LETTERS

Adult T-cell progenitors retain myeloid potential

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During haematopoiesis, pluripotent haematopoietic stem cells are sequentially restricted to give rise to a variety of lineage-committed progenitors. The classical model of haematopoiesis postulates that, in the first step of differentiation, the stem cell generates common myelo-erythroid progenitors and common lymphoid progenitors (CLPs). However, our previous studies in fetal mice showed that myeloid potential persists even as the lineage branches segregate towards T and B cells[1–6]. We therefore proposed the 'myeloid-based' model of haematopoiesis[7,8], in which the stem cell initially generates common myelo-erythroid progenitors and common myelo-lymphoid progenitors. T-cell and B-cell progenitors subsequently arise from common myelo-lymphoid progenitors through myeloid-T and myeloid-B stages, respectively. However, it has been unclear whether this myeloid-based model is also valid for adult haematopoiesis. Here we provide clonal evidence that the early cell populations in the adult thymus contain progenitors that have lost the potential to generate B cells but retain substantial macrophage potential as well as T-cell, natural killer (NK)-cell and dendritic-cell potential. We also show that such T-cell progenitors can give rise to macrophages in the thymic environment *in vivo*. Our findings argue against the classical dichotomy model in which T cells are derived from CLPs; instead, they support the validity of the myeloid-based model for both adult and fetal haematopoiesis.

By using a clonal analysis of lympho-haematopoietic cells in fetal mice, we previously showed the existence of bipotent progenitors of myeloid-T and myeloid-B lineages, in addition to multipotent and unipotent progenitors[1–5]. However, our clonal analysis failed to detect any of the CLP-type progenitors. More recently we discovered the presence of common myelo-lymphoid progenitors in fetal liver[6], and thus came to propose a myeloid-based model of haematopoiesis[7,8], in which myeloid potential is retained in erythroid, T-cell and B-cell branches (Supplementary Fig. 1). By contrast, CLPs have been reported to be present in a bone marrow subpopulation in the adult mouse[9]. To reconcile these disparate findings between fetal liver and adult bone marrow, the concept has emerged that fetal haematopoiesis differs from adult haematopoiesis, with the CLP stage existing only during adult haematopoiesis[10–13].

We decided to examine critically whether the CLP stage exists or not in the developmental pathway from the stem cell to T cells during adult haematopoiesis. Previous studies have reported that a subpopulation of adult thymus cells that has no B-cell potential still retains myeloid and T-cell potential[14–16]. These findings suggest that B-cell potential is shut off earlier than myeloid potential, providing support for the myeloid-based model. However, lacking clonal evidence, these studies failed to provide sufficient impact to change the existing model in haematopoiesis.

We first examined whether early thymic progenitors are able to produce granulocytes and/or macrophages. The $CD4^-CD8^-$ (double-negative) fraction of adult thymus cells was subdivided into $c\text{-}Kit^+CD25^-$, $c\text{-}Kit^+CD25^+$, $c\text{-}Kit^-CD25^+$ subsets, which are designated as DN1, DN2 and DN3 subsets, respectively (Fig. 1a). We cultured DN1, DN2 and DN3 cells from adult thymus, and lineage-marker $(Lin)^-c\text{-}Kit^+Sca\text{-}1^+$ (LKS) cells from bone marrow

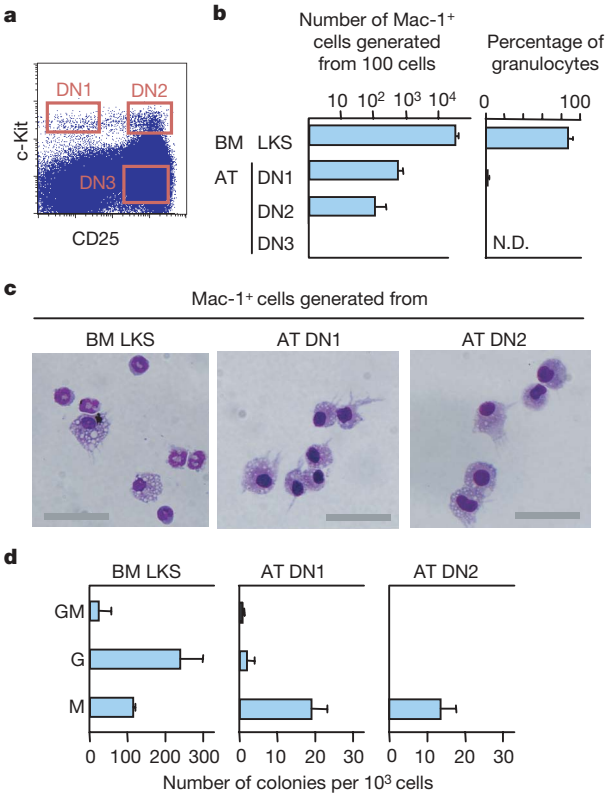

Figure 1 | Early progenitors in the adult thymus retain the potential to generate macrophages. **a**, The c-Kit/CD25 profile of the Lin^- fraction of adult thymus (AT) cells from eight-week-old mice. **b**, **c**, Cells from the indicated subpopulations of AT and LKS bone marrow (BM) cells were cultured (100 cells per well) for seven days with the PA6 stromal cell line in the presence of granulocyte colony-stimulating factor. Numbers of $Mac\text{-}1^+$ cells per well (mean and s.d. for triplicate cultures) are shown (**b**, left panel). Generated $Mac\text{-}1^+$ cells were sorted, spun onto glass slides, and Wright-stained. The proportion of cells with granulocytic morphology (mean and s.d. for triplicate culture) (**b**, right panel) and representative photomicrographs of $Mac\text{-}1^+$ cells (**c**) are shown. Scale bars, 50 μm. **d**, A CFU-C assay was performed with DN1 and DN2 AT cells and also with LKS bone marrow cells. Colony numbers (mean and s.d. for triplicate cultures) per 10^3 cells are shown. GM, colonies containing granulocytes and macrophages; G, granulocyte colonies; M, macrophage colonies.

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as a control, at 100 cells per well, on a monolayer of the stromal cell line PA6, which preferentially supports the generation of myeloid cells[17]. Mac-1$^+$ cells were generated in cultures of DN1 and DN2 cells, although many fewer cells were recovered than from cultures of LKS cells (Fig. 1b). Although LKS progenitors gave rise mainly to granulocytes, only a few were generated from DN1 cells, and no granulocytes at all were generated from DN2 cells (Fig. 1b, c). The results were confirmed by a colony-forming units in culture (CFU-C) assay (Fig. 1d). Similar findings were obtained with fetal thymus progenitors (Supplementary Fig. 2). These results indicate that the myeloid potential of thymic progenitors is primarily specified to macrophages. Expression of the transcription factor PU.1 (also called Sfpi1) has been implicated in the myeloid potential of early T-cell progenitors[18]. Indeed, RT–PCR analysis confirmed that DN1 and DN2 cells express PU.1, but PU.1 is downregulated at the DN3 stage (Supplementary Fig. 3).

To examine T-cell and myeloid potential of thymic progenitors at a single-cell level, we developed a clonal assay system with the stromal cell line TSt-4/DLL1 (ref. 19), which supports the generation of both T cells and myeloid cells (Fig. 2a). As a source of progenitor cells, we used green fluorescent protein (GFP)-transgenic mice ('green mouse')[20]. In this green mouse strain, macrophages and dendritic cells express GFP highly, whereas differentiated T cells are GFP$^-$, making the detection of GFP$^+$ macrophages easier. When 192 DN1 cells were individually cultured with TSt-4 cells, 18 wells containing only macrophages were detected and no B-cell-containing wells were observed (Fig. 2b, middle panel). In co-cultures with TSt-4/DLL1 cells, 136 of 192 wells seeded with single DN1 cells showed growth of progeny cells (Fig. 2b, right panel). Of these 136 wells, 13 contained both T cells and macrophages. Macrophages were seen as large GFP$^+$ cells in these cultures (Fig. 2c). Green cells with dendritic morphology were also seen in almost all macrophage-T wells (Fig. 2d). Because early thymic progenitors are able to produce dendritic cells[21,22], it is important to distinguish macrophages from dendritic cells. A majority of large GFP$^+$ cells in these cultures contained phagosomal vacuoles (Supplementary Fig. 4a), and more than half of these cells were able to engulf latex beads (Fig. 2e), in a manner reminiscent of macrophages. Flow cytometric analysis confirmed the

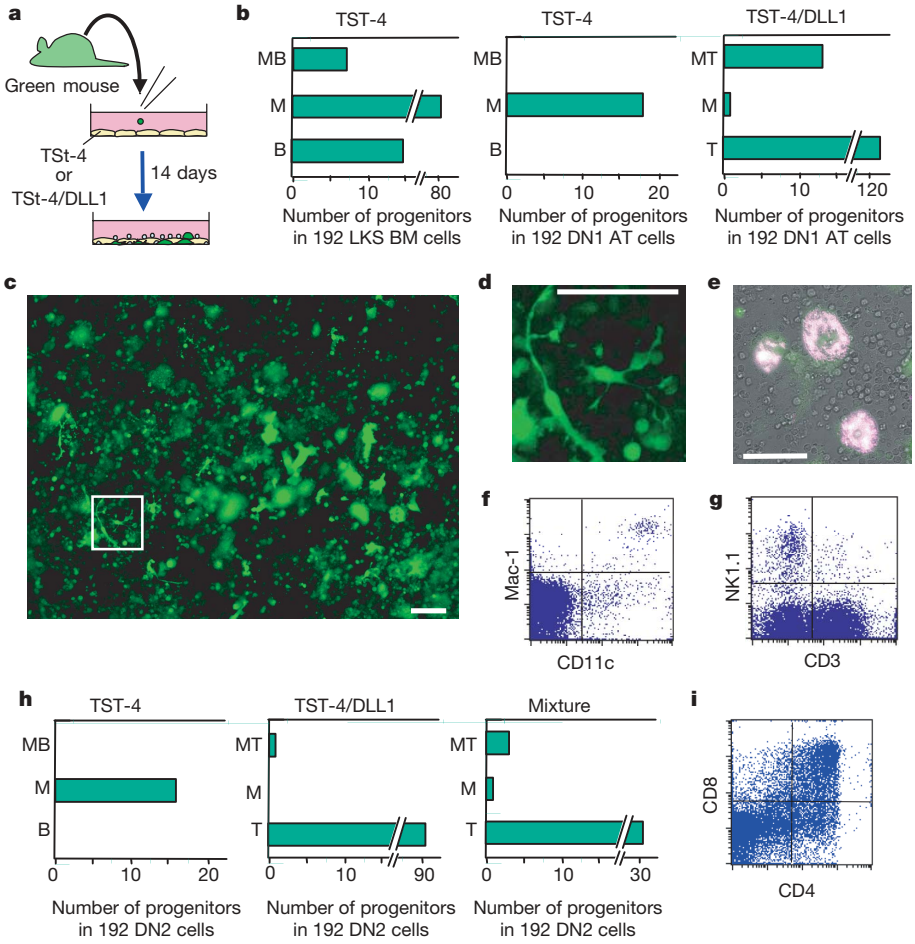

Figure 2 | T-cell progenitors in adult thymus that have lost B-cell potential retain macrophage potential. **a**, Experimental procedure for clonal analysis. Single cells were cultured for 14 days with the stromal cell line TSt-4 or TSt-4/DLL1 in the presence of stem cell factor, interleukin-7 and granulocyte/macrophage colony-stimulating factor. **b**, A total of 192 individual LKS bone marrow (BM) cells (left panel) and 192 DN1 adult thymus (AT) cells (middle panel) were cultured with TSt-4. Individual DN1 AT cells (total 192 cells) were also cultured with TSt-4/DLL1 (right panel). Seeded cells were classified according to the cells generated in each clonal culture: progenitors that generated both macrophages and B cells (MB), both macrophages and T (MT), only macrophages (M), only B cells (B) and only T cells (T). **c**, Photomicrograph of cells in a macrophage-T clone in the TSt-4/DLL1 cultures. Macrophages are seen as large GFP$^+$ cells. Most of the T-lineage cells are GFP$^-$ and are therefore not visible here. In this particular well, the total number of large GFP$^+$ cells (diameter more than 20 μm) was about 100, whereas that of T-lineage cells was about 10^5. Scale bar, 100 μm. **d**, Enlarged image of the area indicated in **c**. Scale bar, 100 μm. **e**, Photomicrograph taken 6 h after the addition of latex beads (1 μm diameter) to cells in a macrophage-T clone. Scale bar, 100 μm. **f**, **g**, Flow cytometric profiles of the cells recovered from a macrophage-T clone. Macrophages express Mac-1 and CD11c (**f**). NK-cell generation was determined by the appearance of CD3$^-$NK1.1$^+$ cells (**g**). **h**, A total of 192 DN2 AT cells were individually cultured with TSt-4 (left panel), with TSt-4/DLL1 (middle panel), or with a mixture of TSt-4 and TSt-4/DLL1 (4:1 ratio) (right panel). **i**, Flow cytometric profile of cells in a macrophage-T clone from a single DN2 cell shown in the right panel of **h**.

presence of Mac-1$^+$ cells in macrophage-T clonal cultures (Fig. 2f). Although these Mac-1$^+$ cells also expressed CD11c, a commonly used marker for dendritic cells, we consider them to be macrophages because they expressed F4/80 highly and showed efficient phagocytic activity (Supplementary Fig. 4c, d). In addition, the Mac-1$^+$CD11c$^+$ cells were much larger than the Mac-1^{low}CD11c$^+$ cells (Supplementary Fig. 4e). The macrophage–T-cell containing wells always contained NK cells (Fig. 2g). Similar results were obtained with fetal thymus progenitors (Supplementary Fig. 5).

We next examined the macrophage potential of DN2 cells, which have been shown to have completely lost B-cell potential, by culturing them with TSt-4 cells. The frequency of macrophage-generating progenitors was nearly comparable to that of the DN1 cells (16 of 192) (Fig. 2h, left panel). By using a mixture of TSt-4 and TSt-4/DLL1cells at a 4:1 ratio, 36 wells showed growth of progeny cells; of these, three showed growth of both T cells and macrophages (Fig. 2h, right panel, Fig. 2i and Supplementary Fig. 6). These results demonstrate that T-cell progenitors retain substantial macrophage potential after shutting off B-cell potential.

We then investigated whether thymic T-cell progenitors can generate macrophages within the thymic environment. Deoxyguanosine-treated fetal thymus lobes, which had been seeded with 200 DN2 cells from a green mouse several days earlier, were transplanted under the kidney capsule of a human (h) CD3εTg mouse[23] (Supplementary Fig. 7a), which completely lacks T-lineage cells as a result of a cell-autonomous defect in early T-cell progenitors[24]. All grafts showed a well-developed thymic architecture (Supplementary Fig. 7b, upper left panel). Macrophages, revealed by staining with anti-F4/80 antibody, were found mainly in the cortex of the graft (Supplementary Fig. 7b, lower left panel), a distribution also seen in the normal thymus[25]. A substantial number of F4/80$^+$ macrophages (about 20%) were GFP$^+$. Such GFP$^+$ macrophages were not seen in control grafts seeded with double-positive thymocytes from green mice (Supplementary Fig. 7c). In a serial section of the thymic lobe reconstituted with DN2 cells, a significant proportion of CD11c$^+$ cells were found to express GFP (Supplementary Fig. 7d), indicating that DN2 cells also can generate dendritic cells in the thymus.

Macrophage–T bipotentiality was further examined at a single-cell level with the same system as described above (Fig. 3a). Fifty adult DN1 cells were tested; reconstitution of thymocytes was observed in 20 lobes. Of these, five lobes showed completely restored thymic organization (Supplementary Fig. 8a, b). A proportion of the F4/80$^+$ cells in these grafts expressed GFP (Fig. 3b), providing clonal evidence that T-cell progenitors are able to give rise to thymic macrophages. In addition, a fraction of the CD11c$^+$ dendritic cells was also GFP$^+$ (Supplementary Fig. 8c).

We next injected DN2 adult thymus cells (1,000 cells) from green mice directly into the thymus of non-irradiated wild-type recipient mice (Fig. 3c). In thymic lobes harvested two weeks later, GFP$^+$ cells were seen mainly in medullary regions (Fig. 3d, upper right panel). GFP$^+$ macrophages were detected in the cortex close to these medullary regions (Fig. 3d, lower left panel).

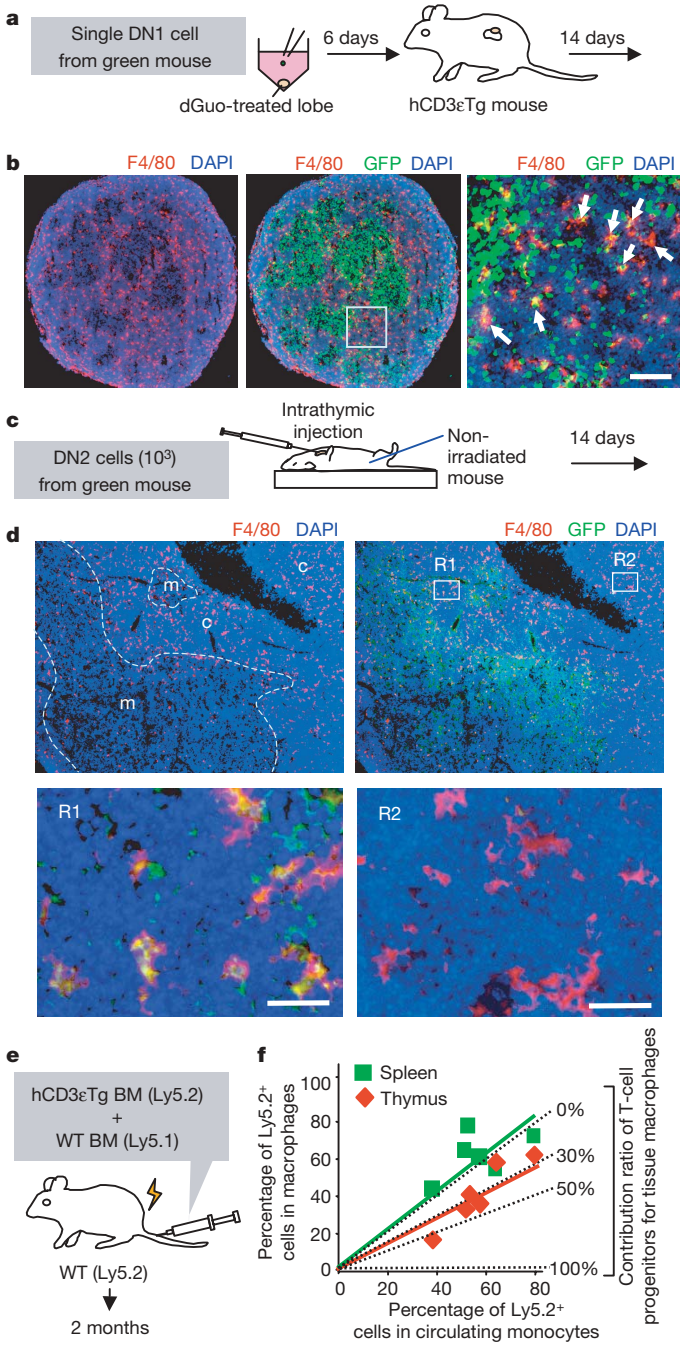

Figure 3 | *In vivo* evidence that T-cell progenitors contribute to the production of thymic macrophages. **a**, DN1 adult thymus cells from a green mouse were individually seeded onto deoxyguanosine-treated lobes and cultured for six days; the lobes were transplanted under the kidney capsule of adult hCD3εTg mice. The mice were killed two weeks later. **b**, A section of the graft was stained with F4/80 monoclonal antibody (red) and counterstained with 4,6-diamidino-2-phenylindole (DAPI; blue). The right panel shows an enlarged image for the gated area in the middle panel. Scale bar, 100 μm. Arrows indicate F4/80$^+$GFP$^+$ cells. **c**, DN2 adult thymus cells (1,000 cells) from a green mouse were injected directly into the thymus of non-irradiated wild-type mice and the thymuses were harvested 14 days later. **d**, A thymic section was stained with anti-F4/80 monoclonal antibody (red) and counterstained with DAPI (blue). The lower panels represent enlarged images of the areas indicated in the upper right panel as R1 (cortical region that is close to the medulla, which contains GFP$^+$ progeny of injected cells) and R2 (control cortical region). Scale bars, 50 μm. **e**, Experimental procedure to examine the contribution of T-cell progenitors to the production of thymic macrophages. A 1:1 mixture (5×10^6 for each) of wild-type bone marrow (BM) cells (Ly5.1) or bone marrow cells from hCD3εTg mice (Ly5.2) were injected intravenously into the tail vein of lethally irradiated wild-type Ly5.2 mice. The chimaeric mice were killed for analysis two months later. **f**, Derivation of a substantial proportion of thymic macrophages from thymic T-cell progenitors. Percentages of Ly5.2$^+$ cells in circulating monocytes (*x* axis) were determined by flow cytometry. The percentage of Ly5.2$^+$ cells among thymic and splenic F4/80$^+$ macrophages was determined by counting the cells in immunohistocheminally stained sections (*y* axis). Data from six chimaeric mice are plotted, and the line for splenic macrophages versus circulating monocytes (green line) and that for thymic macrophages versus circulating monocytes (red line) were fitted by least-squares analysis. The dotted lines represent the predicted outcome assuming that the contribution of T-cell progenitors to macrophage production in the tissue is 0%, 30%, 50% or 100%.

Because the cortex is a site where DN1 and DN2 cells expand[26], it is likely that these cells produce cortical macrophages in addition to T cells. In contrast, just as in other tissues, circulating monocytes may also be a source of thymic macrophages. To estimate the extent that thymic T-cell progenitors contribute to the production of thymic macrophages, we transplanted a 1:1 mixture of wild-type bone marrow cells (Ly5.1) and bone marrow cells from hCD3εTg mice (Ly5.2) into lethally irradiated wild-type Ly5.2 mice (Fig. 3e). Two months later, all thymic T cells were Ly5.1$^+$ as expected, because the Ly5.2$^+$ hCD3εTg bone marrow cells cannot give rise to T-lineage cells, including DN1 and DN2 cells (data not shown). The proportion of Ly5.2$^+$ cells among circulating monocytes fluctuated between recipients and ranged from 40% to 80%. If thymic macrophages are derived exclusively from circulating monocytes, the proportion of Ly5.2$^+$ thymic macrophages should be the same as that of the circulating Ly5.2$^+$ monocytes. In contrast, if thymic macrophages are produced exclusively from double-negative thymocytes, all thymic macrophages should be Ly5.1$^+$. The chimaerism ratios from individual mice for splenic macrophages versus circulating monocytes were plotted (green rectangles in Fig. 3f), and the derived line (green line) through the data points fell along the line representing the identical chimaerism ratio, indicating that almost all splenic macrophages are derived from circulating monocytes. By contrast, the line derived from the data points for the chimaerism ratio for thymic macrophages versus circulating monocytes (red line) had a smaller slope than that of the splenic macrophage line. From the inclination of the line it can be predicted that about 30% of thymic macrophages are derived from thymic progenitors that also can generate T cells.

We have shown here that a large majority of myeloid cells derived from thymic progenitors were macrophages. However, in the accompanying paper[27] the authors show that thymic progenitors produce granulocytes in addition to macrophages, in experiments both *in vitro* and *in vivo*. The inconsistency with the *in vitro* data could be due to a difference in the culture period, because a longer period is required for macrophages to develop in significant numbers. Moreover, flow cytometric analysis used in their *in vivo* studies could detect granulocytes more efficiently than macrophages, whereas the reverse is true for the histological analysis we used.

The present study therefore provides evidence, at a clonal level, for the existence of thymic T-cell progenitors that have lost B-cell potential but still retain macrophage potential (Fig. 4). We show further that this macrophage potential is not merely a phenomenon *in vitro* but occurs *in vivo* in the thymus. Our present findings indicate that the progenitor cell at the branch point of the T-cell and B-cell lineages retains macrophage potential, thus formally proving that the CLP stage is dispensable in the developmental pathway from haematopoietic stem cells to T cells. We propose that the myeloid-based model holds true for both fetal and adult haematopoiesis (Supplementary Fig. 1b). We emphasize that the fetal haematopoiesis described here is not the primitive haematopoiesis that takes place in the yolk sac but belongs in the same category as adult haematopoiesis, which is referred to as 'definitive' haematopoiesis, producing adult-type blood cells in fetal liver and subsequently in bone marrow. Because lineage restriction programmes in haematopoiesis should have been constructed step by step during evolution, it seems logical to assume that the same or at least a similar programme is used regardless of the anatomical site where haematopoiesis occurs.

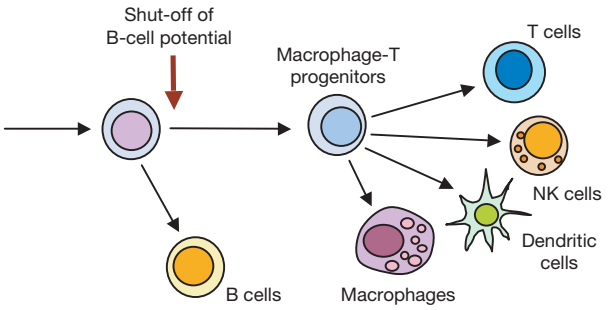

Figure 4 | T-cell progenitors retain macrophage potential after B-cell potential has been shut off. The present study shows that early T-cell progenitors in the adult thymus that have lost B-cell potential still retain a substantial ability to generate macrophages. It is therefore unlikely that the CLP stage exists along the normal physiological pathway towards T cells in adult haematopoiesis. Our present findings argue against the classic model of haematopoiesis in which the CLP is located at the branch point towards T and B-cell lineages, and strongly suggest that the myeloid-based model is applicable to both fetal and adult haematopoiesis.

METHODS SUMMARY

Transgenic mice carrying an enhanced GFP gene ('green mice')[20] were donated by M. Okabe. hCD3εTg mice[23] were donated by C. Terhorst. To assess the developmental potential of progenitors *in vitro*, co-culture with different stromal cells was mainly used. For the *in vivo* experiments, the following methods were used: transplantation of deoxyguanosine-treated fetal thymus lobes, seeded with progenitors to be tested, under the kidney capsule of hCD3εTg mice; intrathymic injection; and an *in vivo* competition assay in which a 1:1 mixture of bone marrow cells from B6 Ly5.1 mice and from hCD3εTg mice (Ly5.2) were injected intravenously into lethally irradiated wild-type Ly5.2 mice. The proportion of Ly5.2$^+$ cells among F4/80$^+$ cells was determined on immunohistochemically stained thymic sections from the chimaeric mice.

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

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

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Author Contributions H.W. performed most of experiments, with the assistance of R.S. and K.K. for immunohistochemical analysis and stromal cell co-cultures, respectively. Experiments shown in Fig. 1 and Supplementary Figs 2 and 3 were performed by K.M., and those in Supplementary Fig. 4d by T.I. Y.K. gave critical advice and comments in designing the experiments and writing the paper. H.K. designed the experiments and wrote the paper.

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METHODS

Mice. C57BL/6 (B6), B6Ly5.1 and Balb/c mice were purchased from CLEA Japan Inc. Transgenic mice on an B6 background carrying an enhanced GFP gene (EGFP Tg mice)[20] were donated by M. Okabe. hCD3εTg mice[23] were donated by C. Terhorst. Embryos at various stages of gestation were obtained from timed pregnancies. The day of observing the vaginal plug was designated as 0 days after coitus.

Antibodies. The following antibodies were purchased from BD Pharmingen: Ly5.1 (A20), Ly5.2 (104), c-Kit (2B8), Sca-1 (D7), erythroid lineage cells (TER119), Mac-1 (M1/70), Gr-1 (RB6-8C5), CD11c (HL3), B220 (RA3-6B2), Thy 1.2 (53-2.1), CD8 (53-6.7), CD4 (H129.19), NK1.1 (PK136), CD3ε (145-2C11), CD19 (1D3), anti-class II (M5/114), F4/80 (BM8), and CD25 (PC61). TER119, Mac-1, Gr-1, B220, CD19, NK1.1, CD3ε, CD4 and CD8 were used as Lin markers. For immunohistochemistry, the following antibodies were used: F4/80, biotinylated CD11c and biotinylated mouse anti-K8 (PROGEN) and ER-TR5 (donated by W. van Ewijk) as primary antibody, followed by goat anti-rabbit IgG-Texas Red conjugate, Alexa Fluor546 goat anti-rat IgG (H+L) conjugate or Alexa Fluor546 streptavidin conjugate (Molecular Probes), as secondary reagents.

Preparation of cells from progenitor populations. Single-cell suspensions of adult thymus cells were prepared from 8–12-week-old B6 mice as described previously[22]. Cells were then treated for 30 min at 37 °C with rabbit complement in the presence of anti-CD8 (3.155) and anti-CD3 (145-2C11) monoclonal antibody. The cells surviving the complement killing were used for cell sorting. The procedure for the preparation of progenitor enriched populations from fetal thymus and fetal liver has been described elsewhere[19]. Bone marrow cells were obtained from femurs of ten-week-old adult mice.

Growth factors. Recombinant murine (rm) stem cell factor (SCF), interleukin (IL)-1α, IL-3, IL-7, Flt-3L, granulocyte colony-stimulating factor (G-CSF), macrophage colony-stimulating factor (M-CSF) and granulocyte/macrophage colony-stimulating factor (GM-CSF) were purchased from R&D Systems.

Co-culture with stromal cells. To assess granulocyte/macrophage potential, 100 cells per well were cultured with PA6 (ref. 17) stromal cells in the presence of granulocyte colony-stimulating factor (10 ng ml^{-1}) for 7 days. Generated cells were counted and then stained with Mac-1; Mac-1^{+} cells sorted by fluorescence-activated cell sorting were centrifuged onto glass slides for Wright staining. For the detection of the B-cell and myeloid potential of progenitors, single cells from green mice were cultured for 14 days with TSt-4 cells in 96-well plates. Generation of macrophages was determined by fluorescence microscopy, and that of B cells was detected by subsequent flow cytometric analysis of CD19 expression. For the detection of T-cell and macrophage potential, TSt-4 cells that had been retrovirally transduced with the murine *Dll-1* gene (TSt-4/DLL1 cells)[19] were used. Medium was supplemented with SCF (1 ng ml^{-1}), IL-7 (0.5 ng ml^{-1}), Flt-3L (5 ng ml^{-1}) and GM-CSF (1 ng ml^{-1}). Generation of macrophages was determined as above, and that of T cells was detected by subsequent flow cytometric analysis. All co-cultures were maintained in RPMI 1640 medium (Sigma) supplemented with 10% (v/v) FCS, L-glutamine (2 mM), sodium pyruvate (1 mM), sodium bicarbonate (2 mg ml^{-1}), non-essential amino acid solution (0.1 mM) (Gibco BRL), 2-mercaptoethanol (50 μM), streptomycin (100 mg ml^{-1}), and penicillin (100 U ml^{-1}).

CFU-C assay. Cells (1,000 cells per dish) were cultured for seven days in triplicate dishes in α-minimal essential medium (GibcoBRL) containing 30% FCS, 1% methylcellulose, 1% bovine serum albumin, 2-mercaptoethanol (50 μM), L-glutamine (1 mM), SCF (10 ng ml^{-1}), IL-3 (10 ng ml^{-1}), GM-CSF (10 ng ml^{-1}), IL-1α (10 ng ml^{-1}), G-CSF (10 ng ml^{-1}) and M-CSF (10 ng ml^{-1}).

Phagocytosis assay. Carboxylate-modified microspheres (FluoSpheres, red, 1 μm; Molecular Probes) were added to the wells that were judged to contain macrophages. After 6 h, cells were gently washed with culture medium. All large GFP^{+} cells (more than 20 μm in diameter) with or without engulfed beads in each clonal culture were counted by fluorescence-microscopic observation. In the experiment shown in Supplementary Fig. 4d, sorted cells were incubated with Fluoresbrite YG beads (1 μm; Molecular Probes) for 6 h in the presence of M-CSF (10 ng ml^{-1}).

Fetal thymus organ culture and kidney subcapsular transplantation. The basic procedure of fetal thymus organ culture under high-oxygen-submersion conditions has been described previously[1,22]. In brief, single deoxyguanosine-treated fetal thymus lobes at 15 days after coitus were placed into wells of a 96-well V-bottomed plate. DN2 adult thymus cells (200 cells per lobe) or DN1 adult thymus cells (one cell per lobe) were cultured for six days with a deoxyguanosine-treated lobe under high-oxygen-submersion conditions in the presence of SCF (1 ng ml^{-1}), IL-7 (0.5 ng ml^{-1}), Flt-3L (5 ng ml^{-1}) and GM-CSF (1 ng ml^{-1}). The medium used for deoxyguanosine treatment and high-oxygen-submersion cultures was the same as used for co-culture with stromal cells. The lobes were then transplanted individually under the kidney capsule of recipient mice.

Intrathymus cell transfer experiment. Intrathymic injection of sorted thymocytes was performed as described previously[22]. DN2 adult thymus cells (1,000 cells) were injected into the thymic lobes of non-irradiated recipient mice.

Bone marrow transplantation experiments. In experiments shown in Fig. 3e, f, a 1:1 mixture of wild-type bone marrow cells (B6 Ly5.1 mice) (5×10^6 cells) and bone marrow cells from hCD3εTg mice (Ly5.2) (5×10^6 cells) was transplanted into lethally irradiated wild-type Ly5.2 mice. The resulting chimaeric mice were killed and analysed two months later. For assessing the chimaerism ratio in macrophages residing in the thymic cortex, the proportion of Ly5.2^{+} cells among F4/80^{+} cells was determined on immunohistochemically stained thymic sections from the chimaeric mice. To establish baseline values for these experiments, we reconstituted lethally irradiated wild-type Ly5.2 recipient mice with wild-type Ly5.1 bone marrow cells (10^7 cells). The proportions of Ly5.2^{+} cells were 36.3% (s.d. 19.1%; $n = 4$) and 31.9% (s.d. 10.6%; $n = 4$) among thymic and splenic macrophages, respectively. For the analysis in Fig. 3f, these proportions were regarded as 0%. The lines for the experimental data were fitted by least-squares analysis.

RT–PCR. RT–PCR was performed as described previously[4]. Primers used are described in the legend to Supplementary Fig. 3. PCR products were subjected to electrophoresis through a 1.2% agarose gel and stained with ethidium bromide.

Immunohistochemistry. Tissues were fixed for 15 min with 4% paraformaldehyde, placed in sucrose gradients, embedded in OCT compound, and snap-frozen in liquid nitrogen. Frozen blocks were cut into serial 5-μm sections and mounted onto MAS-coated microscope slides (Matsunami). Sections were incubated with primary antibodies and washed with PBS/0.01% Tween; this was followed by incubation with the appropriate secondary reagents. Nuclei were counterstained with DAPI (Molecular Probes).