RECEPTOR UBIQUITINATION REGULATES IL-5Rα FUNCTION

A Thesis

by

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MASTER OF SCIENCE

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ABSTRACT

Eosinophils are multifunctional leukocytes implicated in the pathogenesis of inflammatory processes including hypereosinophilic syndrome, eosinophilic esophagitis, and allergic asthma. Due to its role in the initiation and propagation of inflammatory responses, and as modulators between innate and adaptive immunity, eosinophils are emerging as critical cells in both health and disease. Eosinophil activation and inflammatory signaling are dependent on interleukin-5 (IL-5) and signaling by its receptor, IL-5R, comprised of a ligand specific alpha chain (IL-5Rα) and a shared signaling component, βc. Previously, our laboratory demonstrated the critical role of three membrane proximal lysine residues in the cytoplasmic domain of βc for Janus kinase (JAK) binding and optimal receptor ubiquitination, and such binding is responsible for receptor signaling and endocytosis following IL-5 stimulation. However, little is known about whether or not IL-5Rα itself is ubiquitinated or what the molecular determinants are. Since βc and IL-5Rα are both members of the Type I cytokine receptor family, with similar structural features, we hypothesized that IL-5Rα is also ubiquitinated and that lysine residues in homologous regions were important for this process.

To test this hypothesis, we performed site directed mutagenesis on four membrane proximal lysine residues within the cytoplasmic domain of IL-5Rα, generating six mutant receptors expressed in HEK293 cell lines with wild-type βc. Our data revealed that IL-5Rα is ubiquitinated under steady state conditions, highlighting a

novel role of ubiquitin in stabilizing cell surface expression of receptors prior to cytokine stimulation. In addition, the binding of JAKs to IL-5Rα depended on the presence of all four lysine residues, and such binding resulted in optimal ubiquitination under basal conditions. Our most striking finding was the IL-5-induced deubiquitination of IL-5Rα that triggers receptor internalization, with this deubiquitination event being dependent on the presence of the third and fourth lysine residues (Lys^{370} and Lys^{379}).

In sum, these findings reveal a unique regulatory role for ubiquitin in IL-5Rα biology, particularly the significance of receptor deubiquitination following cytokine stimulation. Due to the specific role of IL-5Rα within eosinophil biology, results from this study may provide more insight into IL-5Rα regulation and perhaps help identify novel molecular targets for controlling inappropriate signaling associated with eosinophilic inflammation.

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

INTRODUCTION

Introduction and Background

Eosinophils are leukocytes of the innate immune system characterized by their bi-lobed nuclei and 'brick-red' coloring following eosin-staining[1]. Historically, eosinophils have long been recognized for their cytotoxic, effector function in defense against parasitic infection[2], with recent studies documenting additional host defense against viruses[3,4], fungi[5-7], and bacteria[8]. Recently, great strides have been made in eosinophil biology, widening its role as a pleiotropic, multifunctional leukocyte involved in the initiation and propagation of inflammatory responses, and, interestingly, as modulators between innate and adaptive immunity[9-11]. The limited quantity of eosinophils—typically comprising only 1-6% of white blood cells[12]— is sufficient in light of their ability to secrete various proinflammatory cytokines, chemokines, and lipid mediators, in addition to cytotoxic cationic proteins that are capable of inducing direct tissue damage and dysfunction[9,10].

Unfortunately, certain targets are essentially innocuous, resulting in eosinophil-derived pathologies directly caused by the increased infiltration of activated eosinophils into tissues, such as hypereosinophilic syndrome[13,14], eosinophilic esophagitis[15], eosinophilic colitis[16], and allergic asthma [17,18] (Fig. 1). Due to its significant prevalence, allergic asthma has been studied in great detail over the past twenty-five years[18-24]. Characterized by eosinophilic inflammation within the airways[19], tissue specimens of allergic asthmatics also exhibit marked infiltration of eosinophils into

bronchial mucosa and submucosa[20]. Subsequent studies have been able to relate disease severity directly to the degree of tissue eosinophilia[18,21-24]. As such, eosinophils are the source of proinflammatory mediators that cause airway epithelial cell damage and loss, airway hyperresponsiveness, mucous hypersecretion and airway remodeling following fibrosis and collagen deposition[18,21-24].

Although a number of inflammatory mediators posses the chemotactic activity for eosinophils and, thus, could potentially be involved with tissue eosinophilia in asthma, only interleukin-5 (IL-5) has the ability to regulate the growth, differentiation, and activation of eosinophils[10,25]. As such, several lines of evidence indicate the critical role of IL-5 in the pathophysiology of allergic inflammation and asthma[26-28]. One study found elevated IL-5 levels in both serum and bronchoalveolar fluid of atopic asthmatic patients[26], with a subsequent study finding increased eosinophil numbers and airway hyperresponsiveness in response to IL-5 inhalation[27]. Similar increases in eosinophils, T_H2 cells, and IL-5 were found in bronchial mucosa of biopsies from severe asthmatic patients[28].

Using the mouse asthma model, two individual laboratories further demonstrated the importance of IL-5 in eosinophil accumulation within the asthmatic airway.[29,30] In an earlier study, researchers found that $CD4^+$, rather than $CD8^+$, T-cells mediate eosinophil recruitment in allergic airways, and that $CD4^+$ derived IL-5 is directly responsible for such eosinophil recruitment.[29] A second study provided further confirmation, since eosinophilia, lung damage and airway hyperreactivity were entirely eliminated in IL-5-deficient mice, with the reconstitution of IL-5 restoring allergen-

induced eosinophilia and airway dysfunction.[30] Additionally, administration of a neutralizing IL-5 antibody, prior to antigen inhalation, suppressed airway hyperactivity in the guinea pig[31] and monkey[32] asthma models.

Given the important role of eosinophils in disease pathogenesis, understanding mechanisms that limit the degree of eosinophilic activation are critically important for attenuating eosinophil-derived inflammatory signals. Specifically, much is to be understood about the intracellular signaling that occurs following IL-5-mediated eosinophil activation. Through our increased understanding of the IL-5 receptor biology, our long-term goal with this study is to develop *in vivo* interventional strategies for manipulating eosinophil activity.

Eosinophil activation is critically dependent on IL-5 and signaling by its receptor, IL-5R, comprised of a ligand specific α-chain (IL-5Rα) and a shared signaling component (βc). Our previous studies revealed novel insight into the downregulation of IL-5R, specifically IL-5-dependent βc ubiquitination that is required for IL-5R endocytosis, proteasomal degradation, and optimal signal transduction.[33] Considering that IL-5Rα is the subunit that is specific for IL-5, we sought to explore whether the function of this receptor chain was regulated by ubiquitination, and if so, to identify the molecular determinants regulating this process.

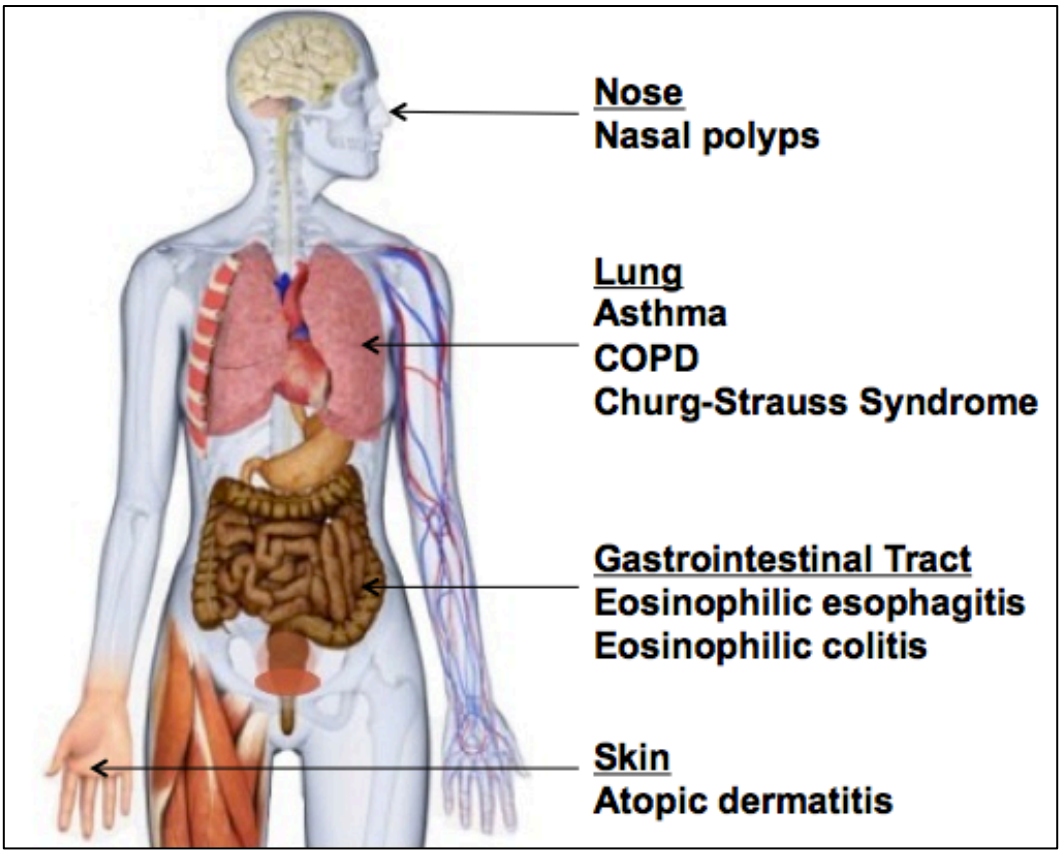


FIGURE 1. **Eosinophils in disease.** The overproduction of IL-5 and eosinophilia are involved in the pathogenesis of specific inflammatory disorders affecting various organ systems throughout the body.

The IL-5, IL-3 and GM-CSF Receptor Complexes

Cytokines are secreted by cells of the hematopoietic lineage, and provide either regulation of hematopoietic cell differentiation and/or induce changes in immune cell function[34,35]. IL-5, interleukin-3 (IL-3), and granulocyte-macrophage colony-stimulating factor (GM-CSF) are members of the short-chain 4-α-helical subset of hematopoietic cytokines, further grouped by their shared use of βc within their heterodimer receptors[36]. The high affinity receptor complexes for each of these cytokines are composed of a ligand specific α-chain (approximately 65 kDa) and the shared, signaling βc subunit (130 kDa)[36] (Fig. 2). Alone, each α-chain binds its respective cytokine with low affinity (K_D = 0.2-100 nM), with the recruitment of βc converting the binding to high affinity (K_D = 100 pM)[37]. Due to the physical and conformational proximity of the subunits, the resulting heterodimerization initiates receptor activation and intracellular signaling for desired physiological effect.

The hematopoietin receptor family is structurally characterized by an extracellular domain, of approximately 200 amino acids, containing two fibronectin type III domains with a conserved membrane proximal WSXWS motif, as well as membrane distal pair of cysteine residues[34-36] (Fig. 3). Although the α-chains exhibit these conserved features, βc has a longer extracellular portion due to an additional pair of fibronectin type III domains. Common to all cytokine receptors, the cytoplasmic domain of the three α-chains and βc express the Box 1 and Box 2 motifs, with the highly conserved Box 1 motif known to be required for JAK kinase binding [38-40]. It is believed

that the significantly longer cytoplasmic domain in βc contains necessary sequences required for the initiation and regulation of signal transduction[36].

Interestingly, GM-CSFα and IL-5Rα exist as soluble external domains generated from the alternative splicing of the *GM-CSFα* and *IL-5Rα* genes[41,42]. These soluble external domains are able to compete with the transmembrane GM-CSFα and IL-5Rα for binding with GM-CSF and IL-5, respectively. However, these receptor soluble isoforms are unable to participate in cytokine signaling, lacking the necessary cytoplasmic domains, with their exact physiological purpose in immunity yet to be determined.

Furthermore, the crystal structure of the extracellular GM-CSF ligand-receptor complex has been solved[43]. Investigators found that the ligand-bound GM-CSFR complex exists as a hexamer (2 alpha chains, 2 βc, and 2 GM-CSF molecules) capable of supporting differentiation signals. Surprisingly, they also found that each hexamer could form a larger dodecamer complex (12-subunit) with another GM-CSFR hexamer for additional signaling that supports proliferation in addition to differentiation signaling. Interestingly, mutations of βc, at the site of dodecamer interface, reduced signaling and function following GM-CSF stimulation, but surprisingly also from IL-3 stimulation [43]. Together these data suggest that the dodecamer formation may in fact be a conserved mechanism utilized by the GM-CSF/IL-3/IL-5 family for optimal receptor activation and signal transduction.

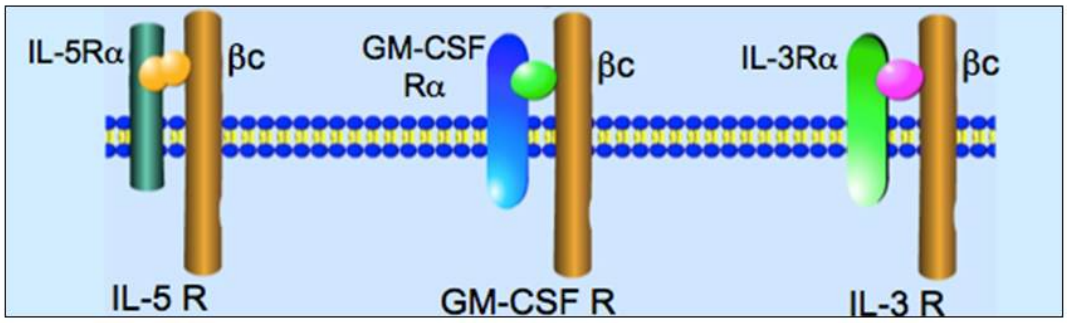


FIGURE 2. **Family of hematopoietic cytokine receptors that share beta-common chain (βc) receptor.** The hematopoietic receptor family is composed of a heterodimer, consisting of a ligand-specific α-chain and beta-common chain (βc), shared among all three α-chain receptors. Compared to the short cytoplasmic domain of the α-chains, βc significantly longer cytoplasmic region aids in its function of cell signaling.

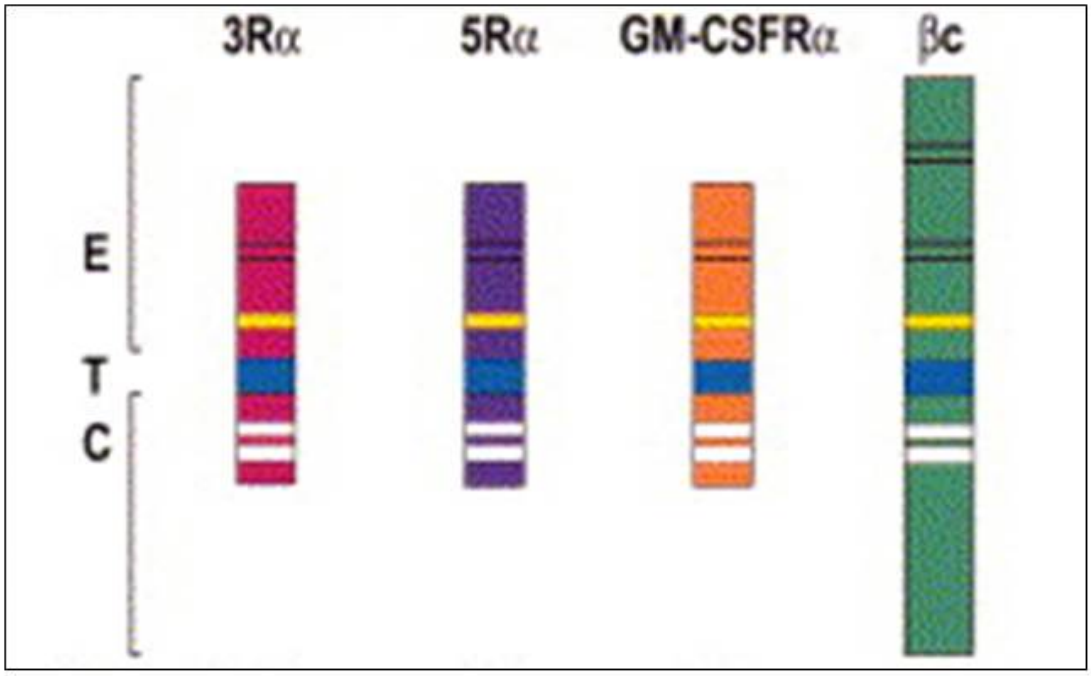


FIGURE 3. **Structural components of βc and ligand-specific α-chains.** (E) Extracellular domains of each α-chain composed of membrane proximal WSXWS motif (yellow), characteristic to Type I cytokine receptors, and a homology module of two fibronectin type III domains with paired cysteine residues (solid black lines). Similar extracellular domain is found in βc, although longer due to the presence of an additional pair of cysteine residues and homology modules. (T) Transmembrane region of receptors (blue). (C) Cytoplasmic domains of α-chain and βc all contain Box 1 and Box 2 motifs (white boxes), although βc cytoplasmic region is notably longer for downstream signaling purposes.[*]

[*] Reprinted with permission from "Biology of common β receptor-signaling cytokines: IL-3, IL-5, and GM-CSF" by Moczygemba MM and Huston DP, 2003. Journal of Allergy and Clinical Immunology, 112, 653-665, Copyright (2003) by Elsevier.

Intracellular Signaling Cascades

Similar to external stimuli such as growth factors and cytokines, IL-5 exerts its biological effects after binding its receptor on the surface of target cells[44-48]. The initiation of signal transduction, following recruitment of βc to the ligand-bound IL-5Rα, ultimately results in the reprogramming of gene expression to alter behavior of target cells. Currently, four primary signaling pathways have been described for IL-5: Janus kinase (JAK)/signal transducer and activator of transcription (STAT) pathway, the mitogen-activated protein kinase (MAPK) pathways, the c-JNK/p38 pathway, and the phosphatidylinositol 3-kinase (PI3-K) pathway[44-48].

The JAK/STAT Pathway

The JAK/STAT pathway is utilized by all cytokines to transmit extracellular signals, through transmembrane receptors, to the promoters of target genes within the nucleus of activated cells[49,50]. Thus, the JAK/STAT pathway is remarkable in achieving transcriptional gene regulation without the use of secondary messengers. Additionally, the evolutionary conservation from slime molds to humans of the JAK/STAT pathway further confirms its biological significance[49].

In the absence of IL-5, JAK1 and JAK2 are constitutively associated with βc and IL-5Rα, respectively[51]. Specifically, a canonical Proline-*X*-Proline motif in the Box 1 region of Type I and II cytokine receptors is required for both JAK kinase binding and activation[38-40]. Following IL-5 binding to IL-5Rα, βc is recruited close enough to IL-5Rα that the two JAK proteins are able to transphosphorylate one another. Previous

studies have shown that the kinase-negative form of JAK2 resulted in both JAK1 and JAK2 inhibition, whereas the kinase-negative form of JAK1 only inhibited JAK1 activity, highlighting the importance of JAK2 as the main signaling kinase [51].

Tyrosine-phosphorylated JAK1 and JAK2 are then activated to phosphorylate tyrosine residues on βc and IL-5Rα, respectively. Specifically, JAK activation results in the phosphorylation of six crucial tyrosine (Y) residues on βc: Y577, Y612, Y695, Y750, Y806, and Y866[44-48]. Of these six residues, Y612, Y695, and Y750 serve as a docking site for the phosphotyrosine-binding SH2 domains of STAT1 and STAT5[49,50]. Once bound to phosphorylated tyrosine residues on the receptor, STAT proteins themselves are activated via JAK-mediated tyrosine phosphorylation. This phosphotyrosine event allows for the dimerization of STAT proteins through interaction of their SH2 domains with one another. Phosphorylated STATs then enter the nucleus via the Ran nuclear import pathway to activate transcription of various proteins involved with eosinophil growth, survival and activation[49,50].

The MAPK Pathways

The MAPK pathways include the extracellular signal-regulated kinase (ERK), c-Jun amino-terminal kinase (JNK) and p38 signaling cascades, responsible for a variety of cell processes such as growth, proliferation and survival[44-48]. The phosphorylation of Y577 on βc by JAK2 results in the recruitment of the cellular substrate Shc, which itself becomes tyrosine phosphorylated. Tyrosine phosphorylation of Shc allows it to interact with the SH2 domain of growth factor receptor-bound adaptor protein Grb2. The

Shc/Grb2 complex associates with the nucleotide exchange factor for Ras, resulting in a successive activation of Ras, Raf-1, MEK and ERK. The downstream targets of the ERK pathway are transcription factors ATF2 ad TCF, which activate c-Fos and c-Jun in order to induce cellular proliferation[52].

The PI3-K Pathway

Upon cytokine stimulation, PI3-K is activated in a tyrosine phosphorylation-dependent manner, with previous studies having found a direct link between IL-3-stimulated βc serine phosphorylation, PI3-K activation, and hematopoietic cell survival[53-55]. Specifically, IL-3 stimulation induces the activation of protein kinase A, which phosphorylates Serine^{585} on βc[54]. The phosphorylated-serine residue then serves as a recruitment site for the adaptor protein 14-3-3, which appears to recruit the regulatory subunit (p85) and catalytic subunit (p110) of PI3-K to the receptor complex. Following PI3-K activation, the secondary messenger phosphatidylinositol 3,4,5-triphosphate (PIP_3) is generated[56,57], resulting in the recruitment and binding of the serine-threonine kinase Ak2/PKB[54]. Ak2/AKB phosphorylation of BAD results in the direct inhibition of apoptosis[55], suggesting that βc Serine^{585} is critical for hematopoietic cell survival.

Signal Termination

To ensure that the execution of specific signals are of adequate strength and delivered for appropriate time, organisms have developed regulatory mechanisms to

safeguard the transient nature of signaling pathways[36]. Currently, several forms of regulatory mechanisms have been discovered for IL-3, IL-5, and GM-CSF receptors[58-61].

One known mechanism involves the activation of cytosolic tyrosine phosphatases, responsible for regulating the ligand-induced phosphorylation of substrates[58]. Specifically, researchers found that the tyrosine phosphatase Src homology protein tyrosine phosphatase 1 (SHP1) was involved in βc downregulation, due to the suppression of cell growth following IL-3 stimulation in SHP1 overexpressed cells[58].

Another mechanism for terminating IL-3, IL-5 and GM-CSF signals is the induction of the suppressors of cytokine signaling (SOCS) family of small SH2-containing proteins[59]. SOCS proteins are known to negatively regulate signaling by blocking the access of STATs to receptor binding site, direct inactivation of JAK kinases, and ubiquitination of signaling proteins for proteasomal degradation[59].

A third mechanism that antagonizes IL-3, IL-5, and GM-CSF signaling is receptor endocytosis and degradation[60,61]. Previous research within the Huston and Moczygemba lab found that βc is first tyrosine phosphorylated and then ubiquitinated in response to IL-5 binding, with such events triggering receptor internalization via endocytosis[60] (Fig. 4). Within the late endosome, the signaling portion of βc undergoes proteasomal cleavage, resulting in the truncated βc product known as βc intracytoplasmic proteolysis (β_{IP}). The remaining IL-5R complex, comprised of β_{IP} and IL-5Rα, are then degraded in the lysosome for signal termination. Interestingly, use of a proteasomal inhibitor prolonged the activation of βc, JAK2, and STAT5, highlighting

the ubiquitin proteasomal pathway as rapid technique in terminating βc signal transduction[60]. Since similar results were found following IL-3 and GM-CSF stimulation, it is possible that the ubiquitin/proteasomal-degradation pathway is a conserved regulatory mechanism used by βc, and possibly by the α-chains.

Ubiquitin, Endocytosis, and Signaling

The ubiquitin/proteasome (Ub/Pr) degradation pathway is the principle mechanism for intracellular protein degradation in the mammalian cytosol and nucleus[62-68]. This proteolytic system is tightly controlled, and alterations in this pathway are associated with the pathogenesis of various diseases such as Parkinson's, Alzheimer's, Huntington's disease, and multiple myeloma[69].

The classical function of the 8kDa protein, ubiquitin, is associated with housekeeping functions, as well as regulation of protein turnover, and antigenic peptide generation[62-64]. However, over the past fifteen years, ubiquitin has garnered the label of 'master regulator' of the cell, wherein protein modification by this molecule has the potential to regulate cellular processes as diverse as endocytosis, trafficking, cell cycle progression, signal transduction, inflammation, apoptosis, neural and muscular degeneration, and transcription[62-64].

Ubiquitin can be covalently linked to itself and to other substrate proteins either as a single molecule (monoubiquitination, monoUb) or a chain of ubiquitin molecules (polyubiquitination, polyUb)[62]. In general, polyUb chains, formed through Lysine^{48} and Glycine^{76} linkages, are attached to substrates destined for proteasome degradation. In

contrast, polyUb chains formed through Lysine^{29} or Lysine^{63} have other non-proteolytic functions in cells, such as transcriptional regulation and membrane trafficking[65-68]. Other types of protein ubiquitination, such as monoUb and multi-monoUb, are involved in at least three distinct cellular functions: histone regulation, endocytosis, and the budding of retroviruses from the plasma membrane[65-68].

The pioneering work of P.P. Di Fiore and colleagues has firmly established that ubiquitin regulates protein transport between membrane compartments, by serving as a sorting signal on protein cargo and through controlling the activity of trafficking machinery[66,67]. MonoUb and Lysine^{63}-linked poly-Ub chains appended to integral membrane proteins serve as regulated signals for internalization into the endocytic pathway[66,67]. Interestingly, our laboratory has detected immunoreactivity of βc with polyclonal antibodies against Lysine^{63}-linked poly-Ub chains, indicating that βc is modified by this type of Ub chain, possibly for endocytic trafficking (unpublished data).

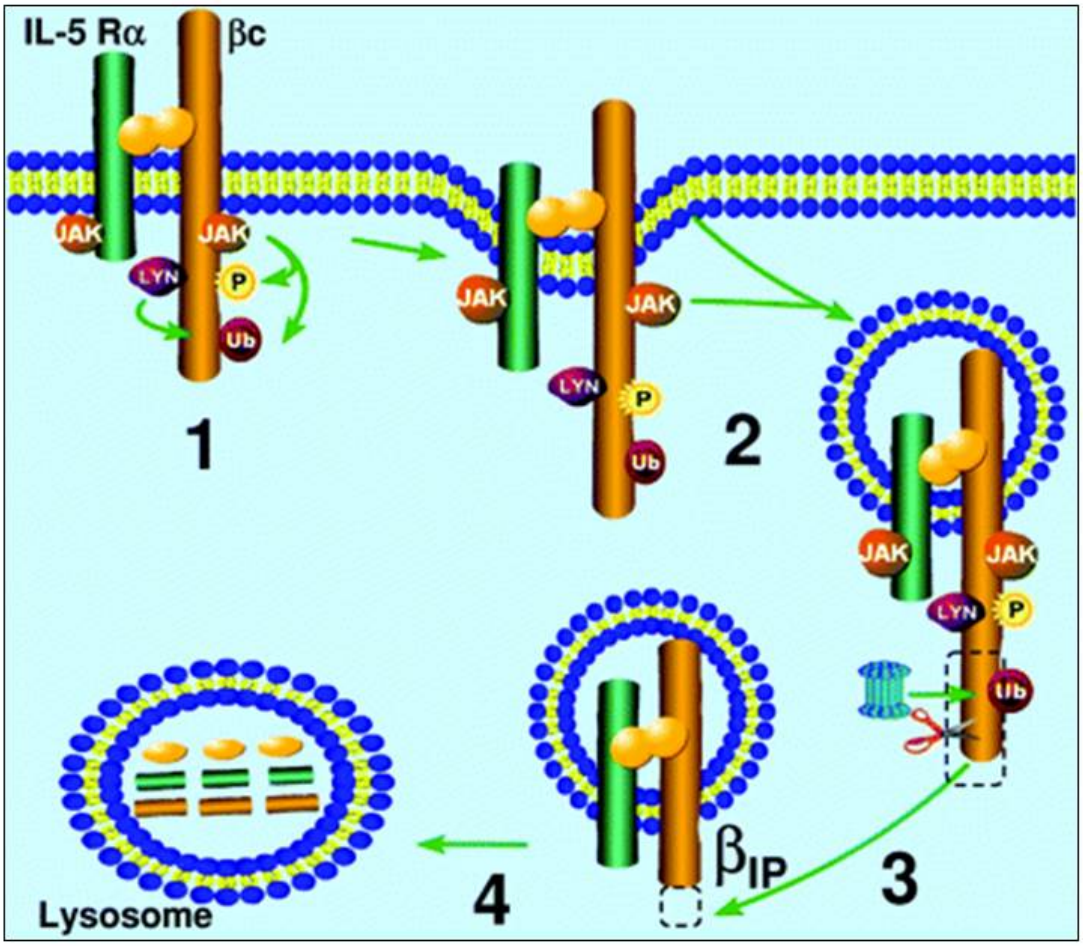


FIGURE 4. **Current model of βc downregulation following IL-5 stimulation.** Step 1: binding of IL-5 to the IL-5R complex results in JAK2/1 and Lyn kinase activation, and subsequent βc ubiquitination (Ub) and tyrosine phosphorylation (P) by the JAK kinases. Step 2: JAK kinase activation triggers the entry of full-length IL-5R complex into the lipid raft endocytic pathway. Step 3: Within the endocytic pathway, proteasomes cleave the signaling portion of βc cytoplasmic domain for signal termination, generating truncated β_{IP} protein as a result. Step 4: Following proteasomal cleavage of βc's signaling cytoplasmic domain, β_{IP} and IL-5Rα are degraded in the lysosome.*

* Reprinted with permission from "JAK kinases control IL-5 receptor ubiquitination, degradation, and internalization" by Moczygemba MM, Huston DP, and Lei JT, 2007. Journal of Leukocyte Biology, 81, 1137-1148, Copyright (2007) by The Society for Leukocyte Biology.

Specific Aims

Although the endocytic pathway has been historically viewed as a mechanism of receptor downregulation, recent studies have shown that signal transduction continues throughout the endocytic route[70-73]. As such, several studies have explored the connection between receptor tyrosine phosphorylation and endocytosis in the transforming growth factor-β receptor, acetylcholine receptor, protease-activated receptor, and tropomyosin receptor kinase A receptors[74-77]. Our previous studies with the IL-5R have shown that inhibition of endocytosis prevents association of pSTAT5 and pMAPK with the IL-5R complex. Thus, entry of IL-5R into the endocytic pathway is another mechanism that permits the interaction of βc with its signaling molecules[78].

Following the discovery that ubiquitin regulates protein transport, and in certain situations directly serve as signals into the endocytic pathway, it was first important to further understand the role of ubiquitin in IL-5R, specifically within the signaling component, βc. Initial studies found that IL-5R signaling was partially terminated due to ubiquitin/proteasomal degradation of βc's signaling component, followed by the lysosomal degradation of the truncated receptor complex[60]. More recent studies in the Moczygemba laboratory have shown that a cluster of three lysine residues, proximal to the plasma membrane, were required for JAK1/2 binding to βc and receptor ubiquitination, and that this binding is required for receptor ubiquitination, endocytosis, and signaling[33].

Since IL-5Rα is the IL-5-specific binding receptor chain for the complete IL-5R, we sought to investigate the interplay of IL-5Rα ubiquitination and IL-5R endocytosis

and signaling. In this study, we will apply similar molecular, cellular, and biochemical techniques to investigate the potential role of ubiquitination in IL-5Rα function. The major goal of this study is to identify specific ubiquitination sites in the cytoplasmic domain of IL-5Rα and define the functional importance of these novel ubiquitination events. Due to the specific and unique expression of IL-5Rα in eosinophils, results from this study have the potential to identify new directions in future therapeutics for eosinophilic disorders. We propose the following specific aims to achieve these goals:

1) Test the hypothesis that IL-5Rα is ubiquitinated and identify key receptor ubiquitination sites
2) Determine the functional significance of IL-5Rα ubiquitination
3) Determine the role of JAK kinases in IL-5Rα ubiquitination

EXPERIMENTAL PROCEDURES

Cell Culture, Materials, and Inhibitors

Human embryonic kidney line, HEK293 (purchased from ATTCC) was kept in DMEM media supplemented with 10% FBS and 10 μg/ml gentamicin. The human erythroleukemic cell line, TF-1, endogenously expresses the IL-5 receptors, and was cloned by limited dilution in media with 5 ng/ml IL-5. The TF1-F11 clone was selected due to its strong proliferative response to IL-5, 1L-3 and GM-CSF. This clone was maintained in RPMI 1640 with 10% FBS, 10 mM β-mercaptoethanol, 10 μg/ml gentamicin, and 5 ng/ml human IL-5. Recombinant human IL-5 was expressed by a baculovirus and purified via affinity chromatography[79].

Cyclohexamide was purchased from Calbiochem and used at a 10 μg/ml concentration. Antibodies used for immunoflourescence microscopy and flow Cytometry were purchased from the following companies: anti-βc (R&D Systems), anti-βc (clone 3D7, BD Biosciences), anti-IL-5Rα (R&D Systems), anti-IL-5Rα (N-20, Santa Cruz Biotechnology), anti-actin (Santa Cruz Biotechnology), anti-JAK1 (Cell Signaling), anti-JAK2 (Cell Signaling), anti-STAT5 (Santa Cruz Biotechnology), anti-Ubiquitin (Santa Cruz Biotechnology), anti-phospho-STAT5 (Millipore), anti-phospho-JAK2 (Cell Signaling), PE-conjugated rat anti-mouse IgG1 (BD Biosciences), and FITC-conjugated donkey anti-goat IgG1 (Santa Cruz Biotechnology).

Construction of IL-5Rα Mutants and Generation of Stable Cell Lines

WT IL-5Rα (GenBank™Accession #NM000564)[80] cloned in a pLentivector as a template for site directed mutagenesis of IL-5Rα cytoplasmic lysines 1-4 to arginine using the multisite site-directed mutagenesis kit (Stratagene) in stepwise manner with specific primers listed in Table 1. All lysine to arginine mutations were sequenced to confirm the amino acid mutation. The mutated cDNA was then cloned into a Virapower pLentivector (Invitrogen), and constructs were fully sequenced. Lentiviral particles containing the mutated receptor were made per manufacturer's instructions, purified using Amicon Ultrafiltration tubes (Millipore), and then titered in HeLa cells for stable transduction. Initially we established a parental cell line of HEK293 expressing WT βc only, to which lentiviral particles were added for transduction of the mutant IL-5Rα. Surviving transduced cells were selected in presence of 6 μg/ml blasticidin, and sorted by flow cytometry for confirmation of dual receptor expression.

TABLE 1. Primers Used for Cloning IL-5Rα K-to-R Mutants

Primer Name	Primer Sequence (5'-3')
IL-5Rα K1R	ATT CTC TCG CTT ATC TGT AGA ATA TGT CAT TTA
IL-5Rα K2R	ATA TGT CAT TTA TGG ATC AGG TTG TTT CCA CCA
IL-5Rα K3R	CCA CCA ATT CCA GCA CCA AGA AGT AAT ATC AAA
IL-5Rα K3,4R	CCA CCA ATT CCA GCA CCA AGA AGT AAT ATC AGA GAT CTC TTT GTA ACC

Cyclohexamide Pulse Chase Assay

WT and mutant IL-5Rα were pretreated with 10 μg/ml Cyclohexamide for one hour. Cells were then stimulated with 10 ng/ml IL-5 and then harvested after 10 minutes, 20 minutes and 30 minutes of stimulation. Afterwards, cells were lysed using 50 μL of RIPA buffer (1% Nonidet P-40, 1% Triton, 0.1% SDS, 0.15 M NaCl, 0.01 M sodium phosphate at pH 7.2, 0.2 mM EDTA, 5 mM *N*-ethylmalemide, 1 mM sodium vanadate, 1 mM PMSF, 1 μg/ml leupeptin, 1 μg/ml pepstatin) for 20 minutes on ice. 25 μg of lysate was resolved by lauryl deodecyl sulfate-PAGE, followed by immunoblot analysis using anti-βc (R&D Systems), anti-IL-5Rα (R&D Systems), and anti-actin (Santa Cruz Biotechnology). Degradation curves were obtained by measuring the band densities using AlphaView software (Alpha Innotech), normalizing βc and IL-5Rα densities using actin band densities. The Cyclohexamide treated, 0-hour cells were designated as 100% with subsequent time points percent densities calculated with this postulation. Curves were drawn with % βc or IL-5Rα desities (*y*-axis) relative to time following IL-5 stimulation (*x* axis).

Flow Cytometry

Expression of IL-5Rα and βc was measured by incubating WT IL-5Rα, IL-5Rα K2R, IL-5Rα K2,3R, IL-5Rα K3,4R, IL-5Rα K2-4R, and IL-5Rα K1-4R HEK293 cell lines (approximately 250,000 cells per tube) in PBS + 2% FBS with anti-IL-5Rα (R&D Systems) and anti-βc (BD Biosciences) for 20 minutes on ice. Afterwards, cells were washed with FITC-conjugated anti-goat IgG and PE-conjugated anti-mouse IgG1

flourophore linked antibodies, respectively. Immediately the labeled proteins were analyzed using an Accuri C6 (Accuri Cytometers) flow cytometer. The data was then analyzed using a CFlow Plus (Accuri Cytometers) software and then graphed using Excel.

IL-5Rα Endocytosis Assay

Following IL-5 stimulation, we developed an assay that measures the loss of cell-surface IL-5Rα in WT and IL-5Rα K2R mutant cell line. First, anti- IL-5Rα (N-20, Santa Cruz Biotechnology)—which doesn't prevent IL-5 binding to the receptor—was added to the pre-chilled cell lines for thirty minutes on ice. Then the cells were washed three times with ice-cold media to remove any unbound antibodies. 10 ng/ml IL-5 was then added to cells, with aliquots of the cells being transferred to 37°C for 5, 10 and 15 minutes. After the respective time at 37°C, receptor internalization was halted by the addition of cold PBS. The remaining IL-5Rα receptors on the cell surface were analyzed by incubating the anti-IL-5Rα bound receptors with FITC-conjugated anti-goat IgG and then measured by flow cytometry. The MFI at 0 minutes (no IL-5 stimulation) represented 100% IL-5Rα expression and the loss of immunoreactivity (MFI) was determined for the subsequent time points.

Immunopercipitation and Immunoblot Assays (IP/IB)

All IP/IB assays were performed as previously described with the following antibodies: IL-5Rα was immunopercipated with anti-IL-5Rα polyclonal goat; IBs were

done with anti-IL-5Rα monoclonal mouse; anti-ubiquitin mAb (P4D1) (Santa Cruz Biotechnology); anti-actin rabbit pAb (Sigma); anti-phosphotyrosine (mAb clone 4G10) and anti-pSTAT5 (Upstate Biotechnology); Anti-STAT5 (BD Biosciences). We visualized proteins after incubating with enhanced chemiluminescence with reagents (GE Healthcare) and images were captured with FuorChem 8000 Imaging System (Alpha Innotech).

Cell Surface Biotinylation Assay

HEK293 cells were grown to 80% confluency in 100 mm plates and cell surface proteins were labeled with a non-permeable sulfo-NHS-SS-biotin reagent (Thermo Fisher). First cells were washed with 10 mL cold PBS pH 8.0 before addition of 10 mL PBS pH 8.0 containing 0.5 mg biotin reagent. Cell surface proteins were labeled with biotin following 30 minutes of rocking at 4°C. Unbound biotin was removed by centrifuging the cells five times with 50 mL PBS glycine, before resuspending the pellet in 10 mL chilled serum containing DMEM media. Cells were split into two tubes, one remaining on ice to inhibit endocytosis and the other being stimulated with 10 ng/ml IL-5 for 30 minutes at 37°C. After washing with PBS, cell pellets were re-suspended in 500 μL of PBS with the addition of 0.5 μg of R&D α-IL-5Rα for two hours on ice to bind all IL-5Rα receptors on the cell surface. Cells were then washed three times with PBS to remove any unbound antibody and then lysed with 300 μL of RIPA lysis buffer. Cell surface immune complexes were then precipitated using 15 μL of Protein G, separated LDS-PAGE, and transferred to Immobilon-P PVDF membranes. Cell surface biotin

labeled proteins was then detected by incubating the membranes with Neutravidin-HRP reagent (Thermo Fisher).

JAK1/2 Knockdown

Cell surface IL-5Rα in wild-type cells were analyzed in the context of a JAK1 and JAK2 knockdown. First cells were split into three 100 mm plates: mock, negative control, and JAK1/JAK2 knockdown. After twenty-four hours of incubation, mock cells received Opti-MEM and lipofectamine 2000 (Invitrogen). In addition, knockdown cells were transfected with SMART pool JAK2 (75 nM) and JAK1 siRNAs (50 nM) whereas negative control cells were transfected with a non-target negative control siRNA. Forty-eight hours post transfection, cells were washed with 10 mL cold PBS pH 8.0, and then lysed with 300 μL of RIPA lysis buffer. In certain experiments, we performed the cell surface biotinylation in the context of JAK1/2 knockdown. Forty-eight hours post transfection, cells were washed with 10 mL cold PBS pH 8.0 and cell surface proteins were biotinylated with the addition of 0.5 mg of biotin to 10 mL cold PBS pH 8.0 following the previous protocol.

JAK2 Overexpression

JAK2 was overexpressed in certain cell lines to see the physiological effect of overexpression on IL-5Rα ubiquitination. Two 100 mm plates were plated per cell line, control pcDNA3 and experimental JAK2 overexpression. Control plates were transfected with gene jammer and 3 μg of empty pcDNA3 with Opti-MEM, whereas

experimental cells were transfected with 3 μg of plasmid JAK2 in pcDNA3 vector (generous gift from Dr. Yu-Lee). Twenty-four hours post transfection cells were washed with cold PBS before resuspending the cell pellet with 300 μL of RIPA lysis buffer. Proteins were separated on an LDS-PAGE before being transferred to an Immobilon-P PVDF membrane for analysis.

RESULTS

IL-5Rα Ubiquitination in TF1 and HEK293 Cells

Previous studies in the Moczygemba lab demonstrated that βc is ubiquitinated in response to IL-5 stimulation[33]. Since the IL-5R complex is a heterodimer of βc and IL-5Rα, we sought to investigate whether IL-5Rα was also ubiquitinated in the human erythroleukemic cell line, TF1, due to its endogenous expression of the receptor complex. To examine the ubiquitination status of IL-5Rα, whole cell lysates of TF1 cells were immunopercipated (IP) with anti-IL-5Rα antibodies and immunoblotted (IB) with anti-ubiquitin antibodies, in the absence (-) or presence (+) of IL-5 (Fig. 5A, *top panel*). IP/IB analysis revealed two highly ubiquitinated smears of differing molecular weights, which we will refer to as upper and lower smears. Interestingly, the upper smear migrated between 97-191 kDa, while the lower smear migrated between 60-75 kDa. Based on the sizes of the smears and the known molecular weights of βc and IL-5Rα, we hypothesized that the upper smear contained ubiquitinated forms of βc co-precipitating with the anti-IL-5Rα immune complexes, and the lower smear contained ubiquitinated forms of IL-5Rα.

Indeed, IB with anti-βc antibodies confirmed its presence in the IL-5Rα immune complexes as a single band in the 97 kDa range rather than a smear, indicating that our βc IB antibodies recognize the non-ubiquitinated form of βc (Fig. 5A, *middle panel*). Similarly, IB with anti-IL-5Rα antibodies confirmed our prediction that the lower smear corresponded to the location of ubiquitinated forms of IL-5Rα (Fig. 5A, *bottom panel*).

Interestingly, TF1 cells had increased levels of ubiquitination in both upper and lower smears following thirty minutes of IL-5 stimulation (Fig. 5A, *top panel, lanes 1 vs 2*). This result of increased ubiquitination in IL-5Rα following IL-5 stimulation is consistent with previous studies of βc ubiquitination in TF1 cells[33].

After confirming IL-5Rα ubiquitination under endogenous conditions, we sought to investigate the ubiquitination status of our model cell system, the human embryonic kidney cell line, HEK293, stably transduced with the IL-5R complex. Although HEK293 do not endogenously express the receptor complex, previous studies using HEK293 cells has shown that the IL-5R complex proceeds with normal signaling patterns and lysosome trafficking following IL-5 stimulation[78,80]. Therefore, the use of HEK293 as our study cell model system will allow for phenotypic analysis of IL-5Rα mutants, relative to WT IL-5Rα, in order to further understand the role of ubiquitination in this receptor.

Similar to TF1 cells, IP/IB analysis of IL-5Rα with anti-ubiquitin antibodies in HEK293 cells— stably transduced with the WT IL-5R complex— showed the presence of two highly ubiquitinated smears (Fig. 5B, *top panel*). After the membranes were stripped and blotted with anti-βc antibodies (Fig. 5B, *middle panel*) and anti-IL-5Rα antibodies (Fig. 5B, *bottom panel*), the upper and lower smears were identified as ubiquitinated forms of βc co-precipitating with anti-IL-5Rα immune complexes and ubiquitinated forms of IL-5Rα, respectively. Although TF1 cells appeared to exhibit increased ubiquitination following IL-5 stimulation (Fig. 5A, *top panel, lanes 1 vs. 2*), unstimulated and stimulated HEK293 cell lines had comparable levels of ubiquitination

(Fig. 5B, *top panel, lanes 1 vs. 2*). This is likely caused by the overexpression of IL-5R subunits in HEK293 cells, which— as we have seen with βc— may result in receptor activation in the absence of ligand[78].

Interestingly, IP/IB analysis with anti-IL-5Rα antibodies shows the presence of two distinct bands in both unstimulated and stimulated HEK293 cells (Fig. 5B, *bottom panel*). Previous findings in that Moczygemba lab found that the upper, higher molecular weight band is glycosylated IL-5Rα, whereas the lower band is most likely an immature, unglycosylated form of IL-5Rα. Following treatment a glycosylation inhibitor, Peptide-*N*-Glycosidase F (PNGaseF), the 'smeary' upper band becomes a tight band that shifts to a faster migrating band, co-migrating with the lower band[60]. Thus, it is likely possible that the higher molecular weight band in our HEK293 cells is most likely glycosylated IL-5Rα, whereas the lower molecular weight band is most likely an immature unglycosylated form of IL-5Rα.

In conclusion, the presence of ubiquitination in TF1 cells confirms that IL-5Rα is in fact ubiquitinated, much like βc, in cells that endogenously express the receptor complex. Additionally, HEK293 cell lines, transduced with WT IL-5R, show comparable levels of ubiquitination and receptor complex expression, allowing us to use this cell line as the model cell system. Consequently, having confirmed IL-5Rα ubiquitinated, we first sought to determine potential ubiquitination sites through sequence analysis of the receptor, in comparison to βc.

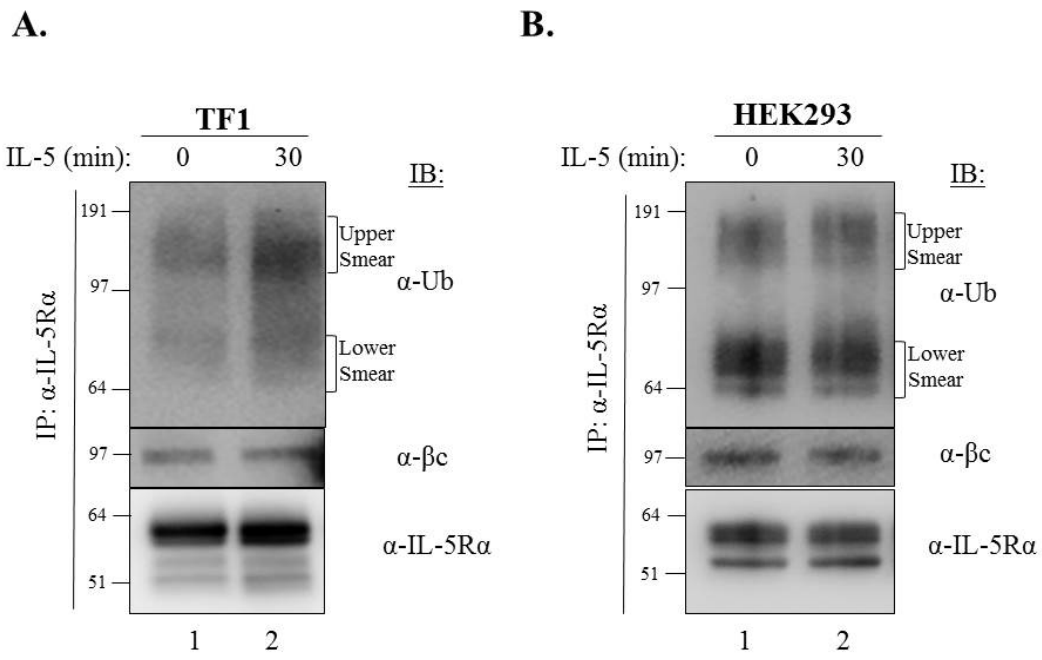


FIGURE 5. **Ubiquitination of WT IL-5Rα in TF1 and HEK293 cell lines.** *A.* TF1 cells endogenously expressing IL-5R complex were left unstimulated (0 min) or stimulated with 10 ng/ml of IL-5 for 30 minutes. Cell lysates were prepared using RIPA buffer, IP with anti-IL-5Rα mAbs, and analyzed by IB with indicated antibodies (n = 3). *B.* Same as in *A,* using HEK293 cells stably transduced with WT IL-5R complex (n = 5).

Construction of IL-5Rα K-to-R Mutants

Established as sole ubiquitination sites, sequence analysis of the cytoplasmic signaling portion of βc indicated the presence of sixteen lysine residues. Through the construction of several mutant βc receptors with different combinations of lysine-to-arginine (K-to-R) mutations, previous research in the Moczygemba lab identified a cluster of three lysine residues, adjacent or within the Box 1 motif, whose presence were required for not only JAK1/2 binding to βc, but also subsequent βc ubiquitination[33]. Having now also confirmed IL-5Rα ubiquitination, we first sought to determine if there are any sequence similarities in the distribution of lysine residues between βc and IL-5Rα. Sequence analysis of the first 22 amino acids in the IL-5Rα cytoplasmic domain—proximal to the cell membrane— indicated a similar K-rich region in the vicinity of the Box 1 motif with comparable spatial distribution to βc.

Four lysine residues were located within or adjacent to the Box 1 motif (Fig. 6A), specifically Lys^{363}, Lys^{370}, Lys^{379}, and Lys^{383}. To determine whether the presence of these K residues were also involved in IL-5Rα ubiquitination, site-directed mutagenesis was performed to substitute each lysine residue with another positively charged amino acid, arginine (R), using IL-5Rα cDNA cloned in a lentiviral vector (Fig. 6B). Since both lysine and arginine are positively-charged residues, we predicted that protein folding of IL-5Rα would not be significantly disrupted following the K-to-R mutation, as observed with similar experiments with βc[33]. At the same time, lysine residues are canonical sites of ubiquitination, whereas arginine residues are not. Six IL-5Rα mutant constructs were generated, each expressing various combinations of K-to-R mutations to

devise single (K1R; K2R), double (K2,3R; K3,4R), triple (K2-4R) and quadruple (K1-4R) mutant IL-5Rα cell lines (Fig. 6B). Lentiviral particles expressing each mutant IL-5Rα receptor were stably-transduced into a HEK293 cell line expressing only WT βc (Fig. 7), and double receptor expression of sorted cells was evaluated by flow cytometry analysis of anti-IL-5Rα-FITC (x-axis) and anti-βc-PE (y-axis) labeled cells (Fig. 8).

Relative to WT expression of both receptor chains (90.8%), specific mutant cell lines had comparable percentages of double-positive cells (Fig. 8)—K1R (97.7%), K2R (94.2%), and K3,4R (93.5%)— allowing us to continue in examining the physiological effects of the K-to-R mutations in the mutant cell lines. Although certain mutants expressed reduced double-positive cells relative to WT—K2,3R (85.7%), K2-4R (75.0%), and K1-4R (86.8%)—this varied expression of double receptors may be caused by 1) inefficient transduction 2) short residence time on cell surface 3) ineffective trafficking to the cell surface.

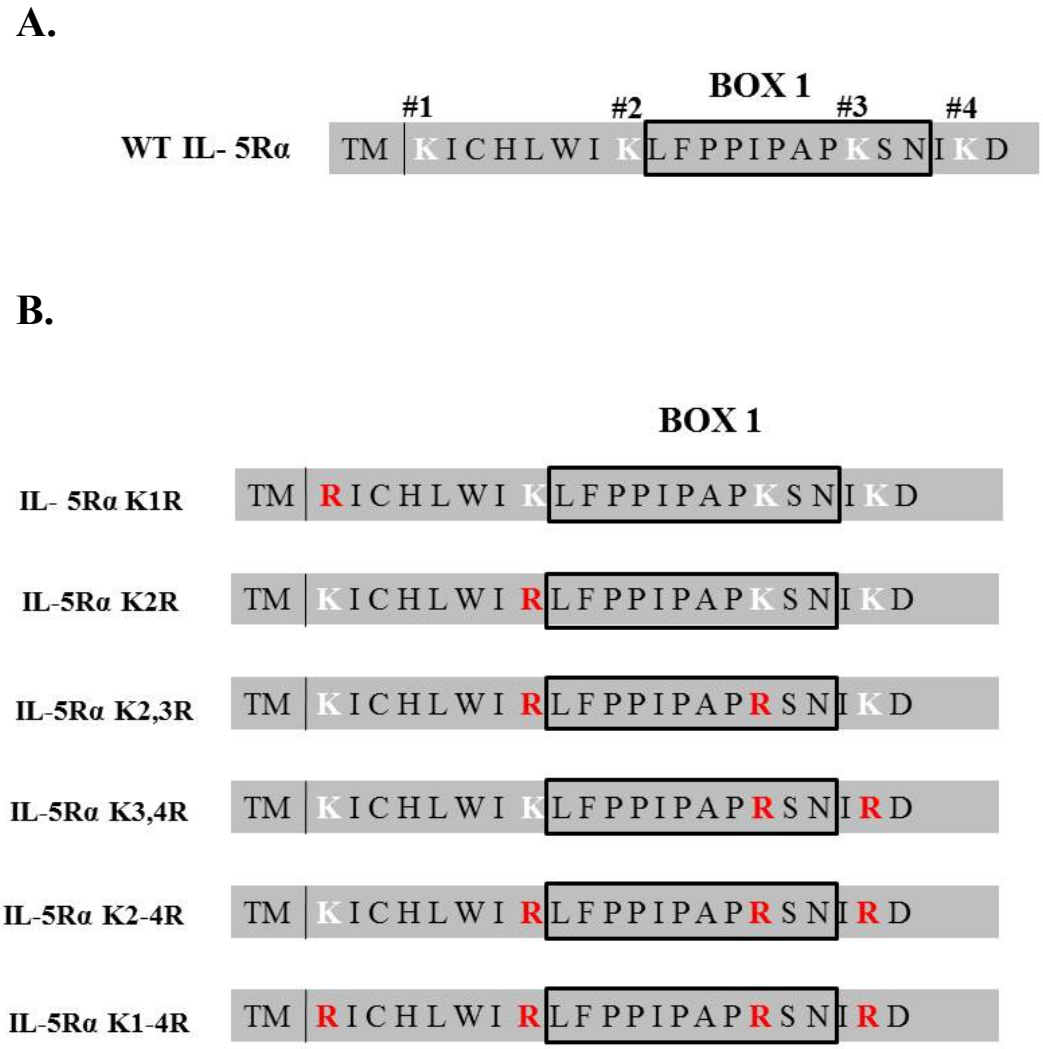


FIGURE 6. **Generation of HEK293 expressing WT and mutant IL-5Rα receptors.** *A,* schematic illustration of the four intracellular lysine residues on WT IL-5Rα located within or adjacent to the Box 1 motif. *B,* illustration of the six IL-5Rα mutant constructs, with different combinations of lysine (K) residues substituted to arginine residues (R) following site-directed mutagenesis. All IL-5Rα constructs (WT and mutants) were cloned in pLentivector, and stably transduced into βc expressing HEK293 cells, after confirming correct K-to-R mutations with sequence analysis. Dual-receptor expressing cell lines were selected in the presence of blasticidin (6 µg/ml) and Geneticin (0.85 mg/ml), and then further sorted to enrich the presence of dual expressing cells. Box indicates location of Box 1 motif; TM, transmembrane domain.

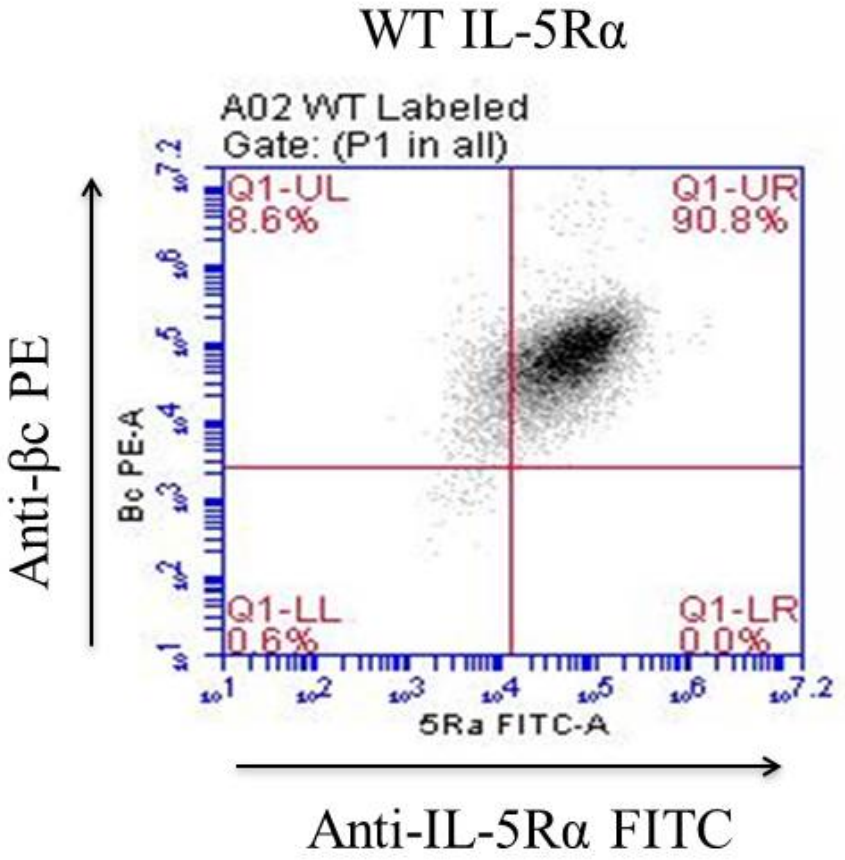


FIGURE 7. **Expression of WT IL-5R heterodimer in HEK293.** Flow cytometry analysis of sorted, dual receptor-expressing HEK293 cells transduced with WT IL-5Rα and βc. Cells were labeled with anti-IL-5Rα-FITC (x-axis) and anti-βc-PE (y-axis) antibodies. Upper right corner indicates presence of double-positive cells with dual receptor expression, i.e. both IL-5Rα and βc.

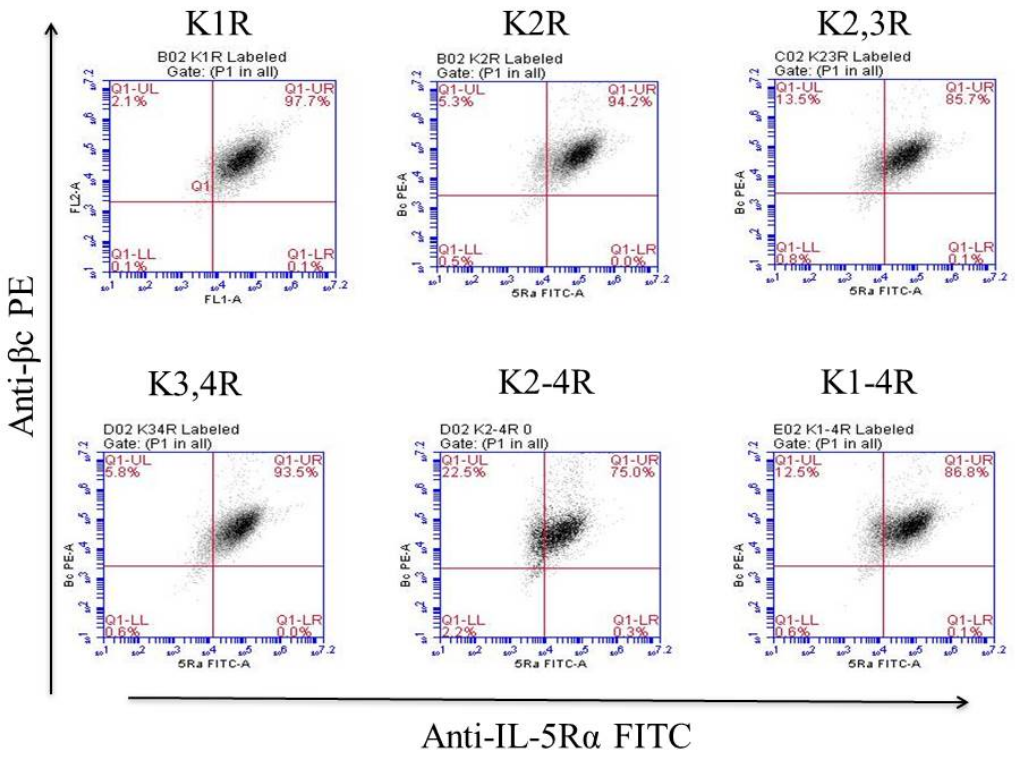


FIGURE 8. **Expression of mutant IL-5R heterodimer in HEK293.** Flow cytometry analysis of sorted, dual receptor-expressing HEK293 cells transduced with mutant IL-5Rα and WT βc. Cells were labeled with anti-IL-5Rα-FITC (x-axis) and anti-βc-PE (y-axis) antibodies. Upper right corner indicates percentage of double-positive cells with dual receptor expression, i.e. both mutant IL-5Rα and WT βc.

Accumulation of IL-5Rα Cell Surface Expression in Certain IL-5Rα K-to-R Mutants

First, we wanted to determine if the IL-5Rα K-to-R mutations affected functional properties of the receptor, such as cell surface expression under steady state conditions. Flow cytometry was used to test our hypothesis that certain mutations would alter the cell surface expression of the receptor. After labeling unstimulated cells with anti-IL-5Rα-FITC antibodies, cell surface expression was measured and we repeated the experiment multiple times to obtain mean fluorescence intensity across cell lines. Interestingly, our results showed that single and double K-to-R mutations (K1R; K2R; K2,3R; K3,4R) resulted in higher cell surface expression relative to WT (Fig. 9; Table 2). In particular, mutant K3,4R had significantly higher cell surface expression relative to WT (p-value: 0.0006). On the other hand, triple and quadruple K-to-R mutations exhibited comparable (K2-4R) or decreased (K1-4R) cell surface expression relative to WT expression, although these results were not significant (Fig. 9; Table 2).

It would be interesting to see if this pattern of cell surface expression was maintained following IL-5 stimulation. Thus, the focus of our remaining study will evaluate the physiological effects of K-to-R IL-5Rα mutations in cells under steady state conditions, and following IL-5 stimulation.

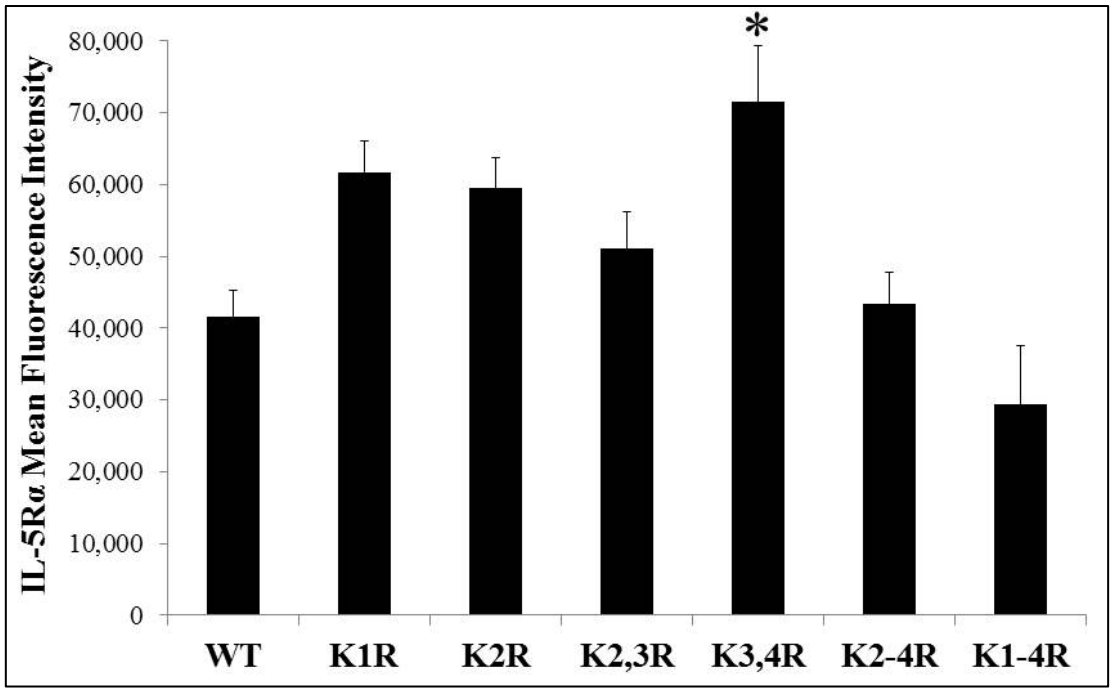


FIGURE 9. **Cell surface expression of WT and mutant IL-5Rα receptors**. Cell surface IL-5Rα in unstimulated WT and mutant cells were labeled with anti-IL-5Rα-FITC antibodies and measured by flow cytometry. Cell surface expression is shown as mean fluorescence intensity (MFI) ± standard error (S.E.). (*) Represents statistically significant data.

TABLE 2. Mean Fluorescence Intensity ± S.E. of WT and Mutant IL-5Rα

IL-5Rα Cell Line	Mean Fluorescence Intensity	Standard Error	Sample Size
WT	41,639.1	3,714.0	19
K1R	61,715	4,272	4
K2R	59,471	4,241	7
K2,3R	51,097	5,040	6
K3,4R	71,521	7,748	8
K2-4R	43,455	4,292	7
K1-4R	29,320	8,181	4

Ubiquitination Levels Directly Correlate with Cell Surface Expression of IL-5Rα Mutants

Previous studies with βc have shown that a cluster of three lysine residues, within the vicinity of the Box 1 motif, is crucial for receptor ubiquitination due to the complete loss of IL-5-dependent βc ubiquitination following K-to-R mutations of these three residues[33]. Based on these results, we hypothesized a similar inverse relationship between IL-5Rα ubiquitination and cell surface expression, i.e. mutants with higher cell surface expression would exhibit decreased ubiquitination, whereas mutants with lower cell surface expression would exhibit increased ubiquitination.

We analyzed total levels of proteins associated with IL-5Rα by immunoprecipitating (IP) IL-5Rα and subsequently immunoblotting (IB) with antibodies against our proteins of interest. Similar to our experiments with TF1 and WT HEK293 cells, upper and lower smears were readily visible following IB with anti-ubiquitin antibodies (Fig. 10, *top panel*). Stripping the membranes and blotting with anti-IL-5Rα (Fig. 10, *second panel*) and anti-βc (Fig. 10, *third panel*) antibodies confirmed the identity of the upper and lower smears as co-immunoprecipitating ubiquitinated-βc and ubiquitinated IL-5Rα, respectively.

To our surprise, we found ubiquitination of both smears to be higher in particular mutants; specifically the IL-5Rα mutants that had increased cell surface expression in previous flow cytometry experiments. In unstimulated cells, single and double mutants had higher IL-5Rα ubiquitination relative to WT (Fig. 10, *top panel, lane 1 vs. lanes 3, 5, and 7*). Following thirty-minutes of IL-5 stimulation, an increase in ubiquitination is

apparent in WT (Fig. 10, *top panel, lanes 1 vs. 2)*, K2R (Fig. 10, *top panel, lanes 3 vs. 4*), and K2,3R (Fig. 10, *top panel, lanes 5 vs. 6*). Interestingly, K3,4R not only had the highest degree of ubiquitination in unstimulated cells, but also lacked a clear increase in ubiquitination following IL-5 stimulation (Fig. 10, *top panel, lanes 7 vs. 8*). Since the K3,4R mutant also had the highest cell surface expression in steady state conditions, it is possible that ubiquitin has a novel role in stabilizing cell surface expression in IL-5Rα.

Furthermore, it appears that mutants with decreased cell surface expression seem to also have decreased ubiquitination under steady state conditions. Specifically, K2-4R (Fig. 10, *top panel, lane 9*) and K1-4R (Fig. 10, *top panel, lane 11*) have ubiquitination expression significantly less than single and double mutants, with ubiquitination in triple and quadruple K-to-R mutants returned to WT levels (Fig. 10, *top panel, lane 1*).

Next, we studied the effects of K-to-R mutations on cell signaling by stripping the membrane and blotting with anti-JAK2 antibodies (Fig. 10, *bottom panel*). There is an obvious increase in JAK2 association with IL-5Rα following IL-5 stimulation in WT (Fig. 10, *bottom panel, lanes 1 vs. 2*), K2R (Fig. 10, *bottom panel, lanes 3 vs. 4*), K2,3R (Fig. 10, *bottom panel*, l*anes 5 vs. 6*), and K3,4R (Fig. 10, *bottom panel, lanes 7 vs. 8*). Furthermore, there is a direct correlation between the extent of JAK2 association with IL-5Rα to the degree of IL-5Rα ubiquitination. Interestingly, in addition to less JAK2 association under basal conditions in K2-4R and K1-4R mutants, there also was no apparent increase in JAK2 association following IL-5 stimulation (Fig. 10, *bottom panel, lanes 9 vs. 10; lanes 11 vs. 12*). These results are most likely caused by the complete, or nearly complete, loss of lysine residues in the Box 1 motif. Although the canonical P-*X*-

P residues within Box 1 were still intact within K2-4R and K1-4R, the loss of three or all four lysine residues within this region may have prevented optimal JAK2 association with the receptor, as was found with βc[33].

In sum, these results formulate the basis of a unique model for IL-5Rα, whereby ubiquitin is linked to its cell surface stability. This is in stark contrast to previous research with βc, which indicated an inverse relationship between βc-ubiquitination and cell surface expression due to the induction of receptor internalization mechanisms triggered by βc-ubiquitination[33]. Although, JAK2 expression appears to mirror IL-5Rα ubiquitination in WCL studies, it will be interesting to see if JAK kinase binding is necessary for optimal IL-5Rα ubiquitination, as found with our βc studies.

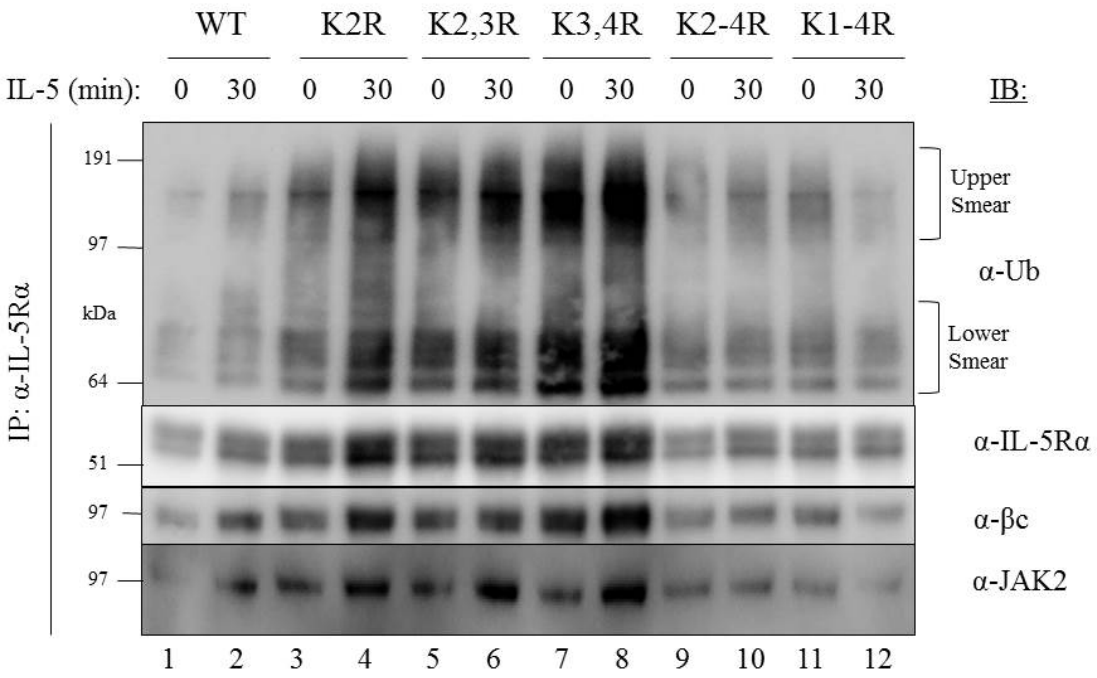


FIGURE 10. **Differential degrees of ubiquitination in IL-5Rα K-to-R mutants.** Stably transduced HEK293 cells expressing WT or mutant IL-5Rα receptors were stimulated with 10 ng/ml of IL-5 for thirty minutes. Lysates were IP with anti-IL-5Rα-mAbs and analyzed by IB with antibodies indicated on right panel.

Dysregulated Cell Surface Expression of IL-5Rα Mutants Partially Explained by Degradation Rates

Due to the similar patterns among sets of mutants, we decided to further compare the consistently highest expressing mutant (K3,4R) and lowest expressing mutant (K1-4R) to WT IL-5Rα for the remainder of the study. We first sought to examine the underlying molecular mechanism of increased or decreased cell surface expression in K3,4R and K1-4R, respectively, by mirroring experiments performed with the βc mutant that also exhibited impaired cell surface expression[33]. Three possible explanations could account for the impaired cell surface expression seen in IL-5Rα mutants: 1) delayed or accelerated protein degradation in K3,4R and K1-4R, respectively 2) decreased or increased endocytosis in K3,4R and K1-4R, respectively 3) accelerated or delayed recycling of K3,4R and K1-4R, respectively.

First, we sought to determine if delayed or accelerated protein degradation was responsible for cell surface expression of K3,4R and K1-4R, respectively, as compared to WT IL-5Rα. To test this hypothesis, we compared degradation rates among the three receptors by pretreating cells with cycloheximide to halt protein synthesis, and then "chased" IL-5Rα protein levels at various time points following IL-5 stimulation by Western blot (Fig. 11A). IB with anti-IL-5Rα antibodies indicates an obvious delay in K3,4R degradation, relative to WT (Fig. 11A, *top panel, lanes 2-4 vs. 6-8*). On the other hand, K1-4R appears to have accelerated degradation rates, relative to WT (Fig. 11A, *top panel, lanes 2-4 vs. 10-12*).

We repeated this experiment several times, and generated degradation curves by measuring IL-5Rα band densities from IBs after normalizing to corresponding actin band densities (Fig. 11B). K3,4R had a significantly slower degradation relative to WT following 30 minutes of IL-5 stimulation (p-value: 0.0083), with 96% of starting K3,4R protein levels remaining compared to 77% of starting WT protein level. This trend of delayed K3,4R degradation continued at 60 minutes of IL-5 stimulation, with 86% of K3,4R protein levels remaining relative to 74% of WT, although differences in degradation were not significant.

Even though at 30 minutes IL-5 stimulation, K1-4R had delayed degradation relative to WT—with 86% of starting protein levels remaining—the mutant appears to have had accelerated degradation rates by 60 minutes of IL-5 stimulation, with 68% of starting K1-4R protein levels remaining relative to 74% of remaining WT protein. While not statistically significant, reduced K1-4R cell surface expression maybe partially attributed to faster degradation rates. These data indicates that K3,4R mutant resulted in a ~10% slower turnover rate, whereas K1-4R mutant resulted in a ~10% faster turnover rate. Yet, these changes in turnover do not entirely explain the strong differences in cell surface expression seen in these two mutants, highlighting the possibility of additional regulatory mechanisms in effect.

During previous studies with βc, a recycling inhibitor, monensin[81,82], was used to determine if increased cell surface expression of mutant βc was due to faster recycling back to the cell surface. It was hypothesized that if this mechanism was responsible for increased cell surface expression, monensin treated βc mutants would result in increased

βc-positive intracellular vesicles with a corresponding reduction in cell surface levels. Due to negligible effects of monensin on the mutant βc receptor, impaired recycling was discounted as a possible mechanism of increased cell surface expression[33]. We hypothesized that the same would be true for IL-5Rα receptors, deciding to focus next on endocytosis as another contributing mechanism of increased or decreased cell surface expression of K3,4R and K1-4R, respectively.

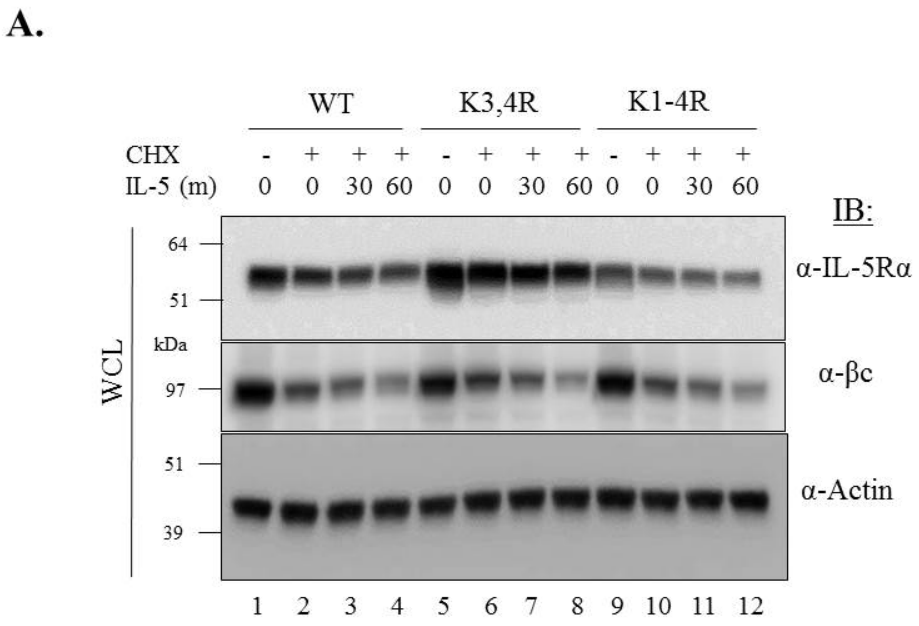


FIGURE 11. **Partial delay in degradation in K3,4R IL-5Rα.** *A,* WT, K3,4R, and K1-4R IL-5Rα were either left untreated (−) or treated (+) with 10 μg/ml of cycloheximide (CHX) for 1h, followed by harvesting cells at the specified time points. Whole cell lysates (50 μg/lane) were analyzed with anti-IL-5Rα, anti-βc, and anti-actin antibodies.

B.

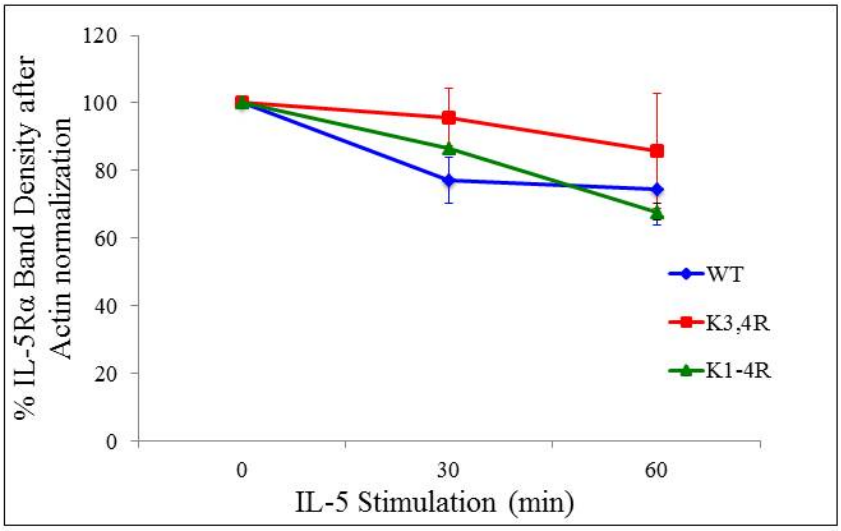


FIGURE 11 CONTINUED. **Partial delay in degradation in K3,4R IL-5Rα.** *B,* Degradation curves were generating by measuring IL-5Rα band densities from IBs and normalized against corresponding actin band densities. The IL-5Rα band density at the 0-h time point (following 1h pretreatment with cycloheximide) was designated as 100% and percent densities of later time points were calculated based on this assumption. Averages of percent densities were used for WT (n = 5), K3,4R (n = 4), and K1-4R (n = 2), with corresponding standard deviation.

IL-5Rα Mutants Display Impaired Endocytosis

The second mechanism we interrogated was whether decreased or increased endocytosis in K3,4R and K1-4R, respectively, explained the differences in cell surface expression. To test this hypothesis, we performed a cell surface biotinylation assay in WT, K3,4R, and K1-4R IL-5Rα expressing cells. All cell surface proteins were first biotinylated and then precipitated with biotin-specific neutravidin beads, before separating proteins on an LDS-PAGE and blotting with antibodies to our proteins of interest. Following IB with anti-IL-5Rα antibodies, unstimulated cells appear to confirm previous flow cytometry and WCL studies (Fig. 12A). Specifically, at steady-state conditions, K3,4R (Fig. 12A, *lane 3*) appears to have increased IL-5Rα cell surface expression relative to WT (Fig. 12A, *lane 1*), whereas K1-4R (Fig. 12A, *lane 5*) exhibits decreased IL-5Rα cell surface levels. After thirty minutes of IL-5 stimulation, differences across the three cell lines are apparent.

In order to better determine the amount of IL-5Rα remaining on the cell surface following IL-5 stimulation, densitometry was performed to obtain band densities at thirty minutes of stimulation, using steady state conditions as an initial reference. Densitometry revealed that 44.8% of WT, 84.8% of K3,4R and 61.77% of K1-4R IL-5Rα remained on the cell surface following thirty minutes of IL-5 stimulation (Fig. 12B). These data indicate that the increased cell surface expression seen in K3,4R may in fact be due to delayed endocytosis, as seen in our previous βc studies[33]. Although the mechanism behind increased cell surface expression maybe comparable between IL-5Rα and βc, it is very interesting to note that our IL-5Rα mutant with increased cell surface

expression also had increased ubiquitination, whereas the βc mutant with increased cell surface expression was ubiquitin-deficient[33].

To gain further insight into the role of ubiquitin just on receptors located on the cell surface, we repeated the experiment and blotted with additional antibodies against our proteins of interest (Fig. 12C). We hypothesized that IP/IB analysis of only cell surface proteins will confirm steady state trends in our previous results with whole cell lysates (Fig. 10)—specifically the role of ubiquitination in stabilizing cell surface expression of IL-5Rα receptors.

Following precipitation with neutravidin beads, we first immunoblotted with anti-IL-5Rα antibodies (Fig. 12C, *top panel*). Although not strongly visible due to exposure, a difference among the three cell lines in unstimulated conditions is apparent, with K3,4R having increased cell surface expression (Fig. 12C, *top panel, lane 3*) and K1-4R having decreased cell surface expression (Fig. 12C, *top panel, lane 5*), relative to WT (Fig. 12C, *top panel, lane 1*). Following thirty minutes of IL-5 stimulation, a decrease in cell surface IL-5Rα expression is somewhat present, yet not as obvious as the previous western blot, again most likely due to exposure.

After stripping the membrane and blotting within anti-ubiquitin antibodies, the ubiquitination of all cell surface proteins at steady-state conditions is visible (Fig. 12C, *second panel*). Mirroring cell surface IL-5Rα expression, ubiquitination of all cell surface proteins appear to be increased in K3,4R (Fig. 12C, *second panel, lane 3*) and decreased in K1-4R (Fig. 12C, *second panel*, *lane 5*), relative to WT IL-5Rα (Fig. 12C, *second panel, lane 1*), under steady state conditions. The most startling finding is the

obvious, and dramatic, deubiquitination of all cell surface proteins following thirty minutes of IL-5 stimulation (Fig. 12C, *second panel, lane 2, 4, 6*). This deubiquitination event is not caused by loss of cell surface receptors, since IL-5Rα expression is still present (Fig. 12C, *top panel*). Although there is a slight reduction in cell surface IL-5Rα receptors following IL-5 stimulation, this decrease is not sufficient to explain the complete loss of ubiquitination. Based on previous WCL IP/IB studies and this cell surface biotinylation experiment, IL-5Rα is indeed ubiquitinated prior to IL-5 stimulation, and a key first step in IL-5 signaling may likely involve a deubiquitination event of all cell surface proteins.

Next, we confirmed βc cell surface expression after stripping the membrane and blotting with anti-βc antibodies (Fig. 12C, *third panel*). βc expression appears to correlate with IL-5Rα expression, with K3,4R having higher expression and K1-4R having lower expression of βc relative to WT, although differences in βc expression is not as visibly apparent.

Lastly, we sought to confirm βc activation at the cell surface by stripping the membrane and blotting with anti-phosphorylated-tyrosine antibodies (Fig. 12C, *fourth panel*). Indeed, IL-5-dependent JAK kinase-activation was also confirmed at the cell surface, with increased tyrosine phosphorylation evident only after 30 minute IL-5 stimulation (Fig. 12C, *fourth panel*, *lanes 2, 4, 6*). Although reduced tyrosine phosphorylation levels were detected in K1-4R (Fig. 12C, *fourth panel*, *lane 6*), this is most likely caused by the decreased levels of βc that co-immunoprecipitated with the

immune complex (Fig. 12C, *third panel*, *lane 6*). Interestingly, the deubiquitination event of cell surface proteins appears to occur alongside JAK kinase signaling.

Taken together, cell surface biotinylation assay provide results that the mechanism of increased cell surface expression in K3,4R and K1-4R may in fact be due to impaired endocytosis (Fig. 12A, 12B). Surprisingly, it also appears that not only are cell surface proteins ubiquitinated in steady state conditions, a massive IL-5-dependent deubiquitination event occurs following IL-5 stimulation (Fig. 12C, *second panel*).

A.

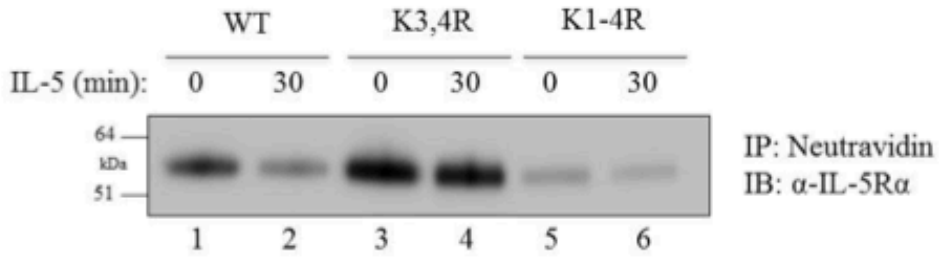
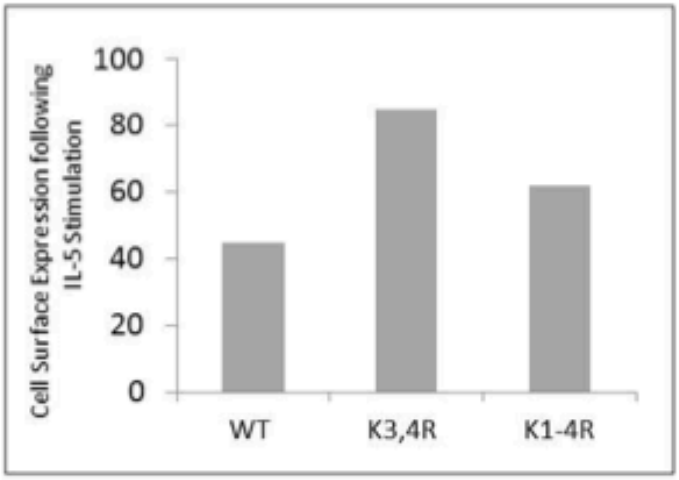


FIGURE 12. **Dramatic de-ubiquitination event following IL-5 stimulation of cell surface proteins.** *A,* WT, K3,4R, and K1-4R IL-5Rα expressing HEK293 cell lines were cell surface labeled with the nonpermeable sulfo-NHS-SS-biotin reagent. Briefly, labeled cells were either left unstimulated or stimulated with 10 ng/ml of IL-5 for thirty minutes. Following cell lysis, lysates incubated with neutravidin beads and all biotinylated cell-surface analyzed by IP with Neutravidin-HRP antibodies, and blotted with anti-IL-5Rα antibodies.

B.



C.

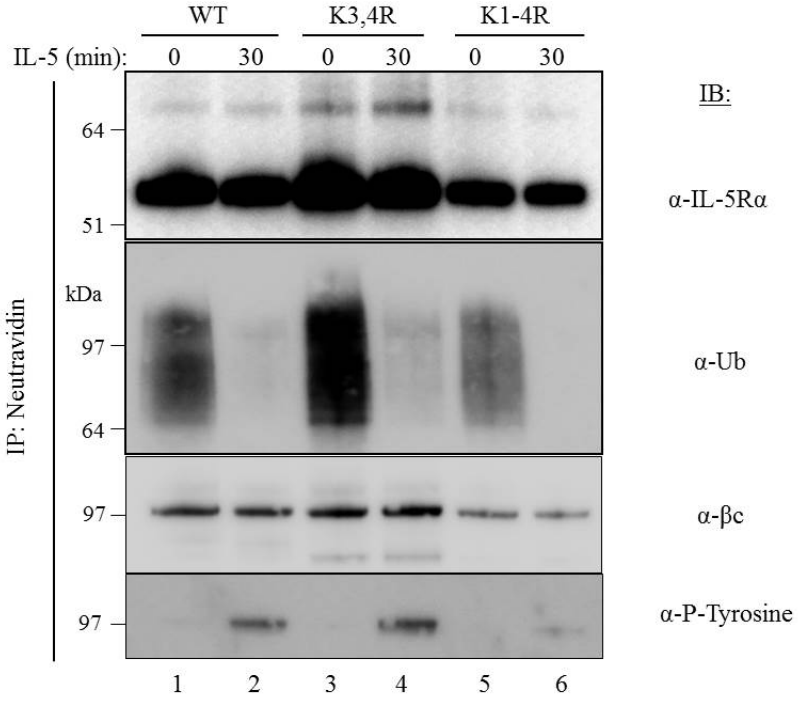


FIGURE 12 CONTINUED. **Dramatic de-ubiquitination event following IL-5 stimulation of cell surface proteins.** *B,* densitometry analysis to quantify the percentage of WT and mutant IL-5Rα receptors remaining on the cell surface following IL-5 stimulation, after using steady state conditions as an initial reference. *C,* repeated the experiment described in *A*, with the membrane stripped and blotted with additional antibodies against proteins of interest.

JAK1 and JAK2 are Necessary for IL-5Rα Ubiquitination

Past research has shown the necessity of the Box 1 motif for JAK kinase binding to Type I and II cytokine receptors, due to reduced binding and activation of JAKs following mutation of these proline residues[38-40]. Previous studies with βc demonstrated that the presence of JAK1 and JAK2 were required for optimal βc ubiquitination, and subsequent signal transduction[33]. Therefore, we decided to investigate whether a similar relationship was present for WT IL-5Rα. We used combinatorial RNAi to simultaneously silence both JAK1 and JAK2 in HEK293 cells stably expressing WT IL-5R complex. If JAK kinases were involved in optimizing IL-5Rα ubiquitination, we hypothesized that JAK knockdown cells will result in a corresponding reduction in IL-5Rα ubiquitination.

First, we wanted to confirm successful knockdown of the JAK proteins (Fig. 13). Relative to scramble control, JAK1 had 77.1% knockdown efficiency at steady state conditions (Fig. 13, *top panel, lane 3 vs. 5*) and 63.9% knockdown efficiency at 30 minute IL-5 stimulation (Fig. 13, *top panel, lane 4 vs. 6*). Furthermore, relative to scramble control, JAK2 had 79.6% knockdown efficiency at steady state conditions (Fig. 13, *second panel, lane 3 vs. 5*) and 65.9% knockdown efficiency at 30 minute IL-5 stimulation (Fig. 13, *second panel, lane 4 vs. 6*).

After confirming successful knockdown, we proceeded to examine the ubiquitination of IL-5Rα by our standard IP/IB analysis in the absence or presence of IL-5. We repeated the knockdown experiment and lysates were immunoprecipitated with anti-IL-5Rα antibodies and then blotted with IL-5Rα to confirm receptor expression

(Fig. 14, *top panel*). Relative to scramble control, IL-5Rα expression was not significantly altered in JAK1/2 knockdown cells.

Next we stripped the membrane and blotted with anti-ubiquitin antibodies. Our data revealed that in the absence of JAKs, IL-5Rα ubiquitination was partially reduced in steady state conditions relative to scramble control (Fig. 14, *bottom panel, lanes 3 vs. 5*). This finding is in contrast with previous βc studies, which found a negligible effect of basal βc ubiquitination in the absence of JAK1/2[33]. Similar to βc results, loss of JAK1/2 expression resulted in a partial decrease in IL-5Rα ubiquitination relative to scrambled siRNA control (Fig. 14, *bottom panel, lanes 4 vs. 6*). Overall, JAK1 and JAK2 gene silencing partially prevented IL-5Rα ubiquitination, although residual ubiquitination of the receptor was still present. Thus, other ubiquitination mechanisms must be involved in supporting a model whereby JAK kinases are required for optimal, but not entire, IL-5Rα ubiquitination.

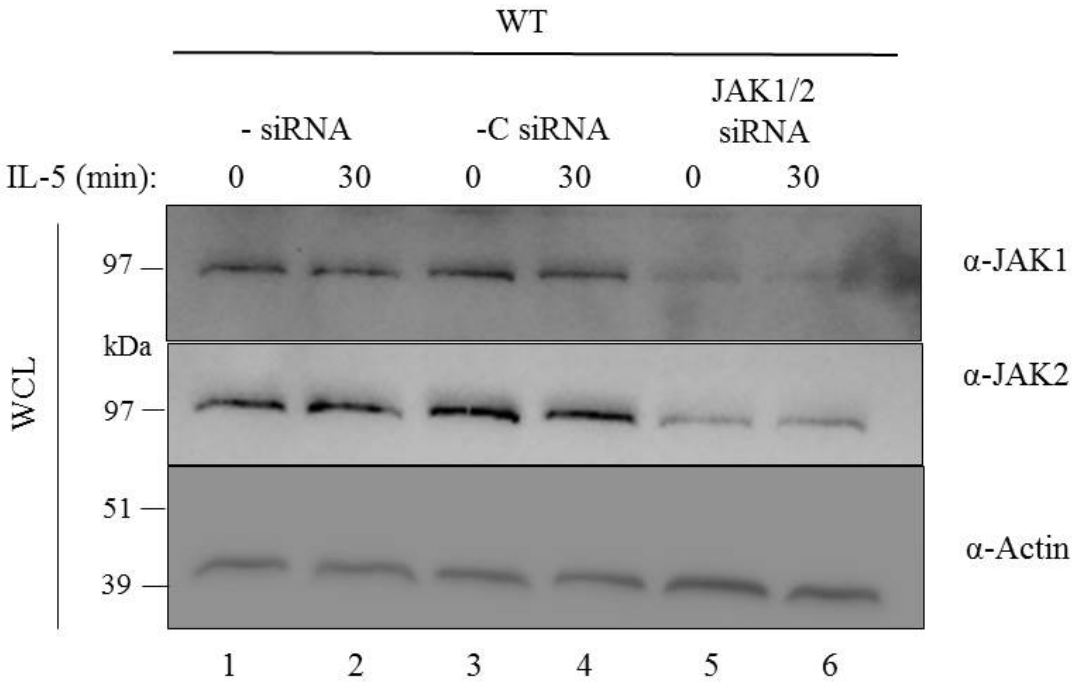


FIGURE 13. **Successful JAK1 and JAK2 knockdown in HEK293 WT IL-5R cells.** HEK293 cells expressing WT IL-5Rs were transfected with both JAK1 and JAK2 siRNAS, or scrambled –C siRNAs for 48h as described under "Experimental Procedures." Whole cell lysates, in the absence or presence of 10ng/ml IL-5, were analyzed by IB with both anti-JAK1 and anti-JAK2 antibodies to determine knockdown efficiencies of JAK1 and JAK2, respectively. Band densities were normalized using corresponding actin band densities. –siRNA was designated as 100% to determine percent knockdown of JAK1 and JAK2.

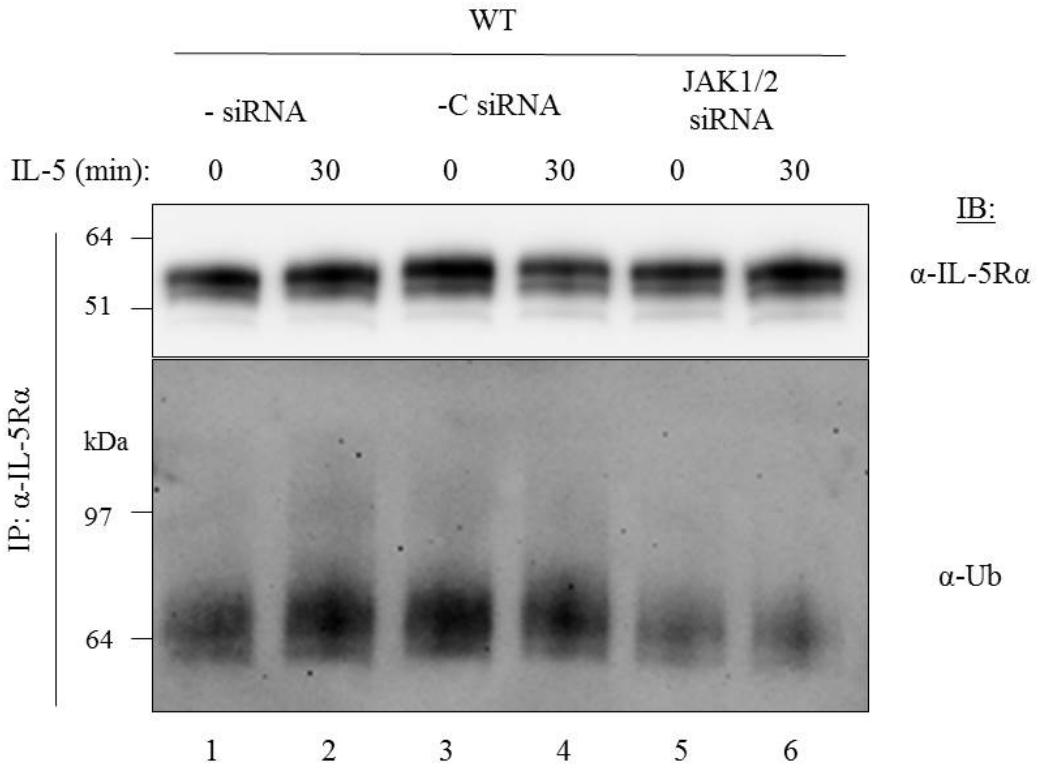


FIGURE 14. **JAK1 and JAK2 are partially involved in regulating IL-5Rα ubiquitination.** HEK293 cells expressing WT IL-5Rs were transfected with both JAK1 and JAK2 siRNAS, or scrambled –C siRNAs for 48h as described under "Experimental Procedures." Whole cell lysates, in the absence or presence of 10ng/ml IL-5, were further analyzed by IB with the indicated antibodies to determine the role of JAK1/2 in IL-5Rα ubiquitination.

JAK-Mediated IL-5Rα Tyrosine Phosphorylation Dependent on Two Key Lysine Residues

We chose to conclude our studies by examining the potential impairment of K-to-R mutations on IL-5 induced cell signaling. Our previous studies with βc receptor showed reduced JAK binding and signaling if lysine residues within or surrounding the Box 1 motif were mutated to arginine[33]. Thus, we hypothesized that our IL-5Rα mutants would exhibit impaired JAK binding and signaling, specifically the K1-4R mutant due to loss of all four lysine residues. To this end, we overexpressed JAK2, using pcDNA3 in the presence of GeneJammer, in both WT and K3,4R IL-5Rα cells. In light of our knockdown experiments, we hypothesized that overexpression of JAK2 would results in enhanced IL-5Rα tyrosine phosphorylation. We first confirmed JAK2 overexpression after immunoprecipitating with anti-IL-5Rα antibodies and immunoblotted with anti-JAK2 antibodies (Fig. 15, *first panel*). Although endogenous levels are difficult to detect, overexpression cell lines have obvious JAK2 expression at steady state conditions (Fig. 15, *first panel*, *lanes 3 and 7*) and following thirty minutes of IL-5 stimulation (Fig. 15, *first panel, lanes 5 and 8*).

Although tyrosine phosphorylation could not be detected in either WT or K3,4R cell lines under endogenous conditions, JAK2 overexpression lanes provided interesting results. In JAK2 overexpressing WT cell lines, two major phosphorylated tyrosine bands are clearly visible (Fig. 15, *third panel, lanes 3 and 4*). As expected, the higher molecular weight is confirmed to be tyrosine phosphorylated βc after stripping the membrane and blotting with anti-βc antibodies (Fig. 15, *fourth panel*). Surprisingly, the

lower molecular weight appears to be tyrosine phosphorylation IL-5Rα, the first scientific evidence of the receptor's tyrosine phosphorylation. In JAK2 overexpressing K3,4R cell lines, βc tyrosine phosphorylation was easily detected in cells overexpressing JAK2 (Fig. 15, third panel, *lanes 7 and 8*). However, there is a distinct absence of IL-5Rα tyrosine phosphorylation even though JAK2 was clearly overexpressed. Thus, this reduction in IL-5Rα tyrosine phosphorylation is not due to the inability of JAK2 to associate with the receptor, but rather a consequence of K3,4R mutagenesis. This result highlights a specific role of the third and fourth lysine residues in optimizing JAK2 signaling.

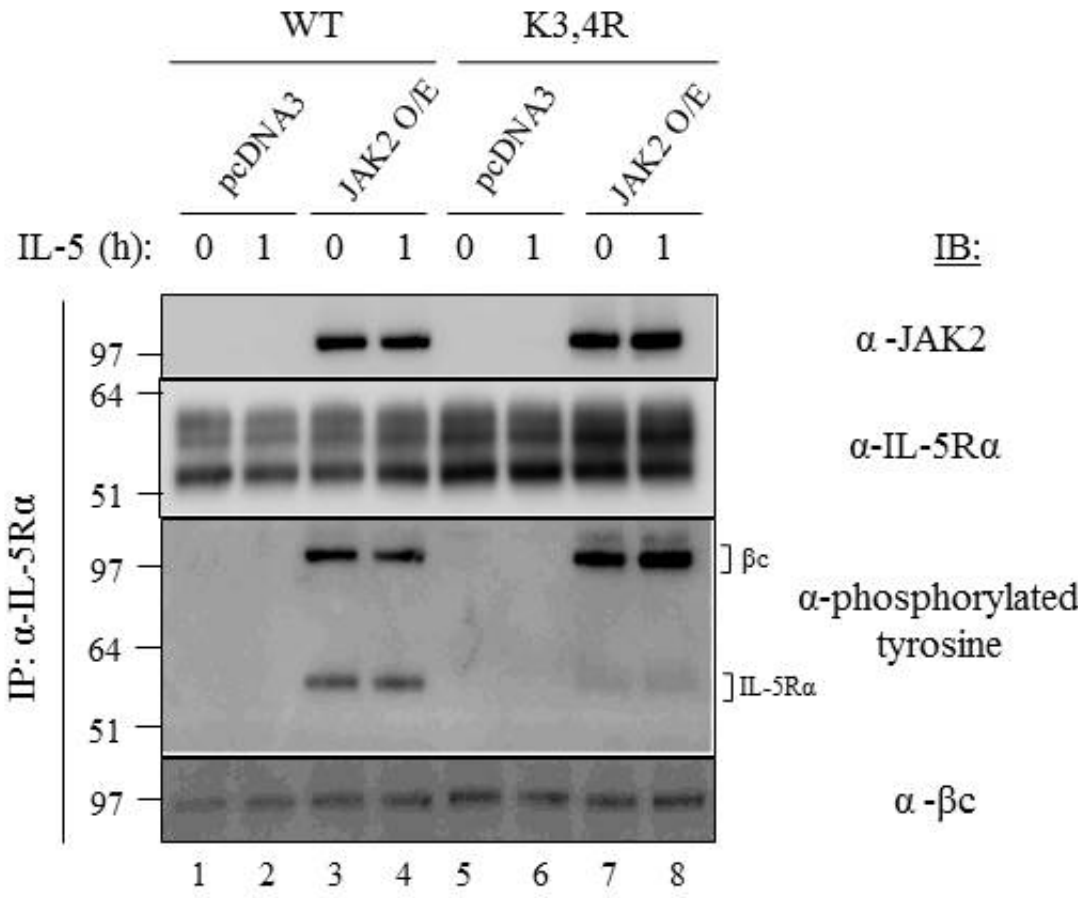


FIGURE 15. **Lack of tyrosine phosphorylation in K3,4R IL-5Rα.** HEK293 cells expressing WT or K3,4R IL-5Rα were transfected with a JAK2 overexpression vector using GeneJammer or with a control vector (pcDNA3). Whole cell lysates from unstimulated and cells stimulated with 10ng/ml IL-5 were immunoprecipitated with anti-IL-5Rα mAbs and analyzed by IB with indicated antibodies. Note the lack of IL-5Rα tyrosine phosphorylation in the third panel, bottom bands (*lanes 3 & 4 vs. lanes 7 & 8*).

DISCUSSION AND CONCLUSIONS

The IL-5 receptor complex is a heterodimer composed of the ligand-specific α-chain, IL-5Rα, and a signaling component, βc, shared with both IL-3Rα and GM-CSFRα[34,35]. Previous studies have shown that IL-5-binding induces βc ubiquitination, which then regulates βc endocytosis and turnover, and this ubiquitination of βc is dependent on the presence of three cytoplasmic lysine residues, Lys^{457}, Lys^{461}, and Lys^{467}[33]. Due to a similar cluster of lysine residues within IL-5Rα, we sought to determine if IL-5Rα is regulated by ubiquitination in our HEK293 cell model system and if so, to identify the functional significance of such ubiquitination.

Based on our current findings, we propose the following model for the ubiquitination of IL-5Rα (Fig. 16): Step 1, under steady state conditions, IL-5Rα is heavily ubiquitinated at the cell surface—by a yet to be determined ubiquitin ligase—with faint levels of βc ubiquitination[33]. Step 2, following ligand stimulation, IL-5Rα is drastically deubiquitinated at the cell surface, perhaps by the USP7 deubiquitinating enzyme (unpublished observations). At the same time, βc is heavily ubiquitinated,[33] most likely by the β-TrCP ubiquitin ligase (unpublished observation), while constitutively associated JAK1/2 kinases are activated to phosphorylate tyrosine residues on both IL-5Rα and βc. Step 3, the activated tyrosine-phosphorylated IL-5R complex is internalized via endocytosis.

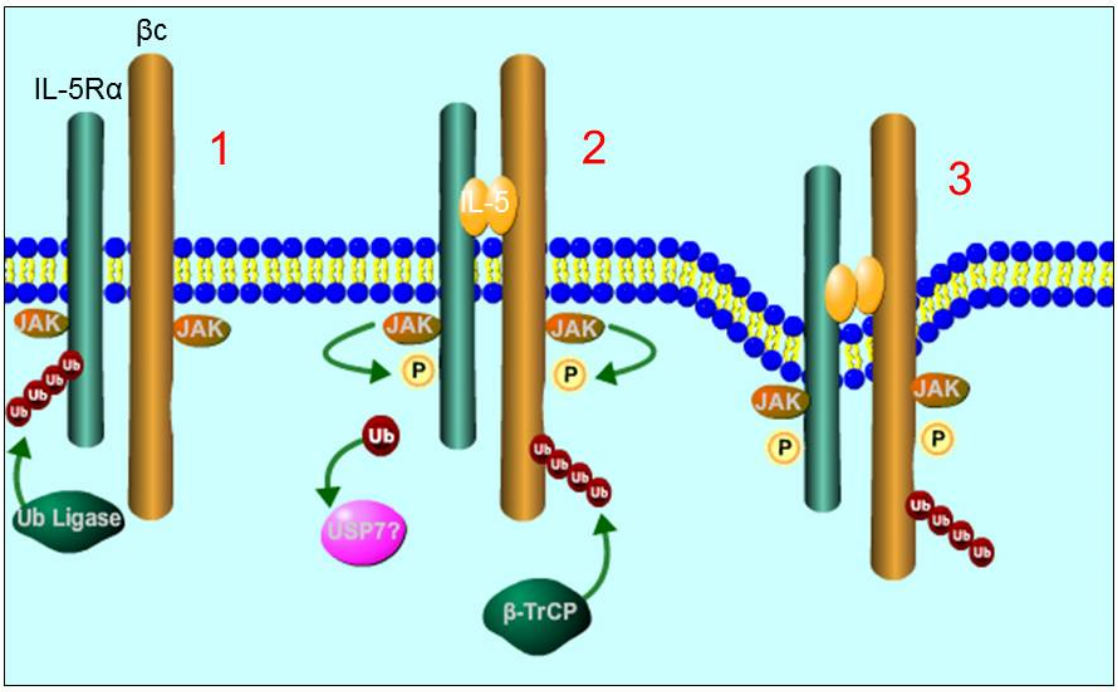


FIGURE 16. **Working model of IL-5Rα and βc at the cell surface prior to and following IL-5 stimulation.** Step 1, under basal conditions, IL-5Rα is highly ubiquitinated at the cell surface, by an unknown ubiquitin ligase, whereas βc has low levels of ubiquitination. Step 2, following IL-5 binding to the receptor complex, IL-5Rα appears to be deubiquitinated by a deubiquitinating enzyme, (perhaps USP7, unpublished observations), while βc is ubiquitinated by the β-TrCP ubiquitin ligase (unpublished observations). In addition, activation of JAK kinases results in tyrosine-phosphorylation of both receptors' cytoplasmic domain. Step 3, the tyrosine-activated receptors with heavily ubiquitinated βc and deubiquitinated IL-5Rα enter the endocytic pathway.

Currently, this is the first report of IL-5Rα ubiquitination to date. Not only is IL-5Rα indeed ubiquitinated, it is also surprising that IL-5Rα is ubiquitinated under steady state conditions. This is in stark contrast to βc, which is dependent on IL-5-induced ubiquitination, with marginal ubiquitination in the absence of IL-5[33]. Our findings with IL-5Rα mutants indicate that the ubiquitination of IL-5Rα stabilizes its expression on the cell surface, prior to ligand stimulation, since mutants with increased basal expression of ubiquitination (Fig. 10) also displayed increased cell surface expression under steady state conditions (Fig. 9). Since βc is not ubiquitinated to the same degree as IL-5Rα under steady state conditions, perhaps its cell surface expression is regulated by another mechanism. It is highly probable that IL-5Rα and βc employ different mechanisms to regulate their cell surface expression, considering each receptor subunit exists independently of each another on the cell surface[36], and dimerize following ligand simulation, a characteristic trait of many cytokine receptors[83,84].

Although previous studies found the cluster of three lysine residues to be crucial in optimizing βc ubiquitination, our data suggests that IL-5Rα lysine residues #1-4 are not likely involved in optimizing IL-5Rα ubiquitination. When all four lysine residues are mutated to arginine in our K1-4R mutant, we see IL-5Rα ubiquitination return to WT levels (Fig. 10). If these four lysine residues were indeed necessary for optimal IL-5Rα ubiquitination, we would expect to see a complete loss of ubiquitination following the quadruple K-to-R mutation. Thus, although both receptors are ubiquitinated, the lysine residues in both IL-5Rα and βc appear to have different roles in receptor ubiquitination.

Although our quadruple mutant returned IL-5Rα ubiquitination to WT levels, our results did in fact indicate that single or double K-to-R mutations—most notably the K3,4R mutant— resulted in significantly increased IL-5Rα ubiquitination, relative to WT, under steady state conditions. Furthermore, ligand stimulation appears to strongly decrease WT IL-5Rα cell surface expression, while cell surface expression of the K3,4R mutant was only slightly reduced, indicating an impairment in IL-5-induced receptor internalization. Therefore, the presence of these lysine residues may have to do with the deubiquitination of IL-5Rα, following IL-5 stimulation, or, contrarily, they might be involved in regulating IL-5Rα endocytosis in steady state conditions.

Interestingly, the increased cell surface expression consistently seen in K3,4R mutant, is most likely caused by the decreased endocytosis of the mutant receptor (Fig. 12). Considering that this mutant also had the highest expression of receptor ubiquitination, our findings suggest that ubiquitination stabilizes IL-5Rα on the cell surface, with a deubiquitination event triggering its endocytosis. These findings are in direct contrast to previous βc results which found that βc ubiquitination is required for its IL-5 induced endocytosis[33]. Additionally, previous βc studies showed that the proteasomal degradation of the signaling, cytoplasmic domain of βc— generating the βc intracytoplasmic proteolysis (β_{IP}) protein—occurs prior to signal termination via lysosomal degradation of the receptor complex[60]. Interestingly, IP/IB analysis showed no presence of a truncated IL-5Rα following cytokine ligation, (Fig. 10), suggesting that the short cytoplasmic tail of IL-5Rα is not an adequate proteasomal substrate. Future studies may entail a lysosomal inhibitor, such as bafilomycin A_1[85], to confirm that IL-

5Rα signal termination requires lysosomal degradation, alongside β_{IP}, even though a preceding proteasomal degradation appears to not be utilized in IL-5Rα.

In general, it is well supported that canonical P-*X*-P residues within the Box 1 motif of Type I and II cytokine receptors are necessary for JAK kinase binding[38-40]. Our studies revealed that a cluster of four lysine residues are also crucial for JAK kinase association, due to the impaired JAK binding to IL-5Rα receptor in K1-4R mutants, even though the P-*X*-P motif was still intact (Fig. 10). Additionally, this reduction of JAK kinase binding correlated with decreased K1-4R ubiquitination, highlighting the potential role of JAK kinases in optimizing receptor ubiquitination, as seen with βc[33]. The role of JAK kinases in optimizing receptor ubiquitination was later confirmed in JAK1/2 knockdown studies where we saw a reduction in WT IL-5Rα ubiquitination in cells that lacked JAK1/2 expression (Fig. 14). However, the presence of residual receptor ubiquitination in JAK1/2 knockdown cells does highlight that although the kinases are necessary for optimal ubiquitination, additional undiscovered mechanisms of ubiquitination are also involved.

Similar findings of the role of lysine residues in JAK kinase binding to cytokine receptors have been made with the IFNα receptor (IFNAR1)[86,87]. In an earlier report, researchers found that a cluster of two lysine residues were necessary for Tyk2 binding to IFNAR1[86]. Subsequent studies found that a different cluster of lysine residues within IFNAR1 were critical for not only receptor ubiquitination, but also endocytosis and turnover[87]. Interestingly, the latter study found that the ubiquitination of these three

lysine residues exposed a tyrosine-linear endocytic motif that, alongside receptor ubiquitination, was required for endocytosis.

Another unexpected finding in our study was the loss IL-5Rα tyrosine phosphorylation in K3,4R mutant cells within JAK2 overexpression studies (Fig. 15). This loss of tyrosine phosphorylation was not due to loss of JAK2 association with IL-5Rα since CO-IP experiments revealed a strong association of JAK2 in IL-5Rα immune complexes. Thus, it is likely that the third and fourth lysine residues, Lys^{370} and Lys^{379}, are involved with optimal JAK2 signaling. Further studies could examine the role of first and second lysine residues by repeating JAK2 overexpression studies with additional mutants, specifically K2R and K1-4R mutant cells.

The most striking finding of our study is the significant IL-5-dependent deubiquitination event of all cell surface proteins in our biotin-labeled precipitation assays (Fig. 12B). Furthermore, in response to cytokine stimulation, the deubiquitination of IL-5Rα appears to correlate with receptor internalization by endocytosis (Fig. 12A). Thus, we propose that following cytokine stimulation, ubiquitinated IL-5Rα is deubiquitinated, and this deubiquitination event triggers endocytosis of the receptor.

Interestingly, the K3,4R mutant had greater levels of ubiquitination in steady state conditions, relative to WT. Following cytokine stimulation, K3,4R mutant had a minimal reduction in ubiquitination and increased cell surface expression, relative to WT, most likely due to impaired endocytosis. Therefore, we hypothesize that the third and fourth lysine residues, Lys^{370} and Lys^{379}, are presumably involved with the association and binding of deubiquitinating enzymes (DUBs) to the activated IL-5Rα.

The K-to-R mutation in this mutant may have prevented DUB association with the activated receptor, and the persistent ubiquitination of K3,4R delaying its internalization and, thus, increasing its residency time on the cell surface.

Future Studies

Our most significant, and unexpected finding, was the deubiquitination event of all cell surface proteins following IL-5 stimulation. Considering that this deubiquitination event was visualized in our HEK293 cell model system, it is first critical to confirm a similar event under endogenous conditions. Thus, repeating the cell surface biotinylation assay using WT TF1 cells would be a necessary preliminary experiment prior to studying the possible players involved with deubiquitination.

Upon examination of the IL-5Rα cytoplasmic sequence, we have found a known deubiquitination enzyme, USP7[88], binding site at amino acid 394-398, specifically an AGSSE motif. Future studies could entail the use of known USP7 inhibitors, such as P22077[89,90], or siRNA to determine if this deubiquitinating enzyme is indeed involved with the major deubiquitination event. If USP7 is indeed involved with this deubiquitination event, then loss of USP7 activity would lead to less IL-5Ra deubiquitination following cytokine stimulation in cell surface biotinylation assays. After blocking USP7 activity, if WT begins to appear more like K3,4R following cytokine stimulation—i.e. more ubiquitination, delayed endocytosis, and prolonged cell surface expression—it is possible to potentially confirm the involvement of Lys^{370} and Lys^{379} in associating with DUBs, specifically USP7.

Relative to WT, the K3,4R mutant had increased ubiquitination and cell surface expression relative to WT, both in the absence and presence of IL-5. Thus, we propose that the third and fourth lysine residues are perhaps involved with USP7 binding to IL-5Rα. To elucidate this question, future studies could repeat CO-IP experiments with WT and K3,4R IL-5Rα, immunoprecipitating with α-IL-5Rα-antibodies and immunoblotting with α-USP7-antibodies. We hypothesize that if the third and fourth lysine residues are indeed involved with USP7 binding, a reduction in USP7 CO-IP will be seen in K3,4R cell lanes.

Additional examination of the cytoplasmic regions of both IL-3α and GM-CSFα shows a similar cluster of lysine residues that may serve as potential ubiquitination sites. Not only could the ubiquitination status of these α-chains be further studied, but also the possible deubiquitination event seen in IL-5Rα. Perhaps deubiquitination of the α-chains is a key regulatory mechanism employed by all three receptors in signal termination.

Concluding Remarks

Although a great deal of progress has been in the functional roles of ubiquitin within the cell, the role of deubiquitination has become a field of interest. For instance, the deubiquitinating enzyme, DUB2A, was found to enhance colony-stimulating factor 3 receptor signaling by attenuating ubiquitin-dependent lysosomal degradation of the receptor[91]. Through the use of DUB inhibitors, such as P22077, it would be very interesting to further elucidate the purpose of deubiquitinating IL-5Rα. If deubiquitination of IL-5Rα is involved with prolonged cell signaling, perhaps inhibition

of its deubiquitination is a possible, more-eosinophil specific therapy against inflammatory disorders involving IL-5 and eosinophils.

Altogether, this study has provided the first indication that IL-5Rα is ubiquitinated. Surprisingly, ubiquitin appears to have a novel role within this receptor, whereby IL-5Rα ubiquitination seems to stabilize its cell surface expression, prior to ligand stimulation. Following IL-5 binding, a very dramatic deubiquitination event of IL-5Rα appears to trigger endocytosis of the receptor, entirely contrary to previous studies with βc[33]. By far, our most significant finding is the drastic deubiquitination event of all cell surface proteins, which has caused us to speculate that deubiquitination events within the IL-5R complex may be just as important as its ubiquitination, and thus future studies will be focused on the deubiquitination of IL-5Rα.

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