Title
The role of STAT5 in hematolymphoid development in vivo

Author
Snow, Jonathan W.

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The Role of STAT5 in Hematolymphoid Development *in vivo*.

by

Jonathan W. Snow

DISSERTATION

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and

UNIVERSITY OF CALIFORNIA BERKELEY

[redacted]

Arma virumque cano, Troiae qui primus ab oris
Italiam, fato profugus, Laviniaque venit
litora, multum ille et terris iactatus et alto
vi superum saevae memorem Iunonis ob iram;
multa quoque et bello passus, dum conderet urbem,
inferretque deos Latio, genus unde Latinum,
Albanique patres, atque altae moenia Romae.

Vergil, *Aeneid*, 1.1-7

Dedication

For my family, the whole lot of them. But especially for my mother, Catherine Snow, who has always been my most important source of knowledge, guidance, and love.

Preface

There are a number of important people I would like to acknowledge for help in the completion of this odyssey of scientific and personal growth. For guidance and support through my education, I am deeply indebted to a number of colleagues and friends. First, I gratefully acknowledge the support of my family. I have a rather large and complicated one, and they have all helped me in too many ways to count.

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I am grateful for the scores of friends from outside science who have provided so much support and laughter at different stages in my life, especially those who moved to San Francisco with me, Kevin Ma, Jeremy Woodlee, Matthew Wiltshire, and Andy Berlind.

I am also grateful for Claudia Grossmann for being a fun and special person and for making my last and perhaps most challenging year of graduate school one of the best.

I acknowledge Dr. Stephen Chan for scientific and life advice, for never hesitating to give me a hard time, and for being a superb brother-in-arms throughout this entire experience. I thank Mauricio Montano for always being willing to provide assistance,

whether for science or otherwise. I thank Jason Kreisberg for useful comments (and many that were specifically designed not to be) and many interesting lunches. I acknowledge Brian F. Eames for helping me take myself less seriously.

Also, I thank the entire Goldsmith lab, and everyone at the Gladstone Institutes, for creating such a fun, stimulating, and open environment for doing science.

I thank Dr. Kevin Shannon for taking a wayward Eph under his wing and the entire Shannon lab for making me feel at home and teaching me much about mouse models and cancer. I express gratitude to Dr. Warner C. Greene and Dr. J. Michael McCune for many insightful thoughts regarding this work and for advice and support for securing my post-doctoral position.

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I thank the UCSF Laboratory Animal Resource Center animal care staff, especially Allan Gray and Milton Griffon, for going above and beyond the call of duty in keeping our animals happy. I also acknowledge the technical assistance of the Gladstone Flow Cytometry Core and the Gladstone Histology Core in the conduct of these experiments.

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I am also grateful for the financial support of the Biomedical Sciences Program, the Dean's Health Sciences Fellowship, and the National Institute of Health.

All of the work described in this dissertation has been submitted to or published in scientific journals. Chapter 2 contains an article published in *Blood*, reproduced with copyright permission from the American Society of Hematology. Chapters 3, 4, and 5, as well as the Appendices I and II, contain manuscripts submitted for possible publication in scientific journals.

The Role of STAT5 in Hematolymphoid Development *in vivo.*

Jonathan W. Snow

Hematolymphoid development is regulated by multiple systems delivering signals from physiologic inputs. Among these are cytokines delivering signals to target cells through activation of cognate cell surface receptors. The role of specific signaling molecules utilized by cytokines in hematolymphoid development in the context of the organism has not been fully elucidated.

We hypothesized that Signal Transducer and Activator of Transcription 5 (STAT5), a signal transduction molecule activated by many cytokine receptors in hematopoiesis, would play a significant role in the efficient performance of this process *in vivo*. Using STAT5A/5B-deficient mice, we found this molecule to be critical in maintaining wild-type levels of multiple blood lineages at steady-state, due to cell-autonomous defects in hematopoietic progenitors, perhaps related to decreased survival of these cells.

Examination of STAT5A/5B-deficient mice expressing a Bcl-2 transgene revealed that ectopic expression of Bcl-2 was not sufficient to rescue all hematopoietic defects seen in STAT5A/5B-deficient mice, indicating alternate biological roles of STAT5 in early hematopoietic progenitors.

Suspecting that defects in cells other than hematopoietic progenitors were contributing to hematopoietic defects in these mice, we found that loss of tolerance in these mice leads to an autoimmune pathology affecting multiple processes including

hematolymphoid development. STAT5's toleragenic role may be contributing to the homeostatic survival of $CD4^+/CD25^+$ regulatory T cells. We were also able to show that hematopoietic defects in STAT5A/5B$^{-/-}$ mice could be rescued with transplant of wild-type bone marrow, indicating that STAT5A/5B-deficiency in non-hematopoietic tissue did not adversely affect hematolymphoid development at steady-state.

Finally, we wished to explore the role of STAT5 as a molecular effector of IL-7 receptor activation in normal lymphocyte development. Using mice expressing an IL-7 transgene, we found a STAT5-dependent role for this cytokine in the preferential development of $CD8^+$ T-cells. In addition, we found that heterozygosity of STAT5 was able to provide protection from the IL-7-induced lymphomas found in these Tg IL-7 mice.

Taken together, these discoveries will add significantly to our understanding of cytokine-dependent signal transduction, in particular that of STAT5, in hematolymphoid development *in vivo*. In addition, these findings will contribute to our understanding of this molecule as a therapeutic target.

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Chapter 1

Introduction

Hematolymphoid Development

Hematolymphoid development can be simply defined as the process through which mature cells of the major blood lineages, including red blood cells, platelets, neutrophils, basophils, eosinophils, mast cells, monocytes / macrpophages, dendritic cells, natural killer cells, B-cells, and T-cells, are derived from a small population of hematopoietic stem cells (HSC) throughout the lifespan of the organism (Fig. 1). Pictured is a simplified schematic, which represents the differentiation of HSC to mature blood cells via intermediate populations, such as the common lymphoid progenitor (CLP), common myeloid progenitor (CMP), granulocyte-monocyte progenitor (GMP), megakaryocyte-erythroid progenitor (MEP), and lineage-committed progenitors. The populations making up the current proposed hierarchy have been defined in recent years by their prospective isolation and functional characterization. However, the paradigm of a three compartment hematopoietic system was put forth over 40 years ago [1] after the ability of bone marrow to provide radioprotection in mice was appreciated [2,3] and the first attempts at human bone marrow transplantation had been initiated [4]. This model proposed a stem cell population, a population of progeny cells with progressively more restricted self-renewal capacity and differentiative potential, and the mature cells of the blood lineages. The first experimental support for these concepts was initiated by *in vivo* spleen colony assays began in the early 1960's [5-7]. In addition, the concepts of a myeloid-lymphoid split, and a further granulocyte-monocyte split from other myeloid lineages, have long been proposed, based on *in vivo* transfer assays [8], *in vitro* colony types observed in response to defined growth factors [9], the existence of multipotential

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The HSC are operationally defined as cells competent to reconstitute the hematopoietic system of a recipient individual [11] and they are unique in two ways: firstly, they possess the potential to differentiate into all hematopoietic lineages (and although considered controversial, perhaps into cells of other tissues [12-16]), and secondly, they have a remarkable, albeit limited, capacity for self-renewal. Indeed, one HSC from a donor animal has the ability to repopulate the hematopoietic system of a lethally irradiated recipient mouse [17] and to give rise to progeny with long-term repopulating ability [18]. The HSC-containing population can be quantified and / or separated using a number of different criteria, most notable of which is that pioneered by I. L. Weissman and colleagues using the presence or absence of various surface proteins to delineate HSC identity. In this paradigm, it is widely accepted that cells fitting a lineage marker dim (Lin^{dim}), $Sca\text{-}1^{+}$, $c\text{-}kit^{+}$ profile are in fact HSC [19]. Other common approaches utilize surface binding of lectins such as WGA [20] or exclusion of vital dyes such as rhodamine 123 [21] and Hoechst3342 [22]. HSC have been isolated in humans as well (reviewed in [23]).

There are a number of assays for testing HSC number / function, which all rely on measuring the long-term output of mature hematopoietic cells. These assays include long-term reconstitution, competitive repopulation, colony-forming-unit-spleen (CFU-S) based assays, long-term culture initiating cell assay (LTCIC), cobble-stone-forming cell assay (CAFC) (reviewed in [24]). The major limitation with these assays is that defects in HSC

or post-HSC progenitors lead to reductions in the read-out and may lead to overdrawn conclusion in terms of the stage of defect.

The HSC population is itself rather heterogeneous and can be separated into at least three subpopulations in mice, the long-term-HCS (LT-HSC), the short term-HSC (ST-HSC), and the multipotenital progenitor (MPP) based on surface expression of other markers Mac-1 and CD4 [25]. The long-term HSC (LT-HSC) type has the ability to fully reconstitute hematopoiesis for the lifespan of the organism. The short term HSC (ST-HSC) has limited self-renewal activity and can only repopulate all hematopoietic lineages for 8-10 weeks, while the multipotenital progenitor (MPP) has no self-renewal activity, and is therefore not a stem cell per se, and can only transiently repopulate multilineage hematopoiesis [25]. In addition, different activation states based on alternate markers, such as CD34 [26] and CD38 [27], and Flt3 [28,29] can be observed. In addition, strain differences can affect the markers used [30].

Post-HSC progenitors include the CLP and CMP, which are restricted to lymphoid and myeloid lineages respectively and have limited, if any, self-renewal potential. CLP have the ability to give rise to B-cells on stromal layers and T-cells in fetal thymic organ cultures (FTOC) as well as B-cells, T-cells, NK cells, and dendritic cells [31,32] *in vivo*. In addition, transplant of this sub-population can provide protection from CMV infection post HSC transplant [33]. CMP have the ability to give rise to *in vitro* colonies consisting of CFU-Mix, burst-forming units-erythroid (BFU-E), CFU-megakaryocyte (CFU-Meg), CFU-megakaryocyte / erythroid (CFU-MegE), CFU-granulocyte / macrophage (CFU-GM), CFU-granulocyte (CFU-G) and CFU-macrophage (CFU-M) as well as cells from all myeloid lineages *in vivo* [34]. In addition, this sub-

population can provide protection against fungal infection post HSC transplant [33]. This cell type gives rise to two progenitor subsets with further restricted myeloid potential, the GMP and the MEP. MEP give rise to CFU-Meg, BFU-E, or CFU-MegE colonies *in vitro* and *in vivo* [34,35]. In addition, these cells are sufficient to provide short-term radioprotection in lethally irradiated mice [36]. GMP give rise to CFU-M, CFU-G, or CFU-GM colonies *in vitro* and granulocytes and macrophages *in vivo* [34]. CMP have been isolated in humans as well [37].

Lineage-committed progenitors undergo specialized terminal differentiation processes in the bone marrow, and in the case of T-cells, in the thymus. The committed progenitors for B-cells [38], T-cells [39], and mast cells [40] have been isolated by surface phenotype, but the analogous progenitors for all other lineages have not been identified to date. Mature cells of the given lineages are highly specialized in both structure and function.

Important caveats to this schematic include the possibility that there are multiple pathways to derive some populations [41]. In addition, the schematic may be more accurately portrayed as a continuum rather than as discreet populations. Also, alternate models, such as the sequential model, have been proposed [42] and the current paradigm is still subject to debate [43-46].

Hematopoiesis demonstrates ontological changes in location and functional output during embryonic development. Fetal hematopoiesis is derived from both extraembryonic and intraembryonic tissue. Extraembryonically derived hematopoiesis is found in the yolk sac at day E7 [47] and is thought to be transient in nature. These hematopoietic cells are derived from the hemangioblast, a precursor that also gives rise to endothelial tissue

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or endothelial cells themselves [14]. The first intraembryonic hematopoietic cells, also found around day E7, are derived from the para-aortic-splanchnopleura (PAS) / aorta-gonad-mesonephros region (AGM) [47], probably from specialized endothelial cells [14]. Starting at day 10, these HSC move to the fetal liver, where they remain until they colonize the bone marrow and to some extent the spleen. As work from multiple groups show, fetal hematopoiesis is likely to follow rules that are similar to those for adult hematopoiesis, but with some distinctions [48]. Fetal liver HSC are defined by alternate markers [49] and are functionally disparate. They possess increased competitive repopulating ability [50]. Also, they possess distinct differentiative potential, as exemplified by the differences in the mature cells produced such as Vγ3 and V4 T-cells, B1a B-cells, and primitive macrophages that cannot be produced by adult HSC [51-54]. Also, differences in potential are demonstrated by the differential lineage relationships of the post-HSC progenitors derived from fetal liver HSC [55-57]. In addition, it is currently unclear whether there is a developmental switch converting fetal HSC to adult HSC or whether they are derived separately [48].

Although some functional aspects of the mammalian hematopoietic system are found in very early organisms from the Kingdom Animalia, the first recognizable forms are observed in organisms possessing true coelums, the Coelenterata, within the bilaterally symmetric organisms. Specialized blood cells that produce and transport oxygen-binding proteins (replacing soluble proteins in hemolymph) are first found in true coelumates, however only sporadically in various Phyla until Chordata.[58].

Cells involved in the professional performance of scavenging and innate immunity arose in coelumates as well. In *D. melanogaster*, there are at least two

"hemocyte" lineages produced during embryogenesis from the mesoderm, and later in a specialized tissue known as the lymph gland, already demonstrating a divergence in production sites during ontogeny [59]. The plasmatocytes are small rounded cells that are able to phagocytose apoptotic cells, foreign material, and pathogens, as well as to produce anti-microbial peptides. The second cell, called the crystal cell, is involved in the encapsulation of pathogens by melanization [60].

In addition, hemostasis, the process of maintaining the integrity of a circulation system, is first noted in these organisms. In *D. melanogaster,* this role is also undertaken by crystal cells. The melanization cascade, although not equivalent to the vertebrate clotting cascade, shares functional and some molecular similarities [61].

It is within the Craniata, of the Phylum Chordata, that hematopoiesis first closely resembles the same process found in mammals. In the jawed fish, *Danio rareo,* there are red blood cells [62] and thrombocytes [63], which share some structural features with mammalian platelets. In addition, *D. rareo* have a clotting cascade analogous to that found in mammalian organisms [64]. Multiple myeloid cell types with segmented nuclei join macrophages in innate immunity [65] and the adaptive branch of the immune system is first evident in jawed vertebrates, the Gnathostomata. It is now speculated that lymphocyte-like cells arose following genome duplication events and that the subsequent ability to rearrange Ig and TCR followed the genomic insertion of a transposase [66]. Although hematopoieis occurs in the kidney, secondary lymphoid organs such as the spleen and thymus are detected in jawed fish [67]. In addition, all Craniata possess a highly regulated vasculature system with endothelial cells [68]. These cells stem from the hemangioblast, a common ancestor with hematopoietic cells [62]. Perhaps most striking

is the presence of the sequential program of hematopoietic development, with stem cells and multipotential progenitors, in these organisms [62].

Many of the molecular mechanisms controlling both the differentiation and function of hematopoietic cells are conserved throughout phylogeny. Transcription factors, which are involved in the specification of stem cells and the commitment and differentiation of various lineages in mammals, such as GATA and RUNX family homologes, are found in both fish and flies [62,69]. Cytokines and cytokine signaling pathways, such as the JAK-STAT pathway [70,71], are also shared among these species. In short, hematopoiesis is both extremely old and conserved in its function, organization, and molecular regulation.

Homeostatic Regulation

Regulation of this rather complex developmental process is carried out by multiple inputs and strives to balance the somewhat opposing goals of hematopoiesis: to maintain a quiescent population of HSC available for continuing hematopoietic production through out the lifespan of the organism and to maintain a dynamic homeostasis in the production of the various hematopoietic cells with their different and opposing functions as dictated by the physiologic needs of the organism. The physiologic requirements of the organism are defined as the size of the peripheral compartment of a given lineage needed to optimally accomplish its function. Homeostasis is maintained by modulating two factors: the rate of production of a given lineage, and the half-life of the mature cells (Figure 2).

The half-life of mature cells of a given lineage at steady-state is highly variable depending on the lineage and ranges from a few hours for mature granulocytes [72], to a few days for platelets [73], to a few weeks for erythrocytes [74,75], and from a few days to up to years for lymphocyte [76-78] in the mouse. The half-life of mature cells can be altered by modulating their survival and in the case of erythrocytes and platelets, their removal by the reticuloendothelial system.

Both the total number of cell produced and the distribution of production among various cell lineages are dictated by the organismal requirements. At steady state, it has been estimated that 2.4×10^8 cells must be generated per day to maintain homeostasis in the mouse [79]. At steady state, distribution of production for a given lineage is inversely proportional to the half-life of the lineage and the size of the compartment, so granulocyte progenitors are the predominant lineage committed population in the bone marrow. Regulation of production is operationally achieved by altering the expansion of multi-potential and / or committed progenitors that are forbearers of the lineage or lineages needed (as well as mature cells in the case of lymphocytes).

Physiological stressors can lead to the alteration of the size of the peripheral compartment, the half-life of mature cells of a given lineage, and the production of mature cells by progenitors. These stressors include genetic lesions and environmental causes. The size of the peripheral compartment of a given lineage can be affected by alterations in the environmentally dictated demand for a given lineage, for example infection or change in pO2. The half-life of mature cells can be affected by the inability of mature cells to respond to appropriate stimuli and abnormalities leading to decreased survival of mature cells, either genetic or acquired. In addition, destruction of cell can be

mediated by non-specific blood loss, selective destruction of mature cells by pathogens, or aberrant immune response. The rate of production of mature cells can be affected by supply of appropriate stimuli and the ability of progenitors to respond to them, defects in the machinery that specifies lineage commitment, defects in the uptake and storage of various molecules necessary for development by support cells or utilization of these molecules by progenitors. In addition, selective destruction of progenitors by pathogens or aberrant immune response can occur as well.

Biologically, the regulation of production consists of controlling the behavior of HSC, post-HSC progenitors, and lineage committed progenitors. For HSC that translates into controlling the rate at which quiescent cells differentiate, self-renew, apoptose, or mobilize into peripheral tissue and home to bone marrow [80]. For post-HSC-progenitors and lineage committed progenitors that translates into controlling the rate at which cells differentiate, proliferate, apoptose, or mobilize into peripheral tissue and home to bone marrow (Figure 3).

Mechanistically, these biological behaviors are regulated utilizing two different approaches. First, hematopoietic cells can be controlled by soluble mediators either in systemic feedback loops or in microenvironments within the sites of hematopoietic production. Soluble mediators include two basic classes of molecules. First, protein mediators include growth factors and chemokines. Cytokines, TGFβ family members, and ligands for receptor tyrosine kinases are involved in positive or negative regulation of proliferation, survival, and differentiation. Chemokines are involved primarily in the regulation of chemotaxis. Second, non-protein molecules are involved in controlling proliferation, survival, differentiation, and chemotaxis. Hematopoietic cells can also be

controlled via cellular interactions with mediators anchored to other cells and to extracellular matrix components (ECM). These mediators include cytokines or chemokines anchored to cells or to ECM, involved in the same regulation as above. In addition, transmembrane ligands for the TNF family of death receptors, and transmembrane adhesion molecules such as integrins, lectins, and sialomucins are involved in the regulation of proliferation, survival, differentiation, and adhesion.

Cytokines and Receptors

One broad class of soluble mediators are the helical cytokines, which comprise a large family of small molecular weight glycoproteins with an anti-parallel four-helix bundle structure with diverse biological activities [81,82]. The study of these molecules as regulators of hematopoiesis was initiated with *in vitro* colony assays in the presence of conditioned media [83,84]. Their subsequent purification and characterization *in vitro*, the elucidation of their physiologic role through the use of purified protein administration, transgenics, and gene targeting in mice, and their clinical utility, has solidified this family of molecules as critical for the efficient regulation of hematolymphoid development. Some features of this family include, pleotropic effects, synergism with other factors, and, often, redundancy of function. Cytokines also tend to act at multiple stages in hematopoietic development. For example, many act on the HSC themselves, such as granulocyte-colony stimulating factor (G-CSF), interleukin-6 (IL-6), interleukin-11 (IL-11), thrombopoietin (TPO), and leukemia inhibitory factor (LIF) [85] Others, such as erythropoietin (EPO), TPO, and G-CSF, act on lineage-committed

progenitors exclusively or in addition to their earlier effects [86-88]. For a comprehensive review of *in vivo* roles of various cytokines see [89].

There are two families of cytokines known as type I and type II. Cytokines that utilize class I cytokine receptors in particular play an indispensable role in controlling hematopoietic development and function. These Class I, or hematopoietin, receptors have the following characteristics. In the extracellular domain, the receptors share a number of conserved cysteine residues and a characteristic WSXWS domain [82,90]. With the exception of these characteristics, cytokine receptors are fairly diverse, and can be subdivided, based on order of oligimerization, structural similarities, and shared subunits, into the GH family, βc family, gp130 family, gp130 related family, and the γc family. Intracellularly, most of the sub-family members possess a conserved multi-domain Jak-binding region and a number of distal tyrosines. Activation of these receptor complexes involves ligand-induced dimerization and conformational shifts [82].

Ligand-induced activation of cytokine receptors has been hypothesized to have a number of potential biological effects on target cells within the hematopoietic system, including regulation of the actions of proliferation, apoptosis, mobilization / homing, and differentiation mentioned above.

Cellular division is the result of a tightly regulated cell cycle in which DNA is doubled and the cellular components divided, resulting in two daughter cells. The cell cycle is regulated by an independently acting system that controls downstream processes corresponding to the different phases of the cell cycle. Quiescent cells are in G0. These cells move into G1 to initiate the protein synthesis required for DNA synthesis. To pass the G1/S transition, cyclin proteins are upregulated by extrinsic and intrinsic factors.

These proteins interact with cyclin dependent kinases, which direct downstream cell cycle processes via phosphorylation of target molecules, including Rb-E2F complexes, releasing its inhibition of entry into the cell cycle, and DNA synthesis machinery. After S-phase, G2 represents a gap in the cycle during which DNA synthesis is checked for completion. Other cyclin / cdk complexes regulate the checkpoint before M-phase, during which the physical act of cell division, mitosis, occurs [91]. Cytokine-mediated control of proliferation occurs at the G1/G0 to S transition, as cells committed to S-phase are largely insensitive to growth factors. Growth factors regulate this transition through upregulation of members of the Cyclin D family to contribute to the formation of cyclin / cyclin dependent kinase (cdk) complexes noted above. In addition, cytokines are known to be involved in the regulation of cdk-inhibitiors of the INK4 class (p16, p19) and KIP/CIP class (p21, p27), which antagonize cdk activity [92].

Mobilization of HSC and progenitors into the periphery and its' converse, homing to hematopoietic niches, are processes with unclear physiologic relevance, but huge clinical applications. Homing of hematopoietic stem and progenitor cells to the bone marrow occurs naturally in development as these cells migrate from the fetal liver beginning at E17 [93] and mobilized progenitors appear to continue to home at low levels throughout life. The process of homing to hematopoietic niches is best understood for bone marrow and it is thought to be regulated by interactions of the chemokine SDF-1 with its receptor CXCR4 [94,95] and upregulation of adhesion molecules, especially β1-integrins (VCAM) [96,97] and sialomucins such as CD34 [98]. Mobilization of stem and progenitor cells into the peripheral blood and secondary sites of hematopoiesis such as the spleen and liver has been known for over 40 years [99] and the physiologic relevance

of this process has been explored [100,101]. Mobilization is generally considered to involve disruption of the same pathways utilized to achieve homing of the cells to hematopoietic niches. Cytokine-mediated control of homing and mobilization consists of increasing or decreasing the expression or function of the chemokine and adhesion molecules that mediate these processes. Administration of a number of cytokines, including G-CSF, interleukin-3 (IL-3), granulocyte / macrophage-colony stimiltaing factor (GM-CSF), TPO, fms-like tyrosine kinase 3 ligand (Flt3L), stem cell factor (SCF), and interleukin-8 (IL-8), as well as myeloablation via radiation and cytotoxic drugs increases the rate of mobilization of these cells. Mobilization via G-CSF has been the most extensively studied and may be a common pathway for mobilization. G-CSF mobilizes neutrophils as well as progenitors. However, this process is not a non-specific release of bone marrow cells, as other cells such as eosinophils are not released [102]. G-CSF is thought to operate indirectly on progenitors by causing the G-CSF receptor-dependent release of proteases. These proteases cleave proteins important for the adhesion of stem and progenitor cells within the bone marrow [103-105], such as VCAM, known to be important in mobilization [106]. In addition other mechanisms may be important, such as the downregulation of SDF-1 concomitant with upregulation its receptor [107,108], or upregulation of other proteases in non-hematopoietic cells [109] or directly impacting function of adhesion molecules [110].

Differentiation can be divided into two processes in hematopoietic development. The first is the decision of differentiation versus self-renewal for stem cells. The regulation of this process likely involves transcription factors that help to maintain "stemness", such as homeobox genes [111,112] and Ikaros family members [113], as

well as extrinsic factors that promote self-renewal division over differentiation division, including members of the Notch [114,115], Wnt [116], TNF-α [117], and bone morphogenic protein (BMP) [118] families. In addition, molecules that maintain quiescence, such as TGF-β [119,120], appear to positively affect stem cell maintenance perhaps through regulation of cdki p21 [121]. The role of cytokines in this process is thought to favor differentiation division [122], although some combinations are reported to expand stem cells [123]. The molecules involved are not well understood and efforts are now being made to define stem cell specific molecules, which may be important [124]. The second process of differentiation is the decision to commit to a specific lineage (or set of lineages) from multiple choices for multi-potential progenitors. Lineage specification is currently thought to involve positive regulation of a specific lineage program [125] concomitant with negative regulation of alternate lineage programs [126] by transcription factors within the cell. Recent evidence supports a model in which multipotential progenitors are "primed", i.e. express lineage specific transcription factors for multiple lineages simultaneously [127-129]. Competing factors are known to antagonize the transcriptional activity of one another at the protein level, hypothetically resulting in dominance of one factor, which is then reinforced. This leads to the downregulation of other factors and the lineage of the dominant factor is chosen at the expense of others [11]. The role of cytokine receptor signaling in differentiation and commitment to a specific lineage path and the subsequent differentiation is a point of much debate. Two paradigms have been proposed and consist of the stochastic and the instructive models. There is compelling evidence for each of these and there is also the possibility that elements of both models may be applicable. The stochastic model states

that the lineage of a progenitor cell is "chosen" in a random manner by the cell and that the ensuing differentiation is an intrinsic program without need for any external signals for its completion. In this model cytokine receptors merely allow the cell to escape apoptosis and to proliferate. Swapping of the cytoplasmic tails of cytokine receptors *in vivo* results in normal lineage commitment in multiple cases [130,131]. Bcl-2 can rescue differentiation of some multipotential progenitor cells in the absence of cytokine-signaling *in vitro* [132] and *in vivo* [133,134]. These data collectively imply a purely survival function for cytokines.

However, there is a large body of evidence that supports an instructive model. The instructive model states that a progenitor cell is instructed to follow a specific lineage path and that the differentiation program will not move to fulfillment in the absence of these receptor-specific signals. This model implies that proliferative, anti-apoptotic, and specific differentiative signals can all be generated by cytokine receptors. There is also a large body of results supporting this model. The combination of cytokines given in the medium can dramatically skew the number of lineage-committed precursors toward a particular lineage [135]. Furthermore, ectopic expression of various cytokines or receptors can result in lineage-specific gene expression in cell lines [136-138] and transdiffierentiation to myeloid lineages in CLP and Pro-T cells [139]. Thus, these findings assign some level of differentiation instruction to certain cytokine receptors.

In addition, some evidence supports a compromise of these two models. Bcl-2 can only rescue specific differentiation phenotypes in another G-CSF-dependent cell line [140]. This result implies that at this stage in lineage commitment, there is an intrinsic "program" that directs some differentiation processes, while another program requires

direct and specific G-CSF receptor signals. Retroviral delivery of a constitutively activated EPO receptor with EPOR, GHR, or c-mpl cytoplasmic tails result in erythrocytosis, while receptors with G-CSFR resulted in half of the animals displaying anemia and neutrophilia [141].

Apoptosis and cytokine-mediated regulation of this process will be discussed in detail in Chapter 3.

Signaling Pathways

Cytokine receptors utilize multiple downstream signaling pathways to effect biological outcomes. Many cytokine receptors utilize catalytic domains located within their cytoplasmic tails to transduce signals [82]. However, cytokine receptors of the Class I family do not possess any nascent enzymatic activity, but instead rely on associated kinases of the Janus (JAK) and Src family for generating intracellular signals. The JAK kinases are required for cytokine receptor signaling as their removal *in vitro* or *in vivo* abolishes all downstream signaling [81]. Upon ligand-induced dimerization, the associated JAK kinases cross-phosphorylate each other, undergo a conformational shift that increases their activity, and phosphorylate the cytoplasmic tyrosine of the cytokine receptors. These tyrosines serve as docking sites for SH2 domains on other signaling molecules, which are then phosphorylated by the JAK kinases and other associated kinases.

Downstream pathways that are recruited and triggered by cytokine receptors include signal transducers and activators of transcription (STAT), the Ras / mitogen activated protein kinase (MAPK) pathway, the phosphatidylinositol 3-kinase (PI3K) /

Akt pathway, and the PI3K / phospholipase C γ (PLCγ) pathway, Src kinases family members, the Pyk2 pathway, and perhaps others not yet characterized (Figure 4) [81,142]. The relative contribution of each of these pathways to the biological outcomes executed by various cytokines *in vivo* requires further study.

Cytokine receptors are also subject to multiple pathways of negative regulation. First, receptor-mediated endocytosis and degradation limit the lifespan of receptors on the cell surface. Second, critical tyrosine residues are dephosphorylated by receptor activated phosphotases, such as SHP. Finally, members of the CIS family interfere with binding of proteins to SH2 domains on the receptor and target various components of the receptor complex for degradation (reviewed in [143]).

How this family of receptors generates specificity in biologic outcomes has been a point of considerable interest. It probably involves the combination of multiple mechanisms including spatial and quantitative limits on availability of the cytokine, restricted receptor expression, and the state of the target cell [81]. In addition, although these pathways are often studied or considered as separate entities, it is important to recognize that they are actually subunits of highly interconnected networks. Signals from multiple different inputs, both within the cytokine receptors family and from other sources activate overlapping sets of pathways that together effect biological outcomes. Processing of the combination of incoming signals likely results from both summation of inputs as well as the temporal characteristics of the pathways [144,145].

STAT Factors

The discovery of the JAK-STAT pathway [146] appeared to offer one mechanism through which cytokine receptors could selectively upregulate distinct target genes and be responsible for specific biological behaviors through the mechanism of distinct intracellular signaling pathways. STATs are cytoplasmically located, latent transcription factors that are activated by JAK kinases and receptor tyrosine kinases. There are seven STAT proteins, denoted STAT1, STAT2, STAT3, STAT4, STAT5A, STAT5B, and STAT6. STAT proteins share some basic structural features, including an N-terminal domain important for tetramerization, a coiled-coil domain, a DNA binding domain, a linker domain, an SH2 domain, and a C-terminal domain containing both a critical tyrosine residue and a transactivation domain. The SH2 domain of STAT proteins is involved in recruitment to phosphotyrosines on the cytoplasmic tails of receptors. Differences in SH2 domain structure are thought to lead to the specificity of STAT-receptor interactions [147,148]. In addition, there is evidence that STATs can be recruited to bind to the JAK kinases directly [142]. Upon binding to a phosphotyrosine motif in an activated receptor complex, the STAT protein is phosphorylated on the C-terminal tyrosine. The molecule then homo-oligimerizes with other STAT molecules allowing translocation into the nucleus perhaps through internalization of a nuclear import signal. Once in the nucleus, STATs bind DNA and increase the transcription of target genes by binding to specific DNA sequence motifs known as the γ-interferon-activated site (GAS). [142,149-151].

STAT proteins are thought to display different target specificity dependent on the dimerization partners and whether dimerization or tetramerization is involved [152]. In addition, STAT proteins often accomplish transcriptional activation in conjunction with

other nuclear factors. Various genes have been shown to be upregulated by STAT molecules, including those involved in cell cycle progression, anti-apoptosis, and regulation of cytokine signaling (reviewed in [153]).

The realization that there were a very limited number of these proteins and that many were activated by multiple cytokine receptors with different functions, reduced the emphasis for the one STAT, one function-model. However, STAT signaling is still thought to be important for contributing to specificity of signaling by cytokine receptors. Specificity of signaling is though to de dependent on the receptor-specific STAT complexes activated, cell-specific expression of STAT proteins, crosstalk between receptors, and interaction with co-factors.

STAT5

One member of the STAT family, STAT5, was first characterized as mediating signal transduction from the prolactin (PRL) receptor [154,155]. This molecule has subsequently been shown to be activated by diverse cytokine receptors involved at multiple levels within the hematopoietic system and in other tissues (Figure 5) in addition to receptor tyrosine kinases and non-receptor tyrosine kinases [156-159]. The widespread engagement of its two isoforms, STAT5A and STAT5B, has implicated STAT5 as a potentially important component of cytokine receptor signaling in hematopoietic cells. Cell culture studies supported a role for STAT5 in hematolymphoid development. For example, STAT5 was shown to be involved in EPO-induced proliferation of TF-1 cells [160] and in IL-2 mediated survival of 32D cells [161]. STAT5 has also been implicated

in both interleuken-3 (IL-3)-induced proliferation and G-CSF-induced differentiation in 32D cells [162] and the proliferation and survival of Baf3 cells [163].

STAT5 is known to upregulate the proteins in hematopoietic cells that are involved in the regulation of proliferation, such as Cyclin D1 [164], c-myc [165], Cyclin D2 [166], and p21 [167]; survival, such as Bcl-x [168-170], Bcl-2 [164], pim-1 [171], and fas [172]; and growth factor signaling, such as oncostatin-M (OSM) [173] and IL-2Rα [174]. STAT5 also upregulates transcription of multiple genes involved in the specific function of non-hematopoietic cells, including β-casein [175], β-lactoglobulin [176], bile acid co-transporter [177], insulin [178], and aromatase [179]. In addition, STAT5 upregulates cytokine–induced suppressor (CIS), a protein involved in the negative regulation of cytokine signaling [180,181]. Naturally occurring forms of STAT5 that are truncated and function as dominant negatives have been found [182-184].

STAT5 is known to interact with a number of nuclear factors, which may add to its function, such as the glucocorticoid receptor and N- myc interactor (NMI) [185,186].

To determine physiologic relevance of STAT5 in cytokine signaling, various gene deficient mice were generated. Mice deficient for either STAT5A [187] or STAT5B [188] were found to exhibit dramatic defects in specific non-hematopoietic tissues, which demonstrated indispensable roles for these two isoforms in mammary gland development, and body growth and liver gene expression, respectively. These models confirmed the role of STAT5 in signal transduction from growth hormone (GH) and PRL receptors that had been demonstrated in cell culture. In addition, these models demonstrated a divergence of function for the two molecules *in vivo*. However, the individual isoform-deficient mice possessed only subtle alterations in the regulation of hematopoietic cells

[189-191], which led to the hypothesis that they could compensate for each other in this context. Despite the expectations that a compound knock-out would reveal importance in hematopoietic development, mice deficient in both the STAT5A and STAT5B isoforms were reported to have no further defects in the production of mature blood cells of various lineages with the exception of decreased numbers of peripheral T-cells [192]. However, some evidence of additional hematologic dysregulation was evident in these animals, such as reduced bone marrow colony counts *in vitro* and notable extramedullary hematopoiesis [192,193]. In addition, marked fetal anemia *in vivo* as well as defects in EPO-dependent production and survival of fetal liver hematopoietic colonies *in vitro* were subsequently reported [194].

A certain expectation of importance for STAT5 in hematolymphoid development had been dictated by its profiles of activation by class I cytokine receptors and extensive *in vitro* data demonstrating its key role in signaling by this family of receptors. However, *in vivo* evidence produced by multiple groups provided an unclear and contradictory picture of the importance of this molecule in hematolymphoid development. Elucidating the role of STAT5 *in vivo* at the start of this work was a critical objective in the fields of cytokine receptor biology and hematopoietic development. This was an important aim, not only for determining the role of STAT5 and the cytokines that activate it in this process, but for furthering our understanding of the molecular mechanisms of cytokine signaling in general. The goal of this project was therefore to extensively characterize the functions of STAT5 *in vivo* using mouse genetics and rigorous assays for hematopoietic development in order to clarify these issues.

Figure Legends

Figure 1. Hematopoietic development proceeds through stages of increasingly restricted developmental potential and proliferative capacity. Pictured is a simplified schematic, which represents the differentiation of HSC to mature blood cells via intermediate populations, such as the common lymphoid progenitor (CLP), common myeloid progenitor (CMP), granulocyte-monocyte progenitor (GMP), megakaryocyte-erythroid progenitor (MEP), and lineage-committed progenitors.

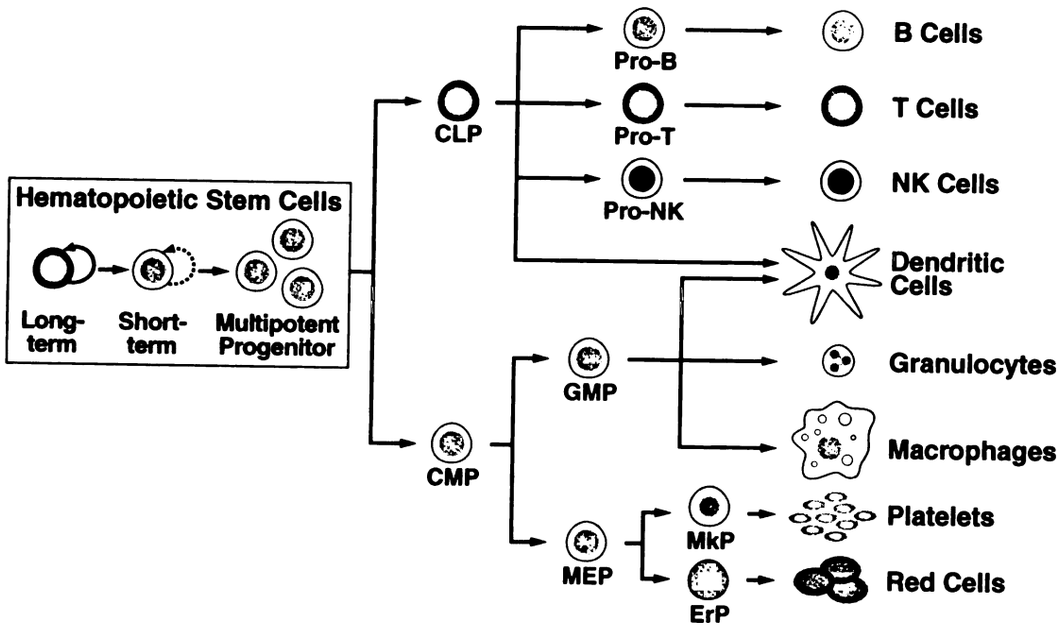
Figure 2. Homeostasis in hematopoietic development is set by the physiologic needs of the organism. The physiologic requirements of the organism is defined as the size of the peripheral compartment of a given lineage needed to optimally accomplish its function (box). Homeostasis is maintained by modulating two factors: the rate of production of a given lineage, and the half-life of the mature cells (arrows).

Figure 3. Hematopoietic cell production is regulated by controlling the biological behavior of progenitor cells. Biologically, the regulation of production consists of controlling the behavior of HSC, post-HSC progenitors, and lineage committed progenitors. That translates into controlling the rate at which quiescent cells differentiate, self-renew, proliferate, apoptose, or mobilize into peripheral tissue and home to bone marrow.

Figure 4. Type I cytokine receptors activate multiple dounstream signal transduction pathways. Downstream pathways that are recruited and triggered by cytokine receptors via their JAK kinases include the signal transducers and activators of transcription (STAT) pathway (black), the Ras / mitogen activated protein kinase (MAPK) pathway (gray), the phosphatidyl-inositol 3-kinase (PI3K) / Akt pathway, and the PI3K / phospholipase C γ (PLCγ) pathway (white), as well as Src kinases family members, the Pyk2 pathway, and others not yet characterized.

Figure 5. STAT5 is activated by a diverse array of cytokine receptors. STAT5 has been shown to be activated by diverse cytokine receptors involved at multiple levels within the hematopoietic system and in other tissues in addition to receptor tyrosine kinases and non-receptor tyrosine kinases (not shown).

Figure 1



Adapted from Reya *et al*, 2001, *Nature*, Vol 414:105

Figure 2

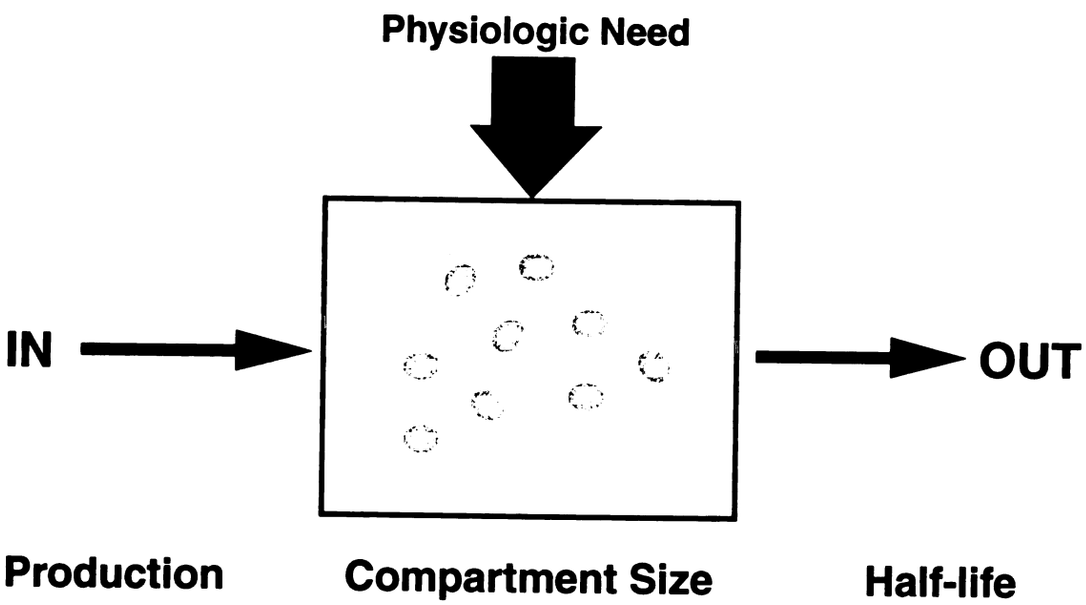
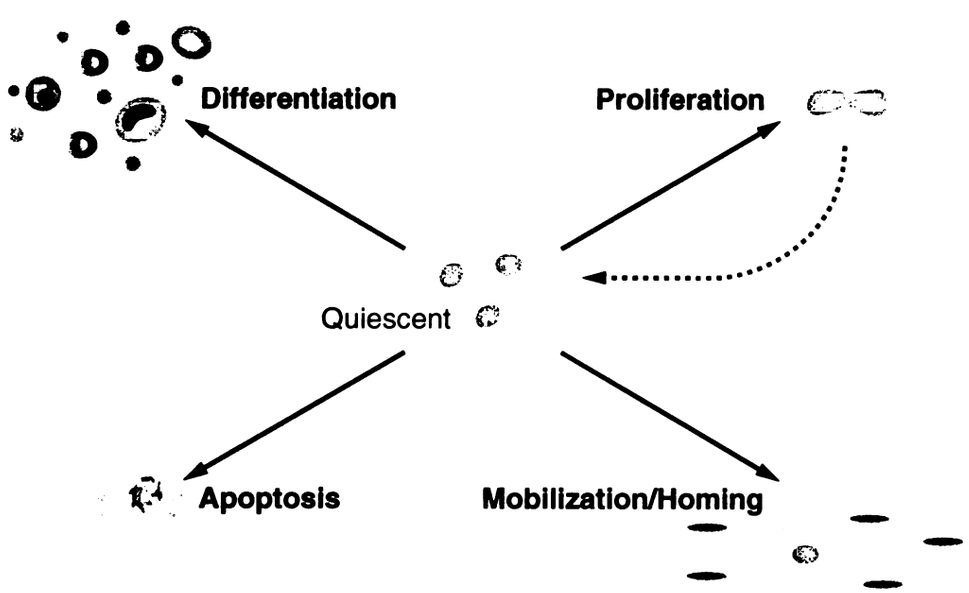

Physiologic Need
IN
OUT
Production
Compartment Size
Half-life

Figure 3



Adapted from Domen *et al*, 2000, *JEM*, Vol 191:253

Figure 4

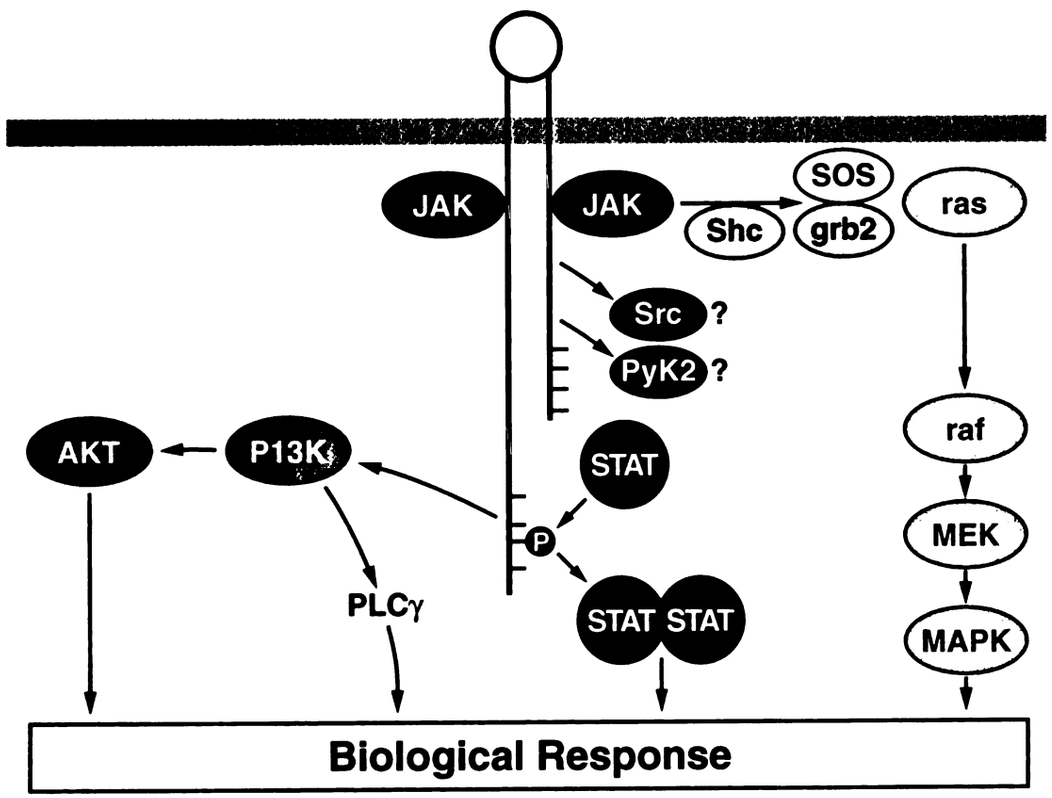

JAK
JAK
SOS
Shc
grb2
ras
Src
?
PyK2
?
raf
AKT
P13K
STAT
MEK
P
PLCγ
STAT STAT
MAPK
Biological Response

Figure 5

Hematopoietic Receptors		Non-Hematopoietic Receptors
EPO	IL-2	Growth Hormone
G-CSF	IL-7	Prolactin
c-MPL	IL-9	Leptin
IL-3	IL-15	
GM-CSF	IL-4	
IL-5	IL-13	

References

1. Cronkite (1959). In: F Stohlman (ed) The kinetics of cellular proliferation. New York: Grune and Stratton

2. Lorenz E, Uphoff D, Reid TR, Shelton E (1951) Modification of irradiation injury in mice and guinea pigs by bone marrow injections. J Natl Cancer Inst. 12:197

3. Urso P, Congdon CC (1957) The Effect of the Amount of Isologous Bone Marrow Injected on the Recovery of Hematopoietic Organs, Survival and Body Weight after Lethal Irradiation Injury in Mice. Blood 12:251

4. Thomas ED, Lochte H. L., Lu W. C., and Ferrebee J. W. (1959) Intravenous Infusion of Bone Marrow in Patients Receiving Radiation and Chemotherapy. New England Journal of Medicine 257:491

5. Wu AM, Till JE, Siminovitch L, McCulloch EA (1967) A cytological study of the capacity for differentiation of normal hemopoietic colony-forming cells. J Cell Physiol 69:177

6. Till JE, McCulloch GA (1961) A direct measurement of the radiation sensitivity of normal mouse bone marrow cells. Radiation Res. 14:213

7. Wu AM, Till JE, Siminovitch L, McCulloch EA (1968) Cytological evidence for a relationship between normal hemotopoietic colony-forming cells and cells of the lymphoid system. J Exp Med 127:455

8. Abramson S, Miller RG, Phillips RA (1977) The identification in adult bone marrow of pluripotent and restricted stem cells of the myeloid and lymphoid systems. J Exp Med 145:1567

9. Metcalf D (1984) The hematopoietic colony stimulating factors. Amsterdam: Amsterdam

10. Spooncer E, Heyworth CM, Dunn A, Dexter TM (1986) Self-renewal and differentiation of interleukin-3-dependent multipotent stem cells are modulated by stromal cells and serum factors. Differentiation 31:111

11. Orkin SH (2000) Diversification of haematopoietic stem cells to specific lineages. Nat Rev Genet 1:57

12. Wagers AJ, Sherwood RI, Christensen JL, Weissman IL (2002) Little evidence for developmental plasticity of adult hematopoietic stem cells. Science 297:2256

13. Wagers AJ, Christensen JL, Weissman IL (2002) Cell fate determination from stem cells. Gene Ther 9:606

14. Orkin SH, Zon LI (2002) Hematopoiesis and stem cells: plasticity versus developmental heterogeneity. Nat Immunol 3:323

15. Dorshkind K (2002) Stem cells and lineage plasticity: the challenge to existing paradigms. Immunol Rev 187:5

16. French SW, Hoyer KK, Shen RR, Teitell MA (2002) Transdifferentiation and nuclear reprogramming in hematopoietic development and neoplasia. Immunol Rev 187:22

17. Smith LG, Weissman IL, Heimfeld S (1991) Clonal analysis of hematopoietic stem-cell differentiation in vivo. Proc Natl Acad Sci U S A 88:2788

18. Morrison SJ, Wandycz AM, Hemmati HD, Wright DE, Weissman IL (1997) Identification of a lineage of multipotent hematopoietic progenitors. Development 124:1929

19. Spangrude GJ, Heimfeld S, Weissman IL (1988) Purification and characterization of mouse hematopoietic stem cells. Science 241:58

20. Visser JW, Bauman JG, Mulder AH, Eliason JF, de Leeuw AM (1984) Isolation of murine pluripotent hemopoietic stem cells. J Exp Med 159:1576

21. Spangrude GJ, Johnson GR (1990) Resting and activated subsets of mouse multipotent hematopoietic stem cells. Proc Natl Acad Sci U S A 87:7433

22. Goodell MA, Brose K, Paradis G, Conner AS, Mulligan RC (1996) Isolation and functional properties of murine hematopoietic stem cells that are replicating in vivo. J Exp Med 183:1797

23. Bonnet D (2002) Haematopoietic stem cells. J Pathol 197:430

24. Domen J, Weissman IL (1999) Self-renewal, differentiation or death: regulation and manipulation of hematopoietic stem cell fate. Mol. Med. Today 5:201

25. Morrison SJ, Weissman IL (1994) The long-term repopulating subset of hematopoietic stem cells is deterministic and isolatable by phenotype. Immunity 1:661

26. Sato T, Laver JH, Ogawa M (1999) Reversible expression of CD34 by murine hematopoietic stem cells. Blood 94:2548

27. Randall TD, Lund FE, Howard MC, Weissman IL (1996) Expression of murine CD38 defines a population of long-term reconstituting hematopoietic stem cells. Blood 87:4057

28. Adolfsson J, Borge OJ, Bryder D, Theilgaard-Monch K, Astrand-Grundstrom I, Sitnicka E, Sasaki Y, Jacobsen SE (2001) Upregulation of Flt3 expression within the bone marrow Lin(-)Sca1(+)c-kit(+) stem cell compartment is accompanied by loss of self-renewal capacity. Immunity 15:659

29. Christensen JL, Weissman IL (2001) Flk-2 is a marker in hematopoietic stem cell differentiation: a simple method to isolate long-term stem cells. Proc Natl Acad Sci U S A 98:14541

30. Spangrude GJ, Brooks DM (1992) Phenotypic analysis of mouse hematopoietic stem cells shows a Thy-1-negative subset. Blood 80:1957

31. Manz MG, Traver D, Miyamoto T, Weissman IL, Akashi K (2001) Dendritic cell potentials of early lymphoid and myeloid progenitors. Blood 97:3333

32. Kondo M, Weissman IL, Akashi K (1997) Identification of clonogenic common lymphoid progenitors in mouse bone marrow. Cell 91:661

33. Brown JM, Weissman IL, Shizuru JA (2001) Immunity to infections following hematopoietic cell transplantation. Curr Opin Immunol 13:451

34. Akashi K, Traver D, Miyamoto T, Weissman IL (2000) A clonogenic common myeloid progenitor that gives rise to all myeloid lineages. Nature 404:193

35. Vannucchi AM, Paoletti F, Linari S, Cellai C, Caporale R, Ferrini PR, Sanchez M, Migliaccio G, Migliaccio AR (2000) Identification and characterization of a bipotent (erythroid and megakaryocytic) cell precursor from the spleen of phenylhydrazine-treated mice. Blood 95:2559

36. Na Nakorn T, Traver D, Weissman IL, Akashi K (2002) Myeloerythroid-restricted progenitors are sufficient to confer radioprotection and provide the majority of day 8 CFU-S. J Clin Invest 109:1579

37. Manz MG, Miyamoto T, Akashi K, Weissman IL (2002) Prospective isolation of human clonogenic common myeloid progenitors. Proc Natl Acad Sci U S A 99:11872

38. Hardy RR, Carmack CE, Shinton SA, Kemp JD, Hayakawa K (1991) Resolution and characterization of pro-B and pre-pro-B cell stages in normal mouse bone marrow. J Exp Med 173:1213

39. Godfrey DI, Kennedy J, Suda T, Zlotnik A (1993) A developmental pathway involving four phenotypically and functionally distinct subsets of CD3-CD4-CD8- triple-negative adult mouse thymocytes defined by CD44 and CD25 expression. J Immunol 150:4244

40. Rodewald HR, Dessing M, Dvorak AM, Galli SJ (1996) Identification of a committed precursor for the mast cell lineage. Science 271:818

41. Montecino-Rodriguez E, Leathers H, Dorshkind K (2001) Bipotential B-macrophage progenitors are present in adult bone marrow. Nat Immunol 2:83

42. Brown G, Bunce CM, Lord JM (1990) Models of haemopoiesis. Leuk Res 14:495

43. Spangrude GJ (2002) Divergent models of lymphoid lineage specification: do clonal assays provide all the answers? Immunol Rev 187:40

44. Rothenberg EV, Dionne CJ (2002) Lineage plasticity and commitment in T-cell development. Immunol Rev 187:96

45. Quesenberry PJ, Colvin GA, Lambert JF (2002) The chiaroscuro stem cell: a unified stem cell theory. Blood 100:4266

46. Allman D, Sambandam A, Kim S, Miller JP, Pagan A, Well D, Meraz A, Bhandoola A (2003) Thymopoiesis independent of common lymphoid progenitors. Nat Immunol 4:168

47. Dzierzak E, Medvinsky A, de Bruijn M (1998) Qualitative and quantitative aspects of haematopoietic cell development in the mammalian embryo. Immunol Today 19:228

48. Bonifer C, Faust N, Geiger H, Muller AM (1998) Developmental changes in the differentiation capacity of haematopoietic stem cells. Immunol Today 19:236

49. Morrison SJ, Hemmati HD, Wandycz AM, Weissman IL (1995) The purification and characterization of fetal liver hematopoietic stem cells. Proc Natl Acad Sci U S A 92:10302

50. Harrison DE, Zhong RK, Jordan CT, Lemischka IR, Astle CM (1997) Relative to adult marrow, fetal liver repopulates nearly five times more effectively long-term than short-term. Exp Hematol 25:293

51. Havran WL, Allison JP (1988) Developmentally ordered appearance of thymocytes expressing different T-cell antigen receptors. Nature 335:443

52. Ikuta K, Kina T, MacNeil I, Uchida N, Peault B, Chien YH, Weissman IL (1990) A developmental switch in thymic lymphocyte maturation potential occurs at the level of hematopoietic stem cells. Cell 62:863

53. Naito M, Umeda S, Yamamoto T, Moriyama H, Umezu H, Hasegawa G, Usuda H, Shultz LD, Takahashi K (1996) Development, differentiation, and phenotypic heterogeneity of murine tissue macrophages. J Leukoc Biol 59:133

54. Hardy RR, Hayakawa K (1991) A developmental switch in B lymphopoiesis. Proc Natl Acad Sci U S A 88:11550

55. Lu M, Kawamoto H, Katsube Y, Ikawa T, Katsura Y (2002) The common myelolymphoid progenitor: a key intermediate stage in hemopoiesis generating T and B cells. J Immunol 169:3519

56. Mebius RE, Miyamoto T, Christensen J, Domen J, Cupedo T, Weissman IL, Akashi K (2001) The fetal liver counterpart of adult common lymphoid progenitors gives

rise to all lymphoid lineages, CD45+CD4+CD3- cells, as well as macrophages. J Immunol 166:6593

57. Traver D, Miyamoto T, Christensen J, Iwasaki-Arai J, Akashi K, Weissman IL (2001) Fetal liver myelopoiesis occurs through distinct, prospectively isolatable progenitor subsets. Blood 98:627

58. Terwilliger NB (1998) Functional adaptations of oxygen-transport proteins. J Exp Biol 201 (Pt 8):1085

59. Lanot R, Zachary D, Holder F, Meister M (2001) Postembryonic hematopoiesis in Drosophila. Dev Biol 230:243

60. Kimbrell DA, Beutler B (2001) The evolution and genetics of innate immunity. Nat Rev Genet 2:256

61. De Gregorio E, Han SJ, Lee WJ, Baek MJ, Osaki T, Kawabata S, Lee BL, Iwanaga S, Lemaitre B, Brey PT (2002) An immune-responsive Serpin regulates the melanization cascade in Drosophila. Dev Cell 3:581

62. Thisse C, Zon LI (2002) Organogenesis--heart and blood formation from the zebrafish point of view. Science 295:457

63. Jagadeeswaran P, Sheehan JP, Craig FE, Troyer D (1999) Identification and characterization of zebrafish thrombocytes. Br J Haematol 107:731

64. Sheehan J, Templer M, Gregory M, Hanumanthaiah R, Troyer D, Phan T, Thankavel B, Jagadeeswaran P (2001) Demonstration of the extrinsic coagulation pathway in teleostei: identification of zebrafish coagulation factor VII. Proc Natl Acad Sci U S A 98:8768

65. Bennett CM, Kanki JP, Rhodes J, Liu TX, Paw BH, Kieran MW, Langenau DM, Delahaye-Brown A, Zon LI, Fleming MD, Look AT (2001) Myelopoiesis in the zebrafish, Danio rerio. Blood 98:643

66. Litman GW, Anderson MK, Rast JP (1999) Evolution of antigen binding receptors. Annu Rev Immunol 17:109

67. Willett CE, Cortes A, Zuasti A, Zapata AG (1999) Early hematopoiesis and developing lymphoid organs in the zebrafish. Dev Dyn 214:323

68. Shigei T, Tsuru H, Ishikawa N, Yoshioka K (2001) Absence of endothelium in invertebrate blood vessels: significance of endothelium and sympathetic nerve/medial smooth muscle in the vertebrate vascular system. Jpn J Pharmacol 87:253

69. Fossett N, Schulz RA (2001) Functional conservation of hematopoietic factors in Drosophila and vertebrates. Differentiation 69:83

70. Lieschke GJ (2001) Zebrafish--an emerging genetic model for the study of cytokines and hematopoiesis in the era of functional genomics. Int J Hematol 73:23

71. Hou SX, Zheng Z, Chen X, Perrimon N (2002) The Jak/STAT pathway in model organisms: emerging roles in cell movement. Dev Cell 3:765

72. Basu S, Hodgson G, Katz M, Dunn AR (2002) Evaluation of role of G-CSF in the production, survival, and release of neutrophils from bone marrow into circulation. Blood 100:854

73. Baker GR, Sullam PM, Levin J (1997) A simple, fluorescent method to internally label platelets suitable for physiological measurements. Am J Hematol 56:17

74. Goodman JWaS, L. H. (1961) Eryhtrocyte life span in normal mice and in radiation bone marrow chimeras. Am J Physiol 200:764

75. Bernstein ESRaSE (1966) Blood and Blood Formation. In: EL Green (ed) Biology of the Laboratory Mouse, 2nd edition. New York: McGraw-Hill Book Company, 351

76. Slifka MK, Antia R, Whitmire JK, Ahmed R (1998) Humoral immunity due to long-lived plasma cells. Immunity 8:363

77. Tough DF, Sprent J (1994) Turnover of naive- and memory-phenotype T cells. J Exp Med 179:1127

78. Hao Z, Rajewsky K (2001) Homeostasis of peripheral B cells in the absence of B cell influx from the bone marrow. J Exp Med 194:1151

79. Cheshier SH, Morrison SJ, Liao X, Weissman IL (1999) In vivo proliferation and cell cycle kinetics of long-term self-renewing hematopoietic stem cells. Proc Natl Acad Sci U S A 96:3120

80. Domen J (2001) The role of apoptosis in regulating hematopoietic stem cell numbers. Apoptosis 6:239

81. Skoda RC (1999) Specificity of signaling by hematopoietic cytokine receptors: instructive versus permissive effects. J Recept Signal Transduct Res 19:741

82. Thompson A (1998) Cytokine Handbook. In, 3rd edition. San Diego: Academic Press, 1017

83. Pluznik DH, Sachs L (1965) The cloning of normal "mast" cells in tissue culture. J Cell Physiol 66:319

84. Bradley TR, Metcalf D (1966) The growth of mouse bone marrow cells in vitro. Aust J Exp Biol Med Sci 44:287

85. Ogawa M, Matsunaga T (1999) Humoral regulation of hematopoietic stem cells. Ann. N.Y. Acad. Sci. 872:17

86. Wu H, Liu X, Jaenisch R, Lodish HF (1995) Generation of committed erythroid BFU-E and CFU-E progenitors does not require erythropoietin or the erythropoietin receptor. Cell 83:59

87. de Sauvage FJ, Carver-Moore K, Luoh SM, Ryan A, Dowd M, Eaton DL, Moore MW (1996) Physiological regulation of early and late stages of megakaryocytopoiesis by thrombopoietin. J. Exp. Med. 183:651

88. Lieschke GJ, Grail D, Hodgson G, Metcalf D, Stanley E, Cheers C, Fowler KJ, Basu S, Zhan YF, Dunn AR (1994) Mice lacking granulocyte colony-stimulating factor have chronic neutropenia, granulocyte and macrophage progenitor cell deficiency, and impaired neutrophil mobilization. Blood 84:1737

89. Bagby GC, Jr., Heinrich MC (2000) Chapter 14: Growth Factors, Cytokines, and the Control of Hematopoiesis. In: R Hoffman (ed) Hematology: Basic Principles and Practice, 3rd edition. New York: Churchill Livingston, 154

90. Bazan JF (1990) Haemopoietic receptors and helical cytokines. Immunol Today 11:350

91. Murray AW, Hunt T (1993) The cell cycle : an introduction. New York: New York

92. Steinman RA (2002) Cell cycle regulators and hematopoiesis. Oncogene 21:3403

93. Blazsek I, Chagraoui J, Peault B (2000) Ontogenic emergence of the hematon, a morphogenetic stromal unit that supports multipotential hematopoietic progenitors in mouse bone marrow. Blood 96:3763

94. Nagasawa T, Hirota S, Tachibana K, Takakura N, Nishikawa S, Kitamura Y, Yoshida N, Kikutani H, Kishimoto T (1996) Defects of B-cell lymphopoiesis and bone-marrow myelopoiesis in mice lacking the CXC chemokine PBSF/SDF-1. Nature 382:635

95. Ma Q, Jones D, Borghesani PR, Segal RA, Nagasawa T, Kishimoto T, Bronson RT, Springer TA (1998) Impaired B-lymphopoiesis, myelopoiesis, and derailed cerebellar neuron migration in CXCR4- and SDF-1-deficient mice. Proc Natl Acad Sci U S A 95:9448

96. Hirsch E, Iglesias A, Potocnik AJ, Hartmann U, Fassler R (1996) Impaired migration but not differentiation of haematopoietic stem cells in the absence of beta1 integrins. Nature 380:171

97. Potocnik AJ, Brakebusch C, Fassler R (2000) Fetal and adult hematopoietic stem cells require beta1 integrin function for colonizing fetal liver, spleen, and bone marrow. Immunity 12:653

98. Healy L, May G, Gale K, Grosveld F, Greaves M, Enver T (1995) The stem cell antigen CD34 functions as a regulator of hemopoietic cell adhesion. Proc Natl Acad Sci U S A 92:12240

99. Goodman JWaHGS (1962) Evidence for Stem Cells in the Peripheral Blood of Mice. Blood 19:702

100. Fliedner TM (1998) The role of blood stem cells in hematopoietic cell renewal. Stem Cells 16 Suppl 1:13

101. Wright DE, Wagers AJ, Gulati AP, Johnson FL, Weissman IL (2001) Physiological migration of hematopoietic stem and progenitor cells. Science 294:1933

102. Ulich TR, del Castillo J, Souza L (1988) Kinetics and mechanisms of recombinant human granulocyte-colony stimulating factor-induced neutrophilia. Am J Pathol 133:630

103. Liu F, Poursine-Laurent J, Link DC (2000) Expression of the G-CSF receptor on hematopoietic progenitor cells is not required for their mobilization by G-CSF. Blood 95:3025

104. Liu F, Poursine-Laurent J, Link DC (1997) The granulocyte colony-stimulating factor receptor is required for the mobilization of murine hematopoietic progenitors into peripheral blood by cyclophosphamide or interleukin-8 but not flt-3 ligand. Blood 90:2522

105. Levesque JP, Takamatsu Y, Nilsson SK, Haylock DN, Simmons PJ (2001) Vascular cell adhesion molecule-1 (CD106) is cleaved by neutrophil proteases in the bone marrow following hematopoietic progenitor cell mobilization by granulocyte colony-stimulating factor. Blood 98:1289

106. Papayannopoulou T, Nakamoto B (1993) Peripheralization of hemopoietic progenitors in primates treated with anti-VLA4 integrin. Proc Natl Acad Sci U S A 90:9374

107. Petit I, Szyper-Kravitz M, Nagler A, Lahav M, Peled A, Habler L, Ponomaryov T, Taichman RS, Arenzana-Seisdedos F, Fujii N, Sandbank J, Zipori D, Lapidot T (2002) G-CSF induces stem cell mobilization by decreasing bone marrow SDF-1 and up-regulating CXCR4. Nat Immunol 3:687

108. Semerad CL, Liu F, Gregory AD, Stumpf K, Link DC (2002) G-CSF Is an Essential Regulator of Neutrophil Trafficking from the Bone Marrow to the Blood. Immunity 17:413

109. Heissig B, Hattori K, Dias S, Friedrich M, Ferris B, Hackett NR, Crystal RG, Besmer P, Lyden D, Moore MA, Werb Z, Rafii S (2002) Recruitment of stem and progenitor cells from the bone marrow niche requires MMP-9 mediated release of kit-ligand. Cell 109:625

110. Levesque JP, Leavesley DI, Niutta S, Vadas M, Simmons PJ (1995) Cytokines increase human hemopoietic cell adhesiveness by activation of very late antigen (VLA)-4 and VLA-5 integrins. J Exp Med 181:1805

111. Antonchuk J, Sauvageau G, Humphries RK (2001) HOXB4 overexpression mediates very rapid stem cell regeneration and competitive hematopoietic repopulation. Exp Hematol 29:1125

112. Antonchuk J, Sauvageau G, Humphries RK (2002) HOXB4-induced expansion of adult hematopoietic stem cells ex vivo. Cell 109:39

113. Nichogiannopoulou A, Trevisan M, Neben S, Friedrich C, Georgopoulos K (1999) Defects in hemopoietic stem cell activity in Ikaros mutant mice. Journal of Experimental Medicine 190:1201

114. Varnum-Finney B, Xu L, Brashem-Stein C, Nourigat C, Flowers D, Bakkour S, Pear WS, Bernstein ID (2000) Pluripotent, cytokine-dependent, hematopoietic stem cells are immortalized by constitutive Notch1 signaling. Nat Med 6:1278

115. Karanu FN, Murdoch B, Gallacher L, Wu DM, Koremoto M, Sakano S, Bhatia M (2000) The notch ligand jagged-1 represents a novel growth factor of human hematopoietic stem cells. J Exp Med 192:1365

116. Austin TW, Solar GP, Ziegler FC, Liem L, Matthews W (1997) A role for the Wnt gene family in hematopoiesis: expansion of multilineage progenitor cells. Blood 89:3624

117. Rebel VI, Hartnett S, Hill GR, Lazo-Kallanian SB, Ferrara JL, Sieff CA (1999) Essential role for the p55 tumor necrosis factor receptor in regulating hematopoiesis at a stem cell level. J Exp Med 190:1493

118. Bhardwaj G, Murdoch B, Wu D, Baker DP, Williams KP, Chadwick K, Ling LE, Karanu FN, Bhatia M (2001) Sonic hedgehog induces the proliferation of primitive human hematopoietic cells via BMP regulation. Nat Immunol 2:172

119. Fortunel N, Hatzfeld J, Kisselev S, Monier MN, Ducos K, Cardoso A, Batard P, Hatzfeld A (2000) Release from quiescence of primitive human hematopoietic stem/progenitor cells by blocking their cell-surface TGF-beta type II receptor in a short-term in vitro assay. Stem Cells 18:102

120. Batard P, Monier MN, Fortunel N, Ducos K, Sansilvestri-Morel P, Phan T, Hatzfeld A, Hatzfeld JA (2000) TGF-(beta)1 maintains hematopoietic immaturity by a reversible negative control of cell cycle and induces CD34 antigen up-modulation. J Cell Sci 113 (Pt 3):383

121. Cheng T, Rodrigues N, Shen H, Yang Y, Dombkowski D, Sykes M, Scadden DT (2000) Hematopoietic stem cell quiescence maintained by p21cip1/waf1. Science 287:1804

122. Zhu J, Emerson SG (2002) Hematopoietic cytokines, transcription factors and lineage commitment. Oncogene 21:3295

123. Bryder D, Jacobsen SE (2000) Interleukin-3 supports expansion of long-term multilineage repopulating activity after multiple stem cell divisions in vitro. Blood 96:1748

124. Phillips RL, Ernst RE, Brunk B, Ivanova N, Mahan MA, Deanehan JK, Moore KA, Overton GC, Lemischka IR (2000) The genetic program of hematopoietic stem cells. Science 288:1635

125. Kulessa H, Frampton J, Graf T (1995) GATA-1 reprograms avian myelomonocytic cell lines into eosinophils, thromboblasts, and erythroblasts. Genes Dev 9:1250

126. Nutt SL, Heavey B, Rolink AG, Busslinger M (1999) Commitment to the B-lymphoid lineage depends on the transcription factor Pax5. Nature 401:556

127. Hu M, Krause D, Greaves M, Sharkis S, Dexter M, Heyworth C, Enver T (1997) Multilineage gene expression precedes commitment in the hemopoietic system. Genes Dev 11:774

128. Cross MA, Enver T (1997) The lineage commitment of haemopoietic progenitor cells. Curr Opin Genet Dev 7:609

129. Miyamoto T, Iwasaki H, Reizis B, Ye M, Graf T, Weissman IL, Akashi K (2002) Myeloid or lymphoid promiscuity as a critical step in hematopoietic lineage commitment. Dev Cell 3:137

130. Stoffel R, Ziegler S, Ghilardi N, Ledermann B, de Sauvage FJ, Skoda RC (1999) Permissive role of thrombopoietin and granulocyte colony-stimulating factor receptors in hematopoietic cell fate decisions in vivo. Proc Natl Acad Sci U S A 96:698

131. Semerad CL, Poursine-Laurent J, Liu F, Link DC (1999) A role for G-CSF receptor signaling in the regulation of hematopoietic cell function but not lineage commitment or differentiation. Immunity 11:153

132. Fairbairn LJ, Cowling GJ, Reipert BM, Dexter TM (1993) Suppression of apoptosis allows differentiation and development of a multipotent hemopoietic cell line in the absence of added growth factors. Cell 74:823

133. Akashi K, Kondo M, von Freeden-Jeffry U, Murray R, Weissman IL (1997) Bcl-2 rescues T lymphopoiesis in interleukin-7 receptor-deficient mice. Cell 89:1033

134. Lagasse E, Weissman IL (1997) Enforced expression of Bcl-2 in monocytes rescues macrophages and partially reverses osteopetrosis in op/op mice. Cell 89:1021

135. Metcalf D (1991) Lineage commitment of hemopoietic progenitor cells in developing blast cell colonies: influence of colony-stimulating factors. Proceedings of the National Academy of Sciences of the United States of America 88:11310

136. Chiba T, Nagata Y, Kishi A, Sakamaki K, Miyajima A, Yamamoto M, Engel JD, Todokoro K (1993) Induction of erythroid-specific gene expression in lymphoid cells. Proceedings of the National Academy of Sciences of the United States of America 90:11593

137. Liboi E, Carroll M, D'Andrea AD, Mathey-Prevot B (1993) Erythropoietin receptor signals both proliferation and erythroid-specific differentiation. Proceedings of the National Academy of Sciences of the United States of America 90:11351

138. Maruyama K, Miyata K, Yoshimura A (1994) Proliferation and erythroid differentiation through the cytoplasmic domain of the erythropoietin receptor. Journal of Biological Chemistry 269:5976

139. Kondo M, Scherer DC, Miyamoto T, King AG, Akashi K, Sugamura K, Weissman IL (2000) Cell-fate conversion of lymphoid-committed progenitors by instructive actions of cytokines. Nature 407:383

140. Rodel JE, Link DC (1996) Suppression of apoptosis during cytokine deprivation of 32D cells is not sufficient to induce complete granulocytic differentiation. Blood 87:858

141. Longmore GD, You Y, Molden J, Liu KD, Mikami A, Lai SY, Pharr P, Goldsmith MA (1998) Redundant and selective roles for erythropoietin receptor tyrosines in erythropoiesis in vivo. Blood 91:870

142. Leonard WJ, Lin JX (2000) Cytokine receptor signaling pathways. J Allergy Clin Immunol 105:877

143. Yasukawa H, Sasaki A, Yoshimura A (2000) Negative regulation of cytokine signaling pathways. Annu Rev Immunol 18:143

144. Pawson T, Saxton TM (1999) Signaling networks--do all roads lead to the same genes? Cell 97:675

145. Jordan JD, Landau EM, Iyengar R (2000) Signaling networks: the origins of cellular multitasking. Cell 103:193

146. Darnell JE, Jr., Kerr IM, Stark GR (1994) Jak-STAT pathways and transcriptional activation in response to IFNs and other extracellular signaling proteins. Science 264:1415

147. Stahl N, Farruggella TJ, Boulton TG, Zhong Z, Darnell JE, Jr., Yancopoulos GD (1995) Choice of STATs and other substrates specified by modular tyrosine-based motifs in cytokine receptors. Science 267:1349

148. Greenlund AC, Morales MO, Viviano BL, Yan H, Krolewski J, Schreiber RD (1995) Stat recruitment by tyrosine-phosphorylated cytokine receptors: an ordered reversible affinity-driven process. Immunity 2:677

149. Bromberg J, Darnell JE, Jr. (2000) The role of STATs in transcriptional control and their impact on cellular function. Oncogene 19:2468

150. Ihle JN (2001) The Stat family in cytokine signaling. Curr Opin Cell Biol 13:211

151. O'Shea JJ, Gadina M, Schreiber RD (2002) Cytokine signaling in 2002: new surprises in the Jak/Stat pathway. Cell 109 Suppl:S121

152. John S, Vinkemeier U, Soldaini E, Darnell JE, Jr., Leonard WJ (1999) The significance of tetramerization in promoter recruitment by Stat5. Mol Cell Biol 19:1910

153. Ward AC, Touw I, Yoshimura A (2000) The Jak-Stat pathway in normal and perturbed hematopoiesis. Blood 95:19

154. Gouilleux F, Wakao H, Mundt M, Groner B (1994) Prolactin induces phosphorylation of Tyr694 of Stat5 (MGF), a prerequisite for DNA binding and induction of transcription. Embo J 13:4361

155. Wakao H, Gouilleux F, Groner B (1994) Mammary gland factor (MGF) is a novel member of the cytokine regulated transcription factor gene family and confers the prolactin response. Embo J 13:2182

156. Grimley PM, Dong F, Rui H (1999) Stat5a and Stat5b: fraternal twins of signal transduction and transcriptional activation. Cytokine Growth Factor Rev 10:131

157. Zhang S, Fukuda S, Lee Y, Hangoc G, Cooper S, Spolski R, Leonard WJ, Broxmeyer HE (2000) Essential Role of Signal Transducer and Activator of Transcription (Stat)5a but Not Stat5b for Flt3-dependent Signaling. J Exp Med 192:719

158. Ryan JJ, Huang H, McReynolds LJ, Shelburne C, Hu-Li J, Huff TF, Paul WE (1997) Stem cell factor activates STAT-5 DNA binding in IL-3-derived bone marrow mast cells. Exp Hematol 25:357

159. Shelburne C, McCoy M, Piekorz R, Sexl V, Gillespie S, Bailey D, Gharse A, Mirmonsef P, M NM, Kashyap M, Wright H, Chong H, Bouton L, Ramirez C, Lantz C, Ryan J (2002) Stat5: an essential regulator of mast cell biology. Mol Immunol 38:1187

160. Chrétien S, Varlet P, Verdier F, Gobert S, Cartron JP, Gisselbrecht S, Mayeux P, Lacombe C (1996) Erythropoietin-induced erythroid differentiation of the human erythroleukemia cell line TF-1 correlates with impaired STAT5 activation. EMBO J. 15:4174

161. Zamorano J, Wang HY, Wang R, Shi Y, Longmore GD, Keegan AD (1998) Regulation of cell growth by IL-2: role of STAT5 in protection from apoptosis but not in cell cycle progression. J Immunol 160:3502

162. Ilaria RL, Jr., Hawley RG, Van Etten RA (1999) Dominant negative mutants implicate STAT5 in myeloid cell proliferation and neutrophil differentiation. Blood 93:4154

163. Nosaka T, Kawashima T, Misawa K, Ikuta K, Mui AL, Kitamura T (1999) STAT5 as a molecular regulator of proliferation, differentiation and apoptosis in hematopoietic cells. Embo J 18:4754

164. Lord JD, McIntosh BC, Greenberg PD, Nelson BH (2000) The IL-2 receptor promotes lymphocyte proliferation and induction of the c-myc, bcl-2, and bcl-x genes through the trans-activation domain of Stat5. J Immunol 164:2533

165. Matsumura I, Kitamura T, Wakao H, Tanaka H, Hashimoto K, Albanese C, Downward J, Pestell RG, Kanakura Y (1999) Transcriptional regulation of the cyclin D1 promoter by STAT5: its involvement in cytokine-dependent growth of hematopoietic cells. Embo J 18:1367

166. Martino A, Holmes JHt, Lord JD, Moon JJ, Nelson BH (2001) Stat5 and Sp1 regulate transcription of the cyclin D2 gene in response to IL-2. J Immunol 166:1723

167. Matsumura I, Ishikawa J, Nakajima K, Oritani K, Tomiyama Y, Miyagawa J, Kato T, Miyazaki H, Matsuzawa Y, Kanakura Y (1997) Thrombopoietin-induced differentiation of a human megakaryoblastic leukemia cell line, CMK, involves transcriptional activation of p21(WAF1/Cip1) by STAT5. Mol Cell Biol 17:2933

168. Silva M, Benito A, Sanz C, Prosper F, Ekhterae D, Nunez G, Fernandez-Luna JL (1999) Erythropoietin can induce the expression of bcl-x(L) through Stat5 in erythropoietin-dependent progenitor cell lines. J Biol Chem 274:22165

169. Chida D, Miura O, Yoshimura A, Miyajima A (1999) Role of cytokine signaling molecules in erythroid differentiation of mouse fetal liver hematopoietic cells: functional analysis of signaling molecules by retrovirus-mediated expression. Blood 93:1567

170. Dumon S, Santos SC, Debierre-Grockiego F, Gouilleux-Gruart V, Cocault L, Boucheron C, Mollat P, Gisselbrecht S, Gouilleux F (1999) IL-3 dependent regulation of Bcl-xL gene expression by STAT5 in a bone marrow derived cell line. Oncogene 18:4191

171. Matikainen S, Sareneva T, Ronni T, Lehtonen A, Koskinen PJ, Julkunen I (1999) Interferon-alpha activates multiple STAT proteins and upregulates proliferation-associated IL-2Ralpha, c-myc, and pim-1 genes in human T cells. Blood 93:1980

172. Van Parijs L, Refaeli Y, Lord JD, Nelson BH, Abbas AK, Baltimore D (1999) Uncoupling IL-2 signals that regulate T cell proliferation, survival, and Fas-mediated activation-induced cell death. Immunity 11:281

173. Yoshimura A, Ichihara M, Kinjyo I, Moriyama M, Copeland NG, Gilbert DJ, Jenkins NA, Hara T, Miyajima A (1996) Mouse oncostatin M: an immediate early gene induced by multiple cytokines through the JAK-STAT5 pathway. Embo J 15:1055

174. Meyer WK, Reichenbach P, Schindler U, Soldaini E, Nabholz M (1997) Interaction of STAT5 dimers on two low affinity binding sites mediates interleukin 2 (IL-2) stimulation of IL-2 receptor alpha gene transcription. J Biol Chem 272:31821

175. Standke GJ, Meier VS, Groner B (1994) Mammary gland factor activated by prolactin on mammary epithelial cells and acute-phase response factor activated by

interleukin-6 in liver cells share DNA binding and transactivation potential. Mol Endocrinol 8:469

176. Streuli CH, Edwards GM, Delcommenne M, Whitelaw CB, Burdon TG, Schindler C, Watson CJ (1995) Stat5 as a target for regulation by extracellular matrix. J Biol Chem 270:21639

177. Ganguly TC, O'Brien ML, Karpen SJ, Hyde JF, Suchy FJ, Vore M (1997) Regulation of the rat liver sodium-dependent bile acid cotransporter gene by prolactin. Mediation of transcriptional activation by Stat5. J Clin Invest 99:2906

178. Galsgaard ED, Gouilleux F, Groner B, Serup P, Nielsen JH, Billestrup N (1996) Identification of a growth hormone-responsive STAT5-binding element in the rat insulin 1 gene. Mol Endocrinol 10:652

179. Zhao Y, Nichols JE, Bulun SE, Mendelson CR, Simpson ER (1995) Aromatase P450 gene expression in human adipose tissue. Role of a Jak/STAT pathway in regulation of the adipose-specific promoter. J Biol Chem 270:16449

180. Matsumoto A, Masuhara M, Mitsui K, Yokouchi M, Ohtsubo M, Misawa H, Miyajima A, Yoshimura A (1997) CIS, a cytokine inducible SH2 protein, is a target of the JAK-STAT5 pathway and modulates STAT5 activation. Blood 89:3148

181. Matsumoto A, Seki Y, Kubo M, Ohtsuka S, Suzuki A, Hayashi I, Tsuji K, Nakahata T, Okabe M, Yamada S, Yoshimura A (1999) Suppression of STAT5 functions in liver, mammary glands, and T cells in cytokine-inducible SH2-containing protein 1 transgenic mice. Mol Cell Biol 19:6396

182. Mui AL, Wakao H, Kinoshita T, Kitamura T, Miyajima A (1996) Suppression of interleukin-3-induced gene expression by a C-terminal truncated Stat5: role of Stat5 in proliferation. Embo J 15:2425

183. Moriggl R, Gouilleux-Gruart V, Jahne R, Berchtold S, Gartmann C, Liu X, Hennighausen L, Sotiropoulos A, Groner B, Gouilleux F (1996) Deletion of the carboxyl-terminal transactivation domain of MGF-Stat5 results in sustained DNA binding and a dominant negative phenotype. Mol Cell Biol 16:5691

184. Wang D, Stravopodis D, Teglund S, Kitazawa J, Ihle JN (1996) Naturally occurring dominant negative variants of Stat5. Mol Cell Biol 16:6141

185. Stocklin E, Wissler M, Gouilleux F, Groner B (1996) Functional interactions between Stat5 and the glucocorticoid receptor. Nature 383:726

186. Zhu M, John S, Berg M, Leonard WJ (1999) Functional association of Nmi with Stat5 and Stat1 in IL-2- and IFNgamma-mediated signaling. Cell 96:121

187. Liu X, Robinson GW, Wagner KU, Garrett L, Wynshaw-Boris A, Hennighausen L (1997) Stat5a is mandatory for adult mammary gland development and lactogenesis. Genes Dev. 11:179

188. Udy GB, Towers RP, Snell RG, Wilkins RJ, Park SH, Ram PA, Waxman DJ, Davey HW (1997) Requirement of STAT5b for sexual dimorphism of body growth rates and liver gene expression. Proc. Natl. Acad. Sci. USA 94:7239

189. Feldman GM, Rosenthal LA, Liu X, Hayes MP, Wynshaw-Boris A, Leonard WJ, Hennighausen L, Finbloom DS (1997) STAT5A-deficient mice demonstrate a defect in granulocyte-macrophage colony-stimulating factor-induced proliferation and gene expression. Blood 90:1768

190. Nakajima H, Liu XW, Wynshaw-Boris A, Rosenthal LA, Imada K, Finbloom DS, Hennighausen L, Leonard WJ (1997) An indirect effect of Stat5a in IL-2-induced proliferation: a critical role for Stat5a in IL-2-mediated IL-2 receptor alpha chain induction. Immunity 7:691

191. Imada K, Bloom ET, Nakajima H, Horvath-Arcidiacono JA, Udy GB, Davey HW, Leonard WJ (1998) Stat5b is essential for natural killer cell-mediated proliferation and cytolytic activity. J. Exp. Med. 188:2067

192. Teglund S, McKay C, Schuetz E, van Deursen JM, Stravopodis D, Wang D, Brown M, Bodner S, Grosveld G, Ihle JN (1998) Stat5a and Stat5b proteins have essential and nonessential, or redundant, roles in cytokine responses. Cell 93:841

193. Moriggl R, Topham DJ, Teglund S, Sexl V, McKay C, Wang D, Hoffmeyer A, van Deursen J, Sangster MY, Bunting KD, Grosveld GC, Ihle JN (1999) Stat5 is required for IL-2-induced cell cycle progression of peripheral T cells. Immunity 10:249

194. Socolovsky M, Fallon AE, Wang S, Brugnara C, Lodish HF (1999) Fetal anemia and apoptosis of red cell progenitors in Stat5a-/-5b-/- mice: a direct role for Stat5 in Bcl-X(L) induction. Cell 98:181

Chapter 2

STAT5 Promotes Multilineage Hematolymphoid Development *in Vivo* Through Effects on Early Hematopoietic Progenitor Cells

Introduction

Cytokines of the type 1 family receptors are themselves catalytically inactive and require the presence of an associated kinase to signal to the cell upon ligand-induced activation. The primary group of kinases thought to provide this role *in vivo* is that of the Janus kinase (JAK) family. One class of downstream molecules that is phosphorylated and activated by these kinases, is the signal transducer and activator of transcription (STAT) family of transcription factors.

One STAT in particular, STAT5, was hypothesized to be extremely important in cytokine signaling transduction *in vivo*, due to its ubiquitous activation and apparent importance in cytokine-mediated responses in vitro. However, mice deficient for one or both isoforms of STAT5 appeared to exhibit only minor hematopoietic defects upon first examination [1-6].

However, some evidence of additional hematologic abnormalities was evident in these animals, such as reduced bone marrow colony counts *in vitro* and notable extramedullary hematopoiesis [6,7]. In addition, marked fetal anemia *in vivo* as well as defects in EPO-dependent production and survival of fetal liver hematopoietic colonies *in vitro* were subsequently reported [8].

The contradictory results of these previous studies led to a controversy in defining the role that STAT5 played in hematolymphoid development *in vivo*. In addition, the difficulty of interpreting subtle phenotypes of gene-deficient mice is well appreciated. We hypothesized that the resolution and rigor of the assays used previously to examine the state of hematolymphoid development in STAT5A/5B-deficient mice *in vivo* might have lead to incomplete assessment of the hematopoietic health of these mice.

The goal of this project was to utilize assays of hematopoietic progenitor function *in vivo* in conjunction with detailed phenotypic analysis of progenitor subsets in STAT5A/5B-deficient mice for an analysis of more breadth and depth in evaluating the role of STAT5 in hematolymphoid development. We found that in contrast to previous results there were steady state hematopoietic abnormalities, including multilineage cytopenias in these mice. In addition, we found a marked reduction in the number and function of early multilineage progenitor cells. Most striking was the complete lack of competitive repopulating potential of HSC from these mice.

These data helped to provide further evidence that STAT5 plays an important role in hematolymphoid development *in vivo*. Although the absence of this molecule does not lead to a complete block in production of mature blood cells, it does play an important role in maintaining the number and function of progenitor cells and normal levels of peripheral blood cells at steady state. These results also underscore the necessity for careful and complete analysis of gene-deficient mice in cases where the molecule may have a non-essential, but significant role.

References

1. Liu X, Robinson GW, Wagner KU, Garrett L, Wynshaw-Boris A, Hennighausen L (1997) Stat5a is mandatory for adult mammary gland development and lactogenesis. Genes Dev. 11:179

2. Udy GB, Towers RP, Snell RG, Wilkins RJ, Park SH, Ram PA, Waxman DJ, Davey HW (1997) Requirement of STAT5b for sexual dimorphism of body growth rates and liver gene expression. Proc. Natl. Acad. Sci. USA 94:7239

3. Feldman GM, Rosenthal LA, Liu X, Hayes MP, Wynshaw-Boris A, Leonard WJ, Hennighausen L, Finbloom DS (1997) STAT5A-deficient mice demonstrate a defect in

granulocyte-macrophage colony-stimulating factor-induced proliferation and gene expression. Blood 90:1768

4. Nakajima H, Liu XW, Wynshaw-Boris A, Rosenthal LA, Imada K, Finbloom DS, Hennighausen L, Leonard WJ (1997) An indirect effect of Stat5a in IL-2-induced proliferation: a critical role for Stat5a in IL-2-mediated IL-2 receptor alpha chain induction. Immunity 7:691

5. Imada K, Bloom ET, Nakajima H, Horvath-Arcidiacono JA, Udy GB, Davey HW, Leonard WJ (1998) Stat5b is essential for natural killer cell-mediated proliferation and cytolytic activity. J. Exp. Med. 188:2067

6. Teglund S, McKay C, Schuetz E, van Deursen JM, Stravopodis D, Wang D, Brown M, Bodner S, Grosveld G, Ihle JN (1998) Stat5a and Stat5b proteins have essential and nonessential, or redundant, roles in cytokine responses. Cell 93:841

7. Moriggl R, Topham DJ, Teglund S, Sexl V, McKay C, Wang D, Hoffmeyer A, van Deursen J, Sangster MY, Bunting KD, Grosveld GC, Ihle JN (1999) Stat5 is required for IL-2-induced cell cycle progression of peripheral T cells. Immunity 10:249

8. Socolovsky M, Fallon AE, Wang S, Brugnara C, Lodish HF (1999) Fetal anemia and apoptosis of red cell progenitors in Stat5a-/-5b-/- mice: a direct role for Stat5 in Bcl-X(L) induction. Cell 98:181

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STAT5 promotes multilineage hematolymphoid development in vivo through effects on early hematopoietic progenitor cells

Jonathan W. Snow, Ninan Abraham, Melissa C. Ma, Nancy W. Abbey, Brian Herndier, and Mark A. Goldsmith

The transcription factor signal transducers and activators of transcription 5 (STAT5) is activated by numerous cytokines that orchestrate blood cell development. Multilineage peripheral blood cytopenias were observed in adult mice lacking both isoforms of STAT5 (STAT5A and STAT5B) as well as accelerated rates of apoptosis in the bone marrow. Although the hematopoietic stem cell (HSC) population was preserved in a number of these mice, the post-HSC progenitor populations were diminished and a marked reduction in functional progenitors (spleen colony-forming units) was detected. Competitive bone marrow transplantation studies in vivo revealed a profound impairment of repopulation potential of STAT5-null HSCs, leading to complete lack of contribution to the myeloid, erythroid, and lymphoid lineages. These abnormalities were associated with heightened proliferation activity in the HSC fraction, suggesting the action of homeostatic mechanisms to maintain sufficient levels of diverse blood cell types for viability. Thus, STAT5 normally sustains the robust hematopoietic reserve that contributes to host viability through crucial survival effects on early progenitor cells. (Blood. 2002;99:95-101)

Introduction

Hematolymphoid development is a complex process controlled by multiple positive and negative regulatory systems to maintain homeostasis. This process begins with hematopoietic stem cells (HSCs), which possess a high proliferative capacity, a differentiative potential encompassing all hematopoietic lineages, and the ability to repopulate the hematopoietic system of a bone marrow–ablated animal for its lifespan. In response to unknown signals, HSCs undergo a differentiation program, yielding cells that have short-term repopulating activity. These cells subsequently give rise to multilineage progenitors that are restricted to either the myeloid (common myeloid progenitor, or CMP) or lymphoid (common lymphoid progenitor, or CLP) lineages.[1,2] Lineage-specific differentiation of these pluripotent cells and further expansion produces mature cells of a given lineage.[3]

Cytokines that use class I cytokine receptors play an indispensable role in controlling hematopoietic development and function. Many of these cytokines act on the HSCs themselves, such as granulocyte colony-stimulating factor (G-CSF), interleukin-6 (IL-6), IL-11, thrombopoietin (TPO), and leukemia-inhibitory factor.[4] Others, such as erythropoietin, TPO, and G-CSF, act on lineage-committed progenitors exclusively or in addition to their earlier effects.[5-7] How this family of receptors generates specificity in biologic outcomes while employing shared intracellular signaling pathways has been a point of considerable interest. The discovery of the JAK-STAT pathway appeared to offer one mechanism through which cytokine receptors could selectively up-regulate distinct target genes and be responsible for specific biologic behaviors. STATs are cytoplasmically located, latent transcription factors that dimerize on phosphorylation by an activated receptor complex, translocate into the nucleus, and increase the transcription of target genes by binding to specific DNA sequence motifs.[8,9] Various genes have been shown to be up-regulated by STAT molecules, including those involved in cell cycle progression, antiapoptosis, and regulation of cytokine signaling.[10]

One member of the STAT family, STAT5, is activated by diverse cytokine receptors involved at multiple levels within the hematopoietic system. The widespread engagement of its 2 isoforms, STAT5A and STAT5B, has implicated STAT5 as a potentially important component of cytokine receptor signaling in this tissue. Cell culture studies have suggested a possible role for STAT5 in hematolymphoid development. For example, STAT5 was shown to be involved in erythropoietin-induced proliferation of TF-1 cells.[11] STAT5 has also been implicated in both the IL-3–induced proliferation and G-CSF–induced differentiation in 32D cells.[12] Surprisingly, therefore, mice deficient in either STAT5A[13] or STAT5B[14] were found to exhibit dramatic defects in specific nonhematopoietic tissues, but only subtle alterations in the regulation of hematopoietic cells.[15-17] Moreover, mice deficient in both the STAT5A and STAT5B isoforms were reported to have no further defects in the production of mature blood cells of various lineages with the exception of decreased numbers of peripheral T cells.[18] However, some evidence of additional hematologic dysregulation was evident in these animals, such as reduced bone marrow colony counts in vitro and notable extramedullary hematopoiesis.[18,19] In addition, marked fetal anemia in vivo as well as defects in erythropoietin-dependent production and survival of fetal liver hematopoietic colonies in vitro were subsequently reported.[20] Finally, an increase in apoptosis of cultured STAT5A/5B-deficient

From the Gladstone Institute of Virology and Immunology, Departments of Microbiology and Immunology, Pathology, and Medicine, University of California at San Francisco, San Francisco, CA.

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Reprints: Mark A. Goldsmith, Gladstone Institute of Virology and Immunology, PO Box 419100, San Francisco, CA 9414-9100; e-mail: mgoldsmith@gladstone.ucsf.edu.

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bone marrow progenitors in the presence of granulocyte-macrophage colony-stimulating factor was also described.[21]

In view of these provocative findings and the fact that compensatory mechanisms may mask the biologic action of a particular molecular component in vivo, we examined the hematologic status of STAT5A/5B-deficient mice in depth. Our studies uncovered an important role for STAT5 in hematopoiesis at an early progenitor stage in vivo. In these mice, marked impairment in hematopoietic potential affecting diverse blood lineages is linked to significant abnormalities in central and peripheral hematolymphoid tissues.

Materials and methods

Handling and characterization of mice

STAT5A/5B$^{-/-}$ mice[18] were obtained from Dr James Ihle (St Jude Children's Research Hospital, Memphis, TN) and back-crossed onto a C57Bl/6 background at least 3 generations. Mice were housed in a pathogen-free rodent barrier facility and received mouse chow and acidified water ad libitum. All studies were performed on 8- to 10-week mice unless otherwise specified, and littermates were always used as wild-type controls. Peripheral blood was obtained via cardiac puncture, and EDTA-treated samples were used for complete blood counts (IDEXX Veterinary Service, Sacramento, CA). Bone marrow was harvested by flushing femurs and tibias into 6 mL phosphate-buffered saline (PBS) containing 2% fetal bovine serum, and cell counts were determined after ACK (NH_4Cl) lysis by trypan blue exclusion.

Flow cytometry

All antibodies were obtained from Pharmingen (San Diego, CA), and the following clones were used: Sca-1 (E13-161.7), Ly-76 (Ter-119), Gr-1 (RB6-8C5), CD3 (145-2C11), CD41 (MWReg30), CD45.1 (A20), CD45.2 (104), B220 (RA3-6B2), and CD11b (M1/70). For blocking nonspecific binding to Fc receptors, purified antibody to CD16/CD32 (2.4G2) was used at 1:100 for 3 minutes. Subsequently, antibodies to surface markers of interest were used at 1:60 dilution. Apoptosis staining was performed by using AnnexinV-GFP[22] (generously provided by Dr Joel Ernst, University of California, San Francisco, CA) in conjunction with antibodies to selected surface markers as well as one of 2 DNA dyes, ToPro (Molecular Probes, Eugene, OR) or 7-AAD (Pharmingen, San Diego, CA). DNA content was assessed by an initial incubation with antibodies to selected surface markers, followed by a 30-minute fixation and permeabilization step using 2% formaldehyde in H_20, a 30-minute exposure to ToPro in the presence of 1 mg/mL RNase (Sigma, St Louis, MO), and fluorescence-activated cell sorter (FACS) analysis using a slow acquisition. All FACS analyses were performed by using a FACScalibur, and all sorting was performed by using a FACSvantage (Becton Dickinson).

Bone marrow transfer studies

Recipient mice were 8-week-old sex-matched C57Bl/6 obtained from Jackson Laboratories (Bar Harbor, ME). Recipients were γ-irradiated from a Cesium source in two 450-rad doses 4 to 5 hours apart. Spleen colony-forming unit (CFU-S) studies were performed as previously described.[23] Briefly, whole bone marrow cells from donor mice were injected via tail vein, spleens were harvested 8 or 12 days after transfer, and macroscopic colonies were enumerated. Mice receiving the transplants were maintained on 2.5 mg/100 mL Sulfatrim Pediatric Suspension (Alpharma, Baltimore, MD). Competitive repopulation studies were performed as previously described.[24] Lin^{dim}Sca-1$^+$ tester cells (CD45.2$^+$), derived from wild-type littermate or STAT5A/5B$^{-/-}$ whole bone marrow, were sorted by using antibodies to Sca-1 and Ter-119, Gr-1, CD3, B220, and CD11b. Competitor whole bone marrow from congenic B6.SLJ (CD45.1$^+$) mice was harvested as above. Tester and competitor cells were mixed at various ratios (see "Inferior competitive repopulating capacity of STAT5A/5B-deficient HSCs") and injected into irradiated recipients, prepared as above. After 8 to 10 weeks, chimeric mice were killed, and peripheral blood, spleen, thymus, and bone marrow were collected and analyzed by FACS for contribution of CD45.1$^+$- and CD45.2$^+$-derived cells to selected lineages.

Homing assay

Cell labeling with 5- and 6-carboxyfluorescein diacetate succinimidyl ester (CSFE) was performed as described previously.[25] Briefly, whole bone marrow from donor mice was labeled in PBS at a final concentration of 15 μM CSFE (Molecular Probes). After 12 minutes at 37°C, further dye uptake was prevented by adding a quarter volume of fetal bovine serum. Cells were washed twice with PBS, and 5 × 10^6 CFSE-labeled cells were injected into the tail vein of recipient mice that had been irradiated 18 hours before injection. Bone marrow was harvested 23 hours after injection, stained with lineage markers and Sca-1 as before, and the number of CFSE$^+$ cells in each subset was enumerated by FACS.

Statistical analysis

Data are presented as mean ± SEM. Statistical significance was assessed by 2-sided Student *t* test.

Results

Adult STAT5A/5B-deficient mice exhibit cytopenias affecting multiple peripheral blood lineages

We characterized the peripheral blood compartment of adult STAT5A/5B-deficient mice and found significant abnormalities in multiple blood lineages. At 8 weeks of age, STAT5A/5B$^{-/-}$ mice exhibited significantly decreased numbers of erythrocytes in peripheral blood compared with wild-type littermate controls (Figure 1A). Platelets, a second myeloid lineage, were also reduced in these mice, although this effect was less profound (Figure 1B). A marked decrease in lymphocyte number in peripheral blood was also observed (Figure 1C); both T cells and B cells were decreased in STAT5A/5B-deficient animals (data not shown). Finally, although no significant difference in peripheral neutrophil counts was detected (Figure 1D), we observed a significant reduction in mature

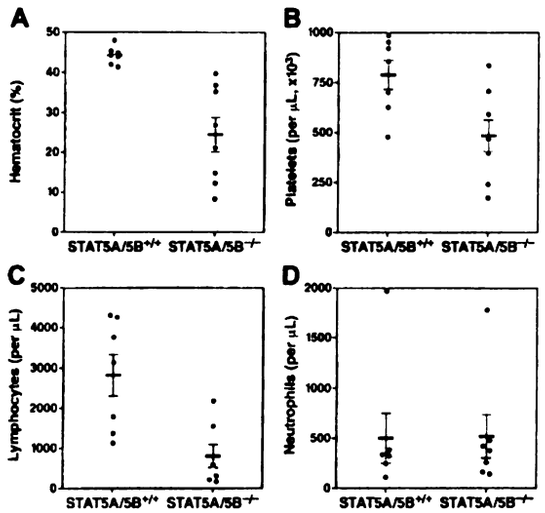

Figure 1. Multilineage cytopenias in adult STAT5A/5B-deficient mice. Complete blood counts were performed on whole peripheral blood from STAT5A/5B$^{-/-}$ and STAT5A/5B$^{+/+}$ mice. (A) Hematocrit ($P < .001$). (B) Platelets ($P = .01$). (C) Lymphocytes ($P = .003$). (D) Neutrophils ($P = 1.0$).

neutrophils (Gr-1^{hi}) in the bone marrow (see below). Thus, the absence of STAT5A/5B was associated with abnormalities in multiple blood cell lineages.

STAT5A/5B-deficient mice have hypocellular bone marrow and a defect within early progenitor cells

Such broad defects in peripheral blood cell numbers in STAT5A/5B-deficient mice could be due to accelerated peripheral consumption or destruction of mature cells, to a lowered capacity to produce mature cells, or to a combination of these 2 mechanisms. The multilineage character of the effects we observed suggested that a primary pathophysiologic defect might be in the bone marrow, where both unique and shared precursors for these cell types exist. Gross examination of bone marrow in STAT5A/5B-deficient mice revealed generalized hypocellularity compared with wild-type mice (Figure 2A). This 2-fold decrease in total nucleated cells in the bone is consistent with the hypothesis that a central defect in the bone marrow is responsible for the pancytopenia observed in the periphery.

Various cytokines that trigger STAT5 activity are critical for the regulation of hematopoiesis at all levels of differentiation, including stem cells, multipotent progenitors, lineage-committed progenitors, and mature blood cells. We therefore sought to determine the specific developmental stage(s) in which the functional effects of STAT5 are manifested in the bone marrow. We used FACS analysis to subset bone marrow cells by expression of canonical surface lineage-defining markers (lin) and Sca-1 (Figure 2B). The HSCs, which are defined as cells that have both the capacity for self-renewal and the ability to reconstitute the multilineage hematopoietic system, are found within the lin^{dim}Sca-1^{+} fraction.[26] This population was increased as a percentage of total nucleated bone marrow cells in STAT5A/B-deleted mice compared with controls but was unchanged in absolute terms (Figure 2C). In contrast, the

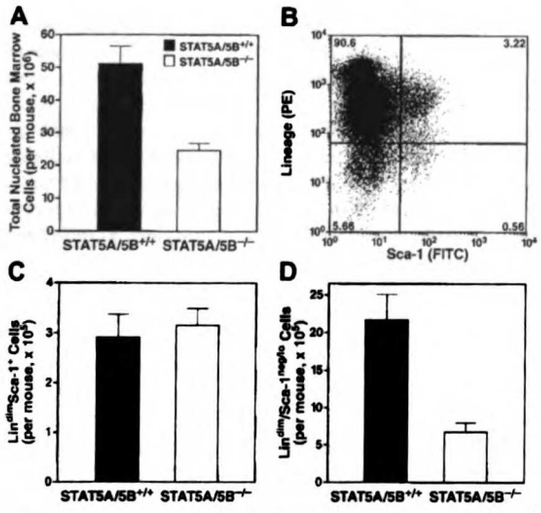

Figure 2. Decreased total nucleated cells in the bone marrow of STAT5A/5B-deficient mice. Total nucleated cells were obtained from both hind legs, and cell counts were determined after red blood cell lysis. Subsetting was performed according to surface expression of lineage-defining markers and Sca-1. Absolute values were generated by multiplying gated percentages by total nucleated cell numbers. (A) Total nucleated cells (P = .002). (B) Representative FACS analysis to quantitate bone marrow subsets. (C) The lin^{dim}Sca-1^{+} compartment, containing HSCs (P = .7). (D) The lin^{dim}Sca-1$^{neg/lo}$ compartment, containing post-HSC progenitors (P = .002). PE indicates phycoerythrin; FITC, fluorescein isothiocyanate.

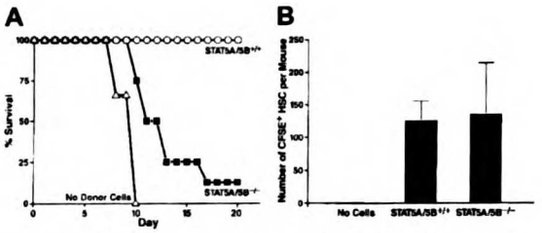

Figure 3. Decreased short-term radioprotective ability of whole bone marrow despite preserved homing of HSCs from STAT5A/5B-deficient mice. (A) Irradiated recipients received either 2.5 × 10^5 STAT5A/5B$^{+/+}$ whole bone marrow cells (n = 6), 2.5 × 10^5 STAT5A/5B$^{-/-}$ whole bone marrow cells (n = 8), or no donor cells (n = 3). Survival rates are shown as a Kaplan-Meyer plot. (B) Irradiated recipients received 5 × 10^6 CFSE-labeled whole bone marrow cells from a STAT5A/5B$^{+/+}$ donor (n = 3) or a STAT5A/5B$^{-/-}$ donor (n = 3). Bone marrow was harvested 23 hours after transplantation, and the absolute number of CFSE$^+$ cells within the HSC (lin^{dim}/Sca-1$^+$) gate per mouse was determined.

lin^{dim}Sca-1$^{neg/lo}$ population, containing CLPs,[2] CMPs,[1] and oligopotent progenitors, was dramatically decreased in STAT5A/5B-deficient animals (Figure 2D). Also, we observed that the absolute number of mature neutrophils (Gr-1^{hi}) in the bone marrow was decreased (wild type = 12.5 × 10^6 ± 4.1 per 2 hind legs and knock-out = 5.5 × 10^6 ± 1.6 per 2 hind legs) as well as progenitors for neutrophils (Gr-1^{int}) (wild type = 10.4 × 10^6 ± 3.0 per 2 hind legs and knock-out = 5.2 × 10^6 ± 1.1 per 2 hind legs), erythrocytes (Ter119$^+$) (wild type = 6.6 × 10^6 ± 0.96 per 2 hind legs and knock-out = 3.3 × 10^6 ± 0.79 per 2 hind legs), and B cells (B220$^+$) (wild type = 12.6 × 10^6 ± 2.1 per 2 hind legs and knock-out = 5.0 × 10^6 ± 0.71 per 2 hind legs). Therefore, the absence of STAT5A/5B results in a marked decrease in lin^{dim}Sca-1$^{neg/lo}$ cells as well as in specific lineage marker-positive cells in the bone marrow despite preservation of the earlier lin^{dim}Sca-1$^+$ HSC.

A number of short-term bone marrow transfer assays were performed to assess the functional capabilities of bone marrow from the STAT5A/5B-deficient mice. Lethally irradiated wild-type mice typically die of hematopoietic failure between 7 and 18 days after irradiation unless they are given new hematopoietic progenitors from a donor animal. Therefore, one functional assay determines the radioprotective ability of whole bone marrow from a donor mouse. A dose of 2.5 × 10^5 transplanted whole bone marrow cells from wild-type littermate control mice provided radioprotection to 100% of lethally irradiated recipient mice for 20 days (Figure 3A). In contrast, the same dose of whole bone marrow from a STAT5A/5B-deficient donor provided radioprotection to only 12.5% of recipient mice through the same period (Figure 3A), demonstrating that these cells have markedly decreased reconstituting capacity as indicated by radioprotective effects. To determine whether this phenotype derives from defects in homing or in postengraftment expansion and hematopoiesis, irradiated recipients were injected with wild-type or STAT5A/5B-deficient whole bone marrow that had been labeled with the membrane dye CFSE. We found that the number of CFSE$^+$ cells in the HSC-containing lin^{dim}/Sca-1$^+$ fraction that had homed to the bone marrow after 23 hours was comparable when whole bone marrow from either STAT5A/5B-deficient or wild-type littermate donors was injected (Figure 3B).

A second, direct, functional assay for progenitor cells is the in vivo CFU-S assay,[23] in which macroscopic colonies in the spleens of irradiated recipients are counted 8 or 12 days after bone marrow transfer.[26] We detected a pronounced reduction in CFU-S (day 12) colonies per donor in the STAT5A/5B-deleted mice relative to wild-type littermate controls (Figure 4A), which indicates an

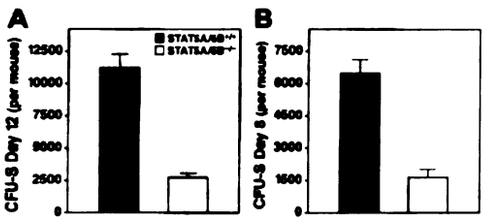

Figure 4. Reduction in CFU-S activity in bone marrow from STAT5A/5B-deleted mice. Whole bone marrow was obtained from the hind legs of donor mice, 7.5×10^4 (day 12) or 2×10^5 (day 8) cells were injected into irradiated recipients, and colonies were counted on the indicated days after transfer. Absolute CFU-S values were calculated by using the total nucleated cell counts from the donor mice. (A) CFU-S day 12 ($P < .001$). (B) CFU-S day 8 ($P < .001$).

abnormality in the early pluripotent progenitors. We observed a similarly dramatic decrease in CFU-S (day 8) per donor in STAT5A/5B$^{-/-}$ mice (Figure 4B), which indicates an abnormality in lineage-committed progenitors. Moreover, spleen colonies derived from STAT5A/5B-deficient donor cells were markedly smaller in size, both macroscopically and microscopically, compared with those produced by wild-type donor cells, reflecting that fewer progeny cells are produced per colony (data not shown). The reduction in both number and size of spleen colonies in these experiments indicates a marked functional impairment in both multilineage progenitors and lineage-committed progenitors in mice lacking STAT5A/5B.

Bone marrow deficiency involves increased apoptosis, rather than decreased proliferation

In principle, the cellular effects described might be caused by various mechanisms, including decreases in the rates of proliferation, survival, or differentiation of selected progenitor populations. We used cell labeling with Annexin-V in conjunction with a DNA dye, To-Pro, to measure rates of apoptosis in the bone marrow. The absence of STAT5A/5B was associated with an increase in the rate of apoptosis of unfractionated bone marrow (31.1% ± 2.65%) relative to wild type (17.6% ± 0.73%). This 2-fold increase in cell death was seen in both the lin$^+$ population as well as the lin^{dim}Sca-1$^{neg/lo}$ population (Figure 5A), whereas the lin^{dim}Sca-1$^+$ compartment showed only a modest increase in the rate of apoptosis (Figure 5A). To measure proliferation, we used ToPro in conjunction with cell surface markers to quantitate DNA content in bone marrow by using DNA content more than 2n as an indirect measure of the proportion of cells undergoing cell cycle. An insignificant increase in the proportion of cells with DNA content more than 2n was observed among the unfractionated whole bone marrow population of STAT5A/5B-deficient bone marrow (17.2% ± 1.79%) in comparison to the wild-type marrow (13.4% ± 1.01%). Lin$^+$ cells likewise showed little change in proliferation activity in STAT5A/5B-deleted mice (Figure 5B). In contrast, both the early lin^{dim}Sca-1$^{neg/lo}$ and the lin^{dim}Sca-1$^+$ subsets exhibited a 2-fold increase in the percentage of cells with DNA content more than 2n in STAT5A/5B-deficient mice (Figure 5B). Together, these findings provide evidence of globally decreased cellular survival (represented as increased apoptosis) in bone marrow cells in the absence of STAT5A/5B, with a concurrent increase in cellular proliferation among the least differentiated hematopoietic cells.

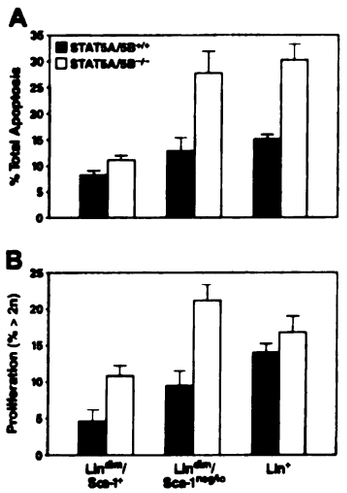

Figure 5. Increases in apoptosis and in the proportion of cells in S/G2/M in bone marrow of STAT5A/5B-deficient mice. Whole bone marrow from the indicated mice was stained for apoptosis or DNA content measurements in conjunction with antibodies to lineage markers and Sca-1. (A) Percentage of apoptotic cells in the lin$^+$ ($P = .04$), lin^{dim}Sca-1$^{neg/lo}$ post-HSC progenitors ($P = .03$), and lin^{dim}Sca-1$^+$ HSC ($P = .04$) subsets in STAT5A/5B$^{-/-}$ and STAT5A/5B$^{+/+}$ mice are shown. (B) Percentage of cells with more than 2n DNA content for the lin$^+$ ($P = .2$), lin^{dim}Sca-1$^{neg/lo}$ post-HSC progenitors ($P = .01$), and lin^{dim}Sca-1$^+$ HSC ($P = .005$) subsets are shown.

Inferior competitive repopulating capacity of STAT5A/5B-deficient HSCs

A rigorous functional test of stem cell fitness is the competitive repopulation assay, which measures the capacity of "tester" HSCs to reconstitute the hematopoietic system of irradiated recipient mice in direct competition with wild-type "competitor" bone marrow cells.[26,27] For the representative experiment shown, we injected 2500 lin^{dim}Sca-1$^+$ tester cells from STAT5A/5B-deficient mice or wild-type littermate control mice, carrying the CD45.2 allele, together with 2×10^5 whole bone marrow competitor cells derived from congenic mice carrying the CD45.1 allele. An additional control group received 2×10^5 whole bone marrow competitor cells alone to establish the level of ablation achieved by the irradiation regimen. After 10 weeks, peripheral blood, thymus, spleen, and bone marrow were harvested for analysis of hematopoietic lineages by using various antibodies to lineage markers and to the 2 alleles of CD45. At this ratio of input cells, which was

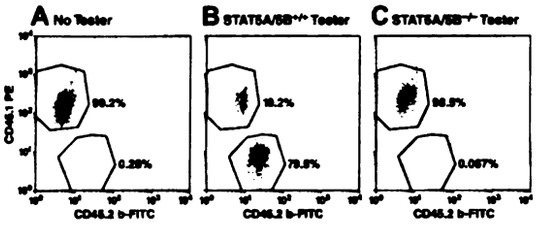

Figure 6. Absence of STAT5A/5B$^{-/-}$ contribution (CD45.2$^+$) to granulocytes (Gr-1$^+$) in peripheral blood after competitive repopulation. Peripheral blood from irradiated recipients receiving competitor cells only (A), competitor cells plus STAT5A/5B$^{+/+}$ tester cells (B), or competitor cells plus STAT5A/5B$^{-/-}$ tester cells (C) were analyzed 10 weeks after initiation of competitive repopulation assay. Peripheral blood was stained for CD45.1, CD45.2, and Gr-1 after red blood cell lysis and shown here are representative dot-plots portraying CD45.1 versus CD45.2 expression within the Gr-1$^+$ gate.

weighted to favor tester cells, wild-type tester cells ($CD45.2^+$) gave rise to approximately 80% of all cellular lineages in all tissues examined, including $Gr\text{-}1^+$ (Figure 6), and other specific lineages within peripheral blood, including T cells ($CD3^+$) and B cells ($B220^+$) (Figure 6A). As is commonly observed, residual radioresistant $CD3^+$ T cells from the recipient mouse are detected in all groups, including those mice that received competitor cells alone (Figure 7A). Likewise, bone marrow cells representing the granulocyte ($Gr\text{-}1^+$), B-cell ($B220^+$), megakaryocyte ($CD41^+$) lineages, and erythroid progenitors ($Ter\text{-}119^+$), as well as thymocytes ($CD3^+$), were predominantly $CD45.2^+$ (Figure 7B). In contrast, at the same input doses, STAT5A/5B-deficient tester cells failed to give rise to significant numbers of cells of any lineage within peripheral blood, spleen, thymus, or bone marrow (Figures 6 and 7 and data not shown); in no case was the $CD45.2^+$ signal greater than that of the background observed in animals not receiving tester cells. Complete competitive failure was also observed in 2 other independent experiments with highly backcrossed donors (G4 and G7, respectively).

Finally, because STAT5A/5B-deficient mice exhibited broad hematopoietic deficiencies, we sought to identify the earliest stage of differentiation at which defects were evident in the competitive repopulation assay. We failed to detect $lin^{dim}Sca\text{-}1^+$ cells, $lin^{dim}Sca\text{-}1^{neg/lo}$ cells, or lin^+ cells derived from STAT5A/5B-deficient cells at levels significantly above background (Figure 7C). Therefore, STAT5A/5B-deficient cells are inferior to wild-type cells in their ability to occupy the limited niche available for stem cells, early pluripotent cells, and oligopotent progenitor cells. The STAT5A/$5B^{-/-}$ HSCs thus failed to compete effectively with wild-type competitor cells in the same tissue environment in the production of progeny cells representing diverse hematolymphoid lineages. Overall, STAT5A/5B-deficient cells exhibit a profound defect in HSCs that is independent of both nonhematopoietic cell genotype and indirect effects mediated by mature cells, such as T cells.

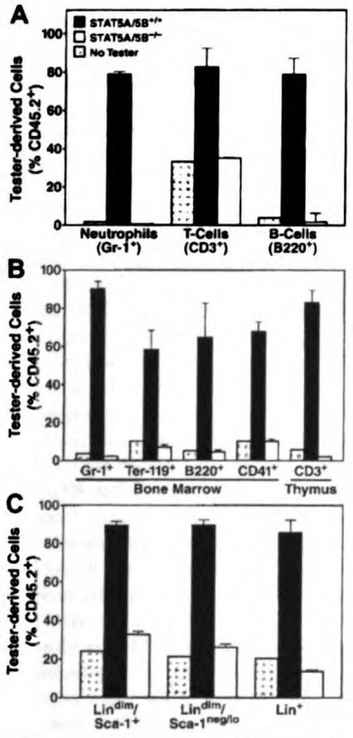

Figure 7. STAT5A/5B-deficient HSCs possess inferior competitive repopulating capacity. Peripheral blood, thymus, and bone marrow were harvested from irradiated recipients 10 weeks after receiving either 2×10^5 competitor cells only ("No Tester," n = 2), 2×10^5 competitor cells plus $STAT5A/5B^{+/+}$ tester cells ("$STAT5A/5B^{+/+}$ Tester," n = 3), or 2×10^5 competitor cells plus $STAT5A/5B^{-/-}$ tester cells ("$STAT5A/5B^{-/-}$ Tester," n = 6). FACS analysis was performed by using lineage markers and CD45.1 and CD45.2, and the results for the indicated lineage-specific subsets are displayed. (A) Peripheral blood cells positive for $Gr\text{-}1^+$ ($P < .001$), $B220^+$ ($P < .001$), or $CD3^+$ ($P = .002$). (B) Cells from hematolymphoid organs, including bone marrow cells expressing for Gr-1 ($P < .001$), B220 ($P = .001$), Ter-119 ($P = .001$), or CD41 ($P < .001$) and thymocytes expressing CD3 ($P < .001$). (C) Bone marrow cells from the lin^+ ($P < .001$), $lin^{dim}Sca\text{-}1^{neg/lo}$ ($P < .001$), and $lin^{dim}Sca\text{-}1^+$ ($P < .001$) subsets; in this case $CD45.2^+$ cells were scored according to $CD45.1^-$ phenotype.

Discussion

The transcription factor STAT5 is activated by multiple and diverse cytokines on binding to their cognate receptors, including several that act on the hematopoietic system. However, mice deficient for STAT5A, STAT5B, or both were reported to have surprisingly subtle deficiencies in hematolymphoid development, including reduction in peripheral T cells,[13,14,18] impaired fetal erythropoiesis,[20] and decreased survival of monocyte progenitors.[21] Because numerous and complex regulatory pathways impinge on hematolymphoid development in vivo, we sought to define the hematologic features of STAT5A/5B-deficient mice to determine whether compensatory mechanisms may mask greater contributions of STAT5 in hematopoiesis in vivo.

The first phase of our characterization revealed multilineage effects in peripheral blood at steady state, including marked decreases in erythrocytes and reduced numbers of circulating platelets. We also observed significant lymphopenia affecting both T cells and B cells. Moreover, although there was no abnormality in levels of peripheral blood neutrophils, a substantial decrease in the pool of mature neutrophils in the bone marrow was observed. In addition, histologic analysis of whole long bones and spleen in STAT5A/5B-deficient mice revealed extramedullary hematopoiesis in the enlarged spleens that was nearly exclusively erythropoietic tissue, as well as an exaggeration of myelopoiesis and few erythropoietic cells within intramedullary tissue in bone (data not shown). Hematologic stress in the mouse characteristically induces a myelopoietic dominance within bone marrow sites and heightened erythropoiesis within extramedullary sites such as the spleen.[28] Therefore our histologic findings may represent further evidence of hematopoietic stress in these mice. We note that our peripheral blood analysis of STAT5A/5B-deficient mice differs from the initial characterization of these mice.[18] One possible explanation is the age of the mice examined, because the influence of maturity has not been studied. Another possible factor is genetic background, because hematopoiesis is likely influenced by numerous strain-specific determinants. In any event, our findings indicate that there are defects in the circulating levels of 3 distinct blood lineages associated with signs of overall hematopoietic stress.

Multilineage cytopenia can be caused by hyperactive consumptive mechanisms broadly affecting peripheral blood cells, by multiple and independent defects affecting production or survival of individual cell types, or by a central bone marrow defect affecting progenitor cells that are common to multiple lineages. The scope of the blood cell abnormalities evident in STAT5A/$5B^{-/-}$ mice was most consistent with a central hematopoietic defect. Although STAT5A/5B-deficient mice possessed marked

bone marrow hypocellularity overall, the cell population containing HSCs ($lin^{dim}Sca\text{-}1^{+}$) was preserved quantitatively, whereas cells of more restricted differentiation potential were profoundly reduced. Collectively, these findings based on cellular representation within the bone marrow strongly suggest that the absence of STAT5A/5B leads to a quantitative loss in the population of cells that contain the CMP,[1] the CLP,[2] oligopotent progenitors, and lineage-committed progenitors.

To complement the flow cytometry data, we performed several in vivo reconstitution assays. The short-term radioprotection assay showed a severe defect in the ability of whole bone marrow from STAT5A/5B-deficient mice to protect a recipient from radiation-induced hematopoietic failure. In addition, we found that the reduction in the functional capability of the bone marrow from STAT5A/5B-deficient mice was not due to decreased homing ability. We used a second assay, the CFU-S assay, to quantitate early progenitor cells on the basis of functional criteria. These experiments revealed a dramatic reduction of hematopoietic colonies derived from STAT5A/5B-deficient animals. These results support our earlier finding that there is a diminution of the population ($lin^{dim}Sca\text{-}1^{neg/lo}$) within the bone marrow reported to contain oligopotent or lineage-committed cells and demonstrate that STAT5A/5B is a regulator of the biology of early progenitor cells.

A decrease in the overall cell numbers in STAT5A/5B-deficient bone marrow could be caused by various mechanisms involving insensitivity of hematopoietic progenitors to one or more cytokines, including a reduction in survival half-life, a decrease in proliferation potential of progenitors, or an impaired execution of differentiation programs. We detected an increase in the rate of apoptosis in both the lin^{+} and $lin^{dim}Sca\text{-}1^{neg/lo}$ fractions of bone marrow in STAT5A/5B-deficient mice. Although these studies do not elucidate the precise mechanism of antiapoptosis, impaired regulation of Bcl-X[20,21] or other antiapoptotic mediators may be operative. We also detected an increased proportion of bone marrow cells in the S/G2/M phases of the cell cycle, a feature present in both the HSC and post-HSC populations. We propose that this increase in the proportion of cycling cells represents part of a compensation mechanism seeking to counter relative ineffective hematopoiesis in STAT5A/5B-deficient mice. Alternatively, slowed rates of progression through cell cycle in vivo might also underlie our results, as prolonged in vitro doubling times have been reported for STAT5A/5B-deficient progenitors.[21] Finally, progenitors from STAT5A/5B-deficient mice have been shown to differentiate fully to mature cells in vitro,[21] implying that there is no absolute loss of differentiation potential of progenitor cells in the absence of STAT5A/5B. Overall, these findings provide evidence of globally decreased cellular survival in bone marrow cells in the absence of STAT5A/5B, with a concurrent increase in the proportion of cycling cells among the least differentiated hematopoietic cells.

To establish definitively the abnormalities in the stem cells of STAT5A/5B-deficient mice, we applied the most rigorous functional assay for stem cell fitness, the competitive repopulation assay.[26,27] Cells from $STAT5A/5B^{-/-}$ animals were unable to give rise to significant numbers of cells of any lineage in any tissue examined, despite being introduced at a dose that yielded widespread reconstitution by $STAT5A/5B^{+/+}$ cells. Additionally, these experiments addressed the cell autonomy of the STAT5A/5B-mediated defect in these cells. Because the $STAT5A/5B^{-/-}$ stem cells compete poorly with wild-type competitor cells in the same biologic environments, the functional defect evidently is independent of both nonhematopoietic cell genotype and indirect effects mediated by mature cells such as T cells.

Thus, these analyses of the role of STAT5 in hematopoiesis in vivo reveal that STAT5A/5B is an important positive factor that promotes HSC fitness and multilineage hematopoiesis. Relative insensitivity to cytokines such as G-CSF, TPO, growth hormone, IL-3, or others that act on progenitors could be responsible through either a reduction in the overall signal intensity or the loss of specific signals mediated by these cytokines. Bone marrow from STAT5A-deficient mice was shown to produce fewer in vitro colonies in the presence of flt3-ligand, a cytokine known to be important for hematopoietic progenitor homeostasis.[29] At the cellular level, STAT5 may provide an antiapoptotic signal that lifts the threshold of survival in the context of internal and external apoptotic, antiapoptotic, proliferative, and differentiative signals. At the organismal level, this effect translates into lower viability of bone marrow hematopoietic cells, which likely results in fewer cells produced per stem cell that enter the differentiation program. In the context of lineage-specific defects shown in our analysis of post-HSC populations and in earlier reports regarding STAT5 deficiency, it remains unknown to what degree the cytopenias seen in these mice are attributable to the HSC, multilineage, or lineage-specific effects. In fact, evidence from some models in which stem cells or multilineage progenitors are affected[30-32] suggests that defects at these stages alone may not induce mulitlineage cytopenias in some contexts. Nevertheless, our findings establish a novel and important role for STAT5 in the regulation of these early hematopoietic cells. This role is quantitative and nonessential, but genetic modifiers may control the degree of severity. These modifying loci could be responsible for multiple compensation mechanisms, such as extramedullary hematopoiesis and increases in bone marrow proliferation, which together allow the organism to achieve levels of hematopoietic production that are compatible with life but reduced nonetheless.

Further studies in these animals may promote better understanding of the molecular pathogenesis of some forms of bone marrow failure. Additionally, STAT5 may act in a similar manner in human hematopoiesis and would thus be an attractive target for therapy in hematologic settings. Cytokine therapy is often used to ameliorate the phenotypes of lineage-specific and multilineage cytopenias, but it may be associated with increased rates of leukemic transformation in some settings.[33] Perhaps directed activation of specific cytokine-mediated intracellular signals would serve to increase blood cell production without a concomitant rise in the rate of transformation.

Acknowledgments

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References

1. Akashi K, Traver D, Miyamoto T, Weissman IL. A clonogenic common myeloid progenitor that gives rise to all myeloid lineages. Nature. 2000; 404:193-197.
2. Kondo M, Weissman IL, Akashi K. Identification of clonogenic common lymphoid progenitors in mouse bone marrow. Cell. 1997;91: 661-672.
3. Weissman IL. Translating stem and progenitor cell biology to the clinic: barriers and opportunities. Science. 2000;287:1442-1446.

4. Ogawa M, Matsunaga T. Humoral regulation of hematopoietic stem cells. Ann N Y Acad Sci. 1999;872:17-23.
5. Wu H, Liu X, Jaenisch R, Lodish HF. Generation of committed erythroid BFU-E and CFU-E progenitors does not require erythropoietin or the erythropoietin receptor. Cell. 1995;83:59-67.
6. de Sauvage FJ, Carver-Moore K, Luoh SM, et al. Physiological regulation of early and late stages of megakaryocytopoiesis by thrombopoietin. J Exp Med. 1996;183:651-656.
7. Lieschke GJ, Grail D, Hodgson G, et al. Mice lacking granulocyte colony-stimulating factor have chronic neutropenia, granulocyte and macrophage progenitor cell deficiency, and impaired neutrophil mobilization. Blood. 1994;84:1737-1746.
8. Bromberg J, Darnell JE Jr. The role of STATs in transcriptional control and their impact on cellular function. Oncogene. 2000;19:2468-2473.
9. Ihle JN. STATs: signal transducers and activators of transcription. Cell. 1996;84:331-334.
10. Ward AC, Touw I, Yoshimura A. The Jak-Stat pathway in normal and perturbed hematopoiesis. Blood. 2000;95:19-29.
11. Chrétien S, Varlet P, Verdier F, et al. Erythropoietin-induced erythroid differentiation of the human erythroleukemia cell line TF-1 correlates with impaired STAT5 activation. EMBO J. 1996;15:4174-4181.
12. Ilaria RL Jr, Hawley RG, Van Etten RA. Dominant negative mutants implicate STAT5 in myeloid cell proliferation and neutrophil differentiation. Blood. 1999;93:4154-4166.
13. Liu X, Robinson GW, Wagner KU, et al. Stat5a is mandatory for adult mammary gland development and lactogenesis. Genes Dev. 1997;11:179-186.
14. Udy GB, Towers RP, Snell RG, et al. Requirement of STAT5b for sexual dimorphism of body growth rates and liver gene expression. Proc Natl Acad Sci U S A. 1997;94:7239-7244.
15. Feldman GM, Rosenthal LA, Liu X, et al. STAT5A-deficient mice demonstrate a defect in granulocyte-macrophage colony-stimulating factor-induced proliferation and gene expression. Blood. 1997;90:1768-1776.
16. Nakajima H, Liu XW, Wynshaw-Boris A, et al. An indirect effect of Stat5a in IL-2-induced proliferation: a critical role for Stat5a in IL-2-mediated IL-2 receptor alpha chain induction. Immunity. 1997;7:691-701.
17. Imada K, Bloom ET, Nakajima H, et al. Stat5b is essential for natural killer cell-mediated proliferation and cytolytic activity. J Exp Med. 1998;188:2067-2074.
18. Teglund S, McKay C, Schuetz E, et al. Stat5a and Stat5b proteins have essential and nonessential, or redundant, roles in cytokine responses. Cell. 1998;93:841-850.
19. Moriggl R, Topham DJ, Teglund S, et al. Stat5 is required for IL-2-induced cell cycle progression of peripheral T cells. Immunity. 1999;10:249-259.
20. Socolovsky M, Fallon AE, Wang S, Brugnara C, Lodish HF. Fetal anemia and apoptosis of red cell progenitors in Stat5a $^{-/-}$5b$^{-/-}$ mice: a direct role for Stat5 in Bcl-X(L) induction. Cell. 1999;98:181-191.
21. Kieslinger M, Woldman I, Moriggl R, et al. Antiapoptotic activity of Stat5 required during terminal stages of myeloid differentiation. Genes Dev. 2000;14:232-244.
22. Ernst JD, Yang L, Rosales JL, Broaddus VC. Preparation and characterization of an endogenously fluorescent annexin for detection of apoptotic cells. Anal Biochem. 1998;260:18-23.
23. Till JE, McCulloch GA. A direct measurement of the radiation sensitivity of normal mouse bone marrow cells. Radiat Res. 1961;14:213-222.
24. Haneline LS, Gobbett TA, Ramani R, et al. Loss of FancC function results in decreased hematopoietic stem cell repopulating ability. Blood. 1999;94:1-8.
25. Oostendorp RA, Audet J, Eaves CJ. High-resolution tracking of cell division suggests similar cell cycle kinetics of hematopoietic stem cells stimulated in vitro and in vivo. Blood. 2000;95:855-862.
26. Domen J, Weissman IL. Self-renewal, differentiation or death: regulation and manipulation of hematopoietic stem cell fate. Mol Med Today. 1999;5:201-208.
27. Harrison DE, Astle CM. Short- and long-term multilineage repopulating hematopoietic stem cells in late fetal and newborn mice: models for human umbilical cord blood. Blood. 1997;90:174-181.
28. Wolf NS, Trentin JJ. Hematopoietic colony studies, V: effects of hematopoietic organ stroma on differentiation of pluripotent stem cells. J Exp Med. 1968;127:205-214.
29. Zhang S, Fukuda S, Lee Y, et al. Essential role of signal transducer and activator of transcription (Stat)5a but not Stat5b for Flt3-dependent signaling. J Exp Med. 2000;192:719-728.
30. Solar GP, Kerr WG, Zeigler FC, et al. Role of c-mpl in early hematopoiesis. Blood. 1998;92:4-10.
31. Kimura S, Roberts AW, Metcalf D, Alexander WS. Hematopoietic stem cell deficiencies in mice lacking c-Mpl, the receptor for thrombopoietin. Proc Natl Acad Sci U S A. 1998;95:1195-1200.
32. Mackarehtschian K, Hardin JD, Moore KA, et al. Targeted disruption of the flk2/flt3 gene leads to deficiencies in primitive hematopoietic progenitors. Immunity. 1995;3:147-161.
33. Freedman MH, Bonilla MA, Fier C, et al. Myelodysplasia syndrome and acute myeloid leukemia in patients with congenital neutropenia receiving G-CSF therapy. Blood. 2000;96:429-436.

Chapter 3

Transgenic Bcl2 is Not Sufficient to Rescue All Hematolymphoid Defects in STAT5A/5B-deficient Mice

Introduction

The relative physiological importance of various biological tasks mediated by cytokines and the specific signaling pathways responsible for these distinct biological outcomes are still areas of intense research. Recent studies have focused on the importance of maintenance of survival as the chief biologic consequence of cytokine signaling. The cytokine dependent, multilineage-potential cell line, FDCP-mix, is rescued for differentiation into multiple lineages with ectopic expression of bcl-2 [1]. Complete rescue of defects in two mouse models lacking IL-7 and M-CSF respectively, by transgenic expression of hBcl-2 provided further in vivo evidence for the paradigm that cytokines act predominantly through the provision of survival signals [2,3]. These studies seemed to indicate that differentiation and proliferation could be regulated by intrinsic factors or by alternate extrinsic factors in the absence of cytokine signaling as long as survival signals were provided ectopically.

Apoptosis is composed of three interconnected pathways that ultimately lead to activation of the caspases, cysteine proteases that cleave cellular targets resulting in programmed cell death of the target cell. The receptor-mediated pathway involves signaling by pro-apoptotic receptors of the TNF receptor family that activate initiator caspases via death-domain interactions, which in turn activate the effector caspases that then destroy the cell. The receptor-mediated pathway is triggered in cells to maintain homeostasis such as through Fas / FasL-dependent destruction of erythroid progenitors by more mature progenitors [4]. The cytotoxic pathway involves the delivery of granzymes to cells by T-cells via perforin-pores in the cellular membrane. These granzymes activate the cellular caspase system. The cytotoxic pathway is utilized to kill

damaged cells such as virally infected cells. The mitochondria-mediated pathway involves the disruption of mitochondrial membrane. This ultimately leads to the release of cytochrome c, which combines with Apaf-1 to initiate effector caspase activation. In addition, mitochondrial dysfunction can lead to alteration in other parameters, such as cellular redox. The mitochondrial pathway can be triggered by multiple pathways linked to the overall health of the cell, such as DNA damage through the p53 pathway, as well as a cell's physiologic value, such as the death by neglect of megakaryocyte progenitors at limiting concentrations of TPO [5]. Pro-survival action of cytokines in hematopoietic progenitors is thought to impinge on regulation of these pathways at multiple levels [6], but most importantly through regulation of the mitochondrial-dependent pathway. Cytokines affect mitochondrial health by modulation of members of the Bcl-2 family either through transcriptional regulation or biochemical modification. This family is composed of pro-survival and pro-apoptotic members, which may act through a putative channel-forming activity [7-11].

Mounting data from multiple groups, using a model deficient for STAT5, indicated that the major role of this molecule in hematolymphoid development *in vivo* was linked to the maintenance of target cell survival. Lineage specific progenitors from both the erythroid and myeloid lineages as well as multilineage progenitors from STAT5A/5B-deficient mice display higher rates of apoptosis [12-16]. Mechanistically, STAT5 is thought to act at the level of Bcl-2 family members by upregulating pro-survival family members, such as Bcl-x, Bcl-2, and A1 [17-21]. Cytokine dependent upregulation of pro-survival Bcl-2 family members has been shown to depend on STAT5

in vitro [13-15]. In fact, overexpression of these molecules *in vitro* can ameliorate the apoptosis observed in selected lineage specific progenitors [14,15].

The goal of the following study was to utilize combinatorial genetics to attempt to complement the loss of STAT5 in with the transgenic expression of Bcl-2. The results indicate that while peripheral blood counts are normalized in STAT5A/5B-deficient mice in the presence of the Bcl-2 transgene, that the majority of defects in the bone marrow, including hypocellularity and inferior competitive repopulating ability are not rescued by the presence of the transgene.

These results indicate that our understanding of the role of STAT5 in cytokine-mediated biological outcomes is incomplete and must be revised to accommodate the importance of STAT5 in promoting other biological pathways.

References

1. Fairbairn LJ, Cowling GJ, Reipert BM, Dexter TM (1993) Suppression of apoptosis allows differentiation and development of a multipotent hemopoietic cell line in the absence of added growth factors. Cell 74:823

2. Akashi K, Kondo M, von Freeden-Jeffry U, Murray R, Weissman IL (1997) Bcl-2 rescues T lymphopoiesis in interleukin-7 receptor-deficient mice. Cell 89:1033

3. Lagasse E, Weissman IL (1997) Enforced expression of Bcl-2 in monocytes rescues macrophages and partially reverses osteopetrosis in op/op mice. Cell 89:1021

4. De Maria R, Testa U, Luchetti L, Zeuner A, Stassi G, Pelosi E, Riccioni R, Felli N, Samoggia P, Peschle C (1999) Apoptotic role of Fas/Fas ligand system in the regulation of erythropoiesis. Blood 93:796

5. Osada M, Komeno T, Todokoro K, Takizawa M, Kojima H, Suzukawa K, Ninomiya H, Abe T, Nagasawa T (1999) Immature megakaryocytes undergo apoptosis in the absence of thrombopoietin. Exp Hematol 27:131

6. Lotem J, Sachs L (2002) Cytokine control of developmental programs in normal hematopoiesis and leukemia. Oncogene 21:3284

7. Antonsson B, Martinou JC (2000) The Bcl-2 protein family. Exp Cell Res 256:50

8. Cory S, Adams JM (2002) The bcl2 family: regulators of the cellular life-or-death switch. Nat Rev Cancer 2:647

9. Battle TE, Frank DA (2002) The role of STATs in apoptosis. Curr Mol Med 2:381

10. Gross A, McDonnell JM, Korsmeyer SJ (1999) BCL-2 family members and the mitochondria in apoptosis. Genes Dev 13:1899

11. Vander Heiden MG, Thompson CB (1999) Bcl-2 proteins: regulators of apoptosis or of mitochondrial homeostasis? Nat Cell Biol 1:E209

12. Snow JW, Abraham N, Ma MC, Abbey NW, Herndier B, Goldsmith MA (2002) STAT5 promotes multilineage hematolymphoid development in vivo through effects on early hematopoietic progenitor cells. Blood 99:95

13. Socolovsky M, Nam H, Fleming MD, Haase VH, Brugnara C, Lodish HF (2001) Ineffective erythropoiesis in Stat5a(-/-)5b(-/-) mice due to decreased survival of early erythroblasts. Blood 98:3261

14. Socolovsky M, Fallon AE, Wang S, Brugnara C, Lodish HF (1999) Fetal anemia and apoptosis of red cell progenitors in Stat5a-/-5b-/- mice: a direct role for Stat5 in Bcl-X(L) induction. Cell 98:181

15. Kieslinger M, Woldman I, Moriggl R, Hofmann J, Marine JC, Ihle JN, Beug H, Decker T (2000) Antiapoptotic activity of Stat5 required during terminal stages of myeloid differentiation. Genes and Development 14:232

16. Shelburne C, McCoy M, Piekorz R, Sexl V, Gillespie S, Bailey D, Gharse A, Mirmonsef P, M NM, Kashyap M, Wright H, Chong H, Bouton L, Ramirez C, Lantz C, Ryan J (2002) Stat5: an essential regulator of mast cell biology. Mol Immunol 38:1187

17. Lord JD, McIntosh BC, Greenberg PD, Nelson BH (2000) The IL-2 receptor promotes lymphocyte proliferation and induction of the c-myc, bcl-2, and bcl-x genes through the trans-activation domain of Stat5. J Immunol 164:2533

18. Nosaka T, Kawashima T, Misawa K, Ikuta K, Mui AL, Kitamura T (1999) STAT5 as a molecular regulator of proliferation, differentiation and apoptosis in hematopoietic cells. Embo J 18:4754

19. Silva M, Benito A, Sanz C, Prosper F, Ekhterae D, Nunez G, Fernandez-Luna JL (1999) Erythropoietin can induce the expression of bcl-x(L) through Stat5 in erythropoietin-dependent progenitor cell lines. J Biol Chem 274:22165

20. Dumon S, Santos SC, Debierre-Grockiego F, Gouilleux-Gruart V, Cocault L, Boucheron C, Mollat P, Gisselbrecht S, Gouilleux F (1999) IL-3 dependent regulation of Bcl-xL gene expression by STAT5 in a bone marrow derived cell line. Oncogene 18:4191

21. Feldman GM, Rosenthal LA, Liu X, Hayes MP, Wynshaw-Boris A, Leonard WJ, Hennighausen L, Finbloom DS (1997) STAT5A-deficient mice demonstrate a defect in granulocyte-macrophage colony-stimulating factor-induced proliferation and gene expression. Blood 90:1768

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Transgenic Bcl-2 is Not Sufficient to Rescue All Hematolymphoid Defects in STAT5A/5B-deficient Mice[ς]

Jonathan W. Snow[1,2], Ninan Abraham[1], Melissa C. Ma[1], Sarah K. Bronson[4], and Mark A. Goldsmith[1,3,5]

[1]*Gladstone Institute of Virology and Immunology, Departments of* [2]*Microbiology and Immunology and* [3]*Medicine, University of California at San Francisco, San Francisco, CA 94114-9100, USA.*

[4]*Department of Cellular and Molecular Physiology*

The Penn State College of Medicine, Hershey, PA 17033-0850

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[5]To whom correspondence should be addressed:

Gladstone Institute of Virology & Immunology

P.O. Box 419100, San Francisco, CA 94141-9100

Phone: 415.695.3775 Fax: 415.695.1364

Email: mgoldsmith@gladstone.ucsf.edu

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Abstract

Cytokines bind high affinity receptors expressed on hematopoietic cells to initiate signaling cascades that regulate differentiation, proliferation, and survival. Previous studies have established a role for STAT5 in transducing survival signals for hematopoietic progenitor cells in response to cytokines. To determine if constitutive expression of a member of the Bcl-2 family of anti-apoptotic proteins could compensate for the loss of STAT5, we utilized combinatorial genetics to generate STAT5A/5B-deficient mice expressing a Bcl-2 transgene. Although Bcl-2 expression restored peripheral blood counts to normal in STAT5A/5B$^{-/-}$ mice, we noted a striking failure of this transgene to correct defects in hematopoietic stem and progenitor cells. These data imply important effects of STAT5 in addition to survival in modulating hematopoietic cell fates.

Introduction

STAT5 is activated by diverse cytokine receptors involved at multiple levels within the hematopoietic system. The widespread engagement of its two isoforms, STAT5A and STAT5B, has implicated STAT5 as a potentially important component of cytokine receptor signaling in this context [1]. In fact, mice deficient in both the STAT5A and STAT5B isoforms were shown to have multiple defects in hematopoietic development affecting all stages of differentiation from the hematopoietic stem cell (HSC) [2-4] to lineage-committed progenitors [5-7]. In addition, some abnormalities in the function of mature hematopoietic cells have been described in mice either singly or doubly deficient for the STAT5 isoforms [7-14].

The biological mechanism responsible for many of these defects, specifically in hematopoietic progenitors, is thought to be a reduction in cytokine-mediated survival of these cells in the absence of STAT5 [15,16]. For example, erythroid progenitors lacking STAT5A/5B exhibited increased apoptosis *in vivo* and *in vitro* in response to erythropoetin (EPO) [5,6]. Similarly, committed myeloid progenitors lacking STAT5A/5B demonstrated increased cell death *in vitro* in response to granulocyte / macrophage-colocy-stimulating-factor (GM-CSF) or interleukin-3 (IL-3) [7]. Finally, early multipotential progenitors in STAT5A/5B-deficient mice exhibited increased apoptosis *in vivo* [2]. The relevant molecular targets of STAT5 in hematopoietic progenitors that are responsible for these survival effects are thought to consist of anti-apoptotic members of the Bcl-2 family of proteins, predominantly Bcl-x [5-7,15-17].

Members of this family of proteins are important for regulating mitochondrial homeostasis, by positively or negatively impacting mitochondrial compartmentalization of cytochrome c [18-20], a major junction point in the cellular decision to apoptose.

Combinatorial genetics to explore whether a survival factor can rescue defects resulting from the loss of a cytokine signaling was first employed to show that the hematopoietic defects of op/op mice and IL-7 receptor-deficient mice could be rescued by transgenic expression of a human Bcl-2 [21,22] and has since been used extensively to interrogate the ability of this family of survival factors to complement loss of cytokine signaling molecules [23-26]. To determine the role of Bcl-2 expression in mediating the contributions of STAT5 to hematopoiesis, we generated STAT5A/5B-deficient mice in which a Bcl-2 transgene was expressed from the constitutive β-actin promoter [27]. We found that ectopic expression of Bcl-2 was not sufficient to rescue all the hematopoietic defects in STAT5A/5B-deficient mice.

Materials and Methods

Handling and Characterization of Mice.

STAT5A/5B$^{-/-}$ mice [8] were obtained from Dr. James Ihle (St. Jude Children's Research Hospital, Memphis, TN) and back-crossed onto a C57Bl/6 background at least 9 generations. Mice with a murine Bcl-2 transgene expressed under the control of human β-actin promoter and Bcl-2-deficient mice have been described[27,28]. Mice were housed in a pathogen-free rodent barrier facility and received mouse chow and acidified water *ad libitum*. All studies were performed on 6–8 week old mice unless otherwise specified, and littermates were always used as wild-type controls. Peripheral blood was obtained via cardiac puncture, and EDTA-treated samples were used for complete blood counts (IDEXX Veterinary Service, Sacramento, CA). Bone marrow was harvested by flushing femurs and tibias into 6 ml phosphate buffered saline (PBS) containing 2% fetal bovine serum (FBS) and 2 mM EDTA, and cell counts were determined after ACK (NH_4Cl) lysis by trypan blue exclusion. Single cell suspensions of spleens were generated by passing them through a 70 μm nylon mesh strainer into 5 ml PBS containing 2% FBS and 2mM EDTA, and cell counts were determined after ACK (NH_4Cl) lysis by trypan blue exclusion.

Flow Cytometry.

All antibodies were obtained from Pharmingen (San Diego, CA), and the following clones were used; Sca-1 (E13-161.7), Ly-76 (Ter-119), Gr-1 (RB6-8C5), CD3 (145-2C11), B220 (RA3-6B2), CD11b (M1/70), c-kit (2B8), CD45.1 (A20), CD45.2 (104),

Bcl-2 (3F11). For blocking non-specific binding to Fc receptors, purified antibody to CD16/CD32 (2.4G2) was used in at 1:100 for 3 minutes. Subsequently, antibodies to surface markers of interest were used at 1:60 dilution. For intracellular staining of Bcl-2, cells were stained for expression of surface markers, then fixed and permeabilized for 1 h at 25° C in 1% paraformaldehyde (PFA) with 0.1% Tween-20, washed, stained for 30 minutes at 25° C with anti Bcl-2 antibody at a 1:5 dilution, then washed. All FACS analyses were performed using a FACSCalibur and all sorting was performed using a FACSVantage (Becton Dickinson).

Competitive Repopulation Studies.

Recipient mice were 8 - 10 week old C57Bl/6 obtained from Jackson Laboratories (Bar Harbor, ME). Recipients were γ-irradiated from a Cesium source in two 450 rad doses 4–5 hrs apart. Transplanted mice were maintained on 2.5 mg/ 100 ml Sulfatrim Pediatric Suspension (Alpharma, Baltimore, MD) in the drinking water. Competitive repopulation studies were performed as previously described.[29] Whole bone marrow ($CD45.2^+$), derived from wild-type littermate or $STAT5A/5B^{-/-}$, with or without the Bcl-2 transgene, were mixed at a 1:1 ratio with competitor whole bone marrow from congenic B6.SLJ ($CD45.1^+$) and injected into irradiated recipients, prepared as above. After 10-12 weeks, chimeric mice were sacrificed and peripheral blood and bone marrow were collected and analyzed by FACS for contribution of $CD45.1^+$ and $CD45.2^+$ derived cells to selected lineages.

Statistical Analysis.

Data are presented as mean ± SEM when appropriate. Statistical significance was assessed by two-sided Student's t-test.

Results

Bcl-2 transgene is expressed in mature cells and progenitor subsets in the bone marrow

To determine the ability of exogenously expressed Bcl-2 to rescue hematopoietic defects in STAT5A/5B-deficient mice, we used mice expressing Bcl-2 under the β-actin promoter to generate STAT5A/5B-deficient mice expressing this transgene. To show that the Bcl-2 transgene under control of the β-actin promoter was expressed in hematopoietic progenitors and mature blood cells, we performed intracellular staining for Bcl-2 on various bone marrow subsets from Bcl-2-deficient mice crossed with the β-actin-Bcl-2 transgenic line [27,28]. We detected robust Bcl-2 expression in lineage-positive bone marrow cells, in the $Lin^{dim}Sca\text{-}1^{neg/lo}c\text{-}kit^{+}$ fraction containing post-HSC, including common lymphoid progenitors (CLP), common myeloid progenitors (CMP), and oligopotent progenitors, and in the $lin^{dim}Sca\text{-}1^{+}c\text{-}kit^{+}$ fraction containing HSC[30] (Fig. 1).

Expression of transgenic Bcl-2 rescues cytopenias in adult STAT5A/5B-deficient mice.

We then examined peripheral blood lineages from $STAT5A/5B^{+/+}$ and $STAT5A/5B^{-/-}$ mice without (-Tg) or with (+Tg) the Bcl-2 transgene. As shown previously, [2,3] $STAT5A/5B^{-/-}$, -Tg mice displayed cytopenia affecting a number of peripheral blood lineages, including a marked drop in hematocrit, platelets, and

lymphocytes. When we examined peripheral blood lineages from STAT5A/5B$^{+/+}$ and STAT5A/5B$^{-/-}$ mice with (+Tg) the Bcl-2 transgene, we found that Bcl-2 expression restored erythrocyte and platelet and lymphocyte numbers to levels equivalent to wild-type in STAT5A/5B$^{-/-}$ mice (Fig 2A-2C). Both erythrocytes and lymphocytes in STAT5A/5B$^{-/-}$, (+Tg) mice demonstrated trends of reduced numbers compared to STAT5A/5B$^{+/+}$ mice with the Bcl-2 transgene that were not statistically significant. The number of neutrophils was not significantly different between STAT5A/5B$^{-/-}$, -Tg mice and STAT5A/5B$^{+/+}$, -Tg mice (Fig. 2D). A similar equivalence was observed in the number of neutrophils in STAT5A/5B$^{-/-}$, +Tg compared to STAT5A/5B$^{+/+}$, +Tg mice (Fig 2D.). Thus the presence of Bcl-2 fully rescues all the values of peripheral blood lineages, supporting the hypothesis that a major role of STAT5 in hematopoiesis is indeed to modulate cell survival.

Expression of the Bcl-2 transgene does not rescue hypocellular bone marrow and incompletely rescues defects in progenitor subsets in STAT5A/5B-deficient mice

To determine whether defects in hematopoietic progenitors observed previously in STAT5A/5B-/- mice would be rescued by the ectopic expression of Bcl-2, we examined the bone marrow of mice without (-Tg) or with (+Tg) transgene. As shown previously, STAT5A/5B-deficient mice possessed a hypocellular bone marrow compared with wild-type mice [2,3] (Fig. 3). This defect in cellularity was not rescued by transgenic expression of Bcl-2 (Fig. 3).

We next performed FACS analysis of bone marrow from mice of each STAT5A/5B and Bcl-2 Tg genotype to quantify various progenitor subsets within the bone marrow by expression of surface lineage-defining markers (Lin), Sca-1, and c-kit. The $Lin^{dim}Sca\text{-}1^{+}c\text{-}kit^{+}$ population was increased, but not to a statistically significantly degree, in STAT5A/5B$^{-/-}$, -Tg mice compared to STAT5A/5B$^{+/+}$, -Tg mice (Fig. 4A). Presence of the Bcl-2 transgene reduced this subset of cells in STAT5A/5B-deficient bone marrow to numbers comparable to wild-type (Fig. 4B). The $Lin^{dim}Sca\text{-}1^{neg/lo}c\text{-}kit^{+}$ population was substantially decreased in STAT5A/5B$^{-/-}$, -Tg mice compared to STAT5A/5B$^{+/+}$, -Tg mice (Fig. 4C). Ectopic expression of Bcl-2 did not restore this subset of cells in STAT5A/5B-deficient bone marrow (Fig. 4D). Thus the presence of the Bcl-2 transgene does not rescue bone marrow cellularity or the number of post-HSC progenitors in STAT5A/5B-deficient mice, while the number of HSC was returned to normal in the these mice.

Inferior competitive repopulating capacity of STAT5A/5B-deficient HSC is not rescued by presence of the Bcl-2 transgene.

We next performed competitive repopulation experiments to determine whether expression of the Bcl-2 transgene would complement the defective competitive repopulating potential of STAT5A/5B-deficient HSC [2,3]. Tester cells of each STAT5A/5B and Bcl-2 Tg genotype were mixed with congenic CD45.1^{+} wild-type C57Bl/6 competitor cells at a 1:1 ratio and injected into irradiated recipients. Peripheral blood and bone marrow were harvested from recipient mice 10 weeks later and the relative contribution of the tester and competitor populations to different hematopoietic

lineages was assessed by FACS. At this ratio of input cells, STAT5A/5B$^{+/+}$, -Tg tester cells (CD45.2^{+}) and STAT5A/5B$^{+/+}$, +Tg tester cells gave rise to greater than 50% of the differentiated cells within peripheral blood, including granulocytes (Gr-1^{+}), T-cells (CD3^{+}), B-cells (B220^{+}), and monocytes (Mac1^{+}/Gr-1^{-}) (Fig. 5A, 5B). By contrast, STAT5A/5B$^{-/-}$, -Tg tester cells were impaired in their ability to repopulate these lineages in recipient mice (Fig. 5A). Expression of the Bcl-2 transgene did not significantly enhance the repopulating potential of STAT5A/5B-deficient bone marrow (Fig. 5B). We observed similar results in peripheral blood assayed at 4 weeks post-transfer (data not shown).

Previous studies demonstrated minimal contribution by STAT5A/5B-deficient tester cells to the Lin^{dim}Sca-1^{+}, HSC-containing, population in competitive repopulation experiments [2]. We observed that the presence of the Bcl-2 transgene did not increase the contribution by STAT5A/5B-deficient tester cells to this compartment and therefore was unable to rescue the restricted ability of STAT5A/5B-deficient HSC to fill a limited "niche" in the bone marrow (Fig. 6A, 6B) as previously described.

Discussion

Previous evidence from multiple groups has established a role for STAT5 in providing survival signals for hematopoietic progenitor cells in response to cytokines [2,5-7]. In particular, STAT5 has been shown to upregulate the transcription of Bcl-2 and Bcl-x [1,17]. In addition, erythroid cell lines unable to activate STAT5 have increased rates of apoptosis [31] and mice deficient for one or both isoforms of STAT5 have increased cell death in specific hematopoietic cells, leading to marked hematopoietic defects [2,5-7]. To determine the extent to which constitutive Bcl-2 expression can substitute for STAT5 in fulfilling its role in hematopoiesis, we examined STAT5A/5B-deficient mice without (-Tg) or with (+Tg) a Bcl-2 transgene, under the control of the β-actin promoter [27].

We have shown that ectopic expression of a Bcl-2 transgene rescues some, though not all of the hematopoietic defects in STAT5A/5B-deficient mice. Restoration of normal peripheral blood counts is consistent with previous data showing an important role for STAT5 in providing protection from apoptosis in lineage-committed progenitors and mature cells through upregulation of Bcl-2 family members. A role for Bcl-2 family members in the survival of erythroid progenitors [32,33] and megakaryocytes [34,35] has been reported and STAT5 has been implicated in contributing to the upregulation of Bcl-x in erythroid progenitors [5,6], granulocyte and macrophage progenitors [7], and mast cell progenitors [17]. In addition, the lifespan of mature T-cells and B-cells has been shown to be sensitive to the level of Bcl-2 family members [24,36,37] and STAT5 may play a role in the physiologic regulation of these molecules [38,39].

Recent studies have also defined a role for STAT5 in multipotential progenitors and in HSC [2,3,7]. There is widespread expression of Bcl-2 family members in multilineage progenitors and HSC (reviewed in [40]) and regulation of these cells depends in part on an apoptosis that can be modulated by levels of Bcl-2 [40-42]. Interestingly, the Bcl-2 transgene failed to rescue defects in these compartments. Thus, while the absence of STAT5 does lead to increased apoptosis in these cells [2,7], it appears that other STAT5-dependent biological responses are of equal importance to in these cells.

These data support the existence of additional functions of STAT5A/5B that are unrelated to promoting survival through Bcl-2 family members. Consistent with this conclusion, STAT5 has been implicated in the regulation of hematopoietic cell proliferation in studies of mice deficient for one or both isoforms [4,10,17,43] and STAT5 has been shown to upregulate genes that positively regulate the cell cycle, such as c-myc and cyclin D1 [39,44]. However, in our initial studies, we found that proliferation did not appear to be affected in the $Lin^{dim}/Sca\text{-}1^{+}$ and $Lin^{dim}/Sca\text{-}1^{-}$ populations from STAT5A/5B-deficient mice by DNA content [2]. In addition, the proportion of cells in cycle and rate through the cycle was not decreased in these populations from STAT5A/5B$^{-/-}$ mice as assessed by BrdU incorporation (data not shown). This evidence against a role for proliferation defects in hematopoietic progenitors in STAT5A/5B-deficient mice could be explained by the heterogeneous nature of the populations examined, which may contain subsets of cells, irrelevant to efficient hematopoietic development, with increased proliferation. In fact, there is a population of $Lin^{dim}/Sca\text{-}1^{+}/ckit^{-}$ cells that is dramatically increased in the bone marrow of STAT5A/5B$^{-/-}$ mice

that appears linked to an autoimmune phenotype observed in these mice (Chapter 4). In addition, loss of STAT5 could result in cell cycle dysfunction that is not measured by our assays [17]. Therefore, impairment of this or some other biological process in the absence of STAT5A/5B may underlie the persistent hematopoietic defects.

It is interesting to note that the Bcl-2-independent role of STAT5 appears largely dispensable in steady-state hematopoiesis for maintaining wild-type peripheral blood counts. It is possible that the addition of hematopoietic stress, such as cytotoxic drugs, might reveal defects in maintaining peripheral blood counts in these mice. It also implies that defects in early progenitors can be overcome in maintaining peripheral blood counts, at least in a non-competitive setting, if later progenitors and mature cells function normally.

It is formally possible that all of the effects of STAT5A/5B are mediated through survival pathways, and that Bcl-2 is unable to substitute for other family members under all circumstances. Indeed, other anti-apoptotic proteins, such as Bcl-x, may be the relevant target molecule in some progenitors [5-7]. However, this seems unlikely, as the interchangeability of these two family members has been shown [45]. Additionally transgenic expression of Bcl-2, or a related family member, would likely not provide protection from cell death caused by a Bcl-2 insensitive mechanism, such as that caused by absence of T-Cell Receptor (TCR) in $Rag1^{-/-}$ mice or by negative selection [46,47]. Bcl-2 is also a well characterized inhibitor of proliferation and may compound proliferation defects resulting from the loss of STAT5 [42,48,49]. Finally, variable hematopoietic effects of transgenic Bcl-2 observed depending on the species origin of the Bcl-2 protein, the promoter controlling the transgene [41,50], and perhaps position effects

may complicate interpretation. However, combinatorial genetics has been used extensively to explore the ability of Bcl-2 to rescue defects resulting from the loss of cytokine signaling as noted above [21-26].

In summary, our data imply activities of STAT5A/5B in HSC and hematopoietic progenitors that are unrelated to its ability to suppress apoptosis. Further study will be necessary to identify other functions of STAT5A/5B targets and specifically how they influence early hematopoietic progenitors.

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Figure Legends

Figure 1. Bcl-2 Transgene under control of the β-Actin promoter is expressed in HSC, post-HSC, and lineage positive cells in the bone marrow. Bone marrow from Bcl-2$^{-/-}$ mice expressing the Bcl-2 Transgene was analyzed using FACS for intracellular Bcl-2 staining in conjunction with surface expression of lineage-defining markers, Sca-1, and c-kit. Samples stained with isotype control (solid line) and anti Bcl-2 antibody (dotted line) are shown.

Figure 2. Multilineage cytopenias in adult STAT5A/5B-deficient mice are rescued by ectopically expressed Bcl-2. Complete blood counts were performed on whole peripheral blood from STAT5A/5B$^{-/-}$ and STAT5A/5B$^{+/+}$ mice without (-Tg) or with (+Tg) the Bcl-2 transgene. (A) Hematocrit. (B) Platelets. (C) Lymphocytes. (D) Neutrophils. For STAT5 A/5B$^{+/+}$, -Tg, n = 7, for STAT5A/5B$^{-/-}$, -Tg, n = 9, for STAT5 A/5B$^{+/+}$, +Tg, n = 7, for STAT5A/5B$^{-/-}$, +Tg, n = 4.

Figure 3. Decreased total nucleated cells in the bone marrow of STAT5A/5B-deficient mice is not rescued by the presence of the Bcl-2 Transgene. Total nucleated cells were obtained from one femur and tibia and cell counts were determined after red blood cell lysis for bone marrow from STAT5A/5B$^{-/-}$ and STAT5A/5B$^{+/+}$ mice without (-Tg) (A) or with (+Tg) (B) the Bcl-2 transgene. For STAT5 A/5B$^{+/+}$, -Tg, n = 3, for

STAT5A/5B$^{-/-}$, -Tg, n = 3, for STAT5 A/5B$^{+/+}$, +Tg, n = 3, for STAT5A/5B$^{-/-}$, +Tg, n = 4.

Figure 4. Presence of the Bcl-2 Transgene incompletely rescues progenitor defects in the bone marrow of STAT5A/5B-deficient mice. Subsetting was performed based on surface expression of lineage-defining markers, Sca-1, and c-kit. Absolute values were generated by multiplying gated percentages by total nucleated cell numbers. The Lin^{dim}Sca-1^{+}/c-kit^{+} compartment, containing HSC, from mice without (-Tg) (A) or with (+Tg) (B) the Bcl-2 transgene and the Lin^{dim}Sca-1$^{neg/lo}$/c-kit^{+} compartment, containing post -HSC progenitors, from mice (-Tg) (C) or with (+Tg) (D) the Bcl-2 transgene are shown. For STAT5 A/5B$^{+/+}$, -Tg, n = 3, for STAT5A/5B$^{-/-}$, -Tg, n = 3, for STAT5 A/5B$^{+/+}$, +Tg, n = 3, for STAT5A/5B$^{-/-}$, +Tg, n = 3.

Figure 5. Transgenic Bcl-2 does not rescue inferior competitive repopulating capacity of STAT5A/5B-deficient bone marrow. Peripheral blood was harvested from irradiated recipients 10 weeks after receiving either 5x10^5 competitor cells only ("No Tester", n=6), 5x10^5 competitor cells plus 5x10^5 STAT5A/5B$^{+/+}$, -Tg tester cells ("STAT5A/5B$^{+/+}$ -Tg Tester", n=6), or 5x10^5 competitor cells plus 5x10^5 STAT5A/5B$^{-/-}$, -Tg tester cells ("STAT5A/5B$^{-/-}$, -Tg Tester", n=6) (A) or 5x10^5 competitor cells only ("No Tester", n=5), 5x10^5 competitor cells plus 5x10^5 STAT5A/5B$^{+/+}$, +Tg tester cells ("STAT5A/5B$^{+/+}$, +Tg Tester", n=5), or 5x10^5 competitor cells plus STAT5A/5B$^{-/-}$, +Tg

tester cells ("STAT5A/5B$^{-/-}$, +Tg Tester", n=8)(B). FACS analysis was performed using lineage markers and CD45.1 and CD45.2, and the results for the indicated lineage-specific subsets are displayed. Peripheral blood cells positive for Mac-1(Gr-1$^-$), Gr-1$^+$, B220$^+$, or CD3$^+$ are shown.

Figure 6. Transgenic Bcl-2 does not rescue inability of HSC from STAT5A/5B-deficient bone marrow to occupy stem cell niches. Bone marrow cells was harvested from irradiated recipients 10 weeks after receiving either 5x10^5 competitor cells only ("No Tester", n=6), 5x10^5 competitor cells plus 5x10^5 STAT5A/5B$^{+/+}$, -Tg tester cells ("STAT5A/5B$^{+/+}$ -Tg Tester", n=6), or 5x10^5 competitor cells plus 5x10^5 STAT5A/5B$^{-/-}$, -Tg tester cells ("STAT5A/5B$^{-/-}$, -Tg Tester", n=6) (A) or 5x10^5 competitor cells only ("No Tester", n=5), 5x10^5 competitor cells plus 5x10^5 STAT5A/5B$^{+/+}$, +Tg tester cells ("STAT5A/5B$^{+/+}$, +Tg Tester", n=5), or 5x10^5 competitor cells plus STAT5A/5B$^{-/-}$, +Tg tester cells ("STAT5A/5B$^{-/-}$, +Tg Tester", n=8)(B). FACS analysis was performed using lineage markers, Sca-1, and CD45.1 and CD45.2. CD45.2$^+$ cells in the Lin^{dim}Sca-1$^+$ subset were scored based on CD45.1$^-$ phenotype.

Figure 1

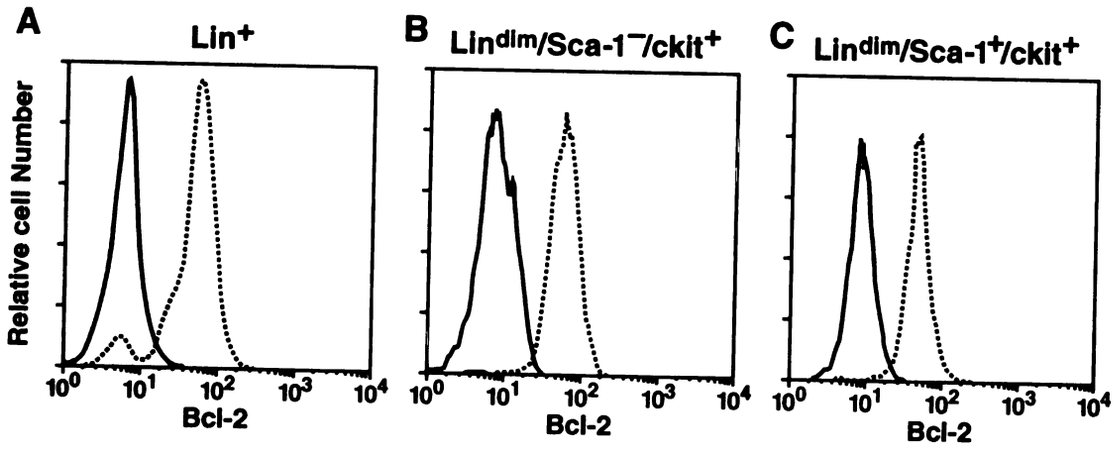

A
Lin+
B
Lindim/Sca-1−/ckit+
C
Lindim/Sca-1+/ckit+
Relative cell Number
10^0 10^1 10^2 10^3 10^4
Bcl-2

Figure 2

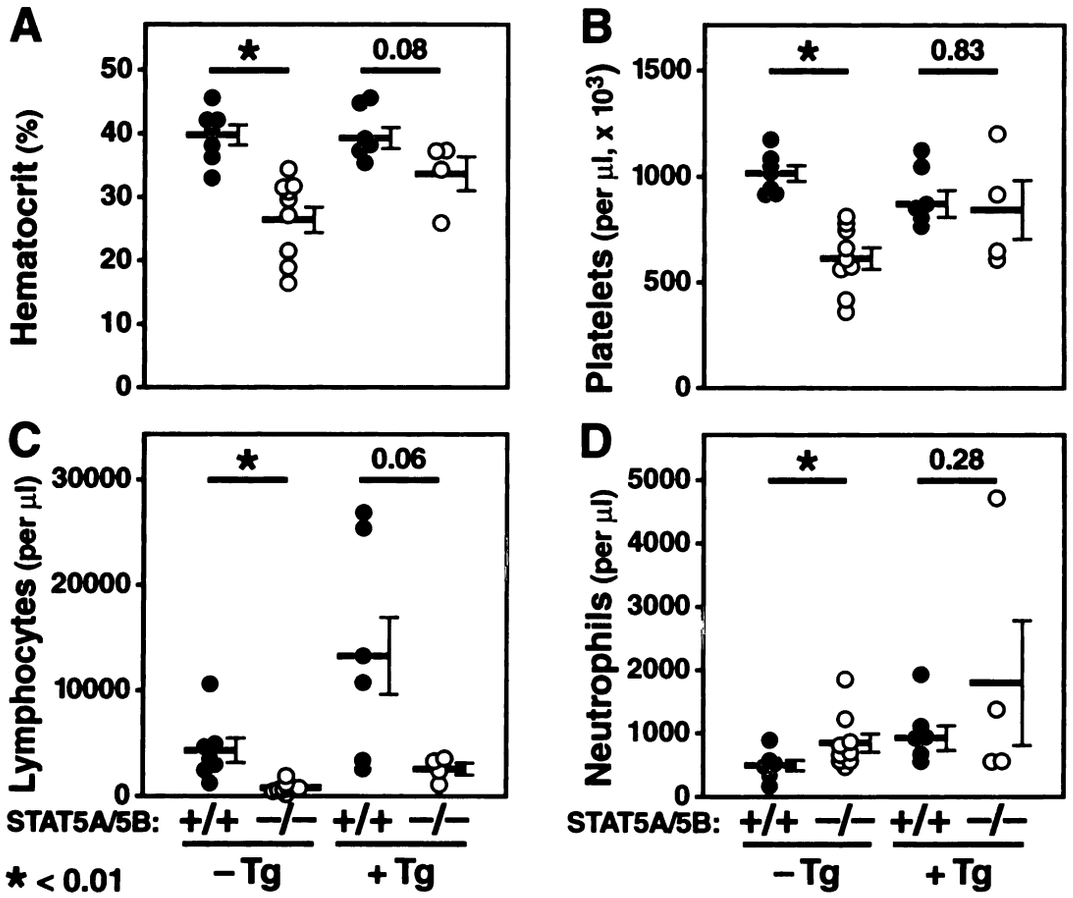

A
Hematocrit (%)
50
40
30
20
10
0
*
0.08
B
Platelets (per μl, x 10³)
1500
1000
500
0
*
0.83
C
Lymphocytes (per μl)
30000
20000
10000
0
*
0.06
D
Neutrophils (per μl)
5000
4000
3000
2000
1000
0
*
0.28
STAT5A/5B: +/+ −/− +/+ −/−
−Tg +Tg
* < 0.01
STAT5A/5B: +/+ −/− +/+ −/−
−Tg +Tg

Figure 3

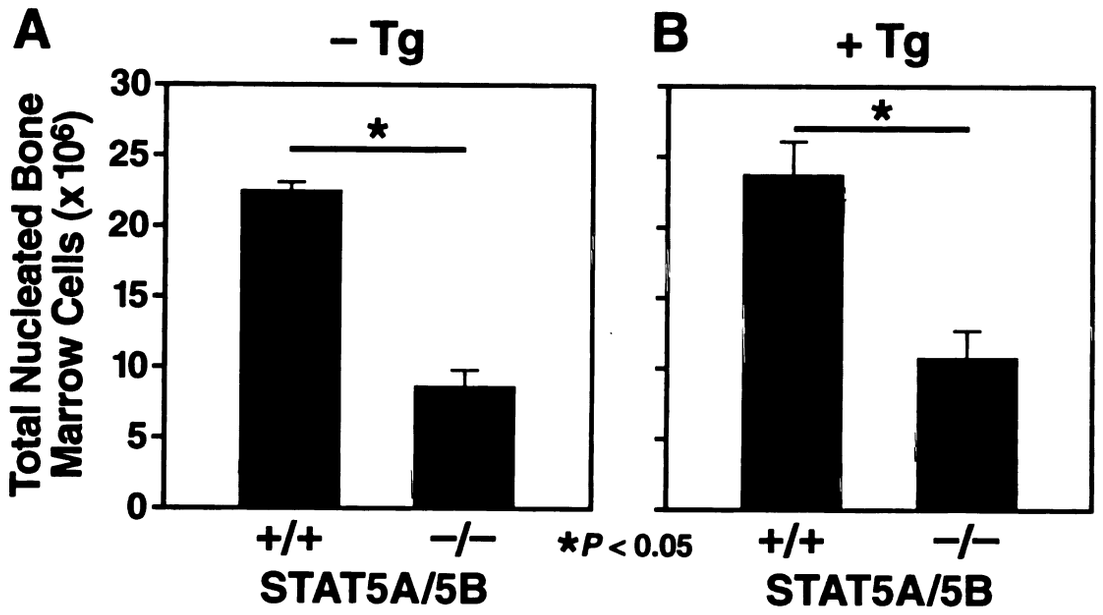

A
– Tg
B
+ Tg
Total Nucleated Bone Marrow Cells (x10⁶)
30
25
20
15
10
5
0
*
*
+/+
–/–
+/+
–/–
*P < 0.05
STAT5A/5B
STAT5A/5B

Figure 4

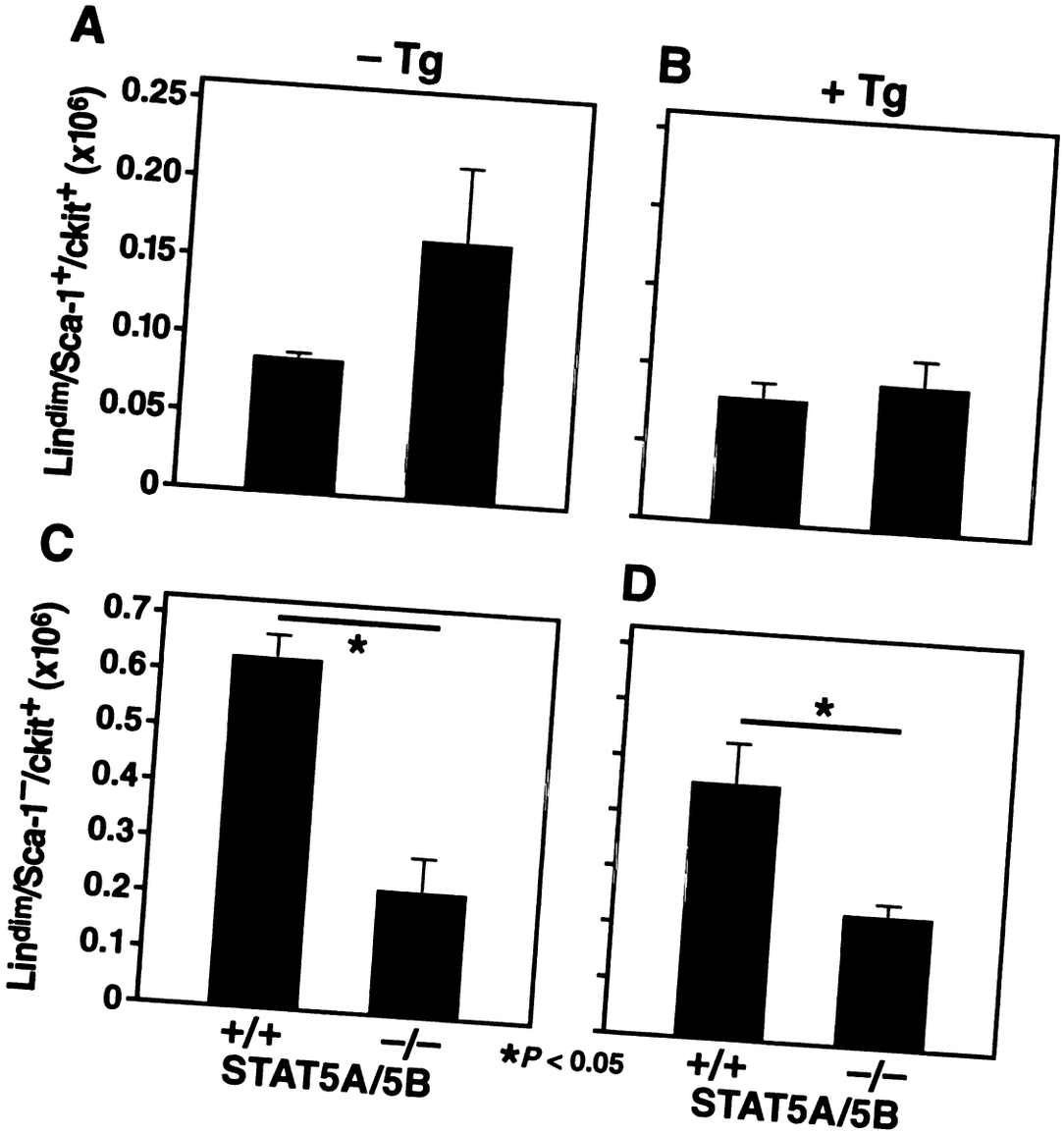

A
– Tg
B
+ Tg
Lin^dim/Sca-1^+/ckit^+ (x10^6)
0.25
0.20
0.15
0.10
0.05
0
C
D
Lin^dim/Sca-1^−/ckit^+ (x10^6)
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
*
*
+/+
−/−
STAT5A/5B
*P < 0.05
+/+
−/−
STAT5A/5B

Figure 5

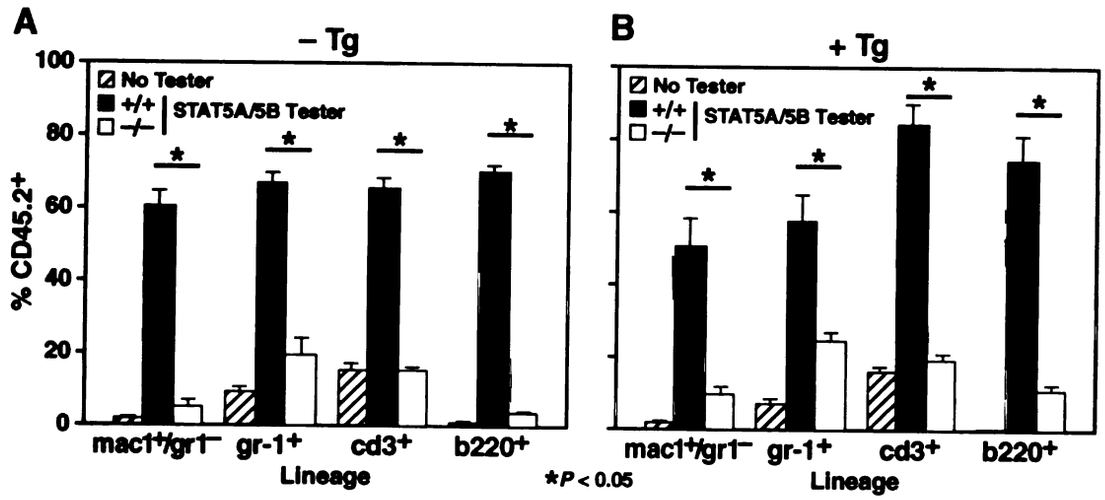
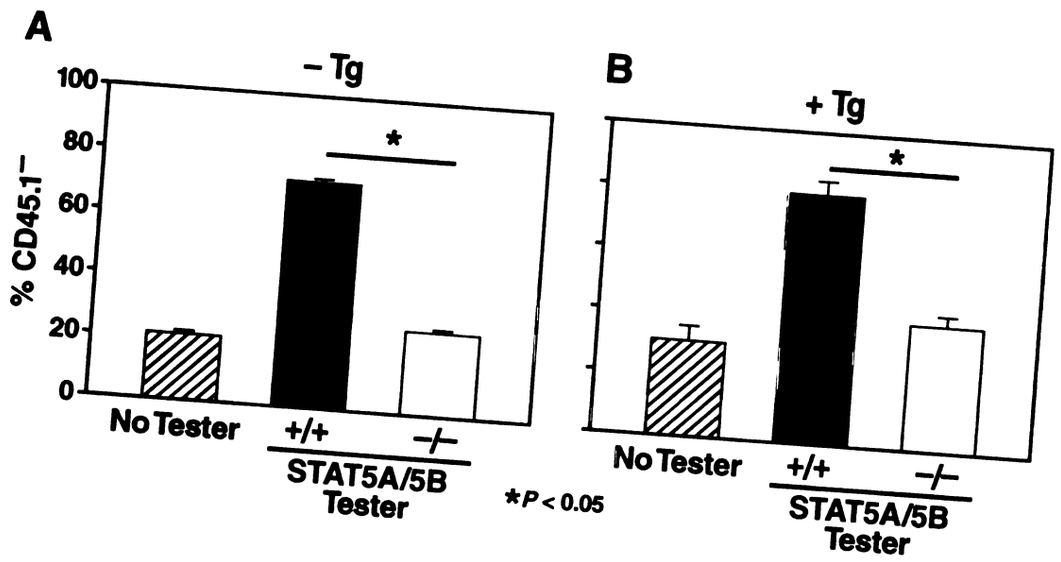

A
– Tg
B
+ Tg
No Tester
+/+
–/–
STAT5A/5B Tester
% CD45.2+
100
80
60
40
20
0
mac1+/gr1–
gr-1+
cd3+
b220+
Lineage
*P < 0.05

Figure 6


A
– Tg
100
80
60
40
20
0
% CD45.1⁻
*
No Tester
+/+
–/–
STAT5A/5B
Tester
*P < 0.05
B
+ Tg
*
No Tester
+/+
–/–
STAT5A/5B
Tester

References

1. Grimley PM, Dong F, Rui H (1999) Stat5a and Stat5b: fraternal twins of signal transduction and transcriptional activation. Cytokine Growth Factor Rev 10:131

2. Snow JW, Abraham N, Ma MC, Abbey NW, Herndier B, Goldsmith MA (2002) STAT5 promotes multilineage hematolymphoid development in vivo through effects on early hematopoietic progenitor cells. Blood 99:95

3. Bunting KD, Bradley HL, Hawley TS, Moriggl R, Sorrentino BP, Ihle JN (2002) Reduced lymphomyeloid repopulating activity from adult bone marrow and fetal liver of mice lacking expression of STAT5. Blood 99:479

4. Zhang S, Fukuda S, Lee Y, Hangoc G, Cooper S, Spolski R, Leonard WJ, Broxmeyer HE (2000) Essential Role of Signal Transducer and Activator of Transcription (Stat)5a but Not Stat5b for Flt3-dependent Signaling. J Exp Med 192:719

5. Socolovsky M, Nam H, Fleming MD, Haase VH, Brugnara C, Lodish HF (2001) Ineffective erythropoiesis in Stat5a(-/-)5b(-/-) mice due to decreased survival of early erythroblasts. Blood 98:3261

6. Socolovsky M, Fallon AE, Wang S, Brugnara C, Lodish HF (1999) Fetal anemia and apoptosis of red cell progenitors in Stat5a-/-5b-/- mice: a direct role for Stat5 in Bcl-X(L) induction. Cell 98:181

7. Kieslinger M, Woldman I, Moriggl R, Hofmann J, Marine JC, Ihle JN, Beug H, Decker T (2000) Antiapoptotic activity of Stat5 required during terminal stages of myeloid differentiation. Genes and Development 14:232

8. Teglund S, McKay C, Schuetz E, van Deursen JM, Stravopodis D, Wang D, Brown M, Bodner S, Grosveld G, Ihle JN (1998) Stat5a and Stat5b proteins have essential and nonessential, or redundant, roles in cytokine responses. Cell 93:841

9. Moriggl R, Sexl V, Piekorz R, Topham D, Ihle JN (1999) Stat5 activation is uniquely associated with cytokine signaling in peripheral T cells. Immunity 11:225

10. Moriggl R, Topham DJ, Teglund S, Sexl V, McKay C, Wang D, Hoffmeyer A, van Deursen J, Sangster MY, Bunting KD, Grosveld GC, Ihle JN (1999) Stat5 is required for IL-2-induced cell cycle progression of peripheral T cells. Immunity 10:249

11. Kagami S, Nakajima H, Kumano K, Suzuki K, Suto A, Imada K, Davey HW, Saito Y, Takatsu K, Leonard WJ, Iwamoto I (2000) Both stat5a and stat5b are required for antigen-induced eosinophil and T-cell recruitment into the tissue. Blood 95:1370

12. Kagami S, Nakajima H, Suto A, Hirose K, Suzuki K, Morita S, Kato I, Saito Y, Kitamura T, Iwamoto I (2001) Stat5a regulates T helper cell differentiation by several distinct mechanisms. Blood 97:2358

13. Van Parijs L, Refaeli Y, Lord JD, Nelson BH, Abbas AK, Baltimore D (1999) Uncoupling IL-2 signals that regulate T cell proliferation, survival, and Fas-mediated activation-induced cell death. Immunity 11:281

14. Sexl V, Piekorz R, Moriggl R, Rohrer J, Brown MP, Bunting KD, Rothammer K, Roussel MF, Ihle JN (2000) Stat5a/b contribute to interleukin 7-induced B-cell precursor expansion, but abl- and bcr/abl-induced transformation are independent of stat5. Blood 96:2277

15. Battle TE, Frank DA (2002) The role of STATs in apoptosis. Curr Mol Med 2:381

16. O'Shea JJ, Gadina M, Schreiber RD (2002) Cytokine signaling in 2002: new surprises in the Jak/Stat pathway. Cell 109 Suppl:S121

17. Shelburne C, McCoy M, Piekorz R, Sexl V, Gillespie S, Bailey D, Gharse A, Mirmonsef P, M NM, Kashyap M, Wright H, Chong H, Bouton L, Ramirez C, Lantz C, Ryan J (2002) Stat5: an essential regulator of mast cell biology. Mol Immunol 38:1187

18. Antonsson B, Martinou JC (2000) The Bcl-2 protein family. Exp Cell Res 256:50

19. Cory S, Adams JM (2002) The bcl2 family: regulators of the cellular life-or-death switch. Nat Rev Cancer 2:647

20. Gross A, McDonnell JM, Korsmeyer SJ (1999) BCL-2 family members and the mitochondria in apoptosis. Genes Dev 13:1899

21. Akashi K, Kondo M, von Freeden-Jeffry U, Murray R, Weissman IL (1997) Bcl-2 rescues T lymphopoiesis in interleukin-7 receptor-deficient mice. Cell 89:1033

22. Lagasse E, Weissman IL (1997) Enforced expression of Bcl-2 in monocytes rescues macrophages and partially reverses osteopetrosis in op/op mice. Cell 89:1021

23. Kondo M, Akashi K, Domen J, Sugamura K, Weissman IL (1997) Bcl-2 rescues T lymphopoiesis, but not B or NK cell development, in common gamma chain-deficient mice. Immunity 7:155

24. Maraskovsky E, Peschon JJ, McKenna H, Teepe M, Strasser A (1998) Overexpression of Bcl-2 does not rescue impaired B lymphopoiesis in IL-7 receptor-deficient mice but can enhance survival of mature B cells. Int Immunol 10:1367

25. Ohteki T, Maki C, Koyasu S (2001) Overexpression of Bcl-2 differentially restores development of thymus-derived CD4-8+ T cells and intestinal intraepithelial T cells in IFN-regulatory factor-1-deficient mice. J Immunol 166:6509

26. Rodewald HR, Waskow C, Haller C (2001) Essential requirement for c-kit and common gamma chain in thymocyte development cannot be overruled by enforced expression of Bcl-2. J Exp Med 193:1431

27. Bronson SK, Plaehn EG, Kluckman KD, Hagaman JR, Maeda N, Smithies O (1996) Single-copy transgenic mice with chosen-site integration. Proc Natl Acad Sci U S A 93:9067

28. Veis DJ, Sorenson CM, Shutter JR, Korsmeyer SJ (1993) Bcl-2-deficient mice demonstrate fulminant lymphoid apoptosis, polycystic kidneys, and hypopigmented hair. Cell 75:229

29. Haneline LS, Gobbett TA, Ramani R, Carreau M, Buchwald M, Yoder MC, Clapp DW (1999) Loss of FancC function results in decreased hematopoietic stem cell repopulating ability. Blood 94:1

30. Wagers AJ, Christensen JL, Weissman IL (2002) Cell fate determination from stem cells. Gene Ther 9:606

31. Bittorf T, Seiler J, Ludtke B, Buchse T, Jaster R, Brock J (2000) Activation of STAT5 during EPO-directed suppression of apoptosis. Cell Signal 12:23

32. Motoyama N, Kimura T, Takahashi T, Watanabe T, Nakano T (1999) bcl-x prevents apoptotic cell death of both primitive and definitive erythrocytes at the end of maturation. J Exp Med 189:1691

33. Wagner KU, Claudio E, Rucker EB, 3rd, Riedlinger G, Broussard C, Schwartzberg PL, Siebenlist U, Hennighausen L (2000) Conditional deletion of the Bcl-x gene from erythroid cells results in hemolytic anemia and profound splenomegaly. Development 127:4949

34. Sanz C, Benet I, Richard C, Badia B, Andreu EJ, Prosper F, Fernandez-Luna JL (2001) Antiapoptotic protein Bcl-x(L) is up-regulated during megakaryocytic differentiation of CD34(+) progenitors but is absent from senescent megakaryocytes. Exp Hematol 29:728

35. Terui Y, Furukawa Y, Kikuchi J, Iwase S, Hatake K, Miura Y (1998) Bcl-x is a regulatory factor of apoptosis and differentiation in megakaryocytic lineage cells. Exp Hematol 26:236

36. Grillot DA, Merino R, Pena JC, Fanslow WC, Finkelman FD, Thompson CB, Nunez G (1996) bcl-x exhibits regulated expression during B cell development and activation and modulates lymphocyte survival in transgenic mice. J Exp Med 183:381

37. Nakayama K, Negishi I, Kuida K, Shinkai Y, Louie MC, Fields LE, Lucas PJ, Stewart V, Alt FW, et al. (1993) Disappearance of the lymphoid system in Bcl-2 homozygous mutant chimeric mice. Science 261:1584

38. Zamorano J, Wang HY, Wang R, Shi Y, Longmore GD, Keegan AD (1998) Regulation of cell growth by IL-2: role of STAT5 in protection from apoptosis but not in cell cycle progression. J Immunol 160:3502

39. Lord JD, McIntosh BC, Greenberg PD, Nelson BH (2000) The IL-2 receptor promotes lymphocyte proliferation and induction of the c-myc, bcl-2, and bcl-x genes through the trans-activation domain of Stat5. J Immunol 164:2533

40. Domen J (2001) The role of apoptosis in regulating hematopoietic stem cell numbers. Apoptosis 6:239

41. Domen J, Gandy KL, Weissman IL (1998) Systemic overexpression of BCL-2 in the hematopoietic system protects transgenic mice from the consequences of lethal irradiation. Blood 91:2272

42. Domen J, Cheshier SH, Weissman IL (2000) The role of apoptosis in the regulation of hematopoietic stem cells: Overexpression of Bcl-2 increases both their number and repopulation potential. Journal of Experimental Medicine 191:253

43. Feldman GM, Rosenthal LA, Liu X, Hayes MP, Wynshaw-Boris A, Leonard WJ, Hennighausen L, Finbloom DS (1997) STAT5A-deficient mice demonstrate a defect in granulocyte-macrophage colony-stimulating factor-induced proliferation and gene expression. Blood 90:1768

44. Matsumura I, Kitamura T, Wakao H, Tanaka H, Hashimoto K, Albanese C, Downward J, Pestell RG, Kanakura Y (1999) Transcriptional regulation of the cyclin D1 promoter by STAT5: its involvement in cytokine-dependent growth of hematopoietic cells. Embo J 18:1367

45. Chao DT, Linette GP, Boise LH, White LS, Thompson CB, Korsmeyer SJ (1995) Bcl-XL and Bcl-2 repress a common pathway of cell death. J Exp Med 182:821

46. Maraskovsky E, O'Reilly LA, Teepe M, Corcoran LM, Peschon JJ, Strasser A (1997) Bcl-2 can rescue T lymphocyte development in interleukin-7 receptor-deficient mice but not in mutant rag-1-/- mice. Cell 89:1011

47. Sentman CL, Shutter JR, Hockenbery D, Kanagawa O, Korsmeyer SJ (1991) bcl-2 inhibits multiple forms of apoptosis but not negative selection in thymocytes. Cell 67:879

48. Mazel S, Burtrum D, Petrie HT (1996) Regulation of cell division cycle progression by bcl-2 expression: a potential mechanism for inhibition of programmed cell death. J Exp Med 183:2219

49. O'Reilly LA, Huang DC, Strasser A (1996) The cell death inhibitor Bcl-2 and its homologues influence control of cell cycle entry. Embo J 15:6979

50. Ogilvy S, Metcalf D, Print CG, Bath ML, Harris AW, Adams JM (1999) Constitutive Bcl-2 expression throughout the hematopoietic compartment affects multiple lineages and enhances progenitor cell survival. Proc Natl Acad Sci U S A 96:14943

Chapter 4

Loss of Tolerance and Autoimmunity Affecting Multiple Organs in STAT5A/5B-deficient Mice

Introduction

The design of the adaptive immune system has evolved to meet a world of pathogens whose antigenic guise cannot be fully known before a first encounter. The system requires that lymphocytes be generated to maximize the number of epitopes seen as foreign by their antigen receptors. The organism uses a system of imprecise recombination within the antigen receptor genes and nucleotide addition and removal at key points that will contact antigen to generate specificity for 25 million [1] never before encountered foreign epitopes by T-cells (and probably similar numbers for B-cells). The danger of this system is found in the fact that random generation of lymphocytes specific for so many epitopes will result in lymphocytes that are specific for self-antigens. When triggered by antigen, autoreactive lymphocytes can attack normal tissue and cause damage via multiple effector mechanisms, including soluble factors, such as cytokines and antibodies, and cell to cell mediators, such as death receptors and cytotoxic killing molecules [2].

The organism has developed a number of physiologic checkpoints to block both the development of autoreactive lymphocytes and the initiation and progression of pathologic adaptive immune responses against normal tissue. Breakdown of multiple checkpoints may be necessary to cause a long-term autoimmune pathology. One mechanism is central tolerance, the process of deletion of lymphocytes that express antigen receptors with high reactivity to self antigen [2]. Central tolerance is not considered to be wholly efficient [3] and peripheral tolerance mechanisms exist in addition [4].

Peripheral tolerance has reached new levels of complexity in recent years and consists of multiple regulatory mechanism acting on a number of checkpoints. First, sequestration is used to keep some self-antigens from ever being seen by the immune system in a process called immunologic ignorance [4].

Second, the innate immune system helps the immune system to direct its activity to situations where pathogens are involved. Cells of the innate immune system are activated by a combination of pattern receptors on innate immune cells [5] and "danger" signals from damaged cells [6]. Innate recognition of a threat is translated to lymphocytes through the antigen presenting cell (APC) system to help regulate whether antigens are seen by the adaptive immune response. Dendritic cells normally perform antigen collection in peripheral tissues, and, matured by microbial products or cellular damage signals [7], present the foreign antigens to T-cells in the context of a second signal from a B7 family member that is required for activation (at least for naïve T-cells)[8]. In the absence of a pathogen dendritic cells are not matured, but circulate carrying self-antigen with no second signal [9]. This leads to T-cell anergy or even deletion in the absence of the second signal [4]. Also, other cells of the innate immune system, such as NK and NKT-cells are thought to play a role in modulating autoimmune initiation and progression, although not well understood [4].

Third, self-reactive thymic escapees are maintained in a quiescent state by one or more populations of regulatory T-cells, currently defined by multiple phenotypes including $CD4^+CD25^+$ and $CD4^+CD45RB^{lo}$. These T-cells are thought to arise in the thymus through intermediate affinity interactions with self-peptide that are not high enough to trigger deletion, but instead render the cells unresponsive in the classical sense,

but able to suppress the activation of self-reactive T-cells in the periphery via suppressor cytokines, cell-cell interactions, or both. In addition, these cells may be derived from conventional αβ T-cells in the periphery as well (reviewed in [10-12]).

Finally, dampening of existing immune responses is utilized to block a normal immune response from becoming a context for initiation of an autoimmune response by epitope spreading. First, negative feedback loops such as IL-2 mediated upregulation of Fas on activated T-cells helps to limit the size of a response [13]. Secondly, phenotypic skewing helps to maintain balance in the Th1/Th2 subset polarization and restrict the character of an immune response [4].

Many cytokines have been implicated in regulating these mechanisms of tolerance *in vivo*. IL-2 has been especially interesting to investigators, as it appears to have such contradictory roles. IL-2 was isolated as a T-cell growth factor and was proposed to positively regulate T-cell numbers *in vivo*. However, as mice deficient for IL-2 or any one of its receptor components spontaneously develop autoimmunity [14-17], it is clearly responsible for more complicated actions *in vivo*. In IL-2 receptor $\beta^{-/-}$ mice, two current models account for the breakdown in tolerance. The first model is that activation of this receptor is important for the upregulation of Fas on activated T-cells leading to activation –induced cell death (AICD) and elimination of self-reactive cells [13,18-20]. The second model is that a reduction in an IL-2-dependent subset of $CD4^{+}/CD25^{+}$ "regulatory T-cells" causes a disruption in the maintenance of peripheral tolerance to multiple self-antigens [12 and refs therein,21].

The downstream signaling components used by the IL-2 receptor in regulating peripheral tolerance *in vivo* have not been elucidated [22-25]. One signal transduction

pathway utilized by the IL-2 receptor to transmit signals into biological actions of target cells is the JAK-STAT pathway, with the major activated STAT being STAT5. Because of this we posited that a loss of tolerance in the adaptive immune system might lead to autoimmune pathology, which might contribute to hematopoietic abnormalities observed in these mice.

The following study was designed to interrogate the role of lymphocytes in the hematopoietic defects observed in STAT5A/5B-deficient mice. We found a striking correlation between T-cell infiltration of the bone marrow and the most severely affected mice in skewing of progenitor subsets. We then found that this infiltration represented a more generalized autoimmune phenomenon affecting multiple organs. Both early death and the pathology observed in these mice could be corrected by the genetic removal of lymphocytes indicating a prominent role of these cells in the etiology of the disease. We found similar pathology extant in IL-2 receptor β-deficient mice, implying that it was STAT5's role downstream of this cytokine that was responsible for the effects observed. We also found a reduction in the IL-2-dependent $CD4^+CD25^+$ regulatory T-cell population in STAT5A/5B-deficient mice, which appeared to be due to decreased survival of these cells. As these cells are critical for the maintenance of peripheral tolerance, it appears that STAT5 is important for the IL-2-mediated maintenance of tolerance *in vivo* through survival effects on these cells.

References

1. Arstila TP, Casrouge A, Baron V, Even J, Kanellopoulos J, Kourilsky P (1999) A direct estimate of the human alphabeta T cell receptor diversity. Science 286:958
2. Janeway CA, Jr., Travers P (1997) Immunobiology: The immune system in health and disease. New York: New York

3. Bouneaud C, Kourilsky P, Bousso P (2000) Impact of negative selection on the T cell repertoire reactive to a self-peptide: a large fraction of T cell clones escapes clonal deletion. Immunity 13:829
4. Walker LS, Abbas AK (2002) The enemy within: keeping self-reactive T cells at bay in the periphery. Nat Rev Immunol 2:11
5. Medzhitov R, Janeway CA, Jr. (2002) Decoding the patterns of self and nonself by the innate immune system. Science 296:298
6. Matzinger P (2002) The danger model: a renewed sense of self. Science 296:301
7. Steinman RM, Nussenzweig MC (2002) Avoiding horror autotoxicus: the importance of dendritic cells in peripheral T cell tolerance. Proc Natl Acad Sci U S A 99:351
8. Croft M, Dubey C (1997) Accessory molecule and costimulation requirements for CD4 T cell response. Crit Rev Immunol 17:89
9. Coyle AJ, Gutierrez-Ramos JC (2001) The expanding B7 superfamily: increasing complexity in costimulatory signals regulating T cell function. Nat Immunol 2:203
10. Sakaguchi S (2000) Regulatory T cells: key controllers of immunologic self-tolerance. Cell 101:455
11. Shevach EM (2002) CD4+ CD25+ suppressor T cells: more questions than answers. Nat Rev Immunol 2:389
12. Maloy KJ, Powrie F (2001) Regulatory T cells in the control of immune pathology. Nat Immunol 2:816
13. Van Parijs L, Refaeli Y, Lord JD, Nelson BH, Abbas AK, Baltimore D (1999) Uncoupling IL-2 signals that regulate T cell proliferation, survival, and Fas-mediated activation-induced cell death. Immunity 11:281
14. Suzuki H, Kündig TM, Furlonger C, Wakeham A, Timms E, Matsuyama T, Schmits R, Simard JJ, Ohashi PS, Griesser H, et al. (1995) Deregulated T cell activation and autoimmunity in mice lacking interleukin-2 receptor beta. Science 268:1472
15. Willerford DM, Chen J, Ferry JA, Davidson L, Ma A, Alt FW (1995) Interleukin-2 receptor alpha chain regulates the size and content of the peripheral lymphoid compartment. Immunity 3:521
16. Sadlack B, Merz H, Schorle H, Schimpl A, Feller AC, Horak I (1993) Ulcerative colitis-like disease in mice with a disrupted interleukin-2 gene. Cell 75:253
17. Nakajima H, Shores EW, Noguchi M, Leonard WJ (1997) The common cytokine receptor gamma chain plays an essential role in regulating lymphoid homeostasis. J Exp Med 185:189
18. Van Parijs L, Ibraghimov A, Abbas AK (1996) The roles of costimulation and Fas in T cell apoptosis and peripheral tolerance. Immunity 4:321
19. Van Parijs L, Biuckians A, Ibragimov A, Alt FW, Willerford DM, Abbas AK (1997) Functional responses and apoptosis of CD25 (IL-2R alpha)-deficient T cells expressing a transgenic antigen receptor. J Immunol 158:3738
20. Van Parijs L, Abbas AK (1998) Homeostasis and self-tolerance in the immune system: turning lymphocytes off. Science 280:243
21. Papiernik M, de Moraes ML, Pontoux C, Vasseur F, Penit C (1998) Regulatory CD4 T cells: expression of IL-2R alpha chain, resistance to clonal deletion and IL-2 dependency. Int Immunol 10:371

22. Lin JX, Leonard WJ (2000) The role of Stat5a and Stat5b in signaling by IL-2 family cytokines. Oncogene 19:2566
23. Nelson BH (2002) Interleukin-2 signaling and the maintenance of self-tolerance. Curr Dir Autoimmun 5:92
24. O'Shea JJ, Ma A, Lipsky P (2002) Cytokines and Autoimmunity. Nature Reviews Immunology 2:37
25. Gaffen SL (2001) Signaling domains of the interleukin 2 receptor. Cytokine 14:63

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Loss of Tolerance and Autoimmunity Affecting Multiple Organs in STAT5A/5B-deficient Mice[1]

Jonathan W. Snow[*,†], Ninan Abraham[*], Melissa C. Ma[*], Brian G. Herndier[‡], Alexander W. Pastuszak[*,4], and Mark A. Goldsmith[*,¶,2]

[*]*Gladstone Institute of Virology and Immunology, Departments of* [†]*Microbiology and Immunology, Pathology*[‡]*, and* [¶]*Medicine, University of California at San Francisco, San Francisco, CA 94114-9100, USA.*

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[2] To whom correspondence should be addressed:
Gladstone Institute of Virology & Immunology
P.O. Box 419100, San Francisco, CA 94141-9100
Phone: 415.695.3775 Fax: 415.695.1364
Email: mgoldsmith@gladstone.ucsf.edu

Abstract

STAT5 has previously been reported to be dispensable for the maintenance of tolerance *in vivo*. However, in examining hematopoiesis in mice lacking both isoforms of STAT5, STAT5A and STAT5B, we noted that a subset of these mice demonstrated dramatic alterations in several bone marrow progenitor populations concomitant with lymphocytic infiltration of the bone marrow. In addition, cellular infiltration affecting the colon, liver, and kidney was observed in these mice. Survival analysis revealed that STAT5A/5B$^{-/-}$ mice exhibited early death. The increased mortality and the pathology affecting multiple organs observed in these mice were abrogated on the RAG1$^{-/-}$ background. In light of the similarities between STAT5A/5B-deficient mice and mice unable to signal through the IL-2R, we hypothesized that the tolerizing role of STAT5A/5B was triggered via activation of the IL-2R. In agreement with this, we found that IL-2Rβ chain-deficient mice exhibited similar hematopoietic abnormalities. As IL-2 signaling is thought to contribute to tolerance through maintenance of a CD4^{+}/CD25^{+} regulatory T-cell population, we examined these cells and observed a numerical reduction in STAT5A/5B$^{-/-}$ mice along with a higher rate of apoptosis. These data provide strong evidence for a requirement for STAT5 in the maintenance of tolerance *in vivo*.

Introduction

Autoreactive T-cells normally are eliminated by central mechanisms of tolerance in the thymus, or are subsequently destroyed or rendered anergic by the complex system of peripheral tolerance. Tolerance in the periphery involves multiple cell types and signaling networks to avoid the generation of cell-mediated or humoral immune responses against self-antigens by lymphocytes that have escaped central tolerance mechanisms [1-3]. Cytokines that utilize type I cytokine receptors, such as IFNγ, IFNα/β, IL-2, IL-4, IL-10, IL-12, play a critical role in this system by regulating the type, strength, and duration of normal immune responses, and in arresting the initiation and progression of pathogenic immune responses [2,4-6]. In particular, the role of IL-2 and its receptor in maintaining tolerance in lymphocytes is well established, as mice deficient for IL-2 or any one of its receptor components spontaneously develop autoimmunity [7-10].

The downstream signaling components used by the IL-2R in regulating peripheral tolerance *in vivo* have not been elucidated [2,11-13]. One signal transduction pathway utilized by the IL-2R to transmit signals into biological actions of target cells is the JAK-STAT pathway. STATs are cytoplasmically located, latent transcription factors that dimerize upon tyrosine phosphorylation by an activated receptor complex, translocate into the nucleus, and modulate the transcription of target genes by binding to specific DNA sequence motifs [14,15]. Disruption of a number of STAT factors such as STAT3 in myeloid cells [16] and STAT6 [17], normally activated by IL-10 and IL-4, respectively, has revealed that the activation of these transcription factors can be important for the maintenance of tolerance by these cytokines. However, the main STAT family member activated by the IL-2R, STAT5, has not been shown to be important in

the maintenance of tolerance *in vivo* [11,12]. Initial characterization of mice deficient for STAT5A/5B detected no evidence of autoimmunity [18], despite the presence of activated lymphocytes in these mice.

STAT5 is activated by diverse cytokine receptors involved at multiple levels within the hematopoietic system. The widespread engagement of its two isoforms, STAT5A and STAT5B, has implicated STAT5 as a potentially important component of cytokine receptor signaling in this context. Surprisingly, therefore, mice deficient for either STAT5A [19] or STAT5B [20] were found to exhibit only subtle alterations in the regulation of hematopoietic cells [21-23]. In contrast, mice deficient for both the STAT5A and STAT5B isoforms, were shown to have multiple defects in hematopoietic development at all stages of differentiation from the hematopoietic stem cell (HSC) [24-26] to lineage-committed progenitors [27-29]. In addition, some abnormalities in the function of mature hematopoietic cells have been described in mice both singly and doubly deficient for the STAT5 isoforms [18,29-35]. These findings provided a basis for considering the possibility that abnormalities in T-cell maturation and or homeostasis in the setting of impaired activation could lead to dysregulation and aberrant cell function.

In our studies of the role of STAT5 in hematopoietic development we noticed significant variability in the expressivity of multiple hematopoietic phenotypes that prompted us to investigate the possibility that defects in cells other than the hematopoietic progenitors contribute to the spectrum of hematopoietic abnormalities in STAT5A/5B-deficient mice. We found that a subset of STAT5A/5B-deficient mice exhibited autoimmune pathology very similar to mice lacking IL-2 or its receptor components, characterized by lymphocytic infiltration of multiple organs, including the

bone marrow. This disease correlated with decreased numbers of $CD4^+/CD25^+$ regulatory T-cells, which undergo apoptosis at increased rates in the absence of STAT5. Our findings provide definitive evidence that STAT5 is critical for the maintenance of tolerance *in vivo*. In addition, our findings indicate that regulatory T-cells require the activation of STAT5, most likely by the IL-2R, to maintain their own homeostasis *in vivo* and that reduction of this population may contribute to the loss of tolerance evident in these mice.

Materials and Methods

Handling and Characterization of Mice.

STAT5A/5B$^{-/-}$ mice [18] were obtained from Dr. James Ihle (St. Jude Children's Research Hospital, Memphis, TN) and back-crossed onto a C57Bl/6 background at least 6 generations. IL-2R$\beta^{-/-}$ mice [7] were generously provided by Dr. Tak Mak (Ontario Cancer Institute, Toronto, Ontario, Canada). RAG1$^{-/-}$ mice were purchased from Jackson Laboratories (Bar Harbor, ME). Mice were housed in a pathogen-free rodent barrier facility and received mouse chow and acidified water *ad libitum*. All studies were performed on 6–8 week old mice unless otherwise specified, and littermates were always used as wild-type controls. Peripheral blood was obtained via cardiac puncture, and EDTA-treated samples were used for complete blood counts (IDEXX Veterinary Service, Sacramento, CA). Bone marrow was harvested by flushing femurs and tibias into 6 ml phosphate buffered saline (PBS) containing 2% fetal bovine serum (FBS) and 2 mM EDTA. Single cell suspensions of spleens were generated by passing them through a 70 μm nylon mesh strainer into 5 ml PBS containing 2% FBS and 2mM EDTA. Cell counts were determined after ACK (NH_4Cl) lysis by trypan blue exclusion.

Flow Cytometry.

All antibodies were obtained from Pharmingen (San Diego, CA), and the following clones were used; Sca-1 (E13-161.7), Ly-76 (Ter-119), Gr-1 (RB6-8C5), CD3 (145-2C11), B220 (RA3-6B2), CD11b (M1/70), c-kit (2B8), CD4 (RM4-5), CD8 (53-6.7), CD25 (7D4), CD62L (MEL-14), CD44 (IM7), Bcl-2 (3F11), BrdU (3D4). For blocking non-specific binding to Fc receptors, purified antibody to CD16/CD32 (2.4G2)

was used in at 1:100 for 3 minutes. Subsequently, antibodies to surface markers of interest were used at 1:60 dilution. Apoptosis staining was performed using Annexin-V-GFP (generously provided by Dr. Joel Ernst, University of California, San Francisco [36]) along with other antibodies to surface markers in 1.5 mM $CaCl_2$. For intracellular staining of BrdU, cells were stained for expression of surface markers, then fixed and permeabilized for 1 h at 25° C in 1% paraformaldehyde (PFA) with 0.1% Tween-20, washed, incubated with 10mg/ml DNAse 1 for 30 minutes at 37° C, washed, stained for 30 minutes at 25° C with anti BrdU antibody at 20 µl per sample with 7 µl FCS, then washed. For intracellular staining of Bcl-2, cells were stained for expression of surface markers, then fixed and permeabilized for 1 h at 25° C in 1% PFA with 0.1% Tween-20, washed, stained for 30 minutes at 25° C with anti Bcl-2 antibody at a 1:5 dilution, then washed. All FACS analyses were performed using a FACSCalibur and all sorting was performed using a FACSVantage (Becton Dickinson).

Histology.

Tissues were prepared by fixation in 1% PFA. Leg bones were decalcified in 19% EDTA for 10 days with changing of solutions every other day. Tissues were then dehydrated and embedded in paraffin. Hematoxilin and eosin staining was performed on 5 µm thick sections.

Statistical Analysis.

Data are presented as mean ± SEM when appropriate. Statistical significance was assessed by two-sided Student's t-test.

Results

Severe alterations in bone marrow progenitor populations in a subset of STAT5A/5B-deficient mice, coincident with infiltration of memory CD4$^+$ and CD8$^+$ T-cells.

FACS analysis of the bone marrow compartment of STAT5A/5B-deficient mice at 6-7 weeks of age revealed a wide range in the severity of alterations in the representation of two progenitor subsets found among cells that have low expression of lineage-commitment markers: (a) the Sca-1$^+$/c-kit$^+$ population, which contains the HSC; and (b) the Sca-1$^-$/c-kit$^+$ post-HSC myeloid progenitors, which include the common myeloid progenitor (CMP), megakaryocyte-erythroid progenitor (MEP), and granulocyte-macrophage progenitor (GMP) [37,38]. Contour plots of c-kit and Sca-1 expression portray the normal distribution of the progenitor subsets from a typical STAT5A/5B$^{+/+}$ animal, and abnormal distribution in a moderately affected STAT5A/5B$^{-/-}$ animal and a severely affected STAT5A/5B$^{-/-}$ animal (as based on the criteria below) (Fig. 1). These plots reveal a dramatic increase in the relative representation of the HSC-containing population as well as a substantial decrease of the post-HSC myeloid progenitor population in the severely affected animal; similar, though less severe, changes were evident in the moderately affected STAT5A/5B-deficient animal. When we examined these subsets in absolute terms, we found that the STAT5A/5B$^{-/-}$ mice could be separated into two groups based on the absolute number of cells in the Sca-1$^+$/c-kit$^+$ HSC containing population. We classified 4/10 of STAT5A/5B$^{-/-}$ mice as severely affected and the remaining 6/10 STAT5A/5B$^{-/-}$ mice as moderately affected. There was a greater than two-fold increase in the Sca-1$^+$/c-kit$^+$ population in the severely affected STAT5A/5B$^{-/-}$ mice compared to either the moderately affected STAT5A/5B$^{-/-}$ mice or STAT5A/5B$^{+/+}$

mice (Table 1). We further found that all STAT5A/5B$^{-/-}$ mice possessed numbers of Sca-1^{-}/c-kit^{+} cells that were well below wild-type littermates. Since the Sca-1^{-}/c-kit^{+} post-HSC myeloid progenitor population represents a direct product of the Sca-1^{+}/c-kit^{+} HSC population, we calculated the number of the post-HSC myeloid progenitors per HSC in each of these groups as another means to represent the severity of the block at this stage of hematopoiesis. Severely affected STAT5A/5B$^{-/-}$ animals had a greatly decreased number of post-HSC myeloid progenitors per HSC (0.684 ± 0.28) compared to moderately affected STAT5A/5B$^{-/-}$ animals (2.02 ± 0.15) or STAT5A/5B$^{+/+}$ animals (6.83 ± 0.69). These data reveal that based on criterion of absolute number of cells in the Sca-1^{+}/c-kit^{+} population, the STAT5A/5B$^{-/-}$ genotype segregates into two fundamental groups.

In order to determine whether abnormalities in mature lymphocytes in a subset of animals might contribute to the existence of a bimodal distribution in STAT5A/5B-deficent mice, we examined lymphocyte subsets within the bone marrow of these mice. FACS analysis revealed that the mice with the most severe alterations in bone marrow progenitor subsets also had a substantial increase in the absolute numbers of memory CD4^{+} (Fig 2A) and CD8^{+} (Fig. 2B) T-cells in the bone marrow compartment (severely affected animals represented by open circles) compared with the moderately affected STAT5A/5B$^{-/-}$ mice (closed circles) and wild-type littermates. There was a four-fold increase in the number of activated / memory CD3^{+}/CD4^{+} (CD62L^{lo}/CD44^{hi}) T-cells [39] in the severely affected animals (36.5 x10^{4} ± 9.7 x10^{4} cells) compared to the moderately affected animals (7.94 x10^{4} ± 2.9 x10^{4} cells, p = 0.0064) and STAT5A/5B$^{+/+}$ animals (6.43 x10^{4} ± 0.69 x10^{4} cells, p = 0.004). Similarly there was a four-fold increase in the

number of activated / memory $CD3^+/CD8^+$ ($CD62L^{lo}/CD44^{hi}$) T-cells in the severely affected animals ($16.3 \times 10^4 \pm 5.7 \times 10^4$ cells) compared to the moderately affected animals ($3.66 \times 10^4 \pm 0.82 \times 10^4$ cells, $p = 0.023$) and the $STAT5A/5B^{+/+}$ animals ($3.63 \times 10^4 \pm 0.38 \times 10^4$ cells, $p = 0.039$). The presence of increased numbers of activated / memory T-cells in bone marrow implied that these cells had been activated and homed to this tissue in response to some foreign or self antigen localized to the bone marrow. The association of this infiltration with striking abnormalities in progenitor subsets implied that the presence of memory T-cells might contribute to the phenotypic alterations observed in the bone marrow.

Cellular infiltration is observed in other organs of severely affected STAT5A/5B-deficient mice.

To determine whether the T-cell infiltration we observed in the bone marrow represented systemic pathology, we performed histological analyses of other organs, including liver, colon, and kidney. We observed significant cellular infiltration in all organs examined from the STAT5A/5B-deficient mice that exhibited increased numbers of T-cells in the bone marrow, as described earlier. The colon was the most severely affected organ, with massive mononuclear and polymorphonuclear cell infiltration of the lamina propria (Fig. 3A). The inflammation was associated with disruption of the architecture of the tissue including elongation of the crypts and widening of the gap between crypts. In the kidney, there was mononuclear cell infiltration of the tubular interstitium (large arrow) and sporadic alterations of glomerular architecture; primarily cell loss and apparent shrinkage (small arrow) were also noted (Fig. 3B). The liver

exhibited mononuclear cell infiltration surrounding the vasculature of the portal triad with few cells seen invading the parenchyma (Fig. 3C). In addition, using blood chemistry analysis, we found that values for Lactose Dehydrogenase (LDH) and Aspartate Aminotransferase (AST) were increased in STAT5A/5B-deficient mice, consistent with liver disease. Levels of blood urea nitrogen (BUN) were increased, while levels of bicarbonate (TCO2) were decreased in STAT5A/5B$^{-/-}$ mice, both consistent with renal disease. Finally, values for albumin were decreased in STAT5A/5B$^{-/-}$ mice, consistent with kidney disease, liver disease, or both (Fig. 4). We did not observe infiltration of either the colon or the kidney in moderately affected STAT5A/5B-deficient mice or in STAT5A/5B wild-type littermates. However, we did observe a cellular infiltration of the liver parenchyma in one of the moderately affected STAT5A/5B-deficient animals, which had the characteristics of extramedullary hematopoietic foci (data not shown). Mononuclear and polymorphonuclear cell infiltration of multiple organs observed coincidentally with histologic destruction of the tissues in affected organs suggested the possibility that a more global breakdown of the maintenance of tolerance might be occurring in STAT5A/5B-deficient mice.

Reduced survival in STAT5A/5B-deficient mice is rescued on an immunodeficient RAG1-deficient background.

We found that STAT5A/5B-deficient mice died spontaneously as early as 6 weeks of age and that greater than 70% (14/19) of these mice were dead by 20 weeks of age (Fig. 5A). Based on the mononuclear cell infiltration we detected in the livers, colons, and kidneys as well as the CD4^{+} and CD8^{+} T-cell infiltration we observed in the bone marrow, we

hypothesized that the premature deaths were linked to infiltration by lymphocytes. To test the hypothesis of a cause and effect relationship, we crossed the STAT5A/5B-deficient mice onto an immunodeficient $RAG1^{-/-}$ background. Preliminary evidence indicated that both $STAT5A/5B^{+/-}$ and $STAT5A/5B^{-/-}$ mice on the $RAG1^{-/-}$ background were more susceptible to infection compared to mice on a $RAG1^{+/+}$ background. This phenomenon was likely due to the neutropenia (Fig. 6C) and NK cell deficiencies [31] observed in these mice that increased the immunocompromised state of the $RAG1^{-/-}$ background. To reduce the confounding effects of infection-related deaths on our assessment of the role of lymphocytes in STAT5A/5B-deficient mice, we measured survival curves for the various STAT5A/5B genotypes on both $RAG1^{+/+}$ and $RAG1^{-/-}$ backgrounds in the presence of antibiotics administered through the drinking water. We observed that while greater than 70% (5/7) of $STAT5A/5B^{-/-}$ mice on the $RAG1^{+/+}$ background died by 20 weeks, only 9% (1/11) of the $STAT5A/5B^{-/-}$ on the $RAG1^{-/-}$ background had succumbed by the same time period (Fig. 5B). Furthermore, examination of euthanized STAT5A/5B-deficient mice on the $RAG^{-/-}$ background revealed no evidence of mononuclear cell infiltration in any organs (data not shown). These data demonstrate that the pathologies observed in multiple tissues are dependent on the presence of lymphocytes. In addition, as the removal of lymphocytes rescued the early death exhibited by STAT5A/5B-deficient mice, these data suggest that the early death is linked to infiltration of these organs by lymphocytes.

Removal of lymphocytes rescues anemia and neutrophilia, but not thrombocytopenia in adult STAT5A/5B-deficient mice.

In order to examine the role that the autoimmune pathology in STAT5A/5B-deficient mice influenced the hematopoietic abnormalities observed in these mice, we examined the peripheral blood of STAT5A/5B$^{-/-}$ and STAT5A/5B$^{+/+}$ mice on a RAG1$^{-/-}$ background. In peripheral blood lineages, we found that STAT5A/5B$^{-/-}$ (RAG1$^{-/-}$) mice showed a slight decrease in erythrocytes compared to STAT5A/5B$^{+/+}$ (RAG1$^{-/-}$) (Fig. 6A). This represents a dramatic rescue of severe anemia in STAT5A/5B$^{-/-}$ mice on a RAG1$^{+/+}$ background. Platelets in STAT5A/5B$^{-/-}$ (RAG1$^{-/-}$) mice showed a greater than 30% decrease compared to STAT5A/5B$^{+/+}$ (RAG1$^{-/-}$) mice (Fig. 6B). Lymphocytes were not assessed on the RAG1$^{-/-}$ background. Finally, neutrophils were decreased modestly in STAT5A/5B$^{-/-}$ (RAG1$^{-/-}$) mice compared to STAT5A/5B$^{+/+}$ (RAG1$^{-/-}$) (Fig. 6C). Removal of lymphocytes in STAT5A/5B-deficient mice results in rescue of severe anemia, no increase in platelets, and a reduction that is not statistically significant, in neutrophils. These data indicate that lymphocytes, probably within the context of the autoimmunity, cause the most severe decreases in erythrocytes in STAT5A/5B$^{-/-}$ mice. However, correction of autoimmunity cannot rescue platelets in STAT5A/5B$^{-/-}$ mice. In addition, it appears that the trend of increased neutrophils in STAT5A/5B$^{-/-}$ mice is due to the presence of lymphocytes.

Removal of lymphocytes does not rescue hypocellular bone marrow and partially rescues defects in progenitor subsets in STAT5A/5B-deficient mice

To examine the effects of the autoimmune phenomenon on hematopoietic progenitors in STAT5A/5B$^{-/-}$ mice, we examined the bone marrow in STAT5A/5B$^{-/-}$ and STAT5A/5B$^{+/+}$ mice on a RAG1$^{-/-}$ background. Removal of lymphocytes by crossing

onto the RAG1$^{-/-}$ background did not increase bone marrow cellularity of STAT5A/5B$^{-/-}$ mice compared to STAT5A/5B$^{+/+}$ mice (Fig. 7A).

We then used FACS analysis to subset bone marrow cells by expression of surface lineage-defining markers (lin), Sca-1, and c-kit. We found no alteration in the fractional representation (Fig. 7B) of the HSC or post-HSC myeloid progenitor subsets in the bone marrow of these mice compared with STAT5A/5B$^{+/+}$ animals on the RAG$^{-/-}$ background. There is a non-significant trend for the HSC containing population to be increased in STAT5A/5B$^{-/-}$ (RAG1$^{+/+}$) mice compared to STAT5A/5B$^{+/+}$ (RAG1$^{+/+}$) mice (Table 1). This population was equivalent in STAT5A/5B$^{-/-}$ (RAG1$^{-/-}$) compared to STAT5A/5B$^{+/+}$ (RAG1$^{-/-}$) in absolute terms (Fig. 7C), representing a correction of the increase in this population found in STAT5A/5B$^{-/-}$ (RAG1$^{+/+}$) compared to STAT5A/5B$^{+/+}$ (RAG1$^{+/+}$) mice (Chapter 3). As shown previously, the post-HSC containing population is substantially decreased in STAT5A/5B$^{-/-}$ (RAG1$^{+/+}$) compared to STAT5A/5B$^{+/+}$ (RAG1$^{+/+}$) mice (Chapter 3). The number of these cells in STAT5A/5B$^{-/-}$ (RAG1$^{-/-}$) remained decreased compared to STAT5A/5B$^{+/+}$ (RAG1$^{-/-}$) mice (Fig. 7D). However, there appeared to be a partial rescue in the number of these cells in the absence of lymphocytes. Correction of the immune mediated effects by crossing onto a RAG1$^{-/-}$ background results in reduction of HSC and partial rescue of post-HSC progenitors.

It is interesting to note that removal of lymphocytes from STAT5A/5B$^{-/-}$ mice by crossing onto a RAG2$^{-/-}$ background does not rescue the competitive repopulation defects [40].

Mice deficient for IL-2Rβ-chain exhibit similar abnormalities in the bone marrow.

Previous reports indicate some hematopoietic progenitor abnormalities in mice lacking IL-2 [41,42], with mononuclear cell infiltration in the colon and liver and anemia in mice deficient for IL-2 [9], IL-2Rα [8], or IL-2Rβ [7] similar to that observed by us in STAT5A/5B-deficient mice. We speculated that, if IL-2 signaling was responsible for the tolerizing effect of STAT5, mice deficient in IL-2 signaling would manifest the same specific bone marrow alterations that we had observed in the STAT5A/5B-deficient mice. Indeed, we found that at 6 weeks of age, mice deficient for IL-2 receptor β exhibited bone marrow abnormalities including T-cell infiltration and alterations in the representation of lineage marker dim progenitor populations. The phenotypes appeared more severe in these mice and displayed a higher penetrance, as all IL-2 receptor β-deficient mice examined were markedly affected (n = 6). FACS contour plots portray the distribution of the progenitor subsets from a wild-type littermate and a typical IL-2 receptor $\beta^{-/-}$ animal (Fig. 8A), which reveal a dramatic increase in the representation of the HSC-containing population and a substantial decrease of the post-HSC myeloid progenitor population in the IL-2 receptor $\beta^{-/-}$ animal. Specifically, IL-2 receptor $\beta^{-/-}$ mice had a greater than eight-fold increase in the absolute number of Lin^{dim} /Sca-1^{+}/c-kit^{+} cells ($59.1 \times 10^4 \pm 6.7 \times 10^4$ cells), compared to IL-2 receptor $\beta^{+/+}$ mice ($7.22 \times 10^4 \pm 0.61 \times 10^4$ cells, $p = 0.00025$) (Fig. 8B). We further noted that these animals had greater than a five-fold lower absolute number of Lin^{dim} /Sca-1^{-}/c-kit^{+} cells ($1.37 \times 10^5 \pm 0.99 \times 10^5$ cells) compared to wild-type littermates ($7.42 \times 10^5 \pm 0.58 \times 10^5$ cells, $p = 0.0019$) (Fig. 8C). These data demonstrate that loss of IL-2 signaling by deficiency in the IL-2 receptor β-chain also result in significant abnormalities in bone marrow progenitors.

As in the STAT5A/5B$^{-/-}$ mice, these abnormalities in the bone marrow were associated with a substantial increase in the absolute numbers of activated / memory $CD4^+$ and $CD8^+$ T-cells in the bone marrow compartment. We detected a greater than 27-fold increase in the absolute number of activated / memory $CD3^+/CD4^+$ ($CD62L^{lo}/CD44^{hi}$) T-cells in IL-2 receptor $\beta^{-/-}$ mice ($174 \times 10^4 \pm 19 \times 10^4$ cells) compared to wild-type littermates ($6.33 \times 10^4 \pm 1.1 \times 10^4$ cells, $p = 0.00013$) (Fig. 8D). We also observed a 46-fold increase in the number of activated / memory $CD3^+/CD8^+$ ($CD62L^{lo}/CD44^{hi}$) T-cells in IL-2 receptor $\beta^{-/-}$ animals ($132 \times 10^4 \pm 54 \times 10^4$ cells) compared to wild-type littermates ($2.84 \times 10^4 \pm 0.37 \times 10^4$ cells, $p = 0.054$) (Fig. 8E). These data showed that IL-2 receptor $\beta^{-/-}$ mice and STAT5A/5B$^{-/-}$ mice exhibited similar hematopoietic abnormalities in the bone marrow. These data, in conjunction with other similarities noted in these animals, suggest that loss of tolerance in these two mouse models results from defects in the same mechanism of maintaining tolerance.

STAT5A/5B-deficient mice have reduced numbers of $CD4^+/CD25^+$ regulatory T-cells in secondary lymphoid organs and in peripheral tissue.

Since the multiorgan autoimmunity seen in the STAT5A/5B$^{-/-}$ mice was strikingly similar to that seen in IL-2R$\beta^{-/-}$ mice, we hypothesized that the same mechanism for disruption of tolerance might be involved in both types of gene-targeted mice. One model to account for the loss of tolerance in these mice is that a reduction in an IL-2-dependent subset of $CD4^+/CD25^+$ "regulatory T-cells" causes a disruption in the maintenance of peripheral tolerance to multiple self-antigens [43-47]. We therefore examined STAT5-deficient mice for the number of T-cells expressing these markers both

in a secondary lymphoid organ, the spleen, and in an affected end-organ, the bone marrow. We observed that there was an approximately four-fold decrease in both $CD25^+$ and $CD25^-$ $CD4^+$ cells in the spleen, such that the relationship of $CD4^+/CD25^+$ to $CD4^+/CD25^-$ cells was preserved (Fig. 9A). In contrast, the absolute number of $CD4^+/CD25^-$ cells in the bone marrow was unaffected by STAT5A/5B-deficiency mice while the absolute number of $CD4^+/CD25^+$ in this tissue was reduced two-fold in $STAT5A/5B^{-/-}$ mice compared to wild-type mice (Fig. 9B). To exclude the possibility that $CD4^+/CD25^+$ cells had down-regulated CD62L and thus represented activated naïve cells that confounded quantitation of regulatory T-cells [48], we also examined the expression of CD62L. We found that 78.1% of $CD4^+/CD25^+$ cells in the spleens of $STAT5A/5B^{+/+}$ animals were $CD62L^{hi}$, while only 33.8% of these cells in the spleens of $STAT5A/5B^{-/-}$ mice were $CD62L^{hi}$ (Fig. 9C). Therefore, by this analysis, there was in fact a relative reduction in the actual number of regulatory T-cells versus non-regulatory T-cells in the spleens of STAT5A/5B-deficient animals. Similar results for CD62L expression were observed in the bone marrow. In accordance with our model and the conclusion of others that IL-2 was important for the regulation of this population [43,45], we found that the number of $CD4^+/CD25^+/$ $CD62L^{hi}$ cells in the spleen of IL-2 receptor $\beta^{-/-}$ mice was reduced compared to wild-type littermates (data not shown). Thus, the overall reduction of regulatory T-cells in $STAT5A/5B^{-/-}$ mice may contribute to a loss of peripheral tolerance in these mice, thereby causing the multiorgan autoimmunity observed in a manner similar to mice that lack all IL-2R signaling.

$CD4^+/CD25^+$ regulatory T-cells have increased rate of apoptosis and decreased Bcl-2 expression in STAT5A/5B-deficient mice.

To determine how STAT5 may contribute to maintenance of $CD4^+/CD25^+$ cells, we examined both proliferation and apoptosis of this subset *in vivo*. We observed an approximately two-fold increase in the proportion of $CD4^+/CD25^+/CD62L^{hi}$ cells in the spleen staining positive for Annexin-V in $STAT5A/5B^{-/-}$ mice compared to wild-type mice (Fig 10A). In addition, *in vivo* labeling with BrdU revealed that the proportion of $CD4^+/CD25^+/CD62L^{hi}$ cells in the spleen that were $BrdU^{hi}$, and thus had incorporated BrdU during DNA synthesis in the periphery, was increased in $STAT5A/5B^{-/-}$ mice compared to $STAT5A/5B^{+/+}$ mice (data not shown). Therefore, despite an apparent ability to undergo homeostatic proliferation in secondary lymph organs, the regulatory T-cell population in $STAT5A/5B^{-/-}$ mice has a higher rate of cell death, which correlates with the reduction in their numbers in these animals. In addition, we also observed an approximately two-fold increase in the proportion of $CD4^+/CD25^+/CD62L^{hi}$ cells in the spleen staining positive for Annexin-V in IL-$2R\beta^{-/-}$ mice compared to wild-type mice (data not shown). To determine whether Bcl-2 might play a role in the regulation of apoptosis in regulatory T-cells, we then examined its expression in these cells by intracellular FACS. We found that nearly all of these $CD4^+/CD25^+/CD62L^{hi}$ cells in the spleen expressed Bcl-2 protein in wild-type mice (Figure 10B, left panel), while fewer than half of $CD4^+/CD25^+/CD62L^{hi}$ cells in the spleen of STAT5A/5B-deficient mice expressed Bcl-2 protein above background (Figure 10B, right panel). An even more striking reduction in the proportion of $CD4^+/CD25^+/CD62L^{hi}$ cells expressing the Bcl-2 protein above background was observed in IL-$2R\beta^{-/-}$ mice compared to wild-type mice

(data not shown). These data indicate that STAT5 contributes to the homeostasis of $CD4^+/CD25^+$ regulatory T-cells in part through survival effects that may be mediated through Bcl-2. Similar results observed in IL-2R$\beta^{-/-}$ mice suggests that IL-2 may be the cytokine that provides the STAT5-dependent survival signals to the regulatory T-cells that are critical for their homeostasis.

Discussion

Since STAT5 is activated by a diverse array of cytokine receptors, we hypothesized that defects that are non-cell-autonomous to early progenitors might contribute to the complex, and variably expressed, hematologic defects in STAT5A/5B-deficient mice. Specifically, we posited that a loss of tolerance in the adaptive immune system might lead to autoimmune-mediated effects on hematopoiesis. Three lines of evidence led us to this hypothesis: (a) an autoimmune etiology has been postulated to be responsible for a number of human diseases that affect the function of hematopoietic progenitor cells and interfere with their ability to produce mature cells of the hematopoietic lineages, such as aplastic anemia, pure red cell aplasia, and paroxysmal nocturnal hemoglobinuria [49-52]; (b) the role of IL-2 and its receptor system in the maintenance of tolerance has been well established [7-10,13], and mice lacking IL-2 manifest hematopoietic abnormalities affecting bone marrow [41,42]; and (c) the importance of STAT5 activation by the IL-2R system has been well documented *in vitro* and *in vivo* [11,12].

In a higher resolution examination of the early progenitor compartment of the bone marrow of STAT5A/5B-deficient mice, we established two phenotypic categories, which we termed moderately affected and severely affected. The two most striking characteristics of the severely affected mice were dramatic increases in the HSC-containing population and low production of post-HSC myeloid progenitors despite the increased HSC population. We found that the severely affected mice exhibited a dramatic increase in the number of activated / memory $CD4^+$ and $CD8^+$ T-cells infiltrating the bone marrow. Since STAT5A/5B$^{-/-}$ mice are lymphopenic, this infiltration of T-cells

represents a heightened recruitment of lymphocytes into this tissue rather than a new equilibrium reached with an increased total T-cell pool. T-cells localized aberrantly in the bone marrow could cause the progenitor effects observed either by inhibiting the productive capacity of HSC or by reducing the number of post-HSC. Further study is required to determine whether these effects are caused by antigen-specific autoimmunity or by bystander effects and whether the effector cells that are responsible for pathology are $CD4^+$ or $CD8^+$ T-cells [4,53-55].

We found that the immune cell infiltration was a more general phenomenon as we observed it in all organs examined, including colon, kidney, and liver. In both the colon and the kidney, this infiltration resulted in architectural abnormalities, which could have resulted in compromised function of these tissues. These severe abnormalities in multiple organs most likely contribute to the 70 % incidence of premature death observed in STAT5A/5B-deficient mice by 20 weeks of age. The longitudinal analysis indicates that the cross-sectional analysis performed at 6 to 7 weeks of age detected the presence of disease in only a fraction of those animals that would ultimately develop pathology. Crossing the STAT5A/5B-deficient mice onto the $RAG1^{-/-}$ background abrogated severe anemia, the incidence of early death, and any visible alterations in bone marrow, colon, kidney, and liver in STAT5A/$5B^{-/-}$ animals. This observation indicated that lymphocytes were necessary for the infiltration disease in these animals, although certainly the complex effects of STAT5 deficiency on other cell types such as the bone marrow progenitors [18,24,25,27-31] may also play a role in the pathology observed in these organs.

The lymphocytic infiltration of the colon and liver, the anemia, as well as the recognized role of STAT5 in IL-2 signaling implicated impaired signaling by this cytokine as the cause of loss of tolerance in STAT5A/5B-deficient mice. In further support of this, all IL-2Rβ$^{-/-}$ mice possessed bone marrow characteristics similar to the severely affected STAT5A/5B$^{-/-}$ mice. In addition, the severity of the abnormalities in the IL-2Rβ-deficient mice was markedly higher compared to the defects in STAT5A/5B-deficient mice. The increased penetrance and severity of pathology in the IL-2Rβ$^{-/-}$ mice might be explained by one or more differences between STAT5A/5B$^{-/-}$ and IL-2Rβ$^{-/-}$ mice. First, loss of STAT5 signaling may not fully disable IL-2 signaling competence, while loss of the β subunit of the IL-2R would have more profound effects on the complex downstream signaling pathways. This distinction might allow persistence of a portion of the toleragenic effect of IL-2R signaling in the STAT5A/5B-deficient mice. Second, loss of STAT5 has more wide ranging effects on lymphocytes than loss of IL-2Rβ [18,24,30,31] that may result in reduced incidence of autoimmunity or a reduced ability of effector populations to cause damage. In any event, it would appear that STAT5 is an important mediator of IL-2-mediated tolerance maintenance.

Currently, loss of tolerance in the absence of IL-2 or any of its receptor components is thought to be due to loss of two tolerizing mechanisms. First, activation of the IL-2R is important for upregulation of Fas and activation-induced cell death (AICD) [6,34,56,57]. Second, there is a requirement for this cytokine in the maintenance of a population of CD4^{+}/CD25^{+} regulatory T-cells [43-45,58,59]. Reduced number and/or function of these regulatory cells have been observed in multiple mouse models of autoimmunity [46,47,60]. Furthermore, such cells possess the capacity to inhibit

responses of $CD4^+CD25^-$ T-cells *in vitro* [61-63]. The dependence of such regulatory T-cells on the presence of IL-2 and intact receptor components has been shown both *in vivo* and *in vitro* [43,45-47]. However, the signaling molecules downstream of IL-2 and its cognate receptor that are important for the regulation of these cells and the mechanisms by which these signals affect cell number are not well understood [13]. A representational loss of $CD4^+/CD25^+$ cells has been reported in the spleens of DO11.10 STAT5A-deficient mice [33], but the relevance of this observation to the role of STAT5 in the maintenance of tolerance has not been explored. In the present study, we found that there were reductions in the number of $CD4^+/CD25^+$ regulatory T-cells in both the spleen and an end-organ tissue, the bone marrow, in STAT5A/5B$^{-/-}$ animals. These data suggest that STAT5 is indispensable for the maintenance of this population *in vivo*, which may contribute significantly to the loss of tolerance in these mice.

Interestingly, although all STAT5A/5B-deficient animals exhibited reduced numbers of these cells, not all of the mice developed autoimmune pathology or succumbed to premature death. This observation fits with evidence that reduction in these regulatory T-cells may not be sufficient to cause autoimmunity [64]. Since many of the phenotypes seen in STAT5A/5B-deficient mice exhibit varying degrees of expression in individual mice, development of autoimmunity may require a convergence of multiple threshold effects

The mechanism by which IL-2 contributes to the maintenance of these cells *in vivo* is under intense investigation. Defects in thymic development, or in peripheral proliferation or survival, could all be involved. Our finding that a greater proportion of $CD4^+/CD25^+/CD62L^{hi}$ regulatory T-cells were apoptotic in STAT5A/5B-deficent

animals compared with littermates implies that STAT5 may be important for the survival of the cells. These data are consistent with earlier work by us and others that showed STAT5 to be an important mediator of survival signals in multiple cell types [24,27-29]. In addition, this hypothesis was further supported by the observation that a smaller proportion of the $CD4^{+}/CD25^{+}/CD62L^{hi}$ cells were positive for Bcl-2 protein, which may be an important target of STAT5 in providing survival signals. IL-2 has been shown to provide anti-apoptotic signals to cell lines *in vitro* [65] and to activate Bcl-2 transcription through STAT5 [66]. These findings are consistent with the known role for STAT5 as a mediator of survival signals in other cell types [27-29]. However, other STAT5-dependent mechanisms could be important for providing increased survival in these cells, such as upregulation of other Bcl-2 family members or the IL-2Rα chain [67]. The finding that these cells in IL-2Rβ$^{-/-}$ mice also have increased apoptosis and decreased expression of Bcl-2 suggest that the IL-2R mediates effects on regulatory T-cells in part though modulation of their survival, perhaps through STAT5-dependent effects. Additionally, these data suggest a novel role of apoptosis in the cytokine-mediated homeostasis of this cellular population [46,47].

Use of the Bcl-2 transgenic mice would be one way to test the hypothesis that Bcl-2-mediated increases in survival can complement for loss of STAT5 in these "regulatory T-cells", increasing their numbers and preventing autoimmunity in STAT5A/5B-deficient mice. Corrections of certain hematologic pathologies in STAT5A/5B-deficient $^{+}$Tg mice, such as the increases in the HSC population and the anemia (Chapter 3), lend some evidence to this hypothesis although further studies are necessary.

Our data support a model in which IL-2 contributes to the survival of $CD4^+/CD25^+/CD62L^{hi}$ regulatory T-cells *in vivo*, through receptor mediated activation of STAT5. The reduction in the number of these cells contributes to loss of tolerance in STAT5A/5B-deficient animals, leading to a lymphocyte-dependent autoimmune disease affecting multiple organs. One sequela of this disease is the lymphocyte-dependent perturbation of the hematopoietic system, contributing to the complex hematologic phenotypes seen in STAT5A/5B-deficient mice.

These data provide the first evidence that STAT5 is important for the maintenance of tolerance *in vivo*, which adds a new function to the growing list that this transcription factor plays in the context of the organism. Whether STAT5 plays its toleragenic role downstream of the IL-2R exclusively, or whether other receptors are involved, must be further explored. In addition, the extent of the cell types involved in STAT5-dependent maintenance of tolerance and the downstream genes through which STAT5 mediates these affects need further study.

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Figure Legends

Figure 1. The HSC and post-HSC abnormalities in STAT5A/5B$^{-/-}$ mice exhibit variable expressivity. Subsetting was performed according to surface expression of lineage markers (Lin), Sca-1, and c-kit for the Lin^{dim}/Sca-1^{+}/c-kit^{+} population, which contains the HSC; and the Lin^{dim}/Sca-1^{-}/c-kit^{+} population, containing post-HSC myeloid progenitors. Representative contour plots showing Sca-1 and c-kit surface expression by lineage marker dim cells for a typical STAT5A/5B$^{+/+}$ animal, a moderately affected STAT5A/5B$^{-/-}$ animal, and a severely affected STAT5A/5B$^{-/-}$ animal.

Figure 2. STAT5A/5B$^{-/-}$ mice with severe hematopoietic abnormalities exhibit bone marrow infiltration by memory CD4^{+} and CD8^{+} memory cells. Subsetting was performed according to surface expression of CD4 or CD8 in combination with CD44 and CD62L. Absolute values were obtained by multiplying gated percentages by total nucleated cell numbers. Closed circles represent animals from the STAT5A/5B$^{+/+}$ group (n = 6) and from the STAT5A/5B$^{-/-}$ Moderate group (n=6), while open circles represent animals from the STAT5A/5B$^{-/-}$ Severe (n=4) group in Table 1. (A) Memory CD4^{+} cells in bone marrow and (B) Memory CD8^{+} cells in bone marrow.

Figure 3. Mononuclear cell infiltration in multiple organs of severely affected STAT5A/5B-deficient mice. Histological analysis of Hematoxilin and Eosin sections from colon (A), kidney (B), and liver (C) of both STAT5A/5B$^{+/+}$ and STAT5A/5B$^{-/-}$ mice that were previously defined as severely affected reveal infiltration of these tissues by mononuclear cells in the STAT5A/5B$^{-/-}$ mice. All images are at 20x magnification.

Figure 4. Blood chemistry analysis reveals dysfunction in multiple organs. Plasma was collected and assays for AST (A) ($p < 0.01$), LDH (B) ($p < 0.05$), BUN (C) ($p < 0.01$), TCO2 (D) ($p < 0.05$), and Albumin (E) ($p < 0.01$), are shown. For STAT5A/5B$^{+/+}$ mice (n=11) and for STAT5A/5B$^{-/-}$ mice (n=13).

Figure 5. An immunodeficient background fully abrogates the lethality of STAT5A/5B deficiency. (A) Survival analysis of STAT5A/5B$^{+/+}$ mice (n=18) and STAT5A/5B$^{-/-}$ mice (n=19). (B) Survival analysis of STAT5A/5B$^{+/+}$ mice (n=9) and STAT5A/5B$^{-/-}$ mice (n=11) on the RAG1$^{+/+}$ background and STAT5A/5B$^{+/+}$ mice (n=14) and STAT5A/5B$^{-/-}$ mice (n=11) on the RAG1$^{-/-}$ background in the presence of antibiotics.

Figure 6. An immunodeficient background rescues anemia and neutrophilia, but not thrombocytopenia in STAT5A/5B deficiency. Complete blood counts were performed on whole peripheral blood from STAT5A/5B$^{-/-}$ and STAT5A/5B$^{+/+}$ mice on the RAG1$^{-/-}$ background. . (A) Hematocrit (STAT5A/5B$^{+/+}$ (RAG$^{-/-}$), n= 11, STAT5A/5B$^{-/-}$ (RAG$^{-/-}$), n=10). (B) Platelets (STAT5A/5B$^{+/+}$ (RAG$^{-/-}$), n= 11, STAT5A/5B$^{-/-}$ (RAG$^{-/-}$), n=10). (C) Neutrophils (STAT5A/5B$^{+/+}$ (RAG$^{-/-}$), n= 6, STAT5A/5B$^{-/-}$ (RAG$^{-/-}$), n=7).

Figure 7. An immunodeficient background does not rescue bone marrow cellularity or progenitor subsets in STAT5A/5B-deficient mice. Total nucleated cells were obtained from one femur and tibia and cell counts were determined after red blood cell

lysis for bone marrow from STAT5A/5B$^{-/-}$ (n=4) and STAT5A/5B$^{+/+}$ (n=4) mice on the RAG1$^{-/-}$ background (A). Subsetting was performed based on surface expression of lineage-defining markers, Sca-1, and c-kit. (B) Representative contour plots showing Sca-1 and c-kit surface expression by lineage marker dim cells for STAT5A/5B$^{+/+}$ mice and STAT5A/5B$^{-/-}$ mice on the RAG1$^{-/-}$ background are shown. Absolute values were generated by multiplying gated percentages by total nucleated cell numbers. The lin^{dim}Sca-1^{+}/c-kit^{+} compartment, containing HSC (C), and the lin^{dim}Sca-1$^{neg/lo}$/c-kit^{+} compartment, containing post -HSC progenitors (D), from STAT5A/5B$^{-/-}$ mice (n=4) and STAT5A/5B$^{+/+}$ mice (n=4) on the RAG1$^{-/-}$ background are shown.

Figure 8. IL-2Rβ-deficient mice manifest severe hematopoietic abnormalities like those in STAT5A/5B-deficient mice. Subsetting and generation of absolute values was performed as above. (A) Representative contour plots showing Sca-1 and c-kit surface expression by lineage marker dim cells for an IL-2Rβ$^{+/+}$ (n=4) and an IL-2Rβ$^{-/-}$ (n=4) animal are shown. Scatter Plots showing absolute values for (B) HSC, (C) Post-HSC myeloid progenitors, (D) Memory CD4^{+} cells in bone marrow, and (E) Memory CD8^{+} cells in bone marrow of both IL-2Rβ$^{+/+}$ and an IL-2Rβ$^{-/-}$ animal are shown.

Figure 9. STAT5A/5B-deficient mice have reduced numbers of CD4^{+}/CD25^{+} regulatory T-cells. Total nucleated cells obtained from either the spleen (STAT5A/5B$^{+/+}$ mice (n=4) and STAT5A/5B$^{-/-}$ mice (n=4)) or the bone marrow (STAT5A/5B$^{+/+}$ mice (n=3) and STAT5A/5B$^{-/-}$ mice (n=4) were analyzed. Subsetting was performed according to surface expression of CD4 and CD25. Plots showing CD4^{+}/CD25^{-} and CD4^{+}/CD25^{+}

subsets in both the spleen (A) and the bone marrow (B) are shown. (C) Surface expression of CD62L on $CD4^+/CD25^+$ cells in the spleen is shown.

Figure 10. Increased apoptosis and decreased Bcl-2 expression in regulatory T-cells from STAT5A/5B-deficent mice. CD4+/CD25+/$CD62L^{hi}$ splenocytes were stained for either the surface expression of Annexin-V (A) or intracellular Bcl-2 (B) with dotted lines representing isotype control and solid lines representing anti-Bcl-2 antibody staining. of $STAT5A/5B^{+/+}$ mice (n=3) and $STAT5A/5B^{-/-}$ mice (n=3).

Table 1. Absolute Number of Cells in Lin^{dim} Progenitor Subsets (x 10^4)[3]

	$Sca\text{-}1^+/c\text{-}kit^+$	$Sca\text{-}1^-/c\text{-}kit^+$
$STAT5A/5B^{+/+}$	4.1 ± (0. 23)	27.7 ± (2.5)
$STAT5A/5B^{-/-}$		
Moderate	5.09 ± (1.2)	9.75 ± (1.8)[a]
Severe	13.4 ± (1.9)[a, b]	8.45 ± (2.8)[a]

[3] Table 1. Absolute number of lin^{dim} progenitor subsets (x10^4).
Total nucleated cells obtained from hind legs. Absolute values were obtained by multiplying gated percentages and total nucleated cell numbers.
[a] $p < 0.05$ compared to $STAT5A/5B^{+/+}$
[b] $p < 0.05$ compared to $STAT5A/5B^{-/-}$ Moderate

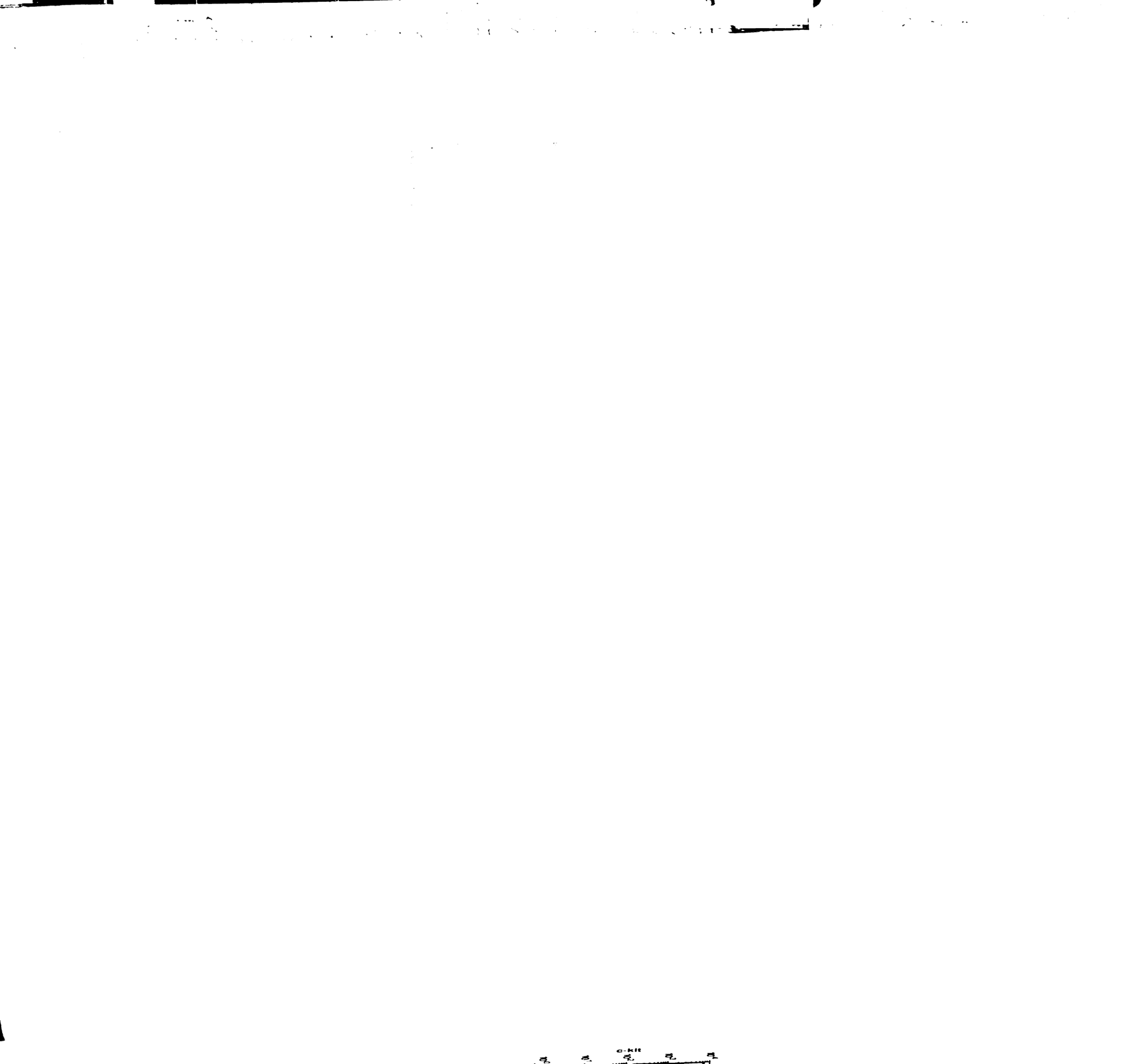


Figure 1

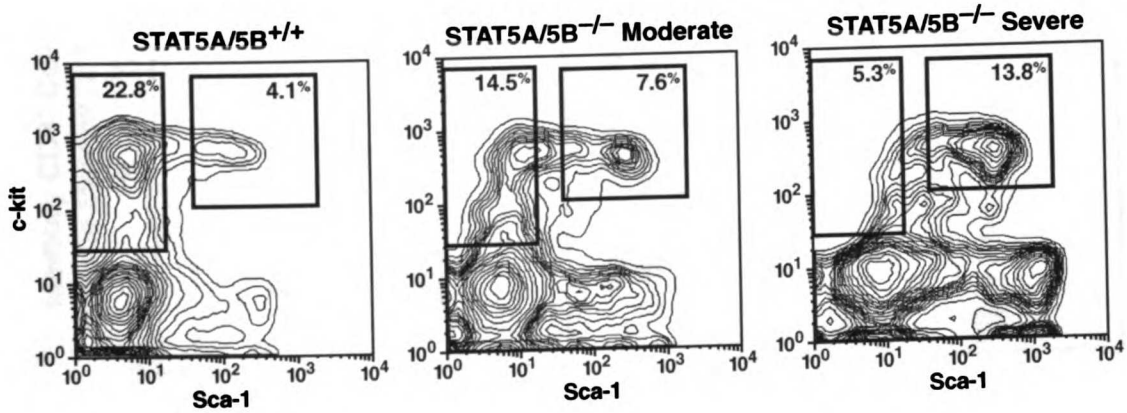

STAT5A/5B+/+
STAT5A/5B−/− Moderate
STAT5A/5B−/− Severe
c-kit
Sca-1
22.8%
4.1%
14.5%
7.6%
5.3%
13.8%

Figure 2

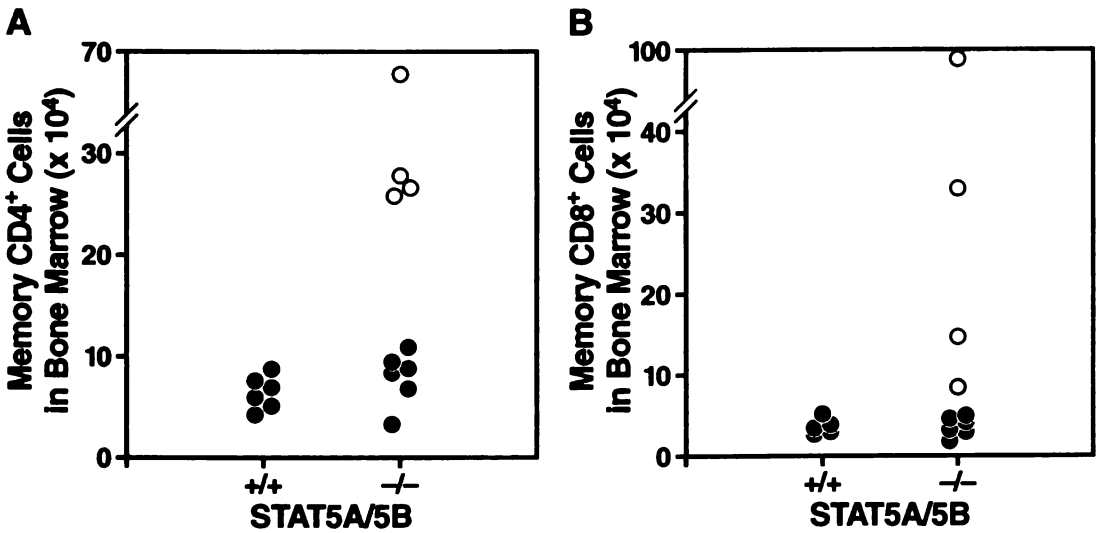

A
Memory CD4+ Cells in Bone Marrow (x 10⁴)
70
30
20
10
0
+/+
−/−
STAT5A/5B
B
Memory CD8+ Cells in Bone Marrow (x 10⁴)
100
40
30
20
10
0
+/+
−/−
STAT5A/5B

Figure 3

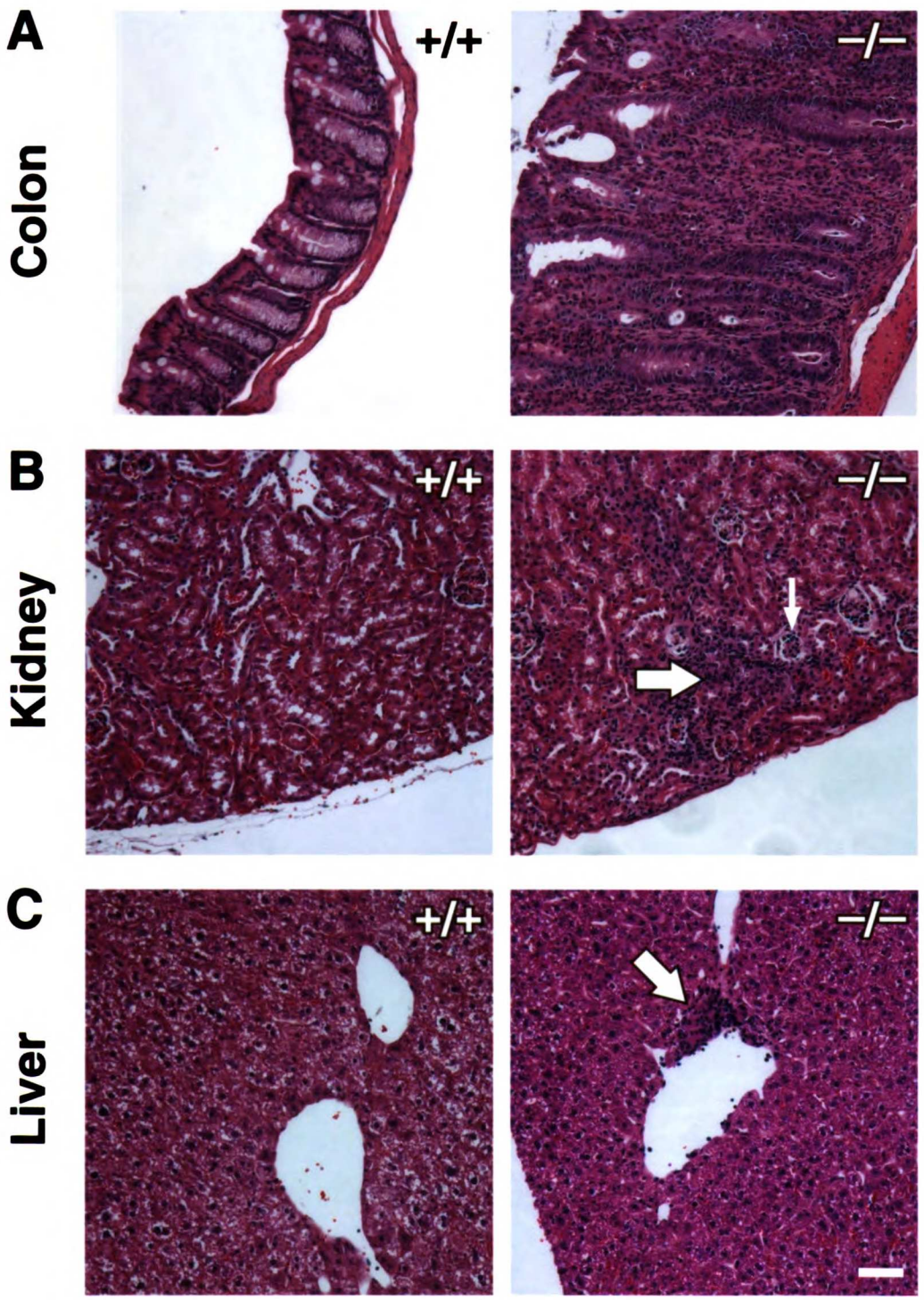


Figure 4

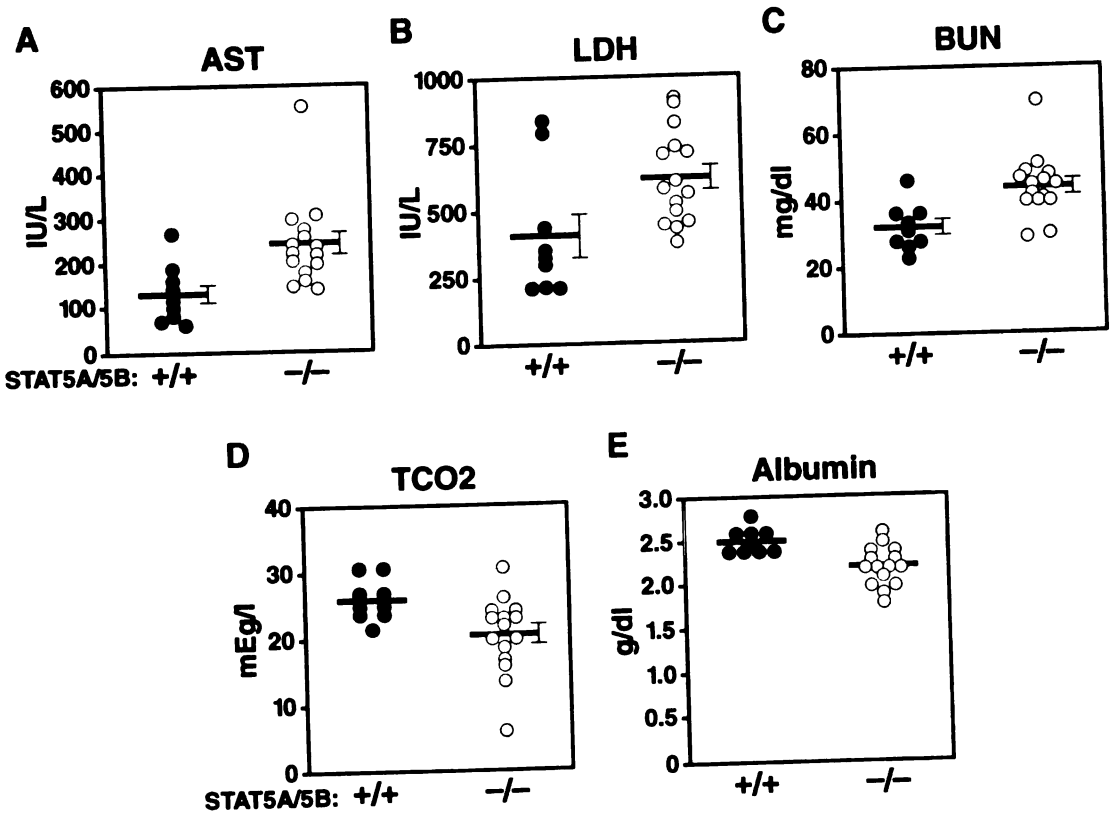

A
AST
600
500
400
300
200
100
0
IU/L
STAT5A/5B: +/+
−/−
B
LDH
1000
750
500
250
0
IU/L
+/+
−/−
C
BUN
80
60
40
20
0
mg/dl
+/+
−/−
D
TCO2
40
30
20
10
0
mEg/l
STAT5A/5B: +/+
−/−
E
Albumin
3.0
2.5
2.0
1.5
1.0
0.5
0
g/dl
+/+
−/−

Figure 5

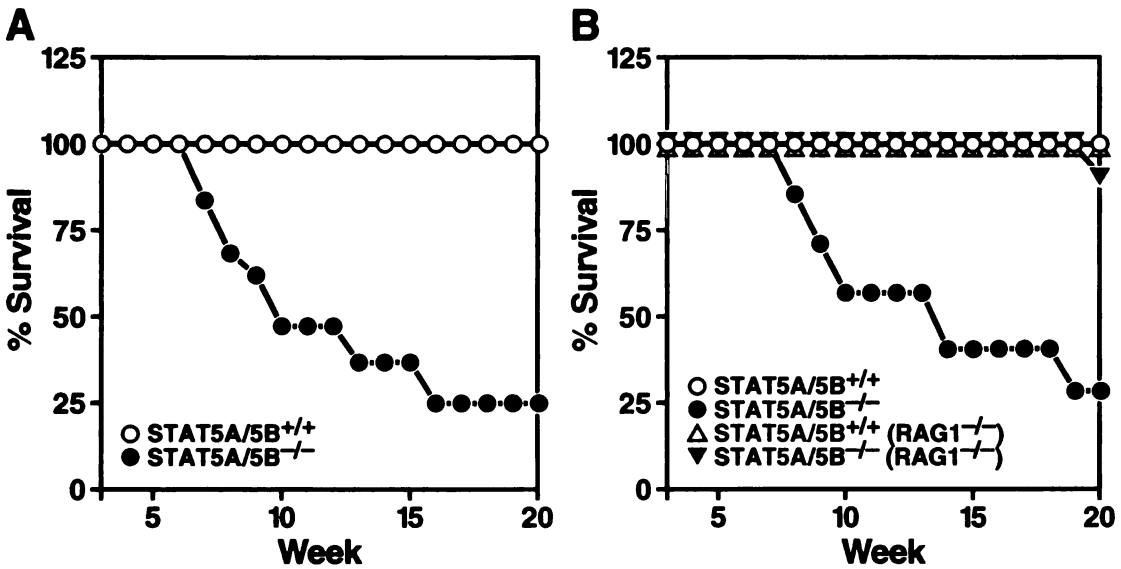

A
125
100
75
50
25
0
% Survival
5
10
15
20
Week
STAT5A/5B+/+
STAT5A/5B−/−
B
125
100
75
50
25
0
% Survival
5
10
15
20
Week
STAT5A/5B+/+
STAT5A/5B−/−
STAT5A/5B+/+ (RAG1−/−)
STAT5A/5B−/− (RAG1−/−)

Figure 6

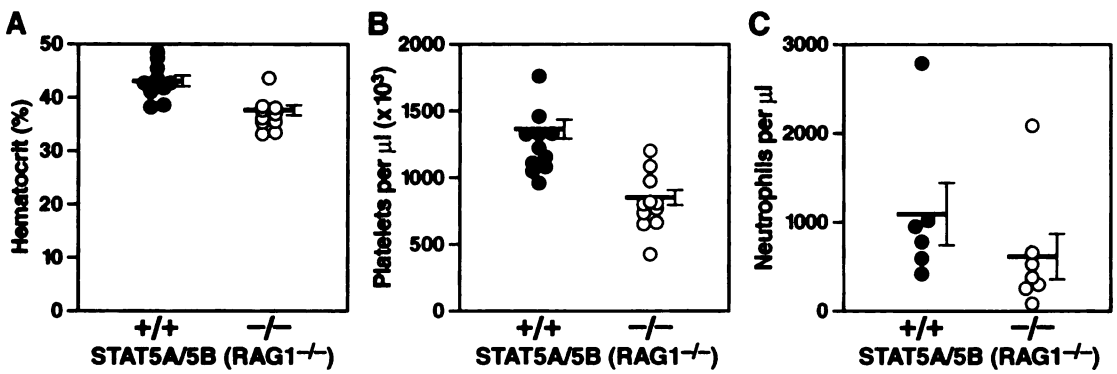

A
Hematocrit (%)
50
40
30
20
10
0
+/+
−/−
STAT5A/5B (RAG1−/−)
B
Platelets per µl (x 10³)
2000
1500
1000
500
0
+/+
−/−
STAT5A/5B (RAG1−/−)
C
Neutrophils per µl
3000
2000
1000
0
+/+
−/−
STAT5A/5B (RAG1−/−)

Figure 7

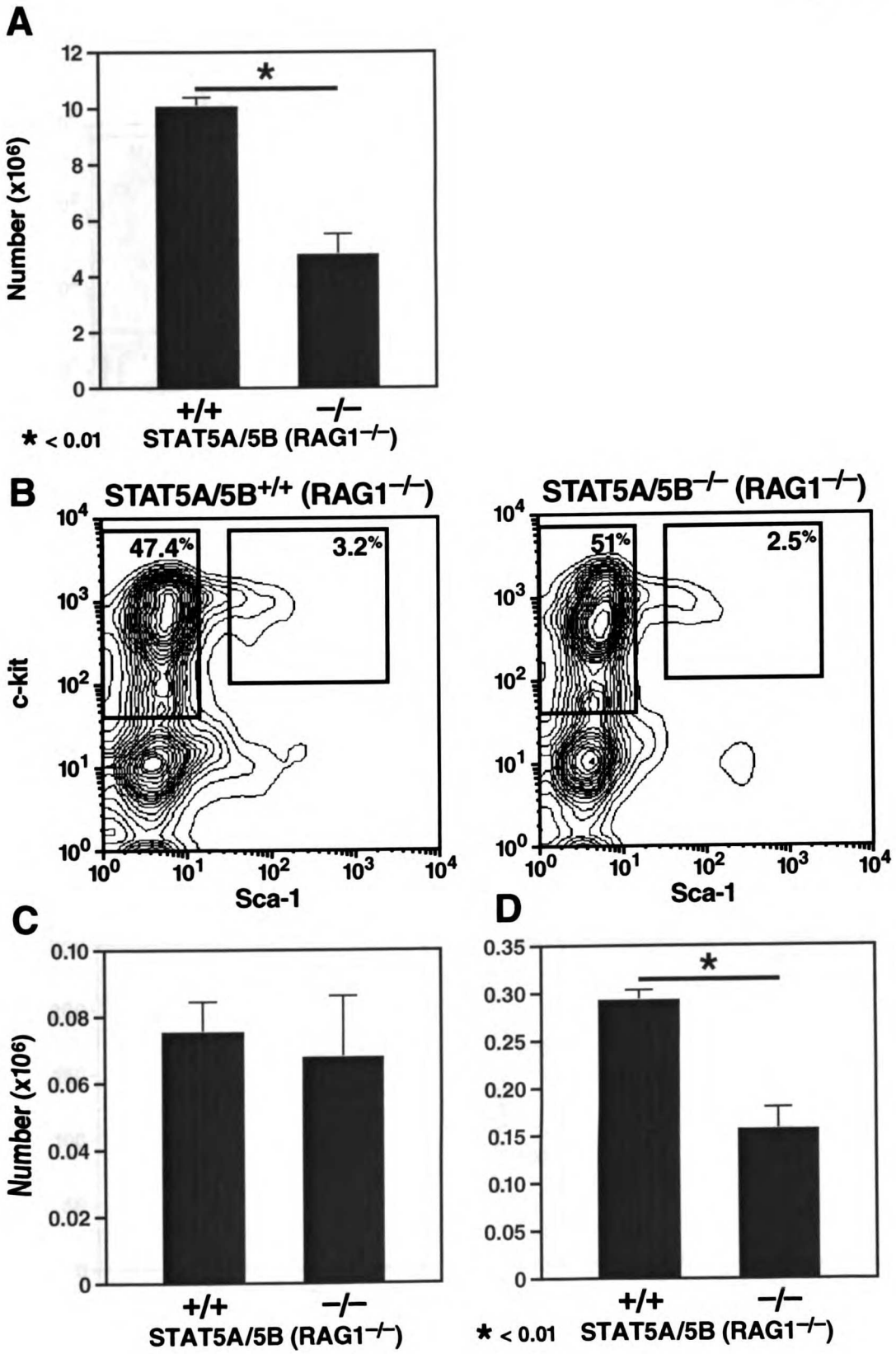

A
Number (x10⁶)
12
10
8
6
4
2
0
*
+/+
−/−
* < 0.01
STAT5A/5B (RAG1−/−)
B
STAT5A/5B+/+ (RAG1−/−)
STAT5A/5B−/− (RAG1−/−)
c-kit
Sca-1
47.4%
3.2%
51%
2.5%
C
D
Number (x10⁶)
0.10
0.08
0.06
0.04
0.02
0
0.35
0.30
0.25
0.20
0.15
0.10
0.05
+/+
−/−
STAT5A/5B (RAG1−/−)
* < 0.01

Figure 8

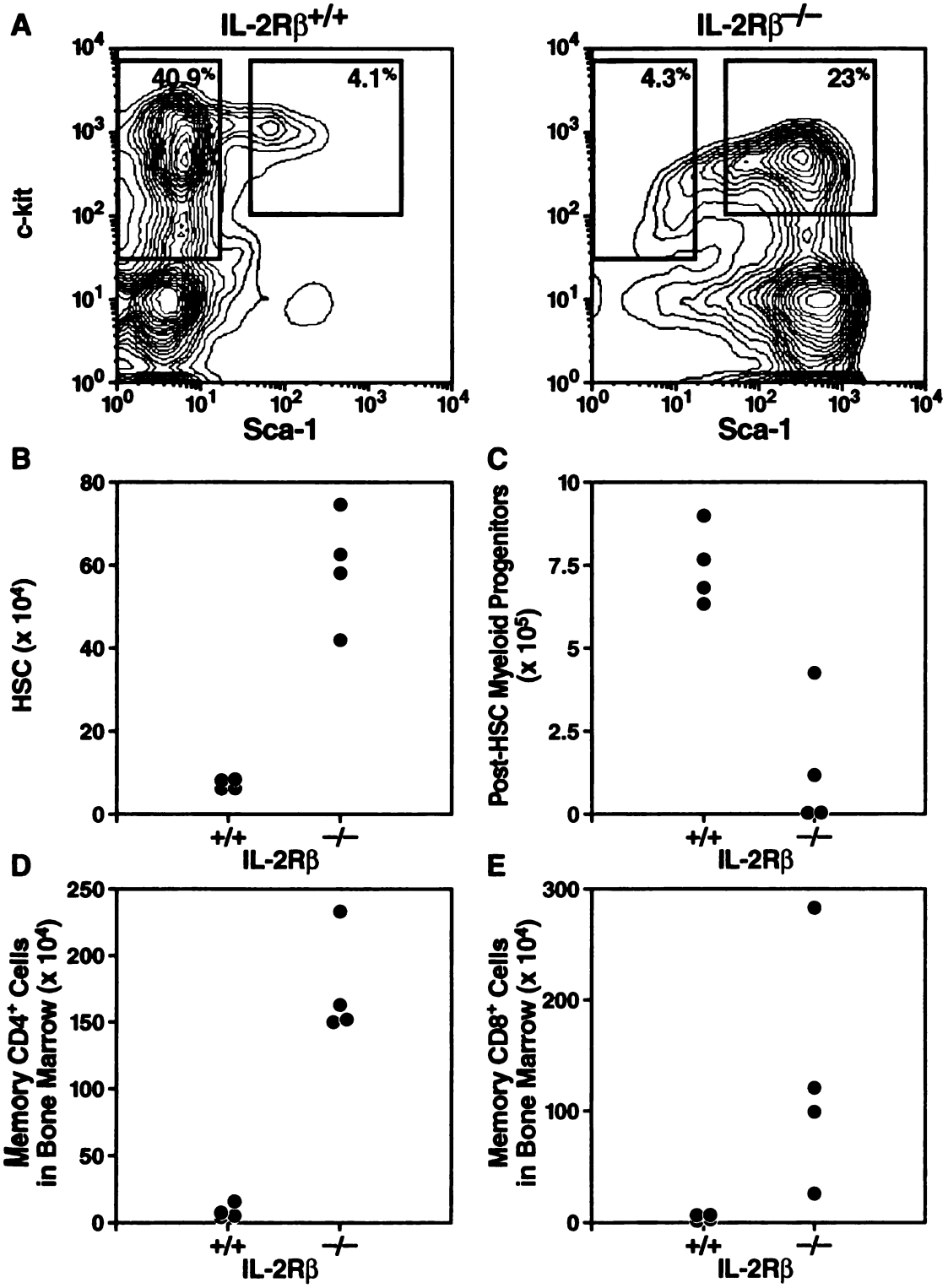

A
IL-2Rβ+/+
IL-2Rβ−/−
c-kit
Sca-1
40.9%
4.1%
4.3%
23%
B
HSC (x 10⁴)
C
Post-HSC Myeloid Progenitors (x 10⁵)
D
Memory CD4⁺ Cells in Bone Marrow (x 10⁴)
E
Memory CD8⁺ Cells in Bone Marrow (x 10⁴)
+/+
−/−
IL-2Rβ

Figure 9

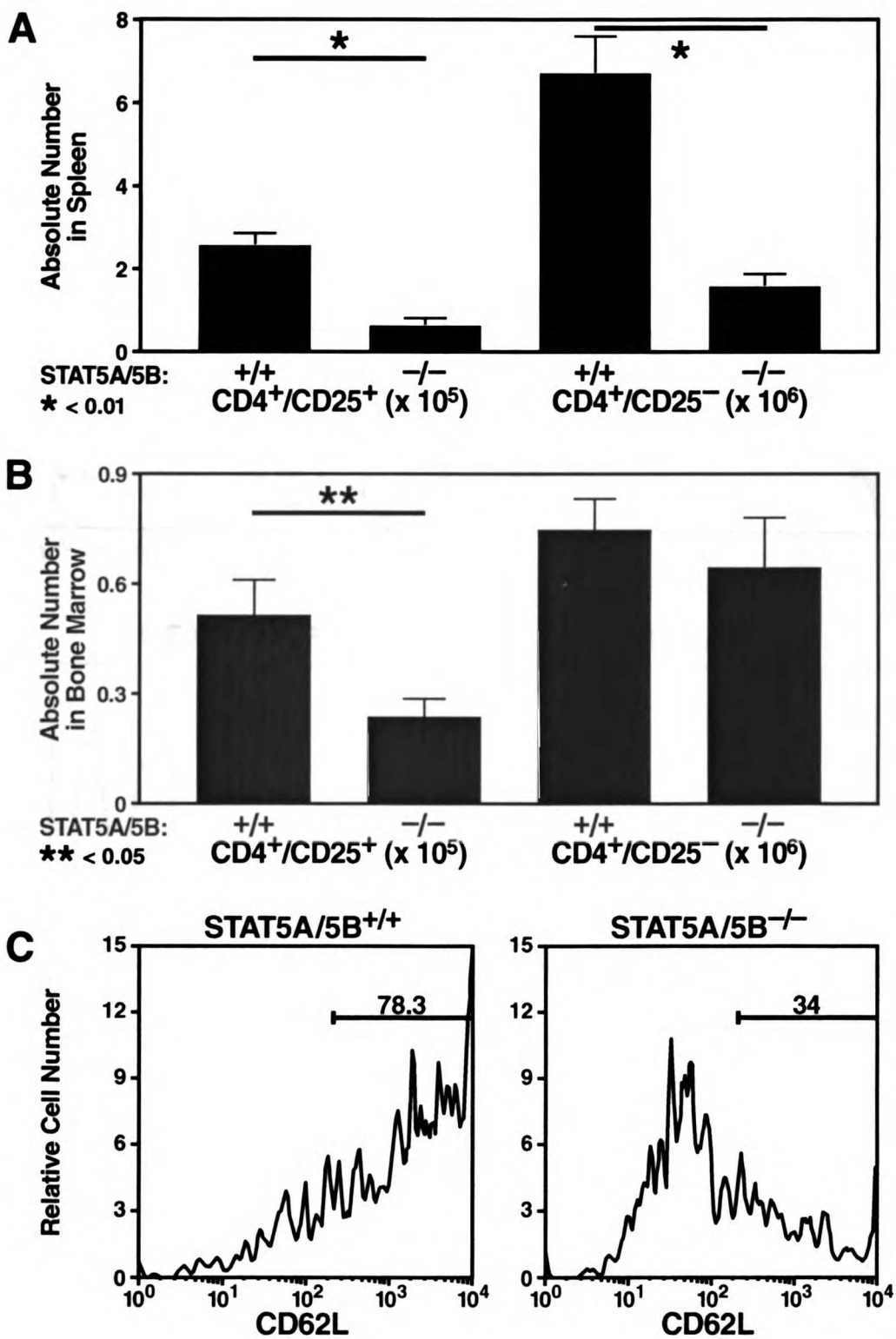
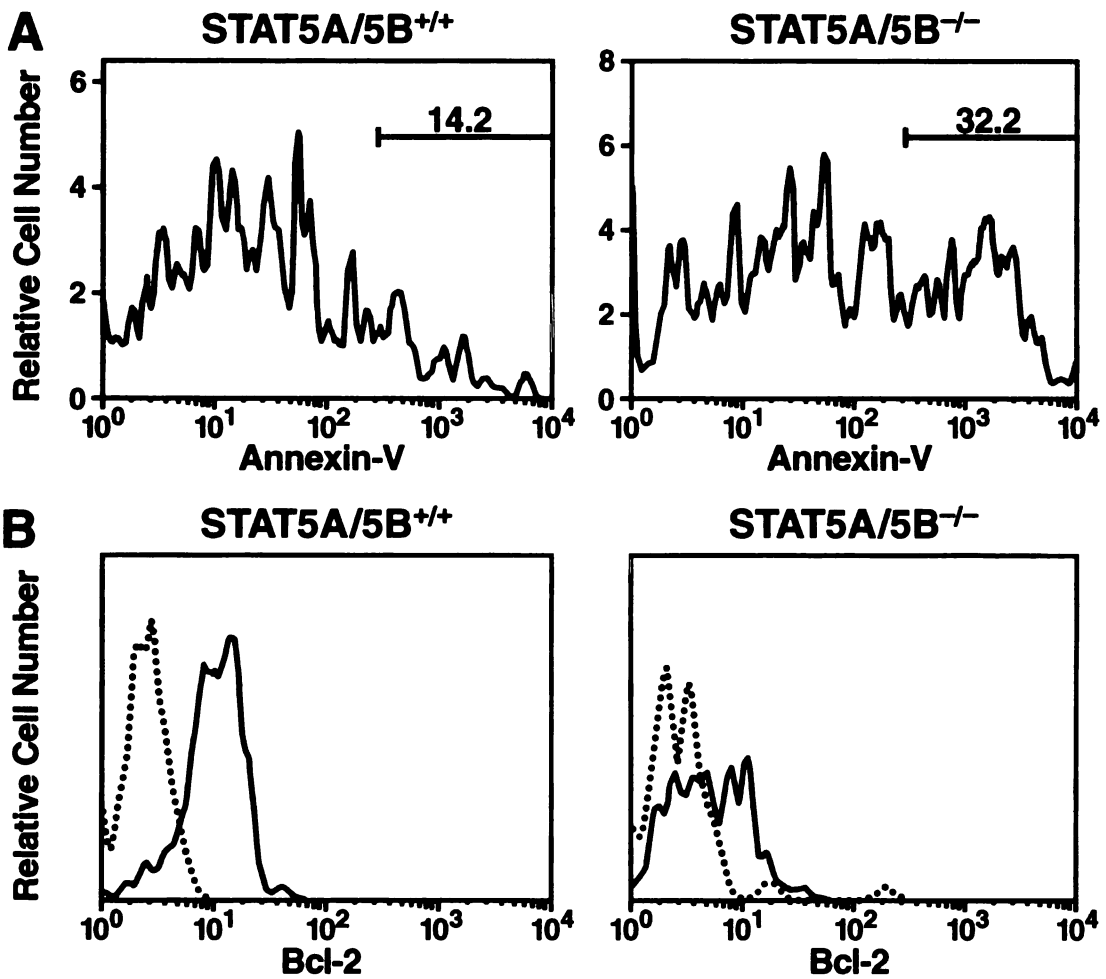

A
Absolute Number in Spleen
8
6
4
2
0
*
*
STAT5A/5B:
+/+
−/−
+/+
−/−
* < 0.01
CD4+/CD25+ (x 10^5)
CD4+/CD25− (x 10^6)
B
Absolute Number in Bone Marrow
0.9
0.6
0.3
0
**
STAT5A/5B:
+/+
−/−
+/+
−/−
** < 0.05
CD4+/CD25+ (x 10^5)
CD4+/CD25− (x 10^6)
C
STAT5A/5B+/+
STAT5A/5B−/−
Relative Cell Number
15
12
9
6
3
0
78.3
34
10^0
10^1
10^2
10^3
10^4
CD62L
CD62L

Figure 10


A
STAT5A/5B+/+
STAT5A/5B−/−
Relative Cell Number
14.2
32.2
Annexin-V
B
STAT5A/5B+/+
STAT5A/5B−/−
Relative Cell Number
Bcl-2

References

1. Marrack P, Kappler J, Kotzin BL (2001) Autoimmune disease: why and where it occurs. Nat Med 7:899

2. O'Shea JJ, Ma A, Lipsky P (2002) Cytokines and Autoimmunity. Nature Reviews Immunology 2:37

3. Durkin HG, Waksman BH (2001) Thymus and tolerance. Is regulation the major function of the thymus? Immunol Rev 182:33

4. O'Garra A, Steinman L, Gijbels K (1997) CD4+ T-cell subsets in autoimmunity. Curr Opin Immunol 9:872

5. Falcone M, Sarvetnick N (1999) Cytokines that regulate autoimmune responses. Curr Opin Immunol 11:670

6. Van Parijs L, Abbas AK (1998) Homeostasis and self-tolerance in the immune system: turning lymphocytes off. Science 280:243

7. Suzuki H, Kündig TM, Furlonger C, Wakeham A, Timms E, Matsuyama T, Schmits R, Simard JJ, Ohashi PS, Griesser H, et al. (1995) Deregulated T cell activation and autoimmunity in mice lacking interleukin-2 receptor beta. Science 268:1472

8. Willerford DM, Chen J, Ferry JA, Davidson L, Ma A, Alt FW (1995) Interleukin-2 receptor alpha chain regulates the size and content of the peripheral lymphoid compartment. Immunity 3:521

9. Sadlack B, Merz H, Schorle H, Schimpl A, Feller AC, Horak I (1993) Ulcerative colitis-like disease in mice with a disrupted interleukin-2 gene. Cell 75:253

10. Nakajima H, Shores EW, Noguchi M, Leonard WJ (1997) The common cytokine receptor gamma chain plays an essential role in regulating lymphoid homeostasis. J Exp Med 185:189

11. Gaffen SL (2001) Signaling domains of the interleukin 2 receptor. Cytokine 14:63

12. Lin JX, Leonard WJ (2000) The role of Stat5a and Stat5b in signaling by IL-2 family cytokines. Oncogene 19:2566

13. Nelson BH (2002) Interleukin-2 signaling and the maintenance of self-tolerance. Curr Dir Autoimmun 5:92

14. Bromberg J, Darnell JE, Jr. (2000) The role of STATs in transcriptional control and their impact on cellular function. Oncogene 19:2468

15. Ihle JN (1996) STATs: signal transducers and activators of transcription. Cell 84:331

16. Takeda K, Clausen BE, Kaisho T, Tsujimura T, Terada N, Forster I, Akira S (1999) Enhanced Th1 activity and development of chronic enterocolitis in mice devoid of Stat3 in macrophages and neutrophils. Immunity 10:39

17. Homann D, Holz A, Bot A, Coon B, Wolfe T, Petersen J, Dyrberg TP, Grusby MJ, von Herrath MG (1999) Autoreactive CD4+ T cells protect from autoimmune diabetes via bystander suppression using the IL-4/Stat6 pathway. Immunity 11:463

18. Teglund S, McKay C, Schuetz E, van Deursen JM, Stravopodis D, Wang D, Brown M, Bodner S, Grosveld G, Ihle JN (1998) Stat5a and Stat5b proteins have essential and nonessential, or redundant, roles in cytokine responses. Cell 93:841

19. Liu X, Robinson GW, Wagner KU, Garrett L, Wynshaw-Boris A, Hennighausen L (1997) Stat5a is mandatory for adult mammary gland development and lactogenesis. Genes Dev. 11:179

20. Udy GB, Towers RP, Snell RG, Wilkins RJ, Park SH, Ram PA, Waxman DJ, Davey HW (1997) Requirement of STAT5b for sexual dimorphism of body growth rates and liver gene expression. Proc. Natl. Acad. Sci. USA 94:7239

21. Feldman GM, Rosenthal LA, Liu X, Hayes MP, Wynshaw-Boris A, Leonard WJ, Hennighausen L, Finbloom DS (1997) STAT5A-deficient mice demonstrate a defect in granulocyte-macrophage colony-stimulating factor-induced proliferation and gene expression. Blood 90:1768

22. Nakajima H, Liu XW, Wynshaw-Boris A, Rosenthal LA, Imada K, Finbloom DS, Hennighausen L, Leonard WJ (1997) An indirect effect of Stat5a in IL-2-induced proliferation: a critical role for Stat5a in IL-2-mediated IL-2 receptor alpha chain induction. Immunity 7:691

23. Imada K, Bloom ET, Nakajima H, Horvath-Arcidiacono JA, Udy GB, Davey HW, Leonard WJ (1998) Stat5b is essential for natural killer cell-mediated proliferation and cytolytic activity. J. Exp. Med. 188:2067

24. Snow JW, Abraham N, Ma MC, Abbey NW, Herndier B, Goldsmith MA (2002) STAT5 promotes multilineage hematolymphoid development in vivo through effects on early hematopoietic progenitor cells. Blood 99:95

25. Bunting KD, Bradley HL, Hawley TS, Moriggl R, Sorrentino BP, Ihle JN (2002) Reduced lymphomyeloid repopulating activity from adult bone marrow and fetal liver of mice lacking expression of STAT5. Blood 99:479

26. Zhang S, Fukuda S, Lee Y, Hangoc G, Cooper S, Spolski R, Leonard WJ, Broxmeyer HE (2000) Essential Role of Signal Transducer and Activator of Transcription (Stat)5a but Not Stat5b for Flt3-dependent Signaling. J Exp Med 192:719

27. Socolovsky M, Nam H, Fleming MD, Haase VH, Brugnara C, Lodish HF (2001) Ineffective erythropoiesis in Stat5a(-/-)5b(-/-) mice due to decreased survival of early erythroblasts. Blood 98:3261

28. Socolovsky M, Fallon AE, Wang S, Brugnara C, Lodish HF (1999) Fetal anemia and apoptosis of red cell progenitors in Stat5a-/-5b-/- mice: a direct role for Stat5 in Bcl-X(L) induction. Cell 98:181

29. Kieslinger M, Woldman I, Moriggl R, Hofmann J, Marine JC, Ihle JN, Beug H, Decker T (2000) Antiapoptotic activity of Stat5 required during terminal stages of myeloid differentiation. Genes and Development 14:232

30. Moriggl R, Sexl V, Piekorz R, Topham D, Ihle JN (1999) Stat5 activation is uniquely associated with cytokine signaling in peripheral T cells. Immunity 11:225

31. Moriggl R, Topham DJ, Teglund S, Sexl V, McKay C, Wang D, Hoffmeyer A, van Deursen J, Sangster MY, Bunting KD, Grosveld GC, Ihle JN (1999) Stat5 is required for IL-2-induced cell cycle progression of peripheral T cells. Immunity 10:249

32. Kagami S, Nakajima H, Kumano K, Suzuki K, Suto A, Imada K, Davey HW, Saito Y, Takatsu K, Leonard WJ, Iwamoto I (2000) Both stat5a and stat5b are required for antigen-induced eosinophil and T-cell recruitment into the tissue. Blood 95:1370

33. Kagami S, Nakajima H, Suto A, Hirose K, Suzuki K, Morita S, Kato I, Saito Y, Kitamura T, Iwamoto I (2001) Stat5a regulates T helper cell differentiation by several distinct mechanisms. Blood 97:2358

34. Van Parijs L, Refaeli Y, Lord JD, Nelson BH, Abbas AK, Baltimore D (1999) Uncoupling IL-2 signals that regulate T cell proliferation, survival, and Fas-mediated activation-induced cell death. Immunity 11:281

35. Sexl V, Piekorz R, Moriggl R, Rohrer J, Brown MP, Bunting KD, Rothammer K, Roussel MF, Ihle JN (2000) Stat5a/b contribute to interleukin 7-induced B-cell precursor expansion, but abl- and bcr/abl-induced transformation are independent of stat5. Blood 96:2277

36. Ernst JD, Yang L, Rosales JL, Broaddus VC (1998) Preparation and characterization of an endogenously fluorescent annexin for detection of apoptotic cells. Anal. Biochem. 260:18

37. Akashi K, Traver D, Miyamoto T, Weissman IL (2000) A clonogenic common myeloid progenitor that gives rise to all myeloid lineages. Nature 404:193

38. Kondo M, Weissman IL, Akashi K (1997) Identification of clonogenic common lymphoid progenitors in mouse bone marrow. Cell 91:661

39. Dutton RW, Bradley LM, Swain SL (1998) T cell memory. Annu Rev Immunol 16:201

40. Bradley HL, Hawley TS, Bunting KD (2002) Cell intrinsic defects in cytokine responsiveness of STAT5-deficient hematopoietic stem cells. Blood

41. Contractor NV, Bassiri H, Reya T, Park AY, Baumgart DC, Wasik MA, Emerson SG, Carding SR (1998) Lymphoid hyperplasia, autoimmunity, and compromised intestinal intraepithelial lymphocyte development in colitis-free gnotobiotic IL-2-deficient mice. J Immunol 160:385

42. Reya T, Contractor NV, Couzens MS, Wasik MA, Emerson SG, Carding SR (1998) Abnormal myelocytic cell development in interleukin-2 (IL-2)-deficient mice: evidence for the involvement of IL-2 in myelopoiesis. Blood 91:2935

43. Papiernik M, de Moraes ML, Pontoux C, Vasseur F, Penit C (1998) Regulatory CD4 T cells: expression of IL-2R alpha chain, resistance to clonal deletion and IL-2 dependency. Int Immunol 10:371

44. Furtado GC, De Lafaille MA, Kutchukhidze N, Lafaille JJ (2002) Interleukin 2 Signaling Is Required for CD4(+) Regulatory T Cell Function. J Exp Med 196:851

45. Malek T, Yu A, Vincek V, Scibelli P, Kong L (2002) CD4 Regulatory T Cells Prevent Lethal Autoimmunity in IL-2Rbeta-Deficient Mice. Implications for the Nonredundant Function of IL-2. Immunity 17:167

46. Maloy KJ, Powrie F (2001) Regulatory T cells in the control of immune pathology. Nat Immunol 2:816

47. Shevach EM (2002) CD4+ CD25+ suppressor T cells: more questions than answers. Nat Rev Immunol 2:389

48. Chatenoud L, Salomon B, Bluestone JA (2001) Suppressor T cells--they're back and critical for regulation of autoimmunity! Immunol Rev 182:149

49. Young NS, Maciejewski J (1997) The pathophysiology of acquired aplastic anemia. N Engl J Med 336:1365

50. Young NS, Abkowitz JL, Luzzatto L (2000) New Insights into the Pathophysiology of Acquired Cytopenias. In: American Society Of Hematology. San Francisco: American Society Of Hematology, 18

51. Croisille L, Tchernia G, Casadevall N (2001) Autoimmune disorders of erythropoiesis. Curr Opin Hematol 8:68

52. Rosti V (2000) The molecular basis of paroxysmal nocturnal hemoglobinuria. Haematologica 85:82

53. Santamaria P (2001) Effector lymphocytes in autoimmunity. Curr Opin Immunol 13:663

54. Vizler C, Bercovici N, Cornat A, Cambouris C, Liblau RS (1999) Role of autoreactive CD8+ T cells in organ specific autoimmune diseases: insight from transgenic mouse models. Immunol Rev 169:81

55. Suri A, Katz JD (1999) Dissecting the role of CD4+ T cells in autoimmune diabetes through the use of TCR transgenic mice. Immunol Rev 169:55

56. Van Parijs L, Ibraghimov A, Abbas AK (1996) The roles of costimulation and Fas in T cell apoptosis and peripheral tolerance. Immunity 4:321

57. Van Parijs L, Biuckians A, Ibragimov A, Alt FW, Willerford DM, Abbas AK (1997) Functional responses and apoptosis of CD25 (IL-2R alpha)-deficient T cells expressing a transgenic antigen receptor. J Immunol 158:3738

58. Suzuki H, Zhou YW, Kato M, Mak TW, Nakashima I (1999) Normal regulatory alpha/beta T cells effectively eliminate abnormally activated T cells lacking the interleukin 2 receptor beta in vivo. J Exp Med 190:1561

59. Wolf M, Schimpl A, Hunig T (2001) Control of T cell hyperactivation in IL-2-deficient mice by CD4(+)CD25(-) and CD4(+)CD25(+) T cells: evidence for two distinct regulatory mechanisms. Eur J Immunol 31:1637

60. Asano M, Toda M, Sakaguchi N, Sakaguchi S (1996) Autoimmune disease as a consequence of developmental abnormality of a T cell subpopulation. J Exp Med 184:387

61. Takahashi T, Kuniyasu Y, Toda M, Sakaguchi N, Itoh M, Iwata M, Shimizu J, Sakaguchi S (1998) Immunologic self-tolerance maintained by CD25+CD4+ naturally anergic and suppressive T cells: induction of autoimmune disease by breaking their anergic/suppressive state. Int Immunol 10:1969

62. Itoh M, Takahashi T, Sakaguchi N, Kuniyasu Y, Shimizu J, Otsuka F, Sakaguchi S (1999) Thymus and autoimmunity: production of CD25+CD4+ naturally anergic and suppressive T cells as a key function of the thymus in maintaining immunologic self-tolerance. J Immunol 162:5317

63. Thornton AM, Shevach EM (2000) Suppressor effector function of CD4+CD25+ immunoregulatory T cells is antigen nonspecific. J Immunol 164:183

64. McHugh RS, Shevach EM (2002) Cutting edge: depletion of CD4+CD25+ regulatory T cells is necessary, but not sufficient, for induction of organ-specific autoimmune disease. J Immunol 168:5979

65. Zamorano J, Wang HY, Wang R, Shi Y, Longmore GD, Keegan AD (1998) Regulation of cell growth by IL-2: role of STAT5 in protection from apoptosis but not in cell cycle progression. J Immunol 160:3502

66. Lord JD, McIntosh BC, Greenberg PD, Nelson BH (2000) The IL-2 receptor promotes lymphocyte proliferation and induction of the c-myc, bcl-2, and bcl-x genes through the trans-activation domain of Stat5. J Immunol 164:2533

67. Kim HP, Kelly J, Leonard WJ (2001) The basis for IL-2-induced IL-2 receptor alpha chain gene regulation: importance of two widely separated IL-2 response elements. Immunity 15:159

Chapter 5

Bone Marrow Transfer Completely Rescues Hematolymphoid Defects in STAT5A/5B-deficient Mice

Introduction

Normal hematopoietic development is absolutely dependent on external stimuli for fulfilling its varied functions, from the maintenance of a quiescent stem cell pool to the homeostatic production of the mature cells of all the blood cell types. These signals are derived from two sources: cells and tissues remote to the site of hematopoietic development, and cells of the microenvironment at the site of hematopoietic development.

Cells that are responsible for providing these signals from both sites are heterogeneous with regards to derivation, function, and signals provided to the hematopoietic progenitor cells. Remote acting cells that produce endocrine-acting molecules in response to local physiologic stimuli are comprised of both hematopoietically derived cells, such as T-cells producing interleukin-3 (IL-3) [1], and non-hematopoietically derived cells, such as kidney cells of the proximal convoluted tubules producing erythropoietin (EPO) [2].

Cells that create the microenvironment found at the sites of primary hematopoiesis are also of hematopoietic and non-hematopoietic origin. The hematopoietic cells include macrophages [3,4], dendritic cells [5], NK cells [6], T-cells [7,8], and feedback regulation, mediated by progenitor cells themselves [9]. The non-hematopoietic cells, which are often termed "stromal cells", include a heterogeneous mix of myo-fibroblasts and endothelial-like cells [10,11]. Current dogma describes the myo-fibroblast cells as a group of mesenchymal cells that include adipogenic cells, chondrogenic cells, osteogenic cells, and the progenitors for these cells, all of which probably contribute to hematopoietic development. It is believed that a single

mesenchymal stem cell (MSC) gives rise to cells of all these lineages in a developmental hierarchy akin to the hematopoietic system. However, interrelationships between these cells remain unclear [12-14]. The endothelial like cells are derived from a common precursor with hematopoietic cells, the hemangioblast, which may or may not remain in adult bone marrow after fetal development [15,16].

Stromal cells have largely been characterized *in vitro* due to their rarity and they are in part defined by their ability to support some aspects of hematopoietic development in culture. This work, in conjunction with histological data and transplant studies, has identified heterogeneity with respect to support characteristics among stromal cells and spatial differences within the bone marrow, supporting the concept that specialized niches exist to help direct hematopoiesis. This hypothesis had been introduced by the work of N. S. Wolf and J. J. Trentin, establishing that microenvironment could play a major role in the regulation of hematopoietic cell differentiation [17]. The differences in hematopoietic progenitor cell distribution observed within the marrow, with lineage-specific microenvironments and a gradient of maturation from the most primitive cells near the endosteum to the mature cells near the central sinus (reviewed in [18]), have also supported these concepts.

Stromal cells are thought to perform their role through a combination of the production of ECM components, both soluble and membrane bound growth factors, and membrane associated adhesion molecules. However, the further characterization of these cells and the applicability of *in vitro* data to *in vivo* function has been hampered by the absence, until recently, of the ability for prospective isolation of pure populations and the

technical difficulty of transplantation experiments with cells that do not readily engraft or repopulate [10,11].

However, the importance of stromal cells in hematopoietic development is undisputed. The classic example *in vivo* is the work of E. A. McCulloch and colleagues, exploring the sl/sl naturally occurring mouse mutation. This case helped to define both the relationship between hematopoietic cells and their stromal environment and the methodology to test stromal effect *in vivo*. In the sl/sl mouse, there is a mutation that results in hematopoietic defects that are not rescued [19] by transplant of wild-type marrow, demonstrating that the defect is not intrinsic to hematopoietic cells [20]. The defect was later characterized as a mutation in the stem cell factor (SCF) gene [21], the protein product of which provides critical survival signals for hematopoietic stem and progenitor cells. In this case, non-hematopoietic stroma inefficiently provides important growth signals to hematopoietic progenitors. The reciprocal mutation in the w/w mouse [19], later found to affect the gene for the receptor of SCF, c-kit, leads to a defect intrinsic to hematopoietic cells [22]. These two cases laid the foundation for the "seed and soil" concept in both clinical and experimental hematology [23]. In addition, the sl/sl case demonstrated that reduction in the number or function of stromal cells in the bone marrow microenvironment adversely affect hematopoietic development and that reciprocal transplants are critical for establishing the contribution by hematopoietic and non-hematopoietic cells to the hematopoietic defects observed in mouse models, especially in the context of germ-line deletion of a gene.

In STAT5A/5B-deficient mice, there are broad hematopoietic defects affecting multiple lineages. Due to the ubiquitous activation of STAT5 in various tissues in the

mouse it appeared likely that defects in non-hematopoietic tissue might influence the hematopoietic phenotype in these mice. Specifically, STAT is activated by multiple receptors, such as those for EGF and PDGF [24-31], that have been shown to act on both the mesenchymal cells and the endothelial cells thought to be important in supporting hematopoietic development at the site of primary hematopoiesis. In addition, effects on hematopoiesis indirectly produced by loss of STAT5 in tissues more distant from the site of hematopoietic development, such as the liver [32,33], could not be ruled out.

In the following study, the effects of the loss of STAT5 in non-hematopoietically derived elements on hematopoietic development are assessed by transplanting wild-type bone marrow into mice deficient for STAT5A/5B. At high doses of transferred marrow, engraftment, repopulation, and steady state hematopoiesis is identical between STAT5A/5B$^{+/+}$ and STAT5A/5B$^{-/-}$ recipients. This result indicates that loss of STAT5 in non-hematopoietic tissue does not adversely affect steady state hematopoiesis.

This study provides evidence that the role of STAT5 in hematolymphoid development is confined to effects that are cell autonomous to hematopoietic cells. These data provide the first evidence that the defects in non-hematopoietic organs and tissues in STAT5-deficient mice do not adversely affect hematolymphoid development. In addition these results show that STAT5 is dispensable for the biology of bone marrow stroma cells, at least with regards to their ability to support hematolymphoid development.

References

1. Niemeyer CM, Sieff CA, Mathey-Prevot B, Wimperis JZ, Bierer BE, Clark SC, Nathan DG (1989) Expression of human interleukin-3 (multi-CSF) is restricted to human lymphocytes and T-cell tumor lines. Blood 73:945

2. Jacobson LO, Goldwasser E, Fried W, Pizak L (1957) The role of the kidney in the production of erythropoietin. Trans Assoc Am Physicians 70:305

3. Sadahira Y, Mori M (1999) Role of the macrophage in erythropoiesis. Pathol Int 49:841

4. Rich IN (1986) A role for the macrophage in normal hemopoiesis. I. Functional capacity of bone-marrow-derived macrophages to release hemopoietic growth factors. Exp Hematol 14:738

5. Wang Q, Zhang W, Ding G, Sun L, Chen G, Cao X (2001) Dendritic cells support hematopoiesis of bone marrow cells. Transplantation 72:891

6. Seaman WE (2000) Natural killer cells and natural killer T cells. Arthritis Rheum 43:1204

7. Pantel K, Nakeff A (1993) The role of lymphoid cells in hematopoietic regulation. Exp Hematol 21:738

8. Broxmeyer HE, Bruns HA, Zhang S, Cooper S, Hangoc G, McKenzie AN, Dent AL, Schindler U, Naeger LK, Hoey T, Kaplan MH (2002) Th1 cells regulate hematopoietic progenitor cell homeostasis by production of oncostatin M. Immunity 16:815

9. Janowska-Wieczorek A, Majka M, Ratajczak J, Ratajczak MZ (2001) Autocrine/paracrine mechanisms in human hematopoiesis. Stem Cells 19:99

10. Nardi NB, Alfonso ZZ (1999) The hematopoietic stroma. Braz J Med Biol Res 32:601

11. Deryugina EI, Muller-Sieburg CE (1993) Stromal cells in long-term cultures: keys to the elucidation of hematopoietic development? Crit Rev Immunol 13:115

12. Bianco P, Riminucci M, Kuznetsov S, Robey PG (1999) Multipotential cells in the bone marrow stroma: regulation in the context of organ physiology. Crit Rev Eukaryot Gene Expr 9:159

13. Bianco P, Gehron Robey P (2000) Marrow stromal stem cells. J Clin Invest 105:1663

14. Bianco P, Riminucci M, Gronthos S, Robey PG (2001) Bone marrow stromal stem cells: nature, biology, and potential applications. Stem Cells 19:180

15. Orkin SH, Zon LI (2002) Hematopoiesis and stem cells: plasticity versus developmental heterogeneity. Nat Immunol 3:323

16. Pelosi E, Valtieri M, Coppola S, Botta R, Gabbianelli M, Lulli V, Marziali G, Masella B, Muller R, Sgadari C, Testa U, Bonanno G, Peschle C (2002) Identification of the hemangioblast in postnatal life. Blood 100:3203

17. Wolf NSaT, J. J. (1967) Hematopoietic Colony Studies; V. Effects of Hematopoietic Stroma on Differentiation of Pluripotent Stem Cells. J. Exp. Med. 127:205

18. Nilsson SK, Johnston HM, Coverdale JA (2001) Spatial localization of transplanted hemopoietic stem cells: inferences for the localization of stem cell niches. Blood 97:2293

19. McCulloch EA, Siminovitch L, Till JE (1964) Spleen Colony Formation in Anemic Mice of Genotype WW. Science 144:844

20. McCulloch EA, Siminovitch L, Till JE, Russell ES, Bernstein SE (1965) The cellular basis of the genetically determined hemopoietic defect in anemic mice of genotype Sl-Sld. Blood 26:399

21. Galli SJ, Zsebo KM, Geissler EN (1994) The kit ligand, stem cell factor. Adv Immunol 55:1

22. Bernstein A, Chabot B, Dubreuil P, Reith A, Nocka K, Majumder S, Ray P, Besmer P (1990) The mouse W/c-kit locus. Ciba Found Symp 148:158

23. (1977) Aplastic anaemia: Seed or soil? Lancet 2:748

24. Gronthos S, Simmons PJ (1995) The growth factor requirements of STRO-1-positive human bone marrow stromal precursors under serum-deprived conditions in vitro. Blood 85:929

25. Kuznetsov SA, Friedenstein AJ, Robey PG (1997) Factors required for bone marrow stromal fibroblast colony formation in vitro. Br J Haematol 97:561

26. Satomura K, Derubeis AR, Fedarko NS, Ibaraki-O'Connor K, Kuznetsov SA, Rowe DW, Young MF, Gehron Robey P (1998) Receptor tyrosine kinase expression in human bone marrow stromal cells. J Cell Physiol 177:426

27. Paukku K, Valgeirsdottir S, Saharinen P, Bergman M, Heldin CH, Silvennoinen O (2000) Platelet-derived growth factor (PDGF)-induced activation of signal transducer

and activator of transcription (Stat) 5 is mediated by PDGF beta-receptor and is not dependent on c-src, fyn, jak1 or jak2 kinases. Biochem J 345 Pt 3:759

28. Gallego MI, Binart N, Robinson GW, Okagaki R, Coschigano KT, Perry J, Kopchick JJ, Oka T, Kelly PA, Hennighausen L (2001) Prolactin, growth hormone, and epidermal growth factor activate Stat5 in different compartments of mammary tissue and exert different and overlapping developmental effects. Dev Biol 229:163

29. Valgeirsdottir S, Paukku K, Silvennoinen O, Heldin CH, Claesson-Welsh L (1998) Activation of Stat5 by platelet-derived growth factor (PDGF) is dependent on phosphorylation sites in PDGF beta-receptor juxtamembrane and kinase insert domains. Oncogene 16:505

30. Kloth MT, Catling AD, Silva CM (2002) Novel activation of STAT5b in response to epidermal growth factor. J Biol Chem 277:8693

31. Guren TK, Abrahamsen H, Thoresen GH, Babaie E, Berg T, Christoffersen T (1999) EGF-induced activation of Stat1, Stat3, and Stat5b is unrelated to the stimulation of DNA synthesis in cultured hepatocytes. Biochem Biophys Res Commun 258:565

32. Teglund S, McKay C, Schuetz E, van Deursen JM, Stravopodis D, Wang D, Brown M, Bodner S, Grosveld G, Ihle JN (1998) Stat5a and Stat5b proteins have essential and nonessential, or redundant, roles in cytokine responses. Cell 93:841

33. Udy GB, Towers RP, Snell RG, Wilkins RJ, Park SH, Ram PA, Waxman DJ, Davey HW (1997) Requirement of STAT5b for sexual dimorphism of body growth rates and liver gene expression. Proc. Natl. Acad. Sci. USA 94:7239

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Bone Marrow Transfer Completely Rescues Hematolymphoid Defects in STAT5A/5B-deficient Mice

Jonathan W. Snow, B.A.[1,2], Ninan Abraham, Ph.D.[1], Melissa C. Ma, B.S.[1], and Mark A. Goldsmith, M.D., Ph.D.[1,3]

[1]*Gladstone Institute of Virology and Immunology, Departments of* [2]*Microbiology and Immunology, and* [4]*Medicine, University of California at San Francisco, San Francisco, CA 94114-9100, USA.*

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To whom correspondence should be addressed:

Gladstone Institute of Virology & Immunology

P.O. Box 419100, San Francisco, CA 94141-9100

Phone: 415.695.3775 Fax: 415.695.1364

Email: mgoldsmith@gladstone.ucsf.edu

Abstract

Objective: STAT5A/5B-deficient mice exhibit defects in multiple cell types and tissues. In particular, the hematopoietic defects in these mice are widespread, affecting multiple lineages and multiple stages of development. Previous studies indicate that deficiencies intrinsic to hematopoietic cells contribute substantially to the observed defects. However, in light of the broad effects of STAT5 in the context of the organism, we sought to investigate the possibility of STAT5-dependent environmental influence of non-hematopoietic origin on hematopoietic development in these mice.

Material and Methods: We transplanted wild-type bone marrow into STAT5A/5B-deficient mice to determine the effects of loss of STAT5 in non-hematopoietic tissue on hematopoietic development.

Results: We observed that transplant of wild-type marrow completely corrects hematopoietic defects in STAT5A/5B-deficient recipient mice, including peripheral blood counts, bone marrow cellularity, and reductions in specific progenitor subsets.

Conclusion: These results indicate that loss of STAT5 in tissues of non-hematopoietic origin does not adversely affect hematolymphoid development at steady state in these mice.

Introduction

We and others have reported multiple hematopoietic defects in mice deficient for both isoforms of STAT5, STAT5A and STAT5B [1-4,5 1,6]. Results have demonstrated that STAT5A/5B-deficient mice manifest a cell-autonomous abnormality in bone marrow progenitor cells [1,6,7]. However, we observed high variability in the severity of hematopoietic phenotypes among STAT5A/5B-deficient mice. In addition, bone marrow transfer (BMT) of STAT5A/5B-deficient bone marrow into wild-type recipients revealed that although hematologic abnormalities could largely be transferred with bone marrow cells, recipients expressed these defects to a lesser degree ([1] and data not shown). Since STAT5 is triggered by a diverse array of cytokine receptors, we hypothesized that defects, which are non-cell-autonomous to early progenitors, might contribute to the complex, and variably expressed, hematologic defects in these mice. Subsequently, we demonstrated a role for STAT5 in the maintenance of tolerance in mice through survival effects on regulatory T-cells. Loss of tolerance in the absence of STAT5 leads to immune mediated effects at multiple levels of hematolymphoid development (Chapter 4). Crossing STAT5A/5B-deficiency onto a $RAG1^{-/-}$ background did not alleviate all of the hematopoietic defects in these mice (Chapter 4) leaving open the possibility that defects in non-hematopoietic cells might play a role in addition to the cell-autonomous defects previously described.

However, no attempts have been made to date to address the possibility that the absence of STAT5 in non-hematopoietic tissue could indirectly contribute to the hematopoietic defects observed in STAT5A/5B-deficient mice. There is published evidence for a role for STAT5 in multiple tissues that could indirectly affect

hematolymphoid development. First, stromal cells in the bone marrow play an indispensable role in the support and fine regulation of hematopoiesis. This heterogeneous population of cells that reside in the bone marrow provides support to hematopoietic cells in the form of soluble factors such as cytokines, extracellular matrix components, and cell-to-cell interactions [8,9]. Defects in the number or function of stromal cells has been shown to adversely affect hematopoiesis in multiple mouse models, including the classic example of anemia in the sl/sl naturally occurring mouse mutation [10]. The maintenance and function of stromal elements in the bone marrow are tightly controlled by mechanisms similar to those by hematopoietic cells, including cytokines such as granulocyte / macrophage-colony stimulating factor (GM-CSF) [11], interleukin-3 (IL-3) [11], epidermal growth factor (EGF) [12-14], platelet-derived growth factor (PDGF) [12-14], and erythropoietin (EPO) [15,16]. STAT5 is known to be activated by these cytokines, including PDGF [17-19], EGF [18,20,21], IL-3 and GM-CSF [22,23], and EPO [24,25]. We hypothesized that partial loss of signaling by these receptors in stromal cells might reduce their ability to provide adequate and appropriate support for hematolymphoid development *in vivo*.

Second, multiple tissues not considered to provide direct support to hematopoietic cells, but with potential physiologic effects on hematolymphoid development are affected by loss of STAT5A/5B, including liver [2,26], bone [27], and adipose tissue [28]. In addition, growth hormone (GH) signaling is severely impaired in STAT5A/5B-deficient mice as is production of its mediator, insulin-like growth factor (IGF-1) [2]. There is some evidence that the pituitary axis provides protection from stress to the hematopoietic system among others via these molecules, through effects on non-hematopoietic tissue

[29-31]. Some or all of these defects may contribute indirectly to the hematopoietic defects observed in these mice.

To address the possibility that defects in tissues of non-hematopoietic origin could compound known cell-autonomous defects of hematopoietic progenitors to generate the phenotypes observed in STAT5A/5B-deficient mice, we utilized BMT studies, thereby creating a model of loss of STAT5 specifically in non-hematopoietic tissue. Bone marrow transplantation has been used extensively to explore the tissue specificity of hematopoietic defects [32-35] in the past.

Methods

Handling and Characterization of Mice. STAT5A/5B$^{-/-}$ mice [2] were obtained from Dr. James Ihle (St. Jude Children's Research Hospital, Memphis, TN). RAG1$^{-/-}$ mice on a C57Bl/6 background were purchased from Jackson Laboratories (Bar Harbor, ME). Mice were housed in a pathogen-free rodent barrier facility and received mouse chow and acidified water *ad libitum*. All studies were performed on 8–10 week mice unless otherwise specified, and littermates were always used as wild-type controls. Peripheral blood was obtained via cardiac puncture or axial artery, and EDTA-treated samples were used for complete blood counts (IDEXX Veterinary Service, Sacramento, CA). Bone marrow was harvested by flushing femurs and tibias into 6ml phosphate buffered saline (PBS) containing 2% fetal bovine serum (FBS), and cell counts were determined after ACK (NH_4Cl) lysis by trypan blue exclusion.

Flow Cytometry. All antibodies were obtained from Pharmingen (San Diego, CA), and the following clones were used; Sca-1 (E13-161.7), Ly-76 (Ter-119), Gr-1 (RB6-8C5), CD3 (145-2C11), CD117/c-kit (2B8), B220 (RA3-6B2), CD11b (M1/70). For blocking non-specific binding to Fc receptors, purified antibody to CD16/CD32 (2.4G2) was used in at 1:100 for 3 minutes. Subsequently, antibodies to surface markers of interest were used at 1:60 dilution.

Bone Marrow Transplant Studies. Donor mice were 6-10 week old C57Bl/6 or congenic B6.SJL-PtrcaPep3b/BoyJ (B6/BoyJ) obtained from Jackson Laboratories (Bar Harbor, ME). Recipients were γ-irradiated from a Cesium source in two 450 rad doses

4–5 hrs apart. For steady state studies, $1x10^6$ whole bone marrow cells were transferred into STAT5A/5B$^{+/+}$(RAG1$^{-/-}$) mice or STAT5A/5B$^{-/-}$(RAG1$^{-/-}$) and hematologic parameters were assessed at 8 weeks post-transfer. Transplanted mice were maintained on 2.5 mg/ 100 ml Sulfatrim Pediatric Suspension (Alpharma, Baltimore, MD) in the drinking water.

Results

White blood cells in the peripheral blood of recipients are donor derived.

In order to assess the effect of loss of STAT5A/5B in non-hematopoietic tissue on hematolymphoid development, we performed BMT of bone marrow cells from wild-type donors into STAT5A/5B$^{+/+}$ and STAT5A/5B$^{-/-}$ mice. We performed these transfers on a RAG1-deficient background to remove effects of T-cells remaining after irradiation, in light of our findings of autoreactive T-cells in STAT5A/5B-deficient mice (Chapter 4). To assess the derivation of leukocytes in the peripheral blood of recipient mice, we transplanted bone marrow from B6/BoyJ congenic mice, expressing the CD45.1 allele, in one set of experiments, and assessed expression of this donor-derived allele on cells of various peripheral blood lineages. In the representative plot displayed, CD45.1 is expressed on the majority of all lymphocytes (Fig. 1A) and of all myeloid cells (Fig. 1B) in both STAT5A/5B$^{+/+}$(RAG1$^{-/-}$) and STAT5A/5B$^{-/-}$(RAG1$^{-/-}$) mice at 8 weeks post transplant, indicating that all hematolymphoid development was donor derived in these mice.

Reduced peripheral blood counts in STAT5A/5B-deficiency can be completely rescued by bone marrow transfer of wild-type cells.

At 8 weeks post transfer, we characterized the peripheral blood compartment of recipient mice, and found no significant alterations of peripheral blood counts in any blood lineages examined. At 8 weeks post-transfer, STAT5A/5B$^{-/-}$(RAG1$^{-/-}$) recipients, receiving BMT from wild-type donors, exhibited equivalent numbers of erythrocytes in peripheral blood compared with STAT5A/5B$^{+/+}$(RAG1$^{-/-}$) littermate control recipients

(Fig. 2A). Platelets were also equivalent in these mice (Fig. 2B). No decrease in lymphocyte number in peripheral blood was observed (Fig. 2C); neither T-cells nor B-cells were decreased in the STAT5A/5B$^{-/-}$(RAG1$^{-/-}$) recipients (data not shown). Finally, no significant difference in peripheral neutrophil counts was detected (Fig. 2D). BMT of wild-type bone marrow cells thus can restore normal hematolymphoid development in STAT5A/5B-deficient mice. In addition, the absence of STAT5A/5B in non-hematopoietic tissue evidently does not contribute to abnormalities in any blood cell lineages observed in STAT5A/5B-deficient mice.

STAT5A/5B$^{-/-}$(RAG1$^{-/-}$) mice receiving wild-type bone marrow cells have normal bone marrow cellularity and normal progenitor subsets.

Gross examination of bone marrow from STAT5A/5B$^{-/-}$(Rag1$^{-/-}$) recipients receiving wild-type BMT revealed equivalent cellularity compared with STAT5A/5B$^{+/+}$(RAG1$^{-/-}$) recipients (Fig. 3A). The absence of a clear decrease in total nucleated cells in the bone marrow indicates that the reduction in total nucleated cells observed in the bone marrow of STAT5A/5B-deficient mice [1,6] can be explained primarily by defects in hematopoietic progenitor cells and their progeny. We used FACS analysis to subset bone marrow cells by expression of canonical surface lineage-defining markers (lin), Sca-1, and c-kit. The hematopoietic stem cells (HSC), which are defined as cells that have both the capacity for self-renewal and the ability to reconstitute the multilineage hematopoietic system, are found within the lin^{dim}Sca-1^{+}c-kit^{+} fraction [36]. This population was unchanged in absolute terms in STAT5A/5B$^{-/-}$(RAG1$^{-/-}$) recipients compared to STAT5A/5B$^{+/+}$(RAG$^{-/-}$) recipients (Fig. 4A). The lin^{dim}Sca-1^{-}c-

kit$^+$population, containing common lymphoid progenitors (CLP),[37] common myeloid progenitors (CMP),[38] and oligopotent progenitors, was also similar in absolute terms in STAT5A/5B$^{-/-}$(RAG1$^{-/-}$) recipients compared to STAT5A/5B$^{+/+}$(RAG1$^{-/-}$) recipients (Fig. 4B). In addition, we observed no difference in the number of progenitors for B-cells (B220$^+$) (Fig. 4A), erythrocytes (Ter119$^+$) (Fig. 4B), or neutrophils (Gr-1^{int}) (Fig. 4C). Although there was a trend of decreased numbers of mature neutrophils (Gr-1^{hi}) in the bone marrow of STAT5A/5B$^{-/-}$(RAG1$^{-/-}$) mice (6.38 ± 1.25 x 10^6) compared to STAT5A/5B$^{+/+}$(RAG1$^{-/-}$) mice (10.3 ± 3.04 x 10^6), this did not turn out to be significant. Finally, there was no difference in the number of mature macrophages (Mac1$^+$/Gr-1$^-$) (STAT5A/5B$^{+/+}$(RAG1$^{-/-}$) = 1.04 ± 0.157 x 10^6, STAT5A/5B$^{-/-}$(RAG1$^{-/-}$) = 1.31 ± 0.075 x 10^6) in the bone marrow. Therefore, transfer of wild-type bone marrow into STAT5A/5B$^{-/-}$(RAG1$^{-/-}$) largely rescued hematopoietic defects observed in STAT5A/5B-deficient mice. Furthermore, these data suggest that the absence of STAT5A/5B in non-hematopoietic tissue does not affect steady state hematolymphoid development.

Discussion

As firmly established by the classic paper by E. A. McCulloch and colleagues examining the cellular basis of the sl/sl defect in hematopoiesis, non-hematopoietic cells are indispensable for contributing to the hematopoietic niche in the bone marrow and for normal hematolymphoid development [10]. In order to determine the role of loss of STAT5A/5B in non-hematopoietic cells on the defects in hematolymphoid development observed in STAT5A/5B-deficient mice, we performed BMT to generate mice with specific loss of STAT5 in non-hematopoietic tissues. After BMT, we observed normal peripheral blood values, as well as rescue of bone marrow cellularity and progenitor subset abnormalities that were previously observed in STAT5A/5B-deficient mice. Thus, STAT5A/5B appears largely dispensable in non-hematopoietic tissues for normal hematolymphoid development.

These findings indicate that the any potential defects in stromal elements in the marrow microenvironment due to the loss of STAT5 are not severe enough to affect their ability to support hematopoiesis at steady-state. The implications of these results are: (1) that the cytokines thought to be important for stromal growth and function *in vitro* may not be relevant for regulating these populations *in vivo*, and / or (2), that STAT5 is dispensable for signaling by these cytokines through their cognate receptors in the setting of stromal cells. Perhaps other signaling components downstream of these receptors are responsible for the effects documented by them *in vitro* or there is redundancy with other signaling molecules *in vivo*.

In addition, known defects in tissues distal to hematopoietic development do not disrupt the function of these tissues in a way that adversely affects hematolymphoid

development. Thus, although the function of the liver, bone, adipose tissue, as well as response by non-hematopoietic tissues to the pituitary axis are known to be disrupted in STAT5A/5B-deficient mice, these defects appear unimportant to the maintenance of normal hematopoietic values in these mice.

Stromal cells have been shown to require the presence of hematopoietic cells for formation of colonies *in vitro* [39]. The studies shown here do not address the role that defects in STAT5A/5B-deficient progenitors could have on stromal cells, which might in turn affect hematopoietic development. In addition, these studies do not address indirect affects of the autoimmunity on hematolymphoid development through immune-mediated damage of non-hematopoietic cells, since we used a $RAG1^{-/-}$ background to avoid this confounding variable. In addition, these studies used high doses of donor bone marrow cells and interrogated hematopoiesis at steady state (i.e. 8 weeks post-transplant), so it is conceivable that limiting doses of bone marrow or examination of these mice under hematopoietic stress conditions, such as 5-FU treatment for progenitor cell depletion, phenylhydrazine treatment for red blood cell depletion, or treatment with monoclonal antibodies for depletion of mature cells of other blood lineages, could illuminate defects not observed in our studies.

It is important to note that although rare donor-derived stromal cells can be found in recipients in both mice and humans, contribution of these cells to hematopoietic development in BMT recipients is thought to be minimal [9,40,41]. These data therefore demonstrate that hematopoietic defects observed in STAT5A/5B-deficient mice can be completely corrected through provision of wild-type bone marrow via BMT. These

results indicate that loss of STAT5 in non-hematopoietic tissue is largely dispensable for normal steady-state hematopoietic development *in vivo*.

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Figure Legends

Figure 1. Complete donor engraftment of peripheral blood lineages in BMT recipient mice. STAT5A/5B$^{+/+}$ (RAG$^{-/-}$) and STAT5A/5B$^{-/-}$ (RAG$^{-/-}$) mice (CD45.2^{+}) were transplanted with wild-type bone marrow from BoyJ mice (CD45.1^{+}) and representative plots of the percent of (A) lymphoid cells (both CD3^{+} and B220^{+} cells) and (B) myeloid cells (both Gr-1^{+} and Mac-1^{+} cells) positive for CD45.1^{+} are shown.

Figure 2. Multilineage cytopenias are rescued in adult STAT5A/5B$^{-/-}$ (RAG$^{-/-}$) mice receiving BMT. Complete blood counts were performed on whole peripheral blood from STAT5A/5B$^{+/+}$ (RAG$^{-/-}$) (n=5) and STAT5A/5B$^{-/-}$ (RAG$^{-/-}$) (n=5) mice transplanted with wild-type bone marrow. (A) Hematocrit ($p = 0.95$). (B) Platelets ($p = 0.65$). (C) Lymphocytes ($p = 0.55$). (D) Neutrophils ($p = 0.48$).

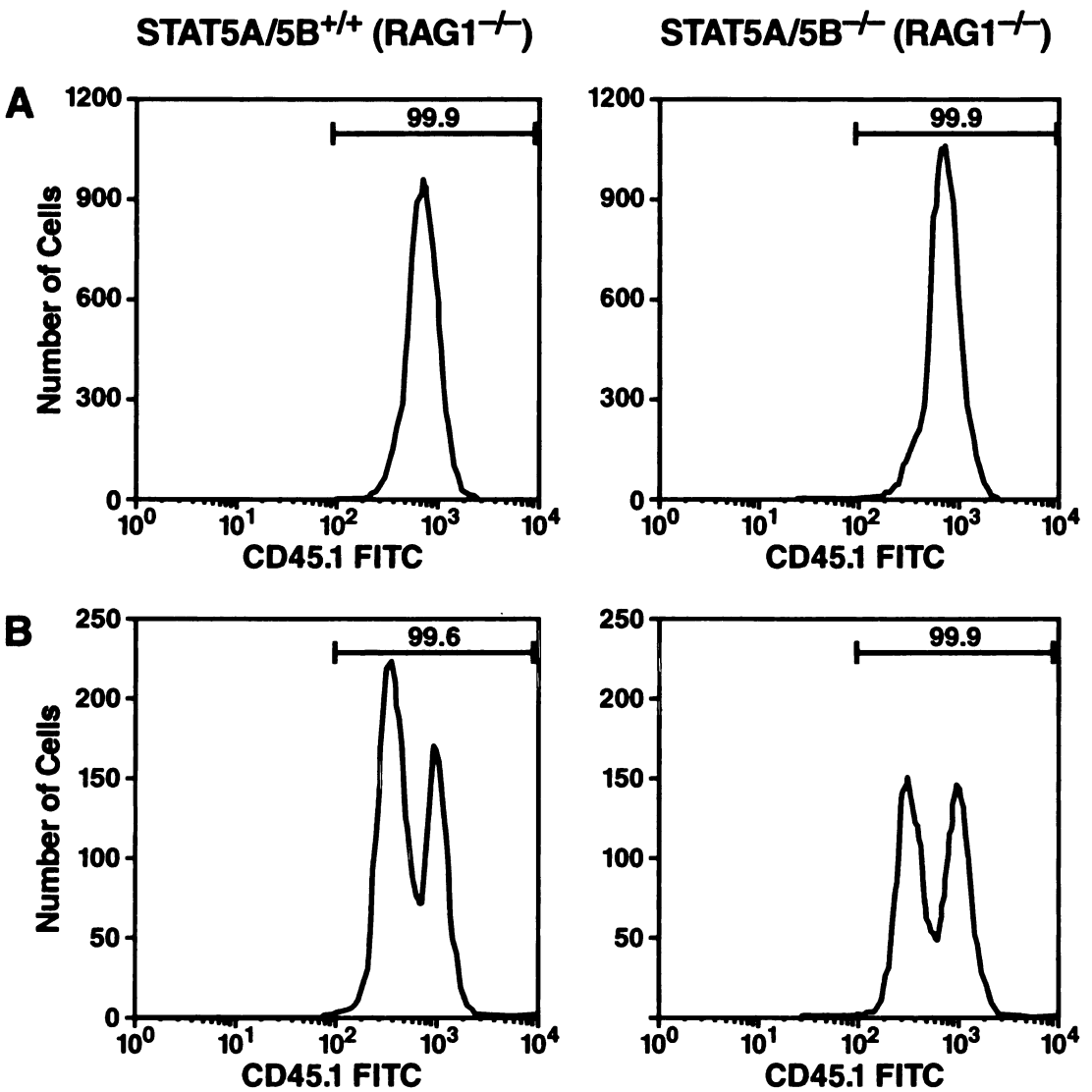
Figure 3. BMT completely rescues total nucleated cells in the bone marrow of STAT5A/5B$^{-/-}$ (RAG$^{-/-}$) mice. Total nucleated cells were obtained from one femur and tibia and cell counts were determined after red blood cell lysis for bone marrow from STAT5A/5B$^{+/+}$ (RAG$^{-/-}$) and STAT5A/5B$^{-/-}$ (RAG$^{-/-}$) mice transplanted with wild-type bone marrow. (STAT5A/5B$^{+/+}$ (RAG$^{-/-}$), n= 5, STAT5A/5B$^{-/-}$ (RAG$^{-/-}$), n=5) ($p = 0.19$).

Figure 4. BMT completely rescues HSC and post-HSC progenitor defects in the bone marrow of STAT5A/5B$^{-/-}$ (RAG$^{-/-}$) mice. Subsetting was performed based on surface expression of lineage-defining markers, Sca-1, and c-kit. Absolute values were generated by multiplying gated percentages by total nucleated cell numbers. The

$Lin^{dim}Sca\text{-}1^{+}/c\text{-}kit^{+}$ compartment, containing HSC, ($p = 0.26$) (A), and the $Lin^{dim}Sca\text{-}1^{neg/lo}/c\text{-}kit^{+}$ compartment, containing post -HSC progenitors, ($p = 0.12$) (B), from $STAT5A/5B^{+/+}$ ($RAG^{-/-}$) and $STAT5A/5B^{-/-}$ ($RAG^{-/-}$) mice transplanted with wild-type bone marrow. ($STAT5A/5B^{+/+}$ ($RAG^{-/-}$), n= 5, $STAT5A/5B^{-/-}$ ($RAG^{-/-}$), n=5).

Figure 5. BMT completely rescues progenitor defects in the bone marrow of $STAT5A/5B^{-/-}$ ($RAG^{-/-}$) mice. Subsetting was performed based on surface expression of lineage-defining markers. Absolute values were generated by multiplying gated percentages by total nucleated cell numbers. B-cell progenitors ($B220^{+}$) ($p = 0.96$), (A), Erythroid progenitors ($p = 0.64$), (B), and neutrophil progenitors ($Gr\text{-}1^{int}$) ($p = 0.21$), (C), from $STAT5A/5B^{+/+}$ ($RAG^{-/-}$) and $STAT5A/5B^{-/-}$ ($RAG^{-/-}$) mice transplanted with wild-type bone marrow are shown. ($STAT5A/5B^{+/+}$ ($RAG^{-/-}$), n= 5, $STAT5A/5B^{-/-}$ ($RAG^{-/-}$), n=5).

Figure 1


STAT5A/5B+/+ (RAG1−/−)
STAT5A/5B−/− (RAG1−/−)
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B
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99.6
99.9
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1200
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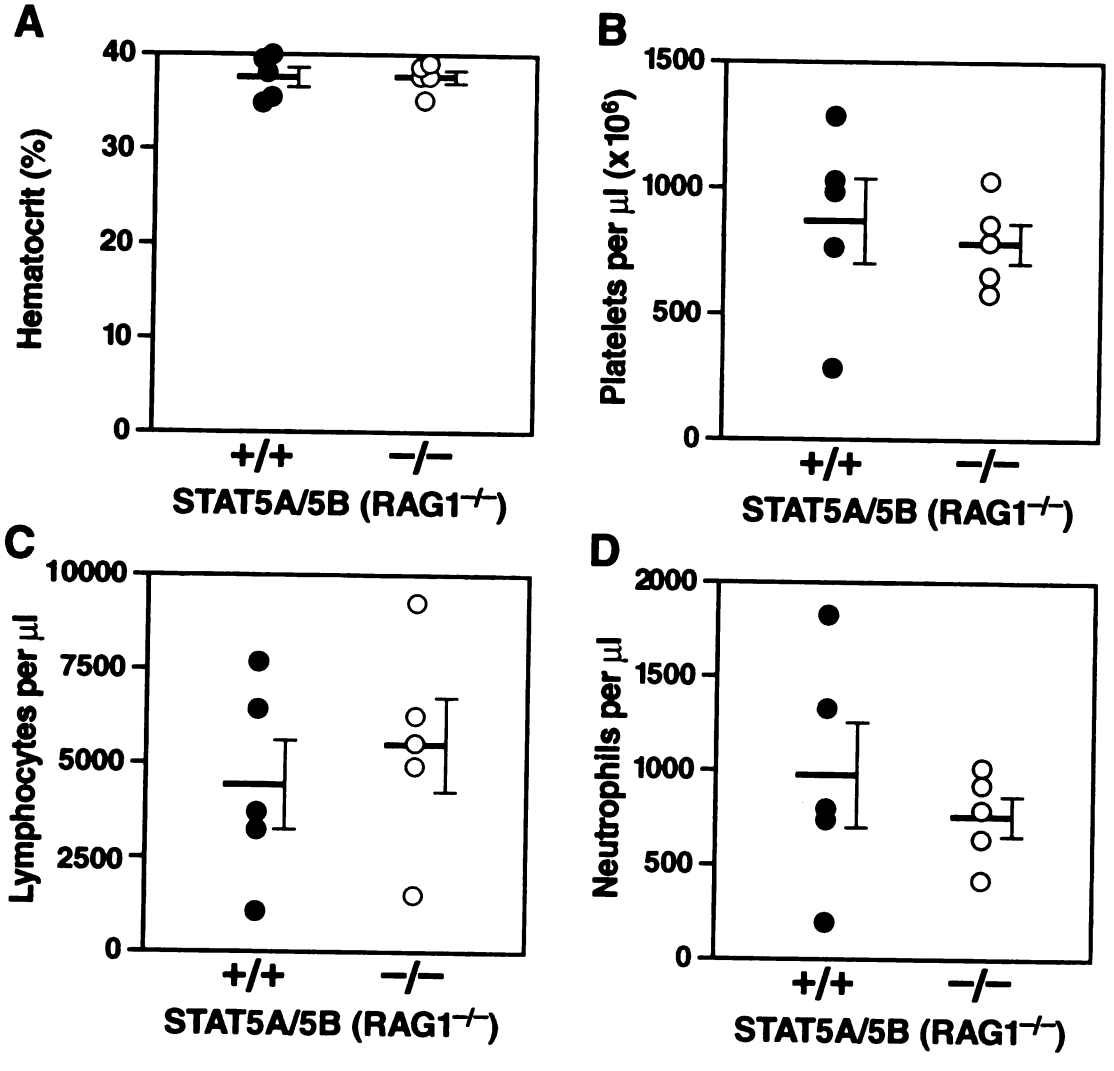
10^1
10^2
10^3
10^4

Figure 2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30
20
10
0
+/+
−/−
STAT5A/5B (RAG1−/−)
B
Platelets per µl (x10⁶)
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1000
500
0
+/+
−/−
STAT5A/5B (RAG1−/−)
C
Lymphocytes per µl
10000
7500
5000
2500
0
+/+
−/−
STAT5A/5B (RAG1−/−)
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1500
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500
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+/+
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STAT5A/5B (RAG1−/−)

Figure 3

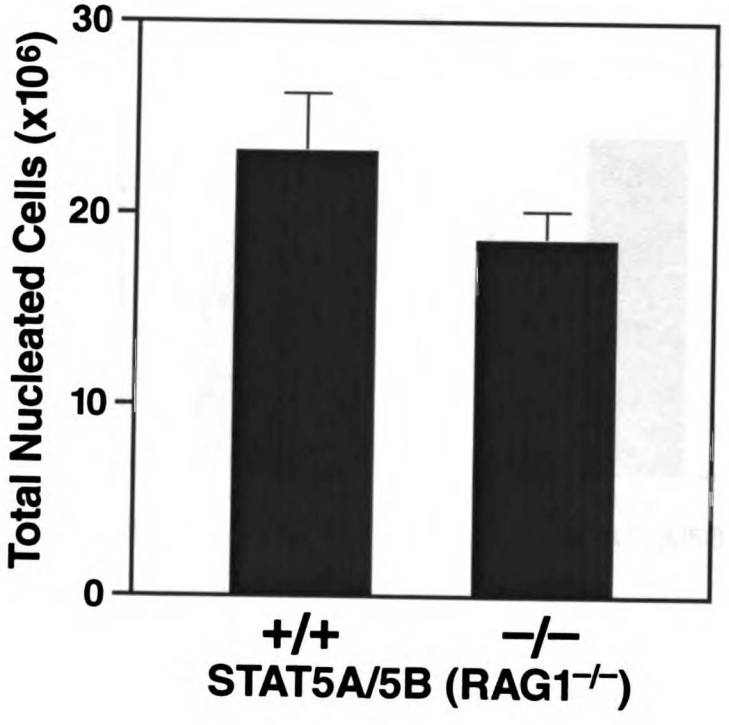
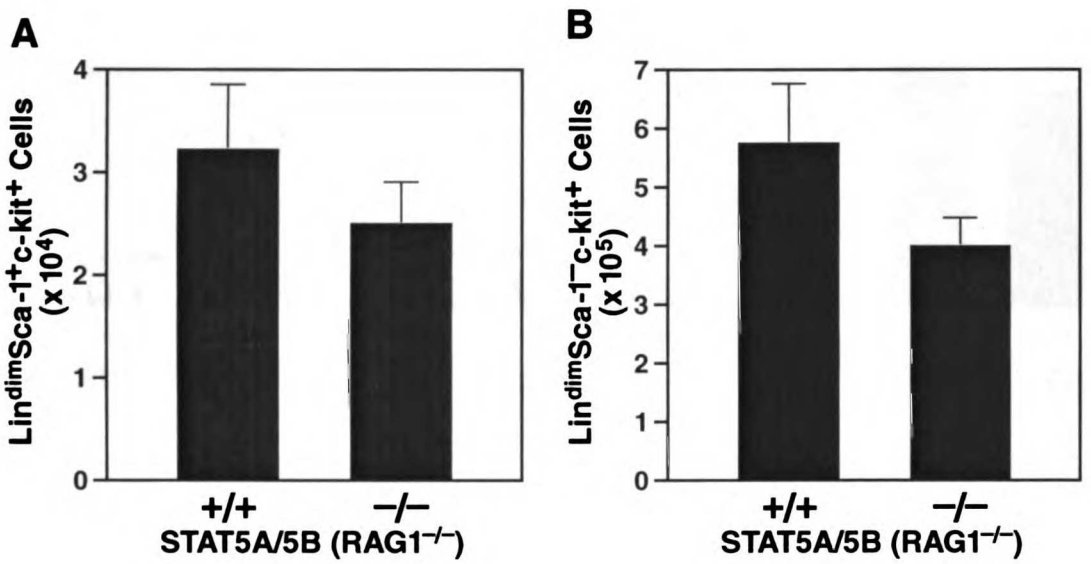


Figure 4


A
B
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Lin^dim Sca-1^+ c-kit^+ Cells (x 10^5)
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+/+
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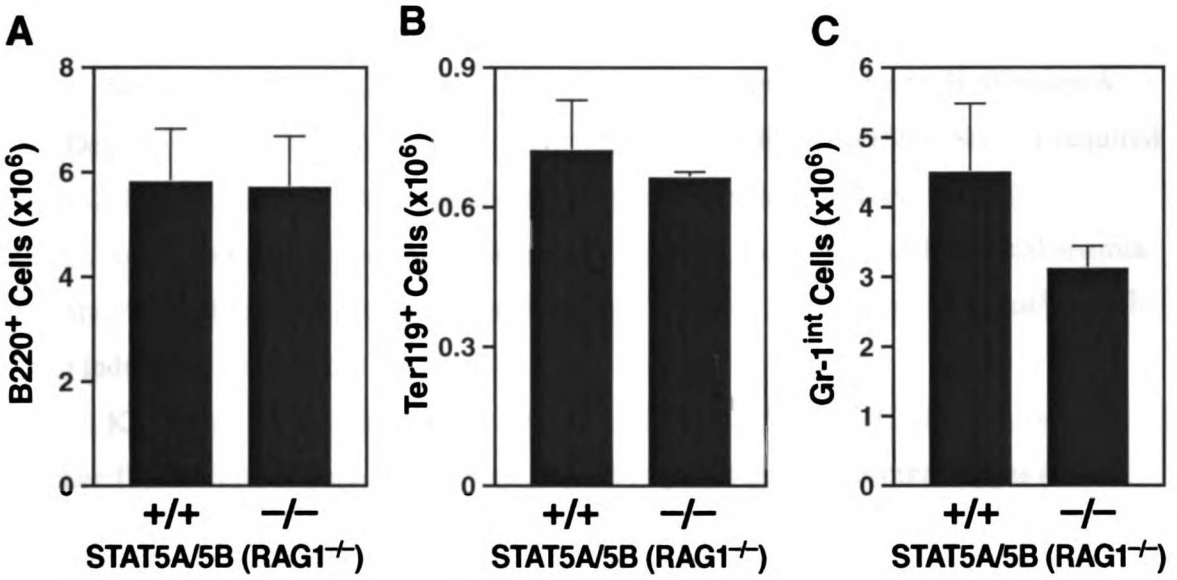
STAT5A/5B (RAG1^−/−)

Figure 5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0
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−/−
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Ter119+ Cells (x10^6)
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0.6
0.3
0
+/+
−/−
STAT5A/5B (RAG1−/−)
C
Gr-1int Cells (x10^6)
6
5
4
3
2
1
0
+/+
−/−
STAT5A/5B (RAG1−/−)

References

1. Bunting KD, Bradley HL, Hawley TS, Moriggl R, Sorrentino BP, Ihle JN (2002) Reduced lymphomyeloid repopulating activity from adult bone marrow and fetal liver of mice lacking expression of STAT5. Blood 99:479

2. Teglund S, McKay C, Schuetz E, van Deursen JM, Stravopodis D, Wang D, Brown M, Bodner S, Grosveld G, Ihle JN (1998) Stat5a and Stat5b proteins have essential and nonessential, or redundant, roles in cytokine responses. Cell 93:841

3. Moriggl R, Topham DJ, Teglund S, Sexl V, McKay C, Wang D, Hoffmeyer A, van Deursen J, Sangster MY, Bunting KD, Grosveld GC, Ihle JN (1999) Stat5 is required for IL-2-induced cell cycle progression of peripheral T cells. Immunity 10:249

4. Socolovsky M, Fallon AE, Wang S, Brugnara C, Lodish HF (1999) Fetal anemia and apoptosis of red cell progenitors in Stat5a-/-5b-/- mice: a direct role for Stat5 in Bcl-X(L) induction. Cell 98:181

5. Kieslinger M, Woldman I, Moriggl R, Hofmann J, Marine JC, Ihle JN, Beug H, Decker T (2000) Antiapoptotic activity of Stat5 required during terminal stages of myeloid differentiation. Genes and Development 14:232

6. Snow JW, Abraham N, Ma MC, Abbey NW, Herndier B, Goldsmith MA (2002) STAT5 promotes multilineage hematolymphoid development in vivo through effects on early hematopoietic progenitor cells. Blood 99:95

7. Bradley HL, Hawley TS, Bunting KD (2002) Cell intrinsic defects in cytokine responsiveness of STAT5-deficient hematopoietic stem cells. Blood

8. Nardi NB, Alfonso ZZ (1999) The hematopoietic stroma. Braz J Med Biol Res 32:601

9. Deryugina EI, Muller-Sieburg CE (1993) Stromal cells in long-term cultures: keys to the elucidation of hematopoietic development? Crit Rev Immunol 13:115

10. McCulloch EA, Siminovitch L, Till JE, Russell ES, Bernstein SE (1965) The cellular basis of the genetically determined hemopoietic defect in anemic mice of genotype Sl-Sld. Blood 26:399

11. Yamada M, Suzu S, Tanaka-Douzono M, Wakimoto N, Hatake K, Hayasawa H, Motoyoshi K (2000) Effect of cytokines on the proliferation/differentiation of stroma-initiating cells. J Cell Physiol 184:351

12. Gronthos S, Simmons PJ (1995) The growth factor requirements of STRO-1-positive human bone marrow stromal precursors under serum-deprived conditions in vitro. Blood 85:929

13. Kuznetsov SA, Friedenstein AJ, Robey PG (1997) Factors required for bone marrow stromal fibroblast colony formation in vitro. Br J Haematol 97:561

14. Satomura K, Derubeis AR, Fedarko NS, Ibaraki-O'Connor K, Kuznetsov SA, Rowe DW, Young MF, Gehron Robey P (1998) Receptor tyrosine kinase expression in human bone marrow stromal cells. J Cell Physiol 177:426

15. Ribatti D, Presta M, Vacca A, Ria R, Giuliani R, Dell'Era P, Nico B, Roncali L, Dammacco F (1999) Human erythropoietin induces a pro-angiogenic phenotype in cultured endothelial cells and stimulates neovascularization in vivo. Blood 93:2627

16. Takahashi T, Kalka C, Masuda H, Chen D, Silver M, Kearney M, Magner M, Isner JM, Asahara T (1999) Ischemia- and cytokine-induced mobilization of bone marrow-derived endothelial progenitor cells for neovascularization. Nat Med 5:434

17. Paukku K, Valgeirsdottir S, Saharinen P, Bergman M, Heldin CH, Silvennoinen O (2000) Platelet-derived growth factor (PDGF)-induced activation of signal transducer and activator of transcription (Stat) 5 is mediated by PDGF beta-receptor and is not dependent on c-src, fyn, jak1 or jak2 kinases. Biochem J 345 Pt 3:759

18. Gallego MI, Binart N, Robinson GW, Okagaki R, Coschigano KT, Perry J, Kopchick JJ, Oka T, Kelly PA, Hennighausen L (2001) Prolactin, growth hormone, and epidermal growth factor activate Stat5 in different compartments of mammary tissue and exert different and overlapping developmental effects. Dev Biol 229:163

19. Valgeirsdottir S, Paukku K, Silvennoinen O, Heldin CH, Claesson-Welsh L (1998) Activation of Stat5 by platelet-derived growth factor (PDGF) is dependent on phosphorylation sites in PDGF beta-receptor juxtamembrane and kinase insert domains. Oncogene 16:505

20. Kloth MT, Catling AD, Silva CM (2002) Novel activation of STAT5b in response to epidermal growth factor. J Biol Chem 277:8693

21. Guren TK, Abrahamsen H, Thoresen GH, Babaie E, Berg T, Christoffersen T (1999) EGF-induced activation of Stat1, Stat3, and Stat5b is unrelated to the stimulation of DNA synthesis in cultured hepatocytes. Biochem Biophys Res Commun 258:565

22. Coffer PJ, Koenderman L, de Groot RP (2000) The role of STATs in myeloid differentiation and leukemia. Oncogene 19:2511

23. Mui AL, Wakao H, Harada N, O'Farrell AM, Miyajima A (1995) Interleukin-3, granulocyte-macrophage colony-stimulating factor, and interleukin-5 transduce signals through two forms of STAT5. J Leukoc Biol 57:799

24. Anagnostou A, Lee ES, Kessimian N, Levinson R, Steiner M (1990) Erythropoietin has a mitogenic and positive chemotactic effect on endothelial cells. Proc Natl Acad Sci U S A 87:5978

25. Gouilleux F, Pallard C, Dusanter-Fourt I, Wakao H, Haldosen LA, Norstedt G, Levy D, Groner B (1995) Prolactin, growth hormone, erythropoietin and granulocyte-macrophage colony stimulating factor induce MGF-Stat5 DNA binding activity. Embo J 14:2005

26. Udy GB, Towers RP, Snell RG, Wilkins RJ, Park SH, Ram PA, Waxman DJ, Davey HW (1997) Requirement of STAT5b for sexual dimorphism of body growth rates and liver gene expression. Proc. Natl. Acad. Sci. USA 94:7239

27. Sims NA, Clement-Lacroix P, Da Ponte F, Bouali Y, Binart N, Moriggl R, Goffin V, Coschigano K, Gaillard-Kelly M, Kopchick J, Baron R, Kelly PA (2000) Bone homeostasis in growth hormone receptor-null mice is restored by IGF-I but independent of Stat5. J Clin Invest 106:1095

28. Fain JN, Ihle JH, Bahouth SW (1999) Stimulation of lipolysis but not of leptin release by growth hormone is abolished in adipose tissue from Stat5a and b knockout mice. Biochem Biophys Res Commun 263:201

29. Welniak LA, Tian ZG, Sun R, Keller JR, Richards S, Ruscetti FW, Murphy WJ (2000) Effects of growth hormone and prolactin on hematopoiesis. Leuk Lymphoma 38:435

30. Dorshkind K, Horseman ND (2001) Anterior pituitary hormones, stress, and immune system homeostasis. Bioessays 23:288

31. Dorshkind K, Horseman ND (2000) The roles of prolactin, growth hormone, insulin-like growth factor-I, and thyroid hormones in lymphocyte development and function: insights from genetic models of hormone and hormone receptor deficiency. Endocr Rev 21:292

32. Harrison DE (1972) Marrow transplantation and iron therapy in mouse hereditary microcytic anemia. Blood 40:893

33. Strom TS, Li X, Cunningham JM, Nienhuis AW (2002) Correction of the murine Wiskott-Aldrich syndrome phenotype by hematopoietic stem cell transplantation. Blood 99:4626

34. Kaminski WE, Lindahl P, Lin NL, Broudy VC, Crosby JR, Hellstrom M, Swolin B, Bowen-Pope DF, Martin PJ, Ross R, Betsholtz C, Raines EW (2001) Basis of hematopoietic defects in platelet-derived growth factor (PDGF)-B and PDGF beta-receptor null mice. Blood 97:1990

35. Simon-Stoos KL, Bloom ML (1999) The hemoglobin-deficit mouse: cure of the anemia following bone marrow transplantation with normal marrow. Exp Hematol 27:162

36. Wagers AJ, Christensen JL, Weissman IL (2002) Cell fate determination from stem cells. Gene Ther 9:606

37. Kondo M, Weissman IL, Akashi K (1997) Identification of clonogenic common lymphoid progenitors in mouse bone marrow. Cell 91:661

38. Akashi K, Traver D, Miyamoto T, Weissman IL (2000) A clonogenic common myeloid progenitor that gives rise to all myeloid lineages. Nature 404:193

39. Friedenstein AJ, Latzinik NV, Gorskaya Yu F, Luria EA, Moskvina IL (1992) Bone marrow stromal colony formation requires stimulation by haemopoietic cells. Bone Miner 18:199

40. Simmons PJ, Przepiorka D, Thomas ED, Torok-Storb B (1987) Host origin of marrow stromal cells following allogeneic bone marrow transplantation. Nature 328:429

41. Awaya N, Rupert K, Bryant E, Torok-Storb B (2002) Failure of adult marrow-derived stem cells to generate marrow stroma after successful hematopoietic stem cell transplantation. Exp Hematol 30:937

Chapter 6

Discussion

Role of STAT5 *in vivo*

Over the course of this thesis project, our understanding of the role of STAT5 in hematolymphoid development *in vivo* has made great progress. First, a cell-autonomous role for STAT5 in the biology of hematopoietic progenitors has been definitively demonstrated. When these studies were undertaken, the importance of STAT5 in mediating the signals of cytokine receptors in the development of hematopoietic cells was thought to be negligible [1]. The first indication that STAT5 was important in mediating cytokine signaling was from work by Socolovsky and colleagues demonstrating its role in erythroid progenitors *in vivo* and *in vitro* [2]. Shortly thereafter, the importance of STAT5 in myeloid progenitors *in vivo* and *in vitro* was demonstrated [3]. However, both of these studies failed to demonstrate defects in steady state production of hematopoietic cells. The studies of our group [4] and of Bunting and colleagues [5] were the first to demonstrate reductions in multiple blood lineages at steady state in the adult. In addition, these two studies were the first to demonstrate defects at the stem and multilineage progenitor cell level, both numerically and functionally. Many of these studies, including our own, implicated STAT5 as primarily mediating its effects through upregulation of pro-survival family members of the Bcl-2 family. However, when combinatorial genetics was used to attempt to demonstrate that ectopically expressed Bcl-2 could complement for the loss of STAT5, we observed that although peripheral blood counts were returned to normal, numerical and functional defects in the bone marrow still prevailed (Chapter 3). These data implied that other biological activities, besides suppression of apoptosis, were dependent on STAT5 acting through molecular targets other than Bcl-2 family members.

It will be important in the future to elucidate the cytokines for which signaling is affected by the loss of STAT5 in hematopoiesis. Mice that have been engineered by knock-in technology to express cytokine receptors selectively unable to activate STAT5 may begin to provide answers to this question. In addition, the biological behaviors involved and the STAT5-dependent transcriptional targets that mediate these activities will also require further study. One potential approach is the use of gene arrays to examine differential regulation of genes at the transcription level. Use of STAT5A/5B compound heterozygotes, which display normal steady state hematopoiesis, but alterations under stress, would avoid differences in gene expression due to alterations in the representation of various populations and the pathology evident in the STAT5A/5B-deficient mice, but would likely not yield all of the relevant target genes.

The range in the severity of the various hematopoietic aspects of the STAT5A/5B-deficient phenotype led us to consider whether effects that were non-cell autonomous for hematopoietic progenitors were impacting hematolymphoid development in these mice. First, mature hematopoietic cells, specifically lymphocytes, participate in the normal regulation of hematopoiesis and can be responsible for perturbations of hematopoiesis in various immunologic disease states. As STAT5 is known to be a critical signaling component of the IL-2 receptor, the importance of STAT5 in transducing the toleragenic signal of IL-2 receptor activation represented an essential question. Structure function analysis of the IL-2Rβ, had previously indicated that regions important for STAT5 activation were dispensable for the toleragenic signal [6]. In addition, initial studies found no evidence of autoimmunity in STAT5A/5B-deficient mice excepting the presence of activated T-cells [7]. Subsequent studies showed a lymphocyte-dependent

extramedullary hematopoiesis [8] and a drop-out of B-cells [9], reminiscent of IL-2-deficient mice [10]. To further address whether a role of STAT5 in tolerance might be affecting hematopoietic development in these mice, we reexamined the hematopoietic compartment with parallel analysis of T-cell subsets, finding a subset of STAT5A/5B-deficient mice displaying T-cell infiltration of the bone marrow that correlated with abnormal skewing of progenitor subsets. In addition, these mice had immune cell infiltrations affecting other organs and premature death that was dependent on the presence of lymphocytes. Thus STAT5 appears to be important for providing a general toleragenic signal. We found that one putative mechanism for providing this signal is through positively regulating cell survival of an IL-2-dependent subset of regulatory T-cells. Further experiments include the use of "add-backs" of regulatory T-cells to attempt to abrogate disease. In addition, our STAT5A/5B-deficient mice with the Bcl-2 transgene could be used to test the hypothesis that suppression of apoptosis in regulatory T-cells is the STAT5-dependent mechanism for their reduction.

Second, non-hematopoietically derived cells are known to provide important signals for the support and regulation of normal hematolymphoid development. The ubiquitous expression of STAT5 and the disruption of multiple tissues in its absence raise the formal possibility that defect in a non-hematopoietic tissue could synergize with defects in hematopoietic progenitors and immune-mediated defects to be responsible for the hematologic phenotypes observed in these mice. We used bone marrow transplant from wild-type donors into STAT5A/5B-deficient mice on a $RAG1^{-/-}$ background to address this possibility and found that BMT largely rescued hematopoietic defects in these mice. Thus, STAT5-deficiency in non-hematopoietic tissue is largely dispensable

for the proper maintenance of hematolymphoid development *in vivo*. This result does not exclude roles for STAT5 in stromal cells and more work will be needed to determine whether this molecule is important for the production, maintenance, and function of these cells *in vivo*. In addition, examination of similar transplant recipients under stress conditions may elucidate defects in hematolymphoid development of non-hematopoietic origin that were not observed in these studies.

Based on its activation by the interleukin-7 (IL-7) receptor, STAT5 was hypothesized to play an important role in normal T-cell development. However, initial characterization of mice deficient in STAT5A/5B detected no differences in thymic development and only alterations in the post-thymic proliferation of T-cells in response to IL-2 [7]. When studies of an IL-7 transgenic model revealed that the overexpression of this cytokine promoted the preferential expansion of CD8 T-cells that appeared be due to an increase in production of these cells in the thymus, STAT5 was considered as a potential effector of this growth factor. In fact, we found that heterozygosity at the STAT5A and 5B loci reduced the CD8 expansion and the increases in Bcl-2 associated with it. Further efforts are required to elucidate whether IL-7 directs the CD8 expansion by increasing survival, altering the lineage commitment signals, by affecting TCR signaling to indirectly impact lineage commitment, or by positively impacting post-commitment $CD8^+$ T-cell development [11].

IL-7 can function as an oncogene in mice, with 100% of mice developing lymphomas by 7 months of age in the IL-7 transgenic model above [12]. STAT5 has been hypothesized to play an extensive role in mediating the signals of oncogenes and has been shown to be important in some mouse models of leukemogenesis [13]. Thus,

STAT5 was considered to be a likely candidate as the signaling molecule responsible for mediating IL-7-dependent tumor promotion in these mice. Indeed, STAT5A and 5B heterozygosity provided a marked delay in the initiation and / or progression of IL-7-dependent lymphomas, indicating that the dose of this signaling effector is limiting for signaling by this cytokine. Haploinsufficiency of signaling mediators in oncogenesis is becoming an important area of research, and has been shown for STAT5A in a model of mammary gland tumors [14]. It will be important to determine how loss of one copy of STAT5A/5B affects the initiation and / or progression of IL-7-induced lymphomas and the target genes responsible.

Physiology of hematopoiesis

One of the key concepts that emerge from this work is the importance of considering whole organism physiology when studying one organ or tissue. This is especially true for hematopoietic cells, which are exquisitely sensitive to pathology in other systems. One mechanism for this sensitivity is the tremendous rate of proliferation and metabolic activity of hematopoietic progenitors as they struggle to fill the physiologic demands of the organism for production of $2.4x10^8$ cells per day in the mouse [15].

Hematopoiesis can be aberrantly affected by multiple types of lesions that indirectly affect progenitors or mature cells as a result of defects in other cells. Erythropoiesis provides an excellent example, as defects in multiple cells and tissues acting in trans on erythroid progenitors and mature cells can cause anemia. These include the inability to produce appropriate stimuli, such as reduction in erythropoietin (EPO)

production by the kidney, improper uptake of various molecules necessary for development, such as iron or folate by the gastrointestinal system, improper storage of various molecules necessary for development by support cells, such as iron stores within the liver, or immune mediated destruction of progenitors and mature cells. Therefore, understanding the defects in erythropoiesis require that contributions resulting from abnormalities in these other tissues must be considered [16].

In addition, the physiology of the organism is such that multiple compensation mechanisms can be utilized to overcome defects in a process such as hematopoietic development. In erythropoiesis, reductions in red blood cells leads to hypoxia, which triggers increased EPO production, which in turn increases the number of erythroid progenitors and erythrocytes produced per progenitor in the bone marrow. In addition, mobilization of erythroid progenitors to sites of extramedullary hematopoiesis can increase the erythropoietic mass of the organism. Finally, decreased clearance of senescent erythrocytes by the reticuloendothelial system can increase the lifespan of erythrocytes to increase number of mature cells. Thus, defects in erythropoiesis may be masked by a state of stress-induced compensation.

This knowledge leads to two important experimental considerations: 1) the importance of using genetic tools, or experimental manipulations, to determine the tissue, stage and ontological specificity of a defect, and 2) the importance of pushing a system with stressors and sensitive assays to uncover important, but subtle functions, in a system with multiple mechanisms for compensation.

Stressors and Sensitive Assays to Demonstrate Subtle Phenotypes

Often disruption of a gene *in vivo* results in a disappointing paucity of phenotype in the physiologic process of interest. Although this can mean that the molecule has never been or is no longer involved in the process in question, two other critical points must be considered when evaluating a subtle phenotype. First, the organism has typically evolved multiple pathways and homeostatic mechanisms to control a given physiologic process. Second, many assays used to interrogate specific physiologic processes are rather blunt and the level of resolution low. Therefore, many molecules that are involved in a specific process are likely to demonstrate rather subtle phenotypes upon disruption. In order to fully understand the status of the physiologic process under investigation in the absence of a molecule, clues of compensation mechanisms must be examined, the most rigorous assays available must be employed, higher resolution assays must be developed, and the organism must be interrogated under stress conditions. In this case sensitive tests, such as the competitive repopulation assay, and the availability of stressors helped to uncover the phenotypes described

Tissue, Stage, and Ontological Specificity of Mouse Models

Use of genetic techniques in a manner that is not temporally or spatially restricted can lead to some caveats with respect to the conclusions drawn. Animals that undergo development with some alteration in the amount or structure of a specific molecule may have adapted in such a way as to mask or alter phenotypes observed. In addition, genetic alterations that affect the whole organism can lead to difficulty in determining the extent of contribution to the phenotypes observed by various tissues.

Use of genetics to remove a specific population or transplantation to isolate specific genetically altered populations can allow for avoiding these difficulties in some cases, as has been done here. However, these cases are limited and ultimately result in imperfect systems, so new genetic strategies to control expression of transgenes or disruption of genes must be employed. New methodologies are now available that that allow for much finer control of these genetic manipulations. These use binary systems, in which the first component is the "effector", under control of a specific spatio-temporal promoter, and the second component the "target", the locus of the gene of interest modified to respond to the "effector". Genes can now be disrupted in a manner under fine control using the Cre and Flp recombinase systems and reversibly turned on and off using a tetracycline sensitive transcription factor [17].

Use of these techniques would be very powerful in future studies of STAT5 *in vivo.* Tissue specific expression of STAT5 transgene on a knock-out background and tissue specific knockouts of this molecule such as has been undertaken with the STAT3 mice [18] would add a higher level of resolution in terms of tissue specificity of various defects observed. For example, selective disruption of STAT5 in various hematopoietic progenitor populations, such as the megakaryocyte-erythroid bipotential progenitor (MEG), the megakaryocyte progenitor (MKp), and the megakaryocyte itself for platelet development, might allow for illumination of which stages of development in a given lineage are most sensitive to STAT5 deletion.

Clinical implications

The implication of growth factors in clinical pathology and their importance for use in treatment of disease imparts significance to work that increases understanding of the molecular mechanisms necessary for transducing receptor activation into biological outcomes. This body of work has firmly established STAT5 as a molecule that is critical for cytokine receptor signaling, downstream of multiple receptors and in regulating multiple biological processes.

Both acquired and congenital situations can result in decreases in the number of mature cells of hematopoietic lineages. These can be divided into disease states related to sequelae of malignancies and the treatment thereof (such as recovery post-stem cell transplant) and cytopenias resulting from other mechanisms (such as an inherited disorder). Both of these classes are often treated with administration of growth factors, such as granulocyte-colony stimulating factor (G-CSF), granulocyte/macrophage-colony stimulating factor (GM-CSF), EPO, thrombopoietin (TPO), and IL-7 [19], all of which activate STAT5. Understanding the role that STAT5 plays in translating the signals of these growth factors is important for understanding the biology of the clinical therapy. In addition improvement of the clinical strategies may involve the selective activation of specific pathways, of which STAT5 may be a good target.

Enhancement of immune responses may be critical for increasing the efficacy of prophylactic strategies to pathogens and tumors, such as vaccines. One mechanism for achieving this goal is through the relaxing of tolerance checkpoints, perhaps through modification of regulatory T-cells [20]. In addition, restriction of immune responses through augmentation of these tolerance-promoting cells could have a critical role in the

clinical control of autoimmune responses or inflammatory conditions that cause pathology [20].

Many hematologic malignancies may be initiated or worsened by aberrant activation of normal or mutated growth factor receptors. Often, STAT5 has been implicated as one downstream signaling pathway of these receptors. Increases in the understanding of the role STAT5 plays in these aberrant growth factor responses and contribution of this molecule to the production and function of normal cells may indicate that STAT5 might be a good target for disrupting signals that are critical for malignant cells without adversely affecting normal development [21].

Conclusion

Cytokine growth factors are indispensable for the maintenance of homeostasis in hematolymphoid development and the function of mature cells. This project has made significant contributions to the understanding of the role of STAT5 as a downstream signaling component of cytokines *in vivo*. Biologically, these results firmly establish STAT5 as a critical effector of cytokines in mediating biological effects at multiple levels of hematolymphoid development and function. In addition, these studies emphasize the necessity of considering the interconnected circuitry of physiology when examining a single organ system or tissue and the importance of utilizing sensitive assays to uncover subtle alterations in a system that has multiple homeostatic mechanisms at its disposal. Thus, this project reinforces the use of genetically modified animal models in elucidating molecular mechanisms of physiology *in vivo*, but demonstrates the need for second and third generation improvements of these techniques. Clinically, these results add to our

understanding of the molecular mechanisms underlying the use of these growth factors in therapeutic applications and identify STAT5 as a potential target for modifying hematolymphoid development, tolerance, and carcinogenesis.

References

1. Teglund S, McKay C, Schuetz E, van Deursen JM, Stravopodis D, Wang D, Brown M, Bodner S, Grosveld G, Ihle JN (1998) Stat5a and Stat5b proteins have essential and nonessential, or redundant, roles in cytokine responses. Cell 93:841

2. Socolovsky M, Fallon AE, Wang S, Brugnara C, Lodish HF (1999) Fetal anemia and apoptosis of red cell progenitors in Stat5a-/-5b-/- mice: a direct role for Stat5 in Bcl-X(L) induction. Cell 98:181

3. Kieslinger M, Woldman I, Moriggl R, Hofmann J, Marine JC, Ihle JN, Beug H, Decker T (2000) Antiapoptotic activity of Stat5 required during terminal stages of myeloid differentiation. Genes and Development 14:232

4. Snow JW, Abraham N, Ma MC, Abbey NW, Herndier B, Goldsmith MA (2002) STAT5 promotes multilineage hematolymphoid development in vivo through effects on early hematopoietic progenitor cells. Blood 99:95

5. Bunting KD, Bradley HL, Hawley TS, Moriggl R, Sorrentino BP, Ihle JN (2002) Reduced lymphomyeloid repopulating activity from adult bone marrow and fetal liver of mice lacking expression of STAT5. Blood 99:479

6. Fujii H, Ogasawara K, Otsuka H, Suzuki M, Yamamura K, Yokochi T, Miyazaki T, Suzuki H, Mak TW, Taki S, Taniguchi T (1998) Functional dissection of the cytoplasmic subregions of the IL-2 receptor betac chain in primary lymphocyte populations. Embo J 17:6551

7. Moriggl R, Topham DJ, Teglund S, Sexl V, McKay C, Wang D, Hoffmeyer A, van Deursen J, Sangster MY, Bunting KD, Grosveld GC, Ihle JN (1999) Stat5 is required for IL-2-induced cell cycle progression of peripheral T cells. Immunity 10:249

8. Zang H, Sato K, Nakajima H, McKay C, Ney PA, Ihle JN (2001) The distal region and receptor tyrosines of the Epo receptor are non-essential for in vivo erythropoiesis. Embo J 20:3156

9. Sexl V, Piekorz R, Moriggl R, Rohrer J, Brown MP, Bunting KD, Rothammer K, Roussel MF, Ihle JN (2000) Stat5a/b contribute to interleukin 7-induced B-cell precursor expansion, but abl- and bcr/abl-induced transformation are independent of stat5. Blood 96:2277

10. Suzuki H, Kündig TM, Furlonger C, Wakeham A, Timms E, Matsuyama T, Schmits R, Simard JJ, Ohashi PS, Griesser H, et al. (1995) Deregulated T cell activation and autoimmunity in mice lacking interleukin-2 receptor beta. Science 268:1472

11. Germain RN (2002) T-cell development and the CD4-CD8 lineage decision. Nat Rev Immunol 2:309

12. Rich BE, Campos TJ, Tepper RI, Moreadith RW, Leder P (1993) Cutaneous lymphoproliferation and lymphomas in interleukin 7 transgenic mice. J Exp Med 177:305

13. Levy DE, Gilliland DG (2000) Divergent roles of STAT1 and STAT5 in malignancy as revealed by gene disruptions in mice. Oncogene 19:2505

14. Ren S, Cai HR, Li M, Furth PA (2002) Loss of Stat5a delays mammary cancer progression in a mouse model. Oncogene 21:4335

15. Domen J (2001) The role of apoptosis in regulating hematopoietic stem cell numbers. Apoptosis 6:239

16. Schnall SF, Berliner N, Duffy TP, Benz Jr. EJ (2000) Chapter 23: Approach to the Adult and Child with Anemia. In: R Hoffman (ed) Hematology: Basic Principles and Practice, 3rd edition. New York: Churchill Livingston, 367

17. Lewandoski M (2001) Conditional control of gene expression in the mouse. Nat Rev Genet 2:743

18. Akira S (2000) Roles of STAT3 defined by tissue-specific gene targeting. Oncogene 19:2607

19. Gordon MS, Sosoman JA (2000) Chapter 51: Clinical Application of Cytokines and Biological Response Modifiers. In: R Hoffman (ed) Hematology: Basic Principles and Practice, 3rd edition. New York: Churchill Livingston, 939

20. Shevach EM (2002) CD4+ CD25+ suppressor T cells: more questions than answers. Nat Rev Immunol 2:389

21. Buettner R, Mora LB, Jove R (2002) Activated STAT signaling in human tumors provides novel molecular targets for therapeutic intervention. Clin Cancer Res 8:945

Appendix I

Interleukin-7 Influences CD4 and CD8 αβ-T-cell Lineage Commitment During Late Thymopoiesis in an Antigen-independent Manner

Introduction

Normal thymopoiesis begins with the seeding of the thymus by common lymphoid progenitors (CLP) and perhaps T-cell restricted progenitors from the bone marrow [1,2]. The α/β thymocyte developmental program begins with the double negative (DN) stages during which NK, dendritic cell, and B-cell potential is lost [1], the T-cell receptor (TCR) β locus is rearranged, and β-selection occurs [3]. Thymocytes with functional TCRβ receptors then rearrange the TCRα locus and proceed to upregulate the co-receptors CD4 and CD8, producing double positive (DP) cells [3]. In this stage, thymocytes whose TCR cannot interact with MHC I or II die by neglect [4], lineage commitment to either a $CD4^+$ or $CD8^+$ single positive (SP) T-cell is determined by positive selection [4], and finally, T-cells with high affinity for self-peptides in the context of MHC are deleted by negative selection [4] or slated to become regulatory T-cells [5].

Lineage commitment, which is critical for producing T-cells whose TCR-MHC specificity match their co-receptor expression (MHC II interacts with CD4 and MHC I interacts with CD8) to generate productive TCR-MHC-co-receptor interactions, is thought to be a accomplished utilizing a combination of instructive and stochastic mechanisms. In the instructive model, different qualitative signals are given by the co-receptor when the correct TCR-MHC interaction occurs, signaling the cell to down regulate the inappropriate co-receptor. In the stochastic or selection model, cells randomly downregulate a co-receptor and those cells that still have a productive TCR-MHC-co-receptor interaction are signaled to survive [6,7].

In addition to signals from TCR-MHC-co-receptor interactions, at least one other molecule, Notch, has been implicated in altering fate decisions in thymocyte development [8]. Although its role is somewhat controversial [9,10], Notch may act by increasing survival of various subsets [11], by actually altering the lineage commitment signals resulting in the downregulation of CD4 expression [8], by affecting TCR signaling to indirectly impact lineage commitment [11], or by positively impacting post-commitment $CD8^+$ T-cell development [12]. *In vitro* data has indicated that IL-7 may play a similar role in lineage commitment decisions in the thymus [13].

In the following study, IL-7, the cytokine that is indispensable for early thymocyte development and T-cell homeostasis in the periphery, is implicated in impacting CD4 / CD8 lineage commitment in a manner analogous to Notch *in vivo*. Transgenic overexpression of IL-7 results in increased CD8 T-cells in the periphery, increased $CD8^+$ SP thymocytes, and increased Bcl2 expression in DP thymocytes. Interestingly, $CD8^+$ T-cells were still produced in the absence of MHC I, indicating that these effects can occur in the absence of the normal positive selection signals. In addition, STAT5, a key mediator of IL-7 signaling *in vitro*, was shown to affect the skewing in $CD8^+$ T-cell production and Bcl-2 expression, implicating it as an important player in this late thymopoietic role of IL-7.

References

1. Rothenberg EV, Dionne CJ (2002) Lineage plasticity and commitment in T-cell development. Immunol Rev 187:96

2. Shortman K, Wu L (1996) Early T lymphocyte progenitors. Annu Rev Immunol 14:29

3. Germain RN (2002) T-cell development and the CD4-CD8 lineage decision. Nat Rev Immunol 2:309

4. von Boehmer H, Teh HS, Kisielow P (1989) The thymus selects the useful, neglects the useless and destroys the harmful. Immunol Today 10:57

5. Shevach EM (2002) CD4+ CD25+ suppressor T cells: more questions than answers. Nat Rev Immunol 2:389

6. Robey EA, Fowlkes BJ, Pardoll DM (1990) Molecular mechanisms for lineage commitment in T cell development. Semin Immunol 2:25

7. Borgulya P, Kishi H, Muller U, Kirberg J, von Boehmer H (1991) Development of the CD4 and CD8 lineage of T cells: instruction versus selection. Embo J 10:913

8. Robey E, Chang D, Itano A, Cado D, Alexander H, Lans D, Weinmaster G, Salmon P (1996) An activated form of Notch influences the choice between CD4 and CD8 T cell lineages. Cell 87:483

9. Yasutomo K, Doyle C, Miele L, Fuchs C, Germain RN (2000) The duration of antigen receptor signalling determines CD4+ versus CD8+ T-cell lineage fate. Nature 404:506

10. Wolfer A, Bakker T, Wilson A, Nicolas M, Ioannidis V, Littman DR, Lee PP, Wilson CB, Held W, MacDonald HR, Radtke F (2001) Inactivation of Notch 1 in immature thymocytes does not perturb CD4 or CD8T cell development. Nat Immunol 2:235

11. Izon DJ, Punt JA, Xu L, Karnell FG, Allman D, Myung PS, Boerth NJ, Pui JC, Koretzky GA, Pear WS (2001) Notch1 regulates maturation of CD4+ and CD8+ thymocytes by modulating TCR signal strength. Immunity 14:253

12. Deftos ML, Huang E, Ojala EW, Forbush KA, Bevan MJ (2000) Notch1 signaling promotes the maturation of CD4 and CD8 SP thymocytes. Immunity 13:73

13. Brugnera E, Bhandoola A, Cibotti R, Yu Q, Guinter TI, Yamashita Y, Sharrow SO, Singer A (2000) Coreceptor reversal in the thymus: signaled CD4+8+ thymocytes initially terminate CD8 transcription even when differentiating into CD8+ T cells. Immunity 13:59

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Interleukin-7 influences CD4 and CD8 αβ-T-cell lineage commitment during late thymopoiesis in an antigen-independent manner[1]

Ninan Abraham[*†], Jonathan W. Snow[*‡], Melissa C. Ma[*], Nigel Killeen[‡], Benjamin E. Rich[§], Mark A. Goldsmith[*¶]

[*]*Gladstone Institute of Virology and Immunology, San Francisco, CA 94141, USA;* [‡]*Department of Microbiology and Immunology, University of California, San Francisco, CA 94141, USA;* [§]*Harvard Skin Disease Research Center, Brigham and Women's Hospital, 77 Avenue Louis Pasteur, Boston, MA 02115, USA; and* [¶]*Department of Medicine, University of California, San Francisco, CA 94141, USA*

Running title: IL-7 influences CD4/CD8 lineage commitment in thymopoiesis

Abbreviations used: TN, triple negative; DP, double positive; SP, single positive; Tg IL-7, transgenic IL-7; WT, wild-type.

[†]To whom correspondence should be addressed:

Gladstone Institute of Virology and Immunology,

P.O. Box 419100, San Francisco, CA 94141-9100, USA

Telephone: (415) 695-3803, fax: (415) 826-1514

Email: nabraham@gladstone.ucsf.edu

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Summary

Interleukin-7 (IL-7) provides survival and proliferation signals essential for T-cell maturation and expansion. Disruption of IL-7 or its receptor causes lymphopenia from a profound block in early thymopoiesis that obscures later events regulated by IL-7. In transgenic mice, overexpression of IL-7 influenced transition from double-positive to single-positive thymocytes. IL-7 induced a polyclonal expansion of mature CD8 SP cells, even in β_2 microglobulin-null mice, that persisted in peripheral blood. Thymocytes undergoing positive selection showed a marked elevation of intracellular Bcl-2 levels. Furthermore, the CD8 SP expansion and induction of Bcl-2 levels were reduced by STAT5A/B haploinsufficiency, suggesting STAT5A/B mediate IL-7 action. IL-7 has a novel antigen-independent influence on lineage commitment of thymocytes during late thymopoiesis *in vivo*.

1 Introduction

In early thymopoiesis, lymphoid progenitors seed the thymus and mature into $CD3^-CD4^-CD8^-$ triple-negative pro-T thymocytes [1]. These progenitors eventually become $CD3^{med}CD4^+CD8^+$ double-positive (DP) immature thymocytes, undergo positive and negative selection and lineage commitment to develop into $CD3^{hi}CD4^+$ single-positive (SP) or $CD3^{hi}CD8^+$ SP cells.

IL-7 is a key growth factor of T- and B-cell progenitors [2], exerts effects on mature peripheral T-cells [3,4], and is produced by thymic and intestinal epithelial cells, bone marrow stromal cells, and keratinocytes [5]. IL-7 is essential in early thymopoiesis [2,6]. In mice lacking IL-7, its α receptor (IL-7Rα), or the common γ chain (γc), pro-T $CD25^+CD44^+CD4^-CD8^-$ cells die, resulting in an early block in thymopoiesis [7]. In αβ T-cells, IL-7/IL-7R deficiency is primarily manifest as absent survival cues since transgenic expression of Bcl-2 can largely rescue αβ T-cells in IL-7R deficient mice [8,9].

Two mechanisms have been proposed to explain how DP thymocytes mature into SP T-cells with matching MHC specificity: the instructive and the stochastic models [10-12]. Furthermore, there are evolving views on whether $CD4^+CD8^+$ DP or $CD4^+CD8^{lo}$ intermediate thymocytes are the immediate precursors of mature CD4 SP and CD8 SP cells. The common precursor of these cells is the likely locus of action of soluble factors that influence lineage commitment. Factors affecting thymocyte development have been studied extensively to elucidate critical control points [13].

Both antigen-dependent and antigen-independent factors may influence thymocyte DP-to-SP transition. Lck activation [14,15] and T-cell receptor (TCR) and

coreceptor signaling [16,17] are antigen-dependent processes implicated in lineage determination. Notch-1 activation is an antigen-independent event that influences lineage commitment toward CD8 SP cells [18], perhaps by inhibiting TCR signal strength [19]. Experiments in thymic organ cultures and bone marrow chimeric animals [20-22] suggested that IL-7 also affects lineage commitment .

The IL-7Rα chain is generally absent in DP thymocytes but is expressed in certain cells undergoing positive selection from the DP to SP stage [23]. Gene disruption models of IL-7 receptor function have elucidated the earliest IL-7 dependent block in T-lymphopoiesis, but did not identify other roles at later stages of thymocyte development. In this study, we examined the *in vivo* role of IL-7 in CD4/CD8 lineage commitment during late thymopoiesis in transgenic mice expressing IL-7 at high levels in the thymus [24].

2 Results

2.1 Peripheral lymphocyte abnormalities

Tg IL-7 mice had more lymphocytes in blood (9497 ± 4081 vs. 3640 ± 787 cells/μl) and markedly more lymphocytes in the spleen (314 ± 67.6 vs. 39 ± 3.4 x 10^6) than wildtype (WT) mice. These abnormalities were evident by 14 wk, well before alopecia or lymphoma progression was detected [24].

FACS analysis of T-cell subsets in the peripheral lymphoid compartment with CD4 and CD8 surface markers revealed an aberrant subset (2.7% of total splenocytes) of DP cells in the spleen; in WT mice, this immature population was largely restricted to the thymus (Fig. 1A). These cells were HSA^{hi}, unlike SP cells in the spleen or thymus (not shown), confirming their immature state. CD8 SP cells were relatively more abundant than CD4 SP cells in Tg IL-7 mice (Fig. 1B). Therefore, expansion of peripheral T-lymphocytes by IL-7 is accompanied by distortions in the relative abundance of individual subsets.

2.2 IL-7 affects lineage commitment during late-stage thymocyte maturation

We next examined IL-7's effect on thymic maturation processes. FACS analysis of thymocytes from WT and Tg IL-7 mice yielded the expected profile in WT mice (Fig. 1C), with a predominance of DP cells and a normal CD4/CD8 SP ratio of ~2 (Fig. 1D). In Tg IL-7 mice, the profile was skewed toward CD8 SP cells (Fig. 1C), with a significantly lower CD4/CD8 SP ratio (Fig. 1D), and a striking increase in CD8 SP cell numbers (Table 1). The expansion of DN cells is consistent with IL-7's role in early thymopoiesis. Tg IL-7 mice had a dramatic decrease in DP thymocytes and a concomitant, but smaller

increase in DP cells in the spleen. Thus, alterations of T-cell subsets in the peripheral blood correlate with alterations in the thymus.

To determine if CD8 SP expansion is IL-7 dependent, we examined IL-7Rα-null mice (IL-7R$^{-/-}$). CD8 SP cells show a relative failure to develop compared with CD4 (Fig. 1C, D). These data from complementary models suggest that IL-7 has a role in late-stage thymocytes, affecting the DP-to-SP transition of CD8 SP cells.

2.3 IL-7 causes expansion of mature, HSA^{lo} thymocytes with preferential, polyclonal increase in CD8 SP thymocytes

Next, we further characterized thymocyte surface marker expression. Subpopulations of IL-7-expanded CD8 SP cells were TCRβ^{hi} CD3^{hi}, HSA^{lo}, and CD8α/β^{+} (not shown) and indistinguishable from mature thymocytes. In IL-7 transgenic mice, HSA^{lo} thymocytes, which have engaged the TCR and downregulated HSA, were increased 20-fold over levels in WT mice and were markedly skewed toward CD8 SP cells (Fig. 2A, B).

Expansion of CD8 SP cells by IL-7 may occur by a general effect on precursors, selective expansion, or transformation and monoclonal expansion. To test this, we analyzed the TCR Vβ representation on thymocytes in Tg IL-7 and WT mice as a measure of clonality. FACS analysis of surface expression of 14 TCR Vβ regions in CD8 SP (Fig. 2C) and CD4 SP (not shown) thymocytes revealed minor perturbations of Vβ representation among CD8 SP thymocytes in Tg IL-7 mice. It is unlikely that peripheral pathologies that arise at later stages account for the skewing toward CD8 SP cells since the CD8 SP subset was similarly expanded in bone marrow chimera recipients at 9

months in the absence of alopecia or lymphoma development (not shown). These data indicate a general, polyclonal expansion of CD8 SP in Tg IL-7 thymocytes.

2.4 IL-7 induced CD8 SP subset expansion in the absence of MHC class I

To test whether IL-7 affects CD8 SP expansion by modulating positive selection during maturation of DP cells, we crossed Tg IL-7 mice with β_2m-null mice. Mice lacking β_2m do not express MHC class I molecules and have dramatically reduced numbers of CD8 SP cells [25] (Figs. 3A, B). Remarkably, Tg IL-7, β_2m-null mice had a significant population of CD8 SP thymocytes (Fig. 3A), comparable to that in Tg IL-7 mice heterozygous for β_2m (Fig. 3B). Thus, IL-7 appears capable of expanding the CD8 SP compartment in the absence of positive selection.

CD8 SP cells were also present in the periphery in β_2m-null mice (Figs. 3C, D) but were not as elevated as in Tg IL-7 mice expressing β_2m. The decreased expansion of CD8 SP cells in the spleen of Tg IL-7, β_2m-null mice (Fig. 3B vs. 3D) suggests that these cells do not respond to peripheral survival signals after exiting the thymus, perhaps due to loss of continued interaction with MHC class I required for normal maintenance [26]. Nevertheless, IL-7-induced expansion of CD8 SP cells in mice lacking MHC class I was evident in a peripheral lymphoid organ as well as the thymus, supporting a model of antigen-independent expansion of CD8 SP cells by IL-7. Interestingly, Tg IL-7, β_2m-null mice had a higher fraction of DP cells in the spleen than Tg IL-7, β_2m mice (18% vs. 6% of total cells; Fig. 3C, and not shown). The absence of β_2m appears to increase the number of IL-7-associated peripheral DP cells.

2.5 Augmented levels of distal effectors of IL-7 signaling in positive selection

We next sought to determine whether IL-7 alters the differentiation, proliferation,

or survival of SP precursors. Pulse-chase labeling of cycling cells *in vivo* with bromodeoxyuridine (BrdU) and cell-cycle analysis with 7-amino-actinomycin D showed that DP thymocytes and DP thymocytes undergoing positive selection turned over at identical rates in Tg IL-7 and WT mice and showed no difference in the fraction of cells entering the cell-cycle (data not shown).

Since IL-7 induces Bcl-2 in receptor-bearing cells [8,27,28], we examined intracellular Bcl-2 expression in thymocytes from Tg IL-7 and WT mice (Fig. 4). Consistent with the high rate of apoptosis in the DP population [29,30], only 1% of DP cells in WT mice expressed Bcl-2. In Tg IL-7 mice, 9% of DP cells expressed Bcl-2. In DP TCRβ^{hi} cells undergoing positive selection, a higher fraction of cells expressed Bcl-2 in both WT mice (10%) and Tg IL-7 mice (30%). Bcl-2-expressing cells were more numerous in CD8, but not CD4, SP cells in Tg IL-7 than in WT mice. Thus, both DP thymocytes in positive selection and CD8 SP cells have increased expression of a survival factor that supports earlier stages of thymopoiesis.

2.6 STAT5A/5B haploinsufficiency alters IL-7-induced expansion of CD8 SP cells and Bcl-2 levels in thymocytes in positive selection

JAK1, JAK3, and STAT5A/B are downstream effectors of IL-7R. We tested the possible importance of STAT5 in IL-7-mediated CD8 SP expansion by crossing Tg IL-7 mice with mice deficient for STAT5A and STAT5B [31]. Mice lacking both STAT5 loci have developmental defects, and many die early. However, compound heterozygotes (STAT5A/5B$^{+/-}$) had a normal appearance and lifespan, no defects in thymocyte development, and a normal distribution of CD4 SP and CD8 SP cells (Figs. 5A, B). Interestingly, IL-7-induced less expansion of the CD8 SP compartment in TgIL-7,

STAT5A/5B$^{+/-}$ than in Tg IL-7 mice on a WT STAT5 background (Fig. 5A, B). Northern analysis showed similar IL-7 expression in both sets of mice (not shown).

STAT5 has been implicated in cytokine-mediated protection from apoptosis [32-34] and provides a proliferation signal during engagement of cytokine receptors [35-37]. We next examined the levels of IL-7-induced intracellular Bcl-2 expression in thymocyte subsets. Bcl-2 levels were significantly lower in DP cells undergoing positive selection in Tg IL-7, STAT5A/5B$^{+/-}$ than in Tg IL-7, STAT5A/5B$^{+/+}$ mice (Figs. 5C, D). Nonetheless, Bcl-2 levels were significantly higher in Tg IL-7 than in non-Tg STAT5A/5B$^{+/+}$ or STAT5A/5B$^{+/-}$ DP cells.

3 Discussion

This study shows that IL-7 promotes CD8 SP expansion during late thymic development. Related observations have been made *in vitro* in rat fetal thymic organ cultures treated with IL-7 [21] and mice reconstituted with bone marrow infected with IL-7 expression vectors [20], although control conditions in both models showed evidence of skewed thymic subsets. Studies of neonatal thymocytes in reaggregate thymic organ cultures also revealed a role for IL-7Rα in lymphocyte expansion after positive-selection, although with more dramatic effect on CD4 than CD8 SP cells [38]. This study could not distinguish between IL-7 and thymic stromal lymphopoietin (TSLP) in mediating post-selection effects via the IL-7Rα receptor [39]. Our Tg IL-7 mouse model shows that IL-7 permits polyclonal expansion of mature CD8 SP cells. The subpopulations in the IL-7-expanded CD8 SP subset (Fig. 1C) did not correspond to an expansion of $CD8^{+}CD4^{lo}$ $TCR\beta^{int}$ transition intermediates [40,41] but bore hallmarks of mature thymocytes. Furthermore, IL-7 expression on a β_2m-null background promoted CD8 SP cell expansion despite the absence of MHC class I–mediated antigen presentation. This observation suggests that IL-7 can circumvent a CD8 SP maturation block in the thymus by inducing an appropriate signal.

One of the signals induced by IL-7 is Bcl-2, a key survival factor [8,27,28]. Forced Bcl-2 expression can substitute for IL-7Rα during early T-cell development [8,9], and the promotion of CD8 SP maturation in Tg Bcl-2 mice [42-44] is strikingly similar to the IL-7-induced expansion of CD8 SP cells. We found that IL-7 enhanced Bcl-2 expression in DP cells undergoing positive selection and in CD8 SP cells without affecting the proliferation or turnover of DP cells or DP cells in positive selection. Taken

together, our data indicate that IL-7 affects the DP-to-SP transition of maturing thymocytes and CD8 SP cells.

We propose a model of thymocyte maturation during lineage commitment in which IL-7 induces survival factors such as Bcl-2, thereby lowering the threshold at which the antigen-induced TCR signal can elicit expansion of CD8 cells that would otherwise fail selection. One possible framework of action is the model of IL-7-induced coreceptor reversal in intermediate thymocytes [22]. IL-7 induced signals may also affect the strength or duration of TCR signals to favor CD8 SP development [16,45]. Whether these mechanisms are applicable or not, our study suggests that IL-7 acts *in vivo* to influence lineage commitment in late thymopoiesis.

STAT5 is another effector of IL-7R function [46,47] that may provide a proliferative or survival signal [32,35-37]. In a STAT5A/B-deficient mouse model, we examined whether STAT5 was necessary for IL-7-induced expansion of CD8 SP cells and induction of Bcl-2 levels in DP thymocytes. IL-7-induced CD8 SP skewing and Bcl-2 expression were reduced in STAT5A/B$^{+/-}$ mice, suggesting that STAT5A and STAT5B are at limiting levels for effects mediated by Tg IL-7 and act upstream of Bcl-2.

The selective effect of IL-7 on CD8 SP expansion is consistent with observations in Tg mouse models based on Bcl-2 and Xiap [42-44,48]. Tg Bcl-2 expansion of CD8 SP thymocytes also occurs in the absence of MHC class I [49]. Indeed, other nonantigen signals can modify thymocyte development. An activated form of Notch-1 skews maturation in favor of CD8 SP cells by a mechanism that is MHC class I-independent [18]. Notch-1 also protects against apoptosis in a T-cell line [50]. Conversely, studies indicate that while the lymphocyte-specific tyrosine kinase Lck is required for formation

of both CD4 and CD8 lineages, a higher level of Lck signaling can favor CD4 SP maturation [14,15]. Moreover, along with c-kit, IL-7Rα expression distinguishes two possible pathways during positive selection [23]. These studies support our finding that cell fate decisions during DP thymocyte maturation are controlled not only by TCR and coreceptor signals but also by other nonantigen secreted regulators via intracellular survival effectors such as STAT5 and Bcl-2.

Splenic lymphocyte subsets in the Tg IL-7 mice were also skewed toward CD8 SP cells. Since IL-7 acts on peripheral lymphocytes, the overrepresentation of CD8 SP cells in the spleen is consistent with IL-7 effects on both thymocyte maturation and peripheral maintenance. This is notably different from Tg Bcl-2 and activated Notch expansion of CD8 SP thymocytes, which do not survive to populate the periphery [18,49,51]. This suggests that IL-7 can sustain CD8 SP cells by a mechanism other than survival induced by Bcl-2 alone. Finally, the DP cells in the spleens of Tg IL-7 mice are probably immature DP cells that escaped the thymus due to deregulation by elevated IL-7 levels in these mice. A similar effect occurred in mice reconstituted with bone marrow infected with IL-7 expression vectors [20]. This escape phenomenon may reflect deregulation of chemotactic or related factors that retain DP cells in the thymus. The number of DP cells in the spleen of Tg IL-7 mice on a MHC class I-null background appears exaggerated and may be due to compromised β_2m-mediated selection, leading to increased DP migration. Overall, both the CD8 SP skewing and the presence of DP cells in the periphery indicate a quantitative action of IL-7 in late thymopoiesis that is both sufficient and necessary.

4 Materials and Methods

4.1 Mice

Transgenic mice expressing murine IL-7 (Tg IL-7) under control of the immunoglobulin heavy chain enhancer and promoter (UP line) [24] were backcrossed with C57/Bl6 mice (The Jackson Laboratory, Bar Harbor, ME) for 4–9 generations. Age-matched, non-Tg littermates were used as controls. IL-7Rα-null mice [52] and β_2-microglobulin (β_2m)-null mice [25] were from the Jackson Laboratory. Heterozygous STAT5A/5B knockout mice with targeted disruption of one allele each of theSTAT5A and STAT5B loci [31] were provided by Dr. James Ihle (St. Jude Children's Research Hospital, Memphis, TN). Mice were housed in a pathogen-free barrier facility and studied at 8–16 wk of age. The study was approved by the Committee on Animal Research (University of California, San Francisco).

4.2 Analysis of peripheral blood

Peripheral blood was obtained by cardiac puncture, and EDTA-treated samples were used for complete blood counts (IDEXX Veterinary Service, Sacramento, CA) and absolute lymphocyte counts.

4.3 Flow cytometry

Thymus and erythrocyte-depleted spleen cell suspensions were prepared in PBS (calcium and magnesium free). Live cells were counted by trypan blue exclusion. Antibodies were from Pharmingen (San Diego, CA). Nonspecific binding to Fc receptors in splenocytes was blocked by preincubating cells with purified antibody to CD16/CD32 (2.4G2; 1:100). Cells were stained for CD4 (clone RM4-5; 1:60) and CD8 (clone 53-6.7; 1:60). TCR Vβ analysis was performed with biotinylated monoclonal antibodies (Pharmingen)

and antibodies against CD4, CD8, and heat-stable antigen (HSA) at 1:60 dilution. Intracellular Bcl-2 was assessed by fixation and permeabilization in 1% paraformaldehyde and 0.1% Tween-20 for 1 h at room temperature and incubation with antibody to Bcl-2 (clone 3F11; 1:5). Fluorescence-activated cell sorter (FACS) analysis was performed with a FACScalibur (Becton Dickinson, San Jose, CA). *p* values were calculated by the *t* test for independent samples.

Figure Legends

Figure 1. IL-7-dependent alterations of T-cell subsets.

(A) FACS analysis of lymphocyte subsets in spleen from WT and Tg IL-7 mice was performed by surface staining of CD4 and CD8. Representative profiles are shown as contour graphs. (B) The ratio of CD4/CD8 SP cells was calculated from the number of CD4 SP and CD8 SP cells. (C) FACS analysis of lymphocyte subsets in thymi from WT, Tg IL-7, and IL-7R$\alpha^{-/-}$ mice was performed by surface staining of CD4 and CD8. Representative profiles are shown as contour graphs. (D) The ratio of CD4/CD8 SP cells was calculated from the number of CD4 SP and CD8 SP cells. Values are mean ± SEM (n = 3/group).

Figure 2. IL-7 overexpression causes expansion of mature HSA^{lo} thymocytes with a preferential, polyclonal effect on CD8 SP cells. (A) Cells from WT and Tg IL-7 thymi were stained with antibodies against HSA, CD4, CD8, and various TCR Vβs. Mature thymocytes visualized by forward scatter (FSC) and HSA staining downregulate HSA, indicated within the circled area. (B) HSA^{lo} CD4 SP and CD8 SP cells from WT or Tg IL-7 mice as a percentage of mature HSA^{lo} cells. (C) TCR Vβ representation within the mature CD8 SP subset (HSA^{lo}CD8^{+} cells). TCR Vβ^{+} thymocytes from WT or Tg IL-7 mice were plotted as a percentage of HSA^{lo}CD8^{+} cells. Values are the mean ± SEM (n = 3/group).

Figure 3. IL-7 induced CD8 SP thymocyte expansion is independent of MHC class I and is manifest in the periphery. (A) Lymphocyte subsets in thymocytes from non-Tg, β_2m-null (left) and Tg IL-7, β_2m-null (right) mice were analyzed by surface staining of CD4

and CD8. Representative profiles are shown as contour graphs. (B) CD8 SP thymocytes plotted as a percentage of total live cells. (C) Lymphocyte subsets in splenocytes from non-Tg, β_2m-null (left) and Tg IL-7, β_2m-null (right) mice were analyzed by surface staining of CD4 and CD8. Representative profiles are shown as contour graphs. (D) CD8 SP splenocytes plotted as a percentage of total live cells. Values are mean ± SEM (n = 3/group).

Figure 4. Intracellular Bcl-2 is elevated in Tg IL-7 DP thymocytes, particularly those in positive selection. Cells from WT and Tg IL-7 thymi were stained with antibodies against CD4, CD8, and TCRβ followed by anti-Bcl-2. Representative histograms showing intracellular Bcl-2 levels in (A) $CD4^+CD8^+$ DP cells, (C) $CD4^+CD8^+$ $TCR\beta^{hi}$ cells and (E) CD8 SP cells from WT and Tg IL-7 mice are shown. The number of intracellular $Bcl\text{-}2^+$ WT and Tg IL-7 cells within the indicated subsets was plotted as a percentage of (B) $CD4^+CD8^+$ DP cells, (D) $CD4^+CD8^+$ $TCR\beta^{hi}$ cells, and (F) CD4 SP and CD8 SP cells. Values are mean ± SEM (n = 3/group).

Figure 5. STAT5A/B haploinsufficiency attenuates Tg IL-7-induced skewing of CD8 SP thymocytes and induction of Bcl-2 in DP thymocytes in positive selection. (A) Lymphocyte subsets in cells from non-Tg, $STAT5A/B^{+/+}$ (upper left), Tg IL-7, $STAT5A/B^{+/+}$ (lower left), non-Tg, $STAT5A/B^{+/-}$ (upper right), and Tg IL-7, $STAT5A/B^{+/-}$ (lower right) thymi analyzed by surface staining of CD4 and CD8. Representative profiles are shown as contour graphs. (B) The ratio of CD4/CD8 SP cells in each group. (C–D) Thymocytes were stained with antibodies against CD4, CD8, and

TCRβ followed by anti-Bcl-2. (C) Representative histograms showing intracellular Bcl-2 levels in $CD4^+CD8^+$ $TCR\beta^{hi}$ cells from non-Tg, $STAT5A/B^{+/+}$ (upper left), Tg IL-7, $STAT5A/B^{+/+}$ (lower left), non-Tg, $STAT5A/B^{+/-}$ (upper right), and Tg IL-7, $STAT5A/B^{+/-}$ (lower right) mice are shown. (D) $CD4^+CD8^+$ $TCR\beta^{hi}$ cells positive for intracellular Bcl-2 were plotted as a percentage of $CD4^+CD8^+$ $TCR\beta^{hi}$ cells. Values are mean ± SEM (n = 3/group).

Table 1: Absolute counts of lymphocyte subsets[a)]

	Thymus				Spleen
Mice	DN	DP	CD4 SP	CD8 SP	DP
WT	6.05 ± 1.12	85.7± 14.9	6.73 ± 1.43	1.88 ± 0.58	0.26 ± 0.03
Tg IL-7	12.1 ± 1.23*	5.95 ± 2.99*	3.91 ± 1.1	6.43 ± 1.14*	13.0 ± 2.97*

a) Values are mean ± SEM (10^6) of six animals in each group. *$p < 0.05$ vs. WT by t test.

(To be positioned near Results Section 2.2)

Figure 1

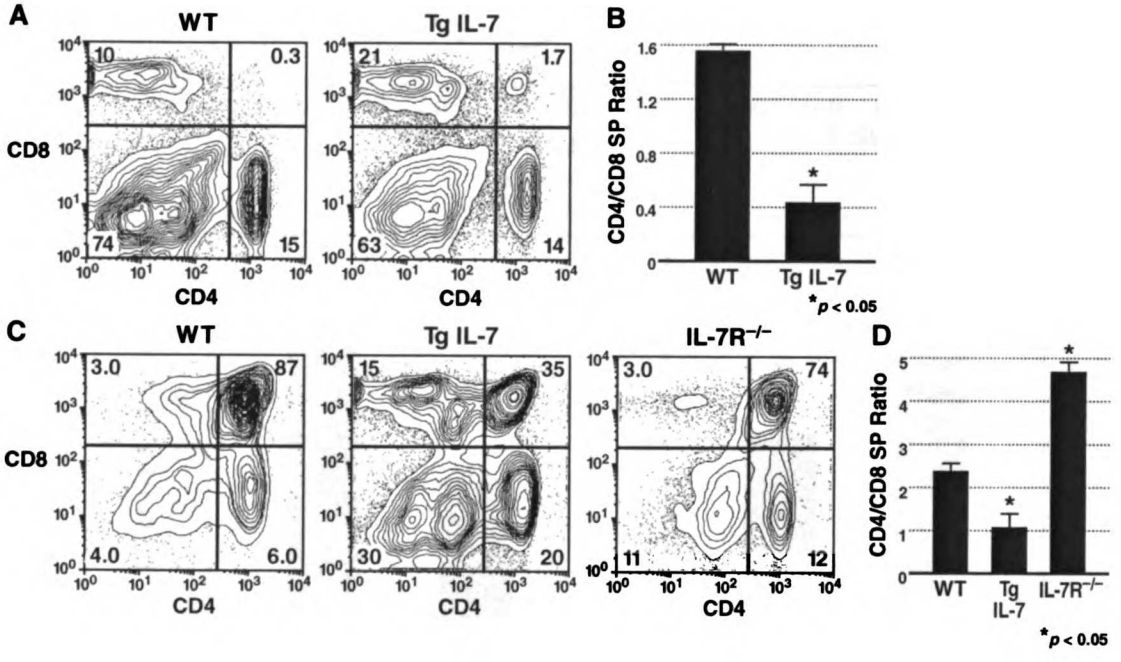

A
WT
Tg IL-7
CD8
CD4
10
0.3
74
15
21
1.7
63
14
B
CD4/CD8 SP Ratio
1.6
1.2
0.8
0.4
0
WT
Tg IL-7
*p < 0.05
C
WT
Tg IL-7
IL-7R−/−
3.0
87
4.0
6.0
15
35
30
20
3.0
74
11
12
D
CD4/CD8 SP Ratio
5
4
3
2
1
0
WT
Tg IL-7
IL-7R−/−
*p < 0.05

Figure 2

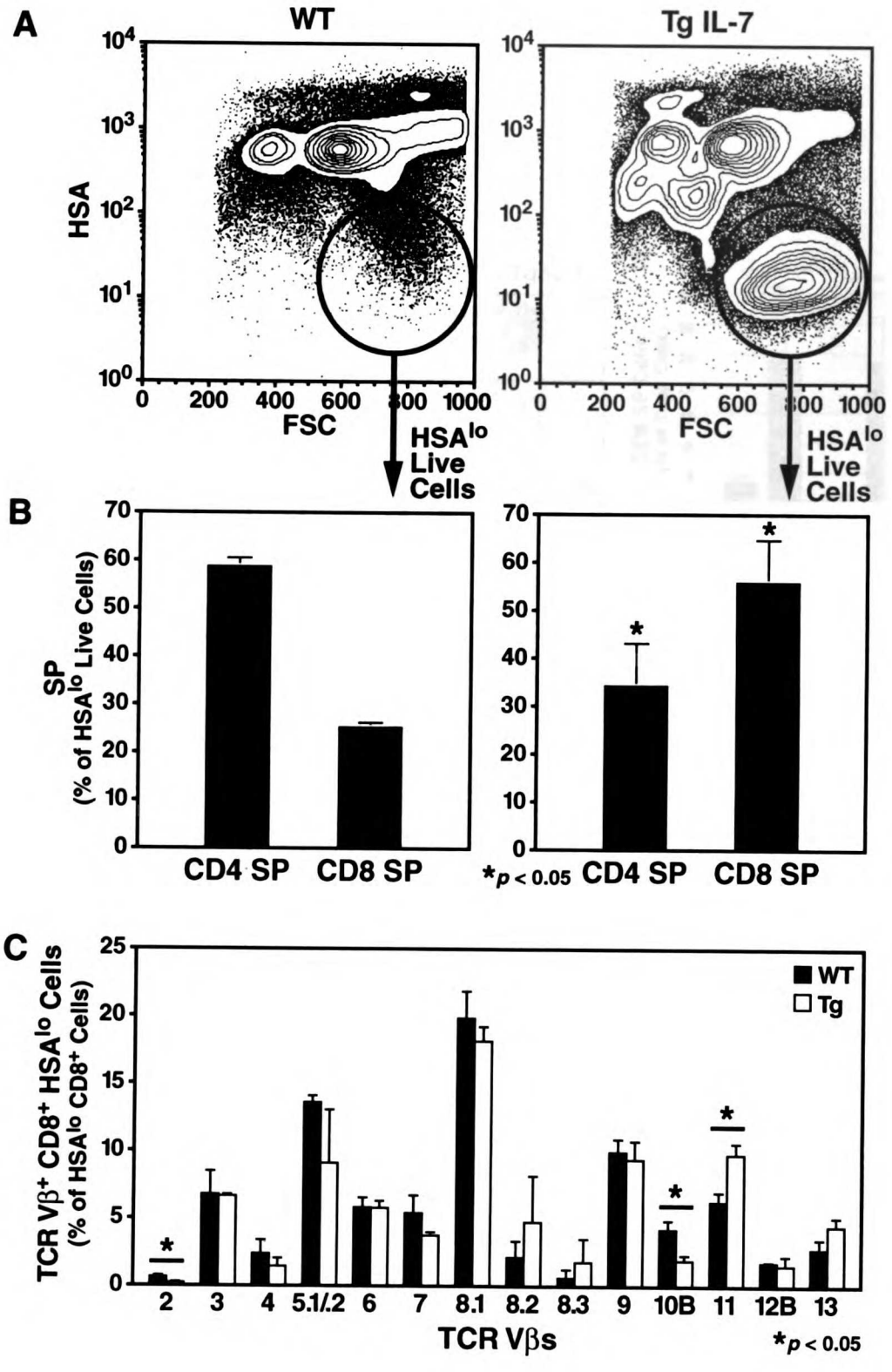

A
WT
Tg IL-7
HSA
FSC
HSA^lo Live Cells
B
SP (% of HSA^lo Live Cells)
CD4 SP
CD8 SP
*p < 0.05
C
TCR Vβ+ CD8+ HSA^lo Cells (% of HSA^lo CD8+ Cells)
WT
Tg
2 3 4 5.1/.2 6 7 8.1 8.2 8.3 9 10B 11 12B 13
TCR Vβs
*p < 0.05

Figure 3

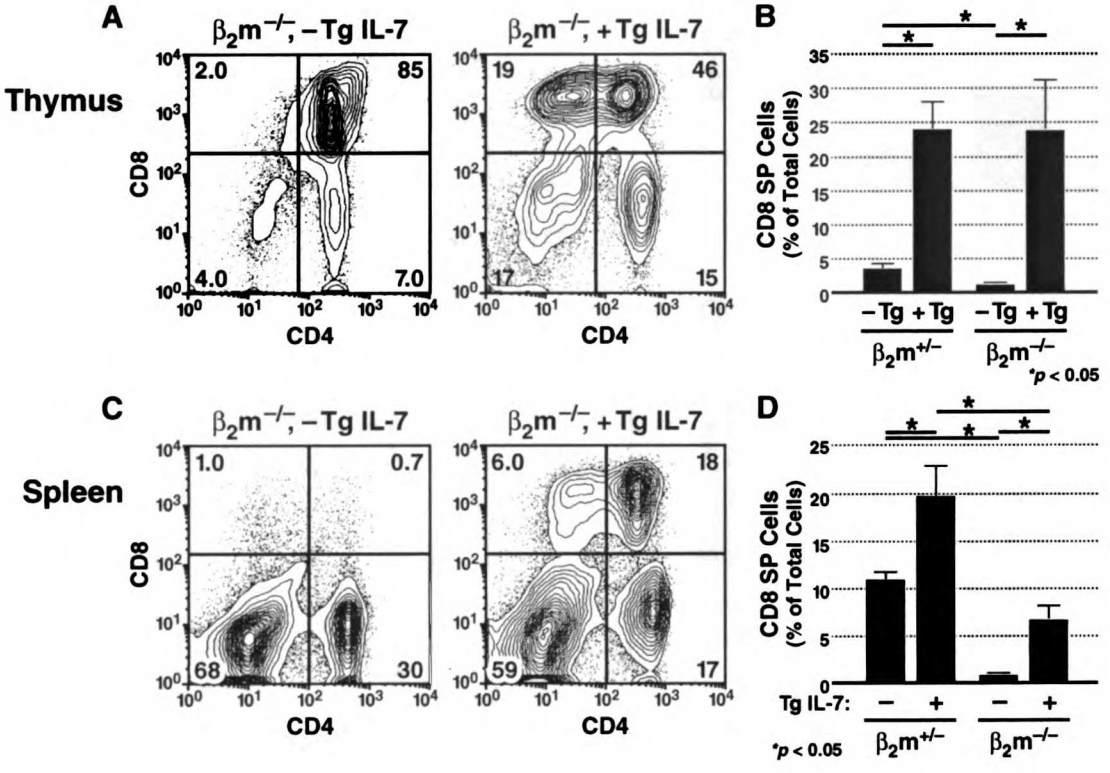

A
Thymus
β2m−/−; −Tg IL-7
β2m−/−; +Tg IL-7
2.0
85
4.0
7.0
19
46
17
15
CD8
CD4
B
CD8 SP Cells (% of Total Cells)
−Tg +Tg −Tg +Tg
β2m+/− β2m−/−
*p < 0.05
C
Spleen
1.0
0.7
68
30
6.0
18
59
17
D
Tg IL-7: − + − +
*p < 0.05

Figure 4

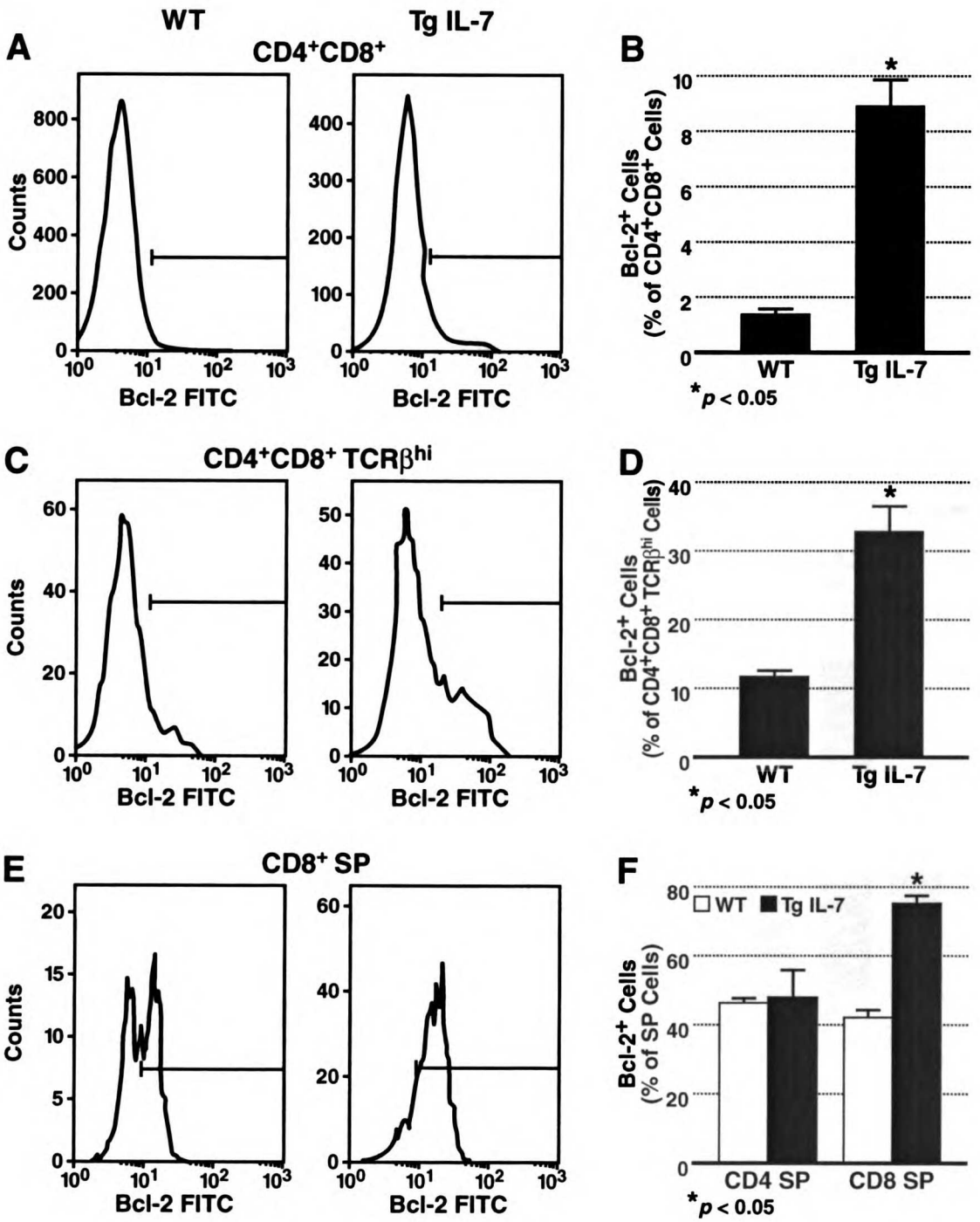
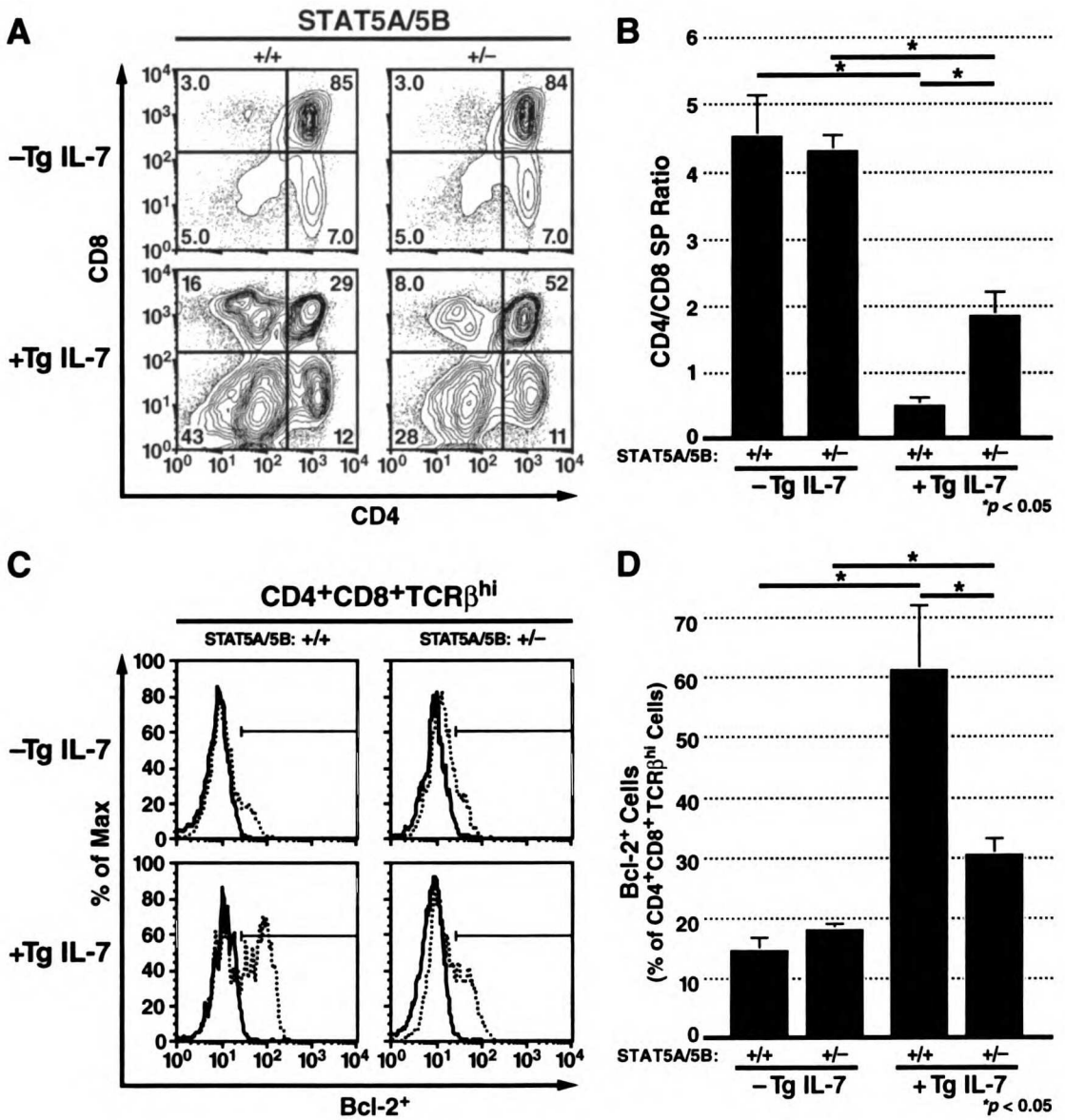

A
WT
Tg IL-7
CD4+CD8+
Counts
Bcl-2 FITC
B
Bcl-2+ Cells (% of CD4+CD8+ Cells)
WT
Tg IL-7
*p < 0.05
C
CD4+CD8+ TCRβhi
D
Bcl-2+ Cells (% of CD4+CD8+ TCRβhi Cells)
E
CD8+ SP
F
WT
Tg IL-7
Bcl-2+ Cells (% of SP Cells)
CD4 SP
CD8 SP

Figure 5


A
STAT5A/5B
+/+
+/–
–Tg IL-7
+Tg IL-7
CD8
CD4
3.0
85
5.0
7.0
3.0
84
5.0
7.0
16
29
43
12
8.0
52
28
11
B
CD4/CD8 SP Ratio
STAT5A/5B:
–Tg IL-7
+Tg IL-7
*p < 0.05
C
CD4+CD8+TCRβhi
STAT5A/5B: +/+
STAT5A/5B: +/–
% of Max
Bcl-2+
D
Bcl-2+ Cells
(% of CD4+CD8+ TCRβhi Cells)

References

1.Wu L, Livak F, Petrie HT (1998) TCR-Independent Development of Pluripotent T-Cell Precursors. In: JG Monroe, Rothenberg EV (eds) Molecular Biology of B-Cell and T-Cell Development. Totowa, N.J.: Humana Press, 285

2.Sudo T, Nishikawa S, Ohno N, Akiyama N, Tamakoshi M, Yoshida H (1993) Expression and function of the interleukin 7 receptor in murine lymphocytes. Proc. Natl. Acad. Sci. USA 90:9125

3.Schluns KS, Kieper WC, Jameson SC, Lefrançois L (2000) Interleukin-7 mediates the homeostasis of naïve and memory CD8 T cells in vivo. Nat. Immunol. 1:426

4.Chazen GD, Pereira GM, LeGros G, Gillis S, Shevach EM (1989) Interleukin 7 is a T-cell growth factor. Proc. Natl. Acad. Sci. USA 86:5923

5.Maeurer MJ, Edington HD, Lotze MT (1998) Interleukin-7. In: AW Thomson (ed) The Cytokine Handbook, 3rd edition. New York, N.Y.: Academic Press, 229

6.Grabstein KH, Waldschmidt TJ, Finkelman FD, Hess BW, Alpert AR, Boiani NE, Namen AE, Morrissey PJ (1993) Inhibition of murine B and T lymphopoiesis in vivo by an anti-interleukin 7 monoclonal antibody. J. Exp. Med. 178:257

7.DiSanto JP, Rodewald H-R (1998) *In vivo* roles of receptor tyrosine kinases and cytokine receptors in early thymocyte development. Curr. Opin. Immunol. 10:196

8.Akashi K, Kondo M, von Freeden-Jeffry U, Murray R, Weissman IL (1997) Bcl-2 rescues T lymphopoiesis in interleukin-7 receptor-deficient mice. Cell 89:1033

9.Maraskovsky E, O'Reilly LA, Teepe M, Corcoran LM, Peschon JJ, Strasser A (1997) Bcl-2 can rescue T lymphocyte development in interleukin-7 receptor-deficient mice but not in mutant rag-1-/- mice. Cell 89:1011

10.Davis CB, Killeen N, Crooks ME, Raulet D, Littman DR (1993) Evidence for a stochastic mechanism in the differentiation of mature subsets of T lymphocytes [see comments]. Cell 73:237

11.von Boehmer H (1996) CD4/CD8 lineage commitment: back to instruction? J. Exp. Med. 183:713

12.Germain RN (2002) T-cell development and the CD4-CD8 lineage decision. Nature Rev Immunol 2:309

13.Killeen N, Irving BA, Pippig S, Zingler K (1998) Signaling checkpoints during the development of T lymphocytes. Curr. Opin. Immunol. 10:360

14.Legname G, Seddon B, Lovatt M, Tomlinson P, Sarner N, Tolaini M, Williams K, Norton T, Kioussis D, Zamoyska R (2000) Inducible expression of a p56Lck transgene reveals a central role for Lck in the differentiation of CD4 SP thymocytes. Immunity 12:537

15.Hernández-Hoyos G, Sohn SJ, Rothenberg EV, Alberola-Ila J (2000) Lck activity controls CD4/CD8 T cell lineage commitment. Immunity 12:313

16.Yasutomo K, Doyle C, Miele L, Fuchs C, Germain RN (2000) The duration of antigen receptor signaling determines CD4+ versus CD8+ T-cell lineage fate. Nature 404:506

17.Bosselut R, Feigenbaum L, Sharrow SO, Singer A (2001) Strength of signaling by CD4 and CD8 coreceptor tails determines the number but not the lineage direction of positively selected thymocytes. Immunity 14:483

18.Robey E, Chang D, Itano A, Cado D, Alexander H, Lans D, Weinmaster G, Salmon P (1996) An activated form of Notch influences the choice between CD4 and CD8 T cell lineages. Cell 87:483

19.Izon DJ, Punt JA, Xu L, Karnell FG, Allman D, Myung PS, Boerth NJ, Pui JC, Koretzky GA, Pear WS (2001) Notch1 regulates maturation of CD4+ and CD8+ thymocytes by modulating TCR signal strength. Immunity 14:253

20.Fraser CC, Thacker JD, Hogge DE, Fatur-Saunders D, Takei F, Humphries RK (1993) Alterations in lymphopoiesis after hematopoietic reconstitution with IL-7 virus-infected bone marrow. J. Immunol. 151:2409

21.Varas A, Vicente A, Jiménez E, Alonso L, Moreno J, Muñoz JJ, Zapata AG (1997) Interleukin-7 treatment promotes the differentiation pathway of T-cell-receptor-alpha beta cells selectively to the CD8+ cell lineage. Immunology 92:457

22.Brugnera E, Bhandoola A, Cibotti R, Yu Q, Guinter TI, Yamashita Y, Sharrow SO, Singer A (2000) Coreceptor reversal in the thymus: signaled CD4+8+ thymocytes initially terminate CD8 transcription even when differentiating into CD8+ T cells. Immunity 13:59

23.Akashi K, Kondo M, Weissman IL (1998) Two distinct pathways of positive selection for thymocytes. Proc. Natl. Acad. Sci. USA 95:2486

24.Rich BE, Campos TJ, Tepper RI, Moreadith RW, Leder P (1993) Cutaneous lymphoproliferation and lymphomas in interleukin 7 transgenic mice. J. Exp. Med. 177:305

25.Koller BH, Marrack P, Kappler JW, Smithies O (1990) Normal development of mice deficient in beta 2M, MHC class I proteins, and CD8+ T cells. Science 248:1227

26.Nesic D, Vukmanovic S (1998) MHC class I is required for peripheral accumulation of CD8+ thymic emigrants. J. Immunol. 160:3705

27.Veis DJ, Sentman CL, Bach EA, Korsmeyer SJ (1993) Expression of the Bcl-2 protein in murine and human thymocytes and in peripheral T lymphocytes. J. Immunol. 151:2546

28.von Freeden-Jeffry U, Solvason N, Howard M, Murray R (1997) The earliest T lineage-committed cells depend on IL-7 for Bcl-2 expression and normal cell cycle progression. Immunity 7:147

29.Hockenbery DM, Zutter M, Hickey W, Nahm M, Korsmeyer SJ (1991) BCL2 protein is topographically restricted in tissues characterized by apoptotic cell death. Proc. Natl. Acad. Sci. USA 88:6961

30.Surh CD, Sprent J (1994) T-cell apoptosis detected in situ during positive and negative selection in the thymus [see comments]. Nature 372:100

31.Teglund S, McKay C, Schuetz E, van DJ, Stravopodis D, Wang D, Brown M, Bodner S, Grosveld G, Ihle JN (1998) Stat5a and Stat5b proteins have essential and nonessential, or redundant, roles in cytokine responses. Cell 93:841

32.Zamorano J, Wang HY, Wang R, Shi Y, Longmore GD, Keegan AD (1998) Regulation of cell growth by IL-2: role of STAT5 in protection from apoptosis but not in cell cycle progression. J. Immunol. 160:3502

33.Socolovsky M, Fallon AE, Wang S, Brugnara C, Lodish HF (1999) Fetal anemia and apoptosis of red cell progenitors in Stat5a-/-5b-/- mice: a direct role for Stat5 in Bcl-X(L) induction. Cell 98:181

34.Kieslinger M, Woldman I, Moriggl R, Hofmann J, Marine JC, Ihle JN, Beug H, Decker T (2000) Antiapoptotic activity of Stat5 required during terminal stages of myeloid differentiation. Genes Dev. 14:232

35.Lord JD, McIntosh BC, Greenberg PD, Nelson BH (2000) The IL-2 receptor promotes lymphocyte proliferation and induction of the c-myc, bcl-2, and bcl-x genes through the trans-activation domain of Stat5. J. Immunol. 164:2533

36.Moriggl R, Topham DJ, Teglund S, Sexl V, McKay C, Wang D, Hoffmeyer A, van Deursen J, Sangster MY, Bunting KD, Grosveld GC, Ihle JN (1999) Stat5 is required for IL-2-induced cell cycle progression of peripheral T cells. Immunity 10:249

37.Van Parijs L, Refaeli Y, Lord JD, Nelson BH, Abbas AK, Baltimore D (1999) Uncoupling IL-2 signals that regulate T cell proliferation, survival, and Fas-mediated activation-induced cell death. Immunity 11:281

38.Hare KJ, Jenkinson EJ, Anderson G (2000) An essential role for the IL-7 receptor during intrathymic expansion of the positively selected neonatal T cell repertoire. J. Immunol. 165:2410

39.Levin SD, Koelling RM, Friend SL, Isaksen DE, Ziegler SF, Perlmutter RM, Farr AG (1999) Thymic stromal lymphopoietin: a cytokine that promotes the development of IgM+ B cells in vitro and signals via a novel mechanism. J. Immunol. 162:677

40.Lucas B, Germain RN (1996) Unexpectedly complex regulation of CD4/CD8 coreceptor expression supports a revised model for CD4+CD8+ thymocyte differentiation. Immunity 5:461

41.Akashi K, Kondo M, Schlageter AM, Weissman IL (1998) T-Cell Development from Hematopoietic Stem Cells. In: JG Monroe, Rothenberg EV (eds) Molecular Biology of B-Cell and T-Cell Development. Totowa, N.J.: Humana Press, 305

42.Sentman CL, Shutter JR, Hockenbery D, Kanagawa O, Korsmeyer SJ (1991) bcl-2 inhibits multiple forms of apoptosis but not negative selection in thymocytes. Cell 67:879

43.Strasser A, Harris AW, Cory S (1991) bcl-2 transgene inhibits T cell death and perturbs thymic self-censorship. Cell 67:889

44.Tao W, Teh SJ, Melhado I, Jirik F, Korsmeyer SJ, Teh HS (1994) The T cell receptor repertoire of CD4-8+ thymocytes is altered by overexpression of the BCL-2 protooncogene in the thymus. J. Exp. Med. 179:145

45.Itano A, Salmon P, Kioussis D, Tolaini M, Corbella P, Robey E (1996) The cytoplasmic domain of CD4 promotes the development of CD4 lineage T cells. J Exp Med 183:731

46.van der Plas DC, Smiers F, Pouwels K, Hoefsloot LH, Löwenberg B, Touw IP (1996) Interleukin-7 signaling in human B cell precursor acute lymphoblastic leukemia cells and murine BAF3 cells involves activation of STAT1 and STAT5 mediated via the interleukin-7 receptor alpha chain. Leukemia 10:1317

47.Lin JX, Migone TS, Tsang M, Friedmann M, Weatherbee JA, Zhou L, Yamauchi A, Bloom ET, Mietz J, John S, Leonard WJ (1995) The role of shared receptor motifs and common Stat proteins in the generation of cytokine pleiotropy and redundancy by IL-2, IL-4, IL-7, IL-13, and IL-15. Immunity 2:331

48.Conte D, Liston P, Wong JW, Wright KE, Korneluk RG (2001) Thymocyte-targeted overexpression of *xiap* transgene disrupts T lymphoid apoptosis and maturation. Proc. Natl. Acad. Sci. USA 98:5049

49.Linette GP, Grusby MJ, Hedrick SM, Hansen TH, Glimcher LH, Korsmeyer SJ (1994) Bcl-2 is upregulated at the CD4+ CD8+ stage during positive selection and promotes thymocyte differentiation at several control points. Immunity 1:197

50.Jehn BM, Bielke W, Pear WS, Osborne BA (1999) Protective effects of notch-1 on TCR-induced apoptosis. J. Immunol. 162:635

51.Deftos ML, Huang E, Ojala EW, Forbush KA, Bevan MJ (2000) Notch1 signaling promotes the maturation of CD4 and CD8 SP thymocytes. Immunity 13:73

52.Peschon JJ, Morrissey PJ, Grabstein KH, Ramsdell FJ, Maraskovsky E, Gliniak BC, Park LS, Ziegler SF, Williams DE, Ware CB, Meyer JD, Davison BL (1994) Early lymphocyte expansion is severely impaired in interleukin 7 receptor-deficient mice. J. Exp. Med. 180:1955

Appendix II

Haploinsufficiency Identifies STAT5 as a Modifier of IL-7-induced Lymphomas

Introduction

In all types of cancer, including those that affect hematopoietic cells, cells undergo mutation in genes involved in the control of proliferation, survival, and genetic stability. In addition, some cancers require further mutations in genes controlling migration [1].

In order to circumvent the mechanisms that control these biological activities, cells undergo mutations in two classes of genes: oncogenes and tumor suppressors. Oncogenes are defined as genes whose products induce oncogenic growth when expressed in the appropriate cell types, and include protein kinases, growth factors, and transcription factors. They usually undergo dominant, gain of function, mutations which increase their activity, such as point-mutations or deletions increasing activity or removing sites of regulation, amplification at the gene or chromosome level, and especially important in leukemia and lymphoma, gene rearrangements resulting in the creation of fusion proteins with altered specificity, activity, localization, transcriptional regulation, and regulation. Tumor suppressors are defined as genes whose products negatively regulate cell growth, and include genes involved in the negative regulation of cell division, survival, and control of repair of DNA damage. They usually undergo recessive, loss of function, mutations which decrease their activity or amount, such as point mutations or deletions [1].

Multiple growth factors are thought to play a role in the initiation or progression of hematologic malignancies. Autocrine-dependent growth has been described for multiple neoplastic cells and cell lines [2]. In addition, overexpression of many growth factors can lead to transformation of hematopoietic cells in mice [3-8]. Interleukin-7 (IL-

7) is thought to be an important growth factor for myscosis fungoides and Sézary syndrome, both forms of cutaneous T-cell lymphoma (CTCL) [9-13]. In addition, IL-7 can support multiple lymphoid cell lines *in vitro* [14,15] and functional IL-7 receptors are expressed in both hematopoietic and non-hematopoietic neoplasms [16]. In fact, some mouse models that overexpress IL-7 support a role for this growth factor in tumorogenesis, displaying lymphoproliferation and lymphomas [8,17-19].

IL-7 engagement of its receptor activates multiple pathways, including the signal transducer and activator of transcription (STAT) 5 (reviewed in [20]) and the role of IL-7 in the models of malignancy above may be mediated through triggering of this molecule. In fact, activation of STAT5 has been seen in a wide variety of malignancies. Tyrosine kinase fusion proteins associated with human leukemia such as BCR/Abl, TEL/JAK2, TEL/PDGFβR, TEL/Abl, NPM/ALK, and HIP1/ PDGFβR activate STAT5 ([21],reviewed in [22]). STAT5 has been shown to be a minor fusion partner for RARα that inhibits differentiation in acute promyelocytic leukemia (APL) [23,24]. In addition, other oncogenes, such as activated Flt3R [25], v-Abl [26], Xmrk [27] and v-Erb [28], are characterized by activation of STAT5. In some cases activation of STAT5 is downregulated by drugs that target these oncogenes, implying a role in tumoregenesis [29-31]}. Constitutively activated STAT5A transforms hematopoietic cell lines to growth-factor independence [32]. STAT5 deficiency has different effects on the progression of tumors in various mouse models of these human diseases, having no effect in some models [33], while reducing transformation and progression in others [34,35].

We used an established transgenic model of over-expression of IL-7 driven by the immunoglobulin enhancer [8] to examine the role of STAT5 in lymphomagenesis

mediated by IL-7. These Tg IL-7 mice manifest a high incidence of T and B lymphomas later in life and exhibit shortened lifespans with 100% mortality by 6 months of age. Tg IL-7 crossed onto an STAT5A/5B$^{+/-}$ background, displayed a striking amelioration of IL-7-induced death. These data suggest that STAT5 levels are dose-dependent and shows that STAT5 haploinsufficiency lessens IL-7 induced mortality. This observation lends support for blockage of STAT5 activation as a potential therapeutic to counter tumor progression in a variety of tumors. It shows a dose-effect that has little effect on normal lymphocyte development but clearly affects IL-7-induced lymphoma progression.

References

1. Rosenberg N, Krontiris TG (2000) Chapter 48: The Molecular Basis of Neoplasia. In: R Hoffman (ed) Hematology: Basic Principles and Practice, 3rd edition. New York: Churchill Livingston, 871

2. Bagby GC, Jr., Heinrich MC (2000) Chapter 14: Growth Factors, Cytokines, and the Control of Hematopoiesis. In: R Hoffman (ed) Hematology: Basic Principles and Practice, 3rd edition. New York: Churchill Livingston, 154

3. Hawley RG, Fong AZ, Ngan BY, de Lanux VM, Clark SC, Hawley TS (1993) Progenitor cell hyperplasia with rare development of myeloid leukemia in interleukin 11 bone marrow chimeras. J Exp Med 178:1175

4. Kovalchuk AL, Kim JS, Park SS, Coleman AE, Ward JM, Morse HC, 3rd, Kishimoto T, Potter M, Janz S (2002) IL-6 transgenic mouse model for extraosseous plasmacytoma. Proc Natl Acad Sci U S A 99:1509

5. Hawley TS, Fong AZ, Griesser H, Lyman SD, Hawley RG (1998) Leukemic predisposition of mice transplanted with gene-modified hematopoietic precursors expressing flt3 ligand. Blood 92:2003

6. Orchard PJ, Detrick RA, Gorden K, Dunbar CE, Vallera DA, Taylor P, McIvor RS, Blazar BR (1993) Retroviral-mediated transfer of the murine interleukin-3 gene

engineered for intracellular retention results in a myeloproliferative syndrome but is associated with circulating interleukin-3 levels. Exp Hematol 21:1245

7. Fehniger TA, Suzuki K, Ponnappan A, VanDeusen JB, Cooper MA, Florea SM, Freud AG, Robinson ML, Durbin J, Caligiuri MA (2001) Fatal leukemia in interleukin 15 transgenic mice follows early expansions in natural killer and memory phenotype CD8+ T cells. J Exp Med 193:219

8. Rich BE, Campos TJ, Tepper RI, Moreadith RW, Leder P (1993) Cutaneous lymphoproliferation and lymphomas in interleukin 7 transgenic mice. J Exp Med 177:305

9. Dalloul A, Laroche L, Bagot M, Mossalayi MD, Fourcade C, Thacker DJ, Hogge DE, Merle-Beral H, Debre P, Schmitt C (1992) Interleukin-7 is a growth factor for Sezary lymphoma cells. J Clin Invest 90:1054

10. Qin JZ, Dummer R, Burg G, Dobbeling U (1999) Constitutive and interleukin-7/interleukin-15 stimulated DNA binding of Myc, Jun, and novel Myc-like proteins in cutaneous T-cell lymphoma cells. Blood 93:260

11. Qin JZ, Kamarashev J, Zhang CL, Dummer R, Burg G, Dobbeling U (2001) Constitutive and interleukin-7- and interleukin-15-stimulated DNA binding of STAT and novel factors in cutaneous T cell lymphoma cells. J Invest Dermatol 117:583

12. Qin JZ, Zhang CL, Kamarashev J, Dummer R, Burg G, Dobbeling U (2001) Interleukin-7 and interleukin-15 regulate the expression of the bcl-2 and c-myb genes in cutaneous T-cell lymphoma cells. Blood 98:2778

13. Poszepczynska E, Bagot M, Echchakir H, Martinvalet D, Ramez M, Charue D, Boumsell L, Bensussan A (2000) Functional characterization of an IL-7-dependent CD4(+)CD8alphaalpha(+) Th3-type malignant cell line derived from a patient with a cutaneous T-cell lymphoma. Blood 96:1056

14. Barata JT, Cardoso AA, Nadler LM, Boussiotis VA (2001) Interleukin-7 promotes survival and cell cycle progression of T-cell acute lymphoblastic leukemia cells by down-regulating the cyclin-dependent kinase inhibitor p27(kip1). Blood 98:1524

15. van der Plas DC, Smiers F, Pouwels K, Hoefsloot LH, Löwenberg B, Touw IP (1996) Interleukin-7 signaling in human B cell precursor acute lymphoblastic leukemia cells and murine BAF3 cells involves activation of STAT1 and STAT5 mediated via the interleukin-7 receptor alpha chain. Leukemia 10:1317

16. Cosenza L, Gorgun G, Urbano A, Foss F (2002) Interleukin-7 receptor expression and activation in nonhaematopoietic neoplastic cell lines. Cell Signal 14:317

17. Uehira M, Matsuda H, Hikita I, Sakata T, Fujiwara H, Nishimoto H (1993) The development of dermatitis infiltrated by gamma delta T cells in IL-7 transgenic mice. Int Immunol 5:1619

18. Williams IR, Rawson EA, Manning L, Karaoli T, Rich BE, Kupper TS (1997) IL-7 overexpression in transgenic mouse keratinocytes causes a lymphoproliferative skin disease dominated by intermediate TCR cells: evidence for a hierarchy in IL-7 responsiveness among cutaneous T cells. J Immunol 159:3044

19. Fisher AG, Burdet C, LeMeur M, Haasner D, Gerber P, Ceredig R (1993) Lymphoproliferative disorders in an IL-7 transgenic mouse line. Leukemia 7 Suppl 2:S66

20. Hofmeister R, Khaled AR, Benbernou N, Rajnavolgyi E, Muegge K, Durum SK (1999) Interleukin-7: physiological roles and mechanisms of action. Cytokine Growth Factor Rev 10:41

21. Nieborowska-Skorska M, Slupianek A, Xue L, Zhang Q, Raghunath PN, Hoser G, Wasik MA, Morris SW, Skorski T (2001) Role of signal transducer and activator of transcription 5 in nucleophosmin/ anaplastic lymphoma kinase-mediated malignant transformation of lymphoid cells. Cancer Res 61:6517

22. Levy DE, Gilliland DG (2000) Divergent roles of STAT1 and STAT5 in malignancy as revealed by gene disruptions in mice. Oncogene 19:2505

23. Maurer AB, Wichmann C, Gross A, Kunkel H, Heinzel T, Ruthardt M, Groner B, Grez M (2002) The Stat5-RARalpha fusion protein represses transcription and differentiation through interaction with a corepressor complex. Blood 99:2647

24. Dong S, Tweardy DJ (2002) Interactions of STAT5b-RARalpha, a novel acute promyelocytic leukemia fusion protein, with retinoic acid receptor and STAT3 signaling pathways. Blood 99:2637

25. Birkenkamp KU, Geugien M, Lemmink HH, Kruijer W, Vellenga E (2001) Regulation of constitutive STAT5 phosphorylation in acute myeloid leukemia blasts. Leukemia 15:1923

26. Gross AW, Ren R (2000) Bcr-Abl has a greater intrinsic capacity than v-Abl to induce the neoplastic expansion of myeloid cells. Oncogene 19:6286

27. Morcinek JC, Weisser C, Geissinger E, Schartl M, Wellbrock C (2002) Activation of STAT5 triggers proliferation and contributes to anti-apoptotic signalling mediated by the oncogenic Xmrk kinase. Oncogene 21:1668

28. von Lindern M, Deiner EM, Dolznig H, Parren-Van Amelsvoort M, Hayman MJ, Mullner EW, Beug H (2001) Leukemic transformation of normal murine erythroid progenitors: v- and c-ErbB act through signaling pathways activated by the EpoR and c-Kit in stress erythropoiesis. Oncogene 20:3651

29. Levis M, Allebach J, Tse KF, Zheng R, Baldwin BR, Smith BD, Jones-Bolin S, Ruggeri B, Dionne C, Small D (2002) A FLT3-targeted tyrosine kinase inhibitor is cytotoxic to leukemia cells in vitro and in vivo. Blood 99:3885

30. Mow BM, Chandra J, Svingen PA, Hallgren CG, Weisberg E, Kottke TJ, Narayanan VL, Litzow MR, Griffin JD, Sausville EA, Tefferi A, Kaufmann SH (2002) Effects of the Bcr/abl kinase inhibitors STI571 and adaphostin (NSC 680410) on chronic myelogenous leukemia cells in vitro. Blood 99:664

31. Wasik MA, Nowak I, Zhang Q, Shaw LM (1998) Suppression of proliferation and phosphorylation of Jak3 and STAT5 in malignant T-cell lymphoma cells by derivatives of octylamino-undecyl-dimethylxanthine. Leuk Lymphoma 28:551

32. Onishi M, Nosaka T, Misawa K, Mui AL, Gorman D, McMahon M, Miyajima A, Kitamura T (1998) Identification and characterization of a constitutively active STAT5 mutant that promotes cell proliferation. Mol Cell Biol 18:3871

33. Sexl V, Piekorz R, Moriggl R, Rohrer J, Brown MP, Bunting KD, Rothammer K, Roussel MF, Ihle JN (2000) Stat5a/b contribute to interleukin 7-induced B-cell precursor expansion, but abl- and bcr/abl-induced transformation are independent of stat5. Blood 96:2277

34. Schwaller J, Parganas E, Wang D, Cain D, Aster JC, Williams IR, Lee CK, Gerthner R, Kitamura T, Frantsve J, Anastasiadou E, Loh ML, Levy DE, Ihle JN, Gilliland DG (2000) Stat5 is essential for the myelo- and lymphoproliferative disease induced by TEL/JAK2. Mol Cell 6:693

35. Ren S, Cai HR, Li M, Furth PA (2002) Loss of Stat5a delays mammary cancer progression in a mouse model. Oncogene 21:4335

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Haploinsufficiency identifies STAT5 as a modifier of IL-7 induced lymphomas

Ninan Abraham[*†], Melissa C. Ma[*], Jonathan W. Snow[*‡], Brian Herndier, Mark A. Goldsmith[*¶]

[*]Gladstone Institute of Virology and Immunology, San Francisco, CA 94141, USA; [‡]Department of Microbiology and Immunology, University of California, San Francisco, CA 94141, USA and [¶]Department of Medicine, University of California, San Francisco, CA 94141, USA

[†]To whom correspondence should be addressed:

Gladstone Institute of Virology and Immunology,

P.O. Box 419100, San Francisco, CA 94141-9100, USA

Telephone: (415) 695-3803, fax: (415) 826-1514

Email: nabraham@gladstone.ucsf.edu

After January 1st, 2003:

Department of Microbiology and Immunology

The University of British Columbia

#300-6174, University Boulevard,

Vancouver, B.C., Canada V6T 1Z3

Telephone: 614) 822-3308, fax: (614) 822-6041

Email: ninan@interchange.ubc.ca

Abstract

The requirement for receptor components and the signaling effector, STAT5A/5B, was assessed genetically in a lymphoma progression model induced by IL-7. This growth factor for T- and B-cell progenitors and mature lymphocytes activates survival and proliferative pathways including Bcl-2, phosphatidylinositol 3-kinase and STAT5. Mice heterozygous for the IL-7Rα subunit over-expressing IL-7 show improved survival compared to wild-type mice. In addition, STAT5A/5B$^{+/-}$ compound heterozygous mice with one targeted allele each of STAT5A and STAT5B show striking amelioration of IL-7-induced mortality and disease progression. STAT5A/5B$^{+/-}$ compound heterozygous mice are otherwise normal in stem cell and lymphocyte development and cellularity. The reduction in STAT5A/5B copy number is accompanied by lower STAT5 protein levels, which suggests that STAT5 haploinsufficiency is a modifier of IL-7 signal strength.

Introduction, Results and Discussion

Cytokines are key regulators of cells by engaging appropriate receptors and triggering downstream signaling events that govern proliferation, survival, differentiation and mobilization. Interleukin-7 (IL-7) is an essential, non-redundant growth factor for early T- and B-cell precursors [1,2] and exerts effects on mature peripheral T cells [3,4]. IL-7 acts on target cells by interacting with the IL-7 receptor (IL-7R), a heterodimer comprised of the IL-7Rα chain and the common γ (γc) chain. In mice lacking IL-7, its α receptor subunit (IL-7Rα), or the γc chain, pro-T cells die in the thymus, resulting in an early block in thymopoiesis, and murine pro-B cells fail to progress resulting in overall lymphopenia (reviewed in [5]). Deficiency in IL-7 signaling accounts for the severe T-cell immunodeficiency seen in human X-linked severe combined immunodeficiency (X-SCID) [6,7] and $T^-B^+NK^+$ SCID [8].

While loss of IL-7 causes immunodeficiency, overexpression of IL-7 results in neoplasias. Mouse models that overexpress IL-7 show phenotypes ranging from increased levels of T and B cells [9] to lymphoproliferation and lymphomas [10-13]. IL-7's involvement in neoplasias is also suggested by its role as a trophic factor for Sezary lymphoma cells [14], its production by chronic lymphocytic leukemia (CLL) cells [15] and Burkitt's lymphoma cells [16], and its elevation in sera of Hodgkin's patients [17].

IL-7 engagement of its receptor activates two main pathways, signal transducer and activator of transcription (STAT) 5 [18,19] of the JAK/STAT pathway and the phosphatidylinositol 3-kinase (PI3-kinase) pathway [20-22]. Both STAT5 and PI3 kinase have been implicated in cell-growth control and survival. Two nearly identical genes, STAT5A and STAT5B, encode two separate polypeptides with similar, but non-identical

functions [23,24]. Tyrosine kinase fusion proteins associated with human leukemia can activate STAT5 in a wide variety of malignancies. For example, BCR/Abl in chronic myelogenous leukemia (CML), TEL/JAK2 in myeloproliferative disease, and TEL/PDGFβR, TEL/Abl, and HIP1/ PDGFβR (reviewed in [25]), as well as v-Abl activate this transcription factor. Constitutively activated STAT5A transforms hematopoietic cell lines to growth-factor independence [26]. Much is known about the molecular events initiating these pathways. Less is known about integration to downstream biology *in vivo*.

The effect of gene dosage on biology is well appreciated in the study of tumour suppressors in predisposing a cell to malignancy. Haploinsufficiency, the concomitant reduction in protein levels, is gaining significance in both tumor suppressor studies [27,28] as well as in transcription factor biology [29]. Human haploinsufficiency of transcription factors can manifest as complex, multisystem syndromes with wide clinical variation, with at least 30 syndromes identified. The extensive catalog of transcription factor families that demonstrate haploinsufficiency does not include members if the STAT family of transcription factors [29]. Our study of effectors that can ameliorate IL-7-mediated lymphoma progression revealed haploinsufficiency of receptor components as well as a transcription factor, STAT5, could significantly modify the consequence of over-expression of IL-7.

IL-7 induced lymphoma progression is affected by IL-7Rα copy number

Mice over-expressing IL-7 under the control of the immunoglobulin enhancer and promoter (Tg IL-7 mice) display accelerated mortality (Figure 1A, left panel) compared

to non-transgenic mice due to T- and B-lymphoma development, with 100% mortality by 6 months of age as described previously [11]. IL-7 induced mortality is completely dependent on IL-7Rα, since mice that have homozygous disruption of IL-7Rα (IL-7Rα$^{-/-}$) show no difference in survival in the absence or presence of the IL-7 transgene (Figure 1A, right panel), with survival well beyond 8 months in all mice. More interestingly, we observed that mice heterozygous for the IL-7Rα subunit (IL-7Rα$^{+/-}$), that have comparable lymphocyte cellularity to IL-7Rα wild-type (IL-7Rα$^{+/+}$) mice (data not shown), exhibited improved survival from IL-7 induced death (Figure 1B). Nearly half of Tg IL-7; IL-7Rα$^{+/-}$ mice are alive at 8 months of age while none of the Tg IL-7; IL-7Rα$^{+/+}$ mice survive to this age.

IL-7 induced lymphoma progression is STAT5A/5B dose-dependent

Since IL-7 activates STAT5, we acquired mice with disruption at both the STAT5A and STAT5B loci [30] to test the necessity of this transcription factor effector in IL-7 signaling. While mice with compound homozygous disruption have multiple non-hematopoietic and hematolymphoid defects [30-38], STAT5A/5B$^{+/-}$ compound heterozygous mice have normal lifespans (Figure 2A, triangles), lymphoid cellularity (Figure 2C), T-cell development (data not shown) and bone marrow precursors that are normal in competitive repopulation assays (unpublished observations). This indicates no detectable consequence of reduction of STAT5 copy number on lymphoid development at steady state. When crossed to Tg IL-7 mice, STAT5A/5B$^{+/-}$ compound heterozygous mice showed a striking amelioration of IL-7-induced mortality (Figure 2A, squares) compared to Tg IL-7; STAT5A/5B$^{+/+}$ mice (Figure 2A, circles). Half of mice lacking a

copy each of STAT5A and STAT5B that over-expressed IL-7 were still alive at 9 months of age while none of the Tg IL-7; STAT5A/5B$^{+/+}$ mice lived beyond 6 months.

We determined that Tg IL-7, STAT5A/5B$^{+/-}$ compound heterozygous mice have equal levels of transgene-derived IL-7 transcript as Tg IL-7, STAT5A/5B$^{+/+}$ mice (data not shown). We examined the level of STAT5 in WT, heterozygous and homozygous STAT5A/5B-null thymocytes to determine whether STAT5 protein levels were affected by gene dosage. Immunoblot analysis shows that STAT5A/5B$^{+/-}$ compound heterozygous mice have reduced levels of STAT5 present in the thymus compared to STAT5A/5B$^{+/+}$ mice (Figure 2B). No polypeptide is detectable in STAT5A/5B$^{-/-}$ compound homozygous mice as expected. These data clearly indicates that STAT5 haploinsufficiency is manifest with reduced STAT5A/5B copy number.

We next assessed IL-7-induced splenomegaly as another indicator of disease progression in these mice. Tg IL-7 mice show a markedly elevated splenocyte cellularity (Figure 2C) compared to non-transgenic littermates. Tg IL-7, STAT5A/5B$^{+/-}$ compound heterozygous mice, however, have significant attenuation of IL-7-induced splenomegaly.

STAT5A/5B haploinsufficiency affects lymphoma progression

Histopathological analysis was performed on thymus, spleen and skin sections from non-transgenic, Tg IL-7; STAT5A/5B$^{+/+}$ and Tg IL-7; STAT5A/5B$^{+/-}$ mice. Over-expression of IL-7 resulted in disorganized thymic architecture, the presence of sub-capsular lymphomas in the spleen with a greatly expanded white pulp, and infiltration of the dermal layers reminiscent of cutaneous lymphoma (data not shown). Tg IL-7, STAT5A/5B$^{+/-}$ mice however, displayed fewer signs of aggressive disease with thymic

architecture that appears normal, spleen with atypical lymphoproliferation but no sign of lymphomas and skin with greatly reduced lymphocyte infiltration (data not shown). These data suggest that STAT5A/5B haploinsufficiency moderates IL-7-induced lymphoma progression.

The categorization of pathways required during tumorigenesis is a promising avenue for identification of novel targets for targeted therapeutics in treating malignancies. Genetic analyses of pathways in tumor models is a powerful means of assessing the usefulness of targeting a given molecule and its potential side effects. STAT5 is known to mediate cytokine-induced survival as well as proliferation in response to various cytokines [39]. Indeed, as an effector of IL-7R function [18,19], it can provide a proliferative or survival signal [40-43].

Haploinsufficiency has gained significance in helping elucidate the importance of specific molecules by genetics. Its role in transcription factors and disease is implicated in several syndromes, involving a broad class of these regulatory factors [29]. For example, haploinsufficiency of AML1 [44] and the Foxo1 forkhead transcription factor [45] have clear effects on stem cell and hepatocyte biology respectively. Dose dependence on receptors such as Follicle-Stimulating Hormone receptor [46] and even the Erythropoietin receptor, a Type I cytokine receptor, [47] is increasingly recognized as physiologically relevant in mice. In addition, dose dependence has been described for the adaptor protein, Grb2, in regulating tissue architecture [48] as well as in polyoma middle T antigen induction of mammary tumors [49]. Few other examples of haploinsufficiency, particularly of transcription factors, affecting tumor progression are known.

We examined whether IL-7 receptor components and effectors, like STAT5, were necessary in IL-7-induced lymphoma progression. IL-7-induced mortality was moderated in mice heterozygous for the IL-7Rα. Since IL-7Rα heterozygous mice have normal lymphoid development and cellularity, this suggests that IL-7Rα can be rate limiting for constitutive IL-7 action, such as in the Tg IL-7 lymphoma model. The effect of loss of one copy each of STAT5A and STAT5B on IL-7-induced lymphomas was similarly striking in ameliorating disease and improving survival. Our data shows that STAT5A/5B$^{+/-}$ mice have a reduction in STAT5 protein levels indicating STAT5 haploinsufficiency in these mice.

We propose that IL-7-induced lymphoma progression is dependent on signal strength that is affected by STAT5 levels. Thus, the STAT5 transcription factor is shown to be limiting for IL-7 in a tumor model. This contrasts with the absence of effect of loss of STAT5 on BCR-Abl transformation of bone marrow and myeloid cells [33]. However, it mirrors the importance of STAT5 for progression of mammary cancer induced by SV40 T antigen [50]. This suggests that inhibition of STAT5 can have differing outcomes in ameliorating disease in different tumor progression models and its utility has to weighed in the lineage concerned. The absence of bone marrow and lymphocyte abnormalities in non-transgenic, STAT5A/5B$^{+/-}$ mice also shows that graded inhibition of STAT5A/5B to ameliorate hematolymphoid diseases that are sensitive to STAT5 levels would have little to no side effects.

Our findings show that a member of the STAT family of transcription factors, STAT5, can be included in the list of transcription factors whose haploinsufficiency can have a biological effect. Questions of whether STAT5A or STAT5B haploinsufficiency

modifies IL-7 action by a qualitative or quantitative mechanism and the potential targets affected need further examination.

Figure Legends

Figure 1. IL-7Rα copy number affects IL-7-induced mortality.

IL-7Rα deficient mice were crossed with Tg IL-7 mice and monitored twice monthly for lesions, lymph-node enlargement and survival over 8 months.

A) Left panel Non-transgenic; IL-7R$\alpha^{+/+}$, black triangles (n=8), Tg IL-7; IL-7R$\alpha^{+/+}$, white squares (n=7). Right panel Non-transgenic; IL-7R$\alpha^{-/-}$, black triangles (n=6), Tg IL-7; IL-7R$\alpha^{-/-}$, white squares (n=9).

B) Tg IL-7; IL-7R$\alpha^{+/+}$, black circles (n=7), Tg IL-7; IL-7R$\alpha^{+/-}$, white squares (n=28), Tg IL-7; IL-7R$\alpha^{-/-}$, black triangles (n=6).

Figure 2. STAT5A/5B haploinsufficiency affects IL-7-induced mortality.

A) IL-7-induced lymphoma progression is STAT5A/5B copy number dependent. STAT5A/5B$^{+/-}$ compound heterozygote targeted mice were crossed with Tg IL-7 mice and and monitored twice monthly for lesions, lymph-node enlargement and survival over 9 months. Non-transgenic; STAT5A/5B$^{+/-}$, black triangles (n=13), Tg IL-7; STAT5A/5B$^{+/-}$, white squares, (n=10), Tg IL-7; STAT5A/5B$^{+/+}$, black circles (n=5).

B) Immunoblot analysis of STAT5 expression in STAT5A/5B targeted mice. Lysates were prepared from thymocytes from the indicated mouse gentotypes, normalized for total protein, resolved by SDS-PAGE, transferred and immunoblotted. Rabbit polyclonal antibodies to STAT5A and STAT5B (kindly provided by Dr. Lothar Henninghausen, NIH, MD) were used together at 1:10000, mouse monoclonal antibody to α-tubulin (Sigma) was used at 1:5000 and visualized with anti-rabbit or anti-mouse HRP using enhanced chemiluminesence.

C) Spleens from the indicated mouse genotypes were removed, weighed, dispersed into single cell suspension. Total cell counts of viable cells was performed by trypan blue exclusion. Non-transgenic; STAT5A/5B$^{+/+}$ (n=6), non-transgenic; STAT5A/5B$^{+/-}$ (n=10), Tg IL-7; STAT5A/5B$^{+/+}$ (n=5), Tg IL-7; STAT5A/5B$^{+/-}$ (n=8).

Figure 1

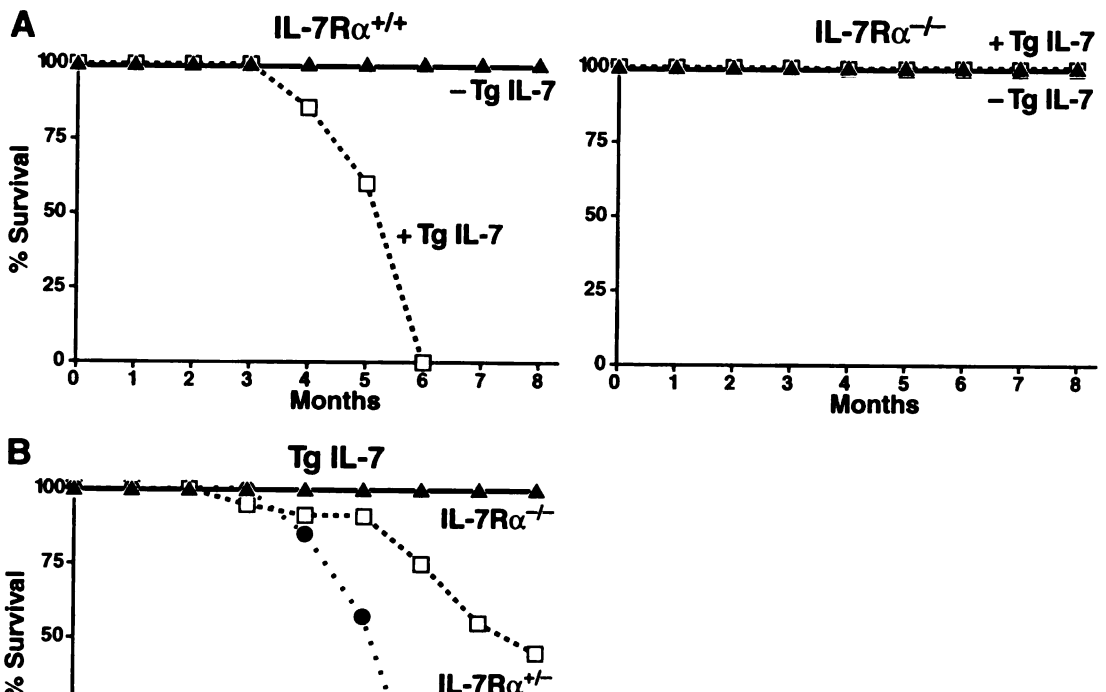
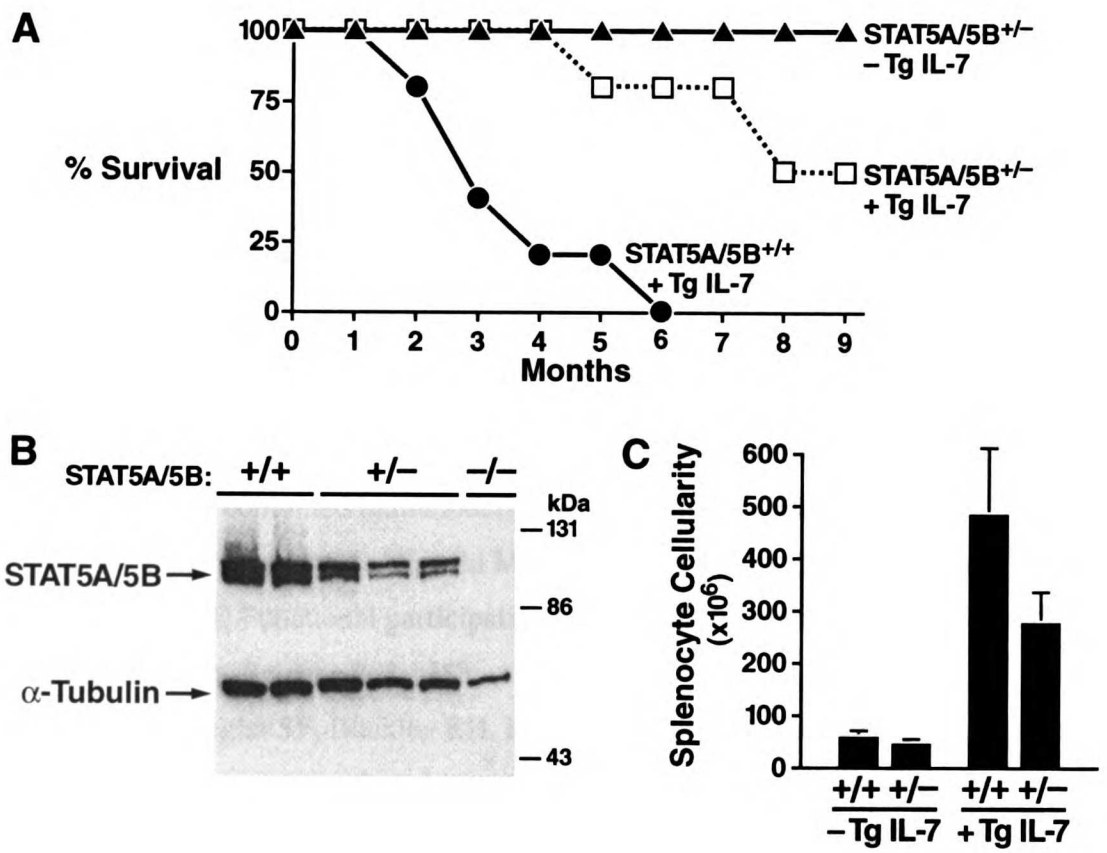

A
IL-7Rα+/+
– Tg IL-7
+ Tg IL-7
% Survival
Months
IL-7Rα–/–
+ Tg IL-7
– Tg IL-7
Months
B
Tg IL-7
IL-7Rα–/–
IL-7Rα+/–
IL-7Rα+/+
% Survival
Months

Figure 2


A
100
75
50
25
0
% Survival
0 1 2 3 4 5 6 7 8 9
Months
STAT5A/5B+/–
– Tg IL-7
STAT5A/5B+/–
+ Tg IL-7
STAT5A/5B+/+
+ Tg IL-7
B
STAT5A/5B: +/+ +/– –/–
kDa
131
86
43
STAT5A/5B
α-Tubulin
C
Splenocyte Cellularity
(x10⁶)
600
500
400
300
200
100
0
+/+ +/–
– Tg IL-7
+/+ +/–
+ Tg IL-7

References

1. Grabstein KH, Waldschmidt TJ, Finkelman FD, Hess BW, Alpert AR, Boiani NE, Namen AE, Morrissey PJ (1993) Inhibition of murine B and T lymphopoiesis in vivo by an anti-interleukin 7 monoclonal antibody. J. Exp. Med. 178:257

2. Sudo T, Nishikawa S, Ohno N, Akiyama N, Tamakoshi M, Yoshida H (1993) Expression and function of the interleukin 7 receptor in murine lymphocytes. Proc. Natl. Acad. Sci. USA 90:9125

3. Schluns KS, Kieper WC, Jameson SC, Lefrançois L (2000) Interleukin-7 mediates the homeostasis of naïve and memory CD8 T cells in vivo. Nat. Immunol. 1:426

4. Chazen GD, Pereira GM, LeGros G, Gillis S, Shevach EM (1989) Interleukin 7 is a T-cell growth factor. Proc. Natl. Acad. Sci. USA 86:5923

5. Hofmeister R, Khaled AR, Benbernou N, Rajnavolgyi E, Muegge K, Durum SK (1999) Interleukin-7: physiological roles and mechanisms of action. Cytokine Growth Factor Rev 10:41

6. Noguchi M, Yi H, Rosenblatt HM, Filipovich AH, Adelstein S, Modi WS, McBride OW, Leonard WJ (1993) Interleukin-2 receptor gamma chain mutation results in X-linked severe combined immunodeficiency in humans. Cell 73:147

7. Kondo M, Takeshita T, Higuchi M, Nakamura M, Sudo T, Nishikawa S, Sugamura K (1994) Functional participation of the IL-2 receptor gamma chain in IL-7 receptor complexes. Science 263:1453

8. Puel A, Ziegler SF, Buckley RH, Leonard WJ (1998) Defective IL7R expression in T(-)B(+)NK(+) severe combined immunodeficiency. Nat Genet 20:394

9. Samaridis J, Casorati G, Traunecker A, Iglesias A, Gutierrez JC, Muller U, Palacios R (1991) Development of lymphocytes in interleukin 7-transgenic mice. Eur J Immunol 21:453

10. Fisher AG, Burdet C, LeMeur M, Haasner D, Gerber P, Ceredig R (1993) Lymphoproliferative disorders in an IL-7 transgenic mouse line. Leukemia 7 Suppl 2:S66

11. Rich BE, Campos TJ, Tepper RI, Moreadith RW, Leder P (1993) Cutaneous lymphoproliferation and lymphomas in interleukin 7 transgenic mice. J Exp Med 177:305

12. Uehira M, Matsuda H, Hikita I, Sakata T, Fujiwara H, Nishimoto H (1993) The development of dermatitis infiltrated by gamma delta T cells in IL-7 transgenic mice. Int Immunol 5:1619

13. Williams IR, Rawson EA, Manning L, Karaoli T, Rich BE, Kupper TS (1997) IL-7 overexpression in transgenic mouse keratinocytes causes a lymphoproliferative skin disease dominated by intermediate TCR cells: evidence for a hierarchy in IL-7 responsiveness among cutaneous T cells. J Immunol 159:3044

14. Dalloul A, Laroche L, Bagot M, Mossalayi MD, Fourcade C, Thacker DJ, Hogge DE, Merle-Beral H, Debre P, Schmitt C (1992) Interleukin-7 is a growth factor for Sezary lymphoma cells. J Clin Invest 90:1054

15. Frishman J, Long B, Knospe W, Gregory S, Plate J (1993) Genes for interleukin 7 are transcribed in leukemic cell subsets of individuals with chronic lymphocytic leukemia. J Exp Med 177:955

16. Benjamin D, Sharma V, Knobloch TJ, Armitage RJ, Dayton MA, Goodwin RG (1994) B cell IL-7. Human B cell lines constitutively secrete IL-7 and express IL-7 receptors. J Immunol 152:4749

17. Trumper L, Jung W, Dahl G, Diehl V, Gause A, Pfreundschuh M (1994) Interleukin-7, interleukin-8, soluble TNF receptor, and p53 protein levels are elevated in the serum of patients with Hodgkin's disease. Ann Oncol 5 Suppl 1:93

18. Lin JX, Migone TS, Tsang M, Friedmann M, Weatherbee JA, Zhou L, Yamauchi A, Bloom ET, Mietz J, John S, Leonard WJ (1995) The role of shared receptor motifs and common Stat proteins in the generation of cytokine pleiotropy and redundancy by IL-2, IL-4, IL-7, IL-13, and IL-15. Immunity 2:331

19. van der Plas DC, Smiers F, Pouwels K, Hoefsloot LH, Löwenberg B, Touw IP (1996) Interleukin-7 signaling in human B cell precursor acute lymphoblastic leukemia cells and murine BAF3 cells involves activation of STAT1 and STAT5 mediated via the interleukin-7 receptor alpha chain. Leukemia 10:1317

20. Dadi HK, Roifman CM (1993) Activation of phosphatidylinositol-3 kinase by ligation of the interleukin-7 receptor on human thymocytes. J Clin Invest 92:1559

21. Dibirdik I, Langlie MC, Ledbetter JA, Tuel-Ahlgren L, Obuz V, Waddick KG, Gajl-Peczalska K, Schieven GL, Uckun FM (1991) Engagement of interleukin-7 receptor

stimulates tyrosine phosphorylation, phosphoinositide turnover, and clonal proliferation of human T-lineage acute lymphoblastic leukemia cells. Blood 78:564

22. Venkitaraman AR, Cowling RJ (1994) Interleukin-7 induces the association of phosphatidylinositol 3-kinase with the alpha chain of the interleukin-7 receptor. Eur J Immunol 24:2168

23. Udy GB, Towers RP, Snell RG, Wilkins RJ, Park S-H, Ram PA, Waxman DJ, Davey HW (1997) Requirement of STAT5b for sexual dimorphism of body growth rates and liver gene expression. Proc Natl Acad Sci USA 94:7239

24. Park SH, Liu X, Hennighausen L, Davey HW, Waxman DJ (1999) Distinctive roles of STAT5a and STAT5b in sexual dimorphism of hepatic P450 gene expression. Impact of STAT5a gene disruption. J Biol Chem 274:7421

25. Levy DE, Gilliland DG (2000) Divergent roles of STAT1 and STAT5 in malignancy as revealed by gene disruptions in mice. Oncogene 19:2505

26. Onishi M, Nosaka T, Misawa K, Mui AL, Gorman D, McMahon M, Miyajima A, Kitamura T (1998) Identification and characterization of a constitutively active STAT5 mutant that promotes cell proliferation. Mol Cell Biol 18:3871

27. Kemkemer R, Schrank S, Vogel W, Gruler H, Kaufmann D (2002) Increased noise as an effect of haploinsufficiency of the tumor-suppressor gene neurofibromatosis type 1 in vitro. Proc Natl Acad Sci U S A 99:13783

28. Quon KC, Berns A (2001) Haplo-insufficiency? Let me count the ways. Genes Dev 15:2917

29. Seidman JG, Seidman C (2002) Transcription factor haploinsufficiency: when half a loaf is not enough. J Clin Invest 109:451

30. Teglund S, McKay C, Schuetz E, van DJ, Stravopodis D, Wang D, Brown M, Bodner S, Grosveld G, Ihle JN (1998) Stat5a and Stat5b proteins have essential and nonessential, or redundant, roles in cytokine responses. Cell 93:841

31. Socolovsky M, Lodish HF, Daley GQ (1998) Control of hematopoietic differentiation: Lack of specificity in signaling by cytokine receptors. Proc. Natl. Acad. Sci., USA in press

32. Socolovsky M, Fallon AE, Wang S, Brugnara C, Lodish HF (1999) Fetal anemia and apoptosis of red cell progenitors in Stat5a-/-5b-/- mice: a direct role for Stat5 in Bcl-X(L) induction. Cell 98:181

33. Sexl V, Piekorz R, Moriggl R, Rohrer J, Brown MP, Bunting KD, Rothammer K, Roussel MF, Ihle JN (2000) Stat5a/b contribute to interleukin 7-induced B-cell precursor expansion, but abl- and bcr/abl-induced transformation are independent of stat5. Blood 96:2277

34. Kieslinger M, Woldman I, Moriggl R, Hofmann J, Marine JC, Ihle JN, Beug H, Decker T (2000) Antiapoptotic activity of Stat5 required during terminal stages of myeloid differentiation. Genes and Development 14:232

35. Bradley HL, Hawley TS, Bunting KD (2002) Cell intrinsic defects in cytokine responsiveness of STAT5-deficient hematopoietic stem cells. Blood 100:3983

36. Bunting KD, Bradley HL, Hawley TS, Moriggl R, Sorrentino BP, Ihle JN (2002) Reduced lymphomyeloid repopulating activity from adult bone marrow and fetal liver of mice lacking expression of STAT5. Blood 99:479

37. Snow JW, Abraham N, Ma MC, Abbey NW, Herndier B, Goldsmith MA (2002) STAT5 promotes multilineage hematolymphoid development in vivo through effects on early hematopoietic progenitor cells. Blood 99:95

38. Shelburne C, McCoy M, Piekorz R, Sexl V, Gillespie S, Bailey D, Gharse A, Mirmonsef P, M NM, Kashyap M, Wright H, Chong H, Bouton L, Ramirez C, Lantz C, Ryan J (2002) Stat5: an essential regulator of mast cell biology. Mol Immunol 38:1187

39. Lin JX, Leonard WJ (2000) The role of Stat5a and Stat5b in signaling by IL-2 family cytokines. Oncogene 19:2566

40. Lord JD, McIntosh BC, Greenberg PD, Nelson BH (2000) The IL-2 receptor promotes lymphocyte proliferation and induction of the c-myc, bcl-2, and bcl-x genes through the trans-activation domain of Stat5. J. Immunol. 164:2533

41. Moriggl R, Topham DJ, Teglund S, Sexl V, McKay C, Wang D, Hoffmeyer A, van Deursen J, Sangster MY, Bunting KD, Grosveld GC, Ihle JN (1999) Stat5 is required for IL-2-induced cell cycle progression of peripheral T cells. Immunity 10:249

42. Van Parijs L, Refaeli Y, Lord JD, Nelson BH, Abbas AK, Baltimore D (1999) Uncoupling IL-2 signals that regulate T cell proliferation, survival, and Fas-mediated activation-induced cell death. Immunity 11:281

43. Zamorano J, Wang HY, Wang R, Shi Y, Longmore GD, Keegan AD (1998) Regulation of cell growth by IL-2: role of STAT5 in protection from apoptosis but not in cell cycle progression. J. Immunol. 160:3502

44. Cai Z, de Bruijn M, Ma X, Dortland B, Luteijn T, Downing RJ, Dzierzak E (2000) Haploinsufficiency of AML1 affects the temporal and spatial generation of hematopoietic stem cells in the mouse embryo. Immunity 13:423

45. Nakae J, Biggs WH, 3rd, Kitamura T, Cavenee WK, Wright CV, Arden KC, Accili D (2002) Regulation of insulin action and pancreatic beta-cell function by mutated alleles of the gene encoding forkhead transcription factor Foxo1. Nat Genet 32:245

46. Danilovich N, Sairam MR (2002) Haploinsufficiency of the follicle-stimulating hormone receptor accelerates oocyte loss inducing early reproductive senescence and biological aging in mice. Biol Reprod 67:361

47. Jegalian AG, Acurio A, Dranoff G, Wu H (2002) Erythropoietin receptor haploinsufficiency and in vivo interplay with granulocyte-macrophage colony-stimulating factor and interleukin 3. Blood 99:2603

48. Saxton TM, Cheng AM, Ong SH, Lu Y, Sakai R, Cross JC, Pawson T (2001) Gene dosage-dependent functions for phosphotyrosine-Grb2 signaling during mammalian tissue morphogenesis. Curr Biol 11:662

49. Cheng AM, Saxton TM, Sakai R, Kulkarni S, Mbamalu G, Vogel W, Tortorice CG, Cardiff RD, Cross JC, Muller WJ, Pawson T (1998) Mammalian Grb2 regulates multiple steps in embryonic development and malignant transformation. Cell 95:793

50. Ren S, Cai HR, Li M, Furth PA (2002) Loss of Stat5a delays mammary cancer progression in a mouse model. Oncogene 21:4335