frequency of Flt3-expressing ETPs from adult mice generated B cells (3.5%–4.5%), whereas no other thymocyte progenitors from adult mice, including Flt3− ETPs, had any detectable B cell potential (Fig. 1b and Supplementary Fig. 2). The frequency of Lin−CD4−CD8α−CD25−c-Kithi ETPs was more than tenfold higher in newborn mice than in adult mice (Fig. 1c) and, most notably, the frequency of Lin−CD4−CD8α−CD25−c-Kithi ETPs with B cell potential was 25% (Fig. 1d and Supplementary Fig. 3). Neither Lin−CD4−CD8α−CD25−c-KithiFlt3hi thymocytes nor downstream populations at CD4−CD8− double-negative stages 2–4 (DN2–DN4) in the neonatal thymus had any B cell potential (Fig. 1d). Lin−CD4−CD8α−CD25−c-Kithi ETPs from newborn mice also produced B cells in vivo when transplanted into irradiated mice deficient in recombination-activating gene 1 (Rag1−/−) but produced only very low numbers of short-lived myeloid cells (Supplementary Fig. 4).

Because B cell activity in the thymus might reflect the presence of cells already committed to the B cell lineage21,22, which overlap with the CD25+CD44+ phenotype of DN1 thymocytes, we did a fate-mapping experiment with mice expressing Cre recombinase from the promoter of the gene encoding the immunoglobulin-associated antigen CD79A (CD79a), in which all committed B cell progenitors and their progeny are labeled with enhanced yellow fluorescent protein23,24. In agreement with published studies23, cells of the B cell lineage, including all CD19+ B220+CD43−c-Kithi pro-B cells, as well as a fraction of Ly6D+ CLPs (Lin−CD19−B220−Sca-1−c-KithiFlt3−IL-7r−Ly6D+), were labeled in the bone marrow (Supplementary Fig. 5a,b). We observed no cells expressing enhanced yellow fluorescent protein among Lin−CD4−CD8α−CD25−c-Kithi ETPs (Fig. 1e) or among Ly6D− CLPs (Lin−CD19−B220−Sca-1−c-KithiFlt3+IL-7r−Ly6D−) or LMPPs (Lin−Sca-1−c-KithiFlt3−b, Supplementary Fig. 5c,b). In addition to producing B cells, Lin−CD4−CD8α−CD25−c-KithiFlt3hi ETPs from newborn mice gave rise efficiently to cells of the T cell, natural killer cell and GM lineages, as demonstrated before with ETPs from adult mice5,6,8,9 (Fig. 1f,g and Supplementary Fig. 6a). In contrast, ETPs from newborn mice were completely devoid of MKE potential (Fig. 1f). ETPs from adult mice lacked megakaryocyte potential as well but, in agreement with published studies6,8,9, had GM potential (Supplementary Fig. 6a). Quantitative gene-expression analysis showed that purified Lin−CD4−CD8α−CD25−c-KithiFlt3hi ETPs from newborn mice expressed many genes associated with granulocytes-monocytes and lymphoid cells but not those associated with megakaryocytes or erythrocytes (Fig. 1h). Single-cell PCR showed that as many as 65% of newborn Lin−CD4−CD8α−CD25−c-KithiFlt3hi ETPs coexpressed genes of granulocytes-monocytes and lymphoid cells, whereas they lacked expression of genes of megakaryocytes and erythrocytes (Fig. 2a).

To establish whether the T cell, B cell and GM potential of ETPs from neonatal mice reflected the existence of a multipotent lympho-myeloid progenitor in the thymus or only a mixture of lineage-restricted progenitors, we assessed the combined lineage potential of single Lin−CD4−CD8α−CD25−c-KithiFlt3hi ETPs. We sorted single ETPs onto OP9 bone marrow stroma to allow each single ETP to proliferate for 54 h, after which we split the expanded cell cultures and transferred them for an additional week to OP9 stroma and OP9 stroma expressing the Notch ligand Delta-like 1 (OP9-DL1 stroma) to promote differentiation into B cells and combined differentiation into T cells and myeloid cells, respectively.

**Figure 2** ETPs have combined T cell, B cell and GM lineage potential. (a) Single-cell analysis of the expression of genes associated with lymphoid cells, myeloid cells and megakaryocytes–erythroid cells, by purified Flt3hi ETPs from newborn mice, among cells that express Kit (left; 96–98% of total cells; n = 176 cells). Right, frequency of ETPs with combined lymphoid-GM gene expression based on coexpression (Ly-GM) of one or more genes of the lymphoid program (Ilt7r and sterile IgH) and myeloid-GM program (Csf1r and Mpo) but not of the MKE program (Gata1 and Epor). (b) Flow cytometry and morphology analysis of a clone from a single Flt3+ETP cell from a newborn wild-type mouse, with combined T cell–B cell (white arrowhead) and myeloid (black arrowhead) lineage potential. Scale bar, 5 μm. (c,d) Cloning frequency (left) of ETPs generating CD45+ cells (open bars) and CD45− cells that are also positive for T cell, B cell and/or GM markers (filled bars), assessed for wild-type mice (c) or vavP–Mcl1-trangenic mice (d). Right, lineage distribution of clones from single ETPs from wild-type mice (c; n = 132 cells) or vavP–Mcl1–trangenic mice (d; n = 167 cells). Data are from two experiments (a,d) or three experiments (b,c; mean and s.d. in a and mean and s.e.m. in c,d).
Although the frequency of ETP-derived clones with detectable GM potential was lower than that of assays in which only the GM differentiation of ETPs was promoted (Fig. 1f), we demonstrated the existence of single Lin−CD4−CD8α−CD25−c-Kit+bFlt3+ ETPs with combined T cell, B cell and GM lineage potential (9.2% of clones with a lineage ‘readout’; Fig. 2b,c and Supplementary Fig. 7a). In fact, we tracked all the GM potential from wild-type ETPs to cells that not only had T cell potential, as demonstrated before⁶,⁷, but also had B cell lineage potential (Fig. 2c). Next we used ETPs purified from mice expressing Mcl1 (encoding the antiapoptotic protein Mcl-1) from the vavP transgenic vector²⁵ to evaluate whether enhanced cell survival could better sustain short-lived myeloid cells in the assay for combined myeloid and T lymphoid development. Whereas the B cell potential in thymuses from neonatal MclI-transgenic mice remained restricted to Lin−CD4−CD8α−CD25−c-Kit+bFlt3+ ETPs (Supplementary Fig. 7c), the frequency of ETPs that generated combined T cell–B cell–GM progeny was 20% of all single ETPs (relative to 9.2% of wild-type ETPs) producing one or more hematopoietic lineages (Fig. 2d and Supplementary Fig. 7b). These findings obtained with single-cell clonal assays established the existence of thymic ETPs with combined T cell, B cell and GM lineage potential.

**Lymphomyeloid ETPs are the most multipotent thymic progenitors**

We next explored whether the Lin−CD4−CD8α−CD25−c-Kit+bFlt3+ ETPs with combined T cell, B cell and GM lineage potential represented the most multipotent progenitors in the neonatal thymus. The lack of detectable MKe potential in Lin−CD4−CD8α−CD25−c-Kit+bFlt3+ ETPs did not rule out the possibility of the presence of rare pluripotent hematopoietic stem cells or progenitor cells in the neonatal thymus. Thus, we first used highly sensitive flow cytometry to investigate the expression of three antigens, CD150 (ref. 26), CD201 (ref. 27) and Mpl28, each with high expression on most if not all HSCs as well as multipotent progenitors with sustained MKe potential. None of these antigens was expressed on Lin−CD4−CD8α−CD25−c-Kit+bFlt3+ ETPs (Fig. 3a). Similar to a subfraction of bone marrow LMPPs, all ETPs expressed Rag1, as assessed through the use of a green fluorescent protein (GFP) reporter under control of the Rag1 promoter²⁹, and most expressed the chemokine receptor CCR9 (Fig. 3b), in agreement with published studies of ETPs, LMPPs and CLPs from adult mice³⁰,³¹. No bone marrow HSCs expressed either the Rag1-driven GFP reporter or CCR9 (Fig. 3b).

Whole thymocytes from neonatal mice transplanted intravenously or intrafemorally (to bypass potential changes in bone marrow–homing properties after entry into the thymus) into irradiated wild-type mice transiently reconstituted T cells and small amounts of B cells (Fig. 3c,d) but failed to sustain any long-term multilineage reconstitution (Fig. 3e,f), in further support of the idea that the postnatal thymus does not contain any HSCs. To enhance the detection of HSCs potentially present in the thymus, we depleted whole-thymocyte samples of CD4+ and CD8+ cells and transplanted these into recipient mice intravenously or intrafemorally (Fig. 3g,h). The absence of long-term...
ETPs are closely molecularly related to bone marrow TSPs

Because our findings indicated that Lin<sup>−</sup>CD4<sup>−</sup>CD8<sup>−</sup>CD25<sup>−</sup>-c-Ki<sup>−</sup>Fli1<sup>−</sup>F<sup>+</sup>ETPs in the neonatal thymus had the same lineage potential as Lin<sup>−</sup>Sca-1<sup>−</sup>-c-Ki<sup>−</sup>Fli1<sup>−</sup>F<sup>+</sup>LMPPs expressing the Rag1-driven GFP reporter (which also had high expression of interleukin 7 receptor α (IL-7Rα); Supplementary Fig. 9a) in the bone marrow<sup>13,32</sup>, we next investigated the molecular relationship between ETPs and IL-7Rα<sup>+</sup> LMPPs and HSCs in the bone marrow of neonatal mice. We also compared ETPs with the next stages of lineage restriction in the thymus: Lin<sup>−</sup>CD4<sup>−</sup>CD8<sup>−</sup>CD25<sup>−</sup>-c-Ki<sup>−</sup>Fli1<sup>−</sup>F<sup>+</sup>DN2 cells, which sustain combined T cell and GM lineage potential but no B cell lineage potential<sup>9,9</sup>, and Lin<sup>−</sup>CD4<sup>−</sup>CD8<sup>−</sup>DN3 cells, which represent the first T cell–restricted progenitors in the thymus<sup>33</sup>. Global gene-expression analysis done as described before<sup>34</sup>, demonstrated that the gene-expression profile of ETPs clustered much closer to that of IL-7Rα<sup>+</sup> LMPPs in the bone marrow than to that of thymic DN2 or DN3 progenitors or bone marrow HSCs. Moreover, the gene-expression profile of LMPPs clustered closer to that of ETPs than to that of HSCs, and that of DN2 cells was closer to that of DN3 cells than to that of ETPs (Fig. 4a). Because CLPs have been suggested to be candidate TSPs<sup>7</sup> and have been shown to not only have lymphoid potential but also sustain some myeloid potential similar to LMPPs<sup>35</sup>, we also compared the molecular profiles of ETPs with those of the two candidate TSP populations in the bone marrow: IL-7Rα<sup>+</sup> LMPPs and Lin<sup>−</sup>CD19<sup>−</sup>B220<sup>−</sup>Sca-1<sup>−</sup>-c-Ki<sup>−</sup>Fli1<sup>−</sup>IL-7Rα<sup>+</sup>Ly<sup>−</sup>D<sup>+</sup> CLPs<sup>36</sup> (Fig. 4b). The molecular profiles of ETPs from neonatal mice clustered closely with those of CLPs as well as those of LMPPs and were more distant from those of HSCs, DN3 cells and also pro-B cells. Moreover, the molecular profiles of ETPs from neonatal and adult mice clustered closely together with those of LMPPs and CLPs (Fig. 4b). These findings established a close molecular relationship between Lin<sup>−</sup>CD4<sup>−</sup>CD8<sup>−</sup>CD25<sup>−</sup>-c-Ki<sup>−</sup>Fli1<sup>−</sup>F<sup>+</sup>ETPs in neonatal mice with those of CLPs and LMPPs.

Figure 4 The gene expression of ETP clusters closer to that of candidate TSPs in the bone marrow than to that of other thymic progenitors. Two- and three-dimensional principal-component analysis of normalized global gene-expression profiles of purified HSCs, IL-7Rα<sup>+</sup> LMPPs, ETPs, DN2 cells and DN3 cells from neonatal mice (n = 3 replicates), and purified HSCs (n = 3 replicates), IL-7Rα<sup>+</sup> LMPPs (n = 3 replicates), CLPs (n = 4 replicates), ETPs (n = 3 replicates), DN3 cells (n = 3 replicates) and pro-B cells (n = 3 replicates) from neonatal mice (b) and ETPs from adult mice (b; n = 2 replicates), with 1,600–2,000 cells per replicate (derivation of gene lists). On the left, (b) numbers in boxes indicate normalized median ± IQR. On the right, (b) y values for each population measured in the first two principal components. Data are representative of two experiments (a) or two to three experiments (b).

Figure 5 ETPs, IL-7Rα<sup>+</sup> LMPPs and CLPs have closely related T cell– and myeloid-lineage transcriptional profiles. Expression of genes associated with the T cell lineage (a) or GM lineage (b) by purified HSCs (n = 6 replicates), IL-7Rα<sup>+</sup> LMPPs (n = 6 replicates), CLPs (n = 4 replicates), ETPs (n = 6 replicates), DN2 cells (n = 3 replicates) and DN3 cells (n = 6 replicates) from neonatal mice, and ETPs from adult mice (n = 2 replicates), with 1,600–2,000 cells per replicate (derivation of gene lists; Online methods and Supplementary Note). Numbers in this image (expression values) were obtained by the robust multivararray average method; darker shading indicates higher expression; lighter shading indicates lower expression. Dendrograms above indicate relationships between samples according to their gene profiles. Data are representative of two to four experiments.
receptors critical for migration to the thymus30,31, these genes showed considerable upregulation in IL-7Rα+ LMPPs, in further support of the idea that LMPPs are TSPs. Collectively, these results demonstrated that ETPs and candidate TSPs such as LMPPs and CLPs had closely related gene-expression profiles, which reinforced the proposal that ETPs are more probably derived from lympho-myeloid–restricted TSPs than from HSCs in the bone marrow.

**DISCUSSION**

Here we have identified ETPs in the neonatal thymus with combined T cell, B cell and GM lineage potential but no MkE lineage potential, and we have demonstrated a close functional and molecular link between ETPs and candidate TSPs in the bone marrow. The observation that ETPs lacked MkE potential was notable for reconciliation of the ongoing debate about the roadmap for commitment to different hematopoietic lineages, as the classical model for such commitment indicates that the first lineage-commitment step of HSCs results in strict separation of the myeloerythroid- and lymphoid-commitment pathways1,14. According to that model, any cell with combined lymphoid and GM potential should also have MkE potential. However, we found that Lin−CD4+CD8α−CD25+ c-KithiFlt3hi ETPs with combined T cell, B cell and GM lineage potential were devoid of megakaryocyte or erythroid lineage potential. These cells coexpressed, at the single-cell level, genes related to lymphoid cells and granulocytes-monocytes, but not megakaryocytes or erythroid cells, similar to LMPPs with identical lineage potentials in the bone marrow13,32,34. Thus, our study has provided further support for a myeloid-based lineage-commitment model2,13,15–17 by demonstrating the existence of T cell–B cell–GM–restricted progenitors in the postnatal thymus. Such cells have been identified before in the bone marrow and fetal liver13,34.
The real frequency of Lin^−CD4^−CD8α^−CD25^−c-Kit^hi^Flt3^hi^ ETPs from neonatal mice with T cell–B cell–GM potential is probably higher than we were able to demonstrate. Analysis of clones grown from single ETPs from newborn mice demonstrated that most ETPs with T cell potential simultaneously had B cell potential, but less than 20% of these also showed GM potential in wild-type mice, although under optimized GM conditions more than 50% of ETPs demonstrated GM potential. We speculated that the lower detection of cells of the GM lineages in the multilineage clonal assay reflected the short lifespan of vulnerable myeloid cells, and in agreement with that, transgenic expression of the antiapoptotic protein Mcl-1 enhanced the generation of myeloid cells from ETPs with combined T cell, B cell and GM potential in neonatal mice, most probably through the enhanced survival of myeloid cells. Our findings also suggest that the T cell–B cell–GM restricted progenitor identified is the most multipotent progenitor in the neonatal thymus, as we did not detect any MkE lineage potential or MkE-specific gene expression among highly purified ETPs. Furthermore, we also demonstrated that there were no phenotypic or in vivo reconstituting HSCs or multipotent progenitors in the neonatal thymus.

Published studies have suggested that the earliest fetal thymic progenitors in the embryo have combined T cell and myeloid lineage potential but no B cell lineage potential10,11, which raises the possibility that the progenitors that seed the embryonic thymus might be distinct from and more committed than those in the postnatal thymus. In contrast to the seeding of the neonatal thymus, which was the focus of our study, it remains unclear if the adult thymus is also seeded with ETPs with combined T cell, GM and B cell lineage potential. As thymopoiesis is much less active in adult thymus than in newborn thymus, it can be predicted that the most multipotent ETPs are much more infrequent in adult thymus than in the neonatal thymus. Although the low frequency of B cell lineage potential of ETPs from adult mice reported before6,20 and confirmed here does not allow definitive demonstration of the combined T cell, GM and B cell lineage potentials of ETPs from adult mice with the present clonal lineage-potential assay, it is notable that rare Lin^−CD4^−CD8α^−CD25^−c-Kit^hi^Flt3^hi^ ETPs were the only thymocytes from adult mice with B cell potential. In addition, we found that ETPs from adult mice, like ETPs from neonatal mice, had GM potential but not megakaryocyte potential, and global gene-expression analysis indicated a close molecular relationship between ETPs from neonatal mice and those from adult mice. Collectively, these data suggest that the adult thymus, like neonatal thymus, might also be seeded by rare T cell–B cell–GM progenitors, which we were unequivocally able to identify in neonatal thymus. Nevertheless, there are distinct differences among HSCs and hematopoietic progenitor cells from fetal, neonatal and adult mice. The regulated migration of TSPs to the thymus might also differ in the fetus, newborn and adult, so it remains possible that the lineage potentials of TSPs from embryos, newborns and adults might be different.

Although our studies have established the extent of ETP multipotentiality (T cell–B cell–GM) and the close phenotypic and molecular relationship of ETPs, LMPPs and CLPs with the same lineage potentials in the bone marrow13,32,34,35, they do not exclude the possibility that other candidate progenitors in the bone marrow might seed the thymus40. A published study has suggested that T cell–GM–restricted progenitors might exist in the bone marrow41, although such progenitors remain to be purified and characterized in further detail. The GM potential of ETPs is limited, and studies have suggested that it has little if any functional relevance to these progenitors’ acting as myeloid progenitors in the thymus35,42. Likewise, it seems unlikely that ETPs have any important physiological role as B cell progenitors. Instead, the importance of these sustained lineage potentials of ETPs is to provide a better understanding of the lineage-restriction steps required for lineage commitment from pluripotent HSC in the bone marrow to a T cell–restricted progenitor in the thymus. Specifically, progenitors with combined T cell–B cell–GM potential, such as LMPPs and CLPs, are derived in the bone marrow from HSCs that have shut down the MkE transcriptional programs and lineage potential. Unlike HSCs, LMPPs and CLPs upregulate CCR9, which enables their transfer to the thymus30,31. Migration to the thymus seems critical for the next T cell lineage–restriction steps, first to a T cell–GM progenitor8,9 and finally to a fully T cell–restricted progenitor.

Our studies have provided new insight into the normal stepwise process of commitment to the T cell lineage in the bone marrow and thymus. In addition, they are also relevant to the clinically, phenotypically and molecularly distinct group of mixed T cell–GM acute lymphoblastic leukemias that are mostly observed in children but also seen in adults, called ‘ETP leukemias’42. Furthermore, the sustained B cell potential of ETPs might explain why the MLL–AF4 fusion oncogene that is highly specific for human B cell malignancies can give rise to B cell malignancies even if targeted to thymic progenitors44.

METHODS

METHODS and any associated references are available in the online version of the paper at http://www.nature.com/natureimmunology/.

Accession code. GEO: microarray data, GSE29382.

Note: Supplementary information is available on the Nature Immunology website.
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ONLINE METHODS

Animals. All mice were bred and maintained at the Oxford Biomedical Services and all experiments were done with the approval of the UK Home Office. Mice with Rag1-driven GFP expression were provided by N. Sakaguchi; mice with eGFP expression driven by the gene encoding lysozyme M were provided by T. Graf; vanP-Mef2–transgenic mice were provided by S. Cory; CD94int/cis/Reh mice (provided by M. Reth), which express Cre from the Cd79a promoter, were crossed with mice (provided by S. Srinivas) expressing enhanced yellow fluorescent protein from the ubiquitously expressed Rosa26 locus. Transgenic mice in which all platelets express eGFP were generated by bacterial artificial chromosome recombinant engineering in bacteria. For this, sequence encoding eGFP followed by a polyadenylation signal was inserted at the initiation codon in exon 1 of Vwf (the gene encoding the von Willebrand factor homolog), thereby replacing exon 2. Vwf-eGFP–transgenic mice were generated by intracytoplasmic sperm injection (C.N. and S.E.W.J., data not shown). Mice were backcrossed for many generations onto a C57BL/6 background.

Transplantation. Thyocytes from newborn-neonatal mice (total thyocytes or thymocyte samples depleted of cells of the CD4+ and CD8+ lineage; from one donor to each recipient) or sorted Flt3+ ETPs from wild-type or backcrossed for many generations onto a C57BL/6 background. thereby replacing exon 2. Vwf-eGFP–transgenic mice were generated by intracytoplasmic sperm injection (C.N. and S.E.W.J., data not shown). Mice were backcrossed for many generations onto a C57BL/6 background.

Purification of stem cells and progenitor cells. A FACSAria II (BD Biosciences) was used for all cell sorting, with a total cell-sorting purity of 99%.

Microarray analysis. Global gene expression was analyzed in HSCs (Lin−Sca-1+c-KithiFlt3−), IL-7Rα+ LMPPs (Lin−Sca-1+c-KithiFlt3hiIL-7Rα−), ETPs (Lin−CD4−CD8−CD25−c-KithiFlt3hi), DN2 cells (Lin−CD44−CD25+c-Kithi), DN3 cells (Lin−CD44−CD25+), CLPs (Lin−CD19−B220−Sca-1−c-KithiFlt3+IL-7Rα−Ly6D+) and pro-B cells (CD19+R20+CD43−c-Kithi) from 1-week-old mice and ETPs from adult mice. For all cell populations, two to four individually sorted samples from different pools of mice were prepared. Cells (1,600–2,000 per sample) were sorted directly into TRIzol reagent and RNA was extracted according to the manufacturer’s instructions (Life Technologies). With the same total amount of input RNA, samples were amplified with a WT-Ovation Pico RNA Amplification System followed by WT Ovation cDNA Biotin Module V2 for cDNA labeling (NuGEN) and fragmentation, and were finally hybridized to Affymetrix Mouse Genome 430 2.0 arrays by standard protocols (Affymetrix) at the Stanford Protein and Nucleic Acid facility. Data were normalized by the robust multi-array averages method of the affy Bioconductor/R package. The R package was also used for subsequent data analysis (PCA and cluster analysis; Supplementary Note).

In vitro cultures. GM and megakaryocyte lineage potential was analyzed as described (Supplementary Table 5). Megakaryocyte potential was additionally evaluated with a Megacollagen-based assay (StemCell Technologies). ETPs (2 × 105) or unfractionated control bone marrow cells (1 × 105) were plated in Megacollagen media supplemented with growth factors (Supplementary Table 5). Megakaryocyte colonies were evaluated after 7 d by acetylsalicylic-acid staining according to manufacturer’s instructions (Sigma).

For the evaluation of erythroid potential, ETPs (5 × 104 to 1 × 105) and Lin−Sca-1+c-KithiFlt3− control bone marrow cells (50) were seeded in complete methylcellulose (GF M3434; StemCell Technologies). Cultures were evaluated after 8–11 d with 2,3-diaminophenol red staining (Sigma) as described.

OP9 and OP9-DL1 stromal cells were provided by A. Cumano. For analysis of individual GM, B cell, T cell and natural killer cell lineage potential, single ETPs were sorted by flow cytometry onto monolayers of OP9 or OP9-DL1 stromal cells as described (Supplementary Table 5). Myeloid lineages formed on OP9 stroma cocultures were evaluated after 6 d by morphology analysis of slides stained with May-Grünwald (Sigma) and Giemsa (Fluka).

For analysis of combined lineage potential, single cells were sorted into individual wells and cultured with human Flt3 ligand, and Chinese hamster ovary cells. The total number of cells plated unless otherwise stated.

Fluojo analysis software (TreeStar) was used for subsequent data analysis. Unfractionated bone marrow thyocytes, or bone marrow cells or thyocytes positively selected for CD117 expression by MACS bead separation with anti-CD117 immunomagnetic beads (Miltenyi Biotec) were used in cell-sorting experiments (cells pooled from groups of mice). Cells were incubated with Fc Block and stained with antibodies to mouse antigens (Supplementary Table 2). Fluorescence-minus-one controls as well as negative populations were used as gate-setting controls.

Microarray analysis. Global gene expression was analyzed in HSCs (Lin−Sca-1+c-KithiFlt3−CD48+CD150+), IL-7Rα+ LMPPs (Lin−Sca-1+c-KithiFlt3hiIL-7Rα−), ETPs (Lin−CD4−CD8−CD25−c-KithiFlt3hi), DN2 cells (Lin−CD44−CD25+c-Kithi), DN3 cells (Lin−CD44−CD25+), CLPs (Lin−CD19−B220−Sca-1−c-KithiFlt3+IL-7Rα−Ly6D+) and pro-B cells (CD19+R20+CD43−c-Kithi) from 1-week-old mice and ETPs from adult mice. For all cell populations, two to four individually sorted samples from different pools of mice were prepared. Cells (1,600–2,000 per sample) were sorted directly into TRIzol reagent and RNA was extracted according to the manufacturer’s instructions (Life Technologies). With the same total amount of input RNA, samples were amplified with a WT-Ovation Pico RNA Amplification System followed by WT Ovation cDNA Biotin Module V2 for cDNA labeling (NuGEN) and fragmentation, and were finally hybridized to Affymetrix Mouse Genome 430 2.0 arrays by standard protocols (Affymetrix) at the Stanford Protein and Nucleic Acid facility. Data were normalized by the robust multi-array averages method of the affy Bioconductor/R package. The R package was also used for subsequent data analysis (PCA and cluster analysis; Supplementary Note).

Single-cell and quantitative PCR. Multiplex single-cell RT-PCR of single cells sorted from ETPs from newborn mice was done as described (Supplementary Table 3). The BioMark 48.48 Dynamic Array platform (Fluidigm) and TaqMan Gene Expression assays (Applied Biosystems) were used as described for multiplex quantitative real-time PCR analysis of sorted populations. For each cell population, two biological replicates (25 cells per replicate; individually sorted samples from different mice) or single cells (88 cells per experiment) were prepared. TaqMan assays were also used (Supplementary Note and Supplementary Table 4).

In vitro cultures. GM and megakaryocyte lineage potential was analyzed as described (Supplementary Table 5). Megakaryocyte potential was additionally evaluated with a Megacollagen-based assay (StemCell Technologies). ETPs (2 × 105) or unfractionated control bone marrow cells (1 × 105) were plated in Megacollagen media supplemented with growth factors (Supplementary Table 5). Megakaryocyte colonies were evaluated after 7 d by acetylsalicylic acid staining according to manufacturer’s instructions (Sigma).

For the evaluation of erythroid potential, ETPs (5 × 104 to 1 × 105) and Lin−Sca-1+c-KithiFlt3− control bone marrow cells (50) were seeded in complete methylcellulose (GF M3434; StemCell Technologies). Cultures were evaluated after 8–11 d with 2,7-diaminofluorene staining (Sigma) as described.

OP9 and OP9-DL1 stromal cells were provided by A. Cumano. For analysis of individual GM, B cell, T cell and natural killer cell lineage potential, single ETPs were sorted by flow cytometry onto monolayers of OP9 or OP9-DL1 stromal cells as described (Supplementary Table 5). Myeloid lineages formed on OP9 stroma cocultures were evaluated after 6 d by morphology analysis of slides stained with May–Grünwald (Sigma) and Giemsa (Fluka). For analysis of combined lineage potential, single cells were deposited (ensured as described above) onto OP9 cells supplemented with human Flt3 ligand, mouse stem cell factor and human IL-7. Approximately 34 h later, cocultures were split and placed onto a secondary layer of OP9 cells with the same conditions to promote B cells and plated onto layer of OP9-DL1 cells with various cytokines (human Flt3 ligand, mouse stem cell factor, human IL-7, human IL-6, mouse IL-3, human granulocyte colony-stimulating factor, mouse granulocyte-macrophage colony-stimulating factor and human colony-stimulating factor 1) to promote differentiation into T cells and myeloid cells. The timing of the first culture period was optimized to ensure the highest frequency possible for all lineages in the combined-lineage-potential assay. One week after transfer to the second culture, clones were evaluated for the presence of CD19+ B cells, CD4+CD8−NK1.1+ and/or Thy-1.2+CD25+NK1.1+ T cells and Mac-1+CD48+myeloid cells. Clones expressing Mac-1 and/or F4/80 were additionally evaluated by morphology. The analysis was optimized to the earliest time point at which T cells could be detected and myeloid progeny would still be possible to detect. However, as the differentiation time is longer for lymphoid cells, the production of myeloid progeny is probably underestimated.

All results of single-cell assays are presented as total frequency relative to the total number of cells plated unless otherwise stated.

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