MOLECULAR BIOLOGY INTELLIGENCE UNIT 20

Alister C. Ward

The Jak-Stat Pathway in Hematopoiesis and Disease

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Dedication

To my wife, Tania, for her unwavering support, and our son, Samuel, for making it all worthwhile.

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PREFACE

Blood cell development—or hematopoiesis—is a complex, multi-step process known to be controlled by a range of extracellular signals, including cytokines and growth factors. The elucidation of the Janus kinase-signal transducer and activator of transcription (Jak-Stat) pathway represents one of the great advances in our understanding of how these various signals can facilitate rapid alterations in gene expression in hematopoietic and other target cells. This book aims to describe the role of the Jak-Stat pathway in the normal development and function of hematopoietic cells, and to describe how perturbations of this pathway contribute to several hematopoietic disorders, including malignancy.

In Chapter One, Tetsuya Nosaka describes the function of the four members of the mammalian Jak tyrosine kinase family and details their role in normal hematopoiesis as revealed by gene targeting. In Chapter Two, Nicholas Cacalano and James Johnston focus more specifically on Jak3, revealing its close association with both interleukin-2 receptor and common gamma chain signaling in hematopoiesis, and the consequences of its ablation in immunodeficiency syndromes. In Chapter Three, Thomas Smithgall explores an exciting new area involving activation of Stats by non-Jak tyrosine kinases: specifically Src, Fes and Btk. In Chapter Four, I detail the function of Stats in normal hematopoietic processes, which is extended by David Frank in Chapter Five to include their role in leukemia and the implications of this for potential therapeutic intervention. In Chapter Six, Erika Bach and Norbert Perrimon describe the role of the Jak-Stat pathway in hematopoiesis and immune responses using Drosophila as a model organism. Finally, in Chapter Seven, Sandra Nicholson and Warren Alexander describe important negative regulators of the Jak-Stat pathway, the SOCS family of proteins, with emphasis on their function in hematopoiesis.

These contributions highlight the central role played by the Jak-Stat pathway in hematopoiesis. Importantly, it lies downstream of receptors for the clinically-relevant cytokines granulocyte colony-stimulating factor, erythropoietin, thrombopoietin, and the interferons. In addition, this pathway is perturbed in a variety of malignancies and hematopoietic disorders. Therefore, it can be safely anticipated that therapeutics currently under development which target this pathway will have wide and important hematological application.

Alister C. Ward

Chapter 1

Jaks and Normal Hematopoiesis

Tetsuya Nosaka

Introduction

Cytokines regulate cell fate including proliferation, differentiation, and apoptosis of hematopoietic progenitor cells, while lineage commitment of the hematopoietic stem cells is determined stochastically. Cytokines exert their specific function through binding to specific members of the cytokine receptor superfamily. The specific expression of these receptors is a vital determinant of hematopoietic cell differentiation. The Jak tyrosine kinases, consisting of four members in mammals: Jak1, Jak2, Jak3, and Tyk2, were each "Just Another Kinase" until their biological functions were unveiled.[1] In 1993, Ihle and colleagues reported that Jak2 associates with the membrane-proximal region of the erythropoietin receptor (EpoR) —which itself lacks a tyrosine kinase domain —and phosphorylates it on tyrosine residues upon Epo stimulation.[2] Meanwhile, Jak1 and Tyk2, and Jak1 and Jak2 were found by genetic complementation experiments using mutant cell lines to be involved in the signaling of the receptors for IFNα/β and IFNγ, respectively.[3-5] Since then Jaks have been recognized as one of the most important tyrosine kinases in cytokine signaling. A number of experiments in vitro have disclosed that Jak family tyrosine kinases are indispensable for signal transduction via the cytokine receptor superfamily. In addition, generation of Jak-deficient mice has revealed the biologically important and nonredundant roles of Jaks in vivo.

Modes of Jak Activation in Cytokine Signaling

The specificity of cytokine-mediated Jak-Stat signaling derives from the specific interaction of the phosphotyrosine of the cytokine receptor and the SH2 domain of the Stat protein.[6,7] Although preferential usage of a Jak for a subunit of the cytokine receptor exists (Table 1), the particular Jak engaged does not appear to determine the specificity of the downstream signaling. In contarst, Stats directly regulate the target gene expression in a cytokine receptor-specific manner.[8-10] On the other hand, since Jak activation is required for all signaling pathways including the Ras/Raf/Mitogen activated protein kinase (MAPK) and phosphatidylinositol 3-kinase (PI3-k)/Akt cascades, loss of Jak function leads to a wide range of aberrant signaling, in contRrst to loss of Stat function which results in a more restricted phenotype.[11]

The patterns of Jak activation in signaling via the cytokine receptor superfamily can be classified into three modes (Fig. 1 and Table 2). In the case of the receptors for Epo and thrombopoietin (TPO) which consist of a single receptor chain, and the receptors which use a common β chain as a single signaling chain (IL-3, IL-5, GM-CSF), Jak2 is activated

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Table 1. Jak requirements for function of cytokines essential for normal hematopoiesis and immune responses against pathogens

IL-6	IL-2	IL-15	IL-7	IFNγ	IFNα/β	IL-12	Epo
gp130	IL-Rβ		IL-7Rα	GR1(α)	AR2(β)	βII	EpoR
			Jak1			Jak2	
	γc			GR2(β)	AR1(α)	βI	
	Jak3			Jak2	Tyk2		

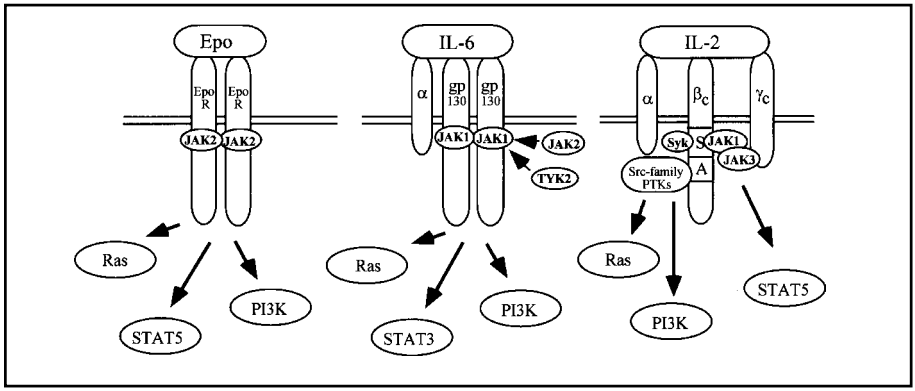

Figure. 1. Three modes of Jak activation through cytokine receptors. **EpoR**: single signaling chain-single Jak. Epo stimulation induces dimerization of EpoR, resulting in autophosphorylation of Jak2. Activated Jak2 phosphorylates EpoR on tyrosine residues, followed by recruitment of Stat5. Then Jak2 phosphorylates Stat5, resulting in dimerization of Stat5. Dimerized Stat5 enters the nucleus to regulate target gene expression. Tyrosine phosphorylation of EpoR also activates both Ras/Raf/MAP kinase and PI3K/Akt pathways. **IL-6R**: single signaling chain-multiple Jaks. IL-6 stimulation induces dimerization of gp130. Although Jak1, Jak2, and Tyk2 are phosphorylated after receptor chain aggregation, only Jak1 plays a major role in phosphorylation of gp130, while Jak2 and Tyk2 are dispensable.[12] The α chain of the IL-6R contributes to the ligand-specific binding of the receptor, since gp130 is shared among the other cytokines. **IL-2R**: multiple signaling chains-multiple Jaks. IL-2 stimulation induces transphosphorylation of Jak1 and Jak3 which associate with a serine-rich region (S) of the β chain and C-terminus of the common γ chain, respectively.[13,14] Activation of both Jak1 and Jak3 is essential for subsequent activation of Stat5 and signal transducing adaptor molecule (STAM) which activates c-*Myc* transcription (not shown).[15] The other tyrosine kinase Syk which associates with a serine-rich region of the β chain, and Src-family tyrosine kinases such as Lck, Fyn, and Lyn which associate with an acidic region (A) of the β chain, are also activated upon IL-2 stimulation.[16] Src-family tyrosine kinases activate pathways of both Ras/Raf/MAP kinase and PI3K/Akt, whereas Syk induces c-*Myc* expression.

via dimerization of the single receptor chain or the common β chain. In the latter case, a ligand-specific α chain does not associate with Jaks and is not directly involved in intracellular signaling. The second mode of activation is typified by receptors of the IL-6 family —IL-6, IL-11, oncostatin M (OSM), leukemia inhibitory factor (LIF), ciliary neurotrophic factor, cardiotrophin-1, and G-CSF—where multiple Jaks are activated with a single signaling chain. However, in this case, only Jak1 appears to be essential for signaling, with activation of Jak2 or Tyk2 not mandatory in spite of the activation of these kinases upon ligand stimulation.[12] The third mode is typified by signaling of the cytokines which use a

Table 2. Three modes of cytokine signaling using Jaks in hematopoietic cells

1. Single signaling chain-single Jak: (Jak2 is used) Epo, TPO, βc family (IL-3, IL-5, GM-CSF)
2. Single signaling chain-multiople Jaks: (Jak1 is essential) IL-6 family (IL-6, IL-11, OSM, LIF), G-CSF
3. Multiple signaling chains-multiple Jaks: γc family (IL-2, IL-4, IL-7, IL-9, IL-15), IFNα/β, IFNγ, IL-10, IL-13, TSLP, IL-12

common γ chain (γ_c) family (IL-2, IL-4, IL-7, IL-9, and IL-15), as well as IFNα/β, IFNγ, IL-10, IL-13, thymic stromal lymphopoietin (TSLP), and IL-12. The receptors for these cytokines consist of multiple signaling chains and multiple Jaks are used. In the case of IL-2 signaling, for example, Jak1 and Jak3 associate with the β chain of the IL-2R and the γ_c chain, respectively,[13,14] and transphosphorylate each other upon the ligand binding. Therefore, deletion of either Jak1 or Jak3 abolishes the response to IL-2. Because Jak activation by auto- or trans-phosphorylation is indispensable for tyrosine phosphorylation of the hemopoietin receptor superfamily in each of the modes described above, Jaks inevitably play critical roles in hematopoiesis.[17,18]

Jak Function Determined by Targeted Gene Disruption

Since cytokines and hematopoietic growth factors are somewhat redundant in their function,[19] mice deficient for single factors generally display no severe developmental abnormalities, except for rare cases such as Epo,[20] stem cell factor,[21,22] IL-7,[23] and platelet derived growth factor.[24-26] Of these, Epo and IL-7 exert their function via receptors with no intrinsic tyrosine kinase activity, and so rely entirely on Jak function. The phenotype of Jak2-deficient mice[27,28] (to be described later) is recapitulated in Epo- or EpoR-deficient mice.[20] Likewise, the phenotype of Jak3-deficient mice[29-31] is very similar to that of IL-7-[23], IL-7R α chain-,[32] or γ_c chain-[33,34]deficient mice.

Jak1 Performs Nonredundant Functions in Three Classes of Cytokine Receptor-Mediated Biological Responses

Activation of Jak1 is essential for signal transduction of class II cytokine receptors as well as those of the γ_c and gp130 famililies. Jak1-deficient mice die within 24 hr of birth, probably by neuronal defects due to impaired gp130-mediated signaling.[35] Hematologically, $Jak1^{-/-}$ mice exhibit severely impaired lymphoid development. B cell differentiation is blocked at the transition step of pro-B to pre-B cells. Thymocyte numbers are greatly reduced, but CD4/CD8 profile is not perturbed. These profiles of lymphoid development are very similar to those of $Jak3^{-/-}$ mice, indicating that IL-7 signaling, which is essential for lymphoid development, requires activation of both Jak1 and Jak3 as obligate partners to one another. In addition, $Jak1^{-/-}$ embryonic fibroblasts fail to respond to IFNα/β and IFNγ, resulting in impaired antiviral activity, and $Jak1^{-/-}$ macrophages fail to respond to IL-10 in inhibiting production of tumor necrosis factor α induced by LPS. Interestingly, colony forming assays using fetal liver cells from $Jak1^{+/+}$ and $Jak1^{-/-}$ embryos revealed that Jak1 is dispensable for G-CSF responses, in contrast to the results obtained from in

vitro experiments.[36] Thus Jak1 appears indispensable for lymphoid development principally mediated by IL-7, and immune responses mediated by IFNs.

Jak2 is Essential for Definitive Erythropoiesis and Signaling of TPO, IL-3, GM-CSF, and IFNγ, but not for the Generation of Lymphoid Progenitors

Jak2 also plays nonredundant roles in signaling via a variety of cytokine receptors. Jak2-deficient mice[27,28] die around day 12.5 postcoitum due to lack of definitive erythropoiesis. The phenotype of Jak2-deficiency is similar to that of Epo or EpoR-deficiency,[20] but the reduction in the numbers of erythrocytes is more severe in $Jak2^{-/-}$ mice than in $Epo^{-/-}$ or $EpoR^{-/-}$ mice. This is because Jak2 is involved not only in signaling of Epo, but also that of TPO which also contibutes to the expansion of early erythroid progenitor cells. Furthermore, $Jak2^{-/-}$ fetal liver cells are unable to trasduce sigrals upon stimulation with GM-CSF, IL-3, and IL-5, whose functions in hematopoiesis are redundant as reported in mice doubly-deficient for common β chain and IL-3 ligand.[37] Jak2 is also required for IFNγ signaling, but not for IFNα/β signaling in vitro and in vivo. Thus fibroblasts from Jak2-deficient embryos are no longer protected by IFNγ from the cytopathic effect of viral infection. On the other hand, fetal liver cells from Jak2-deficient embryos contain lymphoid progenitor cells which can reconstitute lymphoid development in sub-lethally irradiated Jak3-deficient mice, as well as hematopoietic stem cells which co-express CD34 and c-kit. $Jak2^{-/-}$ fetal liver cells and $Jak2^{-/-}$ fibroblasts respond normally to G-CSF and IL-6, respectively. Taken together, Jak2 seems to play a crucial role in definitive erythropoiesis and is essential for signaling through a distinct group of cytokines which are involved in erythroid/myeloid differentiation, but is dispensable for lymphoid development. Other studies have shown that aberrant signaling through Jak2 causes dysregulated cell growth. For example, constitutive activation of Jak2 by oligomerization as a result of a *TEL-Jak2* fusion generated by chromosomal translocation leads to acute lymphoid and chronic myeloid leukemias in humans.[38,39]

Jak3 is Essential for Lymphoid Development

Among the Jak family members, Jak3 is unique in that it is predominantly expressed in hematopoietic tissues, particularly in lymphoid cells, and is only activated by the cytokines of γ_c family.[40-42] In contrast, Jak1,rJak2, and Tyk2 are expressed ubiquitously and involved in signaling of more than two types of cytokine receptors. Mutations in the human Jak3 gene were first reported in 1995 in patients with autosomal recessive SCID[43,44] whose phenotype is indistinguishable from that of X-linked SCID (XSCID) which is caused by mutations in γ_c chain.[45] At the same time, Jak3-deficiency was proven to be sufficient to develop SCID in mice by gene targeting experiments.[29-31] Furthermore, a mutation of γ_c found in a family of patients with a moderate phenotype (XCID) was shown to result in partial loss of Jak3 association,[13] suggesting that the γ_c-Jak3 pathway is pivotal for driving lymphoid development. Jak3-deficient mice show severe lymphopenia (reduction in B and T cell numbers and no NK cells) with tiny thymuses. However, in spite of the extremely low thymic cellularity, $Jak3^{-/-}$ thymocytes do not display any block of differentiation, although the CD4/CD8 ratio is increased. Bone marrow and spleen cells from $Jak3^{-/-}$ mice show markedly diminished numbers of mature B cells and a developmental blockade of B cell differentiation at the pro-B to pre-B stage. However, a few cells with a mature B cell phenotype do exist in $Jak3^{-/-}$ mice. Hence, the B cell differentiation blockade in

Jak3$^{-/-}$ mice is incomplete, in comparison with the complete arrest of lymphoid differentiation observed in recombination activating gene (RAG)-1-[46] or RAG-2-[47] deficient mice. One major functional defect of Jak3$^{-/-}$ lymphocytes is an inability to proliferate upon stimulation with the γ_c family of cytokines. Among these, IL-7 plays a critical role for lymphoid development. Lack of responses to IL-7 results in a failure of lymphoid progenitor expansion in early hematopoietic development. The phenotype of deficiency in γ_c or Jak3 is nearly identical to that in IL-7 except that IL-7$^{-/-}$ mice retain NK cells which respond to IL-15. Jak3$^{-/-}$ splenocytes show impaired response to antigenic stimulation by polyclonal activators such as concanavalin A, anti-CD3 antibody, and lipopolysaccharide (LPS), due to a lack of response to IL-2 and IL-4 that are required for maximal proliferation of T and B cells. However, the frequency of generation of immunologically competent mature lymphocytes can be theoretically increased by expanding early lymphoid progenitor cells as a reservoir, although Jak3$^{-/-}$ mature lymphocytes lack the ability to expand with γ_c family cytokines. For this purpose, IL-3 which is similar to IL-7 in the effect on expansion of early lymphoid progenitors can be used, based on the usage of Jak2 for IL-3 signaling. In fact, IL-3 treatment of new-born Jak3$^{-/-}$ mice partially restores lymphoid development.[48] On the other hand, complete rescue of lymphoid development in Jak3$^{-/-}$ mice is achieved by bone marrow transplantation after retroviral gene transfer of a wild type Jak3 gene into Jak3$^{-/-}$ bone marrow cells.[49] This finding has opened a possibility of gene therapy for Jak3-deficient SCID and γ_c-deficient XSCID patients. In 2000, XSCID patients were reported to be successfully treated for at least 10 months of follow-up period by retroviral gene transfer of the γ_c gene.[50] This is one of the most exciting medical advances which has eventuated from the study of signal transduction via cytokine receptors. Of course, further improvements of the delivery system for more efficient introduction of the deficient gene into hematopoietic stem cells would be required for a complete cure.

A lack of response to IL-15 in Jak3-deficient mice appears to be responsible for the absence of NK cells and intestinal intraepithelial γδ T cells—which is also seen in IL-15-deficient mice.[51] The phenotype of the IL-7 receptor α chain-deficient mice is more severe than that of the Jak3- or IL-7-deficient mice in the aspect of lymphoid development, probably due to abrogation of TSLP signaling which also uses the α chain of the IL-7 receptor together with the TSLP-specific second chain of the receptor (also called δ1/CRLM-2) in conjunction with Jak1 and Jak2, respectively.[52-55] Reconstitution experiments of Jak3$^{-/-}$ mice by interbreeding the transgenic mice expressing Jak3 in the thymus but not in peripheral T cells revealed that Jak3 expression in the thymus restores normal T cell development, but not the function of peripheral T cells.[56] This finding indicates that constitutive expression of Jak3 in peripheral T cells is required to maintain T cell function. Interestingly, transgenic expression of *Bcl*-2 in γ_c-deficient mice rescues T lymphopoiesis, but not B or NK cell development,[57] and retroviral gene transfer of *Bcl*-2 into Jak3$^{-/-}$ bone marrow cells followed by transplantation into Jak3-deficient mice also improves peripheral T cell numbers.[58] These findings suggest that γ_c/Jak3-mediated signaling contributes to survival of T lineage cells. Curiously, Jak3$^{-/-}$ splenic T cells show a spontaneously "activated" phenotype without antigenic stimulation. These cells actively synthesize DNA and proliferate in vivo. Transgenic expression of Jak3 on mature T cells in Jak3-deficient mice rescues the quiescent state of peripheral T cells, suggesting that Jak3 not only contributes to expansion and survival of thymocytes, but also plays an important role for T cell homeostasis.[59] An unexpected expansion of cells of the myeloid lineages caused by Jak3$^{-/-}$ T cells was also reported in Jak3-deficient mice, suggesting that Jak3 is also involved in downregulating a myeloproliferative signal.[60] Finally, immunoglobulin class switching is

impaired in human SCID patients with Jak3 mutations, due to lack of IL-4 response, resulting in readily detectable levels of serum IgM with greatly diminished levels of IgG, IgA, and IgE.[44]

Tyk2 is Required for the Full Response to IL-12, but Plays a Restricted Role in IFNα/β Signaling

The function of Tyk2 was originally demonstrated to be an essential tyrosine kinase for IFNα/β signaling by genetic complementation of a mutant cell line.[3] However, Tyk2-deficient mice[61,62] are developmentally normal and, unexpectedly, $Tyk2^{-/-}$ cells show only partially impaired responses to IFNα/β stimulation. Reduced signaling of IFNα/β in $Tyk2^{-/-}$ cells is overcome by increased amounts of IFNα/β, suggesting that Tyk2 plays only a restricted role in IFNα/β signaling. Another unexpected finding was that the response to IFNγ was also reduced in $Tyk2^{-/-}$ cells, demonstrating a cross-talk between IFNα/β and IFNγ signaling components.[63] It should be noted that this cross-talk is unidirectional; IFNγ signaling depends on IFNα/β signaling, while IFNα/β signaling is not affected by IFNγ signaling as observed in $Jak2^{-/-}$ mice.

In contrast to the semi-redundant role of Tyk2 in IFNα/β signaling, $Tyk2^{-/-}$splenocytes show a markedly decreased IL-12-stimulated production of IFNγ, although it is not completely abolished. On the other hand, IL-12-induced proliferation of $CD3^{+}$ splenocytes is not significantly affected by the absence of Tyk2. Consistent with this finding is that the cytoplasmic region of IL-12RβII alone was shown to be sufficient to deliver a proliferative signal by using Jak2[64] (see Table 1). Thus Tyk2 plays an important role in IL-12-mediated IFNγ production in association with IL-12RβI. However, there is no obligatory requirement for it in IFNα/β signaling in vivo.

Conclusions

Gene targeting experiments in mice has clarified that Jak1, Jak2, and Jak3 play nonredundant and critical roles in cytokine signaling and hematopoietic development, whereas Tyk2 is not absolutely required for any cytokine signaling and is dispensable for the normal development of the hematopoietic system (summarized in Table 3). Now it is quite obvious that the three Jaks are indispensable for normal hematopoiesis, and aberrant signaling of Jak2 or Jak3 due to structural alterations leads to leukemia or immunodeficiency, respectively. This makes Jaks one of the most attractive molecular targets for drug design[65] and gene therapy.[49]

Table 3. Phenotypes of Jak-deficient mice

Gene	Phenotype	Cytokines affected
Jak1	• **defective lymphoid development** • **perinatal lethality**	• **γc family (IL-2, IL-4, IL-7, IL-9, IL-15)** • **gp130 family (IL-6, IL-11, OSM,LIF)** • **class II cytokine receptor family (IFN α/β, IFNγ, IL-10)**
Jak2	• **no definitive erythropoiesis** • **embryonical lethality (day 12.5)**	• **Epo, TPO, IFNγ** • **β_c family (IL-3, IL-5, GM-CSF)**
Jak3	• **SCID** • **dysregulated myelopoiesis**	• **γ_c family (IL-2, IL-4, IL-7, IL-9, IL-15)**
Tyk2	• **reduced antiviral response** • **reduction in IL-12 induced IFNγ production**	• **IFNα/β, IFNγ (due to cross-talk), IL-12**

Cytokines with nonredundant roles in hematopoiesis and immune responses against pathogens are shown in bold.

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CHAPTER 2

Jak3-Dependent Pathways in Hematopoiesis and SCID

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Introduction

Hematopoiesis is a critical developmental process that depends on highly regulated growth and differentiation of pluripotent bone marrow stem cells. The process of hematopoiesis regulates the production of myeloid, erythroid, and lymphoid cells from bone marrow progenitors and maintains the correct balance of different blood cell lineages. The control of hematopoiesis depends on signals delivered through surface receptors of the hematopoietic superfamily present on progenitor cells in response to a complex cocktail of cytokines in the bone marrow microenvironment and peripheral blood.[1-4] Members of the cytokine receptor superfamily lack endogenous tyrosine kinase activity, but bind members of the Jak family of tyrosine kinases including Jak1, Jak2, Jak3, and Tyk2. Jak3 expression is restricted to cells of hematopoietic lineage, and binds specifically to the common gamma chain (γ_c) component of several multisubunit hematopoetic receptor complexes. γ_c-dependent cytokines include IL-2, IL-4, IL-7, IL-9, IL-15 and IL-21. Signaling through all of these receptors requires functional Jak1 and Jak3, and mutation of either kinase or their associated receptor chains ablates functional signal transduction. The importance of this signaling pathway has been underlined by naturally occurring mutations in γ_c or Jak3 which disrupt kinase-receptor interaction and block signal transduction. Naturally occuring mutations in Jak3 or γ_c cause Severe Combined Immmunodeficiency (SCID). This type of SCID is the most common inherited immunodeficiency, appearing in 1:50,000 live births. SCID mice and humans have profound defects in lymphocyte development and development of secondary lymphoid organs. They have a significant block in productive TCR and BCR gene rearrangements and markedly reduced survival of lymphoid progenitor cells.[5-8]

It has been found that Jak3-dependent cytokine signaling is critical for multiple, opposing effects on hematopoietic cells. Jak3 is required for delivering survival and proliferative signals to lymphocyte progenitor cells derived from the bone marrow, by activating cell cycle progression through transcriptional regulation of cell cycle activators, cyclins D1, D2 and D3,[9-12] while simultaneously delivering potent survival signals by increasing expression of the anti-apoptotic Bcl-2 family of proteins and activation of the cell survival kinase Akt.[13] Interestingly, at later stages of lymphocyte development, Jak3 signal transduction ensures proper control of the immune system by delivering critical differentiation

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and cell death signals to mature, activated peripheral T cells. Jak3 is required in the periphery for deletion of self-reactive T cells, generation of memory antigen-specific CD8+ T cells, and for triggering the Fas ligand-dependent activation induced cell death (AICD) pathway, crucial for the proper negative control of immune responses and prevention of autoimmune reactions. Indeed, gene-targeting experiments in mice have demonstrated the dual requirement for Jak3-dependent activation of cell cycle progression and cell survival, as well as for the negative control of lymphocyte activation.[14,15] Jak3, γ_c, and IL-7 receptor deficiency results in profound defects in T, B, and NK cell development and cytokine-dependent proliferative responses of peripheral lymphocytes.[16-20] The T cells that develop in Jak3, γ_c, Stat5a/b, IL-2, and IL-2 receptor knockout mice are activated peripheral T cells, and in some cases, can cause fatal autoimmune disease.[17,20] This chapter will discuss our current knowledge of the multiple roles of Jak3 in hematopoiesis and peripheral lymphocyte function by detailing the function of specific cytokine-activated biochemical pathways in T, B and natural killer (NK) cell development. In addition, we will analyse the results of gene targeting experiments as well as the phenotypes of humans with mutations in Jak3. Finally, the function of individual Jak3-dependent receptors will be discussed through the analysis of mice lacking single receptor chains, but retaining all other Jak3 signaling complexes.

Jak3-Regulated Signal Transduction Pathways

Treatment of cells with cytokines induces a marked increase in total cellular tyrosine phosphorylation. However, members of the cytokine receptor superfamily lack endogenous tyrosine kinase activity, which suggested that the intracellular domains of the signaling chains were non-covalently associated with cytoplasmic tyrosine kinases. In genetic complementation experiments using interferon-non-responsive mutant cell lines, interferon signaling could be reconstituted with cDNAs encoding tyrosine kinases of the Janus (Jak) family.[21,22] Four Jak family members were identified: Jak1, Jak2, Jak3, and Tyk2, all of which are important in signal transduction through a variety of cytokine receptors. In contrast to Jak1, Jak2, and Tyk2, which are ubiquitously expressed in all cell types, Jak3 expression is restricted to cells of hematopoietic lineage, suggesting an important, specific role for this kinase in hematopoiesis.[23-26] Indeed, stimulation of lymphoid cells with several different γ_c-dependent cytokines was found to induce rapid tyrosine phosphorylation of Jak3 as well as other downstream signaling molecules.[27-29] The structures of the receptor complexes that use Jak3 as a signaling component are shown in Figure 1. All of these receptors are heteromeric complexes containing either two or three polypeptide chains.[30] Each receptor contains a ligand-specific high affinity binding chain as well as a chain shared by all members of this subfamily of hematopoietic receptors, p64, which was subsequently named the common gamma chain (γ_c)(Fig. 1)[31-39] An important finding which implicated Jak3 in signal transduction through this family of receptors was the finding that Jak3 was physically associated with γ_c, and immunoprecipitating anti-γ_c antibodies can co-precipitate Jak3 with γ_c.[40,41] The receptors for IL-4, IL-7 and IL-9 are all heterodimers containing γ_c as well as their respective ligand-specific chains that are non-covalently associated with another family member, Jak1. By comparision, the receptors for IL-2 and IL-15 are trimeric complexes which contain ligand-specific components (IL-2Rα and IL-15Rα), as well as two shared chains, γ_c and IL-2Rβ. In these complexes, the shared IL-2Rβ chain is associated with Jak1 (Fig. 1). Signaling through all of these receptors requires functional Jak1 and Jak3, as mutation of either kinase or their associated receptor chains ablates functional signal transduction.[42-46] Although there is only weak sequence

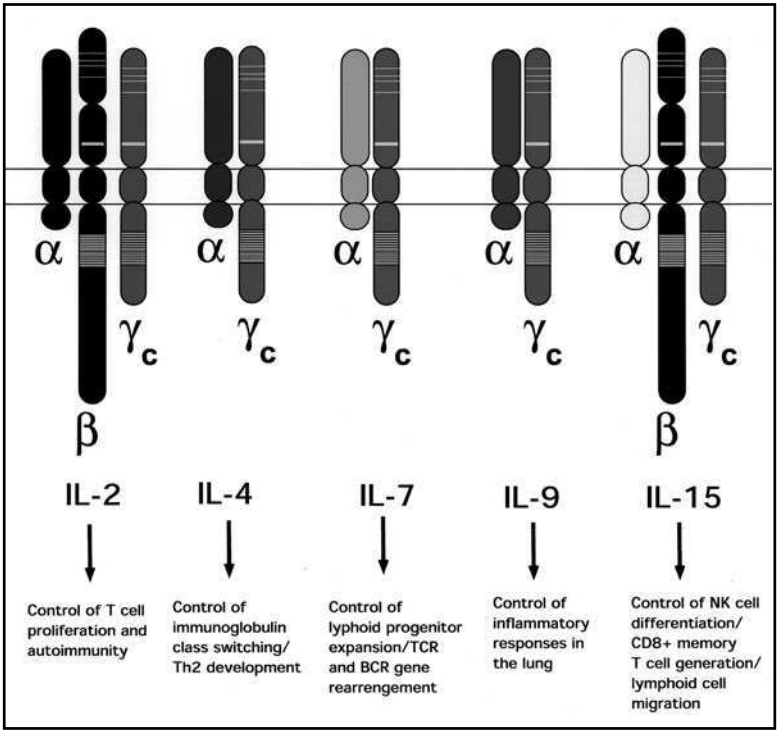

Figure 1. IL-2-related cytokine receptors.

homology among members of the cytokine receptor superfamily, there is a loosely homologous region in the membrane-proximal portion of the cytoplasmic tail, known as the Box1/Box2 motif, which encodes conserved proline-containing motifs that have been shown to be critical for the association of the receptors to the amino-terminal regions of Jak kinases.[47-49] The importance of these sequences have been demonstrated by the identification of naturally occurring mutations in γ_c or Jak3 which disrupt the association and cause human genetic disease.[50]

A critical event in cytokine signal transduction is the Jak kinase-mediated phosphorylation of tyrosine residues on the signaling components of the receptor complex. Phosphorylated tyrosines serve as docking sites for signaling molecules that contain Src homology (SH) 2 and phosphotyrosine binding (PTB) domains and initiate a cascade of protein-protein interactions that are required for the activation of survival and proliferative pathways. Extensive analysis of receptor mutants that fail to activate one or more of these pathways has demonstrated the role of these biochemical signals in cell survival, proliferation, and differentiation at many stages of hematopoietic development.[51-53] Shown in Figure 2 are some of the most well studied signaling pathways activated by cytokine receptors.

Stat Proteins

One of the best studied pathways involves the latent cytoplasmic SH2-containing transcription factors known as Stats (signal transducers and activators of transcription). Early experiments in IFN α/β-non-responsive mutant cell lines that identified Jak kinase

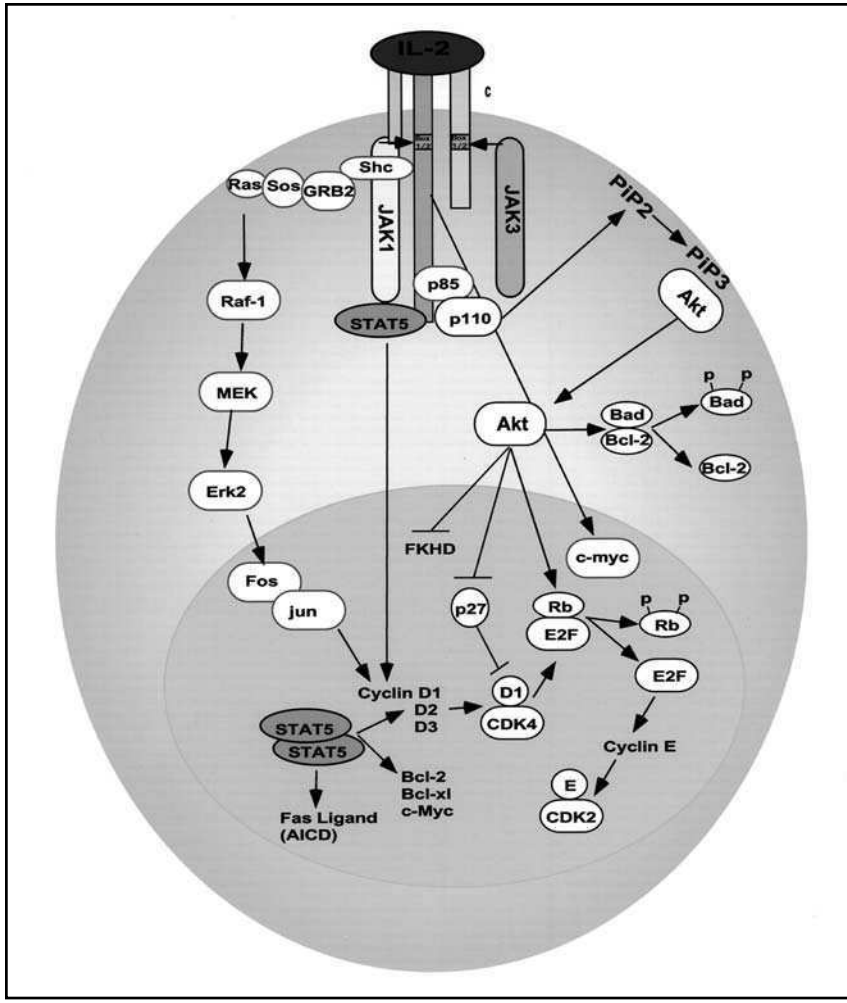

Figure 2. Key signaling pathways in the IL-2 response in T-lymphocytes

family members as critical cytokine signaling components also allowed for the dissection of important signaling pathways downstream of kinase activation. In particular, Stats were also discovered by these genetic complementation experiments, and are critical mediators of cytokine-induced responses.[22,54,55]

There are seven known mammalian Stat molecules (Stats 1-4, 5a, 5b, and 6), each with unique roles in cytokine biology. Figure 2 shows the mechanism of Stat transcriptional activity in response to cytokine stimulation. Specific tyrosine residues on the cytoplasmic tails of the receptor chains (IL-2Rβ, for example) are substrates for receptor-associated Jak kinases and become phosphorylated in response to ligand stimulation. A subset of these phosphotyrosine residues function as binding sites for the SH2 domains of specific Stat molecules, and recruit Stats to the activated receptor.

Once a Stat molecule binds to the receptor, it serves as a substrate for the associated Jaks and becomes phosphorylated on a key C-terminal tyrosine residue near the DNA

Table 1. Stat activation by γ_c-dependent cytokine receptors

Receptor Complex	Stats Activated
IL-2	Stats 1, 3, 5a, 5b
IL-4	Stats 3, 5a, 5b
IL-7	Stat6
IL-9	Stats 3, 5a, 5b
IL-15	Stats 3, 5a, 5b

binding domain. Once activated in this way, the Stat dissociates from the receptor, and, through its SH2 domain, reciprocally binds to another phosphorylated Stat, forming homo- or heterodimers. The dimeric form of Stat molecules, which is capable of DNA binding and transcriptional activation, then translocates to the nucleus and binds specific promoter elements upstream of cytokine-activated genes.[56,57] These DNA elements are known as GAS (IFN Gamma Activated Sequences), since they were originally found upstream of interferon-regulated genes. As shown in Table 1, γ_c/Jak3-dependent cytokine receptors activate overlapping sets of Stats, with the exception of IL-4 which uniquely activates Stat6. Otherwise, IL-2, 7, 9, and 15 all activate various combinations of Stats 1, 3 and 5.[8,56-59] The specificity of Stat activation by individual receptors is determined by the binding affinities of the Stat SH2 domains for the 3-4 amino acid sequence immediately downstream of the receptor phosphotyrosine docking site. Binding studies have shown that Stat5a and Stat5b bind strongly to tyrosines 392 and 510 of the IL-2Rβ chain, but not to the IL-4Rα chain. Conversely, Stat 6 binds specifically to phosphorylated residues Y575, Y603 and Y631 of the IL-4Rα chain, but not to the IL-2 receptor. Thus, IL-2 is known to activate Stats 5a and 5b, while Stat6 is activated only by IL-4 receptor signaling.

Several Stat dependent genes are important for Jak3 mediated hematopoiesis. Stat binding sites have been found in the promoter elements of all D-type cyclins (D1, D2 and D3), which, in complex with cyclin-dependent kinases such as CDK4 and CDK6, induce cell cycle progression.[9-11] Genetic evidence for the importance of Stat activation in cytokine-induced cell cycle progression has been provided by a murine gene knockout model of Stat5 deficiency. Peripheral T cells from mice lacking both the Stat5a and Stat5b isoforms fail to proliferate in response to mitogenic stimuli, such as TCR cross-linking or combined treatment with PMA and ionoMycin in vitro. The failure of these cells to proliferate has been attributed to their inability to induce the expression of the Stat5-dependent genes cyclin D2 and cyclin D3, which are required for G1/S progression in response to cytokine stimulation.[11] In addition, a GAS element ($TTCN_{2\text{-}5}GAA$) has been identified in the promoter region of the c-Myc gene. Recently, it has been found that expression of c-Myc, which activates cell division through several distinct mechanisms (see below) is also regulated by Stats 3 and 5.[60] The genes encoding Bcl-2 and the Bcl-2 family member Bcl-x_L can be induced by Stat5 as well, consistent with the finding that a Stat binding site has been identified in the promoter region of the *Bcl-x_L* gene.[61] The transcription of all these genes can be inhibited by overexpression of dominant negative alleles of Stat5, and enhanced by overexpression of WT or constitutively active forms of Stat5.[62] Thus, Stats

uniquely serve a dual function as cytoplasmic signaling molecules and Jak kinase substrates which can rapidly translate receptor-mediated signals into a transcriptional effect by translocating to the nucleus and directly activating the expression of cytokine-inducible genes.

PI-3 Kinase/Akt

Activation of several survival pathways is a key function of Jak-3-dependent signal transduction. Survival signals, mediated by the serine-threonine kinase survival factor Akt (protein kinase B) have been shown to be a component of multiple cytokine/growth factor signaling mechanisms in various tissues. The activation of Akt activity is linked to upstream events that generate a lipid-based second messenger molecule, phosphatidyl inositol 3,4,5 triphosphate (PIP3).[63,64] Activation of Jak3-dependent cytokine receptors generates docking sites for a heterodimeric lipid kinase, PI-3 kinase (Fig. 2). In the IL-2 system, this enzyme is recruited to the receptor as part of a tripartite complex involving Jak1 and the IL-2Rβ chain.[65] PI-3 kinase is comprised of two subunits, p85, a regulatory molecule, which contains a SH3 domain and two phosphotyrosine-binding SH2 domains. The p85 subunit directs the catalytic domain, p110, to areas of the plasma membrane containing activated receptors. The altered subcellular localization of PI-3 kinase allows the p110 subunit to catalyze the following reaction: PI(4,5)P2→PI(3,4,5)P3, generating a ligand for another class of signaling molecules, one member of which is the serine-threonine-specific phosphotransferase Akt. Akt is recruited to the inner leaflet of the plasma membrane via an interaction between PI(3,4,5)P3 with its amino-terminal pleckstrin homology (PH) domain.[63,64] This interaction stimulates the catalytic activity of Akt, allowing it to phosphorylate and inactivate several pro-apoptotic signaling molecules (Fig. 2). Akt-dependent phosphorylation of key substrates such as Bad generates active Bcl-2 family members, thus supporting cell survival. Bad, a pro-apoptotic molecule, normally heterodimerizes with survival factors Bcl-2 or Bcl-x_L, forming an inactive complex. Phosphorylation of Bad by Akt disrupts dimer formation, and promotes the interaction of Bad with 14-3-3 proteins, which prevents its interaction with Bcl-2. Consequently, active Bcl-2 and Bcl-x_L survival factors are released from inhibition.[66] An additional substrate for Akt is the forkhead family of transcription factors. These transcriptional activators control expression of the pro-apoptotic cysteine proteases of the caspase family. Akt phosphorylates and inactivates forkhead transcription factors, thus decreasing the levels of caspases in the cell and delivering an additional survival signal.[67-69] Finally, Akt has also been shown to promote cell survival by direct phosphorylation and inactivation of caspase-9.[70] The antagonistic effects of Akt on multiple pro-apoptotic molecules such as Bad, caspase-9, and forkhead transcription factors delivers a very potent survival signal through Jak3-dependent cytokine receptors by several independent mechanisms. The positive regulation of Bcl-2 family member expression levels is particularly important in hematopoiesis, since the survival of lymphoid and myeloid precursors depends on tightly regulated Bcl-2 and Bcl-x_L expression levels (see below).

The PI-3 kinase pathway not only promotes cell survival, but is also important in stimulation of cell cycle progression. There is a strong link between activation of PI-3 kinase activity and (i) inactivation of the tumor suppressor protein Rb, resulting in activation of the transcription factor E2F, and (ii) destruction of the cell cycle inhibitor $p27^{kip1}$. IL-2-induced cell cycle progression is accompanied by Rb phosphorylation, activation of D-type cyclin expression, and degradation of $p27^{kip1}$. Interestingly, in one study, all of these effects of IL-2 could be abrogated by expression of dominant-negative (kinase-inactive)

alleles of Akt, as well as by treatment of the cells with the PI-3 kinase inhibitor LY294002.[13] These findings strongly suggest that Akt delivers potent proliferative signals as well as activating multiple survival pathways. It is important to note here that while PI-3 kinase activation is sufficient for E2F activity, it is not sufficient for cell cycle progression. The PI-3 kinase/Akt signal must be integrated with Stat, MAP kinase, and/or Myc activities in order to drive the cell through the G1/S phase of the cell cycle. Thus, it is the combinatorial effects of multiple signaling pathways that allows the cell to exit from the G1 phase of the cell cycle and enter the S phase.

MAP Kinase

As shown in Figure 2, one of the IL-2Rβ chain phosphotyrosine residues provides a docking site for an SH2- and PTB-containing adaptor molecule, Shc. Shc binds to tyrosine residue 338 in the IL-2Rβ chain, and it also docks to specific phosphotyrosines present on signaling chains of all Jak3-dependent cytokine receptors. Through multiple protein-protein interactions (shown in Fig**.** 2) Shc activates the ERK mitogen-activated protein (MAP) kinase pathway. Once bound to the cytokine receptor, Shc becomes phosphorylated by the receptor-associated Jaks which generates a binding site for the adaptor protein Grb2. Grb2 contains Src homology domains in the configuration SH3-SH2-SH3. The phosphotyrosine-binding SH2 domain docks to phosphorylated Shc, while each SH3 domain is bound to a proline-rich region found in the Ras guanine nucleotide exchange factor (GEF) SOS. Once SOS is targeted to an activated receptor, it stabilizes the GTP-bound (activated) form of the small G protein Ras, thus stimulating signal transduction through this pathway (Fig. 2).[4,8]

There are several downstream effectors of the Ras pathway, one of which is the serine/threonine kinase Raf, and the kinases which are immediately downstream of Raf, MEK and the MAP kinases ERK1 and ERK2. Activation of the ERK MAP kinase pathway is yet another mechanism for sending a proliferative signal via cytokine receptors. Several lines of evidence have shown that the Ras pathway is important in cytokine-mediated cell cycle progression. In experiments using tyrosine mutants of the IL-2 receptor beta chain, it was found that mutation of residue 338 to phenylalanine inhibited IL-2-induced proliferation of a murine pro-B cell line by 50%.[51] In addition, dominant-negative alleles of Ras (Ras N17) have been shown to inhibit cytokine-induced proliferation and activation of cyclin D1 expression.[71] Therefore, the experimental evidence suggests that maximal cytokine-induced proliferation of a lymphocyte requires the integration of multiple biochemical signals from the Stat, PI-3 kinase, and Ras/Raf/MAP kinase pathways.

c-Myc

Activation of c-Myc expression strongly correlates with progression from the G1 to S phases of the cell cycle. Myc is a transcription factor that acts as a heterodimer with its binding partner, Max, to transcriptionally regulate multiple targets involved in cytokine-induced cell cycle progression. Myc has been shown to regulate the expression and activity of cell cycle inhibitors $p21^{cip1}$ and $p27^{kip1}$ by several mechanisms.[72,73] Myc decreases $p27^{kip1}$ protein levels by transcriptional activation of the gene encoding CulI, a component of the ubiquitin ligase that targets phosphorylated $p27^{kip1}$ for destruction.[74] In addition, c-Myc has been shown to be a direct transcriptional repressor of $p21^{cip1}$ and $p27^{kip1}$ gene expression. Furthermore, Myc can activate cyclin E/CDK2 complexes by causing their dissociation from $p21^{cip1}$ and $p27^{kip1}$, and sequestering the cell cycle inhibitors

in cyclin D/CDK4 complexes. Myc also can induce cell cycle progression by inducing expression of the bHLH protein Id, which bypasses the Rb tumor suppressor pathway.[72]

The mechanism of *Myc* gene induction by activated cytokine receptors is unclear. It has been shown by several groups that *c-Myc* is a Stat3 and Stat5-inducible gene. A Stat-binding GAS elements have been identified upstream of the *c-Myc* gene, and *Myc* expression can be enhanced by constitutively active forms of Stat5 and repressed by dominant-negative Stat5 mutants.[60,75] However, it has also been demonstrated that Stats are not necessary for *c-Myc* induction, and may function only to synergize with other factors to optimize *Myc* expression. A truncated IL-2Rβ mutant, lacking a Stat5 binding site can still induce *c-Myc* expression even in the absence of Stat phosphorylation.[76] In other systems, it has been shown that expression of *Myc* can be induced by truncated cytokine receptors that fail to activate all known biochemical signal transduction pathways. These receptors retain only the membrane-proximal portion of their cytoplasmic tails, which is required for the binding and activation of Jak kinases.[77] The molecular basis of *Myc* activation by truncated receptors is not presently known.

It has become obvious that multiple stages of hematopoietic cell differentiation are dependent on Jak3-mediated signals, and that Jak3 deficiency results in hematopoietic defects at many stages of development. At the molecular level, activation of cytokine-inducible genes can be attributed to the Stat and MAP kinase pathways, which stimulate the activity of multiple transcription factors that are crucial to cell survival and proliferation. Specificially, Stat molecules, in particular Stat5, transcriptionally activate genes that are critical for driving cells through different stages of the cell cycle, such as cyclins and cyclin-dependent kinases. Without the induction of cyclins D2 and D3, a hematopoietic cell cannot progress through the cell cycle and arrests at an early stage in the proliferative pathway. In addition, the activation of survival pathways, and inactivation of pro-apoptotic molecules is also a critical function of Jak3-dependent cytokine responses. This is accomplished by stimulation of the PI-3 kinase/Akt pathway, which results in the phosphorylation and inactivation of Akt substrates Bad and forkhead transcription factors, both of which are potent pro-apoptotic molecules. The combined antagonism of two cell death mechanisms generates a potent survival signal through this pathway.

Bcl-2

Bcl-2 and Bcl-x_L are both anti-apoptotic molecules that are found associated with the outer mitochondrial membrane and prevent cell death by blocking the Apaf-1-mediated activation of caspase-9 in response to apoptotic stimuli. In contrast, homologous proteins within the same family, such as Bad, Bax, and Bim, are pro-apoptotic proteins that function by forming a heterodimeric complex with either Bcl-2 or Bcl-x_L and inhibiting their cell survival activity.[78-80]

Expression of Bcl-2, Bcl-x_L, and a closely related molecule, BAG-1 can be modulated by Jak3-dependent cytokines,[60,76,81-84] and the role of these survival factors in lymphocyte homeostasis has been demonstrated in murine models of Bcl-2 and Bcl-x_L deficiency. Targeted deletion of Bcl-2 resulted in massive cell death of immature lymphocytes at 4-6 weeks after birth. Bcl-2 deficiency caused apoptotic involution of the thymus and spleen, and T and B lymphocytes disappeared from all lymphoid tissues, suggesting a selective effect of Bcl-2 deficiency on lymphocytes.[85-87] In addition, adoptive transfer of Bcl-$2^{-/-}$ bone marrow-derived stem cells resulted in reconstitution of all non-lymphoid lineages in recipient mice. However, there was a complete failure to produce peripheral T cells, and B cell differentiation was arrested at a very early stage of development (B220-IgM-).

Interestingly, thymocyte development in the reconstituted animals was arrested at the CD4-CD8-CD3- triple-negative stage (TN). There is an accumulation of aberrant CD4+CD8+ cells that have failed to rearrange their TCR genes, suggesting that Bcl-$2^{-/-}$ thymocytes cannot respond to differentiation signals.[88] These results demonstrate that Bcl-2 is essential for the survival and proliferation early B and T cell progenitors and for critical developmental transitions during lymphoid differentiation

Targeted deletion of the Bcl-x_L gene has demonstrated a requirement for this protein in B cell development and sensitivity of lymphocytes to apoptosis. There was a 10-fold decrease in B220+IgM+ peripheral B cells, which suggests a stage-specific role for Bcl-x_L in B cell development.[89] Furthermore, isolated splenic B cells as well as CD4+CD8+ thymocytes from Bcl-x_L-deficient mice displayed a greatly accelerated rate of spontaneous apoptosis in vitro, which strongly suggests that the regulation of a cell's sensitivity to apoptotic stimuli by Bcl-x_L is critical for lymphocyte survival and development.

The processes of B and T cell differentiation are known to be dependent on strictly regulated fluctuations of Bcl-2 and Bcl-x_L expression at critical stages of the developmental program. Detailed analysis of purified thymocyte populations has shown that Bcl-2 levels are high in CD3-CD4-CD8- immature thymocytes and mature CD4+ or CD8+ lineage-committed T cells that subsequently exit the thymus, but CD4+CD8+ DP thymocytes express little Bcl-2. Likewise, immature B220+CD43+ IgM+ Igd- developing B cells also express markedly reduced Bcl-2 levels compared to other developmental stages.[90-95] T and B cells at this stage of development are subject to two critical developmentally regulated processes: positive and negative selection of the antigen receptor repertoire.

Several models of T cell development in TCR transgenic mice have demonstrated that ectopic overexpression of Bcl-2 can result in perturbed T cell development and skewing of the T cell receptor repertoire, supporting the idea that fluctuation of Bcl-2 levels regulates thymocyte differentiation. Several studies have shown that Bcl-2 maintains the survival of thymocytes that express either low affinity or self-reactive receptors that are normally deleted in the thymus. However, levels of Bcl-2 expression that allow survival of thymocytes with defective antigen receptors cannot sustain thymic export and expansion of the autoreactive mature SP thymocytes, and are not sufficient to induce autoimmunity. Interestingly, some self-reactive T cells that can escape negative selection and migrate to peripheral lymphoid organs in Bcl-2 transgenic mice fail to cause disease, because the cells retain their sensitivity to peripheral tolerance mechanisms and are anergic. Therefore, regulated expression Bcl-2 family members is important for survival of early lymphocyte progenitors and efficient positive selection of the T cell repertoire, but does not play a significant role in peripheral tolerance and induction of anergy.[96-104]

Likewise, ectopic expression of Bcl-2 and Bcl-x_L markedly disrupts B cell development and negative selection. High levels of Bcl-x_L caused an accumulation of immature B220+CD43+ pro-B cells and rescued lymphocytes expressing aberrant, self-reactive BCRs which are eliminated in non-transgenic control animals.[105] Bcl-2 transgenes also disrupted the clonal deletion of autoreactive BCRs that are generated during somatic hypermutation and affinity maturation of the antibody response, suggesting that the regulation of anti-apoptotic molecules is critical for proper B cell deletion during lymphopoiesis.[106] Normal mechanisms of self-tolerance and clonal deletion are defective in B cells expressing elevated levels of Bcl-2 and Bcl-x_L, due to enhanced resistance to apoptosis.

Both Bcl-2 and Bcl-x_L are induced by Jak3-dependent cytokines such as IL-2 and IL-7, and can be transcriptionally regulated by Stat5 (Fig. 2).[60,76] Indeed, the *Bcl-x_L* gene contains a Stat5 binding GAS element in its promoter, and *Bcl-2* and *Bcl-x_L* mRNA levels

are increased by constitutively active forms of Stat5 and reduced in the presence of dominant negative Stat5 alleles. But *Bcl-2* levels can be controlled by other cytokine-activated pathways, as IL-2Rβ chain mutants that fail to activate Stat5 increase *Bcl-2* mRNA levels.[76] IL-2 induced *Bcl-2* and *Bcl-x$_L$* expression can be inhibited by the PI-3 kinase inhibitor LY294002, and IL-7-induced cell survival is dependent on an intact PI-3 kinase signaling pathway, but not Stat activation.[107]

Thymocytes from mice lacking Jak3 (see below) expressed elevated levels of the pro-apoptotic Bax protein and reduced levels of Bcl-2. The regulation of Bax expression by Jak3 is likely to regulate thymocyte survival, as thymus cellularity in Jak3$^{-/-}$ Bax$^{-/-}$ double knockout mice was markedly enhanced in comparison to Jak3-deficient controls.[108,109] The importance of maintaining the correct balance of Bcl-2 and Bax levels during positive selection has been demonstrated in mice that overexpress a Bax transgene. Bax transgenic mice have greatly reduced numbers of peripheral T cells, reflecting defects in positive selection of CD4+CD8+ thymocytes. Interestingly, *Bax* transgenic thymocytes undergo accelerated apoptosis upon cytokine withdrawal, but not in response to other apoptotic stimuli, such as α-Fas antibody and TNFα treatment.[110] In addition, it has been shown that IL-7 withdrawal from WT thymocytes results in the translocation of Bax from the cytoplasm to the mitochondria, perhaps activating a cell death program.[111] Thus regulation of Bax expression likely plays an important role in modulating survival signals that are delivered through γ$_c$/Jak3-dependent cytokine receptors.

Likewise, ectopic expression of a *Bad* transgene from a T cell specific CD2 promoter results in greatly decreased thymocyte numbers, skewed T cell repertoire, and accelerated spontaneous apoptosis of immature thymic T cells. Furthermore, expression of Bad is markedly increased in thymocytes undergoing apoptosis in response to γ-irradiation or dexamethasone.[112] Finally, it has been shown that targeted mutation of the pro-apoptotic Bcl-2 family member Bim results in prolonged survival of peripheral lymphocytes, accumulation of self-reactive, activated plasma cells, and fatal autoimmune disease due to autoantibody production. These results suggest that multiple Bcl-2 family members play a role in lymphocyte development, and peripheral lymphocyte homeostasis.

Proper regulation of lymphocyte sensitivity to apoptotic signals is therefore a key function of Jak3-dependent cytokines essential throughout lymphopoiesis, as well as in peripheral B and T cells. The dysregulation of the expression of Bcl-2 family members in Jak3-deficient animals significantly contributes to the developmental and peripheral lymphoid defects observed in these model systems, and is a key molecular determinant of the SCID phenotype.

Models of Jak3 Deficiency

Patients who lack either Jak3 or γ$_c$ present with phenotypically-identical Severe Combined Immunodeficiency (SCID).[43,113-117] The identical phenotype of both γ$_c$ (X-linked) and Jak3 (autosomal) SCID strongly supports the idea that the primary function of Jak3 is to transduce signals from activated cytokine receptors that use γ$_c$ as a signaling component. Thus, both γ$_c$ and Jak3 are components of a common signal transduction system activated by cytokine receptor cross linking.[118] Jak3 deficiency in vivo supports a critical role for Jak3 in both lymphoid development and peripheral B, T, and NK cell function, implying that Jak3 is essential at all stages of lymphocyte development. However, the developmental defects that occur in the absence of Jak3 are specific to cells that are pre-committed to lymphocyte lineages. Bone marrow stem cell number, myeloid and erythroid colony formation as well as monocytes and dendritic cells are unaffected by Jak3 or γ$_c$

deficiency,[46] suggesting that Jak3 has a unique, specific role in the development and function of cells of the immune system.[43,45,46] Jak3 expression has been detected in non-lymphoid and non-myeloid lineages, such as cardiac endothelium and tumors of epithelial origin, suggesting that it may have a function in several different tissues.[119] In addition, Jak3 deficiency results in severe thymic atrophy and involution, charactertized by loss of specific structures formed by the stromal layer. Thymic architecture in γ_c-deficient mice is more wild type in appearance, which suggests that Jak3 has a role in the development of epithelial components of lymphoid tissue.[46,117] However, in spite of the phenotypic differences in murine SCID models, most data have demonstrated that Jak3 likely does not have a function beyond its role as a γ_c-dependent signal transducer in cells of hematopoietic origin.

The developmental pathways of both T and B lymphocytes have been extensively studied in normal animals and in those with specific targeted mutations of various genes that are required for essential developmental transitions. The severe, pleiotropic effects of Jak3 mutations are due to the loss of signal transduction through all γ_c-dependent cytokine receptors. This results in a profound lymphopenia, due to markedly reduced thymocyte numbers and complete loss of NK cell development and γδ T cells. In addition, Jak3 and γ_c-deficiency causes a profound defect in the development of secondary lymphoid organs. There are no detectable Peyer's patches or lymph nodes, with the exception of a small amount of mesenteric lymph node tissue. The loss of these lymphoid structures is likely a secondary effect of defective B and T cell development, and the concomitant reduction in the migration of functional lymphocytes to the periphery.[43,113-117]

There are several distinct stages of T cell development characterized by critical lineage commitment steps, expression of stage-specific surface markers, developmentally-regulated gene expression and enzymatic steps (such as antigen receptor gene rearrangements and expression of the *Rag1* and *Rag2* genes). Figure 3 summarizes the developmental pathway for T cells, and identifies the developmental steps that are sensitive to individual cytokine signals. Pluripotent bone marrow stem cells (CD34+Sca-1+c-kit+Lin-) make an initial lineage commitment deCISion, differentiating into cells that will eventually form all myeloid cells (common myeloid progenitor, CMP) or a precursor that is the common lymphoid progenitor (CLP), that is capable of differentiating into B, T, and NK cells.[18,120,121] Shown in Figure 3 is the CLP that is the progenitor for all T cells, and is characterized by a specific surface profile ($CD4^{lo}$CD44+CD25-c-kit+CD8-CD3-). This cell migrates from the bone marrow to the thymus and differentiates into the earliest T cell progenitor in the thymus, with a surface profile very similar to the CLP. T cell development in the thymus then proceeds through an ordered progression of differentiation events, characterized by cells with a specific pattern of surface marker expression. CD3-CD4-CD8- triple negative (TN) thymocytes undergo TCR gene rearrangement, and begin to express low levels of surface T cell receptor ($CD3^{lo}$). These cells, which are CD44+CD25+ then activate CD4 and CD8 expression and undergo TCR positive and negative selection, a process that results in massive cell death. Cells that successfully complete these developmental stages, then make a CD4 or CD8 lineage commitment step, and exit the thymus as mature, naïve CD4+ or CD8+ single positive (SP) T cells, to seed the periphery. Each stage of development requires specific biochemical events, such as activation of Bcl-2 expression or progression through the cell cycle and proliferation, and many of these events are dependent on Jak3 signal transduction.[120,121]

Gene ablation studies have shown that Jak3 is critical at very early stages in T cell differentiation. Mice lacking Jak3 show a 95% decrease in thymus cellularity. However,

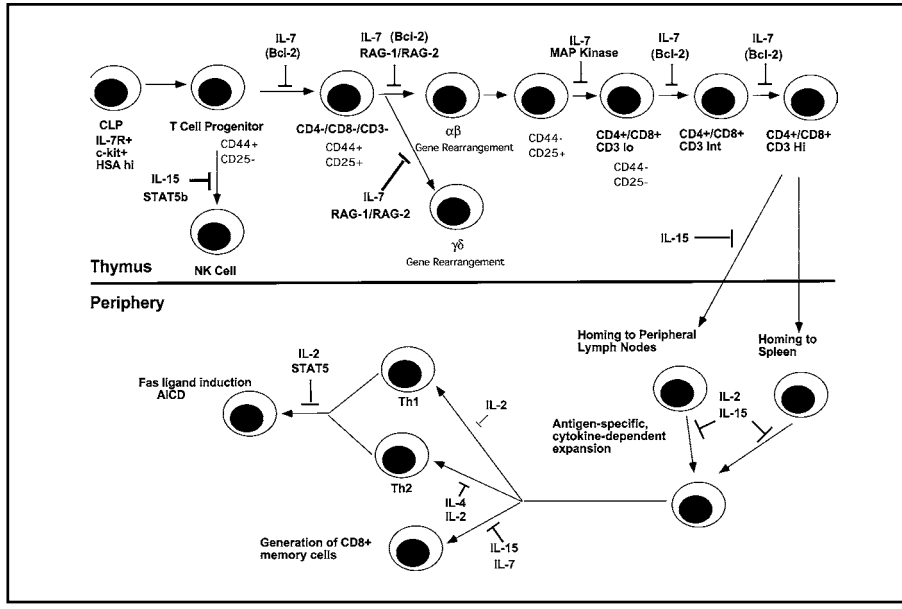

Figure 3. T cell development.

the remaining 5% of thymocytes develop normally, as demonstrated by the presence of the expected ratios of thymocyte subsets (CD4-CD8-, CD4+CD8+, CD4+, CD8-,CD4-CD8+) in Jak3$^{-/-}$ and $\gamma_c^{-/-}$ mice. These data suggest that a major function of Jak3 signaling is to promote the expansion and survival of T cell progenitors, but that cells surviving this transition in the absence of Jak3 develop normally. In support of these findings, experiments in fetal thymic progenitor cells have implicated a critical role for Jak3 in the expansion of pro-T cells that initially seed the thymus.[115] Thymocytes in Jak3-deficient fetuses were virtually undetectable at day 14-15 of gestation, when all of the thymocytes consist of bone marrow-derived progenitors. Only 200 Thy-1+ progenitor T cells were found per thymus in Jak3$^{-/-}$ mice, while WT thymuses contained appoximately 20,000 pro-T cells. Furthermore, the rate of expansion of thymocytes from days 14-18 was identical in Jak3$^{+/-}$ and Jak3$^{-/-}$ animals, indicating that after common lymphoid progenitors migrate from the bone marrow and seed the thymus, they have the same capacity to proliferate and differentiate into mature T cells as WT progenitors.[115]

These findings contrast with the phenotypes of mice with targeteed mutations in genes required for T cell development, such as *Rag1, Rag2, TCRβ*, pre-*Tα*, and components of the TCR signaling complex, which all resulted in a profound block in T cell differentiation at a specific developmental check point (CD3-CD4-CD8- TN stage). This data strongly supports a model in which γ_c/Jak3 signal transduction is critical but not absolutely essential for the survival and expansion of early lymphocyte progenitors. A small number of progenitors in γ_c/Jak3-deficient mice can bypass the signaling defect, populate the thymus and differentiate into mature SP T cells. However, although 5% of developing thymocytes in $\gamma_c^{-/-}$ or Jak3$^{-/-}$ mice can still differentiate into mature T cells, there are additional defects at many developmental transitions in the thymus, due to reduced Bcl-2, increased sensitivity to apoptosis, or defective cell cycle progression, as discussed below.

The earliest stage of T cell development in the thymus, which is represented by a population of pro-T cells that have not yet rearranged their TCR genes and lack surface CD4 and CD8 expression (TN, CD4-CD8-CD3-/CD44+CD25+) is highly sensitive to Jak3-dependent cytokines.[121,122] Jak3 signaling is required at this stage for the rapid proliferation and expansion of these early thymocytes to generate a large pool of cells that will undergo further developmental transitions such as TCR gene rearrangements and positive and negative selection.[18] In agreement with this data, $\gamma_c^{-/-}$ and Jak3$^{-/-}$ thymocytes incorporate BrdU in vivo much less efficiently than WT thymocytes at all stages of development, in particular the pro-T cell stage, indicating that these cells are defective in cell cycle progression and proliferation.[123] The function of Jak3 in rapid expansion of pro-T cells is likely due to signals delivered through the IL-7 receptor, as mice lacking the IL-7 receptor α chain display profound lymphopenia, markedly reduced thymus cellularity, and T cell defects similar to the Jak3 knockout.[125-129] This model is also consistent with the finding that the IL-7 receptor is expressed early in lymphopoiesis, on the surface of the CLP, while other γ_c-dependent cytokine receptors are expressed later in development, suggesting that bone marrow-derived lymphocyte progenitors can respond only to a restricted subset of cytokines.[18] And, as discussed below, IL-7R-deficient thymocytes are arrested at the TN/CD44+CD25+ stage of development, strongly suggesting a vital role for Jak3 and IL-7 in the expansion of T cell progenitors and TN thymocytes and the delivery of signals that are essential to drive the cells through the developmental program.[125-129]

One important finding from these experiments is that there is a critical, specific role for Jak3 signaling in antigen receptor gene rearrangement. Productive rearrangement of TCR beta chain gene CD4-CD8-CD3-/CD44+CD25+ thymic T cell precursors and their subsequent differentiation into CD44-CD25+ thymocytes is a critical developmental transition. Signaling through a pre-T-cell receptor complex composed in part, of the TCR beta chain is essential for all subsequent T cell differentiation steps in the thymus, including positive and negative selection. In Jak3-deficient mice and humans, there is a significant block in productive TCR as well as BCR gene rearrangements. This likely results from decreased lymphocyte survival due to low Bcl-2 levels, and lack of genes required for antigen receptor gene rearrangement.[120-121] Jak3 deficient mice and humans express low levels of the Rag1 and Rag2 proteins, which are components of the site-specific recombinase that is essential for TCR and BCR rearrangement. In support of this model, lymphocyte survival and numbers can be partially rescued by ectopic expression of either *Bcl-2* or productively rearranged *TCR* transgenes.[121,123,124] In addition, it has been shown that one Jak3-dependent cytokine, IL-7, is an important cofactor for *TCR* gene rearrangements.[125-135] Specifically, IL-7 has been shown to induce *Rag1* and *Rag2* expression in purified thymocyes in vitro, and to induce V-DJ gene rearrangements in fetal thymus cell suspensions, while other cytokines, such as IL-2, had no effect.[134] In another study, IL-7 induced the earliest event of TCRβ chain rearrangement, the joining of D and J gene segments, in cultured fetal thymic progenitor cells, and treatment of fetal liver organ cultures with anti-IL-7 or anti-IL-7 receptor antibody inhibited this rearrangement event.[133] Likewise, TCRβ V-DJ gene segment joining was found to be defective in at least one X-SCID patient.[130] Interestingly, recombination of the TCRβ D and J segments, which occurs earlier in development, is not affected by the mutation, suggesting that Jak3-dependent cytokines control very specific events in antigen receptor gene recombination in humans as well as mice. However, the requirement for Jak3 in TCR beta chain gene rearrangement is important, but not absolute, because there is some T cell survival in Jak3-deficient animals, albeit greatly decreased compared to wild-type. Thus, Jak3 greatly increases the efficiency of

productive TCR beta chain gene rearrangements, but some can occur despite low levels of *Bcl-2, Rag1* and *Rag2* expression in the developing thymocytes. In contrast, there appears to be a very strict requirement for Jak3 in TCR gamma chain gene rearrangement, in that Jak3 deficiency results in a complete loss of γδT cells in both mice and humans (see below).[113-117]

As mentioned above thymocytes in γ_c/Jak3$^{-/-}$ mice express reduced levels of Bcl-2 throughout all stages of development.[121] This makes the cells abnormally sensitive to apoptotic stimuli, and likely contributes to the decreased thymus cellularity in Jak3 and γ_c-deficient mice. Bcl-2 levels are particularly reduced in Jak3-deficient TN, CD44-CD25+ cells which are undergoing TCRβ chain rearrangement, which likely contributes to the inefficiency of this process in the absence of Jak3-dependent cytokines (Fig. 3). In particular, it is noteworthy that Bcl-2 levels normally increase during CD4 and CD8 lineage commitment and may be critical for the survival of mature SP T cells that must exit the thymus and migrate to secondary lymphoid organs. The regulation of Bcl-2 expression at this stage of T cell development may explain the effects on CD8+ SP cells in the thymus. As discussed below, Jak3 and γ_c deficient mice contain a population of peripheral CD4+ T cells that can develop in the absence of Jak3 . However, mature CD8+ SP peripheral T cells are almost undetectable, despite normal representation of CD8+ T cells in the thymus.[43,45,46,117] Thus, CD8+ cells apparently differentiate normally in the absence of Jak3, but their export from the thymus and subsequent survival may depend on the upregulation of Bcl-2 or activation of other survival pathways that fails to occur in the absence of Jak3. In support of this, in a model of T cell development, expression of the class I-restricted H-Y antigen-specific TCR rescues thymus cellularity and increases the number of mature CD8+ thymocytes in female γ_c-deficient mice. However, no CD8+ peripheral T cells expressing the transgenic receptor can be detected in the periphery.[137]

Consistent with the biochemical data presented above, lymphopenia in Jak3$^{-/-}$ mice and humans likely results from abnormally low levels of endogenous Bcl-2 expression, leading to reduced cell survival, as well as a lack of Stat5, PI-3 kinase, and MAP kinase-induced cell cycle progression. As a result, Jak3 deficiency results in an early developmental arrest in thymocytes (CD4-/CD8-/CD3- triple negative stage) and, in mice, a block in B-cell development at the preB-proB stage of differentiation (see below).

It is noteworthy that at all stages of thymocyte development, Bcl-2 levels in Jak3$^{-/-}$ cells are markedly reduced compared to wild-type cells. Thus, thymocyte survival during critical developmental transitions is likely to be severely compromised, and probably contributes significantly to the greater than 20-fold reduction in thymic cellularity in Jak3-deficient animals.

In humans, spontaneous, naturally-occurring mutations in Jak3 or γ_c cause Severe Combined Immmunodeficiency (SCID).[113,114,116,139-147] SCID is a relatively rare disorder, occurring in approximately one in 50,000 live births.[139] The common gamma chain gene and the X-SCID genetic locus were found to colocalize to the Xq13.1 chromosomal region, and subsequently, it was shown that many patients with T-B+NK- SCID had mutations in the γ_c gene.[135-140] Extensive analysis of γ_c-deficient patients revealed a wide range of mutations, most of which were single nucleotide substitutions that affect γ_c surface expression, mRNA levels, or ligand binding. Since it had been shown that Jak3 was physically associated with γ_c and activated in response to γ_c-dependent cytokines, patients with autosomal SCID were identified that harbored mutations in the Jak3 gene.[113,114,116,146,147] γ_c/Jak3-defective SCID accounts for over half of all known SCID cases. Consistent with profound defects in lymphoid development and function, SCID patients present with persistent, severe infections early in life and rarely survive beyond 2

years of age without heterologous bone marrow transplants. In the human form of the disease, patients have few, if any peripheral T cells, no NK cells, and normal to elevated levels of non-functional B cells (Fig. 4).

In contrast, a murine model of SCID in which either γ_c or Jak3 expression has been ablated by gene targeting demonstrate a slightly different phenotype from human SCID patients. In the knockout mice, B cell development is arrested at a very early stage of differentiation in the bone marrow (preB—proB transition) (Fig. 4), and, while there is a severe reduction in mature T cells early in life, there is an age-dependent accumulation of activated CD4+ T cells in the periphery.[43,45,46,117] The differences in phenotypes are likely due to species-specific requirements of different lymphoid lineages for Jak3-dependent cytokines during development. Specifically, there is greater IL-7-dependence in murine B cell development compared to humans, in which normal levels of mature B cells are found in the periphery. However, in spite of a higher efficiency of B cell development and normal numbers of peripheral B cells in human SCID patients, these cells are functionally inactive and fail to proliferate in response to surface Ig cross-linking. In addition, $Jak3^{-/-}$ humans fail to produce detectable levels of serum IgG or IgE.[113,114,116] suggesting a role for Jak3 in antigen-driven expansion and immunoglobulin class switching. In contrast, Jak3-deficiency appears to have a more profound effect on T cell development in humans than in mice. This likely reflects a species-specific dependence on signaling through the IL-7 receptor during the early stages of T cell differentiation. While human T cell development is highly sensitive to defects in IL-7 signal transduction, the limited development of CD4+ T cells observed in $Jak3^{-/-}$ mice may be attributed to a Jak3-independent cytokine, TSLP, that serves as an alternate ligand for the IL-7 receptor.[148-150]

In human SCID, various types of mutations have been identified, most of which result in a drastic reduction in γ_c or Jak3 protein levels. However, a few patients have more subtle mutations, in particular, one in γ_c and one in Jak3, that disrupt kinase-receptor interaction, that result in the SCID phenotype.[40,50] Such mutations have been valuable in assessing the function of various domains of γ_c and Jak3[50,151,152] and support the idea that the main function of Jak3 is to transduce signals through the common gamma chain. Interestingly, the presence of normal levels of B cells in the peripheral blood of human SCID patients has provided targets for gene transfer experiments. In cases of γ_c or Jak3 deficiency, retroviral transduction of the WT allele of the mutated gene has completely restored cytokine responsiveness in the patient's cells.[44]

The profound defects in cytokine-dependent growth and expansion of $\gamma_c^{-/-}$ and $Jak3^{-/-}$ lymphoid cells implies that cells expressing the WT forms of these molecules have a selective growth advantage in vivo. In agreement with this idea, peripheral lymphocytes derived from female patients carrying the defective X-linked γ_c allele demonstrate non-random X-chromosome inactivation,[139-143] strongly suggesting thatcells expressing the WT gene expand preferentially in response to Jak3-dependent cytokines. Thus, both humans and mice with γ_c/Jak3 SCID are ideal candidates for gene therapy strategies in which lymphoid precursors are reconstituted with the WT alleles of Jak3 or γ_c. In mice, both of these genetic deficiencies have been corrected by retroviral-mediated gene transfer and re-introduction of infected bone marrow cells.[153-158] Likewise, in vivo gene therapy has been successfully performed in γ_c-deficient humans. Virus-mediated transfer of the WT γ_c gene into bone marrow precursor cells from a SCID patient has allowed for complete reconstitution of the lymphoid compartment, and restoration of normal immune function.[159-161]

Jak3 in Positive and Negative Selection of Thymocytes

One critical function of the thymus beyond its general role in supporting T cell differentiation is the selection of cells expressing the correct repertoire of T cell antigen receptors. After migration of bone marrow-derived T cell precursors to the thymus, and a massive Jak3 cytokine-dependent expansion of pro T cells, there are several distinct, temporally-controlled selection and differentiation events, regulated by a network of signals transduced by multiple cell surface receptors. As shown in Figure 3, the TN CD44+CD25- pro-T cells differentiate into $CD3^{lo}$CD44-CD25+ cells upon productive TCR rearrangement. Cells at this stage activate expression of both CD4 and CD8, and these CD4+CD8+ double positive (DP) cells are subject to positive and negative selection events. The progression through early stages of development is crucial for the selection of cells expressing TCRs of sufficient affinity for self-peptide-MHC complexes that they will expand and proliferate in response to non-self peptides in the periphery during antigenic challenge. On the other hand, the thymus also selects against high affinity TCRs that are self-reactive and capable of generating autoimmune disease.

Positive selection of cells expressing rearranged TCR α and β chain transgenes is normal in $Jak3^{-/-}$ mice.[121,123,136-138] Experiments using transgenes that encode a MHC class I-restricted TCR that recognizes the murine male-specific H-Y antigen have demonstrated that positive thymocyte selection in female animals is unimpaired in the $Jak3^{-/-}$ background.[137] In addition, there is apparently no defect in positive selection of a class-II restricted trangenic TCR as well. $Jak3^{-/-}$ mice expressing transgenes encoding the ova peptide-specific TCR DO11.10 undergo the same degree of positive selection as $Jak3^{+/+}$ mice, supporting a model in which Jak3 signaling is not required for this critical developmental transition in thymocytes.[138]

The role of Jak3 in the negative selection of self-reactive TCRs, is less clear. Some models of thymocyte negative selection suggest that Jak3 deficiency results in defective deletion of autoreactive T cell receptors. For example, thymocytes that express class II-restricted TCRs using the Vβ5 and Vβ11 variable regions are normally deleted in the thymuses of mice that present MMTV-encoded superantigens in conjunction with the I-E MHC molecule. In mice lacking Jak3, deletion of Vβ5 and Vβ11-expressing thymocytes was inefficient when compared to $Jak3^{+/+}$ controls, suggesting that Jak3-dependent cytokines play a role in clonal deletion of these autoreactive T cells.[136] In another study, deletion of Vβ11+ thymocytes in response to the Mtv9 superantigen was inefficient in $\gamma_c^{-/-}$ mice. However, the same study showed that Vβ5+ T cell deletion proceeded normally.[138] In addition, thymocytes from $\gamma_c^{-/-}$ mice that express the ova-specific trasgenic TCR DO11.10 undergo normal levels of negative selection in vitro when cultured with antigenic peptide.[138] Furthermore, in the H-Y TCR transgenic model, there appears to be no defect in negative selection in $Jak3^{-/-}$ mice. In this model, clonal deletion of the H-Y-specific TCR occurs in male animals that express the H-Y antigen in the thymus in the context of H-2Db class I molecules.[137] In both $Jak3^{-/-}$ and control animals, there is a similar distribution of thymocyte subsets, including a drastic reduction of CD4+CD8+ cells that are sensitive to negative selection, and an accumulation of immature CD4-CD8- thymocyes, indicating that negative selection is not altered by Jak3 deficiency.

Thus, negative selection of $Jak3^{-/-}$ thymocytes, under some conditions, is impaired, and may correlate with TCR affinity or MHC background. However, negative selection can occur normally in the absence of Jak3 or γ_c. On the basis of the available data, it is also possible that Jak3 signaling is more important for deletion of self-reactive class II-restricted TCRs than for class I-restricted receptors.

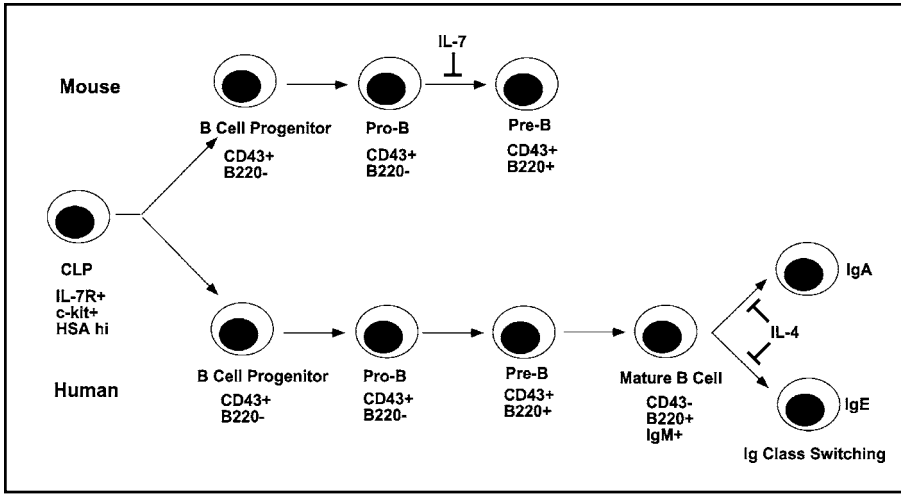

Figure 4. B cell development in mice and humans.

Role of Jak3 in Innate Immunity

Murine models of Jak3 and γ_c deficiency have elucidated the role of Jak3-dependent cytokines in the development of αβ TCR-expressing T cells as well as B lymphocytes. However, these studies have also demonstrated that Jak3 plays a critical role in the development of cells involved in innate immunity, such as γδ T cells, natural killer (NK) cells, and NK T cells.

γδ T Cells

In mice, the earliest and most "primitive" T cells begin to develop at day 15 of gestation, and express an invariant γδ T cell receptor, Vγ3 Vδ1.[127] These cells mature along a defined developmental pathway characterized by expression of specific surface markers. Immature $V\gamma3^{lo}CD24^{hi}$ (heat stable antigen, HSA), differentiate into mature $V\gamma3^{hi}CD24^{lo}$Thy-1+ T cells after productive Vγ gene rearrangement. Some of these cells are found in skin epithelium and are called dendritic epidermal T cells (DETC). DETCs are derived from CD3+CD4-CD8- thymocytes. Alternatively, a subset of γδ T cells, intestinal intraepithelial lymphocytes (iIELs), are found in the mucosa of the small and large intestines and are γδ+CD8α+CD5-Thy-1-, which suggests that they develop extrathymically and likely differentiate within the epithelial lining of the intestine.[46,127] In $Jak3^{-/-}$ mice, all of these cells are absent, suggesting a very early developmental arrest in a common γδ T cell progenitor.[43,45,46,127] Consistent with this model, in addition to the absence of γδ+ DETC and iIELs in Jak3-deficient animals it has been found that there are no γδ+CD3+CD4-CD8- thymocytes in the mutant mice as well (Fig. 3). Detailed phenotypic analysis of bone marrow-derived T cell progenitors has shown that Jak3 deficiency results in a developmental arrest at the immature $V\gamma3^{lo}$ $CD24^{hi}$ stage of differentiation.[127] These findings suggest that Jak3 is required not only for lymphocyte differentiation in primary lymphoid organs, but also for T cells that develop extrathymically. In addition, it has been found that Jak3 deficiency results in complete block in the development of extrathymically-derived TCRαβ-expressing IELs as well.[137] The very early developmental arrest in γδ T cell development is likely due to the role of Jak3 in the proliferation, expansion,

and survival of T cell progenitors as well as its function as a cofactor for *TCR* gene rearrangement. The effects of Jak3 in *TCR* gene rearrangement are likely due to signal transduction through a single cytokine receptor, IL-7R. In support of this idea is the finding that IL-7 induces the expression of the recombinase activating genes *Rag1* and *Rag2*, as well as terminal deoxynucleotidyltransferase (*TdT*), which are essential for *TCR* gene rearrangement and generation of antigen receptor diversity.[134,163] In addition, IL-7 and IL-7R-deficient mice have a profound block of γδ T cells differentiation at a very early stage of development which is indistinguishable from the effects of Jak3$^{-/-}$ deficiency.[164,165] Detailed analysis of developing γδ T cell in Jak3 or IL-7$^{-/-}$ mice has shown that there is a specific block of V and J gene segment rearrangement at the δ_c locus, thus causing the developmental arrest in these cells. In this study, it was found that while all Vγ-Jγ rearrangements were completely blocked in the mutant mice, rearrangements at the γ_c locus were comparable to those seen in WT control animals.[160] Thus, Jak3-dependent signaling enhances αβ and γδ T cell development by acting as a general factor for the survival/expansion/cell cycle progression of T cell progenitors through its effects on Bcl-2 expression, activation of the PI-3 kinase pathway, and Stat-mediated differentiation. Furthermore, Jak3 also plays a very specific role in the regulation of TCR β and γ_c gene rearrangements by inducing the expression of key genes involved in site-specific recombination, and, perhaps by regulating the expression of other cytokine receptor molecules, such as c-Kit, thus affecting multiple stages of T cell differentiation.

NK Cell Development

Natural Killer (NK) cells are derived from a primitive progenitor cell that is pre-committed to the T cell lineage, but can further differentiate into either T cells or NK cells, depending on the cytokines present in the microenvironment. Jak3 has been shown to be essential for the generation of NK lineage cells in the bone marrow and spleen. A critical lineage commitment deCISion occurs at the earliest stages of thymocyte development, in cells that express a surface profile (CD44+CD25-c-kit^{hi}) that is very similar to that of bone marrow T cell precursors. Depending on the cytokine stimulus, these cells will differentiate into either T or NK cells (Fig. 3). Activation of these pro-T cells with IL-7 induces T cell commitment, while IL-15 stimulation triggers NK cell development. In Jak3$^{-/-}$ or $\gamma_c^{-/-}$ mice, staining of splenocytes with antibodies specific for NK lineage surface markers (DX5 and NK1.1) revealed that no mature NK cells are present in the peripheral lymphoid organs of mutant animals. As expected, NK cell cytolytic activity is also absent from splenocytes isolated from Jak3-deficient mice.[43,45,46,117]

B Cell Development

B cell development in the bone marrow proceeds through an ordered sequence of developmental stages characterized by changes in the expression pattern of cell surface markers CD43, B220 (CD45), and the antigen receptor, IgM (Fig. 4). The earliest pre-committed B cell precursors are CD43+B220-, and the development of B cells progresses through the following stages of surface marker expression: CD43+B220-, CD43+B220+, CD43-B220+IgM+. Jak3 deficiency in mice results in a profound block of differentiation at the pro-B (CD43+B220-) to pre-B (CD43+B220+) stages of development. The number of mature, CD43-B220+IgM+ cells among splenocytes of Jak3$^{-/-}$ mice is reduced 10-40 fold compared to WT mice. In the bone marrow, there are no detectable mature B cells, and no CD43+B220+ immature B lymphocytes. However, there seems to be no effect of Jak3 deficiency on the accumulation of early-stage immature CD43+B220- cells.[45] This

strongly suggests that Jak3-dependent cytokines are required to expand early B cell lineage cells and drive their differentiation through later stages of development, in a manner resembling the effects of Jak3 signaling during the early stages of T cell development. There is evidence that the early developmental B cell arrest in Jak3$^{-/-}$ mice is due to loss of signal transduction through the IL-7 receptor. IL-7-deficient mice have a B cell defect similar to Jak3 and γ_c mutant mice.[125,126] In addition, retroviral reconstitution experiments in primary B cells derived from IL-7 receptor- deficient mice have shown that introduction of the IL-7R restores the expansion of B cell progenitors and BCR gene rearrangements.[166] In agreement with data demonstrating that Jak3-dependent cytokines are important cofactors for TCRβ and TCRγ rearrangement, the regulation of the recombinase activating genes *Rag1* and *Rag2* by IL-7R signaling likely plays an important role in the induction of immunoglobulin heavy chain gene rearrangements in B cells.

Role of Jak3 in the Periphery

Due to low thymus cellularity, and greatly reduced numbers of developing T cells available to migrate to peripheral lymphoid organs, at two weeks of age, there are few, if any detectable peripheral T cells in Jak3 or γ_c-deficient mice. However, CD4+ T cells then undergo a dramatic expansion, and by 12 weeks of age the number of splenic T cells in Jak3$^{-/-}$ mice exceeds WT levels by 4-5 fold. This aberrant proliferation of mature T cells is likely due to an imbalance of Jak3-dependent proliferative signals and negative regulatory mechanisms that are required to maintain a homeostatic balance of naïve and activated T cells in secondary lymphoid organs and peripheral blood.

Although CD4+ T cells eventually populate the peripheral lymphoid organs of Jak3$^{-/-}$ mice, they are phenotypically abnormal and fail to respond to mitogenic stimuli and exogenous cytokines in vitro. Peripheral T cells from Jak3-deficient mice have a surface marker profile characteristic of memory/activated T cells. The majority of these cells are CD62L^{lo}CD44^{hi}CD69^{hi}Mel-14^{lo}, suggesting that they are chronically stimulated through their T cell receptors, fail to return to a naïve phenotype, and are not deleted through peripheral tolerance mechanisms.[43,45,46,117] In support of this idea, both WT and Jak3$^{-/-}$ peripheral T cells expand at the same rate in vitro, suggesting that the aberrant accumulation of these cells in vivo is due to a defect in negative regulation of their expansion. Interestingly, rare cases of human Jak3-deficient SCID have been described in which signaling function has not been completely abrogated, allowing a significant population of T cells to develop and seed the periphery.[168-170] T cell from these patients are almost exclusively CD4+, and have an activated/memory phenotype (CD45RO+Fas+HLA-DR+CD62L^{lo}). They are refractory to multiple mitogenic stimuli in vitro, and are susceptible to spontaneous apoptosis. This phenotype is strikingly similar to the murine Jak3 and γ_c knockouts, and demonstrate that, despite subtle differences between human and murine SCID, γ_c/Jak3 signal transduction plays a very similar role in hematopoiesis and lymphoid development across species. It is important to note that neither defects in thymocyte development nor the hyper-expansion of mature T cells in the periphery can be attributed to altered surface expression of T cell receptor components or co-stimulatory molecules. Jak3$^{-/-}$ T cells express normal levels of CD3, TCRα and β chains, CD25, CD28, CTLA-4 and Fas.

Models of peripheral clonal deletion and self tolerance using in vitro systems and Jak3$^{-/-}$ mice expressing TCR transgenes have allowed a detailed analysis of the role of Jak3 in peripheral lymphoid homeostasis. Several studies have demonstrated that Jak3-deficiency does not affect Vβ5+ and Vβ6+ thymocyte deletion in response to endogenous superantigens, as well as clonal deletion of DO11.10 transgenic thymocytes in response to

antigenic peptide in vitro.[138] However, these same experiments have shown that peripheral deletion of Vβ8 1,2 T cells in response to staphlococcus enterotoxin B (SEB) is drastically reduced in Jak3$^{-/-}$ mice.[138] These findings suggest that the accumulation of CD4+ T cells in Jak3 or γ_c-deficient animals is likely due to defects in cytokine-dependent elimination of activated or self-reactive lymphocytes. In support of this model is evidence from TCR transgenic mice showing that the aberrant expansion of mature Jak3$^{-/-}$ T cells is antigen-dependent. Jak3 or γ_c-deficient mice expressing the DO11.10 (ovalbumin peptide-specific) transgenic TCR contain cells that express the Tg receptor that can be identified with an anti-clonotypic antibody, and a subset of T cells expressing endogenous T ell receptors. Using this approach, it has been found thaT cells expressing the non-self-reactive DO11.10 TCR have a surface phenotype characterisitic of naïve, quiescent T lymphocytes. On the other hand, T cells expressing endogenous TCRs have the activated/memory phenotype characteristic of Jak3$^{-/-}$ CD4+ T cells.[167] This strongly suggests that there is an imbalance between antigen-driven proliferative signals and negative regulatory mechanisms which results in the accumulation of high levels of activated peripheral T cells in he Jak3-deficient mice.

This model is supported by data showing that splenic Jak3$^{-/-}$ T cells incorporate much higher levels of BrdU in vivo than cells from WT controls.[167] Surprisingly, although Jak3$^{-/-}$ CD4+ T cells incorporate markedly enhaned levels of BrdU in vivo, suggesting a very high level of proliferation, hese cells are refractory to all polyclonal stimuli tested in vitro. They fail to proliferate or secrete IL-2 in respose to α-TCR antibody, α-TCR+PMA as well as PMA+ionomycin. In contrast, thymocytes from the same mice proliferate in response to PMA+α-TCR antibody or PMA+ionomycin. These findings suggest that Jak3$^{-/-}$ peripheral T cells are exhaustively stimulated by antigen and fail to respond to secondary challenge in vitro, yet continuously proliferate in a TCR-dependent manner to antigens in vivo.[167,171] In support of these findings, proximal vents in TCR signaling are normal in Jak3$^{-/-}$ T cells. Ca^{++} flux and tyrosine phosphorylation of cellular subtrates in response to TCR activation in Jak3-deficient T cells are indistinguishable from WT cells, suggesting that self or enviromental activation can occur in vivo. Inaddition, analysis of TCR usage has shown that Jak3-deficient thymocytes express a normal distribution of T cll receptors, but peripheral T cells expressed a greatly skewed, oligoclonal population of receptors, indicating a preferential antigen-driven expansion of a small number of antgen-reactive clones in the periphery. However, there is a profound reduction in the secretion of IL-2 from these cells when stimulated through the TCR in vitro. This may be due, at least in part, to decreased expression of NFAT and c-Fos, transcription factors that are essential for efficient expression of the IL-2 gene in response to antigen receptor cross-linking.[171]

Interestingly, it has been shown that the survival of naïve and memory T cells is differentially regulated by Jak3-dependent cytokines, and significantly contributes to the accumulation of activated CD4+ peripheral T cells in γ_c/Jak3 KO mice.[172] In adoptive transfer experiments, naïve T cells from $\gamma_c^{-/-}$ mice have a greatly decreased half-life compared to WT controls, but the survival of activated T cells is unaffected by γ_c deficiency, suggesting a selective role fo γ_c-dependent cytokines in the survival of unstimulated T cells. Furthermore, antigen-driven expansion and cytokine production of both naïve and memory T cells in vivo was found to be γ_c-independent.[172] Thus, the selective effects of Jak3-dependent survival signals on different populations of mature T cells results in disrupted peripheral lymphoid homeostasis in γ_c/Jak3 deficient SCID. Defective Jak3 signal transduction causes the accelerated loss of naïve CD4+ T cellsand the preferential expansion of antigen-activated cells in secondary lymphoid organs.

In addition to to defects in mitogenic resones, splenocytes from Jak3 and $_c$-deficient mice are 5-10-fold more sensitive to Fas-mediated apoptosis than WT cells.[167] The enhanced sensitivity of Jak3$^{-/-}$ T cells to anti-Fas-induced apoptosis may be explained by the finding that Fas expression is often elevted in Jak3-deficient T cells. The accelerated cell death defect in these cells is partially corrected by ectopic overexpressionof a Bcl-2 transgene. However, protection from apoptosis by Bcl-2 expression is not complete, which suggests that Jak3dependent cytokines activate additional survival pathways.

The defects in peripheral T cell tolerance and clonal expansion demonstrate an essential role for Jak3 expression in peripheral lymphocytes for cytokine-mediated regulation o lymphoid homeostasis. Two studies have shown that the effects of Jak3 deficiency on peripheral T cell function are not due to defective T cell development, but toa specific role for Jak3 in the periphery. Thymus-specific expression of Jak3 has been achieved using a transgene in which expression of the Jak3 cDNA is driven by the lck proximal promoter.[173,174] In the Jak3$^{-/-}$ background, expression of this transgene ishigh in thymocytes, and is drastically reduced in peripheral T cells. Studies using these mice have shown that while thymu-specific expression of Jak3 rescued thymic cellularity and T cel development, the defects in peripheral T cell expansion and cell cycle progression were identical to non-transgenic Jak3-deficient mice. There were few detectable splenic CD8+ T cells, while CD4 cells gradually accumulated. These peripheral CD4+ T cells expanded to much higher levels than that seen in WT animals, expressed an activated/memory surface phnotype, but were refractory to polyclonal mitogenic stimuli in vitro. This data demonstrates that Jak3 expression in peripheral lymphocytes is essential to maintain a pool of functionally active naïve T cells, and to properly control the proliferation and expansion of antigen-activated lymphocytes in secondary lymphoid organs.

Thus, tere are multiple defects in Jak3$^{-/-}$ T cells in both TCR-induced proliferation as well as cell death mechanisms that ultimately favors a hyper-expansion of CD4+ peripheral lymphocytes in vivo. Paradoxically, while low Bcl-2 levels and high expression of Fas results in icreased sensitivity of Jak3-deficient T cells to Fas-induced apoptosis, defective Fas ligand expression in these cells likely results in resistance to cell death in vivo, which contributes to the accumulation of these cells in secondary lymphoid organs (Fig. 3).

Knockouts of Jak3-Dependent Signaling Components

Clearly, models of Jak3 and γ_c deficiency provide strong evidence that multiple cytokine receptors play an important role at many levels of hematopoietic cell differentiation and development. However, Ja3 dependent signals have been addressed in genetically anipulated mice under conditions where single receptors or cytokines have been ablated by gene targeting while all other Jak3/γ_c-dependent signaling complexes were left intact. What follows is a description of the phenotypes of individual γ_c-dependent cytokine/receptor knockouts, and Table 2 summarizes the results of these gene-targeting experiments.

IL-7

In L-7 knockout mice, the early developmental arrest in T and B cells is identical to that in the Jak3 or γ_c knockout. B cells fail to develop beyond the pro-B cell stage, and there is a 95% decrease in thymus cellularity, due to decreased Bcl-2 expression, as well as defective TCR gene rearrangement.[125,126,170,175] These specific molecuar defects are likely the major consequence of IL-7 signaling deficiency, as ectopic expression of Bcl-2 or rearranged TCR β chain transgenes can at least partially rescue thymus cellularity and thymocyte differentiation in vivo.[177,178] In addition, consistent with Jak3 deficiency, there

also is a greater effect of IL-7 deficiency on γδT cell development (there are no detectable γδ T cells in the periphery), which is likely due to a very strict requirement for IL-7 in γ chain gene rearrangement (Fig.3).

Interestingly, targeted mutation of the IL-7 receptor in mice results in a much more severe T cell defect than Jak3, γ_c or IL-7 deficiency. In IL-7R α chain$^{-/-}$ mice, there is a >99% decrease in thymus cellularity and T cell differentiation is completely blocked at the CD4-/CD8-/CD3- pro-T cell stage of development.[125,126] On the other hand, in Jak3^{--} or IL-7$^{-/-}$ mice, the remaining 5% of surviving thymocytes appear to develop normally, as shown by the expected ratios of CD4+ and CD8+ T cells in the thymic compartment. The severe T cell defects in IL-7R$^{-/-}$ mice strongly suggest an dditional, Jak3-independent function for the IL-7 receptor in T cell development. In support of this idea, the IL-7Rα chain has recently been found to be a component of the receptor for an IL-7-like cytokine, thymic stromal lymphopoietin (TSLP). TSLP hs a biological activity that is very similar to IL-7: it induces proliferation, Bcl-2 expression, and Stat5 activation in pre-T cells.[148-150] Importantly, however, these biological effects occur in the absence of γ_c-dependent Jak3 phosphorylation. These studies suggest that lack of the IL-7 receptor results in severe T and B cell developmental defects due to a combined effect on signal transduction through two critical ligands of the IL-7Rα chain, IL-7 and TSLP. onsistent with these developmental defects in IL-7Rα$^{/-}$ mice, two naturally-occurring IL-7 receptor mutations have been identified in human patients, which result in T-/B+/NK+ SCID. The mutations cause defective surface expression of the IL-7Rα chain, or drastically reduced ligand affinity. In agreement with the murine gene knockout, both of these mutations cause a profound defect in T cell development.[179,180]

Interestingly, models of IL-7 and IL-7 receptor deficiency suggest important differences between mice and humans in the requirement for Jak3-dependent cytokines in lymphocyte development. In the process of T cell development and differentiation, Jak3-dependent cytokines (especially IL-7) appear to be more important in humans than in mice. Jak3$^{-/-}$ mice accumulate CD4+ T clls in peripheral lymphoid organs, while humans with Jak3 mutations have few, if any mature T cells. This may e due in part to the presence of the alternative IL-7R ligand TSLP. Since TSLP is IL-7-like in ts effects on T cells, but is Jak3-independent, the limited T cell development observe in Jak3$^{-/-}$ mice is likely to be due to a low level of thymocyte rescue by TSLP.

In contrast, human TSLP and TSLP receptor (TSLPR) homologs have been shown to promote growth and functon of myeloid cells, in particular dendritic cells, with little if any effect on lymphoid development.[181] As in the murine system, the human TSLP receptor complex is a heterodimer composed of the IL-7Rα chain and a recently described partner, TSLPR, which has significant homology to the common gamma chain. The human forms of TSLP and its receptor are only distantly related to their mouse counterparts (43% and 39% homologous, respectively), suggesting that TSLP may have species-specific effects on different lineages of hematopoietic cells. Human TSLP has been found to strongly induce the production of T cell chemoattractants in monocytes and enhances the differentiation of CD11c+ dendritic cells by inducing the expression of cell-surface costimulatory molecules such as CD40 and CD80. Human TSLPR is expressed primarily on monocytes and dendritic cells, with much lower levels detected on T, B, and NK lineages.

Both human and mouse TSLP appear to induce tyrosine phosphorylation of Stat3 and Stat5 in a Jak kinase-independent manner, suggesting a common alternative signaling pathway in both species. However, while murine TSLP has been demonstrated to induce proliferation and differentiation of B and T cells, human TSLP acts primarily on myeloid

Table 2. Phenotypes of Jak3-dependent cytokine receptor knockouts

Cell Population	Knockout				
	Jak3/ γc	**IL-2**	**IL-4**	**IL-7**	**IL-15**
B cells	•Development arrested at CD43+B220- pro-B stage	•Exhaustive B cell expansion •Elevated IgGl and IgE •Hemolytic anemia	•Low IgG1 •Undetectable IgE •Elevated IgG2 and IgG3	•Development arrested at CD43+B220- pro-B stage	•Defective homing to lymph nodes
αβ	•Cellularity thymocytes	•Unaffected reduced 95%	•Unaffected	•Cellularity	•Unaffected reduced 95%
αβ peripheral T cells	•Defective/ activated	•Activated/ autoimmune CD4+ T cells	•Complete loss of Th2 differentiation	•Defective colony formation •Reduced proliferation/ survival	•Defective homing to lymph nodes •Reduced CD8+ memory T cells
γδ thermocytes	•Absent	•ND	•ND	•Absent	•Unaffected
γδ/αβ IELs	•Absent	•ND	•ND	•Absent	•Unaffected
NK cells	•Absent	•ND	•ND	•Unaffected	•Absent

cells, and it cannot promote growth of B cell precursors from bone marrow in vitro. In addition, it has been found that few human T cell clones coexpress IL-7Rα and TSLPR, indicating that human TSLP does not have a major role in T cell development or function. These data suggest that there is little functional overlap between the human and mouse forms of this novel cytokine. Therefore, it is possible that the differences between murine and human γ_c/Jak3-deficient SCID may be due to the effects Jak3-independent cytokines such as TSLP, which signal through receptors of the hematopoietic superfamily, but may have species-specific effects on the development and function of different hemapietic lineages.

As mentioned above, it is clear that IL-7 is essential to support the proliferation and expansion of very early thymic precursors, including the bone-marrow-derived common lymphoid progenitor cell ($CD4^{lo}$CD3-CD44+CD25-c-kit^{hi}), and that is has a crucial role in the promotion of TCRβ and TCRγ chain rearrangements. In support of this are the findings that the IL-7 receptor is expressed on the surface of the bone marrow-derived CLP,[18,182] suggesting that early in lymphopoiesis can cells respond to IL-7. In addition, IL-7 expression is detected in the subcapsular region of the thymus, where the most immature thymocytes initially migrate. However, there are at least three possible mechanisms through which IL-7 may enhance TCR gene rearrangements in the thymus: IL-7 may actually induce D-J and V-DJ recombination through it effects on the expression of the Rag1 and Rag2 genes; it may also induce the proliferation of pro-T cells in the thymus, to generate a large number of substrates for recombination, or it may selectively induce the expansion and survival of thymocytes that have productively rearranged their TCR genes. Of course, it is possible that IL-7 can use any one or more of these mechanisms to ensure normal T cell development, TCR gene rearrangements and peripheral T cell survival.

IL-7 plays a direct role in the induction of TCR gene rearrangements. Ectopic expression of a *Bcl-2* transgene rescues T cell development in IL-7$^{-/-}$, IL-7Rα chain$^{-/-}$, and Jak3 or γ_c-deficient mice, suggesting that IL-7 functions as a growth factor for bone marrow and thymic T cell precursors. However, *Bcl-2* expression rescues thymus cellularity to only 10%-20% of wild type levels, suggesting that the functionof IL-7 extends beyond its role in progenitor expansion.[176,178] In addition, as mentioned above, treatment of fetal liver thymocytes with IL-7 alone in vitro is sufficient to induce both DJ as well as V-DJ recombination, while other cytokines such as IL-2, have no effect. Importantly, treatment of fetal liver cells with stem cell factor, a ligand of the c-kit tyrosine kinase receptor, known to induce proliferation and expansion of pro-T cells, fails to promote TCRβ chain rearrangements.[134] Since most fetal liver T cell precursors are c-kit+ and proliferate in response to SCF, the inability of this cytokine to replace the function of IL-7 in TCRβ chain recombination suggests that IL-7 has a direct, specific role in the process of T cell receptor gene rearrangement, beyond induction of progenitor expansion.

The effects of IL-7 deficiency on IgH and TCRγ chain rearrangements have been attributed to altered accessibility of the heavy chain and TCR gamma chain loci to the recombination machinery. Production of sterile RNA transcripts of TCR and BCR genes is known to precede rearrangement and is likely required to increase locus accessibility to recombination enzymes by demethylation of the target DNA sequence and the induction of histone acetylation. It has been found that transcripts from the IgH and γ chain loci are greatly reduced or absent in early B cells and thymocytes from IL-7R$^{-/-}$ mice. In both cases, the genomic locus was found to be constitutively methylated, and thus inaccessible to the recombinase, while this region of DNA is unmethylated and undergoes normal rearrangement in IL-7$^{+/+}$ cells. Furthermore, pre-treating the IL-7$^{-/-}$ thymocytes with the deacetylase inhibitor trichostatin A (TSA) can reverse the defect in TCRγ rearrangements.[183]

These results suggest that, in addition to its role in regulating the transcription of the *Rag1* and *Rag2* genes, IL-7 has a highly specific effect on the IgH and TCR γ chain loci and is essential to activate the earliest events in receptor rearrangement Interestingly, in the B cell compartment, it has been shown that the transcription factor Pax5 is required for production of sterile transcripts that leads to BCR recombination events, and, in fact, Pax5$^{-/-}$ mice have a B cell phenotype very similar to the IL-7 knockout. These findings suggest that the accessibility the IgH locus to this transcription factor is a critical step in the induction of BCR gene rearrangements, and that this event is controlled by IL-7 receptor signaling.[183]

Interestingly, some data suggest that IL-7 is also required for peripheral T cell survival, and the maintenance of normal capacity to proliferate in response to T cell receptor cross-linking. IL-7 receptor deficiency results in a 6-7-fold reduction in the number of T cell clones derived from invitro stimulation with α-CD3 and either α-CD4 or α-CD8 antibodies in vitro. In addition, peripheral T cells from IL-7R$^{-/-}$ mice proliferate poorly to allogeneic cell stimulation and undergo accelerated apoptosis when stimulated through their TCRs.[184]

These results suggest that, in addition to its role in the promotion of thymocyte survival and TCR rearrangments in immature thymocytes, IL-7 also functions to maintain a pool of functional, mature peripheral T cells in the secondary lymphoid organs. Thus, the inability of Jak3$^{-/-}$ or $\gamma_c^{-/-}$ peripheral T cells to proliferate in response to multiple polyclonal stimuli in vitro may, in part, be explained by the failure of IL-7 signaling to maintain a population of functionally active mature T cells in the peripher.

IL-2

Gene targeting experiments have revealed a surprising role for IL-2 in the negative regulation of peripheral lymphocyte function and induction of apoptosis, in contrast to its expected role as a T cell growth factor. Ablation of IL-2 signaling has been accomplished genetically by independent disruption of the genes encoding IL-2, IL-2Rα, or IL-2Rβ[185-189] Lines of mice from all three knockouts have very similar phenotypes, characterized by a major defect in control of peripheral lymphocyte expansion, severe, multi-organ inflammatory disease and fatal hemolytic anemia. On the other hand, lack of IL-2 signaling, surprisingly, has little if any effect on B and T cell development. In all knockouts, thymus cellularity as well as the ratio of developing thymocyte subsets are normal. Furthermore, experiments in mice expressing class I or class II-specific transgenic T cell receptors have demonstrated that both positive and negative selection of thymocytes is normal in the absence of IL-2.[20,189] In addition, there is no detectable developmental arrest in the B cell compartment. Normal numbers of pro-B and pre-B cells are detected in the bone marrow of young mutant mice. Furthermore, exit of mature lymphocytes from the thymus or bone marrow, as well as their migration to secondary lymphoid organs such as the lymph nodes or spleen also appear unaffected by the absence of IL-2 signaling. Therefore, it is now believed that IL-2, while a potent T cell growth factor in vitro, does not play any significant role in lymphopoiesis. This is further support for the idea that IL-7 signal transduction is the major cell growth and survival pathway through which lymphocyte progenitors and early committed T and B cells expand and differentiate.

Although T and B cell development in mice lacking IL-2, IL-2Rα and IL-2Rβ chains appears to be normal, lack of IL-2 signaling results in profound defects in peripheral B and T lymphocyte function as a result of uncontrolled CD4+ and CD8+ T cell expansion. Peripheral T cells have a surface phenotype characteristic of activated/memory cells. The lymph nodes of IL-2Rβ$^{-/-}$ mice have high numbers of large, blasting T cells, two-thirds of which are CD69+, whereas all lymph node T cells in WT controls lack CD69 expression.[188] Similarly, the majority of IL-2$^{-/-}$ lymph node T cells express high levels of the activation marker CD44, and a large fraction of colonic T cells from IL-2Rα$^{-/-}$ mice are CD44+CD69+, and incorporate BrdU to a greater extent than cells from WT controls.[186,187] These phenotypes are strikingly similar to Jak3 and γ_c-deficient animals, and suggest that mice lacking IL-2 signaling fail to properly control peripheral T cell activation, which results in the accumulation of large numbers of activated, antigen-reactive proliferating lymphocytes in secondary lymphoid organs.

IL-2$^{-/-}$ and IL-2Rα chain$^{-/-}$ mice have very similar disease phenotypes. Both knockouts result in splenomegaly and lymphadenopathy due to excessive T cell proliferation and overproduction of Th1- and Th2-specific cytokines, with subsequent loss of normal spleen and lymph node architecture. As a result of the hyperproliferation of activated T cells, there is abnormal B cell expansion with concomitant increase in serum IgG1 and IgE levels, anti-self antibody production and hemoytic anemia which is lethal in a significant fraction of the mice. The remaining animals succumb to bone marrow destruction by expanding lymphocytes, as well as massive lymphocytic infiltration of the intestines resulting in fatal inflammatory bowel disease (IBD) at 10 to 25 weeks of age.[185-187]

The phenotype of the IL-2Rβ chain mutant is similar, but differs from the IL-2 and and IL-2Rα chain knockouts in several important respects. These mice also suffer from splenomegaly and lymphadenopathy due to hyper-expansion of peripheral T cells. In addition, IL-2Rβ$^{-/-}$ mice also have elevated anti-nuclear and anti-DNA autoantibody titers.[188] However, autoimmune disease is restricted to hemolytic anemia due to production

of anti-self antibodies. These mice do not develop fatal IBD, but instead suffer from generalized granulocytic infiltration of multiple organs. IL-$2^{-/-}$ and IL-$2R\alpha^{-/-}$ peripheral CD4+ T cells, which retain the intermediate affinity, signaling-competent, IL-$2R\beta/\gamma_c$ receptor heterodimer, can proliferate to mitogenic stimuli in vitro, although responses are reduced 3-10-fold compared to WT controls. The reduced proliferation is likely due to the absence of autocrine stimulation of the cells by endogenously produced IL-2 and induction of cell surface IL-2Rα chain expression. In contrast, IL-$2R\beta^{-/-}$ peripheral CD4+ T cells are much more refractory to stimulation with multiple polyclonal mitogens in vitro. There are barely detectable responses to α-CD3, α-CD3+PMA, as well as PMA+ionoMycin, ConA and SEB.[188]

Interestingly, mature B cells (B220+, sIgM+) disappear from the periphery of IL-$2R\beta^{-/-}$ mice by 6-8 weeks, suggesting that there is an age-dependent reduction of B cell development and exhaustive expansion of mature B cells. In contrast, peripheral B cell numbers in IL-$2^{-/-}$ and IL-$2R\alpha^{-/-}$ mice remain elevated due to uncontrolled proliferation and excessive T cell help. As a result, IL-$2R\beta^{-/-}$ mice fail to mount an antibody response to challenge with hapten-carrier conjugates or VSV infection,[188] while the IL-2 and IL-2Rα chain knockouts respond with antibody titers equal to or greater than WT controls. This finding is supported by data demonstrating that IL-15 is a B cell growth factor in vitro. Thus, loss of the IL-2Rβ chain, but not IL-2 or IL-2Rα would abrogate the effects of IL-15 on B cell development and function in vivo.

In several in vivo models, it has been shown that most of the autoimmune dysfunction in mice lacking IL-2 signaling is due to the hyper-expansion of activated, antigen-reactive CD4+ T cells. Introduction of the IL-2Rβ mutation into nude mice (nu/nu, lacking T cells), or treating the IL-$2R\beta^{-/-}$ mice with anti-CD4 antibody alleviated the hemolytic anemia, restored peripheral B cell numbers, and reduced serum IgG1 and IgE concentrations to wild-type levels.[188] In addition, IL-$2^{-/-}$ mice with the nu/nu mutation do not develop anemia or IBD, and have reduced numbers of activated, blasting peripheral lymphocytes.[190] These data demonstrate that B cell defects in IL-2-deficient mice are not cell autonomous. Elevated serum Ig levels and autoantibody production are an indirect effect of activated CD4+ T cell help and elevated cytokine production on B cell activation and expansion.

In a manner similar to Jak3 and γ_c-deficient mice, the uncontrolled expansion of IL-$2^{-/-}$ CD4+ peripheral T cells is induced by TCR stimulation with self or environmental antigens. This has been demonstrated in mice that express a non-self-reactive transgenic T cell receptor. Peripheral T cells expressing the Tg TCR have a naïve surface phenotype, while those expressing endogenous, antigen-reactive receptors express high levels of activation antigens. Furthermore, expression of the Tg TCR markedly prolongs the liFespan of IL-2-deficient mice, and, in addition, maintenance of IL-$2^{-/-}$ mice in germ-free conditions eliminates IBD. This strongly suggests that repeated encounters with antigen drives the autoimmune phenotypes of animals lacking IL-2 signal transduction.

The severe autoimmune disease in mice lacking IL-2 signal transduction suggests thaT cell death mechanisms are defective, which allow the accumulation of self-reactive T cells. It has been suggested that Fas ligand induction by Stat5 is a critial mechanism of peripheral T cell deletion (Fig. 3). In fact (see above), it has been shown that Jak3 or γ_c-deficient T cells have low surface expression of Fas ligand and are defective in AICD, which contributes to their prolonged liFespan in vivo.[138] However, the role of IL-2 in activation-induced cell death by Stat5-mediated fas ligad expression is somewhat controversial. Retroviral-mediated transfer of IL-2 receptor beta chain mutants into primary T cells

from IL-2Rβ knockout mice has demonstrated that fas ligand expression is dependent on activation of Stat5. Mutants of the β chain that fail to induce Stat5 activity also cannot upregulate Fas ligand expression.[191] However, work from several groups has shown that expression of neither fas nor fas ligand is affected in cells lacking IL-2Rβ[20] Yet, it has been shown that IL-$2^{-/-}$ peripheral T cells fail to undergo apoptosis in response to anti-Fas antibody in vitro.[20,185,186] In addition, deletion of Vβ8+ mature T cells by SEB in vivo is markedly reduced in IL-$2R\alpha^{-/-}$ mice.[187] These findings suggest that IL-2 regulates cell death by mechanisms other than control of fas or fas ligand expression. One such mechanism may involve the modulation of the anti-apoptotic, fas-like decoy receptor c-FLIP, whose expression is known to be down-regulated in response to IL-2, and may be aberrantly regulated in IL-2-deficient animals.[193] In addition, T cells from IL-$2^{-/-}$ mice fail to down-regulate c-FLIP mRNA in response to IL-2,[193,194] and mice expressing a c-FLIP transgene in bone marrow precursors develop autoimmune disease due to prolonged survival of autoreactive peripheral lymphocytes.[195] These findings suggest that regulation of expression of decoy receptors may be a component of IL-2-mediated control of lymphocyte expansion in the periphery, and are consistent with data demonstrating that Fas/Fas ligand-mediated apoptosis is essential for peripheral tolerance and clonal deletion, but is not critical for thymocyte negative selection.[193,196] Another possible mechanism has been suggested by analysis of a naturally-occurring mutation of the IL-2Rα chain in a human patient.[197] The human form of IL-2Rα chain deficiency closely resembles the murine gene knockout. The mutation results in severe inflammatory disease, infiltration of activated CD4+ T cells into several organs, splenomegaly, and enlarged lymph nodes with abnormally high cellularity. Immunohistochemical analysis of tissue sections has revealed that lymph node T cells from the patient have elevated expression of Bcl-2. Thus, the autoimmune dysfunction resulting from IL-2 or IL-2 receptor deficiency may be explained by prolonged survival of activated, self-reactive peripheral T cells which fail to be deleted normally due to high levels of Bcl-2 expression. Bcl-2 levels are likely increased by signaling through Jak3-dependent cytokine receptors such as IL-7 or IL-15, and fail to be properly downregulated due to defective IL-2 signaling.

Targeted deletion of Jak3 as well as IL-2 or its receptor components produce a similar effect on peripheral lympoid homeostasis. All of the mutations result in the accumulation of mature, CD4+ T cells with an activated/memory surface phenotype. Due to the peiotropic effects of Jak3 deficiency on proliferation and signal transduction through multiple receptors, and reduced T and B cell development, Jak$3^{-/-}$ or $\gamma_c^{-/-}$ T cells do not induce severe autoimmune disorders. In contrast, IL-2 deficiency results in the loss f a single signaling pathway that is critical for negative regulation of immune responses, while all other Jak3-dependent signaling complexes remain intact. Thus, autoimmune dysfunction in IL-$2^{-/-}$ mice results from the combined effects of normal expansion and development of immature T and B cells combined with the selective loss of a major lymphod homeostatic control mechanism. These studies demonstrate that a major function of IL-2 in vivo is not to induce T cell growth and differentiation, but rather to induce peripheral lymphocyte deletion and tolerance, as well as to control the expansion of antigen-activated mature T cells.

IL-15

IL-15 was initially characterized as a growth factor for T cells and NK cells.[198] However, unlike most Jak3-dependent cytokines and their receptors, which display a highly tissue-specific pattern of expression, the ubiquitous expression of IL-15 and the IL-15Rα chain raised questions about the function of IL-15 in various tissues of the immune

system, and suggested a pleiotropic role for this cytokine in lymphopoiesis. Consistent with data demonstrating that the IL-2Rβ chain is a signaling component of the IL-15 receptor, IL-2Rβ$^{-/-}$ mice have no functional peripheral NK cells, while IL-2$^{-/-}$ and IL-2Rα chain-deficient mice have normal NK development and function.[185-188] These results strongly suggested that IL-15, and not IL-2 was the major NK cell growth factor in vivo. However, these studies did not exclude additional functions for IL-15 in lymphoid and non-lymphoid cells, because in IL-2Rβ chain gene knockouts, it was not possible to distinguish between IL-2 and IL-15 signaling deficits on the lymphoid defects and autoimmune diseases observed in these mice. The IL-2Rβ knockout suggests additional roles for IL-15 beyond NK cell development, but its role in lymphocyte development in vivo, has until recently, been unclear. In order to clarify the function of this cytokine, IL-15Rα gene targeted mice have been generated, revealing in vivo functions of IL-15 in the development and migration of T, B, and NK cells, the generation of CD8+ memory T lymphocytes, as well as a major role in the development of cells involved in innate immunity.

A major effect of IL-15Rα chain deficiency is a complete lack of functional peripheral NK cells.[199] Splenocytes from IL-15Rα$^{-/-}$ mice displayed no cytolytic activity toward an NK targeT cell line, YAC-1. In addition, IL-15Rα$^{-/-}$ spleen cells were devoid of lymphocytes of the NK lineage, (CD3-/DX5+, and CD3-/NK1.1+), demonstrating that IL-15 is required for the development of natural killer cells (Fig. 3). Interestingly, disruption of the genes encoding the two other components of the IL-15 receptor, IL-2Rβ and γ_c also result in a complete loss of NK cells, strongly supporting a critical role for IL-15 in natural killer cell development and function. As mentioned above (Table 1), IL-15 induces the activation of the transcription factor Stat5. It has been shown that disruption of the gene of one Stat5 isoform, Stat5b, results in a profound decrease NK cell differentiation and proliferation.[200] Thus, the critical role of IL-15 in NK cell development can be attributed, at least in part, to the activation of Stat5b transcriptional activity in lymphoid precursors. However, the target genes activated by Stat5b that induce NK cell differentiation have not yet been identified.

On the other hand, the development of T and B lymphocytes is not significantly affected by IL-15Rα chain deficiency. In IL-15R mutant mice, there is a modest decrease in thymus cellularity (20-25% reduction), and the ratios of different thymocyte subsets are very similar to WT animals. In addition, normal numbers of B220+/IgM+ lymphocytes were found in the fetal livers, bone marrow and spleen of IL-15R-deficient mice, suggesting that IL-15 does not play a critical role in T and B cell differentiation.[199] These observations are consistent with data implicating IL-7 as the primary Jak3-dependent cytokine in expansion of lymphocyte precursors in the bone marrow and for the differentiation of early pre-committed T and B cells.

These gene-targeting experiments have, however, demonstrated a role for IL-15 in the homestasis of peripheral lymphocyte numbers. In mice lacking the IL-15 receptor, there is a 30-80% reduction in peripheral lymphocyte cellularity. However, all lymphoid lineages were affected equally, suggesting a general role for IL-15 in maintaining lymphocyte numbers in the periphery and their proliferation, survival, or migration to secondary lymphoid organs. Additional experiments in IL-15Rα$^{-/-}$ primary lymphocytes have shown that lymph node B and T cells incorporate 20% less BrdU than cells from WT animals, suggesting that IL-15 plays a role in driving proliferation and expansion of peripheral lymphocytes and in the absence of IL-15, lymphocytes are defective in cell cycle progression. Interestingly, an additional function for IL-15 in the migration of mature lymphocytes to lymph nodes has been found. In adoptive transfer experiments, the ability of

labeled IL-15Rα$^{-/-}$ lymphocytes to repopulate the lymph nodes of recipient animals was reduced 2-4-fold compared to cells from WT mice. Interestingly, this defect appears to be specific for lymph node migration, as IL-15Rα$^{-/-}$ cells can repopulate the spleen to the same extent as WT lymphocytes. Thus, this data suggest that IL-15 is not only a growth factor but an important chemotactic attractant that induces B and T cell migration to peripheral lymphoid organs. In addition, it has been shown that IL-15 is critical to the development of cells involved in innate immunity. IL-15Rα$^{-/-}$ mice are deficient in NKT cells as well as TCR γδ intraepithelial T cells (IELs). However, these defects are significant, but not absolute in IL-15 Rα-deficient mice. Reduction in NKT cells and γδT cells is much more severe in Jak3$^{-/-}$ and $\gamma_c^{-/-}$ animals,[43,45,46,117] suggesting that additional Jak3-dependent cytokines can partially compensate for the loss of IL-15 signaling in the development of these lineages.

Interestingly, IL-15 has been shown to be important inthe generation of CD8+ memory effector T cells as well.[199,201,202] IL-15$^{-/-}$ mice have a profound deficiency in CD8+CD44^{hi}CD62^{lo} cells in the periphery, suggesting that signaling through this receptor rescues activated CD8+ T cells from AICD and induces their differentiation into memory cells. This function of IL-15 is in opposition to the effects of IL-2 on activated T cells.[202] As described above, IL-2 appears to be required for sensitizing cells for apoptosis upon secondary antigenic challenge, through induction of Fas ligand expression and possibly down-regulation of Bcl-2 and FLIP expression.

Deletion of the IL-15Rα chain gene has revealed specific, non-redundant functions for IL-15 in NK cell developmental transitions, generation of cells involved in innate immunity, peripheral expansion of T and B lymphocytes, and the delivery of key signals for the differentiation of CD8+ T lymphocytes into memory cells. The function of IL-15 in NK cell development is likely due to its ability to activate the Stat5b transcription factor in developing bone marrow-derived NK cell precursors. Interestingly, T and B cell development is not affected in IL-15Rα chain-deficient mice. IL-15 is required for the expansion of mature peripheral lymphocytes and their ability to migrate to peripheral lymph nodes suggesting that IL-15 plays a key role in the proliferation of mature lymphocytes, targeting their migration to secondary lymphoid organs, and inducing the differentiation of CD8+ memory T cells.

IL-4

Naïve T cells can differentiate into the functionally distinct Th1 and Th2 subsets, which are characterized by defined patterns of lymphokine secretion and effector activity. Th1 cells secrete IL-2 and IFN-γ, and function to promote delayed-type hypersensitivity and cytotoxic responses. Reciprocally, Th2 cells produce IL-4, IL-5 and IL-10, and function to stimulate antibody responses and Ig gene class switching. The differentiation of Th2 cells depends on stimulation of naïve T lymphocytes by the Jak3-dependent cytokine IL-4 (Fig. 3). Targeted disruption of the genes for IL-4 and the IL-4Rα chain resulted in a highly specific defect in Th2 differentiation.[203]

IL-4$^{-/-}$ naïve CD4+ splenic T cells induced to differentiate by treatment wih α-CD3+IL-2 fail to produce Th2-specific cytokines IL-5, IL-10, and IL-3. In addition, infection of mice with the nematode *Nippostrongylus brasiliensis*, which normally induces a strong Th2 response, fails to induce Th2-specific cytokines IL-4, IL-5, IL-9 and IL-10 in IL-4-deficient mice, while production of the Th1-specific cytokine IFN-γ was unaffected or enhanced. Consistent with an important role for IL-4 in immunoglobulin class switching to the IgG1 and IgE isotypes, naïve IL-4$^{-/-}$ mice had undetectable levels of IgG1 and

IgE, while the levels of other isotypes (IgG2a IgG2b, IgG3,and IgA) were unaffected. In addition, the isotype profile in the serum of immunized mice was drastically altered in IL-4$^{-/-}$ mice. IgG1 levels were 10-fold lower and IgG2 and IgG3 isotypes were 100-200-fold higher in immunized IL-4-deficient mice compared to WT controls, demonstrating the role of IL-4 in the regulation of specific immunoglobulin isotypes in the serum.

As mentioned above, IL-4 is the only cytokine that activates the transcription factor Stat6,[56,57] which suggested that Stat6 might be a critical mediator of the unique functions of IL-4. Consistent with this model, targeted disruption of the Stat6 gene resulted in a phenotype strikingly similar to IL-4 deficiency in mice.[204] Naïve T cells from Stat6-deficient mice failed to differentiate into Th2 cells when cultured under permissive conditions for Th2 development (IL-4+anti-IFN-γ), and did not produce IL-4 or IL-5. While peripheral T cells from Stat6$^{-/-}$ mice did not proliferate in response to IL-4, they retained their responsivenes to IL-2 and could differentiate to Th1 cells to the same extent as wild-type controls. In addition, Stat6 deficient mice fail to produce any serum IgE after immunization, a defect which closely resembles the effects of IL-4 deficiency. Thus, Jak3 signal transduction is required for IL-4 induced differentiation of naïve CD4+ T cells into Th2 effector lymphocytes.

IL-9

IL-9, originally identified as a T cell growth factor, is a Th2-specific pro-inflammatory cytokine that is corrlated with pathologic conditions such as asthma and atopy. Mice expressing an IL-9 transgene had several hematopoietic defects such as lymphoma, dysregulated antibody production, and aberrant mastocytosis, suggesting that IL-9 may be involved in many aspects of lymphoid and myeloid cell function. However, targeted mutation of the IL-9 gene has revealed a highly specific role for this Jak3-dependent cytokine in migration and expansion of mast cells, mucin production, and goblet cell hyperplasia in a model of lung inflammation.[205] Mice lacking IL-9 fail to induce the proliferation and expansion of epithelial-derived goblet cells in response to pulmonary granuloma formation. In addition, IL-9-deficient mice induce the migration of 6-fold fewer mast cells into the sites of inflammation, suggesting that IL-9 is an important chemotactic factor for mast cell infiltration into lung granulomas. Importantly, it was also shown that this phenotype is not due to defects in myeloid cell development, as the levels of bone marrow mast cells in IL-9$^{-/-}$ mice were similar to wild-type controls. This strongly suggests that the function of Jak3 in mast cell function is restricted to the responses of mature cells in the periphery. In addition, the finding that epithelial cells express the IL-9 receptor indicates that expansion and hyper secretion of mucus by goblet cells may be due to a direct effect of IL-9 on the epithelium of the lung during inflammatory reactions. Interestingly, there are no other hematopoietic defects in these animals. All lymphoid and myeloid lineages develop normally, and there is no effect of IL-9 deficiency on serum immunoglobulin production, specific antibody responses, or the differentiation of naïve T cells into Th1 and Th2 subsets.[205] This is the most striking example of a role for Jak3 in cells of non-lymphoid lineage.

IL-21

A novel cytokine has recently been identified, as another member of the γ_c signaling family. The gene for the IL-21 receptor was initially identified as an open reading frame encoding signature class I cytokine receptor motifs.[206] Interestingly, its sequence was found to have the greatest homology to the IL-2Rβ chain and the IL-4Rα and IL-9Rα chains. Furthermore, the IL-21 ligand, which was cloned on the basis of its ability to induce

proliferation of an IL-21R+ cell line, has significant homology to IL-15, IL-4 and IL-2, suggesting that this ligand-receptor pair may be a member of the γ_c family.[207] Analysis of IL-21R mRNA has shown that it is expressed exclusively in lymphoid tissue, primarily thymus and spleen. In addition, constitutive surface expression of the IL-21 receptor is limited to specific lymphoid populations, CD23+ peripheral B cells and CD56+ NK cells, and high levels of the IL-21R are expressed on the surface of developing thymocytes. In addition IL-21R expression can be induced in CD4+ peripheral T cells in an activation-dependent manner, much like the IL-2Rα chain, strongly suggesting that it has a role in lymphopoiesis and peripheral immune function.[208,209]

IL-21 has a wide range of activities on lymphoid cells which include induction of proliferation in peripheral B and T cells, and marked enhancement of cytolytic activity in NK cells. Interestingly, the proliferative effects of IL-21 on peripheral T cells depend on a second signal delivered through the TCR , suggesting that the IL-21 receptor expression is TCR-dependent. In addition, it has been found that isolated thymocytes, which express high levels of the IL-21 receptor, fail to proliferate in response to IL-21, which suggests that other molecular events are required to induce cell division, such as the induction of another component of the IL-21 signaling complex or TCR cross-linking. Alternatively, IL-21 may not induce proliferation of thymocytes, but may be a survival or differentiation factor. Similarly, peripheral, mature NK cells do not proliferate in response to IL-21, yet this cytokine can strongly enhance cytolytic effector function and also synergizes with other growth factors to induce the differentiation of lytic NK cells from CD34+ precursors in vitro. These biological activities suggest that IL-21 may function as a γ_c-dependent growth/differentiation factor for lymphoid cells that can also act as a cofactor for the induction of effector function.[209]

While the expression patterns of IL-21 and its receptor, its lymphoid-specific biological activities and its ability to induce phosphorylation of Jak1 and both isoforms of Stat5 strongly suggest that the IL-21 receptor may be the newest member of the γ_c signaling family, further biochemical analysis of its signaling complex and a murine gene knockout of IL-21 or its receptor will likely be required to fully evaluate the molecular mechanism of action and in vivo function of IL-21.

Stat5

Targeted deletion of both isoforms of Stat5, Stat5a and Stat5b has revealed that activation of this transcription factor plays an important role in peripheral T lymphocyte cell cycle progression and NK cell differentiation. Thus, it has been found that loss of signaling through this single pathway is responsible for several of the defects observed in Jak3 and γ_c-deficient mice.

Individual knockout of Stat5a and Stat5b, as well as mice lacking both isoforms have demonstrated redundant and unique roles for these two transcription factors in peripheral lymphoid function. Interestingly, it hs been found that there is no significant defect in B and T cell development in any of the Stat5 knockout mice.[11,210] Mice deficient for both Stat5 isoforms have normal cellularity of the thymus and spleen, as well as normal distribution of T cell subsets in all lymphoid organs. In addition, loss of Stat5 apparently has no effect on B cell differentiation or mature B cell function. Serum immunoglobulin levels and Ig gene class switching from IgM to IgG or IgE are unaffected in the double-knockout(DKO) mice.

However, the Stat5a/b DKO has demonstrated that Stat5 is essential for proper peripheral T cell function.[11] Interestingly, in both Stat5a and Stat5b single knockouts, the surface profile of splenic CD4+ T lymphocytes was characteristic of naïve, quiescent cells,

similar to the phenotype of CD4+ T cells in wild-type mice ($CD62L^{hi}CD44^{lo}CD69^{lo}Mel\text{-}14^{hi}$).[11,210] In addition, proliferation of these cells in response to polyclonal T cell activators in vitro was only mildly reduced compared to WT controls. This effect on proliferation has been attributed to a role of both Stat5 isoforms in the expression of the IL-2Rα chain (CD25), which is defective in cells lacking Stat5a or Stat5b. However, in the DKO, as in the Jak3 and γ_c-deficient mice, CD4+ T cells accumulate in the periphery and have an activated/memory phenotype ($CD62L^{lo}CD44^{hi}CD69^{hi}Mel\text{-}14^{lo}$).[11] In addition, $Stat5^{-/-}$ splenic T cells are refractory to polyclonal proliferative stimuli, such s α-CD3+α-CD28 antibodies, α-CD3+exogenous IL-2, α-CD3+ionoMycin, as well as PMA+ionoMycin in vitro. In addition, cell cycle analysis of purified splenic T cells stimulated with α-CD3/α-CD28/IL-2 revealed that Stat5a/b deficiency caused a S/G2 arrest. Normal cell cycle progression and proliferation could be restored by retroviral transduction of the wild-type Stat5a gene into purified T cells from the DKO mice, demonstrating the essential role of Stat5 in the control of peripheral T cell activation.

Detailed analysis of CD4+ splenic T cells has shown that IL-2 stimulation induced cyclin D2, cyclin D3, and CDK6 expression in WT and Stat5a or Stat5b-deficient T lymphocytes, but not in the Stat5 DKO mice.[11] However, destruction of the cell cycle inhibitor $p27^{kip1}$ in response to anti-CD3 antibody or α-CD3+exogenous IL-2 is normal, suggesting that other Jak3-dependent pathways, such as activation of PI-3 kinase, are still functional in $Stat5^{-/-}$ mice. Thus, it appears that the severe defect in proliferation of $Jak3^{-/-}$ peripheral T cells results from defective expression of cyclins and CDK6.These data also demonstrate the requirement for integration of multiple signal transduction pathways to induce cell cycle progression and proliferation in respnse to Jak3-dependent cytokines.

The abnormal expansion and activated phenotype of peripheral CD4+ T cells from the Stat5a/b DKO mice, which is strikingly similar to that seen in Jak3-deficient animals, likely reflects a role for Stat5 in AICD. Insupport of this model, it has been shown that IL-2Rβ chain mutants that lack the Stat5 docking site but activate all other IL-2-dependent signaling pathways fail to induce he expression Fas ligand on the surface to IL-2-stimulated cells.[191] The phenotype of Stat5 DKO cells resembles that of $Jak3^{-/-}$ peripheral CD4+ T cells, which are refractory to polyclonal stimulation, and are highly susceptible to Fas-mediated apoptosis in vitro, but proliferate rapidly in vivo and are defective in Fas ligand expression.

Studies in Stat5 knockout mice have demonstrated a redundant role for Stat5 isoforms in NK cell development, as well as unique role for Stat5b in peripheral NK cell proliferation and induction of cytolytic activity. In one study, deletion of Stat5b alone resulted in a mild reduction in splenic, mature NK cells (CD3-DX5+),[199] but a profound loss of cytolytic activity. Therefore, Stat5 isoforms have a redundant function in NK cell differentiation from bone marrow or thymic progenitors, but Stat5b uniquely influences peripheral NK cell proliferation and function. This is likely due to the failure of the DKO mice to activate Stat5 in response to the essential NK cell growth factor IL-15.[199,202]

Conclusions

In summary, the cytokines that signal via γ_c/Jak3 plays a critical role as a general homeostatic regulator of lymphoid development and peripheral B and T cell function. They have both positive and negative regulatory roles, and are crucial for the proliferation and expansion of bone marow-derived lymphocyte progenitors as well as immature, developing T, B, and NK cells. In addition, Jak3 is required to promote lineage-specific commitment of Th1 and Th2 CD4+ cells, generation of CD8+ cytolytic effector cells and

memory cells, mature B cells through induction of immunoglobulin class switching, activation of NK cell funtion, and differentiation and migration of mast cells and goblet cells. Finally, γ_c/Jak3 cytokines, in particular IL-2, are also essential for the negative regulation of antigen-specific immune responses in mature peripheral T and B cells by regulating Bcl-2 expression and inducing Fas ligand-dependent programmed cell deathand peripheral tolerance in terminally differentiated effector lymphocytes. Through extensive biochemical and genetic analysis of Jak3 signaling, it has become clear that Jak3 is critical in maintaining a delicate homeostatic balance between lymphocyte proliferation, survival and cell death. This is accomplished through the regulation of a complex network of signalingpathways that affect various enzymatic acivities in the cell as well as the transcriptional activation of many genes involved in cell cycle progression and AICD. Furthermore, the generation of murine gene knockouts of individual Jak3-dependent receptors has allowed a detailed dissection of the role of specific cytokines in hematopoiesis (lymphopoiesis), control of peripheral lymphocyte proliferation and survival, and the development of innate immunity. However,in light of some controversial data regarding the mechanisms of these processes, further analysis of the biochemistry of signal transduction and the genetic models of cytokine deficiencies is still required to fully understand the molecular basis of Jak3/γ_c-dependent biological effects in hematopoiesis and SCID.

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CHAPTER 3

Stat Activation by Src, Fes and Btk Tyrosine Kinases

Thomas E. Smithgall

Introduction

Hematopoiesis is regulated by a diverse group of soluble protein factors known collectively as cytokines. Although the number of cytokines and related hematopoietins is large and continues to grow, the mechanisms by which they activate cellular signaling pathways share several common features. First, the receptors for many of these factors are composed of multiple subunits, which are sometimes shared by more that one cytokine.[1] Well known examples include the IL-6 family of cytokines, which share the gp130 signal transducer,[2] the IL-2 family of cytokines, which signal through a common receptor γ chain,[3] as well as GM-CSF, IL-3, and IL-5, which share a common β subunit.[4] Second, these and most other cytokines induce rapid protein-tyrosine phosphorylation following interaction with their cell surface receptors. Unlike growth factor receptors, however, cytokine receptor subunits lack intrinsic kinase activity and rely instead on cytoplasmic tyrosine kinases to initiate signal transduction. As an example, Figure 1 illustrates the activation of the GM-CSF receptor. GM-CSF induces clustering of the α and β chains of the receptor, resulting in a complex with a 2:2:2 stoichiometry of cytokine to α and β subunits. This oligomeric form of the receptor promotes the juxtaposition of Jaks and other associated tyrosine kinases, leading to kinase autophosphorylation by a trans mechanism similar to that demonstrated for growth factor receptor tyrosine kinases. Structural analyses of inactive and active forms of several tyrosine kinase domains have revealed that autophosphorylation is essential for full catalytic activity (see below).

Once activated, receptor-associated kinases then phosphorylate specific tyrosine residues within the receptor itself. Receptor phosphotyrosine residues create high-affinity binding sites for multiple effector proteins with SH2 and PTB domains. For example, Stat3 binds to phosphorylated receptors through a $Y^{P}XXQ$ motif, which represents a preferred docking sequence for the Stat3 SH2 domain.[5] The recruitment of Stats to the receptor places them in proximity to receptor-associated kinases, promoting phosphorylation of highly conserved tyrosine residues near the C-terminus of the molecule. Once phosphorylated, Stats are rapidly released from the receptor, undergo dimerization, and translocate to the nucleus where they affect the transcription of target genes with Stat-responsive promoter elements.[6,7] Recent crystal structures of Stat1 and Stat3 bound to DNA provide

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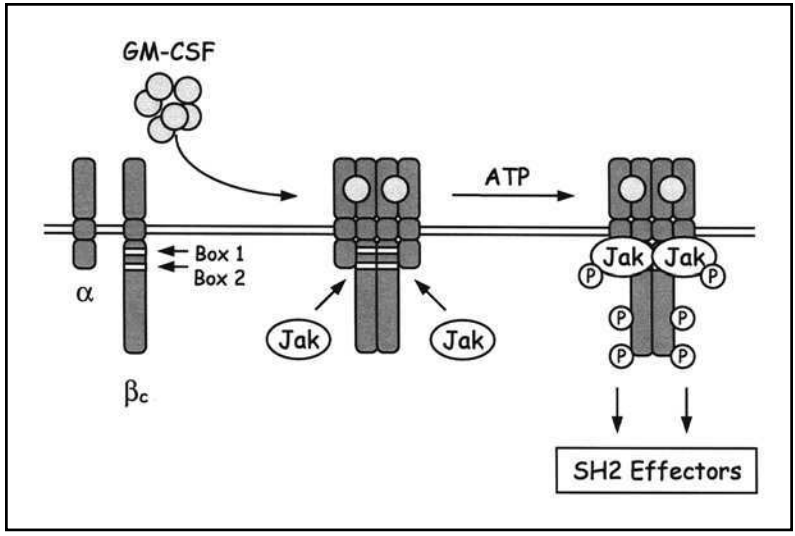

Figure 1. Mechanism of GM-CSF receptor activation. Activation of the GM-CSF receptor illustrates the major features of cytokine-initiated signal transduction. The GM-CSF receptor consists of two subunits, a unique α subunit that confers ligand binding specificity and a common β subunit that is shared with the receptors for IL-3 and IL-5. The β subunit also exhibits box 1 and box 2 homology motifs which are involved in the recruitment of Jak kinases to the membrane-proximal region. Binding of GM-CSF induces the formation of the heteromeric receptor complex shown, resulting in the close positioning of Jak monomers. This arrangement allows for Jak autophosphorylation by a trans mechanism, an essential aspect of Jak kinase activation. Activated Jaks then phosphorylate multiple sites on the receptor β subunit, creating specific docking sites for downstream effector molecules with SH2 and PTB domains which recognize phosphotyrosine residues in a sequence-specific manner. A detailed review of the activation mechanism has been published elsewhere.

direct evidence that tyrosine phosphorylation induces Stat dimerization via reciprocal phosphotyrosine-SH2 interactions.[8,9]

Although Stat activation is most often associated with Jak kinases, a growing body of literature suggests that other cytoplasmic tyrosine kinases may contribute to Stat activation as well. Cytokine receptor activation is not only linked to Jak activation, but to the activation of several other tyrosine kinases, including members of the Src, Fes and Btk families.[10] All of these kinases have been shown to phosphorylate Stats directly in defined expression systems, suggesting that they may contribute to Stat activation under physiological conditions. In addition, studies with specific inhibitors and dominant-negative kinase mutants have suggested nonredundant roles for these kinases in Stat activation. The purpose of this chapter is to review the structure and function of the Src, Fes and Btk tyrosine kinases and present current evidence linking these kinases to Stat activation.

Src Kinases

Structure and Regulation

The Src family of tyrosine kinases currently consists of nine members (c-Src, c-Yes, Fyn, Lyn, Hck, c-Fgr, Blk, Lck, and Yrk), which share the same overall structural organization and appear to be regulated by the same mechanism.[11-13] The c-Src proto-oncogene product serves as the prototype for this kinase family, and its structure is shown in Figure 2.

All Src family members have a short unique N-terminal region, which is followed by SH3 and SH2 domains, a catalytic domain and a negative regulatory tail. Src kinases are myristylated on their unique N-terminal regions, and this modification, together with palmitylation in some cases, serves to target these proteins to the plasma membrane. The SH2 and SH3 domains are essential for control of Src kinase activity and signaling functions in vivo. As described in more detail below, these domains regulate kinase activity through intramolecular interactions with other regions of the protein, and also mediate interactions with upstream receptors as well as downstream substrates, including Stats.

Two intramolecular interactions regulate the activity of Src family kinases in vivo[14] (Fig. 2). The first of these involves the SH2 domain, which binds to a phosphotyrosine residue in the C-terminal tail. The tail tyrosine residue (Tyr 527 in c-Src) is conserved in all members of the Src kinase family, and is phosphorylated by a distinct regulatory kinase known as Csk, for C-terminal Src kinase.[15] Gene targeting experiments strongly suggest that Csk is the master regulator of all Src family kinases.[16,17] Homozygous inactivation of Csk leads to embryonic lethality and a concomitant elevation in overall Src family kinase activity. In contRast, knockouts of individual Src kinases can show more subtle phenotypes, suggesting functional compensation by other family members.[12]

Intramolecular SH2-phosphotyrosine interaction has long been suspected in the negative regulation of Src kinase activity, as mutations of the tail tyrosine residue and the SH2 domain release kinase activity in vivo.[11] Direct proof for this interaction comes from the X-ray crystal structures of c-Src as well as the macrophage-specific family member, Hck.[18-22] All of these structures are of the Csk-phosphorylated, inactive forms, and directly show the tail bound to the SH2 domain. This interaction promotes an inactive, closed conformation in which the large and small lobes of the kinase domain are pushed together. The closed form of the kinase is stabilized by an additional intramolecular interaction of the SH3 domain with a polyproline type II helix formed by a loop connecting the SH2 and kinase domains (termed the SH2-kinase linker). The SH3-dependent interaction revealed by the crystal structure helps to explain earlier observations that mutations within the SH3 domain can also activate Src.[23-25] In addition, more recent work has shown that mutation of proline residues in the linker region also releases the tyrosine kinase and transforming activities of both Hck[26] and c-Src.[27]

The crystal structures of the down-regulated forms of Src kinases predict that SH2 and SH3-dependent interaction with other molecules may disturb intramolecular regulatory interactions and induce kinase activation. Several examples of such stimulatory interactions have been reported, including interactions of c-Src with p130 Cas and related focal adhesion molecules,[28,29] and also of Hck with the Nef protein of HIV-1.[30,31] Nef is a small myristylated protein essential for AIDS progression with an unusually high affinity and specificity for the Hck SH3 domain.[32,33] Nef binds to full-length Hck through its SH3 domain, leading to constitutive Hck activation both in vitro and in intacT cells. As described in a later section, constitutive activation of Src kinases by Nef and other viral proteins can lead to Stat activation and cellular transformation.

Src Kinases Contribute to Growth Factor and Cytokine Signaling

Src family kinases have been implicated in signal transduction by both cytokines and growth factors. Several growth factors have been shown to stimulate Src kinase activity, including EGF, PDGF, and CSF-1.[34] These results are somewhat surprising, given that growth factor receptors possess intrinsic tyrosine kinase activity and are capable of coupling directly to downstream effector molecules. The mechanism of Src kinase activation by

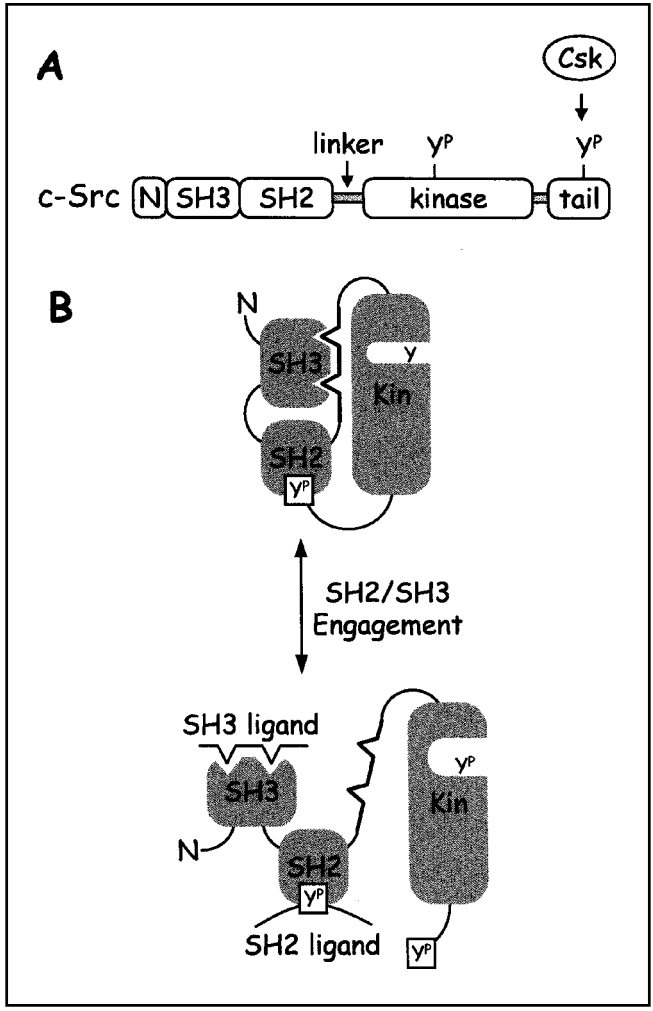

Figure 2. Structural organization and regulation of the Src tyrosine kinase family. A. The overall organization of c-Src consists of a unique N-terminal domain with a site for myristylation (N), followed by SH3 and SH2 domains, an SH2-kinase linker, the kinase domain, and a negative regulatory tail. All Src family members have two major tyrosine phosphorylation sites with opposing actions on kinase activity. The autophosphorylation site, located within the activation loop of the kinase domain, is required for full catalytic function. In contRast, phosphorylation of the negative regulatory tail results in suppression of kinase activity. The tail tyrosine residue is phosphorylated by an independent regulatory kinase known as Csk. B.Intramolecular interactions regulate Src family kinase activity. Structural studies of the down-regulated forms of c-Src and Hck revealed two intramolecular interactions that suppress kinase activity. Tyrosine phosphorylation of the negative regulatory tail by Csk induces intramolecular interaction with the SH2 domain, while the SH3 domain interacts with a polyproline type II helix formed by the linker connecting the SH2 domain and the small lobe of the kinase domain. These interactions stabilize an inactive conformation in which the two lobes of the kinase domain are forced together. Interaction of Src family kinases with SH2 and or SH3 ligands (in the form of receptors, substrates or docking proteins) is sufficient to disrupt these intramolecular interactions, allowing the kinase domain to switch to an active state. The active conformation is stabilized by autophosphorylation within the activation loop. For additional details of this model, see Schindler et al.[20] and Xu et al.[21]

growth factor receptors involves recruitment of Src to the activated, autophosphorylated form of the receptor via the Src SH2 domain. SH2-mediated binding to the receptor may activate Src by displacing the negative regulatory tail, direct phosphorylation by the receptor tyrosine kinase, or both.[11,35]

Whether or not Src kinase activation is required for growth factor signaling is somewhat controversial. On one hand, microinjection of fibroblasts with dominant-negative Src mutants or interfering Src antibodies blocks cell cycle progression in response to PDGF and other growth factors, supporting the idea that Src kinase activation is required for proliferation in response to factor treatment.[34,36,37] However, cells derived from embryos in which the Src, Fyn and Yes genes were deleted by gene targeting still responded fully to PDGF in terms of substrate phosphorylation, *c-Myc* induction, S-phase entry, proliferation and chemotaxis, indicating that Src kinases may not be required for growth factor-initiated signaling in some cell types.[38] The reason for the contRasting results from these two experimental approaches is not completely clear, although dominant-negative Src kinases may interfere with more that just endogenous Src activation.

Multiple members of the Src family are activated by a variety of cytokines, all of which also activate Stats.[13] One example is the myeloid-restricted Src family member Hck, which is expressed primarily in myeloid leukocytes and is also involved in antigen receptor signaling and adhesion-related responses.[39-42] Several studies have implicated Hck in the signal transduction pathways for IL-3, GM-CSF, and LIF.[43-46] Hck associates with the β subunit of the IL-3 receptor,[47] and over-expression of Hck enhances cellular protein-tyrosine phosphorylation following IL-3 treatment.[45] Hck-receptor interaction involves the SH2 and SH3 domains of Hck and the distal portion of the β subunit; this interaction may contribute to kinase activation by disruption of the intramolecular negative regulatory mechanisms described above. Hck has also been shown to interact with gp130, the signal-transducing component of the LIF receptor that is shared with receptors for the IL-6 family of cytokines.[2,44] LIF treatment induces Hck activation, and the portion of gp130 responsible for this effect is also required for the activation of Stat3; both Hck and Stat3 have been implicated in LIF signals essential for embryonic stem (ES) cell self-renewal (see below). In addition to Hck, other members of the Src kinase family have been implicated in hematopoietic cytokine receptor signal transduction, including Fyn, Lyn, Lck, and c-Src itself.[13,45,48-52] As discussed in more detail below, Src family kinases also have the potential to activate Stat signaling directly.

Stat Activation by Src Kinases—Clues from Transformed Cells

Some of the first evidence for Stat activation by members of the Src kinase family comes from studies of fibroblasts transformed by the v-*Src* oncogene. Yu et al[53] first reported that Stat3 was constitutively activated in several fibroblasts cell lines following transformation by v-Src. In contRast, Stats were not activated by transforming oncogenes that operate downstream or independently of tyrosine kinases, such as v-Ras and v-Raf. In a related study, Cao et al[54] also demonstrated strong endogenous Stat3 activation in rat embryo fibroblasts and NIH 3T3 cells expressing v-Src. Using an inducible v-Src expression vector, they demonstrated a tight correlation between v-Src expression and Stat3 tyrosine phosphorylation as well as DNA-binding activity. Src was also demonstrated to associate with Stat3 in vitro and in coprecipitation experiments from rat embryo fibroblasts, providing additional support for a specific interaction between these proteins. Consistent with the studies of Yu et al,[53] Src selectively induced the formation of Stat3 homodimers and did not activate Stat1.

Cao et al[54] also provided evidence that Src activation may be important for coupling the CSF-1 receptor to Stat3 activation. These experiments employed NIH3T3 cells expressing either the wild-type CSF-1 receptor or a mutant form of the receptor lacking an autophosphorylation site essential for binding and activation of Src, Yes, and Fyn. They found that CSF-1 treatment of cells expressing the wild-type receptor led to endogenous Stat3 activation, while cells expressing the mutant receptor were unable to activate Stat3 in response to this growth factor. As a control, both cell lines were shown to respond to PDGF with Stat3 and Stat1 activation. These results suggest that Src may act as an intermediate between the CSF-1 receptor and downstream Stats.

To address the requirement for Stat3 in transformation signaling downstream from v-Src, two recent studies used dominant-negative mutants of Stat3. Turkson et al[55] employed a naturally occurring splice variant of Stat3, known as Stat3β which lacks the C-terminal transactivation domain.[56] They observed that Stat3β completely blocked Src-induced transcription from a Stat3-specific reporter gene construct. As a control for specificity, they showed that Stat3β had no effect on Src-induced transcription from an SRE-based construct that is sensitive to dominant-negative Ras and Raf. They also found that co-expression of Stat3β substantially reduced v-Src transforming activity in NIH3T3 cells. Similar results were reported by Bromberg et al[57] at the same time. Instead of Stat3β, their study used three other dominant-negative Stat3 mutants: Stat3Y, in which the tyrosine phosphorylation site involved in dimerization is converted to phenylalanine; Stat3DB, which has multiple mutations in the DNA binding domain, and Stat3S, in which the serine phosphorylation site is replaced with phenylalanine. Previous studies by this group established that phosphorylation of the serine site is essential for transcriptional activation but does not affect dimerization or DNA binding.[58,59] All three mutant forms of Stat3 significantly reduced v-Src-mediated transformation of NIH3T3 cells in a soft-agar colony-forming assay. In addition, these mutants also prevented Src-dependent transcription from a Stat3 reporter gene. Together with the results of Turkson et al,[55] these data firmly establish a necessary role for Stat3 in Src-mediated fibroblast transformation. One final note concerns the mechanism by which the Stat3Y mutant (the one lacking the tyrosine phosphorylation site) suppresses transformation. While all of the other mutants act downstream of Src (i.e., by blocking the DNA binding or transcriptional activities of endogenous Stat3), this mutant presumably acts to prevent phosphorylation of endogenous Stat3 in the first place. This result provides evidence that interaction of Src with Stat3 may be an important part of the activation mechanism in vivo.

Given that transformation by v-Src correlates with constitutive Stat3 activation, and that Stat3 activation is required for Src signaling in fibroblasts, Bromberg et al[60] decided to address the issue of whether or not active Stat3 alone is sufficient to induce transformation. For these experiments, they produced a constitutive Stat3 dimer that does not require tyrosine phosphorylation for activation. This was accomplished by substituting cysteine for alanine and asparagine residues in the SH2 domain previously shown to be in very close proximity within the X-ray crystal structure. This substitution promoted the formation of functional Stat3 dimers through disulfide linkages in the absence of tyrosine phosphorylation. The resulting mutant, termed Stat3C, retained DNA binding and transcriptional activation properties similar to those of wild-type Stat3 following activation by Src. Stat3C induced transformation of several fibroblasT cell lines which in turn produced tumors following injection into nude mice, although the strength of the transforming signal was weaker than that of v-Src. These results show that Stat3 activation is sufficient to transform fibroblasts, and support a role for constitutive Stat3 activation downstream

of Src or other kinases in oncogenic transformation not only in model systems but in human cancers as well. The role of Stats in oncogenesis has been the subject of several recent reviews.[61,62]

The simplest interpretation of the transformation data described above is that Src kinases may be directly responsible for Stat activation. The ability of Src and Stat3 to form a stable complex strongly suggests that direct activation occurs in vivo. However, accumulating evidence suggests that other kinases may contribute to the activation mechanism in mammalian cells. For example, Campbell et al[63] immunoprecipitated Jak1 from several v-Src-transformed cell lines and observed enhanced phosphotyrosine content compared to untransformed controls. Src itself was observed in the Jak1 immunoprecipitates, suggesting a physical interaction between the kinases. Similar experiments showed possible activation of Jak2 as well, although the effect was less pronounced.

More recent work by Zhang et al[64] shows that Src may cooperate with Jak1 to induce Stat3 activation. Using Sf-9 insecT cells, which presumably lack Jaks or homologs of other mammalian tyrosine kinases, they clearly demonstrated that co-expression with Src is sufficient to induce Stat3 tyrosine phosphorylation and DNA binding, providing strong evidence that Stat3 is a direct Src substrate. In fact, they observed that Src phosphorylates Stat3 much more efficiently than Jak1 in this system. However, highly selective pharmacological inhibitors of either Src or Jak kinases (PD180970 and AG490, respectively) completely blocked Stat3 DNA binding activity in v-Src-transformed fibroblasts. Consistent with these results, the Jak inhibitor as well as a dominant-negative Jak1 mutant blocked v-Src-induced transcription from a Stat3-dependent reporter gene construct. These results suggest that despite the ability of Src to activate Stat3 directly in Sf-9 cells, Src requires Jak1 for maximal Stat3 activation in mammalian cells. Interestingly, these authors also found that the PDGF receptor enhances Stat3 activation by v-Src. This effect does not require the intrinsic tyrosine kinase activity of the receptor, suggesting that it may serve a scaffolding function to bring Src, Jak1 and Stat3 together as part of a complex activation mechanism. The authors propose a model in which Jak kinases create a recruitment site for Stat3 by phosphorylating the cytoplasmic domain of the PDGF receptor. Stat3 is then recruited to the receptor, placing it in proximity to Src, which is then responsible for Stat3 phosphorylation (Fig. 3). Thus v-Src cooperates with Jak1 in Stat3 activation during fibroblast transformation, with the PDGF receptor serving an accessory role as a molecular scaffold. As described in more detail below, c-Src may also contribute to Stat3 activation under physiological conditions of growth factor receptor activation as well.

Activation of Stat Signaling by PDGF—Controversial Role for Src

Work summarized above from v-Src transformed fibroblasts strongly suggests that Src may contribute to Stat3 activation by PDGF and other growth factors. Wang et al[65] investigated this question directly in Balb/c-3T3 fibroblasts, which respond to PDGF with Stat3 homodimer and Stat1-Stat3 heterodimer formation.[66] These investigators demonstrated that the Src family kinase inhibitor PD180970 completely blocks Stat3 activation by PDGF in this system, providing strong evidence that Src is an essential intermediate between the receptor and Stat3. In contRast, PD180970 did not inhibit IL-6-induced Stat3 activation, which is dependent upon Jak kinase activity. PD180970 blocked PDGF-induced entry of the cells into the S phase of the cell cycle, which is surprising given that the compound did not interfere with PDGF-induced activation of the Ras/Erk pathway. Both the Ras/Erk pathway[67] and Stat3[60,68] have been linked to the induction of cyclin D1, which is essential for this phase of the cell cycle.

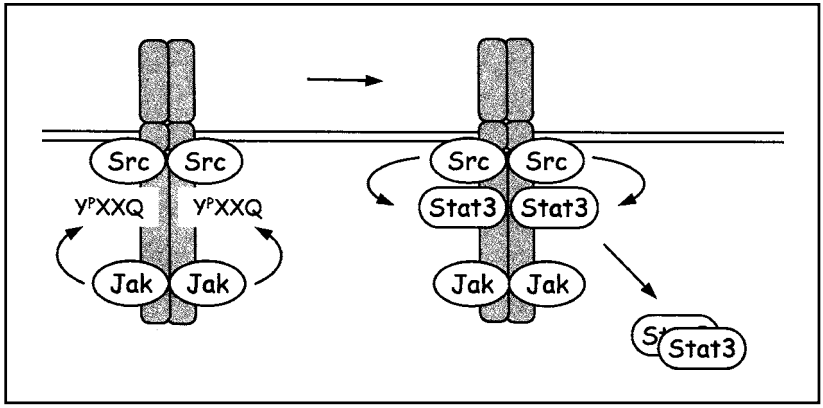

Figure 3. Cooperation of Jak1 and Src kinases in the activation of Stat3. Recent work in v-Src transformed fibroblasts shows that both Jak and Src kinases are required for Stat3 activation and implicate the PDGF receptor as a molecular scaffold in the activation event. These data are fit by the model shown in which receptor activation induces Jak kinase activation by the mechanism described in Figure 1. This leads to the phosphorylation of specific recruitment sites for Stat3, which recognizes $Y^{P}\times\times Q$ motifs through its SH2 domain. Once recruited to the receptor, Stat3 can interact directly with Src kinase, which also associates with and is activated by the PDGF receptor. Src is responsible for direct phosphorylation of Stat3, inducing its release from the receptor, dimerization, nuclear translocation, and transcriptional activation. This model was originally proposed by Zhang et al.[64]

Although inhibition of PDGF-induced Stat3 activation with a Src-selective kinase inhibitor argues for a necessary Src function in PDGF-Stat3 signaling, other studies suggest that Src kinases may not be required. Sachsenmaier et al[69] investigated this question using a panel of PDGF receptor mutants with altered coupling to Src kinases and Stat3. First, they demonstrated that PDGF receptor autophosphorylation sites in the juxtamembrane region of the receptor (Tyr 579 and Tyr 581) were involved in the activation of Stat3 in response to PDGF. Mutagenesis of either of these sites led to a dramatic reduction in Stat3 activation following PDGF treatment. Both of these sites have also been implicated in the recruitment of Src to the receptor through its SH2 domain, leading to Src activation through a mechanism that involves displacement of the negative regulatory tail.[70] They then changed the amino acid sequences surrounding Tyr 579 to ones with higher (Y^{P}EEI) or lower (Y^{P}VNV) affinity for the Src SH2 domain, and tested Stat3 and Src kinase activation by these mutants. The mutants were expressed as chimeRas with the extracellular ligand-binding domain of the CSF-1 receptor to avoid the complication of endogenous PDGF receptor activation. Importantly, the mutant receptor bearing the Y^{P}VNV site was no longer able to bind or activate Src, Yes or Fyn, presumably because of its low affinity for the SH2 domains of these kinases. However, this receptor mutant activated Stat3 as strongly as the wild-type receptor, suggesting that Src kinase activation though these sites is not required for Stat3 activation by the PDGF receptor. This study also describes work done in cell lines derived from mouse embryos lacking Src, Yes and Fyn, the three major Src isoforms expressed in fibroblasts. Stat1 and Stat3 are both activated by PDGF in these cells, providing further evidence that Src kinases are not required for PDGF receptor-induced Stat activation, at least in certain cell types. This result raises the question of the identity of the kinase that is responsible for Stat activation in response to PDGF. The authors speculate that Jak kinases may be important here, because they are strongly activated by PDGF.[66] However, mutanT cell lines lacking individual Jak kinases

are still able to activate Stats in response to PDGF, suggesting some redundancy in Jak-Stat signaling downstream of growth factor receptor tyrosine kinases.[66]

Another possibility is that Stats are direct targets for the PDGF receptor kinase itself. Recent work from Paukku et al support this notion in the context of Stat5 activation by PDGF.[71] They found that the PDGF receptor kinase domain phosphorylated Stat5 and induced its DNA-binding activity in Sf-9 insecT cells, supporting a direct activation mechanism. Consistent with the work of Sachsenmaier et al[69] they also found that PDGF receptor juxtamembrane tyrosine residues 579 and 581 were required for Stat5 activation by the receptor in transfected COS cells. Co-expression of wild-type or kinase-defective Src suppressed the activation of Stat5 in this system, suggesting that these proteins compete for the same SH2 binding sites on the receptor. In contRast, kinase-dead Jaks were without effect, suggesting that they are not required for Stat5 activation. PDGF induced efficient activation of Stat5 in fibroblasts lacking Src or Fyn, indicating that neither of these kinases is required individually for Stat5 activation. The authors conclude that the PDGF receptor itself may directly activate Stat5 following SH2-dependent recruitment to juxtamembrane autophosphorylation sites. However, investigation of Stat5 activation by PDGF in cells devoid of all Src family kinase expression will be required to rule out a requirement for these kinases.

Src May Couple ErbB Family Members to Stat Activation During Tumorigenesis

A growing number of studies support the hypothesis that Src cooperates with the EGF receptor tyrosine kinase family to promote tumorigenesis, and that oncogenic signaling through this pathway involves Stat3 activation. Using murine C3H10T1/2 fibroblasts as a model system, Maa et al found that co-expression of Src and the EGF receptor led to a synergistic increase in cellular transformation measured as colony formation in soft agar and tumor formation in nude mice.[72] Stable complex formation was reported to occur between the two proteins, and the presence of Src led to the phosphorylation of novel receptor tyrosine residues and enhanced phosphorylation of mitogenic substrates. Subsequent studies by this group identified EGF receptor Tyr 845 as one of the Src-dependent sites. Tyr 845 lies in the activation loop of the EGF receptor kinase domain, and phosphorylation of this site is known to stimulate kinase activity by enhancing accessibility of the catalytic cleft to both ATP and substrates.[73] Phosphorylation of this site by Src appears to be essential to release the transforming activity of the receptor. Co-expression of the EGF receptor and a kinase-defective form of Src completely blocks EGF-dependent tumor formation by 10T1/2 fibroblasts and correlates with a lack of Tyr 845 phosphorylation.[73] Taken together, these studies provide strong mechanistic support for the idea that Src is required for the generation of oncogenic signals by the EGF receptor.

A similar relationship may exist between Src and Her2/Neu, a close relative of the EGF receptor that is also upregulated in many breast and ovarian cancers.[74] Increased expression of Her2/Neu, often as a result of gene amplification, correlates with a poor prognosis and a more aggressive tumor phenotype.[75,76] Targeting of Her2/Neu to mammary tissues in transgenic mice leads to the induction of mammary tumors, and these tumors exhibit enhanced c-Src and c-Yes kinase activity compared to the surrounding normal cells.[77,78] As observed for the EGF receptor, Src was found to form a stable complex with Her2, suggesting a cooperative relationship in the transformation process.

As described above, both the EGF receptor and Her2/Neu cooperate with Src in mediating transformation in breast and ovarian cancers. Stat3 may be an important downstream

effector for these kinases, as constitutive activation of Stat3 is a common finding in breast cancer cell lines and primary tumor samples.[79] Interestingly, a recent study has shown that dominant-negative Src completely suppresses Stat3 activation by the EGF receptor and related tyrosine kinases.[80] These studies strongly suggest an essential role for Src in coupling oncogenic signaling from the EGF receptor and Her2/Neu to activation of Stat3 downstream. Stat3 has been shown to induce genes directly involved in cell cycle progression, such as cyclin D, as well as anti-apoptotic genes such as Bcl-x_L.[60,61] Src-dependent activation of these and other genes via Stat3 are likely to contribute to tumor development.

Contribution of Src Kinases to Stat Activation in Cytokine Signaling

Some of the initial evidence suggesting that Src kinases may contribute to Stat activation downstream of cytokine receptors comes from work by Chaturvedi et al[81] These workers employed the myeloid leukemia cell line 32Dcl3, which requires IL-3 for growth and survival, and differentiates into granulocytes following treatment with G-CSF. IL-3 was found to induce activation of Stats 1, 3, and 5 in this cell line. Expression of v-Src in these cells, which transforms them to cytokine-independent growth, induced constitutive activation of the same Stats. These studies also demonstrated formation of a stable complex between v-Src and Stat3, consistent with previous work in v-Src-transformed fibroblasts[54] (see above). IL-3 induced a complex between Stat3 and endogenous Src in 32Dcl3 cells, providing evidence that c-Src contributes to IL-3-mediated Stat3 activation in this system. Complex formation was only observed with Stat3, and not Stat1 or Stat5, despite the constitutive activation of all three Stats in the v-Src transformed cells.

To investigate the structural features of Src that are required for Stat3 activation, Chaturvedi et al[81] created a series of chimeRas between v-Src and v-Fgr. This viral oncogene is an activated form of c-Fgr, a member of the Src kinase family with myeloid-restricted expression.[12] Despite its strong transforming activity in fibroblasts, v-Fgr is unable to promote IL-3-independent growth of 32Dcl3 cells, a result that is surprising given the strong structural homology between the Src and Fgr sequences. ChimeRas containing the Src SH2 and SH3 domains and the Fgr kinase domain retained biological activity and induced Stat activation, while replacing the Src SH2 domain or the SH2 and kinase domains with Fgr-derived sequences blocked both effects. In addition, replacing the Src N-terminal unique domain and SH3 domain with N-terminal v-Fgr sequences also interfered with cytokine-independenT cell growth and Stat activation. These findings led the authors to conclude that the Src SH2 and SH3 domains are essential determinants of both transformation to IL-3 independence and Stat recognition in 32D cells. These results also imply that Stats contribute to the survival and proliferative effects of v-Src in this cell line, which is a reasonable conclusion given the ability of Stat3 and Stat5 to activate genes involved in survival pathways and cell cycle progression.[60,68,82-85]

In a subsequent study, the same group compared the requirement for Src and Jak kinases in the response of 32Dcl3 cells to IL-3.[52] IL-3 induced rapid activation of Jak1, Jak2 and Src in these cells and led to stable Stat3-Src complex formation as described in the previous study.[81] When these experiments were performed with cells stably expressing a kinase-defective form of c-Src, IL-3 failed to induce Stat3 tyrosine phosphorylation and DNA binding activity. The cells also failed to proliferate in response to IL-3, although they did not enter the apoptotic pathway. In contRast, a kinase-defective mutant of Jak2 had no effect on Stat3 activation but accelerated the induction of apoptosis in response to IL-3 withdrawal. Whether or not the presence of dominant-negative Src or Jak2 affected the activation of other Stats was not investigated. Note that the kinase-defective Src mutant

used in these studies formed a stable complex with Stat3; it is possible that this mutant may trap Stat3 and prevent its phosphorylation not only by Src but by other kinases as well.

The Src Family Kinase Hck and LIF-induced Stat3 Activation in Embryonic Stem Cells

Several studies suggest that Hck may mediate Stat3 activation downstream of gp130, the signal transducing subunit associated with the IL-6 family of cytokine receptors.[2,82] In particular, Hck has been implicated in the LIF-induced maintenance of embryonic stem (ES) cell pluripotency. LIF, acting through heteromeric receptors composed of gp130 and a LIF-specific subunit, is sufficient to promote ES cell self-renewal and suppress differentiation.[86] Ernst et al [44,87] demonstrated that LIF induces rapid Hck activation in ES cells. Activation was first apparent 2 minutes after LIF treatment and was sustained for at least two hours. Persistent Hck signaling may contribute to the sustained activation of Stat3 that is essential for the maintenance of ES cell pluripotency.[86,88-90] LIF also induced rapid but transient activation of Jak1 and Jak2 in ES cells which returned to basal levels within 60 minutes. Whether or not Hck and Jak kinases cooperate in Stat3 activation in ES cells is not clear.

Hck activation may be sufficient for the generation of self-renewal signals in ES cells. Using gene-targeting techniques, Ernst et al[44] replaced the wild-type alleles of Hck with a constitutively active mutant in which the negative regulatory tyrosine residue in the C-terminal tail is substituted with phenylalanine. As a result, this mutant is not subject to regulation by Csk and is strongly transforming in rodent fibroblasts.[26] ES cells expressing tail-activated Hck required far less LIF for self-renewal, implicating Hck in the self-renewal signaling pathway. It will be of interest to determine whether Stat3 is constitutively active in ES cells expressing the activated form of Hck.

More recent work provides additional evidence that Hck is essential to LIF/gp130 signaling, Stat3 activation and ES cell self-renewal.[46] This study employed chimeric receptors in which the extracellular ligand-binding domain of the G-CSF receptor was fused to the transmembrane and cytoplasmic domains of gp130. These chimeric receptors respond to G-CSF with gp130 signals for self-renewal,[88,89] allowing for their analysis in ES cells without interference from endogenous gp130. Ernst et al[46] found that mutant gp130 chimeRas lacking C-terminal sequences necessary for ES cell self-renewal did not activate either Hck or Stat3. In contRast, these gp130 sequences were not required for activation of Erk signaling and proliferative responses in a plasmacytoma cell line. Thus Hck and Stat3 appear to contribute predominantly to self-renewal signaling in ES cells. Interestingly, we have recently observed that Stat3 binds directly to Hck. Binding is mediated in part through the Hck SH3 domain, and is sufficient to induce transient Hck activation following co-expression of the molecules in fibroblasts (S. Schreiner and T. Smithgall, manuscript in preparation). Whether or not Hck and Stat3 interact by a similar mechanism in ES cells is currently under investigation.

Constitutive Activation of Src Kinases by Viral Proteins Induces Stat Signaling Downstream

Src kinases are targets for several viral proteins including the Nef protein of HIV-1,[91] the Tip protein of herpesvirus saimiri,[92] as well as the middle T antigen of polyoma virus.[93] Recent work suggests that constitutive Src kinase activation by these proteins induces

Stat activation, which may contribute to cell survival and proliferation. For example, we have observed that constitutive activation of Hck by the Nef protein of HIV-1 induces survival signaling in a macrophage cell line via a Stat3-dependent pathway (Briggs, S., Scholtz, B., Jacque, J.-M., Swingler, S., Stevenson, M., and Smithgall, T. E., J Biol Chem, in press). Nef, a small myristylated protein that is essential for AIDS progression, has no catalytic activity of its own and works by activating multiple intracellular signaling pathways in HIV targeT cells.[91,94] Nef binds with very high affinity and specificity to the Hck SH3 domain.[32,33] This interaction is sufficient for Hck kinase activation both in vitro and in vivo by an SH3 domain displacement mechanism.[26,30,31] We have also observed that Hck strongly activates Stat3 following co-expression of the proteins in 293T cells and Sf9 insecT cells, providing further evidence that these proteins may interact directly in vivo.

A similar phenomenon has been observed in T cell lines expressing the Tip protein of herpervirus saimiri, which has the ability to transform T-cells to an IL-2-independent phenotype.[95] Tip interacts with Lck, a lymphocyte-specific member of the Src kinase family, and strongly induces its tyrosine kinase activity.[92] Lund et al[96] recently observed that Tip induces potent constitutive activation of Stat1 and Stat3 in Jurkat T cells. This effect required the presence of Lck, as Tip was unable to induce Stat activation in a Jurkat variant lacking Lck expression. Co-expression of Tip and Lck in the Lck-deficienT cell line restored Stat1 and Stat3 DNA binding activity, providing a clear link between Tip, Lck, and Stat activation. In related experiments, the authors showed that a recombinant GST-Tip fusion protein was able to precipitate both Stat1 and Stat3 from JurkaT cells, and that this interaction was dependent upon Lck. Addition of [γ-^{32}P]ATP to the Tip-precipitated complexes resulted in Stat3 phosphorylation, suggesting that Stat3 is a direct substrate for Lck in vitro. A subsequent study by this group shows that Lck can directly activate Stat3 in insecT cells, providing further support for this hypothesis.[97] Infection of JurkaT cells with herpesvirus saimiri also led to strong Stat1 and Stat3 activation, providing the first link between a DNA tumor virus and the Stat pathway.[96]

The c-Fes Tyrosine Kinase

Overview

The human c-*Fes* proto-oncogene encodes a protein-tyrosine kinase (Fes) distinct from c-Src, c-Abl and other nonreceptor tyrosine kinases. Originally identified as the cellular homolog of avian (v-Fps) and feline (v-Fes) transforming retroviral oncoproteins, Fes is strongly expressed in myeloid hematopoietic cells and may play a direct role in myeloid differentiation. Fes is also expressed in vascular endothelial cells and has been implicated in angiogenesis. Structurally, Fes consists of a unique N-terminal region, a central SH2 domain and a C-terminal kinase domain. Within the unique region are two coiled-coil oligomerization domains that regulate kinase activity. Fes is linked to growth, differentiation and survival signaling through Ras and other small G proteins, phosphatidylinositol 3-kinase (PI-3K), and Stats. A brief summary of Fes biology and structure-function relationships is presented below followed by data implicating Stat3 as a downstream effector for Fes. For a detailed review of c-Fes and related kinases, see Smithgall et al.[98]

Fes Regulates Growth and Differentiation of Multiple Cell Types

Early studies of c-Fes expression patterns demonstrated a correlation with terminal differentiation of hematopoietic cells of myeloid origin[99-102] Fes is not expressed in immature or differentiation-resistant myeloid leukemia cell lines. On the other hand, Fes is expressed

in cell lines such as HL-60 which undergo terminal differentiation in response to a variety of chemical and biological agents. Fes expression often increases as a function of differentiation, which is largely due to transcriptional activation of the c-*Fes* locus.[99,103] Early work with v-Fps, the avian transforming homolog of c-Fes, established a causative relationship between Fes expression and myeloid differentiation. Chicken bone marrow cells infected with Fujinami sarcoma virus, which carries the v-*fps* oncogene, undergo macrophage differentiation in the absence of macrophage colony-stimulating factor.[104] This result implies that Fes tyrosine kinase activity is sufficient to induce myeloid differentiation. Similar findings have been described recently with activated mutants of human c-Fes, which induce differentiation following expression in myeloid precursor cell lines (see below).

Antisense experiments support a requirement for Fes expression in myeloid differentiation. Suppression of c-Fes expression in HL-60 promyelocytic leukemia cells using antisense oligonucleotides blocks the myeloid differentiation response to phorbol esters and other chemical inducers.[105,106] However, mice in which both normal *Fes* alleles have been replaced with a kinase-defective mutant did not show any remarkable defects in hematopoiesis.[107] These results suggest that Fes may have kinase-independent signaling capabilities, or that Fes function may be complemented in these animals by the closely-related tyrosine kinase, Fer. Supporting the former possibility is the recent observation that animals with null mutations in both c-*Fes* alleles are not born with the expected Mendelian ratios.[108] Surviving *Fes*$^{-/-}$ animals exhibited defects in homoeostasis of both myeloid and lymphoid cells as well as compromised innate immunity. These data suggest that Fes has an essential function in terms of negative feedback regulation of cytokine-dependent hematopoiesis, an effect that may be mediated in part through the Stat3 pathway. This issue is discussed in more detail below.

Other work shows that Fes-induced differentiation may suppress signals for transformation in certain forms of leukemia. In early studies, transfection of the human cell line K-562 with the c-*Fes* gene resulted in growth arrest and differentiation to macrophage-like cells.[103] K-562 cells do not express Fes, but because they are derived from chronic myelogenous leukemia (CML), they exhibit the Philadelphia chromosome translocation and express the p210 form of Bcr-Abl.[109] Bcr-Abl is the constitutively active protein-tyrosine kinase responsible for the initiation of CML.[110] Thus, the Fes signal for differentiation is dominant to the Bcr-Abl signal for growth and survival. More recently, we demonstrated that Fes suppresses Bcr-Abl-induced transformation of murine myeloid leukemia cells to cytokine independence. Fes was observed to interact directly with Bcr-Abl in these studies, suggesting that the presence of Fes may delay the progression of CML.[111] Co-expression of the two kinases resulted in reciprocal trans-phosphorylation, although the impact of this interaction on the kinase activity and downstream signaling by each of the partners has not been worked out. Interestingly, Fes has been shown to interact with and phosphorylate Stat3, which has been implicated in the differentiation of myeloid cells (following). Bcr-Abl, on the other hand, predominantly activates Stat5 which may contribute to its effects on survival signaling.[112,113]

In addition to its potential roles in myeloid hematopoiesis, Fes may influence the development of the vascular endothelium. Using a modified form of Fes carrying the v-Src myristylation signal sequence, Greer et al[114] showed that Fes induces hypervascularity and hemangioma formation in transgenic mice. Myristylation targets Fes to membranes, mimicking the activation of Fes by cytokine receptors. This study also demonstrated endogenous Fes expression in vascular endothelial cells at levels comparable to those detected in myeloid cells. Consistent with these findings are more recent studies showing that Fes is

activated by the angiogenic growth factor FGF-2 in capillary endothelial cells, and that over-expression of Fes is sufficient to induce formation of tube-like structures in these cells in the absence of angiogenic factors.[115] Interestingly, this study demonstrated strong phosphorylation of kinase-defective Fes by Src kinases, providing evidence that Src may couple the FGF receptor to Fes activation downstream.

Fes Tyrosine Kinases: Unique Structure and Regulation

The product of the human c-*Fes* gene is a 93 kDa protein-tyrosine kinase with three distinct structural regions[116] (Fig. 4). These include an N-terminal unique region with at least two coiled-coil forming motifs, an SH2 domain and a C-terminal kinase domain. Fes lacks an SH3 domain and signal sequences for lipid modification associated with Src, Abl and other nonreceptor tyrosine kinases. Fes tyrosine kinase activity is tightly regulated in cells, and recent evidence suggests that the unique N-terminal region plays an important role in negative regulation.

Unique N-terminal Region

One unusual feature of Fes is that its active form exists as a large oligomeric complex.[117] Oligomerization requires the N-terminal region and promotes Fes autophosphorylation by a trans mechanism, a key step in the activation of the kinase domain. Analysis of the unique region with COILS, a computer algorithm that searches for the heptad repeat pattern associated with coiled-coil domains,[118] reveals the presence of two regions with a high probability of forming coiled-coil structures. The presence of two N-terminal coiled-coil-forming sequences suggests several mechanisms for the regulation of Fes tyrosine kinase activity. One possibility is that a cellular protein binds to the coiled-coil regions and suppresses oligomerization. Alternatively, the two coiled-coils may interact in an intramolecular fashion, thus preventing the formation of the active oligomer. Deletion or mutagenesis of the more N-terminal coiled-coil domain activates the tyrosine kinase and biological functions of Fes in vivo, including potent differentiation-inducing activity in myeloid leukemia cell lines.[119] These results suggest that the first coiled-coil domain plays an essential role in the negative regulation of kinase function. In contRast, deletion of the more C-terminal coiled-coil domain impairs the kinase activity and biological function of active Fes mutants, suggesting a more dominant role in oligomerization or downstream signaling.[119] Under physiological conditions, oligomerization may be initiated upon recruitment of multiple Fes molecules to an activated cytokine receptor or to other sites of Fes activation, such as focal adhesions. [120]

SH2 Domain

Although the role of SH2 domains in the tyrosine phosphorylation-dependent recruitment of effector proteins to autophosphorylated growth factor receptors is well known, these domains were first identified within the sequences of Src and other nonreceptor tyrosine kinases. In fact, the SH2 domain of the Fes-related oncoprotein v-Fps was among the first to be described, and was shown to be required for full catalytic activity and to influence host range. Later work established a function for the SH2 domain in v-Fps-induced phosphorylation of transformation-related substrates.[121] More recent studies reveal a similar dual role for the human c-Fes SH2 domain in the regulation of kinase activity and biological function. Deletion of the SH2 domain greatly reduces Fes kinase activity both in terms of substrate phosphorylation as well as autophosphorylation in vitro.[122] On the other hand, replacement of the Fes SH2 domain with that of v-Src releases Fes from negative

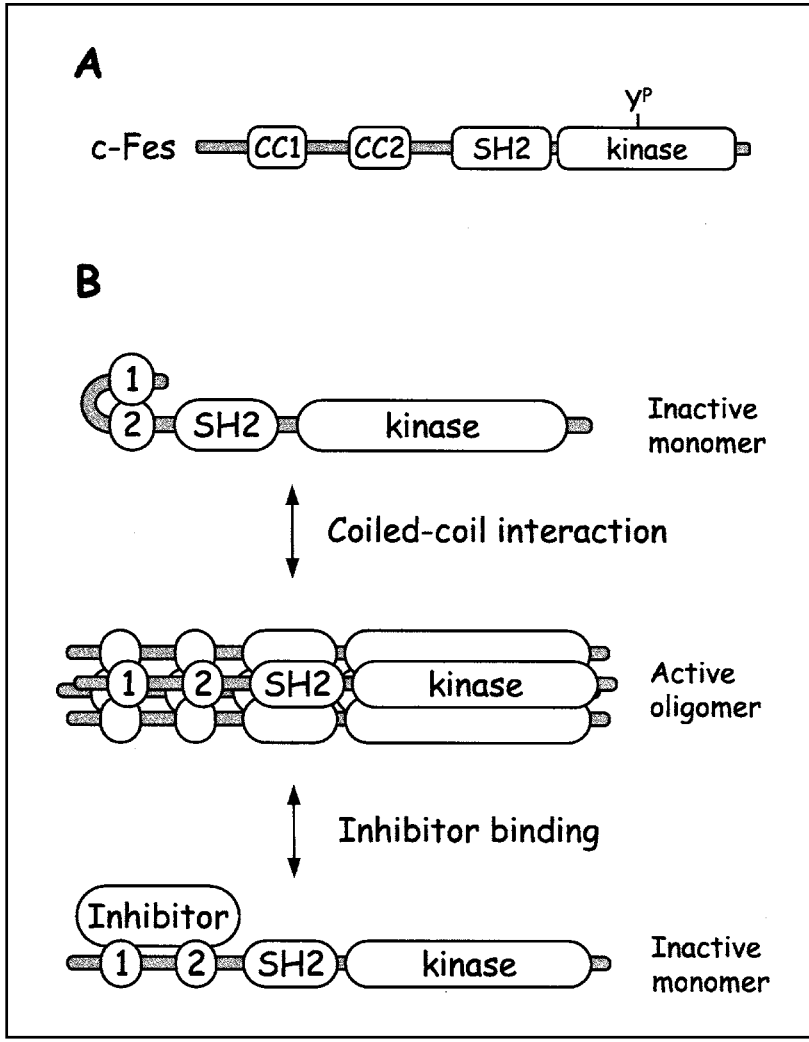

Figure 4. Structural organization and regulation of c-Fes. A. c-Fes is a structurally distinct kinase characterized by a long N-terminal region with two regions of strong homology to coiled-coil oligomerization domains (CC1 and CC2). This unique N-terminal region is followed by SH2 and kinase domains. The major site of autophosphorylation is located in the kinase domain in a region with strong homology to the activation segment of Src and other kinases of known structure. B. Regulation of c-Fes kinase activity by coiled-coil domains. The presence of two coiled-coil domains in the Fes N-terminal region suggests novel mechanisms of kinase regulation. Active Fes forms a large oligomeric complex in vitro and autophosphorylation proceeds by an intermolecular mechanism, suggesting that suppression of oligomerization may be an important aspect of negative regulation. Maintenance of the inactive, monomeric form of the kinase may involve intramolecular coiled-coil domain interaction, interaction with an inhibitory protein that binds to the coiled-coils, or perhaps a combination of the two mechanisms.

regulation in vivo, leading to fibroblast transformation.[120] This Fes/Src SH2 domain chimera also exhibits constitutive localization to focal adhesions, consistent with other studies suggesting that Fes signals from this subcellular compartment in macrophages.[123,124]

Kinase Domain

The Fes catalytic domain is localized to the C-terminal region of the protein and exhibits structural features typical of tyrosine kinases. These include a conserved lysine in the ATP binding pocket (Lys 590) and a major site of tyrosine autophosphorylation (Tyr 713), which lies in a consensus activation loop sequence. Mutagenesis of Tyr 713 to Phe greatly reduces catalytic activity both in vitro and in vivo, suggesting that autophosphorylation is required for full kinase activation. Substitution of Lys 590 with Glu or Arg completely abolishes kinase activity.[107,122,125]

Activation of Fes by Multiple Hematopoietic Cytokines

The strong expression of Fes in myeloid cells has led a number of investigators to test its regulation by hematopoietic cytokines. Using the human cytokine-dependenT cell line TF-1, Hanazono et al[126,127] showed that GM-CSF, IL-3 and erythropoietin stimulated tyrosine autophosphorylation of Fes. Both GM-CSF and IL-3 induced a physical complex between Fes and the β_c subunit shared by the receptors for both of these cytokines. Further evidence for the interaction of Fes with cytokine receptors is provided by Rao and Mufson,[128] who demonstrated that a recombinant GST fusion protein containing the membrane proximal portion of β_c is sufficient to associate with Fes in vitro. Although these results implicate Fes in GM-CSF, IL-3 and erythropoietin signal transduction, they are somewhat controversial as other studies have not been able to demonstrate activation of Fes by erythropoietin or β_c-linked cytokines in other cellular systems.[45,129]

Another study looked at the association of Fes with gp130, the signal-transducing subunit associated with the IL-6 family of cytokines.[130] This study demonstrated rapid autophosphorylation of Fes following stimulation with IL-6.[131] In addition, Fes was observed to form a stable complex with gp130 both in the presence and absence of IL-6, indicative of constitutive Fes-gp130 association. These results implicate Fes in signal transduction by other members of the IL-6 cytokine family, which includes LIF, oncostatin-M, IL-11, and ciliary neurotrophic factor.[2] Fes activation has also been linked to signal transduction by IL-4, a multi-functional cytokine that affects the growth and differentiation of a variety of hematopoietic lineages including macrophages.[132] Using transfected COS cells, this study demonstrated that Fes stably associates with the IL-4 receptor. IL-4 treatment led to enhanced association of Fes with the receptor and stimulated Fes autophosphorylation. Subsequent studies by this group suggest that IL-4-induced activation of Fes may promote its association with PI-3K.[133]

Stat Activation by c-Fes

The first evidence that Fes may contribute directly to Stat activation comes from studies of fibroblasts transformed with v-Fps, the avian transforming homolog of c-Fes. As described above for v-Src, v-Fps induces constitutive activation of Stat3 in transformed cells, suggesting a direct link to the Stat signaling pathway.[79] However, the possibility remained that v-Fps may activate Jaks, Src family members or other endogenous tyrosine kinases which in turn provide the activating signal. To address the issue of whether Stats represent direct substrates for c-Fes, our laboratory compared Stat activation in human embryonic kidney vs. Sf-9 insecT cell backgrounds.[134] We found that Fes strongly activated Stat3 in both cell types, as determined by analysis of tyrosine phosphorylation and DNA-binding activity. The finding that Fes is sufficient to activate Stat3 in insecT cells indicates that it has the potential for Stat activation without a requirement for other tyrosine kinases as intermediates. However, as described in the previous section for v-Src,[64]

the possible contribution of other tyrosine kinases to the activation mechanism in mammalian cells cannot be ruled out. In contRast to Stat3, we observed that Fes was unable to activate Stat5 in insecT cells, despite some degree of activation in mammalian cells. These findings suggest that Fes activates Stat5 via an indirect mechanism in mammalian cells, possibly via endogenous Jak or Src tyrosine kinases.

Potent activation of Stat3 by Fes in transfected human cells suggests that such an event may also be induced by Fes in myeloid hematopoietic cells. As described above, Fes has been linked to myeloid differentiation in both normal and leukemic cell lines [103,119,120] and is activated GM-CSF, IL-3, IL-6 and other hematopoietic cytokines that also induce Stat3 activation.[126,127,131] Brizzi et al[135] found that GM-CSF induced the formation of a multiprotein complex containing Fes, Stat1, Stat3, Jak2 and the β-subunit of the GM-CSF receptor in primary human neutrophils. Similar findings were subsequently reported by Park et al[136] who demonstrated that GM-CSF induces a complex between Fes and Stat3 in TF-1 myeloid leukemia cells by co-immunoprecipitation and sucrose gradient centrifugation techniques. In addition, they found that a kinase-defective mutant of c-Fes suppressed Stat3 tyrosine phosphorylation in response to GM-CSF, suggesting that Fes is required for efficient tyrosine phosphorylation of Stat3 in this cell line. These results agree with later work in mice engineered by gene-targeting techniques to express a kinase-defective form of Fes in place of the wild-type alleles.[107] Bone-marrow-derived macrophages from these animals failed to respond to GM-CSF with Stat3 activation, indicating a possible nonredundant role for c-Fes in coupling GM-CSF to Stat3 signaling. In contRast, these cells still responded normally to IL-6 in terms of Stat3 activation, suggesting that gp130-linked cytokines do not require Fes kinase activity to activate Stat3. Interestingly, Jak2 activation was not affected by the loss of Fes kinase activity, suggesting that activation of Jak2 alone may not be sufficient for Stat3 activation in response to GM-CSF in macrophages.

Several recent studies have strongly implicated Stat3 as a key component of myeloid differentiation, suggesting that Stat3 activation by c-Fes or other kinases in myeloid cells may contribute to the differentiation response. Nakajima et al[137] used dominant-negative Stat3 mutants lacking the tyrosine phosphorylation site (termed Stat3F) or with alanine substitutions for glutamate residues essential for DNA binding (termed Stat3D). Both Stat3 mutants blocked morphological differentiation of M1 myeloid leukemia cells in response to IL-6, demonstrating a requirement for Stat3 in the differentiation pathway. Interestingly, IL-6 is one of the cytokines shown to activate c-Fes.[131] Minami et al[138] observed very similar results with M1 cells over-expressing the identical Stat3F mutant as well as a truncation mutant lacking the C-terminal transcriptional activation domain and major serine phosphorylation site. These Stat3 mutants completely blocked IL-6 and LIF-induced growth arrest and expression of differentiation markers in M1 cells, consistent with the results of Nakajima.[137] Whether direct activation of Stat3 by Fes accounts for its differentiation-inducing activity in myeloid leukemia cells is currently under investigation in our laboratory.

Although Fes does not phosphorylate Stat5 directly in insecT cells, co-expression of these proteins in human cells induced both Stat5 tyrosine phosphorylation and DNA-binding activity.[134] In addition, GM-CSF-induced Stat5 activation was reduced in macrophages from mice expressing Fes genes with targeted inactivating mutations in the kinase domain.[107] These results suggest that Fes may contribute to Stat5 activation under physiological conditions. Woldman et al have established a correlation between Stat5 activation and the differentiation of myeloid leukemia cell lines.[139] They found that monocytic differentiation of human U937 cells with phorbol ester, retinoic acid, or

1α,25-dihydroxy-vitamin D_3 induced strong DNA-binding activity of Stat5. Interestingly, most of these differentiation inducers have also been shown to up-regulate Fes protein levels and kinase activity. Similar results were observed following chemically-induced differentiation of HL-60 promyelocytic leukemia cells. Stat5 activation was also observed in primary cultures of chicken myeloblasts following differentiation to macrophages with chicken myelomonocytic growth factor. In related work, induction of megakaryocytic differentiation by thrombopoietin was shown to correlate with Stat5 activation. This study also identified the cyclin-dependent kinase inhibitor $p21^{WAF1/Cip1}$ as a possible Stat5 target gene.[140] Whether or not Fes activates Stat5 as part of a differentiation signaling pathway in hematopoietic cells will require further investigation.

The Btk Kinase Family

Structural Features and Regulation

Bruton's tyrosine kinase (Btk) is best known as the genetic target in X-linked agammaglobulinemia (XLA), an immunodeficiency disorder caused by a differentiation block in B lymphocyte maturation. XLA is characterized by increased susceptibility to bacterial infections as a result of loss of B lymphocytes and attendant immunoglobulin production.[141] Btk, together with Tec, Itk, Bmx/Etk, and Txk, define a structurally distinct family of cytoplasmic protein-tyrosine kinases. While these kinases share SH3, SH2 and kinase domains in an arrangement similar to Src and Abl, they are the only cytoplasmic tyrosine kinases to contain a pleckstrin homology (PH) domain (Fig. 5). This phosphoinositide-binding domain localizes these kinases (as well as other signaling proteins) to the plasma membrane in response to PI-3K activation.[142] A second unique aspect of the structure of this kinase family is the TH (Tec homology) domain, which is composed of an N-terminal Zn^{++}-binding domain followed by a C-terminal proline-rich motif. Structural studies of Itk demonstrate that the TH proline-rich region binds intramolecularly to the SH3 domain,[143] an interaction that may provide a regulatory function analogous to that observed for Src family kinase SH3 domains. The TH proline rich-region also interacts with the SH3 domains of the Src family kinases Fyn, Lyn and Hck,[144] which may contribute to the interactions of these two kinase families during B-cell receptor-induced Btk activation (see below).

The finding that loss of Btk function leads to profound defects in B-cell development points to a central role for this kinase in signaling pathways controlling B-cell differentiation. In this regard, Btk functions downstream of the B-cell receptor and its activation is dependent upon both membrane targeting via its PH domain as well as transphosphorylation by Src kinases. Current data suggest a model of Btk activation that is initiated following B-cell receptor-induced activation of PI-3K and the generation of phosphatidylinositol-3,4,5-P_3 (PIP_3) at the plasma membrane (Fig. 5). Generation of PIP_3 recruits Btk to the membrane via its PH domain placing it in proximity to Src kinases such as Lyn. The Src kinase then phosphorylates Btk on its activation loop tyrosine residue, leading to activation of the kinase. The kinase then undergoes autophosphorylation of a second site within the SH3 domain. This dually phosphorylated form is fully active. Some XLA mutations map to the Btk PH domain, illustrating the importance of membrane recruitment in the normal activation mechanism. The role of the PH domain in the regulation of Btk kinase activity is the subject of several reviews.[142,145,146]

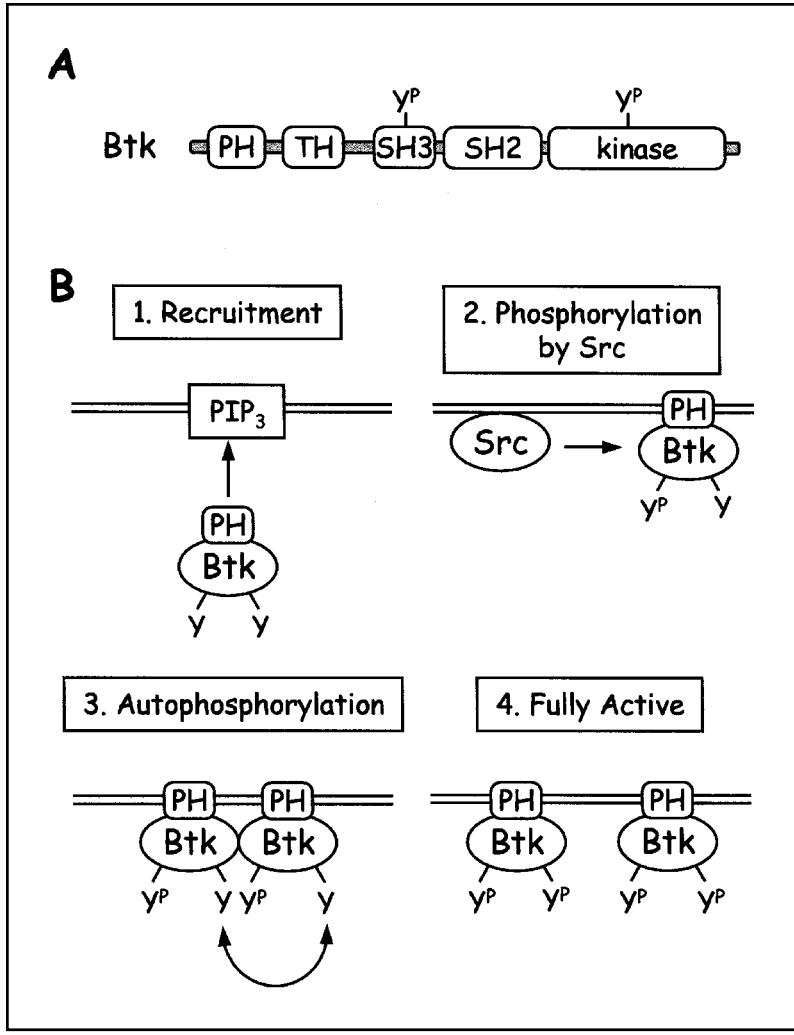

Figure 5. Structural organization and regulation of the Btk kinase family. A. Btk is the prototype of a unique kinase family that consists of an N-terminal PH domain, followed by TH, SH3, and SH2 domains and a C-terminal kinase domain. Btk has two tyrosine phosphorylation sites, one in the kinase domain and a second within the SH3 domain. B.PI-3K and Src are key regulators of Btk kinase activity. Activation of PI-3K and accumulation of phosphatidylinositol-3,4,5,-trisphosphate (PIP_3) at the plasma membrane trigger recruitment of Btk through its PH domain. Recruitment places Btk in proximity to Src family kinases, which are often activated by the same upstream events as PI-3K (e.g., B-cell receptor engagement; see text). Src phosphorylates Btk within its kinase domain activation loop, leading to autophosphorylation within the SH3 domain. The dually phosphorylated form of Btk is fully active. The SH3 domain of the Btk-related kinase Itk interacts in trans with proline-rich sequences in the TH domain,[143] and this interaction may serve to suppress kinase activation. Phosphorylation of the SH3 domain may serve to prevent this interaction and keep the kinase in the active state.

Activation of Btk Kinases by Hematopoietic Cytokines

More recent studies have shown that several members of the Btk family are linked to cytokine receptors. Tec, the first member of the family to be described, was originally identified in hepatocytes[147] and subsequently shown to be expressed in hematopoietic cells.[148] Tec is activated downstream of several cytokine receptors, including those for IL-3,[149] IL-6,[150] SCF,[151] EPO,[152] and G-CSF.[153] Btk has also been shown to constitutively associate with gp130, the signal transducer for the IL-6 family of cytokines.[150] Both Tec and Btk were activated in response to IL-6 in this study. In myeloid cells, IL-3 and EPO have been shown to induce the association of Tec with Vav,[152,153] a hematopoietic signaling molecule that regulates small GTPases of the Rho family. A more recent study has implicated the Bmx kinase as a downstream effector for GM-CSF and IL-3, where it may couple to Stat signaling.[154] This kinase is described in more detail in the next section.

Stat Activation by Bmx/Etk, a Widely-expressed Btk Family Member

The relationship of several Btk family members to cytokine signaling raises the possibility that they may contribute to Stat activation. This appears to be true for Bmx (also known as Etk), which was originally cloned from bone marrow and is expressed in normal and leukemic myeloid cells as well as epithelial and endothelial cell types.[155-157] Recent studies have shown that IL-3, GM-CSF and IL-6 induce Bmx/Etk activation through a PI-3K-dependent mechanism.[154,157] To address the connection of Bmx/Etk to Stat signaling, Saharinen et al[158] co-expressed this kinase with Stats 1, 3, and 5 in COS and insecT cells. They observed Bmx/Etk-dependent tyrosine phosphorylation and DNA binding activity for all three Stats, providing the first evidence that Stats are direct targets for the Btk tyrosine kinase family. Using reporter gene constructs, this study also showed that Bmx/Etk can activate Stat1- and Stat5-dependent transcription as strongly as Jak kinases. Over-expression of Bmx/Etk alone was sufficient to induce Stat-dependent transcription without affecting endogenous Jak kinase activity. However, activation of endogenous Src kinases was not investigated. Based on the relationship of Btk to Lyn in B-cell receptor signaling, it is reasonable to suspect that Src kinases may cooperate with Btk in Stat activation under some circumstances. In this regard, Bmx/Etk has recently been shown to cooperate with Src in transformation of fibroblasts via a Stat3-dependent mechanism (see below).

Wen et al[159] approached the question of Btk-Stat signaling by creating a conditionally active Bmx/Etk construct. First, they deleted a portion of the N-terminal region, encompassing part of the PH domain. The resulting truncation mutant showed constitutive kinase activity in epithelial cells. They then fused the hormone-binding domain of the estrogen receptor to the C-terminal portion of the mutant kinase, placing it under the control of estradiol. Using this Etk-ER construct, they were able to demonstrate an estradiol-dependent induction of kinase activity which correlated with enhanced tyrosine phosphorylation of Stats 1, 3 and 5. They also showed strong induction of gene expression from Stat1 and Stat5 reporter gene constructs, whereas Stat3 responses were much weaker. Etk-ER activation also induced cyclin D1 expression through a Stat5-dependent mechanism, providing evidence that this kinase may contribute to proliferative signaling by multiple cytokines.

Bmx/Etk Cooperates with Src in Stat3 Activation

Recent work by Tsai et al[160] suggests that Btk kinases may couple Src to Stat3 activation and oncogenic transformation in some cell types. Using a 293 cell over-expression system, they demonstrated that v-Src directly phosphorylates Bmx/Etk on Tyr 566 result-

ing in kinase activation. This tyrosine residue is homologous to Btk Tyr 551, the activation loop tyrosine directly phosphorylated by Src kinases as part of the B-cell receptor activation scheme described above. The authors also demonstrated Bmx/Etk-Stat3 complex formation, which was dependent upon an intact Bmx/Etk PH domain. To address the significance of these interactions in vivo, they employed the rat liver epithelial cell line WB which expresses a relatively high level of endogenous Bmx/Etk. A kinase-defective Bmx/Etk mutant was over-expressed in the cells and shown to block v-Src-induced activation of Stat3. The ability of kinase-dead Bmx/Etk to prevent Stat3 activation correlated with suppression of v-Src-induced soft-agar colony formation by WB cells. The overall pattern of cellular protein-tyrosine phosphorylation was not affected by the kinase-dead mutant, leading the authors to suggest that Bmx/Etk specifically couples Src to Stat3 activation in this system.

One interpretation of the data presented above is that Bmx/Etk couples Src kinases to Stat3 activation in the WB epithelial cell line. However, this may represent a cell-type-specific effect, as v-Src induces strong constitutive activation Stat3 in NIH3T3 cells where Etk is expressed at much lower levels relative to WB cells.[160] However, co-expression with Bmx/Etk greatly enhanced the transforming activity of a partially activated Src allele in NIH 3T3 cells, and this effect correlated with enhanced Stat3 activation.[160] Thus Bmx/Etk may cooperate with Src in cell lines in which the two kinases are co-expressed. On the other hand, the finding that kinase-dead Bmx/Etk can interfere with Stat3 activation by v-Src does not only imply that Bmx/Etk is required to couple Src to Stat3. Since Bmx/Etk forms a stable complex with Stat3, the kinase-inactive mutant may form a stable complex with Stat3 in vivo sequestering it from direct phosphorylation by v-Src. It will be of interest to determine whether v-Src is able to induce Stat3 activation in an Etk-null background.

Conclusions

Studies summarized in this chapter show that multiple nonreceptor protein-tyrosine kinases can contribute to the activation of Stats in a variety of cell types. Several lines of evidence strongly suggest that members of the Src, Fes, and Btk kinase families have the potential to activate Stats in vivo. In some situations, Stat activation may be direct, but more often appears to involve cooperation between multiple kinase families. Data reviewed here provide evidence for Src-Jak, Src-Fes, and Src-Btk interactions in physiological signaling pathways as well as those leading to oncogenic transformation. Further investigations will reveal which of these kinases have nonredundant functions in Stat activation in the various cellular contexts in which they are co-expressed. These studies will help to address the important general question of why cytokine and growth factor receptors are often linked to the activation of multiple cytoplasmic tyrosine kinases.

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Chapter 4

Stats and Hematopoiesis

Alister C. Ward

Introduction

There has been a great deal of literature dedicated to the study of Stats in a variety of cell systems. However, many of these studies have been from the perspective of the individual Stats or of those receptors which activate them and have utilized a range of cell systems, not all of them relevant to hematopoiesis. Therefore, I have chosen in this Chapter to not only describe what is known about Stat function generally, but also to focus in more detail on particular hematopoietic processes and those studies which have sought to elucidate the role of Stats therein. In addition, I will attempt to unravel from the vast literature some of likely mechanistic details of how Stats exert their effects in hematopoietic cells, as well as to identify areas where our knowledge base remains lacking.

Stat Proteins

Activation

Activation of Stat proteins occurs downstream of a number of different receptor classes, including cytokine receptors, receptor tyrosine kinases and G-protein coupled receptors.[1] In addition, nonreceptor tyrosine kinases (principally oncogenes) can also activate Stats, often in a constitutive manner.[2] A general picture of Stat activation from cytokine receptors has emerged,[3-5] although there are many variations and exceptions on this basic scheme which was first established for the interferon receptors.[6] Binding of ligand induces clustering of these cell-surface receptors which leads to activation of associated Jak kinases through trans autophosphorylation on tyrosine (Fig. 1). Activated Jaks, or other receptor-associated kinases, then phosphorylate the receptor, creating docking sites for specific signaling proteins, including Stat proteins, which bind via their the Src homology 2 (SH2) domains. These kinases are then able to phosphorylate the Stat proteins on a conserved tyrosine residue at their C-terminus. Subsequently, the Stats form stable homo- and heterodimers by interactions between the SH2 domain of one Stat protein and the phosphotyrosine of another, before translocation to the nucleus, where they influence transcription of target genes by binding to specific regulatory sequences.[4,5]

Specificity

Seven Stat proteins have been identified in mammalian cells, Stats1-6, including Stat5a and Stat5b which are encoded by distinct genes. In addition, different isoforms of several

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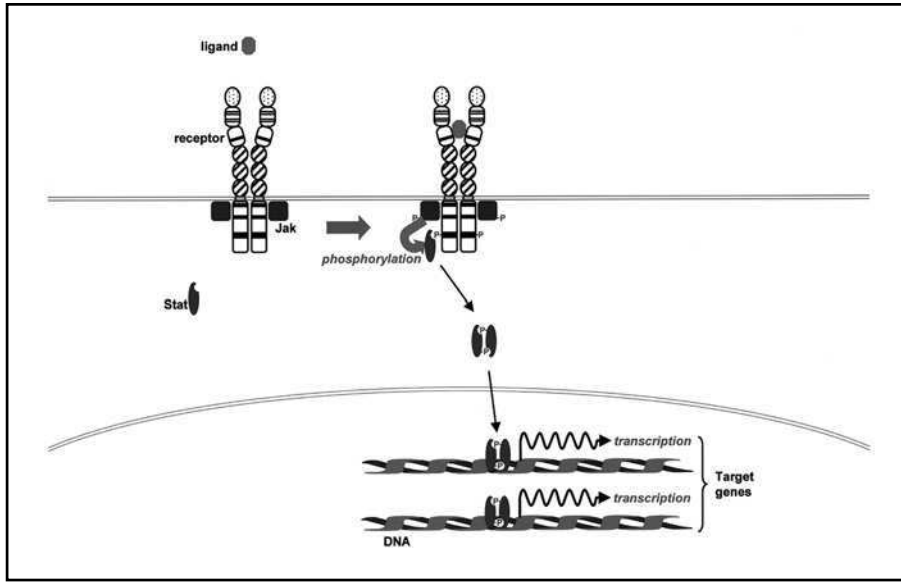

Figure 1. Activation of the Jak-Stat pathway by cytokine receptors. Ligand binding results in receptor dimerisation which activates the associated Jak kinases. These can then phosphorylate the receptor as well as Stat proteins recruited to the activated receptor complex. The activated Stats can then translocate to the nucleus to act on a varitey of responsive genes.

Stats have been identified.[4,7] Specificity in cell signaling is determined in part by the combination of Jaks and Stats activated. However, while a wide range of cytokines, growth factors and other ligands can activate the Jak kinases Jak1, Jak2 and Tyk, and all receptors which share the common gamma chain can activate Jak3,[8] receptors show much more specificity in their ability to recruit and activate Stats (Table 1). Indeed the relatively distinct phenotypes of Stat knock-out mice (Table 2) reinforces this concept. This specificity is partially mediated through recruitment of Stats to particular cytoplasmic receptor tyrosines via their SH2 domains. For example, tyrosine 440 of the IFN-γ receptor α chain cytoplasmic domain serves as a Stat1 binding site,[9] while tyrosines 578 and 606 of the IL-4-R are required for recruitment of Stat6.[10] Similarly, the Y××Q motif has been delineated as a consensus Stat3 docking site, although this Stat can bind to other motifs as well.[11,12] However, several examples of Stat activation not requiring direct docking to receptor tyrosines also exist. For instance, activation of Stat1 by G-CSF or growth hormone and of Stat5 by G-CSF or GM-CSF can occur in the complete absence of receptor tyrosines.[13-17] This may be directly via Jaks which can specifically recruit and phosphorylate Stat1 and Stat5, respectively.[18,19] In addition, there is growing evidence that other receptor components can also act as docking sites for Stats.[16,20] For example, Stat1 is known to be recruited to the IFN-α/β receptor complex by binding to a Stat2 molecule already docked to the activated receptor,[4] while Stat3 can bind to other receptor-associated kinases.[21] In this way, Stat specificity is determined by recruitment to the entire receptor complex, rather than being dictated simply by the linear sequence of each receptor cytoplasmic domain. Furthermore, the particular Jaks and Stats activated may also be dependent on the cell-type or its state of differentiation,[4,22-24] while receptor "cross-talk" may further modify the response elicited. It has been reported, for example, that IL-10 can

Table 1. Hematopoietic activators of Stat proteins

Stat	Activators
Stat1	IFNα, IFNγ, PDGF, ANGII, TSH, MIP-1, RANTES
Stat2	IFNα
Stat3	Il-6, IL-11, OSM, LIF, IL-10, G-CSF, PDGF, Gαo, TSH, MIP-1, RANTES
Stat4	IL-12
Stat5	IL-2, IL-7, IL-9, IL-15, IL-3, IL-5, GM-CSF, EPO, TPO, PDGF
Stat6	IL-4, IL-13

Table 2. Stats in hematopoiesis: phenotypes of mouse knockouts

Stat type	Relevant phenotypes	Cytokines affected
Stat1	Interferon responses absent: • innate immune responses absent • highly sensitive to viral/microbial infection • IFN-responsive genes not activated	IFNs only
Stat2	Type I interferon responses impaired	IFNα/β
Stat3	Embryonic lethal	Many
Stat4	IL-12 responses absent: • ↓ production of high IFNγ, low IFNγRα Th1 cells • ↓ priming for high level IFNγ production • ↓ lymphocyte proliferation • ↓ enhancement of NK cell-mediated cytotoxicity • ↑ Th2 cells	IL-12 only
Stat5ab	Proliferation signaling affected: • ↓ CFU-Mix, Eos, G, GM, Pre-B • ↓ peripheral T cells • NK cells absent	IL-2, IL-3, IL-7, GM-CSF, G-CSF
Stat6	IL-4 responses absent: • absence of IL-4 producing Th2 cells • block in B cell IgE class-switching • ↓ lymphocyte proliferation (partial) • ↓ expression of IL-4-induced cell surface markers	IL-4 only

suppress IFN-mediated Stat activation,[25] IL-4 can inhibit IL-2-mediated Stat5 activation,[26] and cAMP can dampen IL-2-dependent signaling by down-regulating levels of the Jak3 protein itself.[27]

Function

Stats bind to DNA response elements related to the gamma interferon activated site (GAS), a regulatory element in the promoter of interferon γ-inducible genes.[6] However, while the Stat recognition sites are similar they are not identical,[28-30] and so different genes are targeted for induction by different Stats (Table 3). Many natural Stat-responsive elements contain closely adjacent tandem sites, with Stat dimer-dimer (tetramer) interactions

Table 3. Genes induced by Stat proteins

Stat	Genes encoding
Stat1	2,3 dioxygenase; ISG54; IRF-1; CIITA; GBP; mig; p21
Stat3	IL-2Rα; JunB; SAA3; JAB; C-reactive protein; Bcl-x_L; p21
Stat4	FcγRI; IFNγ; IRF-1; MHC class II; CD23
Stat5	CIS; β-casein; pim1; IL-2Rα; osm; p21; cyclin D1
Stat6	FcεRIIa; Cε; Cγ1; Cγ4; IL-4Rα; Bcl-x_L
Stat1/Stat1/p48	GBP
Stat1/Stat2/p48 (ISGF3)	2′,5′ oligoadenylate synthetase; ISG15; ISG54; 6-16; 2,3-dioxygenase

required for maximal transciptional stimulation.[31] Transcriptional activation is achieved, at least partially, via direct association with components of transcriptional machinery, such as the helicase MCM5[32] and the histone transacetylase CBP/p300.[33] In addition, Stats can interact with a range of other transcription factors bound at neighboring sites: for example, Stat1 and Sp1 associate on the ICAM promoter,[34] Stat3, c-Jun and the glucocorticoid receptor (GR) form a complex on the α2-macroglobulin promoter,[35] while Stat5 and GR interact on the β-casein promoter.[36] Finally, Stats can also mediate transcriptional repression at specific promoters.[37-39]

Regulation

While phosphorylation of the C-terminal tyrosine is critical for Stat activation, serine phosphorylation probably also modulates the transcriptional response.[40-42] Indeed, Ser727 of Stat1α was shown to be directly involved in the recruitment of MCM5 as part of interferon γ-induced transcriptional activation.[32] In activation of Stats occurs by tyrosine dephosphorylation by a nuclear phosphatase after which they return to the cytoplasm,[43] or alternatively they may be degraded.[44] The mechanistic details of serine dephosphorylation remain to be elucidated.

Diversity

Naturally-occurring splice variants of Stats occur, such as Stat1β, Stat3β, and Stat5β, which lack a C-terminal activation domain.[37,45,46] These act as dominant-negative in some, but not all, cell types.[37,47,48]. Other forms are generated by specific proteolysis events, such as Stat3γ[49] and Stat5 p80,[50] while mast cells express a specific Stat6 isoform that appears to act as a repressor of IL-4 transcription.[39] In addition, while Stats normally form homodimers, they can also participate in heterodimeric interactions which can further broaden the range of Stat/DNA binding specificities.[4] For example, G-CSF stimulation leads to activation Stat1/3 and Stat3/5 heterodimers as well as Stat3, Stat5 and some Stat1 homodimers.[15,16] Finally, the duration of Stat activation is also able to influence the transcriptional program induced.[4,51]

Role of Stats in Hematopoietic Processes

The results of a number of studies point to a critical role of Stat3 in the differentiation of macrophages and neutrophils, while the role of Stat5 in these cell types more elusive. It

is clearly important for enhancement of proliferation in some cases, and may be important for differentiation in others. In contrast, Stat5 appears to be one of the key factors in mediating differentiation of cells of the eosinophilic, megakaryocytic and erythroid lineages. T cell development, on the other hand, is more complex, with many Stats involved in different aspects of the process. Other hematopoietic processes also require the function of distinct Stat proteins.

Macrophage Development

Yamanaka et al have shown that those regions of the gp130 cytoplasmic domain which are responsible for Stat3 activation are also required for IL-6 and LIF-induced macrophage differentiation of murine myeloid M1 cells.[52] Equivalent results were obtained when the closely-related G-CSF-R receptor was introduced into these cells.[53] Suppression of Stat3 activation, by overexpressing either dominant-negative Stat3[54,55] or SOCS proteins,[56] blocked IL-6 and LIF-induced differentiation of these cells. In the case of the dominant-negative Stat3, overexpression also increased proliferative responses to these ligands, suggesting that Stat3 might have a growth-suppressive function in these cells as well.[54] Stat3 knockout mice are embryonic lethal.[57] Those with Stat3 deleted specifically in macrophages and neutrophils show defects in functional activation of macrophages (see below) suggesting a perturbation in the final stages of differentiation.[58] However, it is worth noting that gene ablation in this case probably occurs quite late in the differentiation process, so that important earlier roles for Stat3 might be missed. In Stat5a$^{-/-}$ mice there was no difference in the proliferation of bone-marrow-derived macrophages at low concentrations of GM-CSF. However, it was reduced at higher doses.[59] In addition, there was a slight decrease in IL-3/GM-CSF colonies in the Stat5 double knockout,[60] suggesting Stat5 might play some minor role in macrophage proliferation and differentiation.

Neutrophilic Development

Both Stat3 and Stat5 have been implicated in the differentiation of neutrophilic granulocytes. Both are activated by one of the key regulators of neutrophilic differentiation, G-CSF, as well as other cytokines important in the development of this lineage, including IL-3 and GM-CSF.[61] Several studies have shown that G-CSF-R mutants which are defective in Stat3 activation are also defective in their ability to mediate neutrophilic differentiation (and survival) of suitable cells.[17,62] In addition, overexpression of dominant-negative forms of Stat3 is able to block G-CSF-induced neutrophilic differentiation of mouse myeloid cell lines LG-M1[63] and 32D.[64] More recent studies have also shown that introduction of a dominant-negative Stat3 into hematopoietic progenitor cells via retroviral transduction can inhibit G-CSF-dependent colony formation.[65] These authors further showed that a constitutively-active Stat3 was able to partially rescue CFU-G formation from a defective G-CSF-R.[65] Studies on other mutant G-CSF-Rs has indicated a positive role for Stat5 in proliferation and possibly survival. Thus, one G-CSF-R form harboring an extracellular mutation that can only weakly activate Stat5 transduces poor signals for proliferation and survival,[66] while a truncated G-CSF-R that constitutively activates Stat5 is hyperproliferative in both primary cells and the 32D cell line.[17,67] Co-expression of dominant-negative Stat5 with this truncated form in 32D cells can partially inhibit G-CSF-mediated proliferation (ACW, unpublished data). In contrast, other investigators have shown that expression of a dominant-negative Stat5A in 32D cells decreased IL-3-dependent proliferation and G-CSF-dependent differentiation without induction of apoptosis, and could also inhibit G-CSF-dependent granulocyte colony formation in vitro.[68]

This is supported to some extent in Stat5a$^{-/-}$Stat5b$^{-/-}$ mice, where both the number and size of CFU-G colonies was somewhat decreased.[60]

Erythroid Development

A strong correlation has been identified between the ability of EPOR receptor forms to activate Stat5 and their ability to induce differentiation in both erythroleukemia ELM-I-1 cells[69] and SKT6 cells.[70] Furthermore, expression of dominant-negative Stat5 is able to suppress EPO-induced differentiation in both cell types.[69,70] Moreover, studies with Stat5-deficient mice have established a vital role for Stat5 activation in the fetal development of erythroid progenitors.[60,71] Embryos from mice with a homozygous deletion of both Stat5 genes are anemic, due to decreased responsiveness to the anti-apoptotic effect of EPO.[71] In addition, introduction of a dominant-negative Stat5 into wild-type fetal liver cells led to increased rates of apoptosis and a decreased net growth rate in response to EPO.[71] By corollary, a constitutively-active Stat5 protected a number of EPO-dependent cell-lines from apoptosis following EPO withdrawal.[72,73] Furthermore adult Stat5a$^{-/-}$Stat5b$^{-/-}$ mice have normal numbers of circulating red blood cells although twice the number of normal progenitors,[60] which may reflect a compensatory mechanism for the reduced survival of these cells.[71] However, the EPOR which best reconstituted erythropoiesis of fetal liver cells derived from EPOR$^{-/-}$ mice correlated with their ability to induce PI 3-kinase, rather than Stat5,[74] suggesting that Stat5 may not be the only factor required.

T Cell Development

Targeted disruption of the Stat3 gene in mice produced early embryonic lethality.[57] Subsequently, T cell-specific Stat3-deficient mice were generated and found to be severely impaired in IL-6-induced T cell proliferation due to enhanced apoptosis.[75] Stat4 knockout mice were defective in the formation of Th1 cells, largely as a result of disrupted IL-12R function.[76,77] Stat5a$^{-/-}$ mice showed impaired peripheral T cell proliferation due to defective IL-2Rα expression,[78] a significant decrease in Th2 cell differentiation as well as impaired development of CD4+CD25+ immunoregulatory T cells.[79] Mice with both Stat5a and Stat5b genes disrupted showed a profound decrease in the number of peripheral T cells due to a lack of Stat5 responses to circulating IL-2 and IL-4.[80,81] Finally, a block in Th2 cell development and IgE class-switching was observed in Stat6 knockout mice.[82,83] Further studies have established a key role for Stat6 in Th2 cell trafficking and effector functions.[84] Indeed introduction of a constitutively-active Stat6 into CD4+ T cells can lead to Th2 differentiation and enhanced cell expansion.[85]

Development of Other Hematopoietic Cells

A number of studies have also highlighted important roles for Stats in the development of other hematopoietic lineages. For example, Stat5a$^{-/-}$ mice are defective in NK cell proliferation.[86] In addition, deletion of a 10 amino acid intracellular region of c-Mpl (the receptor for TPO), both abrogated Stat5 activation and blocked megakaryocytic differentiation of UT-7 cells without effecting proliferation or survival.[87-89] In addition, Stat5 has been implicated in IL-5-mediated differentiation of eosinophils.[50] IL-5 also mediates the survival of mast cells, although in this instance it is through Stat6-mediated inhibition of apoptosis, with expression of a dominant-negative Stat6 blocking this inhibition.[90] Finally, Stat3 activation by gp130 in embryonic stem cells is required to maintain totipotency and suppress differentiation.[91,92]

Innate Immunity

Early studies with mutant cell lines showed that Stat1 was essential for interferon signaling.[93] This was confirmed in Stat1 knockout mice which showed defective innate immunity due to loss of interferon responses.[94,95] Thus, macrophages from these animals are unable to up-regulate nitric oxide production in response to IFNγ, which leads to a high degree of sensitivity to viral and microbial infections. Similarly, Stat2 knockouts showed defective responses to interferon α/β, although this was much less marked in macrophages compared to fibroblasts.[96] However, recent studies have also revealed important Stat-independent pathways leading from IFN receptors.[97] Finally, mice with Stat3 deleted specifically in macrophages and neutrophils show an increased susceptibility to endotoxin shock as well as increased production of inflammatory cytokines.[58] This appears to be due to decreased responsiveness to IL-10, which normally controls the activation of these cells.

Mechanisms of Stat Action

It is clear from the above studies that Stats exert important pleiotropic functions in many hematopoietic processes. This section aims to highlight a number of mechanisms that Stats employ in mediating these effects. Different mechanisms may be employed by the same Stat in different cells, while more than one may operate concurrently in the same cell.

Cell Cycle Progression

There is considerable evidence that Stats contribute to proliferative responses by activating important mediators of cell cycle progression. For example, constitutively-active Stat3 can lead to factor-independent cell proliferation which correlates with increased cyclin D1 levels, with the cyclin D1 promoter able to be directly activated by Stat3.[45] The cyclin D1 gene is positively regulated in a similar manner by Stat5.[98] Another key target for activated Stat5 is the *osm* gene, enforced expression of which is sufficient to produce proliferative responses in hematopoietic cells.[99] There is also evidence that Stat3 and Stat5 can both mediate transcriptional regulation of the proto-oncogenes *c-myc*[100,101] and *pim-1*.[101,102]

Negative Regulatory Functions

Stats also appear to exert a number of negative regulatory effects by inducing cell cycle inhibitors and SOCS proteins, as well as inhibiting expression of cell-cycle regulators. Thus, IL-6 induces the cell-cycle inhibitor $p19^{INK4B}$ in a Stat3-dependent fashion.[103] Similarly, Stat3-induced G1 arrest of 32D cells by G-CSF is mediated in part by induction of cell cycle inhibitor, $p27^{kip1}$,[64] while an EPOR engineered to activate Stat3 induced $p27^{kip1}$ and growth arrest in 32D cells as well as activating integrins and promoting cell adhesion compared to normal proliferative response.[104] Stat5-mediated induction of another the cell cycle inhibitor $p21^{CIP}$ is sufficient for megakaryocytic differentiation by TPO, with Stat5 shown to directly interact with sites within the $p21^{CIP}$ promoter.[89] Finally, Stat1-deficient cells are less susceptible to apoptosis and show increased proliferation and destruction of $p27^{kip1}$.[105] Furthermore, a number of factors, such as EPO, IL-3 and GM-CSF, induce the cytokine-inducible SH2-domain containing protein CIS in a Stat5-dependent manner.[106,107] Indeed, there are Stat binding sites in the *CIS* promoter,[107] expression of dominant-negative Stat5 can suppress its induction,[108] and Stat5 knockout mice show reduced GM-CSF-induced expression of *CIS*.[59] CIS can bind to tyrosine-phosphorylated cytokine receptors and seems to act as a negative regulator of cell

proliferation,[106] which is probably a vital prelude to differentiation. Finally, IL-6-induced repression of the *c-Myc* and *c-myb* genes can be blocked by expression of a dominant-negative Stat3,[54,55] while Stat1-deficient cells show enhanced induction of cyclin A.[105]

Survival—Bcl Family

Another key mechanism by which Stats exert their biological effects is through the induction of a number of anti-apoptotic genes, particularly those of the Bcl-2 family.[109] Activation of Stat5 in response to IL-3[110] or Epo[73] is required for maximal expression of Bcl-x_L and correlates with cell survival. Furthermore, a dominant-negative Stat5 can suppress EPO-induced Bcl-x_L,[106] while constitutively-active Stat5 can induce its expression.[72,101] In addition, Stat5a$^{-/-}$Stat5b$^{-/-}$ mice show decreased levels of expression of this gene, with the induction of Bcl-x_L by EPO demonstrated to be Stat5-dependent via binding to a consensus site located the Bcl-x_L promoter.[71] Similarly, IL-15 prevents apoptosis of mouse mast cells through Stat6-mediated Bcl-x_L expression as determined by dominant-negative Stat6 expression.[90] Furthermore, autocrine activation of IL-6/gp130 in human multiple myeloma cells leads to resistance to apoptosis, and can be blocked by dominant-negative Stat3 which also blocks Bcl-x_L expression.[111] gp130-mediated anti-apoptotic responses in BAF3 cells required Stat3, which also correlated with Bcl-2 induction,[112] while similar responses in human neutrophils are associated with induction of the Bcl-2 family protein Mcl-1.[113] Meanwhile, in Stat5a$^{-/-}$ macrophages, GM-CSF-mediated induction of the Bcl-2 like gene A1 is greatly reduced.[59] The importance of the Bcl-2-family-mediated pathway has been confirmed recently in chicken and mouse cells. Introduction of a dominant-negative Stat5 into chicken myoblasts led to massive apoptosis during differentiation, which could be rescued by Bcl-2. Similarly, bone marrow cells from Stat5a$^{-/-}$Stat5b$^{-/-}$ mice show apoptosis during GM-CSF-dependent maturation, which can be rescued by Bcl-x_L expression.[114]

Unanswered Questions

There are still a number of outstanding issues regarding Stat function that remain to be resolved. These have important implications for our understanding of the Jak-Stat pathway:

1. How can the same Stat do different things in different cell-types?
   For example, Stat3 appears to stimulate differentiation of myeloid cells[63] whereas it suppresses differentiation of ES cells.[91]
2. How do receptors activate different Stats in different cell types?
   For example, IFNγ stimulates Stat3 in neutrophils[37] and Stat1 in eosinophils,[115] while GM-CSF activates Stat1 in eosinophils,[115] but not in neutrophils.[116] This is not due to the availability of the individual Stats.
3. How do Stats form different complexes following stimulation with different factors?
   For example, G-CSF stimulates Stat1, Stat3 and Stat5 homodimers, as well as Stat1/Stat3 and Stat3/Stat5 heterodimers.[46,51,64]
4. What are the mechanisms by which different levels of Stat activation or competition between Stats affects the biological outcome?
   It is apparent from many studies that the relative levels of activation of different Stats is a key determinant of the biological outcome. Thus, high level Stat5 activation in TF1 cells gives proliferation, compared with low level induction which leads to differentiation.[117] Similarly, IL-3, which induces high levels of Stat5,[61] blocks differentiation of BaF3 cells by Epo[118] and 32D cells by G-CSF (ACW, unpublished observations). Moreover, a truncated form of the G-CSF-R that constitutively activates Stat5 leads to proliferation, rather than

differentiation of the 32D cell line.[17] However, there is also competition between different Stats and different isoforms in determining a particular outcome. Therefore, if you treat UT-7/GM cells with GM-CSF there is activation of Stat1α and Stat3 thereby inhibiting EPO-induced (Stat5-mediated) erythroid differentiation, which can be blocked by dominant-negative Stat1 or Stat3.[119] Similarly, overriding Stat3 with a dominant-negative form changes IL-6 or G-CSF signals from differentiation to proliferation,[54,64] while engineering a Stat3 site into the EPOR changes it from proliferation to differentiation.[104]

5. What exact role do the different Stat isoforms play in hematopoiesis?
   For example, there is a change in Stat3 isoform expression during myeloid differentiation of human CD34+ cells.[120] In leukemic cells which fail to differentiate there is both Stat3α and Stat3β, but in terminally-differentiated neutrophils Stat3γ is activated.[49] Indeed, overexpression of Stat3α makes the cells partially resistant to differentiation.[12] Similarly carboxy-terminally truncated forms of Stat5 exist, but preferentially in progenitors.[121,122] Indeed, some studies show that Stat5 processing is required for myeloid maturation.[123] However, other studies show that the processed p80-form of Stat5 increased during G-CSF driven granulocytic maturation of CD34+ cells, but not IL-5-driven eosinophils.[50] Obviously this area requires considerably further study before clear conclusions can be drawn.

Conclusions

Stat proteins are clearly pivotal in mediating a range of hematopoietic processes through their actions on key genes. As a testament of their importance, they are controlled by several layers of specificity, being activated by an array of factors and regulated by diverse mechanisms including phosphorylation status, alternative splicing, specific proteolysis and receptor "cross-talk". Together, this complex control of specificity enables individual hematopoietic cells to instigate the appropriate transcriptional program, and hence biological response, to the myriad of signals it receives at any given time.

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Chapter 5

Stat Signaling in Leukemia: Implications for Pathogenesis and Treatment

David A. Frank

Introduction

Great advances have been made in recent years in understanding the intracellular signaling events which control the survival, proliferation, and differentiation of hematopoietic cells. However, at the same time, relatively little progress has been made in the treatment of leukemias, which are essentially diseases arising from abnormalities in these very same processes. Beginning with the hypothesis that the pathways which lead to survival and proliferation of normal hematopoietic cells are subverted in leukemias, it is possible to dissect the molecular defects in these malignant cells. This knowledge might then allow the design of targeted molecular therapeutic strategies to selectively kill leukemic cells while leaving normal cells relatively unaffected. Abundant evidence has revealed that Stat transcription factors are involved in transducing signals generated by cytokines in controlling physiologic programs of hematopoietic cells. Furthermore, inappropriate Stat activation is seen in essentially every form of human leukemia, albeit resulting from a number of different mechanisms. It appears that redundancy of signaling pathways allows normal cells to compensate for inhibition of Stats, while malignant cells may be dependent on Stat activation. These findings have raised the possibility that inhibitors of Stat signal transduction may be an important component of the next generation of anti-leukemic therapy.

For a variety of reasons, much of the pathbreaking work in studying signal transduction has occurred in hematopoietic cells. This is in part a reflection of the relative ease in obtaining large quantities of purified primary normal and malignant cells both from humans and other organisms, and the ability to generate cell lines for in vitro study. In addition, the myriad steps through which a single pluripotent stem cell can give rise to lineages of great diversity in morphology and function have been well described in both animal and tissue culture models. One principle which has emerged from these studies is that normal hematopoietic cells essentially always require the addition of cytokines to the culture medium for survival and growth. By contrast, many malignant hematopoietic cells have a relative or absolute loss of this growth factor requirement. From this observation it was hypothesized that the signaling pathways activated by cytokines which mediate the growth and survival of normal hematopoietic cells might be activated inappropriately in leukemic cells. Given the prominence of Stats in transducing signals generated by

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cytokine-receptor interactions, these transcription factors became a focus for understanding the pathobiology of hematologic malignancies. Using both primary patient samples and informative tissue culture models, it soon became clear that inappropriate Stat activation was a common occurrence in human leukemia. Research in this area has been exceptionally rewarding, and has contributed greatly to our understanding of the molecular pathogenesis of leukemias. Furthermore, by identifying pathways which distinguish normal hematopoietic cells from malignant ones, it has suggested a new series of therapeutic approaches which can be undertaken with potentially greater efficacy and diminished toxicity.

Human Leukemia

All of the lineages of hematopoiesis are susceptible to neoplastic transformation, but broadly the leukemias are divided into those of lymphoid origin and those of myeloid origin. Myeloid leukemias include cells differentiating into granulocytes and/or monocytes, as well as the uncommon leukemias of precursors of red blood cells and platelets (or the cells from which they are derived, megakaryocytes). This distinction reflects the early differentiation of a hematopoietic stem cell into precursors of lymphocytes or precursors of the other blood elements. Furthermore, the morphology, biology, and treatment of all of the subtypes of myeloid leukemias are fairly similar, though generally distinct from the lymphoid leukemias. The lymphocytic and myeloid leukemias are then further divided into acute and chronic forms (Table 1). This initially reflected the clinical differences seen in patients with leukemia prior to the advent of effective chemotherapy. The acute leukemias, be they lymphoid or myeloid, were characterized by the presence in the blood and bone marrow of very immature cells which were highly proliferative, and these diseases were generally rapidly fatal. The chronic leukemias, by contrast, showed populations of highly mature and differentiated cells in the circulation and were compatible with survivals of many years.

With the introduction of effective chemotherapy in the second half of the 20th century, many patients with acute leukemias could be cured. The most dramatic gains were made in childhood ALL. This disease, which inevitably caused death in children from bleeding or infection, can now be eradicated in the majority of cases. However, the chronic leukemias, despite their often indolent course, remain generally incurable with standard treatments. The general approach to the chemotherapy of patients with leukemia is to administer highly cytotoxic drugs. The goal is to exploit a potential therapeutic ratio in which a drug at a given dose will kill the malignant cells in excess of the normal cells. However, in reality this distinction is often slight, and the drugs administered lead to severe toxicity through the killing of normal cells. Hence, a new approach to anti-leukemic therapy, and anti-cancer therapy in general, is greatly needed.

Stat Activation in Leukemias

General Considerations

From our increased understanding of molecular and cellular processes, it can be hypothesized that inappropriate activation of signaling pathways in leukemia will recapitulate those activated during the normal survival and proliferation of hematopoietic cells. If this is true, it would be expected that only a subset of Stats would be found to be activated in these malignant cells. The only ligand known to induce the tyrosine phosphorylation of Stat2 is interferon (IFN)-α, a cytokine which has prominent anti-viral and anti-proliferative

Table 1. Common forms of human leukemia

	Myeloid	Lymphocytic
Acute	Acute myeloid leukemia (AML) 9700 cases/year 7100 deaths/year	Acute lymphocytic leukemia (ALL) 3200 cases/year 1300 deaths/year
Chronic	Chronic myeloid leukemia (CML) 4400 case/year 2300 deaths/year	Chronic lymphocytic leukemia (CLL) 8100 cases/year 4800 deaths/year

Leukemias can be characterized, based on their cell of origin, as either myeloid—affecting precursors of granulocytes, monocytes, erythrocytes, or megakaryocytes (platelet precursors)—or lymphoid. Furthermore, based on the degree of differentiation of the transformed cell and the biology of the disease, they can be divided into acute and chronic leukemias. Data on estimated number of cases and deaths in the year 2000 are from Greenlee et al.[21]

actions.[1,2] Stat4 and Stat6 appear to be involved primarily in regulating the differentiation of T helper lymphocytes into so-called T_h1 and T_h2 lineages.[3-5] By contrast, a plethora of cytokines which lead to the proliferation of each of the distinct hematopoietic lineages induce the phosphorylation of Stat3 and/or Stat5. Examples include interleukin (IL)-2 for the proliferation of lymphocytes,[6-10] G-CSF,[11,12] IL-3,[13] and GM-CSF[13] for the proliferation of myeloid cells, thrombopoietin for the proliferation of megakaryocytes,[14-16] and erythropoietin for the proliferation of red blood cells.[13] These factors may also promote various differentiation pathways, but always in association with proliferation of precursor cells. Depending on the cellular system, Stat1 activation occurs in conjunction with signals for proliferation[17] or for growth arrest.[18-20] IFN-γ, which can also have anti-viral and cytostatic actions, leads to the activation primarily, and often exclusively, of Stat1. Although Stat1 activation can be associated with cell cycle arrest and the induction of inhibitors of cyclin-dependent kinases, many cytokines which induce the proliferation of hematopoietic cells induce the tyrosine phosphorylation of Stat1 in conjunction with Stat3 and/or Stat5. It is unclear whether Stat1 has different effects when activated along with these other Stat family members. It is known, for example, that Stat1 can form heterodimers with Stat3, and it is possible that through this heterodimerization distinct programs of gene activation can be specifically modulated. Alternatively, the genes activated by Stat1 homodimers may be different in association with the activation of Stat3 or Stat5. Finally, it is conceivable that Stat1 activation acts as a "brake" or homeostatic mechanism to the proliferation that would otherwise be mediated by activated Stat3 or Stat5. Nonetheless, if mutations occurring in leukemic cells lead to a recapitulation of intracellular signals activated during physiologic proliferation, one would expect to detect activated Stat3 and/or Stat5, perhaps in conjunction with Stat1.

Acute Leukemias

Nowhere perhaps is the need for improved therapy more clear than in the acute leukemias, particularly acute myeloid (or myelogenous) leukemia (AML). Among the leukemias, AML has the greatest incidence, with nearly 10,000 new cases yearly in the United States.[21] The vast majority of patients diagnosed with this disease die from it or the complications of its treatment. Finally, the basic therapeutic approach to the treatment of

AML, the combination of an anthracycline and cytosine arabinoside, has not changed in decades. Thus, it is hoped that an increased understanding of the molecular abnormalities underlying this disease might reveal insights into new therapeutic strategies which could be undertaken.

In recent years, several studies have examined the activation of Stats in primary cells isolated from the peripheral blood or bone marrow of patients with acute leukemia. Given the limited material available, it is necessary to use extremely sensitive techniques. Thus the preferred method has been electrophoretic mobility shift assays (EMSAs), using radiolabeled double stranded DNA sequences to which Stat dimers can bind. Two reports in 1996 used this technique to examine Stat activation in primary cells from patients with AML and ALL. Weber-Nordt et al[22] examined 14 patients with AML, and found that in 10 of them Stat3 was activated, in 10 Stat1 was activated, and in one patient Stat5 was activated. These authors reported a higher incidence of Stat5 activation in patients with ALL. Among cells which had previously been frozen they reported that 8 of 15 with B-cell ALL and 3 of 5 with T-cell ALL displayed activated Stat5. Among four patients with ALL whose cells had not been frozen, all displayed Stat5 activation. No data were provided as to Stat3 activation in ALL cells. One of three ALL patients had Stat1 activation. Of 8 patients with acute leukemia studied by Gouilleux-Gruart et al,[23] all had some basal Stat activation detected on EMSA. Of the three patients with ALL, all displayed activation of Stat5, and one also contained activated Stat1. Stat3 activation was found in all 5 patients with AML. Two displayed activation of Stat5 and one showed Stat1 activation in addition to the activation of Stat3. These authors specifically looked for activation of Stat2 and Stat4 and failed to find it in any of their patients. It is also important to note that in neither of these studies was basal activation of Stats found in hematopoietic cells from normal donors, highlighting the abnormality of their detection in leukemic cells. Furthermore, many in vitro models of AML are also characterized by constitutive Stat activation. For example, in two human cell lines displaying features of the megakaryocytic variant of AML, Stat5 is found to be activated.[28]

The largest published series of Stat activation in AML was reported in 1998 by Xia et al.[24] Among 36 patients with newly diagnosed AML, these authors reported that Stat3 was constitutively activated in 10 patients, and Stat5 was activated in eight. An increased level of complexity was revealed in the finding that the leukemic cells in patients with AML often express a truncated form of Stat3, the so-called Stat3β, which lacks 55 amino acids at the carboxy terminus of the protein. Similar β forms of Stat1 and Stat5 have been reported, each of which appears to function in a dominant inhibitory capacity. While Stat1β is known to be encoded by an alternatively spliced mRNA, the Stat3β found in AML cells appears to be generated by proteolytic cleavage of the full length molecule.[25] Presumably these shortened Stats can form dimers with the full length proteins, but by virtue of the absence of their transcriptional activation domain, they render the complex nonfunctional. Thus, it is possible that the expression of these truncated Stats might interfere with differentiation-promoting functions that are mediated by Stats, thereby contributing to leukemogenesis. This is an interesting area whose significance remains to be elucidated. In addition, other modified forms of Stats have been reported to be activated in AML. For example, a variant of Stat1, termed LIL-Stat, has been described, which shares homology with Stat1 at the amino terminus, but not the carboxy terminus.[26] LIL-Stat is also characterized by a unique DNA binding sequence, preferring TTN_5AG to the canonical TTN_5AA recognized by Stat1. LIL-Stat was reported to be activated in 7 of 9 patients with AML.[27]

Although many papers have reported the common finding of Stat activation in primary leukemic cells, there is considerable variability among them. This may reflect a number of difficulties which arise in studying primary human leukemic cells. First, the relatively small number of patients included in any of the studies may increase the chance of skewed findings. Second, a key event in leukemogenesis may be the activation of at least one family member which can support proliferation. Since there are data in tissue culture models that activated Stat3[29] or activated Stat5 can lead to cellular proliferation,[30] it may be important solely that at least one of these proteins is activated. It is quite likely that the pathways to Stat activation in acute leukemias are quite diverse, and thus it may not be critical as to which protein becomes activated to the final event of cellular transformation. A third consideration reflects the clinical variability among these diseases. For example, AML may arise either as a de novo disease, or it may be preceded by a state of bone marrow dysfunction (which is sometimes asymptomatic and undetected) known as myelodysplasia. Patients with myelodysplasia may evolve into AML. This so-called secondary form of AML is often associated with exposure to substances toxic to the bone marrow, has a higher frequency of deleterious chromosomal abnormalities, and is more difficult to treat than primary de novo AML. Thus, one might expect a difference in the pathogenesis of AML depending on whether it arises de novo or evolves from myelodysplasia. Furthermore, the entity of AML is divided into eight principal subtypes reflecting the degree and nature of differentiation of the myeloid blast cells. Again, these subtle differences may reflect different pathogenic events, which might be associated with the activation of distinct signaling pathways.

Although cytotoxic agents have been the mainstay of treatment for acute leukemias since the 1940s, there has been a recent interest in the induction of differentiation as a therapeutic strategy. Modulation of Stats may be an important mechanism of action for agents which modulate leukemic cell differentiation. In a murine model of erythroleukemia, a rare variant of AML, planar polar compounds such as hexamethylenebisacetamide can induce cellular differentiation.[31] During this induced differentiation, Stat5, which is constitutively tyrosine phosphorylated in these cells, becomes dephosphorylated.[32] This would support the model that Stat5 activation is necessary for the maintenance of the leukemic state. However, there may be an added level of complexity to Stat modulation by inducers of differentiation. In another subtype of AML, leukemic cells accumulate in a partially differentiated state known as a promyelocyte. Agents such as retinoic acid can induce the malignant cells to overcome their block in differentiation, and progress to terminally differentiated granulocytes which have an abbreviated life span and then undergo apoptosis.[33] It is known that retinoic acid induced differentiation is associated with alterations in cellular protein tyrosine phosphorylation[34-36] which could affect Stat signal transduction. In fact, retinoic acid appears to cause upregulation of Stat1 signaling,[37-40] and may exert a beneficial effect through Stat1-mediated differentiation and cell cycle arrest. Thus, Stats may play a complex role in determining programs of proliferation, differentiation, and survival in leukemic cells.

Chronic Myeloid Leukemia

Although chronic myeloid (or myelogenous) leukemia (CML) is a relatively rare disease, with an incidence less than half that of AML,[21] it is the form of leukemia which has likely been studied most intensively. The reason for this is the unique molecular abnormality which underlies nearly every case of this disease. Over 40 years ago it was first noted

that an unusual chromosome was found in the hematopoietic cells of patients with this disease,[41] which was found to represent a translocation between chromosomes 9 and 22.[42] This chromosomal translocation led to a fusion of two genes, *c-abl* from chromosome 9 and *BCR* (for breakpoint cluster region) from chromosome 22.[43] The fusion of these two genes led to the expression of a chimeric protein, BCR-Abl, which is a potent tyrosine kinase.[44] Whereas most hematopoietic cells require the addition of cytokines into the medium for in vitro growth, cells in which BCR-Abl has been introduced often grow in the absence of growth factors.[45,46] Furthermore, in animal models, expression of BCR-Abl leads to a leukemic-like syndromes.[47]

Thus CML and the in vitro systems in which BCR-Abl can be analyzed, provided the perfect model to test the hypothesis that malignant transformation of hematopoietic cells involves a recapitulation of the signaling events induced by physiological stimuli for growth. Many myeloid and myeloid-like cell lines require IL-3 for growth in culture. IL-3 is known to stimulate the tyrosine phosphorylation and activation of Stat5. Since introduction of BCR-Abl into these cells obviates the need for the addition of IL-3, it was conjectured that BCR-Abl would lead to the activation of Stat5. In 1996, several groups published this finding which strengthened the hypothesis that Stat activation was important to leukemogenesis.[48-50] Furthermore, the finding that primary cells from patients with CML,[51] and cell lines derived from patients with CML[48,49,52] contained activated Stats, made it clear that this was not an artefact of these tissue culture systems, but likely reflected the pathogenesis of this disease as it occurs in humans.

Although the association of inappropriate Stat activation in leukemia is suggestive of a role for these transcription factors in the pathogenesis of these diseases, more direct experimentation was necessary to prove causality. Models employing BCR-Abl transformed cells proved perfect for this purpose. Employing a cell line into which BCR-Abl and Stat5 constructs could be transfected, Nieborowska-Skorska et al showed that functional Stat5 was necessary for the ability of BCR-Abl to lead to factor independence growth and survival.[53] This was also shown by Sillaber et al[54] who expressed a dominant-interfering form of Stat5 under the control of a promoter regulated by the tetracycline analog doxycycline in BCR-Abl transformed cells. Induction of the dominant interfering form of Stat5 inhibited Stat5-dependent gene transcription in these cells, and led to a decrease in cellular viability and growth. Furthermore, expression of the mutant form of Stat5 made these cells more susceptible to being killed by cytotoxic drugs such as cytarabine and hydroxyurea. This finding may be of significance in considering Stat inhibition as a mode of therapy for treating leukemias, as discussed below. When cells express BCR-Abl, this highly active tyrosine kinase phosphorylates numerous cellular substrates and leads to a myriad of changes in cellular physiology. Thus, it is unlikely that Stat5 activation is sufficient for the transformation of hematopoietic cells induced by BCR-Abl. However, the data generated with the Stat5 mutants clearly demonstrates that Stat5 activation is necessary for transformation of these cells, and that Stat5 might be an appropriate target for therapeutic intervention.

The potential importance of targeting Stat5 in these leukemic cells has been enhanced by recent data. Using a cell line in which BCR-Abl was under the control of an inducible promoter, it has been shown that subsequent genetic events can occur in a cell rendering it transformed, but no longer requiring expression of BCR-Abl.[55] In these subclones, Stat5 remains constitutively activated suggesting that this transcription factor is essential for the maintenance of the malignant state of these cells. This finding in a tissue culture model is supported by clinical data. The BCR-Abl tyrosine kinase inhibitor STI571 is extremely

effective in early stage CML, but less so in more advanced stages of this disease. It is likely that after continued evolution the leukemic cells develop mechanisms independent of BCR-Abl to maintain a malignant state. Hence targeting a transcription factor downstream of BCR-Abl, like Stat5, may be a more effective strategy (Fig. 1).

Another interesting facet of BCR-Abl-induced leukemogenesis has been revealed by the finding that there are two major isoforms of this protein, depending on the exact location of the fusion between the *BCR* and *c-abl* genes. A 210-kDa isoform of BCR-Abl, p210, is found most commonly in CML. By contrast, a smaller form of this chimeric protein of approximately 185 to 190 kDa, p190, is found most commonly in ALL. It has been suggested that although both of these chimeric proteins are highly active tyrosine kinases, some difference in their activity might underlie the biological differences in the diseases in which they are expressed. In fact, evidence has arisen that both qualitative and quantitative differences in Stat activation mediated by these isoforms may be biologically important. Using Ba/F3 cells transfected with constructs encoding each of these proteins, it was shown that despite equal expression of each kinase, the magnitude of tyrosine phosphorylation of Stat5 (and Stat1) occurring in p190-transformed cells was considerably greater than in p210-transformed cells.[50] Thus, the increased proliferation seen in ALL cells could reflect the enhanced activation of Stat5 in cells expressing the p190 form of BCR-Abl. Another reason for the difference in the malignancies associated with these proteins might reflect a difference in the subset of Stats activated by each. It has been reported that in addition to the activation of Stat5, p190 transformation was associated with the tyrosine phosphorylation of Stat6.[48] It is unclear whether this activation of Stat6 has an impact on the biology of these cells. However, these findings indicate that despite the similarity between the p190 and p210 forms of BCR-Abl, they may differ in the activation of downstream targets, and these differences might lead to an alteration in the cellular phenotype they induce.

Chronic Lymphocytic Leukemia

Rapidly growing leukemic cells have been extremely informative in dissecting the molecular pathogenesis of hematologic malignancies. However, the leukemia with the highest prevalence in western societies, chronic lymphocytic leukemia (CLL), has been more difficult to understand. CLL is characterized by the slow accumulation of well differentiated B lymphocytes. In the peripheral blood, the large population of monoclonal lymphocytes has an extremely low growth fraction. Thus, studies of these cells in vitro is quite difficult, and no cell culture models which accurately recapitulate human CLL exist. Based on the indolent nature of most CLL cases in humans, and the quiescent nature of the circulating leukemia cells, it was perhaps not surprising that these cells lacked constitutively activated Stats.[56] However, cytokines clearly play an important role in the survival, proliferation, and differentiation of normal lymphocytes, and nearly all such cytokines, such as IL-2, IL-6, and IFN-γ signal through Stats, particularly Stat1 and Stat3. Given the importance of these Stats in lymphocyte function, and the subtle biological abnormality in CLL, the hypothesis was considered that a different abnormality in Stat function might occur in this disease. Stats require phosphorylation on their unique tyrosine residues for dimerization, nuclear translocation, and DNA binding to occur. However, Stat1 and Stat3 also contain unique serine residues (serine 727 in each) which can become phosphorylated as well. Although there is controversy as to the role this phosphorylation plays in Stat function, a great deal of evidence indicates that phosphorylation on this carboxy terminal serine residue enhances the transcriptional activation mediated by the Stat.[57-59] This would

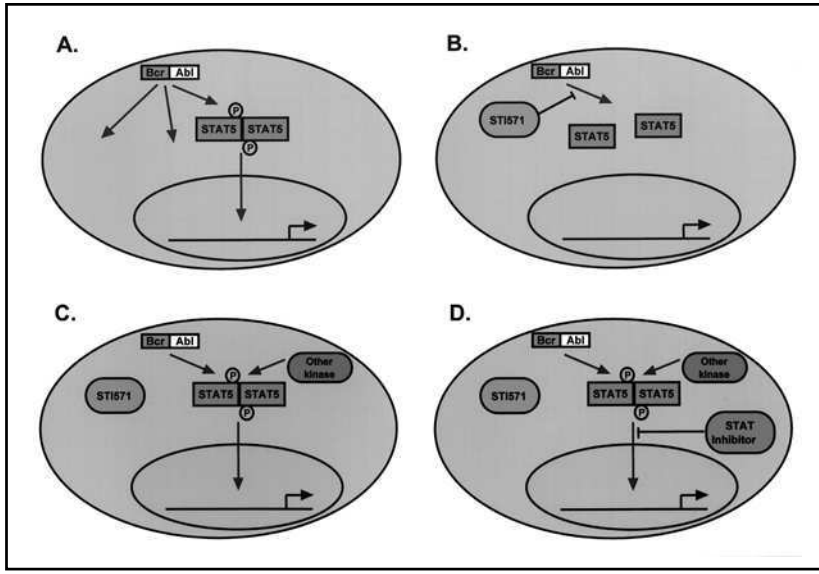

Figure 1. BCR-Abl-mediated leukemic transformation requires Stat5. A. BCR-Abl is a highly active tyrosine kinase which phosphorylates a number of cellular substrates, and activates several signaling pathways, including Stat5. B. Inhibition of BCR-Abl kinase activity by the drug STI571 inhibits all of these pathways, and inhibits cellular proliferation and survival. C. In advanced CML, and in cell culture models, resistance to STI571 can arise secondary to changes in BCR-Abl or the activation of other kinases. However, Stat5 activation appears to be necessary regardless of the mechanism. D. Hence, inhibitors directed at Stat5 may be very important in anti-leukemic therapy.

place Stats at a convergence point of tyrosine kinase and serine kinase pathways, and would allow the gene activation mediated by a Stat to be modulated more closely than a simple "on-off" switch. If a Stat is inappropriately phosphorylated on this serine residue, then although this will not lead to gene activation by itself, it would amplify the response which would otherwise occur in a cell after cytokine stimulation. Given this, the possibility was considered that CLL is characterized by inappropriate Stat serine phosphorylation. Whereas Stat1 and Stat3 occurring in lymphocytes in the peripheral blood of normal donors never display serine phosphorylation under basal conditions, in patients with CLL serine-727 of Stat1 and Stat3 were found to be universally phosphorylated.[56] These findings suggest that inappropriate phosphorylation of Stats on either serine or tyrosine residues may occur in human leukemias.

The consequence of Stat1 and Stat3 serine phosphorylation in CLL cells is not known. However, there is evidence that Stats may be an important target for therapeutic intervention in this disease. The most effective drug against CLL is a purine analogue, fludarabine.[60] As with other nucleoside-based chemotherapeutic drugs, the mechanism of action of fludarabine was suggested to be based on incorporation into nucleic acids, particularly DNA. However, fludarabine is particularly effective against low grade lymphoid malignancies with minimal growth fractions.[65] It seemed unlikely that this drug could be acting through DNA incorporation when less than 1% of CLL cells are in the S phase of the cell cycle at any time. Given the potential importance of Stats in the pathogenesis of CLL, it was considered that fludarabine might be acting by inhibiting these transcription factors. This possibility seemed more plausible in considering that patients treated with fludarabine

show a defect in cellular immunity.[62] This is somewhat akin to what is seen in mice which lack Stat1 through gene targeting.[63,64] In fact, both normal lymphocytes and CLL cells treated with fludarabine show a loss of Stat1 protein and mRNA.[65] A similar loss of Stat1 occurs in the CLL cells of patients treated with fludarabine. These studies provided the first evidence that not only may Stats be playing an important role in the pathogenesis of leukemias, but that Stats might also be an important target for therapeutic strategies.

Adult T-cell Leukemia/Lymphoma

In addition to the major human leukemias discussed in the preceding sections, even uncommon forms of leukemia are characterized by the activation of Stats. Adult T-cell leukemia/lymphoma (ATL), although rare in the United States, is more prevalent in the Caribbean region and in Japan. ATL, which is caused by infection with human T-cell lymphotropic virus I (HTLV-I), can be manifested by an indolent leukemia or lymphoma, or it may present with an aggressive acute leukemia. HTLV-I can infect CD4+ cells in vitro, and lead to their polyclonal expansion, after which dominant clones generally emerge. It is believed that similar events occur in vivo, although with a long latency on the order of decades. Initially, the malignant T cells still require IL-2 for growth. Eventually, however, they become IL-2-independent. Given that IL-2 signals through the activation of Stat transcription factors,[8] it was considered that Stat activation might be a prominent feature of ATL. In fact, HTLV-I transformed T cell lines display constitutive activation of Stat3 and Stat5,[66] Stats which are activated in response to IL-2. By contrast, T cell lines dependent on IL-2 did not show such Stat activation. Stat activation following HTLV-I infection is not only a laboratory finding, but can be found in patients with ATL. Peripheral blood lymphocytes, the majority of which were leukemic, were examined from patients with ATL. Cells from two-thirds of the patients demonstrated activation of Stat3, Stat5, or both.[67] Altered Stat signal transduction in ATL may not be restricted to Stat3 and Stat5. The Stat1 variant LIL-Stat, found to be activated in some patients with AML, was reported to be activated in all of seven ATL patients examined.[68] One of the key viral proteins necessary for HTLV-I infection is tax, which functions as a transcriptional activator. Among the many genes whose expression is induced by tax are a number of cytokines, all of which activate Stats. Thus, one of the mechanisms by which HTLV-I can induce growth of T cells independent of exogenously added cytokines is through induction of an autocrine pathway which leads to Stat activation. Finally, in contrast to CML where it appears that the BCR-Abl tyrosine kinase activates Stats independent of Jak family kinases, activation of Jaks is frequently found in ATL.[66,69] Thus, the mechanism of activation of Stats in diverse leukemias likely varies from the generation of novel mutated or chimeric kinases to the activation of endogenous kinases.

Chronic Myelomonocytic Leukemia

Although it is an uncommon hematologic disorder, chronic myelomonocytic leukemia (CMML) has been instructive in understanding the mechanisms of Stat activation in leukemias. A subgroup of patients with CMML have a characteristic chromosomal translocation involving chromosomes 5 and 12.[70] This results in the fusion of a portion of the β chain of the platelet-derived growth factor receptor (PDGFR) with the Ets family transcription factor, Tel. Tel contains a dimerization domain critical for the transforming ability of these chimeric kinases. The Tel portion of the protein allows dimers to form, thus bringing the kinase domains into juxtaposition. This leads to the activation of the kinase just as the interaction of PDGF with its receptor on the cell surface leads to receptor

dimerization and kinase activation. Transformation of Ba/F3 hematopoietic cells with Tel-PDGFR leads to activation of Stat1 and Stat5.[71] Stat activation can be blocked by pharmacologic inhibitors of the PDGFR kinase but not by AG490, a Jak inhibitor. These results indicated that Stat activation in this model is independent of Jaks. Further evidence that the Tel-PDGFR fusion could directly phosphorylate Stats was provided by experiments in COS cells. When these cells were transfected with Stat5 and the Tel-PDGFR fusion, Stat5 became tyrosine phosphorylated. Other chromosomal translocations involving Tel and tyrosine kinases have been described. For example, translocation of chromosomes 9 and 12, which is seen in B and T cell ALL and atypical CML, generates a fusion of Tel and Jak2.[72-74] This chimeric kinase also leads to leukemic transformation involving Stat activation. Thus, there are multiple mechanisms by which genetic modulation of leukemic cells can lead to inappropriate activation of Stats.

Targeting Stats in Anti-leukemia Therapy

General Considerations

The development of new therapies for leukemia has not kept paced with the advances made in our molecular understanding of these diseases. However, it is understood that there is a lag between the development of a technology and its application to medicine. A clear example of this is the use of monoclonal antibodies. Although the ability to generate hybridomas is over 20 years old, the clinical use of monoclonal antibody therapy is only now beginning to accelerate. A key issue that scientists now face is how to rapidly translate our understanding of signaling abnormalities in leukemias into a new generation of therapies. Before Stat inhibitors could be developed as investigational treatments for human leukemias, two questions need to be considered.

Will Stat Inhibitors Be Effective at Inhibiting Tumor Cell Growth?

Although Stats are phosphorylated inappropriately in every form of leukemia, a key question to be considered is whether Stat activation is necessary for the malignant nature of these cells. Experiments in several models of leukemia suggest that this is so. As was noted above, the introduction of a dominant inhibitory form of Stat5 into cells transformed with BCR-Abl led to a reduction in the growth of the cells, an increase in apoptosis, and an increase in susceptibility to killing by chemotherapeutic agents.[54,75] These studies are supported by other in vitro models of leukemogenesis in which inhibition of Stat5 prevents the development of leukemic clones.[76] Similar results were found in studies on cells from patients with mycosis fungoides or multiple myeloma, both of which are characterized by Stat3 activation.[77-80] While these are not, strictly speaking, leukemias, they represent transformed states of T and B lymphocytes. Inhibition of Stat3 activation in either of these cell types, by any of a variety of approaches, leads to enhancement of apoptosis.[81,82] Analogous findings were seen in a nonhematopoietic tumor, melanoma, in which inhibition of Stat3 function in an animal model was associated with notable tumor regression.[83] Thus, abundant evidence in tumor cell models suggests that Stat inhibition can lead to a loss of viability of malignant hematopoietic cells.

The critical contribution of Stats to leukemic transformation was also demonstrated in an elegant animal model. As was noted earlier, translocation of chromosomes 9 and 12 leads to the production of a Tel-Jak2 fusion protein in several forms of leukemia. The introduction of Tel-Jak2 into lymphoid or bone marrow cells leads to the generation of an

aggressive myeloproliferative and lymphoproliferative disorder.[84] However, if bone marrow from Stat5 deficient mice is used, in which both the Stat5a and Stat5b genes have been inactivated, this syndrome does not develop.[85] It should be noted that not all work is consistent with this finding, however. Analogous studies using mutants of c-abl did not show protection when Stat5-null animals were used.[86] Nevertheless, a distinction can be made between the effects of inhibiting Stats in an animal in which leukemia arises, from the ability of leukemia to form in an animal lacking Stats. A preponderance of evidence would suggest that Stat inhibition will lead to inhibition of tumor cell survival and/or proliferation.

Will Stat Inhibitors Be Toxic to Normal Tissue?

The observation that some abnormality in Stat phosphorylation has been reported in essentially every hematologic cancer raises the possibility that Stat inhibition might be an attractive approach to therapy. However, since Stats are activated by a wide range of physiologic signals, an equally important question to be considered is whether a Stat inhibitor would cause intolerable toxicity. Despite the presence of tumor cell resistance, most of the chemotherapeutic agents now in clinical use are fairly effective at killing tumor cells. The problem in therapy, however, is that normal cells are often equally sensitive to killing by these drugs, so that no therapeutic advantage can be achieved. Despite the sensitivity of malignant cells to inhibition of Stat function, several lines of evidence suggest that normal cells can be quite tolerant of Stat inhibition.

One source of information has been genetic experiments in which specific Stats have been deleted by gene targeting. The three Stats activated most commonly in leukemias, and all forms of human cancer, are Stat1, Stat3, and Stat5.[87] The loss of Stat1 is associated with decreased cellular immunity, but the animals are otherwise viable.[63,64] Similarly, animals lacking both forms of Stat5 show subtle defects in blood cell production, but they too are viable.[88] Mice in which Stat3 has been inactivated by insertional mutagenesis die during embryogenesis.[89] Stat3 is involved in cardiac development, and thus it is unclear whether inhibition of Stat3 in a fully developed animal will also lead to damage to normal organs. However, Stat3 can be inhibited in nontransformed fibroblasts with no ill effects on their proliferation or survival,[90] raising the possibility that Stat3 can be inactivated in normal adult tissue without ill effects. This may reflect an inherent redundancy in signaling pathways found in normal cells which make them tolerant to inhibition of any one pathway. Malignant cells, by contrast, may have become dependent on a small number of signaling pathways for their growth and survival, making them more susceptible to therapeutic inhibition.

Strategies for Stat Inhibition

Given the potential importance of the inhibition of Stat signal transduction in the treatment of human leukemias, consideration can be given to how this can be achieved. One can potentially design inhibitors targetting each of the mechanisms by which Stats can be activated inappropriately—that is, by nonphysiologic pathways (Fig. 2). However, it soon becomes clear that different strategies may have significantly different toxicities.

Receptor Blockade

In certain diseases, such as HTLV-I-induced ATL or multiple myeloma, paracrine or autocrine loops might be important for the establishment of Stat activation. Thus, one can use antibodies to the cytokines or their receptors, or other small molecular antagonists.[91]

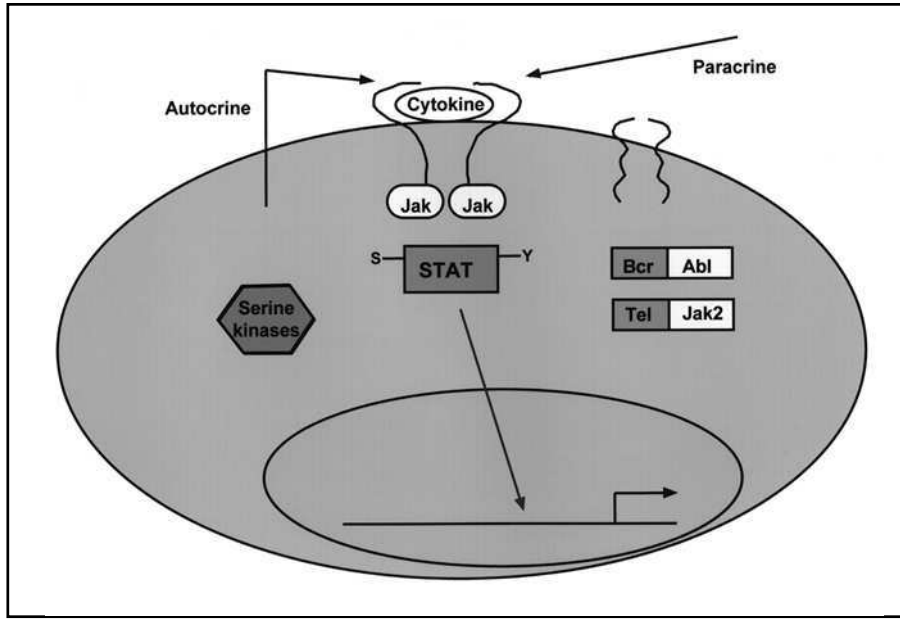

Figure 2. Multiple mechanisms can lead to Stat activation in leukemia. Although Stat activation is a common finding in essentially every form of human leukemia, there are many mechanisms by which this occurs. These include activation of endogenous cytokine receptors through autocrine or paracrine activation, mutations in receptors (such as Flt3), and the generation of mutated, activated tyrosine kinases. These mutated tyrosine kinases which can phosphorylate Stats in leukemia may phosphorylate Stats under physiologic conditions (such as Jak2) or may normally not include Stats among their substrates (such as Abl). In CLL, inappropriate phosphorylation of Stats on serine residues may contribute to the pathogenesis of this disease.

For example, it has been suggested that constitutive Stat3 activation seen in some patients with AML may reflect an IL-6 autocrine loop.[92] Furthermore, addition of an antibody to IL-6 in tissue culture leads to a loss of Stat3 activation. However, such approaches will lead to an inhibition of all of the effects of that cytokine on all cell types, a level of disruption that might be more nonspecific and disruptive than optimal. Nonetheless, IL-6 is not essential for survival in mice,[93] and anti-IL-6 antibody therapy has been attempted in humans.[94] Thus, this approach is at least technically feasible.

Tyrosine Kinase Inhibition

The next level one can consider is to inhibit the kinase responsible for the activated Stats (Table 2). The most dramatic example of this is the use of the compound STI571 in the treatment of CML. This drug is an inhibitor of the BCR-Abl tyrosine kinase (as well as other kinases such as c-abl, c-kit, and the PDGFR). STI571 can rapidly inactivate BCR-Abl and reverse the phosphorylation of a variety of cellular targets including Stat5. STI571 has also induced dramatic improvement in patients with advanced refractory CML. Interestingly, despite the fact that this drug inhibits other kinases, it is remarkably nontoxic. Thus, theoretical concerns about the inhibition of secondary pathways may not be as great of a problem in practice. While this example is proof that a moderately specific kinase inhibitor directed at a chimeric oncoprotein kinase can lead to remarkable benefit, CML is somewhat unique in that essentially every patient with this disease possesses BCR-Abl.

Table 2. Stat tyrosine kinases in leukemia

Kinase	Mutated form in leukemia	Potentially active forms
Jak2	Tel-Jak2	wildtype
Abl	BCR-Abl	v-abl
PDGFR-B chain	Tel-PDGFR	
	HIP-1-PDGFR	
	CEV14-PDGFR	
Flt3	ITD-mutation	wildtype
Jak1		Tel-Jak1
Jak3		Tel-Jak3
Tyk2		Tel-Tyk2

A number of tyrosine kinases have been associated with Stat phosphorylation in leukemia. Activating mutations in leukemia may alter cellular localization (such as BCR-Abl), or may lead to spontaneous dimerization (such as the TEL, Huntington interacting protein-1 (HIP-1), and CEV14 fusions). Mutations in the internal tandem domain (ITD) of Flt3 appear to lead to constitutive kinase activity.

Few if any other examples exist, in which every case of a leukemia is associated with the same activated kinase. Furthermore, as noted previously, STI571 is less effective in more advanced forms of CML in which BCR-Abl-independent mechanisms of Stat activation may occur (Fig. 1). Thus, while inhibition of kinases is clearly feasible, it may be necessary to perform molecular characterizations of a given patient's leukemic cells to determine which kinases should be targeted.

Despite this problem, there is some precedent for the use of Jak kinase inhibitors in certain experimental models of leukemia. In ALL, Stat1 and Stat5 are tyrosine phosphorylated, and Jak2 activation has been described as well. A small molecule of the tyrphostin class, AG490, has been shown to be an inhibitor of Jak2, and perhaps other Jak family members. In a mouse model of ALL characterized by constitutive activation of Jak2, treatment with AG490 was effective at inhibiting leukemic cell growth, while having no significant effects on normal hematopoiesis.[95] Since fusions of all of the Jak family members to Tel can lead to cellular transformation in vitro, the use of Jak inhibitors might be of particular importance.[96] In addition, some patients with Hodgkin's disease, a B cell neoplasm, may have chromosomal amplifications of the gene for Jak2, although the significance of this amplification is uncertain at present.[97] Thus, tyrosine kinase inhibition remains an intriguing strategy for suppressing Stat activity in leukemic cells. As with receptor inhibitors, they may inhibit a variety of other intracellular events, but that may not be as much of a concern in practice as in theory.

Tel-PDGFR, a chimeric kinase which results from a translocation between chromosomes 5 and 12 in many patients with CMML, can phosphorylate Stat5 and Stat1.[71] Expression of this chimeric kinase in lymphoid cells in transgenic mice leads to an aggressive lymphoproliferative disorder.[101] Of note, administration of the PDGFR tyrosine kinase inhibitor CGP57148, which inhibits Stat activation, can suppress hematologic malignancies in these animals. This is of particular interest since the PDGFR can be fused to a number of dimerizing proteins in leukemias, and thus its inhibition may have widespread applicability.[102,103] Finally, the receptor tyrosine kinase Flt3 is mutated in many forms of leukemia. In both patient samples and in vitro models, there is evidence that the mutated receptors possess enhanced tyrosine kinase activity, and lead to cellular proliferation

and transformation in a Stat5-dependent manner.[98-100] Thus, the Flt3 tyrosine kinase may be another important target for kinase-based strategies to inhibit inappropriately activated Stats in leukemias.Thus, drugs which can target a number of tyrosine kinases can inhibit Stat activation and suppress leukemias in a variety of models.

Serine Kinase Inhibitors

Although the importance of tyrosine phosphorylation in the activation of Stats is indisputable, the role that phosphorylation of carboxy-terminal serine residues in Stat1 and Stat3 are unclear. Such phosphorylation is found in the leukemic cells of patients with CLL.[56] Serine phosphorylation modulates the function of Stat1 and Stat3,[57-59] though the effect of this modulation in CLL cells is not yet clear. If this event is important in the pathogenesis of CLL or other forms of leukemia, then pharmacologic inhibition might be a useful approach.[56] It is likely that a number of serine kinases can catalyze the phosphorylation of the conserved carboxy terminus serine in Stat1 and Stat3 (Table 3).[56,104-106] In CLL cells, the kinase inhibitor H7 is able to lead to the rapid dephosphorylation of both Stat1 and Stat3. Incubating the cells with okadaic acid, an inhibitor of protein phosphatase 2A, blocked the ability of H7 to induce the dephosphorylation of serine 727 of these Stats. These data suggest that there is an active phosphorylation-dephosphorylation cycle occurring in these cells, and thus inhibition of the appropriate kinase allows rapid dephosphorylation to occur.

Direct Targeting of Stats

If one wished to inhibit Stat function without perturbing other signaling events, then designing strategies affecting the Stats directly might be of greatest use. Several approaches could be considered. One technique which has been useful in tissue culture models is the use of antisense oligonucleotides to directly deplete the level of a given Stat from cells. Antisense molecules targeting Stat1 have been shown to inhibit mitogenesis in certain models.[17] Similarly antisense oligonucleotides directed to Stat3 can inhibit mitogenesis in lymphoid cells.[107] Experiments are underway regarding the use of antisense molecules in whole animals and in humans, although significant pharmacologic hurdles need to be overcome. Nevertheless, this is one potentially important strategy for interrupting Stat signaling in leukemic cells. Indeed, it is intriguing to consider that pharmacologic agents in current use might work by depleting specific Stats. As noted earlier, the nucleoside analogue fludarabine can induce the specific depletion of Stat1 from malignant and normal cells.[65] This may account for the activity of this drug in low grade lymphoid malignancies (such as CLL), as well as its immunosuppressive effects. It is unclear whether other nucleoside analogues or other chemotherapeutic drugs might work at least in part by inhibiting Stat function.

Another appealing way to target Stats would be to inhibit the function of their SH2 domain (Fig. 3). A small molecule which could bind specifically to this portion of a Stat molecule would have the capability of blocking two critical steps in the activation of a Stat. First, the recruitment of Stats appears to be through an interaction of the SH2 domain of a Stat and a phosphotyrosine residue on a particular receptor or kinase. This is certainly true for Stat phosphorylation mediated by cytokine receptor-Jak complexes.[108,109] Secondly, for Stats to translocate from the cytoplasm to the nucleus, they must from dimers. They do so physiologically through reciprocal SH2-phosphotyrosine interactions.[110] Thus, blockade of the SH2 domain of a Stat would also inhibit the ability of the molecule to

Table 3. Stat serine kinases

p38 Map kinase
Erks
Protein kinase C
cAMP-dependent protein kinase
Calcium, calmodulin-dependent kinases

A number of kinases have been found to be capable of mediating the phosphorylation of Stat1 and/or Stat3 on their conserved carboxy-terminal serine residues.

form dimers, and exclude the Stat from the nucleus. The challenge would be to design molecules which are specific for the SH2 domain of a given Stat without affecting the myriad of other cellular proteins which contain related motifs.

Even if a Stat becomes phosphorylated, forms a dimer, and translocates to the nucleus, one could consider a strategy based on blocking the DNA binding of the Stat dimer. Now that the crystal structure of Stat dimers are available,[111,112] it may be possible to design small molecules which can target these sites and prevent Stat-dependent gene activation from occurring. Finally, it should also be noted that although Stats function as transcription factors, the key target genes they activate to maintain neoplastic cellular function are unknown. As such, identification of the genes activated by Stats in malignant cells may reveal a new level of targets for therapy.

Combination Therapy

One of the major advances that was made in the curative chemotherapy of diseases such as childhood ALL, testicular cancer, and Hodgkin's disease was the combination of effective drugs. Although Stat signal transduction is an appealing target for the development of anti-leukemic therapy, Stat inhibitors may work best when combined with other forms of therapy. For example, in BCR-Abl-mediated cellular transformation the inhibition of Stat5 not only slows cell growth, but makes the cells more prone to undergo apoptosis when treated with a cytotoxic agent.[54] Thus, the combination of a Stat inhibitor and a cytotoxic drug may be a particularly effective regimen. In addition, it is possible that adding a Stat antagonist to a kinase inhibitor such as STI571 may show superior anti-leukemic effects. Thus, the successful introduction of Stat inhibitors into the anti-cancer armamentarium may require a significant amount of clinical research once the basic biology has been elucidated.

Conclusion

It is to be hoped that the next generation of anticancer agents will be rationally designed based on our increasing understanding of the differences between normal and neoplastic cells. The finding that Stat transcription factors are commonly activated in every form of human leukemia suggests that these proteins might be an attractive target. This, coupled with the observation that the inhibition of Stat function in normal cells and tissues is generally well tolerated, should spur the development of Stat inhibitors as a potentially important new family of anti-leukemic agents.

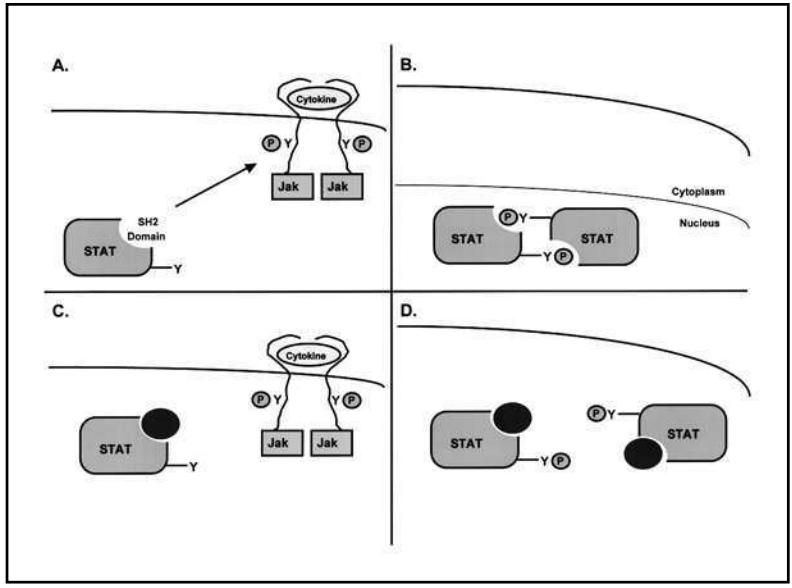

Figure 3. Stat SH2 domains are attractive targets for small molecule inhibitors. A. The SH2 domain of a Stat is necessary for its recruitment to an activated receptor-kinase complex, where it interacts with tyrosine phosphorylated receptor chains. The Stat SH2 domain is likely important for Stat activation by other kinases as well. B. Once Stats are tyrosine phosphorylated, they form dimers through reciprocal phosphotyrosine-SH2 interactions, thereby allowing translocation to the nucleus so that they can interact with their target DNA sequences. C. A small molecule which could bind specifically to a Stat SH2 domain would be able to block Stat signal transduction at two steps. It would block Stat recruitment to tyrosine kinases, thereby inhibiting phosphorylation. D. In addition, even if a Stat became tyrosine phosphorylated, an SH2-directed inhibitor would have the capability of blocking Stat dimerization and nuclear translocation.

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Chapter 6

The Role of the Jak-Stat Pathway in Hematopoiesis and Immune Responses in *Drosophila*

Erika A. Bach and Norbert Perrimon

Introduction

The Janus kinase (Jak)-signal transducer and activator of transcription (Stat) (Jak-Stat) pathway is highly conserved between flies and mammals not only in terms of the structure and function of its components but also in its mechanism of action. In mammals, this pathway plays a central role in blood cell development and in innate and acquired immunity. Recent evidence suggests that the Jak-Stat pathway is also involved in hematopoiesis and immunity in flies. In *Drosophila*, the major hematopoietic cell lineage is highly homologous to the vertebrate myeloid lineage, and both share a common set of factors that direct cell lineage decisions and proliferation. Moreover, both mammals and flies have recently been shown to share conserved signaling cassettes that respond to microbial pathogens during innate immune responses. Thus, mammals and flies are likely to share a common developmental program that directs hematopoiesis and induction of immune responses. In this chapter, we describe *Drosophila* hematopoiesis and immunity and discuss how the Jak-Stat pathway functions in these processes.

In vertebrates, stem cells in the bone marrow give rise to progenitor cells that differentiate into different hematopoietic lineages.[1] Under certain stimuli, the mature cells in these lineages can be induced to divide and differentiate further. Research on *Drosophila* hematopoiesis is in its infancy, however much progress has been made in the last few years in understanding the molecular and genetic events that control this process. In the fruit fly, hematopoiesis occurs in at least two temporally and developmentally distinct stages, embryos and larvae. Progenitor cells differentiate into the major cell types, the plasmatocyte and the crystal cell. The predominant cell type, the plasmatocyte, is homologous to the mammalian macrophage or neutrophil.[2] In contrast, the crystal cell does not have a known vertebrate counterpart.[3] Interestingly, the genes that regulate hematopoiesis in the fly share many features with the genes that regulate mammalian blood cell development. Therefore, it is likely that vertebrates and invertebrates will have a common developmental plan.[4] Because mammals lacking certain Jak and Stat genes exhibit profound hematopoietic defects, this pathway is fundamental to mammalian hematopoiesis.[5-11] If developmentally regulated events are evolutionarily conserved, then this pathway should be involved

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in fly hematopoiesis. In fact, in *Drosophila*, the Jak-Stat pathway is a key regulator of proliferation and differentiation of larval hematopoietic cells.[12-16] However, its role in embryonic hematopoiesis is not known.

In recent years, the immune system of the fly has also received much attention, largely because of the high degree of evolutionary conservation between its immune system and the innate immune system in mammals. Flies mount effective immune responses to bacterial and fungal pathogens by producing anti-microbial peptides and activating hematopoietic cells to phagocytose or encapsulate the invading pathogen.[17] The signaling pathways that control immunity have been the subjects of considerable research. Despite the fact that the Jak-Stat pathway is important in fly hematopoiesis, its role in innate immune responses is currently not known. However, the Toll/IκB/NFκB pathway, which plays a central role in mammalian immunity, is involved in the induction of the several antimicrobial peptides in *Drosophila* immunity.[17] Moreover, the Tl pathway is also important in the regulation of proliferation and differentiation of fly hematopoietic cells.[18,19] Whether the Jak-Stat and Toll/IκB/NFκB pathways are functionally integrated in the control of hematopoiesis and immunity in *Drosophila* has not been clearly established.

The Jak-Stat Pathway

The Jaks and Stats are the key components of an evolutionarily conserved signal transduction pathway. A unique feature of the Jak-Stat pathway is that it transduces extracellular signals directly from the membrane to the nucleus without the need for cascades of second messengers. In the mammalian system, four Jak and seven Stat genes have been identified, and more than 30 cytokines and growth factors have been shown to activate specific combinations of Jak or Stat proteins.[20] In addition to the critical role of this pathway in immune responses, it plays a fundamental role in development of glial cells in the mammalian nervous system, and in cell survival and apoptosis in the mammary epithelium.[21,22] Biochemical analysis in mammalian tissue culture systems has demonstrated that extracellular ligands bind to and activate their cognate cell surface receptors, the cytoplasmic domains of which are constitutively associated with inactive Jaks. Binding of the ligand to the receptor leads to multimerization of the receptor and autoactivation of the Jaks, which then phosphorylate tyrosine residues on the cytoplasmic domain of the receptor. A latent, cytosolic Stat then interacts with the receptor phosphotyrosine residue via its Src homology 2 (SH2) domain and becomes phosphorylated on a conserved C-terminal tyrosine residue by activated Jaks. The tyrosine phosphorylated Stat dissociates from the receptor and forms a homo- or heterodimer with another activated Stat via reciprocal SH2 domain interactions. The activated Stat dimer translocates from the membrane to the nucleus, where it binds to a specific DNA sequence (consensus TTCNNNGAA) and activates the transcription of target genes.[20] In addition to this general paradigm, the Jak-Stat pathway displays additional levels of complexity. For instance, tyrosine kinases other than Jak, such as Src and Abl, can directly phosphorylate Stat molecules and thus activate the pathway.[23] Moreover, in addition to the phosphorylation of the conserved tyrosine residue, mammalian Stats require for induction of a robust transcriptional response the phosphorylation of a conserved C-terminal serine residue.[24] Finally, activated Stats have been shown to act in synergy with several other transcription factors, including NFκB and c-jun, and to interact physically with other transcription factors, including other Stat dimers, the glucocorticoid receptor, and p300/CREB-binding protein.[25-27] Taken together, this suggests that the Jak-Stat pathway may exhibit cross-talk with other signaling pathways to generate biologically relevant responses. In addition, the four Jak and seven Stat proteins

in mammals lead to additional levels of complexity if different homo- or heterodimers affect distinct biological processes. Given the redundancy observed in vivo in mammalian systems, the ability to dissect the Jak-Stat pathway in a genetically tractable organism such as *Drosophila* is extremely valuable.

The *Drosophila* Jak-Stat Pathway

The Jak-Stat pathway in flies was identified by its role in embryonic segmentation.[28] In addition, it has important functions in other processes, including sex determination, spermatogenesis, oogenesis, hematopoiesis and eye development.[29,30] To date, three components of this pathway have been identified genetically: the ligand, *unpaired* (*upd*), the Jak, *hopscotch* (*Hop*) and the Stat, *Stat92E* (Fig. 1). The receptor that activates this pathway has not yet been identified.

The gene *upd* encodes a novel secreted protein that bears strong homology to a gene called Om1E in the closely related fruit fly species *Drosophila ananassae*.[31,32] However, no vertebrate homologs have been identified. The Upd protein has a signal sequence and several potential N-linked glycosylation sites.[31] Although it has a predicted M_r of 47 kDa, Upd displays an apparent M_r of 65 kDa in electrophoretic analysis, presumably due to post-translational modifications such as glycosylation. Tissue culture experiments have indicated that Upd associates tightly with the extracellular matrix, but it is found in the supernatant after the addition of heparin to the media. When added to the fly imaginal disc Clone 8 cell line, recombinant Upd induces phosphorylation and activation of Hop.[31] Additionally, in vivo evidence shows that Upd activates Jak-Stat signaling in the eye.[33] Taken together, these data indicate that Upd is a ligand which activates this pathway in flies.

The *Hop* gene encodes a 120 kDa cytosolic protein tyrosine kinase and shares with other members of the Jak family an overall structural pattern of seven conserved domains.[28,34] Common features of this family include a C-terminal pseudo-kinase domain followed by a *bona fide* tyrosine kinase domain and the absence of SH2 and Src homology 3 (SH3) domains.[28,34] Hop is most similar to human Jak2, with 27% identity overall and higher levels of homology in the kinase and kinase-like domains.[28] The *Drosophila* Stat homolog *Stat92E*, also known as *D-Stat* and *marelle*, encodes an 83 kDa protein that is most similar to human Stat5, with 37% identity overall. Stat92E contains an SH2 domain, a DNA binding domain and the single C-terminal tyrosine residue found in all Stat-like genes.[14,15] This residue is phosphorylated by in vitro activation of the pathway.[15] In contrast, the conserved C-terminal serine residue phosphorylated in mammalian systems is not conserved in Stat92E.[14,15] It is not known whether this results in a change of the functional capabilities of Stat92E relative to mammalian Stats or alters the context of its activation. A detailed analysis of the recently sequenced fly genome revealed that Stat92E is likely to be the only Stat in *Drosophila*.[30] This analysis also revealed no Jak-like kinases other than Hop.

At least three classes of cytosolic proteins that modulate Jak-Stat signal transduction in mammals have homologs in flies. These include two classes of negative regulatory proteins, Protein Inhibitor of Activated Stat (PIAS) and Suppressor Of Cytokine Signaling (SOCS), and one class of positively acting proteins, Signal Transducing Adapter Molecule (STAM). PIAS proteins have zinc-finger domains and bind to and inhibit the activity of specific Stats.[35] In *Drosophila*, a PIAS homolog named *zimp* has been identified by sequence homology. Mutations in *zimp* are recessive lethals, but no further functional data is available. It is therefore not yet clear whether this protein inhibits the activity of Stat92E.[36] SOCS proteins share a highly conserved 40-amino acid C-terminal SOCS domain preceded

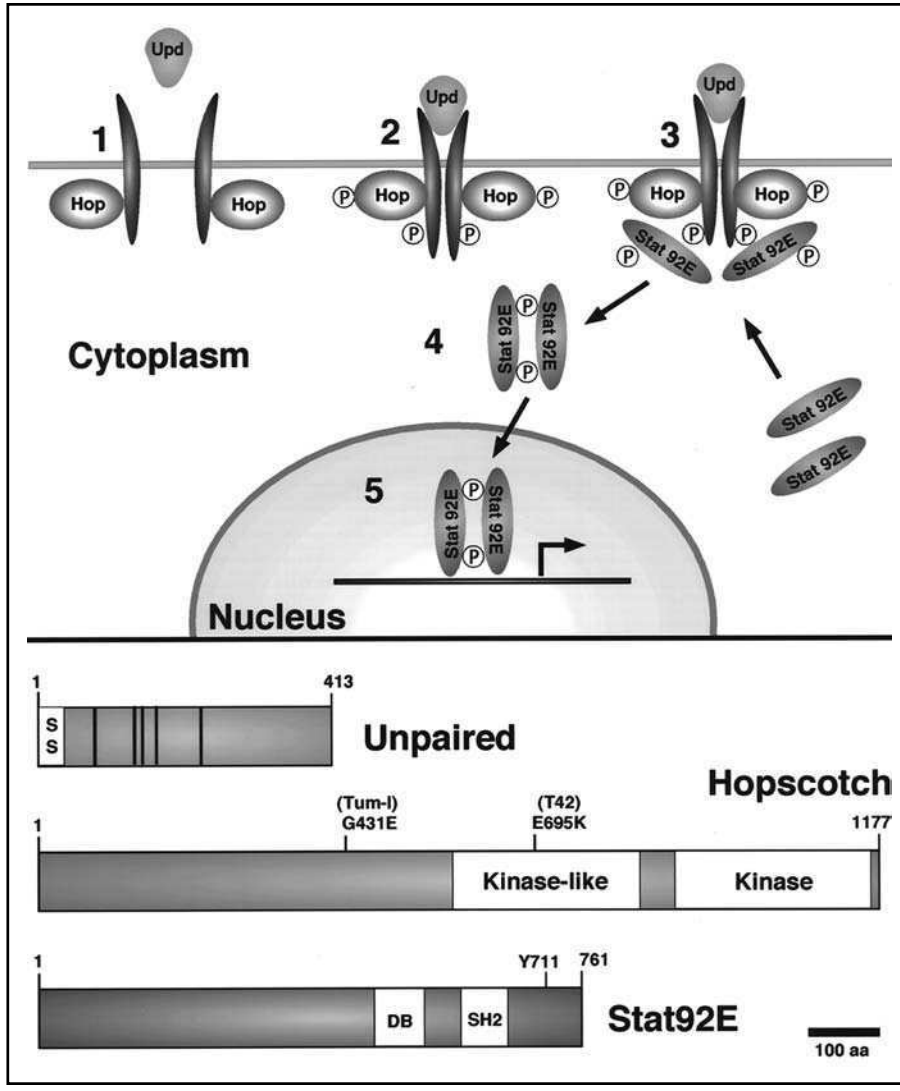

Figure 1. Model of the Drosophila Jak-STAT pathway. See text for details.

by an SH2 domain.[37] They can inhibit cytokine signaling by either binding to the Jak kinase domain or by competing with Stat for a phosphotyrosine binding site on the receptor. In addition, the expression of some SOCS genes is induced by activation of the receptor system they inhibit.[38] There is at least one fly homolog of mammalian SOCS proteins, and it retains sequence identity in both the SH2 domain and regions previously identified as being required for SOCS activity.[37] There are no mutant alleles available for this gene, and therefore its physiological function is not known. Identified as positive regulators of cytokine-dependent signal transduction, STAMs contain a SH3 domain and associate with Jak2 and Jak3.[39] The fly homolog of STAM, also called *stam*, was cloned by homology. Like the SOCS homolog, there are no mutations in the *Drosophila stam* gene.[40] Future

genetic and biochemical characterization of these *Drosophila* homologs may establish these genes as true orthologs of the mammalian proteins.

Dephosphorylation of tyrosine residues on activated Jak and Stat molecules is clearly a crucial regulatory step in turning off the signaling cascade. In mammals, both CD45, a transmembrane protein tyrosine phosphatase (PTP), and SH-PTP1, a hematopoietic cytosolic PTP, are known to dephosphorylate Jaks and negatively regulate cytokine signaling in hematopoietic cells in vivo.[41,42] Although this aspect of Jak-Stat signaling has not been investigated in flies, analysis of the genome indicates that in *Drosophila* there are several transmembrane and cytosolic PTPs that could fill this function.

Components of the *Drosophila* Jak-Stat pathway were originally identified by their roles during embryogenesis. *Hop* is contributed maternally and is expressed uniformly throughout the various stages of embryonic development.[28] *Stat92E* also has a strong maternal contribution and is expressed uniformly during early stages of embryogenesis. However, later its expression resolves into seven and then 14 segmental stripes.[14,15] In contrast, *upd* is not maternally contributed. It is expressed from the zygotic genome in a highly dynamic pattern during embryogenesis. Initially, *upd* is expressed broadly in the trunk; during syncyntial development in a stripe in the head; after cellularization in seven stripes; during gastrulation in 14 stripes; and later in the tracheal pits. The 5' and 3' untranslated regions of the *upd* gene contain at least seven consensus RNA-destabilizing sites, which presumably regulate its highly dynamic expression pattern.[31]

Despite these different patterns of expression the known components of the Jak-Stat pathway exhibit very similar, loss-of-function phenotypes in the embryo. Because of the maternal contributions of *Hop* and *Stat92E*, the roles of these genes in embryonic development can be only assessed in embryos that lack contribution of the specific gene. Such embryos are derived from females with homozygous mutant germlines. The "dominant female sterile technique" allows for the generation of females that have a homozygous mutant germline in an otherwise wild type fly.[43] Embryos that lack maternally contributed *Hop* or *Stat92E*, hereafter referred to as *Hop* and *Stat92E* mutant embryos, can be generated by crossing germline clone females to males that carry one copy of the mutation. *Hop* and *Stat92E* mutant embryos or embryos lacking the zygotic contribution of *upd* exhibit a characteristic defect in segmentation.[14,28,31] In wild type embryos a stereotypic pattern of hairs or denticles decorate the external cuticle, which is secreted by the embryo shortly before hatching (Fig. 2 A). These denticle belts are found in each thoracic and abdominal larval segment. In Jak-Stat mutant embryos the fifth abdominal denticle belt is missing, with variable deletion of the fourth and eighth belts, as well as occasional fusion of the sixth and seventh belts (Fig. 2 B, C, and D).[14,15,28,31]

Hematopoiesis in *Drosophila*

In the fly embryo, hematopoiesis begins when hematopoietic cells, termed hemocytes, arise from the embryonic mesoderm.[44] The precursor hemocytes, called the prohemocytes, first appear at stage 10, about two hours after gastrulation, and express the GATA transcription factor *serpent* (*srp*).[45,46] Vertebrate GATA-1, -2 and -3 are required for erythroid development, definitive hematopoiesis, and T cell development, respectively.[45,47,48] *srp* is essential for *Drosophila* embryonic hematopoiesis and for the formation of the fat body, an insect organ analogous to the mammalian liver.[49] At stage 11, one and a half hours later, there are about 700 prohemocytes as assessed by detection of peroxidasin, a hemocyte-specific extracellular matrix component.[44] Shortly thereafter the prohemocytes migrate throughout the embryo and differentiate into two major cell types: the plasmatocyte

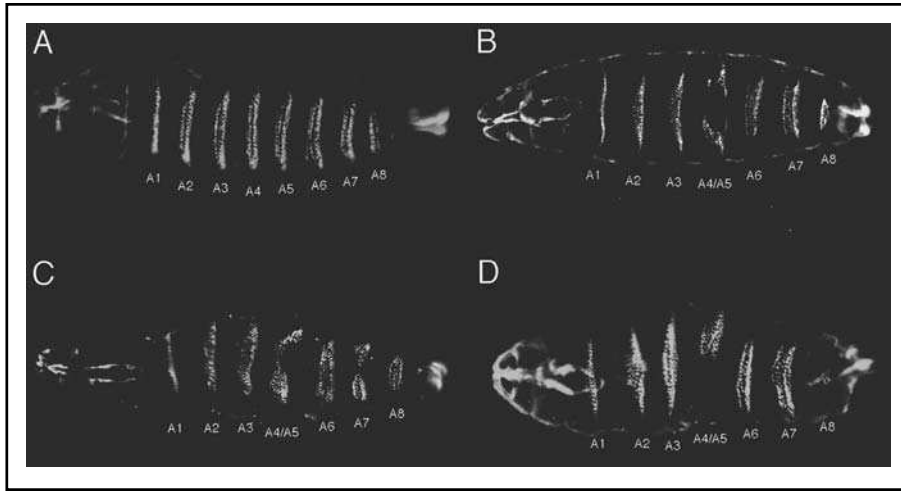

Figure 2. Jak-STAT pathway genes share a similar loss-of-function phenotype in the embryo. See text for details.

and the crystal cell (Fig. 3).[3] Constituting 90% of the embryonic hemocytes, plasmatocytes acquire specific phagocytic capabilities following the expression of Croquemort, the fly ortholog of CD36, a scavenger receptor. Then they begin to act as macrophages in tissue remodeling and phagocytose dead or dying cells.[50,51] Crystal cells contain cytoplasmic crystalline inclusions, however it is not known what functional role they play in the embryo. Recent work has shown that crystal cell differentiation depends upon the transcription factor Lozenge (Lz), a protein containing a Runt domain and with high homology to the mammalian AML1 protein.[52] In mice, *aml1* is required for definitive hematopoiesis, and in humans, the *aml1* locus is the most frequent target of chromosome rearrangement in acute myeloid leukemias.[1] Using genetic analysis, *srp* has been shown to be required for expression of *Lz* and of another transcription factor, *glial cells missing* (*Gcm*), which is necessary for the differentiation of plasmatocytes (Fig. 3).[52] As mentioned above, mice and humans deficient in certain Jak or Stat genes exhibit hematopoietic defects.[5-11] This suggests a role for the Jak-Stat pathway in the proliferation, differentiation or functional activity of embryonic hemocytes in *Drosophila*. However, additional studies are needed to assess this.

At the completion of embryonic development, the embryo hatches and becomes a freely-moving larva. Larval development takes place in three major phases called instars, termed first, second and third, all separated by a molt. At the conclusion of larval development, the third-instar larva forms a pupal case and undergoes metamorphosis into an adult fly. During larval and pupal development, a small number of cells, set aside at the end of embryogenesis and grouped in structures called imaginal discs, undergo extensive rounds of proliferation and give rise to presumably all adult structures. Most of the larval tissue, except the imaginal discs, die and are resorbed by macrophages during metamorphosis.

Like the embryonic hemocytes, the hematopoietic cells in larval, pupal and adult stages perform phagocytic functions. Moreover, they are also involved in the immune response. In larval stages, the primary organ of hematopoiesis is the lymph gland, which is derived from the embryonic mesoderm.[3] The lymph gland is a multi-lobed organ that is associated with the dorsal vessel. Little is known about the ontogeny and function of larval

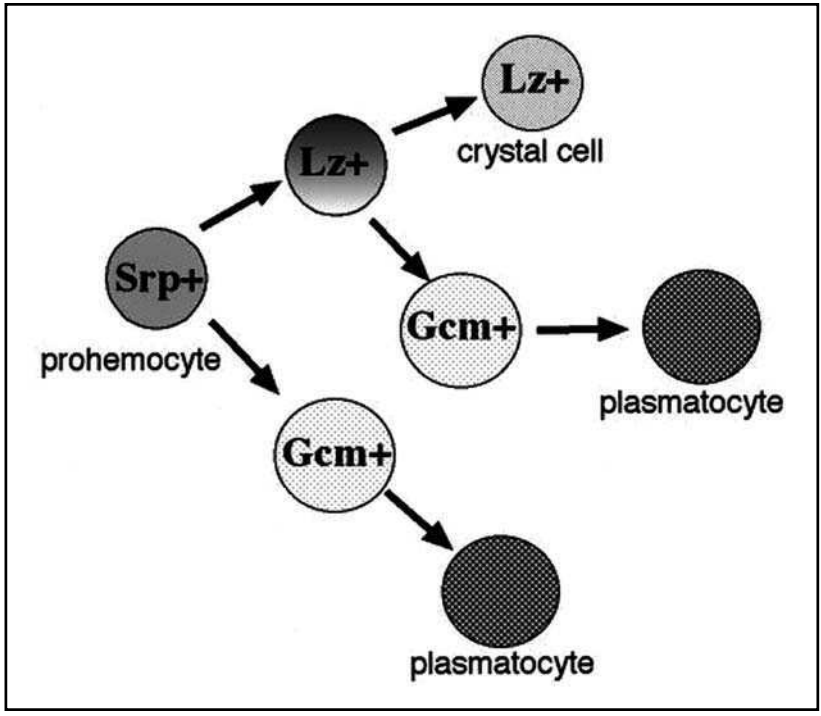

Figure 3. Tentative lineage of embryo hemocytes. Adapted from Lebestky et al.[52] See text for details.

hemocytes. However, a tentative cell lineage has been proposed based on analyses of phenotypic characteristics of hemocytes (Fig. 4).[53,54] The prohemocytes in the lymph gland are thought to self-renew and differentiate into specific types of hemocytes, which are released into the circulatory system, called the hemolymph, possibly via the dorsal vessel. The circulatory system in flies is open, and the internal organs are surrounded by hemolymph. Though rudimentary, the fly circulatory system is similar to the mammalian one as it carries materials throughout the insect. However, unlike its mammalian counterpart, the hemolymph is not involved in exchange of gases.[3] Like the embryonic lineage already discussed, plasmatocytes in the larval hemolymph are small, nonadhesive cells. They constitute 90% of larval hemocytes, and, in response to pupariation, injury or immune challenge, become activated and increase in number and in phagocytic and secretory capabilities.[54] A second type of plasmatocyte, called the podocyte, has also been observed in the larval hemolymph.[3] Larger than a plasmatocyte and exhibiting extensive pseudopodia, a podocyte may represent a transitory state as a plasmatocyte differentiates to a lamellocyte. Alternatively the podocyte may represent an "activated" plasmatocyte described above, analogous to the "activated" macrophage characterized in mammalian systems.[54,55] The largest cell in the hemolymph, lamellocytes are flat cells, the cytoplasm of which is not granular when viewed by light microscopy. Under normal conditions, lamellocytes constitute less than 6% of the larval hemolymph and may represent an even smaller percentage (1%).[54] The ontogeny of these cells is not clear. As mentioned above, it was thought that they differentiated from plasmatocytes.[53] However, currently they are believed to be derived from prohemocytes and to represent a lineage separate from

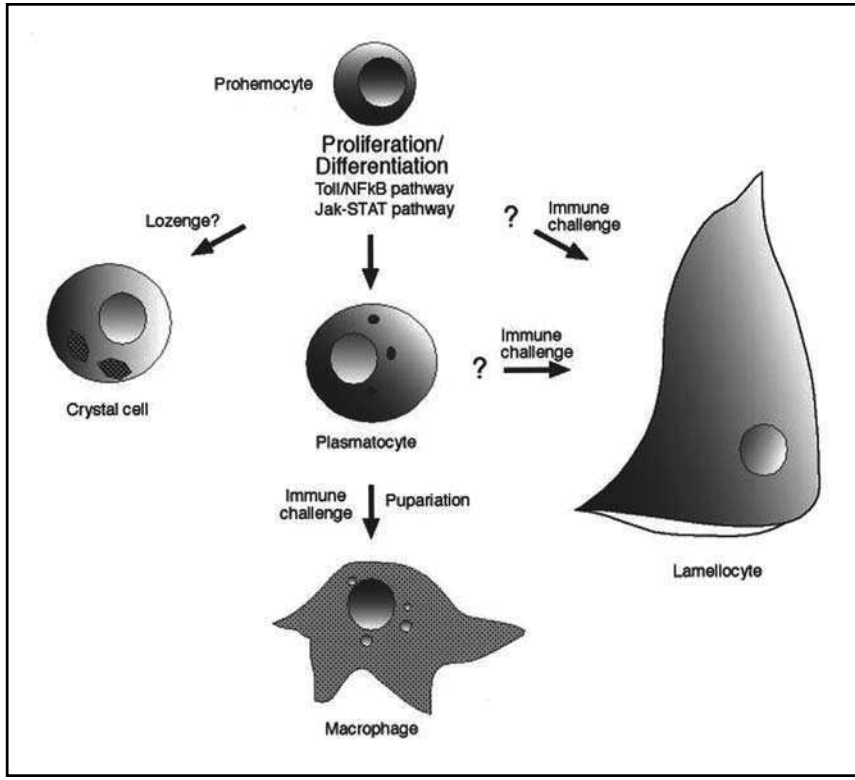

Figure 4. Tentative lineage of larval hemocytes. Adapted from Lanot et al.[54] See text for details.

plasmatocytes.[54] Lamellocytes are thought to encapsulate objects too large to be phagocytosed by forming multi-cell layers around the object or pathogen. The encapsulated object is then melanized by the third major type of larval hemocyte, the crystal cell. Constituting 5% of larval hemocytes, crystal cells are larger than plasmatocytes and have crystalline-like inclusions in their cytoplasm. The inclusions are thought to contain components of the prophenoloxidase cascade, which is involved in melanization of encapsulated objects.[55] Crystal cells in larval hematopoiesis are of unknown origin; however, they appear to be a separate lineage from plasmatocytes as mutants that lack crystal cells, for example *Lz* mutants, have plasmatocytes.[52,56] It remains to be shown whether expression of Lz also directs crystal cell differentiation in larval stages as it does during embryogenesis. However, recent evidence supports this hypothesis. Immunolocalization studies of third-instar larval hemocytes have shown that Lz is expressed in circulating crystal cells, and given the lack of crystal cells in *Lz* mutants, this suggests an autonomous requirement for *Lz* is this lineage.[52] Moreover, Lz-expressing larval precursor hemocytes give rise to all larval crystal cells.[52]

Adult hematopoiesis has not been well studied. No hematopoietic organ has been identified in the adult, and additionally it is not known whether the cells that constitute the adult hemolymph are derived from larval hemocytes or arise during adult life. It has been estimated that the adult hemolymph contains 1000-2000 hemocytes, which are primarily phagocytic plasmatocytes, although podocytes and lamellocytes, but not crystal

cells, have also been observed.[54] However, given that adults are able to melanize foreign objects, crystal cells may exist in low numbers or may be induced to differentiate from precursor cells. Alternatively, a cell type other than the crystal cells may be capable of inducing the melanization reaction in the adult.

Signaling Pathways Involved in Larval Hematopoiesis

Genetics analyses of mutants with abnormal hematopoiesis have provided insights into which signaling pathways are involved in the differentiation of larval hemocytes. Gain-of-function mutations in *Hop* have been the best characterized so far. Two temperature sensitive, dominant, gain-of-function mutations called $hop^{Tumorous\text{-}lethal\ (Tum\text{-}l)}$ and hop^{T42} give rise to hyperactive kinases even at the permissive temperature.[12,13,16] Molecular characterization of these mutations has demonstrated that the $hop^{Tum\text{-}l}$ lesion results in substitution of an amino acid (G341E) that is not conserved among Jak molecules (Fig. 1).[12,13] In contrast, the other mutation hop^{T42} is the result of a single amino acid substitution of a residue within the kinase-like domain (E695K) that is highly conserved among Jak molecules (Fig. 1).[16] Interestingly, the introduction of the E695K mutation into murine Jak2 results in a more active kinase.[13]

At both permissive and restrictive temperatures, these mutants exhibit over-proliferation and precocious differentiation of hemocytes. These animals have greatly increased numbers of plasmatocytes and abundant lamellocytes.[12,13,16,54] In second-instar $hop^{Tum\text{-}l}$ and hop^{T42} larvae, lamellocytes constitute 10-15% of the total hemocyte population, compared with less than 6% in wild type flies raised at the same temperature. Because the total number of hemocytes is higher in $hop^{Tum\text{-}l}$ and hop^{T42} larvae, there are dramatically more total lamellocytes in these flies than in wild type controls.[12,13,16,54] By third instar, the total number of lamellocytes in $hop^{Tum\text{-}l}$ larvae has risen to 50-75% of the total hemocytes, and the plasmatocytes frequently have abnormal intracellular features.[54] At pupariation, lamellocytes are very abundant in the lymph gland in these animals.[54] Lamellocytes in $hop^{Tum\text{-}l}$ and hop^{T42} larvae tend to aggregate into masses that often become melanized, the so-called "melanotic tumors". A "melanotic tumor" appears as a black mass within the body cavity of the fly and can be free floating or attached to tissues. Although the precise constitution of cells in the tumor has not been rigorously tested, it consists of at least two cell types, spherical cells that represent an abnormal type of hemocyte, presumably the plasmatocytes, and lamellocytes.[57] It has been hypothesized that the latter encapsulate the former, after which the aggregate is melanized, presumably by activation of the prophenoloxidase cascade in crystal cells, although this has not been demonstrated.[57] When transplanted into wild type hosts the hypertrophied lymph glands of $hop^{Tum\text{-}l}$ give rise to greatly increased numbers of plasmatocytes and melanotic tumors.[13] Interestingly, mis-expression of the Hop^{+} cDNA in the larval lymph gland causes tumors.[12] These data support the hypothesis that the *Hop* gain-of-function mutations disrupt the signal transduction pathways that control proliferation and differentiation in hematopoietic cells. It is not currently known whether mis-expression of *Stat92E* or *upd* causes tumors. However, reducing the dose of the *Stat92E* gene product can suppress the incidence of precocious differentiation but not the over-proliferation of hemocytes in $hop^{Tum\text{-}l}$ flies.[14,16] This suppression led to the proposal that the Stat branch of the Jak-Stat pathway does not control hemocyte proliferation but instead hemocyte differentiation.[16] Alternatively, it is possible that suppression of differentiation requires less modulation of the pathway than suppression of over-proliferation. Therefore, suppression of the hyperplastic phenotype may require a greater reduction in the dose of *Stat92E*.

Examination of other tissues suggest that the Jak-Stat pathway is not required for general cell proliferation in the fly but may be required in specific developmental contexts.[58] In the presence of maternally derived *Hop* or *Stat92E*, both *Hop* and *Stat92E* homozygote animals die at larval/pupal stages.[14,58] These animals contain the normal number and type of imaginal discs, however the individual discs contain vastly fewer cells than wild type discs at the same developmental stage (unpublished observations).[58] It is not currently known whether the hemolymph of *Hop* or *Stat92E* homozygous mutant larvae contains fewer cells. However, given the role of the Jak-Stat pathway in over-proliferation of hemocytes when hyperactive, it is likely that this pathway will have an important role in normal hematopoiesis in *Drosophila*. Therefore, *Hop* and *Stat92E* homozygous mutants should exhibit decreased numbers of larval hemocytes.

Despite extensive characterization of the *Hop* gain-of-function mutations, our knowledge of the function of the Jak-Stat pathway in larval hematopoiesis is limited. For example, we do not know whether Upd is a ligand for the Jak-Stat pathway in hematopoiesis. Moreover, it is not clear what receptor activates this pathway in hemocytes. Lastly, the downstream targets of this pathway are largely unidentified. In fact, only one has been described. *D-eIF4E*, the *Drosophila* homolog of a eukaryotic initiation factor, is upregulated in $hop^{Tum\text{-}l}$ larvae.[59] However, the biological significance of this observation is unclear.

The Toll (Tl)/IκB/NFκB pathway also plays an important role in larval hematopoiesis in the fly.[19] This pathway was originally discovered as the key player of dorsal-ventral patterning in the embryo. A cascade of serine proteases modify the ligand Spätzle (Spz), which activates Tl. Tl then activates Tube (Tub), an adaptor protein homologous to mammalian MyD88, and Pelle (Pll), a member of the serine/threonine innate immunity kinase family that is homologous to the IL-1 receptor-associated kinase. Pll is then thought to mediate either directly or indirectly the phosphorylation of the IκB homolog in flies, Cactus (Cac). Cac binds to members of the *Drosophila* NFκB/Rel family of transcription factors, Dorsal (Dl) and Dorsal-related immunity factor (Dif), and sequesters them in the cytoplasm. Upon phosphorylation, Cac is targeted to the proteosome for degradation, and Dl and Dif are released and translocate to the nucleus where they can induce transcription by interaction with specific DNA recognition sites in the promoter/enhancer region of target genes.[60]

Mutations in genes in the Tl pathway lead to abnormal hematopoiesis and melanotic tumors. Animals homozygous for null mutations in *Tl*, *Tub* and *pll* have reduced numbers of hemocytes.[18,19] Hyperactivity of the NFκB/Rel pathway, from loss of *cac* and dominant or gain-of-function mutations in *Tl*, such as Tl^{10B}, result in over-proliferation and precocious differentiation of hemocytes.[18,19,61] In cac homozygous mutants, the tumors are suppressed only by mis-expression of the cac^{+} cDNA in the larval lymph gland but not in other larval tissues.[18] Melanotic tumors were also suppressed by loss-of-function mutations in *Tl*, *Tub* and *pll*. These data indicate that, like the Jak-Stat pathway, the *Tl* pathway is involved in the regulation of hemocyte proliferation and differentiation. It will be important to establish the functional relationships between the Jak-Stat and Toll/IκB/NFκB pathways in fly hematopoiesis.

Immune Responses in *Drosophila*

Flies mount immune responses by employing three major lines of defense: (i) the rapid induction of proteolytic cascades that result in melanization and coagulation and production of signaling molecules; (ii) the humoral response in which the fat body produces antimicrobial peptides which are released into the circulatory system; and (iii) the

cellular response in which hemocytes expand and differentiate and participate in phagocytosis and encapsulation.[17] In response to septic injury, antimicrobial peptides are produced rapidly and massively in the larval and adult fat body and by hemocytes themselves and are released immediately into the hemolymph.[17] All of the antimicrobial genes examined to date have κB sites in their promoter region, and it is now clear that *Drosophila* NFκB/Rel proteins are activated in response to infection.[17] It is not known whether the Jak-Stat pathway is involved in the induction of proteolytic cascades or in the humoral response. However, there is evidence indicating that this pathway plays a role in the cellular response.

If there is evolutionary conservation of the integration of signaling cassettes, it is likely that the NFκB pathway will control the activation of the Jak-Stat pathway in certain situations in *Drosophila*. In mammals, the NFκB pathway induces cytokines, such as interferon-β and interleukin-6, which then activate the Jak-Stat pathway to promote effector functions in immune responses.[62,63] In flies, evidence to support this hypothesis has come from a recent analysis an activated allele of *18 wheeler* (*18w*), a member of the Tl receptor family. *18w* is expressed in the fat body, and homozygous mutants for loss-of-function *18w* mutations are more susceptible to infection.[64] 18w has been shown to activate Dif for signaling as in *18w* homozygous mutants, Dif translocation to the nucleus is inhibited.[64] Like *Hop* and *Tl* gain-of-function mutations, constitutive activation of *18w* leads to an over-proliferation and premature differentiation of larval hemocytes.[65] The hyperplastic phenotype observed in animals with the gain-of-function *18w* mutation can be suppressed by reducing the dose of *upd* or *Hop*, but not *Stat92E*, in these larvae.[65] A similar decrease in hemocyte counts is also observed when the dose of Tl pathway genes *Tub*, *pll*, or *cac*, but not *spz* or *Tl*, is reduced.[65] The precocious differentiation of lamellocytes seen in gain-of-function *18w* mutants is suppressed by reducing the dose of *Hop* or *Stat92E*.[61,65,66] In one model that integrates these observations, 18w inactivates Cac via Tub and Pll, thus freeing an NFκB/Rel family member, presumably Dif, to translocate to the nucleus, where it induces the expression of *upd* and of a hypothetical gene called *X* (Fig. 5).[4,65] Upd is then secreted and acts via a plasma membrane receptor to induce proliferation via activation of Hop, and possibly of the raf-Ras-MAPK pathway through a receptor tyrosine kinase.[65] Protein X is also secreted and activates the Hop-Stat92E pathway to induce differentiation of plasmatocytes.[65]

Interestingly, in another insect species, *Anopheles gambiae*, a Stat protein called Ag-Stat is activated in response to bacterial infection.[67] Currently, the mechanism of Ag-Stat activation is not known. Moreover, it is not known whether the activation of Ag-Stat depends upon prior gene induction by the NFκB pathway, although this is unlikely given the short time frame of activation. Importantly, this is the first report that the Jak-Stat pathway is involved in insect immunity. It will be important to examine if this observation is also true in *Drosophila*.

Conclusions

Clearly both the Jak-Stat and Tl/IκB/NFκB pathways are involved in the proliferation and differentiation of hemocytes. However, there are many unanswered questions that presumably will be addressed in the next few years. For instance, it is not known whether the Jak-Stat pathway is involved in proliferation and differentiation of hemocytes during an immune response. Moreover, it is important to clarify if activation by typical stimuli of the Tl pathway in the hematopoietic system results in induction of events controlling hemocyte expansion and differentiation. Then the question of whether the Jak-Stat pathway is downstream of the Tl/IκB/NFκB pathway or rather responds to signals that

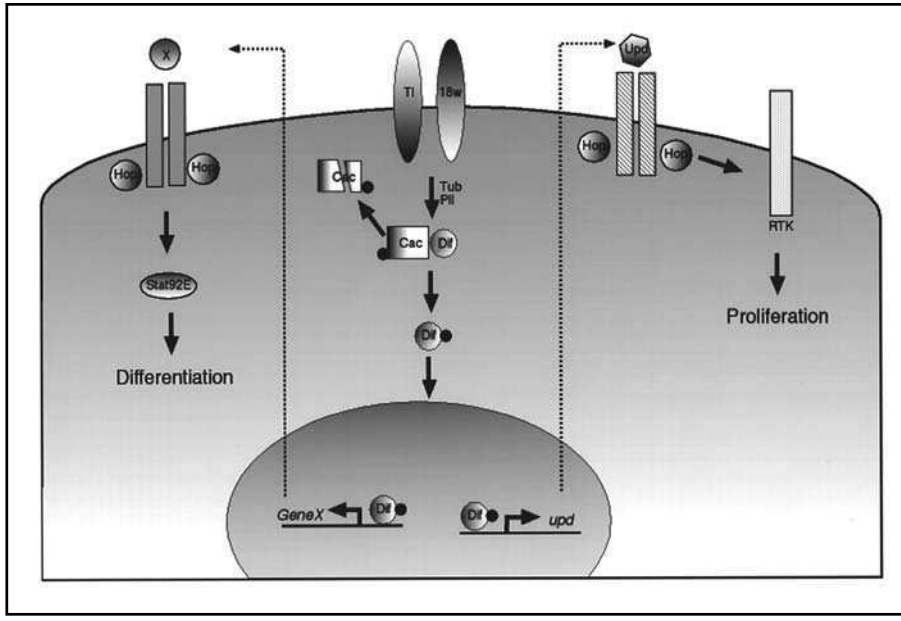

Figure 5. Model of integration of Jak-Stat and Tl pathways. Adapted from Mathey-Prevot and Perrimon[4] and Williams[65] See text for details.

are completely independent of Tl/IκB/NFκB pathway, or both, can be answered. It remains possible that the Jak-Stat pathway responds to signals from the Tl/IκB/NFκB and from other signaling pathways. Clearly, dissection of the fly hematopoietic and immune systems will help elucidate conserved mechanisms of hematopoiesis and immunity that also exist in mammals.

Acknowledgments

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CHAPTER 7

SOCS Proteins in Hematopoiesis

Sandra Nicholson and Warren Alexander

Introduction

The control of hematopoiesis is a tightly regulated process that maintains the numbers of blood cells within narrow normal ranges in healthy individuals with sufficient capacity to allow rapid responses to environmental changes such as infection, tissue injury and changes in oxygen levels. The cytokines are a group of predominantly soluble proteins including the colony stimulating factors (CSF's), interleukins, interferons and growth factors. They are key players in hematopoietic regulation, controlling cell production, maturation and function via interaction with specific cell surface receptors. The resultant receptor activation triggers complex intracellular signalling cascades that ultimately direct the cell's genetic and biological response to cytokine.[1]

The receptors for the majority of cytokines that influence hematopoiesis belong to the hematopoietin or cytokine receptor superfamily, a group of transmembrane proteins that share conserved peptide motifs within their extracellular domains.[2] The cytokine receptors are activated following ligand binding by oligomerisation of receptor subunits. Intracellular signalling is initiated by the resultant aggregation of members of the Jak family of protein tyrosine kinases that are associated with the receptor cytoplasmic domains. The Jaks cross-phosphorylate, resulting in an increase in their intrinsic enzymatic activity allowing them to phosphorylate tyrosine residues within the receptor subunits. Recruitment of a second wave of signalling molecules which dock to receptors on specific phosphorylated tyrosine residues via their SH2 domains ensues.[3,4] Many of these molecules, such as the Stat proteins, are themselves substrates for the Jak kinases. Once phosphorylated they relay a series of signals to the nucleus, resulting in changes to gene transcription.[4]

While the actions of many of the intracellular molecules that drive signal transduction are now well defined, a great deal of recent interest has centered on understanding how signalling cascades are negatively regulated. Several mechanisms have emerged. Protein tyrosine phosphatases regulate signalling by de-phosphorylation of critical tyrosine residues resulting in deactivation of receptors or signalling molecules. Internalization and endocytic trafficking regulate receptor complexes, whilst ubiquitination and degradation of receptors and signalling components through the proteasome provide further levels of control. In addition to these mechanisms, a growing number of specific inhibitors have been identified. One such example is the PIAS family of proteins (Protein Inhibitors of Activated Stats), which appear to bind directly to Stats and inhibit their action.[5] Another

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rapidly expanding field of research involves the SOCS proteins or suppressors of cytokine signalling.

SOCS Proteins

A New Family of Negative Regulators

SOCS-1/SSI-1/JAB was discovered independently by several groups, the different nomenclature reflecting the experimental approaches used by each. SOCS-1 was discovered in a somatic cell genetic screen for suppressors of IL-6 signalling.[6] SSI-1 or Stat-induced Stat inhibitor-1 was discovered on the basis of antigenic similarity between the SSI-1 and Stat SH2 domains,[7] whilst Endo et al,[8] identified JAB (Jak-binding protein) during a yeast two-hybrid screen, using the JH1 kinase domain of Jak2 as bait. This review will use the SOCS nomenclature.

The SOCS-1 gene encodes a 212 amino acid protein with a predicted SH2 domain and sequence similarity to an already known protein, CIS, originally identified as a cytokine inducible SH2 domain-containing protein.[6-8] Database searches revealed six other related proteins (SOCS-2 to SOCS-7).[9] All eight proteins contain a central SH2 domain, a divergent N-terminal region of variable length and a conserved C-terminal 40 amino acid motif which has been termed the SOCS box. This motif consists of two blocks of conserved residues and a consensus based on these was used to identify an extended family of SOCS box containing proteins. This family can be subdivided on the basis of the structural domains upstream of the SOCS box. In addition to the SH2 domain containing proteins, there are currently ten mammalian proteins containing ankyrin repeats (ASB-1 to 10)[10], four proteins containing SPRY domains (SSB-1 to 4), three containing WD40 domains (WSB-1 to 3) and two containing a GTPase domain N-terminal to the SOCS box (rar and Ras-like GTPase) (Figure 1). In several instances SOCS box containing proteins have also been found in lower organisms including C. elegans (SSB/ASB), Drosophila (SOCS/SSB), Xenopus (SSB) and C. intestinalis (SSB)[9] (manuscript in preparation).

SOCS Proteins Form Part of a Classic Negative Feedback Loop

CIS was originally identified as an IL-3-inducible gene and was subsequently shown to be induced by a number of other cytokines including IL-2, IL-3, GM-CSF, erythropoietin (EPO)[11] and growth hormone.[12] Over-expression studies in hematopoietic cell lines suggested that CIS was able to negatively regulate signalling by interacting with IL-3 and EPO receptor phosphotyrosines, thus blocking recruitment of Stat5 to the receptor complex. In addition, Stat5 binding sites were identified in the CIS promoter region, suggesting that CIS was one of the immediate early genes induced by Stat5.[11] In confirmation of this, CIS expression was not observed in Stat5 knockout mice.[13] Similarly, SOCS-1 mRNA expression is induced in response to a number of different cytokines and is highest in the mouse thymus and spleen, where it is expressed primarily in lymphocytes.[6] Like CIS, the promoter region of SOCS-1 contains Stat binding sites, in this case for Stat3 and Stat6,[7] suggesting that SOCS-1 is also transcriptionally regulated by the Jak/Stat pathway. These studies provide compelling evidence that SOCS proteins control cytokine signalling as part of a classical negative feedback loop that blocks the Jak/Stat pathway (Figure 2).

However, as the number of studies examining cytokine induction of SOCS expression expands, an increasingly complex picture emerges. Induction of expression of individual SOCS proteins appears sensitive to the actions of many different cytokines and the

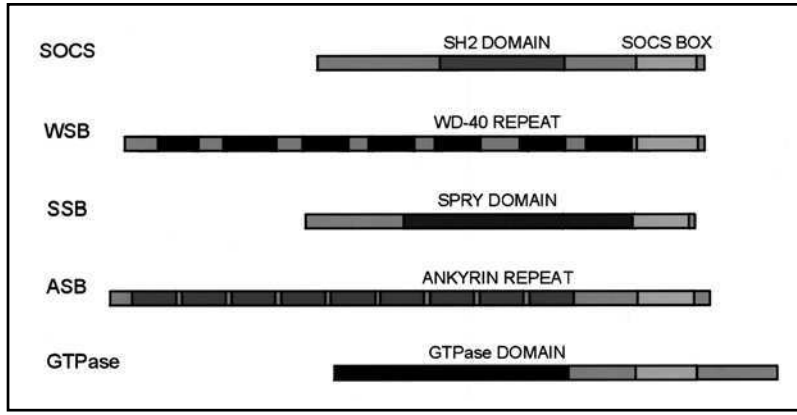

Figure 1. The SOCS family of proteins. Defined by the presence of the SOCS box, a conserved 40 amino acid motif, the family can be divided into 5 subgroups distinguished by the protein domains located N-terminally: SOCS (suppressor of cytokine signalling), containing SH2 domains; WSB, WD40 repeat-containing SOCS box proteins; SSB, SPRY domain-containing SOCS box proteins; ASB, ankyrin repeat-containing SOCS box proteins; and RAR, GTPase-like domain-containing proteins.

patterns of induction of members of the family differ in a cytokine and cell type specific manner. Moreover, the expression of a SOCS protein in response to cytokine is not always a reliable indicator of the capacity to inhibit signalling. For example, in mouse liver, SOCS-1, SOCS-2, SOCS-3 and CIS are induced in response to IL-6, while in M1 myelomonocytic cells only SOCS-1 and CIS are induced.[6] Nevertheless, only SOCS-1 and SOCS-3 appear able to inhibit IL-6 signalling when overexpressed in M1 cells.[14] Accordingly, it should be noted that many of these studies have exploited in vitro and overexpression systems which, while useful, may over-represent the true in vivo actions of the SOCS proteins. Indeed, the indispensable physiological roles and specific actions of the SOCS proteins have only emerged through the combination of biochemical studies and analyses of genetically modified mice (see below).

Mechanisms of Action of SOCS Proteins

Inhibition of the Jak/Stat Pathway

The SOCS proteins can be divided into pairs based on their sequence similarity. SOCS-1 and SOCS-3 are more closely related to each other than to the other SOCS proteins. Similarly, CIS and SOCS-2 are more closely related, as are SOCS-4 and SOCS-5, and SOCS-6 and SOCS-7. In most cases the similarity lies within the SH2 domain and the SOCS box; for example, the SOCS-4 and SOCS-5 SH2 domains share 89% identity at the amino acid level.[9] While SOCS-1 and SOCS-3 appear to have some common modalities of function, it remains to be determined whether these patterns reflect overlapping functions for others of the SOCS protein pairs.

While CIS appears to act by direct interaction with cytokine receptors resulting in blockade of Stat recruitment (Figure 2), the precise mechanisms of SOCS-1 and SOCS-3 action appear distinct and have been the subject of intense recent scrutiny. Endo and colleagues[8] originally identified SOCS-1 by its ability to bind directly to the Jak JH1, or kinase, domain. This group and others went on to demonstrate binding of the SOCS-1

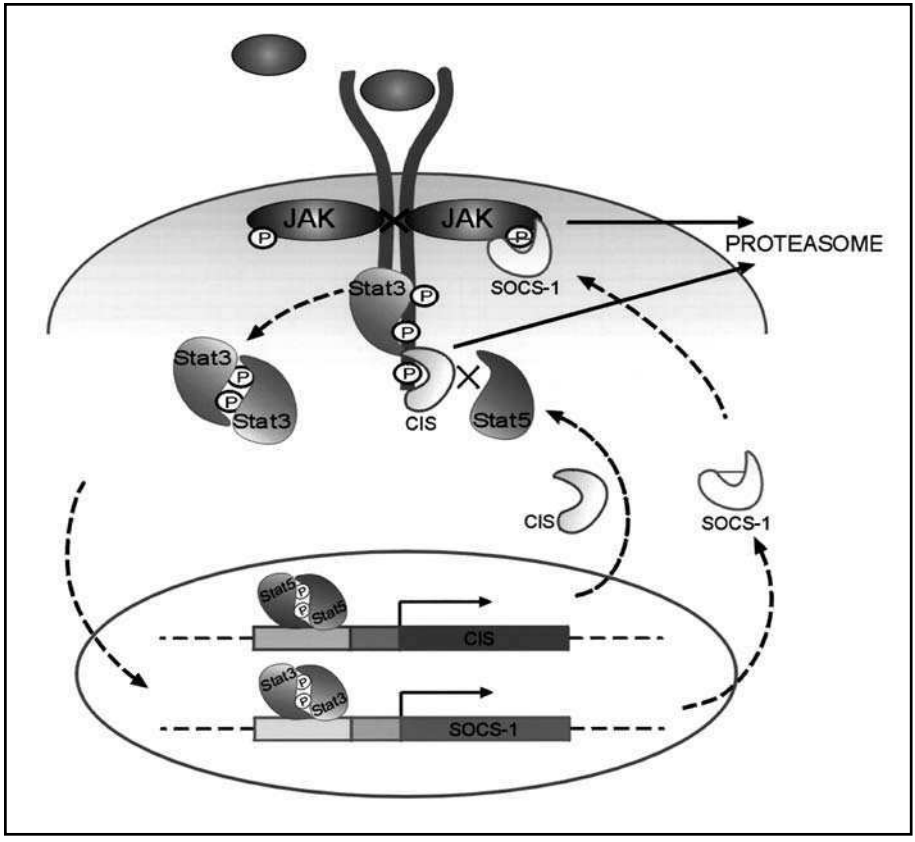

Figure 2. Inhibition of the Jak/Stat pathway by SOCS proteins via a classical negative feedback loop. SOCS gene expression is induced by activated Stats following cytokine stimulation. CIS appears to act by binding phosphotyrosine residues on the activated receptor preventing further Stat recruitment and activation. SOCS-1 is thought to directly bind Jak proteins, inhibiting tyrosine kinase activity. The interaction between the SOCS box and elongins B and C may also target signalling molecules and receptors for proteasome-mediated degradation.

SH2 domain to tyrosine 1007 in the catalytic loop of the Jak2 kinase domain and the subsequent inhibition of Jak enzymatic activity.[8,15,16] Exactly how SOCS-1 effects the inhibition of catalytic activity remains open to debate. Based on weak homology between the SOCS box and the residues surrounding the critical tyrosines within the Jak catalytic loops, Yoshimura and associates have proposed that a 12 amino acid region upstream of the SH2 domain termed the 'kinase inhibitory region' (KIR) acts as a pseudosubstrate, blocking further catalytic activity.[15] This hypothesis is supported by mutational analysis of the region, which demonstrates that while binding to phosphopeptides is retained, mutants in this region are no longer able to inhibit signalling.[15] However, direct evidence is still lacking and the role of the KIR may not be fully clarified until structural analysis of the SOCS-1-Jak complex is completed. Interestingly, the KIR region appears to be loosely conserved between mammalian SOCS-1, SOCS-3, SOCS-4, SOCS-5 and Drosophila SOCS-5,[14] suggesting that a structural or functional role may indeed be conserved.

SOCS-1 and SOCS-3 require an extended SH2 domain for phosphopeptide binding. In both proteins this involves an additional twelve residues N-terminal to the classically defined SH2 domain[15,16] and in the case of SOCS-3, it includes 44 amino acids

C-terminal to the SH2 domain.[16] In each case, mutation of the SH2 phosphotyrosine binding pocket destroys the inhibitory action of the proteins.[14]

Differential Actions of SOCS-1 and SOCS-3

Initial studies by Yoshimura and colleagues suggested that SOCS-1 and SOCS-3 inhibited signalling by the same mechanism: binding directly to the Jak kinases, and considerable evidence has accrued to support this model for SOCS-1. However, while SOCS-3 clearly can bind to Y1007 in the Jak2 JH1 domain,[16] either in vitro or when both are over-expressed, a number of observations suggest a more complex scenario that challenges a simple SOCS-3/Jak interaction model. Consistent with differential mechanisms of SOCS-1 and SOCS-3 action, the kinetics of suppression of Stat3 phosphorylation in response to IL-6 differs between the two proteins.[17] Moreover, in contrast to SOCS-1, SOCS-3 did not inhibit Jak autophosphorylation in an in vitro kinase assay.[14] Finally, SOCS-3 inhibition of Jak1 phosphorylation was enhanced by the presence of the IL-2Rβ.[18] As suggested by this latter observation, receptor subunits appear to have a critical role in mediating SOCS-3 action. SOCS-3 has been shown to bind to tyrosine 757 within the IL-6 signalling subunit gp130 and mutation of that residue reduces the efficacy with which SOCS-3 can inhibit LIF signalling.[19,20] Phosphopeptide binding studies have further shown that the affinity of SOCS-3 for a peptide corresponding to Y757 is a 1000 fold higher than binding to peptides derived from the catalytic loops of the Jak kinases.[20] Together these data suggest that one of the physiological substrates for the SOCS-3 SH2 domain will be Y757 within the gp130 cytoplasmic domain. This raises the interesting conundrum as to how SOCS-3 is able to inhibit Jak phosphorylation and kinase activity, while docked to the receptor. One possibility is that, once recruited to the signalling complex via receptor binding, the N-terminal region is able to bind to the Jak JH1 domain and inhibit kinase activity, as proposed by Sasaki et al.[16]

A functional association of SOCS-3 has also been observed for tyrosine motifs within the long form of the Leptin receptor[21] and the EPO receptor.[22] Sasaki et al,[22] further suggest that SOCS-3 interaction with the EPO receptor involves both the KIR region and the extended SH2 domain. Interestingly, Y757 in gp130,[23] Y985 in the Leptin receptor[24,25] and Y401 in the EPO receptor[26] are also binding sites for the protein tyrosine phosphatase SHP-2. SHP-2 has been suggested to both negatively regulate signalling through its catalytic domain and to act as an adaptor protein by recruiting Grb2 or IRS-1 to receptor complexes.[27] With respect to gp130, the negative regulatory role of SHP2 has largely been inferred by mutation of Y757 within the gp130 cytoplasmic domain.[28-30] Thus, future work will need to resolve the relative contributions of SOCS-3 and SHP-2 to negative regulation of gp130 signal transduction.

The SOCS Box Proteins as E3 Ubiquitin Ligases

The size of the SOCS box (approximately 40 aa) and absence of intrinsic enzymatic activity seemed consistent with a modular domain likely to be involved in protein-protein interactions. The evolutionary conservation of this domain in a diverse array of proteins further suggested that it would mediate a common function within each.[9] Subsequent work by two groups has now linked the SOCS box to the proteasomal degradation pathway through an interaction with Elongins B & C.[31,32]

E3 ubiquitin ligases consist of multimeric protein complexes, which specifically regulate polyubiquitination of proteins, directing their destruction by a protease complex, the

26S proteasome.[33] The Elongin BC complex is known to fulfill two distinct functions. It was initially discovered by its ability to positively regulate the transcriptional activity of the RNA polymerase II complex. This was mediated through an interaction with Elongin A, one of several transcription factors able to enhance RNA polymerase II activity.[34] More recently it has been shown to interact with the von Hippel Lindau (VHL) protein in a complex that contains Cullin 2 and Rbx1, and is thought to target VHL substrates, most likely hypoxia-inducible factors, for degradation through the proteasome.[35,36] The Elongin C interaction sites in Elongin A and VHL had been mapped and a potential binding site was identified in the N-terminal half of the SOCS box. Kamura et al[31] and Zhang et al[32] were able to demonstrate a direct interaction between the SOCS box and the Elongin BC complex, which was lost following mutation of the Elongin C binding consensus.

Striking parallels exist between the SOCS box and the F-box in the phospho-protein ubiquitin ligase complex (PULC). [37] The F-box is also a modular domain found in a wide variety of proteins containing additional protein-protein interaction domains. The F-box binds to Skp1, an Elongin C homologue which in complex with Cullin1, Rbx1 and an E2 ubiquitin ligase (typically cdc34) has been demonstrated to have ubiquitin ligase activity, and targets substrates of the F-box proteins for ubiquitination and degradation. F-box proteins regulate a variety of cellular processes, such as the cell cycle, in organisms as diverse as yeast and humans.

Although ubiquitin ligase activity by the SOCS box complex has yet to be demonstrated, the prevailing evidence suggests that the SOCS box will regulate protein half-lives. However, there is still some controversy as to whether the SOCS proteins themselves will be protected from degradation,[31,38] or whether they will be targeted for destruction by the proteasome.[32] While future work will no doubt resolve this apparent conflict, the strong structural homology with the F-box makes it likely that the SOCS box will play a role in targeting SOCS substrates for degradation.

Physiological Roles of the SOCS Proteins

CIS

Targeted deletion of the CIS gene has been reported to result in a phenotypically normal mouse,[39] suggesting that CIS may be functionally redundant. In contrast, CIS transgenic mice have defects in growth, mammary gland development, NK cells and T-cells, and are remarkably similar in phenotype to Stat5a and Stat5b knockout animals.[40] This suggests that, consistent with the in vitro data, CIS can also act to inhibit the Jak-Stat5 signalling pathway in vivo.

SOCS-1

Germline deletion of the murine gene encoding SOCS-1 resulted in animals that developed a complex and fatal neonatal disease. Mice died within three weeks with low body weight, fatty degeneration of the liver, monocytic infiltration of the lung, pancreas, heart and skin, and a severe depletion of T and B cells.[41,42] The observation that the effects of ablating SOCS-1 strikingly paralleled those seen upon injection of neonatal mice with IFNγ provoked a series of experiments that established a key role for IFNγ in the SOCS-$1^{-/-}$ phenotype. The full spectrum of neonatal disease was prevented in SOCS-$1^{-/-}$ mice by the administration of neutralizing anti-IFNγ antibodies or genetic ablation of IFNγ (Figure 3).[39,43] Subsequent studies suggested that both elevated levels of IFNγ as well as an intrinsic

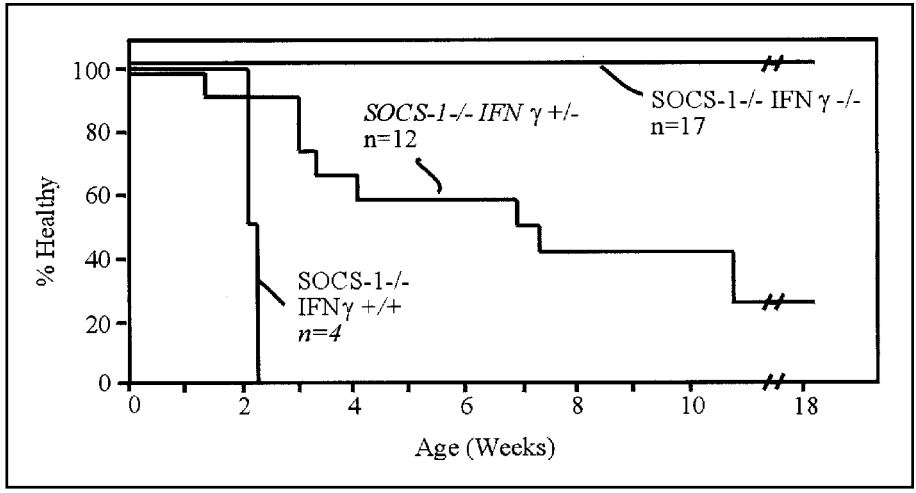

Figure 3. Prevention of lethal neonatal disease in SOCS-1$^{-/-}$ IFNγ$^{-/-}$ mice and amelioration of morbidity in SOCS-1$^{-/-}$ IFNγ$^{+/-}$ mice in comparison with SOCS-1$^{-/-}$ IFNγ$^{+/+}$ mice. Reprinted with permission from Alexander et al. Cell 1999; 98:597-608. [Copyright 1999, Elsevier Science]

hypersensitivity to the cytokine contributed to disease in SOCS-1-deficient animals.[39,43] The increase in IFNγ levels is likely to result from increased T-cell activation, a key anomaly observed in the SOCS-1-deficient mice,[39] and the absence of SOCS-1 in responsive cells allows prolonged IFNγ signalling. These studies imply that SOCS-1 is the key physiological regulator of IFNγ signalling in vivo and as such allows the body to utilize the beneficial immunological actions of IFNγ while avoiding the potentially devastating side effects.

Although these data have provided definitive insights into SOCS-1 function, several outstanding issues remain. For example, the molecular basis for the excess T-cell activation that occurs in the absence of SOCS-1 has not been clarified. Although some evidence implicates SOCS-1 in the negative regulation of the T-cell receptor and IL-2 signalling, this aspect of SOCS-1 action requires considerable further study. Finally, the initial indications that SOCS-1 is induced by, and can inhibit signals from many different cytokines clearly appears to have exaggerated SOCS-1 action in vivo, although the discovery of subtle or redundant functions of SOCS-1 may emerge from further studies. Indeed, hematopoietic progenitor cells from SOCS-1$^{-/-}$ mice are hyper-responsive to GM-CSF in culture assays and the mice themselves contain elevated numbers of macrophage progenitor cells.[42]

SOCS-2

Targeted disruption of the SOCS-2 gene has also revealed a unique physiological role. Until they reach weaning, mice lacking SOCS-2 appear identical to their wild-type littermates. However, at this time their growth rate increases dramatically, resulting in adult SOCS-2$^{-/-}$ mice that are typically 40% heavier than their littermates (Fig. 4). The increase in body size reflects a largely uniform increase in the weight of most organs, as well as increased muscle mass and bone lengths, and is more pronounced in males than in females. There is excess collagen deposition in the skin and altered production of major urinary protein, characteristics previously observed in animals and/or humans with excess

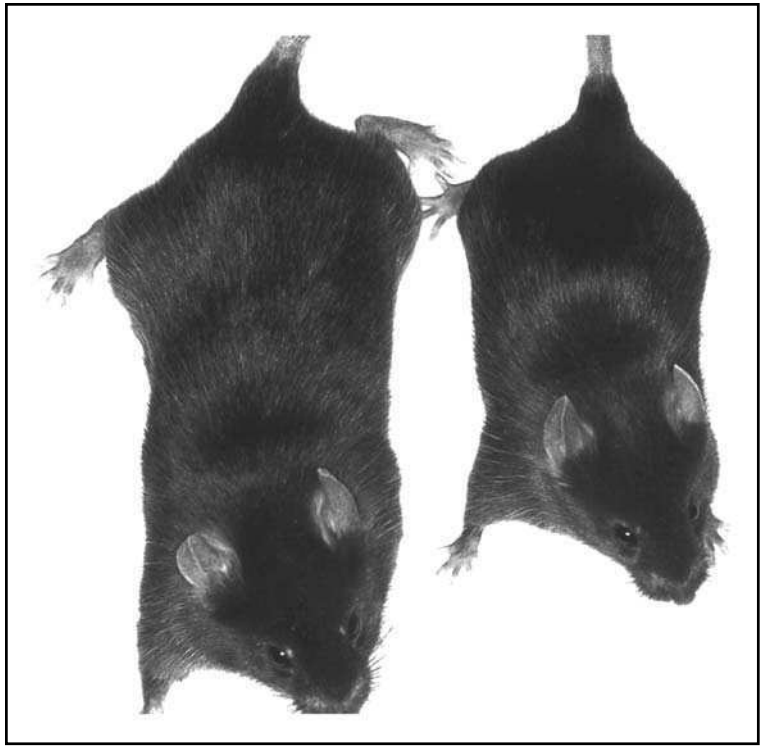

Figure 4. The increase in size of male SOCS-2$^{-/-}$ mouse (left) relative to an age- and sex- matched wild-type animal (right). Reprinted with permission from Metcalf et al. Nature 2000; 405:1069-1073. [Copyright 2000, Nature, http://www.nature.com/]

growth hormone activity. Thus, SOCS-2 appears to be a negative regulator of growth hormone/IGF-1 mediated growth, a conclusion supported by the observation of elevated levels of IGF-I in some organs of SOCS-2-deficient mice.[44] However, the precise mechanism by which SOCS-2 controls these pathways remains to be defined. Although it has been shown that SOCS-2 is induced in response to growth hormone or IGF-I and that SOCS-2 can associate with the IGF-I and growth hormone receptors,[45-47] these studies did not document a convincing effect of SOCS-2 on downstream signalling cascades. Studies of in vivo sensitivity to these cytokines in SOCS-2$^{-/-}$ mice in comparison with normal controls, as well as a more detailed analysis of growth hormone and IGF-I signal transduction in these animals will begin to address the precise mechanism of SOCS-2 regulation of growth. Despite the capacity of several hematopoietic cytokines to induce SOCS-2 expression, hematopoiesis is normal in the absence of this regulator.[44]

SOCS-3

Transgenic over-expression of the SOCS-3 gene under a hematopoietic specific promoter, resulted in a lethal phenotype[48] with embryos that expressed the transgene lacking detectable fetal liver erythropoiesis. Conversely, deletion of the SOCS-3 gene was also embryonic lethal, with viability decreasing after 10-12 days gestation and evidence of marked erythrocytosis.[48] These observations, coupled with evidence that over-expression

of SOCS-3 in cell line models can inhibit erythropoietin signalling,[22] suggest that SOCS-3 is a critical regulator of early erythropoiesis. Over-expression of SOCS-3 has also been shown to inhibit IL-6, LIF, prolactin, growth hormone, IFNγ, IL-4 and IL-9 signalling.[6,45,49-52] The early death of SOCS-3$^{-/-}$ mice has prevented analyses of the effects of SOCS-3 deficiency on signalling by other cytokines and the physiological relevance of these in vitro observations may require more sophisticated gene targeting approaches.

SOCS-4, -5, -6 & -7

The physiological roles of the other SOCS proteins remains obscure. SOCS-5 mRNA is widely expressed and also induced in response to IL-6, but at a much later time point than SOCS-1 or SOCS-3.[9] Over-expression of SOCS-5 has been shown to inhibit IL-6 and LIF signalling, but this effect is only partial compared with that of SOCS-1 or SOCS-3.[14] Induction of SOCS-5 mRNA has also been demonstrated in response to insulin although the ability of SOCS-5 to regulate insulin signalling has not been addressed.[53] It is interesting that SOCS-4, -5, -6 & -7 have much longer N-termini than the other SOCS proteins. It is tempting to speculate that the N-terminal regions in these proteins may have a more complex functional role, possibly as a scaffold to recruit additional proteins into the signalling complex. In this regard, SOCS-7 has been shown to interact with the adaptor proteins Nck, Grb2, Ash, phospholipase-C-γ and the epidermal growth factor receptor,[54] but again to date the functional significance of these interactions remains unknown.

ASBs, SSBs and WSBs

Four members of the ASB family have been cloned and form part of an extensive family comprised of at least ten proteins. The ASBs are highly conserved between human and mouse and show a varied expression pattern, with ASB-1 and -2 mRNA showing a relatively restricted pattern of expression, ASB-3 being ubiquitously expressed and ASB-4 expression restricted to the testes in analyses of mouse organs.[10] The SOCS box proteins containing a WD-40 domain (WSB-1, WSB-2) have been implicated in Hedgehog signalling. Vasiliauskas et al[55] cloned the chick homologue of WSB-1 naming it cSWiP-1, and showed that it was induced in response to Shh protein. Moreover, expression studies localized cSWiP-1 to embryonic sites of Hedgehog activation.[55] These intriguing observations now require extensive biochemical and in vivo confirmation, but have uncovered the first indication of potential roles of members of these less well characterized SOCS box-containing proteins.

Conclusions

The SOCS field has developed rapidly over the last five years. However, while the studies to date have provided vital insights into how these proteins work biochemically as well as function in the body, there are still many aspects that require clarification. Primary among these is the role of the SOCS box and definitive biochemical evidence of its role in protein stability, be it of the SOCS proteins themselves or their associated substrates. In addition, although initial knockout mice studies have defined apparently very specific roles for SOCS-1, SOCS-2 and SOCS-3 in cytokine regulation, outstanding questions regarding the role of these proteins still remain, particularly with regard to possible redundant functions in control of signalling from a more diverse array of cytokines. It will also

be interesting to see whether SOCS-4 to -7 prove to be negative regulators of cytokine signalling. The combined approaches of gene targeting, structural biology and the biochemical characterization of binding partners will be needed to fully address the role of this protein family in cytokine signalling.

It is obvious from the phenotypes of SOCS-deficient mice, that the regulation of the Jak/Stat pathway is critical for normal responses to cytokine. Aberrant activation of the Jak kinases and their substrates has been linked to hematopoietic malignancies and various clinical pathologies (for review refer to Ward et al[56]). Mutation of the SOCS genes has yet to be linked to human disease states, but this may simply reflect the infancy of the field. Already, it is apparent that small molecule mimetics or antagonists of SOCS proteins could have a clinical role in treating human disease involving excess or deficient cytokine action. Furthering our understanding of the biochemical action of the SOCS proteins will hopefully refine the development of therapeutic agents targeting specific signalling molecules involved in the Jak/Stat signalling cascade.

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