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Extrathymic Hemopoietic Progenitors Committed to T Cell Differentiation in the Adult Mouse[1]

Marie-Laure Arcangeli,* Christophe Lancrin,* Florence Lambolez,* Corinne Cordier,† Elke Schneider,‡ Benedita Rocha,* and Sophie Ezine[2]*

The role of the thymus in T cell commitment of hemopoietic precursor is yet controversial. We previously identified a major T cell progenitor activity in precursor cells isolated from bone marrow-derived spleen colonies. In this study, we characterize the properties of these pre-T cells. We demonstrate that they have unique phenotype and can be generated in a total absence of any thymic influence. Indeed, even when studied at the single-cell level, extrathymic T cell-committed precursors express T cell-specific genes. Moreover, these cells are not committed to a particular T cell differentiation pathway because they can generate both extrathymic CD8$\alpha\alpha^+$ intraepithelial lymphocytes and thymus-derived conventional thymocytes. We also compared these pre-T cells with fully T cell-committed thymic progenitors. When tested in vitro or by direct intrathymic transfer, these cells have a low clonogenic activity. However, after i.v. transfer, thymus repopulation is efficient and these precursors generate very high numbers of peripheral T cells. These results suggest the existence of extra steps of pre-T cell maturation that improve thymus reconstitution capacity and that can be delivered even after full T cell commitment. Consequently, our studies identify a source of extrathymic progenitors that will be helpful in defining the role of the thymus in the earliest steps of T cell differentiation. ***The Journal of Immunology,*** **2005, 174: 1980–1988.**

The generation of the majority of mature T cells depends on the differentiation of T cell progenitors in the thymus (1). To ensure this T cell generation, thymus colonization by hemopoietic progenitors is essential. However, it is still highly controversial which progenitors colonize the thymus and where commitment to T cell lineage differentiation occurs. Commitment to lymphocyte differentiation has been associated with the generation of common lymphoid progenitors (CLP)[3] in the bone marrow (BM) (2). These progenitors have lost their myeloid potential and were reported to fully reconstitute the T, B, NK, (2) and DC compartments (3). Indirect evidence suggested that CLP were the immediate precursors of thymocyte populations. Indeed, Notch1-deficient BM precursors, when injected intrathymically (i.t.), generated B cells rather than T cells (4, 5). These results were interpreted as an indication that progenitors colonizing the thymus retained a B cell potential, thus defining them as CLP. Once in the thymus, Notch signals mediated through interactions with the thymus epithelium would then induce local and definitive T/NK cell commitment (6).

Recent evidence challenged the notion that CLP were the direct precursors of immature thymocytes (7). Indeed, comparison of CLP with the most immature thymocyte populations isolated from the thymus (named early T cell progenitor, ETP) showed major differences. First, their phenotype was different (IL-7Rα^+c-*kit*^{low} for CLP and IL-7Rα^-c-*kit*^{high} for ETP). Second, CLP and ETP differed in their thymus reconstitution potential. In contrast to CLP, ETP induced a very prolonged thymus reconstitution, suggesting a higher capacity of self-renewal, and were less differentiated than CLP. These data led to the conclusion that hemopoietic progenitors colonizing the thymus should be distinct from CLP (7), but remain to be identified. Controversies concerning the nature of precursors colonizing the thymus were enhanced by the description of other extrathymic progenitor populations further engaged into T cell commitment than CLP. In the fetus, T/NK progenitors were described in the liver (8) and pre-T cell populations were isolated from the blood (9, 10). In the adult, precursors generating T cells but only traces of B and myeloid cells were isolated from the Lin$^-$Sca1$^+$Thy1.1$^-$ fraction of the BM (11). A fully T cell-committed precursor population, which had not initiated TCR rearrangements but expressed Rag-1, Rag-2, and pre-TCRα (pre-Tα) mRNA, was described in the BM (12, 13). T cell progenitors were also identified in gut cryptopatches (14). Furthermore, we provided evidence for a pre-T cell population (generating only very rare NK cells) isolated from day-12 BM-derived spleen colonies (SC12), originating from adult transplanted BM in irradiated mice (15). The relationship between these different types of pre-T cells, the CLP and the ETP, is far from clear. Each of these progenitor populations was selected and identified by different criteria, which hinders their direct comparison. In many cases, these progenitor populations were frequently not characterized at a single-cell level and thus may be heterogeneous. Moreover, the presence of pre-T cells outside the thymus does not necessarily signify that commitment to T cell differentiation can occur independently from the

*Institut National de la Sant é et de la Recherche Médicale Unit é 591, Institut Necker, †Institut Federatif de Recherches 94, and ‡Centre National de la Recherche Scientifique Unité Mixte de Recherches 8147, Faculté de Médecine René Descartes Paris V, Paris, France

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[2] Address correspondence and reprint requests to Dr. Sophie Ezine, Institut National de la Santé et de la Recherche Médicale Unité 591, Institut Necker, 156 rue de Vaugirard, 75730 Paris Cedex 15, France, E-mail address: ezine@necker.fr

[3] Abbreviations used in this paper: CLP, common lymphoid progenitor; ETP, early T cell progenitor; i.t., intrathymical(ly); BM, bone marrow; SC12, day-12 BM-derived spleen colonies; TX, thymectomized; TN, triple negative; DP, double positive; SP, single positive; pre-Tα, pre-TCRα.

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thymus. First, "pre-T cells" could have been committed already to T cell differentiation in the thymus before seeding the peripheral compartments. Second, such precursors might have differentiated outside the thymus, but nevertheless require the hormonal contribution of the thymus (16). Finally, these extrathymic pre-T cells could belong to an independent T cell lineage, never colonizing the thymus and generating the common type of thymus-derived T cells. Indeed, extrathymic T lymphocytes can be generated in both liver and gut (14). These T cells differ from thymus-derived cells in terms of phenotype, molecular markers, and rules of repertoire selection (14). The pre-T cells isolated outside the thymus could belong exclusively to these particular extrathymic T differentiation pathways.

To address these questions, we took advantage of an experimental system designed by Till and McCulloch (17), which leads to colony formation in spleens from irradiated mice having received a BM graft 12 days before. The clonogenic BM progenitors (so-called CFU-S, CFU in the spleen) seed the spleen reproducing an in vivo limiting dilution assay (18). We have reported before that cells from individual SC12 contained T cell progenitors (19). More recently, we showed that SC12 have unique advantages as a source of T cell-committed precursors (15). Indeed, the yield of precursor cells in these colonies is very high and pre-T cells are very frequent, representing ~40% of Lin^- populations. In the present study, we investigated the role of the thymus in the generation of such progenitors and compared these pre-T cell properties with the T cell precursors that are present in the thymus. We show that SC12 pre-T cells develop in the absence of any thymus influence and represent a unique T cell-committed precursor population.

Materials and Methods

Mice

All mice were bred on a pure C57BL/6 genetic background. Ly5.1 (Thy1.2) mice and Ly5.1 CD3$\epsilon^{-/-}$ mice were purchased from Transgenic Alliance and Centre de Distribution, Typage et Archivage Animal. Ly5.2 (Thy1.2) mice and nude mice were purchased from Centre d'Elevage R. Janvier. Ba (Thy1.1, Ly5.2), Ly5.1 Rag2$^{-/-}$, $\gamma c^{-/-}$Rag2$^{-/-}$ mice were bred and maintained in the animal care facility at the Necker Institute (Paris, France). Four- to 12-wk-old male or female mice were used. Ly5.1 CD3$\epsilon^{-/-}$ mice were thymectomized (TX) 4 wk before BM graft. They were anesthetized and positioned on their back; an incision was made in the skin above the sternum; and the thymus removed with forceps. The skin wound was closed with surgical metal clips.

Abs, flow cytometry, and cell sorting

The following mAbs used for flow cytometry and/or cell sorting were obtained from BD Pharmingen: anti-CD2, anti-CD3, anti-CD4, anti-CD8 (53-6.7), anti-CD8β (H35-172), anti-CD11b/Mac1 (M1/70), anti-CD16/CD32 (24G2), anti-CD19 (1D3), anti-CD24 (M1/69), anti-CD25 (PC61), anti-CD29 (Ha2/5), anti-CD43 (S7), anti-CD44 (1M781), anti-CD45.2/Ly5.2 (104-2.1), anti-CD49d (R1-2), anti-CD49e (5H10-27), anti-CD62-L (MEL14), anti-CD90.1/Thy1.1 (HO22.1), anti-CD90.2/Thy1.2 (53-2-1), anti-CD117/c-Kit (3C1), anti-CD127/IL-7Rα (A7R34, gift from Dr. S.-I. Nishikawa, Kyoto University and RIKEN Center, Kyoto, Japan), anti-NK1.1 (PK136), anti-erythroid (Ter 119), anti-Gr1 (RB6-8C5), anti-TCR (HAM or H57-597), anti-TCR (GL3), anti-β_7 integrin (M293), anti-CD45R (B220; RA3-6B2), and anti-AA4.1. The anti-IgM Ab was obtained from Southern Biotechnologies. These Abs were either directly coupled to FITC, allophycocyanin, PE and PerCP or conjugated to biotin, the later being revealed by streptavidin-RED613 (Invitrogen Life Technologies) or streptavidin-allophycocyanin (BD Pharmingen). A FACSCalibur was used for flow cytometry. Cell sorting was performed on a FACSVantage (BD Biosciences). Data were analyzed with CellQuest software (BD Biosciences).

Generation of SC12 and preparation of triple-negative (TN) cells

Ly5.1 or TX Ly5.1 CD3$\epsilon^{-/-}$ and Ly5.2 hosts were exposed to lethal (1200 rad) whole-body irradiation from a ^{137}Cs source and injected through the retro-orbital sinus with 7×10^4 BM cells from Ly5.2 or Ly5.2 nude and Ly5.1 donors, respectively. Mice were maintained on water containing antibiotics (neomycin sulfate, 1 g/500 ml) for 1 wk. Twelve days after BM injection, the recipients' spleen colonies were excised and pooled. Precursor $CD44^-$ populations were obtained as described before (15). Briefly, the SC12 population was labeled with Abs recognizing $Lineage^+$ cells (Mac1, CD3, TCRβ, CD19, and NK1.1), the Ly5.2 donor population, and CD44. $Ly5.2^+Lin^-CD44^-$ cells were sorted.

Thymocytes were first incubated with unconjugated rat Ab (clone 53-6.7) specific for the $CD8\alpha^+$ cells, and labeled cells were removed with anti-rat IgG-conjugated beads (Dynabead M-450; Dynal); the remaining cells were labeled with Abs recognizing $Lineage^+$ cells (Mac1, 8C5, CD3, TCRβ, TCR$\gamma\delta$, CD8β, and IgM), CD25, and CD44. TN cells were then sorted according to CD44/CD25 expression and processed for cellular analysis, in vitro studies, and gene expression.

In vivo transfer of precursor cells

Precursor cells were injected i.v. or directly into the thymus of recipients (i.t.) as described before (15, 19). Briefly, 4×10^4 precursors cells from SC12 or thymus were resuspended in 200 μl of MEM medium and injected i.v. into sublethally irradiated (600 rad) C57BL/6-Ly5.1 Rag2$^{-/-}$ or in 20 μl and directly transferred into one thymic lobe of sublethally irradiated C57BL/6 recipients.

In vitro cultures

Induction of T cell development was initiated using the OP9-DL1 line described by Schmitt and Zùniga-Pflucker (6). Briefly, the OP9 and OP9-DL1 cells were seeded into 24-well tissue culture plates for mass culture or 96-well flat-bottom plates for limiting dilution analysis (Falcon) the day before the coculture with progenitors (SC12 cells, thymic progenitors). All cultures were performed in presence of 5 ng/ml IL-7 (R&D Systems) and 5 ng/ml Flt3-L (R&D Systems) and fed every 4 days. At the indicated time, progenitor cells were recovered and stained to detect B cells (anti-CD19), T cells (CD4/CD8/TCR$\alpha\beta$), NK cells (NK1.1), and myeloid cells (Mac1). To test the frequency of T cell progenitors among SC12 $CD44^-$ cells, 72 wells of each dilution (100, 50, or 20 cells by well) were established. The presence of $CD4^+CD8^+$ (double-positive, DP) cells was used as evidence for T cell progenitor potential. Each well was observed under an inverted microscope, and proliferating wells were analyzed by FACS for the presence of the DP population. Final analysis was performed using the Poisson distribution by scoring the negative wells (20).

Quantification of donor cells in peripheral T cell pools

Donor T cells were recovered from lymph nodes, spleen, and BM. The lymph nodes were pooled from axillary, inguinal, and mesenteric sites, estimated to represent approximately half of the total body lymph node mass (21). BM recovery was calculated based on the fact that two femora and two tibiae represent 25% of total BM (22). Consequently, the size of the T cell pool was calculated with the following formula: 2× LN + 1× spleen + 4× BM (LN, number of donor T cells collected in lymph nodes).

Reverse transcription single-cell PCR

SC12 $CD44^-$ and SC12 TX $CD44^-$ cells were sorted as single cells or in limiting dilution (Table I) using a FACSVantage flow cytometer equipped with an automatic cell disposition unit (BD Biosciences). Cells in each well were lysed, mRNAs coding for the genes of interest were reverse-transcribed using specific 3′ primers, and cDNAs were amplified in modified nested two-step PCR procedure, as previously described (23). None of the primer combinations amplify genomic DNA. The amplification products were analyzed on 1.5% agarose gel and were visualized by ethidium bromide staining. Primers for HPRT, Rag-1, pre-Tα, and CD3ϵ were described previously (14). GATA3 primers were: 5′-TCA GTG GTT GGA ATG CAG AC-3′ (RT), 5′-CTG GAG GAG GAA CGC TAA TG-3′ (5′ primer); for the second PCR,

Table I. *Cell densities (cells per well) used for the limiting dilution RT-PCR analysis*

	SC12 TX $CD44^-$ Cells
CD3ε	1
GATA3	1
pTα	50, 20, 10
Rag1	1

5′ primers were: 5′-TGG GCT GTA CTA CAA GCT TC-3′, 3′ primers were: 5′-ACA CCA CCT CGA GCT CCT TT-3′.

Results

Hemopoietic precursors can undergo T cell commitment without any thymic influence

BM injection into lethally irradiated mice leads to the generation of spleen colonies. Twelve days later, these colonies still contain abundant lineage-negative (Lin^-) cells that can be subdivided into two major subsets, depending on CD44 expression. SC12 Lin^-CD44^+ cells conserve multiple differentiation potentialities. In contrast, the SC12 Lin^-CD44^- cells (SC12 pre-T or SC12 $CD44^-$ cells) represent an abundant source of pre-T cells. These cells represent ~40% of the Lin^- cells recovered from spleen colonies (15). When transferred in vivo, they reconstitute the thymus more rapidly than CLP and have a very restricted differentiation potential. They can generate neither myeloid nor B cells and repopulate the thymus and the peripheral T cell compartment, producing very few NK cells that can only be detected in the BM (15). When cultured in vitro on OP9 stroma cells (Ref. 24; suitable for B, NK, and myeloid development) we also found that these precursors give rise neither to macrophages nor to B cells, confirming their restricted differentiation potential (Fig. 1). In contrast, Lin^-CD44^+ SC12 cells, cultured in the same conditions, remain capable of differentiating into mature myeloid and B cell populations (Fig. 1).

To evaluate the role of the thymus in the generation of these pre-T cells, spleen colonies were produced by injecting BM cells from nude mice into lethally irradiated and TX Ly5.1 CD3$\epsilon^{-/-}$ hosts. In this experimental setting, the presence of thymus-derived precursors in BM transplants can be excluded. Furthermore, the removal of the thymus in irradiated hosts provides an experimental model in which the environment and the hormonal influence on the formation of SC12 pre-T can be appreciated.

Analysis of SC12 generated this way showed that they yielded a similar number of Lin^- cells with the same proportion of SC12 pre-T cells (Fig. 2*A*) as in colonies generated in euthymic mice (15). SC12 pre-T cells obtained in athymic conditions (SC12 TX

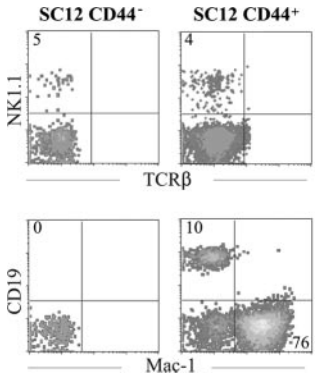

FIGURE 1. In vitro differentiation potential of SC12 $CD44^-$ cells. Lethally irradiated Ly5.1 B6 mice were i.v. grafted with 7×10^4 BM cells from Ly5.2 Ba mice. Twelve days later, spleen colonies were removed. SC12 Lin^-CD44^- cells were sorted, and 1×10^4 cells were seeded into 24-well tissue culture plates containing a confluent monolayer of OP9 stroma cells. Twelve days later, T ($TCR\beta^+$), NK ($NK1.1^+$), B ($CD19^+$), and myeloid ($Mac1^+$) potentialities were examined. SC12 Lin^-CD44^+ cells that can generate T, B, and NK cells in vivo and myeloid cells in vitro (15), were used as positive control of OP9 stroma efficiency. Numbers in quadrants represent the percentage of cells positive for the considered marker. Data represent one experiment of four performed with identical results.

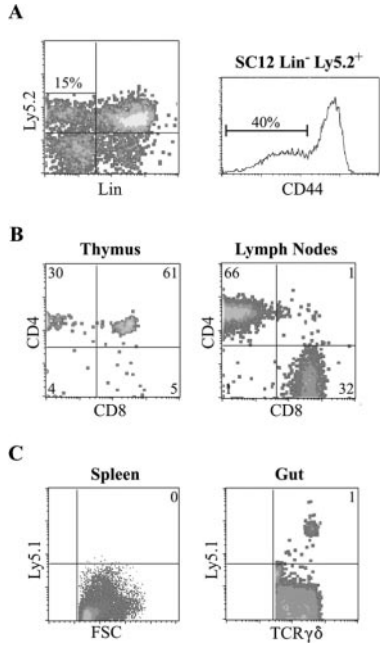

FIGURE 2. Extrathymic origin of SC12 pre-T cell population. Ly5.1 CD3$\epsilon^{-/-}$ TX mice were lethally irradiated and injected i.v. with 7×10^4 BM cells from Ly5.2 nude mice. Twelve days later, spleen colonies were removed. SC12 TX $CD44^-$ cells were sorted and injected i.v. into irradiated Ly5.1 $Rag2^{-/-}$ mice (*A* and *B*). Recipient mice were studied at different time points after injection (4×10^4 cells/mouse). *A,* Gated donor $Ly5.2^+Lin^-$ population (*left*) and CD44 expression in Lin^- cells (*right*). *B,* Donor cells in the thymus (*left*) 2 wk after injection and in the lymph nodes (*right*) 4 wk after transfer. Data represent 1 mouse of 10 analyzed. *C,* B6 Ly5.2 mice were lethally irradiated and injected i.v. with 7×10^4 BM cells from B6 Ly5.1 mice. Spleen colonies were removed, and SC12 $Ly5.1^+Lin^-CD44^-$ cells were sorted and injected i.v. into irradiated $Ly5.2^+$ nude mice. $Ly5.1^+$ donor-type cells are not detected in the spleen (*left*) but are present in the gut (*right*) 4 wk after transfer. One of four mice analyzed is represented.

pre-T cells) reconstitute the thymus very rapidly after i.v. injection into sublethally irradiated Ly5.1 $Rag2^{-/-}$ recipients. At 15 days postgraft, DP and single-positive (SP) thymocytes of donor origin were detected in the thymus (Fig. 2*B*). Kinetics of thymus reconstitution were not different from those of SC12 pre-T cells recovered from euthymic mice. Analysis of the peripheral compartment at 30 days after injection showed that over 99% of donor cells were mature $TCR\alpha\beta^+$ T cells in lymph nodes (Fig. 2*B*), spleen, and BM (data not shown). Neither B nor myeloid cells were detected while a small residue of NK cells was transiently detected in the BM (data not shown), demonstrating that virtually all progenitors are T cell restricted. In athymic conditions, these progenitors maintained the properties of a pre-T cell population after in vivo injection; that is, they were able to colonize the thymus rapidly and had a very restricted T cell potential. Besides, in these functional tests, their properties were identical to those we described for SC12 pre-T cells generated in euthymic mice (15).

To investigate whether the thymus could eventually modify the degree of T cell commitment of individual pre-T cells, we compared the gene expression profiles of individual pre-T cells generated in euthymic mice with those obtained in athymic conditions (in TX mice injected with nude BM). For this purpose, SC12 pre-T cells generated in these two conditions were purified by double cell sorting and individual cells were analyzed for their expression of CD3ϵ, considered to be an early marker of T cell commitment (25); GATA-3, which encodes a transcription factor specific for T

cell commitment (26, 27); Rag-1; and pre-Tα. We found a slightly lower percentage of SC12 CD44$^-$ progenitors expressing CD3ϵ (67 $\pm$ 5% in athymic vs 96 $\pm$ 4% in euthymic (15)) and GATA-3 (42 $\pm$ 5% in athymic vs 70 $\pm$ 6% in euthymic), but the expression of Rag-1 was similar in both situations (49 $\pm$ 6% vs 43%). The percentage of progenitors expressing pre-Tα was also lower in athymic conditions (3 $\pm$ 1% vs 8 $\pm$ 3%). However, in both types of SC12 precursors, CD3ϵ, GATA-3, and Rag-1 were not necessarily expressed by the same cells (Fig. 3). As a consequence, each individual cell expressed at least one of these genes even in athymic conditions. It could be argued that Rag-1 expression is not T cell specific and should not be referred to as a T cell-specific gene in this context. However, it must be noted that this population is unable to generate B cell type and thus, Rag expression rather appears as T cell specific.

We conclude that the majority of pre-T cells generated in the total absence of thymus influence already show molecular markers of T cell commitment, even when studied at the single-cell level, demonstrating that the influence of the thymus is not required for such T cell commitment. However, the presence of a thymus enhances the multiple expressions of T cell-specific genes among individual pre-T cells.

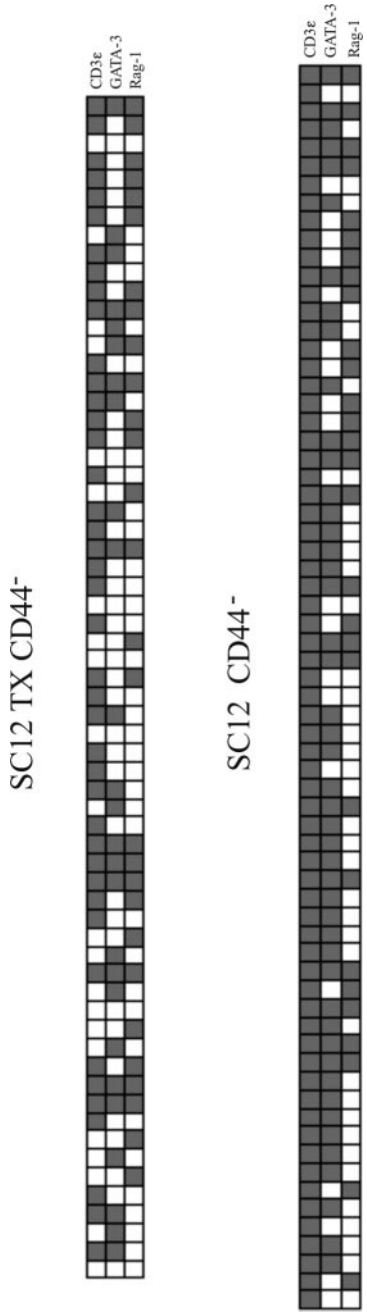

FIGURE 3. T cell-specific gene coexpression among individual SC12 progenitor cells produced in euthymic (SC12 CD44$^-$) and athymic (SC12 TX CD44$^-$) conditions. Individual cells were analyzed for the coexpression of CD3ϵ, GATA-3, and Rag-1 mRNA. A total of 68 SC12 CD44$^-$ and 64 SC12 TX CD44$^-$ cells were studied; 25% (20–30) in SC12 CD44$^-$ (68 single cells analyzed) and 16% (11–21) in SC12 TX CD44$^-$ (64 cells analyzed) of coexpression was scored in each subset. The binomial law permits evaluation of SD.

SC12 pre-T cells require either the thymus or the gut epithelium to generate T cells

Pre-T cell populations may differ in their requirements to further differentiate and generate T cells. Pre-T cells isolated from the adult BM were reported to give rise to peripheral T cells even in the absence of the thymus after injection into athymic mice (13). Extrathymic pre-T cells could also be preferentially committed to the generation of peculiar T cell populations, i.e., the extrathymic T cell types generated in the liver or the gut in athymic conditions. To investigate these two alternatives, SC12 pre-T cells generated in euthymic mice were injected into athymic mice. In these mice, we found no peripheral T cells of donor origin in the spleen (Fig. 2*C*), lymph nodes, and BM (data not shown) 4 wk after transfer. TCR$\gamma\delta$ cells of donor origin could be found in the gut but these populations were rare (Fig. 2*C*). In contrast, these pre-T cells generated abundant peripheral T cells once they had colonized the thymus (15). We conclude that SC12 pre-T cells require additional signals from the thymus or gut epithelium to further differentiate and generate T cells. Besides, they are not preferentially committed to the peculiar extrathymic differentiation pathways, because their capacity to generate T cells in the thymus appeared to be more efficient than their capacity to generate T cells in the gut.

Comparison of phenotype between SC12 pre-T cells and thymus TN populations

To establish possible relationships between SC12 pre-T cells and early thymocytes, we compared CD3$^-$CD4$^-$CD8$^-$ (TN) subtypes and SC12 CD44$^-$ cells for the expression of several surface markers associated with precursor differentiation and commitment. To facilitate this comparison, our data are presented in two different ways. In Table II, we compared all TN subsets and SC12 pre-T cells for several markers. Fig. 4 illustrates the labeling of SC12 CD44$^-$ cells, as compared with isotype controls and CD44$^-$ TN thymocytes studied simultaneously. The labeling of individual TN1/TN2 subtypes is not shown but is summarized in Table II.

As described previously, we found that all TN subtypes were AA4.1$^-$; the few B220$^+$ cells still present among TN1 cells were CD43$^-$. In contrast, all other TN subpopulations as well as SC12 pre-T cells were B220$^-$CD27$^+$CD43$^+$. It has been suggested that this phenotype (B220$^-$AA4.1$^-$CD43$^+$CD27$^+$) identifies precursor cells committed to T cell differentiation. TN3 and TN4 populations were CD2$^+$, but SC12 pre-T cells were CD2$^-$, like TN1 and TN2 subsets. All TN subsets and SC12 cells express CD62L. The latter cells are also more related to mature TN types in terms of heat stable Ag (CD24) expression, which is slightly higher in SC12 CD44$^-$ than in TN1 and TN2 populations, even though it remains lower than in TN3 and TN4 subsets. Both SC12 CD44$^-$ and TN cells expressed CD117 (c-*kit*). In contrast, the expression

Table II. *Phenotype of intrathymic progenitors and SC12 pre-T cells*[a]

Markers	TN1	TN2	TN3	TN4	SC12 CD44$^-$	References
AA4.1	−	−	−	−	−	28
CD2	−	−/low	+	+	−	29
CD43	−	+	+	+	+	30
CD62-L	+	+	+	+	+	31
CD29 (β_1)	+	+	+	+	−	32
CD49d (α_4)	+	+	+	+	+	32
CD49e (α_5)	−	+	+	+	−	32
β/integrin	−/low	+	−	−	+	32
CD24 (HSA)	Low	Low	High	High	High	33, 34
CD27	+	+	+	+	+	7, 35
CD117 (c-Kit)	High	High	Low	Low	Low	7
CD127 (II 7Rα)	−	+	+/low	Low	−	7, 36
CD90.1 (Thy1.1)	Low	Low	+/high	+/high	High	1

[a] −, Absence of staining. +, Positive staining.

of CD127 (IL-7Rα) was very different, TN cells being highly positive, while only a small subset of SC12 pre-T cells expressed the receptor. Furthermore, CD90 (Thy1) expression was much higher in SC12 pre-T cells than in any other TN subset, while CD25 levels were lower among SC12 than among TN2–3, with a very heterogeneous expression pattern in all populations. TN cells express different types of adhesion molecules. All subsets share the expression of both CD49d (α_4 integrin) and CD49e (α_5 integrin) that can associate with several chains depending on the stage of ontogeny. Thus, TN1 cells express solely CD29 (β_1 integrin), TN2 cells coexpress β_1 and β_7, while TN3 cells lose the expression of β_7 but maintain that of β_1. We found that SC12 pre-T cells differed from TN CD44$^-$ cells because they only expressed α_4, but not α_5. Like TN2 cells, they expressed high levels of the β_7 integrin, but no β_1 integrin, in contrast with all TN populations. Therefore, SC12 pre-T cells express mainly $\alpha_4\beta_7$ complexes, while TN cells display a more diverse integrin expression. These results indicate that the SC12 pre-T cell phenotype is not directly related to any stage of intrathymic TN differentiation. Indeed, SC12 pre-T cells resemble TN2 cells by their expression of c-*kit* and the β_7 integrin. However, they share a low expression of IL-7Rα with TN1 cells, while their higher CD24 (heat stable Ag) levels suggest a more advanced differentiation stage. The reduced expression of β_1 integrin, the absence of CD2 expression, and the very high Thy1 expression distinguish this pre-T cell population from any other TN subset. We conclude that the phenotype of SC12 CD44$^-$ cells has features in common, but does not fully overlap with any of the TN subsets.

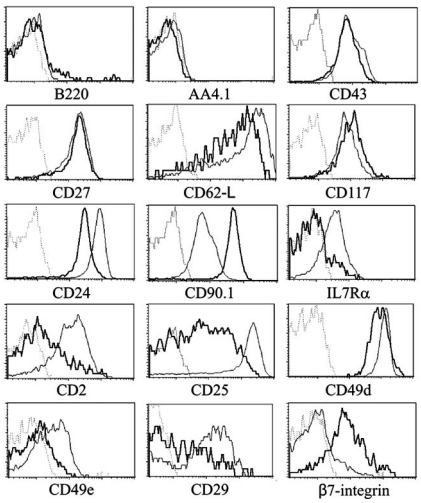

FIGURE 4. Phenotype of SC12 pre-T cells. Thymus and SC12 cells were labeled with Abs recognizing Ly5.2 (donor population), lineage Ags (Ter119, CD3, CD19, Mac1, NK1.1 and TCRβ plus CD8 for thymus cell depletion). Surface expression of indicated Abs in SC12 pre-T cells (bold-lined histograms) and TN Lin$^-$CD44$^-$ cells (thin-lined histograms) are represented with the isotype control (dotted-lined histogram).

Comparison between TN and SC12 cells in terms of thymus reconstitution potential

The differentiation potential of T cell precursors is best defined by the kinetics of DP thymocyte generation from pre-T progenitors and by the length of time during which thymocytes persist after adoptive transfer. To evaluate these parameters, 4×10^4 CD44$^-$ thymocytes or SC12 CD44$^-$ cells were injected i.t. into sublethally irradiated mice. This CD44$^-$ subset was selected based on its restriction potentialities. Indeed, both TN CD44$^-$ and SC12 CD44$^-$ populations are fully T cell restricted. Donor-type cells, identified in recipient mice by the Thy1.1 allotype marker, were evaluated for thymus reconstitution at different time points after i.t. injection (Fig. 5).

TN CD44$^-$ cells generated DP cells in the host thymus 5 days after transfer. The percentage of DP cells increased until day 7 and 8, when they represented 97% of donor-type cells. The percentage of DP cells decreased thereafter, while the proportion of SP increased. By day 21 after transfer, only CD4$^+$CD8$^-$ and CD4$^-$CD8$^+$ SP cells were detectable in the host thymus (Fig. 5*A*). Compared with repopulation by TN CD44$^-$ cells, the generation of DP and SP from SC12 pre-T cells was slightly delayed reaching peak values within 10 days (Fig. 5*A*). Moreover, the SC12 pre-T cell population had a lower expansion capacity because at all time points after transfer, the absolute numbers of recovered thymocytes were 10- to 100-fold less for SC12 than for TN donor cells, and thymus colonization was more transient (Fig. 5*B*). SC12 progeny was no longer detectable in the thymus after day 11 (Fig. 5*B*). Thus, SC12 pre-T cells appear to have a reduced capacity of self-renewal and thus induce a short and very transient wave of thymus repopulation after i.t. transfer, in contrast to TN cells.

The reduced expansion of SC12 pre-T cells as compared with TN CD44$^-$ cells could be explained either by a low frequency of competent T cell progenitors, or by their weak proliferation potential. To discriminate between these possibilities, SC12 CD44$^-$, TN3, and TN4 thymocytes were cultured in limiting dilution on the OP9-DL1 stroma cell line (6), and proliferation and differentiation into DP thymocytes was evaluated at different time points. The

Table III. *T cell progenitor frequency in SC12 pre-T populations*

Progenitor Populations	Frequency^{-1}
TN3[a]	3
SC12 CD44^{-}[b]	71 (63–90)[c]

[a] Single cells from TN3 populations were analyzed in each well ($n = 36$) for the generation of DP cells.

[b] Three limiting dilutions of SC12 CD44^{-} (100, 50, and 20 cells per well, $n \geq 72$) were analyzed for the generation of DP cells.

[c] Statistical analysis was performed using the method of maximum likelihood applied to the Poisson model, and 95% confidence limits are shown in brackets (20).

TN4 populations differentiate immediately, without further proliferation probably because they are already advanced in differentiation. Therefore, they do not grow in vitro, which prevents frequency determinations. In contrast, both TN3 and SC12 proliferated. However, the frequency of competent progenitors differed markedly between both cell types (Table III). In the SC12 CD44^{-} population, ~1 of 71 cells could generate DP cells, whereas 1 of 3 TN3 cells, respectively, was capable of doing so. These results indicate that among SC12 CD44^{-} cells the frequency of competent progenitors defined by their capacity to undergo further differentiation is ~20-fold less in these culture conditions.

Evaluation of SC12 T cell reconstitution potential after i.v. transfer

A low frequency of SC12 pre-T cells giving rise to progeny after i.t. injection or OP9-DL1 stimulation could reflect either a very low T cell reconstitution potential or the requirement of additional extrathymic signals enabling progenitors to differentiate and generate T cells with high clonogenicity. These putative extrathymic signals would not be provided when pre-T cells were injected directly into the thymus or cultured with OP9-DL1 cells. To discriminate between these two explanations, we compared the capacity of SC12 pre-T cells and TN CD44^{-} cells in terms of thymic and peripheral reconstitution after i.v. injection (Fig. 6). Indeed, our preliminary evidence demonstrates that these committed precursors can leave the thymus and recirculate (F. Lambolez, M. L. Arcangeli, B. Rocha, and S. Ezine, manuscript in preparation). Both TN and SC12 pre-T cells generated DP and SP thymocytes. However, the percentages of donor type cells and the CD4/CD8 distribution was variable among individual mice at the same time point, in both groups of mice (Fig. 6*A*); therefore, the absolute number of thymocytes recovered at different time points varied from one host to another (Fig. 6*B*). Such variations have been described when CLP and ETP were i.v. transferred (7) and have been attributed to differences in the receptivity of the thymus to incoming precursors (7, 37). The differences in thymus reconstitution cannot be measured only in cell numbers, because of the variability of thymus receptivity to seeding progenitors after i.v. transfer. The major difference in behavior is actually the capacity to originate a prolonged thymic reconstitution. Indeed, after i.v. injection, SC12 progenitors generate a very prolonged "wave" of thymus reconstitution, considered "typical" of T cell progenitors. In contrast, after i.t. injection they do not behave at all like typical T cell progenitors; e.g., thymus reconstitution is over around day 12. Therefore, after i.v. transfer, the SC12 acquired a self-renewal and expansion capacity that was absent when these cells were directly injected into the thymus. We conclude that SC12 pre-T cells yet require further extrathymic signals to achieve a thymic repopulation.

Besides, we also observed striking differences in the capacity of SC12 and TN progeny to repopulate peripheral T cell pools. SC12 pre-T cell population generates 3 times as much T lymphocytes as TN cells, after i.v. transfer (Fig. 6*C*). With Rag-deficient mice as recipients, peripheral reconstitution is always inferior to normal mice. These differences were permanent, i.e., they were still present two months after precursor transfer (data not shown). These results indicate that splenic pre-T cells, once injected i.v., have a much higher potential to generate mature T cells than more differentiated TN thymocyte subsets.

Discussion

In the present study, we investigated the phenotypical and functional features of a SC12 Lin^{-}CD44^{-} pre-T cell population, isolated from spleen colonies in irradiated mice having received a BM graft 12 days before. We have previously described that SC12 are a unique source of T cell-committed precursors in comparison to BM. Indeed, Lin^{-} cells are particularly abundant in SC12, 40% are pre-T cells. Because of these relatively high frequencies, T cell-committed precursors can be easily recovered and studied.

We provide evidence that SC12 CD44^{-} cells share several characteristics expected from a genuine pre-T cell population. First,

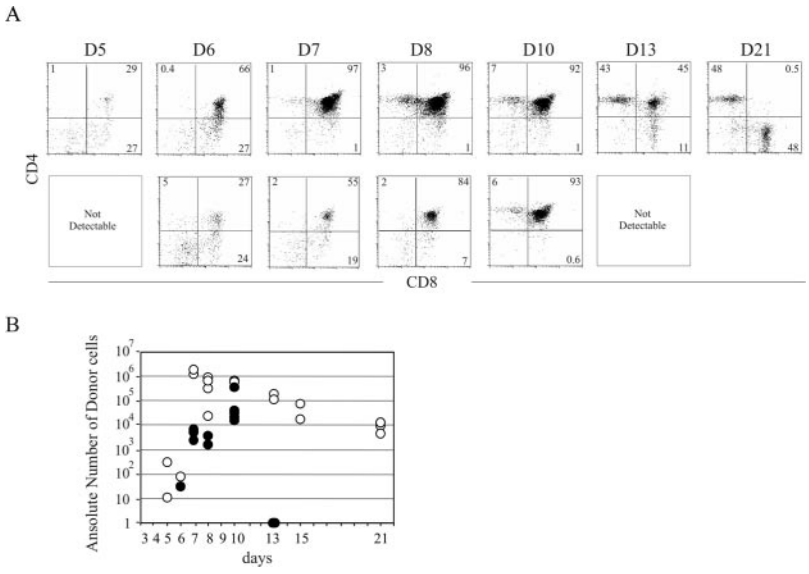

FIGURE 5. Kinetics of thymus reconstitution after intrathymic transfer. Sublethally irradiated B6 Thy1.2^{+} mice were injected i.t. with 4×10^4 Lin^{-}CD44^{-}Thy1.1^{+} cells from thymus or SC12 populations, and studied at different intervals after transfer. *A,* CD4/CD8 expression of donor-type (Thy1.1^{+}) thymocytes generated by TN CD44^{-} thymocytes (*top row*) or by the SC12 pre-T cells (*bottom row*). Between two and four mice were studied at each time point. *B,* Absolute number of donor-type thymocytes recovered in individual mice injected with TN cells (○) or SC12 pre-T cells (●).

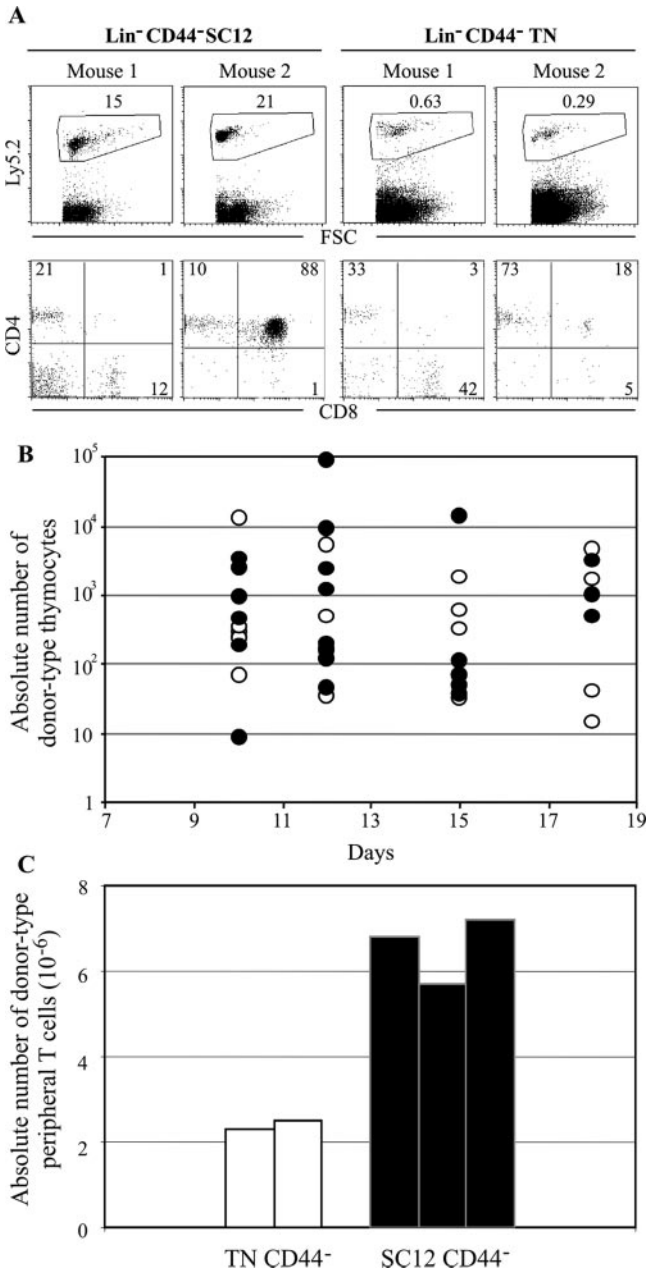

FIGURE 6. Kinetics of thymus reconstitution following i.v. transfer. Sublethally irradiated Ly5.1 Rag2$^{-/-}$ mice were injected i.v. with 4×10^4 Lin$^-$CD44$^-$ cells from thymus or SC12 populations, and studied at different time points after transfer. *A,* Heterogeneity of thymic repopulation 15 days after the transfer; two typical profiles of individual mice in each group of recipient progenitors. *B,* Absolute number of donor-type thymocytes recovered from individual mice injected with TN CD44$^-$ thymocytes (○) or SC12 pre-T cells (●) are represented. *C,* Quantification of the T cell pool in the periphery, 4 wk after i.v. transfer of mice grafted with thymic pre-T (□) or SC12 pre-T cells (■).

they require further differentiation in the thymus or the gut epithelium to generate mature T cells. In this aspect, they differ from the pre-T cells previously reported in the adult BM that can give rise to mature T cells directly (12, 13). Second, they are not exclusively committed to one of the peculiar extrathymic T cell lines, such as the gut intraepithelial CD8$\alpha\alpha$ T lymphocytes or liver-derived extrathymic cells. Indeed, even though they can generate TCR$\gamma\delta^+$CD8$\alpha\alpha^+$ intraepithelial lymphocytes, they are more efficient in producing conventional thymus-derived T cells. Finally, their development occurs without any influence of the thymus because they are produced at similar frequencies and maintain their rapid thymic reconstitution potential even when generated in athymic conditions. Only single-cell analysis revealed some differences concerning the expression of molecular markers of T cell commitment, such as the frequency of CD3ϵ, GATA-3, and pre-Tα expression, which was lower in pre-T cells generated in athymic than in euthymic mice. It is possible that the thymus maintains a hormonal influence on the differentiation of these pre-T cells, similar to that reported for extrathymic T cell generation (14). Despite this potential influence of the thymus, single-cell analysis of multiple gene expression revealed that virtually all "athymic" pre-T cells expressed at least one of the genes implicated in T cell differentiation; i.e., most were probably T cell committed, even in athymic conditions.

Two experimental approaches were used to evaluate the capacity of these precursors to restore T lymphocytes. Surprisingly, they provided different results. Indeed, compared with thymic CD44$^-$ populations, thymus reconstitution was delayed and fewer thymocytes were generated when SC12 pre-T cells were delivered directly into the thymus. Furthermore, in vitro culture with OP9-DL1 revealed that fully committed precursors were much less frequent (~1 of 70) among SC12 pre-T cells than among TN3 populations with a precursor frequency of 1 of 3. It is noteworthy that the frequency of precursors in SC12 pre-T cells is even lower than that reported for CLP (1 of 21) (2). Indeed, SC12 pre-T cells are generated independently of the thymus and, thus, need different signals than conventional thymic progenitors. These low precursor frequencies and reduced growth and differentiation capacities of SC12 pre-T cells delivered directly into the thymus suggested that their capacity to generate thymocytes and peripheral T cells after i.v. injection should also be reduced. Surprisingly, this was not the case, because they colonized the thymus efficiently in these conditions and actually generated a prolonged wave of thymocytes and mature T lymphocytes. The most plausible explanation for this discrepancy is that these pre-T cells yet require additional differentiation steps outside the thymus to acquire efficient clonogenic ability. These results emphasize the necessity for multiple types of assays to characterize the behavior of precursor T cell populations. They also suggest the existence of multiple differentiation steps that may even occur after full T cell commitment.

Comparison of these pre-T cells with previously reported T cell precursors revealed common features as well as major differences. They share the B220$^-$AA4.1$^-$Thy1$^+$c-*kit*$^+$ phenotype with most pre-T cells, but are more like cryptopatch pre-T cells (14) as far as their heterogeneous expression of CD25 and the presence of TCRβ DJ rearrangements (15) are concerned. However, while cryptopatch cells are CD44$^+$ and express high levels of IL-7Rα chain (14), SC12 pre-T cells are CD44$^-$, and only a few express IL-7Rα. They also differ from pre-T cells isolated directly from the adult BM, which are CD44$^+$CD25$^-$ and have no TCRβ-DJ rearrangements (12, 13). Finally, comparison of SC12 pre-T cells with the most abundant TN CD44$^-$ precursor population in normal thymus also revealed clear-cut phenotypic distinctions. Indeed, this TN population expresses higher levels of IL-7Rα and CD25, and cells expressing mRNA coding for Rag-1 and pre-Tα much more frequent than among SC12 cells. It could be argued that SC12 contain a mixture of precursor cell types, the few IL-7Rα^+CD25^{high} SC12 being identical with TN IL-7Rα^+CD25^{high} cells. However, these progenitors differ entirely by their expression of integrins because SC12 cells do not express α_5 integrin in contrast with all TN populations and virtually lack β_1, which is expressed by most TN cells. These results demonstrate that SC12 pre-T cells and TN CD44$^-$ thymocytes are different precursor cell types and that

SC12 pre-T cells represent a unique population, which has not been described as yet.

Taken as a whole, the present and previous studies suggest that, besides CLP, a panoply of pre-T cells with different phenotypic markers, and a different degree of differentiation and T lineage commitment, can be found in the periphery, as well as in the thymus (11–14). At least in the case of SC12 pre-T cells, commitment to the T cell lineage occurred outside the thymus while T cell differentiation requires the thymus (or the gut) or at least Delta-like signals. Whether BM cells seeding the spleen can commit to the T cell lineage during the formation of spleen colonies or whether these cells result from the expansion of a rare pre-T population present in the normal BM, remains unclear. Indeed, the search for an equivalent population (Lin^-CD44^-) in steady state mice, in the BM and the spleen, has proved difficult, because of the extremely rare Lin^-CD44^- cells present and their differentiation stage; however, related pre-T populations are present in peripheral organs (M.-L. Arcangeli and S. Ezine, manuscript in preparation).

These results have several implications. First, they indicate that not all pre-T cells or their precursors migrate directly to the thymus. These populations may not express CCR9 (38, 39) or other chemokine receptors required for migration into the thymus. Alternatively, they may be generated at a time when the thymus is not receptive to incoming precursor (37). In any circumstances, some committed progenitors may remain in the periphery where they may pursue further maturation and T cell commitment. Second, at least experimentally, these heterogeneous precursors can colonize the thymus rapidly after transfer. Therefore, it is possible that thymus colonization is not associated with a particular differentiation stage. Different precursor types at different stages of T commitment may share this ability. To summarize, we provide evidence for a new pre-T cell population in the adult mouse, generated and committed to T cell differentiation outside the thymus and much more efficient in inducing precocious peripheral T cell reconstitution than previously described precursor cells. This extrathymic population requires additional signals before migrating into the thymus, implying that cells at different developmental stages can enter this organ. It is very efficient in repopulating the peripheral compartment due, probably, to multiple stimulation in their initial niche. Finally, like oncostatin transgenic mice (40, 41), SC12 pre-T cells provide an excellent tool for the study of early T cell lineage commitment in the periphery, independently from thymic influence.

Reconstitution of the hemopoietic system in the adult by stem cell grafts has become a current medical practice. After these grafts, the reconstitution of the T cell compartment is morose and inefficient. In the period of time during which the peripheral T cell pool is not reconstituted, grafted patients are highly susceptible to recurrent infections. Thus, the coinjection of pre-T cells able to produce a rapid T cell reconstitution has major potential therapeutic application.

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