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Regulation of CD44 Expression in Human Monocytic Cells and B Cells

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REGULATION OF CD44 EXPRESSION IN HUMAN MONOCYTIC CELLS AND B CELLS

By

Jyoti Prasad Mishra

A thesis submitted to the Faculty of Graduate Studies
in partial fulfillment of the requirements for the degree of
Doctor of Philosophy

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Abstract

CD44, a family of cell adhesion glycoproteins, is an important mediator in events such as tumorigenesis and metastasis, cell migration, and inflammatory responses. The molecular mechanisms in the regulation of CD44 expression are not well understood. In this study, I examined the regulation of CD44 expression in two models systems: human monocytic cells and Burkitt's lymphoma B cells.

Lipopolysaccharide (LPS), a bacterial cell wall component, regulates CD44 expression, and modulates CD44-mediated biological effects in monocytic cells during inflammation and immune responses. In human monocytic cells, LPS and the proinflammatory cytokine, tumor necrosis factor-α (TNF-α) are potent inducers of CD44 expression. To delineate the mechanism underlying LPS- and TNF-α-induced CD44 expression, the previous results in our laboratory showed the differential role of mitogen-activated protein kinase (MAPK) specifically c-Jun N-terminal kinase (JNK) in LPS-induced but not in TNF-α-induced CD44 expression. Therefore, we hypothesized that distinct signaling pathways are involved in the regulation of CD44 expression in a stimulus and cell-dependent manner.

In this study, I investigated the signaling pathway involved in TNF-α-induced CD44 expression in human monocytic cells. I used human promonocytic THP-1 cells, transfected stably with CD14 receptor (THP-1/CD14) as a model system. My results showed the differential involvement of Ca^{2+} signaling molecules, in particular calmodulin (CaM) and CaM-dependent protein kinase-II (CaMK-II), in TNF-α-induced but not in LPS-induced CD44 expression. The CD44 promoter analysis suggests that the distinct transcription factors AP-1 and Egr-1 may be involved in TNF-α- and LPS-induced CD44 expression, respectively. In addition, I demonstrated the selective involvement of Ca^{2+} signaling molecules, mainly CaM and CaMK-II, in TNF-α-induced CD44 expression through the activation of transcription factor AP-1. In contrast, LPS-induced CD44 expression was regulated by JNK MAPK through the activation of Egr-1.

I have also demonstrated that phosphoinositide 3-kinase (PI3K) constitutes a key downstream component of both the signaling pathways involved in the regulation of LPS- and TNF-α-induced CD44 expression in human monocytic cells. My results suggest that the JNK-activated PI3K regulates LPS-induced CD44 expression through the activation of Egr-1, whereas TNF-α induces CD44 expression by a distinct CaM/CaMK-II-activated PI3K through the activation of AP-1 transcription factor. Further, to determine the involvement of the subunit of PI3K, I demonstrated that the regulatory subunit p85α is involved in both LPS- and TNF-α-induced CD44 expression, without involving the catalytic subunit, p110α.

IL-4, a pleotropic cytokine, has been shown to enhance the survival and development of Burkitt's lymphoma (BL) B cells, and induces the expression of various costimulatory molecules including CD44. In addition, CD44 induction and its ability to bind hyaluronan (HA) have been suggested to play a vital role in *in vivo* BL tumor

growth and dissemination. However, the role of IL-4 in the pathogenesis of BL and other B cell malignancies is not clear. In this study, I investigated the regulation of IL-4-induced CD44 expression in an Epstein-Barr virus (EBV)-transformed Burkitt's lymphoma B cell line, BL30/B95-8 cells. The results suggested that IL-4 did not induce the expression of the transcription factors Egr-1 and AP-1. However, IL-4 induced STAT-6 which plays a critical role in CD44 regulation in these cells. I demonstrated that STAT-6 is activated by two distinct signaling pathways namely Jak-1/3 and ERK MAPK and independent of the IRS-2/PI3K pathway.

Therefore, STAT-6 and ERK MAPK may represent an important and novel target for regulation of CD44 expression and CD44-mediated immune responses in B cell proliferation- and maturation-related disease conditions and cancer malignancies. Further, my results also suggest a critical involvement of PI3K in the regulation of LPS- and TNF-α-induced CD44 expression in human monocytic cells, and hence, may represent a potential therapeutic target for inhibiting CD44 expression and consequent CD44-mediated cell migration, inflammation and autoimmune disorders.

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List of Abbreviations

Ab	Antibody
AP-1	Activation protein-1
ATF-2	Activating transcription factor-2
BCR	B cell receptor
BL	Burkitt's lymphoma
bp	Base pair
CaM	Calmodulin
CaMK-II	CaM-dependent protein kinase-II
cIAP	Cellular inhibitor of apoptosis
cDNA	Complementary DNA
cGMP	Cyclic guanosine monophosphate
cpm	Counts per minute
DD	Death domain
DN	Dominant negative
EBV	Epstein-Barr virus
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
Egr-1	Early growth response factor-1
EMSA	Electrophoretic mobility shift assay
ER	Endoplasmic reticulum
ERK	Extracellular-regulated protein kinase
GM-CSF	Granulocyte macrophage colony stimulating factor
HA	Hyaluronan
HEV	High endothelial venule
hr	Hour
ICAM	Intercellular adhesion molecule
IFN	Interferon
Ig	Immunoglobulin
IGF-1	Insulin growth factor-1
IL	Interleukin

IP_3	Inositol 3,4,5 triphosphate
IRS-2	Insulin receptor substrate-2
I.V.	Intravenous
Jak	Janus associated kinase
JAB	JAK binding protein
JNK	c-jun N-terminal kinase
KO	Knock-out
LFA	Lymphocyte function-associated antigen
LPS	Lipopolysaccharide
mAb	Monoclonal antibody
MHC	Major histocompatibility complex
MAPK	Mitogen activated protein kinase
Mut (mt)	Mutant
mRNA	Messenger RNA
NK	Natural killer
nt	Nucleotides
NOD	Non-obese diabetic mouse
PAGE	Polyacrylamide gel electrophoresis
PBMC	Peripheral blood mononuclear cells
PCR	Polymerase chain reaction
PDGF	Platelet-derived growth factor
PI	Phosphatidyl inositol
PIP_2	Phosphatidyl inositol 4,5 bisphosphate
PI3K	Phosphoinositide 3-kinase
PKC	Protein kinase C
PLC	Phospholipase C
PMA	Phorbol 12-myristate 13-acetate
PP	Peyer's patches
PP2C	Protein phosphatase 2C
4PS	IL-4-induced phosphotyrosine substrate
PTK	Protein tyrosine kinase
RA	Rheumatoid arthritis
RANTES	Regulated on activation, normal T cell expressed and secreted

RHAMM	Receptor for hyaluronic acid mediated motility
RIP	Receptor interacting protein
RLU	Relative luciferase unit
RT-PCR	Reverse transcription-polymerase chain reaction
SCID	Severe combined immunodeficiency
SDS	Sodium dodecyl sulphate
SEK	Stress activated protein/ERK kinase
SH2	Src-homology 2 domain
SHP-1	SH2-containing tyrosine phosphatase-1
SOCS	Suppressor of cytokine signaling
SODD	Silencer of death domain
STAT	Signal transducer and activator of transcription
TCR	T cell receptor
TGF	Transforming growth factor
T_h	T helper
TNF-α	Tumor necrosis factor-α
TRADD	TNF receptor-associated death domain
TRAF-2	TNF-receptor associated factor-2
VCAM	Vascular cell adhesion molecule
Wt	Wild type

Chapter I
Introduction

Cell-cell and cell-extracellular matrix (ECM) interactions play a critical role in many biological processes, including the development of an immune response, inflammation, tumor formation and metastasis (1-3). CD44 is among a plethora of adhesion molecules which participate in these processes (4-8). The CD44 proteins are a family of cell surface membrane glycoproteins that are capable of binding to the glycosaminoglycan, hyaluronan (HA), a major component of the ECM (5;8;9). Engagement of CD44 on activated immune cells with HA is a critical event in many physiological and pathophysiological events such as: lymphocyte homing, cell activation and differentiation, cell migration, angiogenesis, wound healing, tumorigenesis and metastasis, and inflammatory responses (10-16). The multiple functions of CD44 have been attributed to the existence of numerous CD44 isoforms that are generated by alternative splicing, as well as by extensive post-translational modifications such as N- and O-linked glycosylation and glycosaminoglycan and chondroitin sulphate addition (17-20). The family of CD44 molecules is widely expressed by a diverse array of cell types including leukocytes, fibroblasts, epithelial cells, keratinocytes, and various stem cells (7;21). The ability of CD44 to bind HA is a tightly regulated process. Although most cells express some form of CD44, not all cells constitutively bind HA. Acquisition of the HA-binding ability of CD44 thus plays a vital role in determining CD44-mediated biological effects (9;22;23). The HA-binding capacity of CD44 has been suggested to be influenced by multiple factors that include structural variations in the CD44 extracellular domain, oligomerization of CD44 on the cell membrane, phosphorylation of its cytoplasmic tail, alterations in the N- and O-linked glycosylation as well as sialidation and sulfation patterns of CD44 (24-28).

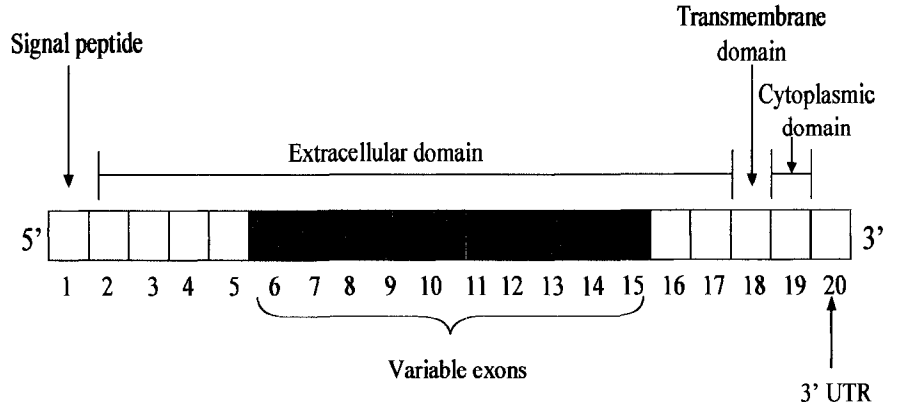
Expression levels, isoform type, and HA-binding ability are usually dependent on the cell type as well as stimuli present in the microenvironment of the cell. The molecular mechanisms underlying the regulation of CD44 transcription, alternative splicing, and post-translational modifications leading to the expression of CD44 as well as the activation of CD44 binding to HA are not well understood (29-31). Regulation of cell-cell and cell-ECM interactions is important in cell migration and in the development and control of inflammatory and immunological events. Because CD44 is an important modulator in these interactions, studies of the regulation of CD44 will lead to further understanding of the mechanisms involved in the generation of immune responses leading to successful clearance of antigen and eventually resolution of inflammatory responses. *The main focus of this study is to examine the signaling pathways induced by cytokines and mitogens that are crucial in the regulation of CD44 expression in human monocytic cells and in human B cells.*

CD44

CD44 gene:

The *CD44* gene, spanning 50-60 kilobases of DNA is located on the short arm of chromosome 11 in humans and chromosome 2 in mouse (7;32;33). A recent search of the DNA databases revealed CD44 orthologues from eleven mammalian species, which share an overall 47-93% identity to the human sequence (34). Pre-messenger RNA (pre-mRNA) of human CD44 consists of a total of 20 exons (Fig. 1.1A), from which a large number of protein products (ranging in sizes from 85 to 200 kDa) are generated as a result of extensive alternative splicing of at least 12 exons (35;36). These include exons 6-15, also called variable exons 1-10 (V1-10), as well as exons 19 and 20 (34;37). The first 5 exons of the gene encode the constant region of CD44 and therefore, are always

A


Signal peptide
Transmembrane domain
Cytoplasmic domain
Extracellular domain
5'
3'
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20
Variable exons
3' UTR

B

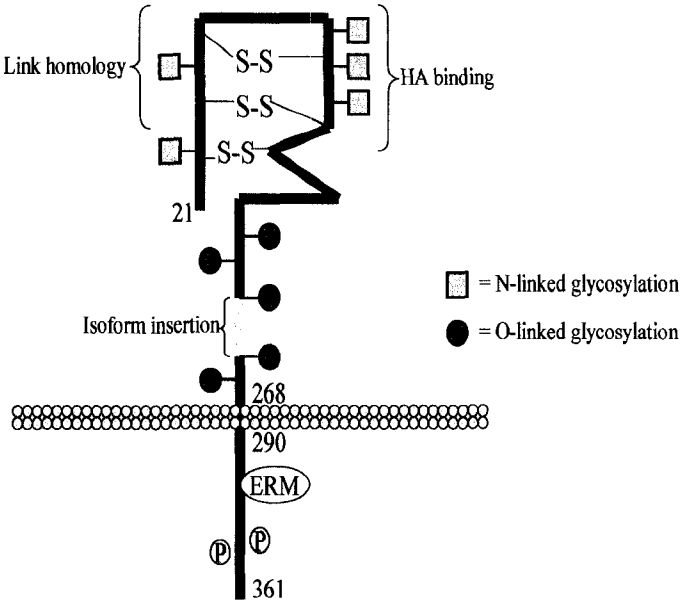

Link homology
S-S
S-S
S-S
HA binding
21
Isoform insertion
= N-linked glycosylation
= O-linked glycosylation
268
290
ERM
P
P
361

Fig. 1.1

Fig. 1.1: CD44 genomic organization and structure (7).

A) Schematic genomic organization of CD44: All exons are represented by the numbered boxes and the 10 variable exons (1-10v) are indicated by the blue shading. The three protein domains of CD44 are as indicated.

B) Schematic structure of the CD44 protein: The CD44 protein exists as a transmembrane glycoprotein. The extracellular domain contains link homology and hyaluronan (HA) binding domains, indicated by the brackets, and also sites for either N- or O-linked glycosylation, represented by the hollow squares or circles, respectively. The site of alternative isoform insertion close to outer membrane is represented by the blue area. The transmembrane domain is flanked by amino acids 268 and 290, as indicated. The presence of two serines with the potential for phosphorylation is indicated by the encircled yellow "P", and the binding site for members of the ezrin-radixin-moesin (ERM) family is indicated by the encircled "ERM".

present in all isoforms. The exons 1-5 and 16-20 are spliced together to form a transcript that encodes the ubiquitously expressed standard isoform (CD44s or CD44H) (34;37). This isoform consists of an N-terminal signal sequence (exon 1), a linked-homology hyaluronan-binding module (exons 2 and 3), a stem region (exons 4,5,16 and 17), a single-pass transmembrane domain (exon 18) and a cytoplasmic domain (exon 20) (34). For other isoforms, the variable exons 6-15 can be alternatively spliced and included within the standard exons at an insertion site between exons 5 and 16 (37). The membrane proximal region of the extracellular domain is encoded by exons 16 and 17, and the majority of exon 18 encodes the trans-membrane region of the protein. Inclusion of exon 19 results in the expression of a short cytoplasmic domain of CD44, whereas exon 20 encodes a longer cytoplasmic tail of CD44 (34;36;38).

***CD44* promoter:**

The region immediately upstream of the site of *CD44* translation initiation functions as a promoter (39-43). Fig. 1.2 shows the genomic nucleotide sequence (-1141 to +53) containing the human *CD44* promoter [GenBank™ accession number AH003670 or M69215] (36;43). The first nucleotide of the translation initiation codon (ATG) of *CD44* is marked as +1 (43). The two nucleotides at positions -136 and -128 serving as the major RNA initiation sites are marked by asterisks (43). TATA or CCAAT boxes were not found in the *CD44* promoter sequences. In addition, unlike the promoter regions of some other adhesion molecule genes (E-selectin, ICAM-1 and VCAM-1) (44;45), NF-κB consensus sequences were not found in the regulatory region of *CD44* (43;46). A hexanucleotide sequence GGGCGG at -298 to -303 bp was found upstream of the transcription initiation sites. This sequence referred to as GC box, has been found

Fig. 1.2: Nucleotide sequence of the *CD44* upstream regulatory region [GenBank™ accession number AH003670 or M69215](43)

The first nucleotide of the ATG codon serving as the translational initiation codon of CD44 is marked as +1. The sequence corresponding to the oligonucleotide for binding of transcription factor is underlined. The two nucleotides at positions -136 and -128 serving as major RNA initiation sites are marked by arrow-heads.

-1141 tggtttgtgg tttttatgaa gagatgtgaa aaaggaagtg tggaatgatg ggatgagaag

-1081 ttgtatgggg aagatgaata gaagaattag gtggttgaat aaaattaaaa ggtgtgtggt

-1021 tggatgaatg aatgagtggg atgatagatg gacctaagtg gttagtggat ggacaggagg

-961 atggatggat gtgagagccc cagaaggaca taaggaaaga tgggtggata gatggatggg

-901 cggatggaag gatatttagg aggatgaatg agcatgtgtg tggagagagg tgcccattca

-841 cactggcttg aacacatggg ttagctgagc caaatgccag ccctatgaca ggccatcagt

-781 agctttccct gagctgttct gccaagaagc taaaattcat tcaagccatg tggacttgtt

-721 attgagggga aaaagaatga gctctccctc tttccacttg gaagattcac caactcccca

-661 cccctcactc cccactgtgg gcacggaggc actgcgccac ccagggcaag acctcgccct

-601 ctctccagct cctctcccag gatatccaac atccctgtga aaccagagat cttgctccag

-541 ccggattcag agaaatttag cgggaaagga gaggccaaag gctgaaccca atggtgcaag

-481 gttttacggt tcggtcatcc tctgtcctga cgccgcgggg ccagcgggag aagaaagcca

-421 gtgcgtctct gggcgcaggg gccagtgggg ctcggaggca caggcacccc gcgacactcc

-361 aggttccccg acccacgtcc ctggcagccc cgattattta cagcctcagc agagcac**ggg**

SP-1, Egr-1 (-293 to -301) AP-2, AP-4, AP-1 (-238 to -243)

-301 **gcgggggcag** aggggcccgc ccgggagggc tgctacttct taaaa**cctct gcgggctgct**

-241 **tagtca**cagc ccccttgct tgggtgtgtc cttcgctcgc tccctccctc cgtcttaggt

-181 cactgttttc aacctcgaat aaaaactgca gccaacttcc gaggc**a**gcct cat**t**gcccag

-121 cggaccccag cctctgccag gttcggtccg ccatcctcgt cccgtcctcc gccggccccc

-61 gccccgcgcc cagggatcct ccagctcctt tcgcccgcgc cctccgttcg ctccggacac

-1 c **atg** gacaag ttttggtggc acgcagcctg gggactctgc ctcgtgccgc tgag +53

Fig. 1.2

within promoters of many cellular genes (lactate dehydrogenase, histones and CREB) and typically bind the transcription factor Sp1 (43;47). Furthermore, several CpGs are scattered within the 650-bp region immediately upstream of the CD44 translation initiation site (43;48;49). Furthermore, binding sites for other transcription factors such as the Egr-1 site at position -293 to -301 bp or the AP-1 site at -238 to -243 bp within the CD44 promoter were shown to be essential for CD44 induction in different cell types (50-54). The binding sites for Egr-1 and AP-1 are shown as underlined and bold nucleotides in Fig. 1.2.

CD44 proteins:

CD44 is comprised of a family of 85-200 kDa type-I transmembrane glycoproteins and can be divided into three domains: the extracellular domain, and the highly conserved transmembrane and cytoplasmic domains (Fig. 1.1B) (7). The extracellular domain is a globular structure generated by disulfide bridging through six conserved cysteine residues. The amino-terminal region is relatively conserved among mammalian species (~85% homology) while the membrane-proximal region is relatively nonconserved (approximately 35-45% sequence similarity among species). This globular structure also contains a region termed the link homology domain as it shares 30% homology with the cartilage link protein. This domain is found on all CD44 isoforms and is responsible for the ability of CD44 to bind HA. Several glycosylation motifs are also present in the region and modulations in N-linked and O-linked glycosylation levels also play a role in the induction of CD44-HA binding. Post-translational modifications of CD44 by sulfation or sialidation are found within the amino terminal and membrane proximal

regions of the extracellular domain and these events can also affect CD44-HA binding (8;24;37).

The membrane proximal region (also called the stem structure) contains the expressed alternatively spliced variant exons. The combination of the link domain with the variant isoform expression as well as the levels and types of glycosylation, determine the potential HA-binding ability of CD44 (24;38). The transmembrane domain consists of 23 amino acids and exhibits 80-90% inter-species homology. It contains 2 cysteine residues at the interface between the transmembrane domain and the cytoplasmic domain, which can potentially be modified with palmitic acid. It has also been suggested that this region may also be responsible for lipid raft association (4;34).

The highly conserved cytoplasmic tail of CD44 contains docking sites for several protein kinases that become activated upon CD44 interaction with HA. Phosphorylation site for phosphoinositide 3-kinase (PI3K) have been identified and it has been demonstrated that CD44 is associated with at least two tyrosine kinases, $p185^{HER2}$ and the c-src kinase (55;56). Both of these kinases have been shown to physically interact with the cytoplasmic tail of CD44: $p185^{HER2}$ via disulfide bonds, and c-src via a specific binding site (57). Binding of HA to CD44 triggers the activation of these kinases. The $p185^{HER2}$ kinase activity is associated with increased tumor cell growth while activation of c-src leads to formation of lipid rafts. In addition to the tyrosine kinases, CD44 has also been shown to be associated with the Rho-like GTPase RhoA. Interaction between CD44 and RhoA has been demonstrated in breast tumor cells and the activation of this kinase is capable of inducing an interaction between CD44 and ankyrin (56). The interaction between CD44 and ankyrin has been shown to affect CD44-HA binding and

may be important for correct membrane display of CD44 so that it can bind to HA and which will lead to HA-induced signal. Another GTPase, Rac1 has also been shown to be activated upon HA binding to CD44 promoting Cdc42, ERK MAPK, and Ca^{2+} signaling (58;59). Thus it appears that signaling proteins associated with the CD44 cytoplasmic domain are responsible for modulating CD44-directed cell adhesion and motility. In support of this, it is known that the cytoplasmic tail of CD44 also interacts with components of the cytoskeleton such as actin and members of the ezrin-radixin-moesin (ERM) family. These ERM proteins are important in the regulation of assembly structure and stability of the cell membrane as well as in the regulation of cell adhesion (34;60;61).

CD44 isoforms:

Thousands of possible combinations of variable exons exist and at least 20 different CD44 isoforms generated by alternative RNA splicing have been described to date (24;62;63). Of these, CD44H, CD44E, and variants containing variable exons V4-7 and V6-7 are best characterized. The smallest isoform, CD44H is the standard or hematopoietic (H) isoform and does not contain variable exons V1-10. All blood cells, including granulocytes, T and B cells, natural killer (NK) cells and cells of the monocytes/macrophage lineage express CD44H (64). CD44E is known as the epithelial (E) variant, containing exons V8-10, and is only weakly expressed in normal epithelial cells. However, it is present in abundance in many carcinomas (65). CD44 V4-7 and V6-7 are metastasis-associated variants; overexpression of these isoforms in non-metastatic rat pancreatic carcinoma and mammary carcinoma confers their metastatic behavior (66). CD44 R1 and R2 expression was detected in normal peripheral blood mononuclear cells (67). Interestingly, V6-containing isoforms have been associated with increased

aggressiveness in human non-Hodgkin's lymphoma (68). CD44 isoform expression was also investigated by our lab in a panel of EBV-negative and EBV-positive Burkitt's lymphoma (BL) B-cell lines. EBV-negative BL cells did not express CD44. In contrast, EBV-positive BL cells expressed CD44 H, R2 and E but not CD44 V6/V7 isoforms, suggesting an association between EBV infection and CD44 isoform induction (69).

CD44 and Hyaluronan Binding

The glycosaminoglycan hyaluronan (HA) is the principle ligand for CD44. It is a high molecular weight, negatively charged, polysaccharide composed of linear repeating units of disaccharide D-glucuronic acid (1-β-3) N-acetyl-D-glucosamine (5). Because HA is found surrounding proliferating and migrating cells, in connective tissues, and in lymphoid tissues, CD44–HA interactions may play an important role in the development of immunological phenomena (5). Other CD44 ligands include fibronectin, collagen, and serglycin; however, interactions between CD44 and HA are well characterized (37;70-72). For initiation of CD44-mediated HA binding, CD44 must first be "activated". Treatment of cells with various agents results in changes in the levels of CD44 expression, alternative splicing, and post-translational modifications of CD44 such that it can recognize its ligand. This property of CD44 has led to the identification of three "subspecies" of CD44: active (ie capable of HA binding), inducible (ie can be induced to bind HA), and inactive (ie those that do not bind HA) (19;23;73-75). Peripheral blood monocytes express abundant cell surface CD44 yet do not bind HA (75;76). However,

recent findings suggest that stimulation of monocytes with LPS up-regulates CD44-mediated HA-binding (75;76).

Several structural properties of CD44 have been shown to influence CD44-HA binding. They are phosphorylation of the cytoplasmic tail, interaction with other cytoskeletal proteins, oligomerization at the cell surface, and structural variations in the extracellular domain (post-translational modifications). However few studies have delineated the exact molecular mechanism by which CD44 is induced to bind HA (8;28;30;77-79).

Post-translational modifications of CD44 have been shown to play an important role in the regulation of ligand binding. Increase in the complexity of the glycosylation state of CD44 is associated with a decrease in CD44-HA binding capacity. Glycosylation of CD44 has also been shown to negatively affect CD44-HA binding (80;81). It has been shown that treatment of lung epithelial derived cancer cells with oncostatin M reduces the amount of N-linked glycosylation thereby inducing CD44-HA binding (74). TNF-α-induced CD44-HA binding in monocytic cells has also been shown to involve a decrease in N-linked glycosylation (19). Not only are the glycosylation levels involved in CD44-HA binding, but also more specifically, sialidation of the glycosyl moieties has been shown to inhibit CD44-HA interactions. Recently, in human monocytic cells, LPS- and TNF-α-induced HA binding have been shown to be regulated by an inducible sialidase; removal of sialic acid residues on the CD44 proteins enables CD44-HA binding (27;82). Further, sulfation of CD44 has also been shown to play a major role in mediating CD44-HA-interactions. Sulfation is thought to affect cell binding by creating a "neoepitope" through the addition of negative charges which then affects the structure of the protein

and therefore is thought to influence ligand binding (26). Treatment of CD14+ PBMC with TNF-α, IFN-γ, LPS, and IL-1β also increased the sulfation of CD44, concurrent with induction of CD44-HA binding. However, the induction of T cell CD44-HA binding by CD3 stimulation was shown not to involve sulfation (83), suggesting that the mechanism involved is dependent not only on the stimulus, but on the cell-type as well (29;83;84).

Because of the diversity in the factors which regulate CD44 expression as well as ligand binding, studies of the maintenance and regulation of this molecule will provide much needed information in the understanding of cell migration under normal and disease conditions.

Biological Functions of CD44

It is very well known that CD44 plays an important role in cell migration (11;14;61;85-88). Cell migration is the body's lifeline in terms of defense against infection and antigenic invasion. CD44 is involved not only in the normal physiologic circumstances but also in the generation of the inflammatory response as well as in the development of cancer (6;89-94). In addition, one report described its involvement during the migration of leukocytes to inflammatory site or during the cancerous process, and not during cell migration under normal circumstances (95). Hence, CD44 is intimately associated with two important pathological conditions inflammation and cancerous process. Therefore, an outline of the role of CD44 in cell migration and consequentially inflammation and the cancerous processes follows.

Role of CD44 in leukocyte-endothelial cell interaction and cell migration:

The most important aspect of the immunological defense system is the migration of lymphocytic cells to infection site or to injury (7;89). In our body, lymphocytes undergo continual recirculation. During this process, they are involved in lymphocyte-endothelial cell interactions leading to diapedesis and homing (96). A specialized structure within lymphoid tissue is composed of endothelial cells, termed the high endothelial venule (HEV) which is not found in non-lymphoid tissues. HEV allows the migration of lymphocytes into the lymphoid tissues from blood (97). Lymphocytes migrate to secondary lymphoid structures (ie lymph nodes, Peyer's patches, tonsils, and spleen) and to extra-lymphoid areas (ie inflamed skin, intestinal mucosa, pulmonary tissues, and joints) in order to clear invading antigens. Adhesion molecules and receptors on both lymphocytes and endothelial cells as well as chemokine expression regulate this entire process. The selectivity involved in motility is directed by the expression of particular adhesion molecules on lymphocytes and their respective receptors on the endothelial cells (96-98).

The interaction between lymphocytic cells and endothelial cells is a multistep process and is described in Fig. 1.3 (99). There are 4 distinct groups of adhesion molecules that are involved in the different steps of cell migration: selectins, integrins, members of the immunoglobulin superfamily, and CD44 (4;100-102). The first step in cell migration has been termed "tethering" and it involves a loose engagement between the lymphocyte and endothelium. Following this, the lymphocyte undergoes "rolling" Whereby, the lymphocyte rolls over the vascular endothelium of postcapillary venules. The rolling interactions are transient and reversible. The selectins are mainly involved in

Fig. 1.3: Overview of the general leukocyte-endothelial interactions during cell migration at an inflammatory site (99).

Chemoattractants and cytokines induced by the inflammatory agents activate the endothelial cells and thus affect receptor expression on the endothelial cell surface as well as on the passing leukocytes. Activation of the cells induces the expression of the necessary adhesion receptors and ligands to initiate leukocyte homing. The proteins responsible for the interactions of the leukocyte with the endothelial cells are depicted above the schematic diagram. Initially cells roll along the activated endothelial vessel wall (indicated by red squire). The selectins in association with CD44 are responsible for the initiation of rolling along the activated endothelial cell surface. Then, cells undergo firm adhesion mediated by the integrins and CD44, followed by diapedesis towards the inflammatory site mediated by integrins and inflammatory agants. ● represents inflammatory agents such as chemokines and cytokines that direct and activate the leukocytes as well as the endothelial cells.

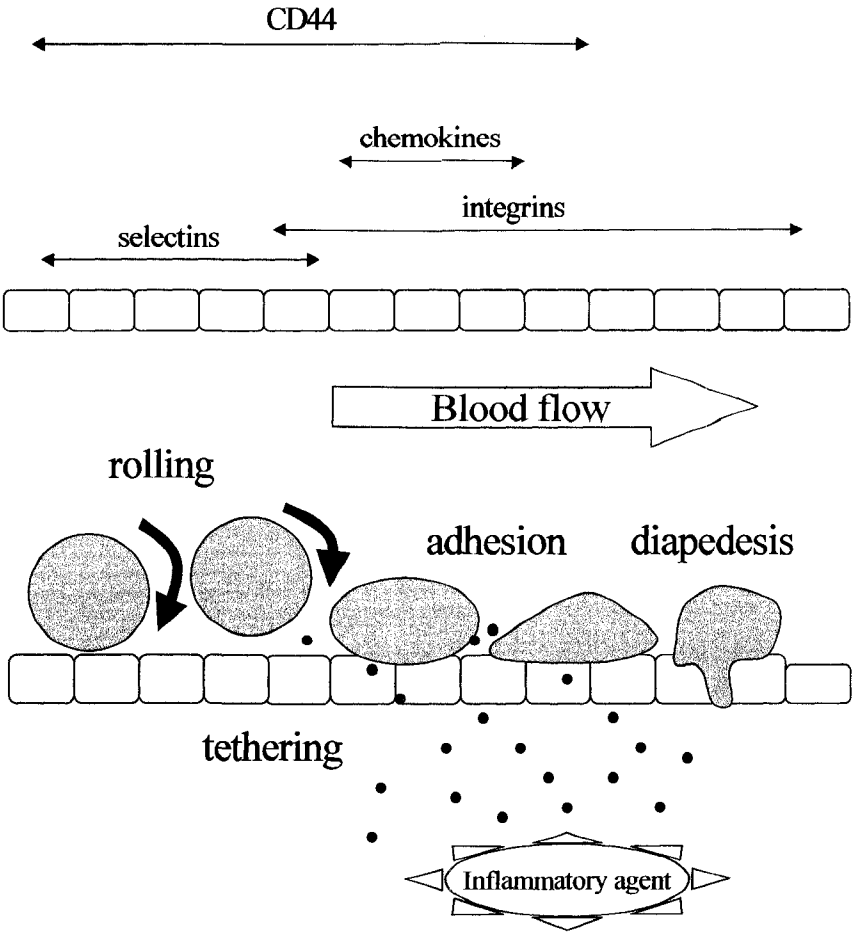

CD44
chemokines
integrins
selectins
Blood flow
rolling
adhesion
diapedesis
tethering
Inflammatory agent

Fig. 1.3

the rolling of blood cells through postcapillary venules in lymphoid tissues via binding to specific carbohydrate groups. Two main types of selectins are L-selectin (expressed on lymphocytes) and E-selectin (expressed on endothelial cells). CD44 has been shown to beinvolved in B and T lymphocytes rolling along cultured endothelial cells through the use of flow chambers designed to mimic physiological flow conditions as well as in the interactions between lymphocytes and tonsillar stroma (103-105).

The next step is stable adherence whereupon a rapid chemokine-induced activation of integrins expressed on the lymphocyte (for example $\alpha_L\beta_2$ also called lymphocyte function-associated antigen-1, LFA-1). The cell becomes stably adherent to the endothelium and although this step is reversible, it can promote the migration of lymphocytes across the vessel wall. Integrins are a heterodimeric family composed of α- and β-subunits and mainly $\alpha_L\beta_2$, $\alpha_4\beta_1$, and $\alpha_4\beta_7$ are involved in stable lymphocyte adherence. Binding of these integrins to the immunoglobulin superfamily members such as intercellular adhesion molecule (ICAM)-1 and ICAM-2 are responsible for the tighter adhesion of cells to the endothelium. The final step is diapedesis where the lymphocyte leaves the bloodstream (106;107). Recent reports indicate that CD44 in association with integrins such as very late antigen-4 (VLA-4, $\alpha4\beta1$) and/or LFA-1 play a crucial role in cell migration during inflammation and cancerous process. Nandi et al. (2004) demonstrated that CD44 in association with VLA-4 forms a bimolecular complex that is required for cell arrest and T cell extravasation during an inflammatory process (107). Similarly, in cancer, it has been reported that CD44 cross-linking induces integrin-mediated adhesion and transendothelial migration of human breast carcinoma cells by upregulating LFA-1 and VLA-4 (108).

Specificity of the migratory and homing interactions is mediated by the unique combinations of adhesion receptor pairs as well as the chemokines and cytokines present in the microenvironment of the cells. The sequence of events combined with the receptor/ligand partners expressed on the leukocytes and endothelial cells dictates the destination of the cells (98;103;109). CD44 has been shown to be directly involved in the homing and rolling interactions between activated lymphocytes and HA-expressing high endothelial venules resulting in the migration of cells to sites of inflammation as well as the metastasis of cancer cells (87;90;109-111). The interaction between CD44 and HA under these circumstances is critical as it costimulates antigen cell receptor-mediated proliferation, cytokine release, integrin activation via outside-in signaling resulting in protein tyrosine kinase activation, which, in turn, serves to regulate CD44-HA binding and CD44-dependent motility (6;14). The role of CD44 and cell migration in terms of cancer and inflammation is discussed in detail below.

Cancer:

Commonly, cancer is viewed as an abnormal and pathogenic growth of altered tissues. The establishment of tumor metastasis requires that cancerous cells acquire new adhesion and migration properties to emigrate from primary sites and colonize in distant organs. Many reports suggest that abnormally elevated CD44 expression has been associated with tumorigenesis and metastatic potential (112-114). As the stroma surrounding tumor cells contains elevated levels of HA, and because, the interaction between CD44 and HA is a critical feature of cell mobility and homing, CD44 is thought to play a key role in the induction of tumor formation and spread. Therefore, interaction of CD44 on cancer cells with HA expressed on the luminal surface of endothelial cells

may serve to promote tumorigenesis or metastasis. Additionally, CD44 interactions with HA expressed in the ECM may also serve to facilitate tumorigenesis as well as tumor metastasis by forming a molecular bridge which could serve as scaffold for tumor growth (103;109).

The type of cancer and the ability of tumor cells to invade various tissues and subsequently undergo metastasis are thought to be associated with particular isoforms of CD44 (115;116). Early studies have demonstrated distinct roles for a number of CD44 isoforms in tumorigenesis. A B16 mouse melanoma expressing CD44H are more aggressive than those not expressing CD44H (117). Additionally, the transfection of a Burkitt's lymphoma cell line, Namalwa, with CD44H induced tumors in nude mice, while injection of cells transfected with CD44E did not form tumors (118). Transfection of murine lymphoma cells with either CD44H or CD44 containing v4-v10 exons showed that the variant-expressing CD44 isoforms exhibited enhanced cellular migration and tumor forming ability when compared to the parental cell lines or those transfected with CD44H (119). Recently, animal experiments which have been conducted targetting CD44 expression and ligand binding by antibodies, antisense siRNA or CD44-soluble proteins resulted in the reduction of malignant activities of various neoplasms (21;90;120-125). The majority of patient studies with respect to CD44 expression attempt to focus on the relationship between CD44 expression and tumorigenicity. It must be stated that in these studies a high expression of CD44 does not always correlate with enhanced tumorigenicity or unfavorable prognosis. For example, increased CD44 expression is not associated in cases of lung (126;127) and skin cancers (116;128) and decreased CD44 expression has been associated with tumorigenic neuroblastoma cell lines (90). However,

in cases of renal, prostate, colorectal and breast cancers, primary cutaneous melanoma, B cell chronic lymphocytic leukemia as well as non-Hodgkin's lymphoma and Burkitt's lymphoma, high levels of CD44 expression are associated with non-favorable outcomes (129-137). Because, the second part (Chapter IV) of my thesis encompasses the regulation of CD44 expression in Burkitt's lymphoma cells, a brief introduction of Burkitt's lymphoma is included below.

Burkitt's lymphoma:

Chapter IV of this study focuses on the regulation of IL-4-induced CD44 expression in Epstein-Barr virus (EBV)-transformed Burkitt's lymphoma cells (BL30/B95-8). Burkitt's lymphomas (BL) are high grade tumors of B cell origin (sIg+, CD19+, CD20+) containing the characteristic c-myc translocation where c-myc is placed under the control of an immunoglobulin promoter (138;139). The translocation causes disregulation in cell cycle control, ultimately resulting in localized, rapidly growing tumors. BL has been classified into two subgroups: endemic (African, EBV+) and non-endemic/sporadic (American, EBV-) (139). In addition to the African Burkitt's lymphoma, EBV is also associated with B cell lymphomas of immunosupressed patients, nasopharyngeal carcinomas, non-Hodgkin's disease, and infectious mononucleosis. EBV is a potent B cell transforming virus where it can latently infect normal B cells resulting in immortalized, non-neoplastic lymphoblastoid B cell lines (B-LCLs) (139;140). As previously mentioned, the majority of patient studies attempt to link CD44 expression as a prognostic marker and in the case of BL, high CD44 expression levels have been observed and have been linked to unfavorable outcomes (130). In contrast, expression of CD44 appears to be lost in EBV-negative Burkitt's lymphoma cells, however upon

infection of BL cells with EBV, CD44 expression can be restored. For example, the BL30 cell line does not express CD44 however when these cells were infected with EBV, they constitutively expressed CD44 (141). CD44 negative BL cells have been useful in studies where CD44 is transfected into the cells and the cell line is subsequently analyzed for CD44-HA interactions and tumorigenicity when injected into mice. For example, CD44 transfected BL cells showed a variable capacity to bind HA and form tumors, depending on the isoform of CD44 transfected (142) and CD44H expressing Namalwa cells induced tumors in nude mice (118).

Inflammation and autoimmune disease:

CD44 expression and ligand binding ability have been demonstrated in the regulation of lymphocyte recruitment to sites of inflammation (87;107;143-146). Several studies have shown that migration of activated T cells to sites of inflammation is dependent on CD44 expression on T cells and is also dependent on HA expression on the endothelial cells (6;87). Studies of the regulation of CD44 expression and HA-mediated binding therefore are important in understanding the mechanisms for the development or control of autoimmune disorders (14;111). This discussion will only focus on the role of CD44 in chronic inflammation as seen in autoimmune disease rather than in acute inflammation, as the majority of evidence suggest a prominent role for CD44 in chronic inflammation (7;110;146-148).

Inflammation is often a key factor in the mechanism of disease processes as well as in response to tissue injury. Release of pro-inflammatory mediators and the recruitment of circulating lymphocytes which become activated at the site of inflammation are two main events of the inflammatory response (149). For example,

activation of T cells in response to antigen causes an influx of immune regulatory cells to the antigenic site, resulting in inflammation (150). Therefore, the inflammatory response is characterized by the release of proinflammatory mediators resulting in aggregation of leukocytes, increased vascular permeability, and tissue damage (89). Firan et al. (2007) suggested that CD44 delineates a population of naturally occurring T regulatory cells containing highly potent suppressor activity that may aid in the tracking of such regulatory function over the course of immune and autoimmune responses (151). A recent study revealed that CD44 is required to clear HA degradation products produced during lung injury; impaired clearance of HA results in persistent inflammation, because, HA fragments utilize both Toll-like receptor (TLR)4 and TLR2 to stimulate inflammatory genes in macrophages (152). Usually the inflammation is resolved by the release of anti-inflammatory factors which clear the inflammatory cells (149). However, failure of the immune system to control inflammation often results in chronic inflammatory disorders, which are the hallmark of autoimmune diseases.

Autoimmune disease can be characterized by high numbers of activated lymphocytes bearing the activated, HA-binding form of CD44 (153). High expression of CD44 appears to be important in the maintenance of autoimmune-induced inflammation by virtue of its ability to bind HA thereby increasing the cell number and propagating the inflammatory response (6). Over the past few years, efforts have been focused on the delineation of the role that activated, HA-adhesive, CD44 plays in autoimmune diseases. Many studies have been performed which take advantage of the fact that specific antibodies directed against CD44 can block its interaction with HA. Using murine models, these types of studies have shown that anti-CD44 antibodies can reduce disease

severity and inflammation. In a murine model of rheumatoid arthritis (RA), it was shown that treatment of mice with blocking antibodies to CD44-HA binding decreased tissue edema and leukocyte extravasation as well as RA-related inflammation and severity (154-156). A role for CD44 has also been demonstrated in the inflammation associated with experimental allergic encephalomyelitis. T cell activation and entry to the brain are key steps in the process of this disease and anti-CD44 antibodies reduced T cell entry to the brain as well as preventing lesion formation in the brain (157). Additionally, in a murine model of autoimmune diabetes using non-obese diabetic (NOD) mice, antibodies to CD44 conferred resistance to the onset of diabetes (158). The same study also illustrated the role for CD44-HA binding by treatment of cells with hyaluronidase, which also had the effect of preventing the onset of disease.

Although the antibodies used may indeed block CD44-HA binding, they may also influence signals generated through CD44, which may themselves reflect changes in CD44 expression and binding capacity. The use of CD44 knockout mice may provide an alternate model for such studies as they enable the *in vivo* study of migration patterns and inflammatory responses induced by autoimmune diseases. It was demonstrated that in CD44-deficient murine models of arthritis, the lymphocyte cell migration to lymph nodes is actually accelerated as observed in the case of super-antigen-induced inflammation (159;160). Therefore, under inflammatory circumstances, CD44-deficient cells exhibit accelerated homing to lymph nodes compared to that of wild type. However, there exists a delay in the migration of CD44-deficient lymphocytes to chronically inflamed joints or tissue; it appears that CD44-HA interactions play a role in the induction and propagation of inflammation at the primary source and that the CD44-HA interactions serve to keep

lymphocytes in circulation and may not play a significant role in sending the cells back to the lymph nodes (159;160).

CD44 has also been shown to be involved in the resolution of lung inflammation through the use of CD44-deficient mice (144;161). Wang et al. (2002) observed that in CD44-deficient mice, inflammatory process was enhanced in *E. coli*-induced pneumonia (161). These studies also indicated that CD44, in addition to the role played in the induction of inflammation in rheumatoid arthritis, it might also play a role in the clearance of inflammation as demonstrated in the resolution of lung inflammation.

Regulation of CD44 Expression

Cytokines and mitogens are important mediators of not only the inflammatory immune response, but also of tumorigenesis and metastasis (149;162-164). Because CD44 plays a role in the migration of immune cells and also CD44 expression and ligand binding are differentially regulated by various cytokines and mitogens, it is important that the effect of these immune modulators be studied. Induction of CD44 expression and ligand binding may occur as two distinct events (27;165;166). It has been demonstrated that, depending on the stimulus and cell type, CD44 expression may be upregulated, but not its ligand binding ability (165). Conversely, it may be possible for the ligand binding capacity of CD44 to be increased, without an increase in CD44 expression, however *in vivo* evidence for such an event has not been shown as yet.

The effect of cytokines on CD44 expression and its ligand binding ability has been investigated in different cell types. IL-5, a key mediator of asthma-related eosinophilic inflammation has been shown to enhance CD44 expression in human

eosinophils (167). As well, IL-5 has been shown to induce CD44-HA binding in murine B cells (168;169). In human hematopoietic progenitor cells, IL-3 and GM-CSF, which direct the leukocyte development, induce CD44-HA binding (170). It has been shown that in normal human T cells, the proinflammatory cytokines IL-12 and IL-18 are capable of modulating CD44-HA interactions (171). In rat epithelial cell lines, IFN-γ has been shown to upregulate CD44 expression but not binding to hyaluronan. Treatment of these cells with TNF-α had no effect on either CD44 expression or binding (172). Our lab previously demonstrated that PMA and IL-4 induce CD44 expression and HA-binding in a Burkitt's lymphoma B cell line, BL30/B95-8 cells (141;173). In human monocytic cells, LPS, TNF-α, and IL-10 also induce the expression of CD44 as well as HA-binding (19;27;75;166).

The role of signaling proteins responsible for the activation of the transcription factors critical to CD44 expression is not well understood. Previously, our lab demonstrated that LPS-induced CD44 expression is regulated by JNK MAPK, and the p38 MAPK is partially involved in the regulation of CD44 expression in TNF-α-stimulated promonocytic THP-1 cells (166). The p38 MAPK is also involved in the generation of TNF-α-induced functionally active hyaluronan adhesive CD44 by activating sialidase in THP-1 cells (27). Ladeda et al. (2001) showed that the activation of the G protein Ral A plays a role in the induction of CD44 in v-src or v-ras transformed NIH3T3 fibroblast cells (174). Further, CD44 expression and binding has also been shown to be upregulated in response to phorbol myristate acetate (PMA), insulin growth factor (IGF)-1, and platelet-derived growth factor (PDGF) in a human neuroblastoma cell line (SK-N-SH) (175). Along with upregulation of CD44 expression, variant isoform

expression was also induced in these cells. In addition, this study also revealed that upregulation of CD44 isoform expression as well as HA-binding was sensitive to specific signaling inhibitors of PI 3-kinase and PKC, thus suggesting a role for these kinases in the regulation of CD44 activation (175).

Examination of the promoter region of CD44 reveals the presence of many putative transcription factor binding sites, however only two factors have been shown to affect the expression of CD44. Of these sites, the consensus site for the early response gene-1 (Egr-1) has been shown to be involved in CD44 expression following B cell receptor (BCR) stimulation in a murine B cell line, WEHI-231 cells, and in hepatocyte growth factor (HGF)-induced melanoma cells (50;52). Following IL-1 stimulation of ECV304 cells (originated from human umbilical vein endothelial cells) demonstrated that AP-1 activity is required for increased CD44 expression (53). Lamb et al. (1997) demonstrated that AP-1 activity is required for increased CD44 expression in Fos- and EGF-transformed cells (54). As well, Foster et al. (2000) have shown that IL-1β stimulation of rat aortic smooth muscle cells induces CD44 expression via activation of AP-1 in cooperation with the architectural protein: high mobility group-I(Y) protein [HMG-I(Y)] (39). A431 cells stimulated with EGF result in AP-1-dependent upregulation of CD44 as well as its colocalization with ezrin to areas of membrane ruffles and microvilli (176). In summary, it appears that Egr-1 in addition to AP-1, plays an important role in the induction of CD44 expression depending on the cell type and stimulation.

Signal Transduction Pathways

Signal transduction pathways are important to most of the vital functions in the cells. Phosphorylation has been shown to be required for the activation as well as transmission of signals (177). Signaling kinases and phosphatases are recruited to the receptor and signals are passed from one protein to another via modulations in phosphorylation, which affect the conformation, activation-state, protein interactions, cellular localization, and sensitivity to degradation (177). Protein kinases are responsible for the covalent attachment of a phosphate to the side chain of either tyrosine and/or serine, or threonine of proteins whereas the protein phosphatases are responsible for removal of the phosphate from those proteins. The active site of the kinase where the phosphorylation occurs is termed the "active loop" and is highly conserved among kinases. As the signal filters through the cytoplasm of the cell, eventually it is transmitted into the nucleus where the activation of transcription factors and repressor/enhancer elements are affected and thus gene transcription is initiated or down-regulated. It is known that the human genome carries between 500-600 protein kinase genes alone, which underlies the potential diversity in the signals capable of being transduced (177).

The signal transduction pathways responsible for modulating CD44 expression are not fully elucidated. As signaling pathways initiated by LPS, and TNF-α as well as IL-4 are the focus of this study, a brief explanation of these molecules and their signaling pathways is included below.

MAPK signaling pathway:

The mitogen-activated protein kinases (MAPK) are a well-conserved family of serine-threonine kinases that are activated in response to several different stimulants such

as growth factors and cytokines as well as cellular responses to stress. Activation of these kinases affects gene expression which can then alter the various cellular functions including cell proliferation and differentiation. In cases where aberrant activation or down-regulation of the pathway occurs, malfunction of the cellular processes can result. This is especially apparent during oncogenesis or infectious disease processes (178-180).

The MAPK signaling cascade is a three-tiered system whereby the MAPKs are activated by dual phosphorylation via the MAPK kinases (MAPKK) and the MAPKK are activated by the MAPKK kinases (MAPKKK). Each of the MAPKs, MAPKKs, and MAPKKKs activate specific kinases as determined by distinct sequence motifs. In order for activation of the MAPK to occur, they must be phosphorylated at the typical threonine in the conserved activation loop as well as at a neighboring tyrosine residue. The MAPKs are phosphorylated in the cytoplasm affecting activation of other kinases, transcription factors, or co-activators (179;180). An outline of the LPS-activated MAPK cascade is illustrated in Fig. 1.5.

In general, activation of a receptor tyrosine kinase results in the association of the adapter protein Grb2 with the cytoplasmic domain of the receptor. This association also recruits the guanine nucleotide exchange factor protein son of sevenless (SOS) to Grb2, which serves to activate Ras, a G-protein, by the exchange of GTP for GDP. Upon Ras activation, Raf binds to Ras (a MAPKKK), creating an anchor to the cell membrane and activating MEK (a MAPKK) via serine phosphorylation, which, in turn, activates the MAPK. There are 7 MEK kinases, MEK 1-7, and there are three main families of MAP kinases, extracellular-regulated protein kinase (ERK)1/2, c-jun-N-terminal kinase (JNK), and p38 based on sequence homology (179;180).

Of the three family members of MAPKs, ERK1/ERK2, also known as p42/44 is the best characterized. The ERK kinases are activated by several factors, for example, cytokines and growth factors. ERK2 is considered to be the prototypic member of the MAPK cascade. Activation of these kinases mainly affects cell growth and differentiation and several stimuli including growth factors, cytokines, viral antigens, carcinogens, and G-protein-coupled receptors are known to activate this arm of the MAPK pathway. The MAPKKK that serve to activate ERK1/ERK2 are members of the Raf family (c-Raf1, B-Raf, and A-Raf), all of which can be activated by Ras. Once those particular MAPKKKs are activated, the MAPKKs, MEK1 and MEK2 serve to activate the ERKs. ERK2 is responsible for activation of D-type cyclins as well as the retinoblastoma protein leading to cell cycle progression. The family of JNK MAPKs includes JNK1, JNK2, and JNK3. JNK1 and JNK2 were originally identified as the kinases responsible for the induction of activation protein-1 (AP-1) activity resulting from their phosphorylation of c-Jun. JNK1 and JNK2 appear to be widely expressed, however JNK3 expression seems to be limited to the brain. The JNK MAPKs are activated mainly by environmental stress, radiation, and growth factors and are known to play a key role in both apoptosis-related and cell survival-related events. These opposite effects of JNK may be explained as these events may be dependent on the stimulant, cell type, and combination of other signaling pathways induced. There is a wide range of upstream kinases responsible for the activation of the JNK MAPKs, including MEKKs, apoptosis signal-regulating kinase 1 (ASK1), TGF-β-associated kinase-1 (TAK-1), and the serine/threonine kinase Tpl-2. The best characterized kinases known to activate the JNKs are MEK4 and MEK7. These dual-specific kinases phosphorylate JNK on Thr183 and Tyr185. JNKs have been shown to

activate several transcription factors including AP-1, activating transcription factor -2 (ATF-2), Elk-1, p53 and c-Myc (179;180).

The p38 MAPK family is comprised of p38α, β, γ, and δ. Of these, the p38α is best characterized. Activation of the p38 MAPKs regulates the expression of number of cytokines, particularly those involved in inflammation, such as IL-10. The p38 MAPKs are activated by cytokines, hormones, stress, and G-protein-linked receptors. It is known that MEK3 and MEK6 are responsible for the activation of the p38 MAPKs, however, it has been recently demonstrated that the scaffolding protein TAK-1 binding protein 1 (TAB1) can activate p38 through the activation of TAK-1 (179;180).

Once activated, the MAP kinases can affect the activation of specific transcription factors for gene transcription. Down-regulation of the induced signaling cascade is as critical to cell function as is activation of this pathway. Specific protein phosphatases function to return the MAPK to inactive states. For example, the phosphatase of activated cells 1 (PAC1) specifically associates with and down-regulates the phosphorylation of p42. Other negative regulators of the MAPK, such as the protein phosphatase type 2C (PP2C) and MAPK phosphatase (MKP)-1 and MKP-2 have been shown to affect the phosphorylation status of p38 and JNK. The complexity of the MAPK pathway is also compounded by the fact that cross-talk occurs between the members of the MAP kinase pathway and the members of the other signaling pathways, in consequence, the control and specificity of the signals trasduced are affected (178;179).

Calcium (Ca^{2+}) signaling pathway:

Ca^{2+} is an almost universal intracellular messenger, controlling a diverse range of cellular processes, such as gene transcription, muscle contraction and cell proliferation

(181;182). It is an essential component to initiate Ca^{2+} signaling pathway activating array of signaling molecules (183). Fig. 1.4 describes the calcium signaling pathway along with the specific pharmacological inhibitors highlighted in color. The Ca^{2+} signaling pathway may also synergize with other signaling pathways which control different cellular functions (184;185). It has been suggested that the calmodulin (CaM), PKC and $p21^{ras}$/PI3K/Akt simultaneously activated by cytoplasmic Ca^{2+} level are involved in the series of signaling events connecting Ca^{2+} second messenger to NFκB activity (186). PKC may integrate two types of signals, one coming from membrane receptors acting through PLC (187) and the second elicited by Ca^{2+} for Ca^{2+} sensitive PKCs (188). The ras pathway acts similarly, integrating membrane receptor signals through G proteins and Ca^{2+} signals through ras-GRF and it may activate PI3K (56;77;189). Since ras activates the MAPKs, Ca^{2+} may activate MAPK through ras by ras GRF. CaM, a Ca^{2+}-activated protein regulates phosphatases and kinases, among which calcineurin and calmodulin kinase-II (CAMK-II) have been reported to be involved in activation of NFκB in neuron cells (186;190). Induction of transcription factors by various stimuli has already been shown to require Ca^{2+} for proper signal transduction. Since Ca^{2+} is upstream of all the major kinase signaling pathways (186;191;192), I investigated the involvement of calcium signaling pathway in induction of CD44 expression by LPS and TNF-α in human monocytic cells.

PI 3-kinase signaling pathway:

PI 3-kinase (PI3K) signaling network is crucial to widely divergent physiological processes that include cell survival, differentiation, proliferation, cell migration and also apoptosis (193;194). The deregulation of these processes influences numerous cellular

Fig. 1.4: Ca^{2+} signaling pathway:

Binding of ligand to respective receptor induces Ca^{2+} influx either through receptor-mediated entry or through voltage-dependent ion channel entry through cell membrane. PLCγ is activated which cleaves PIP_2 to IP_3 and diacylglycerol (DAG). There is also release of calcium from endoplasmic reticulum through binding of IP_3 to its receptor on endoplasmic reticulum. As shown in the figure in arrows, influx of calcium activates calmodulin followed by CaMK-II and calcineurin, which leads to activation of transcription factors resulting in expression of various genes. Calcium ions also activate PKC pathway in association with DAG leading to gene expression. The pharmacological inhibitors used to block the activity of specific Ca^{2+} signaling molecules are highlighted.

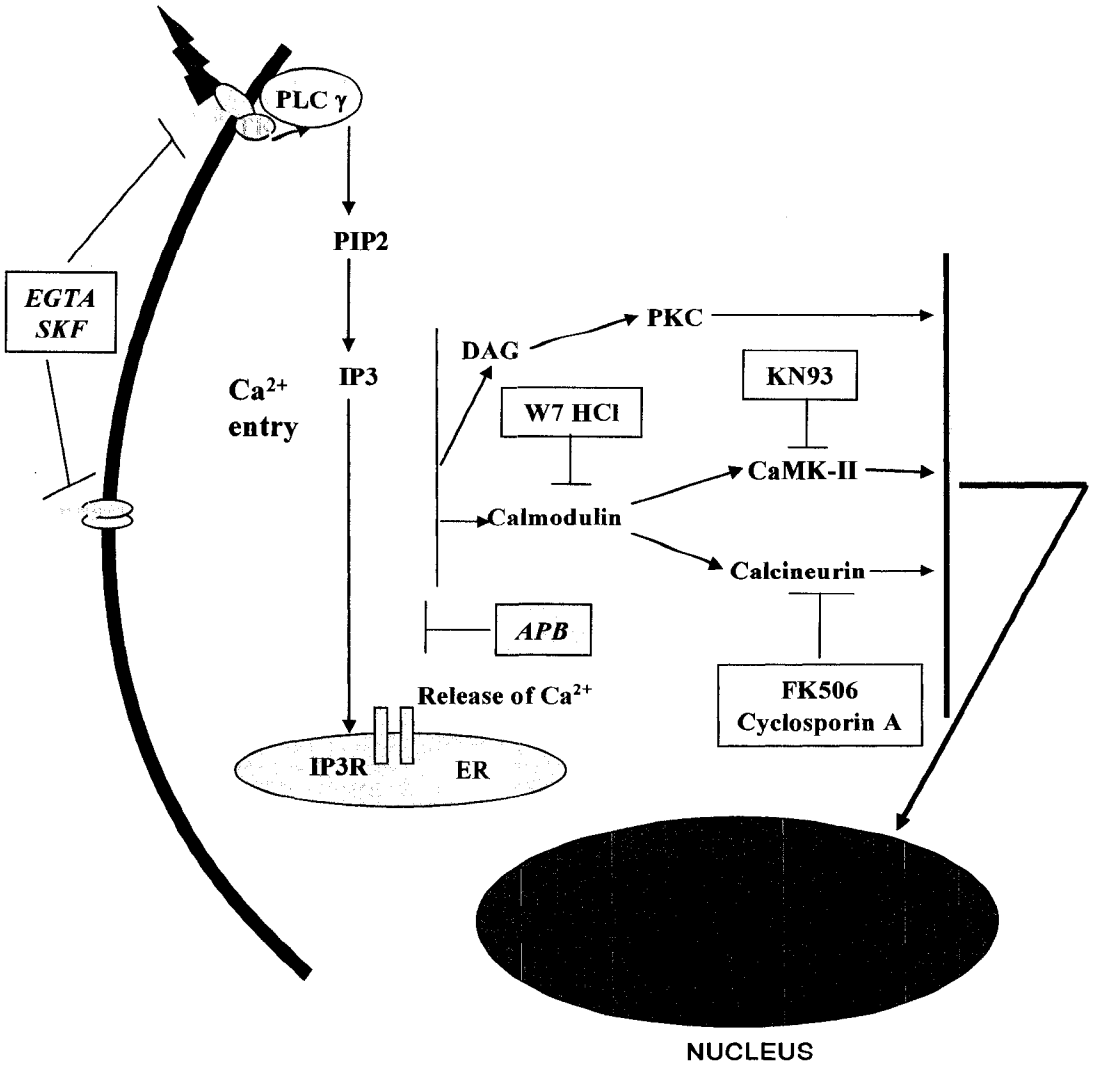

PLC γ
PIP2
EGTA
SKF
Ca2+
entry
IP3
DAG
PKC
KN93
W7 HCl
CaMK-II
Calmodulin
Calcineurin
APB
Release of Ca2+
FK506
Cyclosporin A
IP3R
ER
NUCLEUS

Fig. 1.4

responses which are associated with cancer phenotypes. This family of enzymes is activated by growth factor receptor or by the small GTPase Ras and produces 3' phosphoinositide lipids. PI3K is involved in phosphorylation of phosphatidylinositol (PI) on the third carbon of the inositol ring forming PI(3)P. The phosphorylation of PI(3)P to $PI(3,4)P_2$ and $PI(3,4,5)P_3$ is a part of many signal cascades in a cell (194). The lipid products of PI3K act as second messengers by binding to and activating diverse cellular target proteins, which leads to cell proliferation, differentiation, chemotaxis, survival, and trafficking. $PI(3,4)P_2$ and $PI(3,4,5)P_3$ lipids produced by PI3K are able to bind Akt recruiting it to the plasma membrane. Akt recruitment at the plasma membrane results in a conformational change, which enables the activation loop of the kinase to be phosphorylated on Thr308 by phosphoinositide-dependent protein kinase-1 (PDK-1, which also requires 3-phosphorylated inositol lipids for activation and plasma membrane translocation) and at Ser473 in the C-terminal hydrophobic motif by a kinase (often referred to as PDK-2) (194;195). Phosphorylated Akt targets the proteins involved in cell survival (194;196). Further, PI3K/Akt pathway is also activated by insulin receptor substrate-2 (IRS-2) protein during IL-4 signaling in lymphocytes. Recruitment of IRS-2 to the activated IL-4Rα chain results in its phosphorylation and subsequent activation of downstream signaling proteins, including PI3K/Akt (197;198). The PI3K family is divided into 4 different classes, namely Class Ia, Ib, II, and III based on their structure and substrate specificity. All of them contain one catalytic domain and one regulatory domain. Class Ia enzymes consist of any one of the catalytic units (p110α, p110β, and p110δ) complexed with any one of the regulatory subunits (p85α, p85β, and p55γ). There is only one Class Ib enzyme known and it contains the p110γ catalytic subunit and p101

regulatory subunit. Relatively little is known about the specific functions of Class II and III PI3Ks in non-mammalian (yeast) and mammalian systems (199). However, the role of Class Ia enzymes are well characterized. Class Ia enzymes are activated by tyrosine kinases (e.g. growth factor receptor, antigen receptors), while the class Ib enzyme is activated by G protein coupled receptor (194;199;200).

LPS signaling:

The mitogen, LPS, a component of gram-negative bacterial cell walls, is a potent inducer of monocytes and macrophages, which are key mediators of the innate immune response (201). LPS initially interacts with the soluble LPS-binding protein (LBP) and this complex then interacts with the LPS receptor, CD14 on the surface of the cell membrane (202;203). The signal transduction pathway activated by LPS is depicted in Fig. 1.5. CD14 is a glycosylphosphatidylinositol (GPI)-anchored protein with no transmembrane domain. The signals are transduced through the Toll-like receptor (TLR)4. Toll-like receptors (TLRs) are a family of cell surface receptors that play an important role in protection against viral, bacterial, and protozoal infections. They exist as type-1 transmembrane proteins in animals and are found as cytoplasmic proteins in plants. Up to 11 TLRs have been identified in humans to date(204;205). The human prototypes of these proteins are the TLR2 and TLR4. TLR4 is specific for generating a signal as a result of its association with LPS, CD14 and MD2 (206-208).

LPS interaction with CD14 promotes dimerization of TLR and subsequent recruitment of MyD88, a myeloid differentiation marker that functions as an adapter molecule. MyD88 associates via its c-terminal toll homology domain with the TLR, and via its N-terminal death domain with a serine-threonine protein kinase, IRAK1/4 (IL-1R-

Fig. 1.5: LPS signaling:

LPS binds to LPS binding protein (LBP) which helps transport LPS to CD14. LPS then comes into contact with both TLR4 and MD-2, a small protein associated with the TLR4 ectodomain. The signal is then transmitted to various signaling molecules including MAPKs, PI3K/Akt and Ca^{2+}/CaM/CaMK-II. MAPK signaling cascades are organized hierarchically into three-tiered modules. MAPKs are phosphorylated and activated by MAPK-kinases (MAPKKs), which in turn are phosphorylated and activated by MAPKK-kinases (MAPKKKs). The MAPKKKs are in turn activated by interaction with the family of small GTPases and/or other protein kinases, connecting the MAPK module to cell surface receptors or external stimuli. Further, ERK MAPK is activated by a well known pathway with upstream molecules Ras, C-Raf and MEK1/2. Finally, in the downstream, all the MAPKs such as p38, JNK and ERK are activated to regulate gene expression by activating various transcription factors, for example NFκB, AP-1, Egr-1, SP-1 etc. PI3K/Akt pathway involves PDK to activate Akt. However, many signaling proteins participate in Ca^{2+} signaling pathway-mediated gene expression including CaM and CaMK-II to activate transcription factors for gene expression. The specific pharmacological inhibitors for few signaling molecules used in this study are mentioned in the beige boxes.

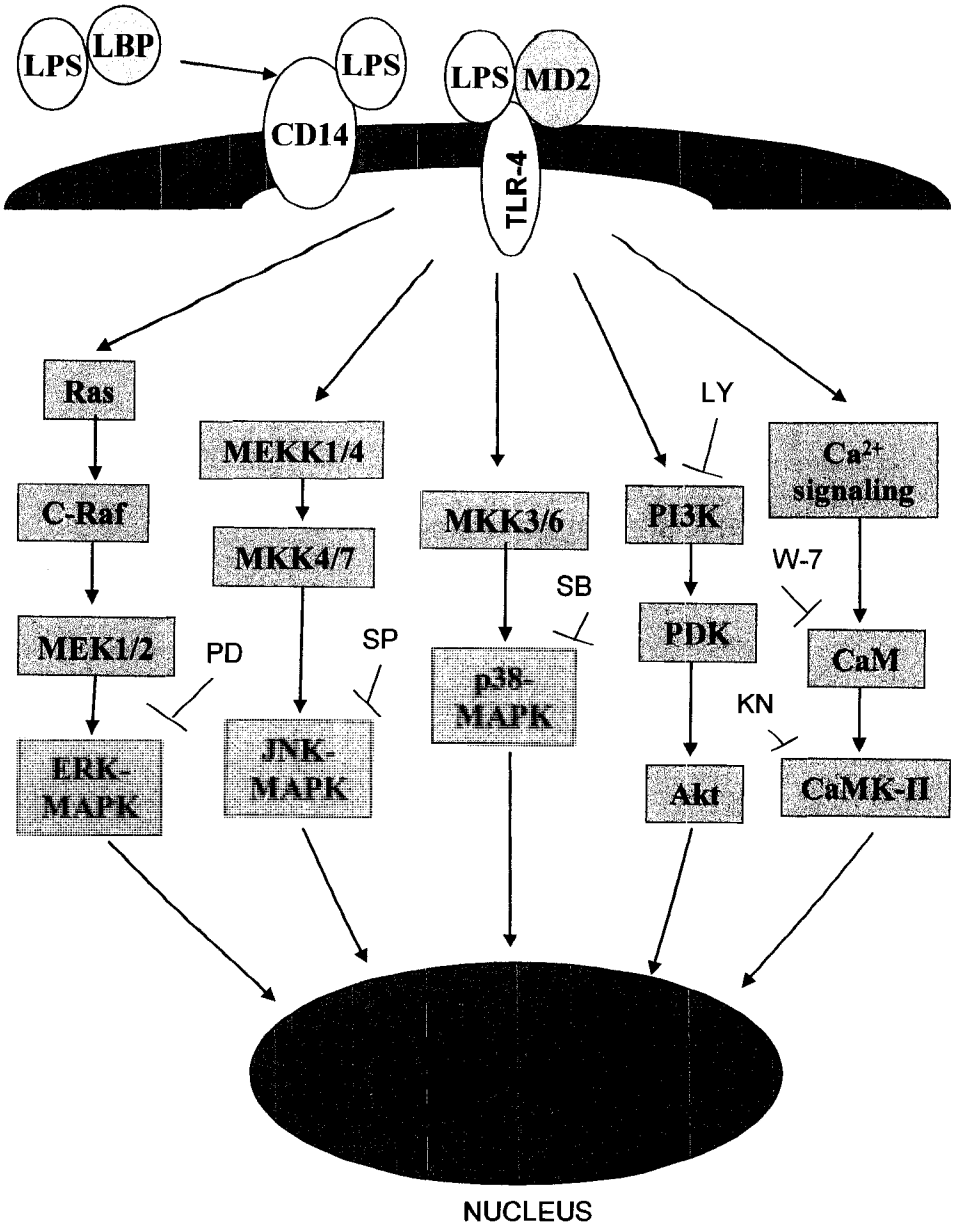

LPS
LBP
LPS
CD14
LPS
MD2
TLR-4
Ras
C-Raf
MEK1/2
ERK-MAPK
PD
MEKK1/4
MKK4/7
JNK-MAPK
SP
MKK3/6
p38-MAPK
SB
LY
PI3K
PDK
Akt
Ca2+ signaling
W-7
CaM
KN
CaMK-II
NUCLEUS

Fig. 1.5

associated kinase). Upon interaction with MyD88, IRAK is hyperphosphorylated and is thought to interact with the TAK-1 binding protein 2 (TAB2), a membrane-associated adaptor protein. This complex results in TAB2 binding to the adaptor protein TNF-receptor-associated factor-6 (TRAF-6). TRAF-6 subsequently interacts with the TAB1, the activator for TAK-1 which leads to NF-κB activation. TRAF-6 also activates the members of the MAPK signaling cascade, p38, p42/44, and JNK. Activation of MAPKs can result in the activation of Egr-1, AP-1, and NFκB (209-211). TRAF-6 activation is required for the induction of TLR4 signals, as DN TRAF-6 abrogates TLR4 signaling. In addition to MyD88-dependent activation of LPS-mediated signals, other signaling pathways may be activated independently of MyD88. For example, activation of the MAPK cascade may occur via the activation of PKC isoforms PKCβ and PKCζ (207). The MyD88-independent signaling pathway was identified in studies using $MyD88^{-/-}$ mice (212;213). The $MyD88^{-/-}$ mice were resistant to LPS-induced death with delayed activation of both NF-κB and the MAPKs pathways. Furthermore, MyD88-deficient cells failed to release proinflammatory cytokines in response to LPS suggesting direct activation of NF-κB and the MAPKs by LPS (212;213). Collectively, these results suggest the existence of a MyD88-independent pathway downstream to LPS signaling.

TNF-α signaling:

TNF-α is a proinflammatory cytokine that is produced by activated immune cells including T cells, monocytes/macrophages, and dendritic cells (214). It mediates numerous inflammatory and immunoregulatory activities. Besides its inflammatory properties, TNF-α is also responsible for the induction of apoptotic and anti-apoptotic signals (214;215). An overview of TNF-α–mediated signaling is depicted in Fig. 1.6.

Fig.1.6: TNF-α signaling:

TNF-α mediated signaling in the cell leads to either cell survival or apotosis. TNF-α binds either the TNF-αR1 or TNF-αR2 and the resulting signals affect many intracellular signaling pathways including caspases, MAPKs, PI3K/Akt and Ca^{2+} signaling pathways. The signals initiated from the death domain (DD)-containing TNF-αR1 are characterized by the release of the inhibitory protein SODD which enables TRADD recruitment to the TNF-αR1. This interaction results in the recruitment of FADD and TRADD-induced activation of FADD affects the activation of Caspase-8 leading to Caspase-3 activation and the induction of apoptosis. The association of TRADD to the TNF-αR1 can also result in the recruitment of the adaptor protein RIP as well as TRAF-2 and FADD. The TRAF-2 kinase is responsible for the activation of JNK which results in the activation of the transcription factors AP-1 and NF-κB. Binding of TNF-α to the TNF-αR2 results in the activation of TRAF-2 only, as these receptors do not contain the death domain, and thus, activation of signaling through this receptor only affects the ultimate activation of various transcription factors including AP-1 and NF-κB.

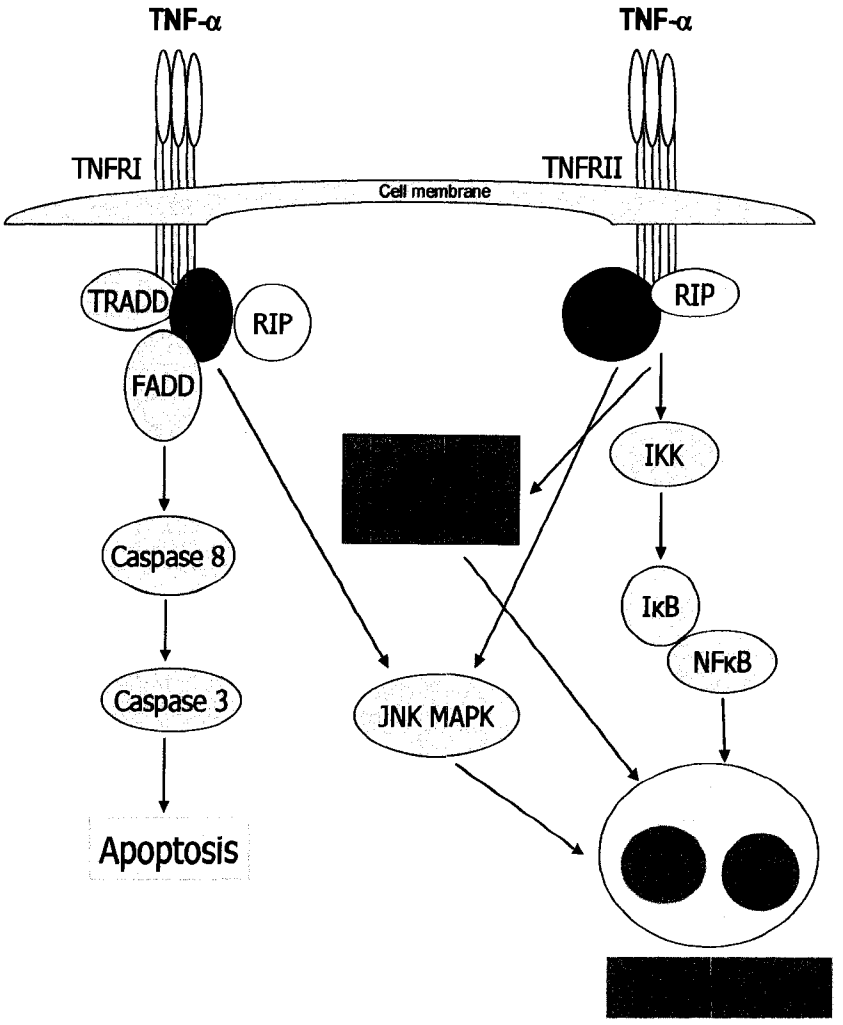

TNF-α
TNF-α
TNFRI
TNFRII
Cell membrane
TRADD
RIP
RIP
FADD
IKK
Caspase 8
IκB
NFκB
Caspase 3
JNK MAPK
Apoptosis

Fig. 1.6

TNF-α-induced signals are generated when TNF-α, which exists as a homotrimer, interacts with the TNF-α receptor units. There are more than 20 members of the TNF-αR super-family which can be divided into two groups: those that contain a death domain, and those that do not. Presence of the death domain leads to caspase activation and therefore the induction of apoptosis. Thus, the presence of two different receptor types can explain the differential roles of TNF-α in either the induction or the prevention of apoptosis (cell survival) (214;215).

The two types of TNF-αR are called TNF-αR1 and TNF-αR2. Notably, binding of TNF-α to TNFR1 causes upregulation of pro-apoptotic signals but it also leads to the translocation of NF-κB into the nucleus, protecting the cell from apoptosis. Whether the end result of TNF-α binding is apoptosis of the cell or protection from apoptosis depends on the cell type and its state of activation. The TNF-αR1 (CD120a) contains the death domain whereas TNF-αR2 (CD120b) does not. Upon binding of TNF-α to TNF-αR1, an inhibitory protein, the silencer of death domains (SODD) is released from the cytoplasmic domain of the receptor. Following the release of SODD, the TNF-αR1 cytoplasmic domain becomes accessible to the TNF receptor-associated death domain (TRADD) adaptor protein. The interaction of TRADD and TNF-αR1 recruits other adaptor proteins to the complex, namely, the receptor-interacting protein (RIP), TNF-αR-associated factor 2 (TRAF2) and Fas-associated death domain (FADD) (216). These proteins function to recruit additional signaling kinases. The recruitment of Caspase-8 by FADD leads to Caspase-3 activation and the induction of apoptosis, whereas TRAF2 attracts the cellular inhibitor of apoptosis protein (cIAP)-1 and -2 which inhibit Caspase-3

activation. The MAP kinase cascade is also thought to be activated through TRAF2 by interacting with MEKs and initiating the rest of the MAP kinase cascade resulting in the activation of p38, p42/44, and JNK. This cascade activates various transcription factors including NF-κB and AP-1 (215;217).

Signals transduced through the TNF-αR2 occur via the activation of TRAF2 whose activation serves to recruit the same MAPK signaling proteins as for the TNF-αR1, but without the activation of the TRADD-dependent apoptosis. Therefore, activation of signaling through TNF-αR2 results in the regulation of NF-κB and AP-1 (215;217).

IL-4 signaling:

IL-4 is a multifunctional cytokine of 12-13 kDa which is produced by T helper type II (Th2) cells, basophills, mast cells, and NK1.1 T cells (218). The biological functions of IL-4 include stimulation of T and B cell proliferation, induction of anti-CD40 dependent IgE class switching, and the upregulation of MHC class-II and CD23 expression on B cells and macrophages (218-220).

The receptors for IL-4 are shown in Fig. 1.7. The IL-4 receptor consists of two receptor chains: the IL-4Rα and the γ common (γc) chain (221). The γc chain is shared by receptors for IL-2, IL-4, IL-7, IL-9, IL-15, and IL-21 (222-225). The IL-4Rα is expressed on all hematopoietic cells, including normal human monocytes, B, and T cells (226-228). Cell lines such as THP-1, COS-7, MonoMac6, plasmacytoma B9, and renal carcinoma have been shown to lack the γc chain expression, but still respond to IL-4, suggesting a potential existence of the type I IL-13R on these cells. Because, IL-4 is capable of binding to the IL-13R type 1 in addition to the IL-4R (228). In fact, non-hematopoietic

Fig. 1.7: IL-4 signaling in lymphocytes:

The binding of IL-4 to its receptor (IL-4R) leads to the activation of signaling pathways including Jak1/3-STAT-6, MAPKs, IRS-2 and PI3K. STAT-6 is an important transcription factor activated by tyrosine phosphorylation by Jak-1 and Jak-3, forms homodimer, and translocates to the nucleus. The homodimer of STAT-6 binds to the promoter to drive the gene expression. IL-4 also activates other transcription factors including Egr-1 in B lymphocytes. Further, activation of IL-4-induced phosphotyrosine substrate IRS-2 triggers other signaling molecules including PI3K leading to the activation of genes responsible for cell proliferation.

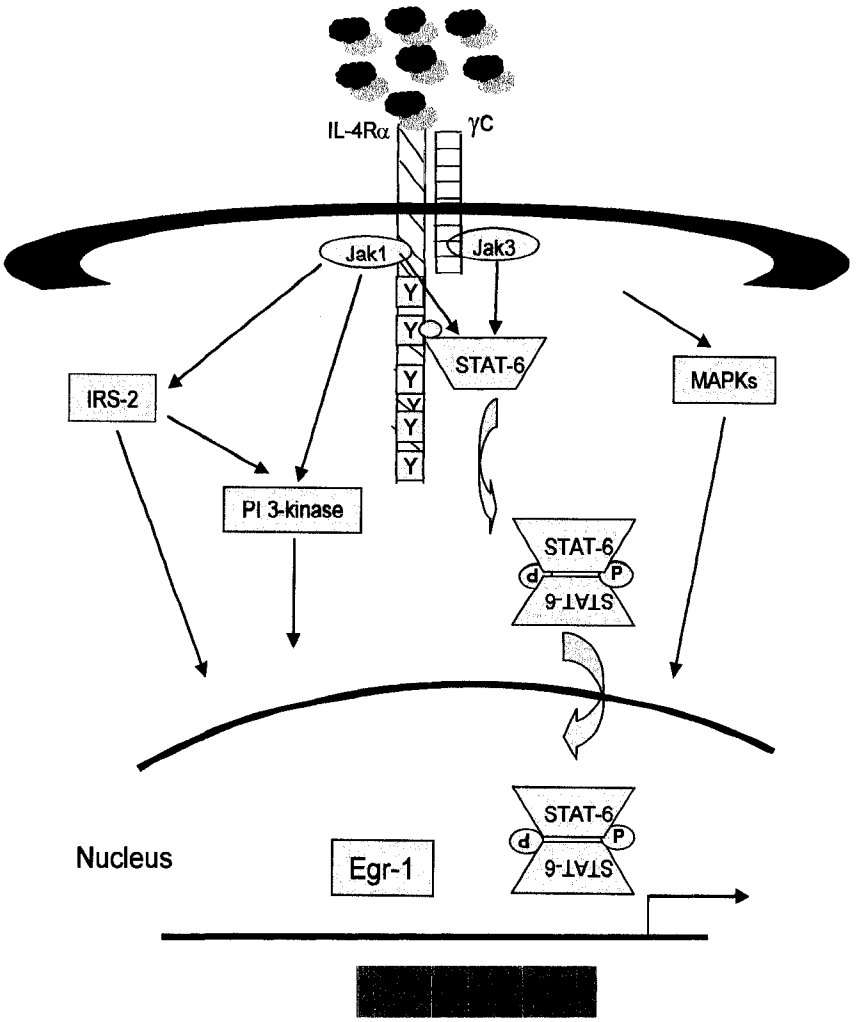

IL-4Rα
γC
Jak1
Jak3
Y
Y
Y
Y
Y
Y
STAT-6
IRS-2
MAPKs
PI 3-kinase
STAT-6
P
P
STAT-6
STAT-6
P
P
STAT-6
Nucleus
Egr-1

Fig. 1.7

cells which lack the γc, have been shown to express the type 1 IL-13 R as a functional IL-4R (226).

The main signaling pathway induced in response to cytokines, including that for IL-4, is the Janus kinase/signal transducers and activators of transcription (Jak/STAT) pathway (Fig. 1.7) (229;230). This pathway involves activation of the Jak tyrosine kinases, of which there are four family members: Jak-1, Jak-2, Jak-3, and Tyk-2. The Jaks are present in the cytosol and normally are specifically associated with particular cytokine receptor chains. The cytokine receptor chains in general have no intrinsic kinase ability and are phosphorylated by tyrosine kinases, such as the Jaks. In the case of IL-4R (Fig. 1.7), Jak-1 associates with IL-4Rα and Jak-3 associates with the γc chain (230). Recently, Jak-2 was shown to associate with the IL-4Rα in monocytic cells (231). The Jaks are autophosphorylated upon ligand binding and then proceed to phosphorylate the STAT proteins. STAT proteins are a family of seven members (STAT-1, -2,-3, -4, -5a, -5b, and -6) that serve as transcription factors. Generally, after activation, the STAT proteins homo or heterodimerize through src-homology (SH)-2 domains, translocate to the nucleus where they site-specifically bind promoter regions of cytokine-responsive genes (229;230).

STAT-6 is specifically activated by IL-4-induced Jak-1 and Jak-3. STAT-6 binds to tyrosine-phosphorylated cytoplasmic domain of the IL-4Rα chain via its SH2 domain and is activated via Jak-mediated tyrosine phosphorylation. This allows STAT-6 to dimerize and translocate to the nucleus where it can bind to a consensus sequence found in the promoters of IL-4-responsive genes such as MHC-II, CD23 and IL-4Rα (218;232). Wick and Berton (2000) showed that STAT-6 is also phosphorylated on serine in normal

mouse B cells in response to IL-4 stimulation and serine phosphorylation is not dependent on tyrosine phosphorylation of STAT-6 (233). Hence, serine phosphorylation may play a role in regulating the ability of STAT-6 to activate transcription. Indeed, other STATs, particularly STAT-1 and STAT-3 have been shown to be phosphorylated on a conserved serine residue within a MAPK consensus sequence in the C-terminus and this phosphorylation significantly potentiates transcriptional activation by these STATs (234;235).

Downregulation of the Jak/STAT pathway after ligand activation is also important in the regulation of signaling. The protein phosphatase Src homology (SH)-2 containing phosphatase (SHP)-1 has been associated with the downregulation of IL-4 induced Jak/STAT activation (236;237). In addition, other cellular inhibitors are responsible for the dephosphorylation of kinases and transcription factors of this pathway. These include the Jak binding protein (JAB) and suppressor of cytokine signaling (SOCS) family of inhibitors that affect activation of specific Jak and STAT proteins (238).

Besides Jak/STAT pathway, the second pathway involves the recruitment of PI3K subunits and activation of downstream signaling molecules such as protein kinase C and Akt (239). This pathway plays a key role in cell survival, cell proliferation and resistance to apoptosis. A recent study suggests that the insulin receptor substrate (IRS)/PI3K pathway participates in the regulation of IL-4-induced expression of the mouse $C\epsilon$ immunoglobulin gene (240). IRS proteins comprise a total of four family members (IRS-1 to -4) with large adaptor protein tyrosine binding (PTB) domain. Among all IRS proteins, IRS-2, in particular, was identified as one of the predominant proteins phosphorylated in response to IL-4 (197;241). Recruitment of IRS-2 to the activated IL-

4Rα chain results in its phosphorylation and subsequent activation of downstream signaling proteins, including PI3K (197;241). In B cells and T cells, PI3K activation is essential for their proliferation and survival driven by cytokines (242).

IL-4 is also known to activate the third major pathway, the MAPK pathway, although in limited cell types such as fibroblasts and keratinocytes (243-245). IL-4 does not activate MAPK pathways in hematopoitic cells with few exception (246-248). A recent report indicates that Ras/ERK MAPK positively regulates Jak-1/STAT-6 activity and IL-4 gene expression in IL-4-treated Jurkat T cells (248). Further, Canfield et al. (2005) demonstrated that IL-4 induced SOCS-3 expression in murine B cells by a mechanism dependent on activation of p38 MAPK (246). In an another study, IL-4 induced the activation of p38 MAPK in murine T cell line, CT6 and pro-B cell line BA/F3 cells, however, no activation of this kinase was observed in IL-4-stimulated RAW264.7 murine macrophages suggesting that the IL-4 regulation of p38 MAPK signaling is dependent on cell type (249).

Thus, these distinct IL-4 signaling pathways can interact to regulate IL-4-induced growth and gene expression, but the mechanisms of signal integration are not well understood.

Hypothesis:

LPS and TNF-α are potent inducers of CD44 expression in human monocytic cells (166). In addition, IL-4, a pleotropic cytokine, has been shown to enhance the survival and development of Burkitt's lymphoma B cells by inducing CD44 expression. Delineation of the signal transduction pathways that are responsible for the regulation of CD44 expression as well as ligand binding are important in gaining a better

understanding of how this family of proteins exerts its functions. Further, resolving the molecular mechanism involved in the regulation of CD44 expression will lead to the identification of key signal transduction proteins and this knowledge may then be used to modulate CD44 expression for therapeutic purpose and may be used in concert with other treatments for cancer or in cases of chronic inflammation.

Previously, we have demonstrated that JNK MAPK regulates LPS-induced but not TNF-α-induced CD44 expression in human monocytic cells (166). The signaling molecules involved in TNF-α-induced CD44 expression remain unknown. Sionov and Naor (1998) reported that calcium and calmodulin were involved in the regulation of PMA-induced CD44 expression in T lymphoma cells (250). In this study, we hypothesize that Ca^{2+} signaling pathway may be involved in the regulation of TNF-α-induced CD44 expression in human monocytic cells. Further, the regulation of IL-4-induced CD44 expression in human B cells is not known. The IL-4 signaling, in general, is believed to be mediated by Jak/STAT-6 and IRS-2/PI3 kinase pathways (251;252). IL-4 has also been shown to activate MAPK pathway (244). Therefore, we hypothesize that a distinct signaling pathway may be involved in the regulation of IL-4-induced CD44 expression.

Therefore, the overall aim of my thesis research proposal was to delineate the molecular mechanisms underlying the regulation of CD44 expression in human monocytic cells in response to LPS and TNF-α, and in Burkitt's lymphoma B cells in response to IL-4.

Rationale: LPS induces inflammatory responses that contribute to the pathogenesis of sepsis, and inflammation. Although the mechanisms that lead to these inflammatory processes remain largely unclear, mononuclear phagocytes play a major role in the

pathogenesis of LPS-induced syndromes. The induction of CD44 expression in monocytic cells may play a critical role in inflammatory responses. LPS and TNF-α are potent inducers of CD44 expression in human monocytic cells. The mechanisms by which LPS/TNF-α induce CD44 expression remain unknown. To delineate the mechanisms underlying LPS- and TNF-α-induced CD44 expression, previously, our laboratory has shown that the role of MAPK specifically JNK MAPK was differentially involved in LPS-induced but not in TNF-α-induced CD44 expression in human monocytic cells (166). In this study, as the objectives of my thesis, I investigated the involvement of signaling pathways in the regulation of TNF-α-induced CD44 expression in human monocytic cells.

IL-4, a pleotropic cytokine, has been shown to enhance the survival and development of Burkitt's lymphoma (BL) B cells, and induces the expression of various costimulatory molecules including CD44. In addition, CD44 induction and its ability to bind HA have been suggested to play a vital role in *in vivo* BL tumor growth and dissemination. However, the role of IL-4 in the pathogenesis of BL and other B cell malignancies is not clear. In this study, I investigated the regulation of IL-4-induced CD44 expression in an Epstein-Barr virus (EBV)-transformed Burkitt's lymphoma B cell line, BL30/B95-8 cells. I wanted to determine the involvement of various signaling molecules including STAT-6 in the regulation of IL-4-induced CD44 expression in BL30/B95-8 cells. The specific research objectives are as follows:

Objectives:

1) To study the role of Ca^{2+} signaling pathway in the regulation of CD44 expression in LPS-/TNF-α-stimulated human monocytic cells

2) To investigate the role of PI3 kinase and its cross-talk with other signaling pathways in LPS- and TNF-α-induced CD44 expression in human monocytic cells

3) To examine the involvement of signaling molecules Jak/STAT, MAPKs, and IRS-2/PI3K and the transcription factor, Egr-1, and STAT-6 in the regulation of IL-4-induced CD44 expression in BL30/B95-8 cells

Chapter II
Materials and Methods

Cell lines and cell culture:

THP-1 cells transfected with a pRc/RSV vector (Invitrogen) containing the CD14 cDNA sequence (THP-1/CD14) were kindly provided by Dr. Richard Ulevitch (The Scripps Research Institute, La Jolla, CA) (51;253;254). The pRc/RSV vector contains RSV promoter to drive the CD14 expression. Basically, THP-1 cells are promonocytic cell line derived from a human acute monocytic leukemia patient. THP-1 cells deficient in the P110α isoform of PI 3-kinase (PI3K) and the control cells, stably transfected with control vectors were a kind gift from Dr Neil E. Reiner from Department of Medicine, University of British Columbia, Vancouver, Canada. In preparation of these stable cell lines, cells were stably transduced with a lentiviral vector expressing short hairpin RNA (shRNA) for the p110α subunit (HR-p110α3) and exhibited complete inhibition of the PI3K p110α isoform expression without affecting the expression of p85 regulatory subunit or that of the p110β and p110γ isoforms. Cells transduced with the control lentiviral vector HRp110α1 exhibited normal levels of p110α isoform (255). Further, the Burkitt's lymphoma cell line, BL30/B95-8, an EBV-negative BL30 cell line infected *in vitro* with EBV, was kindly provided by Dr. E. Kieff (Brigham and Woman's Hospital, Boston, MA) and has been described in our previous work (141;173). This BL cell line used in this study has the characteristic *c-myc* translocation [t(8:14)] (69;141).

Cells were cultured in Iscove's modified Dulbecco's medium (IMDM) (Sigma-Aldrich, St-Louis, MO), supplemented with 10% FBS (Invitrogen, Grand Island, NY), 100 U/ml penicillin, 100 μg/ml gentamicin, 10 mM HEPES, and 2 mM glutamine. The cells were cultured in a T-75 cm^2 flask at 37°C in an incubator containing 5% CO_2 until reaching confluence. Cells were washed in PBS followed by splitting into new flasks

at10^6 cells/ml in complete IMDM media. To avoid cells undergoing too many passages, cells were frozen in 10% DMSO in cryovials under liquid nitrogen and whenever required, cells were thawed immediately following the standard procedure of cell culture.

Reagents:

LPS derived from *Escherichia coli* 0111:B4 (Sigma-Aldrich Canada Ltd., Oakville, ON), human recombinant (r) TNF-α (Biosource, Montreal, Quebec, Canada), rIL-4 (R&D Systems Inc. Minneapolis, MN) and Phorbol 12-myristate 13-acetate (PMA) (Gibco BRL) were purchased for this study. The source and dose of inhibitors used in this study are detailed in Table-2.1. The MAPK inhibitors used in this study are as follows: PD98059 (2′-Amino-3′-methoxyflavone), an inhibitor of MAP/ERK kinase-1, which selectively blocks the activity of ERK MAPK and has no effect on the activity of other serine threonine protein kinases including Raf1, p38 and JNK MAPK (256;257). The pyridinyl imidazole SB202190 [FHPI, 4- (4- Fluoro phenyl) -2-(4-hydroxy phenyl)- 5-(4-pyridyl)1H-imidazol], a potent inhibitor of p38 MAPK, has no significant effect on the activity of ERK or JNK MAPK subgroups (166). SP600125 [Anthra(1,9-cd)pyrazol-6(2H)-one 1,9-Pyrazoloanthrone], a specific JNK inhibitor, is a reversible ATP competitive inhibitor with more than 300-fold selectivity versus related MAPK including ERK1 and p38 (166). The PI3K inhibitor LY294002 [2-(4-Morpholinyl)-8-phenyl-4H-1-benzopyran-4-one], a potent inhibitor that acts on the ATP binding site (258) was purchased from Calbiochem. The following Ca^{2+} signaling inhibitors were employed: EGTA (Ethylene glycol bis(2-aminoethyl ether)-N,N,N'N'-tetraacetic acid), a Ca^{2+} chelating agent (51); SKF-96365 hydrochloride [1-[b-[3-(4- Methoxyphenyl) propoxy] -4-methoxyphenethyl]-1H-imidazole,HCl] specifically inhibits receptor-mediated Ca^{2+}

Table-2.1: Detailed description of pharmacological inhibitors, their source, and doses used in this study

Name of the Inhibitors	Source	Final Concentration	Cellular Targets
SB202190	Calbiochem, San Diego, CA	5-50 μM	p38 MAPK
PD98059	Calbiochem	5-50 μM	ERK MAPK
SP600125	Calbiochem	5-50 μM	JNK MAPK
Jak Inhibitor I	Calbiochem	5-25 nM	Jaks
EGTA	Calbiochem	2-10 mM	Calcium chelator
SKF96365 HCl	Calbiochem	20-100 μM	Receptor mediated Ca^{2+} entry
2-APB	Calbiochem	10-50 μM	IP3 induced Ca^{2+} release From ER
W-7 HCl	Calbiochem	10-50 μM	Calmodulin
KN-93	Calbiochem	5-50 μM	CaMK-II
FK-506	AG Scientific, San Diego, CA	0.5-5 μM	Calcineurin
Cyclosporine A	Sigma-Aldrich	0.5-5 μM	Calcineurin
LY294002	Calbiochem	1-20 μM	PI 3-Kinase

entry (259); 2-APB (2-Aminoethoxydiphenylborate) inhibits inositol (1,4,5) triphosphate (IP3) induced Ca^{2+} release from the ER (260); W-7 hydrochloride [N-(6-Aminohexyl)-5-chloro-1-naphthalenesulfonamide, HCl] is a calmodulin antagonist (261); CaMK-II [(methoxybenzenesulfonyl)]amino-N-(4-chlorocinnamyl)-N-methyl benzylamine] is a specific cell permeable inhibitor of CaMK-II (51); FK-506 (Tacrolimus) interacts with FK-506-binding protein (FKBP), forming a FK-506–FKBP complex, which binds to and blocks calcineurin; and cyclosporine A binds to cyclophilin and inhibits the Ca^{2+} dependent phosphatases (51;262). Jak Inhibitor I (Calbiochem), an inhibitor for Jak-1, Jak-2, Jak-3 and Tyk-2 was employed (263). All other chemicals used for electrophoresis and immunoblot analysis were obtained from Sigma-Aldrich Canada Ltd., Oakville, ON.

Isolation of monocytes from PBMCs:

Monocytes were isolated from peripheral blood mononuclear cells (PBMCs) by automacs negative selection (Miltenyi Biotech Inc. Auburn, CA). Blood was obtained for isolation of PBMCs from healthy volunteers after approval of the protocol by the ethics review committee of the Children's Hospital of Eastern Ontario, Ottawa, Canada. PBMCs were isolated by density gradient centrifugation over Ficoll-Hypaque (Pharmacia Biotech, Piscataway, NJ) as previously described (166). Briefly, the cell layer consisting mainly of mononuclear cells was collected and washed twice in PBS containing 2% EDTA (ethylenediaminetetraacetic acid) followed by incubation with automacs FcR blocking reagent along with biotin antibody cocktail (mixture of anti-CD3,7,16,19,56,123, and 235a antibodies) for 10 min at 4°C. Following incubation, cells were treated with anti biotin microbeads for 15 min at 4°C. Cells were then washed once and subjected to automacs negative selection separation as per the manufacturer's instructions. Cell

populations thus obtained contained more than 95% CD14+ monocytes (191;262). Cells were cultured in complete IMDM media.

Flow cytometry analysis of monocytic cells:

CD44 expression on CD14+ monocytes and on THP-1/CD14 cells was determined by flow cytometry (51;166). Briefly, cells (0.5 X 10^6) were washed once at the time of harvesting with PBS/0.1% sodium azide and aliquoted into polystyrene tubes (Falcon, Lincoln Park, NJ). Cells were double-stained with PE-conjugated anti-CD14 mAbs (BD Biosciences, San Jose, CA) and with FITC-conjugated anti-CD44 mAbs (BD Pharmingen). Autofluorescence and isotype (IgG2b)-matched control antibodies (Becton Dickinson) were also included. 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 and CD44 between different samples was ensured through the use of Calibrite™ Beads (BD Biosciences).

Flow cytometry analysis of BL30/B95-8 cells:

Cells were assayed for CD44 expression by flow cytometry (173). Untreated or treated cells (0.5 X 10^6) with either inhibitor and/or IL-4 were resuspended in PBS with 0.1% sodium azide and were incubated for 15 min at room temperature with either mouse anti-human CD44 mAb (anti-human Leu-44, Becton Dickinson) or isotype-matched control antibodies (Sigma) to analyze CD44 expression. All antibodies were FITC-conjugated. Prior to staining, cell surface Fc receptors were blocked by incubation with aggregated human gamma globulins at 5 mg/ml. The gates were set in accordance with gates obtained with

the isotype-matched control antibodies and mean channel fluorescence (MCF) was determined. 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).

Ca^{2+} influx:

THP-1/CD14 cells were washed with Ca^{2+}-free PBS for 5 min at room temperature and resuspended in Buffer A (RPMI 1640 containing 20 mM HEPES, pH 7). The cells were washed again and resuspended in Buffer A containing 1 mM calcium binding dye Fluo3/AM (Molecular Probes, Eugene, OR) in 1 mM DMSO and 3.75% Pluronic F-127 solution (Sigma) followed by incubation in dark for 45 min at 37°C in shaking water bath (264). The reaction was stopped by adding equal volume of Buffer B (Buffer A containing 5% FBS, pH 7.4) followed by incubation for 15 min at 37°C in water bath. The cells were washed and resuspended in Buffer B at a final concentration of 0.5 X 10^6 cells/ml and analyzed for Ca^{2+} levels by the FACScan flow cytometer (Becton–Dickinson, Franklin Lakes, NJ) equipped with CellQuest software, Version 3.2.1fl. Cell samples were maintained at 37°C during acquisition of data. Intracellular Ca^{2+} levels at baseline and following stimulation with LPS/TNF-α were measured. Ca^{2+} ionophore A23187 (20 mM) and 5 mM EGTA (Sigma) were used as positive and negative controls, respectively.

Northern blot analysis:

Total RNA was extracted with Tri Reagent (Molecular Research Centre, Inc.). Following denaturation with 2.2 M deionized formamide (Fisher Scientific, Pittsburgh, PA), total RNA samples were electrophoresed on a 1.2% MOPS-formaldehyde agarose

gel and transferred onto a Hybond-N (Amersham) nylon membrane. Hybridization was performed overnight at 65°C with ^{32}P-labelled cDNA probes of human Egr-1 (141).

Cell stimulation and Western blot analysis:

THP-1/CD14 cells or primary monocytes (2 X 10^6) were treated with the indicated concentration of inhibitors for 2 hr followed by stimulation with either LPS (1 μg/ml) or TNF-α (10 ng/ml) for 15-60 min for detection of phosphorylation level of signaling molecules by Western blot analysis as described earlier (166;191). Similarly, BL30/B95-8 cells were treated with the indicated concentration of inhibitors for 2 hr followed by stimulation with IL-4 (40 ng/ml) for 15-60 min for detection of signaling molecules activation by Western blot analysis (173). Briefly, cell lysates were prepared by treating the cell pellets 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 orthovandate, and 1 mM EGTA pH 7.7) for 1 hr to lyse the cells, followed by centrifugation for 15 min at 20,000 x g at 4°C. The protein concentration of the supernatants was determined using the Bio-Rad protein determination assay (Bio-Rad Laboratories, Hercules, CA). Total protein (30 μg) was subjected to SDS-PAGE followed by transfer onto a polyvinylidene difluoride (PVDF) membrane (Bio-Rad Laboratory, Hercules, CA). The membranes were probed with either anti-phospho protein antibody or with antibodies against respective protein for control. For example, to detect JNK MAPK, membranes were probed with anti-phospho-JNK antibodies overnight at 4°C. Following incubation, membranes were washed with TBST (8 gm NaCl, 0.2 gm KCl, 3 gm TRIS base with 1 ml Tween-20 in 1 liter of Distilled water, pH 7.4), and then incubated with donkey anti-rabbit polyclonal antibody conjugated to horseradish peroxidase or goat anti-mouse polyclonal antibody

wherever necessary. To control for total protein loading, the membranes were stripped of the primary antibodies and reprobed with rabbit polyclonal antibodies specific for the total p38, ERK, or JNK MAPKs. In brief, the stripping of membranes was done by treating stripping buffer [62.5 mM Tris HCl (pH 6.7), 100 mM 2-mercaptoethanol 2% SDS and 1M DTT)] at 50°C for 30 min with slow rotation followed by washing the membranes with TBST. All immunoblots were visualized by enhanced chemiluminescence (Santa Cruz Biotechnology). All the antibodies used and the sources are presented in Table-2.2.

Measurement of CaMK-II activity:

The CaMK-II assay was performed using a CaMK-II assay kit (Upstate Biotechnology Inc., Mississauga, Ontario, Canada) as per the manufacturer's instructions. THP-1/CD14 cells were pretreated with or without inhibitor for 2 hr followed by stimulation of cells with either LPS or TNF-α for 30 min. Cell pellets were lysed and cytoplasmic extracts were collected as described previously. CaMK-II activity was assayed from cellular proteins utilizing a peptide substrate (KKALRRQETVDAL) specific for CaMK-II. Total protein (200 μg) was added to 10 μl of CaMK-II substrate, 0.4 μM each of peptide inhibitors for PKA and PKC, and 100 μCi of $MgCl_2$-[γ-^{32}P] ATP in ADB II buffer (20 mM MOPS, pH 7.2, 2.5 mM β-glycerol phosphate, 1 mM sodium orthovanadate, 1 mM DTT, and 1 mM $CaCl_2$). The reaction mixture was incubated at 30°C for 10 min, and the phosphorylated substrate was separated from the residual [γ-^{32}P]-ATP using $_P$81 phosphocellulose paper. The papers were washed twice in 0.75% H_3PO_4 and once in acetone for 2 min, and radioactivity was measured by Microbeta

Table-2.2: Description of antibodies and their source

Name of the antibodies	Species	Source
α-phospho-p38 MAPK	Rabbit	Cell Signaling, Danvers, MA
α-p38 MAPK	Rabbit	Santa Cruz Biotechnology, CA
α-phospho-JNK MAPK	Rabbit	Cell Signaling
α-JNK MAPK	Rabbit	Santa Cruz Biotechnology
α-phospho-ERK MAPK	Mouse	Santa Cruz Biotechnology
α-ERK MAPK	Rabbit	Santa Cruz Biotechnology
α-phospho-Akt	Rabbit	Cell Signaling
α-Akt	Rabbit	Cell Signaling
α-phospho-Jak-1	Rabbit	Cell Signaling
α-Jak-1	Rabbit	Cell Signaling
α-phospho-IRS-2	Rabbit	Cell Signaling
α-IRS-2	Rabbit	Cell Signaling
α-phospho-STAT-6	Rabbit	Cell Signaling
α-STAT-6	Rabbit	Cell Signaling
α-rabbit-HRPO	Donkey	Amersham Bioscience, Montreal, Canada
α-mouse-HRPO	Goat	Bio Rad, CA
α-Egr-1 (EMSA)	Rabbit	Santa Cruz Biotechnology
α-cJun (EMSA)	Rabbit	Santa Cruz Biotechnology
α-cFos (EMSA)	Rabbit	Santa Cruz Biotechnology
α-STAT-6 (EMSA)	Rabbit	Santa Cruz Biotechnology

counter (Wallac, Turko, Finland). Blanks to correct for nonspecific binding of [γ-^{32}P]-ATP and its breakdown products to the phosphocellulose paper and controls for phosphorylation of endogenous proteins in the sample were performed. CaMK-II activity was expressed as cpm/μg of protein.

Generation of CD44 promoter and its deletion, and mutant constructs in luciferase reporter vector:

A series of human CD44 promoter fragments (-1109 to +53; GenBank™ accession number AH003670) were amplified from genomic DNA by PCR as described earlier (51). The primers with restriction sites used to amplify the promoter fragments, mutated promoter fragments, and fragment sizes are shown in Table-2.3. The amplification consisted of denaturation at 95°C for 2 min, followed by 30 cycles of denaturation at 95°C for 1 min, annealing at 60°C for 1 min, and extension at 72°C for 1 min, and final elongation at 72°C for 10 min. The amplified fragments were subcloned into the PCRII-TOPO vector, and the sequences were confirmed. The correct insertions were subcloned into *Nhe* I and *Hind* III polylinker site of pGL3B (Promega, Madison, WI) by T4 DNA ligase (New England Biolab) and sequences were confirmed again. All DNA sequencing was performed by the Biotechnology Research Center (University of Ottawa). The Egr-1, AP-1, and STAT-6 mutant constructs were generated either by PCR using specific primers or by a PCR-based site-directed mutagenesis kit (Stratagene, La Jolla, CA) as per manufacturer's instructions.

Transient transfection and measurement of luciferase activity:

The cells were transfected with CD44 promoter constructs by using FuGENE6 transfection reagent (Roche Diagnostics) as per manufacturer's instructions. Briefly, 1 μg

Table-2.3: Primers for amplification of CD44 promoter fragments and sizes of the PCR products generated from human genomic DNA

Primer sequences	Region amplified	Product length
Sense Primers	**bp**	**bp**
5' AAGGCTAGCAGGAAGTGTGGAATGATGG 3'	-1109/+53	1162
5' AAGGCTAGCCAACATCCCTGTGAAACC 3'	-575/+53	628
5' AAGGCTAGCAAAGGCTGAACCCAATG 3'	-505/+53	558
5' AAGGCTAGCCCCCGATTATTTACAGC 3'	-334/+53	387
5' AAGGCTAGCTCTTAAAACCTCTGCGG 3'	-264/+53	317
5' AAGGCTAGCGCTTGGGTGTGTCCTTC 3'	-224/+53	277
5' AAGGCTAGCCACTGTTTTCAACCTCG 3'	-181/+53	234
5' AAGGCTAGCGCCAACTTCCGAGGCAGCCTCATT 3'	-151/+53	204
5' AAGGCTAGCATTGCCCAGCGGACCCCAGC 3'	-130/+53	183

Antisense Primer

5' AACAAGCTTCTCAGCGGCACGAGGCAG 3'

Mutation (mutated bases in bold letter)

Egr-1(wild type)	5' GCGGGGGCAGAG 3'
Egr-1 (mutant)	5' GCGGG**TCT**AGAG 3'
AP-1 (wild type)	5' GCTGCTTAGTCA 3'
AP-1 (mutant)	5' GCTGC**C**TAG**G**CA 3'
STAT-6 (wild type)	5' TGCCTCGGAAGT 3'
STAT-6 (mutant)	5' TGCCTCGG**CT**GT 3'

Oligos for EMSA

Egr-1	5' GCACGGGGCGGGGGCAGAGGGGCC 3'
AP-1	5' GCGGGCTGCTTAGTCACAGCCCCC 3'
Stat-6	5' CAACTTCCGAGGCAGCCTCATTGC 3'

of test plasmid and 0.5 μg of pSV-β-galactosidase vectors (Promega) were incubated for 45 min at room temperature with 4.5 μl of FuGENE6 reagent in 100 μl of serum free IMDM to allow the formation of DNA-liposome complexes. Then the cells (2 X 10^6 cells/well) in a 6 well plate (Falcon) were treated with these complexes and were cultured for 15 hr prior to treatment with inhibitors for 2 hr followed by stimulation with LPS or TNF-α. Cell lysates were subjected to measurement of luciferase and β-galactosidase activities using luciferase and β-galactosidase assay kits (Promega) in a Bio-Orbit luminometer (Fischer, Pittsburg, PA) and spectrophotometer, respectively (51).

Transfection of p85α PI3K siRNA:

Cells (2 X 10^6) were transfected with siRNA SMARTpool PI3K p85α (in THP-1/CD14 cells) or siRNA ERKs MAPK (in BL30/B95-8 cells) and nonspecific control pool (siRNA control) using DharmaFECT™ 2 transfection reagent as per the manufacturer's instructions (Dharmacon) (262). Following transfection, cells were stimulated with either LPS (1 μg/ml)/TNF-α (10 ng/ml) in case of THP-1/CD14 cells or IL-4 (40 ng/ml) in case of BL30/B95-8 cells. Protein lyseates were used in Western blot analysis for pp85α PI3K or pERK1/2. Cells were also collected after 24 hr of stimulation for determination of CD44 expression.

Electrophoretic mobility shift assay (EMSA):

EMSA was performed as per the standard technique of our lab, and as described earlier (51). THP-1/CD14 cells were stimulated either with LPS or TNF-α for various time periods at 37°C to activate Egr-1 or AP-1 transcription factors, and BL30/B95-8 cells were stimulated with IL-4 for STAT-6 activation. To illustrate the effect of inhibitors on transcription factors, cells were pretreated with specific inhibitors 2 hr

before stimulation. Cells were harvested in PBS (pH 7.2) and centrifuged at 200X*g* for 5 min at 4°C. Then, the cells were lysed for 10 min at 4°C with buffer A (10 mM HEPES, 10 mM KCl, 1.5 mM $MgCl_2$, 0.5 mM DTT, and 0.5 mM PMSF (pH 7.9)) containing 0.1% Nonidet P-40. The lysates were centrifuged at 20,000X*g* for 10 min at 4°C. The pellet containing the nuclei was suspended in buffer B (20 mM HEPES, 420 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA and 25% glycerol) at 4°C for 15 min. Both buffers A and B contain DTT (reducing agent), PMSF, and spermidine (protease inhibitors) at concentration 0.5 mM each, as well as 0.15 mM spermine and 5 µg/ml each of aprotonin, leupeptin, and pepstatin A. The supernatant containing the nuclear protein was collected, protein concentration was determined using the Bio-Rad protein determination assay reagent (Bio-Rad, Hercules, CA), and frozen at –80°C till further use. The oligonucleotide sequences corresponding to the Egr-1, AP-1 and STAT-6 binding sites are given in Table-2.3. Two oligonucleotides complementary to each other were annealed to generate a double stranded probe. End labeling was accomplished by treatment of T4 polynucleotide kinase in the presence of [γ-^{32}P] ATP. Nuclear extract samples (5 µg each) were mixed either with ^{32}P labeled AP-1, Egr-1 or STAT-6 binding oligonucleotides for 20 min at room temperature, the reaction mixture was separated on a 5% non-denaturing gel for 90 min. To illustrate specificity of either AP-1, Egr-1 or STAT-6 probe, parallel EMSA reactions were performed with 50-200-fold excess of unlabelled specific and non-specific probes for 20 min prior to the addition of labeled probe. Subsequently, supershift experiments were also performed to identify the transcription factors by using specific mouse anti-Egr-1 or anti-c-Jun (AP-1), and anti-c-

Fos (AP-1) or anti-STAT-6 Abs (0.5-2 μg) (Santa Crutz Biotechnologies) (presented in Table-2.3). The gel was vacuum-dried and subjected to autoradiography for 24-48 hr.

Statistical analysis:

Means were compared using the two-tailed Student's t test. Results are expressed as mean ± standard error of the mean (SEM). P values were calculated using MS Excel computer program.

Chapter III

Regulation of LPS- and TNF-α-induced CD44 expression in human monocytic cells

A) Role of JNK and Ca^{2+} signaling pathways

Introduction

The induction of CD44 expression and it's binding to HA is a key event in the migration of monocytic cells to sites of inflammation or tissue injury (31;89), and as a result, has been suggested to play a role in the pathogenesis of inflammatory and autoimmune diseases (144;160). It is established that mononuclear phagocytes play a key role in the pathogenesis of LPS-induced syndromes and TNF-α, a proinflammatory cytokine, is involved in LPS-induced inflammatory responses (201). Recently, we and others have shown that TNF-α is a potent inducer of CD44 expression and a positive regulator of LPS-induced CD44 expression and CD44-HA interactions in monocytic cells (27;51;75;166).

Alterations in the levels of CD44 expression on mononuclear phagocytes by endotoxins and immunoregulatory cytokines may have profound effects on the migration of immune cells to sites of inflammation and in the development of immune responses. Therefore, understanding the regulation of CD44 expression and characterizing the signal transduction events involved may lead to the development of strategies for the treatment of inflammation, autoimmune diseases, and cancers. During the last few years, the signaling pathways induced in monocytes following engagement of LPS with its cognate CD14-Toll receptor complex have been investigated (265;266). However, the signal transduction events involved in CD44 up-regulation are not well understood. There is some evidence to suggest the involvement of the calcium signaling pathway in CD44 expression on phorbol 12-myristate 13-acetate (PMA)-stimulated T lymphoma cells (250), whereas phosphatidylinositol 3-kinase (PI3K) and protein kinase C (PKC) were

shown to regulate CD44 expression in neuroblastoma cells (175). Our lab has previously demonstrated that the c-Jun N-terminal kinase (JNK) MAPK pathway regulates LPS-induced, but not TNF-α-induced CD44 expression in human monocytic cells (166).

To further define the signaling pathways distinguishing the regulation of LPS- and TNF-α-induced CD44 expression, and with respect to my objective of understanding the molecular mechanisms involved in the regulation of CD44 expression, I examined the role of Ca^{2+} signaling pathway in the upregulation of CD44 expression in human monocytic THP-1/CD14 cells as a model system.

My results suggest a distinct role for the Ca^{2+} signaling pathway, in particular, calmodulin (CaM)/CaM-dependent protein kinase-II (CaMK-II) activation in the regulation of TNF-α-induced, but not LPS-induced CD44 expression. To further understand the involvement of CaM/CaMK-II and JNK in the regulation of CD44 transcription, I analyzed the CD44 promoter to identify the potential transcription factors involved. These results suggest for the first time that TNF-α and LPS induce CD44 expression through two distinct and independent signaling cascades without any evidence of cross-talk between the two pathways. TNF-α regulates CD44 expression specifically by AP-1 through CaM/CaMK-II activation. In contrast, LPS regulates CD44 transcription selectively by Egr-1 through JNK activation (51). The results from these experiments are as follows:

Results

Differential involvement of JNK MAPK and the Ca^{2+} signaling pathways in LPS- and TNF-α-induced CD44 expression, respectively

In this study, I confirmed the earlier observations (166) and demonstrated the involvement of JNK in LPS-, but not in TNF-α-induced CD44 expression in human promonocytic THP-1/CD14 cells. Both LPS and TNF-α induced JNK phosphorylation in THP-1/CD14 cells and this phosphorylation was inhibited by the JNK-specific inhibitor SP600125 in a dose-dependent manner. Furthermore, treatment with SP600125 inhibited LPS-induced CD44 expression in a dose-dependent manner, whereas TNF-α-induced CD44 expression remained unaffected even at the highest dose of SP600125 (Fig. 3.1).

To determine the molecular mechanisms involved, I investigated the role of the Ca^{2+} signaling pathway in the induction of both LPS- and TNF-α-induced CD44 expression. LPS-induced CD44 expression in primary human monocytes is complex and is regulated by the interaction of LPS with its CD14-Toll like receptor complex and endogenously produced cytokines TNF-α and IL-10. (75;166;265;267). The signaling pathways involved in LPS-induced CD44 expression in primary monocytes could not be investigated because of the inherent endogenous IL-10 production following LPS stimulation, and the ability of IL-10 to enhance CD44 expression in monocytes (166). Therefore, I investigated the role of the Ca^{2+} signaling pathway in the regulation of LPS- and TNF-α-induced CD44 expression in IL-10-refractory THP-1/CD14 cells as a model system. I first determined whether LPS and TNF-α activated Ca^{2+} signaling by examining Ca^{2+} influx by flow cytometry using Fluo-3 as a binding dye. Both LPS and TNF-α induced Ca^{2+} influx after 12 min of stimulation; which was inhibited by EGTA, a Ca^{2+}

Fig. 3.1: SP600125 inhibits LPS- but not TNF-α-induced CD44 expression.

THP-1/CD14 cells (0.5 X 10^6/ml) were treated with various concentrations of SP600125 (2.5-50 μM) for 2 hr prior to LPS (1 μg/ml) or TNF-α (10 ng/ml) stimulation for 24 hr. The cells were analyzed for CD44 expression by flow cytometry as described under "Materials and Methods". The results shown are representative of four experiments.

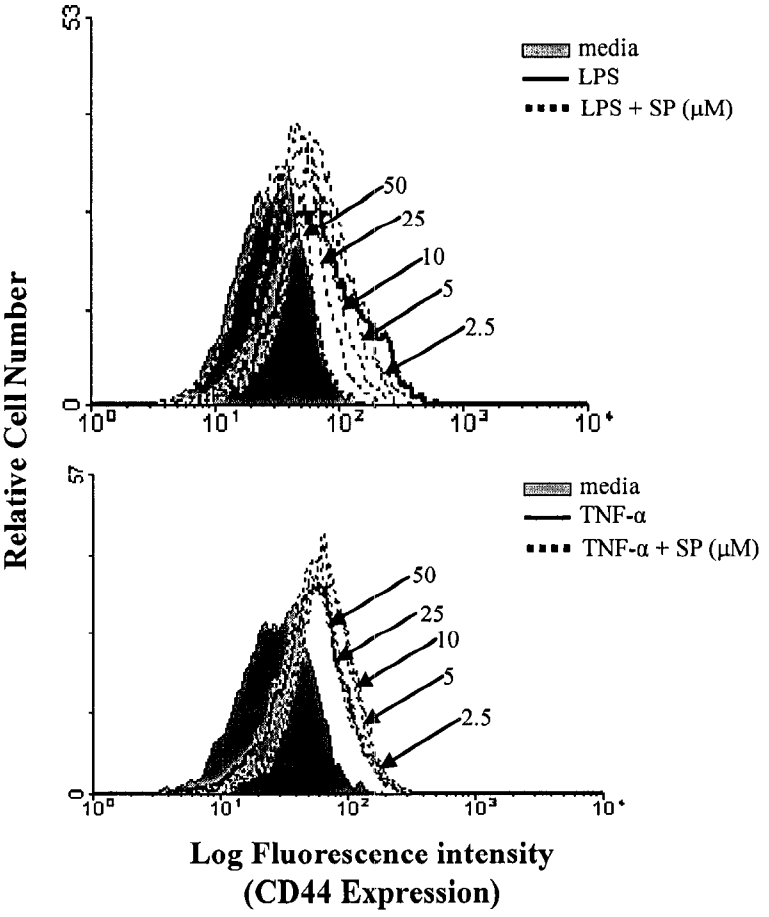

53
media
LPS
LPS + SP (μM)
50
25
10
5
2.5
0
10^0
10^1
10^2
10^3
10^4
57
media
TNF-α
TNF-α + SP (μM)
50
25
10
5
2.5
0
10^0
10^1
10^2
10^3
10^4
Relative Cell Number
Log Fluorescence intensity
(CD44 Expression)

Fig. 3.1

chelator, to basal levels (Fig. 3.2). The Ca^{2+} ionophore A23187 was used as a positive control. To determine the effect of EGTA on CD44 expression, cells were treated with EGTA (2.5-10 mM) for 2 hr prior to stimulation with either LPS or TNF-α for 24 hr. Interestingly, EGTA inhibited TNF-α-induced CD44 expression in a dose-dependent manner without any effect on LPS-induced CD44 expression (Fig. 3.3).

Elevation in cytoplasmic Ca^{2+} concentrations occur following stimulation by diverse stimuli that activate voltage or ligand-gated Ca^{2+} channels in the surface membrane or following release of Ca^{2+} present in intracellular stores, mainly in the endoplasmic reticulum (ER) (182;268). To determine whether Ca^{2+} release from the ER regulates CD44 expression, I used the Inositol 3 phosphate (IP_3) receptor inhibitor, 2-APB, which inhibits the release of Ca^{2+} from the ER by blocking IP3 receptor-gated Ca^{2+} channels (260). Similar to the results obtained with EGTA, 2-APB inhibited TNF-α- but not LPS-induced CD44 expression in a dose-dependent manner (Fig. 3.3). I also investigated the role of receptor-mediated entry of extracellular Ca^{2+} following LPS or TNF-α stimulation by employing SKF-96365, a specific inhibitor for receptor-mediated Ca^{2+} entry (259). Cells were pretreated with SKF-96365 for 2 hr prior to stimulation with either LPS or TNF-α. Interestingly, SKF-96365 inhibited TNF-α- but not LPS-induced CD44 expression in a dose-dependent manner (Fig. 3.3). These results suggest that receptor-mediated Ca^{2+} entry as well as Ca^{2+} release from the ER may be involved in the regulation of TNF-α- but not LPS-induced CD44 expression.

CaM and CaMK-II selectively regulate TNF-α-induced CD44 expression.

CaM, a major Ca^{2+} receptor is present in both cytoplasmic and nuclear compartments. The complex of Ca^{2+}/CaM regulates several downstream targets including

Fig. 3.2: Stimulation of THP-1/CD14 cells with either LPS or TNF-α induces Ca^{2+} influx.

Cells (0.5 X 10^6/ml) loaded with Fluo3/AM were stimulated with either LPS or TNF-α and the resulting Ca^{2+} influx was measured by flow cytometric analysis. Top panel, baseline Ca^{2+} levels in unstimulated cells; second panel, stimulation with LPS followed by the addition of EGTA; third panel, stimulation with TNF-α followed by the addition of EGTA; fourth panel, stimulation with the Ca^{2+} inophore A23187 followed by the addition of EGTA.

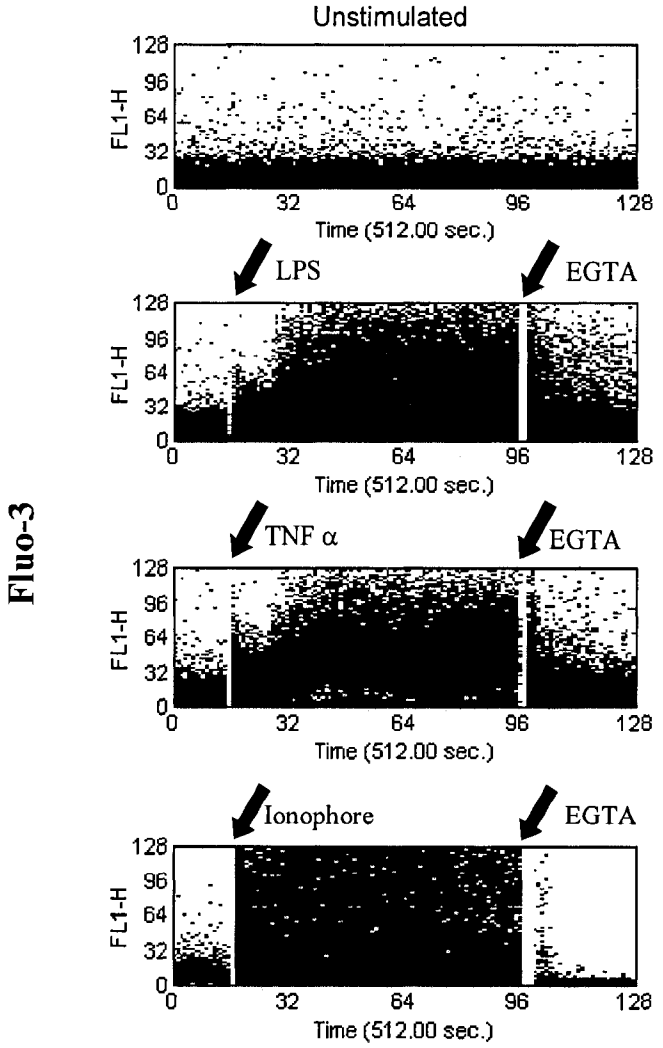

Fluo-3
Unstimulated
FL1-H
128
96
64
32
0
0
32
64
96
128
Time (512.00 sec.)
LPS
EGTA
TNF α
Ionophore

Fig. 3.2

Fig. 3.3: Involvement of Ca^{2+} signaling pathway in TNF-α- but not LPS-induced CD44 expression.

THP-1/CD14 cells (0.5 X 10^6/ml) were treated with various concentrations of EGTA (2.5-10 mM), APB (10-50 μM), or SKF-96365 (10-50 μM) for 2 hr prior to LPS (1 μg/ml) or TNF-α (10 ng/ml) stimulation for 24 hr. The cells were analyzed for CD44 expression by flow cytometry as described under "Materials and Methods". The results shown are representative of four experiments.

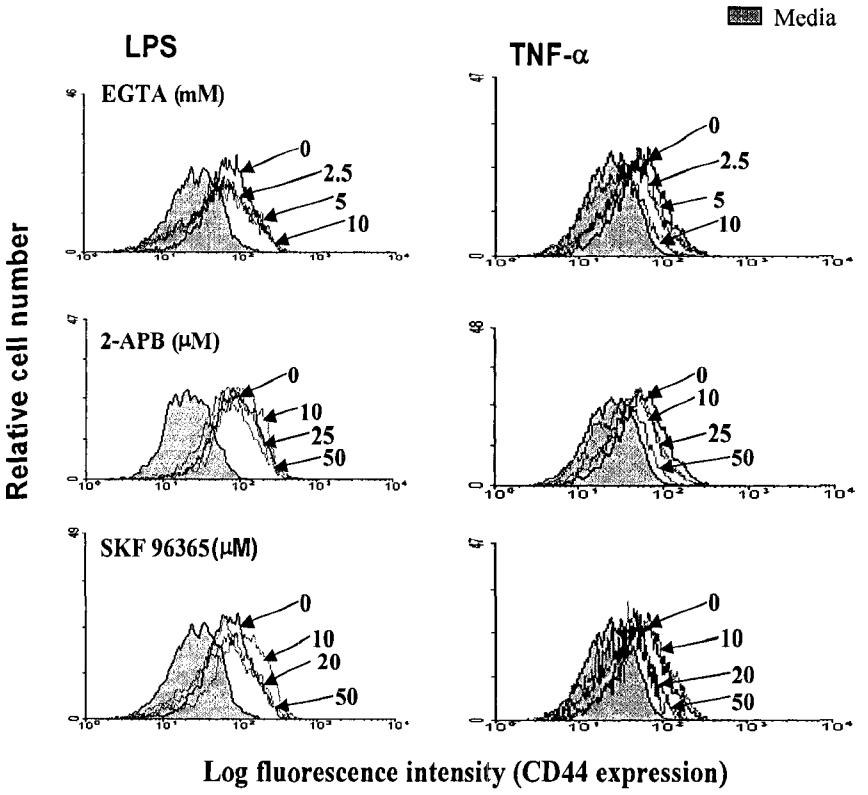

Media
LPS
TNF-α
EGTA (mM)
0
2.5
5
10
2-APB (μM)
0
10
25
50
SKF 96365(μM)
0
10
20
50
Relative cell number
Log fluorescence intensity (CD44 expression)

Fig. 3.3

protein kinases and protein phosphatases (269). To understand the role of CaM, I employed its specific inhibitor, W-7 (186). W-7 inhibited TNF-α-induced CD44 expression in a dose-dependent manner without any effect on LPS-induced CD44 expression (Fig. 3.4B).

One major family of Ca^{2+}/CaM effectors is the CaMKs, which includes a multifunctional CaMK-II that phosphorylates a large number of signaling proteins. To gain further insight into the role of Ca^{2+}/CaM, I examined the involvement of CaMK-II by employing the CaMK-II-specific inhibitor, KN-93 (186;269). Cells were pretreated with KN-93 for 2 hr followed by LPS or TNF-α stimulation. KN-93 inhibited TNF-α- but not LPS-induced CD44 expression in a dose-dependent manner (Fig. 3.4B). To determine the biological activities of W-7 and KN-93, cells were treated with W-7 or KN-93 for 2 hr followed by stimulation with either LPS or TNF-α for 30 min. Both LPS and TNF-α enhanced CaMK-II activity by 2-3-fold and was inhibited by W-7 and KN-93 in a dose-dependent manner (Fig. 3.4A). To determine the specificity of KN93, an inactive analogue of KN93, KN92 was employed. KN92 did not affect TNF-α-induced CD44 expression (data not shown) (191).

To further confirm the involvement of CaMK-II in TNF-α-induced CD44 expression, cells were transfected with a dominant negative CaMK-II plasmid or a control vector (191). CD44 expression was significantly inhibited in cells transfected with the dominant negative CaMK-II plasmid following TNF-α, but not LPS stimulation compared with the cells transfected with the control vector (Fig. 3.4C). In addition, LPS- and TNF-α-induced CaMK-II activity was inhibited by transfecting cells with dominant negative CaMK-II plasmid. Following transfection with the dominant negative CaMK-II

Fig. 3.4: CaM and CaMK-II selectively regulate TNF-α-induced CD44 expression.

A) LPS and TNF-α induce CaMK-II activity in THP-1/CD14 cells: Cells (1 X 10^6/ml) were pretreated with inhibitors for 2 hr followed by stimulation with LPS and TNF-α for 10 min. Cell pellets were lysed and CaMK-II activity was assayed from total cell proteins by employing a specific peptide substrate in the presence of [γ-^{32}P]ATP. The incorporated radioactivity was measured by Microbeta counter. CaMK-II activity was measured as counts/min/μg of protein and calculated as pM.

B) THP-1/CD14 cells (0.5 X 10^6/ml) were treated with various concentrations of inhibitors specific for CaM (W-7, 10-50 μM) or CaMK-II (KN-93, 5-20 μM) for 2 hr prior to LPS (1 μg/ml) or TNF-α (10 ng/ml) stimulation for 24 hr. The cells were analyzed for CD44 expression by flow cytometry. Shaded histogram represents unstimulated cells. The results shown are a representative of four experiments.

C) Dominant negative CaMK-II inhibits TNF-α- but not LPS-induced CD44 expression: THP-1/CD14 cells (2 X 10^6/ml) were transfected with dominant negative (DN) CaMK-II plasmid or a control vector using FuGENE 6 transfection reagent as described under "Materials and Methods". After 8 hr of transfection, cells were stimulated with either LPS (1 μg/ml) or TNF-α (10 ng/ml) for 24 hr followed by analysis for CD44 expression by flow cytometry. The results shown are representative of four independent experiments.

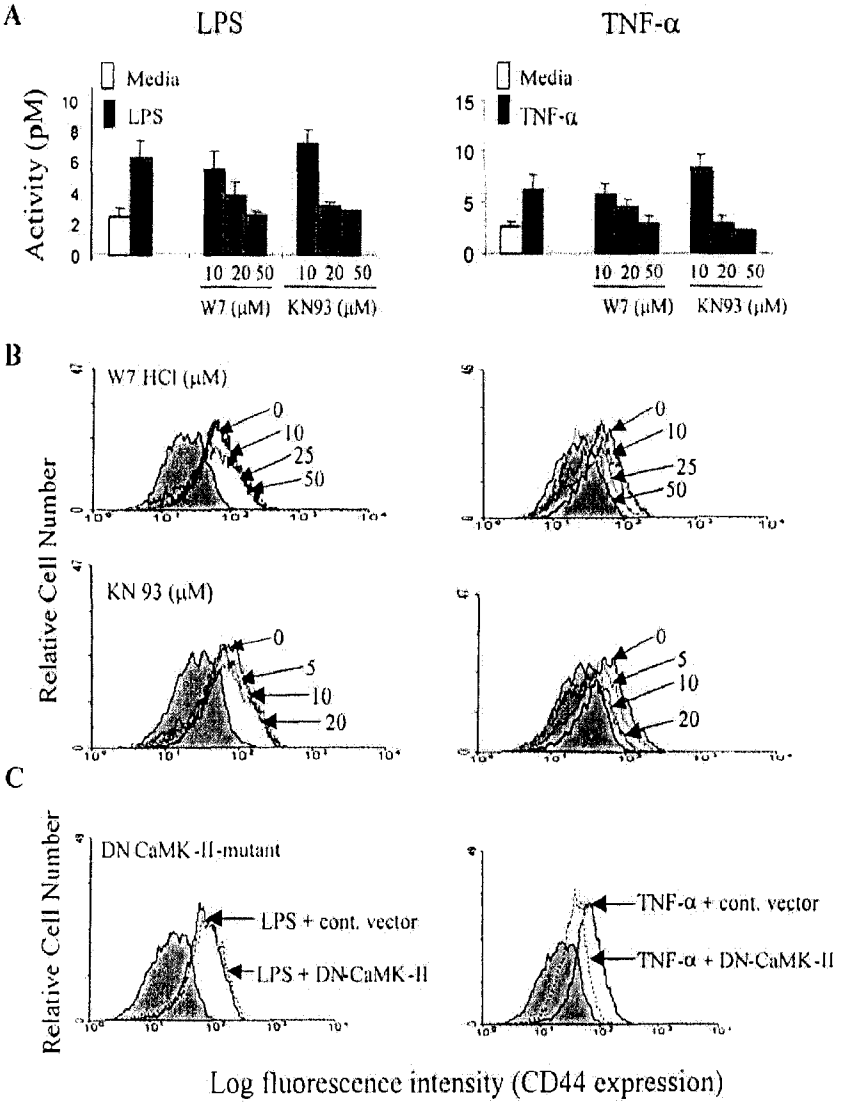

A
LPS
TNF-α
Media
LPS
Media
TNF-α
Activity (pM)
10 20 50 10 20 50
W7 (μM) KN93 (μM)
10 20 50 10 20 50
W7 (μM) KN93 (μM)
B
W7 HCl (μM)
0
10
25
50
KN 93 (μM)
0
5
10
20
Relative Cell Number
C
DN CaMK-II-mutant
LPS + cont. vector
LPS + DN-CaMK-II
TNF-α + cont. vector
TNF-α + DN-CaMK-II
Relative Cell Number
Log fluorescence intensity (CD44 expression)

Fig. 3.4

plasmid, CaMK-II activity was observed as 3 ± 1.2 pM in stimulation with either LPS or TNF-α compared with the activity of 7 ± 1 pM in LPS- and 6 ± 1.5 pM in TNF-α-stimulated cells transfected with the control vector.

Calcineurin is also activated by the binding of Ca^{2+} to CaM, which dissociates the two proteins and allows the catalytic site of calcineurin to become accessible (186). To determine the role of calcineurin, cells were treated with cyclosporine A or FK-506, the calcineurin inhibitors, prior to stimulation with either LPS or TNF-α. Neither cyclosporine-A nor FK-506 inhibited CD44 expression in either LPS- or TNF-α-stimulated cells (Fig. 3.5). Overall, the results suggest that TNF-α-induced CD44 expression may be regulated selectively by CaMK-II through the activation of CaM. It may be noted that none of these inhibitors induced apoptosis at the concentrations used as determined by propidium iodide staining at 24 hr (data not shown). Furthermore, similar results were obtained by using the parental THP-1 cells (166). Because of enhanced LPS-induced responses in THP-1/CD14 cells compared with the parental THP-1 cells (270), and because both THP-1 and THP-1/CD14 cells responded in a similar manner with respect to the involvement of JNK and Ca^{2+} signaling pathways in LPS- and TNF-α-induced CD44 expression, I subsequently employed THP-1/CD14 cells.

Differential involvement of Egr-1 and AP-1 in LPS- and TNF-α-induced CD44 transcription, respectively

The transcription factors involved in CD44 regulation in monocytic cells, and particularly in response to either LPS or TNF-α stimulation are not known. To identify the transcription factors activated by the CaM/CaMK-II and the JNK pathways involved

Fig. 3.5: Calcineurin does not regulate LPS- and TNF-α-induced CD44 expression.

THP-1/CD14 cells (0.5 X 10^6/ml) were treated with various concentrations of inhibitors specific for calcineurin (FK-506, 1-5 μM, and cyclosporine A, 1-5 μM) for 2 hr prior to LPS (1 μg/ml) or TNF-α (10 ng/ml) stimulation for 24 hr. The cells were analyzed for CD44 expression by flow cytometry. Shaded histogram represents unstimulated cells. The results shown are representative of four independent experiments.

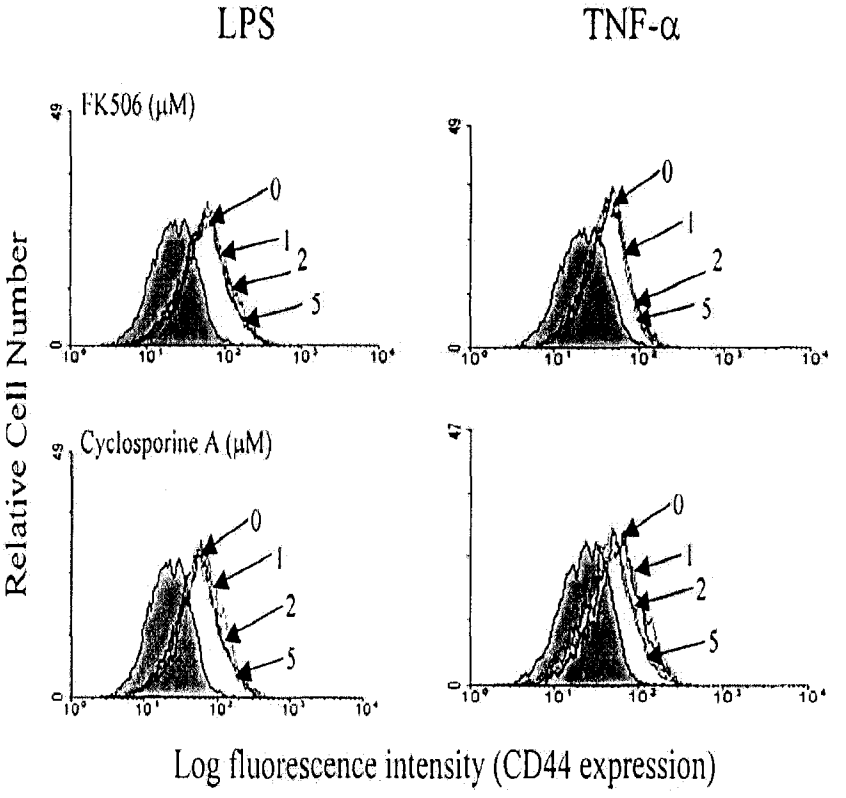

LPS
TNF-α
FK506 (μM)
Cyclosporine A (μM)
0
1
2
5
Relative Cell Number
Log fluorescence intensity (CD44 expression)

Fig. 3.5

in the regulation of TNF-α- and LPS-induced CD44 transcription in monocytic cells, respectively, the human CD44 promoter fragment encompassing nucleotide residues from -1109 to +53 bp relative to the +1 translation start site was cloned, amplified, and subcloned into the *Nhe* I and *Hind* III polylinker site of the luciferase reporter plasmid, pGL3B (pCD44Pr-GL3B). THP-1/CD14 cells were transiently transfected with pCD44Pr-GL3B. After 15 hr, cells were stimulated with either LPS or TNF-α following which relative luciferase activity was assessed. The luciferase activity was detected by 4 hr and peaked at 8 hr following stimulation with either LPS or TNF-α (Fig. 3.6A). The luciferase activity detected in unstimulated cells following 8 hr of culture was 10-15-fold higher compared with the cells transfected with the control plasmid perhaps because of the constitutive expression of CD44 in these cells. The luciferase activity increased by 2.5-3-fold following LPS and TNF-α stimulation compared with unstimulated cells and by 40-50-fold compared with cells transfected with the control plasmid.

To determine the DNA sequences required for CD44 transcription, a series of promoter fragments (from 5' -1109 to 3' +53 bp) were generated by successive deletions starting from the 5'-end. The CD44 promoter fragments were amplified, inserted into pGL3B, and sequenced. The exact size of the amplified product and the location of various transcription factor binding sites identified within the CD44 promoter are depicted in Fig. 3.6B. Examination of the DNA sequences within the CD44 promoter region containing various deletions revealed that deletion of sequences from -1109 to -334 bp had no effect on LPS- and TNF-α-induced luciferase activity compared with the cells transfected with the full-length pCD44Pr-GL3B. However, deletion of sequences from -334 to -224 bp abrogated the LPS- and TNF-α-induced luciferase activity

Fig. 3.6: LPS and TNF-α stimulation induces luciferase activity in THP-1/CD14 cells transfected with a CD44 promoter/luciferase reporter gene construct.

A) THP-1/CD14 cells (2 X 10^6) were transiently cotransfected with 1 μg of either full-length CD44 promoter construct (pCD44PrGL3B) or pGL3B control vector and 0.5 μg of β-galactosidase plasmid. After 15 hr, the transfected cells were stimulated with either LPS (1 μg/ml) or TNF-α (10 ng/ml) for various times followed by the measurement of luciferase and β-galactosidase activities in the cell lysates. Luciferase activity was normalized with β-galactosidase activity to get relative luciferase units. The results shown are mean ± S.D. of three independent experiments performed in triplicate.

B) Transcriptional activities of the CD44 promoter deletion constructs in LPS- and TNF-α-stimulated THP-1/CD14 cells: Cells (2 X 10^6/ml) were transiently cotransfected with various CD44 promoter deletion constructs or control pGL3B vector (1 μg) and β-galactosidase plasmid (0.5 μg). After 15 hr, the transfected cells were stimulated with LPS (1 μg/ml) or TNF-α (10 ng/ml) for another 8 hr followed by measurement of luciferase and β-galactosidase activities. Following normalization, the luciferase activity was calculated as relative luciferase units as described earlier. The results shown are mean ± S.D. of three independent experiments performed in triplicate.

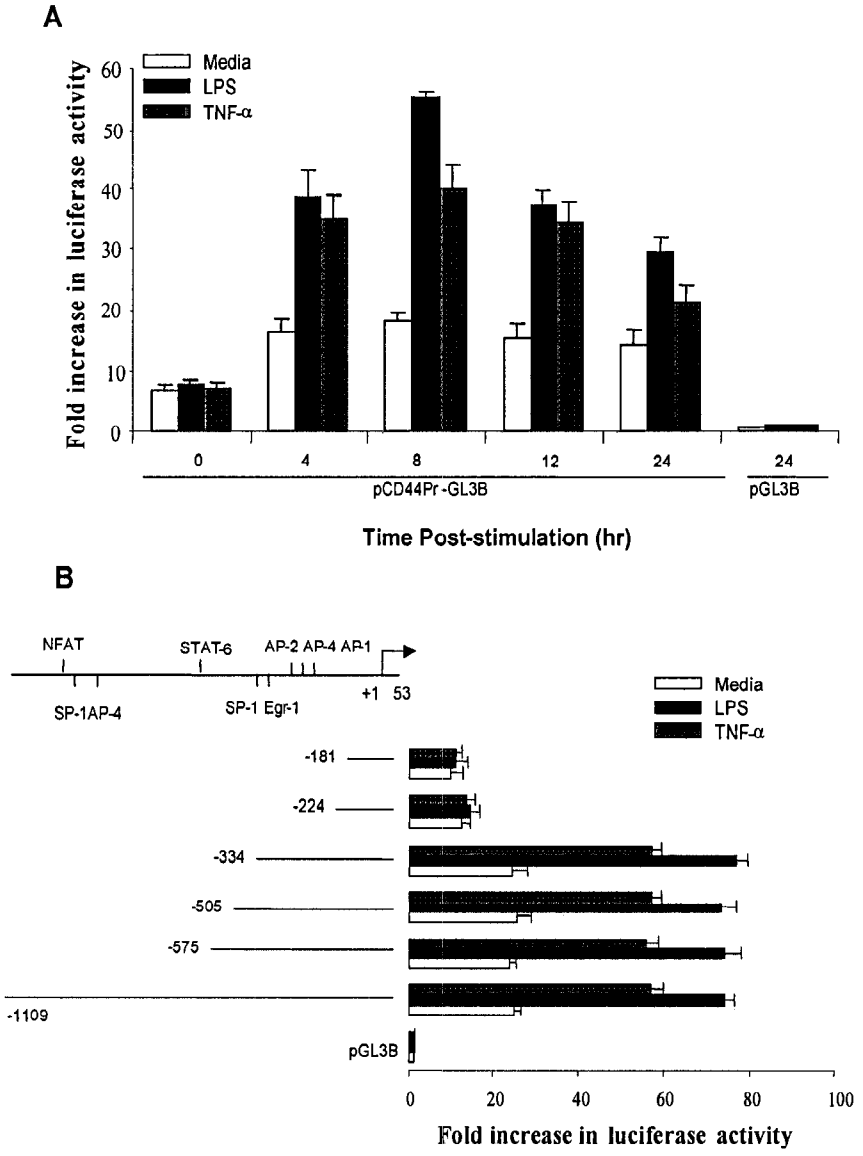

A
Media
LPS
TNF-α
Fold increase in luciferase activity
60
50
40
30
20
10
0
0
4
8
12
24
24
pCD44Pr-GL3B
pGL3B
Time Post-stimulation (hr)
B
NFAT
STAT-6
AP-2 AP-4 AP-1
+1 53
SP-1AP-4
SP-1 Egr-1
Media
LPS
TNF-α
-181
-224
-334
-505
-575
-1109
pGL3B
0
20
40
60
80
100
Fold increase in luciferase activity

Fig. 3.6

significantly ($p<0.001$) suggesting the involvement of the -334 to -224 sequence in LPS- and TNF-α-induced CD44 transcription (Fig. 3.6B).

MAT inspector analysis of the promoter sequence between -334 to -224 revealed the existence of Egr-1 (-293 to -301 bp) and AP-1 (-238 to -243 bp) binding sites (Fig. 3.7A). To delineate the role of Egr-1 and AP-1, a CD44 promoter deletion construct from -264 to +53 bp [pCD44Pr(-264)] containing the AP-1 but not the Egr-1-binding site was generated. LPS stimulation of cells transfected with pCD44Pr(-264) revealed an abrogation of luciferase activity compared with the cells transfected with pCD44Pr(-334) containing both Egr-1 and AP-1 binding sites, suggesting a role for Egr-1 in LPS-induced CD44 transcription (Fig. 3.7B, left panel). To confirm the role of Egr-1, I generated an Egr-1 mutant plasmid, pCD44Pr(-334-Egr-1m). Cells transfected with pCD44Pr(-334-Egr-1m) showed a significant ($P<0.001$) reduction in luciferase activity following LPS stimulation (Fig. 3.7B, left panel) compared with stimulation with TNF-α (Fig. 3.7B, right panel) and to that of the cells transfected with pCD44Pr(-334) containing both Egr-1 and AP-1 sequences (Fig. 3.7B, left panel). Significantly, transfection of cells with pCD44Pr(-264) containing the AP-1 site did not show reduction of luciferase activity upon TNF-α stimulation (Fig. 3.7B, right panel), suggesting that the AP-1-binding sequence may play a key role in TNF-α-induced CD44 transcription. To confirm the role of AP-1, I introduced mutations in the AP-1-binding sequence [pCD44Pr(-264-AP-1m)]. Cells transfected with pCD44Pr(-264-AP-1m) showed significant ($P<0.001$) reduction in luciferase activity following stimulation with TNF-α as compared with cells transfected with pCD44Pr(-264) containing the wild type AP-1 sequence (Fig. 3.7B, right panel).

Fig. 3.7: The effect of mutating the Egr-1 and AP-1 binding sites on CD44 promoter activity in LPS- and TNF-α-stimulated THP-1/CD14 cells.

A) Nucleotide sequence of the CD44 promoter from 5' -334 to -224 bp showing Egr-1 and AP-1 sequences and the superscript indicates mutated nucleotides.

B) THP-1/CD14 cells (2 X 10^6) were transiently cotransfected with 1 μg of either CD44 promoter deletion constructs containing mutant Egr-1 and AP-1 binding sequences or pGL3B and 0.5 μg of β-galactosidase control plasmid. After 15 hr, transfected cells were stimulated with either LPS (1 μg/ml) or TNF-α (10 ng/ml) for 8 hr followed by measurement of luciferase and β-galactosidase activities. Luciferase activity was normalized for β-galactosidase activity to calculate relative luciferase units. The results shown are the mean ± S.D. of three independent experiments performed in triplicate.

C) The effect of mutating either Egr-1 or AP-1 alone, or both Egr-1 and AP-1 binding sites on CD44 promoter activity in LPS and TNF-α stimulated THP-1/CD14 cells: THP-1/CD14 cells (2 X 10^6) were transiently cotransfected with 1 μg of CD44 promoter deletion constructs containing wild type Egr-1 and AP-1 sequences pCD44Pr(-334), mutation at the Egr-1 site alone p(-334-Egr-1m), AP-1 alone, p(-334-AP-1m), both Egr-1 and AP-1 sites p(-334Egr-1m,AP-1m), or control pGL3B and β-galactosidase plasmid (0.5 μg). Following stimulation with either LPS or TNF-α for 8 hr, luciferase activity was calculated as relative luciferase units as described above. The results shown are the mean ± S.D. of three independent experiments performed in triplicate.

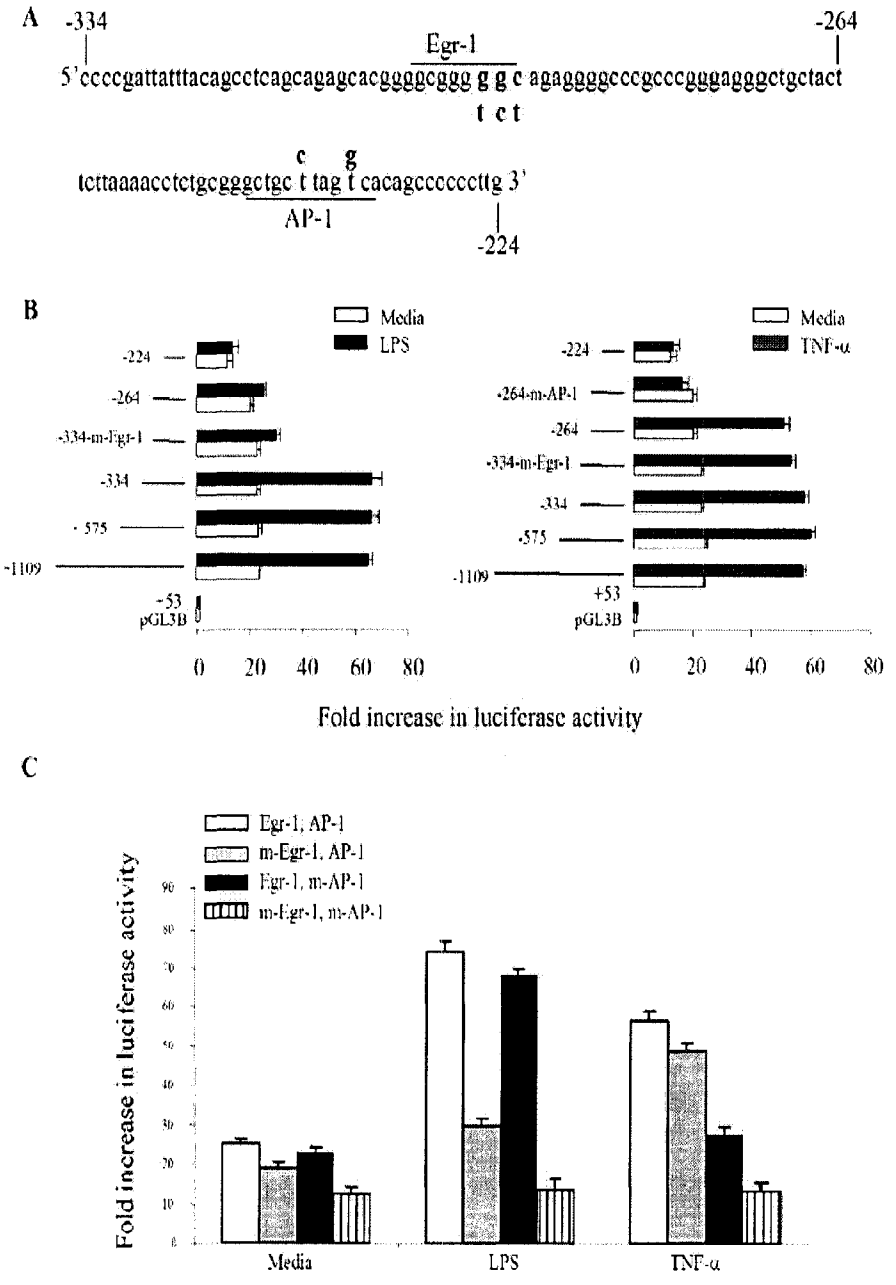

A
-334
-264
Egr-1
5'ccccgattatttacagcctcagcagagcacgggggcggg g g c agagggggcccgcccgggagggctgctact
t c t
c g
tcttaaaacctctgcgggctgc t tag t cacagcccccctg 3'
AP-1
-224
B
Media
LPS
-224
-264
-334-m-Egr-1
-334
-575
-1109
-53
pGL3B
0 20 40 60 80
Media
TNF-α
-224
-264-m-AP-1
-264
-334-m-Egr-1
-334
-575
-1109
+53
pGL3B
0 20 40 60 80
Fold increase in luciferase activity
C
Egr-1, AP-1
m-Egr-1, AP-1
Egr-1, m-AP-1
m-Egr-1, m-AP-1
Fold increase in luciferase activity
Media
LPS
TNF-α

Fig. 3.7

These results suggest that AP-1 and Egr-1 binding sequences may play key roles in the regulation of TNF-α- and LPS-induced CD44 transcription, respectively.

To further confirm the role of Egr-1 and AP-1 in LPS- and TNF-α-induced CD44 transcription, respectively, I generated three additional constructs from pCD44Pr(-334)-GL3B by site-directed mutagenesis. These constructs contained the mutations either in Egr-1 alone [p(-334-Egr-1m)], AP-1 alone [p(-334-AP-1m)], or in both Egr-1 and AP-1 [p(-334-Egr-1m,AP-1m)]. Transfection of cells with p(-334-Egr-1m) containing the mutant Egr-1 sequence alone resulted in significant ($P<0.001$) reduction of luciferase activity following LPS but not TNF-α stimulation compared with the cells transfected with pCD44Pr(-334) containing wild type Egr-1 and AP-1 sequences. Significantly, transfection of cells with p(-334-AP-1m) containing the mutant AP-1 sequence alone resulted in significant ($P<0.005$) reduction of luciferase activity following TNF-α but not LPS stimulation compared with the cells transfected with pCD44Pr(-334). As expected, transfection of cells with p(-334Egr-1m,AP-1m) containing both Egr-1 and AP-1 mutation sequences resulted in abrogation of luciferase activity in both LPS- and TNF-α-stimulated cells (Fig. 3.7C). These results suggest a critical role of Egr-1 and AP-1 in LPS- and TNF-α-induced CD44 transcription, respectively.

TNF-α-induced CD44 expression is selectively regulated by AP-1 through the activation of CaMK-II.

In view of the above results, it was of interest to determine whether TNF-α-induced CD44 expression is regulated by AP-1 through the activation of CaMK-II. To address this question, cells were transfected with pCD44Pr(-334-Egr-1m) containing m-Egr-1 and wild type-AP-1 sequences. The transfected cells were treated for 2 hr with the

inhibitors specific for JNK or Ca^{2+} signaling pathway prior to stimulation with TNF-α for 8 hr. As before, following TNF-α stimulation, a significant 2-3-fold increase in luciferase activity was observed compared with the unstimulated cells. Prior treatment of transfected cells with EGTA, SKF, APB, W-7, and KN-93 decreased the TNF-α-induced luciferase activity in a dose-dependent manner (Fig. 3.8). The luciferase activity is significantly ($P<0.001$) reduced in cells treated with highest dose for all the above five inhibitors (EGTA, SKF, 2-APB, W7, and KN-93) used compared to TNF-α stimulated cells alone. The luciferase activity observed with the highest concentration of the inhibitors was equivalent to the basal activity observed with unstimulated cells. Furthermore, inhibitors for calcineurin (FK-506 and cyclosporine A) and JNK (SP600125) did not affect TNF-α-induced luciferase activity at any concentration. These results suggested that TNF-α-induced CD44-transcription may be regulated by AP-1 through the activation of CaMK-II and not the calcineurin pathway (Fig. 3.8).

To further determine that TNF-α-induced CD44 expression is regulated by AP-1 via CaMK-II, the effect of inhibitors for Ca^{2+} signaling pathway and JNK on the binding of TNF-α-induced AP-1 to its binding site on the CD44 promoter was investigated by EMSA using the ^{32}P-labeled oligonucleotide promoter sequence containing the AP-1 binding site as probe. Maximum binding of AP-1 to the probe occurred by 120 min following TNF-α stimulation (Fig. 3.9A). The identity of a band corresponding to the AP-1 protein-DNA complex was established by competition with cold AP-1 oligonucleotides resulting in abrogation of this band, whereas nonspecific oligonucleotides had no effect on the intensity of this band. Furthermore, treatment of nuclear extracts with anti-c-Fos and anti-c-Jun antibodies resulted in the abrogation of the

Fig. 3.8: Ca^{2+} signaling pathway regulates TNF-α-mediated CD44 promoter activity in THP-1/CD14 cells.

THP-1/CD14 cells (2 X 10^6) were transiently cotransfected with 1 μg of CD44 promoter construct containing mutant Egr-1 binding site [pCD44Pr(-334-Egr-1m)-GL3B] and 0.5 μg of β-galactosidase plasmid and cultured for 15 hr. The transfected cells were stimulated with various concentrations of inhibitors 2 hr prior to stimulation with TNF-α (10 ng/ml) for 8 hr followed by measurement of luciferase activity as described above. The results shown are the mean ± S.D. of two experiments performed in triplicate.

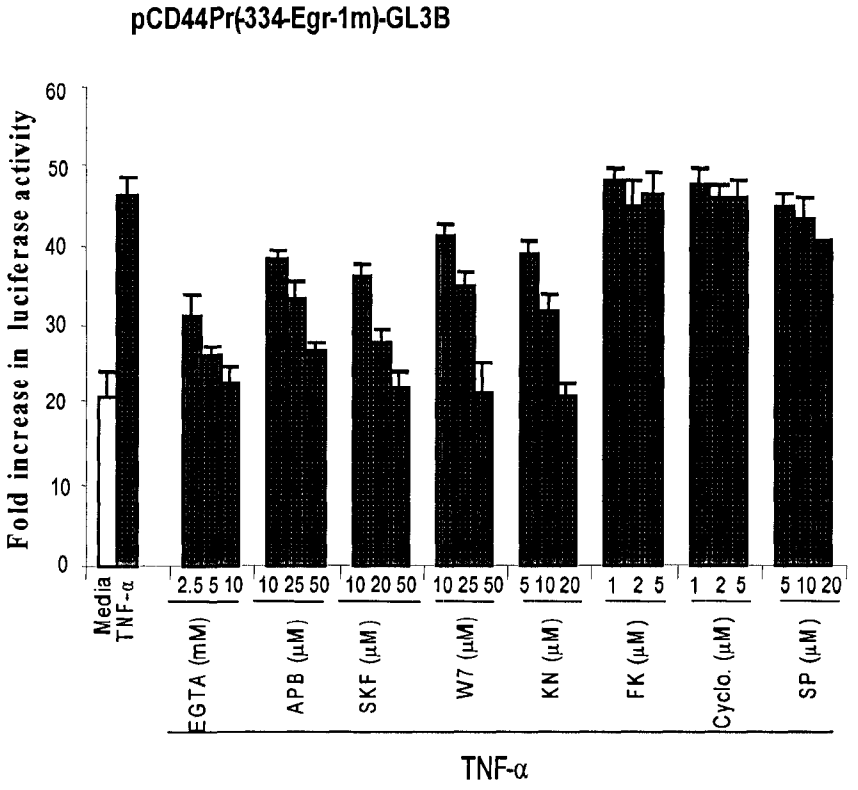

pCD44Pr(-334-Egr-1m)-GL3B
Fold increase in luciferase activity
60
50
40
30
20
10
0
Media
TNF-α
2.5 5 10
10 25 50
10 20 50
10 25 50
5 10 20
1 2 5
1 2 5
5 10 20
EGTA (mM)
APB (μM)
SKF (μM)
W7 (μM)
KN (μM)
FK (μM)
Cyclo. (μM)
SP (μM)
TNF-α

Fig. 3.8

Fig. 3.9: TNF-α stimulation activates the AP-1 transcription factor that is regulated by the Ca^{2+} signaling pathway.

A & B) THP-1/CD14 cells (1 X 10^6/ml) were stimulated with TNF-α (10 ng/ml) (A) or LPS (1 μg/ml) (B) for various times ranging from 30 to 240 min followed by collection of nuclear extracts. Nuclear extracts (5 μg each) samples were probed with ^{32}P-labeled oligonucleotides corresponding to the AP-1-binding sequence of the CD44 promoter. To determine the specificity of AP-1 binding, the nuclear extracts were probed in the presence of different concentrations of unlabeled specific (Sp oligo) or nonspecific oligonucleotides (NS oligo). The nuclear extract samples were also treated with anti-c-Jun or anti-c-Fos antibodies to identify AP-1 specific bands by supershift EMSA. To determine the effect of inhibitors of calcium and JNK MAPKs on TNF-α- and LPS-induced activation of AP-1, cells were treated with different concentrations of either SP600125 or calcium inhibitors (EGTA, W-7, SKF, APB, KN-93 and FK-506) for 2 hr prior to stimulation with either TNF-α or LPS. The complexes were subjected to electrophoresis followed by autoradiography. *Arrows* indicate the AP-1 bands. The experiment shown is representative of three independent experiments.

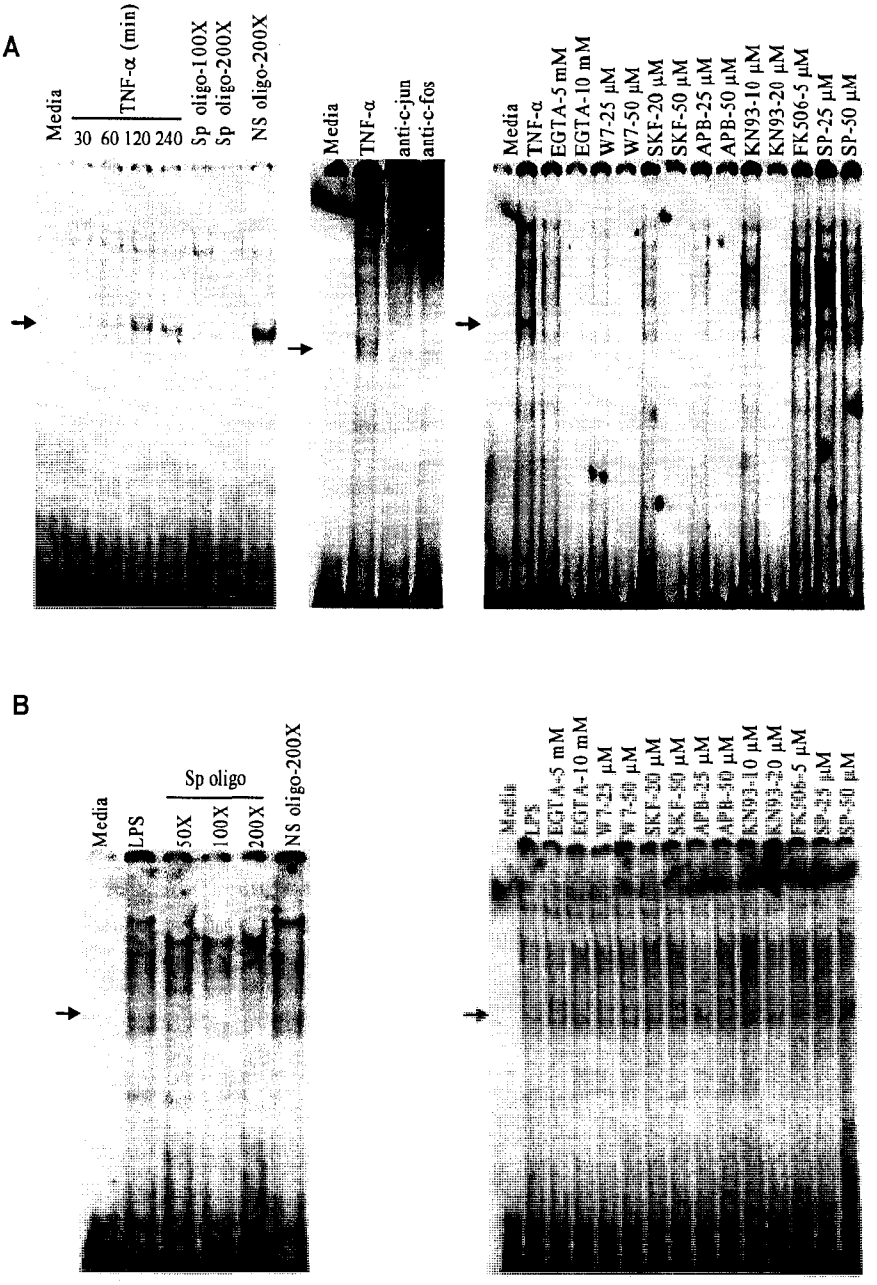

A
Media
30 60 120 240
TNF-α (min)
Sp oligo-100X
Sp oligo-200X
NS oligo-200X
Media
TNF-α
anti-c-jun
anti-c-fos
Media
TNF-α
EGTA-5 mM
EGTA-10 mM
W7-25 μM
W7-50 μM
SKF-20 μM
SKF-50 μM
APB-25 μM
APB-50 μM
KN93-10 μM
KN93-20 μM
FK506-5 μM
SP-25 μM
SP-50 μM
B
Media
LPS
Sp oligo
50X
100X
200X
NS oligo-200X
Media
LPS
EGTA-5 mM
EGTA-10 mM
W7-25 μM
W7-50 μM
SKF-20 μM
SKF-50 μM
APB-25 μM
APB-50 μM
KN93-10 μM
KN93-20 μM
FK506-5 μM
SP-25 μM
SP-50 μM

Fig. 3.9

AP-1-specific band. To determine the effect of the Ca^{2+} signaling pathway inhibitors on the binding of AP-1 to its binding site in the CD44 promoter, cells were treated for 2 hr with various inhibitors prior to stimulation with TNF-α for 2 hr. The Ca^{2+} signaling inhibitors EGTA, SKF, APB, W-7, and KN-93 inhibited AP-1 binding to its probe. As before, the calcineurin and JNK inhibitors (FK-506, cyclosporine A, and SP600125) did not affect the binding of AP-1 (Fig. 3.9A).

To rule out the possibility that the LPS-activated Ca^{2+} signaling pathway does not regulate AP-1 and eventual CD44 transcription, similar gel shift experiments were performed following LPS stimulation. The maximum binding of AP-1 to the ^{32}P-labeled AP-1 oligonucleotide probe occurred at 2 hr following LPS stimulation (data not shown). A major band corresponding to the AP-1-DNA complex was observed and was blocked by competition with specific unlabeled AP-1 oligonucleotides. Pretreatment of cells with either of the inhibitors for Ca^{2+} or the JNK-MAPK pathway did not inhibit the formation of the AP-1 protein-DNA complex (Fig. 3.9B). Taken together, these results show the binding of AP-1 to the CD44 promoter in both LPS- and TNF-α-stimulated THP-1/CD14 cells. However, TNF-α-induced CD44 transcription may be regulated by AP-1 through the activation of CaMK-II and not via the calcineurin or JNK pathway.

Distinct regulation of LPS-induced CD44 expression by Egr-1 through JNK activation

To determine whether LPS-induced CD44 expression is regulated by Egr-1 through JNK activation, cells transfected with pCD44Pr(-334-AP-1m) containing wild type Egr-1, mAP-1 sequences were treated for 2 hr with the JNK and Ca^{2+} signaling pathway inhibitors prior to stimulation with LPS for 8 hr. As before, following LPS

stimulation, a significant 2-3-fold increase in luciferase activity was observed compared with the unstimulated cells. LPS-stimulated luciferase activity was inhibited by SP600125 in a dose-dependent manner. The luciferase activity observed with the highest concentration of SP600125 was similar to the basal activity observed in unstimulated cells and the reduction was significant ($P<0.001$) compared with LPS stimulation alone. However, Ca^{2+} signaling inhibitors (EGTA, SKF, APB, W-7, and KN-93) did not affect LPS-induced luciferase activity (Fig. 3.10). These results suggested that LPS-induced CD44 transcription may be regulated by Egr-1 through the activation of JNK MAPK.

To confirm that LPS-induced CD44 expression is regulated by Egr-1 through JNK activation, the effect of Ca^{2+} signaling pathway and JNK MAPK inhibitors on the binding of LPS-induced Egr-1 to its binding site in CD44 promoter was investigated by EMSA by using the ^{32}P-labeled oligonucleotide probe containing the Egr-1 sequence of the CD44 promoter. LPS induced maximum binding of Egr-1 at 60 min post-stimulation (166). I observed three Egr-1 containing bands as their intensity was abrogated following specific competition with unlabeled oligonucleotides. In contrast, nonspecific oligonucleotides did not affect the intensity of any of the Egr-1 bands. Furthermore, treatment of nuclear extracts with anti-Egr-1 antibodies exhibited abrogation of the top two Egr-1 specific bands. To determine the role of JNK and Ca^{2+} signaling pathways, cells were treated for 2 hr with various inhibitors prior to stimulation with LPS for 2 hr. SP600125 significantly reduced binding of LPS-induced Egr-1 to the labeled probe (Fig. 3.11A), whereas Ca^{2+} signaling inhibitors including EGTA, SKF, APB, W-7, FK-506, cyclosporine A, and KN-93 did not affect the binding, suggesting that LPS-induced CD44 transcription may be regulated by Egr-1 through JNK activation.

Fig. 3.10: JNK MAPK regulates LPS-mediated CD44 promoter activity in THP-1/CD14 cells.

THP-1/CD14 cells (2 X 10^6) were transiently cotransfected with 1 μg of CD44 promoter deletion construct containing mutant AP-1 binding site [pCD44Pr(-334-AP-1m)-GL3B] and 0.5 μg of β-galactosidase plasmid and cultured for 15 hr. The transfected cells were stimulated with various concentrations of inhibitors 2 hr prior to stimulation with LPS (1 μg/ml) for 8 hr followed by measurement of luciferase activity as described above. The results shown are the mean ± S.D. of two experiments performed in triplicate.

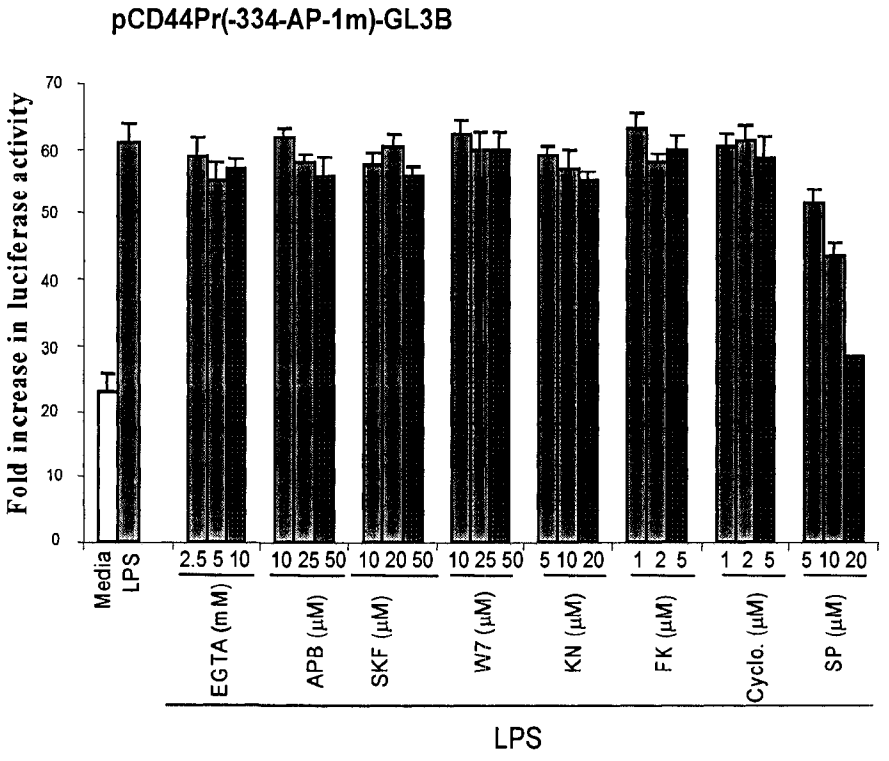

pCD44Pr(-334-AP-1m)-GL3B
Fold increase in luciferase activity
70
60
50
40
30
20
10
0
Media
LPS
2.5 5 10
10 25 50
10 20 50
10 25 50
5 10 20
1 2 5
1 2 5
5 10 20
EGTA (mM)
APB (μM)
SKF (μM)
W7 (μM)
KN (μM)
FK (μM)
Cyclo. (μM)
SP (μM)
LPS

Fig. 3.10

Fig. 3.11: LPS-induced Egr-1 is regulated by SP600125.

A & B) THP-1/CD14 cells (1 X 10^6/ml) were stimulated with LPS (1 μg/ml) (A) or TNF-α (10 ng/ml) (B) for 60 min followed by collection of nuclear extracts. Nuclear extracts (5 μg) were probed with ^{32}P-labeled oligonucleotides containing the Egr-1-binding sequence of the CD44 promoter. To determine the specificity of Egr-1 binding, the nuclear extracts were probed in the presence of different concentrations of unlabeled specific (Sp oligo) or nonspecific oligonucleotides (NS oligo). The nuclear extracts were also treated with anti-Egr-1 antibodies to identify the Egr-1 bands. To determine the effect of inhibitors of calcium and JNK MAPKs on LPS- and TNF-α-induced activation of Egr-1, cells were treated with different concentrations of either SP600125 or Ca^{2+} signaling inhibitors (EGTA, W-7, SKF, APB, KN-93 and FK-506) for 2 hr prior to stimulation with either LPS or TNF-α. The complexes were subjected to electrophoresis followed by autoradiography. Arrows indicate the Egr-1 bands. The experiment shown is representative of three independent experiments.

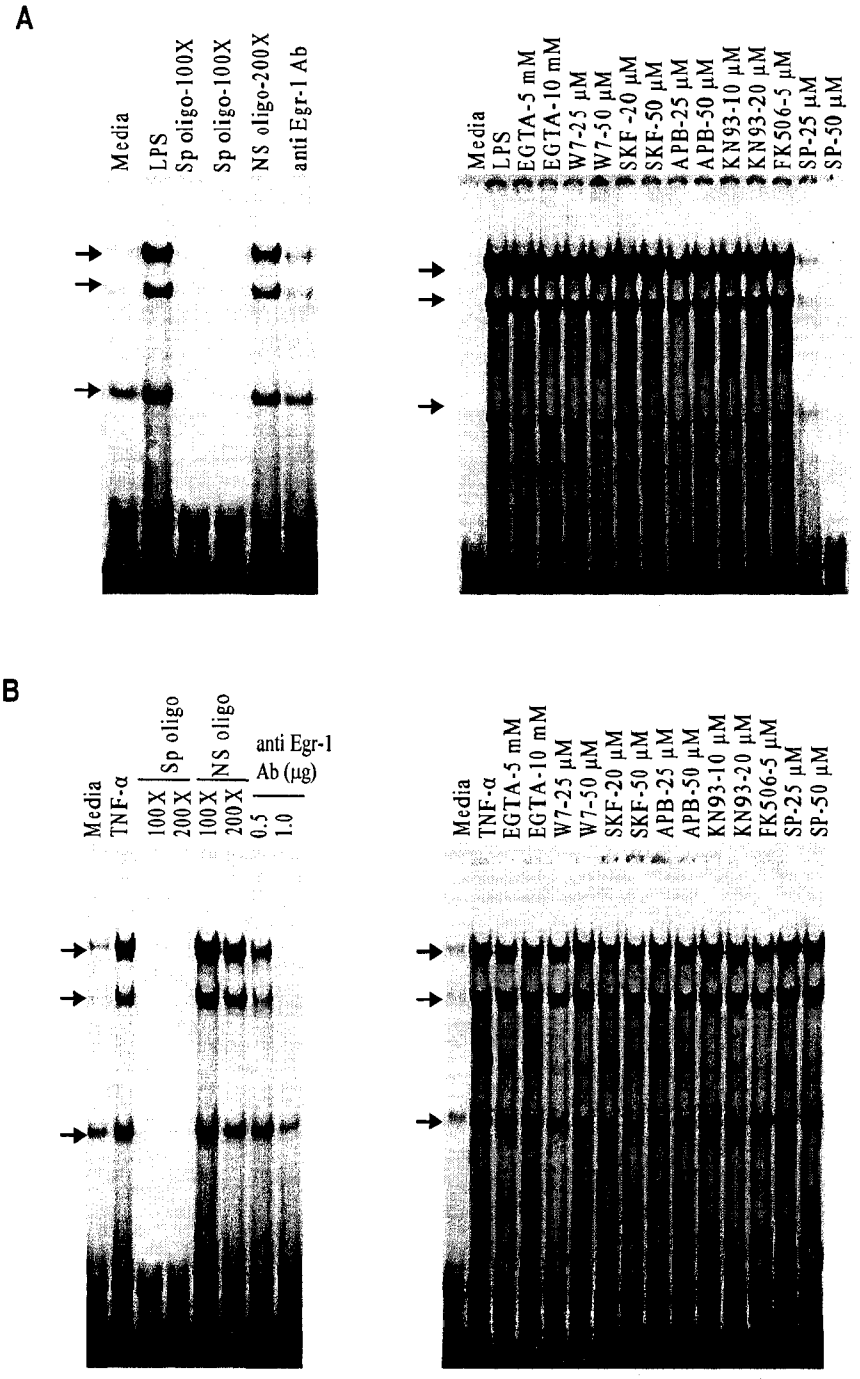

A
Media
LPS
Sp oligo-100X
Sp oligo-100X
NS oligo-200X
anti Egr-1 Ab
Media
LPS
EGTA-5 mM
EGTA-10 mM
W7-25 μM
W7-50 μM
SKF -20 μM
SKF-50 μM
APB-25 μM
APB-50 μM
KN93-10 μM
KN93-20 μM
FK506-5 μM
SP-25 μM
SP-50 μM
B
Media
TNF-α
100X
200X
Sp oligo
100X
200X
NS oligo
0.5
1.0
anti Egr-1
Ab (μg)
Media
TNF-α
EGTA-5 mM
EGTA-10 mM
W7-25 μM
W7-50 μM
SKF-20 μM
SKF-50 μM
APB-25 μM
APB-50 μM
KN93-10 μM
KN93-20 μM
FK506-5 μM
SP-25 μM
SP-50 μM

Fig. 3.11

To rule out the possibility of the Ca^{2+} signaling pathway regulating TNF-α-induced Egr-1 activation, similar gel shift experiments were performed. The results show that as for LPS, three Egr-1 containing bands were observed with maximum binding occurring at 60 min following TNF-α stimulation (51). The intensity of these bands was blocked specifically by competition with cold Egr-1 oligonucleotides. Furthermore, anti-Egr-1 antibodies abrogated the intensity of top two TNF-α-induced Egr-1 bands. Prior treatment of cells with either of the inhibitors for JNK or the Ca^{2+} signaling pathway did not inhibit the formation of Egr-1 bands (Fig. 3.11B). Taken together, the results show the binding of Egr-1 to the CD44 promoter following stimulation with both LPS and TNF-α, however, Egr-1 may play a key role in LPS-induced CD44 transcription in THP-1/CD14 cells.

B) Role of PI 3-kinase pathway

Introduction

Evidence suggests the role of the PI3K pathway in the regulation of cellular activation, chemotaxis and inflammatory responses (193;194). It is well documented that LPS and TNF-α activate PI3K pathway in human monocytic cells (194;262;271-273). As per my results discussed in chapter III, I have demonstrated that LPS- and TNF-α-induced CD44 expression is regulated by two independent signaling pathways in human monocytic cells. LPS-induced CD44 expression is regulated by c-Jun N-terminal kinase (JNK)-activated Egr-1, whereas calmodulin-dependent protein kinase-II (CAMK-II)-activated AP-1 regulates TNF-α-induced CD44 expression (51).

Fichter et al. (1997) suggested that CD44 expression was regulated by PI3K and PKC in neuroblastoma cells (175). Further, PI3K plays a critical role in other biological functions associated with CD44 in different cell types. For examples, the osteopontin-CD44 survival signal involves activation of PI3K/Akt pathway (274), and Ras oncoprotein induces CD44 cleavage through PI3K activation (275). However, the role of PI3K in LPS- and TNF-α-induced CD44 expression in human monocytic cells is not fully understood. ***Therefore, to extend my previous study further to identify the involved other signaling pathways in CD44 regulation, and the possible cross-talk with JNK and calmodulin/CaMK-II, I investigated the role of PI3K.*** My results for the first time suggest that PI3K is involved in the regulation of both LPS- and TNF-α-induced CD44 expression downstream to JNK-MAPK and Ca^{2+}/CAMK-II. Further, LPS- and TNF-α-induced CD44 expression was regulated by the transcription factor Egr-1 and AP-1, respectively through the activation of PI3K.

Results

The p85α regulatory subunit but not p110α catalytic subunit of PI3K regulates LPS- and TNF-α-induced CD44 expression in monocytic cells.

First, I confirmed the earlier observations (51;166) by demonstrating CD44 induction by LPS and TNF-α in promonocytic THP-1/CD14 cells and primary human monocytes (Fig. 3.12A). To understand the role of PI3K/Akt pathway, THP-1/CD14 cells and monocytes were treated with a specific PI3K inhibitor, LY294002, for 2 hr followed by stimulation with LPS or TNF-α. My results showed that LY294002 inhibited LPS- and TNF-α-induced CD44 expression in a dose dependent manner (Fig. 3.12A). Furthermore, LY294002 inhibited the phosphorylation of Akt, an important downstream signaling molecule of PI3K, in both THP-1/CD14 cells and isolated human monocytes (Fig. 3.12B).

Mammalian class-I_A PI3K family members are heterodimers consisting of a regulatory (p85α, p85β, p55 or other splice variants) and a p110 (α, β, or γ) catalytic subunits (194). At first, I investigated the role of the PI3K catalytic subunit p110α in LPS- and TNF-α-induced CD44 expression by employing THP-1 cells deficient in p110α expression (HR-p110α3). THP-1- HR-p110α3 cells expressed normal levels of the p85α regulatory subunit as well as p110β and p110γ isoforms compared to the p110α levels expressed in cells transduced with the control lentiviral vector HRp110α1 (255) (Fig. 3.13A). To determine the role of p110α, HR-p110α3 and HR-p110α1 cells were stimulated with LPS or TNF-α followed by analysis of CD44 expression. Both HR-p110α3 and HR-p110α1 cells induced comparable levels of CD44 expression following

Fig. 3.12: PI3K regulates LPS- and TNF-α-induced CD44 expression in human monocytic cells.

A) THP-1/CD14 cells and primary human monocytes (0.5 X 10^6/ml) were treated with various concentrations of LY294002 for 2 hr prior to stimulation with either LPS (1 μg/ml) or TNF-α (10 ng/ml) for 24 hr followed by analysis of CD44 expression by flow cytometry. The results shown are representative of four experiments.

B) THP-1/CD14 cells and monocytes (2 X 10^6) were treated with either LPS (1 μg/ml) or TNF-α (10 ng/ml) for various time periods (0 to 120 min), and also, cells were treated with LY294002 at varying concentrations for 2 hr prior to stimulation with either LPS or TNF-α. Total protein (30 μg) was subjected to Western blot analysis using either anti-phospho-Akt Ab. The same blots were stripped and reprobed with anti-Akt Ab. The results shown are representative of three experiments.

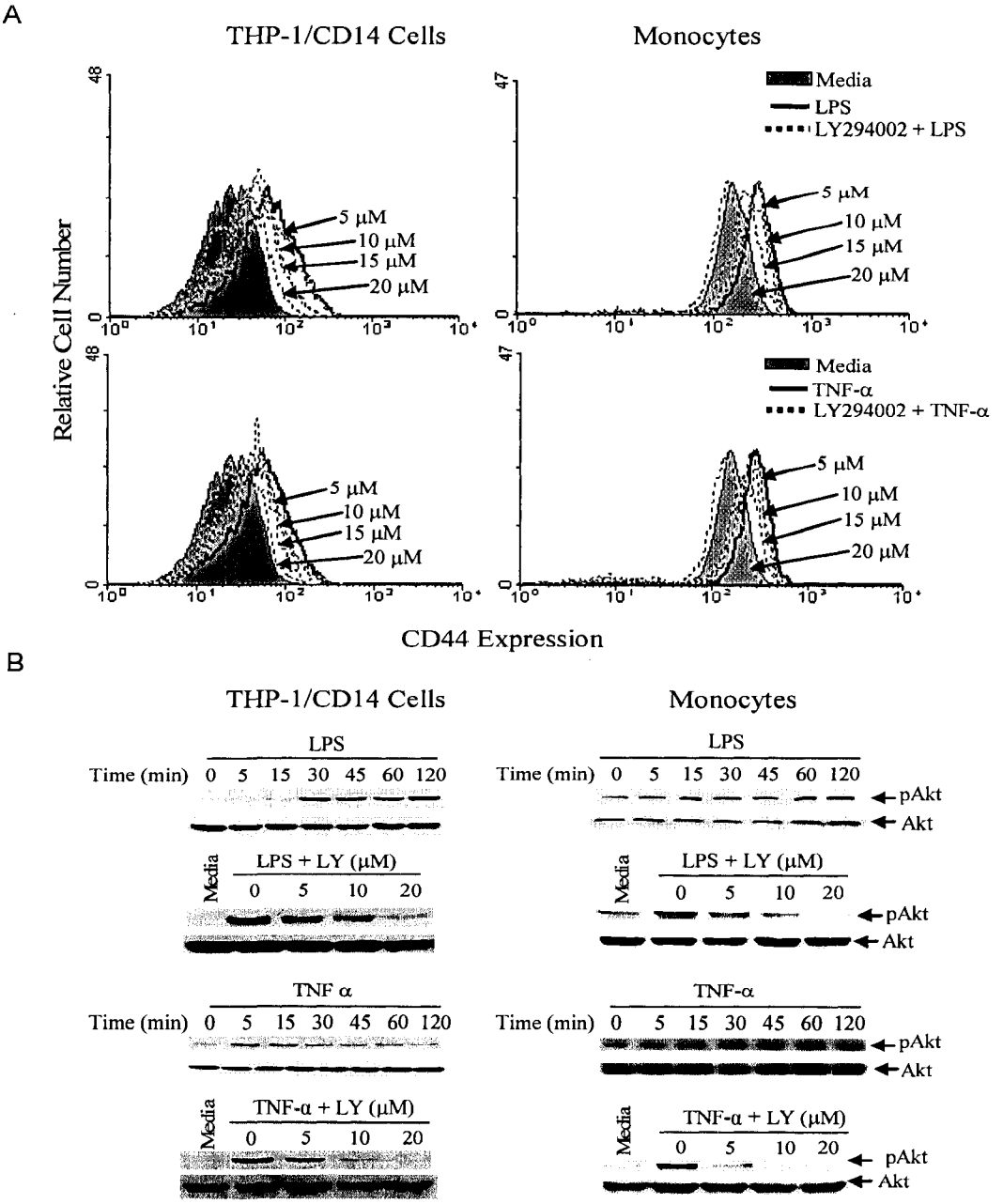

A
THP-1/CD14 Cells
Monocytes
Media
LPS
LY294002 + LPS
5 μM
10 μM
15 μM
20 μM
Relative Cell Number
Media
TNF-α
LY294002 + TNF-α
CD44 Expression
B
THP-1/CD14 Cells
Monocytes
LPS
Time (min) 0 5 15 30 45 60 120
pAkt
Akt
Media
LPS + LY (μM)
0 5 10 20
TNF α
TNF-α
TNF-α + LY (μM)

Fig. 3.12

Fig. 3.13: The p110α subunit of PI3K does not regulate LPS- and TNF-α-induced CD44 expression.

A) The HR-p110α3 THP-1 cells and HR-p110α1 THP-1 cells (2 X 10^6) were stimulated with either LPS (1 μg/ml) or TNF-α (10 ng/ml) for Western blot analysis by using anti-p110α and anti-p85α Abs as per the procedure in Fig. 3.12B. The results shown are representative of three independent experiments.

B) The HR-p110α3 THP-1 cells and HR-p110α1 THP-1 cells (0.5 X 10^6) were stimulated with either LPS (1 μg/ml) or TNF-α (10 ng/ml) for 24 hr followed by analysis of CD44 expression by flow cytometry. The results shown are representative of four experiments.

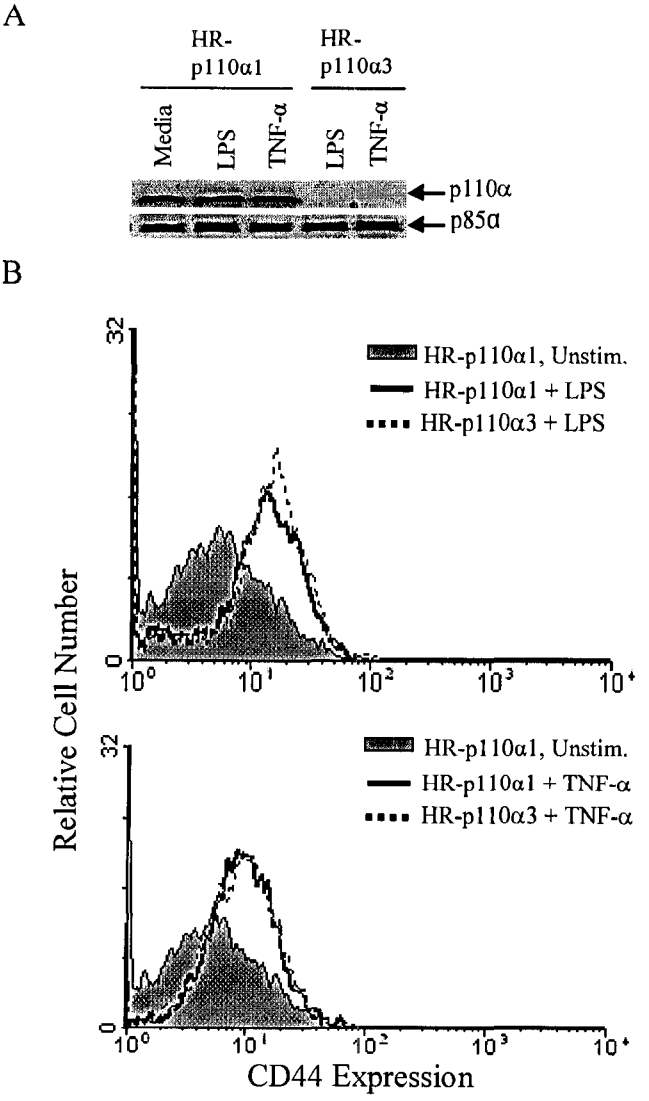

A
HR-p110α1
HR-p110α3
Media
LPS
TNF-α
LPS
TNF-α
p110α
p85α
B
HR-p110α1, Unstim.
HR-p110α1 + LPS
HR-p110α3 + LPS
HR-p110α1, Unstim.
HR-p110α1 + TNF-α
HR-p110α3 + TNF-α
Relative Cell Number
CD44 Expression
32
0
10⁰
10¹
10²
10³
10⁴

Fig. 3.13

LPS and TNF-α stimulation (Fig. 3.13B) suggesting that p110α isoform may not be involved in the regulation of LPS or TNF-α-induced CD44 expression. To understand the role of p85α subunit, THP-1/CD14 cells were transfected with siRNA specific for p85α subunit of PI3K or control vector followed by LPS and TNF-α stimulation. Transfection with p85-siRNA resulted in significant inhibition of LPS- and TNF-α-induced phosphorylation of p85α compared to cells transfected with control vectors without any effect on p110 expression (Fig. 3.14A). Furthermore, cells transfected with p85 siRNA inhibited both LPS- and TNF-α-induced CD44 expression in these cells suggesting the involvement of p85α subunit in CD44 regulation (Fig. 3.14B).

JNK-activated PI3K, but not the Ca^{2+}/CaMK-II-activated PI3K regulates LPS-induced CD44 expression in human monocytic cells.

I confirmed the earlier observations (51;166) by demonstrating the involvement of JNK MAPK in LPS-induced CD44 expression in human monocytic cells by employing SP600125, a specific JNK inhibitor. SP600125 inhibited LPS-induced CD44 expression (Fig. 3.15A) as well as JNK phosphorylation (Fig. 3.15B) in a dose dependent manner in both THP-1/CD14 cells and primary monocytes. It is likely that LPS-induced CD44 expression is regulated by JNK and PI3K as two distinct and independent pathways. Alternatively, JNK and PI3K may functionally cross-talk as a single pathway to regulate CD44 expression. For this, THP-1/CD14 cells and primary monocytes were treated for 2 hr with SP600125 followed by LPS stimulation and analysis for Akt phosphorylation. SP600125 inhibited LPS-induced phosphorylation of Akt in dose dependent manner (Fig. 3.15B). However, prior treatment of cells with LY294002 did not inhibit LPS-induced

Fig. 3.14: The p85α subunit of PI3K regulates LPS- and TNF-α-induced CD44 expression.

A) THP-1/CD14 cells (2 X 10^6) transfected with siRNAs specific for the p85α-PI3K or control vector were stimulated with either LPS (1 μg/ml) or TNF-α (10 ng/ml) for 30 min followed by analysis of phosphorylated p85α by Western blot analysis using anti-phospho-p85α Ab. Same blots were stripped and reprobed sequentially with anti-p85α or anti-p110α Ab. The results shown are representative of three independent experiments.

B) THP-1/CD14 cells (0.5 X 10^6) transfected with siRNAs specific for the p85α-PI3K or control vector were stimulated with either LPS (1 μg/ml) or TNF-α (10 ng/ml) for 24 hr followed by analysis for CD44 expression by flow cytometry. The results shown are representative of four experiments.

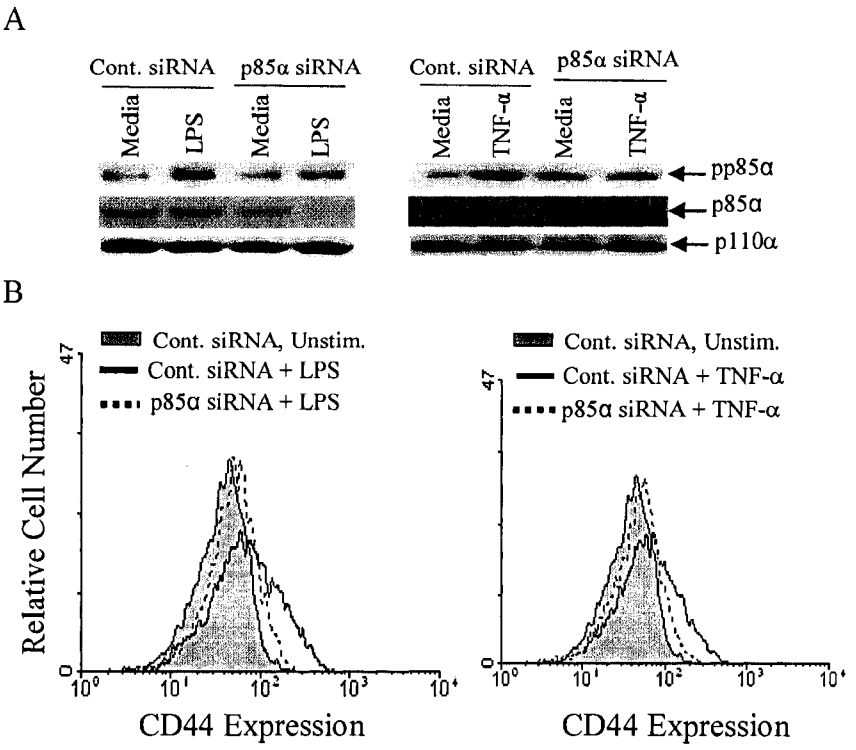

A
Cont. siRNA
p85α siRNA
Media
LPS
Media
LPS
Cont. siRNA
p85α siRNA
Media
TNF-α
Media
TNF-α
pp85α
p85α
p110α
B
Cont. siRNA, Unstim.
Cont. siRNA + LPS
p85α siRNA + LPS
Relative Cell Number
47
0
10⁰
10¹
10²
10³
10⁴
CD44 Expression
Cont. siRNA, Unstim.
Cont. siRNA + TNF-α
p85α siRNA + TNF-α
47
0
10⁰
10¹
10²
10³
10⁴
CD44 Expression

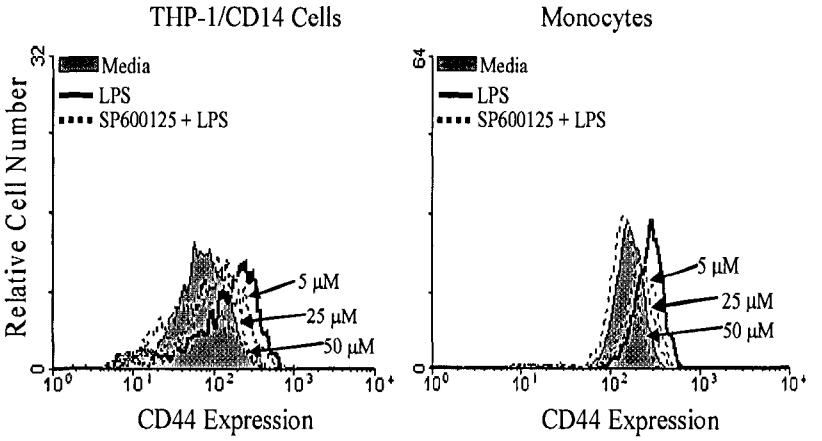
Fig. 3.14

Fig. 3.15: JNK-activated PI3K regulates LPS-induced CD44 expression.

A) THP-1/CD14 cells and monocytes (0.5 X 10^6/ml) were treated with various concentrations of SP600125 for 2 hr prior to LPS (1 μg/ml) stimulation for 24 hr followed by CD44 expression analysis by flow cytometry. The results shown are representative of four experiments.

B) THP-1/CD14 cells and monocytes (2 X 10^6) were treated with SP600125 at varying concentrations for 2 hr prior to LPS (1 μg/ml) stimulation for 30 min followed by analysis of JNK phosphorylation by Western blotting by using anti-phospho-JNK Ab. Same blots were stripped and reprobed sequentially with anti-phospho-Akt, anti-JNK and anti-Akt Abs. The results shown are representative of three independent experiments.

A


THP-1/CD14 Cells
Monocytes
Media
LPS
SP600125 + LPS
Relative Cell Number
32
64
0
5 μM
25 μM
50 μM
10^0 10^1 10^2 10^3 10^4
CD44 Expression

B

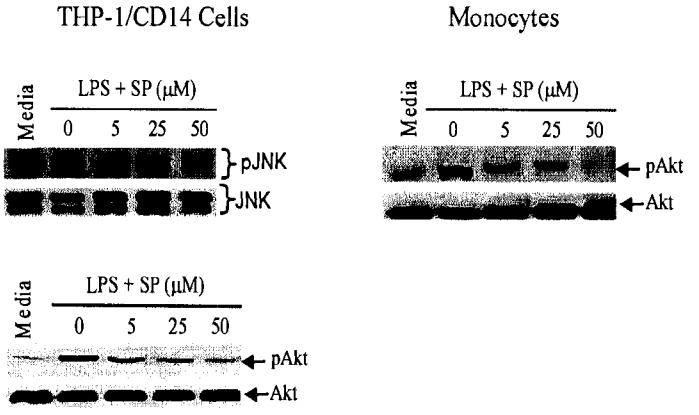

THP-1/CD14 Cells
Monocytes
Media
LPS + SP (μM)
0 5 25 50
pJNK
JNK
pAkt
Akt

Fig. 3.15

JNK activation in both monocytes and THP-1/CD14 cells (Fig. 3.15B) suggesting that JNK-activated PI3K regulates LPS-induced CD44 expression.

I confirmed my previous results (51) and demonstrated that the Ca^{2+} signaling pathway is not involved in LPS-induced CD44 expression by employing a Ca^{2+} chelator, EGTA. EGTA did not inhibit LPS-induced CD44 expression in either THP-1/CD14 cells or monocytes (Fig. 3.16A). Since JNK-activated PI3K regulated LPS-induced CD44 expression independent of Ca^{2+} signaling, it was hypothesized that PI3K and Ca^{2+} signals are activated as independent pathways without any cross-talk between the two pathways. This was investigated by pretreating cells for 2 hr with various concentrations of inhibitors specific for Ca^{2+} signaling followed by LPS stimulation and analysis of phosphorylation of Akt and JNK. Interestingly, the Ca^{2+} signaling inhibitors, EGTA, SKF, APB, W-7, KN-93 and FK-506 suppressed LPS-induced phosphorylation of Akt in a dose-dependent manner with no effect on JNK phosphorylation (Fig. 3.16B) suggesting that PI3K is also activated through Ca^{2+} signaling, however, Ca^{2+}-activated PI3K failed to regulate LPS-induced CD44 expression.

Ca^{2+}/CaMK-II-activated PI3K, but not the JNK-activated PI3K regulates TNF-α-induced CD44 expression.

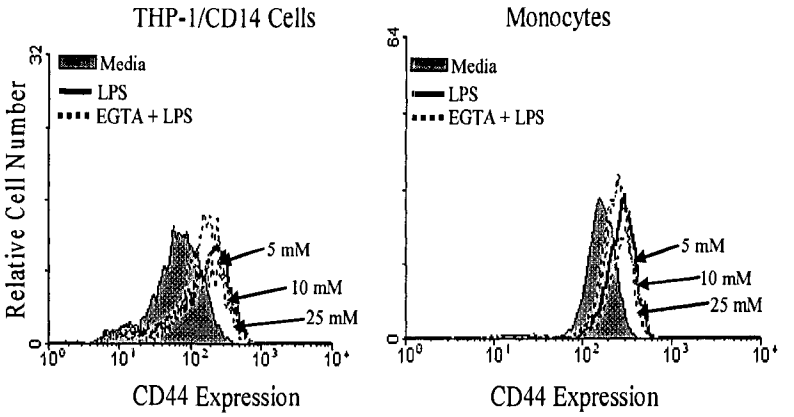
I have previously demonstrated that TNF-α regulates CD44 expression selectively through the activation of Ca^{2+} signaling pathway and in particular the CaM/CaMK-II pathway and independent of the JNK pathway (51). Since PI3K as well as calcium signaling were involved in TNF-α-induced CD44 expression, it was of interest to determine if these two pathways independently and distinctly regulate TNF-α-induced CD44 expression. For this, cells were pretreated for 2 hr with various concentrations of

Fig. 3.16: CaM/CaMK-II-activated PI3K does not regulate LPS-induced CD44 expression.

A) THP-1/CD14 cells and monocytes (0.5 X 10^6/ml) were treated with various concentrations of EGTA for 2 hr prior to LPS (1 μg/ml) stimulation for 24 hr followed by analysis of CD44 expression by flow cytometry. The results shown are representative of four experiments.

B) THP-1/CD14 cells and monocytes (2 X 10^6) were pretreated with indicated concentrations of EGTA, SKF, APB, W-7, KN-93 and FK-506 for 2 hr prior to LPS (1 μg/ml) stimulation for 30 min followed by analysis of JNK phosphorylation by Western blotting by using anti-phospho-JNK Ab. Same blots were stripped and reprobed sequentially with anti-phospho-Akt and anti-Akt Abs. The results shown are representative of three independent experiments.

A


THP-1/CD14 Cells
Monocytes
Media
LPS
EGTA + LPS
Relative Cell Number
5 mM
10 mM
25 mM
CD44 Expression
CD44 Expression

B

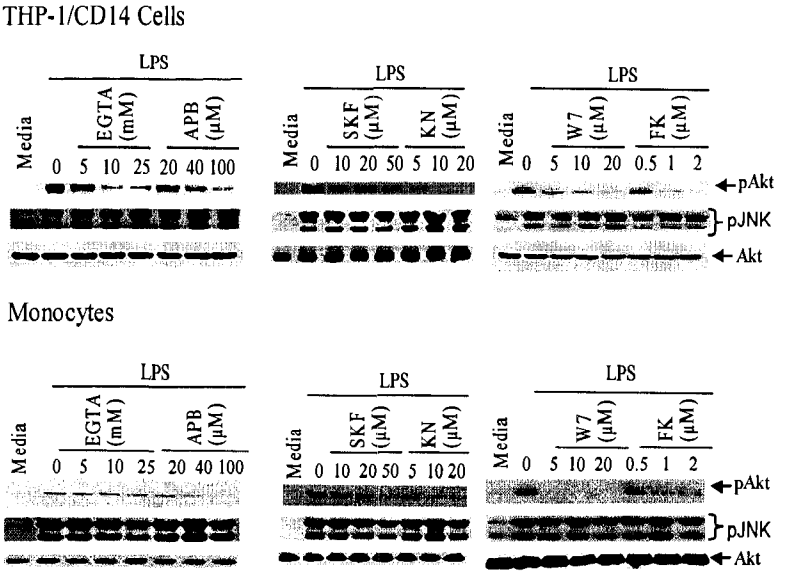

THP-1/CD14 Cells
LPS
Media
EGTA (mM)
APB (μM)
SKF (μM)
KN (μM)
W7 (μM)
FK (μM)
pAkt
pJNK
Akt
Monocytes

Fig. 3.16

inhibitors specific for JNK MAPK and Ca^{2+} signaling pathways followed by TNF-α stimulation, determination of phosphorylation of Akt and JNK, and analysis of CD44 expression. TNF-α-induced CD44 expression was inhibited by EGTA (Fig 3.17A) and other inhibitors specific for the Ca^{2+} signaling including SKF, APB, W-7, KN-93 and FK-506 (data not shown). Interestingly, the Ca^{2+} signaling inhibitors, EGTA, SKF, APB, W-7, KN-93 and FK-506 inhibited phosphorylation of Akt without affecting JNK phosphorylation (Fig. 3.17B). In contrast to the inhibitors specific for Ca^{2+} signaling, TNF-α-induced CD44 expression was not affected by the JNK inhibitor, SP600125 (Fig 3.18A). SP600125 in addition to inhibiting JNK phosphorylation, inhibited Akt phosphorylation in a dose dependent manner in both THP-1/CD14 cells and human monocytes (Fig. 3.18B). These results suggest that TNF-α-induced CD44 expression is regulated selectively by CaM/CaMK-II-activated PI3K, but not by JNK-activated PI3K in human monocytic cells.

JNK-activated PI3K regulates LPS-induced CD44 transcription through Egr-1 activation.

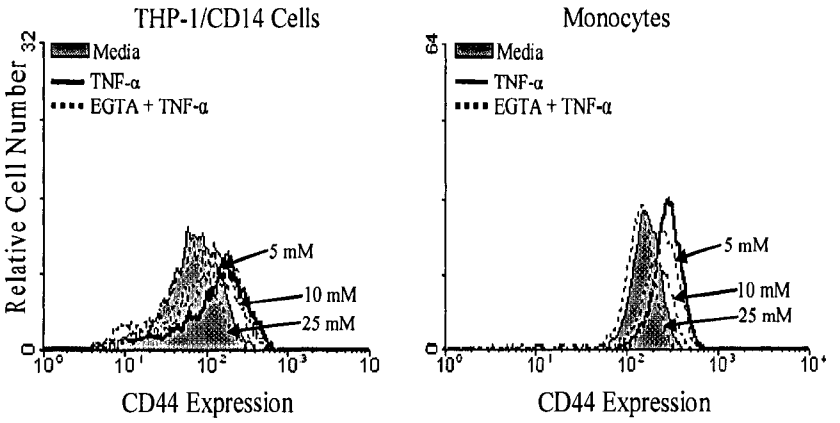
I have previously shown that LPS-induced CD44 transcription is regulated by the transcription factor Egr-1 in human monocytic cells (51). The above results also show that LPS-induced CD44 expression is regulated by the JNK-activated but not the CaM/CaMK-II-activated PI3K. Therefore, it is likely that JNK-activated PI3K may selectively activate Egr-1 to regulate CD44 transcription. Hence, to determine the role of PI3K and Egr-1 in the regulation of LPS-induced CD44 transcription, I generated CD44 promoter mutant constructs in pCD44Pr(-334) containing both wild-type Egr-1 and AP-1

Fig. 3.17: CaM/CaMK-II-activated PI3K is involved in TNF-α-induced CD44 expression in human monocytic cells.

A) THP-1/CD14 cells and monocytes (0.5 X 10^6/ml) were treated with various concentrations of EGTA for 2 hr prior to TNF-α (10 ng/ml) stimulation for 24 hr followed by analysis for CD44 expression by flow cytometry. The results shown are representative of four experiments.

B) THP-1/CD14 cells and monocytes (2 X 10^6) were treated with indicated concentrations of EGTA, SKF, APB, W-7, KN-93 and FK-506 for 2 hr prior to TNF-α (10 ng/ml) stimulation for 15 min followed by analysis of JNK phosphorylation by Western blotting by using anti-phospho-JNK Ab. Same blots were stripped and reprobed sequentially with anti-phospho-Akt and anti-Akt Abs. The results shown are representative of three independent experiments.

A


THP-1/CD14 Cells
Monocytes
Media
TNF-α
EGTA + TNF-α
Relative Cell Number
5 mM
10 mM
25 mM
CD44 Expression

B

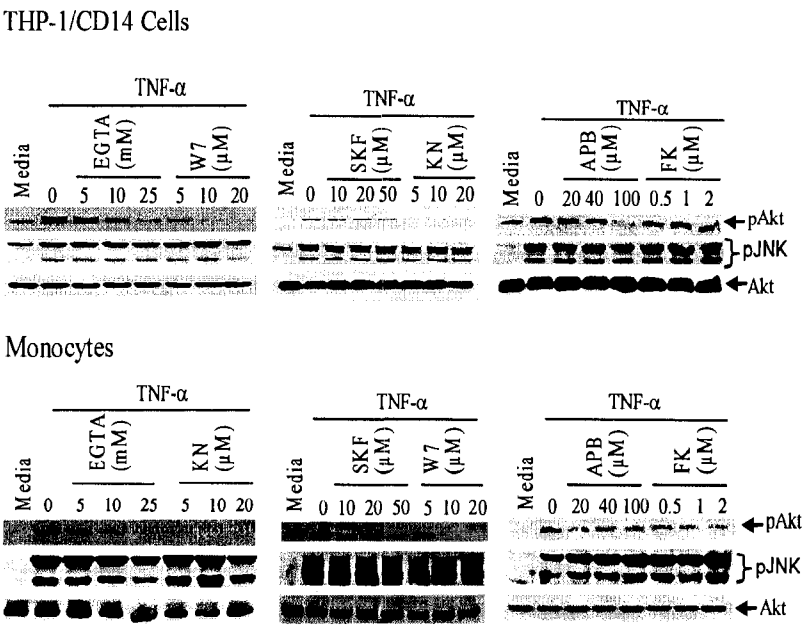

THP-1/CD14 Cells
TNF-α
Media
EGTA (mM)
W7 (μM)
SKF (μM)
KN (μM)
APB (μM)
FK (μM)
pAkt
pJNK
Akt
Monocytes

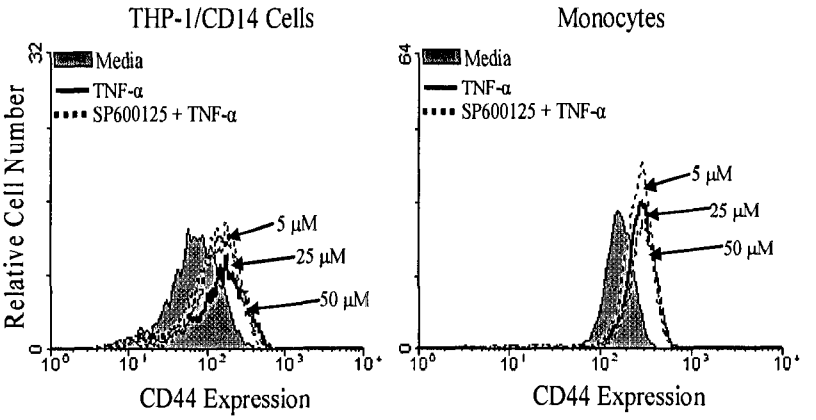
Fig. 3.17

Fig. 3.18: JNK-activated PI3K does not regulate in TNF-α-induced CD44 expression.

A) THP-1/CD14 cells and monocytes (0.5 X 10^6/ml) were treated with various concentrations of SP600125 for 2 hr prior to TNF-α (10 ng/ml) stimulation for 24 hr followed by analysis for CD44 expression by flow cytometry. The results shown are representative of four experiments.

B) THP-1/CD14 cells and monocytes (2 X 10^6) were treated with SP600125 at varying concentrations for 2 hr prior to TNF-α (10 ng/ml) stimulation for 15 min followed by analysis of JNK phosphorylation by Western blotting by using anti-phospho-JNK Ab. Same blots were restripped and reprobed sequentially with anti-phospho-Akt or anti-JNK or anti-Akt Abs. The results shown are representative of three independent experiments.

A


THP-1/CD14 Cells
Monocytes
Media
TNF-α
SP600125 + TNF-α
5 μM
25 μM
50 μM
Relative Cell Number
CD44 Expression
CD44 Expression

B

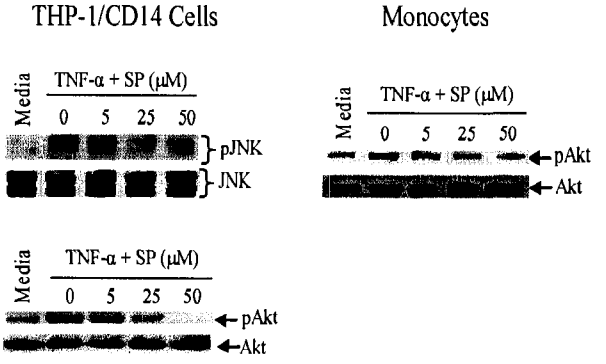

THP-1/CD14 Cells
Monocytes
Media
TNF-α + SP (μM)
0 5 25 50
pJNK
JNK
pAkt
Akt
Media
TNF-α + SP (μM)
0 5 25 50
pAkt
Akt

Fig. 3.18

binding sequences. These constructs contained mutations in either Egr-1 alone [pCD44Pr(-334-Egr-1m)], AP-1 alone [pCD44Pr(-334-AP-1m)] or in both Egr-1 and AP-1 [pCD44Pr(-334-Egr-1m,AP-1m)]. Fig. 3.19A shows the Egr-1 and AP-1 binding nucleotide sequences and their mutations in nucleotide bases, as indicated in bold letters.

To determine whether PI3K regulates Egr-1-mediated LPS-induced CD44 transcription, cells were transfected with the above mentioned CD44 constructs followed by stimulation with LPS for 8 hr and analysis of luciferase activity. As expected, transfection of cells with pCD44Pr(-334) containing both wild-type Egr-1 and AP-1 binding sequences, and transfection with [pCD44Pr(-334-AP-1 m)] resulted in 3-4-fold increase in luciferase activity compared to the unstimulated cells and 30-40-fold compared to the cells transfected with control plasmid. However transfection of cells with promoter constructs pCD44Pr(-334-Egr-1m) containing mutated Egr-1 or with pCD44Pr(-334-Egr-1m,AP-1m) containing both Egr-1 and AP-1 mutated sequences did not enhance LPS-induced luciferase activity compared to the unstimulated cells (Fig. 3.19B).

To determine the role of PI3K in Egr-1 activation, cells were transfected with pCD44Pr(-334-AP-1m) containing wild type-Egr-1 and m-AP-1 sequences. The transfected cells were treated for 2 hr with the inhibitor specific for PI3K, LY294002 prior to stimulation with LPS for 8 hr. Prior treatment of transfected cells with LY294002 decreased the LPS-induced luciferase activity in a dose-dependent manner (Fig. 3.19B). The luciferase activity observed with the highest concentration of inhibitor was equivalent to the basal activity observed with unstimulated cells and the reduction was significant ($P<0.001$) compared with LPS stimulation alone. The above results suggest

Fig. 3.19: LPS-induced CD44 transcription is regulated by PI3K-activated Egr-1, but not by PI3K-activated AP-1.

A) CD44 promoter from 5' -334 to -224 bp shows the Egr-1 and AP-1 binding nucleotide sequences and their mutations in nucleotide bases are indicated in bold letters.

B) THP-1/CD14 cells (2.0 X 10^6) were transiently cotransfected either with various CD44 promoter deletion/mutant constructs or control pGL3B vector and β-galactosidase plasmid. After 15 hr, the transfected cells were stimulated with LPS (1 μg/ml) for 8 hr followed by measurement of luciferase and β-galactosidase activities. Following normalization, the luciferase activity was calculated as relative luciferase units. The results shown are mean ± S.D. of three independent experiments performed in triplicates.

C & D) THP-1/CD14 cells (5 X 10^6) were stimulated with LPS (1 μg/ml) for 60 min. The nuclear extract samples (5 μg) were probed with ^{32}P-labeled oligonucleotides corresponding to either Egr-1-binding (C) or AP-1-binding (D) sequence of the CD44 promoter. To determine the specificity of Egr-1 or AP-1 binding, the nuclear extract samples were probed in the presence of different concentrations of unlabeled specific (Sp oligos) or nonspecific oligonucleotides (NS oligos). The samples were also treated with anti-Egr-1 antibodies or anti-c-Fos or anti-c-Jun antibodies to identify Egr-1 and AP-1, respectively. The Egr-1 specific bands were abrogated by anti-Egr-1 Ab. The supershifted band for AP-1 is indicated by an arrow. To determine the role of PI3K inhibitor, LY294002 on LPS-induced activation of Egr-1 or AP-1, cells were treated with various concentrations of LY294002 for 2 hr prior to stimulation with LPS. The nuclear extracts were subjected to gel shift assays for analysis of Egr-1 and AP-1 bands as described above. The experiment shown is a representative of three independent experiments.

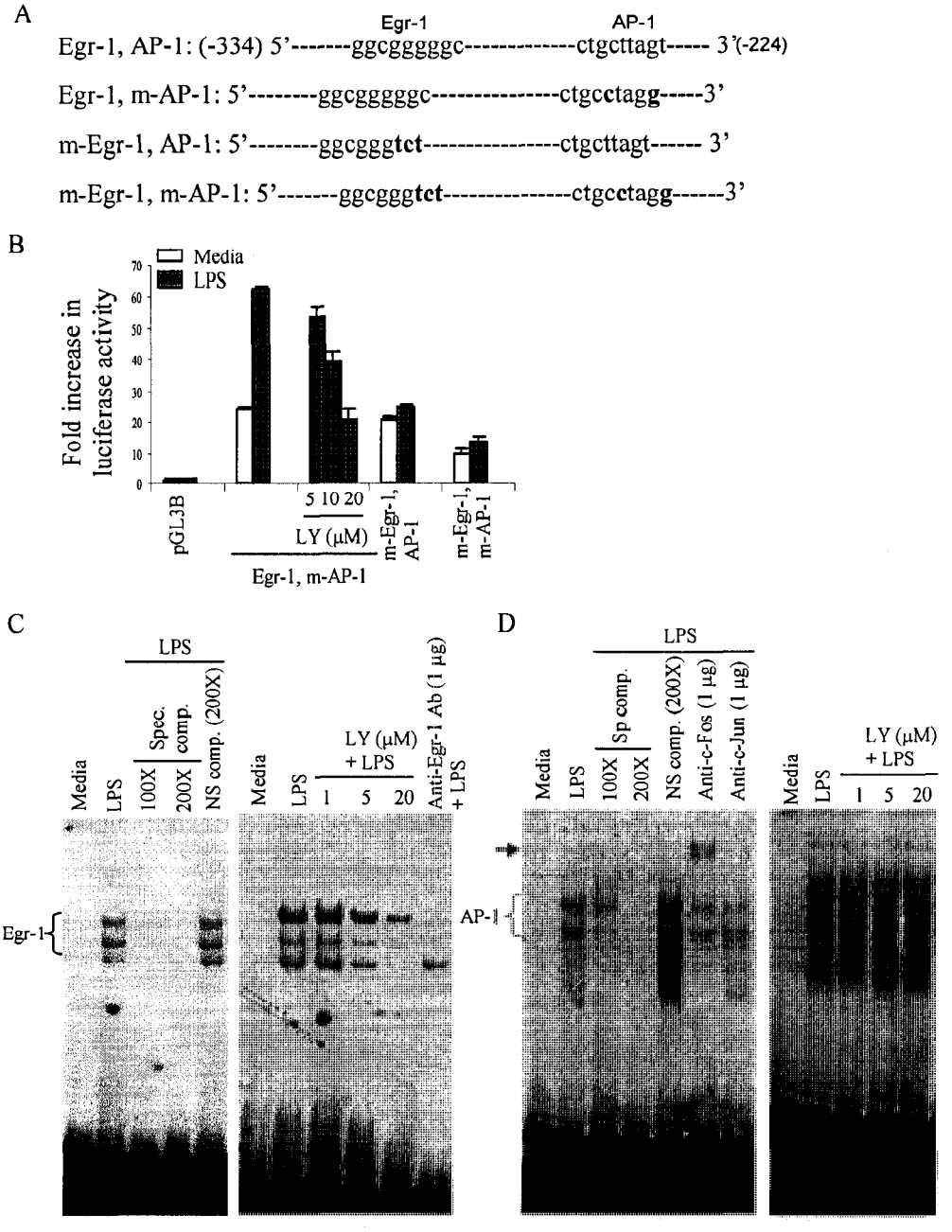

A
Egr-1
AP-1
Egr-1, AP-1: (-334) 5'-------ggcgggggc----------ctgcttagt----- 3'(-224)
Egr-1, m-AP-1: 5'--------ggcgggggc----------ctgcctagg-----3'
m-Egr-1, AP-1: 5'--------ggcgggtct----------ctgcttagt------ 3'
m-Egr-1, m-AP-1: 5'-------ggcgggtct----------ctgcctagg------3'
B
Media
LPS
Fold increase in luciferase activity
pGL3B
5 10 20
LY (μM)
Egr-1, m-AP-1
m-Egr-1, AP-1
m-Egr-1, m-AP-1
C
LPS
Media
LPS
Spec. comp.
100X
200X
NS comp. (200X)
LY (μM) + LPS
1 5 20
Anti-Egr-1 Ab (1 μg) + LPS
Egr-1
D
LPS
Sp comp.
100X
200X
NS comp. (200X)
Anti-c-Fos (1 μg)
Anti-c-Jun (1 μg)
AP-1

Fig. 3.19

that LPS-induced CD44 transcription may be regulated by Egr-1 through the activation of PI3K.

JNK-activated PI3K mediates binding of Egr-1 to its binding site on CD44 promoter.

To confirm that LPS-induced CD44 expression is regulated by Egr-1 through PI3K activation, the effect of PI3K inhibitor on the binding of LPS-induced Egr-1 to its binding site on CD44 promoter was investigated by EMSA by using the ^{32}P-labeled oligonucleotide probe corresponding to the Egr-1 sequence of the CD44 promoter. LPS induced maximum binding of Egr-1 at 60 min post stimulation (data not shown). I observed three Egr-1 containing bands as their intensity was abrogated following specific competition with unlabeled oligonucleotides but not by nonspecific oligonucleotides (Fig 3.19C). Furthermore, treatment of nuclear extracts with anti-Egr-1 antibodies abrogated the top two Egr-1 specific bands (Fig. 3.19C). To determine the role of PI3K, cells were treated for 2 hr with Ly294002 prior to stimulation with LPS for 60 min. LY294002 significantly inhibited the intensity of Egr-1 bands in a dose-dependent manner (Fig. 3.19C). To determine whether LPS-activated PI3K mediates binding of AP-1 to its site on CD44 promoter, similar EMSA experiments were performed following LPS stimulation using the ^{32}P-labeled oligonucleotide probe corresponding to the AP-1 sequence of CD44 promoter. The maximum binding of AP-1 to the ^{32}P-labeled AP-1 oligonucleotide probe occurred at 2 hr following LPS stimulation (data not shown). LPS stimulation revealed two major bands corresponding to the AP-1-DNA complex which were blocked by competition with specific unlabeled AP-1 oligonucleotides but not by nonspecific oligonucleotides (Fig. 3.19D). Furthermore, treatment of nuclear extracts

with anti-c-Fos and anti-c-Jun antibodies resulted in the abrogation of the AP-1-specific bands (Fig. 3.19D). Pretreatment of cells with LY294002 did not inhibit the formation of the AP-1 protein-DNA complex (Fig. 3.19D). These results suggest that LPS-induced CD44 transcription is selectively regulated by Egr-1 through PI3K activation.

Ca^{2+}/CaMK-II-activated PI3K regulates TNF-α-induced CD44 transcription through the activation of transcription factor AP-1.

The TNF-α-induced CD44 expression is regulated by AP-1 (51). The above results also show that CaM/CaMK-II-activated PI3K, but not JNK-activated PI3K regulates CD44 transcription in human monocytic cells. Therefore, we determined if CaM/CaMK-II-activated PI3K selectively activates AP-1 to regulate TNF-α-induced CD44 transcription. Therefore, to understand the role of PI3K and AP-1 in the regulation of TNF-α-induced CD44 transcription, cells were transfected with CD44 promoter constructs containing mutations in either Egr-1 alone [pCD44Pr(-334-Egr-1m)], AP-1 alone [pCD44Pr(-334-AP-1m)] or in both Egr-1 and AP-1 [pCD44Pr(-334-Egr-1m,AP-1m)] followed by stimulation with TNF-α for 8 hr and analysis of luciferase activity. As expected, transfection of cells with pCD44Pr(-334) containing both wild-type Egr-1 and AP-1 binding sequences, and transfection with pCD44Pr(-334-Egr-1m) resulted in 3-4-fold increase in luciferase activity compared to the unstimulated cells and 30-40-fold compared to the cells transfected with control plasmid. However transfection of cells with promoter constructs [pCD44Pr(-334-AP-1m)] containing mutated AP-1 or with pCD44Pr(-334-Egr-1m,AP-1m) containing both Egr-1 and AP-1 mutated sequences did not enhance TNF-α-induced luciferase activity compared to the unstimulated cells (Fig. 3.20A).

Fig. 3.20: TNF-α-induced CD44 transcription is regulated by PI3K-activated AP-1, but not by PI3K-activated Egr-1.

A) The THP-1/CD14 cells (2.0 X 10^6) were transiently cotransfected with either various CD44 promoter deletion/mutant constructs or control pGL3B vector and β-galactosidase plasmid. After 15 hr, the transfected cells were stimulated with TNF-α (10 ng/ml) for 8 hr followed by measurement of luciferase and β-galactosidase activities. The luciferase activity was calculated as relative luciferase units as above. The results shown are mean ± S.D. of three independent experiments performed in triplicates.

B & C) THP-1/CD14 cells (5 X 10^6) were stimulated with TNF-α (10 ng/ml) for 2 hr. The nuclear extract samples were probed with ^{32}P-labeled oligonucleotides corresponding to either AP-1-binding (B) or the Egr-1-binding (C) sequence of the CD44 promoter. The specificities of AP-1 or Egr-1 bands were determined by using unlabeled specific (Sp) or nonspecific oligonucleotides (NS) and by supershift assays as described in the legends of Fig. 3.19. The Egr-1 specific bands were abrogated by anti-Egr-1 Ab. The supershifted band for AP-1 is indicated by an arrow. To determine the role of PI3K on TNF-α-induced activation of AP-1 or Egr-1, cells were treated with various concentrations of LY294002 for 2 hr prior to stimulation with TNF-α. The nuclear extract samples were analyzed for AP-1 or Egr-1 bands by gel shift assays as described above. The experiment shown is representative of three independent experiments.

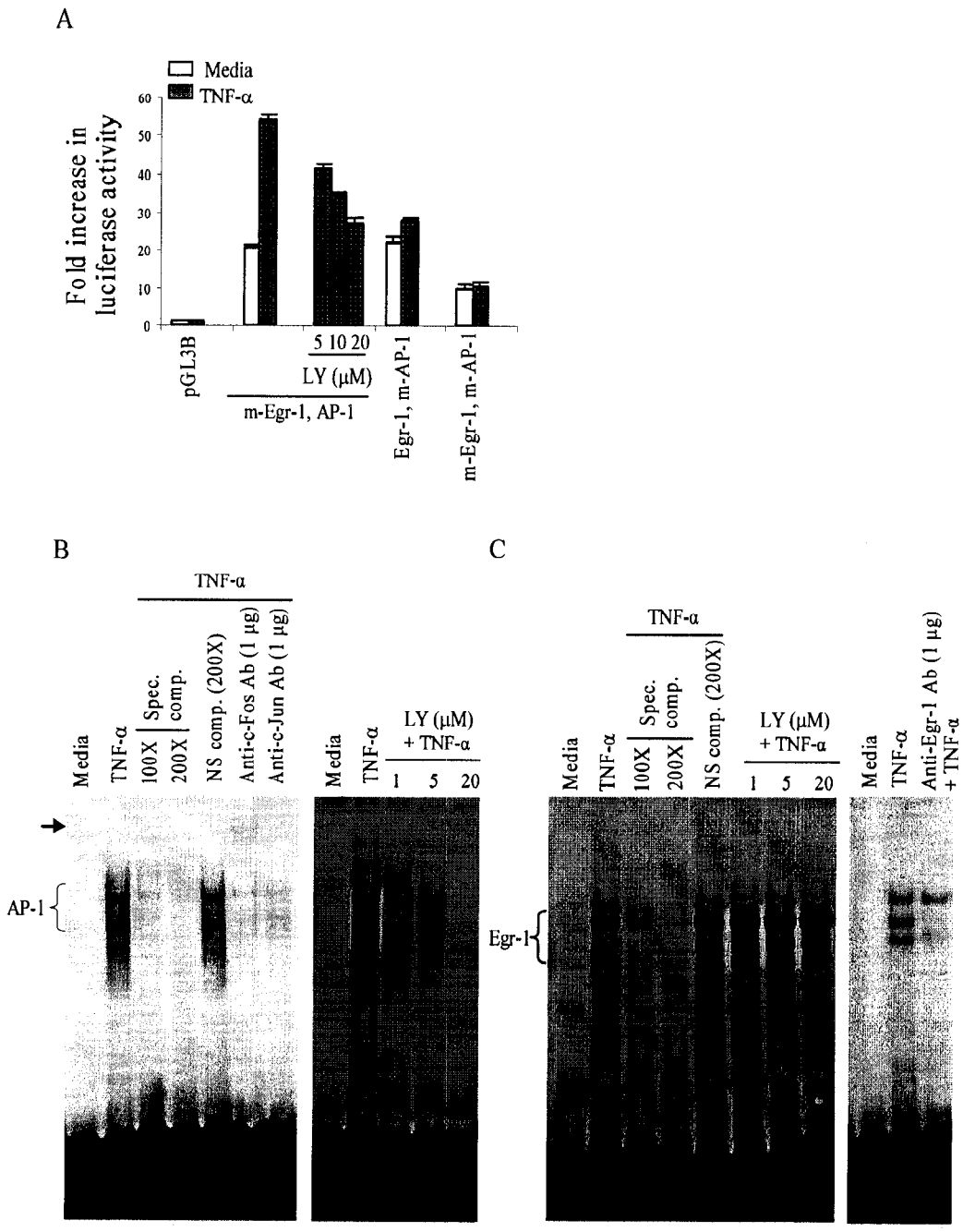

A
Media
TNF-α
Fold increase in luciferase activity
60
50
40
30
20
10
0
pGL3B
5 10 20
LY (μM)
m-Egr-1, AP-1
Egr-1, m-AP-1
m-Egr-1, m-AP-1
B
TNF-α
Media
TNF-α
Spec. comp.
100X
200X
NS comp. (200X)
Anti-c-Fos Ab (1 μg)
Anti-c-Jun Ab (1 μg)
AP-1
Media
TNF-α
LY (μM) + TNF-α
1 5 20
C
TNF-α
Media
TNF-α
Spec. comp.
100X
200X
NS comp. (200X)
LY (μM) + TNF-α
1 5 20
Egr-1
Media
TNF-α
Anti-Egr-1 Ab (1 μg) + TNF-α

Fig. 3.20

To determine the role of PI3K in AP-1 activation, cells were transfected with pCD44Pr(-334-AP-1m) containing wild type-AP-1 and m-Egr-1 sequences. The transfected cells were treated for 2 hr with LY294002 prior to stimulation with TNF-α for 8 hr. As before, following TNF-α stimulation, a significant 3-fold increase in luciferase activity was observed compared with the unstimulated cells. Prior treatment of transfected cells with LY294002 decreased the TNF-α-induced luciferase activity in a dose-dependent manner (Fig. 3.20A). The luciferase activity observed with the highest concentration of inhibitor was equivalent to the basal activity observed with unstimulated cells and the reduction was significant ($P<0.001$) compared with TNF-α stimulation alone. These results suggest that TNF-α-induced CD44 transcription may be regulated by AP-1 through the activation of PI3K.

Ca^{2+}/CaMK-II-activated PI3K mediates binding of AP-1 to its binding site on CD44 promoter in TNF-α-stimulated cells.

To confirm that TNF-α-induced CD44 expression is regulated by AP-1 through PI3K activation, the effect of LY294002 on the binding of TNF-α-induced AP-1 to its binding site on CD44 promoter was investigated by EMSA using ^{32}P-labelled oligonucleotide sequence corresponding to the AP-1 binding sequence of CD44 promoter. Maximum binding of AP-1 to the probe occurred by 120 min following TNF-α stimulation. The identity of a band corresponding to the AP-1 protein-DNA complex was established by competition with cold AP-1 specific and non-specific oligonucleotides (Fig. 3.20B). Furthermore, treatment of nuclear extracts with anti-c-Fos and anti-c-Jun antibodies resulted in the abrogation of the AP-1-specific band (Fig. 3.20B). To determine the effect of the PI3K inhibitor on the binding of AP-1 to its binding site in the

CD44 promoter, cells were treated for 2 hr with LY294002 prior to stimulation with TNF-α. LY294002 inhibited the formation of AP-1-DNA complex (Fig. 3.20B).

To determine whether TNF-α-activated PI3K mediates binding of Egr-1 to its site on CD44 promoter, similar EMSA experiments were performed following TNF-α stimulation using the ^{32}P-labeled oligonucleotide probe corresponding to the Egr-1 sequence of CD44 promoter. As before, the identity of Egr-1 binding was established by competition with specific and non-specific oligonucleotides and by using anti-Egr-1 antibodies. Pretreatment of cells with LY294002 did not inhibit the formation of the Egr-1 protein-DNA complex (Fig. 3.19C).

Taken together, these results suggest that although TNF-α induces the activation of both Egr-1 and AP-1, TNF-α-induced CD44 transcription is selectively regulated by AP-1 through PI3K activation.

Chapter IV
Regulation of IL-4-induced CD44 expression in human B cells: role of STAT-6 and ERK MAPK

Introduction

Interactions of CD44 with its ligand, hyaluronan (HA) play a crucial role in cell migration, inflammation and immune responses. Burkitt's lymphomas (BL) are classified as high grade tumors of B-cell origin, and Epstein-Barr virus (EBV) is associated with the pathogenesis of endemic BL and other pathologies including B cell lymphomas of immunosuppressed individuals, nasopharyngeal carcinomas, Hodgkin's disease and infectious mononucleosis (140;276). BL cells when infected with EBV, a common occurrence in childhood lymphomas, express high levels of CD44 (141). BL and EBV-infected BL B cells produce a number of cytokines including IL-4 has been shown to enhance the survival of BL cells (277), and induce the expression of various costimulatory molecules including CD44, and enhances its binding to HA (141;277). CD44 induction and its ability to bind HA have been suggested to play a vital role in *in vivo* BL tumor growth and dissemination in a nude mouse model (118;278). ***Therefore, understanding the mechanism underlying IL-4-mediated regulation of CD44 expression may have implications for the development of novel strategies for preventing the growth and metastasis of BL cells.***

IL-4 signaling, in general, is believed to be mediated by two major intracellular transduction pathways. One is initiated through the recruitment of Jak/STAT-6 to the IL-4Rα chain which in turn leads to the induction of CD23 and MHC-II and class switch to IgE in B cells (252;279). The second pathway involves the recruitment of insulin receptor substrate-1/2 (IRS-1/2) to the IL-4Rα chain with resulting recruitment of PI3K subunits and activation of down stream signaling molecules such as protein kinase C (PKC) and Akt. This pathway plays a key role in cell survival, cell proliferation and resistance to

apoptosis (198;239;252;280). IL-4 is also known to activate the third major pathway, the mitogen-activated protein kinase (MAPK) family, albeit in limited cell types such as fibroblasts and keratinocytes (243-245). However with few exceptions, IL-4 is not believed to activate the MAPK pathway in general in hematopoitic cells (198;246-248).

During the last few years, the regulation of CD44 expression has been investigated (52;53;281). So far, Egr-1 and AP-1 have been shown to regulate CD44 expression in different cell types (50;281). We have previously shown that LPS-induced CD44 expression is regulated by Egr-1, activated by the c-Jun N-terminal kinase (JNK)-MAPK, whereas TNF-α-induced CD44 expression was regulated by Ca^{2+}/calmodulin/calmodulin-dependent protein kinase-II (CaMK-II)-activated AP-1 in human monocytic cells (51;166). However, the mechanism by which IL-4 regulates CD44 expression remains unknown. In this study, I investigated IL-4-induced regulation of CD44 expression in an Epstein-Barr virus (EBV)-transformed Burkitt's lymphoma cell line, BL30/B95-8 cells. I demonstrate for the first time the involvement of STAT-6 activation through the ERK MAPKs as a novel regulator of IL-4-induced CD44 expression. The results from these experiments are as follows:

Results

CD44 transcription is regulated by STAT-6 in BL30/B95-8 cells.

My results show that IL-4 induced CD44 expression in BL30/B95-8 cells in a dose-dependent manner (Fig. 4.1). Since PMA is a potent inducer of CD44, it was used as a positive control. We and others have previously shown the involvement of Egr-1 transcription factor in the regulation of CD44 expression in several cell types including B cells (50-53). Therefore, to determine the role of Egr-1 in IL-4-induced CD44 expression in human B cells, we examined Egr-1 induction in BL30/B95-8 cells. Interestingly, IL-4 in contrast to PMA, did not activate Egr-1 as determined by EMSA, and Northern blot analysis (Fig. 4.2).

Since, IL-4 is known to induce transcription of several genes through STAT-6 and because two STAT-6 binding sequences were found in the human CD44 promoter by MAT inspector analysis (site # 1 from -136 to -145 bp and site # 2 from -567 to -576 bp, Fig. 4.3), we hypothesized that either one or both STAT-6 binding sites might be involved in IL-4-induced CD44 regulation in human B cells. Therefore, a series of human CD44 promoter fragments (from 5' -1109 to 3' +53 bp) were generated by successive deletions starting from the 5' end. These promoter fragments were cloned, amplified and subcloned into the polylinker site of the luciferase reporter plasmid, pGL3B as described previously (Table-2.3) (51). BL30/B95-8 cells were transiently transfected with pCD44(-1109) followed by stimulation with various concentration of IL-4 and for varying periods of time and subsequently analyzed for luciferase activity. IL-4 induced luciferase activity in a dose dependent manner (Fig. 4.4A). The luciferase activity was detected by 2 hr and

Fig. 4.1: IL-4 induces CD44 expression in BL30/B95-8 cells.

BL30/B95-8 cells (0.5 X 10^6/ml) were treated with various concentrations of IL-4 and PMA for 24 hr followed by analysis for CD44 expression by flow cytometry. The results shown are representative of four experiments.

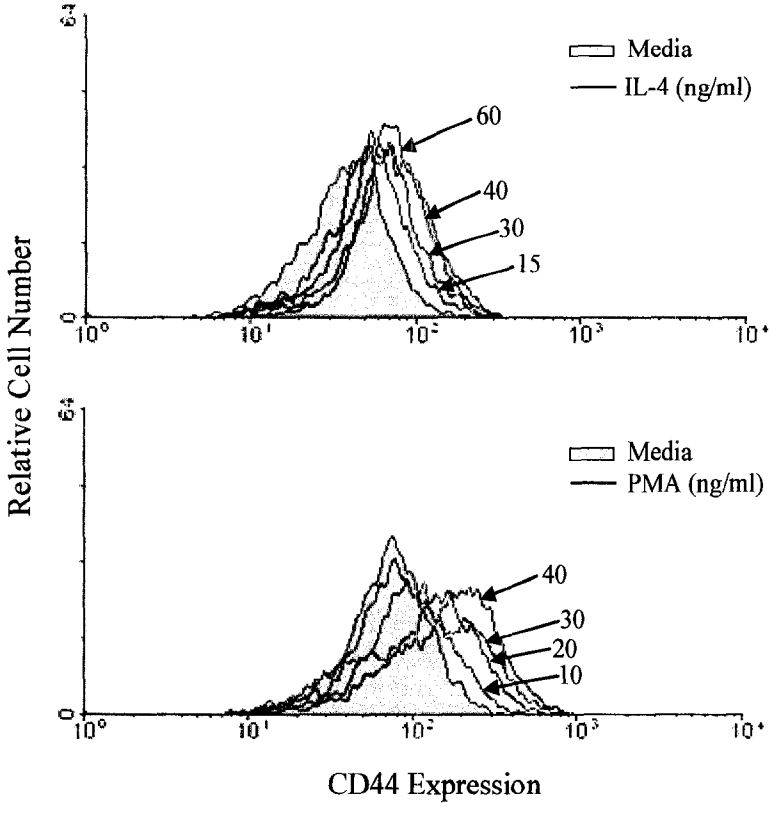

Media
IL-4 (ng/ml)
60
40
30
15
Media
PMA (ng/ml)
40
30
20
10
Relative Cell Number
CD44 Expression

Fig. 4.1

Fig. 4.2: IL-4 does not activate or induce the expression of Egr-1 in BL30/B95-8 cells.

A) BL30/B95-8 cells (1 X 10^6/ml) were treated with IL-4 (40 ng/ml), and PMA (30 ng/ml) as a control for 0 to 24 hr. Total RNA sample was subjected to Northern blot analysis using ^{32}P-labelled cDNA probe specific for human Egr-1. The experiment shown is representative of three independent experiments.

B) BL30/B95-8 cells (1 X 10^6/ml) were stimulated with IL-4 (40 ng/ml) or PMA (30 ng/ml) for 0 to 240 min. Nuclear extract samples (5 μg each) were probed with ^{32}P-labelled oligonucleotides corresponding to the Egr-1-binding sequence of CD44 promoter for gel shift assay. The experiment shown is representative of three independent experiments.

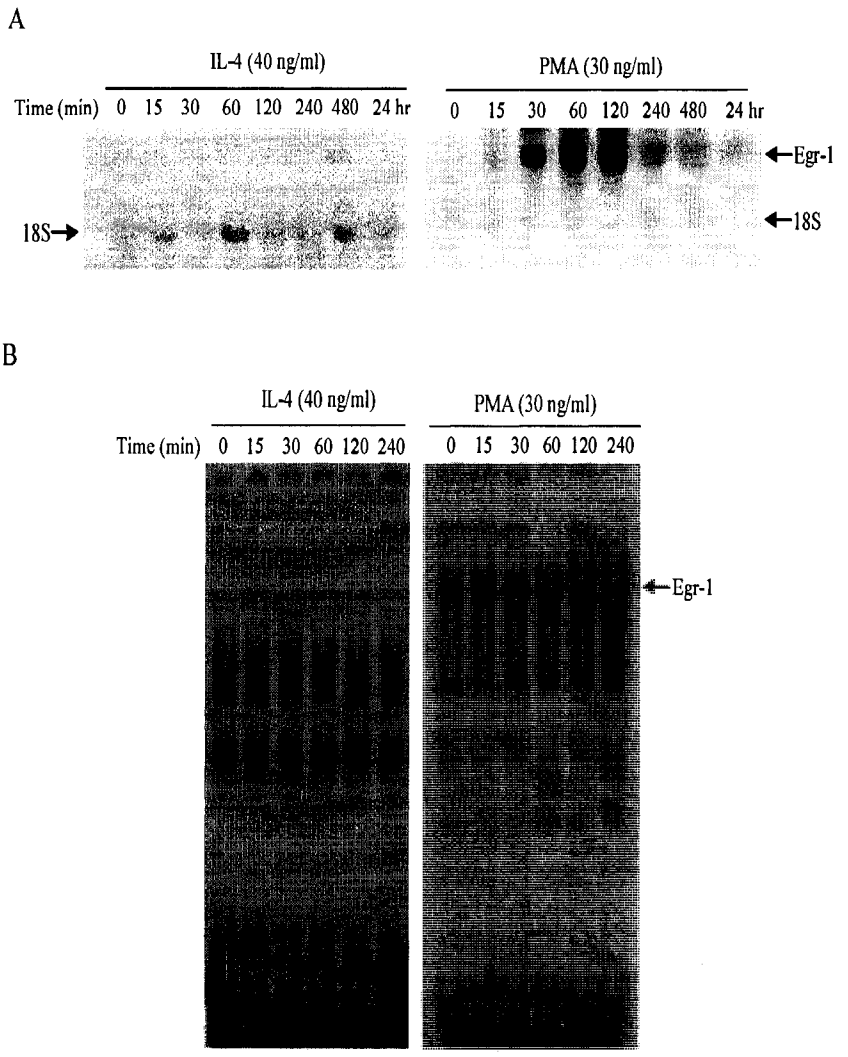

A
IL-4 (40 ng/ml)
PMA (30 ng/ml)
Time (min) 0 15 30 60 120 240 480 24 hr
0 15 30 60 120 240 480 24 hr
Egr-1
18S
18S
B
IL-4 (40 ng/ml)
PMA (30 ng/ml)
Time (min) 0 15 30 60 120 240
0 15 30 60 120 240
Egr-1

Fig. 4.2

Fig. 4.3: STAT-6 (#1 & 2) binding sites in CD44 promoter sequence (-122 to -601) are shown and underlined.

STAT-6 (-567 to -576)

-601 ctctccagct cctctcccag gatat**ccaac atccc**tgtga aaccagagat cttgctccag

Site#2

-541 ccggattcag agaaatttag cgggaaagga gaggccaaag gctgaaccca atggtgcaag

-481 gttttacggt tcggtcatcc tctgtcctga cgccgcgggg ccagcgggag aagaaagcca

-421 gtgcgtctct gggcgcaggg gccagtgggg ctcggaggca caggcacccc gcgacactcc

-361 aggttccccg acccacgtcc ctggcagccc cgattattta cagcctcagc agagcac**ggg**

SP-1, Egr-1 AP-2, AP-4, AP-1

-301 **gcgggggca**g aggggcccgc ccgggagggc tgctacttct taaaa**cctct gcgggctgct**

-241 **tagtca**cagc ccccttgct tgggtgtgtc cttcgctcgc tccctccctc cgtcttaggt

STAT-6 (-136 to -145)

-181 cactgttttc aacctcgaat aaaaactgca gccaac**ttcc gaggca**gcct cattgcccag -122

Site#1

Fig. 4.3

Fig. 4.4: STAT-6 regulates IL-4-induced CD44 transcription.

A & B) BL30/B95-8 cells (2 X 10^6) were transiently cotransfected with 1 μg of either full-length CD44 promoter construct (pCD44PrGL3B) or pGL3B control vector and 0.5 μg of β-galactosidase plasmid. After 15 hr, the cells were stimulated with various concentrations of IL-4 (A) for 0-24 hr (B) followed by the measurement of luciferase and β-galactosidase activities in the cell lysates. Luciferase activity was normalized with β-galactosidase activity and expressed as relative luciferase units (RLU). The results shown are representative of three independent experiments.

C) BL30/B95-8 cells (2 X 10^6) were transiently cotransfected with various CD44 promoter deletion/mutant constructs or control pGL3B vector and β-galactosidase plasmid. After 15 hr, cells were stimulated with IL-4 (40 ng/ml) for 4 hr followed by measurement of luciferase and β-galactosidase activities. The results are shown as fold increase in RLU mean ± S.D. of three independent experiments performed in triplicates. X indicates the mutated (m) STAT-6 binding site. * indicates the p value <0.001. The results shown are representative of three independent experiments.

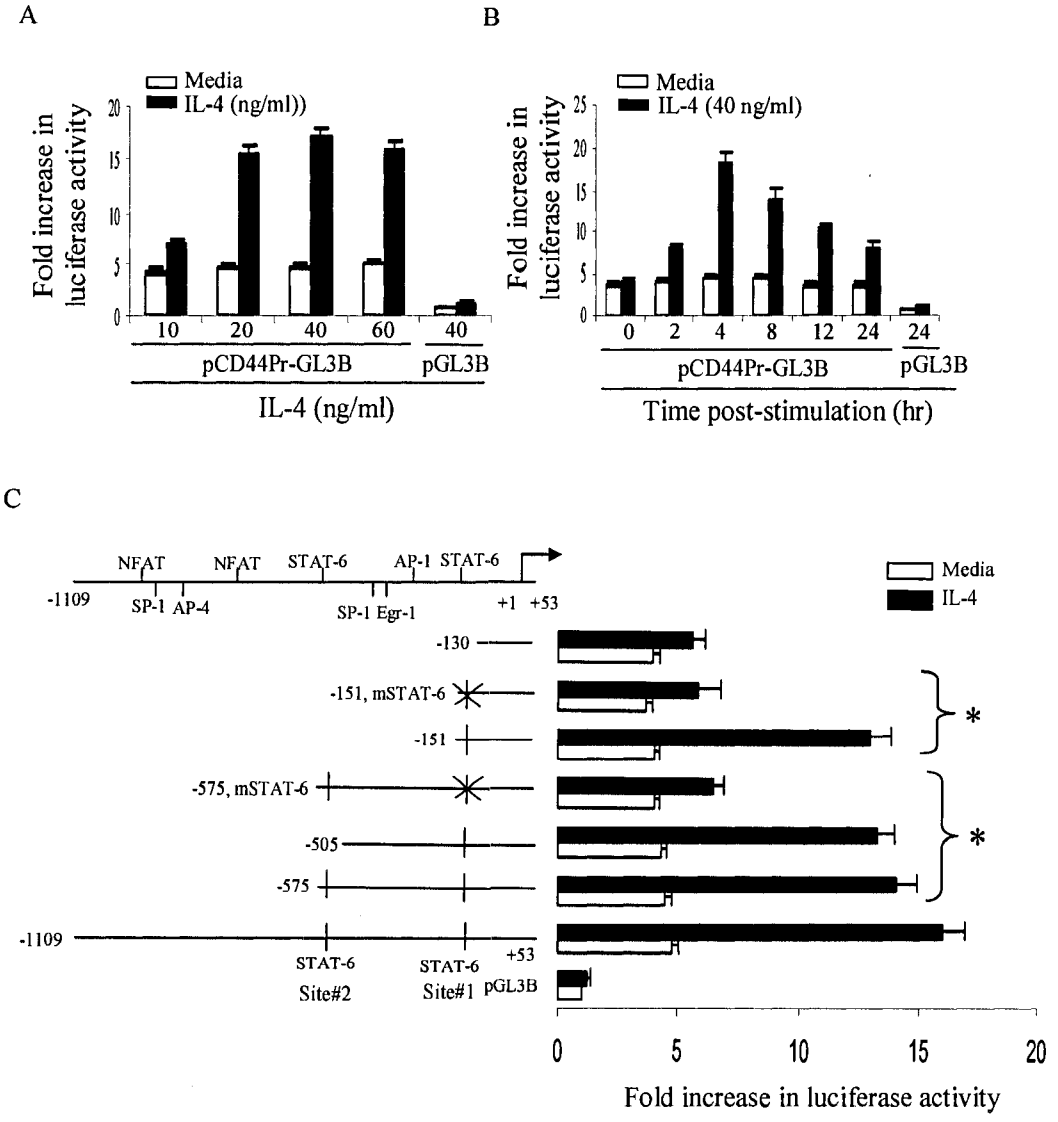

A
B
Media
IL-4 (ng/ml))
Fold increase in luciferase activity
10
20
40
60
40
pCD44Pr-GL3B
pGL3B
IL-4 (ng/ml)
Media
IL-4 (40 ng/ml)
0
2
4
8
12
24
24
Time post-stimulation (hr)
C
NFAT
NFAT
STAT-6
AP-1
STAT-6
-1109
SP-1
AP-4
SP-1
Egr-1
+1
+53
Media
IL-4
-130
-151, mSTAT-6
-151
-575, mSTAT-6
-505
-575
-1109
STAT-6
Site#2
STAT-6
Site#1
+53
pGL3B
*
*
0
5
10
15
20
Fold increase in luciferase activity

Fig. 4.4

peaked at 4 hr (Fig. 4.4B). The luciferase activity detected in unstimulated cells was 4-5-fold higher compared to the cells transfected with the control plasmid probably because of constitutive CD44 expression in these cells. The luciferase activity increased by three-fold following IL-4 stimulation, when compared to the unstimulated cells, and by 15-20-fold, when compared to cells transfected with the control plasmid. Cells transfected with pGL3B alone did not show an increase in luciferase activity following IL-4 stimulation (Fig. 4.4C). To determine the DNA elements required for CD44 transcription, a series of promoter deletion fragments from 5' -1109 to 3' +53 bp were analyzed. The results show that the deletion of sequences from -1109 to -151 bp had no effect on IL-4-induced luciferase activity as compared to cells transfected with the full-length pCD44(-1109). However, deletion of sequences from -151 to -130 bp abrogated IL-4-induced luciferase activity suggesting an involvement of the -151 to -130 sequence in IL-4-induced CD44 transcription (Fig. 4.4C). IL-4-induced luciferase activity in cells transfected with the CD44 promoter construct containing -130 to +53 bp was comparable with the activity observed in unstimulated cells (Fig. 4.4C).

Mat inspector analysis of the promoter sequence between -151 to -130 bp revealed the existence of a STAT-6 binding sequence (-136 to -145 bp, site # 1). To confirm the role of STAT-6, I generated -151 to +53 construct containing a mutated STAT-6 binding sequence (mutated bases shown in Table-2.3). IL-4 stimulation of cells transfected with pCD44(-151-mSTAT-6) revealed significantly decreased luciferase activity that was comparable to the unstimulated control and IL-4-stimulated cells transfected with pCD44(-130) which does not contain any STAT-6 sequences. These results suggest a role for STAT-6 site # 1 in IL-4 induced CD44 transcription (Fig. 4.4C).

To determine if STAT-6 site # 2 (-567 to -576 bp) can drive IL-4-induced CD44 transcription in the absence of a functional STAT-6 site # 1, I generated two plasmids. The plasmid pCD44(-505) did not contain STAT-6 site # 2, whereas the second plasmid was a STAT-6 mutant plasmid pCD44(-575-mSTAT-6) containing wt STAT-6 sequence at site # 2 and mutant STAT-6 sequence at site # 1. Transfection with pCD44(-575-mSTAT-6) exhibited significant reduction in luciferase activity following IL-4 stimulation compared to cells transfected with wt pCD44(-575). Moreover, this luciferase activity was comparable to the activity observed in unstimulated and IL-4-stimulated cells transfected with pCD44(-130) containing none of the STAT-6 sites (Fig. 4.4C). Moreover, IL-4 stimulation of cells transfected with pCD44(-505) containing only STAT-6 site # 1 alone, exhibited luciferase activity that was similar to the cells transfected with pCD44(-575) containing both STAT-6 sequences (Fig. 4.4C). Taken together, these results suggest that STAT-6 site # 1 plays a predominant key role in IL-4-induced CD44 transcription.

The Jak-1-STAT-6 signaling pathway regulates IL-4-induced CD44 expression.

To further determine the role of Jak-1-STAT-6 signaling pathway in IL-4-induced CD44 expression, I first demonstrated that IL-4 activates Jak-1 in BL30/B95-8 cells (Fig. 4.5A). The role of Jak-1 was analyzed by using specific Jak inhibitor, Jak Inhibitor-I. Prior treatment of BL30/B95-8 cells with Jak inhibitor-I Inhibited IL-4-induced phosphorylation of Jak-1 (Fig. 4.5A) and STAT-6 (Fig. 4.5B) and also inhibited IL-4-induced CD44 expression in a dose-dependent manner, as determined by flow cytometry (Fig. 4.5C). To show that IL-4-induced CD44 transcription is regulated by STAT-6

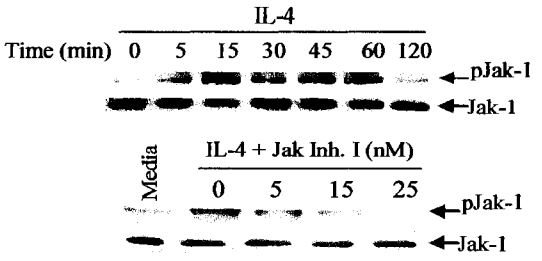
Fig. 4.5: IL-4-induced CD44 expression is regulated by Jak-1/3.

A & B) BL30/B95-8 cells (2 X 10^6) were treated with IL-4 (40 ng/ml) for 0 to 120 min. Cells were also pretreated with Jak Inhibitor I at various concentrations (5-25 nM) for 2 hr followed by IL-4 (40 ng/ml) stimulation for 30 min. Total protein (30 μg) was subjected to SDS-PAGE followed by Western blotting using anti-phospho-Jak-1 and anti-phospho-STAT-6 Ab. For loading controls, the membranes were stripped and reprobed with total anti-Jak-1 and anti-STAT-6 Ab, respectively. The results shown are representative of three independent experiments.

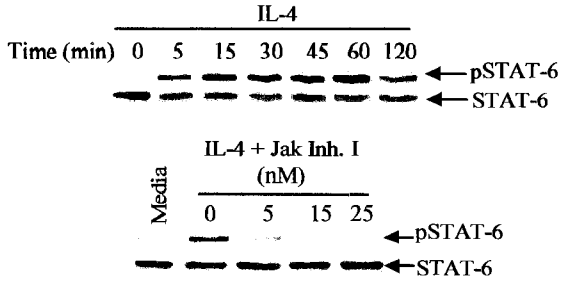
C) BL30/B95-8 cells (0.5 X 10^6/ml) were pretreated with Jak Inhibitor-I at various concentrations (5-25 μM) for 2 hr followed by IL-4 (40 ng/ml) stimulation for 24 hr and analysis of CD44 expression by flow cytometry. The results shown are representative of three independent experiments.

D) BL30/B95-8 cells (2 X 10^6) were transiently cotransfected with CD44 promoter deletion construct (pCD44Pr-151GL3B) and 0.5 μg of β-galactosidase plasmid for 15 hr. The transfected cells were treated for 2 hr with various concentration of Jak Inhibitor-I followed by IL-4 stimulation (40 ng/ml) for 4 hr and measurement of fold increase in RLU as described in the legend of Fig. 4.4. The results shown are representative of three experiments performed in triplicate.

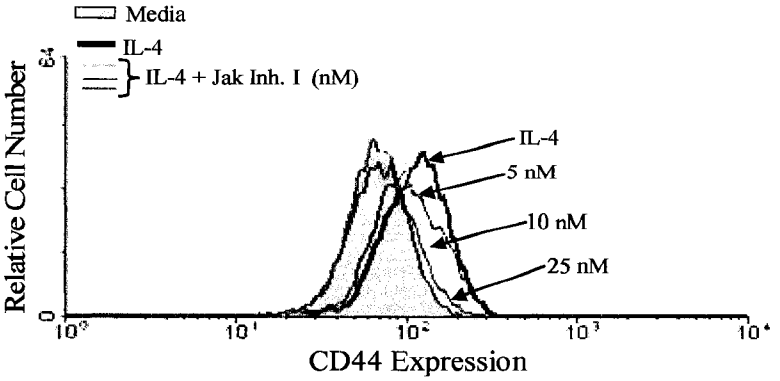
A


IL-4
Time (min) 0 5 15 30 45 60 120
pJak-1
Jak-1
Media
IL-4 + Jak Inh. I (nM)
0 5 15 25
pJak-1
Jak-1

B


IL-4
Time (min) 0 5 15 30 45 60 120
pSTAT-6
STAT-6
Media
IL-4 + Jak Inh. I (nM)
0 5 15 25
pSTAT-6
STAT-6

C


Media
IL-4
IL-4 + Jak Inh. I (nM)
Relative Cell Number
IL-4
5 nM
10 nM
25 nM
CD44 Expression

D

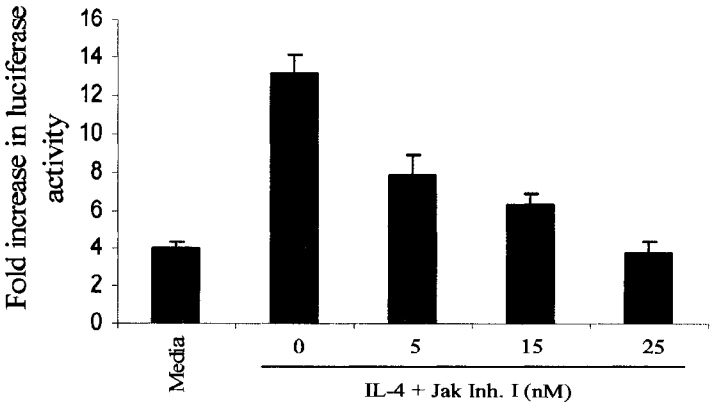

Fold increase in luciferase activity
16
14
12
10
8
6
4
2
0
Media
0
5
15
25
IL-4 + Jak Inh. I (nM)

Fig. 4.5

through Jak-1, cells transfected with pCD44(-151) containing STAT-6 site # 1 were treated for 2 hr with Jak inhibitor-I prior to IL-4 stimulation. IL-4 induced a significant three-fold increase in luciferase activity that was inhibited by Jak Inhibitor-I in a dose dependent manner (Fig. 4.5D). The activity observed with the highest concentration of Jak Inhibitor-I was similar to the basal activity seen in unstimulated cells and and the reduction was significant ($P<0.005$) compared with IL-4 stimulation alone (Fig. 4.5D). The role of Jak-1-activated STAT-6 in CD44 transcription was further confirmed by EMSA. First, I demonstrated that STAT-6 bound to its binding site on the CD44 promoter by using the CD44 promoter STAT-6-binding region as a probe (-126 to -149 bp) (Fig. 4.6A). The specificity of STAT-6-DNA complex was established by competition with specific and non-specific cold STAT-6 oligonucleotides, and by super-shift assays using anti-STAT-6 antibodies (Fig. 4.6A). Treatment of cells with Jak Inhibitor-I prior to IL-4 stimulation inhibited the STAT-6-CD44 promoter complex formation (Fig. 4.6B). These results suggested that IL-4-induced CD44 transcription is regulated by STAT-6 through Jak-1 activation.

IL-4-induced CD44 expression is regulated by the ERK MAPK.

I have previously shown that JNK MAPKs regulated LPS-induced CD44 expression in human monocytic cells (51). Since, IL-4 can activate MAPKs in some cell types (246;247), it was of interest to determine whether IL-4 can activate MAPK pathway in BL cells and if this pathway plays a role in IL-4-induced CD44 expression in B cells. I first demonstrated that in BL30/B95-8 cells, IL-4 induced the phosphorylation of p38, ERK and JNK-MAPKs which was inhibited by their respective inhibitors SB202190,

Fig. 4.6: IL-4 induces the binding of STAT-6 to its binding site in CD44 promoter.

A) BL30/B95-8 cells (1 X 10^6/ml) were stimulated with IL-4 (40 ng/ml) for 15 min. The nuclear extract samples (5 μg each) were probed for binding of STAT-6 to its binding site in the CD44 promoter by using ^{32}P-labelled oligonucleotide probe corresponding to the STAT-6-binding sequence (# 1) in the CD44 promoter by gel shift assay (left panel). To determine the specificity of STAT-6 binding, the nuclear extract samples were incubated with 100-200-fold excess of unlabelled specific and nonspecific oligonucleotides. The samples were also incubated with anti-STAT-6 antibodies to identify STAT-6 specific bands by supershift EMSA (right panel). The arrow indicates the supershifted STAT-6 band. The experiment shown is representative of three independent experiments.

B) IL-4-activated ERK MAPK and Jak-1/3 mediate binding of STAT-6 to its binding site in the CD44 promoter: BL30/B95-8 cells were treated with various concentrations of either Jak Inhibitor-I or PD98059 for 2 hr prior to stimulation with IL-4 (40 ng/ml). The nuclear extract samples (5 μg each) were probed for binding of STAT-6 to its binding site in the CD44 promoter by using ^{32}P-labelled oligonucleotide probe corresponding to the STAT-6-binding sequence (# 1) in the CD44 promoter by gel shift assay as described in the Materials and Methods. The experiment shown is a representative of three independent experiments.

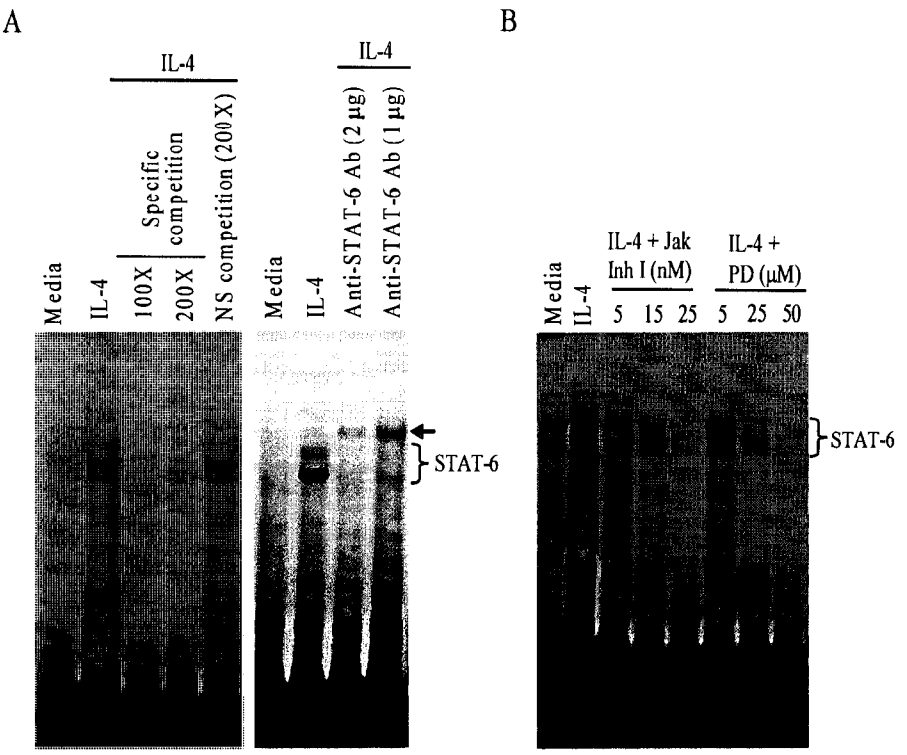

A
IL-4
Specific competition
100X
200X
NS competition (200X)
Media
IL-4
IL-4
Anti-STAT-6 Ab (2 µg)
Anti-STAT-6 Ab (1 µg)
Media
STAT-6
B
IL-4 + Jak Inh I (nM)
IL-4 + PD (µM)
Media
IL-4
5 15 25 5 25 50
STAT-6

Fig. 4.6

PD98059, and SP600125 in a dose-dependent manner (Fig. 4.7A). To determine their role in IL-4-induced CD44 expression, BL30/B95-8 cells were treated with SB202190, PD98059, and SP600125 for 2 hr prior to IL-4 stimulation. In contrast to SB202190 and SP600125, PD98059 inhibited IL-4-induced CD44 expression in a dose-dependent manner (Fig. 4.7B). The role of ERKs was confirmed by transfecting BL30/B95-8 cells with vectors containing ERKs-specific siRNA. Transfection with ERK-siRNA significantly reduced constitutive ERKs as well as IL-4-induced ERKs phosphorylation compared to the cells transfected with the control vectors (Fig. 4.8A). Furthermore, following IL-4 stimulation, CD44 expression was reduced to basal levels in ERK-siRNA-transfected cells compared to the cells transfected with the control vectors, as determined by flow cytometry (Fig. 4.8B). The above results suggest that besides the Jak/STAT-6 pathway, IL-4-induced CD44 expression is regulated by the ERK MAPKs.

ERK MAPK pathway regulates IL-4-induced CD44 expression through STAT-6 activation.

I next determined if IL-4-induced CD44 expression is regulated by two distinct and independent pathways, namely ERK MAPK and Jak-STAT-6 pathways, or by a single ERK-activated STAT-6 pathway. For this, cells were treated for 2 hr with either PD98059 or SB202190 or SP600125 prior to IL-4 stimulation followed by determination of STAT-6 phosphorylation. In contrast to p38 and JNK inhibitors, ERK inhibitor PD98059 inhibited STAT-6 phosphorylation in a dose dependent manner (Fig. 4.9A) suggesting that IL-4-induced CD44 expression is regulated by a single ERK-activated STAT-6 pathway. Moreover, PD98059 did not inhibit the upstream Jak-1 phosphorylation following IL-4 stimulation (Fig. 4.9A).

Fig. 4.7A: IL-4 induces phosphorylation of p38, ERK and JNK MAPKs.

BL30/B95-8 cells (2 X 10^6) were treated with IL-4 (40 ng/ml) for various time periods (0 to 120 min). Cells were also pretreated with SB202190, PD98059 or SP600125 at various concentrations (5-50 μM) for 2 hr followed by Western blot analysis for determination of p38, ERK and JNK phosphorylation by using anti-phospho-p38 (pp38), anti-phospho-ERK (pERK), or anti-phospho-JNK (pJNK) rabbit polyclonal Ab, respectively, as described in the legends of Fig. 4.5 (A & B). The results shown are representative of three independent experiments.

A

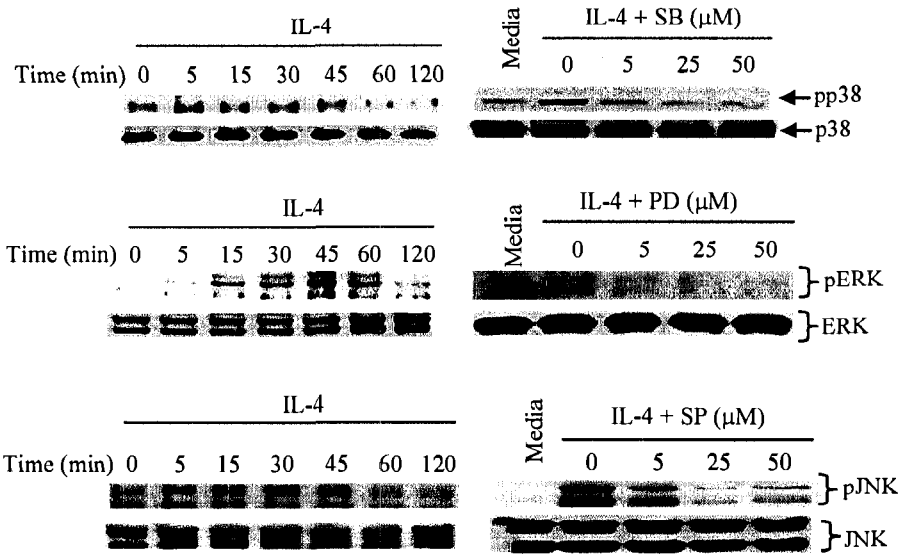

IL-4
Time (min) 0 5 15 30 45 60 120
Media
IL-4 + SB (μM)
0 5 25 50
pp38
p38
IL-4
Time (min) 0 5 15 30 45 60 120
Media
IL-4 + PD (μM)
0 5 25 50
pERK
ERK
IL-4
Time (min) 0 5 15 30 45 60 120
Media
IL-4 + SP (μM)
0 5 25 50
pJNK
JNK

Fig. 4.7

Fig. 4.7B): BL30/B95-8 cells (0.5 X 10^6/ml) were pretreated with SB202190, PD98059, or SP600125 at various concentrations (5-50 μM) for 2 hr before IL-4 stimulation (40 ng/ml) for 24 hr followed by analysis of CD44 expression by flow cytometry. The results shown are representative of three independent experiments.

B

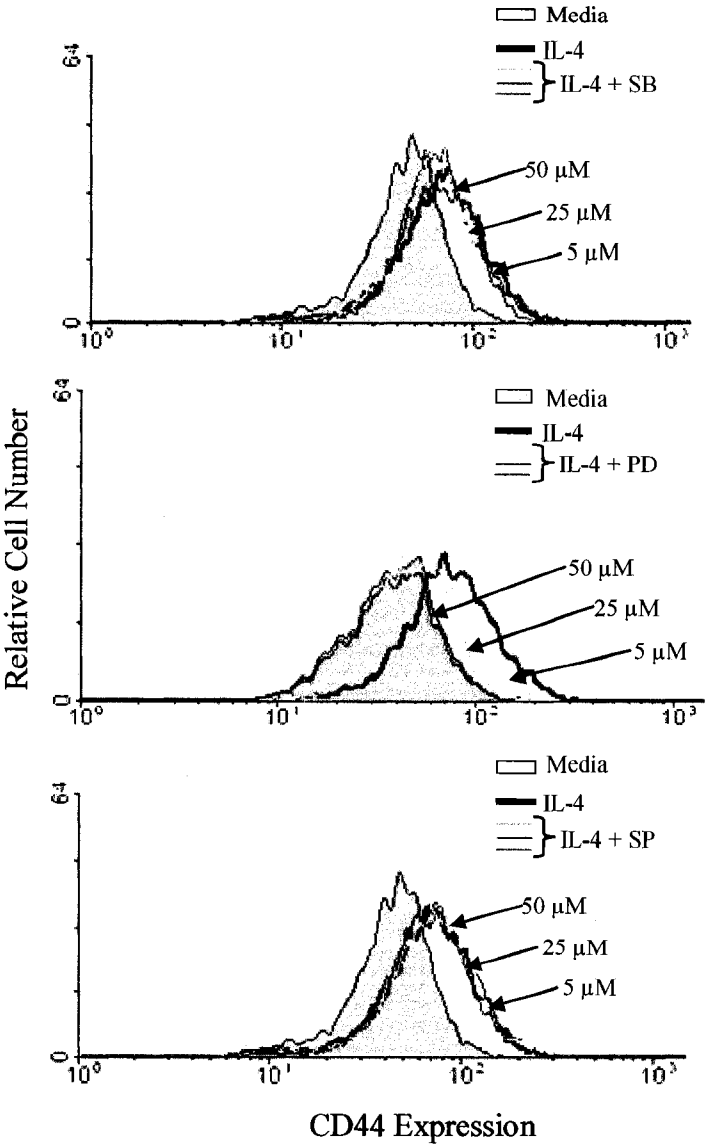
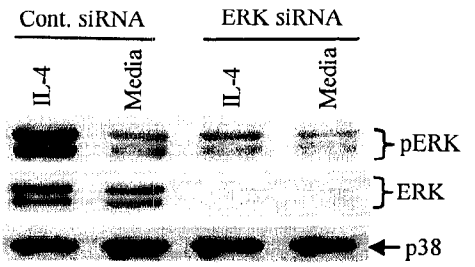

Media
IL-4
IL-4 + SB
50 µM
25 µM
5 µM
Media
IL-4
IL-4 + PD
50 µM
25 µM
5 µM
Media
IL-4
IL-4 + SP
50 µM
25 µM
5 µM
Relative Cell Number
CD44 Expression

Fig. 4.7

Fig. 4.8: ERK-specific siRNA inhibits IL-4-induced CD44 expression.

A & B) BL30/B95-8 cells (2 X 10^6) were transfected with the vectors containing either the ERK specific or control siRNA for 24 hr followed by stimulation with IL-4 (40 ng/ml) either for 45 min for the determination of ERK phosphorylation by Western blotting (A) or for 24 hr for CD44 expression by flow cytometry (B). The results shown are representative of three independent experiments.

A


Cont. siRNA
ERK siRNA
IL-4
Media
IL-4
Media
pERK
ERK
p38

B

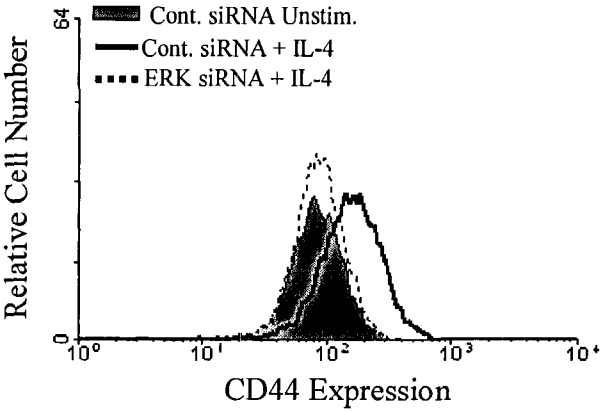

Cont. siRNA Unstim.
Cont. siRNA + IL-4
ERK siRNA + IL-4
Relative Cell Number
64
0
10^0
10^1
10^2
10^3
10^4
CD44 Expression

Fig. 4.8

Fig. 4.9: IL-4-induced STAT-6 phosphorylation is mediated through the ERK MAPKs.

A) BL30/B95-8 cells (1 X 10^6/ml) were treated with SB202190, PD98059 or SP600125 at various concentrations (5-50 μM) for 2 hr before IL-4 stimulation (40 ng/ml). Total protein (30 μg) was subjected to SDS-PAGE followed by Western blotting for analysis of phosphorylation for STAT-6 or Jak-1 by using anti-phospho-STAT-6 and anti-phospho-Jak-1 Abs, respectively as described in the legend of Fig. 4.7A. The results shown are representative of three independent experiments.

B) ERK MAPK and Jak-1/3 regulate IL-4-induced CD44 promoter activity: BL30/B95-8 cells (2 X 10^6) were transiently cotransfected with CD44 promoter deletion construct (pCD44Pr-151GL3B) and 0.5 μg of β-galactosidase plasmid for 15 hr. Cells were treated for 2 hr with various concentrations of SB202190, PD98059 or SP600125 followed by IL-4 stimulation (40 ng/ml) for 4 hr and measurement of fold increase in RLU as described in the legend of Fig. 4.4. The results shown are representative of three experiments performed in triplicate.

C) Jak-1 activates IL-4-induced phosphorylation of ERK: BL30/B95-8 cells (1 X 10^6/ml) were pretreated with various concentrations of Jak Inhibitor-I (5-25 nM) or PD98059 (5-50 μM) for 2 hr followed by IL-4 stimulation (40 ng/ml). Total protein (30 μg) sample was analyzed for ERK or p38 (as a control) phosphorylation by using anti-phospho-ERK or anti-phospho-p38 Ab, respectively by Western blot analysis as described in the legend of Fig. 4.7A. The results shown are representative of three independent experiments.

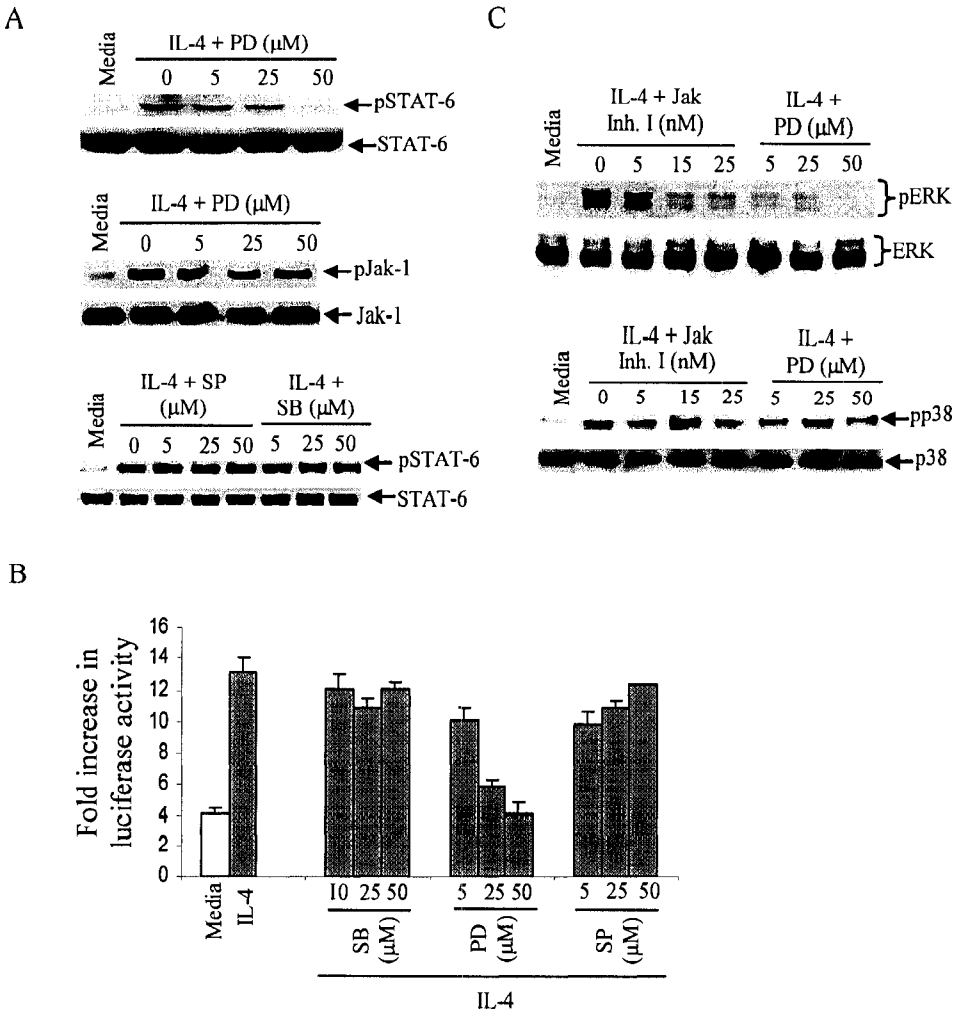

A
Media
IL-4 + PD (μM)
0 5 25 50
pSTAT-6
STAT-6
Media
IL-4 + PD (μM)
0 5 25 50
pJak-1
Jak-1
Media
IL-4 + SP (μM)
IL-4 + SB (μM)
0 5 25 50 5 25 50
pSTAT-6
STAT-6
C
Media
IL-4 + Jak Inh. I (nM)
IL-4 + PD (μM)
0 5 15 25 5 25 50
pERK
ERK
Media
IL-4 + Jak Inh. I (nM)
IL-4 + PD (μM)
0 5 15 25 5 25 50
pp38
p38
B
Fold increase in luciferase activity
0 2 4 6 8 10 12 14 16
Media
IL-4
10 25 50
SB (μM)
5 25 50
PD (μM)
5 25 50
SP (μM)
IL-4

Fig. 4.9

The role of ERK-activated STAT-6 pathway was further determined by transfecting BL30/B95-8 cells with pCD44(-151) containing STAT-6 site # 1 sequence. The transfected cells were treated for 2 hr with either SB202190 or PD98059 or SP600125 prior to IL-4 stimulation. IL-4 stimulation induced a significant 3-fold increase in luciferase activity compared to the unstimulated cells and this effect was inhibited selectively by PD98059 in a dose-dependent manner (Fig. 4.9B). The activity observed with the highest concentration of PD98059 was similar to the basal activity seen in unstimulated cells, and the reduction was significant ($P<0.005$) compared with IL-4 stimulation alone. IL-4-induced luciferase activity was not inhibited by either SB202190 or SP600125 even at the highest concentration of 50 μM (Fig. 4.9B). These results were further confirmed by gel shift assays. Prior treatment of cells with PD98059 inhibited the binding of STAT-6 to the CD44 promoter containing STAT-6 recognition site # 1 (Fig. 4.6B). These results suggested that IL-4-induced CD44 transcription may be regulated by STAT-6 through the selective activation of ERK MAPKs.

IL-4-induced ERK MAPK activation is dependent on Jak-1.

To determine further the functional cross-talk between the Jak-STAT and ERK MAPKs and to determine if ERK MAPK is activated by the upstream Jak-1, cells were treated with the Jak Inhibitor-I for 2 hr prior to IL-4 stimulation followed by analysis of ERK phosphorylation. Jak Inhibitor-I selectively inhibited IL-4-induced ERK but not p38 phosphorylation (Fig. 4.9C) suggesting that IL-4-activated Jak-1 induces the phosphorylation of ERK MAPKs which in turn upregulates phospho-STAT-6. Taken together, these results suggest that IL-4-induced CD44 expression may be regulated by STAT-6 through Jak-1-activated ERK MAPK.

IL-4-induced CD44 expression is not regulated by the IRS-2/PI3K pathway.

The PI3K is another major signaling pathway activated by IL-4 (239;282;283). To determine whether IL-4-activated PI3K regulates CD44 induction, I first demonstrated that IL-4 induced the phosphorylation of Akt which was inhibited following pretreatment of cells with the PI3K inhibitor, LY294002 (Fig. 4.10A). However, LY294002 did not affect IL-4-induced CD44 expression (Fig. 4.10B). Recruitment of IRS-2 to the activated IL-4Rα chain results in its phosphorylation and subsequent activation of downstream p85α PI3K subunit (198;252). IRS-2 is also activated by Jak-1 tyrosine kinase (280;284). Therefore, we determined if IL-4 induced ERK phosphorylation is mediated through the upstream Jak-1/IRS-2 activation. For this, cells pretreated with Jak Inhibitor-1 were stimulated with IL-4 followed by analysis of IRS-2 phosphorylation. IL-4 induced IRS-2 phosphorylation in BL30/B95-8 cells was down regulated by Jak inhibitor-I in a dose-dependent manner (Fig. 4.10C) suggesting that Jak-1 activates IRS-2 in IL-4-stimulated BL30/B95-8 cells. To determine if IRS-2 is influenced ERK MAPK, cells pretreated with PD98059 were analyzed for IL-4-induced IRS-2 and STAT-6 phosphorylation. PD98059 inhibited IL-4-induced STAT-6 phosphorylation without affecting IRS-2 activation (Fig. 4.10C). These results suggest that IL-4-induced STAT-6 activation is mediated by ERK MAPK independent of the IRS-2-PI3K pathway. Furthermore, IL-4-activated IRS-2/PI3K may not regulate CD44 expression in human B cells.

Fig. 4.10: IL-4-induced CD44 expression does not involve IRS-2/PI3K activation.

A) BL30/B95-8 cells (1 X 10^6/ml) were stimulated with IL-4 (40 ng/ml) for 0 to 120 min. Cells were also pretreated with LY294002 at concentrations ranging from 5-25 μM for 2 hr followed by IL-4 stimulation (40 ng/ml) for 30 min. Total protein (30 μg) sample was subjected to Western blot analysis for determination of Akt phosphorylation by using anti-phospho-Akt Ab as described in the legend of Fig. 4.5 (A & B). The results shown are representative of three independent experiments.

B) BL30/B95-8 Cells (0.5 X 10^6/ml) were pretreated with LY294002 at concentrations ranging from 5-25 μM for 2 hr followed by IL-4 stimulation (40 ng/ml) for another 24 hr. Cells were analyzed for CD44 expression by flow cytometry. The results shown are representative of four independent experiments.

C) BL30/B95-8 cells (1 X 10^6/ml) were pretreated with various concentrations of Jak Inhibitor-I (5-25 nM) or PD98059 (5-50 μM) for 2 hr followed by IL-4 stimulation (40 ng/ml). Total protein (30 μg) sample was subjected to Western blot analysis for determination of IRS-2 phosphorylation by using anti-phospho-IRS-2 Ab as described in the legend of Fig. 4.7A. The results shown are representative of three separate experiments.

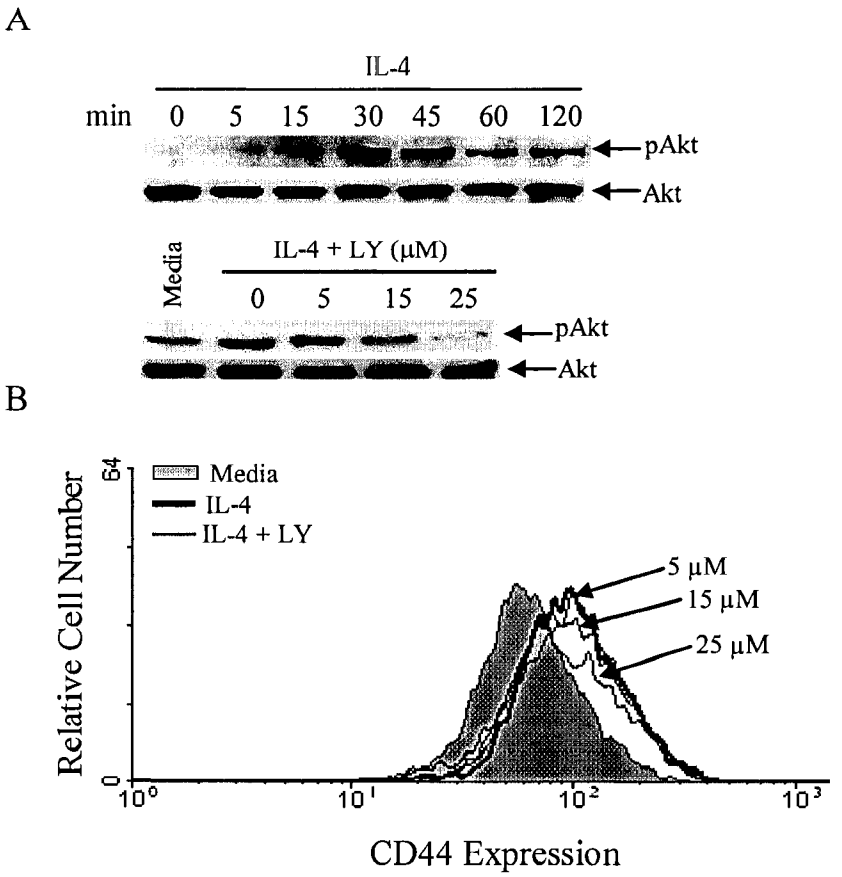

A
IL-4
min 0 5 15 30 45 60 120
pAkt
Akt
Media
IL-4 + LY (μM)
0 5 15 25
pAkt
Akt
B
Media
IL-4
IL-4 + LY
5 μM
15 μM
25 μM
Relative Cell Number
64
0
10⁰
10¹
10²
10³
CD44 Expression
C
IL-4
min 0 5 15 30 45 60 120
pIRS-2
IRS-2
Media
IL-4 + Jak Inh. I (nM)
IL-4 + PD (μM)
0 5 15 25 5 25 50
pIRS-2
IRS-2

Fig. 4.10

Discussion

Regulation of LPS- and TNF-α-induced CD44 expression in human monocytic cells

CD44 may be induced as a result of the signals generated following association of LPS with the LPS-binding protein, and consequential binding of the LPS-LPS-binding protein complex with the CD14-Toll-like receptor-4 complex expressed on cells of the monocytic lineage (267). Our laboratory and others have previously demonstrated that LPS-induced CD44 expression in monocytic cells is mediated, at least in part, by the endogenously produced TNF-α (75;166). Both LPS and TNF-α act as positive regulators of CD44 induction and its binding with HA in monocytic cells, which has been suggested to play a vital role in their migration, and development of immune and inflammatory responses (31;89;144;160). However, the signaling pathways underlying CD44 induction in monocytic cells especially following stimulation with TNF-α are not understood. Our laboratory previously demonstrated the distinct involvement of JNK MAPK in LPS-induced CD44 expression in monocytic cells (166). In this study, I investigated the role of Ca^{2+} signaling pathway and the activation of downstream transcription factors in TNF-α-induced CD44 transcription and compared the signaling cascade activated by LPS in the regulation of CD44 expression in THP-1/CD14 cells as a model system. I show for the first time the involvement of two distinct and independent signaling pathways in the regulation of LPS- and TNF-α-induced CD44 expression. TNF-α-induced CD44 expression was found to be regulated by AP-1 through the activation of the CaM/CaMK-II pathway. In contrast, LPS-induced CD44 transcription was regulated specifically by Egr-1 through the activation of JNK MAPK (Fig. 4.11).

Fig. 4.11: LPS-induced CD44 expression is regulated by JNK-activated Egr-1, and TNF-α-induced CD44 expression is regulated by CaM/CaMK-II-activated AP-1 in human monocytic cells.

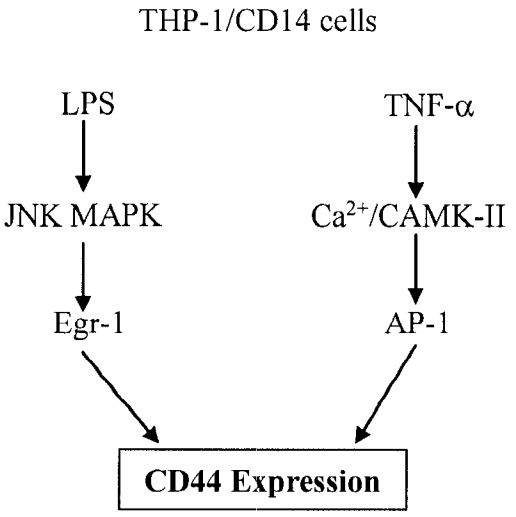

THP-1/CD14 cells
LPS
TNF-α
JNK MAPK
Ca2+/CAMK-II
Egr-1
AP-1
CD44 Expression

Fig. 4.11

Ca^{2+} is an important intracellular messenger in many biological processes (182). Influx of Ca^{2+} through ligand and voltage-gated calcium channels in the plasma membrane together with Ca^{2+} release from endoplasmic reticulum stores results in complex calcium signaling cascades (182;268). Several mechanisms may control Ca^{2+} entry in response to external stimuli including membrane depolarization, activation of intracellular messengers, and depletion of intracellular calcium storage (182;268). The release of Ca^{2+} from internal stores (ER) is controlled by Ca^{2+} itself or by an expanding group of messengers. For example, the IP_3, produced in response to a signal from the membrane lipid phosphatidylinositol, triggers Ca^{2+} release from the ER after binding to the IP_3 receptor (182;268). By employing a number of inhibitors specific for the Ca^{2+} pathway, our results suggested that intracellular release as well as extracellular influx of Ca^{2+} into the cytosol may be involved in the regulation of TNF-α-induced CD44 expression.

CaM, a key signaling protein responsible for integrating the Ca^{2+} signal to transcription factors, is known to regulate cell cycle and related cytoskeletal functions and ion channel activity (269). Following binding to Ca^{2+}, CaM undergoes a conformational change that renders it active and able to recognize and bind target proteins with high affinity (269). Among the possible downstream targets of CaM are calcineurin and CaMK-II (269;285;286). As with other kinases, CaMK-II undergoes autophosphorylation on a threonine residue contained in a phosphopeptide common to it's α and β subunits and converts it into a Ca^{2+}/CaM independent enzyme (269;285). The results obtained by employing specific inhibitors suggested that CaMK-II may act as a key link in TNF-α-induced CaM activation and CD44 expression.

In this study, I also investigated the downstream signaling events responsible for CD44 transcription. The transcription factors involved in the regulation of CD44 expression in monocytic cells have not been well defined. Egr-1 was shown to be involved in phorbol 12-myristate 13-acetate (PMA)-induced CD44 expression in murine B cells and IL-1α-induced CD44 expression in human endothelial cells (50;53). The Egr-1 gene is a prototypic member of a gene family encoding transcription factors that share a conserved zinc finger DNA-binding motif. This gene is induced rapidly and transiently in response to B cell receptor cross-linking or treatment with PMA (287). Recently, AP-1 was shown to regulate CD44 transcription in murine vascular smooth muscle cells in response to stimulation with IL-1β (39). AP-1, a key regulator of the expression of a number of cytokines (288;289), is a heterodimeric transcription factor comprised of members of the Jun (c-Jun, JunB, and JunD) and Fos (c-Fos, Fra-1, Dra-2, FosB, and FosB2) proto-oncogene families (289;290). Members of the Fos and Jun families dimerize via their leucine zipper domain with a variety of transcription factors including cAMP-response element-binding protein/ATF, Maf, NFκB, NFAT, and GR (289;291-293). The results of this study show the differential involvement of Egr-1 and AP-1 in response to two distinct signaling cascades LPS and TNF-α, respectively, in monocytic cells. This was demonstrated by analyzing the luciferase activity in cells transfected with CD44 promoter deletion constructs exhibiting mutations in the binding sites for either Egr-1 and/or AP-1.

CD44 has a relatively high constitutive expression in unstimulated THP-1 cells. It may be noted that the unstimulated cells transfected with CD44 promoter constructs (-224 to +1 bp) containing deleted Egr-1 and AP-1 binding sequences exhibited some

reduction in luciferase activity suggesting that the constitutive basal activity might be mediated by either Egr-1 and AP-1 alone or through their cooperative effects. However, cells transfected with the CD44 promoter construct containing mutated Egr-1 and AP-1 sites did not show complete abrogation of luciferase activity, which indicated some other regulatory elements might play a role in constitutive expression of CD44. The transcription factors responsible for basal CD44 expression/luciferase activity have not been investigated. Analysis of -224 to +1 bp in the CD44 promoter sequence may lead to identification of potential transcription factors responsible for the maintenance of basal levels of CD44 expression. Mat Inspector analysis of the -224 to +1 bp sequence reveals a number of sequences which are similar to the core promoter motifs such as the TATA box required for basal transcription (294;295), in addition to those of NF-1, Elk-1, NFκB, and E2F. It is possible that these transcription factors in concert with Egr-1 and AP-1 transcription factors may regulate basal constitutive expression of CD44.

The findings of a distinct involvement of AP-1 and the upstream CaM/CaMK-II signaling molecules in TNF-α-induced CD44 expression raised the question of how CaMK-II may serve to induce CD44 transcription. Similarly, it was interesting to determine whether JNK activation by LPS resulted in CD44 expression through Egr-1. My results demonstrate that LPS-induced CD44 transcription is regulated specifically by Egr-1 through JNK activation, whereas TNF-α-induced CD44 expression was independently regulated by AP-1 through CaM/CaMK-II activation without any evidence of cross-talk between the two pathways. Although TNF-α induced the activation of Egr-1, it did not regulate CD44 transcription. Furthermore, the JNK inhibitor, SP600125, did not affect the binding of TNF-α-induced Egr-1 to its binding site in the CD44 promoter.

Similarly, LPS induced the activation of AP-1 and upstream calcium signaling proteins including CaM and CaMK-II, activation of these proteins, however, did not affect CD44 transcription. Furthermore, the binding of LPS-induced AP-1 to its binding site on the CD44 promoter was not affected by inhibitors of the CaM/CaMK-II pathway. The molecular mechanism by which LPS- but not TNF-α-induced JNK selectively activates Egr-1 resulting in CD44 transcription or how TNF-α-induced CaM/CaMK-II selectively activates AP-1 leading to CD44 transcription is not understood.

However, in addition to the signal transduction pathways, the molecular mechanisms controlling gene expression involve chromatin remodeling and DNA methylation (48;296-299). Egr-1 is also known to associate with coreceptor proteins such as NAB1 and NAB2 that can repress transactivation of Egr-1 target genes (300). Therefore, it may be possible that the individual signal activates the specific other interacting proteins, which interact with transcription factor, Egr-1 or AP-1 by preventing their access to CD44 promoter (301). Additionally, hypermethylation of the CD44 promoter has been shown to cause silencing of the *CD44* gene (299). It is likely that the inability of LPS and TNF-α to induce CD44 expression through AP-1 and Egr-1, respectively, may be because of the alterations in the methylation status of their binding sites in the CD44 promoter. It is also likely that LPS and TNF-α may activate certain histone deacetylases and chromatin remodeling complexes that may work together to silence AP-1 and Egr-1 binding sites, respectively, of the CD44 promoter. Understanding the role of transcription factor corepressors, the status of CD44 promoter methylation, and chromatin remodeling may provide the potential mechanisms controlling LPS-/TNF-α-mediated CD44 transcription.

In addition, I have also demonstrated that PI3K constitutes a key downstream component of both the signaling pathways involved in the regulation of LPS- and TNF-α-induced CD44 expression. Further, my results for the first time suggest that the JNK-activated PI3K regulates LPS-induced CD44 expression through the activation of transcription factor Egr-1, whereas TNF-α induces CD44 expression by a distinct CaMK-II-activated PI3K through the activation of AP-1 (Fig. 4.12).

TLR4 signaling by LPS activates PI3K signaling pathway which is involved in various cellular functions. For example, PI3K regulates LPS-induced matrix metalloproteinase-9 (271), and IL-12p40 production (262) in human monocytic cells, and nitric oxide (NO) (272) and IL-10 production (273) in murine peritoneal macrophages. Fichter et al. (1997) showed by employing neuroblastoma cells that a mitogenic factor, 12-O-tetradecanoyl phorbol-13-acetate (TPA)- and a neuronal growth factor, insulin like growth factor-1 (IGF-1)-induced CD44 splice variant, CD44v6 expression was regulated by both PI3K, and PKC (175). My results show that the regulation of LPS-induced CD44 expression is through JNK-activated PI3K. We have previously shown that CaM/CaMK-II-activated PI3K regulates IL-12p40 expression in LPS-stimulated human monocytic cells (262). However, a recent study reveals that the regulation of *Mycobacterium* Bacillus Calmette-Guerin (BCG)-induced macrophage-inflammatory protein-2 (MIP-2) is through both PI3K and JNK MAPK in a mouse macrophage cell line RAW264.7 cells. However, they did not study the cross-talk between JNK and PI3K pathways (302). Therefore, the molecular mechanism involved in the activation of PI3K by JNK MAPK and consequent, the regulation of CD44 expression in LPS-stimulated human monocytic cells is not clear at present.

Fig. 4.12: JNK-activated PI3K regulates LPS-induced CD44 expression through Egr-1, and CaM/CaMK-II-activated PI3K regulates TNF-α-induced CD44 expression via AP-1 in human monocytic cells.

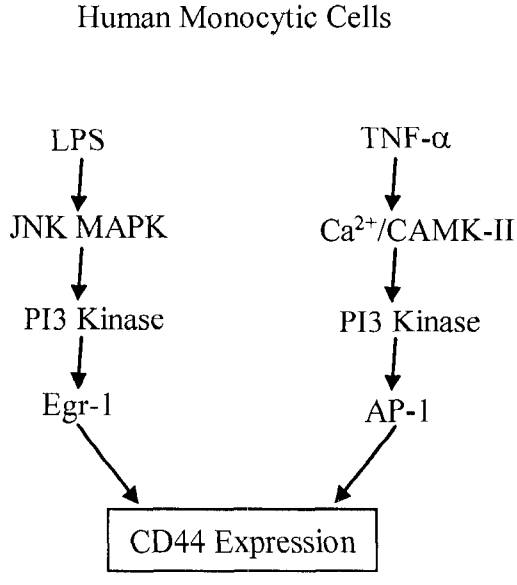

Human Monocytic Cells
LPS
TNF-α
JNK MAPK
Ca2+/CAMK-II
PI3 Kinase
PI3 Kinase
Egr-1
AP-1
CD44 Expression

Fig. 4.12

Class Ia PI3K consists of a 85 kDa regulatory subunit (α, β, and γ isoforms) and a 110 kDa catalytic subunit (α, β, and δ isoforms). Activation of PI3K appears to occur via phosphorylation of tyrosine residues in the Src homology 2 domain of p85, which permits docking of PI3K to the plasma membrane. Interaction of the p85 subunit with phosphorylated YxxM motifs in transmembrane receptors or adaptor molecules results in the recruitment of p85-p110 heterodimers to its substrate phosphatidylinositol 4,5-bisphosphate (PIP_2) that leads to the release of phosphatidylinositol 3,4,5-triphosphate (PIP_3) and eventual phosphorylation of a number of substrates including Akt (193;194). Munugalavadla et al. (2005) reported that p85α subunit of class Ia PI3K is crucial for macrophage growth and migration (303). Further, we previously demonstrated that both p85α and p110α subunits are required in LPS-induced IL-12p40 production in human monocytic cells (262). In this study, my results suggest the involvement of p85α, but not p110α subunit in the regulation of LPS- and TNF-α-induced CD44 expression suggesting the role of other catalytic subunits of p110 other than p110α in this process.

Binding of TNF-α to its receptor (TNFR) leads to a signaling complex, TNFR-associated factor (TRAF)-2/receptor interacting protein (RIP) complex, which activates PI3K pathway (304). It has been reported that the regulation of TNF-α-induced scavenger receptor and foam cell formation are mediated by PI3K in a murine macrophage cell line, J774A.1 cells and in human monocytes derived macrophages (305). However, PI3K is involved in the down regulation of macrophage scavenger receptor expression in TNF-α-induced murine macrophages (305). In this study, my results show that TNF-α-induced CD44 expression is regulated by PI3K in human monocytic cells. Further, the activation of PI3K is mediated by CaM/CaMK-II activation. One study revealed the tuberculosis

toxin lipoarabinomannan blocked phagosome maturation by interfering with Ca^{2+}/CaM-dependent PI3K pathway and membrane trafficking in infected macrophages (306).

My study also revealed that JNK-activated PI3K, but not CaM/CaMK-II-activated PI3K regulated LPS-induced CD44 expression through Egr-1. In contrast, CaM/CaMK-II-activated PI3K, but not JNK-activated PI3K regulated TNF-α-induced CD44 expression via activation of AP-1. The mechanism by which PI3K differentially involved Egr-1 and AP-1 in regulation of CD44 expression in LPS and TNF-α stimulation is not clear at present. However, it may be presumed that different isoforms of PI 3-kinase may play a significant role in this process.

In summary, regulation of CD44 expression in TNF-α- and LPS-stimulated monocytic cells involves a complex sequence of intracellular signaling events. In this study, I show for the first time a role for JNK and Egr-1 in LPS- but not in TNF-α-mediated signaling resulting in CD44 expression involved the selective activation of the AP-1 transcription factor and the Ca^{2+} signaling complex comprised of CaM and CaMK-II. In addition, PI3K constitutes a key downstream component of both the signaling pathways involved in the regulation of LPS- and TNF-α-induced CD44 expression. Finally, my results suggest that PI3K may serve as a potential therapeutic target as anti-inflammatory agent capable of inhibiting CD44 expression and CD44-mediated cell migration.

Regulation of IL-4-induced CD44 expression in human B cells

The transcription factor STAT-6 performs its main role in mediating the biological activities of IL-4 and IL-13, the Th2 type cytokines (251;252). STAT-6 is critically involved in allergic inflammatory diseases such as airway hyper-

responsiveness, eosinophillic inflammation and in the development of protective immunity against gastrointestinal nematodes. Overall, STAT-6 has been shown to play a key role in the regulation of several genes including MHC-II, CD23, IL-4Rα and immunoglobulin class switching to IgE and IgG1 (307-309). Herein, I investigated the role of major signaling pathways and transcription factors involved in the regulation of CD44, in response to IL-4 in human B cells. My results describe a previously unrecognized signaling pathway involving ERK-mediated activation of STAT-6 to regulate IL-4-induced CD44 expression in human BL BL30/B95-8 B cells. There are two STAT-6 binding sites in the CD44 promoter, namely site # 1 at -136 to -145 bp and site # 2 at -557 to -576 bp. By deletion and site-directed mutagenesis, my results clearly suggest a predominant role for STAT-6 at site # 1. Although STAT-6 is known to cooperate with several transcription factors (252;310), neither AP-1 nor Egr-1 was involved in IL-4-induced CD44 transcription in the present study. In fact, IL-4 even failed to induce the expression and activation of Egr-1.

It is interesting to observe that CD44 transcription in different cell types and in response to different stimuli is regulated by a distinct transcription factor. I have previously demonstrated in human monocytic cells that LPS induced CD44 expression through the selective activation of Egr-1, whereas TNF-α-induced CD44 expression in was regulated by AP-1 (51). Egr-1 was also implicated in CD44 transcription in PMA-stimulated B cells and following BCR signaling in WEHI-231 lymphoma B cells (50). In this study, IL-4-induced CD44 transcription in human B cells is shown to be regulated primarily by STAT-6. The molecular mechanism by which LPS, TNF-α and IL-4 induce

CD44 transcription through distinct transcription factors, namely Egr-1, AP-1, and STAT-6, respectively, is not clear.

CD44 is constitutively expressed at relatively high levels in B cells (141). It may be noted that cells transfected with a CD44 promoter construct lacking binding sites for STAT-6, Egr-1 and AP-1 exhibited a significant level of luciferase activity suggesting that the constitutive basal activity is not mediated by STAT-6, Egr-1 or AP-1. The transcription factors responsible for basal CD44 expression are not known. Analysis of CD44 promoter sequence from -130 to +1 bp may lead to the identification of transcription factors responsible for the maintenance of basal levels of CD44 expression.

IL-4 stimulation, in addition to the tyrosine phosphorylation of Jak-1/3 and its substrate STAT-6, is known to activate serine threonine MAPKs in limited cell systems such as myeloid cells, fibroblasts and neuronal cells (243-245). Besides a few reports in different cell types (246-248;311), IL-4 is not known to activate MAPKs in hematopoitic cells and in particular human B cells. Herein, I demonstrated activation of all the three members of the MAPK family in human B cells. My results clearly show that IL-4-induced expression of CD44 in BL cells is regulated through the ERK MAPKs. In addition, IL-4-induced STAT-6 phosphorylation and eventual CD44 expression is regulated by a mechanism requiring cooperation between the Jak/STAT and ERK MAPK pathways. These conclusions are based on the following observations: 1) inhibition of STAT-6 phosphorylation by the ERK and Jak Inhibitor, 2) ERK phosphorylation was inhibited by the Jak inhibitor, 3) Jak-1 and ERK inhibitors down regulated IL-4-induced CD44 promoter activity, binding of STAT-6 to the CD44 promoter and CD44 expression; and 4) ERK specific siRNAs inhibited IL-4-induced CD44 expression. These

observations suggest that IL-4-induced CD44 expression is regulated by STAT-6 through the activation of upstream Jak-1-activated ERK MAPK. However, it is possible that IL-4-induced CD44 expression may be regulated by an alternate ERK-independent Jak-STAT-6 pathway. Although, my results do not support the existence of this pathway, I can not at present rule out this possibility.

Although the precise mechanism by which IL-4-activated Jak-1 induces phosphorylation of ERK and subsequent STAT-6 activation is not clear, an understanding of the cross-talk between the Jak/STAT and receptor tyrosine kinase (RTK)/Ras/MAPKs signaling pathways is beginning to emerge (311;312). IL-4 is known to induce activation of insulin receptor substrate-1/2 (IRS-1/2), the down stream effector of Ras/MAPK pathway, in many cell types (198;280). The activated Jaks may stimulate the Ras pathway by phosphorylating tyrosine residues present on the adaptor proteins such as SHP-2 and Shc which recruit the GRB2 adaptor and stimulate the Ras/MAPK cascade (248;313;314). Jak/STAT signaling can also activate the Ras pathway indirectly through the transcriptional activation of SOCS-3. SOCS-3 binds RasGAP, a negative regulator of Ras signaling, and reduces its activity, thereby promoting the Ras pathway (315).

The functions of STAT-6 is regulated by both tyrosine and serine kinase activities. Although, the role of tyrosine phosphorylation in STAT-6-regulated gene expression is clear, there is evidence to suggest that serine phosphorylation of STATs also regulates their transcriptional activities (251). The phosphoserine sites are localized within proline-rich motifs within the STATs transactivating domains (312). MAPKs can modulate transcriptional activity of STATs by phosphorylating a serine residue at the c-terminus of most members of the STAT family (252;316). The serine727 MAPK

phosphorylational site, Pro-x-Ser-Pro, is positionally conserved in STATs 1, 3, 4, 5a and 5b. STAT-6, on the other hand, does not share this phosphoserine acceptor site with other STAT family members (252;317;318). However, STAT-6 was shown to be serine phosphorylated in response to IL-4 in murine splenic B cells and Ramos BL B cells (233;319). Recently, IL-4 was shown to induce STAT-6 serine but not threonine phosphorylation in primary human T cells (320). Based on phospho-amino acid analysis, this site was identified as serine 756 within the transactivation domain of STAT-6. Whether serine phosphorylation impinges on STAT-mediated gene transcription by influencing tyrosine phosphorylation is not clear. It is believed that a two step activation mode is likely for STAT-1 in which tyrosine phosphorylation by Jak-1 triggers dimerization, nuclear translocation and DNA binding, while a serine kinase regulates the transcriptional potential independent of tyrosine phosphorylation (321). Whether serine phosphorylation influences tyrosine phosphorylation of STAT-6 to affect IL-4-mediated gene transcription is not clear. My results suggest that ERK inhibitor PD98059 inhibited STAT-6 tyrosine phosphorylation, binding of STAT-6 to its binding site on the CD44 promoter and CD44 promoter activity and its transcription. These results clearly suggest a functional cross-talk between serine phosphorylation induced by ERK MAPKs and tyrosine phosphorylation induced by Jak/STAT-6 pathways in response to IL-4 (Fig. 4.13). Since p38 and JNK inhibitors did not influence STAT-6 tyrosine phosphorylation and CD44 transcription, it is likely that STAT-6 may specifically interact with ERK MAPKs. IL-4 also mediates its effects through PI3K, another prominent signaling pathway involving IRS-2, PI3K, and Akt signaling proteins (198;239;248;252;282;283).

Fig. 4.13: IL-4-induced CD44 expression is regulated by STAT-6 activated by Jak-1/3-activated ERK MAPK which is independent of PI3K/IRS-2 pathway.

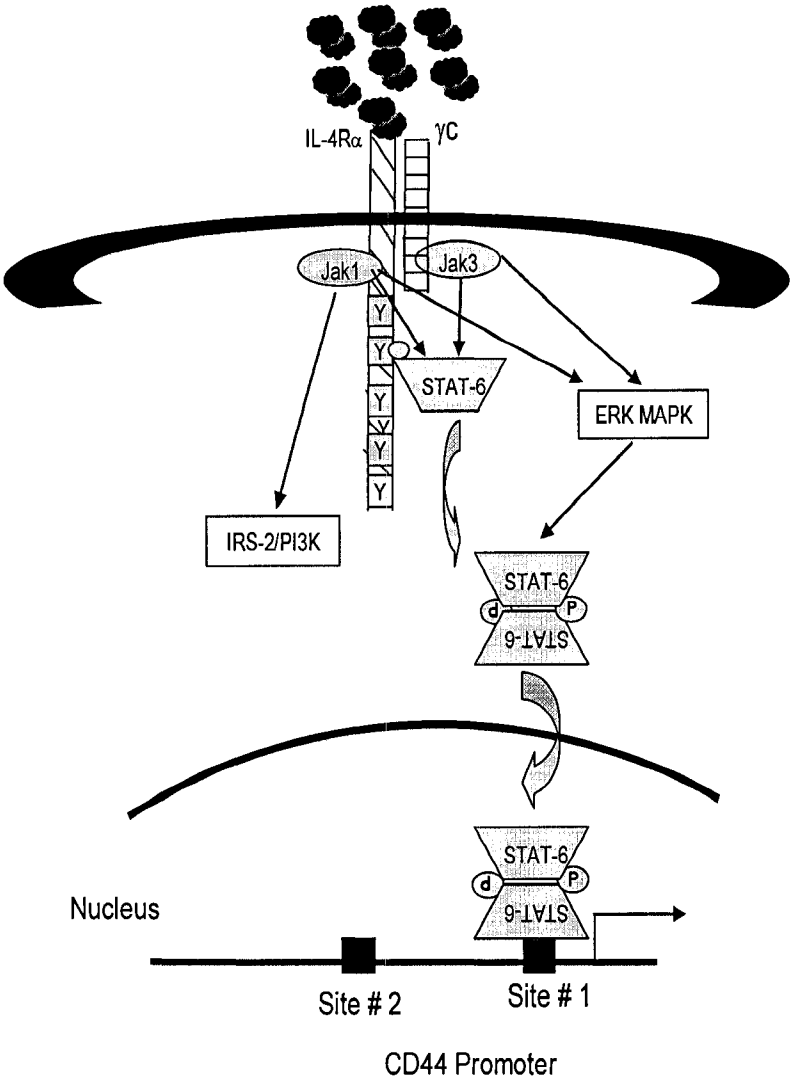

IL-4Rα
γC
Jak1
Jak3
Y
Y
Y
Y
Y
Y
STAT-6
ERK MAPK
IRS-2/PI3K
STAT-6
d
P
STAT-6
STAT-6
d
P
STAT-6
Nucleus
Site # 2
Site # 1
CD44 Promoter

Fig. 4.13

Interestingly, my results failed to implicate the PI3K pathway in IL-4-induced CD44 expression. IL-4 did activate IRS-2 and the Akt proteins in BL30/B95-8 cells. However, inhibition of the PI3K pathway by PI3K-specific inhibitor, LY294002 did not inhibit IL-4-induced CD44 expression in these cells.

In summary, the results of this study demonstrate for the first time the involvement of STAT-6 as a novel transcription factor regulating IL-4-induced CD44 expression in human B cells. Furthermore, IL-4-induced STAT-6 phosphorylation was affected by Jak-activated ERK MAPK pathway. Since, IL-4-induced expression of CD44 has been correlated with increased HA adhesion, inhibition of cell division and reduced tumorigenicity of colorectal carcinoma cells *in vivo* (322-324), this raises the possibility of therapeutic use for IL-4 in this disease. Therefore, STAT-6 and ERK MAPKs may represent important and novel targets for regulation of CD44 expression and CD44-mediated immune responses in B cell proliferation- and maturation-related disease conditions and cancer malignancies.

Chapter V
Concluding remarks, significance, and future direction

The interactions of cell surface receptor, CD44 with its principal ligand hyaluronan (HA) plays a critical role in leukocytes migration during the inflammatory responses as well as during tumorigenesis and metastasis. The expression of CD44 has been reported to be up-regulated in these conditions for CD44-mediated biological functions. To elucidate the molecular mechanism underlying the CD44 expression is an important step to develop the therapeutic strategies. Therefore, one of the most important question is that, what are the possible cellular signaling pathways that determine the regulation of CD44 expression. This constituted the central theme of my thesis research using human monocytic cells and Burkitt's lymphoma B cells as model systems.

LPS, a bacterial cell wall endotoxin, plays a major role in bacterial infections leading to the generation of an inflammatory response as well as sepsis. CD44 is believed to be an important cell surface receptor responsible for the migration of leukocytes to the site of inflammation. To understand the molecular mechanism underlying the regulation of CD44 expression in human monocytic cells, which constituted the first and second objectives of my research proposal, I used one promonocytic cell line, THP-1/CD14 cells and demonstrated that in response to LPS and TNF-α, CD44 expression is induced. In normal conditions, the LPS-induced CD44 expression was mediated by the endogenously produced IL-10 and TNF-α. Because of the inherent difficulties in using normal monocytes as a model system and because, endogenously produced TNF-α and IL-10 can upregulate CD44 expression in an autoregulatory manner, I used the promonocytic THP-1/CD14 cell line as a model system. Further, the THP-1/CD14 cells responded similarly to THP-1 cells, and normal human monocytes with respect to LPS- and TNF-α-induced CD44 expression.

The previous results from our laboratory revealed a distinct role for the JNK MAPK activation in the LPS-induced but not in TNF-α-induced CD44 expression. In this study, my results for the first time suggest a distinct role for the Ca^{2+} signaling pathway, in particular, calmodulin (CaM)/CaM-dependent protein kinase-II (CaMK-II) activation in the regulation of TNF-α, but not LPS-induced CD44 expression. However, the previous study in our laboratory did provide evidence for a partial role of p38 MAPK in TNF-α-induced CD44 expression. They showed that lower concentrations of p38 MAPK inhibitors did partially inhibit TNF-α-induced CD44 expression, but this inhibition was never complete even when high concentrations of inhibitors were employed. I did not examine any interaction between p38 MAPK and calcium signaling pathway in this study. Therefore, the role for p38 in the induction of CD44 expression by TNF-α in relation to Ca^{2+} signaling pathway is not clear at present. However, it may be presumed that p38 MAPK pathway may play a partial role in upstream of Ca^{2+} signaling pathway affecting TNF-α-induced CD44 expression, because the Ca^{2+} signaling inhibitors in higher dose completely inhibited TNF-α-induced CD44 expression.

To further understand the involvement of CaM/CaMK-II and JNK MAPK in the regulation of CD44 transcription, I analyzed the CD44 promoter to identify the potential transcription factors involved. My results suggest for the first time that TNF-α and LPS induce CD44 expression through two distinct and independent signaling cascades without any evidence of cross-talk between the two pathways. TNF-α regulates CD44 expression specifically by AP-1 through CaM/CaMK-II activation. In contrast, LPS regulates CD44 transcription selectively by Egr-1 through JNK activation.

However, both LPS and TNF-α activate JNK and CaM/CaMK-II in human monocytic cells. The findings of a distinct involvement of CaM/CaMK-II signaling molecules in TNF-α-induced but not in LPS-induced CD44 expression raised the question of how CaMK-II may serve to induce TNF-α-induced CD44 transcription. Similarly, it was interesting to understand the distinct involvement of JNK activation by LPS resulted in LPS-induced CD44 expression. My results also demonstrate that LPS-induced CD44 transcription is regulated specifically by Egr-1 through JNK activation, whereas TNF-α-induced CD44 expression was independently regulated by AP-1 through CaM/CaMK-II activation without any evidence of cross-talk between the two pathways. Although TNF-α induced the activation of Egr-1, it did not regulate CD44 transcription. Furthermore, the JNK inhibitor, SP600125, did not affect the binding of TNF-α-induced Egr-1 to its binding site in the CD44 promoter. Similarly, LPS induced the activation of AP-1, activation of these proteins, however, did not affect CD44 transcription. Furthermore, the binding of LPS-induced AP-1 to its binding site in the CD44 promoter was not affected by inhibitors of the CaM/CaMK-II pathway. Understanding the role of transcription factor corepressors, the status of CD44 promoter methylation, and chromatin remodeling may provide the potential mechanisms controlling LPS-/TNF-α-mediated CD44 transcription. Our lab is investigating on the effect of methylation of CD44 promoter in above processes. Other possibilities should also be considered.

In this study, I also demonstrate that phosphoinositide 3-kinase (PI3K) constitutes a key downstream component of both the signaling pathways involved in the regulation of LPS- and TNF-α-induced CD44 expression in human monocytic cells. In these experiments, I used human monocytic cell line, THP-1/CD14 cells as well as the primary

human monocytes. My results suggest that the JNK-activated PI3K regulates LPS-induced CD44 expression through the activation of Egr-1 whereas TNF-α induces CD44 expression by a distinct CaMK-II-activated PI3K through the activation of AP-1. Taken together, my results suggest a critical involvement of PI3K in the regulation of LPS- and TNF-α-induced CD44 expression and hence may represent a potential therapeutic target for inhibiting CD44 expression and consequent CD44-mediated cell migration, inflammation and autoimmune disorders.

Further, my results, by using p85α-specific siRNA, reveal that the regulatory subunit of PI3K, p85α is involved in LPS- and TNF-α-induced CD44 expression in human monocytic cells. In further confirmation, the knock-out animal model may be best for experiments. I also demonstrated that the catalytic subunit, p110α isoform of PI3K is not involved in LPS- and TNF-α-induced CD44 expression by using p110α-knock-out THP-1 cells. Further, in this study, I did not examine the downstream molecules from PI3K to activation of AP-1 in case of TNF-α and from PI3K to activation of Egr-1 in case of LPS stimulation, which may be interesting to find out for better understanding of signaling mechanisms.

In addition to the regulation of CD44 expression in human monocytic cells, I investigated the regulation of IL-4-induced CD44 expression in an Epstein-Barr virus (EBV)-transformed Burkitt's lymphoma cell line, BL30/B95-8 cells. The results suggested that IL-4 did not induce the expression of Egr-1 and AP-1 in these cells. However, IL-4 activates STAT-6 which plays a critical role in IL-4-induced CD44 regulation. I also demonstrated that IL-4-induced STAT-6 is activated by Jak-1/3-activated ERK MAPK and is independent of the IRS-2/PI3K pathway.

IL-4 has been shown to enhance the survival and development of Burkitt's lymphoma (BL) cells. Therefore, STAT-6 and ERK MAPKs may represent an important and novel target for regulation of CD44 expression and CD44-mediated immune responses in B cell proliferation- and maturation-related disease conditions and cancer malignancies. This study also for the first time shows that MAPKs are activated in a human B cell line in IL-4 treatment. It is presumed that IL-4-induced activation of MAPKs in these cells and their implication in upregulation of CD44 expression may have significance in tumor progression in EBV-transformed BL cells. I did not examine the activation of MAPKs in IL-4-activated EBV-negative BL cells. This may provide evidence of a role for EBV infection in ERK MAPK activation and CD44 expression for cancer progression.

It will also be interesting to further confirm the role of above signaling molecules involved in CD44 expression implicated in cell migration and cancer progression by using knock-out animal model.

My studies designed to understand the signaling pathways underlying LPS- and TNF-α-induced CD44 expression in effect have opened a number of avenues and directions that may provide important new insights into the role of CD44 *in vivo*. The delineation of the biochemical signaling pathways regulating CD44 induction by employing JNK knock-out and specific PI3K isoform knock-out animal models, may suggest novel strategies and provide novel targets for therapeutic modulation of CD44. Although anti-CD44 antibodies that specifically target the HA-binding site and inhibit HA-binding have been successfully used in experimental animal models to treat a number of autoimmune and inflammatory disorders, strategies designed to develop novel small

molecule inhibitors based on these studies could provide a more feasible approach for the long term treatment of chronic inflammatory conditions. It will also be interesting to further confirm the role of above signaling molecules involved in CD44-HA binding implicated in cell migration, inflammation and autoimmune diseases. This could be potentially determined by using knock-out animal models. These observations could be applied to animal models of autoimmune disease, such as rheumatoid arthritis, in which CD44-HA interactions have been previously implicated.

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PUBLICATIONS:

- **Mishra J.P.**, Mishra S., and Kumar A. (2008) A key role for phosphoinositide 3-kinase in the regulation of LPS- and TNF-α-induced CD44 expression in human monocytic cells. (Manuscript in preparation)
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Research Papers in Conferences:

- **Mishra J.P.**, Mishra S, and Kumar A. (2007) Phosphoinositide 3-kinase plays a critical role in LPS- and TNF-α-induced CD44 expression in human monocytic cells. 95th AAI Annual Meeting in conjuction with Experimental Biology 2008, April 5-9, 2008, San Diego, California. (Submitted)
- Rahim R.A.A., Gajanayaka N., **Mishra J.P.** and Kumar A. (2007) PI3 kinase and p38 MAPK differentially regulate expression of IL-12 family cytokines (IL-23 and IL-27) in LPS-stimulated human monocytic cells. 50th Anniversary of the Discovery of Interferons, International Society for Interferon and Cytokine Research (ISICR) Annual Meeting, Oxford, England, Sept. 16-19.
- **Mishra J.P.**, Mishra S, and Kumar A. (2007) Phosphoinositide 3-kinase plays a critical role in LPS- and TNF-α-induced CD44 expression in human monocytic cells. 2nd Annual Conference, Canadian Society for Life Science Research (CSLSR), McGill University, Montreal, Quebec, Canada, July 13-14. (In poster Presentation)

- **Mishra J.P.**, Mishra S, and Kumar A. (2007) STAT-6, as a novel regulator of CD44 expression in human B cells. 94th Annual Meeting of the American Association of Immunologist, Miami, US, May 18-22. (In Oral Presentation)
- Mishra S, **Mishra J.P.**, and Kumar A. (2006) A critical role for anti-apoptotic c-IAP2 gene in LPS and TNF-α-induced resistance to HIV-Vpr-mediated apoptosis in human monocytic cells. 2006 AIDS conference, Toronto.
- Mishra S, **Mishra J.P.**, and Kumar A. (2006) A JNK-dependent pathway is required for HIV-Vpr induced apoptosis in human monocytic cells: Involvement of antiapoptotic Bcl2 and c-IAP1 genes. Experimental Biology Annual Meeting, Washington DC, US, April 28-May 2.
- **Mishra J.P.**, Vaithiyanathan S., Mishra S., Prasad R., Mishra A.K., and Kumar R. (2001) Effect of transinoculation of subabul (Leucaena leucocephala) leaves fed goat rumen liquor into sheep rumen on haematobiochemical parameters in sheep. VIII Annual Conference of IAAVR and National Symposium on Animal Health and Production in New Millenium and Indian Veterinary Congress, Punjab Agricultural University, Ludhiana, Punjab, India, Feb. 22-23.
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- Kumar R., **Mishra J.P.**, Sheikh Q., and Vaithiyanathan S. (1998) Growth pattern of condensed tannin degrading bacterium isolated from the rumen of sheep. 3rd Tannin Conference, Bend, Oregon, USA, July 20-25.