A multipotent precursor in the thymus maps to the branching point of the T versus B lineage decision

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Hematopoietic precursors continuously colonize the thymus where they give rise mainly to T cells, but also to B and dendritic cells. The lineage relationship between these three cell types is unclear, and it remains to be determined if precursors in the thymus are multipotent, oligopotent, or lineage restricted. Resolution of this question necessitates the determination of the clonal differentiation potential of the most immature precursors in the thymus. Using a CC chemokine receptor 9–enhanced green fluorescent protein knock-in allele like a surface marker of unknown function, we identify a multipotent precursor present in bone marrow, blood, and thymus. Single cells of this precursor give rise to T, B, and dendritic cells. A more differentiated stage of this multipotent precursor in the thymus has lost the capacity to generate B but not T, dendritic, and myeloid cells. Thus, the newly identified precursor maps to the branching point of the T versus B lineage decision in the hematopoietic lineage hierarchy.

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Abbreviations used: CCR9, CC chemokine receptor 9; CLP, common lymphoid progenitor; DN, double negative; EGFP, enhanced GFP; ETP, early T lineage progenitor; FTOC, fetal thymic organ culture; HSC, hematopoietic stem cell; LSK, $Lin^-Sca\text{-}1^+c\text{-}kit^+$; SCF, stem cell factor; TMP, thymic multipotent precursor.

T lymphocytes that develop in the thymus are derived from a pool of self-renewing, multipotent hematopoietic stem cells (HSCs) that lodge in the bone marrow (1). T cell development in the thymus is replenished continuously by hematopoietic precursors that travel from the bone marrow via blood to the thymus because the thymus does not support precursors with the capacity for self-renewal (2). The nature of these precursors is still controversial. Many hematopoietic precursors in the bone marrow with distinct self-renewal capacities and differentiation potentials generate T cells upon adoptive transfer of irradiated hosts (e.g., HSCs [3], early lymphoid progenitors [4], $Lin^-Sca\text{-}1^+c\text{-}kit^+$ (LSK)$flt3^+$s [5] and common lymphoid progenitors [CLPs; reference 6]), but none of these has been demonstrated to lodge in the thymus. Therefore, it remains to be determined at which level of the hematopoietic lineage hierarchy thymopoiesis branches off. Even the question if thymic precursors are multipotent, oligopotent, or lineage-restricted, and if they commit to the T cell lineage in the bone marrow or in the thymus remains controversial because adult thymic precursors only have been studied on the population level. There is good evidence that hematopoietic precursors in the thymus produce T, B (7, 8), and dendritic cells (9). Whether these cells derive from a single, oligopotent progenitor or from distinct, precommitted precursor cells is unresolved, although the existence of a T/B precursor was suggested by the predominant generation of B cells by Notch1-deficient precursor cells (10, 11). Resolution of these questions necessitates the identification of the most immature hematopoietic precursor in the thymus, and the determination of its clonal differentiation potential.

In 1991, Wu et al. (12) identified the "$CD4^{low}$ precursor" among adult thymocytes which was characterized further as a $Lin^-CD25^-CD44^{hi}$ $c\text{-}kit^{hi}$ cell by others (13). With notable exceptions (14, 15), this population is still viewed as the most immature stage of T cell development which among coreceptor CD4 and CD8 double-negative (DN) thymocytes follows the sequence DN1 ($c\text{-}kit^+CD44^+CD25^-$) to DN2 ($c\text{-}kit^+CD44^+CD25^+$) to DN3 ($c\text{-}kit^-CD44^-CD25^+$) to DN4 ($c\text{-}kit^-CD44^-CD25^-$; references 16–19). Recently, Allman et al. showed that IL-7R-expressing cells among $CD4^{low}$ precursors do not contain T lineage potential and termed the remaining DN1 $Lin^-c\text{-}kit^{hi}IL\text{-}7R^{neg/lo}$ cells "early T lineage progenitors" (ETPs; reference 20). ETPs constitute 87% of $CD4^{low}$ precursors and are functionally

The online version of this article contains supplemental material.

indistinguishable because they contain mainly T lineage precursors and a few B and myeloid progenitors (8, 20, 21). The most immature hematopoietic precursors in the thymus are believed to be contained in the ETP population, because the only population with potent T lineage potential in the blood of adult mice carries the Lin^- $Sca\text{-}1^+$ $c\text{-}kit^+$ (LSK) surface markers that also are found on ETPs (22), but not other presumptive precursors (14, 15). Because 10,000 ETPs can be found in the thymus of an adult mouse, the ETP population is far too numerous to consist homogeneously of thymic precursors (23); the niche that contains thymus repopulating cells is believed to contain only a few hundred cells (24, 25). The large number of these cells per thymus, and the fact that ETPs in the blood cannot be separated from HSCs and other multipotent precursors that are not found in the thymus by conventional surface markers (22) has hampered the investigation of lineage relationships of hematopoietic cells in the thymus. Thus, markers that distinguish functional populations within the ETP population are called for. A recent report confirms that the only DN1 subsets that are less mature than DN2 thymocytes carry the ETP phenotype and distinguishes a DN1a and a DN1b subset by CD24 expression (26). In contrast to ETPs, the DN1a and DN1b subsets lack B potential; this suggests the independent immigration of B precursors and the most immature T lineage progenitors within separate precursor populations. Thus, the identity and the functional properties of the most immature precursors within the large ETP population are unclear.

To investigate whether mature T, B, and dendritic cells derive from a single, oligopotent progenitor or from distinct, precommitted precursor cells, we enhanced GFP (EGFP)-tagged T lineage cells by their expression of the CC chemokine receptor 9 (CCR9) that is expressed exclusively at sites of T cell development (27–29). By following $EGFP^{CCR9}$ expression in heterozygous CCR9-EGFP knock-in mice in which adult αβ-T cell development is indistinguishable from wild-type thymopoiesis, we now identify a thymic precursor that gives rise to T cells, B cells, and dendritic cells on the single cell level. Furthermore, we supply evidence that this progenitor maps to the branching point of the T versus B lineage decision in the hematopoietic lineage hierarchy.

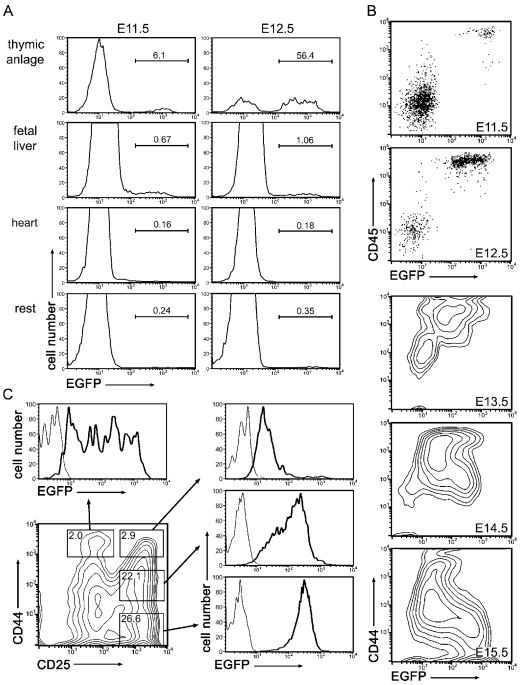

Figure 1. $EGFP^{CCR9}$ expression of early stages of thymopoiesis in heterozygous CCR9-EGFP knock-in embryos. (A) Identification of $EGFP^{CCR9}$-expressing cells in embryonic organs at E11.5 and E12.5 was done by analyzing single cell suspensions of the respective tissues by flow cytometry. The third pharyngeal pouch containing the thymic anlage was analyzed at E11.5. The percentage of $EGFP^{CCR9}$-positive cells is indicated. Cell numbers are plotted as total number of cells. (B) Flow cytometric analysis of the earliest immigrants to the embryonic thymic anlage of heterozygous CCR9-EGFP knock-in embryos was performed for embryonic days 11.5 through 15.5. To avoid contaminating mature cells that were derived from the peripheral blood, the dot plots for E11.5 and E12.5 are gated on lymphoid cells negative for markers of lineage differentiation, including B220, CD3ε, CD4, CD8, CD11b, Gr-1, Ter119, TCRγδ, and NK1.1. Contour plots for E13.5, E14.5, and E15.5 are gated on all lymphoid cells. Note that the dot plots for E11.5 and E12.5 show stainings using anti-CD45 and the contour plots for E13.5, E14.5 and E15.5 show stainings using anti-CD44. (C) $EGFP^{CCR9}$ expression was analyzed in immature stages of adult T cell development. The contour plot shows DN stage thymocytes after electronic exclusion of cells expressing markers of lineage differentiation as in the E11.5 and E12.5 plots in (B). Histogram plots for DN1($CD44^+CD25^-$), DN2($CD44^+CD25^+$), intermediate DN2/DN3($CD44^{low}CD25^+$), and DN3($CD44^-CD25^+$) stage thymocytes of heterozygous CCR9-EGFP knock-in (thick line) and wild-type (thin line) mice are shown. Cell numbers are plotted relative to the channel with the highest number of cells.

RESULTS

Regulation of $EGFP^{CCR9}$ expression during the early steps of T cell development

To reinvestigate the issue of the most immature precursor in the thymus, we sought to develop a novel marker for thymic precursors. Therefore, we tagged T lineage cells by their expression of CCR9 that is expressed exclusively at sites of T cell development (27–29). To this end, we generated mice in which the NH_2-terminal half of the CCR9 coding region is replaced in frame by an EGFP cassette (30). Expression of the tag in the correct cell types was confirmed by FACS analysis of $EGFP^{CCR9}$ in heterozygous CCR9-EGFP knock-in mice, in which adult αβ-T cell development is indistinguishable from wild-type thymopoiesis (Fig. S1, available at http://www.jem.org/cgi/content/full/jem.20050146/DC1). Cytoplasmic EGFP and membrane integral CCR9 can be expected to show widely divergent half-lives. Therefore, we considered $EGFP^{CCR9}$ expression as a T cell precursor identifying tag, just like a surface marker of unknown function. We did not take its presence as evidence for the

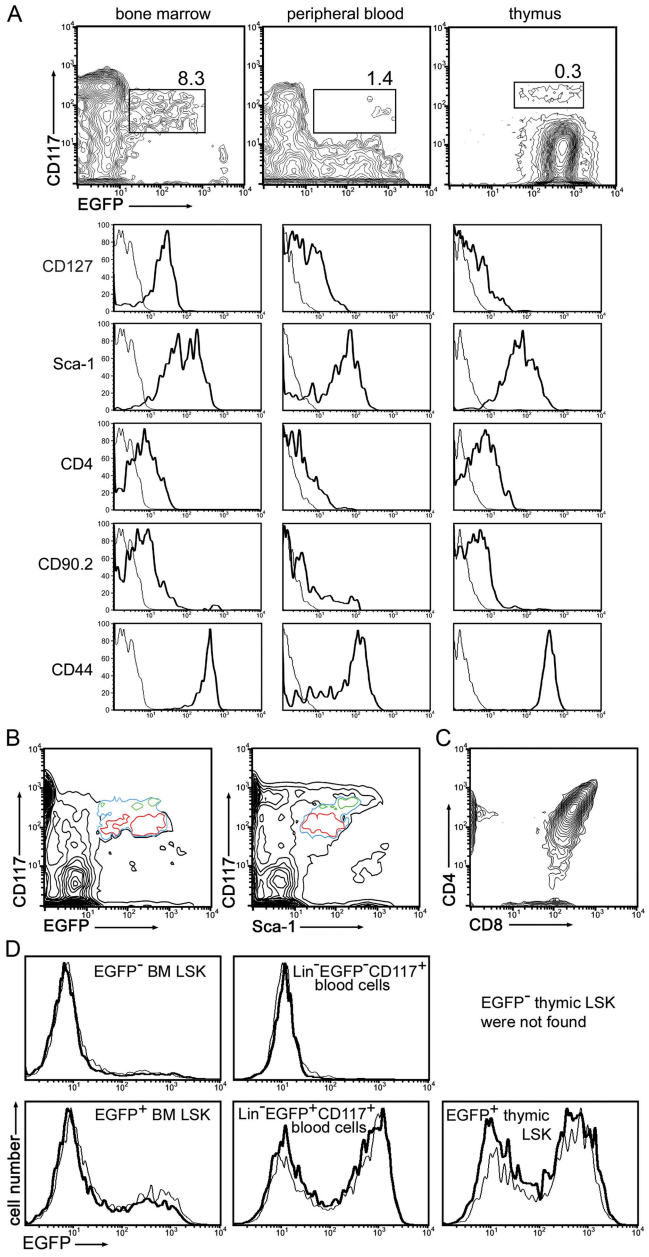

Figure 2. Phenotypic and functional analysis of thymic progenitors found in bone marrow, peripheral blood and thymus of heterozygous CCR9-EGFP knock-in mice. (A) Lin$^-$CD25$^-$CD117$^+$EGFP^{CCR9+} cells found in bone marrow, peripheral blood, and thymus were analyzed for their expression of CD127(IL-7Rα), Sca-1, CD4, CD90.2, and CD44. Contour plots are gated for lymphoid cells negative for B220, CD3ε, CD8, CD25, CD11b, Gr-1, Ter119, TCRβ, TCRγδ, and NK1.1 and histogram plots below show the cells in the indicated gates for each compartment. Cell numbers are plotted relative to the channel with the highest number of cells. (B) A five-color flow cytometric analysis of Lin$^-$CD25$^-$CD117$^+$EGFP^{CCR9+} bone marrow cells is shown in two color-coded contour plots. Both contour plots are gated on lineage marker–negative bone marrow cells, and the location of Lin$^-$CD25$^-$CD117$^+$EGFP^{CCR9+} cells is shown in blue. Within this population the Lin$^-$CD25$^-$CD117$^+$EGFP^{CCR9+}CD127$^{-/low}$ bone marrow cells are shown in green, and the Lin$^-$CD25$^-$CD117$^+$EGFP^{CCR9+}CD127$^+$ cells are shown in red. Lineage markers were defined as in (A). (C) 400 Lin$^-$CD25$^-$CD117$^+$EGFP^{CCR9+} cells isolated from the peripheral blood of heterozygous CCR9-EGFP knock-in mice were FACS sorted as indicated in (A), and intrathymically injected into sublethally irradiated C57BL/6 mice. The contour plot shows the analysis of EGFP-positive thymocytes 19 d after intrathymic transfer. (D) 100 EGFP^{CCR9+} bone marrow LSKs, 100 EGFP^{CCR9+}Lin$^-$CD25$^-$CD117$^+$ blood cells, 100 EGFP^{CCR9+} thymic LSKs, and their respective EGFP^{CCR9-} counterparts were cultured on OP9-DL1 stroma cells for 12 d. Histogram plots are gated on CD19$^-$NK1.1$^-$CD90.2$^+$ cells. The results of two independent experiments are shown for each population.

concomitant presence of CCR9 protein or as evidence for the involvement of CCR9 in thymus homing.

The analysis of heterozygous CCR9-EGFP knock-in embryos revealed that significant amounts of tagged cells were found only in the developing thymic anlage and the fetal liver, the site of embryonic hematopoiesis (Fig. 1 A). At embryonic day 11.5 (E11.5; the time when the first hematopoietic precursors arrive in the thymic anlage), all hematopoietic cells in the developing thymic anlage contained high levels of EGFP^{CCR9} (Fig. 1 B). The first detectable immigrants contained higher amounts of EGFP^{CCR9} than all subsequent stages of development up to the occurrence of CD44-negative DN3/4 thymocytes at E15.5. Already at E12.5, the population expressing EGFP^{CCR9} at the level of E11.5 precursors represents only a small fraction of all EGFP^{CCR9+} cells. EGFP^{CCR9} expression in heterozygous CCR9-EGFP knock-in mice also seems to be regulated tightly during the early steps of adult thymopoiesis. As observed during embryonic thymopoiesis, EGFP^{CCR9} increases in adult, immature thymocytes that progress from the CD44-positive DN2 to the CD44-negative DN3 stage (Fig. 1 C). Adult DN1 thymocytes show all levels of EGFP^{CCR9} expression, from low to high, which indicates phenotypic heterogeneity. EGFP^{CCR9} expression among the DN stages is present throughout at least at low levels.

Identification of EGFP^{CCR9}-expressing thymic progenitors in bone marrow, blood, and thymus

Based on the embryonic analyses, we hypothesized that EGFP^{CCR9} could be used in adult mice as a marker for the most immature precursors in the thymus. Furthermore, we postulated that EGFP^{CCR9} might identify T lineage biased precursors in the peripheral blood. This was crucial since the LSK population has recently been shown to represent the only population with potent T lineage potential in the blood of adult mice (22) but conventional markers could not separate thymic precursors from cells that are absent from the thymus like self-renewing HSCs within this population. Therefore, we investigated EGFP^{CCR9} expressing, thymus repopulating cells in adult bone marrow, blood and thymus. In the bone marrow, we identified a Lin$^-$CD25$^-$CD117$^+$ EGFP^{CCR9+} population that contained all the in vivo thymus repopulating activity among EGFP^{CCR9+} bone marrow cells (Fig. S2, available at http://www.jem.org/cgi/content/full/jem.20050146/

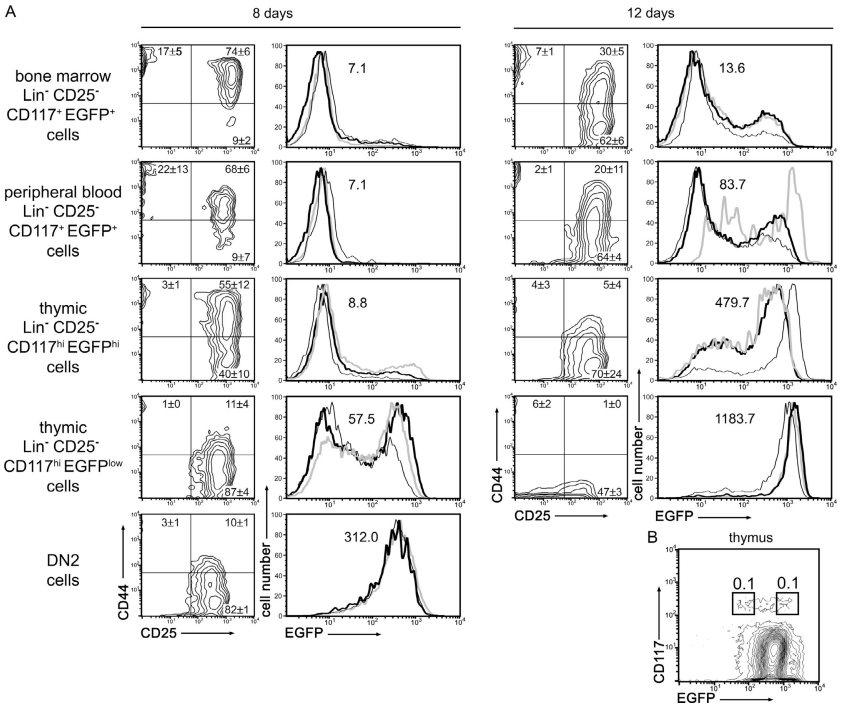

Figure 3. Kinetics of early T cell development in FTOC cultures of Lin$^-$CD25$^-$CD117$^+$EGFP^{CCR9+} thymic precursors isolated from bone marrow, peripheral blood, and thymus. (A) 1,000 cells of the indicated phenotypes were FACS sorted from bone marrow and thymus as shown in Figs. 2 A and 3 B, and cultured together with a dGuo-treated E15.5 fetal thymic lobe. Cultures seeded with peripheral blood Lin$^-$CD25$^-$CD117$^+$EGFP^{CCR9+} cells were sorted as shown in Fig. 2 A and were initiated with 400–800 cells per lobe. FTOCs were analyzed at the indicated time points. Contour plots are gated on Lin$^-$ lymphoid cells and histogram plots are gated on Lin$^-$CD25$^+$ lymphoid cells. The results of three independent experiments for each subset and time point are shown. The median channel of EGFP fluorescence averaged over the three shown experiments is indicated. Coreceptor double-positive cells were found consistently in these cultures after 16 d. (B) The gates used for FACS sorting of thymic Lin$^-$CD25$^-$CD117^{hi}EGFP^{CCR9hi} and Lin$^-$CD25$^-$CD117^{hi}EGFP^{CCR9low} cells that were isolated for the FTOC experiments shown in (A) are indicated. The contour plot was defined as in Fig. 2 A.

DC1). The Lin$^-$CD25$^-$CD117$^+$ EGFP^{CCR9+} population represents a subset of LSK and CLP precursors (Fig. S3, available at http://www.jem.org/cgi/content/full/jem.20050146/DC1). Apart from this population, EGFP^{CCR9+} bone marrow populations were found that lacked detectable T lineage potential, which indicated that EGFP^{CCR9} expression in the bone marrow is not restricted to thymic precursors. Comparing Lin$^-$CD25$^-$CD117$^+$EGFP^{CCR9+} cells in bone marrow, blood, and thymus revealed that only the CD127 (IL-7Rα)-negative to low fraction among bone marrow precursors appeared in blood and thymus (Fig. 2 A), which is consistent with the reported absence of CLPs in blood (22) and thymus (20). All other tested markers suggested phenotypic homogeneity among precursors in the different compartments. Five-color FACS analyses revealed that the CD127(IL-7Rα)-negative to low fraction of bone marrow Lin$^-$CD25$^-$CD117$^+$EGFP^{CCR9+} cells expresses high levels of CD117(c-kit; Fig. 2 B); this is consistent with the phenotype of the same cells in the thymus which homogeneously express high levels of CD117 (Fig. 2 A, top right). Thus, we find evidence for the presence of a Lin$^-$CD25$^-$CD117^{hi} EGFP^{CCR9+}CD127$^{-/low}$Sca-1^{hi}CD4^{low}CD90.2$^{-/low}$CD44$^+$ precursor population in heterozygous CCR9-EGFP knock-in mice that is found in bone marrow, blood, and thymus.

To find support for the idea that EGFP^{CCR9}-tagged blood LSKs are bound for the thymus, we investigated this population in more detail. We detected only 30–60 of these precursors in the blood of one mouse—and consistent with their precursor phenotype—these cells gave rise to thymopoiesis in vivo (Fig. 2 C). Functionally, the culture of blood precursors on OP9-DL1 stroma cells revealed that Lin$^-$CD25$^-$ CD117$^+$EGFP^{CCR9+} blood cells, but not their EGFP^{CCR9-} counterparts, contained T lineage precursors that gave rise to CD90.2$^+$EGFP^{CCR9+} DN3/4 stage thymocytes (compare with Fig. 1 C and Fig. S4, available at http://www.jem.org/cgi/content/full/jem.20050146/DC1) within 12 d (Fig. 2 D). DN3/4 stage thymocytes appeared in cultures of Lin$^-$CD25$^-$ CD117$^+$EGFP^{CCR9-} blood precursors only after 16 d of culture (unpublished data). Although both subsets contain T lineage potential, all rapidly differentiating precursors are contained in the EGFP^{CCR9+} population. This indicated that EGFP^{CCR9+} blood LSKs are biased toward T cell development relative to their EGFP^{CCR9-} counterparts.

Analysis of the kinetics of T cell development of EGFP^{CCR9}-expressing thymic progenitors

Our results suggest that the Lin$^-$CD25$^-$CD117$^+$EGFP^{CCR9+} population represents a thymus repopulating cell that travels to the thymus via the blood. If this were true, one would expect that the thymic counterpart of this population is the most immature precursor in the thymus and that cells isolated from these compartments gave rise to more mature stages of T cell development with similar kinetics. Based on our embryonic analyses we further hypothesized that among thymic Lin$^-$CD25$^-$CD117^{hi} EGFP^{CCR9+} cells, which correspond to the ETP population, the subset expressing the highest levels of EGFP^{CCR9} represented the recent immigrants while cells with lower levels of EGFP^{CCR9} were more mature. Indeed, thymic Lin$^-$CD25$^-$CD117^{hi} EGFP^{CCR9hi} precursors representing ≤20% of ETPs developed in fetal thymic organ culture (FTOC) with kinetics that closely resemble those of circulating precursors from the blood (Fig. 3). In contrast, the wave of developing thymocytes that is generated by the thymic Lin$^-$CD25$^-$CD117^{hi}EGFP^{CCR9low}

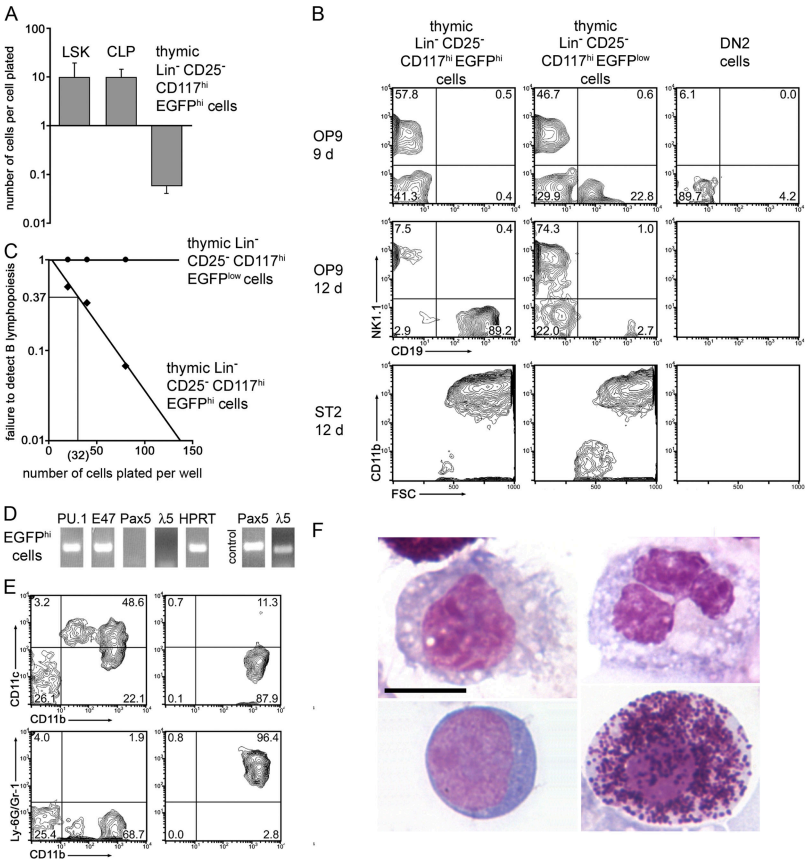

Figure 4. Characterization of the differentiation potentials of thymic Lin$^-$CD25$^-$CD117^{hi} EGFP^{CCR9hi} and EGFP^{CCR9low} cells. (A) 500 cells of the indicated phenotypes were cultured under serum-free conditions on methylcellulose containing IL-7, SCF, and Flt3L for 5 d, after which the number of living cells per cell plated was determined. Mean and standard deviation of three independent experiments are shown for each cell type. (B) Pools of 2,000 cells of the indicated phenotype were cultured on a layer of OP9 or ST2 stromal cells in the presence of IL-7, SCF, Flt3L, and IL-2 and analyzed at the indicated time points. Empty plots indicate absence of living cells. The percentages for the respective quadrants are shown. (C) The frequency of B cell precursors among thymic Lin$^-$CD25$^-$CD117^{hi}EGFP^{CCR9hi} and EGFP^{CCR9low} cells was determined by limiting dilution assays. Both cell types were sorted in pools of 20, 40, and 80 cells ($n = 30$) onto OP9 stromal layers and cultured for 9 and 12 d, respectively. The failure to detect B lymphopoiesis is plotted. (D) RT-PCR analyses on RNA isolated from thymic Lin$^-$CD25$^-$CD117^{hi}EGFP^{CCR9hi} (EGFP^{hi}) cells and total bone marrow cells (control) with primers specific for the indicated transcripts. Mock RT-PCR samples remained negative. (E) Phenotypic analysis of cells derived from a representative culture of Lin$^-$CD25$^-$CD117^{hi}EGFP^{CCR9hi} cells on an ST2 stromal cell layer. No difference was found between cultures of thymic Lin$^-$CD25$^-$CD117^{hi}EGFP^{CCR9hi} and EGFP^{CCR9low} cells. Contour plots on the left are gated on lymphoid cells and show the results of one multiparameter FACS analysis demonstrating the presence of dendritic cells. Contour plots on the right are gated on large, complex, immature myeloid cells and also show the results derived from one multiparameter staining. (F) The morphology of cells in cultures of thymic precursors on ST2 stromal cell layers was determined for a culture that contained predominantly immature myeloid cells as shown in (E, right panels) by staining of cells spun onto a glass slide according to Pappenheim. The majority of cells showed a blast-like morphology (top left) and some resembled bone marrow myeloblasts (bottom left). Few mature granulocytes also were observed (e.g., a neutrophilic granulocyte [top right] and a basophilic granulocyte [bottom right]). Bar, 10 μm.

population had progressed significantly beyond the DN2 stage after 8 d, which indicated a more differentiated state of this subset. Thus, the EGFP^{CCR9} tag allowed us to identify subsets within the ETP population that showed distinct levels of maturity. Specifically, the data demonstrate that adult thymic Lin$^-$CD25$^-$CD117^{hi}EGFP^{CCR9hi} precursors are less mature than their EGFP^{low} counterparts, and that phenotypically identical precursors that show a similar state of immaturity exist in the blood.

Analysis of the differentiation potential of EGFP^{CCR9}–expressing thymic precursors

To address the question of lineage relationship between hematopoietic cells in the thymus, we investigated the growth requirements and the differentiation potential of thymic EGFP^{CCR9}-expressing precursors within the ETP population. Although CLPs and bone marrow precursors contained in the LSK compartment proliferated vigorously in the presence of stem cell factor (SCF), Flt3L, and IL-7 under serum-free conditions as previously reported (5, 6), numbers of thymic Lin$^-$CD25$^-$CD117^{hi}EGFP^{CCR9hi} precursors declined rapidly over a period of 5 d of culture under these conditions (Fig. 4 A). This observation indicated that these progenitors are functionally distinct from other short-term repopulating cells that are found in the bone marrow LSK population (5). Lin$^-$CD25$^-$CD117^{hi}EGFP^{CCR9hi} precursors did proliferate in vitro when they were sorted in pools of 2,000 cells onto layers of the bone marrow stroma cell line OP9 in the presence of SCF, Flt3L, IL-7, and IL-2 (Fig. 4 B). Under these conditions they gave rise to NK and B cells. This is in contrast to the differentiation potential of the recently described ETP subsets, DN1a and DN1b, which do not produce B cells (26) but is consistent with the reported B cell potential when unseparated ETPs were assayed (20). Lin$^-$CD25$^-$CD117^{hi} EGFP^{CCR9hi} precursor–derived B cells consistently required 12 d to develop. In rare cases, pools of 2,000 thymic Lin$^-$CD25$^-$CD117^{hi}EGFP^{CCR9low} cells also produced B cells; however, these cells were detected considerably earlier than in cultures of the EGFP^{CCR9hi} counterpart, supplying further evidence for the more differentiated state of thymic Lin$^-$CD25$^-$CD117^{hi}EGFP^{CCR9low} cells (Fig. 4 B). Limiting dilution assays revealed that only the Lin$^-$CD25$^-$CD117^{hi} EGFP^{CCR9hi} population contained significant capacity to generate B lymphocytes. Although 1 in 32 of these cells gave rise to a colony of B cells on cytokine-supplemented OP9 layers, we did not find a single B cell colony in the cultures of 4,200 thymic Lin$^-$CD25$^-$CD117^{hi}EGFP^{CCR9low} cells (Fig. 4 C). ETPs that contain both of these precursors are believed to consist mainly of T lineage precursors with a few B progenitors. Therefore, our observation could be explained by the presence of a few committed B cell precursors within the Lin$^-$CD25$^-$CD117^{hi}EGFP^{CCR9hi} population. However, RT-PCR analyses of sorted Lin$^-$CD25$^-$CD117^{hi} EGFP^{CCR9hi} cells did not detect transcripts indicative of B cell commitment, such as Pax5 or λ5 (Fig. 4 D). These findings demonstrate that only the most immature precursors in the thymus possess B cell potential, and that committed B precursors are undetectable within this population.

To our surprise, both precursors gave rise to myeloid cells. When we cultured Lin$^-$CD25$^-$CD117^{hi}EGFP^{CCR9hi} and EGFP^{CCR9low} cells as pools of 1,000 cells on the bone marrow stromal cell line ST2 in the presence of SCF, Flt3L, IL-7, and IL-2 we observed the growth of CD11b-positive cells for both types of precursors in nine out of nine experiments (Fig. 4 B). Cytokine-supplemented ST2 cultures from both precursors were indistinguishable in that they contained "myeloid" and "lymphoid" dendritic cells as well as immature myeloid cells, and a few mature granulocytes (Fig. 4, E and F). We then investigated the possibility that thymic precursors induced detectable granulopoiesis in the thymus but, consistent with a previous report (20), could not find significant numbers of cells with the surface markers that are characteristic for common myeloid progenitors, megakaryocyte/erythrocyte lineage-restricted progenitors, and granulocyte/macrophage lineage-restricted progenitors (31) that have been shown to give rise to all myeloid lineages (unpublished data). Therefore, we conclude that although both precursors possess granulocytic differentiation potential in vitro, it is suppressed in the thymic microenvironment in vivo. Collectively, we find that apart from T cells, thymic precursors give rise to B cells, NK cells, dendritic cells, and myeloid cells; this is consistent with most reports (8, 9, 13, 20, 21, 26, 32) but is at odds with another (33). Significantly, the presented data show that only the capacity to generate B cell development ultimately is restricted to the most immature precursor in the thymus, whereas all other lineages still can be produced effectively by a more differentiated precursor. Because the thymic Lin$^-$CD25$^-$CD117^{hi}EGFP^{CCR9hi} progenitor gives rise to mature cells of multiple hematopoietic lineages, we termed it "thymic multipotent precursor" (TMP).

Clonal analysis of thymic multipotent progenitors

Based on the finding that immaturity among thymic precursors correlated with the most diverse differentiation capacity, we investigated the possibility that all hematopoietic lineages that develop in the thymus (namely T cells, B cells, and dendritic cells) are derived from a single precursor. The potential to give rise to granulocytic cells was not investigated further because there is no evidence for ongoing granulopoiesis in the thymus. Alternatively, immigration of committed, lineage-restricted precursors could be the prevailing mode of supply for adult thymopoiesis which would be consistent with the common assumption that immature thymic precursors contain mainly T lineage precursors and a few B and myeloid progenitors (i.e., we investigated the question if thymic precursors commit to the T cell lineage within or outside the thymus). To address this question we cultured thymic precursors on 20:1 mixtures of the OP9 bone marrow stroma cell line (34) and its derivative, the OP9-DL1

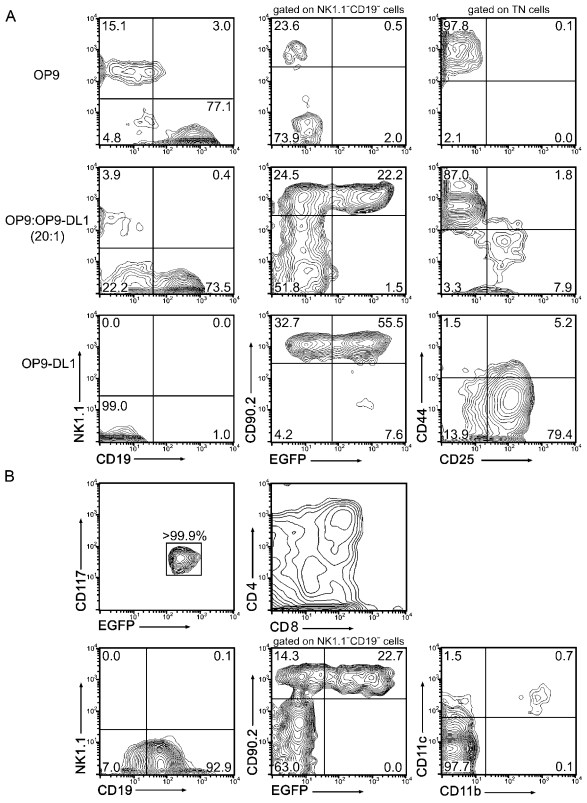

Figure 5. Analysis of the differentiation potential of TMPs on the single cell level. (A) 100 TMPs were cultured on stromal layers of OP9, OP9-DL1, or a mixture of OP9/OP9-DL1 (20:1) in the presence of IL-7, SCF, Flt3L, and IL-2 for 12 d. At that time, cultures were analyzed by FACS. Contour plots in the middle are gated on NK1.1$^-$CD19$^-$ cells found in the bottom left quadrants of contour plots on the left. Contour plots on the right are gated on cells lacking the markers CD4, CD8, and CD3ε (triple-negative cells). The percentages for the respective quadrants are shown. Coreceptor double-positive cells were found in cultures on OP9-DL1 stromal cell layers after 16–19 d. (B) Double-sorted, single TMPs were placed into the wells of a 96-well plate onto OP9/OP9-DL1 (20:1) stromal cell layers and cultured as in (A). The reanalysis of TMPs double-sorted to a purity of >99.9% is shown (top left contour plot). Single TMPs that were transferred after 12 d from the OP9:OP9-DL1 mixture onto a fresh layer of OP9-DL1 cells generated double-positive cells after 16–19 d of culture (top right contour plot). To demonstrate that T, B, and dendritic cells could be derived from a single cell, productive cultures were split after 10 d onto methylcellulose containing IL-1β, IL-3, IL-6, SCF, and Flt3L. OP9/OP9-DL1 cultures were analyzed after 12 d (bottom left and middle panel) and methylcellulose cultures were analyzed 4 d after the split (bottom right panel). The bottom panels show the results of one representative singly-sorted TMP. The bottom middle contour plot is gated on NK1.1$^-$CD19$^-$ cells found in the bottom left quadrant of the bottom left contour plot.

cell line (35). This mixture supports the differentiation of precursor cells into T and B cells in one and the same well (Fig. 5 A). TMPs were double-sorted to a purity of >99.9% and placed as single cells onto OP9/OP9-DL1(20:1) layers in a 96-well format. We consistently found T and B cells in 1 to 3 wells out of 95 wells to which a single TMP had been added (Fig. 5 B and Table I, A1–A3). This experiment demonstrates that the TMP population contains bipotent T/B precursors and that thymic precursors exist that commit to the T cell lineage only after thymus entry. Despite the long period of physiologically unfavorable conditions during cell isolation and double-sorting, ~50% of the double-sorted TMPs gave rise to T cell colonies on OP9-DL1 stromal layers (Table I, B1). Strikingly, single TMPs sorted on OP9/OP9-DL1(20:1) stromal layers frequently gave rise to T cells only, whereas wells that contained only B cells were seen rarely (Table I, A1–A3). This constellation makes the possibility that the T and B cells found in one and the same well originated from two independent, lineage-restricted precursor cells highly unlikely. Single cells giving rise to T and B cells also were found among the few Lin$^-$CD25$^-$CD117$^+$EGFP^{CCR9+} cells in the blood, which further supported the notion of a bipotent T/B precursor colonizing the thymus (Table I, C1 and C2). Again, the low frequency of wells containing only T or only B cells ruled out the possibility that wells containing B and T cells are the result of the erroneous addition of two independent precursors to the same well. To investigate the question if the bipotent T/B progenitor found among TMPs also produced dendritic cells, we split OP9/OP9-DL1(20:1) cultures derived from single TMPs onto methylcellulose containing IL-1, IL-3, IL-6, SCF, and Flt3L in the absence of serum. After 4 to 5 d, these cultures contained small but significant numbers of dendritic cells (Fig. 5 B). This observation is consistent with our previous finding that dendritic cells can be derived in vitro from more mature stages of T cell development (Fig. 4 B), and demonstrates that a single hematopoietic precursor—which can be found among TMPs in heterozygous CCR9-EGFP knock-in mice—gives rise to all three hematopoietic lineages that develop in the thymus.

Finally, we investigated Lin$^-$CD25$^-$CD117^{hi} thymocytes of wild-type mice for bipotent T/B progenitors as the defining feature of TMPs. In a representative experiment we found two progenitors that gave rise to B and T cells among 475 singly sorted cells using the OP9/OP9-DL1(20:1) culture system (Table I, D1). Thus, bipotent thymic progenitors identified in heterozygous CCR9-EGFP knock-in mice also can be found in wild-type mice.

DISCUSSION

The presented data demonstrate for the first time that single hematopoietic precursor cells can be isolated from the adult thymus that give rise to T, B, and dendritic cells (i.e., the three hematopoietic lineages known to develop in the thymus; references 7–9, 36). The fact that all three lineages can be derived from a single precursor indicates that thymic precursors exist that enter the thymus as multipotent progenitors and commit to the T cell lineage only within the thymic microenvironment. That adult thymic precursors have the capacity to give rise to hematopoietic cells of multiple lineages has long been known (8, 9, 13, 20, 21, 26, 32). These

Table I. Single cell analysis of double-sorted thymic precursors

Experiment no.	Cell type of sorted cells	Mouse strain	Stromal layer	No. of single cells sorted	Progenitors for		
					T cells	B cells	T and B cells
A1	TMP	heterozygous CCR9-EGFP knock-in	OP9/OP9-DL1 (20:1)	95	8	0	1
A2	TMP	heterozygous CCR9-EGFP knock-in	OP9/OP9-DL1 (20:1)	95	8	1	3
A3	TMP	heterozygous CCR9-EGFP knock-in	OP9/OP9-DL1 (20:1)	95	7	1	2
B1	TMP	heterozygous CCR9-EGFP knock-in	OP9-DL1	95	47	0	0
C1	peripheral blood $Lin^-CD25^-CD117^+EGFP^+$ cells	heterozygous CCR9-EGFP knock-in	OP9/OP9-DL1 (20:1)	95	0	1	7
C2	peripheral blood $Lin^-CD25^-CD117^+EGFP^+$ cells	heterozygous CCR9-EGFP knock-in	OP9/OP9-DL1 (20:1)	95	4	0	2
D1	$Lin^-CD25^-CD117^{hi}$ thymocytes	wild-type	OP9/OP9-DL1 (20:1)	475	26	3	2

reports suggested that multipotent progenitors enter the thymus, but it had not been demonstrated that the relevant lineages in the thymus all can be derived from a single cell. Our observation that multipotent precursors are found exclusively among the most immature precursors in the thymus and in the peripheral blood supports the idea of a multipotent precursor that repopulates the thymus. These findings change the way we think of precursors in the thymus. Previously, it had been assumed that thymic precursors consist of mainly T lineage cells together with a few B and myeloid progenitors. In line with this notion, a recent report suggested the independent immigration of B precursors and the most immature T lineage progenitors within separate precursor populations (26). In contrast, our data now suggest that thymic precursors in the heterogeneous ETP population (23, 26) represent distinct levels of T cell commitment. The most immature stages are multipotent precursors with the in vitro capacity to give rise to T, B, NK, myeloid/granulocytic, and dendritic cells, and the most differentiated cells are T lineage committed—similar to cells that can be found in the DN2/3 stages.

The isolation of multipotent precursors in the thymus required the identification of the most immature precursor in the thymus within the ETP population. We identify thymic $Lin^-CD25^-CD117^{hi}EGFP^{CCR9hi}$ progenitors, which we term TMPs, as an ETP subset that shows nearly identical developmental kinetics as thymic precursors in the blood, and leaves little space for thymic precursors that might be even more immature. TMPs represent ≤20% of ETPs and are functionally distinct from the remaining ETPs outside of the TMP gate. Only TMPs show nearly identical developmental kinetics as their counterparts in the blood and contain significant B lineage potential. Given the fact that the $CD4^{low}$ precursor and the ETP population are vastly overlapping populations that are functionally indistinguishable from each other, the identification of the TMP represents a major advance in our understanding of early T cell development. We find ~1,000 TMPs per thymus of a 4–5-wk-old mouse which is consistent with the notion that TMPs represent a small subset of the ETPs. The $Lin^-CD25^-CD117^{hi}$ $EGFP^{CCR9low}$ precursor, which represents a closely related, more differentiated version of the TMP, also is contained in the ETP population. It has lost its capacity to produce B cells which indicates that the TMP maps to the branching point of the T versus B lineage decision in the hematopoietic lineage hierarchy. This more differentiated precursor still produces granulocytic cells in vitro, although granulopoiesis is not observed in vivo. This finding suggests that the T versus B cell lineage decision precedes the loss of myeloid potential in early thymic precursors.

A single thymic precursor can give rise to more than 10^5 thymocytes in FTOCs (37), conditions which are presumably far from optimal when compared with the in vivo situation. Thus, less than 1,000 precursors theoretically should be enough to generate the $1–2 \times 10^8$ thymocytes found in young adult mice. There is experimental evidence that the thymic niche that holds the repopulating precursors contains ~200 cells that remained phenotypically uncharacterized (24, 25). This is close to the 1,000 TMPs that we find per thymus of a 4–5-wk-old mouse. The low number of thymic precursor cells that is required to maintain thymopoiesis may explain why the search for the thymus-repopulating precursor has proven to be the search for the proverbial needle in the haystack. Our work introduces the heterozygous CCR9-EGFP knock-in mouse as a tool to study the earliest steps of thymopoiesis. We (30) and others (38, 39) have studied adult αβ-T cell development in homozygous and heterozygous CCR9-deficient mice extensively. No evidence for a nonredundant role for CCR9 in the immigration of hematopoietic precursors into the thymus was found.

In this study we used the CCR9-EGFP knock-in allele like a surface marker of unknown function to identify distinct thymus-repopulating precursors without implying any role for CCR9 in thymus homing. Only two abnormalities have been described in CCR9-deficient mice which affect adult αβ-T cell development; neither are found in the heterozygous EGFP-CCR9 knock-in mice that were used in this study. First, CCR9-deficient bone marrow repopulates the thymus inefficiently in competitive transfer experiments (39). Our own competitive transfer experiments confirm this finding, but show no significant difference for heterozygous mice (Table S1, available at http://www.jem.org/cgi/content/full/jem.20050146/DC1). Second, immature thymocytes do not home to the subcapsular microenvironment in CCR9-deficient thymi but do so in heterozygous CCR9-EGFP knock-in and wild-type mice (30). Furthermore, the finding that bipotent precursors give rise to T and B cell development, a capacity that characterizes the TMP in heterozygous CCR9-EGFP knock-in mice, is also found among wild-type $Lin^-CD25^-CD117^{hi}$ thymocytes further supports the validity of the CCR9-EGFP knock-in model.

Although the characterization of thymic precursors in bone marrow and blood helped us to identify immature precursors in the thymus and to investigate their differentiation potential, many questions concerning thymus repopulation remain. Our experiments do not rule out the possibility that TMP-independent precursors exist in the thymus because we followed cells that expressed the $EGFP^{CCR9}$ tag exclusively. Thus, precursors from distinct levels of the hematopoietic hierarchy may enter the thymus independently of TMPs, and these may be precommitted to one or the other lineage.

In summary, our work identifies a rare thymic precursor that gives rise to all hematopoietic lineages found in the thymus. It maps to the branching point of the T versus B lineage decision—a key developmental position in hematopoietic lineage maps. The study of this cell will add significantly to our understanding of hematopoiesis because this is the cell type that makes the T versus B cell fate decision in the context of the thymic microenvironment under physiologic conditions.

MATERIALS AND METHODS

Mice. BALB/c, C57BL/6, C57BL/6-Ly5.1, RAG2-deficient, CCR9-EGFP knock-in, and RAG2-deficient CCR9-EGFP knock-in mice were kept under specific pathogen-free conditions in the mouse facility of the Max-Planck-Institute for Immunobiology. Embryos were obtained from timed pregnant mice and the day of the vaginal plug was counted as day 0.5 of pregnancy. 3–6-wk-old mice were used for experiments unless otherwise indicated. Because the highest numbers of TMPs were recovered from 4–5-wk-old mice, such mice were used for sorting TMPs from bone marrow, blood, and thymus. Experimentation and animal care was in accordance with the guidelines of the Max-Planck-Institute for Immunobiology.

Flow cytometric analysis. Cells were prepared and stained as described previously (40). Peripheral blood was obtained from the axilla of anesthetized mice. Bone marrow and blood cells were incubated with 1 μg mAb 2.4G2 per 10^6 cells (provided by M. Lamers, Max-Planck-Institute for Immunobiology, Freiburg, Germany) for 10 min on ice before staining. Dendritic cells and immature myeloid cells derived from ST2 cultures were incubated with a 1:5 dilution of normal rat serum and 1 μg 2.4G2 for 10 min on ice before staining. The following PE, PE-Cy5, PE-Cy7, APC, AlexaFluor647, or biotin-conjugated monoclonal antibodies were used (clone names given in parentheses) from BD Biosciences: anti-CD3ε (145–2C11), anti-CD4 (GK1.5), anti-CD8α (53–6.7), anti-CD8β (H35-17.2), anti-CD11b (M1/70), anti-CD11c (HL3), anti-CD25 (PC61), anti-CD44 (IM7), anti-CD45 (30-F11), anti-CD90.2 (53–2.1), anti-TCRβ; (H57–597), anti-TCRγδ (GL3), anti-NK1.1 (PK136), anti-Ter119 (Ly-76), and anti-Gr1 (RB6-8C5); from eBioscience: anti-CD117 (2B8), anti-CD127 (A7R34), anti-Sca-1 (D7); and from Caltag: anti-IgM (polyclonal). The biotin label was visualized using SA-PE-Cy5 (Invitrogen) or SA-APC-Cy7 (eBioscience) conjugates. Stainings were analyzed on a FACSCalibur or an LSRII machine (both obtained from BD Biosciences).

Cell sorting and RT-PCR analysis. To deplete single cell suspensions of lineage marker–positive cells before cell sorting, thymocytes and bone marrow cells were incubated with unlabeled anti-CD8 (169.4.2, provided by M. Lamers) and with unlabeled mAbs directed against Ter119, B220 and Gr-1 (all BD Biosciences), respectively. Lineage marker–positive cells were depleted using goat anti–rat IgG-conjugated paramagnetic beads and MACS separation CS columns according to the manufacturer's recommendations (Miltenyi Biotec). The purified cells were stained and sorted on a high-speed FACS sorter (MoFlo; DakoCytomation) to a purity of >98%. For single cell isolation, sorted cells were sorted a second time. The secondary sort was done in single cell sort mode into 96-well plates at a flow rate of 5–20 cells/s. Purity of double-sorted cells was determined for each experiment and consistently was >99.9%. RT-PCR on RNA isolated from 10,000 sorted cells of each subset was done as previously reported (41) using primers described earlier (42, 43). All mock RT-PCR samples remained negative.

Adoptive transfer experiments. Thymi of injected mice were analyzed 22 d after i.v. injection by FACS analysis. Intrathymic transfers of 400 cells sorted from the blood were done together with 15,000 RAG2-deficient carrier cells in a volume of 10 μl into anesthetized C57BL/6 mice that had been sublethally irradiated with 450 rad 24 h earlier. Thymi of intrathymically transferred mice were analyzed after 19 d.

Fetal thymic organ culture. FTOCs were set up as described previously (13). Because of the low numbers of $Lin^-CD25^-CD117^+EGFP^{CCR9+}$ cells that can be isolated from the peripheral blood, these cells were cultured in FTOCs together with 5,000 RAG2-deficient thymocytes. The rare cases in which either Balb/c (from the embryonic thymus) or RAG2-deficient carrier cells repopulated an FTOC could be identified by the $EGFP^{CCR9}$ expression found in DN2- and DN3-stage thymocytes derived from EGFP-CCR9 knock-in mice. DN2 thymocytes in these mice show low-level $EGFP^{CCR9}$ expression (Fig. 1 C).

Cytospin and hematologic staining. Cells were spun onto glass slides and stained according to Pappenheim. In brief, cells were fixed in May-Grünwald solution (Sigma-Aldrich) for 2 min at RT, washed first with distilled water, and then tap water before 15 min of staining with a 1:20 dilution of Giemsa solution (Sigma-Aldrich).

In vitro cultures of thymic progenitor cells. For the culture of thymic progenitor cells layers of OP9 (provided by M. Kondo, Duke University, Durham, NC) and OP9-DL1 (provided by J.C. Zuniga-Pflücker, University of Toronto, Toronto, Ontario, Canada) stromal cells and mixtures of OP9 and OP9-DL1 cells were plated at 2×10^3 cells per 96-well plate 48 h before progenitors were sorted. Irradiated (3000 rad) ST2 bone marrow stromal cells were seeded at 10^4 cells per 96-well plate. 24 h before cell sorting, the cell culture medium was replaced by RPMI 1640 containing 10% FCS, 2 mM L-glutamin, 100 U/ml penicillin, 100 μg/ml streptomycin, 1 mM sodium pyruvate, and 2×10^{-5} M β-mercaptoethanol. Thymic progenitors were sorted onto the different stromal cell layers, and progenitors were cultured in the presence of recombinant, murine IL-7, Flt3L, SCF (10

ng/ml each; R&D Systems), and 50 ng/ml recombinant, human IL-2 (Peprotech) at 37°C in a humidified chamber and 5% CO_2. At the indicated time points, cultures were analyzed by FACS. For ST2 cultures, adherent hematopoietic cells were detached by incubation with PBS/0.3% BSA/5 mM EDTA for 10 min to generate single cell suspensions. LSKs ($Lin^-CD127^-Sca\text{-}1^{hi}CD117^{hi}$), CLPs ($Lin^-CD127^+Sca\text{-}1^{low}CD117^{low}$), and TMPs were cultured on methylcellulose containing IL-7 (10 ng/ml; Methocult M3630, StemCell Technologies Inc.) supplemented with recombinant SCF (100 ng/ml) and Flt3L (20 ng/ml; both obtained from R&D Systems). Differentiation of thymic progenitors toward dendritic cells was achieved by splitting OP9/OP9-DL1 (20:1) cultures at day 10 onto methylcellulose (Methocult M3231, StemCell Technologies Inc.) supplemented with recombinant, murine IL-1β (5 ng/ml), IL-3 (50 ng/ml), IL-6 (10 ng/ml), SCF (30 ng/ml), and Flt3L (30 ng/ml; all R&D Systems). Cells were analyzed by FACS after 4 d in culture.

Online supplemental material. Fig. S1 identifies EGFP-expressing cells in heterozygous EGFP-CCR9 knock-in mice. Fig. S2 characterizes the in vivo thymus-repopulating capacity of $EGFP^{CCR9+}$ populations in the bone marrow. Fig. S3 shows the fraction of $EGFP^{CCR9}$-expressing cells among CLPs and LSKs. Fig. S4 demonstrates that $NK1.1^-CD90.2^+EGFP^{CCR9+}$ found in OP9-DL1 cultures represent DN3/4 stage thymocytes. Table S1 shows the results of competitive transfer experiments of wild-type versus heterozygous EGFP-CCR9 knock-in bone marrow. Online supplemental material is available at http://www.jem.org/cgi/content/full/jem.20050146/DC1.

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