Homeostatic Regulation of Interleukin-4-Mediated Cell Signaling

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HOMEOSTATIC REGULATION OF INTERLEUKIN-4-MEDIATED CELL SIGNALING

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Dedicated to the Inspiration, Encouragement and Perseverance of my Parents
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HOMEOSTATIC REGULATION OF INTERLEUKIN-4-MEDIATED CELL SIGNALING
RIKHIA CHAKRABORTY

ABSTRACT

Cytokines are intracellular messengers that activate multiple signaling pathways and regulate cell survival, differentiation, migration, apoptosis and immune responses. We are investigating the homeostatic control of cytokine-mediated cell signaling using interleukin-4 (IL-4) as a model cytokine. Protein tyrosine phosphorylation is essential for cytokine-dependent signal transduction through the Janus Kinase-Signal Transducer and Activator of Transcription (Jak-STAT) pathway. Therefore, protein tyrosine dephosphorylation is a key mechanism providing the homeostatic control of the pathway. Here, we have shown for the first time that immediately following ligand-dependent activation, IL-4 receptor induces an intracellular calcium flux via insulin receptor substrate (IRS)-phosphoinositide 3-kinase (PI3K)-phospholipase C (PLC)-γ pathway which, in turn, induces protein kinase C (PKC)-dependent activation of NAD(P)H oxidase (NOX)5 that generates reactive oxygen species (ROS). IL-4 also induces NOX1-mediated ROS production via IRS-PI3K-Ras-related C3 botulinum toxin substrate (RAC) 1 pathway. Moreover, we have also demonstrated that IL-4-generated ROS, in turn, promote IL-4 receptor activation by oxidatively inactivating protein tyrosine phosphatase 1B (PTP1B) that physically associates
with and deactivates IL-4 receptor. In addition, we have shown that antioxidant enzymes peroxiredoxin II, IV and VI (Prx II, IV, VI) physically interact with the IL-4 receptor and become oxidized by IL-4 generated ROS, thereby neutralizing IL-4 generated ROS and compromising ROS-mediated amplification of IL-4 signaling. Cytokine-activated Jak-STAT pathway is believed to operate from cell surface to the nucleus via DNA-protein and protein-protein interactions without involving any second messengers. Here, we demonstrate, for the first time to our knowledge, a role for second messengers (ROS) in the amplification of Jak-STAT signal transduction in IL-4-stimulated cells. Further, our data suggests that redox status of cells controls the magnitude of IL-4 receptor activation and signal transduction.
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<td>IRS-1/2</td>
<td>Insulin receptor substrate-1/2</td>
<td></td>
</tr>
<tr>
<td>ITIM</td>
<td>Immunoregulatory tyrosine-based inhibitory motif</td>
<td></td>
</tr>
<tr>
<td>JAK</td>
<td>Janus Kinase</td>
<td></td>
</tr>
<tr>
<td>IP</td>
<td>Immunoprecipitation</td>
<td></td>
</tr>
<tr>
<td>LIF</td>
<td>leukemia inhibitory factor</td>
<td></td>
</tr>
<tr>
<td>MAPKs</td>
<td>Mitogen activated protein kinase</td>
<td></td>
</tr>
<tr>
<td>MDM</td>
<td>Monocyte-derived macrophages</td>
<td></td>
</tr>
<tr>
<td>MEK1</td>
<td>Mitogen activated protein kinase kinase 1</td>
<td></td>
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<tr>
<td>MEF</td>
<td>Mouse embryonic fibroblast</td>
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<tr>
<td>MHC</td>
<td>Major histocompatibility complex</td>
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<tr>
<td>MMR</td>
<td>Macrophage mannose receptor</td>
<td></td>
</tr>
<tr>
<td>NAC</td>
<td>N-acetyl-L-cysteine</td>
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<tr>
<td>NADPH</td>
<td>Reduced nicotinamide adenine dinucleotide</td>
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<tr>
<td>NFκβ</td>
<td>Nuclear factor κβ</td>
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<tr>
<td>NOX</td>
<td>NADP(H) oxidase</td>
<td></td>
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<tr>
<td>OSM</td>
<td>Oncostatin M</td>
<td></td>
</tr>
<tr>
<td>Abbreviation</td>
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<tr>
<td>PHOX</td>
<td>Phagocytic oxidase</td>
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<td>PI3K</td>
<td>Phosphatidylinositol 3'-kinase</td>
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<td>PKC</td>
<td>Phosphotyrosine-binding</td>
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<td>PTK</td>
<td>Protein tyrosine kinase</td>
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<tr>
<td>PTP</td>
<td>Protein tyrosine phosphatase</td>
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<tr>
<td>PV</td>
<td>Pervanadate</td>
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<tr>
<td>RAC</td>
<td>Ras-related C3 botulinum toxin substrate</td>
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<td>ROS</td>
<td>Reactive oxygen species</td>
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<td>RTK</td>
<td>Receptor tyrosine kinase</td>
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<tr>
<td>SAPK</td>
<td>Stress-activated protein kinase</td>
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<tr>
<td>SH2</td>
<td>Src homology 2</td>
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<tr>
<td>SHP</td>
<td>Src homology containing phosphatase</td>
<td></td>
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<tr>
<td>SOD</td>
<td>Signal transducer and activator of transcription</td>
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<tr>
<td>SP-1</td>
<td>Specificity protein 1</td>
<td></td>
</tr>
<tr>
<td>STAT</td>
<td>Signal transducer and activator of transcription</td>
<td></td>
</tr>
<tr>
<td>TCL</td>
<td>Total cell lysate</td>
<td></td>
</tr>
<tr>
<td>TNF</td>
<td>Tumor necrosis factor</td>
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<tr>
<td>TPO</td>
<td>Thrombopoietin</td>
<td></td>
</tr>
<tr>
<td>TYK</td>
<td>Tyrosine kinase</td>
<td></td>
</tr>
<tr>
<td>VCAM-1</td>
<td>Vascular cell adhesion molecule 1</td>
<td></td>
</tr>
<tr>
<td>WCE</td>
<td>Whole cell extract</td>
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</table>
CHAPTER I

INTRODUCTION

1.1. Cytokines

Cytokines are intracellular glycoprotein messengers, which activate multiple signaling pathways and regulate cell survival, differentiation, migration, apoptosis, and immune responses (Arai et al., 1990). The nomenclature of cytokines is based on their cellular sources, i.e. monokines (produced by mononuclear phagocytes), lymphokines (produced by lymphocytes) and interleukins (produced by leukocytes) (Rozwarski et al., 1994; Abbas and Litchman, 2003; Haque and Sharma, 2006). However, subsequent investigations have shown that a large number of cells produce interleukins, and secreted interleukins have a plethora of actions on different target cells. Cytokines are characterized by rapid secretion and very limited time of action either in a paracrine or autocrine fashion (Abbas and Litchman, 2003). Pleiotropy and redundancy render them very important in regulating multiple physiological functions, including the secretion and activity of other cytokines (Abbas and Litchman, 2003). They regulate their downstream effects through specific cell
surface receptors, where expression of the receptor largely determines the sensitivity of a particular cytokine action (Arai et al., 1990; Miyajima et al., 1992; Abbas and Litchman, 2003; Kishimoto et al., 1994).

Cytokines are divided into four classes based on protein structures. They are hematopoietin (Type 1), IFN (Type 2), chemokine (Type 3) and TNF (Type 4) (Goldsby et al., 2003; Haque and Sharma, 2006). Cytokine include interleukins (1 through 35) (Doan et al., 2007), erythropoietin (EPO), thrombopoietin (TPO), prolactin (PRL), growth hormone (GH), interferons (IFNs, both type I and II), granulocyte colony stimulating factor (G-CSF), granulocyte macrophage colony stimulating factor (GM-CSF), leptin, leukemia inhibitory factor (LIF), oncostatin M (OSM), ciliary neurotrophic factor (CNTF), cardiotrophin-1 (CT-1), cardiotrophin-like cytokine (CLC) and tumor necrosis factor (TNF) (Haque and Sharma, 2006).

Cell signaling by majority of cytokines are mediated by tyrosine-phosphorylation of cognate receptors and downstream signaling proteins, which is initiated upon ligand binding to its cognate receptor. The physiologic control of cytokine receptor activation is primarily mediated by reciprocal activation of receptor-associated protein tyrosine kinases (PTKs) and protein tyrosine phosphatases (PTPs).

1.2. Interleukin-4 (IL-4)

IL-4 was discovered by Maureen Howard, William Paul (Howard and Paul, 1982) and Ellen Vietta in 1982 (Isakson et al., 1982). The nucleotide sequence for human IL-4 was determined four years later confirming its similarity to a
mouse protein called B-cell stimulatory factor-1 (BCSF-1) or B cell growth factor (BCGF) (Rabin et al., 1986). IL-4 belongs to the hematopoietin family and is secreted by activated Th2-lymphocytes, basophils and mast cells (Nelms et al., 1999; Haque and Sharma, 2006).

1.3. Structure of IL-4

IL-4 is a ~15-19 kDa soluble glycoprotein. Like all other hematopoietins, IL-4 has a four parallel α-helix (A, B, C, and D) bundle structure, connected by loops, AB and CD (Powers et al., 1992; Smith et al., 1992; Walter et al., 1992; Wlodawer et al., 1992; Muller et al., 1994; Muller et al., 1995) (Figure 1). Specific regions of A and C primarily serve as binding epitope for IL-4 while interacting with high affinity receptor IL-4Rα (Kruse et al., 1993; Letzelter et al., 1998). Specifically glutamic acid (Glu9) and arginine (Arg88) residues of IL-4 is responsible for ligand binding to IL-4Rα (Wang et al., 1997). On the other hand interaction of IL-4 with the gamma common chain (γc) is mediated by defined regions located on helices A and D of IL-4 (Kondo et al., 1993; Kruse et al., 1993; Russell et al. 1993; Giri et al., 1994; Leonard et al., 1994; Letzelter et al., 1998; Asao et al., 2001). The interaction of IL-4 with the IL-13Rα1 is similar to its interaction with IL-4Rα chain, with the exception of an extra amino-terminal immunoglobulin-like domain (D1) in the former that allows formation of sheet-like contacts with the dorsal surfaces of IL-4 (LaPorte et al., 2008).
1.4. Functions of IL-4

Biological functions of IL-4 in hematopoietic cells are very prominent. IL-4 induces differentiation of Th2 cells (Figure 1.2) from naïve CD4$^+$ T cells (Nelms et al., 1999) and suppresses Th1 differentiation and macrophage activation (Le Gros et al., 1990; Hsieh et al., 1992; Kopf et al., 1993; Nelms et al., 1999). Studies performed in IL-4- and STAT6 knockout mice show significant changes in Th2 differentiation in helminth infection model (Nelms et al., 1999). The Th2 cell in turn produces IL-4 and number of other cytokines including IL-5, IL-9, IL-13, and IL-25. The subsequent major function of IL-4 and IL-13 is to induce proliferation and differentiation of B cells. During immunoglobulin class-switching, IL-9 favors the development of mast cells and IL-5 is involved in the development of eosinophils (Figure 1.2) (Nelms et al., 1999).

IL-4 signaling transcriptionally upregulates the expression of IL-4Rα chain, major histocompatibility complex (MHC) class II and IgE receptor (CD23) genes in B cells (Noelle et al., 1984; Defrance et al., 1984; Ohara and Paul, 1988). In B lymphocytes, IL-4 is responsible for immunoglobulin class switching, IgE and IgG1 in mouse and IgE and IgG4 in humans (Coffman et al., 1986; Vitetta et al., 1985; Gascan et al., 1991; Schindler et al., 1994; Zamorano and Keegan, 1998). IL-4- and IL-4Rα knockout mice show significant decrease in IgE production along with weak response during eosinophil-mediated host defense (Kuhn et al., 1991; Noben-Trauth et al., 1997). IL-4 suppresses cell-mediated immune responses by opposing IFNγ effects on macrophages (Mokoena and Gordon, 1985; Gordon, 2003). Also, in primary monocyte-derived macrophages (MDMs),
IL-4 signaling is associated with increased endocytosis through macrophage mannose receptor (MMR) (Montaner et al., 1999).

Figure 1.1: Crystal Structure of Human Interleukin-4. Structure accessed from Protein Data Bank (PDB: 2INT) and rendered using Pymol. The structure of IL-4 is characterized by antiparallel juxtaposed α–helices and two end-to-end loops. (Adapted from J Biol Chem (1992) 267, 20371-20376).

Functions of IL-4 on non-hematopoietic cells are not very well documented. Recent reports have shown that IL-4 suppresses apoptosis in glioma cells (Rahaman et al., 2005) and in colon cancer stem cells (Francipane et al., 2008), whereas in in vitro and in vivo models of human lung cancer IL-4 shows significant antiproliferative effects (Topp et al., 1993). Other groups have shown overexpression of IL-4Rα in renal cell carcinoma, squamous cell
carcinoma of the head and neck, malignant glioma, AIDS associated Kaposi's sarcoma and in breast cancer cell lines (Obiri et al., 1993; Puri et al., 1994; Obiri et al., 1994; Husain et al., 1997). Other than cancer cells, IL-4 largely modulates cells in the respiratory tract. IL-4 induces mucin 4 (MUC4) expressions in bronchoalveolar cells, whereas in nasal epithelial cells it induces MUC8 expression (Seong et al., 2002; Damera et al., 2006). IL-4 has been implicated to have decisive roles in asthma and allergy. It has been shown to cause bronchoalveolar lavage (BAL) fluid eosinophilia, airway hyperreponsiveness (AHR) and airway goblet cell hyperplasia in an IL-13 deficient allergic asthma model system (Perkins et al., 2006). In non-hematopoietic endothelial cells, IL-4 selectively suppresses E-selectin expression and upregulates vascular cell adhesion molecule 1 (VCAM1) in the presence of another cytokine, tumor necrosis factor alpha (TNFα) (Thornhill et al., 1991; Bennett et al., 1997).

1.5. IL-4 Receptor Complex

1.5.1. IL-4 Receptor Complex Components and Expression

IL-4 exerts its pleiotropic functions by activating intracellular signaling cascades through Type I (hematopoietic cells) and Type II (non-hematopoietic cells) receptor complexes (Nelms et al., 1999) (Figure 1.3). The type I IL-4 receptor complex consists of the IL-4Rα chain and the common γc chain (Mueller et al., 2002). The type II IL-4 receptor complex consists of the IL-4Rα and the IL-13Rα1 chain (primary receptor chain for IL-13) (Callard et al., 1996).
Figure 1.2: IL-4 Functions as a Master Regulator of Allergic Inflammation.
The membrane proximal regions of IL-4 receptor subunits associate with tyrosine kinases of the Janus family (Janus kinase {Jak 1–3} and tyrosine kinase {TYK2}). IL-4Rα associates with Jak1 and γc with Jak3 (Dubois et al., 1998), whereas IL-13Rα1 interacts with either Jak2 or TYK2, but not Jak3 (Nelms et al., 1999). Dimerization of the receptor subunits hyperactivates Jak, leading to phosphorylation of tyrosine residues in the cytoplasmic domain of IL-4Rα (Nelms et al., 1999). These phosphotyrosines then act as docking sites for signaling molecules containing protein tyrosine binding domains (PTBs) or Src homology 2 (SH2) domains (Nelms et al., 1999). IL-4Rα chain also functions as a receptor component for another Th2 cytokine, IL-13. Thus, type II IL-4 receptor complex also functions as the receptor for IL-13. This receptor complex have been found to be expressed in brain, lung, muscle, placenta, kidney, epithelium and endothelium (Ohara and Paul, 1988; Lowenthal et al., 1988; Mehrotra et al., 1998; Doucet et al., 1998; van der Velden et al., 1998; Henriques et al., 1998). Type II complexes are absent in T-cells, but are expressed in macrophages and B-lymphocytes (Nelms et al., 1999; Jiang et al., 2000).
Figure 1.3: Structure of IL-4-receptor complexes.

(i) The type I complex involves IL-4Rα associated with tyrosine kinase Jak1 and dimerizes with common chain γc associated with Jak3. Found mainly in hematopoietic cells.

(ii) The type II complex involves IL-4Rα associated with tyrosine kinase Jak1 and dimerizes with IL-13Rα1, which associates with either Jak2 or TYK2. Found mainly in non-hematopoietic cells.

1.5.2. Extracellular Domains of IL-4Rα Chain

Like other hematopoietin receptor superfamily members, IL-4Rα chain consists of well-characterized fibronectin type III (FnIII) domains (D1&D2), connected by a linker domain (Mueller et al., 2002). Both D1 and D2 are composed of approximately 100 amino acid residues and form an immunoglobulin-like fold with seven beta strands organized into two beta sheets (Bazan, 1990; Haque and Sharma, 2006). A conserved Try-Ser-Try-X-Try-Ser (Try - tryptophan, Ser - serine and X - any amino acid) motif is present in the D2 domain and is a characteristic of all Type I cytokine receptors (Mueller et al., 2002).

The crystal structure of Type I receptor complex reveals that the linker segment between D1 and D2 is composed of beta-sheets L2 and L5. In D1, these loops are flanked by L1 and L3, whereas in D2 it is flanked by L6 (Mueller et al., 2002). IL-4 binds in the groove (or elbow) formed between D1 and D2, and L2 and L6 interact with the bound IL-4 molecule (Meuller et al., 2002). The specific amino acid residues of IL-4Rα chain that interacts with IL-4 are Tyr-13, Ala-71, Tyr-183 and Tyr-127 of avocado cluster I and Asp-72 of avocado cluster II (Mueller et al., 2002).

1.5.3. Intracellular Domains of IL-4Rα Chain

The cytoplasmic domain of IL-4Rα chain also has precisely defined sub-domain structures. Membrane proximal region or box I motif is predicted to be important for interaction with Jak1 (Nelms et al., 1999). The box I motif is acidic
in nature and mainly characterized by a proline rich sequence (Nelms et al., 1999). Box I motif is followed by an insulin IL-4 receptor (I4R) motif (spanning amino acid residues 437-557 of the IL-4Rα), which closely resembles similar sequence elements in insulin like growth factor (IGF-1) and insulin receptors (Keegan et al., 1994b). The intracellular region of the IL-4Rα chain between residues 557 and 657 contains three conserved tyrosine residues at positions 575, 603, and 631, which serve as gene induction domain (Ryan et al., 1996). Upon phosphorylation, these residues recruit STAT6. The membrane distal portion of the IL-4Rα consists of an immunoregulatory tyrosine-based inhibitory motif (ITIM) (Ile/Val-x-Tyr-x-x-Leu) that contains a conserved tyrosine residue at position 713 (Zamorano et al., 1998) (Figure 1.4).

1.6. IL-4 Signaling through the IL-4 Receptor

Binding of IL-4 to the IL-4Rα chain results in the recruitment of either γc or IL-13Rα1 to form the Type I or Type II receptor complex, respectively (Russell et al., 1993; Murata et al., 1998). IL-4Rα, γc and IL-13Rα1 remain constitutively bound to Jak1, Jak3 and Jak2 (or Tyk2), respectively (Johnston et al., 1994; Murata et al., 1996). Formation of the IL-4 receptor complex brings two Jak molecules to an appropriate proximity allowing them to trans-phosphorylate the invariant tyrosine residues located in their activation loops. This increases the catalytic activities of Jaks, which in turn overrides the receptor-associated PTP activity and initiates tyrosine phosphorylation-induced activation of IL-4Rα (Figure
1.3 and Figure 1.4). These phosphotyrosines then act as docking sites for signaling molecules.

![Diagram of IL-4Rα Chain]

**Figure 1.4: Structure of Human IL-4Rα Chain.**

Tyrosine residues represent docking sites of different signaling proteins. The membrane distal portion of the IL-4Rα consists of an immunoregulatory tyrosine-based inhibitory motif (ITIM) (Ile/Val-X-Tyr-X-X-Leu) that contains a conserved tyrosine residue at position 713.

*(Adapted from Annu Rev Immunol (1999). 17, 701-38).*
containing protein tyrosine binding domains (PTBs) or Src homology 2 (SH2) domains, whereby proteins bind to an invariant stretch of Asn-X-X-Tyr<sub>497</sub> (Asn - asparagines, Tyr - tyrosine and X - any amino acid) (Trub et al., 1995).

Phosphorylation of Tyr-497 leads to the binding and phosphorylation of IRS-1/2 molecules as well as other PTB domain containing proteins such as Shc, FRIP and DOK (Keegan et al., 1994a; Keegan et al., 1994b; Nelms et al., 1998), further resulting in interaction with the p85 subunit of phosphatidylinositol 3'-kinase (PI3K) (Nelms et al., 1998). p110 catalytic subunit of PI3K is activated following this interaction (Nelms et al., 1999). Detailed investigations using pharmacological inhibitors and p85<sup>α</sup>−/− mice have revealed the indispensable role of PI3K in IL-4-dependent growth and survival (Nelms et al., 1999). These observations have led to the paradigm that the I4R motif is the growth promotion domain.

On the other hand signal transducer and activator of transcription 6 (STAT6) is recruited as a result of phosphorylation of tyrosine residues 575, 603, and 631 by activated Jaks. STAT6 is subsequently phosphorylated at Tyr-641, which results in its homodimerization (Mikita et al., 1996; Darnell, 1997) and translocation to the nucleus. In the nucleus, activated STAT6 dimers bind to promoters of IL-4-responsive genes harboring the TTC-N4-GAA GAS (IFNγ activated sequences) (Ihle, 1996) (Figure 1.5). STAT6 is responsible for regulating the expression of a wide array of genes, including CD23, IL-4Rα, IgE and IgG1 in mice, IgE and IgG4 in human, and E4 binding protein-4 (E4BP4) (Vietta et al., 1985; Coffman et al., 1986; Defrance et al., 1987; Ohara and Paul,
Additionally, activated Jaks also phosphorylates Tyr-713 in the ITIM (Zamorano et al., 1998). Experimental evidences have shown ITIM to be involved in negative regulation by binding to tyrosine and lipid phosphatases (Muta et al., 1994; Burshtyn et al., 1996). Studies have revealed that SRC homology-domain 2 (SH₂) containing phosphatase-1 (SHP-1) is involved in the negative regulation of IL-4-dependent activation (Haque et al., 1998; Hanson et al., 2003), suggesting the association of a tyrosine phosphatase with the Tyr-713 residue in the ITIM. Interestingly, SHP-1 has also been shown to interact with IL-4Rα (Imani et al., 1997).

1.7. Multiple Mechanisms of Homeostatic Regulation of IL-4 Signaling

IL-4 executes multiple functions by activating two major (IRS-PI3K, Jak-STAT6) and two minor (MAPK, SHC-GRB2) pathways. IL-4 signaling is regulated by complex homeostatic mechanisms (Haque and Sharma, 2006). IL-4 signaling, like most other cytokine signaling pathways, is limited in duration and magnitude (Haque and Sharma, 2006); hence requiring a tight regulation of biochemical events that activate and amplify the signal initiated by ligand binding to the cognate receptor and additional mechanisms that attenuate the signaling pathways.
1.7.1. Negative Regulation of IL-4 Signaling

The Jak-STAT pathway is negatively regulated at multiple levels (Starr and Hilton, 1999).

1.7.1.1. Decoy Receptors

A receptor antagonist may compete with the cytokine for receptor occupation or a decoy receptor may compete with the functional receptor for cytokine binding, and block the initiation of cytokine signaling. Alternate splicing of mRNA generates receptor transcripts that encode extracellular domain containing leader signals but not transmembrane domains, and are secreted by producer cells. Soluble IL-4Rα detected in biological fluids is produced by an alternate splicing of mRNA (Chilton and Fernandez-Botran, 1993; Heaney and Golde, 1996; Levine, 2004). It has been shown that IL-13Rα2 can also inhibit IL-4 signaling through the type II receptor complex comprised of IL-4Rα and IL-13Rα1 (Rahaman et al., 2002).

1.7.1.2. Protein Tyrosine Phosphatases (PTPs)

Protein tyrosine phosphorylation is a dynamic and reversible biochemical events responsible for the regulation of multiple intracellular signaling pathways (Tonks, 2006), including the Jak-STAT signaling pathway. In this event, the forward reaction is driven by PTKs and the reverse reaction by PTPs (Tonks, 2006).
PTPs are receptor-like and non-receptor enzymes possessing highly conserved catalytic domains specific for phosphotyrosine hydrolysis, which make them attractive molecules in the regulation of receptor tyrosine kinases-mediated signal transduction (Haj et al., 2003). PTPs contain essential cysteine residues in a signature active site motif [His-Cys-X-X-Gly-X-X-Arg(Ser/Thr)] (His = histidines, Cys = cysteine, Gly = glycine, Arg = arginine, Ser = serine, Thr = threonine, and X = any amino acid residue) that must be in a reduced state for proper catalytic activity (Maehama et al., 2001; Kwon et al., 2004).

Given the important roles played by PTPs in signal attenuation and data obtained from earlier studies, Haque et al. predicted the existence of separate cohorts of phosphatase, PTP-x that dephosphorylates the Jaks, the cytokine receptors and PTP-y, which dephosphorylates the STAT molecules that have already translocated to the nucleus (Haque et al., 1995; Haque and Sharma, 2006). Subsequent work by the same group has elucidated Shp-1, an SH2 domain containing PTP, being responsible for attenuating the Jak-STAT pathway; likely acting as PTP-x. (Haque et al., 1995; Haque et al., 1998; Haque and Sharma, 2006). Work from other laboratories have identified CD45 as a phosphatase important in downregulating Jak-STAT signaling in hematopoietic cells (Irie-Sasaki et al., 2001; Hermiston et al., 2003; Haque and Sharma, 2006). TC-PTP has been indicated to be the phosphatase responsible for downregulating the Jak-STAT pathway in non-hematopoietic cells (ten Hoeve et al., 2002; Bourdeau et al., 2005).
Figure 1.5: Schematic Representation of IL-4-dependent Major Signaling Pathways.

1.7.1.3. Ubiquitin-mediated Proteosomal Degradation

Proteolytic degradation of signaling proteins also causes attenuation or termination of cytokine signals. Ubiquitin mediated proteolysis also downregulates Jak-STAT signaling, even though much remains unknown about the physiological significance of these proteolytic events (Nelms et al., 1999).

1.8. Reactive Oxygen Species (ROS): Role as Second Messengers

Under normal physiological conditions, signal transduction pathways mediated by cytokines and growth factors are limited in both magnitude and duration (Haque and Sharma, 2006). Over the last few years regulation of cytokine mediated signal amplification has been a major area of interest, where some of the reports suggest reactive oxygen species (ROS) as being a major factor in the determination of magnitude of signal amplification (Sundaresan et al., 1995; Bae et al., 1997; Finkel, 1998; Rhee, 1999; Ushio-Fukai et al., 1999). It has been shown that non-phagocytic cells produce ROS in response to cytokines and growth factors (Rhee et al., 2000) and inhibition of ROS generation causes signal attenuation of insulin, PDGF, EGF and angiotensin II pathways (Sundaresan et al., 1995; Bae et al., 1997; Finkel, 1998; Rhee, 1999; Ushio-Fukai et al., 1999). The current work is perhaps the first report where we are documenting the evidences of ROS contributing to IL-4-mediated signal amplification. The work also provides evidence for a role of ROS as second messengers in Jak-STAT signaling, which was long believed to proceed only by DNA-protein and protein-protein interactions without the involvement of any second messengers.
ROS consist of a variety of reduced form of oxygen, including superoxide anion (O$_2^-$), hydrogen peroxide (H$_2$O$_2$) and hydroxyl radical (OH‘) (Finkel, 1998; Rhee, 1999; Lambeth, 2004). ROS are inevitably generated in all organisms living in aerobic conditions. Growth factors and cytokines execute their actions by binding specific cell-surface receptors that initiate intracellular signaling cascades (Leaman et al., 1996; Darnel, 1997; Brivanlou and Darnell, 2002). By inactivating phosphatases, reactive oxygen species can amplify signal transmission or possibly alter the phosphorylation status of signaling kinases (Denu and Tanner, 1998). PTP1B possesses a low pKa active site Cys215 (Lohse et al., 1997) rendering it sensitive to cellular ROS (Denu and Tanner, 1998; Rhee et al., 2005). Specifically, it has been shown that ROS can reversibly inactivate PTPs in cells that are stimulated by cytokines or growth factors (Lee et al., 1998; Meng et al., 2002; Kwon et al., 2004; Rhee et al., 2005). The mechanism of PTP1B inactivation by ROS involves the reversible oxidation of the catalytic cysteine, thereby resulting in an inactive sulfenic acid intermediate (Kwon et al., 2004). Cys 215 may therefore serve as a regulatory switch, whereby in the presence of ROS, the tyrosine phosphatase activity is reversibly inhibited by the formation of a sulfenic acid (-SOH) intermediate at residue 215. The consequence of this event is the upregulation of receptor tyrosine kinase (RTK)-dependent signal transduction. Phosphatase activity can be restored following the removal of ROS by various cellular antioxidant systems, including catalase, glutathione peroxidases, peroxiredoxins and superoxide dismutases (Margis et al., 2008).

*Signal amplification by ways other than ligand binding can positively cooperate*
with the canonical signaling molecules in upregulating IL-4 signaling, thus making it imperative to investigate the role of ROS in the IL-4 signaling pathway.

1.8.1. Sources of ROS

The sources of ROS, such as O$_2^\cdot$-, OH$,\, H_2O_2$ and others, can be either of exogenous or endogenous origins. Endogenously, ROS are produced by NADPH oxidases, lipoxygenase, xanthine oxidase, and cytochrome P450 (Rhee et al., 2003). Heavy metal ions, $\gamma$-radiation, and UV light can also induce ROS formation. One of the main sources of ROS in many cell types is the mitochondrial respiratory chain (Rhee et al., 2003).

NADPH oxidase (NOX) was originally identified as a phagocytic leukocyte specific enzyme, but subsequently similar oxidase complexes have been characterized in non-phagocytic cells, such as vascular endothelial, smooth muscle cells and fibroblasts (Zulueta et al. 1995). In phagocytes, Nox is activated by chemokines or phagocytic particles, which induce assembly of cytosolic subunits, Ras-related C3 botulinum toxin substrate 2 (Rac2), p47 phagocytic oxidase (p47phox) and p67 phagocytic oxidase (p67phox) with membrane-bound components, p22 phagocytic oxidase (p22phox) and glycoprotein 91 phox (gp91phox), to generate O$_2^-$ from O$_2$ and NADPH (Lambeth, 2004). This so called respiratory burst leads to the release of ROS from the cell, which is an important feature of immune defense against bacterial and fungal infections (Holmes et al. 1967). Released O$_2^-$ may be converted to H$_2$O$_2$, which in turn is a
substrate for myeloperoxidase, which subsequently produces hypochlorous acid and other strong oxidants (Winterbourn et al. 2000).

1.8.2. NADP(H) Oxidases (NOX): The ROS Generators

It was long believed that phagocytes were the only source of intracellular ROS generation. But detection of low amounts of ROS in non-phagocytic cells was reported using sensitive and quantitative detection tools (Lambeth, 2004). Use of small molecule inhibitors showed that a flavoprotein (similar to gp91phox) is responsible for intracellular ROS generation in non-phagocytic cells (Lambeth et al., 2000; Lambeth, 2004). Until now, seven different isoforms of NOXes/DUOX are known (Lambeth, 2004). The first of the NOX homologues of gp91, NOX1 was cloned from colonic epithelial cell complementary DNA library in 1999 (Suh et al., 1999). NOX 3 and NOX 4 were next identified (Geiszt et al., 2000; Shiose et al., 2001). Both NOX3 and NOX4 generate high levels of ROS when exogenously expressed in cells (Geiszt et al., 2000; Shiose et al., 2001; Geiszt et al., 2003). NOX1, on the other hand, require co-expression of regulatory subunits to show comparable amounts of ROS generation when expressed in cells (Banfi et al., 2003; Geistz et al., 2003).

Given the regulated amounts of ROS expressed in non-phagocytic cells, it might be rationalized that intracellular ROS are involved in regulatory or signaling pathways (Lambeth, 2004). ROS can participate in post-translational modification of proteins and have regulatory effects on signal transduction pathways. One such example is ROS-mediated reversible oxidation of the active site cysteine in
tyrosine phosphatases and PTEN (phosphatase and tensin homologue) (Lee et al., 1998). Subsequently the oxidized cysteine hydrolyses the 3-phosphate group from the bioactive lipid phosphatidylinositol 3,4,5-trisphosphate (Lee et al., 1998), inhibiting both tyrosine phosphatases and PTEN. Most of the ROS-mediated effects are reversible, thus providing a viable option for ROS to participate in signaling and regulatory pathways.

1.8.2.1. Types of NOXs

All NOX family members are transmembrane proteins that transport electrons across biological membranes to reduce oxygen to superoxide. In accordance with this preserved function, there are conserved structural properties of NOX enzymes that are common to all family members. Starting from the COOH terminus, these conserved structural features include 1) an NADPH-binding site at the COOH terminus, 2) a FAD-binding region in proximity of the most COOH-terminal transmembrane domain, 3) six conserved transmembrane domains and 4) four highly conserved heme-binding histidines, (two in the third and two in the fifth transmembrane domain). Additional features, such as EF hands, an additional NH₂-terminal transmembrane domain, and/or a peroxidase homology domain, are limited to some of the family members and are discussed below (Lambeth et al., 2004) (Figure 1.6).

The nature of domains present in NOXs in addition to the basic gp91phox domain forms the basis of classification of NOX into three basic classes. NOX1, NOX3 and NOX4 all resemble gp91phox in size and structure (Lambeth et al.,
2000). All three also possess the electron transfer centers required for electron transfer from NADPH to molecular oxygen to form $O_2^\cdot\cdot$. Subsequently, two $O_2^\cdot\cdot$ molecules react to generate $H_2O_2$. The presence of a calmodulin-like domain, containing four calcium binding sites, at the amino-terminal region of gp91phox constitutes NOX5 (Banfi et al., 2001). Banfi et al. has shown that ionomycin, a calcium ionophore, can activate exogenously expressed NOX5 in cells (Banfi et al., 2001). The DUOX enzymes differ from NOX5 by having an additional amino terminal peroxidase homology domain (Lambeth, 2004; De DeKen et al., 2000). The peroxidase domains of DUOX and myeloperoxidase are homologous, but the former also has an inherent myeloperoxidase activity (Zeng and Fenna, 1992). DUOX is thus capable of both generation and elimination of ROS by its own peroxidase activity.

1.8.3. Regulation of NOX-dependent ROS Production

Intricate regulation by regulatory subunits is the landmark of NOX-mediated ROS generation (Figure 1.7). Phox is regulated by membrane-associated p22phox. gp91phox and p22phox form a complex, called flavocytochrome b$_{558}$, whereby the association complementarily stabilizes each other (Lambeth, 2004). The C-terminal proline rich sequence of p22phox interacts with the cytosolic regulatory subunit p47phox via the two SRC-homology 3 (bis-SH3) domains (Ago et al., 2003; Groemping et al., 2003). p67phox, p40phox and the GTPase RAC (RAC1 and/or RAC2) are the other cytosolic regulatory subunits (Vignais et al., 2002). Assembly of the membrane
and cytosolic regulatory subunits with gp91phox activates the enzyme (Lambeth et al., 2000). In the inactive state p47phox does not interact with the p22phox, which is achieved by the bis-SH3 domain and an autoinhibitory region in p47phox (Ago et al., 2003). In the active state, p40phox and p67phox normally complex with p47phox in the cytoplasm.

RAC is kept in an inactivated state by the inhibitory protein RhoGDP-dissociation inhibitor. Activation of NOX occurs through different steps that cumulatively result in the assembly of the membrane and cytoplasmic components with gp91phox, thereby triggering ROS generation (Han et al., 1998; Nisimoto et al., 1999). The rate-limiting step in $\cdot O_2$ generation is the electron transfer between NADPH and bound flavin adenine dinucleotide (FAD), which is potentiated by the p67phox activation domain (Nisimoto et al., 1999).

NOX4 is usually constitutively active and does not require cooperative effect of regulatory subunits (Geistz et al., 2000). It has been suggested that NOX4 activity is either regulated by its expression level or by direct post-translational modification. Given the calcium binding domains in NOX5 and the DUOX enzymes (DUOX 1 and DUOX 2), their regulation is mediated by intracellular calcium levels (Dupuy et al., 1999; Banfi et al., 2001).

As is evident, cells have evolved detailed and distinct regulatory mechanisms of intracellular ROS generation. This might explain the important role of intracellular levels of ROS in modulating different signal transduction pathways.
Figure 1.6: Structure of NOX and DUOX Family of Enzymes.

(Adapted from Curr Opin Hematol (2002) 9, 11–17)
1.8.4. Effects of ROS

In multicellular organisms, ROS-induced damages to DNA can be very harmful since they can lead to mutations, which are then passed on to future cell generations. However, more commonly ROS damage evokes apoptosis or necrosis, which can be very severe and extensive leading to major tissue damage (Raffray and Cohen, 1997). More importantly, ROS has been historically known to be produced by phagocytic cells in order to mount an immune response to microbial infections (Holmes et al. 1967). ROS generated in non-phagocytic cells as a result of growth factor and cytokine signaling has been shown to manipulate the functions of transcription factors, protein kinases and phosphatases, phospholipases, ion channels and G-proteins (Burdon, 1995; Thannickal and Fanburg, 2000; Martindale and Holbrook, 2002). Reversible inactivation of PTPs and PTENs by posttranslational oxidative modifications in cells treated with cytokines and growth factors perhaps account for the most important role of ROS in cell signaling pathways (Rhee et al., 2005). Intracellular ROS have also been associated with stress-activated protein kinase (SAPK) and protein kinase C (PKC) activities (Janssen-Heiningger et al. 1999, Liu et al. 2000). These provide tools for wide regulatory possibilities in cell growth, proliferation, apoptosis, differentiation and cellular metabolism. Several transcription factors have been reported to be redox sensitive e.g. nuclear factor κβ (NFκβ), activator protein 1 (Ap1) and specificity protein 1 (Sp1) (Toledano & Leonard 1991, Li et al. 1994, Ammendola et al. 1994). Clearly, further studies are needed to reveal
the detailed role and mechanisms of the elevated ROS concentrations during cell signaling processes.

1.9. Oxidative Damage by ROS and Need for ROS Elimination

Protein oxidation results from the reaction of ROS with both amino acid side chains and peptide backbone (Berlett et al., 1997). Oxidative damage to proteins can take place within almost all amino acids; being the most reactive with sulfur amino-acid containing proteins (Berlett et al., 1997). Certain oxidations of proteins are reversible; oxidative modification of cysteine and methionine can be reversed by enzymatic systems like glutaredoxin/glutathione/glutathione reductase systems or thioredoxin/thioredoxin reductase systems. The reversibility of protein oxidation is very important in cell signaling pathways and it is considered one of the main mechanisms of redox signaling (Droge, 2002). Changes in the local redox state of protein sulfhydryls lead to conformational changes that, depending on the protein, can either decrease or increase DNA binding activity, or promote protein complex formations which are necessary for signal transduction or transcription to proceed (Droge, 2002). Irreversibly oxidized proteins mainly constitute hydroxylated and carbonylated amino acid derivates. These moieties are chemically stable; hence, protein carbonyl groups are used as biomarkers of oxidative stress. Accumulation of protein carbonyls has been observed in Alzheimer's disease, diabetes and inflammatory bowel disease, among others (Dalle-Donne et al., 2003). Oxidized proteins are generally less active, and expose hydrophobic amino acids at their surface, leading to changes in protein conformation and activity (Friguet, 2006).
Elimination of mildly oxidized proteins is preferentially done by the 20s proteasome in an ATP-and ubiquitin-independent manner (Grune et al., 1995; Grune et al., 2003). However, strong protein oxidation and/or a strong oxidative stress condition lead to proteasome inhibition (Grune et al., 1995). Accumulation of damaged proteins due to proteasome inhibition has been associated with metabolic perturbations, aging and different pathologies including Parkinson’s and Alzheimer’s diseases (Ciechanover, 2005; Reed et al., 2007), but little is known about proteasome inhibition in carcinogenesis.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Regulator(s)</th>
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<tbody>
<tr>
<td>NOX1</td>
<td>p22phox</td>
</tr>
<tr>
<td>NOX2</td>
<td>p22phox</td>
</tr>
<tr>
<td>NOX3</td>
<td>p22phox</td>
</tr>
<tr>
<td>NOX4</td>
<td>p22phox</td>
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<tr>
<td>NOX5</td>
<td></td>
</tr>
<tr>
<td>DUOX1</td>
<td></td>
</tr>
<tr>
<td>DUOX2</td>
<td></td>
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</tbody>
</table>

Figure 1.7: Regulator proteins of NOX and DUOX Family of Enzymes.

1.9.1. Elimination of ROS

ROS are detoxified by antioxidants, including non-enzymatic molecules like carotenoids, vitamin C and vitamin A, and distinct enzymatic systems (Margis et al., 2008). There are four key redox regulation systems: the superoxide dismutases, catalase, glutathione peroxidases and the thioredoxin peroxidases (Flohe and Ursini, 2008).

1.9.1.1. Superoxide Dismutase (SODs)

SODs degrade the highly reactive \(O_2^-\) to the less reactive \(H_2O_2\) (Mates et al., 1999).

\[
O_2^- + O_2^- + 2H^+ \xrightarrow{SOD} H_2O_2 + O_2
\]

There are three predominant forms of SODs in humans. Copper-zinc SOD (CuZnSOD) preferentially localized in the cytosol, manganese SOD (MnSOD) present exclusively in the mitochondrial matrix, and extracellular SOD (ECSOD) (Mates et al., 1999). MnSOD is indispensable as evident by postnatal fatality of MnSOD\(^{-/-}\) knockout mice within 3 weeks of birth. In comparison, CuZnSOD\(^{-/-}\) and ECSOD\(^{-/-}\) mice develop normally (Carlsson et al., 1995). Cells become resistant to hyperoxia, cigarette smoke, cytokines, irradiation and oxidants when MnSOD is ectopically overexpressed in them (St. Clair et al., 1991; Lindau-Shepard et al., 1994; St. Clair et al., 1994). The other prevalent antioxidant enzymes, catalase, glutathione peroxidases (GPxs) and peroxiredoxins (Prxs) are responsible for detoxifying the \(H_2O_2\) produced as a result of SOD activity.
1.9.1.2. Catalase

Catalase was originally identified by Jons Jacob Berzelius (Berzelius, 1836) and Lowe named it in 1900 (Lowe, 1900). It is located mostly in the peroxisomes, and to some extent in cytoplasm and mitochondria (Hillar et al., 2000).

\[
\text{H}_2\text{O}_2 \xrightarrow{\text{CATALASE}} \text{H}_2\text{O} + \frac{1}{2}\text{O}_2
\]

Because it can evolve molecular oxygen, catalase is known to degrade hydrogen peroxide by dismutation (Zamocky et al., 2008). Catalase interacts with NADH/NADPH, which inhibits its inactivation but does not contribute to the catalytic activity of the enzyme (Kirkman and Gaetani, 1984). Catalase can render both protective or damaging effects on the cells in a context-dependent fashion, a fact elucidated by the observation that catalase can generate ROS in response to UVB radiations in keratinocytes (Heck et al., 2003).

1.9.1.3. Glutathione Peroxidases (GPxs)

This system uses NADPH as an ultimate electron donor and requires selenium as a cofactor (Mates et al., 1999). It contains a key enzyme belonging to a pyridine nucleotide disulfide oxido-reductases family and a small redox-reactive peptide. Glutathione reductase reduces glutathione, which in turn controls the redox status of cells. Other components in this system include glutaredoxin (Grx), a small protein that specifically removes glutathione molecules from mixed disulfides formed between glutathione and other molecules. GPxs have different isoforms, each
being different locations (Fourquet et al., 2008). GPx1 and GPx2 are intracellular but GPx2 is found only in the colonic epithelium, whereas GPx1 has a more divergent distribution. GPx3 is found mainly in the plasma (Schwaab et al., 1998). GPx4 is widely expressed, but is limited by the fact that its catalytic power is effective only on hydroperoxides integrated in membranes (Kelner and Montoya, 1998). GPx5 is a secreted protein found exclusively in the epididymis (Rigaudiere et al., 1992). GPx6 has been identified in humans and pigs and is found in the olfactory epithelium (Thisse et al., 2003). GPx1 was identified in the cytosol, nucleus and mitochondria; GPx2 accumulates in the cytosol and nucleus; GPx3 is a secreted protein also found in the cytosol, whereas GPx4 is present in the nucleus, cytosol, mitochondria and in membrane bound form (Herbette et al., 2007). More recently, a new phospholipid hydroperoxide glutathione peroxidase (PHGPx) was described in mammals, which incorporates cysteine instead of selenocysteine in the conserved catalytic motif, and was named as NPGPx (non-selenocysteine PHGPx) (Utomo et al., 2004). NPGPx has a low catalytic efficiency in vitro. It is widely expressed, with prominent expression levels in the mammary glands (Utomo et al., 2004).

The thioredoxins (Trxs) and GSH are important reductants in cells. Trxs have two cysteine residues separated by two amino acids at their catalytic site. Sulfhydryl groups of these cysteine residues undergo reversible oxidation providing reducing power to the substrate. In the case of the thioredoxin system, thioredoxin reductase (TrxR) reduces thioredoxin with electrons originating from
NADPH, which in turn transfers reducing equivalents to numerous substrate proteins, including peroxiredoxin (Prx, also called thioredoxin peroxidase and Tpx) and ribonucleotide reductase. Trx and TrxR are localized in the mitochondria and cytosol. The secreted form of Trx can bind to the outer plasma membrane (Balcewicz-Sablinska et al., 1991).

Being a tripeptide, GSH belongs to a family of low molecular weight antioxidants (Casagrande et al., 2002). The cysteine residue of GSH is responsible for its redox status and it non-enzymatically forms conjugates with reactive electrophilic compounds such as aldehydes or peroxides. The GSH and Trx systems were originally considered as two distinct systems, but there is a clear interaction between them as GSH can inactivate Trx (Casagrande et al., 2002). Oxidation of GSH can be reversed by GSH reductase.

1.9.2. Peroxiredoxins

Peroxiredoxins (Prxs), are a ubiquitous group of antioxidant proteins (Chae et al., 1994a; Chae et al., 1994b; Mates et al., 1999) (Figure 1.8). They do not require any co-factors, unlike the heme-dependent catalase and the selenium-dependent glutathione peroxidases. Prxs are very well conserved across species and have been reported to be present in yeast, plants, animals, and in most eubacteria and archaea (Wood et al, 2003). The basic catalytic mechanism by which Prxs resolve peroxide in the cell is through oxidation of the active cysteine residue (also called the peroxidatic cysteine residue) in Prx to sulfenic acid. This is subsequently followed by a second step whereby the
sulfenic acid is recycled back to the thiol group. The second step is also what distinguishes the different groups of catalytic Prxs (Chae et al., 1994a; Ellis and Poole, 1997a; Ellis and Poole, 1997b; Choi et al., 1998). They are broadly classified into 3 groups: typical 2-Cys Prxs; atypical 2-Cys Prxs; and 1-Cys Prxs. Their importance in maintaining cellular homeostasis is underlined by their abundant expression in cells (Moore et al., 1991; Chae et al., 1999). Prxs have recently been indicated to regulate and modulate cytokine-induced hydrogen peroxide levels, which in turn mediate downstream signaling pathways responsible for cell proliferation, differentiation, and apoptosis (Hofmann et al., 2002; Fuji and Ikeda, 2002). Prxs expression is high also in thyroid, oral and breast carcinomas (Yanagawa et al. 1999; Yanagawa et al. 2000; Noh et al. 2001; Karihtala et al. 2003). Mouse models support the idea for an important role for Prxs in several diseases. Prx level alterations have also been noted in Creutzfeld-Jacob disease, Alzheimer’s disease, Pick’s and Down syndromes (Kim et al., 2001; Nicolls et al., 2003; Sanchez-Font et al., 2003).

**Much still remains to be elucidated about the intricate regulation of Prxs expression and associated peroxidase activity.** Convincing evidence for a role for $H_2O_2$ in cellular signaling exists as discussed, but the regulation of its concentration is poorly understood. As the concentrations needed for signaling are thought to be low, it seems unlikely that catalase could be the enzyme responsible for $H_2O_2$ removal. The catalytic efficiency of Prxs is weaker than that of GPxs or catalase, but the optimal $H_2O_2$ concentration for Prxs is relatively low, and for this reason, these enzymes are now considered as potential regulators of
endogenously produced $H_2O_2$ (Kang et al., 1998; Kang et al., 2004, Chang et al., 2004).

<table>
<thead>
<tr>
<th>Enzymes</th>
<th>Isoforms</th>
<th>Subcellular Location</th>
</tr>
</thead>
<tbody>
<tr>
<td>Catalase</td>
<td></td>
<td>Peroxisome</td>
</tr>
<tr>
<td>Superoxide Dismutase</td>
<td>SOD1, SOD2, SOD3</td>
<td>Cytosol, Mitochondria</td>
</tr>
<tr>
<td>Glutathione Peroxidase</td>
<td>Gpx1, Gpx2, Gpx3, Gpx4, Gpx5, Gpx6</td>
<td>Cytosol, Nucleus, Mitochondria</td>
</tr>
<tr>
<td>Peroxiredoxin</td>
<td>PRX I-VI</td>
<td>Cytoplasm, Plasma membrane, Mitochondria, Peroxisome.</td>
</tr>
</tbody>
</table>

**Figure 1.8: Major Antioxidant Enzymes, Isoforms and Subcellular Localization.**

The overall structure of the different Prxs is similar and it is the secondary structures that differentiate it into different isoforms. These secondary elements are also responsible for determining the oligomeric state in which the Prxs exists, which in turn defines their actions. 2-Cys Prxs seem to present as homodimers, but may also form heterodimers and several dimers may further group to form decamers (Schroder et al. 2000, Harris et al. 2001, Wood et al. 2002). Prxs
oligomerization is dependent on ionic strength, pH, magnesium/calcium concentrations and most of all, the redox state of the catalytic Cys. Peroxidase activity of Prxs is regulated in part by phosphorylation, cysteine oxidation, and limited proteolysis (Wood et al., 2003).

1.9.2.1. Peroxiredoxin I

Human Prx I is a 22 kDa protein expressed ubiquitously throughout the body (Prosperi et al. 1993, Prosperi et al. 1994, Shau et al. 1994, Pahl et al. 1995). Earlier, it was believed that Prx I was only present in the cytosol, but recent investigations have indicated nuclear, mitochondrial and peroxisomal localization (Immenschuh et al. 2003). Serum, oxidative stresses normally induce Prx I, whereas H$_2$O$_2$ induces it in select cell types. (Ishii et al., 1993; Prosperi et al., 1993; Prosperi et al., 1994; Kim et al., 2000). Prx I$^{-/-}$ mice suffer from hemolytic anemia, showing the importance of Prx I in erythrocyte antioxidative defense (Immenschuh et al., 2003). Prx I$^{-/-}$ mice also develop spontaneous lymphomas, sarcomas and carcinomas (Neumann et al., 2003).

1.9.2.2. Peroxiredoxin II

Prx II is a 22 kDa protein (Harris & Naeem 1981; Kim et al., 1988; Moore et al., 1990; Prosperi et al., 1994; Shau et al., 1994) abundantly expressed in erythrocytes. It shows close resemblance to Prx I in its structure, subcellular location and catalytic properties (Lim et al., 1994). Prx II is anti-apoptotic. Exogenous expression of Prx II leads to resistance against cisplatin, irradiation, serum starvation, ceramide and etoposide (Zhang et al., 1997; Park et al., 2000b;
Chung et al., 2001). Prx II inhibits NFκB activation after TNF-α or H₂O₂ treatment (Kang et al., 1998; Kang et al., 2004). siRNA-mediated knockdown of Prx II makes cells more susceptible to apoptotic stimulus and Prx II⁻/⁻ mice develop hemolytic anemia (Lee et al., 2003).

1.9.2.3. Peroxiredoxin III

Prx III (Tsuji et al., 1995) is a mitochondrial enzyme that plays a significant role in the elimination of ROS produced by aerobic metabolism.

1.9.2.4. Peroxiredoxin IV

Prx IV is synthesized and secreted as an inactive intracellular precursor (31 kDa), which subsequently becomes activated (27 kDa) upon translocation to its extracellular location (Okado-Matsumoto et al., 2000). The exact location and function of Prx IV is under debate. It is reported to be exclusively intracellular in cultured rodent cells (Wong et al., 2000). Exogenous expression of Prx IV in these cells eliminates ROS production by the EGF or p53-mediated pathways (Wong et al., 2000). Prx IV is unique among the peroxiredoxins in that it exhibit cytokine like properties. Prx IV exposure to cells induces iNOS in a receptor-mediated mechanism (Haridas et al., 1998). c-Jun N-terminal kinase is also induced by Prx IV and in turn induces proliferation in fibroblasts. Prx IV is anchored on the extracellular surface by heparin sulfate and its expression and activity is regulated by the prevailing redox status of the extracellular milieu. (Okado-Matsumoto et al., 2000).
1.9.2.5. Peroxiredoxin V

Prx V is the smallest (17 kDa) of the Prx families. Prx V is localized in the cytoplasmic, nuclear and organellar milieu (Knoops et al., 1999, Seo et al., 2000, Zhou et al., 2000). It has the capability to bind DNA and repress RNA polymerase III catalyzed transcription in vitro, however the in vivo significance of this ability has yet to be elucidated (Kropotov et al., 1999). Prx V is unique in that it is the only one of human Prxs known to act also as a peroxynitrite reductase (Dubuisson et al., 2004).

1.9.2.6. Peroxiredoxin VI

Prx VI is exclusively expressed in the cytosol (Nagase et al., 1995; Jin et al., 1997; Kim et al., 1997; Kim et al., 1998; Phelan et al., 1998). Prx VI belongs to the group of 1-Cys Prxs and is not reduced by Trx, but rather GSH (Fisher et al., 1999, Fratelli et al., 2002, Manevich et al., 2004). It not only has peroxidase but also possesses phospholipase A2 activity and has been implicated in repairing oxidized lipids (Manevich et al., 2002). Prx VI−/− mice present a shorter life span, severe tissue damage and increased protein oxidation compared to normal mice (Wang et al., 2003). Oxidative stress in the rat lung is known to induce Prx VI expression (Kim et al., 2003).

Detailed knowledge of the importance of ROS in signal amplification can only be achieved by a precise understanding of the intricate regulatory mechanisms of intracellular ROS-mediated signal transduction. The specificity of signaling pathways is tightly regulated by the production and elimination of
intracellular messengers. It can be rationalized that such regulation will be required for ROS, which if not eliminated can lead to deleterious effects including DNA damage and oxidation of proteins. Enzymatic systems exist to reverse such potentially deleterious effect. This reversibility of protein oxidation is very important in cell signaling pathways and is considered as one of the primary mechanisms of redox signaling (Droge, 2002). To better elucidate the various facets of intracellular ROS, further investigation is necessary to understand the mechanisms of ROS elimination.
CHAPTER II

IL-4-INDUCED GENERATION OF INTRACELLULAR REACTIVE OXYGEN SPECIES PROMOTES IL-4-MEDIATED CELL SIGNALING

2.1. Abstract

IL-4 executes pleiotropic functions by activating two major intracellular signaling pathways: the IRS-PI3K pathway that controls the growth of target cells, and the Jak-STAT6 pathway that activates the transcription of genes involved in a variety of immune responses. Under normal physiological conditions, signal transduction through these pathways is limited in both magnitude and duration. The pathways are activated by tyrosine phosphorylation of IL-4 receptor-associated Jaks, receptor subunits, IRS-1/2 and STAT6. Here, we show that immediately following ligand-dependent activation, the IL-4 receptor induces an intracellular calcium flux via IRS-PI3K-PLC-γ pathway which, in turn, induces PKC-dependent activation of NAD(P)H oxidase (NOX5) which
generates reactive oxygen species (ROS). IL-4 also induces NOX1-mediated ROS production via the IRS-PI3K-RAC1 pathway. However, ROS are not required for the initiation of IL-4 receptor activation but it actively participates in promoting receptor activation and signal amplification.
2.2. Introduction

ROS have long been recognized as toxic derivatives of diverse metabolic reactions. Only in the last few years it has become evident that the role of these oxidants is not limited to just being a byproduct but rather they have a significant role in the regulation of cytokine and growth factor signaling (Rhee et al., 2000). Redox-mediated regulation of IL-4 has not been extensively investigated. This is perhaps the first time when oxidant mediated amplification of IL-4 mediated signaling is being reported.

Our laboratory has previously demonstrated that a non-physiologic oxidant pervanadate causes IL-4 receptor activation in the absence of ligand engagement (Haque et al., 1997). Recent studies have shown that ROS are mainly produced by the NOX family enzymes, in response to cytokine or growth factor stimulation of cells (Rhee et al., 2000). That oxidants can facilitate or mimic cytokine action and ROS are generated in response to cytokine stimulation of its target cells have led to the hypothesis that ROS may serve as second messengers in the cytokine signaling cascade.

NOX catalyzes the NADPH-dependent reduction of molecular oxygen to generate superoxide $\text{O}_2^-$, which can be dismuted to generate $\text{H}_2\text{O}_2$ (Lambeth, 2004). Flavocytochrome b558 is the catalytic core of the phagocyte NADPH oxidase and consists of a large glycoprotein gp91phox (or NOX-2) and a small protein p22phox. The other components of the NADPH oxidase are cytosolic proteins, namely p67phox, p47phox, p40phox and Rac (Lambeth, 2004). To
date, seven members of human NOX/DUOX family (NOX-1, NOX-2, NOX-3, NOX-4, NOX-5, DUOX-1 and DUOX-2) have been identified; the founding member being NOX2. The identification of NOX-1 was quickly followed by the cloning of NOX-3, NOX-4, and NOX-5. In parallel, two very large members of the NOX family were discovered, namely DUOX-1 and DUOX-2. The physiological functions of NOX dependent ROS generation are under investigation and require further characterization. The activation of NOX1, NOX2 and NOX3 requires specific regulatory subunits, whereas the activation of NOX5, DUOX1 and DUOX2 requires calcium and no regulatory subunit(s). NOX4 activity is mostly constitutive but can be activated by p22phox (Lambeth, 2004; Lambeth et al., 2007).

IL-4 activates two types of receptors. The type I receptor, comprised of the Jak1 bound IL-4Rα and the Jak3 bound γc (Nelms et al., 1999). Many non-hematopoietic cells do not express γc and Jak3, where IL-4Rα associates with Jak2 (or TYK2) bound IL-13Rα1 to form the type II receptor (Nelms et al., 1999). Binding of IL-4 to IL-4Rα induces Jak1 mediated phosphorylation of multiple tyrosine residues in the cytoplasmic region of IL-4Rα. These, in turn, couple and activate two major downstream pathways: the IRS-PI3K, which promotes the growth of target cells, and STAT6, which activates the transcription of IL-4-responsive genes (Haque and Sharma, 2006; Nelms et al., 1999).

Here, we demonstrate that ROS are generated immediately after IL-4 stimulation of both hematopoietic and non-hematopoietic cells, which promotes IL-4 receptor activation and subsequent signal transduction. Activated IL-4
receptor generates ROS by IRS-PI3K-mediated, calcium-dependent and -independent activation of NOX5 and NOX1 respectively. We also show that IL-4 increases intracellular calcium flux, which is required for NOX5 activation.
2.3. Materials and Methods

2.3.1. Cells and Reagents

A549, 293T and NIH 3T3 cells were maintained in DMEM, supplemented with 10% fetal calf serum (FCS). All recombinant cytokines were purchased from R&D Systems. Apocynin, DPI, AG490, LY294002, wortmannin, U0126, BAPTA-AM, U73122, Go6976 and calphostin C were obtained from Calbiochem. Nifedipine and heparin were purchased from Sigma. Anti-phospho (Tyrosine641)-STAT6 and anti-phospho-tyrosine PY100 were purchased from Cell Signaling Technology, and anti-V5 was from Invitrogen. Anti-STAT6, anti-β-actin, anti-FLAG and anti-goat HRP were obtained from Santa Cruz Biotechnology.

2.3.2. Intracellular ROS Assays

ROS were measured fluorimetrically using CM-H$_2$DCFDA probe (Myhre et al., 2003; Choi et al., 2005). Briefly, adherent cells were washed twice with Hank’s balanced salt solution (HBSS), incubated with 5 µM CM-H$_2$DCFDA in HBSS for 10 min at 37°C in 5% CO$_2$, washed and stimulated with indicated cytokine at 37°C under a Leica DMIRB inverted microscope (Leica Microsystems) equipped with a Retiga EXi Cooled CCD Camera (Q Imaging, Burnby), with a Ex$_{490nm}$ and standard fluorescein filter. Images were taken at every 10 sec, and fluorescence signals were quantified using Image Pro Plus Software (Media Cybernatics). Each value in relative fluorescence units (RFU) represents mean ± SE of three independent measurements; each measurement represents the
mean fluorescence signals of five equivalent fields (approximately same number of cells). To measure ROS for longer

time periods (>5 min), cells were loaded with CM-H$_2$DCFDA, trypsinized, resuspended in HBSS, and changes in fluorescence signals were measured using flow cytometry (FACScan, Beckton-Dickinson). ROS produced by non-adherent cells were measured using Victor$^2$ Wallace Mutichannel Reader (Perkin Elmer) using Ex$_{495nm}$ and Em$_{535nm}$ filters.

### 2.3.3. Electrophoretic Mobility Shift, Supershift and Luciferase Assays

EMSA was performed using 10 µg proteins of whole cell extracts and 0.2 ng of $^{32}$P-labeled high-affinity STAT6-specific probe (Haque et al., 2000). Cell extracts were incubated with 2 µg of normal rabbit IgG or anti-STAT6 antibody prior to incubation with 0.2 ng of radiolabeled oligonucleotide duplex for 20 min for super-shift assay, or with 10 ng or 50 ng of unlabeled wild-type or mutant oligonucleotide duplex for competition assay. The sequences for the sense strand of the oligonucleotide are: 5’-GATCGCTCTTCTTCCCAGGAACTCA-ATG-3’ (wild-type) and 5’-GATCG-CTCTTCTTCCCAGGGGCTCAATG-3’ (mutant) with the conserved N6-GAS sequence underlined (Haque et al., 2000), and the mutated nucleotides italicized. The DNA-protein complexes were resolved on native 6% polyacrylamide gels, dried and visualized after incubation at -80°C. Luciferase assay was performed using the Dual Luciferase Assay Kit (Promega) as per the manufacturer’s instructions.
2.3.4. Cloning, Site-directed Mutagenesis and Plasmid Constructs

Murine IL-4Rα cDNA was isolated from a NIH3T3 cell-derived cDNA library by PCR, and cloned in pcDNA3.1/V5-HisA (Invitrogen). The constructs for p22phox (wild-type and P156Q mutant), NOX1, NOXA1, NOXO1, NOX4 and NOX5L and p22phox shRNA (specific and scramble) are described (Kawahara et al., 2005). The wild-type (WT) and mutant (C174S) PTEN constructs were obtained from Dr. Charis Eng, and wild-type (WT) and mutant (T17N) RAC1 constructs from Dr. Olga Stenina. All point mutants were generated using Quick Change XL Site-directed Mutagenesis Kit (Stratagene) following the manufacturer’s instructions. DNA oligonucleotides encoding shRNA sequences with loop sequence (5’-TTC- AAGAGA-3’) were cloned into pSuper-Neo (OligoEngine) according to the manufacturer’s instructions. The specific target sense sequences were: 5’-GAATTAGGCAAAAGTGGGTT-3’ for NOX1, 5’-AAGAGCGATTCTTTGCCCTAT-3’ for NOX5L, 5’-CGGGAAGACACAGTTCA-TGTACTT-3’ for PTEN, 5’-GAACACCGGCTCTTCTGCT-3’ for PLCγ1, and 5’-AATCCTGA-CTTCCGAGA-3’ for PLCγ2. The scramble versions of the target sequences were generated using siRNA Wizard (InVivogen), and cloned in pSuper-Neo vector (OligoEngine). All constructs were verified by nucleotide sequencing.
2.3.5. Transfection

A549 and 293T cells were transfected using Lipofectamine 2000 (Invitrogen) and calcium phosphate co-precipitation respectively (Haque et al., 2000) with transfection efficiencies of 70-80% and ~90% respectively.

2.3.6. RT-PCR

For determination of mRNA levels, 1.0 µg of total RNA was used for first-strand cDNA synthesis according to the manufacturer’s protocol (Invitrogen). One-tenth of cDNA was used as a template for 35 cycles of PCR using specific primer sets listed in Table I.

2.3.7. RAC1 Activation Assay

A549 cells were treated with 20 µM LY294002 or DMSO (vehicle) for 2 hr after which the cells were treated with IL-4 (20 ng/ml) for 5 min, or left untreated. Cell lysates were used to detect active RAC1 using the RAC1/Cdc42 Activation Assay Kit (Millipore) following the manufacturer’s instructions.

2.3.8. Measurement of Cytoplasmic Calcium Flux

For measurement of cytoplasmic calcium flux, cells were incubated with the calcium indicator dye, Fluo4-AM (1 µM), for 30 min in complete DMEM, according to the manufacturer’s instructions (Molecular Probes). Before measurement of fluorescence signals, cells were washed twice with phenol red-free DMEM, and incubated for another 15 min for complete hydrolysis of intracellular AM-esters. Images of cells were taken both before and after IL-4
stimulation, at every 2 sec for 6 min at 37°C. Images were obtained with Leica SP-2 AOBS confocal microscope (Leica-Microsystems) using an HCX PL APO 40X/1.25 NA oil immersion objective lens (Murata et al., 2004), with minor modifications. Fluo4 was excited with the 488 nm line of an argon laser, and its fluorescence emission was recorded between 500-550 nm using the built-in spectrophotometer. Illumination intensity was kept to a minimum (50 µW) to avoid phototoxicity, and the pinhole was set to two Airy units. Fluorescence signals were quantified using Leica confocal software (Leica-Microsystems).

2.3.9. Statistical and Densitometric Analyses

All experiments were performed at least three times. Statistical differences between different groups were determined using paired Student’s t-test. EMSA and immunoblot signals were quantified using ImageQuant (Molecular Dynamics).
Table I: PCR Primer Sets with Expected Sizes of the PCR Products.

<table>
<thead>
<tr>
<th>Gene Name</th>
<th>Expected Size (bp)</th>
<th>Primer Sequences A: Human Cells</th>
<th>Expected Size (bp)</th>
<th>Primer Sequences B: Mouse Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>NOX1</td>
<td>235</td>
<td>Forward: 5'-GGAGCAGGAATTGGGGTCAC3' Reverse: 5'-TTGCTGTCCCATCCGGTGAG-3'</td>
<td>431</td>
<td>Forward: 5'-TGCGCTGTGATAGCGAGGAGTTC3' Reverse: 5'-GACCTTCATCGCTGCGGTT3'</td>
</tr>
<tr>
<td>NOX2</td>
<td>549</td>
<td>Forward: 5'-TTGCTGTCCCATCCGGTGAG-3' Reverse: 5'-GCCAGACTAGATGTTGAGTGTGCT-3'</td>
<td>301</td>
<td>Forward: 5'-TGCTGCTGATAGCGAGGAGTTC3' Reverse: 5'-GACCTTCATCGCTGCGGTT3'</td>
</tr>
<tr>
<td>NOX3</td>
<td>457</td>
<td>Forward: 5'-GGATCGGAGTCACTCCCTTCGCTG-3' Reverse: 5'-ATGAACACCTCTGGTGAGTCAGCTGA-3'</td>
<td>492</td>
<td>Forward: 5'-TGCTGCTGATAGCGAGGAGTTC3' Reverse: 5'-GACCTTCATCGCTGCGGTT3'</td>
</tr>
<tr>
<td>NOX4</td>
<td>285</td>
<td>Forward: 5'-CTCAGCGGAAGTCACTAGCTGTG-3' Reverse: 5'-AGAGGAACACGAACTAGCTGTTA-3'</td>
<td>521</td>
<td>Forward: 5'-TGCTGCTGATAGCGAGGAGTTC3' Reverse: 5'-GACCTTCATCGCTGCGGTT3'</td>
</tr>
<tr>
<td>NOX5</td>
<td>238</td>
<td>Forward: 5'-ATCAAGCGGGCCCGCTTTTAC-3' Reverse: 5'-CTCATTTGTGACACTGCTGGCTGAGC-3'</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NOX5L</td>
<td>557</td>
<td>Forward: 5'-CGGCCATCTGCTGCTGCGGCTG-3' Reverse: 5'-CCGCTGTACGAGTCAAACTTTC-3'</td>
<td></td>
<td></td>
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<tr>
<td>DUOX1</td>
<td>144</td>
<td>Forward: 5'-GATGCTCTCCGCTCCCACTG-3' Reverse: 5'-GAGAAGACTGTTGGAAGG-3'</td>
<td>760</td>
<td>Forward: 5'-GACCTTCATGAAAGACCCACCGGA-3' Reverse: 5'-GACCTTCATGAAAGACCCACCGGA-3'</td>
</tr>
<tr>
<td>DUOX2</td>
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<td>590</td>
<td>Forward: 5'-AGCTGCCATGAGATGATCTGGAG-3' Reverse: 5'-AGCTGCCATGAGATGATCTGGAG-3'</td>
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<tr>
<td>PTEN</td>
<td>176</td>
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<tr>
<td>PLCγ1</td>
<td>188</td>
<td>Forward: 5'-CCAGGACACTTACCAGGTGGTC-3' Reverse: 5'-GAAGACTGAAAGGAGACTC-3'</td>
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<tr>
<td>PLCγ2</td>
<td>189</td>
<td>Forward: 5'-TCCACCACCGTGTAATGATGAT-3' Reverse: 5'-TGCGGCCGATTTCTTATGAG-3'</td>
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<td></td>
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<tr>
<td>β-ACTIN</td>
<td>209</td>
<td>Forward: 5'-CATGATGCTGCTCCACACGCG-3' Reverse: 5'-CTCTCTAAATGTCGACGCGAT-3'</td>
<td>426</td>
<td>Forward: 5'-AAGGCCAAGCTGAAAGACCACT-3' Reverse: 5'-ACCGCTGCTTGGCAATAGTGATGA-3'</td>
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</table>


2.4. Results

2.4.1. IL-4 Generates ROS that Promote Intracellular Signal Transduction

We have previously demonstrated that IL-4 receptor-associated PTP activity is susceptible to inactivation by PV, which is an exogenous oxidant (Haque et al., 1997). Here, we demonstrate for the first time, that IL-4 stimulation of A549 cells generated endogenous oxidants, ROS, within 10 sec (Figures 2.1 & 2.2A) and reached a peak at ~15 min; declining thereafter (Figure 2.2B). Although the fluorescence probe, CM-DCFH$_2$-DA used for ROS measurement, can also detect reactive nitrogen species (RNS), pretreatment of cells with an inhibitor of nitric oxide synthase, L-NAME (Kubes et al., 1991), did not reduce IL-4-generated fluorescence intensity, indicating that in response to IL-4, A549 cells did not generate RNS (Figure 2.3A). To examine if NOX family members were involved in IL-4-induced ROS production, ROS were measured in A549 cells pretreated with diphenylene iodonium (DPI), an inhibitor of flavoprotein (Cross and Jones, 1986), and apocynin, an inhibitor of NOX activity (Fu et al., 2006). Both the inhibitors completely blocked IL-4-induced ROS production (Figure 2.3A) suggesting that NOX-family enzymes were involved in this process. Importantly, these inhibitors also significantly reduced IL-4-dependent STAT6 activation as measured by EMSA (Figure 2.3B), as well as subsequent gene expression, as determined by luciferase assay (Figure 2.4). Similar observations were made in mouse primary splenocytes (Figures 2.5A & B) and in other cells of both human and mouse origins (data not shown). The specificity of DNA-protein complexes in EMSA was confirmed by competition with an excess of unlabeled
DNA probe, and by super-shift using a STAT6-specific antibody (data not shown). Collectively, these data suggest that IL-4 induces ROS production in all cell types examined, and ROS induce amplification but not initiation of IL-4 signaling.

2.4.2. IL-4 Activates NOX through the IRS-PI3K Pathway

To identify the biochemical pathways through which ROS were generated in IL-4-stimulated cells, we used A549 as a model cell line. Binding of IL-4 to its receptor activates the IRS-PI3K, STAT6 and RAS-MAPK pathways in some cells (Haque and Sharma, 2006; Nelms et al., 1999). Pretreatment with Jak inhibitor, AG490 (Meydan et al., 1996), or PI3K inhibitors, LY294002 and wortmannin (Fukuchi et al., 2000) but not MEK1 inhibitor, U0126 (DeSilva et al., 1998), completely blocked ROS production by IL-4, suggesting that Jak and PI3K activities were required for IL-4-induced ROS production (Figure 2.6A). AG490, LY294002 and wortmannin, but not U0126, also significantly inhibited IL-4-dependent STAT6 activation (Figure 2.6B), further suggesting that ROS promote IL-4-dependent signal transduction.

To confirm the roles of PI3K and STAT6 in IL-4-induced ROS generation, we undertook the following approach. IL-4 signals in A549 cells through the type II receptor (Haque and Sharma, 2006; Nelms et al., 1999). Since the binding of IL-4 to its primary receptor, IL-4Rα, is species-specific (Ohara and Paul, 1987), A549 (human) cells did not respond to murine IL-4 (data not shown). IL-13Rα1, which serves as a secondary receptor chain in type II IL-4 receptor, in A549 cells does not exhibit species specificity (Nelms et al., 1999). We isolated cDNA of
murine IL-4Rα, confirmed its functionality (Figure 2.7), and generated a mutant receptor (Tyr500Phe) which is deficient in activating the IRS-PI3K pathway (Nelms et al., 1999). When expressed in A549 cells, the wild-type murine IL-4Rα efficiently supported murine IL-4-induced ROS generation, whereas the mutant IL-4Rα (Tyr500Phe) failed to do so (Figure 2.8), confirming that IRS-PI3K couples the IL-4 receptor to the ROS-generating system. In addition, inhibition of PTEN expression by shRNA (Figure 2.9, upper panel) significantly enhanced IL-4-induced ROS production (Figure 2.10), and subsequent STAT6 activation (Figure 2.9, lower panel) in A549 cells. Further, overexpression of wild-type, but not a catalytically inactive mutant PTEN (C124S) (Myers et al., 1997), significantly inhibited IL-4-induced ROS generation in A549 cells (Figure 2.11). Taken together, these results confirm the requirement of PI3K activity in IL-4-induced ROS generation and downstream signaling.

Murine IL-4Rα did not support the activation of human STAT6, in 293T cells (Figure 2.7), suggesting that IL-4Rα-STAT6 interaction is also species-specific. Consistent with this, in response to murine IL-4 treatment, A549 cells expressing the murine IL-4Rα did not activate endogenous (human) STAT6 but efficiently supported ROS generation (Figure 2.8). Moreover, cycloheximide did not alter IL-4-induced ROS generation in A549 and other cells (data not shown). Taken together, these data clearly indicate that IL-4Rα-mediated ROS generation does not require either STAT6 activation or new protein synthesis.
2.4.3. IL-4 Activates NOX1 and NOX5

The next question was which of the NOX-family members were involved in IL-4-induced ROS production? We found that NOX1, NOX4 and NOX5L (long form) were predominantly expressed in A549 cells (Figures 2.12 and 2.13). Overexpression of NOX1 and NOX5L but not NOX4 in A549 cells significantly increased IL-4-induced ROS generation (Figure 2.14A) as well as STAT6 activation (Figure 2.14B). Further, inhibition of NOX1 expression by shRNA (Figure 2.20) significantly compromised IL-4-induced ROS generation (Figure 2.15), and STAT6 activation (Figure 2.21) in A549 cells. NOX1 activation requires its regulatory subunits, p22phox, NOXA1, NOXO1 and RAC1 (Lambeth et al., 2007). We found that IL-4-induced ROS generation in A549 cells was significantly increased by overexpression of p22phox (Figure 2.16) and markedly compromised by either overexpression of a dominant-negative mutant p22phox (P156Q) (Kawahara et al., 2005) or shRNA-mediated inhibition of p22phox expression (Figure 2.16). In addition, reconstitution of NOX1 complex in A549 cells by overexpression of NOX1, NOXO1 and NOXA1 significantly increased IL-4-induced ROS generation (Figure 2.17). Moreover, IL-4-induced ROS generation was inhibited by overexpression of a dominant negative mutant (N17) RAC1 (Cool et al., 1998) (Figure 2.18). Further, IL-4 stimulation of A549 cells significantly increased RAC1 activation, which was markedly compromised by inhibition of PI3K activity (Figure 2.19). Collectively, these data demonstrate that IL-4 activates NOX1 complex through the IRS-PI3K-RAC1 pathway.
Here, we also demonstrated the involvement of NOX5 in IL-4-induced ROS production and STAT6 activation by overexpression (Figures 2.14A & B) and silencing of NOX5 gene, in A549 cells (Figures 2.15; 2.20; 2.21). Since NOX5L activation requires binding of calcium to its EF hands (Banfi et al., 2004; Lambeth et al., 2007), we examined if IL-4-induced ROS generation was dependent on intracellular calcium flux. Pretreatment of A549 cells with BAPTA-AM, a general calcium chelator (Tsien, 1980), or heparin, an inhibitor of IP3-receptor-mediated calcium flux (Seuwen and Boddeke, 1995) but not nifedipine, a blocker of L-channel-mediated calcium flux (Reid et al., 1997), significantly inhibited IL-4-induced ROS generation (Figure 2.22A) and STAT6 activation (Figure 2.22B). Therefore, it was important to determine if IL-4 stimulation of cells increased cytoplasmic calcium flux. Using Fluo-4AM (Murata et al., 2004), an increase in cytoplasmic calcium flux was detected by confocal microscopy within 5 sec of IL-4 stimulation of A549 cells, which continued to increase for ~180 sec, and reached a plateau thereafter (Figures 2.23 & 2.24). The kinetics of this calcium flux correlated with that of IL-4-induced ROS generation (Figure 2.2A). Next, pretreatment of A549 cells with PLC-γ inhibitor, U73122 (Stam et al., 1998), or inhibitors of diacylglycerol (DAG)-dependent PKC activity, calphostin C (Jarvis et al., 1994) and Go6976 (Gschwendt et al., 1996), significantly inhibited IL-4-induced ROS generation (Figure 2.25). Further, shRNA-mediated inhibition of PLCγ1 and PLCγ2 expression significantly reduced IL-4-induced ROS production (Figure 2.26A), and subsequent STAT6 activation (Figure 2.26B), in A549 cells. Collectively, these results demonstrate that activated IL-4 receptor induces an
intracellular calcium flux via IRS-PI3K-PLC-γ pathway which likely induces DAG- and calcium-dependent PKC-mediated activation of NOX5L to generate ROS.
Figure 2.1: IL-4 Generates ROS in A549 cells.
Cells were loaded with 5 µM CM-DCHF-DA, stimulated with IL-4 (20 ng/ml) under fluorescence microscope and pictures taken.

Figure 2.2: Quantitation of IL-4 Induced ROS in A549 cells.
(A) ROS generation is rapid in the early phases of IL-4 action. CM-DCHF-DA loaded A549 cells were stimulated with IL-4 (20 ng/ml) and fluorescence intensities were quantified. Values in relative fluorescence units (RFU) represent mean ± SE, (n=3).
(B) ROS Level Peaks at ~15 min after IL-4 stimulation of A549 Cells. ROS were measured by flow cytometry after IL-4 stimulation of cells for indicated lengths of time. The values in RFU are plotted as mean ± SE (n=3).
Figure 2.3: IL-4 Generates ROS by NOX Activation.

(A) A549 cells were pretreated for 2 hr with apocynin (500 µM), DPI (10 µM) or L-NAME (50 µM), and ROS were measured following IL-4 stimulation. RFU represent mean ± SE, (n=3) (** indicates P< 0.01).

(B) Blockade of ROS generation inhibits IL-4-dependent STAT6 activation. A549 cells were pretreated with apocynin, DPI or DMSO as described above, and treated with IL-4 (20 ng/ml) for indicated times. Cell extracts were subjected to EMSA and relative signal intensities (RSI) were quantified using ImageQuant software.

Figure 2.4. Blockade of ROS Generation Inhibits IL-4-dependent Gene Expression.

A549 cells were transfected with 4.0 µg DNA of STAT6-responsive luciferase construct. After 48 hr, cells were pretreated with 500 µM apocynin (or DMSO) for 2 hr, stimulated with IL-4 (20 ng/ml) for indicated periods, and luciferase activity measured. Luciferase activities in arbitrary units (AU) are plotted as mean ± SE, (n=3) (** = P < 0.01; NS = not significant).
Figure 2.5: IL-4-dependent ROS Generation Requires Jak and PI3K Activities.

(A) A549 cells were pretreated with AG490 (50 µM for 6 hr), LY294002 (20 µM for 2 hr), wortmannin (10 nM for 2 hr), U0126 (25 µM for 2 hr), or DMSO, and IL-4-induced ROS production measured. RFU are plotted as mean ± SE, (n=3).

(B) Blockade of Jak or PI3K activity inhibits IL-4-induced STAT6 activation. A549 cells were pretreated with the inhibitors described above, and stimulated with IL-4 (20 ng/ml) for 5 min. Cell extracts were subjected to EMSA, and RSI quantified.
Figure 2.6: IL-4 Generates ROS by NOX Activation in Mouse Primary Splenocytes.

(A) Splenocytes were pretreated for 2 hr with apocynin (500 µM) and ROS measured following IL-4 stimulation. RFU represent mean ± SE, (n=3) (**) indicates P< 0.01.

(B) Blockade of NOX activity inhibits IL-4-dependent STAT6 activation in mouse primary splenocytes. Cells were pretreated with apocynin (500 µM) for 2 hr, and treated with IL-4 (20 ng/ml) for indicated times. Cell extracts were subjected to EMSA and RSI quantified.
Figure 2.7: Murine IL-4R\(\alpha\) Activates Murine but not Human STAT6

293T cells were transfected with 2.0 \(\mu\)g of murine IL-4R\(\alpha\) (lanes 3, 5 and 7) or vector (lanes 4 and 6) along with (i) 2.0 \(\mu\)g of human STAT6, 2.0 \(\mu\)g of \(\gamma\)c and 2.0 \(\mu\)g of Jak3 (lanes 3 and 4), (ii) 2.0 \(\mu\)g of murine STAT6, 2.0 \(\mu\)g of \(\gamma\)c and 2.0 \(\mu\)g of Jak3 (lanes 5 and 6) or (iii) only 2.0 \(\mu\)g murine STAT6 (lane 7). For controls, cells were transfected with (iv) 2.0 \(\mu\)g of human IL-9R\(\alpha\), 2.0 \(\mu\)g of human STAT5A, 2.0 \(\mu\)g of human \(\gamma\)c and 2.0 \(\mu\)g of Jak3 (lane 1), or (v) with 2.0 \(\mu\)g of human STAT6 (lane 2). After 48 hr, cells were stimulated for 30 min with human IL-4 (20 mg/ml), murine IL-4 (20 ng/ml) or human IL-9 (10 ng/ml) as indicated. Cell extracts were subjected to EMSA using radiolabeled probes specific for STAT5 (lane 1) or STAT6 (lanes 2-7).
Figure 2.8: PI3K Activation is required for IL-4-induced ROS Generation.

A549 cells were transfected with 1.0 µg of murine wild-type IL-4Rα, mutant IL-4Rα (Y500F), or vector. After 48 hr, IL-4-generated ROS were measured and RFU plotted as mean ± SE, (n=3) (** indicates P < 0.01)

Figure 2.9: Inhibition of PTEN Expression Increases IL-4-dependent STAT6 Activation.

A549 cells were transfected with 4.0 µg of shRNA (or scrambled) construct for PTEN. After 48 hr, total RNA was isolated and steady-state PTEN mRNA levels determined by RT-PCR (upper panel). Alternatively, 48 hr post-transfection, cells were stimulated with IL-4 (20 ng/ml) for 5 min. Cell extracts were subjected to EMSA and RSI quantified (bottom panel).
Figure 2.10: Inhibition of PTEN expression increases IL-4-induced ROS generation.
A549 cells were transfected with 4.0 µg of shRNA (or scrambled) construct for PTEN. After 48 hr, IL-4 generated ROS were measured and RFU plotted as mean ± SE, (n=3) (** indicates P < 0.01).

Figure 2.11: PTEN Over-expression Significantly Inhibits IL-4-dependent ROS Generation
A549 cells were transfected with 3.0 µg of vector, wild-type or mutant PTEN (C174S) along with 1.0 µg of murine IL-4Rα plasmid. After 48 hr, murine IL-4-generated ROS were measured and RFU plotted as mean ± SE, (n=3) (** indicates P < 0.01).
Figure 2.12: Different NOX Genes are Expressed in A549 cells.
Total RNA from A549 cells was used for RT-PCR using NOX-specific primers (upper panel), or for β-actin (lower panel). The expected sizes (bp) of PCR products are indicated below, and non-specific products are indicated by asterisks.

Figure 2.13: A549 cells Express NOX5L.
The expression of NOX2 and NOX5L (and β-actin as control) was checked using RT-PCR. The expected sizes (bp) of the individual products are indicated.
Figure 2.14: NOX1 and NOX5 are Involved in IL-4-dependent ROS Production

(A) Overexpression of NOX1 and NOX5, but not NOX4, increases IL-4-dependent ROS generation. A549 cells were transfected with 1.0 µg of murine IL-4Rα along with 3.0 µg of NOX1, NOX4 and NOX5 (or appropriate vector controls) plasmid. After 48 hr, murine IL-4-generated ROS were measured and RFU plotted as mean ± SE, (n=3) (* = P < 0.05; ** = P < 0.01).

(B) Overexpression of NOX1 and NOX5, but not NOX4, increases IL-4-dependent STAT6 activation. A549 cells were transfected with 1.0 µg of murine IL-4Rα along with 3.0 µg of NOX1, NOX4 and NOX5 (or appropriate vector control) plasmid. After 48 hr, cell extracts were subjected to EMSA and RSI quantified.

Figure 2.15: Inhibition of NOX1 and NOX5 Expression Reduces IL-4-dependent ROS Generation.

A549 cells were transfected with 4.0 µg of shRNA constructs for NOX1, NOX5 or their scrambled versions. After 48 hr, IL-4 generated ROS were measured and RFU plotted as mean ± SE, (n=3). (* = P < 0.05).
Figure 2.16: p22phox Activity Regulates IL-4-induced ROS Production.

A549 cells were transfected with 1.0 µg of murine IL-4Rα along with 3.0 µg of wild-type p22phox, mutant p22phox (P156Q), specific shRNA (N or C forms) or scrambled controls/ corresponding vector plasmids. After 48 hr, murine IL-4-generated ROS were measured and RFU plotted as mean ± SE, (n=3) (* = P < 0.05; ** = P < 0.01).

Figure 2.17: NOXO1 and NOXA1 are Involved in IL-4-mediated ROS Production

Overexpression of NOXO1 or NOXA1 increases IL-4-dependent NOX1-mediated ROS production. A549 cells were transfected with 1.0 µg of murine IL-4Rα along with NOX1, NOXO1 and NOXA1, singly or in combination (total 3.0 µg plasmid). After 48 hr, murine IL-4-generated ROS were measured and in RFU plotted as mean ± SE, (n=3) (** = P < 0.01).
2.18: RAC1 is Involved in IL-4-induced ROS Production.

A549 cells were transfected with 1.0 µg of murine IL-4Rα along with 3.0 µg of wild-type or dominant-negative mutant (N17) RAC1 constructs. After 48 hr, murine IL-4-generated ROS were measured and RFU plotted as mean ± SE, (n=3) (* = P < 0.05).

Figure 2.19: PI3K Activity is required for IL-4-induced RAC1 Activation.

A549 cells were treated with 20 µM LY294002 for 2 hr after which the cells were treated with IL-4 (20 ng/ml) for 5 min, or left untreated. Cell lysates were analyzed for detection of active (upper panel) or total (lower panel) RAC1, by Western analyses.
Figure 2.20: Steady-state Levels of NOX1 and NOX5L mRNAs after shRNA-mediated Inhibition.

A549 cells were transfected with 4.0 µg of shRNA constructs for NOX1, NOX5 or their scrambled versions. After 48 hr, steady-state levels of NOX1 and NOX5L mRNAs were determined by RT-PCR.

Figure 2.21: Inhibition of NOX1 and NOX5 Expression Reduces IL-4-dependent STAT6 activation.

A549 cells were transfected as in (Figure 2.20). After 48 hr, cells were stimulated with murine IL-4 (20 ng/ml) for 5 min. Cell extracts were subjected to EMSA and RSI quantified.
Figure 2.22: IL-4 Induces Calcium Flux which Increases ROS Production.

(A) IL-4-dependent ROS generation is inhibited by blocking IP3-receptor-mediated calcium release. A549 cells were treated for 2 hr with BAPTA-AM (30 μM), heparin (1 μM), nifedipine (0.1 nM) or DMSO, and IL-4-generated ROS measured and RFU plotted as mean ± SE, (n=3) (** = P < 0.01).

(B) Inhibitors of IP3-receptor-mediated calcium release compromise IL-4-dependent STAT6 activation. A549 cells were pretreated as in (A), treated with IL-4 (20 ng/ml) for 5 min. Cell extracts were subjected to EMSA and RSI quantified.

Figure 2.23: Quantitation of IL-4-stimulated Cytoplasmic Calcium Flux.

A549 cells were loaded with 1 μM Fluo4-AM and changes in cytoplasmic calcium levels measured using live-cell confocal microscopy. The arrow indicates the time when the medium was replaced by fresh medium containing 20 ng/ml of IL-4 or 1 μM of ionomycin (as positive control). The experiment was performed five times and data from one representative experiment shown.
Figure 2.24: IL-4-stimulation Increases Cytoplasmic Calcium Flux.
A549 cells treated as above (Figure 2.23) and confocal microscopic pictures taken. The experiment was performed five times and data from one representative experiment is shown.

Figure 2.25: IL-4-mediated ROS Generation Is Dependent on PLC-γ and DAG-dependent PKC Activities.
A549 cells were pretreated for 2 hr with U73122 (2 µM), calphostin C (10 µM), Go6976 (1 µM) or DMSO, and IL-4-induced ROS production measured. RFU are plotted as mean ± SE, (n=3). (* = P < 0.05; ** = P < 0.01).
Figure 2.26: Inhibition of PLC-γ1 or PLC-γ2 Expression by shRNA Reduces IL-4-dependent ROS Generation and STAT6 Activation.

(A) A549 cells were transfected with 4.0 µg of shRNA or scrambled constructs for PLC-γ1 or PLC-γ2. After 48 hr, IL-4 generated ROS were measured and RFU plotted as mean ± SE, (n=3) (* = P < 0.05; ** = P < 0.01).

(B) Inhibition of PLC-γ1 or PLC-γ2 expression increases IL-4-dependent STAT6 activation. A549 cells were transfected as in (A). After 48 hr, PLC-γ1 and PLC-γ2 transcript levels were determined by RT-PCR (upper panel). Alternatively, 48 hr post-transfection, cells were stimulated with IL-4 for 5 min. Cell extracts were subjected to EMSA and RSI quantified.
2.5. Discussion

In this study we have demonstrated, for the first time that immediately following IL-4 engagement the activated IL-4 receptor produced ROS that, in turn, increased the magnitude of receptor activation and consequent signal transduction, in the absence of *de novo* protein synthesis.

We identified biochemical pathways that coupled an activated IL-4 receptor to ROS producing enzymes NOX1 and NOX5. The mechanism of activation has been best characterized for NOX2, the founding member of the family. In response to appropriate stimuli, a membrane-bound flavocytochrome \((b_{558})\) composed of NOX2 (gp91phox) and p22phox forms an activation complex by recruiting at least three cytoplasmic proteins which includes p67phox, p47phox, (p40phox in some cases) and RAC2 (Lambeth, 2004; Lambeth et al., 2007). NOX1 also forms an activation complex with p22phox, RAC1, NOXO1 (p67phox homolog) and NOXA1 (p47phox homolog) (Lambeth, 2004; Lambeth et al., 2007). Inhibition of p22phox and RAC1 activities by dominant-negative mutants, or RNAi-mediated silencing of p22phox significantly reduced IL-4-mediated ROS generation. On the other hand, ROS generation was significantly increased by overexpression of NOXO1, NOXA1, p22phox and RAC1, suggesting the involvement of these components in IL-4-mediated NOX1 activation. This corroborates an earlier report that demonstrated IL-4-dependent RAC1 activation in human keratinocytes (Wery-Zennaro et al., 2000).

NOX5 is a calcium-dependent enzyme that does not require any regulatory subunits (Banfi et al., 2001; Banfi et al., 2004). Both overexpression
and RNAi-mediated gene silencing data suggested the involvement of NOX5L in IL-4-dependent ROS generation. Further, IL-4-dependent ROS generation was significantly compromised by inhibitors of cytoplasmic calcium flux, suggesting that calcium flux is required for NOX5 activation by IL-4. However, it was not known if IL-4 induces calcium flux. Using the calcium indicator dye, Fura-2, we could not detect IL-4-induced calcium flux (data not shown). An earlier study also failed to detect IL-4-dependent calcium flux (Ho et al., 1994). Using Fluo-4AM, whose fluorescence intensity increases >100-fold upon calcium binding (Harkins et al., 1993), we demonstrated, for the first time, that IL-4 induced an immediate cytoplasmic calcium flux in A549 cells. Interestingly, IL-4-dependent ROS generation was compromised by inhibitors of DAG-dependent PKCs. A recent study has shown that activation of NOX5 is regulated by an unidentified PKC-mediated phosphorylation of specific serine/threonine residues (Jagnandan et al., 2007). A number of previous studies have focused on DAG- and calcium-independent PKC-mediated regulation of IL-4 signaling (Duran et al., 2004; Ho et al., 1994; Ikizawa et al., 1994). Our results suggest a role for classical PKCs which depend on both DAG and calcium, in IL-4-mediated cell signaling. Moreover, IL-4-dependent ROS generation was compromised by PLC-γ inhibition. Two studies have demonstrated the role for PLC-γ1 in IL-4 signaling (Ikizawa et al., 1994; Ikizawa and Yanagihara, 2000), whereas another report has implicated a role of PC-PLC, but not PLC-γ, in IL-4 signaling (Zamorano et al., 2003). This may be due to the use of mouse cells in the latter study, which do not express NOX5 (Banfi et al., 2001).
In summary, we have shown that activated IL-4 receptor generated ROS in an IRS-PI3K-mediated, calcium-dependent and -independent activation of NOX5L and NOX1, respectively. We also demonstrated that IL-4 increased intracellular calcium flux, which is required for NOX5L activation.

The next logical goal was to understand the biochemical basis of ROS-mediated amplification of IL-4 signaling. More specifically, it will be important to identify the PTP, which negatively regulate IL-4 signaling, and to investigate if ROS generated by activated IL-4 receptor oxidatively inactivates the PTP activity.
CHAPTER III

IL-4-STIMULATION GENERATES ROS, WHICH CATALYTICALLY INACTIVATES PTP1B AND UPREGULATES RECEPTOR ACTIVATION

3.1. Abstract

The physiologic control of cytokine receptor activation is primarily mediated by reciprocal activation of receptor-associated PTKs and PTPs. We have shown in chapter II that following ligand-dependent activation, IL-4 receptor induces an intracellular calcium flux via IRS-PI3K-PLC-γ pathway which, in turn, induces PKC-dependent activation of NOX5 that generates reactive oxygen species (ROS). Given our earlier findings, it was imperative to understand the biochemical basis of ROS-mediated IL-4 signal amplification. More specifically, it was important to identify the PTP and to investigate if ROS generated by activated IL-4 receptor oxidatively inactivate the IL-4 receptor-associated PTP activity. We show here that ROS promote IL-4 receptor activation by oxidatively inactivating PTP1B, which physically associates with and deactivates IL-4
receptor. However, ROS are not required for the initiation of IL-4 receptor activation. ROS generated by activated erythropoietin receptor also promote IL-4 signaling. These data reveal that inactivation of receptor-associated PTP-activity by cytokine-generated ROS is a physiologic mechanism for the amplification of cytokine receptor activation in both cis and trans, unfolding a novel means of cytokine signaling cross-talk.
3.2. Introduction

PTPs are a family consisting of a wide variety of receptor-like and non-transmembrane enzymes. PTPs serve as important regulators for many different signaling pathways and are characterized by remarkably high in vivo substrate specificity. Much though remains to be elucidated about the regulatory mechanisms determining the activity of PTPs. Recent evidence suggests that at least some PTPs can be regulated by oxidative inactivation.

Protein tyrosine phosphorylation is a reversible and tightly regulated biochemical event. The forward and reverse reactions are catalyzed by receptor-associated Jaks and protein tyrosine phosphatases (PTPs) respectively (Haque and Sharma, 2006; Heldin, 1995). In the absence of cytokine engagement, receptor-associated PTP activity dominates over the Jak activity, thereby holding the receptor in an inactive state (Fischer et al., 1991; Haque and Sharma, 2006). Engagement of cytokine causes aggregation of receptor chains, bringing their associated Jak molecules into an appropriate proximity. This allows the trans-phosphorylation of Jaks on signature tyrosine residues, which increases their catalytic activities, thereby promoting the forward reaction and stabilizing the receptor activation (Haque and Sharma, 2006; O'Shea et al., 2002).

This mechanism of receptor activation is well established for the majority of cytokines including IL-4. However, in principle, inactivation of receptor-associated PTPs could be an alternate means of receptor activation. The
catalytic cysteine residue of PTPs are susceptible to oxidative inactivation (Rhee et al., 2000; Tonks, 2005; Tonks, 2006).

Our laboratory has previous reported that, vanadium peroxide (pervanadate) causes irreversible oxidation of catalytic Cys of PTPs, which in turn increases the tyrosine phosphorylation of many cytokine-signaling proteins in the absence of cytokine-stimulation of cells. In a subsequent study, we have demonstrated that pervanadate-mediated inactivation of IL-4 receptor-associated PTP activity, which controls the Jak1 function, leads to a ligand-independent activation of STAT6. These results strongly suggest that IL-4 receptor-associated PTP activity functions as a primary negative regulator of the signaling pathway, and the PTP activity is regulated by the redox status of the receptor microenvironment.

All PTPs contain an eleven amino acid signature motif [(Ile/Val)-His-Cys-X-Ala-Gly-X-X-Arg-(Ser/Thr)-Gly] (X is any amino acid), in which the Cys is essential for catalysis. It is shown that the Cys-thiolate anion (Cys-S\(^{-}\)) is more susceptible to oxidation by H\(_2\)O\(_2\) than Cys-SH (Denu and Dixon, 1998; Denu and Tanner, 1998). The catalytic Cys of PTPs can be readily oxidized by H\(_2\)O\(_2\) and other oxidants. Given that thiolate anion (PTP-Cys-S\(^{-}\)) is the reactive form of the enzyme, peroxide-mediated oxidation of signature motif Cys can inactivate the catalytic function of the PTPs.

To this end, our laboratory, and others, have previously identified SHP-1 and CD45 as negative regulators of this pathways (Haque et al., 1998; Yamada et al., 2002). However the PTPs investigated are expressed exclusively in hematopoietic cells (Irie-Sasaki et al., 2001). Since functional IL-4 receptor is
expressed in almost all cell types (Nelms et al., 1999), it is important to identify ubiquitously expressed PTPs that dephosphorylate and deactivate IL-4 receptor.

In the previous chapter, we demonstrated that ROS are generated immediately after IL-4 stimulation of both hematopoietic and non-hematopoietic cells, which promote IL-4 receptor activation and subsequent signal transduction. Here, we show that this is mediated by ROS-dependent oxidative inactivation of PTP1B, a ubiquitously expressed PTP that physically associates with and deactivates IL-4 receptor. Further, we provide evidence that ROS generated by another cytokine, EPO, markedly amplify the activation of IL-4 receptor and consequent signal transduction in the same cells, suggesting that ROS can mediate cytokine signaling cross-talk.
3.3. Materials and Methods

3.3.1. Cells and Reagents

A549, 293T and NIH 3T3 cells were maintained in DMEM, supplemented with 10% fetal calf serum (FCS). Immortalized MEFs from Ptpn1<sup>+/+</sup> and Ptpn1<sup>-/-</sup> mice were maintained in DMEM supplemented with 15% FCS (Buckley et al., 2002). Single cell suspensions were prepared from mouse spleens, and cultured in IMDM supplemented with 10% FCS. Cells obtained by flushing the mouse femur bone marrow were cultured in complete DMEM supplemented with 10% FCS and 20% L929-conditioned medium (as a source of M-CSF) to differentiate them into macrophages (Haque et al., 1998). To develop mast cells, the bone marrow-derived cells were cultured for 3-4 weeks in RPMI supplemented with 10% FCS, 10 mM HEPES, 1X MEM essential amino acids, 1 mM sodium pyruvate, 10 µM β-mercaptoethanol and 20% WEHI-3 conditioned medium (as a source of IL-3), and cells were used when more than 95% of them stained positive with toluidine blue (Yuan et al., 1998).

All recombinant cytokines and anti-oxy-PTP were purchased from R&D Systems. Apocynin, DPI, AG490, LY294002 and wortmannin were obtained from Calbiochem. Nifedipine, heparin and anti-FLAG conjugated agarose beads were purchased from Sigma. All antibodies used for T cell isolation, differentiation and analyses were from BD Pharmingen. Anti-phospho (Tyrosine641)-STAT6 and anti-phospho-tyrosine PY100 were purchased from Cell Signaling Technology, and anti-V5 was from Invitrogen. Anti-STAT6, anti-β-actin, anti-FLAG and anti-goat HRP were obtained from Santa Cruz Biotechnology and anti-PTP1B was
from BD Transduction. Anti-mouse HRP and anti-rabbit HRP were purchased from Amersham Biosciences. All animal experiments were conducted according to the guidelines of our Institutional Animal Care and Use Committee.

3.3.2. Intracellular ROS Assay

ROS were measured fluorimetrically using CM-H$_2$DCFDA probe (Choi et al., 2005; Myhre et al., 2003). Briefly, adherent cells were washed twice with Hank’s balanced salt solution (HBSS), incubated with 5 µM CM-H$_2$DCFDA in HBSS for 10 min at 37°C in 5% CO$_2$, washed and stimulated with indicated cytokine at 37°C under a Leica DMIRB inverted microscope (Leica Microsystems) equipped with a Retiga EXi Cooled CCD Camera (Q Imaging, Burnby), with a Ex$_{490nm}$ and standard fluorescein filter. Images were taken at every 10 sec, and fluorescence signals were quantified using Image Pro Plus Software (Media Cybernatics). Each value in relative fluorescence units (RFU) represents mean ± SE of three independent measurements; each measurement represents the mean fluorescence signals of five equivalent fields (approximately same number of cells). To measure ROS for longer time periods (>5 min), cells were loaded with CM-H$_2$DCFDA, trypsinized, resuspended in HBSS, and changes in fluorescence signals were measured using flow cytometry (FACScan, Beckton-Dickinson). ROS produced by non-adherent cells were measured using Victor$^2$ Wallace Mutichannel Reader (Perkin Elmer) using Ex$_{495nm}$ and Em$_{535nm}$ filters.
3.3.3. Electrophoretic Mobility Shift, Super-shift and Luciferase Assays

EMSA was performed using 10 µg proteins of whole cell extracts and 0.2 ng of ^32^P-labeled high-affinity STAT6-specific probe (Haque et al., 2000). Cell extracts were incubated with 2 µg of normal rabbit IgG or anti-STAT6 antibody prior to incubation with 0.2 ng of radiolabeled oligonucleotide duplex for 20 min for super-shift assay, or with 10 ng or 50 ng of unlabeled wild-type or mutant oligonucleotide duplex for competition assay. The sequences for the sense strand of the oligonucleotide are: 5'-GATCGCTTCTTCCCAGGAATCTCA-ATG-3' (wild-type) and 5'-GATCGCTTCTTCCCAGGGCTCAATG-3' (mutant) with the conserved N6-GAS sequence underlined (Haque et al., 2000), and the mutated nucleotides italicized. The DNA-protein complexes were resolved on native 6% polyacrylamide gels, dried and visualized after incubation at -80°C. Luciferase assay was performed using the Dual Luciferase Assay Kit (Promega) as per the manufacturer's instructions.

3.3.4. Cloning, Site-directed Mutagenesis and Plasmid Constructs

Murine IL-4Rα cDNA was isolated from a NIH3T3 cell-derived cDNA library by PCR, and cloned in pcDNA3.1/V5-HisA (Invitrogen). For BRET assay, human IL-4Rα was cloned in pRLuc-N3, and STAT6 and PTP1B (D181A) in pGFP^2^·N2 (Perkin Elmer). All point mutants were generated using Quick Change XL Site-directed Mutagenesis Kit (Stratagene) following the manufacturer's instructions.
DNA oligonucleotides encoding shRNA sequences with loop sequence (5'-TTC- AAGAGA-3') were cloned into pSuper-Neo (OligoEngine) according to the manufacturer’s instructions. The specific target sense sequences were: 5'-GAATTAGGCAAAAGTGGGTT-3' for NOX1, 5'-AAGAGCGATTCTTTGCCCTAT-3' for NOX5L, 5'-CGGGAAGACAAGTTCA-TGTACTT-3'. The scramble versions of the target sequences were generated using siRNA Wizard (InVivogen), and cloned in pSuper-Neo vector (OligoEngine). All constructs were verified by nucleotide sequencing.

3.3.5. Transfection and Generation of Stable Clones

A549 and 293T cells were transfected using Lipofectamine 2000 (Invitrogen) and calcium phosphate co-precipitation respectively (Haque et al., 2000) with transfection efficiencies of 70-80% and ~90% respectively. Stable clones of immortalized PTP1B-deficient MEFs were generated by selection with 150 µg/ml hygromycin B (Invitrogen) for 2 weeks.

3.3.6. RT-PCR

For determination of mRNA levels, 1.0 µg of total RNA was used for first-strand cDNA synthesis according to the manufacturer’s protocol (Invitrogen). One-tenth of cDNA was used as a template for 35 cycles of PCR using specific primer sets listed in Table I.
3.3.7. *In Vitro* T Cell Differentiation

Single-cell suspensions of mouse lymph node cells were incubated with FITC-conjugated anti-CD8, anti-B220, anti-MHC Class II (Fh2d) and anti-FcγR, followed by incubation with anti-FITC microbeads (Miltenyi Biotec). The negative fraction obtained after magnetic separation using an LS magnetic column (Miltenyi Biotec) contained >95% CD4⁺ T cells. These cells were stained with PE-conjugated anti-CD44, and naïve CD4⁺ T cells were sorted using a FACS Vantage SE cell sorter (Becton Dickinson). 1 x 10⁶ purified naïve CD4⁺ T cells were cultured in 12-well plates in the presence of 3 µg/ml of anti-CD3 and 3 µg/ml of anti-CD28, with 5 X 10⁶ T-cell depleted splenocytes. For Th1 cell differentiation, murine IL-12 (10 ng/ml) and anti-IL-4 (10 µg/ml) were added to the culture media. For Th2 cell differentiation, murine IL-4 (1000 U/ml) was added along with anti-IL-12 (10 µg/ml) and anti-IFN-γ (10 µg/ml). After 72 hr, the cells were harvested and restimulated with immobilized anti-CD3 (3 µg/ml) and anti-CD28 (3 µg/ml) for 6 hr in the presence of 2 µM monensin (Calbiochem) for the last 2 hr. The cells were fixed with 4% paraformaldehyde, permeabilized in buffer (PBS containing 0.1% BSA, 0.5% Triton X-100), and stained with FITC-conjugated anti-IFN-γ and PE-conjugated anti-IL-4. The expression of intracellular cytokines was determined using a FACSCalibur (Becton Dickinson), and analyzed using a FlowJo software (Treestar).
3.3.8. Chemical Cross-linking, Immunoprecipitation and Immunoblotting

Chemical cross-linking, immunoprecipitation and immunoblotting of proteins were performed as described (Maiti et al, 2005).

3.3.9. BRET Assay

BRET assay was performed following the method of Pfleger and Eidne (Pfleger and Eidne, 2006). Briefly, 293T cells were transfected with 1.0 µg of the donor plasmid, pIL-4Rα-LUC-N3 and 3.0 µg of an acceptor plasmid pGFP²-N2 (empty vector), STAT6-GFP²-N2 or PTP1B-D181A-GFP²-N2, using Lipofectamine 2000 (Invitrogen). After 48 hr, cells were washed with PBS and collected in BRET² buffer (Dulbecco’s PBS supplemented with 0.05 g/l CaCl₂, 0.05 g/l MgCl₂ and 0.1 g/l glucose), washed twice, and resuspended at 2.5 x 10⁷ cells/ml. Cell suspensions in 96-well plates (50 µl/well) were stimulated 20 ng/ml of IL-4 or left untreated, followed by addition of DeepBlueC (Perkin Elmer). Fluorescence signals were measured at every 30 sec using Victor³ Wallace Multichannel Reader (Perkin Elmer) with Ex₃95nm and Em₅10nm. For each time point, BRET ratio was calculated as: (Fluorescence₅10nm/Luminescence) – (fluorescence₅10nm for donor only cells/Luminescence). Each point represents mean ± SE (n=3).

3.3.10. ROS-Induced Oxidation of PTP1B

PTP1B oxidation was measured as described (Persson et al., 2004), with minor modifications. To eliminate spontaneous PTP1B oxidation during cell lysis,
buffers were extensively deoxygenated by bubbling nitrogen gas, and cell lysis was performed in an anaerobic chamber with a continuous flow of nitrogen gas. After indicated treatments, cells were washed with deoxygenated cold PBS, and lysed using deoxygenated lysis buffer (20 mM Tris, pH 7.5, 1% NP-40, 10% glycerol, 1 mM benzamidine and 1.4 µg/ml aprotenin) in the presence or absence of 100 mM iodoacetic acid (IAA). IAA alkylates all cysteines except the reversibly oxidized ones. Cell lysates were immunoprecipitated using anti-PTP1B, immobilized on protein G sepharose beads, and incubated with 10 mM DTT to reduce the reversibly oxidized cysteine of PTP1B which was subsequently oxidized irreversibly to cysteine-sulfonic acid by incubation with 100 µM PV for 1 hr at 4°C. The samples were then used for immunoblotting with a monoclonal antibody raised against a peptide corresponding to the conserved PTP active site (‘Val-His-CysSO$_3$H-Ser-Ala-Gly’) in which the catalytic cysteine (C215 in PTP1B) is irreversibly oxidized to cysteine-sulfonic acid.

3.3.11. Statistical and Densitometric Analyses

All experiments were performed at least three times. Statistical differences between different groups were determined using paired Student’s t-test. EMSA and immunoblot signals were quantified using ImageQuant (Molecular Dynamics).
3.4. Results

3.4.1. PTP1B Downregulates IL-4 Receptor Activation

Since ROS produced by IL-4 promote the activation of its receptor, we hypothesized that ROS generated by activated IL-4 receptor may oxidatively inactivate IL-4 receptor-associated PTP activity. Previously we and others have identified SHP-1 and CD45, which are exclusively expressed in hematopoietic cells, as negative regulators of IL-4 signaling (Haque et al., 1998; Yamada et al., 2002). IL-4 induced ROS generation in all cell types examined. Therefore, it was important to identify a ubiquitously expressed PTP that deactivates IL-4 receptor. To this end, overexpression of a panel of ubiquitous PTPs in 293T cells identified PTP1B as a potential candidate which significantly inhibited IL-4-dependent STAT6 activation (Figure 3.1). Further, overexpression of wild-type but not catalytically inactive mutant PTP1B (C215S), markedly inhibited IL-4-dependent STAT6 activation (Figure 3.2) and subsequent gene expression in 293T cells (Figure 3.3). This was confirmed using immortalized embryonic fibroblasts derived from PTP1B−/− mice (Buckley et al., 2002; Elchebly et al., 1999) which exhibited significantly heightened STAT6 activation, in both magnitude and duration, in response to IL-4 stimulation (Figure 3.4). Moreover, IL-4-dependent STAT6 activation was inhibited when PTP1B was knocked-in in PTP1B−/− MEFs (Figure 3.5). IL-13 utilizes the type II IL-4 receptor for cell signaling (Nelms et al., 1999). We found that IL-13-dependent STAT6 activation was significantly increased in PTP1B−/− MEFs (Figure 3.6).
PTPs may exhibit overlapping, redundant functions in cytokine-mediated cell signaling. Since SHP-1 and CD45 inhibit IL-4 signaling in hematopoietic cells (Haque et al., 1998; Yamada et al., 2002), it was important to examine if PTP1B would also inhibit IL-4 signaling in hematopoietic cells. Figure 3.7 shows that IL-4-dependent STAT6 activation was markedly increased in primary splenocytes derived from PTP1B−/− mice. Consistent with the results, PTP1B-deficiency also increased IL-4-induced ROS generation in both MEFs (Figure 3.8) and splenocytes (Figure 3.9). Further, when PTP1B was knocked-in to PTP1B−/− MEFs, IL-4-induced ROS production was significantly reduced (Figure 3.8). PTP1B-deficiency also increased ROS production by IL-4 in mouse primary macrophages, mast cells and T cells, and by IL-13 in MEFs, splenocytes and macrophages (Figure 3.10). Taken together, these data demonstrate that PTP1B plays a non-redundant role in the negative regulation of IL-4 and IL-13 signaling in hematopoietic and non-hematopoietic cells.

Next, we asked whether PTP1B-deficiency would favor the differentiation of naïve T helper cells to a Th2 lineage. We observed that CD4+CD44low (naïve) T cells isolated from lymph nodes of PTP1B−/− mice produced 2.3- and 1.4-fold enhancement in the levels of IL-4 under neutral and Th2-skewed conditions, respectively, compared with wild-type cells (Figure 3.11). These data suggest that PTP1B negatively controls Th2 differentiation of naïve T helper cells in vitro. However, we also noted that under Th1-skewed condition, PTP1B−/− CD4+CD44low T cells increased the level of IFN-γ production by 1.4-fold, (Figure 3.11). Th1 differentiation is governed by IL-12- and IFN-γ signaling (Nelms et al.,
PTP1B binds to, and dephosphorylates Jak2, thereby attenuating IFN-γ signaling (Myers et al., 2001). Although PTP1B-mediated downregulation of IL-12 signaling has not been demonstrated directly, Jak2 and TYK2, that are required for IL-12-mediated cell signaling (Haque and Sharma, 2006), are shown to be potential substrates for PTP1B. Therefore, it was not unexpected that PTP1B-deficiency increased the number of IFN-γ producing cells under Th1-skewed condition (Figure 3.11).

Next, to determine if PTP1B dephosphorylates IL-4 receptor, we used a chimeric receptor, EPOR-IL-4Rα, composed of the extracellular and transmembrane domains of the murine EPOR and the cytoplasmic domain of the human IL-4Rα (Figure 3.12A). This receptor, when co-expressed with STAT6 in 293T cells (which do not express endogenous, functional STAT6), forms homodimer upon EPO-binding, and induces STAT6 activation (Haque et al., 2000). PTP1B significantly inhibited EPO-dependent tyrosine phosphorylation of EPOR-IL-4Rα, and subsequent activation of STAT6 in 293T cells (Figure 3.13), suggesting that PTP1B might interact with IL-4Rα, Jak1 or STAT6. However, a previous study has shown that Jak1 does not bind to PTP1B (Myers et al., 2001).

To determine if PTP1B binds to IL-4Rα and/or STAT6, either wild-type PTP1B or substrate-trapping mutant PTP1B (D181A) (Flint et al., 1997), was co-expressed with EPOR-IL-4Rα and/or STAT6, in 293T cells. Subsequent co-immunoprecipitation and Western analyses, however, failed to detect any association of PTP1B with either EPOR-IL-4Rα or STAT6 (data not shown). To address the possibility of a weak and dynamic interaction occurring between
these proteins, cell lysates were prepared in the presence of the cross-linking agent, dithio[succimidyl propionate] (DSP), prior to immunoprecipitation (Maiti et al., 2005). We found that PTP1B formed a complex with the cytoplasmic domain of IL-4Rα, but not STAT6 (Figure 3.14). Further, using deletion mutants of EPOR-IL-4Rα (Figure 3.12B), we demonstrated that PTP1B interacted with a region of IL-4Rα harboring the STAT6-docking tyrosine residues (Nelms et al., 1999) (Figure 3.12C).

Bioluminescence resonance energy transfer (BRET) assay is a powerful tool for the detection of weak and dynamic protein-protein interactions in live cells (Pfleger and Eidne, 2006). Using this assay, an interaction between PTP1B (D181A) and IL-4Rα was detected even in the absence of IL-4 stimulation, which was increased in the presence of IL-4 (Figure 3.15). These results confirmed the in vitro cross-linking data indicating PTP1B physically associates with IL-4Rα.

3.4.2. ROS Inactivate PTP1B by Oxidation of Its Catalytic Cysteine, and Serve as a Mediator of Cytokine Cross-Talk

ROS-mediated oxidative inactivation of PTP1B has been demonstrated both in vitro (Salmeen et al., 2003; van Montfort et al., 2003), and in vivo by insulin and EGF (Lee et al., 1998; Mahadev et al., 2001; Meng et al., 2002). Since we found that IL-4 induced ROS production (Figure 2.1), and that PTP1B deactivated IL-4 receptor, it was important to examine if IL-4-generated ROS could cause oxidative inactivation of PTP1B. Using a monoclonal antibody raised against oxidized PTP-active site (Persson et al., 2004), we observed a time-
dependent oxidation of the catalytic cys215 of PTP1B in A549 cells after stimulation with IL-4 (Figure 3.16) or IL-13 (Figure 3.17). Further, pretreatment of these cells with apocynin or LY294002 that completely inhibited IL-4-mediated ROS production significantly reduced the oxidation of PTP1B (Figure 3.18). Moreover, shRNA-mediated reduction of NOX1 or NOX5 expression, which significantly decreased IL-4-induced ROS generation, inhibited IL-4-dependent oxidation of PTP1B (Figure 3.19). IL-4 also induced a time-dependent oxidation of PTP1B in primary mouse splenocytes (Figure 3.20A), and primary bone marrow-derived macrophages (Figure 3.20B). These results clearly demonstrate that ROS-mediated amplification of IL-4 (or IL-13) signaling is, in part, due to oxidative inactivation of PTP1B, in both primary and immortalized cells.

Under physiologic settings, multiple cytokines may act on a single cell that expresses its cognate receptor(s). Since ROS are small, diffusible radicals or molecules, it is possible that oxidative inactivation of PTP1B by other cytokine-generated ROS may amplify the activation of IL-4 receptor in the same cell. To examine this possibility, the mutant murine IL-4Rα (Tyr500Phe) that did not generate ROS but supported STAT6 activation, in response to murine IL-4 stimulation, was expressed in A549 cells. Upon simultaneous treatment of these cells with EPO and murine IL-4, a significant increase (1.2-fold) in STAT6 activation was observed (Figure 3.21). EPO did not activate STAT6 (Figure 3.21) but induced ROS production in these cells (Figure 3.22). These results suggest that EPO-generated ROS can act in trans to promote IL-4 signaling in the same cell. Further, to examine if EPO or other cytokines can promote IL-4 signaling
through its endogenous receptor, A549 cells were pretreated with EPO or TNF-α followed by treatment with IL-4, in the presence or absence of PI3K inhibitor, LY294002. Both EPO and TNF-α markedly promoted the activation of endogenous IL-4 receptors in A549 cells (Figure 3.23). Similar observations were made in mouse primary splenocytes where ROS generated by IL-3 and TNF-α (Figures 3.24A & B) significantly increased IL-4-dependent STAT6 activation (Figures 3.25A & B). Of note, LY294002 completely blocked EPO- and TNF-α-induced ROS generation in A549 cells, but failed to completely compromise IL-3- and TNF-α-induced ROS production in primary splenocytes (Figures 3.25), suggesting that IL-3 and TNF-α may also induce ROS production by PI3K-independent mechanism(s). Importantly, these were consistent with trans-activation levels of IL-4 signaling in the respective cells.

Collectively, these results demonstrate, for the first time, that ROS produced by activation of other cytokine receptors were able to enhance the activation of IL-4 receptor in the same cells, suggesting that ROS can serve as a physiologic mediator of cross-talk between different cytokine receptors.
Figure 3.1: PTP1B Negatively Controls IL-4 Signaling

Overexpression of PTP1B inhibits IL-4-dependent STAT6 activation. 293T cells were transfected with 0.1 µg of STAT6 expression construct along with 4.0 µg of PTP1B, TC-PTP, SHP-1 (as a positive control), SHP-2 construct or respective empty vector. After 48 hr, cells were treated with IL-4 (20 ng/ml) for 30 min, and cell extracts were subjected to EMSA and Western analyses.

Figure 3.2: Phosphatase Activity of PTP1B is required for Inhibition of IL-4 dependent STAT6 Activation.

293T cells were transfected with 0.1 µg of STAT6 expression construct along with 4.0 µg of wild-PTP1B, phosphatase-dead mutant PTP1B (C215S) or vector. After 48 hr, cells were treated with IL-4 (20ng/ml) for 30 min, and cell extracts were subjected to EMSA.
Figure 3.3: PTP1B Inhibits IL-4- and IL-13-dependent Gene Expression.

293T cells were transfected with 0.1 µg of STAT6 expression plasmid and 4.0 µg of STAT6-responsive luciferase construct, along with indicated amounts of wild-type or phosphatase-dead mutant PTP1B (C251S), or with 5.0 µg of vector. After 24 hr, transfected cells were split in 1:3; and treated for 18 hr with IL-4 (10 ng/ml), IL-13 (10 ng/ml), or left untreated. Normalized luciferase activities in arbitrary units (AU) are plotted as mean ± SE, (n=3) (** = P < 0.01).
Figure 3.4: PTP1B Deficiency Increases IL-4-dependent STAT6 Activation in MEFs.

Immortalized MEFs derived from PTP1B+/+ and PTP1B−/− mice were treated with IL-4 (20 ng/ml) for the indicated periods, and cell extracts were subjected to EMSA or Western analyses, and RSI quantified.

Figure 3.5: Knocking-in of PTP1B in PTP1B−/− MEFs Reduces IL-4-dependent STAT6 Activation.

Immortalized PTP1B−/− MEFs were stably transfected with either vector or PTP1B, and clones were selected in the presence of hygromycin B. Selected pools were treated with IL-4 (20 ng/ml) for 30 min, and cell extracts were subjected to Western analyses and EMSA.
Figure 3.6: PTP1B-deficiency Increases IL-13-dependent STAT6 Activation in MEFs.

Immortalized MEFs derived from PTP1B\textsuperscript{+/+} and PTP1B\textsuperscript{-/-} mice were treated with IL-13 (10 ng/ml) for the indicated periods. Cell extracts were subjected to EMSA and RSI quantified.

<table>
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<th>PTP1B\textsuperscript{-/-}</th>
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</tr>
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</table>

Figure 3.7: PTP1B-deficiency Increases IL-4-dependent STAT6 Activation in Mouse Primary Splenocytes.

Splenocytes from PTP1B\textsuperscript{+/+} and PTP1B\textsuperscript{-/-} mice were treated with IL-4 (20 ng/ml) for the indicated periods, cell extracts were subjected to EMSA and RSI quantified.

<table>
<thead>
<tr>
<th>IL-4 (min)</th>
<th>PTP1B\textsuperscript{+/+}</th>
<th>PTP1B\textsuperscript{-/-}</th>
</tr>
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<tbody>
<tr>
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<td>30</td>
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<tr>
<td>60</td>
<td>1.0 1.0 0.8 0.7 0.0 1.3 1.4 1.4</td>
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<tr>
<td>120</td>
<td>1.0 1.0 0.8 0.7 0.0 1.3 1.4 1.4</td>
<td></td>
</tr>
</tbody>
</table>
Figure 3.8: PTP1B-deficiency Increases IL-4-dependent ROS Generation in MEFs.

Immortalized MEFs derived from PTP1B$^{+/+}$ and PTP1B$^{-/-}$ mice, and representative clones of PTP$^{-/-}$ MEFs stably transfected with PTP1B (or vector) were examined for IL-4-induced ROS production. RFU are plotted as mean ± SE, (n=3) (* = P < 0.05; ** = P < 0.01).

Figure 3.9: PTP1B-deficiency Increases IL-4-dependent ROS Generation in Mouse Primary Splenocytes.

Splenocytes isolated from PTP1B$^{+/+}$ and PTP1B$^{-/-}$ mice were examined for IL-4-induced ROS production in RFU plotted as mean ± SE, (n=3) (** = P < 0.01).
Figure 3.10: PTP1B-deficiency Results in Increased ROS Production by IL-4 or IL-13.

(A) Immortalized MEFs derived from PTP1B<sup>+</sup>/+ and PTP1B<sup>-/-</sup> mice were examined for IL-13-induced ROS production, and RFU plotted as mean ± SE, (n=3) (** = P < 0.01).

(B) Primary splenocytes derived from PTP1B<sup>+</sup>/+ and PTP1B<sup>-/-</sup> mice were examined for IL-13-induced ROS production, and RFU plotted as mean ± SE, (n=3) (** = P < 0.01).

(C & D) Primary macrophages derived from bone marrows of PTP1B<sup>+</sup>/+ and PTP1B<sup>-/-</sup> mice were examined for IL-4- (C) and IL-13- (D)-induced ROS production, and RFU plotted as mean ± SE, (n=3) (** = P < 0.01).

(E) Primary mast cells derived from bone marrows of PTP1B<sup>+</sup>/+ and PTP1B<sup>-/-</sup> mice were examined for IL-4-induced ROS production, and RFU plotted as mean ± SE, (n=3) (** = P < 0.01).
Naïve CD4+ T cells derived from lymph nodes of PTP1B+/+ and PTP1B−/− mice were examined for IL-4-induced ROS production, and RFU plotted as mean ± SE, (n=3) (** = P < 0.01).

Figure 3.11: PTP1B-deficiency Increases Number of IL-4-producing CD4+ T cells.

Naïve CD4+ T cells were purified from lymph nodes of PTP1B+/+ and PTP1B−/− mice, and cultured with T-cell depleted syngenic irradiated spleen cells as a source of antigen-presenting cells, at a ratio of 1:5, for 72 hr under conditions to induce Th0, Th1 or Th2 differentiation. Intracellular IL-4 and IFN-γ levels were determined following re-stimulation with anti-CD3 and anti-CD28 antibodies.
Figure 3.12: PTP1B Associates with the Cytoplasmic Domain of IL-4Rα.

(A) Schematic representation of the EPOR-IL-4Rα chimeric receptor. The numbers in parentheses indicate the corresponding amino acids of human IL-4Rα.

(B) Schematic representation of the EPOR-IL-4Rα deletion mutants.

(C) PTP1B interacts with a specific region of IL-4Rα. 293T cells (1.2 x 10^6 cells per 10-cm plate) were transfected with 4.0 µg of PTP1B (D181A) (tagged with FLAG) along with 2.0 µg of empty vector, wild-type or individual deletion mutant of EPOR-IL-4Rα (tagged with V5). After 48 hr, cell lysates were prepared in the presence of DSP, and proteins cross-linked to PTP1B were immunoprecipitated using anti-FLAG antibody. The immunoprecipitated complexes (and cell lysates) were decross-linked by boiling in the presence of 2-mercaptoethanol, and subjected to Western analyses.
Figure 3.13: PTP1B Deactivates IL-4Rα.
293T cells were transfected with 50 ng of STAT6 and 5.0 µg of chimeric EPOR-IL-4Rα-V5, along with 5.0 µg of PTP1B or vector. After 48 hr, cells were treated with EPO (5 U/ml) for 5 min, and cell extracts were subjected to EMSA. Immunoprecipitate derived from 1.0 mg protein or 50 µg of cell lysate proteins were analyzed by Western blotting. The experiment was repeated twice and similar results were obtained.

![Table showing protein expression](image)

Figure 3.14: PTP1B Physically Associates with the Cytoplasmic Domain of IL-4Rα.
In lanes 1 to 6, 293T cells were transfected with EPO-IL-4Rα-V5 (2 µg), untagged STAT6 (2 µg) and 4 µg of wild-type PTP1B, mutant PTP1B (D181A) or empty vector. In lanes 7 to 12, cells were transfected with 2 µg of STAT6-V5 and 4 µg wild-type PTP1B, mutant PTP1B (D181A) or empty vector. After 48 hr, cells were treated with EPO (5 U/ml) or IL-4 (20 ng/ml) for 5 min. Cell lysates were prepared in the presence of DSP and immunoprecipitated (IP) with anti-FLAG antibody, decross-linked and subjected to Western analyses along with decross-linked total lysates.
Figure 3.15: PTP1B Interacts with IL-4Rα in Live Cells.

For BRET assay, 293T cells were transfected with 1.0 µg of the donor plasmid, pIL-4Rα-Luc-N3 and 3.0 µg of an acceptor plasmid pGFP2-N2 (empty vector), STAT6-GFP2-N2 or PTP1B-D181A-GFP2-N2 using Lipofectamine 2000 (Invitrogen). After 48 hr, cells were used for BRET assay, and the calculated BRET ratios plotted as mean ± SE, (n=3).
Figure 3.16: Oxidation of PTP1B in IL-4-stimulated A549 Cells.

Cells were treated with 20 ng/ml of IL-4 for indicated lengths of time, with 3 mM H$_2$O$_2$ (positive control) for 5 min, or left untreated. Cell lysates were prepared in the presence (upper panel) or absence (lower panel) of iodoacetic acid (IAA), immunoprecipitated using anti-PTP1B antibody, and the immune complexes were subjected to Western analysis using a monoclonal antibody that recognizes the oxidized form of PTP1B.

Figure 3.17: Oxidation of PTP1B in IL-13-stimulated A549 Cells.

Cells were treated with 10 ng/ml of IL-13 for indicated lengths of time, with 3 mM H$_2$O$_2$ (positive control) for 5 min, or left untreated. Cell lysates were prepared in the presence (upper panel) or absence (lower panel) of iodoacetic acid (IAA), immunoprecipitated using anti-PTP1B antibody, and the immune complexes were subjected to Western analysis using a monoclonal antibody that recognizes the oxidized form of PTP1B.
### Figure 3.18: Blockade of ROS Production Inhibits PTP1B Oxidation in IL-4-stimulated Cells.

A549 cells were treated with 500 μM apocyanin, 20 μM LY294002 or DMSO for 2 hr prior to measuring IL-4-mediated oxidation of PTP1B. The upper and lower panels represent results derived from cell lysates prepared in the presence and absence of IAA respectively. The experiment was repeated two times and similar results were obtained.

<table>
<thead>
<tr>
<th>IL-4</th>
<th>DMSO</th>
<th>DMSO</th>
<th>Apocyanin</th>
<th>LY294002</th>
<th>H₂O₂</th>
<th>+ IAA</th>
<th>- IAA</th>
</tr>
</thead>
</table>

### Figure 3.19: Blockade of ROS Production Inhibits PTP1B Oxidation in IL-4-stimulated Cells.

A549 cells were transfected with 4.0 μg of shRNA or scrambled constructs for NOX1 or NOX5. After 48 hr, cells were treated for 5 min with IL-4 (20 ng/ml), left untreated (negative control) or treated with 3 mM H₂O₂ (positive control) for 5 min. Cell lysates were prepared in the presence (upper panel) or absence (lower panel) of IAA, and PTP1B oxidation measured.
Figure 3.20: IL-4-generated ROS Induce PTP1B Oxidation in Mouse Primary Hematopoietic Cells.

(A) Splenocytes were stimulated with IL-4 for indicated lengths of time or with 3 mM H$_2$O$_2$ (positive control) for 5 min. Cell lysates were prepared in the presence (upper panel) or absence (lower panel) of IAA, and PTP1B oxidation measured.

(B) Bone marrow-derived macrophages were stimulated with IL-4 for indicated lengths of time or with 3 mM H$_2$O$_2$ (positive control) for 5 min. Cell lysates were prepared in the presence (upper panel) or absence (lower panel) of IAA, and PTP1B oxidation measured.
Figure 3.21: EPO-generated ROS Promote IL-4-dependent STAT6 Activation.
A549 cells were transfected with 1.0 µg of murine IL-4Rα (Y500F), along with 3.0 µg of murine STAT6 plasmid. After 48 hr, cells were treated for 5 min with EPO (5U/ml) and murine IL-4 (20 ng/ml), either singly or in combination. Cell extracts were subjected to EMSA and RSI quantified.

Figure 3.22: Cytokine-induced ROS Generation in A549 Cells.
A549 cells were pretreated for 2 hr with LY294002 (20 µM) or DMSO, and ROS production measured after stimulation with IL-4 (20 ng/ml), EPO (5U/ml) or TNF-α (10 ng/ml). RFU plotted as mean ± SE, (n=3).
Figure 3.23: TNF-α and EPO-generated ROS Promote Endogenous IL-4 Receptor Activation in A549 cells.

Cells pretreated with LY294002 (20 μM) or DMSO for 2 hr, were stimulated for 3 min with EPO (5U/ml) or TNF-α (10 ng/ml) followed by treatment with human IL-4 (20 ng/ml) for 2 min. Cell extracts were subjected to EMSA, and RSI quantified.

<table>
<thead>
<tr>
<th></th>
<th>DMSO</th>
<th>LY294002</th>
</tr>
</thead>
<tbody>
<tr>
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Figure 3.24: Cytokine-induced ROS Generation in Mouse Primary Splenocytes.

(A) Mouse primary splenocytes were pretreated for 2 hr with LY294002 (20 μM), apocynin (500 μM) or DMSO, and ROS were measured after 1 min stimulation with IL-4 (20 ng/ml) and TNF-α (10 ng/ml). RFU plotted as mean ± SE, (n=3).

(B) Mouse primary splenocytes were pretreated for 2 hr with LY294002 (20 μM), apocynin (500 μM) or DMSO, and ROS were measured after 1 min stimulation with IL-4 (20 ng/ml) and IL-3 (10 ng/ml). RFU plotted as mean ± SE, (n=3).
Figure 3.25: TNF-α- and IL-3-generated ROS Promote Endogenous IL-4 receptor Activation in Mouse Primary Splenocytes.

(A) After pretreatment for 2 hr with LY294002 (20 µM) cells were treated for 3 min with TNF-α (10 ng/ml) or IL-3 (10 ng/ml) followed by treatment with mouse IL-4 (20 ng/ml) for 2 min. Cell extracts were subjected to EMSA and RSI quantified.

(B) After pretreatment for 2 hr with apocynin (500 µM), cells were treated for 3 min with TNF-α (10 ng/ml) or IL-3 (10 ng/ml) followed by treatment with mouse IL-4 (20 ng/ml) for 2 min. Cell extracts were subjected to EMSA and RSI quantified.
3.5. Discussion

In the previous chapter, we have demonstrated, for the first time that immediately following IL-4 engagement, activated IL-4 receptor produced ROS that, in turn, increased the magnitude of receptor activation and consequent signal transduction, in the absence of de novo protein synthesis. Subsequently, we have defined the biochemical mechanisms by which IL-4 generated ROS. Here, we have identified PTP1B, as a ubiquitously expressed PTP, that associated with IL-4 receptor and that was oxidatively inactivated by IL-4-generated ROS. Finally, we have uncovered a novel mechanism of ROS-mediated cytokine signaling cross-talk, which may provide a basis for side effects associated with cytokine therapies. Although IL-4-generated ROS oxidized PTP1B, which deactivated IL-4 receptor, ROS did not initiate receptor activation, but significantly enhanced IL-4-dependent receptor activation.

Here, we demonstrate, for the first time, that PTP1B is a physiological target of IL-4-generated ROS in both hematopoietic and non-hematopoietic cells. Using PTP1B-deficient fibroblasts and splenocytes, we clearly demonstrate that PTP1B functions as a negative regulator of IL-4 receptor activation and subsequent signal transduction. However, SHP-1 and CD45, which negatively regulate IL-4 signaling in hematopoietic cells (Haque et al., 1998; Yamada et al., 2002), could also be potential targets of IL-4-generated ROS.

PTP1B inactivates Jak2 and TYK2 and TC-PTP inactivates Jak1 and Jak3 (Myers et al., 2001). We found that overexpression of PTP1B, but not TC-PTP inhibited IL-4-mediated STAT6 activation. Further, PTP1B inhibited IL-4 signaling in
hematopoietic cells (splenocytes) that primarily signal through the type I IL-4 receptor composed of Jak1-associated IL-4Rα and Jak3-associated γc (Nelms et al., 1999). Also, PTP1B inhibited the activation of the chimeric EPO-IL-4Rα receptor that, associates only with Jak1. The protein-protein interaction studies using chemical cross-linking agent and BRET assay revealed that PTP1B interacted with Jak1-associated IL-4Rα even in the absence of IL-4 stimulation where IL-4Rα did not form complex with IL-13Rα1-associated Jak2/TYK2. While, it is possible that PTP1B may target Jak2/TYK2 in type II receptor complex, our results clearly demonstrated that IL-4Rα was a primary target of PTP1B. This was further supported by our results that PTP1B did not interact with STAT6, and that STAT6 activation was not required for IL-4-dependent ROS generation, which is regulated by PTP1B.

ROS are diffusible molecules that inactivate PTPs including PTP1B (Rhee et al., 2000). Using IL-4 as a model cytokine, here we uncover a novel cellular mechanism underlying the amplification of cytokine receptor activation and signal transduction, by addressing how ROS generated by IL-4 receptor to promote its own activation as well as the activation of other cytokine receptors in the same cells. ROS-mediated cytokine signaling cross-talk may be dependent on the proximity of the different cytokine (or growth factor) receptors, and their susceptibility to regulation by oxidative inactivation of common PTPs. Another level of regulation may be provided by the specificity of the antioxidant proteins utilized by different receptors for elimination of cytokine-mediated ROS, allowing the regeneration of reversibly inactivated PTPs and restoration of normal homeostasis of cellular
cytokine signaling. Unfolding of the ROS-mediated cytokine signaling cross-talk may explain the causes of side effects of cytokine therapies in a variety of human diseases.
4.1. Abstract

We have previously shown that interleukin (IL)-4 induces the production of ROS, which, in turn, promote receptor activation by oxidatively inactivating PTP1B that deactivates the receptor. Here, we show that the redox state of cells regulated the magnitude of IL-4 receptor activation and signal transduction. Specifically, antioxidant enzymes superoxide dismutase (SOD)2, catalase and glutathione peroxidase (GPx)1, peroxiredoxin (Prx) II, Prx IV and Prx VI served as negative regulators of IL-4 signaling. Moreover, Prx II, Prx IV and Prx VI but not SOD2, catalase or GPx1, formed complexes with IL-4 receptor. We also demonstrate that Prx II was oxidatively inactivated by IL-4-generated ROS. Furthermore, Prx II-deficiency upregulates the oxidative inactivation of PTP1B in IL-4 stimulated cells. Collectively, these data suggest that antioxidant enzymes restore the homeostatic
control of IL-4 signaling by scavenging ROS, thereby acting as physiological negative regulators of IL-4 signaling pathways.
4.2. Introduction

IL-4, an immunomodulatory cytokine, produced by activated Th2 lymphocytes, basophils and mast cells, regulates a variety of immune responses including induction of Th2-differentiation, immunoglobulin class-switching, B cell proliferation, suppression of Th1-differentiation and macrophage activation among others (Nelms et al., 1999; Haque and Sharma, 2006). IL-4 executes these pleiotropic functions by engaging its transmembrane receptor which activates two major intracellular signaling pathways, IRS-PI3K and STAT6. Protein tyrosine phosphorylation, a reversible reaction that is tightly regulated in mammalian cells, functions as a ‘signaling switch’ in cytokine-dependent signal transduction (Nelms et al., 1999). In a resting cell, the ‘signaling switch’ remains off due to receptor-associated PTP activity which dominates over the PTK-catalyzed forward reaction (Tonks, 2006). Upon IL-4 engagement, IL-4Rα which remains constitutively associated with Jak1, recruits γc a secondary receptor to the complex, to form the type I receptor complex. IL-13Rα1 recruitment forms the type II receptor complex. The γc and IL-13Rα1 remain constitutively bound to Jak3 and Jak2 (or Tyk2) respectively. The formation of IL-4 receptor complex brings two Jak molecules to an appropriate proximity allowing them to trans-phosphorylate each other at the invariant tyrosine residues located in their activation loops (Nelms et al., 1999). This increases the catalytic activities of the Jaks, which then override the receptor-associated PTP activity and initiate tyrosine phosphorylation in IL-4Rα (Nelms et al., 1999). We have shown in Chapter II that immediately following its activation, IL-4 receptor activates the IRS-PI3K-NOX pathway to generate ROS including $\text{O}_2^–$ and
H$_2$O$_2$. In Chapter III, we have shown that ROS, in turn, promote IL-4 receptor activation and signal transduction, by oxidatively inactivating PTP1B that physically associates with and deactivates the receptor. We have also demonstrated in Chapter III that ROS generated by other cytokine receptors, including those for EPO, TNF-α or IL-3, also promote IL-4 receptor activation in the same cell.

The magnitude and duration of a given cytokine action are tightly regulated by physiologic requirements. This relies on a delicate balance between physicochemical events that initiate and amplify the activation of cytokine receptor and subsequent signal transduction and those which attenuate and inactive the processes. Because, ROS are found to amplify the IL-4 signaling, we have asked whether cellular antioxidants can dampen the IL-4 signals, and how an IL-4 stimulated cell eliminates ROS, which would otherwise produce oxidative damage to the cell.

Mammalian cells are well equipped with antioxidant enzymes that eliminate ROS and prevent oxidative damages. For example, superoxide dismutase (SOD) converts O$_2^\cdot$ to H$_2$O$_2$, which is further converted to H$_2$O and O$_2$ by catalase. The glutathione system complements the catalase by providing the reducing equivalents (Cantin et al., 1991). GPx uses reduced GSH that serves as the electron donor and is converted to glutathione disulphide (GSSG), which is subsequently converted to reduced GSH by glutathione reductase (GR) (Meister, 1983; Deneke, 1989). GR obtains electrons from NADPH that is produced by the hexose monophosphate shunt system (Meister, 1983; Deneke, 1989).
Another family of proteins called peroxiredoxins (Prx's) eliminates low levels of H$_2$O$_2$ produced by mammalian cells in response to growth factors and cytokine stimulation of cells (Wood et al., 2003; Rhee et al., 2005). Prxs are thiol-containing peroxidases which are conserved from bacteria to mammals (Wood et al., 2003; Rhee et al., 2005). Prxs reduce hydroperoxides and peroxynitrite (produced by the reaction of NO [nitric oxide] and O$_2^•−$) with electrons donated by physiologic thiols like thioredoxin (Rhee et al., 2005). Mammals express six Prx proteins, Prx I-VI. Based on the number and location of Cys residues that participate in the catalysis, Prxs are classified in three subgroups: typical 2-Cys, atypical 2-Cys and 1-Cys (Wood et al., 2003; Rhee et al., 2005). The typical 2-Cys subgroup is comprised of four family members, Prx I-IV, which are differentially distributed in subcellular compartments (Wood et al., 2003; Rhee et al., 2005). Prx I and Prx II are located in the cytosol, Prx III in mitochondria and Prx IV in the extracellular space as well as cytosol (Wood et al., 2003; Rhee et al., 2005). The atypical 2-Cys subgroup is comprised of Prx V, which is located in mitochondria and peroxisomes and Prx VI, which is abundant in the cytosol (Rhee et al., 2005). The catalytic Cys may be hyper-oxidized to Cys-sulfenic acid, which in the case of 2-Cys Prxs is slowly reduced to the active thiol form in an ATP-dependent process (Rhee et al., 2005).

Here we show that antioxidant enzymes including SOD2, catalase, GPx1 and Prx’s (II, IV and VI) suppress IL-4 receptor activation and subsequent signal transduction, thereby restoring the homeostatic control of IL-4 receptor activation, signal transduction and subsequent gene expression by eliminating ROS produced by IL-4 stimulation of cells.
4.3. Materials and Methods

4.3.1. Cells and Reagents

Human embryonic kidney cell line 293T was cultured in DMEM supplemented with 10% heat-inactivated fetal bovine serum (Cellgro, Herndon, VA, USA), 2 mM L-glutamine and 50 mg/liter of penicillin and streptomycin. The immortalized PrxII\textsuperscript{−/−} and PrxII\textsuperscript{+/+} mouse embryonic fibroblasts were cultured in DMEM supplemented with 15% heat-inactivated fetal bovine serum (FBS) (Hyclone, USA), 2 mM L-glutamine and 50 mg/liter of penicillin and streptomycin, as described (Kang et al., 1998; Kang et al., 2004). The primary splenocytes derived from PrxII\textsuperscript{−/−} and PrxII\textsuperscript{+/+} mice were cultured in DMEM supplemented with 10% FBS (Hyclone, USA), 2 mM L-glutamine and 50 mg/liter of penicillin and streptomycin. Recombinant human and murine IL-4 were purchased from R&D Systems.

For the isolation of primary splenocytes, 5 female mice of each genotype, 6-12 weeks of age, were sacrificed and whole spleen was transferred to a 6-cm petri dish containing 3 ml of culture medium. The spleen was cleaned and teased into approximately 1 mm pieces. The cell clumps were transferred to a sterile 15 ml centrifuge tube, and the large tissue clumps were allowed to settle and the supernatant was transferred to a fresh 15 ml tube containing 10 ml of culture medium, and centrifuged at 750 g for 5 minutes at 25 °C to pellet the splenocytes. The pelleted splenocytes were washed twice with fresh culture medium by centrifugation, and resuspended (10\textsuperscript{7} cells/ml) in culture medium (RPMI-1640 + 10% FBS + 50 U/ml penicillin + 50 µg/ml streptomycin + 2mM glutamine + 1mM sodium pyruvate + 2.5 µM β-mercaptoethanol).
4.3.2. Cloning, Site-Directed Mutagenesis and Plasmid Constructs

Human Prx 1 and Prx II cDNA constructs were obtained as kind gifts from Dr. Sue Goo Rhee (Kang et al., 1998). The human Prx IV and Prx VI cDNA constructs were purchased from Origene Technologies (Rockville, MD). They were used as a template for PCR amplification and cloning in pcDNA3-HA in the BamHI-EcoRI sites. All point mutants were generated using Quick Change XL Site-directed Mutagenesis Kit (Stratagene) following the manufacturer’s instructions. For generation of stable clones in MEFs, wild-type and mutant Prx I and Prx II constructs were excised from pcDNA3-HA and cloned in HindIII-XhoI sites of pcDNA3.1/Hygro+ (Invitrogen). The FLAG-tagged mouse Gpx1 construct was a kind gift from Dr. Roger A Sunde (Weiss and Sunde, 1998). The human catalase, SOD1 and SOD2 cDNA constructs were purchased from Open Biosystems (Huntsville, AL) and used as template for PCR amplification and cloning in pcDNA3.1/V5-HisA (HindIII-Xba1 sites for catalase, and HindIII-Xhol sites for SOD1 and SOD2). All constructs were verified by nucleotide sequencing. Please refer to Table II for a complete list of primers that were used to clone the various antioxidant enzymes.

4.3.3. Transient Transfection and Generation of Stable Clones

For electrophoretic mobility shift assays (EMSA), 293T cells (10^6 cells/10 cm plate) were transiently transfected with the indicated plasmid DNA using the calcium phosphate method as described previously (Haque, 1997). A549 cells were transfected using Lipofectamine 2000 reagent (Invitrogen Carlsbad, CA) according to the manufacturer’s instructions. All analyses were performed 48h post transfection. For generating stable clones, we transfected immortalized Prx II^-/- MEF
cell lines with either vector or PrxI, Prx II, PrxI(C52S), PrxI(C83S), PrxI(C52/83S) or PrxII(C51S) using Lipofectamine 2000 (Haque et al., 2000), followed by 2 weeks of selection with 150 µg/ml hygromycin B (Invitrogen).

4.3.4. EMSA and Luciferase Assay

EMSA was performed using 10 µg proteins of whole cell extracts and 0.2 ng of $^{32}$P-labeled high-affinity STAT6-specific probe (Haque et al., 2000). Luciferase assay was performed as described previously (Haque et al., 1997).

4.3.5. Intracellular ROS Assay

ROS were measured fluorimetrically using CM-DCFHDA probe, as described previously in Chapter II.

4.3.6. Immunoprecipitation and Immunoblotting

Cells were harvested and incubated in lysis buffer for 30 min on ice. The lysates (500 µg protein per IP) were pre-cleared by incubation with 50 µl antiMouse IgG Beads from Mouse TrueBlot™ (eBioscience Inc, San Diego, CA) and control IgG (2 µg, Santa Cruz) for 1 h to remove nonspecific binding. The lysates were then incubated with the primary antibody overnight at 4°C. 50 µl anti-Mouse IgG Beads from Mouse TrueBlot™ (eBioscience Inc, San Diego, CA) was then added and the samples were incubated for an additional 1 h. The anti-Mouse IgG Beads from Mouse TrueBlot™ was collected by centrifugation, washed and then boiled in sample buffer. The precipitated proteins were resolved by SDS-PAGE and detected by immunoblotting. Mouse TrueBlot™ ULTRA: Horseradish Peroxidase (HRP) antimouse IgG was used as secondary antibody.
4.3.7. Immunostaining

PrxII expression in primary BMMC's derived from PrxII<sup>+/+</sup> cells was assessed by fluorescence microscopy. The cells were seeded on glass coverslips. When the cells were 70–80% confluent, they were fixed with cold methanol for 10 min at –20 °C. Fixed cells were washed three times with PBS and blocked by incubation in PBS with 3% goat serum for 1 h at room temperature. The cells were washed once with 0.3% PBS/goat serum and incubated overnight at 4 °C with the anti–PrxII (diluted 1:100) and anti-IL-4R (diluted 1:50) antibodies. The coverslips were washed three times with PBS and incubated for 1 h at room temperature with biotinylated secondary antibody and streptavidin-conjugated eFluor605 (red) for IL4Rα and secondary antibody conjugated with Alexafluor 488 (green) for Prx isoforms. Immunofluorescence images were obtained using a confocal laser-scanning microscope (Olympus).

4.3.8. ROS-mediated Oxidation of PTP1B and Prx

Briefly, after the indicated treatments, cell lysates were prepared using degassed buffers in the presence and absence of 100 mM iodoacetic acid (IAA) that irreversibly alkylated each reduced Cys in all proteins except for the catalytic cysteines of Prx II, which was reversibly oxidized to Cys-sulfenic acid (Cys-SOH) by IL-4 or H₂O₂ (positive control) treatment of cells. Individual Prx II containing both reversibly oxidized (Cys-SOH) and irreversibly alkylated Cys residues was immunoprecipitated and treated with DTT to reduce the Cys51-SOH. After removal of unused DTT, Cys51-SH was irreversibly oxidized to Cys-SO₃H (Cys-sulfonic acid) by treatment with pervanadate. This reaction product was then detected by Western
analysis using an antibody which recognizes both sulfenic and sulfonic forms of Prx II with high specificity (Woo et al., 2003a; Woo et al., 2003b).

4.3.9. Statistical and Densitometric Analyses

All experiments were performed at least three times, and data of one representative experiment are shown. Statistical differences between different groups were determined using paired Student’s t-test. EMSA and Western blot signals were quantified using ImageQuant (Molecular Dynamics).

<table>
<thead>
<tr>
<th>Purpose</th>
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<th>Primer Sequences</th>
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<td>Prx I</td>
<td>Forward: 5'-GGCCCCCGGGATCCGATCTCCAGGAAAAAATGCAAAAAATTGG-3' Reverse: 5'-CGAGCTTCTGATATTCTACATTGCTCTTACAGAATTTCTGC-3'</td>
</tr>
<tr>
<td></td>
<td>Prx II</td>
<td>Forward: 5'-GGCCCCCGGGATCCGAGCTCCGGACGTCATAGGTTGGAGAAATATTCG-3' Reverse: 5'-CGAGCTTCTGATATTCTACATTGCTCTTACAGAATTTCTGC-3'</td>
</tr>
<tr>
<td></td>
<td>Prx IV</td>
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</tr>
<tr>
<td></td>
<td>Prx VI</td>
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</tr>
<tr>
<td><strong>Point Mutation</strong></td>
<td>Prx I</td>
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</tr>
<tr>
<td>Prx I</td>
<td>(C52S)</td>
<td>Forward: 5'-GCCCTCTTAGACCTACCTTACTTGTGTGCTGCCAGAGATGACATTGGCTTT-3' Reverse: 5'-GCAAAGCTATACTTGTGAGAGAATCTCTTAGGGAAAC-3'</td>
</tr>
<tr>
<td>Prx I</td>
<td>(C83S)</td>
<td>Forward: 5'-GCCCTCTTAGACCTACCTTACTTGTGTGCTGCCAGAGATGACATTGGCTTT-3' Reverse: 5'-GCAAAGCTATACTTGTGAGAGAATCTCTTAGGGAAAC-3'</td>
</tr>
<tr>
<td>Prx II</td>
<td>(C51S)</td>
<td>Forward: 5'-GCCCTCTTAGACCTACCTTACTTGTGTGCTGCCAGAGATGACATTGGCTTT-3' Reverse: 5'-GCAAAGCTATACTTGTGAGAGAATCTCTTAGGGAAAC-3'</td>
</tr>
<tr>
<td>Prx II</td>
<td>(C172S)</td>
<td>Forward: 5'-GCCCTCTTAGACCTACCTTACTTGTGTGCTGCCAGAGATGACATTGGCTTT-3' Reverse: 5'-GCAAAGCTATACTTGTGAGAGAATCTCTTAGGGAAAC-3'</td>
</tr>
<tr>
<td><strong>Cloning in pcDNA3.1/V5-HisA</strong></td>
<td>CAT</td>
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</tr>
<tr>
<td></td>
<td>SOD1</td>
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</tr>
<tr>
<td></td>
<td>SOD2</td>
<td>Forward: 5'-TGCTAGATGAAAGCTTGGCAACAGCTGACAGCCGCGGGATC-3' Reverse: 5'-TGCTAGATGAAAGCTTGGCAACAGCTGACAGCCGCGGGATC-3'</td>
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4.4. Results

4.4.1. Redox State of Cells Controls the Magnitude of IL-4-induced ROS Generation, Receptor Activation, Signal Transduction and Gene Expression

We have demonstrated in Chapter III that ROS generated by activated receptors of EPO, IL-3 or TNF-α markedly amplified, in trans, the activation of IL-4 receptor and consequent signal transduction. This ROS-mediated cytokine cross-talk, which likely occurred due to the diffusion of ROS from the site of generation to the surrounding environment, raised the possibility that the redox state of a cell may control the magnitude of IL-4 (or other cytokine) receptor activation and subsequent signal transduction. To address this possibility, we took both pharmacologic and molecular genetic approaches.

We measured IL-4-induced STAT6 activation and ROS generation in A549 cells pretreated with buthionine-S,R-sulphoximine (BSO) (Friedman et al., 1989), a glutathione (reduced) depleting agent, 3-amino-1,2,4-trizole (ATZ) (Kingma et al., 1996), an inhibitor of catalase, sodium diethylthiocarbamate trihydrate (DETC) (Vanin et al., 1999), an inhibitor of SOD and N-Acetyl Cysteine (NAC) (Dobashi et al., 2001), a scavenger of ROS (Figure 4.1A & B). We observed that BSO, ATZ and DETC significantly increased IL-4-induced activation of STAT6, as measured by EMSA (Figure 4.1B). In consistence with this, NAC markedly decreased IL-4-induced ROS generation and STAT6 activation (Figure 4.1A & B). However, IL-4-induced ROS generation was not significantly changed by BSO, ATZ or DETC (Figure 4.1A).
Next, A549 cells were transiently transfected with expression constructs of catalase, GPx1 and SOD2 and 48 h post-transfection, IL-4-induced ROS generation and STAT6 activation were measured (Figure 4.2A & B). Figure 4.2B reveals that IL-4-induced STAT6 activation was markedly reduced in cells over-expressing these antioxidant enzymes. Over expression of catalase and GPx1 but not SOD2 moderately reduced the amounts of IL-4-generated ROS in A549 cells (Figure 4.2A). In chapter II, we have demonstrated that diphenylene iodonium, an inhibitor of flavoprotein activity which blocks ROS production by the mitochondrial electron transport chain and NOX/DUOX family enzymes, also reduced IL-4-induced activation of STAT6. Taken together, these data suggest that alterations in cellular antioxidants may regulate the magnitude of IL-4-dependent signal transduction. To confirm this notion, we measured IL-4-induced ROS generation and STAT6 activation in embryonic fibroblasts derived from catalase-knockout and wild type mice (Figure 4.3A & B). We observed that catalase-deficiency significantly increased IL-4-induced ROS generation and STAT6 activation. The change in STAT6 activation measured by EMSA was confirmed by Western analysis for tyrosine-phosphorylated STAT6 (Figure 4.3B, *middle panel*). Moreover, MEFs derived from GPx1-knockout and catalase and GPx1-double knockout mice also exhibited significant increase in IL-4-induced ROS generation and STAT6 activation, when compared with wild-type MEFs (Figure 4.4A & B; Figure 4.5A & B). Collectively, these data reveal that antioxidant enzymes are involved in IL-4 receptor activation and signal transduction.
The above observations prompted us to examine whether Prx family enzymes are involved in IL-4 receptor activation and signal transduction. IL-4 receptor activation occurs on the cytoplasmic side of the plasma membrane. Four Prx’s, namely Prx I, II, IV and VI, which are reported to be expressed in the cytosol, were overexpressed in A549 cells, and measured IL-4-induced ROS generation and STAT6 activation measured (Figure 4.6A & B). We found that Prx II, IV and VI but not Prx I significantly reduced the levels of IL-4-generated ROS and IL-4-induced STAT6 activation (measured by EMSA and Western analysis). Figure 4.6B (bottom panel) revealed that the exogenous Prx proteins were expressed at comparable levels. Furthermore, we found that overexpression of Prx II, IV and VI, but not Prx I, significantly attenuated IL-4-dependent STAT6-responsive gene expression in a time-dependent fashion, as measured by luciferase reporter assay (Figure 4.7). This reporter construct has been described previously in chapter II. Taken together, these data clearly suggest that Prx II, IV and VI act as negative regulators of IL-4 signaling.

To further define the role of Prx II in IL-4 signaling, we treated MEFs and primary splenocytes derived from Prx II-KO (Choi, 2005; Lee, 2003; Moon, 2004) and wild-type mice. Cells were treated with IL-4 and STAT6 activation measured by EMSA and western analysis. The data revealed that Prx II-deficiency markedly upregulated IL-4-dependent STAT6 activation in both MEFs (Figure 4.8, A & B) and splenocytes (Figure 4.8C & D). Moreover, we found that Prx II-deficient cells produced significantly greater amounts of ROS than wild-type cells in response to IL-4 stimulation (Figure 4.8A & C). These data confirmed that Prx II acts as a negative
regulator of IL-4-dependent signal transduction in both hematopoietic and non-hematopoietic cell.

4.4.2. Prx II, IV and VI Physically Associate with IL-4Rα

To understand the spatial, biochemical and structural basis of antioxidant enzyme-mediated negative regulation of IL-4 receptor activation and signal transduction, we performed the following experiments.

Mouse bone marrow-derived macrophages grown on coverslips were stimulated with murine IL-4 for 5 min or left untreated. Fixed cells were stained with anti-IL-4Rα and anti-Prx antibodies, as indicated. Prx localization was detected by green fluorescence and IL-4Rα chain by red fluorescence; yellow fluorescence represented their co-localization. Figure 4.9 revealed that IL-4Rα co-localized with Prx II, IV and VI, but not Prx I, in IL-4-stimulated mouse bone marrow-derived macrophages. In the absence of IL-4 stimulation, co-localization of IL-4Rα with none of these antioxidant enzymes was detected in murine bone marrow derived macrophages.

In humans, Prx I and Prx II have 91% homology and 78% identity in their amino acid sequences (Figure 4.10). A recent study has demonstrated that Prx I functions well as a molecular chaperone than as a peroxidase, whereas Prx II is a better peroxidase (Lee, 2007). This differential function of Prx I is attributed to the presence of unique Cys83 and by the observation that a mutant Prx I, in which Ser replaces Cys83 (Prx I C83S), start behaving as a Prx II protein. These structure-function studies were performed with purified Prx proteins (Lee, 2007).
To explore why Prx I failed to interact with and/or downregulate IL-4 receptor, we generated the mutant Prx I (C52S), in which the catalytic Cys\textsubscript{52} was replaced by a Ser, Prxl (C83S), in which the catalytic Cys\textsubscript{83} was replaced by a Ser, Prxl (C52/83S), in which both catalytic Cys\textsubscript{52} and Cys\textsubscript{83} were replaced by Ser and PrxII (C51S), in which the catalytic Cys\textsubscript{51} was replaced by a Ser constructs (Figure 4.11). We stably transfected immortalized Prx II\textsuperscript{−/−} MEF cell lines with either vector or Prxl, Prx II, Prxl(C52S), Prxl(C83S), Prxl(C52/83S) or PrxII(C51S), and clones were selected in the presence of 150 µg/ml hygromycin B. We found after knocking in wild type PrxII in Prx II\textsuperscript{−/−} MEF, there was a substantial downregulation of activated STAT6 signal which was comparable to the STAT6 levels in Prx II\textsuperscript{+/+} MEFs (Figure 4.12, lane 4), but failed to do so (Figure 4.12, lane 5) where the transgene expressed was PrxII(C51S). In addition when Prxl(C83S) was knocked in (Figure 4.12, lane 6), IL-4-mediated STAT6 activation was downregulated, which was not seen with the wild type Prxl construct (Figure 4.12, lane 9).

To examine whether Prx II, IV and VI physically associates with the IL-4 receptor complexes, co-immunoprecipitation (Co-IP) experiments were performed using 293T cells which were transiently transfected with V5-tagged human IL-4R\textalpha{} and HA-tagged Prx I, II, IV or VI. We found that Prx II (Figure 4.13, lane 6), Prx IV (Figure 4.13, lane 8) and VI (Figure 4.13, lane 9) but not Prx I (Figure 4.13, lane 2) formed complexes with the IL-4R\textalpha{}. We found that Prx I C83S formed a complex with the IL-4R\textalpha{}, while the catalytically inactive mutants Prx I C52S and C52S/C83S were unable to interact (Figure 4.13, lanes 4 and 5). A catalytically inactive Prx II C51S which failed to downregulate IL-4-induced STAT6 activation, was also unable to bind
(Figure 4.13, lane 7). A similar Co-IP experiment was performed to determine whether catalase, GPx1 or SOD2 formed complexes with the IL-4Rα; revealing that none of these antioxidant proteins was present in the IL-4 receptor complex (Figure 4.14).

4.4.3. IL-4-generated ROS induces oxidative inactivation of Prx II and Prx II-deficiency upregulates PTP1B oxidation in IL-4 stimulated cells

The catalytic Cys of Prx II, like those of other Prx’s, may be hyper-oxidized to Cys-sulfenic acid, which is slowly reduced to the active thiol form in an ATP-dependent process (Rhee et al., 2005). Because Prx II formed complex with IL-4Rα, we examined whether the catalytic Cys51 of Prx II was oxidized by IL-4-generated ROS. The N-terminal conserved catalytic Cys of Prx I through Prx IV is located within a conserved motif, Asp-Phe-Trp-Phe-Val-Cys-Pro-Thr-Glu-Ile (Chae et al., 1994b). We treated A549 cells with IL-4 or H2O2 for 5 min (as a positive control). Oxidation of the catalytic Cys51 of Prx II was measured using an antibody raised against a peptide including the oxidized catalytic Cys residue conserved in Prx I through Prx IV, following the method of Woo et al. (Woo et al., 2003a; Woo et al., 2003b). Our experiments revealed that IL-4-generated ROS induced the oxidative inactivation of Prx II in A549 cells (Figure 4.15).

Because Prx II-deficient cells, which produced higher levels of ROS compared to wild-type cells upon IL-4 stimulation, exhibited an enhanced activation of STAT6 (Figure 4.14), we reasoned that Prx II deficiency would lead to enhanced PTP1B oxidation by IL-4. To test this possibility, we treated MEFs derived from wild-type and Prx II-deficient mice with IL-4 or H2O2 (positive control), and measured the
oxidation levels of PTP1B, as described earlier in Chapter III. The data show that Prx II deficiency indeed led to a marked increase in IL-4-induced oxidation of PTP1B, suggesting that Prx II-dependent negative regulation of IL-4 signaling was mediated by elimination of IL-4-generated ROS which oxidatively inactivated PTP1B (Figure 4.16A & B).

Figure 4.1: Effects of Inhibitors of Antioxidant Enzymes and N-Acetyl Cysteine on IL-4-induced ROS Generation and STAT6 Activation.

(A) NAC downregulates IL-4-induced ROS generation. A549 cells were pretreated for 2 hr with BSO (50 μM), ATZ (20 mM), DETC (20 μM), NAC (5 mM), or DMSO, and ROS were measured after IL-4 (20 ng/ml) stimulation. RFU represent mean ± SE (n=3).

(B) Treatment with NAC significantly downregulates IL-4-dependent STAT6 activation. A549 cells were pretreated as in (A), and treated with IL-4 (20 ng/ml) for 5 min. Cell extracts were subjected to EMSA and RSI quantified.
Figure 4.2: Ectopic Expression of Global Antioxidant Proteins Reduces IL-4-dependent ROS Generation and STAT6 Activation.

(A) Overexpression of antioxidant proteins downregulates IL-4-mediated ROS generation. A549 cells were transfected with 4.0 µg of catalase, Gpx1, SOD2, or empty vector. After 48 hr, IL-4-mediated ROS were measured and RFU plotted as mean ± SE (n=3).

(B) IL-4-mediated STAT6 activation is downregulated following overexpression of catalase, Gpx1, or SOD2 in cells. A549 cells were transfected as in (A). After 48 hr, cells were stimulated with IL-4 (20 ng/ml) for 5 min. Cell extracts were subjected to EMSA and RSI quantified.
Figure 4.3: Catalase Deficiency Increases IL-4-induced ROS Generation and STAT6 Activation.

(A) Catalase deficiency increases IL-4-induced ROS generation in MEFs. Catalase-deficient (Cat\(^{-/-}\)) and wild-type (Cat\(^{+/+}\)) MEFs were treated with IL-4 (20 ng/ml), ROS measured and RFU plotted as mean ± SE (n=3; "**" indicates p < 0.05).

(B) Catalase deficiency increases IL-4-dependent STAT6 activation. Catalase-deficient (Cat\(^{-/-}\)) and wild-type (Cat\(^{+/+}\)) MEFs were treated with IL-4 (20 ng/ml) for 5 minutes, cell extracts were subjected to EMSA or immunoblot analysis (with anti-phospho-Tyrosine641-STAT6) and RSI quantified. Immunoblot analysis with anti-STAT6 served as loading control.
Figure 4.4: Gpx1 Deficiency Increases IL-4-induced Signaling.

(A) Gpx deficiency increases IL-4-induced ROS generation in MEFs. Gpx1-deficient (Gpx1⁻/⁻) and wild-type (Gpx1⁺/⁺) MEFs were treated with IL-4 (20 ng/ml) for 5 minutes and ROS measured and RFU plotted as mean ± SE (n=3).

(B) Gpx1 deficiency increases IL-4-dependent STAT6 activation. Gpx1-deficient (Gpx1⁻/⁻) and wild-type (Gpx1⁺/⁺) MEFs were treated with IL-4 (20 ng/ml) for 5 minutes, cell extracts were subjected to EMSA and immunoblot analysis (with anti-phospho-Tyrosine641-STAT6) and RSI quantified. Immunoblot analysis with anti-STAT6 served as loading control.
Figure 4.5: Catalase and Gpx1 Double Knockout Mice Show Increased IL-4 Signaling.

(A) Catalase and Gpx1 deficiency co-operatively increases IL-4-induced ROS generation in MEFs. Catalase and Gpx-deficient (Cat\(^{-/-}\) Gpx\(^{-/-}\)) and wild-type MEFs were treated with IL-4 (20 ng/ml) for 5 minutes and ROS were measured and RFU plotted as mean ± SE (n=3; **** indicates p < 0.01).

(B) Catalase and Gpx1 deficiency increases IL-4-dependent STAT6 activation. Catalase and Gpx-deficient (Cat\(^{+/+}\) Gpx\(^{+-}\)) and wild-type MEFs were treated with IL-4 (20 ng/ml) for 5 minutes, cell extracts were subjected to EMSA or immunoblot analysis (with anti-phospho-Tyrosine641-STAT6) and RSI were quantified. Immunoblot analysis with anti-STAT6 served as loading control.
Figure 4.6: Prx II, Prx IV, and Prx VI but not Prx I Downregulates IL-4 Signaling.

(A) Overexpression of Prx II, Prx IV and Prx VI, but not Prx I downregulates IL-4-mediated ROS generation. A549 cells were transfected with 4.0 mg of Prx I-HA, Prx II-HA, Prx IV-HA, Prx VI-HA, or empty vector. After 48 hr, IL-4-generated ROS were measured and RFU plotted as mean ± SE (n=3; *** indicates p < 0.05; **** indicates p < 0.01).

(B) IL-4-mediated STAT6 activation is downregulated following overexpression of Prx II, Prx IV and Prx VI, but not Prx I. A549 cells were transfected as in (A). After 48 hours cells were stimulated with IL-4 (20 ng/ml) for 5 minutes, cell extracts were subjected to EMSA and immunoblot analysis as indicated and RSI quantified.
Figure 4.7: Prx II, Prx IV, and Prx VI Downregulates STAT6-responsive Gene Expression.

A549 cells were co-transfected with 2.5 µg of Prx I, Prx II, Prx IV, or Prx VI and 2.5 µg of STAT6-responsive luciferase construct (TPU474), or empty vector (TPU222). After 24 hr, transfected cells were stimulated with IL-4 (20 ng/ml) for indicated lengths of time or left untreated, and luciferase activity was measured. Normalized luciferase activities in arbitrary units (AU) plotted as mean ± SE (n=3).
Figure 4.8: Prx II is a Negative Regulator of IL-4 Signaling.

(A) Prx II deficiency increases IL-4-induced ROS generation in MEFs. Prx II-deficient (Prx II\(^{-/-}\)) and wild-type (Prx II\(^{+/+}\)) MEFs (immortalized) were treated with IL-4 (20 ng/ml). ROS were measured and RFU plotted as mean ± SE (n=3; "*" indicates p < 0.05).

(B) Prx II deficiency increases IL-4-dependent STAT6 activation in MEFs. Prx II-deficient (Prx II\(^{-/-}\)) and wild-type (Prx II\(^{+/+}\)) MEFs (immortalized) were treated with IL-4 (20 ng/ml) for 5 minutes. Cell extracts were subjected to EMSA and immunoblot analysis (with anti-phospho-Tyrosine641-STAT6), and RSI were quantified. Immunoblot analysis with anti-β-actin served as loading control. Immunoblot analysis with anti-Prx II confirmed the knock-out status of the Prx II\(^{-/-}\) MEFs.

(C) Prx II deficiency increases IL-4-induced ROS generation in mouse primary splenocytes. Splenocytes isolated from Prx II\(^{-/-}\) and Prx II\(^{+/+}\) were treated with IL-4 (20 ng/ml) for 5 minutes, ROS were measured and RFU plotted as mean ± SE (n=3; "**" indicates p < 0.05).

(D) Prx II deficiency increases IL-4-dependent STAT6 activation in mouse primary splenocytes. Splenocytes isolated from Prx II\(^{-/-}\) and Prx II\(^{+/+}\) were treated with IL-4 (20 ng/ml) for 5 minutes. Cell extracts were subjected to EMSA and immunoblot analysis (with anti-phospho-Tyrosine641-STAT6) and RSI quantified. Immunoblot analysis with anti-STAT6 served as loading control. Immunoblot analysis with anti-Prx II confirmed the knock-out status of the splenocytes isolated from Prx II\(^{-/-}\) mice.
Figure 4.9: Prx II, Prx IV, Prx VI, but not Prx I Co-localizes with the IL-4 Receptor.

Bone marrow derived primary macrophages from wild-type mice, grown on coverslips, were stimulated with murine IL-4 (20 ng/ml) for 5 min or left untreated. The cells were stained with anti-mouse IL-4R\textsubscript{α} antibody and with Prx isoform specific antibodies. For detection of Prx isoforms, rabbit polyclonal primary antibodies was used followed by secondary antibody conjugate d with Alexafluor 488 (that gives green signal). For detection of IL-4R\textsubscript{α}, cells were incubated with rat monoclonal primary antibody followed by incubation with biotinylated secondary antibody and streptavidin-conjugated eFluor605 (that gives red fluorescence). The slides were mounted using Vectashield with DAPI (to stain the nuclei with blue). Images were obtained with original magnification set at 63X, with representative images shown here. The slides were visualized using a fluorescence microscope. The different signals detected are: IL-4Ra (red), Prx II (green) and nuclei (blue). The yellow regions represent areas of co-localization.
Figure 4.10: Schematic Representation of the Prx I and Prx II Protein.

Figure 4.11: Schematic Representation of the Prx I and Prx II Deletion Mutants.

The mutants were generated using Quick Change XL Site-Directed Mutagenesis Kit (Stratagene) following the manufacturer’s instructions.
(A) Immortalized Prx II-/- MEFs were stably transfected with either vector or Prx I, Prx II, Prxl(C52S), Prxl(C83S), Prxl(C52/83S) and PrxII(C51S), and clones were selected in the presence of hygromycin B. Representative stable clones of Prx II-/- MEFs were selected and was examined for IL-4-induced ROS production. RFU are plotted as mean ± SE, (n=3) (* = P < 0.05; ** = P < 0.01).

(B) Stable clones were made as described in (A). Selected pools were treated with IL-4 (20 ng/ml) for 5 min and cell extracts subjected EMSA, and RSI quantified.
Figure 4.13: Prx II, Prx IV, Prx VI, but not Prx I Physically Associates with the IL-4 Receptor.

293T cells were co-transfected with V5-tagged human IL4Ra (2.0 mg) and 4.0 mg wild-type Prx I-HA, mutant Prx I(C83S)-HA, mutant Prx I(C52S)-HA, double mutant Prx I(C52/C83S)-HA, wild-type Prx II-HA, mutant Prx II(C51S)-HA, Prx IV-HA, Prx VI-HA, or empty vector constructs. After 48 hr, cell lysates (1 mg protein) were immunoprecipitated using anti-V5, or anti-HA and the immune complexes subjected to immunoblot analysis with anti-HA and anti-V5 antibody, as indicated.
Figure 4.14: Specificity of Prx II is maintained by Direct Interaction with IL-4 Receptor.

293T cells were co-transfected with human IL4Rα-V5 (2.0 mg) along with catalase, Gpx, SOD2, Prx II-HA, or empty vector (4 mg) constructs. After 48 hr, cell lysates (1 mg protein) were immunoprecipitated using anti-V5, and immune complexes subjected to immunoblot analysis with a cocktail of anti-catalase, anti-SOD2, anti-Gpx1 and anti-Prx II antibodies.
IL-4-stimulation oxidizes Prx II, but not Prx I. A549 cells were treated for 5 minutes with 20 ng/ml of IL-4, with 3 mM H$_2$O$_2$ (positive control), or were left untreated. Cell lysates were prepared in the presence (upper panel) or absence (lower panel) of IAA and immunoprecipitated with anti-Prx I or anti-Prx II. Immune complexes were subjected to immunoblot analysis using a monoclonal antibody raised against oxidized Prx active site.

**Figure 4.15: IL-4 Induces Prx II Oxidation.**
Fig 4.16: Prx II-deficiency Results in Increased IL-4-induced PTP1B Oxidation.

Immortalized MEFs (A) and splenocytes (B) derived from PrxII+/+ (wild type) and PrxII−/− mice were treated for 5 minutes with 20 ng/ml of IL-4, with 3 mM H₂O₂ (positive control), or were left untreated. Cell lysates were prepared in the presence (upper panel) or absence (lower panel) of IAA and immunoprecipitated with anti-PTP1B, and the immune complexes were subjected to immunoblot analysis with a monoclonal antibody that recognizes oxidized-PTP active site.
4.5. Discussion

Cell signaling by hematopoietin and interferon family of cytokines is mediated by tyrosine phosphorylation of their receptors and cognate downstream signaling proteins (Haque and Sharma, 2006). Tyrosine phosphorylation is a reversible protein modification in which the forward reaction is catalyzed by PTK and the reverse reaction by PTP. Previous work from our laboratory and others has demonstrated that cytokine receptor associated PTP-activity plays a primary role in the negative regulation of cytokine receptor activation, and subsequent signal transduction and gene expression (Haque et al., 1995; Haque et al., 1998; Haque and Sharma, 2006; Haque et al., 1997; Pao et al., 2007). In chapter III, we have shown that PTP1B dephosphorylated and deactivated the IL-4 receptor in both hematopoietic and non-hematopoietic cells. We have also shown that IL-4 stimulation produced ROS which oxidatively inactivated PTP1B, thereby promoting IL-4 receptor activation and subsequent signal transduction.

Since ROS are capable of diffusing away from the sites of generation and produce oxidative damage to cells, we hypothesized that cellular antioxidant enzymes might participate as scavengers of IL-4-generated ROS. In testing this hypothesis, we found that cellular antioxidant enzymes including SOD2, catalase, GPx1, Prx II, Prx IV and Prx VI were able to attenuate ROS-mediated amplification of IL-4 receptor activation and its downstream signaling pathways. Interestingly, our data also revealed that among these enzymes, Prx II, Prx IV and Prx VI functioned as specific scavengers of IL-4-generated ROS by forming complexes with activated IL-4 receptor. In contrast, SOD2, catalase and GPx1
did not co-localize or physically associate with the activated IL-4 receptor, thereby functioning as global antioxidants, likely protecting cells from oxidative damage and preventing ROS-mediated cytokine cross-talks. Therefore, these findings suggest that the global redox state of cells may regulate the magnitude of amplification of IL-4 receptor activation which is mediated by IL-4- or other cytokine-generated ROS.

Currently experiments are underway to confirm this notion. If this notion is found to be correct, then the redox state of cells will be reflected in the outcome of cytokine action. Of note, our laboratory found that hypoxia, which also generated ROS, promoted cytokine-dependent activation of STAT3 in glioblastoma multiforme cells (unpublished data). Herein, we presented data that clearly demonstrated the specificity of the ROS-scavenging function of Prx II, Prx IV and Prx VI in IL-4 stimulated cells, by detecting their presence in the activated IL-4 receptor complex. Consistent with our findings, a recent report has shown that Prx II physically associates with activated the PDGF receptor and attenuates its activation (Choi et al., 2005). We have shown that Prx II became oxidatively inactivated by IL-4-generated ROS, thus decreasing the amounts of ROS generated by IL-4 stimulated cells. Oxidation of catalytic Cys51 in Prx II is required for its ROS-scavenging activity (Rhee et al., 2000; Rhee et al., 2005). So, the observation described above is consistent with the observed increase in the amount of ROS detected in IL-4 stimulated, Prx II-deficient cells compared with wild-type cells. Importantly, these correlated well with the increase in STAT6
activation, a readout of IL-4 receptor activation (Haque and Sharma, 2006; Nelms et al., 1999).

To further understand the structural basis of the ROS-scavenging function of Prx II in IL-4 stimulated cells, we compared the roles of two highly homologous (91% homology, and 78% identity in amino acid sequences) Prx proteins, namely Prx I and Prx II. Although both the proteins were localized in the cytoplasm, Prx I failed to associate with the IL-4 receptor complex and scavenge IL-4-generated ROS. We hypothesized that this was due to structural feature(s) unique to the Prx I protein. The catalytic (peroxidase) activity of Prx I or Prx II, in the presence of H$_2$O$_2$, is mediated by the formation of a transient intermolecular disulfide bond between the catalytic Cys (residue 52 in Prx I and 51 in Prx II) and the ‘resolving’ Cys (residue 173 in Prx I and 172 in Prx II); the disulfide bond is then reduced to Cys-SH by thioredoxin which functions as a disulphide oxidoreductase (Lee et al., 2007; Rhee et al., 2000; Rhee et al., 2005). In contrast, the chaperone activity of Prx I is mediated by the formation of a decameric structure, which is stabilized by the formation of a disulfide bond between Cys83 of Prx I (Lee et al., 2007). Thus, Prx I exhibits more chaperone function than peroxidase activity, whereas Prx II, due to the absence of Cys which is equivalent Cys83 of Prx I, functions more as a peroxidase than a chaperone. This conclusion is based on the structural studies using the purified proteins (Lee et al., 2007). Our data using mutant forms of Prx I (C83S, C52S, C52S/C83S) and Prx II (C51S) revealed that the peroxidase activity of Prx II was required for its interaction with the IL-4 receptor complex, and that the Ser substitution of Cys83 transformed Prx
I to a functional Prx II. These observations were then substantiated by the restoration of Prx II function in Prx II-deficient cells by knocking in either the wild-type Prx II or the C83S mutant Prx I. Moreover, co-immunoprecipitation data confirmed that the C83S mutant Prx I acted as a functional Prx II by physically associating with the IL-4 receptor complex. Currently studies are underway to understand the roles of Prx proteins in vivo in IL-4-dependent immune responses including T helper cell differentiation and T cell receptor signaling, using Prx KO mice.

Based on the findings described in this thesis, we may conclude that inactivation of receptor-associated PTP-activity by cytokine-generated ROS is a physiologic mechanism for the amplification of cytokine receptor activation in both cis and in trans, which is negatively regulated by antioxidant enzyme-mediated elimination of ROS in cytokine-stimulated cells, and thus, antioxidant enzymes restore the homeostasis of cytokine signaling in mammalian cells.
CHAPTER V
SUMMARY AND SIGNIFICANCE

The objective of this study was to elucidate the molecular mechanisms underlying homeostatic regulation of IL-4 mediated cell signaling. To accomplish this, we have demonstrated that immediately upon binding with its cognate receptor, IL-4 generated ROS in variety of cell types through IRS-PI3K-Rac1-NOX1 pathway. In addition, we also found that in human A549 cells, IL-4 receptor induced an intracellular calcium flux via IRS-PI3K-PLC-γ pathway, which in turn induces PKC-dependent activation of NOX5L that generated reactive oxygen species (ROS). However, ROS were not required for the initiation of IL-4 receptor activation but they actively participated in promoting receptor activation and signal amplification. Additionally, we have identified a ubiquitously expressed...
phosphatase, PTP1B that associated and negatively regulated IL-4 receptor activation and subsequently became oxidatively inactivated by IL-4-mediated ROS. Further, we found that ROS generated by other cytokine receptors also promoted IL-4 receptor activation. These data reveal that inactivation of receptor-associated PTP-activity by cytokine-generated ROS is a physiologic mechanism for the amplification of cytokine receptor activation both in *cis* and in *trans*, unfolding a novel means of cytokine cross-talk. In addition, we have shown that antioxidant enzymes, including SOD2, catalase, GPx1, Prx II, Prx IV and Prx VI, reduced the steady state level of ROS produced by IL-4. Unlike, SOD2, catalase and GPx1, Prx II, IV and VI physically interacted with IL-4Rα and thereby prevented the inactivation of PTP1B by catalytically reducing ROS.

It was long conceived that the cytokine-activated Jak-STAT pathway operates directly from the cell surface to the nucleus via DNA-protein and protein-protein interactions without involving any second messengers. This study unfolded, for the first time to our knowledge, a role for second messengers (ROS) in the amplification of the IL-4-activated Jak-Stat signaling pathway. Another interesting aspect of this study was cytokine crosstalk, which holds high potential and may help us understand the underlying side effects of cytokine therapies in a variety of diseases.

Further, antioxidant enzyme-mediated catalytic reduction of ROS generated by IL-4-stimulation of cells reiterates the importance of antioxidant proteins for maintaining cellular homeostasis. IL-4 has crucial role in the pathogenesis of allergic diseases including the allergic asthma that affects about
16.2 million people in the US and 130 million more in rest of the world. This study provides new insights into the mechanisms of IL-4 signaling, which may help design new therapies for these diseases.
Figure 5.1: Working Model of Redox Regulation of IL-4-mediated Cell Signaling.
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