The role of Janus Kinase 3 in CD4+ T Cell Homeostasis and Function: A Dissertation

THE ROLE OF JANUS KINASE 3 IN CD4+ T CELL HOMEOSTASIS AND FUNCTION

A Dissertation Presented

By

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Abstract

This dissertation addresses the role for Janus Kinase 3 (Jak3) in CD4+ T cell homeostasis and function. Jak3 is a protein tyrosine kinase whose activity is essential for signals mediated by the γc dependent cytokines IL-2, -4, -7,-9,-15, and -21. Previous data have demonstrated that peripheral CD4+ T cells from Jak3-deficient mice have a memory phenotype and are functionally impaired in both proliferative and IL-2 responses *in vitro*. Interestingly, Jak3/γc activity has been previously shown to play a role in the prevention of T cell anergy.

These studies were initiated to more precisely define the role for Jak3/γc cytokines in the prevention of T cell anergy and the maintenance of functional CD4+ T cell responses. We began to address this question by assessing global gene expression changes between wild type and Jak3-/- CD4+ T cells. These data indicate that Jak3-/- CD4+ T cells have an increase in gene expression levels of inhibitory surface receptors as well as immunosuppressive cytokines.

Further analyses confirmed that Jak3-deficient T cells express high levels of PD-1, secrete a Tr1-type cytokine profile following direct ex vivo activation, and suppress the proliferation of wild type T cells in vitro. These characteristics indicate that CD4+ Jak3-/- T cells share properties with regulatory T cell subsets that have an important role in peripheral tolerance and the prevention of autoimmunity.

We next addressed whether these regulatory characteristics were T cell intrinsic or rather the result of expanding in a Jak3-deficient microenvironment characterized by a number of immune abnormalities and a disrupted splenic architecture. Jak3-/- CD4+ T cells proliferate *in vivo* in a lymphopenic environment and selectively acquire regulatory T cell characteristics in the absence of any additional activation signals. While the precise mechanism by which Jak3-deficient T cells acquire these characteristics remains unclear, our data indicate that one important component is a T cell-intrinsic requirement for Jak3 signaling.

These findings indicate several interesting aspects of T cell biology. First, these studies, demonstrate that the homeostatic proliferation of CD4+ T cells is not dependent on signaling via γc-dependent cytokine receptors. And, second, that the weak activation signals normally associated with homeostatic expansion are sufficient to drive Jak3-/- T cells into a non-conventional differentiation program. Previous data indicate that, for wild type T cells, signaling through both the TCR as well as γc-dependent cytokine receptors promote the homeostatic proliferation of T cells in lymphopenic hosts. Since Jak3-/- T cells are unable to receive these cytokine signals, their proliferation is likely to be wholly dependent on TCR signaling. As a consequence of this TCR signaling, Jak3-/- T cells proliferate, but in addition, are induced to up regulate PD-1 and to selectively activate the IL-10 locus while shutting off the production of IL-2. Since this fate does not occur for wild type T cells in a comparable environment, it is likely that the unique differentiation pathway taken by Jak3-/-

T cells reflects the effects of TCR signaling in the absence of γc-dependent cytokine signaling.

Interestingly, wild type T cells undergoing homeostatic expansion in lymphopenic hosts show many common patterns of gene expression to freshly-purified unmanipulated Jak3-/- T cells. For instance, microarray analysis of gene expression in wild type CD4+ T cells after lymphopenia induced homeostatic expansion show a similar pattern of upregulation in surface markers (PD-1 and LAG-3), and cytokine signaling molecules (IL-10 and IFN-γ cytokine, receptors, and inducible gene targets) to that of Jak3-/- CD4+ T cells immediately ex vivo. These data suggest that the process of homeostatic proliferation normally induces immune attenuation and peripheral tolerance mechanisms, but that full differentiation into a regulatory T cell phenotype is prevented by γc-dependent cytokine signals.

Taken together these data suggest that Jak3 plays an important role in tempering typical immune attenuation mechanisms employed to maintain T cell homeostasis and peripheral tolerance.

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Attributions

All CD4+ CD44-high T cell populations were isolated by the UMASS Sorting Facility.

Microarray hybridizations were performed by the UMASS Affymetrix Core Facility.

Dr. Joe Maciaszek and Dr. SK Kim assisted with tail vein injections for experiments presented in Chapter VI.

Dr. Hai Nyguen performed the Western Blot for Foxp3 expression shown in Figure 36.

Amanda Prince and Min Shi performed the real-time PCR confirmation of microarray data shown in Supplemental Data 7 and 8.

CHAPTER I.

INTRODUCTION

T Lymphocytes and the Immune System

The goal of the immune system is to vigorously and appropriately react against foreign pathogens while remaining completely inert to self. Therefore, the immune system must be equipped with a highly sophisticated surveillance mechanism to detect diverse antigens and to protect the host organism from invading pathogens and altered cells (e.g., virus-infected and tumor cells) as well as equally elaborate mechanisms to appropriately attenuate immune responses and ignore self antigens.

While the immune system is composed of many different cell types armed with a myriad of effector functions, T lymphocytes are one of the most crucial elements of the adaptive immune system and critically influence the outcome of a response against any given pathogen.

Given their importance for the proper function of the immune system, lymphocytes need to be tightly regulated throughout their lifetime. Any breach in the regulation of lymphocyte development or function can have catastrophic consequences. This can be illustrated by the occurrence of autoimmune disease and allergy which typically result from inappropriate lymphocyte responses against self-tissues or innocuous substances. To minimize the occurrence of immune dysregulation, there are a number of safeguards in place such that every stage of lymphocyte development and function requires transition through tightly controlled checkpoints. These checkpoints take place at various stages throughout the life of a T cell, such as during development and activation, and are regulated by a variety of cell surface receptors, intracellular signaling molecules and soluble mediators.

Adaptive Immune Responses and Positive Costimulation

Adaptive immune responses depend on the recognition of antigen by specific receptors that are expressed on the surface of T and B lymphocytes [1]. The TCR specifically recognizes antigens that have been processed into peptides and are presented in the context of the major histocompatibility complex (MHC) on the surface of antigen presenting cells (APC) [2,3]. CD8+ T cells are restricted to the recognition of peptides presented by MHC class I while CD4+ T cells are restricted to MHC class II [4-6]. The interaction between a TCR and peptide-MHC alone however is not sufficient for complete activation of naïve T cells, and in the absence of necessary secondary signals may result in apoptosis or unresponsiveness. The knowledge that naïve CD4+ and CD8+ T cell stimulation is dependent on signals from two discrete receptor ligand interactions has long been appreciated and is referred to as the two signal model of T cell activation . The first signal, as mentioned above, is mediated via the TCR upon recognition of a cognate peptide-MHC. The engagement of a family of structurally related costimulatory receptors with their ligands provides signal two [7]. The B7:CD28 pathway mediates the most potent positive costimulatory signal leading to T cell activation, cytokine secretion, and T cell expansion [8]. CD28 is constitutively expressed on T cells and interacts with B7.1 and B7.2 ligands expressed on activated or mature APCs [9]. CD28 mediated promotion of a T cell response is thought to rely primarily on IL-2 secretion and subsequent signaling via the IL-2 receptor (IL-2R) resulting in the down regulation of p27kip1 cdk inhibitor by activation of PI3/PKB and ultimately the induction of cell cycle

progression at two distinct points, early G1 phase and at the G1/S transition [10,11]. However, further data suggested that there is also an IL-2 independent component to CD28 costimulation. This was shown by *in vitro* stimulation of T cells with α-CD3 + α-CD28 in the presence or absence of IL-2 neutralizing antibodies [10]. The results from these experiments indicate that the role of CD28 costimulation in regulating T cell cycle entry and progression through the G1 phase is actually mediated by an IL-2-independent mechanism which results in the activation of cyclin D2-associated cdk4/cdk6 and cyclin E-associated cdk2. Subsequent progression into the S phase was found to be mediated via both IL-2-dependent and IL-2-independent mechanisms since in the absence of IL-2 (+IL-2 neutralizing antibodies) the majority of the T cells were arrested at the G1/S transition (IL-2 dependence), but, a significant fraction of them (40%) progress to S phase (IL-2 independence).

Although CD28 is necessary for optimal activation of naïve T cells, the same does not appear true of memory and effector T cell responses. This is based on observations that antigen specific memory T cells stimulated in the presence of B7.1/B7.2-deficient APCs can proliferate in the absence of B7 at high antigen concentration, while naive cells are significantly impaired. The absence of B7 did not impair cytokine production in memory cells, while naive cells exhibited defective IL-2 production. These results support the notion that the effector function of memory cells is not dependent on costimulation through CD28:B7 [12].

The fact that memory T cells appear less dependent on CD28 for costimulation suggested the existence of a more complex network of regulatory pathways that would potentially involve additional receptor-ligand pairs. This idea was first supported by the identification of the inducible costimulator (ICOS) and its ligand B7-homolog (B7-H) which was found to stimulate both CD4 and CD8 T cell responses [13,14], and, has been further established by the continually growing number of costimulatory molecules which can either positively or negatively regulate T cells responses (Table 1).

Another perplexity of CD28 function is that while stimulation through the T-cell receptor and CD28 induces efficient T cell activation, it also leads to the expression of surface receptors, such as CTLA-4, that act to negatively regulate T cell function. Such self maintained attenuation mechanisms are crucial to the maintenance of controlled immune responses and prevention lymphoproliferative and autoimmune diseases.

Adaptive Immune Responses and Negative Costimulation

The B7:cytotoxic T-lymphocyte antigen-4 (CTLA-4) and the more recently identified programmed death ligand/programmed death-1 (PD-L:PD-1) interactions both define pathways which appear to function as negative regulators of lymphocyte activation.

CTLA-4 (CD152), in contrast to the positive effects of CD28:B7 interactions, has inhibitory effects on T cells and is important for the attenuation of T cell responses [15,16].

Table 1. **The B7-CD28 superfamily**

Ligand	Expression pattern	Receptor	Function
B7.1/B7.2	activated APC	CD28/CTLA-4	T cell activation/ tolerance
B7h (B7RP1)	B cells, macs, inflamed non-lymphoid	ICOS	Cytokine production, humoral immunity and tolerance
B7H1 B7H2	Activated T cells and B cells	PD-1	Inhibition of T cell proliferation and cytokine; tolerance
B7H3	Lymphoid and non-lymphoid	Unknown	>IFNγ production, cellular immunity
B7S1	Lymphoid and non-lymphoid	Unknown	Inhibition of T cell proliferation and IL-2
B7x	Activated B and T	BTLA	Inhibition of T cell proliferation and IL-2

Table 1. The B7-CD28 Superfamily.

The B7–CD28 superfamily provides critical secondary signals that serve to regulate the activation, inhibition and fine tuning of T-cell responses. The growing number of receptor-ligand pairs of the B7:CD28 family, as well as their tightly controlled tissue-specific and temporal expression, establishes a complex network of regulatory pathways that synergistically achieves proper T cell function and homeostasis.

CTLA-4 shares ~30% homology with CD28, and binds the same ligands, B7.1 and B7.2, on APCs. CTLA-4 is expressed uniquely on activated T cells, and its upregulation is dependent on CD28 costimulation. Importantly, CTLA-4 antagonizes the modulatory events mediated by CD28 signals, including T-cell proliferation and IL-2 production. The strongest evidence for the regulatory role of CTLA-4 comes from the phenotype of CTLA-4-deficient mice. These mice die at 2 to 4 weeks of age from a lymphoproliferative disease that is characterized by massive T cell activation and expansion which has pathogenic effects on multiple organs [17,18]. Furthermore, lymphoproliferative disease manifestation requires CD28 engagement as demonstrated by the fact that blockade of CD28:B7 with the fusion protein CTLA-4-Ig [19,20] or breeding mice deficient in both CTLA-4 and B7 ligands prevents disease [21].

While CTLA-4 appears to be a primary player in the negative regulation of T cell responses, the newly identified B7:CD28 superfamily molecules PD-L:PD-1 also possesses the ability to negatively regulate lymphocytes. The precise role for PD-L:PD-1 pathway in T cell function, the signaling pathways involved, and how PD-L:PD-1 differs from the B7:CTLA-4 pathway are just beginning to be elucidated (Table 2, adapted from [22]).

PD-1 was first characterized in a T cell hybridoma undergoing apoptosis, and accordingly was named PD-1 (programmed death 1) [23,24]. However, further studies indicated that expression is associated with cellular activation, not death, since transfection of PD-1 into cells or crosslinking with PD-1 specific antibodies does not result in apoptosis [24-26]. In

Table 2. **Comparison of CTLA-4 and PD-1** (adapted from [23])

Characteristic	CTLA-4	PD-1
Ligands	B7-1, B7-2	PD-L1,PD-L2
	Receptor Expression	
T cells	Activation induced expression	Activation induced expression
B cels	No	Up-regulated upon activation
Myeloid cells	No	Up-regulated upon activation
	Ligand expression lymphoid	
Location	B, T, DC, and monocytes	PD-L1 expressed on B, T, DC, and monocytes, PD-L2 on DCs. PD-L1 and -L2 expressed on epithelial cells in thymus, spleen and LN.
Regulation	B7-1 late activation induced B7-2 constitutive, induced by LPS, Ig, CD40, cytokine	Both induced by activation and IFN-γ treatment of monocytes and DCs.
	Function	
Proliferation and IL-2	Impairs	Impairs
Effector cytokine	Impairs	Impairs
Cell cycle	Block in G1-S	Block in G1-S
Tolerance	CD4+ peripheral tolerance	Putative role in B/T cell peripheral tolerance
Knock-out phenotype	Onset of phenotype by 2-3 weeks	Late onset phenotype (weeks - months).
	Early lethality due to multi-organ T cell infiltrates and tissue destruction.	Late lethality in 50% of mice associated with glomerulonephritis, arthritis, cardiomyopathy
	Defects in T cell proliferation, Th cytokine production, T cell cycle progression	Defects in T, B and myeloid cell proliferation.

Table 2. The newly identified B7:CD28 superfamily receptor-ligand pair PD-1:PD-L1 negatively regulates lymphocytes. While CTLA-4 appears to be a primary player in the negative regulation of T cell responses, the newly identified B7:CD28 superfamily receptor-ligand pair PD-1:PD-L1 also possesses the ability to negatively regulate lymphocytes. The precise role for PD-L:PD-1 pathway in T cell function, the signaling pathways involved, and how PD-L:PD-1 differs from the B7:CTLA-4 pathway are just beginning to be elucidated. The analysis of receptor and ligand expression patterns, as well as the phenotype of CTLA-4 or PD-1-deficient mice, would strongly suggest that these pathways serve mutually exclusive roles in the modulation of the immune system. One possibility is that the B7:CTLA-4 pathway functions primarily to attenuate, limit, and/or extinguish naive T-cell activation in secondary lymphoid organs. The PD-L1:PD-1 pathway, on the other hand, may primarily attenuate, limit, and/or extinguish secondary immune responses or T-, B-, and myeloid cell activation and effector function at sites of inflammation in the periphery.

contrast to CD28 and CTLA-4 which are predominately expressed in T cells, PD-1 is expressed by activated CD4+ and CD8+ T cells, B cells and myeloid cells [23 25].

PD-1−/− mice have been generated and provide convincing evidence for the inhibitory role of PD-1 [27-29]. Mice deficient in PD-1 are characterized by splenomegaly due to increases in myeloid and B-cell numbers [27]. B cell responses to α-IgM crosslinking are increased, but T-cell responses to α-CD3 appear normal. PD-1−/− mice develop autoimmune manifestations that differ based on the genetic background of the mouse. The PD-1-/- C57BL/6 strain develops progressive arthritis and lupus-like glomerulonephritis associated with high IgG3 deposition [28]. PD-1−/− mice generated on a Balb/c background B develop dilated cardiomyopathy with a 50% mortality rate [29]. Importantly, introduction of the RAG-2 mutation in PD-1−/− Balb/c mice prevents disease development, implicating T and/or B cells in the process.

PD-L1 and PD-L2 have been identified as two novel B7 homologues that interact with PD-1 [30-33]. PD-1 specifically associates with PD-L1 and PD-L2, and not with other B7:CD28 superfamily members. In contrast to B7-1 and B7-2, which are restricted to hematopoietic cells, the expression pattern PD-L1 and PD-L2 is diverse and has been detected in both lymphoid and non-lymphoid compartments [30,34,35]. PD-L1 and PD-L2 are upregulated upon activation or IFN-γ treatment of human monocytes and dendritic cells [30-33]. PD-L1 expression is induced on T cells stimulated via the TCR or by mitogen, and is constitutive on myocardial and microvascular endothelial cells [32]. Additional studies have shown that PD-L1 expression on an endothelial cell line is strongly upregulated by IFN-α, β

or γ [35]. PD-L1 upregulation on endothelial cells is abolished in IFN-γ-deficient mice, although constitutive expression is not affected.

Although T cells do not appear defective in PD-1 deficient mice, evidence that PD-1:PD-L1 plays a negative regulatory role in T cell function stems from many other lines of investigation. First, T cell proliferation and cytokine production by both resting and previously activated CD4+ and CD8+ T cells are inhibited by PD-L1–Ig or PD-L2–Ig fusion proteins coupled to α-CD3 coated beads [30,31,36]. Importantly, the inhibitory effect was not observed when PD-1−/− T cells were stimulated with α-CD3 mAb + PD-L1–Ig. This result indicates that the inhibitory signal was transduced by PD-1. Second, naïve CD8+ 2C-TCR transgenic T cells stimulated with cells expressing MHC class I and PD-L1, had significantly inhibited proliferative response and inhibition was PD-L1 dependent [36]. Exogenous IL-2, but not a CD28 costimulatory signal, could overcome PD-1:PD-L1 mediated inhibition. . PD-1 mediated inhibition of CD4+ T cells has also been shown [36]. However, CD4+ T cell inhibition could be overcome by a strong CD28 signal, possibly due to higher IL-2 production by CD4+ T cells [30,31,36]. In addition to CD28 costimulation, the effect of ICOS costimulation and cytokine receptor signaling on PD-1 inhibition have been determined [37]. In these studies, T cells were stimulated with α-CD3 or α-CD3/PD-L1.Fc coated beads in the presence or absence of ICOS or various cytokines (IL-4, IL-7, IL-15, and IL-21) and proliferation was measured. Interestingly, only IL-2, IL-7, and IL-15 could restore T cell proliferation in α-CD3/PD-L1.Fc stimulated cells. ICOS, IL-4 or IL-21 could not reverse PD-1 mediated inhibition [37]. These results show the capacity of the PD-L:PD-1 pathway to

antagonize a strong costimulatory signal when antigenic stimulation is weak or limiting and indicate a key role for the PD-L:PD-1 pathway in downregulating T-cell responses particularly in the absence of appropriate cytokine signaling.

The negative effect the PD-L:PD-1 pathway has on lymphocyte function suggests a potential role in peripheral tolerance. Polymorphisms in PD-1 have recently been associated with susceptibility to systemic lupus erythematosus and type I diabetes which further implicates PD-L:PD-1 in peripheral tolerance [38-40]. Consistent with this, experiments have shown that PD-1 mediated inhibitory signals can regulate both the induction and progression of experimental autoimmune encephalomyelitis [41] and autoimmune diabetes in non-obese diabetic (NOD) mice [38]. High PD-1 ligand expression has been detected on various tumors and is thought to correlate with an increased ability to escape immune surveillance [42]. Consistent with this notion, therapeutic blockade of PD-L1 leads to tumor regression but this can be PD-1-dependent or independent depending on tumor type [42-46].

Taken together, these data suggest a central role for PD-1 in peripheral tolerance as well as a possible role in tumor escape mechanisms.

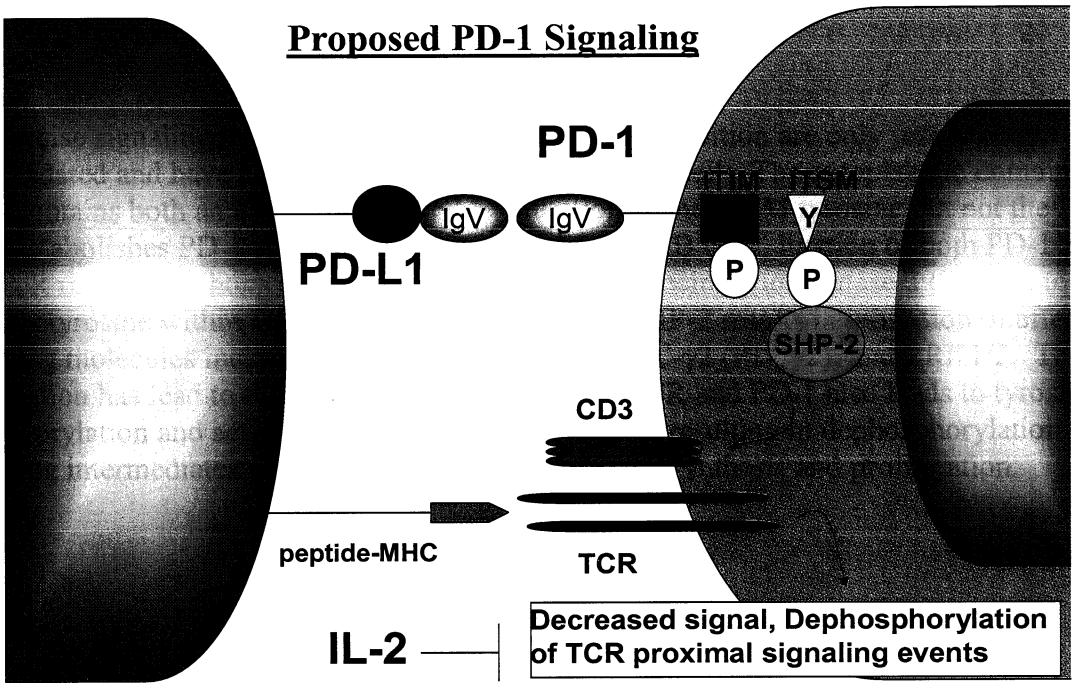
In T cells, PD-L:PD-1 interactions lead to cell cycle arrest in G_0–G_1 and a reduction in IL-2 synthesis, but not cell death [31,36]. The precise signaling requirements for PD-1 mediated inhibition are only just beginning to be elucidated and have been more clearly addressed in B cells. The cytoplasmic domain of PD-1 contains two tyrosines, one of which forms part of an Immunoreceptor Tyrosine-based Inhibition Motif (ITIM; V/IxYxxL/V) and the other forms an Immunoreceptor Tyrosine-based Switch Motif (ITSM; TxYxxV/I) [23,24,47].

Mutation of the ITSM tyrosine but not the ITIM tyrosine abolishes PD-1-mediated inhibitory activity In B cells, ligation of both PD-1 and the BCR recruits src homology 2-domain-containing tyrosine phosphatase 2 (SHP-2) to the phosphotyrosine within the ITSM (Figure 1, adapted from [22]). The activation of SHP-2 results in inhibition of effector signaling molecules including Igβ, Syk, phospholipase C-γ2 (PLCγ2), and ERK1/2 [48]. This observation has lead to the model that ligation of the TCR and PD-1 also leads to tyrosine phosphorylation and activation of SHP-2, resulting in dephosphorylation of signaling intermediates and ultimately reduced cytokine synthesis and proliferation.

Despite all of this data, the precise role of the PD-1:PD-L pathway in the immune system and how this interplays with the CTLA-4 pathway and other regulatory signals remains unclear. However, the analysis of receptor and ligand expression patterns, as well as the phenotype of CTLA-4 or PD-1-deficient mice, would strongly suggest that these pathways serve mutually exclusive roles in the modulation of the immune system. One possibility is that the B7:CTLA-4 pathway functions primarily to attenuate, limit, and/or extinguish naive T-cell activation in secondary lymphoid organs. The PD-L1:PD-1 pathway, on the other hand, may primarily attenuate, limit, and/or extinguish secondary immune responses or T-, B-, and myeloid cell activation and effector function at sites of inflammation in the periphery [13].

CD4+ T Cell Differentiation

Figure 1. Proposed PD-1 Signaling (adapted from [22])



*** Induced upon T cell activation and Upregulated on anergic T cells**

Sharpe AH, Freeman GJ. 2002.. *Nat Rev Immunol* 2: 116-

Figure 1. Proposed model of PD-1 signaling.

The precise signaling requirements for PD-1 mediated inhibition are only just beginning to be elucidated and have been more clearly addressed in B cells. The cytoplasmic domain of PD-1 contains both an ITIM and an ITSM. Mutation of the ITSM tyrosine but not the ITIM tyrosine abolishes PD-1-mediated inhibitory activity In B cells, ligation of both PD-1 and the BCR recruits src homology 2-domain-containing tyrosine phosphatase 2 (SHP-2) to the phosphotyrosine within the ITSM. The activation of SHP-2 results in inhibition of effector signaling molecules including Igβ, Syk, phospholipase C-γ2 (PLCγ2), and ERK1/2. This observation has lead to the model that ligation of the TCR and PD-1 also leads to tyrosine phosphorylation and activation of SHP-2 and/or SHP-1, resulting in dephosphorylation of signaling intermediates and ultimately reduced cytokine synthesis and proliferation.

After successful T cell activation, T cells rapidly undergo clonal expansion and differentiation into effector cells. Programmed differentiation controlled by the induction of unique sets of genes leads to highly polarized immune responses that are tailored to the specific invading pathogen. Naïve CD4+ T helper (Th) cells can differentiate into at least two major functional classes of effector T cells—Th1 and Th2 [49]. Th1 effector cells are responsible for cell mediated immunity and primarily secrete IFN-γ and IL-2 as well as TNF-α. Th2 cells are critical for extracellular mediated immunity and are characterized by the secretion of high levels of IL-4, as well as IL-5, -6, -9, -10, and -13. While many factors can influence the differentiation fate of a T cell including antigen dose, avidity of interaction, and costimulation, it is the cytokine milieu present at the time of activation that is thought to be most critical. Specifically, the cytokines IL-12 and IL-4 are key determinants in the Th differentiation pathway. Signaling through the IL-12 family, in concert with antigen stimulation, leads to the induction of a T box transcription factor, T-bet, that is essential for Th1 development and effector function [50-53]. Similarly, IL-4 instructs a T cell to differentiate along a Th2 pathway by the induction of a zinc finger transcription factor, GATA-3 [54-56].

T Regulatory Cells and Mechanisms of Peripheral Tolerance

While the vast majority of naïve CD4+ T cells will develop into Th1 or Th2 effector T cells upon activation, it is becoming increasingly more evident that there are distinct alternative T cell differentiation pathways possible [57-64]. One such CD4+ subset, are a

population of effector T cells with regulatory function [57-64]. Recently, these alternate T cell subsets have been the focus of intense study due to the fact that they appear to be essential for immune homeostasis and peripheral tolerance. T regulatory cells (Tr/Treg) can originate in the thymus or develop from activated peripheral T cells. "Natural" (Tr-n) Tr cells are CD4+ cells that constitutively express CD25, originate in the thymus and have a poorly understood, contact-dependent, cytokine-independent mechanism of action[61,65,66]. Tr-n cells, in addition to CD25, are defined by markers such as CTLA-4 [67,68], GITR [69-71] and FOXP3 [66,72,73]. FOXP3, in a fashion similar to that of T-bet and GATA-3, is critical for the development and function of Tr-n cells [66,72,73]. Several characteristics of Tr-n cells have emerged from *in vitro* studies (reviewed in [61]) resulting in the idea that Tr cells are anergic in terms of proliferation and IL-2 production and suppress other cells by direct cell contact, which requires neither the IL-10 or TGF-β they characteristically produce [74-76]. Interestingly, however, polyclonal CD25+CD4+ T cells proliferate, expand and acquire amplified regulatory capacity *in vivo* when they are transferred into RAG-/-or IL-2 receptor β-deficient mice, indicating that their anergic state can be reversed under certain in vivo conditions [77-80].

At least two other CD4+ Tr subsets exist and are generated in the periphery as opposed to the thymus. These subsets are generally referred to as "acquired" T regulatory cells (Tr-a). Tr-a cells are also considered anergic and may function via both cytokine and cell contact dependent mechanisms *in vivo*. One of these, T regulatory type-1 (Tr1) cells, produce predominantly IL-10 and to varying degrees IFN-γ and TGF-β [62,81,82]. Additionally, Tr1 cells do not express Foxp3 and are instead induced by stimulation in the presence of

immunosuppressive factors such as IL-10,Vitamin D3 plus dexamethasone and/or by a tolerogenic subset of DC [63,64,82-85]. Another Tr-a subset was identified following the induction of oral tolerance and produces predominantly TGF-β. These cells were named T helper-3 (Th3), or more appropriately T regulatory type -2 (Tr2) cells [86-88].

Interestingly, the PD-L:PD-1 negative regulatory pathway has been implicated in the generation of Tr2 cells [89]. This was shown by testing graft rejection after C57BL/10 hearts were transplanted into the CBA mice CBA (H-2k) mice pretreated with alloantigen plus intratracheal delivery of C57BL/10 (H-2b) splenocytes in the absence or presence of blocking antibodies specific for PD-1, PD-L1, or PD-L2. Pretreatment with antigen plus intratracheal delivery of C57BL/10 splenocytes prolonged graft survival significantly and was due to the generation of TGF-β producing Tr2 cells. In contrast, anti-PD-1 or anti-PD-L1 mAb, but not a-PD-L2, abrogated the prolongation of graft survival [89]. Therefore, in the context of this study, PD-1-PD-L1 is essential for induction of regulatory cells by intratracheal delivery of alloantigen. It remains to be determined what the role of PD-1:PD-L1 is in T regulatory cell induction and function. It is an intriguing possibility that negative signals mediated by this pathway are important for the generation of T cells with regulatory characteristics.

Whether T cells develop helper or regulatory function following antigen stimulation is multi-factorial and depends upon the affinity of the antigen for the T cell receptor (TCR), the costimulating properties of the antigen-presenting cells, and the cytokines in the microenvironment. Intermediate or high-affinity binding favors Th or Tr-n differentiation,

while altered peptide ligands with decreased binding affinity may favor the development of Tr-2 cells [90,91]. Additionally, antigen-presenting cells (APC), dendritic cells (DC) in particular, have a major role in T cell differentiation and this is dictated heavily by the cytokines they produce [92]. For example, specialized subsets of dendritic cells with unique cytokine and costimulatory properties, DC1 and DC2, are thought to control the generation of Th1 and Th2 cells, respectively [93,94]. Moreover, evidence suggests that either immature DC or IL-10 secreting dendritic cell subsets (DCr) direct naive T cells to a Tr1 subtype [94-98]. Since the production of IL-10 by the Tr1 cells could then program the generation of new tolerogenic DC (DCr) from progenitors, a self-maintaining negative regulatory loop may ensue which would require positive signals to reverse [98,99].

Sufficient evidence exists for T cell mediated regulation of peripheral tolerance and immune pathologies [100]. Insight into the mechanisms governing the suppression of immune reactivity may be gained by the study of regulatory T cells which appear dedicated to maintaining peripheral tolerance to self-antigens and preventing harmful immunopathological conditions. However, it is still not well understood how many different types of regulatory T cells might exist, how they develop and function *in vivo,* and whether their primary role is in peripheral tolerance.

The maintenance of peripheral tolerance is essential for the homeostasis of the immune system. Thus, the immune system has evolved numerous mechanisms to achieve peripheral tolerance. These include the negative regulation of T cell signaling through molecules such as CTLA-4 and PD-1 and the development of regulatory T cells, as well as

apoptosis and anergy. All of these mechanisms can act to modulate or down regulate immune responses at various times, locations and circumstances. Although tolerance can be defined and achieved in a number of different ways, the classical definition is that tolerance is an actively maintained state of unresponsiveness to a specific antigen in an animal previously exposed to the same antigen [101-103]. The *in vitro* definition, for which the term anergy is used, is defined as the inability of T cells to produce IL-2 and proliferate upon restimulation with antigen [101-104]. Anergy can be induced in naïve helper T cells when TCR engagement occurs in the absence of CD28 costimulation/IL-2 and is preventable by TCR/CD28 costimulation [103] as would be predicted from the 2-signal model of T cell activation. The 2 signal requirement then is also applicable *in vivo*. But it is important to point out, that the true mechanism and correlation of *in vitro* anergy to *in vivo* immune tolerance is not yet well understood. Clonal deletion, T cell apoptosis, and induction of immunoregulatory T cell subsets are some of the *in vivo* mechanisms that might substitute for anergy

Whether T cells are induced to become anergic, develop T helper or regulatory function, or undergo apoptosis following antigen stimulation is multifactorial and depends upon the affinity of the antigen for the TCR, the co-stimulating properties of the APC, and the cytokine milieu present within the micro-environment. One of the most important signaling pathways downstream of cytokine receptors in T lymphocytes is the Janus Kinase (Jak)-Signal Transducer and Activator of Transcription (STAT) pathway. It is therefore not

surprising that the JAK-STAT pathway has previously been implicated in influencing the fate of a T cell upon stimulation [10,105-111].

The goal of this thesis work is to more clearly elucidate the role for one of the members of the Jak family, Jak3, in CD4+ T cell homeostasis and function.

Cytokines---the universal T cell language

JAKS AND STATs

The Janus family of non-receptor tyrosine kinases is composed of four members, Jak1, Jak2, Jak3 and Tyk2, that constitutively associate with cell surface cytokine and growth factor receptors [112-114]. The signaling events mediated through Jaks are initiated through a related set of receptors [115]. The receptors can be composed of one to three chains (α, β, γ,) that can interact with one or more Jaks (Table 3, adapted from [116]).

Structurally Jaks are composed of seven regions of homology termed JH1 through JH7 [112] (Figure 2A). There are two kinase-like domains, JH1 and JH2. Mutational studies have indicated that functional catalytic activity lies primarily within the JH1 domain. The function of the pseudokinase JH2 domain is unclear as some studies have shown it is necessary for protein activity and others indicate that it is an inhibitory

domain [117-119]. Further studies have suggested that the JH2 domain serves as a potential docking site for the STAT transcription factors [118]. The exact function of the other five

Table 3. Cytokine specific JAK and STAT activation (adapted from[116])

Ligands	Jak kinases	STATs
IFN family		
IFN-α/β	Tyk2, Jak1	Stat1, Stat2, (Stat3, Stat4, Stat5)
IFN-γ	Jak1, Jak2	Stat1, (Stat5)
IL-10	Tyk2, Jak1	Stat3
IL-19	?	?
IL-20	?	Stat3
IL-22	?	Stat3, (Stat5)
gp130 family		
IL-6	Jak1, Jak2	Stat3, Stat1
IL-11	Jak1	Stat3, Stat1
OSM	Jak1, Jak2	Stat3, Stat1
LIF	Jak1, Jak2	Stat3, Stat1
CNTF	Jak1, Jak2	Stat3, Stat1
NNT-1/BSF-3	Jak1, Jak2	Stat3, Stat1
G-CSF	Jak1, Jak2	Stat3
CT-1	Jak1, Jak2	Stat3
Leptin	Jak2	Stat4
IL-12	Tyk2, Jak2	Stat4
IL-23	?	Stat4
γC family		
IL-2	Jak1, Jak3	Stat5, (Stat3)
IL-7	Jak1, Jak3	Stat5, (Stat3)
(TSLP)a	?	Stat5
IL-9	Jak1, Jak3	Stat5, Stat3
IL-15	Jak1, Jak3	Stat5, (Stat3)
IL-21	(Jak1), Jak3	Stat3, Stat5, (Stat1)
IL-4	Jak1, Jak3	Stat6
(IL-13)a	Jak1	Stat6, (Stat3)
IL-3 family		
IL-3	Jak2	Stat5
IL-5	Jak2	Stat5
GM-CSF	Jak2	Stat5
Single chain family		
EPO	Jak2	Stat5
GH	Jak2	Stat5, (Stat3)
PRL	Jak2	Stat5
TPO	Jak2	Stat5
Receptor tyrosine kinases		
EGF	(Jak1, Jak2)	Stat1, Stat3, Stat5
PDGF	(Jak1, Jak2)	Stat1, Stat3
CSF-1	(Tyk2, Jak1)	Stat1, Stat3, Stat5
G-protein coupled receptors		
AT1	Jak2	Stat1, Stat2

a- Bind to related but γc independent receptors.

Table 3. The Janus family of non-receptor tyrosine kinases is composed of four members, Jak1, Jak2, Jak3 and Tyk2.

The Janus family of non-receptor tyrosine kinases is composed of four members, Jak1, Jak2, Jak3 and Tyk2 that constitutively associate with cell surface cytokine and growth factor receptors. The signaling events mediated through Jaks are initiated through a related set of receptors. The receptors can be composed of one to three chains (α, β, γ,) that can interact with one or more Jaks resulting in the ability of this family of proteins to mediate a myriad of pleiotropic cellular functions.

Figure. 2 Structure of Jak and STAT proteins

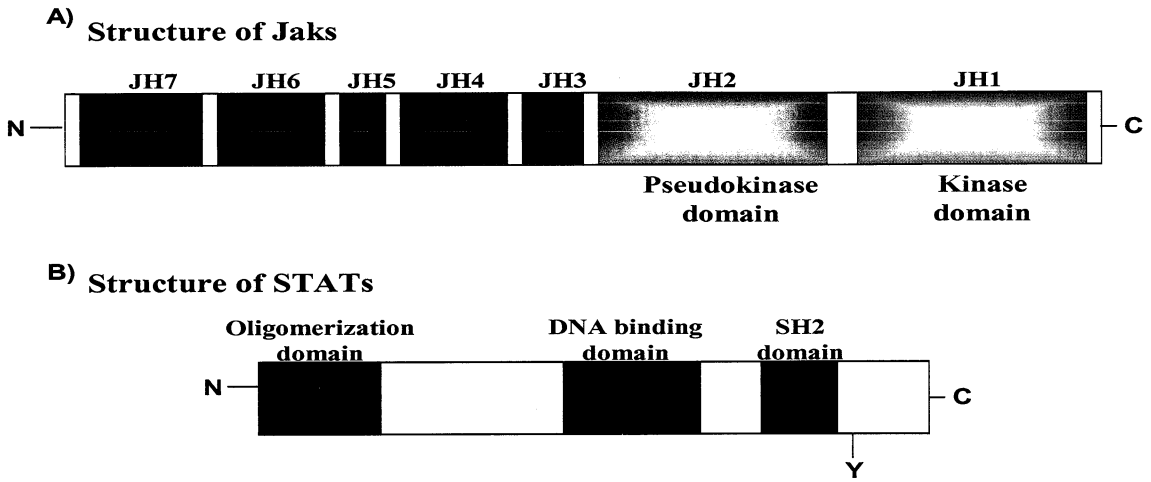


Figure 2. Structure of Jaks and STATs.

A) Jaks are composed of seven regions of homology termed JH1 through JH7. There are two kinase-like domains, JH1 and JH2. Mutational studies have indicated that functional catalytic activity lies primarily within the JH1 domain. The function of the pseudokinase JH2 domain is unclear as some studies have shown it is necessary for protein activity and others indicate that it is an inhibitory domain. Further studies have suggested that the JH2 domain serves as a potential docking site for the STAT transcription factors. The exact function of the other five blocks of homology (JH3 through JH7) remains unclear, however, the N-terminal region has been shown to bind cytokine receptors.

B) The STAT family of transcription factors is composed of seven members, STAT1, STAT2, STAT3, STAT4, STAT5a and STAT5b, and STAT6. All the STAT proteins share common functional domains, including a Src-homology 2 (SH2) domain, oligomerization domain and DNA binding domain. The Src-homology 2 (SH2) domain recognizes phosphorylated tyrosine residues within the cytoplasmic tails of receptor subunits. Upon STAT recruitment to a phosphorylated/activated receptor the tyrosine residue (Y) in the SH2 domain is itself phosphorylated. The activation of STAT proteins then results in homodimerization of STAT proteins which is mediated by the oligodimerization domain. After homodimerization, STAT proteins are translocated to the nucleus where they mediated target gene transcription via the DNA binding domain. The highly homologous family of STAT transcription factors controls the expression of many genes and cellular functions. The pleiotropic nature of the STAT family of proteins is achieved by differences in the SH2 domain docking site recognition and DNA binding domains which allow for selective binding to receptors and DNA targets respectively

blocks of homology (JH3 through JH7) remains unclear, however, the N-terminal region has been shown to bind cytokine receptors [120-123].

The essential role for Jaks in cytokine receptor signaling was first illustrated by experiments which showed crosslinking IL-2,-4, or -7 resulted in Jak3 phosphorylation and that cell lines deficient in Jaks fail to respond to interferons (IFNs) [124,125]. The role for Jaks in cytokine signaling was further confirmed by cytokine receptor mutations that abolished Jak binding and the transfection of dominant negative forms of Jak into cell lines [126-129].

STAT proteins are downstream from Jaks in cytokine mediated signaling and one of the primary functions of Jaks is the activation of STATs. The STAT family of transcription factors is composed of seven members, STAT1, STAT2, STAT3, STAT4, STAT5a and STAT5b, and STAT6 [130]. All the STAT proteins share common functional domains, including a Src-homology 2 (SH2) domain that recognizes phosphorylated tyrosine residues within the cytoplasmic tails of receptor subunits (Figure 2B). The highly homologous family of STAT transcription factors controls the expression of many genes and cellular functions. The pleiotropic nature of the STAT family of proteins is achieved by differences in the SH2 domain docking site recognition and DNA binding domains which allow for selective binding to receptors and DNA targets respectively [114,115,131,132].

Receptor occupation by cytokine leads to dimerization of the receptor subunits and the activation of Jaks via auto- and trans-phosphorylation events [113,130,133] (Figure 3). Once activated, Jaks phosphorylate the cytokine receptor chains which generates a docking site for the SH2 domain of STATs [134,135]. Upon STAT association with the receptor, Jaks

Figure 3.

Jak-STAT signaling pathway

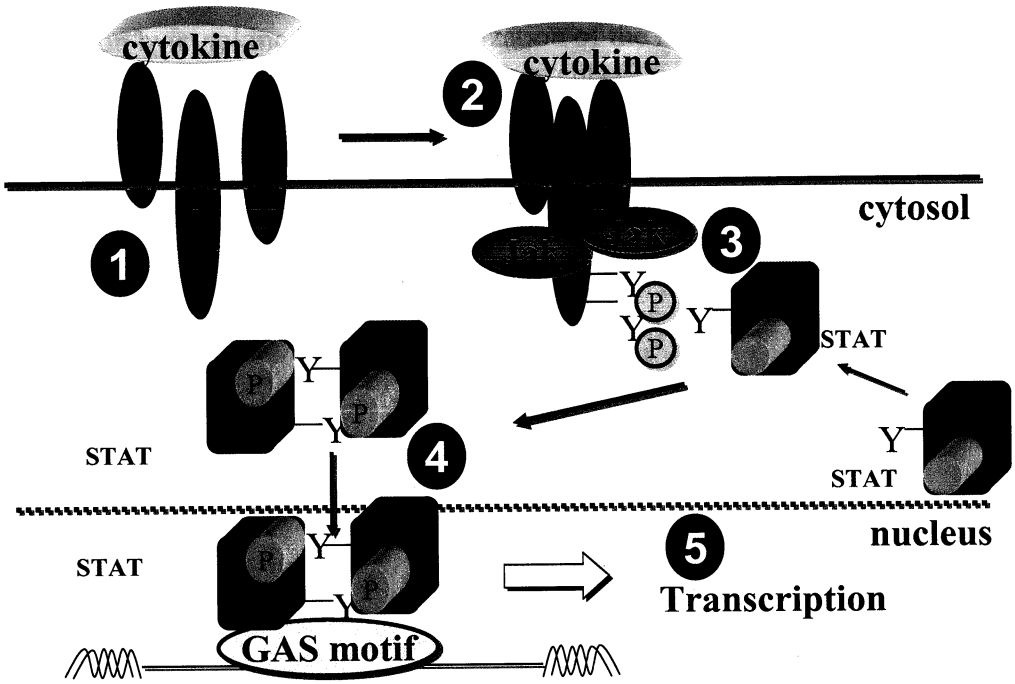


Figure 3. The JAK-STAT Signaling Pathway.

(1)Receptor occupation by cytokine leads to dimerization of the receptor subunits and the activation of Jaks via auto- and trans-phosphorylation events **(2)**. Once activated, Jaks phosphorylate the cytokine receptor chains which generate a docking site for the SH2 domain of STATs **(3)**. Upon STAT association with the receptor, Jaks phosphorylate and activate STAT **(3)** leading to STAT homodimerization **(4)** and translocation into the nucleus where target gene activation will occur **(5)**.

phosphorylate and activate STAT leading to STAT homodimerization and translocation into the nucleus where target gene activation will occur [136,137]. While the cytokine driven dimerization and activation of STATs are well accepted, there are alternate possibilities. Homodimerization of STATs is critical for their function but other forms may exist and participate in transcriptional activation. First, the N-terminal domain of the STAT protein has been shown to function in oligomerization of STAT dimers leading to tetramer formation and even higher ordered oligomerization [138]. Interestingly, STAT N-terminus dimerization that is independent of cytokine receptor-mediated signals and phosphorylation has recently been shown to occur *in vivo* [139]. The role these dimers play in STAT activity is uncertain but might include the promotion of interactions with cytokine receptors, STAT homodimer formation and/or phosphorylation.

Jak proteins have additionally been implicated in the activation of the extracellular signal-regulated kinase subfamily of the mitogen-activated protein kinases (ERK/MAPK) pathway as well as PI-3 kinase activity following cytokine binding [140,141 142,143 144]. Thus, Jak mediated signals appear to function as important upstream modulators of both the STAT and the ERK/MAPK signaling pathways as well as PI-3 kinase activity.

Similar to the down regulation of TCR driven signals by CTLA-4 and PD-1, cytokine mediated signals are also tightly controlled and attenuated in order to maintain proper balance and function of the immune system. Negative regulation of Jak-STAT mediated cytokine signals is accomplished by several mechanisms including endosomal degradation of

Jak/receptor complexes through receptor-mediated endocytosis, and the regulation by the SOCS family of cytokine suppressors and tyrosine phosphatases [131,145-149].

Janus Kinase 3

Jak3 is unique among the members of the Janus kinases family because it is preferentially expressed in hematopoietic cells, where it constitutively binds to the cytokine receptor γ_c chain [126,127,150,151]. The family of cytokines that are Jak3/γc dependent include IL-2, -4, -7, -9, -15, and -21. Signals mediated by these cytokines have a myriad of effects on lymphocytes (Figure 4) and are critical for T cell development and homeostasis and at all phases of effector function (Figure 5, reviewed in and adapted from [152]).

Initially, the notion that Jak3 mediates signals through γc receptors was based on the observation that Jak3 deficient mice had a phenotype that was identical to that of mice lacking the γ_c chain [153,154]. The essential role for both Jak3 and γc was illustrated by the fact that defects in either of these molecules leads to severe combined immunodeficiency (SCID) in both humans and mice [153-158]. Additionally, by characterizing the phenotype of mice deficient in single γ_c-cytokine or γ_c-cytokine receptor subunits, it has been possible to more clearly address the role for specific γc cytokines and signaling mediators during lymphocyte development, homeostasis, and function (Table 4).

Jak3 deficient mice exhibit many immune abnormalities which lament the importance of this signaling pathway in the proper function of the immune system. First, mature B cells

Figure 4. **Jak3 dependent cytokine signaling**

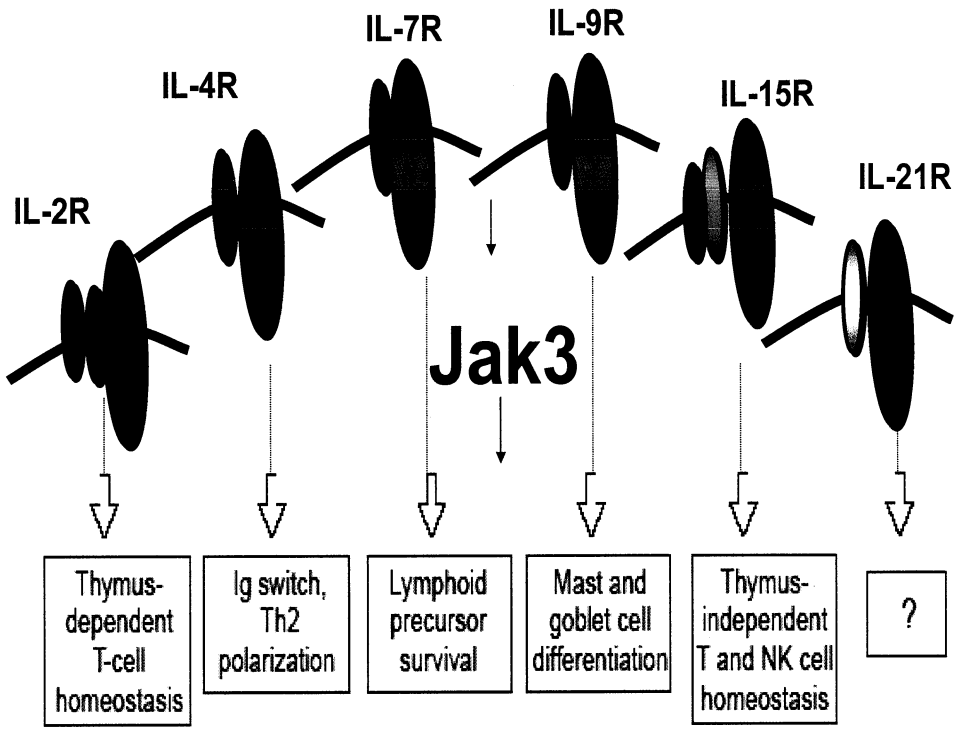


Figure 4. γc mediated cytokine signaling is critical for the development and function of the immune system.

The family of cytokines that are Jak3/γc dependent include IL-2, -4, -7, -9, -15, and -21. Signals mediated by these cytokines have a myriad of effects on the immune system including the survival of lymphoid precursor subsets; B cell development, survival and immunoglobulin class switching; T cell development, survival, differentiation, and homeostasis; and NK cell development.

Figure. 5

γc signals influence the proliferation & survival of naïve, effector and memory T cells (adapted from [152])

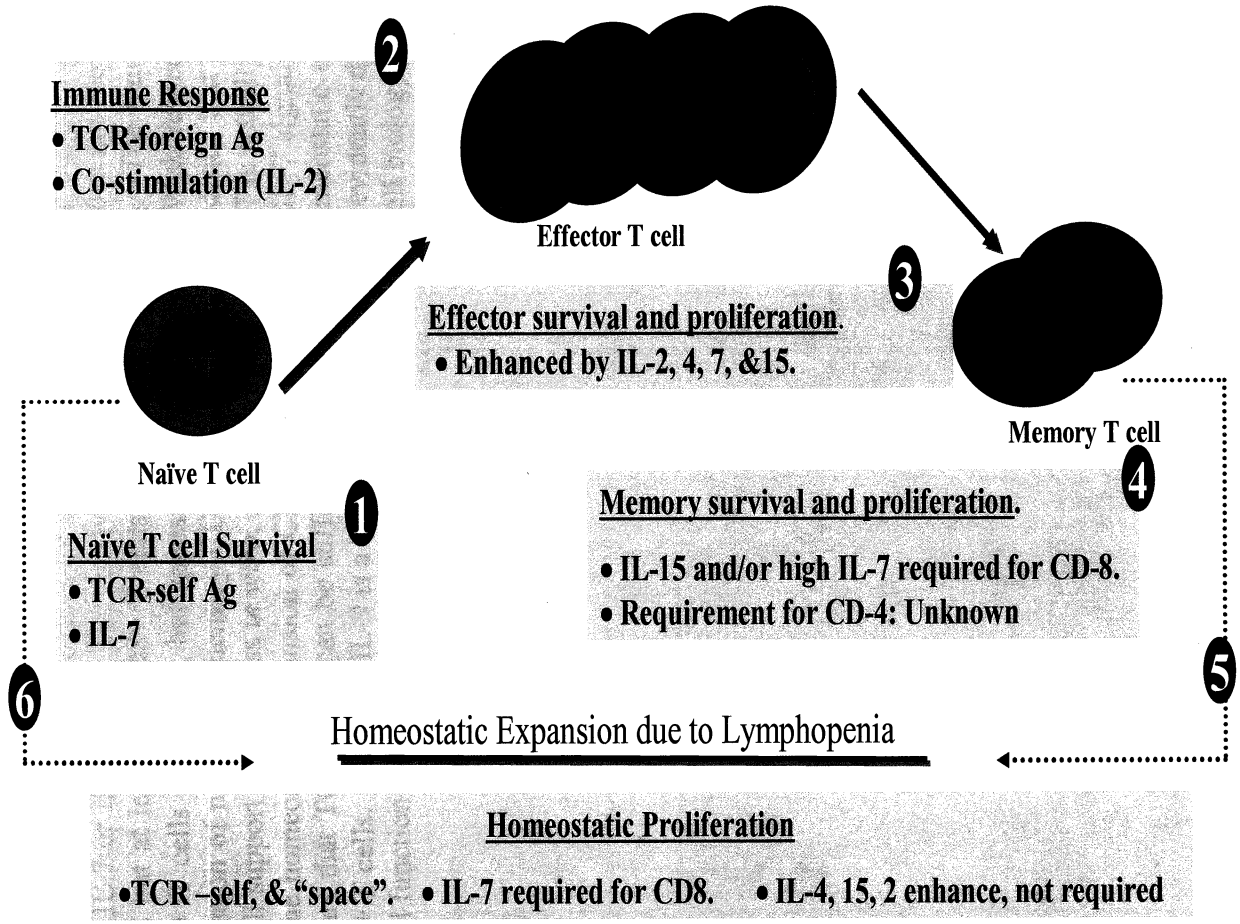


Figure 5. γc factors influence the survival and proliferation of naïve, effector, and memory T cells.

(1)IL-7 is the most critical cytokine signal for T cell development and naïve T cell survival. Although both IL-4 and IL-7 have been implicated in supporting naive T-cell survival *in vitro*, IL-7 along with TCR-MHC interactions appear to have the dominant role *in vivo*. **(2)** To achieve T cell receptor specific stimulation interaction with foreign Ag-MHC plus co-stimulatory signals mediated ultimately by IL-2 are necessary. **(3)**Activated or effector T cells do not require γc cytokines for survival and proliferation but both are enhanced by IL-2, IL-4, IL-7 or IL-15. **(4)** The survival of memory T cells does not require interactions with MHC but at least for CD8+ T cells is dependent on IL-15. The requirement for memory CD4+ T cells is unclear, but appears γc independent. **(5)**With respect to the homeostatic expansion of naïve and memory CD8+ T cells, there is evidence that IL-4, IL-7 and IL-15 can all support this process *in vitro*, although again only IL-7 is actually required *in vivo*. (6) For the homeostatic expansion of CD4+ T cells, TCR and IL-7 signals are important but it appears that TCR alone can be sufficient to support the homeostatic expansion of memory CD4+ T cells. Although IL-9 is a T cell growth factor, it is evidently dispensable for normal T-cell function and homeostasis, and the role of IL-21 in T cell biology is still unclear.

Table 4. γc cytokines, their receptors and function

Ctyokine	Receptors	Producer Cells	Actions	Effect of cytokine or receptor knockout
IL-2	CD25(α) CD122(β) CD132(γc)	T cells	T cell growth factor, proliferation	IL-2: deregulated T cell proliferation IL-2α: incomplete T development IL-2β: increased T cell autoimmunity IL-2γc: SCID
IL-4	CD124 CD132(γc)	T cells, mast cells	B cell activation, IgE switch, suppresses Th1 cells	IL-4: decreased IgE synthesis
IL-7	CD127 CD132(γc)	Non T cells	Growth of pre B and pre T cells	IL-7 early thymic and lymphocyte expansion imparied
IL-9	IL-9R CD132(γc)	T cells	Mast cell enhancement, stimulates Th2 cells	ND
IL-15	IL-15R CD122 (IL-Rβ) CD132(γc)	Non T cells	Stimulates growth of intestinal epithelium, T cells and NK cells	
IL-21				

Table 4. γc mediated cytokine signaling is critical for the development and function of the immune system---lessons learned from mice deficient in γc cytokines or receptors. The phenotype of mice deficient in single γ_c-cytokine or γ_c-cytokine receptor subunits has lead to a more clear understanding of the role(s) for specific γc cytokines and signaling mediators during lymphocyte development, homeostasis, and function and underscores the importance of the γc family of cytokines in immune system development and function.

in Jak3-deficient mice are absent due to developmental arrest at the pro-B cell stage [153,154]. This defect has also been observed in mice that lack the γ_c and IL-7-mediated signals, indicating an essential role for IL-7 in murine B cell development [159,160]. This is consistent with data that IL-7 regulates events that are thought to be important in B cell development such as transcription and rearrangement of the IgH gene locus, and expression of anti-apoptotic factors, such as Bcl-2 [161,162].

NK cell development is also impaired and these cells are absent in the peripheral lymphoid organs of Jak3-/- mice. NK cell development appears to be dependent on IL-15 since mice deficient in IL-15, but not IL-7 or IL-2 also have a block in NK cell development. Additionally, when the effect of either IL-2, -7 or -15 on NK cell differentiation was assessed, only IL-15 could induce NK cell differentiation from bone marrow progenitor cells [163,164], correlating with the requirement for IL-15 in NK development.

In addition to defects in B and NK cells, Jak3 deficient mice have abnormalities in T cell development, homeostasis, and function. The thymus of a Jak3-/- mice is drastically reduced in cellularity to approximately one tenth the size of wild type. A reduction is thymic cellularity is also seen in γc deficient mice and can be at least partially explained in both cases by the fact that there are fewer numbers of thymocyte progenitors. In addition to fewer progenitor numbers, there may be defects in both survival and progression through the stages of T cell development in Jak3 deficient mice. This is suggested by data from IL-7 -/- and γc -/- mice. In the absence of IL-7, transition from the pro-T cell stage to the pre-T cell

stage is blocked [160,165]. The block may at least in part be due to insufficient survival signals in the absence of γc signaling. Evidence for this is illustrated by the fact that in γc-/- mice, upregulation of Bcl-2 expression typically required to transition from DN $CD44^+CD25^-$ to DN $CD44^+CD25^+$ and from DP TCR^{lo} to DP TCR^{int} is reduced [161,166]. Furthermore, higher apoptotic indexes have been shown in DN and $CD4^+/CD8^+$ SP thymocytes in γ_c-/- mice, correlating with reduced levels of Bcl-2 expression [167]. In total, this data suggests that γ_c-mediated signals, especially IL-7, play a vital role in T cell development which may depend on induction of Bcl-2 to provide anti-apoptotic signals during development [168].

Mice deficient in γ_c, Jak3, IL-7R, and IL-15 completely lack γδ T cells [169,170]. Interestingly, γ chain rearrangement in thymocytes could not be detected in the thymus of these mouse models. This suggests that γc function, in addition to mediating survival signals, is required for the rearrangement of the TCRγ locus [169,170]. Thus, the importance of γc signals in the development and function of T cells expands beyond mediating survival signals and is additionally required for the rearrangement and expression of the TCRγ chain. Whether γc mediated signals are required for γδ T cell survival and the specific γc cytokine necessary for the survival of γδ T cells is unclear. In support of a role for γc in survival, γδ T cell numbers are still reduced in γc-deficient mice crossed to a transgenic mouse with a functionally rearranged TCRγ gene [169]. In contrast to this, other research has shown that high copy transgenic expression of the TCRγ chain in IL-7R-/- mice results in the presence of normal numbers of γδ T cells. These results are consistent with the idea that the γc/IL-7R

signals are essential for the rearrangement of the TCRγ chain but not for the survival of γδ T cells [171].

Despite a reduction in number of thymic progenitors, and absence of γδ T cells, the stages of αβ T cell development in γ_c-, Jak3-, and IL-7-deficient mice progress normally suggesting that Jak3-mediated signals are important but not essential for murine αβ T cell development [107,153,172].

In the peripheral lymphoid organs of Jak3-/- mice there are wild type numbers of αβ T cells [153-155]. Interestingly, however, these cells are predominantly CD4+ T cells with a virtual absence of CD8+ T cells. Moreover, the CD4+ T cells have a phenotype consistent with previously activated or memory T cells in that they are large and express surface markers associated with prior activation in particular CD44 (CD44-high, CD25-low, and CD69-low) [153]. The absence of naïve peripheral T cells in Jak3-/- mice is consistent with the notion that Jak3 mediated signals through IL-7 are essential for survival of both naïve CD4+ and CD8+ T cells [173,174].

In Jak3 neonatal mice (d.10), there are reduced numbers of peripheral CD4+ T cell but these cells accumulate over time (d. 25) [153]. This suggests that Jak3-/- CD4+ T cells undergo expansion in the periphery, leading to an increase in overall CD4+ T cell numbers and the acquisition of a memory phenotype. The skewed CD3 spectratype observed in Jak3-/- T cells suggests that activation and expansion of Jak3-/- T cells bearing heterogeneous TCRs is oligoclonal and thus dependent on receptor specific stimulation [175]. However, there are alternative hypotheses that are not necessarily mutually exclusive. Accumulation of

CD4+ CD44hi T cells in Jak3 deficient mice may be due to lymphopenia induced homeostatic expansion. A lymphopenic condition leading to an increased degree of homeostatic expansion may occur in Jak3-/- mice due to a reduction in the number of thymic emigrants as well as an increase in naïve peripheral T cell death in the absence of γc signals. Furthermore, IL-2 signals have been implicated in the development of CD4+CD25+ regulatory T cells which are critical in peripheral tolerance and homeostasis [176,177]. A reduced population of CD4+CD25+ T cells could then account for increased or uncontrolled expansion of T cells in Jak3 deficient mice.

The absence of naïve CD4+ and CD8+ T cells and marked reduction in mature CD8+ T cells in Jak3-/- is not unexpected since γc cytokines have been shown to be essential in the survival of these T cell subsets. Numerous studies have demonstrated that signals mediated by IL-7 but not others, are essential for the survival of naïve $CD4^+$ and $CD8^+$ T cells [173,174]. Additionally, IL-7 is required for the homeostatic proliferation of naïve CD8+ and may augment these events in CD4+ T cells [173,174,178-180]. Both IL-15 and IL-7 are thought necessary for the survival and homeostatic proliferation of $CD8^+$ memory T cells [178,181-183]. Consistent with the phenotype in Jak3 deficient mice, both the survival and homeostatic proliferation of memory $CD4^+$ T cells have been shown to be independent of γ_c cytokines [174,178]. However, both survival and homeostatic proliferation of CD4+ T cells can be enhanced in the presence of IL-7 and possibly other γc mediated signals [109,184].

Although there are abundant numbers of CD4+ T cells in Jak3 deficient mice, these cells are functionally defective. In addition to the fact that Jak3-/- CD4+ T cells are

predominately memory or CD44-high T cells, these cells fail to proliferate or secrete IL-2 in response to either mitogenic or TCR stimulation and thus appear to be anergic *in vitro*. Despite this, previous data indicated that Jak3-/- T cells were not entirely unresponsive in that upon *in vitro* stimulation these cells could induce transcripts for other cytokines, including IL-10 and IFN-γ [185].

The overall objective of this thesis work was to gain further insight into the role Jak3 plays in the homeostasis and function of CD4+ T cells. Specifically, the present studies were initiated to begin to address the molecular mechanisms which lead to atypical CD4+ T cell function (i.e. loss of proliferation and IL-2 production) following expansion *in vivo* in the absence of Jak3. Studying these events will not only provide a more clear understanding of the role Jak3 plays in the immune system, but importantly, will shed light on potential mechanisms of immune tolerance or attenuation when there is incomplete or inappropriate T cell stimulus. Thus, an additional goal of these studies was to determine what functional capacities, if any, in vivo expanded Jak3 deficient T cells either maintain or develop. To this end, the specific aims addressed in the thesis work are as follows:

1. Chapter Three: "The upregulation of inhibitory receptors on Jak3-/- CD4+ T cells along with a concomitant down-regulation of transcription factors and TCR linked cell cycle factors." In chapter three, potential molecular mechanisms involved in Jak3-/- mediated atypical T cell function were assessed by determining global differences in gene expression between functional wild type CD4+ CD44-high T cells and non-functional Jak3-/- CD4+

CD44-high T cells. Global gene expression was determined by gene array analysis. Differential gene expression between these responsive and non-responsive cell types suggested that Jak3 deficient CD4+ CD44-high T cells have an increase in RNA expression for receptors known to be inhibitory to T cells including PD-1, LAG-3 and TJ6. In addition, the expression of two transcription factors which are important for proper T cell function, SATB1 and LKLF, were decreased. We also observed a decrease in the expression of two kinases, Pim-1 and Cis, which have been implicated in TCR mediated cell cycle induction. Despite this however, there was not a global defect in the expression of cell cycle proteins and to the contrary we observed an upregulation in the expression of basal cell cycle factors.

2. Chapter Four: "The role of the PD-1:PD-L1 co-inhibitory pathway and other B7:CD28 superfamily molecules in the unresponsiveness of Jak3-/- CD4+ CD44-high T cells." Given the upregulation of PD-1 mRNA expression in Jak3-/- CD4+ CD44-high T cells, we began to investigate the potential contribution of this pathway in down modulating the function of Jak3-/- T cells. Our results suggest that both the PD-1 receptor and its ligand, PD-L1, are highly up-regulated at the protein level in the Jak3-/- splenic microenvironment. Up-regulated PD-1 receptor expression is limited to the CD4+ splenocytes whereas PD-L1 expression is present within the CD4 negative subset of splenocytes. Importantly, the PD-L1+ Jak3-/- splenocytes can mediate inhibition of *in vitro* stimulated Jak3+/- CD4+ T cells suggesting the PD-L1 on these cells is functional and that this fraction of cells can interact with T cells. Analysis of the expression of other B7:CD28 members, such as CTLA-4,

before and after in vivo T cell stimulation revealed that there was a general defect in the expression of other costimulatory molecules in Jak3-/- T cells. The absence of CTLA-4 in Jak3-/- T cells further supports the notion that the PD-1:PD-L1 pathway is a primary negative pathway which is acting to suppress the function of Jak3 deficient T cells.

3. Chapter Five: "Jak3-/- CD4+ CD44-high T cells have characteristics of Tr1 regulatory T cells." An additional interesting trend observed by microarray analysis was the expression of a unique cytokine profile in Jak3-/- CD4+ CD44-high T cells. Based on RNA expression, Jak3-/- CD4+ CD44-high T cells more highly induced an immunosuppressive panel of cytokines than Jak3+/- CD4+ CD44 high T cells. This cytokine profile which included IL-10, IFN-γ, and TGF-β is shared by subsets of T cells known to have T cell regulatory function indicating the possibility that Jak3-/- CD4+ T cells had differentiated into regulatory-like T cells. To more thoroughly investigate this possibility we measured both cytokine production and the ability of Jak3 -/- T cells to inhibit the proliferation of Jak3+/- CD4+ T cells. Our results suggest that Jak3-/- CD4+ T cells have regulatory qualities which based on current literature is likely to be most consistent with a Tr1 subset of regulatory T cells.

4. Chapter Six: "The phenotypic defects of Jak3-/- CD4+ T cells are cell autonomous." In addition to abnormal T cell function, Jak3 deficient mice are characterized by an absence of B cells, NK cells, γδ T cells and also lack CD8αα dendritic cells. These

gross abnormalities in Jak3-/- mice ultimately lead to a disruption in splenic architecture and the potential loss of interactions necessary for proper T cell function. Therefore, the absence of or defect in immune accessory cells could be responsible for the Jak3-/- T cell phenotype. To test this hypothesis we isolated CD4 SP thymocytes from Jak3 deficient mice which have been previously determined to function comparably to wild type with regards to proliferation and IL-2 production. Jak3-/- CD4 SP thymocytes were injected intravenously into RAG-/- mice where expansion and differentiation could occur in the presence of functionally replete immune accessory cells that have intact Jak3 signaling. In summary of this data, Jak3-/- CD4 SP thymocytes which expand in the presence of Jak3+ accessory cells develop a phenotype consistent with peripheral T cells isolated from unmodified 8 week old Jak3-/- mice suggesting that the T cell defects are cell intrinsic.

5. Chapter Seven: "Comparison of Global Gene Expression Patterns in wild type, Jak3-/-, STAT5ab-/- and lymphopenia-induced homeostatically expanded CD4+ CDD44-high T cells." The global gene expression patterns of STAT5ab-/- CD4+ CD44-high cells and Jak3-/- CD4+ CD44-high cells suggest a role for IL-10 mediated disruption of RAS-RAF mediated MAPK activation in the inhibition of IL-2 production in Jak3-/- peripheral CD4+ T cells. By expanding the populations of CD4+ CD44-high T cells in our microarray analyses to include CD4+ CD44-high T cells from STAT5ab deficient mice which have defects in proliferation but not IL-2 production and thus are only partially unresponsive, we were able to observe a dichotomy between gene expression and induction of genetic programs that are

potentially responsible for the defect in IL-2 production versus the defect in proliferation in Jak3-/- T cells. This data indicates a distinct down regulation in Mef-2C and Elk-1 uniquely in the Jak3-/- CD4+ T cells. Mef-2C and Elk-1 are downstream targets of the RAS-RAF mediated MAPK pathway and are important in the establishment of the AP-1 complex and thus IL-2 synthesis. Since, IL-10 has been determined to specifically block RAS-RAF activation; these data establish a link between the induction of immunosuppressive genetic programs mediated by IL-10 and potentially TGF-β and IFN-γ and the unresponsive phenotype of Jak3-/- CD4+ CD44-high T cells. We next hypothesized that upon in vivo expansion Jak3-/- T cells underwent typical attenuation mechanisms necessary to maintain control of immune responses such as PD-1 induction. However in contrast to a wild type T cell, in the absence of Jak3/γc signals these attenuation mechanisms dominate and thus lead to unresponsiveness in Jak3 deficient T cells. Consistent with this, microarray analysis of CD4+ CD44-high T cells derived from RAG2-/- mice that received wild type CD4+ T cells 3 weeks prior, display similar upregulation of PD-1. In addition, homeostatically expanded CD4+ T cells have moderate increases in expression of IL-10, TGF-β, and IFN-γ. Interestingly, these T cells do not exhibit decreased expression of Mef-2C and Elk-1.

In total, this work suggests that the importance of Jak3 in CD4+ T cells is to overcome or temper typical attenuation mechanisms. In the absence of Jak3 mediated signals, CD4+ T cells can undergo primary expansion and IL-2 production. However, this will result in the induction of modulatory factors such as surface receptors and cytokines

which act to shut down the response. In the absence of positive signals mediated by Jak3, the switches that shut down the immune response cannot be overcome and therefore dominate. The maintenance of these negative signaling pathways then reset genetic programs which lead to the differentiation of T cells with regulatory characteristics.

Chapter II.

MATERIALS and METHODS

MATERIALS and METHODS:

Mice:

Jak3-/- and Jak3+/- mice [153] have been described previously. Mice have been backcrossed to C57BL/6 a minimum of 8 generations. Jak3-/- mice and littermate controls were used at 8-10 weeks of age and maintained in a specific-pathogen-free facility. RAG2-/- mice (Taconic, Germantown, NY) were used for the adoptive transfer experiments.

T cell purification:

Spleens or thymuses were removed from 8- to 10-wk-old Jak3-/-, and littermate controls. *For CD4+ T cell purification,* after RBC lysis of total splenocytes, single-cell suspensions were incubated with α-CD4-coated magnetic microbeads and passed through AutoMACS LS^+ columns according to the manufacturer's protocol (Miltenyi Biotec, Auburn, CA).To isolate CD4+ CD44-high T cells the resulting CD4+ enriched population of cells were then stained with antibodies to CD4 (α-CD4- FITC, BD PharMingen, San Diego, CA) and CD44 (α-CD44-CyChrome (Cy), BD PharMingen, San Diego, CA) and sorted for CD4+ CD44-high cells on a BD Biosciences FACStar. *For CD4 single positive (SP) T cell enrichment,* after RBC lysis of total thymocytes, single cell suspensions were incubated with anti-CD8-coated magnetic microbeads and passed through AutoMACS LS+ columns as previously described. The negative fraction was then used as enriched CD4+ thymocytes. T cell

isolation methods used typically yielded CD4+ CD44-high peripheral T cells with greater than 95% purity and thymic CD4 SP cells of approximately 70% purity.

Abs and flow cytometry:

Cells were stained with the indicated Abs in HBSS supplemented with 3% FCS for 20 min on ice. Cells were then washed and analyzed on a BD Biosciences FACSCalibur. Data were analyzed using CellQuest software (BD Immunocytometry Systems, San Jose, CA). The Abs and flow cytometry reagents used were α-CD4-FITC, α-CD44-CyChrome (Cy), (BD PharMingen, San Diego, CA), and α-PD-1-PE, α-PD-L1-PE, α-armenian hamster IgG-PE, α-rat IgG2a,λ-PE,(eBioscience, San Diego, CA).

Microarray:

For *RNA isolation*, total RNA extraction from purified $CD4^+$ CD44-high T cells was performed with TRIzol (Gibco/BRL, Gaithersburg, MD) according to the manufacturer's instructions. An additional purification of the total RNA was performed using RNeasy spin columns (Qiagen, Germany). cRNA preparation and subsequent steps leading to hybridization and scanning of the mU74Av.2 GeneChip Arrays were carried out according to the manufacturer's instructions (Affymetrix™, Santa Clara, CA). Briefly, 5-10 μg of each total RNA sample were converted into double-stranded cDNA using a cDNA synthesis kit (SuperScript Choice, Gibco/BRL) with a special $oligo(dT)_{24}$ primer containing a T7 RNA polymerase promoter site added 3' of the poly T tract (Genset). Subsequently, biotin-labeled anti-sense cRNA was generated from the cDNA sample by in vitro transcription reaction (IVT) using the ENZO™ BioArray HighYield RNA Transcript Labeling Kit. The labeled

cRNA was purified using RNeasy spin columns (Qiagen, Germany). Approximately twenty micrograms of each cRNA sample were fragmented by mild alkaline treatment, at 94 °C for 35 min and then used to prepare the hybridization cocktail. A mixture of four control cRNAs for bacterial and phage genes was included in the hybridization cocktail (*bioB*, *bioC*, *bioD*, and *cre*, at 1.5, 5, 25, and 100 pM, respectively) to serve as tools for comparing hybridization efficiency between arrays and for relative quantization of measured transcript levels. A biotinylated oligonucleotide, B2, was also added to the hybridization cocktail, which hybridized to unique features at the center and four corners of each chip to facilitate accurate orientation and mapping of the probe sets. Additionally, standard Test GeneChip arrays (Affymetrix, Santa Clara, CA) were performed on all samples prior to mU74Av.2 hybridization as an additional means of testing sample integrity. ***Oligonucleotide array hybridization and scanning*** was performed at the University of Massachusetts Medical Center Affymetrix Core Facility in accordance with the manufacture's instructions. Briefly, the sample cocktails were heated to 99 °C for 5 min, equilibrated to 45 °C for 5 min, and clarified by centrifugation (14,000*g*) at room temperature for 5 min. Aliquots of each sample (15 μg of fragmented cRNA in 300 μl of hybridization cocktail) were hybridized to U74A GeneChip arrays at 45 °C for 16 h in a rotisserie oven set at 60 rpm. The arrays were then washed with non-stringent wash buffer (6 × SSPE, 0.01% Tween 20) and stringent wash buffer (100 mM MES, 0.1 M[Na^+], and 0.01% Tween 20), stained with R-phycoerythrin Streptavidin (Molecular Probes), washed again, and read by the GeneArray Scanner (Agilent Technologies). ***For Oligonucleotide array data analysis,*** initial data analysis was

performed by Affymetrix Microarray Suite (MAS) 4.0 software. Initial absolute analyses for gene expression were performed without scaling while subsequent comparison analysis files were created by scaling all data sets to a uniform value (so-called Target Signal, 250) to normalize all probe sets. Pair-wise comparisons between each control (Jak3+/-) and experimental (Jak3-/-) sample were carried out. During a comparison analysis, each probe set on the experiment array (Jak3-/-) was compared to its counterpart on the baseline array (Jak3+/-), and the change *p*-value calculated by the Wilcoxon's signed-rank test. All arrays were performed in triplicate. Reproducibility between replicates was measured on a gene per gene basis for genes of interest and globally using Genespring™ 6.1 software. In Genespring, global gene scaling was first performed for all samples on all 12,422 genes contained within the mU74Av.2 array(s). For similarity analysis, the target sample (Jak3-/-) was compared to replicate samples and similarity measured using the Genespring Find Similar Sample feature. For this comparison, all genes on the array were considered and similarity determined using a weighting coefficient of 0.25. In this analysis, a correlation value of 1 = perfect match, -1 = opposite and 0 = no match. Additional comparison analyses, clustering and heat map plot generation was also performed using GeneSpring™ software.

Real-time quantitative PCR:

Purification of $CD4^+$ CD44-highT cells was performed as previously described. Total RNA was isolated using either TRIzol (Gibco/BRL) or the Qiagen RNeasy kit (Valencia, CA) according to the manufacturer's protocol. After DNase treatment (Promega, Madison, WI), 1

μg of total RNA was reverse transcribed into cDNA using Superscript II and Random Hexamers (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. Real-time quantitative PCR amplification was performed on a Bio-Rad iCycler using SYBR Green PCR Core Reagents (PE Applied Biosystems, Foster City, CA). To quantify the amount of cDNA for an individual transcript, SYBR Green fluorescence was measured at the end of each cycle. The cycle threshold (C_t), the cycle at which exponential growth of the PCR product is first detected, was determined for known concentrations of plasmid DNA, and a standard curve was created. Template copy numbers were calculated for each sample by interpolating the C_t values on the standard curve using the iCycler software. All samples and standards were run in triplicate for any given experiment. The value of PD-1 was normalized to β-actin by dividing the average copy number of the respective transcript by the average copy number of β-actin in the respective sample. The data was then represented graphically as relative units. ***The PCR was as follows:*** templates were initially denatured at 95°C for 10 min followed by 40 cycles of denaturation at 95°C for 20 s, 25 s of primer annealing at 62°C for β-actin and PD-1, and lastly a 72°C extension for 25 s. Primers were: β-actin sense, 5'-CGAGGCCCAGAGCAAGAGAG-3', antisense, 5'-CGGTTGGCCTTAGGGTTCAG-3'; PD-1 sense 5'-TGGAACCGCTCTGATCTC TGG-3', antisense, 5'-GGTTTAGGG GCTGGTTGTTGC-3'. Specific products were verified by melt-curve analysis and gel electrophoresis. For the generation of standard curves, a plasmid containing a cDNA clone for β-actin (gift from R. Gerstein, University of Massachusetts Medical School, Worcester,

MA) was used. A 180-bp fragment of PD-1 (145–324) was cloned into pGEM-T Easy (Promega) for use in the generation of a PD-1 standard curve.

Adoptive transfer:

CD4 SP thymocytes from Jak3+/- or Jak3-/- thymus were isolated by AutoMACS separation as previously described. 1 x 10^6 CD4 SP thymocytes were injected intravenously into RAG2-/- (Taconic, Germantown, NY) mice. After 2, 4, 6, and 8 weeks spleens from i.v. injected or control mice were harvested and analyzed.

T cell stimulation:

Either total splenic CD4+ or CD4+CD44-high cells were purified as described above. 5 x 10^5 cells were plated with between 2.5 and 10 μg/ml immobilized α-CD3 (clone 145-2C11, PharMingen, San Diego, CA). Cells were stimulated between 6h and 72h depending on experiment. For co-culture experiments target cells were mitomycin-C treated and stimulated at a 1:1 target/responder ratio at a total cell density of 5 x 10^5.

In vitro proliferation assay:

Cells were stimulated as described previously. [^{3}H] Thymidine (NEN, Boston, MA) was added at 1μCi/well at 36 h and incubated for an additional 16 h, plates were harvested on a Tomtec Harvester 96 (Orange, CT), and [^{3}H] thymidine incorporation was quantified on a Trilux microbeta counter (PerkinElmer, Wellesley, MA).

ELISA:

5×10^5 cells were stimulated with 10ug/ml plate-bound α-CD3 for 18-22 hours in a 96 well plate. Cells were removed by centrifugation and the supernatant was serially diluted and assayed for the presence of IL-2 (OptEIA™ Mouse IL-2 Set), IFN-γ (OptEIA™ Mouse IFN-γ Set), IL-4 (OptEIA™ Mouse IL-4 Set), IL-5(OptEIA™ Mouse IL-5 Set), IL-10 (OptEIA™ Mouse IL-10 Set), (PharMingen, Sand Diego, CA) and TGF-β (Emax Immunoassay System), (Promega, Madison WI) according to the manufacturer's protocol. The absorbance was read at 450 nm using an ELISA reader (EL 340 from Bio-Tek Instruments).

***Intracellular cytokine staining*:**

T cells (5×10^5) were cultured with 10μg/ml plate bound α-CD3 for 6h and 16 h in a 96-well plate. Golgi Stop and/or Golgi Plug (BD PharMingen, San Diego, CA) were added for the last 2 h. The cells were stained with α-CD4 FITC for 30 min, fixed for 20 min, then permeabilized, and stained intracellularly with either α -IL-2-PE or α-IL10-PE according to the Cytofix/Cytoperm kit protocol (BD PharMingen). Cells were immediately analyzed by flow cytometry.

Chapter III.

Results

Global Differential Gene Expression in CD4+ CD44-high T cells from Jak3+/- versus Jak3 deficient mice.

Differences in global gene expression between CD4+ CD44-high Jak3+/- and Jak3-/- T cells

As an initial approach to determining the effects of T cell activation in the absence of Jak3-dependent cytokine signals, we used microarray analysis to compare global patterns of gene expression between Jak3-/- and Jak3+/- CD4+ CD44-hi cells immediately following ex vivo isolation. The differential expression of 12,400 genes was determined using Affymetrix GeneChip array mU74Av.2. Our criteria for determining significance was that changes in gene expression should be >2.9-fold (as determined by Test GeneChip results), have p-values of less than or equal to 0.05, and should be reproducible among triplicate experiments. From these analyses, we found that Jak3-/- T cells had approximately 149 genes up-regulated 3 fold or higher and 208 genes 3 fold down-regulated compared to Jak3+/- T cells. These changes spanned many functional categories relevant to proper T cell activation and function including cell surface markers/receptors, cell cycle/apoptosis/survival, cytokine signaling, and transcription/translation (Table 5 and Supplemental (Supp.) 1-6).

In general, there was an increase in inhibitory cell surface receptor gene expression (PD-1, LAG-3, TJ6) and a decrease in transcription/translation factors known to have a positive role in T cell function (LKLF, SATB1). Surprisingly, even though Jak3-/- CD4+ T cells do not proliferate *in vitro* we did not detect any global down regulation of genes involved in basal cell cycle events and instead saw an up-regulation of many of these genes (Cyclin A1,B1 and Lamin B1) (Table 5, Cell Cycle). Consistent with our previous data that

Table 5. Differntial Gene Expression in Jak3-/- CD4+ CD44-high T cells

Jak3+/- CD4+ CD44-hi		Jak3-/- CD4+ CD44-hi		Jak3+/- vs. Jak3-/- CD4+ CD44-hi		Gene Information	
Signal	Abs Call	Signal	Abs Call	Diff Call	Fold Change	Accession #	Description
							Cell Surface Markers/ Receptors
192.8	P	605.2	P	I	3.1	M31226	immune suppressor factor TJ6
275.4	P	756.3	P	I	3.2	X67914	PD-1, B7 family
253.8	P	985.9	P	I	4.1	X98113	Lymphocyte-activation gene 3
							Cell Cycle
358.4	P	1057.3	P	I	4.5	X75483	Cyclin A2
90.9	A	406.3	P	I	3.4	X64713	cyclin B1
1165.7	P	4248.9	P	I	4	X66032	cyclin B2
140.9	P	464.5	P	I	3.3	M35153	Lamin B1
138.8	A	639.7	P	I	4.9	L29480	serine/threonine kinase (sak-b)
151.5	P	591.7	P	I	3.9	AF013166	serine/threonine kinase (nek2)
271.9	P	-60.2	A	D	3.4	M13945	Proviral integration site 1 (pim-1)
							Cytokine Signaling
-59.1	A	277.4	P	I	3.1	K00083	Interferon gamma
5021.1	P	15461.8	P	I	3.1	AF065947	small inducible cytokine A5 (RANTES)
-17.3	A	108.1	P	I	3.1	M37897	interleukin 10
33.8	A	375.3	P	I	3.4	L12120	Interleukin 10 receptor, alpha
31.3	A	3028.4	P	I	22.8	X62502	MIP-1b macrophage inflammatory protein
30	P	199.4	P	I	3.5	L19932	Transforming growth factor, beta
							Transcription/Translation
1405	P	143.9	A	D	7.2	U25096	Kruppel-like factor LKLF
1287.6	P	168.5	P	D	7.6	U05252	nuclear matrix attachment protein SATB1

Table 5. Differential Gene Expression in CD4+ CD44-high Jak3-/- T cells. Global gene expression was compared between Jak3+/- and Jak3-/- CD4+ CD44-high T cells by microarray. Experimentally adjusted Signals for each sample are shown as is an absolute Present (P) or Absent (A) call which was determined by analysis in MAS 4.0. The difference call (Diff Call) between samples are expressed as Increases (I) and Decreases (D) in gene expression and the level of change in gene expression indicated as fold change. These data were determined by analysis in MAS 4.0 and further confirmed by GeneSpring analyses. These results are representative of triplicate experiments performed.

Jak3-/- CD4+ T cells produced transcripts for certain cytokines [185], we detected the increased expression of the mRNAs encoding IFNγ, IL-10, IL-10Rα, Mip-1β, and TGF-β (Table 5, Cytokine Signaling).

Cell Surface marker/Receptors.

T lymphocytes depend on signals from their surroundings for proper activation, homeostasis and survival. These signals can be modified then, not only by changes in the release of chemical mediators from surrounding cells, but also by differential expression of receptors on the surface of a T cell. Based on our comparison of global gene expression there were several changes in expression of surface markers/receptors on CD4+ CD44-high Jak3-/-T cells compared to CD4+ CD44-high Jak3+/- T cells (Table 5, Surface Markers). Strikingly, several of these changes were increases in surface receptors known to inhibit T cell activation or function. Moreover, many of these same changes in surface markers in Jak3-/- T cells have been correlated to anergic T cells, regulatory T cells or both. First, immune suppressor factor TJ6 was 3.1 fold increased. TJ6 has been previously shown to be induced on T cells during T cell activation, anergy and pregnancy and is thought to attenuate the T cell response to a developing fetus. Programmed death receptor-1 (PD-1) and lymphocyte activating gene-3 (LAG-3) were also shown to be upregulated on Jak3-/- CD4+CD44-high T cells by a fold change of 3.5 and 4.1 respectively and this was confirmed

by real-time PCR analysis (Supp.7). PD-1 is a recently identified member of the B7:CD28 signaling family of molecules [14,30]. Interaction with its ligand, PD-L1 (B7-H1) is inhibitory to T cells and results in decreased proliferation and IL-2 secretion [36] . PD-1 is thought to play a role in peripheral tolerance because mice deficient in PD-1 develop lymphoproliferative and autoimmune pathologies [186]. Furthermore, PD-1+ T cells in rheumatoid arthritis (RA) synovial fluid are enriched, and phenotypic analysis suggests that these cells constitute a unique anergic T cell subset which play a role in disease progression [187]. Additionally, studies have investigated the roles of PD-1 and its ligands, PD-L1 and PD-L2, in the induction of regulatory cells by intratracheal delivery of alloantigen. Interestingly, PD-1-PD-L1 interaction has been shown to be essential for induction of this type of regulatory T cell [89]. LAG-3 is a CD4-related, activation-induced cell surface molecule that binds to MHC class II with high affinity [188-190]. Similarly to PD-1, the cross-linking LAG-3 results in a dramatic reduction in proliferation and IL-2 production as well as decreased calcium flux [191-194]. In addition, recent studies suggest that T cells expressing LAG-3 display regulatory function both *in vivo* and *in vitro* [194]. These results suggest multiple pathways that may lead to the development of unresponsive T cells in Jak3 deficient mice.

Cell Cycle.

There were many differentially expressed cell cycle associated genes upregulated in Jak3-/- CD4+ CD44-high T cells (Table 5, Cell cycle). These included changes in members

of the cyclin (A2, B1, and B2) and lamin (B1) family. Cyclins and lamins are critical factors in the initiation of DNA synthesis and cell cycle progression [195]. The upregulation of many pro-cell cycle genes in Jak3-/- T cells indicates that while these cells are non-proliferative *in vitro*, they are capable of cell division *in vivo* and are potentially turning over at a higher rate than Jak3+/- T cells. This is consistent with BrdU labeling experiments which demonstrate the in vivo proliferative capacity of Jak3-/- T cells [110,196].

The expression of cell cycle associated serine/threonine kinases were differentially regulated in Jak3-/- CD4+ CD44-high T cells including pim-1, sak-b, and nek-2 (Table 5, Cell cycle). Kinases play essential roles in regulation of the cell cycle and proliferation. Sak-b and nek-2 which are non-cell specific cell cycle regulators were increased while Pim-1 expression was decreased in Jak3-/- T cells. Interestingly, Pim-1 has been shown to be specifically induced upon TCR cross linking and synergize with the TCR in mediating cell differentiation and survival signals [197] . Moreover, forced expression of pim-1 reconstitutes thymic cellularity in mice lacking IL-7 or γc [197]. These results suggest that while Jak3-/- T cells may be receiving proliferative signals, in the absence of Jak3, these T cells fail to induce factors involved in TCR mediated, T cell specific proliferative signals, like pim-1, and therefore may result in an incomplete activation pathway ultimately leading to unresponsiveness.

Cytokine Signaling.

Cytokines have essential roles in the development and control of immune responses. The biological functions of cytokines mainly depend on cytokine mediated gene activation or repression. Changes in expression of cytokine genes then can act as an indicator of the types of signals a T cell has received and how a T cell has differentiated. Based on microarray data, CD4+CD44-high Jak3-/- T cells upregulate the expression of IFNγ, IL-10, IL-10Rα, Mip-1β, and TGF-β (Table 5, Cytokine Signaling) in addition to many IFN-γ, IL-10 and TGF-β-related genes which include the receptors and down stream inducible targets of these cytokine signaling pathways (Table 6, Cytokine Inducible). IFN-γ, IL-10 and TGF-β are cytokines that have potent immunomodulatory and suppressive properties [81,198,199]. While IFN-γ promotes inflammation [200], IL-10 and TGF-β are considered anti-inflammatory cytokines that have potent immunomodulatory and suppressive properties [201,202]. Secretion of large amounts of IFN-γ is a defining feature of Th1 cells and functions to directly promote cell mediated immunity [49]. IL-10 exerts several immune stimulating, as well as inhibitory effects [203]. CD4+ Th2 cells provide the primary source of IL-10 in the immune system and thus, IL-10 plays a role in humoral immunity [55]. IL-10 also plays a key regulatory role in the immune system as has been demonstrated by IL-10 mediated dampening of both Th2- and Th1-associated diseases as well as the fact that to date most T cell subsets associated with regulatory function produce primarily IL-10 [82,204]. TGF-β has complex and pleiotropic effects on T cells including the inhibition of T-cell proliferation, the development of cytotoxic T cells, and the differentiation of Th1 and 2 lineages [205-207]. However, under certain conditions, TGF-β has also been found to stimulate

TABLE6. Cytokine Inducible Genes

Jak3+/- signal	Jak3+/- Call	Jak3-/- signal	Jak3-/- Call	-/- VS. +/- Diff Call	-/- VS. +/- Fold Change	Accession	Description
							INTERFERON GAMMA RELATED
1355	P	6369.3	P	I	4.7	M21065	Interferon regulatory factor 1
1007.8	P	3517.9	P	I	3.5	M63630	predicted interferon inducible GTP binding protein (IRG-47)
289	A	1098.2	P	I	3.8	U19119	interferon inducible G-protein-like LRG-47
680.8	P	3404.1	P	I	5.1	U51992	Interferon dependent positive acting transcription factor 3 gamma
801.9	P	2566.8	P	I	3.2	AJ007972	GTPI protein
-87.3	A	323.1	A	I	3.7	U69599	Interferon gamma receptor
585.3	P	4155.6	P	I	7.1	M31419	Interferon activated gene 204
548.1	P	25481	P	I	10	AF022371	Interferon activated gene 203
							TRANSFORMING GROWTH FACTOR RELATED
6.5	A	61.9	P	I	9.5	L40459	Latent transforming growth factor beta binding protein 3
-11.9	A	40.6	P	I	3.6	D76446	TAK1 (TGF-beta-activated kinase)
124.5	A	598	P	I	4.8	AF064088	transcription factor GIF (gamma inducible factor)
34.4	A	156.6	P	I	4.5	AV245229	Runx2
-12.1	A	39	P	I	3.2	D32072	Transforming growth factor, beta receptor II
30	P	199.4	P	I	6.6	L19932	Transforming growth factor, beta induced
40.3	P	209.6	P	I	5.2	X62940	Transforming growth factor beta 1 induced transcript 4
							IL-10 RELATED
781.2	P	2521.6	P	I	3.2	U53696	Interleukin 10 receptor, beta
300.3	P	988.1	P	I	3.2	U88328	suppressor of cytokine signalling-3 (SOCS-3)
118.8	P	1080.5	P	I	9.1	X94151	CC chemokine receptor-5 (CCR5)
-8.3	A	41.1	P	I	4.9	U29678	Chemokine (C-C) receptor 1 (CCR1)

Table 6. Differential Gene Expression in CD4+ CD44-high Jak3-/- T cells.

Global gene expression was compared between Jak3+/- and Jak3-/- CD4+ CD44-high T cells by microarray. Experimentally adjusted Signals for each sample are shown as is an absolute Present (P) or Absent (A) call which was determined by analysis in MAS 4.0. The difference call (Diff Call) between samples are expressed as Increases (I) and Decreases (D) in gene expression and the level of change in gene expression indicated as fold change. These data values were determined by analysis in MAS 4.0, specific data mining was however first performed using GeneSpring clustering and pathway analyses. These results are representative of triplicate experiments performed.

T cells, partly by preventing apoptosis, but also by inducing proliferation [201,202,205,208]. Interestingly, TGF-β secretion is not associated with either differentiated Th1 or Th2 cells, but is a potent immunoregulatory cytokine that contributes to the function and generation of most regulatory T cell subsets [209].

Transcription/translation

Signaling events tightly control the differentiation, activation and function of most cell types including T cells by activating or repressing transcription factors. It was therefore of interest to determine changes in expression of transcriptional and translational regulators in CD4+ CD44-high Jak3-/- T cells since such factors can influence the functional activity of these cells. Microarray analysis comparing Jak3+/- CD4+ CD44-high T cells to Jak3-/- CD4+ CD44-high T cells revealed a decrease in gene expression of two transcription factors, LKLF (Lung Krüppel Like Factor) and SATB1 (Special A-T rich Binding Protein-1) which have been previously shown to have important roles in T cell function (Table 5, Transcription and Translation and Supp.8). LKLF encodes a nuclear DNA-binding transcription factor of the Krüppel zinc finger family and is expressed in a range of tissues [210]. In T cells it is highly expressed in resting cells and quiescent memory cells, but the mRNA and protein are rapidly downregulated during T-cell activation and this is thought to be mediated by γc cytokines IL-2 and -7 [211,212]. LKLF-knockout mice die as embryos from defects in blood vessel walls, and have lung defects [213]. However, RAG-2 blastocyst -LKLF-

knockout embryonic stem cell chimeras yield mice with few CD4 or CD8 T cells in the periphery which are hyperactivated, suggesting that LKLF plays an essential role in maintaining T cells in a quiescent state [213]. A decrease in expression of LKLF in CD44-high Jak3-/- CD4 T cells compared to CD44-high Jak3+/- T cells may be solely due to the inability of Jak3-/- T cells to receive γc signals. A deficiency in LKLF could result in the inability of Jak3-/- T cells to maintain a quiescent state accounting for the accumulation of CD44-high T cells and potentially leading to exhaustion or unresponsiveness. SATB1 is a cell type specific nuclear protein that recruits chromatin-remodeling factors that regulate numerous genes during thymocyte differentiation and T cell activation [214]. In different contexts, SATB1 can function as a repressor of transcription or as an activator [215]. Additionally, SATB1 is induced upon T cell stimulation in peripheral T cells as an IL-2 dependent immediate early gene product [216], and has been implicated in the modification of IL-2Rα, IL-7Rα, PD-1, c-myc and CD8 alpha gene loci in T cells [214,217]. Decreased expression of SATB1 mRNA in CD4+ T cells may alter the expression of activation genes, such as c-myc and PD-1, and result in the inability of Jak3-/- to cells to proliferate and secrete IL-2.

In summary of our initial gene expression profiling of Jak3+/- CD4+ CD44-high versus Jak3 -/- CD4+ CD44-high T cells, we demonstrate that in Jak3-/- T cells there was an increase in inhibitory cell surface receptor gene expression (PD-1, LAG-3, TJ6) and a decrease in transcription/translation factors known to have a positive role in T cell function (LKLF, SATB1). Surprisingly, even though Jak3-/- CD4+ T cells do not proliferate *in vitro* we did

not detect any global down regulation of genes involved in basal cell cycle events and instead saw an up-regulation of many of these genes (Cyclin A1,B1 and Lamin B1) (Table 5, Cell Cycle). Consistent with our previous data that Jak3-/- CD4+ T cells produced transcripts for certain cytokines [185], we detected the increased expression of the mRNAs encoding IFNγ, IL-10, IL-10Rα, Mip-1β, and TGF-β (Table 5, Cytokine Signaling). In total, this data suggested that the Jak3-/- CD4+ T cells were not merely anergic but rather had differentiated into a subset of T cell that no longer produced IL-2. This hypothesis will be further discussed and investigated in Chapter IV.

Chapter IV.

Results

A) The role of the PD-1:PD-L1 co-inhibitory pathway and other B7:CD28 or TNFR superfamily molecules in the unresponsiveness of Jak3-/- CD4+ CD44-high T cells.

Increased expression of PD-1 in Jak3 deficient T cells

Of the cell surface receptors upregulated in Jak3-/- T cells, PD-1 is the most well-characterized in terms of its expression and function. Additionally, the known negative regulatory effect of PD-1:PD-L1 on T cells suggests the intriguing possibility that this pathway may contribute to the development of unresponsive T cells in Jak3-deficient mice. Therefore we focused on confirming the differential expression of PD-1 between Jak3+/- and Jak3-/- T cells and on examining the functional consequences of this altered expression. To confirm the increased expression of PD-1 in T cells deficient in Jak3 we first measured relative RNA levels in CD4+ CD44-high Jak3-/- T cells compared to CD4+ CD44-high Jak3+/- T cells by Real-time PCR. CD4+ T cells were enriched from either Jak3+/- or Jak3-/- spleens and then sorted to at least 95% purity for CD44-high expression. As shown in Fig. 6A, PD-1 RNA expression was approximately 3 times more abundant in sorted Jak3-/- T cells than Jak3+/-. This increase in PD-1 RNA expression correlates with the difference seen by microarray (Table 5, Surface Markers). Next, to confirm that the increase in RNA expression in Jak3-/- T cells translates into increased surface protein, we measured the expression of PD-1 on CD4+ CD44-high Jak3-/- T cells compared to CD4+ CD44-high Jak3+/- T cells by flow cytometry. Total splenocytes were isolated from either Jak3+/- or Jak3-/- mice and stained for CD4, CD44, and PD-1. As shown in Fig. 6B, PD-1 is significantly up-regulated on the surface of Jak3-/- CD4+ CD44-high T cells, 60% compared to only 10% in Jak3+/- CD4+ CD44-high cells. The upregulation of PD-1 suggests that it is

Figure 6. PD-1 expression in Jak3-/- mice

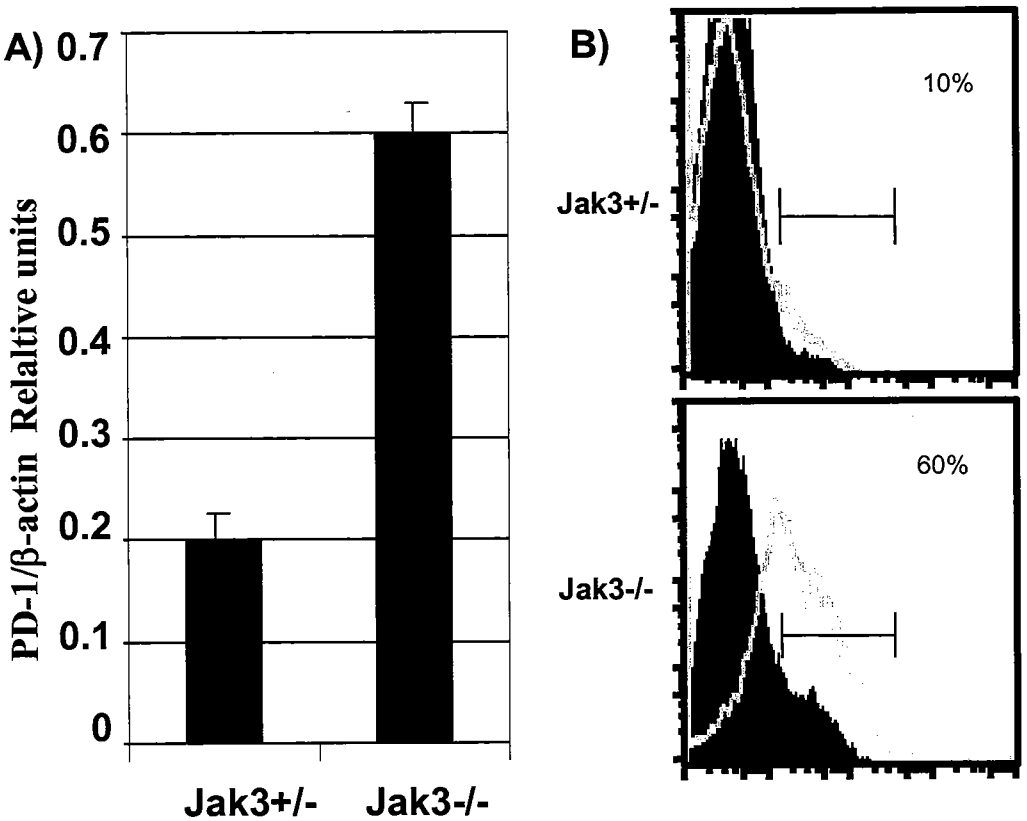


Figure 6. Jak3-/- CD4+CD44-high peripheral T cells have increased levels PD-1 mRNA and protein.

A) Purification of $CD4^+$ CD44-high splenic T cells from either Jak3+/- or Jak3-/- mice was performed as described previously. Total RNA was isolated using either TRIzol or the Qiagen RNeasy kit according to the manufacturer's protocol. After DNase treatment, 1 μg of total RNA was reverse transcribed into cDNA using Superscript II and Random Hexamers according to the manufacturer's protocol. Real-time quantitative PCR amplification was performed on a Bio-Rad iCycler using SYBR Green PCR Core Reagents. To quantify the amount of cDNA for an individual transcript, SYBR Green fluorescence was measured at the end of each cycle for known concentrations of plasmid DNA and used to generate a standard curve. All samples and standards were run in triplicate for any given experiment and represented as mean relative unit (+/-SD). The value of PD-1 was normalized to β-actin by dividing the average copy number of the respective transcript by the average copy number of β-actin in the respective sample. The data was then represented graphically as relative units. Data is representative of two independent experiments performed.

B) Total splenocytes were isolated as described previously from either Jak3+/- (top panel) or Jak3-/- (lower panel) and were stained with the indicated Abs in HBSS supplemented with 3% FCS for 20 min on ice. Cells were then washed and analyzed on a BD Biosciences FACSCalibur. Data were analyzed using CellQuest software. The Abs and flow cytometry reagents used were α-CD4-FITC, α-CD44-CyChrome,and α-PD-1-PE, α-armenian hamster IgG-PE. Cells are shown gated based on live FSC/SSC and CD4+ CD44-high staining. Percent positive cells are indicated in the histograms. **Black filled**= α- Armenian hamster IgG isotype control, **Gray line**=α-PD-1. Data is representative of four independent experiments performed using 3 to four mice per genotype.

available to mediate negative signals to Jak3-/- T cells which could potentially lead to the unresponsive state of these cells.

Increased expression of PD-L1 in the periphery of Jak3 deficient mice

Since the function of PD-1 as a negative regulator of T cells is dependent on ligand binding, we were interested in determining the expression levels of PD-1 ligands in Jak3-/- mice. Two ligands for PD-1 have been described, PD-L1 and PD-L2. To accomplish this, the levels of PD-L1and PD-L2 were compared on Jak3-/- and Jak3+/- splenocytes by flow cytometry analysis. Total splenocytes from either Jak3 +/- or Jak3-/- mice were stained with antibodies to CD4, and PD-L1 and PD-L2. The expression of PD-L2 was comparable between Jak3-/- and Jak3+/- splenocytes (Figure 7). However, as shown in Fig. 8A, the expression of PD-L1 is dramatically increased in the splenic microenvironment of Jak3-deficient mice. Strikingly, 85% of Jak3-/- splenocytes express PD-L1 compared to only 26% of the comparable subset of Jak3+/- splenocytes. This increase is specific to the T cell-negative compartment, as CD4+ T cells from Jak3+/- and Jak3-/- show comparable PD-L1 expression (~15% of cells; Figure 8B). The increased expression of PD-L1 in the Jak3-/- spleen further suggested the potential that Jak3-/- T cells may be negatively-regulated by the PD-1-PD-L1 pathway.

PD-L1+ Jak3-/- splenocytes can inhibit wild type T cell function

Figure 7. PD-L2 expression on total splenocytes from Jak3+/- or Jak3-/- mice

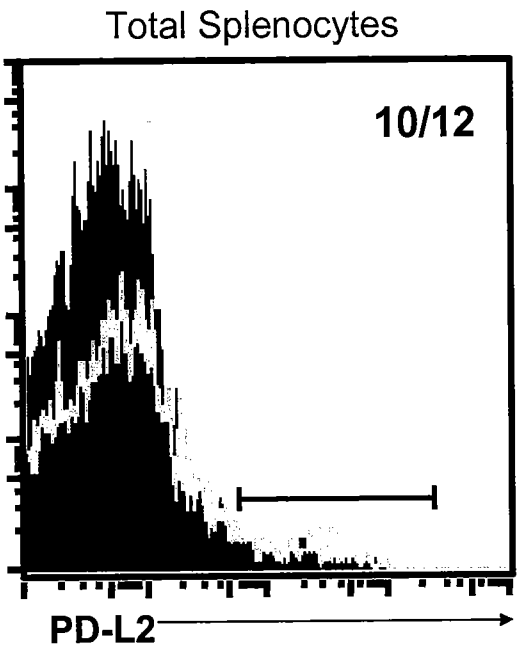
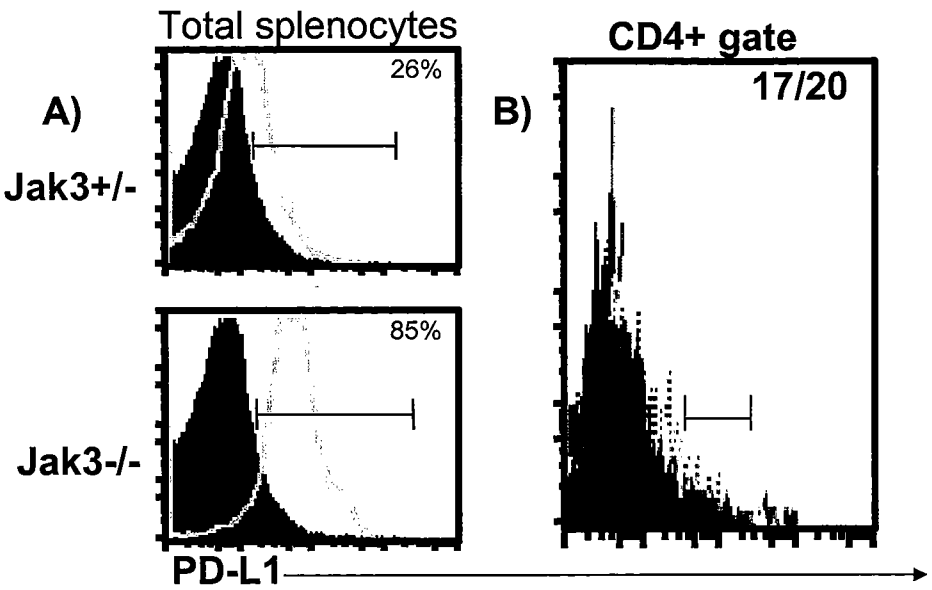


Figure 7. PD-L2 is expressed comparably on splenocytes from Jak3+/- or Jak3-/- mice. Total splenocytes were isolated as described previously from either Jak3+/- (black shaded histogram) or Jak3-/- (gray line) and were stained with the indicated Abs in HBSS supplemented with 3% FCS for 20 min on ice. Cells were then washed and analyzed on a BD Biosciences FACSCalibur. Data were analyzed using CellQuest software. The Abs and flow cytometry reagents used were α-PD-L2-PE and α-rat IgG2a,λ-PE (not shown). Cells are shown gated on live cells based on FSC/SSC. Percent positive cells are indicated in the histograms and are shown as percent positive Jak3+/- over percent positive Jak3-/- in each. Data is representative of two independent experiments performed.

Figure 8. PD-L1 expression in Jak3-/- mice



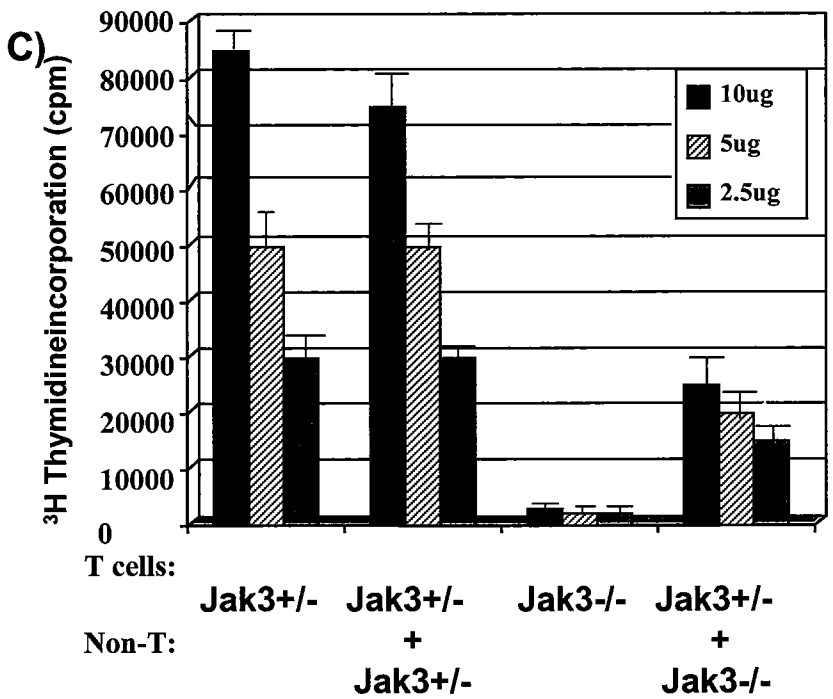


Figure 8. Increased population of PD-L1+ cells in the periphery of Jak3-deficient mice can mediate inhibition of wild type cell proliferation.

A) & B) Total splenocytes were isolated as described previously from either Jak3+/- (top panel) or Jak3-/- (lower panel) and were stained with the indicated Abs in HBSS supplemented with 3% FCS for 20 min on ice. Cells were then washed and analyzed on a BD Biosciences FACSCalibur. Data were analyzed using CellQuest software. The Abs and flow cytometry reagents used were α-CD4-FITC, α-PD-L1-PE and α-rat IgG2a,λ-PE. Cells are shown gated on live cells based on FSC/SSC. Percent positive cells are indicated in the histograms. **Black filled**= α-rat IgG2a,λ isotype control, **Gray line**=α-PD-L1.
C) Total Jak3-/- splenocytes were isolated and then depleted of CD4+ T cells. The resulting population of PD-L1+ Jak3-/- (85-90% PD-L1+) cells were mitomycin-C treated and cultured at a 1:1 ratio with Jak3+/- CD4+ T cells for 36h on either 2.5 **(gray bar)**, 5 **(hatched bar),** or 10 **(black bar)** μg/ml α-CD3 antibody-coated plates at a total cell density of 5 x 10^5. Jak3+/- CD4+ T cells or Jak3-/- CD4+ T cells alone, and Jak3+/- CD4+ T cells co-cultured with T cell depleted Jak3+/- splenocytes were treated similarly as controls. On day 3, cells were harvested and proliferation was determined by ^{3}H-thymidine incorporation. The values are the mean counts per minute (+/- SD) of triplicate determinations. Results are representative of five experiments performed. Significance of these results was determined by student's t test to be for (Jak3 +/- & Jak3 +/-) at 10ug $p < 0.04$, 5ug $p < 0.03$, 2ug $p < 0.03$; and for (Jak3 +/- & Jak3 -/-) at 10ug $p < 0.003$, 5ug $p < 0.01$, 2ug $p < 0.007$.

Since PD-L1 can mediate negative signals to T cells, we next determined whether the PD-L1+ Jak3-/- cells could mediate T cell inhibition. CD4+ T cells were depleted from Jak3-/- total splenocytes. The resulting population of PD-L1+ Jak3-/- (85-90%) cells were mitomycin-C treated and cultured at a 1:1 ratio with Jak3+/- T cells for 36h in 96 well plates coated with 2.5, 5, or 10 μg/ml α-CD3. Proliferation was measured by ^{3}H incorporation. As shown in Figure 8C, PD-L1+ Jak3-/- cells inhibit Jak3+/- T cell proliferation while Jak3+/- T cell depleted splenocytes have no effect. To specifically determine whether PD-L1 was mediating the inhibitory effect, the above experiments were repeated with the addition of blocking antibodies to either PD-L1 alone or in combination with blocking antibodies to PD-L2, a secondary ligand for PD-1. As shown in Fig. 9A, the addition of PD-L1 blocking antibodies to co-cultured cells greatly reduced the inhibitory effect of the PD-L1+ Jak3-/- cells on Jak3+/- T cells. Additionally, treating co-cultures with blocking antibodies to both PD-L1 and PD-L2, resulted in reduced inhibition but the reduction was not significantly different from treatment with α-PD-L1 alone. Finally, to address whether secreted factors might be contributing to the inhibitory effects of the PD-L1+Jak3-/- cells, we performed co-culture experiments in transwell plates. As shown in Fig. 9B, the physical separation of PD-L1+ Jak3-/- cells from Jak3+/- T cells resulted in the inability of the PD-L1+ Jak3-/- cells to inhibit T cell proliferation. Importantly, these results suggest that the PD-L1 expressed on Jak3-/- splenocytes is functional and that the particular cell type expressing PD-L1 can interact with and mediate inhibition of T cells.

Figure 9. Inhibition of WT CD4+ T cells is mediated by PD-L1

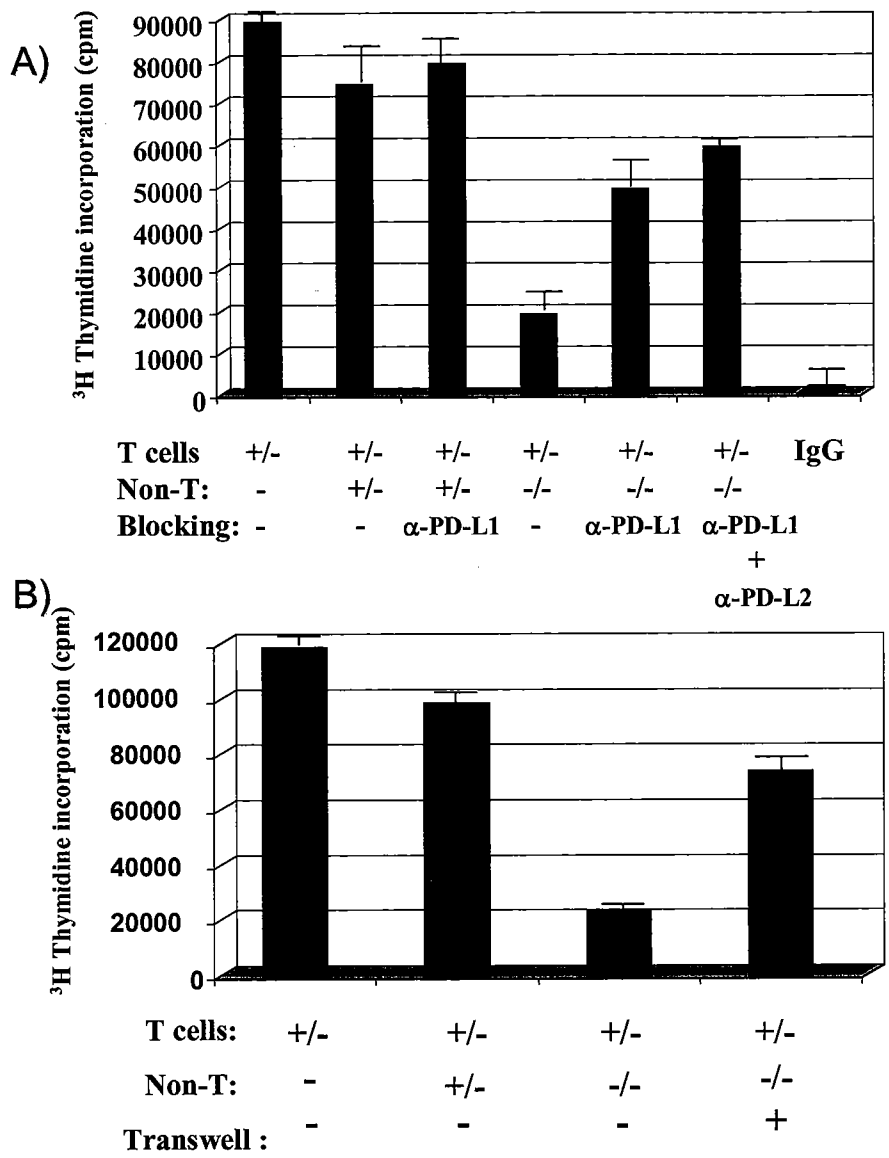


Figure 9. Inhibition of wild type CD4+ T cells by Jak3-/- PD-L1+ CD4-negative splenocytes is dependent on PD-L1 and cell-to-cell contact.

A) Stimulation of CD4+ Jak3+/- T cells co-cultured at a 1:1 ratio with CD4-PD-L1+Jak3-/- cells was performed as described previously with 10μg/ml plate-bound α-CD3 antibody in addition to the presence of blocking antibodies to PD-L1 alone or to PD-L1 plus PD-L2. The control groups shown are Jak3+/-CD4+ T cells alone (+/-), Jak3 +/- CD4+ T cells & Jak3+/- T cell depleted splenocytes (+/- &+/-), or Jak3 +/- CD4+ T cells & Jak3+/- T cell depleted splenocytes in addition to a blocking antibody against PD-L1 (+/- &+/- & α-PD-L1). The experimental groups are Jak3 +/- CD4+ T cells & Jak3-/- T cell depleted splenocytes (+/- &-/-), or Jak3 +/- CD4+ T cells & Jak3+/- T cell depleted splenocytes in addition to blocking antibody against PD-L1 (+/- &-/- & α-PD-L1) or PD-L1 and PD-L2 (+/- &-/- & α-PD-L1& α-PD-L2). On day 3, cells were harvested and proliferation was determined by ^{3}H-thymidine incorporation. The values are the mean counts per minute (+/- SD) of triplicate determinations. Results are representative of five experiments performed. Significance of these results was determined by student's t test to be $p < 0.01$ for (+/- &-/- & α-PD-L1), $p < 0.002$ (+/- &+/- & α-PD-L1) and $p < 0.03$(+/- &-/- & α-PD-L1& α-PD-L2).

B) 2.5 x 10^5 Jak3+/- CD4+ T cells (+/-) were cultured in either standard 96 well plates (-) or transwell plates (+) coated with 10μg/ml α-CD3 antibody. 2.5 x 10^5 T cell depleted splenocytes from either Jak3 +/- (+/-) or Jak3-/- (-/-) mice were co-cultured with the Jak+/- CD4+ T cells. On day 3, cells were harvested and proliferation was determined by ^{3}H-thymidine incorporation. The values are the mean counts per minute (+/- SD) of triplicate determinations. Results are representative of three experiments performed. Significance of these results was determined by student's t test to be $p < 0.05$ for (+/- &+/- transwell+), $p < 0.002$ (+/- &-/- transwell+).

Origin of PD-L1+ splenocytes in Jak3-deficient mice

Given the interesting finding that there is increased expression of PD-L1 on Jak3-/- CD4-negative splenocytes and that these cells can mediate inhibitory signals to wild type T cells, it was next of interest to us to address the nature of the cell type(s) expressing PD-L1.

While, Jak3-/- mice lack many of the cell subsets typically residing in the spleen such as B, NK, γδ T, and CD8αα DC cells ([153,154] and unpublished data M. Wallace) they display overt splenomegaly. Previous data from our lab indicated that many of the cells accumulating in Jak3-/- spleens were lineages that appeared to be hematopoietic progenitors (unpublished data, M. Wallace). Interestingly, PD-L1 but not PD-L2 expression has been observed on subsets of thymocytes, bone marrow derived pre-B and myeloid cells, and, notably, a significant proportion of the most immature lineage-marker negative and c-Kit-positive bone marrow cells [34]. This led us to investigate whether the PD-L1+ Jak3-/- splenocytes were lineage negative progenitor-like cells. To this end, freshly isolated splenocytes from Jak3-/- mice were stained with FITC-conjugated monoclonal antibodies specific for lineage markers including Thy 1.2, B220, TER-119, Gr-1, Mac-1, CD19,and DX-5, APC-conjugated α-c-kit mAb, and biotinylated-α -PD-L1 mAb, followed by PE-conjugated streptavidin. After the lineage (Lin) negative region was defined (representative plot shown in Figure 10A), the expression profile of c-kit and PD-L1 was determined. A significant portion (50%) of the Lin- PD-L1+ cells was in fact c-Kit+ (Figure 10B). As shown in Figure 11A&B, c-Kit+ cells are generally present at a higher frequency in Jak3-/-

Figure 10. Proportion of lin- PD-L1 + cells that are c-kit +

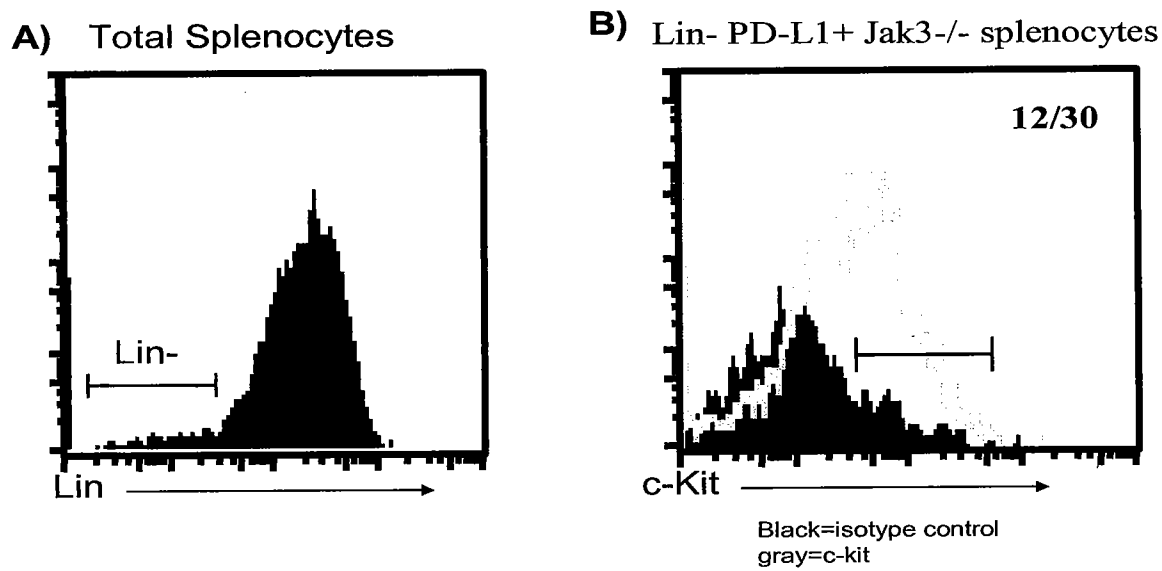


Figure 10. A proportion of Jak3-/- PD-L1+ splenocytes are c-Kit+.

A) & B) Freshly isolated splenocytes from Jak3-/- mice were stained with FITC-conjugated monoclonal antibodies specific for lineage markers including Thy 1.2, B220, TER-119, Gr-1, Mac-1, CD19,and DX-5 in addition to an APC-conjugated α-c-kit mAb (gray line) or isotype control (black shaded histogram), and biotinylated-α -PD-L1 mAb, followed by PE-conjugated streptavidin. After the lineage (Lin) negative region was confined (representative plot shown in **A)**, cells were gated on PD-L1+ staining and the expression profile of c-kit is shown in **B)**. Cells are shown gated on live cells based on FSC/SSC. Percent positive cells are indicated in the histogram and are shown as percent positive isotype control over percent positive Jak3-/-. Data is representative of two independent experiments performed.

splenocytes compared to Jak3+/-, particularly in the CD4-negative subsets (R6/R7 and R8/R9). Surprisingly, we detect that a portion of Jak3-/- splenocytes were CD4 negative, TCRαβ + and c-kit+ (30%; Figure 11B, R8/R9) of which 60% also express PD-L1+ (Figure 11C). Since in Jak3-/- mice the population of CD8+ peripheral T cells is absent (R9 gate ≤ 1% CD8+), these CD4 negative TCRαβ+ cells are a rare subset of CD4/CD8-double negative, TCRαβ + (DNTC), c-kit+,PD-L1+ cells.

While conventional splenic T cells are typically CD4+ or CD8+, unusual populations of DNTC have been described. DNTCs exist in wild type mice comprising ~1% of the splenic population and in higher proportions at sites of extrathymic T cell development such as the liver and appendix [218]. Additionally, DNTCs have been described in several mouse models. The most well characterized of these is the DNTCs that exist in abundance in the spleens of lpr mice [219]. Interestingly, in lpr mice and wild type mice, extrathymically derived DNTC have been shown to originate from c-Kit+ Lin- cells in the appendix and liver [220]. Based on these reports, one hypothesis is that the unique splenic subset of PD-L1+ c-Kit+ DNTCs in Jak3-/- mice is extrathymically derived. Although, PD-L1 expression has, to our knowledge, not been associated with extrathymically derived T cells, PD-L1 expression has been shown on the most immature c-kit+ CD4/CD8-DN thymocyte population in wild type mice. It is unlikely that the DNTCs in Jak3-/- mice represent a population of thymic derived developing T cells since at this stage (DN, c-kit+) of thymocyte development TCRβ gene rearrangement and expression has typically not occurred [221-223].

Figure 11. The expression of c-kit on different subsets of total splenocytes

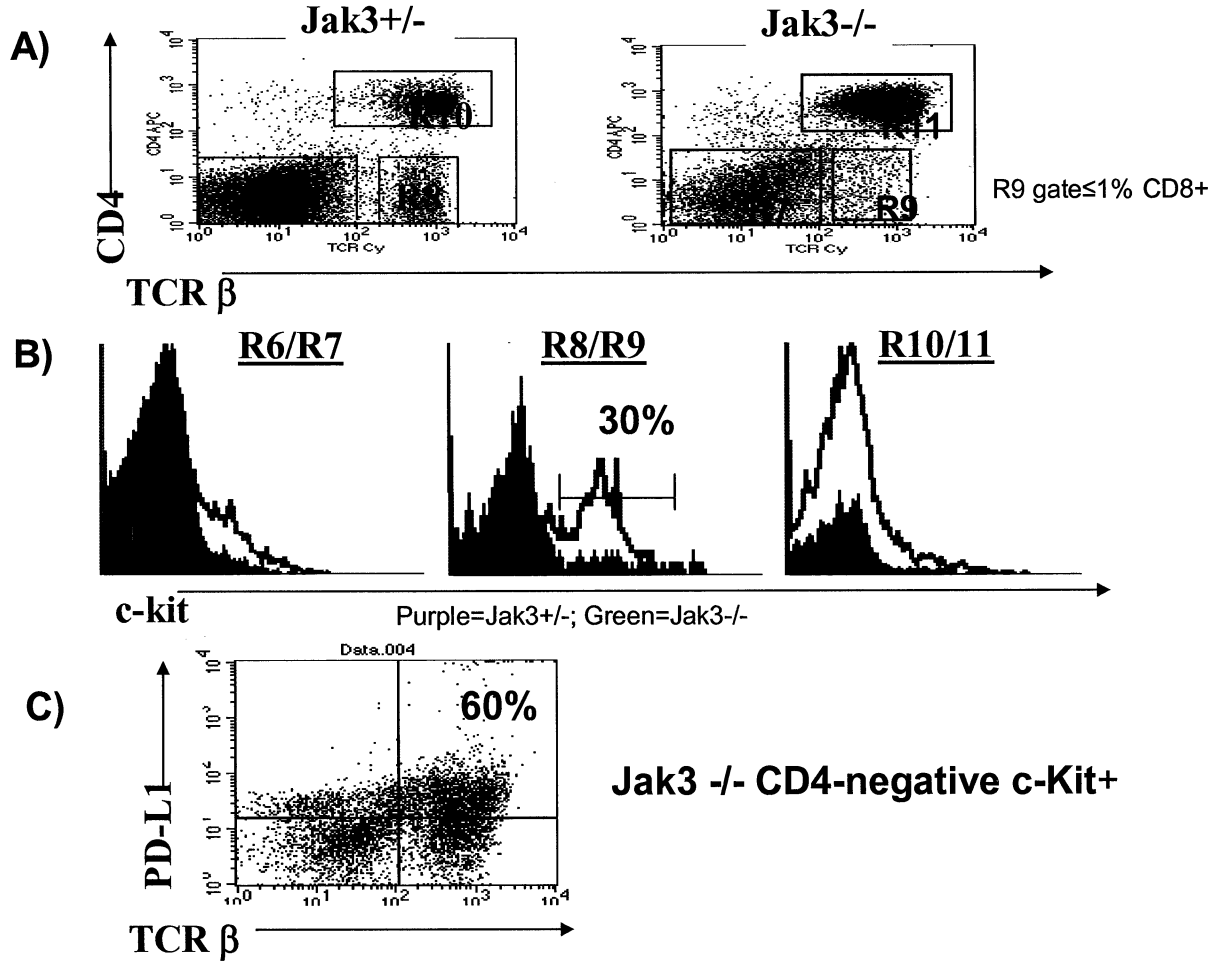


Figure 11. c-Kit+ cells are more abundant in Jak3-/- mice.

Total splenocytes were isolated as described previously from either Jak3+/- or Jak3-/- and were stained with the indicated Abs in HBSS supplemented with 3% FCS for 20 min on ice. Cells were then washed and analyzed on a BD Biosciences FACSCalibur. Data were analyzed using CellQuest software. **A) & B)** The Abs and flow cytometry reagents used were α-CD4-FITC or α-CD8-FITC, α-TCRβ-Cy, α-c-Kit –APC and α-PD-L1-PE. Cells are shown gated on live cells based on FSC/SSC. And in **B)** are additionally gated on CD4- and TCRβ- (left panel R6=Jak3+/-, R7=Jak3-/-) or CD4+ cells (right panel R10=Jak3+/-, R11=Jak3-/-). In **(C)** Total splenocytes were depleted of CD4+ T cells by AutoMACS separation and the resulting population of cells stained with Abs to CD4, TCR-β, c-Kit and PD-L1. Cells are shown gated on live, CD4-, c-Kit+. Data is representative of three independent experiments performed.

Finally, an alternative hypothesis to the origin of Jak3-/- DNTCs is that they are mature peripheral intrathymically derived T cells that have down-regulated the co-receptor. In corroboration with this idea, our microarray data demonstrated, at least for Jak3-/-CD4+ T cells, a decrease in the transcript levels for the gene encoding SATB1. SATB1 is a known IL-2 immediate early gene product that mediates chromatin accessibility [214,216] and, further, has been specifically implicated in the control of CD8α re-expression after T cell activation induced co-receptor internalization [214,217]. Thus, it is possible that PD-L1+ Jak3-/- DNTCs represent CD8+Jak3-/- T cells that have down-regulated the CD8 co-receptor and in the absence of Jak3 mediated IL-2 signals do not express SATB1 and therefore re-expression of the CD8 receptor is impaired. In support of this hypothesis, some studies have noted that peripheral populations of DNTC may derive directly from CD8+ T cell precursors based primarily on the evidence that CD8 alpha gene demethylation could be detected in these cells [224]. The expression of c-Kit was not determined in these studies and it is unclear the mechanism by which a mature peripheral αβ+ T cell would express c-Kit except possibly in the case of malignant transformation[225]. Based on this, the hypothesis that Jak3-/- DNTC are extrathymically derived is favored.

Decreased expression of CTLA-4 and other co-stimulatory molecules on Jak3-/- CD4+ T cells

Our data thus far suggests the potential correlation between PD-1:PD-L1 upregulation and the inability of Jak3-/- CD4+ T cells to proliferate and secrete IL-2. However, many proteins have either co-stimulatory and/or co-inhibitory effects which lead to the modification of T cell receptor signals. For example, in addition to PD-1, CTLA-4 has inhibitory effects on T cells and is considered essential in the attenuation of T cell responses [15,16]. Moreover, receptors outside of the B7:CD28 family have been shown to synergize with and modify T cell receptor signals. The tumor necrosis family receptor (TNFR) superfamily molecules (OX40, 4-1BB, CD27, and CD30) appear to have an important role in initiating and sustaining T cell responses distinct to that of CD28 (reviewed in [226,227]). We focused our attention on OX40 and 4-1BB since their role in T cell function has been better characterized than other TNFR family members. For example, crosslinking either OX40 or 4-1BB in concert with the TCR, has been shown to augment the secretion of cytokines and the proliferation of CD4+ and CD8+ T cells. Engagement of these receptors is thought to provide late-acting signals that enhance survival and total effector cell numbers at the peak or expansion phase of both primary and secondary immune responses [228,229].

We next addressed whether there were any changes in expression of CTLA-4, OX40, or 4-1BB on freshly isolated or *in vitro* stimulated CD4+ CD44-high T cells from either Jak3-/- or Jak3+/- mice. As shown in Figure 12A, a lower percent of Jak3-/- CD4+ T cell express CTLA-4 immediately ex vivo. At 20 hr after stimulation (Figure 12A), there is a slight increase in the population of Jak3-/- CD4+ T cells expressing CTLA-4 but the proportion of cells is still significantly reduced compared to that on stimulated Jak3+/-

controls (17% vs. 46%). Similarly, the percent of OX40 and 4-1BB positive T cells are reduced in Jak3-/- samples at both 0 hr and 20 hr compared to Jak3+/- controls (Figure 12B), although this reduction is not to the degree seen for CTLA-4 at 20 hr after T cell stimulation.

The decrease in CTLA-4 protein expression on the surface of Jak3-/- CD4+ T cells is consistent with a 6 fold reduction in CTLA-4 gene expression which we detected by microarray analysis (Supp. 4). Importantly, this suggests that the PD-1:PD-L1 pathway is a primary inhibitory cascade effecting the Jak3-/- CD4+ T cells. Moreover, a reduction in TNFRs could result in a decrease in important positive costimulatory signals in Jak3-/- CD4+ T. A reduction in TNFR signals might be particularly detrimental in Jak3-deficient mice since the more potent costimulation mediated by CD28/IL-2 will proceed, but possibly be less effective, in the absence of Jak3. Thus, secondary, and possibly redundant, positive costimulatory molecules like TNFRs would be more critical in influencing the fate of the T cell response.

These data presented in this chapter suggest an imbalance between positive and negative costimulation which ultimately favors the inhibition of T cell responses in Jak3-/- CD4+ CD44-high T cells.

Figure 12. The expression of CTLA-4 and TNF family co-stimulatory molecules after stimulation

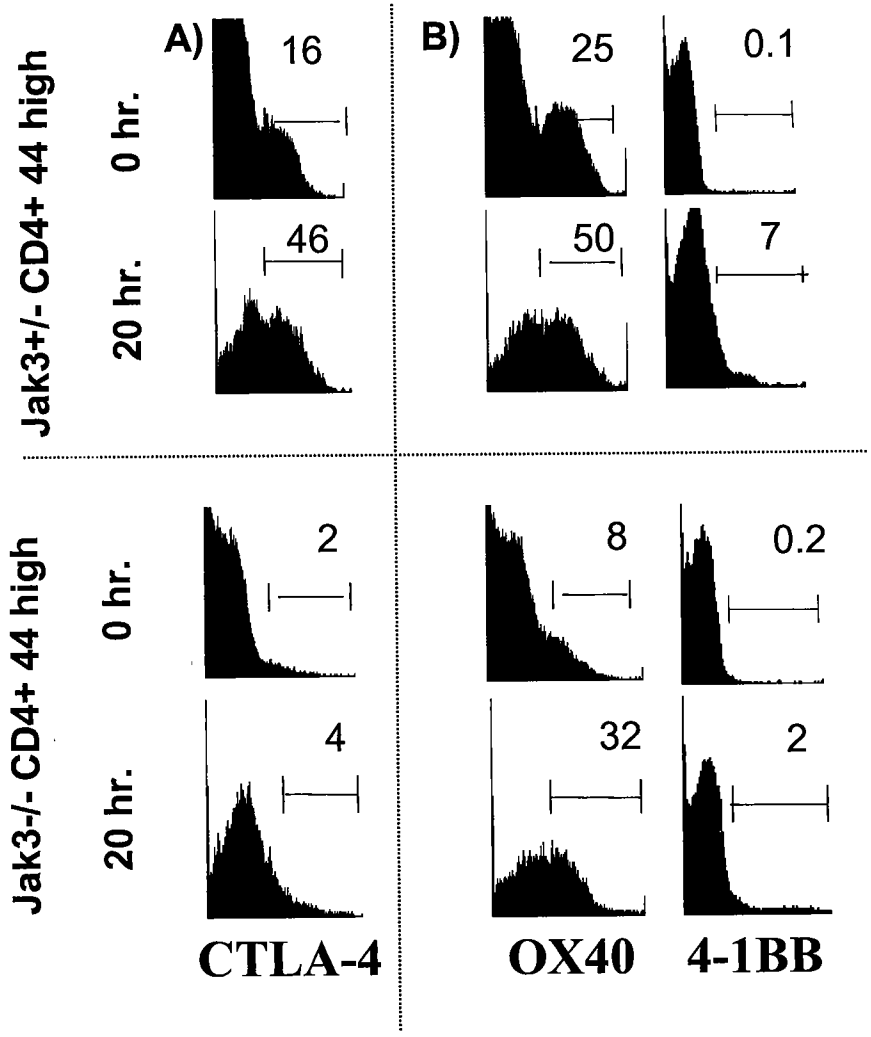


Figure 12. Reduced Expression of CTLA-4, 4-1BB, and OX40 on Jak3-/- CD4+ CD44-high peripheral T cells.

Purification of $CD4^+$ CD44-high splenic T cells from either Jak3+/- or Jak3-/- mice was performed as described previously. Cells were pooled from eight to ten Jak3+/- or Jak3-/- mice. 5×10^5 cells were stimulated with 10μg/ml plate-bound α-CD3 for 24 hours in a 96 well plate. Stimulated cells (24 hr) along with freshly isolated unstimulated controls (0 hr) were then stained with the indicated Abs in HBSS supplemented with 3% FCS for 20 min on ice. Cells were then washed and analyzed on a BD Biosciences FACSCalibur. Data were analyzed using CellQuest software. Abs used were biotinylated-α -CTLA4, 4-1BB or OX40 mAb, followed by APC-conjugated streptavidin. Cells are shown gated on live cells based on FSC/SSC and the percent positive cells are indicated in the upper right of each histogram. Data is representative of two independent experiments performed.

Chapter IV.

Results

B) Jak3-/- CD4+ CD44-high T cells have characteristics of Tr1 regulatory T cells.

Skewed cytokine profile of in vitro stimulated Jak3 deficient T cells

The microarray data on cytokine gene expression (Table 1) by freshly isolated Jak3-/- CD4+ T cells correlated well with our previous findings showing induction of IL-10, IFN-γ and TGF-β mRNA in Jak3-/- T cells stimulated *in vitro*. Additionally, this general cytokine profile is consistent with that described for some regulatory CD4+ T cell subsets. To determine whether Jak3-/- T cells actually secrete increased levels of these cytokines, CD4+ CD44-high T cells were isolated from Jak3+/- or Jak3-/- mice and were stimulated *in vitro* for 18-22h with 10ug/ml α-CD3 antibody. Supernatants from stimulated cells were collected and assayed for IL-2, -4, -5, -10, IFN-γ, and TGF-β by ELISA. Figure 13 shows the results of two independent experiments indicating that Jak3-/- T cells indeed secrete significantly higher levels of IL-10, IFN-γ and TGF-β compared to Jak3+/- controls. Furthermore, these cells secrete no detectable IL-2, and reduced amounts of IL-5 and IL-4. These findings demonstrate that Jak3-/- CD4+ CD44-hi T cells are not conventional Th1 or Th2 cells, but instead, secrete a panel of cytokines associated with a subset of regulatory CD4+ T cells.

Jak3 deficient T cells can suppress the proliferation of wild type T cells

As mentioned previously, immunomodulatory cytokines such as IL-10, IFN-γ, and TGF-β can promote immune suppression, and potentially play a role in the suppressive function of some subsets of regulatory T cells *in vivo* [81,84,209]. Additionally, PD-1 expression

Figure 13. Jak3-/- CD4+ T cells secrete immunosuppressive cytokines

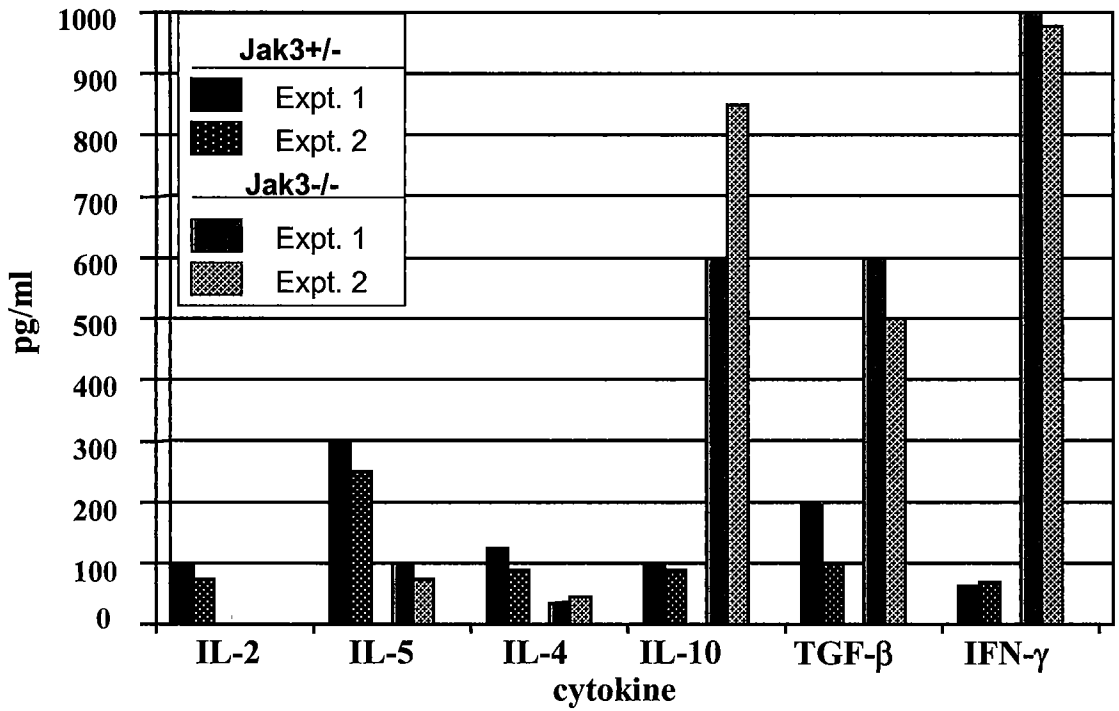


Figure 13. Jak3 -/- CD4+CD44-high peripheral T cells secrete immunosuppressive cytokines.

Purification of $CD4^+$ CD44-high splenic T cells from either Jak3+/- or Jak3-/- mice was performed as described previously. 5×10^5 cells were stimulated with 10μg/ml plate-bound α-CD3 for 18-22 hours in a 96 well plate. Cells were removed by centrifugation and the supernatant was serially diluted and assayed for the presence of IL-2, IFN-γ. IL-4 IL-5, IL-10, (OptEIA™, PharMingen) and TGF-β (Emax Immunoassay System, Promega), according to the manufacturer's protocol. The absorbance was read at 450 nm using an ELISA plate reader. The values are the mean pg/ml cytokine production of triplicate experiments performed. The results are shown for two independent experiments with five to six mice used per group for stimulated CD4+ T cells from either Jak3+/- **(black bars)** or Jak3-/- **(gray bars)**.

has been associated with both anergic and IL-10 producing regulatory T cell subsets [89,187,230]. Therefore, we were interested in determining whether Jak3 -/- T cells could suppress the proliferation of wild type CD4+ T cells. Total CD4+ T cells were isolated from either Jak3+/- or Jak3-/- mice. 2.5×10^5 Jak3+/- CD4+ T cells were cultured in 96 well plates coated with 10ug/ml α-CD3 antibody. At 24 or 48 hours of culture, 2.5×10^5 mitomycin-C treated Jak3 +/- or 2.5×10^5 Jak3-/- CD4+ T cells were added to the responder T cells. In addition, 5×10^5 Jak3+/- or Jak3-/- CD4+ T cells were stimulated in isolation as controls. On day 3, cells were harvested and proliferation was determined by ^{3}H-thymidine incorporation. When Jak3 -/- T cells were added to the control responder T cells at the initiation of the culture, or 24 hours later, there was no apparent effect on responder T cell proliferation (Figure 14 and data not shown). Interestingly, however, when Jak3-/- T cells were added on day 2 of culture, Jak3+/- T cell proliferation was inhibited by approximately 50%. The fact that Jak3-/- T cells rapidly die following *in vitro* culture [231] may account for the inability of these cells to inhibit when they are added early on in the stimulation period. Finally, to determine if the inhibitory function of Jak3-/- T cells is mediated by PD-1 or IL-10, we added blocking antibodies into the co-culture experiments. Blocking either PD-1 or IL-10 with up to 5μg/ml of antibody had only a minimal effect on the inhibition mediated by Jak3-/- T cells (Figure 15).

Figure 14. Jak3-/- CD4+ T cells can suppress WT T cell proliferation

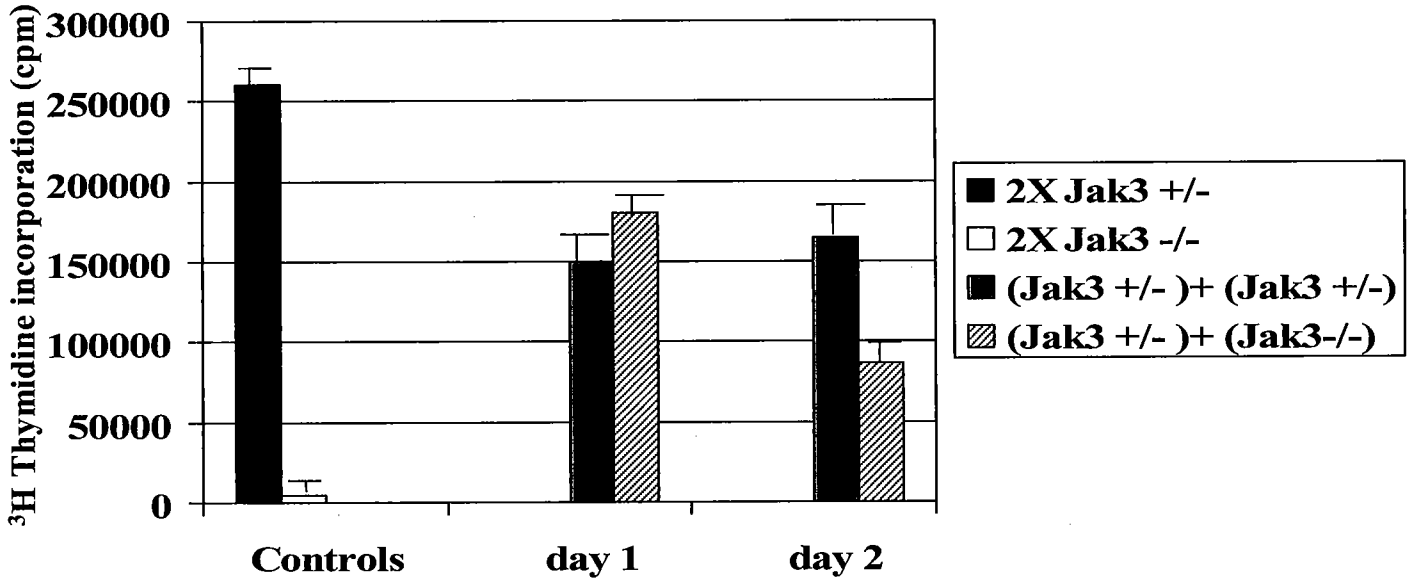


Figure 14. Jak3-deficient T cells can suppress the proliferation of wild type T cells.

2.5×10^5 Jak3+/- CD4+ T cells were cultured in 96 well plates coated with 10μg/ml α-CD3 antibody. At 24 (day 1) or 48 (day 2) hours of culture, 2.5×10^5 mitomycin-C treated Jak3 +/- (light gray bar) or 2.5×10^5 Jak3-/- CD4+ T (hatched bar) cells were added to the responder T cells. In addition, 5×10^5 Jak3+/- (dark gray bar) or Jak3-/- (white bar) CD4+ T cells were stimulated in isolation as controls. On day 3, cells were harvested and proliferation was determined by ^{3}H-thymidine incorporation. The values are mean count per minute (+/- SD) of triplicate determinations. Data is representative of eight experiments performed. Significance of experiments was determined by student's t test to be $p < 0.003$ 2XJak3+/-; $p < 0.001$ 2XJak3-/-; $p < 0.02$ (Jak3+/-) + (Jak3+/-) day 1; $p < 0.009$ (Jak3-/-) + (Jak3-/-) day 1; $p < 0.01$ (Jak3+/-) + (Jak3+/-) day 2; $p < 0.009$ (Jak3-/-) + (Jak3-/-) day 2;.

.

Figure 15. Blocking either PD-1 or IL-10 has only negligible effects on Jak3-/- CD4+ T cell mediated inhibition of Wild type T cell proliferation

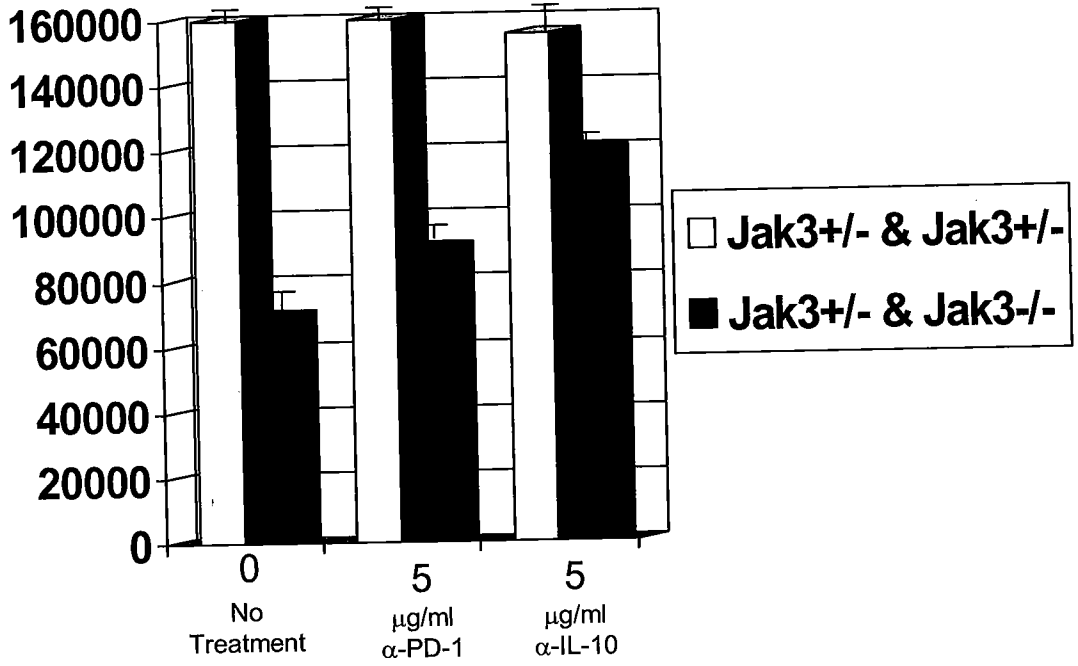


Figure 15. Suppression of wild type CD4+ T cells by Jak3-/- CD4+ T cells is not reversed by blocking either PD-1 or IL-10.

2.5×10^5 Jak3+/- CD4+ T cells were cultured in 96 well plates coated with 10μg/ml α-CD3 antibody. After 48 hours of culture, 2.5×10^5 mitomycin-C treated Jak3 +/- (light blue bar) or 2.5×10^5 Jak3-/- CD4+ T (dark blue bar) cells were added to the responder T cells alone or in addition to 5μg/ml blocking antibodies to PD-1 or IL-10. In addition, 5×10^5 Jak3+/- or Jak3-/- CD4+ T cells were stimulated in isolation as controls (not shown). On day 3, cells were harvested and proliferation was determined by ^{3}H-thymidine incorporation. The values are mean count per minute (+/- SD) of triplicate determinations. Data is representative of three experiments performed. $p < 0.001$ for no treatment group, $p < 0.005$ for anti-PD-1 treatment group, $p < 0.02$ for anti-IL-10 treatment group.

Chapter IV.

Results

C) The phenotypic defects in Jak3-/- CD4+ T cells are cell autonomous.

Jak3-deficient CD4+ thymocytes undergo homeostatic proliferation and acquire a "regulatory" T cell phenotype in Rag2-deficient hosts

Since mice lacking Jak3 have pleiotropic defects resulting in numerous immune abnormalities, it is difficult to determine whether aberrations in T cell function are strictly T cell-intrinsic, or alternatively, is partially dependent on other aspects of the Jak3-/- environment. To examine this issue we began with Jak3-/- thymocytes, which are, by all criteria, developmentally normal [175,185]. Additionally, Jak3-/- thymocytes do not secrete IL-10 nor do they display increased expression of either PD-1 or PD-L2 (Figure 16 and 17), indicating that the functional defects observed in mature Jak3-/- T cells are acquired post emigration from the thymus.

To address whether the immunosuppressive phenotype of Jak3-/- CD4+ T cells is T cell-intrinsic, CFSE labeled Jak3+/- or Jak3-/- thymocytes were adoptively transferred into Rag2-/- mice. Rag2-/- mice were chosen as recipients to mimic the lymphopenic environment normally encountered by Jak3-/- thymocytes as they enter the periphery. At 2, 4, 6, and 8 weeks post adoptive transfer, splenocytes were harvested from Rag2-/- mice that received Jak3+/- (Jak3+/- AT) or Jak3-/- (Jak3-/- AT) thymocytes. At all time points, loss of CFSE was assessed and absolute T cell numbers were calculated as an estimate of proliferation. Initially, CD4+ Jak3-/- thymocytes proliferated (representative CFSE shown in Figure 18) and accumulated (Figure 19) to a similar degree as Jak3+/- CD4+ T cells. However, by 6wks post adoptive transfer, there is a decline in Jak3-/-AT T cell numbers

Figure 16. Cyotkine secretion by Jak3+/- or Jak3-/- thymocytes stimulated with PMA

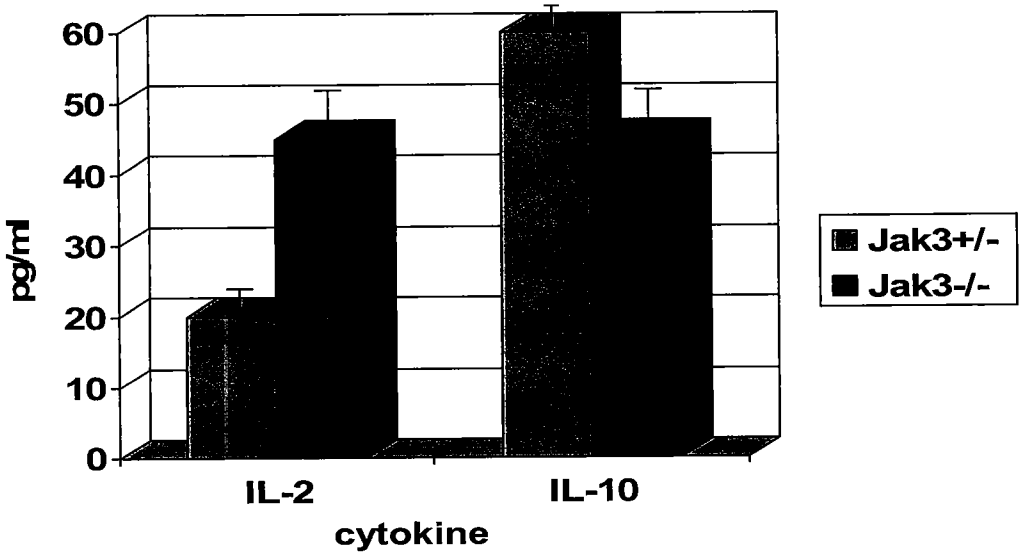


Figure 16. Jak3-/- thymocytes secrete wild type levels of IL-2 and IL-10 in response to stimulation.

Total thymocytes were isolated from either Jak3+/- or Jak3-/- mice and stimulated in 96 well plates in the presence of 1μg/ml PMA + 5ng/ml anti-CD3 for 36 hours. Cells were removed by centrifugation and the supernatant was serially diluted and assayed for the presence of IL-2 or IL-10 according to the manufacturer's protocol. The absorbance was read at 450 nm using an ELISA plate reader. The results are shown for stimulated thymocytes from either Jak3+/- (gray bars) **or Jak3-/-** (black bars). **Data is representative of one experiment performed. The values graphed are the mean pg/ml cytokine production (+/- SD) of triplicate determinations.**

Figure. 17 PD-1 and PD-L1 expression is comparable on Jak3+/- and Jak3-/- CD4 SP thymocytes

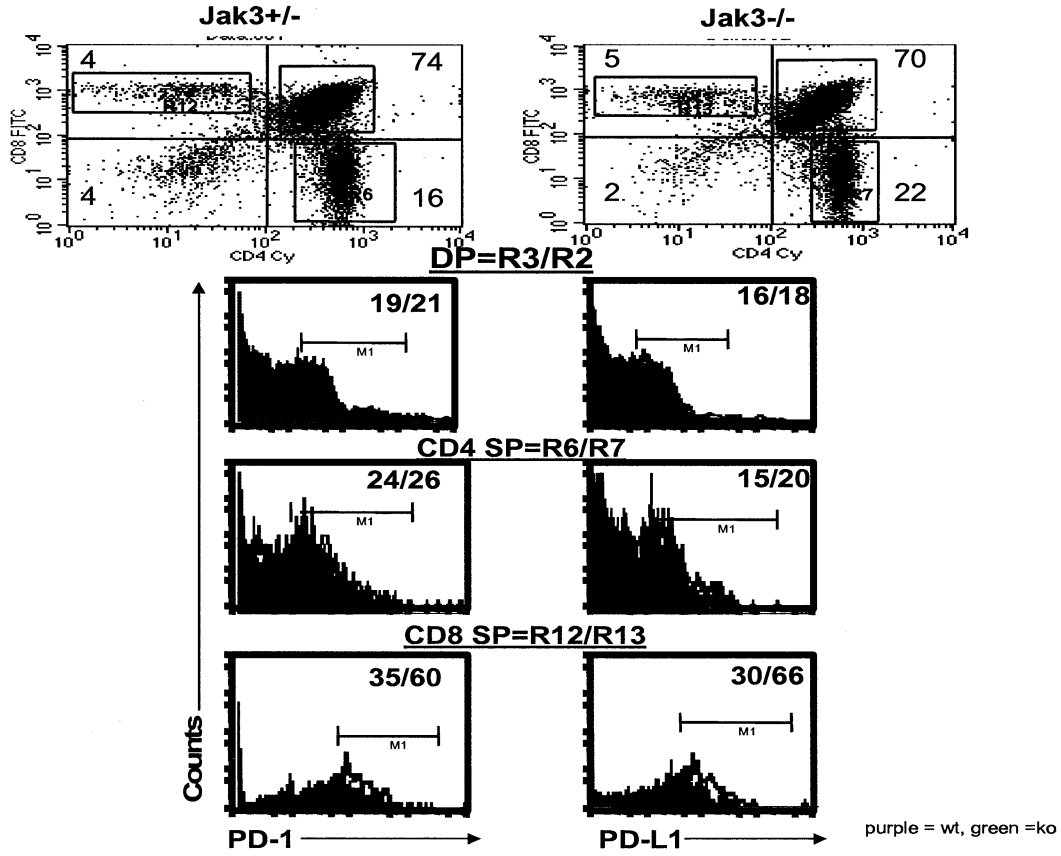


Figure 17. PD-1 and PD-L1 expression is unaltered on Jak3-/- thymocyte subsets.

Total thymocytes were isolated from either Jak3+/- (purple shaded histograms) or Jak3-/- mice (green line) and stained with the indicated Abs in HBSS supplemented with 3% FCS for 20 min on ice. Cells were then washed and analyzed on a BD Biosciences FACSCalibur. Data were analyzed using CellQuest software. Antibodies used were α-CD4-FITC, α-CD8-APC, α-PD-1-PE, biotinylated α-PD-L1 followed by Cy-conjugated streptavidin. Cells are shown gated on live cells based on FSC/SSC. Percent positive cells are indicated in the histogram and are shown as percent positive Jak3+/- over percent positive Jak3-/-. Data is representative of three independent experiments performed.

Figure 18. Jak3 -/- CD4+ T cells proliferate comparably to Jak3+/- CD4+ T cells in a lymphopenic host based on loss of CFSE

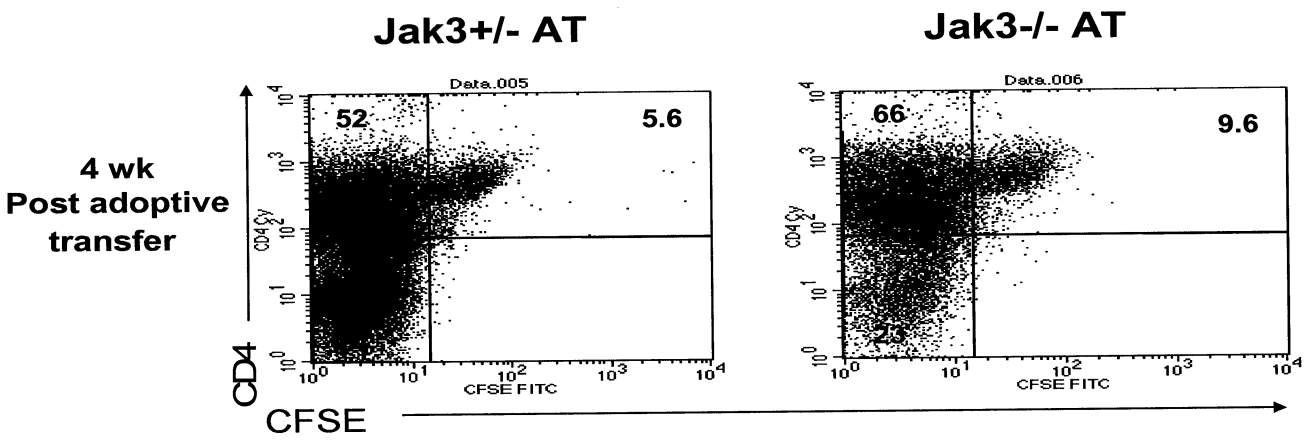


Figure 18. Jak3-/- CD4+ thymocytes proliferate equally to Jak3+/- CD4+ thymocytes in a lymphopenic host based on loss of CFSE.

Jak3+/- CD4 SP or Jak3-/- CD4 SP thymocytes were CFSE labeled and 1 x 10^6 cells were adoptively transferred by intravenous injection into the tail vein of Rag2 deficient hosts. At various time points post adoptive transfer, 4 weeks is shown, proliferation was measured based on loss of CFSE. At 4 weeks, total splenocytes were isolated from Rag2 deficient hosts that received either Jak3+/- (left panel) or Jak3-/- (right panel) and stained with antibodies to CD4 in HBSS supplemented with 3% FCS for 20 min on ice. Cells were then washed and analyzed on a BD Biosciences FACSCalibur. Data were analyzed using CellQuest software. Cells are shown gated on live cells based on FSC/SSC. Percent CFSE positive cells are indicated in the upper right. CFSE+/- gating was done using CFSE labeled *in vitro* stimulated cells. Data is representative of one experiment performed.

Figure 19. Jak3-/- CD4+ T cells initially accumulate comparably to Jak3+/- CD4+ T cells based on absolute cell numbers

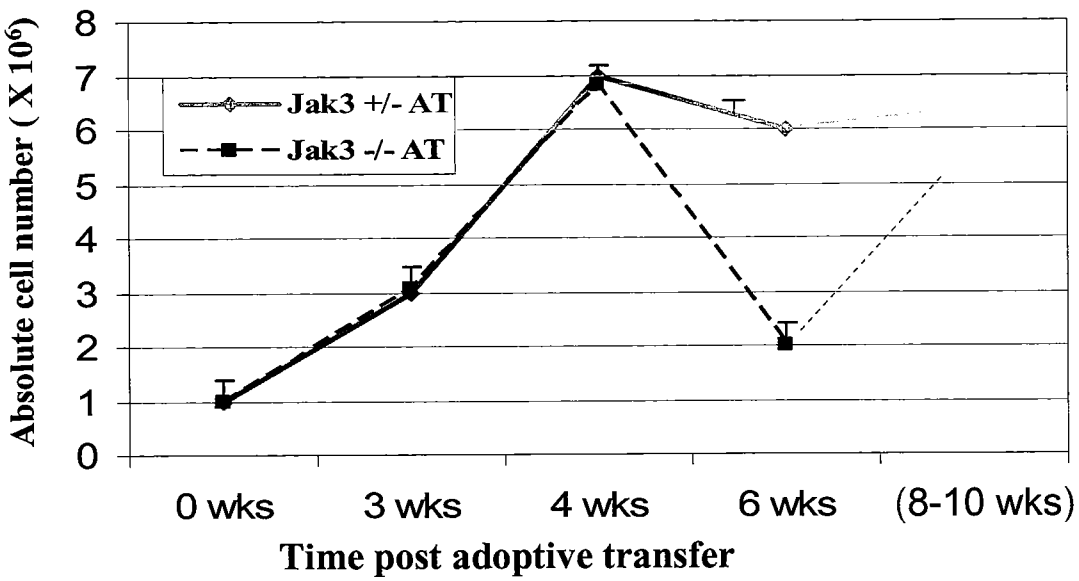


Figure 19. Jak3-deficient CD4+ thymocytes undergo homeostatic proliferation in Rag2-deficient hosts.

Two, four, and 6 weeks following adoptive transfers, spleens from RAG2-/- recipients i.v. injected with either Jak3+/- (Jak3+/- AT, gray line) or Jak3-/- (Jak3-/- AT, black line) CD4+ SP thymocytes were harvested and analyzed. Total splenocytes were stained with antibodies to CD4. The absolute number of CD4+ T cells was obtained by calculating this number from the absolute number of total lymphocytes times the CD4% as determined by flow cytometry analysis. Data are means for four mice at each time point, and are representative of two independent experiments.

(Figure 19). Interestingly, at 8 wks post adoptive transfer we begin to see a rise in Jak3-/- AT T cell numbers from the 6 wk drop, however this is based on a very small sample size and will have to be repeated.

We next determined the cytokine profile of either Jak3+/- AT or Jak3-/- AT CD4+ T cells. At two, four, six and eight week time points, purified splenocytes from adoptive transfer recipients were stimulated *in vitro*, and IL-2 and IL-10 production was determined by ELISA and/or intracellular cytokine staining (Figure 20 and Figure 21). Surprisingly, when CD4+ T cells were stimulated two weeks post adoptive transfer, comparable IL-2 secretion was detected in both Jak3+/- AT and Jak3-/- AT T cells. As shown in Fig. 20, at the 2 week time point, *in vitro* stimulated Jak3-/- AT T cells secrete approximately 1100 pg/ml IL-2 which was strikingly similar to the IL-2 production detected in Jak3+/- AT T cells (950 pg/ml). This amount of IL-2 production is in stark contrast to the undetectable levels of IL-2 production typically seen in non-transferred Jak3-/- CD4+ T cells isolated from mature Jak3 deficient mice and stimulated immediately ex vivo (Figure 13). After 4 and 6 weeks post adoptive transfer, IL-2 production in *in vitro* stimulated Jak3-/- AT T cells begins to decrease, falling to 400pg/ml (Fig. 20). However, IL-2 secretion in stimulated Jak3+/- AT T cells is consistently maintained at approximately 1000pg/ml over all time points tested. Interestingly, by 8 weeks, intracellular cytokine staining demonstrated that IL-2 production in Jak3-/- AT cells declined (0.4%) compared to 4 weeks (2.3%, data not shown) and is indistinguishable to that of non-transferred Jak3-/- in vitro stimulated T cells (0.5%) (Figure 21). Jak3-/- AT cells also produce IL-10 in response to *in vitro* stimulation at all time

Figure 20. Jak3-/- CD4+ T cells acquire immunosuppressive characteristics after expansion in Rag2 deficient hosts

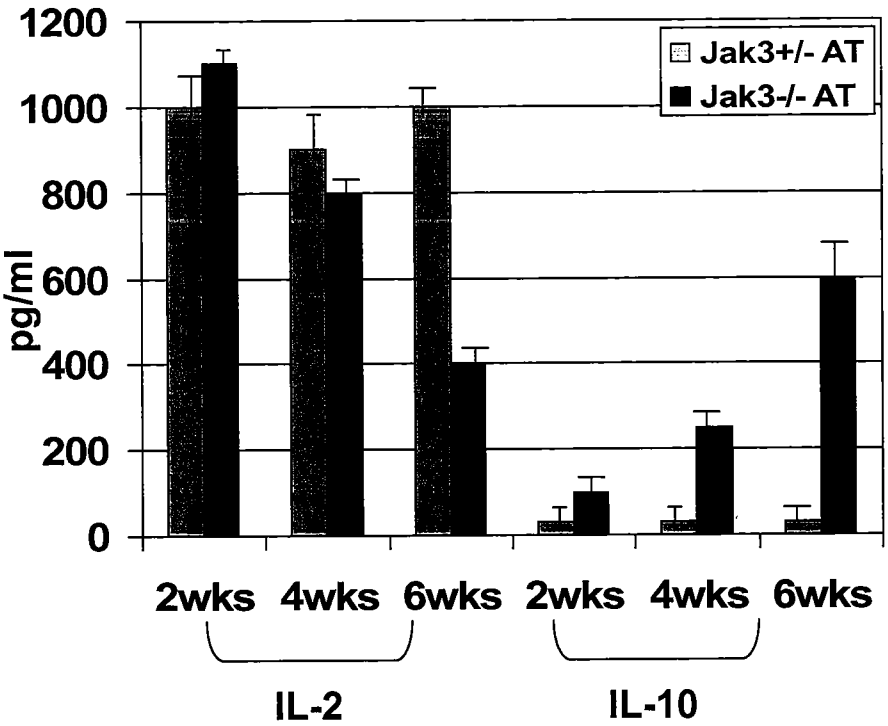


Figure 21. Cytokine analysis of peripheral T cells 8 wks after thymocyte adoptive transfer

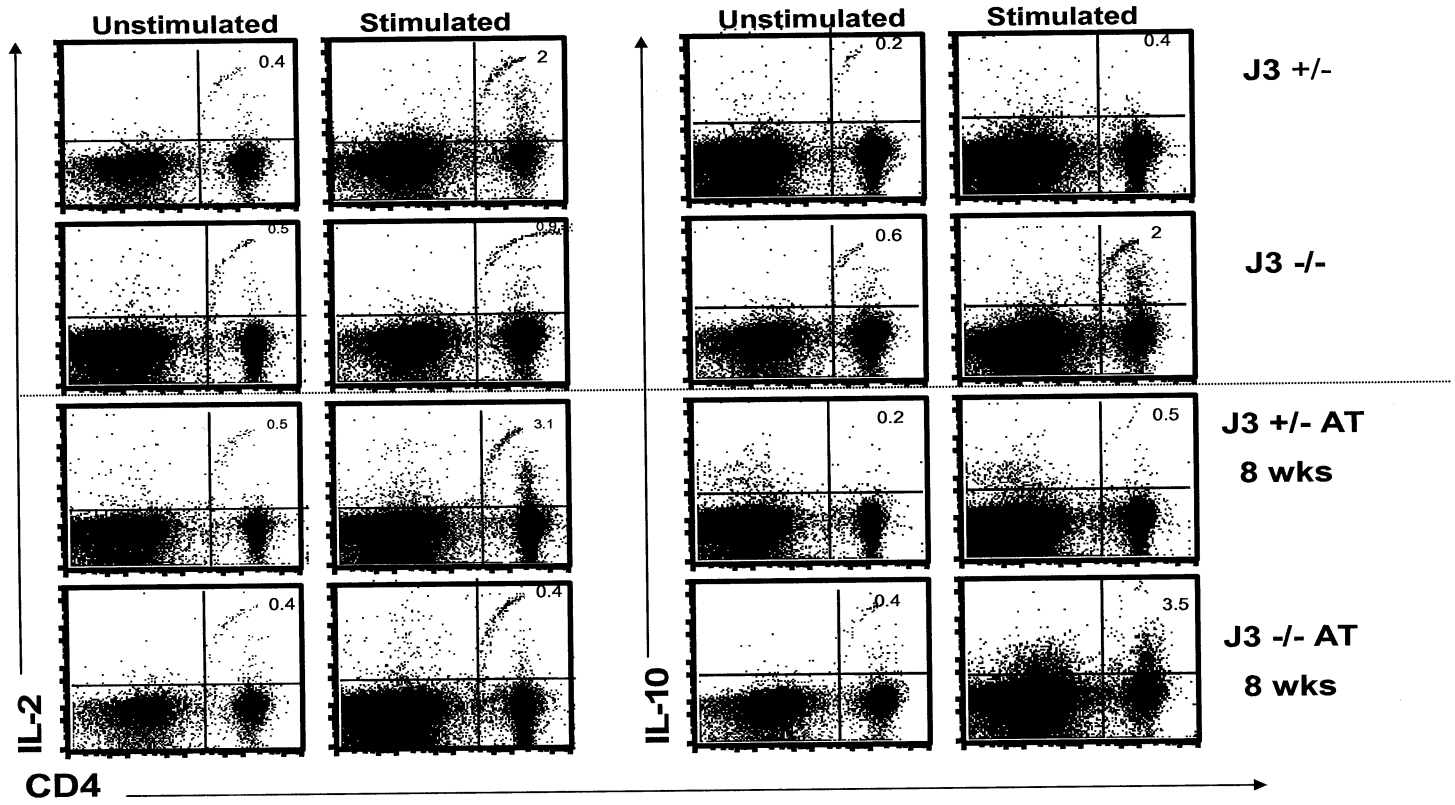


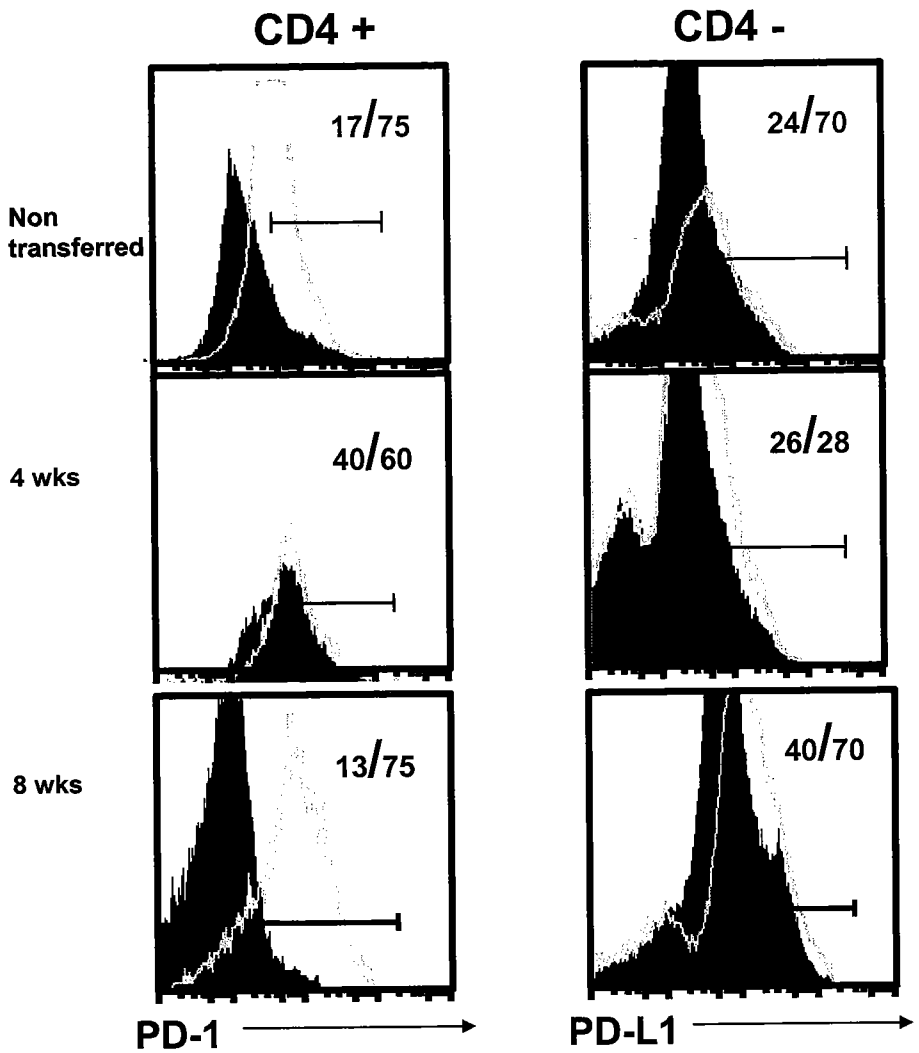
Figure 20 and Figure 21. Jak3-deficient CD4+ thymocytes acquire a "regulatory" T cell phenotype in Rag2-deficient hosts.

Two, 4, and 6 weeks following adoptive transfers, spleens from RAG2-/- recipients i.v. injected with either Jak3+/- (Jak3+/- AT, gray bar) or Jak3-/- (Jak3-/- AT, black bar) CD4+ SP thymocytes were harvested. 5 x 10^5 cells per well were stimulated with 10μg/ml plate-bound α-CD3 for 6-22 hours in a 96 well plate. The presence of IL-2 or IL-10 in each sample was measured by ELISA or Intracellular cytokine staining based on manufacturer's protocol. Data is representative of one experiment performed on 3 to 4 mice per group. ELISA results are represented as the mean of triplicate determinations (+/- SD).

points tested (Fig. 20). Interestingly, in contrast to the negative trend seen for IL-2 production, the levels of IL-10 production in Jak3-/- AT cells increase over time from 100pg/ml at two weeks post adoptive transfer to 600pg/ml by 6 weeks post adoptive transfer (Fig. 20). Additionally, by 8 weeks, ELISA results show that Jak3-/- AT CD4+ T cells secrete increased levels of IFN-γ and TGF- β similar to that seen for CD4+ Jak3-/- T cells (data not shown). This clearly suggests that the propensity of Jak3-/- CD4+ T cells to produce immunosuppressive cytokines is T cell intrinsic.

We then addressed whether changes in PD-1 or PD-L1 expression occurred in the adoptive transfer recipients. Jak3+/- AT or Jak3-/- AT cells were harvested at four and eight weeks and stained with antibodies to CD4 and either PD-1 or PD-L1. At four weeks post adoptive transfer, PD-1 expression is increased on Jak3+/- AT (40% positive) CD4+ T cells compared to non-transferred Jak3+/- CD4+ T cells (17% positive) (Figure 22, CD4+). Jak3-/- AT CD4+ T also express PD-1 (60% positive) at four weeks post transfer. At eight weeks, PD-1 expression declines on Jak3+/- AT CD4+ T cells, but remains at high levels on Jak3-/- AT CD4+ T cells (Fig. 22). Interestingly, at four weeks following adoptive transfer, PD-L1 expression on the non-T cell compartment of both Jak3+/- AT and Jak3-/- AT (26% and 28%) was similar to non transferred Jak3+/- (24%) (Figure 22, CD4-). However, by eight weeks, PD-L1 increased on both CD4-negative cells in both types of recipients, but was dramatically higher in the Jak3-/-AT recipients. These data suggest the interesting possibility that the Jak3-/- CD4+ T cells are inducing the expression of PD-L1 on other cell types, which

Figure 22. PD-1 and PD-L1 expression is increased after proliferation in Rag2 deficient hosts



Black=Jak3+/-AT, gray+=Jak3-/-AT

Figure 22. PD-1 and PD-L1 expression is altered on splenocytes in Rag2-deficient hosts.

Four and 8 weeks following adoptive transfers, spleens from RAG2-/- recipients i.v. injected with CD4+ SP thymocytes from either Jak3+/- (middle and bottom panel, black shaded histogram) or Jak3-/- (middle and bottom panel, gray line) or non transferred control splenocytes from Jak3+/- (top panel, black shaded histogram) or Jak3-/- (top panel, gray line) were harvested and analyzed. Total splenocytes were stained with antibodies to CD4, PD-1, and PD-L1. Cells are shown gated based on live FSC/SSC and either CD4+ or CD4- surface staining. Percent positive cells are indicated in the histograms and are shown as percent positive Jak3+/- over percent positive Jak3-/- in each. Data represents two independent experiments performed at each time point. For each experiment, three to four mice were used per group.

may in turn function to negatively regulate the T cells, thereby establishing a self-maintaining negative regulatory loop.

In addition, these data indicate that the unresponsive and immunosuppressive characteristics of CD4+ Jak3-/- T cells are cell autonomous. Importantly, we observe that initially following adoptive transfer, CD4+ Jak3-/- T cells are capable of IL-2 production when activated, but that this capability is lost over time. One possibility is that only a subset of CD4+ Jak3-/- T cells is capable of IL-2 production, and that these cells fail to survive in the absence of Jak3. Alternatively, the continuous drive to proliferate due to lymphopenia, the negative signals arising from increased levels of PD-1/PD-L1, and the lack of Jak3 signaling, together might drive the naïve IL-2 producing CD4+ T cells to differentiate into an effector subset that acquires the immunosuppressive qualities we observe with adult Jak3-/- peripheral CD4+ T cells.

In summary, the data presented in this chapter establish the following; First, PD-1 and PD-L1 expression levels are increased in the Jak3-/- microenvironment. Interestingly, PD-1 upregulation is found on the CD4+ T cell compartment whereas PD-L1 is detected primarily on CD4-negative splenocytes. Moreover, PD-L1+ Jak3-/- splenocytes can diminish the proliferative capacity of wild type CD4+ T cells, suggesting that the cell type expressing PD-L1 can interact with and mediate negative signals to T cells. This suggests the intriguing possibility that PD-1:PD-L1 may play a role in the unresponsive (lack of proliferation and IL-2 production) phenotype of Jak3-/- CD4+ T cells. It is unlikely however

that PD-1:PD-L1 alone can account for the total atypical phenotype including IL-10 production and T regulatory function. The primary evidence for this is that PD-1 is typically upregulated upon T cell receptor stimulation events that would lead to a productive immune response and generate IL-2 producing T cells with typical effector function. Consistent with this, our experiments in which CD4 SP thymocytes from Jak3+/- or Jak3-/- mice were transferred into RAG2-/- mice demonstrate that initially both Jak3+/- and Jak3-/- CD4+ T cells upregulate PD-1 and to a lesser degree PD-L1 is upregulated on CD4-negative splenocytes. Interestingly though, uniquely in RAG2-/- mice that received Jak3-/- CD4+ T cells, the upregulation of PD-1 is maintained and these CD4+ T cells ultimately take on an IL-10 producing T cell phenotype. In contrast, the PD-1 expression on transferred Jak3+/- T cells returns to levels typical of unmanipulated wild type CD4+ splenocytes and these cells maintain the ability to produce IL-2. This point suggests that it is not merely the upregulation of PD-1 but prolonged and continuous signaling via PD-1 that may lead to unresponsiveness. Further, this data suggests that signals through the γc may be important in the down regulation of the PD-1 receptor. However, as demonstrated, during this period of PD-1 expression the Jak3-/- CD4+ T cells additionally produce IL-10. The effects of IL-10 as well as the IFN-γ and TGF-β shown to be produced by CD4+ T cells from an unmodified Jak3-/- mouse on the differentiation of Jak3-/- CD4+ T cells into a T cell subset with regulatory characteristics cannot then be ruled out. One possibility is that all of these factors--proliferation in the absence of all γc signals, increased/prolonged signaling via PD-1:PD-L1, and potential gene modifications by IL-10,IFN-γ and TGF-β—synergistically lead to the

atypical CD4+ T cell phenotype in Jak3-/- mice. The role of IL-10, IFN-γ, and TGF-β and the effects of these cytokines on gene expression in Jak3-/- CD4+ T cells will be further addressed in the Chapter V.

Chapter V.

Results

Comparison of Global Gene Expression Patterns in wild type, Jak3-/-, STAT5ab-/- and lymphopenia-induced homeostatically expanded CD4+ CD44-high T cells.

Le poulet ou l'oeuf *--- (The chicken or the egg)*

The last chapter of this thesis will discuss data from gene array analyses which compared global gene expression amongst four populations of CD4+ CD44-high peripheral T cells isolated from wild type (Jak3+/- or STAT5ab+/-), Jak3-deficient, STAT5ab-deficient, or wild type CD4+ T cells that underwent lymphopenia induced homeostatic expansion.

Because the impetus for these experiments and the data analyses are complicated, this chapter begins with slightly more detail regarding the rationale behind these experiments as well as some detail regarding analysis. Furthermore, while we had some specific objectives in mind when we began these experiments, and these will be highlighted below, the data will be presented and discussed more from the general view of T cell fitness with some regard to specifically addressing the mechanism(s) by which Jak3-/- CD4+ are unable to proliferate and secrete IL-2 *in vitro*. The term fitness is used broadly to include basal cellular functions such as cell cycle and apoptosis as well as pathways specifically important to T cell function such as cytokine activity and the mitogen activated protein kinase pathway.

Rationale

The underlying theme throughout this thesis work is that the interplay of many cell surface signaling molecules and signaling mediators, some of which are mediated by cytokine, together lead to T cell activation--- a tip in the balance of these signals will lead to

alternative outcomes such as, deletion, unresponsiveness, and/or the development of T cells with regulatory or suppressive effector functions.

Since T cell activation is an elaborate network of intricately balanced signals emanating from TCR, cytokine, and other co-stimulatory molecules, discriminating between important downstream mediators and cellular targets of the many positive acting or negative acting receptors present on a T cell is difficult. This is particularly true in the case of the T cell receptor and γc-dependent cytokine signaling, since T cell activation will actually induce γc/IL-2 cytokine gene expression as well as other cytokines and their receptors. These cytokines then directly augment TCR signals and T cell activation. This fact makes the separation of TCR signals and γc-cytokine signals a challenge.

Moreover, the stimulation of these various receptors expressed by T cells are known to result in the activation of many redundant signal transduction pathways, and in the activation of, some distinct, but also many redundant transcription factors. Accordingly, it can be assumed that triggering any of these receptors should result in the expression of some redundant cellular target genes. In fact, TCR and IL-2 receptors activate some shared signaling pathways, such as the Ras-Raf-MAP kinase and PI-3 kinase/Akt/p70 S6 kinase pathways [232]. However, there are differences, such as the STAT pathway, which is uniquely regulated by cytokines [233].

Many previous studies have shown the specific induction of at least a subset of genes in T cells directly by IL-2 such as those encoding the IL-2Rα-chain, cyclin D2, SOCS1, CIS1, Pim-1, c-myc Bcl-XL, BCL-2, c-fos, and c-jun [234-236]. Further studies corroborated

this and in addition showed that γc-cytokines IL-2, IL-7, and IL-15 all induce very similar patterns of gene expression in T cells [237]. Consistent with these data our original microarray analyses comparing Jak3+/- and Jak3-/- CD4+ T cells showed a decrease in transcripts encoding Cis1, pim-1,and Bcl-2 (Table 5).

While IL-2/γc cytokines can induce these genes in T cells, most of these genes can be induced by multiple regulatory elements. For example, one of these genes, the IL-2Rα gene is regulated by at least five positive regulatory regions (PRRs) [238-242]. PRRI is a T cell receptor response element /NF-κB binding site that is required for IL-2Rα promoter activity in response to PHA or PMA, whereas PRRIII and PRRIV are both required for IL-2-induced IL-2Rα induction [238-242]. A fifth element is a CD28 response element [242]. Thus, in the IL-2Rα gene, different enhancer-like elements differentially respond to different stimuli. The coexistence of antigen and cytokine response elements in other genes as well might account for the highly overlapping gene expression profiles previously reported between γc-dependent cytokine signaling, PI-stimulation and lymphocyte receptor specific stimulation in B and T cells. In this regard, IL-15 and T cell receptor stimulation were recently shown to induce many of the same genes in CD8+ memory T cells [243].

All of the aforementioned factors significantly complicate the interpretation of much of our microarray data generated by comparing CD4+CD44-high T cells from Jak3+/- mice to CD4+ CD44-high T cells from Jak3-/- mice. Changes in gene expression could be the direct result of Jak3 dependent dysregulated signaling, or rather, the result of indirect effects on the general robustness of T cell receptor signals at points of which these signaling

pathways converge. Additionally, some genes known to be induced by γc cytokines might not be down-regulated in Jak3-/- CD4+ T cells because they are compensated for by signals through the TCR alone or the IL-2 independent effects of CD28. For example, TCR driven homoeostatic expansion signals mediated to Jak3-/- CD4+ T cells resulting from the lymphopenia associated with the Jak3 deficiency could induce some similar sets of genes as γc signals. Since the major role of γc signals, like IL-2, in T cell activation is to augment survival and proliferation other signals that initiate cell cycle and survival in T cells will potentially have cellular targets that overlap with cytokines, as has been shown to be the case for T cell receptor signals.

In the Jak3-deficient model, our data suggests that the issue of determining the Jak3/γc dependent events important in T cell responsiveness becomes even more complicated. First, Jak3-/- deficient T cells secrete a number of immunosuppressive cytokines (Fig. 13) which based on gene expression data act in an autocrine manner, directly inducing de novo gene expression in Jak3-/- CD4+ T cells (Table 6). Second, the upregulated expression and potential increase in interaction of PD-1:PD-L1 in Jak3-/- mice (Fig.6 & Fig. 8), may play a role in the unresponsiveness of Jak3-/-CD4+ T cells and could also lead to altered gene expression. Data regarding the precise downstream effector molecules that facilitate PD-1 driven inhibition in T cells and the cellular targets of these molecules is lacking and the general effects of PD-1:PD-L1 signaling could have on gene induction or repression in Jak3-/- CD4+ T cells is unclear.

Therefore, addressing the question of which Jak3 dependent gene targets are important in influencing T cell responsiveness and/or lead to the immunosuppressive phenotype in Jak3-/- CD4+ T cells is confounded by at least three potential factors: expansion/TCR signals that overlap with γc cytokine signals, signaling via PD-1:PD-L1, and signals mediated by IL-10, TGF-β and IFN-γ. Like all cells, the genetic profile of Jak3-/- T cells reflects the whole of its "experiences". Therefore, deciphering between cause or effect and thus "which came first" becomes a challenge. To begin to clear up these rather complicated possibilities, we decided to perform further microarray analyses, generating thousands of additional data points. The underlying goal was to eliminate some of the variables discussed above by essentially "normalizing" those variables using populations of CD4+ T cells that shared some of the "experiences" but lacked others. For example, comparing CD4+ Jak3-/- expression profiles to CD4+ T cells that also lacked γc signaling, underwent some homeostatic expansion, but did not induce immunosuppressive cytokines would provide us with more clear information on which gene expression modifications were related more specifically to the γc signaling deficiency as opposed to the increased signaling via immunosuppressive cytokine-receptor pairs.

To this end, we isolated highly purified (≥95%)CD4+ CD44-high peripheral T cell populations from either STAT5ab-deficient mice as well as wild type CD4+ splenocytes that had acquired a memory-CD44-high phenotype as a result of lymphopenia induced homeostatic expansion after adoptive transfer in a Rag2-/- host. The wild type CD4+ T cells that expanded in a Rag2-/- host will here in be referred to as HPH (homeostatically

proliferated CD44-high) CD4+ T cells. HPH cells were isolated at 3 weeks post adoptive transfer. At the 3 week time point, the majority of the CD4+ T cells were CD44-high (80%) and remained responsive to *in vitro* stimulation based on proliferation and IL-2 production (data not shown).

STAT5 T cells were chosen for several reasons. First, STAT5 it is directly activated by Jak3 and is essential in mediating the induction of γc/IL-2 responsive genes in T cells. Activation of Jak3 will lead to the phosphorylation of specific residues in the cytoplasmic domains of the IL-2Rβ. These phosphotyrosine motifs serve as docking sites for the SH2 domain in STAT5. Upon binding to the receptor complex, STAT5 will become phosphorylated, dimerize and translocate into the nucleus inducing the transcription of its target genes [244]. STAT5 exists in two different forms, STAT5a and STAT5b, encoded by separate genes. The two genes are highly homologous but differ in the C-terminus region [245]. STAT5 is expressed in a variety of tissues and although its biological effects are still incompletely understood, it is known to be of crucial importance for IL-2 mediated T cell proliferation. The second reason for choosing STAT5ab-deficient mice was that the phenotype of the peripheral CD4+ T cell is remarkably similar to Jak3-/- CD4+ T cells. In both of these knock-out models, the peripheral CD4+ T cells are virtually all CD44-high, memory-like and do not proliferate in response to stimulation. It should be noted, with regards to both of these attributes, the phenotype is more drastic in Jak3-deficient T cells. In contrast to Jak3-/- T cells, STAT5ab-/- CD4+ splenocytes do secrete IL-2 in response to in *vitro* stimulation. Additionally, the STAT pathway is activated uniquely by cytokines [233].

Based on this, comparing gene expression profiles of CD4+CD44-high T cells from Jak3-/- versus STAT5ab-/- mice will address the dysregulated gene expression that is uniquely associated with the absence of Jak/γc/STAT5 signaling in T cells (=similar changes in gene expression between Jak3-/- and STAT5ab-/- as compared to wild type), and differences in gene expression that might account for the inability of Jak3-/- CD4+ T cells to produce IL-2 (=dissimilar changes in gene expression Jak3-/- vs. STAT5ab-/-). Importantly, to our knowledge STAT5ab-deficient T cells did not induce immunosuppressive cytokines and therefore the induction of gene expression by these cytokines would not further complicate these analyses. Unfortunately, this gene expression comparison may not necessarily exclude potential effects of homeostatic expansion since the STAT5ab deficiency is likely to also lead to at least partial lymphopenia, leading to the memory CD44+ phenotype in the STAT5ab-/- CD4+ T cells. However, there is some evidence to suggest that the "degree" of homeostatic expansion that occurs in CD4+ T cells from STAT5ab-/- is less than that of Jak3-/- mice. First, the thymus cellularity is much greater in STAT5ab-/- than Jak3-/- mice and therefore there are a higher number of thymic emigrants in STAT5ab-/- mice. Second, in the periphery of STAT5ab-/- mice there are a greater number of CD8+ T cells and more naïve CD4 and CD8 T cells [246-248]. Together these points suggest that the periphery of STAT5ab-/- mice is more replete than that of Jak3-/- mice resulting in less expansion of the CD4+ T cells.

The comparison of gene expression patterns in Jak3-/-CD4+ CD44-high T cells to HPH CD4+ T cells will allow the potential effects of homeostatic driven proliferation on

gene expression patterns (=similar changes between Jak3-/- vs. HPH) to be accounted for and separated from those effects related more specifically to the complete absence of γc signaling (=similar changes in Jak3 and STAT compared to HPH).

Global Gene Array Analyses

Global gene array analyses were performed using MAS 4.0 and Genespring 6.1 software. Biological replicates were performed for all populations to account for random variability in gene expression and due to technical procedures. Therefore we were first interested in determining the similarity between our replicate samples for each CD4+CD44-high population and assessing the similarity between Jak3+/- and Stat5ab+/-. Similarity was measured using the Genespring Find Similar Sample feature which calculates the degree of similarity between the samples. For this comparison, all genes 12,422 (excluding Affymetrix controls) on the mU74Av.2 array were considered. The similarity measured used was Pearson correlation, and therefore a correlation coefficient of 1 indicates a perfect match, -1 = opposite and 0 = no match. The results from this analysis indicated a high degree of correlation between all replicates within in each group with 0.100 to 0.050 (10-5%) variation in the calculated correlation coefficient, thus, there was at least 90-95% similarity amongst replicates (reflected in Fig. 23). In addition, by this measure Jak3 and Stat5ab heterozygous mice appear indistinguishable and for the purpose of these analyses are considered identical.

Figure 23. Correlation between Jak3-/- CD4+ T cells and other CD4+ T cell populations based on global gene expression

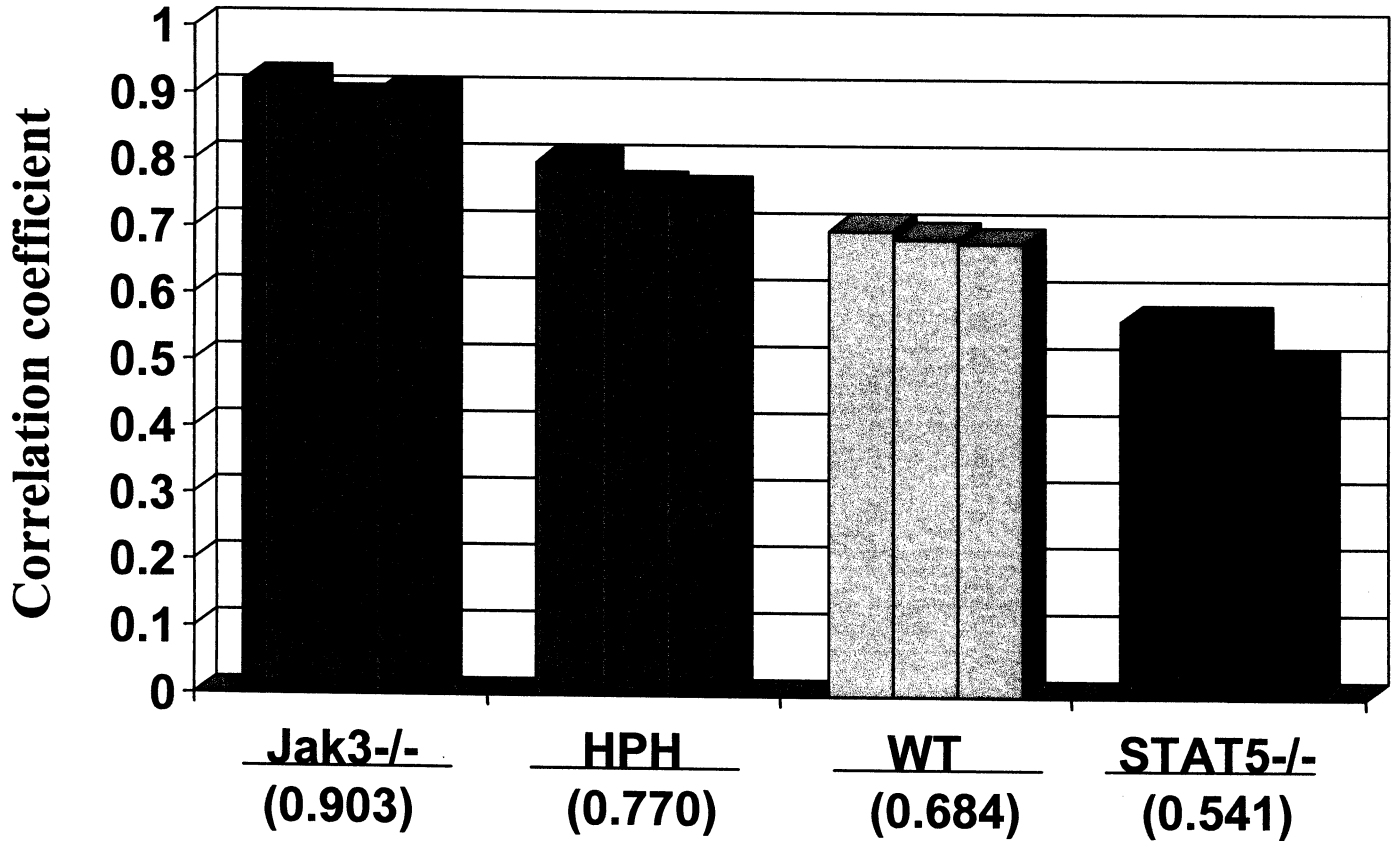


Figure 23. Correlation between Jak3-/- CD4+ T cells and other CD4+ T cell populations based on global gene expression levels.

A)The Genespring Find Similar Sample tool was used to determine the global similarity in gene expression levels between replicate samples performed within each population of CD4+ cells and between Jak3-/- CD4+ CD44-high T cells and wild type (WT), STAT5ab-/-, and HPH CD4+CD44-high population. For this comparison, all 12,422 (excluding Affymetrix controls) genes on the mU74Av.2 array were considered. Pearson correlation was used as the similarity measure, and therefore a correlation coefficient of 1 indicates a perfect match, -1 = opposite and 0 = no match. The data represents triplicate experiments performed as shown on the graph.

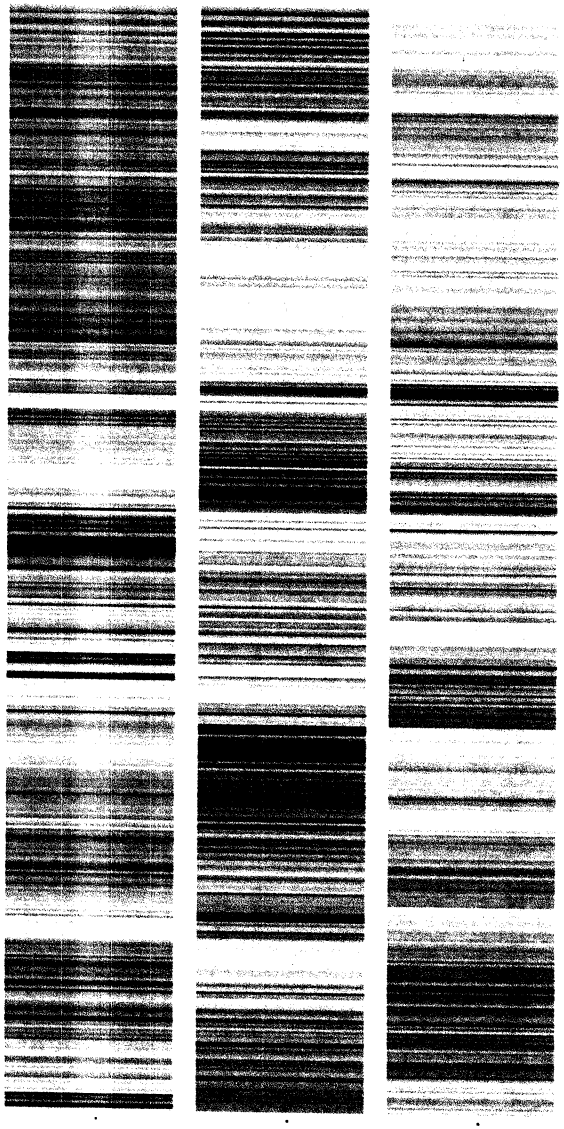
Measuring global similarity between samples also provided a unique way to determine the similarity in gene expression changes on a global level between wild type, Jak3-/-, STAT5ab-/-, and HPH CD4+ T cells. Interestingly, when Jak3-/- CD4+CD44-high T cells are set as the target and all other samples (wild type, STAT5ab-/-, HPH) are ranked according to highest degree of similarity to the target (Jak3-/-), Jak3-/-CD4+ T cells are most similar based on global changes in gene expression patterns to HPH CD4+ T cells with a coefficient of 0.770 or 77% similarity. Strikingly, the Jak3-/- CD4+ T cells are least similar to STAT5ab-/- CD4+ T cells (0.541, Fig. 23). This pattern can be further appreciated by representing the global changes in gene expression as a heat map plot (Fig.24) where changes in gene expression are indicated by a change in color.

For the generation of this heat map plot and all other comparison analyses presented here, the level of gene expression detected in wild type CD4+ CD44-high T cells was set as baseline gene expression level (WT gene expression level intensity on a per gene basis =1) data was then log transformed. Therefore, changes in gene expression levels in the experimental samples (Jak3-/-, STAT5ab-/-, or HPH) is a ratio of experimental gene expression over baseline gene expression, or the equivalent of fold change. The normalized data was then filtered based on the following criteria: p-value ≤ 0.05, with a present or marginal detection flag in at least one of the 4 samples being compared. These criteria generated a gene list of approximately 3,500 genes. Gene groups and clustering classifications (i.e. cell cycle, MAPK, etc) are based on GO ontology groups or defined

KEGG pathways. In some cases, the change in expression of specific genes was determined by searching the data with the GeneSpring Find Gene tool.

Figure 24. **Global gene expression patterns of Jak-/-, STAT5ab-/- and HPH CD4+ T cells**

A)



B)

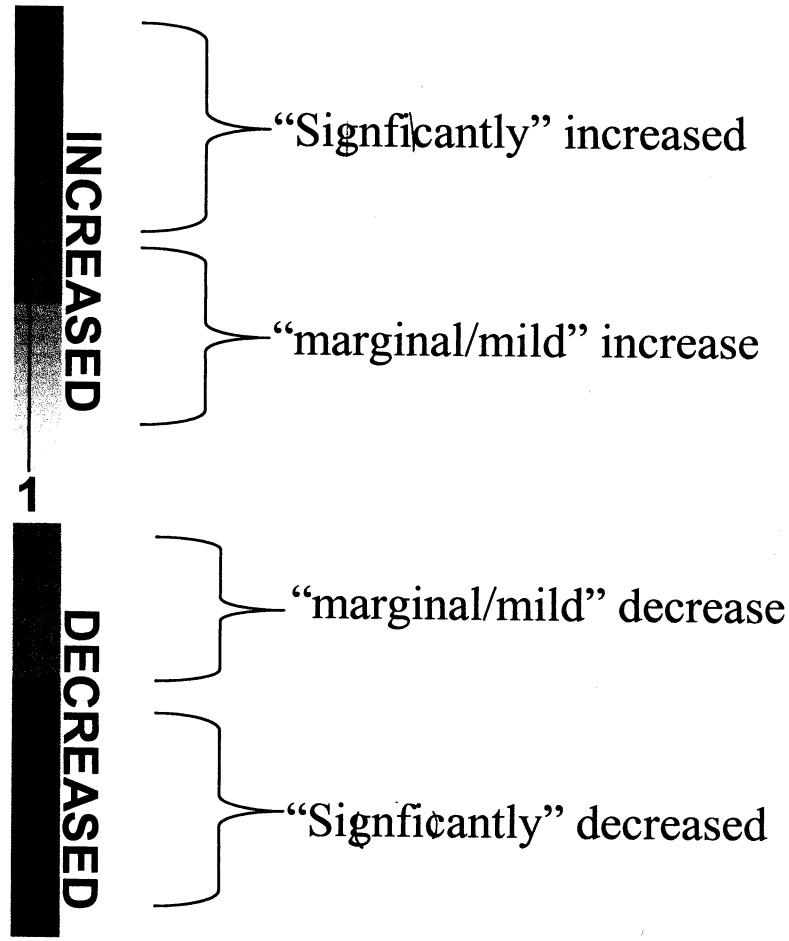


Figure 24. Variations in Global Gene Expression patterns in Jak3-/-, STAT5ab-/- and HPH CD4+CD44-high T compared to wild type.

A) A heat map plot of global gene expression patterns across CD4+ T cell populations was generated in GeneSpring. Each line/box represents one gene. Change in gene expression between CD4+ populations can be read by following the line/box across from left (WT) to right (Jak3-/-, STAT5-/-, HPH). Change in gene expression is represented as a shift in color from yellow. After data normalization wild type CD4+ CD44-high T cells were set as baseline gene expression level on a per gene basis. Expression level in the comparison population of CD4+ T cells from Jak3-/-, STAT5ab-/- or HPH is indicated as a change in color.

B) For an increase in gene expression, the color will become "hotter" (orange/red). To represent a decrease in gene expression the color becomes "cold" (gray/green/blue). Yellow reflects no change in gene expression. In general, gene expression will be described as marginally or significantly changed depending on the magnitude of color shift which is directly proportional to the magnitude of the change in expression level. The range in gene expression changes will change for each heat map plot and will be listed in the corresponding figure legend. All changes in gene expression were determined to be significantly significant by GeneSpring software based on Wilcoxon's Rank test or Standard Correlation ($p<0.05$) for triplicate experiments performed.

Cell cycle/Negative Regulation of Cell cycle/Cell cycle arrest

The interesting finding that Jak3-/- CD4+ CD44-high T cells had a global gene expression profile most similar to HPH CD4+ CD44-high T cells lead us to hypothesize that a great majority of this similarity might be due to more active proliferation i.e. homeostatic expansion occurring in Jak3-/- and HPH populations compared to either wild type or STAT5ab-/- CD4+CD44-high T cells. An increase in proliferation could be reflected by changes in gene expression of cell cycle related mRNAs. Consistent with this hypothesis, there is a vast degree of similarity in cell cycle gene expression in Jak3-/- and HPH CD4+ T cells (Fig. 25 A, and gene list Supp. 9). The general trend reflects an upregulation of many cell cycle genes in Jak3-/- (50%) and HPH (36%) and interestingly a down-regulation in STAT5ab-/- (65%, 25% upregulated). To be sure that the upregulation of cell cycle genes associated with Jak3 and HPH samples was not biased towards genes that would ultimately impair or arrest cell cycle events, we next looked at changes in gene expression amongst negative regulators of cell cycle and cell cycle arrest genes. As shown in (Fig. 25 B & C, and gene lists Supp. 10 & 11), negative cell cycle factors such as cyclin dependent kinase inhibitors p27 and p21 are not up-regulated in Jak3-/- or HPH. The up-regulation of cell cycle factors in Jak3-/- and HPH is specific to pro-mitotic genes such as lamin and cyclin family genes. The fact that Jak3-/- CD4+ T cells have a higher degree of cell cycle gene induction than STAT5ab-/- and the gene expression pattern is similar in HPH CD4+ T cells, adds merit to the notion that STAT5ab-/- are undergoing less homeostatic expansion.

Figure 25. Increased expression of many cell cycle genes in Jak3-/- CD4+ CD44-hi T cells

*Groups are based on GO biological Processes

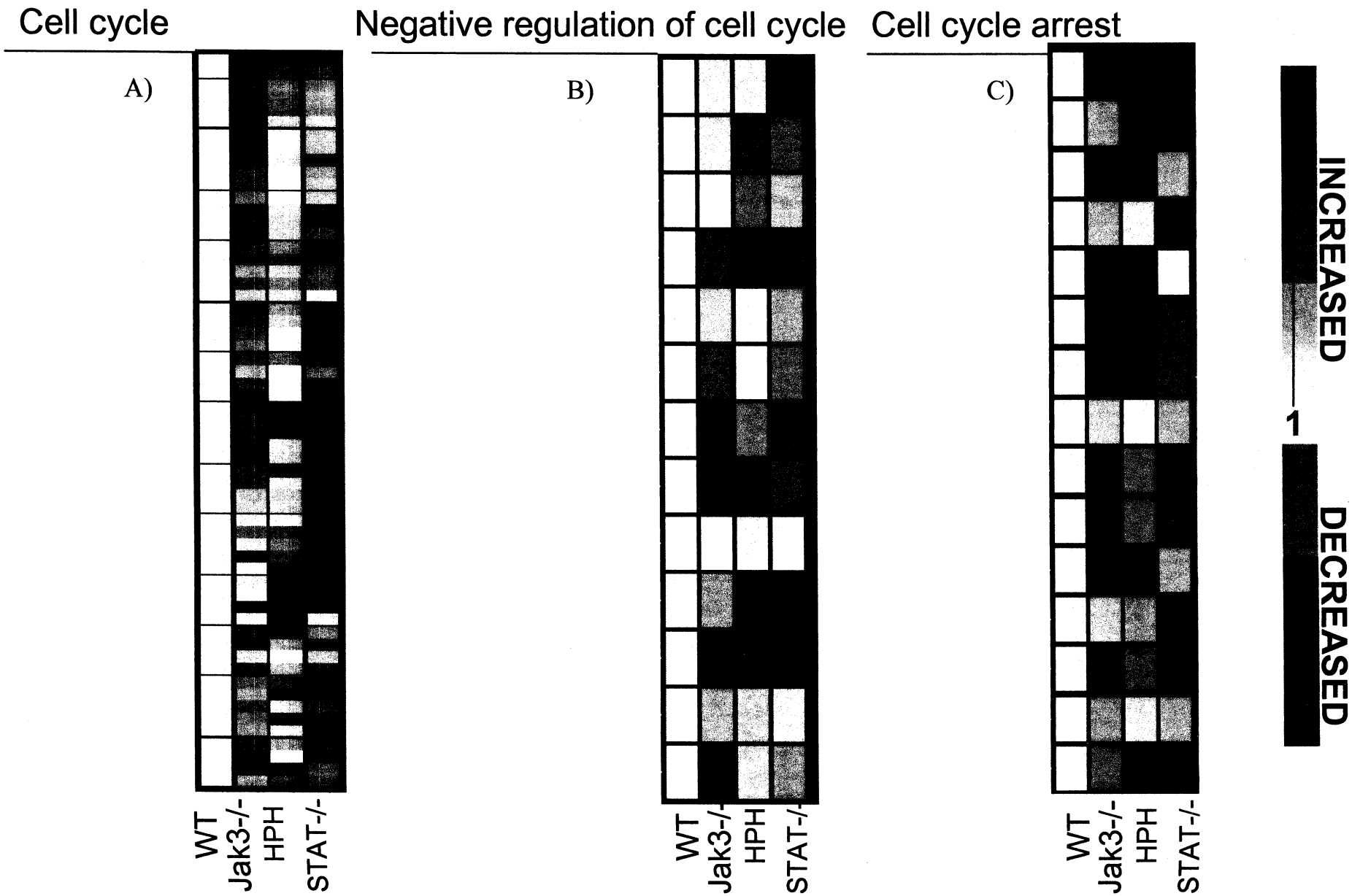


Figure 25. An increase in the expression of cell cycle genes in Jak3-/- CD4+ CD44-high T cells.

Gene expression patterns of genes involved in **A**) Cell cycle **B)** Negative regulation of Cell Cycle or **C)** Cell cycle arrest were clustered based on similarity. Similarity was measured by standard correlation ($p<0.05$). Gene classifications are based on the Gene Ontology Consortium (GO) gene annotations. Information on the genes contained in each group can be found in Supplemental Data 9-11. Gene expression data for CD4+ CD44-high T cells is listed in the following order: WT, Jak3-/-, HPH, STAT5ab-/-. Heat map plot expression level ranges are for A) +/- 6; for B) +/- 4 and C) +/- 3. Data is representative of triplicate experiments performed.

Cytokine

Our second hypothesis leading into these experiments was that, neither STAT5ab-/- or HPH CD4+ T cells would have an immunosuppressive cytokine profile that we have described previously for Jak3-/- CD4+ T cells. To assess this, we looked at the changes in gene expression in Jak3, STAT, or HPH compared to wild type CD4+ T cells for IFN-γ, TGF-β and IL-10. As shown in figure 26 A, in contrast to the up-regulation of these cytokines in Jak3-/- CD4+ T cells, STAT5ab-/- CD4+ show a decrease in transcripts that encode these cytokines. Surprisingly, HPH CD4+ T cells share the upregulated pattern of gene expression for IFN-γ and TGF-β (Fig. 26 A). IL-10 is also upregulated but only mildly. Since we had previously shown that in Jak3-/- CD4+ T cells many IFN-γ, TGF-β, and IL-10 related/inducible genes were also upregulated (Table 6), we next wanted to address whether we could detect any of these same gene inductions in either STAT5ab or HPH. The expression profile of these genes were compiled in Genespring using the Find Gene Tool and depicted as a heat map plot. Consistent with the changes in gene expression for cytokine specific genes in Jak3-/- CD4+ T cells, there is an upregulation of these cytokine inducible genes in HPH (50% compared to 80% in Jak3-/-). In contrast, there is a clear down-regulation of these genes in STAT5ab-/- (Fig. 26 B).

Cytokine Activity

Figure 26. Homeostatic Expansion of WT CD4+T cells also results in the induction IFN-γ, TGF-β, IL-10 and some related genes

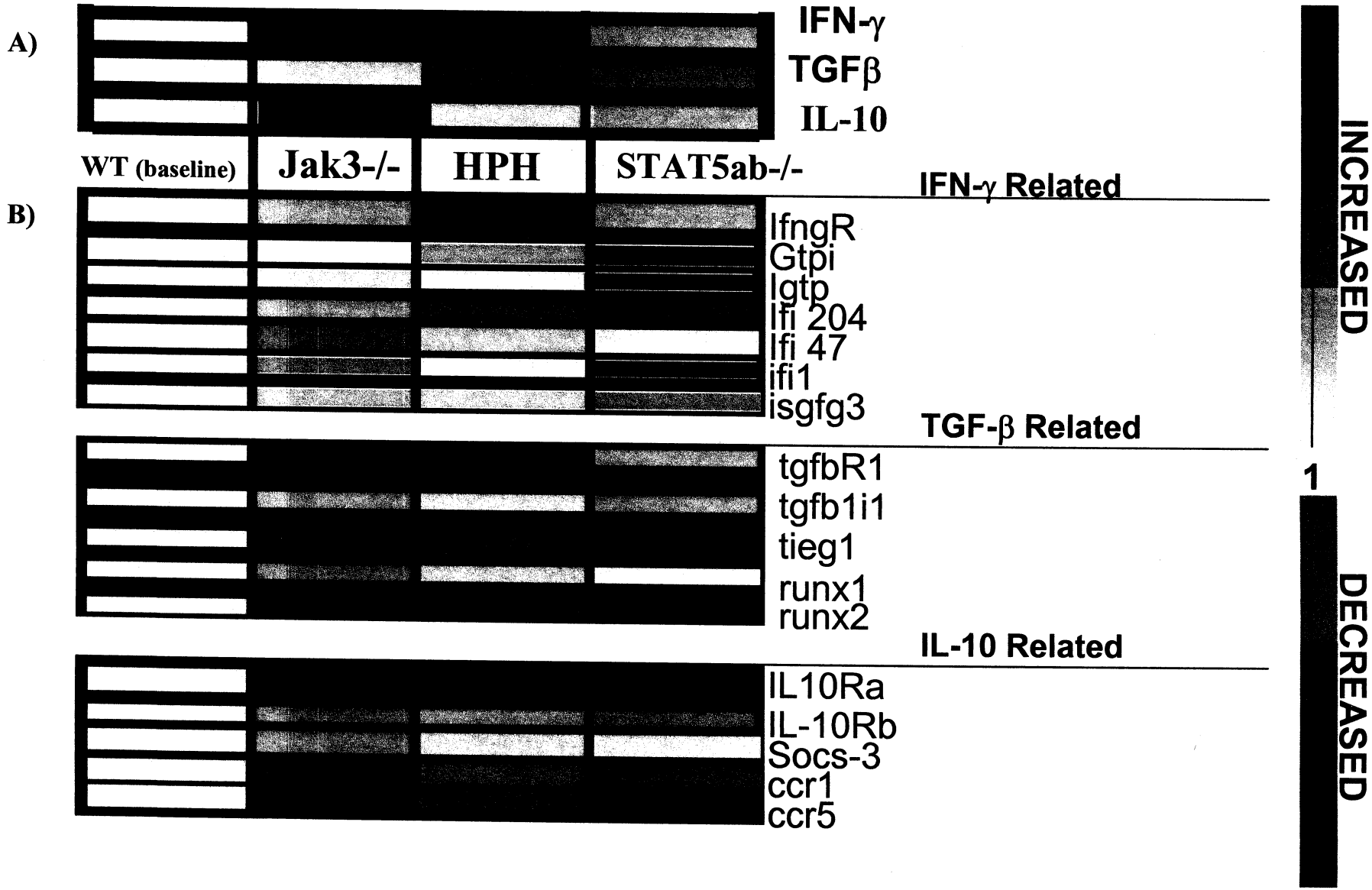


Figure 26. Homeostatic Expansion of WT CD4+ T cells also results in the induction of IFN-γ, TGF-β, IL-10 and related genes.

A) Expression levels of transcripts encoding IFN-γ, TGF-β, or IL-10 from either Jak3-/-, HPH, or STAT5ab-/- were plotted as a heat map in GeneSpring.
B) IFN-γ TGF-β IL-10 related gene classifications are based on signaling pathways compiled by and based on Gene Ontology (GO) Consortium. Gene expression data for CD4+ CD44-high T cells is listed in the following order: WT, Jak3-/-, HPH, STAT5ab-/-. All heat map plot Expression levels in A) and B) range from +10 to -10. Data is representative of triplicate experiments performed.

IFN-γ, TGF-β and IL-10 and the expression of cytokines in T cells could suggest a number of things about both T cell function and differentiation, we next wanted to address whether we could detect any other unusual pattern of cytokine gene activity. For this analysis we grouped the cytokine activity genes based on similarity in gene expression patterns amongst the four populations in CD4+ T cells using the k-means clustering tool in GeneSpring. k-means clustering divides genes into groups based on their expression patterns. Standard correlation was used to measure similarity in expression and the data was clustered into 5 groups (around 5 centroids) after 100 iterations. The gene expression levels of all of the genes in one sample that are clustered together are averaged and therefore the trend in gene expression per cluster group is depicted as a single line on each graph (Figure 27, Supp.12-16). The striking general pattern that can be detected in this data is that in four (Group1, 2, 3, 4) out of the 5 groupings, the pattern of cytokine gene activity is most similar between Jak3-/- and HPH CD4+ T cells. This equates to 81% similarity between Jak3-/- and HPH cytokine activity gene expression. In every group (Fig. 27, group 1-5), the pattern of gene expression in STAT5ab-/- CD4+ T cells is the reciprocal of Jak3-/- CD4+ T cells. Some of the genes represented overlap with those previously noted, such as an increase in IL-10 (Group 2, Fig. 27, Supp. 13) and IFN-γ (Group 3 Fig. 27, Supp. 14) in Jak3-/- and HPH CD4+ CD44-high T cells. Additionally, this analysis reveals an upregulation of genes such as IL-12 α/β, IL-11, IL-15, INF-α, and MIP-1β in Jak3-/- and HPH (Group 2 & 3). And these are down-regulated in STAT5ab-/-. Interestingly, genes that are increased in STAT5ab-/- and HPH

Figure 27. Cytokine activity genes grouped by expression level-5k- means Cluster

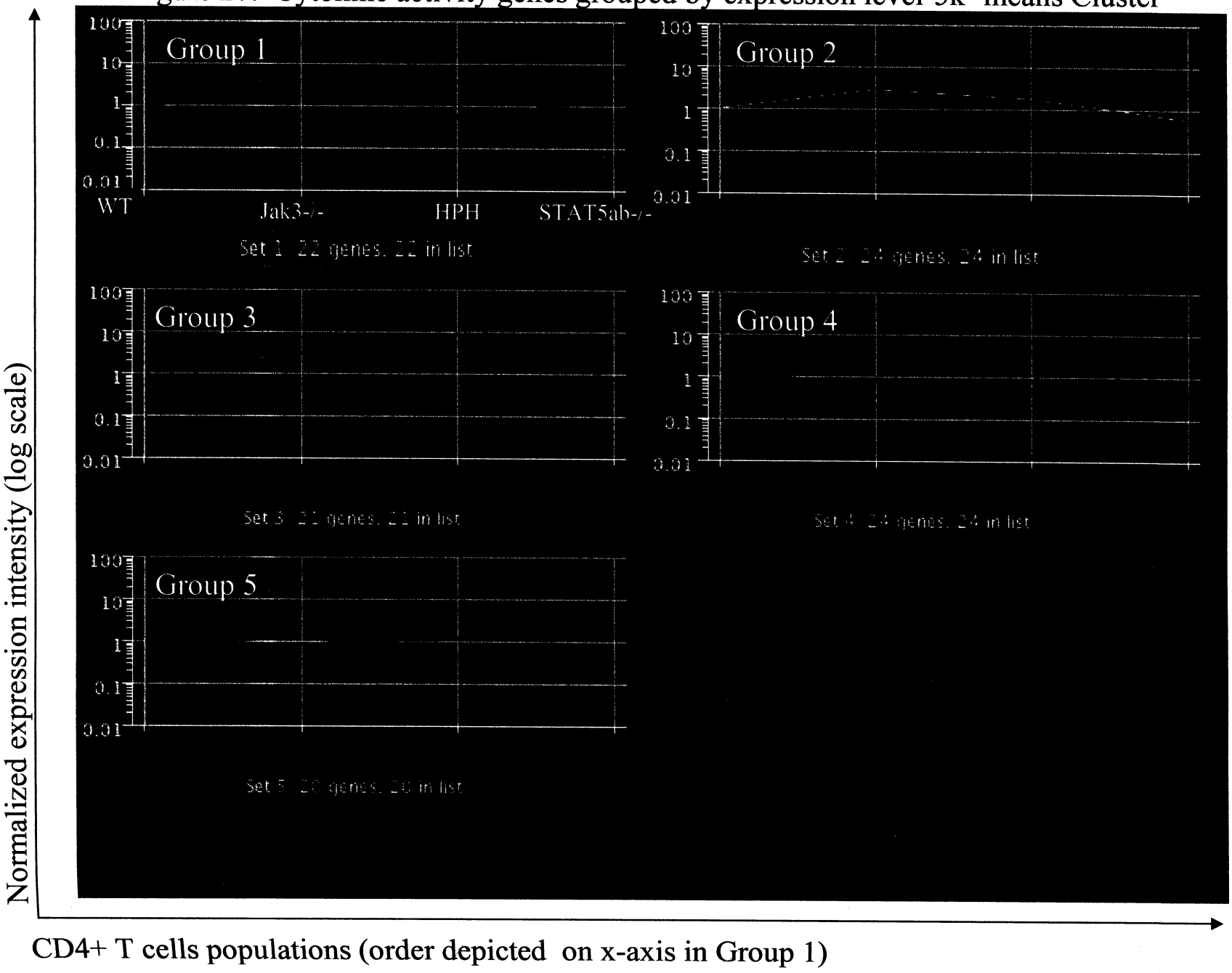


Figure 27. Cytokine activity genes grouped by gene expression patterns.

Cytokine Activity genes were based on GO gene annotations and classifications. Genes with similar patterns of gene expression were determined by k-means clustering around 5 centroids after 100 iterations using standard correlation as the similarity measure ($p<0.05$). The line on the graph represents the average pattern of gene expression within each group. The number of genes classified in each group is listed below the graphs. Information on the genes contained with in each group can be found in Supplemental Data 12-16. Data is representative of triplicate experiments performed.

but decreased in Jak3-/- include those that encode γc cytokines IL-7 and IL-2 (Group 5, Supp. 16).

Apoptosis Regulators

Because of the importance of γc signaling in T cell survival and activation induced cell death and the known role for Jak3/γc/STAT5 signals in the induction of anti-apoptotic factors Bcl-2 and Bcl-Xl, we next looked at genes relating to the regulation of apoptosis. As shown in Fig. 28 A, there is a 70% similarity in gene expression changes between Jak3 and HPH CD4+ CD44-high T cells. STAT5 shares about 48% of these same changes. If we then sub-categorize the apoptosis regulators based on apoptosis inhibitory activity (Fig. 28 B) or apoptosis activator activity (Fig. 28 C), there is a distinct down regulation of apoptosis activators in STAT5ab-/- CD4+ T cells while genes such as PTEN and Bax are upregulated uniquely in Jak3-/- CD4+. In terms of apoptosis inhibitor activity, there is a high degree of similarity across all genes in all three samples including a down-regulation of Bcl-2 and Bcl-xl compared to the baseline, but also an upregulation of many Birc/survivin/IAP apoptosis inhibitor family members. Furthermore, by viewing the apoptosis related genes within the context of the apoptotic pathway (Figure 28 D), the changes in apoptosis gene expression between the CD4+ populations can be summarized. In general, there is an increase in pro-apoptotic genes (Bad, Bid) and a decrease in anti-apoptotic (Bcl-2, Bcl-xl) genes of the intrinsic pathway amongst all three CD4+ populations compared to wild type. Regarding the extrinsic apoptotic pathway, Jak3-/- CD4+ and HPH CD4+ T cells similarly upregulate both

pro-apoptotic (Caspases) and anti-apoptotic (Birc/survivin/IAP) factors. STAT5ab-/- CD4+ T cells share the upregulation in many of the extrinsic apoptotic regulators.

Figure 28 A. Apoptosis Regulators

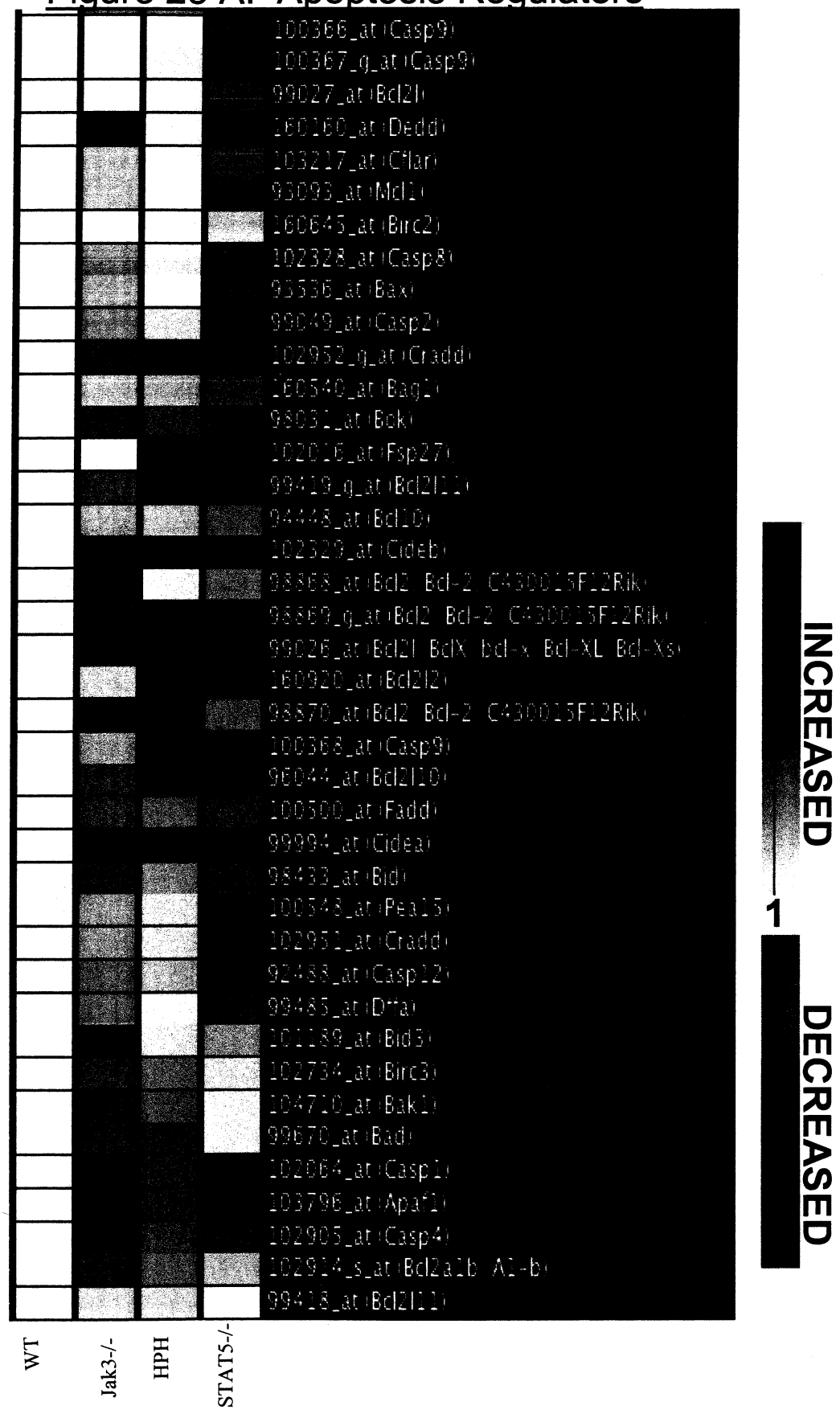


Figure 28 B. Apoptosis Inhibitors

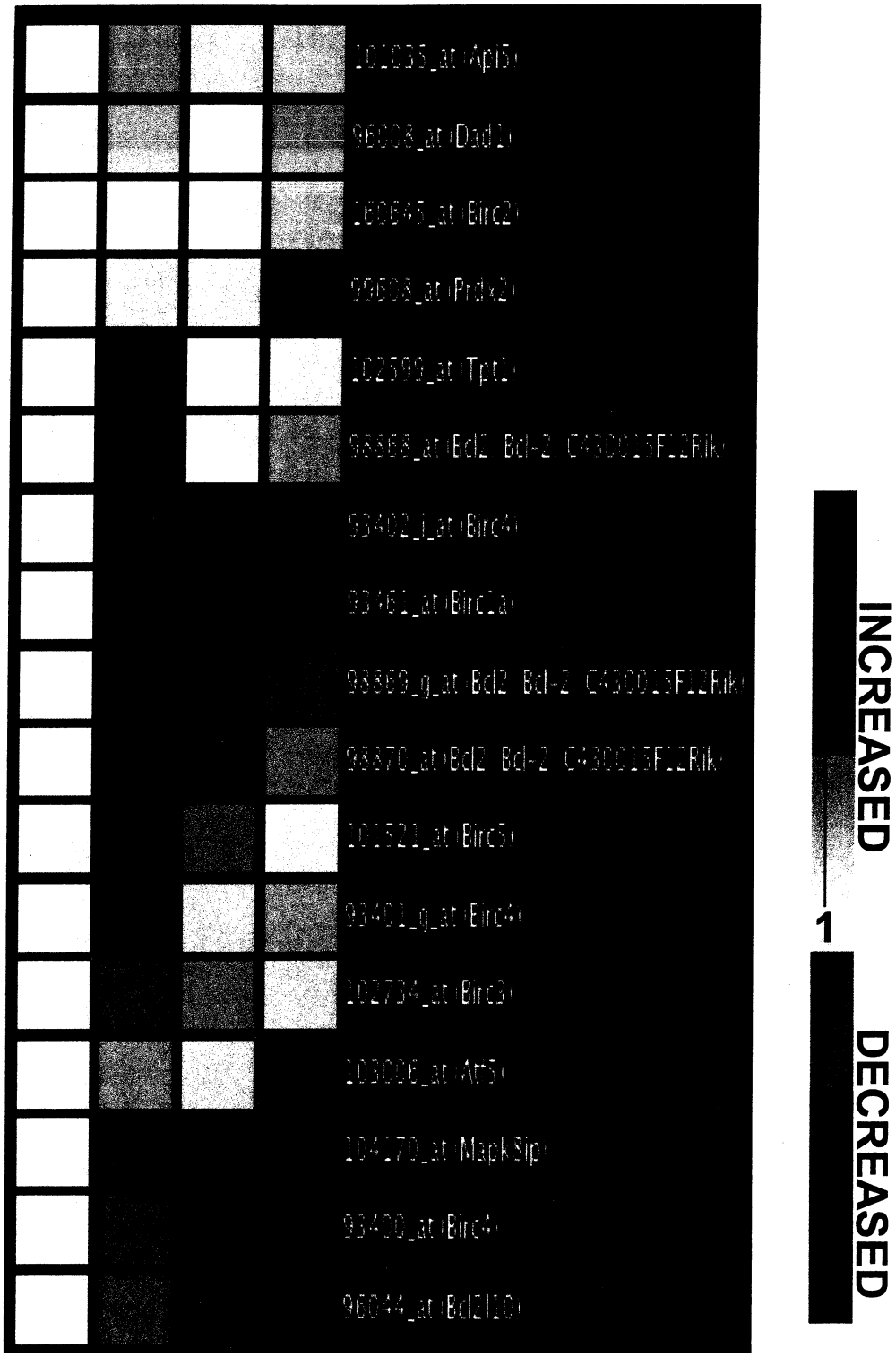
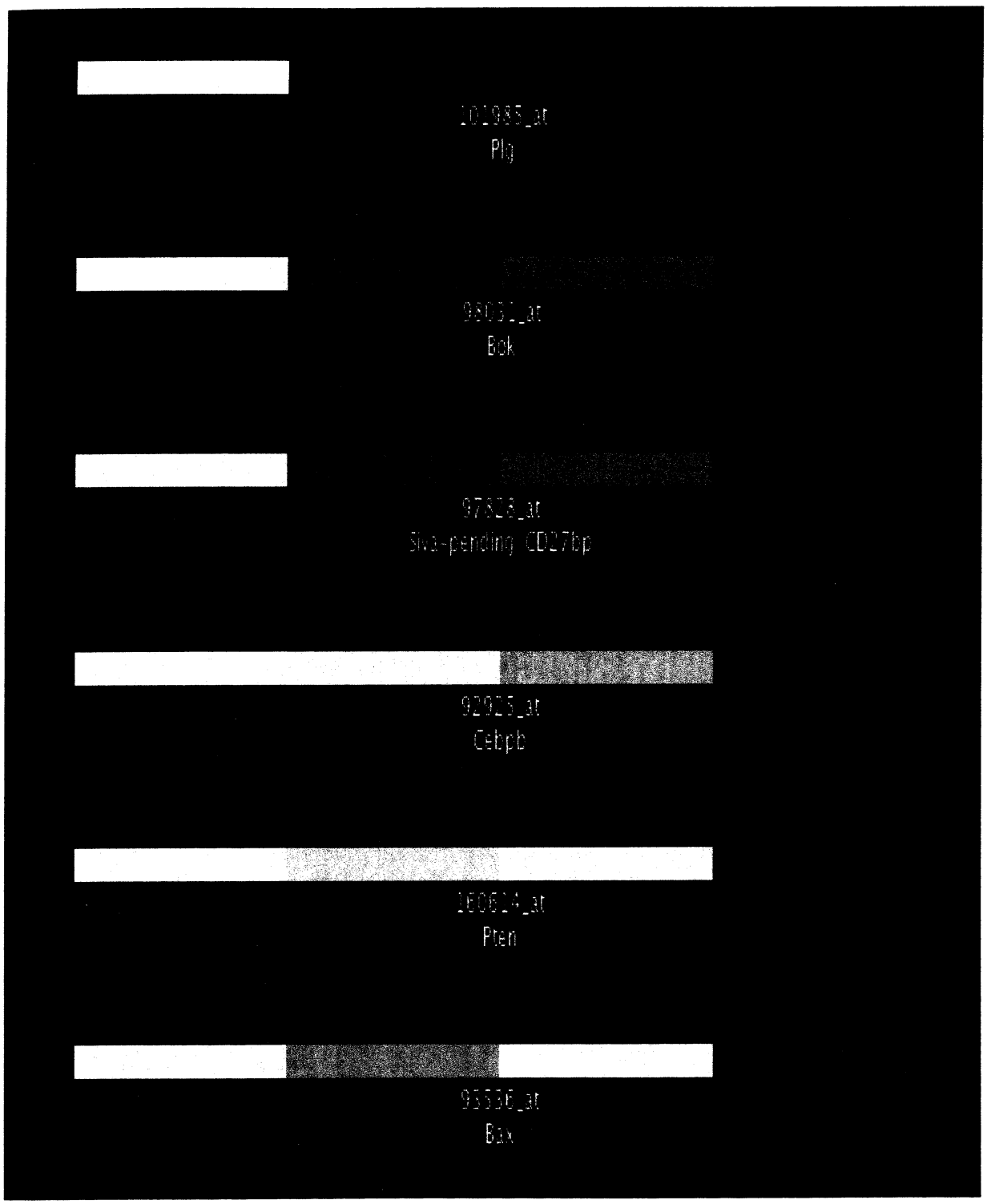
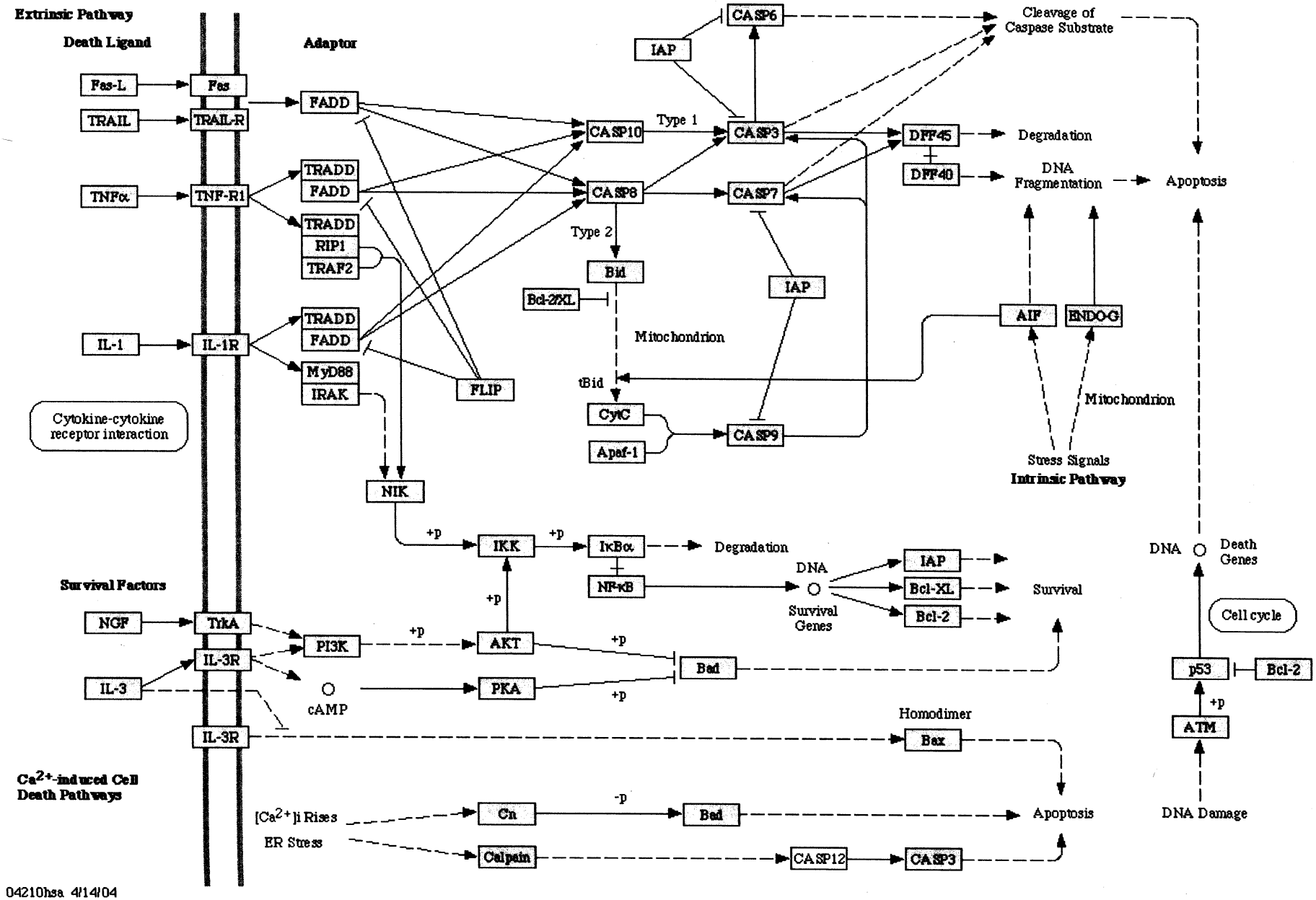


Figure 28 C. Apoptosis Activators



APOPTOSIS

Figure 28D.



APOPTOSIS Figure 28E.

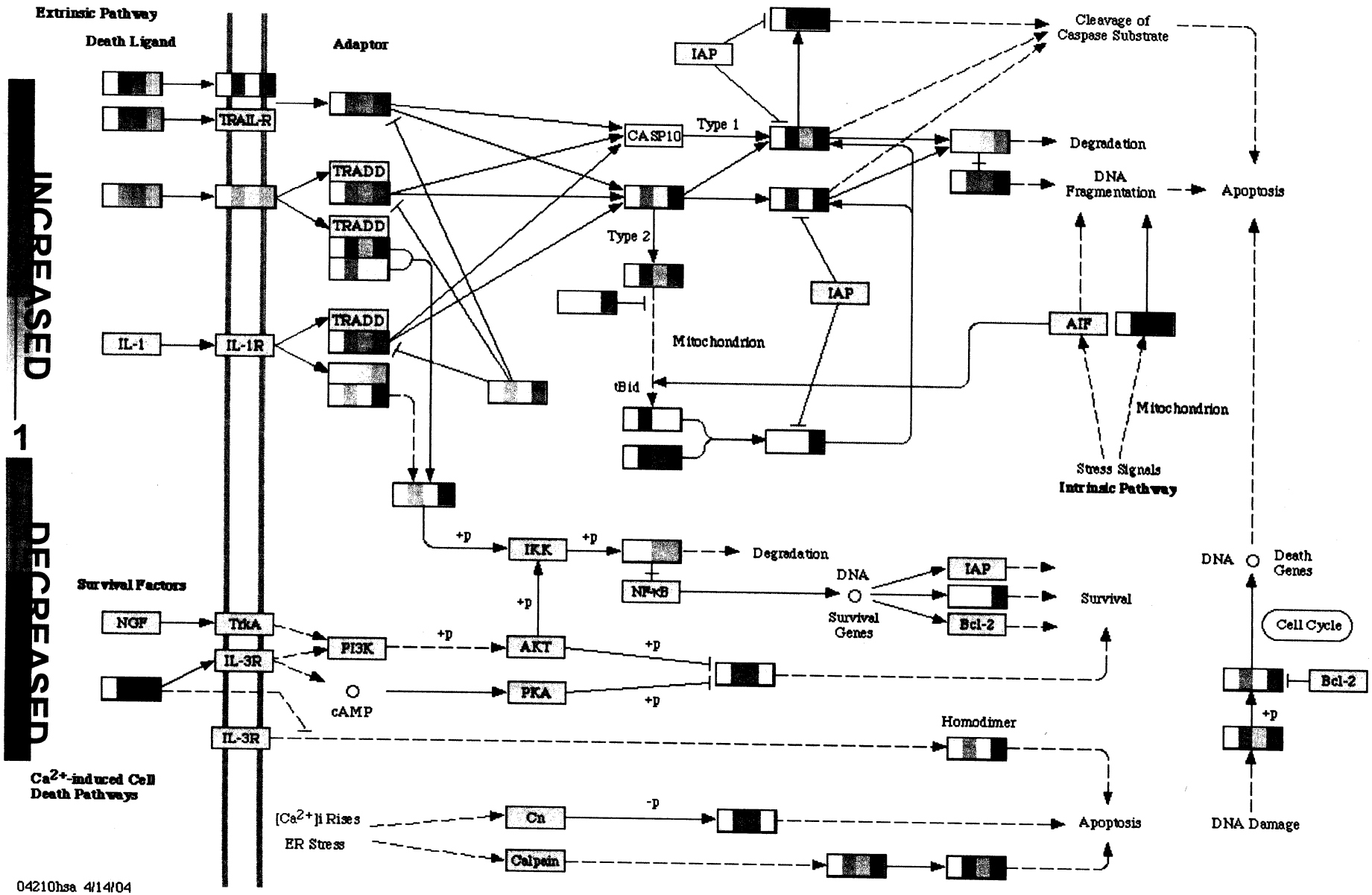


Figure 28. Gene expression patterns of genes involved in Apoptosis.

A) Gene expression patterns of genes involved in Apoptosis Regulation **B)** Apoptosis Activation or **C)** Apoptosis Inhibition were clustered based on gene expression pattern similarity. Similarity was measured by standard correlation ($p<0.05$). Gene classifications are based on the Gene Ontology Consortium (GO) gene annotations. The Affymetrix identification number and gene name is listed to the right or below each gene. Data is representative of triplicate experiments performed.
D) The legend for gene names and locations corresponding to the Kyoto Encyclopedia of Genes and Genomes (KEGG) derived Apoptosis pathway.
E) Gene expression data for CD4+ CD44-high T cells is listed in the following order: WT, Jak3-/-, HPH, STAT5ab-/-. Gene expression data corresponding to the CD4+ T cell populations is represented as a standard heat map plot and merged with the KEGG derived Apoptosis pathway. Data is representative of triplicate experiments performed.

Specific mediators of T cell activation and IL-2 production:

Protein tyrosine kinases, Protein kinase C, MAPK and NF-κB signaling family

Due to the fact that Jak3-/- and STAT5ab-/- T cells do not proliferate and Jak3-deficient CD4+ T cells are additionally unable to secrete IL-2, we were next interested in investigating whether we could detect any differences in gene expression patterns in known mediators of T cell activation and IL-2 production.

The sequence of events which ultimately lead to T-cell activation and IL-2 production involve a number of protein kinases which lead to the phosphorylation of a myriad of substrates. These proximal signaling pathways involve protein tyrosine kinases (PTKs) of the Src, Syk, and Tec families [101]. The src-family kinases p56lck (Lck) and p59fyn (Fyn) are among the first signaling molecules to be activated downstream of the T cell receptor (TCR). This leads to the activation of Syk family members such as ZAP-70, and Syk substrates (Vav1, Vav3, SLP-76, LAT) ZAP-70-mediated LAT phosphorylation then leads to the recruitment and activation of Tec family kinases [249]. Tec family kinases, Itk, Rlk and Tec are important for the full activation of phospholipase C-gamma1 (PLC-gamma1) leading to the production of diacylglycerol (DAG) and activation of protein kinase C (PKC). The novel (PKC) isoform, PKC θ is expressed in a relatively selective manner in T lymphocytes. PKC θ is essential for mature T cell activity. The requirement of PKC θ for T cell activation, proliferation, and cytokine production reflects the essential role of this molecule in inducing signaling pathways, such as the MAP kinase and IKK pathways, leading to the activation of

the transcription factors AP-1 and NF-κB in a T cell-specific manner [250]. The transcription factors AP-1 and NF-κB along with the nuclear factor and activator of transcription (NF-AT) directly promote the induction and synthesis of IL-2 [251]. To investigate whether we could detect any global genetic changes that might lead to the defects in Jak3-/- or STAT5ab-/- we looked at gene expression patterns amongst groups of PTK/PKC signaling molecules, MAP Kinases, and NF-κB related genes. The data is presented in detail for genes within in each group and then a summary of the data along with some discussion is included at the end of this chapter.

PTK &PKC

There were a total of 218 genes contained within the PTK/PKC ontological group which included the members of the Src, Syk, and Tec families and many of the signaling intermediaries discussed above as well as all of the PKC isoforms. Interestingly, 68% of the genes had similar expression patterns in CD4+ T cells from Jak3-/-, HPH, and STAT5ab-/-, 25% of these genes were up-regulated and 43% downregulated (Fig. 29 A). Within these regions of similarity are upregulated genes encoding many factors important for T cell activation and IL-2 production including, itk, rlk, Ras, Rho, Zap-70, Vav, LAT, PI3K p85 regulatory subunit and the PKC isoforms gamma and delta. PKC θ however is slightly down-regulated and Grb2 significantly down-regulated uniquely in STAT5ab-/- CD4+ CD44-high T cells (Fig. 29 B).

Figure 29. Gene Expression Pattern of PTK/PKC.

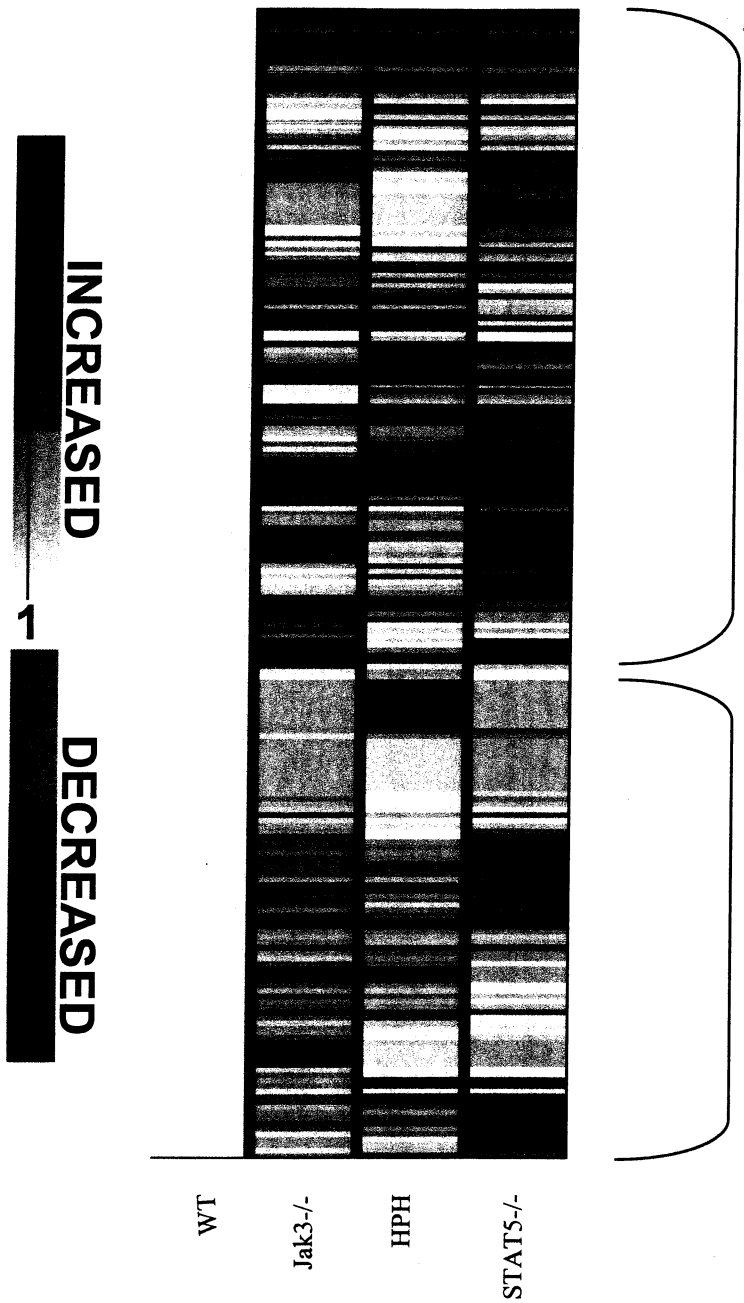


Figure 29. Gene Expression patterns of protein tyrosine kinases and protein kinase C isoforms involved in T cell activation and IL-2 production.

Gene expression patterns of genes encoding PTKs and PKCs were clustered based on gene expression pattern similarity. Similarity was measured by standard correlation ($p < 0.05$). Gene classifications are based on the Gene Ontology Consortium (GO) gene annotations.
A) Region of similarity between all four CD4+ populations contains many PTKs important in T cell activation (itk, rlk, Ras, Rho, Zap-70, Vav, LAT, PI3K p85) ($p< 0.05$).
B) Region of genes decreased uniquely in STAT5ab-/- CD4+ T cells including PKC θ and Grb2 ($p<0.05$). Data is representative of triplicate experiments performed.

MAPK

There are three major groups of MAP kinases (MAPK) in mammalian cells including the extracellular signal-regulated protein kinases (ERK), the p38 MAP kinases, and the c-Jun NH2-terminal kinases (JNK) (Fig. 30, adapted from [252]). In general, the MAPKs are activated by MAPK kinases (MAPKK), which are in turn activated by several different MAPKK kinases (MAPKKK). Different upstream signals can lead to the activation of MAPKKK but small G proteins have a major role. For example, the Erk pathway can be activated by Ras via the Raf group of MAPKKK. However, the p38 and Jnk MAPK are activated by the Rho family GTPases such as Rac and Cdc42. MAPKs play important roles in many aspects of T lymphocyte biology including T cell development, activation/IL-2 production, and differentiation/effector functions (reviewed in [252]) .

To assess changes in gene expression patterns in either MAPKKKK (11 genes), MAPKKK (11 genes), MAPKK/MAPKK (23 genes) or the transcription factors (21 genes) downstream of these signaling molecules we utilized the KEGG derived MAPK signaling pathway provided by Genespring (Figure. 31 & 32).

Of the 11 MAPKKKK genes represented only 3 (27%; NFI, GLK, GCK) show down-regulation in Jak3-/- CD4+ T cells, and two of these are also decreased in HPH CD4+ T cells (NFI, GLK) (Fig. 32 A). STAT5ab-/- CD4+ T cells have decreased transcripts for 6 of the 11 MAPKKKKs 4 of which are uniquely down-regulated, but only moderately ($\leq$~1.5 fold; TAB2, PKC, PKA).

Figure 30. Map kinases of the erk, p38, JNK Pathways (adapted from)

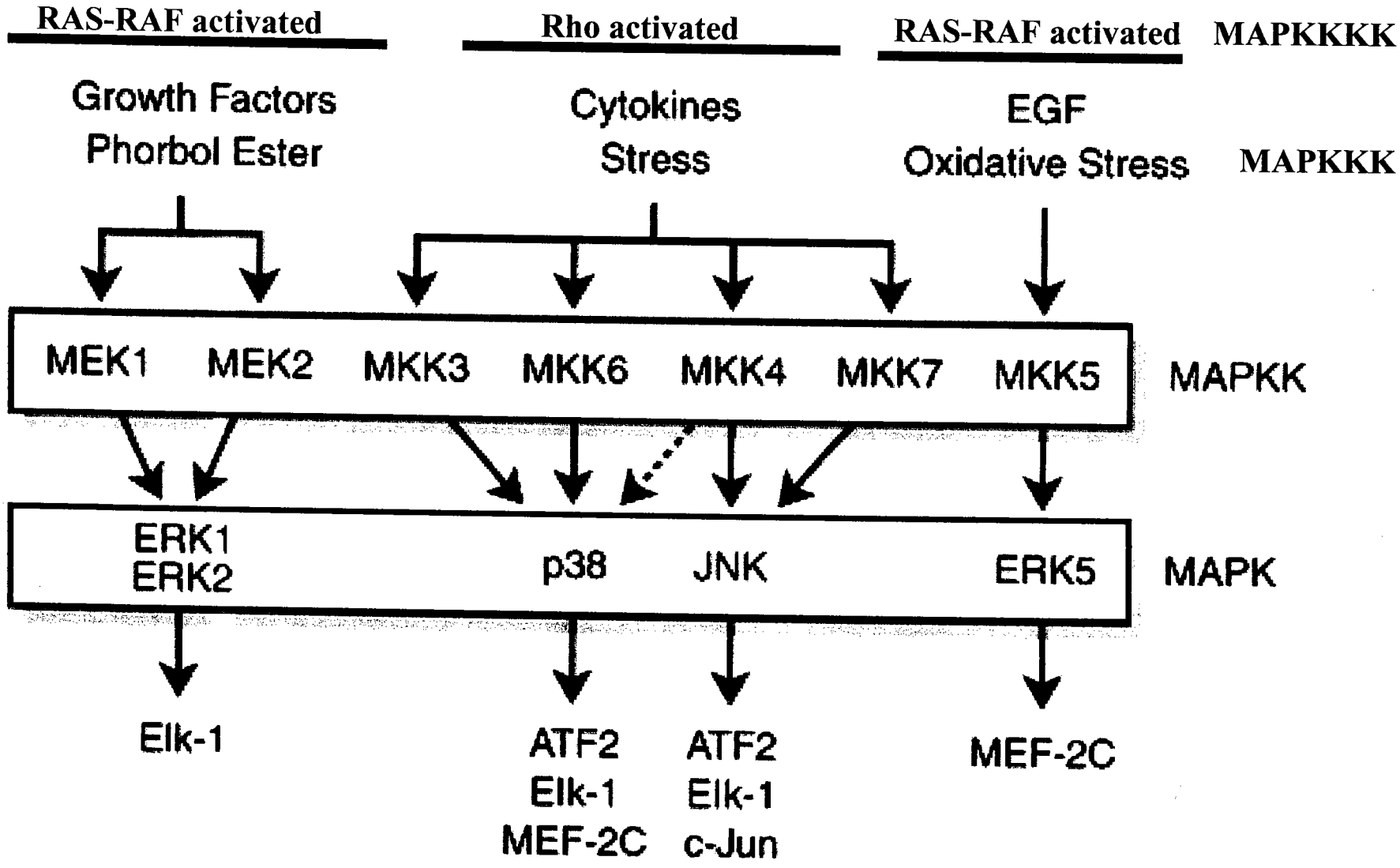


Figure 30. Map kinase of the Erk, p38, and JNK Pathways (adapted from [246]). There are three major groups of MAP kinases (MAPK) in mammalian cells including the extracellular signal-regulated protein kinases (ERK), the p38 MAP kinases, and the c-Jun NH2-terminal kinases (JNK). In general, the MAPKs are activated by MAPK kinases (MAPKK), which are in turn activated by several different MAPKK kinases (MAPKKK). Different upstream signals can lead to the activation of MAPKKK but small G proteins have a major role. For example, the Erk pathway can be activated by Ras via the Raf group of MAPKKK. However, the p38 and Jnk MAPK are activated by the Rho family GTPases such as Rac and Cdc42. MAPKs play important roles in many aspects of T lymphocyte biology including T cell development, activation/IL-2 production, and differentiation/effector functions.

MAPK SIGNALING PATHWAY

Figure 31.

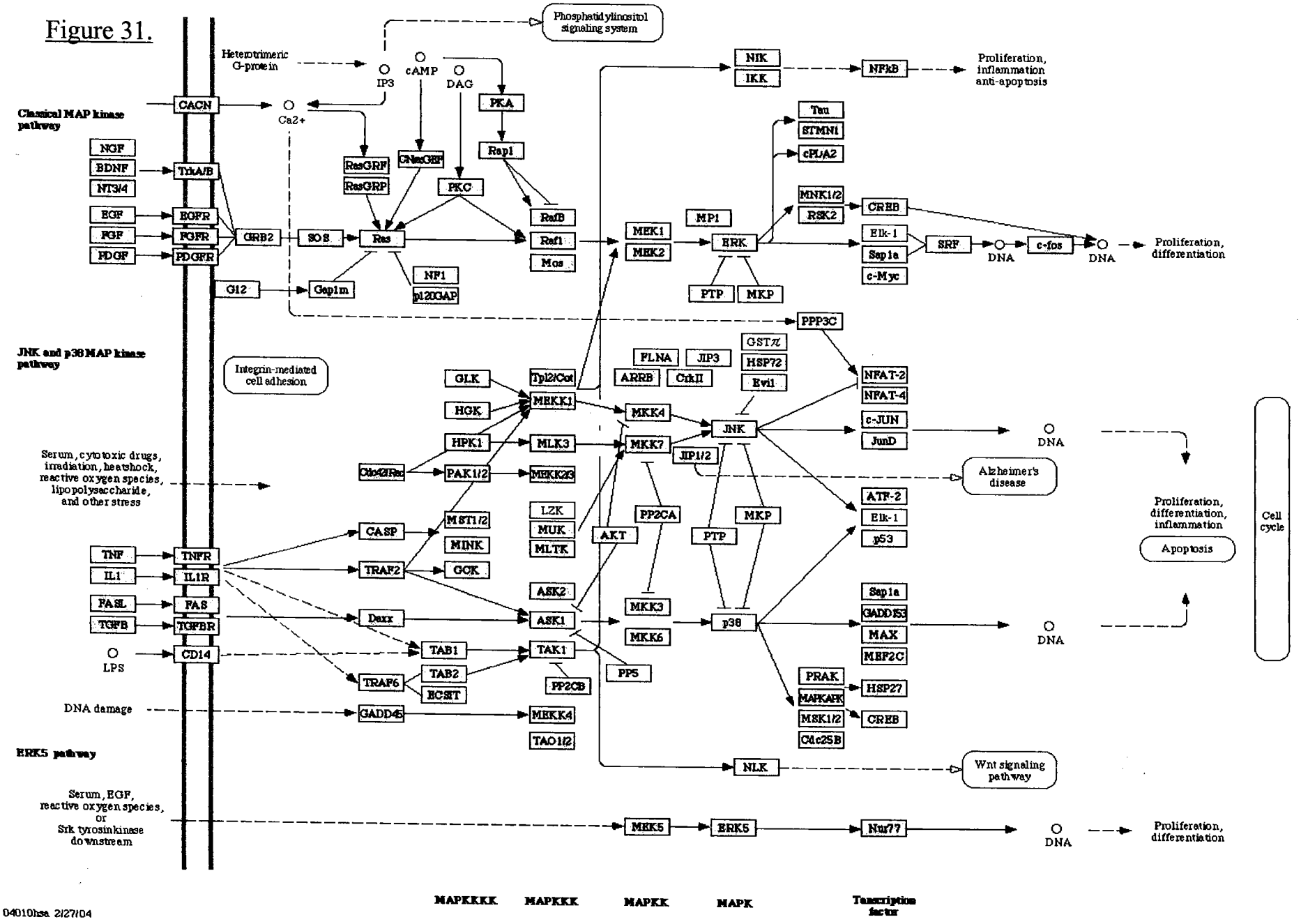


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Figure 31. KEGG derived MAPK Signaling Pathway.

The legend of the gene names and locations in the MAPK signaling pathway.

Figure 32.

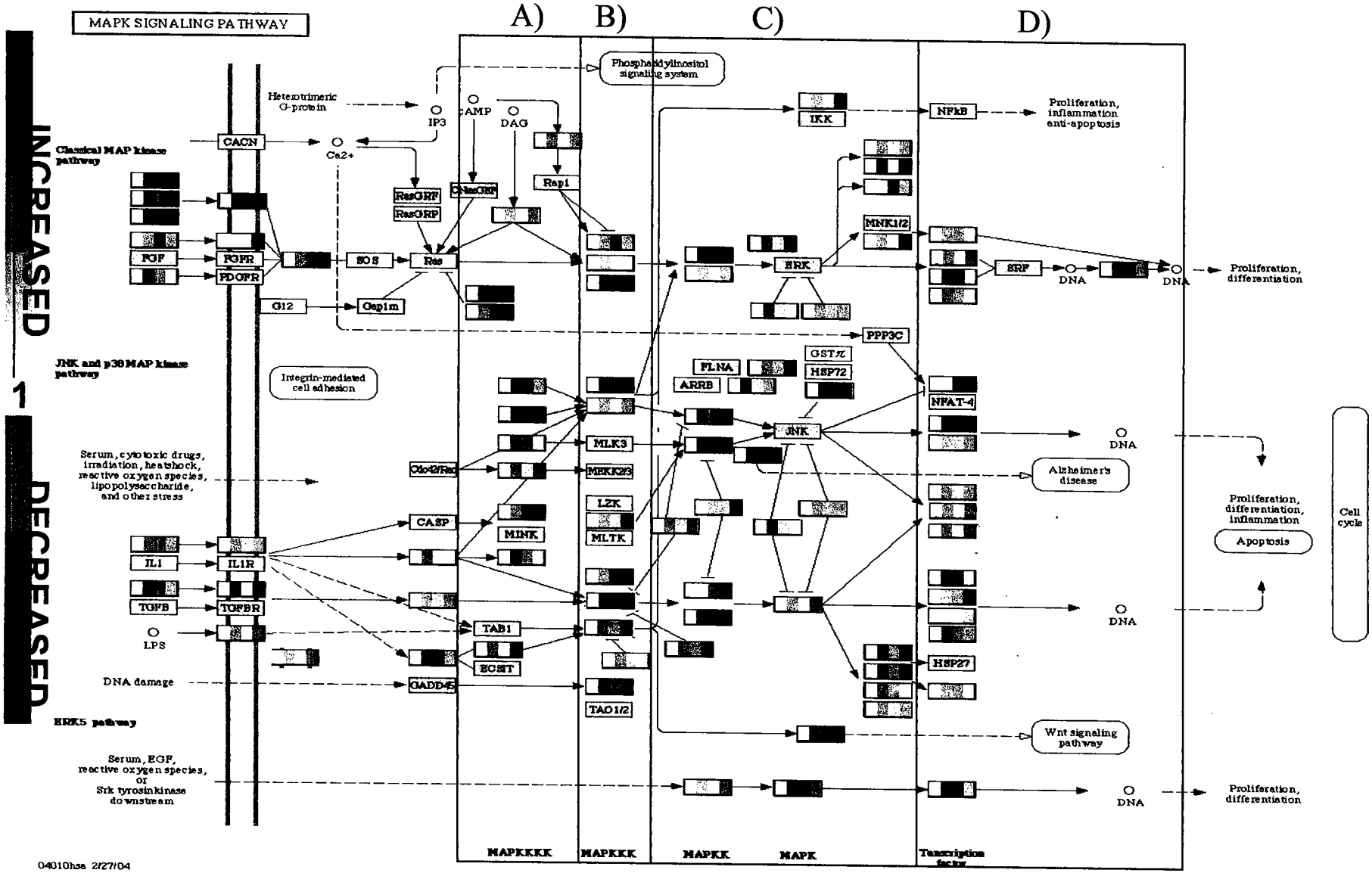

MAPK SIGNALING PATHWAY
A)
B)
C)
D)
INCREASED
1
DECREASED
Classical MAP kinase pathway
JNK and p38 MAP kinase pathway
ERK5 pathway
Heterotrimeric G-protein
IP3
cAMP
DAG
Ca2+
CACN
FGF
FGFR
PDGFR
G12
Gαi1m
RasGRF
RasGRP
SOS
Ras
Rap1
Phosphatidylinositol signaling system
IKK
NFkB
Proliferation, inflammation anti-apoptosis
MNK1/2
ERK
SRF
DNA
Proliferation, differentiation
PPP3C
GSTπ
HSP72
FLNA
ARRB
NFAT-4
JNK
Integrin-mediated cell adhesion
Serum, cytotoxic drugs, irradiation, heatshock, reactive oxygen species, lipopolysaccharide, and other stress
Cdc42/Rac
MLK3
MEKK2/3
LZK
MLTK
MINK
CASP
Alzheimer's disease
Proliferation, differentiation, inflammation
Apoptosis
Cell cycle
IL1
IL1R
TGFB
TGFBR
LPS
TAB1
ECSIT
DNA damage
GADD45
TAO1/2
HSP27
Wnt signaling pathway
Serum, EGF, reactive oxygen species, or Src tyrosinkinase downstream
MAPKKKK
MAPKKK
MAPKK
MAPK
Transcription factor
04010hsa 2/27/04

Figure 32. MAPK Signaling Pathway.

Gene expression data for CD4+ CD44-high T cells is listed in the following order: WT, Jak3-/-, HPH, STAT5ab-/-. Gene expression data corresponding to the CD4+ T cell populations is represented as a standard heat map plot and merged with the KEGG derived MAPK pathway. Data is representative of triplicate experiments performed ($p<0.05$).

Of the MAPKKKs depicted, 3 are similarly decreased in JAK3-/- and STAT5ab-/- CD4+ T cells (MEKK4,Cot, MOS) and 2 of these are also decreased in HPH CD4+ T cells (Cot, MOS; Fig. 32 B). Interestingly, one gene uniquely down-regulated in STAT5ab-/- CD4+ T cells but upregulated in Jak3-/- T cells, is a TGF-β inducible gene (TAK1) which plays a role in the activation of NF-κB.

Of the 23 MAPKK and MAPKs shown, most have an upregulated pattern of gene expression and only the transcript encoding Evil is uniquely down-regulated in Jak3-/- CD4+ T cells. In the STAT5ab-deficient T cells there is a more decreased trend (~45%) in MAPKK/MAPK gene expression but again many of these reflect only a moderate down regulation. Some genes uniquely down-regulated in STAT5ab-/- CD4+ T cells include NLK and NIK which lead to Wnt signaling and NF-KB activation respectively [253].

The transcription factors down-stream of the MAP kinase family molecules will ultimately lead to proliferation and differentiation of cells by inducing specific target genes. While many of the MAP kinases were upregulated or uneffected in Jak3-/- CD4+ T cells, surprisingly, 50% of the MAP kinase related transcription factors are either mildly (c-myc, p-53, Max, Nur77,Elk-1) or significantly (c-fos, PRAK, MAPKAPK,Sap1a, MEF-2C) down-regulated. Interestingly, some of these decreases in gene expression are shared with STAT5ab-/- T cells suggesting the possibility that the induction of these genes might be dependent on γc cytokine signaling (PRAK, MAPKAPK, Max, p53). c-Jun which is known to be an IL-2 responsive gene is down-regulated in Jak3-/-, STAT5ab-/- and HPH CD4+ T cells.

NF-kB

NF-κB is important for the expression of a wide variety of genes that are involved in the control of the immune response and in the regulation of cellular proliferation and survival. NF-κB is downstream of the MAP kinase pathway, and along with the nuclear factor and activator of transcription (NF-AT) and AP-1, directly promotes the induction and synthesis of IL-2 (reviewed in [251]). One of the key steps in the activation of the NF-κB pathway is the stimulation of the inhibitor of κB kinases (I-κB), via the I-κB kinases IKK.

Therefore, we next looked at gene expression patterns in molecules important in the activation of the NF-κB. The 96 genes included in the NF-κB ontological group were clustered by k-means clustering as previously described. Of the 5 clusters, two of them (Group 1 and 4) contained genes that had a decreased pattern of gene expression in Jak3-/- and STAT5ab-/- CD4+ T cells compared to wild type and HPH (Figure. 33 and Supp. 17 & 20). This suggests that a high proportion of the NF-κB related genes are down-regulated in Jak3 and STAT5ab-/- CD4+ T cells (46%). Most of these contained within Group 1 (Fig. 33, Supp. 17) are immunoglobulin genes and therefore would not effect the proliferation or activation status of the CD4+ T cells. The one interesting exception is a decrease in the gene encoding NF-ATc1 which plays a role in IL-2 production. Of the 3 remaining clusters of genes, the gene expression pattern in Jak3-/- CD4+ T cells is comparable to wild type and/or HPH CD4+ T cells. In contrast, two additional groups (Group2 and Group 5, Fig. 33, Supp. 18 & 21) both contain additional genes which are

Figure 33. NF-kB signaling pathway genes grouped by expression level-5k- means Cluster

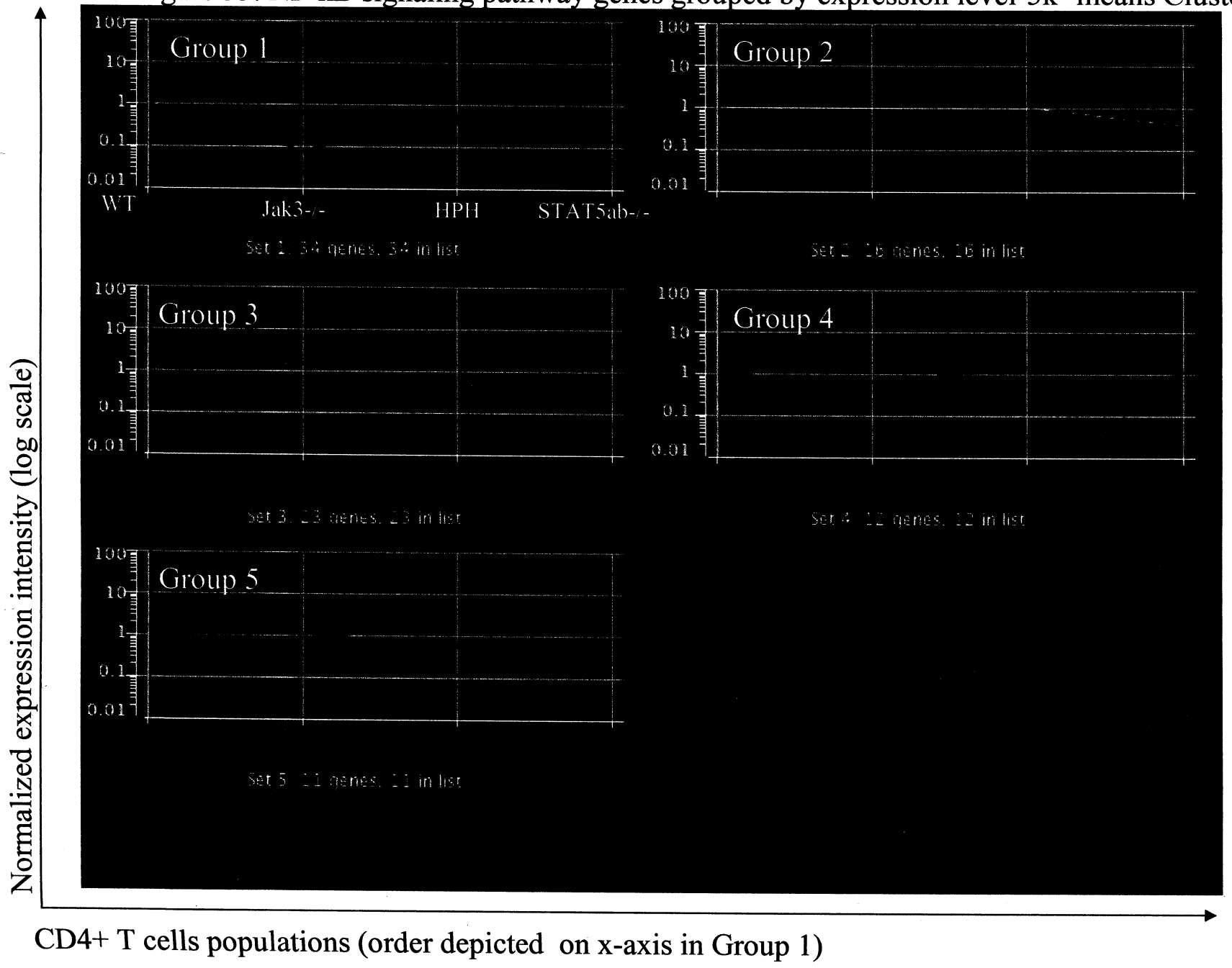


Figure 33. NF-κB signaling pathway genes grouped by expression pattern.

NF-κB signaling related genes were based on GO gene annotations and classifications. Genes with similar patterns of gene expression were determined by k-means clustering around 5 centroids after 100 iterations using standard correlation ($p < 0.05$) as the similarity measure. The line on the graph represents the average pattern of gene expression within each group. The number of genes classified in each group is listed below the graphs. Information on the genes contained with in each group can be found in Supplemental Data 17-21. Data is representative of triplicate experiments performed.

down-regulated in STAT5ab-/- CD4+ but not Jak3-/- CD4+ T cells. Some of these genes have an important role in NF-κB activation including IKKβ, NF-κB1 precursor (p105), NF-κB2 precursor (p100) and RelA.

Summary of changes in gene expression among specific mediators of T cell activation and IL-2 production

Our analysis of gene expression patterns amongst protein tyrosine kinases and PKC signaling molecules which included many of the important TCR proximal signal molecules suggests that in the absence of γc signaling the induction of these genes remains intact. Moreover, Jak3 deficient T cells do not show gross signs of down-regulation among the MAP kinase family of molecules (26%). Instead there is an upregulation in 62% of the genes (MAPKKKK (7/11), MAPKKK (7/11), MAPKK/MAPK (14/23)). While, STAT5ab-/- CD4+ T cells show a much greater degree of down-regulation (48%, 26% were upregulated), the majority of these are of approximately 2 fold or less compared to wild type. Although many of these upstream signaling mediators do not appear decreased at the gene expression level in Jak3-/- CD4+ T cells, some of the transcription factors which they induce are down-regulated (50%). Of these, the down-regulation of c-myc, MEF-2C, Elk-1, c-fos, and c-jun in Jak3-/- CD4+ T cells could all have a negative impact on in T cell proliferation and IL-2 production. For example, MEF-2C and Elk-1 have been shown to augment IL-2 production and c-fos/c-jun make up the AP-1 complex which directly binds to and promotes

transcription of the IL-2 gene [254,255]. Interestingly, we did not detect a down-regulation of NF-κB in Jak3-/- CD4+ T cells. It is unclear why the upstream MAPK related mediators appear in tact, but only a subset of the transcription factors downstream which promote IL-2 production are affected in Jak3-/- CD4+ T cells. One intriguing possibility is that the additional cytokines produced by the Jak3-/- CD4+ T cells are influencing these pathways in various ways. In support of this idea, IL-10 signaling has been reported to block the Ras-Raf mediated MAPK pathway and potentially disrupts the induction and activation of MEF-2C and Elk-1 [256]. TGF-β on the other hand stimulates the MAPK pathway and NF-KB activation via TAK1 (transforming growth factor beta activated kinase-1) ([253,257,258], Supp. 22) which was shown to be upregulated in Jak3-/- CD4+ T cells by microarray analysis (Fig. 32 B).

Interestingly, with the exception of c-jun and some components of the NF-κB pathway, the STAT5ab-/- CD4+ T cells which produce IL-2 *in vitro*, do not have decreases in genes encoding many of the transcription factors known to promote IL-2 gene activity. The fact that c-myc and c-fos are not down-regulated in STAT5ab-/- CD4+ T cells is surprising since both of these genes have been previously shown to be IL-2 responsive genes. This suggests that there is a compensatory mechanism for the induction of these genes or alternatively that signal events proximal to STAT5 in the Jak-STAT pathway suffice to induce the expression of these genes. Additionally, a newly generated STAT5ab-deficient mouse model has suggested the possibility that the STAT5ab-/- mice used in these experiments may only be a partial knock-out, or knock-down (personal communication, J.

O'Shea). Despite this possibility, many of the genes known to be downstream of γc-cytokines such as pim-1, cis-1, LKLF, c-fos, Bcl-2, and SATB1 were decreased similarly in Jak3-/- and STAT5ab-/- CD4+ T cells suggesting that in fact STAT5ab-/- T cells had sufficient impairments in Jak-STAT signaling and thus were an appropriate cell type to use for these experiments.

In summary, this chapter highlights gene expression changes detected between wild type CD4+ CD44-high T cells, homeostatically expanded wild type CD4+ CD44-high T cells, and CD4+ CD44-high T cells isolated from Jak3-/-, and STAT5ab-deficient mice. These microarray analyses demonstrate several interesting points regarding Jak3-deficient T cells. First, genes known to be downstream of IL-10, IFN-γ and TGF-β were upregulated in Jak3-/- T cells. The induction of genes by these cytokines suggests that these immunosuppressive cytokines may be directly influencing the fate of and/or shaping the regulatory-like qualities displayed by Jak3-/- T cells as described in Chapter 4. Consistent with the notion that these cytokines play a role in the regulatory qualities of Jak3-/- T cells, MEF-2C and ELK-1 were decreased in Jak3-/- T cells and IL-10 has been shown to block IL-2 production via disruption of MEF-2C and ELK-1 activity. A second interesting conclusion from these experiments was that wild type T cells undergoing homeostatic expansion in lymphopenic hosts show many common patterns of gene expression to freshly-purified unmanipulated Jak3-/- T cells. For instance, microarray analysis of gene expression in wild type CD4+ T cells after lymphopenia induced homeostatic expansion show a similar pattern of upregulation in surface markers (PD-1 and LAG-3), and cytokine signaling

molecules (IL-10 and IFN-γ cytokine, receptors, and inducible gene targets) to that of Jak3-/- CD4+ T cells immediately ex vivo. In fact, using standard correlation as a similarity measure, there is a high correlation amongst global changes in gene expression between wild type homeostatically expanded CD4+ T cells and Jak3-/- CD4+ T cells with a positive correlation coefficient of 0.770 on a scale from zero to one. Despite, the number of similarities in gene expression between Jak3-/- and HPH CD4+ T cells, HPH T cells remain responsive *in vivo* where as Jak3-/- CD4+ T cells develop regulatory T cell characteristics . These data suggest that the process of homeostatic proliferation normally induces immune attenuation mechanism, but that full differentiation into a regulatory T cell phenotype is prevented by γc-dependent cytokine signals.

Chapter VI.

DISCUSSION

In this study we demonstrate that Jak3-deficient T cells express high levels of PD-1, secrete a Tr1-type cytokine profile following direct ex vivo activation, and suppress the proliferation of wild type T cells in vitro. These characteristics indicate that CD4+ Jak3-/- T cells share properties with regulatory T cell subsets that have an important role in peripheral tolerance and the prevention of autoimmunity [61,82]. While the precise mechanism by which Jak3-deficient T cells acquire these characteristics is currently unclear, our data indicate that one important component is a T cell-intrinsic requirement for Jak3 signaling. When Jak3-/- naïve thymocytes were adoptively transferred into lymphopenic Rag2-/- mice, these cells proliferated and selectively acquired regulatory T cell characteristics in the absence of any additional activation signals.

These findings indicate several interesting aspects of T cell biology. First, these adoptive transfer studies, in addition to the increase in cell cycle specific mRNAs detected by microarray analysis in freshly isolated Jak3-/- CD4+ CD44-high, demonstrate that homeostatic proliferation of CD4+ T cells is not dependent on signaling via γc-dependent cytokine receptors. In fact, over the first four weeks following adoptive transfer, the population of Jak3-/- T cells expanded comparably to that of wild type T cells. It was only at the later time points of six to eight weeks that Jak3-/- cell numbers began to decline in the adoptive transfer recipients, strongly suggesting a defect in survival, rather than proliferation. This latter notion is consistent with previous studies suggesting that γc-dependent cytokines such as IL-7 play a more critical role in naïve CD4+T cell survival than proliferation [184,259]. Regarding survival of memory CD4+ T cells, our microarray analysis demonstrated a 70%

similarity in expression patterns among apoptosis regulator genes in Jak3-/- and HPH CD4+ CD44-high T cells. Although there was an increase in pro-apoptotic genes (Bad, Bid) and a decrease in anti-apoptotic genes (Bcl-2, Bcl-xl), many survival factors of the Birc/survivin/IAP were upregulated in Jak3-/- CD4+ and some in STAT5ab-/-CD4+ T cells. This may indicate that the survivin family of anti-apoptotic molecules plays an important role in the survival of memory CD4+ T cells. The similar gene expression pattern detected in HPH CD4+ supports this notion.

A second important conclusion from the adoptive transfer experiments is that the weak activation signals normally associated with homeostatic expansion are sufficient to drive Jak3-/- T cells into a non-conventional differentiation program. Previous data indicate that, for wild type T cells, signaling through both the TCR as well as γc-dependent cytokine receptors promote the homeostatic proliferation of T cells in lymphopenic hosts. Since Jak3-/- T cells are unable to receive these cytokine signals, their proliferation is likely to be wholly dependent on TCR signaling. As a consequence of this TCR signaling, Jak3-/- T cells proliferate, but in addition, are induced to upregulate PD-1 and to selectively activate the IL-10 locus while shutting off the production of IL-2. Since this fate does not occur for wild type T cells in a comparable environment, it is likely that the unique differentiation pathway taken by Jak3-/- T cells reflects the effects of TCR signaling in the absence of γc-dependent cytokine signaling.

Interestingly, wild type T cells undergoing homeostatic expansion in lymphopenic hosts show many common patterns of gene expression to freshly-purified unmanipulated

Jak3-/- T cells. For instance, microarray analysis of gene expression in wild type CD4+ T cells after lymphopenia induced homeostatic expansion show a similar pattern of upregulation in surface markers (PD-1 and LAG-3), and cytokine signaling molecules (IL-10 and IFN-γ cytokine, receptors, and inducible gene targets) to that of Jak3-/- CD4+ T cells immediately ex vivo. In fact, using standard correlation as a similarity measure, there is a high correlation amongst global changes in gene expression between wild type homeostatically expanded CD4+ T cells and Jak3-/- CD4+ T cells with a positive correlation coefficient of 0.770 on a scale from zero to one. These data suggest that the process of homeostatic proliferation normally induces immune attenuation and peripheral tolerance mechanism, but that full differentiation into a regulatory T cell phenotype is prevented by γc-dependent cytokine signals.

In addition to these T cell-intrinsic defects, defects in the Jak3-dependent functions of other hematopoietic compartments in Jak3-/- mice may contribute to the development of Jak3-/- T cells with regulatory characteristics. This is a particularly intriguing idea since antigen-presenting cells such as dendritic cells (DC) play a major role in T cell differentiation and tolerization. For example, specialized subsets of DC's are thought to control the generation of Th1 and Th2 cells, termed DC1 and DC2 respectively [92,93,260]. Some studies have also suggested that a third dendritic cell subset (DCr) that secretes IL-10, rather than IL-12, directs naive T cells to a Tr1 subtype [81,85,98,261].

Our data demonstrate that non-T cell compartments present in Jak3-deficient mice are modified and may be mediating inhibitory effects on Jak3-/- T cells. Specifically, we find

that PD-L1 is highly expressed on CD4-negative Jak3-/- splenocytes, and further, that these cells can inhibit proliferative responses of wild type T cells in *in vitro* co-culture assays. Interestingly, studies by others have shown that PD-L1 expressed on immature dendritic cells (iDC) as well as mature dendritic cells (mDC) can mediate inhibitory signals to T cells, resulting in reduced IL-2 production and proliferation [36,262]. In this system, T cell activation could be enhanced by the addition of PD-L1 blocking antibodies; interestingly this enhancement was most pronounced with weak APC, such as iDCs and IL-10-pretreated mDCs, and less pronounced with strong APC such as mDCs. Finally, additional data demonstrate a central role for PD-L1 in the regulation of induction and progression of autoimmune diabetes in the NOD mouse [38]. These findings are consistent with the hypothesis that PD-L1 expressed on DCs can reduce the stimulatory capacity of the DCs, and further suggest a role for the PD-1/PD-L1 pathway in the regulation immune responses.

Finally, based on these data, we envision the following model to account for the alternative T cell differentiation pathway taken by Jak3-/- CD4+ T cells (Figure 34). Following emigration from the thymus, naïve Jak3-/- CD4+ T cells are induced to undergo homeostatic proliferation, due to the lymphopenic environment associated with the Jak3 deficiency. The drive to proliferate may also be further facilitated by the inability of Jak3-/-

Figure 34. Model of events leading to alternative CD4+ T cell differentiation in Jak3-/- mice.

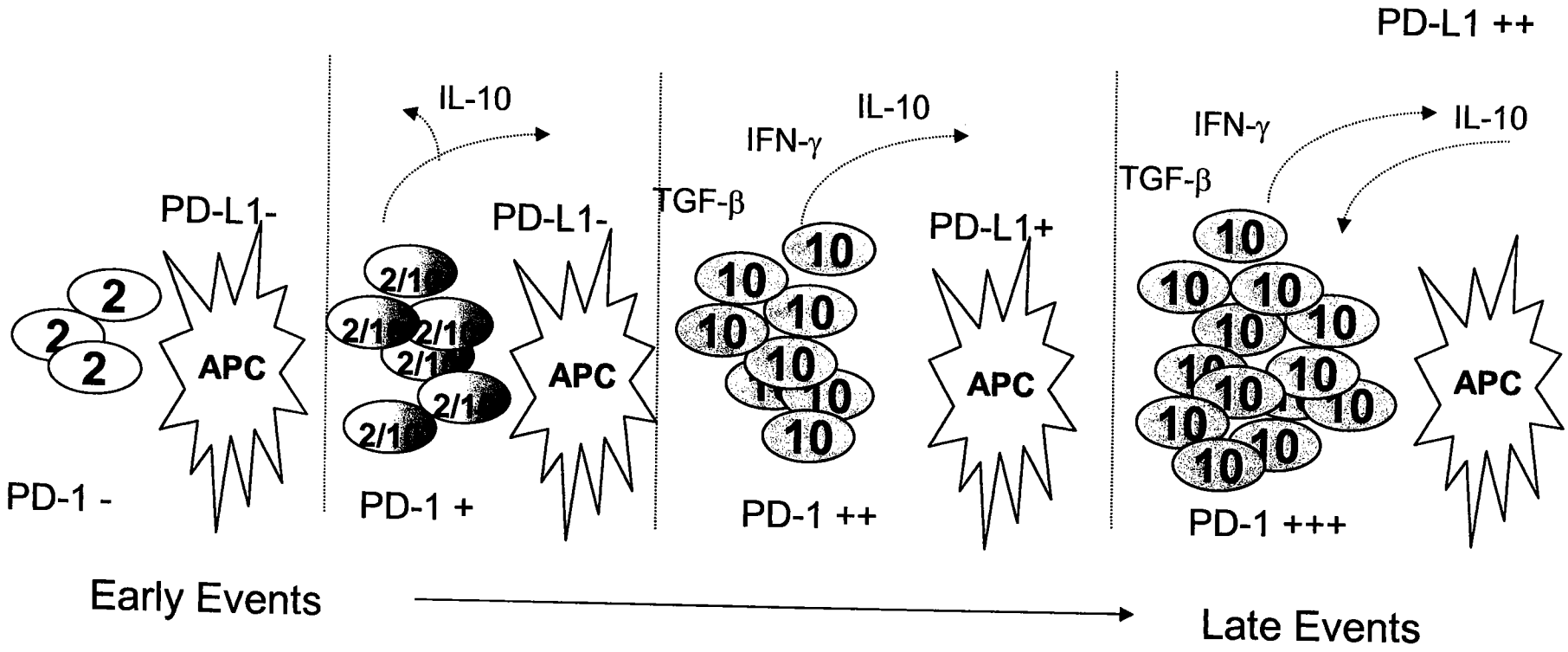


Figure 34. A Model of events leading to an alternative pathway of CD4+ T cell differentiation upon T cell activation in the absence of Jak3.

Following emigration from the thymus, naïve Jak3-/- CD4+ T cells are induced to undergo homeostatic proliferation, due to the lymphopenic environment associated with the Jak3 deficiency. These activation/proliferation signals, coupled with the complete absence of signaling through γc-dependent cytokine receptors, induces Jak3-/- T cells to upregulate PD-1 expression and IL-10 production and to lose the ability to produce IL-2. These PD-1+, IL-10-producting T cells then modify the APCs in their environment, promoting the up-regulation of PD-L1 and the expression of IL-10 by these cells, and thereby establishing a self-reinforcing feedback loop that perpetuates this immunosuppressive environment.

CD4+ T cells to induce the expression of the transcription factor LKLF which is important in maintaining T cell quiescence [213]. LKLF has been previously reported to be regulated by γc-dependent cytokines and in support of this we also found LKLF gene expression to be decreased in STAT5ab-/- CD4+ T cells.

These activation/proliferation signals, coupled with the complete absence of signaling through γc-dependent cytokine receptors, induce Jak3-/- T cells to upregulate PD-1 expression and IL-10 production and to lose the ability to produce IL-2. These PD-1+, IL-10-producting T cells then modify the APCs in their environment, promoting the up-regulation of PD-L1 and the expression of IL-10 by these cells, and thereby establishing a self-reinforcing feedback loop that perpetuates this immunosuppressive environment.

Several lines of evidence support this model. First, the loss of Jak3 and STAT5 phosphorylation has been implicated in models of tumor-induced immunosuppression. In this system, ovarian carcinoma cells were shown to suppress CD8+ T cell proliferation, and to induce these T cells to express IL-10; furthermore, these effects were dependent on the disruption of Jak3 signaling [263]. Second, *in vitro* studies have demonstrated that stimulation of T cells with high-affinity peptide ligands favors their differentiation into T-helper or Tr-n lineages, while, stimulation with lower affinity altered peptide ligands favors the development of Tr1 or Tr2 cells [90,101]. This latter situation may be comparable to the activation signals received during homeostatic expansion, or may result from reduced TCR signaling due to PD-1-mediated inhibition and/or the absence of γc-mediated signals.

An alternative explanation for the initial activation signals that drive the expansion of Jak3-/- T cells is that, in addition to a lymphopenic environment, there is a lack of Tr-n regulatory T cells. Several reports demonstrate that IL-2R signals are critical for Tr-n maturation [176,264], and as a consequence, Jak3-/- mice lack the subset of CD4+CD25+ T cells base on expression of both CD25 and FOXP3 ([177] and Fig. 35 & 36). The absence of these regulatory T cells may result in a lymphoproliferative syndrome, similar to that described for IL-2Rβ-deficient mice [79,176,265]. However, since Jak3-/- T cells acquire immunosuppressive traits subsequent to this lymphoproliferation, no lethal autoimmunity results, as it does for mice lacking IL-2R signaling.

While several possibilities may exist regarding the stimuli that initiate the *in vivo* differentiation process of Jak3-/- T cells, in the long-term, the outcome is identical. Jak3-/- T cells differentiate down an alternative pathway leading to the establishment of an immunosuppressive population of T cells and APCs in Jak3-/- mice.

These data highlight many potential factors leading to alternative CD4+ T cell differentiation in Jak3-/- CD4+ T cells including proliferation/expansion signals, PD-1:PD-L1 interactions, and the autocrine and paracrine effects of IL-10, TGF-β, and IFN-γ. Many of these same phenotype changes occur in wild type T cells undergoing homeostatic expansion and therefore alternate T cell differentiation is uniquely dependent on the absence of Jak3. In accord with this, one would expect to find a difference in γc responsive genes that could account for the fact that the Jak3-/- CD4+ T cells take on these unique characteristics but HPH CD4+ cells do not. Counter to this, our microarray data

Figure 35. Jak3-/- mice have a reduced population of CD4+ CD25+ cells

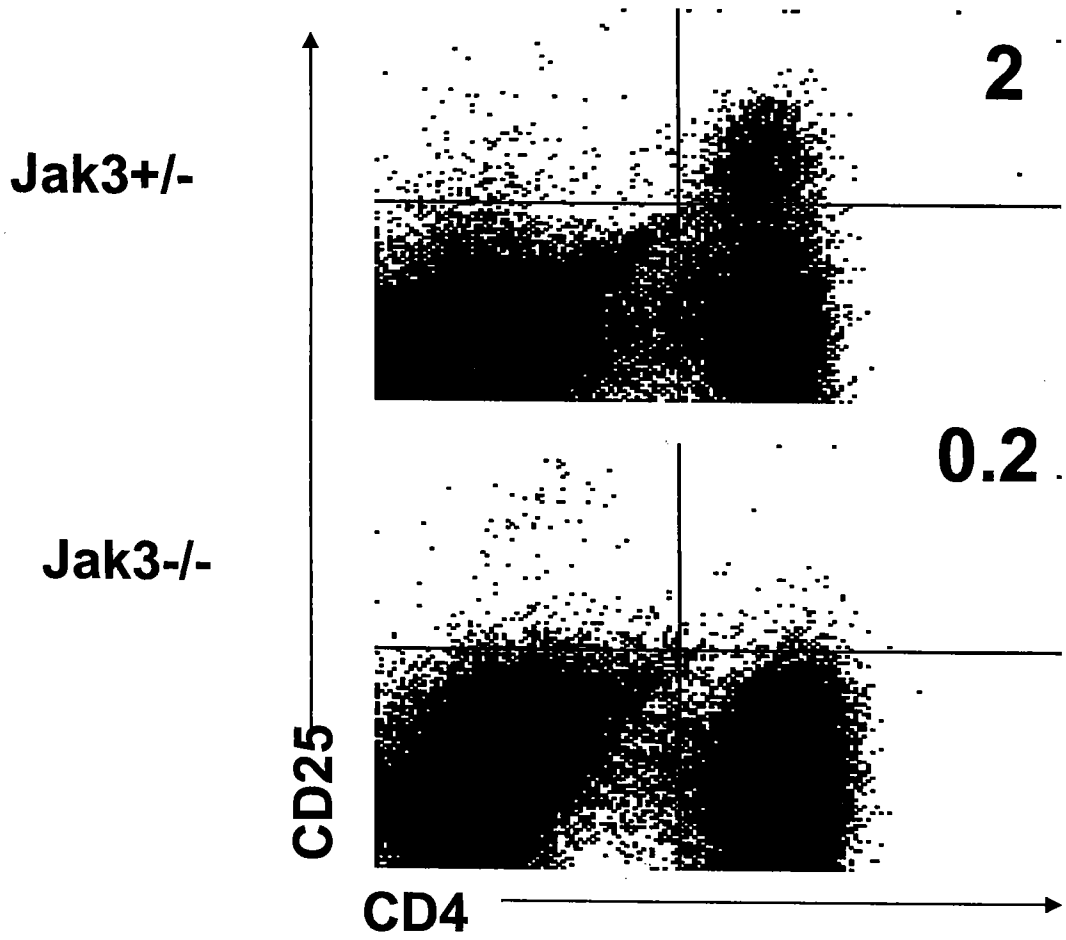


Figure 35. Jak3-/- mice lack CD4+ CD25+ splenocytes.

Splenic T cells from either Jak3+/- (top) or Jak3-/- (bottom) were isolated as described previously. Total splenocytes were stained with the indicated α-CD4-FITC and α-CD25-PE in HBSS supplemented with 3% FCS for 20 min on ice. Cells were then washed and analyzed on a BD Biosciences FACSCalibur. Data were analyzed using CellQuest software. Cells are shown gated on live cells based on FSC/SSC and the percent positive cells are indicated in the upper right of each histogram. Data is representative of one experiment performed on three to five mice per genotype.

Figure 36. Jak3-/- splenocytes do not express Foxp3

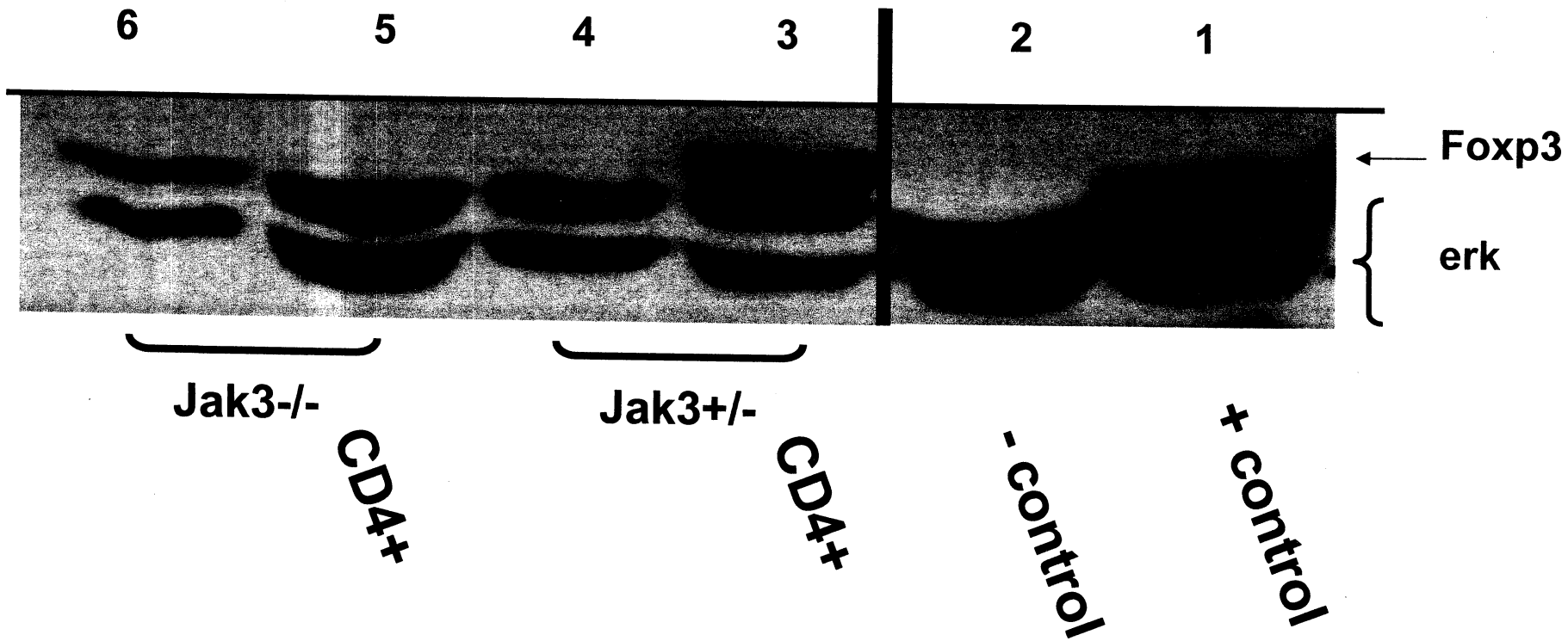


Figure 36. Jak3-/- splenocytes do not express Foxp3.

Total splenocytes from either Jak3+/- or Jak3-/- were isolated and CD4+ T cell populations were enriched by auto-MACS separation. Proteins from the enriched CD4+ T cells (lane 3 or 5) and the negative auto-MACS fraction containing CD4-negative splenocyte populations (lane 4 and 6) were separated by SDS-PAGE and blotted with an antibody specific for Foxp3 along with an α-Erk loading control. Protein lysates from COS cells transfected with retroviral vector expressing Foxp3 (lane 1) or empty vector (lane 2) are shown as positive and negative control, respectively. The experiment was performed one time with three to four mice used per group.

demonstrated a high degree of similarity in global gene expression patterns between HPH and Jak3-/- CD4+ T cells including the down-regulation of many γc-responsive genes (Bcl-2, Bcl-XL, c-fos, c-jun). The exception was among some transcription factors, two γc responsive cytokines, LKLF and SATB1, as well as the transcription factors Grail and Tbr2/Eomesodermin.

SATB1 is a particularly attractive target responsible for the acquisition of these alternative traits in Jak3-/- T cells since it is a T cell specific matrix attachment region-chromatin remodeling factor that can orchestrate both repression and activation of a number of genes simultaneously [215]. SATB1 is also down-regulated in STAT5ab-/- CD4+ T cells. Since STAT5ab-/- T cells do not have an immunosuppressive cytokine profile or do they display appreciable T cell regulatory function when co-cultured with wild type CD4+ T cells (Figure 37), SATB1 cannot fully account for the unique features of Jak3-/- CD4+ T cells.

The two transcription factors we found to be uniquely upregulated in Jak3-/- CD4+ T cells and not in HPH or STAT5ab-/- CD4+ T cells may provide some interesting additional insight. Both the T-box transcription factor Tbr-2/eomesodermin and the anergy related transcription factor GRAIL (Supp. 5 and preliminary RT-PCR data A. Prince) are increased specifically in Jak3-/- CD4+ T cells.

GRAIL was recently shown to be induced specifically upon T cell anergy [266]. Interestingly, GRAIL is an E3 ubiquitin ligase proposed to function in anergy by degrading proteins important in T cell function. That GRAIL functions at the protein modification level

Figure 37. The effect of STAT5ab-/- T cells on the proliferation of WT T cells.

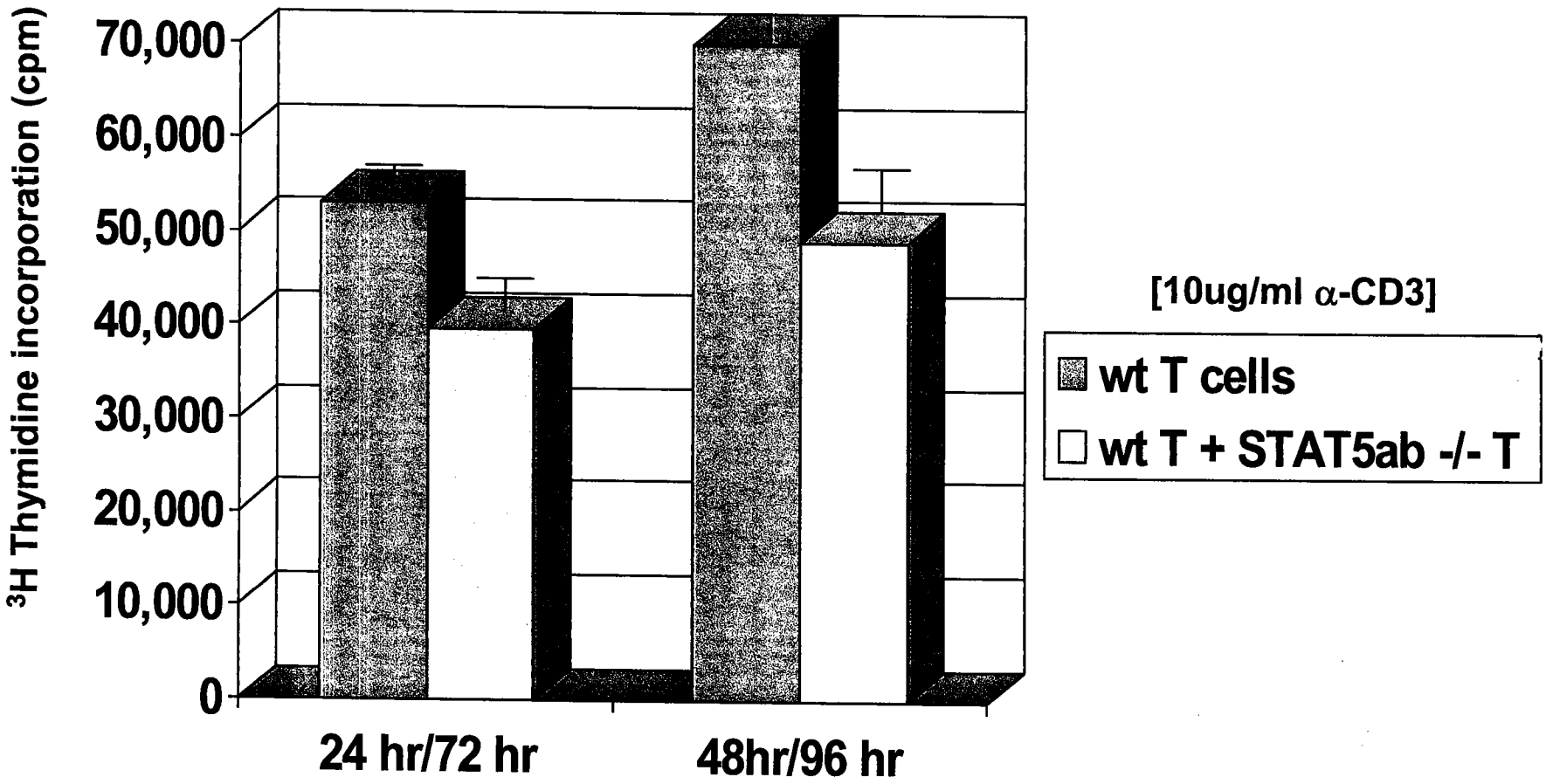


Figure 37. STAT5ab-/- CD4+ T cells have negligible suppressive effects on the proliferation of wild type CD4+ T cells.

2.5×10^5 STAT5ab+/- CD4+ T cells were cultured in 96 well plates coated with 10μg/ml α-CD3 antibody. At 24 (day 1) or 48 (day 2) hours of culture, 2.5×10^5 mitomycin-C treated STAT5ab+/- (gray bar) or 2.5×10^5 STAT5ab-/- CD4+ T (light gray bar) cells were added to the responder T cells. In addition, 5×10^5 STAT5ab+/- or STAT5ab-/- CD4+ T cells were stimulated in isolation as controls (data not shown). Cells were harvested after 48 hours of co-culture and proliferation was determined by ^{3}H-thymidine incorporation. Data is represented as the average total counts per minute (+/-SD) of triplicate determinations for one experiment performed.

would further explain our inability to detect many gene expression changes unique to Jak3-/- CD4+ T cells.

Eomesodermin (Eomes) has recently been implicated as important in effector functions of CD8+ T cells [267]. Specifically, by using dominant negative constructs, it was demonstrated that Eomes is important in the induction of IFN-γ and granzyme B and thus necessary for CD8+ cytolytic killing. Interestingly, not only do the Jak3-/- CD4+ T cells produce IFN-γ at the RNA and protein level but we have also detected a dramatic increase in transcripts encoding Granzymes B and K (Supp. 1) and an increase in and FasL (Supp. 2). This suggests the intriguing possibility that Jak3-/- CD4+ T cells mediate suppression of wild type CD4+ T cells not solely by the upregulation of immunosuppressive cytokines and/or PD-1 but also via a mechanism of T cell fratricide.

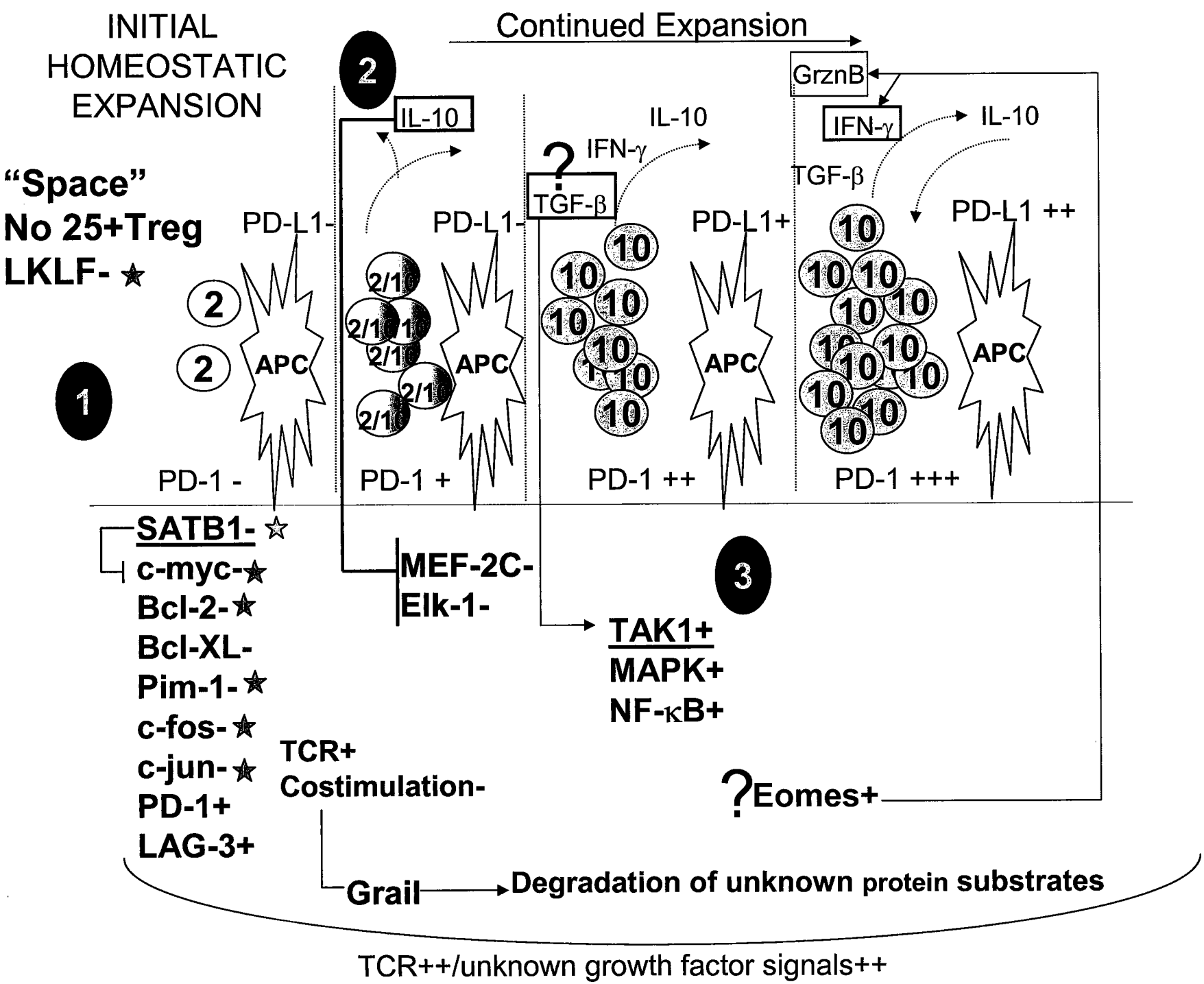
In addition to the cellular and functional changes in Jak3-/- CD4+ T cells described in Chapter Four including increased PD-1:PD-L1 expression and the production of IL-10, IFN-γ, and TGF-β, the microarray data presented in Chapter Five begins to highlight potential genetic and molecular modifications that in parallel with the increase in PD-1:PD-L1 and immunosuppressive cytokine activity may account for the alternative T cell differentiation pathway taken by Jak3-/- CD4+ T (Figure 38).

In summary of these data we envision the following possible model of molecular events that collaboratively lead to alternative T cell differentiation in Jak3-/- CD4+ T cells. First, as naïve Jak3-/- CD4+ T cells are induced to undergo homeostatic proliferation they are unable to receive signals through IL-2/γc cytokines in the absence of Jak3. The absence of γc

signals directly results in the inability of Jak3-/- CD4+ T cells to induce genes typically important for survival, proliferation and IL-2 production (SATB1-,c-myc-,Bcl-2-, Pim-1-,c-fos-,c-jun-). Despite this, these cells can initially secrete IL-2 and are still able to undergo proliferation *in vivo*. The ability to proliferate with only incomplete signals may in part be due to the absence of CD25+Tr-n regulatory T cells, the inability to maintain quiescence in the absence of LKLF, and/or an abundance of TCR and other growth signals that in the lymphopenic environment are sufficient to induce proliferation. Even though these factors may lead to the induction of cell cycle events, they do not provide the Jak3-/- T cells with "complete" activation signals and an anergic/unresponsive genetic program ensues resulting in the induction of genes such as GRAIL+. In addition, these proliferation signals, coupled with the complete absence of signaling through γc-dependent cytokine receptors, induce Jak3-/- T cells to induce IL-10 cytokine production. IL-10 signals block the RAS-RAF arm of the MAPK pathway leading to the complete inhibition of IL-2 production via reduced levels of the transcription factors MEF-2C and ELK-1. TGF-β is induced in Jak3-/- T cells by a mechanism that is unclear. TGF-β may in part contribute to the survival and proliferation of Jak3-/- CD4+ T cells by activating NF-κB pathway via TAK1 induction (TGF-β activated kinase 1). Finally, eomesodermin may play a role in the induction and/or production of IFN-γ as well as Granzyme B which may contribute to the suppressive qualities of Jak3-/-CD4+ T cells. The mechanism by which Eomes is induced in Jak3-/-CD4+ T cells is unclear. One possibility is that SATB1, which can act as either a transcriptional activator or repressor, typically represses the eomesodermin gene locus.

Since SATB1 gene expression is decreased in Jak3-/- CD4+ T cells, this may facilitate accessibility of the Eomes gene locus. In accord with this idea, SATB1 has been shown to repress the transcriptional activity of a number of brain specific transcripts with 40-60% homology to Eomesodermin based on BLAST results.

The findings presented in this thesis in total indicate several interesting aspects of T cell biology. First, the proliferation and accumulation of Jak3-/- CD4+ T cells after adoptive transfer into a lymphopenic host, in addition to the increase in cell cycle specific mRNAs detected by microarray analysis in freshly isolated Jak3-/- CD4+ CD44-high, demonstrate that homeostatic proliferation of CD4+ T cells is not dependent on signaling via γc-dependent cytokine receptors. A second important conclusion from the adoptive transfer experiments is that the weak activation signals normally associated with homeostatic expansion are sufficient to drive Jak3-/- T cells into a non-conventional differentiation program. Finally, the alternative differentiation program in Jak3-/- CD4+ T cells correlates with PD-1 expression and IL-10 production and differentiation may be further influenced by changes in gene expression as a result of PD-1:PD-L1 and immunosuppressive cytokine signals. In addition to these gene modifications, the changes in gene expression detected by microarray and RT-PCR analyses in transcription factors such as SATB1, Eomes, and GRAIL may play additional roles in the alternative T cell phenotype of Jak3-/- CD4+ T cells although further investigation is required. Additionally, this data suggests the intriguing possibility these factors also play a role in the maintenance and/or induction of wild type regulatory T cell subsets.


INITIAL
HOMEOSTATIC
EXPANSION
Continued Expansion
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No 25+Treg
LKLF-
1
2
2
2
APC
PD-L1-
PD-1 -
IL-10
2/1
PD-L1-
PD-1 +
?
IFN-γ
TGF-β
IL-10
10
PD-L1+
PD-1 ++
GrznB
IFN-γ
IL-10
TGF-β
PD-L1 ++
PD-1 +++
SATB1-
c-myc-
Bcl-2-
Bcl-XL-
Pim-1-
c-fos-
c-jun-
PD-1+
LAG-3+
MEF-2C-
Elk-1-
3
TAK1+
MAPK+
NF-κB+
TCR+
Costimulation-
?Eomes+
Grail
Degradation of unknown protein substrates
TCR++/unknown growth factor signals++

Figure 38. Events and molecular mechanisms that in parallel account for alternative T cell differentiation in Jak3-/- CD4+ T cells.

1) As naïve Jak3-/- CD4+ T cells are induced to undergo homeostatic proliferation they are unable to receive signals through IL-2/γc cytokines. The absence of γc signals directly results in the inability of Jak3-/- CD4+ T cells to induce genes typically important for survival, proliferation and IL-2 production (SATB1-,c-myc-,Bcl-2-,Bcl-XL-,Pim-1-,c-fos-,c-jun-). Despite this, these cells can initially secrete IL-2 and are still able to undergo proliferation *in vivo*. The ability to proliferate with only incomplete signals may in part be due to the absence of CD25+Tr-n regulatory T cells, the inability to maintain quiescence (LKLF-), and/or an abundance of TCR and other growth signals that in the lymphopenic environment are sufficient to induce proliferation. Even though these factors may lead to the induction of cell cycle events, they do not provide the Jak3-/- T cells with "complete" activation signals and an anergic/unresponsive genetic program ensues (GRAIL+). **2)** In addition, these proliferation signals, coupled with the complete absence of signaling through γc-dependent cytokine receptors, induce Jak3-/- T cells to induce IL-10 cytokine production. IL-10 signals will block the RAS-RAF arm of the MAPK pathway leading to the complete inhibition of IL-2 production (MEF-2C-, ELK-1-). **3)** TGF-β and Eomesodermin are induced by mechanisms that are unclear. TGF-β may in part be contributing to the survival and proliferation of Jak3-/- CD4+ T cells by activating MAPK and NF-κB pathway via TAK1 (TGF-β activated kinase 1). Eomesodermin may play a role in the induction and/or production of IFN-γ as well as Granzyme B which may contribute to the suppressive qualities of Jak3-/-CD4+ T cells.

★ Indicates a gene that has been demonstrated to be IL-2/γc responsive

Chapter VII.

Supplemental Data

Supplemental#1. Protease/Kinase

Increased

gene	Fold change
Caspase 11	3.2
Spi6 (PI-9)	4.2
Map kinase kinase 3	4.6
FIN16	6
Spi2	6.9
Caspase 3	7
Granzyme B	22.7
Granzyme K	30.7

Decreased

gene	Fold change
cathepsin L	3
kallikrein	3.7
cathepsin H	4.4

Supplemental #2 Cell Cycle/Apoptosis

Increased

gene	change
Dynein	3
MNS-1	3.1
MCM	3.2
centromere protein A (Cenp-A)	3.2
kinesin heavy chain member 4	3.2
Lamin B1	3.3
cyclin B1	3.4
Rad51	3.8
nek2	3.9
cyclin B2	4
Cdc45	4.3
kinesin related mitotic motor	4.4
cyclin A2	4.5
CPP32	4.7
FasL	4.8
Sak-b	4.9
cdc2, p34	4.9
polo like kinase (plk)	5
survivin (TIAP)	5.1
MAD2	5.6
cdc6	6.1

Decreased

gene	change
Bcl-2	3.6
MyD116	4.3
Ena-VASP like (Evl)	6.5

Supplemental #3 Cytokines

Increased

gene	change
IFN-gamma	3.1
small inducible cytokine A5	3.1
IL-10 Ralpha	3.4
small inducible cytokine A3	12.2
MIP-1beta	22.8

Decreased

gene	change
CIS-1	3.3
IL-3 Rbeta	4
IL-6 R alpha	4.5
EBI-3	5.1
IL-7R	7.1

Supplemental #4 Surface Molecules/Receptors

Increased

gene	fold change
TJ6	3.1
MHC class I	3.2
integrin alpha 4 (CD49d)	3.4
PD-1	3.5
LAG-3	4.1
CD7	4.9
CCR5	7.3
CTLA-2 alpha & beta	16

Decreased

gene	fold change
TCR gamma	3.2
VCAM-1	3.3
PIM-1	3.4
EBI-1 (EBV induced 1)	3.8
TCR beta	4.5
CCR-6	4.5
neuropillin	5
Ly6C	6.1
CTLA-4	6

Supplemental #5 Transcription/Translation

Increased

gene	change
Ngfi-A binding protein 1 (NAB1)	3.3
MRF-1	3.4
acidic nuclear phosphoprotein p32	3.7
Dp1	4
Mad3	4.4
GIF	4.7
Trans-acting factor 4 (Sp4)	4.9
Tbr2/Eomesodermin	15.7

Decreased

gene	change
BKLF	3.2
TIS11	3.7
E1-A repressor (CREG)	4
transcription factor 7	4.1
LMO4	4.5
inhibitor of DNA binding 3 (Id)	5.1
MHC class III region RD	5.7
LKLF	7.2
SATB1	7.6

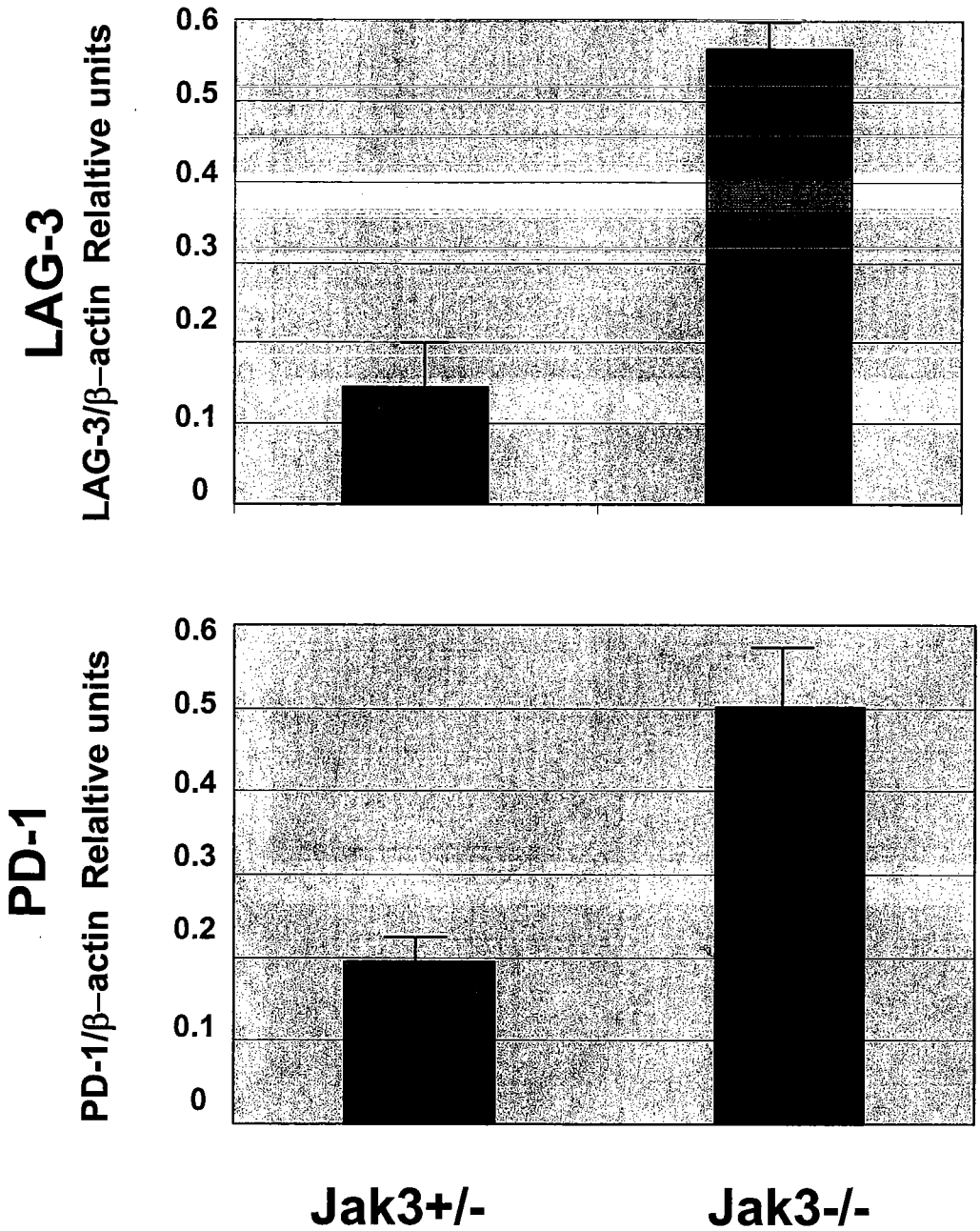
Supplemental #6 Miscellaneous

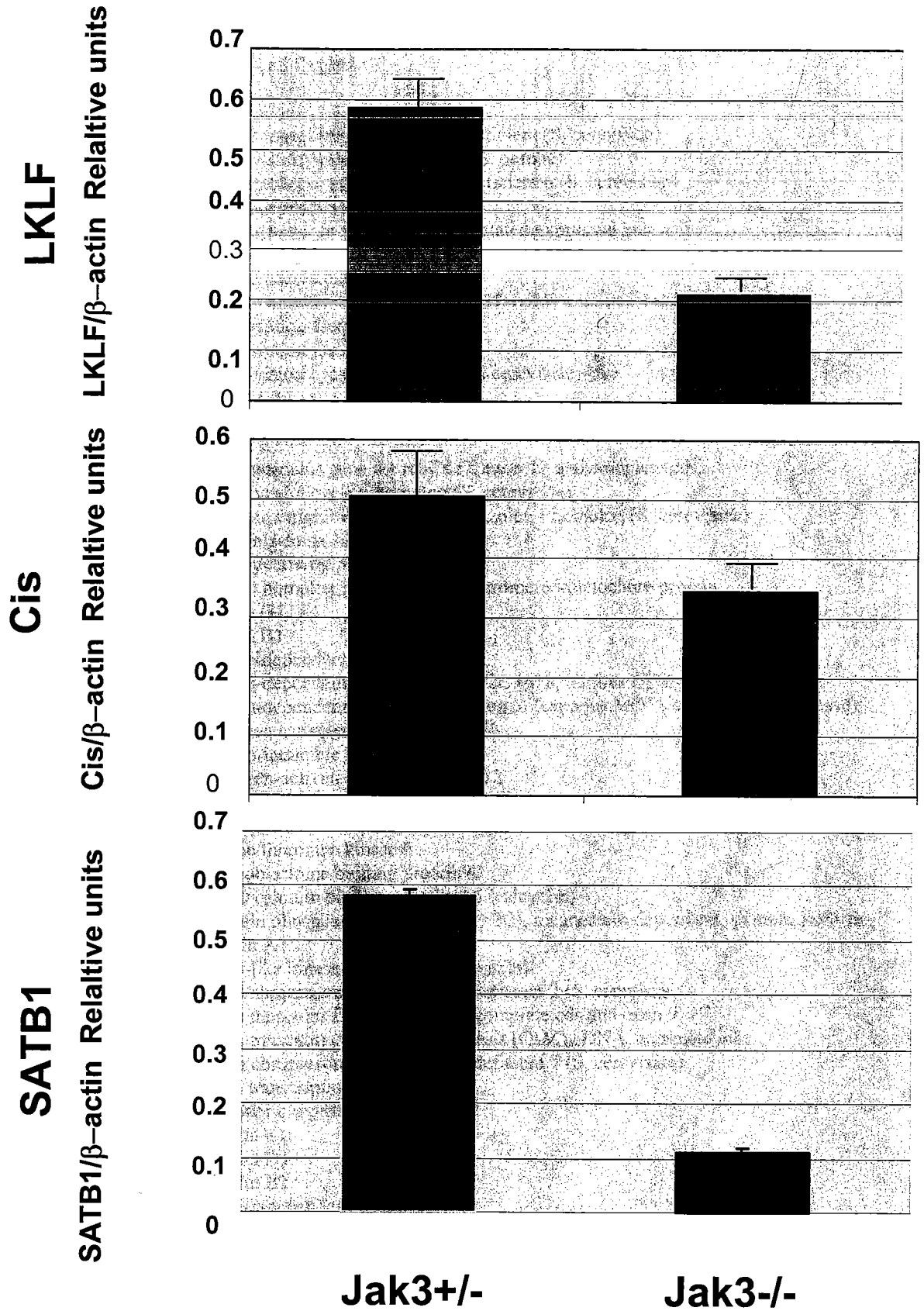
Increased

gene	change
Ki-67	3.1
diazepam binding inhibitor	3.1
Sid1334p	3.5
cystatin like metastasis	3.6
WW domain binding protein	4
flotillin	4
importin alpha	5
Nup107	6.3
GAG	6.8

Decreased

gene	change
cyctochrome c oxidase	3
Mtv 7 sag	3
Notch 2	3.1
MD3	3.1
phospholipid transfer protein	3.3
Isocitrate dehydrogenase 2	3.3
Hsp 70	4.3
Granulin	4.9
MRP-8	5.5
osteosarcoma oncogene	6.6
carbonic anhydrase II (CAII)	6.6
microsomal epoxide hydrolase	6.9


LAG-3
LAG-3/β–actin Relaltive units
0.6
0.5
0.4
0.3
0.2
0.1
0
PD-1
PD-1/β–actin Relaltive units
0.6
0.5
0.4
0.3
0.2
0.1
0
Jak3+/-
Jak3-/-


LKLF
LKLF/β–actin Relaltive units
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
Cis
Cis/β–actin Relaltive units
0.6
0.5
0.4
0.3
0.2
0.1
0
SATB1
SATB1/β–actin Relaltive units
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
Jak3+/-
Jak3-/-

Supplemental Data 9.--- Cell Cycle

Gene Name	Description
100062_at	mini chromosome maintenance deficient (S. cerevisiae)
100128_at	cell division cycle 2 homolog A (S. pombe)
100149_at	mini chromosome maintenance deficient 6 (S. cerevisiae)
100444_at	cyclin-dependent kinase 5
100885_at	NIMA (never in mitosis gene a)-related expressed kinase 2
101180_at	ataxia telangiectasia mutated homolog (human)
101834_at	mitogen activated protein kinase 3
101900_at	cyclin-dependent kinase inhibitor 2B (p15, inhibits CDK4)
101958_f_at	transcription factor Dp 1
101959_r_at	transcription factor Dp 1
102403_at	cell division cycle 45 homolog (S. cerevisiae)-like
102781_at	cyclin L2
102963_at	E2F transcription factor 1
103034_at	cyclin E1
103797_at	Mus musculus gene for muCdc7, exon 12 and complete cds.
103821_at	cell division cycle 6 homolog (S. cerevisiae)
104097_at	budding uninhibited by benzimidazoles 1 homolog (S. cerevisiae)
104476_at	retinoblastoma-like 1 (p107)
104598_at	dual specificity phosphatase 1
104757_at	ZW10 homolog (Drosophila), centromere/kinetochore protein
160127_at	cyclin G1
160159_at	cyclin B1
160538_at	cyclin-dependent kinase 4
160638_at	cyclin-dependent kinase inhibitor 2C (p18, inhibits CDK4)
160658_at	cyclin-dependent kinase 7 (homolog of Xenopus MO15 cdk-activating kinase)
160763_at	mitogen activated protein kinase 13
92310_at	serum-inducible kinase
92323_at	mitogen-activated protein kinase 12
92481_at	CHK2 checkpoint homolog (S. pombe)
92631_f_at	calmodulin 3
92639_at	serine/threonine kinase 6
92647_at	retinoblastoma binding protein 4
92770_at	S100 calcium binding protein A6 (calcyclin)
92879_at	protein phosphatase 1G (formerly 2C), magnesium-dependent, gamma isoform
92911_at	cyclin A1
93099_f_at	polo-like kinase homolog (Drosophila)
93112_at	mini chromosome maintenance deficient 2 (S. cerevisiae)
93253_at	Mus musculus DNA for ERK2, complete cds and exon 9.
93293_at	Mus musculus calmodulin synthesis (CaM) cDNA, complete cds.
93356_at	mini chromosome maintenance deficient 7 (S. cerevisiae)
93405_at	E2F transcription factor 3
93413_at	telomeric repeat binding factor 2
93712_at	cyclin T1
94231_at	cyclin D1
94294_at	cyclin B2
94411_at	cyclin-dependent kinase 2
94521_at	cyclin-dependent kinase inhibitor 2D (p19, inhibits CDK4)

94819_f_at	cyclin I
94820_r_at	cyclin I
95527_at	chromatin assembly factor 1, subunit A (p150)
95617_at	retinoblastoma-like 2
95805_at	cell division cycle 2 homolog (S. pombe)-like 1
96498_at	disrupted meiotic cDNA 1 homolog
96522_at	calmodulin 1
97182_at	cyclin E2
97504_at	cyclin D2
97531_at	G0/G1 switch gene 2
97844_at	regulator of G-protein signaling 2
98469_at	serine/threonine kinase 12
98478_at	cyclin G2
98979_at	menage a trois 1
98996_at	serine/threonine kinase 18
99000_at	mitogen-activated protein kinase 7
99005_at	RAD1 homolog (S. pombe)
99073_at	cyclin F
99186_at	cyclin A2
99522_at	germ cell-specific gene 2
99558_at	cyclin C
99632_at	MAD2 (mitotic arrest deficient, homolog)-like 1 (yeast)
99920_at	telomeric repeat binding factor 1

Supplemental Data 10.---Cell Cycle Arrest

Gene Name	Description
100278_at	cyclin-dependent kinase inhibitor 1B (P27)
100951_at	polycystic kidney disease 2
101429_at	DNA-damage inducible transcript 3
102264_at	schlafen 1
102292_at	Mus musculus GADD45 protein (gadd45) gene, complete cds.
102395_at	peripheral myelin protein
94337_at	growth arrest specific 2
94338_g_at	growth arrest specific 2
94809_at	tumor susceptibility gene 101
94813_at	growth arrest specific 1
95471_at	cyclin-dependent kinase inhibitor 1C (P57)
96801_at	adenylate kinase 1
97375_at	polycystic kidney disease 1 homolog
98067_at	cyclin-dependent kinase inhibitor 1A (P21)
98789_at	cyclin-dependent kinase inhibitor 2A

Supplemental Data 11.---Negative Regulation of Cell Cycle

Gene Name	Description
101457_at	Janus kinase 2
102012_at	src family associated phosphoprotein 2
102264_at	schlafen 1
160300_at	trefoil factor 1
92471_i_at	schlafen 2
92472_f_at	schlafen 2
92959_at	fyn-related kinase
93104_at	M.musculus btg1 mRNA.
93948_at	non-catalytic region of tyrosine kinase adaptor protein 2
94809_at	tumor susceptibility gene 101
96987_at	myeloid/lymphoid or mixed lineage-leukemia translocation to 7 homolog (Drosophila)
98299_s_at	schlafen 3

Supplemental 12.—Cytokine Activity Group 1

Gene Name	Description
100112_at	chemokine (C-X-C motif) ligand 12
101164_at	tumor necrosis factor (ligand) superfamily, member 4
102559_at	Mus musculus bone morphogenetic protein 2 (BMP-2) gene, complete cds.
102630_s_at	lymphotoxin A
102802_at	interleukin 18
103037_at	cardiotrophin 1
160511_at	chemokine (C-X-C motif) ligand 12
160567_at	growth differentiation factor 9
160995_at	RIKEN cDNA E430037F08 gene
92283_s_at	interleukin 4
92415_at	amino acid feature: putative glycosylation sites, aa 139, 161, 293; amino acid feature: transmembrane domain, aa 82 .. 103; amino acid feature: transmembrane domain, bp 299 .. 361; Mus musculus 4-1BB ligand mRNA, complete cds.
92459_at	chemokine (C-C motif) ligand 8
92476_at	growth differentiation factor 3
92982_at	bone morphogenetic protein 8a
93456_r_at	Mus musculus BMP-4 gene, complete cds.
93717_at	chemokine (C-C motif) ligand 12
94086_at	interleukin 3
94142_at	colony stimulating factor 3 (granulocyte)
94145_at	interferon beta, fibroblast
94848_at	Mus musculus migration inhibitory factor (Mif) gene.
95349_g_at	chemokine (C-X-C motif) ligand 1
98772_at	chemokine (C-X-C motif) ligand 5

Supplemental 13.---Cytokine Activity Group 2

Gene Name	Description
100319_at	interleukin 10
100574_f_at	glucose phosphate isomerase 1
100671_at	Mouse alpha-interferon gene, complete cds.
100773_at	interleukin 12a
100779_at	interleukin 12b
101403_at	chemokine (C-C motif) ligand 25
101619_at	Mus musculus gene for interferon alpha 8 protein precursor, complete cds.
101788_f_at	Mouse gene for interferon alpha 1 (MuIFN-alpha 1).
102149_f_at	precursor; Mouse interferon alpha-7 gene, complete cds, clone pPL608.
102736_at	JE protein precursor; Mouse platelet-derived growth factor-inducible protein (JE) gene, complete cds.
161037_at	interleukin 15
92266_at	interleukin 11
92578_at	small inducible cytokine subfamily E, member 1
92960_at	leukemia inhibitory factor
94112_at	tumor necrosis factor (ligand) superfamily, member 10
94146_at	M.musculus MIP-1b gene for macrophage inflammatory protein 1b.
94166_g_at	chemokine (C-C motif) ligand 1
94755_at	interleukin 1 alpha
96419_f_at	putative; Mouse gene for interferon alpha 6 (Mu IFN-alpha 6).
96537_at	Mus musculus ARS component B gene, exons 1-3.
96574_at	P40 T-cell and mast cell growth factor precursor; Mouse P40 T-cell and mast cell growth factor (mP40) gene, complete cds.
97113_at	tumor necrosis factor (ligand) superfamily, member 6
97783_at	chemokine (C-C motif) ligand 17
99801_at	nodal

Supplemental 14.---Cytokine Activity Group 3

Gene Name	Description
100327_at	precursor; Mouse LT lymphotoxin (LT) gene, complete cds.
101136_at	tumor necrosis factor (ligand) superfamily, member 8
101436_at	chemokine (C-X-C motif) ligand 9
101450_at	colony stimulating factor 1 (macrophage)
102148_f_at	IFN-alpha-1-9; Mouse interferon-alpha (IFN-alpha-1-9) gene, complete cds.
102218_at	interleukin 6
102310_at	chemokine (C-C motif) ligand 22
102424_at	chemokine (C-C motif) ligand 3
103199_at	chemokine (C motif) ligand 1
104099_at	peptidoglycan recognition protein
104571_at	platelet factor 4
92284_r_at	interleukin 4
93243_at	bone morphogenetic protein 7
94165_at	chemokine (C-C motif) ligand 1
95348_at	chemokine (C-X-C motif) ligand 1
98318_at	Mus musculus CD70 gene, exon 1 & 2 & joined CDS.
98406_at	chemokine (C-C motif) ligand 5
98600_at	S100 calcium binding protein A11 (calizzarin)
99334_at	interferon gamma
99393_at	bone morphogenetic protein 5
99915_at	amino acid feature: cytoplasmic domain, aa 220..248; amino acid feature: soluble growth factor, aa 100..181; amino acid feature: transmembrane domain, aa 192..219; Mus musculus amphiregulin gene, complete cds.

Supplemental 15.---Cytokine Activity Group 4

Gene Name	Description
100573_f_at	glucose phosphate isomerase 1
101160_at	chemokine (C-X-C motif) ligand 2
101317_f_at	Mus musculus interferon alpha-B (IFN-alpha-B) gene, complete cds.
101325_r_at	fibrosin 1
101789_i_at	Mouse gene for interferon alpha 4 (Mu IFN-alpha 4).
101790_f_at	Mouse gene for interferon alpha 4 (Mu IFN-alpha 4).
101814_at	Mus musculus bone morphogenetic factor 11 (Bmp11) gene, exon 3 and complete cds.
101815_at	BMP-10; Mus musculus bone morphogenetic protein 10 (Bmp10) gene, exon 2 and complete cds.
102025_at	chemokine (C-X-C motif) ligand 13
102029_at	interleukin 16
102345_at	endometrial bleeding associated factor
102929_s_at	FMS-like tyrosine kinase 3 ligand
103486_at	interleukin 1 beta
104388_at	chemokine (C-C motif) ligand 9
104436_at	interleukin 1 family, member 5 (delta)
104646_at	Mus musculus MIC-1 gene, exon 1 and joined CDS.
93066_at	acrosomal protein epithelin/granulin precursor glycoprotein; Mus musculus gene for acrogranin, complete cds.
93416_at	tumor necrosis factor (ligand) superfamily, member 11
93455_s_at	bone morphogenetic protein 4
93917_at	tumor necrosis factor (ligand) superfamily, member 12
94717_f_at	Mouse gene for interferon alpha 2 (Mu IFN-alpha 2).
96968_at	limitin
98793_at	growth differentiation factor 5
99349_at	Mus musculus CTLA8 gene, complete cds.

Supplemental 16---Cytokine Activity Group 5

Gene Name	Description
101657_at	bone morphogenetic protein 8b
101791_f_at	Mouse gene for interferon alpha 5 (Mu IFN-alpha 5).
102629_at	Mus musculus TNFA gene for tumor necrosis factor alpha, exon 4, strain:NOD.
102705_at	interleukin 2
102940_at	lymphotoxin B
92372_at	bone morphogenetic protein 6
92485_at	bone morphogenetic protein 15
92849_at	chemokine (C-C motif) ligand 6
92948_at	Mouse gene for granulocyte-macrophage colony stimulating factor (GM-CSF).
93858_at	chemokine (C-X-C motif) ligand 10
94168_at	interleukin 13
94761_at	M.musculus cytokine gene.
95557_at	bone morphogenetic protein 1
97155_at	growth differentiation factor 8
97519_at	secreted phosphoprotein 1
97709_at	interleukin 7
97734_at	thrombopoietin
98008_at	chemokine (C-X3-C motif) ligand 1
98374_at	Murine gene for interleukin 5 (eosinophil differentiation factor).
98812_at	tumor necrosis factor (ligand) superfamily, member 5

Supplemental 17.---NF-kB pathway Group 1

Gene Name	Description
100299_f_at	immunoglobulin kappa chain variable 28 (V28)
100682_f_at	immunoglobulin kappa chain variable 8 (V8)
101329_f_at	immunoglobulin kappa chain variable 8 (V8)
101331_f_at	immunoglobulin kappa chain variable 8 (V8)
101347_at	immunoglobulin kappa chain variable 8 (V8)
101616_at	precursor; Mouse Ig rearranged kappa-chain mRNA, clone AN08K.
101633_at	precursor polypeptide (AA -20 to -5) (195 is 1st base in codon); Mouse VK gene for kappa light chain variable region and J4 sequence.
101640_f_at	Ig kappa chain precursor VJ5-region; Mouse Ig kappa chain 7B6 mRNA, V-region (VJ5) of monoclonal phOx-specific antibody from a hybridoma.
101656_f_at	immunoglobulin kappa chain variable 5 (V5 family)
101718_f_at	immunoglobulin kappa chain variable 8 (V8)
101720_f_at	immunoglobulin kappa chain variable 8 (V8)
102076_at	Mus musculus IgVk aj4 gene.
102154_f_at	immunoglobulin kappa chain variable 8 (V8)
102155_f_at	Mouse Ig aberrantly rearranged kappa-chain region downstream of J2 gene, from plasmacytoma 3886.
102156_f_at	putative; Mus castaneus IgK chain gene, C-region, 3' end.
102157_f_at	Ig allele 91A3 V-region kappa chain; Mouse Ig V-kappa10-Ars-A kappa chain gene, complete cds.
102209_at	nuclear factor of activated T-cells, cytoplasmic 1
102585_f_at	immunoglobulin kappa chain variable 8 (V8)
160991_at	RIKEN cDNA 2400004O09 gene
93086_at	immunoglobulin kappa chain variable 8 (V8)
93227_f_at	immunoglobulin kappa chain variable 8 (V8)
93395_g_at	ectodysplasin-A
95943_at	ESTs
96670_at	RIKEN cDNA 0610025I19 gene
96971_f_at	Mouse DNA for Ig-kappa light chain V-J kappa 5 joining region (cell line CH2).
96972_f_at	put. Ig kappa precursor; Mouse DNA for Ig-kappa light chain V-J kappa 5 joining region.
96974_at	M.musculus Ig Vkappa-HNK20 gene.
96975_at	M.musculus Ig Vkappa-PCG-4 gene.
96992_r_at	Mus musculus immunoglobulin light chain V-region (VJ) gene for immunoglobulin light chain kappa.
97564_f_at	Mus musculus immunoglobulin kappa light chain variable region gene, partial cds.
97566_f_at	Mus musculus hybridoma BDI-1 immunoglobulin kappa light chain variable region (V kappa) gene, partial cds.
97567_f_at	Mus musculus hybridoma WLA-2C4 immunoglobulin kappa light chain variable region (V kappa) gene, partial cds.
98813_at	reticuloendotheliosis oncogene
99369_f_at	Mus musculus immunoglobulin kappa light chain variable region precursor (Vk10c) gene, partial cds.

Supplemental 18.---NF-kB pathway Group 2

Gene Name	Description
100683_r_at	immunoglobulin kappa chain variable 8 (V8)
101330_f_at	Mus musculus anti-DNA immunoglobulin light chain IgG, antibody 373s.116, partial cds.
101641_at	Mouse Ig L-chain gene variable region, complete cds.
102430_at	myeloid differentiation primary response gene 88
103328_at	TRAF family member-associated Nf-kappa B activator
103614_at	nuclear factor of kappa light polypeptide gene enhancer in B-cells 2, p49/p100
104755_at	TNFAIP3 interacting protein 1
161903_f_at	molecule possessing ankyrin-repeats induced by lipopolysaccharide
93087_r_at	immunoglobulin kappa chain variable 8 (V8)
93394_at	ectodysplasin-A
93416_at	tumor necrosis factor (ligand) superfamily, member 11
93706_at	inhibitor of kappaB kinase beta
94149_at	kappa B and Rss recognition component
97651_at	polymerase (DNA directed), kappa
97813_at	v-rel reticuloendotheliosis viral oncogene homolog A (avian)
99405_at	immunoglobulin kappa chain variable 8 (V8)

Supplemental 19.---NF-kB pathway Group 3

Gene Name	Description
101332_at	immunoglobulin kappa chain variable 38(V38)
101346_at	immunoglobulin kappa chain variable 8 (V8)
101348_at	immunoglobulin kappa chain variable 8 (V8)
101554_at	Mus musculus I kappa B alpha gene, exons 2-6, partial cds.
101632_at	tumor necrosis factor receptor superfamily, member 11a
101826_at	M.musculus extrachromosomal DNA for V kappa and J kappa coding joint (clone pKDE 35).
101902_at	recombining binding protein suppressor of hairless (Drosophila)
103255_at	Tnf receptor-associated factor 5
104042_at	solute carrier family 35, member B1
160760_at	protein tyrosine phosphatase, receptor type, K
161371_r_at	protein tyrosine phosphatase, receptor type, K
162042_i_at	v-rel reticuloendotheliosis viral oncogene homolog A (avian)
162307_at	AV007877 Mus musculus 18-day embryo C57BL/6J Mus musculus cDNA clone 1110007A08, mRNA sequence.
92493_at	recombining binding protein suppressor of hairless-like (Drosophila)
93074_g_at	nuclear factor of activated T-cells, cytoplasmic 2
94006_at	5-azacytidine induced gene 2
94196_at	inhibitor of kappaB kinase gamma
96224_at	beta-transducin repeat containing protein
97975_at	nuclear factor of activated T-cells, cytoplasmic 3
98988_at	molecule possessing ankyrin-repeats induced by lipopolysaccharide
99065_at	casein kappa
99070_at	conserved helix-loop-helix ubiquitous kinase
99396_at	immunoglobulin kappa chain variable 8 (V8)

Supplemental 20.---NF-kB pathway Group 4

Gene Name	Description
101727_at	nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, epsilon
103091_at	avian reticuloendotheliosis viral (v-rel) oncogene related B
104149_at	nuclear factor of kappa light chain gene enhancer in B-cells inhibitor, alpha
92301_at	inhibitor of kappaB kinase epsilon
93212_at	butyrate-induced transcript 1
93213_at	immunoglobulin kappa chain variable 28 (V28)
93226_i_at	immunoglobulin kappa chain variable 8 (V8)
93393_at	ectodysplasin-A
96254_at	DnaJ (Hsp40) homolog, subfamily B, member 1
96969_at	coding sequence; Protein sequence is in conflict with the conceptual translation; Mouse immunoglobulin variable gene V kappa-24 encoding amino acids -4 to 95.
99886_at	opioid receptor, kappa 1
99982_at	nuclear factor of kappa light chain gene enhancer in B-cells inhibitor, beta

Supplemental 21.---NF-kB pathway Group 5

Gene Name	Description
101731_at	RS ORF2 (bp 588-293) first start codon is located at base 427.; putative; Mouse Ig germline kappa-chain 'recombining sequence' (RS).
104474_s_at	opioid receptor-like
161162_at	nuclear factor of activated T-cells, cytoplasmic 3
162372_f_at	avian reticuloendotheliosis viral (v-rel) oncogene related B
92584_at	RIKEN cDNA 2410003M04 gene
93073_at	nuclear factor of activated T-cells, cytoplasmic 2
93075_r_at	nuclear factor of activated T-cells, cytoplasmic 2
94448_at	B-cell leukemia/lymphoma 10
97569_at	Mus musculus hybridoma 3C4 immunoglobulin kappa light chain variable region (V kappa) gene, partial cds.
97570_at	Mus musculus immunoglobulin kappa light chain variable region (Igk-V22) gene, partial cds.
98427_s_at	nuclear factor of kappa light chain gene enhancer in B-cells 1, p105

Chapter VIII.

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