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Identification of an IL-7-Dependent Pre-T Committed Population in the Spleen

Laetitia Gautreau,* Marie-Laure Arcangeli,* Valérie Pasqualetto,* Anne-Marie Joret,* Corinne Garcia-Cordier,† Jérôme Mégret,† Elke Schneider,‡ and Sophie Ezine2*

Several extrathymic T cell progenitors have been described but their various contributions to the T cell lineage puzzle are unclear. In this study, we provide evidence for a splenic Lin−Thy1.2+ T cell-committed population, rare in B6 mice, abundant in TCRα−/− mice, CD3ε−/−, and nude mice, and absent in IL-7- and Rag-2-deficient mice. Neither B nor myeloid cells are generated in vivo and in vitro. The incidence of these pre-T cells is under the control of thymus and/or mature T cells, as revealed by graft experiments. Indeed, IL-7 consumption by mature T cells inhibits the growth of these pre-T cells. Moreover, the nude spleen contains an additional Lin−Thy1.2+CD25+ subset which is detected in B6 mice only after thymectomy. We establish that the full pre-T cell potential and proliferation capacity are only present in the c-kit−/− fraction of progenitors. We also show that most CCR9+ progenitors are retained in the spleen of nude mice, but present in the blood of B6 mice. Thus, our data describe a new T cell lineage restricted subset that accumulates in the spleen before migration to the thymus. The Journal of Immunology, 2007, 179: 2925–2935.

The T cell progenitors develop from bone marrow (BM) hemopoietic stem cells. However, to ensure continuous renewal of the T cell compartment, BM progenitors must seed the thymus which, in turn, implies that they have to pass through the blood stream beforehand. The mechanisms governing progenitor entry into the thymus are poorly defined but depend probably on signals emanating from the thymus itself. It has been suggested that both refractory and permissive periods control pre-T cell homing (1). Indeed, engraftment of thymocyte precursors occurs with a periodicity of 4 wk, during which the availability of i.t. binding sites is optimal. The accessibility of niches ready to receive progenitors depends on the size of the double-negative (DN) CD4−CD8− cell pool already present (2). For instance, the thymus of IL-7Rα−/− mice (that contains only 2% of DN cells) is restored to normal cellularity after intra-thymic (i.t.) transfer of wild-type (wt) BM cells. In contrast, the thymus of Rag-2−/− mice (composed exclusively of DN cells) is weakly reconstituted under the same conditions. Moreover, according to a recent report, interaction between P-selectin (expressed by thymic endothelium) and its ligand PSGL-1 (expressed on lymphoid progenitors) facilitates engraftment of T cell progenitors in the thymus (3). The authors therefore suggested that P-selectin expression is regulated by the number of i.t. progenitors.

Thus, active signals are probably required for efficient thymus seeding by T cell progenitors. It is also possible that circulating progenitors have to reach a critical level and/or a specific maturation stage before they can enter the thymus. The identity of the progenitors (both in terms of phenotype and differentiation stage) which home to the thymus is still subject to intense debate, explaining why the list of potential candidates has grown steadily in recent years.

Progenitors with predominantly T cell potential (but also associated with B, NK, and myeloid activities) have been identified in mouse BM and blood. They belong to the Lin−Sca-1+ c-kit+ (LSK) subset and express additional, specific Ags: Flt3+CD27+IL-7Rα+ for the earliest lymphoid progenitors (4); Thy1.1+CD62L+ for LSK CD62L+ precursors (thought to represent the last stage of differentiation in the BM before thymus colonization (5, 6)); and VCAM-1+ for the multipotent progenitor subset reported recently by Kondo and colleagues (7). The common lymphoid progenitors (CLPs) (Lin−Sca-1−/− c-kit−/− IL-7Rα−) (8) are restricted to the lymphoid lineage, mainly engaged in B cell development (9) and, therefore, probably do not contribute to thymus colonization. However, this point has been recently challenged; in vitro, CLPs are able to quickly adopt a DN2 phenotype, suggesting that this population could indeed colonize the thymus after a BM transplant (10). Thus, it is still not clear whether the T cell progenitors described above belong to a common differentiation pathway or follow distinct maturation routes. Whatever the answer, it is clear that very few cells are needed for thymus colonization. Rather than attempting to trace these cells directly, one way to learn more about the identity of this minute population is to explore how T cell progenitors are regulated.
A progenitor having recently seeded the thymus probably belongs to the earliest T cell progenitor (ETP) c-IgRhi IL-7RαDN1 population (11) and resembles the CCR9hi subset described by Benz and Bleul (12). In CCR9-GFP knockin mice, CCR9hi ETP multipotent cells (present in the BM and the blood) generate a CCR9pop subset, which completely lacks B cell potential in the thymus. Thus, the CCR9hi ETP subset is considered as the colonizing population.

To date, several studies have provided evidence of extrathymic pre-T cells. A Lin- Thy1.2hiCD44hiCD25− fully committed T cell population (CTP) was described in the BM of B6 mice (13, 14); following i.v. transfer to athymic nude mice, this CTP was able to generate T cells via an extrathymic pathway (15). Recently, following i.v. transfer to athymic nude mice, this CTP was described in the BM of B6 mice (13, 14); more, we have reported a Lin-Thy1.2hiCD44hiCD25− pre-T cell population (CTP) described by Strober and colleagues, suggesting that the two populations might belong to the same pathway (16). In gut cryptopatches, Lin-Thy1.1 IL-7Rα+CD25− cells have been identified as precursors of intraepithelial lymphocytes (IELs) (17). Furthermore, we have reported a Lin-Thy1.1 CD44hi CD25− pre-T cell population in colonies formed in the spleen of lethally irradiated mice 12 days after BM transfer, referred to as “SC12” colonies (18). We established that this T cell-restricted population did not depend on the presence of the thymus (19).

Hence, having identified a pre-T cell population in SC12, we sought to determine whether a similar population was present in the spleen of nonirradiated mice. In this study, we report on the identification and regulation of a T cell lineage-committed Lin-Thy1.2+ population that accumulates in the spleen. This subset is under the control of the thymus and/or mature T cells that may regulate the availability of the IL-7 needed for progenitor generation.

Materials and Methods

Mice

All mice were bred on a pure C57BL/6 (B6) genetic background. Ly5.1 mice and Ly5.1 CD3ε−/− mice were purchased from Transgenic Alliance and Centre de Distribution, Typage, Archivage Animal. Ly5.2 mice were purchased from Centre d’Elevage R. Janvier and nude (nu/nu) mice were bought from Centre de Distribution or Taconic. C57BL/6a (Ba), IL-7Rα−/−, Ly5.1 Rag-2−/−, and Ly5.1 Rag-2−/− γc−/− mice were bred and maintained in the animal care facility in the Necker Institute.

Thymectomy and mature T cell grafts

Mice were thymectomized (Tx) between 4 and 12 wk before analysis, as described elsewhere (18). In brief, mice were anesthetized and placed on their back; an incision was made in the skin above the sternum and the thymus was removed with forceps. The skin was closed with metal surgical clips. The completeness of thymectomy was confirmed at autopsy. Animal experiments were approved by our institutional animal care and use committee.

Lymph node (LN) and/or spleen cells were recovered from B6 (Thy1.1, Ly5.2), B6 (Thy1.2, Ly5.2), or IL-7Rα−/− (Thy1.2, Ly5.2) mice, as appropriate. T cells (2 × 10⁶) were i.v. injected into nude or Ly5.1 CD3ε−/− mice (Thy1.2). The number of donor-type cells was estimated in the LNs, spleen, and BM 5, 30, and 60 days after transfer. The host spleen was analyzed for the presence of Lin-Thy1.2+ progenitors.

Antibodies, flow cytometry, and cell sorting

The following mAbs used for cytometry and/or cell sorting were obtained from BD Pharmingen: anti-CD2 (LEA-2, RM2–5), anti-CD3 (145-2C11), anti-CD4 (RM-4–5), anti-CD8α (30–3), anti-CD8β (H3–172), anti-CD11a/αβ (2D7), anti-CD11b/Mac-1 (M1/70), anti-CD16/CD32 (FcγRIIa, 2.4G2), anti-CD18/CD2 (Ly-6, GL3), anti-CD29/IL-2Ra (H–2D), anti-CD44 (H-CAM, 1M781), anti-CD45.2/2C10 (104–2.1), anti-CD49d/44 (1B2–5), anti-CD71 (E3/16), anti-CD90.2/Thy1.2 (53.2.1), anti-CD117/kit (stem cell factor receptor), 2B8, anti-CD127/IL-7Ra (A7R34, a gift from Dr. S.-I. Nishikawa, Kyoto University and RIKEN Center, Kyoto, Japan), anti-CD162/PSGL-1 (2PH1), anti-NK1.1 (PK136), anti-Sca-1 (stem cell Ag-1, E13–161.7), anti-erythroid (TER119), anti-Ly-6G/Gr1 (RB6–8C5), anti-LPAM-1/a4b7 (D4/10), anti-TCRβ (HAM and the anti-CD45.1/Ly5.1 (A20-7.1) mAbs were obtained from Southern Biotech. The anti-CDw199/CRC9 (242503) mAb was obtained from R&D Systems. All the above-mentioned mAbs were directly coupled to FITC, allophycocyanin, PE, and PerCP, or conjugated with biotin (the latter being revealed by streptavidin-allophycocyanin or streptavidin-PE/CY7; BD Pharmingen). A FACSCalibur (BD Biosciences) was used for flow cytometry, whereas cell sorting was performed on a FACS Vantage upgraded with DIVA software (BD Biosciences). Data were analyzed using CellQuest and CellQuestPro software packages (BD Biosciences).

For cell sorting, spleen cells were first incubated with unconjugated TER119 and Ly-6G/Gr1 (RB6–8C5) rat mAbs, which are specific for erythroid and myeloid cells, respectively. Positive cells were magnetically depleted with sheep anti-rat IgG-conjugated beads and sheep anti-mouse IgG-conjugated beads (Dynabeads M-450; Dynal Biotech). The remaining cells were labeled with Abs against the Ly5.2, Thy1.2, and lineage Ags (CD3, CD19, Mac-1, NK1.1, and TCRβ). For analysis of triple-negative (TN) CD3−CD4−CD8− cells in the thymus, cell suspensions were first incubated with unconjugated TER119, CD5 (53-7.3), and CD8α (Ly5.2) rat Abs. Positive cells were removed magnetically with sheep anti-rat IgG-conjugated beads (Dynabeads M-450; Dynal Biotech) and the negative fraction was labeled with Abs against CD25, CD4, and lineage Ags (Mac-1, 8C5, NK1.1, TCRβ, CD8β, and CD19).

In vivo transfer of precursor cells

Recipient mice were lethally irradiated (600 rad) and test cells were injected i.t. or i.v., as described in detail elsewhere (18). In brief, 4 × 10⁶ precursor cells were resuspended in 200 μl of medium for i.v. injection into Ly5.1 Rag-2−/− mice, whereas 2 × 10⁶ precursor cells in 20 μl were directly transferred into the thymic lobe of Ly5.1 B6 recipients. One or two months after i.v. injection, Ly5.2+ donor cells were recovered from the LNs, spleen, BM, and thymus of recipient mice. LN cells were pooled from lymphoid and myeloid cells, respectively. Positive cells were stained with FITC-anti-IgM and the anti-CD45.1/CD45.2 rat mAbs, stained with PE-anti-CD19, and subsequently stained with an allophycocyanin-conjugated anti-TCRβ (2.4G2) mAb. The number of donor T cells collected in the thymus was calculated using the following formula: 2ⁿ × n/n = 2ⁿ, in which n is the number of donor T cells collected in lymph nodes; n is the number of donor T cells collected in the spleen; n is the number of donor T cells collected in the bone marrow; and n is the number of donor T cells collected in the thymus. The number of donor T cells in the bone marrow was determined by co-transfer with congenitally priors. All cultures were performed in the presence of 1 ng/ml IL-7 (R&D Systems) and 5 ng/ml Flt-3L (R&D Systems) and fed every 4 days. At the indicated time, progenitor cells were recovered and stained to detect B cells (CD19), T cells (CD4/CD8/TCRβ), NK cells (NK1.1/TCRβ), and myeloid cells (Mac-1). A propidium iodide exclusion assay was used to screen out dead cells.

Results

Identification of a lymphoid progenitor population in the spleen

We have previously described a phenotype that defines committed T cell progenitors in the spleen after a BM graft: Lin-Thy1.2−CD25− (18, 19). Based on these findings, we sought to establish whether or not this population is present in unmanipulated wt B6 mice. We analyzed the lineage negative (Lin−) compartment of spleen cells by using appropriate markers (Mac-1, NK1.1, CD3ε, TCRβ, CD19, and TER119) to exclude mature cells and to identify the progenitor population. The Lin− population represented 0.34 ± 0.19% (n = 9) of the total spleen cells in B6 mice and could be divided into a major Thy1.2− subset (92.2 ± 3.7% (n = 9)) and a minor Thy1.2+ (7.8 ± 3.7% (n = 9)) subset (Fig. 1A). Lin−Thy1.2+ populations were also present in the BM and the blood (Fig. 1A), although expression of the CD25− subset varied;
in the BM, most Thy1.2\(^+\) cells were CD25\(^-\) but these were far more rare in the blood and the spleen.

We analyzed nude mice to determine whether or not the thymus has an influence on the presence of these populations. Using the same procedure as for B6 mice, we found that the Lin\(^-\) population in nude mice represented \(1.0\%\) of the total spleen cells and was significantly higher than in B6 mice (\(t\) test, \(p < 0.005\)) (Fig. 1A). Most spleen cells belonged to the Lin\(^-\)Thy1.2\(^+\) subset, whereas Thy1.2\(^+\) cells constituted \(18.9\%\) of the Lin\(^-\) cells in 4- to 12-wk-old nude mice (Fig. 1A). This last value reached \(39.0\%\) in older mice and was statistically different than younger mice (\(t\) test, \(p < 0.002\)). The BM and blood profiles looked similar to those observed in B6 mice (Fig. 1A). However, we were able to detect an increased CD25\(^+\) subset in the spleen.

**FIGURE 1.** Phenotypic characterization of progenitors in the B6 mouse and the nude mouse. A, BM, blood, and spleen cells from B6 and nude mice were labeled for lineage (Mac-1, NK1.1, CD3, CD19, TCR\(\beta\), and Ter119), Thy1.2, and CD25 Ags. Results show the gate for Lin\(^-\) cells realized in the spleen (upper panel) and the Lin\(^-\) gated population in the BM, blood, and spleen (lower panel). The data are representative of at least five independent experiments. Numbers in quadrants indicate the percentages of each population.

**FIGURE 2.** Phenotypic characterization of progenitors in the spleen. Spleen cells from B6 and nude mice were labeled for lineage (Mac-1, NK1.1, CD3, CD19, TCR\(\beta\), and Ter119) Ags. Surface Ag expression on Lin\(^-\) cells was analyzed as a function of Thy1.2 expression. The results show the Lin\(^-\) gated population. The data are representative of at least five independent experiments. Numbers in quadrants indicate the percentages of each population.
Hence, our results show that the Lin⁻/Thy1.2⁺ population we identified in the spleen was more abundant in the nude mouse and, when compared with the B6 mouse, contained a prominent CD25⁺ subset. We then focused on defining how this population was represented in various immunodeficient mouse strains. Data from CD3ε⁻/⁻, TCRα⁻/⁻, Rag-2⁻/⁻, Rag-2⁻/⁻ yc⁻/⁻, and IL-7⁻/⁻.
mice are presented in Fig. 1B. They revealed that the Thy1.2+ population was rare in Rag-2−/− mice and absent in IL-7−/− and Rag-2−/−γc−/− mice, suggesting that the Rag machinery and IL-7 are important for maintenance of this population. The high number of pre-T cells in CD3ε−/− mice, compared with Rag-2−/− mice, suggested that, probably, TCRβ-rearranged subsets exist among the Lin− Thy1.2+ population whose survival/proliferative signals are unclear and will have to be defined. Most extrathymic pre-T cells described by us and others present some TCRβ rearrangements: Thy1+ c-kitlow cells in the fetal blood (23), CTP in the BM (13), and SCID (18). Moreover, one cannot exclude that the presence of mature B cells plays a major role in shaping the environment of the spleen in CD3ε−/− mice. In contrast, CD3ε−/− and TCRα−/− mice contained abundant Lin− Thy1.2+ cells (Fig. 1B), as did nude mice. The highest number of progenitors in TCRα−/− mice is most probably due to the production of IL-7 in the thymus. Indeed, these mice differentiate until the double-positive (DP) stage (in contrast to CD3ε−/− mice) and therefore, the thymic epithelium is stimulated to produce IL-7 (24, 25). However, with age, IL-7 production is decreased (26). Thus, less cytokine is available in periphery, reducing the number of progenitors in the spleen of TCRα−/− mice (Fig. 1B). Thus, the Lin− Thy1.2+ population was best represented in T cell-deficient mice and rare in B6 animals.

To further characterize these splenic Lin− Thy1.2+ populations, we phenotyped them and compared the profiles obtained in B6 and nude mice with those from specific hemopoietic subsets (Fig. 2). We phenotyped them and compared the profiles obtained in B6 and nude mice with those from specific hemopoietic subsets (Fig. 2). Most progenitors expressed CD44 and Thy1.2, whereas this characteristic was less represented in B6 progenitors. This finding was also reported for a BM pre-T population (13). Thus, most B6 progenitors were CD44−/−IL-7Rα−/− c-kit−/− CD2−/−/−Sca−/−/−, whereas nude progenitors differed in terms of c-kit and Sca-1 expression and were mostly CD2+.

Taken as a whole, these data revealed that B6 and nude Lin− Thy1.2+ progenitors are potential lymphoid progenitors and might constitute a novel population.

Absence of myeloid and B cell potentials among Lin− Thy1.2+ progenitors

Phenotype characterization revealed that Lin− Thy1.2+ progenitors resembled committed lymphoid progenitors, rather than LSKs. Hence, we examined the potential of B6 and nude splenic progenitors by transferring them i.t. (2 × 106 cells) or i.v. (4 × 106 cells) to appropriate recipients.

Nude Lin− Thy1.2+ progenitors grafted in the thymus generated progeny from day 8 onwards, as did the Lin− Thy1.2+ progenitor population (Fig. 3A). At this time point, the progeny of the

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* Lin− Thy1.2+ or Thy1.2+ populations isolated from the spleen of nude mice were i.v. injected into sublethally irradiated Ly5.1 Rag-2−/− recipient mice.

Data show the total numbers of T (2 × Lin− + 1 × spleen + 4 × BM), B, NK and myeloid cells (1 × spleen + 4 × BM) recovered in mice injected with 4 × 106 Lin− Thy1.2+ or Thy1.2+ cells one month after transfer.

Data show the total numbers of T (2 × Lin− + 1 × spleen + 4 × BM), B, NK and myeloid cells (1 × spleen + 4 × BM) recovered in mice injected with 4 × 106 Lin− Thy1.2+ or Thy1.2+ cells two months after transfer.

Thy1.2+ population had reached the CD4+CD8+ (DP) stage of differentiation—unlike the Thy1.2− cells, which mostly remained CD4−CD8− (DN) (data not shown). The respective changes over time in the progenitor populations were quite different. By day 12–13, DP cells were abundant and SP cells were detected among the progeny of Lin− Thy1.2+ cells; few TCRy+ cells were present within the CD4−CD8− window (data not shown); at day 25, the DP pool was exhausted and CD4+ or CD8+ TCRαβ+ (SP) cells constituted the majority of donor cells in the thymus. In contrast, T cell differentiation of the Lin− Thy1.2+ subset was much slower, because a high percentage of DP cells was still present at day 30 and thus indicated the persistence of progenitors (data not shown). Indeed, the Thy1.2− subset generated the most cells between days 12 and 15 postgraft and declined thereafter, whereas the Thy1.2+ progeny continued to increase (Fig. 3A). One can therefore conclude that the Lin− Thy1.2+ subset is more enriched

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in T cell progenitors than the Thy1.2− subset and engendered a shorter wave of thymic repopulation, which is indicative of a pre-T cell-enriched population with limited renewal potential. We tested this hypothesis by assessing the full hemopoietic differentiation potential of these subsets.

In i.v.-grafted recipients of Lin−/Thy1.2−/H11002 or Thy1.2+/H11001 or Thy1.2+/H11002 nude progenitors, TCRβ+/H9251/H9252 high T cells were recovered in peripheral compartments, such as spleen (Fig. 3B), LN (data not shown), and BM (data not shown). In Lin−/Thy1.2−-recipient mice, no B, myeloid, or NK cells were detected, whereas the Thy1.2− population generated T cells as well as B, NK, and myeloid cells (Fig. 3B). The data in Table I show that in the five grafted mice, Thy1.2− cells gave rise mainly to T and myeloid cells 1 mo and 2 mo later (Table I). In contrast, the Thy1.2+ population from nude mice essentially generated T cells; very few NK cells were detected in the BM only 1 mo after transfer (Table I). These data demonstrate that the nude Lin−/Thy1.2− population is T cell committed, whereas the Lin−/Thy1.2−/H11002 subset is multipotent. Similar studies were performed on splenic B6 Lin−/H11002 progenitors (Table II), showing that NK and T cells derived from Lin−/Thy1.2−/B6 progenitors and
that the Thy1.2− population is multipotent. We noticed that the T cell pool was greater when nude splenic T cell precursors were grafted compared with B6 precursors (Tables I and II). These populations found in a B6 control mouse, Lin− cells expanded reasonably well on the OP9-DL1 stroma but only after a certain time lag (Fig. 4A). Comparative studies (run side-by-side with identical initial cell numbers) indicated that the TN3 population differentiated earlier than B6 or nude splenic pre-T cells: DP cells were generated from thymic progenitors at day 4 of culture, whereas nude Thy1.2− cells produced a significant amount of DP cells much later, around day 11 (data not shown). Moreover, for identical time points, more DP were produced by nude progenitors than B6 ones; a CD4/CD8 expression profile on OP9-DL1 stroma is shown in Fig. 4B at day 15 of culture. A few NK cells were detected at 3 wk in B6 and nude cultures (Fig. 4B); this suggests that NK potentiality is repressed by Notch signaling, as reported by others (28).

Thus, like its B6 counterpart, the nude Lin− Thy1.2− population lacks B and myeloid potential. Apart from their common pre-T potential, both pre-T populations are differentially represented in the spleen (with five times fewer progenitors in B6 (0.03 × 10^6 ± 0.02 (n = 9)), compared with nude spleen (0.15 × 10^6 ± 0.09 (n = 9)), t test, p < 0.005), suggesting that they might develop under the pressure of distinct and as yet undefined microenvironments.

**T lineage-restricted potential in the nude spleen**

Given that the Thy1.2− population retained a weak NK potential, we decided to purify this subset. Further stainings revealed that CD25− cells were almost all CD44+ CD2− and mainly c-kitlow (Fig. 5A).
Therefore, we subdivided the CD25+ subset according to c-kit expression and assessed the resulting in vitro cultures, as described above. Our data show that c-kitlow cells proliferated very well and generated only T cells on OP9-DL1 (data not shown) and OP9-DL4 like TN cells (Fig. 5B). In contrast, c-kit− cells developed poorly and generated NK cells during the culture (Fig. 5B). These results demonstrate that Lin− Thy1.2+ CD25+ c-kitlow represents the phenotype of the T cell-committed population in the spleen.

Phenotypic analysis within the Lin− Thy1.2+ subset of B6 mice revealed that wt mice presented the same CD25/c-kit profile as nude mice: ~60% of CD25+ cells were c-kitlow, whereas few CD25− cells expressed the c-kit Ag (Fig. 5C). However, sufficient numbers of these precursors were not available in the spleen of B6 mice to realize such cultures.

**In the absence of a thymus, the pre-T population accumulates in the spleen**

Our data had confirmed that the nude spleen can maintain pre-T cells within the CD25+c-kitlow subset. However, this subset is rare in B6 spleen, suggesting that the presence of a competent thymus hampered the generation and/or expansion of this subset. To investigate the role of the thymus, we analyzed the spleen of B6 mice at 2 to 3 mo after thymectomy. Fig. 6A reveals that the absolute number of Lin− Thy1.2+ CD25+ cells was significantly greater in Tx mice (7 × 103 ± 2 × 103 (n = 10)) than in normal B6 controls (3 × 103 ± 0.3 (n = 5)) (t test, p < 0.05). Hence, the thymus might directly or indirectly control the emergence of the CD25+ progenitor subset. This role could potentially be attributed to thymic progenitors or mature T cells. Nevertheless, we cannot exclude the possibility that the CD25+ subset accumulates in the spleen if it is unable to seed the thymus.

**Mature T cells inhibit the development of splenic pre-T cells**

Analysis of mutant and Tx mice had suggested that the thymus and/or mature T cells might exert negative feedback on the Lin− Thy1.2+ population (Figs. 1B and 6A). To test this hypothesis, we injected around 2 × 106 T cells i.v. into Ly5.1 CD3e−/− mice and recovered their spleen 5, 30, or 60 days later. To establish whether IL-7 had a role in the process, a set of mice was grafted with IL-7Rb−/− T cells in the T cell-deficient host, mature adoptively transferred T cells underwent an expansion phase during the first 5 days and reached a plateau thereafter. Indeed, donor-type T cells represented between 2 and 10% of the spleen cell population during the study (data not shown). Furthermore, the number of Lin− cells in the spleen was greater in reconstituted mice than in un.injected controls (data not shown). The three independent experiments are reported in Fig. 6. A, B, and C. Fig. 6B represents the ratio of Lin− Thy1.2+ to Lin− Thy1.2− cell numbers at different time points after grafting mature T cells sourced from normal B6 mice or IL-7Rα−/− mice. Our data show that this ratio decreased in CD3e−/− recipients of normal T cells, relative to control mice and recipients of IL-7Rα−/− T cells (t test, p < 0.05). This reduction was also observed in terms of absolute cell numbers and was significant at 60 days (t test, p < 0.05) (Fig. 6C). However, the reduction was not complete and stabilized at the level of pre-T cells found in unmanipulated B6 mice. Hence, mature T cells do inhibit the generation of pre-T cells in the spleen; this inhibition is driven by IL-7 consumption by mature T cells, so pre-T cells can accumulate in the absence of mature T cells.

**Adhesion molecule expression on pre-T cells from the spleen**

In principle, the pre-T population depicted in the spleen should also be specified by its integrin, selectin, and chemokine receptor expression. These molecules (involved in cell migration through
FIGURE 7. Adhesion molecule expression on nude splenic Lin−Thy1.2+ CD25+ and CD25− cells. Spleen cells from nude mice were labeled for lineage, Thy1.2, and CD25 Ags. Expression of several adhesion molecules (α4, α5, β1, α4β7, αL, β2, PSGL-1, and CCR9) was studied on the two splenic subsets as a function of CD25 expression. The black and the gray lines represent the CD25+ and CD25− subsets, respectively. The numbers indicate the percentage of negative (M1) and positive (M2) cells. The data are representative of at least three independent experiments.

Discussion

Thymopoiesis is maintained by the influx of progenitors from the bloodstream. These progenitors have been described in the BM and the blood and are thought to colonize the thymus (27). However, the exclusive role of the thymus in shaping T cell-restricted precursors is subject to debate. In this study, we show that pre-T cells are present in the spleen. This T cell-committed population, which is present in wt B6 mice (0.03 × 106 ± 0.02 (n = 9)) and more abundant in nude mice (0.15 × 106 ± 0.09 (n = 9)) (t test, p < 0.05), is dependent on RAG-2 expression, is Lin−Thy1.2+, and is predominantly CD44IL-7RαSca-1+. It differentiates transiently in the thymus and produces only mature T cells following an i.v. graft. This splenic pre-T population expresses α4β1, α5β1, α4β7, αL, β2, PSGL-1, and CCR9, indicating a capacity to circulate, and to colonize the thymus and the gut. We demonstrate that the incidence of splenic pre-T cells is negatively regulated by the graft of B6 mature T cells (but not IL-7Rα−/− T cells). Hence, Lin−Thy1.2+ pre-T cells are IL-7 dependent and under the control of the thymus and mature T cells. Moreover, we have identified a Lin−Thy1.2+ CD25+ c-kitlow subset in the spleen of nude mice, which contains the full pre-T cell potential and proliferation capacity. This latter population accumulates in the spleen of B6 mice after Tx, suggesting that this pre-T subset might be indeed able to colonize the thymus.

Phenotypic studies of Thy1, c-kit, and Sca-1 expression revealed that the pre-T cells we have identified in the spleen resemble the pre-T population we characterized after BM graft in spleen colonies (SC12) (18, 19). Moreover, these markers are present with the same intensity on pre-T cells from the BM (15, 32) and from adult (16) and fetal blood (23), suggesting that they are stable markers for this lineage. In contrast, IL-7Rα and CD44 are expressed in a steady state but not after BM graft, indicating that these receptors could be modulated over the course of differentiation into pre-T cells. Antigenic modulation could be organ-specific, indeed, CCR9+ Lin−Thy1.2+ progenitors are abundant in the blood of B6 mice but rare on nude circulating progenitors. In contrast, CCR9+ progenitors are retained in the spleen in the absence of a thymus.

Intrathymic differentiation proceeds according to four major differentiation steps defined by CD44/CD25 expression. Expression of CD25 on the splenic pre-T population indicates that the latter enters the thymus at the DN2 stage. In addition to its phenotypic characteristics (CD44, CD25, IL-7Rα, and Thy1 expression), its hematopoietic potential and molecular profile (data not shown) resemble those of TN2 cells. Indeed, less specialized progenitors probably enter at the DN1 stage; once in the niches, they receive the appropriate molecules for driving T cell differentiation. NK development is repressed by Notch signaling prior to the DN2 stage (33) and thus only the T cell potential is retained. This mechanism is confirmed by our culture of splenic Lin−Thy1.2+ c-kit+ cells on an OP9-DL1 stroma, on which the NK potential is considerably repressed (Fig. 4). However, we succeed in purifying a population exclusively restricted to the T cell lineage within the CD25+ c-
Table III. Characteristics of pre-T cells according to their location

<table>
<thead>
<tr>
<th>Cell Surface Phenotype</th>
<th>Additional Information</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>BM (B6, nude)</td>
<td>Lin^Thy1^high c-kitlow Sca-1^− IL-7Ra^low^ CD44^high^ CD25^− (few CD25^+^ cells)</td>
<td>Generate mature TCRαβ^+^ cells via an extrathymic pathway</td>
</tr>
<tr>
<td></td>
<td>CD2^+^ CD16^inh^ CD24^inh^ CD5^+^ CD122^-</td>
<td>Regulation by mature T cells (in vitro)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Prethymic origin</td>
</tr>
<tr>
<td>Blood (pTA/hu CD25 Tg)</td>
<td>Lin^Thy1^+ c-kitlow Sca-1^− IL-7Ra^low^ CD44^+^ Fli-3^- B220^- CCR9^- PSGL-1^-</td>
<td>Need the thymus to generate TCRαβ^+^ cells</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Notch independent commitment</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Prethymic origin</td>
</tr>
<tr>
<td>Spleen (B6, nude)</td>
<td>Lin^Thy1^high c-kitlow Sca-1^− IL-7Ra^low^ CD44^+^ CD2^- CD16^+^ CD122^- Fli-3^- B220^- PSGL-1^- a4β1^- a5β1^- a4β7^- c^+^-CCR9^-</td>
<td>Need the thymus to generate TCRαβ^+^ cells</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Generate TCRγδ^+^ cells in IEL in Tx mice</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Increased in nude spleen and after Tx</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Regulation by mature T cells (in vivo)</td>
</tr>
<tr>
<td>SC12 (BM graft)</td>
<td>Lin^Thy1^+ c-kitlow Sca-1^− IL-7Ra^− CD44^- CD25^+/- a4β7^-</td>
<td>Need the thymus to generate TCRαβ^+^ cells</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Generate TCRγδ^+^ cells in IEL in absence of a thymus</td>
</tr>
<tr>
<td>Blood (fetus)</td>
<td>CD3ɛ Thy1^+^ c-kitlow IL7Ra^-</td>
<td>DJβ rearrangement in a fraction of cells</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Prethymic origin</td>
</tr>
</tbody>
</table>

*int, intermediate.

**Kitlow subset**, suggesting that it is the latter that migrates to the thymus (Fig. 5). In addition to Notch signals, numerous molecular events take place from TN2 stage on: *Pit1* is one of the genes shut off at this transition and *Gata3* and the E protein/Id ratio seem to be essential for T cell specification (Ref. 34 and our unpublished data). Hence, identification of the cascade of molecular events driven by, to date, undefined signals will be of major importance.

In terms of other T cell precursors reported outside the thymus, a Lin^Thy1^2^high^CD44^high^CD25^- population isolated from the BM of B6 mice was shown to generate only T cells through an extrathymic pathway, although in vitro studies on an OP9 stroma were not performed (15). However, the BM counterpart in the nude mouse was unable to generate T cells in vivo, probably due to a BM defective stroma, as suggested by the authors (32). In contrast, we demonstrated that the pre-T population in nude spleen is able to reconstitute the T cell compartment efficiently. Thus, in nude mice, T cell commitment is absent in the BM (because of a defective stroma) but always occurs in the spleen, suggesting that the microenvironment and an appropriate signal density (Delta-1 and Delta-4 ligands are expressed by stromal cells in the spleen; our unpublished observations) are maintained. Interestingly, both splenic and medullary pre-T cells are under the control of mature T cells. The maturation of the BM T cell precursors is arrested in vitro by coculture with mature T cells (35), and we have shown that the splenic pre-T population is negatively regulated in vivo by mature T cells via the uptake of IL-7. This inhibitory feedback prevents BM pre-T cell maturation and splenic pre-T cell expansion/survival. Hence, T cell precursors are tightly controlled. Thus, although DN populations are in charge of controlling the entry of progenitors (2), more mature T cells are responsible for the hematopoietic regulation of progenitors in the periphery (this work and Ref. 13).

Very recently, another pre-T population has been described in the blood (16). Phenotypic and functional analogies between this latter population, the CTP in the BM and splenic pre-T suggest that all three populations might belong to a common pathway (Table III). These studies demonstrate that T cell commitment (characterized by Thy1 up-regulation) can occur in the BM and in the spleen. These pre-T populations can recirculate in the blood and colonize the thymus and the gut. The location-specific characteristics of the pre-T populations identify major markers that will help establish the different steps in the pre-T lineage.

The IL-7-dependent T cell-committed population that we have identified in the spleen could represent a “rescue precursor population” in the event of rapid elimination of the mature T cell compartment (acquired immune-deficiency syndrome, myeloablation, and stem cell transplantation). Further characterization should enable us to elaborate potential therapeutic tools for accelerating thymus seeding after a BM graft.

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**Disclosures**

The authors have no financial conflict of interest.

**References**


