

Stat5 Is Required for IL-2-Induced Cell Cycle Progression of Peripheral T Cells

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Summary

Many cytokines activate two highly homologous Stat proteins, 5a and 5b. Mice deficient in both genes lack all growth hormone and prolactin functions but retain functions associated with cytokines such as erythropoietin. Here, we demonstrate that, while lymphoid development is normal, Stat5a/b mutant peripheral T cells are profoundly deficient in proliferation and fail to undergo cell cycle progression or to express genes controlling cell cycle progression. In addition, the mice lack NK cells, develop splenomegaly, and have T cells with an activated phenotype, phenotypes seen in IL-2 receptor β chain-deficient mice. These phenotypes are not seen in mice lacking Stat5a or Stat5b alone. The results demonstrate that the Stat5 proteins, redundantly, are essential mediators of IL-2 signaling in T cells.

Introduction

Cytokines regulate a variety of physiological responses through their interaction with receptors of the cytokine receptor superfamily. These receptors couple ligand binding to the transphosphorylation and activation of one or more of the receptor-associated *Janus* protein tyrosine kinases (Jaks) (Ihle, 1995). Following their activation, the Jaks phosphorylate the receptors and a variety of signaling proteins that are recruited to the receptor complex. Common substrates of Jak tyrosine phosphorylation are one or more members of the signal transducers and activators of transcription (Stats) (Darnell et al., 1994, 1997). Among the seven mammalian family members, the two highly related Stat5 gene products, Stat5a and Stat5b, have been of particular interest because of the broad spectrum of cytokines that induce their

tyrosine phosphorylation and activation. Our initial interest in the Stat5 proteins was based on their activation by cytokines that affect the myeloid lineages including erythropoietin (Epo), thrombopoietin (Tpo), interleukin-3 (IL-3), and granulocyte-macrophage colony-stimulating factor (GM-CSF). However, the Stat5 proteins are also activated by a variety of cytokines that affect lymphoid lineages. For example, IL-7, which is required for the early amplification and survival of lymphoid lineage progenitors, activates the Stat5 proteins (Lin et al., 1995), as do a number of cytokines affecting various lymphoid functions, including IL-2, IL-4, IL-9, IL-13, and IL-15 (Fujii et al., 1995; Gaffen et al., 1995; Gilmour et al., 1995; Hou et al., 1995; Wakao et al., 1995). The role for activation of the Stat5 proteins by the IL-2 receptor has been examined by introducing the IL-2 receptor chains into immortalized, myeloid lineage cells (Zamorano et al., 1998). Under these conditions, Stat5 was found to not be required for cell cycle progression. More recently, transgenic mice have been derived with IL-2 receptor β chain mutants that suggested that the region required for Stat5 activation was not essential for most peripheral T cell functions (Fujii et al., 1998).

We have previously described the derivation and characterization of mice that lack Stat5a, Stat5b, or both genes (Teglund et al., 1998). Viable mice were obtained in each case, including mutant mice that lacked both Stat5 proteins. The most evident phenotypes were associated with the loss of growth hormone and prolactin functions. Indeed, most of the phenotypic changes seen in prolactin receptor (Ormandy et al., 1997)- and growth hormone receptor (Zhou et al., 1997)-deficient mice were evident in the Stat5a/b mutant mice. Conversely, the hematopoietic lineages were largely unaffected, suggesting that in the responses to cytokines such as Epo, the activation of the Stat5 proteins is either not important or is redundant to other signaling pathways.

The initial characterization of the lymphoid lineages also indicated the lack of any alterations in the normal production of T or B lineage cells. However, as described in this manuscript, analysis of mature peripheral T cells demonstrated a profound inability to proliferate in response to T cell receptor ligation in the presence or absence of IL-2. The T cell defects are only revealed when both Stat5a and Stat5b are disrupted, indicating that the two Stat5 proteins function interchangeably in T cells. In this regard, the conclusions derived from Stat5a/b-deficient mice concerning the roles of the Stat5 proteins in peripheral T cells are substantially different from those derived from studies of mice deficient in only Stat5a (Nakajima et al., 1997a) or Stat5b (Imada et al., 1998), which failed to take into consideration the high degree of homology of the two Stat5 proteins. The inability to enter the cell cycle and proliferate is associated with a loss in the ability to express cyclin D2 and D3 as well as cdk6. Taken together, the phenotype of peripheral T cells from Stat5a/b-deficient mice resembles that of IL-2 receptor β chain-deficient mice, revealing an unexpected, crucial role for Stat5 in IL-2 signaling in peripheral T cells.

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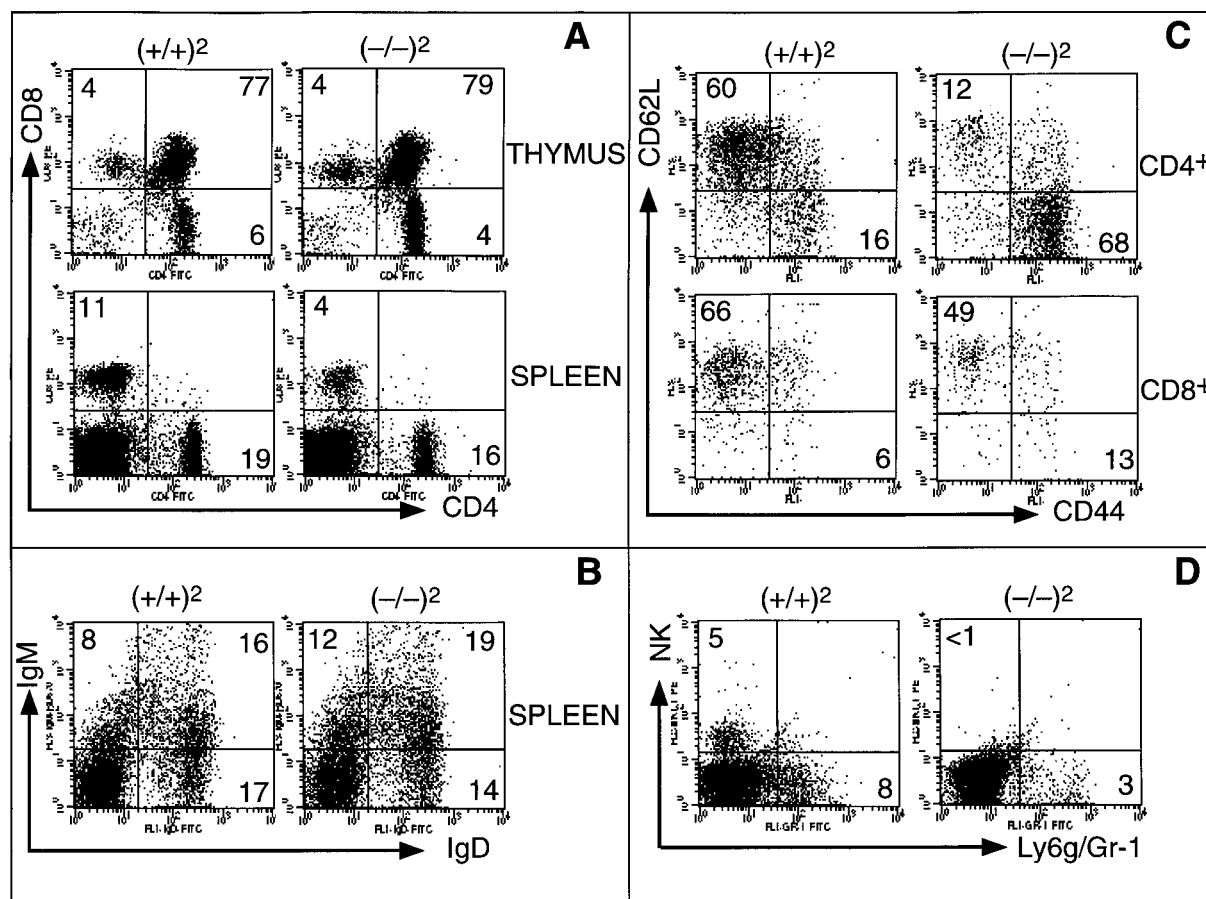


Figure 1. Flow Cytometric Analysis of Wild-Type and STAT5a/b Mutant Mice

(A) Thymic and splenic lymphocytes were obtained from individual 5-week-old Stat5a/b wild-type and mutant mice and stained with FITC-conjugated RM-4-4 MAb to CD4 and PE-conjugated 53-6.7.2 MAb to CD8.

(B) Spleen cells were also stained with FITC-conjugated 11-26c.2a MAb to mouse IgD and PE-conjugated goat anti-mouse IgM. The stained cells were then analyzed on a Becton-Dickinson FACScan in two-color mode using CellQuest software.

(C) Splenic T cells from 6-week-old Stat5a/b wild-type and mutant mice were stained with the PE-conjugated anti-CD4 (RM-4-4) or anti-CD8 (53-6.7.2) and counterstained with MAb FITC-IM7 to CD44 and biotinylated MAb MEL-14 to CD62L. The biotinylated antibody was then developed with streptavidin-red-670. The cells were analyzed in three-color mode. A gate was set on CD4⁺ and CD8⁺ cells and the expression of the activation markers assessed. T cells with an activated/memory phenotype are CD44^{high} and CD62L^{low} and naive T cells are CD44^{low} and CD62L^{high}.

(D) Spleen cells were stained with PE-conjugated 2B4 MAb for NK cells and FITC-conjugated RB6-8C5 MAb to the mouse granulocyte/neutrophil marker Ly6G/Gr-1.

Results

Normal Development of the Lymphoid Lineages in Stat5A/B Mutant Mice

To assess the contributions of Stat5a, Stat5b, or the two proteins in a redundant way to normal lymphoid development, a number of parameters were examined in 3- to 5-week-old mice. With regard to gross anatomy of lymphoid organs, there were no apparent defects in any of the mutants. The size and cellularity of the thymi and spleens from young mice were comparable among all the mutant mice and similar to those observed in control wild-type mice. However, as noted below, a fraction of the Stat5a/b mutant mice develop splenomegaly with age. Serum immunoglobulin levels were normal, both with regard to the concentrations and representation of the various isotypes (data not shown). As indicated in Figures 1A and 1B, the phenotypic properties

and the distribution of single- and double-positive T cells were also comparable to normal mice. In the periphery, the numbers of B cells were unaffected and both CD4 and CD8 single-positive T cells were present.

However, in the Stat5a/b mutant mice there were detectable changes in the T cell populations relative to controls or to Stat5a or Stat5b mutant mice. For example, there was a decrease in CD8 single-positive cells relative to CD4 single-positive cells (Figure 1A) with time and, also with time, a decrease in the total number of peripheral T cells. We also detected an increase over time in the expression of the activation antigen, CD44, on CD4⁺ and CD8⁺ lymphocytes, and a decrease in the percentage of T cells expressing L-selectin (CD62L) (Figure 1C). In addition, peripheral T cells were larger and had the morphology of activated T cells (data not shown). There was also an absence of NK cells (Figure 1D) and, consistent with this, there was no detectable

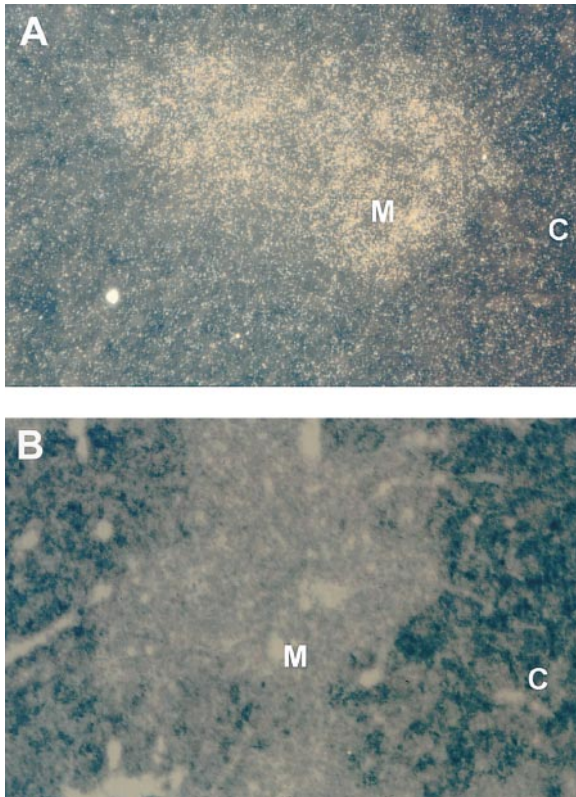


Figure 2. Expression of *CIS* in the Thymus Is Dependent upon the Stat5 Proteins

Thymi from wild-type (A) and Stat5a/b mutant (B) mice were fixed and embedded as described in Experimental Procedures. In situ hybridization was carried out essentially as described (Angerer and Angerer, 1992) using an [α - 32 P] UTP labeled antisense RNA probe transcribed from a plasmid containing a fragment (216 bp) of the murine cDNA for *CIS*. Tissue sections were counterstained with toluidine blue and a dark-field image obtained. The medullary (M) and cortical (C) regions of the thymi are indicated.

in vitro NK cytotoxic activity in the presence or absence of IL-12 or IL-15 (data not shown). Importantly, this phenotype is similar to that observed in mice lacking the IL-2 receptor α chain (Willerford et al., 1995) or the IL-2 receptor β chain (Suzuki et al., 1995). The results were consistent with the hypothesis that the Stat5 proteins are not required for the normal differentiation of the lymphoid lineages but may be required for peripheral T cell functions.

Stat5a, or Stat5b, Is Required for *CIS* Expression in Thymocytes

The absence of any effect on lymphoid numbers or thymocyte differentiation might suggest that another Stat protein, or another signaling pathway, could function redundantly to the Stat5 proteins in thymocyte development. We explored this possibility by examining the expression of a Stat5 regulated gene in the thymus. Previous studies have shown that the cytokine-induced SH2-containing gene (*CIS*) is transcriptionally regulated by the Stat5 proteins (Yoshimura et al., 1995). As illustrated in Figure 2, *CIS* expression was readily detected by in situ hybridization in the thymus of wild-type mice

and, unexpectedly, expression was primarily localized to the medullary regions of the thymus. This pattern of expression was also seen in the Stat5a and Stat5b mutant mice (data not shown). However, *CIS* expression was not detected in the thymus of a Stat5a/b mutant mouse (Figure 2). Thus, either Stat5a or Stat5b is required for the expression of *CIS* during T cell differentiation in the thymus, but, importantly, no additional pathways exist that are capable of inducing *CIS* expression in medullary thymocytes.

Inability of Peripheral T Cells from Stat5-Deficient Mice to Proliferate in Response to Anti-CD3 and IL-2

To assess the functionality of peripheral T cells, proliferation assays and cell cycle analysis were employed. Splenic lymphocytes were stimulated with anti-CD3 at various concentrations or with a limiting amount of IL-2 in the presence of varying concentrations of IL-2. As illustrated in Figure 3A, increasing concentrations of anti-CD3 induced the proliferation of splenic lymphocytes from wild-type mice as well as with lymphocytes from Stat5a- or Stat5b-deficient mice. However, there was a dramatically reduced proliferative response with cells from mice lacking both Stat5a and Stat5b at all of the anti-CD3 concentrations examined. In addition, splenic lymphocytes from Stat5a/b, but not Stat5a or Stat5b, mutant mice failed to significantly respond to increasing concentrations of IL-2 in the presence of anti-CD3 (Figure 3B). Importantly, increasing the concentrations of IL-2 to superphysiological levels (1000 units/ml), which would eliminate the required upregulation of the IL-2 receptor α chain, also failed to induce a proliferative response with cells from Stat5a/b mutant mice (Willerford et al., 1995). Similar results were obtained with FACS purified T cells from spleen or lymph nodes (data not shown). Consistent with the thymidine incorporation data, cell cycle analysis of Thy1.2 purified T cells demonstrated an inability of Stat5a/b-deficient cells to enter the cell cycle and accumulate S/G2 phase cells following stimulation with anti-CD3 and anti-CD28 in the presence of IL-2 (Figure 3C).

In addition to the response to anti-CD3 and IL-2, there was no proliferation in the response of Stat5a/b mutant T cells to ConA or to a combination of PMA and ionomycin (data not shown). There was also a comparable reduction in the response of Stat5a/b mutant cells to a combination of anti-CD3, anti-CD28, and increasing concentrations of IL-2 (data not shown). The results suggest that the Stat5 proteins are essential for T cell proliferation although Stat5a and Stat5b can fulfill this requirement in a completely redundant manner.

We also examined the proliferative response of B cells. In these experiments, spleen cells were stimulated with either LPS or anti-IgM, with or without IL-4, and thymidine incorporation was assessed after 3 days in culture. In contrast to the results obtained with T cells, there were no detectable differences between any of the mutant and wild-type control mice (data not shown). Therefore, the Stat5 proteins are not required for B cell differentiation or function in a nonredundant manner to other signaling pathways.

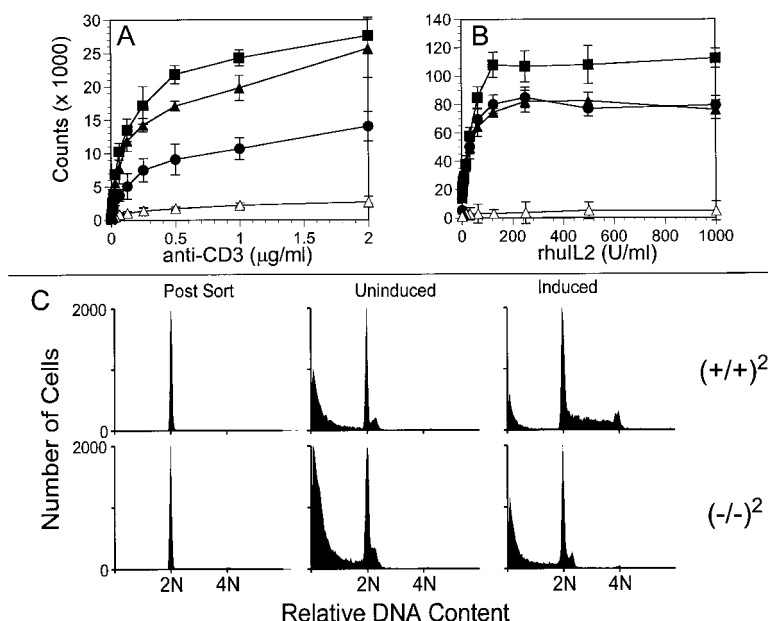


Figure 3. Peripheral T Cells from Stat5a/b-Deficient Mice Fail to Proliferate following Stimulation

(A) Splenic lymphocytes from wild-type mice (closed triangle), Stat5b-deficient mice (closed circle), Stat5a-deficient mice (closed square), or mice deficient in both Stat5a and Stat5b (open triangle) were stimulated with increasing concentrations of anti-CD3.

(B) The samples were as in (A) and the cells were stimulated with increasing concentrations of IL-2 in the presence of anti-CD3 (0.2 µg/ml).

(C) Thy1.2 positive cells were purified by FACS from splenic lymphocytes and were >98% Thy1 positive. The cells were cultured with plate-bound anti-CD3 and anti-CD28 and IL-2 (500 units/ml) or in media alone. The cells were incubated with propidium iodide at 48 hr or at 0 hr and examined by FACS for DNA content.

Peripheral T Cell Defects Are Rescued by Introducing Stat5 with Retroviral Vectors

The defect in peripheral T cells we detected was much more severe than suggested from studies of Stat5a-deficient mice (Nakajima et al., 1997a), Stat5b-deficient mice (Imada et al., 1998), or mutant IL-2 receptor β chain transgenic mice (Fujii et al., 1998), and suggested a more central role for the Stat5 proteins in T cell proliferation. Alternatively, the absence of Stat5a and Stat5b could alter T cell differentiation. Another possibility was that during the targeted disruption of the Stat5 genes other genes were affected that are responsible for the T cell phenotype. To distinguish among these possibilities, we assessed the ability to rescue T cell function by introducing Stat5 into lymphocytes with a retroviral vector. Splenic lymphocytes from wild-type or mutant mice were incubated for 2 days in the presence of anti-CD3 and IL-2 with a cell line producing a retroviral vector expressing wild-type Stat5a. As a control, lymphocytes were also incubated with a Stat5a that was inactivated by mutation of the tyrosine (Y⁶⁹³) required for phosphotyrosine-mediated dimer formation. Both viruses contained the green fluorescent protein (GFP) gene as a marker. Following initial activation and infection, the cells were incubated for 7–10 days and analyzed for cell markers and proliferation (Figure 4). After 7–10 days, cultures of lymphocytes from Stat5a/b-deficient mice exposed to the control virus, or the mutant-Stat5a(Y⁶⁹³F)-expressing virus contained few cells (data not shown). However, the cultures exposed to the Stat5a-expressing vector had increased cell numbers that were similar to the numbers seen in cultures of wild-type lymphocytes. Staining of lymphocytes from wild-type mice for the expression of GFP indicated that relatively few of the cells were infected (Figure 4A, left panel). In contrast, approximately 82% of the lymphocytes from cultures of Stat5a/b-deficient peripheral T cells expressed GFP (Figure 4A). Among the cells, approximately 90% expressed CD8, while 10% were CD4 positive, comparable

to the distribution seen in cultures of lymphocytes from wild-type mice (data not shown). The differences between the percentage of GFP positive lymphocytes from cultures of wild-type or Stat5a/b-deficient peripheral T cells are consistent with the hypothesis that only with restoration of Stat5 can the peripheral T cells from Stat5a/b-deficient mice proliferate in culture. Consistent with this hypothesis, Stat5a/b-deficient lymphocytes expressing the Stat5a-GFP virus proliferated in response to anti-CD3 and IL-2 comparable to cultures of lymphocytes from wild-type mice (Figure 4B). Therefore, a transcriptionally active Stat5 dimer is essential for the proliferative response of peripheral T cells.

T Cell Receptor Activation of CD25 and Cytokine Production Are Unaffected by the Absence of Stat5 Proteins

The activation of peripheral T cells and cell cycle progression requires signals derived from both the T cell receptor and the IL-2 receptor. Engagement of the T cell receptor is required for the increased expression of the IL-2 receptor α chain (CD25) and the formation of a high-affinity IL-2 receptor. As illustrated in Figure 5A, the ability of anti-CD3 to induce the expression of the IL-2 receptor α chain on CD4⁺ or on CD8⁺ (data not shown) lymphocytes was comparable between wild-type and Stat5a/b mutant lymphocytes. In addition, the expression of the IL-2 receptor β chain and the common γ_c chain were not altered in any of the mutant mice (data not shown). The induction of Fas ligand expression, which relies on IL-2 signaling (Refaeli et al., 1998), was also not altered in lymphocytes from Stat5a/b mutant mice (data not shown).

Engagement of the T cell receptor on CD4⁺ T cells activates signaling pathways that control the production of cytokines. As shown in Figure 5B, peripheral lymphocytes from both wild-type and Stat5a/b mutant mice were capable of producing cytokines in response to anti-CD3. However, the Stat5a/b-deficient lymphocytes

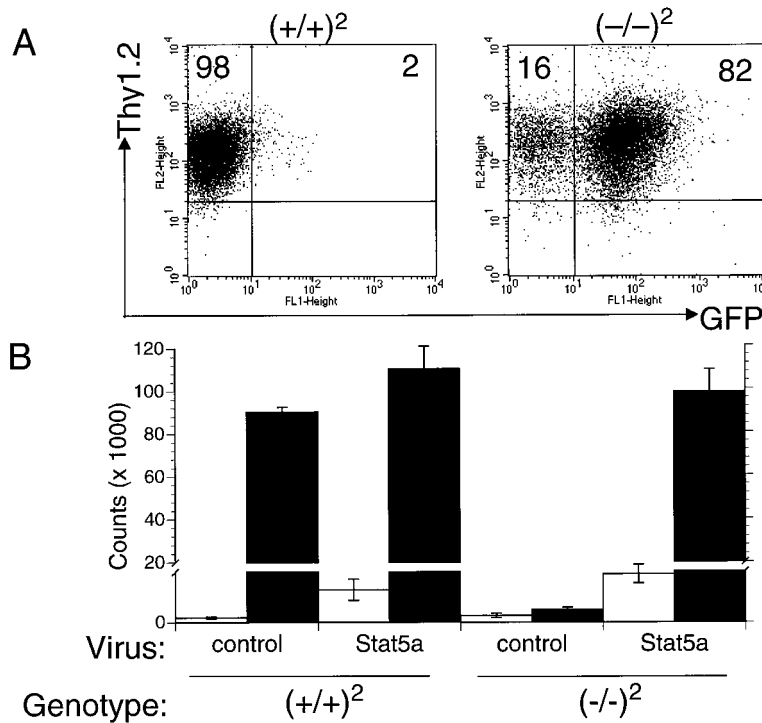


Figure 4. Rescue of Peripheral T Cell Function by the Retroviral Transduction of Stat5a. Splenic lymphocytes from wild-type mice or Stat5a/b-deficient mice were incubated with an MSCV based retrovirus capable of expressing green fluorescent protein (GFP) and Stat5a or with a control virus expressing only GFP for 2 days in the presence of anti-CD3 (2.5 μ g/ml) and IL-2 (500 units/ml). The cells were subsequently expanded for 5 days in the presence of IL-2. (A) Cells from cultures of Stat5a/b-deficient lymphocytes, incubated with the Stat5a/GFP virus, were analyzed for expression of Thy-1 and GFP. Lymphocytes from cultures of wild-type peripheral T cells are shown on the left while the lymphocytes from Stat5a/b-deficient peripheral T cells are shown on the right. Approximately 82% of Thy 1.2 positive cells from cultures of peripheral T cells from Stat5a/b-deficient mice expressed GFP. (B) Viable cells from the cultures were collected and proliferation in response to anti-CD3 (2.5 μ g/ml) in the presence of IL-2 (500 units/ml) was examined by ³H-thymidine incorporation. The open bars indicate the responses in the absence of anti-CD3 and IL-2 while the closed bars indicate the response in the presence of anti-CD3 and IL-2. The viral constructs utilized and the genotype of the cells are indicated at the bottom.

consistently produced less IL-2 and more IFN- γ than control lymphocytes. Comparable results were obtained by RT-PCR and by analysis of cytokine-producing cells with an ELISPOT assay (data not shown). The change in cytokine production reflects the predominance of CD62L⁻/CD44⁺ T cells in the mutant mice; these T cells primarily produce IFN- γ (Kanegane et al., 1996). Together, the results demonstrate that the signal transduction through the T cell receptor is intact in the Stat5a/b-deficient mice.

Previous studies have suggested that Stat5a contributes to IL-2-mediated regulation of CD25 in activated T cells (Nakajima et al., 1997a). To confirm this observation, we examined the ability of IL-2 to sustain CD25 expression on activated T cells from our various mutant strains. Unexpectedly, there was no defect in maintenance of CD25 expression on lymphocytes from Stat5a-deficient mice (Figure 5C) nor was there a defect on lymphocytes from Stat5b-deficient mice (data not shown). However, the expression of CD25 on lymphocytes from Stat5a/b-deficient mice was not maintained in the presence of IL-2 consistent with a role for the Stat5 proteins, redundantly, in CD25 expression at this stage of lymphocyte differentiation.

The expression of CD25 also occurs during early lymphoid differentiation, during a phase of development in which expansion of the lymphoid progenitors is dependent upon IL-7. The ability of IL-7 to activate Stat5 suggested the possibility that at this stage of lymphocyte development, CD25 expression may also require Stat5. However, normal numbers of CD25⁺ double-negative thymocytes were present in thymi from Stat5a/b-deficient mice (data not shown).

Stat5 Proteins Are Required for the Expression of Genes that Regulate the Cell Cycle

The activation of peripheral T cells is associated with changes in the levels of a number of proteins that are involved in cell cycle progression. For example, the activation of peripheral T cells results in the loss of the cell cycle kinase inhibitor, p27, a response that is attributed to IL-2 signaling and hypothesized to be critical for entry into the cell cycle (Nourse et al., 1994). As illustrated in Figure 6A, activation of lymphocytes from either wild-type or Stat5a/b-deficient mice resulted in a comparable loss of p27. Therefore, p27 turnover is not regulated by Stat5 but may be regulated by another IL-2 receptor-dependent signaling pathway or through the T cell receptor.

The activation of peripheral T cells is also associated with the induction of the expression of cyclin D2, cyclin D3, and Cdk6 (Ajchenbaum et al., 1993; Lucas et al., 1995), which are required for G1 cell cycle progression. As shown in Figure 6A, cyclin D2, cyclin D3, cyclin A, and Cdk6 were induced in splenic T cells from wild-type mice by treatment with anti-CD3 in the presence or absence of exogenously added IL-2. In addition, the levels of cyclin E increased following stimulation of wild-type cells. Similarly, the ability to induce the expression of cyclin D2, cyclin D3, cyclin A, and Cdk6 was unaltered in peripheral lymphocytes from Stat5a or Stat5b mutant mice (Figure 6B). However, the induction of these proteins did not occur in lymphocytes from Stat5a/b mutant mice following stimulation with anti-CD3 alone or in the presence of exogenously added IL-2 (Figure 6A). In addition, the increases in cyclin E levels seen in wild-type cells were not observed in the Stat5a/b-deficient cells.

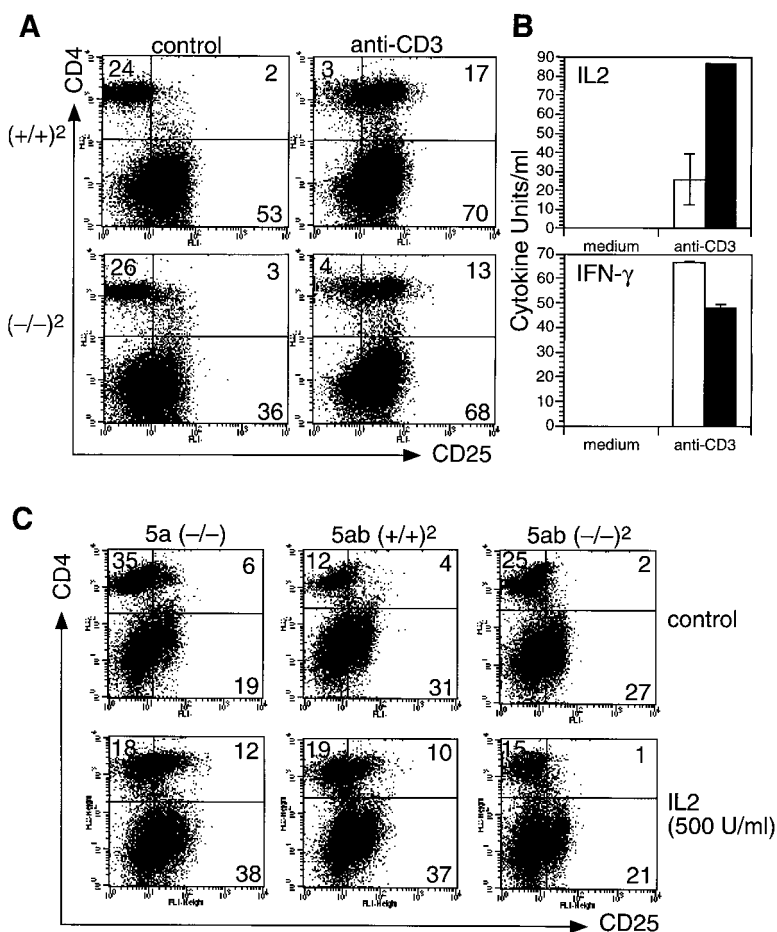


Figure 5. CD25 Expression and Cytokine Production Are Not Altered but IL-2 Sustained Expression of CD25 Is Lost in Stat5a/b-Deficient Mice

(A) Splenic lymphocytes from control (top) or Stat5a/b-deficient mice (bottom) were incubated overnight in the presence or absence of anti-CD3 (2 μg/ml). The cells were subsequently examined by FACS for the expression of CD4 and CD25.

(B) Lymph node lymphocytes were obtained from either control or Stat5a/b-deficient mice and were incubated in either media alone or with anti-CD3 (4 μg/ml) for 48 hr. The supernatants were subsequently collected and the levels of cytokines were determined by ELISA assays.

(C) Cells from Stat5a/b wild-type [5AB(+/+)], Stat5a mutant [5A(-/-)], and Stat5a/b mutant mice [5AB(-/-)] were cultured for 48 hr with plate-bound anti-CD3 (10 μg/ml) for 24 hr, washed, and then cultured an additional 48 hr in new plates with fresh medium alone (control) or supplemented with 500 U/ml recombinant human IL-2. CD25 expression on CD4⁺ spleen cells was analyzed by FACS after staining with FITC-PC61 Mab.

In contrast, the expression of cdk4 and cdk2 were not dependent upon Stat5.

Stat5a/b Mutant Mice Develop Splenomegaly with Age

The Stat5a/b mutant mice, but not the Stat5a or Stat5b mutant mice, were also observed to develop splenomegaly with age. As indicated in Figure 7A, approximately one fourth of the mice had spleens that were 4- to 10-fold larger than normal at 4-5 weeks of age and this increased to approximately half of the mice at 10-12 weeks of age. Histologically, the structure of the enlarged spleens was detectably altered as illustrated by a comparison of a spleen from a wild-type animal (C) and a spleen from a Stat5a/b mutant animal with splenomegaly (D). The altered size and morphology was characterized as a hyperplasia of the red pulp with extensive extramedullary hematopoiesis. This increase included the granulocytic and megakaryocytic lineages, but the erythroid elements were particularly numerous. To further examine the lineages of cells contributing to the splenomegaly, FACS analysis and colony assays were done. Remarkably, the expanded cell population lacked any of the markers examined including T cell lineage markers (CD3, CD4, CD8, and Thy1) or B cell lineage markers (B220, IgM, and IgD). Myeloid lineage markers (Gr1, Nk1, pan Nk marker, CD11b, and CD34) were also not increased, but there was a dramatic increase in

Ter119 positive cells, a marker for early erythroid lineage cells (Figure 7E). Consistent with the histological data, colony assays revealed a dramatic increase in Epo responsive colonies.

Discussion

The results demonstrate an unexpected and remarkably specific requirement for the Stat5 proteins in cell cycle progression of peripheral T cells in response to T cell receptor ligation and IL-2 receptor activation. Moreover, the lack of any phenotype in the Stat5a or Stat5b mutant T cells demonstrate that either protein can function redundantly in supporting peripheral T cell proliferation. A role for the Stat5 proteins in T cell proliferation was unexpected based on the existing literature. In particular, the activation of Stat5 by the IL-2 receptor was shown to require the carboxy-terminal sequences of the β chain, a region that is not required for a proliferative signal in a myeloid cell line (Fujii et al., 1995). More recently, it was concluded that Stat5 was involved in IL-2-mediated protection from apoptosis but was not involved in cell cycle progression (Zamorano et al., 1998). However, both studies involved reconstitution of the IL-2 receptor in immortalized myeloid cell lines and, as previously demonstrated by the phenotype of Stat5-deficient mice (Teglund et al., 1998), myeloid cells do not require Stat5 for proliferation.

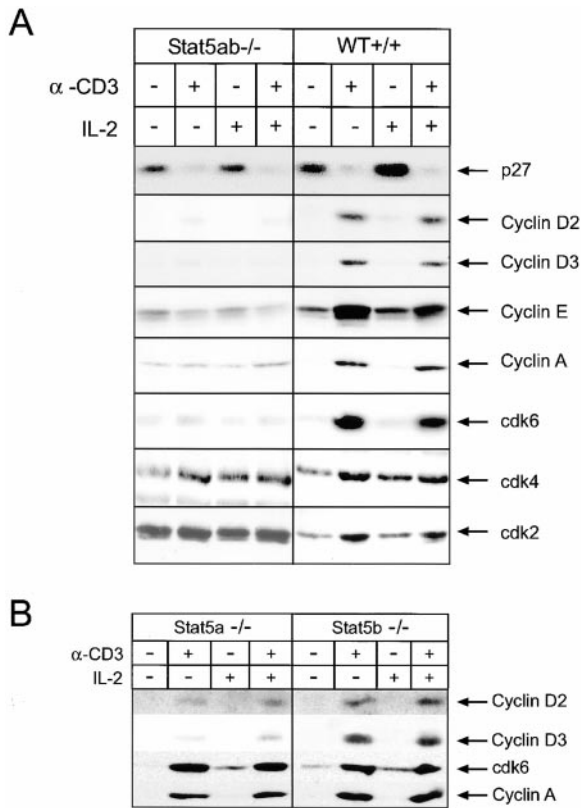


Figure 6. Lack of Induction of Expression of Cell Cycle Related Genes in T Cells from Stat5a/b-Deficient Mice

(A) Splenic lymphocytes were obtained from control or Stat5a/b-deficient mice and were cultured in media, media containing anti-CD3 (2.5 μg/ml), media containing IL-2 (100 units/ml), or both anti-CD3 and IL-2 as indicated. Following 18 hr of culture, the cells were collected and the levels of the various proteins assessed by Western blotting. (B) The samples were treated as in (A) but the splenocytes were obtained from Stat5a- or Stat5b-deficient mice.

A potential role for Stat5 activation by the IL-2 receptor has also been examined by deriving transgenic lines of mice expressing mutant receptors on the IL-2 receptor β chain-deficient background (Fujii et al., 1998). Transgenic lines lacking the membrane distal region of the receptor, which has been implicated in Stat5 activation as well as the activation of other signaling pathways, have a subtle phenotype that includes a lack of NK cells, γδ T cells, and a reduced proliferation at low concentrations of IL-2. Based on the phenotype of the Stat5a/b-deficient mice, it must be concluded that some degree of activation of Stat5 proteins was occurring in the mice expressing the truncated receptor either through the mutated β chain or through another receptor-associated protein. In this regard, it should be mentioned that the lack of Stat5 activation in Jak3-deficient T cells precludes a contribution to Stat5 activation by the T cell receptor complex (our unpublished data).

Because of the unexpected nature of the defect, we considered the possibility that the absence of one or both Stat5 proteins might influence differentiation rather than peripheral T cell function directly. Even more indirectly, as with any gene disruption, it was possible that

the phenotype was due to secondary effects of the genetic manipulations. That these possibilities are not relevant is demonstrated by the ability to rescue peripheral T cell function by introducing Stat5a with retroviral constructs. As illustrated in the studies presented here, rescue of peripheral T cell proliferation occurred with the wild-type protein but not with a mutant that lacks the critical tyrosine for phosphorylation and dimer formation. This approach is currently being used to explore the role of dimer-dimer formation or serine phosphorylation for Stat5 function in T cells.

A previous study (Nakajima et al., 1997a) concluded that Stat5a played a minor role in IL-2-mediated IL-2 receptor α chain induction and that this function indirectly affected IL-2-induced proliferation. More recently, the same group described relatively minor alterations in NK cell functions in Stat5b-deficient mice (Imada et al, 1998). Our results and conclusions are significantly different from those reported. Specifically, we have not found any evidence for a unique role of Stat5a relative to Stat5b in peripheral T cell function, including either IL-2 regulation of CD25 or NK cell function. The reason for this difference is not known but may be due to a partial effect of the disruption of the Stat5a gene on the level of expression of the adjacent Stat5b gene in the Nakajima et al. (1997a) studies. Indeed, in the Nakajima et al. (1997a) studies there was a decrease of Stat5b levels in some mice; however, the authors concluded that this did not correlate with phenotype. This critical issue was not addressed in the studies of Stat5b-deficient mice. Irrespective, the studies with our independently derived mutant strains demonstrate that the conclusion that Stat5a and Stat5b have unique, nonredundant functions in T cells is incorrect.

The most significant difference between the Nakajima et al. (1997a) studies and our studies is the conclusion that the effects of Stat5, either individually or redundantly, on IL-2 receptor α chain expression can explain the decreased proliferation of peripheral T cells. As demonstrated here, the profound inability of peripheral T cells from Stat5a/b-deficient mice to proliferate cannot be rescued by concentrations of IL-2 that would bypass the requirement for α chain expression. Whether the decreased proliferation reported by Nakajima et al. (1997a) is due to a decrease in α chain expression or is due to a decrease in the levels of Stat5 to a point that it becomes limiting is an unanswered question.

Recent studies have also examined the role of Stat3 activation in T cell functions through the generation of mice that lack Stat3 in the lymphoid lineages (Takeda et al., 1998). Consistent with the results presented, we have observed little, if any, activation of Stat3 in response to IL-2 although Stat3 is strongly activated by IL-6. It should also be noted that the levels of Stat3 are only slightly reduced in Stat5a/Stat5b-deficient mice (Teglund et al., 1998). The low proliferative response of peripheral T cells to IL-6, in combination with anti-CD3, has precluded us from determining whether this response is also affected in Stat5a/Stat5b-deficient mice. Irrespective, Takeda et al. (1998) concluded that Stat3 was only required for prevention of apoptosis through a Bcl-2 independent pathway. Therefore, it is likely that Stat3 functions are quite distinct from those of the Stat5 proteins in peripheral T cells.

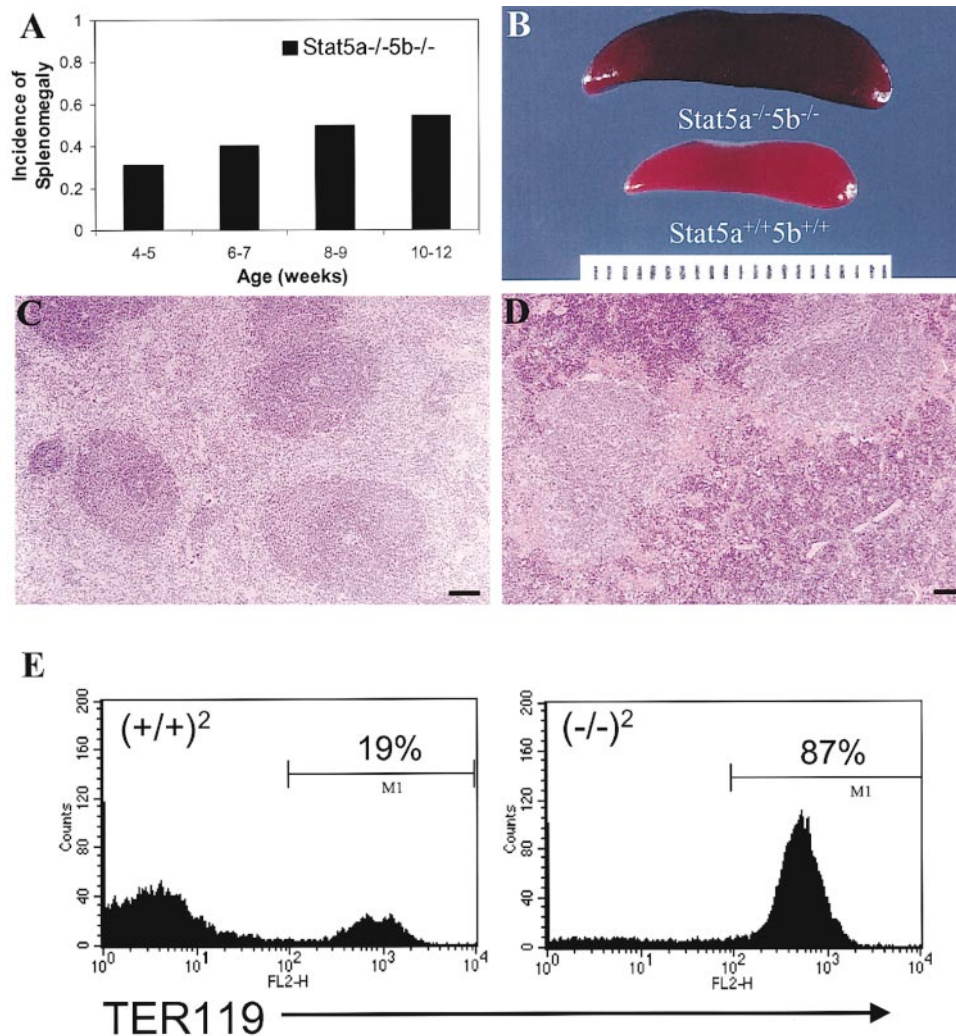


Figure 7. Splenomegaly Develops in Stat5a/b-Deficient Mice

(A) Incidence of splenomegaly in Stat5a^{-/-}5b^{-/-} mice at 4–5 weeks (n = 16), 6–7 weeks (n = 20), 8–9 weeks (n = 14), and 10–12 weeks of age (n = 22).

(B) Spleen from an 8-week-old Stat5a^{-/-}5b^{-/-} mouse (top) and wild-type littermate control (bottom).

(C and D) Hematoxylin-eosin stained sections (4 μm) of spleens in (B); bar scale = 100 μm.

(E) FACS analysis of splenocytes from a Stat5a/b-deficient mouse with splenomegaly.

The lymphoid lineage phenotypes displayed by the Stat5a/b-deficient mice have many similarities with those of mice in which the IL-2 receptor β chain is disrupted (Suzuki et al., 1995). Similar to the β chain mutant mice, thymocyte development was normal. The two mutant strains are also similar in the profound effect of the mutations on the response of T cells, including the responses to PMA and ionomycin that would have been predicted to bypass the IL-2 receptor pathway. Also similar to the β chain mutants, the Stat5a/b mutant mice develop a myeloproliferative disease, although the IL-2 receptor β chain mutants were reported to have a predominance of the granulocytic lineage. Also similar to the IL-2 receptor β mutants, NK cells are not present (Suzuki et al., 1997). However, the two mutants differ in the absence of the B cell defects in Stat5a/b mutant mice that are found in the β chain mutants. These included

elevated IgG₁ and IgE and the development of autoimmune antibodies. It is possible that the differences are related to the expression of the IL-2 receptor on non-T cells, including B cells, which do not require the activation of the Stat5 proteins for their functional responses.

Like the peripheral T cells from the IL-2 receptor β chain-deficient mice, Stat5a/b mutant mice increasingly with age have phenotypically activated T cells. Interestingly, this phenotype has been observed in a number of conditions of immunodeficiency including that associated with the absence of Jak3 (Saijo et al., 1997), the IL-2 receptor common γ chain (Nakajima et al., 1997b), and the IL-2 receptor α chain (Willerford et al., 1995), as well as the IL-2 receptor β chain as mentioned above. The similarity would suggest that the Stat5 proteins play an essential role in IL-2-mediated negative selection of autoreactive T cells.

Our results demonstrate that genes that have been implicated in cell cycle progression are regulated by the Stat5 proteins in the response to IL-2. Previous studies demonstrated that cyclin D2 and D3, but not D1, were induced following T cell activation (Ajchenbaum et al., 1993). The ability of cyclosporin A to block this induction suggested a role for IL-2 signaling. The possibility that cyclin D2 and/or D3 are regulated in T cells by the Stat5 proteins is supported by the observation that prolactin, which strongly induces Stat5 activation, induces cyclin D2 and D3 expression in the rat pre-T-lymphoma cell line, Nb2 (Hosokawa et al., 1994). Moreover, the promoters for both cyclin D2 and D3 contain Stat5 binding sites (our unpublished data). Therefore, it will be important to further determine whether D2 and D3 are immediate early genes in T cells and therefore potentially directly regulated by the Stat5 proteins.

The results also suggest that the Stat5 proteins may be involved in the regulation of the Cdk6 gene. Previous studies have shown that Cdk6 is present as the major cyclin D2 and D3 associated kinase in T lymphocytes and that its increased accumulation is one of the earliest detectable events after mitogen stimulation (Lucas et al., 1995). However, the observation that the increased Cdk6 expression was not inhibited by cyclosporin A or FK506 suggested that the increase was independent of IL-2 signaling. In contrast, the regulation of p27 levels in lymphocytes from Stat5a/b mutant mice was normal. Regulation of p27 levels occurs both at the protein level and at the transcriptional level. In both cases, the ability of cyclosporin A to block the downregulation has implicated a role for IL-2 signaling events. Our data would suggest that if IL-2 signaling is involved in p27 regulation, it involves a Stat5 independent pathway.

In summary, the derivation of Stat5a/b-deficient mice has revealed an unexpected but essential role for Stat5 in the regulation of peripheral T cell activation by IL-2. With this information, efforts can be now focused on the mechanisms by which Stat5 regulates cell cycle progression of activated T cells. The results also suggest that drugs that target Stat5 function may have utility in controlling peripheral T cell expansion and function.

Experimental Procedures

T Cell Isolation, Culture, and Activation Conditions

Splenic T cells were isolated from 8- to 12-week-old wild-type or Stat5a/b-deficient mice without splenomegaly. In brief, fresh isolated organs were meshed and passed through a cell strainer (70 μ m; Becton Dickinson) to separate fibrous tissue. The splenic cell solution was kept in T cell culture media. The media consisted of RPMI 1640, 10% fetal bovine serum (HyghClone), 10 mM Hepes (pH 7.0), 2 mM L-glutamine, (1 \times) nonessential amino acids, 1 mM sodium pyruvate, and 50 μ M 2-mercaptoethanol. The T cells were centrifuged and a red blood cell lysis was performed for 5 min using a lysis buffer (pH 7.3) containing 150 mM NH₄Cl, 1 mM KHCO₃, and 0.1 mM Na₂EDTA at 10⁶ cells/ml. Lysis was stopped by adding an excess (4 \times) of T cell media and centrifugation, followed by three subsequent wash and centrifugation steps with T cell culture media. The splenic cells were resuspended at 10⁶ cells/ml culture media. T cell receptor activation after CD3 ligation was induced by addition of the monoclonal antibody 145.2C11 (Pharmingen) into the culture media (1 μ g/ml culture media) if not detailed differently. Recombinant human IL-2 (Boehringer Mannheim) was added at 100 units/ml medium if not stated otherwise.

In Situ Hybridization

For in situ hybridization, thymi were fixed with 4% paraformaldehyde (Sigma), and 6 μ m sections were prepared from frozen tissue samples. In situ hybridization was carried out essentially as described (Angerer and Angerer, 1992) using [γ -³³P]UTP-labeled antisense RNA probes transcribed from a plasmid containing murine cDNA fragments of *Cis* (216 bp). Tissue sections were counterstained using toluidine blue (Sigma).

Protein Analysis

Harvested cells were washed twice with PBS and lysed in Tween-20 buffer containing 50 mM Hepes buffer (pH 7.5), 0.1% Tween-20, 150 mM NaCl, 1 mM EDTA, 20 mM β -glycerophosphate, 0.1 mM sodium orthovanadate, 1 mM sodium fluoride, 10 μ g/ml each aprotinin and leupeptin (both from Sigma), and 1 mM PMSF. After brief sonication, lysates were cleared of nuclear debris by centrifugation at 14,000 g for 15 min. Protein concentrations were determined using the BCA-kit as recommended by the manufacturer (Pierce). To assess expression levels of cyclin D2 and cyclin D3, 200 μ g total protein/sample was electrophoretically resolved on a 12.5% polyacrylamide gel containing SDS and transferred onto NitroPure membranes (MicronSeparationsInc.). Membranes were either probed with a rat monoclonal antibody directed against cyclin D2 (34B1-3) or directed against cyclin D3 (18B6-10) followed by rabbit anti-rat IgG (Vallance et al., 1994). Sites of antibody binding were detected using protein A-conjugated horse-radish peroxidase (EY Laboratories) with the ECL chemiluminescent detection kit (Amersham). To assess expression levels of p27^{Kip1}, cyclin E, cyclin A, and the cyclin dependent kinases cdk4, cdk6, and cdk2, 100 μ g total protein/sample was electrophoretically resolved on a 12% polyacrylamide gel containing SDS and transferred onto NitroPure membranes. The membranes were either probed with affinity-purified antiserum directed against the full-length mouse p27 (Kato et al., 1994) or affinity-purified antibodies against cyclins or cyclin-dependent kinases (Santa Cruz). Sites of antibody binding were detected using protein A-conjugated horse-radish peroxidase as described above.

Flow Cytometry

Thymus and spleen obtained from individual mice were made into single cell suspensions in PBS supplemented with 1% bovine serum albumin (PBS/BSA). Nonspecific staining was minimized by blocking with 10% normal mouse serum in PBS/BSA. The cells were then stained with cocktails of MAb's in PBS/BSA conjugated to fluorescein isothiocyanate (FITC), phycoerythrin (PE), or biotin for 30 min at 4°C. The cells were washed with PBS/BSA, and the biotinylated antibodies were developed with streptavidin conjugated to red 670 (Life Technologies, Gaithersburg, MD). All MAb's with the exceptions of anti-CD25 and anti-IgM were purchased from Pharmingen (San Diego, CA) and can be referenced in their current catalog. The FITC-anti-CD25 MAb clone PC61 was purchased from Accurate Chemical and Scientific Corp. (Westbury, NY) and the anti-mouse IgM from Southern Biotechnology Associates, Inc. (Birmingham, AL). The cell surface expression of the markers was analyzed in a Becton-Dickinson FACScan in two- or three color mode using CellQuest software.

Proliferation Responses

Single cell suspensions of spleen cells in SMEM (Life Technologies, Gaithersburg, MD) supplemented with 10% fetal bovine serum (Atlanta Biologicals, Norcross, GA), L-glutamine, penicillin, streptomycin, gentamycin (all from Life Technologies), sodium bicarbonate (Sigma, St. Louis, MO), essential and nonessential amino acids (Life Technologies), and 2-mercaptoethanol (Sigma) were plated in 96-well round-bottom plates (Corning, Corning, NY) at a density of 1 \times 10⁵ per well in 100 μ l. Stimuli were added as indicated at a range of concentrations to assess dose dependency. The stimuli were anti-CD3 (145.2C11), anti-CD28 (37.51) (both antibodies from Pharmingen, San Diego, CA), recombinant human IL-2 (Chiron, Emeryville, CA), and recombinant murine IL-4 (R&D Systems, Minneapolis, MN). Proliferation was assessed by measuring radioactivity using a Matrix 9600 beta counter (Packard, Meriden CT) 18 hr after adding 1 μ Ci/well ³H-thymidine (Amersham Life Sciences, Arlington Heights, IL).

Retroviral Constructs

A bicistronic retroviral vector, using the murine stem cell virus (MSCV) long terminal repeats, was utilized for expression of Stat5a or Stat5a(Y⁶⁹³F). The methods utilized for preparation of the constructs and methods for obtaining high-titered viral stocks have been described in detail (Persons et al., 1997, 1998). Splenic cells were isolated as above and cocultured on irradiated (1500 rads) ecotropic producer cell lines for 48 hr in the presence of 3 μ g/ml anti-CD3, 500 units/ml of IL-2, and 6 μ g/ml polybrene (Sigma). Semiconfluent cultures were divided 1:4 and subcultured with 500 units/ml of IL-2. For proliferation assays, cells were washed three times in T cell media and cultured at 2×10^5 per well in 100 μ l with media alone or anti-CD3 and IL-2 as above. After 24 hr, ³H-thymidine was added for an additional 24 hr and the incorporation determined as above.

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