

IL2R $\beta$  T450 PHOSPHORYLATION IS A POSITIVE REGULATOR FOR  
RECEPTOR COMPLEX STABILITY AND ACTIVATION  
OF SIGNALING MOLECULES

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## **Dedication**

A mi madre. Su amor y su apoyo incondicional hicieron posible éste logro.

(To my mother. Her unconditional love and support made this accomplishment possible).

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by

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DISSERTATION

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## Abstract

Homeostasis of the immune system is required for proper defense against pathogenic insult. Cells of the innate and adaptive compartments provide a strictly regulated response to clear infections while allowing for self-tolerance. Dysregulation of the components of the immune system can lead to immunodeficiency, autoimmunity, and cancer. Key players of the immune response are T, B, and NK cells, which become strongly activated by IL2 through its receptor. The  $\beta$  subunit of the receptor becomes tyrosine, serine, and threonine phosphorylated upon induction with IL2. Phosphorylation of tyrosine residues has been extensively studied, however, the putative regulatory role of serine and threonine phosphorylation on IL2 signaling has yet to be characterized. The first objective of this research was to identify novel phosphorylation sites on IL2R $\beta$ . Using immunoprecipitation combined with LC-MS/MS five novel IL2R $\beta$  phosphorylation sites, S268, T394, T450, S484, and S512 were identified. In an attempt to characterize T450 and S512 phosphosites, phosphospecific antibodies were generated and found to be reliable new tools to elucidate the role of T450 and S512 in IL2 signal transduction. Using these phosphospecific antibodies, the phosphorylation status of T450 in response to physiological stimuli was investigated. The phosphorylation of IL2R $\beta$  T450 occurred in multiple cell types, including primary human PBMCs, with profiles indicating a general mechanism of IL2R $\beta$  activation. Kinase/phosphatase inhibition studies along with siRNA or purified phosphatases allowed for the identification of ERK1/2 as the kinases that phosphorylate the receptor while PP1 was the phosphatase that mediates its dephosphorylation. Finally, IL2 induced IL2R $\beta$  T450 was found to have a positive regulatory role as demonstrated in a HEK293 reconstitution system using WT or amino acid substitution within IL2R $\beta$ . We assessed the effect of IL2 induced T450 phosphorylation in the assembly of the IL2R complex and the activation of key signaling molecules by immunoprecipitation and Western blot analysis. Phosphorylation of T450 was found to be important for IL2R complex formation, recruitment of JAK3, full activation of STAT5 and AKT. These results suggest a new target to modulate T-cells with therapeutic potential.

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# **CHAPTER 1**

## **Introduction**

## 1.1 IMMUNE RESPONSE AND LYMPHOCYTE ACTIVATION

Homeostasis of the immune system is required for proper defense against pathogenic insult. The first line of defense, innate immunity, is mediated through cells of myeloid origin, including granulocytes, monocytes/macrophages, and natural killer (NK) cells, while the adaptive immune response relies on the activation of B and T lymphocytes and their development into mature functioning antibody producing and effector cells respectively. Cells of the innate and adaptive compartments provide a strictly regulated response to clear infecting agents while allowing for self-tolerance [1]. Dysregulation of the components of the immune system can lead to immunodeficiency, autoimmunity, and cancer [2].

B and T lymphocytes require activation in order to differentiate and proliferate. T cells become activated through the T cell receptor (TCR) complex which includes molecules for antigen recognition, adhesion, and signal transduction. The TCR recognizes an antigen associated to the major histocompatibility complex (MHC) molecule on antigen presenting cells (APC). Adhesion and signal transduction is mediated by the cluster of differentiation (CD) 4 and CD8 on T cells as well as the co-stimulatory receptor CD28, which binds to its ligand B7 (CD80) on APC [1]. These and other co-stimulatory interactions induce the production and secretion of cytokines like interleukin 2 (IL2) which bind their corresponding receptors in an autocrine or paracrine manner. Together, these events provide the three signals required for full activation of T cells and initiation of signaling cascades that drive differentiation, proliferation, and cell survival [3].

B cell activation relies on antigen recognition by the B cell receptor (BCR). Immunoglobulins (Ig) on its membrane bind the antigen and induce phosphorylation of immunoreceptor tyrosine-based activation motifs (ITAMs) on IgG $\alpha$  and IgG $\beta$  peptides. This initiates signaling events that lead to transcription of genes involved in B cell proliferation and differentiation. B cells can also function as APC. Once the antigen is internalized and processed, it is presented to T helper cells through a MHC class II molecule. The T helper cell will then produce and secrete cytokines that will stimulate proliferation and differentiation of B cells [4]. Other important players of immune response are NK cells. They have the ability to recognize and

kill infected as well as cancer cells without stimulation by antigen and are considered part of innate immune response [5] although recent reports have confirmed memory cell-like functions, suggesting a role in adaptive immunity [6]. NK cells, like T and B cells, are strongly activated by IL2.

## **1.2 INTERLEUKIN 2**

IL2, originally called T cell growth factor, is a cytokine produced and secreted primarily by activated helper T ( $CD4^+$  T) cells in secondary lymphoid organs, although it can also be expressed in other cell types including cytotoxic ( $CD8^+$  T), NK, dendritic, and mast cells [7, 8]. Its structure is comprised of four alpha helices with a molecular weight of 15 kDa [9]. IL2 activates different subsets of T cells that include helper, regulatory, suppressor, and cytotoxic [10]. In addition, IL2 induces B cell proliferation and differentiation, as well as Ig secretion [10, 11]. Moreover, IL2 promotes differentiation and a heightened activity of NK cells [12] [10] and activation induced cell death (AICD) of antigen activated T cells after clearance of antigen [13]. The main transcription factors involved in IL2 gene regulation include nuclear factor of activated T-cells (NFAT), activating protein 1 (AP-1), NF-KappaB, CREB/CREM and BLIMP [14-16]. One of the main targets of IL2's signal transduction is the gene forkhead box P3 (FOXP3) on Treg cells; blockade of this signaling hinders development and function of Tregs which results in autoimmunity [17]. The cellular responses to IL2 stimulation are mediated through the IL2 receptor (IL2R) complex and cellular effectors unique to each cell type.

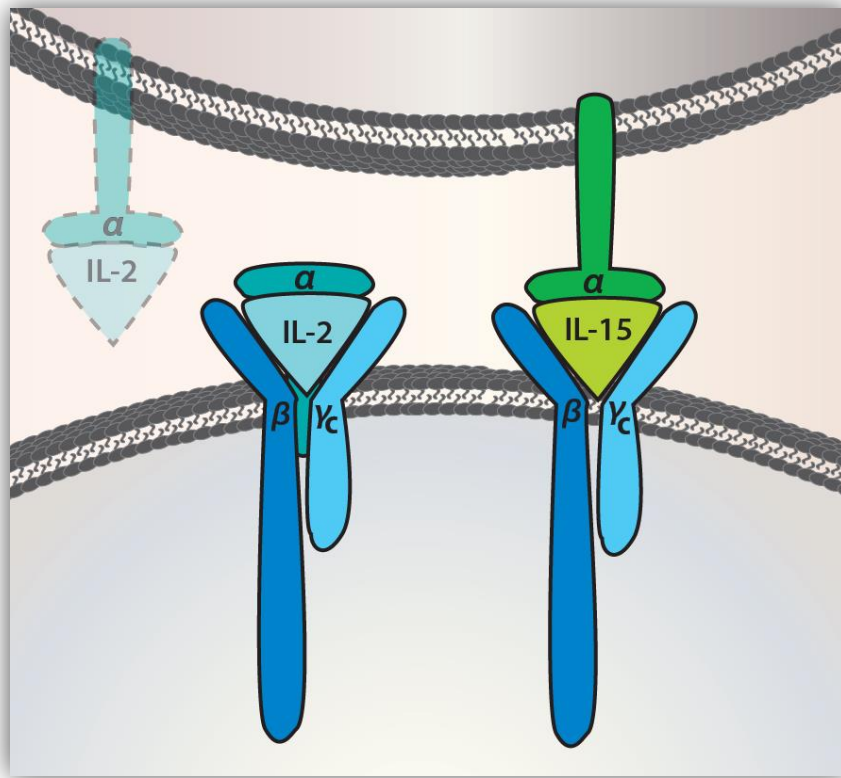
## **1.3 IL2 RECEPTOR**

The IL2R is composed of three subunits or chains:  $IL2R\alpha$  (CD25),  $IL2R\beta$  (CD122), and  $\gamma_c$  (CD132). Subunit  $\alpha$  is considered to be the low affinity receptor ( $K_d \approx 10$  nM) [18] and is expressed in primitive T cells prior to undergoing TCR $\beta$  rearrangement, and on mature B cells after activation of the BCR [19]; expression of  $\beta$  and  $\gamma_c$  form the intermediate affinity IL2R ( $K_d \approx 1$  nM) that is observed on resting T cells, macrophages, NK cells and immature dendritic cells [20] [21]; the

highest binding affinity ( $K_d \approx 10$  pM) occurs when  $\alpha$ ,  $\beta$ , and  $\gamma_c$  subunits bind IL2, as is observed on activated T cells [18, 22] and mature DCs [21].

Binding of IL2 to the high affinity IL2R possibly occurs in a stepwise fashion: first, the  $\alpha$  subunit of the receptor binds and induces a conformational change within IL2 which heightens its ability to bind the  $\beta$  subunit by twofold; after recruitment and binding of the IL2/IL2R $\alpha$  complex to the  $\beta$  subunit, the  $\gamma_c$  subunit is brought to close proximity and binds the complex through interaction with IL2 and the  $\beta$  chain [23]. The biological effects of IL2 *in vivo* appear to be mediated by the high affinity receptor since studies of IL2 or IL2R $\alpha$  deficient mice show a similar phenotype that includes enlarged lymph nodes, severe anemia, increased erythropoiesis, and bowel disease [20]; T cells were found to be responsible for such conditions, since IL2 $^{-/-}$  nude mice failed to develop these diseases [24], pointing to the negative regulation of the peripheral lymphoid compartment as the main role of IL2/IL2R *in vivo* [20].

IL2R $\alpha$  is an affinity conferring chain of the IL2R for IL2. The  $\alpha$  chain is not involved in signal transduction due to a short cytoplasmic tail [25] and it is not shared with any other cytokine receptors, although it is structurally similar to IL15R $\alpha$  [16, 26]. IL2R $\beta$  on the other hand is involved in signal transduction and it is shared with the IL15R [26, 27]. IL2R  $\gamma$  is also involved in signal transduction and it is known as  $\gamma_c$  or the gamma common chain since it is shared by the receptors for IL4, IL7, IL9, IL15 and IL21, also known as the IL2 family cytokines [20, 22, 28]. Both the IL2R $\beta$  and the  $\gamma_c$  chain belong to the type I cytokine receptor family: they have two fibronectin III domains, form two disulfide bonds in the N-terminal domain, and contain the WSXWS motif near the transmembrane region[22]. Neither the  $\beta$  subunit nor the  $\gamma_c$  chain contain intrinsic catalytic activity and they both require activation by receptor-associated kinases to initiate signal transduction. The  $\beta$  and  $\gamma_c$  subunits of the IL2 receptor are shared with the IL15 receptor (Fig. 1.1).



**Figure 1.1: IL2 and IL15 share the IL2R  $\beta$  and  $\gamma_c$  subunits.** IL2R $\alpha$  and IL15R $\alpha$  confer affinity to IL2 and IL15 respectively. IL2 acts in an autocrine or paracrine fashion and can bind the receptor in *cis* or *trans*, while IL15 has to be presented in *trans* by IL15R $\alpha$  to the intermediate affinity IL2R.

The fully activated IL2R complex, including IL2, can be endocytosed by a clathrin-independent dynamin-dependent mechanism that involves PI3K [29]. After endocytosis occurs, the  $\alpha$  subunit is presumably transported through the early endosomal compartment and recycled back to the plasma membrane. IL2R $\beta/\gamma_c$  subunits are transported through the early endosomal compartment possibly for recycling and to late endocytic vesicles and lysosomes for degradation [30, 31].

### 1.3.1 IL2 Receptor Structure

#### *IL2R $\alpha$*

IL2R $\alpha$  is a 55 kDa, 272 amino acids (AA) glycoprotein encoded on chromosome 10p14-15 [10, 32]. It is expressed on monocytes as well as on T, NK, and mature B cells after BCR stimulation [16]. IL2R $\alpha$ 's extracellular domain is composed of 219 AA, and it contains two “sushi-like” (small  $\beta$ -sheet sandwich) domains separated by a linker domain that is bent -90° [33]. The transmembrane domain contains 19 AA and the cytoplasmic domain 13 AA, rendering it unable to participate in signal transduction [25].

#### *IL2R $\beta$*

IL2R $\beta$  is encoded by 10 exons that span nearly 21 Kb localized on chromosome 22q13.1 [16, 34, 35]. It is expressed on neutrophils, T, B, NK, monocytes and some dendritic cells. Factors that stimulate its expression include IL2, IL4, phytohemagglutinin (PHA), anti-CD3, anti-CD28, a combination of anti-CD2 and anti-CD28 antibodies, and protein kinase C activator phorbol 12-myristate 13-acetate [16]. IL2R $\beta$  is a transmembrane protein of 551 AA in length with a molecular weight of 75kDa. It does not contain intrinsic catalytic activity, hence requires activation by receptor-associated kinases for initiation of signal transduction. It belongs to the type-I cytokine receptor family; the extracellular domain which spans from AA 1 to 240 [36] contains two fibronectin-III domains which are characterized by a  $\beta$ -sandwich sheet, connected by a helical

linker bent at about 90 degrees. The amino terminal domain forms two disulfide bonds and it contains the WSXWS motif near the transmembrane domain [20, 22] (Fig. 1.2).

The cytoplasmic domain of IL2R $\beta$  is divided into three main regions: membrane proximal, A (acidic) and H (half). The membrane proximal region, also called S region, is rich in serine residues and contains two motifs common to hematopoietic cytokine receptors named box 1 (LKCNTPDPS) and box 2 (PLEVLERDKV) to which Janus tyrosine kinase (JAK) 1 and JAK3 bind [20, 37, 38]. A variable region called V box connects these two domains [37]. Both Box 1 and 2 are important for JAK1 binding and activation [38] while deletion of Box 2 eliminates activation of Jak1 and Jak3 and the signaling events downstream of the receptor [20] (Figure 1.2).

The A (acidic) region of the receptor contains four tyrosine residues: Y338, Y355, Y358, and Y361. Upon activation and dimerization of IL2R, Y338 on the  $\beta$  chain becomes phosphorylated and serves as a docking site for molecules that recognize phosphotyrosine-binding domains (PTB), like the adaptor molecule SHC [20]. In addition, the A region contains the SRC-family tyrosine kinases Lck, Fyn, and Lyn binding sites [20, 37, 39]. The A region can be deleted from the receptor without affecting proliferation and JAK activation as long as the H region is present [20]. However, NK cells from mice that express IL2R $\beta$  that lack the A region do not exert their cytotoxic function and T cells show enhanced proliferation due to sustained tyrosine phosphorylation of  $\beta$  chain and longer binding of Signal Transducer and Activator of Transcription (STATs) to DNA [40]. In addition, the A region is needed for IL2 mediated inhibition of erythropoietin induced globin gene expression [41]. These results suggest a negative regulatory function for the A region (Figure 2B).

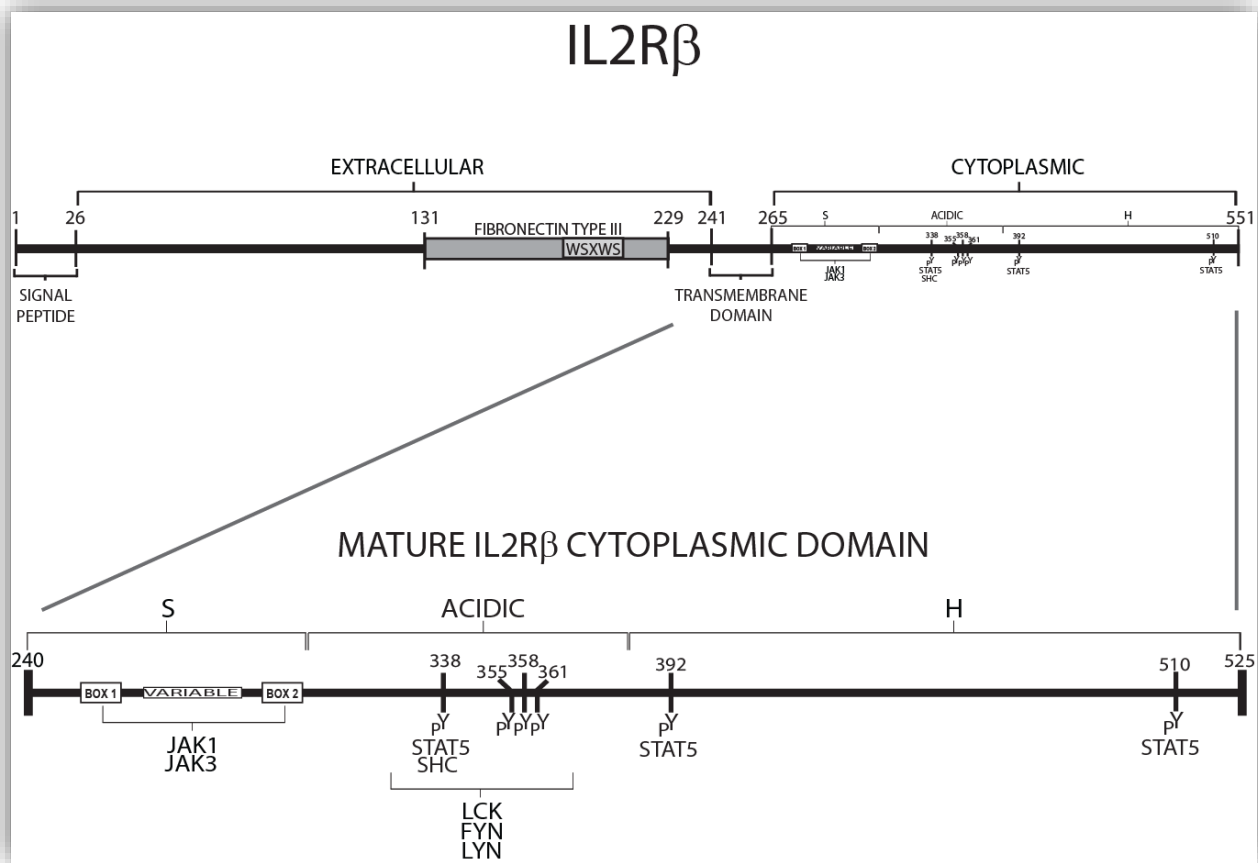
The H region comprises half of IL2R $\beta$  cytoplasmic domain. It contains two tyrosine residues Y392 and Y510, which are phosphorylated and serve as docking sites for STAT5 [20, 37]. Mice that express IL2R $\beta$  lacking the H region fail to produce NK cells,  $\gamma\delta$  T cells, and their T cells do not respond to low concentrations of IL2 presumably because STAT5 and STAT3 activation is impaired, leading to down-regulation of IL2R $\alpha$  [40]. Later studies showed that a

minimum receptor including Box 1 and 2 along with one STAT5 docking site and the region between Y392 and Y510 is essential for expression of IL2R $\alpha$  [42] (Figure 2B).

Mice that fail to express IL2R $\beta$  die at about 12 weeks of age, after developing autoimmune hemolytic anemia; they present an irregular immunoglobulin profile, dysregulated T cell activation and B cell differentiation, and lack NK and CD4<sup>+</sup> CD25<sup>+</sup> regulatory T cells [16].

### ***Gamma Common Chain***

The gamma common chain ( $\gamma_c$ ) is a 64 KDa protein composed of 369 amino acids encoded on chromosome Xq13 [20]. It is expressed on NK, B, CD4<sup>+</sup> and CD8<sup>+</sup> T cells, neutrophils, granulocytes, monocytes and macrophages [20]. Like IL2R $\beta$ , the  $\gamma_c$  chain belongs to the type-I cytokine receptor family, has fibronectin-III domains, three disulfide bonds [22], the WSXWS motif, and contains no intrinsic catalytic activity [20]. The cytoplasmic domain of  $\gamma_c$  contains a box 1 motif and a region with slight homology to box 2 from the  $\beta$  chain. This region is necessary for JAK3 binding and activation, which is not affected by deletion of the rest of the  $\gamma_c$  chain [20]. The  $\gamma_c$  chain has a greater interaction with IL2R $\beta$  than with  $\alpha$  or IL2 [22]. Mutations in the  $\gamma_c$  chain lead to X-linked severe combined immunodeficiency (X-SCID) in humans [43]. Mice lacking the  $\gamma_c$  chain exhibit under developed thymus, a small B cell population and no NK,  $\gamma\delta$  cells, gut-associated lymphoid tissue, dendritic epidermal T cells, or peripheral lymph nodes [16].



**Figure 1.2: IL2R $\beta$  Domain Architecture.** Representation of IL2R $\beta$  chain. Cytoplasmic domain shows known phosphorylation sites. Numbers indicate amino acid residues of human IL-2R $\beta$ . Putative binding sites for JAK1, JAK3, STAT5 and SHC along with binding sites for LCK, FYN, and LYN are indicated.

## **1.4 IL2 AND ACTIVATION OF SIGNAL TRANSDUCTION PATHWAYS**

Signal transduction pathways initiated following binding of IL2 to the receptor include JAK/STAT, RAS/RAF/MEK/ERK, and PI3K/AKT [20, 31]. The signaling cascades involved in these pathways are possible through phosphorylation; the addition of a phosphate group to a substrate by a kinase is an essential post-translational modification event that regulates a large number of proteins. Phosphorylation promotes activation of enzymes and can be regarded as an “on/off” switch in signal transduction pathways due to its reversible and transitory character. In addition, phosphorylation can alter a protein’s cellular localization and substrate binding affinity. About one third of the human proteome can be phosphorylated [44] and it is estimated that 2-4% of the genome codes for kinases and phosphatases, the enzymes in charge of de-phosphorylating proteins [45].

### **1.4.1 JAK/STAT Pathway**

Engagement of IL2 to the IL2R induces activation of the JAK/STAT pathway. When IL2 comes in close proximity to the receptor, JAK1 and JAK3 are recruited and bind IL2R $\beta$  and  $\gamma_c$  respectively. Once activated through autophosphorylation they tyrosine phosphorylate the receptor, providing docking sites for STATs. STATs bind phosphorylated tyrosines on the receptor through their SRC homology 2 (SH2) domains, become tyrosine and serine phosphorylated, and form homo- or heterodimers which translocate to the nucleus to initiate gene transcription [3].

#### ***JAKs***

Many cytokines signaling pathways are dependent upon JAKs. There are four JAK family members: JAK1, JAK2, JAK3, and TYK2. All are ubiquitously expressed except JAK3 which is confined to immune cells, mostly those of lymphoid origin. They have seven JAK homology (JH) domains. Their kinase activity is harbored in the JH1 domain, while JH2 contains a pseudokinase that regulates kinase activity and binds substrates. JH3 through JH7 are involved in JAK/receptor

binding, with JH4 containing a SH2 domain that binds to a phosphotyrosine on different proteins [3].

JAK1 and JAK3 are the only JAKs involved in the IL2R signaling. There is constitutive association of JAK1 to IL2R $\beta$  and JAK3 to the  $\gamma_c$  chain through the box 1 and box 2 motifs in the membrane proximal region [20]. After stimulation of the receptor with IL2, JAK1 and JAK3 are activated via autophosphorylation and then tyrosine phosphorylate the receptor to initiate a cascade of signaling events. *In vitro* studies have revealed that if JAK3 loses its catalytic activity, JAK1 cannot become activated; although if JAK1 is inactive, JAK3 can still become activated [20]. Studies in mice showed that JAK1 deficiency renders B and T cells unable to respond to survival signals, which leads to a SCID phenotype due to low numbers of mature T and B cells, pre B cells, and thymocytes. Most mice with a JAK1 knockdown die perinatally [3, 46].

JAK3 is essential for lymphoid tissue function. It is expressed in T, B, NK, and monocytic cells [47]. Disruption of JAK3 catalytic activity *in vivo* results in a lower level of tyrosine phosphorylation of IL2R $\beta$ , JAK1, SHC, and STAT5 [48]. JAK3 associates with and activates the  $\gamma_c$  to initiate signal transduction. Deficiency in its expression in humans or mutations in its kinase, pseudokinase or the SH2 like domain that prevent its phosphorylation, produce a phenotype similar to the one caused by mutations in the  $\gamma_c$  chain. Signaling of IL4, IL7, IL9, IL15 and IL21 is severely affected and SCID is developed [48, 49].

Tyrosine phosphorylation of the IL2R and JAKs, allows for recruitment, docking and activation of STATs transcription factors that regulate genes associated with proliferation and survival of cells (Fig. 1.3).

### ***STATs***

STATs are a family of transcription factors localized in the cytoplasm that require phosphorylation of specific tyrosine residues that bind a reciprocal STAT SH2 domain to become activated, dimerize and translocate to the nucleus to regulate transcription. STAT proteins contain six domains: an N-terminal domain, a coiled coil domain, important for protein-protein interaction,

DNA binding domain, linker domain, an SH2 domain for docking to receptor and dimer binding, and a trans-activation domain which recruits coactivators and promotes transcriptional activity [50]. There are seven STAT proteins: STAT1, STAT2, STAT3, STAT4, STAT5A, STAT5B, and STAT6 [3]. STAT2, STAT4, and STAT6 are involved in specific functions of B and T cells. STAT1, STAT3, STAT5A, and STAT5B contribute to cell cycle progression and protection against apoptosis of lymphocytes. The main STATs involved in IL2R signaling are the highly homologous STAT5A and STAT5B [37]. After activation of the IL2R and recruitment and activation of JAK1 and JAK3, STAT5 proteins dock on phosphorylated Y338, Y392, and Y510 within IL2R $\beta$  and become tyrosine and serine phosphorylated themselves [51]. Mutants lacking these tyrosine residues are not able to fully activate STATs even though JAK1 and JAK3 present normal function and activation [20]. STAT5A is phosphorylated on Y-694 while STAT5B at Y-699. After activation, STAT5A and STAT5B are released from the receptor and form homo or heterodimers that translocate to the nucleus and in conjunction with other elements and co-activators, initiate transcription of genes involved in regulation of cell cycle like cyclin D1 and D2, the cyclin-dependent kinase (CDK) inhibitor p21WAF/Cip1 and p27kip, as well as the antiapoptotic BCL-XL and BCL-2 [51]. In addition, STAT5 is involved in the transcription of IL2R $\alpha$ , and the apoptotic inducing Fas ligand (FasL), which is implicated in AICD [37].

Studies with STAT5 knockout mice have revealed defective cytokine induced proliferation of T and NK cells on animals that do not express STAT5A with females failing to lactate. When STAT5B is knocked down, male mice are smaller and the liver gene expression profile is comparable to their female counterpart [51]. Knockdown of both STAT5A and STAT5B results in smaller mice and infertile females. NK cells are absent [52], T cells fail to proliferate in response to IL2, and autoimmunity is developed [3] because of lack of T regulatory cells to maintain self-tolerance.

### ***JAK/STAT Pathway Regulation***

Regulation of the JAK/STAT pathway occurs through several mechanisms. Some regulatory molecules like suppressor of cytokine signaling (SOCS) block the interaction of phosphotyrosine residues on receptors with JAKs or STATs. Other, like protein tyrosine phosphatases (PTPs) have the ability to dephosphorylate such residues, inactivating the receptor. In addition, there are several post-translational modifications that regulate activity of the proteins or target them for degradation (Figure 3).

There are eight members of the SOCS family of proteins, SOCS1-SOCS7 and cytokine inducible SH2 domain protein (CIS). SOCS1-SOCS3 and CIS are induced by cytokines and regulate JAK/STAT by negative feedback inhibition. SOCS proteins contain a conserved SH2 domain within the variable amino terminal, and a SOCS box in its carboxyl end. They act by recognizing and binding phosphotyrosine residues through their SH2 domain. SOCS1 and SOCS3 have been shown to be involved in regulation of immune function; they inhibit activity of JAKs and STATs respectively by binding to the cytokine receptor docking sites [53]. In addition, SOCS3 is phosphorylated by JAK1, inhibits phosphorylation and activation of JAK1 and STAT5, and prevents IL2 induced cell proliferation [54]. The protein inhibitor of activated STATs (PIAS) family of proteins regulates STATs by preventing their binding to DNA or by acting as ligases to facilitate SUMOylation [55].

JAKs are regulated by the protein tyrosine phosphatases SHP1, SHP2, CD45, PTP1B and T-cell PTP (TCPTP). SHP1 and SHP2 contain an SH2 domain and both are capable of acting on JAK1. The transmembrane tyrosine phosphatase CD45 has been shown to dephosphorylate all the JAKs while TCPTP acts on JAK1 and JAK3 [53]. The phosphatase SHP2 can also act on STAT5, reverting it into its inactive form and out of the nucleus. In addition, it removes phosphates from serine and tyrosine residues on STAT1 [53]. Protein phosphatase 2A (PP2A) is involved in the regulation of the JAK3/STAT5 pathway by dephosphorylating serine residues on JAK3 and STAT5, and serine/threonine on IL2R $\beta$ ; blockade of PP2A results in augmented serine/threonine phosphorylation and reduced tyrosine phosphorylation [56].

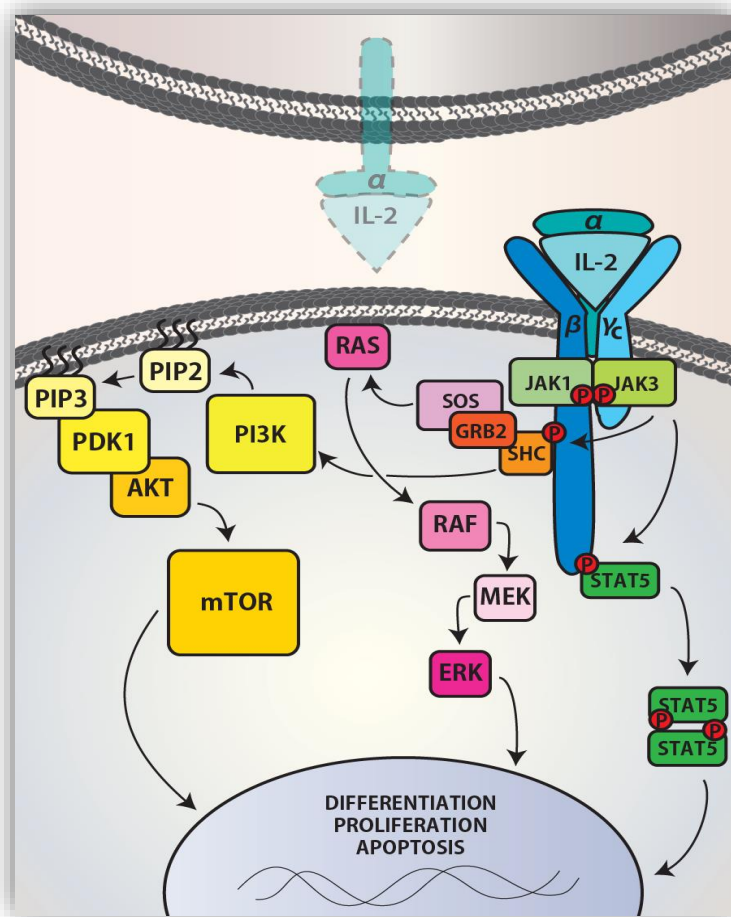
Post-translational modifications other than phosphorylation have also been reported to be involved in the JAK/STAT pathway, including ubiquitylation and ISGylation (the conjugation of interferon stimulated gene 15). JAK2 and  $\gamma_c$  chain can be ubiquitinated [53, 57] while JAK1 and STAT1 can be ISGylated [53]. Although ubiquitylation and ISGylation are involved in regulation of several pathways, more studies are needed in order to determine their role in JAK/STAT signaling.

STATs can not only be tyrosine and serine phosphorylated, ubiquitylated and ISGylated, but also acetylated, methylated and SUMOylated [53]. All of these post-translational modifications are involved in their regulation. However, the extent to which they regulate the different STATs remains to be elucidated.

#### **1.4.2 RAS/RAF/MEK/ERK Pathway**

Engagement of IL2 to IL2R results in recruitment of JAKs, which phosphorylate tyrosine residues on themselves and on the  $\beta$  and  $\gamma$  subunits of the receptor. These phosphorylated tyrosines not only serve as docking sites for STATs, but are also recognized by other proteins. Phosphorylation of tyrosine residue 338 on IL2R $\beta$  allows for recognition and binding of the adaptor molecule SHC through its PTB domain. SHC is activated through tyrosine phosphorylation and recruits the adaptor protein growth factor receptor-bound protein 2 (GRB2), and son of sevenless (SOS), a nucleotide exchange factor that promotes exchange of GDP to GTP on RAS to activate it [37]. Once RAS is activated, the serine/threonine kinase RAF is recruited and activates MEK, another serine/threonine kinase that phosphorylates and activates mitogen activated protein kinase (MAPK) and extracellular signal regulated protein kinase (ERK), which then phosphorylate and activate different transcription factors involved in cell survival and apoptosis. ERK activates the transcription factor MYC, involved in cell proliferation and chromatin remodeling; JUN and FOS, involved in differentiation, proliferation, and apoptosis; TOB, an antiproliferative transcription factor; and MAP kinase-interacting serine/threonine kinase (MNK) among others [58]. The anti-apoptotic genes BCL-2 and BCL-XL are induced through

MAPK, which is also involved in activation of the PI3K pathway [37]. *In vitro* studies revealed that the A region on IL2R $\beta$  has been found to be indispensable for SHC, RAS, and MAPK activation and induction of the proto-oncogenes C-FOS and C-JUN; however, activation of SHC appears to be enough for the signaling cascade to occur [20]. The RAS/RAF/MEK/ERK pathway activates several genes involved in



**Figure 1.3: Activation of IL2 dependent pathways.** Upon binding of IL2 to the high affinity IL2R, JAK1 and JAK3 are activated via autophosphorylation and then tyrosine phosphorylate the receptor. This allows for recruitment, docking and activation of STAT transcription factors, which dimerize and translocate to the nucleus to regulate genes associated with proliferation and cell survival. Phosphorylation of tyrosine residue 338 on IL2R $\beta$  allows for recognition by the adaptor molecule SHC through its PTB domain. SHC recruits GRB2 and SOS to activate RAS, which recruits RAF to activate MEK, which in turns activates MAPK and ERK, which transcribe genes involved in cell survival and apoptosis. The PI3K/AKT pathway is also activated through SHC. Once PI3K is activated it phosphorylates PIP2 to make PIP3, which recruits PDK1 to phosphorylate AKT. Target proteins phosphorylated by AKT include transcription factors involved in cell cycle control, tumor suppression, and proliferation.

proliferation and cell survival although some studies *in vitro* have shown their signals to be redundant with signals activated through IL2 like the JAK/STAT pathway (Figure 3).

Negative regulation of the RAS/RAF/MEK/ERK pathway is far from straightforward since the components of the pathway are controlled by different factors. Activated ERK induces feedback phosphorylation of SOS to prevent RAS from being activated and signal transduction to occur. RAF-1 is regulated by auto-inhibition and serine phosphorylation through AKT. In addition, the RAS family member RAP1 binds RAF-1 and prevents its interaction with RAS. The regulatory molecule 14-3-3 has also been implicated in RAF-1 regulation and RAF Kinase Inhibitor Protein (RKIP) prevents RAF-1 from phosphorylating MEK by preventing their interaction. ERK1 and 2 are inactivated and anchored in the cytosol by the protein tyrosine phosphatases PTP-SL and HePTP [59]. Additionally, SHC has been found to interact with ERK, regulating its activation in the absence of stimuli [60].

### **1.4.3 PI3K/AKT Pathway**

In addition to activation of JAK/STAT and RAS/RAF/MEK/ERK pathways, signaling of IL2 through IL2R activates the phosphatidylinositol 3-kinase (PI3K)/AKT pathway, which is involved in signaling for proliferation and cell survival. Once PI3K is activated it phosphorylates phosphatidylinositol-4,5-diphosphate (PIP2) to make phosphatidylinositol-3,4,5-triphosphate (PIP3), which recruits phosphoinositide dependent protein kinase 1 (PDK1) to phosphorylate AKT. Target proteins phosphorylated by AKT include Checkpoint Kinase 1 (CHK1), involved in cell cycle control; Murine Double Minute (MDM2), a regulator of p53 tumor suppressor; BCL-2 Associated Death Promoter (BAD); Forkhead Box O (FOXO) family of transcription factors, involved in proliferation and stress tolerance; Tuberous Sclerosis Complex 2 (TSC2), implicated in tumor suppression; and Mammalian Target of Rapamycin (MTOR), a serine/threonine kinase critical for cell growth and survival [61] (Fig. 1.3). In addition, AKT can stimulate activity of NFκB to allow transcription of genes involved in cell survival. Induction of PI3K by IL2 also

regulates the activity of the transcription factor E2F, which acts on dihydrofolate reductase and cyclin E genes, which regulate cell cycle progression from G1 to S; In addition, it upregulates of p70 S6 kinase activity through MTOR [61], which phosphorylates the S6 component of the 40S ribosomal subunit, hence increasing transcription of genes related to cell division [37]. The PI3K/AKT pathway activates different sets of genes on different cells [37]. Importantly, the PI3K pathway is differentially activated by IL2 and IL15. IL2 [62].

Negative regulation of the PI3K/AKT pathway is mainly accomplished by the phosphatase and tensin homologue deleted on chromosome 10 (PTEN), a protein and lipid phosphatase in charge of dephosphorylating PIP3 to PIP2 [63]. Absence or malfunction of PTEN leads to a constitutive activation of the PI3K/AKT pathway and has been linked to the development of cancer [63]. Other factors that may be involved in the regulation of the PI3K/AKT pathway include SH2 containing inositol phosphatase (SHIP), which is able to dephosphorylate PIP3, and carboxyl terminal modulating protein (CTMP), which binds AKT to reduce its activity [63].

#### **1.4.4 Other Kinases Involved in IL2 Signaling**

Although JAKs are the main tyrosine kinases involved in IL2R signaling, other tyrosine kinases have been found to be associated with the receptor, including lymphocyte-specific protein tyrosine kinase (LCK), spleen tyrosine kinase (SYK), and the tyrosine kinase LYN [20]. LCK is mainly involved in TCR signaling; however, it has been found to be constitutively associated to the A region of IL2R $\beta$  and its activation is independent of JAK3, and although it does heighten STAT activation it could be through a different receptor other than IL2R [64]. SYK on the other hand, requires JAK3 for activation, which appears to be mediated by JAK1. In addition, SYK is dispensable for IL2 induced activation of STAT [64]. LYN is expressed mainly in myeloid and B cells, and it is involved in the activation of several proteins including PI3K, MAPK, JAKs, STATs, and SYK. LYN interacts with IL2R $\beta$  and JAK3 in Human T-cell leukemia virus type 1 (HTLV-1) transformed cell lines and it is believed to aid in the sustained activation of JAK/STAT [65].

The JAK/STAT, PI3K/AKT, and RAS/RAF/MEK/ERK pathways are not straightforward processes. Crosstalk between these pathways complicates their regulation, which means that they are not simple linear paths, but modify each other's cascade.

## **1.5 SIGNIFICANCE AND HYPOTHESIS**

Dysregulation of the components of the immune system can result in disease. Proper activation of the interleukin 2 (IL2) signaling pathway is crucial for regulation of immunity. Deficiency of IL2 or any of the interleukin 2 receptor (IL2R) components, including IL2R $\beta$  results in severe autoimmunity mainly due to lack of natural T regulatory cell development [66]. Dysregulation of IL2/IL2R has also been associated to autoimmune diseases. T cells from systemic lupus erythematosus (SLE) patients show defective IL2 production [67], while CD25<sup>+</sup> B cells from SLE and rheumatoid arthritis (RA) patients show a heightened expression of IL2R $\beta$  [68]. Polymorphisms on the IL2 receptor  $\beta$  have been associated with rheumatoid arthritis, type I diabetes, Graves disease, multiple sclerosis [69-71], human visceral leishmaniasis [72], and myasthenia gravis [73]. In addition, IL2R $\beta$  and  $\gamma$  subunits but not IL2 are expressed in the human papilloma virus-associated cervical cancer cell lines CALO and INBL; they show comparable phosphorylation of the  $\beta$  subunit by endogenous c-Kit and exogenous IL2, which initiates the JAK3/STAT5 signaling pathway that drives proliferation or anti-apoptotic signals in these cells [74]. Thus, such findings point to a possible involvement of IL2R signaling in cervical cancer.

The  $\beta$  subunit of the IL2R is shared with the IL15 receptor complex, which is essential for natural killer (NK) cell development and survival. No circulating NK cells and abnormalities of T and B cell function due to downregulation of IL2R $\beta$  were observed in peripheral blood mononuclear cells (PBMCs) from an infant boy with symptoms of severe combined immunodeficiency (SCID) [75]. IL15 signaling is needed for proper function and/or development of macrophages, dendritic cells, monocytes, NK-T cells and intestinal intraepithelial lymphocytes [76]. Over-expression of IL15 has been linked to several diseases including Celiac disease, inflammatory bowel diseases, multiple and systemic sclerosis, and rheumatoid arthritis, while its

deficiency appears to heighten susceptibility to some infectious diseases [76]. Additionally, a recent study shows that reducing the expression of IL2/IL15R $\beta$  through small interfering RNA significantly reduced clinical signs of disease in rats with adjuvant-induced arthritis [77]. This underlines the great potential that IL2R $\beta$  has as a therapeutic target to treat autoimmune diseases.

IL2 and IL15 signaling mediated by the  $\beta$  subunit of the receptor is crucial for homeostasis of the immune system. The positive regulation of IL2 signaling through tyrosine phosphorylation of the  $\beta$  subunit has been extensively studied, it allows for recruitment and docking of different proteins to become tyrosine and serine/threonine phosphorylated. These sites have been mapped and their effects in signal transduction extensively studied. However, the negative regulation that might occur through serine and threonine phosphorylation of this receptor has yet to be characterized. Work from our lab confirmed serine and threonine phosphorylation of IL2R $\beta$  which has a direct impact on IL2 induced activation of  $\beta$ , JAK3 and STAT5 [56]. Serine and threonine phosphorylation appear to play an important role in IL2R complex formation and its dysregulation can disrupt homeostasis of the immune system. Therefore, there is a critical need to characterize the phosphorylation of serine and threonine sites in order to have a better understanding of the regulation of the IL2 signaling pathway and its putative role in disease.

The objective of this dissertation is to elucidate novel regulatory mechanisms governing IL2 signaling through IL2R $\beta$ . The evidence of serine and threonine phosphorylation of IL2R $\beta$  and its importance for IL2R complex formation is the foundation for the proposed studies. **We hypothesize that phosphorylation of specific serine and threonine residues on IL2R $\beta$  regulates its signaling capacity and impacts T-cells function.** To address this hypothesis, we first mapped the serine and threonine sites that become phosphorylated on IL2R $\beta$  by using large-scale immunoprecipitation coupled to LC-MS/MS. We found thirteen sites to become phosphorylated in response to IL2 or CA treatment. We choose two sites for characterization: the proline flanked T450, and S512, which is in close proximity to an important STAT5 binding site. The generation of phosphospecific antibodies provided a reliable tool to assess the functional role of the phosphosites as well as the kinase/phosphatase that regulates them. T450 was found to

become phosphorylated upon induction of IL2 and IL15, which lead us to believe it had a positive regulatory role in their signal transduction.

The knowledge gathered in this dissertation provides insight into the modulation of IL2 signal transduction through threonine phosphorylation of IL2R $\beta$ . It brings us one step closer to understanding the fine tune regulation of the IL2 signaling pathway.

## **CHAPTER 2**

### **Identification of Novel Serine/Threonine Phosphosites in IL2R $\beta$**

## 2.1 INTRODUCTION

IL2 signal transduction is initiated by its engagement to the IL2R, a heterotrimeric complex consisting of  $\alpha$ ,  $\beta$ , and  $\gamma$  subunits. Activation of IL2R $\beta$  occurs through the phosphorylation of its tyrosine residues, which allows for recruitment and docking of proteins like STATs and SHC [20] and the subsequent activation of downstream signaling molecules involved in proliferation, survival and apoptosis [3]. Tyrosine phosphorylation of IL2R $\beta$  and its importance on IL2 signal transduction has been extensively studied. However, the regulation that might occur through serine and threonine phosphorylation of this receptor has yet to be characterized. Previous work from our group showed that blocking the serine/threonine phosphatases PP1 and PP2A with CA increases serine and threonine phosphorylation of IL2R $\beta$ . In addition, CA treatment blocks IL2 induced tyrosine phosphorylation of  $\beta$  and the association of the IL2R complex [56]. This strongly suggests that phosphorylation of serine and threonine plays an important role in the activation of IL2R $\beta$ . Dysregulation of such phosphorylation could disrupt the homeostasis of the immune system.

In order to understand the role of serine and threonine phosphorylation in IL2R $\beta$  we first sought to identify the residues that become phosphorylated in the receptor under different stimuli. IL2 has been shown to induce not only tyrosine, but also serine and threonine phosphorylation of  $\beta$  while CA treatment blocks the serine/threonine phosphatases PP1 and PP2A, allowing for phosphorylation of such residues [56]. The strategy employed here consisted of immunoprecipitation coupled to LC-MS/MS analysis. To obtain the  $\beta$  chain in its native environment we used the NK-like cell line YT, which shows high expression of IL2R [78] and provided an excellent source of  $\beta$  for large-scale immunoprecipitation, while mass spectrometry recognizes phosphorylated peptides at a picomol concentration without the need of extensive purification [79]. A second strategy consisted on overexpression of IL2R $\beta$  via a large-scale transfection of HEK293 cells with the receptor.

## **2.2 MATERIALS AND METHODS**

### **2.2.1 Cell Culture, Transfection and Treatment**

The human NK-like cell line YT and the adherent HEK293 cell line were maintained in RPMI 1640 (Thermo Scientific Inc.) medium with 10% fetal bovine serum (FBS) (Atlanta Biologicals), 2 mM L-glutamine (Cellgro), and penicillin-streptomycin (50 IU/ml and 50 µg/ml, respectively) (Cellgro) (complete media) at 37 °C with 5% CO<sub>2</sub>. YT cells were made quiescent by growing to exhaustion ( $> 5 \times 10^5$  cells/mL) and then stimulated with 10,000 IU of human recombinant IL2 (NCI Preclinical Repository) for 10 minutes, or treated with 100 nM CA (Sigma-Aldrich) for 15 minutes. Treatments were performed at 37 °C using  $20 \times 10^6$  cells per treatment. Untreated, unstimulated cells were used as control. HEK293 cells were transfected with 36 µg of plasmid pcDNA3.1/GS human IL2R $\beta$  (Invitrogen) per confluent T-150 flask. Cells were harvested 48 h post transfection and were incubated with or without 100 nM CA for 15 minutes. Two confluent T-150 flasks were used per treatment. Transient transfections of HEK293 cells were performed with Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions.

### **2.2.2 Solubilization of Proteins, Immunoprecipitation, Western Blot and Mass Spectrometry Analysis**

Cells were pelleted and solubilized in lysis buffer (10 mM Tris-HCl (pH 7.6), 5 mM EDTA (pH 8.0), 50 mM NaCl, 30 mM Na<sub>4</sub>P<sub>2</sub>O<sub>7</sub>, 50 mM NaF, 1 mM Na<sub>3</sub>VO<sub>4</sub>, 1% Triton X-100) containing 1 mM phenylmethylsulfonyl fluoride (PMSF), 5 µg/ml aprotinin, 2 µg/ml leupeptin, and 1 µg/ml pepstatin A, and rotating at 4 °C for 1 h. Whole cell lysates were clarified by centrifugation (15,000x g, 15 min, 4 °C). For immunoprecipitation reactions, supernatants were rotated with 3 µg of  $\alpha$ -IL2R $\beta$  mouse monoclonal antibody clone 561 [80] for 2 hours at 4 °C. Immune complexes were captured by incubation with Protein A-Sepharose beads (Rockland Immunochemicals) rotating for 1 h at 4 °C. The beads were then washed three times with ice cold lysis buffer and eluted by boiling 5 min in 4 x SDS sample buffer (50 mM Tris-HCl (pH 6.8), 100 mM dithiothreitol, 2% SDS, 0.02% promophenol blue, 10% glycerol, pH 6.8). Samples were resolved in 10% SDS-PAGE and visualized by Coomassie blue staining. Concentration of the

eluted samples was performed using PTFE membrane centrifugal filter units (Millipore). An aliquot (5%) of each sample was simultaneously ran in the gel, transferred to polyvinyl-difluoride (PVDF) membrane and blocked with 1% bovine serum albumin (BSA) for 1 h at room temperature. Western blot analysis was performed by incubating the membrane with  $\alpha$ -IL2R $\beta$  polyclonal antibody C-20 (Santa Cruz Biotechnology) overnight at 4 °C. Assays were developed with horseradish peroxidase-conjugated goat anti-rabbit IgG (heavy plus light chains; KPL) and visualized by using enhanced chemiluminescence and x-ray film. Bands corresponding to IL2R $\beta$  were excised from Coomassie blue stained gel and sent to the Taplin Biological Mass Spectrometry Facility (Harvard University) analysis through liquid chromatography-tandem mass spectrometry (LC-MS/MS). Any protein with three or more unique peptide matches was considered confidently identified. Manual examination of the spectra as well as the probability-based score Ascore were used to determine phosphorylation site localization [81].

## **2.3 RESULTS**

### **2.3.1 Large-scale Immunoprecipitation of IL2R $\beta$ and Phosphosite Mapping**

Transmembrane proteins have proven to be difficult to study due to their low abundance biological membranes. To circumvent this, a large-scale immunoprecipitation of the receptor was performed to obtain the concentration of IL2R $\beta$  required for LC-MS/MS analysis. For each sample,  $10^9$  YT cells per treatment were used for immunoprecipitation of  $\beta$ . Cells were left untreated, stimulated with IL2 for 10 min or treated with CA for 15 min. IL2R $\beta$  was immunoprecipitated and the samples separated by 10% SDS-PAGE and visualized by Coomassie blue staining (Fig. 2.1A). A small aliquot of the sample (5%) was analyzed by Western blot for tyrosine phosphorylation and total IL2R $\beta$  (Fig. 2.1B) to ensure IL2 induced activation of the receptor. IL2 stimulation induced tyrosine phosphorylation of the receptor. In addition, a reduction in IL2R $\beta$  mobility was observed in the sample stimulated with IL2 as well as the CA treated sample, a phenomenon frequently observed in phosphorylated proteins [56]. Representative data of several experiments is shown. The bands corresponding to non-stimulated (75-KDa), IL2-

stimulated (80-KDa), and CA-treated (100-KDa) IL2R $\beta$  were excised, subjected to trypsin and Asp-N digestion, and analyzed by liquid chromatography-tandem mass spectrometry. YT Spectra analysis using the Sequest search algorithm revealed a combined protein coverage of 61% for the non-treated sample, 84% for IL2 stimulated, and 99% for CA treated cells, and the identification of several novel IL2R $\beta$  phosphorylated peptides. In an attempt to confirm the results in a different system, a large-scale transfection of HEK293 with IL2R $\beta$  was performed as indicated in the Methods section. Cells were left untreated or incubated with CA for 15 minutes. IL2R $\beta$  was immunoprecipitated and the samples processed and analyzed as described for YT cells. The protein coverage was 90% for the untreated sample and 85% for the CA treatment. Several phosphorylation sites were also identified with this strategy. The location of the novel phosphorylation sites in the receptor is shown in (Fig. 2.1C). The diagram includes the novel phosphorylation sites found in the present study as well as residues previously reported to be phosphorylated and important binding sites for signal transduction. Table 2.1 shows a summary of treatments, coverage, and phosphosites identified employing each of the two strategies.

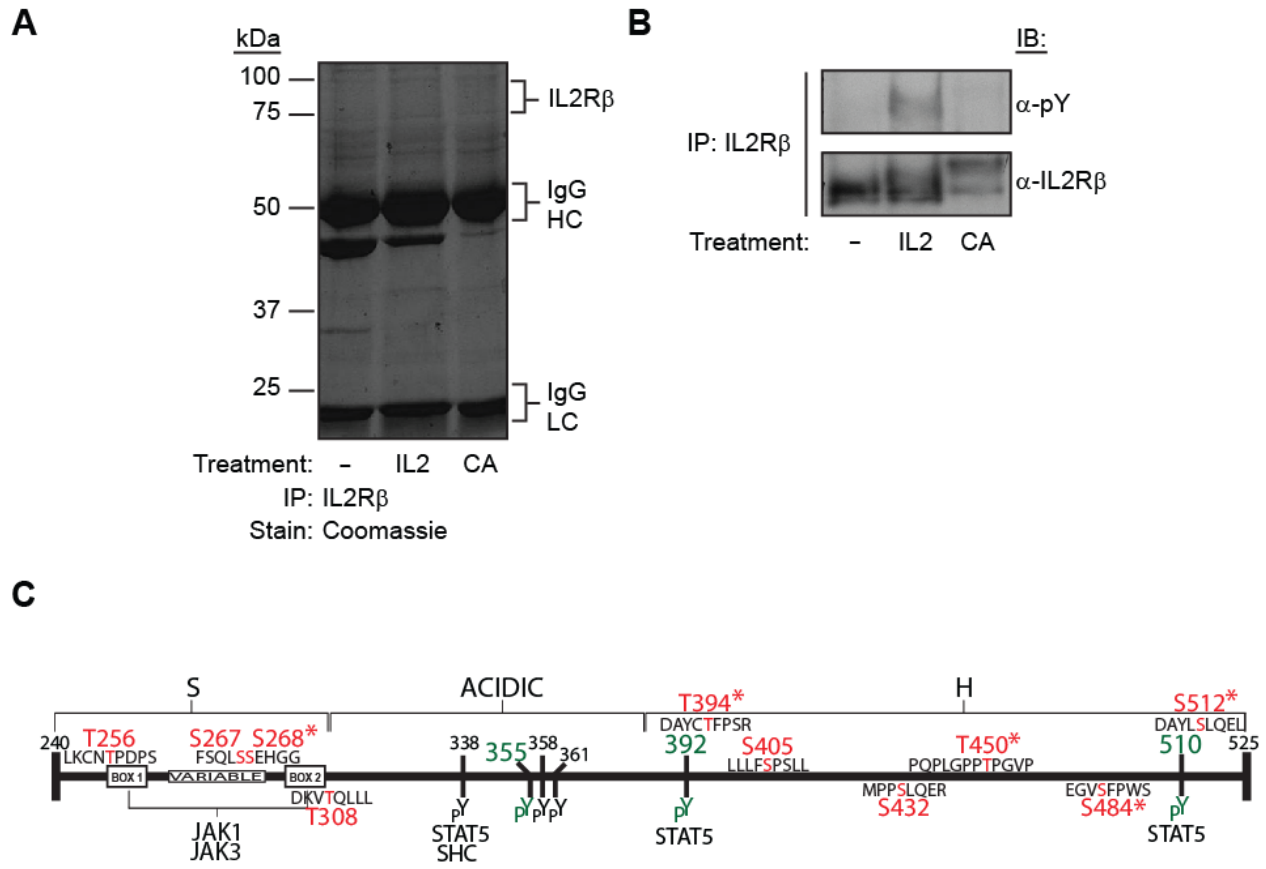
### **2.3.2 Mass Spectra and Conservation of Two Novel Phosphorylation Sites Selected for Characterization**

The strategy employed in section 2.3.1 lead to the identification of 13 residues that become phosphorylated in the cytoplasmic domain of IL2R $\beta$ . The tyrosine residues Y355, Y392 and Y510 were identified in this study and have already been characterized [82]. Ten serine and threonine residues were also identified as phosphosites, five of which have been previously reported in the literature, however, their identification was through proteomic analysis, meaning that no site specific methods were employed in order to characterize them [83]. Here we report S268, T394, T450, S484, and S512 as novel IL2R $\beta$  phosphorylation sites. Of special interest were T450 and S512.

Threonine-450 was found to be phosphorylated in most of our samples. In addition, it is a proline-flanked threonine and the PPTP motif is a known ERK1 and ERK2 substrate [84]. Tandem mass spectrum for the peptide DWDPQPLGPPTPPGVDPDVFQPPPELVLR, which contains the

phosphorylated IL2R $\beta$  T450 residue (underlined) from IL2 stimulated samples, is shown in Fig. 2.2A. This phospho-threonine was identified in YT cells that were untreated, IL2 stimulated, and CA treated. In addition, this site was identified in IL2R $\beta$  of transfected HEK293 cells treated with CA. Comparable spectra was obtained for the peptide containing T450 from the all the samples (data not shown). To determine the extent of T450 conservation, human IL2R $\beta$  protein sequence was aligned with IL2 receptor beta from other species. T450 as well as the surrounding amino acid sequence was found to be well conserved among eight different species, mainly primates, and was found to be replaced by a serine in mice (Fig. 2.2B). Data suggests that phosphorylation of T450 could be a result of an evolutionary recent gain-of-function mutation related to a specialized activity of the more complex immune system in higher organisms.

The second site of interest S512 was found to be phosphorylated in IL2R $\beta$  from CA treated YT cells. This phosphosite is in close proximity to Y510, an important STAT5 binding site. Serine 512 is localized in a SLQE motif, a known CK2 substrate [85]. Tandem mass spectrum for the peptide DAYLSLQELQGQDPTHLV, which contains the phosphorylated IL2R $\beta$  S512 residue (underlined) from CA treated samples, is shown in Fig. 2.3A. To determine the extent of S512 conservation among different species, human IL2R $\beta$  protein sequence was aligned with IL2 receptor beta from other species. S512 as well as the surrounding amino acid sequence was found to be well conserved among thirteen different species, including primates, bovine, marsupials and mouse (Fig. 2.3B).

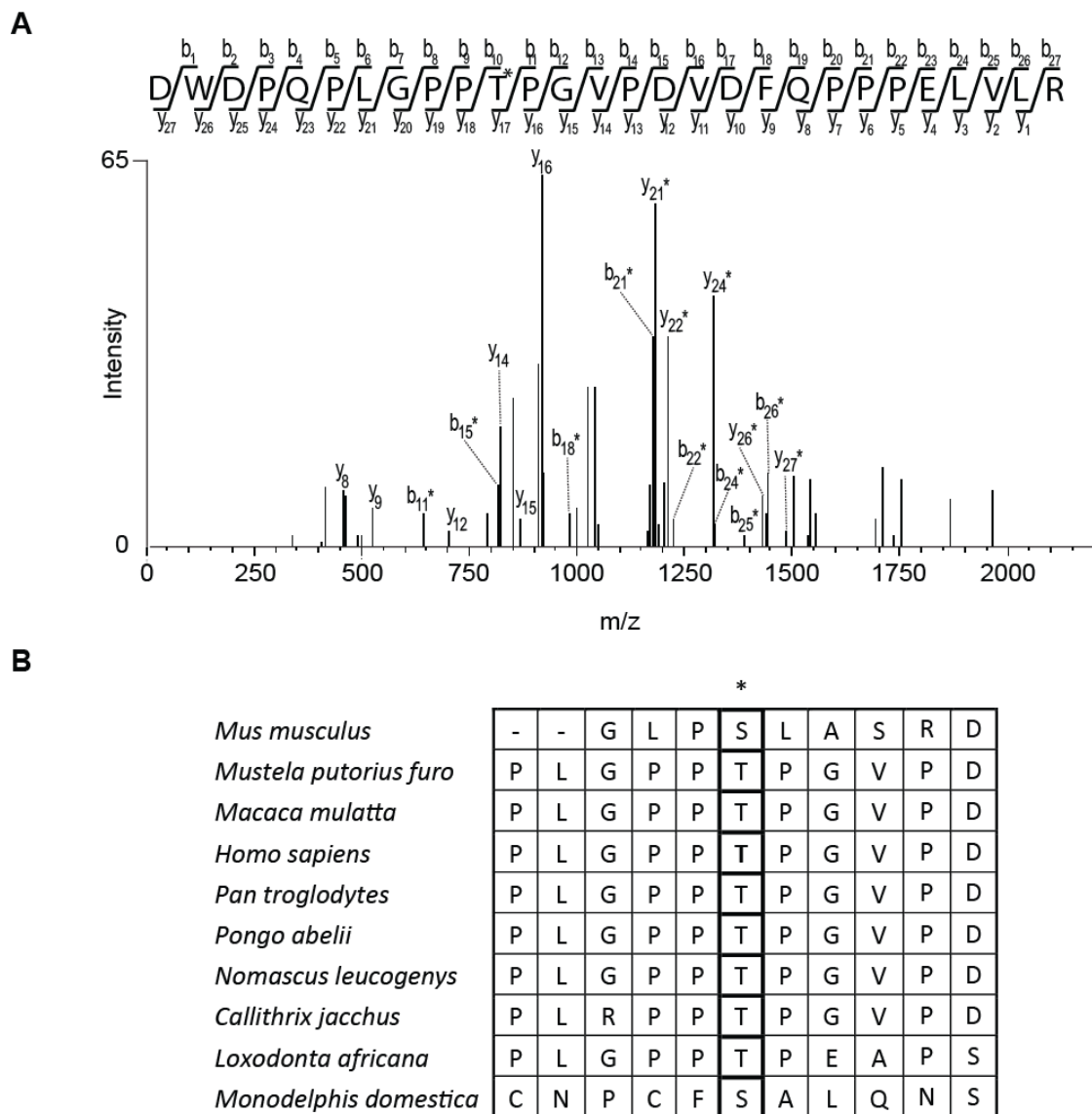


**Figure 2.1: Identification and mapping of novel phosphorylation sites in human IL2R $\beta$ .**

YT cells were left untreated (-), stimulated with IL2 for 10 min (+) or treated with CA (100 nM) for 15 min. Cell lysates were immunoprecipitated (IP) with  $\alpha$ -IL2R $\beta$  and separated by SDS-PAGE. One set was Coomassie blue-stained (A), and the other was immunoblotted (IB) (B) with  $\alpha$ -phospho-Tyr ( $\alpha$ -pY), or  $\alpha$ -IL2R $\beta$  antibodies. HC, heavy chain; LC, light chain. C, Cytoplasmic domain architecture of human IL2R $\beta$ . Tyrosines identified in the present study are in green. Serine and threonine phosphorylation sites are in red and the amino acid sequence surrounding the residue is indicated. Novel serine/threonine sites are designated with an asterisk. Numbers indicate amino acid residues of human IL2R $\beta$ . The binding sites for JAK1, JAK3, STAT5 and SHC are shown.

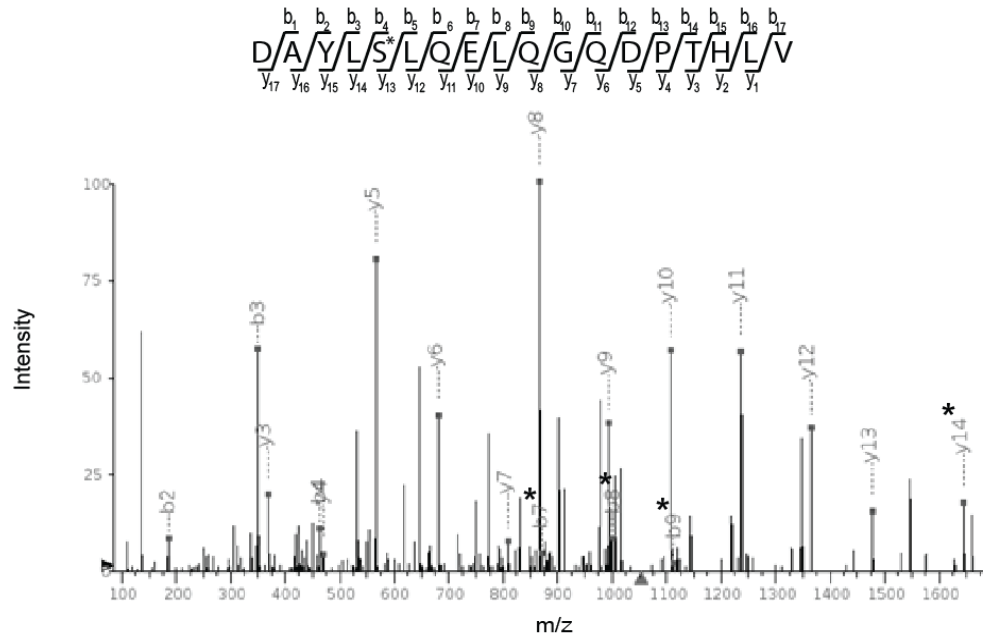
**Table 2.1: LC-MS/MS identification of phosphorylation sites in IL2R $\beta$ .**

Treatment	YT		HEK293	
	Coverage	Phospho residues	Coverage	Phospho residues
None	61%	-	90%	T256, S267, S268
IL2	84%	S405, T450, Y355, Y392, Y510	-	-
CA	99%	T308, T394, S432, T450, S484, S512	85%	T256, S267, S268, T394, S432, T450, S484



**Tandem mass spectra and sequence conservation of IL2R $\beta$  T450.** A, tandem mass spectra of a monophosphorylated peptide showing site localization of T450 on IL2 stimulated YT cells, as indicated by *asterisks*. B, amino acid sequence alignment of the region surrounding T450 (*asterisk*) from different organisms using CLUSTAL OMEGA program.

**A**



**B**

	*										
<i>Mus musculus</i>	T	D	A	Y	L	S	L	Q	E	L	Q
<i>Cricetus griseus</i>	T	D	A	Y	L	S	L	Q	E	L	Q
<i>Ailuropoda melanoleuca</i>	T	D	A	Y	L	S	L	Q	E	L	Q
<i>Equus caballus</i>	T	D	A	Y	L	S	L	Q	E	L	Q
<i>Bos taurus</i>	T	D	A	Y	L	S	L	Q	E	L	Q
<i>Macaca mulatta</i>	T	D	A	Y	L	S	L	Q	E	L	Q
<i>Homo sapiens</i>	T	D	A	Y	L	S	L	Q	E	L	Q
<i>Pan troglodytes</i>	T	D	A	Y	L	S	L	Q	E	L	Q
<i>Pongo abelii</i>	T	D	A	Y	L	S	L	Q	E	L	Q
<i>Callithrix jacchus</i>	T	D	V	Y	L	S	L	Q	E	L	Q
<i>Loxodonta africana</i>	T	D	A	Y	L	S	L	Q	E	L	Q
<i>Monodelphis domestica</i>	T	G	A	Y	L	S	L	G	E	L	Q
<i>Ornithorhynchus anatinus</i>	A	G	A	Y	L	S	L	R	E	L	Q
<i>Salmo salar</i>	C	S	G	Y	L	T	I	K	E	L	Q
<i>Danio rerio</i>	G	S	G	Y	L	T	L	K	E	M	Q

**Tandem mass spectra and sequence conservation of IL2R $\beta$  S512.** A, tandem mass spectra of a monophosphorylated peptide showing site localization of S512 on CA treated YT cells, as indicated by *asterisks*. B, amino acid sequence alignment of the region surrounding S512 (*asterisk*) from different organisms using CLUSTAL OMEGA.

## 2.4 DISCUSSION

In an effort to gain insight into the complex regulation of IL2 signaling at the receptor level, mapping of serine and threonine phosphorylation sites on IL2R $\beta$  was pursued. The strategy of large-scale immunoprecipitation of  $\beta$  followed by identification of phosphopeptides through LC-MS/MS revealed thirteen sites to be differentially phosphorylated in IL2R $\beta$  in response to diverse stimuli (Figures 2.1, 2.2, 2.3 and Table 2.1). These findings provide insight into the putative role for serine and threonine phosphorylation for IL2 signaling.

Phosphorylation of proteins is a major post-translational modification employed by cells for transmitting signals. It can alter a protein's cellular localization and substrate binding affinity and regulate processes like differentiation, proliferation and cell cycle. For example, engagement of IL2 to its receptor results in the recruitment and activation of the protein tyrosine kinases JAK1 and JAK3, which phosphorylate each other and specific tyrosines in the cytoplasmic domain of the receptor providing docking sites for SH2 domain containing proteins like SHC and STATs. In this way initiation of several signaling pathways including JAK/STAT, PI3K, and MEK/ERK occurs [3]. The general focus of study has been the positive regulation of signaling through tyrosine phosphorylation of proteins, which was regarded as an "ON/OFF" switch for activation. However, the importance of serine and threonine phosphorylation in cytokine signaling has been recently highlighted. Several proteins involved in IL2 signaling become not only tyrosine, but also serine and threonine phosphorylated. One example is STAT5, which requires not only tyrosine, but also serine phosphorylation of specific residues for maximum transcriptional activation [86]. In addition, CA induced serine/threonine phosphorylation has been shown to regulate IL2 signaling at multiple levels, including formation of the receptor complex and activation of downstream signaling molecules [56].

We were able to successfully identify several phosphorylated residues in the cytoplasmic domain of IL2R $\beta$  in response to different stimuli. Through phosphoamino acid analysis, our group had reported that IL2R $\beta$  becomes not only tyrosine, but also serine and threonine phosphorylated in response to IL2. In addition, inhibition PP2A heightened serine and threonine phosphorylation

and diminished IL2 induced tyrosine phosphorylation of the  $\beta$  chain of the receptor [56]. Here S405 and T450 were identified to become phosphorylated in response to IL2 stimulation. In addition, serines 267, 268, 432, 484, and 512 as well as threonine 256, 308, 394 and 450 were found to be phosphorylated following CA treatment. Threonine 450 and serine 512 were not previously characterized and were chosen for further examination. Amino acid sequence alignment of the region surrounding each of the phosphosites from different organisms revealed conservation among different species. Interestingly, S512 was found to be more highly conserved than T450.

In conclusion, using immunoprecipitation coupled to mass spectrometry five novel phosphosites in IL2R $\beta$  were identified. In order to elucidate the potential regulatory role of the IL2 induced T450 and the CA induced S512 in IL2 signaling, phospho-specific polyclonal antibodies were generated against those phosphoresidues. The characterization of such antibodies is the focus of the next chapter.

## **CHAPTER 3**

### **Characterization of $\alpha$ -T450 and $\alpha$ -S512 IL2R $\beta$ Antibodies**

### **3.1 INTRODUCTION**

Serine and threonine phosphorylation of proteins has been proven to be a key player in the regulation of signaling. According to our investigation, IL2R $\beta$  T450 becomes phosphorylated in response to IL2. In addition, CA induced inhibition of phosphatases results in phosphorylation of S512. In order to verify phosphorylation of T450 and S512 in IL2R $\beta$  and to elucidate their role in IL2 signaling, we developed phosphospecific polyclonal antibodies against those sites. This chapter is focused on the characterization of the  $\alpha$ -pT450 and  $\alpha$ -pS512 IL2R $\beta$  antibodies by testing their efficacy through ELISA, specificity through dot blot analysis and peptide competition assays, functional affinity through surface plasmon resonance (SPR), and application through immunoprecipitation of protein as well as total cell lysates testing through western blot. These antibodies represent new tools that will help to more precisely define the function of T450 and S512 phosphorylation.

### **3.2 MATERIALS AND METHODS**

#### **3.2.1 Generation of Phosphospecific Antibodies**

The  $\alpha$ -pT450 IL2R $\beta$  rabbit polyclonal antibody was custom-generated by GenScript (Piscataway, NJ) using the immunogen LGPP(pT)PGVPDLVDFC (where pT indicates phosphothreonine) coupled to keyhole limpet hemocyanin. The  $\alpha$ -pS512 IL2R $\beta$  rabbit polyclonal antibody was generated using the immunogen DAYL(pS)LQELQGQDPC in a similar fashion. Four rabbits were immunized per phosphopeptide. Efficacy of serum from final bleeds was tested through enzyme-linked immunosorbent assay (ELISA) by the company. According to their protocol, wells were coated with 100  $\mu$ l of either phosphopeptide or non-phosphopeptide at 4  $\mu$ g/ml. Peroxidase conjugated  $\alpha$ -rabbit IgG (H&L) goat antibody was used as the secondary antibody.

#### **3.2.2 Surface Plasmon Resonance**

*In vitro* studies to determine the functional affinity between phosphopeptide or non-phosphopeptide and either pT450 IL2R $\beta$  or pS512 IL2R $\beta$  anti-serum were performed on a Biacore T100 instrument (GE Healthcare). Phosphopeptide LGPP(pT)PGVPDLVDFC and non-

phosphopeptide LGPPTPGVPDLVDFC (T450) as well as phosphopeptide DAYL(pS)LQELQGQDPC and non-phosphopeptide DAYLSLQELQGQDPC (S512), were modified with PDEA and covalently linked to a carboxymethylated dextran (CM5) sensor chip via surface thiol immobilization. Aqueous solution of the analytes (pT450 or pS512 IL2R $\beta$  anti-sera) at different dilutions (1:10, 1:20, 1:40, 1:100, 1:200, 1:400 and 1:1000) were injected through the flow cells at 30  $\mu$ L/min for 600 seconds at 25 °C. Dissociation was allowed for 600 seconds and regeneration was performed with 50 mM NaOH for 360 seconds at a flow rate of 30  $\mu$ L/min. Kinetic data was analyzed using the Biacore evaluation software. To determine the binding kinetics of the serum to the peptides, a bivalent analyte model was used. Functional affinity was determined using a 1:1 steady state model.

### **3.2.3 Dot Blot Analysis and Peptide Competition**

Phosphopeptides and non-phosphopeptides were diluted in water and blotted in decreasing concentration (1000 ng, 100 ng, 10 ng, 1 ng, and 0.1 ng) into methanol activated PVDF membranes. The membranes were allowed to dry, re-activated and then blocked in 1% BSA 1 h at room temperature followed by incubation with phosphospecific antibodies. Peptide competition assay was performed by incubating the phosphospecific antibodies with increasing concentrations (0.1  $\mu$ g, 1  $\mu$ g, 10  $\mu$ g, and 50  $\mu$ g) of either the phosphopeptide or the non-phosphopeptide for 2 h rotating at room temperature before probing dot blotted membranes.

### **3.2.4 Cell Culture, Transfection and Treatment**

The human HEK293 cell line was maintained as described in section 2.2.1. Cells were grown to 90% confluency in a 6-well plate and transfected with 1  $\mu$ g of plasmid pcDNA3.1/GS human IL2R $\beta$  (Invitrogen). Cells were harvested 48 h post transfection and were incubated with or without 100 nM CA for 1 h at 37 °C. Transient transfections of HEK293 cells were performed with Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions.

### **3.2.5 Solubilization of Proteins, Immunoprecipitation and Western blot analysis**

Cells were pelleted, solubilized, and clarified as described in section 2.2.2. For immunoprecipitation reactions, supernatants were rotated with 3  $\mu$ g of  $\alpha$ -IL2R $\beta$  mouse monoclonal antibody clone 561 [80] for 2 hours at 4 °C. Immune complexes were captured by incubation with Protein A-Sepharose beads (Rockland Immunochemicals) rotating for 1 h at 4 °C. The beads were then washed three times with ice cold lysis buffer and eluted by boiling 5 min in 2 x SDS sample buffer (50 mM Tris-HCl (pH 6.8), 100 mM dithiothreitol, 2% SDS, 0.02% promophenol blue, 10% glycerol, pH 6.8). Samples were resolved in 10% SDS-PAGE, transferred to polyvinyl-difluoride (PVDF) membrane and blocked with 1% bovine serum albumin (BSA) for 1 h at room temperature. Western blot analysis was performed by incubating the membrane with the custom generated  $\alpha$ -pT450 IL2R $\beta$  rabbit polyclonal antibody 2 h at room temperature. Assays were developed with horseradish peroxidase-conjugated goat anti-rabbit IgG (heavy plus light chains; KPL) and visualized by using enhanced chemiluminescence and x-ray film. For reblotting, polyvinylidene difluoride membranes were incubated with stripping buffer (100 mM  $\beta$ -mecaptoethanol, 2% SDS, 62.5 mM Tris-HCL, pH 6.7) at 55 °C for 30 min, blocked, and then reprobed with  $\alpha$ -IL2R $\beta$  C-20 (Santa Cruz Biotechnology) rabbit polyclonal antibody overnight at 4 °C.

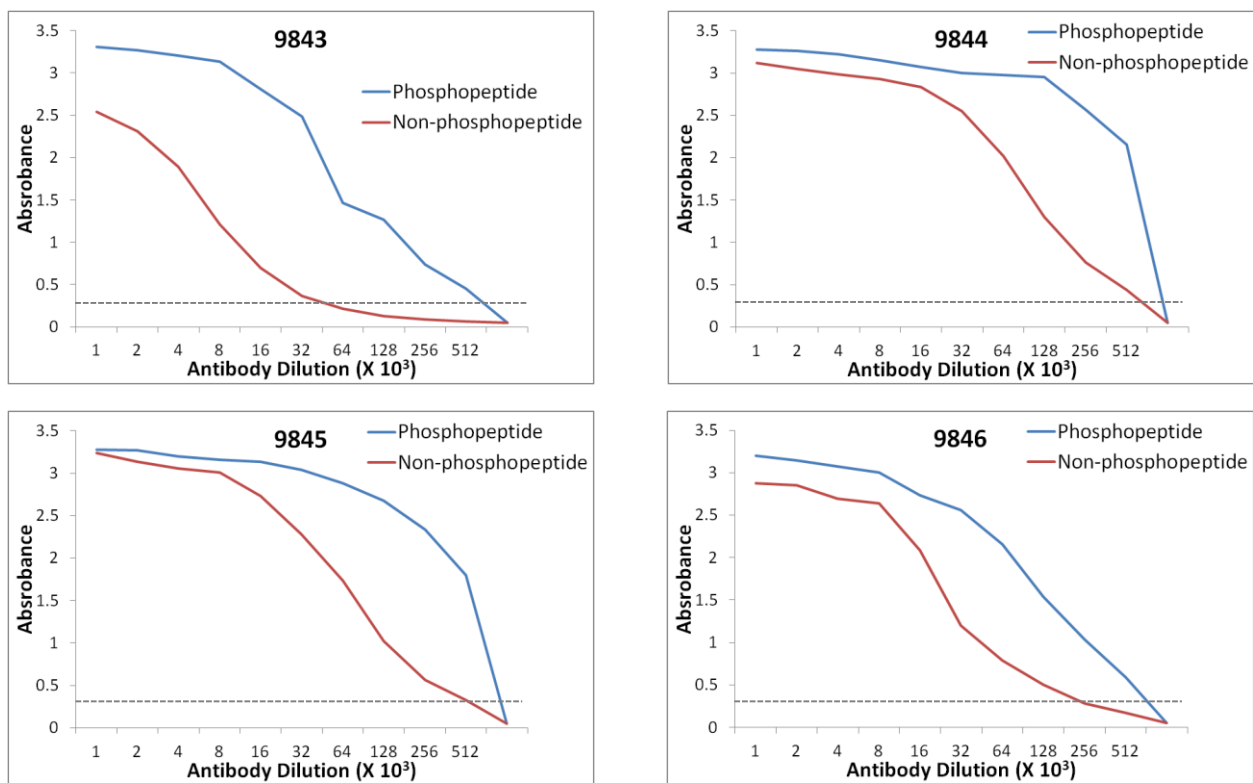
## **3.3 RESULTS**

### **3.3.1 $\alpha$ -pT450 IL2R $\beta$ Antiserum Preferentially Recognizes Phosphorylated Peptide**

Phosphorylation of T450 on IL2R $\beta$  was identified through mass spectrometry analysis and may represent a novel mechanism of regulation of IL2 signaling. To verify that IL2R $\beta$  is phosphorylated at threonine 450 and to investigate the regulatory role of this phosphorylation site, a phospho-specific polyclonal antibody was generated. Four rabbits were immunized with the phosphopeptides and are represented by the numbers 9843, 9844, 9845, and 9846. Final bleed antisera were analyzed by ELISA and specificity to phosphopeptide (blue) versus non-phosphopeptide (red) was confirmed for all four rabbits (Fig 3.1).

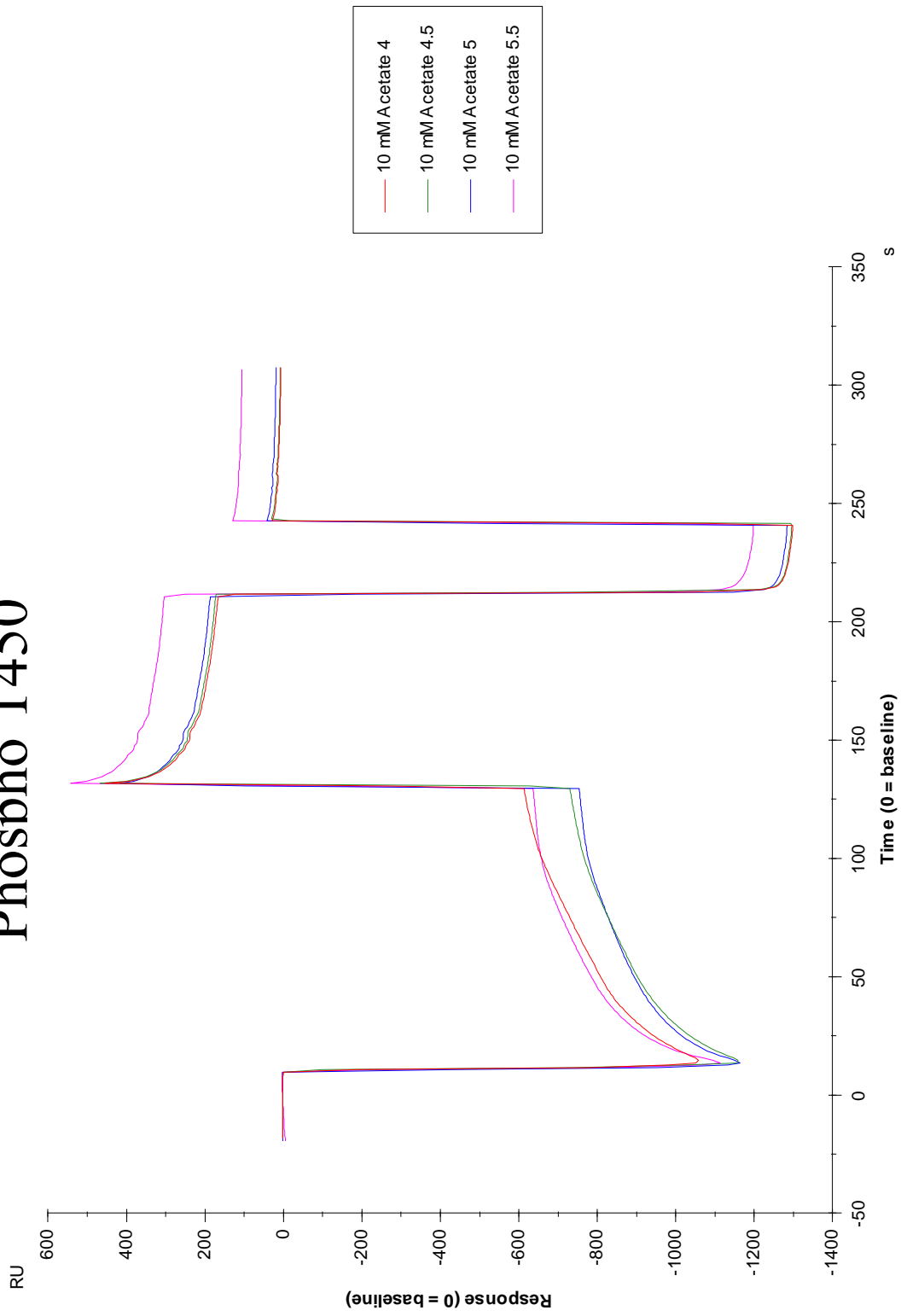
The affinity of 9846 antisera to the phosphopeptide versus the non-phosphopeptide was further tested by SPR. As shown in figure 3.2, the highest pre-concentration of both the phosphopeptide and non-phosphopeptide on the CM5 chip was obtained using a pH 4 buffer. Similar amount of ligands (phosphopeptide and non-phosphopeptide) were immobilized on the dextran surface (Table 3.1). Increasing dilutions of the antiserum were tested for binding response to the immobilized phosphopeptide and non-phosphopeptide. The concentration of the antibody was assigned to 10  $\mu\text{g/mL}$  on the Biacore, however, for the purpose of this assay the values are represented as arbitrary units. Binding curves show higher response (resonance units) for the phosphopeptide versus the non-phosphopeptide for all the dilutions tested (Fig 3.3). In addition, dissociation of the antiserum occurs more rapidly from the non-phosphopeptide versus the phosphopeptide. Binding kinetics was determined using a bivalent analyte model. Expected values are shown in black and obtained values are in red. We observed a higher association rate constant of the antiserum to the phosphopeptide versus the non-phosphopeptide. In addition, the dissociation rate constant of the antiserum from the phosphopeptide was about half of that for the non-phosphopeptide (Fig. 3.4). The functional affinity of the antibody was tested by using a 1:1 steady state model and the dissociation equilibrium constant ( $K_D$ ) of the antiserum was 105 nM for the phosphopeptide and 596 nM for the non-phosphopeptide (Fig. 3.5). Representative data from three independent experiments are shown. Statistical significance was determined using Student's *t* test (\*,  $p < 0.05$ ; \*\*,  $p < 0.01$ ) and the difference in association, dissociation and equilibrium constants between the phosphopeptide and non-phosphopeptide were found to be statistically significant.

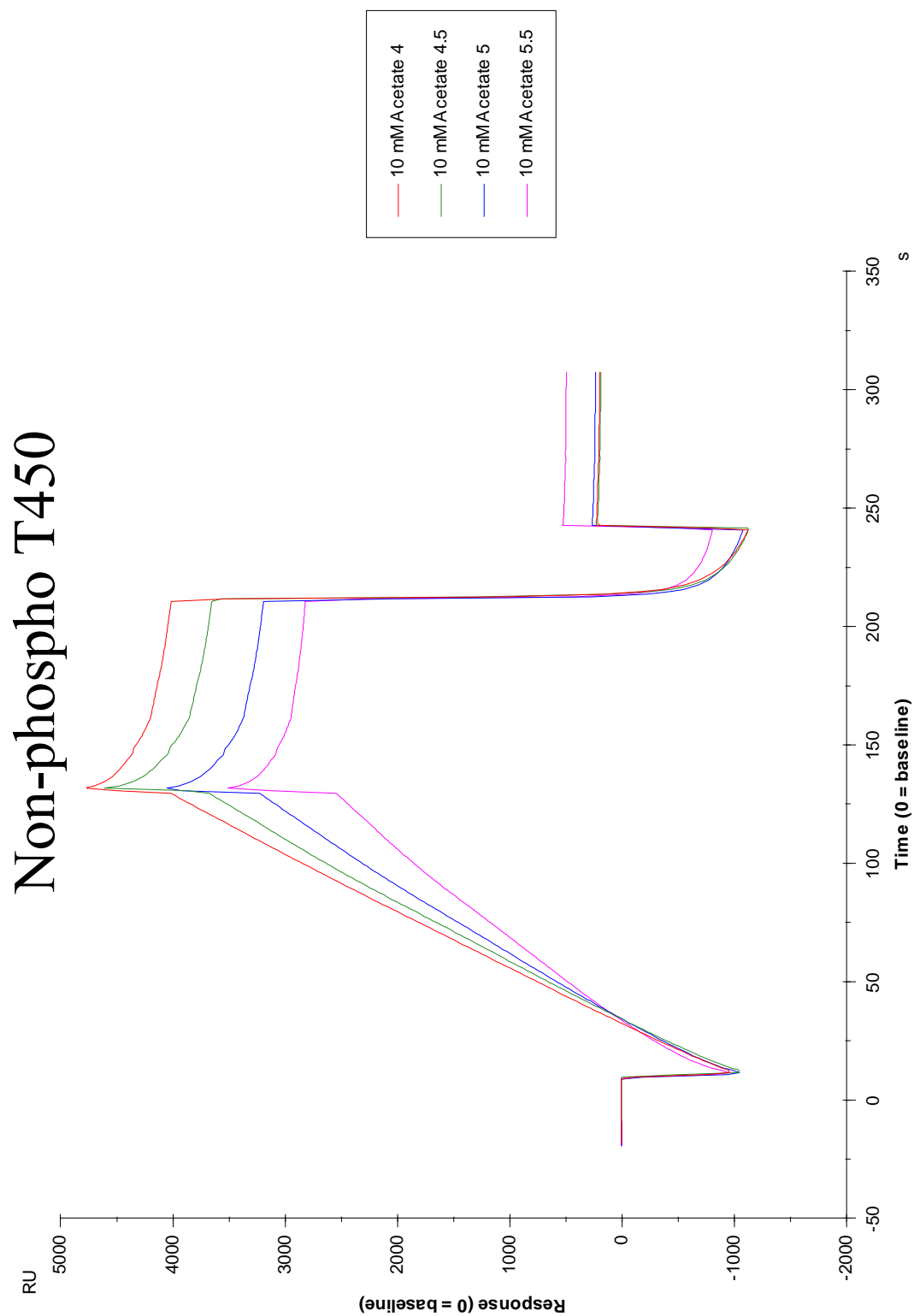
To determine whether the IL2R $\beta$  phospho-specific antibody cross-reacts with the non-phosphorylated form of the peptide, dot blot analysis with the immunizing phospho-peptide and the corresponding non-phosphorylated peptide was performed (see section 3.2.1 for sequences). Increasing amounts of pT450 or T450 peptides were spotted on to PVDF membranes and



**Figure 3.1: pT450 IL2R $\beta$  antisera has specificity towards phosphopeptide.** Four rabbits, 9843, 9844, 9845, and 9846, were immunized with the phosphopeptide. Increasing dilutions of each antiserum were analyzed through ELISA. Specificity of the antisera to phosphopeptide (blue) and non-phosphopeptide (red) is shown.

# Phospho T450

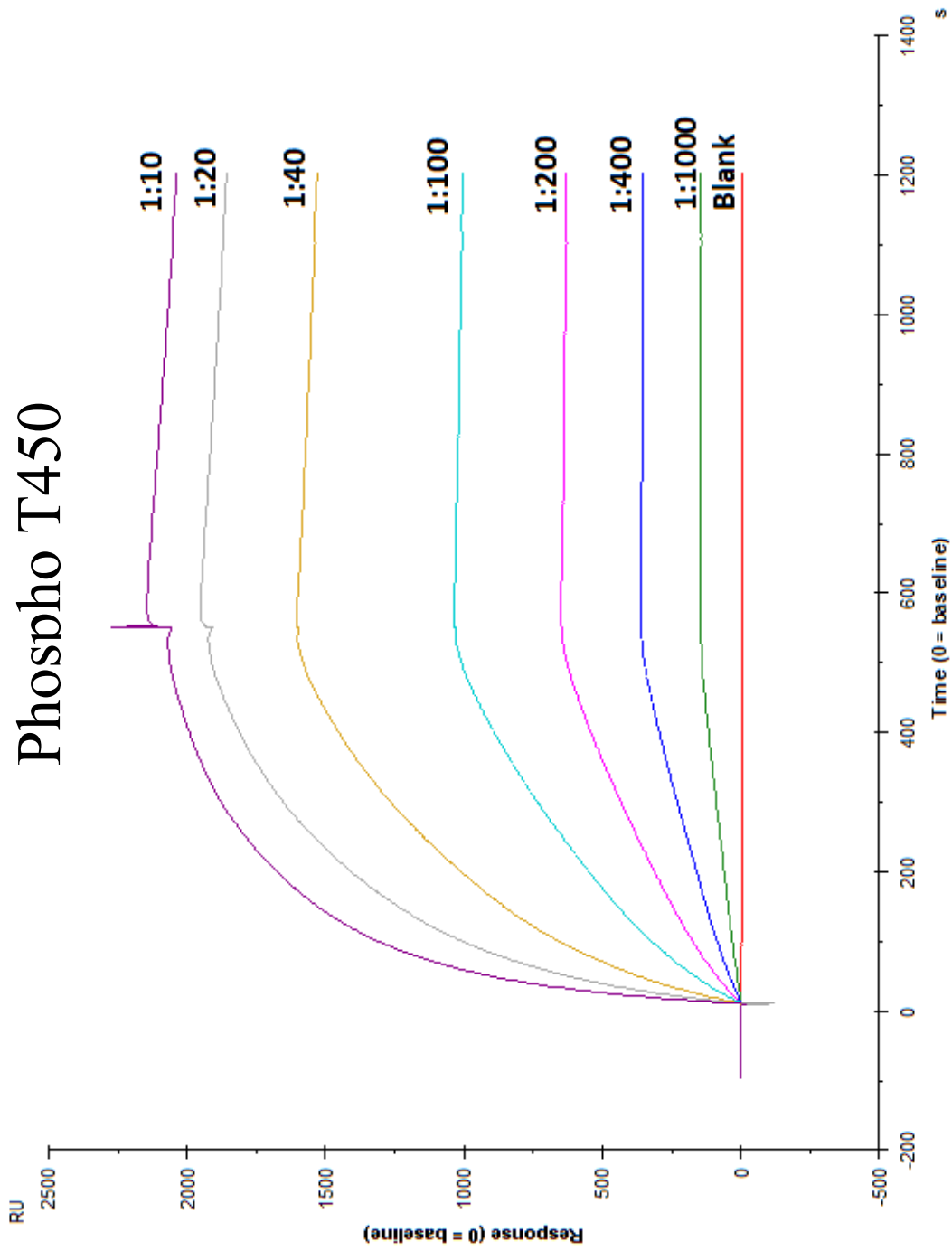


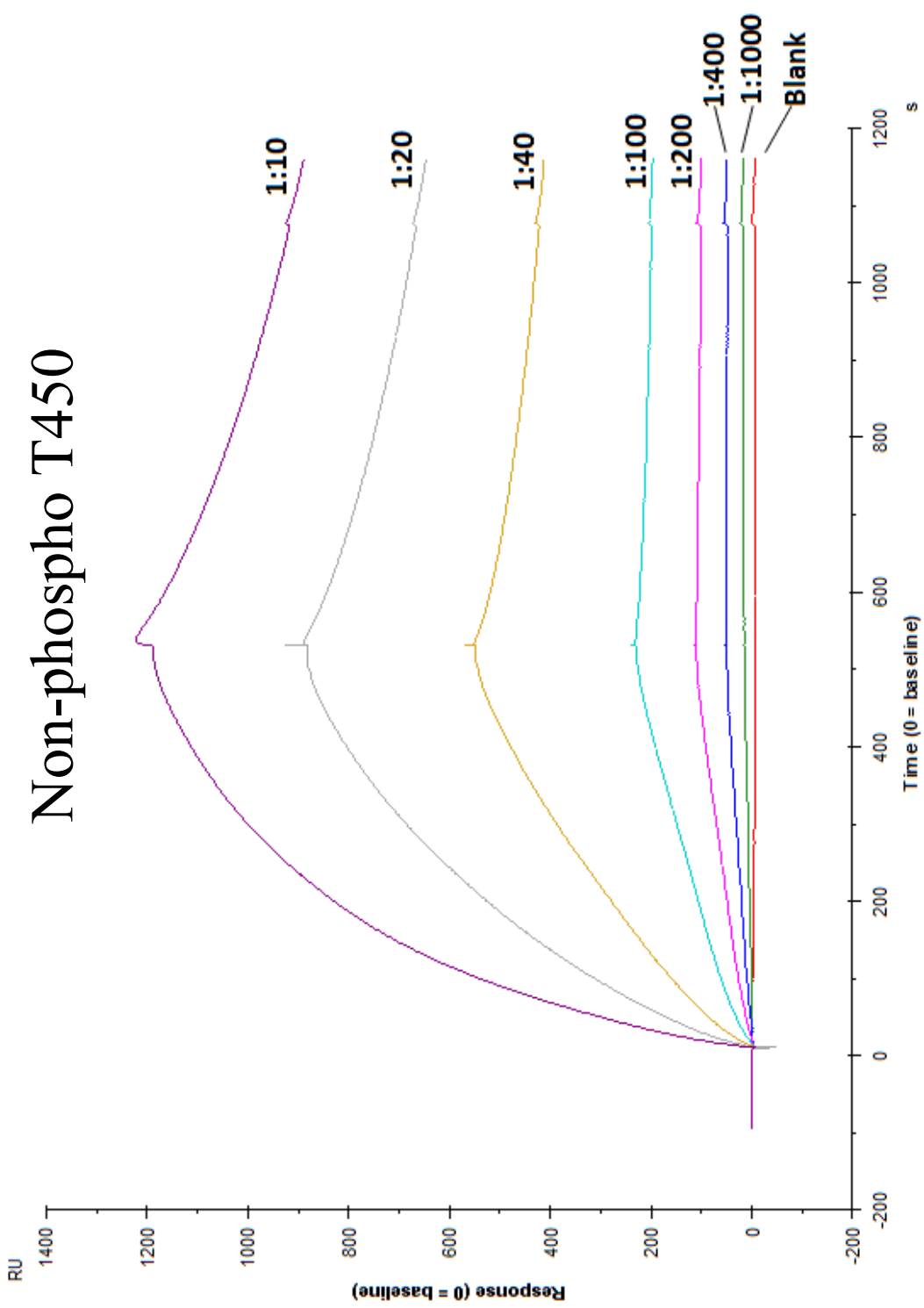


**Figure 3.2: Phosphopeptide and non-phosphopeptide for immobilization.** Scouting for immobilization of pT450 (top) and T450 (bottom) was performed by pre-concentrating the phosphopeptide or the non-phosphopeptide on the dextran surface of the CM5 chip using 10 mM Acetate buffer at pH 4 (red), 4.5 (green), 5 (blue), and 5.5 (pink).

Flow Cell	Ligand	Response Bound (RU)
1	-	-
2	IL2R $\beta$ T450	210.7
3	-	-
4	IL2R $\beta$ pT450	219.9

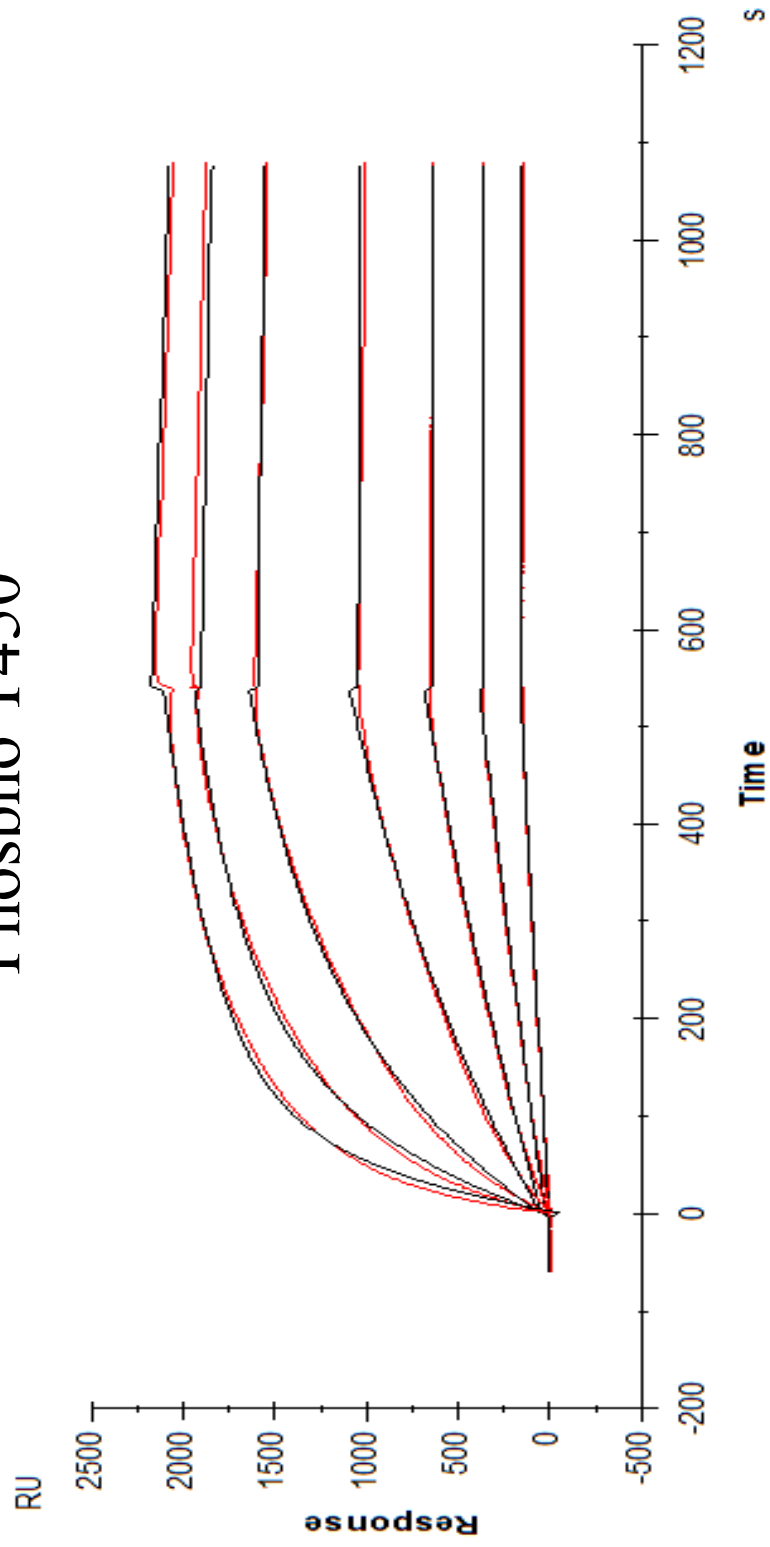
**Table 3.1: Similar levels of phospho T450 and non-phospho T450 were immobilized for SPR analysis.** Phosphopeptide and non-phosphopeptide were immobilized on the dextran surface of the CM5 chip using 10 mM Acetate buffer at pH 4. Flow cells 1 and 3 were used as controls. Non-phosphopeptide was immobilized on flow cell 2 while the phosphopeptide was immobilized on flow cell 4.





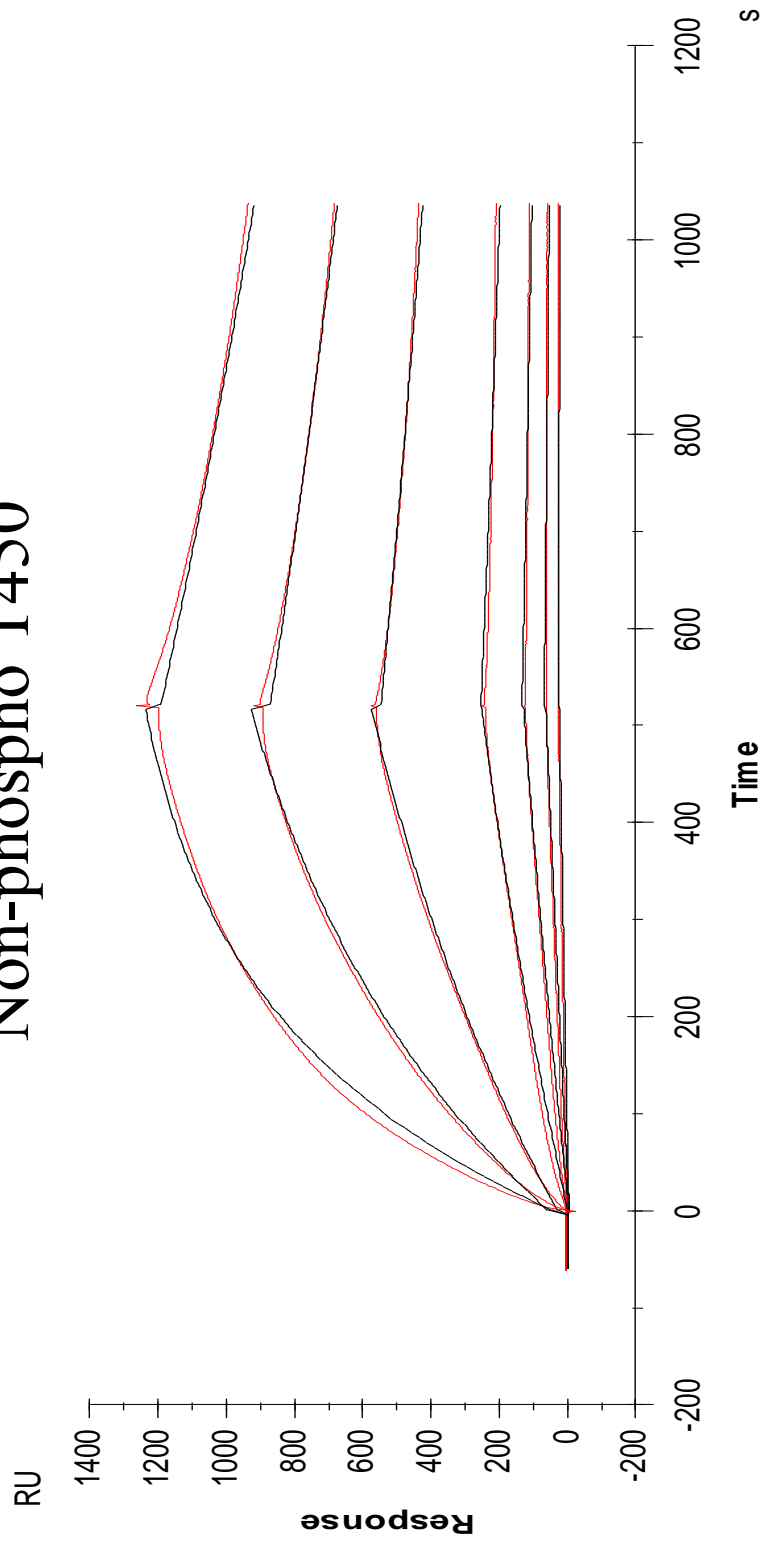
**Figure 3.3: IL2R $\beta$  pT450 antiserum shows higher binding response to phosphopeptide versus non-phosphopeptide.** Increasing dilutions (purple 1:10, grey 1:20, yellow 1:40, aqua 1:100, pink 1:200, blue 1:400, green 1:1000 and red blank) of the antiserum were tested for binding response to the immobilized phosphopeptide and non-phosphopeptide. Samples were injected for 600 seconds and then allowed to dissociate for 600 seconds.

# Phospho T450



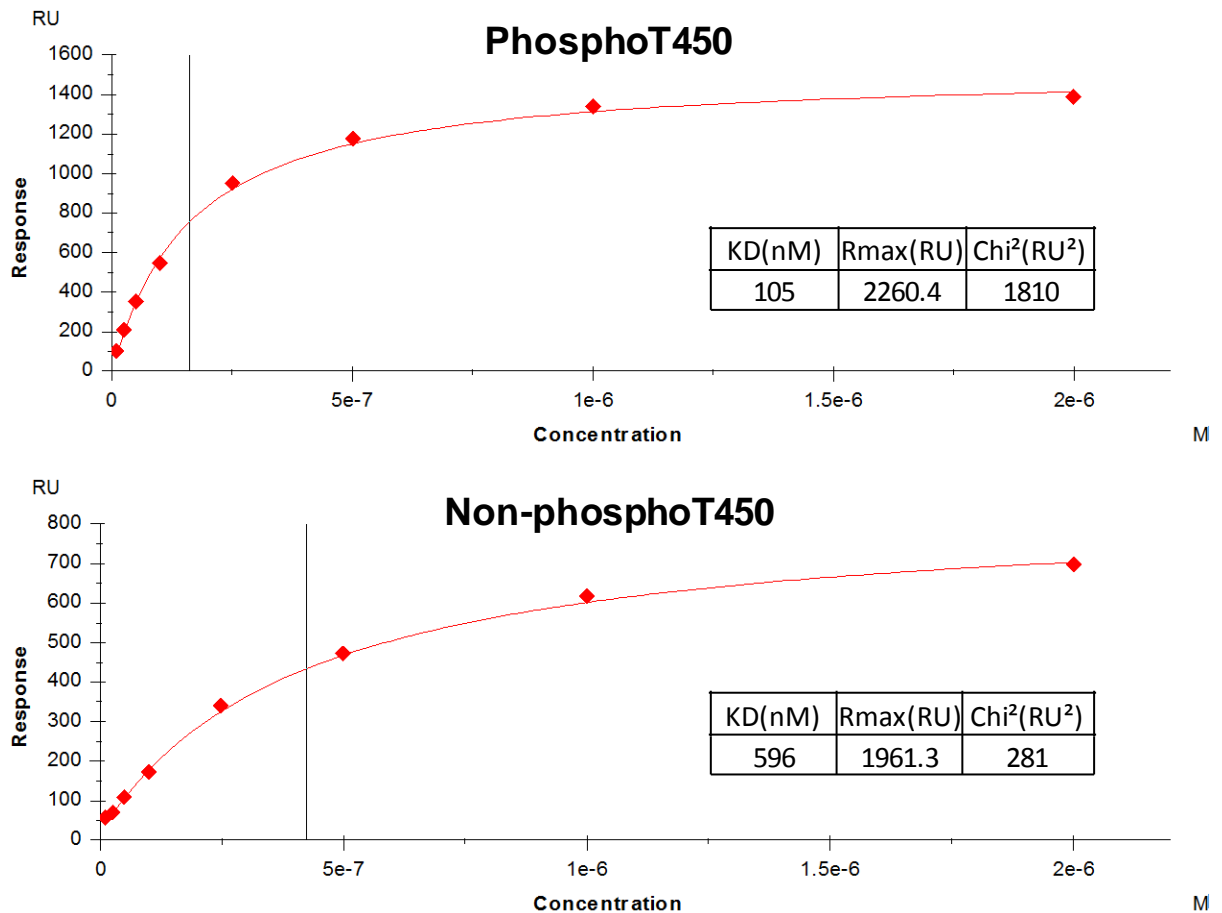
Ka(1/Ms)	Kd(1/s)	Rmax(RU)	Chi <sup>2</sup> (RU <sup>2</sup> )
4295	2.818E-04	3294.6	584

# Non-phospho T450



Ka(1/Ms)	Kd(1/s)	Rmax(RU)	Chi <sup>2</sup> (RU <sup>2</sup> )
2064	5.146E-04	1491	103

**Figure 3.4: IL2R $\beta$  pT450 antiserum binding kinetics.** Binding kinetics was determined using a bivalent analyte model. Expected (black) and obtained (red) values are shown along with the association and dissociation rate constants.



t-test		
Ka	Kd	KD
0.0070725	0.018467054	0.009166

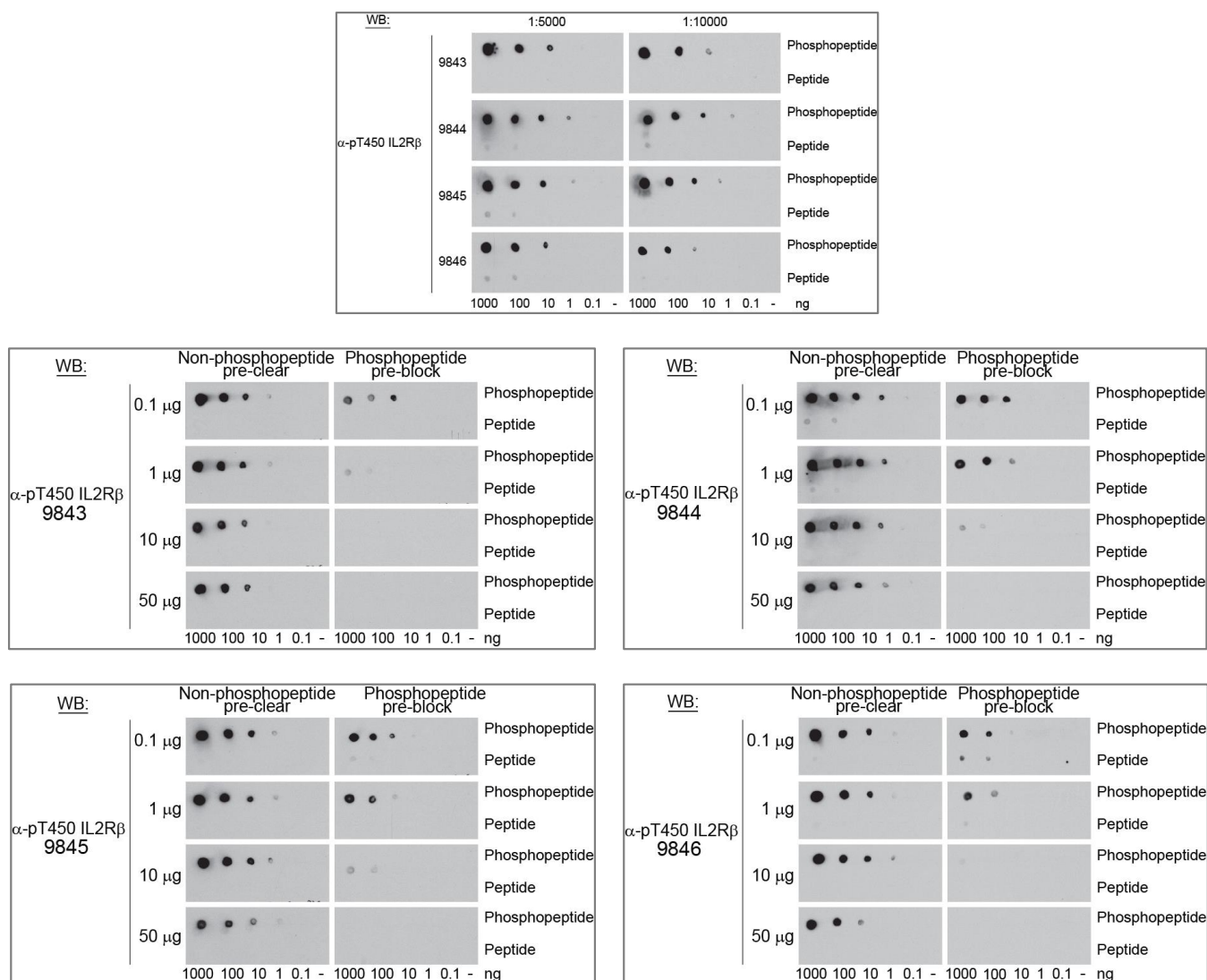
	Ka	Kd	KD
Phospho T450	4586 ± 275.57*	1.926E-4 ± 7.793E-5**	1.147E-7 ± 8.737E-9**
Non-phospho T450	2228.33 ± 144.58	5.377E-4 ± 2.225E-5	5.173E-7 ± 6.827E-8

\* p < 0.05 \*\* p < 0.01

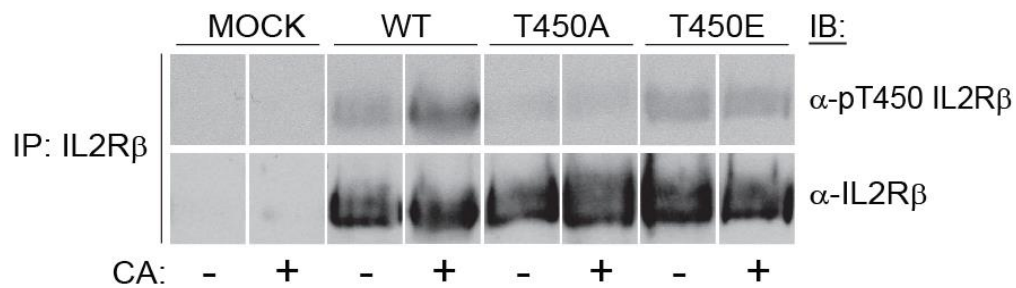
**Figure 3.5: IL2Rβ pT450 antiserum functional affinity to phosphopeptide.** The functional affinity of the antibody was tested by using a 1:1 steady state model. Dissociation equilibrium constant (KD) of the antiserum to the phosphopeptide and non-phosphopeptide is shown. Statistical significance was determined using Student's *t* test (\*,  $p < 0.05$ ; \*\*,  $p < 0.01$ ) using data from three experiments and the difference in association, dissociation and equilibrium constants between the phosphopeptide and non-phosphopeptide were found to be statistically significant.

immunoblotted with  $\alpha$ -pT450 IL2R $\beta$  at 1:5000 and 1:10000 dilutions. For both concentrations, the  $\alpha$ -pT450 IL2R $\beta$  antibody primarily recognized the phosphorylated peptide but not its non-phosphorylated counterpart, indicating that this phospho-specific antibody does not cross-react significantly with the non-phosphorylated peptide (Fig. 3.6 top panel). In addition, a peptide competition assay was performed to confirm specificity of the antibody. Dot blotted membranes were probed using  $\alpha$ -pT450 IL2R $\beta$  pre-incubated with increasing amounts of the non-phosphopeptide or phosphopeptide as indicated. The  $\alpha$ -pT450 IL2R $\beta$  phospho-antibody was specifically blocked by the phospho-, but not the non-phosphopeptide in a dose-dependent manner (Fig. 3.6, middle and bottom panel).

To further characterize this phospho-specific antibody, HEK293 cells were transfected with cDNA encoding WT IL2R $\beta$ , T450A or T450E mutants. 48 hrs post-transfection cells were treated with 100 nM CA for 1 h and IL2R $\beta$  immunoprecipitated, resolved in SDS-PAGE, transferred to PVDF membrane and examined for phosphorylation of T450 by Western blot. As shown in Fig. 3.7, the  $\alpha$ -pT450 IL2R $\beta$  antibody recognizes CA treated WT IL2R $\beta$  but not the T450A mutant. The level of recognition of the T450E mutant by the antibody was comparable to non-treated WT, however, CA induced phosphorylation signal is lost on both mutants. Reprobing these blots with  $\alpha$ -IL2R $\beta$  antibody confirmed similar IL2R $\beta$  expression. This data confirms the specificity of our phospho-specific antibody towards the T450 phosphorylated form of IL2R $\beta$  in Western blot analysis.



**Figure 3.6: IL2R $\beta$  pT450 recognizes phosphorylated peptide and is specific.** Antibodies were tested by dot blot analysis using decreasing concentrations of IL2R $\beta$  T450 and pT450 peptides spotted on to PVDF membranes and incubated with two different dilutions of the anti-serum as indicated (top panel). For peptide competition analysis, the polyclonal pT450 IL2R $\beta$  antibody was pre-blocked for 2 h at room temperature with increasing concentrations of the non-phosphopeptide or phosphopeptide as indicated (middle and bottom panel).

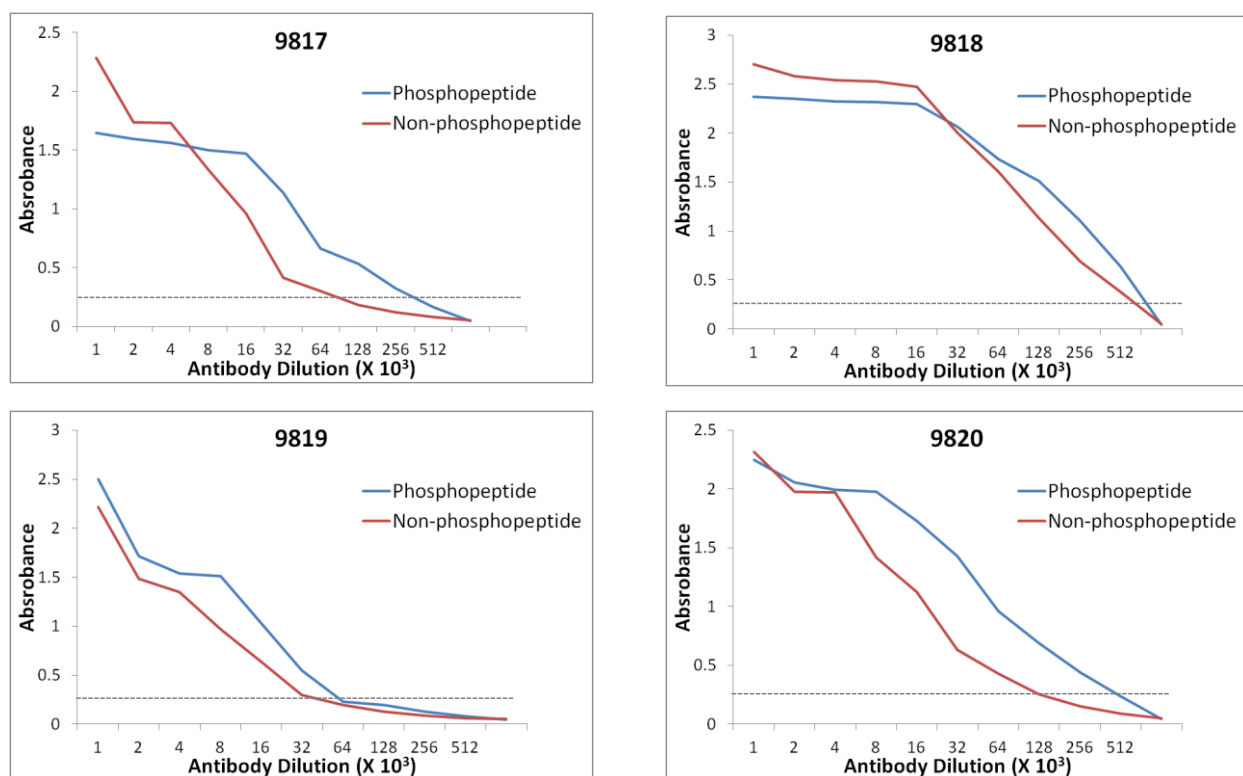


**Figure 3.7: Anti-IL2Rβ pT450 antibody recognizes CA treated WT IL2Rβ.** HEK293 cells were transfected with plasmids for WT IL2Rβ, T450A or T450E mutants. At 48 h post-transfection cells were incubated in the absence or presence of CA (100 nM) for 60 min. IL2Rβ was immunoprecipitated (IP), separated by SDS-PAGE, and immunoblotted (IB) with α-pT450 IL2Rβ. Membrane was stripped and reprobed with α-IL2Rβ.

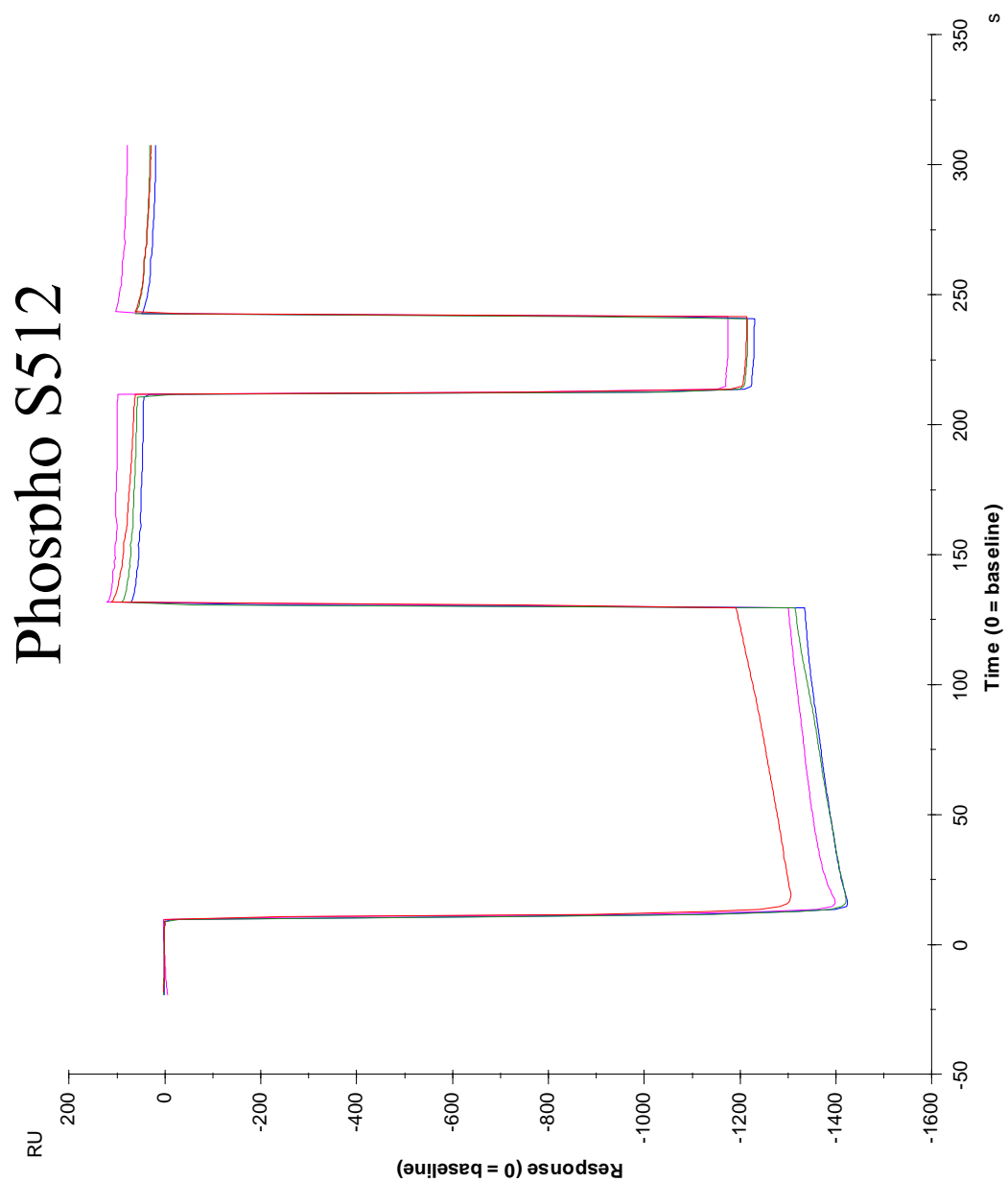
### 3.3.2 $\alpha$ -pS512 IL2R $\beta$ Antiserum Preferentially Recognizes Phosphorylated Peptide

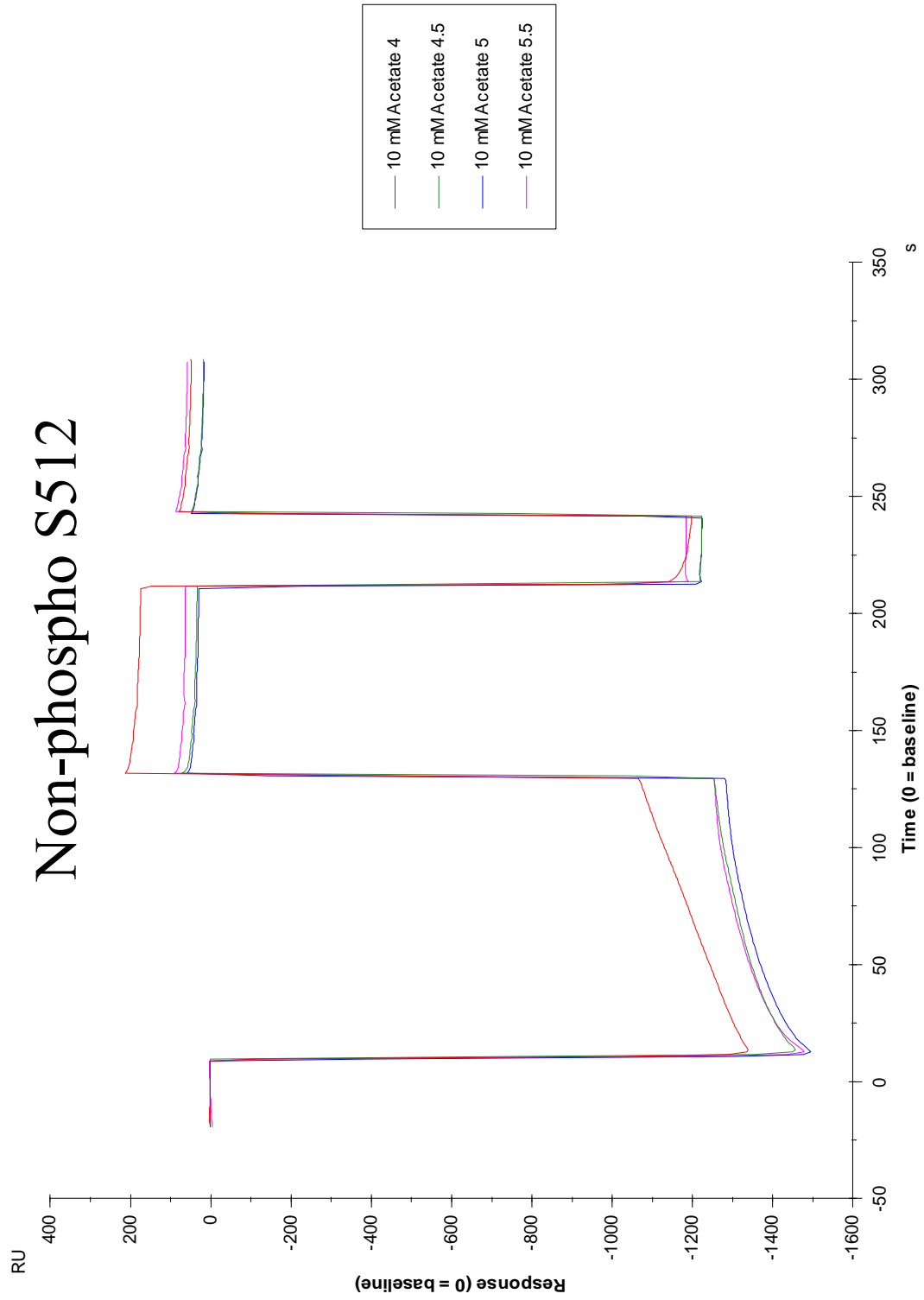
Phosphorylation of S512 on IL2R $\beta$  was identified through mass spectrometry analysis and may represent a novel mechanism of regulation of IL2 signaling. To verify that IL2R $\beta$  is phosphorylated at threonine S512 and to investigate the regulatory role of this phosphorylation site, a phospho-specific polyclonal antibody was generated. Four rabbits were immunized with the phosphopeptides and are represented by the numbers 9817, 9818, 9819, and 9820. Final bleed antisera were analyzed by ELISA and limited specificity to phosphopeptide (blue) versus non-phosphopeptide (red) was observed for all four rabbits (Fig 3.8).

The affinity of 9818 antisera to the phosphopeptide versus the non-phosphopeptide was further tested by SPR. As shown in figure 3.9, the highest pre-concentration of both the phosphopeptide and non-phosphopeptide on the CM5 chip was obtained using a pH 4 buffer. Similar amount of ligands (phosphopeptide and non-phosphopeptide) were immobilized on the dextran surface (Table 3.3). Increasing dilutions of the antiserum were tested for binding response to the immobilized phosphopeptide and non-phosphopeptide. The concentration of the antibody was assigned to 10  $\mu$ g/mL on the Biacore, however, for the purpose of this assay the values are represented as arbitrary units. Binding curves show higher response (resonance units) for the phosphopeptide versus the non-phosphopeptide for all the dilutions tested (Fig 3.10). In addition, dissociation of the antiserum occurs more rapidly from the non-phosphopeptide versus the phosphopeptide. Binding kinetics was determined using a bivalent analyte model. Expected values are shown in black and obtained values are in red. We observed a higher association rate constant of the antiserum to the phosphopeptide versus the non-phosphopeptide. In addition, the dissociation rate constant of the antiserum from the phosphopeptide was 3 log smaller than that for the non-phosphopeptide (Fig. 3.11). The functional affinity of the antibody was tested using a 1:1 steady state model and the dissociation equilibrium constant ( $K_D$ ) of the antiserum was found to be 162.3 nM for the phosphopeptide and 424.4 nM for the non-phosphopeptide (Fig. 3.12). Representative data from three independent experiments are shown. A t-test was performed and



**Figure 3.8: pS512 IL2R $\beta$  antisera shows limited specificity towards phosphopeptide.** Four rabbits represented by the numbers 9817, 9818, 9819, and 9820, were immunized with the phosphopeptides. Increasing dilutions of each antiserum were analyzed through ELISA. Specificity of the antisera to phosphopeptide (blue) and non-phosphopeptide (red) is shown.



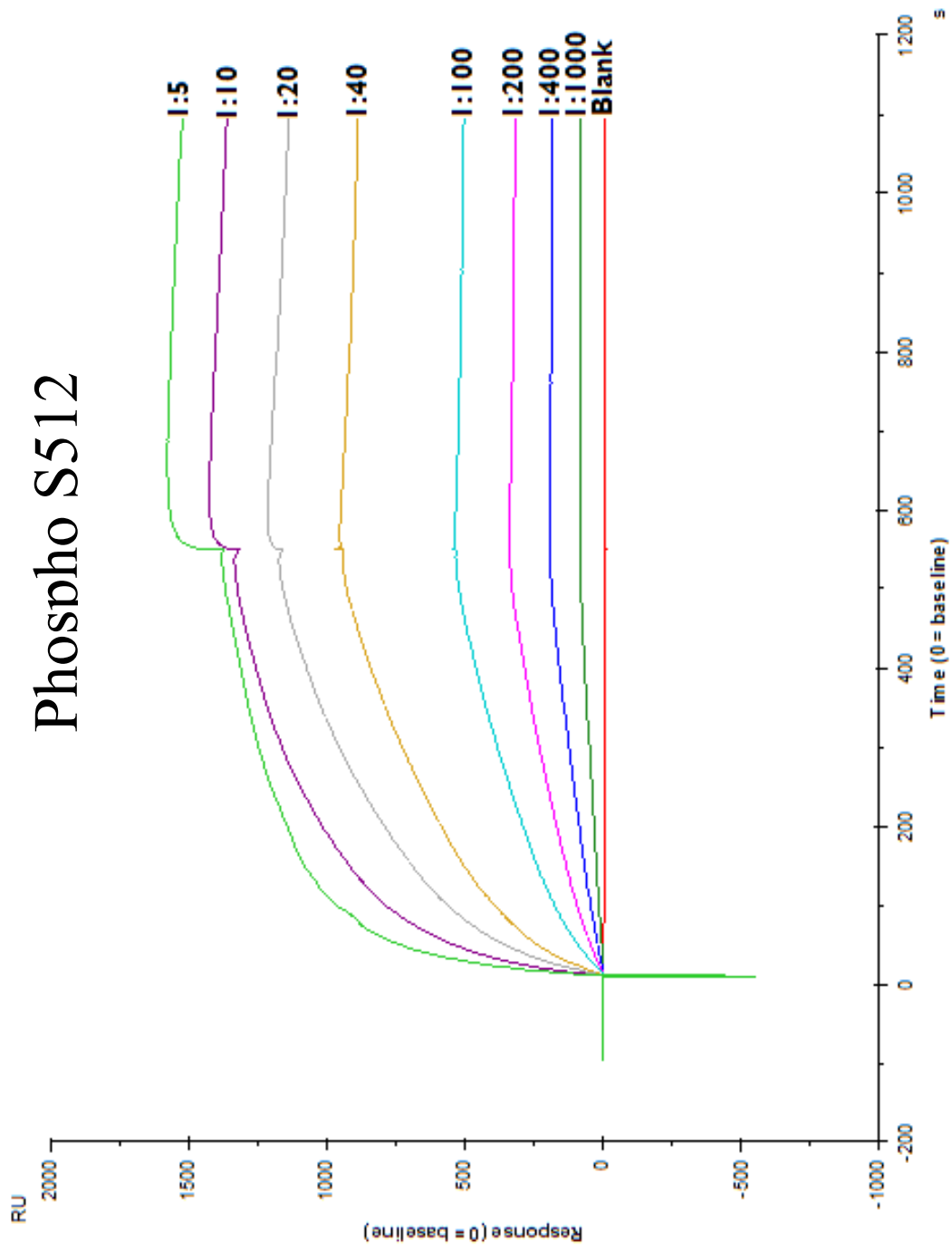


**Figure 3.9: Phosphopeptide and non-phosphopeptide for immobilization.** Scouting for immobilization of pS512 and S512 was performed by pre-concentrating the phosphopeptide or the non-phosphopeptide on the dextran surface of the CM5 chip using 10 mM Acetate buffer at pH 4 (red), 4.5 (green), 5 (blue), and 5.5(pink).

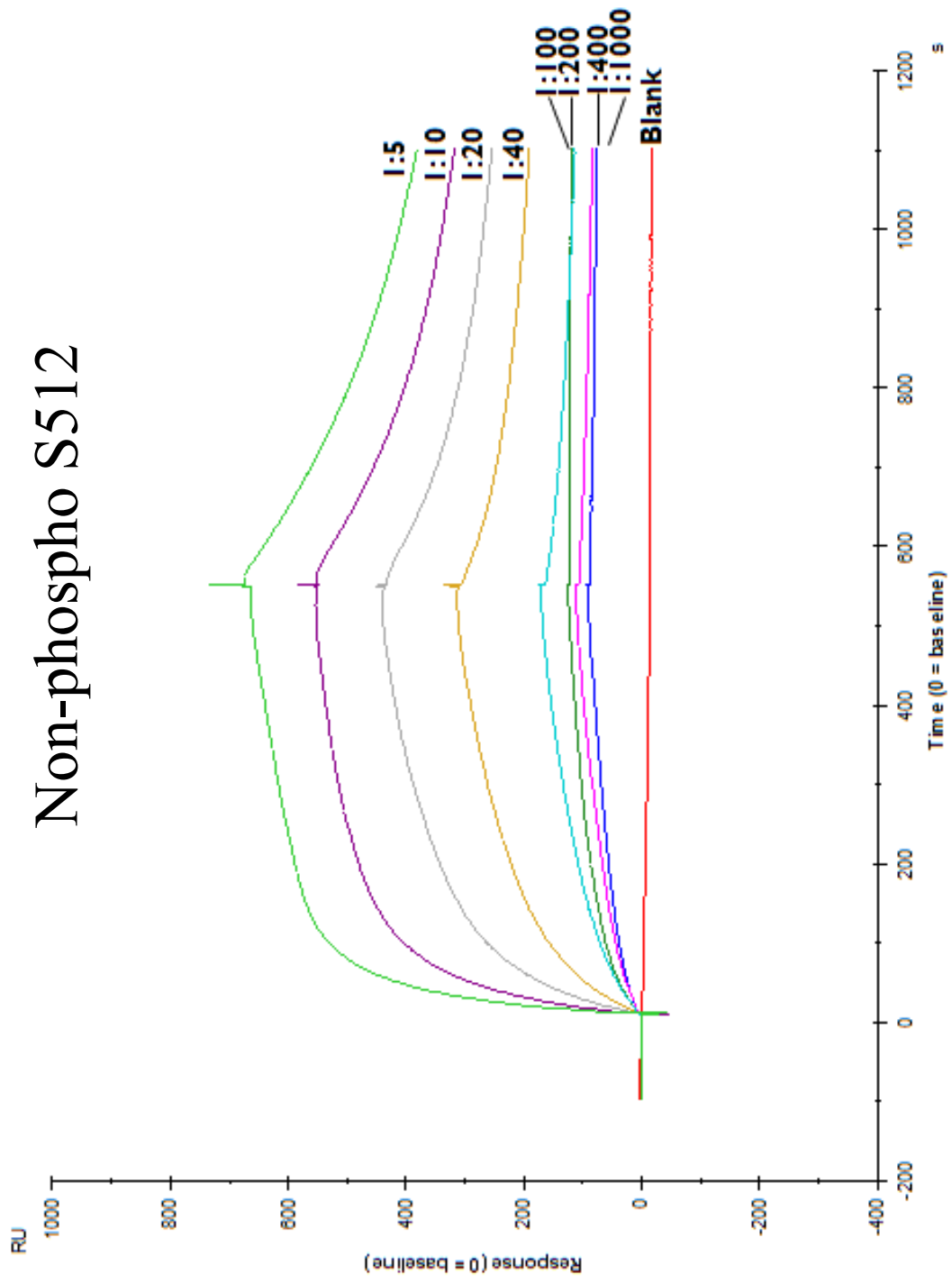
Flow Cell	Ligand	Response Bound (RU)
1	-	-
2	IL2R $\beta$ S512	209.5
3	-	-
4	IL2R $\beta$ pS512	159.3

**Table 3.2: Similar levels of phospho S512 and non-phospho S512 were immobilized for SPR analysis.** Phosphopeptide and non-phosphopeptide were immobilized on the dextran surface of the CM5 chip using 10 mM Acetate buffer at pH 4. Flow cells 1 and 3 were used as controls. Non-phosphopeptide was immobilized on flow cell 2 while the phosphopeptide was immobilized on flow cell 4.

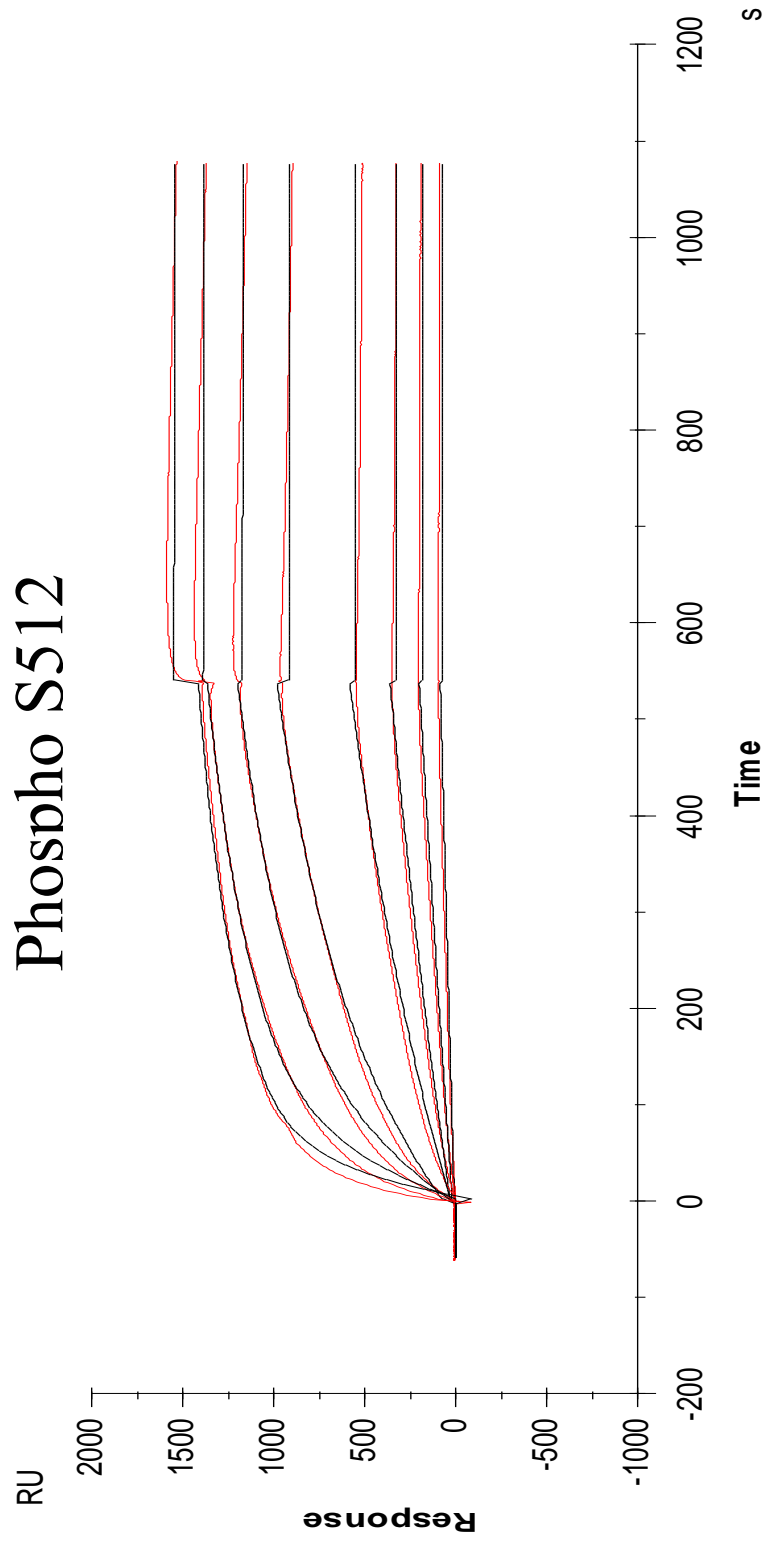
# Phospho S512

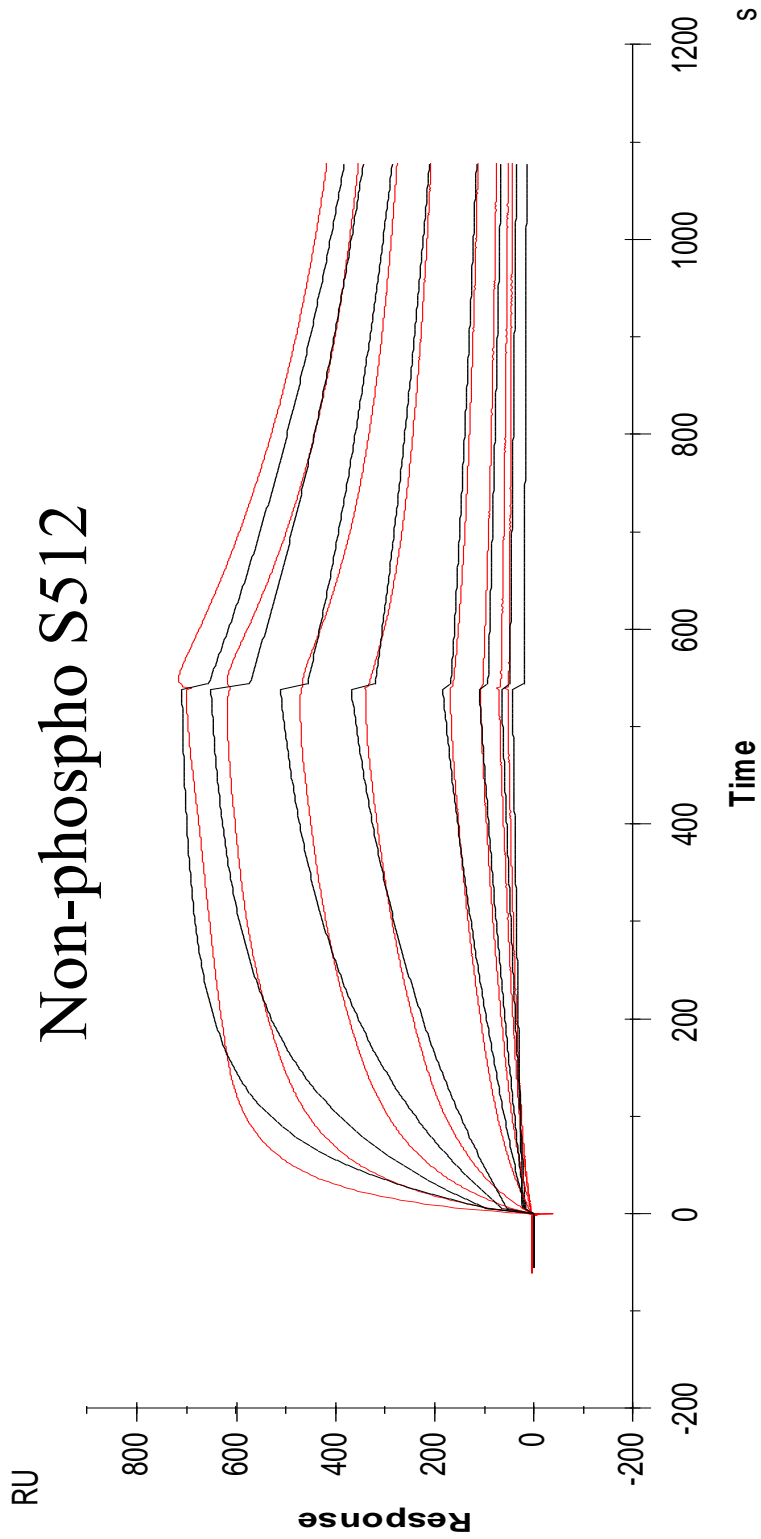


# Non-phospho S512



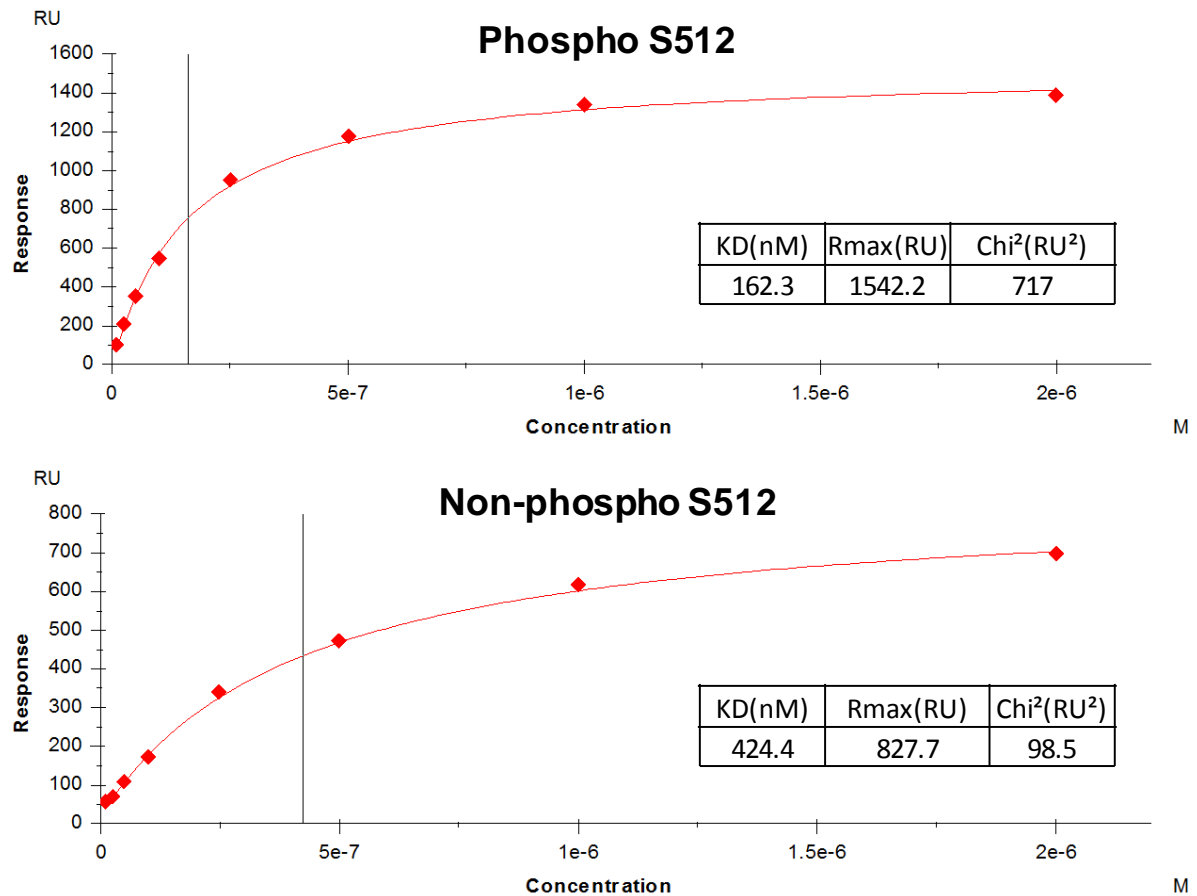
**Figure 3.10: IL2R $\beta$  pS512 antiserum indicates higher binding response to phosphopeptide versus non-phosphopeptide.** Increasing dilutions (green 1:5, purple 1:10, grey 1:20, yellow 1:40, aqua 1:100, pink 1:200, blue 1:400, dark green 1:1000, and red blank) of antiserum were tested for binding response to the immobilized phosphopeptide and non-phosphopeptide. Samples were injected for 600 seconds and then allowed to dissociate for 600 seconds.





## Non-phospho S512

**Figure 3.11: IL2R $\beta$  pS512 antiserum binding kinetics.** Binding kinetics was determined using a bivalent analyte model. Expected (black) and obtained (red) values are shown along with the association and dissociation rate constants.



t-test		
Ka	Kd	KD
0.0015816	0.002446251	0.098355

	Ka	Kd	KD
Phospho S512	3955 ± 399.7**	7.991E-6 ± 8.5E-6**	1.713E-7 ± 8.238E-9
Non-phospho S512	3262 ± 391.8	1.285E-3 ± 1.068E-4	4.35E-7 ± 1.468E-7

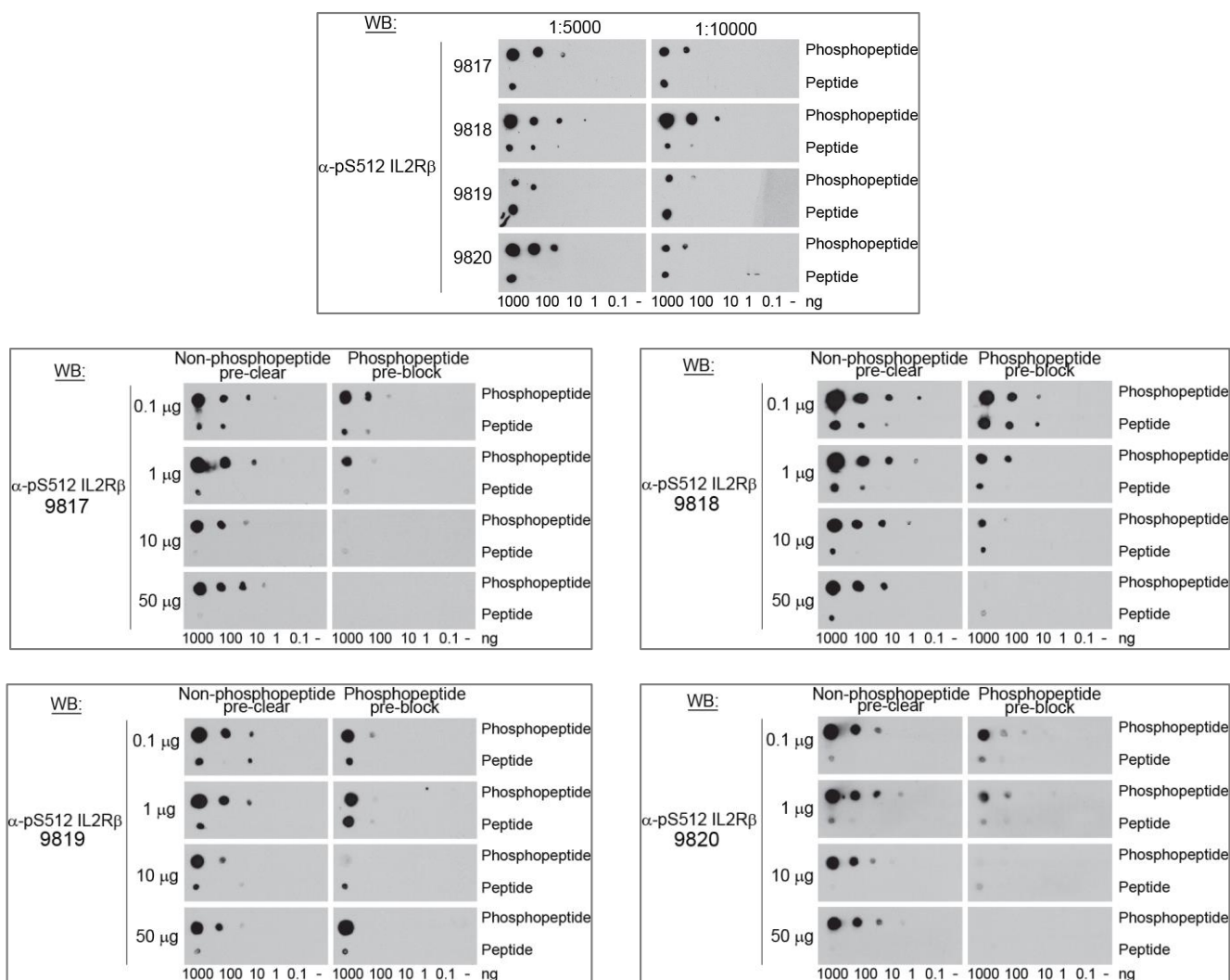
\* p < 0.05    \*\* p < 0.01

**Figure 3.12: IL2Rβ pS512 antiserum functional affinity to phosphopeptide.** The functional affinity of the antibody was tested by using a 1:1 steady state model. Dissociation equilibrium constant ( $K_D$ ) of the antiserum to the phosphopeptide and non-phosphopeptide is shown. Statistical significance was determined using Student's *t* test (\*,  $p < 0.05$ ; \*\*,  $p < 0.01$ ) using data from three experiments and the difference in association and dissociation constants between the phosphopeptide and non-phosphopeptide were found to be statistically significant.

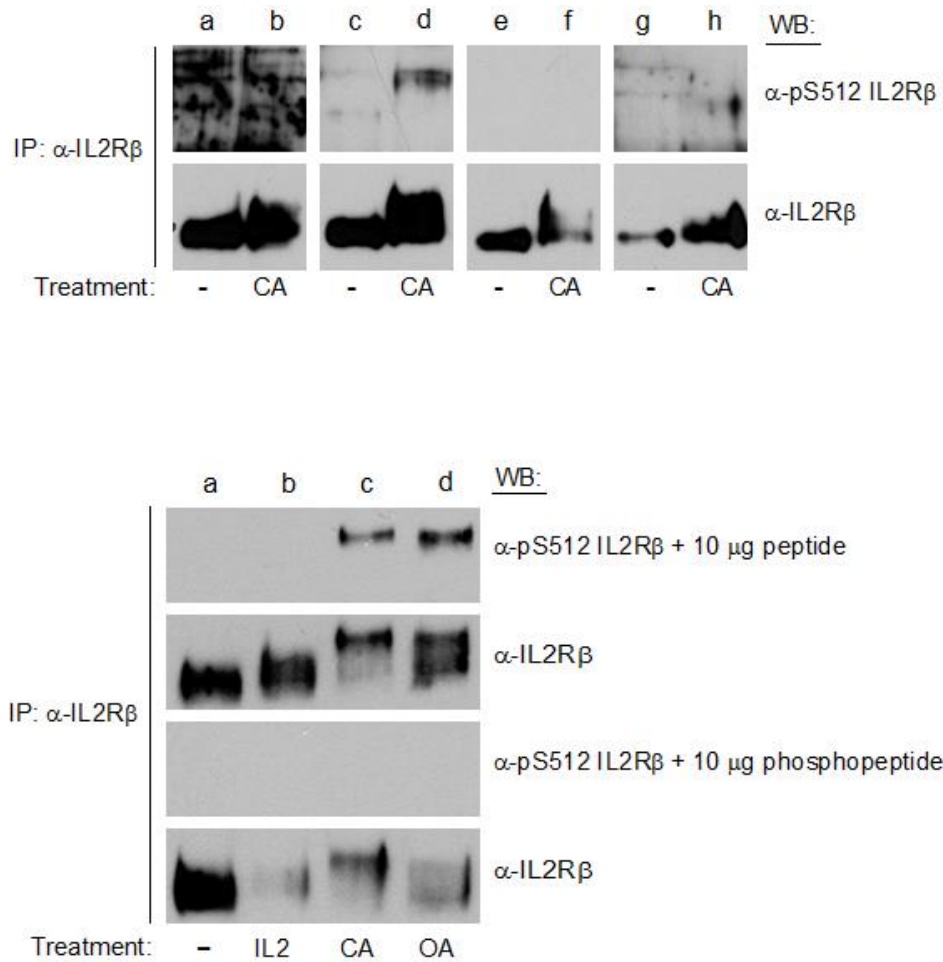
the difference in association, dissociation and equilibrium constants between the phosphopeptide and non-phosphopeptide were found to be statistically significant (Table 3.2).

To determine whether the S512 IL2R $\beta$  phospho-specific antibody cross-reacts with the non-phosphorylated form of the peptide, dot blot analysis with the immunizing phospho-peptide and the corresponding non-phosphorylated peptide was performed (see section 3.2.1 for sequences). Increasing amounts of pS512 or S512 peptides were spotted on to PVDF membranes and immunoblotted with  $\alpha$ -pS512 IL2R $\beta$  at 1:5000 and 1:10000 dilutions. For both concentrations, the  $\alpha$ -pS512 IL2R $\beta$  antibody primarily recognized the phosphorylated peptide but not its non-phosphorylated counterpart, indicating that this phospho-specific antibody does not cross-react significantly with the non-phosphorylated peptide (Fig. 3.13, top panel). In addition, a peptide competition assay was performed to confirm specificity of the antibody. Dot blotted membranes were probed using  $\alpha$ -pS512 IL2R $\beta$  pre-incubated with increasing amounts of the non-phosphopeptide or phosphopeptide as indicated. The  $\alpha$ -pS512 IL2R $\beta$  phospho-antibody was specifically blocked by the phospho-, but not the non-phosphopeptide in a dose-dependent manner (Fig. 3.13, middle and bottom panel).

To further characterize this phospho-specific antibody, YT cells ( $20 \times 10^6$ ) were treated with 100 nM CA for 15 min and IL2R $\beta$  immunoprecipitated, resolved in SDS-PAGE, transferred to PVDF membrane and examined for phosphorylation of S512 by Western blotting. As shown in Fig. 3.14 (top panel), the  $\alpha$ -pS512 IL2R $\beta$  9818 antibody recognizes IL2R $\beta$  from CA treated but not from the non-treated cells. Reprobing these blots with  $\alpha$ -IL2R $\beta$  antibody confirmed similar IL2R $\beta$  expression. In a similar fashion, YT cells were stimulated with IL2, treated with CA or OA, or left untreated and IL2R $\beta$  immunoprecipitated and samples analyzed by Western blot using antibody previously blocked with phosphopeptides or cleared with peptide. Results show that the 9818 antibody recognizes IL2R $\beta$  from CA and OA treated cells. However when the antibody is pre-incubated with the phosphopeptide, the signal is lost (Fig. 3.14, bottom panel). This data confirms the specificity of our phospho-specific antibody towards the S512 phosphorylated form of IL2R $\beta$  in Western blot analysis.



**Figure 3.13: IL2Rβ pS512 recognizes phosphorylated peptide and is specific.** Antibodies were tested by dot blot analysis using decreasing concentrations of IL2Rβ S512 and pS512 peptides spotted on to PVDF membranes and incubated with two different dilutions of the anti-serum as indicated (top panel). For peptide competition analysis, the polyclonal pS512 IL2Rβ antibody was pre-blocked for 2 h at room temperature with increasing concentrations of the non-phosphopeptide or phosphopeptide as indicated (middle and bottom panel).



**Figure 3.14: IL2Rβ pS512 antibody 9818 recognizes IL2Rβ from CA and OA treated YT cells and the signal is blocked by the phospho- but not the non-phosphopeptide.** YT cells were incubated with or without CA (100 nM) for 15 min at 37 °C. IL2Rβ was immunoprecipitated (IP), separated by SDS-PAGE, and Western blotted (WB) as indicated (upper panel). For the peptide competition assay (lower panel), cells were left untreated, stimulated with IL2 (10<sup>3</sup> IU, 5 min), treated with CA (100 nM, 15 min) or OA (250 nM, 1 h). IL2Rβ was immunoprecipitated (IP) and samples were analyzed through Western blot using pS512 IL2Rβ antibody 9818 previously incubated with 10 μg of either the phosphopeptide or the non-phosphopeptide.

### 3.4 DISCUSSION

In order to study the importance of the two novel IL2R $\beta$  phosphorylation sites identified through mass spectrometry T450 and S512, rabbit polyclonal phosphospecific antibodies were generated. The strategy for characterization of the antibodies which included ELISA, SPR, dot blot, and Western blot of immunoprecipitated IL2R $\beta$  revealed specificity of our antibodies to be used in Western blot analysis of both immunoprecipitated proteins and total cell lysates. Both  $\alpha$ -pT450 and  $\alpha$ -pS512 IL2R $\beta$  antisera preferentially recognized their corresponding phosphorylated peptide; displayed higher binding response, stronger binding kinetics and functional affinity, they were also specifically blocked by the phospho- but not non-phosphopeptide. In addition, the  $\alpha$ -IL2R $\beta$  pT450 antibody recognized CA treated WT IL2R $\beta$  but not the phosphodeletion mutant T450A. Both  $\alpha$ -pT450 and  $\alpha$ -pS512 IL2R $\beta$  proved to be useful for Western blot analysis of immunoprecipitated protein and total cell lysates.

These findings support the use of phosphospecific antibodies as reliable new tools for investigating the functional role of T450 and S512 in IL2 receptor signal transduction, its mechanism of phosphorylation and dephosphorylation.

## **CHAPTER 4**

**IL2R $\beta$  T450 is a Novel IL2 and IL15 Induced Phosphosite**

**Regulated by ERK1/2 and PP1**

## **4.1 INTRODUCTION**

IL2 and IL15 share the  $\beta$  and  $\gamma_c$  subunits of their receptors and utilize JAK/STAT molecules for signaling. The result is an overlap of some cellular functions since both cytokines induce the proliferation of B and T cells and the generation of cytotoxic T lymphocytes and NK cells. However, IL2 and IL15 also play distinct roles necessary for homeostasis of the immune system. The primary function of IL2 is both the expansion and elimination of self-reactive T-cells while IL15 supports the survival of memory T-cells [87]. Activation of IL2R $\beta$  by IL2 and IL15 occurs through JAK mediated phosphorylation of its tyrosine residues, which allows for recruitment and docking of proteins like STATs and SHC [20] and the subsequent activation of downstream signaling molecules involved in cell proliferation, survival and apoptosis [3]. Through phosphoamino acid analysis, our group has shown threonine phosphorylation of the receptor in response to IL2 [56], although the specific regulatory role it might play on signaling remains to be elucidated. The focus of this chapter was to determine whether IL2R $\beta$  is phosphorylated at T450 in response to physiological stimuli. Several human malignant hematopoietic cell lines along with non-tumorigenic primary lymphocytes were stimulated with IL2 or IL15 and phosphorylation of T450 assessed by Western blot using the  $\alpha$ -pT450 IL2R $\beta$  antibody. In addition, using kinase/phosphatase inhibitors along with siRNA and purified phosphatases, we investigated the kinases and phosphatases involved in mediating such phosphorylation/dephosphorylation events. We provide evidence that T450 phosphorylation is mediated by ERK1/2 in response to IL2 and IL15 and dephosphorylated by PP1.

## **4.2 MATERIALS AND METHODS**

### **4.2.1 Cell Culture and Treatment**

The human YT, KIT225, HUT102, HH, MT2, and SUPT1 cell lines were maintained in RPMI 1640 medium containing 10% fetal bovine serum (Atlanta Biologicals), 2 mM L-glutamine, and penicillin-streptomycin (50 mg/ml) (complete RPMI). KIT225 cells were supplemented with

100 IU/ml human recombinant IL2 (NCI Preclinical Repository). Human peripheral blood mononuclear cells (PBMCs) from healthy donors (Research Blood Components) were purified by isocentrifugation (Ficoll-Hypaque), and activated with phytohemagglutinin (PHA) (10 µg/ml) for 72 h as previously described [88]. Quiescent PBMCs or malignant hematopoietic cell lines were stimulated with human recombinant IL2 ( $10^3$  IU) or human recombinant IL15 (0.49 µg/ml) (NCI Preclinical Repository) at 37 °C for the indicated times. Kinase and phosphatase inhibition studies with wortmannin (Calbiochem), KT5720 (Sigma), tofacitinib and trametinib (Selleck Chemicals), okadaic acid (OA; Millipore), tautomycin (TAU; Santa Cruz), cyclosporin A (CSA; Sigma) and ERK Inhibitor II – FR180204 (Santa Cruz) were performed for 1 h while calyculin A (CA; Invitrogen) treatment was performed for 15 minutes at 37 °C at the indicated concentrations.

#### **4.2.2 Solubilization of Proteins, Immunoprecipitation, and Western Blot Analysis**

Cells were pelleted, lysed and subjected to immunoprecipitation and Western blot analysis as previously reported [89]. For all samples, total protein was determined by the bicinchoninic acid method (Pierce). Western blot assays were developed with horseradish peroxidase-conjugated goat anti-mouse immunoglobulin G (IgG; heavy plus light chains) or goat anti-rabbit IgG (heavy plus light chains; KPL) and visualized by using enhanced chemiluminescence and X-ray film. For reblotting, polyvinylidene difluoride membranes were incubated with stripping buffer (100 mM β-mecaptoethanol, 2% SDS, 62.5 mM Tris-HCl, pH 6.7) at 55 °C for 30 min, blocked, and then reprobed. The anti-phosphothreonine 450 (α-pT450) IL2Rβ rabbit polyclonal antibody was custom-generated by GenScript (Piscataway, NJ) using the immunogen LGPP(pT)PGVPDLVDFC (where pT indicates phospho-threonine) coupled to keyhole limpet hemocyanin. The anti-JAK3 [90] antibody was used as previously described. A monoclonal mouse antibody made against the IL2Rβ chain (561-IgG2,) was a generous gift from Dr. Richard Robb [91]. The anti-phospho-Tyr<sup>694</sup> STAT5a and Tyr<sup>699</sup> STAT5b (pYSTAT5) monoclonal antibody,

anti-STAT5 polyclonal antibody, anti-phospho-p44/42 MAPK (ERK1/2) Thr<sup>202</sup>/Tyr<sup>204</sup> polyclonal antibody and anti- p44/42 MAPK (ERK1/2) polyclonal antibody were purchased from Cell Signaling Technology and were used according to the manufacturer's protocol along with anti-phosphotyrosine monoclonal 4G10 ( $\alpha$ -pY) antibody (Upstate Biotechnology), anti-GAPDH monoclonal antibody (Fitzgerald), and the anti-IL2R $\beta$ /anti-IL2R $\gamma$  chain antibodies (Santa Cruz Biotechnology).

#### **4.2.3 siRNA-mediated Silencing of ERK1 and ERK2**

ERK1 (SMARTpool catalog number M-003592-03-0010) and ERK2 (SMARTpool catalog number M-003555-04-0010) as well as control non-targeting (siGENOME Non-Targeting siRNA Pool #1 catalog number D-001206-13-20) siRNA were purchased from Dharmacon. Transfection of YT cells was carried out by electroporation using the Nucleofection System by Amaxa according to manufacturer's instructions. Briefly, YT cells ( $5 \times 10^6$ ) were suspended in 100  $\mu$ l of transfection solution V and transfected with 750 nM of ERK1, 1  $\mu$ M ERK2, 750 nM of ERK1 and 1  $\mu$ M ERK2, or 1.750  $\mu$ M control siRNA using the O-017 program. Transfected cells were immediately diluted with pre-warmed complete RPMI and cultured for 48 h. Cells were then quieted for 24 h in 1% FBS media before incubation with or without IL2 as indicated.

#### **4.2.4 In Vitro Dephosphorylation Assay**

YT cells ( $20 \times 10^6$ ) were incubated with or without 100 nM CA for 15 min prior to solubilization in lysis buffer (1% Triton X-100, 10 mM Tris-HCl pH 8.0, 50 mM NaCl). Total cell lysates were incubated without or with 0.5 units of purified PP1 or PP2A (Millipore) at 37 °C for 60 min according to the manufacturer's instructions. The reactions were stopped by addition of sample buffer containing 125 mM Tris-HCl, pH 6.8, 10%  $\beta$ -mercaptoethanol, 9.2% SDS, 0.04% bromophenol blue, 20% glycerol, and boiled for 5 min. Samples were resolved by SDS-PAGE and phosphorylation of T450 was measured by Western blot analysis.

## 4.2 RESULTS

### 4.2.1 Phosphorylation of T450 IL2R $\beta$ Occurs In Vivo and Displays Rapid Kinetics

To determine whether IL2R $\beta$  is phosphorylated at T450 in response to physiological stimuli, and kinetic rate compared to tyrosine phosphorylation, YT cells were subjected to an IL2 stimulation time course (0-60 min). Endogenous IL2R $\beta$  was immunoprecipitated and each sample divided and examined for phosphorylation with either  $\alpha$ -pY or  $\alpha$ -pT450 IL2R $\beta$  antibodies (Fig. 4.1A). IL2 stimulation resulted in phosphorylation of tyrosine and T450 within 30 seconds and attained maximal levels at approximately 2.5 minutes. T450 phosphorylation signal continued to increase and reached a maximum at 10 minutes post IL2 stimulation. A reduction in tyrosine and T450 phosphorylation began at 30 minutes post-stimulation. In order to evaluate a putative pleiotropic role of T450 phosphorylation, YT cells were stimulated with IL15, harvested between 0 and 60 min and immunoprecipitated IL2R $\beta$  examined for phosphorylation with  $\alpha$ -pT450 IL2R $\beta$ . IL15 displayed similar phosphorylation profiles compared to IL2. Maximum phosphorylation of IL2R $\beta$  T450 occurred within 2.5 minutes, and was sustained for 30 minutes (Fig. 4.1B). To determine whether IL2 and IL15 induced phosphorylation of T450 is isolated to the acute lymphoblastic lymphoma NK-like cell line, YT, other human malignant hematopoietic cell lines including the HTLV-1 positive cutaneous T-cell lymphoblast HUT102 [92], cutaneous T-cell lymphoma HH [93], T-cell chronic lymphocytic leukemia KIT225, HTLV-1 positive T-cell leukemia MT2 [94], and the T-cell lymphoblastic leukemia SUPT1 were made quiescent by growing to media exhaustion and then stimulated with IL2 or IL15 for 5 min. Endogenous IL2R $\beta$  was immunoprecipitated and resolved in SDS-PAGE for analysis through Western blot. Both IL2 and IL15 were able to induce phosphorylation of IL2R $\beta$  at T450 in all human malignant hematopoietic cell lines (Fig. 4.1C). To test whether T450 is phosphorylated in non-tumorigenic primary lymphocytes, PHA-activated primary human peripheral blood mononuclear cells (hPBMCs) were made quiescent and then stimulated with IL2 or IL15 (Fig. 4.1D). Both cytokines were able to induce IL2R $\beta$  T450 phosphorylation. IL2 and IL15 induced phosphorylation was detectable within 5 min of stimulation. Phosphorylation induced by IL2 returned to basal levels at

15 min. These data suggest that IL2R $\beta$  T450 phosphorylation may be important for diverse biological functions mediated by IL2 and IL15. Thus, the phosphorylation of IL2R $\beta$  T450 occurred in multiple cell types, including primary human PBMCs, with activation profiles indicating a general mechanism of IL2R $\beta$  activation.

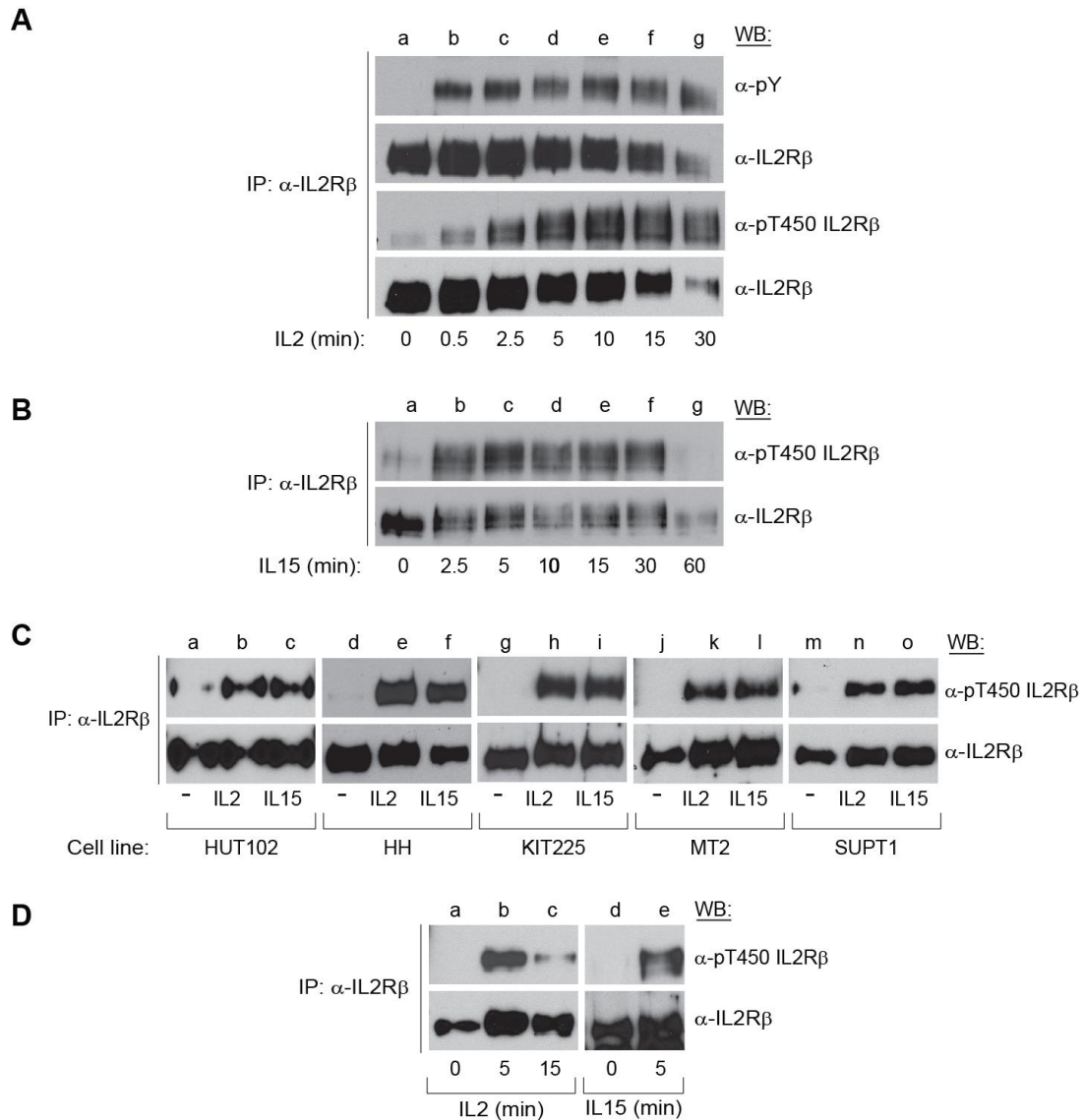
#### **4.2.2 Inhibition of JAK and MEK Kinases Disrupts IL2 Mediated T450 IL2R $\beta$ Phosphorylation**

To identify the putative kinase(s) responsible for phosphorylating IL2R $\beta$  T450, YT cells were incubated with inhibitors of candidate serine-threonine kinases prior to IL2 stimulation. The candidate kinases were chosen based on the main pathways activated by IL2: JAK/STAT, PI3K, and MEK/ERK. In addition, the intracellular domain of IL2R $\beta$  was analyzed using the PhosphoMotif Finder tool of the Human Protein Reference Database [95] for consensus serine/threonine kinase substrate motifs. Of interest were ERK1 and ERK2, which were reported to phosphorylate proline flanked threonine residues and are activated by IL2. We found that blockade of JAK activation (tofacitinib) resulted in abrogation of IL2 induced T450 phosphorylation in a dose dependent manner. In contrast, inhibition of PI3K (wortmannin) or PKA (KT5720) had no visible effect even at high doses (Fig. 4.2A). However, treatment with the highly specific MEK inhibitor trametinib, resulted in a significant loss of IL2 induced T450 phosphorylation in a dose dependent manner that corresponded with a loss of ERK1/2 activation (Fig. 4.2B). Interestingly, IL2 induced tyrosine phosphorylation of IL2R $\beta$  was unaffected by trametinib treatment, although the shift in electrophoretic mobility of the receptor reverted to its unstimulated form (Fig. 4.2B). Additionally, inhibition of ERK resulted in a reduction of phosphorylation of IL2 induced T450. Taken together, these results suggest that IL2R $\beta$  T450 phosphorylation is dependent upon IL2 induced activation of the MEK/ERK pathway.

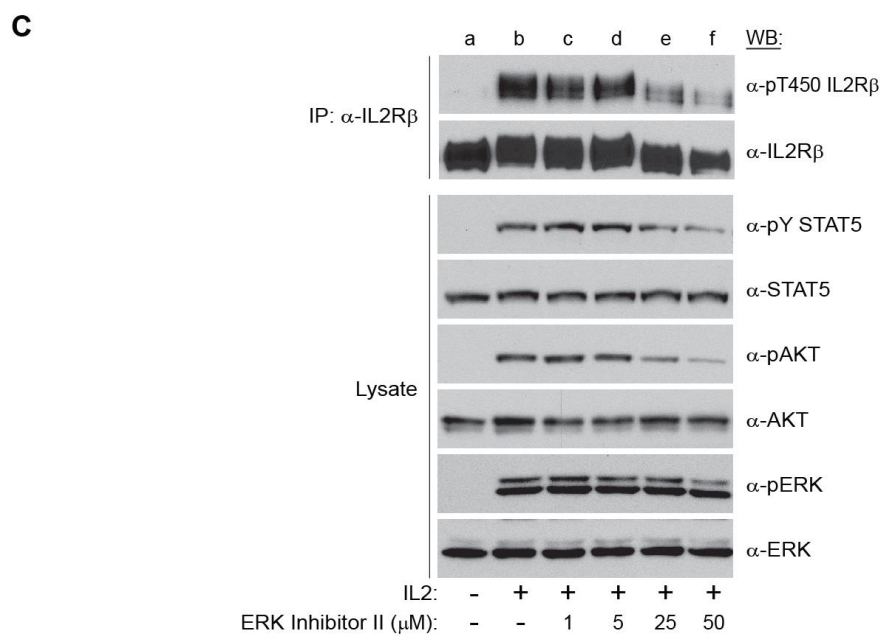
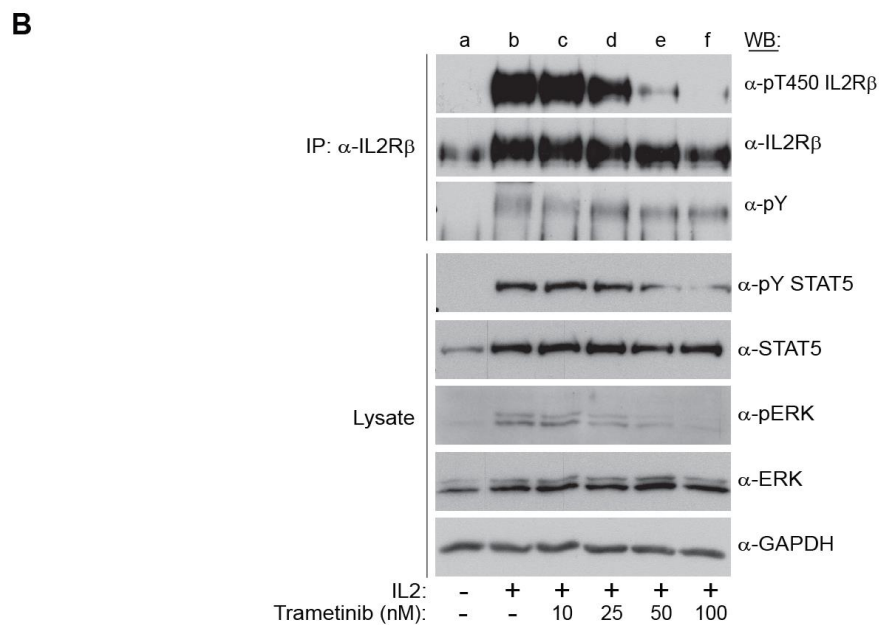
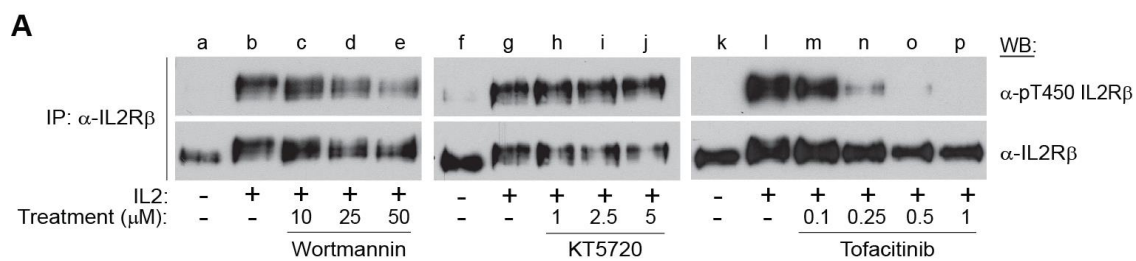
#### **4.2.3 siRNA-mediated Knockdown of ERK1 and ERK2 Inhibits IL2 Induced IL2R $\beta$ T450 Phosphorylation**

To confirm their role in phosphorylation of T450, siRNA mediated knockdown of ERK1 and ERK2 was performed in YT cells. First, increasing concentrations (100, 250, and 500 nM) of

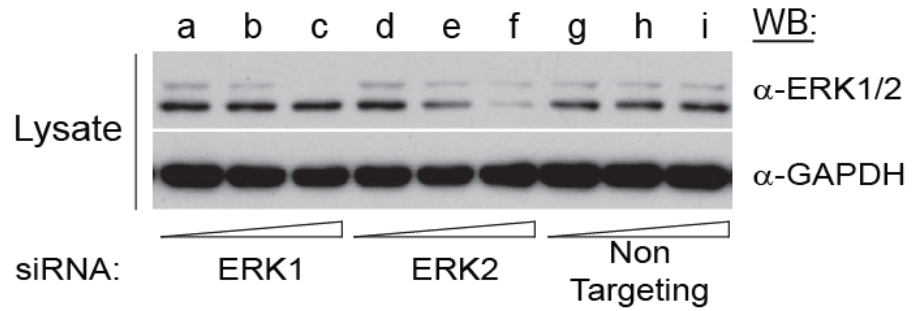
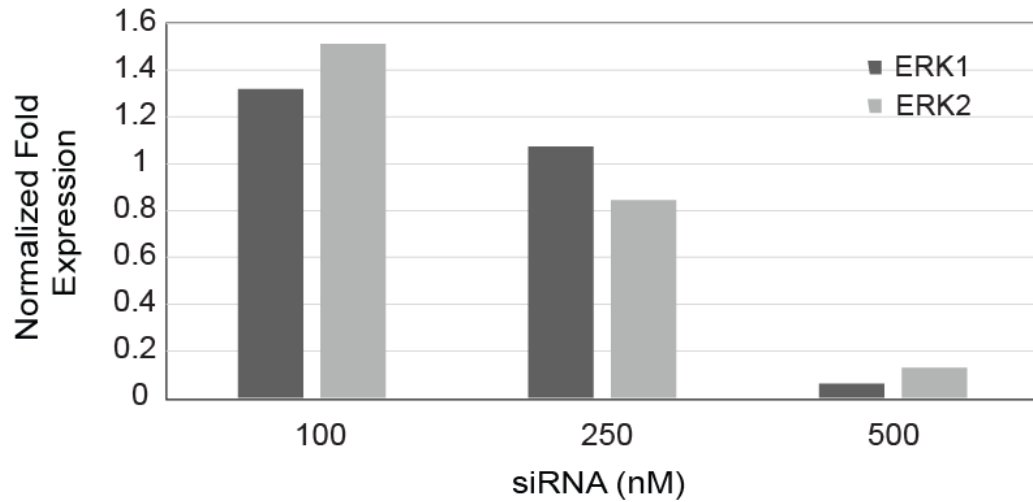
ERK1, ERK2, or non-targeting control siRNA were delivered into YT cells via electroporation in order to determine the ideal concentration for ERK1/2 knockdown. At 72 h post transfection, cells were harvested and lysates analyzed by Western blot for ERK1/2 and GAPDH. As shown in figure 4.3A, a reduction of ERK1 and ERK2 was observed with increasing concentrations of their corresponding siRNA but not with the non-targeting control. We were able to obtain about 1-fold reduction of ERK1 and ERK2 protein levels relative to GAPDH with 500 nM of siRNA (Fig. 4.3B). For the T450 phosphorylation studies, YT cells were transfected with a combination of ERK1 and ERK2 or non-targeting siRNA. Cells were stimulated with IL2 for 5 min 72 h post-transfection and phosphorylation of IL2R $\beta$  and STAT5 along with ERK1/2 knockdown was determined by Western blot analysis of total cell lysates. ERK1 and ERK2 specific siRNA significantly reduced protein levels of ERK1 and ERK2 as demonstrated in Fig. 4.4A, upper panel. Concomitantly, knockdown of both ERK1 and ERK2 correlated with the loss of IL2 induced phosphorylation of IL2R $\beta$  T450 while phosphorylation of STAT5 confirmed successful stimulation of the cells with the cytokine and total STAT5 and GAPDH confirmed equal loading of protein. Densitometric analysis of ERK1/2 expression normalized to GAPDH showed about a 0.5-fold decrease of ERK1/2 siRNA treated cells compared to non-targeting control (Fig. 4.4B lower panel) In addition, we analyzed IL2 induced pT450 signal and we observed a 0.6-fold reduction in cells transfected with a combination of ERK1/2 siRNA compared to the non-targeting control (Fig. 4.4C). These findings demonstrate that ERK1 and ERK2 expression has a direct effect on IL2 induced phosphorylation of IL2R $\beta$  T450. Additionally, the temporal relationship between IL2 induced phosphorylation of IL2R $\beta$  on tyrosine versus threonine 450 with rapid tyrosine phosphorylation and more protracted threonine phosphorylation kinetics is consistent with an activation of the IL2R and the MEK/ERK pathway prior to phosphorylation of IL2R $\beta$  T450 (Fig. 4.1A).



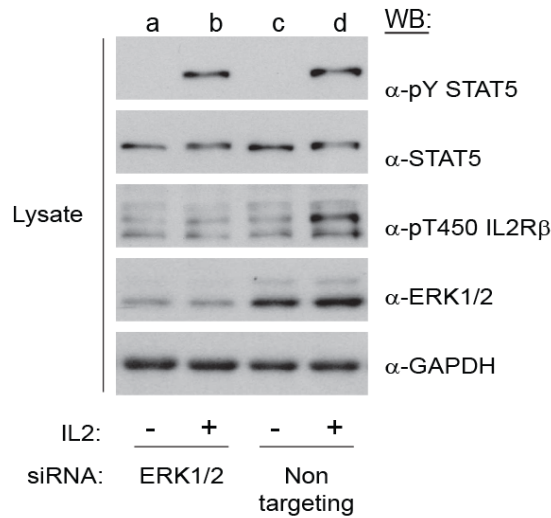
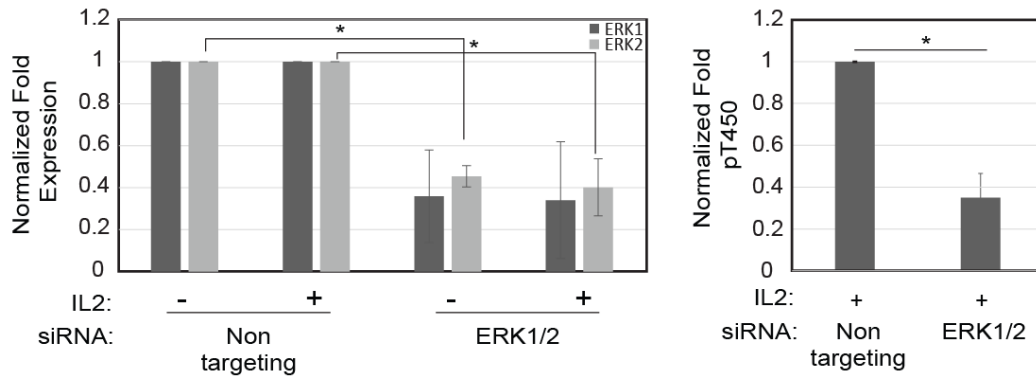
**FIGURE 4.1: Phosphorylation of T450 IL2R $\beta$  displays rapid kinetics and is inducible by IL2 and IL15 in several malignant hematopoietic cell lines as well as in non-tumorigenic primary lymphocytes.** T cells were grown to exhaustion and then stimulated with IL2 ( $10^4$  IU) (A) or IL15 (0.49 mg/mL) (B) for the indicated times. C, The indicated cell lines were grown to exhaustion and then stimulated for 5 min. D, quiescent PHA-activated human PBMCs were stimulated with IL2 and IL15 and the cells were harvested at the indicated time points. IL2R $\beta$  was immunoprecipitated (IP), separated by SDS-PAGE, and immunoblotted (IB) as indicated. Representative data from three independent experiments are shown.



**FIGURE 4.2: Inhibition of JAK, MEK, and ERK kinases inhibits IL2 mediated T450 IL2R $\beta$  phosphorylation.** YT (*A*) cells were grown to exhaustion, treated with increasing concentrations of the indicated inhibitors for 60 min and then stimulated with or without IL2 ( $10^3$  IU) for 5 min. Cell lysates or immunoprecipitated (*IP*) IL2R $\beta$  were separated by SDS-PAGE and immunoblotted (*WB*) as indicated.

**A****B**

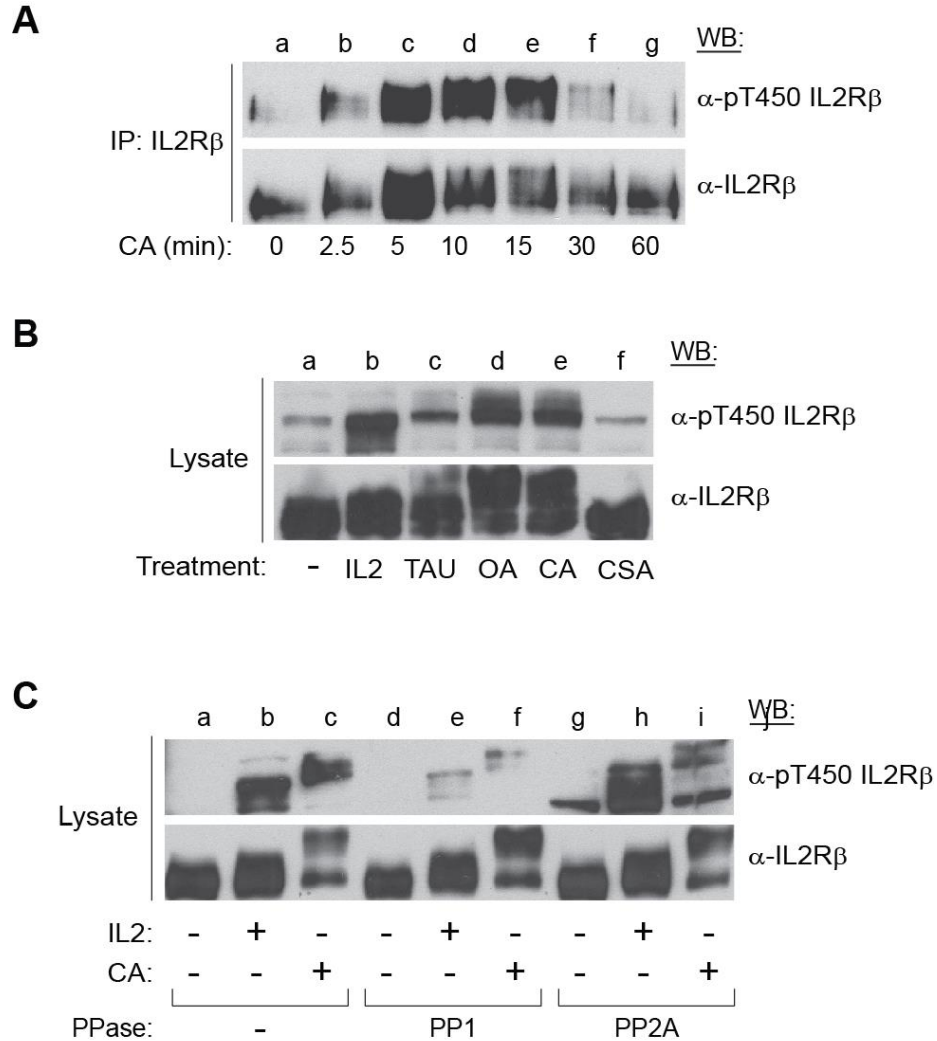
**Figure 4.3: siRNA mediated silencing of ERK1 or ERK2 in YT cells.** *A*, YT ( $5 \times 10^6$ ) cells were nucleofected with ERK1-specific siRNA, ERK2-specific siRNA, or non-targeting control siRNA (100-500 nM). 72 h post-nucleofection cells were harvested, cell lysates (10 mg) separated by SDS-PAGE and analyzed by Western blot (*WB*) as indicated. Band intensities were normalized to GAPDH using densitometric analysis and fold decrease plotted for each treatment.

**A****B**

**FIGURE 4.4: Knockdown of ERK1/2 kinases inhibits IL2 mediated T450 IL2Rβ phosphorylation.** *A*, YT ( $5 \times 10^6$ ) cells were nucleofected with ERK1-specific siRNA (750nM), ERK2-specific siRNA (1mM), a combination of both ERK1 and ERK2-specific siRNA or with a non-targeting control siRNA (1.75mM). At 72 h post-nucleofection cells were harvested and incubated with or without IL2 ( $10^3$  IU) for 5 min at 37 °C. Cell lysates (10 mg) were separated by SDS-PAGE, and immunoblotted (*WB*) as indicated. Representative data from three independent experiments are shown. Band intensities were normalized to GAPDH using densitometric analysis and fold decrease plotted for each treatment. Values represent the mean  $\pm$  S.D of ERK1/2 expression or T450 phosphorylation signal. Statistical significance was determined using Student's *t* test. \*,  $p < 0.05$ .

#### 4.2.4 PP1 Negatively Regulates Phosphorylation of IL2R $\beta$ T450

Our group has shown that inhibition of PP1 and PP2A with calyculin A induces electrophoretic mobility shift in IL2R $\beta$  characteristic of phosphorylated proteins [56]. In the present study, mass spectrometry analysis of IL2R $\beta$  revealed T450 to be phosphorylated in CA treated samples. To characterize the effect of PP1 and PP2A inhibition on T450 phosphorylation, a kinetic analysis of CA treatment was performed. YT cells were treated with 100 nM CA for the indicated times. IL2R $\beta$  was immunoprecipitated from soluble cell lysates, separated by SDS-PAGE, and subjected to Western blot analysis with  $\alpha$ -pT450 IL2R $\beta$  antibody (Fig. 4.5A). Consistent with our mass spectrometry data, CA treatment induces phosphorylation of IL2R $\beta$  T450 with rapid kinetics. Phosphorylation of T450 was observed as early as 5 min, the signal was sustained until 15 min and returned to basal levels after 60 minutes post-stimulation. The membrane was stripped and reprobed for total IL2R $\beta$  to ensure equal gel loading. To elucidate the primary phosphatase responsible for regulation of IL2R $\beta$  T450 phosphorylation, inhibitors with opposing specificity toward PP1 or PP2A were investigated. For this analysis, YT cells were left untreated, stimulated with IL2, or incubated with inhibitors of PP1 (1  $\mu$ M TAU), PP2A (250 nM OA), PP1/PP2A (100 nM CA), or PP2B (250 nM CSA). Cell lysates were separated by SDS-PAGE and phosphorylation of IL2R $\beta$  T450 analyzed by Western blot (Fig. 4.5B). Indeed, stimulation with IL2 and treatment with OA, CA, and to a lesser extent TAU, but not CSA, resulted in phosphorylation of IL2R $\beta$  T450. To further differentiate the role of PP2A from PP1 in the regulation of T450 phosphorylation, an *in vitro* phosphatase assay was performed. YT cells were left untreated, stimulated with IL2, or treated with 100 nM CA for 15 min. Cell lysates were subjected to *in vitro* dephosphorylation using purified PP1 or PP2A enzymes prior to SDS-PAGE and Western blot analysis. As shown in Fig. 4.5C, dephosphorylation using purified PP1, but not PP2A, reversed the IL2 and CA induced phosphorylation of T450. Taken together, these data indicate that inhibition of PP1 but not PP2A mediates dephosphorylation of IL2R $\beta$  T450 *in vivo* and that PP1 directly dephosphorylates it *in vitro*.



**Figure 4.5: PP1 regulates IL2R $\beta$  T450 phosphorylation.** *A*, YT cells were left untreated or incubated with CA (100 nM), lysed, IL2R $\beta$  immunoprecipitated (IP) and analyzed through Western blot (WB) using  $\alpha$ -pT450 IL2R $\beta$ . The membrane was stripped and reblotted for total IL2R $\beta$  as indicated. *B*, YT cells were left untreated, stimulated with IL2 ( $10^4$  IU) for 5 min, treated with TAU (1 mM), OA (250 nM), CSA (250 nM) for 1 h, or CA (100 nM) for 15 min. Cell lysates were separated by SDS-PAGE, and Western blotted (WB) as indicated. *C*, YT cells were incubated in the absence or presence of IL2 ( $10^4$  IU) for 5 min, or CA (100 nM) for 15 min. Cells lysates were left untreated or subjected to dephosphorylation using 0.5 units of purified PP1 or PP2A for 60 min at 37 °C before separation by SDS-PAGE and Western blot analysis as indicated (WB).

## 4.2 DISCUSSION

Serine and threonine phosphorylation have been found to elicit positive and negative regulatory effects on signal transduction [96], hence the importance to have a better understanding of its functional role in IL2 signaling. In this chapter, the phosphorylation of a specific threonine residue (T450) in IL2R $\beta$  in response to stimuli by IL2 and IL15 was assessed. The regulatory pathway governing the phosphorylation and dephosphorylation of such residue in order to describe the mechanism behind this phenomenon was also investigated. The strategy employed consisted of cell stimulation with IL2 or IL15 and assessment of phosphorylation by Western blot using our novel phosphospecific antibody  $\alpha$ -pT450 IL2R $\beta$ . To elucidate the regulators of IL2R $\beta$  T450 phosphorylation we used commercially available inhibitors of several kinases activated by IL2 coupled with siRNA knockdown of our main candidate ERK1/2. In addition, inhibitors to different protein phosphatases revealed two candidates for the regulation of T450 phosphorylation, which was further specified by *in vitro* dephosphorylation using purified phosphatases.

Tyrosine phosphorylation of IL2R $\beta$  is mediated by JAKs and has been proven to be essential for IL2 signal transduction while serine and threonine phosphorylation of the receptor has only been recently reported to be an important negative regulator of such signaling [56]. Here we found that IL2R $\beta$  is phosphorylated at T450 in response to both IL2 and IL15 in several malignant hematopoietic cell lines. In addition, T450 is phosphorylated in non-tumorigenic primary lymphocytes when stimulated with IL2 or IL15. We also found that treatment with the highly specific MEK inhibitor trametinib, resulted in loss of IL2 induced T450 phosphorylation in a dose dependent manner that corresponded to loss of ERK1/2 activation (Fig. 4.2B). siRNA mediated knockdown of ERK1 and ERK2 correlated with the loss of IL2 induced phosphorylation of IL2R $\beta$  T450, confirming thus a role for these kinases in such phosphorylation. In addition, treatment with OA, CA, and to a lesser extent TAU, but not CSA resulted in phosphorylation of IL2R $\beta$  T450. An *in vitro* dephosphorylation revealed that PP1 was able to reverse IL2 and CA induced phosphorylation of T450.

In summary, phosphorylation of T450 is induced by IL2 and IL15 with rapid kinetics attaining peak levels at 2-10 min. In addition, IL2R $\beta$  T450 phosphorylation is dependent upon IL2 induced activation of the MEK/ERK pathway and ERK1 and ERK2 expression has a direct effect on IL2 induced phosphorylation of IL2R $\beta$  T450 while inhibition of PP1 mediates dephosphorylation of IL2R $\beta$  T450 *in vivo* and directly dephosphorylates it *in vitro*. Taken together, these data suggest that IL2R $\beta$  T450 phosphorylation may be important for diverse biological functions mediated by IL2 and IL15 with activation profiles indicating a general mechanism of IL2R $\beta$  activation. Identifying the functional role of IL2R $\beta$  phosphorylation will take us one step closer to understanding IL2 signaling and is the subject of the next chapter.

## **CHAPTER 5**

### **IL2R $\beta$ T450 Phosphorylation Modulates IL2 Signaling and STAT5 Transcriptional Activity**

## 5.1 INTRODUCTION

IL2 induced tyrosine phosphorylation of IL2R $\beta$  allows for recruitment and docking of different proteins to become tyrosine, serine, and threonine phosphorylated. It has been proven to be an important mechanism of activation of JAK/STAT, PI3K and MEK/ERK signaling pathways and a vehicle for cell proliferation. Threonine phosphorylation of the receptor on the other hand has only been recently described. Two sites, T79 and T256 were reported to be phosphorylated through proteomic discovery-mode mass spectrometry [83] while our group confirmed through phosphoamino acid analysis threonine phosphorylation in response to IL2 stimulation and CA treatment [56]. To our knowledge, there are no reports of site specific methods of identification of threonine phosphosites on IL2R $\beta$ , much less characterization and description of functional role. In this part of our study, we sought to investigate the functional role of IL2R $\beta$  T450 phosphorylation. Ross et al. reported that serine and threonine phosphorylation appear to act as negative regulators for IL2 signaling. Blocking the serine/threonine phosphatase PP2A increased serine/threonine phosphorylation and reduced IL2 induced tyrosine phosphorylation of the  $\beta$  subunit [56]. Here we provide evidence of T450 phosphorylation in response to IL2 and IL15 which was reduced by blocking ERK1/2 activation using the MEK specific inhibitor trametinib or the ERK specific inhibitor FR18024 (ERK inhibitor II). IL2 induced tyrosine phosphorylation of the receptor was found to be independent of T450 phosphorylation since blocking the latter did not have a noticeable effect on the former (Fig. 4.1), which lead us to believe T450 phosphorylation might play a positive regulatory role on IL2 signal transduction.

In order to determine the role of IL2 induced IL2R $\beta$  T450, a HEK293 reconstitution system was employed using either WT or a phospho-deletion (T450A) mutation of IL2R $\beta$ . We assessed the effect of IL2 induced T450 phosphorylation in the assembly of the IL2R complex and the activation of key signaling molecules by immunoprecipitation and Western blot analysis. In addition, we used a STAT5B luciferase reporter assay in order to assess putative transcriptional activity differences caused by the phospho-deletion mutant compared to WT. We show that

phosphorylation of T450 is important for IL2R complex formation and full activation of IL2 signaling which leads to impaired STAT5B transcriptional activity.

## **5.2 MATERIALS AND METHODS**

### **5.2.1 Plasmids, Site-directed Mutagenesis, and Transfection of HEK293 Cells**

The pcDNA3.1/GS human IL2R $\beta$  and IL2R $\gamma$  expression plasmids were purchased from Invitrogen. The pcDNA3.1 human JAK3 was obtained as previously described [97]. STAT5b cDNA (OriGene) was generated as previously described [86]. The  $\beta$ -casein-luciferase reporter plasmid was used as reported previously [89]. The pCMV- $\beta$ -galactosidase expression plasmid is described in [98]. Mutant forms of IL2R $\beta$  were prepared using the QuikChange site-directed mutagenesis kit (Stratagene) according to the manufacturer's instructions. The primers used for the IL2R $\beta$  mutation were as follows: T450A (5'- TGGGGCCTCCCGCCCCAGGAGTC -3'); T450E (5'- CCCTGGGGCCTCCCGAGCCAGGAGTCCCAGA -3'). All subclones and mutations were verified by DNA sequencing at the Genomic Analysis Core Facility of the Border Biomedical Research Center at The University of Texas at El Paso. Transient transfections of HEK293 cells were performed with Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions.

### **5.2.2 Solubilization of Proteins, Immunoprecipitation, and Western Blot Analysis**

Cells were pelleted, lysed and subjected to immunoprecipitation and Western blot analysis as described in section 2.2.2. For reblotting, membranes were incubated with stripping buffer as described in section 3.2.5. Immunoprecipitation was performed using the  $\alpha$ -IL2R $\beta$  monoclonal antibody 561 [91]. The anti-phospho-Tyr<sup>694</sup> STAT5a and Tyr<sup>699</sup> STAT5b (pYSTAT5) monoclonal antibody,  $\alpha$ -STAT5 polyclonal antibody,  $\alpha$ -phospho-p44/42 MAPK (ERK1/2) Thr<sup>202</sup>/Tyr<sup>204</sup> polyclonal antibody,  $\alpha$ -p44/42 MAPK (ERK1/2) polyclonal antibody,  $\alpha$ -phospho-AKT and  $\alpha$ -AKT antibodies were purchased from Cell Signaling Technology and were used according to the manufacturer's protocol along with anti-phosphotyrosine monoclonal 4G10 ( $\alpha$ -pY) antibody

(Upstate Biotechnology), anti-GAPDH monoclonal antibody (Fitzgerald), and the anti-IL2R $\beta$ /anti-IL2R $\gamma$  chain antibodies (Santa Cruz Biotechnology).

### **5.2.3 Luciferase/ $\beta$ -galactosidase**

Subconfluent HEK293 cells in 10-cm dishes were transfected with the following plasmids: WT or mutant IL2R $\beta$  (6 $\mu$ g),  $\gamma_c$  (6  $\mu$ g), JAK3 (0.5  $\mu$ g), STAT5B (3  $\mu$ g),  $\beta$ -casein-luciferase reporter (3  $\mu$ g), and pCMV- $\beta$ -gal (1  $\mu$ g). Cells were stimulated with or without 10<sup>4</sup> IU/ml IL2, transferred to 96-well plates (10,000 cells per well) and incubated for 3 h at 37 °C. Luciferase and  $\beta$ -gal activities were measured using ONE-Glo Luciferase and Beta-Glo Assay systems (Promega) respectively following the manufacturer's instructions.

## **5.3 RESULTS**

### **5.3.1 Phosphorylation of T450 is Required for Optimal IL2 Receptor Complex Formation**

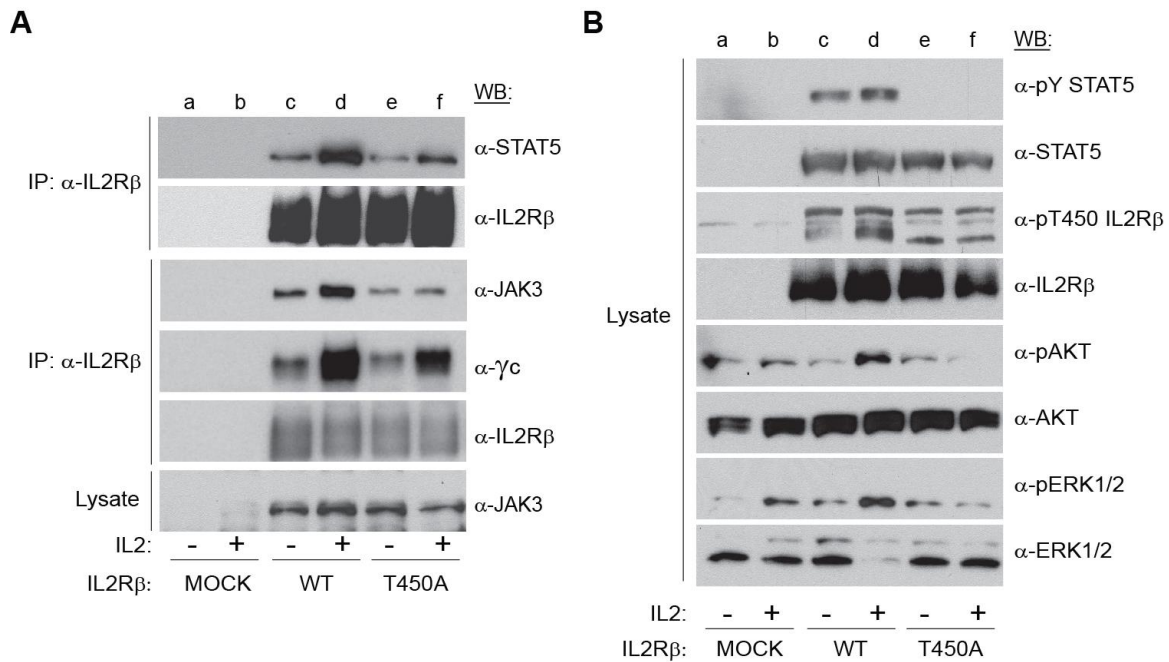
IL2 promotes the formation of a heterotrimeric receptor complex that consists of two essential subunits, IL2R $\beta$ , the  $\gamma_c$ , and the affinity modulating subunit  $\alpha$ . Failure in the assembly of IL2R $\beta$  and  $\gamma_c$  upon stimulation of IL2 results in the blockade of downstream signaling components [99] [100] [56]. Previous work from our group had shown that CA induced serine and threonine phosphorylation disrupted the IL2 induced IL2R complex formation and activation of downstream targets [56]. To determine the role of IL2 induced IL2R $\beta$  T450 phosphorylation in the assembly of the IL2R complex, a HEK293 reconstitution system was employed using phospho-deletion (T450A) mutation of IL2R $\beta$ . Plasmids encoding  $\gamma_c$ , JAK3, and STAT5B were co-transfected into HEK293 cells with WT or T450A forms of IL2R $\beta$ . At 48 h after transfection, cells were made quiescent and then left untreated or stimulated with IL2 for 10 min. The IL2R $\beta$  subunit was immunoprecipitated from soluble lysates and probed for the association of  $\gamma_c$ , JAK3 and STAT5B by Western blot analysis. IL2 stimulation resulted in co-immunoprecipitation of JAK3, STAT5B and  $\gamma_c$  with WT IL2R $\beta$ . Importantly, IL2 induced association of JAK3, STAT5 and  $\gamma_c$  with the T450A mutated  $\beta$  was reduced as shown in the co-immunoprecipitation studies (Fig. 5.1A). To further investigate the effects of the T450 phospho-deletion mutant on IL2 signaling, we analyzed

whole cell lysates for activation of key proteins of each of the IL2 induced pathways: JAK/STAT, PI3K, and MEK/ERK (Fig. 5.1B). We found constitutive STAT5 tyrosine phosphorylation in cells transfected with  $\gamma_c$ , JAK3, STAT5 and WT IL2R $\beta$ . However, such phosphorylation appears to be slightly induced by IL2 stimulation. Importantly, tyrosine phosphorylation of STAT5 was lost in cells transfected with the phospho-deletion mutant (T450A). Activation of the PI3K pathway was assessed in terms of phosphorylation of its downstream target AKT. IL2 induced phosphorylation of AKT was observed in cells transfected with WT but not in cells expressing the T450A mutant.

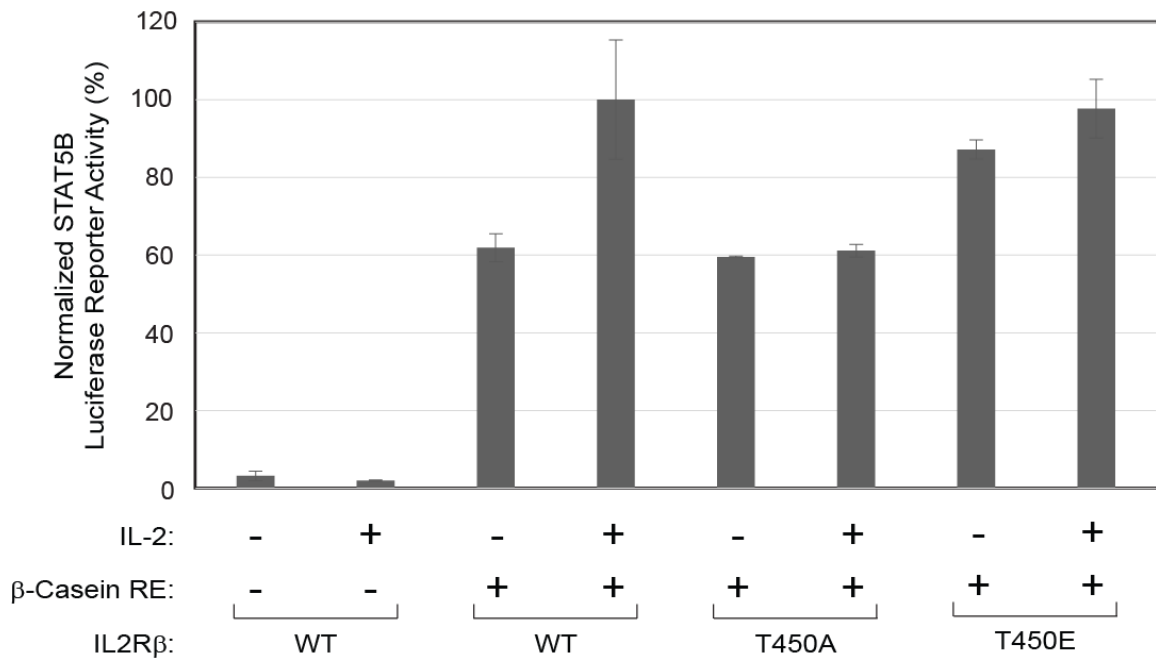
Similarly, IL2 induced phosphorylation of ERK1/2 was detected in cells transfected with the WT receptor, but not with the phospho-deletion mutant. These results suggest that phosphorylation of T450 is required for the assembly of the IL2R $\beta$  complex in addition to regulating the phosphorylation of the downstream signaling molecules STAT5, AKT, and ERK1/2.

### **5.3.2 Phosphorylation of T450 is Important for Maximum IL2 Induced STAT5B Transcriptional Activity**

STAT5A and STAT5B transcription factors become activated in response to several growth factors and cytokines, including IL2, which induces tyrosine phosphorylation of the receptor and provides docking sites for STATs to bind through their SH2 domains. Bound STATs become activated through tyrosine and serine phosphorylation, form dimers, and translocate to the nucleus to activate genes related to differentiation and proliferation of cells (31). To determine if phosphorylation of IL-2R $\beta$  T450 is important for STAT5B transcriptional activity in vivo, luciferase reporter assays were performed using the HEK293 reconstitution system. Plasmids encoding  $\gamma_c$ , JAK3, and STAT5B were co-transfected into HEK293 cells with WT, T450A, or T450E forms of IL-2R $\beta$ , along with a  $\beta$ -casein-firefly luciferase reporter, and a  $\beta$ -gal reporter construct. At 48 h post-transfection, cells were made quiescent and then left untreated or stimulated with IL-2 for 3 h. Luciferase activity was normalized to  $\beta$ -gal activity and change calculated in reference to IL-2 stimulated WT IL-2R $\beta$  samples. Upon IL-2 stimulation, the IL-2R $\beta$  T450A



**Figure 5.1: IL2R $\beta$  T450 phosphorylation is a positive regulator for IL2 complex stability and activation of signaling molecules in reconstituted HEK293 cells.** *A*, HEK293 cells were transfected with  $\gamma_c$ , JAK3, STAT5B and IL2R $\beta$  (WT or T450A), incubated for 48 h in complete media, followed by incubation 48 h in 1% FBS media. The cells were then stimulated without (-) or with IL-2 (+) for 10 min, cells were lysed, the IL-2R $\beta$  was immunoprecipitated (IP) before separation by SDS-PAGE and Western blot analysis as indicated (IB). Total cell lysate (10mg) was also probed (*B*) by Western blot with the indicated antibodies.



**Figure 5.2: IL2R $\beta$  T450 phosphorylation is required for maximal IL2 induced STAT5B transcriptional activity.** HEK293 cells were transfected with IL2R $\beta$  (WT or T450A),  $\gamma_c$ , JAK3, STAT5B,  $\beta$ -casein-luciferase, and pCMV- $\beta$ -galactosidase. 48 h post-transfection, cells were incubated with or without IL2 for 3 h and luciferase activity measured and normalized to  $\beta$ -gal activity. Each treatment was performed in triplicates. Results are presented as the mean  $\pm$  S.D.

mutant showed a 40% decrease in STAT5B luciferase reporter activity relative to WT IL-2R $\beta$  (Fig. 5.2). Additionally, we found that the phospho-mimetic mutation (T450E) displayed transcriptional activity comparable to wild type. These data suggests that IL-2R $\beta$  T450 phosphorylation is important for effective transduction of IL-2 signal and STAT5B transcriptional activity.

## 5.4 DISCUSSION

In an attempt to determine the functional role of phosphorylation of IL2R $\beta$  T450, we reconstituted the IL2R system in HEK293 cells. We assessed the differences in response to IL2 of the WT receptor versus the T450A mutant by co-immunoprecipitation and Western blot analysis. In addition, STAT5B transcriptional activity in response to IL2 signaling through the WT receptor versus the phospho-deletion mutant was assessed by using a luciferase reporter assay.

T450 is located in the H region of the IL2R $\beta$ , which also contains Y392 and Y510. Upon stimulation with IL2, these tyrosines become phosphorylated and serve as docking sites for STAT5 [20] [37]. Studies in mice showed that removal of the H region results in a lack of NK cells,  $\gamma\delta$  T cells, and T cells that fail to respond to low concentrations of IL2, which results in downregulation of IL2R $\alpha$  [40]. By using IL2R $\beta$  chimeras it was later shown that although STAT5 is able to become activated, deleting the region between Y392 and Y510, where T450 is located, abolishes expression of IL2R $\alpha$  [42]. This suggests a positive regulatory role for that region of the receptor.

In line with those studies, we found a putative positive regulatory role for the phosphorylation of T450. It appears to be important for maximal IL2 induced IL2R complex formation and activation of the downstream signaling molecules STAT5B, AKT, and ERK1/2. Such impaired activation coincides with the observed decrease in STAT5B transcriptional activity as demonstrated in the luciferase reporter assay. Taken together, this data provides evidence that in addition to tyrosine phosphorylation, threonine phosphorylation of IL2R $\beta$  regulates IL2 signal transduction.

## **CHAPTER 6**

### **Overview and Future Directions**

## 6.1 OVERVIEW

Homeostasis of the immune system is required for proper defense against pathogenic insult. IL2 is essential to maintain homeostasis and its signaling provides activation of T, B, and NK cells. IL2 signal transduction occurs through the IL2R, which becomes tyrosine phosphorylated upon induction with the cytokine, providing a site for binding and activation of downstream molecules. Serine and threonine phosphorylation of the receptor has been shown to occur upon inhibition of the serine/threonine phosphatases PP1 and PP2A. Such phosphorylation was implicated in negative regulation of IL2 signaling and formation of the IL2R complex. We sought to investigate the putative regulatory role that specific serines and threonine have in IL2 signaling.

The first objective of this dissertation was to map the serine and threonine phosphorylation sites on IL2R $\beta$ . The strategy of large-scale immunoprecipitation of  $\beta$  followed by identification of phosphopeptides through LC-MS/MS revealed thirteen sites to be differentially phosphorylated in IL2R $\beta$  in response to diverse stimuli (Figures 2.1, 2.2, 2.3 and Table 2.1). **We identified S405 and T450 to become phosphorylated in response to IL2 stimulation. In addition, serines 267, 268, 432, 484, and 512 as well as threonine 256, 308, 394 and 450 were found to be phosphorylated with CA treatment.** The novel IL2 inducible threonine 450 and serine 512 were chosen for further examination and amino acid sequence alignment of the region surrounding each of the phosphosites from different organisms revealed conservation among different species. Interesting, S512 was found to be more highly conserved than T450. Thus, we identified a novel IL2 inducible threonine phosphorylation site in human IL2R $\beta$ .

In order to verify that IL2R $\beta$  is phosphorylated at T450 and S512 and to investigate the regulatory role of this phosphorylation sites, phospho-specific polyclonal antibodies were generated. The strategy for characterization of the antibodies including ELISA, SPR, dot blot, and Western blot of immunoprecipitated IL2R $\beta$  revealed that both  $\alpha$ -pT450 and  $\alpha$ -pS512 IL2R $\beta$  antisera preferentially recognize their phosphorylated peptide; they show higher binding response, stronger binding kinetics and functional affinity to and are specifically blocked by phospho- but not non-phosphopeptide. In addition, both  $\alpha$ -pT450 and  $\alpha$ -pS512 IL2R $\beta$  proved to be useful for

western blot analysis of immunoprecipitated protein and total cell lysates, which provided us with an excellent tool for the characterization of our phosphosites.

In the second part of our study, we assessed phosphorylation of T450 in IL2R $\beta$  in response to stimuli by IL2 and IL15. We also investigated the regulatory pathway governing the phosphorylation and dephosphorylation of such residue. The strategy employed consisted on stimulation of cells with IL2 or IL15 and assessment of phosphorylation through Western blot using our phosphospecific antibody  $\alpha$ -pT450 IL2R $\beta$ . To elucidate the regulators of IL2R $\beta$  T450 phosphorylation we used commercially available inhibitors of several kinases activated by IL2 coupled to siRNA knockdown of our main candidate ERK1/2. In addition, inhibitors to different protein phosphatases revealed two candidates for the regulation of T450 phosphorylation, which was further specified by *in vitro* dephosphorylation using purified phosphatases. We found that **IL2R $\beta$  is phosphorylated at T450 in response to both IL2 and IL15 in several malignant hematopoietic cell lines and in non-tumorigenic primary lymphocytes.** We also found that treatment with the specific MEK inhibitor trametinib, resulted in loss of IL2 induced T450 phosphorylation in a dose dependent manner that corresponded to loss of ERK1/2 activation (Fig. 4.2B). Inhibition of ERK by FR180204 also resulted in loss of IL2 induced T450 phosphorylation. **siRNA mediated knockdown of ERK1 and ERK2 inhibited IL2 induced IL2R $\beta$  T450 phosphorylation.** To further delineate the regulation of T450 phosphorylation, cells were treated with inhibitors against protein phosphatases and the data indicate that inhibition of **PP1 but not PP2A or PP2B induces phosphorylation of IL2R $\beta$  T450 *in vivo* and that PP1 directly dephosphorylates it *in vitro*.**

Taken together, these data suggest that IL2R $\beta$  T450 phosphorylation may be important for diverse biological functions mediated by IL2 and IL15 with activation profiles indicating a general mechanism of IL2R $\beta$  activation. To determine the functional role of phosphorylation of IL2R $\beta$  T450, we reconstituted the IL2R system in HEK293 cells and assessed the differences in response to IL2 of the WT receptor versus the T450A mutant by co-immunoprecipitation and Western blot analysis. In addition, STAT5B transcriptional activity in response to IL2 signaling through the WT

receptor versus the phospho-deletion and phospho-mimetic mutant was assessed by using a luciferase reporter assay. We found that **phosphorylation of IL2R $\beta$  T450 is important for maximum IL2 induced IL2R complex formation and activation of the downstream signaling molecules STAT5B, AKT, and ERK1/2**. In addition, we observed a decrease in STAT5B transcriptional activity as demonstrated in the luciferase reporter assay. Taken together, this data provides evidence that in addition to tyrosine phosphorylation, **threonine phosphorylation of IL2R $\beta$  regulates IL2 signal transduction**.

In conclusion, we were able to identify novel phosphosites in IL2R $\beta$  and successfully generated and characterized polyclonal antibodies for two of those sites. The novel phosphosite T450 is IL2 and IL15 inducible and regulated by ERK and PP1. Phosphorylation of T450 is important for IL2R complex formation and effective IL2 signal transduction which influences STAT5 transcription activity.

## **6.2 FUTURE DIRECTIONS**

### **Determine involvement of T450 phosphorylation in disease**

Dysregulated phosphorylation of proteins plays a critical role in several cancers, including hematopoietic malignancies. Therefore, the T450 phosphorylation status could be explored in leukemia and lymphoma patient samples as well as samples from patients with autoimmune diseases like lupus and rheumatoid arthritis. Cell lysates from these samples could be assessed for phosphorylation of T450 by Western blot using our novel phosphospecific antibodies.

### **Comparative analysis of IL2R $\beta$ T450 and tyrosine phosphorylation**

Analysis of downstream effects of T450 compared to tyrosine phosphorylation of the  $\beta$  subunit of the receptor can be performed using threonine and tyrosine phospho-deletion mutants. Tyrosines 338, 392, and 510 could be mutated to phenylalanine alone, or in combination with a T450A mutant and its effects compared to each other or to wild type.

### **Investigate the functional role of S512 phosphorylation on IL2 signal transduction**

Regulation of IL2 signaling has yet to be explored for S512. Studies performed indicate that S512 does not become phosphorylated with IL2 or IL15 stimulation, however treatment with CA does result in phosphorylation of the site. Crosstalk between IL2R $\beta$  and the T-cell receptor could be explored by assessing S512 phosphorylation during T-cell activation. In addition, phosphorylation of this site in response to other stimulants including G-protein coupled receptor agonists could be explored.

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## Abbreviations

AP-1, activator protein-1; APC, antigen presenting cell; Bap37, B cell receptor associated protein of 37 kDa; ConA, concanavalin A; C-Raf, Ras activated factor C; CsA, cyclosporin A; DAG, diacylglycerol; Foxp3, forkhead box protein 3; Grb2, growth factor receptor bound protein 2; hT, human T cell; HTLV, human T cell leukemia virus; IFN- $\gamma$ , interferon gamma; Ig, immunoglobulin; IL, interleukin; IP3, inositol 1,4,5-trisphosphate; ITAM, immunoreceptor tyrosine kinase activation motif; JAK, Janus tyrosine kinase; LAT, linker of activated T cells; Lck, lymphocyte specific protein tyrosine kinase; MHC, major histocompatibility complex; MS, mass spectrometry; MW, molecular weight; NCBI, National Center for Biotechnology Information; NFAT, nuclear factor of activated T cells; NF- $\kappa$ B, nuclear factor  $\kappa$ -B; p53, protein 53; PBMC, peripheral blood mononuclear cells; PHA, phytohemagglutinin; PIP2, phosphoinositol 4,5 biphosphate; PKC, protein kinase C; PMA, phorbol 12-myristate 13-acetate; PTB, phosphotyrosine binding; IL2, Interleukin 2; IL2R, IL2 receptor; NK, natural killer; PBMC, peripheral blood mononuclear cells; SCID, severe combined immunodeficiency; SLE, systemic lupus erythematosus; PP2, protein phosphatase; IP, immunoprecipitation; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; LC-MS/MS, liquid chromatography tandem mass spectrometry; FBS, fetal bovine serum; IU, international unit; TCR, T cell receptor; MHC, major histocompatibility complex; APC, antigen presenting cells; CD, cluster of differentiation; BCR, B cell receptor; ITAMs, immunoreceptor tyrosine-based activation motifs; Ig, immunoglobulin; AICD, activation induced cell death; NFAT, nuclear factor of activated T-cells; AP-1, activating protein 1; FOXP3, forkhead box P3;  $K_d$ , dissociation constant; AA, amino acids; PTB, phosphotyrosine-binding domains; SHC, SRC homology 2 domain containing; SRC, sarcoma; X-SCID, X-linked SCID; JH, JAK homology; SH2, SRC homology 2; CDK, cyclin-dependent kinase; FasL, Fas ligand; SOCS, suppressor of cytokine signaling; PTPs, protein tyrosine

phosphatases; CIS, cytokine inducible SH2 domain protein; PIAS, protein inhibitor of activated STATs; GRB2, Growth Factor Receptor-bound Protein 2; MAPK, mitogen activated protein kinase; ERK, extracellular signal regulated protein kinase; MNK, MAP kinase-interacting serine/threonine kinase; RKIP, RAF kinase inhibitor protein; PI3K, phosphatidylinositol 3-kinase; PIP2, phosphatidylinositol-4,5-diphosphate; PIP3, phosphatidylinositol-3,4,5-triphosphate; PDK1, phosphoinositide dependent protein kinase 1; CHK1, Checkpoint Kinase 1; MDM2, murine double minute; BAD, BCL-2 Associated Death Promoter; FOXO, Forkhead Box O; TSC2, Tuberous Sclerosis Complex 2; MTOR, mammalian target of rapamycin; PTEN, phosphatase and tensin homologue deleted on chromosome 10; SHIP, SH2 containing inositol phosphatase; CTMP, carboxyl terminal modulating protein; LCK, lymphocyte-specific protein tyrosine kinase; SYK, spleen tyrosine kinase; HTLV-1, Human T-cell leukemia virus type 1; PKA, protein kinase A; LC-MS/MS, liquid chromatography tandem mass spectrometry; PVDF, polyvinylidene difluoride; PCR, polymerase chain reaction; ELISA, enzyme-linked immunosorbent assay; SPR, surface plasmon resonance.

## **Vita**

Blanca E. Ruiz Medina earned her Bachelor of Science degree in Microbiology from The University of Texas at El Paso (UTEP) in 2004. She went on to work in industry and in 2010 joined the doctoral program in the Department of Biological Sciences with an emphasis on cancer.

While pursuing her degree, Dr. Ruiz worked as a teaching assistant and then research assistant in the Department of Biological Sciences. She was a guest speaker in the Biology Senior Seminar course for two consecutive semesters and participated in various outreach programs, including serving as a science fair judge at many elementary and middle schools located in our mostly Hispanic community. She enjoys speaking with prospective students about the many opportunities offered at UTEP and career opportunities in the field of science.

Dr. Ruiz was the recipient of several awards including the Woman's Auxiliary Fellowship (2013-2014), various travel scholarships to present her research findings at national and international conferences, including Society for Advancement of Hispanics/Chicanos and Native Americans in Science National conference for two consecutive years. Moreover she has presented her work on cancer at a Research Centers in Minority Institutions International Symposium on Health Disparities, in San Juan, Puerto Rico, and the Minority Health and Health Disparities Grantees' Conference held in Baltimore, Maryland.

Work from Dr. Ruiz's dissertation, IL2R $\beta$  T450 Phosphorylation is a Positive Regulator for Receptor Complex Stability and Activation of Signaling Molecules, supervised by Dr. Robert A. Kirken, will be submitted for publication in a top tier peer-reviewed scientific journal this year. She plans to continue her exciting cancer research as a post-doctoral fellow at UTEP.

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