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Molecular Mechanisms for IL-10-Induced CD-14 Expression in Human Monocytic Cells

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# MOLECULAR MECHANISMS FOR IL-10-INDUCED CD14 EXPRESSION IN HUMAN MONOCYTIC CELLS

by

Ali Akbar Rahim Rahimi

A thesis submitted to the Faculty of Graduate Studies
in partial fulfilment of the requirements for the degree of
**Masters of Science.**

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*Your file Votre référence*
*ISBN: 0-494-01587-X*
*Our file Notre référence*
*ISBN: 0-494-01587-X*

## ABSTRACT

IL-10, an immunoregulatory cytokine with biological effects primarily on inhibition of inflammatory responses, has also been shown to stimulate a variety of functions including CD14 expression on human monocytic cells. CD14, a receptor for lipopolysaccharide (LPS), plays a critical role in the synthesis of proinflammatory cytokines by LPS-stimulated monocytic cells. Herein, I show that LPS-induced CD14 expression on monocytic cells may be mediated by endogenously produced IL-10. In this study, I have investigated the molecular mechanisms by which IL-10 enhances CD14 expression in normal human monocytes and a promyelocytic HL-60 cells as a model system. IL-10 induced the phosphorylation of PI3K and p42/44 extracellular signal-regulated kinase (ERK) MAPKs. By employing specific inhibitors for PI3K (LY294002) and ERK MAPKs (PD98059), I provide evidence that LY294002 either alone or in conjunction with PD98059 inhibited IL-10-induced phosphorylation of STAT1 and consequently CD14 expression. However, IL-10-induced STAT3 activation remained unaffected under these conditions. Furthermore, LY294002 and PD98059 inhibited the binding of STAT1 transcription factor to its binding site in the CD14 promoter. Finally, STAT1 siRNA inhibited IL-10-induced CD14 expression. Taken together, results show for the first time that IL-10-mediated CD14 upregulation may be mediated by STAT1 activation independently of STAT3. Furthermore, IL-10-activated STAT1 may be regulated through PI3K either alone or in concert with the ERK MAPKs.

## DEDICATION

I dedicate this thesis to my dear wife, Nahid for all her love, endless patience, support and encouragement throughout the course of my studies.

## ACKNOWLEDGEMENTS

I would like to gratefully acknowledge the supervision of Dr. Ashok Kumar during this work. I thank Dr. Marko Kryworuchko, Dr. Katrina Gee and Dr. Masoud Ghorbani for numerous helpful discussions, help with experimental setup and general advice; in particular I would like to thank Wilfred Lim. Wei Ma, Sasmita Mishra, Jyoti Mishra, Ali Azizi and all other respected lab mates for their assistance with all types of technical problems - at all times. From the staff, Rita Frost, Sue Aucoin, and Angela are especially thanked for their care and attention.

I would especially like to thank my thesis advisory committee members Dr. Karen Copeland, Dr. Maya Kozlowski, and Dr. Jonathan Angel for their great advice throughout the course of my study.

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## LIST OF ABBREVIATIONS:

| | |
|---|---|
| aa | amino acid |
| AP-1 | activator protein-1 |
| APCs | antigen presenting cells |
| Ba/F3 | mouse pro-B cell line |
| Bcl-2 | B cell lymphoma-2 |
| BCRF1 | viral IL-10, vIL-10 |
| bp | base pair |
| CD | cluster of differentiation |
| CD14 | specific receptor for LPS |
| cIL-10 | host cellular IL-10 |
| CMIR | cell mediate immune response |
| CMV | cytomegalovirus |
| cmvIL-10 | cytomegalovirus IL-10 |
| CRF2 | cytokine receptor family 2 |
| CSFs | colony-stimulating factors |
| CSIF | cytokine synthesis inhibiting factor |
| DAG | diacyl-glycerol |
| DCs | dendritic cells |
| DNA | deoxy ribonucleic acid |
| EBV | Epstein Barr virus |
| EGF | epidermal growth factor |
| EHV2 | equine herpes virus type 2 |
| EMSA | electrophoretic mobility shift assay |
| ERK | external signal-regulated kinase |
| FACS | fluorescence activated cell sorting |
| FACScan | fluorescence-activated cell scan |
| FcR | Fc receptor |
| FCS | fetal calf serum |
| FITC | fluorescein isothiocyanate |
| G proteins | guanine nucleotide-binding proteins |
| G-CSF | granolocyte-colony stimulating factor |
| GM-CSF | granolocyte monocyte-colony stimulating factor |
| GMT | geometric mean titers |
| GPCRs | G protein-coupled receptors |
| hIL-10 | human Interleukin-10 |
| HRP | horseradish peroxidase |
| IκB | inhibitors of κB |
| IBD | inflammatory bowel disease |
| ICAM-1 | intercellular cell adhesion molecule-1 |
| IFN | interferon |
| IgG | immunoglobulin G |
| IL-10 KO | IL-10 knockout |
| IL | interleukin |
| IL-10R | interleukin-10 receptor |

| | |
|---|---|
| IL-1Ra | IL-1 receptor agonist |
| IMDM | Is cove's modified Dulbecco's modified Eagle's medium |
| IRS-2 | insulin receptor substrate-2 |
| JAK | janus family tyrosine kinase |
| JNK/SAPK | c-Jun kinase/stress activated protein kinase |
| kb | kilo base |
| kDa | kilo Dalton |
| LIF | leukemia inhibitory factor |
| LPS | lipopolysaccharide |
| mAb | monoclonal antibody |
| MAP | mitogen-activated protein |
| MAPKs | mitogen-activated protein kinases |
| M-CSF | monocyte-colony stimulating factor |
| MDDCs | monocyte-derived dendritic cells |
| MDMs | monocyte-derived macrophages |
| MEK | MAPK/ERK kinase |
| MEKK | MEK kinase |
| MFI | mean fluorescence intensity |
| MHC | major histocompatibility complex |
| mIL-10 | mouse IL-10 |
| MIP-1 | macrophage inflammatory protein-1 |
| MIP-1α | macrophage inflammatory protein 1α |
| mRNA | messenger ribonucleic acid |
| NF-κB | nuclear factor κB |
| NK cells | natural killer cells |
| NLS | nuclear localization signal (from cytoplasm to nuclear) |
| NO | nitric oxide |
| $O2^-$ | super anion oxide |
| PBMCs | peripheral blood mononuclear cells |
| PBS | phosphate buffered saline |
| PE | phycoerythrin |
| PGE2 | prostaglandin E2 |
| PH | pleckstrin homology |
| PI3K | phosphoinositide-3-kinases |
| PTB | phosphotyrosine-binding domains |
| PTKs | protein tyrosine kinases |
| PVDF | polyvinylidene fluoride |
| RANTES | regulated upon activation normal T-expressed and secreted |
| rhIL-10 | recombinant human IL-10 |
| RNA | ribonucleic acid |
| RNAse | ribonuclease |
| RTK | receptor-type tyrosine kinase |
| PTKs | receptor tyrosine kinases |
| SH2 | Src homology-2 |
| siRNA | small interfering RNA |
| SLE | systematic lupus erythematosus |

| | |
|---|---|
| SOS | guanine nucleotide exchange factor |
| STAT | signal transducer and activator of transcription |
| TF1 | human erythroleukemia cell line |
| Th1 | T helper 1 cells |
| Th2 | T helper 2 cells |
| TNF-α | tumor necrosis factor-α |
| TNFR | tumor necrosis factor receptor |
| Tyk2 | tyrosine kinase 2 |
| vIL-10 | viral IL-10 |

# CHAPTER 1:

# INTRODUCTION

## Background

Monocytes/macrophages are professional phagocytes capable of inducing adaptive immunity by presenting antigens to T cells. Mononuclear phagocytes are widely recognized as cells that play a central role in the regulation of immune and inflammatory activities as well as in tissue remodeling. The execution of these activities is mediated by complex and multifactorial processes, which involve products secreted by activated macrophages. These low molecular-weight proteins are produced by virtually all cells of the innate and adaptive immune systems and, in particular, by T helper (Th) cells and monocytes/macrophages, acting non-enzymatically in picomolar to nanomolar concentrations to regulate host cell function. Their major functional activities are concerned with the regulation of the development and behavior of immune effector cells such as activation, proliferation, differentiation and migration [1]. Cytokines serve as chemical messengers within the immune system, although they also communicate with certain cells in other systems, including those of the nervous system. Thus, they can function in an integrated fashion to facilitate homeostasis. By contrast, they also play a significant role in driving hypersensitivity and inflammatory responses and in some cases they can promote acute or chronic distress in tissues and organ systems [1].

In general, monocyte activation in response to bacterial endotoxin or lipopolysaccharide (LPS) interaction with its receptor, CD14, induces pro-inflammatory (IL-1, TNF-α, etc.) and anti-inflammatory (IL-10, soluble TNF-R, IL-1Rα) cytokines [2-4]. Interleukin (IL)-1, IL-6, IL-12 and Tumor Necrosis Factor (TNF)-γ are important cytokines produced by monocytic cells which contribute to pathophysiologic changes associated with several acute and chronic inflammatory conditions, including septic

shock, autoimmune diseases and Acquired Immunodeficiency Syndrome (AIDS) [5]. Activated monocytic cells also produce a number of regulatory molecules that are believed to prevent the excessive production of pro-inflammatory mediators including TNF-α and IL-6 [6]. One of the important regulatory molecules is IL-10. There are more than 4200 reports of research involving IL-10 during past decade. IL-10 was first described as cytokine synthesis inhibitory factor (CSIF). IL-10 has indirect inhibitory effect on cytokine production by both T cells and natural killer cells (NK cells), via inhibition of accessory cell (monocytes/macrophages) function. IL-10 inhibited a wide range of activated monocytes/macrophages functions, including monokine synthesis, nitric oxide (NO) production, and expression of major histocompability (MHC) class II and co-stimulatory molecules such as IL-12 and B7.1/B7.2 [7]. Since IL-10 is a pleiotropic cytokine, it has also been shown to have stimulatory effects on the expression of various surface molecules. IL-10 treated monocytes and macrophages display an enhanced expression of IgG-Fc receptors (CD16, CD32, and CD64) as well as scavenger receptors (CD163 and CD14) [8]. The main aim of this study was to understand the molecular signaling pathways delivered following interaction of IL-10 with its receptors that result in enhanced expression of CD14; therefore, a detailed description of these molecules is described below.

**IL-10 Protein, Gene, and Expression**

Human IL-10 (hIL-10) is a 178 aa polypeptide in length with an 18 aa signal sequence and a 160 aa mature segment. Its molecular weight is approximately 18 kDa (monomer) [9]. The mouse IL-10 (mIL-10) and hIL-10 genes are encoded by five exons

on the respective chromosomes 1. Activation of IL-10 gene expression results in ~2 kb (hIL-10) and ~1.4 kb (mIL-10) mRNAs [7]; a ~1 kb mRNA was also seen in a mouse Th2 clone [10;11].. Cells known to express IL-10 include $CD8^+$ T cells, microglia, $CD14^+$ monocytes, Th2 $CD4^+$ cells, keratinocytes, hepatic stellate cells, melanoma cells, activated macrophages, NK cells, dendritic cells, B cells ($CD5^+$ and $CD19^+$), mast cells and eosinophils and to a certain extent $CD4^+$ Th1 cells [9]. IL-10 is also produced by a wide variety of $EBV^+$ B cell line [12] and B cells from systemic lupus erythematosus (SLE) patients. IL-10 induces B cells from SLE patients to secrete immunoglobulins [13;14].

IL-10 gene homologes have been found in Epstein-Barr virus (EBV), equine herpes virus type 2 (EHV2), poxvirus Orf, and human cytomegalovirus (CMV) genomes [10;11;15-17]. Viral IL-10 (vIL-10) is a 17-kDa nonglycosylated polypeptide that is expressed during the lytic phase of virus infection [18;19] and exhibits only a subset of host cell IL-10 (cIL-10) activities *in vitro* and *in vivo* [11;12;20-25]. The EBV, EHV2, and poxvirus Orf virus genomes encode viral homologues of IL-10 genes that lack introns. Excluding the signal sequences, the hIL-10 and EBV (vIL-10) amino acid sequences are 84% identical, with most differences in the N-terminal 20 amino acids[26].

Recombinant human IL-10 (rhIL-10) is an 18 kDa polypeptide, which is not glycosylated. hIL-10 is active on both mouse and human cells, but mIL-10 is not active on human cells. The mIL-10 and hIL-10 genes are composed of five exons and are located on the respective chromosomes 1. Transcription generates predominantly single mRNAs of approximately 1.4 kilo-bases (kb) mIL-10 or 2 kb hIL-10. mIL-10 production stimulated by lipopolysaccharide (LPS) in vivo was not inhibited by cyclosporin, where

anti-CD3 stimulated IL-10 expression was. Suggesting that stimulation of T cell IL-10 production (anti-CD3) could be inhibited by cyclosporin, whereas monocyte IL-10 expression (LPS) could not. These data suggested different cell type-specific regulatory mechanisms of IL-10 expression[26].

**IL-10 Receptor**

IL-10 mediates its biological effects following interaction with its receptor which is exposed on most of the cell types including T cells, B cells and monocytic cells. The receptor for the IL-10 is member of the class II cytokine receptor family (CRF2). The functional receptor is heterodimer composed of type 1 or α (R1) chain and type 2 or β (R2) chain. At a minimum, it would appear that there are at least two α and two β chains involved in each signaling receptor complex. In general, the α subunit is characterized as the ligand-binding molecule and the β subunit as being the signal transducing subunit [9]. Human IL-10R1 is a 90-110 kDa; type 1 transmembrane glycoprotein that is expressed on hemopoietic and nonhemopoietic cells. The open reading frame encodes a 578 aa protein that contains a 21 aa signal sequence, a 215 aa extracellular region, a 25 aa transmembrane segment, and a 317 aa cytoplasmic domain. There are two fibronectin type III motifs within the extracellular region and signal transducer and activator of transcription-3 (STAT3) docking site plus a JAK1 association region within the cytoplasmic domain [9]. Human IL-10R2 is a 60 kDa, 325 aa glycoprotein that contains a 201 aa extracellular region, a 29 aa transmembrane segment, and a 76aa cytoplasmic domain. Its cytoplasmic domain is associated with tyrosine kinase 2 (Tyk2) kinase [9]. IL-10 receptor mediated pathways are discussed on page 6.

**Biological activities of IL-10**

IL-10 is a pluripotent cytokine with potent effects on numerous cell populations, in particular circulating and resident immune cells, as well as epithelial and some other parenchymal cells. IL-10 significantly modulates expression of cytokines, soluble mediators and cell surface molecules on cells of myeloid origin. These changes have important consequences for the functions of these cells in activating and maintaining immune and inflammatory responses. IL-10 strongly inhibits the production of IL-1α, IL-1β, IL-6, IL-8, IL-12, Granolocyte Monocyte Colony Stimulating Factor (GM-CSF), G-CSF, M-CSF, TNF- α, MIP-1 α, MIP-1β, MIP-2, RANTES and leukemia inhibitory factor (LIF) by activated monocytes/macrophages [27]. IL-10 also inhibited expression of MHC II antigens, CD54, B7.1 and B7.2 on monocytes, even following induction of these molecules by IL-4 or IFN-γ. Down regulation of these stimulatory or co-stimulatory molecules significantly affected the T cell-activating capacity of monocytes as antigen-presenting cells (APCs). Up-regulation of Fc receptor (FcR) by IL-10 enhanced the capacity of monocytes/macrophages to phagocytose opsonized particles, bacteria or fungi, but IL-10 reduced the ability of the cells to kill the ingested organisms by decreasing the generation of super oxide anion (O2-) and nitric oxide (NO). IL-10 inhibited production of prostaglandin E2 (PGE2), another pro-inflammatory mediator[26]. However, IL-10 enhanced production of IL-1Rα and expression of soluble P55 and P75 tumor necrosis factor receptor (TNFR), indicating that IL-10 induces a shift from production of pro-inflammatory to anti-inflammatory mediators. IL-10 exhibits stimulatory effects on B cell growth and differentiation [28-30], Naïve $IgD^+$ B cells, when activated with anti-CD40 antibodies, secrete IgG1 and IgG3 in the presence of IL-

10[31], indicating a critical role of IL-10 in isotype switching and B cell differentiation. Similarly, IL-10 enhances the transcription and production of IgG4 and IgE by B cells [32]. IL-10 acts as an autocrine growth factor for Ly-$1^+$ B cells, which are important in murine models of autoimmune disease [33].

**Regulation of IL-10 production:**

The above observations suggest that the effects of IL-10 are quite complex and considering IL-10 as immunosuppressive and anti-inflammatory might be an oversimplification.

Regulation of IL-10 production is a complex process and is not well understood. Studies on the kinetics of cytokine production have revealed that IL-6 and IL-12 along with IL-1, IL-2, TNF-α, GM-CSF, G-CSF and IFN-γ are the first cytokines synthesized following activation of PBMC by mitogens [34;35]. IL-10 is produced later and down-regulates the production of cytokines synthesized earlier as well as its own production [36-38]. It has been shown that IL-10 produced by different cell types is differentially regulated. IL-12 induces IL-10 production by T cells whereas TNF-α induces IL-10 production in monocytes [39-42].

The molecular mechanisms responsible for regulating the production of IL-10 by various cell types have been studied in the recent past. A recent report has indicated that IL-10 production is dependent on protein tyrosine kinases (PTK) and protein kinase C (PKC) activation in a murine cell line (28). In addition, factors that elevate cAMP have been suggested to be involved in the regulation of monocytic IL-10 synthesis, primarily at the mRNA level [43]. Recently, it has been suggested that p38 MAPK is involved in

the regulation of IL-10 production (31). However, very little is known about the regulation of the hIL-10 gene and the involvement of MAP kinases in this process. To gain insight into hIL-10 gene regulation, Wei et al, have employed promonocytic THP-1 cells that produce IL-10, IL-12 and TNF-α following LPS stimulation as do normal human monocytes. Using mutagenesis, they analyzed the promoter sequence of the hIL-10 gene and presented evidence that an element located at -650 bp, encompassing a Sp1 consensus sequence is involved in the transcription of the hIL-10 gene [44]. This was further demonstrated by introducing a mutation in the Sp1 consensus sequence that abrogated IL-10 promoter activity. Furthermore, the Sp1 transcription factor is induced by LPS stimulation and is selectively regulated by the p38 MAP kinase. Brightbill *et al.* demonstrated the involvement of a non-consensus Sp1-like sequence in mIL-10 expression using RAW264.7, a murine macrophage cell line. IL-10 transcription can be regulated by transcription factors SP1 and SP3, which are expressed constitutively by many different cell types [45].

**CD14**

CD14 is a glycoprotein with 356 amino acids, encoded on chromosome 5q 23-31[46]. Membrane CD14 is expressed in mature myeloid cells, to a varying extent in most human tissue macrophages, and at very low numbers on B-lymphocytes, basophils, mammary cells, placental trophoblasts and gingival fibroblasts [47]. The number of CD14 on the surface of myeloid cells is closely related to the maturity of cells. There are different reports about the number of CD14 molecules on monocytes varying from 6000 to 190,000 on human monocytes [47]. CD14 was initially established as a surface marker,

membrane CD14 (mCD14), to define myeloid cells or monocyte differentiation [48], but in 1990 its putative main function as a receptor for LPS was discovered [49]. Transgenic mice over expressing human CD14 were hypersensitive to the effects of LPS [50]. Two soluble forms of CD14 are constitutively generated, the 55 kDa and the 49 kDa. The membrane/ soluble CD14 ratio depends on the presence of the 8 C-terminal amino acids. CHO cells transfected with a full-length $CD14_{1-356}$ cDNA strongly express mCD14, but release only a few nano-grams of sCD14/$10^6$ cells/day [51]. In contrast, CHO cells transfected with a CD14 truncated by eight amino acids have only a weak surface CD14 expression, but release 1000-fold more sCD14. sCD14 is abundant in serum, in cerebrospinal fluid as well as in a truncated form in urine from patients with proteinuria [52]. Moreover, part of CD14 proteins is intracellular and it is detectable by a specific ELISA method [47].

**CD14 ligands**

LPS binds to CD14 in a native state or in aggregates with a 60-kDa serum glycoprotein, named the LPS-binding protein (LBP), CD14 receptor [52]. In addition to LPS, several other agents have been shown to interact with CD14, for example: cell wall compounds from gram-negative and gram-positive bacteria as well as for fungi or spirochetes (*Borrelia burgdorferi*) [53]. Also, purified lipophilic or polysaccharide components such as muramyl dipeptide, peptidoglycan, lipoarabinomannan, or phospholipids interact with CD14 [52]. In addition to these ligands are several protein ligands, including heat shock protein 60 (HSP60), HSP70, and the fusion glycoprotein from respiratory syncytial virus (RSV). CD14 expression can be regulated by endotoxins

and a number of immunoregulatory cytokines. It has been shown that IL-4 and IL-13 can down-regulate CD14 expression on monocytes [52;54] and that TNF-α and IL-1 are able to up-regulate CD14 expression on monocytes [52]. In this study I have shown that hrIL-10 also can induce CD14 up-regulation on normal human monocytes and human promonocytic HL-60 cells.

**Biological functions of CD14**

CD14 plays an essential role in the internalization and detoxification of LPS. Also CD14 can interact with several microorganisms' components and activate monocytes to eliminate those particles [47;52;55;56]. An additional function of CD14 on monocytes is its role in cell-cell interaction, and also inhibition of the adhesion of monocytes to activated endothelial cells [57;58]. Additionally, some investigations have described the role of CD14 and *Porphyromonas gingivalis* in the development of periodontal diseases. Interestingly, bone resorption was found to be inhibited by anti-CD14 mAbs and by antisense CD14 oligonucleotides in mouse embryonic calvarial cells [59]. Moreover, there are several lines of evidences that suggest the possible role of CD14 in the induction or suppression of programmed cell death. Activation of monocytes and up-regulation of CD14 expression results in survival of the cells, while IL-4 or phospholipase C mediated CD14 reduction promoted cell death [52;60].

Septicemia, trauma and severe burning result in reduction of CD14 numbers on peripheral monocytes [61;62], on the other hand peripheral monocytes of patients with rheumatoid arthritis showed CD14 expression [63]. CD14 expression down-regulation

might occur in case of administration of therapeutic substances such as Glucocorticosteroids and Novobiocin [47].

**Signal transduction pathways**

Since the main aim of my research project is to understand the signaling pathways involved in the stimulatory effects of IL-10 on CD14 induction in human monocytic cells, I will describe briefly the major signaling pathways activated following interaction of ligand with its receptor. Activation of kinases in cytosolic domain of the receptor and G protein activation are two major types of signal transduction pathways. In general, cytokine receptors have protein kinase activity in its cytosolic domain. The receptor kinases are activated when ligand binds to its extra-cellular domain. The kinase phosphorylates its own cytoplasmic domain; this auto-phosphorylation enables the receptor to associate with and activate a target protein, which in turn acts upon new substrates within the cell. The most common kinase receptors are tyrosine kinases, but there are also some serine/threonine kinase receptors. An alternate type of signal transduction through G protein activation occurs when the inactive form of the G protein, the trimer bound to GDP, gets converted to the active form by replacement of GDP with GTP and dissociation of the G protein into a single subunit carrying GTP and a dimer of the two other subunits. [64].

**The JAK/STAT signaling pathways**

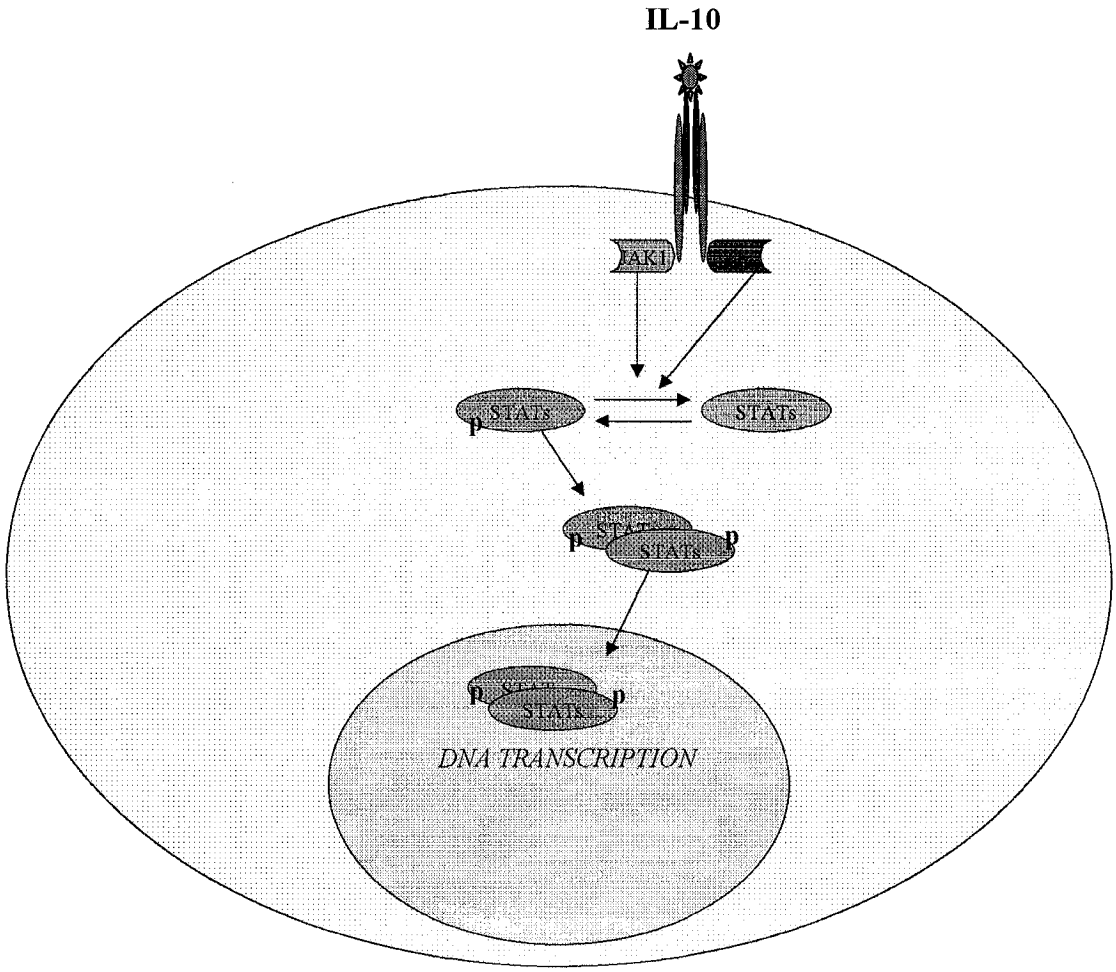
Although cytokines activate multiple signaling pathways, the activation of Jak-STAT pathway plays an important role in mediating their biological effects. The Jak-

STAT pathway triggered by a number of cytokines including IL-10 and IFN-γ allows rapidly the transduction of an extracellular signal into the nucleus. In general, the interaction of a cytokine with its ligand-binding receptor α subunit is the first step in the formation of a signaling-competent receptor complex. This process involves the oligomerization of the ligand-bound subunit with either another subunit or a separate, signal-transducing β subunit [65;66]. This oligomerization initiates the process of signal transduction by activation of the receptor-associated Janus family tyrosine kinases (JAKs) through cross-phosphorylation (Fig. 1-1) Immediate targets of the activated JAKs are the cytoplasmic portions of the receptors and receptor associated proteins. The tyrosine phosphorylated sites become docking elements for Src homology 2 (SH2)- and phosphotyrosyl-binding domain-containing proteins present in the membrane or the cytoplasmic compartment. Prominent among these are the signal transducer and activator of transcriptions (STATs). Receptor-recruited STATs are phosphorylated on a single tyrosine residue in the carboxyl terminal portion. The modified STATs are released from the cytoplasmic region of the receptor subunits to form homodimers or heterodimers through reciprocal interaction between the phosphotyrosine of one STAT and the SH2 domain of another. Following dimerization, STATs rapidly translocate to the nucleus and interact with specific regulatory elements to induce target gene transcription [67]. Seven members of the STAT family of transcription factors have been identified in mammalian cells: STAT1, STAT2, STAT3, STAT4, STAT5a, STAT5b, STAT6. STAT isoforms lacking regions of the c-terminal domain have a competitive dominant-negative effect on gene induction mediated by the STAT pathway, counteracting the effects of the full-

length isoform STATα [68-71]. A representative example of the different STAT3 isoforms is described in table 1 [67].

**Fig. 1-1.** The JAK/STAT signaling pathway

Upon binding ligand, receptor-associated JAKs become activated and mediate phosphorylation of specific receptor tyrosine residues. This leads to the recruitment of specific STATs, which are then also tyrosine-phosphorylated. Activated STATs are released from the receptor, dimerize, translocate to the nucleus, and bind to STAT binding site in DNA and activate transcription of specific genes.




**JAK/STAT Signaling Pathway**

| STAT3 isoforms | Description | MW (kDa) |
|---|---|---|
| STAT3α | Full length | 92 |
| STAT3β | Missing c-terminal transactivation domain; functionally distinct, either dominant negative or altered binding | 83 |
| STAT3γ | Lacking tyrosine residue; can still be recruited to tyrosine-phophorylated receptor proteins by the binding function of the remaining SH2 domain but signaling by the receptor complex terminates | 72 |
| STAT3δ | Unknown | 64 |

**Table 1. STAT3 isoforms** [67]

Constitutive activation of STAT1, STAT3, and STAT5 has been demonstrated to be associated with malignant transformation induced by various oncoproteins [71-73] and also it has been reported in a number of malignant cell lines and human cancers [74]. Dimerization of STAT proteins in the cytoplasm by phosphotyrosine-SH2 interaction is a critical step in STAT activation and subsequent gene transcription. Ideal candidates to interfere with dimerization would be SH2-like peptides recognizing phosphotyrosine residues of the STATs or small molecule peptide mimetics with phosphotyrosine residues that specifically bind to SH2 sequence of STATs. Disruption of STAT3 dimerization by the SH2 domain-binding phosphotyrosyl peptide, PYpLKTK, was demonstrated to block STAT3-mediated DNA binding activity, gene regulation, and cell transformation in vitro and in vivo [75]. Because STAT proteins are directly and selectively targeted, nonspecific side effects are theoretically expected to be much less than with other strategies that block STAT upstream signaling [67].

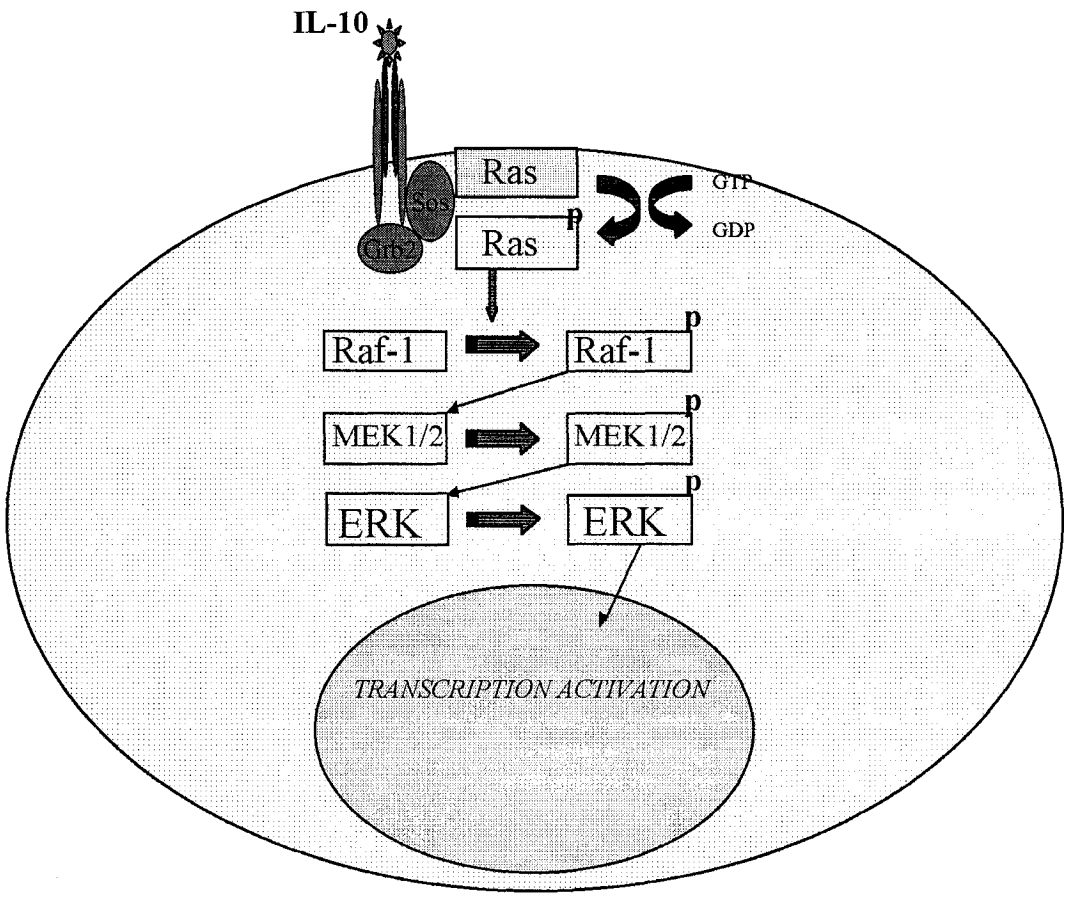
**The Mitogen Activated Protein Kinases (MAPKs) Signaling Pathway**

The best characterized pathway that is initiated by receptor tyrosine kinases passes through the activation of a monomeric GTP-binding protein to activate a cascade of cytosolic kinases. In mammalian cells, the cascade is often initiated by activation of a tyrosine kinase receptor, such as EGF or PDGF receptors. The receptor activates the Ras pathway by means of an "adaptor" protein. The activation of Ras leads to the activation of the Raf Ser/Thr kinase, which in turn activates the kinase MEK. In general, MEK phosphorylation induces MAPK activation and it leads to the phosphorylation of transcription factors that trigger changes in cell phenotype varying from growth to differentiation, depending on the cell type (Fig. 1-2). Other targets for the kinases include cytoskeletal proteins that may directly influence cell structure [64]. MAPKs are key players in cellular response such as proliferation, differentiation, and apoptosis [76]. The three main families of MAPKs are the extracellular signal-regulated protein kinase (ERK1 and ERK2), the C-jun N-terminal kinases (JNKs), and p38 MAPK/stress-activated protein kinases. ERKs respond to mitogens and growth factors that regulate cell proliferation and differentiation, whereas JNK and p38 MAPKs are predominantly activated by stress and inflammatory cytokines (IL-1β and TNF-α) [76].

It is generally believed that activation of Ras/Raf/MAP kinases does not occur following interaction of IL-10 with its receptor. In some studies, however, this pathway has been shown to be inhibited [77]. In one study, IL-10 was shown to activate p38 MAPK in murine macrophages that was implicated in IL-10-induced expression of heme-oxigenase [78]. In another study, IL-10 has been shown to increases the amount of activated ERK MAPKs in factor-dependent cell progenitor-1/Mac-1 cells (FDCP cells)

**Fig. 1-2.** The ERK MAPKs signaling pathway

The MAPKs signaling cascade can be initiated upon ligand binding to a receptor tyrosine kinase receptor. Upon activation of the receptor the adaptor molecule Grb2 recruits Sos which in turn recruits small G proteins such as Ras, Rac, and Cdc42 to the inner plasma membrane. The small G protein is phosphorylated and activated upon the exchange of GTP for GDP and subsequently activates a MAPK kinase (MEK 1-7). The MEK activated is specific to the activated upstream small G protein. In case of ERK MAPKs pathway, the small G protein Ras can activate the Raf-1 which then activate the MEK1&2 responsible for ERK MAPKs activation.




**ERK MAPKs Signaling Pathway**

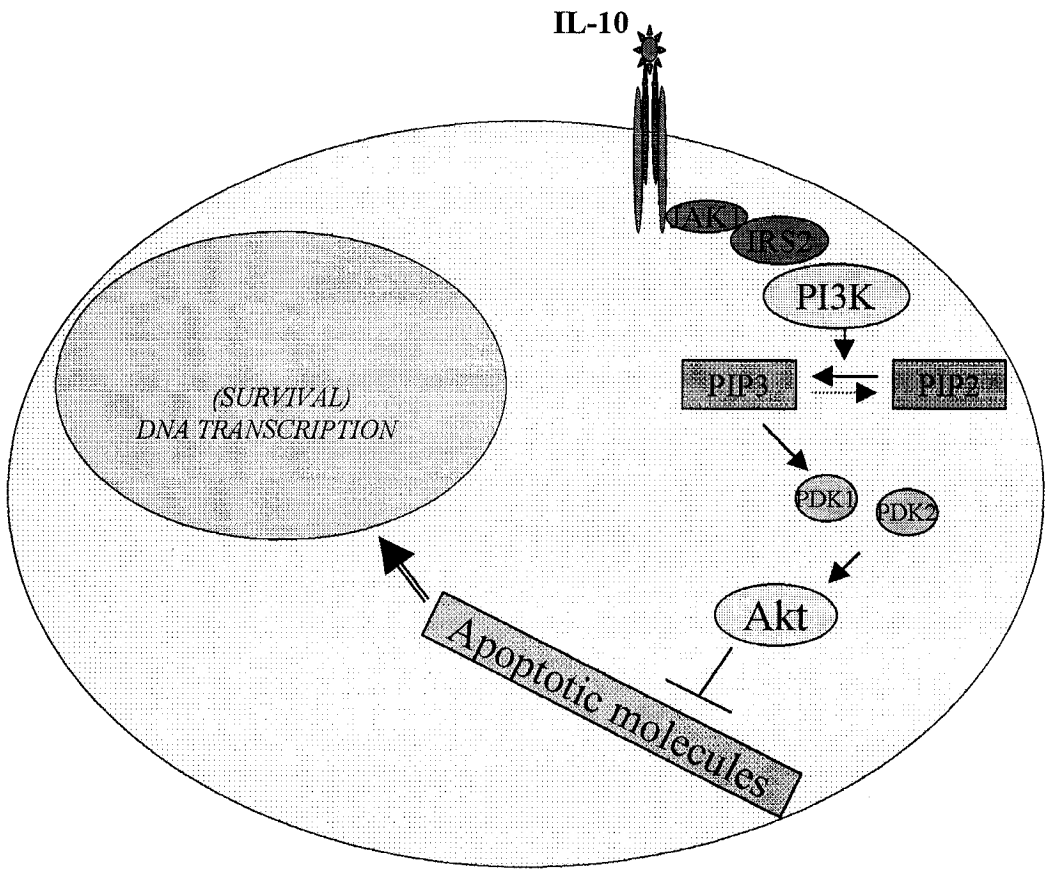
[79]. In our lab, it has been shown previously that IL-10 selectively induces the activation of ERK MAPKs in human monocytic cells [4]. The signaling events involved in specifically activating ERK MAPKs are poorly understood. There is evidence to suggest that ERK MAPKs can be activated by the JAK1/2 in IFN-g-mediated signaling pathway. However, the role of ERK MAPKs in IL-10 mediated effects is also not clear at present. Zhou et al. have shown that the increase in ERK MAPKs activity was inhibited in a dose-dependent manner by PI3K inhibitors, Wortmannin or LY294002, and they showed that these inhibitors have no effect on IL-10 mediated STAT3 activation [79].

**The Phosphatidylinositol 3-kinases (PI3K) Signaling Pathway**

Over the past decade, research on PI3K has demonstrated that this family of enzymes contains important regulators of cellular signaling. They are activated by G-protein-coupled receptors or receptors with an intrinsic or associated protein tyrosine kinase activity and/or proteins that are tyrosine phosphorylated in response to extra cellular stimuli [80]. Another way in which phosphatidylinositol 3-kinases are activated is by a direct interaction with the small GTPase Ras [81;82]. In response to extracellular stimuli, phophoinositides are phosphorylated on the 3-position of the inositol ring by PI3K [83;84]. The products of PI3K, namely phophatidylinositol(3)monophosphate [PtdIns(3)P] or PIP, phosphatidylinositol(3,4)biphosphate [PtdIns(3,4)P] or PIP2, and phosphatidylinositol-(3,4,5)triphosphate[PtdIns(3,4,5)P] or PIP3, govern many cellular events, such as cell growth and survival, cytoskeletal remodeling and the trafficking of intracellular organelles (Fig. 1-3) [83-85]. The PI3Ks are divided into four classes,

**Fig. 1-3.** The Phosphoinositide 3-kinase (PI3K) signaling

PI3K represents a major signaling transducer involved in a variety of cellular responses. It is a heterodimers consisting of p85 and p110. Activation of PI3K catalyzes the phosphorylation of phosphatidylinositol biphosphate (PIP2). The phosphorylated lipids (PIP3) activate phosphatidylinositol dependent kinases 1 and 2 (PDK 1&2) resulting activation and phosphorylation of Akt, a serine-threonine kinase. This allows Akt to mediate a variety of cellular responses such as protection of cells from apoptosis.




**PI3-Kinases Signaling Pathway**

referred to $I_A$, $I_B$, II and III, on the basis of their structural characteristics and substrate specificity[85].

Class I phosphatidylinositol 3-kinases generate PtdIns(3)P, PtdIns(3,4)P2, and PtdIns(3,4,5)P3 and consist of four mammalian p110 catalytic isoforms (p110α, β, γ, and δ) which associate with the p85-family of regulatory subunits, except for p110γ which binds to a p101 adaptor [86;87]. The majority of tyrosine-kinase coupled transmembrane receptors can activate class Ia PI3K. The well-known characterized down stream target for class I PI3K is protein kinase B (PKB). PKB is also known as Akt, the mammalian homologue of the viral oncogene (v-Akt). Akt is activated by two phosphorylation events, both of which are most likely induced by the phosphoinositide dependent kinase, PDK1, following PI3-kinase activation. Downstream of Akt lie a number of enzymes implicated as effectors of the actions of insulin on glucose metabolism and protein synthesis, and of PI3-kinase signaling on cell survival and cellular proliferation [88]. The phosphatidylinositol 3-kinases of class II generate PtdIns(3)P and PtdIns(3,4)P2 are 170-210 kDa. Enzymatically, the class II kinases are distinguished by a virtual inability to phosphorylate PtdIns(4,5)P2 in vitro. A further, distinguishing feature of these enzymes is that they are predominantly membrane-associated, in contrast to class I kinases, which, in the resting state, are cytoplasmic [88]. The class III enzymes only produce PtdIns(3)P [80;89]. Crowley et al. have shown that IL-10 activates PI3-kinase in both primary monocytes and a murine mast cell line, D36 [90]. Also they have shown inhibition of IL-10 mediated proliferation in these cells by using inhibitors of PI3K, Wortmannin and LY294002. However, PI3K activation was not required for IL-10 suppression of lipopolysaccharide-induced TNF-α production [90]. Another study in primary culture of

mouse astrocytes has been shown that IL-10 in isolation induces Akt phosphorylation, but interestingly this study showed that IL-10 attenuate IL-1β-induced Akt phosphorylation [91].

**Signal Transduction in response to IL-10**

As mentioned above, IL-10 exerts bipolar inhibitory as well as stimulatory biological activities. The signaling molecules that mediate the biological effects of IL-10, and especially those involved in inhibitory or stimulatory activities are not well understood. Mouse pro-B cell (Ba/F3) and human erythroleukemia cell (TF1) lines transfected with wild type and mutant IL-10R have been used to study IL-10R function. The most studied of IL-10 signaling has been the JAK/STAT system. Like IFN-γ, IL-10 induces tyrosine phosphorylation of tyrosine kinases JAK1 and TYK2. In addition, tyrosine phosphorylated, DNA binding STAT1, STAT3 and, in lymphocytes, STAT5 were detected. Both STAT activation and biologic responses to IL-10 require the presence of at least 1 of 2 tyrosine residues (Y427 & Y477) in the mIL-10R cytoplasmic domain. These two tyrosines appear to be substantially functionally redundant with respect to IL-10-induced short-term proliferation and STAT activation, and have been implicated directly in recruitment of STAT3 to IL-10R. IL-10 induces transcription and expression of STAT3-responsive gene [26]. The ability of IL-10 to inhibit TNF-α production requires the presence of STAT3 and JAK1[92].

The molecular mechanism by which IL-10 mediates its inhibitory effects is not well understood. IL-10 has been shown to mediate its inhibitory effects on proliferation in murine macrophages through the activation of STAT3 [93;94]. Recently, IL-10 has

been shown to increase the expression of the cell cycle inhibitor, cyclin-dependent kinase inhibitor $p19^{INK4D}$ in macrophages. IL-10-induced expression of $p19^{INK4D}$ was later shown to be dependent on the activation of STAT3, indicating that STAT3-dependent activation of $p19^{INK4D}$ constitute an important component of the mechanism by which IL-10 inhibits macrophage proliferation [95].

A few studies suggested involvement of other signaling pathways. IL-10 reportedly inhibits nuclear factor- kB (NF-kB) activation in response to macrophage activation, but activates activating protein-1 (AP-1) and NF-kB in $CD8^+$ T cells. IL-10 induced Bcl-2 expression in $CD34^+$ progenitors and germinal center B cells, and also activated c-fos expression in human B cells [26]. The inhibitory effects of IL-10 in anti-CD3-antibody-stimulated lymphocytes has been suggested to be mediated by down-regulating the TAP1 family of transporter proteins, which are responsible for transporting peptides from the endoplasmic reticulum to the cell membrane [96]. In monocytes, IL-10 has also been shown to activate P85 phosphatidylinositol-3 (PI-3) and P70 S6 kinases [26]. The complex interaction between signaling molecules with respect to IL-10 is not fully understood and needs further investigation.

Cytokine signals transduced by the JAK/STAT pathway is, at least in part, negatively regulated by a family of endogenous JAK inhibitor proteins referred to as suppressors of cytokine signaling (SOCS) or cytokine-inducible SH2 (CIS) proteins [97]. At the present time, the SOCS family contains eight members of related proteins that share a common modular organization of an SH2 domain followed by a short motif called a SOCS box [98]. There is sequence homology between all family members (particularly in the SOCS box and SH2 domain) [98]. Both SOCS1 and SOCS3 have the kinase

inhibitory region (KIR) in their amino-terminal region, which is proposed to function as a pseudosubstrate to inhibit JAK tyrosine kinase activity. A three-dimensional model of the SOCS1-JAK2 complex has been predicted in which SOCS1 binds directly to the activation loop of JAK through the SH2 domain and the SOCS3 SH2 domain binds to cytokine receptors. Tyr757 of gp130, Tyr985 of the leptin receptor, and Try401 of EPO receptor are binding sites for the SOCS3 SH2 domain [99-101]. IL-10 and LPS potently induce expression of SOCS3. However, in SOCS3 $^{-/-}$ macrophages, LPS and IL-10-induced responses were normal, suggesting that SOCS3 is not a physiological regulator of the signaling cascades initiated by these agents [102;103]. Many reports have indicated that SOCS3 is induced by various inflammatory cytokines, such as IL-6, IL-12, IFN-γ and IL-10 [97;104], and that it negatively regulates those cytokine actions as well as STAT functions. Moreover, SOCS3 is induced by IL-1 and TNF-α as well as LPS [97]. In macrophages lacking the SOCS3 binding site in gp130, LPS induced pro-inflammatory cytokine, TNF-α, was suppressed [102]. The SOCS3 protein was strongly induced by both IL-6 and IL-10 in the presence of LPS, but selectively inhibited IL-6 signaling. This selective inhibition was due to SOCS3 binding the IL-6 receptor, gp130, but not the IL-10 receptor [102]. High levels of SOCS3 expression and inhibition of IL-6 mediated STAT3 activation has been shown in different studies [102;105]. Moreover, it has been suggested that, in hepatocytes, STAT1 and STAT3 negatively regulate one another through induction of SOCS proteins [106]. But the precise roles of SOCS3 in IL-10 mediated STATs activation is not clarified.

## HYPOTHESIS AND RATIONALE

As mentioned above, the molecular mechanism regulating the inhibitory effects of IL-10 on various biological functions in a number of cell types have been investigated. It has been suggested that activation of STAT3 by IL-10 plays a critical role in mediating the inhibitory effects of IL-10 in various model systems [107;108]. However, there is little information regarding the molecular mechanism by which IL-10 exerts its stimulatory effects. It has been suggested that IL-10 may mediate its stimulatory effects through the activation of PI3K pathway [90]. In our lab, it has been previously demonstrated that LPS, a component of Gram-negative bacterial cell walls, induced CD14 expression through endogenously produced IL-10 [109]. CD14 is a primary LPS receptor expressed on cells of myelocytic lineage including monocytes/macrophages, dendritic cells and neutrophils. The interaction of LPS with cell surface CD14 on human monocytes plays a critical role in the activation of these cells during sepsis. CD14 is one of the few surface receptors whose expression is enhanced by IL-10 on human monocytes. The signaling molecules involved in the up-regulation of CD14 expression by monocytic cells in response to IL-10 are not known. Therefore, the main aim of this study was to understand the molecular mechanism underlying the stimulatory effects of IL-10 on CD14 expression in human monocytic cells. **I hypothesized that IL-10 may employ a distinct signaling pathway to enhance the expression of CD14 on monocytic cells.**

**Main Aim**

To investigate the molecular mechanisms by which IL-10 induces up-regulation of membrane bound CD14 by employing normal human monocytes and human promyelomonocytic HL-60 cells as model system.

**Objectives**

1. To identify the MAPKs (ERK, JNK, and p38) involved in IL-10-induced CD14 expression in normal human monocytes and HL-60 cells.
2. To investigate the role of PI3K and their downstream anti-apoptotic molecule, Akt, involved in the up-regulation of CD14 in response to IL-10 in normal human monocytes and HL-60 cells.
3. To understand the regulatory effect of JAK/STAT signaling pathway molecules, especially STAT3 and STAT1 in IL-10-induced CD14 expression in normal human monocytes and HL-60 cells.

# CHAPTER 2:

# MATERIALS AND METHODS

**Cell lines, cell culture, and reagents**

HL60, a promyelocytic cell line derived from a human acute promyelocytic leukemia patient was obtained from the American Type Culture Collection (ATCC, Rockville, MD). Cells were cultured in Iscove's Modified Dulbecco's Medium (IMDM) (Sigma-Aldrich, St. Louis, MO) supplemented with 10% fetal bovine serum (FBS) (Invitrogen/Life Technologies, Grand Island, NY), penicillin (100 U/mL), gentamicin (100 μg/mL), HEPES (100 mM) and glutamine (2 mM). PD98059, an inhibitor of mitogen-activated protein ERK kinase-1 (MEK1) that selectively blocks the activity of ERK MAPKs [76;110] was purchased from Calbiochem (San Diego, CA). The pyridinyl imidazole, SB202190, a potent and specific inhibitor of p38 MAPK [44;76] was also purchased from Calbiochem. LY294002, a cell permeable, potent and specific phosphatidylinositol 3-kinases (PI3Ks) inhibitor [111] was also obtained from Calbiochem (San Diego, CA). Lipopolysaccharide (LPS) derived from *E. coli 0111:B4* (Sigma-Aldrich Canada Ltd., Oakville, ON, Canada), and recombinant human IL-10 (rhIL-10) (R&D Systems Inc.; Minneapolis, MN) were also purchased. All other chemicals used for Western blotting were obtained from Sigma-Aldrich.

**Monocyte isolation and cell stimualtion**

Peripheral blood mononuclear cells (PBMCs) were prepared from blood obtained from healthy adult volunteers following approval of the protocol by the Ethics Review Committee of the Children's Hospital of Eastern Ontario. The PBMCs were isolated by density gradient centrifugation over Ficoll Hypaque (Amersham Biosciences, Piscataway, NJ), as previously described [4]. Briefly, the cell layer containing mononuclear cells was collected and washed three times in PBS. Purified, nonactivated monocytes were isolated

by negative selection by depletion of T cells and B cells using magnetic polystyrene M-450 Dynabeads coated with Abs specific for CD2 (T cells, NK cells) and CD19 (B cells) (Dynal Biotech, Oslo, Norway), as described earlier [4;112]. Briefly, PBMCs (10 x $10^6$ /ml) were incubated with CD2 and CD19 Dynabeads for 20 minutes on ice with constant mixing, followed by magnetic removal of the dynabead:cell rosettes. $CD2^+CD19^+$ cells were separated magnetically from the $CD2^-CD19^-$ cells. $CD2^-CD19^-$ cells were incubated at 37°C / 5% $CO_2$ for 2 hours following which nonadherent cells were removed. The adherent mononuclear cells obtained contained <1% $CD2^+$ T cells and $CD19^+$ B cells as determined by flow-cytometric analysis [4;112].

**Cell stimulation**

To determine the effect of p38, ERK MAPKs, JNK, STAT3, and PI3K specific inhibitors on IL-10-induced CD14 expression, monocytes (1 x $10^6$ cells/ml) and HL-60 cells (1 x $10^6$ cells/ml) were incubated with various concentrations of the MEK-1 inhibitor PD98059 (5 to 50 μM), the p38 MAP kinase inhibitor, SB202190 (10 to 50 μM), STAT3 inhibitor, PYpLKTK (0.02 to 0.6 mM), or PI3K inhibitor, LY294002 (2 to 20 μM) for 2 hours in 12 well culture plates (Falcon, Becton-Dickinson, Frankland Lakes, NJ). Cells were left untreated or stimulated with LPS (1 μg/ml) or IL-10 (10 ng/ml) for 24 hours in the presence or the absence of the above mentioned inhibitors. The cells were then analyzed for CD14 expression by flow cytometry. All experiments were performed at least three times and the figures show representative experiments. Human monocytes and HL-60 cells continuously exposed for 24 hours to LY294002 alone or in

combination with PD98059 in the presence of 10% FBS, and without a change of medium, were >95% viable by microscopic analysis based on Trypan blue staining.

**Flow cytometric analysis**

CD14 expression on monocytes and HL-60 cells was determined by flow cytometry as previously described [109]. Briefly, cells were washed once at the time of harvesting with PBS/0.1% sodium azide and stained with 3 μl of PE-conjugated anti-CD14 mAbs (BD Biosciences, San Jose, CA). Autofluorescence and isotype matched control antibodies, IgG2b (BD Biosciences), were also included. Analysis of CD14 expression was performed on CD14+ monocytes. The gates were set in accordance with the gates obtained with the isotype matched control antibodies. Data were acquired on a BD FACScan Flow Cytometer and analyzed using the WinMDI version 2.8 software package (J. Trotter, Scripps Institute, San Diego, CA). Validity of comparisons in the expression levels of CD14 between different samples was ensured through the use of Calibrite™ Beads (BD Biosciences).

**Western blot analysis**

Phosphorylation of MAPKs (p38, ERK and JNK), PI3K downstream substrate, Akt, and also STAT3 and STAT1 phosphorylation was determined by Western blot analysis using phospho-MAPKs, phospho-Akt and phospho-STATs specific antibodies, respectively, according to the standard procedures as previously described [44;112]. Normal human monocytes and HL-60 cells were pretreated with indicated concentrations of inhibitors followed by stimulation at 37°C / 5% $CO_2$ for 0-60 min with either 1 μg/ml

of LPS or 10 ng/ml of rhIL-10. Subsequently, cells were immediately placed in ice followed by washing with cold PBS. Briefly, cell lysates were prepared by lysing the cell pellets at $4^0$C for 30-45 minutes with lysis buffer (50 mM HEPES pH 7.5, 150 mM NaCl, 10% glycerol, 1% Triton X-100, 1.5 mM $MgCl_2$, 100 mM NaF, 100 mM sodium orthovanadate, and 1 mM EGTA pH 7.7), followed by centrifugation for 20 min at 11500 x g and 4°C. The protein concentration of the supernatants was determined using the Bio-Rad protein determination assay (Bio-Rad laboratories, Hercules, CA). Total cell proteins were subjected to electrophoresis on 12% polyacrylamide SDS-PAGE gels and the proteins were transferred onto polyvinylidene difluoride (PVDF) membranes (BioRad). The membranes were probed with either rabbit anti-human- phospho-p38 (Thr180/Tyr182) mAb (New England Biolabs, Mississauga, Ontario, Canada), mouse anti-human-phosho-ERK MAPKs (Thr202/Tyr204) mAb (Santa Cruz, Biotechnologies, Inc., Santa Cruz, CA), mouse anti-human phospho-JNK (Thr183/Tyr185) antibodies (Santa Cruz, Biotechnologies, Inc., Santa Cruz, CA), rabbit anti-human-phospho-Akt (Ser473) (Cell Signaling Technology), rabbit anti-human-phospho-STAT3 (Tyr705) (Cell Signaling Technology), rabbit anti-human-phospho-STAT1 (Tyr701) (Cell Signaling Technology), followed by horseradish peroxidase conjugated goat anti-rabbit or goat anti-mouse polyclonal antibodies (Bio-Rad). The membranes were stripped of the primary antibodies by incubating the blots in stripping buffer (62.5 mM Tris-HCl, pH 6.7, 100mM 2ME, 2% SDS, and 710 µl DTT) for 30 min and reprobed with rabbit polyclonal antibodies specific for the unphosphorylated forms of either p38, ERK, JNK MAPKs (Santa Cruz), or Akt, STAT3, STAT1 (Cell Signaling Technology) essentially as

described earlier [44;112]. All immunoblots were visualized by enhanced chemiluminescence (ECL, Amersham, Baie d'Urfe, PQ, Canada).

**Transfection of small interfering RNA (siRNA)**

HL-60 cells were seeded in 12 well plates (Falcon) at a concentration of 0.25 x $10^6$ cells/ml in serum-free IMDM media. Cells were transfected with either 1 μg of small interfering RNAs specific for STAT1 or control vector (Panomics, Redwood City, CA) using FuGene 6 (Roche Applied Biosciences, Laval, Quebec, Canada) as described by the manufacturer. The siRNA for STAT1 was a mix of up to three different plasmids. Briefly, the cells were washed once in sterile PBS and then resuspended in serum-free IMDM media. Fugene 6 was allowed to complex with 1 μg of the siRNA plasmids in a total volume of 100 μl of serum free IMDM media for 15 min prior to drop-wise addition to the cell culture. Following transfection, cells were incubated in serum-free media for 6 hours and were cultured overnight for an additional 16 hours with 2% fetal calf serum. IL-10 (10 ng/ml) was added 24 hours after transfection and following an additional 24 hr period, the cells were harvested and analyzed for CD14 expression by flow cytometry.

**Electrophoretic mobility shift assays (EMSA)**

EMSA were performed as per the standard technique and as described earlier [4;112]. Cells ($10^7$) were harvested in Tris-EDTA-saline buffer pH 7.8 and centrifuged at 200 x g for 5 min at 4°C. The cells were lysed for 10 min at 4°C with buffer A (10 mM of HEPES, 10 mM of KCl, 1.5 mM of $MgCl_2$, 0.5 mM of DTT, 0.5 mM of PMSF, pH 7.9) containing 0.1% Nonidet P-40. The lysates were centrifuged at 20,000 x g for 10 min at

4°C. The pellet containing the nuclei was suspended in buffer B (20 mM of HEPES, 420 mM of NaCl, 1.5 mM of $MgCl_2$, 0.2 mM of EDTA, and 25% glycerol) at 4° C for 15 min. Both buffers A and B contained the proteolytic inhibitors including DTT, PMSF, and spermidine at concentrations of 0.5 mM each, as well as 0.15 mM of spermine, and 5 μg/ml each of aprotinin, leupeptin, and pepstatin. The supernatant containing the nuclear proteins was collected and frozen at -80° C. Nuclear proteins (5 μg) were mixed for 20 min at room temperature with $^{32}$P-labeled STAT1 oligonucleotide probes, and the complexes were subjected to non-denaturing 5 % PAGE for 90 min. The oligonucleotide sequences corresponding to the STAT1 binding sites in the CD14 promoter was as follows: 5'-(TGC AAT ATT TCC AGG AAA GGA AGG)-3'. To illustrate specificity of nuclear factor binding for STAT1 probes, parallel EMSA reactions were incubated with 50 to 200 fold excess of cold unlabelled probe. Subsequently, supershift analyses were also performed to identify the bands specific for STAT1 transcription factor by using specific rabbit anti-STAT1 polyclonal antibodies (Santa Cruz). Briefly, nuclear extracts were incubated with the STAT1 oligonucleotides in the presence of anti-STAT1 antibodies or control antibodies at a final concentration of 0.5-2 μg. The bound and unbound $^{32}$p labelled oligonucleaotides were resolved by gel electrophoresis as described above. The gel was dried and exposed to Kodak X-ray films.

**Statistical analysis**

Means were compared using the two-tailed Student's t test. Results are expressed as mean ± standard error of the mean (SEM).

# CHAPTER 3:

# RESULTS

**LPS-induced CD14 expression is regulated by endogenously produced IL-10, in normal human monocytes**

In this study, I confirmed our laboratory earlier observations that stimulation of normal human monocytes with LPS enhanced CD14 expression (Fig. 3-1). It has been suggested that IL-10 is the major cytokine that regulates LPS-induced CD14 expression in LPS-stimulated human monocytes [109]. To determine whether endogenously produced IL-10 regulates LPS-induced CD14 expression, specific antibodies are employed for IL-10Rα (0.1 μg/ml). These antibodies are capable of neutralizing the biological activity of 10 ng/ml of IL-10, as described previously, and as recommended by the manufacturer. Anti-IL-10Rα antibodies inhibited the LPS-induced CD14 expression (Fig. 3-2).

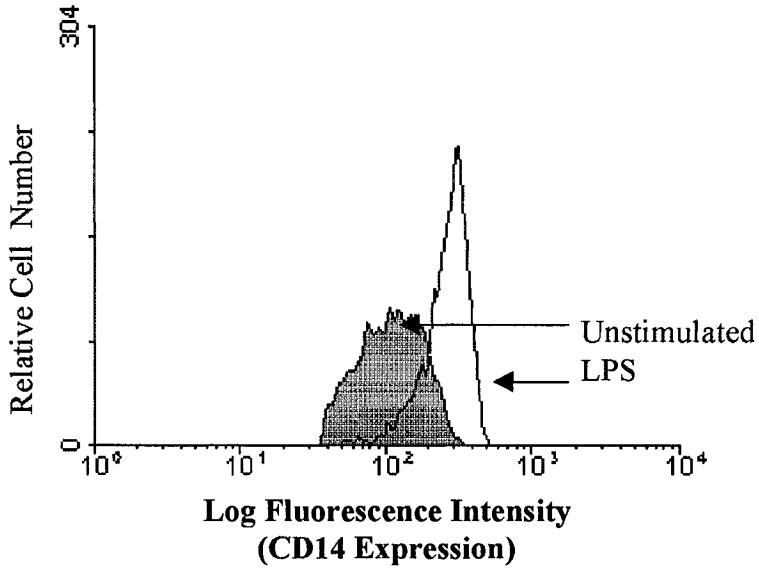
Our laboratory has also previously demonstrated that LPS-induced IL-10 production is regulated via the activation of p38 MAPKs [44]. To determine whether inhibition of IL-10 production by p38 inhibitor SB202190 will inhibit CD14 expression, LPS-induced monocyte CD14 expression and IL-10 production was investigated using p38 specific inhibitor SB202190. As shown previously [44], LPS strongly induced p38 phosphorylation in monocytes, which could be abolished by pre-treatment with the p38 inhibitor SB202190 (Fig. 3-3A). Prior treatment of monocytes with SB202190 inhibited CD14 expression in a dose dependent manner (Fig 3-3B). Furthermore, SB202190 and SB203580 significantly inhibited IL-10 production in a dose dependent manner (Fig 3-4A&B) whereas ERK MAPKs specific inhibitor did not (Fig. 3-4C). In addition, stimulation of normal monocytes with recombinant IL-10 enhanced CD14 expression.

Unstimulated cells expressed CD14 to considerable levels and IL-10 stimulation was able to further up-regulate its expression (Fig 3-5). Taken together, these observations clearly

**Fig. 3-1.** LPS induces CD14 expression in human monocytes

A. Purified normal human monocytes ($1\times10^6$ / ml) were stimulated with LPS (1 μg /ml) and then after 24 hours analyzed for CD14 expression by flow cytometry. The result shown is a representative histogram.
B. Purified normal human monocytes ($1\times10^6$ / ml) from five different individuals were cultured for 24 hours in the presence or absence of LPS (1μg / ml) and then analyzed for expression of CD14 by flow cytometry.

**A**


304
0
Relative Cell Number
$10^0$
$10^1$
$10^2$
$10^3$
$10^4$
Unstimulated
LPS
Log Fluorescence Intensity
(CD14 Expression)

**B**

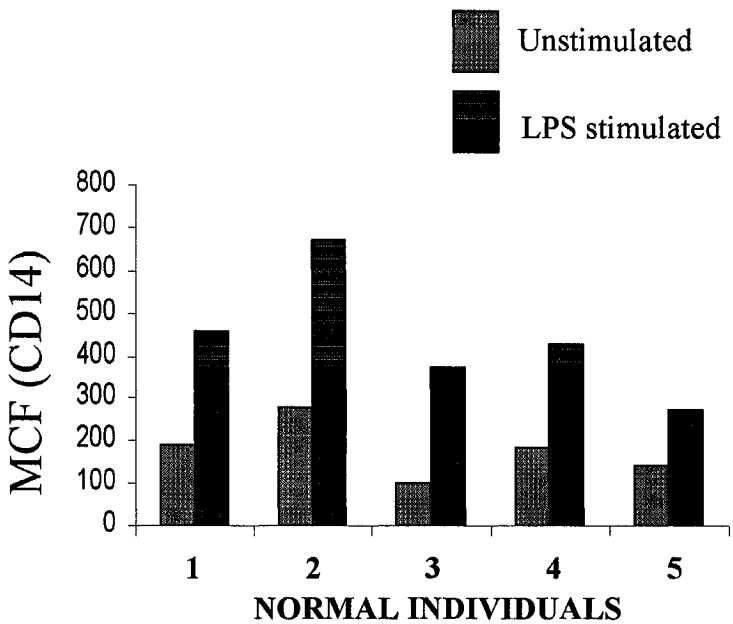

Unstimulated
LPS stimulated
MCF (CD14)
800
700
600
500
400
300
200
100
0
1
2
3
4
5
NORMAL INDIVIDUALS

**Fig. 3-2.** LPS induces CD14 expression in human monocytes via IL-10

Normal human monocytes were treated with anti-IL-10Rα (0.1 μg / ml) or with isotype matches control antibody followed by stimulation with LPS for 24 hours. Shaded histogram represents untreated cells. Bold line indicates LPS-stimulated cells in the presence of control antibody whereas broken line represents LPS-stimulated cells treated with anti-IL-10Rα antibody. The cells were stained with PE-labeled mouse anti human CD14 antibodies. CD14 expression was analyzed by flow cytometry. The experiment shown is a representative of three different experiments.

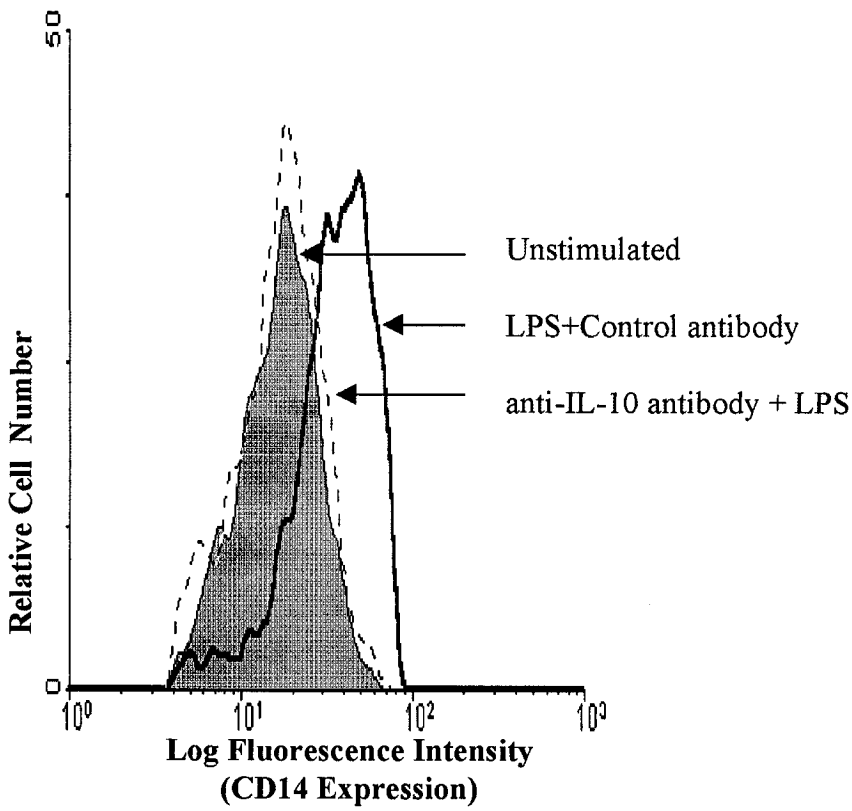
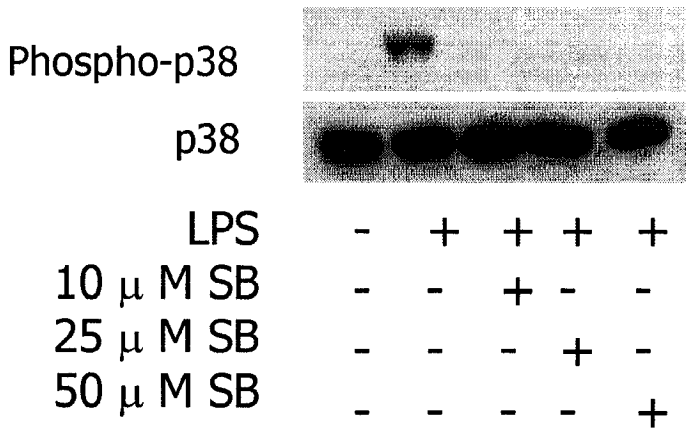

50
0
Relative Cell Number
$10^0$
$10^1$
$10^2$
$10^3$
Log Fluorescence Intensity
(CD14 Expression)
Unstimulated
LPS+Control antibody
anti-IL-10 antibody + LPS

**Fig. 3-3.** Role of p38 MAPK in LPS-induced CD14 expression in human monocytes

A. LPS stimulation induces P38 MAPK phosphorylation in purified normal human monocytes, which were pretreated ($1\times10^6$ / ml) with SB202190 at varying concentrations ranging from 0 to 50 μM for 2 hours prior to LPS (1 μg / ml) stimulation for 10 minutes. Crude protein extracts (50 μg) were subjected to SDS-PAGE followed by Western blot analysis. The membranes were blotted with anti-phospho-P38 antibodies, and to control for loading, the membranes were stripped and probed with anti-P38 antibodies.
B. The P38 MAPK inhibitor modulates LPS-induced CD14 expression. Purified normal human monocytes ($1\times10^6$ / ml) were pretreated with SB202190 at varying concentrations ranging from 0 to 50 μM for 2 hours prior to LPS (1 μg / ml) stimulation for 24 hours. CD14 expression was then analyzed by flow cytometry. The histogram shown is a representative of five different experiments performed.

**A**


Phospho-p38
p38
LPS - + + + +
10 μ M SB - - + - -
25 μ M SB - - - + -
50 μ M SB - - - - +

**B**

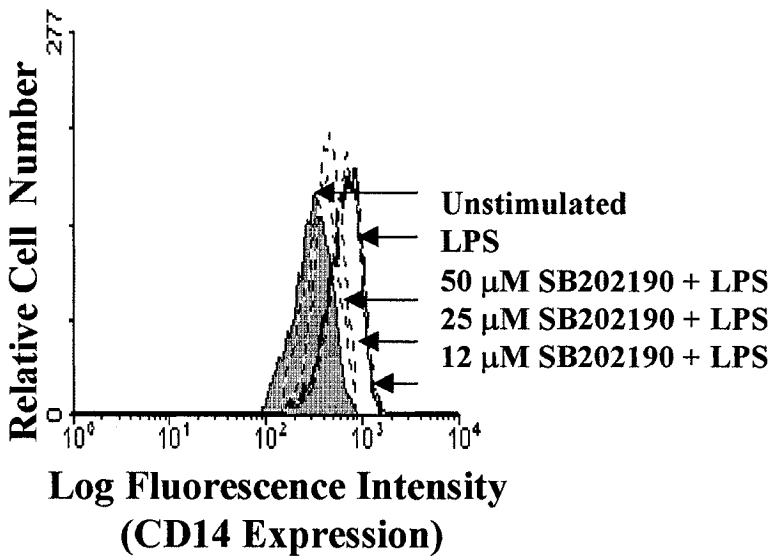

Relative Cell Number
277
0
$10^0$ $10^1$ $10^2$ $10^3$ $10^4$
Unstimulated
LPS
50 μM SB202190 + LPS
25 μM SB202190 + LPS
12 μM SB202190 + LPS
Log Fluorescence Intensity
(CD14 Expression)

**Fig. 3-4.** Role of p38 MAPK in LPS-induced IL-10 production in human monocytes

Purified normal human monocytes ($1\times10^6$ / ml) were pretreated with either SB202190 (A) or SB203580 (B) or PD98059 (C) at varying concentrations ranging from 0 to 50 μM for 2 hours. Supernatants were harvested and analyzed by ELISA for IL-10 production. The histograms shown are representative of five different experiments performed. ELISA results are represented as a mean ± SD of five experiments.

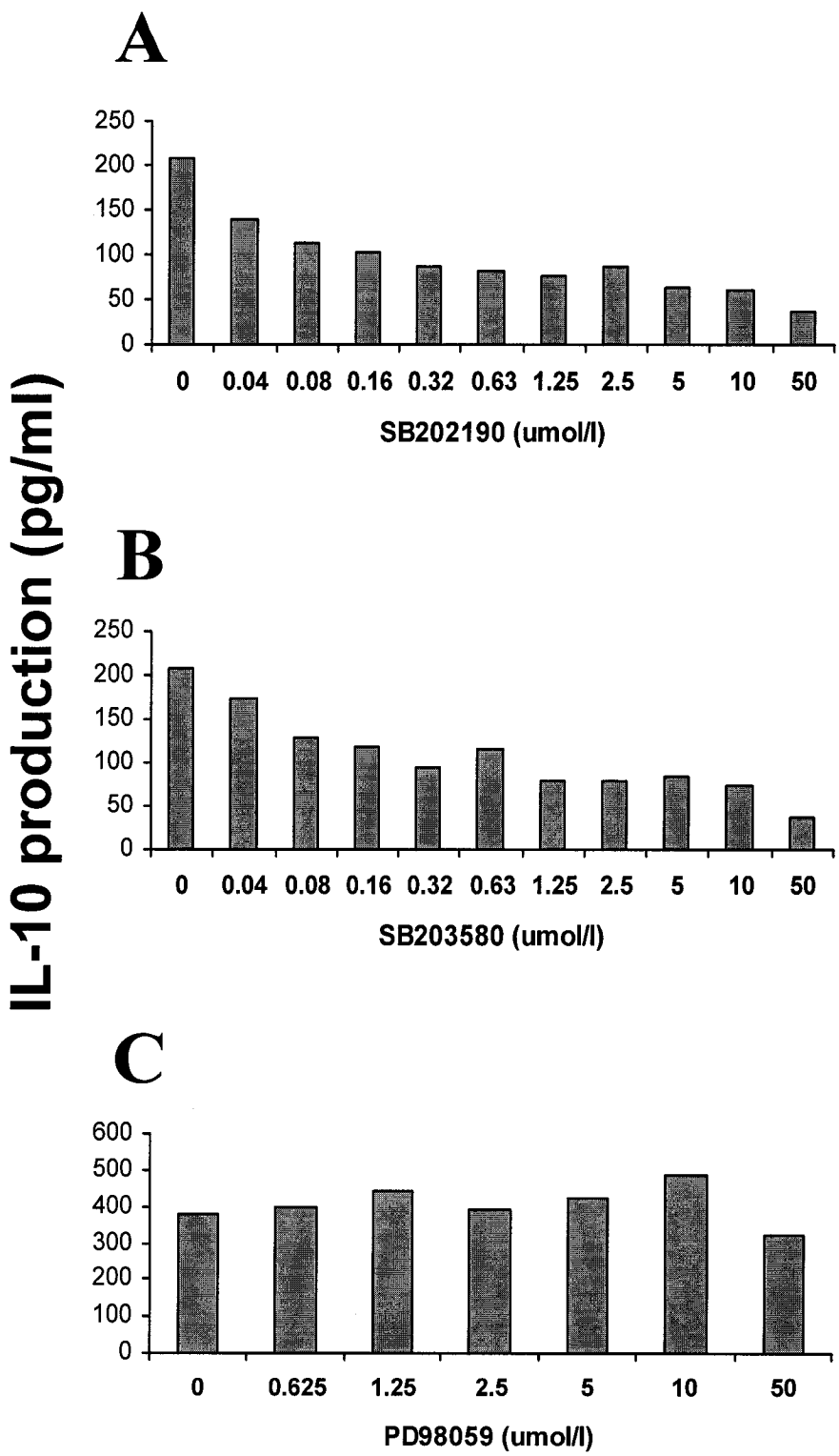
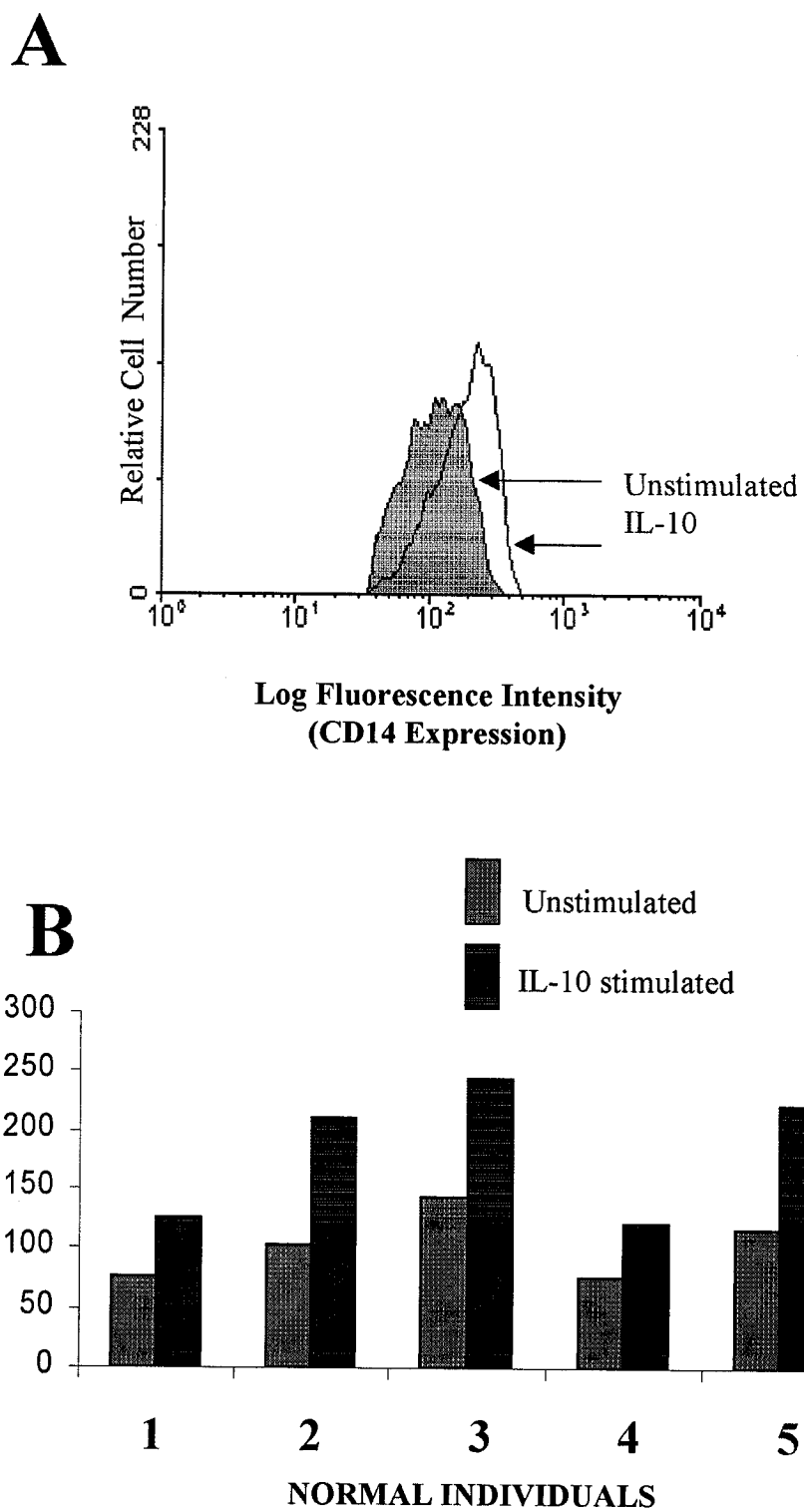

A
250
200
150
100
50
0
0
0.04
0.08
0.16
0.32
0.63
1.25
2.5
5
10
50
SB202190 (umol/l)
B
250
200
150
100
50
0
0
0.04
0.08
0.16
0.32
0.63
1.25
2.5
5
10
50
SB203580 (umol/l)
IL-10 production (pg/ml)
C
600
500
400
300
200
100
0
0
0.625
1.25
2.5
5
10
50
PD98059 (umol/l)

**Fig. 3-5.** IL-10 induces CD14 expression in human monocytes

A. Purified normal human monocytes ($1\times10^6$ / ml) were stimulated with rhIL-10 (10 ng / ml) and then after 24 hours analyzed for CD14 expression by flow cytometry. The result shown is a representative histogram.
B. Purified normal human monocytes ($1\times10^6$ / ml) from five different individuals were cultured for 24 hours in the presence or absence of rhIL-10 (10ng / ml) and then analyzed for expression of CD14 by flow cytometry.


A
228
Relative Cell Number
0
$10^0$
$10^1$
$10^2$
$10^3$
$10^4$
Unstimulated
IL-10
Log Fluorescence Intensity
(CD14 Expression)
B
Unstimulated
IL-10 stimulated
MCF (CD14)
300
250
200
150
100
50
0
1
2
3
4
5
NORMAL INDIVIDUALS

suggest that endogenously produced IL-10 following LPS stimulation plays an important role in LPS-induced CD14 expression.

**Role of MAP kinases in IL-10-induced upregulation of CD14 expression in normal human monocytes and HL-60 cells.**

To understand the molecular mechanism involved in the regulation of IL-10-induced CD14 expression in human monocytic cells, I wished to reproduce the stimulatory effects of IL-10 on CD14 expression in a model system involving human monocytic cell line. Therefore, a number of human monocytic cells such as THP-1, U937 and HL-60 for IL-10 responsiveness and mimic CD14 induction analyzed. Among these cells, HL-60 cells alone were found to be responsive to IL-10 and demonstrated CD14 up-regulation (Fig 3-6). It may be noted that HL-60 cells were heterogenous and only 5-10 % of cells responded to IL-10-stimulation depending on their state of cell growth and differentiation.

The MAPKs play a major role in LPS- and cytokine-mediated induction of several cell surface receptors. It was, therefore, of interest to identify the members of the MAPKs family that are involved in IL-10-induced regulation of CD14 expression in monocytic cells. At first, I investigated the involvement of p38 and ERK MAPKs by examining their activation following stimulation of both normal human monocytes (Fig. 3-7; 3-8) and HL-60 cells (Fig. 3-9) with IL-10. IL-10 enhanced the phosphorylation of ERK MAPKs in both monocytes (Fig. 3-8A) and HL-60 cells (Fig. 3-9A; 3-10) but had no effect on p38 and JNK phosphorylation. The role of ERK MAPKs has been studied through the use of specific kinase inhibitors. For example, PD98059 has been shown to

**Fig. 3-6.** IL-10 induces CD14 expression in HL-60 cells

A. HL-60 cells ($1\times10^6$ / ml) were stimulated with rhIL-10 (10 ng / ml) and then after 24 hours analyzed for CD14 expression by flow cytometry. The result shown is a representative histogram.
B. HL-60 cells ($1\times10^6$ / ml) were cultured for 24 hours in the presence or absence of rhIL-10 (10ng / ml) and then analyzed for expression of CD14 by flow cytometry.

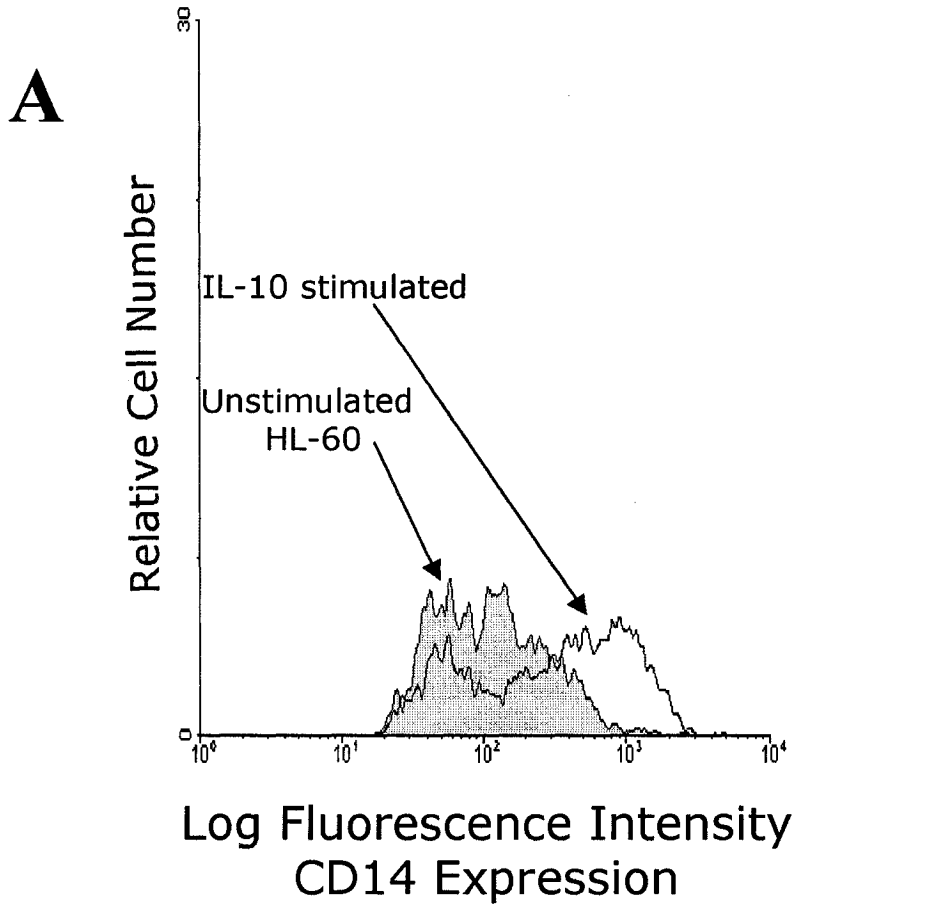
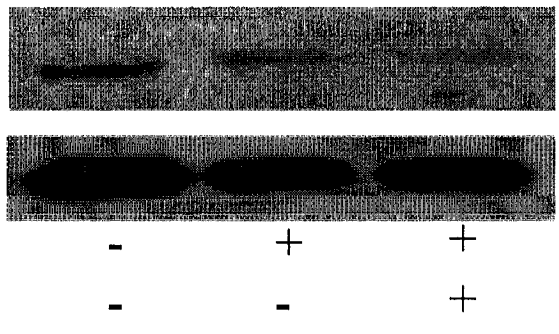

A
Relative Cell Number
IL-10 stimulated
Unstimulated
HL-60
Log Fluorescence Intensity
CD14 Expression
B
Unstimulated
IL-10 stimulated
MEAN CHANNEL
FLUORESCENCE (CD14)
300
250
200
150
100
50
0
1
2
3
4
5
HL-60 cell lines

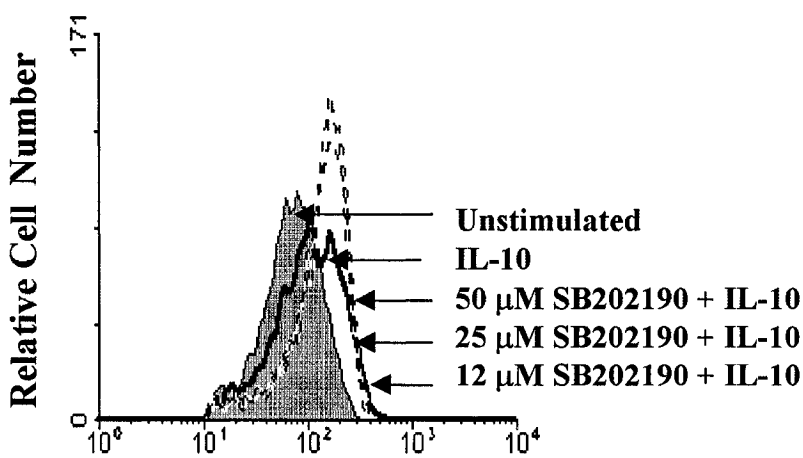
**Fig. 3-7.** Role of p38 MAPK in IL-10 mediated CD14 expression in human monocytes

A. IL-10 stimulation induces P38 MAPK phosphorylation in purified normal human monocytes, which were pretreated ($1\times10^6$/ml) with SB202190 at varying concentrations (0 to 50 μM) for 2 hours prior to IL-10 (10 ng / ml) stimulation for 10 minutes. Crude protein extracts (50 μg) were subjected to SDS-PAGE followed by Western blot analysis. The membranes were blotted with anti-phospho-P38 antibodies, and to control for loading, the membranes were stripped and probed with anti-P38 antibodies.
B. The P38 MAPK inhibitor doesn't modulate IL-10-induced CD14 expression. Purified normal human monocytes ($1\times10^6$/ml) were pretreated with SB202190 at varying concentrations (0 to 50 μM) for 2 hours prior to IL-10 (10 ng / ml) stimulation for 24 hours. CD14 expression was then analyzed by flow cytometry. The histogram shown is a representative of three different experiments performed.

**A**




**B**

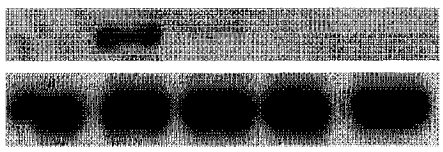



**Log Fluorescence Intensity**
**(CD14 Expression)**

**Fig. 3-8.** Role of ERK MAPKs in IL-10 mediated CD14 expression in human monocytes

A. IL-10 stimulation induces ERK MAPKs phosphorylation in purified normal human monocytes, which were pretreated ($1\times10^6$/ml) with PD98059 at varying concentrations ranging from 0 to 50 μM for 2 hours prior to IL-10 (10 ng/ml) stimulation for 30 minutes. Crude protein extracts (50 μg) were subjected to SDS-PAGE followed by Western blot analysis. The membranes were blotted with anti-phospho-ERK MAPKs antibodies, and to control for loading, the membranes were stripped and probed with anti-ERK MAPKs antibodies.
B. The ERK MAPKs inhibitor partially modulates IL-10-induced CD14 expression. Purified normal human monocytes ($1\times10^6$/ml) were pretreated with PD98059 at varying concentrations ranging from 0 to 50 μM for 2 hours prior to IL-10 (10 ng /ml) stimulation for 24 hours. CD14 expression was then analyzed by flow cytometry. The histogram shown is a representative of five different experiments performed.

**A**

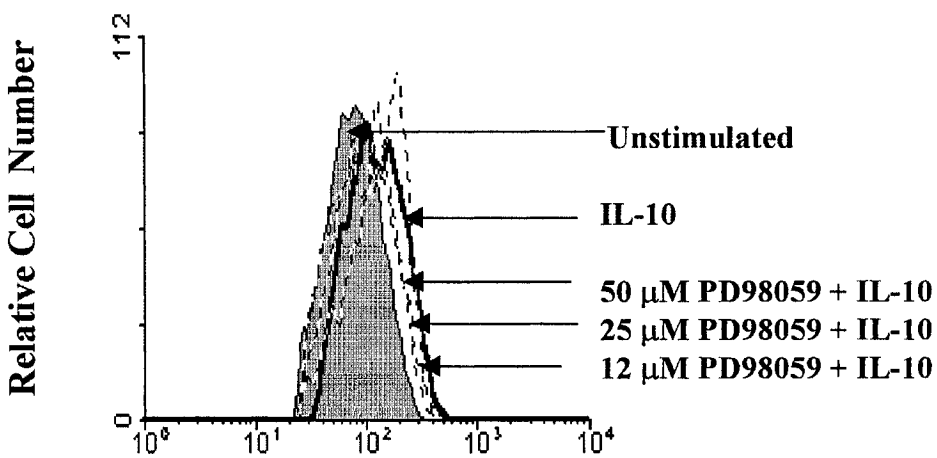


P-ERK

ERK

| | | | | | |
|---|---|---|---|---|---|
| - | + | + | + | + | IL-10 |
| - | - | + | - | - | PD 10 μM |
| - | - | - | + | - | PD 25 μM |
| - | - | - | - | + | PD 50 μM |

**B**




**Log Fluorescence Intensity**
**(CD14 Expression)**

**Fig. 3-9.** Role of ERK MAPKs in IL-10-induced CD14 expression in HL-60 cells

A. IL-10 stimulation induces ERK MAPKs phosphorylation in HL-60 cells, which were pretreated ($1\times10^6$ / ml) with PD98059 at varying concentrations ranging from 0 to 50 μM for 2 hours prior to IL-10 (10 ng/ml) stimulation for 30 minutes. Crude protein extracts (50 μg) were subjected to SDS-PAGE followed by Western blot analysis. The membranes were blotted with anti-phospho-ERK MAPKs antibodies, and to control for loading, the membranes were stripped and probed with anti-ERK MAPKs antibodies.
B. The ERK MAPKs inhibitor partially modulates IL-10-induced CD14 expression. HL-60 cells ($1\times10^6$ / ml) were pretreated with PD98059 at varying concentrations ranging from 0 to 50 μM for 2 hours prior to IL-10 (10 ng/ml) stimulation for 24 hours. CD14 expression was then analyzed by flow cytometry. The histogram shown is a representative of five different experiments performed.

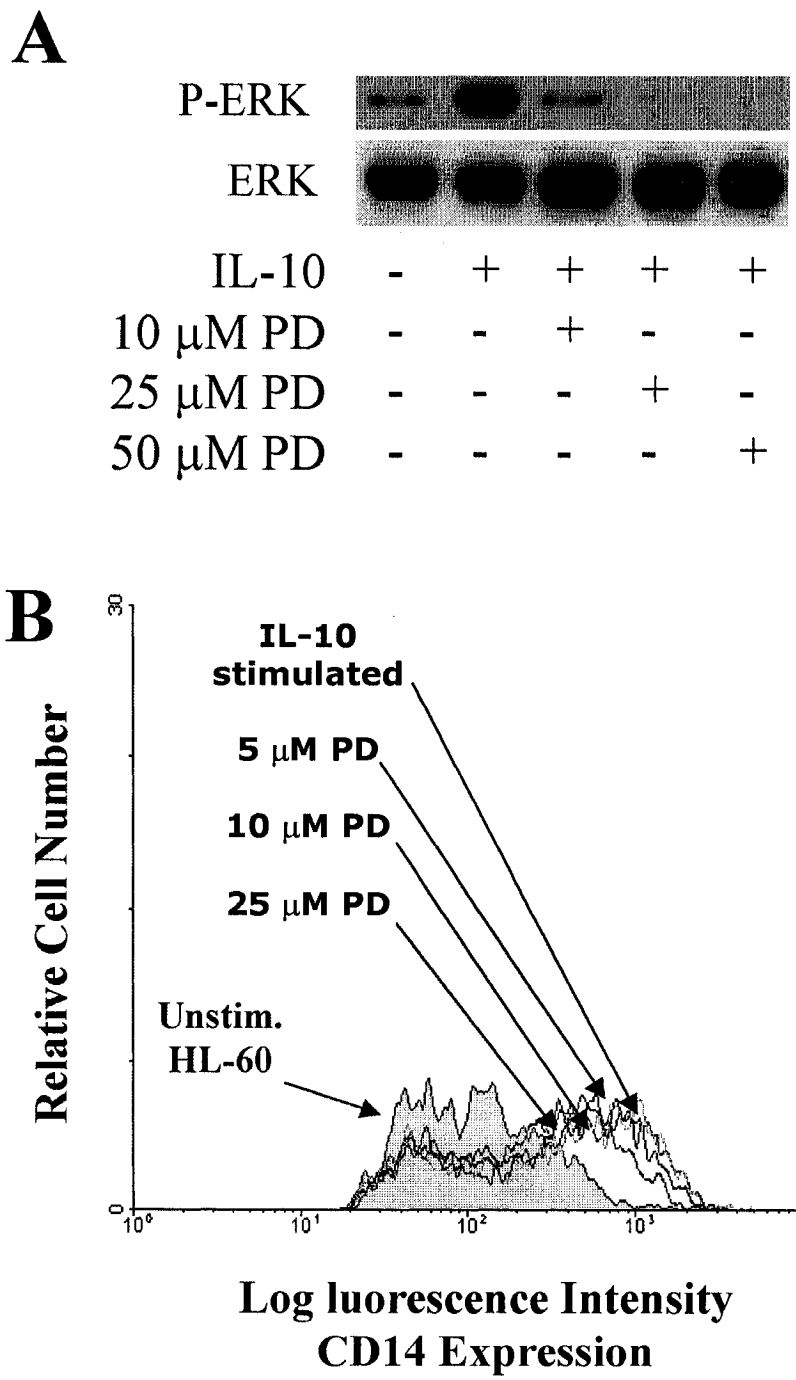
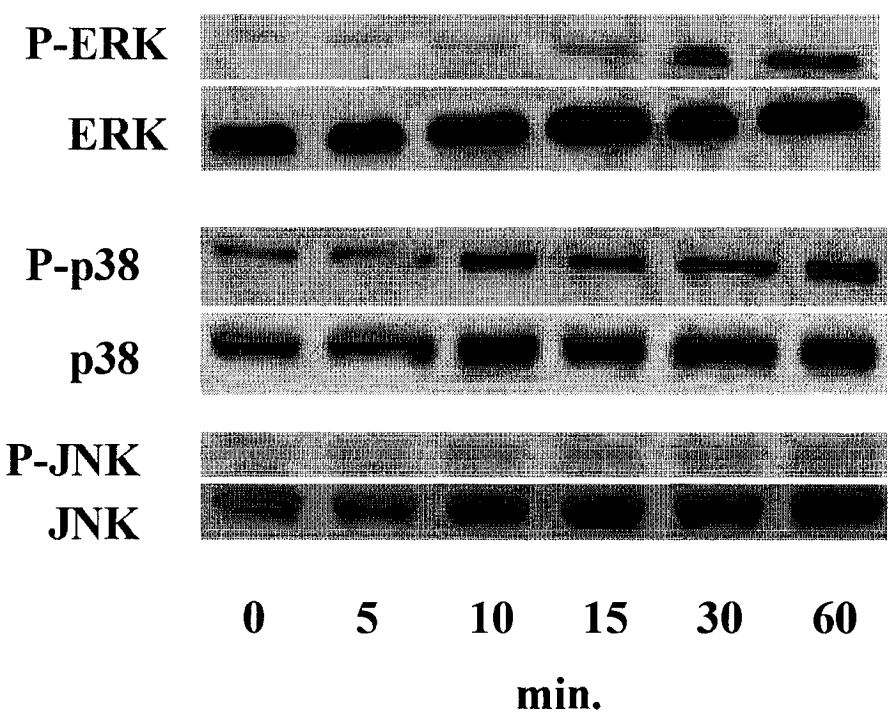

A
P-ERK
ERK
IL-10 - + + + +
10 μM PD - - + - -
25 μM PD - - - + -
50 μM PD - - - - +
B
Relative Cell Number
30
0
IL-10 stimulated
5 μM PD
10 μM PD
25 μM PD
Unstim. HL-60
10⁰ 10¹ 10² 10³ 10⁴
Log luorescence Intensity
CD14 Expression

**Fig. 3-10.** IL-10 induces selectively the activation of ERK MAPKs in HL-60 cells

HL-60 cells ($1\times10^6$ / ml) were stimulated with rhIL-10 (10 ng/ml) in a time course experiment from 0 to 60 minutes. Crude protein extracts (50 μg) were subjected to SDS-PAGE followed by Western blot analysis. The membranes were blotted with anti-phospho-p42, anti-phospho-p38, and anti-phospho-JNK MAPKs antibodies. To control for loading, the membranes were stripped and probed with anti-p42, anti-p38, and anti-JNK antibodies, respectively.


P-ERK
ERK
P-p38
p38
P-JNK
JNK
0
5
10
15
30
60
min.

specifically inhibit ERK MAPKs mediated signaling [4;113]. To confirm that in this system that PD98059 inhibited the phosphorylation of ERK MAPKs, freshly isolated monocytes and HL-60 cells were pretreated with these inhibitors for 2 hours followed by stimulation with IL-10 for 10 minutes in monocytes and 30 minutes in HL-60 cells. Prior treatment of monocytes (Fig. 3-8A) and HL-60 cells (Fig. 3-9A) with PD98059 resulted in the inhibition of IL-10-induced ERK MAPKs phosphorylation in a dose-dependent manner.

To determine the role of ERK MAPKs in IL-10-induced CD14 expression, monocytes and HL-60 cells were pretreated with PD98059 for 2 hours before IL-10 stimulation for 24 hours followed by analysis for CD14 expression by flow cytometry. PD98059 slightly inhibited CD14 expression in a dose dependent manner in monocytes (MCF from 347 to 320) (Fig. 3-8B) and HL-60 cells (MCF from 248 to 214)(Fig. 3-9B).

**Synergistic action of the PI3K and ERK MAPKs pathways in the up-regulation of CD14 by IL-10**

The above observations suggested that signaling molecules other than ERK MAPKs either independently or in synergy with the ERK MAPKs regulates IL-10-induced CD14 expression. IL-10 has been shown to activate the PI3K pathway [90]. To investigate the role of PI3K, I employed PI3K-specific inhibitor, LY294002. First, I determined whether IL-10 can induce the activation of Akt in our cell system and if so whether LY294002 can inhibit the activation of Akt in both normal monocytes and HL-60 cells. IL-10-induced Akt activation was determined by stimulating cells with IL-10 for varying periods of time followed by analysis of Akt activation by employing anti-phospho-Akt antibodies with Western blot analysis. Results show that IL-10 induced the

activation of Akt in both monocytes (Fig. 3-11A) and HL-60 cells (Fig. 3-11B). Maximum Akt phosphorylation was observed at 60 minutes post-stimulation with IL-10 of normal monocytes and HL-60 cells. Similarly, LY294002 inhibited IL-10-induced Akt phosphorylation in a dose dependent manner in both normal monocytes and HL-60 cells (Fig. 3-12A; 3-13A).

To understand the role of PI3K in IL-10-induced CD14 expression, both normal monocytes and HL-60 cells were treated with LY294002 for 2 hours prior to stimulation with IL-10 for 24 hours followed by analysis of CD14 expression by flow cytometry. Results show that treatment of normal monocytes (Fig. 3-12B) and HL-60 cells (Fig. 3-13B) with increasing concentration of LY294002 partially inhibited IL-10-induced CD14 expression. Since partial inhibition of CD14 expression was observed with both ERK MAPKs and PI3K specific inhibitors, I hypothesized that ERK MAPKs and PI3K may synergistically regulate IL-10-induced CD14 expression. For this, both monocytes and HL-60 cells were pretreated with varying concentrations of either LY294002 alone or in combination with PD98059 for 2 hours prior to stimulation with IL-10 followed by analysis of Akt phosphorylation and CD14 expression. Interestingly, the combination of ERK MAPKs and PI3K inhibitors completely abolished the increase in CD14 expression induced by IL-10, suggestive of a synergistic effect between these two pathways in both human monocytes (Fig. 3-14) and HL-60 cells (Fig. 3-15; 3-16).

**Fig. 3-11.** IL-10 induces Akt phosphorylation in human monocytes and HL-60 cells

A. IL-10 induces Akt phosphorylation in purified normal human monocytes ($1\times10^6$ /ml). The cells were stimulated with rhIL-10 (10 ng / ml) in a kinetic experiment from 0 to 60 minutes.
B. IL-10 induces Akt phosphorylation in HL-60 cells ($1\times10^6$/ml). The cells were stimulated with rhIL-10 (10 ng/ml) in a kinetic experiment from 0 to 60 minutes. The cell extracts were analyzed for Akt phosphorylation by Western blot analysis as described in materials and methods. The results shown are representative of three experiments performed.

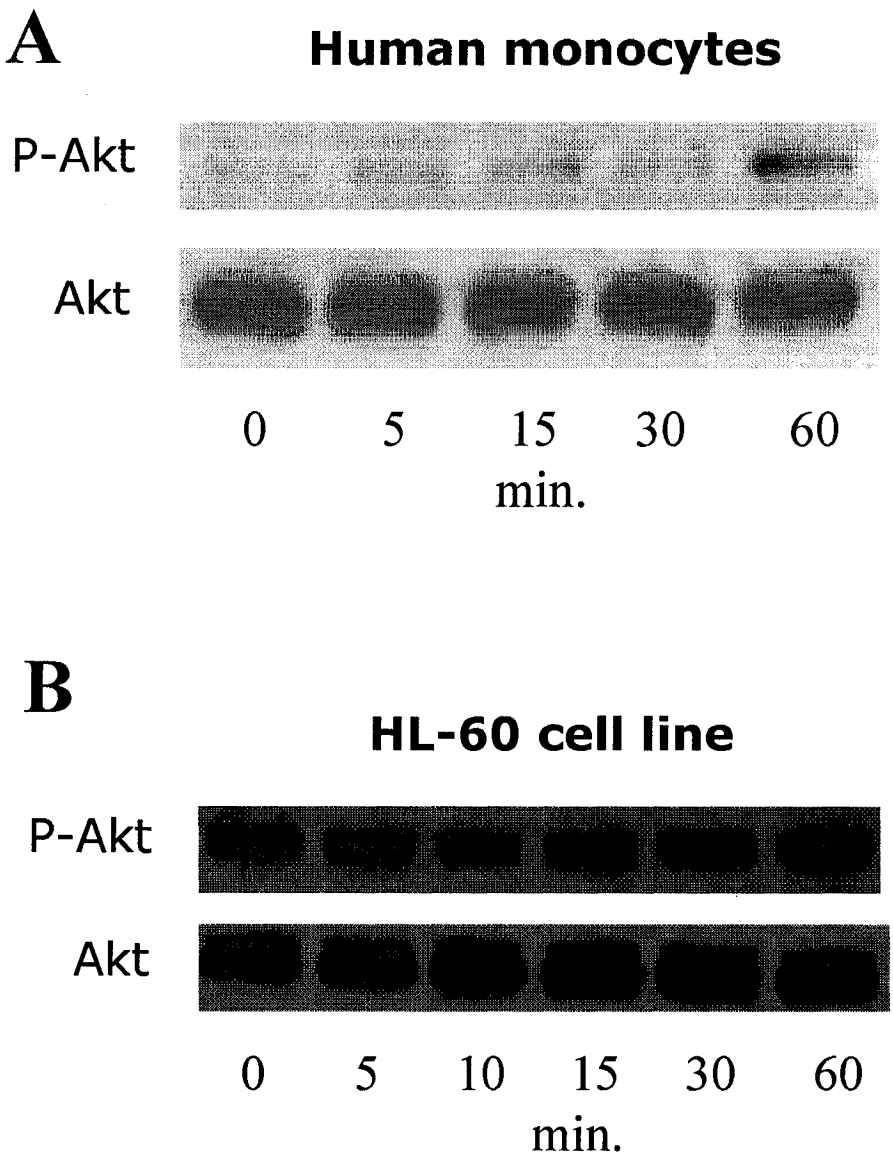
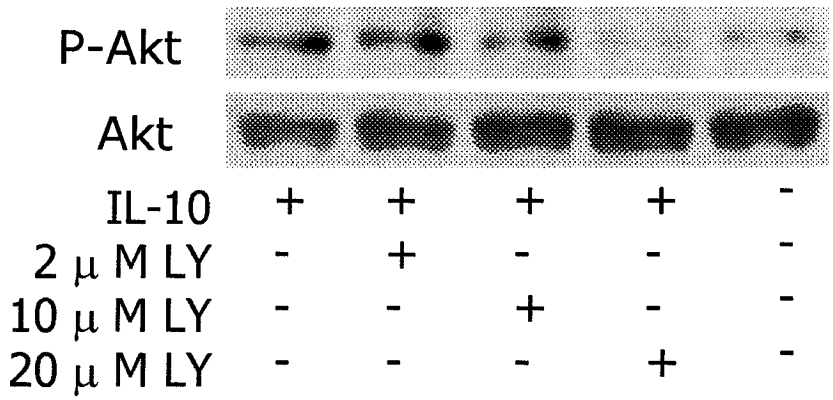

A
Human monocytes
P-Akt
Akt
0
5
15
30
60
min.
B
HL-60 cell line
P-Akt
Akt
0
5
10
15
30
60
min.

**Fig. 3-12.** Role of PI3K in IL-10 mediated CD14 expression in human monocytes

A. IL-10 stimulation induces Akt phosphorylation (down stream protein kinase of PI3K signaling pathway) in purified normal human monocytes, which were pretreated ($1\times10^6$/ml) with LY294002 at varying concentrations ranging from 0 to 20 μM for 2 hours prior to IL-10 (10 ng/ml) stimulation for 60 minutes. Crude protein extracts (50 μg) were subjected to SDS-PAGE followed by Western blot analysis. The membranes were blotted with anti-phospho-Akt antibodies, and to control for loading, the membranes were stripped and probed with anti-Akt antibodies.
B. The PI3K inhibitor partially modulates IL-10-induced CD14 expression. Purified normal human monocytes ($1\times10^6$ /ml) were pretreated with LY294002 at varying concentrations ranging from 0 to 10 μM for 2 hours prior to IL-10 (10 ng/ml) stimulation for 24 hours. CD14 expression was then analyzed by flow cytometry. The histogram shown is a representative of four different experiments performed.

**A**


P-Akt
Akt
IL-10 + + + + -
2 μ M LY - + - - -
10 μ M LY - - + - -
20 μ M LY - - - + -

**B**

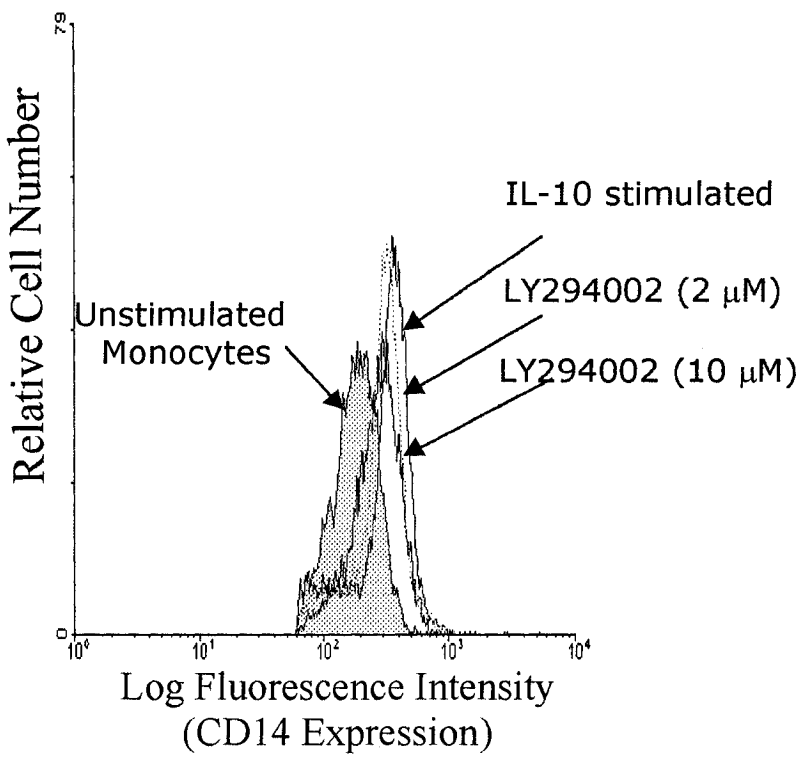

Relative Cell Number
79
0
IL-10 stimulated
LY294002 (2 μM)
LY294002 (10 μM)
Unstimulated Monocytes
10⁰ 10¹ 10² 10³ 10⁴
Log Fluorescence Intensity
(CD14 Expression)

**Fig. 3-13.** PI3K partially regulates IL-10-induced CD14 expression in HL-60 cells

A. IL-10 stimulation induces Akt phosphorylation (down stream protein kinase of PI3K signaling pathway) in HL-60 cells, which were pretreated ($1\times10^6$/ml) with LY294002 at varying concentrations ranging from 0 to 10 μM for 2 hours prior to IL-10 (10 ng/ml) stimulation for 15 minutes. Crude protein extracts (50 μg) were subjected to SDS-PAGE followed by Western blot analysis. The membranes were blotted with anti-phospho-Akt antibodies, and to control for loading, the membranes were stripped and probed with anti-Akt antibodies.
B. The PI3K inhibitor partially modulates IL-10-induced CD14 expression. HL-60 cells ($1\times10^6$/ml) were pretreated with LY294002 at varying concentrations ranging from 0 to 10 μM for 2 hours prior to IL-10 (10ng/ml) stimulation for 24 hours. CD14 expression was then analyzed by flow cytometry. The histogram shown is a representative of four different experiments performed.

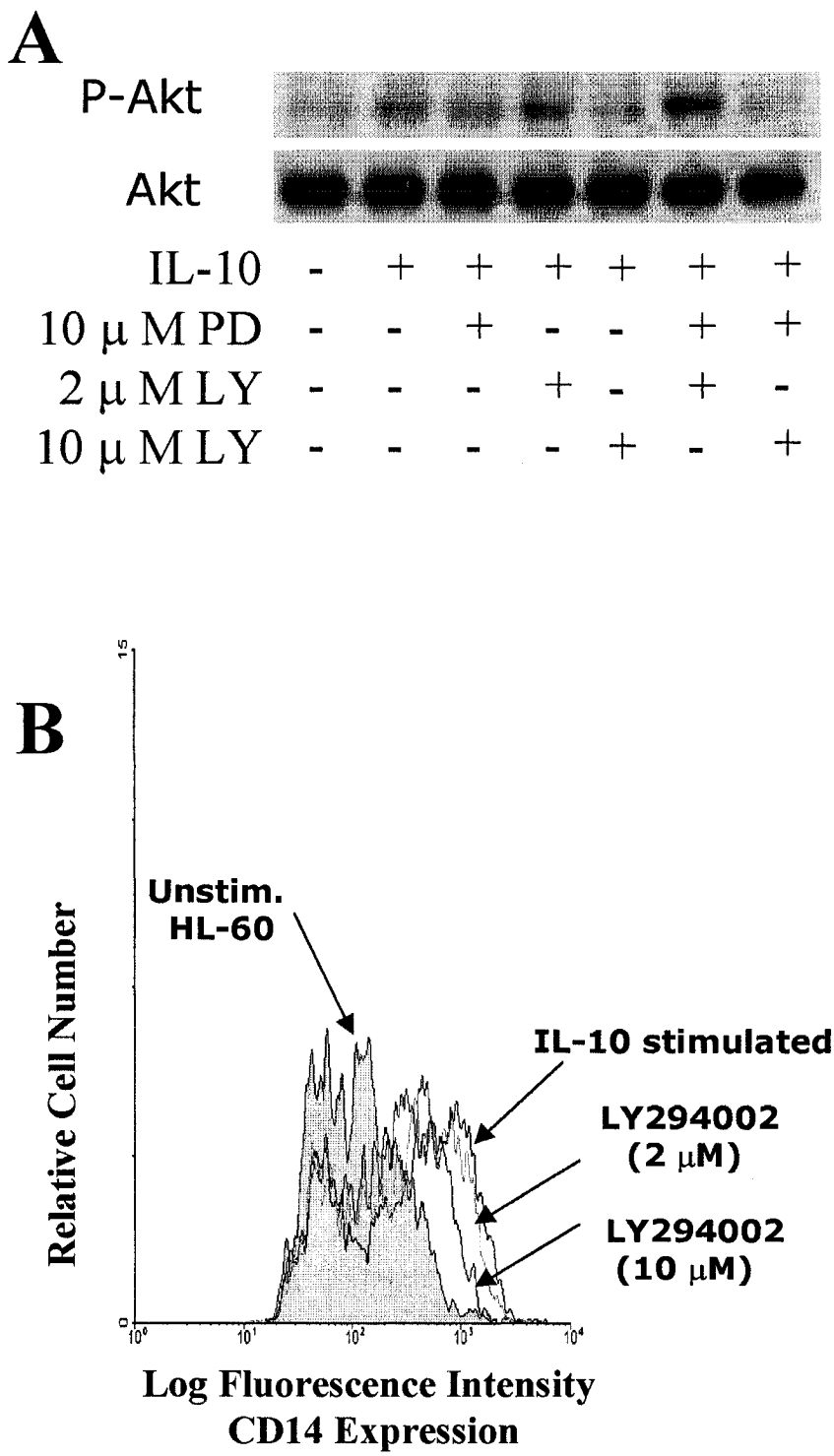
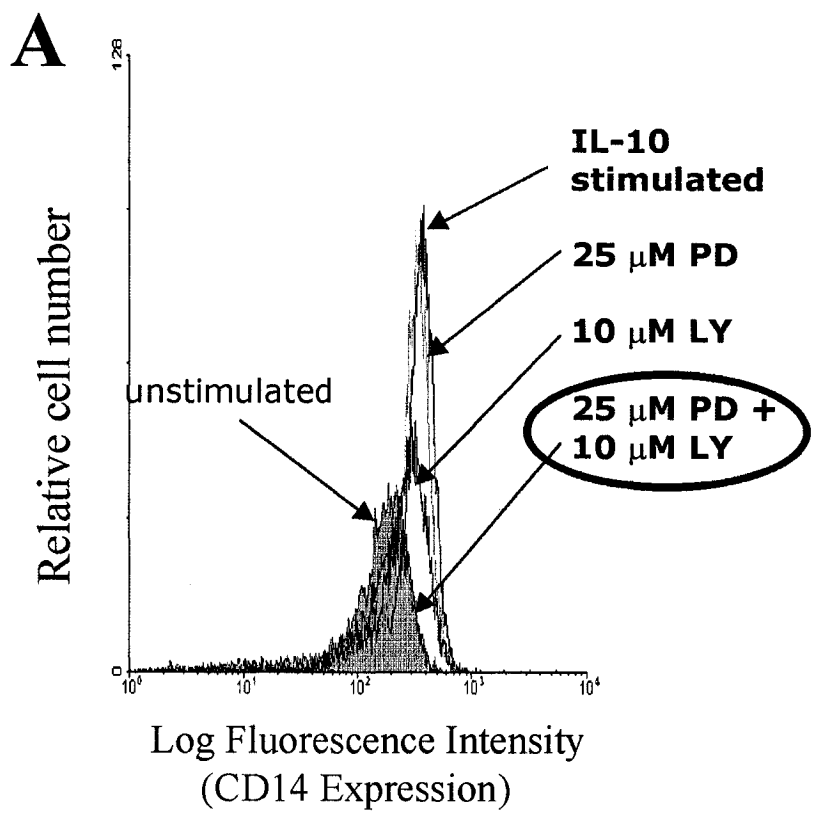

A
P-Akt
Akt
IL-10 - + + + + + +
10 μM PD - - + - - + +
2 μM LY - - - + - + -
10 μM LY - - - - + - +
B
Relative Cell Number
Unstim. HL-60
IL-10 stimulated
LY294002 (2 μM)
LY294002 (10 μM)
Log Fluorescence Intensity
CD14 Expression

**Fig. 3-14.** PI3K and ERK MAPKs synergistically regulate IL-10-induced CD14 expression in human monocytes

A. The ERK MAPKs and PI3K inhibitors synergistically inhibit IL-10-induced CD14 expression. Purified normal human monocytes ($1\times10^6$ / ml) were pretreated with 25 μM PD98059, 10 μM LY294002 alone, and in combination of them for 2 hours prior to IL-10 (10 ng / ml) stimulation for 24 hours. CD14 expression was then analyzed by flow cytometry. The histogram shown is a representative of three different experiments performed.
B. Results shown are the MCF data are represented as a mean ± SD of three different experiments performed.


A
Relative cell number
IL-10 stimulated
25 μM PD
10 μM LY
unstimulated
25 μM PD + 10 μM LY
Log Fluorescence Intensity
(CD14 Expression)

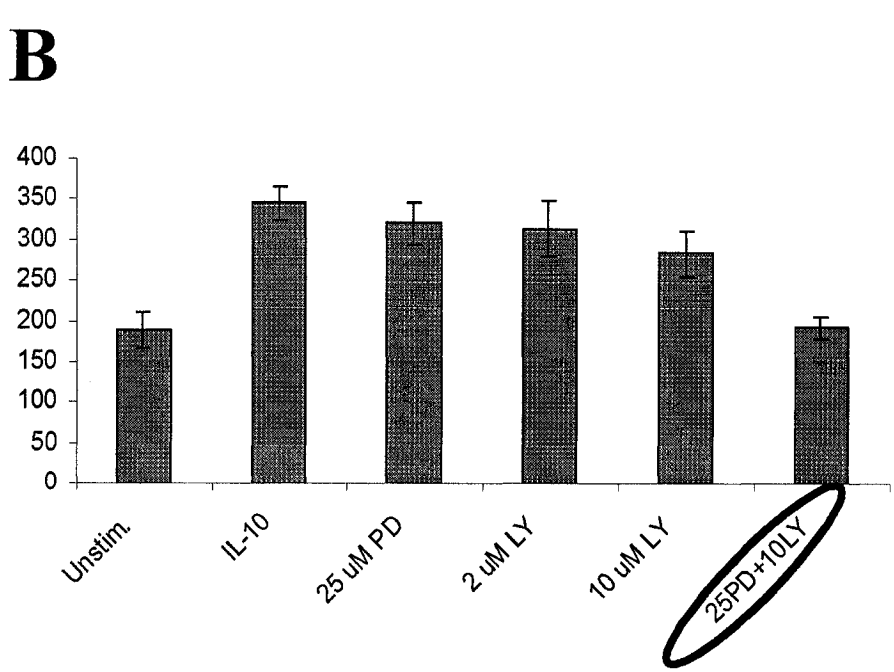

B
400
350
300
250
200
150
100
50
0
Unstim.
IL-10
25 uM PD
2 uM LY
10 uM LY
25PD+10LY

**Fig. 3-15.** PI3K and ERK MAPKs synergistically regulate IL-10-induced CD14 expression in HL-60 cells

A. The effect of different concentration of PD98059 and LY294002 combination on IL-10-induced CD14 expression in HL-60 cells. HL-60 cells ($1\times10^6$ / ml) were pretreated with 10 μM PD98059, 2 and 10 μM LY294002 alone, or in combination of them for 2 hours prior to IL-10 (10 ng / ml) stimulation for 24 hours. CD14 expression was then analyzed by flow cytometry. The histogram shown is a representative of six different experiments performed.

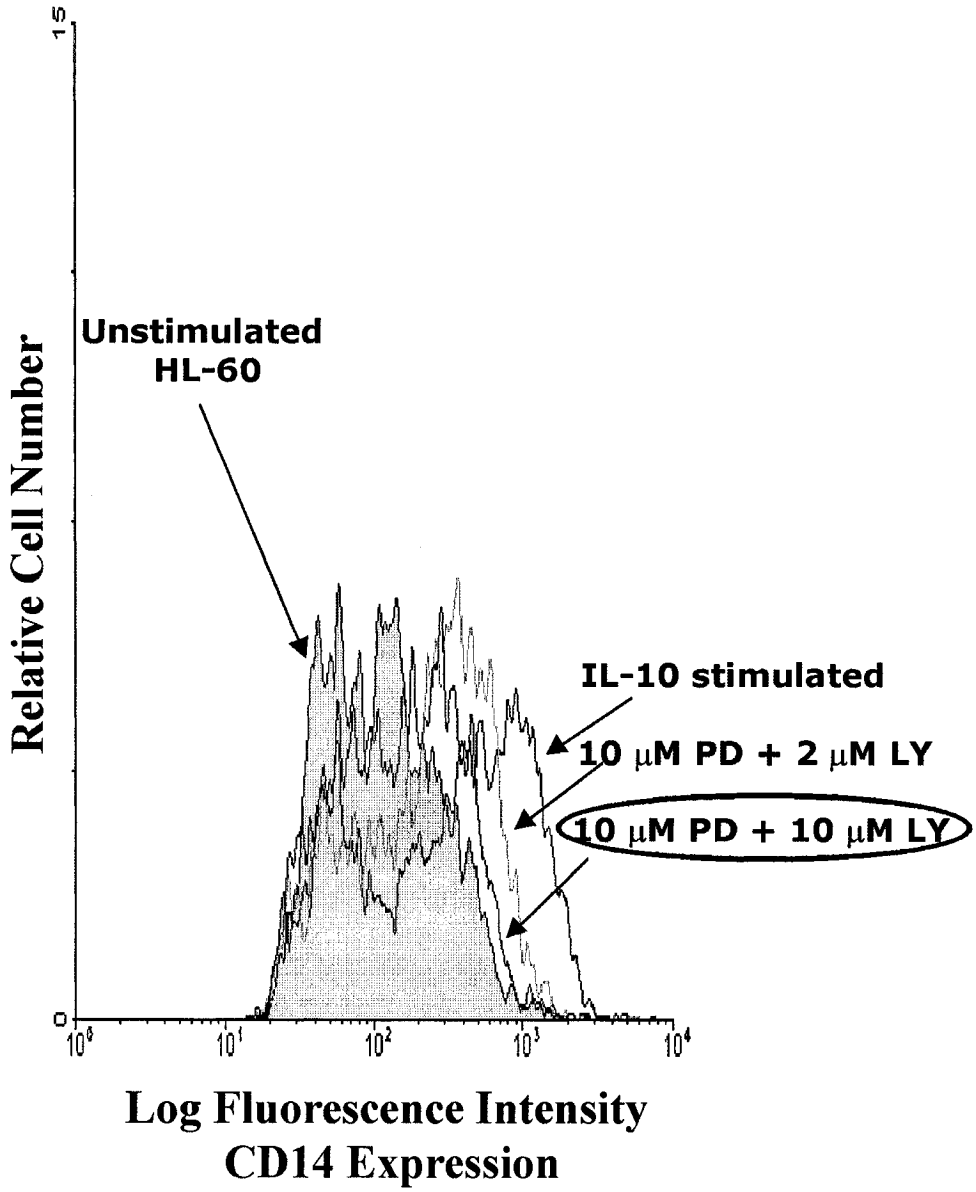
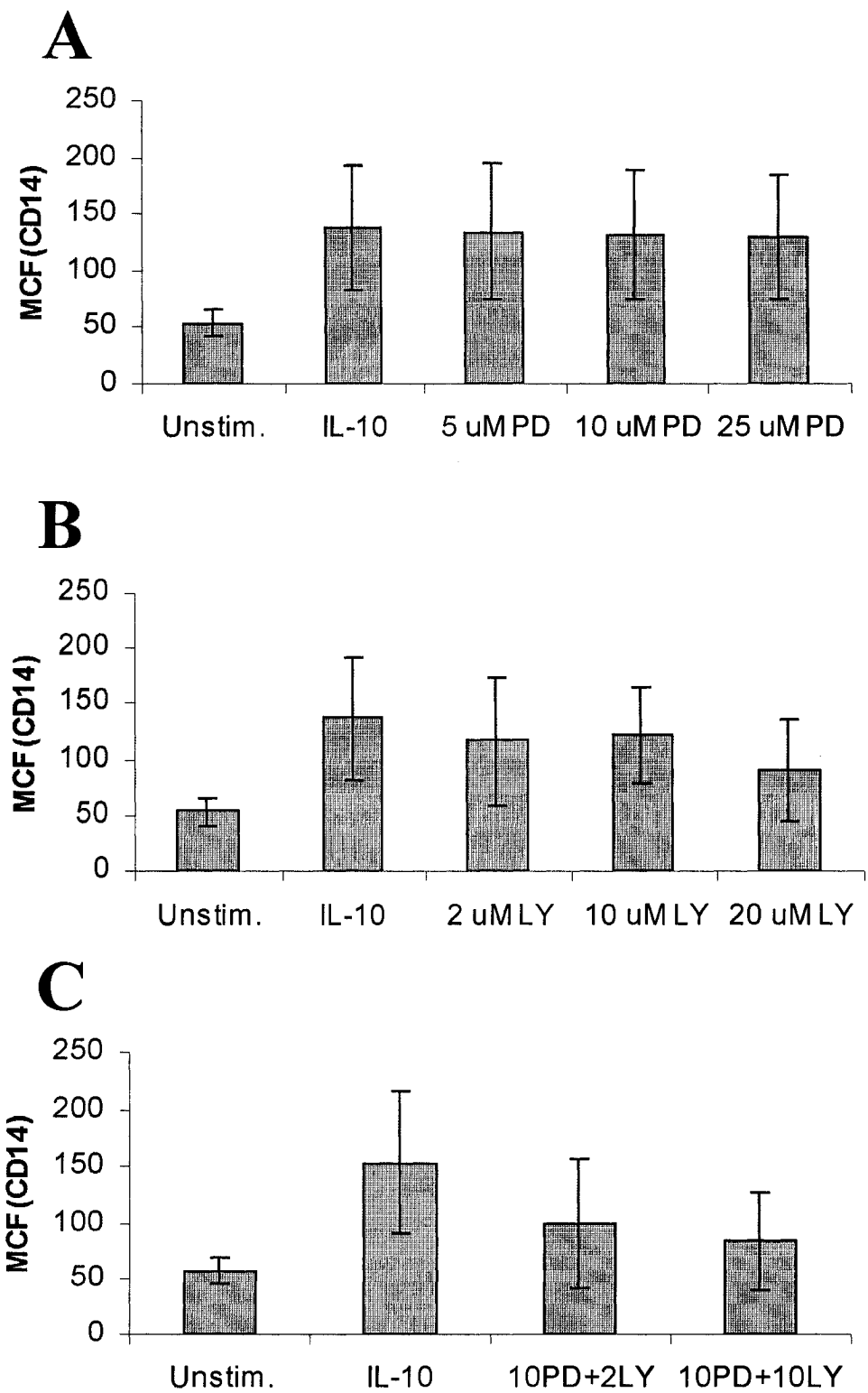

Relative Cell Number
15
0
Unstimulated
HL-60
IL-10 stimulated
10 μM PD + 2 μM LY
10 μM PD + 10 μM LY
10⁰
10¹
10²
10³
10⁴
Log Fluorescence Intensity
CD14 Expression

**Fig. 3-16.** PI3K and ERK MAPKs synergistically regulate IL-10-induced CD14 expression in HL-60 cells

Effect of ERK MAPKs and PI3K inhibitors on IL-10-induced CD14 expression in HL-60 cells ($1\times10^6$ / ml) which were treated with various concentrations of either PD98059 (A) or LY294002 (B) alone or in combination (C) for 2 hours prior to stimulation with rhIL-10 (10 ng / ml). After 24 hours, cells were analyzed by flow cytometry for the expression of CD14. MCF data are represented as a mean ± SD of six different experiments performed.


A
250
200
150
100
50
0
MCF(CD14)
Unstim.
IL-10
5 uM PD
10 uM PD
25 uM PD
B
250
200
150
100
50
0
MCF(CD14)
Unstim.
IL-10
2 uM LY
10 uM LY
20 uM LY
C
250
200
150
100
50
0
MCF(CD14)
Unstim.
IL-10
10PD+2LY
10PD+10LY

**IL-10-induced CD14 expression is not regulated via the activation of STAT3**

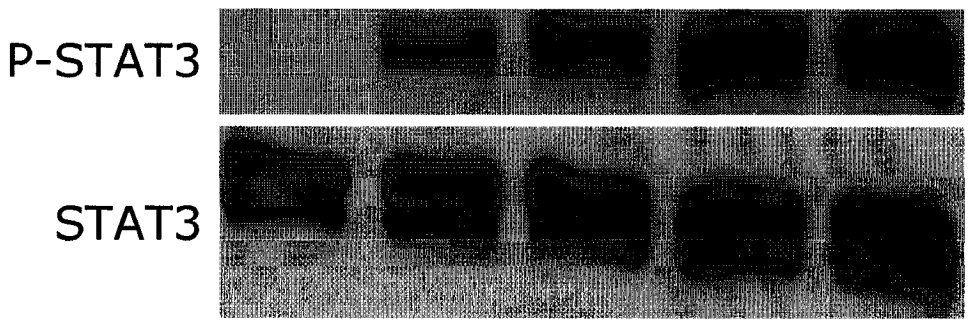
IL-10 has been shown to regulate its effects primarily through the activation of STAT3 [95;114;115]. Therefore, it was of interest to determine whether IL-10-induced CD14 expression is regulated independently by STAT3 activation or through the ERK/PI3K mediated activation of STAT3. To determine if IL-10-induced STAT3 activation is mediated through the activation of PI3K and/or ERK MAPKs, both normal monocytes as well as HL-60 cells were treated with various concentrations of PD98059 or LY294002 either alone or in combination with varying concentrations of LY294002 and PD98059 for 2 hours prior to stimulation with IL-10 following which cells were analyzed for STAT3 phosphorylation. Results show that IL-10 induced the phosphorylation of STAT3 in both normal monocytes (Fig. 3-17A) and HL-60 cells (Fig. 3-17B) and PD98059 either alone or in combination with LY294002 at any concentration did not down-regulate the phosphorylation of STAT3 in both normal monocytic cells (Fig. 3-18) as well as HL-60 cells (Fig. 3-19). To determine the involvement of STAT3 in IL-10-induced CD14 expression, I employed a specific inhibitor of STAT3 (PYpLKTK). HL-60 cells were treated with varying concentrations of STAT3 inhibitor for 2 hours prior to the stimulation with IL-10. Analysis of CD14 expression after IL-10 stimulation revealed that STAT3 inhibitor did not influence CD14 expression at any concentration (Fig. 3-20).

**Fig. 3-17**. IL-10 induces STAT3 phosphorylation in human monocytes and HL-60 cells

IL-10 induces STAT3 phosphorylation in purified normal human monocytes (A) ($1\times10^6$ / ml) and HL-60 cells (B). The cells were stimulated with rhIL-10 (10 ng / ml) in a kinetic experiment from 0 to 60 minutes. The cell extracts were analyzed for STAT3 phosphorylation by Western blot analysis as described in materials and methods.

**A**

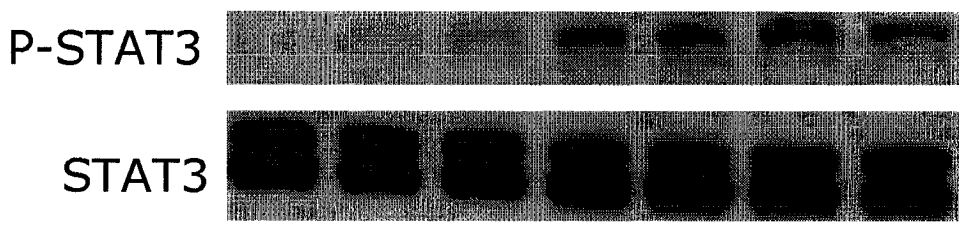
**Human Monocytes**




**0 5 15 30 60**
**min.**

**B**

**HL-60 cells**




**0 5 10 15 30 60 120**
**min.**

**Fig. 3-18.** ERK MAPKs and PI3K specific inhibitors do not inhibit STAT3 activation in human monocytes

A. Cells ($1\times10^6$ / ml) were pretreated with PD98059 at varying concentrations ranging from 0 to 50 μM for 2 hours prior to IL-10 (10 ng / ml) stimulation for 30 minutes and 60 minutes. Crude protein extracts (50 μg) were subjected to SDS-PAGE followed by Western blot analysis. The membranes were blotted with anti-phospho-STAT3 (P-STAT3) antibodies, and to control for loading, the membranes were stripped and probed with anti-STAT3 (STAT3) antibodies. The Western blot shown is a representative of four different experiments.
B. Cells ($1\times10^6$ / ml) were pretreated with either LY294002 or PD98059 at varying concentrations ranging from 0 to 25 μM for 2 hours prior to IL-10 (10 ng / ml) stimulation for 30 minutes. STAT3 phosphorylation was determined as described above. The Western blot shown is a representative of three different experiments.

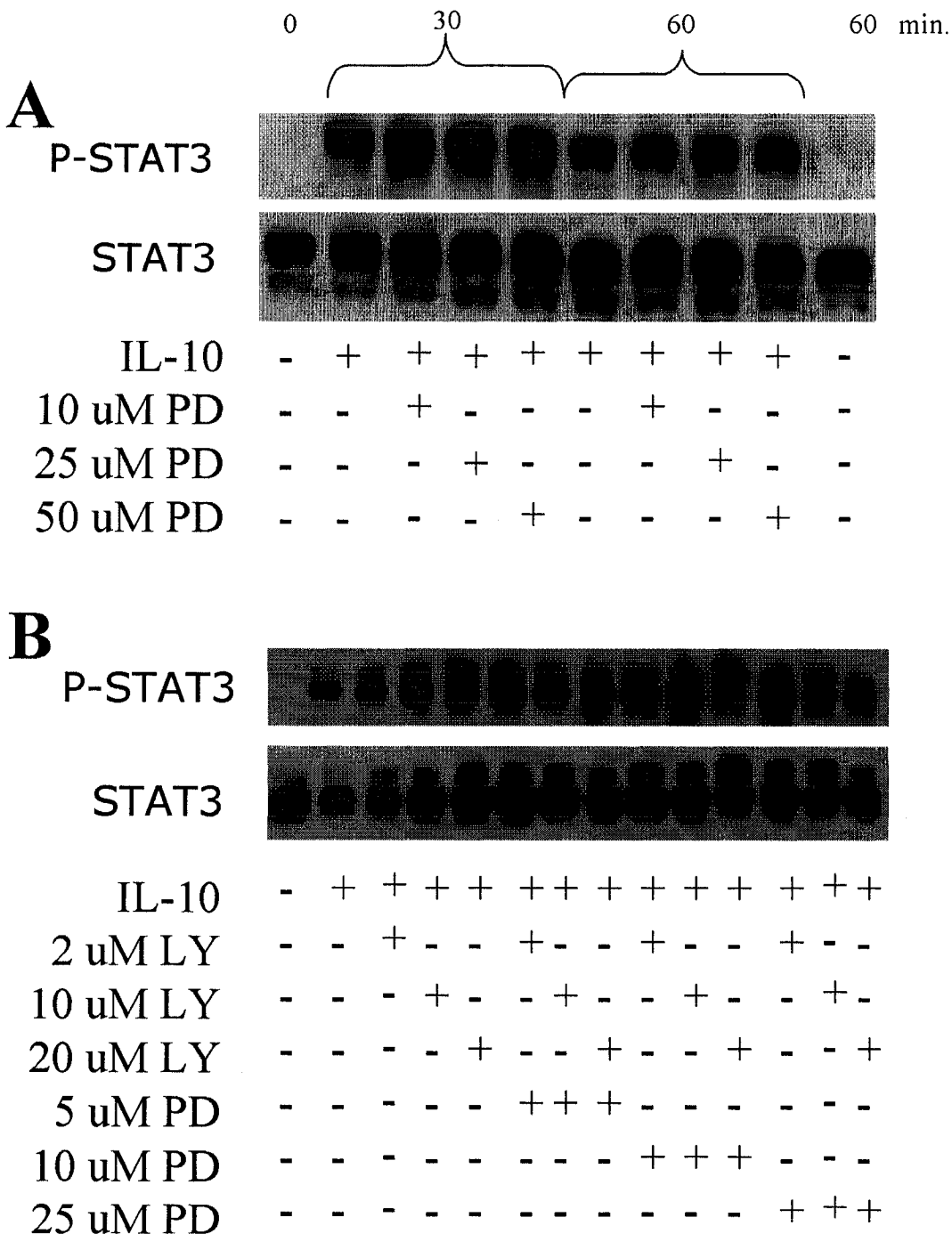

A
0
30
60
60 min.
P-STAT3
STAT3
IL-10 - + + + + + + + + -
10 uM PD - - + - - - + - - -
25 uM PD - - - + - - - + - -
50 uM PD - - - - + - - - + -
B
P-STAT3
STAT3
IL-10 - + + + + + + + + + + + +
2 uM LY - - + - - + - - + - - + - -
10 uM LY - - - + - - + - - + - - + -
20 uM LY - - - - + - - + - - + - - +
5 uM PD - - - - - + + + - - - - - -
10 uM PD - - - - - - - - + + + - - -
25 uM PD - - - - - - - - - - - + + +

**Fig. 3-19.** ERK MAPKs and PI3K specific inhibitors do not inhibit STAT3 activation in HL-60 cells

A. Cells ($1\times10^6$ / ml) were pretreated with either PD98059 or LY294002 at varying concentrations ranging from 0 to 25 μM for 2 hours prior to IL-10 (10 ng / ml) stimulation for 30 minutes.
B. Cells ($1\times10^6$ / ml) were pretreated with a combination of LY294002 and PD98059 at varying concentrations ranging from 0 to 25 μM for 2 hours prior to IL-10 (10 ng / ml) stimulation for 30 minutes. The STAT3 phosphorylation was determined as described in legends to figure 3.17. The Western blot shown is a representative of three different experiments.

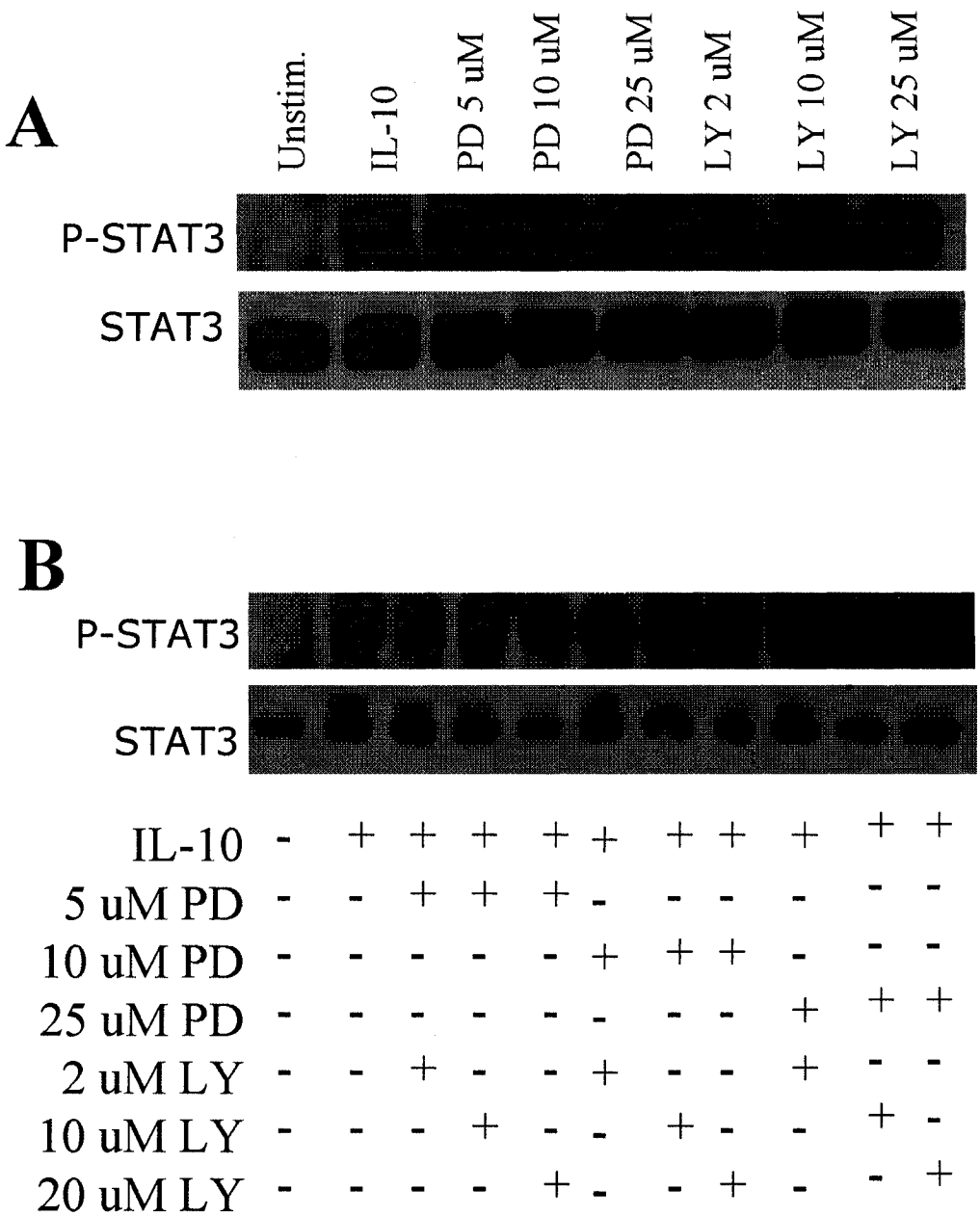
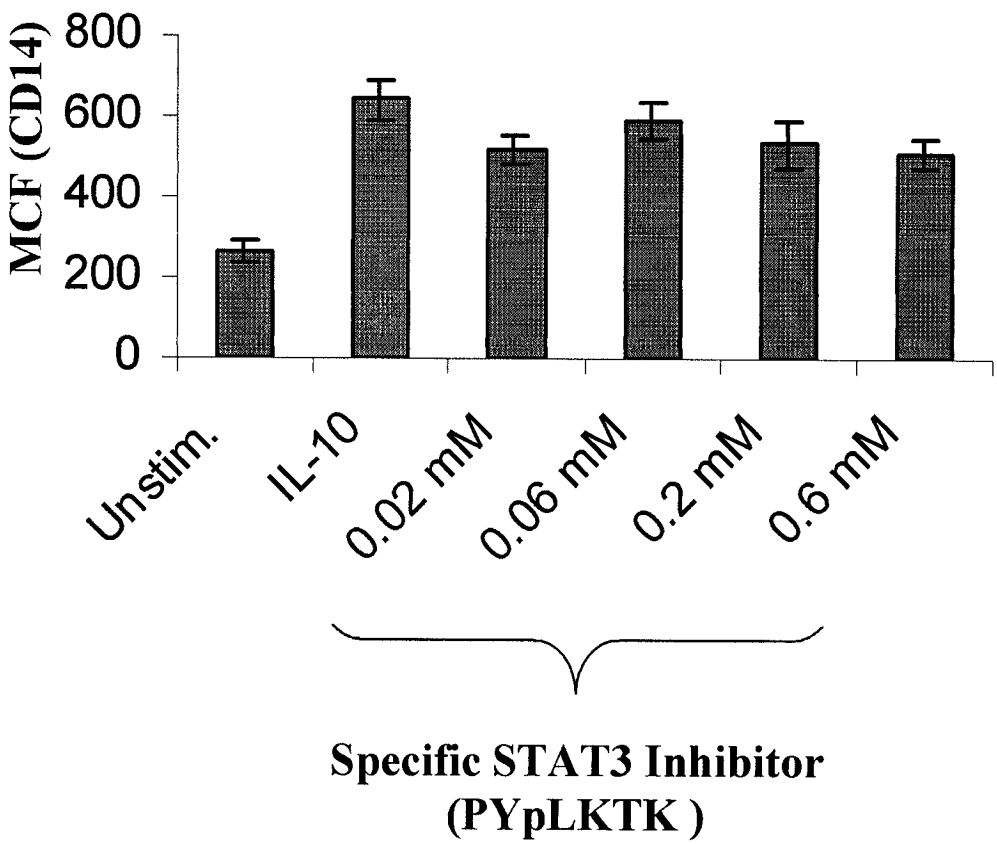

A
Unstim.
IL-10
PD 5 uM
PD 10 uM
PD 25 uM
LY 2 uM
LY 10 uM
LY 25 uM
P-STAT3
STAT3
B
P-STAT3
STAT3
IL-10 - + + + + + + + + + +
5 uM PD - - + + + - - - - - -
10 uM PD - - - - - + + + - - -
25 uM PD - - - - - - - - + + +
2 uM LY - - + - - + - - + - -
10 uM LY - - - + - - + - - + -
20 uM LY - - - - + - - + - - +

**Fig. 3-20.** STAT3 specific inhibitor does not modulate IL-10-induced CD14 expression in HL-60 cells.

Cells ($1\times10^6$ / ml) were pretreated with PYpLKTK at varying concentrations ranging from 0 to 0.6 mM for 2 hours prior to stimulation with IL-10 (10 ng / ml). After 24 hours, cells were analyzed by flow cytometry for the expression of CD14. MCF data are represented as a mean ± SD of two different experiments.


MCF (CD14)
800
600
400
200
0
Unstim.
IL-10
0.02 mM
0.06 mM
0.2 mM
0.6 mM
Specific STAT3 Inhibitor
(PYpLKTK )

**IL-10-induced CD14 expression involves the activation of STAT1**

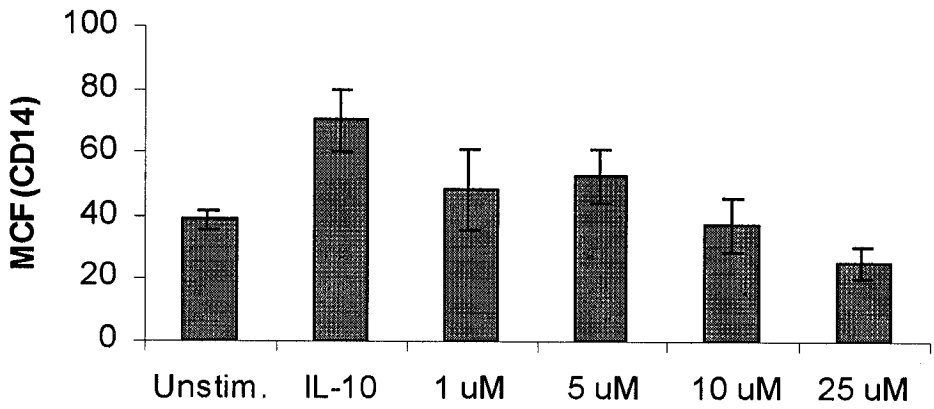
To determine that IL-10 induces CD14 expression through JAK/STAT activation, I employed a tyrosine kinase inhibitor, AGL2592, which inhibits the tyrosine phosphorylation of a number of proteins [116] which in turn these proteins inhibits tyrosine phosphorylation and DNA binding of STAT proteins [117]. HL-60 cells were treated with varying concentrations of AGL2592 for 2 hours (Fig. 3-21A) and 4 hours (Fig. 3-21B) prior to the stimulation with IL-10. Analysis of CD14 expression after IL-10 stimulation revealed that JAK/STAT signaling pathway may involved in IL-10-induced CD14 expression. IL-10 has also been shown to activate STAT1 through the JAK1/Tyk2 signaling pathway [95;114;115]. However, the role of STAT1 in IL-10-mediated biological effects is not known. So, I hypothesized that IL-10 may regulate CD14 expression through the ERK/PI3K mediated activation of STAT1. In a similar experimental design, both normal monocytes and HL-60 cells were treated with various concentrations of PD98059 either alone or in combination with varying concentrations of LY294002 for 2 hours prior to stimulation with IL-10 following which cells were analyzed for STAT1 phosphorylation. IL-10 induced the phosphorylation of STAT1 in both normal monocytes and HL-60 cells (Fig. 3-22) and LY294002 significantly inhibited STAT1 phosphorylation in a dose dependent manner in both human monocytes (Fig. 3-23) and HL-60 cells (Fig. 3-24). Furthermore, LY294002 when used in combination with PD98059 completely inhibited STAT1 phosphorylation in a dose dependent manner (Fig. 3-23; 3-24). These results suggest that IL-10-induced STAT1 activation and not STAT3 activation is mediated through the activation of PI3K alone as well as through both ERK MAPKs and PI3K.

To further determine that IL-10 induces CD14 expression through STAT1 activation, HL-60 cells were transfected with either the STAT1 siRNA plasmid or the control plasmid. First, I determined whether transfection with STAT1 siRNA plasmid affected the expression of STAT1 and the phosphorylation of STAT1 following IL-10 stimulation. Cells transfected for 24 hours were stimulated with IL-10 for 25 minutes and analyzed for STAT1 expression and STAT1 phosphorylation. Results show that transfection of HL-60 cells with STAT1 siRNA plasmids inhibited IL-10-induced STAT1

**Fig. 3-21.** Phospho-tyrosine inhibitor modulates IL-10-induced CD14 expression in HL-60 cells.

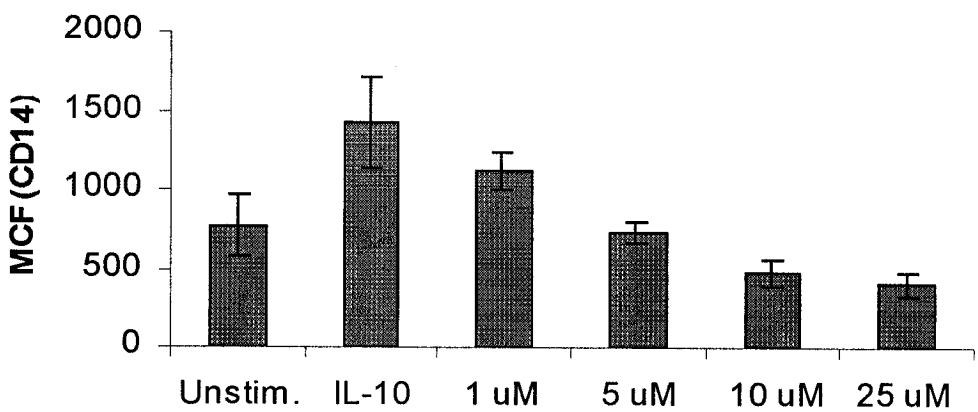
Cells ($1\times10^6$ / ml) were pretreated with AGL2592 at varying concentrations ranging from 0 to 25 μM for 2 hours prior to stimulation (A) and 4 hours prior to stimulation (B) with IL-10 (10 ng / ml). After 24 hours, cells were analyzed by flow cytometry for the expression of CD14. MCF data are represented as a mean ± SD of five different experiments.

**A**




AGL2592
2 hours pre incubation

**B**



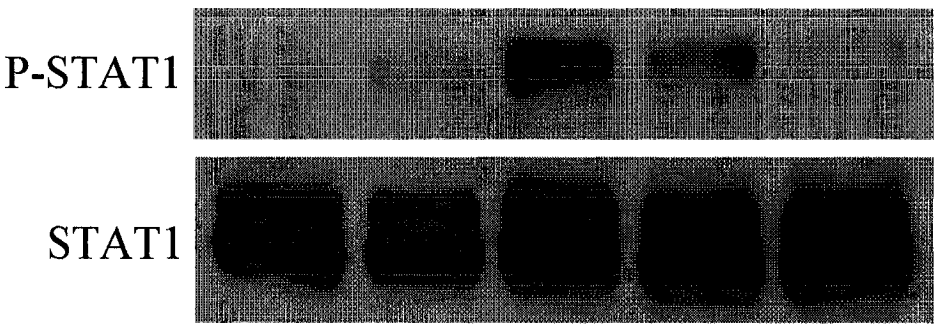

AGL2592
4 hours pre incubation

**Fig. 3-22.** IL-10 induces STAT1 phosphorylation in human monocytes and HL-60 cells

Purified normal human monocytes (A) and HL-60 cells (B) ($1\times10^6$ / ml) were stimulated with rhIL-10 (10 ng / ml) in a kinetic experiment from 0 to 60 minutes. The cell extracts were analyzed for STAT1 phosphorylation by Western blot analysis as described in legend to 3.17. The results shown are representative of three different experiments performed.

**A**

**Human Monocytes**




**B**

**HL-60 cells**

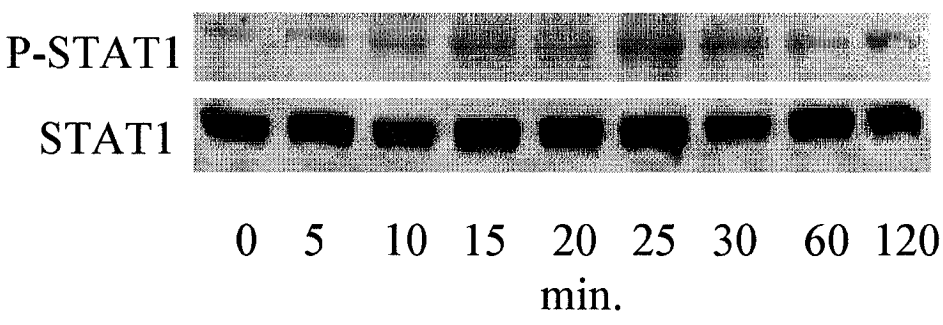
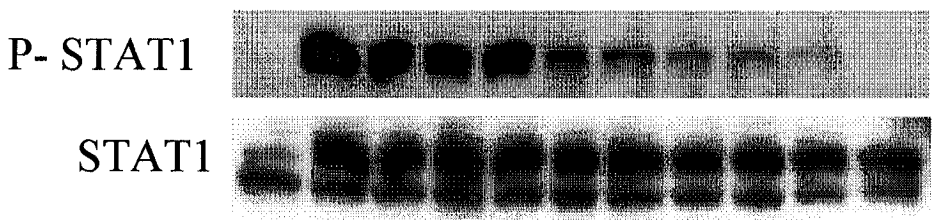


**Fig. 3-23.** ERK MAPKs and PI3K specific inhibitors inhibit IL-10-induced STAT1 activation in human monocytes

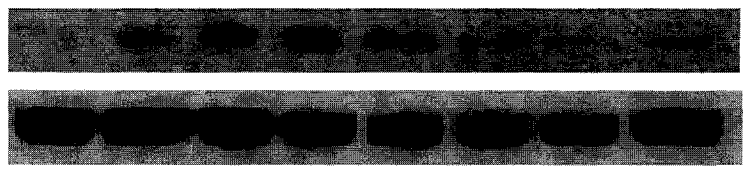
Cells ($1\times10^6$ / ml) were pretreated with either PD98059 or LY294002 alone or in combination at varying concentrations ranging from 0 to 25 μM for 2 hours prior to IL-10 (10 ng / ml) stimulation for 15 minutes. Crude protein extracts (50 μg) were subjected to SDS-PAGE followed by Western blot analysis. The membranes were blotted with anti-phospho-STAT1 (P-STAT1) antibodies, and to control for loading, the membranes were stripped and probed with anti-STAT1 (STAT1) antibodies. The Western blot shown is a representative of three different experiments.




| | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| IL-10 | - | + | + | + | + | + | + | + | + | + | - |
| 10 uM PD. | - | - | + | - | - | - | + | + | - | - | - |
| 25 uM PD | - | - | - | + | - | - | - | - | + | + | - |
| 2 uM LY | - | - | - | - | + | - | + | - | + | - | - |
| 10 uM LY | - | - | - | - | - | + | - | + | - | + | - |

**Fig. 3-24.** ERK MAPKs and PI3K specific inhibitors inhibit IL-10-induced STAT1 activation in HL-60 cells

IL-10 stimulation induces STAT1 phosphorylation in HL-60 cells. Cells ($1\times10^6$ / ml) were pretreated with either PD98059 or LY294002 alone or in combination at varying concentrations ranging from 0 to 25 μM for 2 hours prior to IL-10 (10 ng / ml) stimulation for 25 minutes. Crude protein extracts (50 μg) were analyzed for STAT1 phosphorylation by Western blot analysis. The Western blot shown is a representative of three different experiments.




| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| IL-10 | - | + | + | + | + | + | + | + |
| 10 uM.PD | - | - | - | - | + | + | - | - |
| 25 uM PD | - | - | + | - | - | - | + | + |
| 10 uM LY | - | - | - | + | + | - | + | - |
| 20 uM LY | - | - | - | - | - | + | - | + |

phosphorylation as well as STAT1 expression compared to the cells transfected with the control plasmids (Fig. 3-25A). To determine whether STAT1 inactivation influenced CD14 expression, transfected cells were analyzed for CD14 expression 24 hours after transfection by flow cytometry. IL-10 failed to induce CD14 expression in cells transfected with STAT1 siRNA plasmid. In contrast, IL-10 induced the expression of CD14 in cells transfected with the control plasmid (Fig. 3-25B).

**LY294002 and PD98059 inhibit STAT1 binding to the CD14 promoter in IL-10-stimulated HL-60 cells**

To further confirm the role of STAT1 in the regulation of IL-10-induced CD14 expression in monocytic cells, I investigated whether IL-10 stimulation of HL-60 cells induced the binding of STAT1 transcription factor to its binding site present in human CD14 promoter. STAT1 binding site exists in the human CD14 promoter at - 126 bp relative to the +1 transcription site of CD14. HL-60 cells were stimulated with IL-10 over a period of time ranging from 0 to 120 minutes (0, 15, 30, 45, 60, and 120 min.), and then the nuclear extracts were analyzed in a gel shift assay for binding to STAT1 oligonucleotides probe corresponding to the STAT1 binding site in the CD14 promoter. The results show that significant binding of STAT1 to the STAT1 oligonucleotides in the form of two bands (1 and 2) occurred 30 to 45 minutes following stimulation of HL-60 cells with IL-10. The binding of STAT1 as indicated by band-1 was completely blocked by competition with cold STAT1 oligonucleotides indicating its specificity (Fig. 26A lanes 1 and 2). However, the binding of STAT1 as indicated by band 2 was unaffected even if the cold oligonucleotides was used in excess of 100 fold higher concentration

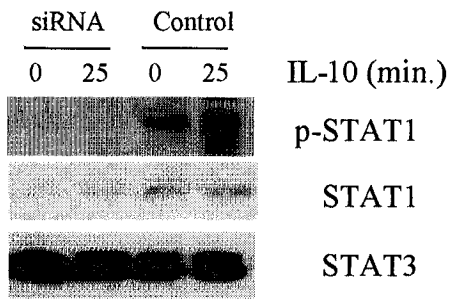
(Fig. 26A lanes 3 and 4) indicating the STAT1 specificity of the band 1. In contrast, non-specific oligonucleotides unlike the STAT1 oligonucleotides, failed to compete for binding of STAT1 transcription factors (band-1) to the labeled oligonucleotides probe (Fig. 26A lane 5). In addition, supershift analyses were performed to confirm the identity of STAT1 transcription factor by using specific mouse anti-STAT1 antibodies. Incubation of nuclear extracts obtained from HL-60 cells with oligonucleotides probes and anti-STAT1 antibodies significantly abrogated STAT1 specific band when added 2 μg anti-STAT1 antibodies (Fig. 26B lane 5).

To determine whether PI3K inhibitor, LY294002, and ERK MAPKs inhibitor, PD98059, inhibited the binding of STAT1 to its binding site in the CD14 promoter, HL-60 cells were treated with either LY294002 alone or in combination with PD98059 in various concentrations for 2 hours prior to IL-10 stimulation for 30 minutes followed by the analysis of STAT1 binding to its oligonucleotides probe. The results show that LY294002 inhibited the binding of STAT1 in a dose dependent manner whereas this binding was inhibited partially by PD98059. However, LY294002 and PD98059 when added together completely inhibited the binding of STAT1 to its probe. In fact, the intensity of this binding was even lower than that observed in untreated cells (Fig. 26C). Taken together, the results suggest that IL-10-induced CD14 transcription may be regulated by STAT1 through the activation of PI3K either alone or synergistically with ERK MAPKs.

**Fig. 3-25.** IL-10-induced CD14 expression is dependent on STAT1 activation

A. HL-60 cells were transiently transfected with either vectors containing small interfering (si)RNAs or control vectors followed by analysis of IL-10-induced STAT1 phosphorylation and expression. The membrane was initially blotted using phospho-specific anti-STAT1 antibodies and was stripped and reprobed with non-phospho-STAT1 antibodies to examine STAT1 expression levels. The membrane was stripped and reprobed with anti-STAT3 antibodies as a control for equal loading.
B. HL-60 cells were transiently transfected with either vectors containing siRNAs or control vectors followed by analysis of IL-10-induced CD14 expression.

**A**


siRNA
Control
0
25
0
25
IL-10 (min.)
p-STAT1
STAT1
STAT3

**B**

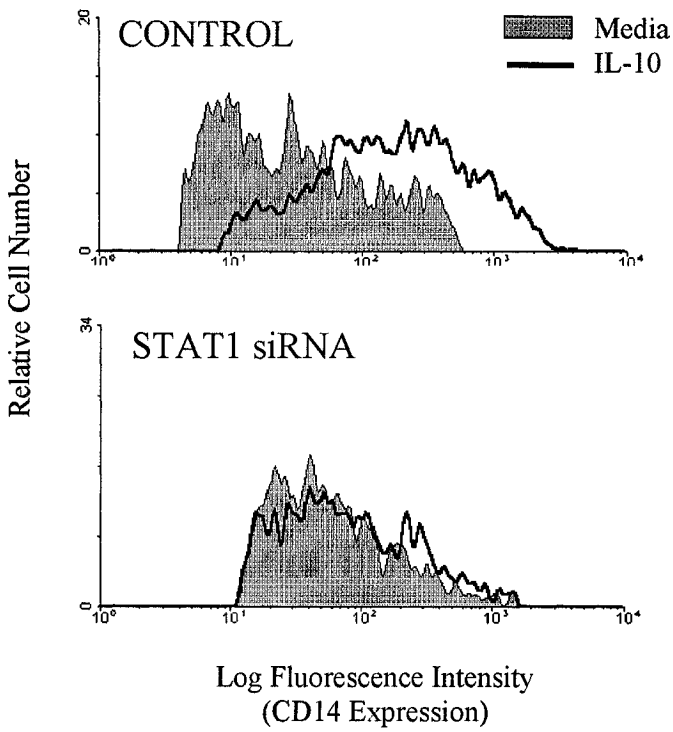
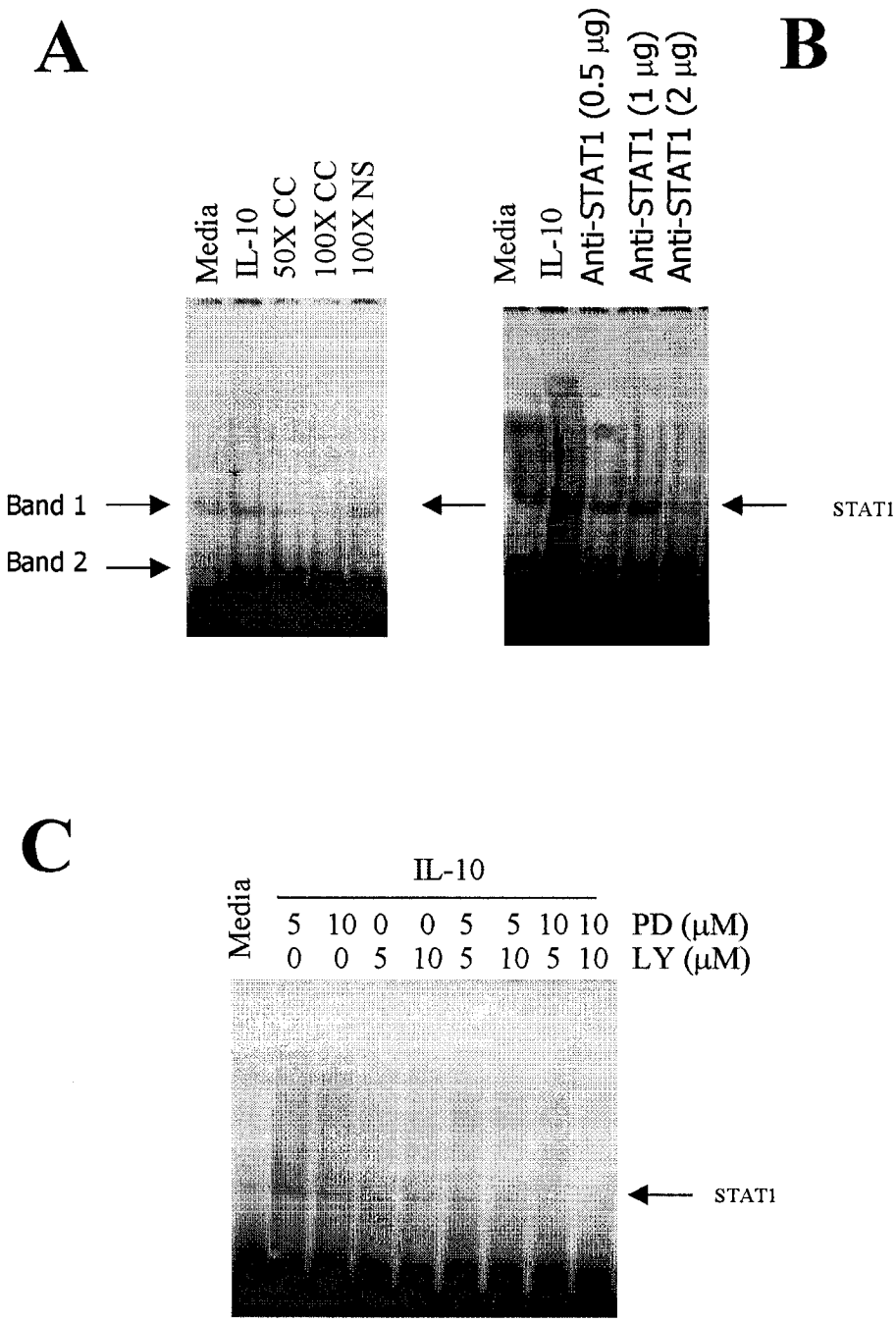

CONTROL
Media
IL-10
STAT1 siRNA
Relative Cell Number
Log Fluorescence Intensity
(CD14 Expression)

**Fig. 3-26.** IL-10 induced STAT1 activation is dependent on ERK MAPKs and PI3K activation

A. HL-60 cells were treated with IL-10 (10 ng/ml) for 15 to 120 minutes and the nuclear lysates were analyzed for STAT1 activation by EMSA (left blot). In order to show specificity the nuclear extracts were incubated with 50X and 100X cold oligonucleotides probes for STAT1 (left blot, lanes 3 and 4). As a negative control, the nuclear extract was also incubated with a non-specific probe (left blot, lane 5). Supershift analysis was also performed (right blot). Nuclear extracts were incubated with increasing concentrations of STAT1 antibody. The STAT1-specific band is indicated by the arrow.
B. Cells were pretreated with varying concentrations of either PD98059 or LY294002 alone or in combination followed by IL-10 stimulation for 30 minutes. The nuclear extracts were examined for STAT1 activation by EMSA analysis.


A
B
Media
IL-10
50X CC
100X CC
100X NS
Media
IL-10
Anti-STAT1 (0.5 μg)
Anti-STAT1 (1 μg)
Anti-STAT1 (2 μg)
Band 1
Band 2
STAT1
C
IL-10
Media
5 10 0 0 5 5 10 10 PD (μM)
0 0 5 10 5 10 5 10 LY (μM)
STAT1

# CHAPTER 4:

# DISCUSSION

IL-10 is a cytokine that exhibits primarily immunosuppressive effects on the synthesis of inflammatory cytokines and the co-stimulatory molecules. However, it has also been shown to have stimulatory effects on the expression of a number of surface molecules including FcγR and CD14 on human monocytic cells. In the recent past, molecular mechanisms by which IL-10 exerts its inhibitory effects have been investigated. In most of the instances, IL-10-induced activation of STAT3 has been shown to play a major role in the inhibition of cell proliferation and synthesis of proinflammatory cytokines [93;95;118-120]. However, in contrast to STAT3, the role of STAT1 in regulating IL-10-mediated biological effects remains unknown. In the current study, I investigated the signaling pathways involved in IL-10-induced stimulatory effect of CD14 expression in human monocytic cells. The results show for the first time that IL-10 induces CD14 expression by a mechanism dependent on STAT1 and independent of STAT3 activation. Furthermore, IL-10-induced activation of STAT1 is mediated through the activation of PI3K alone as well as through the synergistic effect of PI3K and ERK MAPKs.

LPS, a potent monocytic mitogen, stimulates myelomonocytic cells following its association with the plasma LPS-binding protein (LBP), and through binding of the LPS/LBP complex with the CD14/Toll like receptor-4 (TLR-4) complex [49;121;122]. CD14, a primary LPS receptor is a 53 kD GPI-linked cell surface antigen expressed on monocytic cells and to a lesser extent on neutrophils. The role of CD14 in various disease states has been studied [109;123-127]. Elevated levels of circulating soluble CD14 (sCD14) have been identified in patients with inflammatory conditions such as systemic lupus erythematosus, chronic active hepatitis, and septic shock. In Human immuno-

deficiency virus (HIV) infection, up-regulated expression of mCD14 on monocytes and alveolar macrophages and increased levels of sCD14 have been reported. Elevated levels of sCD14 were found to correlate with disease progression in HIV infection [125]. The findings of enhanced CD14 expression following IL-10 treatment raise questions as to whether enhanced CD14 expression acts as an anti-inflammatory and negative regulator of LPS-induced synthesis of proinflammatory cytokines by neutralizing the endotoxin (LPS) and decreasing the susceptibility of the host to bacterial infections. Therefore, it is important to understand the molecular mechanism by which IL-10 up-regulates the surface expression of CD14 on monocytic cells.

Interaction of IL-10 with its receptor complex initiates a cascade of events leading to the tyrosine phosphorylation of JAK1 and Tyk2 that is followed by the recruitment of STAT3 and STAT1 in monocytic cells [118;119]. Activation and recruitment of STAT3 to IL-10R1 is essential in manifestation of a number of the biological effects of IL-10 on B cells and macrophages [93;95;119;128]. STAT3 is directly recruited to two tyrosine residues (Tyr 427 and Tyr477) in the intracellular domain of IL-10R1 following binding to IL-10. Binding of STAT3 to these tyrosine residues is required for receptor function and activation of STAT3 but for STAT1 suggesting that STAT1 is not directly recruited to the IL-10 receptor complex. It has recently become clear that a number of cytokine receptors that activate JAK/STAT signaling pathway can also cause activation of other signaling molecules especially the PI3K signaling proteins. For example, JAK1 activated by IL-4, IFN-α, IL-10, and Oncostatin-M can cause the activation of IRS-2 and PI3K signaling pathways [79;129-131]. In this study, I show that IL-10 stimulation of normal human monocytes and HL-60 induces the activation of survival enzyme Akt, the

signaling molecule downstream of PI3K. The PI3K inhibitor, LY294002, inhibited specifically IL-10-induced Akt activation. It has been suggested that Akt activation by IL-10 is required to promote cell proliferation, but not for IL-10 to suppress the synthesis of pro-inflammatory cytokines such as TNF-α and IL-1β [90;132]. Recently, IL-10 was also shown to promote the survival of astrocytes by a mechanism that depends on PI3K activation. My studies showing the involvement of PI3K in IL-10-induced CD14 expression support the concept that PI3K activation may be involved in the stimulatory effects of IL-10 and not for the inhibitory effects of IL-10 on the synthesis of pro-inflammatory cytokines. However, further studies are needed to address the role of PI3K as a key mediator of IL-10-induced stimulatory effects.

There is evidence to suggest that PI3K interact with the JAK/STAT proteins. STAT3 was found to act as an adaptor molecule in signal transduction from the type 1 interferon receptor. STAT3 bound to a conserved sequence at Tyr 527 and Tyr 538 of IFN-R and underwent IFN-dependent tyrosine phosphorylation at residues Tyr 656 and Tyr 705. The p85 subunit of the PI3K, which activates a series of serine kinases, bound to the phosphorylated STAT3 and subsequently underwent tyrosine phosphorylation [133]. Activated PI3K can then promote serine phosphorylation of STAT3 which is critical for the formation of stable STAT3 homodimers required for translocation into the nuclei [133]. Similarly, PI3K and its effector kinase Akt play an important role in the serine phosphorylation of STAT1 (Serine727) and in the activation of gene expression in response to IFN-g [134]. In this study, I show for the first time the involvement of PI3K in IL-10-induced activation of STAT1. I have shown that PI3K inhibitor LY294002 inhibited the activation of IL-10-induced STAT1 but had no effect on the activation of

STAT3. However, the sequence of events leading to the binding of PI3K to IL-10-induced STAT1 needs to be elucidated.

The finding that IL-10 induces the activation of ERK MAPKs in both normal human monocytes and HL-60 cells was unexpected. IL-10 induced the phosphorylation of ERK MAPKs within 15 minutes, which peaked at 60 min. Furthermore, IL-10-induced ERK MAPKs activation could be inhibited by its inhibitor, PD98059, in a dose dependent manner indicating the specificity of ERK MAPKs activation by IL-10. However, IL-10 failed to induce the activation of either p38 or JNK MAPK at any time following stimulation. Whether IL-10-induced ERK MAPKs activation has a broader role to play in IL-10 signaling is not clear at present. In one study, IL-10 was shown to activate p38 MAPK in murine macrophages that was implicated in IL-10-induced expression of heme oxigenase [78]. Results show that PD98059 did inhibit IL-10-induced CD14 expression albeit partially. However, in synergy with the PI3K inhibitor, it abolished IL-10-induced-STAT1 activation and CD14 expression. The signaling events involved in specifically activating ERK MAPKs are poorly understood. There is evidence to suggest that ERK MAPKs can be activated by the JAK1/2 in IFN-g-mediated signaling pathway. The signaling events involved in the activation of ERK MAPKs in IL-10 signaling need to be investigated.

STAT1, the transcription factor critically involved in IFN-γ-mediated signaling pathway plays an important role in IFN-γ-mediated anti-proliferative responses, immune surveillance and tumor suppression. The role of STAT1 in biological responses has been studied in STAT1 knock out mice. $STAT1^{-/-}$ mice develop normally. However, STAT1 deficient mice fail to elicit biological responses to IFN-γ or IFN-α, exhibit impaired

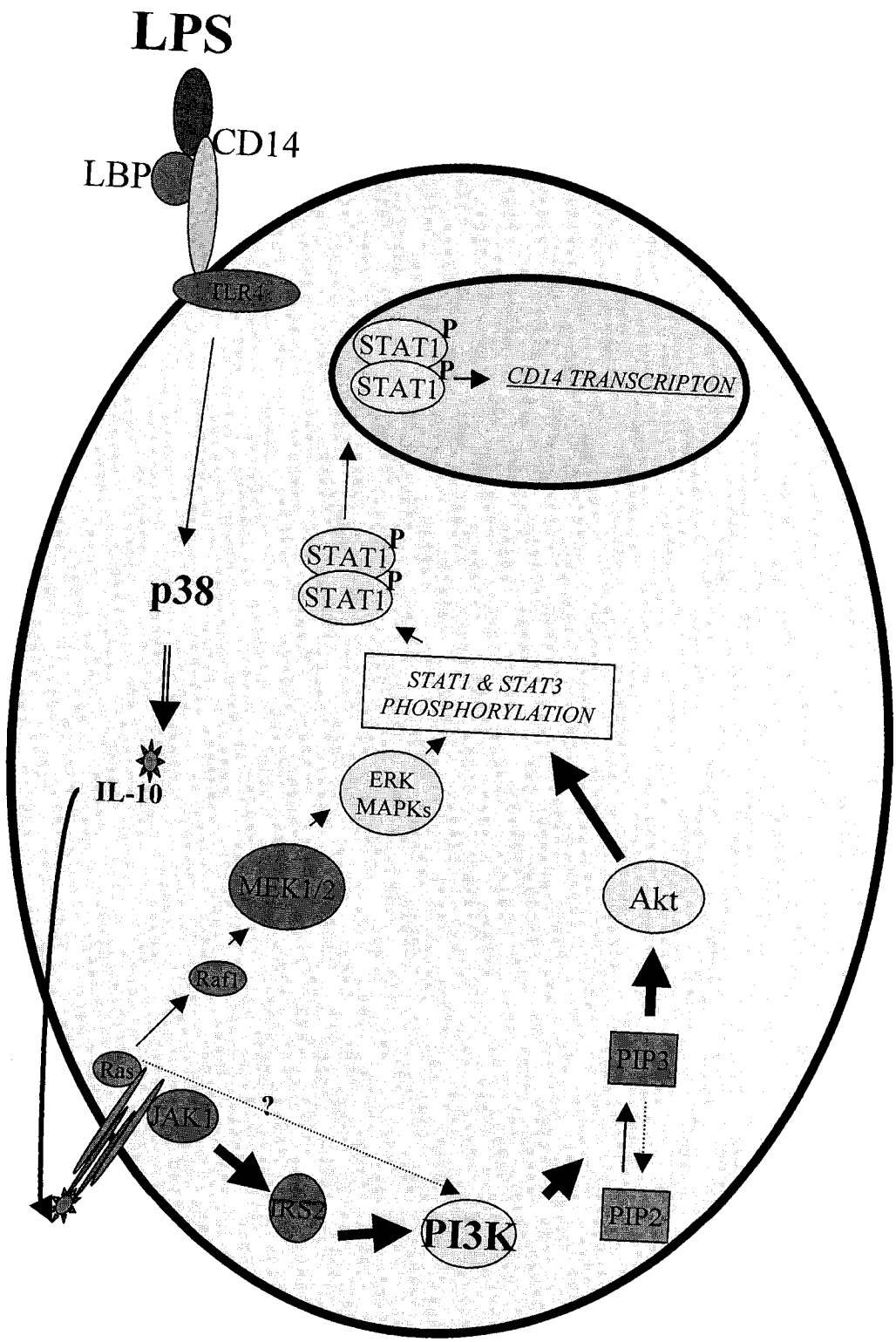
development of regulatory T cells, and develop tumors rapidly and greater frequency following challenge with chemical carcinogens. Interestingly, macrophages from STAT1 null mice remain responsive to IL-10, and other cytokines such as EGF, which activate STAT1. Furthermore, over expression of dominant negative STAT1 did not block IL-10-induced effects in a macrophage cell line. My results show for the first time that the stimulatory effect of IL-10 on CD14 expression is mediated by the specific activation of STAT1 and in a STAT3 independent manner.

The signaling molecules and the transcription factors involved in the regulation of CD14 transcription are not well understood. CD14 promoter has been cloned and its active sites responsible for transcription in response to LPS have been elucidated [135;136]. CD14 expression has been shown to be regulated primarily by the Sp-1 transcription factor in human monocytic cells and by AP-1 and Sp-1 in rat hepatocytes [135;136]. The results clearly show that IL-10-induced CD14 expression is regulated by STAT1 transcription factor through the activation of PI3K and ERK MAPKs in both normal human monocytes and HL-60 cells. The role of STAT1 was demonstrated by employing STAT1 siRNA and gel shift assays. STAT1 binding site exists on the CD14 promoter. By employing gel shift assays, I also show that IL-10 can induce the binding of STAT1 to its binding site on the CD14 promoter, and this binding can be inhibited significantly by PI3K inhibitors alone and in combination with ERK MAPKs inhibitor. Although results show the involvement of STAT1 in IL-10-induced CD14 transcription, the involvement of other transcription factors particularly Sp-1 can not be ruled out. STAT proteins have been demonstrated to associate physically and functionally with co-activator proteins such as CBP, p300, and BRCA1. STAT1 also co-operates with both general (SP-1) and inducible (AP-1, NFκB) transcription

factors [137-144]. Whether STAT1 induces CD14 expression in response to stimulation with IL-10 by directly binding to the CD14 promoter or through the cooperative action with transcription factors AP-1 and/or SP-1 remains to be understood.

**Fig. 4-1.** Overview of the signaling pathways responsible for the induction of CD14 expression in monocytic cells.

Treatment of monocytic cells with LPS induces the expression of endogenous IL-10. Endogenous IL-10 is capable of inducing CD14 expression. The signaling pathway responsible for the LPS-induced expression of IL-10 involves the activation of p38 MAPK and IL-10 itself induces ERK MAPKs and PI3K activation leading to STAT1 phosphorylation. Dimerization and translocation of phosphorylated STAT1 triggers CD14 transcription.


LPS
CD14
LBP
TLR4
STAT1
P
STAT1
P
CD14 TRANSCRIPTON
p38
STAT1
P
STAT1
P
STAT1 & STAT3
PHOSPHORYLATION
IL-10
ERK
MAPKs
MEK1/2
Akt
Raf1
PIP3
Ras
?
TAK1
IRS2
PI3K
PIP2

Monocyte

# CHPATER 5:

# CONCLUDING REMARKS, SIGNIFICANCE, AND FUTURE DIRECTIONS

The interaction of the glycoprotein, CD14, and its ligand (LPS) have been shown to play a critical role in the synthesis of proinflammatory cytokines (IL-1β, TNFα, etc) and anti-inflammatory cytokines (IL-10 and IL-1Rα). IL-10 is a pleotropic immunoregulatory cytokine which has inhibitory and also stimulatory effects on human monocytic cells. It has been shown that IL-10 induces CD14 expression on monocytes. The molecular mechanisms by which IL-10 enhances CD14 expression in monocytic cells is the major and central question in my thesis research. Based on this question, I started to identify the role of MAPKs (JNK, p38, and ERK) involved in IL-10-induced CD14 expression in normal human monocytes and HL-60 cells as a model system (the first objective). MAPKs are one of the best-characterised pathway that is initiated by receptor tyrosine kinases. MAPKs are key players in cellular response such as proliferation and differentiation. My results show that IL-10 selectively induces ERK MAPKs phosphorylation and there are no effect on JNK and p38 MAPKs activation mediated by IL-10. Using ERK MAPKs specific inhibitor, PD98059, slightly reduced CD14 expression on the monocytic cells. These results suggested that I should look for other pathways to find more significant involved pathway. So I continued my work by looking for PI3K activation in CD14 regulation, because of their important regulatory role in cellular signaling (the second objective). My results show that IL-10 induces PI3K phosphorylation and leading phosphorylation of the important downstream effector of PI3K, Akt. Treatment the cells with specific inhibitor of P110 catalytic subunits proteins of PI3K, LY294002, partially downregulate CD14 expression on the cells. Interestingly, treatment the cells with combination of LY294002 and PD98059 significantly have synergistic downregulatory effect on IL-10-induced CD14 expression. These resutls

suggest that PI3K and ERK MAPKs may involve in CD14 expression. The next step was finding the involved transcription factor (the third objective). It was known that the JAK/STAT pathway triggered by IL-10 and allows rapidly the transduction of an extracellular signal into the nucleus. So as we expected IL-10 mediated STAT3 and STAT1 phosphorylation occurred in the cells. The interesting point is that specific inhibitors of ERK MAPKs and PI3K do not have effect on STAT3 phosphorylation but have inhibitory effect on STAT1 phosphorylation. Moreover, STAT3 specific inhibitor, PYpLKTK, does not have effect on CD14 expression; meanwhile, STAT1 siRNA has significant downregulatory effect on CD14 expression on these cells. Finally, LY294002 and PD98059 inhibited the binding of STAT1 transcription factor to its binding site in the CD14 promoter. Taken together, my resutls for the first time show that IL-10-mediated CD14 upregulation may be mediated by STAT1 activation independently of STAT3. Furthermore, IL-10-activated STAT1 may be regulated through PI3K either alone or in concert with the ERK MAPKs.

To continue this research proposal to confirm these results and also to use the benefits of the results in clinic, I think the next suggested steps would help us to draw a complete pathway of IL-10-induced CD14 expression on monocytic cells:

- Using the dominant negative PI3K (p110 catalytic subunits) and/or ERK MAPKs cells to further confirm the role of these enzymes in CD14 expression.
- To study the sequence of events leading to the binding of PI3K to IL-10-induced STAT1.

- To determine the role of STAT1 in the regulation of IL-10-mediated effects by employing STAT1 knockout animal models.
- To study whether PI3K and/or STAT1 mediated stimulatory effect of IL-10 on other molecules such as NK cell activation, B cell activation and differentiation, FCγR up-regulation, and HIV-replication.

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