A Profound Deficiency in Thymic Progenitor Cells in Mice Lacking Jak3

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A Profound Deficiency in Thymic Progenitor Cells in Mice Lacking Jak3

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Humans and mice with genetic deficiencies that lead to loss of signaling through common γ-chain (γc)-containing cytokine receptors have severe defects in B and T lymphocytes. In humans, these deficiencies lead to a complete absence of T cells, whereas in mice, small thymuses give rise to normal numbers of peripheral T cells. We have examined the first wave of developing T cells in Jak3−/−, IL-7 Rα−/−, and IL-7Rα−/−/pre-T cell fetal mice, and have found a near absence of thymic progenitor cells. This deficiency is highlighted by the complete inability of Jak3−/−/pre-T cell progenitors to reconstitute T cell development in the presence of competing wild-type cells. These data clearly demonstrate a strong common basis for the T cell deficiencies in mice and humans lacking γc/Jak3 signaling pathways. The Journal of Immunology, 2000, 165: 3680–3688.

T cell development in the thymus is dependent on several independent signaling pathways. Of these, cytokine receptor signals are among the least well understood. Studies of gene-targeted mice have provided much of the evidence implicating specific cytokines in αβ T cell, γδ T cell, or NK cell development. For instance, mice lacking IL-7 or components of the IL-7R signaling pathway (i.e., IL-7Rα, common γ-chain (γc), or Jak3) fail to develop γδ T cells (1–7). IL-15R signaling has been shown to be critical for NK cell development, as IL-15Rα−/−, γc−/−, and Jak3-deficient mice all lack NK cells (4–10). In contrast, no single cytokine has been found to be essential for αβ T cell development in the mouse. Instead, mice lacking IL-7R signaling components have a small thymus, containing ~5% of the normal number of cells, but eventually develop a sizeable compartment of peripheral T cells (4–8, 10–12).

The role of IL-7R signaling in γδ T cell development has been established. Numerous studies now document a lack of TCR Vγ gene transcription, and hence rearrangement, in IL-7−/−, IL-7Rα−/−, γc−/−, or Jak3−/− mice (13–17). For the TCR γ gene, IL-7R signaling appears to be involved in regulating locus accessibility by inducing demethylation and histone acetylation of the gene (15, 16). Thus, at the molecular level, IL-7 plays a direct role in allowing the recombination machinery access to the TCR γ gene locus, thereby promoting the maturation of γδ T cells. However, no similar findings have been reported for the TCR α or β gene loci, indicating that IL-7R signaling plays an alternative role in αβ T cell development.

Some clues about the role of IL-7R signaling in αβ T cell development have been provided by studies of thymocyte survival in gene-targeted mice, and in mice reconstituted with a bcl-2 transgene (18–21). These studies demonstrated that IL-7−/−, IL-7Rα−/−, and γc−/− mice had reduced numbers of pre-T cells, and furthermore, that pre-T cell survival could be partially restored in IL-7Rα−/− and γc−/− mice by ectopic expression of the antiapoptotic gene, bcl-2. The role of this cytokine receptor signaling pathway in promoting T cell survival at the pre-T cell stage is also supported by studies of γc−/−/pre-Tα−/− double knockout mice. In these mice, thymocyte numbers are reduced ~4000-fold, and no mature T cells develop (22).

Although these studies have suggested an important role for a γc-dependent cytokine receptor signal, presumably that of IL-7R, in the transition from the pro-T to the pre-T cell stage in the thymus, the precise role of IL-7R signaling in αβ T cell development remains controversial. In part this is due to discrepancies in reports characterizing the profiles of early thymocyte subsets in adult IL-7−/−, IL-7Rα−/−, γc−/−, and Jak3−/− mice, all of which lack signaling through the IL-7R. For instance some studies observe a complete lack of pre-T cells among adult CD3−/− triple negative (TN) thymocytes (11), whereas others find an overrepresentation of both the earliest progenitor cell and the pre-T cell stages of maturation (20). In addition, expression of the bcl-2 gene in these knockout mice was not universally capable of restoring normal numbers of thymocytes, and in one system, only led to a 4-fold increase in total thymocyte cell numbers (21). Thus the precise role of IL-7R signaling and the stage of thymocyte development at which these signals are most important remain unresolved.

In addition to these concerns, T cell development in Jak3−/−, γc−/−, IL-7−/−, or IL-7Rα−/− mice does not resemble the phenotype observed in other mice with defects at the pre-T cell stage in the thymus. For instance, several mouse mutants with disruptions of genes involved in formation of the pre-TCR (e.g., TCRβ, pre-Tα, RAG-1, RAG-2) or in pre-TCR signaling (e.g., Lck, SLP-76, CD3ζ, Zap-70/Syk) have reduced thymocyte numbers, but also display a prominent block in T cell development at the CD3−/− stage (23–31). In these cases, mutant thymuses routinely contain a dramatically reduced proportion of CD4−/− thymocytes. In contrast, mice with deficiencies in γc/Jak3 signaling pathways have reduced thymocyte numbers, similar to those in mutants lacking pre-TCR signals, but have nearly normal proportions of the four major CD4/CD8 thymocyte subsets (4–8, 10). In particular, these mutant thymuses contain normal percentages of CD4+ cells. Thus, the underlying defect in T cell development in Jak3−/− or
γc− mutant mice is unlikely to be caused by abnormalities solely at the pre-T cell stage of maturation.

To address the specific T cell defect in the absence of γc/Jak3 signaling pathways, we have examined the first wave of T cells developing in the fetal thymus of Jak3−/−, IL-7−/−, and IL-7Rα−/− mice. Our results indicate that these three lines of mice share a common defect, which is a near absence of the earliest stage of thymic progenitor cells. Furthermore, this defect can completely account for the low thymocyte numbers observed in the adult knockout mice. In addition, our data indicate that this defect does not result solely from a failure of progenitor cells to home to the thymus. Finally, we observe that Jak3−/− progenitor cells are incapable of differentiating into T cells when placed in competition with wild-type progenitor cells. This latter observation recalls the complete absence of T cells in human X-linked SCID or Jak3-SCID patients (32–35), and indicates that the underlying defect in humans and mice lacking the γc/Jak3 signaling pathway is much more similar than was previously anticipated.

Materials and Methods

Mice and timed pregnancies

Jak3−/−, IL-7−/−, and IL-7Rα−/− mice have been described previously (7, 11, 12) and were bred and maintained under specific pathogen-free conditions. IL-7Rα−/− mice were a kind gift of Rachel Gerstein (University of Massachusetts Medical School, Worcester, MA), and IL-7−/− mice were a kind gift of Richard Murray (DNAX, Palo Alto, CA). For adoptive transfer experiments, congenic B6-Ly5.2/Cr (CD45.1+) mice were purchased from the National Cancer Institute-Frederick Cancer Institute (Frederick, MD). Timed pregnancies in Jak3−/−, IL-7−/−, and IL-7Rα−/− lines were set up by mating homozygous knockout males with heterozygous females, so that each litter contained 50% heterozygous and 50% homozygous knockout embryos. The day of plugging was counted as embryonic day 0. Fetal embryos were typed by PCR analysis on fetal liver DNA using the following primer sequences: Jak3 sense, 5′-GTC AAC TTT GGC AGC GTG GAG CTG TGG TGC CGC-3′; and antisense, 5′-CTG GCC CAT AGC TGA CTC CCC GCT ACT GTA-3′; IL-7 sense, 5′-TGA TTC TCT TCT TCT AAT AGG AA-3′; and antisense, 5′-AGT GAG TAG TCC ACT CTC AC-3′; and IL-7R sense, 5′-GTT TTT ATA GCC TTA TGT CAA G-3′; and antisense, 5′-CTT TTA GCA GTG AAA TTA GCC TCA ACT C-3′; and Neo sense, 5′-ATT GAA CAA GAT GGA TTG CAC-3′; Neo antisense, 5′-CGT CCA GAT CAT CCT GAT C-3′.

Cell suspension preparation

Adult thymocyte suspensions were prepared by gently pressing the thymus between two frosted slides. Fetal thymocyte suspensions were prepared in Eppendorf tubes using a glass eppi-pesle. Bone marrow cells were prepared by flushing the marrow from femurs with cold medium (RPMI 1640) plus 5% FCS. Bone marrow cells were washed once in medium, counted, and then washed twice in cold PBS before injection.

Abs and flow cytometry

Each fetal thymus was stained individually. The following mAbs were used: anti-CD4-FITC, anti-CD8-PE, anti-CD44-FITC, anti-CD8-PE, anti-CD68-Cy-Chrome, anti-CD44-Cy-Chrome, anti-CD25-biotin, anti-CD25-PE, anti-Thy1.2-FITC, anti-Thy1.2-APC, anti-CD45.1-PE, and anti-CD45.2-FITC. Detection of biotinylated Abs was performed with streptavidin-FITC, streptavidin-Cy-Chrome, or streptavidin-APC. All Abs were purchased from PharMingen (San Diego, CA). Cells were analyzed on a FACScan/Caliber flow cytometer, and data were analyzed with CellQuest software (Becton Dickinson, Mountain View, CA).

Analysis of adult CD3−4−8− (TN) thymocytes

Adult thymus (1 × 10^7 total cells) were incubated with a panel of biotinylated Abs: anti-CD3-biotin, anti-CD4-biotin, anti-Gr-1-biotin, anti-IgM-biotin, anti-Thy1.1-biotin, and anti-Mac-1-biotin for 30 min at 4°C. Cells were washed and then incubated with anti-CD8-FITC, anti-B220-FITC, anti-CD44-Cy-Chrome, anti-CD25-PE, and streptavidin-FITC for 30 min at 4°C. Adult TN cells were gated on the FITC-negative population, and 10,000 events were collected. Cells and data were analyzed as above.

Apopotosis analysis

Apopotosis analysis was performed according to the published protocol (36). Briefly, 7-aminocinnamycin D (7AAD; Sigma, St. Louis, MO) was dissolved in acetone and diluted to 200 μg/ml in PBS. Fetal thymocytes from individual thymus were stained with anti-CD44-biotin for 30 min at 4°C. The cells were washed and then incubated with anti-Thy1.2-APC, anti-CD25-PE, and streptavidin-FITC for an additional 30 min at 4°C. The cells were washed twice and then incubated with 7AAD (20 μl for fetal day 14 and 15 thymocytes, 50 μl for fetal day 16–18 thymocytes) for 20 min at 4°C in the dark. Cells were pelleted, the supernatant was removed, and the cells were resuspended in 100 μl PBS with 5% FCS and 0.02% sodium azide and analyzed immediately by flow cytometry.

Intrathymic injections

Four-week-old B6-Ly5.2/Cr (CD45.1+) mice were irradiated with 750 rad. Approximately 6 h later, they were injected i.v. with 0.5 × 10^7 syngeneic bone marrow cells. The recipients were then anesthetized with ketamine hydrochloride (1.20 mg/100 g body weight). The sternum was exposed following a mid-line incision in the upper thoracic region. A small longitudinal incision in the upper third of the sternum exposed the tops of both thymic lobes. Each lobe was injected twice with 10 μl of bone marrow cells (4 × 10^7 total cells) using a 1-ml syringe equipped with a 27-gauge needle attached to a Tridak Stepper (Indicon, Brookfield Center, CT). The wound was then closed with surgical staples, and the animals were maintained in specific pathogen-free conditions on antibiotic water. The recipients were sacrificed ~4 week postinjection, and their thymocytes were stained with the indicated Abs and analyzed by flow cytometry.

Results

Jak3-deficient animals have a profound deficit in thymic progenitor cells

Initial studies of T cell development in Jak3− and γc−/− deficient mice revealed that thymic cellularity was reduced by 10- to 100-fold, even though the pattern of CD4 and CD8 expression was nearly normal (4–8, 10). To determine the underlying cause of this thymocytopenia in Jak3−/− mice, we chose to analyze thymocytes from staged fetal embryos. The advantage of this approach is that the first wave of developing T cells can be followed, allowing a more direct assessment of any developmental blocks or delays. Furthermore, this system allows one to examine T cell maturation before the establishment of steady-state conditions, as is found in the adult thymus.

To accomplish this end, Jak3−/− and Jak3−/− mice were mated, and embryos were harvested on fetal days 14–18 of gestation. From these matings, each litter contained 50% Jak3−/− and 50% Jak3−/− embryos, providing internal controls for the analysis. Our initial efforts to analyze the earliest stages of fetal thymocyte development, I.e., gestation days 14 and 15, immediately indicated that thymocytes from the Jak3−/− embryos were virtually undetectable. Not only were total cell yields from these samples extremely low, but in addition, few of the recovered cells were lymphocytes. To overcome this difficulty, stained thymocytes from three to five individual Jak3−/− fetuses were pooled before analysis. This allowed us to examine the phenotype of thymocytes recovered from the Jak3−/− embryos.

Two interesting findings emerged from these studies. First, we discovered that fetal day 14 Jak3−/− thymuses have only ~200 Thy1+ cells per thymus, in contrast to the ~20,000 present in Jak3−/− littermates (Fig. 1A). This finding indicates that even as early as fetal day 14, when all of the lymphoid cells in the thymus are thymic progenitor cells or pro-T cells (see below), Jak3−/− mice have a severe deficit in thymic lymphoid cell numbers. Second, by following the kinetics of thymocyte expansion from fetal days 14–18, we found that rates of thymocyte expansion were identical between Jak3−/− and Jak3−/− littermates (Fig. 1A). This observation suggests that, once present, Jak3−/− thymic progenitor cells have the same capacity to differentiate into mature T cells as...
control (Jak3+/−) progenitor cells, and furthermore, that any subsequent defects in T cell development are not likely to be responsible for the profound reduction in thymocyte cell numbers seen in adult Jak3−/− mice.

To determine whether the reduction in thymocyte cell numbers observed in fetal day 14 Jak3−/− embryos was due to a loss of IL-7R signaling, we examined fetal day 14 thymocytes from IL-7−/− and IL-7RA−/− mice. As shown in Fig. 1B, fetal thymocytes from both of these knockout lines showed the identical deficit in lymphoid cell numbers as those from Jak3−/− mice. These data are consistent with those previously reported by Crompton and colleagues (37), and indicate that a lack of IL-7R signaling is responsible for the 100-fold reduction in thymic progenitor cell numbers observed in fetal day 14 Jak3−/− embryos.

To assess the phenotype of cells present in the thymus of fetal day 14 Jak3−/−, IL-7−/−, and IL-7RA−/− embryos, thymocytes were stained with Abs to Thy1, CD44, and CD25. CD44 and CD25 are particularly useful for characterizing the early stages of T cell maturation, as these Abs divide CD3−4−8− thymocytes into four sequential subsets, thymic progenitor cells (CD44+/2+), pre-T cells (CD44+/2−), pre-T cells (CD44+/2+), and late pre-T cells (CD44−/2−) (38). Thymic progenitor cells have been shown to be multipotent, capable of giving rise to T, B, and NK cells (39), whereas pre-T cells are committed to the T cell lineage (40, 41). Pre-T cells are undergoing TCRβ-chain rearrangement and expression, which leads to pre-TCR selection, thymocyte proliferation, and differentiation into late pre-T cells (For review, see Refs. 42 and 43).

To characterize the thymocyte subsets present in the fetal day 14 embryos, thymocytes from individual fetuses were isolated, stained, and analyzed by flow cytometry. As described above, samples from multiple mutant embryos were pooled before analysis to provide a visible number of cells. Fig. 2A shows that Jak3−/−, IL-7−/−, and IL-7RA−/− thymuses have an extremely low percentage of Thy1+ cells compared with their heterozygous littermates. However, interestingly, CD25 vs CD44 analysis of these Thy1+ cells indicates normal proportions of thymic progenitor (CD44+CD25−) and pre-T (CD44−CD25+) cells in each of the mutant thymocyte samples (Fig. 2B). Thus, although overall cell numbers are extremely low (~100-fold reduced) in Jak3−/−, IL-7−/−, and IL-7RA−/− fetal day 14 thymuses, the differentiation of thymic progenitor cells into pro-T cells appears to proceed at the normal rate and with the normal frequency.

Kinetic analysis of Jak3−/− fetal thymocytes indicates a developmental lag at the pre-T cell stage

As previous studies of T cell development in adult γc−, IL-7−/−, IL-7RA−/−, and Jak3−/− mice indicated a defect in the transition from the pro-T to the pre-T cell stage of maturation (4, 10–12, 44), we were interested in determining whether this defect was also visible during fetal thymic maturation. In addition, by examining thymocytes from sequential days of fetal development, we can also assess any changes in the kinetics of T cell maturation at these early stages. For this analysis, thymocytes were isolated from Jak3−/− or Jak3+/− embryos at days 15–18 of gestation, and were stained with Abs to Thy1, CD44, and CD25, or Thy1, CD4, and CD8. As shown in Fig. 3A, nearly half of the thymocytes (~50%) from fetal day 15 control (Jak3+/−) embryos have progressed to the pre-T cell stage (CD25+CD44−) and beyond (CD44−CD25−). By fetal day 16, only a small fraction of thymic progenitor and pro-T cells remain, and this number decreases progressively over the next 2 days of fetal gestation. In Jak3−/− fetal thymuses, the kinetics of T cell maturation are delayed by ~1 day. For instance, the CD44/CD25 profile of Jak3−/− fetal day 16 thymocytes shows a substantial number of CD44−CD25+ cells, and thus more closely resembles the pattern seen at fetal day 15 in control embryos. This modest delay in the kinetics of thymocyte maturation continues for the next 2 days of gestation.

The delayed kinetics of maturation of Jak3−/− fetal thymocytes can also be seen by examining CD4 and CD8 expression. As shown in Fig. 3B, ~20% of control (Jak3+/−) thymocytes are CD4+8+ by fetal day 16 and the vast majority are CD4−8+ by fetal day 17. In contrast, the proportion of CD4+8+ thymocytes in Jak3−/− embryos at fetal day 17 is similar to that seen in fetal day 16 control thymuses. Interestingly, T cell maturation in the Jak3−/− embryos has caught up with the control embryos by fetal day 18, at which time point ~80% of thymocytes in both samples are CD4+8+ cells. This observation is consistent with the fact that the CD4/CD8 profile of adult thymocytes is very similar between Jak3+/− and Jak3−/− animals (see below).

Although analyses of fetal thymocytes provide a useful view of the kinetics of T cell development, it is also informative to examine the steady-state situation. Therefore, we characterized the early Jak3−/− thymocytes by staining adult thymuses (40, 41), fetal day 14 thymuses (42, 43), and then quantified by flow cytometry. As shown in Fig. 4, ~20% of adult thymocytes are CD4+8+ by fetal day 16 and the vast majority are CD4−8+ by fetal day 17. In contrast, the proportion of CD4+8+ thymocytes in Jak3−/− embryos at fetal day 17 is similar to that seen in fetal day 16 control thymuses. Interestingly, T cell maturation in the Jak3−/− embryos has caught up with the control embryos by fetal day 18, at which time point ~80% of thymocytes in both samples are CD4+8+ cells. This observation is consistent with the fact that the CD4/CD8 profile of adult thymocytes is very similar between Jak3+/− and Jak3−/− animals (see below).
stages of T cell maturation in adult Jak3<sup>−/−</sup> and Jak3<sup>+/−</sup> mice. As previously described, staining with Abs to CD4 and CD8 indicates that the overall pattern of the four major thymocyte subsets is extremely similar between Jak3<sup>−/−</sup> and control animals, despite the severe reduction in Jak3<sup>−/−</sup> thymocyte cell numbers (Fig. 4A; Refs. 5–8). However, examination of TN cells with Abs to CD44 and CD25 demonstrates several striking and interesting differences in early thymocyte stages between Jak3<sup>−/−</sup> and Jak3<sup>+/−</sup> mice. For instance, Jak3<sup>−/−</sup> mice have an increased proportion of pro-T cells (CD44<sup>−</sup>CD25<sup>−</sup>), suggesting a defect in the transition from the pro-T to the pre-T cell stage (Fig. 4B). This observation is consistent with previous reports of adult TN thymocytes from <i>g<sub>c</sub></i>, IL-7<sup>−/−</sup>, and IL-7Rα<sup>−/−</sup> mice (4, 10–12, 44). In addition, Jak3<sup>−/−</sup> thymocytes are depleted for the cells in transition between the pre-T and late pre-T cell stages (i.e., CD44<sup>+</sup>CD25<sup>int</sup> cells), and furthermore, exhibit an increased level of CD25 expression on both pro-T and pre-T cells. The increased CD25 expression has, in other systems, been attributed to the lack of proliferation of pre-T cells, as this proliferation is believed to result in the loss of CD25 surface expression by dilution (25, 26, 30). This observation is consistent with a loss of Jak3<sup>−/−</sup> thymocytes after successful TCRβ-chain rearrangement.

This analysis of TN thymocytes from adult Jak3<sup>−/−</sup> mice supports the findings from previous studies indicating that IL-7R signaling plays an important role in the maturation or survival of pre-T cells (18–21, 44). Nonetheless, given the extremely small size of the Jak3<sup>−/−</sup> thymus, these shifts in the proportions of early thymocyte stages are quantitatively minor compared with the absolute reduction in cell numbers (e.g., a 3-fold increase in the percentage of early pre-T cells (CD44<sup>+</sup>CD25<sup>+</sup>) among Jak3<sup>−/−</sup>-TN thymocytes vs a 20-fold reduction in absolute cell numbers). Thus, even the analysis of adult TN thymocytes highlights the profound reduction in thymic progenitor cells that is so clearly visible upon analysis of fetal Jak3<sup>−/−</sup> mice.

**Increased apoptosis of Jak3<sup>−/−</sup> fetal thymocytes**

One possible explanation for the reduced cell numbers in both Jak3<sup>−/−</sup> fetal and adult thymuses is that thymic progenitor cells may be more prone to apoptosis in the absence of IL-7R signaling. This possibility is consistent with studies indicating that IL-7 can promote the survival of thymic progenitor cells, as well as pro- and pre-T cells, during in vitro culture (45). This study and others (18–21) suggested that IL-7R induces expression of antiapoptotic genes such as <i>bcl-2</i> or <i>bcl-2</i> family members, thus promoting cell survival. To test this possibility, we examined the fraction of apoptotic cells among fetal thymocytes from Jak3<sup>+/−</sup> and Jak3<sup>−/−</sup> mice at days 14–18 of gestation. For these experiments, fetal thymocytes were isolated, stained with Abs, and then incubated with 7-AAD. Fig. 5 shows that the percentage of Thy1<sup>+</sup> cells that are 7-AAD<sup>+</sup> is elevated in Jak3<sup>−/−</sup> fetal thymi at each day analyzed,
with the exception of fetal day 18. Interestingly, though, the increased proportion of apoptotic cells among Jak3

\(2/2\) thymocytes (\(\sim 2\)-fold) is roughly constant from fetal days 14 through 17. These data indicate that the absence of \(g_c/Jak3\) signaling pathways leads to decreased survival of fetal thymocytes throughout their maturation. However, as thymic progenitor cells (CD44

\(1/CD25^2\)) are not a rapidly proliferating subset of TN thymocytes (46), this nearly 2-fold decrease in cell survival is unlikely to account for the 100-fold decrease in thymic progenitor (CD44

\(1/CD25^2\)) cells found in fetal Jak3

\(2/2\), IL-7

\(2/2\), and IL-7R

\(a^2/2\) mice.

Intrathymic injection of Jak3

\(2/2\) bone marrow cells does not restore normal T cell development

In addition to increased apoptosis, two other possible explanations might account for the reduced numbers of thymic progenitor cells observed in the absence of IL-7R signaling. One possibility is that thymic progenitor cells may be dependent on IL-7 for their low level of proliferation in the thymus. This seems unlikely, as in vitro culture experiments indicate that IL-7 promotes the survival, but not the proliferation, of thymic progenitor cells (45). Due to the extremely small numbers of thymocytes (\(\sim 200/\)thymus) present in fetal day 14 Jak3

\(2/2\) embryos, we were unable to perform routine cell cycle analysis or BrdU labeling experiments to examine this possibility. The low numbers of Jak3

\(2/2\) fetal thymocytes presented a particular technical problem for these specific experiments, as DNA analysis requires a step of cell fixation followed by permeabilization. These treatments, which are quite harsh to the cells, generally reduce cell yields substantially. In the case of the Jak3

\(2/2\) fetal day 14 thymocytes, no cells could be recovered for analysis following these treatments. Thus, we could not directly address the possibility that reduced thymic progenitor cell proliferation is responsible for the low thymocyte numbers in the absence of IL-7R signaling. However, due to the fact that thymic progenitor cells (CD44

\(^+/CD25^-\)) are not a rapidly dividing subset of TN thymocytes (46), and furthermore, cannot be induced to proliferate in vitro in response to IL-7 (45), it seems unlikely that reduced proliferation could account for the 100-fold decrease in progenitor cells found in Jak3

\(2/2\) mice.

Instead, we addressed a second possible explanation for the 100-fold decrease in thymic progenitor cells seen in Jak3

\(2/2\) mice, that Jak3-deficient bone marrow progenitor cells have a defect in homing to the thymus. This possibility is consistent with our observations of relatively normal distributions of all thymocyte subsets, but a profound reduction in total thymocyte numbers. To address this possibility, wild-type or Jak3

\(2/2\) bone marrow cells were directly injected into the thymus of irradiated wild-type C57BL/6 (CD45 congenic) recipients, thus bypassing the need for progenitor cells to home to the thymus. These experiments were performed in three groups. One group of recipient mice received only Jak3

\(2/2\) bone marrow cells, a second group received only Jak3

\(2/2\) bone

\(\text{FIGURE 3.} \) Jak3

\(2/2\) fetal thymocytes exhibit delayed kinetics of T cell maturation. On the indicated days of fetal development, thymocytes from fetal embryos were stained with Abs to Thy1.2, CD44, and CD25, or Thy1.2, CD4, and CD8, and analyzed by flow cytometry. Dot-plots of CD44 vs CD25 (A) and CD4 vs CD8 (B) staining for each day of fetal development are shown. All dot plots show gated Thy1.2

\(^+\) cells. Numbers indicate the percentage of cells in each quadrant. Each dot plot is representative of an individual embryo, except for fetal days 15–16, where Jak3

\(2/2\) thymocytes were pooled from two to four embryos before analysis. For a given day of development, the data are representative of experiments performed on three to five independent litters.
marrow cells, and a third group received a 1:1 mixture of Jak3<sup>+/+</sup> and Jak3<sup>−/−</sup> bone marrow cells by intrathymic injection. This latter experiment was performed to test the ability of Jak3<sup>−/−</sup> cells to compete with wild-type progenitor cells for reconstituting T cell development in the thymus. All recipient mice also received 0.5 × 10<sup>6</sup> syngeneic (CD45.1<sup>+</sup>) bone marrow cells i.v. to aid in reconstituting myeloid and erythroid lineage cells after irradiation.

As shown in Table I, Jak3<sup>−/−</sup> bone marrow cells injected intrathymically are extremely poor at reconstituting T cell development. When 4 × 10<sup>5</sup> Jak3<sup>+/+</sup> or Jak3<sup>−/−</sup> bone marrow cells are injected intrathymically, the thymus is restored to normal size (~2 × 10<sup>6</sup> cells) after 4 wk, in contrast to the small size of the thymus (<10<sup>5</sup> cells) in mice that received no reconstituting cells. However, when the intrathymically injected bone marrow is Jak3<sup>−/−</sup>, virtually none of the reconstituting cells are donor derived, whereas the vast majority of cells are of donor origin after intrathymic injection of Jak3<sup>−/−</sup> bone marrow. In the former case, it is likely that the large thymus size is due to reconstitution of T cell development by the small number of host-type bone marrow cells injected i.v. in these mice.

The inability of Jak3<sup>−/−</sup> bone marrow progenitor cells to reconstitute T cell development in the thymus when placed in competition with wild-type bone marrow cells is also evident when Jak3<sup>−/−</sup> (CD45.2<sup>+</sup>) and wild-type (CD45.1<sup>+</sup>) bone marrow cells are mixed 1:1 before intrathymic injection. In this case, again virtually none of the thymocytes present after 4 wk are derived from the Jak3<sup>−/−</sup> donor cells. In contrast, when Jak3<sup>−/−</sup> bone marrow cells (CD45.2<sup>+</sup>) are mixed 1:1 with wild-type bone marrow (CD45.1<sup>+</sup>), approximately half as many of the resulting thymocytes are CD45.2<sup>+</sup> as when the Jak3<sup>−/−</sup> cells are injected alone into the thymus of the irradiated recipients. These experiments indicate that direct intrathymic injection of Jak3<sup>−/−</sup> bone marrow cells cannot overcome the defect in T cell development, arguing that failure to home to the thymus is not their primary underlying deficiency.

**Jak3<sup>−/−</sup> thymic progenitor cells fail to differentiate in the presence of competing wild-type cells**

Most interestingly, phenotypic analysis of the donor-derived thymocytes following intrathymic injection of Jak3<sup>−/−</sup> or Jak3<sup>−/−</sup> bone marrow cells indicates that the development of Jak3<sup>−/−</sup> progenitor cells is completely blocked in the presence of wild-type competitors. As shown in Fig. 6A, Jak3<sup>−/−</sup> bone marrow cells, injected intrathymically, reconstitute all stages of T cell maturation in normal proportions and numbers. This is true both for overall thymocyte CD4/CD8 profiles, as well as for the earliest thymocyte stages. In striking contrast, the few donor-derived thymocytes present after intrathymic injection of Jak3<sup>−/−</sup> bone marrow are predominantly CD4<sup>−</sup> 8<sup>−</sup> cells, and furthermore, are all arrested at the CD4<sup>−</sup>CD25<sup>+</sup> progenitor cell stage of maturation. Thus, in the presence of one-eighth the number of wild-type bone marrow cells injected i.v., the Jak3<sup>−/−</sup> bone marrow cells, even when placed directly into the thymus, fail to differentiate past the thymic progenitor cell stage. Strikingly, this phenotype is likely to be identical with that of human SCID patients with deficiencies in γc/Jak3 signaling pathways, as these individuals completely lack thymocytes (35, 47, 48).

**Discussion**

In this report, we demonstrate that Jak3<sup>−/−</sup>, IL-7<sup>−/−</sup>, and IL-7Rα<sup>−/−</sup> mice have a severe deficit in thymic progenitor cell numbers, starting as early as fetal day 14. We suggest that this defect is the major underlying cause of the low thymocyte numbers in adult mice lacking γc/Jak3 signaling pathways. The initial characterization of Jak3<sup>−/−</sup> mice by Park et al. revealed a dramatic
bone marrow cells. All animals receiving intrathymic injections also received 0.5
3
by demonstrating that the small number of progenitor cells present
10 6 CD45.1
3
underlying cause of the T cell development defect observed in
distence that the low numbers of progenitor cells are the major un-
etics as control cells, and differentiate into mature T cells with
reduction in fetal thymocyte numbers in IL-7R
(5). An earlier study by Crompton and colleagues also indicated a
involved in generating normal numbers of thymic progenitor cells
lyzed by flow cytometry to determine the percentage of CD45.2-positive (donor-
from recipients were counted and stained with Ab to CD45.1 and CD45.2 and ana-
Table I. THYMIC PROGENITOR CELL DEFECT IN JAK3-DEFICIENT MICE

<table>
<thead>
<tr>
<th>Source of Donor Bone Marrow Cells</th>
<th>Total No. of Thymocytes Recovered</th>
<th>Percent CD45.2-Positive Thymocytes</th>
</tr>
</thead>
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<tr>
<td>No Cells</td>
<td>9.5 x 10^6</td>
<td>NA\textsuperscript{a}</td>
</tr>
<tr>
<td>Jak3\textsuperscript{−/−} (CD45.2\textsuperscript{−/−}) 4 x 10^6 cells</td>
<td>7.2 x 10^7</td>
<td>49</td>
</tr>
<tr>
<td>Jak3\textsuperscript{−/−} (CD45.2\textsuperscript{−/−}) 4 x 10^6 cells</td>
<td>1.4 x 10^8</td>
<td>0.1</td>
</tr>
<tr>
<td>Jak3\textsuperscript{−/−} (CD45.2\textsuperscript{−/−}) 2 x 10^6 cells +</td>
<td>3.6 x 10^8</td>
<td>33</td>
</tr>
<tr>
<td>Jak3\textsuperscript{−/−} (CD45.1\textsuperscript{−/−}) 2 x 10^6 cells</td>
<td>1.8 x 10^8</td>
<td>9</td>
</tr>
<tr>
<td>Jak3\textsuperscript{−/−} (CD45.2\textsuperscript{−/−}) 2 x 10^6 cells</td>
<td>1.3 x 10^8</td>
<td>0.2</td>
</tr>
<tr>
<td>Jak3\textsuperscript{−/−} (CD45.1\textsuperscript{−/−}) 2 x 10^6 cells</td>
<td>1.6 x 10^8</td>
<td>0.1</td>
</tr>
<tr>
<td>Jak3\textsuperscript{−/−} (CD45.2\textsuperscript{−/−}) 2 x 10^6 cells</td>
<td>1.7 x 10^8</td>
<td>0.1</td>
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<tr>
<td>Jak3\textsuperscript{−/−} (CD45.1\textsuperscript{−/−}) 2 x 10^6 cells</td>
<td>2.3 x 10^8</td>
<td>0.1</td>
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<tr>
<td>Jak3\textsuperscript{−/−} (CD45.2\textsuperscript{−/−}) 2 x 10^6 cells</td>
<td>1.7 x 10^8</td>
<td>&lt;0.1</td>
</tr>
<tr>
<td>Jak3\textsuperscript{−/−} (CD45.1\textsuperscript{−/−}) 2 x 10^6 cells</td>
<td>1.8 x 10^8</td>
<td>&lt;0.1</td>
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</table>

\textsuperscript{a} Congenic B6-LY5.2 mice (positive for CD45.1) recipients were irradiated and injected within 6 h with 4 x 10^6 Jak3\textsuperscript{−/−} or Jak3\textsuperscript{−/−} (CD45.2\textsuperscript{−/−}) bone marrow cells from the indicated donor intrathymically. A third group of animals received a 1:1 mixture of 2 x 10^6 Jak3\textsuperscript{−/−} or Jak3\textsuperscript{−/−} (CD45.2\textsuperscript{−/−}) plus 2 x 10^6 Jak3\textsuperscript{−/−} (CD45.1\textsuperscript{−/−}) bone marrow cells. All animals receiving intrathymic injections also received 0.5 x 10^6 CD45.1\textsuperscript{−/−} (host origin) bone marrow cells \textit{i.e.} four weeks postransfer thymocytes from recipients were counted and stained with Ab to CD45.1 and CD45.2 and analyzed by flow cytometry to determine the percentage of CD45.2-positive (donor-origin) thymocytes.

\textsuperscript{a} NA, Not applicable.

reduction in Lin\textsuperscript{−} ckit\textsuperscript{−} CD4\textsuperscript{+} CD8\textsuperscript{−} cells in the adult thymus, providing the first indication that yc/Jak3 signaling pathways might be involved in generating normal numbers of thymic progenitor cells (5). An earlier study by Crompton and colleagues also indicated a reduction in fetal thymocyte numbers in IL-7R\textsuperscript{−/−} mice (37). Our analysis of fetal thymocyte development extends this finding by demonstrating that the small number of progenitor cells present in the Jak3\textsuperscript{−/−} thymus are able to expand with the identical kinetics as control cells, and differentiate into mature T cells with only a slight delay in timing. These data provide compelling evidence that the low numbers of progenitor cells are the major underlying cause of the T cell development defect observed in Jak3\textsuperscript{−/−} mice.

The precise reason for the low numbers of thymic progenitor cells in Jak3\textsuperscript{−/−}, IL-7\textsuperscript{−/−}, and IL-7R\textsuperscript{−/−} mice is currently unresolved, although we have addressed several possible explanations. One possibility was that, in the absence of IL-7R signaling, thymic progenitor cells fail to proliferate and/or are more prone to apoptosis. Although we could not directly test the proliferative status of thymic progenitor cells in these mutant mice due to their low cell numbers, it is unlikely that reduced proliferation accounts for the \~100-fold decrease in progenitor cell numbers. This is because CD44\textsuperscript{+} CD25\textsuperscript{−} thymic progenitor cells, under normal circumstances, are not expanding (46). Instead, proliferation at the latter stages of pro- and pre-T cells is responsible for the large increase in early thymocyte numbers, an expansion that occurs identically in Jak3\textsuperscript{−/−} and Jak3\textsuperscript{−/−} fetal thymuses. We did observe a 2-fold increase in the proportion of apoptotic cells in Jak3\textsuperscript{−/−} fetal thymuses at each day of fetal gestation analyzed (days 14–18). However, this uniform increase in apoptosis across all the stages of early T cell development is also unlikely to account for the 100-fold decrease in thymic progenitor (CD44\textsuperscript{+} CD25\textsuperscript{−}) cells, again, because the progenitor cells are a nondividing population. Finally, we addressed the possibility that progenitor cells developing elsewhere fail to home to the thymus. Direct intrathymic injection of Jak3\textsuperscript{−/−} bone marrow cells, thereby circumventing the need for homing to the thymus, did not restore normal T cell development. Instead, Jak3\textsuperscript{−/−} bone marrow cells displayed an extremely poor ability to reconstitute the thymus in these irradiated recipients. Although these data do not completely rule out a possible defect in homing, they indicate the existence of profound developmental defects that are independent of thymic homing.

On the basis of these data, we propose that Jak3\textsuperscript{−/−} mice have a defect in generating lymphoid progenitor cells in the fetal liver or bone marrow, before their migration to the thymus. This explanation would account for all of our observations, and is consistent with reports of IL-7R expression on the common lymphoid progenitor cell in the bone marrow (49). In addition, this explanation suggests the interesting possibility that the defects in T cell and B cell development in Jak3\textsuperscript{−/−}, yc\textsuperscript{−/−}, IL-7\textsuperscript{−/−}, and IL-7R\textsuperscript{−/−} mice arise from a single common root, although this possibility is inconsistent with reports of normal numbers of pro-B cells in IL-7R\textsuperscript{−/−} mice (50). Alternatively, a bone marrow progenitor cell committed to the T cell lineage may be more dependent on IL-7R signaling than its B cell counterpart, and other growth factor signals may play a more critical role for the B cell progenitor.

In addition to the putative major role of IL-7 in generating lymphoid progenitor cells, IL-7 also plays an important role in promoting the survival of early thymocytes at all stages of maturation.

\textbf{FIGURE 6}. Jak3\textsuperscript{−/−} thymic progenitor cells fail to differentiate in the presence of competing wild-type cells. Congenic B6-LY5.2 mice (positive for CD45.1) were irradiated and injected intrathymically with 4 x 10^6 Jak3\textsuperscript{−/−} or Jak3\textsuperscript{−/−} (CD45.2\textsuperscript{−/−}) bone marrow cells. Four-week postransfer thymocytes from recipient mice were stained with Abs to CD45.1, CD45.2, CD4, CD8, CD44, and CD25 and analyzed by flow cytometry. The dot plots were gated on CD45.2\textsuperscript{+} cells and are representative of the animals analyzed in Table I. The CD44/CD25 plots were also gated on TN cells as described in Materials and Methods. The percentage of cells in each quadrant is indicated.
For instance, there is substantial evidence in the literature to support the requirement for γc/Jak3 signaling pathways, in particular IL-7R signaling, at the pro-T cell (CD44+CD25+) stage of development to promote cell survival (3, 45, 51). In particular, IL-7R signaling has been shown to induce bcl-2 expression and promote entry into the cell cycle at this stage (20). Finally, constitutive expression of bcl-2 in IL-7Rγc−/− and γc− mice could partially restore thymocyte numbers, indicating that one of the principal functions of IL-7R signaling is to promote cell survival (18, 19, 21).

The generation of double knockout mice has also provided interesting clues to the role(s) of γc/Jak3 signaling pathways in early T cell development. For instance, mice lacking both γc and c-kit are completely devoid of thymocytes, whereas c-kit-deficient mice alone have only a 5-fold reduction in thymocyte cell numbers (52, 53). As IL-7R and c-kit are coexpressed on thymic progenitor cells and pro-T cells (54, 55), these data suggest that the two receptors can partially compensate for each other during early T cell development. These data provide an interesting contrast to a more recent study that examined T cell development in mice lacking both γc and the pre-TCRα-chain. These double knockout mice also had a compounded defect in thymocyte numbers and differentiation, including a more severe block at the TN stage of maturation than that seen in either of the single knockout mice (22). However, this phenotype is likely to be the sum of the individual defects. The absence of γc causes a decrease in thymic progenitor cells, which leads to a large reduction in the numbers of cells going through the subsequent stages of T cell maturation. The loss of the pre-TCR signal then even further reduces the numbers of cells progressing through the TN compartment. Coupled with an additional decrease in the survival of pre-T cells due to lack of IL-7R signaling, virtually no cells in these double knockout mice make it to the CD4+8+ stage. Nonetheless, γc/pre-Trα double knockout mice do have detectable thymocytes that progress through to the pro-T cell (CD44+CD25+) stage, in contrast to γc/c-kit double knockout mice, which are totally devoid of thymocytes (22, 52). Together, these studies highlight the important role of γc/Jak3 signaling pathways at the earliest stages of T cell maturation.

Perhaps the most striking finding in this report is the Jak3−/− progenitor cells to reconstitute T cell development in the presence of competing wild-type cells. Specifically, intrathymic injection of Jak3−/− bone marrow cells together with wild-type bone marrow cells into irradiated recipients revealed that the Jak3−/− cells were completely blocked at the thymic progenitor stage of development (CD44+CD25+). The most likely explanation for this result is that the small numbers of progenitor cells in the Jak3−/− bone marrow, or developing from the Jak3−/− bone marrow, were out-competed for the limiting numbers of progenitor cell niches in the thymus by the wild-type cells. An additional factor may also be the reduced survival potential of Jak3−/− pro-T and pre-T cells, a problem that may be further compounded by competition with wild-type cells for limiting niches in the subcapsular region of the thymus. The interesting implication of this observation is that it forces a reconsideration of the dramatically different phenotypes observed in mice vs humans lacking γc/Jak3 signaling pathways. Human X-linked SCID (γc−) and Jak3-SCID patients are completely devoid of T cells and have undetectable numbers of thymocytes (35, 47, 48). In contrast, mice with these deficiencies have normal numbers of peripheral T cells, a nearly normal distribution of thymocyte subsets, and a ~20-fold reduction in thymocyte cell numbers. At face value, these disparate observations suggest that γc/Jak3 signaling pathways have a very different role in mouse vs human T cell development. However, the fact that Jak3−/− thymic progenitor cells are completely blocked in their maturation and incapable of giving rise to any mature T cells in the presence of competing wild-type cells strongly suggests that these mutant progenitor cells are intrinsically defective and poised on the brink of being totally nonfunctional. The difference between the two species may be as simple as a slightly higher level, or longer time of expression, of the c-kit receptor on murine progenitor cells, thus providing a meager compensation for the lack of IL-7R signaling. Thus, the profound deficiency in T cell development in human X-linked SCID or Jak3-SCID compared with the more moderate phenotype in the comparable mouse mutants may be due to a scant difference in ability of the earliest thymic progenitor cells to survive and differentiate.

Acknowledgments

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References


