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Developmental Defects of Lymphoid Cells in Jak3 Kinase-Deficient Mice

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Summary

Jak3 is a tyrosine kinase mediating cytokine receptor signaling through the association with the common γ chain of the cytokine receptors such as IL-2, IL-4, IL-7, IL-9, and IL-15. Unlike other members of the Jak family, the expression of Jak3 is highly restricted in hematopoietic cells. To elucidate in vivo function of Jak3, Jak3-deficient mice were generated by homologous recombination. Mice homozygous for *Jak3* null mutation showed severe defects, specifically in lymphoid cells. B cell precursors in bone marrow, thymocytes, and both T and B cells in the spleen drastically decreased, although these defects were significantly recovered as aging occurred. Peripheral lymph nodes, NK cells, dendritic epidermal T cells, and intestinal intraepithelial γδ T cells were absent. Normal number of hematopoietic stem cells in bone marrow from Jak3-deficient mice and the similar capability to generate myeloid and erythroid colonies as wild-type mice indicated specific defects in lymphoid stem cells. Furthermore, the abnormal architecture of lymphoid organs suggested the involvement of Jak3 in the function of epithelial cells. T cells developed in the mutant mice did not respond to either IL-2, IL-4, or IL-7. These findings establish the crucial role of Jak3 in the development of lymphoid cells.

Introduction

The family of Janus kinases, including Jak1, Jak2, Tyk2, and Jak3, are nonreceptor tyrosine kinases and play important roles in cytokine signal transduction through the association with various cytokine receptors (Ihle and Kerr, 1995; Ihle et al., 1994). Cytokine receptors do not contain intrinsic kinase activity, and thus, the activation of Jaks associated with these receptors is one of the earliest biochemical events upon binding of cytokines onto their specific receptors and has been shown to be crucial for transducing the growth signal (Johnston et al., 1994; Witthuhn et al., 1994, Kawamura et al., 1994). Although Jaks have no typical SH2 and SH3 domains of src-type tyrosine kinases, they contain distinct Jak homology (JH) regions with two unique tandemly arranged kinase domains, a kinase domain and a kinase-like domain.

Unlike other members of the Jak family kinases that are expressed ubiquitously, the expression of Jak3 is highly restricted to the hematopoietic cell lineage (Johnston et al., 1994; Witthuhn et al., 1994). Jak3 has been found to be associated with the common γ chain (γc) of the receptors for interleukin-2 (IL-2), IL-4, IL-7, IL-9, and IL-15 (Takeshita et al., 1992; Kondo et al., 1993, 1994; Russell et al., 1993, 1994; Noguchi et al., 1993a; Giri et al., 1994). All of these cytokines and receptors are essential for differentiation, growth, and function of lymphocytes. Jak3 associates with γc constitutively and is activated upon binding of these cytokines to the receptors, and thereafter transduces growth signal.

The functional importance of Jak3 in the lymphoid system has been extensively investigated in vitro. When the anergy state of T cells was induced, the addition of cytokines such as IL-2, IL-4, and IL-7 together with antigen could prevent the anergy induction through activation of Jak3 (Boussiotis et al., 1994). Furthermore, Migone et al. (1995) reported that the transition to IL-2-independent growth of human T cell lymphotrophic virus I (HTLV-I)-transformed cells can be attributed to the constitutively activated Jak3–Stat5 pathway (Migone et al., 1995). Activation of the Jak–Stat pathway is also responsible for the transformation of pre-B cells by v-*abl* (Danial et al., 1995). Finally, both Jak1 and Jak3 have been shown to be required unequivocally to activate downstream of the IL-2 receptor for T cell growth (Miyazaki et al., 1994). These studies suggest that the defects in the Jak3 signaling pathway result in dysregulation of immune function and eventually in in vivo pathogenesis. It has been shown that the mutation in γc caused X-linked severe combined immunodeficiency (XSCID) in patients. Patients with the mutation of the *Jak3* gene have recently been shown to have similar immunodeficiency (Macci et al., 1995).

To define the in vivo function of Jak3, especially for development of lymphocytes, as well as to create a new type of immunodeficiency model mice, we have generated

mice deficient for the expression of the *Jak3* gene. Mice homozygous for *Jak3* null mutation revealed severe impairment in development of T and B cells in lymphoid tissues. Although the Jak3 is also expressed in nonlymphoid tissues, the defect was observed exclusively in lymphoid tissues and the overall phenotype of Jak3-deficient mice was similar to that of γc-deficient mice. However, importantly, we found some differences between γc knockout and Jak3-deficient mice, especially on the architecture of thymus.

Results

Generation of Jak3-Deficient Mice

We isolated an approximately 11 kb genomic DNA fragment containing the whole *Jak3* cDNA sequence from a 129 genomic library. Since the exon–intron boundaries of the genomic locus of the *Jak3* gene have not been determined yet, a targeting vector in which a 7.8 kb genomic fragment containing the JH regions, the kinase-like domain, and a part of the kinase domain was replaced by the *neo* resistance gene was designed in order to make the null mutation of the *Jak3* gene (Figure 1A). The targeting construct was transfected into E14.1 embryonic stem (ES) cells. Two independent ES clones possessing the specific homologous recombination in one allele of the *Jak3* gene were obtained and injected into C57BL/6 blastocysts. Chimera mice were mated with C57BL/6 mice and the offspring heterozygous for the disrupted *Jak3* gene, which were derived from both of the two ES clones, were generated. These heterozygous mice were intercrossed to produce homozygotes for the *Jak3* mutation. The genotypes

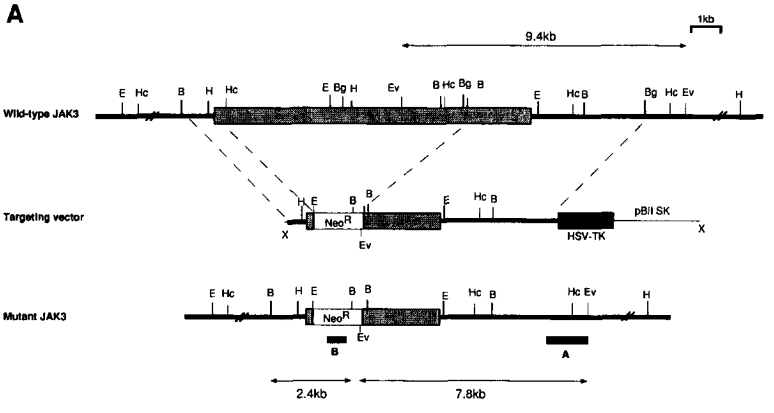


Figure 1. Production of Jak3-Deficient Mice

(A) Partial map of *Jak3* gene locus and targeting vector. The *Jak3* genomic locus including exons and introns is indicated by a shaded rectangle. The exon–intron structure of the *Jak3* gene has not been defined yet. At both right and left ends, the scale is interrupted. The targeting vector in pBluescript is constructed by replacing a 7.8 kb genomic region of the *Jak3* gene, where Jak homology regions, the kinase-like domain, and the 5′ portion of the kinase domain are located, with a *neo*-resistance gene labeled as Neo^R. The targeting vector was linearized by digestion with XbaI and transfected into E14.1 ES cells. The lowest line is an expected *Jak3* mutant locus after homologous recombination takes place. Restriction endonucleases indicated above are abbreviated as the following: B, BamHI; E, EcoRI; Bg, BglII; H, HindIII; Hc, HincII; Ev, EcoRV; X, XbaI. HSV-TK, thymidine kinase gene from herpes simplex virus.

(B) Southern blot analysis of tail DNA. The *Jak3* genomic DNA of offspring born from heterozygous mutant parents was analyzed with Southern blot analysis. The tail DNA was digested with EcoRV and hybridized with the probe A in (A), which was derived from the franking region outside of the homologous recombination locus. A wild-type and a mutant allele shows 9.4kb and 7.8kb, respectively. The probe B was used to confirm the single integration event of the targeting vector. The representative pattern of wild-type ($^{+/+}$), heterozygous ($^{+/-}$), and homozygous ($^{-/-}$) mice is shown.

(C) RT–PCR analysis for Jak3 transcript. The cDNAs derived from total RNA of bone marrow cells were subjected to PCR analysis for null mutation of *Jak3* in mutant mice. The primers used in PCR were designed from the cDNA sequence corresponding to the kinase domain of Jak3. PCR was done for β-actin as a control. Both the PCR condition and primer sequences are described in Experimental Procedures.

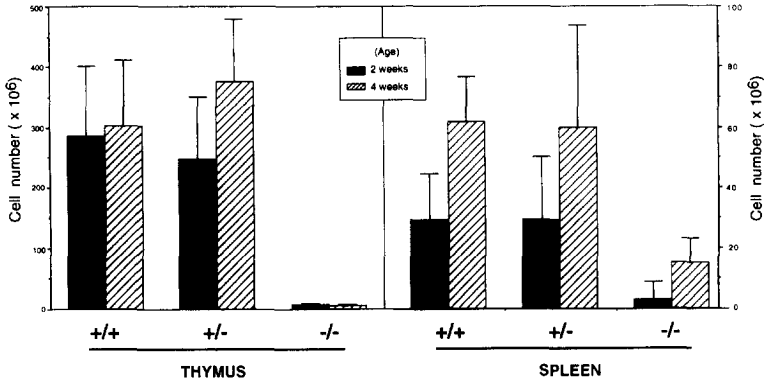


Figure 2. Cellularity of Thymus and Spleen in Jak3-Deficient Mice

The cell numbers of thymocytes and splenocytes from 2- and 4-week-old wild-type($^{+/+}$), heterozygous mutant($^{+/-}$), and homozygous mutant($^{-/-}$) mice were counted. Each bar represents the average values for seven mice. SD is indicated by the error bar.

of wild-type ($^{+/+}$), heterozygous ($^{+/-}$), and homozygous ($^{-/-}$) mice were identified by Southern blotting (Figure 1B) and polymerase chain reaction (PCR) (data not shown). The null mutation of *Jak3* in the homozygous mutant mice was confirmed by the lack of the Jak3 transcript by RT–PCR (Figure 1C).

Developmental Defects of Lymphoid Cells in Jak3-Deficient Mice

The Jak3$^{-/-}$ mice were born normally in close Mendellian expectations, developed in an indistinguishable manner from wild-type littermates, and showed no gross abnormality. We have not observed any significant phenotypic difference between wild-type and heterozygous mice in histological and flow cytometric analysis, therefore, we present data on wild-type and Jak3$^{-/-}$ mice in this report.

The cell numbers in lymphoid organs from Jak3$^{-/-}$ mice of various ages were compared with those in the wild-type littermates. Both the size and cellularity of thymocytes from Jak3$^{-/-}$ mice were drastically reduced as compared with those of the wild-type littermates. The thymus of Jak3$^{-/-}$ mice contained 30–60 times fewer thymocytes than those from wild-type mice (Figure 2). Whereas the number of the thymocytes from the wild-type mice was 300×10^6 as the mean, that of the mutant mice varied considerably but did not exceed 15×10^6. The developmental defect of lymphocytes in the spleen was not so severe as in the thymus. Nevertheless, Jak3-deficient mice showed reducing cellularity in the spleen as well as variations in number: the Jak3$^{-/-}$ mice possessed only 4–10 times fewer splenocytes than the littermate wild-type mice (Figure 2). In 5-week-old Jak3$^{-/-}$ mice, the size of spleen was still smaller than that of wild-type mice. This is clearly different from the pathology of γc knockout mice, which showed no significant reduction in size (Cao et al., 1995).

Histological Analysis of Lymphoid Organs in Jak3-Deficient Mice

Both 2- and 4-week-old Jak3$^{-/-}$ mice were analyzed histologically and were compared with heterozygous and wild-type mice. Macroscopically, homozygous mice showed severe hypoplasia in thymus, from mild to moderate in spleen, and severe in mesenteric lymph nodes, whereas heterozygotes showed no abnormality in thymus, spleen, and peripheral lymphoid organs by macroscopic and microscopic examinations. Although the Jak3 expression was also detectable in nonlymphoid tissues, including kidney, lung, and liver (Takahashi and Shirasawa, 1994), no macroscopic or microscopic abnormalities were observed in these nonlymphoid tissues of Jak3$^{-/-}$ mice.

The histological sections of hypoplastic thymus from 2- and 4-week-old Jak3$^{-/-}$ mice basically showed the same abnormalities. The size of both medulla and cortex were reduced markedly (Figure 3B). The histology of cortex was clearly characterized by the decreased number of thymocytes, which made corticomedullary junction unclear and densely condensed epithelial structure in the cortex (Figure 3D). These histological features generally resemble those of the γc-deficient mice (Cao et al., 1995), except that Jak3-deficient mice showed more distinct hypoplasia in thymic medulla. Interestingly, we failed to identify Hassall's corpuscles in the thymic medulla of Jak3$^{-/-}$ mice, which were readily identified in γc knockout mice (Cao et al., 1995). To analyze the structure of epithelia further, the expression of class II molecule and cytokeratin in thymic epithelia were analyzed. As shown in Figure 3B, thymic epithelial cells expressed the comparable level of class II molecule in both medulla and cortex of Jak3$^{-/-}$ mice, suggesting that thymic epithelial cells of Jak3$^{-/-}$ mice may be functionally mature as antigen-presenting cells. The immunostaining of cytokeratin, however, showed the marked reduction in reactivities in the medullar structure of Jak3$^{-/-}$ mice (Figures 3C, 3D). In the control mice (Figure 3C), thymic epithelial cells in medulla exhibited the characteristic networks of cytokeratin and the positive immunoreactivity for Hassall's corpuscle (an arrow in Figure 3C). In Jak3-deficient mice, however, no formation of networks nor the immunoreactivities for Hassall's corpuscles were found by the immunostaining for cytokeratin. These results suggest that the depletion of Jak3 signaling pathway in thymic epithelia may lead to the structural abnormality.

In the spleen of 2-week-old Jak3$^{-/-}$ mice, surface immunoglobulin M$^+$ (IgM$^+$) B cells were hardly detected (Figure 3F), while mature white pulps were organized in heterozygous and wild-type mice (Figure 3E). The histological data showing B cell depletion in the spleen of 2-week-old mice coincided well with flow cytometric analysis (Figure 4). In the spleen of 4-week-old Jak3$^{-/-}$ mice, the white pulps, which were mainly composed of surface IgM$^+$ B cells and

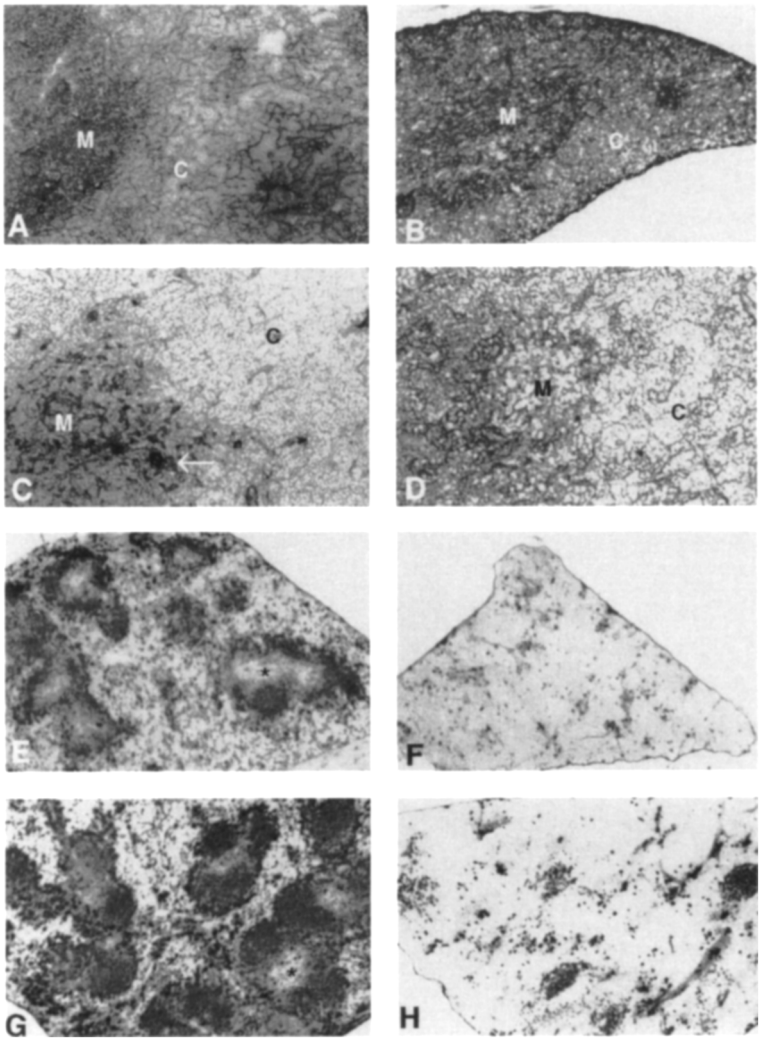


Figure 3. Immunohistological Analysis of Thymus and Spleen from Jak3-Deficient Mice

Sections of thymus (A–D) and spleen (E–H) from 2-week-old (E, F) or 4-week-old (A–D, G, H) wild-type (A, C, E, G) and Jak3$^{-/-}$ mice (B, D, F, H) were analyzed. Immunostainings for MHC class II (A, B), cytokeratin (C, D), and surface IgM (E–H) were performed. M, medulla; C, cortex. An arrow in (C) indicates the positive staining of cytokeratin in a Hassal's corpuscle. The asterisk denotes PALS in the spleen of wild-type mice. Original magnification, 30× in A–B, 100× in C–D, 30× in E–H.

CD4$^+$ T cells, appeared to be scattered, although they were severely diminished in number and size (see Figure 3H). Jak3-deficient mice showed a comparable level of CD4 reactivities in the aberrant form of white pulps to 4-week-old wild-type mice (data not shown). The clear structure of periarteriolar lymphoid sheath (PALS) was absent in Jak3$^{-/-}$ mice (see Figure 3H) as compared with wild-type mice (asterisks in Figures 3E, 3G). This may be due to the hypoplasia of surface IgM$^+$ B cell population surrounding PALS; alternatively, functionally immature CD4$^+$ T cells in PALS may fail to stimulate surrounding B cells sufficiently enough to form mature white pulps. We failed to detect the peripheral lymph nodes in Jak3$^{-/-}$ mice.

Flow Cytometric Analysis of Lymphocytes in *Jak3* Mutant Mice

Lymphocytes in various differentiation stages were analyzed by flow cytometry. We analyzed 10-day- (2-week-) and 4-week-old mice. In spite of the severely reduced cell numbers of thymocytes in Jak3$^{-/-}$ mice, the overall expression profiles of CD4 and CD8 on the remaining cells was similar to those of wild-type mice (Figure 4A). The expression level of CD3 and TCR$\alpha\beta$ on the cell surface of each subpopulation of thymocytes was also similar among wild-type and Jak3$^{-/-}$ mice (data not shown). However, we found significant differences in the staining profiles in CD4 and CD8 single-positive (SP) thymocytes from Jak3$^{-/-}$ mice. The increasing ratio of CD4$^+$ versus CD8$^+$ SP cells and the down-regulation of the HSA expression in all subpopulations (CD4$^-$CD8$^-$, CD4$^+$CD8$^+$, CD4$^+$CD8$^-$, CD4$^-$CD8$^+$) were observed in Jak3$^{-/-}$ mice (data not shown). Both alterations have been observed in γc-deficient mice (Cao et al., 1995).

Although the profile of the CD4/CD8 subpopulation of thymocytes from Jak3$^{-/-}$ mice was similar to that from wild-type mice, no T cells were detected in the spleens from 2-week-old Jak3$^{-/-}$ mice (Figure 4B). Staining of splenocytes for CD4, CD8, Thy1, and CD3 was almost negative in the mutant mice, whereas T cells from wild-type littermates mice were positively stained (Figure 4B). Since there were mature SP cells in the thymus of Jak3$^{-/-}$ mice, we postulated that T cells would appear in the spleen as aging occurred. This was indeed the case. Staining for CD4, CD8, and Thy1 revealed the emergence of T cells in the

spleen from 4-week-old Jak3$^{-/-}$ mice. Being consistent with the observation in the thymus, the ratio of CD4/CD8 of splenocytes from Jak3$^{-/-}$ mice increased significantly, and this increase of CD4$^+$ T cells was also age dependent.

As shown in Figure 4B, the splenocytes from Jak3$^{-/-}$ mice showed dramatic decrease in number of B220$^+$ surface IgM$^+$ mature B cells. Developmental impairment of B cells was more severe than that of T cells in the spleen. B cell generation in bone marrow follows ordered stages as characterized by the cell surface markers CD43 and B220: CD43$^+$B220$^-$, CD43$^+$B220$^+$, and CD43$^-$B220$^+$ (Hardy et al., 1991). Therefore, we analyzed the lymphoid cell populations in bone marrow, especially for the expression pattern of CD43, B220, and surface IgM. In Jak3$^{-/-}$ mice, most of the mature B220$^+$IgM$^+$ B cells as well as CD43$^-$B220$^+$ cells containing the majority of pre-B cells dramatically decreased in mutant mice (10- to 40-fold less than wild-type mice) (Figure 4C). In contrast, the number of the most immature CD43$^+$B220$^-$ cells was not altered significantly in the bone marrow of Jak3$^{-/-}$ mice. We also analyzed natural killer (NK) cells by staining with DX5 monoclonal antibody (MAb). Whereas NK cells were readily detected in the splenocytes of wild-type mice, no NK cells were stained in the splenocytes from Jak3$^{-/-}$ mice (Figure 4D).

Developmental Defect of Lymphoid Stem Cells in Jak3$^{-/-}$ Mice

Since development of immature and mature T and B cells was severely impaired in Jak3$^{-/-}$ mice, we then analyzed the stem cell population in bone marrow, thymus, and spleen to answer the question whether the number and function of the hematopoietic stem cells were impaired by the depletion of Jak3. Lin$^-$c-*kit*$^+$ double-negative (DN) cells, the precursor of thymocytes, were hardly detected in the thymus of Jak3$^{-/-}$ mice, while those in wild-type mice were readily observed (Figure 5). In contrast, the approximately similar number of hematopoietic stem cells, characterized as Lin$^-$c-*kit*$^+$Sca-1$^+$ cell population, was present in bone marrow of the mutant mice, suggesting that only lymphoid stem cells had defects to develop lymphocytes (Figure 5). This was further confirmed by the analysis of the spleen. The number of stem cells in the spleen was not changed between the wild-type and Jak3$^{-/-}$ mice (Figure 5, legend) in spite of the fact that the total number of splenocytes was reduced and the development of both T and B cells was severely impaired (Figure 5). To prove directly that the ability of the hematopoietic stem cells was not affected by the *Jak3* mutation, stem cell colony assay was performed (Table 1). The result clearly demonstrated that the FACS-sorted Lin$^-$c-*kit*$^+$Sca-1$^+$ cells in the bone marrow from both wild-type and Jak3-deficient mice generated approximately the same numbers of colonies of myeloid, macrophage, and erythroid lineages. These data clearly demonstrated that the number and function of the hematopoietic stem cells in both the bone marrow and the spleen from Jak3$^{-/-}$ mice were not altered and nevertheless there were specific defects of lymphoid cell development.

Absence of DETC and i-IELs in Jak3$^{-/-}$ Mice

We next analyzed the effect of Jak3 deficiency on the development of T cells in epithelia of skin and intestine. Epidermal sheets from ear skin of Jak3$^{-/-}$ and wild-type mice were examined for the development of Thy1$^+$ dendritic epidermal T cells (DETC) by immunohistochemical staining. Whereas DETC was brightly stained with anti-TCR$\gamma\delta$ MAb in wild-type mice, we failed to detect any $\gamma\delta^+$ DETC in epidermal sheets from the mutant mice (Figure 6).

Intestinal intraepithelial lymphocytes (i-IEL) were also examined by both immunohistochemical and flow cytometrical analysis. In wild-type mice, $\gamma\delta^+$ T cells, CD4$^+$ T cells, CD8$^+$ T cells, and surface IgM$^+$ B cells were localized in the small intestinal villi by immunohistochemical study. In Jak3$^{-/-}$ mice, lymphocytes were almost completely absent except for the reduced number of CD4$^+$ T cells (Figures 7A–7H). To analyze the population in detail, i-IELs were isolated and stained with MAbs against CD4, CD8, CD3, TCR$\alpha\beta$, and TCR$\gamma\delta$ (Figure 7I). The number of both CD8$\alpha^+\beta^-$ and CD8$\alpha^+\beta^+$ cells were dramatically reduced in Jak3$^{-/-}$ mice. While most of the $\gamma\delta^+$ T cells were absent, some $\alpha\beta^+$ T cells were developed. Consistent with the immunohistochemical results (Figure 7D), only CD4$^+$ T cells were readily detectable in the mutant mice.

Functional Impairment of Lymphocytes in Jak3$^{-/-}$ Mice

Since thymocytes from Jak3$^{-/-}$ mice expressed the surface TCR–CD3 complex at a comparable level to those from wild-type mice, we analyzed the functional competence of these cells. Because normal thymocytes can not respond well to polyclonal activators such as concanavalin A (ConA) or phorbol 12-myristate 13-acetate (PMA) in the absence of exogenous addition of cytokines, we were able to utilize this system to analyze the effect of cytokines in supporting the cell growth. Thymocytes from 4-week-old wild-type, heterozygous, and homozygous *Jak3* mutant mice were stimulated with ConA or PMA in the presence or absence of cytokines, IL-2, IL-4, and IL-7. As shown in Figure 8, whereas there was no proliferation of thymocytes with PMA in the absence of any cytokines, the addition of either IL-2, IL-4, or IL-7 induced marked proliferation of thymocytes from wild-type and heterozygous mice. However, those cells from Jak3$^{-/-}$ mice did not respond to either of the cytokines at all, in spite of the fact that those cells exhibited proliferation upon stimulation with PMA plus ionophore at a comparable level to those from wild-type and heterozygous mice. ConA responses showed similar results (Figure 8). It is noteworthy that thymocytes from Jak3$^{-/-}$ mice did proliferate upon stimulation with ConA and anti-CD3 MAb, although these cells did not enhance the response in the presence of added cytokines. Another important finding was the difference of the proliferative responses between wild-type and heterozygous mice. Jak3$^{+/-}$ mice showed reproducibly half the response of that of wild-type mice. The results suggest that there is gene–dose effect by the level of Jak3 expression.

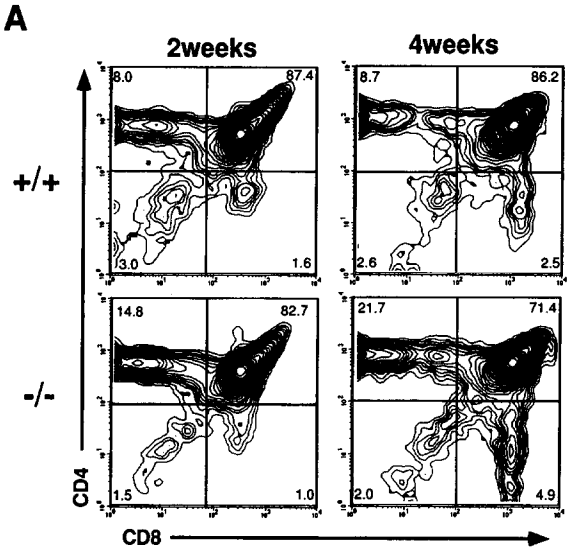
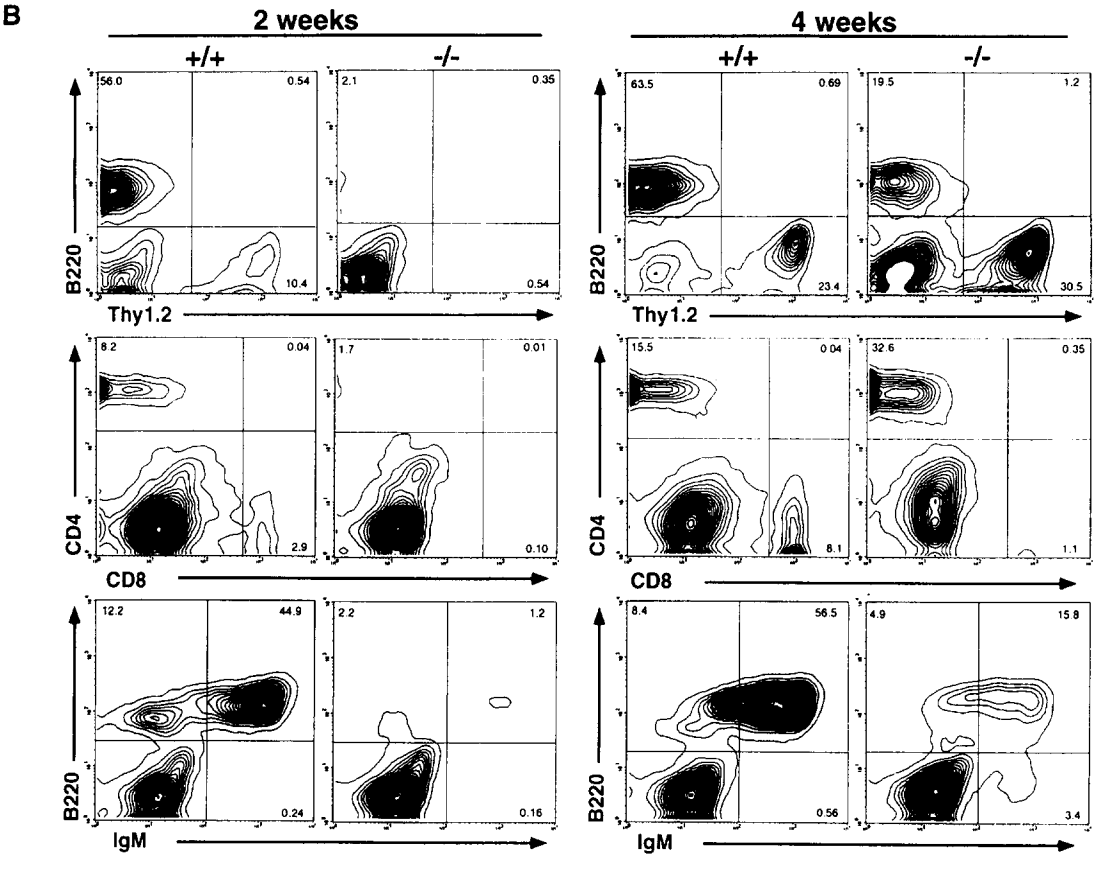


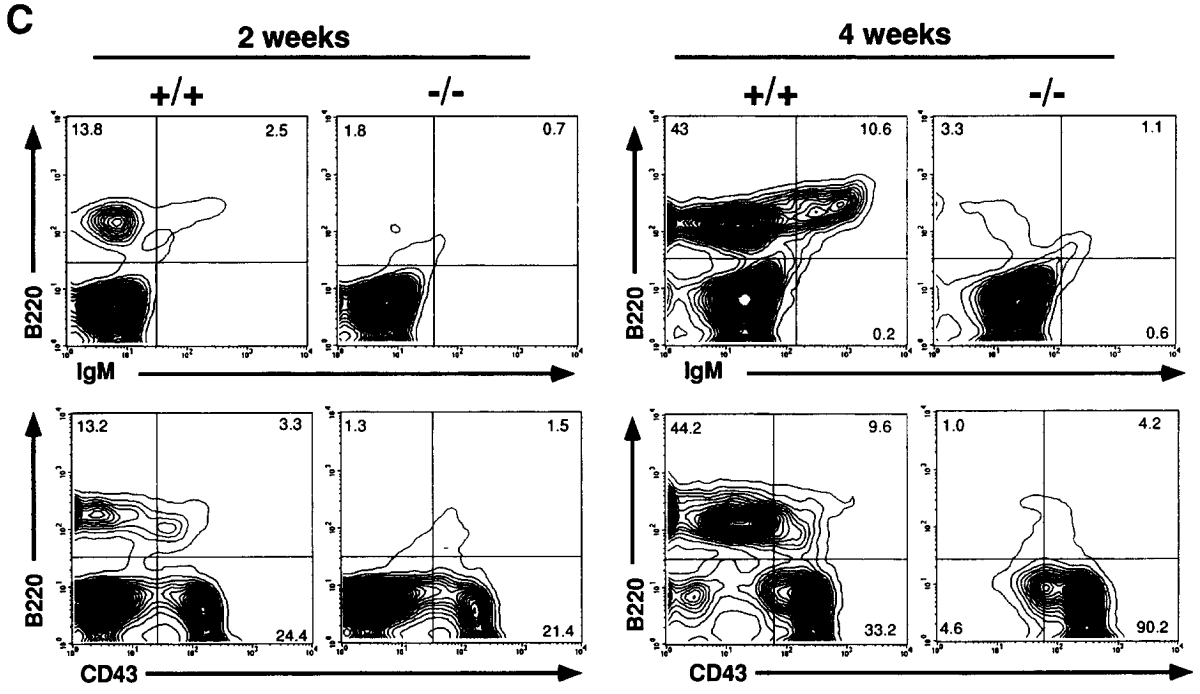
Figure 4. Flow Cytometric Analysis of Thymoctes, Splenocytes, Bone Marrow Cells, and NK Cells from Jak3-Deficient Mice

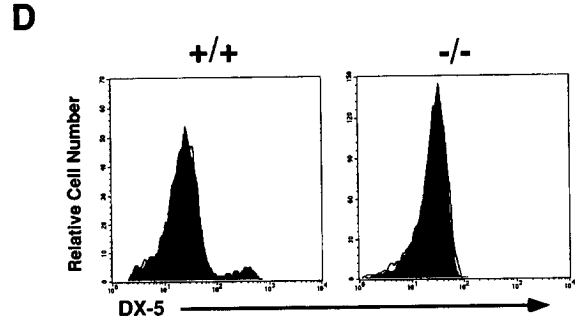
Thymocytes (A), splenocytes (B), bone marrow cells (C), and NK cells (D) from 10 day- (2-week-) and 4-week-old wild-type ($^{+/+}$), homozygous mutant ($^{-/-}$) mice were analyzed. Cells (1–5 × 10^5) were stained with specific MAbs indicated. Splenocytes and bone marrow cells were preincubated with anti-FcR MAb 2.4G2 to minimize nonspecific staining. The surface IgM^-, $CD4^-$, $CD8^-$ cells of splenocytes were gated and analyzed for NK cells. Stained cells (1 × 10^5) were collected in a FACScan flowcytometer and analyzed with a LYSIS II program. The percentages of the events are indicated as numbers at each rectangle. The difference of the fluorescence intensities in 2- and 4-week-old mice reflects experimental variations.



Discussion

Present analysis of Jak3-deficient mice demonstrated the crucial role of Jak3 in development of lymphocytes. Jak3 has been shown to be responsible for transducing growth signals (Johnston et al., 1994; Witthuhn et al., 1994; Miyazaki et al., 1994; Tanaka et al., 1994). However, in the IL-2/IL-2R system for example, the IL-2R complex has been shown to be associated with various tyrosine kinases; not only with Jak3 but also with Jak1 (Miyazaki et al., 1994), syk (Minami et al., 1995), and Lck (Hatakeyama et al., 1991; Minami et al., 1993). Whether these tyrosine kinases have redundancy for signal transduction and the in vivo function of each kinase for transmitting growth signal through these cytokine receptors have not been determined yet. Our results clearly demonstrated Jak3 is crucial for development of lymphocytes but not for nonlymphoid cells, because the lack of Jak3 resulted in severe defects





on both T and B cell differentiation; Jak3 is responsible for growth signals, since even small number of T cells were developed in Jak3-deficient mice, but these cells failed to respond to either IL-2, IL-4, or IL-7. It should be stressed that Jak3 deficiency with keeping all other tyrosine kinases intact abolished cytokine responses by all IL-2, IL-4, and IL-7, indicating no functional redundancy in these systems.

While T and B cell development was externally normal in mice deficient for IL-2, IL-4, or both (Schorle et al., 1991; Kuhn et al., 1991; Sadlack et al., 1993, 1994), IL-7R-deficient mice revealed severe impairment of both T and B cell development (Peschon et al., 1994). IL-7R was expressed on surface IgM^- immature B lineage cells in bone marrow and in the DN thymocytes. In IL-7R-deficient mice, B cell maturation was arrested at the stage of early pro-B cells and the cellularity of the thymus was approximately 1% of normal, and thymic development was arrested at DN stage. Accordingly, it is likely that the impairment of T and B cell development observed in Jak3-deficient mice is largely due to the defect in signaling through IL-7R, which has been discussed also for γc-deficient mice (Cao et al., 1995). Indeed, $IL\text{-}7R^+$ cells could not develop in Jak3-deficient mice (data not shown), since almost all $IL\text{-}7R^+$ cells in bone marrow are $B220^+$ and most of $B220^+$ cells were absent in bone marrow from the mutant mice. However, thymocytes, especially $CD4^+$ SP cells, and peripheral T cells as well as small number of B cells increased as aging occurred. Furthermore, the normal profile of thymic CD4/CD8 subsets in $Jak3^{-/-}$ mice indicates that once thymic progenitor cells are seeded in the thymus, T cells develop normally in the absence of Jak3. In addition, thymocytes from $Jak3^{-/-}$ mice could exhibit significant, though low, proliferation upon stimulation with ConA or anti-CD3 MAb without addition of cytokines. These data suggest the possibility that there is another growth pathway(s) independent of the γc–Jak3 pathway, possibly involving some new cytokines to support the growth.

Jak3-deficient mice increased $CD4^+$ T cells specifically as aging occurred. This increase was observed in thymus, spleen, and also small intestine (i-IEL). In the thymus, although $CD8^+$ SP thymocytes also increased slowly, the increase of $CD4^+$ SP thymocytes were much faster and selectively expanded. This might be explained by selective signal requirement for distinct population. It is possible to assume that $CD4^+$ T cells have redundancy for utilizing other members of Jak family, or that $CD4^+$ cells possess another specific growth mechanism. Alternatively, SP thymocytes might be generated without cell division in the absence of Jak3. In any case, $CD4^+$ T cells in the thymus, spleen, and i-IEL from Jak3-deficient mice may provide a possible clue to find a new mechanism for growth.

Jak3 is expressed not only in lymphoid tissues but also in other tissues, such as lung, kidney (Takahashi and Shirasawa, 1994), mesangial cell (Takahashi et al., 1995), glo-

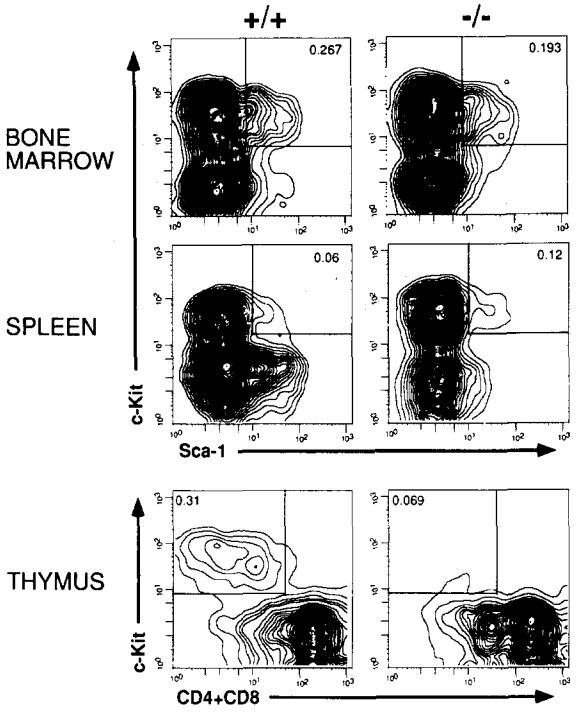


Figure 5. Analysis of the Stem Cells in Bone Marrow, Spleen, and Thymus

The Lin$^-$ cells were incubated with FITC–anti-c-*kit* MAb and APC–Sca-1 MAb. The percentages of c-*kit*$^+$Sca-1$^+$ cells in bone marrow (top) and spleen (middle) are indicated in the figure. The absolute numbers of the Lin$^-$c-*kit*$^+$Sca-1$^+$ cells in the spleen were 7.2 × 10^3 ($^{+/+}$) and 6.5 × 10^3 ($^{-/-}$), respectively. The reason why the number of stem cells of the spleen from Jak3$^{-/-}$ mice was higher than that in wild-type mice was due to the decrease of the total number of splenocytes from Jak3$^{-/-}$ mice. Thymocytes were stained with FITC–c-*kit* MAb and Texas red–CD4 and –CD8 MAbs. The parentage of CD4^{lo}CD8^{lo}c-*kit*$^+$ cells were shown at the bottom.

Table 1. Colony Assay

	Number of colonies/200 cells				
Sca-1$^+$ c-Kit$^+$Lin$^-$ cells from	GEMM	GM	G	BFU-E	Total
JAK3$^{+/+}$	22.5	20.3	2.0	10.5	55.3
JAK3$^{-/-}$	20.5	17.0	3.0	9.5	50.0

Culture was performed in the presence of 20 ng/ml of SCF, IL-3, IL-6, and EPO. Data represent mean of quadruplicate cultures seeded with 200 Sca-1$^+$, c-Kit$^+$Lin$^-$ cells derived from bone marrow.
GEMM, granulocyte, erythrocyte, macrophage and megakaryocyte; GM, granulocyte and macrophage; G, granulocyte; BFU-E, burst-forming unit (erythroid).

merular epithelial cells (T. T. and T. S., unpublished data), and thymic epithelial cells (Kuwata et al., 1995). Since some of these tissues or cell lines apparently lacked γc and failed to respond to cytokines, IL-2, IL-4, IL-7, or IL-15, one may speculate that Jak3 is involved in other signaling receptors in nonlymphoid tissues and that Jak3-deficient mice might show additional abnormalities other than immune deficiency. However, our histological analysis of Jak3-deficient mice showed no obvious abnormality in these tissues and the defects were exclusively restricted to immune system. This may be explained by the possibility that functional redundancy for Jak3 signal transduction exists in nonlymphoid tissues, whereas no alternative signal pathway can replace the γc–Jak3 signal pathway, or the possibility that Jak3 plays a role in the normal development of the immune system, while it only shows abnormal phenotypes in pathological state of nonlymphoid tissues. Further investigation on Jak3-deficient mice is required to test these possibilities. Accordingly, Jak3 deficiency may share the same phenotypes as γc knockout mice. Indeed, most of the defects observed in Jak3$^{-/-}$ mice were common

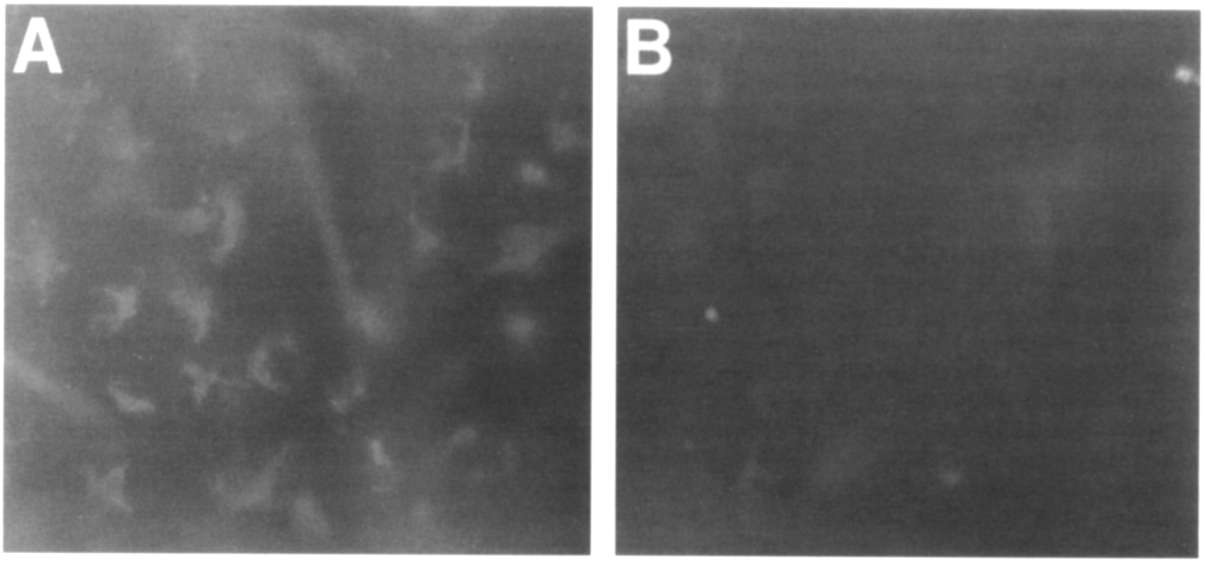
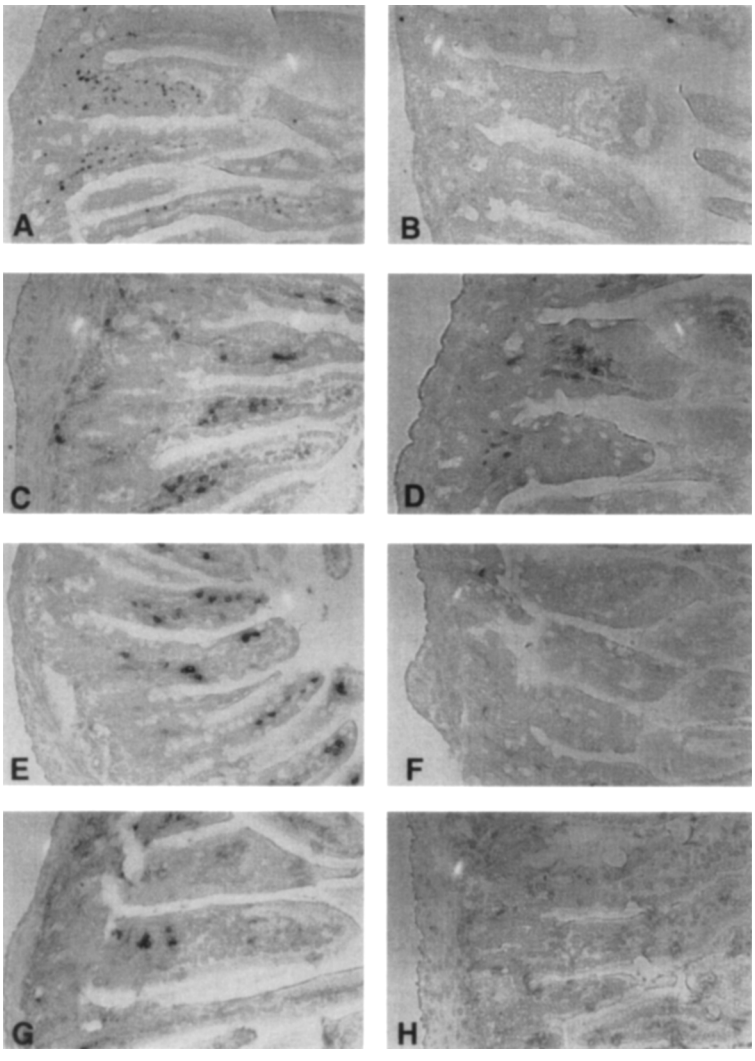


Figure 6. Immunohistological Analysis of DETC Cells

Epidermal sheets containing DETC cells were prepared from ear skin of wild-type (A) and Jak3-deficient (B) mice. The DETC cells were stained with FITC–anti-TCRγδ MAb and were analyzed by immunofluoresence microscope. Original magnification, 40×.



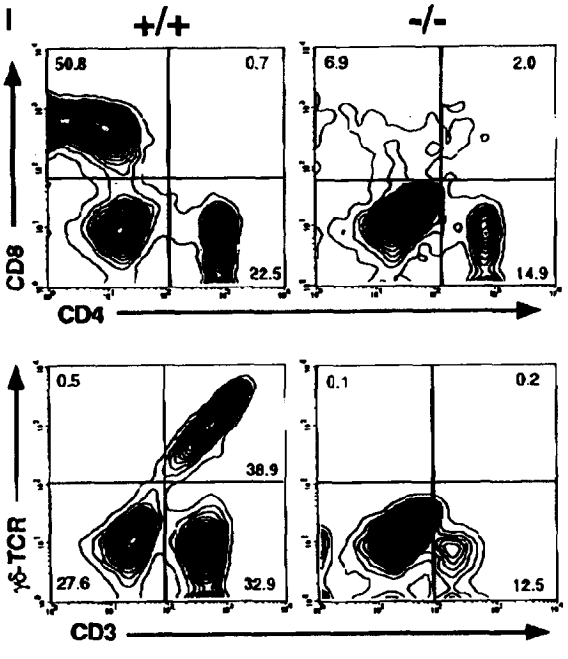


Figure 7. Immunohistological and Flow Cytometric Analysis of i-IEL in Small Intestine

(A–H) Immunohistological analysis of i-IEL. Lymphocytes from small intestine of 4-week-old wild-type (A, C, E, G) and Jak3$^{-/-}$ (B, D, F, H) mice were analyzed. Immunostaining for TCRγδ (A, B), CD4 (C, D), CD8 (E, F), and surface IgM (G, H) were shown. Original magnification, 100×.

(I) Flow cytometric analysis of i-IEL. The i-IELs were isolated from 4-week-old wild-type (+/+) and Jak3-deficient (-/-) mice by using the percoll gradient method as described in Experimental Procedures. Cells were gated for lymphocytes based on the forward- and side-scatter profiles. The numbers indicate the percentages of cells in each quadrant.

to those in γc-deficient mice, such as developmental defects of T and B cells; while both mutant mice have severe defect in B cells, human XSCID and T–B$^+$ SCID possessed normal B cell development; the absence of NK cells, DETC, and γδ$^+$ i-IEL; CD4$^+$ T cells increased as aging occurred as compared with CD8$^+$ T cells or B cells; mature T cells failed to respond to IL-2, IL-4, or IL-7.

However, we found an important difference between γc knockout and Jak3-deficient mice on the role of thymic epithelia cells and the structure of the thymus. We found that thymus in Jak3$^{-/-}$ mice was atrophic, the medullary–cortex junction became unclear, and Hassall's corpuscles were absent, which was confirmed by keratin and class II staining. This was a sharp contrast with the pathology of γc knockout mice, which showed a cortical rim of small lymphocytes and the presence of Hassall's corpuscles in the medulla (Cao et al., 1995). Taken together with the fact that Hassall's corpuscles are composed of thymic epithelial cells and that Jak3 is strongly expressed in cultured thymic epithelial cells in addition to thymocytes (unpublished data; Kuwata et al., 1995), Jak3 may play a role in signal transduction systems of thymic epithelial cells for

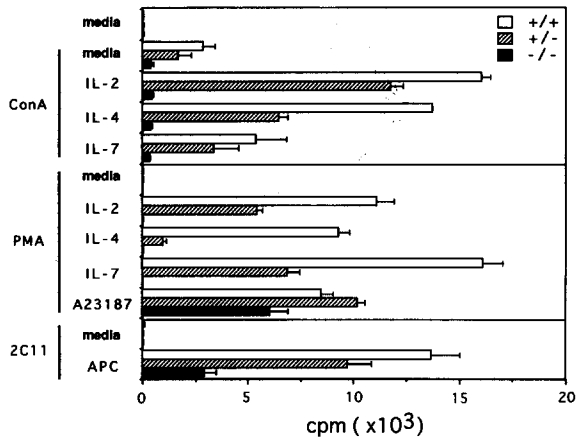


Figure 8. Proliferative Response of Thymocytes from Jak3-Deficient Mice

Thymocytes (5 × 10^4) from 4-week-old wild-type ($^{+/+}$), heterozygous ($^{+/-}$), and homozygous ($^{-/-}$) mutant mice were stimulated with ConA (2.5 μg/ml) or PMA (10 ng/ml) in the presence of IL-2, IL-4, or IL-7 for 48 hr, pulsed with 1 μCi of [^{3}H]thymidine for the final 8 hr to evaluate the proliferative responses against cytokines. As for 2C11 stimulation, autologous splenocytes (2.5 × 10^5) isolated from the littermate wild-type mice were used as accessory cells. [^{3}H]thymidine uptake with 2C11 plus APC alone in the absence of thymoctes was 1600 ± 286, 1476 ± 166, and 1815 ± 192 cpm for wild-type, heterozygous, and homozygous mutant mice, respectively. The results are shown as mean ± SD from triplicate cultures.

the support of thymocyte development. The depletion of Jak3 in thymic epithelia may lead to the structural abnormality. It is of interest to know whether the hypoplatic thymus of Jak3-deficient mice could regenerate when mature T cells are transferred and localized in the dysplatic medulla. In this respect, Jak3-deficient mice would provide a good model for study of the intrinsic and extrinsic regulation of thymic epithelial structure.

Interestingly, we found another significant difference between Jak3$^{-/-}$ mice and γc knockout mice. The proliferative responses to IL-2, IL-4, or IL-7 exhibited the gene–dose effect; the response of T cells from Jak3-heterozygous mice were reproducibly half of that from wild-type mice. Such dose effects can not be observed in γc-deficient mice due to the single allele of γc on X-chromosome, in even wild-type mice. To explain the observed gene–dose effect in Jak3$^{-/-}$ mice, one can speculate that intracellular kinase like Jak3 may associate with a half amount of target molecules when only a half of the kinase is produced.

Present analysis showed novel insights in a couple of aspects with which previous analysis on γc knockout mice have not dealt. The first one is the analysis of hematopoietic stem cells. We answered the question at which stage T cell development was blocked in Jak3-deficient mice. We analyzed hematopoietic stem cells in bone marrow as well as T cell precursors in the thymus. Despite the fact that the number of stem cells in bone marrow as characterized by Lin$^-$c-*kit*$^+$Sca-1$^+$ was indistinguishable between wild-type and Jak3$^{-/-}$ mice and that approximately the same number of all colonies other than lymphoid lineage were generated from the stem cells in bone marrow of Jak3$^{-/-}$ mice as those of wild-type mice, the number of CD4^{lo}CD8^{lo}c-*kit*$^+$ cells known as the early T cell precursors in thymus (Wu et al., 1991) was reduced to almost the background level. These results strongly suggest that Jak3 is involved in the maintenance and growth of T cell precursors in thymus but not in bone marrow. In other words, it is most likely that Jak3 plays a crucial role in the proliferation, function, or both of lymphoid stem cells. The fact that although the cellurality of Lin$^-$c-*kit*$^+$Sca-1$^+$ cells in the spleen was not changed by Jak3 deficiency, no T and B cells were developed in the spleen of young mutant mice also supports the idea that lymphoid stem cells were specifically damaged by Jak3 deficiency.

The second was the analysis of IEL population. We analyzed i-IEL population by histological sections and flow cytometry. The latter analysis demonstrated that γδ$^+$ T cells as well as CD8$^+$ T cells were completely absent and only small number of CD4$^+$ αβ$^+$ T cells were present in the intestine of Jak3$^{-/-}$ mice. Selective survival of CD4$^+$ T cells in Jak3$^{-/-}$ mice was also confirmed by the accumulation of those cells in thymus and spleen. Approximately half of the γδ$^+$ i-IEL were thought to be developed extrathymically. However, regardless of thymic and extrathymic development, all γδ$^+$ i-IEL required Jak3 signals for their development. This was also true for DETC cells. Collectively, NK cells, DETC cells, and γδ$^+$ i-IEL may have special requirement for their growth that is sensitive to *Jak3* mutation.

In human patients, the mutation in γc results in XSCID (Noguchi et al., 1993b). It has recently shown that the mutation or deletion of the *Jak3* gene induced autosomal recessive T–B$^+$ SCID (Macci et al., 1995). Although there are obvious differences between human patients and γc or Jak3-deficient mice, such as development of B cells, the Jak3-deficient mice provide a new type of SCID model mice and can be used for the model of gene therapy of autosomal recessive T–B$^+$ SCID.

Experimental Procedures

Cloning of *Jak3* Gene and Construction of the Targeting Vector

A murine *Jak3* genomic gene was isolated from a 129 genomic library prepared in EMBL3, using a probe (a full-length *Jak3* cDNA) derived from the rat *Jak3* cDNA (Takahashi and Shirasawa, 1994). The targeting vector, pK1, was constituted of a long arm (6.5 kb BglII–BglII fragment) and a short arm (1 kb PCR fragment) by using the following primers: 5′-GCTGCTATGCCTACTTTTGT-3′ for the upstream primer and 5′-TAAATCCTCAGCCAAGTAGT-3′ for the downstream primer), a *neo*-resistant gene from pKJ2 (Ohno et al., 1993), and the HSV–*tk* gene from pIC19R/MC1–TK (Mansour et al., 1988) as described in Figure 1A.

Generation of Jak3-Deficient Mice

E14-1 ES cells were cultured as previously described (Ohno et al., 1993). Of the targeting vector pK1, 25 μg was linearlized by XbaI digestion, and introduced by electroporation into 2.5 × 10^7 E14–1 cells as described (Kuhn et al., 1991). After selection in the presence of 150 μg/ml of G418 (GIBCO) and 2 μM gancyclovir (a gift from Syntex, Incorporated), surviving colonies were screened for homologous recombination by Southern blotting. DNA derived from ES cells were digested with EcoRV using a probe BglII–EcoRV fragment (Figure 1A, probe A). The positive clones were confirmed by BamHI digestion with

a *neo* gene as a probe. The ES clones carrying the disrupted *Jak3* gene were injected into C57BL/6 blastocysts isolated at day 3.5 of gestation, and blastocysts were subsequently transferred into the uteri of day 2.5 pseudopregnant BDF1 foster mothers. Resulted chimeras were bred with C57BL/6, and F1 mice carrying the mutated *Jak3* gene were intercrossed. F2 offsprings were analyzed for the genotype by Southern blotting to identify wild-type, heterozygous, and homozygous mice.

RT-PCR and Western Blotting

Total RNA was prepared from single cell suspensions of bone marrow cells, according to the procedure described previously (Chomczynski and Sacchi, 1987). cDNA were synthesized by reverse transcriptase (GIBCO) and PCR was performed using Jak3-specific primers (5′-CCAGACCAGCAGAGGGACTT for the top strand, and 5′-CCAAAGCGAACAGCAGTAGGC for the bottom strand) and β-actin specific primers (5′-TGGAATCCTGTGGCATCCATGAAAC, and 5′-TAAAACGCAGCTCAGTAACAGTCCG). The PCR condition was as follows: 94°C for 5 min, with 33 cycles for Jak3; and 25 cycles for β-actin at 94°C for 1 min, 57°C for 1 min, and 72° C for 1 min 30 s, then 72°C for 10 min.

Flow Cytometric Analysis

Cells from thymus, spleen, bone marrow, and small intestine (IEL) were incubated with fluorescence- or biotin-conjugated antibodies and analyzed on a FACScan (Becton Dickinson) using lysis II software. Cells ($1–5 \times 10^5$) were analyzed for each sample and viable cells were gated by propidium iodide exclusion. For multicolor analysis, cells were first incubated with anti-Fc receptor MAb 2.4G2 (Unkeless, 1979) to prevent nonspecific staining. The following antibodies were used: CD4 (RM 4-5), CD8α (53-6.7), CD8β (53-5.8), CD3ε (145-2C11), TCRαβ (H57-597), TCRγδ (GL-3), Thy1 (53-2.1), CD45R/B220 (RA3-6B2), surface IgM (R6-60.2), IL-2Rβ (TMβ1), Mac-1 (M1/70), Gr-1 (RB6-8C5), red cells (TER119). These antibodies were purchased from PharMingen (San Diego, California). Anti-IL-2Rγ MAbs (TUGm3) were provided by Dr. K. Sugamura. For the staining of NK cells, anti-NK MAb DX5 (a gift from Dr. L. Lanier) and fluorescein isothiocyanate (FITC) goat anti-rat antibody were used for indirect staining.

For analysis of hematopoietic stem cells, all lineage marker–positive cell were depleted from bone marrow cells by incubating with biotinylated anti-lineage markers (Mac-1, Gr-1, B220, CD4, CD8, and TER119) and with streptavidin-conjugated magnetic beads. These lineage marker–negative (Lin^-) cells were stained with MAbs against Sca-1 and c-*kit* and analyzed by FACStar Plus, using the FACS/DESK software.

Immunohistochemistry

For the immunostaining of class II, keratin, surface IgM, CD4, CD8, γδ TCR, 5 μm cryostat sections of thymus, spleen, and intestine from Jak3 homozygous, heterozygous, and wild-type mice were fixed in acetone at −20°C for 10 min. Nonspecific binding of avidin and biotin was prevented by using an ABC blocking kit (Vector Laboratories, Burlingame, California). Endogenous peroxidases were inhibited by a 20 min incubation with 0.3% hydrogen peroxide and then the sections were blocked with 10% goat serum and incubated with primary antibodies at 4°C overnight. Primary antibodies used in this study were antibodies against keratin (AE1/AE3, Boehringer Mannheim, Federal Republic of Germany), μ (Cappel, West Chester, Pennsylvania), CD4 and CD8 (PharMingen), MHC class II (Y3P, a gift from Dr. Kasai) and anti-γδ TCR MAb (3A10, a gift from Dr. S. Tonegawa). Dilution of primary antibodies were 1:100 for anti-class II, anti-keratin, and anti-μ; 1:50 for anti-CD4, 1:300 for anti-CD8, and 1:3 for anti-γδ TCR antibodies. The sections labeled by biotinylated antibodies (anti-class II and anti-μ) were washed with PBS and reacted with avidin–biotin complex (Vector Laboratories). The sections labeled by primary antibody were incubated with biotinylated anti-mouse IgG (Nichirei Company, Limited, Tokyo) or anti-rat IgG (Vector Laboratories) antibody at room temperature for 60 min followed by avidin–biotin complex. The peroxidase activity was developed with 0.05% 3,3′-diaminobenzidine and 0.01% H_2O_2 in 50 mM Tris–HCl (pH 7.6) for 2 min. The sections were counterstained by 4% methyl green.

Proliferation Assay

For lymphokine-dependent cell proliferation analysis, thymocytes from wild-type, heterozygous, and homozygous mice were cultured at a concentration of 5×10^4/well in 96-well round-bottomed plates, stimulated with RPMI, ConA (2.5 μg/ml), or ConA plus IL-2 (20 ng/ml), IL-4 (20 ng/ml), IL-7 (10 ng/ml), PMA (10 ng/ml), or PMA plus IL-2, IL-4, IL-7, and A23187 (300 ng/ml). Cells were also stimulated with anti-CD3 (2C11) MAb in the presence of 2.5×10^5 irradiated (3000 rads) splenocytes as accessory cells. Cells were cultured for 48 hr, pulsed with 1 μCi of [^{3}H]thymidine (20–40 Ci/mmol, ICN, Costa Mesa, California) for the last 8 hr, harvested, and ^{3}H uptake was measured with a μβ liquid scintillation counter. All assays were performed in triplicate.

Stem Cell Colony Assay

Lin^-, $Sca\text{-}1^+$, c-*kit*$^+$ cells were sorted from bone marrow of 2-week-old mice and 200 sorted cells were seeded on 35 mm culture plates containing α medium (GIBCO), 30% fetal calf serum, 50 μM 2-mercaptoethanol (Sigma), 1% deionized bovine serum (Sigma). 20 ng/ml murine stem cell factor, 20 ng/ml IL-3 , or 10 U/ml human erythropoitin were added for different colonies. Plates were incubated at 37°C in the presence of 5% CO_2. After 14 days of culture, the number and size of various colonies were analyzed. To confirm colony types, cell morphology was determined by staining of isolated colonies with May–Grunwald–Giemsa.

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