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Identification Of A Novel Cytokine Inducible Stat5 Phosphoserine Site (ps193) That Positively Regulates Its Transcriptional Activity And Is Found Constitutively Activated In Certain Hematopoietic Cancers

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IDENTIFICATION OF A NOVEL CYTOKINE INDUCIBLE STAT5 PHOSPHOSERINE SITE
(PS193) THAT POSITIVELY REGULATES ITS TRANSCRIPTIONAL ACTIVITY AND IS FOUND
CONSTITUTIVELY ACTIVATED IN CERTAIN HEMATOPOIETIC CANCERS

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by

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Abstract

Hematological malignancies such as leukemia and lymphoma, can develop from aberrant changes in the cell signaling molecules to drive their uncontrolled cellular proliferation and differentiation. Activation, maturation, expansion and differentiation of T cells are critically regulated by the dynamics of various transcription factors activated by a variety of cytokines. Additionally, multiple effector molecules that mediate these T cells dependent signals include the JAK (Janus Kinase)-STAT (Signal transducer and activator of transcription) cascade. These signaling proteins are activated in response to a broad array of cytokines. Constitutively activated JAKs and STATs have been described in several T cell malignancies. A growing body of evidence also suggests that dysregulation of STAT5 may be a major contributor to this event and promote the development of certain types of T cell cancers while the conserved c-terminal domain and tyrosine phosphorylation sites within STATs are important for their dimerization and transcriptional activity, serine phosphorylation has been identified in the constitutively activated STAT molecules in various cancer patient samples. Based on tandem mass-spectroscopic analysis, we have identified a novel STAT5S193 site that undergoes phosphorylation in a cytokine dependent manner. The central hypothesis of this thesis was to elucidate a cytokine dependence of this novel phosphorylation site and determine its function related to transcriptional activity and T cell responses. This site was inducibly phosphorylated in response to IL-2, IL-7, IL-9 and IL-15. Moreover the phosphor-kinetics of the STAT5pS193 were rapid and attained maximal phosphorylation within 15 min and was cytoplasmically localized in the inactive state but nuclear upon activation.

In the second objective of this research, we have investigated key regulatory pathways that control the phosphorylation and dephosphorylation state of STAT5 S193. Based on the inhibitor studies we identified that STAT5 S193 phosphorylation is an mammalian target of rapamycin (mTOR) dependent manner however protein phosphatase 2A (PP2A) negatively regulates its phosphorylation. A cell based (HEK293) reconstitution showed that STAT5pS193 was independent on JAK3 activation. However HEK-293 reconstitution system suggests that this site is not required for receptor recruitment, tyrosine phosphorylation, dimerization nor nuclear translocation of STAT5. However, EMSA and luciferase assay suggest that phosphorylation of STAT5 S193 positively regulates the transcriptional and functional activity of STAT5. Collectively we can conclude that this novel phospho-serine site is a positive regulator of the functional activity of STAT5 in an mTOR and PP2A dependent manner in human lymphocytes.

Given this data, hematopoietic tumors were evaluated for constitutively phosphorylated STAT5 S193. Indeed several established tumor cell lines and primary cancer samples were found to be phosphorylated. These data suggest that STAT5 S193 phosphorylation may be important for the oncogenic activity of STAT5 and may therefore serve as a therapeutic target for controlling those types of cancer.

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Abbreviations

ALL, acute lymphoblastic leukemia; α -pY, anti-phosphotyrosine; APC, antigen presenting cell; CML, chronic myelogenous leukemia; CRAC, calcium-release-activated calcium channel; CA, calyculin A; DAG, diacylglycerol; EGF, epidermal growth factor; ITAM, immunoreceptor tyrosine-based activation motif; γ_c , gamma common cytokine; HTLV-1, Human T-lymphotropic virus Type I; IL-2, interleukin 2; IS, immunological synapse; JAK, Janus Kinase; EMSA, electro-phoretic mobility shift assays; HL, Hodgkin lymphoma; Lck, lymphocyte specific protein tyrosine kinase; LAT, linker of activated T cells; MHC, major histocompatibility complex; MAPK, mitogen-activated protein kinase; mTOR, mammalian target of Rapamycin; MS, mass spectrometry; MW, molecular weight; NFAT, nuclear factor of activated T cells; NLS, nuclear localization signal; OA, okadaic acid; PBMC, peripheral blood mononuclear cell; PDGF, platelet-derived growth factor; PHA, phytohemagglutinin; PIAS, protein inhibitor of activated STAT; PLC- γ , phospholipase C γ ; PP2A, protein phosphatase 2A; PI3K, phosphoinositide 3-kinase; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel-electrophoresis; SMAC, supra molecular activation cluster; SOCS, suppressor of cytokine signaling; STAT, signal transducer and activator of transcription; TAU, tautomycin; TCR, T cell receptor; T_{reg}, regulatory T cell; ZAP-70, ζ -chain-associated protein kinase of 70 kDa;

Chapter 1: Biological relevance of the JAK-STAT pathway in immune function and role in hematopoietic malignancies

1.1 MAMMALIAN IMMUNE SYSTEMS AND HEMATOPOIETIC MALIGNANCIES

The immune system, our principal defense repertoire, shields us from a wide range of pathogenic invasions as well as promotes hyperactive responses of the host. Although innate immunity is our first line of protection against foreign pathogenic invasion or aberrant cellular activities, it does not have unlimited capacity to modulate itself following mutagenic immune challenges. The adaptive immune system generates combinatorial collections of clonally diverse lymphocytes that can potentially ward off any kinds of pathogenic invaders (Cooper MD 2010). Principal components of the adaptive immune system include T cells, B cells and the participation of various phagocytic and dendritic cells. Clonal populations of T and B cells are generated with the help of professional antigen presenting cells to coordinate our immune response. Another interesting feature of the adaptive immune system is its ability to maintain memory cells which can rapidly respond to recurring infection.

This complex mammalian immune system is fine-tuned so as not to be hyperactive or auto reactive towards its host. The development and maturation of immune cells are critically dependent on various micro-environmental factors such as cytokines, interaction with different types of lymphoid derived cells, signaling machineries and self-tolerance towards auto-antigens. Whenever these micro-environmental factors, extrinsic or intrinsic, become dysregulated it can lead to either hyper or hypo-active adaptive immune cells. As a result of which, the host is susceptible to different kinds of diseases such as severe combined immunodeficiency (SCID) (Elhasid R 2002), human immunodeficiency, rheumatoid arthritis, cancer (Becher 2007), diabetes mellitus type I (Akira Shimada 2003) and lupus erythematosus (Lau 2003). Aberrant

proliferation of human lymphocytes can result in various types of leukemias, lymphomas and myelomas. An estimated 139,860 people in the United States were diagnosed with these three cancers in 2009 alone (Staudt LM 2002). New cases of leukemia, Hodgkins and non-Hodgkins lymphoma and myeloma account for 9.5 percent of the 1,479,350 new cancer cases diagnosed in the United States this year (Staudt LM 2002). Approximately 53,240 people died in the United States in 2009 due to these cancers accounted for almost 9.5 percent of total cancer related deaths. Among these, leukemia is the fifth most common among men and seventh most common among women (Staudt LM 2002).

With an ever growing population suffering from blood related diseases, it is of paramount important to know the etiology of these cancers. There are currently 40 different types of blood cancer that can be broadly classified into two major categories-leukemias and lymphomas (Staudt LM 2002; Guerrouahen 2010). In the case of leukemia, there is an aberrant proliferation of anomalous white blood cells that migrate into the circulation. On the other hand, lymphomas, a solid neoplasia of the lymph nodes, causes the lymph node to expand (Jaffe 2001; Staudt LM 2002). Based on the specific cell type, leukemia can be classified into two categories-lymphocytic and myelogenous leukemia. Usually lymphocytic leukemia is related to aberrant proliferation of T and B cells while myelogenous is associated with uncontrolled growth of myeloid cells such as erythrocytes, megakaryocytes and macrophages.

Leukemias can persist as acute or chronic phases. In the acute phase, there is a rapid proliferation of immature cells that lead to an overpopulated bone marrow while the chronic phase is indolent and often asymptomatic (Jaffe 2001). Cytotoxic chemotherapeutic treatments are often found to be suitable for the acute phase however only bone marrow transplantation is one of the few treatment options for the chronic phase (El-Helw 2005). Various types of

leukemia have been predominantly identified among young children between the ages 0-10 and aged people >55 (INHLFPF 1993). With respect to lymphomas, they can migrate from one lymph node to another or distant organs via the lymphatic system. Lymphomas have been classified into two groups: Hodgkin's lymphomas (HLs) and non-Hodgkin's lymphomas (NHLs)(INHLFPF 1993). There are five subtypes of HLs and about 30 subtypes of NHLs. Hodgkin's disease occurs predominantly among young adults (16-34 yrs) and aged people >55 years however NHL is more likely to occur in older people (S. 1966; Staudt LM 2002). HL disease develops from a single abnormal B lymphocyte lineage. NHL may derive from abnormal B or T cells that can be distinguished by their unique genetic markers (G. 2002).

1.2 THREE SIGNALING PATHWAYS REQUIRED TO ACTIVATE, MATURE AND EXPAND NAIVE T CELLS

Immunological response occurs within the adaptive immune system by a multistep process where naïve T cells first interact with antigen presenting cells (APCs) to undergo clonal expansion. Interaction between T cells and APCs occurs in the thymus, secondary lymphoid organs and peripheral tissues to promote their development, maintenance, activation, helper and effector function (Cannon JL 2002; Becher 2007). Complete activation of T cells and their clonal expansion towards a specific immune response and effector phase requires three consecutive signals. Signal I and II are generated by the immunological synapse (IS) formed between the T cell receptor (TCR) of naïve T cells and the peptide-major histocompatibility complex (MHC) of APCs. Supramolecular activation clusters (SMACs) are the mature form of IS where TCR engages with either MHC class I or II on APCs to deliver signal I that leads to activation of the tyrosine kinase Lck. Subsequently Lck phosphorylates tyrosine residues within the immunoreceptor tyrosine-based activation motifs (ITAMs) of the invariant CD3 and ζ -chains to facilitate the recruitment and activation of ζ -chain-associated protein kinase of 70 kDa (ZAP-

70) (Burkhardt JK 2008; Smith-Garvin JE 2009). A series of the adapter proteins undergo phosphorylation by activated ZAP-70 that promotes recruitment of phospholipase c (PLC- γ), phosphoinositide 3-kinases (PI3K), IL-2-inducible Tcell kinase (Itk) and Ras to the activated TCR complex. Activated PLC- γ hydrolyzes phosphatidylinositol-4,5 biphosphate into diacylglycerol (DAG) and inositol 1, 4, 5-triphosphate (IP3) (Smith-Garvin JE 2009). IP3 binds to its cognate receptor IP3R and induces the release of intracellular calcium from intracellular stores. Subsequently the opening of calcium-release-activated calcium (CRAC) channels in the plasma membrane leads to sustained increases in intracellular calcium levels (Wang Y 2000; Huang GN 2008). Binding of calcium to calmodulin leads to activated calcineurin that dephosphorylates certain phosphoserine residues in nuclear factor of activated T cells (NFAT) protein exposing its nuclear localization signal (NLS) upon which it can translocate to the nucleus and bind to specific response elements to induce the transcription of genes such as interleukin-2 (IL-2) and IL-2R α subunit (Wang Y 2000; RS. 2001).

Costimulation of T cells is mediated by an accessory signal II that is generated by the engagement of T cell costimulatory receptors. Engagement of CD28 receptor on T cells with B7-1/B7-2 on APCs can activate the jun kinase, JNK that is required for the formation of activator protein 1(AP1) complex for induction of IL-2 and IL-2R gene transcription (Lenschow DJ 1996; Greenfield EA 1998). Extensive experiments suggest that failure to induce signal II leads to reduced activation levels of the MAPKs extracellular-signal-regulated kinase 1 (ERK1) and/or ERK2 (Altman A 2000; Coudronniere N 2000). In turn, minimal RAS activation occurs that leads to reduced expression and upregulation of proteins yielding an anergic state unable to generate following full activation of T cells.

Complete induction of Signal I and II is required for the production and secretion of gamma common cytokines- IL-2, 4, 7, 9, 15 and 21 (DiSanto JP 1996). Several studies suggest that IL-2 mediated signal III activates JAK3-STAT5 pathway that subsequently induces the expression of various cell cycle regulators to execute the expansion and followed by differentiation of T cells (Lin JX 2000). (Shown in Fig. 1.1)

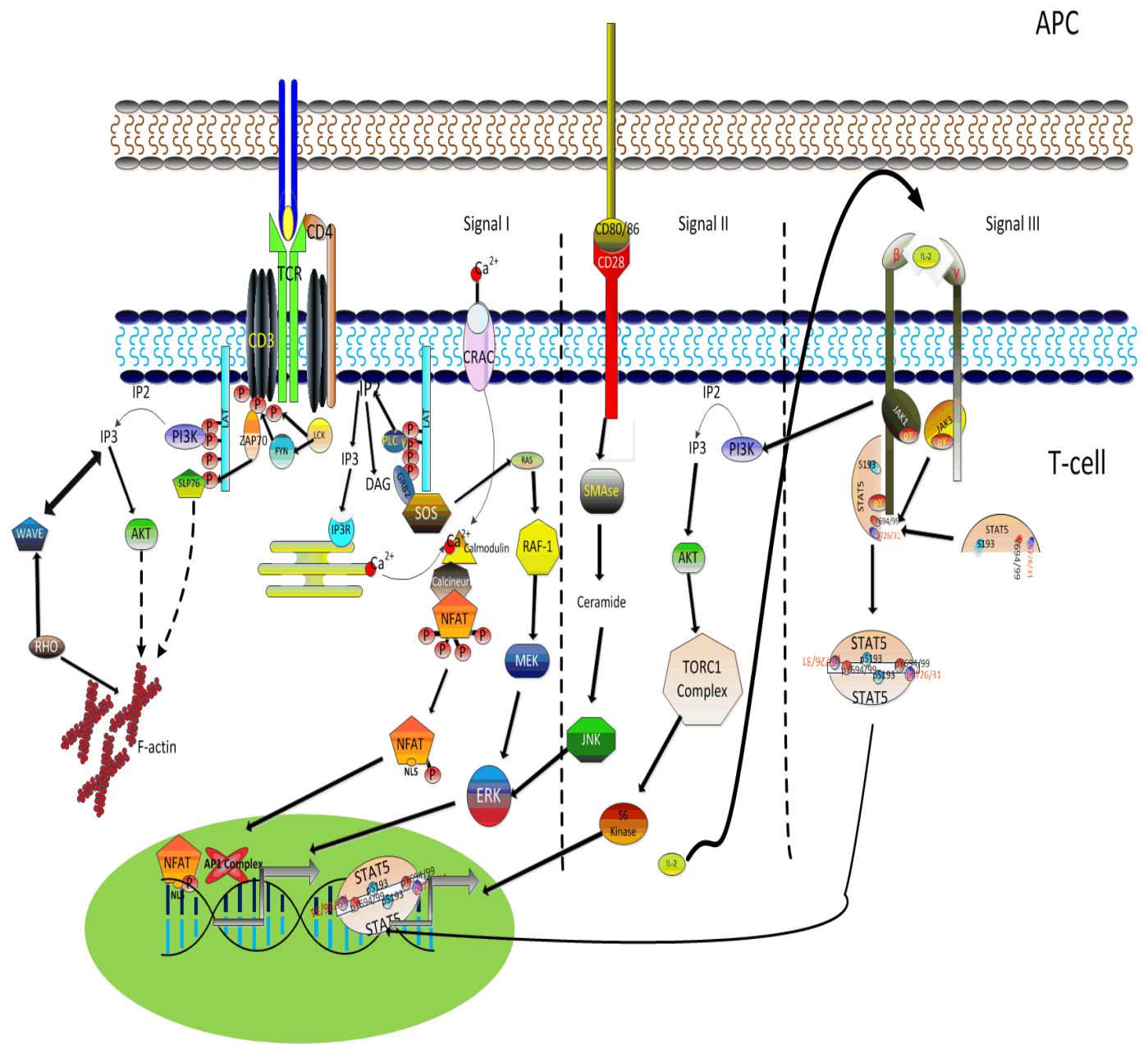


Figure 1.1: Model for the maturation of naïve T cells by APC.

Supramolecular activation cluster (SMAC) is generated between naïve T cells and APC. There are three signals to activate, mature and expand naïve T cells. Signal I is generated by the cluster formation between peptide-MHC complex of APC and CD3 receptor of naïve T cells. Signal II is generated by the interaction between CD80/86 of APC and CD28 of naïve T cells. Signal I and II are required for the complete activation of T cells that drives NFAT activation

and transcription of interleukin-2 (IL-2). IL-2 drives signal III by the activation of JAK3-STAT5 pathway to proliferate activated T cells.

1.3 BACKGROUND INFORMATION OF JAK-STAT PATHWAY

Despite decades of research and drug development, we are unable to elucidate a suitable answer for the exact cause and treatment of certain types leukemia and lymphoma as multiple levels of dysregulation have been identified in various types of blood cancer (S. 1966; Baldus 2006). Potential limitations of the standard chemotherapeutic measures and inadequacy of suitable treatments have motivated researchers to explore newer and less toxic therapeutic agents to uncouple their cellular targets to prevent the atypical growth of blood cells. Henceforth, there should be more rational approaches to understanding the etiology of hematological malignancies and to identify effective cellular targets. Activation, maturation, and such pleiotropic pathways that can control such events belong to the JAK-STAT pathway to drive differentiation of many types of cells including lymphocytes (Rawlings 2004; J. 2007).

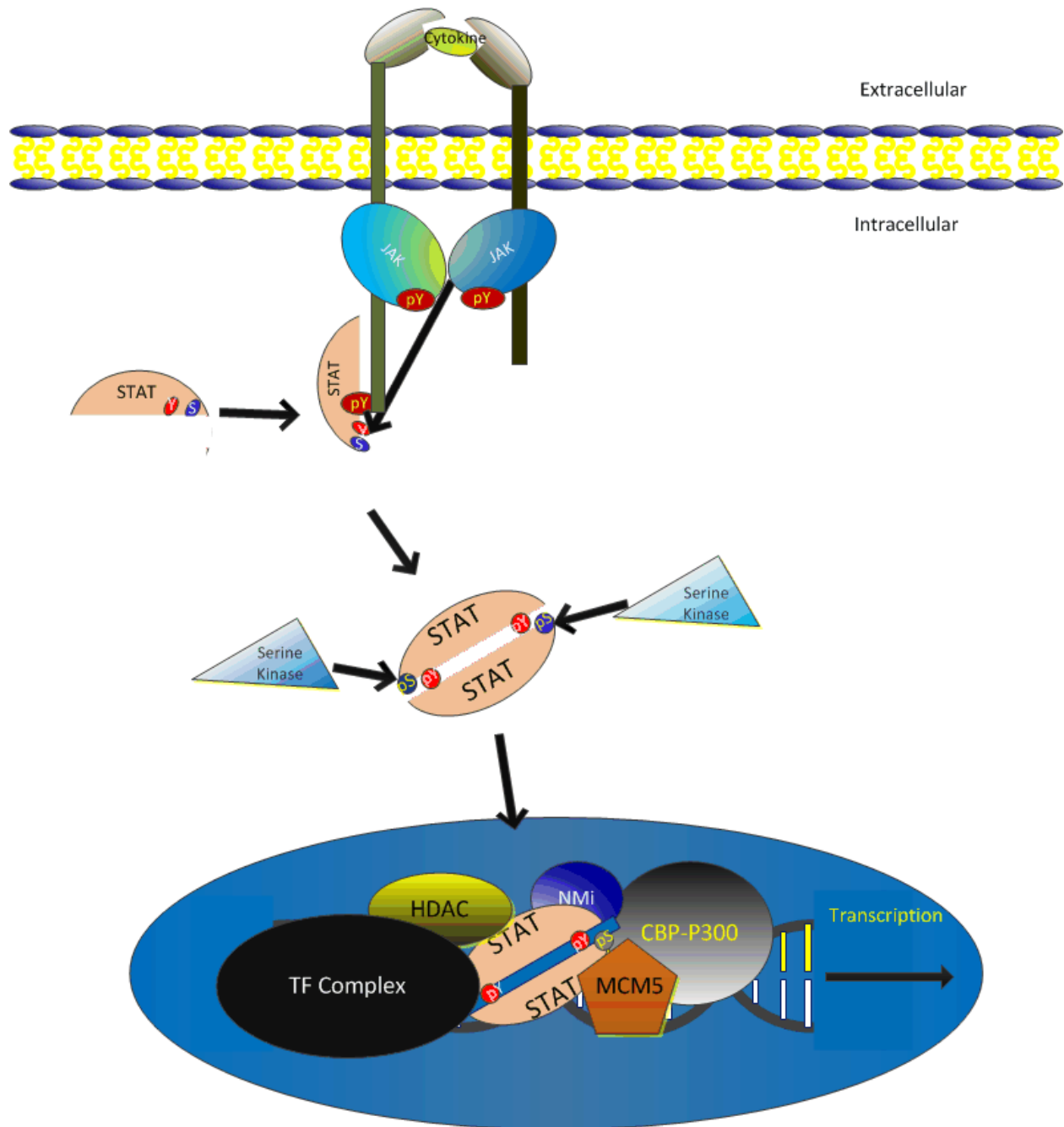
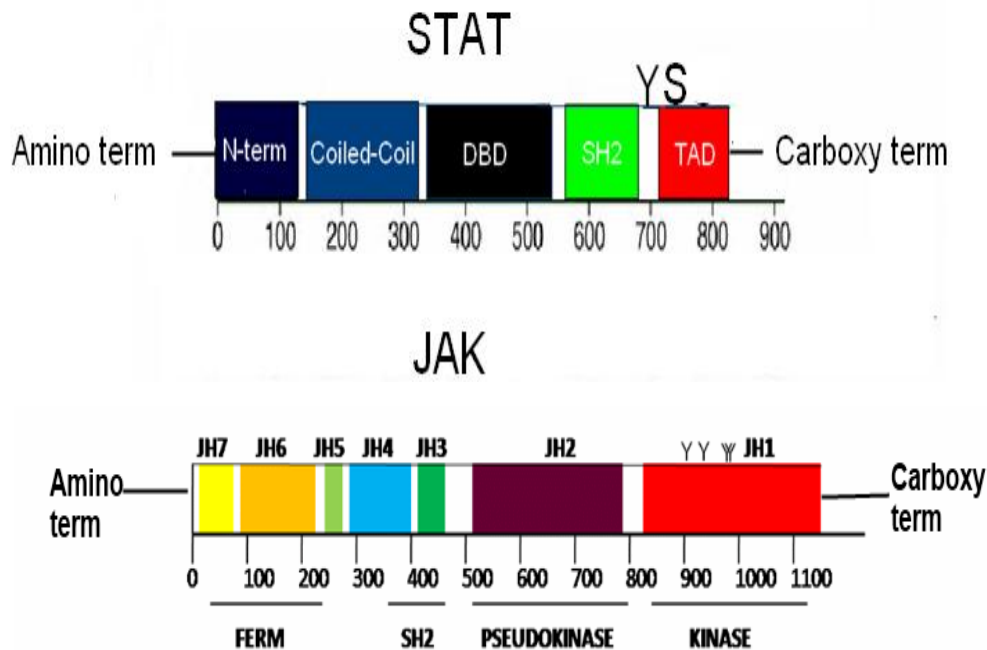


FIGURE 1.2: Schematic illustration of the canonical JAK-STAT pathway.

A schematic representation of the Janus kinase (JAK)–signal transducer and activator of transcription (STAT) pathway. The activation of JAKs after cytokine stimulation

results in the phosphorylation of STATs, which then dimerize and translocate to the nucleus to activate gene transcription.

One such pleiotropic pathway is the JAK-STAT cascade, which plays an important role in hematopoiesis and cellular differentiation via a broad spectrum of cytokines, growth factors and hormones (Leonard WJ 1998; Benekli 2003). Cytokine receptors do not harbor intrinsic kinase domains. However, upon ligand binding, the receptor undergoes a change in structural conformation which leads to activation of the pre-associated JAK molecules with these receptors (Gadina M 2001). In response, JAK molecules are auto activated and they phosphorylate certain key residues on the cytoplasmic domain of the receptor upon which STAT molecules dock and subsequently become phosphorylated by JAK at a conserved tyrosine residue at the C-terminus promoting dimerization and their translocation to the nucleus. STAT molecule can bind specific response elements to induce the transcription of STAT specific genes (Fig. 1.2). However, STAT molecules also undergo activation by growth factors which have autocatalytic activity such as PDGF, EGF which in turn directly phosphorylate STAT molecules to drive a similar event (Valgeirsdóttir S 1998).



Seven mammalian STATs range in size from 120-130 KDa. All of them contain five domains- amino terminal, coiled-coil, DNA binding domain, src homology 2 (SH2) and transactivation domain (TAD). Tyrosine and serine residues at the carboxy-terminal ends are conserved in all STATs. However JAKs are comparatively larger and range from 120-130 KDa. Seven JAK homology domains (JH) JH1-JH7 exist from carboxy to amino terminal ends.

STATs were originally identified within the interferon receptor pathway. STATs 1 & 2 which subsequently expanded to seven mammalian STATs (STAT1,2,3,4,5A,5B and 6) that are highly homologous in several regions and function to regulate diverse biological response in various tissues under activation of many distinct cytokines, hormones and growth factors (O' Shea, 2002). Seven mammalian STATs have six domains which composed of an amino

terminal domain, a coiled-coil domain, DNA binding domain (DBD), a linker domain, an SH2 domain and a transactivation domain (TAD)(Reich N.C. 2006).

The mammalian STAT family proteins have molecular weights ranging from 80-113 kDa and sequences which code for up to 851 amino acids (Copeland NG 1995). Chromosomal locations of human STAT genes are scattered on three loci. *STAT1* and *STAT4* are located in chromosome 2 however *STAT2* and *STAT6* map to chromosome 12 (Copeland NG 1995). While *STAT3*, *STAT5A* and *STAT5B* cosegregate and map to chromosome 17 (S. 1999). Similarly mouse *STATs* are located on different chromosomes. Based on this information, it has been suggested that the human STAT family has undergone tandem duplication of an ancestral locus followed by dispersion of the linked loci to different chromosomes (Reich N.C. 2006). Finally, the *STAT5A* and *STAT5B* genes have arisen due to gene duplication that occurred after this translocation (S. 1999).

1.4 EVOLUTIONARY PERSPECTIVE OF THE JAK-STAT PATHWAY

From an evolutionary perspective, JAK-STAT pathway is present in both vertebrates and invertebrates. A STAT-like protein, Dd-STAT, was discovered in *Dictyostelium discoideum* which regulates stalk cell differentiation (Kawata T 1997). The genomic sequence of the mosquito *Anopheles gambiae* and the worm *Caenorhabditis elegans*, also express one STAT gene most closely related to human STATs (Rawlings 2004). This data indicate that this pathway is highly conserved across phyla and plays critical and diverse biological roles in simple and complex model organisms. One such example is in case of the fruit fly, *Drosophila melanogaster*, where D-STAT is critical for segmentation, eye development, cell growth, haematopoiesis, sex determination and tracheal development (Hombría JC 2002).

The family of mammalian JAKs consists of four distinct (protein's) JAK1, JAK2, JAK3 and TYK2 that are relatively large tyrosine kinases with approximate molecular weights of about 120-135 kDa. Expression of JAK3 is restricted to lymphoid tissue while other JAKs are ubiquitously expressed. JAKs are constitutively expressed at high levels in NK cells and inducible in T cells, B cells and myeloid cells (Leonard WJ 1998). JAKs have a tyrosine kinase and pseudokinase domains in tandem that are unique among non-receptor tyrosine kinases. They are located in segments of homology known as JAK homology domains (JH). All JAKs have seven homology domains (JH1 to JH7), JH1 harbors the catalytic domain, JH2 pseudokinase domain and 300 amino acid residues from N terminus forms a FERM domain (four point1, ezrin, radixin and moesin) that is used to dock to the receptor. However in certain receptors such as interferon α/β R, EpoR, PrlR, and oncostatin M (OSM)R, this domain provides a chaperone function, influencing plasma membrane expression of the receptor (Leonard WJ 1998; J. 2007). Although many cytokine receptors have common chains and activate common JAKs, it is still a matter of speculation about the modus operandi to transmit these signals in cytokine specific manner to regulate unique genes that can be up regulated to execute their specific functions (Gadina M 2001; Ross JA 2007).

1.5 BIOLOGICAL IMPORTANCE OF JAKS AND STATS BASED ON KNOCKOUT MOUSE MODELS

The JAK/STAT pathway regulates a diverse array of biological processes. All four JAKS and seven STATs members have been genetically deleted in murine systems, in addition to the creation of conditional alleles for genes whose loss of function leads to embryonic or perinatal lethality (Ross JA 2007). JAK1^{-/-} mice display defects in lymphoid cell development and perinatal death possibly due to disruption of JAK1-dependent signaling pathways important for cytokine/growth factor receptor subfamilies, including IFNs, TCGFs (i.e. IL-2, IL-4, IL-7,

IL-9, IL-15, and IL-21), and gp130 subunit cytokines (i.e. IL-6, IL-11, LIF, OSM, CNTF, and CT-1 (Ross JA, 2007). JAK2 which is widely recognized as a key mediator of erythropoiesis through multiple hematopoietic factors, including erythropoietin (EPO), thrombopoietin, IL-3, and IL-5 (Parganas E., 1998), its knockout model shows embryonic lethality which is likely due to a failure of erythropoiesis. The JAK3 knockout, an important molecule for complete T cells activation via the signal 3 pathway (Ross JA, 2007), exhibits a severe combined immunodeficiency (SCID). However in the case of TYK2 which is activated by IFN α/β and IL-12, instead showed hypersensitivity towards pathogens, and similar to IL12 signaling defects. In addition to being a mediator for pro-inflammatory cytokine signaling, TYK2 has been identified as an important regulator for the signaling and expression of the immunosuppressive cytokine IL-10 (Shaw M. H., 2006).

As our understanding of the many roles that JAK play in cellular function, it has become important to explore the functional role of its primary substrate STATs. Great insight has been gained by generating murine knockouts models (S. 1999; Rawlings 2004). As stated earlier, STAT1 undergoes activation by IFN α/β and IFN γ . *STAT1* knockout mice revealed selective signaling defects in their response to both Type-I and Type-II IFNs and other cytokines and growth factors, including growth hormone, interleukin-2 (IL-2), EGF, and angiotensin (S. 1999). These studies revealed that IFN α and IFN γ induced expression of major histocompatibility complex (MHC) class II protein, as well as interferon regulatory factor-1 (IRF-1), guanylate-binding protein 1 (GBP-1), MHC class II transactivating protein (CIITA), and complement protein C3 were absent or diminished in *STAT1* KO mice (Decker T 1997). Besides increased viral infection and poor response of T cells towards IFN- α/β activation, STAT2 was proactive or prevented the apoptosis of T cells in response to IFNs.

Unlike other members of STAT family, *STAT3* KO mice have embryonic lethality in which embryos showed rapid degeneration between 6.5 days post coitum (dpc) and 7.5 dpc (Copeland NG 1995). *STAT3* deficient T cells showed complete impairment of IL-6 mediated proliferation as well as partial defect in IL-2 induced cell proliferation. Macrophages and neutrophils isolated from *STAT3* KO mice showed aberrant inflammatory responses which resulted in the development of chronic enterocolitis (S. 1999; Bromberg J 2000). *STAT3* plays an active role to induce apoptosis of mammary epithelial cells during involution with a somewhat reciprocal function with respect to the function of *STAT5* (DA. 1999; Bowman T 2000). Thus decreased apoptosis of epithelial cells delayed involution after weaning and severely reduced expression of insulin-like growth factor binding protein 5.

STAT4 and *STAT6* have been linked with the developments of T_H1 and T_H2 cells upon activation by IL-12 and IL-4 respectively (S. 1999). *STAT4* KO mice showed impaired IL-12-mediated increases in IFN- γ production, cellular proliferation, and NK cell cytotoxic activity of lymphocytes was impaired (S. 1999). Similarly *STAT6* KO mice showed defects in IL-4-mediated increases in surface expression of MHC class II and IL-4 receptor α chain, cellular proliferation, and IgE class switching (S. 1999).

Although *STAT5A* and *STAT5B* share 96% sequence identity, they have functional differences based on their knockout models. Impaired lobulo-alveolar outgrowth during pregnancy and defective lactation was observed in *STAT5A* knockout females. Besides breasts tissues, the biological importance of *STAT5A* KO was also found in prostate epithelium where it was characterized by acinar cyst formation, local disorganization and shedding of the epithelial cells to the glandular lamini (Wei L 2008). In contrast, *STAT5B* knockout females did not show significant impairment in mammaryogenesis. Conversely *STAT5B* knockout males showed a loss

of sexually dimorphic pattern as a result of impaired pituitary growth hormone response. *STAT5A* and *STAT5B* double knockout female mice showed complete infertility due to the impaired development of functional corpora lutea in the ovary (S. 1999). From the immune system perspective, germline amino terminal truncated *STAT5A* and *STAT5B* suggests to us that *STAT5A* and *5B* are not indispensable for T or B cell development (Wei L 2008). However peripheral B cell populations and bone marrow precursors were less in number but not removed and CD4⁺ T cell populations were increased at the expense of CD8⁺ T cells (Wei L 2008). Complete deletion of *STAT5A/B* locus showed > 99% perinatal lethality and survivors are highly runted and anemic. Phenotypes of these mice showed that B cells development were blocked at the prepro B cell stage and thymocyte population is profoundly reduced at the double negative stage and NK cells are completely absent in these mice (Wei L 2008). In addition to these phenotypes, thymocyte numbers were markedly reduced to 98% and abolished TCR γ rearrangement. In the case of regulatory T cells (T_{reg}), interleukin-2 (IL-2) which plays an important role in its development and homeostasis (Wei L 2008). Human devoid of either IL-2 or its receptor subunits is often associated with autoimmune disorders. *STAT5A* and *5B* deficient mice showed a reduction of T_{reg} population in both thymus and periphery. From these studies it is clear that *STAT5A* and *5B* are critically important for lymphoid cells development, maturation and differentiation (Wei L 2008).

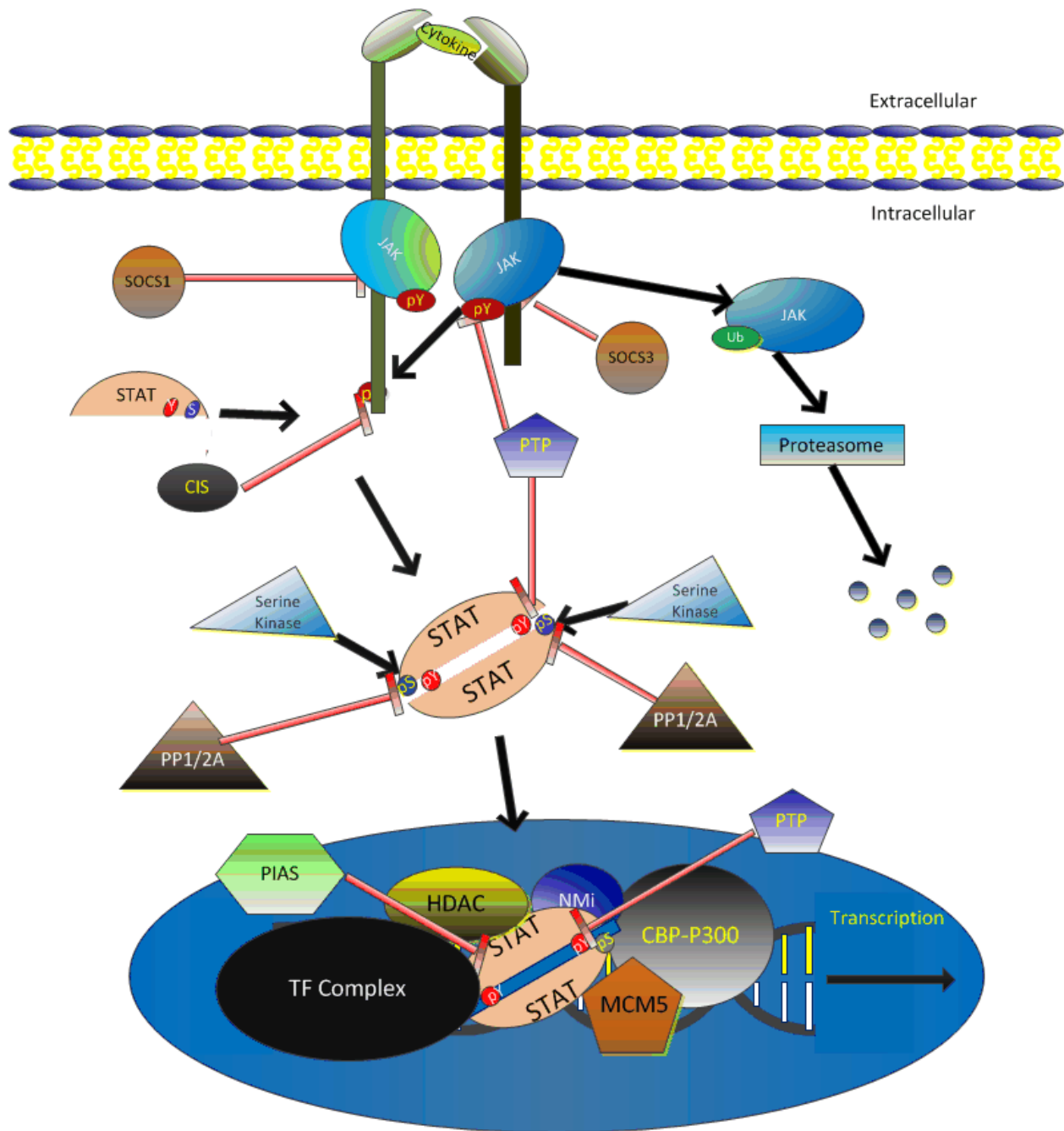


FIGURE 1.4: Schematic illustration of negative regulators of JAK-STAT pathway.

The Janus kinase (JAK)–signal transducer and activator of transcription (STAT) pathway undergoes multiple levels of regulations. Suppressor of cytokine signalling (SOCS) proteins, protein tyrosine phosphatases (PTPs), such as SRC homology 2 (SH2)-domain-containing PTP1

(SHP1), SHP2, CD45 and T-cell PTP (TCPTP), and ubiquitin-mediated protein degradation inhibit JAKs. STATs can be negatively regulated by PTPs (such as PTP1B and TCPTP) in the cytoplasm, and by PIAS (Protein inhibitor of activated STAT) proteins, as well as PTPs (such as TCPTP and SHP2), in the nucleus (Shuai K 2003).

1.6 NEGATIVE REGULATORS OF JAK-STAT PATHWAY

Genetic deletion of JAKs and STATs has dramatically increased our understanding of these proteins (S. 1999). They also showed highly specific roles in controlling different immune responses. Activation of this pathway results in transient gene transcription. It is equally important to “turn-off” the JAK-STAT pathway after activation. Negative feedback loops are activated to suppress this pathway and to prevent malignant transformation and to maintain proper cell homeostasis. Current research suggests that the JAK-STAT pathway can be negatively governed at multiple levels by distinct proteins. Key regulators include the suppressor of cytokine signalling (SOCS) proteins and the recently discovered protein inhibitor of activated STAT (PIAS) family, various protein tyrosine phosphatases (PTPs) and serine/threonine phosphatase (Shuai K 2003). In addition to these classical regulators, STAT molecules can be post-translationally modified via other cell-signaling pathways to positively or negatively regulate this pathway (Reich N.C. 2006).

Biochemical and genetic studies indicate that SOCS proteins play important physiological roles in the negative feedback loop of the JAK-STAT pathway. It is interesting that these proteins do not perturb the initial activation of this pathway. SOCS protein levels are low in unstimulated cells; however they can become rapidly induced and elevated following cytokine activation. SOCS family of proteins is comprised of eight members: CIS (cytokine-inducible SH2 domain protein) and SOCS1–SOCS7 (Valentino L 2006). Ubiquitin-mediated degradation

of the receptor by binding to elongins B and C, known components of a ubiquitin E3 ligase, is the classical approach to suppress cytokine signaling however they can directly inhibit JAKs (Zhang JG 1999). These proteins contain a carboxy -terminal SOCS box flanked by a classical Src homology 2 (SH2) domain while amino terminal end harbors a Kinase Inhibitory Region (KIR). KIR plays the critical role in blocking JAK catalytic activity by its SH2 domain which binds to the activation loop of the catalytic domain through any phosphorylated JAKs tyrosine residue (Zhang JG 1999). SOCS3 mechanism of action for inhibiting JAKs is slightly different. It first binds to the receptor, then to JAKs. Another member of this family CIS directly competes for STAT docking sites on the receptor to negatively regulate this pathway.

JAKs and STATs are tyrosine and serine phosphorylated proteins. Thus another mechanism to negatively regulate STATs is via tyrosine phosphatases such as SHP1, SHP2, CD45, PTP1B, T-cell PTP (TCPTP) and a receptor tyrosine phosphatase CD45 which act on this pathway through dephosphorylation. These phosphatases are characterized by the presence of two SH2 domains amino-terminal to the canonical 250 amino-acid-long tyrosine phosphatase domain (Valentino L 2006). Usually these cytoplasmic phosphatases bind to the receptor or activated JAK molecules to dephosphorylate their substrate (Valentino L 2006). SHP1 is predominantly expressed in hematopoietic cells however SHP2 is ubiquitously expressed; sharing 55% sequence homology. Receptor tyrosine phosphatase CD45 is abundantly expressed in hematopoietic cells and directly binds to all JAKs to dephosphorylate and inactivate them (Shuai K 2003). Mice deficient in CD45 showed hyperphosphorylated JAK1 and JAK3 molecules that was associated with a loss of antigen responses in T and B lymphocytes. Similarly phospho-tyrosine phosphatase 1B (PTP1B) and T cell protein tyrosine phosphatase (TC-PTP) are ubiquitously expressed. PTP1B preferentially dephosphorylates JAK2 and TYK2

while TC-PTP interacts principally with JAK1 and JAK3 as per their sequences recognition (Shuai K 2003).

A newly recognized family of proteins regulates STAT function is Protein Inhibitors of Activated STATs (PIAS) which consists of PIAS1, PIAS3, PIASx and PIASy. Recent studies indicate that PIAS have a small ubiquitin-like modifier (SUMO)-E3-ligase activity (Shuai K 2005). Co-immunoprecipitation studies suggest that PIAS3 and PIASx interact with STAT3 and STAT4 respectively, while PIAS1 and PIASy are able to interact with STAT1 (Liu B 2001; Shuai K 2005). These PIAS-STAT interactions negatively regulate the activity of a STAT(s) within the complex. From the current results, it has been suggested that PIAS facilitates the recruitment of the histone deacetylases (HDACs) to repress the DNA binding activity of STATs (Liu B 2001).

Additional negative regulatory molecules also exist to downregulate the JAK-STAT pathway. Indeed posttranslational modification can occur as well. Recently JAK1 has been identified to undergo modification by an interferon-stimulated gene 15 (ISG15) that is a ubiquitin like protein member (Malakhova OA 2003). However ISGylation can positively regulate JAK1 activity by UBP43 (another name is ubiquitin-specific protease18, USP18) which removes ISG15 from JAK1 to regulate its activity (Malakhova OA 2003). In addition to serine/threonine phosphatase can also play an active role to negatively regulate the JAK-STAT pathway through dephosphorylation of conserved phosphorylated serine residues as well as in the cytoplasmic domains of its cognate receptor. Recently our laboratory reported that IL-2 mediated JAK3-STAT5 pathway is negatively regulated by protein phosphatase type 2A (PP2A) group of phosphatases that occurs at multiple levels (Ross JA 2010). Previous studies also suggested that blocking PP1 and PP2A can reduce STAT3 and STAT6 induction profiles (Ross

JA 2010). Overall, these negative regulatory pathways provide detailed insights about the downregulation of JAKs and STATs that might play an important role in aberrant activation but also provide a place to search where the hyperactive JAKs and STATs have been identified in patient samples.

1.7 FUNCTIONAL CHARACTERIZATION OF hSTAT5

STAT5 regulates IL-2 mediated expansion of T cells and the development of B cells from prepro stages (Wei L 2008). It is critically required for lymphoid cells development, expansion and thus represents a therapeutic target to treat in certain blood cancers. Although there are numerous targets in blood cancer therapy, STAT5 has been gained insights as a novel biomarker tool to get a suitable answer for this. STAT5 exists in two forms due to gene duplication and share 91% sequence identity in human and mouse. This data suggests their evolutionary importance in biological functions (Teglund S 1998; Grimley PM 1999; S. 1999). These STAT5 isoforms share a highly conserved coiled-coil region, DNA binding domain, linker region and SH2 domain however carboxy-terminal of these two proteins show the maximum divergence (Teglund S 1998). The SH2 domain of STAT5 is highly conserved by STATs indicating that they share similar mechanisms of receptor docking and dimerization. The amino terminal domain of STAT5A and 5B facilitates stabilization of STAT5 dimers to form tetramer (Soldaini E. 2000). Another reason for tetrameric structure is two gamma activated sequence (GAS) motifs are positioned tandem with a 6bp space due to non-consensus motif (Soldaini E. 2000). These two isoforms share overlapping biological roles under cytokine stimulation however specific functions of these two proteins have also been identified. STAT5A and 5B undergo homo or hetero dimerization in different tissues. STAT5 undergoes activation by broad spectrum of stimulants such as cytokines, growth factors and hormones that include IL-2, IL-3, IL-5, IL-7,

IL-9, IL-15, granulocyte-macrophage colony-stimulating factor, erythropoietin, growth hormone, thrombopoietin, epidermal growth factor and platelet-derived growth factor (Teglund S 1998; Ross JA 2007). In case of IL-2 mediated activation of STAT5, it binds with the help of its SH2 domain to either phosphorylated Tyr-392 or Tyr-510 of IL-2 receptor beta chain as shown by Gel-shift assay (Gaffen SL 1996).

Among the five structurally and functionally conserved domains of STATs, the coiled-coil domain adjacent to the amino-terminal domain plays an important role in interaction with various co-activators as well as co-repressors to regulate STAT5 transcriptional activity. In case of STAT5A and 5B, the coiled-coil domain that ranges from 138-330 amino acids contains four anti-parallel α -helical regions that are connected by helical turns. Helices 1 and 2 are comparatively larger than helices 3 and 4. These helices provide flexibility in structural bending that might provide suitable interactions with various proteins. The coiled-coil domain has been well characterized in STAT3 where this domain is critically important for SH2 mediated docking on the receptor by Asp 170 and to a lesser extent Lys 177 (Soldaini E. 2000) as well as Arg 214/215 is required for nuclear translocation (Ma J 2003). However very limited research has been done on the STAT5 coiled-coil domain. Presently it is known that helix 2 of the STAT5 coiled-coil domain interacts with a chaperone called N-myc interactor (NMI) that stabilizes interaction with the transcriptional coactivator CREB binding protein (CBP)/p300 (Zhu M 1999). From loss of functional studies it has been found that NMI positively regulates STAT5 transcriptional activity (Zhu M 1999). Although STAT5 does not have a classical NLS signal, usually a stretch of several basic amino acids, a recent finding suggest that helix 1 of STAT5 in the coiled-coil domain (amino-acids from 142-149) may interact with various transporter molecules to regulate nuclear translocation of both phosphorylated and unphosphorylated

proteins (Reich 2008). As this domain is juxtaposed to the DNA binding domain of STAT5, it could be further investigated to determine which types of regulators might play a role in regulating STAT5 transcriptional activity.

The DNA binding domain of STAT5 is the largest domain (a.a. 332 to 583). This domain contains 11 stranded β -sheets with an immunoglobulin-type of fold that are anti-parallel to each other (Chen X 1998). These β -sheets run parallel to the major axis of this domain which is perpendicular to the DNA structure. The result is one side of these sheets facing towards the DNA molecule (Chen X 1998). STAT1 bound to DNA has been crystallized in the tyrosine phosphorylated form (Chen X 1998). Here the DNA binding region has been divided into four segments. Segment 1 connects with the major groove via a positively charged Lys-336 that goes inside the major groove. Segment 2 is connected distantly by Arg-378 to the phosphate group (Chen X 1998). Segment 3 connects with the minor groove and to keep in contact with the major groove with the aid of a phosphate group. The last segment on the carboxy-terminal end associates with the DNA binding domain is critical for DNA recognition. This segment has been found in deep contact with the major groove by Asn-460 (Chen X 1998). Another important feature of this domain is that it facilitates the stabilization of the dimer through side chains and backbones of the various residues including Asn-361, Val-362, His-363, Met-364, and Asn-365 belonging to the loop joining β b- β c as well as Ser-449 and Ser-452 belonging to the loop joining β f- β g (Chen X 1998).

The SH2 domain harbors (a.a. 593-670) three β strands followed by two α -helices ($\alpha\beta\beta\alpha$) (Chen X 1998; Neculai D 2005). The first β sheet contains a positively charged Arg residue that facilitates STAT5 docking to the tyrosine phosphorylated residue of its IL-2R β

chain (Neculai D 2005). In addition, the SH2 domain is important for reciprocal dimerization process for a fully active STAT5.

The carboxy-terminal end of STAT molecules i.e., distal to the phosphorylated tyrosine residue, shows lower frequency of conserved amino acids (Copeland NG 1995). Of note, however is a conserved PMSP motif between localized position 720-730 for STAT1, and 4, a SSPD motif for STAT6 located around 750-760 (Wang Y 2004) and a PSP motif for STAT5A and 5B (Kovarik, 2000). Based on serine to alanine mutations e.g. - in case of STAT1 S to A mutation caused 80% reduction in interferon-gamma (IFN- γ)-induced transcription factor activity (Kovarik, 2000). Similarly for STAT3, the mutation resulted in a 50% reduction of IFN- α response (Zilong Wen, 1995). STAT4 which undergoes activation by interleukin-12 during T-helper 1 differentiation (T_H1) differentiation and produces IFN- γ , failed to restore these functions following a mutation of S721A (Morinobu A 2002).

In the case of STAT5 which exists in two isoforms, STAT5A and 5B, also contain PSP motif at 726 and 731, respectively. Supportive evidence in the form of functionally relevant pro-B and T cell lines lends credence to our findings, as delivery of carboxyl terminal-deleted STAT5 variants lacking these serine residues inhibits cytokine-driven cell proliferation and cell cycle gene expression. Previous studies showed that phosphorylation of the serine sites were critical for maximal growth hormone-regulated gene transcription (Park SH, 2001) and suitable duration of DNA binding activity (Iwan Beuvink, 2000) to regulate gene expression. Although the importance of the serine residue within the PSP motif of STAT5 was characterized based on cell specific experimental outcomes, it is critical to assess the functional role of phosphorylated serine (726/731) residue of STAT5 via *in vivo* studies. Recent studies suggest that the TAD

domain is important in mammary gland development especially milk production as well as survival of mammary epithelial cells in transgenic mice (Iavnilovitch E 2006).

1.8 STAT5 REPRESENTS A THERAPEUTIC TARGET FOR CANCER THERAPY

Thus we have discussed the functional importance of distinct domains of hSTAT5 molecule. Given the critical role of JAKs and STATs in normal and abnormal functions, it is important to know the correlation between hematological malignancies and this pathway. STAT5 is considered a key player in various cell dependent oncogenic transformations as they undergo constitutive activations in patient samples derived from a variety of cell types (Bowman T 2000). Previous studies from our lab found the lymphoblastic lymphoma derived cell line YT, showed depletion of STAT5 but not STAT3 facilitates the survival of the survival of the lymphoid tumors (Nagy ZS 2006).

New insight into the molecular pathogenesis of various types of blood cancer suggests that multiple abnormalities, as well as rare mutations, correlate with an anomalous cell phenotype. Although there are several available drugs on the market to therapeutically treat certain blood cancers, there is a steady increase in the death rate among the American population for hematological malignancies. This trend suggests that we still need to explore more unique and specific drug targets as well as identify small molecules with less toxic side effects to treat these tumors.

Compelling evidences from several studies suggest that STAT5 is a critical regulator of lymphoid development as well as its transformation. STAT5 can regulate the expression of anti-apoptotic genes such as *Bcl-X_L* as well as certain types of cell cycle regulators including *D type cyclins* (Moriggl R 1999; Nagy ZS 2009). Constitutively activated STAT5 has been frequently identified in various cancer patient samples such as myeloid leukemia where approximately 66%

of the cases showed aberrantly activated STAT5 (Schepers H 2007; Hoelbl A 2010). Persistently activated STAT5 potentiates cell self-renewal and differentiation capacity of hematopoietic cells and confers their leukemic potential (Schepers H 2007). In vitro studies with the leukemic cell line *STAT5A/Bfl/flMxCre* cells that had targeted ablation of *STAT5A/B* showed that cells lost their ability to transform and often undergo apoptosis or show a complete arrest (Hoelbl A 2010). These and other studies indicate that STAT5 is important for tumor initiation as well progression of this disease. Several new strategies have been investigated by generating monoclonal antibodies against cytokines Sant7 (Tassone P 2005), tyrosine kinase inhibitor AG490 (Kirken RA 2001), NC1153 (Stepkowski SM), PD180970 (Corbin AS 2004), antisense oligonucleotides and dominant negative isoforms of STAT5.

Constitutively serine phosphorylated STAT5 molecules are widely identified in numerous cancer patient samples such as breast carcinoma, leukemias, lymphomas, prostate cancer, melanoma and head and neck cancers. One puzzling question exists as to whether STAT5 can be considered as a drug target to uncouple T-cell mediated diseases? Concurrent induction of multiple signaling pathways occurs in response to stimulation by growth factors, cytokines or oncoproteins and it is hard to understand the root cause of each cancer. This evidence suggests that it is an uphill task from a therapeutic point of view. Although aberrant activation of STAT5 has been mechanistically linked with various cancers such as chronic myelogenous leukemia (CML) and myeloproliferative diseases induced by TEL-JAK2 (Yu H, 2004), a suitable therapy to regulate its activity is still a significant challenge in cancer research. Most strategies focus on different steps of the effector pathway such as blocking the receptor with a specific antibody, tyrosine kinase inhibitor, modulation of phosphatases and protein modulators that amplify the signaling cascade remain to be determined.

1.9 RATIONAL AND HYPOTHESIS

To understand the molecular and biological mechanisms of aberrantly activated STAT5 signaling pathway is of paramount importance to delineate a suitable therapeutic strategy to treat leukemic blasts. Cell signaling machineries are a complicated process as they undergo spontaneous cross-talk amongst themselves involving multiple pathways. In the era of global comprehensive phosphoproteomic analysis, as well as mapping of other key post translational modifications, STAT5 should be explored to better understand its role in cancer. The objective of this dissertation is to identify possible novel regulatory pathways effecting STAT5 and to characterize the functional importance of these modifications. These results in turn or represents a suitable therapeutic target to regulate aberrantly activated STAT5 molecules. **The central hypothesis to be investigated is that cytokines induce novel phosphorylation sites within STAT5 that are functionally important. We will address this hypothesis in the following three specific aims**

Specific Aim 1: Identify, confirm and characterize novel cytokine dependent phosphorylation sites in human STAT5.

Specific Aim 2: Investigate the regulatory pathways governing STAT5 S193 phosphorylation in human lymphocytes.

Specific Aim 3: Determine the functional and potential biological significance of S193 phosphorylation for STAT5 function.

CHAPTER 2: Identify, confirm and characterize novel cytokine dependent phosphorylation sites in human STAT5

2.1 INTRODUCTION

STAT5 is a critical regulator of signal three mediated expansion of T lymphocytes. It is widely expressed in other tissue such as breast, hepatocytes, prostate and neck tumors (Wakao H 1995; Leong PL 2002; Hosui A 2009; Gu L 2010). Since the earliest discovery of this protein as a mammary gland factor, two isoforms due to gene duplication events are known to exist. Various studies suggest that aberrantly activated STAT5 plays a role in various malignancies (Friedbichler K 2010). There are potentially numerous factors involved in promoting constitutive activation of STAT5 molecules. As of now, few residues in STAT5 have been identified and associated with posttranslational modification. Several studies have suggested that tyrosine kinases such as JAKs and Srcs are not the only enzymes that interact with STATs. Supporting evidences indicate that serine/threonine kinases such as p38, ERK1/2 also interact with this pathway and phosphorylate critical serine residues responsible for regulating its transcriptional activity (Wang LH 1999; Nagy ZS 2002; Rawlings 2004). Dual phosphorylation is required for fully activated STAT molecules. From these studies it has been suggested that putative phosphorylation sites in STAT5 should be more fully investigated to understand their participation in the JAK3-STAT5 pathway.

Mass spectroscopic instrumentation (QTOF, LC-MS/MS) has gained importance in the recent era for its identification of subtle post-translational modifications in proteins. However, we still need to use this technology to understand the detailed mechanism of action both in the cytoplasm as well as in the nucleus and to identify probable cross-talk with different kinds of cell-signaling pathways.

2.2 MATERIALS AND METHODS

2.2.1 CELL CULTURE

Human NK cells YTs were grown in RPMI 1640 (Thermo Scientific Inc.) containing 10% Fetal Bovine Serum (FBS) (Atlanta Biologicals), 2 mM L-glutamine (Cellgro), and penicillin-streptomycin (50 IU/ml and 50 µg/ml, respectively) (Cellgro) at 37 °C with 5% CO₂.

2.2.2 IMMUNOPRECIPITATION ANALYSIS

Human NK cells were grown to mid-log phase phase. Cells were counted with a hemocytometer and 20 x 10⁶ cells were aliquoted per centrifuge tube. Cells were stimulated without (-) or with (+) 10,000 IU/ml IL-2 for 10 min and lysed in a lysis buffer (10mM Tris, 5mM EDTA, 50mM NaCl, 30 mM Na₄P₂O₇, 50 mM NaF, 1 mM Na₃VO₄, 1% Triton X-100) containing 2 µg/ml leupeptin, 1 µg/ml pepstatin A, and 1 mM phenylmethanesulphonyl fluoride (PMSF) at 4 °C for 30 min. Lysates were harvested after centrifugation at 20,854 g for 30 min into new tubes and were quantitated by Bicinchonic acid (BCA) protein assay (Pierce). lysate (5mg) was transferred to 15 ml conical tubes from IL-2 (-) and IL-2 (+) and 1 mg lysate transferred into two separate centrifuge tubes. STAT5B antibody (lot # 2337) generated by our group to the 15 carboxy-terminal amino acids of STAT5B were aliquoted into each lysate and rotated for 3 hrs at 4 °C. Subsequently, 100 µl of slurry of protein A sepharose beads (30%) were added to those lysates to capture the protein-antibody complex with rotation for 45 min at 4 °C. Samples were pelleted by centrifugation at 20,854 g for 15 min at 4 °C. The beads were transferred into 1.5 ml centrifuge tubes to simplify washing with lysis buffer. Each tube was washed three times with 1ml lysis buffer containing 1% Triton-X100 (Sigma Aldrich). Excessive lysis buffer was drawn away by cotton and 45 µl 2X sample buffer (20% Glycerol, 10% 2-Mercaptoethanol, 4.6% SDS, 0.125 M Tris, 0.004% Bromophenol blue) was added to

each centrifuge tube. Samples were heated to 100 °C in a water-bath for 5 min followed by centrifugation at 20,854 g for 1 min. Samples were separated on 7.5% SDS-PAGE gel for 4 hrs at 25 mA to separate the proteins based upon their molecular weights. Then gel was dissected into two parts with one part was stained with Coomassie blue R350 (BIORAD) as per the standard protocol and the other section transferred onto polyvinylidene fluoride (PVDF) membrane. Western-blot was performed as per the standard protocol with either α -pY (Millipore) or α -pS726/31 STAT5 (Sigma Genosys) or α -STAT5 (Sigma Genosys) (Ross JA 2010).

2.2.3 CLUSTALW ANALYSIS

Potentially homologous sequences to human STAT5A were recovered from GenBank using BLAST with default parameter settings (BLOSUM 62). The searches were restricted to a number of representative mammals (*Monodelphis*, *Rattus*, *Mus*, *Bos*, *Macaca*, *Pan*) as well as vertebrate outgroup taxa (*Gallus*, *Danio*). Restricting the search to the genomes of individual species facilitated the recovery of potential paralogs.

All sequences were aligned using CLUSTALW (progressive alignment). Phylogenetic analysis of the aligned sequences was carried out using the maximum likelihood (with Jones-Thornton-Taylor model of amino acid substitution) modules in the PHYLIP package, run over 100 bootstrap replicates. Multiple alignments were displayed using Dot Plot, while the tree output files from PHYLIP were reformatted with MrEnt.

2.2.4 DOT BLOT ANALYSIS

Polyclonal antibody to detect STAT5 pS193 was generated against the phosphopeptide CLAQL(pS)PQERL that would minimize the cross-reaction with other proteins and

phosphorylated serine sites in human STAT5 and other STATs. Rabbits were immunized with phosphopeptide. After the third bleed (77 days) sera were collected from those rabbits and purified against phosphopeptide as well as non-phosphopeptide bound column, positively and negatively respectively. To understand the specificity of the polyclonal anti-pS193, different doses of phosphopeptide and non-phosphopeptide were blotted onto the PVDF membrane. The membrane was then incubated with 1:5000 polyclonal α -pS193 overnight at 4 °C and followed the standard Western-blotting.

2.2.5 CELLS STAINING

YT cells were grown to mid-log phase and then 1×10^8 cells were aliquoted per centrifuge tube and carried out different treatment as indicated for each experiment. Approximately 2×10^5 cells were bound to the surface of the slide by Cytopro (Wescor) at a speed of 250 g for 5 min. Cells were then fixed on the slide by immersed in cold (-20 °C) methanol for 5 min. Fixed cells were washed with 1X phosphate-buffered-saline (PBS) (Cellgro). Then cells were permeabilized with 0.2% Triton-X (Sigma Aldrich) and blocked with 2% Bovine serum Albumin (BSA) (Calbiochem) for 1 hr. Cells were stained sequentially with α -pYSTAT5 (Upstate) and α -STAT5pS193 for 1 hr individually and washed three times with wash buffer (PBS+0.05% Tween-20) after the first primary antibody then three times more followed by staining with the second primary antibody. Primary antibodies were stained with Cy2 (Green) conjugated donkey-anti mouse or Cy3 (Red) conjugated donkey-anti rabbit (Jackson Immune Research) at 1:400 dilution for 1 hr. Cells were washed with wash buffer for 5 min and stained with DAPI (Calbiochem) for 10 min at 1:800. After 10 min, cells were washed two additional times. Finally mounting media (Calbiochem) was used to fix cells and

covered with a coverslip. Images were obtained using Carl Zeiss microscope LSM 500 and PASCAL software.

2.2.6 PEPTIDE COMPETITION ASSAYS

Purified anti-STAT5 pS193 (3 μ g) was pre-blocked with 100 ng-10 μ g of phosphopeptide and non-phosphopeptide separately for 1 hr at 4 $^{\circ}$ C. YT cells were stimulated with 10,000 IU/ml IL-2 for 15 min and approximately 2×10^5 cells were plated on the slide with the help of Cytopro (Wescor). Cells were stained with the previously mentioned protocol. Primary STAT5pS193 antibody was preblocked with different doses of phosphopeptide or non-phosphopeptides.

2.2.7 PURIFICATION OF HUMAN T CELLS FROM BUFFY COAT

Peripheral blood mononuclear cells (PBMCs) were isolated from the supplied buffy-coat (Innovative Research). A sterile density gradient medium of Ficoll-paque Plus (GE Healthcare) was used to purify lymphocytes from the buffy-coat. Blood was first diluted with sterile PBS in the following ratio (30 ml 1X PBS+ 5 ml blood), then 35 ml of diluted blood was loaded over 15 ml of Ficoll-paque Plus and centrifuged at a speed 931 g for 30 min at room temperature. Lymphocytes were collected from the interface of the plasma and Ficoll-paque layer. Lymphocytes were washed two times with 1x sterile PBS at 524 g for 10 min at room temperature. Once cells were counted and resuspended in complete RPMI media as previously mentioned. Next day cells were washed 2 times with media and activated with Phytohemagglutinin (1 μ g/ml) for 72 hrs. Cells were stripped by bubbling cells with CO₂ in the media and keep in quiescent for 48 hrs in RPMI 1640 with 1% FCS, penicillin-streptomycin and

glutamine. Then quiescent cells were stimulated with different kinds of cytokines and stained as per the protocol mentioned previously.

2.2.8 SURFACE PLASMON RESONANCE

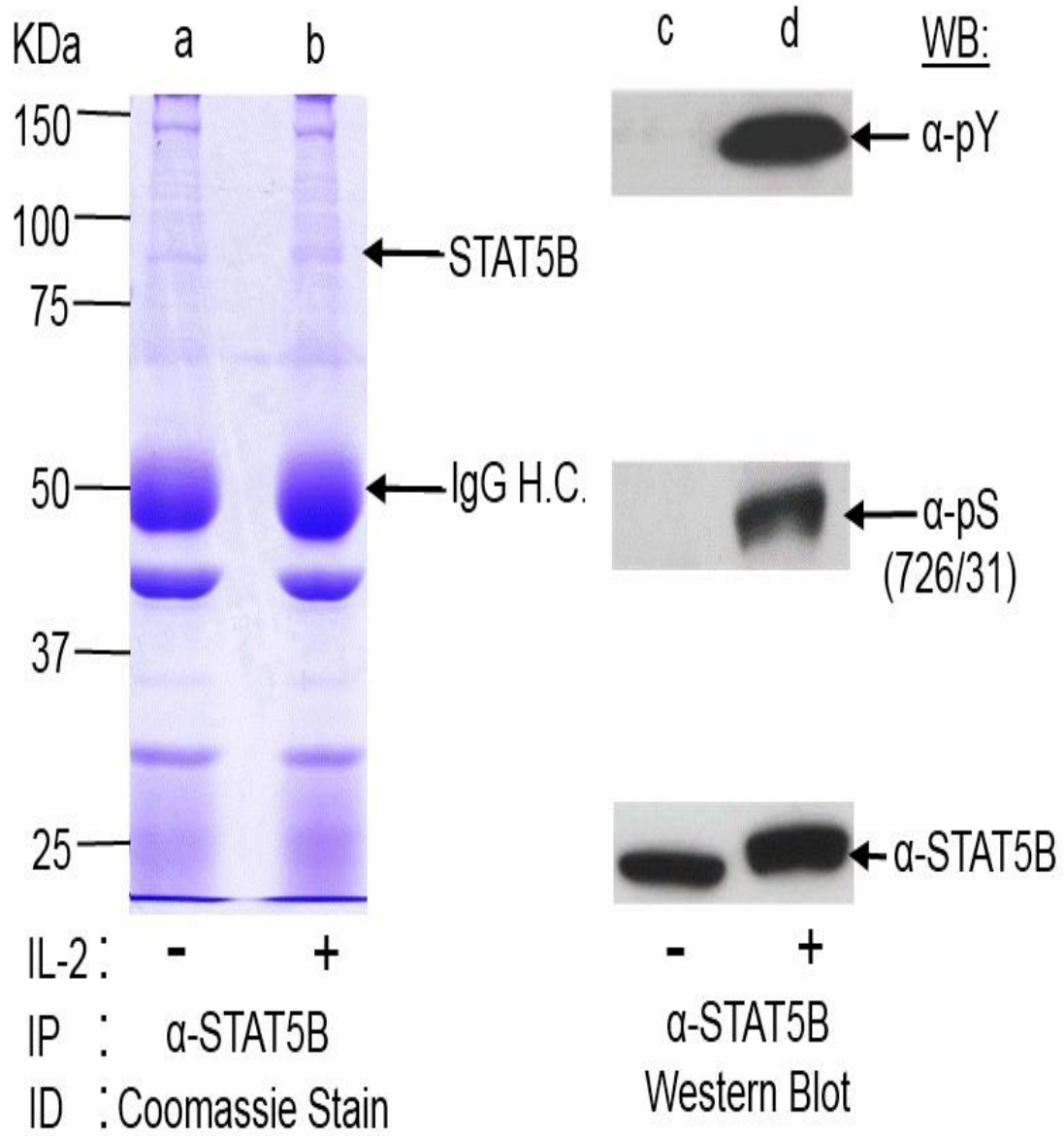
Surface plasmon resonance (SPR) based instruments measure the change in the refractive index near a sensor chip to determine the ligand and analyte interaction. The binding affinity between peptide (phospho or non-phospho) and affinity purified α -STAT5pS193 were performed on a BIAcore T100 instrument (GE Healthcare). Ligands, phosphopeptide CLAQL(pS)PQERL and non-phosphopeptide CLAQLSPQERL, were covalently linked to a carboxymethylated dextran (CM5) sensor chip via amine coupling. Aqueous solution of the analyte α -STAT5pS193 at different dilutions (10 μ g/ μ l, 25 μ g/ μ l, 50 μ g/ μ l, 100 μ g/ μ l and 250 μ g/ μ l) were injected in BIAcore running buffer (10 mM HEPES, pH 7.4, 150 mM NaCl, 1 mM EDTA, and 0.005% P-20 surfactant (Pharmacia)) through the flow cells at a specific flow rate (10 μ l/min) and a contact time (300 seconds) at 25 $^{\circ}$ C. As the analyte binds to the ligand, the change in refractive index was measured in real time and the result plotted as resonance units (RUs) versus time (a sensorgram). Kinetic data were evaluated using the BIAcore evaluation software. To determine the affinity constant, Langmuir binding model was used where 1:1 interaction between analyte and immobilized ligand was assumed and two-state reaction, including binding of analyte with ligand followed by conformational change, occurred. Scatchard plot was used for graphical representation of the data as it was consistent with the Langmuir binding isotherm.

2.3 RESULTS

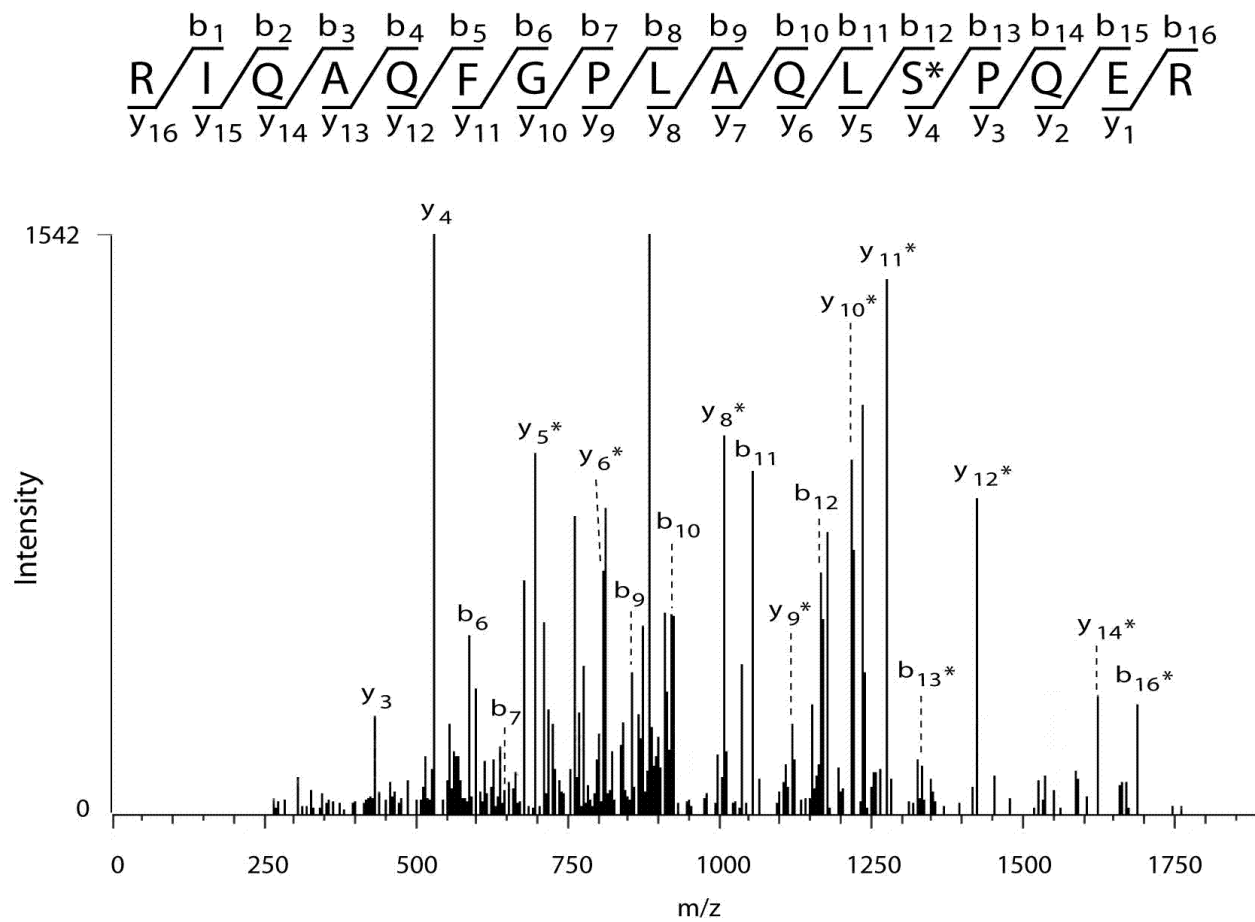
2.3.1 CYTOKINE DEPENDENT PHOSPHORYLATION OF A NOVEL SERINE SITE IN hSTAT5

Previous studies from our lab identified a proline juxtaposed S726 in hSTAT5A and S731 in hSTAT5B to undergo IL-2 dependent phosphorylation (Nagy ZS 2002). Proline flanked serine PSP or PMSP motifs are now known to occur in different STATs and play an active role in the transcriptional activities of STATs (RA. 1997; Morinobu A 2002; Shen Y 2004). As of now, few sites have been mapped in hSTAT5 to undergo posttranslational modifications. Limited knowledge about multiple levels of hSTAT5 regulation exists. In Fig 2.1 (A), YT cells were stimulated without (-) or with IL-2 for 10 min and immunoprecipitated with STAT5B. STAT5B showed slightly upward shift due to IL-2 driven phosphorylation (Fig 2.1 A lane b). Similarly the IL-2 induced tyrosine (Y699) and serine (731) phosphorylations of STAT5 were validated by western blot (Fig 2.1 A lane d) with specific antibodies respectively. Subsequently excised bands (Fig 2.1A lanes a and b) of STAT5B from the commassie stained gel were analyzed to determine putative phosphorylation sites by liquid chromatography tandem mass spectrometry (LC-MS/MS). The result was 85% mass spectrometry coverage of hSTAT5B and two phosphorylation sites (S193 and Y699) were identified in an IL-2 dependent manner (Fig. 2.1 B). Phosphorylation of Y699 is known to be important for STAT5 dimerization and activation; however S193 was not previously reported. Figure 2.1 (B) tandem MS spectra showed that b13 and b16 fragments positively correlate with S193 phosphorylation as indicated by asterisk. Sequence alignment studies with other STATs indicate that the site is located in the coiled-coil domain in between coil1 and coil2 specifically within the helical turn (Fig. 2.1 C).

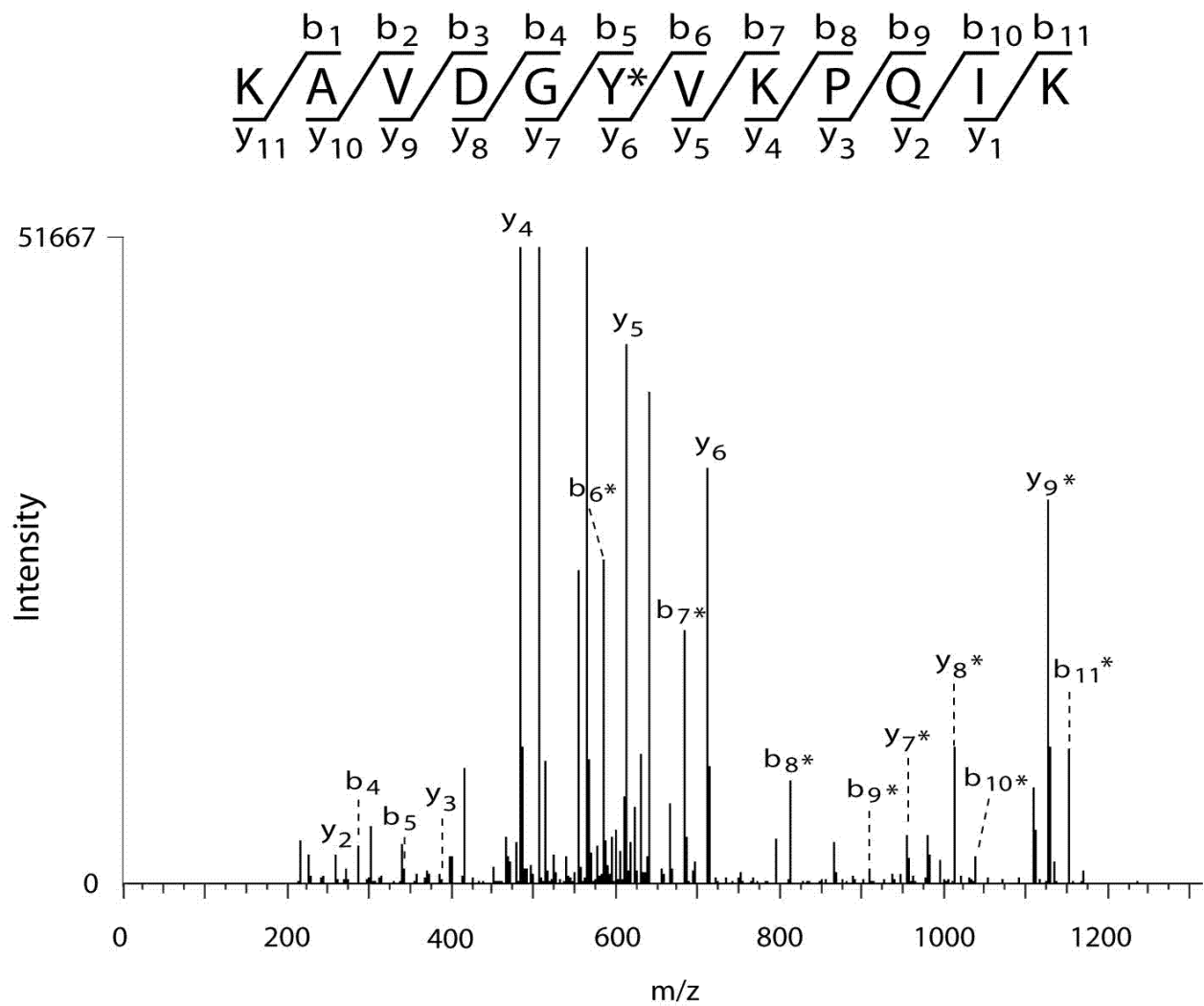
A)



B)



MS/MS spectra of the peptide containing pS193



MS/MS spectra of the peptide containing pY699

C)

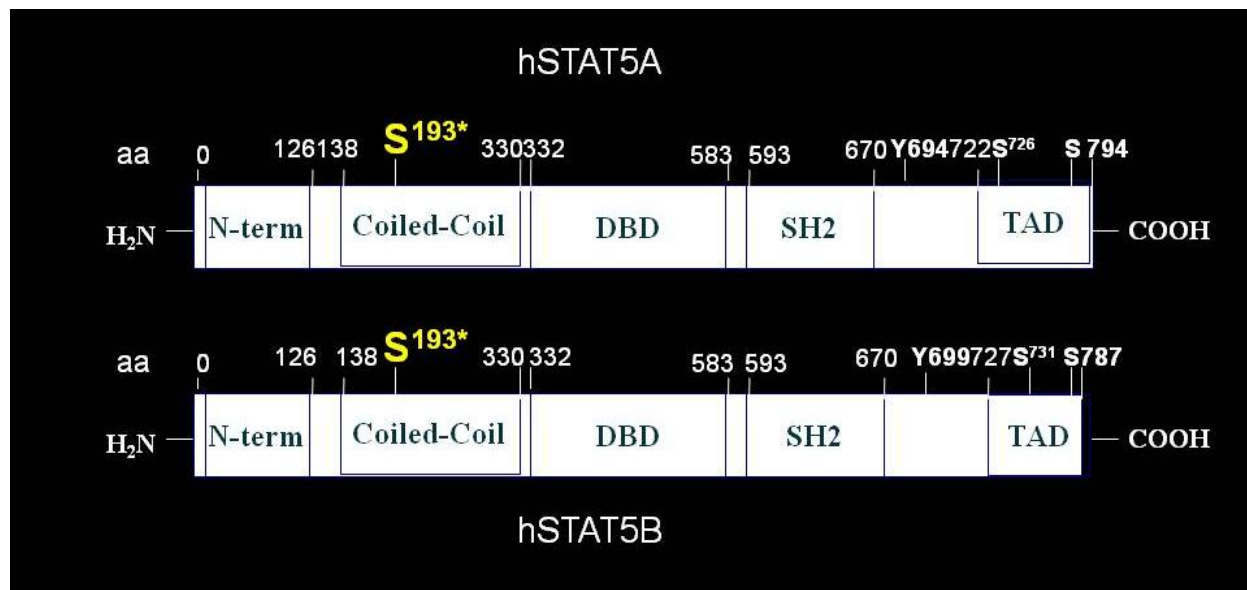


FIGURE 2.1: Identification of cytokine dependent novel phosphorylated serine residue in hSTAT5.

A) Human Natural Killer cells were stimulated with IL-2 for 15 min, harvested, lysed and STAT5B proteins were immunoprecipitated (IP) from soluble lysates with α -STAT5B. Two sets of immunoprecipitates were separated on the same 7.5% SDS-PAGE gel. One set was Coomassie stained (Lanes a & b) and another set (Lanes c & d) was Western blotted with either α -pY or α -pS(726/731) or α -STAT5B antibodies. B) Tandem mass spectra of monophosphorylated peptides showing site identified S193 (upper panel) or Y699 (lower panel), as indicated by asterisks. C) Schematic illustrations of human STAT5A and 5B domains with known and newly identified serine (asterisks) and previously known tyrosine phosphorylation site. Numbers indicate amino acid residues of human STAT5. S193 is located

in the coiled-coil domain within the helical turn between coil 1 and coil2. Representative data from two independent experiments are shown.

2.3.2 EVOLUTIONARY IMPORTANCE OF NOVEL S193 IN hSTAT5 IN COMPARISON TO THE NEAREST PRIMATES AND PHYLOGENETICALLY DISTANTLY RELATED SPECIES

Human STAT5A and 5B protein sequence has been aligned with other human STATs. S193 is only conserved in STAT3 and STAT6; however the surrounding sequence has been partially conserved in other STATs (Fig. 2.2 A). Alignment of human STAT5 sequences with the closest mammalian species suggest that S193 is conserved only in monkey and chimpanzee where in non-primates such as rat, mouse or guinea pig, the site has been modified to an asparagine (N) (Fig. 2.2 B and C). Corresponding regions in STAT5s within primates and non-primates show almost 90% conservation suggesting the importance of this primary sequence in STAT5, suggesting its functional importance. To understand its evolutionary significance, the STAT5 sequence was aligned with evolutionary primitive species that have STAT molecules such as *Drosophila*, *Xenopus*, *Dictyostelium* and *Arabidopsis* (Fig. 2.2 D and E). Clustal alignment studies showed that *Xenopus* harbors the serine residue in its STAT protein, possibly an aberrant mutation since this site was not found in other primitive species. From these phylogenetic studies it can be concluded that the serine residue is predominantly found in primates but not in non-primates, this site may therefore have a functional role in primates but is dispensable to non-primates or lower eukaryotes.

A)

STAT1 185	D	F	K	C	K	T	L	Q	N	R	E	-	-	E	T	N	200
STAT2 185	F	R	Y	K	-	-	-	-	A	K	G	L	D	P	H	Q	200
STAT3 185	D	F	N	Y	K	T	L	K	S	Q	N	N	Q	S	V	T	200
STAT4 185	F	D	R	Y	K	T	I	Q	T	M	D	-	-	-	-	Q	200
STAT5A 185	Q	F	A	Q	L	A	Q	L	S	P	Q	E	R	L	S	R	200
STAT5B 185	Q	F	A	Q	L	A	Q	L	S	P	Q	E	R	L	S	R	200
STAT6 185	E	A	G	Q	V	L	S	H	S	L	I	E	T	P	A	N	200

B)

<i>Homo sapiens</i>	STAT5A 185	Q	F	A	Q	L	A	Q	L	S	P	Q	E	R	L	S	R	200
<i>Pan troglodytes</i>	STAT5A 185	Q	F	A	Q	L	A	Q	L	S	P	Q	E	R	L	S	R	200
<i>Macaca mulatta</i>	STAT5A 185	Q	F	G	P	L	A	Q	L	S	P	Q	E	R	L	S	R	200
<i>Mus musculus</i>	STAT5A 185	Q	F	A	Q	L	G	Q	L	N	P	Q	E	R	M	S	R	200
<i>Rattus norvegicus</i>	STAT5A 185	Q	F	G	Q	L	A	Q	L	N	P	Q	E	R	M	S	R	200
<i>Bos taurus</i>	STAT5A 185	Q	F	A	Q	L	A	Q	L	N	P	Q	E	R	L	S	R	200
<i>Monodelphis domestica</i>	STAT5A 185	Q	F	S	Q	L	A	Q	L	S	P	Q	E	R	L	T	R	200
<i>Danio rerio</i>	STAT5.1 185	Q	L	S	S	L	A	T	L	P	P	A	D	R	Q	L	R	200
<i>Danio rerio</i>	STAT5.2 185	Q	I	V	S	L	A	S	L	P	P	T	E	R	A	Q	R	200

C)

<i>Homo sapiens</i>	STAT5B 185	Q	F	A	Q	L	A	Q	L	S	P	Q	E	R	L	S	R	200
<i>Pan troglodytes</i>	STAT5B 185	Q	F	A	Q	L	A	Q	L	S	P	Q	E	R	L	S	R	200
<i>Macaca mulatta</i>	STAT5B 185	Q	F	G	P	L	A	Q	L	S	P	Q	E	R	L	S	R	200
<i>Mus musculus</i>	STAT5B 185	Q	F	A	Q	L	G	Q	L	N	P	Q	E	R	M	S	R	200
<i>Rattus norvegicus</i>	STAT5B 185	Q	F	A	Q	L	A	Q	L	N	P	Q	E	R	M	S	R	200
<i>Bos taurus</i>	STAT5B 185	Q	F	A	Q	L	A	Q	L	N	P	Q	E	R	L	S	R	200
<i>Gallus gallus</i>	STAT5B 185	Q	F	S	Q	L	S	Q	L	G	P	Q	E	R	L	S	R	200
<i>Monodelphis domestica</i>	STAT5B 185	Q	F	S	Q	L	S	Q	L	S	P	Q	E	R	L	T	R	200
<i>Danio rerio</i>	STAT5.1 185	Q	L	S	S	L	A	T	L	P	P	A	D	R	Q	L	R	200
<i>Danio rerio</i>	STAT5.2 185	Q	I	V	S	L	A	S	L	P	P	T	E	R	A	Q	R	200

D)

<i>Homo sapiens</i>	STAT5A	Q	F	A	Q	L	A	Q	L	S	P	Q	E	R	L	S	R
<i>Pan troglodytes</i>	STAT5A	Q	F	A	Q	L	A	Q	L	S	P	Q	E	R	L	S	R
<i>Macaca mulatta</i>	STAT5A	Q	F	G	P	L	A	Q	L	S	P	Q	E	R	L	S	R
<i>Mus musculus</i>	STAT5A	Q	F	A	Q	L	G	Q	L	N	P	Q	E	R	M	S	R
<i>Rattus norvegicus</i>	STAT5A	Q	F	G	Q	L	A	Q	L	N	P	Q	E	R	M	S	R
<i>Bos taurus</i>	STAT5A	Q	F	A	Q	L	A	Q	L	N	P	Q	E	R	L	S	R
<i>Monodelphis domestica</i>	STAT5A	Q	F	S	Q	L	A	Q	L	S	P	Q	E	R	L	T	R
<i>Xenopus laevis</i>	STAT	Q	F	T	Q	L	S	Q	L	S	P	Q	E	R	I	P	R
<i>Drosophila melanogaster</i>	STAT92E	Q	H	Q	I	M	Q	S	L	N	E	G	N	C	A	N	A
<i>Caenorhabditis elegans</i>	STAT	L	Q	M	V	I	A	Q	L	E	Q	Q	Q	R	L	E	N
<i>Dictyostelium discoideum</i>	STAT	N	F	N	T	T	T	T	T	T	T	T	S	N	N	N	N
<i>Arabidopsis thaliana</i>	STAT	N	R	V	F	E	V	F	T	G	Y	R	A	D	E	V	L

E)

<i>Homo sapiens</i>	STAT5B	Q	F	A	Q	L	A	Q	L	S	P	Q	E	R	L	S	R
<i>Pan troglodytes</i>	STAT5B	Q	F	A	Q	L	A	Q	L	S	P	Q	E	R	L	S	R
<i>Macaca mulatta</i>	STAT5B	Q	F	G	P	L	A	Q	L	S	P	Q	E	R	L	S	R
<i>Mus musculus</i>	STAT5B	Q	F	A	Q	L	G	Q	L	N	P	Q	E	R	M	S	R
<i>Rattus norvegicus</i>	STAT5B	Q	F	A	Q	L	A	Q	L	N	P	Q	E	R	M	S	R
<i>Bos taurus</i>	STAT5B	Q	F	A	Q	L	A	Q	L	N	P	Q	E	R	L	S	R
<i>Monodelphis domestica</i>	STAT5B	Q	F	S	Q	L	S	Q	L	S	P	Q	E	R	L	T	R
<i>Xenopus laevis</i>	STAT	Q	F	T	Q	L	S	Q	L	S	P	Q	E	R	I	P	R
<i>Drosophila melanogaster</i>	STAT92E	Q	H	Q	I	M	Q	S	L	N	E	G	N	C	A	N	A
<i>Caenorhabditis elegans</i>	STAT	L	Q	M	V	I	A	Q	L	E	Q	Q	Q	R	L	E	N
<i>Dictyostelium discoideum</i>	STAT	N	F	N	T	T	T	T	T	T	T	T	S	N	N	N	N
<i>Arabidopsis thaliana</i>	STAT	N	R	V	F	E	V	F	T	G	Y	R	A	D	E	V	L

F)

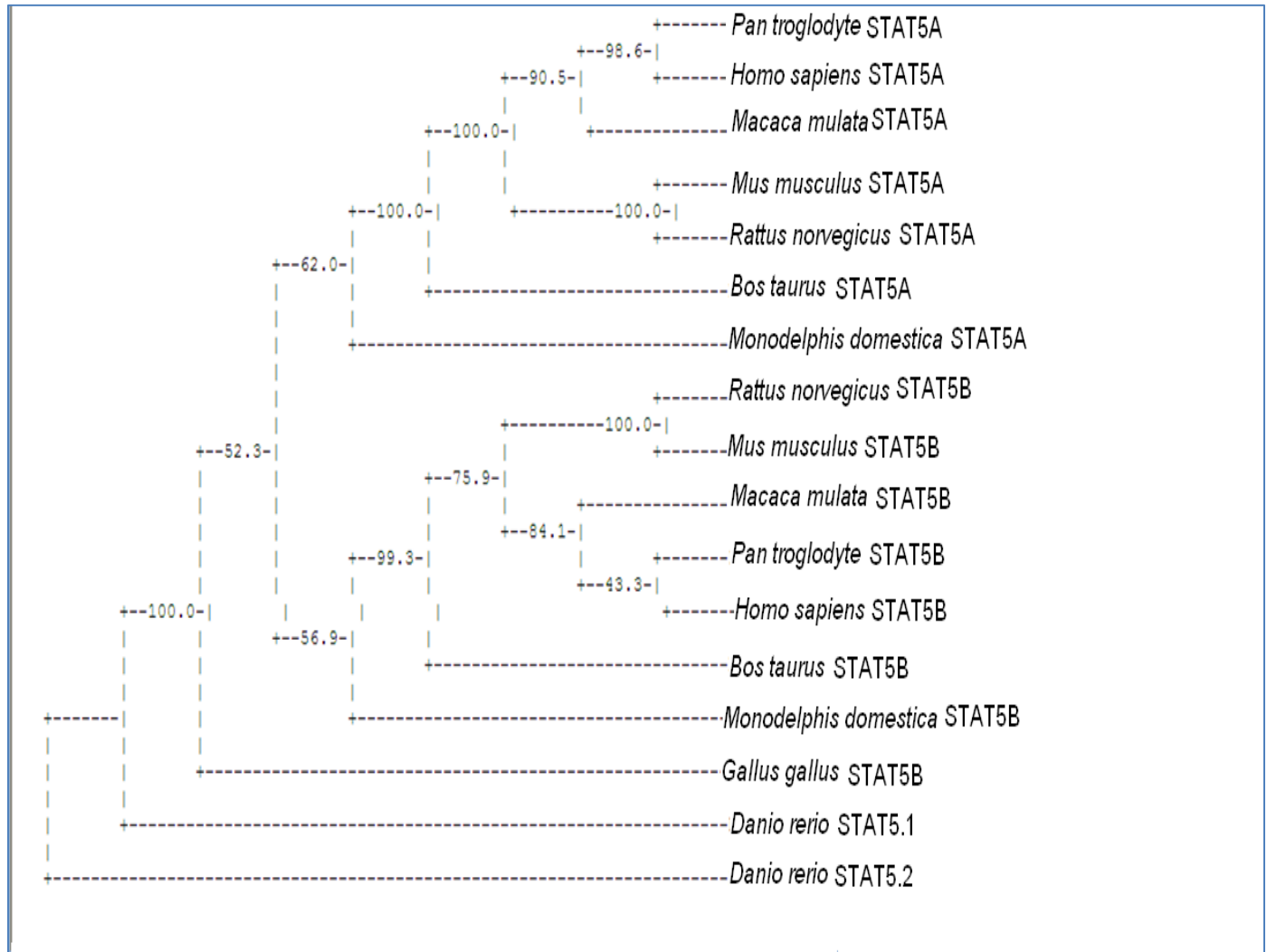


FIGURE 2.2: Evolutionary conservation of STAT5S193 to understand the phylogenetic importance of novel S193 in human STAT5.

A) Alignment of hSTAT5 protein sequences corresponding to regions surrounding S193 of hSTAT1, 2, 3, 4, 5A, 5B and 6 indicated that S193 is only conserved in hSTAT3, 5 and 6. B) and C) Alignment of hSTAT5A and 5B protein sequences corresponding to region surrounding S193 to other closest mammalian species comparing with phylogenetically distantly related avian (*Gallus gallus*), marsupial (*Monodelphis domestica*) and fish (*Danio rerio*) respectively.

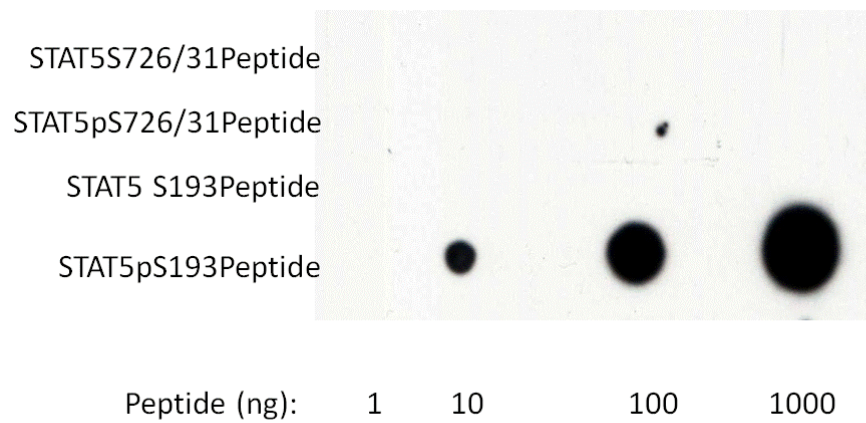
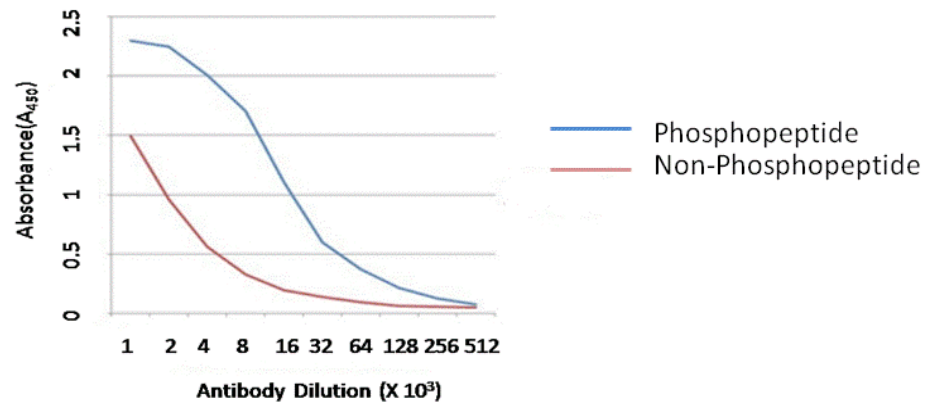
D) and E) Alignment of human STAT5A and STAT5B proteins sequence with STAT sequences of evolutionary primitive organisms respectively. F) Bootstrap analysis shows the phylogenetic divergences of STAT5s between primates and non-primates among mammals. *Danio rerio* was considered as an outgroup to represent a negative control. Representative data from two independent experiments are shown.

2.3.3 POLYCLONAL PHOSPHO-SER193-SPECIFIC ANTISERUM PREFERENTIALLY DETECTS THE hSTAT5pS193

Since phosphorylation of S193 in STAT5 may represent a novel mechanism of regulating STAT5 function, therefore we sought to characterize this site further. Since no commercially available antibody exists, polyclonal antibodies were raised against the phosphopeptide sequence CLAQL(pS)PQERL in rabbit. Polyclonal antibodies represent a diverse population of antibodies that have varied avidity towards a given epitope. Thus we subjected our sera to double affinity purification against both non-phospho and phosphopeptides. To determine the efficacy of the purified antibody to recognize the antigen, enzyme linked immunosorbant assays (ELISA) and Dot blot assays were performed. Indeed this antibody preferentially detected the phosphopeptide in competition with the non-phosphopeptide (Fig. 2.3 A) and Dot blot assay also showed that the antibody does not detect STAT5pS72/31, another site in STAT5 that undergoes phosphorylation in an IL-2 dependent manner. Next surface plasmon resonance was performed using the ligand and analyte in an *in vitro* system BIACORE T100. The binding studies showed that the polyclonal phospho-Ser193- specific antiserum binds approximately 20 times stronger to phosphopeptide in comparison to non-phosphopeptide (Fig. 2.3 B). Fig 2.4 represents a peptide competition assay where the polyclonal phospho-Ser193-specific antiserum was preblocked with either

phosphopeptide or non-phosphopeptide at different doses for 1 hr at 4 °C. IL-2 mediated YT cells were stained with the antibody that were preblocked with different doses of peptides. From this experiment, it has been found that phosphopeptide blocked the recognition specificity of the polyclonal antibody in a dose dependent manner as a result of which 10 µg phosphopeptide completely blocked antibody binding (Fig. 2.4 second row lane c) however similar doses of non-phosphopeptide did not modify the recognition specificity of this antibody. STAT5 can reside in the cytoplasm or nucleus. To determine whether STAT5pS193 preferentially exist in one of these compartments, confocal imaging was utilized here. Tyrosine phosphorylated STAT5 (Fig. 2.4 upper row) showed STAT5 undergoes IL-2 mediated activation that was considered as a positive control. Colocalization studies (Fig. 2.4, fourth row) corroborated the polyclonal antibody recognized STAT5pS193 as it merges with STAT5pYSTAT5. Moreover DAPI staining (Fig. 2.4. third row) represented cells did not undergo any apoptotic or necrotic death.

A)



B)

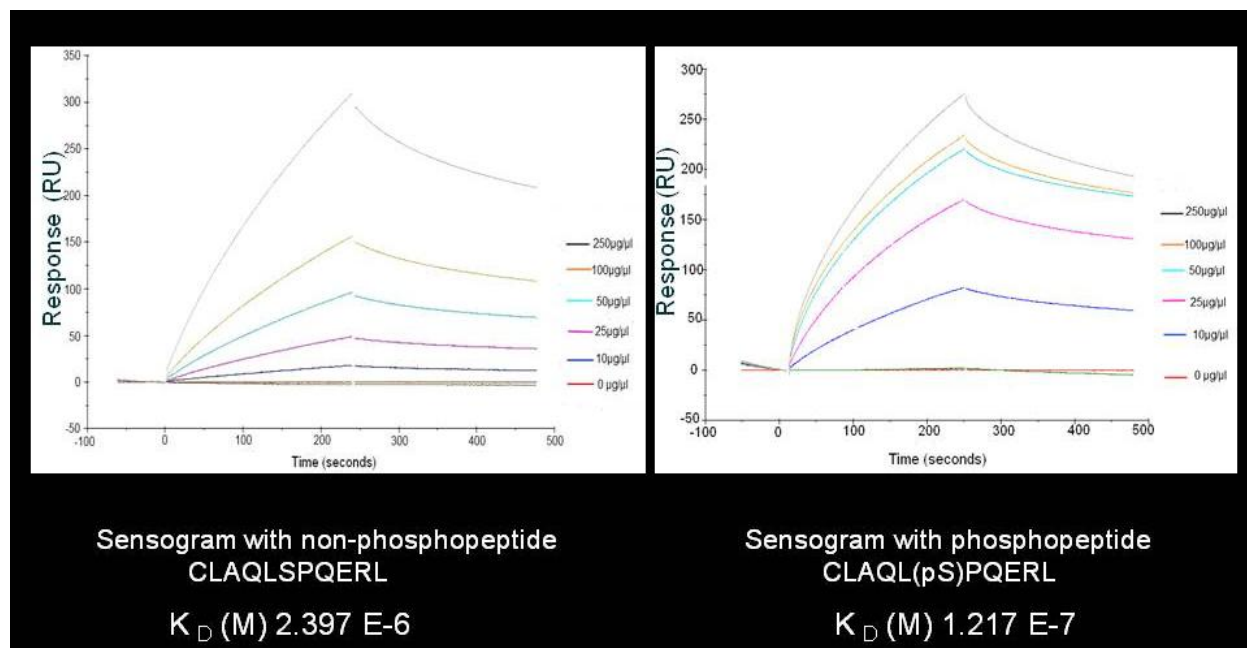


FIGURE 2.3: Characterization of polyclonal phospho-Ser193-specific antiserum.

A) Phospho-specific polyclonal STAT5 antibody to pS193 is target specific as shown by ELISA in the top panel and Dot Blot Assay to the bottom panel. In case of ELISA, increasing amounts of antibody concentrations were used against S193 and pS193 peptides and measured the absorbance at 450 nM. For Dot Blot Assays, increasing amounts of S193 and pS193 peptides were blotted on the PVDF membrane and tested for recognition by rabbit anti-pS(193)STAT5 by Western Blotting. B) S193 and pS193 peptides were prefixed on the CM5 (carboxymethylated) chip by amine coupling. Different dilutions of antibody were run through them at a constant flow rate and contact time to study the binding kinetics that was measured by Surface Plasmon Resonance. Representative data from two independent experiments are shown.

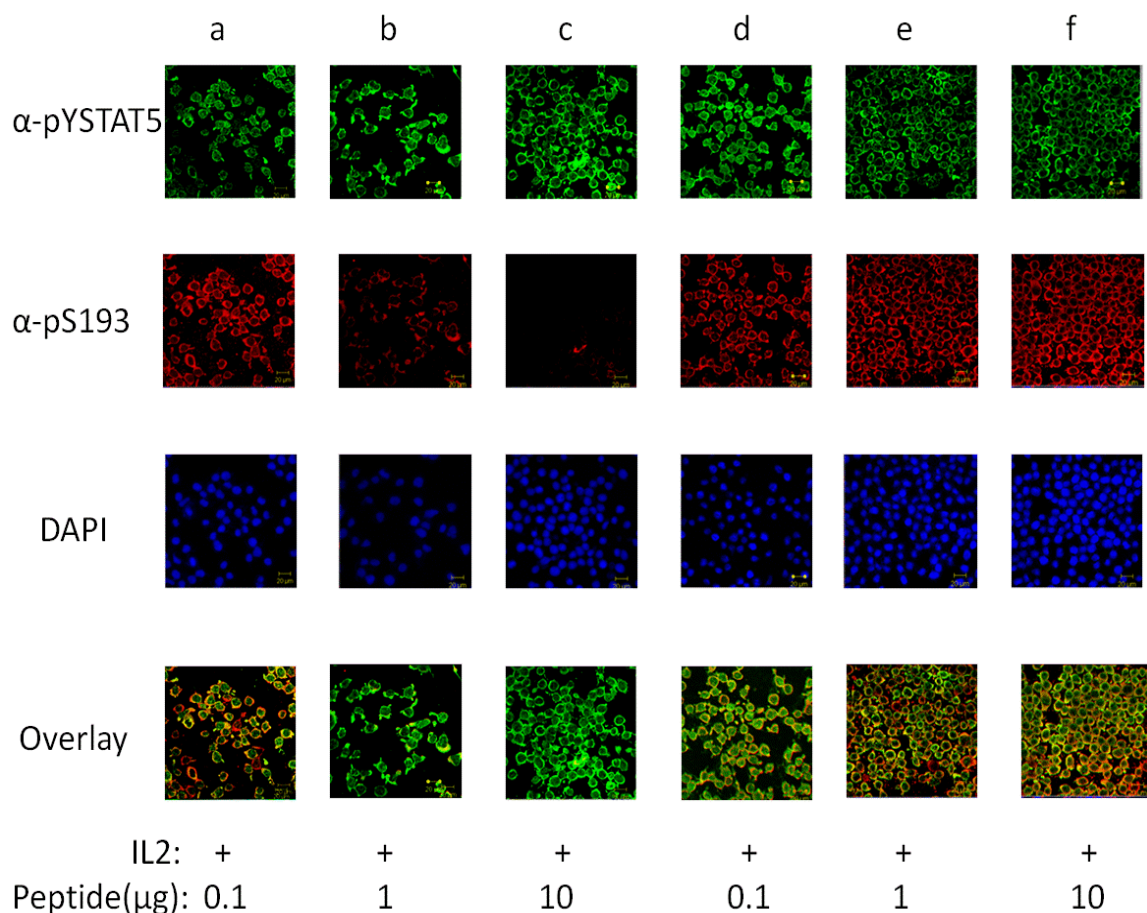


FIGURE 2.4: Phosphopeptide blocks antibody recognition of STAT5 pS193 in a dose dependent manner in human NK cells.

Human NK cells, stimulated with IL-2 for 15 min. Subsequently stained with α -pYSTAT5 (upper row), α -pS193 (second row) and DAPI (third row). Overlay (bottom row) shows that co-localization of pYSTAT5 and STAT5pS193 co-localize. Polyclonal α -STAT5pS193 pre-blocked with different doses of phosphopeptide (lanes a-c) and non-phosphopeptide (lanes d-f) for 1hr at 4 $^{\circ}$ C followed by staining of IL-2 induced human NK cells. Representative data from three independent experiments are shown.

2.3.4 POLYCLONAL PHOSPHO-SER193-SPECIFIC ANTISERUM PREFERENTIALLY DETECTS STAT5 S193 PHOSPHORYLATION BUT NOT STAT3 S727 OR PUTATIVE S193 PHOSPHORYLATION.

The closest homolog (80% sequence similar) of STAT5 is STAT3 that is widely expressed in human lymphoid cells. IL-2 stimulation also activates STAT3 (Fung MM 2003) as shown in Fig. 2.5 lane e that shows Y705 phosphorylation that plays important role in STAT3 activation, dimerization and nuclear translocation to carry out STAT3 induced genes transcription. Alignment studies show that STAT3 is also present serine at position 193 and there is a possibility to undergo phosphorylation in an IL-2 dependent manner. To determine whether polyclonal phospho-Ser193-specific antisera cross-reacts with either STAT3 S193 or previously identified STAT3 S727 phosphorylated site (Schuringa JJ 2000), hPBMCs were stimulated with either IL-2 or IL-6 for 15 min. Cells were fixed as well as lysed to carry out cells staining and western blot respectively. Confocal studies showed (Fig. 2.5 lane c second row) that the antibody does not cross react with STAT3 S193 or S727 phosphorylation upon IL-6 stimulation as we see only basal level of detection in comparison to IL-2 mediated staining of STAT5pS193 (Fig. 2.5 lane b second row). As pY705 STAT3 antibody cannot stain cells so we have shown western blots of pY705 and total STAT3 upon no stimulation (Fig. 2.5 lane d), IL-2 (Fig. 2.5 lane e) and IL-6 (Fig. lane f). It showed that IL2 stimulation activates STAT3 Y705 phosphorylation in hPBMCs. Overall this study concluded that polyclonal phospho-Ser193-specific antiserum is specific for detecting STAT5 S193 phosphorylation.

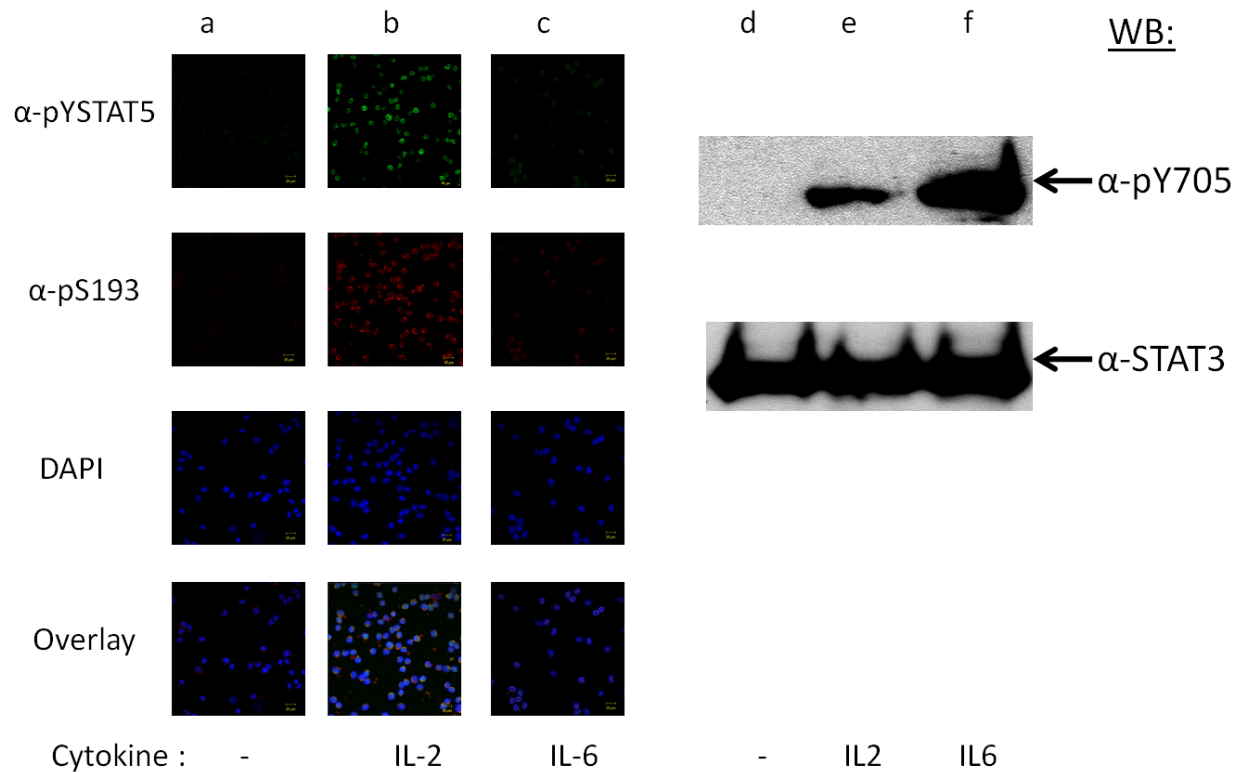


FIGURE 2.5: Polyclonal phospho-Ser193-specific antiserum specifically detects STAT5 S193 phosphorylation but not STAT3 S727 or STAT3 S193 phosphorylation in hPBMCs.

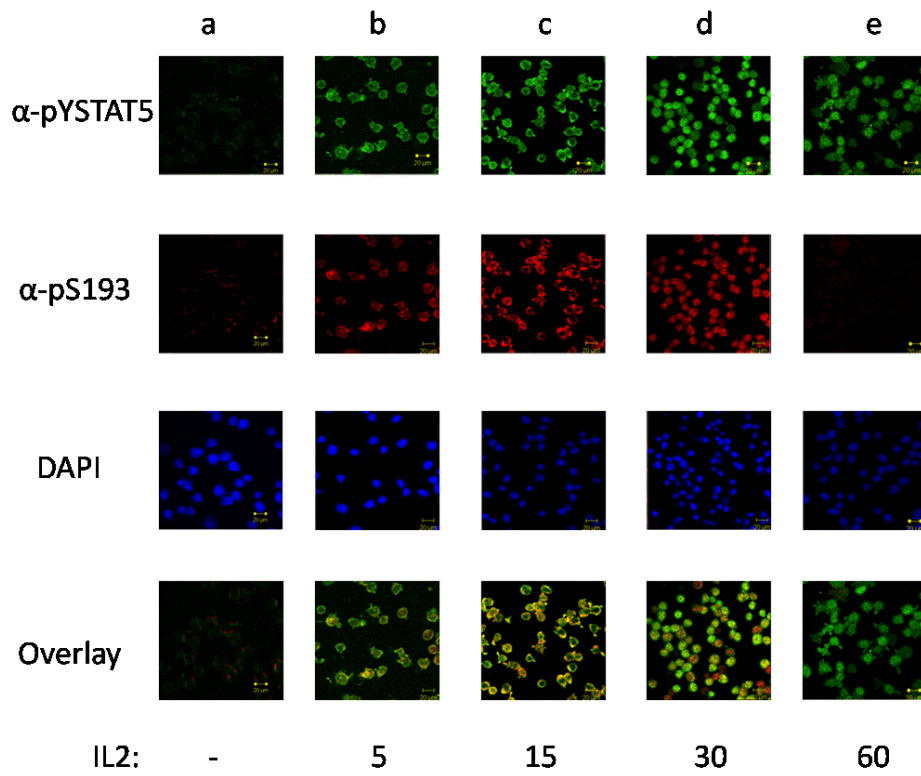
A) Human NK cells were stimulated without (-) (lane a) or with IL-2 (+) from 0 to 60 min (lanes b-e) and fixed with cold methanol. Cells were then stained with anti-STAT5pS193 to determine the phospho-kinetics of STAT5 S193 phosphorylation and sub-cellular localization. In these images α -pYSTAT5 was stained as green (upper row), α -pS193 stained the STAT5 pS193 as red (second row), and DAPI stained the nucleus as blue (third row) and overlay showed the colocalization of pYSTAT5 and STAT5pS193 indicated as yellow (bottom row). B) Quiescent PHA-activated human T cells were stimulated with medium (-) (lane a) or IL-2 (+)

from 0-90 min (lanes b-f) and fixed with cold methanol. Then cells were stained with anti-STAT5pS193 to determine the phospho-kinetics of STAT5 S193 phosphorylation and sub-cellular distribution. C) Quiescent PHA-activated human T cells were stimulated with medium (-) (lane a) or IL-2 (lane b), 7 (lane c), 9 (lane d) or 15 (lane e) for 15 min and fixed with cold methanol. Cells were then stained with anti-pS193 (STAT5) to determine the phospho-kinetics of STAT5 S193 phosphorylation and sub-cellular distribution monitored. Representative data from three independent experiments are shown.

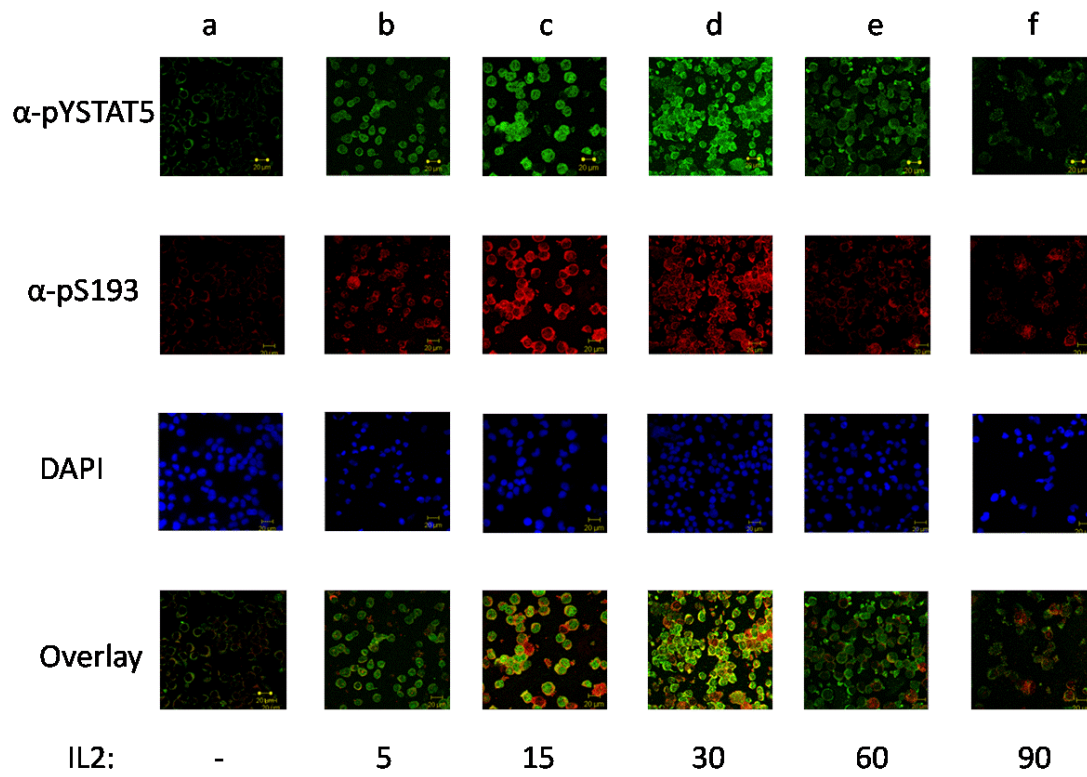
2.3.5 PHOSPHOKINETICS ANALYSIS OF STAT5S193 PHOSPHORYLATION AND ITS SUB-CELLULAR DISTRIBUTION FOLLOWING ACTIVATION BY GAMMA COMMON CYTOKINES

STAT5 is a critical regulator of lymphoid cell proliferation and development. Mass spectroscopy analysis identified a novel serine site (S193) that undergoes phosphorylation in an IL-2 dependent manner. The kinetics of its activation was subsequently measured using our unique phospho-STAT5 specific antibodies. Human NK cells and primary human peripheral blood mononuclear cells were stimulated with IL-2 from 0-60 min and 0-90 min; respectively. As shown in Figures 2.6 A) and B) the phosphorylation of STAT5S193 was detectable within 5 min of stimulation and at 15 min STAT5pS193 was predominantly cytoplasmic based on Fluorescent confocal microscopy images. At 30 min, STAT5pS193 was distributed in both cytoplasm and nucleus. This data suggests that STAT5S193 phosphorylation occurs in the cytoplasm, and that STAT5pS193 can translocate to the nucleus. Cytokines that utilize the IL-2 receptor gamma chain shared (e.g. IL-4, 7, 9 and 15) were all capable of inducing the STAT5 S193 phosphorylation. These data suggest that phosphorylation of STAT5S193 may be important for a diverse set of biological functions; IL-7 appeared to be the weakest activation in these cells (Fig. 2.6 C lane c), required by gamma common cytokines.

A)



B)



c)

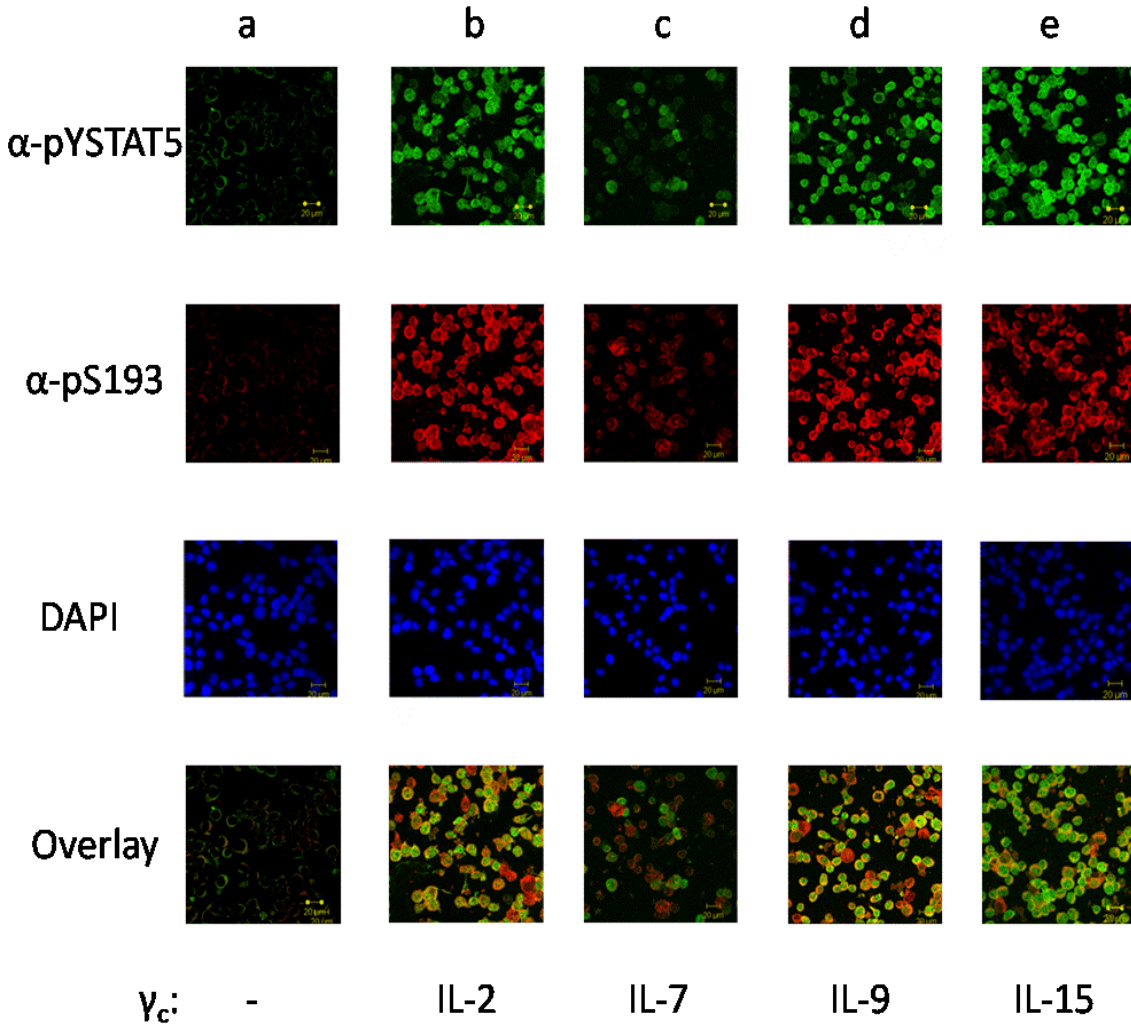


FIGURE 2.6: Phospho-kinetics study of STAT5 S193 in YT and normal human T cells using γ_c cytokines.

A) Human NK cells were stimulated without (-) (lane a) or with IL-2 (+) from 0 to 60 min (lanes b-e) and fixed with cold methanol. Cells were then stained with anti-STAT5pS193 to determine the phospho-kinetics of STAT5 S193 phosphorylation and sub-cellular localization. In these images α -pYSTAT5 was stained as green (upper row), α -pS193 stained the STAT5 pS193 as red (second row), and DAPI stained the nucleus as blue (third row) and overlay

showed the colocalization of pYSTAT5 and STAT5pS193 indicated as yellow (bottom row). B) Quiescent PHA-activated human T cells were stimulated with medium (-) (lane a) or IL-2 (+) from 0-90 min (lanes b-f) and fixed with cold methanol. Then cells were stained with anti-STAT5pS193 to determine the phospho-kinetics of STAT5 S193 phosphorylation and sub-cellular distribution. C) Quiescent PHA-activated human T cells were stimulated with medium (-) (lane a) or IL-2 (lane b), 7 (lane c), 9 (lane d) or 15 (lane e) for 15 min and fixed with cold methanol. Cells were then stained with anti-pS193 (STAT5) to determine the phospho-kinetics of STAT5 S193 phosphorylation and sub-cellular distribution monitored. Representative data from three independent experiments are shown.

2.4 DISCUSSION

The JAK3-STAT5 pathway has gained importance in lymphoid cell mediated diseases. STAT5 has been previously identified to be aberrantly activated in various types of cancers including leukemias, lymphomas, prostate, breast, head, neck and colon. Often, STAT5 is tyrosine phosphorylated within these samples. Limited research of STAT5 serine phosphorylation is known however phospho-serine sites were found to be an important regulator of transcriptional activity in other STATs (Park S.H. 2001; Friedbichler K 2010). As discussed earlier, mass spectrometry (Fig 2.1 (A) & (B)) identified a novel phosphoserine site STAT5pS193 located in the helical turn between coil 1 and coil2. ClustalW analysis (Fig. 2.2 (A) & (B)) against other hSTATs within this region suggested that this residue is only conserved in STAT3 and STAT6. When STAT5 sequences were aligned across primates and non- primates, the serine site was also found in monkey and chimpanzee however this residue was not conserved in non-primate mammals such as mouse and rat. From this phylogenetic analysis we conclude that STAT5pS193 may have some important role in primates where this

site could modulate STAT5 activity. Moreover, the surrounding region in both primates and non-primates was highly conserved (90%) suggesting that this region has important functional significance.

Confocal microscopy and phosphokinetic analysis (Fig 2.6 (A) &(B)) revealed that this site undergoes phosphorylation in the cytoplasmic region in both human NK cells and PBMCs and that the STAT5pS193 translocates to the nucleus. Duration of STAT5pS193 was transient in comparison to STAT5pY699 as there was no detectable STAT5pS193 at 60 min after cytokine stimulation. Conversely, tyrosine phosphorylation was still present at a low level. These data suggest that STAT5 S193 phosphorylation is not a prerequisite for tyrosine (Y694/699) phosphorylation, receptor recruitment or dimerization while it is known that gamma common cytokines are important for lymphoid cells development and maturation as they all can activate the JAK3-STAT5 pathway. Defective signaling mediated by gamma common cytokines can lead to lymphopoiesis and severe combined immunodeficiency (SCID). Gamma common cytokine studies (Fig. 2.6 (C)) suggested us that phosphorylation of STAT5S193 is not restricted to IL-2 but the phosphorylation of this site is also mediated by other cytokines too.

Chapter 3: Investigate the regulatory pathways governing STAT5 S193 phosphorylation in human lymphocytes

3.1 INTRODUCTION

Reversible processes of dephosphorylation and phosphorylation are critically important to regulate STAT5 functional activities. Numerous studies have suggested that serine/threonine kinases play active roles in T cells maturation, development and differentiation (Kirken RA 2001; Smith-Garvin JE 2009; Friedbichler K 2010). To determine the putative serine/threonine kinase that phosphorylates STAT5 S193 one would be required to screen of more than 500 serine/threonine kinases in the human kinome family. Loss of functional kinase activity by RNA interference is currently available commercially but has a high false positive rate and off-target effects that limit its effectiveness. The rational approach to screen the potential STAT5pS193 serine/threonine kinase(s) is to identify those kinases that are characterized as proline directed serine/threonine kinase as this site has been flanked by a proline residue. Few kinases have been characterized as proline directed, but include mitogen-activated protein kinase/extracellular signal-regulated kinase (MEK) (Haq R 2002), phosphatidylinositol 3-kinase (PI3K) (Fung MM 2003) and mammalian target of rapamycin (mTOR) (Kim JH 2009) that might phosphorylate STAT5 S193.

Negative regulatory pathways are equally active to maintain homeostasis in normal healthy cells once signal is transmitted to the nucleus. To downregulate the JAK3-STAT5 pathway, SOCS, PIAS and protein tyrosine phosphatases have been well characterized by several studies (Shuai K 2003). Recently serine/threonine phosphorylation at the receptor level as well as downstream of this pathway gained huge importance to control STAT5 activity upon

cytokine stimulation (Ross JA 2010). The dephosphorylation of STAT5pS193 is an important aspect so that STAT5 should not become hyperactive in normal healthy cells. We need to identify the putative serine/threonine phosphatase(s) to delineate the negative regulator of STAT5pS193 dephosphorylation.

3.2 MATERIALS AND METHODS

3.2.1 SERINE/THREONINE KINASE INHIBITORS

For proline directed serine/threonine kinase-inhibitor experiments, cells were preincubated for 1 hr with DMSO as a mock control or varying concentrations of PD98059 (NewEngland Biolabs), Rapamycin (Calbiochem), staurospoine (Sigma Aldrich) wortmannin (Calbiochem) or PP242 hydrate (Sigma Aldrich) and were then prefixed at -20 °C methanol for 5 min and then washed with 1X phosphate-buffered-saline solution. These pretreated cells were then stained and imaged as previously mentioned in the earlier chapter. Images were developed in the Carl Zeissmicroscopy LSM 500 and PASCAL software.

3.2.2 PHOSPHATASE INHIBITORS

PP1 and PP2A inhibitor calyculin A (CA) (Sigma Aldrich), PP2A inhibitors okadaic Acid (OA), fostriecin (Sigma Aldrich) and PP1 inhibitor tautomycin (TAU) (Sigma Aldrich) were incubated for 1 hr at 37 °C YT cells were treated with the recommended doses. Subsequently cells were stained and analyzed as previously described.

3.3 RESULTS

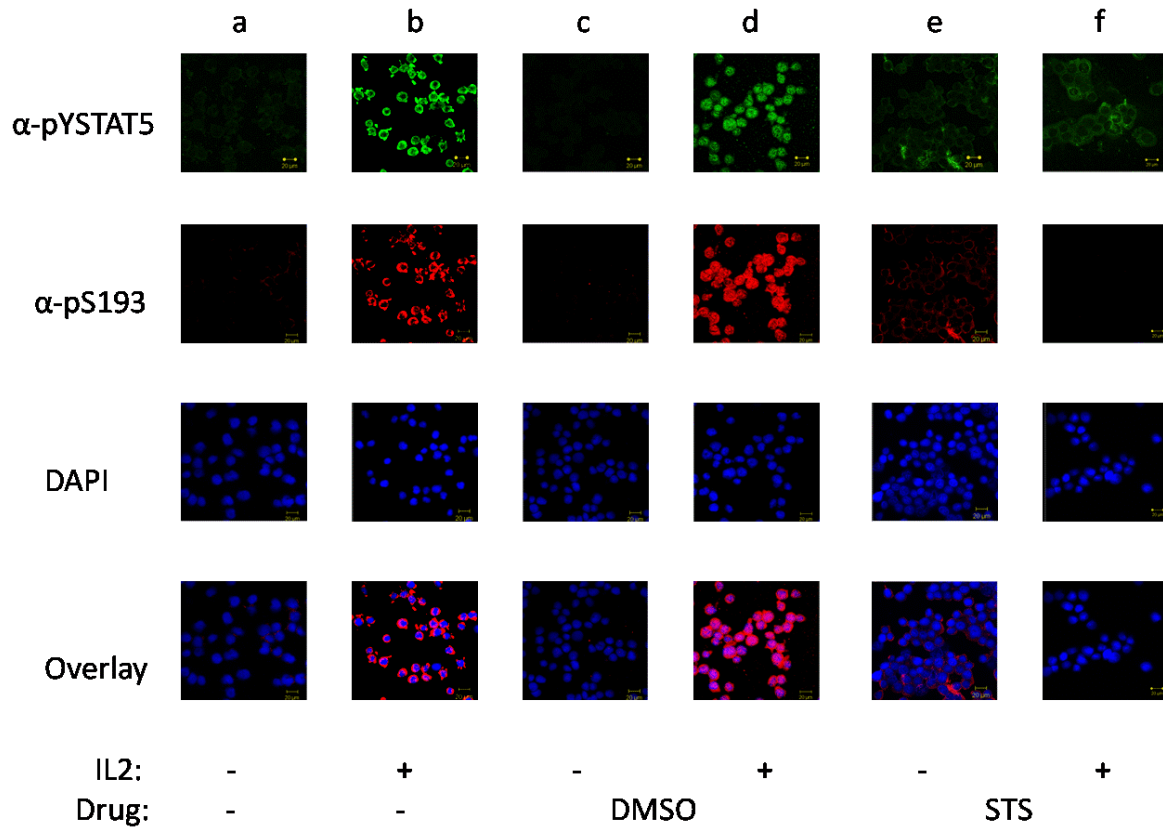
3.3.1 A STAUROSPORINE SENSITIVE PROLINE DIRECTED SERINE/THREONINE KINASE MEDIATES STAT5S193 PHOSPHORYLATION

Staurosporine is a well-established pan-kinase inhibitor which competitively binds to the ATP binding pocket (Prade L 1997). To determine whether STAT5 S193 was phosphorylated by a staurosporine sensitive serine/threonine kinase the following study was performed. Fig. 3.1 lanes (e) & (f) showed that pretreatment of YT cells with 50 nM staurosporine completely blocked STAT5 S193 phosphorylation compared to untreated controls (lanes a and b) or vehicle treated cells (lanes c and d). Unfortunately the specificity of this drug is not clear as it also inhibits JAK3 which resulted in a loss of STAT5 tyrosine phosphorylation was also inhibited (Fig. 3.1 upper panel) which complicates the data. Nonetheless this data does suggest that the STAT5 S193 kinase is staurosporine sensitive.

Previous reports by other groups have suggested that STAT serine kinase may depend on RAS-RAF-MEK and PI3K-mTOR pathways (Nagy ZS 2002; Fung MM 2003). Targeting these kinases with different inhibitors was valuable in this analysis. To corroborate this, we performed a dose and time course with the mTOR inhibitor, rapamycin, shown in Fig. 3.2 (A), (B). Rapamycin treated YT cells showed that STAT5pS193 was decreased in a dose dependent manner (Fig. 3.2A second panel) while tyrosine phosphorylation of STAT5 was not altered (Fig. 3.1B upper panel). To confirm this finding, YT cells were treated with another mTOR inhibitor PP242 hydrate which resulted in a similar response but required slightly higher drug concentrations (Fig. 3.2C

second panel). Moreover, STAT5 S193 phosphorylation was inhibited by rapamycin in a time-dependent manner with maximal inhibition occurring at 1 hr (Fig. 3.2B). Specifically, the inhibitory effect of rapamycin was detectable at 60 min pretreatment (Fig. 3.1 B lane d). All of these experiments suggest that STAT5 S193 phosphorylation may be dependent upon the mTOR pathway for its regulation directly or indirectly.

A)



B)

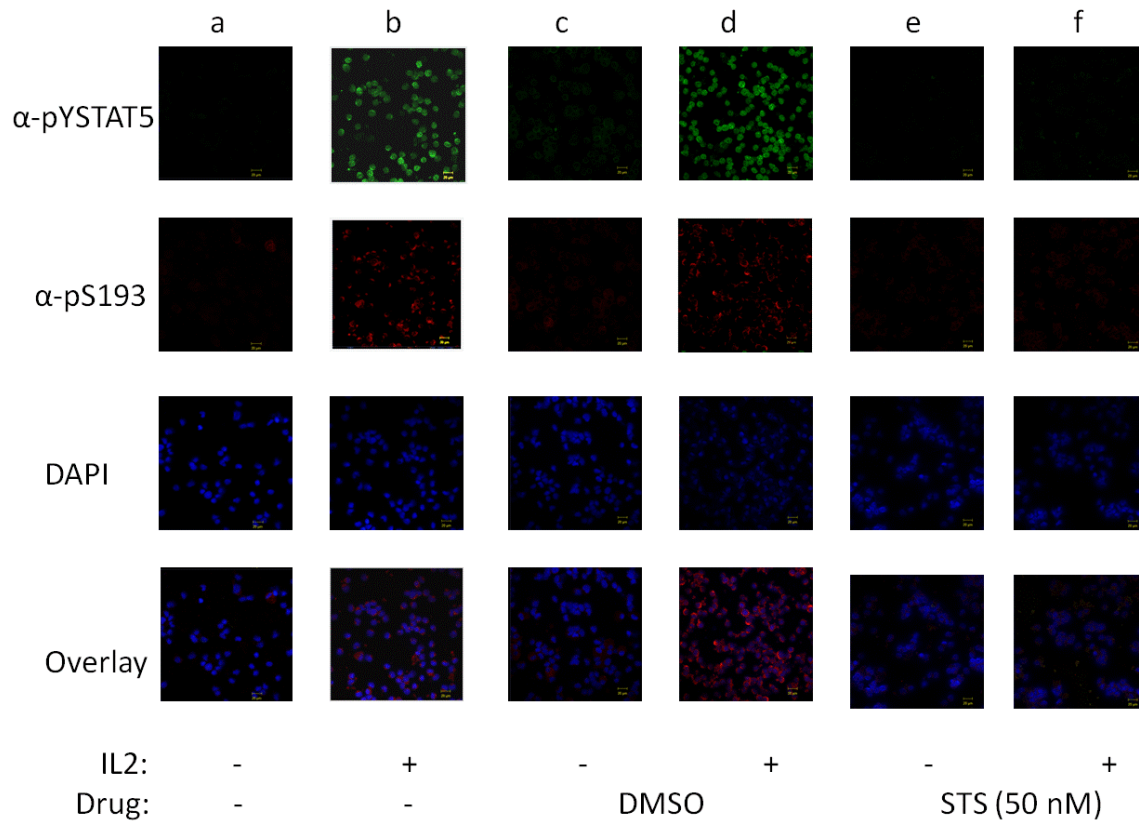
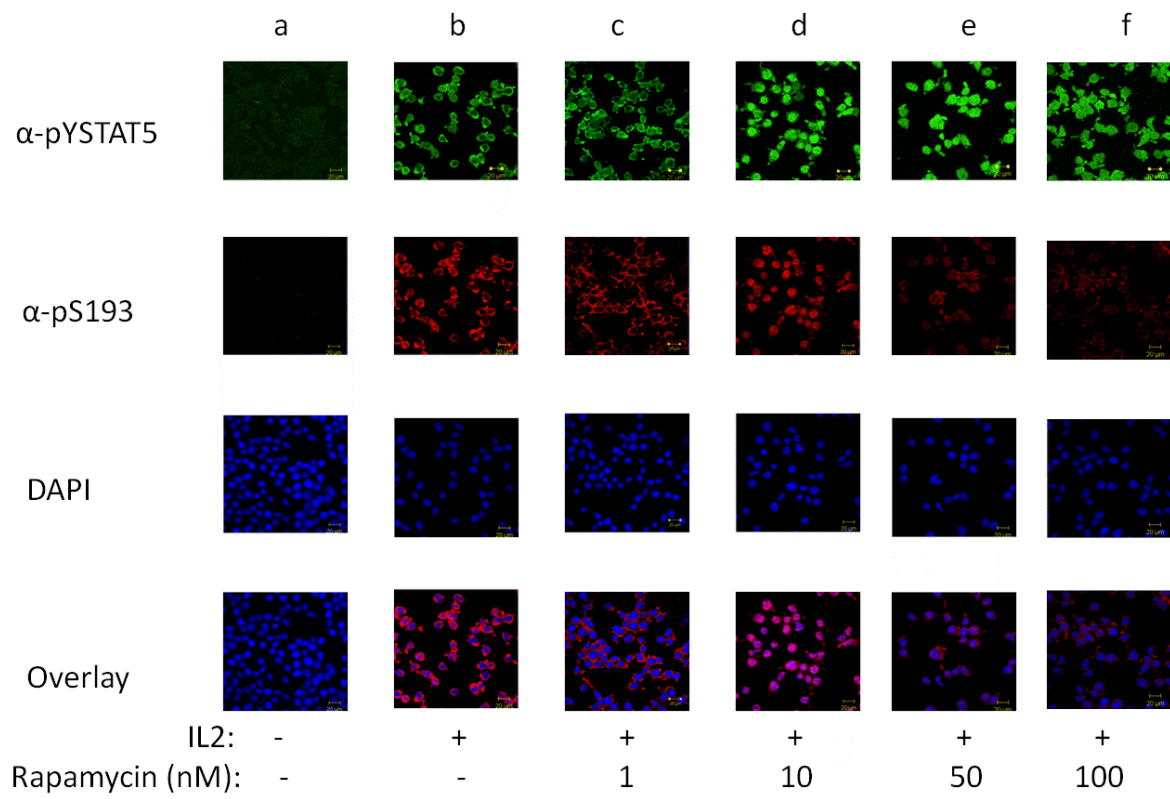


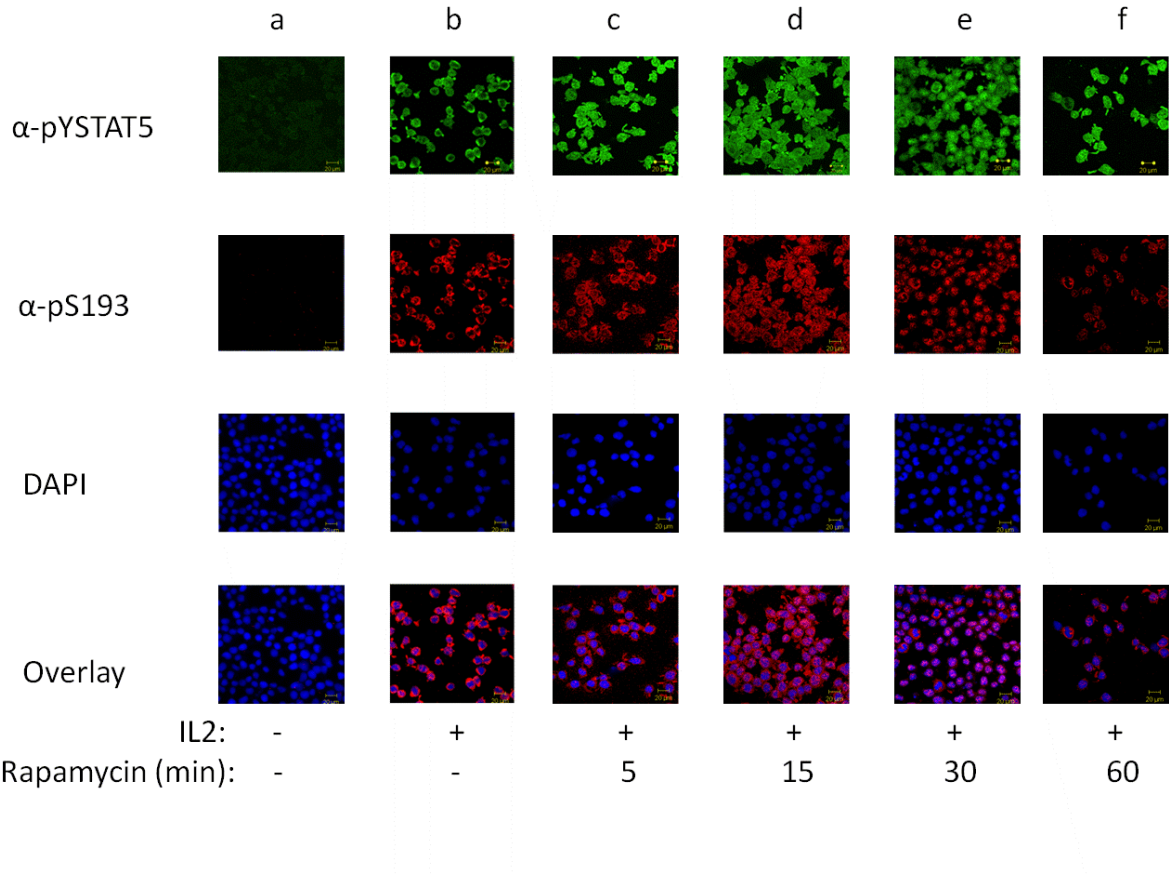
FIGURE 3.1: A staurosporine sensitive serine kinase phosphorylates STAT5 S193.

Human NK cell line, YT, were pretreated with 50 nM staurosporine (STS) for 1 hr followed stimulated without (-) or with (+) IL-2 for 15 min. In this diagram α-pYSTAT5 (upper row) was stained the activated STAT5 as green, α-pS193 (second row) stained the STAT5 pS193 as red, DAPI (third row) stained the nucleus as blue and overlay (fourth row) showed the colocalization of STAT5pS193 and DAPI. Representative data from three independent experiments are shown.

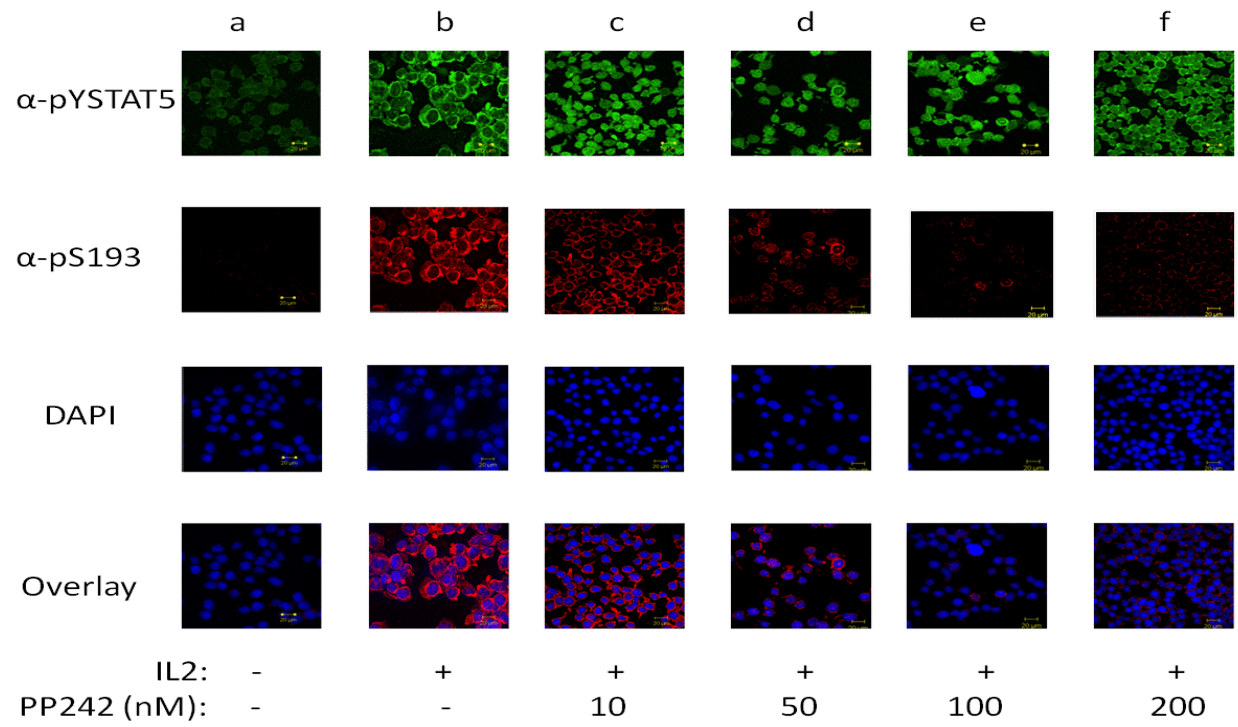
A)



B)



c)



D)

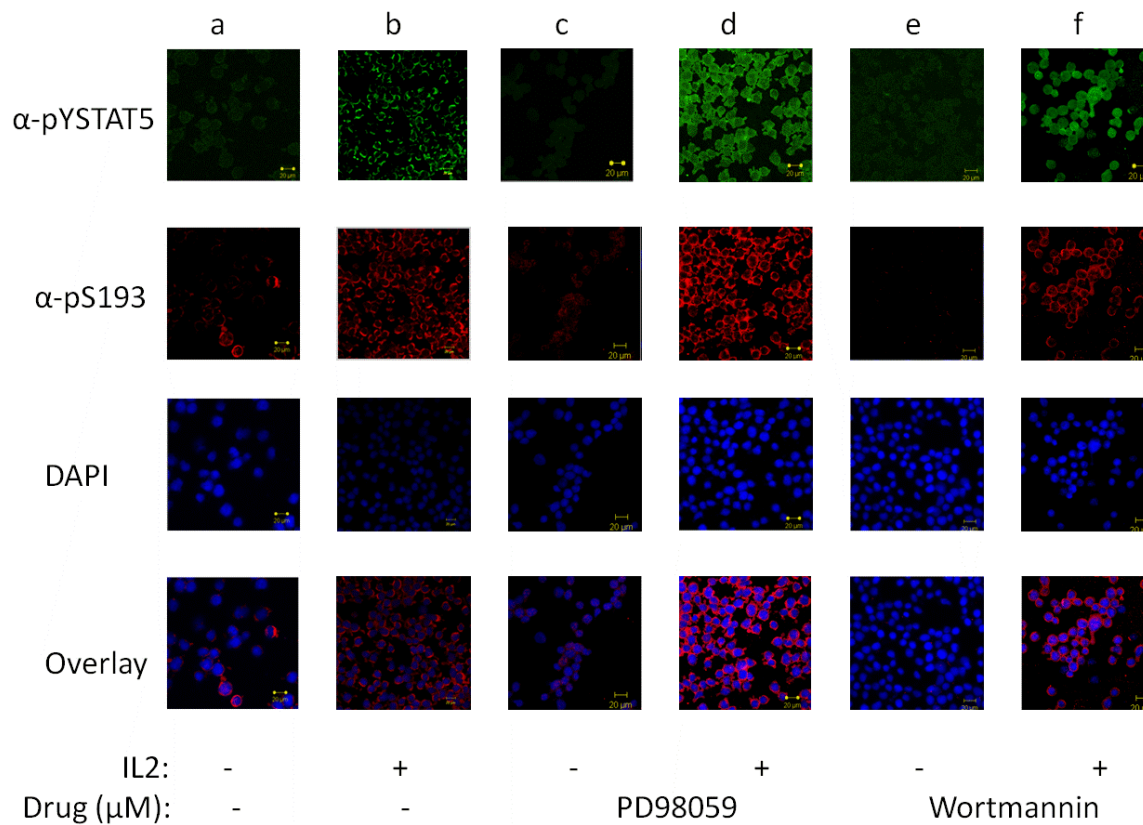


FIGURE 3.2: STAT5S193 undergoes phosphortylation in an mTOR dependent manner in human NK cells.

A) YT cells were left untreated (lanes a and b) or pretreated with 1-100 nM rapamycin (lanes c-f) for 1hr followed by stimulated with 100 nM IL-2 (lanes b-f) for 15 min at 37 °C. B) Cells were left untreated (lanes a and b) or treated with 100 nM rapamycin for 5-60 min (lanes c-f), prior to stimulation with 100 nM IL-2 for 15 min (lanes b-f). C) Cells were left untreated (lanes a and b) or pretreated with 10-200 nM PP242 hydrate for 1 hr followed by stimulation with IL-2 for 15 min (lanes b-f) as indicated. D) Cells were left untreated (lanes a and b) or pretreated with 250 μM PD98059 (lanes c and d) or 50 μM wortmannin (lanes e and f) for 1hr

followed by stimulated with 100 nM IL-2 (lanes b-f) for 15 min at 37 °C. Cells were fixed by cold methanol (-20 °C) and α -pYSTAT5 (upper row) stained the activated STAT5 as green, α -pS193 (second row) stained the STAT5 pS193 as red, DAPI (third row) stained the nucleus as blue and overlay (fourth row) showed the colocalization of STAT5pS193 and DAPI. Representative data from three independent experiments are shown.

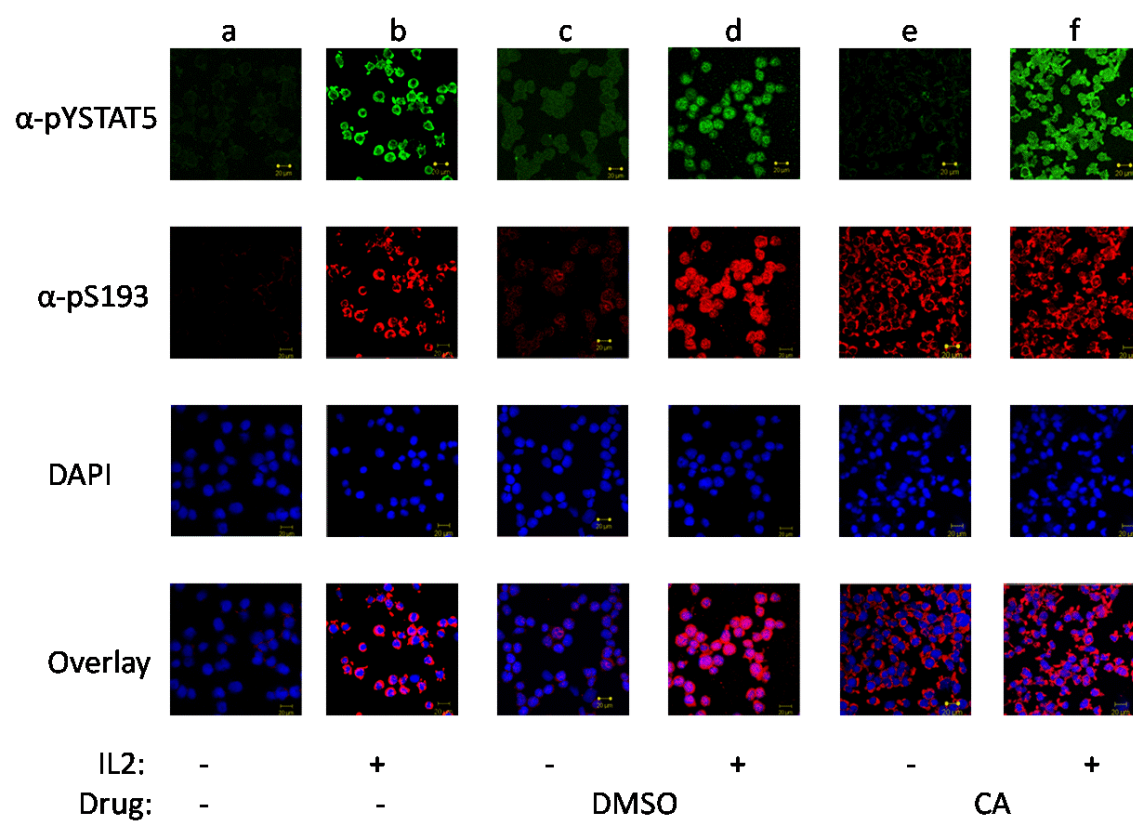
3.3.2 SERINE/THREONINE PHOSPHATASE PP2A FAMILY NOT PP1 SELECTIVELY DEPHOSPHORYLATES STAT5PS193

Tyrosine phosphatases as negative regulators of the JAK-STAT pathway have been well characterized by several studies however recent studies showed that serine/threonine phosphatase also played active role to control this pathway (Ross JA 2010). Calyculin A (CA) is a serine/threonine phosphatase inhibitor that disrupts PP1 and PP2A (Ishihara H 1989). As shown in Fig 3.3 (A), YT cells were pretreated with either DMSO (vehicle) (lanes c and d) or 50 nM CA (lanes e and f) for one hr followed by without (-) or with (+) IL2 for 15 min. Cells staining with polyclonal α -STAT5pS193 showed basal levels of phosphorylation and dephosphorylation occur. Blockade of PPP family of serine/threonine phosphatases by CA, inhibited the basal level of dephosphorylation (second panel lane e) resulting in constitutively active STAT5pS193 however lanes a and c did not display any basal level STAT5S193 phosphorylation. Another outcome of this experiment was that the sub-cellular distribution of STAT5pS193 from CA treated cells was localized to the cytoplasm as the colocalization images indicated a very low level of red in the nucleus. Based on this data, it can be concluded that phosphorylation of STAT5S193 occurred in the cytoplasmic domain of cells. Lane f showed that pre-treatment with CA did not block the tyrosine (Y694/699) phosphorylation of STAT5

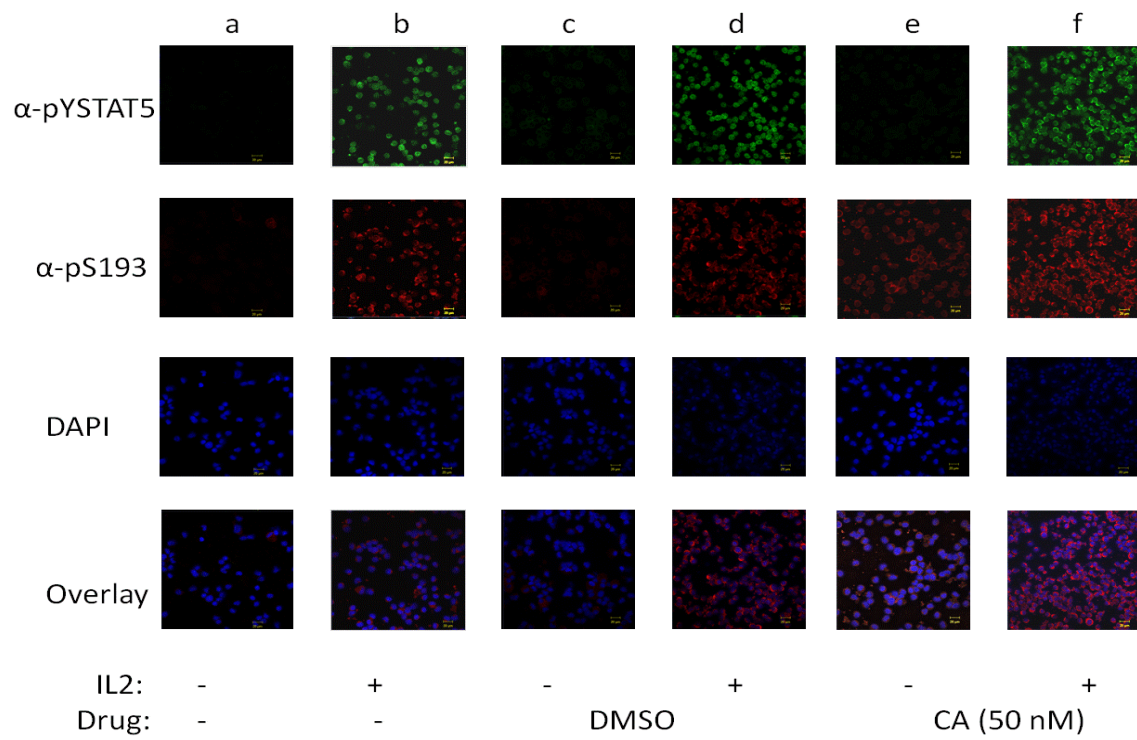
nor its sub-cellular localization. Similar experiments were performed in hPBMCs with identical doses and pretreatment time with CA (Fig.3.3 (B)) and we saw similar outcome.

To gain further insight whether PP1 or PP2A dephosphorylates STAT5pS193, PP1 or PP2A specific inhibitors were administered. Fig 3.3 (C) showed that OA (lanes c and d) and Fostriecin (lanes e and f) which preferentially block PP2A group phosphatases, inhibited the dephosphorylation of STAT5pS193. Conversely, the PP1 specific inhibitor TAU (lanes g and h) did not block the basal level of dephosphorylation of STAT5pS193. These experiments suggest that PP2A group but not PP1 is the principal serine/threonine phosphatase that negatively regulates the STAT5pS193. We also conducted similar experiments in hPBMCs (Fig. 3.3 (D)) with similar doses of inhibitors, treatment time and there is no significant change observed.

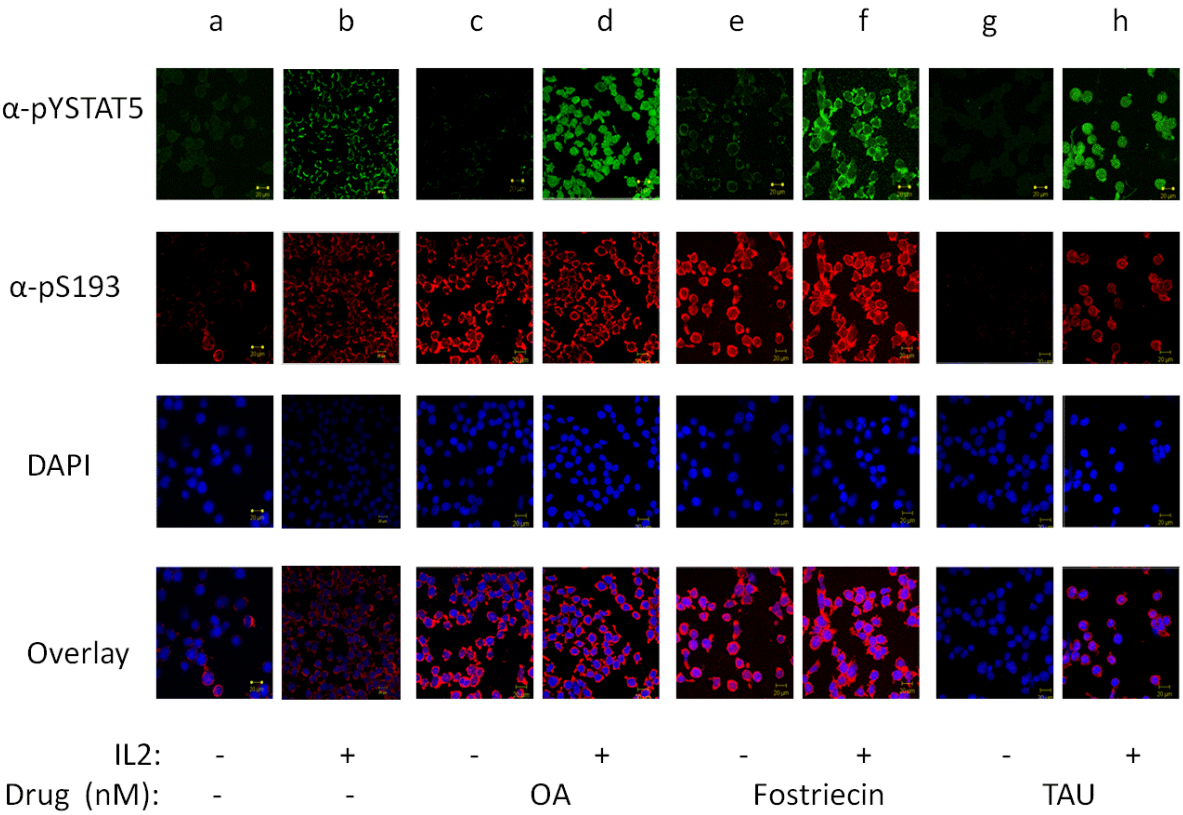
A)



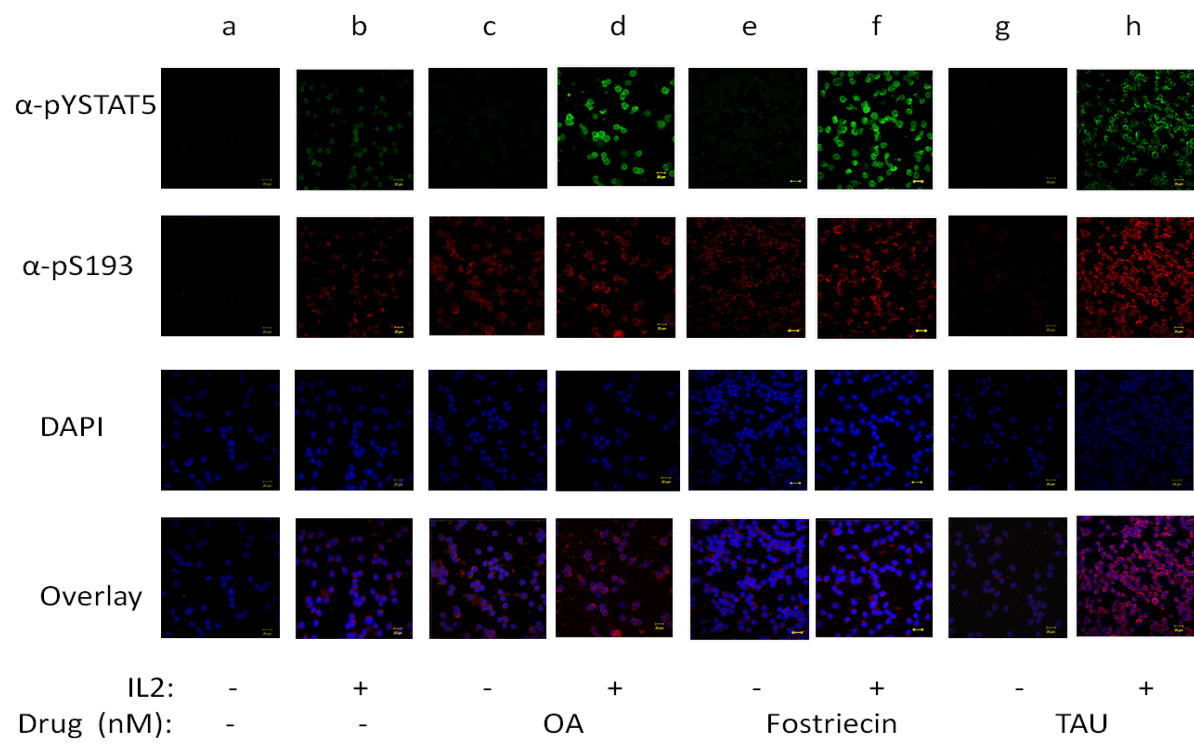
B)



c)



D)



E)

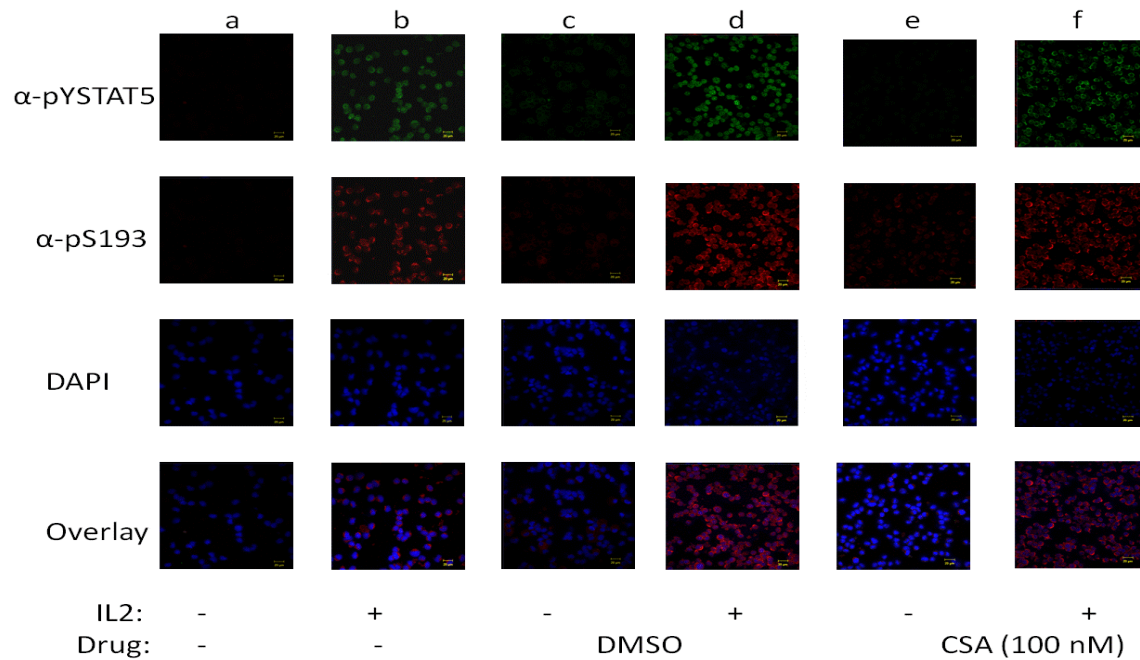


FIGURE 3.3: PP2A negatively regulates STAT5 S193 phosphorylation in human lymphocytes.

A) Human NK cells and B) hPBMCs were stimulated with medium (-) or IL-2 (+) for 15 min and pretreated with 50 nM CA for 1 hr. Lanes (a) and (b) untreated cells, (c) and (d) DMSO treated cells and (e) and (f) CA treated cells stimulated without (-) or with (IL-2) for 15 min. C) Human NK cells pretreated with 50 nM PP2A inhibitor OA (lanes c and d), 25 nM Fostriecin (lanes e and f) and 1 μ M PP1 inhibitor TAU (lanes g and h) for 1 hr followed by without (-) or with 100 nM IL-2 for 15 min. Cells in lanes a and b do not undergo any drug treatment and they are stimulated without (-) or with IL-2 (+) respectively for 15 min. D) Human PBMCs pretreated with 10 nM PP2A inhibitor OA (lanes c and d), 25 nM Fostriecin (lanes e and f) and 1 μ M PP1 inhibitor TAU (lanes g and h) for 1 hr followed by without (-) or with 100 nM IL-2 for 15 min. Cells in lanes a and b do not undergo any drug treatment and they are stimulated

without (-) or with IL-2 (+) respectively for 15 min. E) Human PBMCs pretreated with 100 nM PP2B inhibitor CSA (lanes e and f), DMSO (lanes c and d) and no treatment (lanes a and b) for 1 hr followed by without (-) or with (+) 100 nM IL-2 for 15 min. Cells in lanes a and b do not undergo any drug treatment and they are stimulated without (-) or with IL-2 (+) respectively for 15 min. Representative data from three independent experiments are shown.

3.4 DISCUSSION

Results from these studies provide new insights regarding the positive and negative regulator of the JAK3-STAT5 pathway. The most important aspect of this chapter was to investigate the putative proline directed serine/threonine kinase that competent to phosphorylate STAT5S193. Staurosporine studies (Fig.3-2 (A)) suggested that the putative kinase is staurosporine sensitive. To further investigate the putative serine/threonine kinase we screened kinases known to interact with other STATs and identified as potential proline directed serine/threonine kinases. Kinase specific inhibitor studies screened indicated that it is dependent on a mTOR pathway, but not MAPK or PI3K. Dose curve, time course and treatment with a second inhibitor that preferentially blocks mTOR were also effective (Fig 3-2 (B), (C)). This evidence suggests us that phosphorylation of STAT5S193 is dependent on mTOR regulated whether directly or indirectly is not known. Further studies such as co-immunoprecipitation of mTOR with STAT5 or pull down assays might clarify the association.

Negative regulatory mechanisms of this pathway suggest that the serine/threonine phosphatase family plays an important role in the recruitment of STAT5 to the receptor as well as activation of this pathway. Basal levels of STAT5 S193 phosphorylation were constitutive and PP2A mediated the dephosphorylation process of STAT5. CA inhibitor studies (Fig. 3-1 (A)) suggest us that the dephosphorylation of STAT5pS193 is mediated by PP1 or PP2A. Subsequent studies (Fig. 3-1 (B), (C)) with PP1 specific inhibitor Tautomycin and PP2A specific inhibitor Okadaic acid and Fostriecin substantiated that this negative regulation is performed by PP2A.

Chapter 4: Determine the functional and potential biological significance of S193 phosphorylation for STAT5 function

4.1 INTRODUCTION

Chapters two and three were predominantly concerned with the identification of a novel phosphoserine residue in STAT5S193 that may serve a regulatory role important for maintaining cell function. Herein, it is important to understand on how the phosphorylation of STAT5S193 modulates one of several functional activities of STAT5 that define cell differentiation, growth and survival. Moreover, it would be important to investigate whether this site undergoes constitutive phosphorylation in hematological malignant patient samples as well as Human T-lymphotropic virus Type I (HTLV-1) transformed cell lines since constitutively active STAT5 is known to be. Previous studies on serine phosphorylations for different STATs, including STAT5, suggested that serine phosphorylation of STAT5 (726/31) is required for the maximal transcriptional activation (Yamashita H 1998; Kee Chuan Goh 1999; Park S.H. 2001). Cell based studies showed that whenever a conserved and phosphorylate serine site was mutated to alanine the transcriptional activity of STAT was significantly reduced. However, these serine sites were localized to the transcriptional activation domain of STATs which we now know regulates transcription. Our novel STAT5 S193 is located in the coiled-coil domain of STAT5 distal to this region, which may interact with various co-regulators. Potential functional roles may include receptor recruitment, dimerization, nuclear translocation, DNA binding activity and transcriptional activity. To investigate the functional importance STAT5S193, we therefore reconstituted JAK-STAT5 minimal machinery in HEK-293 cells by incorporating IL-2R β , IL-2R γ , hJAK3 and hSTAT5. Phosphorylation of STAT5S193, its mutant on transcriptional

activity was measured by EMSA and luciferase assays compared with wild type STAT5. Furthermore, both T and B cells leukemia patient samples were screened to understand the potential clinical significance of this site and whether it may be hyperactive in these samples.

4.2 MATERIALS AND METHODS

4.2.1 CELL CULTURE

Human Embryonic Kidney 293 (HEK-293) cells were grown in RPMI 1640 containing 10% Fetal Bovine Serum (FBS) (Atlanta Biologicals), 2 mM L-glutamine (Cellgro), and penicillin-streptomycin (50 IU/ml and 50 µg/ml, respectively) (Cellgro) at 37 °C with 5% CO₂.

4.2.2 STAT5 SITE DIRECTED MUTAGENESIS

Human STAT5 cDNA was purchased from the Origene and used for the template and subcloned into pCMV-FLAG (Agilent). Point mutations were made using a QuickChange site-directed mutagenesis kit (Stratagene). Primers were designed to mutate S193A (5'-GCTGGCCCAGCTGGCCCCC-3'), S193E (5'-CCGCTGGCCCAGCTGGAAC-3'), S726/731A (5'-GGACCAGGCCCCGGCCCCAG-3'), S193AS726A and S193AS731A as per the requirements of Stratagene Kit. Primer products were transformed into high competent cells XL-1 blue and grow the mutants. Sequences were verified by the DNA sequencing core.

4.2.3 JAK3-STAT5 PATHWAY RECONSTITUTION SYSTEM

Semi-confluent HEK-293 cells were transfected in 10 cm dish with the following plasmids 6µg hIL2 receptor β (IL-2Rβ), 6µg hIL2 receptor γ (IL-2Rγ), 250 ng hJAK3 and 3 µg hSTAT5 or its mutant forms such as hSTAT5 S193A or hSTAT5 S193E. Cells were incubated with each plasmid for 24 hrs at 37 °C with complete media followed by replacement with 1% FBS containing complete media to keep in under quiescent conditions. After 48 hr cells were

shoot off from the plate and centrifuged at 560 g for 10 min. Then cells were resuspended in 2ml media and aliquoted into two centrifuge tubes with 1 ml each. Cells were stimulated with 10,000 IU/ml IL-2 or without for 15 min. Then media was harvested and pellets were lysed with 1% Triton-X containing lysis buffer for 30 min at 4 °C as previously described. Supernatants were harvested into fresh tubes and quantitated the protein concentration by BCA protein assay as mentioned previously. Proteins (10 µg) were loaded per well and separated by 7.5% SDS-PAGE gel and transferred to PVDF membrane and Western-blotted with α -pYSTAT5 (Millipore) to monitor tyrosine phosphorylation induction of STAT5.

4.2.4 LUCIFERASE ASSAYS

The β -casein-luciferase reporter plasmid was generated by cloning a triple repeat of the STAT5 consensus site corresponding to the β -casein gene promoter (5'-AGATTTCTAGGAATTCAATCC-3') into the pGL3-Promoter vector (Promega) using SacI and XhoI restriction sites (Cheng H 2008). HEK 293 cells containing the IL-2R component and hJAK3 were transfected with either luciferase or empty plasmid vector and normalization against the pCMV- β gal vector in transfected cells. At 48 hrs post transfection, cells were stimulated without or with 10,000 IU/ml IL-2 for 6 hrs. Luciferase activity was measured using the Dual Light luciferase assay kit (Applied Biosystem).

4.2.5 NUCLEAR EXTRACTION AND ELECTROPHORETIC MOBILITY SHIFT ASSAYS

HEK-293 were reconstituted cells with either WT STAT5B or STAT5B S193A or STAT5B S193E. Cells were stimulated without (-) or with (+) 10,000 IU/ml IL-2 for 30 min at 37 °C. Supernatants were harvested and pellets washed two times with 1X PBS (Cellgro) and lysed using Buffer A (10 mM HEPES (pH 7.9), 10 mM KCL, 10 mM EDTA, 100 mM DTT,

0.4 % IGEPAL, 5 µg/ml aprotinin, 2 µg/ml leupeptin, 1 µg/ml pepstatin A, 1 mM PMSF) and rocked for 10 min at 4 °C. Cells lysates were centrifuged at 20,854 g for 5 min and supernatants transferred to new tubes. The pellet was washed twice with 1X PBS to discard cytoplasmic impurities. The buffer B (20 mM HEPES (pH 7.9), 400 mM NaCl, 1 mM EDTA, 10% Glycerol , 100 mM DTT, 5 µg/ml aprotinin, 2 µg/ml leupeptin, 1 µg/ml pepstatin A, 1 mM PMSF) then vortexed for 2hrs at 4 °C. Nuclear extracts were quantitated by BCA protein method. Nuclear extracts (5 µg) were pre incubated with binding buffer, poly DIDC for 1 hr at 4 °C. Oligos of the β-casein promoter element was radio-labeled with [³²P]-γ ATP and incubated with nuclear extracts with radio-labeled oligo for 10 min at room temperature and separated on non-denaturing gels for 2 hrs. The gel was subsequently dried at 70 °C for an hour and exposed to X-ray film overnight at -80 °C and the autoradiograph developed by a Kodak automated film developer.

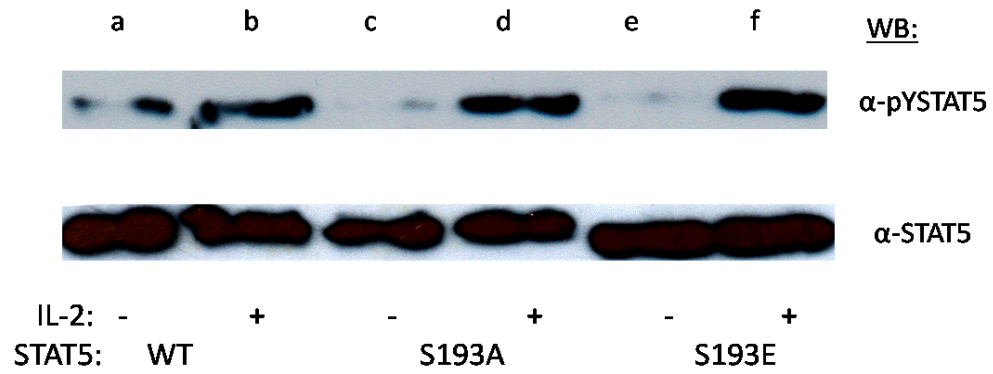
4.3 RESULTS

4.3.1 S193A MUTATION HAS NO EFFECT ON TYROSINE (Y694/699) PHOSPHORYLATION OR NUCLEAR TRANSLOCATION IN HEK-293 RECONSTITUTED WITH IL-2R COMPLEX.

HEK-293 cells do not express endogenous STAT5, JAK3, IL-2Rβ or IL-2Rγ. Therefore a reconstitution system was generated by transfecting the plasmids for activations of JAK3-STAT5 pathway to measure STAT5 activation. Fig 4.1 (A) lane d showed that the STAT5S193A mutant did not cause any change in the tyrosine phosphorylation status of STAT5. Moreover, STAT5pS193 was able to be recruited to the receptor and dimerized. Reblot with α-STAT5B (shown in the lower panel of Fig 4.1(A)) represented equivalent loading of proteins in all of the lanes a-f. To study the effect of STAT5 on sub-cellular distribution, we stimulated cells without (-) or with (+) IL-2 for 30 min and stained the cells

with α -pYSTAT5 on α -STAT5 as shown in Fig. 4.1 (B). Lane a in Fig. 4.1(B) showed IL-2 induced sub-cellular distribution of pY-STAT5. Upper two panels of lane a in Fig. 4.1 (B) showed the distribution of wt STAT5 and compared to that next two panels did not exhibit any significant change in the distribution of STAT5S193A mutant in lane a of Fig. 4.1(B) in an IL-2 dependent manner. Also phosphomimetic mutant STAT5 S193E represented similar pattern as shown in lower two panels in lane a. Similarly total STAT5 distribution (lane b) of wild type and mutants and colocalization of induced and total STAT5 (represents as yellow in lane c) were similar. Although STAT does not have any classical NLS signal, this experiment suggest us that STAT5S193 is not important for nuclear translocation. Confocal analysis also provided valuable information that total STAT5 was predominantly in cytoplasmic portion of the cells however activated STAT5 was distributed in both nucleus and cytoplasm.

A)



B)

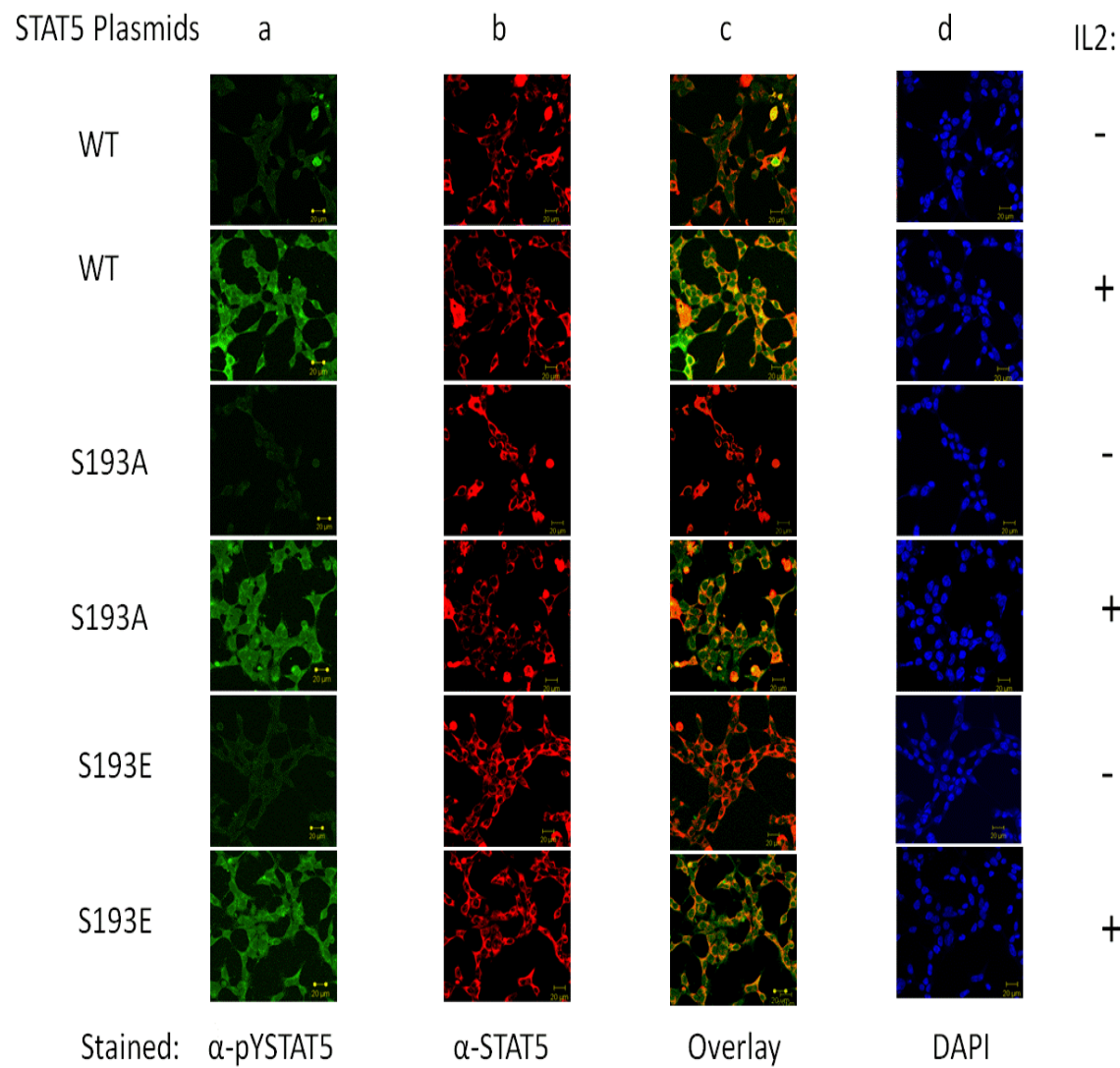


FIGURE 4.1: hSTAT5 S193 phosphorylation does not affect its recruitment to the receptor, activation and dimerization.

A) S193A does not affect IL2 induced STAT5 activation and pY(694) in HEK-293 reconstitution system. HEK-293 cells were transfected with IL-2R β , IL-2R γ , JAK3 and STAT5

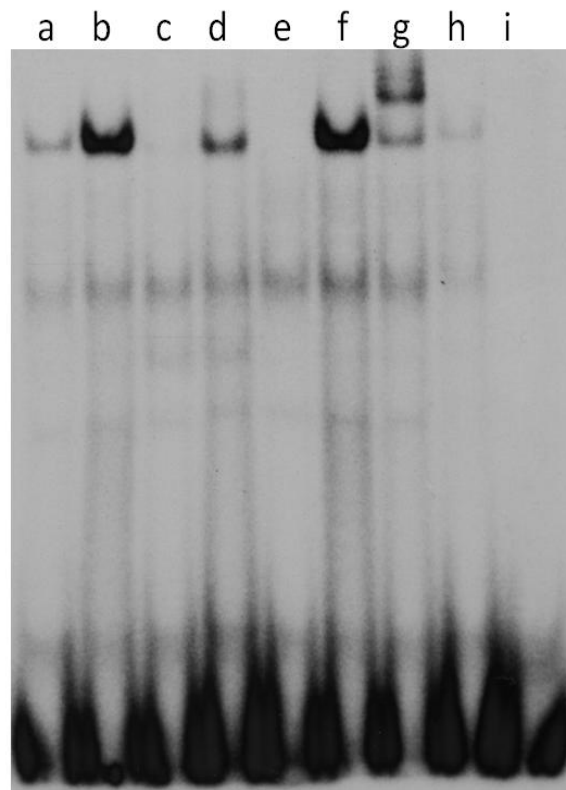
(WT, S193A, S193E) and incubated for 48 hrs. Cells were stimulated without (-) or with IL-2 (+) for 15 min. STAT5 Wt (lanes a and b), STAT5 S193A (lanes c and d) and STAT5 S193E (lanes e and f) were blotted with α -pYSTAT5 (upper panel) or total STAT5 (lower panel). B) Sub-cellular distribution of wild types or mutant STAT5s without (-) or with (+) IL-2 was shown in confocal images. Wt STAT5 (upper two panels), STAT5 S193A (middle two panels) and STAT5 S193E (lower two panels) were stained with α -pYSTAT5 (lane a), α -total STAT5 (lane b), overlay between red and green (lane c) and DAPI (lane d).

4.3.2 S193A SHOWS REDUCED DNA BINDING CAPACITY AND CONSEQUENT TRANSCRIPTIONAL ACTIVITY OF STAT5

Electrophoretic-mobility shift assays (EMSA) were used to determine the DNA binding activity of STAT5 to a β -casein response element. Oligonucleotides were radio-labelled with [32 P]. Next, nuclear extracts were isolated from reconstituted cells treated without (-) or with (+) IL-2 and incubated with the radiolabelled probe. This complex was separated within a non-denaturing gel to determine its DNA binding activity. From Fig. 4.2 (A) the S193A mutation significantly decreased STAT5-DNA binding activity (lane d) stimulated by IL-2. To mimic the phosphorylated states of STAT5pS193 we generated phosphomimetic (STAT5S193E). In Fig. 4.2 lanes e and f STAT5S193E mutant partially recovered the DNA binding activity to the radiolabelled probe. The Supershift of STAT5-DNA complex occurred by an amino-terminal STAT5 antibody (lane g) while lane h showed no supershift when rabbit IgG was used as a negative. Lane I was void of any nuclear extract as an additional negative control. From this experiment we can conclude that phosphorylation of STAT5S193 is required for optimal DNA binding activity.

Next to see if reduced DNA binding correlated with decreased transcriptional activity, the last figure showed the transcriptional activity of STAT5 in luciferase assays. Fig. 4.2 (B) lanes c and d revealed the IL-2 induced wild type STAT5 transcriptional activity however lanes e & f showed STAT5S193A mutant decreased the transcriptional activity in comparison to wild type so it correlates with the EMSA data as shown in Fig. 4.2 (A). Lanes g & h showed phosphomimetic STAT5S193 partially recovered the binding activity of STAT5. Lanes i & j showed also partial reduction of STAT5 when there was a mutation of S731A. Lanes k and l showed IL-2 mediated response of transcriptional activity of the double mutant containing STAT5S193S731A and comparing individual mutant analysis it can be concluded that STAT5S193A has more significant role in its transcriptional activity in comparison to another mutant STAT5S731A.

A)



IL2:	-	+	-	+	-	+	+	+
Antibody:	-	-	-	-	-	-	N20	IgG
STAT5:	WT		S193A		S193E		WT	WT -
Probe:	+	+	+	+	+	+	+	+

B)

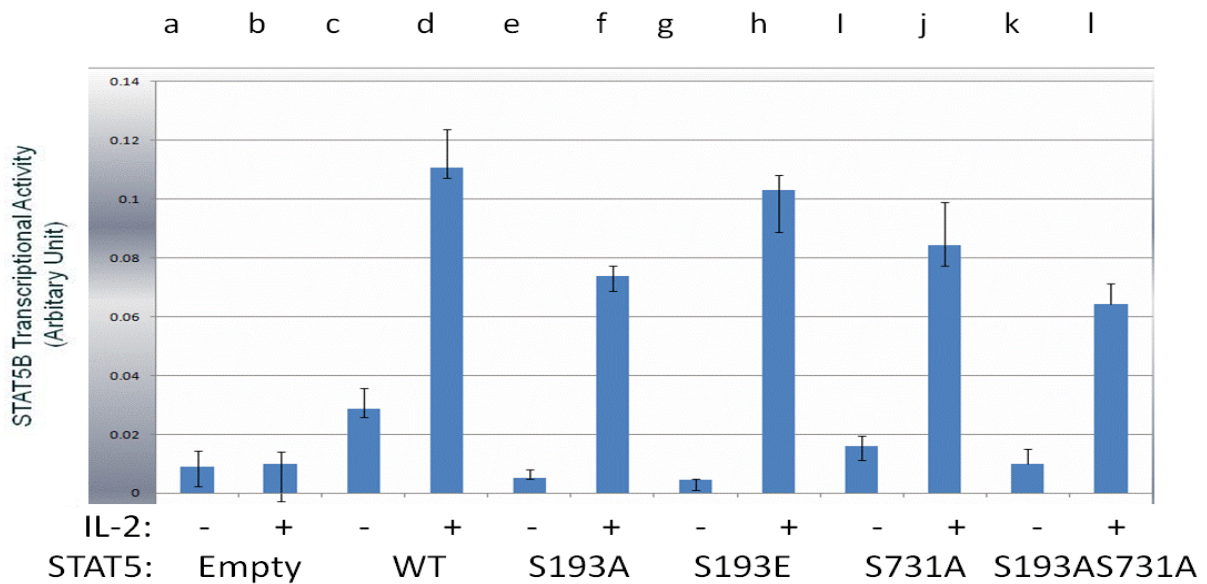


FIGURE 4.2: hSTAT5 S193 phosphorylation site positively regulates its transcriptional activity.

A) Nuclear extracts of IL-2 without (-) or with (+) treated HEK-293 reconstituted cells transfected with either Wt or mutant STAT5 incubated with radiolabelled oligonucleotide. Migrations of Wt STAT5-DNA complex (lanes a and b), STAT5 S193A-DNA complex (lanes c and d), phosphomimetic STAT5S193E-DNA complex (lanes e and f) were shown in non-denaturing gel. Similarly nuclear extract (Wt STAT5) were co-incubated with N-terminal directed α -STAT5 (lane g) or normal rabbit serum (lane h). B) In 10 cm dish, HEK-293 cells were transfected with either wt STAT5B (lanes c and d) or STAT5B S193A (lane e and f) or STAT5B S193E (lanes g and h) or STAT5B S731A (lanes i and j) or STAT5B S193A S731A

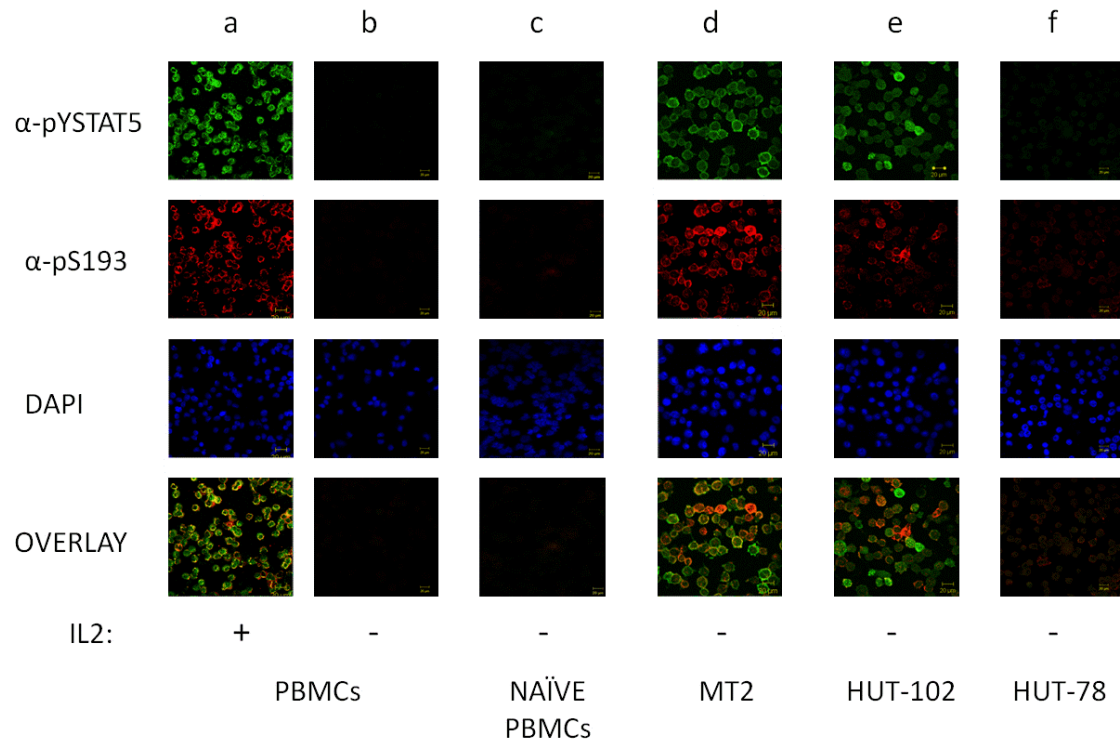
(lanes k and l), stimulated by without (-) or with (+) 100 nM IL-2 for 8 hrs. Control (CTRL) cells were transfected with identical amounts of empty vectors (lanes a and b). At 48 hrs posttransfection, Cells were lysed with ABI lysis buffer, and the luciferase activities were measured in luminoscan. Data was normalized by β -galactosidase. Representative data from three independent experiments are shown.

4.3.3 IDENTIFICATION OF CONSTITUTIVELY PHOSPHORYLATE STAT5 S193 IDENTIFIED IN T-ALL PATIENT SAMPLES AND HTLV-1 TRANSFORMED T CELL LINES

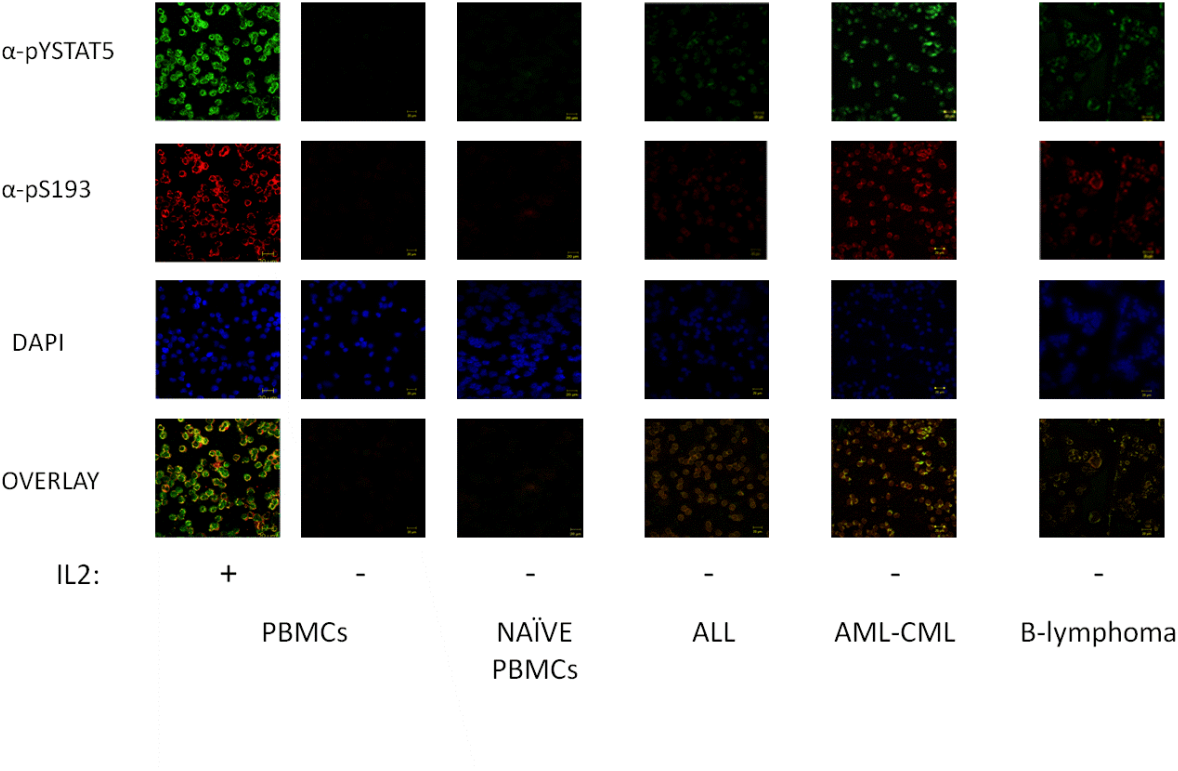
Elevated levels of STAT5 transcriptional activity has been identified in a significant number of diverse malignant patient samples and tumor cell lines. To correlate hyperactive STAT5 with constitutive STAT5 S193 phosphorylation, HTLV-1 transformed cell lines and patient with hematological cancers cell lines were screened. Similarly HTLV-1 transformed pathogenic T cells, MT2, HUT 102, were stained to determine constitutive STAT5 S193 phosphorylation. We found that MT2, HUT 102 (Fig. 4.3 (A) second row, lanes d and e) showed that STAT5 S193 undergoes phosphorylation without IL-2 however non HTLV-1 transformed HUT 78 did not represent similar result. To determine whether STAT5 has any active role in various types of hematopoietic cancers by aberrant activation, patient samples were analyzed. While screening patient samples, sustained pYSTAT5, STAT5pS193 were identified in acute leukemia (Fig. 4.3 (B) lane d), AML-CML (Fig. 4.3 (B) lane e) and T-lymphoma (Fig. 4.3 (B) lane f), T-ALL (Fig. 4.3 (C) lane d). From cell viability assays to study the efficacy of various kinase inhibitors to understand the mechanism of aberration of T-ALL, we found that Gleevec showed significant death of this malignant cell type. Interestingly post Gleevec treated T-ALL patient showed remarkable drop in malignant cell types and we isolated PBMCs from blood where we identified that constitutive pYSTAT5 and STAT5pS193 were

sharply decreased. It suggests us that aberrant proliferation of these cells might be caused by hyperactive STAT5. Similarly CML patient samples (Fig. 4.3 (C) lane f) no aberrantly active STAT5. Finally we made a list to (table 5.1) represent the results of 14 patient samples. It was found that hyperactive STAT5 is not only restricted to lymphoid progenitors but also myeloid type too. Another outcome of this screening showed that aberrantly active STAT5 is predominantly found in acute stage rather than chronic ones.

A)



B)



C)

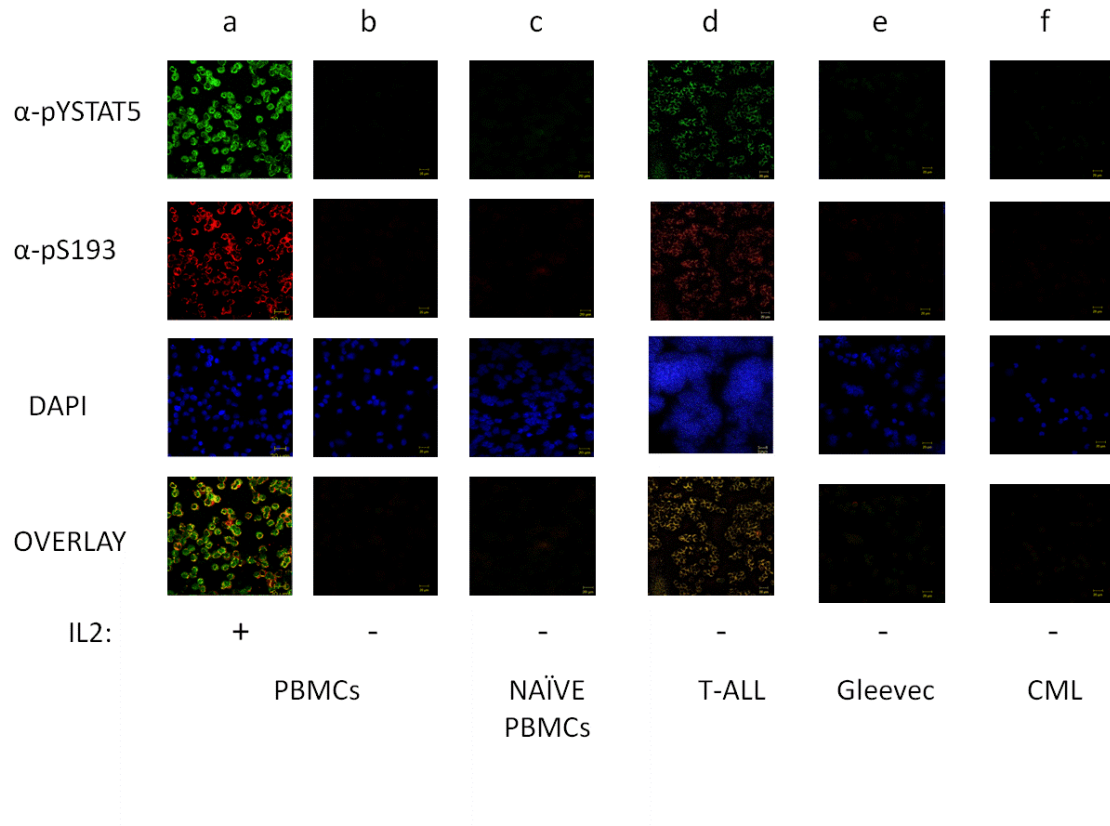


FIGURE 4.3: Constitutive STAT5 S193 phosphorylation identified in HTLV-1 transformed malignant T cell lines and leukemia patient samples.

A) MT2 (lane d), HUT 102 (lane e) and HUT 78 (lane f) cells were fixed with methanol (-20°C) on the slides. α -pYSTAT5 (upper row) stained the activated STAT5 as green, α -pS193 (second row) stained the STAT5 pS193 as red, DAPI (third row) stained the nucleus as blue and overlay (fourth row) showed the colocalization of STAT5pS193 and STAT5pY694/99. B) PHA activated then quiescent PBMCs stimulated without (-) or with IL-2 for 15 min (lanes a and b), naïve PBMCs (lane c), AL (lane d) and AML-CML (lane e) and T-lymphoma patient samples were fixed with methanol (-20°C). C) Similarly T-ALL (lane d), post Gleevec treated T-ALL

(lane e) and CML (lane f) patient samples were stained just like the previous figure. Representative data from two independent experiments are shown.

Table 1.1 Determination of constitutive pYSTAT5, STAT5 pS193 and total STAT5 in different types of hematopoietic cancers.

Patient No.	Cancer Type	pYSTAT5	STAT5 pS193	Total STAT5
1	T-ALL	+	+	+
2	ALL	+	+	+
3	AML-CML	+	+	+
4	HCL	+	+	+
5	B-Lymphoma	+	+	+
6	AMML-JMML	+	+	+
7	CML	-	-	+
8	HL	-	-	+
9	NHL	-	-	+
10	JMML	-	-	+
11	B-ALL	-	-	+
12	ALL	-	-	+
13	AMoL	-	-	+
14	AMoL	-	-	+
15	AML	-	-	+

4.4 DISCUSSION

Chapter four results suggest a functional role for STAT5S193 phosphorylation in mediating STAT5 activity. In HEK-293 reconstitution studies we found that mutant STAT5S193A did not have any effect (Fig. 4.1 (A)) on IL-2 inducible tyrosine phosphorylation, recruitment of STAT5 to the receptor nor its ability to dimerize. Sub-cellular distribution analysis (Fig. 4.1(B)) in HEK-293 reconstitution system indicates total STAT5 distributed to both cytoplasm and nucleus similar compared to wild type. STAT5S193 did not reside in the nuclear localization signature sequence of STAT5. Moreover DNA binding analysis (Fig 4.2 (A)) by EMSA and transcriptional reporter assays (Fig 4.2 (B)) suggest that phosphorylation of STAT5S193 is required for the DNA binding in an IL-2 inducible manner. Phosphomimetics of STAT5S193E completely recovered DNA binding activity visualized in STAT5 S193A mutants and partially rescued its transcriptional activity. Previous studies from our lab identified another serine site (S726/731) to undergo phosphorylation in an IL-2 dependent manner and important for transcriptional activity (Nagy ZS 2002). Also we made a mutant STAT5S731A and performed the luciferase assay. Transcriptional activity of STAT5 was similar to effect of STAT5 S193A mutant. From these results, we conclude that STAT5pS193 plays a regulatory role in the functional activity of STAT5 however whether this is a structural modulation STAT5 S193A causes weaker STAT5-DNA binding affinity complex or transcriptional activity to some weak interaction with co-activator or strong interaction with co-repressor cannot be concluded as further investigation is required with the purified mutant STAT5 S193A. Moreover, leukemia patient samples and HTLV-1 transformed malignant cell lines studies suggest that STAT5 S193 phosphorylation is required to maintain hyperactive STAT5.

Chapter 5: Overview and potential clinical significances of the research

5.1 OVERVIEW

T cells are critically important in adaptive immune system to modulate our defense system against new and old-age infections. Although T cells undergo different stages of development such as activation, proliferation and differentiation, the JAK3-STAT5 pathway is critically important for expansion of T cells by the signal three mediated pathway. Although there is limited understanding concerning the STAT5 induced gene expression and its targets. It is known that STAT5 regulates the expression of anti-apoptotic genes such as BCL-X_L (Dumon S 1999) and cell cycle regulators such as Cyclin D (Moriggl R 1999; Martino A 2001). Partial or complete STAT5 knockouts mice have demonstrated that differentiation of B cells, distribution of CD4⁺ and CD8⁺ T cells, T_{reg} and NK cells development are dependent on STAT5 (O' Shea 2002). Molecular regulators of STAT5 activation at the receptor, cytoplasmic and nuclear levels are being elucidated. Additionally posttranslational modification of STAT5 has not been thoroughly investigated with today's most sensitive technologies such as mass spectrometry.

To fully understand phospho-regulatory roles and their effect on modulating hSTAT5 activity, we extensively mapped the protein isolated from immune cells stimulated without and with IL-2. Chapter two discussed the strategy for identifying a novel phospho-serine regulatory site STAT5 pS193. Upon characterization the phosphokinetics of its activation, sub cellular localization and regulation by γ_c common cytokines including IL-2, 7, 9 and 15 we were able to mediate this event. LC-MS studies on trypsin digested hSTAT5 were able to map with 85% coverage of hSTAT5. We identified two sites, 1) a novel STAT5S193 and 2) previously identified Y699; both as being phosphorylated (Fig. 2.1 B). ELISA, Dot-blot assays, surface

plasmon resonance and peptide competition assays suggested that polyclonal STAT5pS193 antiserum preferentially detects the phosphopeptide CLAQLpSPQERL and not the non-phosphopeptide (Fig. 2.3, 2.4). In addition to this, the polyclonal antibody does not detect STAT3 S193 or S727 phosphorylation upon IL-6 stimulation that provides an additional level of specificity of this antibody to detect STAT5 S193 phosphorylation. Subsequently human NK cells and peripheral blood mononuclear cells were stimulated with IL-2 to determine the phosphokinetics profile of STAT5pS193 activation. Phosphokinetic analysis indicated that STAT5S193 phosphorylation occurs in the cytoplasm and attains the maximal phosphorylation within 15 minutes post cytokine stimulation by IL-2 as well as IL-7, 9 and 15 (Fig. 2.6). Similar results were found in primary human T cells. Gamma common cytokines stimulation suggests that STAT5S193 phosphorylation has diverse biological functions and it is not restricted to IL-2 mediated STAT5 signaling process. Among these cytokines, IL-7 mediated stimulation showed the lowest phosphorylation of STAT5S193.

Chapter three predominantly focused on the positive and negative regulatory pathways that govern the phosphorylation and dephosphorylation of STAT5S193. To identify potential serine/threonine kinase, we screened kinases that were identified as proline directed serine/threonine kinase and known to “cross-talk” with the JAK-STAT pathway. Several studies demonstrated that MAPK, PI3K and mTOR phosphorylate serine residues that are proline flanked and also previously shown to phosphorylate serine residues in STAT3 and STAT1 (Kee Chuan Goh 1999; Nguyen H 2001). From this rationale, we focused on those kinases and cells were pre-treated with different types of kinase specific inhibitors including PD98059 (MEK inhibitor), wortmannin (PI3K inhibitor) and rapamycin (mTOR inhibitor) in a dose and time dependent manners to uncouple these pathways (Fig. 3.2). Subsequently cells stained with

polyclonal α -STAT5pS193 showed that phosphorylation of STAT5S193 may be due to mTOR dependence, since rapamycin and PP242 reduced STAT5 S193 phosphorylation. This data indicates that mTOR may be responsible for mediating this event. Moreover PI3K and MAPK do not appear to be involved in mediating this event.

It is important to balance positive and negative regulatory pathways to control cell homeostasis. Negative regulators of JAK-STAT pathway include SOCS, PIAS, and protein tyrosine phosphatases. Recently our lab showed that serine/threonine phosphatase PP2A regulates the JAK3-STAT5 pathway (Ross JA 2010). Similarly pretreatment with PP2A inhibitors such as Okadaic acid and Fostriecin at a biologically relevant doses showed that PP2A controls the dephosphorylation of STAT5pS193 but not PP1 or PP2B (Fig 3.3).

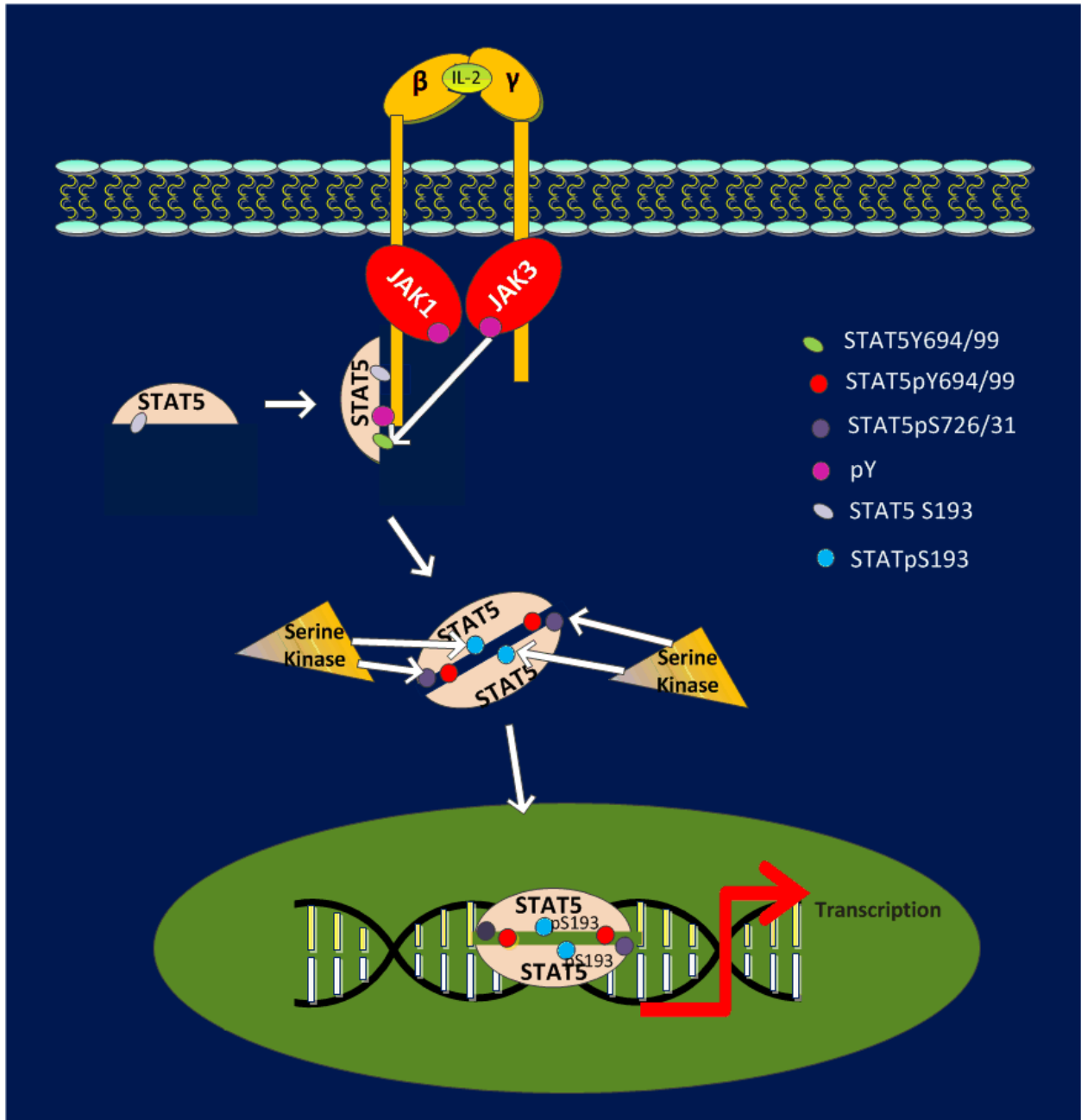
Finally the work in chapter four was focused on understanding of the functional importance of STAT5 pS193. Reconstitution studies in HEK-293 cells where there is no endogenous expression of IL-2R β , IL-2R γ , JAK3 and STAT5. We constructed mutant versions of STAT5S193 to S193A and a phosphomimetic S193E to study the effects on STAT5. It was found that S193A mutation does not block the phosphorylation of the conserved tyrosine residue of STAT5 in the transactivation domain (Fig. 4.1 A). Confocal data suggest that S193A did not have any effect on sub-cellular distribution of STAT5 (Fig. 4.1 B). However when the DNA binding studies were performed by EMSA it was found that S193A weakened the STAT5 binding activity in β -casein response elements (Fig. 4.2 A). Apart from that transcriptional activity of STAT5 was measured by luciferase assay where S193A mutation also decreased its transcriptional activity that corroborated our EMSA analysis (Fig. 4.2 B). Hyperactive STAT5 has been frequently identified in various types of cancers. From the cell staining studies on

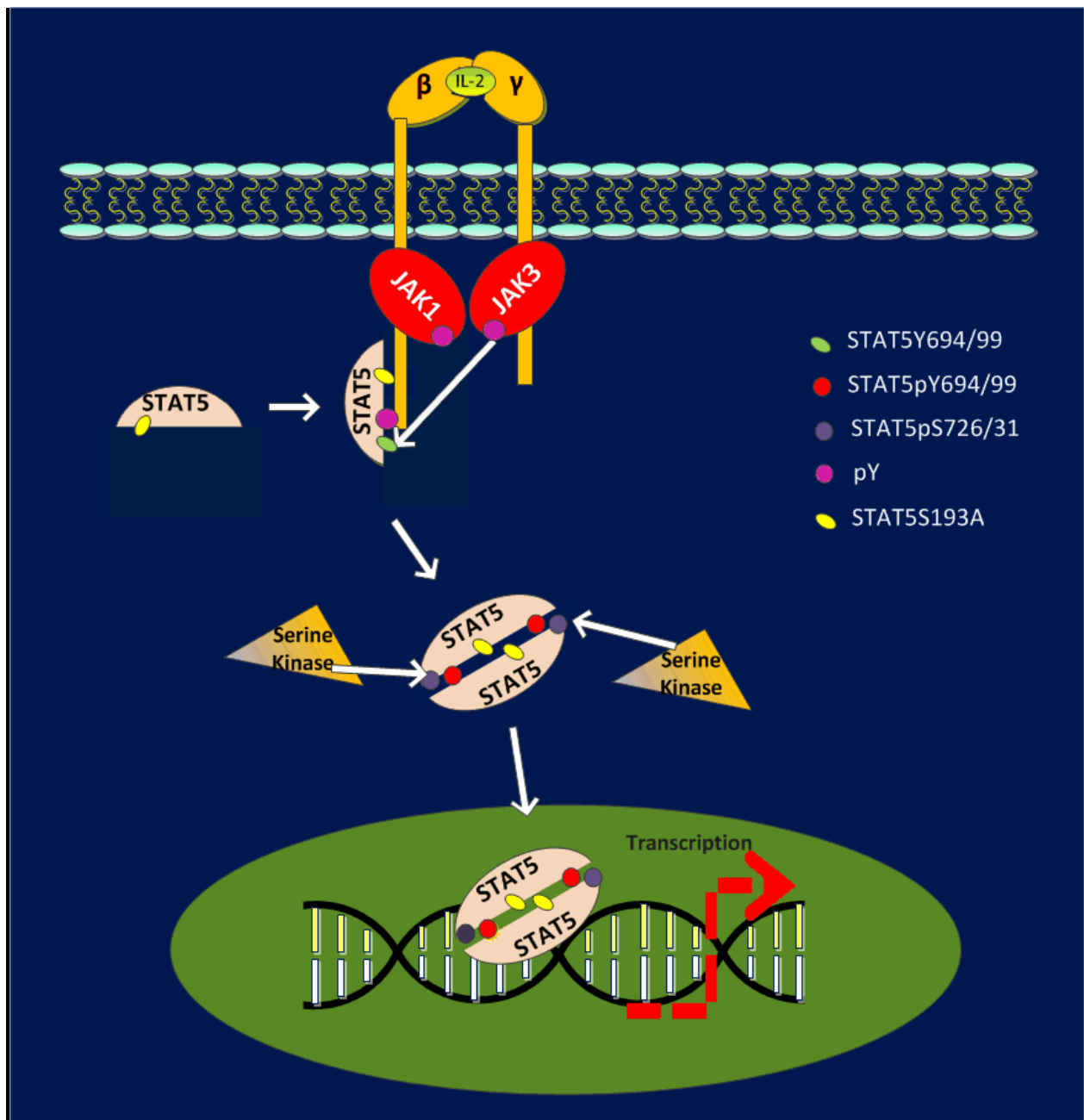
hematopoietic cancer patient samples it has been concluded that constitutively active STAT5 is present in both lymphoid and myeloid progenitor cells (Fig 4.3).

5.2 PROPOSED MODEL

Fig. 5.1 represents the IL-2 inducible HEK-293 reconstitution system. HEK293 cells were transfected with hIL-2R β , hIL-2R γ , JAK3 and STAT5 at a desired proportion to induce JAK3-STAT5 pathway in an IL-2 dependent manner. The STAT5 S193A mutant showed comparable tyrosine (Y694/699) phosphorylation to wild type. It suggests that the STAT5 S193A mutation does not affect its docking to the IL-2R β chain and JAK3 can phosphorylate the conserved tyrosine (Y694/99) residue to undergo activation and dimerization. Moreover sub-cellular distribution analysis suggested that the STAT5 S193A mutant is not localized to the nuclear localization signal (NLS) nor nuclear export signal (NES) as confocal images did not exhibit any change of distribution of total and activated STAT5. In the second specific aim, regulatory pathways have been investigated to identify putative serine/threonine kinase and phosphatase to phosphorylate and dephosphorylate STAT5 S193 respectively. Previous studies to screen serine/threonine kinase revealed that MEK, PI3K and mTOR phosphorylates proline flanked serine residues in STATs. Based on this rationale, YT cells were pre-treated with PD98059 (MEK inhibitor) or wortmannin (PI3K inhibitor) or rapamycin (mTOR inhibitor) followed by IL-2 stimulation showed that STAT5 S193 undergoes phosphorylation in an mTOR dependent manner either directly or indirectly. To investigate the putative negative regulator, we focused on the PPP family of serine/threonine phosphatases as our lab already showed that PP2A plays an active role for JAK3-STAT5 activation (Ross JA 2010). PP1 and PP2A inhibitor calyculin A treated cells exhibited that there is a continuous process of phosphorylation and dephosphorylation of STAT5 S193 and either PP1 or PP2A regulates the dephosphorylation of

STAT5 S193. Subsequently tautomycin (PP1 inhibitor) or okadaic acid and fostriecin (PP2A inhibitor) treated cells concluded that PP2A acts as a negative regulator for STAT5pS193. Whether phosphorylation of this residue making some structural change of the activated STAT5 as a result of which facilitating the interaction with co-activator(s) (NMI, CBP) or weakening the interaction with co-repressor is yet to be discovered.





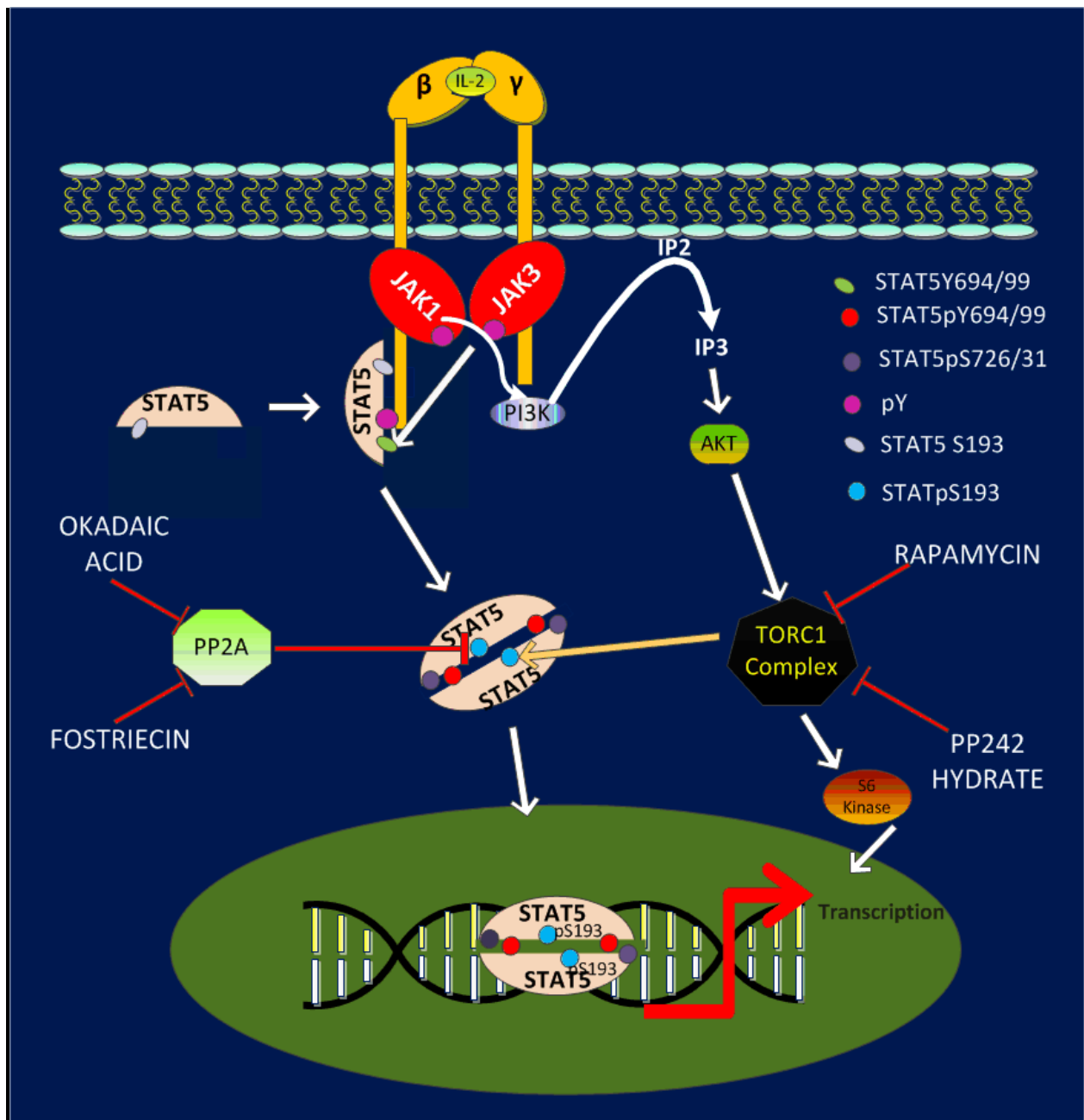


FIGURE 5.1: Schematic representation of the novel STAT5^{pS193} positively regulates its transcriptional activity in an mTOR and PP2A dependent manner.

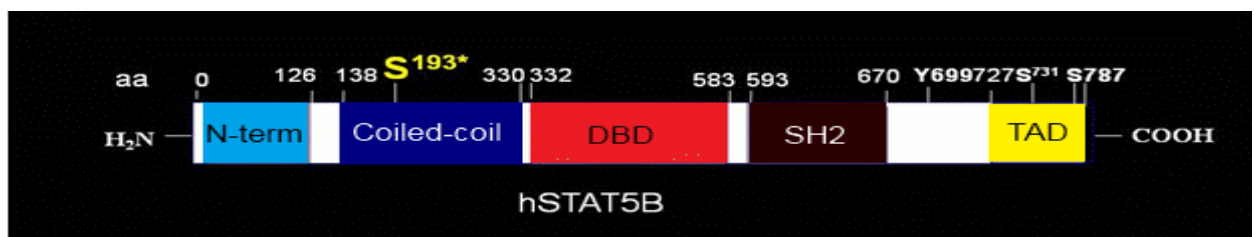
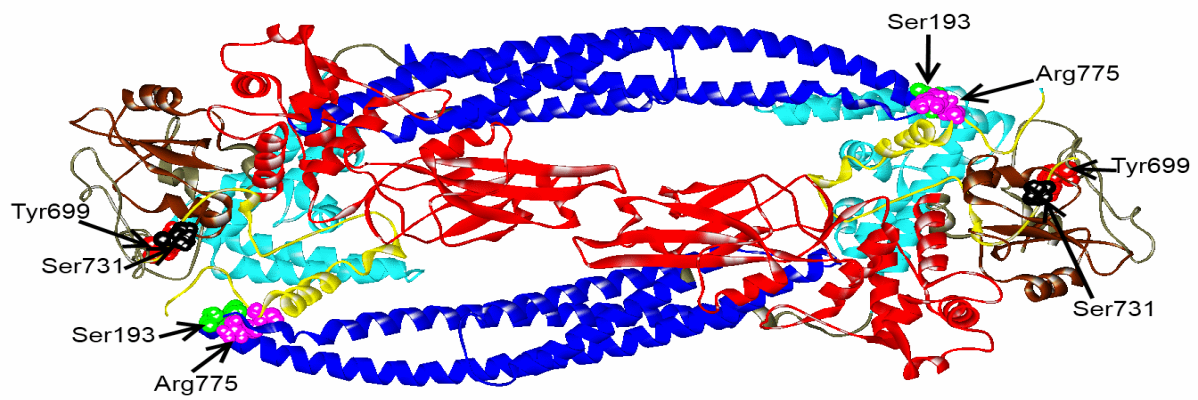
HEK-293 cells were transfected with hIL-2R β , hIL-2R γ , hJAK3 and STAT5 wt or STAT5 S193A or S193E. After 48 hrs post-transfection, cells were stimulated with IL-2 to induce JAK3-STAT5 pathway in this reconstituted system. Based on the functional studies on

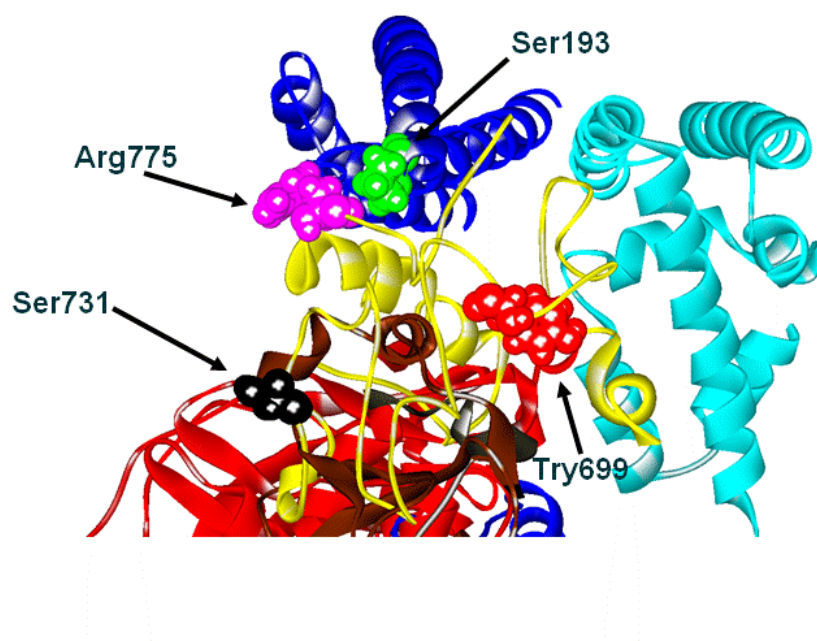
HEK-293 reconstitution system, STAT5 S193 does not play any regulatory role to the receptor docking or tyrosine (Y694/699) phosphorylation or nuclear translocation upon IL-2 stimulation. IL-2 stimulation in lymphocytes upregulates several pathways such as JAK3-STAT5, PI3K-AKT and MAPK. Inhibitor studies by targeting different proline directed serine threonine kinases to identify putative kinase(s) showed that STAT5 S193 phosphorylation is an mTOR dependent process. To balance the phosphorylation, serine/threonine phosphatases were screened by various inhibitors specific PP1 or PP2A. From this screening, it is identified that STAT5pS193 dephosphorylation is PP2A mediated process.

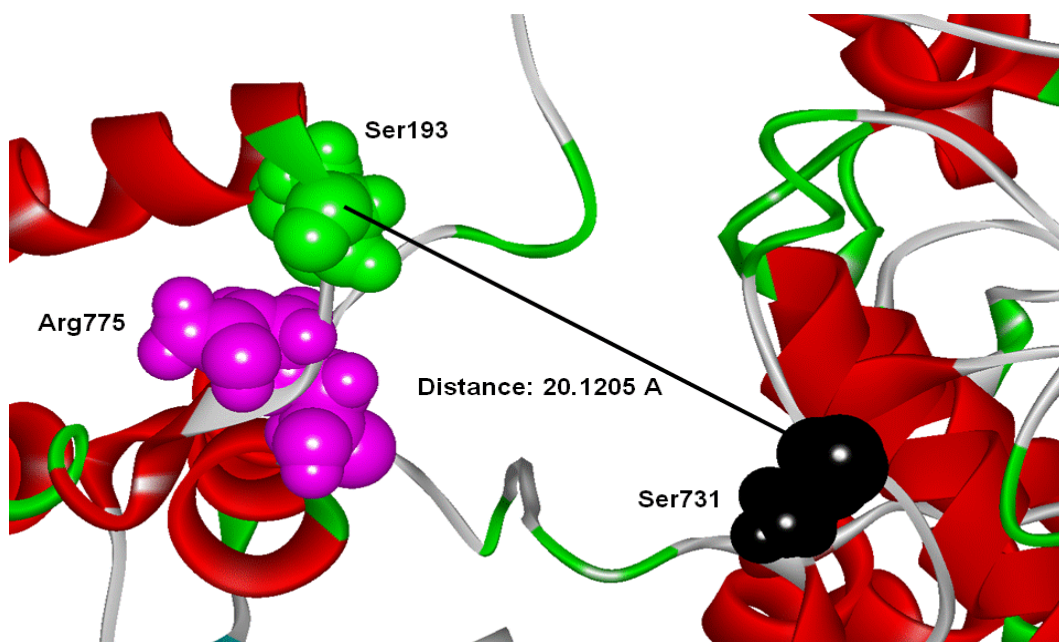
5.3 COMPUTATIONAL PREDICTION OF THE MECHANISTIC ROLE OF STAT5 S193 PHOSPHORYLATION IN DNA BINDING AND TRANSCRIPTIONAL ACTIVITY OF THE PHOSPHORYLATED STAT5 DIMER

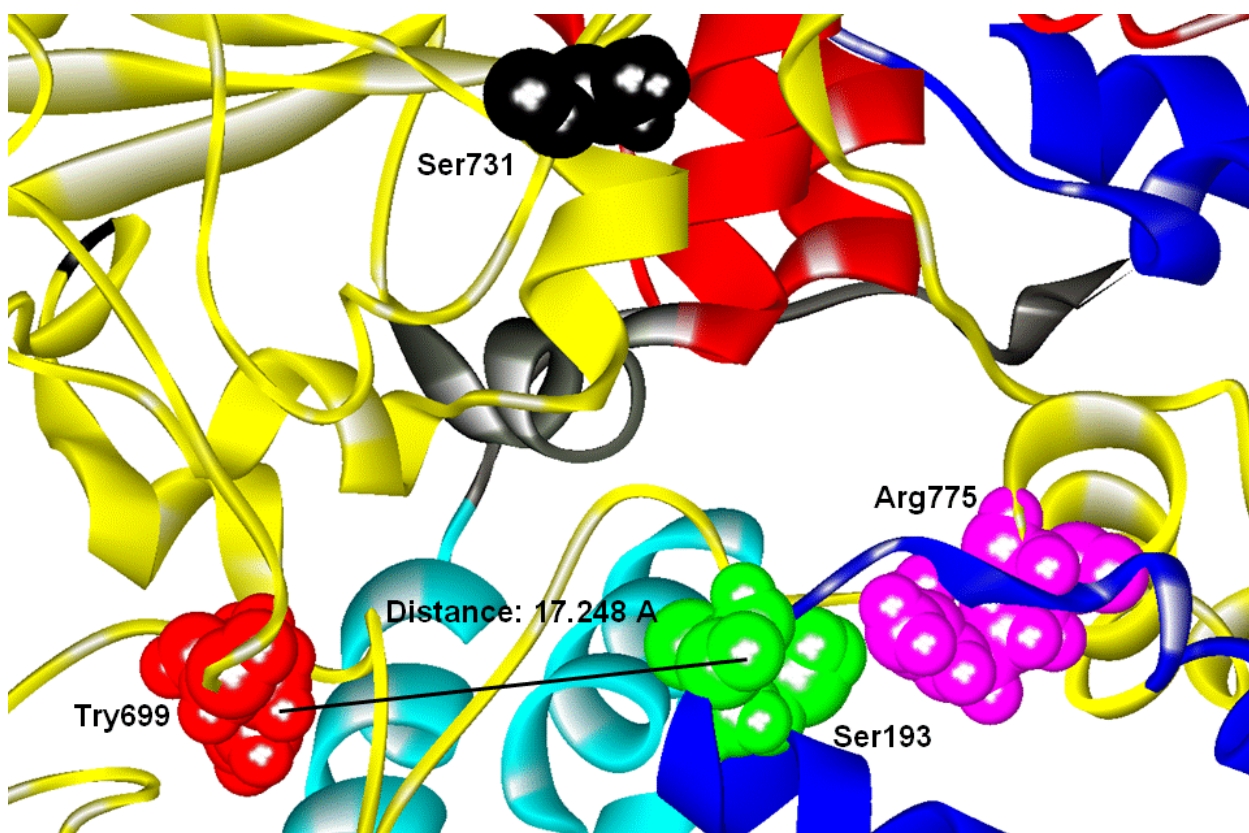
STAT5 forms reciprocal dimerization where two monomers are anti-parallel to each other upon stimulation either by cytokines, hormones or growth factors. Using computational software, the phosphorylated STAT5 dimer has been predicted considering the lowest state of energy as well as simulating the human body temperature and pH conditions. The location of STAT5 S193 is in the helical turn between coil 1 and coil 2 of the coiled-coil domain. STAT5 contains five domains- amino terminal (cyan), coiled-coil (navy blue), DNA binding domain (red), Src homology 2 (brown) and transactivation domain (yellow). The orientation of DNA is along the Z axis of this dimer, which means it is perpendicular to the current plane. STAT5 undergoes phosphorylation in Y694/99 and S726/731 in a cytokine dependent manner that was already established. Y694/99 residue is important for its dimerization, translocation to the nucleus and transcriptional activity. On the other hand, S726/731 regulates its transcriptional activity upon phosphorylation. Based on the predicted structure, the novel STAT5 S193 is located closest to the Arg 775 of the other monomer at a distance of 5.17562 Å. This distance is

not sufficient for a normal interaction between these two residues. From the electron density map, it has been found that when STAT5 S193 is phosphorylated, the side chain of S193 changes its conformation as a result of which the distance between the phosphate group and the imino group of Arg 775 changes to 2.71159 Å. This distance causes a Vander Waal interaction between S193 and Arg 775 to facilitate the formation of a salt bridge between them to form a clip on both sides of the STAT5 dimer to provide a stronger binding with the DNA response element. In addition to this, STAT5 S193 is only 17.208 Å and 20.1205 Å from Y694/99 and S726/31 respectively. The transactivation domain of STATs has been characterized as interactor with various coactivators and corepressors inside the nucleus. The vibration and interaction that the protein exerts to the interacting proteins might bring these four residues of STAT5 close enough to regulate its transcriptional activity.









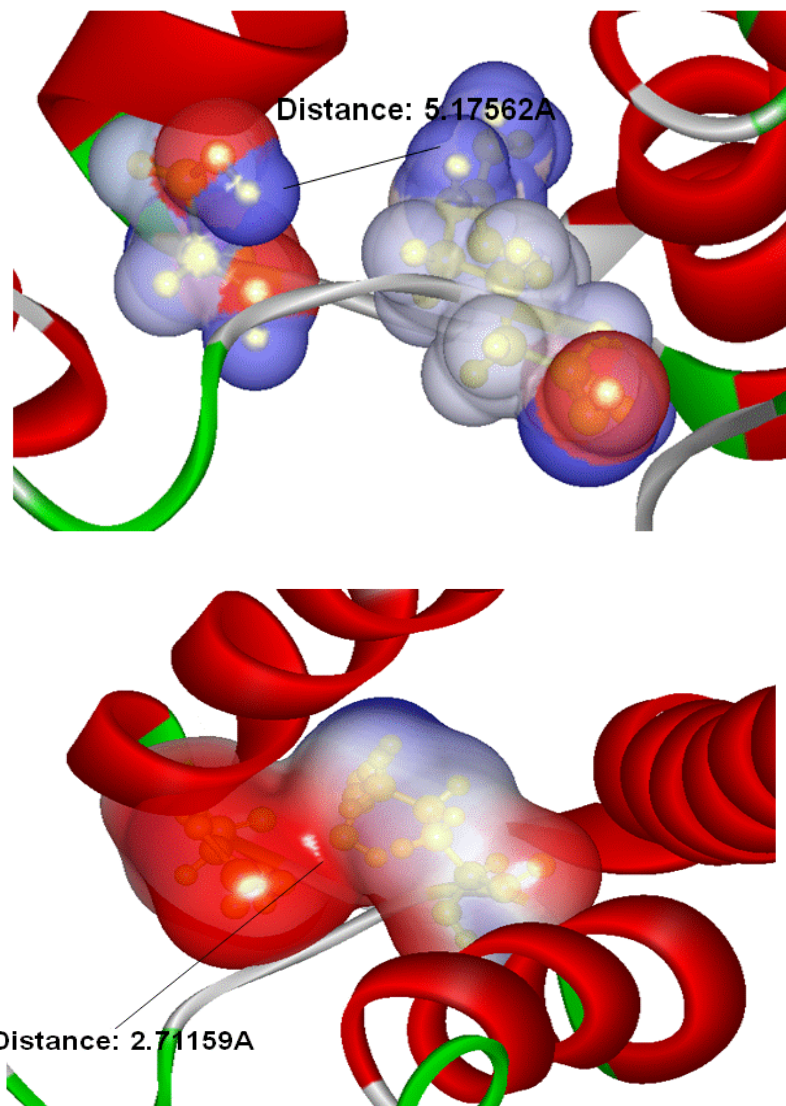


FIGURE 5.2: Computationally predicted functional role of STAT5 S193 in DNA binding and transcriptional activity.

Phosphorylated STAT5 dimer showed the location of Tyr 699, Ser 193, Ser 731 that undergo phosphorylation in a cytokine dependent manner. Upon STAT5 S193 phosphorylation, it becomes close to Arg 775 that might facilitate to form a salt bridge. In addition to this, S193 is located only 17.208 Å and 20.1205 Å from Y694/99 and S726/31 so they might interact under molecular vibration or interacting with other proteins.

5.4 FUTURE DIRECTIONS

5.4.1 DETERMINE STAT5 S193 UNDERGOES PHOSPHORYLATION FOLLOWING STIMULATION BY HORMONES AND OTHER GROWTH FACTORS

STAT5 undergoes induction by growth hormone; prolactin and androgen mediated signaling in hepatic, breast and prostate cancer cell lines respectively. To determine whether STAT5 S193 undergoes phosphorylation, different tissues specific primary and cancer cells will be stimulated by selected hormones for different time points. Subsequently cells will be fixed and stained with α -pYSTAT5, α -STAT5pS193 and DAPI to determine whether STAT5 S193 phosphorylation occurs in these tissues to understand its importance in broad spectrum roles in STAT5 transcriptional regulation.

5.4.2 DETERMINE THE SPECIFIC PP2A PHOSPHATASE

From chapter two, we had determined PP2A negatively regulates STAT5S193 phosphorylation. However PP2A belong to a group of phosphatases within the PPP family of serine/threonine phosphatases. These enzymes typically contain one regulatory, one catalytic and scaffold subunit. As per the varied regulatory PP2A domain specificity is defined. To determine the specific PP2A enzyme, a loss of function approach to screen using SMARTPOOL technologies (DHARMACON). We need to validate the knockdown by performing reverse transcriptase-PCR (RT-PCR) to see the downregulation of messenger-RNA level as well as the

protein expression level by immune-blotting with specific antibody. We need to normalize the data with respect to housekeeping gene expression to make sure that RNAi is not showing any “off-target effect”. Subsequently to validate the RNAi screening *in vitro* phosphatase assay will be performed. STAT5 needs to be purified from the whole cell lysate by immunoprecipitation and purified phosphatase enzyme will be added to the tube for a specific incubation time and the reaction will be stopped by adding 2X sample buffer. Then sample will be separated on 7.5% SDS-PAGE gel and gel will be stained coomassie dye and the band will be excised to analyze for LC-MS/MS for the determination of phosphorylation of STAT5 S193.

5.4.3 CHARACTERIZATION OF MTOR DEPENDENT ENZYME

Pretreatment with various proline-directed serine/threonine kinase inhibitors suggested the phosphorylation of STAT5S193 depends upon mTOR pathway. However mTOR exists in two forms; TORC1 and TORC2 complexes. Our approach to decipher which one act on the STAT5 would be to knockdown TORC1 and TORC2 complexes individually, stimulate the cells with IL-2 and monitor phosphorylation of STAT5S193. This experiment might indicate which TOR complex phosphorylates this site. Further validation of the kinase complex will be done by *in vitro* kinase assay. Synthetic peptide of 11 mer corresponding to that area will be incubated with the pre-screened kinase as per the indicated time. Then the reaction could be stopped by adding a specific buffer followed by dot blot assay on PVDF membrane. Then the membrane will be blotted with α -pS193 to validate the putative kinase by a loss in phosphorylation signal.

5.4.4 DETERMINE THE FUNCTIONAL IMPORTANCE OF STAT5S193 PHOSPHORYLATION IN LYMPHOCYTES

In chapter four we generated HEK-293 reconstitution system that can activate the JAK3-STAT5 pathway in an IL-2 dependent manner. However the HEK-293 reconstitution system might express proteins distinct from normal lymphoid cells. To determine the functional importance of STAT5pS193 in lymphoid cells, we could generate STAT5 KO cell lines. To generate this type of cell lines, a small population of RNAi targeting vector made against STAT5 would be required to be tested in lymphocytes using nucleofection technologies. At 48 hrs post-transfection periods STAT5 expression would be assessed both at the mRNA level by RT-PCR and protein level by Western blot analysis on STAT5 S193 phosphorylation.

If the cells are able to proliferate in the absence of STAT5 then we would need to generate stable cell lines via lentiviral packaged system containing shRNA. Next cells would be electroporated with STAT5 S193A plasmid with a modified CDNA to avoid the RNAi target. Then certain functional assays such as luciferase assays to determine the transcriptional activity, EMSA assays could be performed to determine DNA binding capacity and qRT-PCR to elucidate whether there is any change in the STAT5 inducible gene expression due to this mutation. If we could generate a stable KO or knock-in with the STAT5 S193A mutant in Naïve lymphoid cells then would be able to perform proliferation as well as differentiation assays to determine whether the phosphorylation of this site is important in lymphoid cell development.

5.4.5 IDENTIFY INTERACTING PROTEINS WITH PHOSPHORYLATED STAT5S193 RESIDUE

It is clear that from the functional studies (e.g. EMSA as well luciferase assays) STAT5 S193 positively regulates DNA binding and transcriptional activity (Fig. 4.2). However this site does not appear to have any effect on the conserved tyrosine phosphorylation proximal to the transactivation domain for its dimerization and ability to translocate to the nucleus. To better understand the weakened DNA binding activity due to STAT5 S193A mutant, we would purify both wild type and the STAT5 mutant proteins. Consequently, we would perform binding studies with pure wt or mutant proteins and the β -casein response element to understand structural modulation has any effect on binding with respect to the response element. If we do not observe any change in binding of wild type versus the mutant we might conclude that the mutant version of STAT5 has some interaction with either co-activator proteins or other co-repressors would be assessed using MS/MS. To determine which interacting protein associate with STAT5pS193 we would perform yeast two hybrid assays where we would insert a fragment of the STAT5 region as the bait to screen potential interacting protein. Subsequently we could validate these proteins with help of GST pull down assays, co-immunoprecipitation or cross-linking studies. Another approach would be to utilize peptide pull down assays where lymphoid cells are stimulated without or with IL-2 and coupled phospho or non-phospho peptides to a matrix.

5.5 CLINICAL SIGNIFICANCE OF THIS RESEARCH

To consider STAT5 as a viable drug target, one of the most critical issues to be found out is whether a target hypothetically associated with a disease necessarily represents an appropriate point for new drug intervention (Bromberg J 2000; Buettner R 2002). STAT5 has been widely

identified as constitutively activated in acute lymphoblastic leukemia (ALL), acute myeloid leukemia (AML), chronic myeloid leukemia (CML), breast carcinoma, colon adenocarcinoma and prostate cancer patient samples and tumor cell lines (Haura EB 2005). Several functional studies regarding serine phosphorylation of STATs suggest that it is important for DNA binding, immune responses, cofactor association and transformation (Buettner R 2002; Haura EB 2005). Consistent with its critical role in oncogenesis, sustained activation of STAT5 in various tumor models and patient samples indicates a point of convergence between tyrosine and serine kinase(s) signals. Pharmacological or genetic intervention of constitutive STAT5 signaling may offer an effective therapeutic approach in certain types of cancer cells such as prostate and mammary epithelial cells. Presently decoy oligonucleotides can be used to preferentially bind with this one rather than DNA response elements. However this might completely block the normal biological activities of STAT5 and it can lead to off-target effects. From combinatorial screening of peptides library, peptides can be designed to block the dimerization, recruitment to the receptor.

However it is really challenging to apply these strategies in *in vivo* conditions as STAT5 might be targeted in normal healthy cells. Concurrent induction of multiple signaling pathways due to stimulation by cytokines, growth factors or hormones makes difficult to find a suitable drug target. Therapeutic approaches need to be reconsidered where aberrantly activated STAT5 would be down regulated to an extent where it returns normal cellular status rather than completely depleting it. STAT5 S193 phosphorylation does not block the phosphorylation of the conserved tyrosine residue of its transactivation domain. Apart from that STAT5pS193 positively regulating the transcriptional activity and binding to the response elements. If we are able to generate a monoclonal antibody to block STAT5pS193, it will decrease the

hyperactivated STAT5 to a moderate level. However it should not completely abrogate the biological functions of STAT5. We would also need to know whether this site STA5S193 also undergoes phosphorylation in distinct tissue specific cell lines as STAT5 undergoes constitutive activation in breast, prostate and colon cancer so would be utilized as biomarker in various malignancies.

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Curriculum Vitae

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