

A human postnatal lymphoid progenitor capable of circulating and seeding the thymus

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Identification of a thymus-seeding progenitor originating from human bone marrow (BM) constitutes a key milestone in understanding the mechanisms of T cell development and provides new potential for correcting T cell deficiencies. We report the characterization of a novel lymphoid-restricted subset, which is part of the lineage-negative CD34⁺CD10⁺ progenitor population and which is distinct from B cell-committed precursors (in view of the absence of CD24 expression). We demonstrate that these Lin⁻CD34⁺CD10⁺CD24⁻ progenitors have a very low myeloid potential but can generate B, T, and natural killer lymphocytes and coexpress *recombination activating gene 1*, terminal deoxynucleotide transferase, *PAX5*, *interleukin 7 receptor α* , and *CD3 ϵ* . These progenitors are present in the cord blood and in the BM but can also be found in the blood throughout life. Moreover, they belong to the most immature thymocyte population. Collectively, these findings unravel the existence of a postnatal lymphoid-polarized population that is capable of migrating from the BM to the thymus.

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T cell production is maintained throughout life by thymic seeding by BM-derived progenitors, although this process does decline in intensity with age (1). The identification of postnatal lymphoid progenitors is of crucial importance for both basic and applied research, because they may help us to understand and correct defects in T cell development. In humans, the nature of the progenitors that seed the adult thymus is poorly known, mainly because of experimental limitations. Most of our knowledge derives from extensive *in vitro* and *in vivo* studies performed in mice. Weissman's model proposes that B, NK, and T lymphoid cells are derived from a common lymphoid progenitor (CLP). Indeed, a population of mouse BM cells harboring a Lin⁻c-kit^{lo}terminal deoxynucleotide transferase (TdT)⁺

IL-7R α^{+lo} phenotype has been shown to be devoid of any erythroid and myeloid potential while maintaining lymphoid differentiation capacity and, thus, has been referred to as a CLP (2, 3). However, CLPs were never detected in the blood (4), and subsequent investigations revealed that BM precursors other than CLPs might seed the thymus. Krueger et al. identified a CLP2 population that can efficiently seed the thymus after intravenous injection (5). Moreover, within the most primitive Lin⁻Sca1⁺c-kit⁺ hematopoietic progenitors, several populations were shown to have both the capacity to home to the thymus and generate T cells (6–9). Inside the thymus, several canonical T progenitors—the early thymic progenitors (ETPs) and two subsets of the DN1 population—could be derived from thymic seeding progenitors (4, 8, 10). Collectively, these findings suggest that different types of BM progenitors can reconstitute the thymus under experimental conditions (4), but the relative

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contributions from each population to the physiological thymic input remain difficult to assess.

Two groups have identified lymphoid progenitors in human umbilical cord blood (11–13). The minor population of CD34^{hi}CD45RA^{hi}CD7⁺ hematopoietic progenitor cells described by Haddad et al. in the fetal BM and in the cord blood is T/NK lineage polarized and might correspond to candidate prethymocytes (12, 13). This population is present in the fetal BM but declines around birth, leaving open the question as to which type of progenitor seeds the thymus thereafter.

20 yr ago, Hokland et al. isolated Lin⁻CD10⁺ progenitors from fetal BM. After culture on irradiated feeder thymocytes, these CD10⁺ progenitors underwent the primary steps in T cell differentiation (14). More recently, Galy et al. refined the phenotypic characterization of this population and demonstrated that a Lin⁻CD34^{hi}CD45RA⁺CD10⁺ population is also present in the BM after birth. These progenitors lacked erythroid, myeloid, and megakaryocytic potential but contained a broad B, T, and NK cell and DC differentiation potential, suggesting that this population might correspond to the human postnatal CLP (15). However, further studies concluded that BM CD19⁻CD34⁺CD10⁺ cells were relatively B cell committed, based on the presence of partial DJ_H rearrangements and gene expression profile analysis (16–18). It must be noted that these studies did not evaluate the T cell potential of this cell subset, mainly because of the lack of an efficient *in vitro* T cell differentiation assay at the time (19, 20). Moreover, the ability of this CD10⁺ population to seed the thymus was not assessed.

Comparing the characteristics of progenitors isolated from BM to those of immature precursors present in the thymus should help identify the human T lymphocyte progenitors. Interestingly, early studies showed that a fraction of CD2⁻ thymocytes does express CD10 (21). Later, thymus-seeding progenitor candidates were identified in the most immature population of the postnatal thymus, characterized by its CD34⁺CD4⁻CD8⁻CD3⁻ triple-negative phenotype (22). Several groups fulfilled the characterization of the early steps of postnatal thymopoiesis and showed that the most immature thymocytes were also CD1a⁻, CD7⁺, and CD38^{dim} and were able to generate T, B, and NK cells, DCs, and myeloid cells *in vitro* (23–26). Thus, the thymus-seeding progenitor should probably form part of the CD34⁺CD1a⁻CD7⁺ thymic population (27).

In this report, we set out to identify a postnatal lymphoid progenitor present in the cord blood as well as in the BM, the blood, and the thymus at various ages. We were able to isolate and characterize a subset of the lymphoid-restricted Lin⁻CD34⁺CD10⁺ population that displays the expected characteristics of a postnatal, physiological B, NK, and T cell progenitor.

RESULTS AND DISCUSSION

Lin⁻CD34⁺CD10⁺ progenitors can be detected throughout the postnatal period

To evaluate the presence of lymphoid progenitors postnatally, CD10 and CD7 expression were analyzed after gating

on Lin⁻CD34⁺ cells both in cord blood and BM at various ages (Fig. S1, A and B, available at <http://www.jem.org/cgi/content/full/jem.20071003/DC1>). Although cord blood contained both CD7⁺ and CD10⁺ cells, only CD10⁺ cells were detected in all BM samples. We showed that the majority of Lin⁻CD34⁺ cells were CD10⁺ in the cord blood (59.5 ± 19.6%) and in BM from donors <10 yr of age (64 ± 18.6%), and that the proportion of this cell population decreased to 19 ± 13.9% in donors >10 yr of age (Fig. S1 C). The proportion of CD10⁺ cells was estimated to be 1 in 350 and 1 in 1,170 mononuclear cells in the BM of donors <10 or >10 yr of age, respectively. Thus, in contrast to what we and others observed during the fetal period, when both CD10⁺ and CD7⁺CD45RA⁺ progenitors coexist (11–13, 18), our phenotypic characterization of postnatal samples indicates that only CD10⁺ progenitor cells are present during this period (even up to 57 yr of age).

Postnatal CD10⁺ progenitors have no erythroid and very limited myeloid differentiation potential but exhibit a strong lymphoid potential

CD10⁻ and CD10⁺ cells sorted from BM and cord blood samples were cultured in semisolid medium to assess their myeloid and erythroid potential. CD10⁻ progenitors exhibited a high erythroid clonogenic potential and a lower myeloid one, and was also able to generate a significant proportion of mixed colonies (Table S1, available at <http://www.jem.org/cgi/content/full/jem.20071003/DC1>). In contrast, under the same culture conditions, no colonies grew from CD10⁺ progenitors. This result was confirmed by coculture on MS5 stromal cells (Table S2). Collectively, these results indicate that the erythro-myeloid potential of postnatal CD10⁺ progenitors is very low as compared with CD10⁻ progenitors.

We next tested the lymphoid potentials of CD10⁺ and CD10⁻ progenitors by culture in B and NK cell conditions. Irrespective of donor age, CD10⁺ progenitors sorted from either cord blood or BM gave rise to high numbers of CD19⁺ and CD56⁺ cells, whereas CD10⁻ progenitors generated only a few lymphoid cells (Fig. S2, A and B, available at <http://www.jem.org/cgi/content/full/jem.20071003/DC1>). The T cell differentiation potential of CD10⁺ and CD10⁻ progenitors was tested by using co-cultures on OP9-hDelta1 stromal cells. CD10⁺ cells generated T lymphocytes more efficiently than CD10⁻ cells regardless of their ontogenic origin (BM or cord blood; Fig. S2 C). In addition, kinetics of T cell differentiation were different between both populations of progenitors. Indeed, CD4⁺CD8⁺ double-positive and mature $\gamma\delta$ T cells were detectable after 3 wk of culture of cord blood-sorted CD10⁺ progenitors (Fig. S2 D). In contrast, CD10⁻ progenitors gave rise to low numbers of immature and mature T cells in the same culture conditions. Collectively, our results show that postnatal CD10⁺ cells have a very high lymphoid potential in contrast to CD10⁻ cells, thus confirming the data published by Galy et al. (15).

CD24 expression on postnatal CD10⁺ progenitors enables the isolation of a B cell–restricted subpopulation and the purification of a CD10⁺CD24⁻ subset exhibiting a broad lymphoid potential

The full lymphoid potential of CD10⁺ progenitors demonstrated by Galy et al. in 1995 (15) was later challenged by several studies concluding that these cells were committed to the B cell differentiation pathway (12, 16–18). Thus, the expression of specific lymphoid precursor markers, CD45RA, CD62L, IL-7R α , CD25 (not depicted), and CD24 (Fig. 1 A), was analyzed to evaluate the degree of heterogeneity of the CD10⁺ subset. We observed that CD24 expression divided the CD10⁺ population into two distinct cell types. To begin with, a high proportion of CD10⁺ progenitors expressed CD24. Moreover, irrespective of donor age, CD24 expression correlated with higher CD10 expression levels, particularly in BM samples from young donors (Fig. 1 A). We analyzed the expression of CC chemokine receptor (CCR) 7 and 9, which are involved in homing to the thymus in mice (6, 28), at the surface of the

various Lin⁻CD34⁺ subpopulations (Fig. 1 B). Only a minority of CD10⁻ and CD10⁺CD24⁻ cells express CCR9. On the other hand, CCR7 was detected on the surface of a significant fraction of CD10⁺CD24⁻ cells, whereas it was completely absent on both other subpopulations.

Comparative analysis of CD10⁺CD24⁻ and CD10⁺CD24⁺ populations also unraveled distinct differentiation potentials. In bulk cultures, CD10⁺CD24⁻ cells gave rise to B, NK, and T cells (Fig. 2, A and B). In addition, they exhibited an enriched DC potential (Fig. 2 C). To note, a few *IgH* DJ and VDJ rearrangements were detected in CD10⁺CD24⁻ cells (Table S3, available at <http://www.jem.org/cgi/content/full/jem.20071003/DC1>), whereas they were absent in CD10⁻ progenitors.

NK progenitor frequency remained constant with age (1 in 40 for cord blood, 1 in 68 for <10 yr of age, and 1 in 58 for >10 yr of age; Fig. 2 D). In contrast, the cord blood CD10⁺CD24⁻ fraction demonstrated a high T cell potential (1 in 20) that decreased with age (1 in 82 and 1 in 165 in <10- and >10-yr-of-age BM samples, respectively), and a low B cell potential (1 in 87) that increased with age (1 in 12 and 1 in 25 for <10- and >10-yr-of-age BM samples, respectively; Fig. 2 D). These frequencies, together with the presence of complete *IgH* VDJ (which was never observed in human mature T cells), suggest that the CD10⁺CD24⁻ population might still be heterogeneous and may consist of real lymphoid progenitors as well as more committed precursors. Additional markers may help to resolve this heterogeneity and to test the lineage potentials in clonal assays.

In contrast to CD10⁺CD24⁻ progenitors, CD10⁺CD24⁺ cells appeared exclusively restricted to the B cell lineage. Indeed, in bulk cultures, CD10⁺CD24⁺ cells fully differentiated into CD19⁺ cells but were unable to give rise to any other lineages (Fig. 2 A), even when plated at higher cell numbers (not depicted). *IgH* DJ and VDJ rearrangements were found much more frequently in the CD10⁺CD24⁺ population as compared with CD10⁺CD24⁻ cells (Table S3).

Collectively, these results indicate that CD24 expression in Lin⁻CD34⁺CD10⁺ progenitors defines a major cell population that is completely restricted and already committed to B cell lineage, whereas the full B, NK, and T cell potential previously described for CD10⁺ cells (15) can be ascribed to the CD10⁺CD24⁻ subset. Thus, the subdivision of CD10⁺ progenitors into CD24⁻ and CD24⁺ subsets probably accounts for the controversial results described in the Introduction, because the vast majority of CD10⁺ progenitors express CD24 and are thus B cell committed. It is noteworthy that, in mice, CD24 expression also coincides with the presence of a B cell potential both in the BM and in thymocyte populations (8, 29). The current model of postnatal hematopoiesis based on segregation between lymphoid and myeloid pathways in mice may be similar in humans (2, 30).

Postnatal CD10⁺CD24⁻ progenitors are present in the blood and the thymus

Lin⁻CD34⁺CD10⁺ cells were found in the blood of both pediatric and adult donors at an average frequency of 1 in 1,540

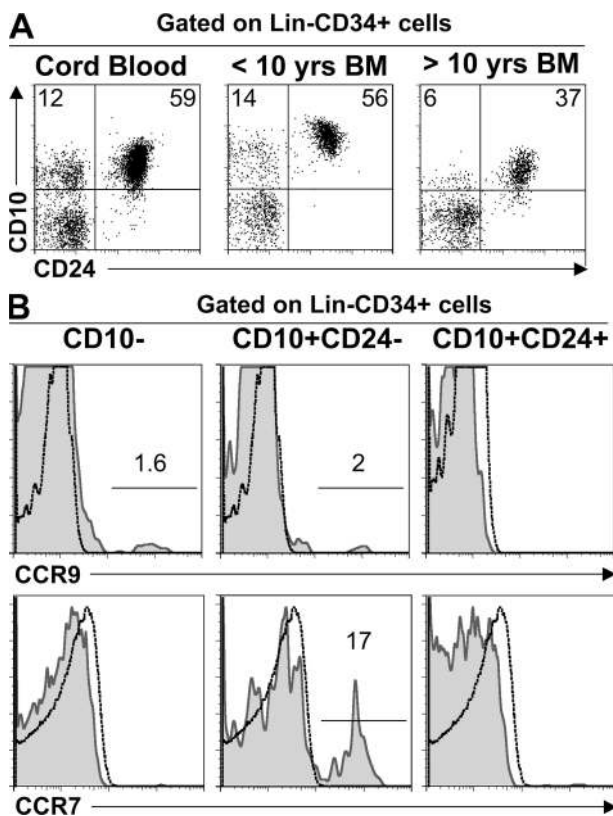


Figure 1. Flow cytometry analysis of cord blood and postnatal BM Lin⁻CD34⁺ progenitors. Mononuclear cells from postnatal BM and CD34⁺-enriched cord blood were stained with a combination of lineage (Lin) markers and anti-CD34, anti-CD10, and anti-CD24 antibodies. (A) Representative analysis of CD10 and CD24 expression of gated Lin⁻CD34⁺ cells from cord blood and BM of young (<10 yr of age) and older (10–60 yr of age) donors. (B) CCR9 and CCR7 expression of gated Lin⁻CD34⁺CD10⁻, Lin⁻CD34⁺CD10⁺CD24⁻, and Lin⁻CD34⁺CD10⁺CD24⁺ cells (shaded histogram). The dotted line indicates control isotypes. Numbers in A and B represent percentages of cells.

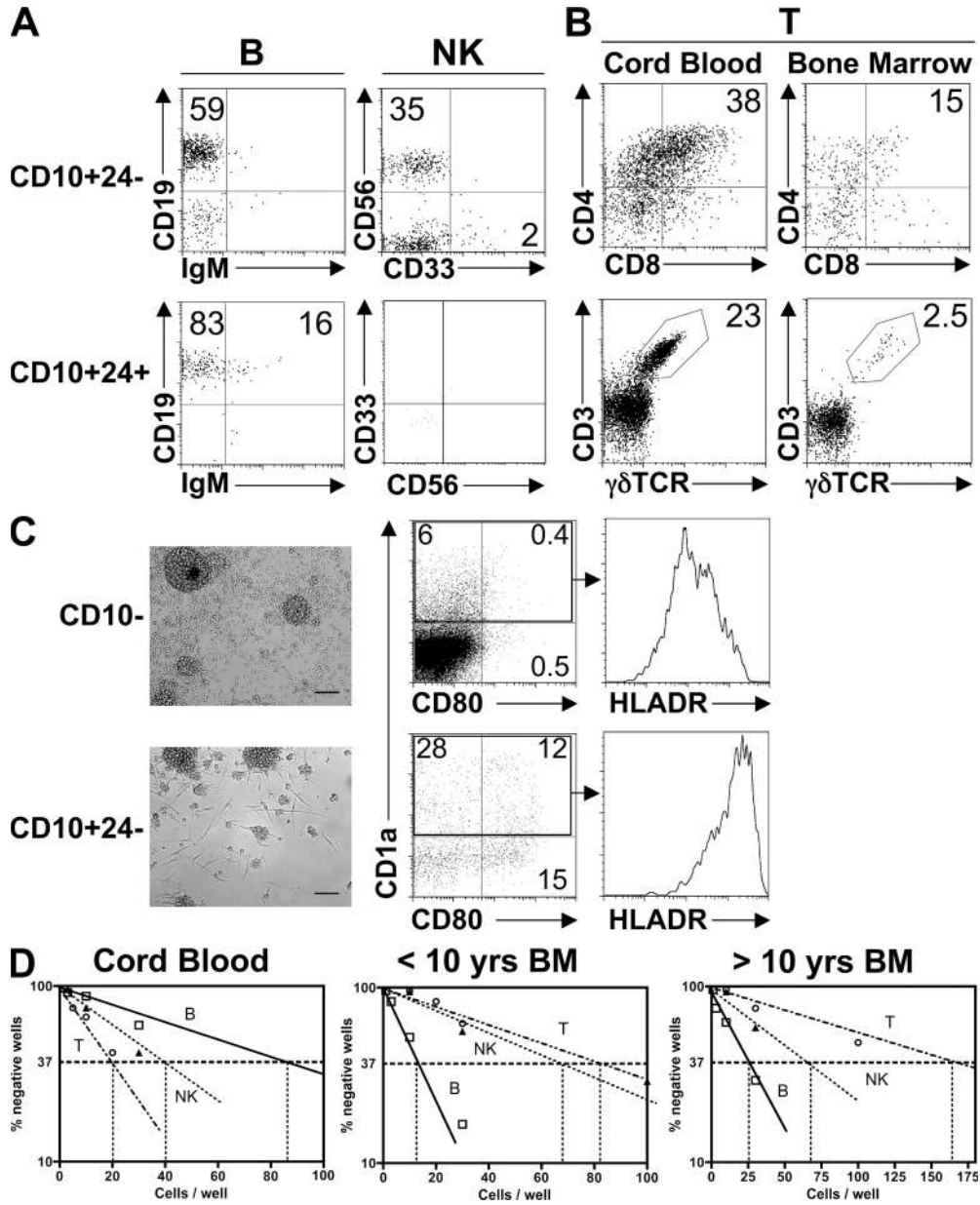


Figure 2. Differentiation potential of CD10⁺CD24⁻ and CD10⁻CD24⁺ progenitors. (A) Flow cytometry analysis of 3-wk cultures on MS5 stromal cells in B or NK cell differentiation assays of CD10⁺CD24⁻ and CD10⁻CD24⁺ progenitors sorted from <10-yr-old BM. The data shown represent six independent experiments performed on cord blood (*n* = 2) and <10-yr-old (*n* = 2) and >10-yr-old (*n* = 2) BM samples. (B) CD4, CD8, CD3, and γδTCR expression analysis of 5-wk cultures on OP9-hDelta1 stromal cells of CD10⁺CD24⁻ progenitors sorted from cord blood or pediatric BM. The data shown represent five independent experiments performed on cord blood (*n* = 2) and <10-yr-old (*n* = 2) and >10-yr-old (*n* = 1) BM samples. After 5 wk, we recovered an average of 6,000 and 200 T cells (CD4⁺CD8⁺ and γδ T cells) from 1,000 CD10⁺CD24⁻ progenitors sorted from cord blood and BM samples, respectively. (C) Lin⁻CD34⁺CD10⁺ progenitors exhibit a DC potential. (left) A representative 10-d culture in DC differentiation conditions from cord blood-sorted CD10⁻ and CD10⁺CD24⁻ progenitors is shown. Bar, 10 μm. In the same culture conditions, CD10⁺CD24⁺ cells did not survive. (right) Flow cytometry analysis of 10-d cultures of CD10⁻ and CD10⁺CD24⁻ progenitors. HLA-DR expression is analyzed on CD1a⁺-gated cells. Numbers in A–C represent percentages of cells. (D) Limiting dilution assay analysis of CD10⁺CD24⁻ progenitors sorted from cord blood (*n* = 2) and <10-yr-old (*n* = 1) and >10-yr-old (*n* = 1) BM. After 4–5 wk of culture in 96-well plates, means of 20,000, 3,250, and 560 viable cells were recovered from 100 CD10⁺CD24⁻ progenitors sorted from cord blood, pediatric, and adult BM samples, respectively.

and 1 in 5,700 mononuclear cells, respectively (Fig. 3 A). CD10⁺ also constituted a sizeable proportion of Lin⁻CD34⁺ cells recovered from the thymus (Fig. 3 C). Neither adult

blood- nor thymus-derived Lin⁻CD34⁺CD10⁺ cells expressed CD24 (unpublished data), suggesting that B cell-committed CD10⁺CD24⁺ cells do not circulate, in contrast to their

CD10⁺CD24⁻ counterparts. To characterize more precisely the CD10⁺CD24⁻ progenitor in the thymus, we performed colabeling with CD7, one of the earliest markers associated with T cell differentiation (27). It is interesting to note

that portion of the thymic Lin⁻CD34⁺CD10⁺ cells expressed the CD7 marker and that the acquisition of CD7 correlated with the progressive loss of CD10 expression (Fig. 3 C). CD10⁺CD24⁻CD7⁻ cells (hereafter referred to as

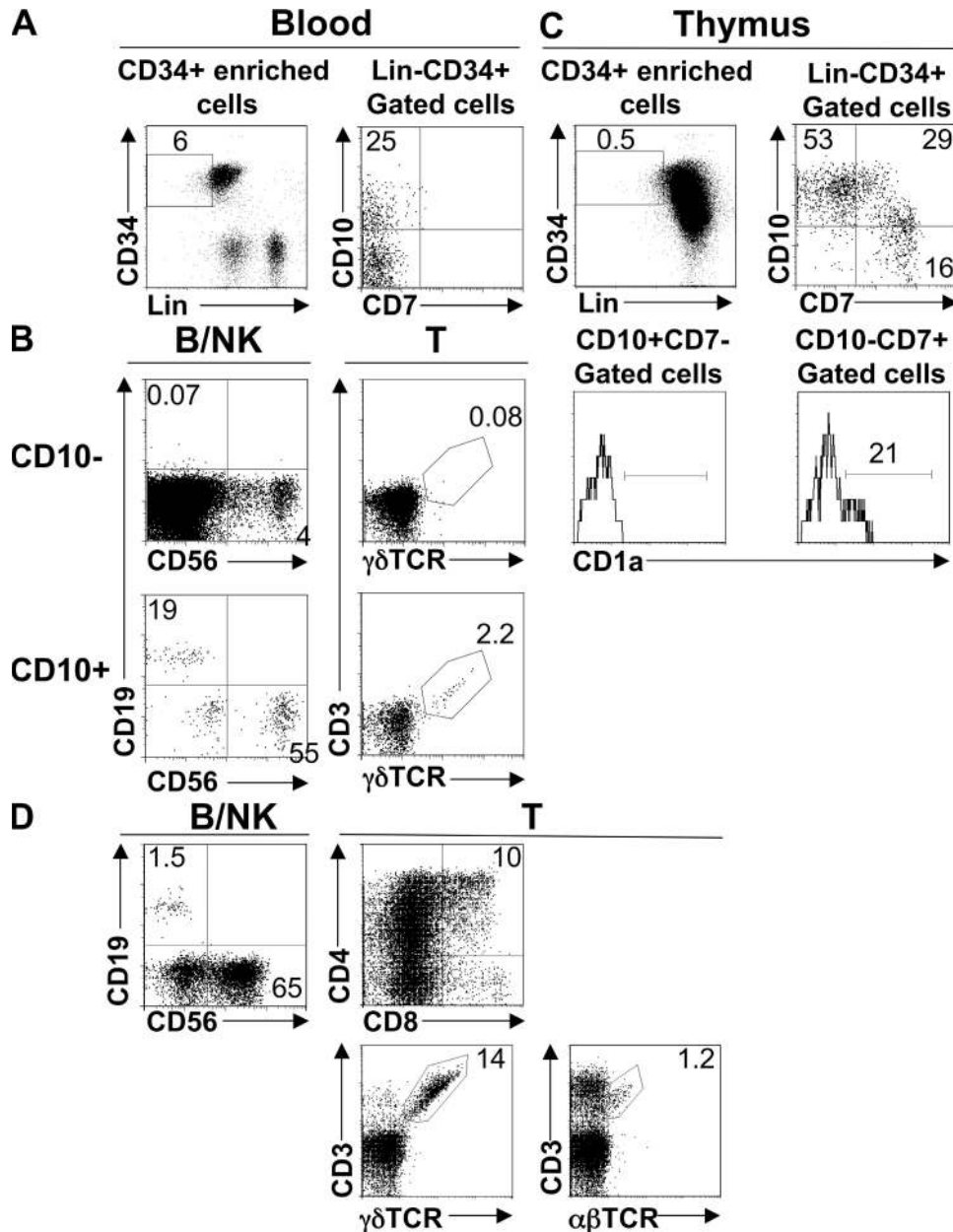


Figure 3. Flow cytometry analysis and differentiation potential of Lin⁻CD34⁺ cells in adult blood and postnatal thymus. (A) CD34⁺ enriched cells isolated from the blood of an adult healthy donor were stained with a combination of lineage (Lin) markers and anti-CD34, anti-CD7, and anti-CD10 antibodies. Data are representative of 5 pediatric and 11 adult blood samples. (B) CD10⁺ and CD10⁻ progenitors recovered from adult blood were plated in B/NK (left) or T (right) cell differentiation conditions. The culture was analyzed using flow cytometry. The results of one out of four experiments are shown. (C) CD34⁺-enriched cells from postnatal thymus were stained with a combination of lineage (Lin) markers and anti-CD34, anti-CD7, anti-CD1a, and anti-CD10 antibodies. (left) Analysis of Lin and CD34 expression in CD34⁺-enriched thymocytes. (right) CD10/CD7 expression on gated Lin⁻CD34⁺ cells is shown. CD1a expression is analyzed on Lin⁻CD34⁺CD10⁺CD7⁻ and CD7⁺ cells (histogram, bottom). Data are representative of 25 analyzed thymuses. (D) The lymphoid potential of Lin⁻CD34⁺CD10⁺ thymic progenitors. (left) Up to 8,000 Lin⁻CD34⁺CD10⁺CD7⁻ thymic progenitors were plated in B/NK cell differentiation conditions and analyzed after 3 wk. (right) Flow cytometry analysis of a representative 5-wk culture on OP9-hDelta1 stromal cells from postnatal thymus-sorted Lin⁻CD34⁺CD10⁺CD7⁻ cells. After 5 wk, we recovered an average of 4,000 T cells (CD4⁺CD8⁺ and γδ T cells) from 500 CD10⁺CD7⁻ progenitors sorted from thymus samples. Numbers in A–D represent percentages of cells.

CD10⁺CD7⁻ cells) thus constitute a weak portion of total CD34⁺ thymic cells, explaining why they were not identified in previous studies (23, 24).

Blood-sorted CD10⁺ cells exhibited a significant lymphoid potential as compared with their CD10⁻ counterparts. Indeed, no erythro-myeloid colonies were detected after semi-solid cultures (not depicted), whereas they gave rise to B, NK, and T lymphocytes when cultured in appropriate conditions (Fig. 3 B).

When cultured in conditions of erythro-myeloid differentiation, thymic CD10⁺CD7⁻ cells were found to have no erythroid and very low myeloid potential (unpublished data). Conversely, they retained the ability to differentiate into CD56⁺ NK and CD19⁺ B cells (Fig. 3 D), although B cell potential required the seeding of a high number of progenitors (~10⁴ cells) and was thus much lower than the ones found in the blood-, cord blood-, and BM-derived counterparts. When cultured on OP9-hDelta1 cells, thymic CD10⁺CD7⁻ cells were able to generate CD4⁺CD8⁺ double-positive cells as well as $\gamma\delta$ and $\alpha\beta$ T cells (Fig. 3 D). Hence, like their BM counterparts, blood and thymic CD10⁺CD7⁻ cells are highly lymphoid restricted. We examined the T cell differentiation potential of CD10⁺CD7⁻ and CD10⁻CD7⁺ thymocytes (Table I). CD10⁺ cells down-regulated the CD10 marker within 5 d and then started to express CD7 and CD5. Double-positive CD4⁺CD8⁺ and $\gamma\delta$ TCR⁺CD3⁺ T cells were detected from day 25 on. CD7⁺ cells generated T cells much faster, because CD4⁺CD8⁺ and $\gamma\delta$ TCR⁺CD3⁺ T cells were already detected at day 15. Together with these kinetics of differentiation, the thymic-resident CD10⁺CD7⁻ cells lacked CD1a (Fig. 3 C) and expressed only low levels of CD38 (not depicted). In contrast, a portion of thymic CD10⁻CD7⁺ cells was CD1a⁺ (Fig. 3 C). These results suggest a continuum between CD10⁺CD7⁻, CD7⁺CD1a⁻, and CD7⁺CD1a⁺ cells. CD10⁺ cells precede the CD7⁺ stage in the thymus and may therefore constitute the earliest thymic progenitor in the postnatal period (24, 26).

Table I. Kinetics of T cell differentiation of CD10⁺CD7⁻ and CD10⁺CD7⁺ thymus-sorted progenitors

Days of culture	Markers	Plated progenitor cells (% ^a)	
		CD10 ⁺ CD7 ⁻	CD10 ⁻ CD7 ⁺
5	CD10	3	0
	CD7	33.5	91
	CD5	15.5	83
15	CD7	89.5	98
	CD5	66.5	97
	$\gamma\delta$ TCR/CD3	0	0.4
25	CD4/CD8	0.25	3.4
	$\gamma\delta$ TCR/CD3	2.7	54
	CD4/CD8	2.4	20

^aMean percentages of CD10⁺, CD7⁺, CD5⁺, $\gamma\delta$ TCR⁺CD3⁺, and CD4⁺CD8⁺ double-positive cells after 5, 15, and 25 days of culture on OP9-hDelta1 stromal cells (measured in two independent experiments).

Gene expression profiling of CD10⁺ progenitor cells

To further characterize the CD10⁻, CD10⁺CD24⁻, and CD10⁺CD24⁺ cell populations, we studied the expression of several genes involved in hematopoietic differentiation. Using our previously described multiplex RT-PCR method, which enables the simultaneous analysis of multiple genes in very small samples (31), we set up four distinct multiplex assays to simultaneously monitor the expression of two or three lineage-specific genes, together with the 28S ribosomal RNA gene as an endogenous control (Fig. 4).

Irrespective of their ontogenic origin, CD10⁻ cells were the only ones to express the *GATA1* transcription factor (Fig. 4, A and B). These cells frequently expressed the surrogate light chain of the pre-BCR (*Vpre-B*), whereas other lymphoid-specific genes were mostly absent. CD10⁺CD24⁺ cells expressed lymphoid- (*IL-7R α* , *RAG1*, and *TdT*) and B cell lineage- (*PAX5*, *EBF1*, and *Vpre-B*) specific genes but did not express the T cell-specific genes *CD3 ϵ* or the pre-TCR α chain (*pT α*). In contrast, the CD10⁺CD24⁻ cells displayed a broad lymphoid profile because they highly expressed *CD3 ϵ* as well as B cell lineage-specific genes. Nevertheless, unlike mouse CLP-2, they did not express pT α (5) and resemble more, in terms of gene expression profile as well as differentiation potential, CCR9⁺ multipotent progenitors and early lymphoid progenitors (ELPs) (6, 7). Interestingly, only the CD24⁻ fraction of the CD10⁺ progenitors (which exhibits the broadest lymphoid potential) concordantly expressed *IL-7R α* and γ c (unpublished data). This result fits with several studies showing that acquisition of *IL-7R α* by the earliest human progenitors is accompanied by an increase in lymphoid potential (3, 32, 33), and that CD34⁺CD19⁻*IL-7R α* ⁺ cells do not express CD24 (32). It remains to be investigated whether the *IL-7R α* -associated cytokines *IL-7* and thymic stromal lymphopoietin play a role in the generation, survival, and/or proliferation of the CD10⁺CD24⁻ population.

Thymus-isolated CD10⁺CD7⁻ cells expressed genes that are common to both the T and B cell differentiation pathways but showed evidence of T cell commitment (Fig. 4 C). Interestingly, they express *IL-7R α* chain, a result that contrasts with the absence of the *IL-7R α* chain observed in mouse ETP (8, 10). Compared with their CD10⁺CD24⁻ BM and cord blood counterparts, thymic CD10⁺CD7⁻ cells rarely expressed B cell-specific genes, whereas the activity of all T cell-specific genes increased: *CD3 ϵ* , *GATA3*, and *pT α* . It is noteworthy that gene expression profiling of the thymic CD10⁺CD7⁻ population revealed down-regulation of B cell-specific genes, an observation that correlates with a decreased ability to generate B cells in vitro. Conversely, we observed an up-regulation of the T cell-specific genes. These singular features match the signals triggered by the thymic niche (i.e., the Notch signal), which may program the ETPs into T cell development (34, 35).

Collectively, our results show that CD10⁺CD24⁻ progenitors are present in the cord blood and in the BM throughout life, and that cells with a similar phenotype circulate and are present in the thymus. Moreover, we have demonstrated that

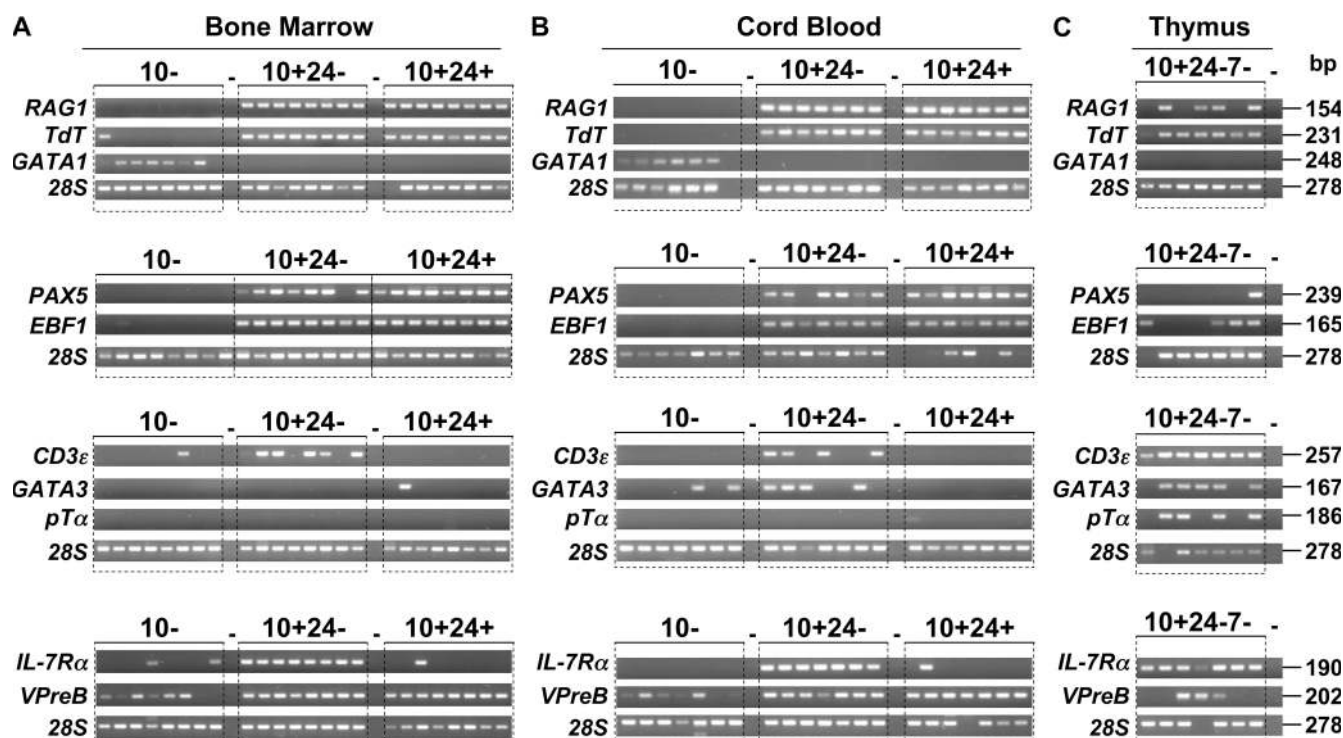


Figure 4. Gene expression profile of CD10⁻, CD10⁺CD24⁻, and CD10⁺CD24⁺ progenitors. (A) BM or (B) cord blood Lin⁻CD34⁺CD10⁻, Lin⁻CD34⁺CD10⁺CD24⁻, and Lin⁻CD34⁺CD10⁺CD24⁺ populations and (C) thymic Lin⁻CD34⁺CD10⁺CD24⁻CD7⁻ cells were sorted directly into PCR tubes at 10 cells per well. RT followed by a quadruplex or triplex amplification PCR assay was performed. Each well was duplicated to perform a second qualitative PCR, and seven or eight representative wells are presented for each population. DNA fragment sizes are indicated. —, negative control.

CD10⁺CD24⁻ cells derived from BM and thymic CD10⁺CD7⁻ cells display very similar features in terms of their differentiation potential, which is lymphoid biased, and RAG, TdT, CD3ε, and IL-7Rα expression levels. This observation strongly suggests that the thymic CD10⁺CD7⁻ population directly derives from BM CD10⁺CD24⁻ cells. We therefore suggest that CD10⁺CD24⁻ progenitors constitute a thymus-seeding population and may replace CD7⁺CD45RA⁺ cells in the postnatal period. Further characterization of CD10⁺CD24⁻ lymphoid progenitors and analysis of their expansion capacity should pave the way for their enrichment and potential use in a variety of immune restoration scenarios.

MATERIALS AND METHODS

Human cells and tissues. Mononuclear BM cells corresponded to the unused, healthy residues of allogeneic, hematopoietic stem cell harvests. Cord blood samples were harvested on delivery of full-term, healthy pregnancies at the Intercommunal Hospital (Creteil, France) and the Saint-Vincent de Paul Hospital (Paris, France) after obtaining written, informed consent of the mothers according to French legislation and ethical guidelines. Blood samples were obtained from healthy adult donors after obtaining written, informed consent from the French Blood Transfusion Institute (Paris, France). Human thymic tissue was obtained from children aged from 1 d to 10 yr of age undergoing cardiac surgery. Experimental procedures with human thymic fragments were approved by the human research Ethics Committees at the Oswaldo Cruz Foundation and the Necker Children's Hospital (Paris, France).

Phenotypic characterization and sorting of progenitors. CD34⁺ cells were isolated using an indirect CD34 microbead kit and a separator (VarioMACS;

Miltenyi Biotec), according to the manufacturer's instructions. Purified CD34⁺ cells or mononuclear BM cells were stained with anti-CD34–allophycocyanin (APC; 8G12). The lineage (Lin) PE-conjugated antibody cocktail contained antibodies against CD2 (S5.2), CD3 (SK7), CD4 (SK3), CD8 (SK1), CD19 (J4.119), CD20 (L27), CD56 (NCAM16.2), CD13 (SJ1D1), CD14 (MφP9), CD15 (MMA), CD16 (NKP15), CD33 (HIM3-4), and CD235a (Ga-R2). Additional markers were used for both phenotypic analysis and sorting: anti-CD10–PE-Cy7 (H10a), anti-CD7–FITC (M-T701), anti-CD1a–APC (HI149), anti-CCR7–PE-Cy7 (3D12), purified anti-CCR9 (112509), and anti-CD24–FITC (ML5). All antibodies were obtained from BD Biosciences, Beckman Coulter, or R&D Systems.

Stained cells were either analyzed with a flow cytometer (FACSCalibur) and CellQuest software, or sorted on a cell sorter (FACSVerse; all from BD Biosciences). The purity of sorted cells was routinely ~98%.

In vitro differentiation protocols. DC potential was assessed as previously described (36). Cultures were analyzed by flow cytometry using a combination of anti-CD14–FITC, anti-CD1a–APC (HI149), ViaProbe (7-amino-actinomycin D, to exclude dead cells from the analysis; BD Biosciences), and anti-CD80–PE (L307.4) or anti-HLA-DR–FITC (clone G46-6).

B and NK cell differentiations were performed on a confluent stroma of nonirradiated MS5 cells (DSMZ ACC 441), as previously described (13). Cultures were analyzed by flow cytometry using a combination of anti-CD19–APC, anti-IgM–PE, and either anti-CD45–PerCP or ViaProbe for B cell differentiation and a combination of anti-CD56–PE and anti-CD45–PerCP to evaluate NK cell differentiation.

T lymphoid potential was tested by plating candidate progenitors on OP9 stromal cells (American Type Culture Collection) transduced with a defective retrovirus containing a double cassette expressing the human Notch ligand Delta1 (hDelta1) and GFP. The co-culture was performed as previously described (19, 20). After 3–5 wk, a fraction of the culture was

analyzed by flow cytometry using the following antibodies: anti- $\gamma\delta$ TCR-PE (IMMU510), anti- $\alpha\beta$ TCR-PE (T10B9.1A-31), anti-CD3-APC (SK7), anti-CD8-PE, and anti-CD4-APC (SK3). ViaProbe was added to all stainings. In some cases, intermediate flow cytometry analyses were performed by staining cultures with anti-CD5-FITC and anti-CD7-PE. As previously described, the OP9-hDelta1 assay consistently and rapidly gave rise to a high number of $\gamma\delta$ T cells, with $\alpha\beta$ T cells usually detected after 7 wk of culture (19).

Limiting dilution assays. Cells were seeded at 1, 3, 10, 30, and 100 cells per well into 24 wells (for each concentration) of either MS5- (for B and NK cell differentiation) or OP9-hDelta1-coated 96-well plates (for T cell differentiation) and cultured as described in the previous section. The presence of CD45⁺CD19⁺ B, CD45⁺CD56⁺ NK, or CD4⁺CD8⁺ and CD3⁺ $\gamma\delta$ TCR⁺ cells was determined by flow cytometry in each well. Data are presented as percentages of negative wells according to the initial number of cells plated per well. An exponential curve fit was drawn for each type of precursor. The proportion of lymphoid precursors was calculated by linear regression analysis (on the basis of a Poisson distribution) (37).

Multiplex RT-PCR. Multiplex RT-PCR analysis was performed according to the method published by Peixoto et al. (31), with minor modifications. Our primers were designed using Primer3 software (frodo.wi.mit.edu), and primer compatibility for the multiplex analysis was assessed using Amplify freeware (version 3.1.4; engels.genetics.wisc.edu/amplify/). The absence of competition in the multiplex reactions was validated using quantitative PCR and by comparing individual and multiplex gene amplification. The primers selected for the PCR reactions are listed in Table S4 (available at <http://www.jem.org/cgi/content/full/jem.20071003/DC1>). Sorted cells were collected in individual PCR tubes containing PBS-diethyl pyrocarbonate (Sigma-Aldrich) and stored at -80°C . After cell lysis, RNA was reverse transcribed using three or four gene-specific 3' primers. The first amplification PCR was subsequently performed in the same wells by addition of a premixed PCR buffer containing 3' and 5' primers for all three or four genes and amplified for 15 cycles. The products of the first amplification were split into new PCR tubes using 4% of the first-round PCR product per reaction. This second PCR was performed separately for each individual gene using seminested primers. PCR products were resolved on a 2.5% agarose ethidium bromide gel and were sequenced to confirm specificity.

Statistical analysis. Data are expressed as mean \pm SD and were statistically analyzed using an unpaired, two-tailed Student's *t* test with an α risk of 0.05.

Online supplemental material. Fig. S1 shows flow cytometry analysis of cord blood and postnatal BM progenitors. Fig. S2 illustrates the lymphoid potential of CD10⁺ and CD10⁻ progenitors. Table S1 shows the colony-forming potential of CD10⁻ and CD10⁺ progenitors. Table S2 shows the generation of myeloid cells from CD10⁻ and CD10⁺ BM-sorted cells after culture on MS5 stromal cells. Table S3 shows the status of IgH recombination in BM-sorted CD10⁻, CD10⁺CD24⁻, and CD10⁺CD24⁺ cells. Table S4 shows the various sets of primers used for multiplex RT-PCR assays. Supplemental material and methods provides information about in vitro differentiation protocols and VDJ recombination. Online supplemental material is available at <http://www.jem.org/cgi/content/full/jem.20071003/DC1>.

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