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Requirement for Jak3 in Mature T Cells: Its Role in Regulation of T Cell Homeostasis

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The tyrosine kinase Jak3 plays a key role in transducing signals from the IL-2, -4, -7, -9, and -15 receptors. Mice lacking Jak3 exhibit a profound, early block in both B and T cell development. To examine the mechanisms whereby Jak3 influences T cell function, we have reconstituted thymic development in Jak3−/− animals by introducing a Jak3 transgene in which expression was driven by the lck proximal promoter. Thymic reconstitution required Jak3 kinase activity, as catalytically inactive Jak3 did not restore early thymic development. Furthermore, the thymus-restricted expression pattern of the transgene allowed us to assess the requirement for Jak3 in peripheral T cells. In these mice, loss of Jak3 expression was associated with a failure to proliferate in response to antigen receptor cross-linking, the accumulation of T cells manifesting an activated cell surface phenotype, and an increased CD4/CD8 ratio among peripheral T cells, all of which are characteristics that were observed in Jak3−/− animals. Finally, we present data which suggest that peripheral T cells proliferate more rapidly in vivo and also undergo apoptosis more rapidly, upon loss of Jak3. Hence Jak3 exerts effects on mature peripheral T lymphocytes, as well as on thymocytes, resulting in the proper maintenance of circulating, quiescent cells.


The Janus family of protein tyrosine kinases (JAKs), 3 together with the signal transducers and activators of transcription (STATs), mediate signals from cytokine and growth hormone receptors. Among the four known members of the JAK family (Jak1, Jak2, Jak3, and Tyk2), Jak3 is unique in that its expression is limited to hemopoietic cells. T and B lymphocytes as well as myeloid cells express Jak3, and detailed studies suggest that this pattern of expression is developmentally regulated (1, 2). In mice, Jak3 is expressed at its highest level in the thymus and at lower levels in the bone marrow and spleen (1).

Jak3 is associated with the common γ-chain (γc), a signaling component shared by the IL-2,-4,-7,-9, and -15 receptors (3–5). In each case, receptor-ligand interaction, or crosslinking with anti-cytokine receptor Abs, induces phosphorylation of Jak3 on tyrosine residues (3, 4, 6–9) and activates its catalytic activity (6, 8). Recent reports have further confirmed that Jak3 plays an indispensable role in relaying the signals generated by these cytokines, some of which are absolutely necessary for the normal development and function of lymphocytes. The human immunodeficiencies XSCID (X-linked severe combined immunodeficiency) and XCID (X-linked combined immunodeficiency), both marked by a dramatic reduction in the number of circulating functional lymphocytes, are associated with mutations in ζc that diminish its interaction with Jak3 (4, 10, 11). Mutations that result in reduced Jak3 expression have also been associated with the SCID syndrome (12, 13). In mice, targeted disruption of the gene encoding ζc results in a severe deficiency of B cells and a hypoplastic thymus. Moreover, peripheral lymphocytes in these animals do not respond to cytokines that stimulate ζc, and these cells bear high levels of surface markers that normally appear following Ag receptor-induced activation (14, 15). These features are duplicated in mice that lack Jak3, strongly suggesting that Jak3 directly mediates signals from ζc (16–18).

Targeted disruption of the genes encoding unique chains of cytokine receptors, such as the IL-2 receptor α- (19) and β-chains (20) or the IL-7 receptor (21), has demonstrated that these cytokine receptors play distinct roles during lymphocyte development. Disruption of the unique IL-7R chain results in a profound reduction in the number of immature and mature T and B cells, the effect of an early block in lymphocyte development (21). In contrast, IL-2R α- or β-chain deficiency results in the polyclonal expansion of lymphocytes that are nonresponsive to IL-2, with increased surface expression of activation markers such as CD69 or CD25 (19, 20). Jak3-deficient mice exhibit characteristics similar to both the early, IL-7 receptor deficiency and the late, IL-2 receptor deficiency. However, since an early developmental block may alter the functional capacity of mature lymphocytes, defining the role of Jak3 in the late stages of lymphocyte development has been difficult.

In a reconstitution system, Thomis and Berg (22) recently showed that catalytically active Jak3 is required for early cellular expansion in the thymus and that Jak3 is required for proliferation of mature T cells stimulated in vitro. We have used a similar approach to restore Jak3 expression selectively in immature thymocytes or in peripheral T cells of mice that lack endogenous Jak3. Analyses of T cell development and function in these reconstituted mice confirm the findings reported earlier by Thomis and Berg (22). More importantly, we demonstrate that restoration of Jak3 expression in mature T cells, driven by the lck distal promoter,
corrects peripheral T cell defects without increasing thymic cellularity significantly. Our results demonstrate that continuous expression of Jak3 is absolutely required for maintenance of T cell number and function. Moreover, we provide evidence supporting the view that Jak3, perhaps by regulating Fas expression, controls apoptotic responses in T cells.

Materials and Methods

Assembly of transgene constructs

Jak3 transgenes were constructed by juxtaposing a 3.7-kb NotI/RcaI fragment of the mouse Jak3 cDNA (6) with the lck proximal promoter in the p1017 vector (23) or the lck distal promoter vector pW120 (24). The cloned fragment includes the Jak3 coding sequences, the initiation and termination codons, and 60 bp of 5′ untranslated region and 360 bp of 3′ untranslated region (6). Transgenes encoding a catalytically inactive version of Jak3 were generated by introducing a Lys-to-Glu point mutation at codon 851 in the cDNA, thereby disrupting a pivotal residue for phosphate transfer. This mutant form of Jak3 failed to autoprophosphorylate in response to IL-2 treatment when expressed in COS cell transfectants (data not shown).

Mice

Transgenic animals were generated by injecting Jak3 constructs into (C57BL6 × DBA)F1 embryos as previously described (25). The resulting pups were screened for integrated transgene DNA by Southern blot analysis of tail DNA using a radiolabeled hGH gene fragment (24). Transgene-positive founders were backcrossed with C57BL/6 mice through multiple generations to establish transgenic lines.

For reconstitution experiments, two p1017 wild-type transgenic lines with differing levels of transgene expression (Tg<sub>low</sub> and Tg<sub>high</sub>), one pW120 wild-type Jak3 line, and one mutant Jak3 transgenic line (Tg<sub>KE</sub>) were crossed with Jak3<sup>−/−</sup> mice (17). Progeny from these crosses were analyzed by Southern blotting or PCR for genotype at the endogenous Jak3 locus and for inheritance of the hGH-containing transgene. Southern blot analysis was performed as described (17). The PCR screen was designed to amplify the HygR cassette and to distinguish untargeted genomic sequences from targeted sequences. Two independent sets of primers were used for this assay: Hyg<sup>5′</sup> primer, 5′-CGA CGT CTG TCG AGA AGT TTC TGA, and Hyg<sup>3′</sup> primer, 5′-CAG GCC ATG TAG TGT ATT GAC CGA, were derived from HygR gene sequences; 5′ Jak3 genomic primer derived from the coding sequences 5′ of the HygR insertion site, 5′-AGG AOC CTT GCCA TGT GCT CCT TCC, and 3′ Jak3 genomic primer, which extends across an exon-intron boundary 3′ of the HygR insertion site, 5′-AAC AGG CAG GAT GCC TGA AGG GAT. Transgene-positive Jak3 heterozygotes (Jak3<sup>+/−</sup>Tg<sup>−</sup>) were further crossed to yield progeny on a Jak3<sup>+/−</sup>/, or −/− background.

Analysis of Jak3 transgene expression

Total cellular lysates were prepared from thymocytes and splenocytes in lysis buffer (1% Triton X-100, 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM PMSF, 1 µg/ml aprotinin, 1 µg/ml leupeptin). 10<sup>6</sup> cell equivalent lysates were resolved using 7.5% SDS-PAGE, transferred onto nitrocellulose (Hybond-C, Amersham, Arlington Heights, IL), and blocked with 1% BSA (Tris-saline buffer, pH 7.5, 0.1% Tween-20). The membrane was incubated at room temperature in polyclonal rabbit antiserum generated against a Jak3 polypeptide (6) at a 1:2000 dilution, followed by horseradish peroxidase-conjugated donkey anti-rabbit Ig (at 1:3000) as the secondary detection reagent (Amersham). Horseradish peroxidase activity was visualized by chemiluminescence according to the manufacturer’s instructions (Dupont, Boston, MA).

Flow cytometry

Single-cell suspensions of thymocytes, splenocytes, and lymph node cells were prepared in RPMI 1640 containing 3% FCS. Cells were treated in hypotonic saline solution to lyse RBCs, then stained as described previously (25). The following conjugated mAbs and secondary reagents were purchased from Caltag, San Francisco, CA: phycoerythrin (PE)-conjugated anti-CD4 (CT-CD4), FITC-conjugated anti-CD8α (CT-CD8α), PE-conjugated streptavidin, and tricolor-conjugated streptavidin. Biotinylated anti-CD3 (2C11), biotinylated anti-CD69 (H1.2F3), and PE-conjugated anti-Fas (Jo2) were purchased from PharMingen, San Diego, CA. Biotinylated anti-human IgG Fab<sub>1</sub>, was purchased from Jackson Immunoresearch Laboratories, West Grove, PA. Murine Fas-Fc<sub>β</sub> reagent was a generous gift from the Imnunex Corporation (Seattle, WA) and was used as described (26). For apoptosis assays, cells were incubated in PBS containing 20 ng/ml of 7-amino actinomycin D (7AAD; Molecular Probes, Eugene, OR), washed, fixed in 1% paraformaldehyde, and analyzed as described (27). Data were collected in list mode files on a FACScan flow cytometer (Becton Dickinson, Mountain View, CA) using Lysis II software (Becton Dickinson) and analyzed using Reproman software (TrueFacts Software, Seattle, WA).

In vivo BrdU uptake and staining

Mice were injected i.p. with 1.6 mg of BrdU (Sigma, St. Louis, MO) in 200 µl of PBS twice over a 4-h interval. At 42 h after the second injection, spleens were harvested and single-cell suspensions were prepared. Cells were stained with biotinylated anti-CD4 and PE-conjugated anti-CD8 mAbs followed by streptavidin-tricolor as the secondary detection reagent. Cells were then fixed and stained with FITC-conjugated anti-BrdU reagent (Becton Dickinson) according to the protocol described by Tough and Sprent (28).

Results

Assembly of Jak3 transgene constructs and generation of transgenic mouse lines

The effects of Jak3 deficiency are apparent at early stages of T cell development. To restore Jak3 expression in developing thymocytes, we used the lck proximal promoter to drive the expression of transgenes encoding either wild-type or catalytically inactive Jak3. Transgene constructs were assembled by inserting wild-type or mutant forms of the Jak3 cDNA into the p1017 vector (Fig. 1A, 29). Transgenic founders were generated and backcrossed with C57BL6 mice through multiple generations to establish independent lines.

Two wild-type transgenic lines with differing levels of transgene expression (designated Tg<sub>low</sub> and Tg<sub>high</sub>) and one mutant transgene line (Tg<sub>KE</sub>) were bred with the Jak3<sup>−/−</sup> mice to obtain progeny bearing the Jak3 transgene in the Jak3 wild-type, heterozygous, or homozygous-null genetic background. We evaluated the levels of Jak3 expression by immunoblotting total cellular lysates with anti-Jak3 antisera. Figure 1B shows the relative levels of transgene expression in Jak3<sup>+/−</sup> or Jak3<sup>−/−</sup> mice. Translational products derived from the transgene locus increased the observed abundance of Jak3 in all three genotypic backgrounds, creating a wide range of Jak3 expression. As expected for transgenes driven by the thymus-specific lck proximal promoter, the levels of reconstituted Jak3 were highest in thymocytes and decreased dramatically in the periphery. This is most evident in Jak3<sup>−/−</sup> transgenic mice. While transgene-derived Jak3 protein is detectable in splenocytes of the Tg<sub>high</sub> line (lane 16), it is no longer detectable in splenocytes of the Tg<sub>low</sub> line or the Tg<sub>KE</sub> line (lanes 15 and 20). RNA analysis indicated that transcripts from the transgene locus were present at a low level even in splenocytes from Jak3<sup>−/−</sup>Tg<sub>low</sub> and Jak3<sup>−/−</sup>Tg<sub>KE</sub> mice, suggesting that a diminished level of transgene product may still be present in the peripheral T cells of these mice (data not shown).

Wild-type, but not catalytically inactive, Jak3 restores T cell numbers

Jak3<sup>−/−</sup> mice possess small thymus containing 0.5 to 10% of the normal number of thymocytes (16–18). This decrease in cellularity is thought to result from the absence of appropriate proliferative signals in early thymocytes and is reminiscent of the phenotype observed in IL-7-null (30), IL-7R-null (21), or γc-null (15) mutant mice. Figure 2A demonstrates that the wild-type Jak3 transgene restores thymic cellularity in Jak3<sup>−/−</sup> mice, resulting in a 10- to 100-fold increase in cell number. Judged by the average number of total thymocytes, low level Jak3 transgene expression (Tg<sub>low</sub>) in Jak3<sup>−/−</sup> animals appears as efficient as high level expression (Tg<sub>high</sub>) in driving this early expansion, not a surprising result.
given that the level of Jak3 in Jak3<sup>−/−</sup>-Tg<sub>low</sub> mice exceeds that in Jak3<sup>+/−</sup> thymocytes by 6.4-fold (Fig. 1B, lanes 1 and 5). Interestingly, thymocyte number is unaltered in Jak3 heterozygotes by transgene expression, despite a substantial increase in total Jak3 abundance (data not shown). These data suggest that expression of wild-type Jak3 at quite high levels is not injurious. More importantly, the catalytically inactive version of Jak3 (Tg<sub>KE</sub>) was completely ineffective in restoring thymus cellularity. Thus, Jak3 catalytic activity is required to achieve normal thymic cellularity.

To investigate whether expression of the Jak3 transgene alters thymocyte development, we analyzed thymocyte expression patterns for the CD4 and CD8 coreceptors (Fig. 2B), as well as those for CD3, CD69, and CD25 (data not shown). Neither wild-type nor KE mutant Jak3 proteins affected development of Jak3<sup>+/−</sup> or Jak3<sup>−/−</sup> thymocytes. However, as previously observed (16–18), the ratio of CD4 to CD8 single positive thymocytes increased in Jak3<sup>−/−</sup> mice between 4 and 7 wk of age (lower left), a phenomenon also seen in γ<sup>−/−</sup> mice (15). This increase was reversed by expression of the wild-type Jak3 transgene (lower right). The cause of this skewing in CD4:CD8 ratio is unknown at present; however, this effect appears to be exaggerated in the periphery, as will be discussed later.

Splenitic and lymph node cellularity of Jak3<sup>−/−</sup> mice is reduced, due to a block in early B cell development, in addition to reduction in the number of mature T cells generated in the thymus (16–18). As shown in Figure 2C, total splenic lymphocyte cellularity is enhanced by expression of the wild-type Jak3 transgene due to a selective increase in T cell numbers. Flow cytometric analysis of splenocytes also indicates that B220<sup>+</sup> B cells remain under-represented even in mice with a high level of Jak3 transgene expression (data not shown).

Although Jak3 protein is not detectable in Jak3<sup>−/−</sup>-Tg<sub>low</sub> splenocytes, increased numbers of mature T cells exit the thymus and appear in the spleen and lymph nodes. Surprisingly, these animals develop splenomegaly by 7 wk of age. In contrast, Jak3<sup>−/−</sup>-Tg<sub>high</sub> animals maintain normal splenic cellularity. Further analysis revealed that the splenomegaly in Jak3<sup>−/−</sup>-Tg<sub>low</sub> mice results from a fivefold increase in the representation of Mac-1<sup>+</sup> cells, as well as normalization of T cell numbers. While the mechanism underlying this accumulation of myeloid cells in the spleens of Jak3<sup>−/−</sup>-Tg<sub>low</sub> mice remains unknown, it appears that the loss of Jak3 expression in otherwise normal T cells can profoundly affect leukocyte homeostasis.

Loss of Jak3 correlates with the development of an activated phenotype among peripheral T cells

Previous analyses of Jak3- or IL-2R-deficient mice indicated that peripheral lymphocytes in these animals spontaneously develop an "activated" phenotype in vivo, while mounting minimal proliferative responses to in vitro stimulations. T cells in these animals manifest increased surface expression of CD25 (16), CD69 (16, 20), or CD44 (16, 19), markers that typically appear following Ag receptor-mediated activation, and these activated cells accumulate with age. As shown in Figure 3, a larger fraction of CD4<sup>+</sup> splenic T cells from Jak3<sup>−/−</sup> mice bears high levels of CD69. In Jak3<sup>−/−</sup>-Tg<sub>low</sub> mice, the same effect is observed, albeit with somewhat delayed kinetics. Moreover, T cells from Jak3<sup>−/−</sup>-Tg<sub>low</sub> mice failed to proliferate in response to TCR cross-linking or mitogens (data not shown). In contrast, peripheral T cells from Jak3<sup>−/−</sup>-Tg<sub>high</sub> animals do not express high levels of activation markers at age 7 wk or even when obtained from a 20-week old animal (data not shown), suggesting that the low level of Jak3 (60% of Jak3<sup>−/−</sup>-Tg<sup>−</sup> control; Fig. 1B, lanes 11 and 16) retained in peripheral T cells in these mice is sufficient to establish and/or maintain the resting state of circulating T cells. Interestingly, the
In mice as in other species, the ratio of CD4 to CD8 T cells is tightly regulated (31). However, increased CD4:CD8 T cell ratios are characteristically observed in mice deficient in the γc, IL-2Rα- and β-chains or Jak3 (16–18). Thus, in Jak3-deficient animals, this ratio often exceeds 10:1 (Fig. 4A, lower left), a value much higher than the normal 2:1 ratio (upper left). High level expression of the Jak3 transgene restores the normal CD4:CD8 ratio in splenic T cells in Jak3<sup>−/−</sup> animals (lower right) but has no effect on Jak3<sup>+/+</sup>-control cells (upper right and middle). Low level transgene expression also restores the normal ratio initially (data not shown); however, the ratio increases significantly in older animals (lower middle), suggesting that continuous presence of Jak3 is required to maintain the balance between the CD4 and CD8 compartments.

Loss of Jak3 enhances peripheral expansion of T cells

The increased CD4:CD8 ratio in Jak3<sup>−/−</sup> mice may be caused by preferential expansion of the CD4<sup>+</sup> population or by preferential elimination of the CD8<sup>+</sup> population. Figure 4B indicates that splenic CD4<sup>+</sup> T cells accumulate much more rapidly, compared with CD8<sup>+</sup> T cells, in Jak3<sup>−/−</sup> mice (filled squares), while the numbers of CD4<sup>+</sup> and CD8<sup>+</sup> T cells in Jak3<sup>+/+</sup>-controls are maintained at relatively constant levels over time (unfilled circles). Expression of the Jak3 transgene at low levels augments accumulation of CD8<sup>+</sup> T cells (crossed squares), although it does not prevent accumulation of CD4 T cells. These data suggest that asymmetric expansion of cells results in the increased ratio between the CD4 and CD8 compartments. To examine this phenomenon in more detail, we assessed BrdU incorporation by splenic T cells in vivo. As shown in Figure 4C, markedly increased incorporation of BrdU is observed for the Jak3<sup>−/−</sup> splenocytes compared with the Jak3<sup>+/−</sup> controls, suggesting that Jak3<sup>−/−</sup> T cells are actively synthesizing DNA and are proliferating. Furthermore, our data clearly demonstrate that CD8<sup>+</sup> T cells in Jak3<sup>−/−</sup> mice incorporate BrdU as well as do their CD4<sup>+</sup> counterparts. These data demonstrate that preferential expansion of CD4<sup>+</sup> T cells cannot by itself explain the observed skewing of the CD4:CD8 ratio.

Loss of Jak3 correlates with Fas and FasL up-regulation

Although both CD4<sup>+</sup> and CD8<sup>+</sup> T cells proliferate in vivo in Jak3<sup>−/−</sup> mice, in seemed possible that these populations might differ with respect to their ability to undergo apoptosis. To address this possibility, we first compared the relative levels of Fas expression on splenocytes from Jak3<sup>−/−</sup> mice and Jak3<sup>+/−</sup> controls (Fig. 5A). Both CD4 and CD8 T cells from Jak3<sup>−/−</sup> mice express high levels of Fas relative to cells from Jak3<sup>+/−</sup> mice. This is not surprising, given that activated T cells up-regulate Fas (32). When these cells were subsequently treated with anti-CD3 in an overnight culture, Jak3<sup>−/−</sup> controls readily up-regulated Fas, while Jak3<sup>−/−</sup> cells, which express maximal levels of Fas before stimulation, fail to increase Fas expression further (data not shown). These effects correlate with the relative levels of Jak3 protein, as T<sup>+</sup> cells from Jak3<sup>−/−</sup>/low-reconstituted cells displayed an intermediate level of Fas. Similar profiles were seen when p75 TNF receptor levels were analyzed (data not shown). These findings, in conjunction with results already discussed, suggest that T cells become activated spontaneously in vivo following the loss of Jak3 and that these activated T cells up-regulate CD69, CD44, Fas and p75 TNFR.

To investigate whether Fas-mediated apoptosis leads to loss of T cells in Jak3<sup>−/−</sup> mice, we analyzed CD4<sup>+</sup> and CD8<sup>+</sup> splenic T cells for the expression of FasL. Remarkably, CD8<sup>+</sup> T cells from Jak3<sup>−/−</sup> mice express high levels of FasL, while CD4<sup>+</sup> T cells in the same mice maintain basal levels of FasL expression (Fig. 5B). In fact, a fraction of CD4<sup>+</sup> T cells in Jak3<sup>−/−</sup> animals appear to have down-regulated FasL expression. This distinction between FasL expression in CD4<sup>+</sup> vs CD8<sup>+</sup> T cells is consistent with published data indicating that the level of FasL expression on activated CD8<sup>+</sup> splenic T cells is roughly 10-fold higher than that found on

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**FIGURE 2.** T cell numbers are dramatically increased in reconstituted Jak3-null animals. A. Total thymocyte numbers are represented as the percentages of the control (Jak3<sup>+/−</sup>/Tg<sup>−</sup>). Jak3 genotype at the endogenous locus (Jak3<sup>+/+</sup> or Jak3<sup>−/−</sup>) and reconstituting transgenic lines (Tg<sup>−</sup>, Tg<sup+a</sup>, Tg<sup+b</sup>, or Tg<sup>KE</sup>) are indicated. Number of animals analyzed (n) and SEM value are also shown with each bar. B. Analysis of CD4 and CD8 coreceptor expression pattern. Total thymocytes were stained with fluorochrome-conjugated monoclonal reagents and analyzed by flow cytometry (see Materials and Methods). Lower panels represent CD4 and CD8 profiles of thymocytes from transgene-negative and reconstituted (Tg<sup>+</sup>) Jak3-null animals. Upper panels are those of transgene-negative and transgene-positive Jak3<sup>+/+</sup> control animals. Numbers represent the percentage of cells in each quadrant. C. The numbers of splenic mononuclear cells, T cells, and B cells are represented as percentages of control (Jak3<sup>+/−</sup>/Tg<sup>−</sup>) values.
CD4+ T cells (33). CD8+ T cells from Jak3−/− mice reconstituted with low levels of the Jak3 transgene (Jak3−/−Tglow) display an intermediate level of FasL expression, indicating that regulation of FasL expression is sensitive to the abundance of Jak3.

Finally, we analyzed ex vivo splenocytes for apoptosis. The percentage of apoptotic cells was approximately twofold higher among the CD8+ T cells from Jak3−/−Tg− mice, an effect that disappeared in the Jak3−/−Tglow cells (Fig. 5C). Low levels of Jak3 transgene expression significantly lowered the proportion of apoptotic cells. These data suggest that CD8+ T cells, while expanding at higher than normal levels in the Jak3−/− animals, become highly susceptible to apoptosis in the absence of Jak3. Furthermore, the increased surface expression of Fas and FasL in vivo suggests that the ongoing apoptosis may be at least partially mediated via a Fas-dependent mechanism.

**Amelioration of peripheral defects correlates with Jak3 abundance in mature T cells**

Even though the peripheral T cell defects in reconstituted animals correlated roughly with Jak3 protein abundance in those cells, it was formally possible that recent thymic emigrants (with high levels of Jak3 protein) displaced any resident peripheral T cells (which have lost Jak3 protein) that were undergoing phenotypic changes, thereby masking the accumulation of abnormal cells. To address this question, we thymectomized Jak3−/−Tghigh mice and monitored the pool of peripheral T cells over time. Before surgery, at 11 wk of age, T cells from Jak3−/−Tghigh mice exhibited normal CD4/CD8 ratios and normal profiles of surface CD69 protein (data not shown). After the surgery, we analyzed the PBL of unthymectomized and thymectomized Jak3−/−Tghigh, Jak3−/−Tghigh, and Jak3−/− animals by flow cytometry at 2-wk intervals. We observed no significant change of CD4/CD8 T cell ratios (Fig. 6A) or surface expression of CD69 (Fig. 6B) in thymectomized animals compared with their unthymectomized littermates, even at 6 wk post-thymectomy. Immunoblot analysis of splenic T cells indicated that Jak3 abundance did not decrease in thymectomized animals (Fig. 6C), suggesting that expression from the Jak3 transgene locus persisted in the periphery.

We also tested the possibility that an earlier developmental defect was responsible for the peripheral T cell anomaly associated with the lack of Jak3 by crossing Jak3−/− animals with mice that express wild-type Jak3 transgene under the control of the ick distal promoter (23). The thymi of the progeny from this cross, Jak3−/−DTg+, remained small (10% of Jak3+/− controls and in the range of Jak3−/−Tg−), indicating that the early requirement for Jak3 had not been restored (Fig. 7A). Despite its inability to reconstitute thymocyte numbers, the distal promoter-driven expression of Jak3 considerably reduced the accumulation of splenic T cells with high levels of surface CD69 (Fig. 7B). Total Jak3 abundance in splenocytes of Jak3−/−DTg+ animal was threefold over Jak3−/−Tg− control, as judged by immunoblotting (data not shown). These data suggest that developing thymocytes that lack Jak3 are not automatically committed to express high levels of CD69 and CD44 after they exit the thymus. Instead, Jak3 expression maintains the quiescent state of peripheral T cells.

**Discussion**

Catalytic activity of Jak3 is required to establish normal numbers of proliferating, immature thymocytes

Activation of JAKs in response to IFNs and growth factors has been associated with phosphorylation and nuclear translocation of STAT factors (reviewed in Refs. 34–36). The actual sequence of events immediately following receptor engagement remains unresolved at the molecular level; however, a current model supports the view that activated JAKs phosphorylate tyrosine residues in cytokine receptor molecules as well as those in STAT factors (reviewed in Refs. 34–36). The actual sequence of events is thought to occur at restoring IFN-inducible gene expression or antiviral cytopathic activities (37). Although the kinase-negative mutant Jak1 was shown to induce low levels of gene expression in this system, its ability to complement the overall response was significantly lower than that observed using wild-type Jak1. Our study, like the previous analysis by Thomis and Berg (22), demonstrates that Jak3 catalytic activity is absolutely required for normal thymocyte development. In this regard, it is interesting that high level expression of catalytically inactive Jak3 did not in any way impair lymphocyte development (data not shown). We infer from this that comparatively little Jak3-derived signal is required to sustain normal lymphocyte function. The reconstitution of peripheral T cell function that we observe is consistent with this inference.
Requirement for Jak3 can be correlated with distinct cytokines that regulate T cell development and function

The normal maturation and functional response of a T cell requires orchestrated cytokine signals (38–40). Loss of Jak3 leads to a complex phenotype, presumably reflecting defects in the function of cytokines that utilize γc. The earliest defect, relating to the severe reduction in lymphocyte numbers, resembles loss of the IL-7 signal, which is indispensable for generation of proliferating immature lymphocytes. Jak3 transgene expression under the control of the lck proximal promoter successfully restores this early signal, increasing the number of T cells. In this environment, selective loss of Jak3 in the periphery appears to affect T cell functions in a manner consistent with the loss of an IL-2 signal. IL-2 is thought to provide a costimulatory signal in the Ag receptor-mediated response, as well as in regulating the course of activation responses. This requirement is distinct from the early one, because restoring Jak3 expression in immature thymocytes does not prevent peripheral T cells from becoming spontaneously activated.

During maturation, thymocytes undergo multiple rounds of replication as they transit from the CD4−CD8− (double negative) to CD4+CD8+ (double positive) stage (reviewed in Ref. 41). A developmental block at this stage has been correlated with reduction of thymic cellularity and an increase in the proportion of double negative thymocytes, as has been observed in Rag-1- or Rag-2-deficient mice (42, 43), or in mice lacking p56

\[ \text{src} \] and p59

\[ \text{fyn} \] function (44–46). Given that the small number of thymocytes that remains in Jak3−/− mice develops normally from the double negative to the double positive stage, the paucity of thymocytes in these mice almost certainly reflects a requirement for Jak3 at an earlier stage. Indeed the number of Lin−c−kit+/CD4+CD8− cells, thought to be thymocyte precursors, was reportedly reduced in
In reconstituted Jak3\(^{-/-}\) mice, transgene-derived Jak3 protein selectively restores the T cell compartment, while other compartments of the hemopoietic system continue to suffer from Jak3 deficiency. Total numbers of B cells in the spleens and lymph nodes of transgene-positive (both Tg\(^{high}\) and Tg\(^{low}\)) Jak3\(^{-/-}\) mice are approximately 1 to 2% of the Jak3\(^{+/+}\) control and are often lower than those of their transgene-negative Jak3\(^{-/-}\) littermates (5–10%). We suspect that in the process of T cell expansion, mature B cells that completely lack Jak3 may suffer a competitive disadvantage. It is also possible that circulating T cells in some way promote B cell death in the periphery. A similar scenario has been described for IL-2R \(\beta\)-chain-deficient mice (20) in which an age-dependent reduction in B cell numbers was prevented by in vivo administration of anti-CD4 mAb.

**Jak3 regulates the activation of mature peripheral T cells**

Prior studies demonstrate a pivotal role for Jak3 in delivering IL-2R-derived signals. Therefore, it is not surprising that those T cells that mature successfully in Jak3\(^{-/-}\) mice fail to proliferate following TCR stimulation. Our experiments demonstrate that this nonresponsiveness does not reflect a developmental anomaly, since levels of Jak3 that are fully sufficient to restore normal thymocyte numbers still cannot entirely correct the proliferative defect in peripheral T cells (data not shown). Thus, the replicative response of peripheral T cells to TCR stimulation is regulated by Jak3 abundance.

Curiously, T cells that lack Jak3 nevertheless accumulate in vivo and, by several criteria, appear activated, in that they express high levels of surface CD69 and CD44 protein. Similar observations have been made in mice lacking \(\gamma_c\), or the IL-2R \(\alpha\)- or \(\beta\)-chain. In Jak3\(^{-/-}\) mice, the accumulation of these activated cells can be detected at 3 wk of age, and it increases thereafter. The accumulation of CD4\(^+\)CD69\(^+\) T cells in reconstituted mice varies as a function of relative levels of Jak3 protein. While the initial event responsible for activation remains undefined, our study clearly demonstrates that the continuous presence of a threshold level of Jak3 is necessary to prevent this accumulation of activated cells in the periphery.

Several observations support the view that activation of Jak3-deficient T cells occurs after migration from the thymus. First, augmented expression of CD69 and CD44 protein. Second, provision of Jak3 intrathymically via the p1017-Jak3 transgene (Tg\(^{high}\)) reconstituted normal thymocyte development, but failed to prevent the accumulation of activated cells. While it might be imagined that constant production of normal cells by the Jak3\(^{-/-}\)Tg\(^{high}\)-reconstituted thymus displaced any abnormal cells that might otherwise accumulate, thymocyte-reconstituted Jak3\(^{-/-}\)Tg\(^{high}\) mice retained normal peripheral T cell profiles, indicating that reconstitution of Jak3 expression in the periphery, a result of “leaky” promoter activity in this compartment, was responsible for the ameliorating effect. Finally, expression of Jak3 under the control of the distal \(lck\) promoter, which directs expression primarily in peripheral T cells, suppressed the generation of activated T cells without appreciably improving thymocyte development (Fig. 7).

Therefore, although Jak3 mediates cytokine-induced proliferation, cells lacking Jak3 respond to a stimulus in the periphery that yields both illegitimate activation and replicative expansion.

**Jak3 regulates T cell homeostasis**

Jak3 deficiency in the periphery engenders in T cells several abnormal features that are seemingly paradoxical. As discussed above, loss of Jak3 impairs in vitro proliferation of mature T cells in response to mitogens or TCR stimulation, while it promotes...
their constitutive activation in vivo. Loss of Jak3 also results in the asymmetric accumulation of CD4⁺ T cells, while it initiates peripheral proliferation of both CD4⁺ and CD8⁺ compartments.

Although the surface expression of many T cell activation markers increases with the loss of Jak3, only FasL expression differed between CD4⁺ and CD8⁺ T cells. While CD8⁺ T cells expressed higher levels of FasL, its receptor molecule, Fas, is expressed at high levels on both CD4⁺ and CD8⁺ T cells of Jak3⁻/⁻ mice, a result consistent with the known increase in Fas expression typically observed following TCR stimulation. FasL expression, however, is especially dramatic in stimulated CD8⁺ cells (33). Our data both confirm the differential expression of FasL on activated CD4⁺ vs CD8⁺ T cells in Jak3⁻/⁻ mice and suggest a mechanism for the augmented CD4:CD8 ratio in such animals.

Engagement of Fas with FasL triggers an apoptotic signal (47, 48). Moreover, mice lacking either of these genes manifest a lymphoproliferative disorder, suggesting that Fas-mediated cell death marks a normal end point in the course of lymphocyte activation (reviewed in Refs. 49–53). Since apoptosis occurs readily in Jak3⁻/⁻ cells (Fig. 5C), we conclude that alternative mechanisms must exist to sensitize these lymphocytes. More to the point, Jak3-deficiency (as demonstrated here) encompasses a broad range of signaling defects, resulting in profound abnormalities in splenic T cell homeostasis.

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