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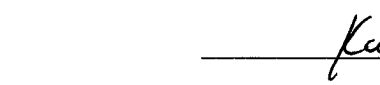 — (AUTEUR) SIGNATURE (AUTHOR)

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THE MOLECULAR MECHANISM FOR THE REGULATION OF CD44 EXPRESSION AND GENERATION OF HYALURONAN-ADHESIVE CD44 IN HUMAN MONOCYTIC CELLS AND HUMAN B CELLS

by

Katrina Gee

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

Adhesion molecules are vital in cell-cell and cell-extracellular matrix interactions. The regulation of adhesion molecule expression and activity plays a critical role in the physiology of many immunological events under both physiological and pathophysiological conditions. Among the many adhesion proteins, the CD44 family of cell adhesion glycoproteins, is an important mediator in events such as tumorigenesis and metastasis, cell migration, angiogenesis, wound healing, and inflammatory responses. The regulation of CD44 expression as well as activation of binding to its ligand, hyaluronan (HA), are tightly regulated phenomena. The molecular mechanisms and cell signaling events behind the regulation of CD44 expression and HA binding events are not well understood. In this study, I examined the regulation of CD44 expression and HA binding in two models systems: human monocytic cells and human B cells.

LPS, a bacterial cell wall component, regulates CD44 expression and may modulate CD44-mediated biological effects in monocytic cells during inflammation and immune responses. In this study, I show that in normal human monocytes, LPS and LPS-induced cytokines IL-10 and TNF-α enhance CD44 expression. To delineate the mechanism underlying LPS-induced CD44 expression, I investigated the role of mitogen-activated protein kinases (MAPK), p38, p42/44 extracellular signal-regulated kinase (ERK), and c-Jun N-terminal kinase (JNK) by employing specific inhibitors. I demonstrate the partial involvement of p38 MAPK in TNF-α-induced CD44 expression in both monocytes and the promonocytic THP-1 cell line. However, neither p38 nor p42/44 MAPKs were involved in IL-10-induced CD44 expression in monocytes. To further dissect the TNF-α and LPS-induced signaling pathways regulating CD44 expression independent of IL-10-mediated effects, I utilized IL-10-refractory THP-1 cells as a model system. Herein, I showed that CD44 expression induced by the LPS-mediated pathway predominantly involved JNK activation. This conclusion was based on results derived by transfection of THP-1 cells with a dominant-negative mutant of stress-activated protein/Erk kinase 1 (SEK1), and by exposure of cells to JNK inhibitors dexamethasone and SP600125. All these treatments prevented CD44 induction in LPS-stimulated, but not in TNF-α-stimulated, THP-1 cells. Furthermore, I showed that CD44 induction may involve JNK-dependent Egr-1 activation in LPS-stimulated monocytic cells. Taken together, these results suggested a predominant role of JNK in LPS-induced CD44 expression in monocytic cells.

Interaction of CD44 with HA in monocytic cells also plays a critical role in cell migration, inflammation, and immune responses. Most cell types express CD44 but do not bind HA and the biological functions of CD44 have been attributed to the generation of the functionally active, HA-adhesive form of this molecule. Although LPS and cytokines induce HA-adhesive CD44, the molecular mechanism underlying this process remains unknown. In this study, I showed that LPS-induced CD44-mediated HA (CD44-HA) binding in monocytes is regulated by endogenously produced TNF-α and IL-10. Furthermore, p38 activation was required for LPS- and TNF-α-induced, but not IL-10-induced, CD44-HA-binding in normal monocytes. To dissect the signaling pathways regulating CD44-HA-binding independently of cross-regulatory IL-10-mediated effects, again the IL-10-refractory promonocytic THP-1 cells were employed. LPS-induced CD44-HA-binding in THP-1 cells was regulated by endogenously produced TNF-α. My results also suggest that lysosomal sialidase activation may be required for the acquisition

of the HA-binding form of CD44 in LPS- and TNF-α-stimulated monocytic cells. Studies conducted to understand the role of MAPKs in the induction of sialidase activity revealed that LPS-induced sialidase activity was dependent on p42/44 MAPK-mediated TNF-α production. Blocking TNF-α production by PD98059, a p42/44 inhibitor, significantly reduced the LPS-induced sialidase activity and CD44-HA binding. Subsequently, TNF-α-mediated p38 MAPK activation induced sialidase activity and CD44-HA-binding. My results suggest that TNF-α-induced p38 MAPK activation may regulate the induction of functionally active HA-binding form of CD44 by activating sialidase in LPS-stimulated human monocytic cells.

IL-4 and IL-13, cytokines with similar biological effects may influence growth and progression of B cell tumors through regulation of cell surface molecules, such as CD44, are important in intercellular communications. In this study, I demonstrated that IL-4 and IL-13 exhibited differential effects on CD44 expression and binding to hyaluronan in BL30/B95-8, a Burkitt's lymphoma (BL), and MK3.31, an Epstein-Barr virus transformed normal human B cell line (B-LCL). Studies conducted to understand the molecular mechanisms underlying this differential effect show that IL-4 induced phosphorylation of JAK1, JAK3, and STAT6 in BL30/B95-8 cells and of JAK3 and STAT6 in MK 3.31 cells. In contrast, IL-13 failed to induce the phosphorylation of JAK kinases or STAT6 proteins in these cell lines. The inability of BL30/B95-8 cells to respond to IL-13 was attributed to the loss of expression of IL-13R subunits α1 and α2, a finding confirmed for a number of other BL cell lines examined.

Taken together, my results indicate the diversity in the regulation of CD44 expression and HA-binding ability and can be applied to further investigations leading to prevention of tumorigenesis as well as in the control of inflammation.

DEDICATION

I dedicate this thesis to my husband, Stephen, for his never-ending support and encouragement throughout the course of my studies.

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LIST OF ABBREVIATIONS

$[Ca^{2+}]_i$	Intracellular calcium ions
A	Adenosine
aa	Amino acid
Ab	Antibody
anti-IgM Abs	Antibodies directed against surface immunoglobulin M
BL	Burkitt's lymphoma
B-LCL	B-lymphoblastoid cell lines; normal human B cells transformed *in vitro* with EBV
bp	Base pair
cAMP	Cyclic adenosine monophosphate
cGMP	Cyclic guanosine monophosphate
CD44 V	CD44 isoforms containing variable exons
cIAP	Cellular inhibitor of apoptosis
cDNA	Complementary DNA
cGMP	Cyclic guanosine monophosphate
cpm	Counts per minute
CRG	Cytokine response gene
CS	Chondroitin sulfate
CTL	Cytotoxic T lymphocyte
dATP	Deoxyadenosine triphosphate
dCTP	Deoxycytidine triphosphate
dGTP	Deoxyguanosine triphosphate
dTTP	Deoxythymidine triphosphate
DD	Death domain
EBNA	Epstein-Barr nuclear antigen
EBV	Epstein-Barr virus
ECM	Extracellular matrix
Egr-1	Early growth factor response gene-1
ERK	Extracellular-related kinase
GAG	Glycosaminoglycan
GM-CSF	Granulocyte macrophage – colony stimulating factor
GTP	Guanosine triphosphate
HA	Hyaluronan
HB-EGF	Heparin-binding epidermal growth factor
HEV	High endothelial venule
HS	Heparin sulfate
I.V.	Intravenous
ICAM	Intercellular adhesion molecule
IFN	Interferon
Ig	Immunoglobulin
IGF-1	Insulin growth factor-1
IL	Interleukin
IL-2R	Interleukin-2 receptor
IL-4R	Interleukin-4 receptor

IL-13R	Interleukin-13 receptor
JAK	Janus associated kinase
JAB	JAK binding protein
JNK	c-jun N-terminal kinase
Kb	Kilobase
LECAM	Lymphocyte endothelial cell adhesion molecule
LFA	Lymphocyte function-associated antigen
LMP	Latent membrane protein
LPS	Lipopolysaccharide
mAb	Monoclonal antibody
MHC	Major histocompatibility complex
MAPK	mitogen-assiociated protein kinase
MAPKK	MAPK kinase
MAPKKK	MAPKK kinase
MEK	MAP kinase/ERK kinase
MKP	MAPK phosphatase
mRNA	Messenger RNA
NF-κB	Nuclear factor-κB
NHL	Non-Hodgkin's lymphoma
NK	Natural killer
nt	Nucleotides
NOD	Non-obese diabetic mouse
PAC1	Phosphatase of activated cells 1
PBMC	Peripheral blood mononuclear cells
PDGF	Platelet-derived growth factor
PHA	Phytohemagglutinin
PK	Protein kinase
PMA	Phorbol 12-myristate 13-acetate
PP	Peyer's patches
PP2C	Protein phosphatase 2 C
PTK	Protein tyrosine kinase
PWM	Pokeweed mitogen
RA	Rheumatoid arthritis
RANTES	Regulated on activation, normal T cell expressed and secreted
RHAMM	Receptor for hyaluronan acid mediated motility
RT-PCR	Reverse transcription-polymerase chain reaction
SAC	*Staphylococcus aureus* Cowan strain I
SH2	Src-homology 2 domain
SHP-1	SH2-containing tyrosine phosphatase-1
SEK	stress activated protein/ERK kinase
SCID	Severe combined immunodeficiency
SOCS	Suppressor of cytokine signaling
SODD	Silencer of death domain
sIg	Surface immunoglobulin
T	Thymidine
TCR	T cell receptor

TGF	Transforming growth factor
T_h	T helper
TNF-α	Tumor necrosis factor-α
TRADD	TNF receptor-associated domain
TRAF	TNF-associated factor-2
VCAM	Vascular cell adhesion molecule

CHAPTER 1:

INTRODUCTION

Background

The CD44 proteins are a family of cell surface membrane glycoproteins that are capable of binding to hyaluronan (HA), a major component of the extracellular matrix (ECM). Engagement of CD44 on activated immune cells with HA is a critical event in many physiological and pathophysiological events such as: tumorigenesis and metastasis, cell migration, angiogenesis, wound healing, and inflammatory responses (1-4). The multiple functions of CD44 have been attributed to the existence of numerous CD44 isoforms that are generated by alternative mRNA splicing, as well as by extensive post-translational modifications such as N- and O-linked glycosylation and glycosaminoglycan addition. The family of CD44 molecules is widely expressed by a diverse array of cell types including leukocytes, fibroblasts, epithelial cells, and keratinocytes. The ability of CD44 to bind HA is a tightly regulated process (2,5-9). Although most cells express some form of CD44, not all cells constitutively bind HA (6). Acquisition of the HA-binding ability of CD44 thus plays a vital role in determining CD44-mediated biological effects. 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, (2,9-11) alterations in the N- and O-linked glycosylation as well as sialidation and sulfation patterns of CD44. (10-13)

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. 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 behind the generation of immune responses leading to successful clearance of antigen and to resolution of inflammatory responses. **The main focus of this study is to examine the signaling pathways induced by cytokines and mitogens that are crucial to the regulation of CD44 expression and activation using normal human monocytes and B cells as model systems.**

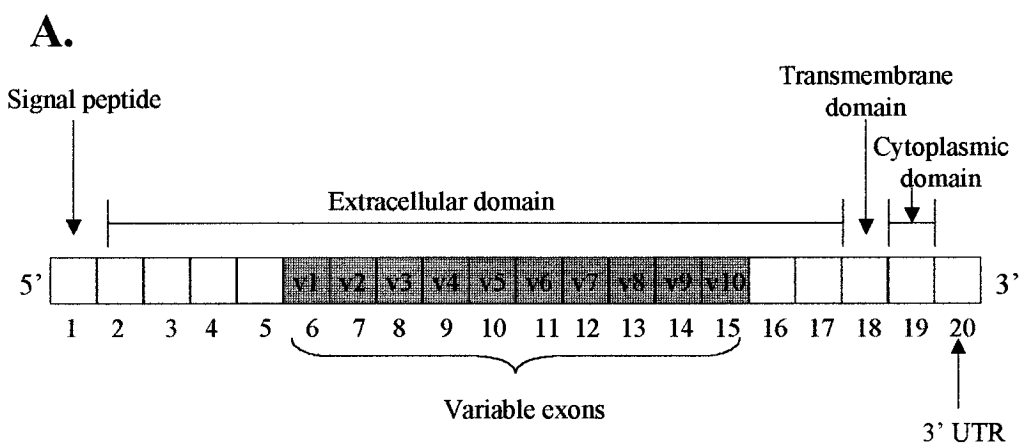
Structure of CD44:

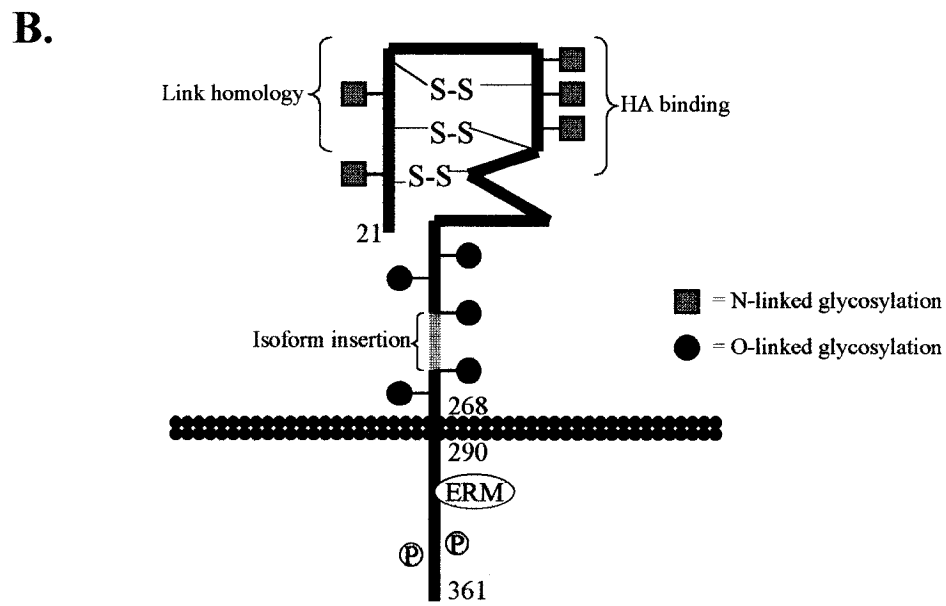
Genomic:

Located on human chromosome 11 (14) and murine chromosome 2 (15), the single CD44 gene spans 50 kb of human DNA and contains 20 exons. As illustrated in Fig. 1-1, 20 exons are encoded by the CD44 DNA, 12 of them are alternatively transcribed (16-18). These include exons 6-15, also called variable exons 1-10, as well as exons 19 and 20. The first 5 exons of the gene encode the constant region of CD44 and therefore are always transcribed. 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 (2). Inclusion of exon 19 results in expression of a short cytoplasmic domain of CD44, whereas exon 20 encodes a longer cytoplasmic tail of CD44 (2).

Fig. 1-1: CD44 structure and genomic organization.

A. Genomic organization of CD44. Exons are represented by the numbered boxes. The 10 variable exons (1-10v) are as indicated by the blue shading. The three protein domains of CD44 are as indicated. B. Structure of the CD44 protein. The CD44 protein exists as a transmembrane glycoprotein. The link homology and hyaluronan (HA) binding domains are indicated by the brackets. Sites of either N- or O-linked glycosylation are represented by the hollow squares or circles respectively. The site of alternative isoform insertion is represented by the blue area. Amino acids 268 and 290 flank the transmembrane domain as indicated. The two serines with the potential for phosphorylation are 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".


A.
Signal peptide
Transmembrane domain
Cytoplasmic domain
Extracellular domain
5'
v1 v2 v3 v4 v5 v6 v7 v8 v9 v10
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

CD44 Isoforms:

Hundreds of possible combinations of variable exons exist and 20 different CD44 isoforms have been identified at the cDNA level (2). Of these, CD44H, CD44E, and variants containing variable exons V4-7 and V6-7 are the best characterized. CD44H is the smallest, and is the standard, or hematopoietic (H) isoform and does not contain any of the variable exons (2). 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 (19). CD44 V4-7 and V6-7 are metastasis-associated variants; overexpression of these isoforms in non-metastatic rat pancreatic carcinoma and mammary carcinoma confers metastatic behavior (20).

Protein.

CD44 exists as a type I membrane protein and can be divided into 3 domains: the extracellular domain, and the highly conserved transmembrane and cytoplasmic domains (Fig. 1-1) (1,2). The extracellular domain is a globular structure generated by disulfide bridging through six conserved cytosine residues and the amino-terminal end of this domain is well conserved (85%) (21,22). This globular structure also contains a region termed the link homology domain as it shares 30% homology with the cartilage link protein (19). This domain is found on all CD44 isoforms and is responsible for the ability of CD44 to bind HA (22-24). 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 (10,12,25-28). 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 (13,29,30).

The membrane proximal region, also called the stem structure, of the extracellular domain is not as well conserved as the N-terminal end and is where the alternatively spliced variants are expressed (31,32). 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. The HA-binding ability of CD44 is discussed in depth in Chapter 1, page 8.

The transmembrane domain consists of 23 aa 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 (1,33). It has also been suggested that this region may also be responsible for lipid raft association (34,35).

The highly conserved cytoplasmic tail of CD44 contains docking sites for several protein kinases that become activated upon CD44 interaction with HA. Phosphorylation sites for protein kinase (PK) C have been identified (2,36,37) and it has been demonstrated that CD44 is associated with at least two tyrosine kinases, $p185^{HER2}$ and the c-src kinase (38-40). 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. Binding of HA to CD44 triggers the activation of these kinases. $p185^{HER2}$ kinase activity is associated with increased tumor cell growth while activation of c-src leads to formation of lipid rafts (36,39-41). In addition to the tyrosine kinases, CD44 has also been shown to be associated with the Rho-like GTPase RhoA (38,42,43). 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 (41,44). The interaction between CD44 and ankyrin has been shown to affect CD44-HA binding and may be important for correct membrane display of CD44 such that it can bind to HA, as well as for HA-induced signaling to occur (34,45-47). Another GTPase, Rac1 has also been shown to be activated upon HA binding to CD44 (44,48). 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 (1,2,37,48,49). 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 (49).

CD44 and HA Binding

As mentioned previously, the glycosaminoglycan hyaluronan is the principle ligand for CD44 (50-52). It is a high molecular weight, negatively charged, protein polysaccharide composed of linear repeating units of disaccharide D-glucuronic acid (1-β-3) N-acetyl-D-glucosamine (2,53). 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 (2,52,53). Other CD44 ligands include fibronectin (54,55), collagen (54,56,57), and serglycin (58,59); however, interactions between CD44 and HA are the best characterized. For initiation of CD44-mediated HA binding, CD44 must first be "activated" (22,24,50,60).

Treatment of cells with various agents results in changes in CD44 expression levels, 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: those that are active (ie capable of HA binding), those that are inducible (ie can be induced to bind HA), and those that are inactive (ie those that do not bind HA) (10).

Several properties have been shown to influence CD44-HA binding. Among them 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) (24). However few studies have delineated the exact molecular mechanism by which CD44 is induced to bind HA.

Post-translational modifications of CD44 have been shown to play an important role in the regulation of ligand binding. Increases in the complexity of the glycosylation state of CD44 is associated with a decrease in CD44-HA binding capacity; glycosylation of CD44 has been shown to negatively affect CD44-HA binding (25,28). As previously stated, both N- and O-linked glycosylation sites are present in the extracellular domain; modulations in these glycosylation sites have been shown to be responsible for the induction of HA-binding (10,12,25-28). 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 (61). TNF-α-induced CD44-HA binding in monocytic cells has also been shown to involve a decrease in N-linked glycosylation (62).

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. Sialidases are a family of exoglycosidases whose enzyme activity results in the removal of sialyl residues from glycosylated proteins (63,64). Sialidase activity has been very well studied in the context of influenza virus infections however, similar sialidases also exist in humans. In fact deficiencies in production of the lysosomal sialidase enzyme result in an autosomal recessive disease termed sialidosis as well as galactosialidosis where patients suffer from tissue accumulation and urinary excretion of sialylated oligosaccharides and glycolipids (63-65). Studies have demonstrated the involvement of sialidases in cell surface adhesion proteins and their recognition and binding capacity of their ligands. For example, in HL60 cells, desialidation of β1-integrin was shown to reveal the ligand binding site on the integrin (66). Recently in human monocytic cells, LPS-induced HA binding was shown to be regulated by an inducible sialidase; removal of sialic acid residues on the CD44 proteins enables CD44-HA binding (29,61).

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 (67). Treatment of SR91 cells with TNF-α was shown to induce CD44-HA binding via sulfation of CD44 (30,67). As well, 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 (68). However, the induction of T cell CD44-HA binding by CD3 stimulation was shown not to involve

sulfation, suggesting that the mechanism involved is dependent on not only the stimulus, but the cell-type as well (68).

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:

Perhaps the most central role for CD44 is in lymphocyte cell migration. Cell migration is the body's lifeline in terms of defense against infection and antigenic invasion. It is involved in not only normal physiologic circumstances but also in the generation of the inflammatory response as well as in the development of cancer, two conditions with which CD44 is intimately associated (69-78). CD44 has been shown to be involved in the migration of cells to sites of inflammation or during the cancerous process, and not during cell migration under normal circumstances (69). Therefore an outline of the role of CD44 in cell migration and consequentially inflammation and the cancerous process follows.

Cell Migration

Perhaps the most important facet of the immunological defense system is the exquisite mobility of lymphocytic cells during the clearance of infections or response to injury. Lymphocytes undergo continual recirculation and in doing so participate in lymphocyte-endothelial cell interactions leading to diapedesis and homing (76,79). The mature lymphocytes cycle from the blood, into secondary lymphoid structures (ie lymph

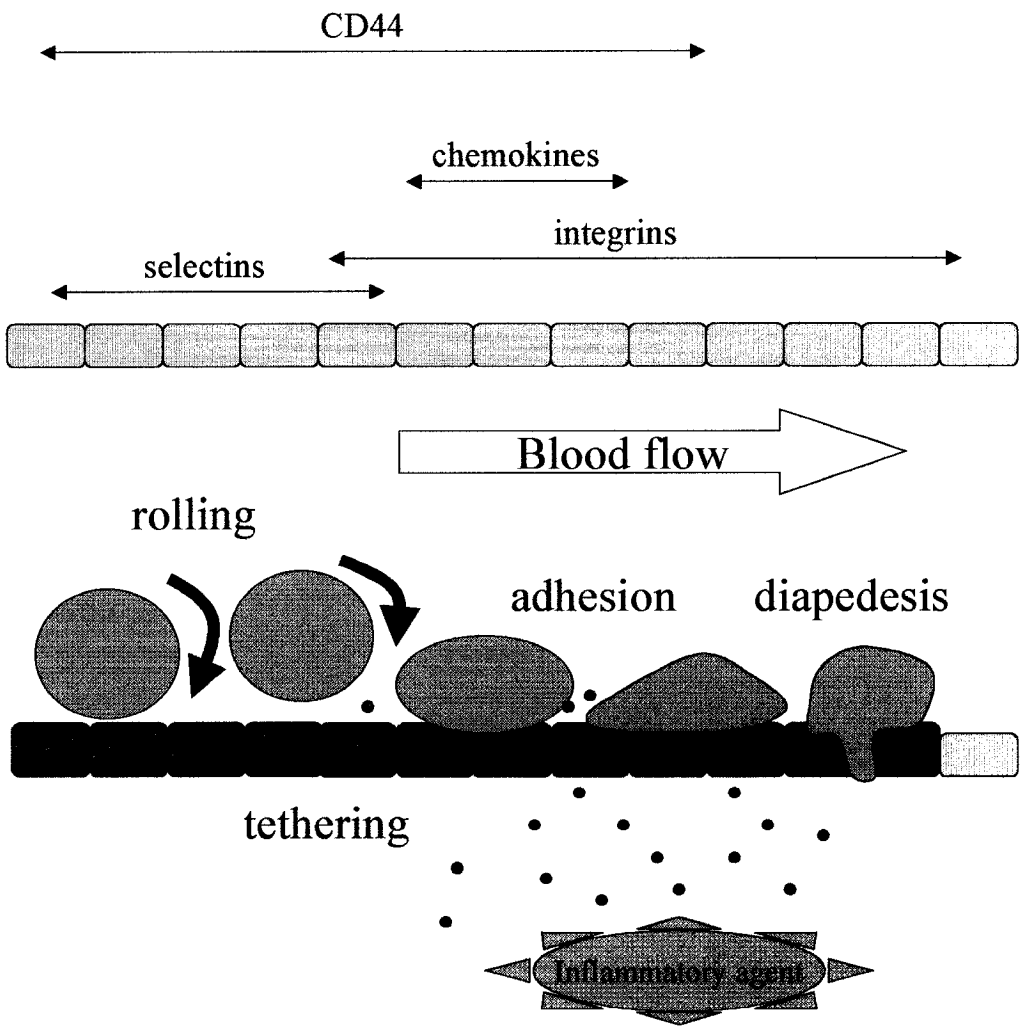
nodes, Peyer's patches, tonsils, and spleen) and back to the blood through the lymphatic system (80). A specialized structure composed of endothelial cells, termed the high endothelial venule (HEV) is found within lymphoid, but not in non-lymphoid, tissues and it allows the migration of lymphocytes into the lymphoid tissues (80). As well as undergoing continual recirculation under normal conditions and during an immune response, lymphocytes also migrate to extra-lymphoid areas (ie inflamed skin, intestinal mucosa, pulmonary tissues, and joints) in order to clear invading antigens (80). Adhesion molecules and receptors on both lymphocytes and endothelial cells as well as chemokine expression regulate this entire process (80-83). The selectivity involved in motility is directed by the expression of particular adhesion molecules on lymphocytes and their respective receptors on the endothelial cells (80-83).

General properties of lymphocyte-endothelial interactions

The interaction between lymphocytic cells and endothelial cells is a multistep process and is described in Fig. 1-2. There are 4 distinct groups of adhesion molecules that are involved in the different steps of cell migration: selectins, integrins, members of the immunoglobulin superfamilly, and CD44 (1,84-86). 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 the rolling of blood cells through postcapillary venules in lymphoid tissues via binding to specific carbohydrate groups. Two main selectins are L-selectin (expressed on lymphocytes) and

Fig. 1-2: Overview of the general leukocyte-endothelial interactions during cell migration at an inflammatory site (reproduced from (13)).

Chemoattractants and cytokines induced by the inflammatory agent activate the endothelial cells and thus affect receptor expression on the endothelial cell surface as well as on the passing lymphocytes. Activation of the cells induces the expression of the necessary adhesion receptors and ligands to initiate lymphocyte homing. The proteins responsible for the interactions of the leukocyte with the endothelial cells are depicted above the diagram. Initially cells "roll" along the activated endothelial vessel wall (indicated by ▬). The selectins are responsible for the initiation of rolling along the activated endothelial cell surface. Cells undergo firm adhesion mediated by the integrins followed by diapedesis towards the inflammatory agent. ● 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

E-selectin (expressed on endothelial cells). CD44 has been shown to be involved in B and T lymphocyte 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 (76,87,88).

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 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 (89,90). Binding of these integrins to the immunoglobulin superfamilly 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.

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 (76). The sequence of events combined with the receptor/ligand partners expressed on the leukocytes and endothelial cells dictates the destination of the cells (80). 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 (73,74,77). 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 (76). The role of CD44 and cell migration in terms of cancer and inflammation is discussed in detail below.

Cancer

Abnormally elevated CD44 expression has long been associated with tumorigenesis and metastatic potential (2,91-94). As the stroma surrounding tumor cells contains elevated levels of HA (95), 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 motility by forming a molecular bridge which could serve as scaffold for tumor growth (76).

The type of cancer and the ability of tumor cells to invade various tissues and subsequently undergo metastasis is thought to be associated with particular isoforms of CD44, however there does not appear to be a strict correlation between isoform expression and tumor progression (96). Early studies have demonstrated distinct roles for a number of CD44 isoforms in tumorigenesis. Gunthert et al showed that in a rat model CD44v6 confers metastatic potential to a non-metastasizing pancreatic andenocarcinoma cell line (20). Hart et al (1991) shows that a B16 mouse melanoma expressing CD44H are more aggressive than those not expressing CD44H (97). Additionally, Sy et al showed that 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 (98). Transfection of murine lymphoma cells with either CD44H or CD44 containing v4-v10 exons showed that the variant-containing CD44 isoforms exhibited enhanced cellular migration and tumor forming ability when compared to the parental cell lines or those transfected with CD44H (99). Recently animal experiments have been conducted that target CD44 expression and ligand binding by antibodies, antisense, or CD44-soluble proteins resulting in the reduction of malignant activities of various neoplasms (74,100). 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 (101,102) and skin cancers (103-105) and decreased CD44 expression has been associated with tumorigenic neuroblastoma cell lines (106). However, in cases of renal cancer as well as non-Hodgkin's lymphoma and Burkitt's lymphoma high levels of CD44 expression are associated with non-favorable outcomes (74,107-109). Because the second part (Chapter 4) of my thesis encompasses the regulation of CD44 in Burkitt's lymphoma (BL), a brief introduction of BL is included below.

Burkitt's lymphoma:

Chapter 4 of this study focuses on the effect of the regulation of CD44 expression and activation in Burkitt's lymphoma (BL) and in Epstein-Barr virus (EBV)-transformed lymphoblastiod B cell lines (B-LCL) (110,111). Burkitt's Lymphoma 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

(112). The translocation causes disregulation in cell cycle control, ultimately resulting in localized, rapidly growing tumors. Burkitt's lymphoma has been classified into two subgroups: endemic (African, EBV+) and non-endemic/sporadic (American, EBV-) (112). 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 factor where it can latently infect normal B cells resulting in immortalized, non-neoplastic lymphoblastiod B cell lines (B-LCLs) (113-115). 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 (109). In contrast, expression of CD44 appears to be lost in EBV-negative Burkitt's lymphoma cells (116), however upon infection of BL cells with EBV, expression of CD44 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 (111). 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 (117) and CD44H expressing Nalmalwa cells induced tumors in nude mice (98).

Inflammation and Autoimmunity

CD44 expression and ligand binding ability has been demonstrated in the regulation of lymphocyte recruitment to sites of inflammation (92,118-121). 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 (92,118). 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. 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 suggests a prominent role for CD44 in chronic inflammation.

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 (122). For example, activation of T cells in response to antigen causes an influx of immune regulatory cells to the antigenic site, resulting in inflammation (123). Therefore, the inflammatory response is characterized by the release of proinflammatory mediators resulting in aggregation of leukocytes, increased vascular permeability, and tissue damage (75). Usually the inflammation is resolved by the release of anti-inflammatory factors which clear the inflammatory cells (122). However, failure of the immune system to control inflammation often results in chronic inflammatory disorders, which are the hallmark of autoimmune diseases.

Autoimmune diseases can be characterized by high populations of activated lymphocytes bearing the activated, HA-binding form of CD44 (92,124). 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. 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 (125-127). A role for CD44 has also been demonstrated in the inflammation associated with experimental allergic encephalomyelitis (128). 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 (128). Additionally, in a murine model of autoimmune diabetes using non-obese diabetic (NOD) mice antibodies to CD44 conferred resistance to the onset of diabetes (129). 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 (see Box 1.1) 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. For example, 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 (130,131). 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 (130,131).

Box 1:1 CD44 KO mice:
CD44 knock-out mice tend to have relatively mild phenotypes. They show abnormalities in various functions such as:

1. myeloid progenitor migration
2. bone-marrow colonization
3. homing of lymphocytes to lymph nodes or thymus longer survival of activated T cells resulting in a resistance to hepatitis
4. impaired maintenance of lactation post-partum and accelerated uterine involution

CD44 has also been shown to be involved in the resolution of lung inflammation through the use of CD44-deficient mice (132,133). CD44-deficient mice succumbed to non-infectious, induced lung inflammation. However, it was possible to reverse the inflammatory response upon reconstitution of CD44 into the mice (132). Similarly, Wang et al observed that *E. coli*-induced pneumonia in CD44-deficient mice resulted in enhanced inflammation (133). These studies indicated that, in addition to the role played in the induction of inflammation in RA disease, CD44 might also play a role in the clearance of inflammation as demonstrated in the resolution of lung inflammation.

Regulation of CD44 Expression and Ligand Binding:

Cytokines and mitogens are important mediators of not only the inflammatory immune response, but also of tumorigenesis and metastasis. Because CD44 plays a role in the migration of immune cells and because CD44 expression and ligand binding is 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). It has been demonstrated that, depending on the stimulus and cell type, CD44 expression may be upregulated, but not its ligand binding ability (27). 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.

Cytokine/ mitogen regulation of CD44: Signaling proteins and transcription factors involved in the control of CD44 expression

The effect of cytokines on CD44 expression and binding ability has been investigated in different cell types. IL-5, a key mediator of asthma-related eosinophillic inflammation has been shown to enhance CD44 expression in human eosinophils (134). As well, IL-5 has been shown to induce CD44 binding in murine B cells (5,135). In human hematopoietic progenitor cells IL-3 and GM-CSF, cytokines which direct leukocyte development, induce CD44-HA binding (136). 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 (137). 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 (138). We have

previously demonstrated that PMA and IL-4 induce CD44 expression and HA-binding in a Burkitt's lymphoma cell line (110,111). In human monocytic cells, LPS, TNF-α, and IL-10 induce the expression of CD44 as well as HA-binding (26,30,62,139).

The role of signaling proteins responsible for the activation of the transcription factors critical to CD44 expression is not well understood. Transformation of cells using v-src or v-ras also has the effect of increasing CD44 expression. Laleda et al (2001) showed that the activation of the G protein Ral A plays a role in the induction of CD44 in these transformed cells (140). 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) (141). Along with upregulation of CD44 expression, variant isoform expression was also induced. This study also showed 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 (141).

Examination of the promoter region of CD44 reveals the presence of many putative transcription factor binding sites, however only a few have been shown to affect the expression of CD44. Of these sites, the consensus site for the early response gene (Egr-1) has been shown to be involved in CD44 expression following B cell receptor (BCR) stimulation in the murine B cell line WEHI-231 (142) and following IL-1 stimulation of the human endothelial cell line ECV304 (143). Lamb et al (1997) demonstrated that AP-1 activity is required for increased CD44 expression in Fos- and EGR-transformed cells (144). 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)) (145). 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 (146). In summary, it appears that AP-1 in addition to EGR-1, plays an important role in the induction of CD44 expression depending on the cell type and stimulation.

Signal Transduction

General Introduction

Phosphorylation has been shown to be required for the activation as well as transmission of signals (147). Signaling kinases and phosphatases are recruited to the receptors 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. Protein kinases are responsible for the covalent attachment of a phosphate to the side chain of either tyrosine, 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 (147). 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 (147). A general overview of the main intracellular signaling pathways are depicted in Fig. 1-3. The signal

transduction pathways responsible for modulating CD44 expression and ligand binding are not fully elucidated. As signaling pathways initiated by IL-4 and IL-13 as well as LPS, and TNF-α are the focus of this study, a brief explanation of these molecules and their signaling pathways is included below.

Signaling via IL-4 and IL-13: The JAK/STAT signaling pathway

IL-4 and IL-13 are 12-13 kDa pleiotropic cytokines sharing 30% homology at the amino acid level. IL-4 is produced by T helper type II (Th2) cells, basophills, and mast cells (148). 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 (148-150). IL-13 is also produced by activated Th2 cells and has overlapping but not identical biological functions when compared to IL-4 (148,151-154). IL-13 induces most of the same responses as IL-4 with the exception of IL-4-dependent induction of Th2 cells from Th0 cells (155). IL-13 has been suggested to play a distinct role in allergic inflammation (156).

The receptors for IL-4 and IL-13 are shown in Fig. 1-4A. The IL-4 receptor is thought to consist of two receptor chains: the IL-4Rα and the γ common chain (157). The γ common chain is shared by receptors for IL-2, IL-4, IL-7, IL-9, IL-15, and IL-21 (155,158-160). Two IL-13Rα chains have been identified; IL-13Rα1 was originally cloned from mice (161) whereas IL-13Rα2 was cloned from a human renal cell carcinoma cell line, Caki-1 and shares homology with the IL-5Rα chain (162). The IL-

Fig. 1-3: General Intracellular Signalling pathways

There exist many different intracellular signalling pathways in different cell types. This diagram illustrates some of the main signalling pathways and how they may be activated in lymphocytic cells. Signals transduced via the membrane receptor tyrosine kinase receptors can activate the phospholipase C (PLC)-mediated pathway that can result in the activation of protein kinase C (PKC) and calcium-induced (Ca^{2++}) signals. Signals transduced through membrane receptor tyrosine kinase receptors can also induce the activation of the phospho-inositol-3 kinase (PI3-k) pathway as well as induction of the MAPK cascade. Signalling through the MAPK cascade is discussed in more detail in Fig. 1-5. The main cytokine-induced signalling pathway is the JAK/STAT pathway (see Fig.1-4), but in addition to activation of that pathway, cytokines have also been shown to induce PI3-k- and MAPK-induced signals.

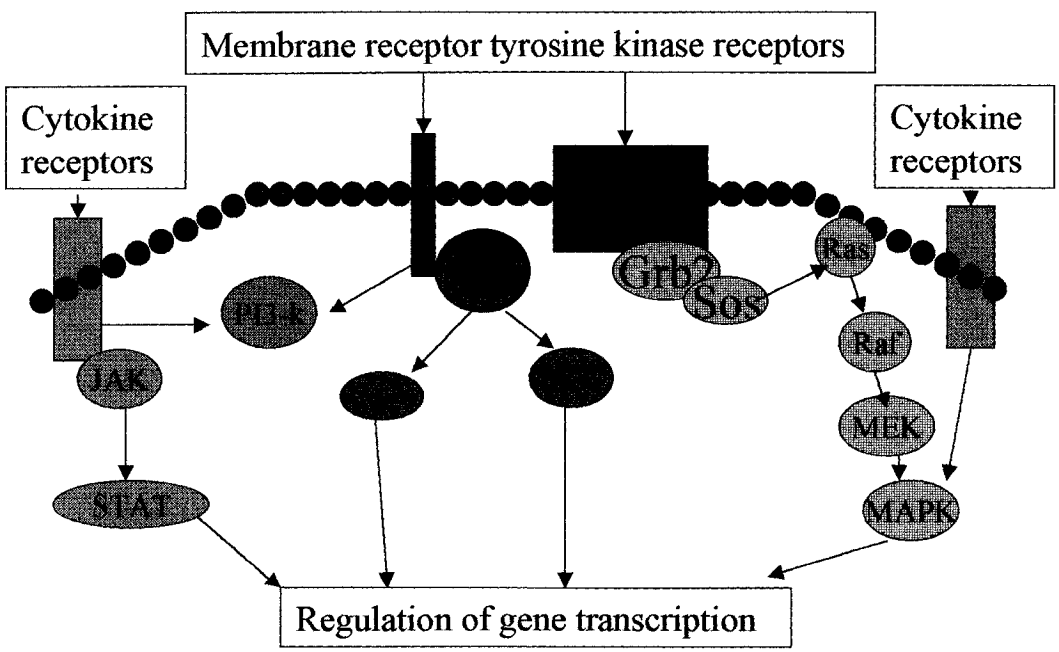
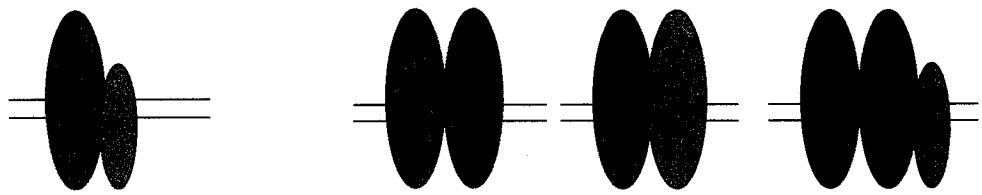

Membrane receptor tyrosine kinase receptors
Cytokine receptors
Cytokine receptors
PI3-k
Grb2
SOS
Ras
Raf
MEK
MAPK
JAK
STAT
Regulation of gene transcription

Fig.1-4: IL-4 and IL-13 receptors and the JAK/STAT pathway.

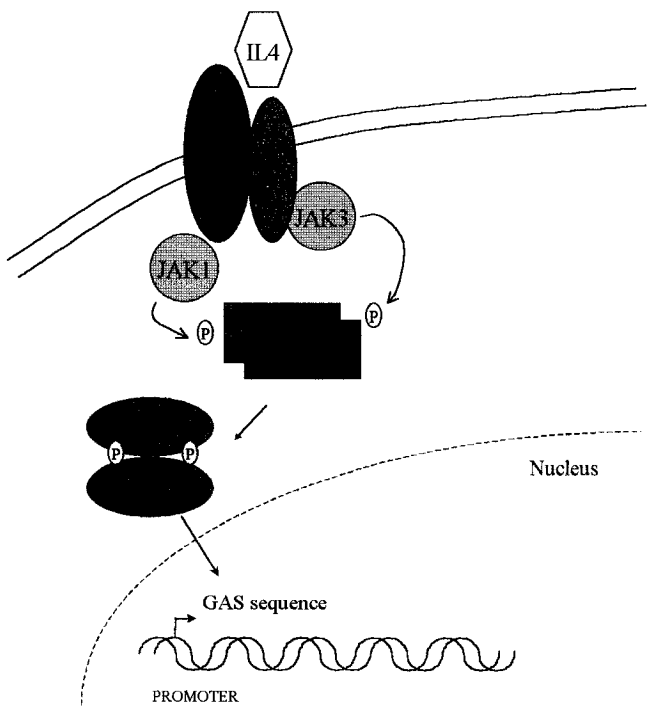
A. The IL-4R is composed of two chains, the IL-4Rα chain and the γ common chain. B. There is up to three different combinations for the IL-13R complex, all of which contain the IL-13 binding receptor chain, IL-13Rα1. The type I receptor is composed of the IL-4Rα and IL-13Rα chains. In this case, both IL-4 and IL-13 are capable of binding this receptor form. The Type II receptor is comprised of the IL-13Rα1 chain and the IL-13R2. The type III receptor may be composed of the Type I receptor chains as well as the γ common chain. B. IL-4, upon binding to the IL-4Rα receptor chain induces signals that radiate from the IL-4Rα chain as well as the γ common chain which is recruited upon IL-4 binding. Once the receptor chains are activated by IL-4, JAK1, which associates with the IL-4Rα chain, and JAK3, which associates with the γ common chain, are activated and function to phosphorylate the transcription factor STAT6, which dimerizes and translocates to the nucleus where is activates gene transcription via binding to the INF-γ associated sequence (GAS) found in STAT6-responsive genes.

A. IL-4R

B. IL-13R



C. IL-4-induced JAK/STAT signaling



13Rα2 chain has a short cytoplasmic tail, indicating that it is most likely a non-signalling entity. However, when expressed alone, it has a high binding affinity for IL-13 (163). The IL-13Rα1 has a low binding affinity for IL-13 on its own, but when complexed with the other receptor chains, IL-4Rα or IL-13Rα2, it acquires a high binding affinity for IL-13 (156). Therefore, there are three potential receptors for IL-13: the type I is composed of the IL-4Rα and IL-13Rα1, the type II is composed of IL-13Rα1 and IL-13Rα2, and the type III is composed of IL-4Rα, γc, and IL-13Rα1 (157,164-167). IL-4 is capable of binding to the IL-13R type 1 in addition to the IL-4R.

The IL-4R composed of the IL-4Rα and γc chains is expressed on all hematopoietic cells, including normal human monocytes, B, and T cells (167-169). 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 (169), suggesting a potential existence of the type I IL-13R on these cells. In fact, non-hematopoietic cells which lack the γc, have been shown to express the type 1 IL-13R as a functional IL-4R (167).

The IL-13R system appears to be selectively expressed, depending on the cell type. For example, no functional IL-13R has been found on the surface of human or mouse T cells (153,170). However, the IL-13Rα1 chain has been found to be expressed intracellularly in T cells, but the IL-13Rα2 has not been detected (170). Monocytes have been shown to express the IL-13Rα1 chain as have tonsillar B cells, naïve and memory B cells (171). Expression of the IL-13Rα2 chain appears to be variable, but has been shown to be expressed on non-hematopoietic cells, such as fibroblasts (157). The IL-

13Rα2 chain has also been found to be expressed on colon cancer and ovarian cancer cell lines (157,165).

The main signaling pathway induced in response to cytokines, including that for IL-4 and IL-13, is the Janus kinase/signal transducers and activators of transcription (JAK/STAT) pathway (172-176). This pathway involves activation of the JAK tyrosine kinases, of which there are four family members: JAK1, JAK2, JAK3, and Tyk2. 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. 1-4B), JAK1 associates with the IL-4Rα and JAK3 associates with the γc chain (174,177). Recently, JAK2 has been shown to associate with the IL-4Rα in monocytic cells (178). The JAKs are autophosphorylated upon ligand binding and then proceed to phosphorylate the STAT proteins. STAT proteins are a family of seven members (STAT1, 2, 3, 4, 5a, 5b, and 6) that serve as transcription factors. STAT6 is specifically activated by IL-4-induced JAK1 and JAK3 and has been shown to be activated by IL-13 (176). Generally, after activation, the STAT proteins homo or heterodimerize by association through src-homology (SH)-2 domains, translocate to the nucleus where they site-specifically bind promoter regions of cytokine-responsive genes (173,176). STAT6 homodimerizes and binds to a consensus sequence found in the promoters of IL-4-responsive genes such as CD23 and IL-4Rα. The IL-13 JAK/STAT signalling pathway is not as clearly defined as that for IL-4; there is evidence for STAT 6 activation, as well as for Tyk 2 phosphorylation in IL-13 stimulated B cells (179). In monocytic cells, IL-13

has been shown to induce the activation of JAK2 and Tyk2, as well as STAT1α, STAT3, STAT5a , STAT5b and STAT6 (178).

Downregulation of the JAK/STAT pathway after ligand activation is also important in the regulation of signalling. The protein phosphatase Src homology (SH)-2 containing phosphatase (SHP)-1 has been associated with the downregulation of IL-4 induced JAK/STAT activation (180,181). 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 signalling (SOCS) family of inhibitors that affect activation of specific JAK and STAT proteins (182).

MAP kinase signalling:

The mitogen-activated protein (MAP) kinases 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 rates of cell proliferation and differentiation, as well as cell motility and apoptosis. 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.

The MAP kinase (MAPK) signaling cascade is a three tiered system whereby the MAPK are activated by dual phosphorylation via the MAPK kinases (MAPKK) and the MAPKK are activated by the MAPKK kinases (MAPKKK) (183). Each of the MAPKs, MAPKKs, and MAPKKKs activate specific kinases as determined by distinct sequence motifs (183). In order for activation of the MAPK to occur, they must be phosphorylated

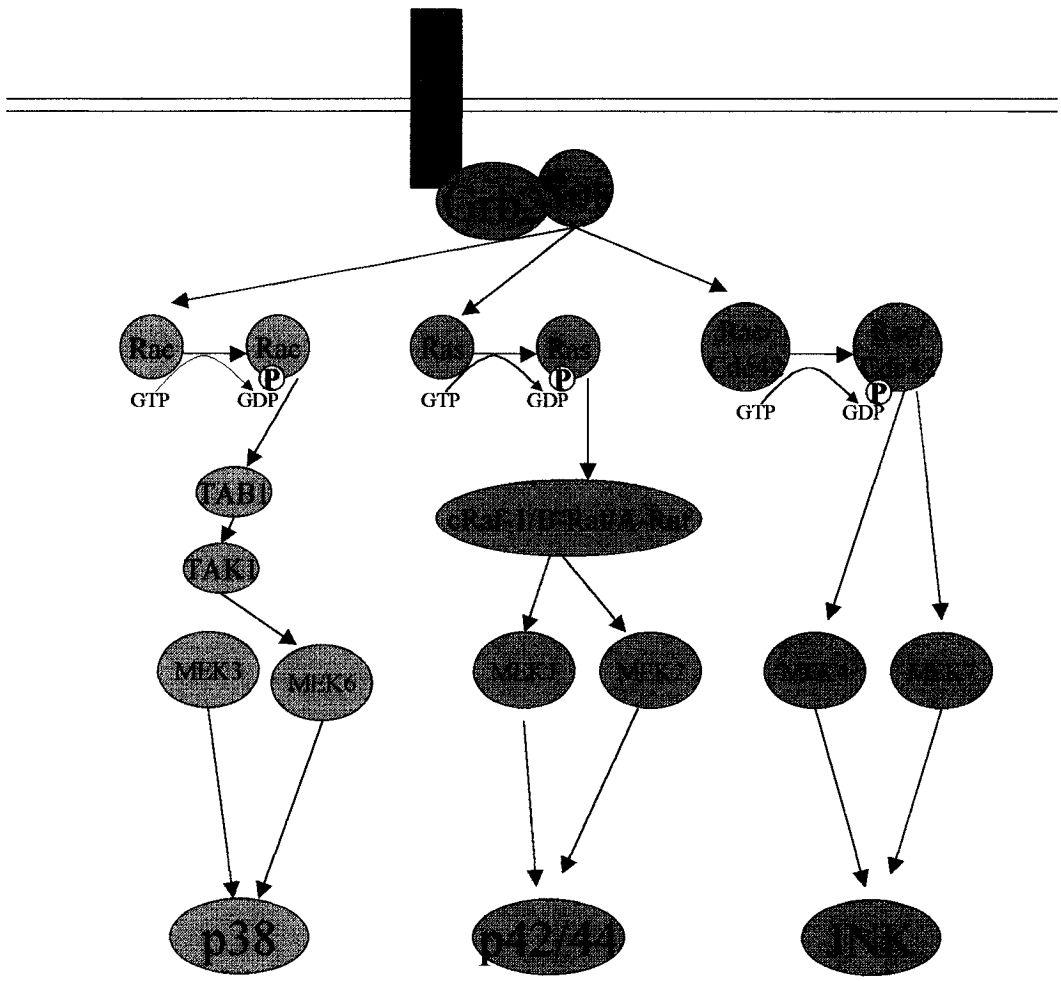
at the typical threonine in the conserved activation loop as well as at a neighboring tyrosine residue (147,184). The MAPKs are phosphorylated in the cytoplasm and translocated to the nucleus to affect activation of other kinases, transcription factors, or co-activators (185). A general outline of the 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 (183). 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-related kinase (ERK)1/2, c-jun-N-terminal kinase (JNK), and p38 based on sequence homology (147,183).

Of the three family members of MAPKs, ERK1/ERK2, also known as p42/44, are the best characterized. The ERK kinases are activated by several factors, for example, cytokines and growth factors (183). ERK2 is considered to be the prototypic member of the MAP cascade (147). 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 (183). 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

Fig.1-5: The MAP kinase cascade.

The MAPK signaling cascade can be initiated upon ligand binding to a receptor tyrosine kinase (RTK) receptor. Upon activation of the receptor the adaptor molecule Grb2 recruits Sos which in turn recruits small G-proteins such as Ras, Rac, and Cdc42 to the inner plasma membrane. The small G protein is phosphorylated and activated upon the exchange of GTP for GDP and subsequently activates a MAPKK (MEK1-7). The MEK activated is specific to the activated upstream small G protein. For example, in the p42/44 pathway, the small G protein Ras can activate the MAPKKKs cRaf-1, B-Raf, or A-Raf which then activate the MEK1 and 2 responsible for p42/44 activation. The small G proteins Rac and Cdc42 are responsible for inducing the activation of the MEK4 and MEK7 which activate the JNK MAPKs. In addition to the activation of the JNK signaling pathway, Rac can also activate the p38 MAPK pathway through the induction of interaction of the adaptor protein TAB1 with the TAK1 kinase which subsequently activates the MEK3/6 upstream of p38.


Rac
Rac
P
GTP
GDP
Ras
Rac
P
GTP
GDP
GTP
GDP
TAB1
TAK1
MEK3
MEK6
p38
p42/44
JNK

retinoblastoma protein leading to cell cycle progression (147). The family of JNK MAPKs includes JNK1, JNK2, and JNK3 (186). JNK1 and JNK2 were originally identified as the kinases responsible for the induction of AP-1 activity resulting from their phosphorylation of c-jun (187). 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 (187). 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 (187). There is a wide range of upstream kinases responsible for the activation of the JNK MAPKs, including MEKK's, apoptosis signal-regulating kinase 1 (ASK1), TGF-β-associated kinase (TAK-1), and the serine/threonine kinase Tpl-2 (187). The best characterized kinases known to activate the JNKs are MEK4 and MEK7 (183). These dual-specific kinases phosphorylate JNK on Thr 183 and Tyr185 (187). JNK has been shown to activate several transcription factors including activating transcription factor -2 (ATF-2), Elk-1, p53 and c-Myc (187).

The p38 MAPK family is comprised of p38α, β, γ, and δ. Of these, the p38α is the best characterized. Activation of the p38 MAPKs regulates the expression of a number of cytokines, particularly those involved in inflammation, such as IL-10 (183,188). The p38 MAPKs are activated by cytokines, hormones, stress, and G-protein-linked receptors (183). 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 (189).

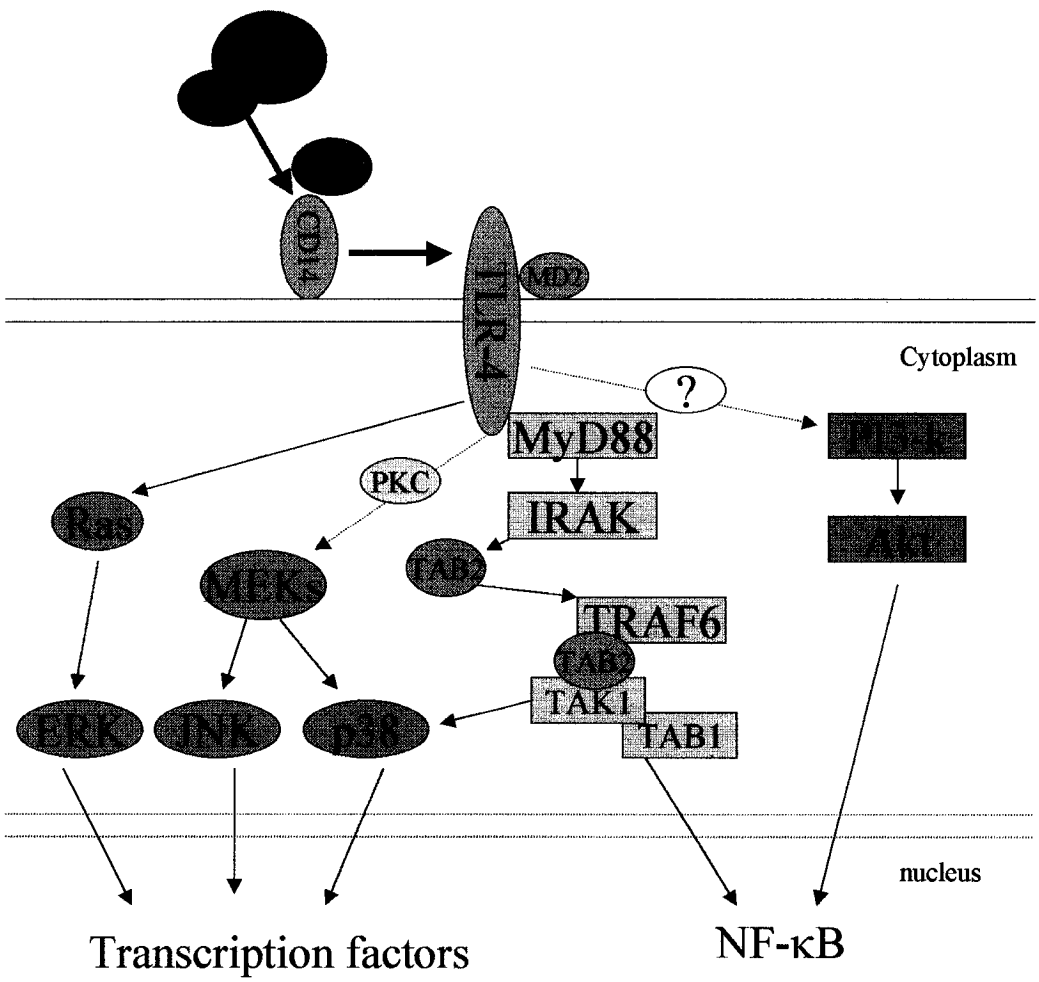
Once activated, the MAP kinases translocate to the nucleus where they can affect the activation of specific transcription factors. Down-regulation of the induced signaling cascade is as critical to cell function as is activation of this pathway. Specific protein phosphatases function to return the MAPK to inactive states. For example, the phosphatase of activated cells 1 (PAC1) specifically associates with and down-regulates the phosphorylation of p42 (190). 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 (184,191,192) 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 such that the control and specificity of the signals trasduced are affected.

Signaling via LPS

LPS, a component of gram-negative bacterial cell walls, is a potent mitogen for monocytic cells. 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 (193). The signal transduction pathway activated by LPS is depicted in Fig. 1-6. CD14 does not transmit signals, as it is a glycosylphosphatidylinsitol (GPI)-anchored protein with no transmembrane domain. The signals are transduced through the Toll-like receptor (TLR)-4 (see box 1-2) (193). The interaction with LPS and CD14 also results in the necessary recruitment of a secreted accessory protein MD2 which interacts with the

Fig.1-6: LPS mediated signals.

LPS stimulation activates several signaling pathways and transcription factors. LPS binds to the serum LPS binding protein (LBP) and is transferred to the CD14 at the cell surface. LPS then interacts with the signaling receptor Toll-like receptor (TLR)-4 and the accessory protein MD-2. The TLR-4-associated MyD88 interacts with IRAK, resulting in the hyperphosphorylation of IRAK. IRAK then interacts with the membrane associated TAB2 protein and transports it to a complex consisting of activated TRAF6 and TAB2 associatedTAK1 and TAB1 proteins. This signalosome can then induce the activation of the NF-kB transcription factor. LPS-induced signals can also induce the activation of the MAPK cascade either independently or dependently of MyD88 involvement. For example, the Ras-activated ERK signaling pathway may be activated by TLR-4-induced activation of Ras. Activation of the MEKs responsible for JNK and p38 activation may occur through activation of PKC. MyD88-dependent activation of the p38 pathway is known to occur via the activation of TAK1 as described for the MAPK signaling cascade in Fig. 1-5. PI3-kinase may also be activated via TLR-4 through unknown intermediate signaling kinases, as indicated by the encircled "?" .


CD14
TLR4
MD2
Cytoplasm
?
MyD88
PKC
IRAK
Ras
MEKs
TAB2
TRAF6
TAB2
TAK1
TAB1
ERK
JNK
p38
PI3-K
Akt
nucleus
Transcription factors
NF-κB

extracellular domain of the TLR4 (194,195). LPS interaction with CD14 promotes dimerization of TLR and subsequent recruitment of MyD88, a myeloid differentiation marker that functions as an adapter molecule (195,196). 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, IRAK (IL-1R-associated kinase) (196). 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 (197). This complex results in TAB2 binding to the adaptor protein TNF-receptor-associated factor-6 (TRAF-6) (197). TRAF-6 subsequently interacts with the TAB1, the activator for TAK-1 which leads to NF-κB activation (197). TRAF-6 also activates the members of the MAPK signalling cascade, p38, p42/44, and JNK (198-200). Activation of p38 and JNK MAPKs can result in the activation of NFκB (200,201). TRAF-6 activation is required for the induction of TLR4 signals as deletion of TRAF6 abrogates TLR4 signaling (197). 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 (193).

Box 1-2: Toll-like receptors:
Toll-like receptors (TLRs) are a family of cell surface reseptors that play an important role in protection afainst viral, bacterial, and protozoal infections. They exist as type 1 transmembrane proteins in animals and are found as cytoplasmic proteins in plants. Up to 9 TLRs have been identified in humans. 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.

TNF-α:

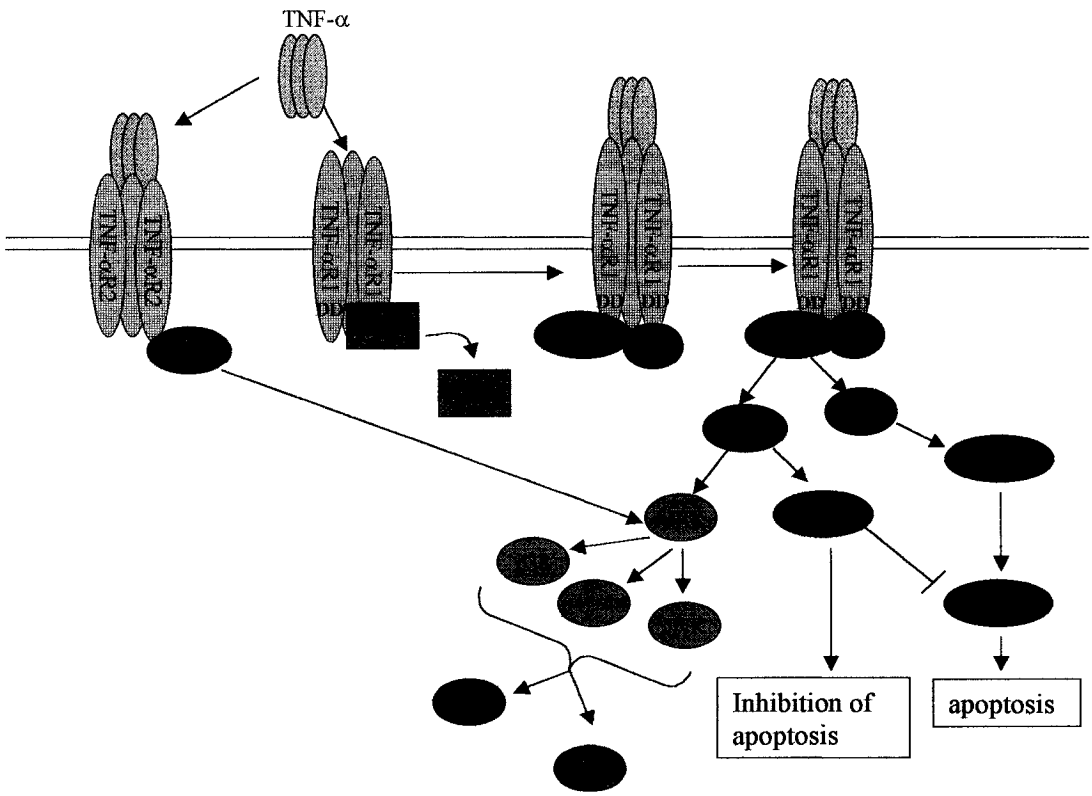
TNF-α is a proinflammatory cytokine that is produced by activated T cells, monocytes/macrophages, and dendritic cells. Besides its inflammatory properties, TNF-α

is also responsible for the induction of apoptotic and anti-apoptotic signals (202). An overview of TNF-α–mediated signaling is depicted in Fig. 1-7. 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 superfamilly which can be divided into two groups: those that contain a death domain, and those that do not (202). 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 prevention of apoptosis.

The two types of TNF-αR are called TNF-αR1 and TNF-αR2. 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 TNR-αR1 cytoplasmic domain becomes accessible to the TNF receptor-associated death domain (TRADD) adaptor protein. The interaction of TRADD and TNR-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) (203). 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 (202,204), whereas TRAF2 attracts the cellular inhibitor of apoptosis protein (cIAP)-1 and -2 which inhibit Caspase-3 activation (203). 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. From

Fig.1-7: TNF-α mediated signals

TNF-α binds either the TNF-αR1 or TNF-αR2 and the resulting signals affect many intracellular 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 TNFαR1 can also result in the recruitment of the adaptor protein RIP as well as TRAF2 and FADD. The TRAF2 kinase is responsible for the activation of p38, p42/44, and JNK through the activation of MEKs which results in the activation of the transcription factors AP-1 and NF-κB. TRAF2 also affects the apoptotic pathways of the cell by activating cIAP which functions to inhibit Caspase-3 activation. Binding of TNF-α to the TNF-αR2 results in the activation of TRAF2 only, as these receptors do not contain the death domain, and thus, activation of signaling through this receptor only affects the ultimate activation of AP-1 and NF-κB.


TNF-α
TNF-αR2
TNF-αR1
DD
Inhibition of apoptosis
apoptosis

this cascade the activation of the transcription factors NF-κB (205) and AP-1 (206-208) has been demonstrated.

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 (202). Therefore activation of signaling through TNF-αR2 results in the regulation of NF-κB and AP-1 (209-211).

I, and others, have demonstrated that LPS and TNF-α have been shown to be potent stimulants that induce CD44 expression and the generation of HA-adhesive CD44 in monocytic cells (139,212,213). In addition, I have shown that IL-4, a cytokine involved in B cell activation and differentiation, induced CD44 expression and HA-binding in B cell lines (110). Delineation of the signal transduction pathways that are responsible for the regulation of CD44 expression as well as ligand binding are important to gaining a better understanding of how this family of proteins exerts its functions. Resolving the molecular mechanism behind the regulation of CD44 will lead to the identification of key signal transduction proteins and this knowledge may then be used to modulate CD44 expression in a therapeutic manner and may be used in concert with other treatments for cancer or in cases of chronic inflammation.

HYPOTHESIS, AIMS, AND OBJECTIVES

HYPOTHESIS and OVERALL AIM: Distinct signaling pathways are involved in the regulation of CD44 expression as well as those proteins that regulate the generation of the HA-adhesive, functionally active form of CD44. Thus, in a stimulus and cell-dependent manner, specific signaling cascades are activated and affect CD44 expression and activation.

The overall aim of my thesis research proposal is to delineate the molecular mechanisms underlying the regulation of CD44 expression and the generation of functionally active HA-adhesive CD44 in human monocytes and B cells in response to cytokines and mitogens.

Rationale 1: LPS induces inflammatory responses that contribute to the pathogenesis of sepsis, inflammation, and a number of autoimmune diseases including rheumatoid arthritis. 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 of the HA-adhesive CD44 phenotype in monocytes may play a critical role in inflammatory responses. LPS-induced CD44 expression and HA-binding has been suggested to be mediated primarily by TNF-α. The mechanisms by which LPS/TNF-α induce CD44 expression and induce adhesive CD44 remain unknown. I used purified human monocytes and promonocytic THP-1 cells and show that LPS and TNF-α induced CD44 expression in both cell types. The advantage of using the THP-1 cells is that they behave in a similar fashion as human monocytes with respect to the effects of LPS/TNF-α on CD44. As a first objective of my thesis, I investigated the role of MAP kinases, and transcription factors in the regulation

of CD44 expression and in the generation of adhesive CD44 in LPS/TNF-α-stimulated monocytic cells. The specific research objectives are as follows:

1. To study the role of intracellular signaling molecules, in particular the MAPKs, in the regulation of CD44 expression and in the induction of the adhesive CD44 phenotype in LPS/TNF-α-stimulated human monocytic cells. Specifically;

   A. To identify the MAPKs involved in LPS and TNF-α mediated effects on CD44 expression in human monocytic cells

   B. To investigate the role of MAPKs involved in the generation of functionally active, HA-adhesive, CD44 in response to LPS and TNF-α in human monocytic cells

Rationale 2: The regulation of CD44 expression and HA binding on human B cells has not been well documented. Previous work from our laboratory indicated that CD44 is differentially regulated in response to cytokines and mitogens in human B cells, EBV-transformed human B cells, and on a series of Burkitt's lymphoma cell lines. Therefore as the second objective of my thesis, I investigated the effect of a panel of mitogens and cytokines on the regulation of CD44 expression and binding in Burkitt's lymphoma and EVB-transformed B cells. Results from these experiments revealed that IL-4 and IL-13 have differential roles in the regulation of CD44 expression and binding. The specific objectives are as follows:

2. To examine the role of cytokines and mitogens in the regulation of CD44 expression and HA-binding in human B cells, using Burkitt's lymphoma and EBV-transformed B cells as model systems. Specifically:

A. To identify which cytokines and mitogens affect CD44 expression and ligand binding in BL and B-LCL cells.

B. To identify the molecular mechanism involved in the differential regulation of CD44 expression in response to IL-4 and IL-13 in Burkitt's lymphoma and EBV-transformed B cell lines.

CHAPTER 2:

MATERIALS AND METHODS

Cell lines and cell culture

Normal human B cells from different donors were transformed *in vitro* with EBV to generate the heterogeneous lymphoblastoid B cell lines (B-LCL), namely MK3.31, MK4.15, and MK5.26, according to the standard protocol described elsewhere (111). 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 previous work (214,215). Raji, Jijoye, and EB3 are EBV-positive BL B cell lines that were obtained from the American Type Culture Collection (ATCC, Rockville, MD). The BL cell lines CA46, ST486, and Ramos are EBV-negative BL cell lines that were also obtained from ATCC. All of the EBV-positive and EBV-negative BL cell lines used in this study have the characteristic *c-myc* translocation [t(8:14)]. P3HR1 cells are BL cells infected with the EBNA-2 deleted strain of EBV. The EBV-positive IM cell line was generated from peripheral blood mononuclear cells (PBMC) of a patient diagnosed with infectious mononucleosis at the Children's Hospital of Eastern Ontario (CHEO), Ottawa, Ontario, Canada. Human synovial fibroblasts were provided by Dr. H. Birnboim (Cancer Research Institute, Ottawa, Ontario, Canada). Human erythroleukemic TF-1 cells were obtained from ATCC. THP-1, a promonocytic cell line derived from a human acute lymphocytic leukemia patient was obtained from the American Type Culture Collection (Manassas, VA). All cells were cultured in Iscove's Modified Dulbecco's Medium (IMDM) (Sigma, St. Louis, MO) supplemented with 10% fetal bovine serum (FBS) (Gibco BRL, Burlington, Ontario, Canada), penicillin (100 U/mL) and gentamicin (1 μg/mL). For a summary of all the cell lines used in this study refer to Table 2-1.

Table 2-1: Summary of cell lines used in this study.

Name	Cell type/Origin	Comments
MK3.31 MK4.15 MK5.26	Heterogeneous lymphoblastoid B cell lines	EBV^+ B95-8 EBV strain
BL30/B95-8	Burkitt's lymphoma cell line	EBV^+ B95-8 EBV strain
Raji		EBV^+
Jijoye		
EB3		EBV^-
CA46		
ST486		
Ramos		
IM	Derived from infectious mononucleosis patient	EBV^+
TF-1	Human erythroleukemic cell line	IL-13-responsive
THP-1	Human promonocytic cell line derived from an acute lymphocytic leukemia patient	Non-responsive to IL-10

Reagents

Recombinant human interleukin (IL)-2, IL-4, IL-5, IL-6, IL-10, IL-13, tumor necrosis factor (TNF)-α, tumor growth factor (TGF)-β, and interferon (IFN)-γ were purchased from R&D Systems Inc. (Minneapolis, MN). IL-12 was a kind gift from Dr. Maurice Gately (Hoffman LaRoche, Nutley, NJ). Phorbol 12-myristate 13-acetate (PMA) (Gibco BRL), *Staphylococcus aureus* Cowan strain 1 (SAC) (Calbiochem La Jolla CA), pokeweed mitogen (PWM) (Sigma), and Phytohemagglutinin (PHA) (Gibco) were also purchased. PD98059 (Calbiochem, San Diego, CA), an inhibitor of MEK1 kinase, selectively blocks the activity of ERK MAPK and has no effect on the activity of other serine-threonine protein kinases including Raf-1, p38 and JNK MAPKs, or PKC and PKA (216,217). The pyridinyl imidazole SB202190 (Calbiochem), a potent inhibitor of p38 MAPK, has no significant effect on the activity of the ERK or JNK MAPK subgroups (216,217). SB202474, an inactive analog of SB202190, was also purchased from Calbiochem. Lipopolysaccharide (LPS) derived from *E. coli 0111:B4* (Sigma-Aldrich Canada Ltd., Oakville, ON, Canada), as well as human IL-10Rα and human TNF-αR1 antibodies (R&D Systems) capable of neutralizing the biological activities of IL-10 and TNF-α, respectively, were also purchased. Sialidase from *Arthrobacter ureafaciens,* sialidase inhibitor, 2,3-dehydro-2-deoxy-N-acetylneuraminic acid (2 Neu-Ac), and all other chemicals used for Western blotting were purchased from Sigma-Aldrich.

Isolation of human peripheral blood B cells

Human B cells were isolated from PBMC by employing anti-CD19 mAb-conjugated immunomagnetic M-450, Pan-B Dynabeads (Dynal A.S., Oslo, Norway), as described

previously (6). PBMCs were isolated from the blood of healthy adult volunteers following approval of the protocol by the Ethics Review Committee of the Children's Hospital of Eastern Ontario, Ottawa, Ontario, Canada. PBMCs were isolated by density-gradient centrifugation over Ficoll-Hypaque (Amersham Biosciences, Piscataway, NJ) as previously described (218,219). The B cells were purified by incubating PBMC for 30 min on ice with anti-CD19 antibody-conjugated immunomagnetic beads at a bead to target cell ratio of 4:1. Detachment of B cells from beads was accomplished by incubating the complexes for 1 hr at room temperature with 1 unit of CD19-Detachabeads per $5x10^6$ cells. The purity of the isolated cells was assessed by staining with PerCP-conjugated mouse anti-human CD20 mAb for B cells, fluorescein isothiocyanate (FITC)-conjugated mouse anti-human CD3 mAb for T cells, phycoerythrin (PE)-conjugated mouse anti-human CD14 mAb for monocytes and mouse anti-human CD16 mAb (all from Becton Dickinson, Mountain View, CA) for NK cells. The purified B cells contained fewer than 2% T cells, NK cells, and monocytes. Isolation of B cells by negative selection, described elsewhere, was found not to differ from positively selected cells with respect to CD44 isoform expression after stimulation (220) and therefore the positive selection method was used in this study.

Isolation of monocytes from PBMCs

Purified, non-activated monocytes were obtained by a negative selection procedure involving depletion of T cells and B cells using magnetic polystyrene M-450 Dynabeads (Dynal, Oslo, Norway) coated with antibodies specific for CD2 (T cells) or CD19 (B cells), as described earlier (218,219). Briefly, PBMCs (10×10^6 /ml) isolated as described

above were resuspended with CD2 and CD19 Dynabeads and were incubated for 30 min on ice with constant mixing. Cells were incubated at 37°C for 2 hr following which non-adherent cells were removed. The adherent mononuclear cells obtained contained less than 1% CD2+ T cells and CD19+ B cells as determined by flow cytometry.

B Cell stimulation

Cells were serum-starved for 12 hr prior to stimulation, harvested and resuspended in serum-free media followed by treatment with either IL-4 (40 ng/mL; R&D Systems, Minneapolis, MN) or IL-13 (50 ng/mL; R&D Systems) for either 24 hr or for times ranging from 30 sec to 1 hr. Cell stimulation for immunopreciptiation was stopped by placing pelleted cells on ice, which were then washed and resuspended in lysis buffer before centrifugation at 14,000 x g at 4° C for 20 min. The cell pellets were frozen at -80° C.

Monocytic cell stimulation and collection of culture supernatants

To determine the effects of p38 and p42/44 MAPK inhibitors on CD44 expression, CD44-mediated HA-binding, and IL-10 and TNF-α production, purified human monocytes (1 x 10^6/ml) and THP-1 cells (0.5 x 10^6/ml) were incubated in 24-well culture plates (Falcon, Becton Dickinson, Franklin Lakes, NJ). Cells were treated for 24 hr with either LPS (1 μg/ml), IL-10 (10 ng/ml), or TNF-α (10 ng/ml) in the presence or the absence of MAPK inhibitors. Cell supernatants were frozen at –80° C and thawed at the time of analysis. Cells were analyzed for CD44 expression and CD44-mediated HA-binding, and the supernatants analyzed for IL-10 and TNF-α production by ELISA.

B cell flow cytometry

Cells were assayed for CD23, IL-13Rα1, and CD44 expression by flow cytometry (6). Untreated or treated cells ($2x10^5$) were resuspended in PBS, 0.1% sodium azide and were incubated for 10 min at room temperature with either mouse anti-human CD44 mAb (anti-human Leu-44, Becton Dickinson), mouse anti-human CD23 mAb (Becton Dickenson), mouse anti-human IL-13Rα1 mAb (Cell Science Inc., Norwood, MA), or isotype-matched control antibodies (Sigma); all antibodies were FITC-conjugated with the exception of the anti-IL-13Rα1 antibodies. The IL-13Rα1 mAb were conjugated with bitoin and were consequently detected with PE-conjugated streptavidin (Sigma). Cells were analyzed for the expression of CD44 isoforms containing exons V3, V6, and V9 expression (30). These cells (2×10^5) were incubated with primary mouse anti-human CD44 V3 and CD44 V6 (R&D Systems Inc.) and mouse anti-human CD44 V9 (kindly provided by Dr. M. Zoller, Heidelberg, Germany), as well as mouse IgG1, IgG2a, and IgG2b isotype matched control antibodies (Sigma) (5 μg/ml in PBS containing 0.1% sodium azide for 10 min at room temperature). Following incubation, cells were washed once in PBS–0.1% sodium azide and incubated with 5 μg/ml FITC-conjugated goat anti-mouse antibodies (Jackson ImmunoResearch Laboratories Inc., West Grove, PA) for 10 min at room temperature. 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). Experiments shown were representative of at least five different trials.

Monocytic cell flow cytometric analysis

CD44 expression on CD14+ monocytes and on THP-1 cells was determined by flow cytometry (6,111,219). Briefly, cells 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. 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). Validity of comparisons in the expression levels of CD14 and CD44 between different samples was ensured through the use of Calibrite™ Beads (BD Biosciences).

Hyalruonan (HA) adhesion assay

CD44-mediated binding assays were performed as described previously (6,111). Briefly, stimulated cells ($2x10^7$ cells/mL) were resuspended in IMDM-10% FBS and were pulsed for 1.5 hr with ^{51}Cr (sodium chromate, Amersham, Buckinghamshire, UK) at a concentration of 300 $\mu Ci/10^6$ cells. After 3 washes, cells were aliquoted at a concentration of $2x10^6$ cells/mL into 96-well microtiter plates (NUNC, Denmark) which had been coated overnight with 1 mg/mL of either human umbilical cord hyaluronan

(Sigma) or, as a negative control, chondroitin sulfate C (Calbioshem-Novabiochem Corp. San Diego, CA). Cells were incubated for 1.5 hr at 37^{0}C and were washed 6 times with warm IMDM-10% FBS to remove non-adherent cells. The adherent cells were lysed with 1N HCl and radioactive counts were measured by scintillation counting using a Microbeta counter (Wallac, Turku, Finland). The percentage of adherent cells was determined by the following equation:

$$\text{adherent cells (\% cpm total input)} = \frac{\text{cpm adherent -spontaneous release cpm} \times 100}{\text{total input cpm-spontaneous release cpm}}$$

B cell immunoprecipitation and western blot analysis

Immunoprecipitation and Western blot analysis were performed according to standard procedure (188). IL-4- or IL-13-treated cell pellets were lysed for 30 min on ice in 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 and 1 mM EGTA pH 7.7) followed by centrifugation at 14000 x g at 4°C for 20 min. Protein concentrations were determined for the collected supernatants with the Bio-Rad protein estimation kit (Bio-Rad, Hercules, CA) as described by the manufacturer. Lysates were precleared for 1 hr at 4°C with protein A sepharose 4B beads (Amersham Pharmacia Biotech, Uppsala, Sweden). Total protein lysates (2 mg) were incubated for 2 hr at 4°C with protein A sepharose beads and polyclonal rabbit antibodies specific for JAK1, JAK2, JAK3, Tyk2, or STAT6 (Santa Cruz Biotechnology, CA). Immune complexes were washed with lysis buffer three times by centrifugation at 14000 x g for 5 min at 4°C. After the washes, the complexes were resuspended in Laemmli sample buffer (Bio-Rad) containing 5% beta-mercaptoethanol.

The samples were boiled for 5 min and subjected to electrophoresis on 8% SDS-polyacrylamide gels. The electrophoresed proteins were transferred to Immobilon^{TM}-P membranes (Millipore, Bedford, MA) and the membranes were probed for phosphorylated JAK1, JAK2, JAK3, Tyk2, and STAT6 proteins using the mouse anti-phosphotyrosine monoclonal antibody 4G10 (Upstate Biotechnology, Lake Placid, NY). Levels of phosphorylated protein where compared to total immunoprecipitated protein by reprobing blots with the respective anti-JAK or anti-STAT6 antibodies. Proteins were detected using gamma ^{125}I – protein A (Mandel Scientific, Guelph, Ontario, Canada).

Monocytic cell western blot analysis

Phosphorylation of p38 or p42/44 ERK MAPKs in monocytic cells was determined by Western blot analysis using phospho-MAPK-specific antibodies as previously described (110,188,219). Cells were stimulated at 37°C for 0-15 min with either LPS, IL-10, or TNF-α. Cells were then placed in ice and washed with cold PBS. Cell pellets were lysed and protein concentration was determined following the same procedure as described for the immunoprecipitation experiments. Total cell proteins (50μg) were subjected to electrophoresis on 8% polyacrylamide SDS gels and the proteins were transferred onto polyvinylidene difluoride (PVDF) membranes (BioRad). The membranes were probed with either rabbit anti-human-phospho-p38 mAb (New England Biolabs, Mississauga, Ontario, Canada) or mouse anti-human-phosho-p42/44 mAb (Santa Cruz, Biotechnologies, Inc., Santa Cruz, CA), followed by horseradish peroxidase conjugated goat anti-rabbit or goat anti-mouse polyclonal antibodies (BioRad). The membranes were stripped and reprobed with rabbit polyclonal antibodies specific for the unphosphorylated

forms of either p38 or p42 MAPKs (Santa Cruz), as described earlier. These immunoblots were visualized by enhanced chemiluminescence (ECL, Amersham, Baie d'Urfe, PQ, Canada).

RNA extraction and reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA from $2x10^6$ cells was extracted using Tri Reagent (Molecular Research Center, Inc. Cincinnati, OH), as described (111). Total RNA (1 μg) was reverse-transcribed at 42°C for 1 hr in a 20 μL reaction mixture containing 2.5 μM random hexamers, 5 mM $MgCl_2$, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1 mM each of dGTP, dATP, dTTP, and dCTP, 20 units of RNAse inhibitor, and 500 units of M-MLV reverse transcriptase (Perkin Elmer, Foster City, CA). The cDNA equivalent to 100 ng of RNA was added to standard PCR mixtures containing 100 pmol of oligonucleotide primers. The primer sequences used were as follows: IL-13Rα1: sense-5'GGA GCC AGC TCA AAT TGT AG-3'; antisense-5'-ACA CGG GAA GTT AAA GGC A-3'; IL-13Rα2: sense-5'-GGA GAG AGG CAA TAT CAA GG-3'; antisense-5'-GGC CAT GAC TGG AAA CTG-3' (221). The primers were synthesized at the Molecular Genetics Laboratory (CHEO, Ottawa, Ontario, Canada). Amplification was carried out in a Perkin Elmer DNA Thermal Cycler 480 using a hot start technique. Thirty cycles of 94°C for 1 min, 59°C for 1 min, and 72°C for 1 min 30 sec were performed. The amplified PCR products were electrophoresed through 1.2% agarose gels and subjected to Southern transfer onto a Hybond-N nylon membrane (Amersham). Sequential hybridization was performed overnight at 65°C using ^{32}P-labeled oligonucleotide probes specific for IL-13Rα1 and IL-13Rα2 chains in hybridization buffer (6X sodium chloride/sodium citrate (SSC), 5X

Denhardt's solution, 0.5% sodium dodecyl sulfate (SDS), 50 nM sodium phosphate, and 0.25 mg/mL salmon sperm DNA). Following washes in 2 X SSC, the membranes were exposed to X-ray film for 1-7 days.

Measurement of monocyte IL-10 and TNF-α production in culture supernatants by ELISA

IL-10 and TNF-α were measured by employing two different monoclonal antibodies (mAb) recognizing distinct epitopes as described earlier. Briefly, the plates (Nunc Immunomodules, Roskilde, Denmark) were coated overnight at 4°C with the primary antibody (either anti-IL-10 mAb #18551D from BD Pharmingen, Mississuaga, Ontario, Canada; final concentration of 5 μg/ml, or anti-TNF-α mAb #AHC3712 from Biosource; final concentration of 5 μg/ml) prepared in coating buffer (0.04 M Na_2CO_3, 0.06 M $NaHCO_3$, pH 9.6). The plates were washed with PBS/Tween 20 and blocked with PBS/10% FBS. IL-10 or TNF-α were detected using a second biotinylated mAb prepared in PBS/10% FBS (anti-IL-10 antibody #18562D from Pharmingen, final concentration of 4 μg/ml; anti-TNF-α antibody #AHC3419 from Biosource, final concentration of 4 μg/ml). Streptavidin-peroxidase was used at a final concentration of 1:1000 (Jackson Immuno Research, West Grove, PA). The color reaction was developed by *0*-phenylenediamine (Sigma-Aldrich, Canada) and hydrogen peroxide, and the absorbance was read at 450 nm. IL-10 (R&D Systems) and TNF-α (BioSource) were used as standards and IL-10 and TNF-α concentrations in the culture supernatants were calculated using the Microplate Manager 4.0 software (Bio-Rad Hercules, CA). The level of sensitivity for both IL-10 and TNF-α production by ELISA was 16 pg/ml.

Transient transfection

THP-1 cells were transfected with either a pcDNA-3 plasmid expressing a dominant-negative (DN) mutant of MAPK kinase 4/stress-activated protein/Erk kinase 1 (MKK-4/SEK1, provided by Dr. J. R. Woodget, Princess Margaret Hospital, Toronto, Ontario, Canada) or a control pcDNA-3 plasmid (188). Prior to transfection, 10 μg of plasmid was incubated with 10 μl of LIPOFECTAMINE reagent (Life Technologies Inc., Burlington, ON) in 200 μl of OPTI-MEM I Reduced Serum Medium (Life Technologies) for 45 min to allow formation of DNA-liposome complexes. These complexes were added to the cell suspension (1.5 x 10^6 cells/ml) in each well and cultured for 24 hr. Cells were stimulated with 1 μg/ml of LPS for another 24 hr followed by analysis for CD44 expression. Two positive control vectors, pcDNA3.1/His/LacZ (Invitrogen^{Tm} life technologies, Carlsbad, CA) and pSV-β-galactosidase (Promega) were employed for determination of transfection efficiency by using a commercially available β-Gal Staining Kit (Invitrogen^{Tm}) as per the manufacturer's instructions. An average of 18% cells were found to be transfected.

Gel mobility shift assays.

Gel mobility assays were performed as per the standard technique and as described earlier (188). Cells (1 x 10^7) were harvested in Tris-EDTA-saline buffer pH 7.8 and centrifuged at 200 x g for 5 min at 4°C. The cells were lysed for 10 min at 4°C with buffer A (10 mM HEPES, 10 mM KCl, 1.5 mM $MgCl_2$, 0.5 mM DTT, 0.5 mM PMSF, pH 7.9) containing 0.1% Nonidet P-40. The lysates were centrifuged at 20 000 x g for 10 min at 4°C. The pellets containing the nuclei were suspended in buffer B (20 mM HEPES, 420 mM NaCl,

1.5 mM $MgCl_2$, 0.2 mM EDTA, 25% glycerol) at 4° C for 15 min. Both buffers A and B contained the proteolytic inhibitors at 0.5 mM each of DTT, PMSF, and spermidine, as well as 0.15 mM spermine, and 5 μg/ml each of aprotinin, leupeptin, and pepstatin. The supernatants containing the nuclear proteins were collected and frozen at -80°C. Nuclear proteins (5 μg) were mixed for 20 min at room temperature with ^{32}P-labeled Egr-1 oligonucleotide probes, and the complexes were subjected to non-denaturing 17 % PAGE for 90 min. The gels were dried and exposed to X-ray film. The Egr-1 oligonucleotide sequence used was: 5'-(GGA TCC AGG GGG GGC GAG CGG GGG CGA)-3' (Santa Cruz).

Determination of sialidase activity

Measurement of endogenous sialidase activity in LPS- or TNF-α-treated THP-1 cells was performed according to optimal conditions (29). Cells (15 x 10^6) were washed with PBS and resuspended in ice-cold buffer containing 0.25M sucrose, 1mM EDTA, and 0.2 mM PMSF. The cell suspension was sonicated on ice for 10 sec on a low setting (7% amplitude) (Vibracell™; Sonics and Materials Inc, Newtown, CT) followed by centrifugation at 25 000 x g for 15 min at 4 °C. The resulting supernatant was used to determine the lysosmal sialidase activity. Protein quantification of the supernatant was performed using the BioRad protein determination kit as described above. For the determination of lysosomal sialidase activity, 200 μg of total protein was mixed with 40 nmol of 4-methylumbelliferyl-α-*N*-acetyl-D-neuraminic acid (4-MU-NeuAc) (Sigma-Aldrich), the lysosmal sialidase-specific substrate, 10 μmol sodium acetate buffer pH 4.6, and 200 μg BSA in a total volume of 200 μl. The sialidase reaction was allowed to

proceed for 1 hr at 37°C and was terminated by the addition of 0.25 M glycine NaOH pH 10.4. Released 4-MU was measured fluorometrically (Perkin Elmer) in a sodium carbonate buffer at an excitation wavelength of 365 nm and emission wavelength of 448 nm as described previously (222). One unit of sialidase was defined as the amount of enzyme which catalyzed the release of 1 nmol of sialic acid/hr. The measurement of lysosmal sialidase activity was optimized using various concentrations of total protein (25, 50, 100, 150, 200 μg). The maximal sialidase activity was detected when 200 μg of total cellular proteins was used. Furthermore, the optimal incubation time for sialidase reaction was determined to be 1 hr.

Lysosomal sialidase activity was determined in THP-1 cells stimulated with LPS or TNF-α for varying periods of time. The sialidase activity for these experiments was expressed as a measure of fluorescence intensity (FI). To determine the involvement of MAPKs in the LPS- or TNF-α-induced lysosomal sialidase activity, cells were treated with MAPK inhibitors (SB202190 and PD98059) at concentrations ranging from 5 to 50 μM for 2 hr prior to stimulation with either LPS or TNF-α for 16 hr. The cells were harvested and analyzed for sialidase activity. In order to clearly illustrate the changes observed upon treatment with the inhibitors, the inhibition of sialidase activity by SB202190 or PD98059 was measured as a percentage change (Δ) of the sialidase activity (units) with respect to that observed with LPS or TNF-α stimulated cells. This was calculated as follows:

$$\frac{\text{Sialidase activity (unit) in cells treated with MAPK inhibitor} \pm \text{LPS or TNF-}\alpha}{\text{Sialidase activity (unit) in cells treated with LPS or TNF-}\alpha \text{ alone}} \times 100$$

For the determination of plasma membrane sialidase activity, 200 μg of total cellular protein were mixed with 60 mmol of bovine mixed gangliosides type II (Sigma), the membrane sialidase-specific substrate, 10 μmol sodium acetate buffer pH 4.6, 0.2 mg of Triton-X-100, and 200 μg BSA in a total volume of 200 μl. The mixture was incubated at 37°C for 1 hr and the released sialic acid was measured by the Aminoff method (223). Briefly, the reaction mixture was incubated with 0.2 ml of periodate reagent (25 mM periodic acid in 0.125N H_2SO_4) for 30 min at 37°C followed by reduction with 0.2 ml sodium arsenite (2% sodium arsenite in 0.5 N HCl) for 2 min. Following reduction 2 ml of 0.1M thiobarbituric acid was added and the mixture was heated for 7.5 min. Following cooling, 5 ml of acid butanol (butanol containing 5% v/v of 12 N HCl) was added to the reaction mixture, mixed, and centrifuged to separate the aqueous and organic phases. The color reaction in the butanol layer was measured by reading the absorbance in a spectrophotometer at a wavelength 549 nm. Membrane sialidase activity was expressed as ng of sialic acid released/ 200 μg of total protein. Purified sialic acid (Sigma) at concentrations ranging from 0-40 μg was used to generate a standard curve using the Aminoff method. The membrane sialidase activity was determined by comparing the absorbencies obtained from the cell samples to the absorbencies measured from the standard curve.

Analysis of soluble and membrane-associated lysosomal sialidase activity - To determine whether the lysosomal sialidase was localized in the cell membrane or in the soluble cytosol fraction, the cells were stimulated with LPS or TNF-α for 16 hr. The cell pellet was washed and treated with 1% digitonin for 45 min on ice followed by centrifugation at 14,000 x g for 20 min at 4°C. The supernatant constituted the soluble

cytosol fraction, whereas the pellet represented the membrane fractions. The membrane fraction was solubilized in a non-ionic detergent, 1% NP-40. The lysosomal sialidase activity was determined in both the cytosol and membrane fractions as per the methods described above.

Statistical analysis

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

CHAPTER 3:

Studies on the regulation of CD44 expression and HA-binding by LPS and TNF-α in monocytic cells

INTRODUCTION

The lipopolysaccharide component of endotoxin, derived from Gram-negative bacterial cell walls, induces inflammatory responses that contribute to the pathogenesis of sepsis, inflammation, and a number of autoimmune diseases including rheumatoid arthritis. Although the mechanisms that lead to these inflammatory processes remain largely unclear, monocytic cells play a major role in the pathogenesis of LPS-induced syndromes. In response to LPS, monocytes express large quantities of pro-inflammatory cytokines, as well as several co-stimulatory/adhesion molecules including B7, ICAM-1, VCAM-1, and CD44 (62,224,225). The over-expression of pro-inflammatory cytokines, co-stimulatory and adhesion molecules is a hallmark of inflammatory responses.

Modulation of expression and CD44-HA-binding in monocytic cells by endotoxins and immunoregulatory cytokines may have profound effects on the migration of monocytes to sites of inflammation and on the development of immune responses. Several cytokines have been shown to influence CD44 expression in different cell types (6,111,226). For example, in murine B cells, IL-5 enhances CD44-mediated binding to HA (28), whereas IL-3 and GM-CSF mediate this effect on human hematopoietic progenitor cells (136). The signal transduction events involved in CD44 upregulation are not well understood. CD44 expression has been shown to be regulated via calmodulin/Ca^{2+} signaling pathways in PMA-stimulated T lymphoma cells (227), whereas PI3-kinase and protein kinase C were shown to regulate CD44 expression in neuroblastoma cells (228). Recently, Egr-1 was implicated in the transcription of CD44 in murine B cells (142) and in the endothelial cell line ECV304 (143).

The ability of CD44 to bind HA is a tightly regulated process and is highly dependent on cell type, state of cell activation and differentiation (2,5,6,9,111). Although

most cells express some form of CD44, not all cells constitutively bind HA (6,111,229). Acquisition of the HA-binding ability of CD44 thus plays a vital role in determining CD44-mediated biological effects. 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, and phosphorylation of its cytoplasmic tail (2,9-11,16,20). It is also well documented that alterations in the N- and O-linked glycosylation patterns of CD44 play a vital role in the regulation of its binding to HA (10-12,28). Recently, an inducible sialidase was shown to influence CD44-HA-binding of lipopolysaccharide (LPS)-stimulated human monocytic cells (29).

Peripheral blood monocytes express abundant cell surface CD44 yet do not bind HA (62,230). TNF-α, a proinflammatory cytokine, was shown to be an important positive regulator of LPS-induced CD44-HA interactions in these cells (62). Furthermore, ligation of CD44 with HA has been shown to induce a number of proinflammatory cytokines including TNF-α (231,232). Therefore, the acquisition of HA-binding capacity by CD44 expressed on monocytic cells could be critical for their participation in inflammatory responses. Hence, understanding the signaling pathways governing the regulation of CD44 expression, and the synthesis of the HA-adhesive form of CD44, may lead to the development of strategies for the treatment of autoimmune diseases and cancer.

There is very little information available regarding the molecular mechanisms involved in the regulation of CD44 expression and of HA adhesive, functionally active CD44 expression (228). In this study, I examined the mitogen-activated protein kinase (MAPK) transduction pathways involved in both the regulation of CD44 expression and

the synthesis of the HA-adhesive, functionally active form of CD44 in LPS-stimulated normal human monocytes and in promonocytic THP-1 cells. As discussed in Chapter 1, the MAPKs are serine-threonine protein kinases that include p38, p42/44 ERKs, and JNK (233,234). LPS and TNF-α have been shown to induce the expression of several cellular genes via activation of all three classes of MAPKs (188,219,233,235-237).

With respect to my first objective of understanding of the molecular mechanisms involved in the regulation of CD44 expression, I demonstrate that CD44 expression is upregulated in normal human monocytes following stimulation with LPS as well as with IL-10 and TNF-α, which are produced endogenously following LPS stimulation. I examined the MAP kinase signaling pathways induced by LPS, IL-10, and TNF-α in normal human monocytes. My results revealed a partial role for p38 MAPK in LPS- and TNF-α-induced expression of CD44 in monocytic cells. In contrast, IL-10-induced CD44 expression did not involve p38 or p42/44 MAPK activation. To further dissect the TNF-α- and LPS-induced signaling pathways regulating CD44 expression independent of IL-10-mediated effects, I utilized IL-10 refractory THP-1 cells as a model system. I provide evidence that CD44 expression induced by the LPS pathway is mediated through JNK signaling. Furthermore, my results suggest that CD44 expression consequent to LPS treatment of human monocytes may require JNK-dependent activation of Egr-1.

The second objective of this study involved understanding the regulation of hyaluronan-adhesive CD44 in human monocytic cells. My results show that LPS-induced CD44-HA-binding in normal monocytes is regulated by endogenously produced TNF-α and IL-10. Studies designed to delineate the role of MAPKs revealed that activation of p38 was required for LPS- and TNF-α-induced expression of the HA-adhesive CD44. In

contrast, IL-10-induced CD44-HA interactions were not mediated through either p38 or p42/44 activation. To further dissect the TNF-α- and LPS-induced signaling pathways independent of IL-10-mediated effects, I utilized IL-10 refractory promonocytic THP-1 cells as a model system. I show that production of TNF-α following LPS-stimulation of THP-1 cells involves p42/44 MAPK activation, and endogenously produced TNF-α is required for the induction of CD44-mediated HA binding. My results also show that sialidase activation is required for the acquisition of HA-binding capacity by CD44 in LPS- and TNF-α-stimulated monocytic cells. These results prompted me to investigate a role for p38 and p42/44 in LPS- and TNF-α-induced sialidase activity resulting in the synthesis of the HA-binding form of CD44. LPS-induced sialidase activity was dependent on p42/44 MAPK-mediated TNF-α production. Blocking TNF-α production by PD98059, a p42/44 inhibitor, significantly reduced the LPS-induced sialidase activity and CD44-HA binding. Subsequently, TNF-α-mediated p38 MAPK activation induced sialidase activity and CD44-HA-binding. Taken together, my results suggest a key role for TNF-α in regulating the synthesis of HA-adhesive CD44 by inducing sialidase activity through p38 MAPK activation in LPS-stimulated monocytic cells.

I will present the results from these experiments in two sections; the first section will deal with the data on the regulation of CD44 expression in monocytic cells and the second section will deal with the data on the regulation of CD44-HA binding in monocytic cells.

RESULTS:

Objective 1 A: Role of MAPK in the Regulation of CD44 Expression by Lipopolysaccharide (LPS) and Tumor Necrosis Factor-α in Human Monocytic Cells

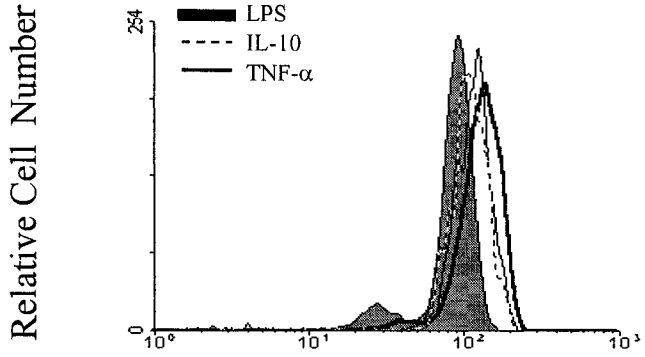
LPS, IL-10, and TNF-α upregulate CD44 expression on the surface of normal human monocytes

Stimulation of normal human monocytes with LPS results in the release of pro- and anti-inflammatory cytokines (IL-1, IL-6, TNF-α, IL-10), and induces the expression of various cell surface receptors including CD44 (62,225,238). To confirm that stimulation of normal human monocytes with LPS resulted in CD44 upregulation, monocytes were isolated by negative selection as described in the materials and methods section and the cells were cultured with LPS (1μg/ml) for 24 hr and analyzed for CD44 expression by flow cytometry (Fig. 3-1). As seen in Fig. 3-1, LPS induced enhanced expression of CD44. The histogram shown is a representative from 5 different donors. It has been suggested that TNF-α and IL-10 are the major cytokines that regulate interaction of CD44 with its ligand HA in human monocytes (26,62). Since TNF-α and IL-10 are produced in response to LPS stimulation of these cells, the ability of exogenously added cytokines to increase expression on monocytes was also investigated. Cells were isolated as described above and were cultured with either TNF-α (10 ng/ml) or IL-10 (10 ng/ml) for 24hr. Both TNF-α and IL-10 increased CD44 expression on normal human monocytes. (Fig. 3-1). To determine whether LPS-induction of IL-10 and TNF-α are responsible for the LPS-induced CD44 upregulation, antibodies specific for human IL-10Rα (0.1 μg/ml) and human TNF-αR1 (5 μg/ml) as well as isotype-matched control antibodies were added to cultures of monocytes in concert with LPS and the cells were culture for 24 hr. These antibodies were capable of neutralizing the biological activity of 1 ng/ml of IL-10 and TNF-α respectively, as recommended by the supplier (R&D Systems). The results show that anti-IL-10Rα and anti-TNF-αR1 antibodies

Fig. 3-1: LPS, IL-10, and TNF-α enhance CD44 expression in normal human monocytes.

A. Purified normal human monocytes (0.5 x 10^6/ml) were stimulated with LPS (1 μg/ml), IL-10 (10 ng/ml), or TNF-α (10 ng/ml) for 24 hr. and analyzed for CD44 expression by flow cytometry. B. LPS treated normal human monocytes were treated with either anti-IL-10Rα (0.1 μg/ml, upper panel) , anti-TNF-αR1 (5 μg/ml, middle panel), or by anti-IL-10Rα (0.1μg/ml) plus anti-TNF-αR1 (5 μg/ml) (lower panel) or with isotype matches control antibodies. Shaded histograms represent untreated cells. Bold lines indicate LPS-stimulated cells in the presence of control antibodies whereas broken lines represent LPS-stimulated cells treated with anti-IL-10R and anti-TNF-αR antibodies. The cells were double-stained with PE-labeled mouse anti-human CD14 antibodies and with FITC-labeled mouse anti-human CD44 antibodies. CD44 expression was analyzed on CD14+ monocytes by flow cytometry. The experiments shown are representative of five different experiments.

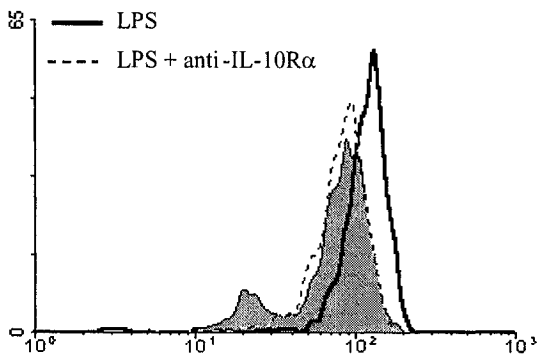
A.

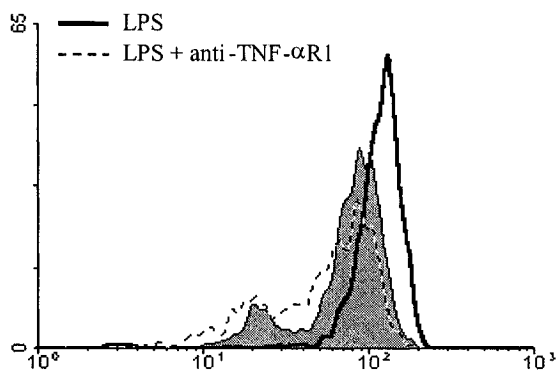


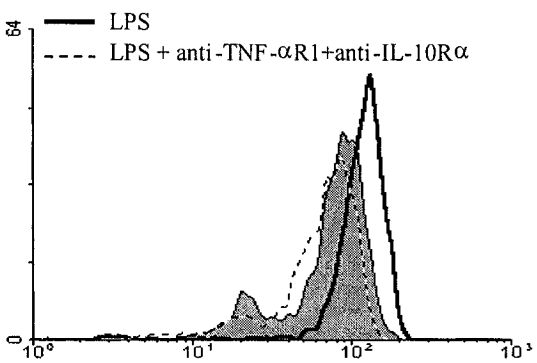
Relative Cell Number

Log Fluorescence Intensity (CD44 Expression)

B.







Relative Cell Number

Log Fluorescence Intensity (CD44 Expression)

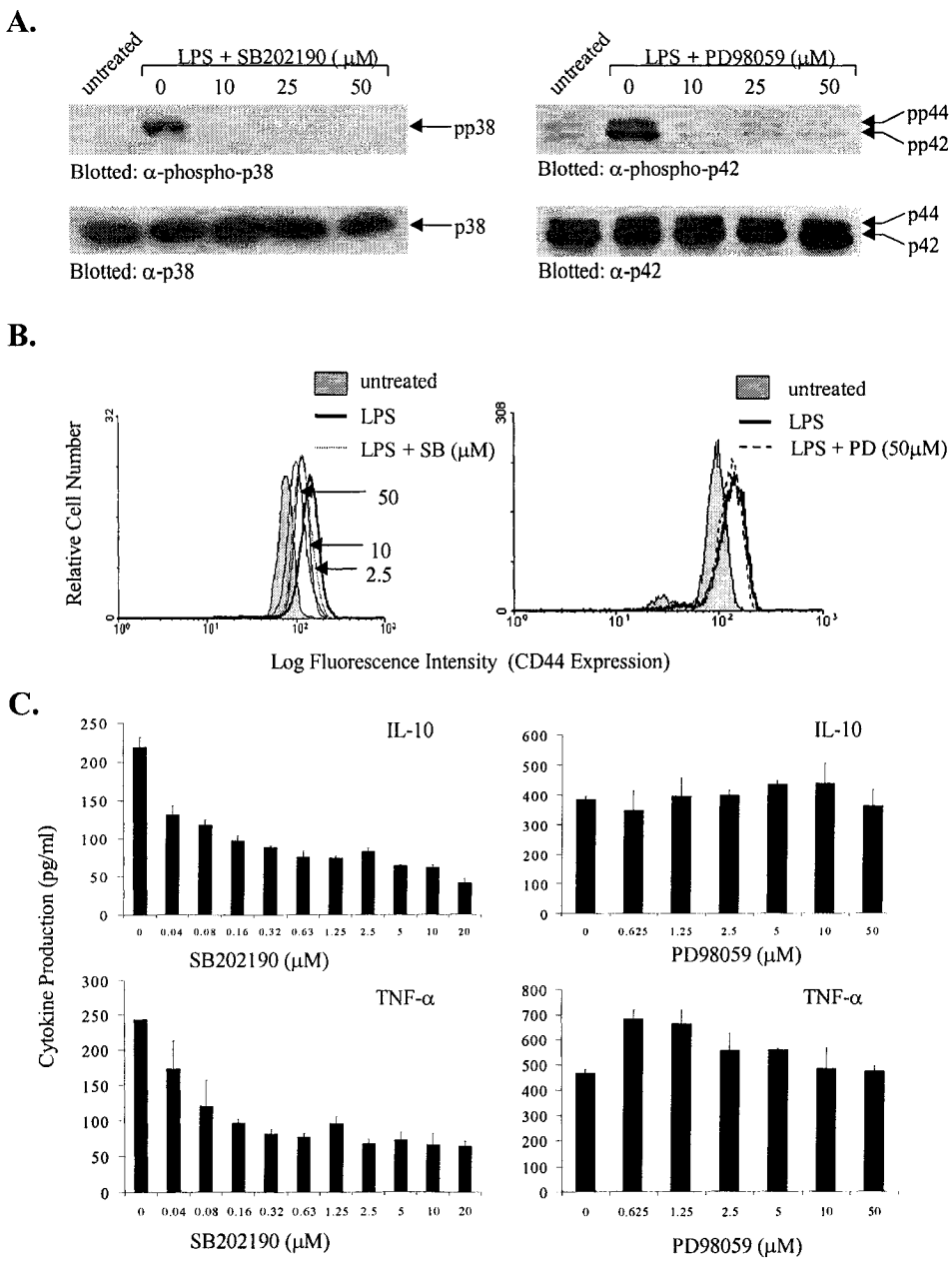
inhibited the LPS-induced CD44 expression but not by the control antibodies (Fig. 3-1). In summary, these results indicate that LPS-induced CD44 upregulation is mediated by the LPS-induced cytokines TNF-α and IL-10.

Role of p38 MAP kinases in LPS-induced upregulation of CD44 expression

The MAP kinases are known to play a major role in the LPS- and cytokine-mediated induction of several cell surface molecules (188,239-242). It was, therefore, of interest to identify the specific members of the MAP kinase family that are involved in the LPS, IL-10, and TNF-α-induced regulation of CD44 expression in monocytic cells. First the involvement of p38 and p42/44 ERK MAP kinases was investigated by examining their activation following stimulation of monocytes with LPS. Isolated monocytes were cultured in the presence of LPS (1μg/ml) for 10 min followed by Western blot analysis using phospho-specific antibodies to either p38 or p42. LPS induced the phosphorylation of both p38 and p42/44 (Fig. 3-2A). The role of p38 and p42/44 has been studied through the use of specific kinase inhibitors: SB202190 for p38-, and PD98059 for p42/44-mediated signaling (216,217). To confirm that SB202190 and PD98059 inhibited the phosphorylation of p38 and ERKs respectively in this system, freshly isolated monocytes were pretreated with these inhibitors for 2 hr followed by stimulation with LPS for 10 min. The lysates were then subjected to the same Western blotting conditions as described above. The results show that prior incubation of monocytes with SB202190 or PD98059 resulted in the inhibition of LPS-induced phosphorylation of p38 and p42/44 ERKs, respectively, (Fig.3-2A).

Fig. 3-2. Effect of LPS on normal human monocytes.

A. LPS stimulation induces p38 and p42/44 MAP kinase phosphorylation in purified human monocytes. Purified normal human monocytes (1.0 x 10^6/ml) were pretreated with either SB202190 or PD98059 at varying concentrations ranging from 0 to 50 μM for 2 hr prior to LPS (1 μg/ml) stimulation for 10 min. Crude protein extracts (50 μg) were subjected to SDS-PAGE followed by Western blot analysis. The membranes were blotted with either anti-phospho-p38 (pp38) antibody or anti-phospho-p42 (pp42) antibody, and to control for loading, the membranes were stripped and reprobed with either anti-p38 (p38) or anti-p42 (p42) respectively. The p38 MAP kinase inhibitor modulates LPS-induced CD44 expression (B) and TNF-α and IL-10 production (C) in normal human monocytes. Purified normal human monocytes (0.5 x 10^6/ml) were pretreated with either SB202190 or PD98059 at varying concentrations ranging from 0 to 50 μM for 2 hr prior to LPS (1 μg/ml) stimulation for 24 hr. CD44 expression was then analyzed by flow cytometry. Supernatants were harvested and analyzed by ELISA for TNF-α and IL-10 production. The histogram shown is a representative of five different experiments performed. ELISA results are represented as a mean ± SD of five experiments.


A.
untreated
LPS + SB202190 (μM)
0
10
25
50
pp38
Blotted: α-phospho-p38
p38
Blotted: α-p38
untreated
LPS + PD98059 (μM)
0
10
25
50
pp44
pp42
Blotted: α-phospho-p42
p44
p42
Blotted: α-p42
B.
untreated
LPS
LPS + SB (μM)
50
10
2.5
Relative Cell Number
untreated
LPS
LPS + PD (50μM)
Log Fluorescence Intensity (CD44 Expression)
C.
IL-10
TNF-α
Cytokine Production (pg/ml)
SB202190 (μM)
PD98059 (μM)

To determine the role of p38 and p42/44 MAP kinases in the LPS-mediated induction of CD44, monocytes were treated with SB202190 or PD98059 at various concentrations for 2 hr before stimulation with LPS for 24hr and analyzed for CD44 expression by flow cytometry. SB202190 partially inhibited CD44 expression at doses as low as 10 μM (Fig. 3-2B). Complete inhibition was not observed even if 25 and 50 μM of SB202190 were used (Fig. 3-2B). When cells from five donors were used in separate experiments, variations in CD44 expression levels resulted ie, the data in Table 3-1 does not show a significant inhibition at 10 μM; however, at doses of 25 and 50 μM, the partial effect of SB202190 is significant ($p<0.05$). Treatment of cells with SB202474, an inactive analogue of SB202190, for 2 hr prior to LPS stimulation did not influence CD44 expression (data not shown), thus confirming the specificity of SB202190. In contrast, PD98059 did not inhibit CD44 expression even at doses of 50 μM (Fig. 3-2B, Table 3-1), indicating that the p42/44 pathway is not directly involved in the regulation of LPS-induced CD44 expression. Doses higher than 50 μM were not used as these concentrations were cytotoxic as determined by the trypan blue exclusion test (data not shown).

Role of TNF-α and IL-10 LPS-induced upregulation of CD44 expression

Because LPS stimulation of primary monocytes also results in the production of cytokines such as IL-10 and TNF-α and because these cytokines affect CD44 expression, it is possible that the downregulation of CD44 expression observed following treatment with SB202190 may be due to the inhibition of endogenous IL-10 and/or TNF-α production. Therefore, the effects of MAP kinase inhibitors on IL-10 and TNF-α production in LPS-stimulated monocytes were evaluated. Preincubation with SB202190,

Table 3-1: Effects of p38 and p42/44 MAPK inhibitors on LPS-, TNF-α-, and IL-10-induced CD44 expression in normal monocytes

Treatment	Media	CD44 expression (MCF) following stimulation in the presence of MAPK Inhibitors (μM)					
		0	2.5	5	10	25	50
LPS + SB202190	206.3 ± 50.0	389.0 ± 50.1 *	410.2 ± 26.7	431.4 ± 26.3	354.8 ± 48.6	315.7 ± 35.6 *	264.7 ± 62.2 *
LPS + PD98059	206.3 ± 50.0	389.0 ± 50.1 *	406.1 ± 79.3	376.5 ± 33.2	461.0 ± 42.5	422.0 ± 46.7	422.5 ± 78.5
TNF-α + SB202190	328.4 ± 47.5	529.3 ± 118.6 *	513.2 ± 12.5	536.8 ± 43.7	499.2 ± 84.4	401.6 ± 52.1 *	345.2 ± 40.2 *
TNF-α + PD98059	328.4 ± 47.5	529.3 ± 118.6 *	549.1 ± 27.0	575.7 ± 61.8	535.7 ± 138.5	606.5 ± 30.9	558.5 ± 90.3
IL-10 + PD98059	281.8 ± 45.3	665.0 ± 69.4 *	N.D.	649.5 ± 24.7	657.5 ± 16.3	654.0 ± 60.8	551.0 ± 22.6

Normal monocytes (1.0 x 10^6/ml) were pretreated with SB202190 or PD98059 for 2 hr at the concentrations indicated prior to stimulation with either LPS (1 μg/ml), TNF-α (10 ng/ml), or IL-10 (10 ng/ml) for 24 hr. CD44 expression (MCF ± SD; n=5) was analyzed by flow cytometry. *, p 0.05; ND, not determined.

but not PD98059, inhibited LPS-induced IL-10 as well as TNF-α production in a dose-dependent manner (Fig. 3-2C). The same cells were used for CD44 expression as well as IL-10 and TNF-α production analyses in the experiments shown in Fig 3-2B and 2C. These results suggest that the partial inhibition of LPS-induced CD44 expression by SB202190 may be, at least in part, due to the decreased endogenous production of TNF-α and/or IL-10.

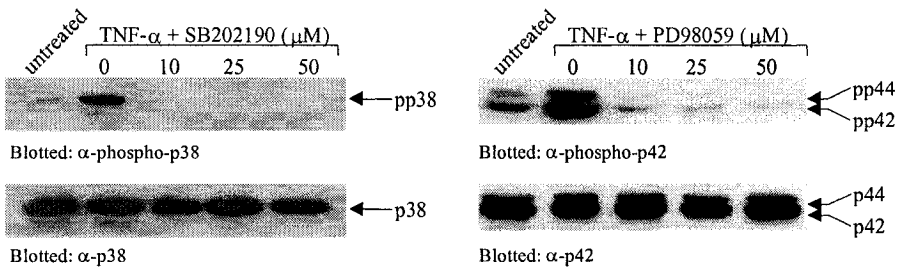
Role of p38 MAPK in TNF-α-induced CD44 expression

It is likely that IL-10 and/or TNF-α induced by stimulation of monocytes with LPS may upregulate CD44 expression by activating either p38 or p42/44 MAPKs. To clarify the role of p38 and p42/44 MAPKs in TNF-α-induced CD44 expression, the effects of the MAPK inhibitors on TNF-α-induced CD44 upregulation were examined. Isolated monocytes were pretreated with varying concentrations of either SB202190 or PD98059 for two hr followed by treatment with LPS (1μg/ml) for 10 min. Lysates were then subjected to Western blot analysis as before. Similar to the LPS-induced activation of p38 and p42/44 (Fig. 3-2A), SB202190 and PD98059 significantly reduced TNF-α-induced phosphorylation of p38 and p42/44 ERK kinases, respectively, in normal monocytes (Fig. 3-3A). Similar to the results obtained for LPS-induced CD44 expression (Fig. 3-2A), neither SB202474 (data not shown) nor PD98059 influenced CD44 expression. SB202190 partially inhibited CD44 expression at doses as low as 10 μM (Fig. 3-3B). Complete inhibition was not observed even if 25 and 50 μM of SB202190 were used (Fig. 3-3B). The results from five different donors are shown in Table 3-1. Because of large variations in the level of CD44 expression on monocytic cells among individuals, the data in Table 3-1 does not show a significant inhibition at 10 μM;

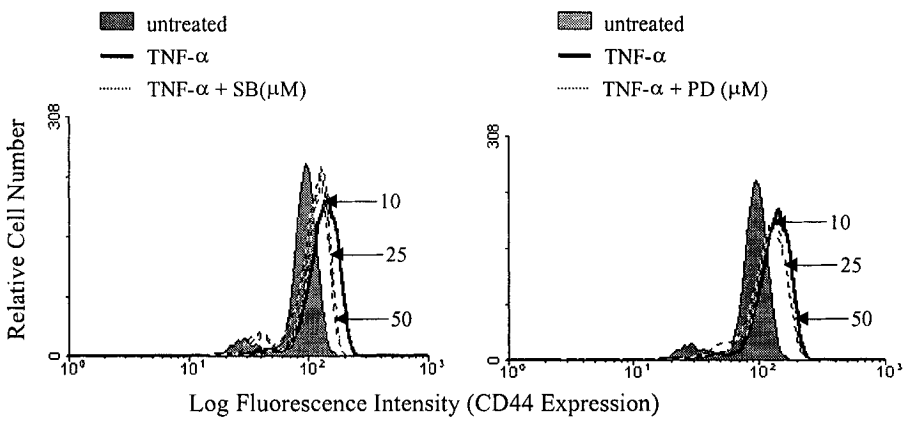
Fig. 3-3. Effect of TNF-α on normal human monocytes

A. TNF-α induces p38 and p42/44 MAP kinase phosphorylation in purified human monocytes. Purified normal human monocytes (1.0 x 10^6/ml) were pretreated with either SB202190 or PD98059 at varying concentrations ranging from 0 to 50 μM for 2 hr prior to TNF-α (10 ng/ml) stimulation for 10 min. Crude protein extracts (50 μg) were subjected to SDS-PAGE followed by Western blot analysis. The membranes were blotted with either anti-phospho-p38 (pp38) antibody or anti-phospho-p42 (pp42) antibody. To control for loading, the membranes were stripped and reprobed with anti-p38 (p38) or anti-p42 (p42) antibodies. B. Effect of p38 and p42/44 MAP kinase inhibitors on TNF-α-induced CD44 expression in normal human monocytes. Purified normal human monocytes (0.5x10^6/ml) were pretreated with either SB202190 or PD98059 at varying concentrations ranging from 0 to 50 μM for 2 hr prior to TNF-α (10 ng/ml) stimulation for 24 hr. CD44 expression was then analyzed by flow cytometry. The experiment shown is representative of five different experiments.

A.


untreated
TNF-α + SB202190 (μM)
0
10
25
50
pp38
Blotted: α-phospho-p38
p38
Blotted: α-p38
untreated
TNF-α + PD98059 (μM)
0
10
25
50
pp44
pp42
Blotted: α-phospho-p42
p44
p42
Blotted: α-p42

B.


untreated
TNF-α
TNF-α + SB(μM)
Relative Cell Number
308
0
10
25
50
Log Fluorescence Intensity (CD44 Expression)
untreated
TNF-α
TNF-α + PD (μM)
308
0
10
25
50

however, at doses of 25 and 50 μM, the partial effect of SB202190 is significant ($p<0.05$). These results suggest that LPS-induced CD44 expression may be partially regulated by the enhanced production of TNF-α which is p38 MAP kinase-dependent. Furthermore, although the interaction of TNF-α with its receptor activated both the p38 and p42/44 MAPK pathways, p38 may play a partial role in TNF-α-induced CD44 expression.

IL-10-induced CD44 expression does not involve p38 or p42/44 activity

To determine the role of p38 and p42/44 MAP kinases in IL-10-mediated CD44 expression, it was first determined whether IL-10 could activate p38 or p42/44 ERK MAP kinases. Stimulation of normal monocytes with IL-10 induced the phosphorylation of p42/44, but not of p38 MAP kinase. Furthermore, IL-10-induced activation of p42/44 ERK was inhibited by PD98059 in a dose-dependent manner (Fig. 3-4A). However, p42/44 ERK was not found to be involved in IL-10-induced CD44 expression since incubation of monocytes with PD98059 did not affect IL-10-induced CD44 expression (Fig. 3-4B, Table 3-1).

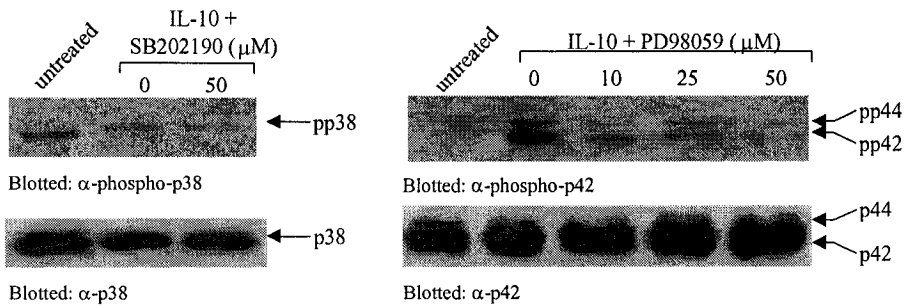
Role of p38 MAP kinase in LPS-and TNF-α-induced CD44 expression in THP-1 cells

LPS-induced CD44 expression in normal human monocytes is complex and regulated by interaction of LPS with its CD14/Toll-like receptor complex as well as by the interaction between IL-10 and TNF-α and with their corresponding receptor complexes. Because of the inherent endogenous production of IL-10 in LPS-stimulated monocytic cells, the role of p38 and p42/44 MAPK in LPS- and TNF-α-induced CD44 expression could not be easily identified. To investigate the role of MAP kinases in LPS-induced CD44

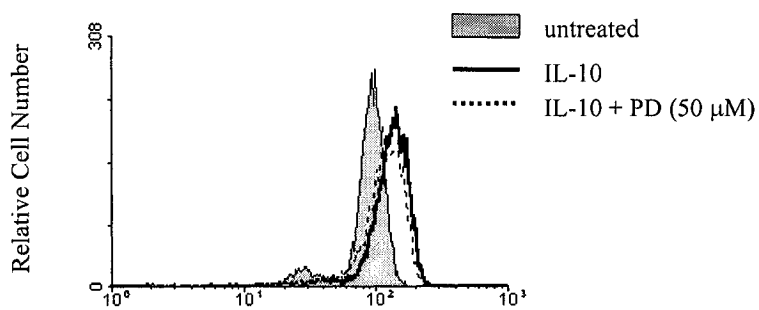
Fig. 3-4. Effect of IL-10 on normal human monocytes.

A. Effect of IL-10 on p38 and p42/44 MAP kinase phosphorylation in purified human monocytes. Purified normal human monocytes (1.0 x 10^6/ml) were pretreated with either SB202190 or PD98059 at varying concentrations ranging from 0 to 50 μM for 2 hr prior to IL-10 (10 ng/ml) stimulation for 10 min. Crude protein extracts (50 μg) were subjected to SDS-PAGE followed by Western blot analysis. The membranes were blotted with either anti-phospho-p38 (pp38) antibody or anti-phospho-p42 (pp42) antibody. To control for loading, the membranes were stripped and reprobed with anti-p38 (p38) or anti-p42 (p42) antibodies. B. The p42/44 ERK MAP kinase inhibitor does not prevent IL-10-mediated down-regulation of CD44 expression in normal human monocytes. Monocytes (1.0 x 10^6/ml) were treated with 50 μM PD98059 for 2 hr prior to stimulation with IL-10. After 48 hr, cells were analyzed by flow cytometry for the expression of CD44. The experiment shown is representative of five different experiments.

A.


untreated
IL-10 +
SB202190 (μM)
0
50
pp38
Blotted: α-phospho-p38
p38
Blotted: α-p38
untreated
IL-10 + PD98059 (μM)
0
10
25
50
pp44
pp42
Blotted: α-phospho-p42
p44
p42
Blotted: α-p42

B.


Relative Cell Number
308
0
10⁰
10¹
10²
10³
untreated
IL-10
IL-10 + PD (50 μM)
Log Fluorescence Intensity (CD44 expression)

expression independent of IL-10, IL-10 refractory THP-1 cells were used as a model system. THP-1 cells responded in a similar manner as monocytes with respect to CD44 induction following a 24hr stimulation with either LPS or TNF-α, but did not respond to IL-10 (Fig. 3-5). When I investigated the role of MAP kinases in LPS-induced CD44 expression, my results revealed that LPS induced the activation of p38 and p42/44 ERK MAP kinases; this activation was inhibited by their respective specific kinase inhibitors (Fig. 3-6A). As for primary monocytes, treatment of THP-1 cells with low doses of SB202190 (10 μM) prior to LPS stimulation resulted in only a partial inhibition of CD44 expression (Fig. 3-6B, Table 3-2, $p < 0.05$). Complete inhibition was not observed even if 25 and 50 μM of SB202190 were used (Fig. 3-2B, Table 3-2). In contrast, LPS-induced CD44 expression in THP-1 cells was not inhibited by either SB202474, an inactive analogue of SB202190 (data not shown), nor by the p42/44 inhibitor PD98059 (Fig. 3-6B, Table 3-2), thereby eliminating the role of p42/44 signaling in LPS-induced CD44 expression and suggesting the involvement of p38, albeit in a partial manner.

Similar to normal human monocytes, LPS may induce CD44 expression in THP-1 cells via activation through either the CD14-mediated pathway or by endogenously produced TNF-α. LPS-induced TNF-α production has been shown to be mediated, at least in part, via the activation of p38 MAP kinase in normal human monocytes [(241) and Fig. 3-2]. It was, therefore, of interest to determine whether the inhibition of p38 MAP kinase by SB202190 would result in the loss of TNF-α production in THP-1 cells concomitant with CD44 inhibition. The results show that SB202190 did not inhibit LPS-induced TNF-α production in THP-1 cells (Fig. 3-6C, and Table 3-2).

Fig. 3-5. LPS and TNF-α enhance CD44 expression in THP-1 cells.

THP-1 cells (0.5 x 10^6/ml) were treated with LPS (1 μg/ml), IL-10 (10 ng/ml), or TNF-α (10 ng/ml) for 24 hr. Following the stimulation, cells were examined for CD44 surface expression by flow cytometry. The experiment shown is representative of five different experiments.

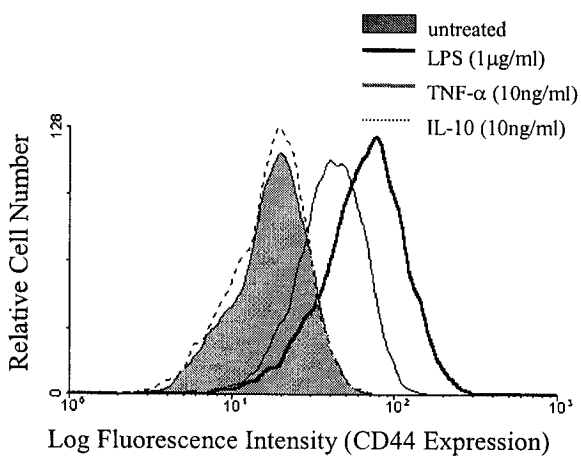

untreated
LPS (1μg/ml)
TNF-α (10ng/ml)
IL-10 (10ng/ml)
Relative Cell Number
128
0
10⁰
10¹
10²
10³
Log Fluorescence Intensity (CD44 Expression)

Fig. 3-6. Effect of LPS on THP-1 cells.

A.LPS stimulation induces p38 and p42/44 MAP kinase phosphorylation in THP-1 cells. Cells (1.0 x 10^6/ml) were pretreated with either SB202190 or PD98059 at varying concentrations ranging from 0 to 50 μM for 2 hr prior to LPS (1 μg/ml) stimulation for 10 min. Crude protein extracts (50 μg) were subjected to SDS-PAGE followed by Western blot analysis. The membranes were blotted with either anti-phospho-p38 (pp38) antibody or anti-phospho-p42 (pp42) antibody. To control for loading, the membranes were stripped and reprobed with anti-p38 (p38) or anti-p42 (p42) antibodies. The western blot shown is a representative of five different experiments. Effect of p38 and p42/44 MAP kinase inhibitors on LPS-induced CD44 expression (B) and TNF-α production (C) in THP-1 cells. Cells (0.5 x 10^6/ml) were treated with various concentrations of either SB202190 or PD98059 for 2 hr prior to stimulation with LPS. After 24 hr, cells were analyzed by flow cytometry for the expression of CD44. Supernatants were harvested and analyzed for TNF-α production by ELISA. ELISA data are represented as a mean ± SD of five different experiments.

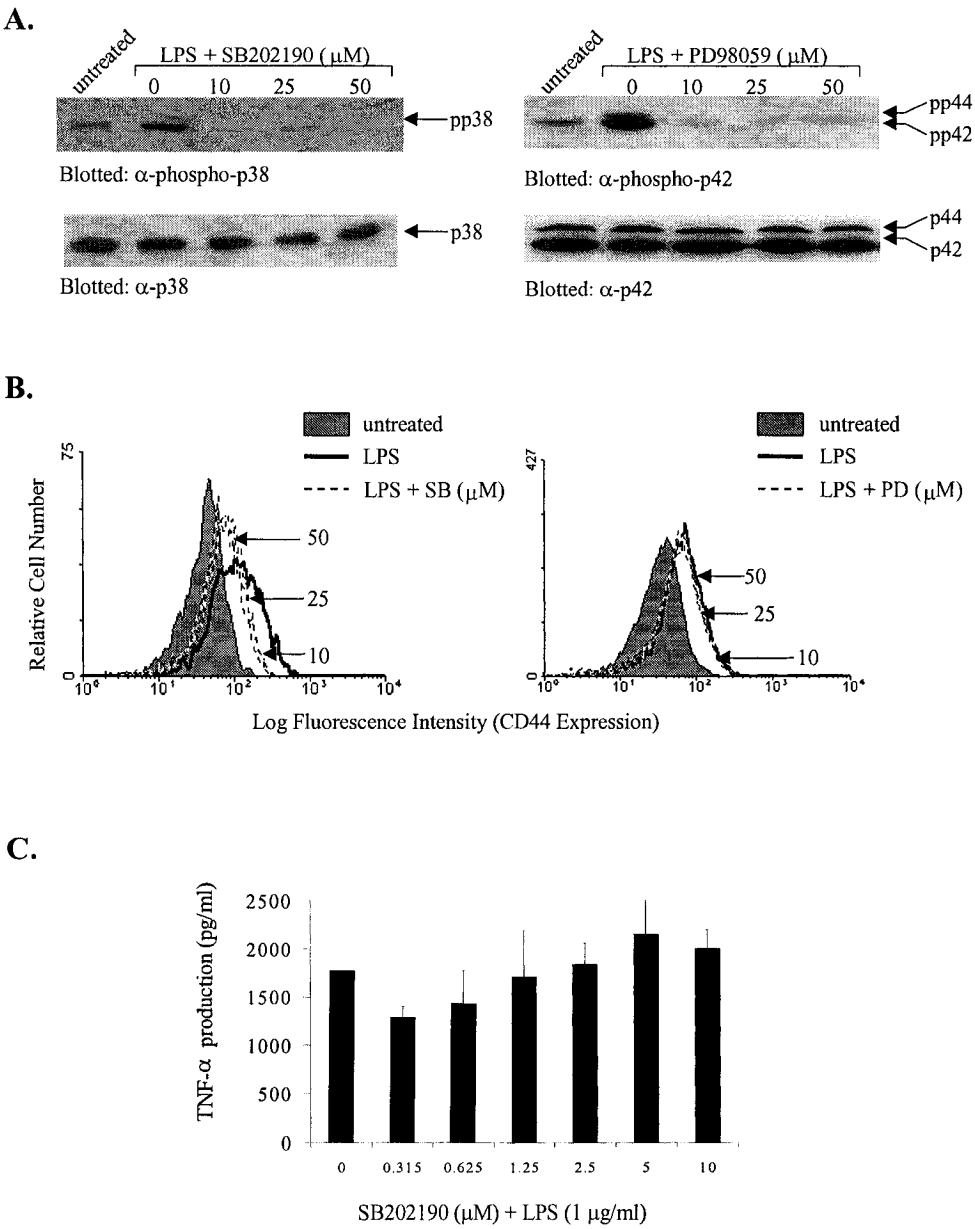

A.
untreated
LPS + SB202190 (μM)
0
10
25
50
pp38
Blotted: α-phospho-p38
p38
Blotted: α-p38
LPS + PD98059 (μM)
pp44
pp42
Blotted: α-phospho-p42
p44
p42
Blotted: α-p42
B.
untreated
LPS
LPS + SB (μM)
LPS + PD (μM)
Relative Cell Number
50
25
10
Log Fluorescence Intensity (CD44 Expression)
C.
TNF-α production (pg/ml)
2500
2000
1500
1000
500
0
0.315
0.625
1.25
2.5
5
SB202190 (μM) + LPS (1 μg/ml)

Table 3-2: Effects of p38 and p42/44 MAPK inhibitors on LPS- and TNF-α–induced CD44 expression in THP-1 cells.

Treatment	Media	CD44 expression (MCF) following stimulation in the presence of MAPK Inhibitors (μM)				
		0	5	10	25	50
LPS + SB202190	61.3 ± 12.1	309.3 ± 50.0 *	395.4 ± 4.7	265.0 ± 77.8	151.3 ± 13.1 *	134.0 ± 14.2 *
LPS + PD98059	58.0 ± 10.7	277.6 ± 15.9 *	267.2 ± 15.4	280.0 ± 42.7	257.8 ± 32.5	257.3 ± 34.5
TNF-α + SB202190	46.3 ± 8.8	86.3 ± 12.3 *	83.5 ± 4.5	78.0 ± 12.1	72.5 ± 9.6 *	57.3 ± 11.5 *
TNF-α + PD98059	37.8 ± 5.3	87.8 ± 10.6 *	76.5 ± 3.5	70.1 ± 39.8	75.3 ± 3.4	71.9 ± 4.1

THP-1 cells (0.5 x 10^6/ml) were pretreated with SB202190 or PD98059 for 2 hr at the concentrations indicated prior to stimulation with either LPS (1 μg/ml) or TNF-α (10 ng/ml) for 24 hr. CD44 expression was analyzed by flow cytometry (MCF ± SD; n=5). *, p 0.05; ND, not determined.

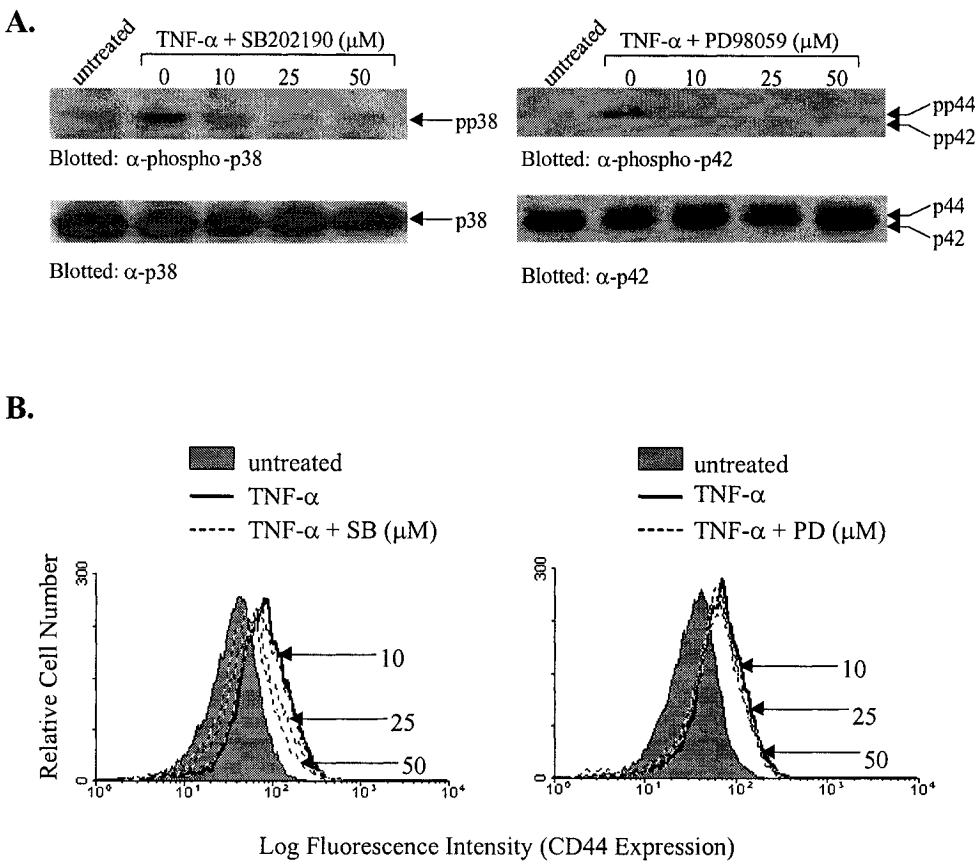
As for normal monocytes, it is likely that LPS-induced CD44 expression in THP-1 cells may be regulated via the activation of the p38 and p42/44 MAP kinase following interaction of endogenously produced TNF-α with the TNF-α receptor. To determine the role of p38 and p42/44 MAP kinases in TNF-α-induced CD44 expression, it was first demonstrated that TNF-α induced the phosphorylation of both p38 and p42/44 in THP-1 cells, and that this phosphorylation was inhibited by their respective inhibitors SB202190 and PD98059 in a dose-dependent manner (Fig. 3-7A). Treatment of THP-1 cells with 25 and 50 μM of SB202190 prior to stimulation with TNF-α resulted in a partial inhibition of CD44 expression (Fig. 3-7B, Table 3-2, $p<0.05$). In contrast, TNF-α-induced CD44 expression in THP-1 cells was not affected by either SB202474 (data not shown) or PD98059 (Fig. 3-7B, Table 3-2). Taken together, these results suggest that both LPS and TNF-α enhance CD44 expression, at least in part, through the activation of p38 MAP kinase in both primary monocytes and THP-1 cells.

JNK MAP kinase plays a distinct role in LPS-induced but not in TNF-α-induced CD44 expression in THP-1 cells

The partial involvement of p38 and the lack of involvement of p42/44 MAP kinases in LPS- and TNF-α-induced CD44 expression lead to the examination of the role of JNK, the third major member of the MAP kinase family. To assess the role of JNK, experiments using the glucocorticoid dexamethasone, an inhibitor of JNK activation were performed (243). It was examined whether LPS or TNF-α could induce JNK phosphorylation in THP-1 cells, and whether dexamethasone could inhibit its phosphorylation. Both LPS and TNF-α induced the phosphorylation of JNK in THP-1

Fig. 3-7. Effect of TNF-α on THP-1 cells.

A. TNF-α induces p38 and p42/44 MAP kinase phosphorylation in THP-1 cells. Cells (1.0 x 10^6/ml) were pretreated with either SB202190 or PD98059 at varying concentrations ranging from 0 to 50 μM for 2 hr prior to TNF-α (10 ng/ml) stimulation for 10 min. Crude protein extracts (50 μg) were subjected to SDS-PAGE followed by Western blot analysis. The membranes were blotted with either anti-phospho-p38 (pp38) or anti-phospho-p42 (pp42) antibodies. To control for loading, the membranes were stripped and reprobed with anti-p38 (p38) or anti-p42 (p42) antibodies respectively. B. Effect of p38 and p42/44 MAP kinase inhibitors on TNF-α-induced CD44 expression in THP-1 cells. Cells (0.5x10^6/ml) were pretreated with either SB202190 or PD98059 at varying concentrations ranging from 0 to 50 μM for 2 hr prior to TNF-α (10 ng/ml) stimulation for 24 hr. CD44 expression was then analyzed by flow cytometry. The experiment shown is representative of five different experiments.


A.
untreated
TNF-α + SB202190 (μM)
0
10
25
50
pp38
Blotted: α-phospho-p38
p38
Blotted: α-p38
untreated
TNF-α + PD98059 (μM)
0
10
25
50
pp44
pp42
Blotted: α-phospho-p42
p44
p42
Blotted: α-p42
B.
untreated
TNF-α
TNF-α + SB (μM)
Relative Cell Number
300
0
10
25
50
Log Fluorescence Intensity (CD44 Expression)
untreated
TNF-α
TNF-α + PD (μM)
300
0
10
25
50

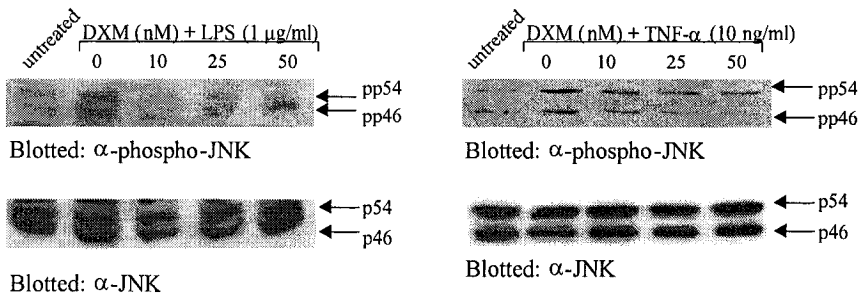
cells and this activation was inhibited in a dose-dependent manner by pretreatment of the cells with increasing doses of dexamethasone (Fig. 3-8A). Furthermore, treatment of THP-1 cells with dexamethasone prior to stimulation with LPS resulted in complete abrogation of CD44 expression in a dose-dependent manner (Fig. 3-8B). In contrast, dexamethasone did not influence TNF-α-induced CD44 expression in THP-1 cells at any concentration (Fig. 3-8B). These results show that JNK signaling may be necessary for LPS-, but not for TNF-α-induced CD44 expression. Recently, a specific JNK inhibitor, SP600125, has become commercially available (244). To confirm the involvement of JNK, THP-1 cells were pretreated with varying concentrations of SP600125 prior to stimulation with LPS or TNF-α. SP600125 inhibited JNK phosphorylation induced by LPS and TNF-α in a dose dependent manner (Fig.3-9A). Consistent with the results obtained with dexamethasone, SP600125 inhibited LPS-induced CD44 expression in a dose dependent manner, whereas TNF-α-induced CD44 expression remained unaffected (Fig 3-9B).

To further determine the role of JNK MAP kinase in LPS-induced CD44 expression, THP-1 cells were transiently transfected with either a dominant-negative SEK1 plasmid (DN SEK1) or with a control plasmid pcDNA3. Activation of SEK1 is responsible for the induction of the JNK signaling cascade and the presence of DN SEK1 effectively blocks JNK activation (245-247). The DN SEK1 kinase becomes activated following stimulation of cells with either LPS or TNF-α, and would compete with the endogenous SEK1 kinase for interaction with JNK, essentially blocking JNK activity. Because the DN SEK1 is expressed in excess, endogenous SEK1 is out-competed for JNK binding. THP-1 cells transfected for 12 hr with either the DN SEK1 or the control

Fig. 3-8. Effect of dexamethasone on CD44 induction in THP-1 cells.

A. Dexamethasone inhibits LPS-induced and TNF-α-induced phosphorylation of JNK kinase in THP-1 cells. THP-1 cells (1.0 x 10^6/ml) were treated with dexamethasone at varying concentrations ranging from 0 to 50 nM for 2 hr prior to stimulation with LPS (1 μg/ml) or TNF-α (10 ng/ml) for 10 min. Total cell proteins were analyzed for phosphorylation of JNK MAP kinase using an anti-phospho-JNK rabbit polyclonal antibody. To control for protein loading, the membranes were stripped and reprobed with anti-JNK rabbit polyclonal antibodies. B. Dexamethasone inhibits LPS-induced but not TNF-α-induced CD44 expression in THP-1 cells. Cells (0.5 x 10^6/ml) were treated with various concentrations of dexamethasone ranging from 0 to 50 nM for 2 hr prior to stimulation with LPS followed by flow cytometric analysis of CD44 expression. The experiment shown is representative of five different experiments.

A.


untreated
DXM (nM) + LPS (1 μg/ml)
0
10
25
50
pp54
pp46
Blotted: α-phospho-JNK
p54
p46
Blotted: α-JNK
untreated
DXM (nM) + TNF-α (10 ng/ml)
0
10
25
50
pp54
pp46
Blotted: α-phospho-JNK
p54
p46
Blotted: α-JNK

B.

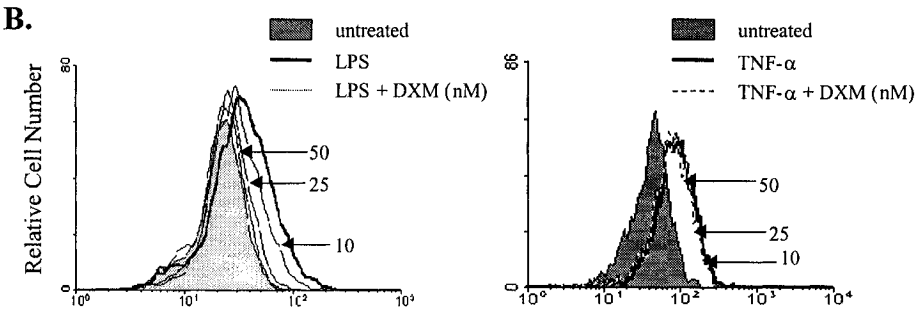
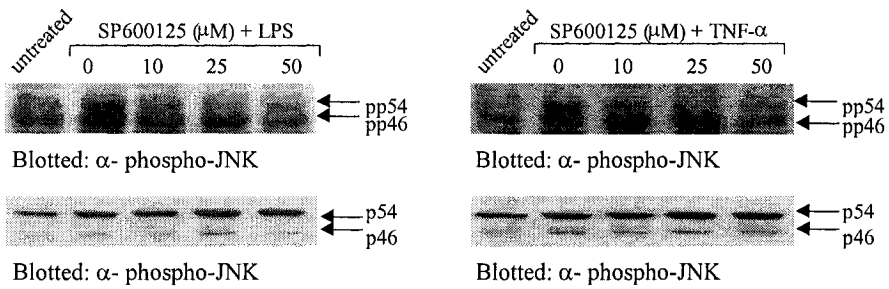

Relative Cell Number
untreated
LPS
LPS + DXM (nM)
50
25
10
untreated
TNF-α
TNF-α + DXM (nM)
50
25
10
Log Fluorescence Intensity (CD44 Expression)

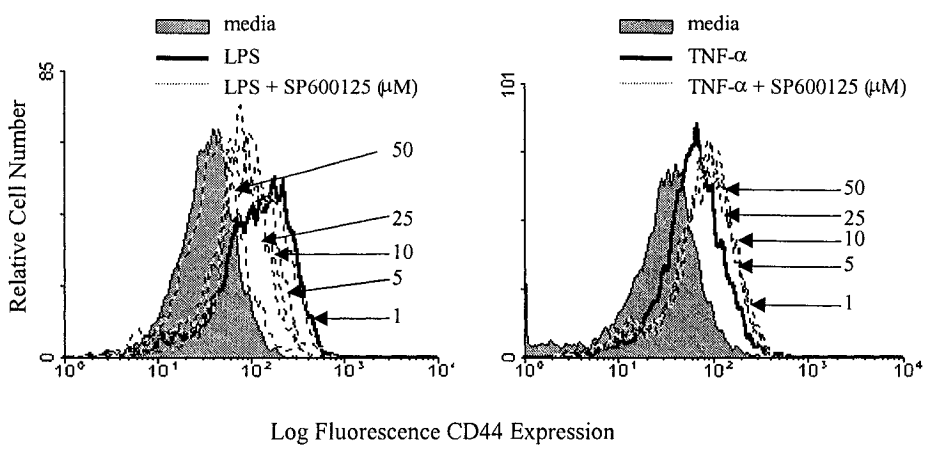
Fig. 3-9. Effect of SP600125 on CD44 induction.

A. SP600125 inhibits LPS-induced and TNF-α-induced phosphorylation of JNK MAPK in THP-1 cells. THP-1 cells (1.0 x 10^6/ml) were treated with SP600125 at varying concentrations ranging from 0 to 50 μM for 2 hr prior to stimulation with LPS (1 μg/ml) or TNF-α (10 ng/ml) for 10 min. Total cell proteins were analyzed for phosphorylation of JNK MAP kinase using an anti-phospho-JNK rabbit polyclonal antibody. To control for protein loading, the membranes were stripped and reprobed with anti-JNK rabbit polyclonal antibodies. B. SP600125 inhibits LPS-induced but not TNF-α-induced CD44 expression in THP-1 cells. Cells (0.5 x 10^6/ml) were treated with various concentrations of SP600125 ranging from 0 to 50 μM for 2 hr prior to stimulation with LPS followed by flow cytometric analysis of CD44 expression. The experiment shown is representative of two different experiments.

A.


untreated
SP600125 (μM) + LPS
0
10
25
50
pp54
pp46
Blotted: α- phospho-JNK
p54
p46
Blotted: α- phospho-JNK
untreated
SP600125 (μM) + TNF-α
0
10
25
50
pp54
pp46
Blotted: α- phospho-JNK
p54
p46
Blotted: α- phospho-JNK

B.


media
LPS
LPS + SP600125 (μM)
Relative Cell Number
85
0
50
25
10
5
1
media
TNF-α
TNF-α + SP600125 (μM)
101
0
50
25
10
5
1
Log Fluorescence CD44 Expression

plasmid which does not contain any inserted DNA, were stimulated with LPS and analyzed for JNK phosphorylation after 10 mins of stimulation and CD44 expression after 24 hr of stimulation. The optimal time period of 12 hr post-transfection prior to LPS-stimulation was chosen based on the results of a series of experiments in which cells were stimulated with LPS 2, 4, 8, 12, or 24 hr post-transfection (data not shown). LPS stimulation of THP-1 cells transfected with the DN SEK1 revealed reduced JNK phosphorylation compared to that observed in cells transfected with the control plasmid (Fig 3-10A). LPS stimulation of THP-1 cells transfected with the DN SEK1 plasmid revealed significantly lower expression of CD44 compared to that of the LPS-stimulated cells transfected with the control vector (Fig. 3-10B). In contrast, transfection of THP-1 cells with DN SEK1 did not impact TNF-α- induced CD44 expression (Fig. 3-10B). The effect of DN SEK1 on CD44 expression was specific since the expression of surface CD14 and IL-10 production remained unaffected following LPS stimulation (data not shown). These results suggest a distinct role for JNK-mediated signals in LPS-induced CD44 expression in THP-1 cells. In contrast, TNF-α-induced regulation of CD44 expression did not involve JNK activation in these cells.

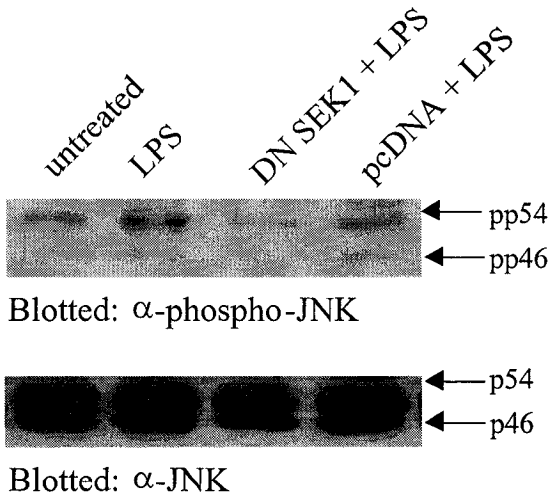
Egr-1 binding to the CD44 promoter in LPS-stimulated THP-1 cells is regulated by JNK MAPK

Egr-1 has been shown to play a role in CD44 expression in human B cells and endothelial cells (248,249). Although the role of Egr-1 in the regulation of CD44 expression in human monocytic cells has not been demonstrated, it was of interest to investigate whether LPS and TNF-α induce Egr-1 activation in THP-1 cells and whether LPS-induced Egr-1 activation can be inhibited by dexamethasone and a specific inhibitor of

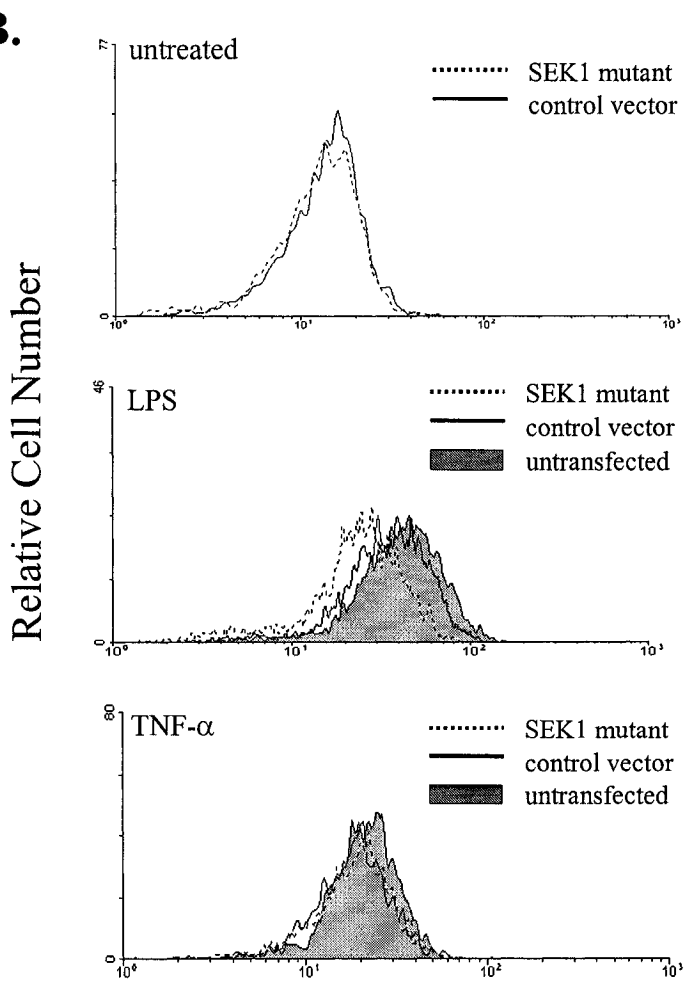
Fig. 3-10. Effect of DN SEK1 transfection on CD44 induction.

A. JNK phosphorylation is inhibited by LPS stimulation in DN SEK1 transfected THP-1 cells. Cells were transfected with either a DN SEK1 kinase or with control vector followed by stimulation with LPS for 10 min. Crude protein extracts (50 μg) were subjected to SDS-PAGE analysis followed by Western blot analysis. The membranes were blotted with an anti-phospho JNK rabbit polyclonal antibody, and to control for equal loading of proteins, the membranes were stripped and reprobed with anti-JNK rabbit polyclonal antibody. B. A Dominant-negative mutant of MKK-4 selectively inhibits LPS-induced, but not TNF-α-induced, CD44 expression in THP-1 cells. THP-1 cells were transfected with either a dominant-negative SEK1 kinase, or with a control vector. Transfected cells were stimulated with either LPS (1 μg/ml) or TNF-α (10 ng/ml) for 24 hr. Following the incubations, cells were analyzed by flow cytometry for CD44 surface expression. The experiment shown is representative of three different experiments.

A.


untreated
LPS
DN SEK1 + LPS
pcDNA + LPS
pp54
pp46
Blotted: α-phospho-JNK
p54
p46
Blotted: α-JNK

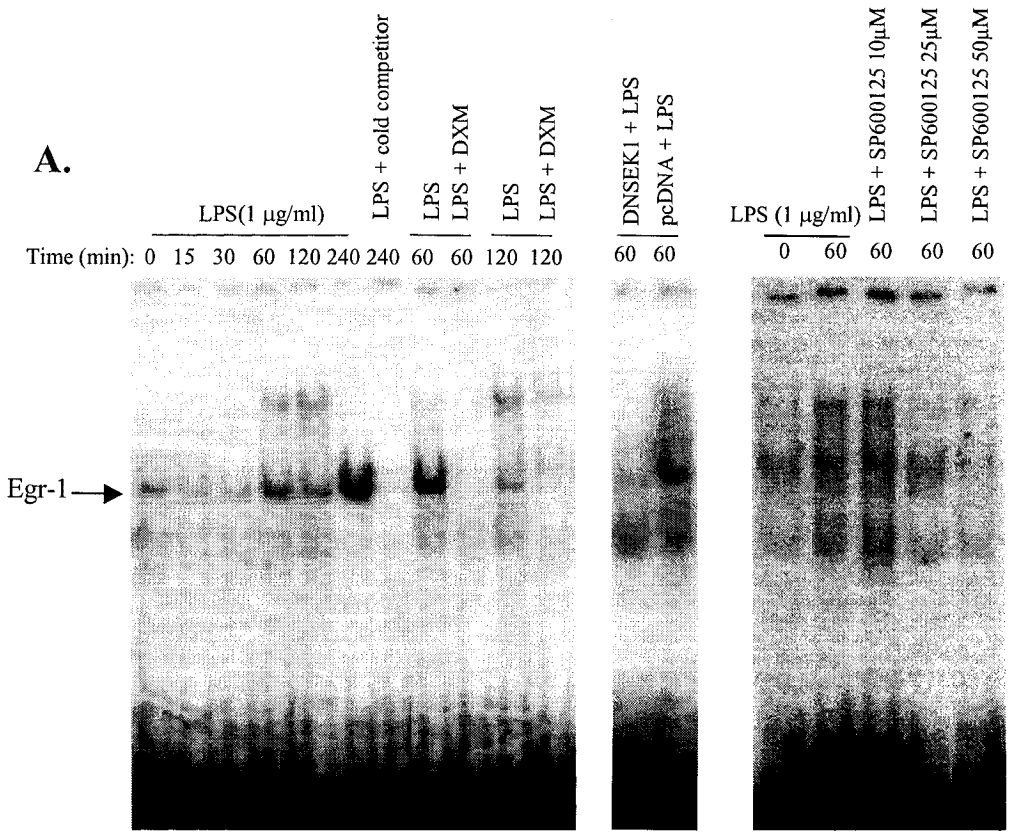
B.

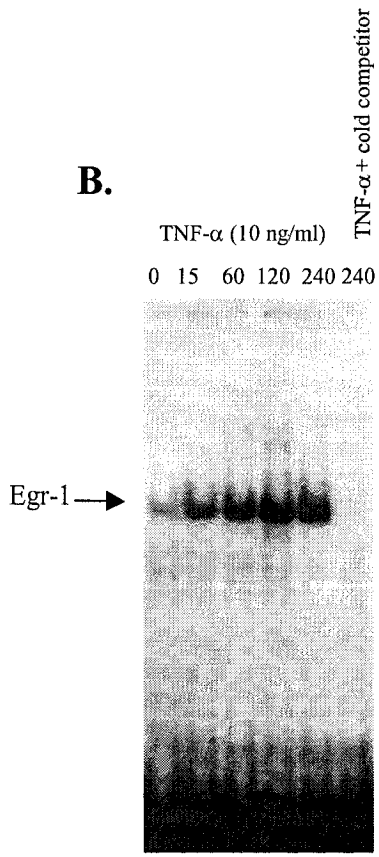

Relative Cell Number
untreated
SEK1 mutant
control vector
LPS
SEK1 mutant
control vector
untransfected
TNF-α
SEK1 mutant
control vector
untransfected
Log Fluorescence Intensity (CD44 Expression)

JNK. Cells were stimulated with LPS or TNF-α over a period of time ranging from 0 to 240 min and the nuclear extracts were analyzed for binding to the Egr-1 oligonucleotide probe by a gel shift assay. Maximum binding of Egr-1 to the Egr-1 oligonucleotide sequence occurred at 60 to 240 min following stimulation with LPS and TNF-α (Fig. 3-11A, B). A major band was observed that corresponded to the Egr-1 DNA-protein complex that was blocked by competition with cold Egr-1 oligonucleotides, indicating its specificity. Incubation of THP-1 cells with dexamethasone or SP600125 for 2 hr prior to stimulation with LPS inhibited Egr-1 binding to the oligonucleotide containing the Egr-1 sequence (Fig 3-11A). It was also shown that LPS stimulation of THP-1 cells transfected with the DN SEK1, in contrast to cells transfected with the control plasmid, showed decreased binding of Egr-1 to the Egr-1 oligonucleotide sequence (Fig 3-11A). Since treatment of THP-1 cells with dexamethasone, SP600125 as well as transfection with DN SEK1 plasmid did not affect TNF-α-induced CD44 expression in my studies, the effect of dexamethasone or SP600125 on TNF-α–induced Egr-1 binding activity was not investigated. These results show that dexamethasone and SP600125 regulate LPS-induced Egr-1 activation, suggesting a role for JNK in Egr-1 activation and possibly for CD44 induction in monocytic cells following LPS stimulation.

Fig. 3-11. Dexamethasone, dominant-negative SEK 1, and SP600125 inhibit LPS-induced activation of the Egr-1 transcription factor in THP-1 cells.

THP-1 cells were stimulated with LPS (1 μg/ml) (A) or TNF-α (10 ng/ml) (B) for various times ranging from 15 to 240 min followed by centrifugation and collection of cell pellets. To determine the effects of dexamethasone and SP600125 on LPS-induced activation of Egr-1, cells were treated with dexamethasone (50 nM) or SP600125 (10-50μM) for 2 hr prior to stimulation with LPS (1 μg/ml). To determine the effect of JNK on Egr-1 activation, THP-1 cells were transfected with either a vector containing a DN SEK1 or control vector, followed by treatment with LPS for 60 min. To perform the gel shift assay, nuclear extracts were harvested from the cell pellets obtained at each time point. Nuclear extracts containing 5 μg of protein were incubated for 1 hr with ^{32}P-labeled oligonucleotides corresponding to the consensus sequence for Egr-1. To determine the specificity of Egr-1 transcription factor binding, the nuclear extracts were incubated with 100 fold unlabeled oligonucleotides (cold competitor) corresponding to the consensus sequence of Egr-1. The complexes were subjected to electrophoresis followed by autoradiography. The experiment shown is representative of three experiments.


A.
LPS(1 μg/ml)
LPS + cold competitor
LPS
LPS + DXM
LPS
LPS + DXM
DNSEK1 + LPS
pcDNA + LPS
LPS (1 μg/ml)
LPS + SP600125 10μM
LPS + SP600125 25μM
LPS + SP600125 50μM
Time (min): 0 15 30 60 120 240 240 60 60 120 120 60 60 0 60 60 60 60
Egr-1


B.
TNF-α (10 ng/ml)
TNF-α+ cold competitor
0 15 60 120 240 240
Egr-1

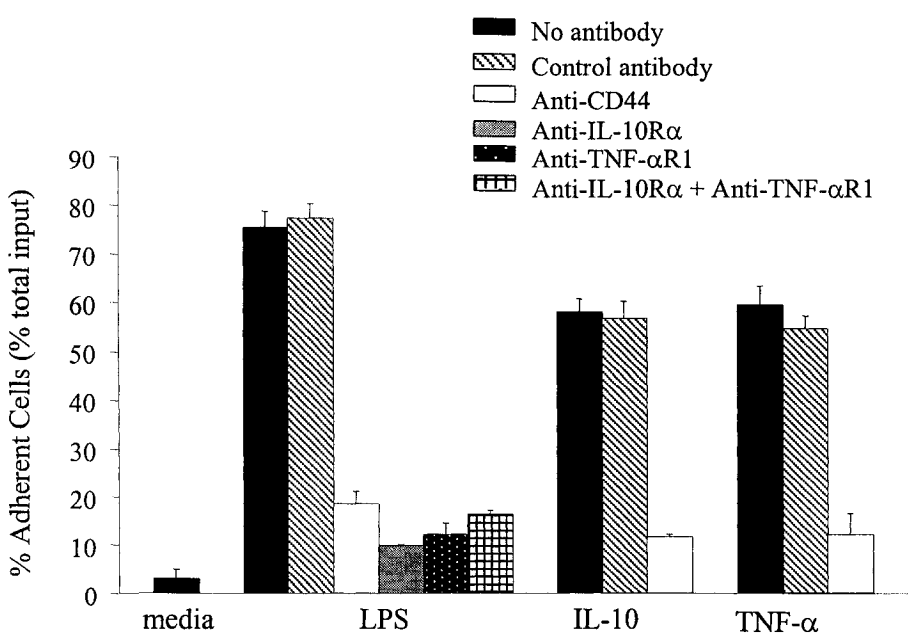
Objective 1 B: Role of MAPKs in the generation of Functionally Active Hyaluronan-Adhesive CD44 in LPS- and TNF-α-Stimulated Human Monocytic Cells

LPS-induced CD44-mediated HA-binding is regulated by endogenously produced IL-10 and TNF-α in normal human monocytes

In the preceding section of my thesis, I demonstrated the role of JNK in the regulation of CD44 expression in LPS-stimulated but not in TNF-α-stimulated monocytic cells. In this section of my thesis, I investigated the role of MAPKs in the generation of functionally active HA-adhesive CD44 in LPS-stimulated monocytic cells. I confirmed that stimulation of normal monocytes with LPS enhanced CD44-mediated HA-binding (Fig. 3-12). It has been suggested that TNF-α and IL-10 are the major cytokines that regulate interaction of CD44 with HA in LPS-stimulated human monocytes (26). To determine whether endogenously produced IL-10 and TNF-α regulate LPS-induced CD44-mediated HA-binding, I employed antibodies specific for IL-10Rα (0.1 μg/ml) and TNF-αR1 (5 μg/ml). These antibodies are capable of neutralizing the biological activity of 1 ng/ml of IL-10 and TNF-α, respectively, as described previously (139), and as recommended by the manufacturer. Anti-IL-10Rα and anti-TNF-αR1 antibodies inhibited the LPS-induced CD44-HA-binding (Fig. 3-12). Furthermore, stimulation of monocytes with exogenous TNF-α and IL-10 enhanced CD44-HA-binding (Fig. 3-12) in a dose dependent manner (data not shown). To determine whether the binding of cells to HA was mediated through CD44, monocytic cells stimulated with either LPS, TNF-α, or IL-10 were cultured with anti-CD44 antibodies prior to incubation with HA. Treatment of monocytic cells with anti-CD44 antibodies inhibited LPS-, TNF-α-, and IL-10-induced HA-binding (Fig. 3-12).

Fig. 3-12. LPS, IL-10, and TNF-α enhance CD44-mediated HA-binding in normal human monocytes.

Purified normal human monocytes (0.5 x 10^6/ml) were stimulated with LPS (1 μg/ml), IL-10 (10 ng/ml), or TNF-α (10 ng/ml) for 24 hr. The LPS-treated normal human monocytes were also incubated with either anti-TNF-αR1 (5 μg/ml), anti-IL-10Rα (0.1 μg/ml), or with anti-IL-10Rα (0.1μg/ml) and anti-TNF-αR1 (5 μg/ml) together for 24 hr. As negative antibody controls, the cells were also incubated with isotype-matched control antibodies. To control for the specificity of CD44-HA interactions, the cells were incubated with anti-CD44 antibodies that block the CD44-HA interaction. Following stimulation, the cells were analyzed for binding to HA-coated plates. % adherent cells are presented as the mean of triplicate samples ± SD. The results shown are representative of three experiments.


No antibody
Control antibody
Anti-CD44
Anti-IL-10Rα
Anti-TNF-αR1
Anti-IL-10Rα + Anti-TNF-αR1
% Adherent Cells (% total input)
90
80
70
60
50
40
30
20
10
0
media
LPS
IL-10
TNF-α

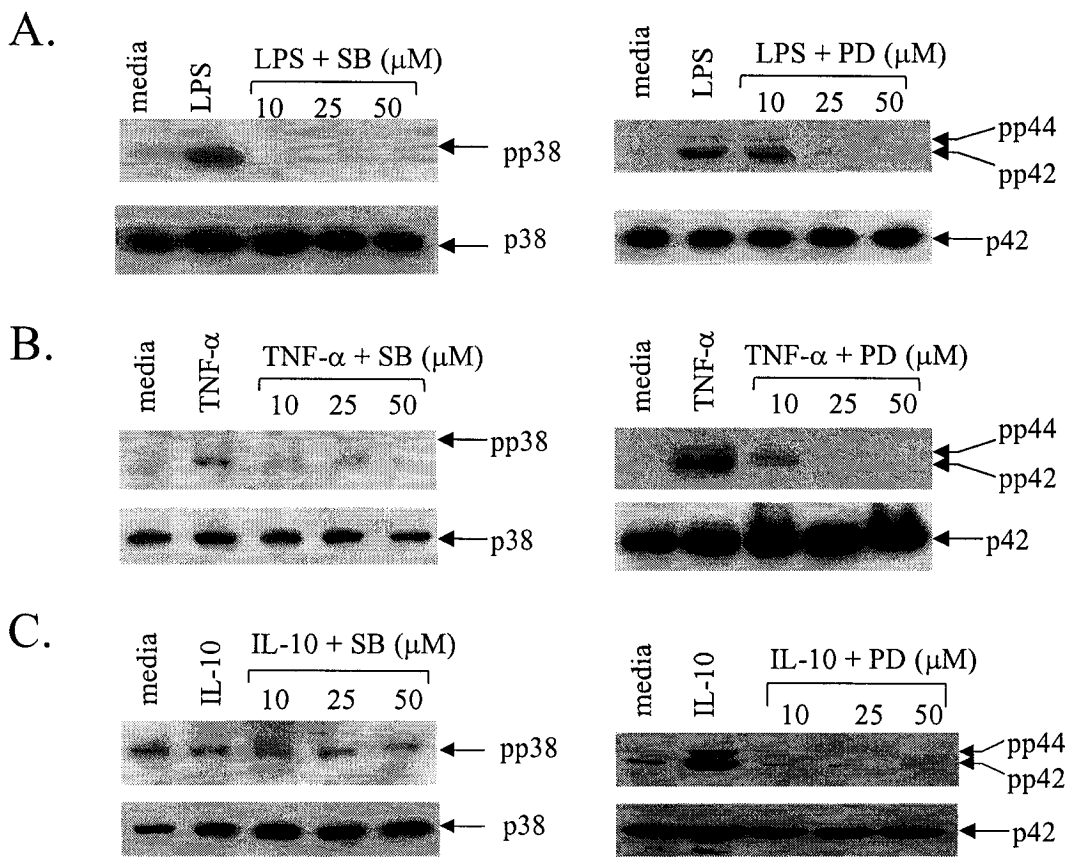
LPS- and TNF-α-induced CD44-mediated HA-binding involves the activation of p38 MAPK

The MAPKs play a major role in LPS- and cytokine-mediated induction of several cell surface receptors including CD44 (139,188,233-237,242). It was, therefore, of interest to identify the members of the MAPK family that are involved in LPS-, IL-10, and TNF-α-induced regulation of CD44-HA-binding in monocytic cells. My results revealed the involvement of p38 and p42/44 MAPKs by examining their activation following stimulation of monocytes either with LPS, TNF-α or IL-10. LPS and TNF-α induced the phosphorylation of both p38 and p42/44 ERKs (Fig. 3-13 A, B). In contrast, IL-10 enhanced the phosphorylation of p42/44 ERKs but had no effect on p38 phosphorylation (Fig. 3-13 C). As mentioned previously, the role of p38 and p42/44 ERKs has been studied through the use of specific kinase inhibitors: SB202190 for p38-, and PD98059 for p42/44-mediated signaling (217). To confirm that in this system SB202190 and PD98059 inhibited the phosphorylation of p38 and ERKs, respectively, freshly isolated monocytes were pretreated with these inhibitors for 2 hr followed by stimulation with either LPS, TNF-α or IL-10 for 10 min. Prior treatment of monocytes with SB202190 or PD98059 resulted in the inhibition of both LPS- and TNF-α-induced p38 and p42/44 ERKs phosphorylation, respectively (Fig. 3-13 A, B). Similarly, pretreatment of monocytes with PD98059 prior to IL-10 stimulation inhibited p42/44 phosphorylation in a dose-dependent manner (Fig. 3-13 C).

To determine the role of p38 and p42/44 MAPKs in LPS-induced CD44-HA-binding, monocytes were pretreated with increasing doses of SB202190 or PD98059 for 2 hr before LPS stimulation for 24 hr. Cells were analyzed for CD44-mediated HA-binding by the HA adhesion assay as described in the materials and methods chapter.

Fig. 3-13. Activation of p38 and p42/44 MAPK phosphorylation in purified normal monocytes in response to LPS, TNF-α, and IL-10.

Cells (1.0 x 10^6/ml) were pretreated with either SB202190 or PD98059 at varying concentrations ranging from 0 to 50 μM for 2 hr prior to (A.) LPS (1 μg/ml), (B.) TNF-α (10 ng/ml), or (C.) IL-10 (10 ng/ml) stimulation for 10 min. Crude protein extracts (50 μg) were subjected to SDS-PAGE followed by Western blot analysis. The membranes were blotted with either anti-phospho-p38 (pp38) antibody or anti-phospho-p42 (pp42) antibody, and to control for protein loading, the membranes were stripped and reprobed with either anti-p38 (p38) or anti-p42 (p42) antibodies respectively. The western blots shown are representative of five different experiments.


A.
media
LPS
LPS + SB (μM)
10 25 50
pp38
p38
media
LPS
LPS + PD (μM)
10 25 50
pp44
pp42
p42
B.
media
TNF-α
TNF-α + SB (μM)
10 25 50
pp38
p38
media
TNF-α
TNF-α + PD (μM)
10 25 50
pp44
pp42
p42
C.
media
IL-10
IL-10 + SB (μM)
10 25 50
pp38
p38
media
IL-10
IL-10 + PD (μM)
10 25 50
pp44
pp42
p42

CD44-HA-binding was significantly inhibited at doses as low as 5 μM of SB202190 (Fig. 3-14). Treatment of monocytes with SB202474, an inactive analogue of SB202190, for 2 hr prior to LPS stimulation did not influence CD44-HA-binding (data not shown), thus confirming the specificity of SB202190. PD98059 did not inhibit CD44 binding at doses up to 50 μM (Fig. 3-14), indicating that the p42/44 pathway is not involved in the regulation of LPS-induced CD44-HA-binding.

To determine whether the inhibition of CD44-HA-binding observed following treatment with SB202190 of LPS-stimulated monocytes was due to the inhibition of endogenous IL-10 and/or TNF-α expression, I evaluated the effects of MAPKs inhibitors on IL-10, and TNF-α production. SB202190, but not PD98059, inhibited LPS-induced IL-10 as well as TNF-α production in a dose-dependent manner (Table 3-3). The same cells were analyzed for HA-binding as well as for IL-10 and TNF-α production. These results suggest that the inhibition of LPS-induced CD44-mediated HA-binding by SB202190 may be, at least in part, due to the decreased endogenous production of TNF-α and/or IL-10.

It is likely that the IL-10 and TNF-α produced following LPS stimulation of monocytes may upregulate CD44-HA-binding by activating either p38 or p42/44 in the case of TNF-α or p42/44 in the case of IL-10. To determine whether p38 and p42/44 MAPKs play a role in TNF-α-induced CD44-HA-binding, monocytes were pretreated with either p38 or p42/44 inhibitors for 2 hrs followed by TNF-α stimulation. Similar to the results obtained for LPS-induced CD44-HA-binding (Fig. 3-14 A), neither SB202474

Fig. 3-14. The p38 MAPK inhibitor modulates LPS- and TNF-α-induced CD44-mediated HA-binding in purified normal monocytes.

Cells (0.5 x 10^6/ml) were pretreated with either SB202190 or PD98059 at varying concentrations ranging from 0 to 50 μM for 2 hr prior to (A) LPS (1 μg/ml), (B) TNF-α (10 ng/ml), or (C) IL-10 (10 ng/ml) stimulation for 24 hr. CD44 mediated-HA-binding was analyzed by the HA adhesion assay. % adherent cells are presented as the mean of triplicate samples ± SD. The results shown are representative of three experiments.

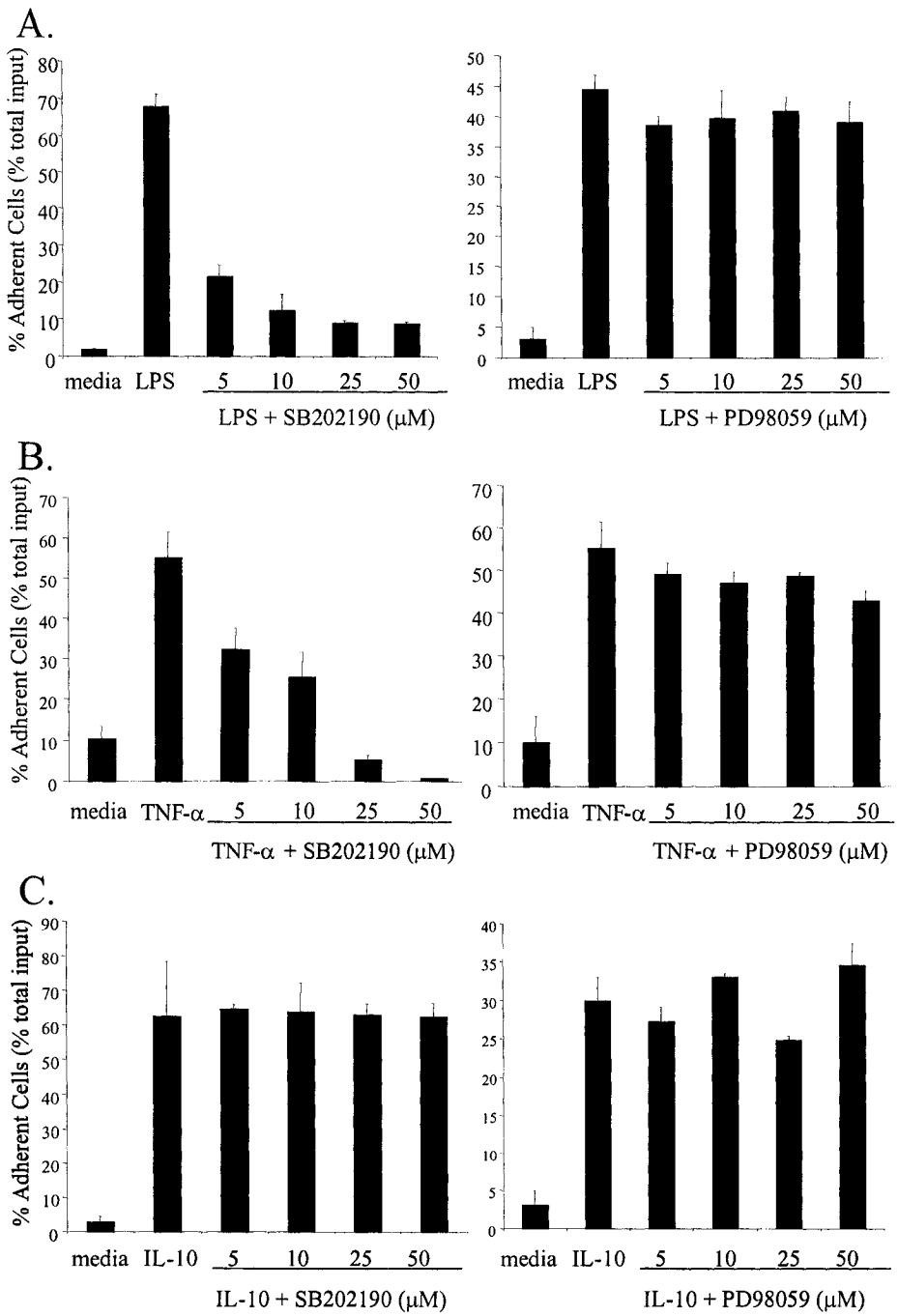

A.
% Adherent Cells (% total input)
media
LPS
5
10
25
50
LPS + SB202190 (μM)
LPS + PD98059 (μM)
B.
TNF-α
TNF-α + SB202190 (μM)
TNF-α + PD98059 (μM)
C.
IL-10
IL-10 + SB202190 (μM)
IL-10 + PD98059 (μM)

Table 3-3: Effect of p38 and p42/44 MAPK inhibitors on LPS-induced TNF-α and IL-10 production (pg/ml) in normal human monocytes.

Cytokine	Treatment	Cytokine production following LPS stimulation in the presence of MAPK Inhibitors (μM)						
		0	0.625	1.25	2.5	5	10	25
IL-10	LPS + SB202190	218.5 ± 13.4	75.5 ± 7.8 *	74.0 ± 2.8 *	83.2 ± 4.2 *	64.1 ± 1.4 *	62.6 ± 2.8 *	41.4 ± 5.7 *
	LPS + PD98059	386.2 ± 11.3	348.5 ± 65.8	397.5 ± 58.7	401.5 ± 14.8	435.0 ± 12.7	438.5 ± 67.9	363.5 ± 54.4
TNF-α	LPS + SB202190	244.5 ± 9.1	76.3 ± 5.1 *	95.7 ± 10.6 *	67.3 ± 6.8 *	72.4 ± 11.8 *	66.1 ± 15.6 *	64.6 ± 7.1 *
	LPS + PD98059	467 ± 15.6	681.5 ± 37.4	662.5 ± 55.9	556.5 ± 68.6	558.5 ± 4.9	485 ± 80.6	475.5 ± 19.1

Normal human monocytes (1.0 x 10^6/ml) were pretreated with SB202190 or PD98059 for 2 hr at the concentrations indicated prior to stimulation with LPS (1 μg/ml) for 24 hr followed by analysis of IL-10 and TNF-α production by ELISA (pg/ml ± SD; n=5). *, p 0.05. Unstimulated cells produced less that 16 pg/ml of IL-10 or TNF-α in the culture supernatants.

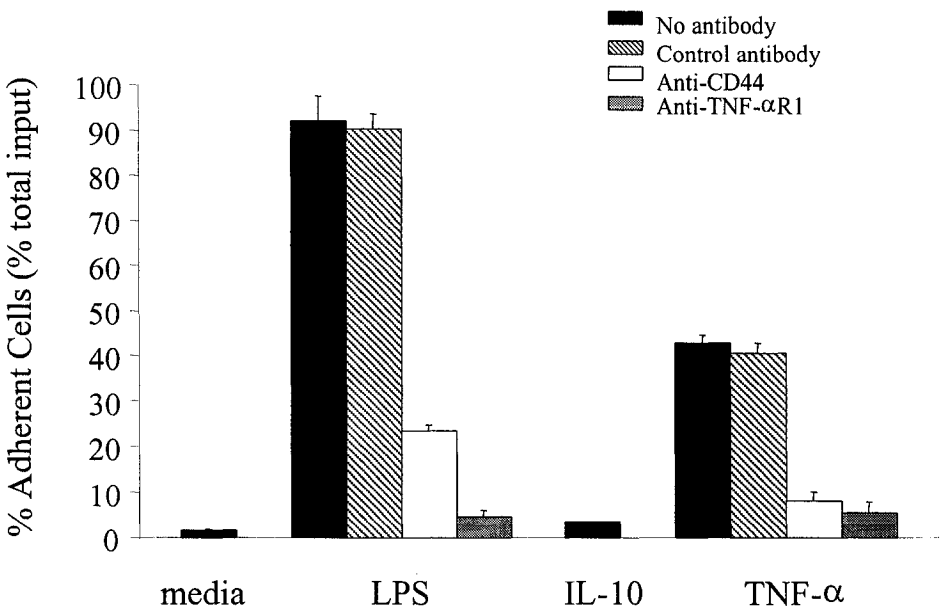
(data not shown) nor PD98059 influenced TNF-α-induced CD44-HA-binding. In contrast, SB202190 significantly inhibited CD44-HA-binding at doses as low as 5 μM and complete inhibition was observed at doses of 25 μM (Fig. 3-14 B). Furthermore, PD98059 did not inhibit IL-10-induced CD44-mediated HA-binding (Fig. 3-14 C). These results suggest that p38 may play a role in both LPS- and TNF-α-induced CD44-mediated HA-binding.

LPS-induced CD44-HA-binding in THP-1 cells is regulated by endogenously produced TNF-α

LPS-induced CD44-HA-binding in normal monocytes is a complex process which is regulated by the interaction of LPS with its CD14/Toll-like receptor (TLR-4) complex, as well as by the interaction between IL-10 and TNF-α with their corresponding receptors (218,225,250). Because of the inherent endogenous production of IL-10 in LPS-stimulated monocytes, the role of p38 and p42/44 MAPK in LPS- and TNF-α-induced CD44-HA-binding could not be easily distinguished. To investigate the role of MAPKs in LPS-induced CD44-HA-binding independent of endogenous IL-10, I again employed IL-10-refractory THP-1 cells as a model system. THP-1 cells responded in a similar manner as monocytes with respect to CD44-HA-binding following stimulation with either LPS or TNF-α (Fig. 3-15). Furthermore, treatment of LPS-stimulated THP-1 cells with anti-TNF-αR1 antibodies inhibited CD44-HA-binding, suggesting that LPS-induced CD44-HA-binding is regulated by endogenously produced TNF-α (Fig. 3-15). In addition, treatment of THP-1 cells with anti-CD44 antibodies inhibited LPS- and TNF-α-induced HA-binding (Fig. 3-15).

Fig. 3-15. LPS and TNF-α enhance CD44-mediated HA-binding in THP-1 cells.

THP-1 cells (0.5 x 10^6/ml) were treated with LPS (1 μg/ml), IL-10 (10 ng/ml), or TNF-α (10 ng/ml) for 24 hr. LPS-treated cells were also incubated with anti-TNF-αR1 antibodies (5 μg/ml). As negative antibody controls, the cells were also incubated with isotype-matched control antibodies. To control for the specificity of CD44-HA interactions, the cells were incubated with anti-CD44 antibodies that block the CD44-HA interaction. Cells were then subjected to the HA adhesion assay. % adherent cells are presented as the mean of triplicate samples ± SD. The results shown are representative of three experiments.


No antibody
Control antibody
Anti-CD44
Anti-TNF-αR1
% Adherent Cells (% total input)
100
90
80
70
60
50
40
30
20
10
0
media
LPS
IL-10
TNF-α

LPS- and TNF-α-induced CD44-HA-binding in THP-1 cells involves differential activation of p38 and p42/44 MAPKs

To examine the role of p38 and p42/44 MAPKs in LPS- and TNF-α-induced CD44-HA-binding, my results show that SB202190 and PD98059 inhibited both the LPS- and TNF-α-induced phosphorylation of p38 and p42/44 MAPKs, respectively (Fig. 3-16). In contrast to the normal monocytes (Fig. 3-14), treatment of LPS-stimulated THP-1 cells with SB202190 did not inhibit CD44-HA-binding. However, PD98059 significantly inhibited CD44-HA-binding at doses as low as 10 μM (Fig 3-17 A). Because LPS treatment of THP-1 cells results in the production of endogenous TNF-α, we examined whether SB202190 or PD98059 affected TNF-α expression. Supernatants from cells treated with either SB202190 or PD98059 obtained from the above-mentioned experiments were analyzed for TNF-α production. The results show that in contrast to SB202190, PD98059 significantly inhibited LPS-induced TNF-α production at doses as low as 1 μM (Table 3-4).

The above results suggested that the induction of CD44-mediated HA-binding observed in LPS-stimulated THP-1 cells is regulated by endogenously produced TNF-α via the activation of p42/44 MAPK. This was confirmed by analysis of CD44-HA-binding in LPS-stimulated cells in which TNF-α production was blocked by graded doses of PD98059 followed by reconstitution with a constant concentration of TNF-α (10 ng/ml) (Fig. 3-17 B). Alternatively, LPS-stimulated cells in which TNF-α production was blocked by pretreatment with a constant dose of 25 μM of PD98059, could bind HA when exposed to increasing concentrations of TNF-α (0.01-10 ng/ml) (Fig. 3-17 B). The

Fig. 3-16. LPS and TNF-α stimulation induce p38 and p42/44 MAPK phosphorylation in THP-1 cells.

Cells (1.0 x 10^6/ml) were pretreated with either SB202190 or PD98059 at varying concentrations ranging from 0 to 50 μM for 2 hr prior to (A) LPS (1 μg/ml) or (B) TNF-α (10 ng/ml) stimulation for 10 min. Crude protein extracts (50 μg) were subjected to SDS-PAGE followed by Western blot analysis. The membranes were blotted with either anti-phospho-p38 (pp38) antibody or anti-phospho-p42 (pp42) antibody. To control for protein loading, the membranes were stripped and reprobed with anti-p38 (p38) or anti-p42 (p42) antibodies, respectively. The western blot shown is a representative of five different experiments.

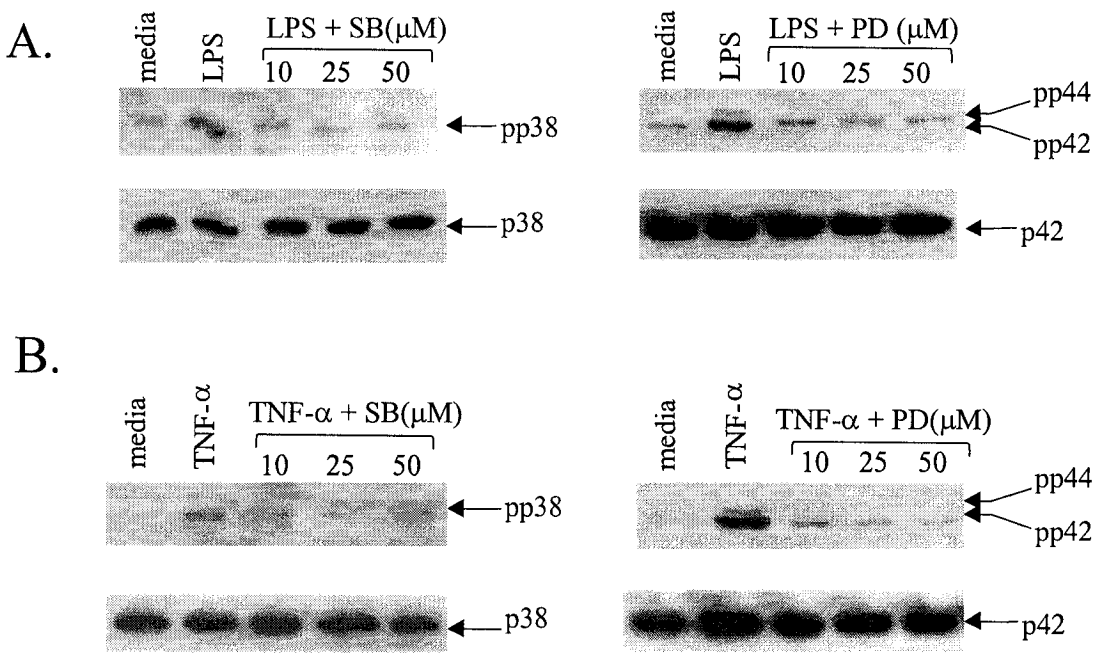

A.
media
LPS
LPS + SB(μM)
10 25 50
pp38
p38
media
LPS
LPS + PD (μM)
10 25 50
pp44
pp42
p42
B.
media
TNF-α
TNF-α + SB(μM)
10 25 50
pp38
p38
media
TNF-α
TNF-α + PD(μM)
10 25 50
pp44
pp42
p42

Fig. 3-17. Role of p38 and TNF-α in LPS-induced CD44-HA binding

A. Effect of p38 and p42/44 MAPK inhibitors on LPS-induced CD44-mediated HA-binding in THP-1 cells. Cells (0.5 x 10^6/ml) were treated with various concentrations of either SB202190 or PD98059 for 2 hr prior to stimulation with LPS. After 24 hr, cells were analyzed by the HA adhesion assay. % adherent cells are presented as the mean of triplicate samples ± SD. The results shown are representative of three experiments.
B. Effect of exogenous TNF-α on PD98059-and LPS-treated THP-1 cells. Left panel: THP-1 cells ($0.5x10^6$/ml) were pretreated with PD98059 at varying concentrations ranging from 0 to 50 μM for 2 hr prior to LPS (1 μg/ml) or TNF-α (10 ng/ml) stimulation for 24 hr. CD44-HA-binding was analyzed by the HA adhesion assay. Right panel: THP-1 cells ($0.5x10^6$/ml) were pretreated with 50 μM of PD98059 for 2 hr prior to LPS (1 μg/ml) and varying doses of TNF-α (0.01 to 10 ng/ml) stimulation for 24 hr. CD44-HA-binding was then analyzed by the HA adhesion assay. % adherent cells are presented as the mean of triplicate samples ± SD. The results shown are representative of three experiments. C. Effect of p38 and p42/44 MAPK inhibitors on TNF-α-induced CD44-mediated HA-binding in THP-1 cells. Cells ($0.5x10^6$/ml) were pretreated with either SB202190 or PD98059 at varying concentrations ranging from 0 to 50 μM for 2 hr prior to TNF-α (10 ng/ml) stimulation for 24 hr. CD44-HA-binding was analyzed by the HA adhesion assay. % adherent cells are presented as the mean of triplicate samples ± SD. The results shown are representative of three experiments.

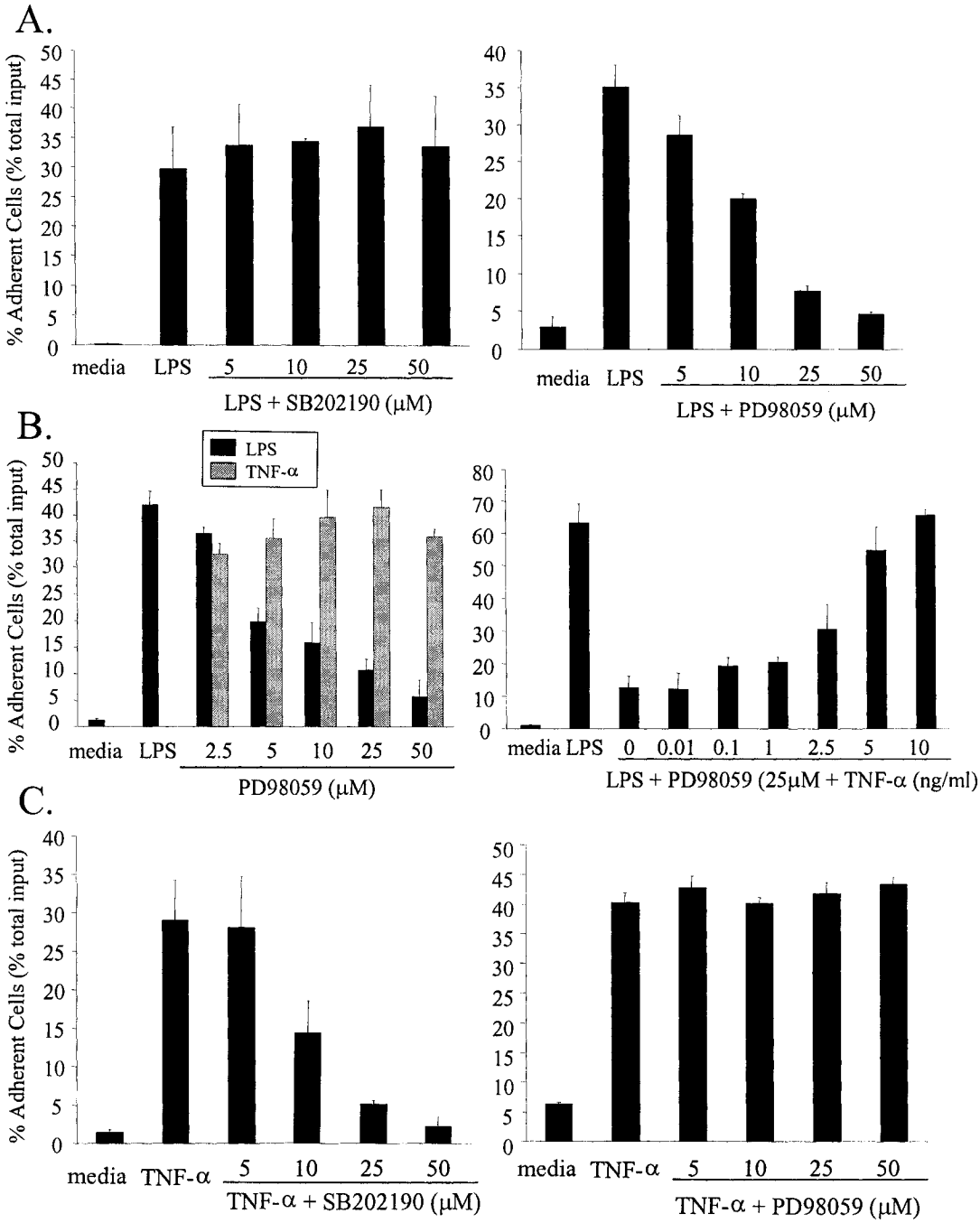

A.
% Adherent Cells (% total input)
media
LPS
5
10
25
50
LPS + SB202190 (μM)
LPS + PD98059 (μM)
B.
LPS
TNF-α
PD98059 (μM)
0
0.01
0.1
1
2.5
LPS + PD98059 (25μM + TNF-α (ng/ml)
C.
TNF-α
TNF-α + SB202190 (μM)
TNF-α + PD98059 (μM)

Table 3-4: Effects of p38 and p42/44 MAPK inhibitors on LPS-induced TNF-α production in THP-1 cells.

Treatment	TNF-α production following LPS stimulation in the presence of MAPK Inhibitors (μM)						
	0	0.625	1.25	2.5	5	10	25
LPS + SB202190	1772.2 ± 26.4	1428.6 ± 138.5	1707.4 ± 282.1	1837.6 ± 230.7	2156.2 ± 225.6	2006.8 ± 195.1	2677.1 ± 36.4
LPS + PD98059	2293.6 ± 207.1	964.4 ± 29.6 *	109.6 ± 24.4 *	72.1 ± 10.6 *	73 .9 ± 8.4 *	69.8 ± 69.9 *	66.1 ± 5.9 *

THP-1 cells (0.5 x 10^6/ml) were pretreated with SB202190 or PD98059 for 2 hr at the concentrations indicated prior to stimulation with LPS (1 μg/ml) for 24 hr. TNF-α production was analyzed by ELISA (pg/ml ± SD; n=5). *, p 0.05. Unstimulated cells produced less that 16 pg/ml of TNF-α in the culture supernatants.

results show that exogenous addition of TNF-α reversed the PD98059-induced inhibition of CD44-HA-binding, suggesting that LPS-activated p42/44 MAPK may play a critical role in TNF-α production which in turn may induce CD44-mediated HA binding.

Studies performed to delineate the involvement of MAPKs in TNF-α-induced CD44-HA-binding revealed that similar to the results observed in normal monocytes, CD44-mediated HA binding in THP-1 cells was regulated by p38 MAPK. Treatment of THP-1 cells with SB202190 prior to stimulation with TNF-α resulted in inhibition of CD44-mediated HA-binding in a dose dependent manner (Fig. 3-17 C). In contrast, TNF-α-induced CD44-HA-binding was not affected by either SB202474 (data not shown) or PD98059 (Fig. 3-17 C). These results suggest that although LPS-induced CD44-HA-binding is dependent on endogenously produced TNF-α via p42/44 activation, TNF-α-induced CD44-mediated HA-binding requires the activation of p38 MAPK.

Involvement of sialidase in the regulation of LPS- and TNF-α-induced CD44-HA-binding

It was recently shown that an inducible lysosomal sialidase could regulate LPS-induced CD44-HA-binding in THP-1 cells (29). In view of my results suggesting the involvement of p38 and p42/44 MAPK in TNF-α- and LPS-induced CD44-HA-binding in THP-1 cells, respectively, I hypothesized that LPS- and TNF-α-induced CD44-HA-binding is regulated by sialidase, whose activity may be influenced by the MAPK signal transduction pathway. Therefore, I first investigated whether LPS- and TNF-α-induced CD44-HA-binding requires the induction of sialidase activity. I treated normal monocytes and THP-1 cells with increasing doses of purified sialidase in order to determine if desialidation of the pre-existing surface CD44 could induce an increase in

CD44-HA-binding. The results show that doses as low as 0.1 U/ml of sialidase were sufficient to induce CD44-HA-binding in both normal monocytes and in THP-1 cells (Fig. 3-18). To determine the validity of the sialidase preparation, the sialidase enzyme (0.8 U/ml) was boiled for 10 min prior to incubation with the ^{51}Cr labeled monocytes as well as THP-1 cells. The results show that the boiling of sialidase significantly inhibited HA binding as compared to the binding observed with the native sialidase (Fig. 3-18). We also treated both monocytes and THP-1 cells with 0.8 U/ml of sialidase in the presence and absence of either anti-CD44 antibodies or isotype matched control antibodies. Anti-CD44 antibodies inhibited HA binding induced by the treatment of cells with sialidase suggesting that sialidase-induced HA binding is mediated via the CD44-HA interactions (Fig.3-18). To determine that sialidase inhibitor could neutralize the biological activity of the sialidase preparation, 0.8 U/ml of sialidase was treated with either 0.25, 1.0 or 4.0 mM of sialidase inhibitor 2-Neu-Ac for 30 min followed by incubation with normal monocytes and THP-1 cells. The results show that concentrations as low as 0.25 mM of 2-Neu-Ac significantly neutralized HA binding in both THP-1 cells (Fig. 3-18B) and monocytes (Fig. 3-18A) compared to the binding observed following treatment of cells with the sialidase enzyme alone.

To demonstrate that LPS- and TNF-α-induced sialidase activity enhances CD44-HA-binding, normal monocytes and THP-1 cells were treated with sialidase inhibitor, 2,3-dehydro-2-deoxy-N-acetylneuroaminic acid (2-Neu-Ac), for 2 hr prior to stimulation with either LPS or TNF-α, followed by analysis of HA-binding. The sialidase inhibitor prevented both LPS- and TNF-α-induced CD44-HA-binding in a dose-dependent manner in both normal monocytes and THP-1 cells (Fig. 3-19).

Fig. 3-18. Role of sialidase in LPS- and TNF-α-induced CD44-HA binding.

Addition of exogenous sialidase enzyme induces CD44-mediated HA-binding in purified normal monocytes (A) and THP-1 cells (B). Left panel: Unstimulated monocytes and THP-1 cells (0.5 x 10^6/ml) were labeled with Cr^{51} as described in the experimental procedures section following which the cells were treated with increasing doses of sialidase enzyme (0.1-0.8 U/ml) for 30 min. The cells were washed prior to addition to the HA-coated plate. In addition, sialidase treated cells were incubated in the presence of either anti-CD44 antibodies or isotype matched control antibodies. Right Panel: To determine the validity of the sialidase preparation, the sialidase enzyme (0.8 units/ml) was boiled for 10 min prior to incubation with the ^{51}Cr labeled cells. To determine that the sialidase inhibitor could neutralize the biological activity of sialidase preparation, 0.8 units/ml of sialidase was treated with either 0.25, 1.0 or 4.0 mM of sialidase inhibitor 2-Neu-Ac for 30 min. % adherent cells are presented as the mean of triplicate samples ± SD. The results shown are representative of three experiments.

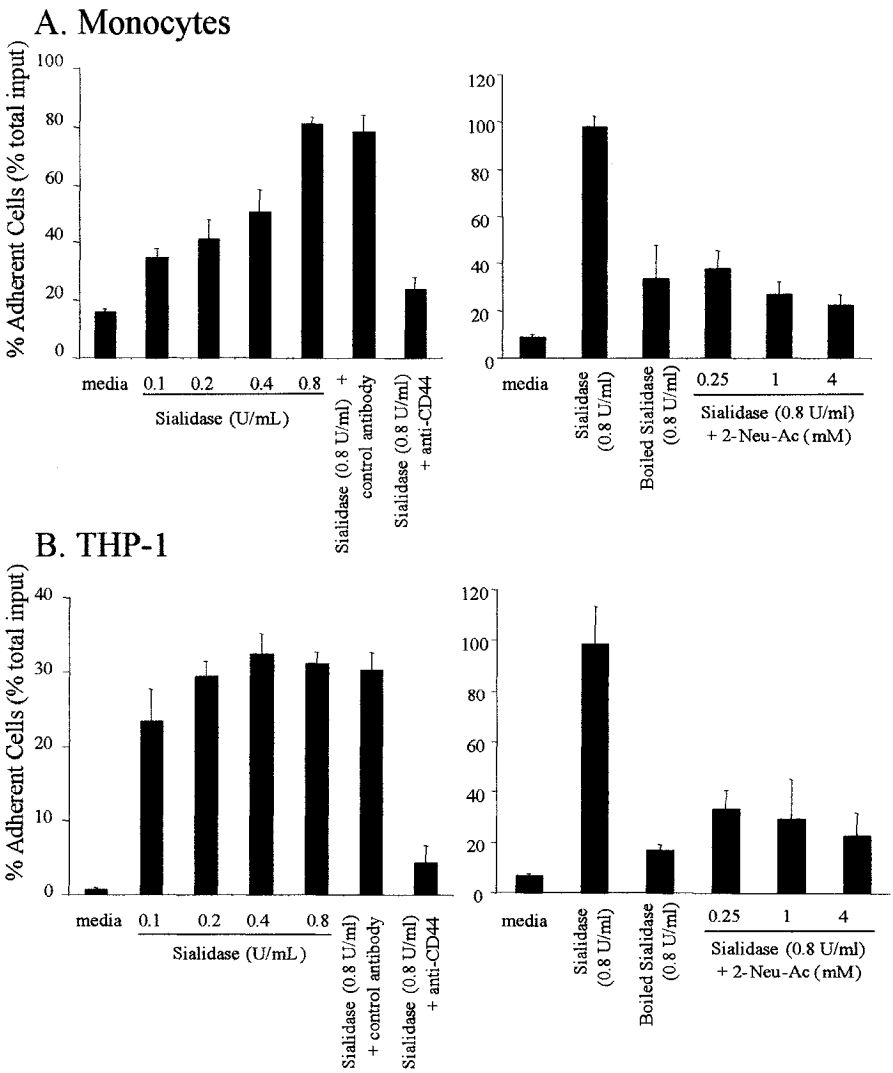
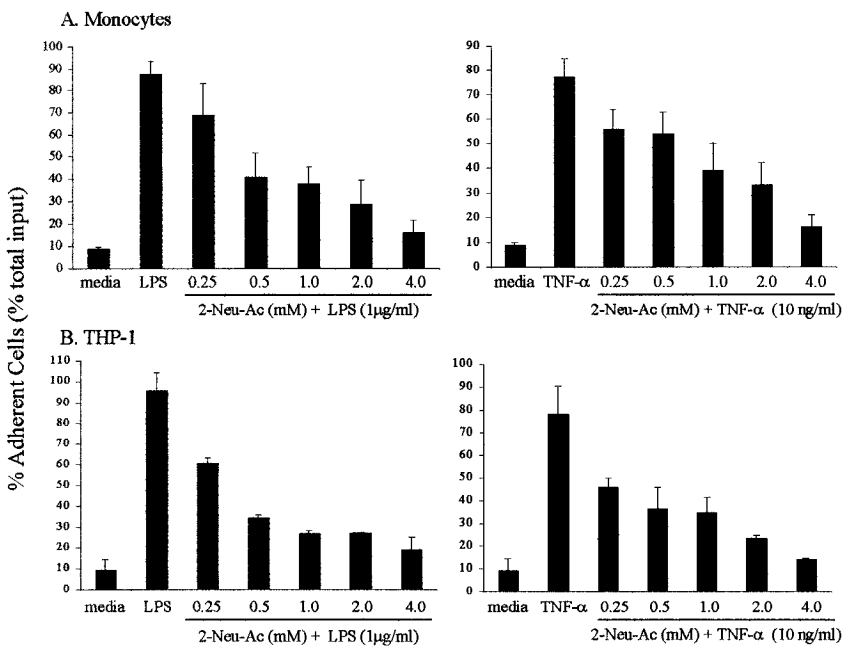

A. Monocytes
% Adherent Cells (% total input)
100
80
60
40
20
0
media
0.1
0.2
0.4
0.8
Sialidase (U/mL)
Sialidase (0.8 U/ml) + control antibody
Sialidase (0.8 U/ml) + anti-CD44
120
100
80
60
40
20
0
media
Sialidase (0.8 U/ml)
Boiled Sialidase (0.8 U/ml)
0.25
1
4
Sialidase (0.8 U/ml) + 2-Neu-Ac (mM)
B. THP-1
% Adherent Cells (% total input)
40
30
20
10
0
media
0.1
0.2
0.4
0.8
Sialidase (U/mL)
Sialidase (0.8 U/ml) + control antibody
Sialidase (0.8 U/ml) + anti-CD44
120
100
80
60
40
20
0
media
Sialidase (0.8 U/ml)
Boiled Sialidase (0.8 U/ml)
0.25
1
4
Sialidase (0.8 U/ml) + 2-Neu-Ac (mM)

Fig. 3-19. Involvement of sialidase activity in THP-1 cells treated with LPS and TNF-α.

Treatment of purified normal monocytes (A) and THP-1 cells (B) with a sialidase inhibitor reduces both LPS- and TNF-α-induced CD44-mediated HA-binding. Cells were pretreated with varying doses of 2-Neu-Ac sialidase inhibitor (0.5-4 nM) for 2 hr prior to addition of LPS (1 μg/ml) or TNF-α (10ng/ml). Following a 24 hr incubation, HA-binding was analyzed by the HA adhesion assay. % adherent cells are presented as the mean of triplicate samples ± SD. The results shown are a representative of three experiments.


A. Monocytes
% Adherent Cells (% total input)
media
LPS
0.25
0.5
1.0
2.0
4.0
2-Neu-Ac (mM) + LPS (1μg/ml)
media
TNF-α
0.25
0.5
1.0
2.0
4.0
2-Neu-Ac (mM) + TNF-α (10 ng/ml)
B. THP-1
media
LPS
0.25
0.5
1.0
2.0
4.0
2-Neu-Ac (mM) + LPS (1μg/ml)
media
TNF-α
0.25
0.5
1.0
2.0
4.0
2-Neu-Ac (mM) + TNF-α (10 ng/ml)

To further demonstrate that a sialidase activity was induced following stimulation with LPS and TNF-α, I performed a fluorescence-based sialidase activity assay for lysosomal sialidase. Because high numbers of cells are required for determining sialidase activity ($15x10^6$ cells per time point), we could not analyze sialidase activity in normal monocytes and so used THP-1 cells instead. THP-1 cells were stimulated with either LPS or TNF-α for various times followed by measurement of sialidase activity. Induction of sialidase activity was observed as early as 8 hr post-treatment but a statistically significant increase was observed at 12 and 16 hr post-stimulation (Fig. 3-20 A). To determine whether induction of sialidase activity is associated with HA-binding, THP-1 cells were treated with LPS or TNF-α for various times. The induction of sialidase activity was concurrent with the time-dependent activation of CD44-HA-binding in response to LPS or TNF-α (Fig. 3-20 B).

To determine whether the LPS- or TNF-α-induced lysosomal sialidase was membrane-bound or in the soluble cytosol fraction, the cells were stimulated with LPS or TNF-α for 16 hr. The cell pellet was treated with 1% digitonin. The resulting supernatant constituted the soluble cytosol fraction, whereas the pellet represented the membrane fractions. The membrane fraction was solubilized in a non-ionic detergent, 1% NP-40. The lysosomal sialidase activity was determined in both the cytosol and membrane fractions. The results show that the LPS- and TNF-α-stimulation of THP-1 cells induced a significant increase in the lysosomal sialidase activity that was localized in the digitonin-solubilized cytosol fraction (Fig. 3-20C). In contrast, the lysosomal sialidase activity detected in the NP-40-solubilzed membrane fraction was not increased following TNF-α stimulation of THP-1 cells, whereas this activity was reduced in THP-1 cell

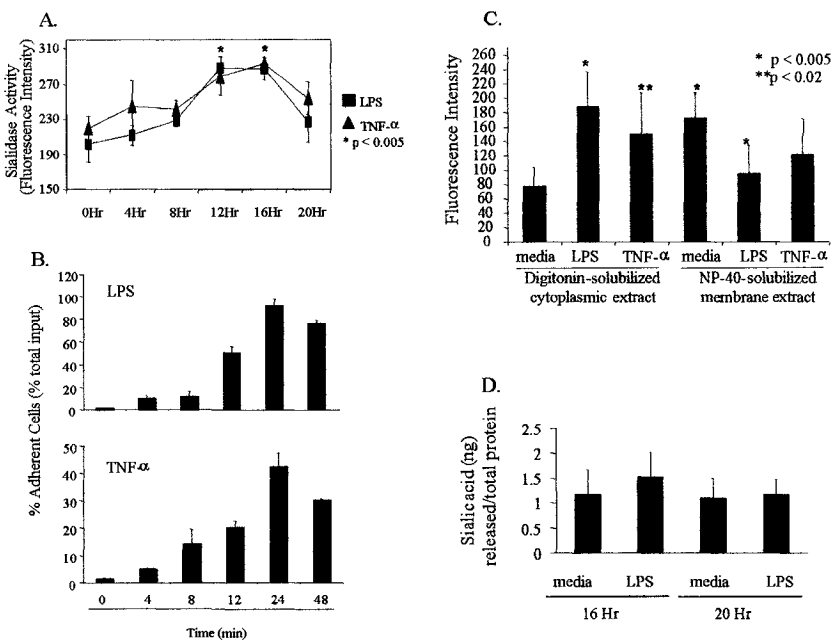
Fig. 3-20. Induction of sialidase activity in THP-1 cells by LPS and TNF-α.

A. LPS and TNF-α induce lysosomal sialidase activity in THP-1 cells. THP-1 cells were treated with either LPS or TNF-α for times ranging from 0-20 hr. At each time point, cells were pelleted, washed once in PBS and analyzed for lysosomal sialidase activity. Results are presented as the mean fluorescence intensity of three separate experiments ± SD. The increase in activity at 12 and 16 hr was determined to be statistically significant ($p \leq 0.01$), and is indicated by *.

B. LPS and TNF-α induce a time-dependent induction of HA-binding in THP-1 cells. THP-1 cells were treated with either LPS (1 μg/ml) or TNF-α (10 ng/ml) for times ranging from 0-48 hr. Cells were analyzed for HA-binding by the HA adhesion assay. % adherent cells are presented as the mean of triplicate samples ± SD. The results shown are representative of three experiments.

C. LPS- and TNF-α-induced lysosomal sialidase activity is localized in the cytosol. THP-1 cells (15 x 10^6 cells/ml) were stimulated with LPS (1 μg/ml) or TNF-α (10 ng/ml) for 16 hr. The cell pellet was treated with 1% digitonin. Following centrifugation, the supernatant constituted the soluble cytosol fraction, whereas the pellet represented the membrane fractions. The membrane fraction was solubilized in a non-ionic detergent, 1% NP-40. The lysosomal sialidase activity was determined in the cytosol and membrane fractions by fluorometry. Results are presented as the mean fluorescence intensity of three separate experiments ± SD. The increase in activity in the digitonin-solubilized fraction was determined to be statistically significant as indicated by *.

D. Membrane sialidases are not induced in LPS-stimulated THP-1 cells. THP-1 cells (15 x 10^6 cells/ml) were stimulated with LPS (1 μg/ml) for 16 hr. Cells were sonicated on ice for 10 sec on a low setting followed by centrifugation. The resulting supernatant was used to determine the membrane-bound sialidase activity as described in the materials and methods section. The results are expressed as ng of sialic acid residues released/ 200μg total protein.


A.
Sialidase Activity (Fluorescence Intensity)
310
270
230
190
150
0Hr
4Hr
8Hr
12Hr
16Hr
20Hr
LPS
TNF-α
* p < 0.005
B.
% Adherent Cells (% total input)
LPS
TNF-α
0
4
8
12
24
48
Time (min)
C.
Fluorescence Intensity
* p < 0.005
**p < 0.02
media
LPS
TNF-α
media
LPS
TNF-α
Digitonin-solubilized cytoplasmic extract
NP-40-solubilized membrane extract
D.
Sialic acid (ng) released/total protein
media
LPS
media
LPS
16 Hr
20 Hr

stimulated with LPS. These results suggest that LPS- and TNF-α-induced lysosomal sialidase activity was localized in the cytosol fraction.

I also determined whether membrane sialidase were induced in response to LPS or TNF-α stimulation of THP-1 cells by using the membrane sialidase-specific substrate, bovine mixed gangliosides type II as described in the experimental procedures section. The results show that stimulation of THP-1 cells with LPS (Fig. 3-20D) or TNF-α (data not shown) did not induce the membrane sialidase activity.

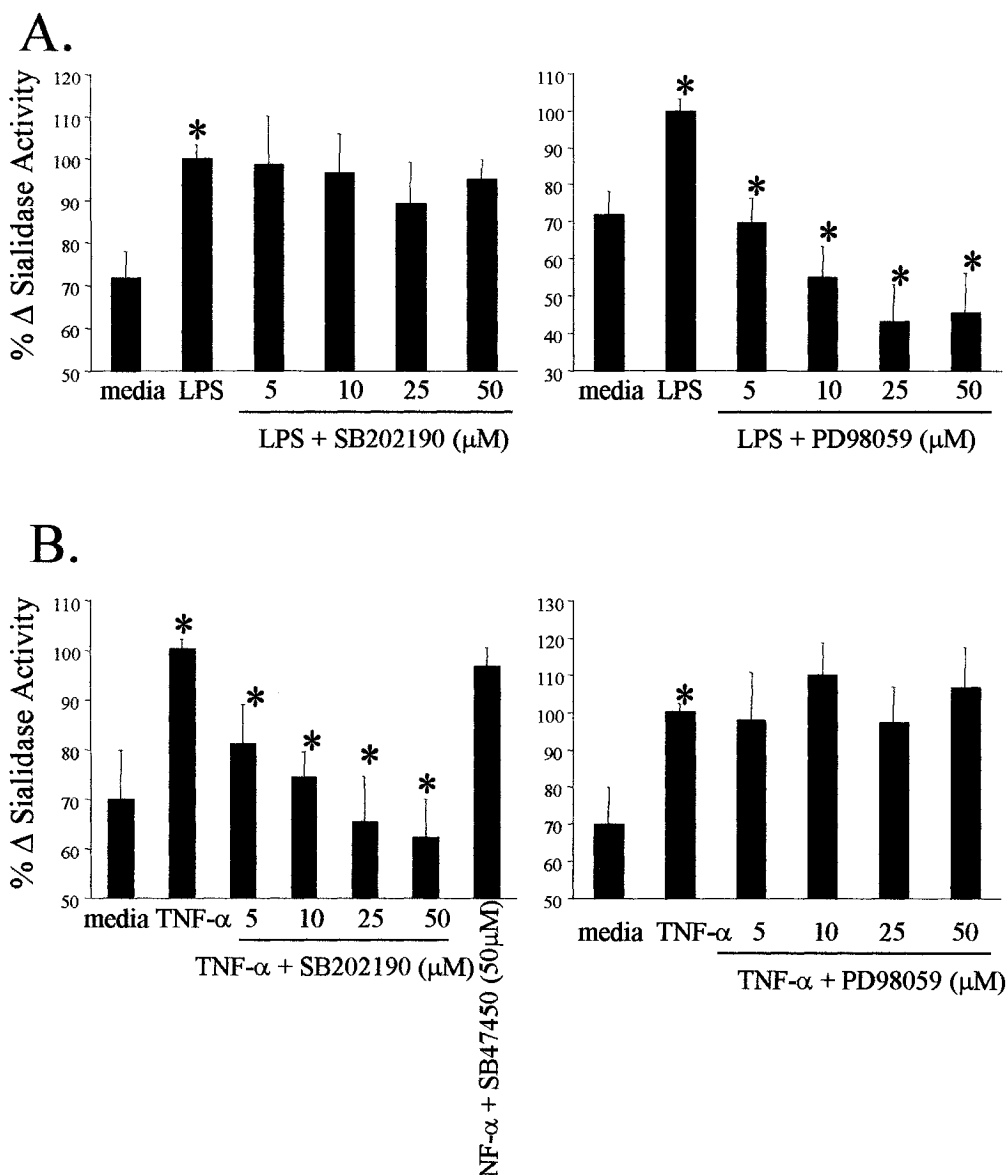
TNF-α-induced sialidase activity involves p38 MAPK activation in THP-1 cells

Because induction of sialidase activity was found to be associated with CD44-mediated HA-binding, we determined whether LPS- and TNF-α-induced sialidase activity involved the activation of p38 or p42/44 MAPKs. Cells were pretreated with various concentrations of either SB202190 or PD98059 for 2 hr prior to stimulation with LPS or TNF-α for 16 hr followed by measurement of sialidase activity. In LPS-stimulated cells, PD98059 significantly reduced LPS-induced sialidase activity in a dose-dependent manner, whereas SB202190 did not have any effect (Fig. 3-21 A). However, in TNF-α-stimulated cells, SB202190 significantly reduced TNF-α-induced sialidase activity in a dose-dependent manner, whereas PD98059, and SB202474, the inactive analogue of SB202190, failed to inhibit sialidase activity (Fig. 3-21 B). Taken together, these results suggest that both LPS and TNF-α induce lysosomal sialidase activity in THP-1 cells. Furthermore, the LPS-induced sialidase activity and consequent CD44-HA-binding may be regulated by endogenously produced TNF-α through p42/44 activation. In contrast, TNF-α-induced sialidase activity resulting in CD44-mediated HA-binding may be dependent on p38 MAPK activation.

Fig. 3-21. Effect of MAPK inhibitors on LPS- and TNF-α-induced sialidase activity.

A. LPS-induced sialidase activity in THP-1 cells is inhibited by PD98059. Cells were pretreated for 2 hr with increasing doses of either SB202190 or PD98059 (5-50 μM) prior to 16 hr incubation with LPS (1μg/ml). Cells were analyzed for sialidase activity and the results presented are the mean change in sialidase activity of six separate experiments ± SD. *, indicates $p \leq 0.01$.

B. TNF-α-induced sialidase activity in THP-1 cells is inhibited by SB202190. Cells were pretreated for 2 hr with indicated concentrations of either SB202190 or PD98059 (5-50 μM) prior to 16 hr incubation with TNF-α (10 ng/ml). Cells were analyzed for sialidase activity and the results presented are the mean change in sialidase activity of six separate experiments ± SD. *, indicates $p \leq 0.01$.


A.
% Δ Sialidase Activity
media
LPS
5
10
25
50
LPS + SB202190 (μM)
LPS + PD98059 (μM)
B.
% Δ Sialidase Activity
media
TNF-α
5
10
25
50
TNF-α + SB202190 (μM)
TNF-α + SB47450 (50μM)
TNF-α + PD98059 (μM)

DISCUSSION

It is well established that CD44-mediated hyaluronan interactions play a vital role at multiple steps in cell migration and the generation of immune responses including inflammation (3,4). LPS is a major inducer of CD44 expression and may modulate CD44-mediated biological effects in monocytes during inflammation (62). One of the main objectives of my thesis was to understand the molecular signaling pathways involved in LPS-induced CD44 expression and in the synthesis of the functionally active hyaluronan-adhesive form of CD44 in human monocytic cells. I primarily investigated the role of MAP kinases in human monocytic cells stimulated with either LPS or the cytokines produced endogenously following LPS stimulation that have been implicated in the regulation of CD44 expression and CD44-HA interactions. I have used primary human monocytes and the promonocytic THP-1 cell line as a model system to understand the signaling pathways involved in CD44 expression and the induction of the HA-binding form of CD44. The proinflammatory cytokine, TNF-α, has been shown to positively regulate CD44 expression and CD44-HA interactions in human monocytic cells. I confirmed these results in my model systems that TNF-α acts as the primary cytokine that regulates LPS-induced CD44 expression and CD44-HA interactions in both primary monocytes and THP-1 cells. Furthermore, my results suggest that CD44 expression and the synthesis of the HA-binding form of CD44 are two independent events that are regulated by two distinct MAPKs in LPS-stimulated monocytic cells. The results reveal the distinct involvement of JNK MAP kinase in the LPS-mediated but not in the TNF-α-mediated pathway resulting in CD44 induction. In addition, I provided evidence for the partial involvement of p38 MAP kinase in TNF-α-induced CD44 expression in both normal monocytes and THP-1 cells. In contrast, TNF-α activated p38 MAPK played a

critical role in the generation of LPS-induced HA-adhesive CD44. It was also demonstrated that acquisition of the HA-binding form of CD44 involved sialidase activation in both LPS- and TNF-α-stimulated monocytic cells. In addition, p38 MAPK activation was required for TNF-α-induced sialidase activity and consequent CD44-HA-binding. Taken together, the results suggest a key role for TNF-α in regulating the generation of HA-adhesive CD44 by inducing sialidase activity through p38 MAPK activation in LPS-stimulated monocytic cells. These results will be discussed in two sections: first, the differential role of JNK MAPK in LPS- and TNF-α-induced CD44 expression followed by the role of TNF-α-induced p38 MAPK activation in LPS-induced CD44-HA-binding and the activation of sialidase.

Differential role of JNK MAPK in LPS- and TNF-α-induced CD44 expression

LPS induces its biological effects through a complex process and may involve signaling via a CD14/LPS/TLR complex as well as through the cytokines produced endogenously following LPS stimulation. My results reveal that LPS-induced CD44-expression is mediated primarily by endogenously produced IL-10 and TNF-α. I provide evidence for the involvement of p38 MAP kinase, at least in part, in TNF-α-induced CD44 expression in both normal monocytes and THP-1 cells. In contrast, neither p38 nor p42/44 MAP kinases were found to be involved in IL-10-induced CD44 expression in monocytes. To dissect the TNF-α-/LPS-induced signaling pathway regulating CD44 expression independent of IL-10, we utilized IL-10 refractory THP-1 cells as a model system. My results reveal the distinct involvement of JNK MAP kinase in the LPS-mediated but not in the TNF-α-mediated pathway resulting in CD44 induction.

LPS signaling through CD14 has been shown to involve Toll-like receptors-4 (TLR-4) which associate with CD14 (194,195). LPS interaction with CD14 promotes dimerization of TLR and subsequent recruitment of MyD88, a myeloid differentiation marker which functions as an adaptor molecule (194,196). MyD88 associates via its c-terminal toll homology domain with TLR, and via its N-terminal death domain with the serine-threonine protein kinase, IRAK (196). Upon interaction with MyD88, IRAK is autophosphorylated and binds to TRAF-6. TRAF-6 subsequently activates TAK-1 and MEKK, members of the MAP kinase signaling cascade (196,200,251). This interaction activates the NFκB complex in addition to the p38 MAP kinase (200,252). The LPS-mediated signaling pathway has been depicted in Fig 1-6 in Chapter 1.

I have shown that the p42/44 ERK MAP kinase was not involved in the regulation of CD44 expression induced either by LPS, IL-10, or TNF-α. However, SB202190, a p38 inhibitor, partially prevented LPS-induced CD44 expression. Since SB202190 inhibited LPS-induced IL-10 and TNF-α production, down regulation of CD44 expression may be attributed to a block in the production of these cytokines. TNF-α is a pleiotropic cytokine that has growth modulatory, cytotoxic and inflammatory activities (253). Interaction of TNF-α with its receptor activates complex intracellular signaling pathways, including protein tyrosine kinase-dependent cascades, leading to the activation of transcription factors such as NFκB, AP1, etc. (236,254). TNF-α has been shown to activate all three types of MAPKs in different cell types (236,237). The signaling pathway activated following interaction of TNF-α with its receptors has been described in detail in Fig 1-7 in Chapter 1. However, the role of intracellular signaling molecules, especially the MAP kinases, in TNF-α-induced CD44 expression remains largely unknown. My results

consistently show partial inhibition of CD44 expression by the p38 inhibitor in both monocytes and THP-1 cells. The partial inhibition of CD44 induction by SB202190 in both cell types was observed at low doses of 10 μM, and a complete inhibition was not observed even when concentrations of 25 and 50 μM of SB202190 were used. Considering the complex mechanism underlying CD44 regulation, partial inhibition of CD44 expression by SB202190 may be attributed to other signaling molecules that may co-operate with the members of p38 MAPK pathway in TNF-α-induced CD44 expression. Since high concentrations of SB202190 may non-specifically inhibit some kinases (255), the possible role of these kinases, howsoever minor, in the observed partial inhibition of CD44 expression cannot be ruled out.

The signaling molecules that mediate the biological effects of IL-10, especially those involved in IL-10-mediated CD44 upregulation, are not well understood. IL-10 has been shown to mediate its inhibitory effects on murine macrophages through the activation of STAT3 (256,257). Recently, IL-10 was shown to increase the expression of the cell cycle inhibitor, cyclin-dependent kinase inhibitor $p19^{INK4D}$ in macrophages. IL-10-induced expression of $p19^{INK4D}$ was later shown to be dependent on STAT3 activation (258). Herein, I show that IL-10 is capable of inducing p42/44 but not p38 activation. Our lab has also shown that IL-10 can induce the activation of p42/44 but not p38 or JNK in another human monocytic cell line, HL60, which expresses IL-10 receptors (data not shown). The role of p42/44 in IL-10-meditated effects is not known. However, our preliminary observations have suggested that it can act in concert with PI-3 kinase to regulate IL-10-mediated effects (data not shown). p42/44 was not found to be involved in IL-10-induced CD44 expression in THP-1 cells. At present, studies are ongoing to

understand the involvement of PI3-kinase in association with p42/44 in CD44 expression. Whether IL-10-induced CD44 expression is also mediated by STAT-3-dependent activation of cyclin-dependent kinase inhibitors $p19^{INK4D}$ needs to be investigated.

The role of MAPKs in LPS- and TNF-α-induced CD44 expression could not be easily studied in normal human monocytes because endogenously produced IL-10 upregulates CD44 expression independent of p38 and ERK kinases. IL-10-refractory THP-1 cells provide a useful model to study the LPS-mediated signaling events in CD44 expression without the additional effects of endogenously produced IL-10. The results show the distinct involvement of JNK in LPS-induced CD44 expression. This conclusion is based on results derived by using the specific JNK inhibitor, SP600125, as well as following transfection of THP-1 cells with a dominant-negative (DN) SEK1 kinase. Two MAPKKs, MKK4 and MKK7, have been found to be the primary activators of JNK (259,260). MKK4 is an essential component of the JNK signal transduction pathway and disruption of the MKK4 gene blocks JNK activity (261). DN SEK1 has also been shown to act as a specific inhibitor of the JNK signal transduction pathway (245-247). However, it was recently shown that MKK4 can also activate p38 MAPK (260,262). Although p38 MAPK can be modulated by MKK4, the p38 MAPK inhibitor had only a partial effect on CD44 expression. This is in contrast to the significant reduction of LPS-induced CD44 expression observed following transfection of DN SEK1. Furthermore, transfection of THP-1 cells with DN SEK1 plasmid did not influence TNF-α-induced CD44 expression. These results suggest a key role for JNK and/or its specific upstream kinases and regulators in LPS-induced CD44 induction.

To determine the role of JNK, dexamethasone, an anti-inflammatory glucocorticoid and inhibitor of JNK/SAPK activation (243) which mediates its biological effects on cytokine production primarily by complexing with AP-1, thus downregulating AP-1 activity was initially employed (263,264). Furthermore, dexamethasone has been shown to inhibit AP-1 activity by interfering with JNK phosphorylation (243,265). In this study, dexamethasone inhibited LPS- and TNF-α-induced phosphorylation of JNK. However, dexamethasone treatment reduced CD44 expression to basal levels in LPS-stimulated, but not in TNF-α-stimulated cells. Similar results were obtained by employing SP600125, a specific JNK inhibitor. These results may also suggest a role for JNK in LPS-, but not in TNF-α-, mediated CD44 upregulation.

The JNK MAP kinase pathway includes JNK1, JNK2, and JNK3 (186). JNK1 and JNK2 are widely expressed in several tissues, whereas JNK3 is more selectively expressed in the brain, the testis, and the heart. The JNK3 gene has been shown to be involved in neuronal cell death (266), whereas JNK1 and JNK2 have been implicated in Th1/Th2 cell differentiation (267,268). JNK1 has also been shown to regulate the development of T cell-mediated immunity against *Leishmania major* infections in an experimental mouse model (269). Whether JNK1 or JNK2 regulate LPS-induced CD44 expression needs to be investigated.

The findings of this study raise the question of how activation of JNK and its upstream regulators may serve to induce CD44 expression. To understand the signaling events down stream of JNK MAPK activation responsible for CD44 gene transcription, the identity of potential transcription factors was investigated. Recently, Egr-1 was shown to be involved in PMA-induced CD44 expression in murine B cells (142) and also

in IL-1α-induced CD44 expression in human endothelial cells (143). The Egr-1 gene is a prototypic member of a gene family encoding transcription factors which share a conserved zinc finger DNA-binding motif (270). This gene is induced rapidly and transiently in response to B cell receptor cross-linking or treatment with PMA (271). It has been shown that both p38 and JNK MAPKs are involved in Egr-1 activation (272). Although the role of Egr-1 in the regulation of CD44 expression in human monocytic cells has not been clearly demonstrated, the results suggest that both LPS- and TNF-α-induce Egr-1 activation in THP-1 cells. Furthermore, treatment of cells with dexamethasone, SP600125, or transfection of cells with DN SEK1 plasmids reduced Egr-1 activation in LPS-stimulated THP-1 cells, suggesting a role for JNK in LPS-induced Egr-1 activation. However, the molecular mechanism by which TNF-α induces Egr-1 activation, as well as the role of Egr-1 in CD44 expression in human monocytic cells needs to be investigated.

The role of p38 MAPK activation in LPS- and TNF-α-induced CD44-HA-binding

The second component of my thesis involved studying the role of MAPKs signaling pathways mediating the synthesis of the functionally active, HA-binding form of CD44 in LPS-stimulated human monocytic cells. My results show that endogenously produced TNF-α plays a vital role in LPS-induced CD44-mediated HA binding in human monocytic cells. Furthermore, I show for the first time that TNF-α-activated p38 MAPK plays a critical role in the generation of LPS-induced HA-adhesive CD44. We also demonstrated that acquisition of the HA-binding form of CD44 involves sialidase activation in both LPS- and TNF-α-stimulated monocytic cells. In addition, p38 MAPK

activation was required for TNF-α-induced sialidase activity and consequent CD44-HA-binding.

As discussed earlier, regulation of LPS-induced CD44-mediated HA-binding in monocytic cells may involve a combination of signals delivered by the interaction of LPS with the CD14/TLR complex as well as by the interaction of endogenously produced cytokines with their cognate receptors. The results of this study suggest that LPS-induced CD44-mediated HA-binding in primary monocytes may be regulated by the endogenously produced TNF-α and IL-10. This was addressed by employing antibodies specific for IL-10 and TNF-α receptors, as well as by the use of specific MAPK inhibitors which blocked LPS-induced TNF-α and IL-10 production. Both the antibodies and inhibitors effectively prevented LPS-induced CD44-HA-binding.

To understand the molecular mechanism underlying the regulation of CD44-HA-binding in LPS-stimulated monocytic cells, the role of p38 and ERK MAPKs was examined. The results consistently demonstrated a significant inhibition of LPS- and TNF-α-induced HA-binding by the p38 inhibitor, SB202190, in normal monocytes. The inhibition of HA-binding by SB202190 was specific as these effects were observed at concentrations ranging from 5-10 μM, thus virtually excluding their non-specific effects (273). However, IL-10-induction of the HA-binding form of CD44 did not involve either p38 or p42/44 MAPK activation. As discussed in the preceding section, IL-10 has been shown to mediate its inhibitory effects by inducing the expression of the cell cycle inhibitor cyclin-dependent kinase inhibitor $p19^{INK4D}$ in macrophages through STAT3 activation (256-258). Whether IL-10-induced CD44-HA-binding is regulated by STAT3 activation needs to be investigated.

As for the role of MAP kinases in CD44 expression, the role of MAPKs in LPS- and TNF-α-induced CD44-HA interactions could not be precisely studied in normal monocytes because endogenously produced IL-10 upregulated HA-binding independently of p38 and ERKs. Therefore the THP-1 cells were again used as the model system. In THP-1 cells, endogenously produced TNF-α was found to play a critical role in LPS-induced CD44-mediated HA-binding. Contrary to the results obtained with normal monocytes, LPS-induced TNF-α production, as well as CD44-mediated HA-binding in THP-1 cells, were regulated by p42/44 MAPK. Since LPS-induced TNF-α production in these cells was inhibited by PD98059, a p42/44 inhibitor, the effects of this inhibitor on LPS-induced HA-binding were attributed to the blockage of endogenously produced TNF-α. This was confirmed by exogenous addition of TNF-α to the THP-1 cells in which LPS-induced TNF-α production was simultaneously blocked by PD98059. The fact that exogenous TNF-α restored CD44-HA-binding suggested that LPS-induced CD44-HA-binding observed in THP-1 cells is regulated by endogenously produced TNF-α via the activation of p42/44 MAPK. Similar to the results obtained with normal monocytes, TNF-α-induced CD44-HA-binding in THP-1 cells was found to be dependent on p38 activation.

Activation of p38 MAPK has been shown to play a role in the regulation of cellular and cytokine genes (236,237,274). For example, T cell activation with specific antigen or staphylococcal antigens has been shown to induce p38 activation resulting in TNF-α production (242). It has also been demonstrated that p38 MAPK regulates IL-1 (242), IL-6 (275), TNF-α (242), and IL-10 (188) production in human monocytes through the activation of the CD14 receptor. Differential involvement of p38 and p42/44

MAPKs in LPS-induced TNF-α production in normal monocytes and THP-1 cells, respectively was observed. The molecular mechanisms for the observed differences in TNF-α regulation in THP-1 cells and primary monocytes are not clear at present. However, both p38 and p42/44 MAPKs have been shown to regulate TNF-α expression depending on the cell types and stage of cell differentiation/maturation (276,277).

The CD44 molecule possesses several N- and O-linked glycosylation sites in its extracellular domain (278) so that the biological functions of CD44 may be regulated by post-translational modifications such as glycosylation (10-12,28). Sialidation of CD44 has been shown to regulate CD44-HA-binding of LPS-stimulated monocytic cells (29). Sialidase is a key enzyme in the metabolism of glycoproteins and glycolipids. Mammalian sialidases have been classified into three types depending on subcellular localization and enzymatic properties. These types, located in the cytosol, in lysosomes and in plasma membranes, have been cloned and characterized (279-281). Lysosomal sialidases catalyze the intralysosomal degradation of sialoglycoconjugates by releasing terminal sialic acids from their oligosaccharide chains (282). The desialidation of these glycoconjugates is a crucial event leading to the modulation of cellular functions in numerous physiological and pathological processes. Aberrant sialidation in cancer cells is generally accepted as a characteristic feature associated with metastatic potential (283). Human lysosomal sialidase has also been shown to be involved in two genetically distinct lysosomal storage disorders, sialidosis and galactosialidosis (284,285).

In this study, it was shown that TNF-α-induced CD44-HA-binding might also involve the activation of lysosomal sialidase in both normal monocytes and THP-1 cells. I showed that LPS- and TNF-α-induced lysosomal sialidase activity resides in the cytosol

fraction and not in the cell membranes. Furthermore, the ganglioside-specific membrane sialidases are not induced in THP-1 cells following stimulation with either LPS or TNF-α. The role of MAPKs in the regulation of cytokines/co-stimulatory molecules has been documented (139,188,219,233-237,242). However, there is little information regarding the involvement of signaling molecules, particularly MAPKs, in regulating sialidase activity. Calcium activation has been shown to stimulate sialidase activity; for example, chelators (EDTA) and certain metals (Cu^{2+}/Zn^{2+}) have been shown to inhibit some sialidases (63). Herein, we show for the first time that TNF-α-induced lysosomal sialidase activity is regulated by p38 MAPK in THP-1 cells. In contrast, LPS-induced sialidase activity was dependent on p42/44 MAPK-mediated TNF-α production. Blocking TNF-α production by PD98059, a p42/44 inhibitor, significantly reduced the LPS-induced sialidase activity and CD44-HA binding. Subsequently, TNF-α-mediated p38 MAPK activation induced sialidase activity and CD44-HA-binding. Although the data suggests a prominent role for sialidase activity in the generation of HA-adhesive CD44, the contribution of other factors such as sulfation of CD44 (30,68) cannot be ruled out in my model system, and needs to be investigated. Since a large number of molecules undergo post-translational sialidation, it is not clear whether regulation of sialidation by p38 is unique to the generation of HA-adhesive CD44, or whether the same signaling pathways are involved in post-translational sialidation of other molecules as well.

My results from this study on CD44 induction and generation of a CD44 protein capable of binding HA suggest that these are two independent events that are regulated by distinct MAPK signaling pathways. This conclusion is based on the following observations. First, low concentrations of SB202190 inhibited LPS- and TNF-α-induced

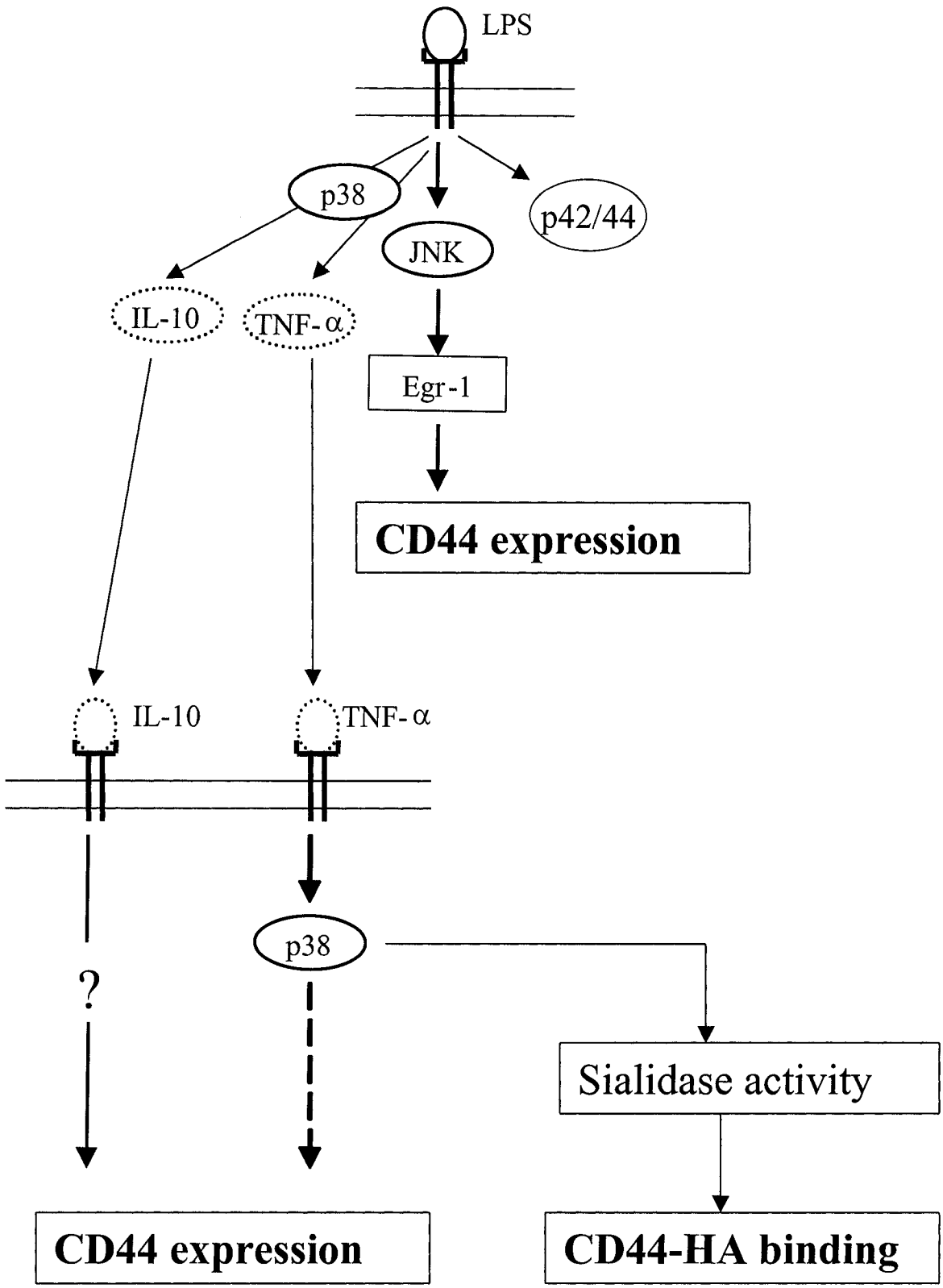
CD44-HA-binding, but not CD44 expression, in normal monocytes. Second, PD98059 completely inhibited LPS-induced CD44-HA-binding in THP-1 cells without any effect on the level of CD44 expression. Conversely, SB202190, a p38 inhibitor, partially inhibited LPS-induced CD44 expression without any effect on HA binding. Whether JNK activation is also involved in the generation of the HA-adhesive CD44 phenotype remains to be investigated.

In summary, regulation of CD44 expression and HA-binding in LPS-stimulated monocytic cells involves a complex sequence of intracellular signaling events (Fig 3-22). The results reveal that the regulation of CD44 expression and HA binding in monocytic cells involves cross-talk between the signals delivered by the interaction of LPS with the CD14/Toll receptor complex and by the interaction of IL-10 and TNF-α with their respective receptor complexes. In this study, it was shown for the first time that there exists a role for JNK in the LPS-mediated, but not in the TNF-α-mediated, signaling pathway resulting in CD44 induction. In contrast, p38 MAP kinase was found to partially regulate TNF-α-induced CD44 expression in both normal monocytes and in THP-1 cells. It was also shown that activation of JNK MAP kinase may result in the activation of Egr-1, the transcription factor implicated in CD44 transcription. JNK MAPK, therefore, represents an important and novel target for anti-inflammatory JNK inhibitors capable of inhibiting CD44 expression. Additionally, my results show for the first time the involvement of p38 MAPK in the generation of HA-adhesive, functionally active CD44 in LPS- and TNF-α-stimulated monocytic cells. Using THP-1 cells as a model system evidence is provided that TNF-α-induced CD44-HA-binding may involve induction of sialidase activity which is regulated by p38 MAPK. The signaling pathways involved in

LPS-, IL-10-, and TNF-α-induced CD44 expression and the generation of the HA-adhesive CD44 are depicted in Fig 3-22. Taken together, these results suggest that the induction of CD44 and the generation of the HA-binding form of CD44 are two independent events that are regulated by distinct signaling pathways. Induction of CD44 expression may involve the activation of JNK MAPK whereas activation of p38 MAPK may be required for the generation of HA-adhesive CD44 in LPS-stimulated human monocytic cells. These studies provide a basis for the identification of molecular players in CD44 induction and CD44-mediated HA-binding that may help in designing targeted drug therapy for inflammatory diseases.

Fig. 3-22. Overview of the signalling pathways responsible for the induction of CD44 expression and CD44-HA-binding in monocytic cells.

Treatment of monocytic cells with LPS induces the expression of CD44 and CD44-HA binding in addition to the endogenous cytokines, IL-10 and TNF-α. These two cytokines are also capable of inducing CD44 expression and HA-binding. The signalling pathway responsible for the LPS-induced expression of CD44 involves the activation of JNK MAPK. However, the LPS-induced expression of TNF-α and IL-10 involves that activation of p38 MAPK. TNF-α-induced CD44 expression partially involves p38 MAPK activation as indicated by the dashed line, whereas the IL-10 induced CD44 expression does not involve any of the three main MAPK members. Regulation of CD44-HA binding induced by LPS is via the endogenously expressed TNF-α. TNF-α induces CD44-HA interactions via the activation of sialidase which occurs through the activation of p38 MAPK.


LPS
p38
p42/44
JNK
IL-10
TNF-α
Egr-1
CD44 expression
IL-10
TNF-α
p38
?
Sialidase activity
CD44 expression
CD44-HA binding

CHAPTER 4:

Studies on the regulation of CD44 expression and HA-binding in response to IL-4 and IL-13 in human B cells

INTRODUCTION

In the previous chapter my results suggested the involvement of two distinct MAPK signaling pathways in the regulation of LPS-induced CD44 expression and LPS-induced CD44-HA binding in human monocytic cells. In this chapter, I extended previous observations on the regulation of CD44 expression and binding in human B cell lines using Epstein Barr virus (EBV) transformed lymphoblastiod B cell lines (B-LCL) as well as EBV-negative and EBV-positive Burkitt's lymphoma (BL) cell lines.

Burkitt's lymphomas (BL) are classified as high-grade tumors of B cell origin cytogenetically characterized by chromosomal translocations that place the *c-myc* oncogene under the regulatory control of one of the immunoglobulin genes, resulting in constant cell cycling (112). This disease, which involves localized tumor formation and growth in extranodal sites, is regarded as one of the most rapidly growing human tumors (112,115). EBV, a B cell-transforming herpes virus, 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 (113,115). Infection of primary human B cells *in vitro* with EBV readily results in the outgrowth of immortalized non-neoplastic lymphoblastoid B cells (B-LCL). BL and B-LCL cells produce a number of cytokines that may act in an autocrine manner to regulate their growth and differentiation.

The effect of cytokines and ECM molecules present in the microenvironment on CD44-mediated adhesion of cells, in particular, BL and B-LCL cells, to HA remains poorly understood. In human endothelial cells, TNF-α upregulated the expression of CD44 while TNF-α and IFN-γ modulated the expression of CD44 V6 and CD44 V9 on

the promonocytic THP-1 and myelomonocytic U-937 cell lines (286). TNF-α has also been shown to enhance binding of human monocytes to HA and this binding was inhibited by IL-4 and IL-13 (62). In murine B cells, IL-5 enhanced CD44-mediated binding of HA (287). These observations suggest that like CD44 isoform expression, the recognition of HA by CD44 and its modulation by cytokines is dependent on cell type and state of cellular activation.

The modulation of CD44-HA recognition by cytokines in human B cells has not been well investigated. We have previously shown that EBV-negative, BL cells do not express CD44 whereas many EBV-positive BL cells, and B-LCL cells express a number of CD44 isoforms (220). Modulation of CD44-HA interactions especially by cytokines may have implications with respect to the localization of leukemic B cells in target tissues and formation of tumors. In the first part of this study, I examined the effect of cytokines, particularly the ones involved in B cell activation on CD44 expression and ligand binding using a series of B-LCL and EBV-positive BL cell lines. Among the cytokines and mitogens tested, IL-4 alone or in conjunction with PMA induced CD44 expression and HA-binding in BL30 BL cells infected *in vitro* with EBV, designated as BL30/B95-8 cells but not in the B-LCL cells. These observations are described in the first part of the results section in this chapter.

IL-4, a pleiotropic immune regulatory cytokine, is produced by Th2 cells, basophills, and mast cells (148). 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, (148-150). IL-13, another pleiotropic cytokine, is also produced by activated Th2 cells and has

overlapping but not identical biological functions when compared to IL-4 (148,151-153,288). IL-4 and IL-13 share 25-30% amino acid homology and their similar biological roles have been attributed to shared receptor components (153,289). The structure and biological function of IL-4 and IL-13 receptors and the binding affinities of their respective ligands have been described in detail in Fig.1-4 in Chapter 1. IL-4 and IL-13 mediate their biological effects through the activation of JAK/STAT signaling pathways (172), the details of which have also been described in Fig.1-4 in Chapter 1(174-176). The IL-13 signaling pathway is not as clearly defined as that for IL-4 but likely depends on the cell type and the combination of IL-4 and IL-13 receptor chains present on the cell surface.

The role of IL-4 and IL-13 in the *in vivo* function of normal B cells, as well as the *in vitro* and *in vivo* behavior of B cell malignancies, is not well understood. There is evidence to suggest that IL-4 modulates the expression of co-stimulatory molecules such as CD23 and HLA-DR in normal human B cells and B-LCL cells (148-150,290-292). IL-4 has also been suggested to participate in the survival and development of BL cells (293). However, the role of IL-13 on BL and B-LCL growth and development is not clear. To further clarify the differential roles of IL-4 and IL-13 observed in B-LCL and BL30/B95-8 cells, I investigated the biological effects of IL-4 and IL-13 on these cells using BL30/B95-8 cells and a B-LCL line, MK3.31, as model systems. IL-4 enhanced the expression of CD23, CD44, and CD44-mediated binding to HA in BL30/B95-8 cells. In MK3.31 B-LCL cells, IL-4 upregulated CD23 expression but did not affect CD44 expression or CD44-mediated HA binding. In contrast to IL-4, IL-13 did not influence any of these biological responses in either BL30/B95-8 or MK3.31 B-LCL cells. To

understand the molecular mechanism underlying the differential effects of IL-4 and IL-13 on BL and B-LCL B cells, I investigated the JAK/STAT signaling pathway. IL-4 induced the activation of distinct JAK/STAT proteins in BL30/B95-8 BL and MK3.31 B-LCL cells. In contrast, IL-13 failed to activate the JAK/STAT signaling pathway in BL30/B95-8 cells. The failure of BL30/B95-8 cells to respond to IL-13 stimulation and activation of JAK/STAT signaling was attributed to the loss of IL-13Rα1 expression, a finding that was confirmed on a panel of EBV-infected and EBV negative BL cell lines. These observations are described in the second part of the results section in this chapter.

RESULTS

Objective 2 A: Differential regulation of CD44-HA interactions in Burkitt's lymphoma and EBV-transformed lymphoblastiod B cells by IL-4

Differential CD44–HA Interactions in Burkitt's Lymphoma and Normal Human B Cells Transformed in Vitro with EBV

CD44 molecules must be activated in order to bind to their extracellular matrix ligand, HA (2). Therefore, to study CD44–HA interactions in transformed human B cells, EBV-positive BL-30/B95-8 BL cells and MK3.31 B-LCL cells were stimulated with a series of mitogens including LPS, SAC, PMA, PHA, and PWM for 24 hr and analyzed for their ability to recognize HA by the ^{51}Cr-based cell adhesion assay as described in the materials and methods chapter. Unstimulated cells did not exhibit binding to HA in either cell line. Stimulation of both cell lines with LPS, SAC, or PWM had no significant effect on the CD44-mediated HA recognition of these cells (Fig. 4-1). In contrast, PMA stimulation induced strong CD44-mediated HA recognition in BL-30/B95-8 cells (6.8 ± 0.4% in unstimulated vs 46.2 ± 8.4% in PMA stimulated cells) but not in MK3.31 cells (5.0 ± 1.2% in unstimulated cells vs 2.8 ± 0.3% in PMA stimulated cells). PHA stimulation also increased CD44-HA binding in only BL-30/B95-8 cells (6.8 ± 0.4% in unstimulated cells vs 17.0 ± 0.9% in PHA stimulated cells, Fig. 4-1). However, none of the mitogens tested enhanced CD44-mediated HA binding in the MK3.31 cell line (Fig.4-1) and other B-LCL cell lines (data not shown).

PMA-induced adhesion of BL-30/B95-8 cells was dose dependent, reaching a plateau at concentrations of 0.8 ng/ml of PMA (Fig. 4-2A). Kinetics experiments revealed that optimal binding occurred between 12 and 24 h of stimulation (61.0 ± 0.3% 67.0 ± 0.3%) and was reduced by 120 h post stimulation to nearly background levels (8.0 ± 1.2%, Fig. 4-2B). To determine whether the differential CD44–HA interactions observed with this set of cell lines were applicable to normal human B cells, and other BL and B-LCL cell lines, a panel of such cells were tested for HA binding in response to

Fig. 4-1. Differential adhesion of BL-30/B95-8 BL and MK3.31 B-LCL cells to hyaluronan following stimulation with mitogens.

Cells ($1x10^6$/ml) were stimulated for 24 h with LPS, SAC, PMA, PHA, and PWM followed by analysis of cell adhesion to HA-coated plates as described previously. Adherent cells (% cpm input) are presented as the mean of triplicate samples ± standard deviation (SD). The results shown are representative of at least three separate experiments.

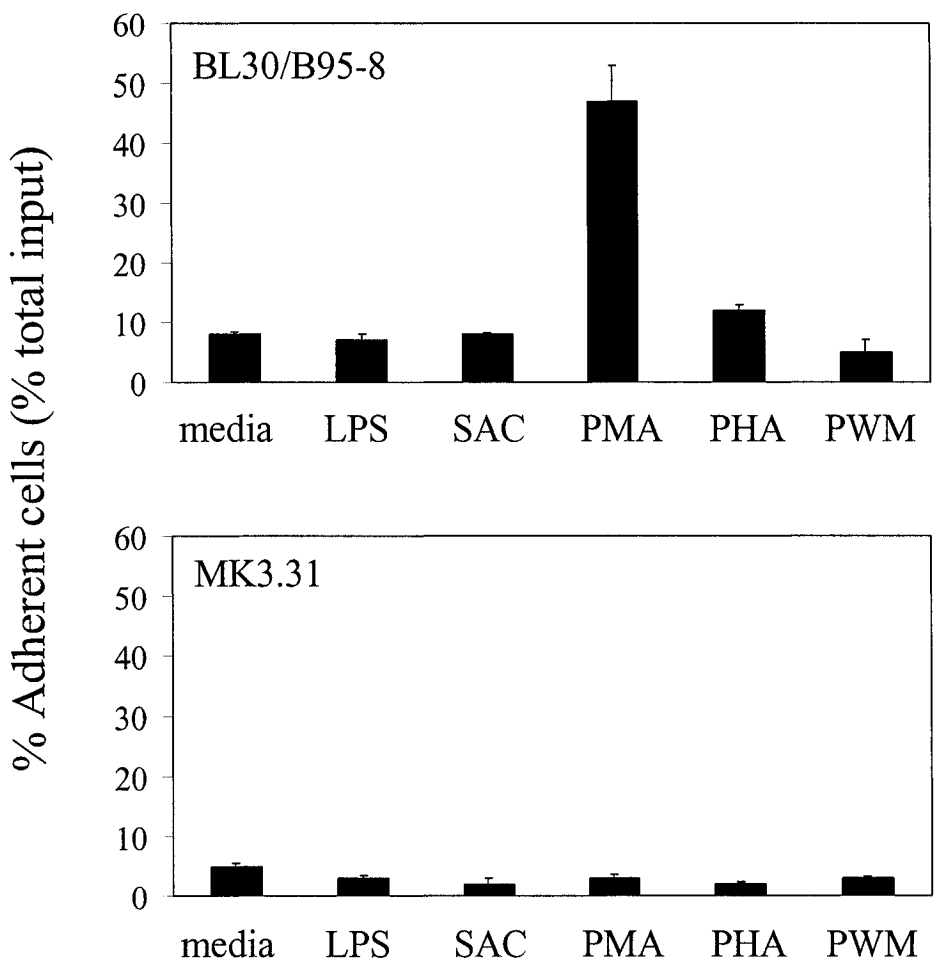
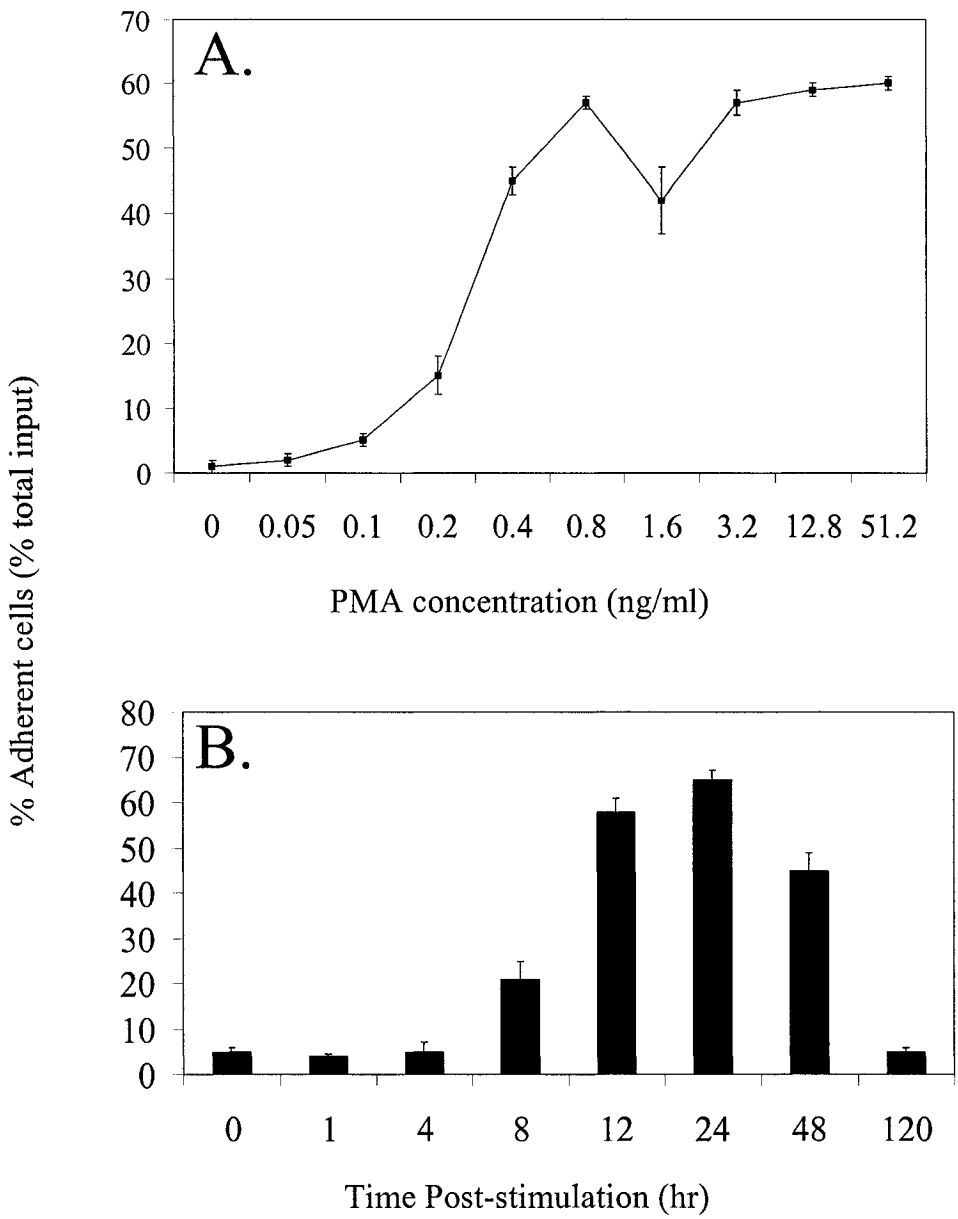

% Adherent cells (% total input)
BL30/B95-8
MK3.31
60
50
40
30
20
10
0
media
LPS
SAC
PMA
PHA
PWM

Fig.4-2. PMA-induced adhesion of BL-30/B95-8 to HA is dose- and time-dependent.

Cells (1 x 10^6/ml) were stimulated for 24 h with increasing concentrations of PMA (0.05–51.2 ng/ml) (A.) For time course experiments (B), cells were stimulated with 20 ng/ml of PMA. Cell adhesion to HA was performed as described in the legend to Fig. 1. Adherent cells (% cpm input) are presented as the mean of triplicate samples ± SD. The results shown are representative of at least three separate experiments.


A.
70
60
50
40
30
20
10
0
0 0.05 0.1 0.2 0.4 0.8 1.6 3.2 12.8 51.2
PMA concentration (ng/ml)
% Adherent cells (% total input)
B.
80
70
60
50
40
30
20
10
0
0 1 4 8 12 24 48 120
Time Post-stimulation (hr)

PMA stimulation (Fig. 4-3). Normal purified B cells isolated from peripheral blood following stimulation with PMA exhibited strong HA binding. The mean HA binding for PMA stimulated normal human B cells from 13 different donors was 51.0 ± 8.3%. In contrast, upon *in vitro* transformation with EBV (B-LCLs), HA binding could no longer be induced in response to PMA stimulation. This was observed in all the B-LCLs tested, including MK3.31, MK4.15, MK5.26, and MK6.1, all generated from different donors (Fig.4-3), and also from several other similarly derived cell lines, the data for which are not shown (3.6 ± 2.0% mean HA binding). However, variable results were obtained in BL cell lines. Unlike in BL-30/B95-8 cells, PMA stimulation of EBV-positive BL cells Jijoye and EB3 failed to induce HA recognition when compared to unstimulated cells. EBV-positive IM cells obtained from the PBMCs of a patient with infectious mononucleosis strongly recognized HA in response to PMA (0.2 ± 0.01% in unstimulated cells vs 17.8 ± 2.4% in stimulated cells) (Fig 4-3). These results suggest that *in vitro* transformation of normal human B cells with EBV abrogated their ability to bind HA following PMA stimulation. In contrast, BL-30/B95-8 cells, which, like B-LCLs, are infected with wild-type EBV and express the same set of CD44 isoforms (220), bind HA following PMA stimulation. The inability of Jijoye and B-LCL cells to bind HA was not due to the lack of CD44 expression as these cells expressed relatively high levels of CD44 on their surface (Fig. 4-4).

IL-4 Induces CD44-Mediated Binding of HA in BL-30/B95-8 BL Cells

Further studies investigating the effect of cytokines involved in B cell activation, proliferation, or differentiation on the regulation of HA recognition in these two cell types were pursued. BL-30/B95-8 BL and MK3.31 B-LCL cells were stimulated with a

Fig. 4-3. Adhesion of a panel of BL and B-LCL cells to HA.

Cells (1×10^6/ml) were stimulated for 24 h with 20 ng/ml of PMA. Cell adhesion to HA was performed as described in the legend to Fig. 4-1. Adherent cells (% cpm input) are presented as the mean of triplicate samples ± SD. The results shown are representative of at least three separate experiments. BL-30, the EBV-negative and CD44-negative BL cell line (30), was used as a negative control. Values at least twofold above this background level (5.1%) were considered significant in all adhesion experiments.

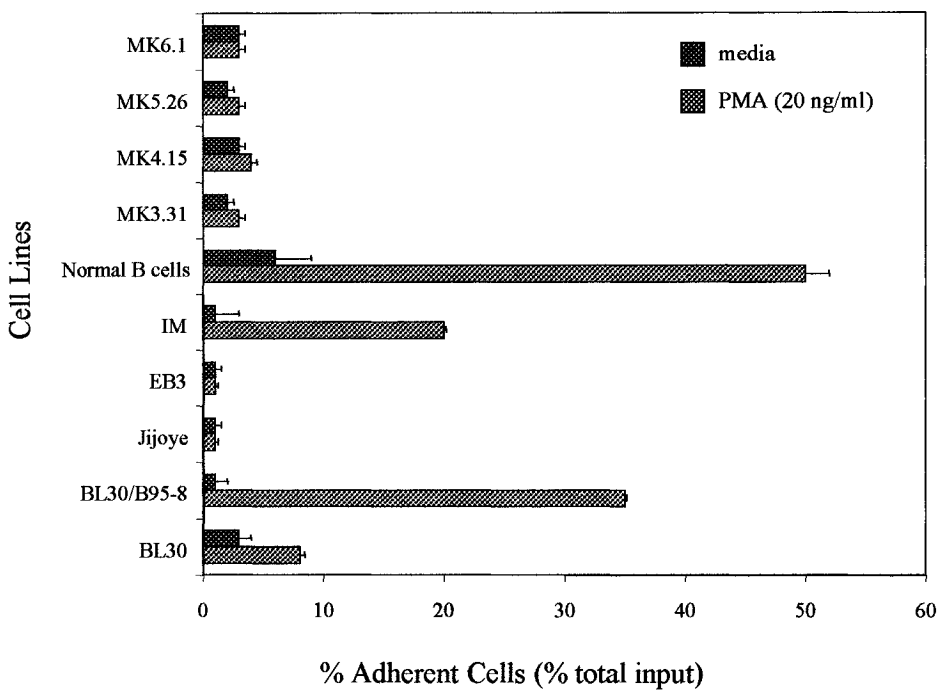
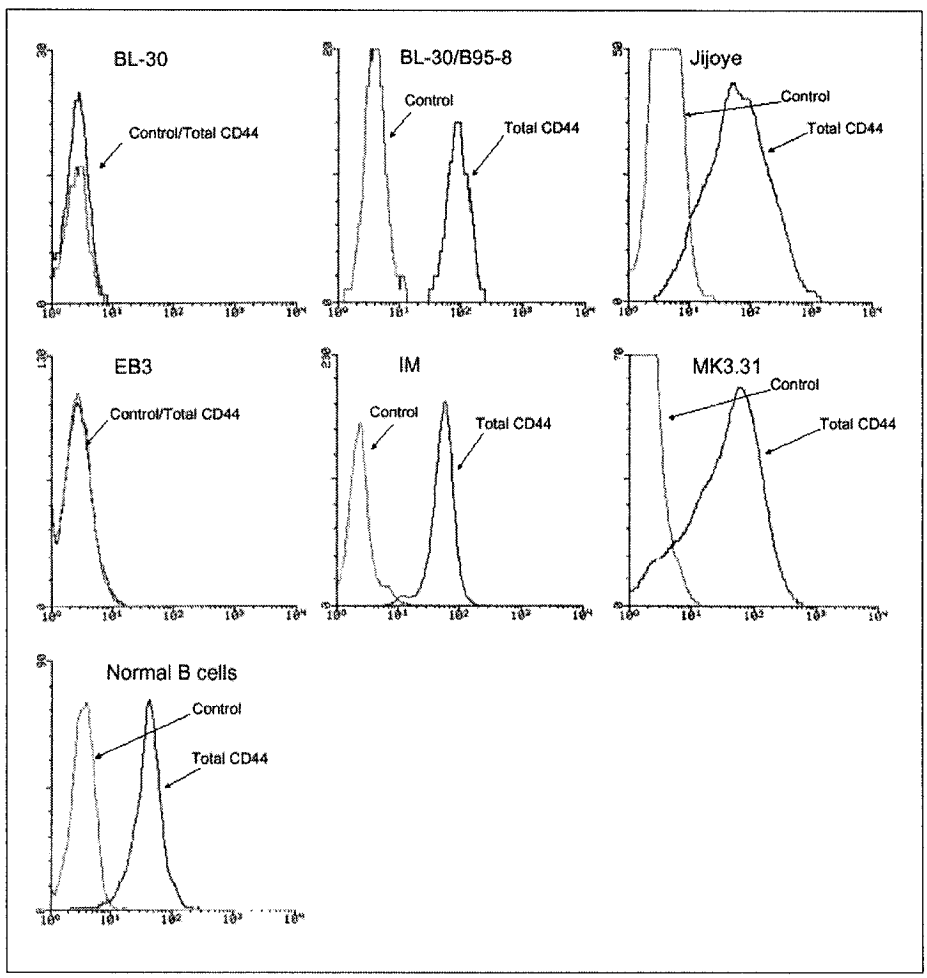

MK6.1
MK5.26
MK4.15
MK3.31
Normal B cells
IM
EB3
Jijoye
BL30/B95-8
BL30
media
PMA (20 ng/ml)
Cell Lines
0
10
20
30
40
50
60
% Adherent Cells (% total input)

Fig. 4-4. Analysis of total CD44 expression in BL, B-LCL, and normal human B cells by flow cytometry.

BL cell lines (BL-30, BL-30/B95-8, Jijoye, and EB3), IM, a B lymphoblastoid cell line generated from a patient with infectious mononucleosis, the *in vitro* EBV transformed B-LCL, MK3.31, and normal human B lymphocytes were stained with either anti-CD44 or isotype-matched control antibodies. The histograms are identified by an arrow as Total CD44 or Control, respectively.


BL-30
Control/Total CD44
BL-30/B95-8
Control
Total CD44
Jijoye
Control
Total CD44
EB3
Control/Total CD44
IM
Control
Total CD44
MK3.31
Control
Total CD44
Normal B cells
Control
Total CD44
Relative Cell Number
Log Fluorescence Intensity

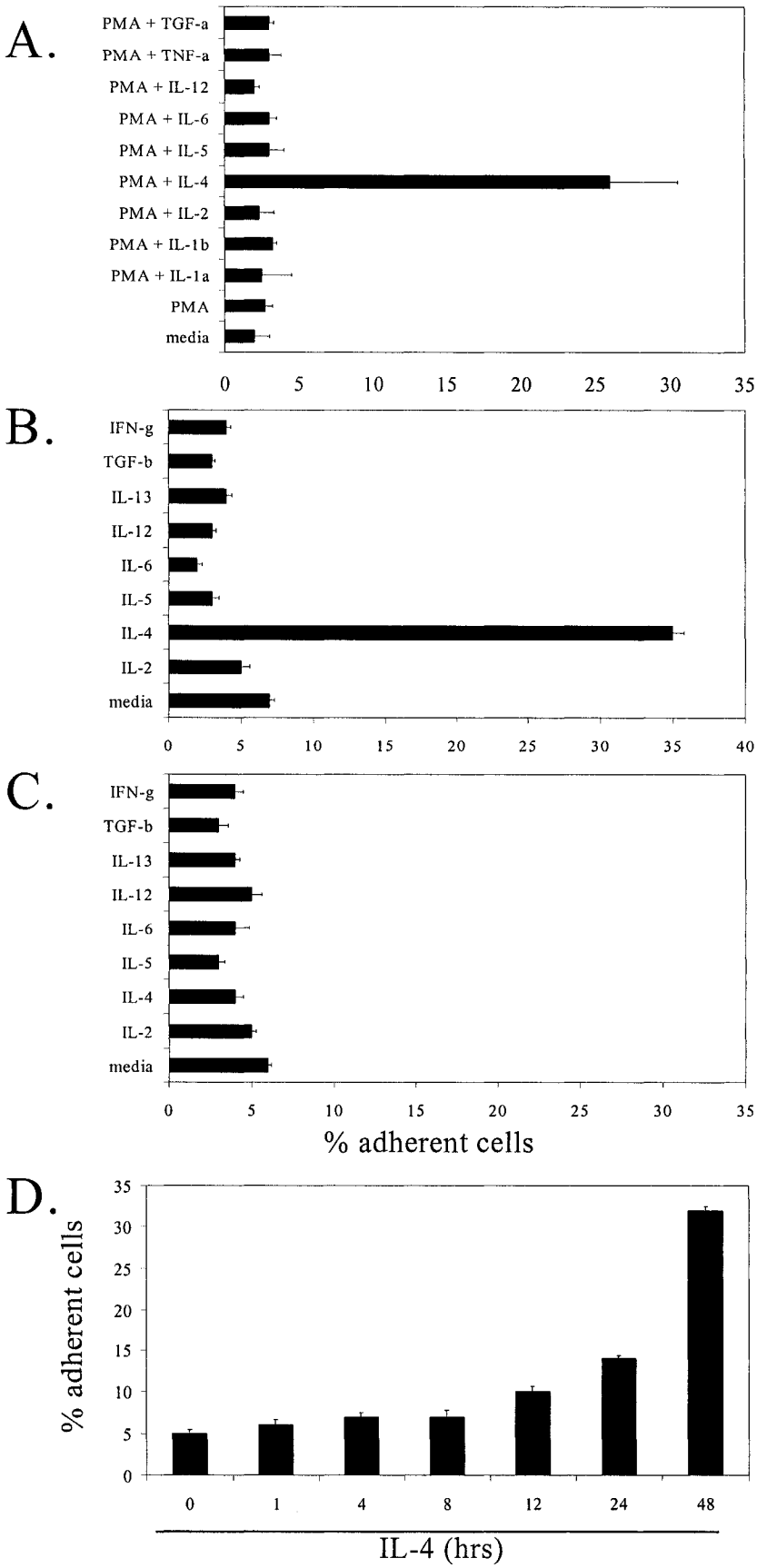
panel of cytokines, in the presence or in the absence of suboptimal concentrations (0.1 ng/ml) of PMA, as defined in Fig. 4-2. In contrast to the other cytokines tested, which included IL-1α, IL-1β, IL-2, IL-5, IL-6, IL-10, IL-12, IL-13, TNF-α, TGF-α, TGF-β, and IFN-γ, only IL-4 was able to induce strong HA binding in BL-30/B95-8 cells. This effect was observed in the presence (26.5 ± 4.4%) or in the absence (33.6 ± 0.1%) of suboptimal concentrations of PMA compared to cells suboptimally stimulated with PMA alone (2.8 ± 0.2%) or unstimulated cells (7.2 ± 0.4%), as illustrated in Figs. 4-5A and 4-5B, respectively. The binding observed in BL-30/B95-8 cells was mediated by CD44, as anti-CD44 antibodies inhibited this effect (37.1% in untreated cells versus 9.41% in treated cells). IL-4-induced binding to HA in BL-30/ B95-8 cells was detectable as early as 12 h following stimulation (Fig. 4-5D). Of particular note, IL-13, which shares receptor components of its receptor with IL-4 (35, 36), was not able to induce CD44-mediated adhesiveness to HA in any of the BL cells (Fig. 4-5B). IM cells exhibited a responsiveness similar to that of BL-30/B95-8 cells with respect to HA recognition following stimulation with IL-4 alone or in concert with suboptimal concentrations of PMA (data not shown). None of the cytokines tested induced CD44-mediated binding to HA in the MK3.31 cell line (Fig 4-5C).

PMA and IL-4 Upregulated CD44 Isoform Expression in BL-30/B95-8 Cells But Not in B-LCL Cells

The detection of total CD44 expression may have masked the effects of IL-4 and IL-13 on the ability to induce distinct CD44 isoforms. Therefore, in order to investigate whether IL-13 was capable of inducing changes in CD44 isoform expression, I examined

Fig. 4-5. Effect of mitogens and cytokine on CD44-HA binding in BL30/B95-8 and MK3.31 cells.

BL30/B95-8 cells (1 x 10^6/ml) were stimulated for 48 h with a variety of cytokines in the presence (A) or in the absence (B) of 0.1 ng/ml of PMA. MK3.31 cells were also treated with a variety of cytokines in the absence of suboptimal doses of PMA(C). D. Time course (0–48 h) for the adhesion of BL-30/B95-8 cells to HA following stimulation with IL-4 (30 ng/ml) alone. Cell adhesion to HA was performed as described in the legend to Fig. 4-1. Adherent cells (% cpm input) are presented as the mean of triplicate samples ± SD. The results shown are representative of at least three separate experiments.


A.
PMA + TGF-a
PMA + TNF-a
PMA + IL-12
PMA + IL-6
PMA + IL-5
PMA + IL-4
PMA + IL-2
PMA + IL-1b
PMA + IL-1a
PMA
media
0 5 10 15 20 25 30 35
B.
IFN-g
TGF-b
IL-13
IL-12
IL-6
IL-5
IL-4
IL-2
media
0 5 10 15 20 25 30 35 40
C.
IFN-g
TGF-b
IL-13
IL-12
IL-6
IL-5
IL-4
IL-2
media
0 5 10 15 20 25 30 35
% adherent cells
D.
% adherent cells
35 30 25 20 15 10 5 0
0 1 4 8 12 24 48
IL-4 (hrs)

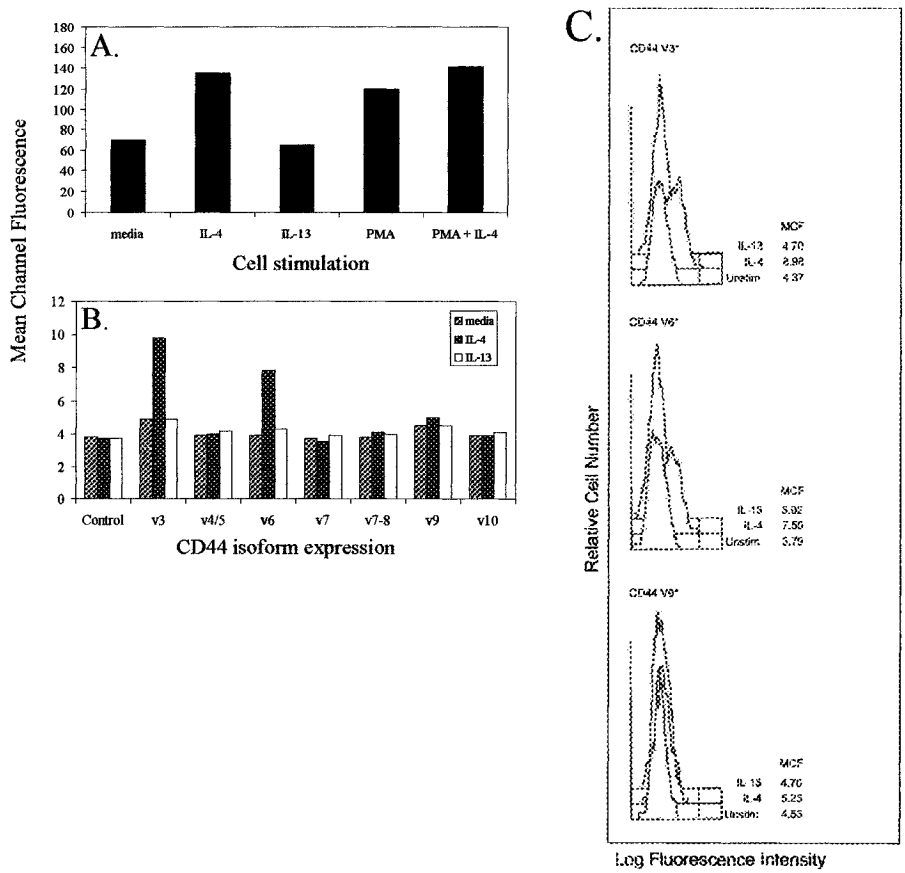
the expression levels of specific CD44 isoforms on IL-4- and IL-13-treated BL30/B95-8 and MK3.31 cell lines.

Unstimulated BL and B-LCL cells expressed low levels of CD44 isoforms as detected by exon specific monoclonal antibodies (<5 median channel fluorescence (MCF) values). Therefore, superimposition of flow cytometry histograms and the observation of a cell population shift in log fluorescence with a change of greater than 3 MCF values were regarded as significant in these cases. This is illustrated in the representative histograms of Fig. 4-6C. Stimulation of BL30/B95-8 cells with IL-4 alone significantly upregulated total CD44 (Fig 4-6A) and CD44 V3 and V6 expression (Fig. 4-6B and Fig. 4-6C). Interestingly, IL-13 (Figs 4-6A and 4-6C) was unable to upregulate total CD44 expression or isoform expression in BL30/B95-8 cells. Induction of novel CD44 isoforms was also investigated by RT-PCR analysis and amplicons were detected by hybridization with ^{32}P-labelled oligonucleotides specific for exons V2-10, as described elsewhere (220). Stimulation of cells with PMA, IL-4 or both did not induce the expression of a novel CD44 isoform ((111) and data not shown). Furthermore, neither PMA nor IL-4 was able to upregulate CD44 isoform expression on any of the B-LCLs tested, as detected by flow cytometry (Fig. 4-6A and data not shown). Therefore, although IL-4 stimulation was capable of inducing isoform expression, IL-13 treatment exhibited a complete lack of effect on CD44 isoform expression further illustrating the starck contrast between the effects of IL-4 and IL-13 as well as the differences between the BL and MK cell lines.

Taken together, these results show that there is a distinct difference between the response of the BL30/B95-8 and MK cells with respect to CD44 expression and HA

Fig. 4-6. The effect of IL-4 and IL-13 on CD44 isoform expression

A. Modulation of CD44 isoform expression on BL-30/B95-8 cells by treated with IL-4, IL-13, PMA, or PMA + IL-4. As indicated, CD44 exon-specific or isotype-matched control monoclonal antibodies (control) were used. Median channel fluorescence (MCF) values were used for comparison of CD44 expression levels and the results shown are representative of at least three separate experiments. B. Modulation of CD44 varient 3 (v3), v4/5, v6, v7, v7-8, v9, or v10 isoform expression in BL-30/B95-8 cells by IL-4 and IL-13. BL-30/B95-8 cells were stimulated with IL-4 (40 ng/ml) or IL-13 (40 ng/ml) and compared to unstimulated cells by flow cytometry. The MCF values corresponding to the described stimulation condition are listed for comparison of expression levels. The results shown are representative of at least three separate experiments. Representative histograms of CD44v3, v6, and v9 expression in response to IL-4 or IL-13 in BL30/B95-8 cells are shown in panel C.


A.
Mean Channel Fluorescence
180
160
140
120
100
80
60
40
20
0
media
IL-4
IL-13
PMA
PMA + IL-4
Cell stimulation
B.
media
IL-4
IL-13
12
10
8
6
4
2
0
Control
v3
v4/5
v6
v7
v7-8
v9
v10
CD44 isoform expression
C.
CD44 V3*
CD44 V6*
CD44 V9*
MCF
IL-13 4.70
IL-4 8.98
Unstim 4.37
MCF
IL-13 5.02
IL-4 7.56
Unstim 5.79
MCF
IL-13 4.76
IL-4 5.25
Unstim 4.55
Relative Cell Number
Log Fluorescence Intensity

binding in response to two similar cytokines, IL-4 and IL-13. In the next series of experiments, I investigated the molecular mechanism responsible for the differential effect of IL-4 and IL-13 on BL and B-LCL cells with respect to CD44 expression and CD44-mediated HA binding.

Objective 2 B: Molecular mechanisms to understand the differential effect of IL-4 and IL-13 on CD44 expression on BL30/B95-8 and B-LCL cell lines

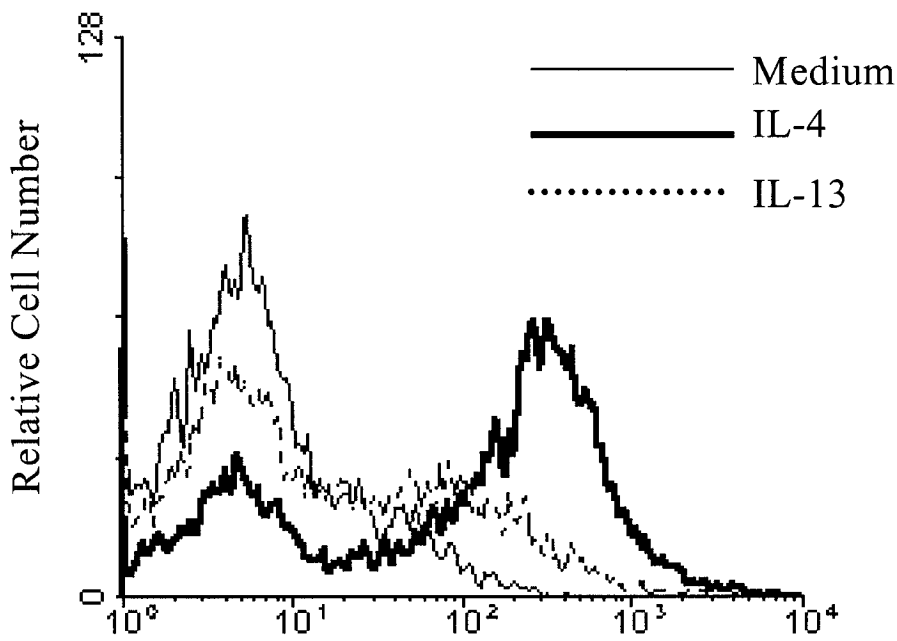
Differential effects of IL-4 and IL-13 on CD23 and CD44 expression in EBV-transformed normal human B cells and BL30/B95-8 BL cells

The results from the previous section suggested that although IL-4 and IL-13 exhibit similar biological effects on B cell biology, IL-4 and IL-13 exhibited differential effects on CD44 expression and CD44-mediated HA binding in BL B cells. Furthermore, IL-13 failed to induce CD44 expression on any of the BL or B-LCL cell lines. These observations suggest that normal human B cells either following EBV-transformation or during the generation of BL malignancies may loose IL-13-mediated biological effects.

In order to more fully investigate the differential effect observed between IL-4 and IL-13 and because it is well established that both IL-4 and IL-13 induce CD23 expression in normal human B cells, I measured the induction of CD23 as a positive control for cytokine activity and cell responsiveness. Therefore, human B cells were isolated as described in the materials and methods section and were treated for 24 hr with IL-4 or IL-13 followed by flow cytometric analysis for CD23 expression. Fig. 4-7 shows a representative histogram obtained from one of five different donors. Both IL-4 and IL-13 induced the expression of CD23 in these cells. The induction of CD23 and CD44 by these cytokines on B cells transformed *in vitro* with EBV (MK cells) and on EBV-infected BL30/B95-8 BL cells was then analyzed (Fig 4-8). Cells were treated with either IL-4 or IL-13 for 24 hr followed by analysis of CD23 and CD44 expression by flow cytometry analysis. IL-4, but not IL-13, induced CD23 expression on the B-LCLs. However, none of the B-LCL cells exhibited CD44 upregulation upon stimulation with IL-4 or IL-13. Representative flow cytometric results from MK3.31 cells are shown in Fig. 4-8A. The effects of IL-4 and IL-13 on BL-30/B95-8 BL cells was examined next.

Fig. 4-7: CD23 expression is increased in response to IL-4 and IL-13 in normal human B cells.

Human B cells were isolated from a healthy donor by using positive selection with CD19+ magnetic beads as described in the materials and methods chapter. The cells (0.5 x 10^6) were either left untreated or were treated with IL-4 (40 ng/mL) and IL-13 (50 ng/mL) for 48 hr. Cells were then harvested and analyzed for CD23 expression by flow cytometry. The histogram shown is a representative of one of five different donors.

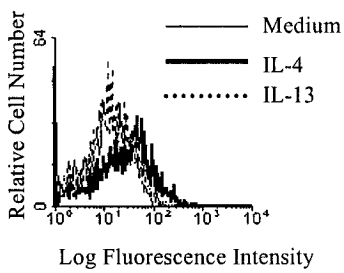


CD23 Expression

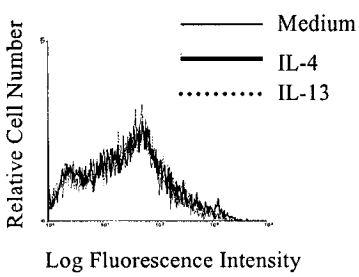
Fig. 4-8: IL-4 and IL-13 exert differential effects on CD23 and CD44 expression in BL and B-LCL cell lines.

MK3.31 and BL30/B95-8 cell lines were stimulated with IL-4 (40 ng/mL) and IL-13 (50 ng/mL) for 24 hours. CD23 and CD44 expression were measured by flow cytometry and the histograms shown are representative of at least five different experiments. A. MK3.31 cells: upper panel CD23 expression; lower panel: CD44 expression. B. BL30/B95-8 cells: upper panel CD23 expression; lower panel: CD44 expression.

A. MK3.31

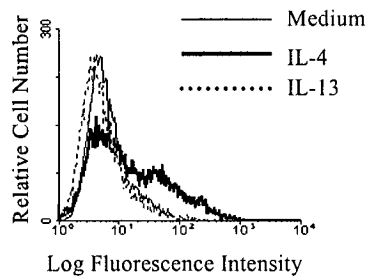


CD23 Expression

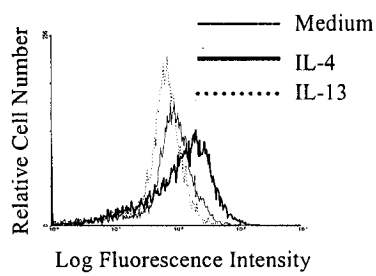


CD44 Expression

B. BL30/B95-8



CD23 Expression



CD44 Expression

IL-4 enhanced both CD23 and CD44 expression on BL30/B95-8 cells, whereas IL-13 failed to influence the expression of either of these surface molecules (Fig. 4-8B).

In summary, normal human B cells responded to IL-13 with increased expression of CD23, but after EBV transformation there is a loss of IL-13-mediated effects on expression of this molecule. At the same time IL-4 mediated effects were preserved with respect to CD23 expression. In BL30/B95-8 BL cells, IL-4 induced increased expression of both CD23 and CD44 expression, but as with EBV-transformed cells, IL-13 failed to elicit any effects. These results are summarized in Table 4-1.

Effect of IL-4 on the activation of the JAK/STAT pathway in BL30/B95-8 and MK3.31 cells

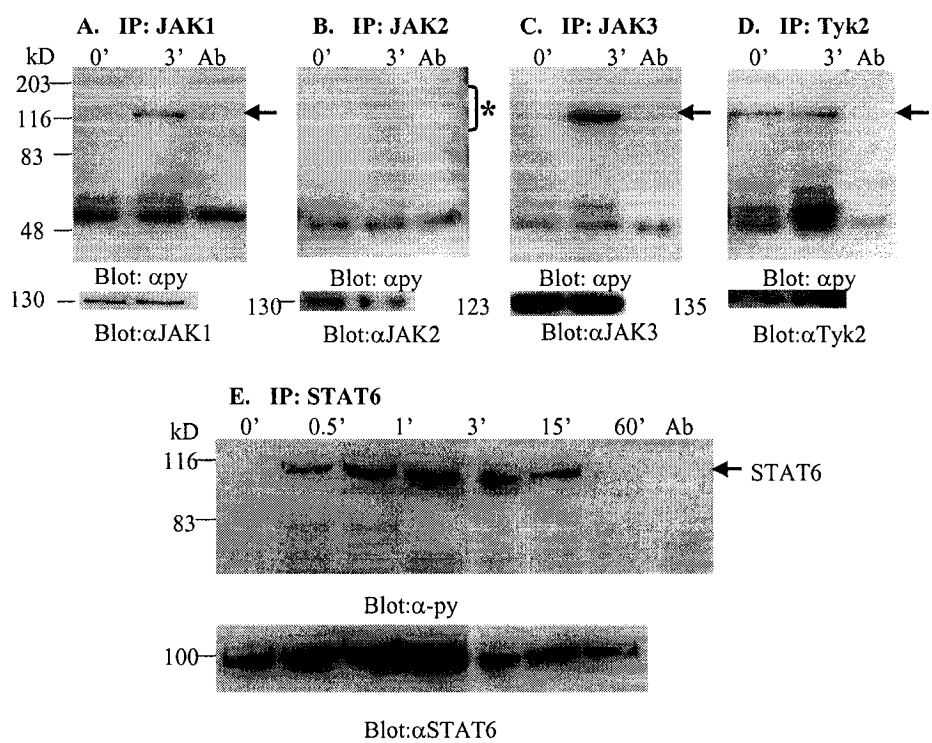
Next, the possibility that the distinct effects of IL-4 on BL30/B95-8 BL and MK3.31 B-LCL cells were due to distinct signaling pathways was investigated. The main pathway induced by cytokine signaling involves the activation of JAK and STAT proteins (172-176). Therefore, the tyrosine phosphorylation status of JAK1, JAK2, JAK3, Tyk2, and STAT6 in response to IL-4 in BL30/B95-8 and MK3.31 cells was examined. BL30/B95-8 cells were cultured in serum-free media overnight followed by stimulation with IL-4 (40 ng/ml) for 3 min. Lysates were prepared and immunoprecipitated with antibodies specific to the above-mentioned members of the JAK/STAT pathway. Subsequent Western blot analysis was performed using an antibody directed against phosphorylated tyrosine. IL-4 induced tyrosine phosphorylation of JAK1 and JAK3 in BL30/B95-8 cells (Fig. 4-9A and C). A basal level of JAK3 phosphorylation was observed in unstimulated cells (Fig. 4-9C). In contrast, IL-4 did not induce

Table 4-1: Summary Flow Cytometry Results from Figures 4-7 and 4-8.

Cell type	Stimulation	CD23 responsiveness	CD44 responsiveness
Normal human B cells	IL-4	Up-regulation	N/A
	IL-13	Up-regulation	N/A
BL30/B95-8	IL-4	Up-regulation	Up-regulation
	IL-13	No effect	No effect
MK3.31	IL-4	Up-regulation	No effect
	IL-13	No effect	No effect

Fig. 4-9: IL-4 activates the JAK/STAT pathway in BL30/B95-8 cells.

Cells were treated with IL-4 (40 ng/mL) for the time points indicated. Cell lysates were immunoprecipitated with antibody specific for JAK1 (A), JAK2 (B), JAK3 (C), Tyk2 (D), and STAT6 (E). Membranes were initially blotted with anti-phosphotyrosine (αpy) mAb, then stripped and reprobed with antibody homologous to the immunoprecipitating antibody to control for the amount of protein immunoprecipitated. "Ab" denotes a control immunoprecipitation reaction consisting of protein A sepharose beads, Ab, and lysis buffer, in the absence of lysate. Arrows indicate phosphorylated proteins. "*" indicates the point of migration of unphosphorylated kinase.


A. IP: JAK1
B. IP: JAK2
C. IP: JAK3
D. IP: Tyk2
kD
0'
3'
Ab
203
116
83
48
*
Blot: αpy
130
123
135
Blot:αJAK1
Blot:αJAK2
Blot:αJAK3
Blot:αTyk2
E. IP: STAT6
0'
0.5'
1'
3'
15'
60'
Ab
116
83
STAT6
Blot:α-py
100
Blot:αSTAT6

phosphorylation of JAK2 (Fig. 4-9B). Tyk2, on the other hand, was constitutively phosphorylated and its phosphorylation was not augmented in response to IL-4 (Fig.4-9D). STAT6 is the major JAK substrate activated by IL-4 in B cells (173,176). Therefore, the activation of STAT6 in response to IL-4 in the BL30/B95-8 cells was investigated. STAT6 was phosphorylated in response to IL-4 as early as 30 sec and decreased 1 hr post-stimulation (Fig. 4-9E). In MK3.31 cells, IL-4 induced phosphorylation of JAK3 (Fig. 4-10C) but did not affect the activation status of JAK1 and JAK2, which remained unphosphorylated (Fig. 4-10A and B). Similar to the results obtained for BL30/B95-8 cells, Tyk2 was constitutively phosphorylated and IL-4 did not further enhance its phosphorylation (Fig. 4-10A-D). Furthermore, in the MK3.31 cells, STAT6 was also strongly phosphorylated in response to IL-4 (Fig. 4-10E).

Effect of IL-13 on the activation of the JAK/STAT pathway in BL30/B95-8 and MK3.31 cells

The failure of IL-13 to induce CD23 and CD44 expression and HA binding in BL30/B95-8 and MK3.31 cells was also investigated at the level of the JAK/STAT pathway. Similar to the experiments with IL-4, I cultured the cells in serum-free media overnight followed by treatment with IL-13 (50 ng/ml) for 3 min. The lysates were then subjected to the same immunoprecipitation conditions as described for the IL-4 experiments. IL-13 treatment of BL30/B95-8 cells did not result in phosphorylation of JAK1, JAK2, JAK3, Tyk2, or STAT6 (Fig. 4-11A-E). To demonstrate that the IL-13 was biologically active and capable of activating STAT6, phosphorylation of STAT6 in an

Fig. 4-10: IL-4 activates JAK3 and STAT6 in MK3.31 cells.

MK3.31 cells were stimulated with IL-4 (40 ng/mL) for various time points. Cell lysates were immunoprecipitated with antibody specific for JAK1 (A), JAK2 (B), JAK3 (C), Tyk2 (D), and STAT6 (E). Blots were probed with anti-phosphotyrosine, then stripped and reprobed with antibodies homologous to the immunoprecitiating antibody (lower panels). "*" indicates the point of migration of unphosphorylated kinase.

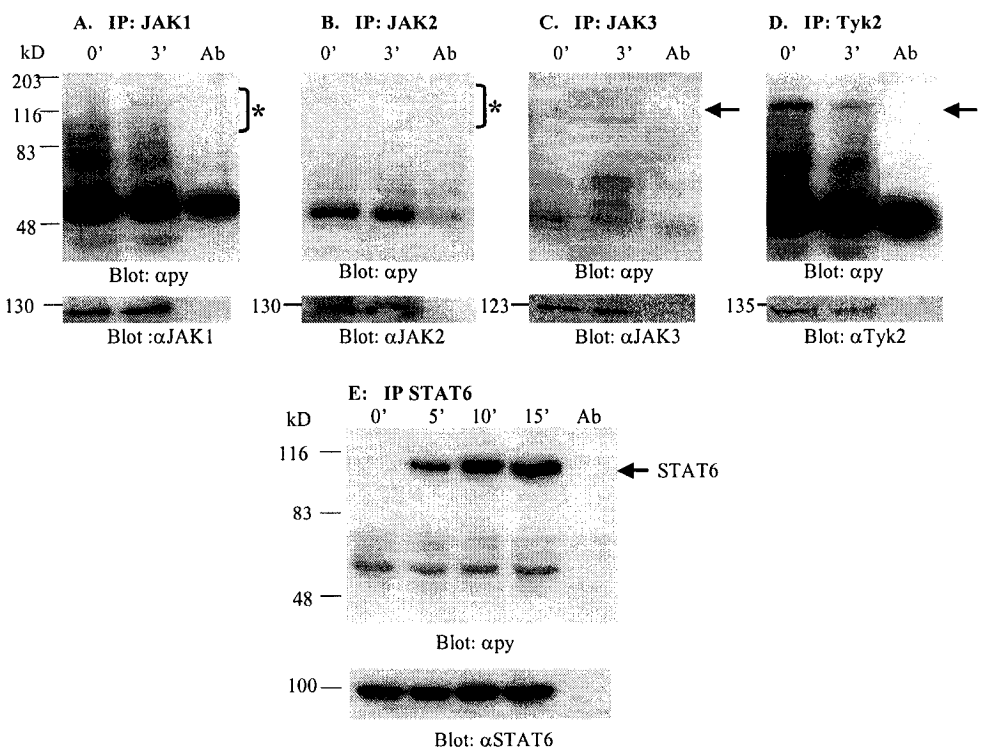
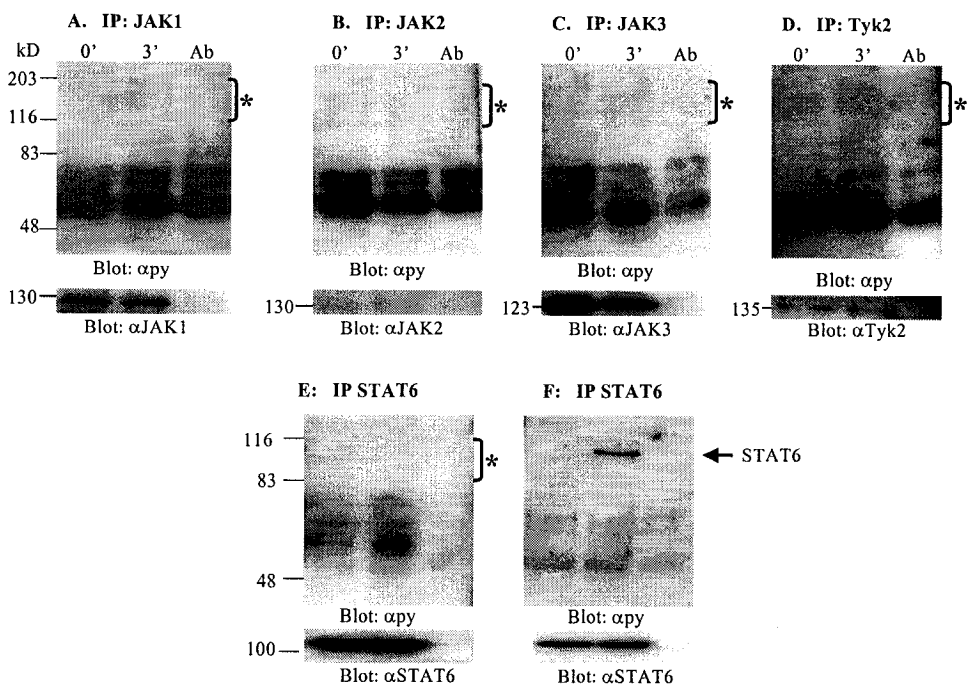

A. IP: JAK1
kD
0'
3'
Ab
203
116
83
48
*
Blot: αpy
130
Blot :αJAK1
B. IP: JAK2
0'
3'
Ab
*
Blot: αpy
130
Blot: αJAK2
C. IP: JAK3
0'
3'
Ab
Blot: αpy
123
Blot: αJAK3
D. IP: Tyk2
0'
3'
Ab
Blot: αpy
135
Blot: αTyk2
E: IP STAT6
kD
0'
5'
10'
15'
Ab
116
83
48
STAT6
Blot: αpy
100
Blot: αSTAT6

Fig. 4-11: IL-13 activates STAT6 in TF-1 cells but not in BL30/B95-8 cells.

BL30/B95-8 cells were treated with IL-13 (50 ng/mL) for various time points. Membranes were treated as described for Fig. 4. BL30/B95-8 lysates were immunopreciptated with antibodies specific for JAK1 (A), JAK2 (B), JAK3 (C), Tyk2 (D), and STAT6 (E+F). TF-1 cell lysates (F) were used as a positive control for IL-13 biological activity. "*" indicates the point of migration of unphosphorylated kinase.


A. IP: JAK1
kD
0'
3'
Ab
203
116
83
48
*
Blot: αpy
130
Blot: αJAK1
B. IP: JAK2
0'
3'
Ab
*
Blot: αpy
130
Blot: αJAK2
C. IP: JAK3
0'
3'
Ab
*
Blot: αpy
123
Blot: αJAK3
D. IP: Tyk2
0'
3'
Ab
*
Blot: αpy
135
Blot: αTyk2
E: IP STAT6
116
83
48
*
Blot: αpy
100
Blot: αSTAT6
F: IP STAT6
STAT6
Blot: αpy
Blot: αSTAT6

IL-13 responsive cell line, TF-1 (Fig. 4-11F) was examined (179). Similar to the results in the BL30/B95-8 cells, IL-13 stimulation of MK3.31 cells also failed to induce tyrosine phosphorylation of JAK1, JAK2, JAK3, or Tyk2 kinases (Fig. 4-12 A-D). As was observed in the case of IL-4-stimulated BL30/B95-8 and MK3.31 cells, Tyk2 was constitutively phosphorylated in MK3.31 cells, with no further increase in phosphorylation in response to IL-13 (Fig. 4-12D). Furthermore, low levels of constitutive STAT6 phosphorylation were observed in MK3.31 cells and when the STAT6 antiphosphotyrosine blot was overexposed for 7 days, no increase above the basal levels of phosphorylation was apparent after 15 min of stimulation (Fig. 4-12E). Similar results were observed for MK4.15 and MK5.26 cells (data not shown).

Expression of IL-13 receptor in BL and B-LCL cells

The absence of phosphorylation of the JAK/STAT proteins in BL30/B95-8 and MK3.31 cell lines in response to IL-13 suggested the presence of a defect in IL-13 receptor expression or function. Expression of IL-13Rα1 and IL-13Rα2 message in both cell lines was examined by RT-PCR analysis using IL-13Rα1 and IL-13Rα2-specific primers. PHA-stimulated PBMCs were used as a positive control. A band of 509 bp corresponded to the IL-13Rα1 and a band of 1088 bp corresponded to the IL-13Rα2. BL30/B95-8 cells did not show IL-13Rα1 or IL-13Rα2 specific bands (Fig. 4-13A-B). However, IL-13Rα1 message was detected in MK3.31 cells, whereas IL-13Rα2 was not. Similar results were obtained for MK4.15 and MK5.26 (data not shown). To further investigate whether the absence of IL-13 receptor chains was not restricted to BL30/B95-8 cells, other BL cells were examined for the expression of IL-13Rα1 and IL-13Rα2. None of the BL cells examined including EBV-negative CA46, ST486 and Ramos, and

Fig. 4-12: IL-13 does not activate JAK1, JAK2, JAK3, Tyk2, or STAT6 in MK3.31 cells.

Cells were treated with IL-13 (50 ng/mL) for various time points. Cell lysates were immunoprecipitated with antibodies specific for: JAK1 (A), JAK2 (B), JAK3 (C), Tyk2 (D), and STAT6 (E). The STAT6 blot anti-phosphotyrosine blot (E) was overexposed (7 days). "*" indicates the point of migration of unphosphorylated kinase.

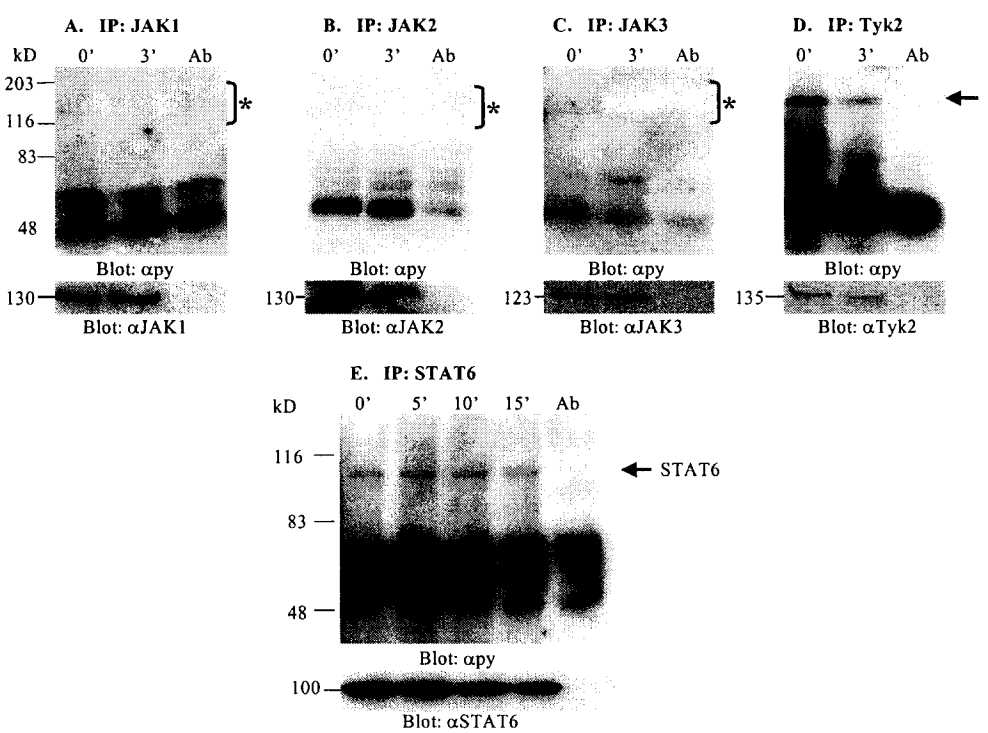
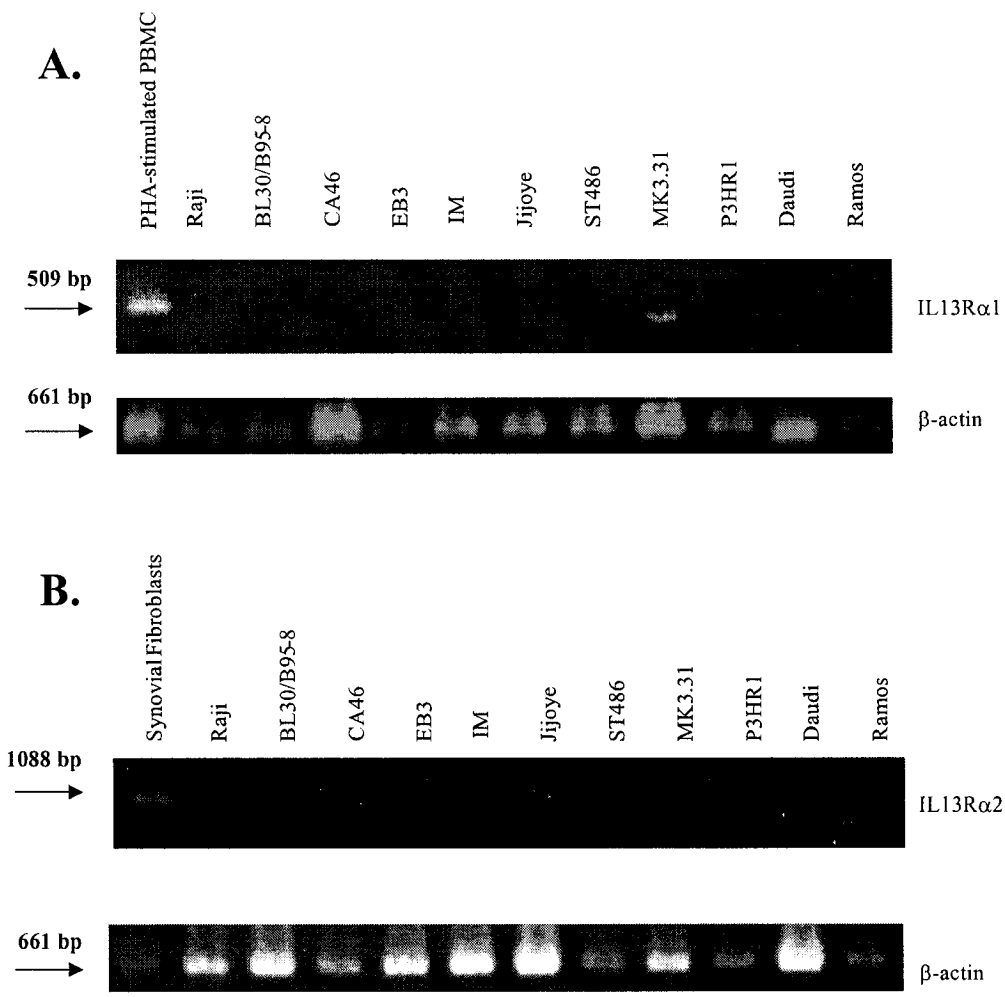

A. IP: JAK1
kD
0'
3'
Ab
203
116
83
48
*
Blot: αpy
130
Blot: αJAK1
B. IP: JAK2
0'
3'
Ab
*
Blot: αpy
130
Blot: αJAK2
C. IP: JAK3
0'
3'
Ab
*
Blot: αpy
123
Blot: αJAK3
D. IP: Tyk2
0'
3'
Ab
Blot: αpy
135
Blot: αTyk2
E. IP: STAT6
kD
0'
5'
10'
15'
Ab
116
83
48
STAT6
Blot: αpy
100
Blot: αSTAT6

Fig. 4-13: IL-13Rα1 is expressed in MK3.31 cells and neither IL-13Rα1 nor 2 are expressed in BL cell lines.

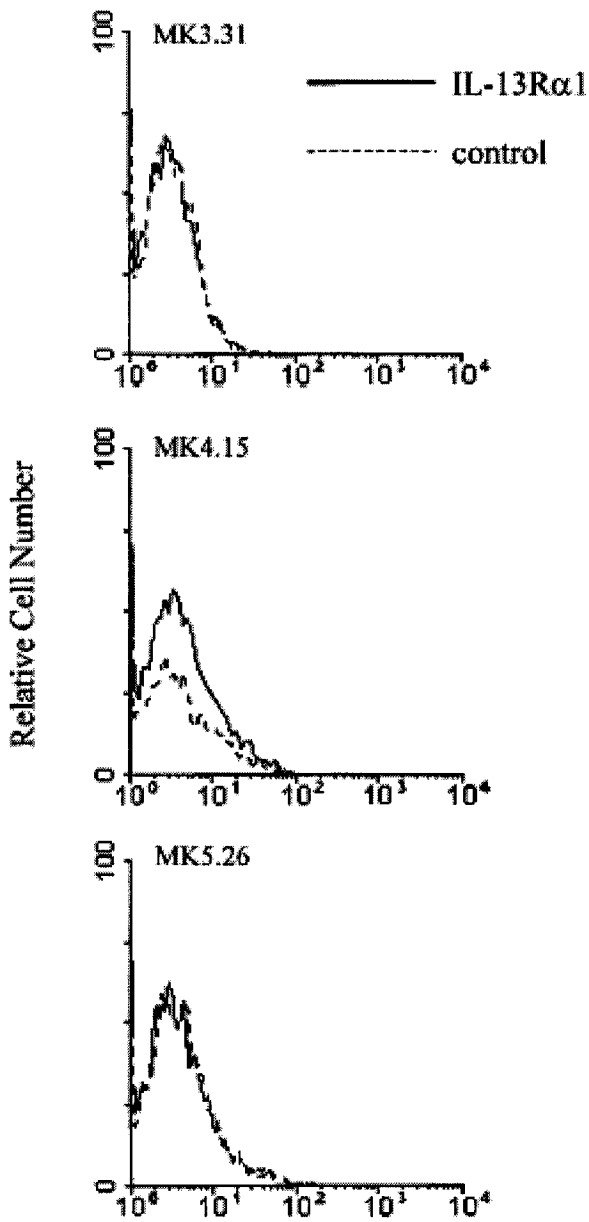
Cellular RNA was subjected to RT-PCR analysis. A. Cells were analyzed for IL-13Rα1 expression. B. Cells were analyzed for IL-13Rα2 expression. Corresponding β-actin blots are shown in the lower panels.


A.
PHA-stimulated PBMC
Raji
BL30/B95-8
CA46
EB3
IM
Jijoye
ST486
MK3.31
P3HR1
Daudi
Ramos
509 bp
IL13Rα1
661 bp
β-actin
B.
Synovial Fibroblasts
Raji
BL30/B95-8
CA46
EB3
IM
Jijoye
ST486
MK3.31
P3HR1
Daudi
Ramos
1088 bp
IL13Rα2
661 bp
β-actin

EBV-positive Raji, Jijoye, EB3, P3HR1 and IM cells expressed either IL-13Rα1 or IL-13Rα2 (Fig. 4-13A-B). Because IL-13Rα1 expression was demonstrated in the MK3.31 cell line by RT-PCR analysis, the expression of the protein on the surface of the three EBV-transformed B-LCL cell lines MK3.31, MK4.15, and MK5.26 was examined by flow cytometry. Cells were stained with a commercially available antibody specific to the IL-13Rα1 chain. This antibody was able to detect IL-13Rα1 expression on CD14+ normal human monocytes (data not shown) however, the MK series of B-LCL cell lines were negative (Fig. 4-14). Taken together, the results show that neither EBV positive nor EBV negative BL cells express either of IL-13R α1 or α2 chains. In contrast, the MK series of B-LCL cells express IL-13Rα1 mRNA, but they do not express IL-13Rα1 on their cell surface membrane.

Fig. 4-14. The IL-13Rα1 chain is not expressed at the surface of MK series of B-LCL cells.

Cells were stained with a biotin conjugated primary mouse anti-human IL-13Rα1 mAb and counterstained with streptavidin-conjugated with PE (solid line). The dashed line indicates staining with isotype matched control antibody.



IL-13Rα1 Expression

DISCUSSION

Differential effect of IL-4 and IL-13 on CD44 induction and CD44-mediated HA interactions in BL and B-LCL cells

In this study, we investigated the regulation of CD44-HA interactions in transformed human B cells. The results show that the ability of CD44 to recognize HA is dependent on the mode of activation and state of transformation of human B cells. Amongst the mitogens, PMA, and amongst the cytokines, IL-4 alone induced strong HA recognition in the EBV-positive BL cells, BL-30/B95-8 and *in vivo* transformed B (IM) cells. In contrast, *in vitro* EBV-transformed normal human B cells (B-LCL) failed to recognize HA following PMA stimulation, highlighting the stark contrast between BL30/B95-8 and B-LCL B cells.

IL-4 is a pleuripotent cytokine that plays a central role in B cell activation, growth and differentiation (294). In addition, IL-4 induces the expression of a number of cell surface molecules including CD23 and HLA class II molecules on B cells (294), and a VCAM-1 like adhesion molecule on endothelial cells (295,296). In EBV-positive BL and B-LCL cells, IL-4 induces the expression of IgE germ line transcripts without inducing IgE synthesis (292), and expression of CD23, class II molecules and adhesion molecules LFA-1 and LFA-3 (290,291). In this report, we describe the novel effect of IL-4 on CD44 expression and CD44-mediated HA adhesion in human BL-30/B95-8 BL cells. Keeping in view the important role of CD44 in the ability of Burkitt's lymphoma cells to form tumors in nude mice (297), IL-4 induced CD44-HA interactions in BL30/B95-8 cells may have implications with respect to tumorigenesis in individuals exhibiting immunosuppression and a predominance of Th2 cytokines. This hypothesis is further supported by the observation that B-LCL cells, which fail to recognize HA even after

stimulation with the potent mitogen PMA, or IL-4, do not induce tumors in nude mice (298).

Cytokines have been reported to modulate CD44 expression and CD44 mediated HA binding in various cell types,(5,62,136,286). Adhesive interactions between CD34+ hematopoietic progenitor cells were regulated by GM-CSF, IL-3 and stem cell factor (136). Interestingly, these cytokines enhanced CD44-mediated adhesive interactions within 15 minutes of stimulation, a phenomenon similar to that described for certain anti-CD44 antibodies (1,299), indicating alterations in the structure/conformation of CD44 molecules. IL-4-induced CD44 expression and CD44-mediated HA binding in BL30/B95-8 cells was observed 12 hrs after stimulation, a time sufficient to allow cell surface expression of newly synthesized CD44 molecules. It has been shown in our laboratory that IL-4 induces CD44 proteins with slightly reduced molecular weight, indicative of a reduction in their glycosylation ((111) and data not shown). Whether IL-4 induces alterations in the glycosylation of other cell surface and secretory molecules is not understood. There is, however, one report demonstrating that IL-4 reduces the complexity of N-glycosylation of secretory IgA when used in conjunction with IL-5 (300), strengthening our contention that it may effect similar modifications to CD44 in BL-30/B95-8 cells and possibly to other molecules. IL-4 and IL-13 exert similar biological effects on B cells such as the enhancement of proliferative responses to IgM and CD40 cross-linking, upregulation of MHC class II and CD23 expression, and anti-CD40 dependent IgE class switching (288,301-303). This has been attributed, at least in part, to the shared IL-4α and γ_c components of their receptors (304,305). Yet unlike IL-4, IL-13 neither upregulated CD44 expression nor induced HA adhesion in BL30/B95-8

cells at days 1, 2, and 4 post-stimulation, even at concentrations of up to 200 ng/mL (data not shown). PMA or IL-4 also did not enhance CD44 expression or CD44-mediated HA binding in B-LCL cells even though BL30/B95-8 and B-LCL cells are infected with wild type EBV and express similar CD44 isoforms (220). I investigated the molecular mechanism for the distinct effects of IL-4 and IL-13 in terms of CD44 expression and CD44 mediated HA adhesion in BL and B-LCL cells and is discussed in the following section. However, the molecular mechanism by which B-LCL cells, in contrast to BL cells, fail to bind HA following stimulation with PMA remains to be investigated. It is possible that *in vitro* EBV transformation of B cells inactivates a vital element in the intracellular signalling pathway such as the transcription factor Egr-1 which has been shown to mediate CD44 induction following PMA stimulation of murine B cells (142). Alternatively, the *c-myc* translocation not found in B-LCLs, may contribute to CD44-mediated HA adhesion. Transfection of an activated *c-myc* gene into B-LCL cell lines has been shown to yield cells with many of the phenotypes attributed to BL cells including CD10 and CD38 expression and the propensity to undergo apoptosis (306). However, PMA and IL-4 were able to induce CD44 expression and CD44-HA binding in IM cells, the *in vivo* transformed human B cells. The status of IM cells with respect to *c-myc* translocation and other genetic alterations is being investigated.

CD44-mediated HA binding may be attributed in part to the level of CD44 expression (1). A minimum threshold level of CD44 expression that is necessary for cells to bind HA has been suggested (307). This may explain at least in part the variability in binding of various BL cells to HA. Although EB3, Raji, and BJAB/B95-8 BL cells are infected with EBV, these BL cells did not express CD44. Enhanced expression of CD44

H by PMA and/or IL-4 was correlated with CD44-mediated HA binding. However, the adhesion of BL cells to HA may not exclusively depend upon the level of CD44 expression as BL cells such as Jijoye and B-LCL cells constitutively express relatively high levels of CD44 (220) yet do not exhibit spontaneous binding to HA. These cells may express a predominantly inactive form of CD44 molecule and stimulation with PMA or IL-4 may induce the expression of active CD44 molecules on the cell surface. This is evident by the detection of new CD44 mRNA synthesis within 4 hrs of stimulation by RT-PCR analysis and alterations in the molecular weight of CD44 (111) and data not shown). The induction of CD44 isoforms containing specific variable exons may also be a determinant for binding to HA. For example, V6 and V9 expression may be required for recognition of HA by activated human T cells (308). Whether induction of novel isoforms by BL cells following stimulation with either PMA or IL-4 plays any role in binding to HA remains to be investigated.

Increases in the complexity of N-linked glycosides, addition of sialic acid and keratan sulfate to CD44 have generally been found to reduce HA recognition (11,12,25,28-30,62). It has been previously shown that IL-4 induced alterations in the elctrophoretic mobility of CD44 (6), and enhanced binding to HA following treatment of BL30/B95-8 cells with tunicamycin, an agent that inhibits N-linked glycosylation ((111) and data not shown), further suggest that alterations in CD44 glycosylation status may be important in the induction of HA binding in these cells. Clustering and homodimerzation of CD44 molecules on the cell surface induced by PMA as observed in a recent study (309) may also enhance HA recognition by BL cells. However, we cannot rule out the involvement of other mechanisms such as the interaction with elements of the

cytoskeleton via the cytoplasmic domain, phosphorylation of the cytoplasmic domain and interaction with other ligands either extracellular or at the cell surface (1,2).

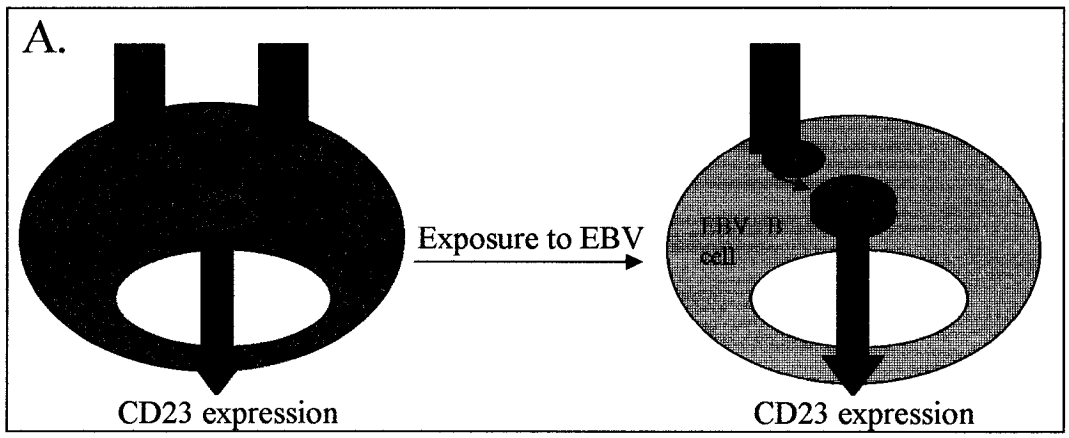
In summary, the present data suggest that CD44 expression and CD44-mediated binding to HA in BL30/B95-8 BL cells is upregulated by the B cell differentiation cytokine, IL-4, through a mechanism that may include a combination of increased CD44 expression levels, or a reduction in CD44 H glycosylation. This mechanism does not come into play for B-LCL cells. Although we have not observed this phenomenon in all BL cell lines, the results obtained with BL30/B95-8 and IM cells may be representative of BL cell lines transformed *in vivo* and may have implications in the establishment of tumors. Furthermore, the novel effect of IL-4 on CD44-HA interactions in BL30/B95-8 cells provides a model system to further study the molecular mechanisms responsible for the regulation of CD44 expression and CD44-mediated HA binding in human B cells. Taken together, these findings suggest that IL-4 in addition to its role in B cell activation, proliferation and differentiation may in parallel influence the adhesive interactions between transformed B cells and ECM molecules, which may affect their traffic and localization within different tissues and organs.

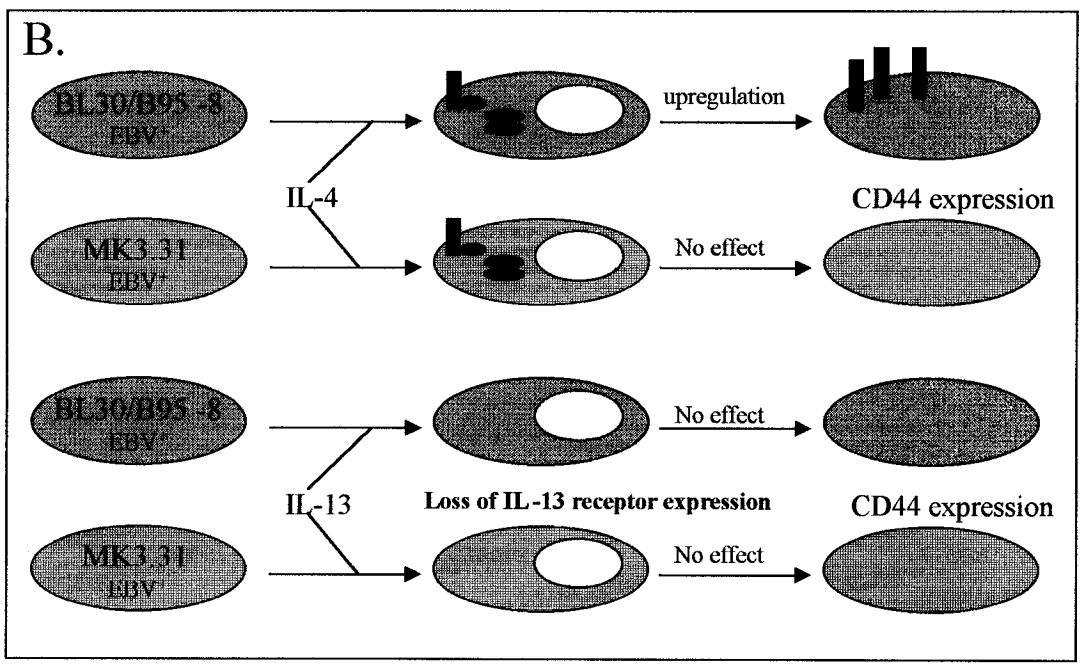
Loss of IL-13 receptors may account for the loss of IL-13-mediated biological effects including CD44 expression and CD44-mediated HA interactions in BL and B-LCL cells.

Transformation of normal human B cells with EBV and the infection of BL B cells with EBV is accompanied by the altered expression of several cell surface molecules (310-314) and this may result in distinct biological effects in response to cytokines. As mentioned earlier, IL-4 and IL-13 exhibit similar biological effects on B cell growth and regulation such as the upregulation of CD23 and MHC-II expression as well as the induction of IgE production (148-150,153,154). I studied the effect of IL-4 and IL-13 on a B lymphoblastoid cell line (B-LCL), MK3.31, and on an *in vitro* EBV-infected BL B cell line, BL30/B95-8. IL-4 induced CD23 and CD44 expression as well as CD44-mediated binding to HA on BL30/B95-8 cells, and CD23 expression on MK3.31 cells. In contrast, IL-13 failed to exhibit these biological effects on either BL30/B95-8 or the MK series of B-LCL cells (Fig.4-8). To understand the molecular mechanisms underlying the differential effects of the two cytokines, the activation of the JAK/STAT signaling pathway in the two B cell lines was compared. It was shown that IL-4 induced the phosphorylation of JAK3 and STAT6 in both BL30/B95-8 and MK3.31 cell lines, yet induction of JAK1 phosphorylation occurred only in the BL30/B98-5 cells and not in the MK3.31 cells. In addition, IL-13 failed to induce the phosphorylation of any JAK kinases or STAT6 proteins in either of these cell lines. The inability of BL30/B95-8 cells to respond to IL-13 may, at least in part, be due to the loss of expression of the IL-13R subunit α1, a finding confirmed for a number of other BL cell lines examined (Fig 4-13). A summary of these results is presented as a model in Fig 4-15.

Fig. 4-15. Model of IL-4/IL-13 responses in BL and B-LCL cells.

Normal human B cells express both IL-4 and IL-13 receptors and respond to both cytokines as indicated by the increase in CD23 expression. The effect of IL-4 and IL-13 on CD23 expression in human B cells is well documented as is the involvement of the JAK/STAT pathway, particularly that for STAT6. Transformation of human B cells with EBV results in, among other things, a loss of expression of the IL-13 receptor, and therefore a lack of IL-13 effects on CD23 expression. The IL-4 receptor remains intact, as does the response of the cells to IL-4 with respect to CD23 expression. B. Treatment of both BL and B-LCL cells with IL-4 results in activation of the JAK/STAT pathway, however only the BL cell line responded with respect to an increase in CD44 expression and HA binding. Both cell types did not respond to IL-13, as there are no IL-13 receptors expressed on either cell types.


A.
Exposure to EBV
EBV+ B cell
CD23 expression
CD23 expression


B.
BL30/B95-8
EBV+
upregulation
IL-4
CD44 expression
MK3.31
EBV+
No effect
BL30/B95-8
EBV+
No effect
IL-13
Loss of IL-13 receptor expression
CD44 expression
MK3.31
EBV+
No effect

B-LCL cells, in contrast to BL cells, do not form tumors in humans or in experimental animals (298). The lack of tumorigenicity of B-LCL cells may be explained at least in part by their inability to bind HA. Since HA, a component of the ECM, is a natural ligand for the CD44 receptor (2), CD44-HA interactions may play an important role in cell-cell and cell-ECM interactions and functions as an adhesive protein in such events as lymphopoiesis, leukocyte extravasation into inflammatory sites, and tumorigenesis (2,70,71,315). CD44 has also been suggested to play a vital role in *in vivo* Burkitt's lymphoma (BL) tumor growth and dissemination in a nude mouse model (93,316). In this study, I confirmed previous observations that IL-4 enhanced CD44 expression and CD44-mediated HA binding in BL30/B95-8 BL cells, but not in MK B-LCL cells (111). In view of the critical role of CD44 in the ability of BL cells to form tumors in nude mice (316), IL-4-induced CD44-HA interactions in BL30/B95-8 cells may have implications with respect to tumorigenesis in individuals exhibiting immunosuppression and a predominance of Th2 cytokines.

This is the first report showing that IL-4, but not IL-13, induces the activation of the JAK/STAT pathway in BL30/B95-8 BL cells. IL-4 induced the tyrosine phosphorylation of distinct JAK proteins in BL30/B95-8 BL and MK3.31 B-LCL cells. A subtle difference between the two cell lines was observed in that IL-4 induced phosphorylation of JAK1 in the BL30/B95-8 cells but not in the MK3.31 cells. My results are in agreement with previous reports which show that IL-4 induces phosphorylation of JAK3 proteins in B-LCL cells (317,318). Whether differences in IL-4-induced activation of JAK1 in these two cell types may account for the differences in IL-4-induced CD44 expression and CD44-mediated HA binding is not clear. In view of

the variable response of IL-4 with respect to JAK1 phosphorylation observed in different B-LCL cell lines (317), this pathway alone may not account for differential CD44 expression, and alternate signaling proteins may be involved in the differential regulation of CD44 in these cell lines. Increased phosphorylation of Tyk2 in either BL30/B95-8 or MK cell lines was not observed in contrast to some reports that show that IL-4 induces Tyk2 phosphorylation in B-LCL cells (317). A constitutive phosphorylation of Tyk2 in BL30/B95-8 and MK3.31 cells was observed, however, and is in agreement with previous reports demonstrating this in B-LCL cells (317,318). The significance of constitutive phosphorylation of the Tyk2 kinase in B-LCL cells is not known. Recently, it was suggested that the JAK/STAT pathway is activated in response to transformation of T cells with HTLV (319,320). In addition, the EBV latent membrane protein- (LMP)-1 has been shown to interact with JAK3, leading to enhanced tyrosine auto/transphosphorylation of JAK3 (321). Therefore, it is possible that EBV transformation of B cells may induce constitutive Tyk2 phosphorylation in these cells. It is also possible that Tyk2 phosphorylation is dependent on cell type and state of cell activation.

In this study I also show that transformation of normal human B cells with EBV results in the loss of IL-13-mediated effects, such as CD23 expression, while at the same time preserving the biological effects of IL-4. This was also evidenced by the failure of IL-13 to activate the JAK/STAT signaling pathway in the MK series of B-LCL cells and the lack of expression of an IL-13 receptor. The existence of other IL-13 receptor chains has been suggested, but none so far have been identified. IL-13 mediates its biological effects by activating JAK/STAT signaling molecules in normal human B cells,

monocytes, and several other cell types including renal cell carcinoma, ovarian cell carcinoma, B9 mouse plasmacytoma, and human colon carcinoma cell lines (322). However, IL-13 exposure did not result in the phosphorylation of JAK1, JAK2, JAK3, or STAT6 in MK cells. The molecular mechanism for the failure in the activation of JAK/STAT signaling proteins in B-LCL cells is not clear. Although the MK3.31 cells expressed IL-13Rα1 mRNA, none of the MK series of B-LCL cells expressed IL-13Rα1 protein on their cell surface as determined by flow cytometry. It is likely that very few IL-13Rα1 molecules are present on the cell surface membrane but are not evident as they are below the threshold of detection by flow cytometric analysis. Since IL-13 did not induce the activation of any of the JAK proteins or of STAT6 in MK3.31 cells, it is reasonable to conclude that IL-13 receptors on the MK series of B-LCL cells are non-functional, even though a small number may be expressed. This is akin to T cells, which characteristically do not respond to IL-13 even though these cells express IL-13Rα mRNA (148,153,171). IL-13Rα has been shown to exist as a soluble protein in T cells, but it is not expressed on the cell surface (171). It is possible that EBV transformation of B cells affects either the expression pattern or the processing of the IL-13R proteins; the receptor in such cases may exist as a message, cytoplasmic protein, or as a non-functional entity on the cell surface. It is also possible that the presence of IL-13Rα1 alone may not confer IL-13 responsiveness; another receptor or intercellular component may be required in order to activate the JAK/STAT signaling pathway. These results are in contrast to some previously published reports showing IL-13-induced activation of STAT6 in some B-LCL cells (317,318). The reason for the differences in the IL-13 signaling pathways of different B-LCL cells is not clear, but may be due to the differences in the generation of

B-LCL cells. Nonetheless, these findings are consistent with the observations that IL-13 has not been shown to influence the biological effects including the expression of any cell surface markers on B-LCL cells.

Most B cell malignancies consist of a clonal expansion of B cells arrested at a particular stage in their development and differentiation. BL cells, which resemble germinal center B cells, are characterized by a chromosomal translocation placing the *c-myc* gene under the control of the immunoglobulin gene promoter. While oncogenic activation due to translocations involving *c-myc* or other B cell receptor genes may give survival or proliferative advantages to malignant cells, various B cell growth and differentiation factors such as IL-4 and IL-13 may participate in the development of Burkitt's lymphomas. IL-4 has been suggested to play a vital role in the survival of BL B cells (293). IL-13, which is produced by various malignant B cell types including non-Hodgkin's lymphoma, chronic B cell leukemia, follicular lymphoma, and Burkitt's lymphoma B cells (323,324), induces proliferative responses in lymph node B cells from non-Hodgkin's lymphoma (325) and protects B cells from chronic lymphocytic leukemia (326). My results show for the first time that IL-13 does not induce the phosphorylation of any of the JAK kinases or STAT6 proteins in BL30/B95-8 cells, resulting from a loss of the IL-13R complex. These results were further confirmed using several BL cells lines including EBV-negative (CA46, ST-486, Ramos) and EBV-positive (Raji, EB3, Jijoye, P3HR-1, Daudi) cells, none of which expressed IL-13 Rα1 or IL-13 Rα2 mRNA. Whether the loss of IL-13R complex subunits in BL cells is a consequence of their transformation is not clear. It has been suggested that immature B cells do not express IL-13 receptors (150). In view of the observations that EBV-negative BL cells did not

express IL-13R, it is likely that loss of IL-13R expression may have occurred during B cell development and differentiation. Loss of IL-13R in BL cells may not, therefore, be a consequence of B cell transformation, and BL cells may represent transformed, immature IL-13R-negative B cells arrested during development.

In summary, I have demonstrated that Burkitt's lymphoma B cells lack IL-13 receptor α1 and α2 chains, findings which may account for the lack of IL-13-mediated effects including induction of CD23 and CD44 expression. The absence of tyrosine phosphorylation of the JAK and STAT6 proteins in response to IL-13 also excludes the possibility of the presence of an unidentified IL-13 receptor subunit that may associate with the IL-4Rα chain to form a functional IL-13 receptor in BL cells. In contrast, EBV-transformed B cells expressed the IL-13Rα1 message but not detectable surface protein, an observation that explains the absence of IL-13-mediated effects in these cells. The results of this section have been summarized in a model in Fig. 4-15. The significance of the specific loss of IL-13-mediated effects in BL and B-LCL cells in the maintenance of the transformed phenotype is not clear. These results suggest that IL-13, in contrast to IL-4, may not play a role in the growth and differentiation of BL and B-LCL B cells. The capacity of IL-4 alone to induce changes in CD44 expression or CD44-mediated HA binding in BL cells may further suggest its vital role in the development and localization of BL B cells within different tissues and organs.

CHAPTER 5:
CONCLUDING REMARKS, SIGNIFICANCE, AND FUTURE DIRECTIONS

The interactions of the glycoprotein, CD44, and its ligand hyaluronan (HA) have been shown to play a major role in cell migration during the inflammatory response as well as during tumorigenesis and metastasis. The ability of CD44 to bind HA is the crucial mechanism underlying the biological functions attributed to CD44. One of the most important questions is what are the possible cellular and biochemical signaling pathways that determine both the regulation of CD44 expression and how CD44 acquires the potential to bind HA. This constituted the central theme of my thesis research using human monocytic cells and B cells as a model system.

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 lymphocytes to the site of inflammation. To understand the molecular mechanism underlying the regulation of CD44 expression in human monocytic cells, which constituted the first objective of my research proposal, I used normal human primary monocytic cells and demonstrated that in response to LPS, CD44 expression is induced. Furthermore, 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 because endogenously produced TNF-α and IL-10 can upregulate CD44 expression in an autoregulatory manner, I used the promonocytic THP-1 cell line as a model system. The THP-1 cells responded similarly to normal human monocytes with respect to LPS-induced CD44 expression. My results revealed for the first time a distinct role for the JNK MAPK activation in the LPS-induced but not in TNF-α-induced CD44 expression. However, my results did provide evidence for a partial role of p38 MAPK in

both LPS- and TNF-α-induced CD44 expression. Lower concentrations of p38 MAPK inhibitors did partially inhibit LPS- and TNF-α-induced CD44 expression consistently, but this inhibition was never complete even when high concentration of inhibitors were employed. The role for p38 in the induction of CD44 expression by LPS and TNF-α is not clear from my studies, however it may cooperate with other signaling pathways to affect CD44 expression.

I further investigated the involvement of transcription factors that may be activated by JNK MAPK resulting in CD44 induction. My results suggest that LPS-induced CD44 expression may involve the Egr-1 transcription factor through JNK MAPK activation. These results suggest that distinct signaling pathways may be involved in LPS and TNF-α-induced CD44 expression in human monocytic cells.

These observations raise a number of questions regarding the role of signaling molecules in LPS- and TNF-α-induced CD44 expression. First, what signaling pathways are involved in TNF-α-induced CD44 expression in human monocytic cells? Our preliminary results do suggest that PI3-kinase may be involved in both LPS- and TNF-α-induced CD44 expression (data not shown). Second, although Egr-1 activation has been shown to be involved in the induction of CD44 expression in B cells and endothelial cells, whether LPS- and TNF-α-induced CD44 expression also involves Egr-1 activation in human monocytic cells remains to be investigated. This question assumes significance in view of our preliminary observations that a transcription factor other than Egr-1 is involved in IL-4-induced CD44 expression in Burkitt's lymphoma B cells. Furthermore, AP-1 activation has also been implicated in CD44 induction in a number of cell types. In order to clarify the role of transcription factors in the regulation of CD44 expression, I

have generated a series of CD44 promoter constructs. I am in the process of identifying the transcription factors and their cognate binding sites responsible for CD44 induction in response to stimulation with LPS and TNF-α in human monocytic cells and in response to stimulation of BL cells with IL-4.

The molecular signaling pathways governing the regulation and generation of the HA-binding form of CD44 in human monocytic cells as well as any other cell type are not known. This question constituted the second objective of my thesis. Similar to the results obtained with LPS-induced CD44 expression, I observed that endogenously produced TNF-α was involved in the generation of the HA-binding form of CD44 in primary human monocytic cells as well as THP-1 cells. In contrast to the results obtained for CD44 induction, the TNF-α-induced p38 MAPK activation was found to regulate LPS-induced CD44-mediated HA-binding. These results clearly demonstrated for the first time that CD44 induction and the generation of HA-adhesive form of CD44 are two independent events that are regulated by distinct signaling pathways.

My studies did not identify the signaling pathways responsible for either IL-10-induced CD44 expression or CD44-mediated HA binding in monocytic cells, however, the results do suggest that none of the MAPKs are involved. Whether the JAK/STAT pathway is activated following interaction of IL-10 with its receptor and this regulates CD44 expression or CD44-mediated HA binding needs to be investigated.

I further investigated the role of TNF-α-induced p38 MAPK underlying the generation of HA-adhesive CD44 isoform following stimulation of monocytic cells with LPS. Desialidation of the CD44 receptor is one of the post-translational mechanisms known to induce CD44-HA binding. My results revealed that in human monocytic cells,

both LPS and TNF-α can induce lysosomal sialidase activity that is both necessary and sufficient to induce the generation of the HA-binding form of CD44. I have also demonstrated that LPS- and TNF-α-induced sialidase activity resided in the soluble cytoplasmic component and not in the membrane component of the cells (data not shown). Furthermore, my results suggested a novel role for p38 MAPK in the TNF-α-mediated induction of sialidase activity that resulted in the generation of HA-adhesive form of CD44. Taken together, these results suggest for the first time the pivotal role for TNF-α-induced p38 MAPK activation in the induction of lysosomal sialidase activity and the generation of HA-adhesive functionally active CD44 phenotype in LPS-stimulated human monocytic cells. Whether this is the case for all cell types, including the IL-4-induction of CD44 expression and HA-binding in the BL30/B95-8 cells, needs to be addressed.

In addition to the role played by lysosomal sialidases in the generation of HA-adhesive CD44, the other post-translational modifications of CD44 such as sulfation and N- and O-linked glycosylation may also be involved. Further experiments are necessary in order to determine whether the addition of sulfation or modulation of N- or O-linked glycosylation are involved in the generation of HA-adhesive CD44 and whether these effects are modified by the MAPK pathways in monocytic cells.

It will also be interesting to determine if the p38 MAPK is involved in biological functions attributed to CD44 such as cell migration, inflammation and autoimmune diseases. This could be potentially determined by using p38 knockout mice. These observations could be applied to animal models of autoimmune disease, such as rheumatoid arthritis, in which CD44-HA interactions have been previously implicated.

Thus, my studies, designed to understand the signaling pathways underlying LPS- and TNF-α-induced CD44 expression and generation of HA-adhesive CD44 phenotype, in effect have opened a number of avenues and directions that may provide important new insights into the role of CD44 *in vivo*. Further delineation of the biochemical signaling pathways regulating CD44 induction and the generation of HA-adhesive phenotype, by employing human monocytic cell lines and CD44 and specific MAPKs 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 disorders.

CD44 has also been shown to play a role in the pathogenesis of human cancers, in particular, in the tumorigenesis of Burkitt's Lymphomas (BL). Cytokines present in the microenvironment of the tumors may affect tumor growth, formation, and metastasis. The first objective of this chapter of my thesis was to investigate the effects of various mitogens and cytokines on CD44 expression and binding in normal human B cells, EBV-transformed lymphoblastoid B cells and EBV-infected BL B cell lines. I observed that in response to PMA, CD44 expression and HA binding were increased in EBV-infected Burkitt's lymphoma, BL30/B95-8, cell line. Of a panel of cytokines tested, I identified IL-4 as one cytokine that could induce both CD44 expression and HA-binding in BL30/B95-8 cells. However, EBV-transformed B-LCL cells were found to be refractory to the CD44-inducing effects of PMA and IL-4. In contrast, IL-13 a cytokine sharing

similar characteristics with IL-4, did not induce CD44 expression or CD44-HA binding in either cell type. Therefore, these results demonstrated a differential role for IL-4-induced CD44 expression and HA binding in EBV-infected BL30/B95-8 and B-LCL cell lines. In addition, none of these cell lines were found to respond to the biologically active IL-13. Taken together, these results suggested that EBV-transformation of normal human B cells results in the preferential loss of IL-13-mediated biological effects. Furthermore, B-LCL cells responded to stimulation with IL-4 but, unlike BL30/B95-8 cells, selectively lost the ability to induce CD44 expression or CD44-mediated HA binding.

To understand the differential effects on CD44 expression and HA binding observed within the two cell lines, as well as within the two similar cytokines, as the second objective of Chapter 4 of my thesis, I examined the signaling pathways induced by IL-4 and IL-13 in the BL30/B95-8 and MK3.31 cell lines. IL-4-induced activation of JAK3, and STAT6 in both cell types, suggesting that the defect in CD44 induction in B-LCL cells by IL-4 could not be explained at the level of the JAK/STAT pathway. Further studies are needed to investigate the molecular mechanism regulating the differential induction of CD44 expression and CD44-mediated HA binding in B-LCL cells and BL30/B95-8 cells. It is tempting to speculate that other IL-4-induced pathways may be affecting the activation of CD44 expression and binding in these cell lines. For example, similarly to TNF-α-induced CD44 expression, IL-4-induced activation of PI3-kinase may be involved. IL-4 had been shown to induce the activation of this kinase and currently studies are underway to determine if PI3-k plays a role in IL-4-induced CD44 expression in human B cells. Additionally, STAT6 activation may not be sufficient to induce CD44 expression and may require the activation of other transcription factors that may not be

present or active in the B-LCL cell line but are functional, or active in the BL cell line. It may also be possible that the control of CD44 transcription is altered in some way in the B-LCL cell line that is not present in the BL cell line. Studies have shown that methylation of the CD44 promoter region is responsible for the down-regulation of CD44 expression in neuroblastoma and prostate cancer cells. Thus it is possible that in the MK3.31 cells that the CD44 promoter may be affected in this way.

The absence of IL-13-mediated effects on CD44 expression and CD44-HA binding in the BL30/B95-8 and MK3.31 cell lines was characterized by the lack of IL-13-induced JAK/STAT signals. Because I was unable to detect any activation of this pathway, I examined the expression levels of IL-13 receptor chains present in these cells. The lack of both receptor chains on the BL30/B95-8, as well as on a panel of BL cell lines, explained the inability of these cells to respond to IL-13. Although the MK series of cell lines expressed mRNA levels of the IL-13Rα1 chain, this receptor component was not expressed on the cell surface, explaining why these cells also did not respond to IL-13. With respect to the ability of IL-13 to induce CD44 expression and HA binding, it would be interesting to examine if, upon transfection of the IL-13 receptor components, the BL30/B95-8 and MK3.31 cells would then respond to IL-13 with induced CD44 expression and HA binding.

In summary, diverse effects of stimulants on CD44 expression and ligand binding exist and are dependent on the signaling pathways induced in the cells. These studies may provide a basis for further investigations in the regulation of CD44 expression versus CD44-mediated HA-binding in other cell types and model systems. The understanding of the molecular mechanisms of the regulation of CD44 expression and ligand binding may

lead to the development of anti-cancer or anti-inflammatory strategies by targeting various aspects of CD44-mediated actions.

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Publications:

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Protein (AP-1) in Human IL-12p40 Gene Transcription in the Lipopolysaccharide-Stimulated Monocytic Cells. *The Journal of Immunology.*

Presentations:

1) Differential Regulation of CD44 expression by Interleukin-4 and Interleuikin-13 in Human B Cell Lines. Departmental Seminar, October 23, 1998.
2) Differential Regulation of CD44 Expression by Lipopolysaccharide (LPS) and TNF-α in Human Monocytic Cells: Distinct Involvement of c-Jun N-Terminal Kinase in LPS-Induced CD44 Expression. Departmental Seminar, November 18, 2000.
3) TNF-α Induces Functionally Active Hyaluronan-Adhesive CD44 by Activating Sialidase Through p38 Mitogen-Activated Protein Kinase in LPS-Stimulated Human Monocytic Cells Departmental Seminar, January 17, 2003

Abstracts:

1) K.Gee, M. Kozlowski, and A Kumar. Differential Regulation of CD44 Expression by IL-4 and IL-13 in Human B Cell Lines. Department of Immunology, Microbiology and Biochemistry, University of Ottawa. April 9, 1999.
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