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By Ivan Lupov

Entitled
"ACQUIRED STAT4 DEFICIENCY AS A CONSEQUENCE OF CANCER CHEMOTHERAPY"

For the degree of Master of Science

Is approved by the final examining committee:

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# ACQUIRED STAT4 DEFICIENCY AS A CONSEQUENCE OF CANCER CHEMOTHERAPY

A Thesis

Submitted to the Faculty

of

Purdue University

by

Ivan Lupov

In Partial Fulfillment of the

Requirements for the Degree

of

Master of Science

May 2011

Purdue University

Indianapolis, Indiana

# ACKNOWLEDGMENTS

I would like to thank Dr. Hua-Chen Chang for accepting me in her lab. She has fully equipped me with the skills I will need to succeed in my future training as a scientist. I would also like to thank Dr. Stephen Randall for challenging me intellectually, inside and outside lecture hall, Dr. Michael Robertson for his instrumental support and mentorship throughout the completion of this project and of course, the wonderful and irreplaceable technician Ling Han.

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# LIST OF ABBREVIATIONS

| | |
|---|---|
| -D | No drug Added |
| C | Control cells from normal PBMCs |
| CC | Coiled-coiled domain |
| CAR | Carmustine |
| CD4+ | Marker for T helper type I lymphocytes (also abbreviated Th1) |
| CD8+ | Marker for CTL |
| CD56+ | Marker for NK cells |
| ChiP | Chromatin immunoprecipitation experiment |
| CVB3 | Coxsackievirus B3 |
| CTL | Cytotoxic T Lymphocyte |
| DBD | DNA binding domain |
| DC | Dendritic Cells |
| EAE | Experimental autoimmune encephalomyelitis |
| ETO | Etoposide |
| Hlx | Homeobox protein HB24 – transcription factor in activated lymphocytes |
| IFNα | Interferon alpha |
| IFNγ | Interferon gamma |
| IL | Interleukin |
| IL#R | Interleukin # Receptor |
| ISG | Interferon Stimulated Gene – ubiquitin-like protein (same as ISG15) |
| JAK | Janus Associated Kinase |

| | |
|---|---|
| LK | Linker domain |
| LPS | Lipopolysaccharide |
| P | Patient cells |
| P#H | Particular patient after high dose of chemotherapy treatment |
| pY | Phosphorylated form of the amino acid tyrosine |
| pS | Phosphorylated form of the amino acid serine |
| PBMC | Peripheral Blood Mononuclear Cells |
| PIAS | Protein Inhibitors of Activated STATs |
| MHC | Major Histocompatibility Complex (Type I or Type II) |
| MS | Multiple sclerosis |
| NK | Natural Killer Cells (see also CD56+) |
| RA | Rheumatoid Arthritis |
| RT-PCR | Real Time Polymerase Chain Reaction |
| S | Standard Dose Chemotherapy |
| SLE | Systemic lupus erythematosus |
| SLIM | STAT-interacting, LIM domain processing protein |
| STAT | Signal Transducer and Activator of Transcription |
| SOCS | Suppressors Of Cytokine Signaling |
| SUMO | Ubiquitin-like molecule that modifies proteins |
| TAD | Transcriptional activation domain |
| TC45 | Nuclear phosphatase involved in deactivating STAT1 |
| Th1 | See CD4+ |
| Y | Designates the amino acid tyrosine |

# ABSTRACT

Lupov, Ivan M.S., Purdue University, May 2011. Acquired STAT4 deficiency as a consequence of cancer chemotherapy. Major Professor: Hua-Chen Chang.

Signal Transducer and Activator of Transcription 4 (STAT4) is an important transcription factor activated by IL-12 signaling. Activated STAT4 is essential for Th1 cell differentiation, a process characterized by increased potential for interferon (IFN)-γ production. Defective IFN-γ production due to STAT4 deficiency occurs after autologous stem cell transplantation for lymphoma.

We have investigated the mechanisms of post-transplant STAT4 deficiency. The tumor-bearing state is ruled out to be the cause because STAT4 levels were not significantly different in peripheral blood mononuclear cells (PBMCs) obtained from lymphoma patients prior to treatment and healthy control subjects. The magnitude of the decrease in STAT4 levels corresponded with increasing intensity of chemotherapeutic treatment in vivo. Furthermore, treatment of normal PBMC cultures or a natural killer (NK) cell line with chemotherapy drugs in vitro also resulted in reduced STAT4 protein and reduced IL-12-induced IFN-γ production. Chemotherapy drugs are shown to have no impact on the stability of *STAT4* mRNA, while steady-state levels of *STAT4*transcripts are decreased in lymphoma patients.

Our findings demonstrated that chemotherapeutic drugs up-regulate the ubiquitination rates of the STAT4 protein, which in turn promotes its degradation via the proteasome-mediated pathway. Treatment with the proteasome inhibitor bortezomib largely reversed the chemotherapy-induced STAT4 deficiency. Thus, acquired STAT4 deficiency in lymphoma patients is a consequence of treatment with chemotherapy. These results have important implications for design of optimal immunotherapy for lymphoma.

# CHAPTER 1. LITERATURE REVIEW

The purpose of this literature review is to provide an overarching background on what has been discovered about the structure and function of the transcription factor called Signal Transducer and Activator of Transcription 4 (STAT4). The information will demonstrate the ever expanding breath of knowledge about the mechanism by which STAT4 brings changes to the repertoire of an entire cell – modulating the expression of a wide rage of genes and in the process driving the systematic differentiation of what's commonly known as T-helper 1 (Th1) cells. Th1 cells are crucial in mounting a proper immune response to a variety of intracellular pathogens and viruses, as well as contributing to the tumor surveillance by the immune system. Gaining greater understanding of what is the molecular structure and function of STAT4, how is it activated and its' function regulated will help us understand the importance of its' biological function. Furthermore, this information will help emphasize the significance of STAT4 deficiency not only for lymphoma patients, which are the object of our study, but for the ability to have a proper functional immune system in general.

STAT4 is member of a family of transcription factors that are commonly referred to as STATs(*1-4*). Initially, about two decades ago, what piqued the scientific community's interest in these transcription factors was their specific response to various extracellular signals and most importantly their direct impact on gene expression that

circumvented the need for secondary messengers. Currently, there are 7 known members of the STAT family that serve distinct biological functions from development to immunity(*5-8*).

## 1.1. STAT4 Structure and Expression

a. Structure

STAT4 shares a well conserved protein structure with the rest of the STAT family transcription factors. There are 6 distinct domains (Figure 1C) – N-terminal ($NH_2$), coiled-coil (CC), DNA binding (DBD), linker (LK), SH2, tyrosine activation (Y) and transcriptional activation domain (TAD) (*5, 6*). Each one of these domains has distinct role in the overall function of each STAT molecule. In order to understand the specific role of each one of these, it is important to delineate the main steps in STAT4 activation and signaling, while leaving the details of the pathway to the following section of the paper.

In general, all STAT molecules are found latent in the cytoplasm (Figure 1A). When outside signaling molecule (ex: IL-12) binds to a transmembrane receptor (IL-12R) it induces a conformational change that allows the recruitment of the Janus family of receptor associated kinases or JAKs. In turn, these JAK kinases phosphorylate the receptor making it a docking site for a STAT molecule (STAT4 in the case of IL-12 stimulation) (Figure 1A). When the respective STAT binds to the phosphorylated part of the receptor, it becomes, in turn, phosphorylated and thus activated. It gets released from the docking site and forms a homodimer with another activated STAT molecule(*4*).

These partnering transcription factors then migrate to the nucleus where they either activate transcription of genes or they form heterodimers with other activated STATs allowing for greater variability in the DNA binding capability(Figure 1A)(*9*).

It has been previously reported that the N-terminus of STAT4 consists of the first 123 amino acids of the overall 748 amino acid long protein sequence (Figure 1B). The amino acids of N-terminus are said to come together and form a hook-like structure (*10, 11*). This structure has been reported as crucial in 2 key functional characteristics of STAT4 – it is needed for the IL-12 receptor mediated phosphorylation and for allowing cooperative binding to DNA sequences in association with other activated forms of STATs (*12, 13*). Recently, researchers have challenged the commonly accepted model of STAT activation by showing that STAT1 and STAT4 can form homodimers prior to activation and that the N-terminal domain is essential for the process (*14, 15*).

These findings about the function of the N-terminus demonstrate a rather prevalent issue within the STAT research field. Investigators have often taken the high level of homology among the STAT family members as an indicator of similarity in function. There is mounting evidence, as will be indicated later in this review, emphasizing the need to consider the potential existence of much greater specificity than has previously been envisioned (*16, 17*).

The C-terminal domains of all STATs contain three individually characterized segments – SH2, tyrosine activation (Y) and transcriptional activation domains (TAD)(Figure 1B). The SH2 domain of all STATs is important for allowing binding to the JAK-tyrosine (Y) phosphorylated receptor. When the respective JAK, in turn,

phosphorylates the receptor associated STAT molecule, then the SH2 domain functions in driving the reciprocal homo or hetero dimerization of the STATs. Thus, each partner docks to the other partner's phosphorylated site via their respective SH2 domains. (*18*)

In order for STAT4 to get activated, released and partnered with another STAT4 molecule it first has to be phosphorylated on the 693$^{rd}$ tyrosine residue of its protein sequence (Figure 1B)(*19*). Furthermore, STAT4, like other STAT members, can also be phosphorylated on the 721$^{st}$ serine amino acid residue via the activation of the p38/MKK pathway (Figure 1B) (*20*). This has been shown to be complementing the full transcriptional activity of activated STAT4, that is in addition to the effects of the IL-12 receptor mediated signaling pathway. (*20-23*).

The third domain that is located within the C-terminus of all STAT molecules is the transcriptional activation domain (TAD). Beyond its ostensible role in activating gene expression, it's the site of alternative splicing that leads to formation of different isoforms. By convention, the full protein structure is referred to as α, while the shorter spliced isoforms are termed β. Currently, the isoforms of STAT1,3, 4 and 5 have been sequenced and their function elucidated (*2, 7, 24-26*). The functional characteristics of STAT4β isoform have generated interesting variations in activation and function. So far the STAT4β has been shown to be phosphorylated and thus activated in response to growth hormones like estrogen (*27*). STAT4β has also been shown to differ from its alpha isoform by the number of genes that it activates. There are 29 unique genes activated by the beta isoform that are not activated by the alpha form, thus demonstrating their inherent capability of mediating IL-12 responses differently (*7*).

So far, the coiled-coil (CC) domain of STATs (Figure 1B) does not have a clearly defined functional characteristic. The evidence has pointed in the direction of it playing a role in either regulating protein half-life independent of the proteasome (*28*), interacting with other non-STAT transcription factors (*29*), mediating the receptor binding and subsequent activation (*30*), or aiding with the nuclear transport of the activated form (*31*). All of these findings have been reported after extensive investigation of either STAT 1, 2 or 3 but not STAT4.

b. Cellular and Tissue Expression of STAT4

STAT4, unlike other transcription factors such as STAT3, is expressed predominantly within the hematopoietic lineage (*32, 33*). Within the lymphoid branch of the hematopoietic cells, STAT4 is expressed in Th1, CTL and NK cells (*19, 34, 35*). Within Th1 type, STAT4 is required for the proper cellular differentiation - as the lack of either IL-12R or STAT4 results in the loss of Th1 phenotype, traditionally associated with reduced IFNγ secretion (*34-37*). Even though, the evidence has firmly established the aforementioned paradigm, researchers have also shown the presence of a STAT4 independent pathway that leads to a proper development of Th1 cells (*38*).

A significant portion of the initial research focused on the role of STAT4 in T and NK cells alone, while recent findings have shown that STAT4 is expressed in activated monocytes (activated by LPS or IFNγ), mature dendritic cells (DC), connective tissue-type mast cells, and B cells – three of which belong to the myeloid lineage (except B

cells)(*39-42*). In activated monocytes and mature DC, STAT4 is activated not by IL-12, as is the case with Th1 and NK cells, but by IFNα and other cytokines (*39, 43*).

Going beyond hematopoietic cell lineage, STAT4 expression has also been confirmed in the testis (*33*) as well as human vascular endothelial cells and human vascular smooth muscle cells (*44-47*). Researchers have made interesting headway in elucidating the mechanism by which STAT4 expression in vascular endothelial cells might guide an inflammatory response (*44*).

## 1.2. Mechanisms of STAT4 regulation

As the importance of proper cytokine signaling continues to be revealed, ever greater information surfaces about the regulatory mechanism that keeps the process under strict control. Because Th1 cells need to mount a quick response to stimuli like IL-12, different pathways must be in place to limit the impact and prevent exaggerated outcome, which is equally damaging to the propriety of the immune response.

There are two known mechanisms regulating transcriptional expression of STAT4 – epigenetic control or regulation by other transcription factors. The epigenetic control of STAT4 transcription has been linked to the DNA methylation levels of its promoter, where hypermethylation has an inhibitory role, while hypomethylation has the opposite effect (*48*). In T cells, STAT4 expression is reported to be under the control of the transcription factor Ikaros, while in DCs the expression is controlled by the activity of NF-κB and AP-1, which are also transcription factors (*43, 49*).

There are 4 known players that have been implicated in the control of STAT4 protein activity – PIASx, SLIM, SOCS3 and Hlx. Prior to explaining the function of each regulatory modulator, it is essential to point out that in order to fulfill their role as STAT4 regulators (with the exception of Hlx) there needs to be a specific post-translation modification of the STAT4 protein. The three main types of modifications that have been linked to the JAK/STAT pathway are ubiquitination, sumoylation (SUMO), and ISGylation (ISG15) (*50-52*).

Regardless of the difference in terminology, all three follow a pathway that's very similar to the traditionally established ubiquitin mediated proteasome degradation – where an E1 enzyme binds to an ubiquitin molecule and transfers it to an E2 conjugating enzyme. The substrate specificity always comes from the E3 enzyme that recognizes its target and facilitates the ubiquitin transfer from the E2 to the target. Once ubiquitinated, the target is destined for degradation through the 26S proteasome (*53*). Ubiquitination has been long implicated in the regulation of JAK/STAT pathway – as early as few years after the initial discovery and even though research has grown tremendously in this area, much more remains (*54*).

Protein-inhibitors of activated STATs (PIAS) were initially discovered at a time when the information about the importance of STAT1 signaling was rapidly growing while the knowledge of its regulation was completely missing (*55*). There are four members of the PIAS family that are currently recognized – PIAS1, PIAS3, PIASy and PIASx. Co-immunoprecipitation experiments have confirmed that three of the PIAS inhibitors have specific STAT partners – PAIS1 pairs with STAT1, PIAS3 with STAT3,

and PIASx with STAT4 (*56-58*). Even thought PIAS inhibitors possess the ability to directly modify their targets by sumoylation, the full function of PIASx has been shown to depend on the recruitment of yet unknown deacetylase (*58-60*).

SLIM (or STAT-interacting LIM domain possessing protein) is the direct link between ubiquitin-mediated proteasome degradation and the total STAT4 protein levels. It has been shown that SLIM is an ubiquitin E3 ligase that specifically marks STAT1 and STAT4 for proteasome degradation (*61*). This was further verified by the phenotype of SLIM deficient mice, which had greater levels of STAT1 and STAT4 correlating with greater IFNγ secretion (*61*). Tanaka et al have also shown that in addition to the aforementioned function, SLIM might be involved in inhibiting STAT4 tyrosine phosphorylation via recruitment of yet unidentified adaptor molecule (*61*). Furthermore, the researchers do not dismiss the idea of SLIM as a monoubiquitin modifier that bypasses the proteasome and instead serves as a localization signal (*61*). Regardless of the limited evidence in favor of STAT specific E3 ligases, evidence implicating the proteasome in regulating either total or activated STATs have been mounting (*62, 63*). All of these scenarios provide an exciting future in understanding the role of ubiquitination in JAK/STAT signaling.

Suppressors of cytokine signaling (SOCS) are the most extensively studied group of proteins that are involved in regulating JAK/STAT signaling (*64*). Their expression is typically minimal unless the cells are stimulated with specific cytokines(*65*). Increase in expression eventually leads to deactivating the cytokine stimulated signaling. There are numerous mechanisms involved by which SOCS actually suppress the JAK/STAT

pathway. They either bind directly to the respective JAK and competitively prevent STATs from binding to the receptor, or associate with specific E3 ligase in designating for destruction different components of the signaling pathway (*66-69*). Furthermore, SOCS molecules themselves can be ubiquitinated and marked for degradation thus eliminating the inhibitory signal on the JAK/STAT pathway (*70*). The signal that prompts proteasome degradation is phosphorylation of SOCS by various receptor associated kinases (JAKs) that are in turn activated by different cytokines (*71*).

Evidence has demonstrated a direct interaction between STAT4 and SOCS3 (*68*). The evidence has shown that SOCS3 binds to the IL-12R in a way that prevents the recruitment of STAT4 via its SH2 domain (*68, 72*). In the same sense, SOCS3 has been shown to be upregulated in Th2 cells (characterized by lack of IFNγ secretion and STAT4 activation) as a way of ensuring proper cellular differentiation (*72, 73*).

The Hlx transcription factor is expressed in both Th1 and NK cells. It has been found that Hlx accelerates dephosphorylation and proteasome-mediated degradation of Y-693 form of STAT4 in NK cells (*74, 75*). The finding was only consequential, meaning that the function of Hlx in NK cells is linked to the reduced form of the activated STAT4. The mechanism by which Hlx achieves this effect is yet to be elucidated. It has been suggested that since Hlx can not directly bind to the phosphorylated tyrosine on the activated form of STAT4, it likely recruits another phosphatase or displaces activated STAT4 from its target DNA sequences, which in turns exposes it to the work of phosphatases residing in the nucleus (*74*). If the information gathered from studying STAT1 is an indication of what could hold true for STAT4, it

will not be unreasonable to expect a substrate specific phosphatase to also be involved in the regulatory process (*76, 77*). Furthermore, the effect of Hlx on NK cells needs to be reconciled with its opposite effects in Th1 cells where it is involved in inducing IFNγ secretion which demands activation of STAT4 (*74, 78*)

### 1.3. STAT4 Activation and Signal Transduction

Prior to activation of the latent STAT4 found within the cytoplasm of CTL, Th1, and NK cells, there are a couple of important trigger events involving a ligand stimulation and kinase-mediated receptor activation.

First, the cytokine IL-12 binds to its heterodimeric transmembrane receptor that consists of two chains – β1 and β2 (*79-81*). Because IL-12R belongs to the cytokine family of receptors it does not have an inherent enzymatic activity. Instead, upon ligation with IL-12, it undergoes a conformational change which allows the recruitment of the receptor associated kinases Tyk2 and Jak2. IL-12 β1 interacts with Tyk2 while IL-12R β2 interacts with Jak2 (*82-84*). Both receptor associated kinases undergo autophosphorylation, which is followed by the phosphorylation of specific tyrosine residues on the respective chains of the IL-12 receptor (*85-87*). In humans, Jak2 phosphorylates the tyrosine that is the 800$^{th}$ amino acid in the IL-12Rβ2 subunit which becomes the docking site for STAT4 (*88*). In mice, the scenario is slightly more complicated due to the presence of additional number of tyrosines within the amino acid sequence of the IL-12Rβ2 receptor – all of which seem to bind equally well to STAT4 (*89*).

Once STAT4 binds to the phosphorylated IL-12R, it in turn becomes phosphorylated by JAK2 (*82*). These events lead to the release of STAT4, to the subsequent homo- or heterodimerization and the eventual nuclear import where STAT4 finally fulfils its function as an activator of cellular transcriptional activity (*4*). It is interesting that proliferative abilities of lymphocytes have been explained by STAT4's function as a modulator of the cyclin dependent kinase inhibitor $p27^{Kip1}$expression (*90*).

In addition to IL-12, STAT4 –in NK and T cells, can be activated by several other cytokines, namely IFNα, IL-2, IL-23, IL-21, IL-15, IL-18 in both mice and humans, while IL-4 can activate STAT4 in mouse NK cells(*91-95*). The activation can be achieved independently by stimulation with a single cytokine – such as IL-2 alone, IFNα alone etc, or it can be achieved synergistically where the simultaneous presence of two cytokines – such as IL-21 and IL-15, is required for full activation.

### 1.4. STAT4 Translocation

The paucity of information regarding the mechanism of nuclear localization of STAT4 is interesting. A lot of research has been done on the mechanism of STAT1(*17*) and to a large extent of STAT2 and 3 (*96*), while there is only one report that has delineated a selectively enhanced nuclear translocation of STAT4 but the pathway is yet to be worked out (*97*). The initial presumption was that understanding the mechanism of STAT1 should be enough to help us understand the mechanism of all other STAT family members. As Reich et al has clearly pointed out in their review of STAT nuclear trafficking, the uniqueness of each STAT calls for a lot greater level of research (*98*).

Even though what we know about the nuclear localization of STAT1 might not necessarily apply to STAT4, for the purpose of understanding the general process, here is a minimal outline of the STAT1 nuclear localization steps.

First, the phosphorylated form of STAT1 has been discovered to bind to a specific nuclear importer protein – importin α5 (*99, 100*). The target of STAT1 has been shown to be one particular amino acid in the protein sequence of importinα5, as a mutation in that particular amino acids was sufficient to abrogate its nuclear import (*101*). Once inside the nucleus, STAT1 remains associated with importinα5 until STAT1 binds to its designated DNA sequences, leading to the release importinα5(*98*).

Research has identified the nuclear phosphatase TC45 as the main agent responsible for the deactivation and the recycling of STAT1 back to the cytoplasm (*76, 77*). It will be incorrect to infer based on these studies, that only the tyrosine phosphorylated form of STAT1 gets localized in the nucleus. It has been shown that there are two pathways of STAT nuclear localization– phosphorylation dependent and independent pathways (*102, 103*). The importance and the potential utilization of these differential pathways for therapeutic purposes has not been explored but do provide novel directions.

## 1.5. Mechanism of Gene Regulation by STAT4

The most cutting edge research on STAT4 is currently focused on the events within the nucleus, namely the transcriptional changes directly associated with the activated form of STAT4.

In order to bring about changes in cellular differentiation STAT4 has been shown to specifically bind in excess of 4000 genes (*104*). As the relative importance of each target gene is yet to be studied in depth, it is worth mentioning some of STAT4's well established gene targets within the context of T cell differentiation, namely IFNγ (*105-107*), IL18r1 (*108*), IL-12Ra (*109*), IL-12Rβ2 (*110*), Tpl2 (*111*) and Furin (*112*).

Newer techniques such as chromatin immunoprecipitation that's followed by microarray analysis (also referred to as ChiP-on-chip) and large scale sequencing of factors bound to transcription factors (also referred to as ChiP-seq) are going to provide further insight into the complexity of the STAT4 mediated T cell differentiation process (*113*).

The two main areas that are receiving the most attention in studying STAT4's ability to regulate gene expression are the histone acetylation and DNA methylation profiling during Th1 differentiation – partly a consequence of the advancement in the field of epigenetics. Since a digression into an explanation of this rapidly growing field of epigenetics, will distract from the main focus of this paper, several great reviews on the histone code, DNA methylation and the epigenetics of Th1 differentiation are included herein as background information (*114-118*).

In short, histones are small proteins, found in the nucleus, that form tight complexes with DNA called nucleosomes. Depending on the positioning and binding interaction of the histones with the DNA, they could either promote or inhibit the transcription of various genes. Furthermore, evidence has shown a plethora of post-translational modifications of the histones such as acetylation, methylation, and

ubiquitination. Histone methylation is rapidly being evaluated as the means by which a cell can reprogram its function and pass it to the next generation without having to make permanent changes to its actual DNA code.

Considering the enormity of the task of having to re-model all nucleosomes during cellular differentiation, STAT4 has been deservedly implicated in promoting epigenetic markers that favor gene transcription (*104*). Furthermore, STAT4 has been shown to interact with several important players for modulating gene transcription like Brg1, Dnmt3a and CREB-binding protein (*119-121*).

Brg1 is a member of the mammalian nucleosome remodeling complex which has the ability of activating or inhibiting gene expression in association with a host of associated-factors. It has been shown that the activity of Brg1 is dependent on STAT4 during Th1 differentiation (*119*). In a mechanism similar to Brg1, the histone acetyltransferase CREB-binding protein has also been implicated in STAT4 driven gene expression, where the N-terminal domain of STAT4 is important for the interaction (*120*).

Another way for STAT4 to mediate the expression of its target genes involves inhibiting the recruitment of the DNA methyltransferase – Dnmt3a. The absence of Dnmt3a prevents methylation of the DNA, relaxes the nucleosomes and allows for greater transcription (*121*).

Lastly, it is important to acknowledge, when attempting to understand the processes involved in activation and inhibition of transcription, that the mere binding or the strength of binding by STAT4 to a target gene does not automatically mean greater

level of gene expression (*113*). Indubitably, a great deal of mystery remains around epigenetic means of cellular differentiation but major breakthroughs have already made a good deal of progress.

## 1.6. Roles of STAT4 in Diseases

Due to its role as a primary driving force of Th1 differentiation, STAT4 has been implicated in a variety of inflammatory and autoimmune diseases.

The most therapeutically promising aspect of the STAT4 signaling pathway is its potential role in cancer immunotherapy (*122, 123*). Interleukin 12 (IL-12), as the primary activator of latent STAT4, has long been proven to cause tumor death in a variety of murine models such as melanomas, sarcomas and mammary, colon and renal carcinomas (*124*). Its antitumor effects are the main consequence of activating the primary mediator of IFNγ secretion – namely STAT4 (*125, 126*).

Some of the reasons why STAT4 mediated IFNγ secretion is vital for the IL-12 induced anti-tumor immunity is because endogenous IFNγ has been shown to induce apoptosis in tumor cells and bacterially infected monocytes (*127, 128*), to enhance MHCI and MHCII expression, and to augment CTL and NK cell cytotoxicity (*129, 130*). Even though, direct IL-12 administration has been proven to have extensive toxicity and diminished effects upon repetitive treatments, it is still a promising pathway in designing novel strategies for cancer immunotherapy (*131, 132*).

One of the autoimmune diseases in the pathogenesis of which STAT4 has been implicated is multiple sclerosis (MS). The mouse model that is used in studying the disease is called experimental autoimmune encephalomyelitis (EAE) (*133*). Lovastatin, a

member of a class of drugs called statins, was initially reported to have anti-inflammatory effects that ameliorate the symptoms of EAE (*134*). Lovastatin, among other things, has been found to reduce phosphorylation of STAT4 by inhibiting the function of the upstream activating enzymes – Jak2 and Tyk2 (*135*). More recent findings has shown that the onset and the severity of the disease vary according to which STAT4 isoform is present in the mouse model (*136*).

In addition to lovastatin, there several other drugs that have a reportedly inhibitory role on the activation of STAT4 in different disease settings. For example, the drug lisofylline has been found to inhibit STAT4 activation and thus reduce β-cell destruction in non-obese diabetic mice – where the importance of STAT4 has been previously established (*137*). Other drugs that have inhibitory role on STAT4 activation are thiols, curcumin, and rapamycin (*89*).

STAT4 also plays a regulatory role in rheumatoid arthritis (RA) and systemic lupus erythematosus (SLE) (*138-140*). These findings have brought an interesting immunological question – how do Th1 cells participate in antibody-mediated diseases? Studies are yet to elucidate the exact mechanism, but the secret may rest with the function of the T follicular helper cells (Tfh).

Tfh cells reside in the germinal centers of secondary lymphoid organs where B cells undergo isotype switching, somatic hypermutation and differentiation into antibody secreting plasma cells. It has been reported that the differentiation of Tfh cells is dependent on IL-12 secretion by DCs which are resident in the germinal centers (*141*). Thus IL-12/STAT4 axis is critical in the proper differentiation of Tfh cells and

consequently important for the B cell growth, differentiation and antibody production. Further insight into the STAT4-mediated Tfh cell differentiation could be crucial in understanding various autoimmune diseases.

In addition to RA and SLE, STAT4 has been implicated in the pathology of a variety of other diseases. It is important in clearing the intracellular protozoan parasite *Toxoplasma gondii* (*111, 142*), *Leishmania (L.)mexicana* and its relative *Leishmania (L.) major* (*143*), the intracellular *Mycobacterium tuberculosis* and *Mycobacterium avium* (*144, 145*). STAT4 activation has been ascribed an active role in sustaining chronic intestinal inflammation (*146, 147*) as well as mediating IL-13 driven murine asthma model (*148*).

Recently, STAT4 has also been identified as a risk factor for systemic sclerosis but more concrete details about how it contributes to sclerosis are to be investigated (*149, 150*). Furthermore, the development of arteriosclerosis in cardiac transplant recipients has been attributed to up-regulated activation of STAT4 and IFNγ (*151-153*). It has been suggested that the elevated levels of these Th1 associated factors, could be due to defective TGFβ secretion (*154, 155*).

In viral immunity, it has been shown that STAT4 is important in limiting the replication of coxsackievirus B3 (CVB3), which leads to the development of myocarditis (*156, 157*). Therefore, scientists are encouraged, when devising anti-inflammatory treatment plans that target lowering Th1 response, to consider the dangers of increased chronic inflammatory heart disease as a side effect.

## 1.7. Summary

The role of STAT4 in IL-12 mediated secretion of IFNγ by Th1, CTL and NK cells has been firmly established. The evidence in support of STAT4's importance in mounting a proper immune response towards pathogens and tumors continues to increase. Significant information about the molecular structure and expression of STAT4 has already been compiled and it points to a much greater level of specificity among various members of the STAT family than was previously ascribed. The means by which STAT4 is involved in re-programming and committing cell differentiation to the Th1 lineage is an exciting and rapidly growing field. Understanding the mechanisms involved would provide new tools for designing treatments for a variety of human diseases.

# CHAPTER 2: MATERIALS AND METHODS

## 2.1. Blood Samples, Cell Cultures, and Cell Lines

Collection of blood samples was approved by the Institutional Review Board at Indiana University Medical Center and written informed consent was obtained from each study subject. Blood samples were obtained from patients with Hodgkin's or non-Hodgkin's lymphoma before and after treatment with standard chemotherapy or high-dose chemotherapy and autologous PBSCT. Standard dose chemotherapy regimens included rituximab, cyclophosphamide, vincristine, and prednisone with (R-CHOP) or without (R-CVP) doxorubicin. High-dose chemotherapy regimens included cyclophosphamide, carmustine, and etoposide (CBV) and carmustine, etoposide, cytarabine, and melphalan (BEAM). Control PBMCs were obtained from healthy volunteer donors. Aliquots of PBMCs were cryopreserved in liquid nitrogen. NKL, a human NK cell line, was grown in culture as previously described (*158*). Activated PBMCs were obtained by culturing PBMCs in medium containing PHA (2.5 μg/mL) and IL-2 (50 U/mL) for 3 days in a 5% $CO_2$ incubator at $37^oC$. Activated PBMCs and NKL cells were incubated in medium with or without chemotherapeutic agents for 2-3 days. For some experiments, NKL cells were incubated with or without 5.2 nM bortezomib simultaneously with the addition of corresponding chemotherapeutic drugs for 2 days. In

other experiments, activated PBMCs were incubated with or without 5-aza-dC (2.5 ng/mL) for 1 day after incubation with or without chemotherapeutic agents.

### 2.2. Cytokines, Antibodies, Chemotherapy Drugs, and Other Reagents

Recombinant human IL-2 was obtained from Chiron (Emeryville, CA) and recombinant human IL-12 from PeproTech (Rocky Hill, NJ). Fluorochrome-conjugated monoclonal antibodies recognizing human CD4 and CD8, Alexa 647-conjugated streptavidin, and anti-STAT4 monoclonal antibody for immunoblot were obtained from BD Biosciences (San Jose, CA). Biotin-labeled anti-STAT4 antibody was purchased from R&D Systems (Minneapolis, MN), anti-ubiquitin monoclonal (SC-130410) and anti-STAT4 polyclonal (SC-486) antibodies from Santa Cruz Biotechnology (Santa Cruz, CA). Protein A agarose beads were obtained from Millipore (Temecula, CA). Bortezomib was obtained from Millennium Pharmaceuticals (Cambridge, MA). Phytohaemagglutinin (PHA), actinomycin D, cycloheximide, MG132, 5-aza-2'-deoxycytidine (5-aza-dC), prednisolone, cyclophosphamide, doxycycline, cisplatin, carmustine, and etoposide were purchased from Sigma-Aldrich (St. Louis, MO).

### 2.3. Analysis of STAT4 Protein and RNA Levels

STAT4 mRNA and protein levels (see also Section 2.9) were analyzed using real time PCR and immunoblotting analysis, respectively (*159, 160*). For flow cytometric analysis of STAT4 protein levels, cells were surface stained with CD4-FITC and CD8-PE, washed, fixed with 4% paraformaldehyde, permeabilized with 0.1% saponin, and

stained with a biotin labeled anti-STAT4 antibody followed by Alexa 647-conjugated streptavidin. Histogram represents the STAT4 expression gated on 5000 events of live CD4 or CD8 positive cells using the WinMDI software.

### 2.4. Analysis of Differential Cell Type Proliferation in Response to In vitro Stimulation

PBMCs from four healthy individuals were stimulated in vitro with IL-2 (50U/mL) and PHA (2.5µg/mL) for a period of 3 days. Subsequently, the cells were surface stained with either CD3-APC, CD4-FITC and CD8-PE or CD3-APC,CD56-PE and CD16-FITC. The results were compared to the surface staining of unstimulated cells from the same individuals. Results were gated on 10,000 live events and the data is presented as the percentage of each cell type among all events.

### 2.5. Assessment of *STAT4* mRNA and Protein Half-life

Lymphoma patient or control subject PBMCs were incubated with or without actinomycin D at 1 µg/mL for 0, 2, 4, and 6 hours in a 5% $CO_2$ incubator at $37^oC$. RNA was extracted and the first-strand cDNA was synthesized followed by real time PCR (*161*). The half-life of *STAT4* mRNA from each sample was calculated accordingly (*161*).

NKL cells were treated without or with carmustine or etoposide for 2-3 days. Cells were incubated with cycloheximide at 80 µg/mL for 0, 2, 4, 6, 8, and 18 hours. The amount of STAT4 and GAPDH protein were determined using Western blot analysis. STAT4 protein levels were normalized to GAPDH, and the half-life was calculated (*162*).

## 2.6. Immunoprecipitation and Analysis of Ubiquitin-conjugated STAT4 Protein

NKL cells were incubated with or without carmustine and etoposide for 2 days. Total protein lysates were extracted as described (*107*). MG132 at concentration of 20 μM was added to the cell lysis buffer (already containing protease inhibitors) to stall the activity of proteasome and prevent the further degradation of protein (*163*). Total protein extracts (2 mg) were incubated for 4 hrs at 4$^{o}$C with slow rotation with anti-ubiquitin monoclonal antibody from Santa Cruz Biotechnology (Santa Cruz, CA). Protein A agarose beads were added and incubated for another 2 hours at the same condition. The immunoprecipitated proteins were analyzed using western blot with anti-STAT4 polyclonal (SC-486) antibodies from Santa Cruz Biotechnology (Santa Cruz, CA).

## 2.7. Evaluation of IFN-γ Production

Cells treated with carmustine or etoposide were incubated for 24 hours in medium alone or medium containing IL-12. Supernatant IFN-γ protein levels were measured using ELISA as previously described (*159, 160*).

## 2.8. Gene Expression of STAT4 Isoforms

PBMCs from healthy individuals were stimulated with IL-2 (50U/mL) and PHA (2.5μg/mL) for 3 days. Cell pellets were collected and RNA was extracted by Trizol. The samples were separated in 2% agarose – from Sigma-Aldrich (St. Louis, MO), and visualized with ethidium bromide – from Genesee Scientific (San Diego, CA). See

Appendix B and C for the exact sequence of the primers used to detect STAT4α and STAT4β isoforms.

## 2.9. STAT4 Expression in Murine NK Cells

The mice for each STAT4 isoform are in Stat4$^{-/-}$ background of C57BL/6, the generation of which has been previously explained (*34*). The cDNA for each isoform was cloned within the CD2/LCR promoter (*7, 12*). The spleen from each mouse was obtained and the red blood cells were lysed in RBC Lysis buffer for 5min.The NK cells were magnetically separated (positive isolation) using CD49b (DX5) MicroBeads (Miltenyi Biotec Inc., Auburn, CA) according to the manufacturer's instructions.

Protein analysis: Isolated NK cells were washed in 1x Phosphate Buffered Saline and re-suspended in cold Igepal protein lysis buffer containing protease and phosphatase inhibitors (DTT, pepstatin, aprotonin, benzamidine, leupeptin, iodoacetimide, AEBSF, sodium vanidate, beta-glycerol, and sodium flouride). After 15min incubation, the solution was centrifuged (14K RPM for 10min), protein was collected and the concentration was measured using BIORAD Protein Assay kit based on the Bradford method. From each sample – 10μg were separated on 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE). The protein bands were electrophoretically transferred (at 200mV overnight) onto a nitrocellulose membrane (Whatman GMbH). The membranes were blocked for 1 hour in 3% by weight of dry non-fat milk in phosphate buffered saline (PBS). Primary monoclonal STAT4 antibody (BD Biosciences) was added (2μg/mL)and membrane was left to incubate at 4ºC overnight.

The membrane was washed with 1xPBS+Tween20 solution 3 times for 10 min, and the secondary antibody – peroxidase–conjugated goat anti-mouse immunoglobulin G (1:3000), was added in 3% by weight of dry non-fat milk in PBS for 2 hours. The nitrocellulose was washed again with PBS+Tween20 3 times and the bands were visualized with a Western Lightning Chemiluminescence Reagent Plus (Perkin Elmer, Wellesley,MA).

### 2.10. Statistical Analysis

P-values were determined using the PASW Statistics (IBM-SPSS, Chicago, IL) with a two-sided test and a p-value $\leq 0.05$ was considered statistically significant.

# CHAPTER 3.ACQUIRED STAT4 DEFICIENCY AS A CONSEQUENCE OF CANCER CHEMOTHERAPY

## 3.1. Introduction

Signal Transducer and Activator of Transcription 4 (STAT4) is required for IL-12 mediated biological functions including the differentiation of T helper type I (Th1) cells and IFN-γ production (*34, 35, 159*). IL-12 has potent antitumor activity in preclinical models (*122, 164-166*). IL-12-mediated antitumor effects are dependent on the production of IFN-γ (*125, 126*). In a Th1-mediated inflammatory environment, IFNγ has pleiotropic effects such as promoting antitumor immunity and antimicrobial activity. IFN-γ induces apoptosis in tumor cells and bacterially infected monocytes (*127, 128*), enhances major histocompatibility class I and II expression, and augments cytotoxic T lymphocyte (CTL) and NK cell cytotoxicity (*129, 130*)

In the context of IL-12-based immunotherapy, it was observed that IFN-γ production in vivo was markedly defective in patients with lymphoma who had undergone autologous peripheral blood stem cell transplantation (PBSCT). Moreover, PBMCs obtained from patients after PBSCT were profoundly deficient in IFN-γ production after direct stimulation with IL-12 in vitro. We have subsequently shown that defective IFN-γ production in this setting is due to a selective deficiency in STAT4 (*159, 160*). STAT4 deficiency may impair not only IL-12-based immunotherapy, but any

therapeutic approach that requires Th1 immunity or optimal production of IFN-γ. The molecular mechanisms responsible for the observed deficiency of STAT4 are not known.

Unlike several other STAT proteins (e.g., STAT1 and STAT3), which appear to be constitutively expressed in many tissues, STAT4 expression is mostly restricted to hematopoietic cells (*32, 33*). STAT4 is weakly expressed by resting T cells and is upregulated after T cell activation (*19*). STAT4 expression is maintained during development of Th1 cells, but is down-regulated during development of Th2 cells (*37*). Human NK cells constitutively express STAT4, but STAT4 protein levels can be increased or decreased in NK cells after cytokine stimulation (*167, 168*). Despite abundant evidence that STAT4 expression is subject to tight regulation, the mechanisms that control STAT4 expression in physiologic or pathologic conditions have not been well characterized. Previous studies indicate that transcriptional silencing of the STAT4 gene due to hypermethylation of its promoter region and proteasome-dependent degradation of STAT4 protein can decrease STAT4 expression in human lymphocytes (*48, 63*). We have undertaken studies to elucidate the mechanisms of STAT4 deficiency in patients with lymphoma.

## 3.2. Results

### 3.2.1.STAT4 Deficiency is a Consequence of Chemotherapy Treatment and is Not Due to Lymphoma Tumor Burden

We have previously demonstrated that STAT4 protein levels are decreased in PBMCs obtained from lymphoma patients after PBSCT (*160*). The observed STAT4 deficiency could be due to the tumor-bearing state per se or could occur as a consequence of the therapy for lymphoma. To address this question, STAT4 protein levels were analyzed in PBMCs obtained from patients with active lymphoma who had not received any therapy. Levels of STAT4 protein in PBMCs of untreated lymphoma patients were not significantly different ($P>0.05$) from those in PBMCs of healthy controls after bands were normalized to β-actin levels (Figures 2 and 4B). Therefore, the presence of tumor burden does not result in STAT4 deficiency in patients with lymphoma.

We hypothesized that STAT4 deficiency is caused by the chemotherapy used to treat lymphoma. To test this hypothesis, STAT4 levels were analyzed in PBMCs collected from lymphoma patients before and after they had received the initial chemotherapy used to treat their lymphoma. STAT4 protein levels were decreased after standard dose chemotherapy treatment compared to pre-treatment levels (Figures 3 and 4B). This result indicates that chemotherapy exposure contributes to STAT4 deficiency in the PBMCs of lymphoma patients.

We next wanted to evaluate whether or not the type of chemotherapy regimen correlates with the degree of reduction in STAT4 expression. PBMCs were obtained from

4 patients who underwent high dose chemotherapy and PBSCT. The reduction in STAT4 protein levels was more severe in patient samples after high-dose chemotherapy (Figure 4).

### 3.2.2. Acquired STAT4 Deficiency in Normal PBMCs Treated In vitro With Chemotherapy Drugs

To directly test our hypothesis that chemotherapy drugs cause STAT4 deficiency, PBMCs obtained from normal healthy control subjects were incubated in vitro with chemotherapeutic agents. Carmustine and etoposide were used in our experiments, as these agents are commonly included in the high-dose chemotherapy regimens that are associated with profound STAT4 deficiency after PBSCT. The levels of STAT4 protein detected in activated PBMCs incubated in vitro with etoposide or carmustine were significantly decreased in comparison to levels detected in cells cultured in medium alone(Figure 5A). In contrast, the levels of STAT3 protein were not affected by the presence of chemotherapy drugs in both – patient samples and in vitro treatment with chemotherapeutic drugs (Figures 3, 4A and 5A). The levels of STAT4 mRNA were also diminished in activated PBMCs treated in vitro with etoposide or carmustine (Figure 5B).The reduced STAT4 protein levels in treated PBMCs were detected by immunoblotting of whole cell lysates. To confirm the relevance of the in vitro experiments on the STAT4 levels after incubation with high dose related agents, wild type mice weresubjected to treatment with either DTIC or Etoposide (Figure 6). Indeed,

the similarity in magnitude of STAT4 reduction further substantiates the contribution of these agents in the acquired deficiency among post treatment patients with lymphoma.

Even though the in vitro studies and mice treatments make a strong case for the chemotherapeutic treatment as the causative agent of STAT4 deficiency, these studies cannot elucidate whether the deficiency is more or less severe in particular subsets of lymphocytes. To address this question, the effect of chemotherapy drugs on STAT4 expression in different cell types was analyzed by flow cytometry. STAT4 protein levels were reduced in both CD4+ (Th1) and CD8+ T (CTL) cell subsets after chemotherapy exposure (Figure 7).

Prior to flow cytometric analysis, PBMCs from healthy individuals was stimulated with the growth promoting cytokines – IL-2 and PHA. Stimulation was deemed necessary to mimic the in vivo conditions among lymphoma patients, where chemotherapy reduces overall lymphocyte counts demanding greater proliferation as a way of counteracting the reduction. Because NK cells are not responsive to PHA and IL-2 is not sufficient to provide a strong proliferation signal to this cell type, the presence of NK cells within stimulated PBMCs had to be evaluated (Figure 8). Flow cytometric analysis showed that indeed IL-2 and PHA had little impact on the proliferation of NK cells which was demonstrated by their reduced numbers as a percentage of the total cells analyzed (Figure 8).

Therefore, the effect of chemotherapeutic agents on STAT4 expression by NK cells was examined using a human NK cell line, NKL (*158*). Western blot analysis

demonstrated reduced STAT4 protein levels in NKL cells treated with carmustine or etoposide for 2 days (Figure 9A).

To determine the potential functional consequences of chemotherapy-induced STAT4 deficiency, we measured IFN-γ production by NK cells incubated with or without chemotherapy drugs. The levels of IFN-γ secreted by IL-12-stimulated NKL cells were significantly lower in the presence of either carmustine or etoposide (Figure 9B). Thus, the partial reduction of STAT4 protein levels in NK cells exposed to chemotherapy drugs is associated with impaired IFN-γ production after cytokine stimulation.

### 3.2.3. *STAT4* mRNA Stability is Not Affected by Chemotherapy

Our results indicate that both mRNA and protein levels of STAT4 were diminished in cells exposed to chemotherapy drugs. The reduction in *STAT4* mRNA levels could be due to decreased transcription of the *STAT4* gene and/or decreased stability of the *STAT4* mRNA. We measured the half-life of *STAT4* mRNA in PBMCs obtained from control subjects or chemotherapy-treated lymphoma patients. The mean half-life of *STAT4* mRNA from lymphoma patient PBMCs obtained after PBSCT (3.967 hours) was not significantly different (P=0.13) than that of control subject PBMCs (2.797 hours; Figure 10B). Thus, although steady-state levels of *STAT4* mRNA are decreased after chemotherapy treatment of lymphoma patients (Figure 10A) (*160*), the *STAT4* mRNA stability was not affected by high-dose chemotherapy.

It has been shown that DNA methylation in the proximal promoter region plays a direct role in the regulation of *STAT4* transcriptional activity (*48*). To determine whether

DNA methylation-related chromatin remodeling is involved in decreased *STAT4* transcripts after chemotherapy exposure, we used 5-aza-dC to inhibit de novo DNA methylation in both normal PBMCs and NKL cells treated with or without chemotherapeutic drugs. Despite increases in *STAT4* gene expression following 5-aza-dC treatment of cells incubated with carmustine or etoposide, the total level of STAT4 protein is minimally rescued upon 5-aza-dC treatment (Figures 11A and 11B).

### 3.2.4. Ubiquitin-mediated Proteasomal Degradation of STAT4 in Chemotherapy-treated Cells

Our results suggest that, despite the decreased *STAT4* mRNA levels we observed in lymphoma patients, the transcription based mechanism of regulating STAT4 expression post-chemotherapy treatment was unaffected as evidenced by the rescued levels of STAT4 mRNA after 5-aza-dC treatment. Since these rescued levels never translated into restored protein levels, we hypothesized that reduced stability of the STAT4 protein is the dominant mechanism of chemotherapy-induced STAT4 deficiency. To test this hypothesis, we measured the half-life of STAT4 protein in human NK cells cultured in the presence or absence of chemotherapy drugs. Consistent with this hypothesis, the half-life of STAT4 protein was found to be significantly reduced in NKL cells treated with carmustine or etoposide (Figures 12A and 12B).

Ubiquitin-mediated proteasomal degradation has been implicated in the regulation of tyrosine phosphorylated (*7*) as well as total STAT4(*61*). To evaluate the role of the ubiquitin-mediated degradation pathway in regulation of STAT4 protein, the levels of

ubiquitinated STAT4 protein were determined in NKL cells treated without or with carmustine and etoposide. The ratios between ubiquitin-conjugated STAT4 and total STAT4 are substantially higher in cells treated with chemotherapy drugs as compared to those incubated in medium alone (Figure 13).

To test the hypothesis that chemotherapy-induced reduction in STAT4 protein stability is due to proteasomal degradation, human NK cells were incubated with or without the proteasome inhibitor bortezomib in the presence of either carmustine or etoposide. The magnitude of the decrease in STAT4 protein levels following chemotherapy exposure was greatly reduced in the presence as compared to the absence of bortezomib (Figures 14A, 14B, and 9A). This result confirms that the proteasome pathway is involved in STAT4 protein degradation after chemotherapy exposure.

We next evaluated whether restored STAT4 protein by bortezomib will circumvent the previously observed defective IFN-γ production by NK cells treated with carmustine or etoposide. The production of IFN-γ was determined following IL-12 stimulation for 1 day. Results showed that bortezomib was capable of rescuing IFN-γ production to the levels produced by cells without receiving any chemotherapy (Figure 15). This further supports the importance of STAT4 in IL-12 mediated IFN-γ production. In addition, circumventing STAT4 deficiency by Bortezomib has the potential for enhancing the efficacy of IL-12 immunotherapy as well as any therapeutic regimen that requires Th1 immunity for production of IFN-γ.

## 3.3. Discussion

We have previously demonstrated that STAT4 deficiency contributes to impaired IFN-γ production in lymphoma patients after autologous PBSCT (*160*). However, the mechanisms responsible for this STAT4 deficiency have not been previously elucidated. In this study, we have shown that STAT4 protein levels are normal in PBMCs obtained from treatment-naïve patients with active lymphoma. These results confirm that STAT4 deficiency in patients is acquired and not an inherited condition that predisposes them to develop lymphoma. Furthermore, these data refute the possibility that acquired STAT4 deficiency is due to the lymphoma-bearing state. Thus, the mechanism of STAT4 deficiency presumably differs from that of the acquired ζ chain deficiency previously identified in lymphocytes of patients with advanced cancer(*169*).

In comparison to the levels seen in PBMCs obtained from lymphoma patients before treatment, STAT4 protein levels were significantly diminished in PBMCs obtained after conventional standard dose chemotherapy. Moreover, STAT4 protein levels were even more strikingly reduced in PBMCs obtained from lymphoma patients after high-dose chemotherapy and autologous PBSCT. Consistent with this result, STAT4 protein levels declined significantly in normal activated PBMCs and wild type treated mice after exposure to chemotherapy drugs used in high-dose regimen in vitro (Figures 5 and 6). These findings suggest that acquired STAT4 deficiency in lymphoma patients is due to cytotoxic chemotherapy and that the severity of the deficiency is directly related to the type of chemotherapy regimen. In contrast to STAT4, levels of STAT3 (Figures 3, 4A and 5A) and STAT1 do not change significantly in chemotherapy-treated cells.

Therefore, the decrease in STAT4 expression is selective and cannot be ascribed to a generalized condition of cellular stress or cytotoxic damage induced by chemotherapy drugs.

Chemotherapy-induced STAT4 deficiency was seen in multiple lymphocyte populations, including CD4 T cells, CD8 T cells, and NK cells. These results suggest that anti-tumor immune responses may be impaired after treatment with systemic chemotherapy. STAT4 protein levels were significantly diminished but generally still detectable in cells exposed to chemotherapy drugs in vitro or in vivo. Nevertheless, the degree of STAT4 deficiency caused by chemotherapy is sufficient to profoundly inhibit IL-12-induced IFN-γ production (Figure 9B). Therefore, the partial STAT4 deficiency occurring after chemotherapy is likely to be functionally relevant.

Chemotherapy-induced STAT4 deficiency could be due to reduced transcript levels of the *STAT4* gene, reduced stability of *STAT4* mRNA, impaired translation of *STAT4* mRNA into protein, or post-translational modifications of STAT4 protein that reduce its stability. We found that *STAT4* mRNA levels were reduced in PBMCs of lymphoma patients after PBSCT even though *STAT4* mRNA stability did not appear to be affected (Figures 10A and 10B). This result prompted the hypothesis that chemotherapy causes epigenetic modifications of the *STAT4* gene that inhibit its transcription. Hypermethylation of CpG islands located 3’ to the core promoter of the *STAT4* gene have been shown to inhibit its promoter activity (*48*). We therefore examined the effect of the DNA methyltransferase inhibitor 5-aza-dC on STAT4 expression in chemotherapy-treated cells. Compatible with our hypothesis, *STAT4* mRNA levels were significantly

higher in cells incubated with carmustine or etoposide plus 5-aza-dC compared to levels in cells incubated in carmustine or etoposide alone (Figure 11A). However, STAT4 protein levels were only minimally increased after 5-aza-dC treatment (Figure 11B). Therefore, post-translational regulation of STAT4 protein levels may be the dominant mechanism of STAT4 deficiency after chemotherapy treatment.

Indeed, we found that the half-life of STAT4 protein was significantly reduced in cells exposed to chemotherapy drugs (Figures 12A and 12B), suggesting that degradation of STAT4 protein is enhanced by chemotherapy. Ubiquitin-mediated proteasomal degradation has been implicated in the regulation of tyrosine-phosphorylated STAT4 as well as total STAT4 (*7, 61*). Furthermore, the H2.0-like homeobox 1 protein (HLX1) promotes proteasome-dependent STAT4 down-regulation in NK cells after IL-12 stimulation (*74*). We observed increased ubiquitination of STAT4 in cells treated with carmustine or etoposide, suggesting that chemotherapy drugs reduce the stability of STAT4 protein at least in part by promoting proteasomal degradation of STAT4. Compatible with this hypothesis, we found that the proteasome inhibitor bortezomib can substantially rescue STAT4 protein in cells exposed to chemotherapy drugs (Figures 14A and 14B). Bortezomib is used for the treatment of relapsed or refractory mantle cell lymphoma patients (*170*)and can be safely given after autologous PBSCT(*171*). Therefore, it is clinically feasible to administer bortezomib also to non-Hodgkin's lymphoma patients in attempts to ameliorate chemotherapy-induced STAT4 deficiency.

The mechanism by which chemotherapy drugs target STAT4 for ubiquitination and proteasomal degradation remains to be identified. It has been shown that STAT-

interacting LIM protein (SLIM) promotes ubiquitination and degradation of STAT4 in mice (*61*). However, SLIM also promotes the proteasomal degradation of STAT1 as well as STAT4. In contrast to STAT4, we have found that STAT1 levels are not reduced in PBMCs obtained from patients after PBSCT or in normal activated PBMCs treated with chemotherapy drugs in vitro. Thus, it is not likely that SLIM mediates the acquired STAT4 deficiency we have demonstrated.

Becknell et al. have reported that cycloheximide treatment leads to increased STAT4 protein levels in the human NK cell line NK-92(*74*). Similarly we have observed that the levels of STAT4 protein are increased in PBMCs treated with cycloheximide for less than 3 hours. These results suggest that a labile protein can suppress STAT4 protein levels in human cells. We speculate that chemotherapy drugs can induce the expression of a labile ubiquitin E3 ligase that participates in the selective ubiquitination of STAT4 protein, leading to its proteasomal degradation. Further investigation is necessary to test this hypothesis.

Considering the reports detailing the differential structure and function of the β isoform STAT4 (*7*), we ventured to explore whether STAT4β might have a distinct role during and post chemotherapeutic treatment. For that purpose, we obtained three kinds of transgenic mice – $STAT4^{-/-}$, STAT4α and STAT4β. We magnetically separated the NK cells and probed for STAT4 after in vitro IL-12 stimulation. Unfortunately, no STAT4 expression was found in these NK cells (Figure A1) nor was any IFNγ secreted as was indicated by ELISA (data not shown). The most likely explanation is that the CD2/LCR promoter does not drive the expression of the inserted cDNA in the NK cell lineage. This

is further supported by the presence of STAT4αin cells from the spleen of STAT4α transgenic mice (Figure A1).

In order to further our ability of detecting STAT4 isoforms, we used primers that distinguish between each type in analyzing cDNA from healthy PBMCs (Figures A2, B1, C1). Further studies are needed in elucidating the extent of its expression and any variance in stability that might allow it to assume dominant function over the α isoform in stress conditions such as those induced by high dose chemotherapy.

Systemic combination chemotherapy, with or without rituximab, is currently the mainstay of initial treatment for patients with lymphoma. Moreover, high-dose therapy and autologous PBSCT is the treatment of choice for eligible patients with relapsed or refractory lymphoma. We have shown both standard dose and high-dose chemotherapy cause a selective, acquired STAT4 deficiency leading to impaired IFN-γ production. Sufficient IFN-γ production has been shown to be required for effective cellular immunity to tumors in many experimental models. Optimal immunotherapy concurrent with or following chemotherapy for lymphoma will require strategies that can ameliorate or circumvent chemotherapy-induced STAT4 deficiency.

### 3.4. Future Directions

Due to the observed partial STAT4 restoration in post-chemotherapy treated cells, it is worth asking the question – could another ubiquitin mediated degradation pathway be involved in regulating STAT4 protein levels? The field of autophagy has been attracting ever greater interest from the scientific community with its complex crosstalk

between various vesicular pathways some of which are know to mediate proteasome independent protein degradation (*172*).Generally, ubiquitin mediated degradation by macroautophagy has been considered to be non-specific yet greater understanding of the pathway could provide new insight in what promises to be a new realm in protein regulation. It will be interesting to explore whether some of the main macroautophagy inhibitors do indeed impact STAT4 levels, Furthermore, the potential interaction among known macroautophagy proteins such p62 (also known as sequestosome 1) (*173, 174*) with STAT4 in promoting the turnover of its ubiquitinated form is worth pursuing. Results from such interaction could redefine our understanding of how immune cells detect changes in their intracellular homeostasis and how that might impact capability of mounting an immune response.

# FIGURES

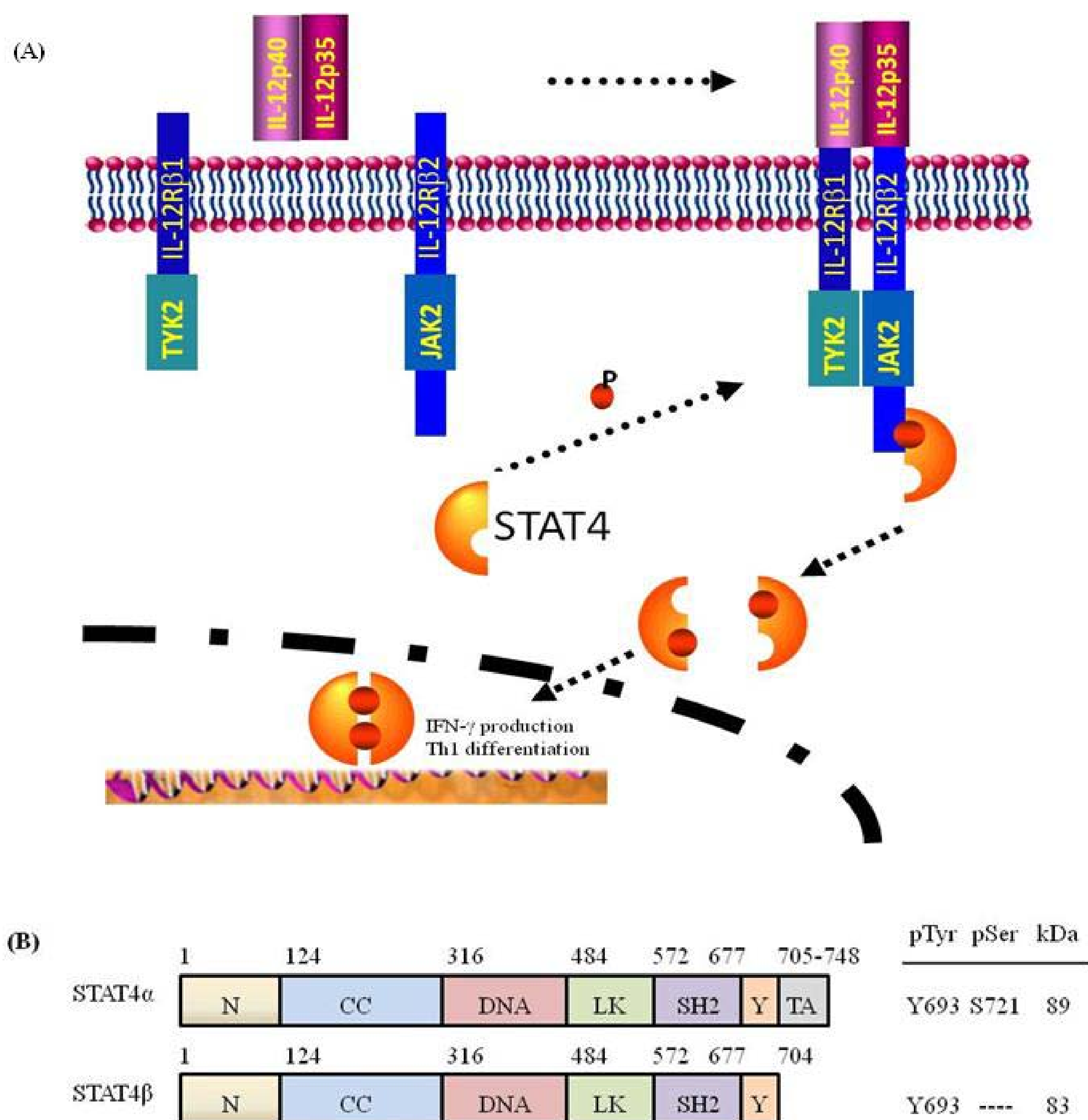


**Figure 1. General information about the STAT4 signaling pathway and the specific structures of both STAT4 isoforms. (A)** When a cytokine binds to its receptor, it causes conformational change in its cytoplasmic fragment, which leads to the recruitment of receptor associated kinases of the JAK family. JAKs phosphorylate themselves and tyrosine residues (Y) on the receptor, which become docking sites for STAT proteins. Binding of STATs leads to their tyrosine phosphorylation (Y) and activation. They migrate to the nucleus and activate transcription of specific genes. **(B)** Schematic representation of the protein domains of STAT4 isoforms: N – amino terminal domain, CC – coiled-coil domain, DNA – DNA binding domain, LK – linker domain, SH2 – Src homology domain, Y – phosphotyrosyl tail segment, and TA – transactivation domain. Also listed are the serine (pSer) and tyrosine (pTyr) phosphorylation sites and the molecular weight in kDa.

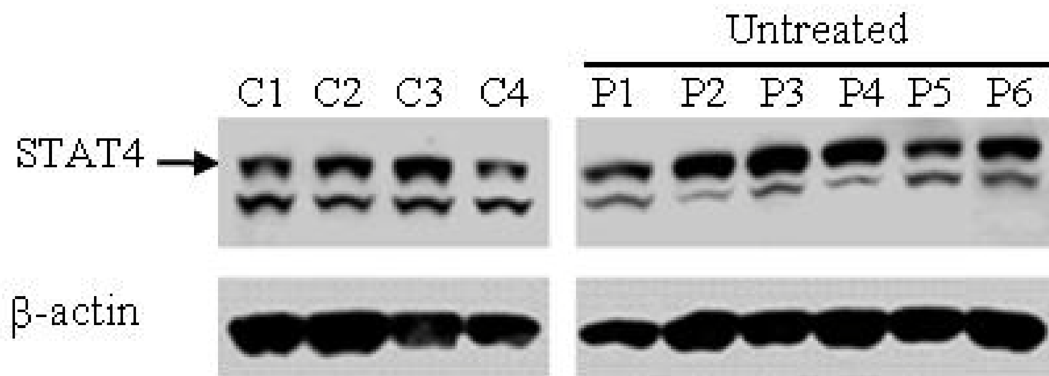


**Figure 2. Expression of STAT4 in PBMCs obtained from lymphoma patients before chemotherapy.** STAT4 protein expression was analyzed by immunoblotting of PBMCs from 4 normal controls (C1-C4) and 6 untreated lymphoma patients (P1-P6). Samples from normal controls and patients were run in separate gels, while exposure was done at the same time. The indicated upper STAT4 band detected with anti-STAT4 monoclonal antibody was confirmed with anti-STAT4 polyclonal antibody (*160*).

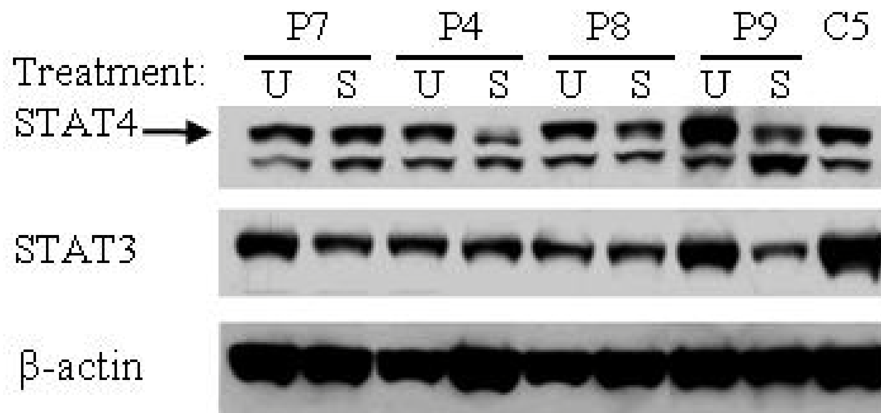


**Figure 3. Expression of STAT4 in PBMCs obtained from lymphoma patients after standard dose of chemotherapy.** Immunoblot comparison of STAT4 expression in untreated lymphoma patient PBMCs (U), three weeks after their first cycle of standard dose chemotherapy (S), and in PBMC from healthy individuals (C).

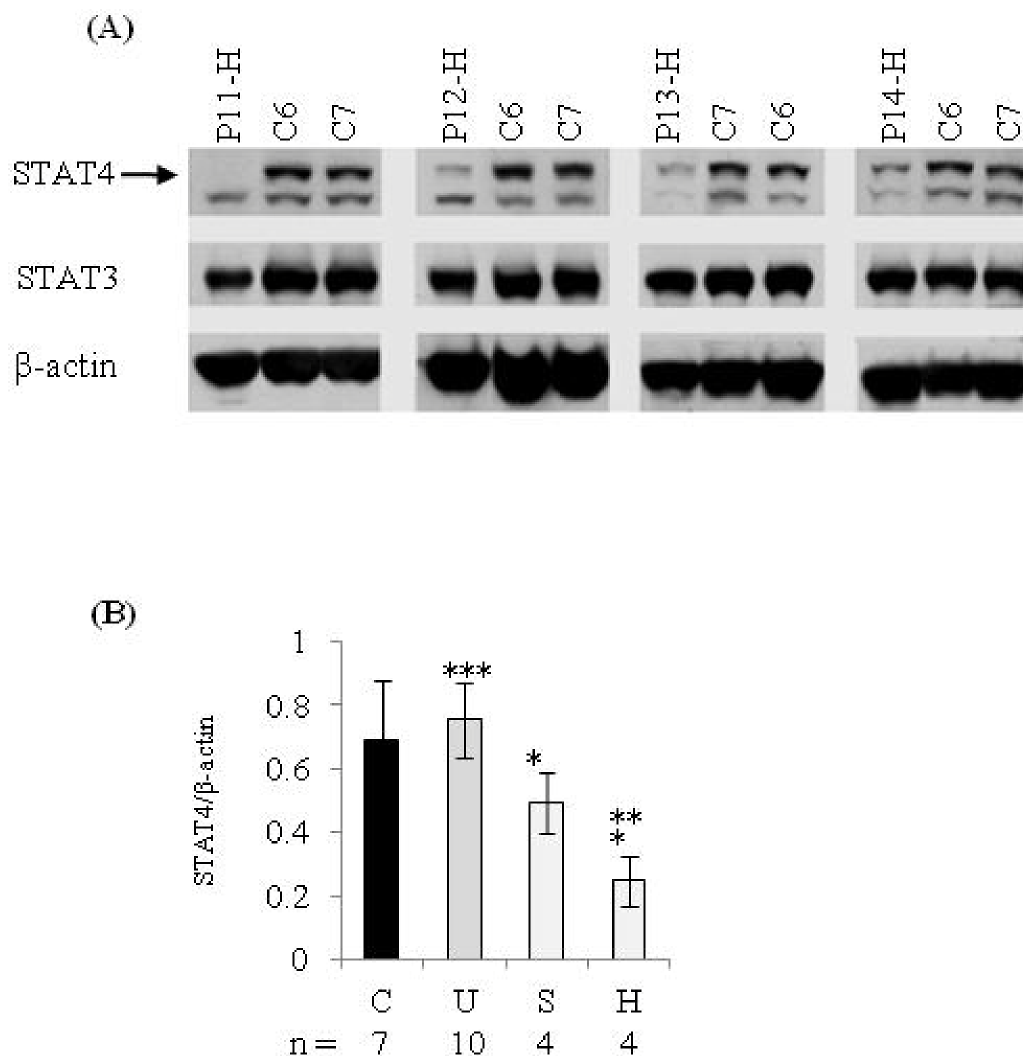


**Figure 4. Expression of STAT4 in PBMCs obtained from lymphoma patients after high dose chemotherapy. (A)** Analysis of STAT4 expression in PBMCs obtained from lymphoma patients after high-dose chemotherapy (CBV or BEAM) and autologous PBSCT (designated by the letter "H" for high-dose). **(B)** The levels of STAT4 protein from **Figures 1, 2, and 3A** were quantified by the densitometry of the corresponding bands, normalized to endogenous control β-actin using the NIH ImageJ program, and presented as mean ± SD from all normal and patient samples. (*$P<0.05$, relative to normal controls (C) or untreated lymphoma patients (U); **$P<0.05$, relative to patients receiving standard dose chemotherapy (S); ***$P>0.05$, relative to normal controls).

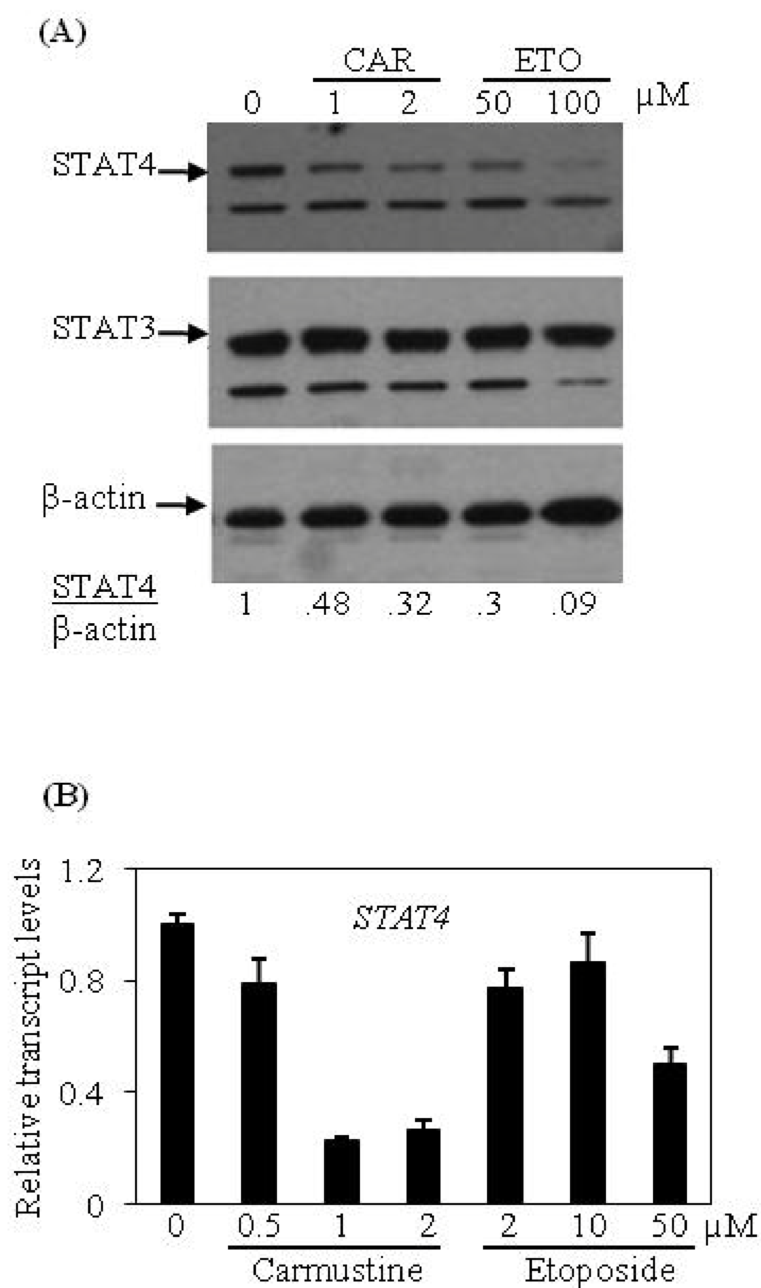


**Figure 5. Expression of STAT4 in cells treated in vitro with chemotherapeutic drugs associated with high dose treatment.** PBMCs from healthy individuals stimulated with IL-2 and PHA, were treated with the indicated concentrations of *2* µM etoposide (ETO) and *50* µM carmustine (CAR). RNA was extracted and the first-strand cDNA was synthesized from the cells. STAT4 expression was analyzed using immunoblot **(A)** and real time PCR **(B)**.

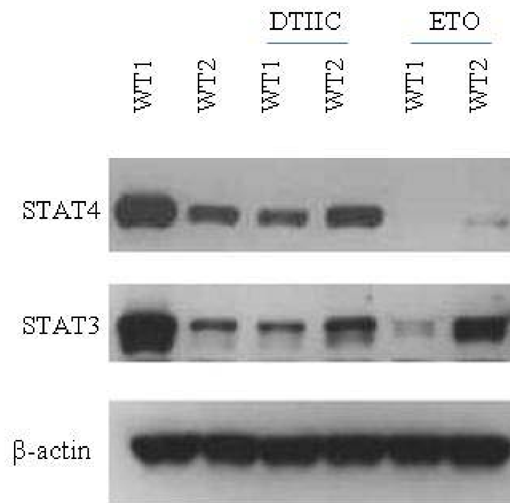


**Figure 6. Effects on mouse STAT4 protein expression as a result of in vivo exposure to chemotherapeutic drugs.** $CD4^+$ T cells were isolated from spleens of wild type (WT) mice using positive selection with $CD4^+$ magnetic beads as it is described in Materials and Methods. The collected cells were treated in vivo with the designated chemotherapy drugs: DTIC and etoposide (ETO). Total protein extracts were separated on SDS-PAGE followed by immunoblotting.

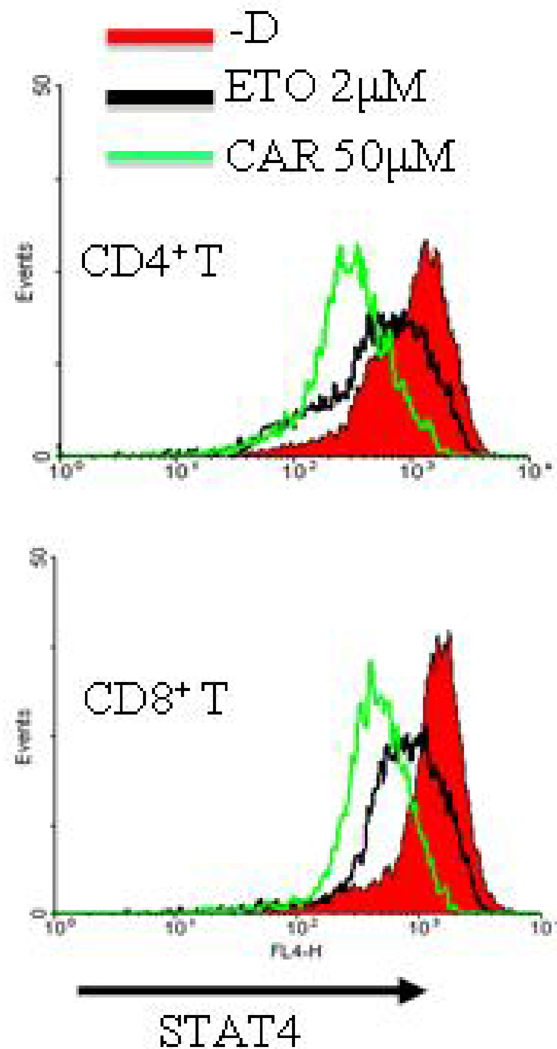


**Figure 7. Expression of STAT4 among different T lymphocytes after in vitro treatment with chemotherapeutic drugs.**STAT4 protein levels in individual cell types from normal PBMC treated above were analyzed using flow cytometry as described in Material and Methods. Histograms represent the STAT4 expression gated on 5000 events of live CD4 or CD8 positive cells using the WinMDI software.

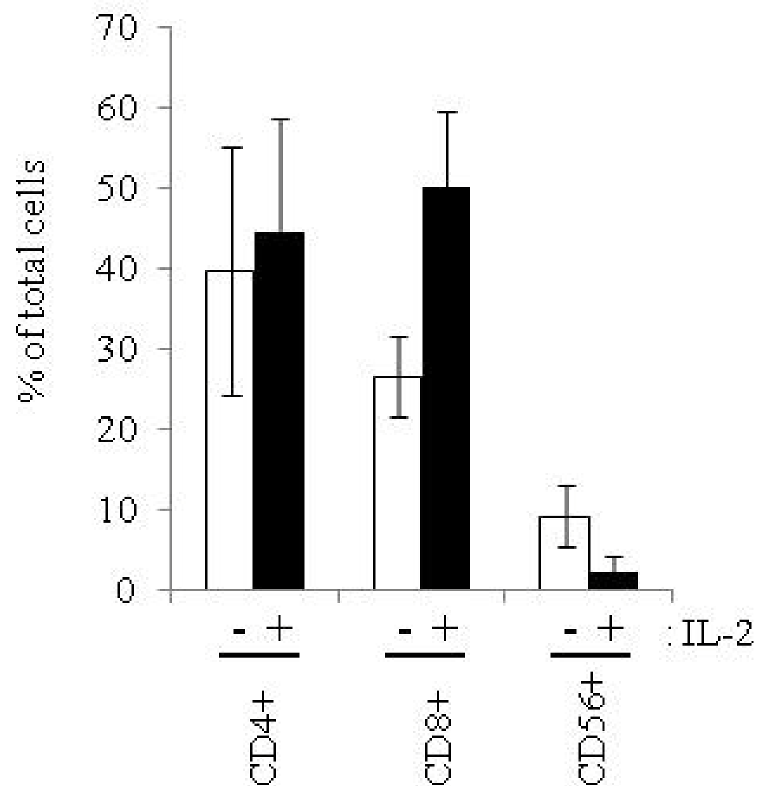


**Figure 8.Relative amounts of different cell types after in vitro stimulation with IL2 and PHA of PBMCs from healthy individuals.** These are results of a flow cytometric analysis which was conducted as described in the Materials and Methods section. The bar graph represents percentages of each cell type before and after stimulation with IL-2 (50U/mL) and PHA (2.5μg/mL) from four different healthy individual PBMCs. Each condition was gated on 10,000 live cells of each type – $CD4^+$ and $CD8^+$are markers for T lymphocytes, while $CD56^+$is a general marker for total NK cell populations.

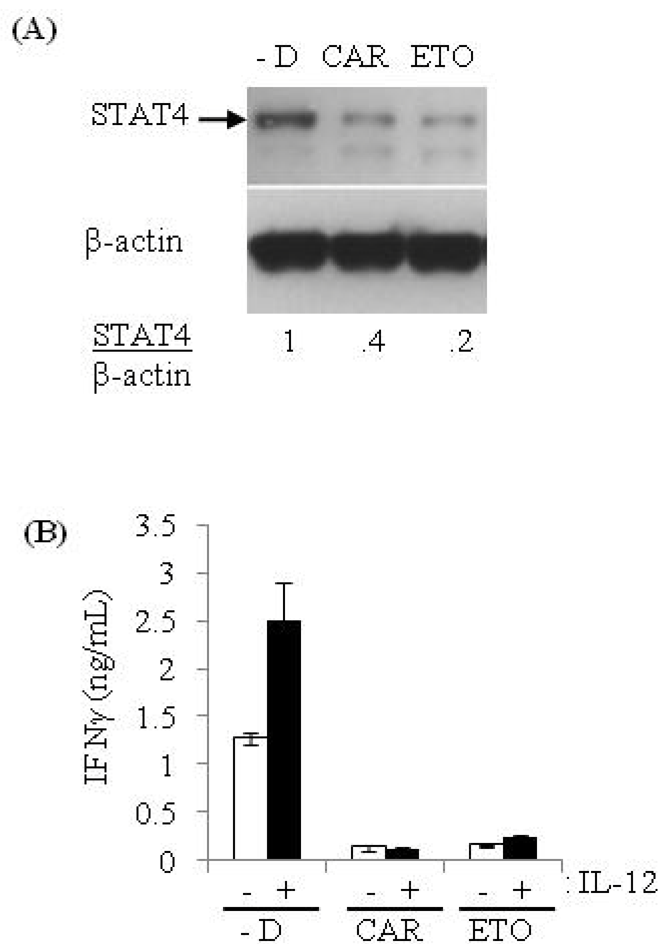


**Figure 9. Effects of in vitro chemotherapy treatment on NK cell population. (A)** NKL cells were treated with *50* µM carmustine (CAR) and *2* µM etoposide (ETO) for 2-3 days. STAT4 protein levels were analyzed using western blotting. **(B)** NKL cells treated as described in **(A)** were incubated with medium alone or medium containing IL-12 (2 ng/ml) for 1day. The cell-free supernatants were analyzed for IFNγ production using ELISA. The data is presented as mean± SD from 3 independent experiments.

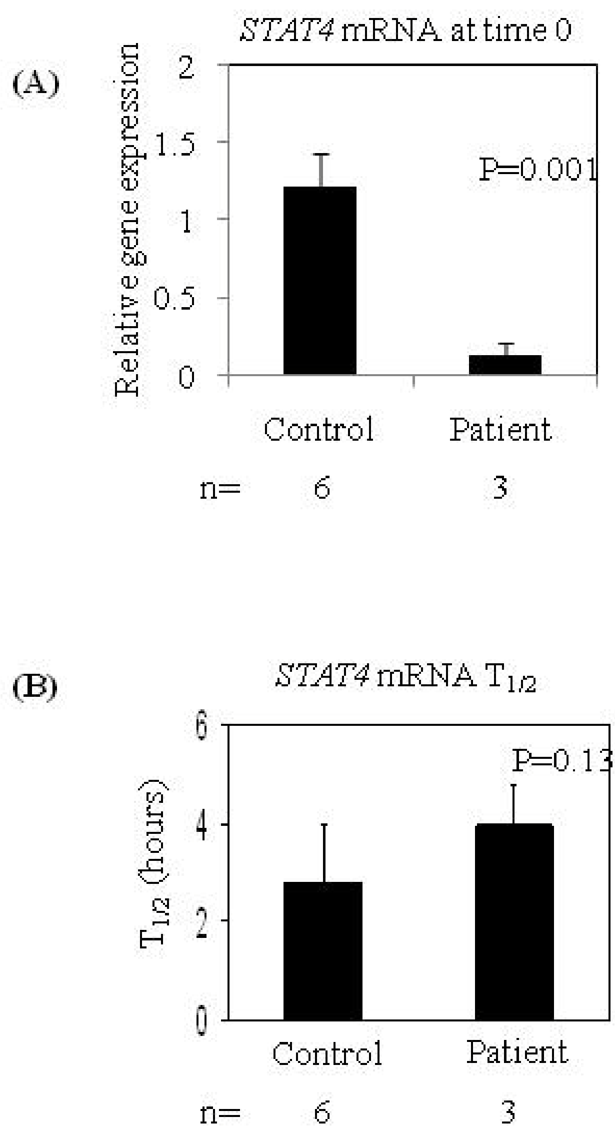


**Figure 10. *STAT4* mRNA levels and half-life of *STAT4* mRNA in PBMCs.** PBMCs obtained from 6 healthy control subjects and 3 lymphoma patients after high-dose chemotherapy and PBSCT were treated with and without actinomycin D at 1μg/ml for 0, 2, 4, and 6 hrs in a 5% $CO_2$ incubator at 37ºC. RNA was extracted, and the first-strand cDNA was synthesized followed by the real time PCR (*161*). The half-life of *STAT4* mRNA from each sample was calculated accordingly (*161*). Results shown are mean ± SD.

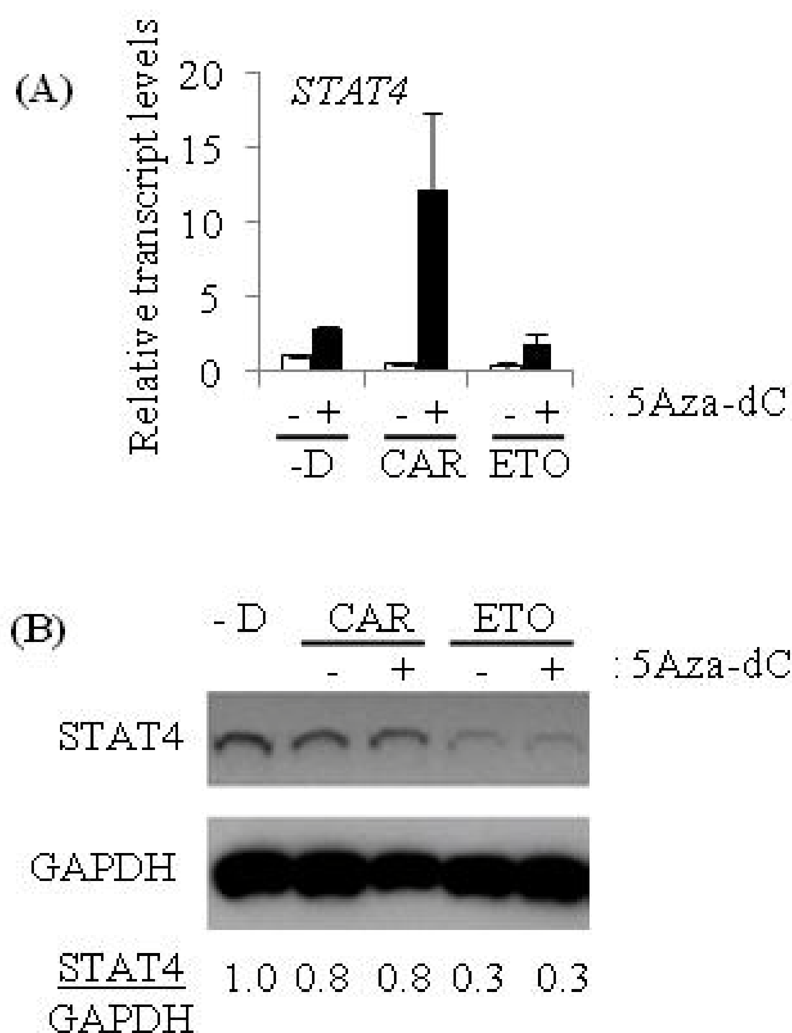


**Figure 11. Analysis of methylation based regulation of STAT4 via 5-Azacitidne treatment.** PBMCs from healthy individuals were activated and subsequently treated with carmustine and etoposide as described in Figure 2A. Following the 3 day drug incubation, cells were washed and re-suspended in medium containing the methylation inhibitor 5-Azacitidine (5-Aza-dC) at 2.5 ng/mL. Cell pellets were collected after 1 day incubation followed by RNA extraction and gene expression analysis using real time PCR **(A)** or protein analysis using western blotting **(B)**.

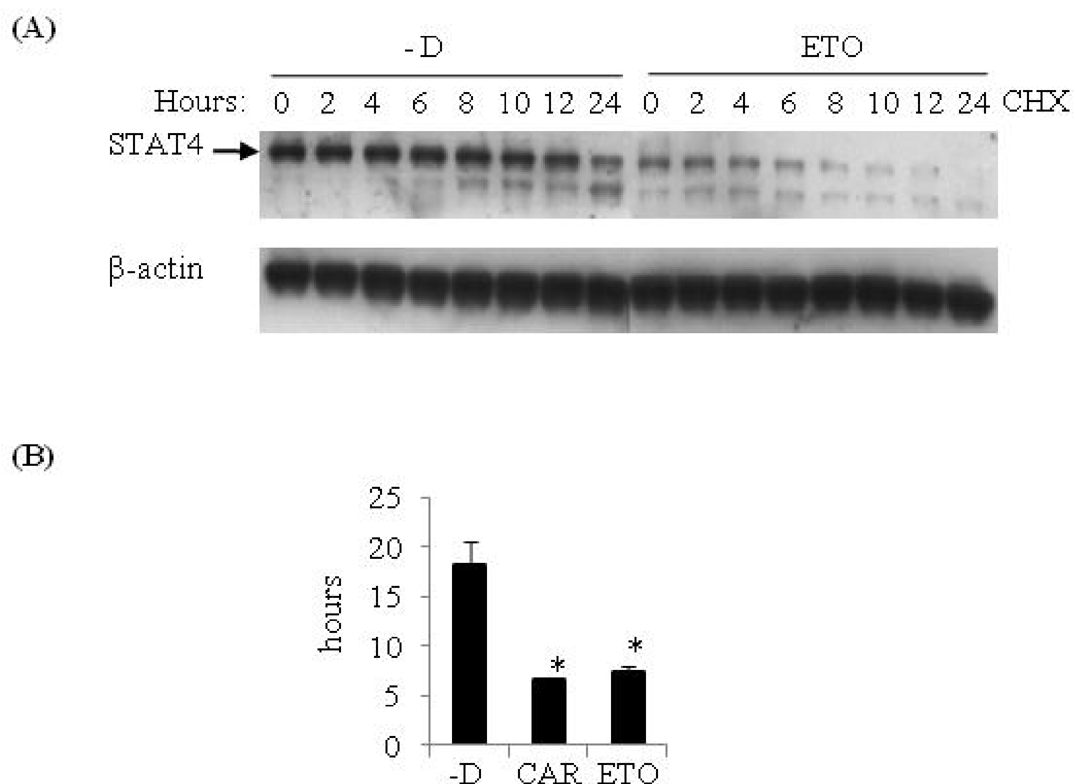


**Figure 12. Chemotherapy drugs reduce STAT4 protein half-life.(A)**NKL cells treated with *50* μM etoposide (ETO) or *2* μM carmustine (CAR) for 2 days. Subsequently, the cells were washed and incubated with cycloheximide (CHX) for 0, 2, 4, 6, 8, 10, 12, and 24 hours. STAT4 protein expression was analyzed using western blotting. **(B)**The levels of STAT4 protein were determined by the densitometry of the corresponding bands normalized to the endogenous control – β-actin, using the NIH ImageJ program (Figure A3). The half-life of STAT4 protein was calculated accordingly, and results shown are mean ± SD from a total of 3 independent experiments.

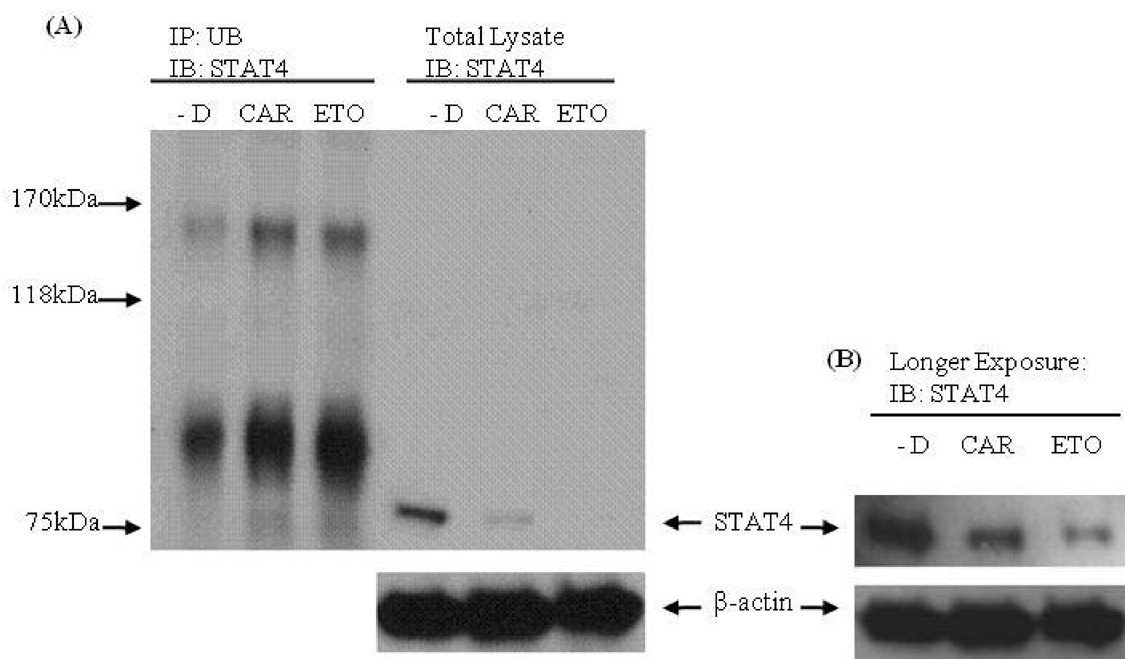


**Figure 13.Ubiquitin-mediated proteasomal degradation as the cause of chemotherapy induced STAT4 deficiency.** NKL cells were treated with 50$\mu$M carmustine (CAR) or 2$\mu$M etoposide (ETO) for 2 days. Ubiquitin-conjugated STAT4 protein levels were analyzed using immunoprecipitation (IP) of whole cell lysates (2 mg) with anti-ubiquitin monoclonal antibody followed by immunoblotting (IB) with anti-STAT4 antibody (left side of panel A). Total STAT4 protein levels were analyzed using immunoblotting from 10 μg of whole cell lysates (right side of panel A and longer exposure of the same lanes in panel B). The results are representative of 3 independent experiments.

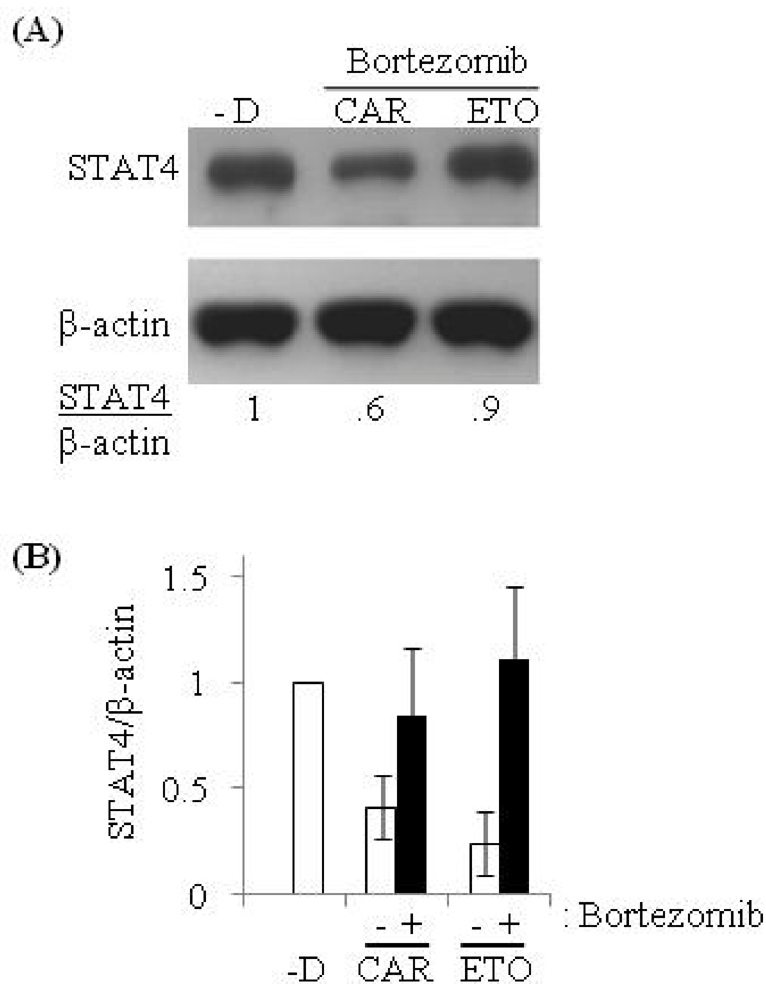


**Figure 14. Rescuing the levels of STAT4 via inhibition of the proteasome machinery.(A)** NKL cells were incubated with the proteasome inhibitor bortezomib at 5.2 nM simultaneously with either 50$\mu$M carmustine (CAR) or 2$\mu$M etoposide (ETO) for 2 days. STAT4 protein levels were determined using western blotting. The results are representative of 3 independent experiments. Ratio of total STAT4 to β-actin is indicated below.**(B)** Densitometric analysis of STAT4 protein levels in NKL cells treated with carmustine and etoposide in the presence or absence of bortezomib. The result is presented as the averaged ratio of STAT4 to β-actin from 2 independent experiments as mean ± SD.

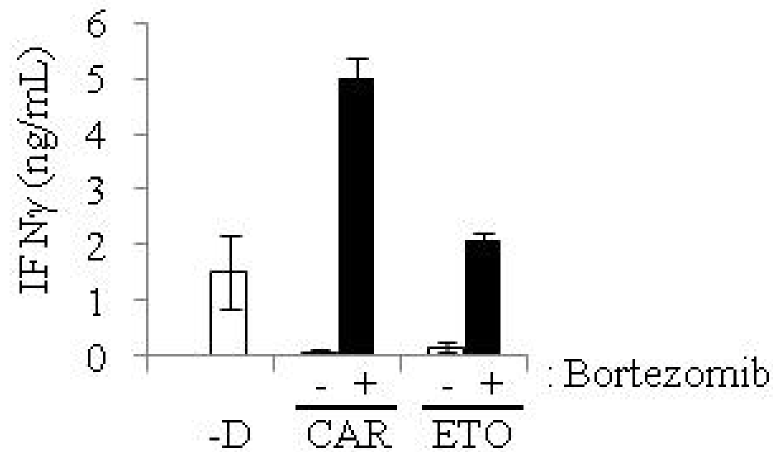


**Figure 15. Restoring IFNγ secretion in post chemotherapy treated cells with Bortezomib.** NKL cells were treated with either 50$\mu$M carmustine (CAR) or 2$\mu$M etoposide (ETO) for 2 days. Subsequently, cells were washed and stimulated with IL-12 at 2ng/ml for 1 day. The IFNγ levels in the cell supernatants were evaluated using ELISA. Results are averaged from 2 independent experiments as mean ± SD.

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APPENDICES

Appendix A

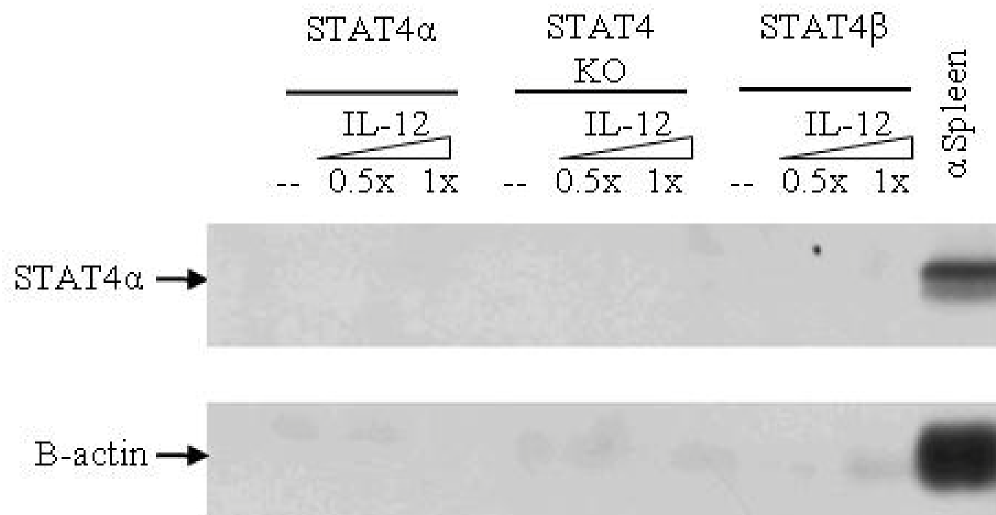



**Figure A1. Expression of STAT4α and STAT4β isoforms in the NK cells from STAT4 transgenic mice.** Mouse NK cells were magnetically separated from the spleen of each mouse type as described in Materials and Methods. Cells were stimulated with IL-12 for 2 days, the protein was collected and resolved on SDS-PAGE.

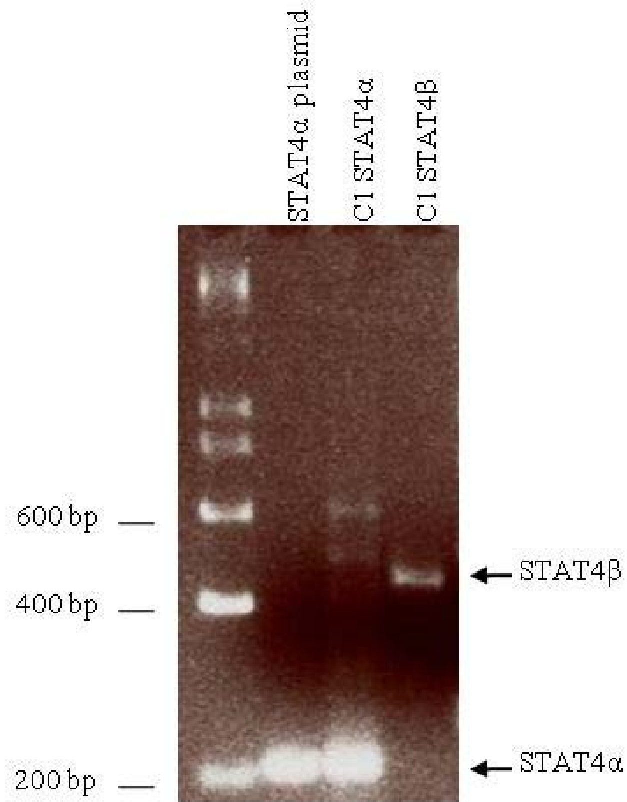
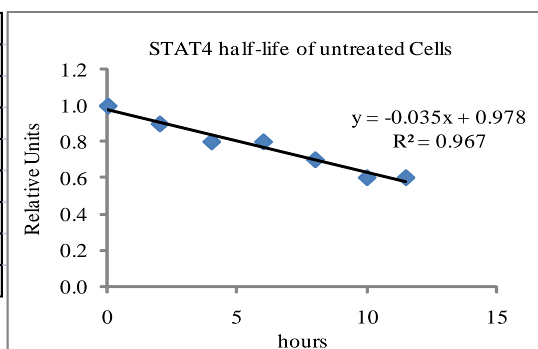



**Figure A2. Detecting STAT4 isoform transcripts in human PBMCs.** PBMCs from healthy individual were stimulated in vitro with IL-2 and PHA for 3 days. The cell pellets were collected, the first-strand cDNA was synthesized and the expression analysis of either STAT4α or STAT4βwas conducted by PCR. The primer sequences differentiating the two isoforms are shown in Appendix A1.

| time (in hours) | Condition | Stat 4 | β-actin | (Stat4)/(β-actin) | Reltive Units |
|---|---|---|---|---|---|
| 0 | No CXM -D | 7295.58 | 4867.99 | 1.5 | 1.0 |
| 2 | w/ CXM -D | 6631.87 | 4688.75 | 1.4 | 0.9 |
| 4 | w/ CXM -D | 6471.75 | 5739.04 | 1.1 | 0.8 |
| 6 | w/ CXM -D | 6064.04 | 4930.21 | 1.2 | 0.8 |
| 8 | w/ CXM -D | 6340.46 | 6302.63 | 1.0 | 0.7 |
| 10 | w/ CXM -D | 5869.04 | 6172.51 | 1.0 | 0.6 |
| 11.5 | w/ CXM -D | 5654.16 | 6416.80 | 0.9 | 0.6 |

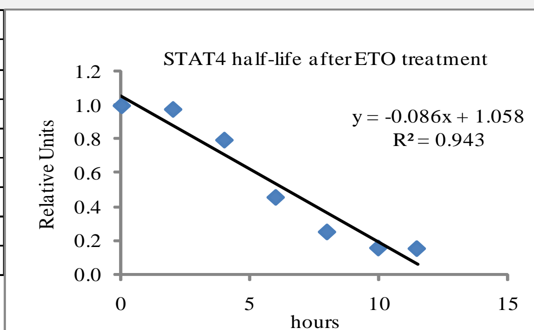
| No Drug | |
|---|---|
| time (in hours) | Reltive Units |
| 0 | 1.0 |
| 2 | 0.9 |
| 4 | 0.8 |
| 6 | 0.8 |
| 8 | 0.7 |
| 10 | 0.6 |
| 11.5 | 0.6 |




t half-life ≈ 14

| time (in hours) | Condition | Stat 4 | β-actin | (Stat4)/(β-actin) | Reltive Units |
|---|---|---|---|---|---|
| 0 | No CXM ETO | 9088.062 | 11094.23 | 0.8 | 1.0 |
| 2 | w/ CXM ETO | 8792.234 | 10974.7 | 0.8 | 1.0 |
| 4 | w/ CXM ETO | 7156.234 | 10961.99 | 0.7 | 0.8 |
| 6 | w/ CXM ETO | 4255.698 | 11329.11 | 0.4 | 0.5 |
| 8 | w/ CXM ETO | 2441.234 | 11706.99 | 0.2 | 0.3 |
| 10 | w/ CXM ETO | 1544.527 | 11806.99 | 0.1 | 0.2 |
| 11.5 | w/ CXM ETO | 1278.355 | 10005.82 | 0.1 | 0.2 |

| ETO | |
|---|---|
| time (in hours) | Reltive Units |
| 0 | 1.0 |
| 2 | 1.0 |
| 4 | 0.8 |
| 6 | 0.5 |
| 8 | 0.3 |
| 10 | 0.2 |
| 11.5 | 0.2 |




t half-life ≈ 6

**Figure A3. Calculating STAT4 protein half-life using the NIH ImageJ program.** The intensity of the bands from Figure 12A were scanned and converted to a numerical unit using the NIH ImageJ software. The intensity from each time point was normalized to endogenous β-actin and the reduction in STAT4 protein was expressed relative to the initial time point (t=0 hours). The equation from the linear graph was used to calculate the time that it takes for the initial amount of STAT4 to be reduced by 50%.

## Appendix B

```
  1 AAGGCTACTCAAGGAGAGTAGGAGGAGGCTAGGTCAGGAAGGGCAAGCACTGGTGGCCTA
    ............................................................
    ............................................................

 61 CCCAGGACTTGGCCCAACCCTCTGGGTCAGGTCACCCTCGTCGGAGTGAGCGGACCCCGC
    ............................................................
    ............................................................

121 TGGAATTGGAGCCCAGTAAGGTCTATGAGTTTGGTGTTTTGATCCTGGCTACCCATCCCT
    ............................................................
    ............................................................

181 TCCATCCCAGGGGTGGTTCACCAGGCTGAGTGGAGCCTTATACTAGGGAGAGAGGAAGCT
    ............................................................
    ............................................................

241 GAAGAACTGGGCTCCAGCATGTCTCAGTGGAATCAAGTCCAACAGTTAGAAATCAAGTTT
    ..................ATGTCTCAGTGGAATCAAGTCCAACAGTTAGAAATCAAGTTT
    ..................-M--S--Q--W--N--Q--V--Q--Q--L--E--I--K--F-

301 TTGGAGCAGGTGGATCAATTCTATGATGACAACTTTCCCATGGAAATTCGGCATCTGTTG
 43 TTGGAGCAGGTGGATCAATTCTATGATGACAACTTTCCCATGGAAATTCGGCATCTGTTG
 15 -L--E--Q--V--D--Q--F--Y--D--D--N--F--P--M--E--I--R--H--L--L-

                Y
361 GCCCAATGGATYGAAAATCAAGACTGGGAGGCAGCTTCTAACAATGAAACCATGGCAACG
103 GCCCAATGGATTGAAAATCAAGACTGGGAGGCAGCTTCTAACAATGAAACCATGGCAACG
 35 -A--Q--W--I--E--N--Q--D--W--E--A--A--S--N--N--E--T--M--A--T-
```

**Figure B1. Primer sequences for analyzing gene expression of STAT4α.**The numbers on the left side of the sequence panels designate the position of each nucleotide in the STAT4 transcript. The letters in the third row designate the position of each amino acid in the STAT4 protein. The numbers differ slightly based on the type of sequencing used to generate them - as indicated in GenBank.

```
421 ATTCTTCTTCAAAACTTGTTAATACAACTGGATGAACAGTTAGGTCGTGTTTCCAAAGAG
163 ATTCTTCTTCAAAACTTGTTAATACAACTGGATGAACAGTTAGGTCGTGTTTCCAAAGAG
 55 -I--L--L--Q--N--L--L--I--Q--L--D--E--Q--L--G--R--V--S--K--E-


481 AAAAACCTACTCTTGATACACAATCTAAAAAGAATTAGGAAGGTCCTTCAGGGAAAATTT
223 AAAAACCTACTCTTGATACACAATCTAAAAAGAATTAGGAAGGTCCTTCAGGGAAAATTT
 75 -K--N--L--L--L--I--H--N--L--K--R--I--R--K--V--L--Q--G--K--F-

                                       R
541 CATGGAAATCCAATGCATGTAGCTGTGGTTATTTCRAACTGTTTAAGGGAAGAGAGGAGA
283 CATGGAAATCCAATGCATGTAGCTGTGGTTATTTCAAACTGTTTAAGGGAAGAGAGGAGA
 95 -H--G--N--P--M--H--V--A--V--V--I--S--N--C--L--R--E--E--R--R-

    R
601 RTATTGGCTGCAGCCAACATGCCTGTCCAGGGGCCTCTAGAGAAATCCTTACAAAGTTCT
343 ATATTGGCTGCAGCCAACATGCCTGTCCAGGGGCCTCTAGAGAAATCCTTACAAAGTTCT
115 =I=-L--A--A--A--N--M--P--V--Q--G--P--L--E--K--S--L--Q--S--S-


661 TCAGTTTCAGAAAGACAGAGGAATGTGGAGCACAAAGTGGCTGCCATTAAAAACAGTGTG
403 TCAGTTTCAGAAAGACAGAGGAATGTGGAGCACAAAGTGGCTGCCATTAAAAACAGTGTG
135 -S--V--S--E--R--Q--R--N--V--E--H--K--V--A--A--I--K--N--S--V-

                                             R
721 CAGATGACAGAACAAGATACCAAATACTTAGAAGATCTGCARGACGAATTTGACTACAGG
463 CAGATGACAGAACAAGATACCAAATACTTAGAAGATCTGCAAGACGAATTTGACTACAGG
155 -Q--M--T--E--Q--D--T--K--Y--L--E--D--L--Q--D--E--F--D--Y--R-


781 TATAAAACAATTCAGACAATGGATCAGAGTGACAAGAATAGTGCCATGGTGAATCAGGAA
523 TATAAAACAATTCAGACAATGGATCAGAGTGACAAGAATAGTGCCATGGTGAATCAGGAA
175 -Y--K--T--I--Q--T--M--D--Q--S--D--K--N--S--A--M--V--N--Q--E-

                           W
841 GTTTTGACACTGCAGGAAATGCTWAACAGCCTCGATTTCAAGAGAAAGGAGGCTCTCAGT
583 GTTTTGACACTGCAGGAAATGCTTAACAGCCTCGATTTCAAGAGAAAGGAGGCTCTCAGT
195 -V--L--T--L--Q--E--M--L--N--S--L--D--F--K--R--K--E--A--L--S-


901 AAAATGACCCAAATCATCCATGAGACAGACCTGTTAATGAACACCATGCTCATAGAAGAG
643 AAAATGACCCAAATCATCCATGAGACAGACCTGTTAATGAACACCATGCTCATAGAAGAG
215 -K--M--T--Q--I--I--H--E--T--D--L--L--M--N--T--M--L--I--E--E-

                    R
961 CTGCAAGACTGGAAGCRGCGGCAGCAAATCGCCTGCATCGGGGGTCCACTCCACAATGGG
703 CTGCAAGACTGGAAGCGGCGGCAGCAAATCGCCTGCATCGGGGGTCCACTCCACAATGGG
235 -L--Q--D--W--K-=R=-R--Q--Q--I--A--C--I--G--G--P--L--H--N--G-
```

**Figure B1. Primer sequences for analyzing gene expression of STAT4α. (continued…)**

```
                                                   M
1021 CTCGACCAGCTTCAGAACTGCTTTACACTATTGGCAGAAAGTMTTTTCCAACTGAGAAGG
 763 CTCGACCAGCTTCAGAACTGCTTTACACTATTGGCAGAAAGTCTTTTCCAACTGAGAAGG
 255 -L--D--Q--L--Q--N--C--F--T--L--L--A--E--S-=L=-F--Q--L--R--R-

1081 CAATTGGAGAAACTAGAGGAGCAATCTACCAAAATGACATATGAAGGTGATCCCATTCCA
 823 CAATTGGAGAAACTAGAGGAGCAATCTACCAAAATGACATATGAAGGTGATCCCATTCCA
 275 -Q--L--E--K--L--E--E--Q--S--T--K--M--T--Y--E--G--D--P--I--P-

1141 ATGCAAAGAACTCACATGCTAGAAAGAGTCACCTTCTTGATCTACAACCTTTTCAAGAAC
 883 ATGCAAAGAACTCACATGCTAGAAAGAGTCACCTTCTTGATCTACAACCTTTTCAAGAAC
 295 -M--Q--R--T--H--M--L--E--R--V--T--F--L--I--Y--N--L--F--K--N-

1201 TCATTTGTGGTTGAGCGACAGCCATGTATGCCAACCCACCCTCAGAGGCCGTTGGTACTT
 943 TCATTTGTGGTTGAGCGACAGCCATGTATGCCAACCCACCCTCAGAGGCCGTTGGTACTT
 315 -S--F--V--V--E--R--Q--P--C--M--P--T--H--P--Q--R--P--L--V--L-

1261 AAAACCCTAATTCAGTTCACTGTAAAACTAAGGCTACTAATAAAATTGCCAGAACTAAAC
1003 AAAACCCTAATTCAGTTCACTGTAAAACTAAGGCTACTAATAAAATTGCCAGAACTAAAC
 335 -K--T--L--I--Q--F--T--V--K--L--R--L--L--I--K--L--P--E--L--N-

1321 TATCAGGTAAAGGTTAAGGCATCAATTGACAAGAATGTTTCAACTCTAAGCAACCGAAGA
1063 TATCAGGTAAAGGTTAAGGCATCAATTGACAAGAATGTTTCAACTCTAAGCAACCGAAGA
 355 -Y--Q--V--K--V--K--A--S--I--D--K--N--V--S--T--L--S--N--R--R-

1381 TTTGTACTTTGTGGAACTAATGTCAAAGCCATGTCTATTGAAGAATCTTCCAATGGGAGT
1123 TTTGTACTTTGTGGAACTAATGTCAAAGCCATGTCTATTGAAGAATCTTCCAATGGGAGT
 375 -F--V--L--C--G--T--N--V--K--A--M--S--I--E--E--S--S--N--G--S-

1441 CTCTCAGTAGAATTTCGACATTTGCAACCAAAGGAAATGAAGTCCAGTGCTGGAGGTAAA
1183 CTCTCAGTAGAATTTCGACATTTGCAACCAAAGGAAATGAAGTCCAGTGCTGGAGGTAAA
 395 -L--S--V--E--F--R--H--L--Q--P--K--E--M--K--S--S--A--G--G--K-

1501 GGAAATGAGGGCTGTCACATGGTGACTGAAGAACTTCATTCCATAACGTTTGAAACACAG
1243 GGAAATGAGGGCTGTCACATGGTGACTGAAGAACTTCATTCCATAACGTTTGAAACACAG
 415 -G--N--E--G--C--H--M--V--T--E--E--L--H--S--I--T--F--E--T--Q-

                             *
1561 ATCTGCCTCTATGGCCTGACCATAGATTTGGAGACCAGCTCATTGCCTGTGGTGATGATT
1303 ATCTGCCTCTATGGCCTGACCATAGATTTGGAGACCAGCTCATTGCCTGTGGTGATGATT
 435 -I--C--L--Y--G--L--T--I-=D=-L--E--T--S--S--L--P--V--V--M--I-
```

**Figure B1. Primer sequences for analyzing gene expression of STAT4α. (continued…)**

```
1621 TCCAATGTCAGTCAGTTACCTAATGCTTGGGCATCCATCATTTGGTACAACGTGTCAACC
1363 TCCAATGTCAGTCAGTTACCTAATGCTTGGGCATCCATCATTTGGTACAACGTGTCAACC
 455 -S--N--V--S--Q--L--P--N--A--W--A--S--I--I--W--Y--N--V--S--T-

                                        Y   M
1681 AACGATTCCCAGAACTTGGTTTTCTTTAATAATCCYCCMCCTGCCACATTGAGTCAACTA
1423 AACGATTCCCAGAACTTGGTTTTCTTTAATAATCCTCCACCTGCCACATTGAGTCAACTA
 475 -N--D--S--Q--N--L--V--F--F--N--N--P--P--P--A--T--L--S--Q--L-

1741 CTGGAGGTGATGAGCTGGCAGTTTTCATCGTACGTTGGTCGTGGTCTTAACTCAGATCAA
1483 CTGGAGGTGATGAGCTGGCAGTTTTCATCGTACGTTGGTCGTGGTCTTAACTCAGATCAA
 495 -L--E--V--M--S--W--Q--F--S--S--Y--V--G--R--G--L--N--S--D--Q-

1801 CTCCATATGCTGGCAGAGAAGCTTACAGTCCAATCTAGCTACAGTGATGGTCACCTCACC
1543 CTCCATATGCTGGCAGAGAAGCTTACAGTCCAATCTAGCTACAGTGATGGTCACCTCACC
 515 -L--H--M--L--A--E--K--L--T--V--Q--S--S--Y--S--D--G--H--L--T-

1861 TGGGCCAAGTTCTGCAAGGAACATTTACCTGGTAAATCATTTACCTTTTGGACATGGCTT
1603 TGGGCCAAGTTCTGCAAGGAACATTTACCTGGTAAATCATTTACCTTTTGGACATGGCTT
 535 -W--A--K--F--C--K--E--H--L--P--G--K--S--F--T--F--W--T--W--L-

1921 GAAGCAATATTGGATCTAATTAAGAAACACATTCTTCCCCTTTGGATTGATGGGTATGTC
1663 GAAGCAATATTGGATCTAATTAAGAAACACATTCTTCCCCTTTGGATTGATGGGTATGTC
 555 -E--A--I--L--D--L--I--K--K--H--I--L--P--L--W--I--D--G--Y--V-

                                 Y
1981 ATGGGCTTTGTTAGCAAAGAGAAGGAAYGGCTGTTGCTAAAGGATAAAATGCCTGGCACC
1723 ATGGGCTTTGTTAGCAAAGAGAAGGAACGGCTGTTGCTAAAGGATAAAATGCCTGGCACC
 575 -M--G--F--V--S--K--E--K--E-=R=-L--L--L--K--D--K--M--P--G--T-

2041 TTTTTATTAAGATTCAGTGAAAGCCATCTCGGAGGAATAACTTTCACCTGGGTGGACCAT
1783 TTTTTATTAAGATTCAGTGAAAGCCATCTCGGAGGAATAACTTTCACCTGGGTGGACCAT
 595 -F--L--L--R--F--S--E--S--H--L--G--G--I--T--F--T--W--V--D--H-

2101 TCTGAAAGTGGGGAAGTGAGATTCCACTCTGTAGAACCCTACAATAAAGGCCGGTTGTCT
1843 TCTGAAAGTGGGGAAGTGAGATTCCACTCTGTAGAACCCTACAATAAAGGCCGGTTGTCT
 615 -S--E--S--G--E--V--R--F--H--S--V--E--P--Y--N--K--G--R--L--S-

2161 GCTCTGCCATTCGCTGACATCCTGCGAGACTACAAAGTTATTATGGCTGAAAACATTCCT
1903 GCTCTGCCATTCGCTGACATCCTGCGAGACTACAAAGTTATTATGGCTGAAAACATTCCT
 635 -A--L--P--F--A--D--I--L--R--D--Y--K--V--I--M--A--E--N--I--P-
```

**Figure B1. Primer sequences for analyzing gene expression of STAT4α. (continued…)**

```
2221 GAAAACCCTCTGAAGTACCTATATCCTGACATTCCCAAAGACAAAGCCTTCGGTAAACAC
1963 GAAAACCCTCTGAAGTACCTATATCCTGACATTCCCAAAGACAAAGCCTTCGGTAAACAC
 655 -E--N--P--L--K--Y--L--Y--P--D--I--P--K--D--K--A--F--G--K--H-


2281 TACAGCTCTCAGCCTTGCGAAGTTTCAAGACCAACAGAAAGGGGTGACAAAGGTTATGTT
2023 TACAGCTCTCAGCCTTGCGAAGTTTCAAGACCAACAGAAAGGGGTGACAAAGGTTATGTT
 675 -Y--S--S--Q--P--C--E--V--S--R--P--T--E--R--G--D--K--G--Y--V-


2341 CCTTCTGTTTTTATCCCCATCTCAACAATCCGAAGTGATTCAACAGAGCCACATTCTCCA
2083 CCTTCTGTTTTTATCCCCATCTCAACAATCCGAAGTGATTCAACAGAGCCACATTCTCCA
 695 -P--S--V--F--I--P--I--S--T--I--R--S--D--S--T--E--P--H--S--P-


2401 TCAGACCTTCTTCCCATGTCTCCAAGTGTGTATGCGGTGTTGAGAGAAAACCTGAGTCCC
2143 TCAGACCTTCTTCCCATGTCTCCAAGTGTGTATGCGGTGTTGAGAGAAAACCTGAGTCCC
 715 -S--D--L--L--P--M--S--P--S--V--Y--A--V--L--R--E--N--L--S--P-

                                                        M
2461 ACAACAATTGAAACTGCAATGAAGTCTCCTTATTCTGCTGAATGACAGGATAMACTCTGA
2203 ACAACAATTGAAACTGCAATGAAGTCTCCTTATTCTGCTGAATGA...............
 735 -T--T--I--E--T--A--M--K--S--P--Y--S--A--E--*-...............


2521 CGCACCAAGAAAGGAAGCAAATGAAAAAGTTTAAAGACTGTTCTTTGCCCAATAACCACA
     ............................................................
     ............................................................

                                                   R
2581 TTTTATTTCTTCAGCTTTGTAAATACCAGGTTCTAGGAAATGTTTGACRTCTGAAGCTCT
     ............................................................
     ............................................................


2641 CTTCACACTCCCGTGGCACTCCTCAATTGGGAGTGTTGTGACTGAAATGCTTGAAACCAA
     ............................................................
     ............................................................

                  W
2701 AGCTTCAGATAAWCTTGCAAGATAAGACAACTTTAAGAAACCAGTGTTAATAACAATATT
     ............................................................
     ............................................................


2761 AACAGAAGA
     .........
     .........
```

**Figure B1. Primer sequences for analyzing gene expression of STAT4α. (continued…).** The line which begins with the nucleotide number 2221/1963 contains the forward primer sequence for STAT4α (shaded in gray), while the line beginning with 2401/2143 contains the sequence of the reverse primer (shaded in gray).

## Appendix C

```
  1 AAGGCTACTCAAGGAGAGTAGGAGGAGGCTAGGTCAGGAAGGGCAAGCACTGGTGGCCTA
    ............................................................
    ............................................................

 61 CCCAGGACTTGGCCCAACCCTCTGGGTCAGGTCACCCTCGTCGGAGTGAGCGGACCCCGC
    ............................................................
    ............................................................

121 TGGAATTGGAGCCCAGTAAGGTCTATGAGTTTGGTGTTTTGATCCTGGCTACCCATCCCT
    ............................................................
    ............................................................

181 TCCATCCCAGGGGTGGTTCACCAGGCTGAGTGGAGCCTTATACTAGGGAGAGAGGAAGCT
    ............................................................
    ............................................................

241 GAAGAACTGGGCTCCAGCATGTCTCAGTGGAATCAAGTCCAACAGTTAGAAATCAAGTTT
    ..................ATGTCTCAGTGGAATCAAGTCCAACAGTTAGAAATCAAGTTT
    ..................-M--S--Q--W--N--Q--V--Q--Q--L--E--I--K--F-

301 TTGGAGCAGGTGGATCAATTCTATGATGACAACTTTCCCATGGAAATTCGGCATCTGTTG
 43 TTGGAGCAGGTGGATCAATTCTATGATGACAACTTTCCCATGGAAATTCGGCATCTGTTG
 15 -L--E--Q--V--D--Q--F--Y--D--D--N--F--P--M--E--I--R--H--L--L-

               Y
361 GCCCAATGGATYGAAAATCAAGACTGGGAGGCAGCTTCTAACAATGAAACCATGGCAACG
103 GCCCAATGGATTGAAAATCAAGACTGGGAGGCAGCTTCTAACAATGAAACCATGGCAACG
 35 -A--Q--W--I--E--N--Q--D--W--E--A--A--S--N--N--E--T--M--A--T-

421 ATTCTTCTTCAAAACTTGTTAATACAACTGGATGAACAGTTAGGTCGTGTTTCCAAAGAG
163 ATTCTTCTTCAAAACTTGTTAATACAACTGGATGAACAGTTAGGTCGTGTTTCCAAAGAG
 55 -I--L--L--Q--N--L--L--I--Q--L--D--E--Q--L--G--R--V--S--K--E-
```

**Figure C1. Primer sequences for analyzing gene expression of STAT4β isoform.** The meaning of the numbers on the left side of the sequence panels are explained in Figure B1.

```
 481 AAAAACCTACTCTTGATACACAATCTAAAAAGAATTAGGAAGGTCCTTCAGGGAAAATTT
 223 AAAAACCTACTCTTGATACACAATCTAAAAAGAATTAGGAAGGTCCTTCAGGGAAAATTT
  75 -K--N--L--L--L--I--H--N--L--K--R--I--R--K--V--L--Q--G--K--F-

                                        R
 541 CATGGAAATCCAATGCATGTAGCTGTGGTTATTTCRAACTGTTTAAGGGAAGAGAGGAGA
 283 CATGGAAATCCAATGCATGTAGCTGTGGTTATTTCAAACTGTTTAAGGGAAGAGAGGAGA
  95 -H--G--N--P--M--H--V--A--V--V--I--S--N--C--L--R--E--E--R--R-

     R
 601 RTATTGGCTGCAGCCAACATGCCTGTCCAGGGGCCTCTAGAGAAATCCTTACAAAGTTCT
 343 ATATTGGCTGCAGCCAACATGCCTGTCCAGGGGCCTCTAGAGAAATCCTTACAAAGTTCT
 115 =I=-L--A--A--A--N--M--P--V--Q--G--P--L--E--K--S--L--Q--S--S-

 661 TCAGTTTCAGAAAGACAGAGGAATGTGGAGCACAAAGTGGCTGCCATTAAAAACAGTGTG
 403 TCAGTTTCAGAAAGACAGAGGAATGTGGAGCACAAAGTGGCTGCCATTAAAAACAGTGTG
 135 -S--V--S--E--R--Q--R--N--V--E--H--K--V--A--A--I--K--N--S--V-

                                              R
 721 CAGATGACAGAACAAGATACCAAATACTTAGAAGATCTGCARGACGAATTTGACTACAGG
 463 CAGATGACAGAACAAGATACCAAATACTTAGAAGATCTGCAAGACGAATTTGACTACAGG
 155 -Q--M--T--E--Q--D--T--K--Y--L--E--D--L--Q--D--E--F--D--Y--R-

 781 TATAAAACAATTCAGACAATGGATCAGAGTGACAAGAATAGTGCCATGGTGAATCAGGAA
 523 TATAAAACAATTCAGACAATGGATCAGAGTGACAAGAATAGTGCCATGGTGAATCAGGAA
 175 -Y--K--T--I--Q--T--M--D--Q--S--D--K--N--S--A--M--V--N--Q--E-

                            W
 841 GTTTTGACACTGCAGGAAATGCTWAACAGCCTCGATTTCAAGAGAAAGGAGGCTCTCAGT
 583 GTTTTGACACTGCAGGAAATGCTTAACAGCCTCGATTTCAAGAGAAAGGAGGCTCTCAGT
 195 -V--L--T--L--Q--E--M--L--N--S--L--D--F--K--R--K--E--A--L--S-

 901 AAAATGACCCAAATCATCCATGAGACAGACCTGTTAATGAACACCATGCTCATAGAAGAG
 643 AAAATGACCCAAATCATCCATGAGACAGACCTGTTAATGAACACCATGCTCATAGAAGAG
 215 -K--M--T--Q--I--I--H--E--T--D--L--L--M--N--T--M--L--I--E--E-

                     R
 961 CTGCAAGACTGGAAGCRGCGGCAGCAAATCGCCTGCATCGGGGGTCCACTCCACAATGGG
 703 CTGCAAGACTGGAAGCGGCGGCAGCAAATCGCCTGCATCGGGGGTCCACTCCACAATGGG
 235 -L--Q--D--W--K-=R=-R--Q--Q--I--A--C--I--G--G--P--L--H--N--G-

                                               M
1021 CTCGACCAGCTTCAGAACTGCTTTACACTATTGGCAGAAAGTMTTTTCCAACTGAGAAGG
 763 CTCGACCAGCTTCAGAACTGCTTTACACTATTGGCAGAAAGTCTTTTCCAACTGAGAAGG
 255 -L--D--Q--L--Q--N--C--F--T--L--L--A--E--S-=L=-F--Q--L--R--R-
```

**Figure C1. Primer sequences for analyzing gene expression of STAT4β isoform. (continued …)**

```
1081 CAATTGGAGAAACTAGAGGAGCAATCTACCAAAATGACATATGAAGGTGATCCCATTCCA
 823 CAATTGGAGAAACTAGAGGAGCAATCTACCAAAATGACATATGAAGGTGATCCCATTCCA
 275 -Q--L--E--K--L--E--E--Q--S--T--K--M--T--Y--E--G--D--P--I--P-

1141 ATGCAAAGAACTCACATGCTAGAAAGAGTCACCTTCTTGATCTACAACCTTTTCAAGAAC
 883 ATGCAAAGAACTCACATGCTAGAAAGAGTCACCTTCTTGATCTACAACCTTTTCAAGAAC
 295 -M--Q--R--T--H--M--L--E--R--V--T--F--L--I--Y--N--L--F--K--N-

1201 TCATTTGTGGTTGAGCGACAGCCATGTATGCCAACCCACCCTCAGAGGCCGTTGGTACTT
 943 TCATTTGTGGTTGAGCGACAGCCATGTATGCCAACCCACCCTCAGAGGCCGTTGGTACTT
 315 -S--F--V--V--E--R--Q--P--C--M--P--T--H--P--Q--R--P--L--V--L-

1261 AAAACCCTAATTCAGTTCACTGTAAAACTAAGGCTACTAATAAAATTGCCAGAACTAAAC
1003 AAAACCCTAATTCAGTTCACTGTAAAACTAAGGCTACTAATAAAATTGCCAGAACTAAAC
 335 -K--T--L--I--Q--F--T--V--K--L--R--L--L--I--K--L--P--E--L--N-

1321 TATCAGGTAAAGGTTAAGGCATCAATTGACAAGAATGTTTCAACTCTAAGCAACCGAAGA
1063 TATCAGGTAAAGGTTAAGGCATCAATTGACAAGAATGTTTCAACTCTAAGCAACCGAAGA
 355 -Y--Q--V--K--V--K--A--S--I--D--K--N--V--S--T--L--S--N--R--R-

1381 TTTGTACTTTGTGGAACTAATGTCAAAGCCATGTCTATTGAAGAATCTTCCAATGGGAGT
1123 TTTGTACTTTGTGGAACTAATGTCAAAGCCATGTCTATTGAAGAATCTTCCAATGGGAGT
 375 -F--V--L--C--G--T--N--V--K--A--M--S--I--E--E--S--S--N--G--S-

1441 CTCTCAGTAGAATTTCGACATTTGCAACCAAAGGAAATGAAGTCCAGTGCTGGAGGTAAA
1183 CTCTCAGTAGAATTTCGACATTTGCAACCAAAGGAAATGAAGTCCAGTGCTGGAGGTAAA
 395 -L--S--V--E--F--R--H--L--Q--P--K--E--M--K--S--S--A--G--G--K-

1501 GGAAATGAGGGCTGTCACATGGTGACTGAAGAACTTCATTCCATAACGTTTGAAACACAG
1243 GGAAATGAGGGCTGTCACATGGTGACTGAAGAACTTCATTCCATAACGTTTGAAACACAG
 415 -G--N--E--G--C--H--M--V--T--E--E--L--H--S--I--T--F--E--T--Q-

                                 *
1561 ATCTGCCTCTATGGCCTGACCATAGATTTGGAGACCAGCTCATTGCCTGTGGTGATGATT
1303 ATCTGCCTCTATGGCCTGACCATAGATTTGGAGACCAGCTCATTGCCTGTGGTGATGATT
 435 -I--C--L--Y--G--L--T--I-=D=-L--E--T--S--S--L--P--V--V--M--I-

1621 TCCAATGTCAGTCAGTTACCTAATGCTTGGGCATCCATCATTTGGTACAACGTGTCAACC
1363 TCCAATGTCAGTCAGTTACCTAATGCTTGGGCATCCATCATTTGGTACAACGTGTCAACC
 455 -S--N--V--S--Q--L--P--N--A--W--A--S--I--I--W--Y--N--V--S--T-
```

**Figure C1. Primer sequences for analyzing gene expression of STAT4β isoform. (continued …)**

```
                                               Y   M
1681 AACGATTCCCAGAACTTGGTTTTCTTTAATAATCCYCCMCCTGCCACATTGAGTCAACTA
1423 AACGATTCCCAGAACTTGGTTTTCTTTAATAATCCTCCACCTGCCACATTGAGTCAACTA
 475 -N--D--S--Q--N--L--V--F--F--N--N--P--P--P--A--T--L--S--Q--L-

1741 CTGGAGGTGATGAGCTGGCAGTTTTCATCGTACGTTGGTCGTGGTCTTAACTCAGATCAA
1483 CTGGAGGTGATGAGCTGGCAGTTTTCATCGTACGTTGGTCGTGGTCTTAACTCAGATCAA
 495 -L--E--V--M--S--W--Q--F--S--S--Y--V--G--R--G--L--N--S--D--Q-

1801 CTCCATATGCTGGCAGAGAAGCTTACAGTCCAATCTAGCTACAGTGATGGTCACCTCACC
1543 CTCCATATGCTGGCAGAGAAGCTTACAGTCCAATCTAGCTACAGTGATGGTCACCTCACC
 515 -L--H--M--L--A--E--K--L--T--V--Q--S--S--Y--S--D--G--H--L--T-

1861 TGGGCCAAGTTCTGCAAGGAACATTTACCTGGTAAATCATTTACCTTTTGGACATGGCTT
1603 TGGGCCAAGTTCTGCAAGGAACATTTACCTGGTAAATCATTTACCTTTTGGACATGGCTT
 535 -W--A--K--F--C--K--E--H--L--P--G--K--S--F--T--F--W--T--W--L-

1921 GAAGCAATATTGGATCTAATTAAGAAACACATTCTTCCCCTTTGGATTGATGGGTATGTC
1663 GAAGCAATATTGGATCTAATTAAGAAACACATTCTTCCCCTTTGGATTGATGGGTATGTC
 555 -E--A--I--L--D--L--I--K--K--H--I--L--P--L--W--I--D--G--Y--V-

                                Y
1981 ATGGGCTTTGTTAGCAAAGAGAAGGAAYGGCTGTTGCTAAAGGATAAAATGCCTGGCACC
1723 ATGGGCTTTGTTAGCAAAGAGAAGGAACGGCTGTTGCTAAAGGATAAAATGCCTGGCACC
 575 -M--G--F--V--S--K--E--K--E-=R=-L--L--L--K--D--K--M--P--G--T-

2041 TTTTTATTAAGATTCAGTGAAAGCCATCTCGGAGGAATAACTTTCACCTGGGTGGACCAT
1783 TTTTTATTAAGATTCAGTGAAAGCCATCTCGGAGGAATAACTTTCACCTGGGTGGACCAT
 595 -F--L--L--R--F--S--E--S--H--L--G--G--I--T--F--T--W--V--D--H-

2101 TCTGAAAGTGGGGAAGTGAGATTCCACTCTGTAGAACCCTACAATAAAGGCCGGTTGTCT
1843 TCTGAAAGTGGGGAAGTGAGATTCCACTCTGTAGAACCCTACAATAAAGGCCGGTTGTCT
 615 -S--E--S--G--E--V--R--F--H--S--V--E--P--Y--N--K--G--R--L--S-

2161 GCTCTGCCATTCGCTGACATCCTGCGAGACTACAAAGTTATTATGGCTGAAAACATTCCT
1903 GCTCTGCCATTCGCTGACATCCTGCGAGACTACAAAGTTATTATGGCTGAAAACATTCCT
 635 -A--L--P--F--A--D--I--L--R--D--Y--K--V--I--M--A--E--N--I--P-
```

**Figure C1. Primer sequences for analyzing gene expression of STAT4β isoform. (continued …)**

```
2221 GAAAACCCTCTGAAGTACCTATATCCTGACATTCCCAAAGACAAAGCCTTCGGTAAACAC
1963 GAAAACCCTCTGAAGTACCTATATCCTGACATTCCCAAAGACAAAGCCTTCGGTAAACAC
 655 -E--N--P--L--K--Y--L--Y--P--D--I--P--K--D--K--A--F--G--K--H-


2281 TACAGCTCTCAGCCTTGCGAAGTTTCAAGACCAACAGAAAGGGGTGACAAAGGTTATGTT
2023 TACAGCTCTCAGCCTTGCGAAGTTTCAAGACCAACAGAAAGGGGTGACAAAGGTTATGTT
 675 -Y--S--S--Q--P--C--E--V--S--R--P--T--E--R--G--D--K--G--Y--V-


2341 CCTTCTGTTTTTATCCCCATCTCAACAATGTGAGTAATGTTAGTCACATGTGAAATATTT
     TTATAAAAAGCTTTCCTATAGGAGATTTAAAGGTAGAGCAGAGTACACATAACTGAGAAC
     AAAGCATTGTAATGTGCAATGTCCCATTTCCTTTAATACATAAGGCTAGCCTTCAGGGCA
     CACTTACCACAATCTATTGTGCCTAAAATTATAAAATTCCCCTTTTATATGCCATATATG
     CCACAGTAAGTTGAGTGTTCTGATATGAAATGATGAATTAGATAACTCAATGTCACAAAT
     AGATGAAGCCCTAGAAATGAGTTCCTGACATAGTAAGTCACCGTGAACTATTATTATTTT
     TTAATCCTTGTCCATATTGACCTTGTTATCTCTTTAAG

                                  CCGAAGTGATTCAACAGAGCCACATTCTCCA
2083 CCTTCTGTTTTTATCCCCATCTCAACAATCCGAAGTGATTCAACAGAGCCACATTCTCCA
 695 -P--S--V--F--I--P--I--S--T--I--R--S--D--S--T--E--P--H--S--P-


2401 TCAGACCTTCTTCCCATGTCTCCAAGTGTGTATGCGGTGTTGAGAGAAAACCTGAGTCCC
2143 TCAGACCTTCTTCCCATGTCTCCAAGTGTGTATGCGGTGTTGAGAGAAAACCTGAGTCCC
 715 -S--D--L--L--P--M--S--P--S--V--Y--A--V--L--R--E--N--L--S--P-

                                                         M
2461 ACAACAATTGAAACTGCAATGAAGTCTCCTTATTCTGCTGAATGACAGGATAMACTCTGA
2203 ACAACAATTGAAACTGCAATGAAGTCTCCTTATTCTGCTGAATGA...............
 735 -T--T--I--E--T--A--M--K--S--P--Y--S--A--E--*-...............


2521 CGCACCAAGAAAGGAAGCAAATGAAAAAGTTTAAAGACTGTTCTTTGCCCAATAACCACA
     ............................................................
     ............................................................

                                                     R
2581 TTTTATTTCTTCAGCTTTGTAAATACCAGGTTCTAGGAAATGTTTGACRTCTGAAGCTCT
     ............................................................
     ............................................................
```

**Figure C1. Primer sequences for analyzing gene expression of STAT4β isoform. (continued …).** The line which begins with the nucleotide number 2221/1963 contains the forward primer sequence for the STAT4β. The primer's sequence is the same as the forward primer for STAT4α forward. The thymine at position 2351 (based on the top row sequencing) is the site of intron retention that is unique to the beta isoform. The reverse primer is located within the same beta specific segment and it is highlighted in grey. Highlighted "TC" – indicates the site of beta specific intron retention.

```
2641 CTTCACACTCCCGTGGCACTCCTCAATTGGGAGTGTTGTGACTGAAATGCTTGAAACCAA
     ............................................................
     ............................................................

                   W
2701 AGCTTCAGATAAWCTTGCAAGATAAGACAACTTTAAGAAACCAGTGTTAATAACAATATT
     ............................................................
     ............................................................


2761 AACAGAAGA
     .........
     .........
```

**Figure C1. Primer sequences for analyzing gene expression of STAT4β isoform. (continued …)**