The stream of precursors that colonizes the thymus proceeds selectively through the early T lineage precursor stage of T cell development

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T cell development in the thymus depends on continuous colonization by hematopoietic precursors. Several distinct T cell precursors have been identified, but whether one or several independent precursor cell types maintain thymopoiesis is unclear. We have used thymus transplantation and an inducible lineage-tracing system to identify the intrathymic precursor cells among previously described thymus-homing progenitors that give rise to the T cell lineage in the thymus. Extrathymic precursors were not investigated in these studies. Both approaches show that the stream of T cell lineage precursor cells, when entering the thymus, selectively passes through the early T lineage precursor (ETP) stage. Immigrating precursor cells do not exhibit characteristics of double-negative (DN) 1c, DN1d, or DN1e stages, or of populations containing the common lymphoid precursor 2 (CLP-2) or the thymic equivalent of circulating T cell progenitors (CTPs). It remains possible that an unknown hematopoietic precursor cell or previously described extrathymic precursors with a CLP, CLP-2, or CTP phenotype feed into T cell development by circumventing known intrathymic T cell lineage progenitor cells. However, it is clear that of the known intrathymic precursors, only the ETP population contributes significant numbers of T lineage precursors to T cell development.

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Abbreviations used: CLP-2, common lymphoid precursor 2; CTP, circulating T cell progenitor; DN, double negative; EGFP, enhanced GFP; ETP, early T lineage precursor; pIpC, poly(I:C); TMP, thymic multipotent progenitor.

The question of which hematopoietic precursor homes to the thymus under physiological conditions has been a matter of considerable debate (1, 2). Several progenitors have been described as potential adult thymus-homing precursors: (a) the $CD4^{lo}$ precursor, defined as a $CD8^-$ $CD25^-$ $CD2^-$ $CD4^{low}$ Thy-1^{low} thymocyte (3); (b) the early T lineage precursor (ETP), defined as a Lin^- $CD44^+$ $CD25^-$ $CD117^+$ thymocyte (4); (c) the common lymphoid precursor 2 (CLP-2), defined in a preTCRα:: huCD25 transgenic mouse as a Lin^- $CD19^-$ $B220^+$ $CD117^-$ $huCD25^+$ cell that is found in the bone marrow and the thymus (5); (d) the L-selectin$^+$ precursor, defined as a Lin^- Sca-1^+ c-kit^+ Thy1.1^- $CD62L^+$ bone marrow cell (6); (e) the double-negative (DN) 1c, DN1d, and DN1e precursors, defined as CD24-high CD117-intermediate, $CD24^+$ $CD117^-$, and $CD24^-$ $CD117^-$ DN1 thymocytes, respectively (7); (f) the thymic multipotent progenitor (TMP), defined in a CCR9–enhanced GFP (EGFP) knock-in mouse as a Lin^- $CD44^+$ $CD25^-$ $CD117^+$ $CCR9^{EGFP+}$ cell present in bone marrow, blood, and thymus (8); (g) the circulating T cell progenitor (CTP), defined in a preTCRα::huCD25 transgenic mouse as a Lin^- $huCD25^+$ $CD44^+$ $CD117^{low\ to\ negative}$ $CD90^{high}$ cell present in the blood (9); and (h) the $CCR9^+$ multipotent precursor, defined as a Lin^- Sca-1^+ c-kit^+ $Flt3^+$ Thy1.1^- $CCR9^+$ bone marrow cell (10). The identification of these precursors as thymic precursors is based, for the most part, on their virtue of having T cell lineage potential in in vivo transfer assays or in vitro culture systems. The possibility of several precursors entering the thymus simultaneously, the uncertainty concerning the relative contribution to differentiating thymocytes, and the functional role of these precursors have hampered progress in the field. Studies that examine the previously described thymus-homing precursors under physiological conditions are needed.

Over time, the limitations of in vivo transfer assays and in vitro culture systems in identifying thymus-homing precursors have become

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increasingly evident. In in vitro assays, T cell lineage potential has been detected in many bone marrow precursors, with unknown relevance for thymus colonization. Also, currently available in vivo assays that use the intravenous transfer of hematopoietic precursors poorly mimic thymus homing in an unmanipulated animal: in irradiated recipients, donor T cell lineage precursors have been shown to home to the thymus not before 6 d after bone marrow transfer (11) and to commit to the T cell lineage in lymph nodes and the spleen (12), making it impossible to say what the actual phenotype of the thymus-settling precursor is. The transfer of hematopoietic precursors into unirradiated mice suffers from the difficulty that recipients can be analyzed either early or late for the identification of immigrants or their differentiation potential, respectively, but not for both. By the intravenous transfer of bone marrow cells, a comparison of thymus-homing precursors has recently concluded that CLP-2 precursors migrate to the thymus more efficiently than any other bone marrow–derived progenitor, and that this pronounced thymus tropism makes it so difficult to detect these cells in the blood (13). Whether the CLP-2 precursors that entered the thymus subsequently differentiated to T lineage cells was not investigated. To assess the overall importance of CLP-2 precursors, this analysis would be of particular interest because CLP-2s have been found to have a lower T cell lineage potential than ETPs (5). Thus, there is a need for assays that investigate thymus-homing precursors under more physiological conditions to identify the thymic precursor or precursors that represent the stream or the channel through which T cell development progresses.

In this study, we have investigated which of the previously described thymus-homing precursors enters the thymus rapidly and efficiently. Because all relevant thymus-settling precursors must eventually appear in the thymus, we concentrated on cells isolated from the thymus. We investigated thymocytes with an ETP, TMP, DN1c, DN1d, and DN1e phenotype, as well as thymocyte populations that contain the thymus equivalent of the CTP and the CLP-2, for their ability to give rise to T cells by two independent approaches, thymus transplantation and inducible lineage tracing. These novel model systems obviate the need for the intravenous transfer of bone marrow cells. Extrathymic precursors were not investigated. Both assays identify the ETP population among the previously described precursors as the precursor through which the stream of precursors replenishes T cell development in the thymus. Although our data do not rule out the possibility that a currently unknown stream of hematopoietic precursors or previously described extrathymic precursors with a CLP, CLP-2, or CTP phenotype feed into T cell development by circumventing the known intrathymic T cell lineage progenitors, it is clear that in comparison to the other known thymic precursors, only the ETP population contributes significant amounts of T cell lineage precursors to T cell development. Furthermore, we identify the $Flt3^{low}$ $CCR9^{EGFP+}$ LSK population as the potential source of thymus-homing precursors in the bone marrow.

RESULTS

T cell lineage precursor emigration from the bone marrow is selective

The process of emigration of bone marrow precursors into the blood is poorly understood but critical for thymus colonization, because only hematopoietic precursors that leave the bone marrow under physiological conditions will ultimately reach the thymus. It was important to learn more about this process, because thymus-homing experiments that transfer bone marrow cells intravenously are based on the assumption that all precursors are able to leave the bone marrow and are found in blood just as they are isolated from the bone marrow. In trying to identify the T cell lineage precursors that constitute the stream of hematopoietic progenitors, we investigated qualitative differences (i.e., the presence or absence of T lineage precursors) between phenotypically identical populations in bone marrow and peripheral blood.

Because the most efficient thymus-settling precursors are thought to lack the expression of lineage markers, we systematically investigated Lin^- precursors in the bone marrow and blood of heterozygous CCR9-EGFP knock-in mice. We have previously shown that these mice have normal T cell development and that the $CCR9^{EGFP}$ marker allows the separation of functionally distinct T cell lineage precursor subsets (8). To examine the presence of T cell lineage precursors in a given subpopulation, we cultured 100 sorted cells on the bone marrow stromal cell OP9 engineered to overexpress the Notch ligand Delta-like 1 (OP9-DL1)(14) and determined the presence of T cell lineage–committed DN3/4 cells 12 and 19 d after culture initiation. No T cell lineage precursors were found in Lin^+ peripheral blood leukocytes (unpublished data). Lin^- blood leukocytes were separated according to their CD117 and $CCR9^{EGFP}$ expression into four populations (Fig. 1 A). Although Lin^- $CD117^-$ cells are largely devoid of T cell lineage precursors that develop on OP9-DL1 cells, the rare Lin^- $CD117^+$ $CCR9^{EGFP+}$ population (representing an estimated 30–60 cells per mouse) contains T cell lineage precursors that give rise to committed T cells already after 12 d (Fig. 1 B). Consistent with our previous work (8), the Lin^- $CD117^+$ $CCR9^{EGFP-}$ population in the blood lacks this activity: day 12 T lineage precursor activity was not detected in >2,000 Lin^- $CD117^+$ $CCR9^{EGFP-}$ blood cells. This population contains a slowly differentiating, presumably hematopoietic stem cell–like precursor activity because it takes an additional 7 d until the first few wells with committed T cell lineage cells can be detected (Fig. 1 B). Significantly, the corresponding Lin^- $CD117^+$ $CCR9^{EGFP-}$ population in the bone marrow does contain T cell lineage precursors that give rise to committed T cells after 12 d. Thus, there is a qualitative difference between phenotypically indistinguishable precursor populations in the bone marrow and blood. Although the basis for this difference remains to be determined, the experiment demonstrates that the ability of bone marrow precursors to enter the blood stream needs to be considered for meaningful thymus-homing assays. We conclude that experiments that use the intravenous transfer of sorted bone marrow

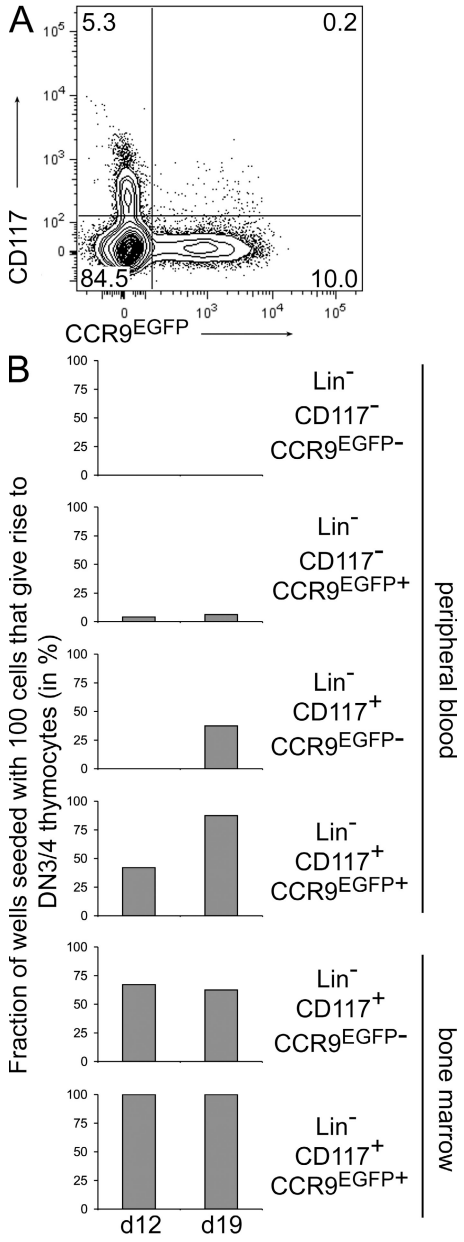


Figure 1. The emigration of T cell lineage precursors from bone marrow to blood is selective. (A) Lin$^-$ blood cells of heterozygous CCR9-EGFP knock-in mice were fractionated according to their CD117 and CCR9^{EGFP} expression. The plot is gated on Lin$^-$ cells. Percentages are indicated. (B) Lin$^-$ fractions with the indicated phenotypes were isolated from blood (as shown in A) and from bone marrow, and were cultured as groups of 100 cells on OP9-DL1 stromal layers for 12 and 19 d. The fraction of wells that contained T lineage–committed DN3/4 thymocytes is plotted for each time point. At least 1,200 cells cultured as 100 cells per well were assayed for each population and time point. For the analysis of T lineage precursor activity of Lin$^-$ CD117$^+$ CCR9^{EGFP-} blood cells at day 12, >2,000 cells cultured as 100 cells per well were analyzed.

populations to identify thymus-settling precursors may be misleading by investigating precursors that do not normally leave the bone marrow microenvironment. To identify the T cell lineage precursors that constitute the stream of hematopoietic precursors that lead to mature T cells, we therefore chose two independent models of thymus homing that avoid the intravenous transfer of bone marrow cells and exclusively investigate precursors that leave the bone marrow under physiological conditions.

A thymus transplantation model identifies ETPs as the precursor through which the stream of progenitors proceeds

To identify the precursors among previously described populations through which the stream of T lineage precursors proceeds, we analyzed the earliest T cell lineage precursors that enter a transplanted newborn thymus. To this end, we transplanted the newborn thymi of mice expressing CD45.1 (referred to as CD45.1$^+$ donor thymi) under the kidney capsule of 4–6-wk-old heterozygous CCR9-EGFP knock-in mice on a CD45.1/CD45.2 F1 background (referred to as CCR9^{EGFP}/+//CD45.1/CD45.2 F1 recipients). Mature donor thymus-derived T cells were readily detectable in recipient mice 3–4 d after transplantation, indicating that thymic emigrants rapidly established access to the recipient's circulation (unpublished data).

Consistent with the fact that during transplantation and subsequent reconnect the flow of precursors was interrupted, the number of immature precursors in the donor thymi declined up to day 9 after transplantation (Fig. 2 A). From then on, donor thymi were rapidly repopulated with recipient-derived immature thymocytes in the expected order: ETP followed by DN2, DN3, and DN4 (Fig. 2 A). Thus, in contrast to the disturbed order of repopulation by immature thymocytes found in irradiated recipients (12), the transplantation model reflects the physiological order of early T cell development. No evidence was found for a colonization of the graft by recipient-derived T cell lineage–committed DN2/3 precursors that circumvented the ETP stage (15) up to day 7 after transplantation. This finding suggested that the ETP stage is an obligatory step for the earliest precursors that colonize the graft in the transplant model. However, the data do not rule out that colonization by T cell lineage–committed DN2/3 precursors occurred at a later time point. Analyzing the DN1 population in donor thymi in detail, we found that the fraction of donor ETPs that makes up ~80% of DN1 thymocytes in a newborn thymus declined after transplantation to ~3% of all DN1 thymocytes on day 11 after transplantation. Conversely, the fraction of recipient ETPs within DN1 thymocytes of transplanted thymi increased from 1% on day 5 to 25% on day 11 after transplantation (Fig. 2 B). Interestingly, the recipient ETP population that was the first to enter donor thymi expressed high levels of CCR9^{EGFP}, consistent with the notion that these cells represent the most immature ETP population (Fig. 2 B, day 5) (8, 16). We have termed this the thymic multipotent precursor population because it contains precursors that give rise to T, B, and dendritic cells on the single-cell level (8). ETPs on subsequent days contained increasing fractions of cells that express intermediate to low

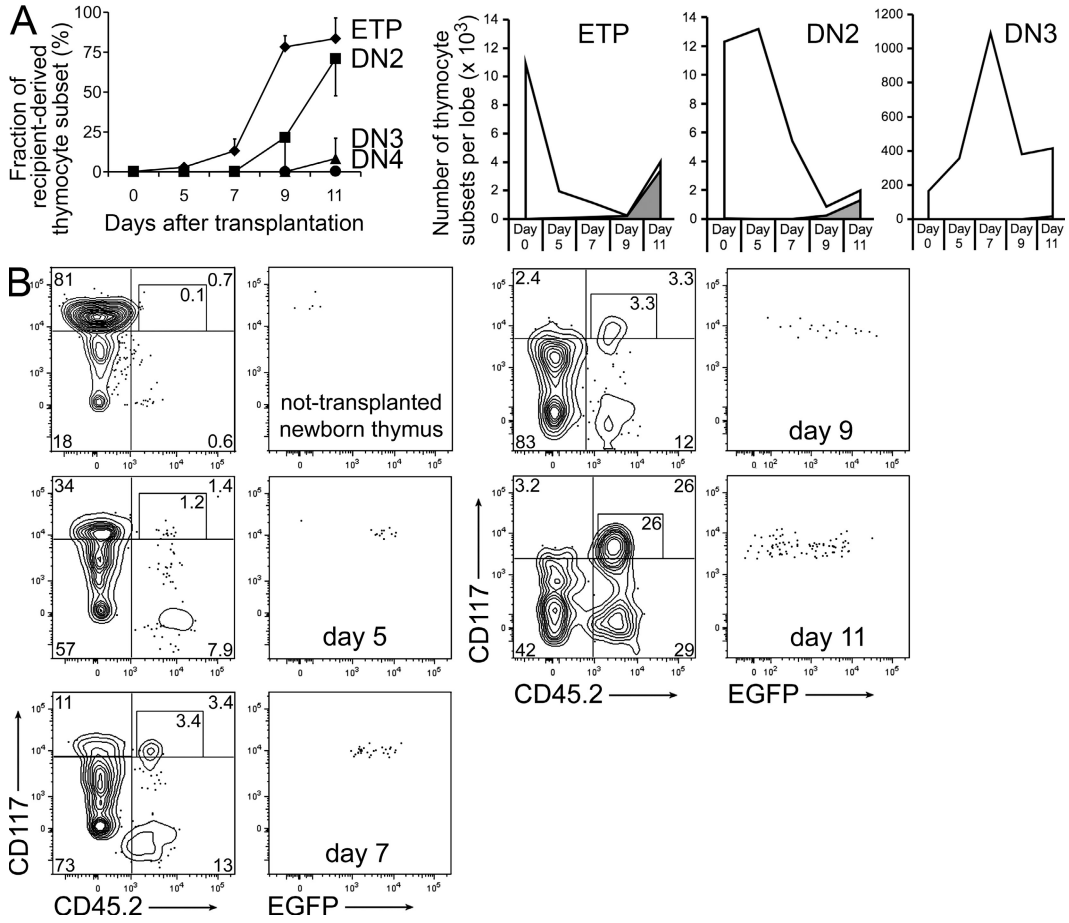


Figure 2. A thymus transplantation model for the identification of thymus-settling precursors. (A) CD45.1$^+$ newborn donor thymic lobes were transplanted under the kidney capsule of CCR9^{EGFP}/+//CD45.1/CD45.2 F1 recipient mice. The fraction, as well as the absolute number, of recipient thymocytes found in the ETP, DN2, DN3, and DN4 populations in the transplanted lobes are plotted against the time after transplantation. (right) The stacked area plots display the absolute number and the relative contribution of recipient (gray area) and donor (white area) cells per transplanted lobe for each thymocyte subset. Three lobes were analyzed per time point. Data are means ± SD. (B) Flow cytometric analyses show the Lin$^-$ CD44$^+$ CD25$^-$ DN1 subset present in a newborn CD45.1$^+$ thymus, and 5, 7, 9, and 11 d after transplantation of such a thymus under the kidney capsule of a CCR9^{EGFP}/+//CD45.1/CD45.2 F1 recipient mouse (FACS plots on the left of each column). FACS plots on the right of each column are gated on recipient, CD45.2$^+$ ETPs, as shown in FACS plots on the left of each column. Percentages are indicated.

levels of CCR9^{EGFP}, suggesting that these cells are more mature stages within the ETP population (Fig. 2 B, days 7, 9, and 11). In this model, transplanted thymi contained small amounts of recipient blood leukocytes that were mainly lineage marker–positive but appeared also as CD117$^-$ CD45.2$^+$ cells in the DN1 population (Fig. 2 B). Using serial transplantation, we found that precursor homing does not occur simply by exposing newborn thymi to recipient blood but requires 48 h of graft resident time, presumably for vascular reconnect (Fig. S1, available at http://www.jem.org/cgi/content/full/jem.20072168/DC1). Thus, the thymus transplantation approach represents a model of thymic precursor homing in which blood-derived precursors that left the bone marrow under physiological conditions initiate T cell development in the transplant.

To identify the precursors among the previously described populations that constitute the stream of hematopoietic precursors entering transplanted newborn thymi, we fractionated Lin$^-$ CD45.2$^{low/positive}$ transplant thymocytes by FACS sorting. Because all relevant thymus-settling precursors must eventually appear in the thymus, we sorted thymocytes with the ETP, TMP, DN1c, DN1d, and DN1e phenotypes. Extrathymic precursors were not studied with this approach. Because CTP and CLP-2 can be directly identified only in a transgenic mouse model that was not available to us (5, 9), we investigated the thymocyte populations that contain the CTP and the CLP-2 for their T cell lineage precursor activity. This approach allowed us to detect a potential stream of precursors in this population even if it would not be possible to attribute it to the previously described precursor. Because the CD4^{lo} precursor and the L-selectin$^+$ precursor are defined by a set of surface markers that is shared by the ETP population, these precursors were not investigated independently. Apart from the CTP, all

Table I. T cell lineage precursor frequency in early stages of T cell development isolated from CD45.1+ newborn donor thymic lobes either 4 or 6 d after transplantation under the kidney capsule of CCR9^{EGFP}/+//CD45.1/CD45.2 F1 recipient mice

Time of sorting after transplantation	Sorted cell type	T cell progenitor frequency^{-1} (95% confidence interval)	
		CD45.2+ CCR9^{EGFP+} (recipient-derived)	CD45.2− CCR9^{EGFP-} (donor-derived)
Day 4	EGFP-pos ETP[a]	24.5 (5.8–103.7)	5.4 (2.6–11.6)
	ETP	43.5 (10.6–176.8)	2.5 (1.6–4)
	DN1c	∞	17.5 (2.4–129.3)
	DN1d	∞	∞
	DN1e	∞	1,323 (320–5,481)
	CLP-2	∞	∞
	CTP	∞	168 (40.5–698)
Day 6	EGFP-pos ETP	5.9 (3.8–9.2)	∞
	ETP	22.2 (14.2–34.5)	28 (17.2–45.6)
	DN1c	∞	37.7 (12.1–117.5)
	DN1d	∞	439 (107–1,800)
	DN1e	∞	∞
	CLP-2	∞	58.8 (25.4–135.9)
	CTP	∞	∞

[a]4 d after transplantation, the vast majority of ETPs are donor derived, and a defined EGFP+-recipient ETP population that can be gated for is not discernible.

precursors studied in this paper have been shown to be present in the thymus. Assuming that CTPs isolated from the thymus are functionally equivalent to CTPs in the blood, we included the CTP in our analysis.

T precursors were sorted from newborn thymi after they had been under the kidney capsule of the recipient for either 4 or 6 d and then cultured on OP9-DL1 stromal layers. CD45.2^{low} cells were included in these sorts because hematopoietic precursors express only low levels of CD45 compared with mature leukocytes, and the concomitant sorting and culture of CD45.2− donor precursors serves as a positive control. In FACS analyses of these cultures, precursors of donor and recipient origin can be easily distinguished by CD45.2 staining and EGFP expression (Fig. S2, available at http://www.jem.org/cgi/content/full/jem.20072168/DC1). Sorting thymocytes from whole thymus lobes, the first T cell lineage precursors were detectable on day 4 after transplantation (Table S1). Each thymocyte precursor type was sorted from transplant-derived thymocytes equivalent to nine complete lobes. This was done for transplants that had been in place for 4 and 6 d, respectively. The T cell lineage precursor frequency was determined (Table I) from the complete dataset (Table S1) by assuming a Poisson distribution. Consistent with the literature, T cell lineage–committed DN3/4 cells developed from all tested precursor populations (Table I). This included the population containing the CTP, suggesting that the thymus equivalent of the CTP may indeed be present in the thymus and possess T cell lineage potential. However, all recipient-derived (i.e., immigrating) T cell lineage precursors were found in the ETP population or in the ETP subset that was gated for EGFP expression. Because 4 d after transplantation the vast majority of ETPs are donor derived (the day 5 panel of Fig. 2 B suggests at least a 24-fold [34% donor vs. 1.4% recipient] excess of donor vs. recipient ETPs) and, because of low numbers of cells, a defined recipient ETP population is not discernible, several donor-derived EGFP− ETPs were inadvertently seeded into culture wells despite gating for expression of EGFP (Table I). With a discernible recipient ETP population occurring 6 d after transplantation, separation was stringently achieved.

The data indicate that ETP but not DN1c, DN1d, DN1e, CLP-2, or the thymus equivalent of the CTP constitute the stream of the earliest T cell lineage precursors that colonize the thymus. Because for any given precursor all precursors of that phenotype found in nine complete lobes were cultured and analyzed for each time point, this assay directly assessed the precursors' relative contribution to T cell development. In other words, precursors are detected in this assay in relation to their contribution to the stream of immigrating precursor cells and not for their absolute potential to repopulate the thymus like in an intravenous transfer model using sorted precursors. It is unknown, however, if ETP, DN1c, DN1d, DN1e, CLP-2, and CTP contain all thymus-settling T cell lineage progenitors that feed into T cell development. Others may exist outside of the populations investigated in this study. Although our data do not rule out the possibility that a currently unknown stream of hematopoietic precursors or previously described extrathymic precursors with a CLP, CLP-2, or CTP phenotype feed into T cell development by circumventing the known intrathymic T cell lineage progenitors, it is clear that in comparison to the other known thymic precursors only the ETP population contributes significantly to T cell development. Although all of the non-ETP precursors contained T cell lineage precursor activity as well, which is

consistent with their previous description as T lineage precursors, the data demonstrate that this activity cannot be detected in the graft early after transplantation. This suggests that either these precursors develop from ETPs later or that they colonize the thymus infrequently or inefficiently. It was often argued that immigrating precursors might rapidly change their phenotype as they enter the thymus (e.g., a precursor may enter as a CLP-2 and rapidly convert to an ETP). No evidence for such a behavior was found. If rapid phenotype switching occurred within the thymus, one would expect to find T lineage potential not only in the ETP but also in at least one other precursor subset.

An inducible lineage-tracing approach indicates that among the previously described precursors only the ETPs represent a stream of precursors that give rise to committed T cell lineage cells

It could be argued that what we can learn from the transplantation model is limited by the possibilities that (a) a newborn thymus may attract different T cell lineage precursors compared with an adult thymus, that (b) the structures through which hematopoietic precursors enter a transplanted thymus may be distinct from those of the orthotopic thymus, and that (c) the stress associated with transplantation may lead to the mobilization of hematopoietic precursors that do not normally colonize the thymus. To build a model that is even closer to the physiological situation, we developed an inducible lineage-tracing approach that allows the observation of cohorts of developing hematopoietic precursors as they pass from the bone marrow to the thymus. In a first step, we genetically engineered mice that express Cre recombinase constitutively under the control of the genomic Flt3 locus (Fig. S3, available at http://www.jem.org/cgi/content/full/jem.20072168/DC1). In these mice, stages of B and T cell development in Flt3::Cre transgenic mice show reporter gene recombination consistent with irreversible marking of cells at the LSK stage, reflecting the physiological Flt3 expression pattern in this multipotent precursor population.

To be able to follow waves of T cell development in mice as they proceed from the bone marrow to the thymus and develop toward T cell lineage–committed double-positive thymocytes, we generated, in a second step, mice that expressed a tamoxifen-inducible Cre recombinase (CreERT2; reference 17) under the control of the abovementioned Flt3 genomic locus as a transgene. Two independent Flt3::CreERT2//R26R-EYFP mouse lines were produced in which $EYFP^+$ (i.e., recombined) blood leukocytes appeared only after intraperitoneal injection of tamoxifen but not after injection of carrier alone. Upon intraperitoneal injection of a single 3-mg dose of tamoxifen, we found that a wave of $EYFP^+$ hematopoietic precursors moved through all stages of T cell development. Higher doses of tamoxifen were not used because they were found to be associated with disturbances of the cellular composition of the lineage-negative compartment in the bone marrow. Transgenic mice were dosed and killed 1, 3, and 5 wk later, and the relevant precursors were investigated for $EYFP^+$ cells (i.e., recombined stages) by FACS. The levels of recombination for three representative mice shown in Fig. 3 A reveal that the fraction of recombined, $EYFP^+$ cells was highest in $Flt3^+$ LSKs and ETPs at 1 wk after injection, in DN3/4 thymocytes 3 wk after injection, and in single-positive thymocytes 5 wk after injection. Consistent with the results from mice with constitutive Flt3::Cre expression (Fig. S3), there was no evidence for recombined thymocytes beyond the DN3/4 stage at 1 wk after injection, confirming the absence of Cre activity beyond the LSK stage also in the inducible Cre transgenic lines.

After the injection of 3 mg tamoxifen, the highest level of recombination at any given stage of T cell development did not exceed 5% (Fig. 3 A). In this inducible lineage-tracing experiment, we viewed the irreversibly marked precursors that appeared only after tamoxifen injection as an "indicator" or "sentinel" cohort of cells. This sentinel cohort was followed over time as the stream of T lineage precursors homes to the thymus and matures therein. As shown in Fig. 3 A, genetically marked sentinel cells progressed through the known stages of T cell development in the expected order. The fact that maximal recombination was stable around 5% for all mice analyzed at any time point of analysis (i.e., regardless of which stage the wave had progressed to) indicated that the absolute number of sentinel cells expanded and collapsed at the same rate as the unmarked cells. Collectively, marked sentinel cells appeared to behave just like unmarked thymocytes and could therefore be used to follow T lineage precursors as they homed to and matured within the thymus under physiological conditions.

To identify the precursors among the previously described populations through which the stream of hematopoietic precursors progresses toward mature T cells, we followed the sentinel cells over time. For this to be achieved, a >5% recombination rate was not required because this is well within the range of detection for EYFP-marked cells by FACS analysis. Following the 5% irreversibly marked sentinel population to the thymus, we concentrated on the leading edge of the cohort of recombined cells. This approach appeared sensible, because the median lifetime of Flt3-expressing LSK bone marrow cells is unknown and recombined $Flt3^+$ LSK cells could still be detected 5 wk after a single injection of tamoxifen (Fig. 3 A). It is therefore possible that smaller waves of recombined precursors may follow the first cohort investigated in this study. We analyzed the previously described thymic precursor subsets that are thought to contain the thymus-settling precursor for EYFP expression 1 and 2 wk after injection by multicolor flow cytometry. These time points were chosen because R26R-EYFP locus recombination and maximal EYFP protein expression in vivo takes 48–72 h and because high-level recombination is still found in the $Flt3^+$ LSK population at 1 wk after injection (Fig. 3 A). These $EYFP^+$ $Flt3^+$ LSKs would be expected to move on to the thymus before week 3, when the majority of recombined cells have reached the DN3/4 stage. Consistent with the transplantation model, we found genetically marked cells in

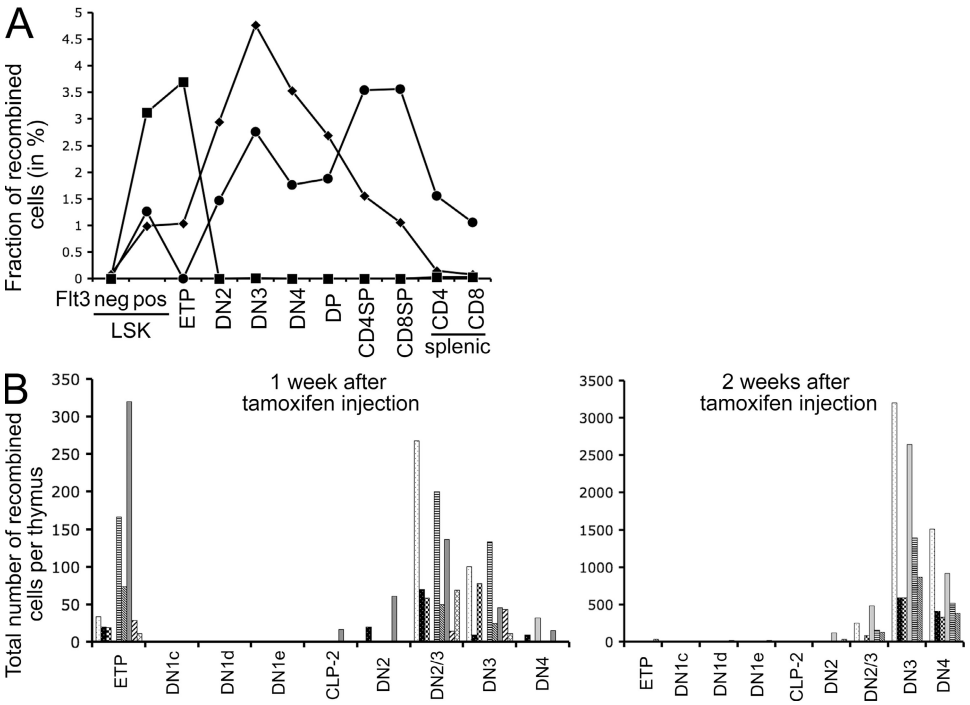


Figure 3. An inducible lineage-tracing approach shows that the stream of precursors proceeds from the bone marrow selectively through the thymic ETP population. (A) The level of recombination for the indicated stages of T cell development is shown for one representative Flt3::CreERT2//R26R-EYFP mouse at 1 wk (squares), one representative mouse at 3 wk (diamonds), and one representative mouse at 5 wk (circles) after tamoxifen injection. Each line represents the data from one mouse. The 3-mg 4-hydroxy-tamoxifen dose was titrated such that a single injection did not lead to detectable changes in the hematopoietic system. (B) The absolute number of recombined, EYFP$^+$ thymic precursors in Flt3::CreERT2//R26R-EYFP mice at 1 and 2 wk after a single 3-mg tamoxifen injection are shown. Each bar represents one mouse.

the ETP population of eight out of nine mice early after wave initiation. In contrast, marked cells were found in the other T lineage precursors only in one mouse, with a few recombined cells in the gate that contains the CLP-2 precursors (absolute cell counts per thymus are shown in Fig. 3 B; and fractions of recombined cells are shown in Fig. S4, available at http://www.jem.org/cgi/content/full/jem.20072168/DC1). The data demonstrate that the majority of genetically marked cells found among the previously described precursors passes through the ETP but not the DN1c, DN1d, and DN1e stages nor the population containing the CLP-2 stage. Extrathymic precursors were not studied with this approach. Although significant absolute numbers of marked DN2 and DN3 cells were already found 1 wk after tamoxifen injection (Fig. 3 B), these represent only a minute fraction of DN2 and DN3 thymocytes (Fig. S4), which is consistent with the idea that these cells represent the leading edge of the marked cohort of cells. As with the transplantation model, the data do not rule out the possibility that a currently unknown stream of hematopoietic precursors feeds into T cell development by circumventing the progenitors tested in this study. Again, no evidence for the possibility that thymus-settling precursors rapidly change their phenotype within the thymus was found. If they did, the investigation of thymus-settling precursors at different time points after the genetic marking of the cohort of homing cells would be expected to find marked cells in several phenotypically distinct populations. Collectively, both the transplantation model and the inducible lineage-tracing model indicate that the stream of hematopoietic precursors, when it first enters the thymus, proceeds selectively through the ETP stage.

Notch1-deficient T cell lineage precursors are detectable in the ETP population

After the identification of the ETP population as the stage through which significant numbers of T cell lineage precursors progress on their way to mature T cells, we asked if in the absence of Notch1, the master regulator of T cell lineage commitment (18), thymus-settling precursors still reach the ETP stage. We have previously shown in competitive transfer experiments with wild-type bone marrow that Notch1-deficient bone marrow reconstitutes thymic B but not T cells (19), which led us to the question if Notch1-deficient T cell lineage precursors enter the thymus at all and, if they do, what their phenotype would be. Notch1-deficient bone marrow precursors injected into the thymus fail to generate T cell lineage cells and either die or acquire a non-T fate (18). Therefore, if ETPs represented a significant part of the stream of immigrating T cell lineage precursors, as suggested by the data above, they would be expected to contain the Notch1-deficient T cell lineage precursors that subsequently fail to thrive. To demonstrate that Notch1-deficient ETPs contain T lineage

potential, we made use of the fact that Notch1-deficient T cell precursors, when cultured on OP9-DL1, use Notch2 to differentiate into T lineage–committed cells (20). CCR9-EGFP knock-in mice were bred onto the Notch1$^{lox/lox}$//MxCre background that allows the inducible deletion of Notch1 (19), and as expected, injecting these mice with pIpC (poly(I:C)) led to the almost complete elimination of the ETP population after 4 wk (Fig. 4 A), consistent with a previous report (21). Sorting single Notch1-deficient CCR9^{EGFP+} ETPs from Notch1$^{lox/lox}$//MxCre//CCR9^{EGFP}/+ mice 4 wk after pIpC injection onto OP9-DL1 stromal layers resulted in 17 positive T cell cultures out of 90 wells in one experiment and in 4 positive T cell cultures out of 39 wells in another (Fig. 4, A and B). For the majority of these T cell cultures, genotyping of the resulting DN3/4 thymocytes confirmed the absence of Notch1 in the precursor that initiated the culture (Fig. 4 C). Thus, consistent with our previous finding that T cell lineage precursors commit only after they arrive in the thymus (16), the data indicate that Notch1-deficient T cell lineage precursors do still enter the thymus and are detectable in the population that contributes significantly to the stream of immigrating T cell lineage precursors.

Identification of a rare bone marrow population as the closest relative to the TMP

Finally, we investigated the potential source of the stream of thymus-homing precursors in the bone marrow that enters as ETPs. We considered the identification of a bone marrow equivalent of the earliest ETP (i.e., the TMP), an important piece of the puzzle because the identification of a phenotypical and functional equivalent of the TMP in the bone marrow would support the notion that the TMP is a thymus-settling precursor and argue against the possibility that the TMP is an intermediate stage between an as yet uncharacterized thymus-settling precursor and more mature stages of T cell development. Several bone marrow populations have been postulated to be the precursors for the thymus-settling progenitor based on phenotype and T cell lineage potential (6, 10, 22). The challenge is to identify a bone marrow cell that shows the same level of maturity and the same bias toward T cell development as intrathymic precursors. Studying developmental kinetics of precursors has been effectively and widely used to determine precursor maturity (4, 7, 23), but a bone marrow cell that develops with the same kinetics as a progenitor isolated from the thymus has not been described. The presented data characterize this stream of precursors as Lin$^-$ CD117$^+$ Sca-1$^+$ CCR9^{EGFP+} cells because all CD117$^+$ cells in the thymus are Sca-1$^+$. Furthermore, the lineage-tracing experiments suggested that the direct source of thymic ETPs had passed through a Flt3$^+$ stage and should therefore present as a highly recombined population in the Flt3 lineage trace model (Fig. S3). To investigate the recombination status of LSK subsets, we crossed constitutive Flt3::Cre//R26R-EYFP lineage trace mice onto a heterozygous CCR9-EGFP knock-in background. As expected, all CCR9^{EGFP-} LSK subsets in CCR9^{EGFP}/+//Flt3::Cre//R26R-EYFP mice showed

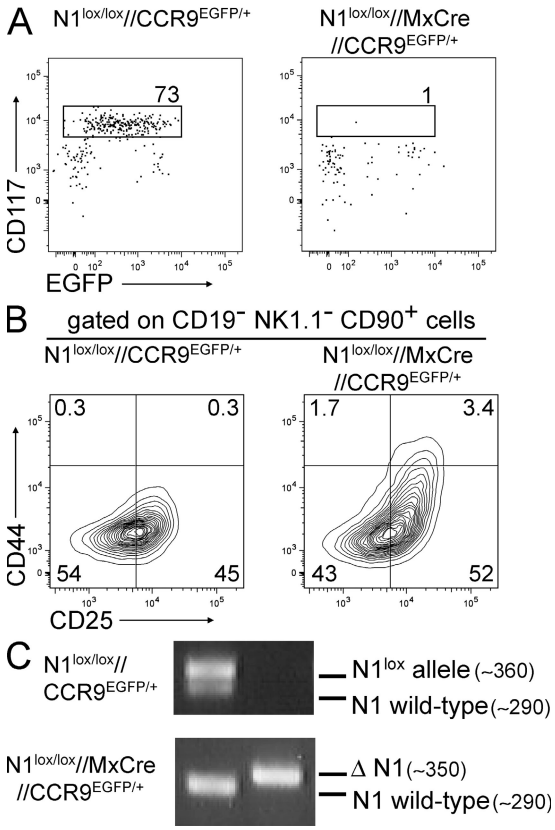


Figure 4. The ETP population in mice after Notch1 ablation contains Notch1-deficient T cell lineage precursors. (A) In the absence of Notch1, only very few ETPs can be isolated for the detection of T cell lineage precursors. The FACS plots are gated on Lin$^-$ CD44$^+$ CD25$^-$ DN1 thymocytes derived from Notch1$^{lox/lox}$//CCR9^{EGFP}/+ and Notch1$^{lox/lox}$//MxCre//CCR9^{EGFP}/+ mice 2 wk after pIpC injection. Percentages are indicated. (B) Single TMPs were sorted from Notch1$^{lox/lox}$//CCR9^{EGFP}/+ and Notch1$^{lox/lox}$//MxCre//CCR9^{EGFP}/+ mice 4 wk after pIpC injection on OP9-DL1 stromal layers and cultured for 12–14 d. Cells falling into the right half of the ETP gate shown in A were considered TMPs. DN3/4 thymocytes developed on OP9-DL1 stroma even in the absence of Notch1, as previously described (reference 20). Percentages are indicated. (C) Genomic PCR was performed on DNA purified from the OP9-DL1 culture of DN3/4 thymocytes generated from a single TMP of a Notch1$^{lox/lox}$//CCR9^{EGFP}/+ and a Notch1$^{lox/lox}$//MxCre//CCR9^{EGFP}/+ mouse 4 wk after pIpC injection. Two sets of primers were used, with one detecting the wild-type (N1 wild-type) and the floxed (N1^{lox} allele) Notch1 allele (left lane) and the other detecting the deleted allele of Notch1 (Δ N1; right lane). Because the added hematopoietic cells did not carry a wild-type Notch1 allele, this band is thought to be derived from OP9-DL1 stroma. PCR fragment sizes in base pairs are given in parenthesis.

lower levels of recombination than their CCR9^{EGFP+} counterparts (Fig. 5), suggesting that the CCR9^{EGFP+} LSKs are more closely related to the ETPs in the thymus. To our surprise, we found in this analysis that a rare Flt3^{low} CCR9^{EGFP+} LSK population showed the highest level of recombination. These high levels of recombination, together with the fact that CCR9^{EGFP+} ETPs also express low levels of Flt3 (16, 21), made this rare population an excellent candidate for the immediate bone marrow precursor of the most immature ETPs,

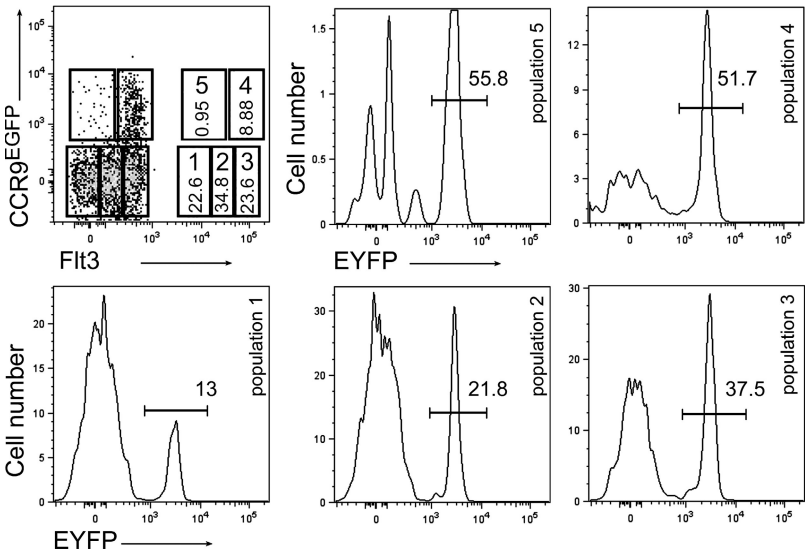


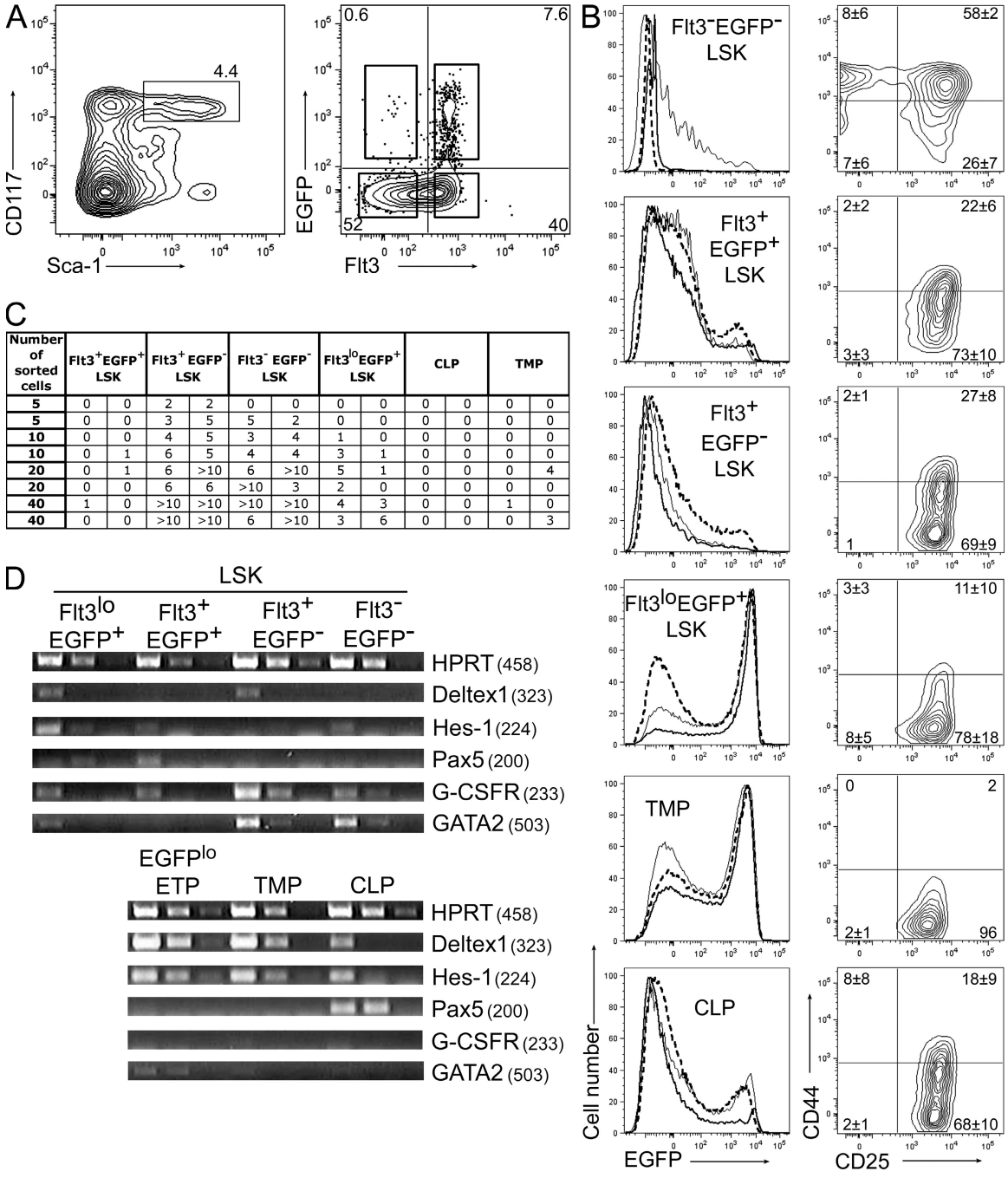
Figure 5. A rare Flt3^{low} CCR9^{EGFP+} LSK population shows the highest level of recombination among LSK subsets in the Ftl3-driven lineage-tracing model. The LSK population was analyzed by FACS in CCR9^{EGFP}/+//Flt3::Cre//R26R-EYFP mice. The top left plot is gated on LSK bone marrow cells, and the histogram plots are gated on the indicated regions of interest. The fraction of EYFP^{+}, recombined cells is shown for each subset. Percentages are indicated.

the TMPs. Several pieces of data support this notion. First, the careful analysis of the blood indicated that CCR9^{EGFP+} LSKs express only low levels of Flt3 (Fig. S5, available at http://www.jem.org/cgi/content/full/jem.20072168/DC1), suggesting that Flt3^{low} CCR9^{EGFP+} LSKs are able to leave the bone marrow. Second, the fractionation of bone marrow LSKs (Fig. 6 A) showed that Flt3^{low} CCR9^{EGFP+} LSKs (representing ~1,000 cells per 3–4-wk-old mouse) contain the highest T cell lineage precursor frequency compared with the other LSK subsets (Table II). Third, Flt3^{low} CCR9^{EGFP+} LSKs develop with kinetics that are indistinguishable from those of the TMP, whereas all other precursors require more time to reach the DN3/4 stage (Fig. 6 B), and fourth, they contain a low level of myeloid precursor activity that is also seen in the TMP (Fig. 6 C). Fifth, the Flt3^{low} CCR9^{EGFP+} LSK population is the only population that expresses detectable levels of both Notch target genes Deltex1 and Hes-1 (Fig. 6 D). In contrast, the Flt3^{+} CCR9^{EGFP+} LSK population expresses detectable levels of Pax-5 but not Deltex1 and Hes-1, suggesting a closer relationship to early B cell stages. Consistently, this population contains the highest B lineage potential among all four subsets (Table II). Collectively, the data indicate that a rare bone marrow population exists that shares the phenotypical as well as most of the functional characteristics of the most immature ETP subset, the TMP population. Specifically, the B cell precursor frequency among TMPs is significantly lower than that of Flt3^{low} CCR9^{EGFP+} LSKs, which is consistent with the previous finding that precursor maturation within the ETP population correlates with the loss of B cell potential (8, 21). Thus, only the most immature cells among TMPs are predicted to reach a comparable B cell precursor frequency. In conclusion, the Flt3^{low} CCR9^{EGFP+} LSK precursor in the bone marrow is phenotypically identical to the TMP based on the expression of the discriminatory markers lineage, Sca-1, CD117, CCR9^{EGFP}, Flt3, and Flt3::Cre-mediated recombination. The fact that the Flt3^{low} CCR9^{EGFP+} LSK precursor develops with the same kinetics as the TMP sets it apart from all previously described bone marrow precursors postulated to represent the thymus-settling precursor. Furthermore, the existence of a TMP equivalent in the bone marrow supports the notion that the TMP is a thymus-settling precursor and argues against the possibility that the TMP is an intermediate stage between an as yet uncharacterized thymus-settling precursor and more mature stages of T cell development.

DISCUSSION

T cell development in the thymus relies on continuous colonization by precursors from the bone marrow. In this study, we have investigated thymus colonization by two independent experimental strategies that avoid both irradiation of recipients as well as the transfer of bone marrow precursors. The data show that an incoming wave of thymus repopulating precursors proceeds exclusively through the ETP but not the DN1c, DN1d, or DN1e stages, nor through the population containing the CLP-2. In addition, we find no evidence for thymus colonization by a population that would be predicted to contain the thymic equivalent to the CTP. Thus, for the currently known thymic precursors, the findings provide strong evidence for thymic colonization by a single type of precursor rather than multiple independent precursors.

The colonization of the thymus by bone marrow–derived precursors is a complex process. Experimental models investigating this process must avoid deviating from the physiological situation as much as possible to produce meaningful results. The relevance of most previously described



Number of sorted cells	Flt3⁺EGFP⁺ LSK		Flt3⁺ EGFP⁻ LSK		Flt3⁻ EGFP⁻ LSK		Flt3ˡᵒEGFP⁺ LSK		CLP		TMP	
5	0	0	2	2	0	0	0	0	0	0	0	0
5	0	0	3	5	5	2	0	0	0	0	0	0
10	0	0	4	5	3	4	1	0	0	0	0	0
10	0	1	6	5	4	4	3	1	0	0	0	0
20	0	1	6	>10	6	>10	5	1	0	0	0	4
20	0	0	6	6	>10	3	2	0	0	0	0	0
40	1	0	>10	>10	>10	>10	4	3	0	0	1	0
40	0	0	>10	>10	6	>10	3	6	0	0	0	3

Figure 6. Flt3^{low} CCR9^{EGFP+} LSKs are phenotypically identical and functionally closely related to TMPs. (A) LSKs were fractionated according to their Flt3 and CCR9-EGFP expression. The FACS plot on the left is gated on Lin$^-$ cells, and the plot on the right is gated on LSK cells (as shown in the plot on the left). LSK subpopulations were sorted from the indicated regions. Percentages are indicated. (B) 100 cells of the indicated precursors were sorted onto OP9-DL1 stroma, cultured for 9 d, and analyzed by FACS. The levels of CCR9-EGFP and CD44 expression are indicative of the maturity of each precursor, as T lineage–biased precursors are expected to progress faster, whereas precursors without a T lineage bias or those biased toward a non-T fate will take longer to develop to DN3 thymocytes. The histogram plots are gated on DN2/3 thymocytes in which CCR9-EGFP expression increases with the progression from DN2 to DN3 thymocytes (reference 8). The results of three independent wells are shown in each histogram plot. Contour plots are gated on Lin$^-$ CD90$^+$ cells, and the numbers in each quadrant indicate the mean frequency of each population from three independent experiments. (C) The results of a representative clonogenic myeloid progenitor assay are shown for a 96-well plate seeded with 5, 10, 20, or 40 cells of the indicated precursors. The number of detectable myeloid colonies for each well is given. (D) Semiquantitative RT-PCR was performed on fivefold dilutions of cDNA prepared from the indicated precursors with primers specific for the indicated transcripts. Primers specific for hypoxanthin-phosphoribosyl-transferase (HPRT) transcript were used as a loading control. PCR fragment sizes in base pairs are given in parenthesis.

Table II. T and B cell progenitor frequency determined for LSK subsets by limiting dilution assays on OP9-DL1 and OP9 stromal cell layers, respectively

Cell type	T cell progenitor frequency^{-1} (95% confidence interval)	B cell progenitor frequency^{-1} (95% confidence interval)
Flt3^{low} CCR9^{EGFP}-pos LSK	6.5 (5.1–8.5)	4.3 (3.2–5.7)
Flt3-pos CCR9^{EGFP}-pos LSK	8.6 (6.6–11.1)	2.4 (1.9–3.1)
Flt3-pos CCR9^{EGFP}-neg LSK	16.9 (12.6–22.6)	15.3 (11–21.5)
Flt3-neg CCR9^{EGFP}-neg LSK	32.7 (23.3–46)	21.2 (14.7–30.6)
CLP	9.1 (7–11.8)	1.7 (1.4–2.1)
TMP	3.1 (2.5–3.8)	64.4 (37.3–111.4)

thymus-homing precursors has been established by experiments in which large numbers, mostly several hundred thousands, of the precursor were transferred intravenously into hosts and tested for their ability to enter the thymus. It is obvious that this assay is far from physiological, and the data presented in this study emphasize that this setup runs the risk of investigating bone marrow precursors that are confined to the bone marrow microenvironment. Most of the short-term thymus-homing experiments found that all of the tested precursors enter the thymus, albeit with different efficacies (7, 13), whereas long-term experiments analyzing thymus reconstitution have shown selectivity for specific precursors (24) without being able to pinpoint the immigrating precursor. The thymus-homing models presented in this study have several advantages over intravenous transfer models because (a) only T cell lineage precursors that enter the blood are investigated, (b) precursor immigration and progression to more mature stages of thymocytes can be monitored, (c) immigration of several precursors can be investigated in one and the same mouse, and (d) the relative contribution of each precursor to the stream of precursors can be assessed. The inducible lineage-tracing model appears closest to the physiological situation, as the only required intervention is a single injection of tamoxifen.

It is important to point out, however, that the data do not exclude the possibility that precursors outside the DN1 and the populations containing the CLP-2 and the CTP home to the thymus to give rise to thymocytes. The aim of the present study was the direct comparison of the known precursors and their relative contribution to T cell development. As information on new, independent precursors becomes available, their contribution will have to be investigated. For the previously described T lineage precursors, however, it is clear that the stream passes through the ETP stage but not through the DN1c, DN1d, or DN1e stages, nor through the populations containing the CLP-2 or the thymic equivalent of the CTP. For this conclusion, the use of the pre–T cell receptor reporter mouse in which CLP-2s and CTPs have been originally characterized (5, 9) was not required, because populations that entirely contain these subsets showed no T lineage precursor activity in the transplant model. For example, CLP-2s have been described to be a subset of Lin$^-$ CD19$^-$ B220$^+$ CD117$^-$ cells that can also be found in the thymus (5). Because the culture of all Lin$^-$ CD19$^-$ B220$^+$ CD117$^-$ cells that could be isolated from nine newborn thymic lobes did not produce committed T cells of recipient origin in the transplant model, a significant contribution to the stream of precursors by CLP-2s can be excluded. It could be argued that the known lower T cell lineage potential of CLP-2s (5) might have been below the level of detection. However, the lineage-tracing approach that does not depend on the T cell lineage potential as a read-out also argues against a significant contribution by CLP-2s.

The fact that Notch1-deficient T cell lineage precursors can be isolated from the rare cells found in the TMP gate of mice rendered Notch1 deficient through MxCre-mediated recombination further supports the notion that the stream of thymus-colonizing precursors proceeds through the ETP stage. Furthermore, the finding adds significantly to our understanding of the role of Notch in thymus colonization, as it shows that Notch1-deficient precursors enter the thymus as T cell lineage precursors with the phenotype of TMPs. Given the turnover of T cell lineage precursors within a few days (Fig. 2 B), all thymic precursors that recombined their Notch1 locus at the time of injection will have moved on to more mature stages of T cell development 4 wk later. Only long-lived precursors in the bone marrow would be expected to give rise to recombined offspring at that time point. Thus, it appears reasonable to assume that T cell lineage precursors isolated from the thymus 4 wk after pIpC injections have recombined their Notch1 locus in the bone marrow. That these Notch1-deficient TMPs give rise to B cells remains an intriguing possibility.

The identification of the TMP/ETP population as the precursor through which significant numbers of precursors progress has led us to investigate the source of precursors in the bone marrow. We were surprised to find that the Flt3^{low} CCR9^{EGFP+} LSK population, a population so small that it could easily be discounted for numbers too low to be investigated, turned out to be phenotypically identical and functionally closely related to TMPs. Taking the presented data together, in our view this Flt3^{low} CCR9^{EGFP+} LSK represents the best candidate for the bone marrow source of T cell lineage precursors homing to the thymus. Most striking is its ability to rapidly give rise to committed DN3/4 thymocytes in culture, which sets it apart from all other bone marrow populations.

MATERIALS AND METHODS

Mice. Mice were kept under specific pathogen-free conditions in the mouse facility of the Max-Planck-Institute of Immunobiology. 3–5-wk-old mice were used for experiments unless indicated otherwise. Experimentation and animal care were in accordance with the guidelines of the Max-Planck-Institute of Immunobiology. A sequence encoding Cre or CreERT2 (17) recombinase together with a kanamycin cassette flanked by frt sites was introduced into bacterial artificial chromosome (BAC) RP24-145C8 (BACPAC Resources Center) by ET cloning according to the manufacturer's recommendations (Gene Bridges). In a deviation from the protocol, negative selection was performed by selecting for kanamycin-sensitive colonies after transfecting the Flp recombinase–containing plasmid 706Flp:tet (Gene Bridges). Proper recombination and removal of the kanamycin cassette were confirmed by sequencing before the BAC was injected into C57BL/6 pronuclei, according to standard protocols.

Flow cytometric analysis, cell sorting and culture of hematopoietic precursors, and RT-PCR. FACS analysis and cell sorting and culture of hematopoietic precursors on stromal cell layers were performed as previously described (8). Stainings were analyzed on a flow cytometer (LSR II; BD Biosciences), and cells were sorted using a MoFlo (Dako) or a FACSAria (BD Biosciences). Precursors were cultured on methylcellulose (MethoCult M3434; StemCell Technologies Inc.), according to the manufacturer's recommendations. RT-PCR was performed as previously described (16).

Thymus transplantation model. Two to three CD45.1$^+$ newborn thymic lobes per kidney were placed under the kidney capsule of anesthetized CCR9^{EGFP}/+//CD45.1/CD45.2 F1 recipient mice. For recipient T lineage precursor identification, single-cell suspensions were prepared from pooled transplanted lobes, and the equivalent of nine complete lobes were stained and sorted on OP9-DL1 for each analyzed precursor. ETP precursors were gated as Lin$^-$ (where the lineage cocktail included anti-CD3, anti-CD8, anti-CD11c, anti-CD11b, anti-TCRβ, anti-TCRγδ, anti-NK1.1, anti-Ter119, anti-Gr1, and anti-B220) CD44$^+$ CD25$^-$ CD117$^+$ cells, TMP precursors were gated as Lin$^-$ CD44$^+$ CD25$^-$ CD117$^+$ CCR9^{EGFP+} cells, DN1c precursors were gated as Lin$^-$ CD44$^+$ CD25$^-$ CD24-high CD117-intermediate cells, DN1d precursors were gated as Lin$^-$ CD44$^+$ CD25$^-$ CD24$^+$ CD117$^-$ cells, DN1e precursors were gated as Lin$^-$ CD44$^+$ CD25$^-$ CD24$^-$ CD117$^-$ cells, the population containing CLP-2 precursors was gated as Lin$^-$ (replacing anti-B220 with anti-CD19 in the cocktail described earlier in this section) CD19$^-$ B220$^+$ CD117$^-$ cells, and the population containing CTP precursors was gated as Lin$^-$ CD44$^+$ CD117^{low} to negative CD90-high cells.

Inducible lineage-tracing model. Flt3::CreERT2//R26R-EYFP mice were injected with a single dose of 3 mg 4-hydroxy-tamoxifen (Sigma-Aldrich) before the analysis of T lineage precursors in the bone marrow and the thymus. For each precursor, at least 2 × 10^6 lymphoid cells were acquired and analyzed for recombined, EYFP$^+$ cells.

Notch1 ablation. Notch1-deficient hematopoiesis was analyzed by injecting Notch1$^{lox/lox}$//CCR9^{EGFP}/+ and Notch1$^{lox/lox}$//MxCre//CCR9^{EGFP}/+ mice intraperitoneally four times with 200 μg pIpC (InvivoGen) at 2-d intervals. The primers used for genotyping of cultured precursors were VM64A (5′-ggaagctactgacttagtagg-3′), VM65A (5′-gagtgttctgtcctgagtgg-3′), and 327 (5′-ctgttcgcaggcatctccag-3′).

Online supplemental material. Fig. S1 describes the serial transplantation experiment that investigates the time point at which recipient precursors enter the transplanted newborn thymus in the transplantation model. Fig. S2 shows the phenotype of T cell lineage cells derived from single precursors of transplanted newborn thymi after culture on OP9-DL1 cells. Fig. S3 describes the generation and characterization of transgenic mice that constitutively express Cre under the control of the Flt3 locus. Fig. S4 shows the fraction of genetically marked intrathymic precursors in the inducible lineage trace model 1 and 2 wk after tamoxifen injection. Fig. S5 shows a FACS analysis of Flt3^{low} CCR9^{EGFP+} LSK in the blood. Online supplemental material is available at http://www.jem.org/cgi/content/full/jem.20072168/DC1.

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