# REGULATION OF HYALURONAN BINDING BY THE CELL ADHESION MOLECULE CD44 IN MYELOID CELLS

BY

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#### ABSTRACT

CD44 is a cell adhesion molecule that mediates cell-cell and cell-ECM interactions through an association with hyaluronan, the most characterized ligand for CD44. CD44 and HA are ubiquitously expressed, but they do not constitutively interact. At the onset of this work, multiple factors were reported to correlate with the activation and conversion of CD44 to an HA-binding form in various cell types however, minimal data linked specific factors and associated molecular mechanisms to particular physiological stimuli.

It has been documented that pro-inflammatory agents induce monocytes to bind to HA. CD44-HA interactions have been implicated in cell adhesion and cell migration events during an inflammatory response and in the pathogenesis of inflammatory disorders. The research described herein aimed to understand the stimuli and corresponding cellular mechanisms that regulate CD44-mediated HA-binding in myeloid cells under inflammatory conditions. The experiments were conducted using a human myeloid progenitor cell line and primary *ex vivo* human monocytes and murine bone marrow-derived macrophages.

Resulting from this investigation, two independent mechanisms that control HA-binding in myeloid cells in response to inflammatory agents were elucidated. One mechanism involves the sulfation of the extracellular domain of CD44. Sulfated CD44 has enhanced reactivity with HA on a cellular and molecular level. Furthermore, a shift in sulfate distribution on CD44 favouring O- and N-linked glycans occurred following cellular activation with inflammatory stimuli, correlating with the induction of HA-binding. A change in sulfate incorporation and distribution on CD44 may encourage conformational changes to CD44 that boost avidity and/or affinity for HA. The second mechanism that was identified to potentially regulate HA-binding in myeloid cells involves an intracellular association between the cytoplasmic domain of CD44 and the cytoskeleton of the cell. A novel CD44-phospho-ERM complex co-localizes to F-actin rich membrane protrusions to promote HA-binding. A mechanism of receptor clustering by the intracellular domain of CD44 likely enhances CD44 avidity for HA.

Evidence presented in this thesis demonstrates that the regulatory mechanisms determine different binding affinities for HA that, in turn, confer different adhesive or migratory phenotypes to myeloid cells. In light of recent data that proposes a conflicting role for CD44, that is, in both the onset and resolution of an inflammatory response, this work proposes that the induced interaction between CD44 and HA can support both cell adhesion to, and migration within, the ECM. This provides evidence that CD44 can play a dual and opposing role in an inflammatory response by promoting an initial infiltration of cells to the tissue to generate the inflammatory response, and to promote subsequent cellular adhesion of cells that 'clean-up' and down-regulate the response.

Overall, this research offers insight into the complexity of the molecular regulation of HAbinding by the cell adhesion molecule CD44 and implicates CD44-HA interaction in the onset and resolution of inflammation.

## **REGULATION OF HYALURONAN BINDING BY THE CELL ADHESION MOLECULE CD44 IN MYELOID CELLS**

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# LIST OF ABBREVIATIONS

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4	
aa	amino acid
Ab	antibody
Ag	antigen
APC	antigen presenting cell
APS	adenosine 5'-phosphosulfate
Asn	asparagine
Arg	arginine
ATCC	American Type Culture Collection
ATP	adenosine triphosphate
ATPase	adenosine triphosphatase
β-actin	beta isoform of actin
BADGal	benzyl-2 acetamido 2-deoxy- $\alpha$ -D galactopyranoside
BM	bone marrow
BMDM	bone marrow-derived macrophages
bp	base pair
BHK	baby hamster kidney cell
BSA	bovine serum albumin
CAM	cell adhesion molecule
CAMK II	Ca <sup>2+</sup> / calmodulin dependent protein kinase II
cAMP	cyclic adenosine monophosphate
CCR5	C-C chemokine receptor 5
CD	cluster of differentiation
CD44H	CD44 haematopoietic, or standard, form
CD44s	CD44 standard, or haematopoietic, form
CD44v	CD44 variant form
CD44.1	antigen for murine allele CD44 <sup>a</sup>
CD44.2	antigen for murine allele CD44 <sup>b</sup>
cDNA	complementary DNA
CHST-2	carbohydrate sulfotransferase 2
CHX	cycloheximide

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Ci	curie
CS	chondroitin sulfate
Cys	cysteine
cyt	cytoplasmic (domain)
CXCR4	CXC chemokine receptor 4
ddH <sub>2</sub> O	double distilled water
DABCO	1,4-Diazabicyclo [2.2.2] octane
DBA-2	dilute brown non-agouti mouse strain
DMEM	Dulbecco's modified eagle medium
DMSO	dimethylsulfoxide
DNA	deoxyribonucleic acid
DTT	dithiothreitol
ECL	enhanced chemiluminescence
ECM	extracellular matrix
EDTA	ethylenediaminetetraacetic acid
EGF	epithelial growth factor
EHS	Engelbroth-Holm-Swarm (mouse sarcoma)
ERM	ezrin, radixin, moesin
Fab	(monovalent) antigen-binding fragment of an Ig
F(ab') <sub>2</sub>	(divalent) antigen-binding fragment of an Ig
FACS	fluorescence activated cell sorting
FAK	focal adhesion kinase
F-actin	filamentous (polymerized) actin
F <sub>c</sub>	fragment, crystallizable of an Ig (non antigen-binding fragment)
F <sub>c</sub> R	F <sub>c</sub> receptor
FCS	fetal calf serum
FERM	protein <u>f</u> our.one, <u>e</u> zrin, <u>r</u> adixin, <u>m</u> oesin (homology domain)
FITC	fluorescein isothiocyanate
FL	fluorescein
FL-HA	fluorescein-conjugated hyaluronan
FL-HA-binding	the ability (of a cell or receptor) to bind FL-HA
Fuc	fucose

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gravitational acceleration
gram
glycosaminoglycan(s)
galactose
GDP dissociation inhibitor
guanine diphosphate
N-acetylglucosamine
N-acetylglucosamine-6-O-sulfotransferase 3, 4
glucose
glycosylation-dependent cell adhesion molecule 1
granulocyte, macrophage colony stimulating factor
glycoprotein
glycosyl sulfotransferase 2
guanosine triphosphatase
hour(s)
hyaluronan or hyaluronic acid
the ability (of a cell or receptor) to bind HA
low HA-binding
high HA-binding
Hank's balanced salt solution
high walled endothelial venule
human immunodeficiency virus
human leukocyte antigen (human MHC)
horseradish peroxidase
heparan sulfate
herpes simplex virus
human unbilical vein endothelial cell
intercellular cell adhesion molecule 1, 2, 3
interferon gamma
immunoglobulin
immunoglobulin isotype G
immunoglobulin isotype M
interleukin 1 alpha or beta, 3, 4, 5,10, 13

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kDa	kilodalton
Lac	lactose
LacNAc	N-acetyl lactosamine
LAD	leukocyte adhesion deficiency
LCCM	L929 cell conditioned media
Leu	leucine
Lewis x (Le <sup>x</sup> )	$Gal\beta \rightarrow 4(Fuc\alpha \rightarrow 3)GlcNAc$
LFA-1	lymphocyte function associated antigen 1
LPS	lipopolysaccharide
Lys	lysine
M	molar
mAb	monoclonal antibody
MAdCAM-1	mucosal addressin cell adhesion molecule 1
MCP-1	monocyte chemoattractant protein 1
M-CSF	macrophage colony stimulating factor
MDCK	Madin-Darby canine kidney cell
mg	milligram
MHC II	major histocompatability complex class 2
min	minute(s)
MIP-1 $\alpha$ , $\beta$	macrophage inflammatory protein 1 alpha or beta
ml	milliliter
MLR	mixed lymphocyte reaction
mm	millimeter
mM	millimolar
mRNA	messenger ribonucleic acid
μCi	microcurie
μg	microgram
μl	microliter
n	number (of experiments)
NAc	N-acetyl
nd	not determined
ng	nanogram

.

NMR	nuclear magnetic resonance
NF2	neurofibromatosis gene 2
NK cell	natural killer cell
PAGE	polyacrylamide gel electrophoresis
PAPS	3'-phosphoadenosine 5'-phosphosulfate
PBM	peripheral blood monocyte
PBMC	peripheral blood mononuclear cell
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PE	phycoerythrin
PG	proteoglycan
РНА	phytohemagglutinin
Phospho-ERM	phosphorylated ezrin, radixin, moesin
PIP2 (PtdIns 4,5 P2)	phosphatidylinositol 4,5-bis-phosphate
РКА	cAMP-dependent protein kinase
ΡΚС-α,θ	protein kinase C alpha, omega
РМА	phorbol myristate acetate
PMSF	phenylmethylsulfonyl fluoride
PNGaseF	peptide:N-glycosidase F
рр	predicted protein
PSGL-1	P-selectin glycoprotein ligand 1
PVDF	polyvinylidene difuloride
r	recombinant
R	receptor
Rho-GDP	Rho guanine diphosphate
Rho-GDI	Rho GDP dissociation inhibitor
RNA	ribonucleic acid
ROCK I / II	Dho kinaca I II
	Kho khase I, II
rpm	revolutions per minute
rpm RPMI	revolutions per minute Roswell Park Memorial Institute
rpm RPMI RT-PCR	Roswell Park Memorial Institute reverse transcriptase polymerase chain reaction

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sec	second(s)
SDS	sodium dodecyl sulfate
Ser	serine
Sialyl Lewis x (Le <sup>x</sup> )	sialylated Lewis x (human blood group antigen)
SK2	sulfurylase kinase 2 (PAPS synthetase)
SVEC	small vascular endothelial cell
SV40	simian virus 40
TBS	Tris-buffered saline
TBST	Tris-buffered saline Tween 20
TCA	trichloroacetic acid
TCS	tissue culture supernatant
Thr	threonine
TNF-α	tumor necrosis factor alpha
TPA	12-tetradecanoylphorbol-13-acetate
Tris	tris(hydroxymethyl)aminomethane
TSG-6	tumor necrosis factor alpha stimulated gene 6
TX-100	Triton X-100
Tyr	tyrosine
U	unit
WASP	Wiskott Aldrich syndrome protein
VCAM-1	vascular cell adhesion molecule 1
VLA-4	very late antigen 4
xyloside	p-nitrophenyl β-D-galactopyranoside

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# Introduction

1

#### **1.1 CELL ADHESION AND MIGRATION**

#### 1.1.1 Cell Adhesion and migration in the immune system

Cell adhesion and migration are deeply rooted in an array of processes required for embryonic development, maintenance of tissue architecture as well as neuronal network formations, and immune responses (1). Within the immune system, adhesion and migration are critical events for hematopoietic cell survival, cell differentiation, cell activation, and immune regulation in the processes of hematopoiesis, lymphocyte activation, lymphocyte re-circulation, and the extravasation of blood leukocytes to sites of inflammation. These events are mediated by transient cell-cell and cell-ECM interactions, and require progenitor cells, naïve and mature leukocytes to present non-adherent, non-migratory phenotypes under certain circumstances and alternatively, acquire adhesive or migratory properties. Inter-conversion between adherent and migratory states is highly regulated. Deviations from these regulatory procedures may drive the aforementioned processes towards pathological conditions associated with cancer cell invasion, metastasis and a number of immune and inflammatory disorders (2).

#### 1.1.1i Adhesive events in hematopoiesis

The development of mature blood cells in the bone marrow occurs in discrete microenvironments consisting of stromal cells, the extracellular matrix (ECM), and a milieu of regulatory cytokines (reviewed in 3). Components of the bone marrow ECM are produced by stromal cells and include fibronectin, hemonectin, thrombospondin, collagens, laminin, and the glycosaminoglycans (GAGs) heparin sulfatate (HS), chondroitin sulfate (CS), dermatan, and hyaluronic acid (HA, reviewed in 4, 5). Undifferentiated, pluripotent progenitor cells receive instructions for survival and differentiation from the surrounding microenvironment via regulatory cytokines, direct cell-cell interactions with bone marrow stromal cells, and an interaction of these cells with components of the ECM (6-8). The compulsory cell-cell and cell-ECM interactions are mediated by growth factor-growth factor receptor contact and the engagement of cell adhesion molecules with cell bound ligands or ECM components (reviewed in 9). For example, the adhesive contacts for early B-cell development in the bone marrow involves the engagement of stromal cell membrane-bound stem cell factor (SCF) and the SCF receptor expressed on early B cells. Subsequent differentiation requires an association between IL-7, secreted by stromal cells, with the IL-7 receptor (IL-7R) expressed on late pro-B and pre-B cells (10). Such relationships provide a model system for the elucidation of adhesive contacts that control the development of other progenitor lineages.

### **1.1.1ii** Activation of T lymphocytes by adhesion to APCs

While traversing the cortical region of lymph nodes, naïve T lymphocytes survey the surfaces of multiple antigen presenting cells (APCs). These transient T cell-APC contacts are mediated by cell surface adhesion molecules, in particular, integrins and members of the immunoglobulin (Ig) superfamily (reviewed by 11, 12). The interaction allows T cells to scan a variety of peptides presented by MHC molecules on APCs in search of a specific peptide to which the T cell is reactive. Identification of a specific peptide antigen initiates a stream of events, beginning with a tight association between the antigenic peptide / MHC molecule and the T cell receptor (TCR) (13). Signal transduction events originating from the TCR induce changes in cell adhesion molecules to stabilize intercellular adhesion and promote 'immunological synapse' formation at the APC-T cell interface (14, 15). An intimate and sustained adhesion between the two cells persists for several days and culminates in T cell proliferation, activation, and differentiation into effector cells (reviewed in 16).

#### **1.1.1iii** The leukocyte cell adhesion cascade

In the immune system, leukocytes reside in and cross between the circulatory and lymphatic systems (reviewed in 17). Naïve T and B lymphocytes constantly re-circulate between the blood and secondary lymphoid tissues in a process termed lymphocyte trafficking, or homing. In this case, the naïve lymphocytes exit the circulation by binding to high endothelial venules (HEV) in the lymph node (18). In an inflammatory response, neutrophils, monocytes, and activated T lymphocytes are induced to leave the circulation at a point juxtaposed to the damaged tissue by engaging the activated blood vessel endothelial cells (19). Although the cells that extravasate through the HEV and through the inflamed endothelium are different, the process of transmigration at either site occurs by a similar pattern of events summarized in what is known as the leukocyte cell adhesion cascade (20, 21). This cascade describes the process of leukocyte extravasation as a 5 step progression from the viewpoint of the blood leukocytes starting when the cells first 'tether' to the endothelium (step 1), 'roll' with decreasing velocity (steps 2 and 3), 'firmly adhere' (step 4), and subsequently 'transmigrate' across the endothelium (step 5). Figure 1.1 illustrates the adhesion cascade of blood monocytes in the context of an inflammatory response depicting the 5 steps mentioned above plus two additional steps to include the migration of monocyte-derived macrophages within the inflamed tissue and their exit to the lymphatic system. Each step of the process is regulated by cell adhesion molecules and corresponding ligands (discussed in section 1.1.2). Since the original model was proposed, many aspects of the cascade have been investigated, and in particular, the mechanisms pertaining to stages 1 through 4 (tethering to firm adhesion) have been unraveled and will be highlighted in the following sections of this chapter. A few molecules have been implicated in mediating step 5, transmigration, yet there are many unanswered questions regarding adhesion molecule participation and regulation in this step of the process and beyond (reviewed in 22). Few pieces of work have described the migration of cells at the inflammatory site, what determines the longevity of cells at the site, what initiates and controls their exit from the inflamed site, what drives cell movement, and what cell adhesion molecules are involved. Certainly this part of the process also requires regulated cell adhesion and migration events that if awry, may contribute to the pathogenesis of chronic inflammation.

## 1.1.2 Cell Adhesion Molecules (CAMs)

Cell adhesion molecules are a family of membrane glycoproteins that mediate cell-cell or cell-ECM contact for the purpose of intercellular communication, cellular activation, adhesion or migration (reviewed in 23, 24). Members include cadherins, Ig superfamily proteins, integrins, selectins, (to be discussed in brief below), and CD44 (discussed in sections 1.2 through to 1.5). Figure 1.2 illustrates the cell adhesion molecules and ligands implicated in leukocyteendothelial cell interactions.

#### **1.1.2i** Cadherins

Neural (N)-cadherin, epithelial (E)-cadherin, and placental (P)-cadherin are primarily responsible for cell-cell adhesion in cellular junctions through the formation of homophilic,  $Ca^{2+}$  dependent contacts (1, 25-27). Although this group of adhesion molecules is extensively implicated in maintaining tissue architecture and in cellular interactions during embryonic development (reviewed in 28), family members also have been implicated in mediating other processes. N-cadherin was reported to mediate hematopoietic cell-stromal cell interactions in the bone marrow (29). Further, the loss of cadherins between inflamed endothelial cells contributes to the process of leukocyte transmigration by creating space between the endothelial cells for leukocytes to pass (30). Small GTPases may regulate the loss in localized cadherin expression as cadherins, CD44, and integrins are lost from the intercellular junctions when Rho or Rac function is blocked (30). A recent report showed that increased expression of E-cadherin in a murine



FIGURE 1.1 The extended Leukocyte Cell Adhesion and Migration Cascade: trafficking during an inflammatory response. Leukocyte movement to an inflammatory site is described by a cascade of adhesion and migration events. The first three steps involve selectin-mediated tethering (1), rolling (2), and slow rolling (3), followed by chemokine-activated, integrin-mediated firm adhesion (4) of leukocytes to the activated vascular endothelium. Mechanisms that regulate leukocyte transmigration (5) into the underlying basement membrane, migration within the assaulted tissue (6), and exit to the lymphatic system (7) are less understood. Here, the scenario involves the movement of blood monocytes, illustrates their differentiation in the tissue, and the participation of monocyte-derived macrophages in innate and adaptive immunity. A similar paradigm of lymphocyte homing to HEV occurs under normal immune conditions. mammary carcinoma cell diminished CD44-HA-binding and blocked cell spreading on an HA substratum, CD44-mediated branching morphogenesis, and tumor cell invasion, suggesting that a link between E-cadherin expression and CD44 function controls tumor progression (31).

### **1.1.2ii** Immunoglobulin (Ig) superfamily

Members of the immunoglobulin (Ig) superfamily are calcium-independent transmembrane glycoproteins and, as the name implies, members share structural features with Ig molecules (reviewed in 1, 23, 32). Family members include Neural Cell Adhesion Molecule (NCAM) expressed on neuronal cells, as well as molecules expressed on endothelial cells including Intercellular Cell Adhesion Molecule (ICAM), Vascular Cell Adhesion Molecule-1 (VCAM-1), Platelet Endothelial Cell Adhesion Molecule-1 (PECAM-1), and Junctional Cell Adhesion Molecule (JAM). Typically, the molecules bind to other Ig superfamily members or to integrins (section 1.1.2iii). ICAM-1 (CD54) and VCAM-1 are expressed on endothelial cells and are integrin receptors for Leukocyte Function-Associated Antigen-1 (LFA-1) or Very Late Antigen-4 (VLA-4). These interactions mediate the firm adhesion of leukocytes to the endothelium, as depicted in stage 4 of the transmigration cascade (Figure 1.1). PECAM-1 and JAM reside primarily in intercellular tight junctions and form homotypic pairs between neighbouring endothelial cells to maintain endothelial integrity, but also to assist in stage 5 of the cascade, the extravasation of leukocytes (33, 34). Inflammatory cytokines regulate the cellular distribution of Ig superfamily molecules on the endothelium that in turn are thought to regulate transendothelial leukocyte migration (34, 35). Following prolonged exposure to TNF- $\alpha$ , the molecules VCAM-1, ICAM-1, ICAM-2,  $\beta$ 1 and  $\beta$ 3 integrin redistribute from the apical surface to the intercellular junction of human umbilical vein endothelial cells (HUVEC, 36). JAMs were redistributed to the HUVEC apical surface following exposure to TNF-a and IFN- $\gamma$ ; therefore, JAM is thought to negatively regulate transendothelial migration (37). This report concurs with previous results in which blocking antibodies to JAM specifically inhibited monocyte migration in vitro and chemokine-induced infiltration in vivo (34).

## 1.1.2iii Integrins

Integrins constitute a large family of transmembrane glycoproteins, each molecule formed by non-covalently linked heterodimers of one  $\alpha$  and one  $\beta$  subunit. Subfamilies are divided based on common  $\beta$  subunits that associate with different  $\alpha$  subunits. The leukocyte  $\beta$ 2 integrin subfamily includes 4 different heterodimers, namely, CD11a/CD18 (LFA-1) primarily on T

and B lymphocytes and monocytes, CD11b/CD18 (Mac-1) exclusive to neutrophils and monocytes, and CD11c/CD18 and CD11d/CD18 which are primarily expressed on dendritic cells and macrophages (reviewed in 38). Integrins mediate cell contact to extracellular matrix proteins of the basement membrane or to cell ligands, such as Ig superfamily molecules on endothelial cells. Ligand recognition may or may not require a three amino acid, RGD, peptide epitope on integrins (39). Although constitutively expressed, integrins require activation to engage ligands, and the interaction is weakly dependent on the divalent cations, Ca<sup>2+</sup> and Mg<sup>2+</sup> (40). Integrins are capable of both 'outside-in' and 'inside-out' signaling, and are therefore involved in two-way communication with the extracellular environment.

#### **1.1.2iv** Selectins

Selectins are expressed on the surface of leukocytes (L-selectin/CD62L), platelets (Pselectin/CD62P), and activated endothelial cells (E-selectin/CD62E and P-selectin) (reviewed in 41, 42). The N-terminal, extracellular domain of selectins has structural similarity to calcium-dependent (C-type) lectins and like lectins, selectins are carbohydrate-binding proteins. Selectins engage ligands containing fucosylated carbohydrate epitopes, in particular, sialvated Lewis x mucins (reviewed in 43). L-selectin is expressed on leukocytes as are the ligands for E- and P-selectin, such as PSGL-1 (P-selectin Glycoprotein Ligand) that is constitutively expressed on all lymphocytes, monocytes, eosinophils, and neutrophils. Ligands for L-selectin include GlyCAM-1 (Glycosylation-Dependent Cell Adhesion Molecule), CD34, and MAdCAM-1 (Mucosal Addressin Cell Adhesion Molecule). L-selectin ligands, with the exception of GlyCAM-1, are expressed on the endothelium along with E- and P-selectin. Interactions between selectins and their ligands are responsible for leukocyte rolling on the endothelium (44). During the process of homing, constitutively expressed L-selectin on lymphocytes, and L-selectin ligands on the endothelium mediate rolling. In an inflammatory response, P-selectin is rapidly induced on the surface of the activated endothelium and engages PSGL-1 to promote neutrophil rolling and recruitment. The details of selectin regulation will be discussed later in this chapter.



**FIGURE 1.2 Molecules involved in leukocyte adhesion and migration.** Subsets of the depicted receptors and associated ligands mediate rolling and adhesion of either lymphocytes on endothelial cells of the HEV during the process of lymphocyte homing, or of leukocytes on the activated vascular endothelium overlaying inflamed tissue. N-linked glycosylation is noted by  $\bullet$ , O-linked glycans by  $\sim \sim$ , and sulfated molecules with a star \*.

## 1.1.3 Leukocyte transmigration at HEV and inflammatory sites

The immune system is divided between different regions of the body, yet through a coordinated effort it functions as a single entity. Lymphatic tissues are connected to one another by the lymphatic and circulatory systems. Leukocytes are a major cellular component of the immune system and by transit between the blood and lymph, leukocytes provide a means by which immunological information and function can be integrated. The mechanisms that control leukocyte extravasation (to HEV and inflammatory sites) govern the critical process of crossover from the vasculature. As mentioned, the process of extravasation is reliant on the expression and activation of different cell adhesion molecules described above (21). The following section is a general discussion of the process of leukocyte extravasation in two contexts; lymphocyte recirculation under normal immune conditions and leukocyte emigration during an inflammatory response. The subsequent section (section 1.1.3i) looks at the regulation of leukocyte extravasation in these two scenarios with respect to adhesion molecule reactivity and in particular, posttranslational sulfate addition.

Under normal immune conditions, the process of lymphocyte re-circulation enables cells to circulate through the blood and lymph, surveying for infection or injury. During an immune response, the process of re-circulation offers additional advantages to immune system function (45, 46). For example, through dissemination of effector and memory cells, re-circulation supports widespread systemic and long-term immunity that originated from a localized antigen. Further, coalescing of T and B lymphocytes in specific lymphoid organs coordinates different facets of the immune system during activation and down regulation of the response. While the re-circulation of lymphocyte re-circulation. Despite the fact that re-circulation occurs in the absence of cellular stimulation by antigen or activation of the immune system, the re-circulate preferentially through the gut compared to cells isolated from the efferent lymph that prefer to re-circulate through peripheral lymph nodes. A subset of cell surface molecules, termed vascular addressins, interact with selectins or integrins expressed on the lymphocyte surface and are responsible in part for the tissue-specific trafficking of lymphocytes (47).

A major facet of research has focused on the regulatory mechanisms specific to lymphocyte recirculation through secondary lymphoid organs (reviewed in 20, 45). In this process,

specialized post-capillary venules, termed high endothelial venules (HEV), serve as the port of entry to lymph nodes and Peyer's patches for naïve T and B lymphocytes in the circulation. Endothelial cells of the HEV are cuboidal, have discontinuous junctions between adjacent cells, and express various cell adhesion molecules on the luminal surface. The surfaces of human and mouse HEV express a determinant detected by a mAb, MECA-79. This antibody blocks L-selectin dependent lymph node attachment of lymphocytes to HEV in vitro and in vivo (48). The addressing that support re-circulation to peripheral lymph nodes, namely GlyCAM-1 (49), CD34 (50, 51), podocalyxin (52), and Sgp200, contain this MECA-79 epitope and interact with L-selectin. All L-selectin-reactive addressins possess an epitope recognized by the mAb, MECA-79 (53, 54), and while the MECA-79 epitope is a predictor of L-selectin reactive sites, not all L-selectin ligands express the MECA-79 determinant (55, 56). Subsequent to leukocyte rolling that is mediated by L-selectin and reactive ligands, integrins such as LFA-1 are activated. An interaction between LFA-1 and ICAM-1-expressing lymphocytes results in firm adhesion of lymphocytes to the endothelium. Cellular transmigration events downstream from integrin-mediated adhesion are less understood; however, they have been cited to require the redistribution of adhesion molecules, either away from or towards intercellular endothelial junctions.

Inflammation was first enunciated by Celsus as 'rubor, calor, dolor, tumor' (redness, heat, pain, swelling), the symptoms of inflammation. Today we recognize that the 'inflammatory process' (57) giving rise to these symptoms is the primary host defense against tissue insult, injury, and invasion by infectious agents. The response is initiated by a combination of tissue damage plus infection (58, 59), and it is mounted by the combined effort of various cell types that quickly and effectively eradicate the irritant, then actively resolve the inflammation and repair the surrounding tissue. Although the initial control of infection or injury requires sentinel cells, primarily mast cells and macrophages stationed in the tissue (reviewed by 60), much attention in inflammation research has focused on the recruitment of leukocytes from the blood (reviewed in 61, 62). Unlike lymphocyte homing to HEV, the inflammatory response. If blood cells continue to transmigrate, the tissue is subject to chronic, debilitating inflammatory disorders. Thus, the migration of leukocytes must be under strict control to promote healing while preventing disease.

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Elucidation of the mechanisms that initiate or stop leukocyte migration has been enthusiastically researched to further an understanding of chronic inflammatory disorders (reviewed in 63). The result of such work demonstrates that the vascular endothelium is different from the endothelium lining of HEV. Activation of the vascular endothelium is a hallmark of inflammation-induced cell transmigration (reviewed in 64, 65). Inflammatory cytokines released from the assaulted tissue promote vascular permeability between overlying blood vessel endothelial cells. This relaxation in cellular junctions facilitates the infiltration of blood leukocytes that release pro-inflammatory cytokines to perpetuate the response. Similar to lymphocyte rolling on HEV, leukocyte rolling on the activated endothelium is controlled by vascular selectins that bind to complementary carbohydrate ligands (20, 66, 67). The MECA-79 epitope has been identified on HEV-like vessels at sites of chronic inflammation (18, 68, 69). Although the expression of MECA-79 epitopes on the endothelium implicates L-selectins in mediating rolling, the engagement of P-selectin and PSGL-1 is the primary interaction that regulates leukocyte rolling at inflammatory sites. The expression of P-selectin on the endothelium is induced by cytokines and chemokines released at the afflicted site or assault inflicted in the tissue. Firm adhesion is consequent to rolling and the chemokine-induced activation of integrins (reviewed in 70, 71). Interference with  $\beta 2$  integrin function is an efficient ways to block leukocyte recruitment in many experimental forms of inflammation (72). In vivo, intravital microscopic studies suggest that LFA-1 is a pivotal  $\beta^2$  integrin in firm leukocyte adhesion (73) through an interaction with ICAM-1 or ICAM-2 (reviewed in 74). CD18-deficient mice have severe inflammatory defects, and human patients lacking CD18 suffer from a syndrome known as leukocyte adhesion deficiency type 1 (LAD-1, 75). VLA-4 is a  $\beta 1$  integrin expressed on human monocytes, eosinophils, lymphocytes, and mouse neutrophils. VLA-4 can mediate both leukocyte rolling (76-78) and firm adhesion by interacting with endothelial VCAM-1, providing an alternative pathway to  $\beta 2$  integrinmediated adhesion (79). Successive stages of lymphocyte emigration proceed as suggested by the leukocyte adhesion cascade. The absence of JAM promotes monocyte transit across the stimulated endothelium, yet the details concerning downstream events have not been elucidated.

#### 1.1.3i Posttranslational sulfation

Sulfation is a post-translational enzymatic reaction catalyzed by sulfotransferases present in the golgi and cytoplasm. This group of enzymes catalyzes the transfer of a sulfate group from an

activated donor to a hydroxyl or amino group of the acceptor molecule (Figure 1.3, step 3). The cellular sulfate donor, 3'-phosphoadenosine 5'-phosphosulfate (PAPS, 80), is generated in the cytoplasm and actively transported into the golgi (81). PAPS is generated by the dual action of an ATP sulfurvlase, which synthesizes adenosine 5' phosphosulfate (APS), and an APS kinase which adds an ATP-derived phosphate to the 3' position of APS (see Figure 1.3, steps 1 and 2). The sulfotransferases are analogous to kinases as PAPS is to ATP. In animal cells, the enzymatic activities of the ATP sulfurylase and APS kinase are carried within a single protein, called PAPS synthase (PAPSS) or sulfurylase kinase (82, 83), encoded by the genes denoted PAPSS1 or PAPSS2 in humans and SK1 or SK2 in mice. Without PAPS, posttranslational sulfation does not occur. A natural missense mutation in the SK2 gene has been identified in mice to cause a defect in PAPS synthesis and posttranslational sulfation. These mice presented with a condition known as brachymorphism, a consequence of the undersulfation of cartilage proteoglycans (84). Inhibition of posttranslational sulfation can be fashioned in vitro with the compound, sodium chlorate. Chlorate is an analogue of sulfate and competes for binding to the ATP sulfurylase (85); thus, sodium chlorate inhibits the generation of PAPS and the sulfation of molecules.

The golgi-bound sulfotransferases should not be confused with cytosolic sulfotransferases that act on small molecule substrates and metabolic end products where sulfation leads to molecular inactivation or elimination (86). Golgi-bound sulfotransferases add sulfate to protein tyrosine residues or carbohydrates on glycoproteins, proteoglycans, and glycolipids (87). The incorporation of sulfate in this circumstance generates recognizable epitopes for extracellular matrix proteins, cell surface receptors, or viruses (87, 88). An investigation of selectin ligands has demonstrated the importance of sialylated and sulfated residues for their recognition by selectins (89-91). All three selectins (E-, L-, and P-selectin) recognize carbohydrates within a sialyl Lewis x group on glycoproteins and/or glycolipids (reviewed in 92). Sialyl Lewis x is a tetrasaccharide found on circulating cells that is comprised of sialic acid, galactose, fucose, and N-acetyl-glucosamine. The engagment of L- or P-selectin with ligands is dependent on sulfation. For example, the tyrosine sulfation of PSGL-1 is critical for recognition by P-selectin (93-95). HEV and HEV-like vessels have been shown to incorporate radiolabeled sulfate on macromolecules (96) catalyzed by an HEV-specific sulfotransferase, SUT-1 (81).



**FIGURE 1.3 Pathway of sulfate incorporation into glycoproteins, glycolipids, or proteoglycans.** The bifunctional enzyme, SK (murine) or PAPSS (humans), catalyzes the synthesis of adenosine phosphosulfate (APS, 1) using endogenous or radiolabeled sulfate then phosphorlyates APS (2) to generate PAPS (3), the cellular sulfate donor. A family of sulfotransferases (4) transfers the sulfate (radiolabeled or not) to sugars or tyrosine residues producing sulfated glycoproteins, glycolipids, and proteoglycans (5). Addition of sodium chlorate (NaClO<sub>3</sub>) inhibits the action of the ATP sulfurylase, thus, blocks the generation of PAPS and inhibits the generation of sulfated molecules.

Unlike P-selectin, the regioselective sulfation of O-glycans is important for L-selectin reactivity (90, 97). The critical sulfation site on HEV-expressed ligands, GlyCAM-1 and CD34, is at the carbon-6 (C6) position of galactose and N-actelyglucosamine of a sialyl Lewis x determinant on core-2 based O-glycans (98, 99). Despite the common sialyl Lewis x carbohydrate recognition motif for selectins, sulfation on O-glycans, tyrosine residues, or a lack of sulfation denotes ligand specificity. Antibodies have been generated by R. Kanangi against sulfated Lewis x isoforms also to help identify the distinguishing features of the different selectin ligands (100). One antibody (mAb G72) that recognizes 6-sulfo Lewis x-containing epitopes was reactive against human HEV (100), establishing that these antibodies have the potential to identify particular Lewis x determinants *in vivo*. Antibodies with specificity for various sulfated moieties were donated by R. Kannagi for this study (described in detail in chapter four) and were used *in vitro* to identify sulfated epitopes on myeloid cells.

#### **1.2 CD44 FUNCTION**

CD44 is another member of the cell adhesion molecule family, and CD44 is thought to mediate interactions in the immune system in a fashion similar to other CAMs. Like selectins, CD44 has been implicated in the regulation of leukocyte rolling along the vascular endothelium (101), and like integrins and Ig superfamily molecules, CD44 has been implicated in hematopoietic progenitor adhesion to bone marrow stromal cells during hematopoiesis (102-106). Despite the similarities in function to the selectin and integrin cell adhesion molecules, the expanding complexity of CD44 function, activation, and regulation remain to be understood. Apart from its role as an adhesion molecule, CD44 may function as a co-stimulatory receptor on T lymphocytes, transmitting signals in co-operation with CD3 that are required for cellular activation (107-111). CD44 has been implicated in cell adhesion and signaling because of evidence that CD44 can mediate homophilic leukocyte-leukocyte interactions (112, 113), leukocyte-bone marrow stromal cell interactions (114), and leukocyte-endothelial cell interactions under both static (115, 116) and flow conditions (101, 117). These cell-cell and cell-ECM interactions implicate CD44 in embryogenesis, lymphopoiesis, lymphocyte activation, progenitor homing, angiogenesis, wound healing, leukocyte rolling, leukocyte extravasation at inflammatory sites, and tumour metastasis; however, the precise role of CD44 in these processes is not clear. Most often, the involvement of CD44 has been attributed to the engagement of CD44 with the glycosaminoglycan, hyaluronic acid (HA, reviewed in 118, 119,
120), the most extensively characterized ligand of CD44 (121). The structure of CD44 and HA and the details concerning their interaction will be discussed in detail in sections 1.3, to 1.5.

# 1.2.1 CD44 gene-knock out mice

CD44 gene-knock out (CD44<sup>-/-</sup>) mice have no obvious developmental or functional abnormalities (122). An analysis of immune cell development and function in CD44<sup>-/-</sup> mice did not reveal any significant defects (122), suggesting a non-essential role for CD44 in normal development and immune function. A comparison of lymphocyte function between CD44-deficient mice and wild type mice suggested that normal Ig class switching occurs in B cells and cognate T:B cell interactions are not critically altered in the CD44-deficient mouse. *In vitro*, lymphocyte proliferation assays were comparable between CD44<sup>+/+</sup> and CD44<sup>-/-</sup> T lymphocytes challenged with soluble anti-CD3 mAb, staphylococcal enterotoxin B, concanavalin, or cross-linked with anti-CD3 mAb. In addition, there was no difference between the functioning of CD44<sup>+/+</sup> and CD44<sup>-/-</sup> T lymphocytes in mixed lymphocyte reactions (MLRs), primary *ex vivo* cytotoxicity assays, delayed-type hypersensitivity tests, and memory-T cell development. CD44-gene knockout mice did, however, have increased myeloid progenitors in the bone marrow, decreased numbers in the spleen, and developed an exaggerated granuloma response to *Crytosporidium parvum* infection. These results suggest that CD44<sup>-/-</sup> mice have defects in monocyte and macrophage development and function.

In general, when gene-deficient mice show no obvious phenotypic differences from wild type mice, the function of the absent molecule is often and mistakenly regarded as being redundant. At most, it can be concluded that the function of CD44 in development is degenerate. Degeneracy is an advantageous biological principle that underlies selectional processes such as evolution and the immune response since it can be compensatory for defects and allows the system to adapt changes. Proper functioning of each facet of the immune system is imperative for execution of a complex host defense. As an assurance, the immune system utilizes a series of checkpoints (for example, in regulation of the cell cycle), confirmatory signals (for example, co-stimulation of T lymphocytes) and degeneracy to avoid the potentially severe consequences of inappropriate activation or malfunction (for example, autoimmune disease and immunodeficiency). In biological systems, the most obvious example of degeneracy is the third codon of nucleotide triplets that code amino acids. In the immune system, different proteins perform the same enzymatic reaction, such as families of kinases and phosphatases,

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different classes of antibodies recognize the same antigen, and different cell adhesion molecules share ligands and participate in the same steps in the leukocytes adhesion cascade. This design allows the immune system to tolerate loss or malfunction of particular molecules.

Perhaps other molecules can compensate for the absence of CD44 during the development of CD44-gene knockout mice. Other CAMs or HA-binding proteins such as RHAMM, LYVE-1, neurocan, versican, aggrecan, link proteins, and TSG-6, with structural and functional properties similar to CD44 may provide such a compensatory role. Despite the potential for overlap in the functions of CD44 with other CAMs, and the rather innocuous phenotype of the CD44-gene knock out mouse, CD44 function has been identified as an integral player in several key processes. Neutralizing anti-CD44 Abs and more recent data evaluating disease models in the CD44-deficient mouse unequivocally support a role for CD44 in lymphopoesis, myelopoeisis, and most prominently in inflammatory diseases (123).

# 1.2.2 Implication of CD44 function in myelopoiesis and lympho-hemopoiesis

CD44 is highly expressed on early myeloid progenitor cells (124), and myeloid progenitor cells in the CD44-deficient mice were observed to have a defect in egress from the bone marrow, sparking an interest in CD44 function in hematopoiesis. Since this report, CD44 has been shown to mediate the adhesion of immature B cells to bone marrow stromal cells. Anti-CD44 mAbs block the progenitor cell-stromal cell adhesion in vitro and in turn, block lymphohemopoiesis (102) and myelopoiesis (125). In contrast, anti-CD44 mAbs that augment cell interactions support hematopoiesis (126, 127). CD44 may influence hematopoiesis through an interaction with HA in the bone marrow or via the expression of variant forms of CD44 (128). The evidence that CD44-HA interactions mediate lymphopoiesis and myelopoiesis is supported by the reports that HA is an abundant GAG in the bone marrow ECM, and that exogenous HA has been shown to enhance hematopoiesis (129). Furthermore, cytokines can regulate the proportion of CD44<sup>+</sup> hematopoietic progenitor cells that bind to HA (130). With respect to isoform usage, CD44v10 was reported to mediate an interaction between progenitor cells and stromal cells (131), CD44v7 mediates hematopoietic progenitor cell homing (131, 132), and CD44v4-10 was shown to regulate human myelopoiesis (125). Through HA- or isoformdependent binding, CD44 is involved in mediating cell-cell interactions in the bone marrow that regulates hematopoiesis.

### **1.2.3 Implication of CD44 function in migration**

The majority of evidence to suggest that CD44 plays a major role in leukocyte-endothelial cell adhesion comes from *in vitro* studies on T lymphocytes. This particular role for CD44 was vigorously investigated following identification of an anti-CD44 mAb (Hermes-1) that could block the adhesion of leukocytes to frozen sections of mucosal lymph nodes (133). Subsequently, anti-CD44 mAbs were shown to delay leukocyte infiltration at sites of inflammation, but had no effect on lymphocyte re-circulation, suggesting a specific role for CD44 in inflammation, rather than homing. To recapitulate the conditions of migration at inflammatory sites, activated T lymphocytes have been shown *in vitro* to mediate rolling on HA and murine endothelial SVEC-4 cells under flow conditions (101). Anti-CD44 antibodies or HA blocked the rolling of T cells in these studies.

Migratory T cells have been described to adopt a polarized shape with an extended membrane ruffle, or lamellipodia, at the leading (forward) edge and at the opposite end, a protrusion called a uropod. The uropod is believed to anchor the cell to the substratum while the front edge of the cells creeps forward and receives directional cues. The uropod structure is described in detail by Delpozo *et al* (134). Of note, CD44 is sequestered to the tail (uropod) of the migratory T cell along with CD43, ezrin, and microtubules, while F-actin is found in the tips of the lamellipodia. The separation of cell adhesion molecules, the dramatic remodeling of the actin cytoskeleton and polarization are most often associated with a migratory phenotype.

### 1.2.4 Implication of CD44 function in inflammatory disorders

*In vitro*, anti-CD44 antibodies that block the engagement of HA also retarded the development of murine inflammatory disorders including inflammatory bowel disease (IBD), collagen- and proteoglycan-induced arthritis (135), experimental autoimmune encephalomyelitis (EAE, 136), and a delayed type hypersensitivity response in the skin (137). Conversely, anti-CD44 antibodies that promote CD44-HA interactions were shown *in vitro* to exacerbate disease pathogenesis in a murine model of arthritis (138). More recently, both a pan-specific and neutralizing antibody against CD44 suppressed lung inflammation and inhibited antigen-induced experimental asthma (139). Leukocyte recruitment, Th2 cytokine production and normal enhancement of HA were reduced in the antibody-treated mice (139). CD44 has also been shown to control the recruitment of leukocytes in atherosclerosis (140-142). *In vitro*, CD44 was implicated in myeloid cell adhesion at inflammatory sites by Maiti *et al* (116). In

this report, the pro-inflammatory cytokine, TNF- $\alpha$ , induced CD44-mediated cell adhesion to murine endothelial SVEC-4 cells that was blocked by anti-CD44 antibodies or hyaluronidase, suggesting that a CD44-HA-CD44 sandwich bridges the leukocyte and endothelial cell. One *in vivo* study demonstrated that a specific isoform, CD44v7, was responsible for chronic inflammatory colitis (143). Taken together, these results implicate CD44 in promoting an inflammatory response (144), however, CD44 also has been suggested to dampen the inflammatory response. In mice with *Escherichia coli*-induced pneumonia, CD44-deficiency accelerates neutrophil migration *in vivo*, and *in vitro*, CD44-deficient neutrophils crawl though matrices nearly twice as fast as wild type neutrophils, implicating CD44 in limiting the inflammatory response to *E. coli*. Another study reported that CD44<sup>-/-</sup> mice developed an exaggerated response to bleomycin-induced lung injury (145). Here the presence of leukocytes, chemokines, and HA were enhanced in the lung which was attributed to an impaired clearance of apoptotic cells by CD44-deficient macrophages (145). This result was supported by *in vitro* studies in human macrophages showing that CD44 mediates phagocytosis of apoptotic neutrophils and granulocytes but not lymphocytes (146).

It is difficult to determine which study best defines the role of CD44 in inflammation. The opposing role for CD44 is most striking when it has been suggested in the same context. For example, in lung inflammation, Katoh *et al* (139) uses anti-CD44 Abs to suppress lung inflammation whereas Teder *et al* (145) report that lung inflammation is exaggerated in CD44-deficient mice. In the study by Katoh *et al*, the analysis was performed 24 hr after inflammation was induced, yet, in the study by Teder *et al*, cells were analyzed 4 days after inflammation was induced. This opens up the possibility that CD44 may play different, opposing roles in acute and chronic inflammation. CD44 may be involved in leukocyte recruitment initially in the response, but may also be required to resolve the response. This concept will be explored further in the later chapters of this thesis.

# **1.3 CD44 STRUCTURE**

CD44 is a type I transmembrane cell adhesion glycoprotein that is broadly expressed on leukocytes, fibroblasts, keratinocytes, endothelial cells, and epithelial cells (reviewed in (118, 147, 148). CD44 is synthesized as a 37 kDa core protein but extensive posttranslational modifications including the addition of glycosaminoglycans (GAGs), N- and O-linked glycans, and the sulfation of these carbohydrates yields a final molecular mass ranging from 80 to 200

kDa. An increase in molecular mass also can be attributed to the alternative splicing of approximately 10 exons of CD44 (v1-10), giving rise to multiple isoforms of CD44 (CD44v) with added sequence in the membrane proximal region. These higher molecular mass isoforms are expressed by endothelial cells, epithelial cells, activated lymphocytes, and some tumor cells, but are not abundant on the cell surface (reviewed in (149). The majority of CD44 expressed on leukocytes and fibroblasts is the standard (or hematopoietic) form, referred to as CD44H or CD44s, that is devoid of differentially spliced exons and resolves to 85 - 90 kDa by SDS-PAGE. CD44H consists of an ~270 amino acid extracellular domain, an ~21 amino acid transmembrane region and an ~72 amino acid cytoplasmic domain (reviewed in 118, 148). Figure 1.4 is a cartoon of CD44H portraying the prominent features of the molecule.

#### **1.3.1 Extracellular domain**

The amino-terminal, extracellular domain of CD44 is comprised of amino acids 21 - 182 (according to reference 150) of which the terminal 168 residues are highly conserved, having 80 - 90% sequence identity among mammalian species. This conserved region contains six disulfide-bonded cysteine residues, five consensus sequences for N-glycosylation, and has sequence identity to Link modules, which are common to a family of extracellular proteins that bind hyaluronan (151). The proposed structure of the Link homology domain is based on an NMR image of one Link module-containing hyaluronan-binding protein, tumor necrosis factorstimulated gene-6 (TSG-6, 152). These results revealed a structure very similar to that of the calcium dependent (C-type) lectins such as mannose binding protein and the selectins. As stated, the homologous region of the hyaladerins lies in the amino terminal end, similar to the location of the carbohydrate-binding domain on selectins. Mutagenesis of sites on a CD44immunoglobulin fusion protein (CD44-Ig) implicated the amino terminal region of CD44 in binding to HA. Two particular residue pairs, Arg41 and Tyr 42, and Arg 78 and Tyr 79, are important for HA-binding and are clustered in the center of the proposed HA-binding site (153). Other important sites in this region are Lys 38, Lys 68, Asn 100, Asn 101, and Tyr 105, residues that are thought to align in a continuous ridge along the surface of the protein (154). Two  $B(X_7)B$  domains, where B is a basic residue (Lys or Arg) and  $X_7$  represents seven nonacidic amino acids including one Arg or Lys, are found in the extracellular domain link module and are required for HA-binding (151). Furthermore, the majority of N-linked glycosylation sites on CD44 are present in the link domain, a modification shown to affect the HA-binding



# FIGURE 1.4 Structural Features of CD44.

Amino acids are numbered according to human CD44 sequence reported by Stamenkovic et al 1989.



ability of CD44. Outside the link homology domain, the membrane proximal region (amino acids 183 - 268) is least conserved, only having ~35% sequence identity between mouse and human CD44. Two conserved Ser-Gly motifs for GAG addition are found within this region as well as multiple sites for O-glycosylation and the insertion site for the addition of amino acids associated with CD44v isoforms created by the alternative splicing of exons. The variability of the membrane proximal region is amplified by additional GAG and glycosylation consensus sequences on the isoforms. In spite of the variability, modification to the membrane proximal region of CD44 also has been shown to affect HA-binding (148, 155, 156) and enhance CD44-transfected AKR1 cell adhesion to HA while discouraging rolling (157).

### **1.3.2** Transmembrane and cytoplasmic domain

The transmembrane and cytoplasmic (CD44cyt) domains of CD44 are highly conserved having 80 - 90% sequence identity in mammalian species. CD44 in fibroblasts is insoluble in nonionic detergent, such as Triton X-100, that has been attributed to the transmembrane domain (158). It has been speculated that insolubility results because CD44 is associated with the actin cytoskeleton (159, 160) or with lipid rafts (158), the latter of which are typically associated with membrane receptor signaling (161). The transmembrane region can influence HA-binding by promoting the self-association of CD44 (162). A cysteine residue in the transmembrane region was shown to mediate a disulfide bond-dependent dimerization of CD44 in Jurkat T cells upon PMA stimulation (163).

The cytoplasmic domain of CD44 has no enzymatic activity, does not contain tyrosine residues but does have six serine residues, four of which are highly conserved (164). The phosphorylation of serine residues enables CD44cyt to engage in protein-protein interactions with 14.3.3 domain-containing intracellular proteins. CD44cyt has also been reported to engage other signaling molecules, in particular Src family kinases (165). CD44cyt has been reported to interact with actin-binding proteins (149, 159) that will be discussed further in chapter five. Various cytoplasmic domain-deletion mutants of CD44 expressed in T cell lines or in transfected COS cells reduce or abolish HA-binding (115, 158, 166-168) and can prevent the migration of transfected melanoma cells on HA (169). The cytoplasmic tail of CD44 can affect the localization of CD44 within a cell. For example, in confluent epithelial cell culture, CD44 is excluded from the apical region and localizes to the basolateral surface (170) that is mediated by a dipeptide, LV, in the cytoplasmic domain (171).

### 1.4 CD44 LIGANDS

CD44 can bind to a wide range of ligands, of which hyaluronan (HA) is the most extensively characterized ligand (121, and reviewed in 148). Besides HA, CD44 binds to other components of the ECM including fibronectin, collagen type I, IV, XIV, and chondroitin sulfate (CS). An interaction between CD44 and these ligands implies a function for CD44 that is associated with cell-ECM contacts. For example, CD44 can mediate melanoma cell migration and invasion through an association with type I and type IV collagen (172, 173). Isoforms of CD44 may have distinct binding partners, for example, CD44v isoforms interact with osteopontin (174), a cytokine-like molecule with adhesive and migratory functions. In this case, CD44v isoforms may co-operate with  $\beta1$  integrins to bind to osteopontin (175).

The ligand-binding face on CD44 is in the amino-terminal link homology, HA-binding region. A mutation in this region abolishes an interaction with chondroitin sulfate (CS), suggesting that CD44 engages HA and CS in a similar manner (176). However, GAGs also interact with one another, making ligand binding outside the link homology domain on CD44H or CD44v forms possible. Specifically, CS-modified forms of CD44 have been reported to bind to  $\beta$ 1 integrin ligands such as fibronectin (177) and collagen XIV (178) and other molecules such as serglycin (179, 180) and CS-modified invariant chain (181) bind to CD44 via CS side chains. Certain isoforms of CD44 (CD44V4-7, CDD44v4-10, and CD44v10) bind to CS-modified forms of CD44 (125, 176, 182). Modification of CD44 by CS or heparan sulfate (HS) glycosaminoglycans can complicate the function of CD44 as CS and HS provide additional binding sites for molecules such as MIP-1 $\beta$  (183), matrix metalloproteinase 9 (MMP-9, 184), and heparin binding growth factors (185), known to participate in cell adhesion, migration, and invasion.

# 1.4.1 Hyaluronic Acid (HA)

HA is a repeating disaccharide of  $\beta(1-4)$ -D-glucuronic acid- $\beta$ -(1-3)-N-acetyl-D-glucosamine, illustrated in Figure 1.5. HA is secreted as a large polymer (comprised of the disaccharide repeats), and it is the only non-sulfated glycosaminoglycan. Because HA is extremely hydrophilic and was initially recognized to participate in tissue remodeling and regeneration, HA was characterized as a space-filling molecule. HA has received more recent attention for its profound effect on cell behaviour. HA is present virtually in all vertebrate tissues as a component of the extracellular matrix (ECM). HA levels in the ECM are regulated by cellular



**FIGURE 1.5 The structure of hyaluronic acid (HA).** HA is a repeating disaccharide, represented above as one disaccharide that may be repeated 'n' number of times where n can range from 1 to over 1000.

synthetases, hyaluronidases, and receptor-mediated endocytosis of HA (186). The concentration of HA is enriched in cartilage and synovial fluids (186, 187), transiently increased in the skin during a delayed-type hypersensitivity reaction (188), and elevated in the extracellular matrix coincident with periods of rapid cell proliferation, aggregation, and migration, events that are associated with embryogenesis, wound healing, leukocyte-endothelial cell interactions, and tumor metastasis (reviewed in 186, 187). HA is secreted from cells, but HA can bind to surface receptors to mediate HA-induced effects and cell-cell interactions (189). CD44 commonly binds to HA that is secreted as a high molecular mass polymer (~ $10^6$  kDa) although there is evidence that hyaluronidase-digested, small chain sugars bind to CD44 for the purpose of outside-in signal transduction (190-198).

The hyaladherins are a family of receptors for HA that includes CD44, RHAMM, LYVE-1, TSG-6, hyaluronectin, and Layilin to name a few (186). This family of receptors shares the Link homology domain, a conserved structure that is similar to, and potentially evolved from, the C-type lectin domain. The emergence of Link-module containing molecules and the synthesis of hyaluronan may have evolved during a similar period early in the evolution of metazoan organisms (199). Among the hyaladerins, RHAMM was identified in migratory cells, and it was shown to interact with HA in T cell lines and malignant cells (201). The interaction was associated with the activation of signaling pathways involving pp60<sup>c-src</sup> and p125<sup>FAK</sup> to promote cell locomotion (202). Another HA receptor, LYVE-1, is a lymph-specific receptor for HA that is completely absent from blood vessels, but was shown to co-localize with HA on the luminal face of the lymph vessel wall (203). Although the hyaladherins have a similar binding domain for HA, the molecular details of HA-binding are distinct for each protein (200).

### **1.5** REGULATION OF HA-BINDING

In the majority of biological processes in which the function of CD44 has been implicated, the involvement of CD44 has been attributed to an interaction with HA. The ability of CD44 to bind to hyaluronan varies with the cell type, state of cellular maturation and activation such that not all cells expressing CD44 will bind to HA. Cells are categorized into three groups with respect to hyaluronan-binding ability of CD44. The first group includes cells that constitutively express CD44 in a form that always interacts with HA. The second class of cells does not bind to HA but can convert CD44 into a ligand-binding form following appropriate cellular activation. For example, HA-binding by PBM can induced by inflammatory agents such as

TNF- $\alpha$ , IFN- $\gamma$ , IL-1, and LPS (204-206) but not Th2 type cytokines, IL-4 and IL-13 (204). Further, TNF- $\alpha$  stimulates the micro- but not macro-vasculature-endothelial cells to bind to HA (207). The third class of cells express CD44 in a form that does not engage HA and cannot be induced to do so. The strict regulation of ligand-engagement is a common characteristic of cell adhesion molecules. Given that CD44 and HA are constitutively and ubiquitously expressed and dysfunctional regulation may ablate or cause an inappropriate spatial or temporal interaction between CD44 and HA, strict regulation is necessary to prevent these improper cell behaviours that often contribute to disease pathogenesis.

### 1.5.1 Regulation of HA-binding by the extracellular domain of CD44

The extracellular domain of CD44 contains sites for extensive posttranslational modifications. The addition of GAGs, glycosylation, sialylation, and the incorporation of alternative exons are modifications that influence HA-binding (208). Although the specific mechanisms associated with particular modification are not known, HA-binding may be altered if the induced posttranslational modification adjusts CD44 conformation, distribution, clustering, or charge.

#### **1.5.1i** Regulation of HA-binding by expression and alternative splicing of CD44

Many reports have highlighted the importance of CD44 receptor density on the cell surface as a simple but effective manner by which the avidity for HA can be boosted (209). The induction of CD44 expression has been reported to occur following IL-1-induced activation of the Egr-1 transcription factor (210, 211) and *de novo* protein synthesis (212). Alternative splicing of the CD44 gene occurs in antigen- or mitogen-stimulated T cells (213, 214) or PBM cultured *in vitro* in the presence of autologous human serum and coincides with HA-binding by these cells (205, 206, 215). *In vivo*, CD44v6-containing variant forms were transiently expressed in T cells, B cells, and macrophages after antigenic stimulation (216). CD44 isoform expression and HA-binding also is observed in infiltrating monocytes isolated from cutaneous inflammatory sites and in alveolar macrophages (206, 215). Additional sites for glycosylation and GAG modification on CD44v forms may induce conformational changes to CD44 or vary CD44-CD44 interactions on the cell surface to influence HA-binding. The cell surface expression of CD44 and cellular binding to HA are not linear, also suggesting that a threshold level of CD44 is required to engage HA.

**1.5.1ii** Regulation of HA-binding by sialylation, glycosylation, and GAG addition It has been reported that LPS induced HA-binding and increased sialidase activity in a human monocyte cell line (THP-1, 217) and in peripheral blood monocytes (218). Use of a sialidase inhibitor, 2-deoxy-2,3-dehydro-N-acetylneuraminic acid, reduced HA-binding in this system, correlating a decrease in cell surface sialic acid residues with enhanced HA-binding (217). Exposure of human PBM to the pro-inflammatory cytokine, tumor necrosis factor-alpha (TNF- $\alpha$ ), induced a 5 - 10 kDa decrease in the apparent molecular mass of CD44 that was not observed in untreated or IL-4-treated PBM, suggesting that TNF- $\alpha$  induces posttranslational changes to CD44. TNF- $\alpha$  also induced HA-binding in PBM thereby correlating the change in the molecular mass of CD44 with an increase in ligand reactivity (219). Comparison of resident and elicited murine macrophages indicated a 5 - 10 kDa increase in the molecular mass of CD44 that was attributed to changes in N-linked glycosylation, but this was not correlated with HA-binding (220). Induction of HA-binding in B cells by IL-5 correlates with a decrease in N-glycosylation of CD44 (221) that also was shown to occur after stimulation of lung epithelial derived cancer cells with oncostatin M (222). In T lymphocytes, both in vitro and in vivo activation by antigen or mitogen can transiently induce HA-binding (223-225) which may (216, 226) or may not (225) be accompanied by the expression of higher molecular mass isoforms of CD44. Inflammatory cytokines IL-1 $\alpha$ , IL-4, and TNF- $\alpha$  have been shown to evoke changes in chondroitin sulfate and heparan sulfate on CD44 that was also reported in other systems where GAG addition affected HA-binding (218, 219, 227-229). Clearly, regulation of posttranslational modification to the extracellular domain of CD44 does affect HA-binding and is cell type and activation-state specific.

# 1.5.1iii Regulation of HA-binding by sulfation

The importance of sulfation for mediating a molecular and cellular interaction was first demonstrated for selectin-selectin ligand recognition. At the onset of this investigation, sulfation was beginning to receive credit for the regulation of receptor-ligand interactions besides those involving selectins, such as for chemokine receptors, viruses, and CD44. A report proposed that TNF- $\alpha$  induced the sulfation of CD44 in a CD33<sup>+</sup> leukemic SR91 cell line to regulate HA-binding and an interaction with a murine endothelial cell line (116). Results to be presented in this thesis will show that sulfation on CD44 regulates HA-binding induced in monocytes and macrophages by inflammatory agents. The complexity of sites for sulfate

incorporation was reported in the SR91 cell line (230) and was unraveled in PBM, the results of which will be presented in chapter four.

### 1.5.2 Regulation of HA-binding by the cytoplasmic domain of CD44

It was first speculated that CD44 associates with the cytoskeleton when CD44 was observed in the cleavage furrow, in filopodia, and in microvilli (164, 231, 232). As stated previously, the cytoplasmic domain of CD44 is important for HA-binding and is required for melanoma cell migration. Cross-linking CD44 or engagement with HA likely causes 'outside-in' signaling to the cytoskeleton since changes in cell spreading, lamellipodia, membrane ruffling, and elevated Rac GTPase activity were observed in cells following CD44-ligation (233). In fact, inhibitors of actin polymerization prevented the induced morphological changes, suggesting that the cytoplasmic domain of CD44 is attached to the actin cytoskeleton and influences cytoskeletal rearrangement. It follows that the actin cytoskeleton could in turn influence the ligand binding function of CD44.

## **1.5.2i** Interaction of CD44 with the cytoskeleton

The mode by which CD44 is connected to the cytoskeleton is not known although actin-binding proteins, primarily ezrin, radixin, moesin (ERM), and ankyrin were reported to associate with CD44 in epithelial cells, fibroblasts, and T cells *in vitro* (149, 159, 234-236). These data provide evidence that an interaction can occur, but the results fail to link the CD44-ERM interaction to CD44 function, be it signal transduction or ligand reactivity.

## 1.5.2ii Interaction of CD44 with ERM proteins

The FERM protein family includes protein band <u>4</u>.1, <u>ezrin</u> (237), <u>radixin</u> (238), <u>moesin</u> (239), and the related tumor suppressor proteins, merlin (neurofibromatosis gene 2 (NF2) 240, 241) and DAL-1 (differentially expressed in adenocarcinoma of the lung-1, 242). Ezrin is most broadly expressed in mammalian cells whereas moesin is quantitatively dominant in leukocytes (243) and is the only ERM protein in platelets (244). Redundant function of these proteins in mammalian cells has impeded the functional analysis of ERM proteins, which may yield clearer results in *Drosophila* that express only moesin (245, 246).

As depicted in Figure 1.6, ERM proteins have 3 distinct domains. The N-terminal FERM region interacts with membrane glycoproteins. The FERM region and the F-actin-binding C-





(Figure adapted from Bretscher et al, Nature Reviews; Molecular Cell Biology 2002)

FIGURE 1.6 ERM protein structure and regulation. Ezrin, radixin, and moesin proteins share common structural and functional characteristics. The upper panel shows the 3 highly conserved protein domains that constitute ERM proteins; the N-terminus (N-ERMAD) that binds to membrane proteins, the central coiled-coil region, and the C-terminus (C-ERMAD) that is essentially an F-actinbinding domain and contains the regulatory PIP<sub>2</sub>-binding site (PP) and conserved threonine residue (T567, T564, T568). The lower panel depicts the negative self-regulation of ERMs through a head-to-tail intramolecular interaction (dormant ezrin). An 'open' and interactive conformation is promoted following PIP<sub>2</sub> binding and/or threonine phosphorylation (a) and subsequent interaction of the opposing termini of ERM proteins with membrane proteins and F-actin (b). terminus flank a central hydrophobic coiled-coil domain (247, 248). ERM proteins are concentrated in particular regions of the cell, such as microvilli and focal adhesions, where Factin associates with the plasma membrane (249). The ERM protein family serves to crosslink the actin cytoskeleton and the plasma membrane (reviewed in 250, 251). It is predicted that the N- and C-termini form intra- or inter-molecular associations under normal physiological conditions yielding that render ERM proteins in an inactive conformation (248, 252-254). The head-to-tail intramolecular interaction may be disrupted either by PIP<sub>2</sub> binding (255) to a conserved region in the C-terminal domain, or through the phosphorylation of a conserved threonine residue (256), or by both events (reviewed in 251). Binding of PIP<sub>2</sub> or phosphorylation are triggered by changes in the cellular environment and convert ERMs to a linear, 'open' conformation that is capable of engaging F-actin and membrane proteins (253, 257). A conformational change following the phosphorylation of the conserved threonine residue or binding of PIP<sub>2</sub> is a mechanism previously shown to regulate vinculin activity (258).

It was first reported that CD44 interacts with ERM proteins in a baby hamster kidney cell line, BHK-1 by Tsukita *et al* (159). Subsequently, results show that CD44 interacts with ERM proteins in epithelial cells and fibroblasts *in vitro* and colocalizes with ERM proteins in L cells (159). The interaction of ERMs and CD44 has been reported *in vitro* under low ionic strength conditions (159) or in the presence of PIP<sub>2</sub> under physiological salt concentrations (259).

### 1.5.3 Regulation of HA-binding in response to physiological stimuli

As indicated above, several factors can act independently or in concert to influence the ability of CD44 to bind to HA (reviewed in 120, 260). Different cell types potentially utilize combinations of these transcriptional and posttranslational changes to regulate HA-binding in response to various extracellular signals and cellular activation. Thus, it is a challenge to identify the cellular changes that occur in response to one physiological stimulus that regulate CD44-mediated HA-binding in various cell types in particular states of maturation or activation. To this date, several reports link the activation of HA-binding to alterations in CD44 expression and structure, but these reports fail to show a direct relationship between the induced modification and ligand binding or the underlying mechanism.

# 1.5.4 Regulation of HA-binding in myeloid cells

CD44 mediated HA-binding has been reported in many human myeloid cell lines (reviewed in 261), in human peripheral blood cells (204, 206, 215, 217), and in mouse macrophages (205, 218). As stated above, cytokine-activated monocytes bind to HA. Some factors have been correlated with, but not proven to regulate, HA-binding in activated monocytes. These factors include glycosylation, sialylation, sulfation, isoform expression, and CD44 aggregation (reviewed in 113 and discussed above). It is not understood how inflammatory mediators induce particular modifications, the signal transduction events involved, or the manner in which modifications to CD44 influence HA-binding ability.

#### **1.6 RESEARCH OBJECTIVES**

CD44 is involved in an array of diverse biological processes likely by mediating cell-cell or cell-ECM interactions through an association with hyaluronan. Prior to this work, it was clear that both CD44 and HA are ubiquitously expressed yet do not constitutively interact. Thus, the engagement must be induced then broken with both events requiring regulation. HA-binding to CD44 is complex, and is predicted to involve multivalent binding events that can be affected by numerous factors such as the quantity, density, and activation state of CD44. It has become clear that neither a single activation signal nor an individual modification to CD44 can regulate HA-binding in all cell types or under all circumstances. This body of work aimed at pinpointing particular modifications resulting from a single stimulus in the regulation of HA-binding by CD44 on monocytes, and it was conceived based on a number of key findings.

First, *ex vivo* peripheral blood monocytes were reported to bind low levels of HA which could be augmented *in vitro* by the inflammatory cytokines TNF- $\alpha$ , IL-1, IFN- $\gamma$ , or LPS (204, 206) whereas Th2 type cytokines, IL-4 and IL-13, inhibit this up-regulation (204). Tissue macrophages from the lung and *in vitro* or *in vivo* activated monocytes also can bind HA (205, 206). These finding show that cells of the myeloid lineage can be induced to bind to HA and at least for monocytes, pro-inflammatory cytokines act as the stimulus. This work was followed by a report that TNF- $\alpha$  induced CD44-HA dependent adhesion of a CD33<sup>+</sup> leukemic SR91 cell line to an endothelial cell line (116). CD44 immunoprecipitated from radiolabeled SR91 cells was sulfated. Both the expression and sulfation of CD44 increased following TNF- $\alpha$  stimulation. The induced sulfation but not up-regulation was blocked by a sulfation inhibitor, which also reduced HA-binding. Although TNF- $\alpha$  altered the HA-binding ability of CD44 in peripheral blood monocytes as reported in the first series of studies, no mechanism was proposed. Based on the second report, the sulfation of CD44 is a potentially novel, inducible mechanism to regulate HA-binding in primary monocytes.

It has been demonstrated that CD44-HA interactions mediate physiological contact between progenitor cells and bone marrow cells during lympho-hemopoiesis (102) and myelopoiesis (125), and between leukocytes and endothelial cells during leukocyte extravasation under inflammatory conditions (101, 224). A human CD34<sup>+</sup> myeloid progenitor KG1a cell line was reported to constitutively bind to HA (262) and cluster CD44 into a membrane protrusion (263). It is well documented that activated, migratory T cells acquire transient HA-binding capacity and polarize, extending a uropod into which CD44 is sequestered (264). Therefore, the sequestration of CD44 and the role of the cytoskeleton in HA-binding by myeloid cells either in the bone marrow or in the periphery are of particular interest as a control mechanism.

The first objective of this research was to establish which of the reported modifications to CD44 affect pro-inflammatory cytokine-induced HA-binding in monocytes with particular attention given to the role of sulfation and the cytoskeleton in this process. This analysis was performed with the human CD34<sup>+</sup> myeloid progenitor cell line, KG1a, primary human monocytes, and primary murine bone marrow-derived macrophages. Emanating from this research, CD44 expression, sulfation and an intact F-actin cytoskeleton were identified as factors that effect HA-binding in the myeloid progenitor cell line and in cytokine-induced PBM and BMDM.

The hypothesis is that the sulfation of CD44 and the cytoskeleton would be key regulators of CD44 function in myeloid cells; thus, the second aspect of the project aimed at understanding the manner by which these factors contributed to HA-binding. To determine the extent of sulfation on CD44 and the general locale on carbohydrates or tyrosine residues of CD44, CD44 was immunoprecipitated from cells radiolabeled with [<sup>35</sup>S]-sulfate. A sulfation inhibitor was used to block sulfate addition in cells. Increasing concentrations of the inhibitor were used to decrease the sulfation on CD44 and compare the effect on cellular HA-binding as well as the ability of sulfate-reduced CD44 to bind to HA in a direct binding assay.

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The KG1a cell line was the primary cell type used to determine how the cytoskeleton influences CD44-ligand binding because the KG1a cells bind constitutively to HA and spontaneously extend unique pseudopod structures to which CD44 is localized. Inhibitors of microfilaments and microtubules were used to determine the effect of cytoskeletal components on CD44 localization by confocal microscopy and HA-binding by flow cytometry. A yeast-two-hybrid analysis was previously performed with the cytoplasmic domain of CD44 (Li and Johnson, unpublished data) yielding few cytoskeleton linker proteins that would be responsible for tethering CD44 to cytoplasmic filaments. Thus, the presence of potential linker proteins was probed by western blot method, the cellular distribution of candidate proteins and CD44 was assessed by co-immunoprecipitation. Culminating from this work, a mechanism for the regulation of HA-binding by sulfation and the F-actin cytoskeleton are proposed in chapters four and five.

The final research aim was to understand the functional significance of a CD44-HA interaction in pro-inflammatory-stimulated monocytes. Given that HA is produced in large amounts at sites of inflammation, and that CD44 has been implicated in the pathogenesis of multiple inflammatory disorders (reviewed in 123), the CD44-HA interaction could occur in activated monocytes to facilitate migration within inflamed tissues and myeloid progenitor motility in the bone marrow. Chapter six discusses a three-dimensional matrix system by which myeloid cells were analyzed for CD44-HA-mediated cell adhesion and migration.

Herein, the results of this investigation are presented and their contribution to our understanding of HA-binding regulation in activated myeloid cells is discussed.

# CHAPTER TWO

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# **Materials and Methods**

### 2.1 REAGENTS

# 2.1.1 Fluorescein-HA (FL-HA)

Rooster comb HA (Sigma-Aldrich Canada, Oakville, ON) was conjugated to fluorescein according to the method of de Belder and Wik (265).

## 2.1.2 Primary antibodies

# 2.1.2i Anti-CD44 antibodies

Mouse monoclonal antibody, 3G12, against human CD44 (266) was provided by G. Dougherty (U. of California, Los Angeles, CA). 3G12 was used for flow cytometry and western blotting. Purified rat anti-human CD44 mAb, Hermes-1 (267), was a gift from S. Hemmerich (Antibody Solutions Inc., Palo Alto, CA), and used to block FL-HA binding. Rat anti-human and antimouse CD44 mAb, IM7.8.1 (IM7), was from J. Lesley and R. Hyman (147) as was the rat antimouse CD44 mAb, IRAWB14.4, that was used to induce FL-HA binding (115). Mouse antibodies against human CD44 (BU52; The Binding Site, San Diego, CA) were used for immunohistochemistry. Antibody concentrations for use in flow cytometry or immunohistochemistry are described in sections 2.3.1 and 2.3.2iib.

# 2.1.2ii Antibodies against cell surface receptors

Mouse antibodies against human CD45 (Caltag Laboratories, Burlingame, CA), human CD34 (from P. Lansdorp, 268) and human CD40 (G28.5; ATCC# HB-9110) as well as rat anti-mouse F4/80 TCS (tissue culture supernatant; ATCC HB198) and mouse TCS (mAb I3/2, 147) were used for either flow cytometric analysis and/or immunohistochemistry at dilutions described in section 2.3.1 for flow cytometry and section 2.3.2iib for fluorescence microscopy. Phycoerythrin (PE)-conjugated mouse anti-human CD14, PE-conjugated mouse anti-human CD86 (B7.2) and PE-conjugated isotype control were purchased from Caltag Laboratories for use in flow cytometry analysis (section 2.3.1). PE-conjugated mouse anti-human HLA-DR from Becton Dickinson (Mississauga, ON) was used at dilutions described in section 2.3.1 for flow cytometry.

## 2.1.2iii Antibodies against cytoplasmic proteins

Rabbit antisera against human ezrin/radixin/moesin (ERM; Cell Signaling Technologies, Mississauga, ON) and phospho-ezrin(Thr567)/radixin(Thr564)/moesin(Thr558) (Phospho-

ERM; Cell Signaling Tech.) were used for immunohistochemistry at dilutions described in section 2.3.2iib or for western blot analysis at dilutions described in 2.4.4i.

# 2.1.2iv Antibodies against sulfo-containing epitopes

R. Kannagi (Aichi Cancer Center, Nagoya, Japan) provided TCS containing mouse IgM (mAbs 2F3, AG105, AG107, SU59, G270-16, L4L4-8, G72; described in (100, 269-273) against a series of sulfated Lewis x and sulfated LacNAc epitopes that will be explained in detail in chapter four. TCS containing monoclonal mouse IgM against 6-sulfo LacNAc (274) was provided by E.P. Rieber (U. of Gottingen, Gottingen, Germany). TCS was used neat or at a maximum 1/8 dilution for flow cytometry analysis (section 2.3.1).

# 2.1.2v Purification and biotinylation of anti-human CD3 mAb

The anti-human CD3 mAb, OKT3 (ATCC# CRL 8001; Rockville, MD) was purified over a protein G column. Purified anti-CD3 mAb was biotinylated with EZ link NHS-LC-biotin (Pierce, Rockford, IL) according to manufacturer's instructions.

## 2.1.3 Secondary antibodies

Fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse, goat anti-rat and horseradish peroxidase (HRP)-conjugated goat anti-rabbit Abs were purchased from Jackson Immunoresearch Laboratories Inc. (Mississauga, ON). HRP-conjugated goat anti-mouse Abs and PE-conjugated streptavidin (SA) were from Southern Biotechnology Associates Inc. (Birmingham, AL). FITC- and HRP-conjugated goat F(ab')<sub>2</sub> anti-mouse IgM antibodies were from Jackson Immunoresearch Laboratories Inc.. Alexa 488-conjugated goat anti-mouse and anti-rabbit antisera, Alexa 594-conjugated goat anti-mouse antisera and Texas Red phalloidin were obtained from Molecular Probes (Eugene, OR). HRP-conjugated goat anti-fluorescein antibody (Rockland Immunochemicals, Gilbertsville, PA) was used in far western blot analysis (section 2.4.4ii). All other antibodies were used for flow cytometry (section 2.3.1), western blotting (section 2.4.4i) or immunohistochemistry (section 2.3.2iib) at dilutions described in the individual methods.

#### **2.2 Cell culture**

### 2.2.1 General cell culture protocols

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### 2.2.1i Growth Medium

Cells were cultured at 37°C in 5% CO<sub>2</sub> in complete RPMI-1640 or DMEM (Cell Culture Facility, University of California San Francisco, San Francisco, CA or Invitrogen Life Technologies, Burlington, ON) supplemented with 10% FCS (HyClone, Logan, UT or Invitrogen Life Technologies), 1 mM sodium pyruvate, 2 mM L-glutamine (Invitrogen Life Technologies) and 50  $\mu$ M  $\beta$ -mercaptoethanol (Sigma-Aldrich Canada), unless otherwise stated.

# 2.2.1ii Freezing cells

 $5 \times 10^6$  mammalian cells were suspended in 0.5 ml of 90% FCS / 10% DMSO, slow frozen to -80°C and transferred to liquid nitrogen (lN<sub>2</sub>) for storage.

#### 2.2.1iii Detaching adherent cells

Growth media was aspirated from adherent cells and replaced with versene or 0.25% trypsin-EDTA (Invitrogen Life Technologies) for 10 min at 37°C in 5% CO<sub>2</sub> to detach adherent cells from the culture dish. Cells were collected and washed twice in PBS prior to further manipulation. Note that versene, not trypsin-EDTA, was always used to detach cells intended for flow cytometry analysis.

#### **2.2.1iv** *Determining live cell concentration by trypan blue exclusion*

A representative aliquot of suspension cells or detached adherent cells was combined 1:1 with 0.4% trypan blue stain (Invitrogen Life Technologies). Live, unstained cells were counted on a hemacytometer, and the concentration of viable cells was determined.

#### 2.2.2 Culture and stimulation of SR91 cells

The human leukemic cell line SR91 (275) was maintained in RPMI / 10% FCS at 2 - 8 x  $10^5$  cells/ml. 1 x  $10^6$  cells/ml were seeded into 6-well tissue culture (TC) dishes in the presence or absence of 10 ng/ml of human recombinant TNF- $\alpha$  (R&D Systems, Minneapolis, MN) for 24 hr.

# 2.2.3 Sorting KG1a cells based on HA-binding capacity

The human myeloid CD34<sup>+</sup> progenitor cell line KG1a (ATCC #CRL246.1 276) was maintained in RPMI / 10% FCS. Approximately 1 x  $10^7$  cells were washed in PBS then labeled with FL-HA as described for flow cytometry analysis. Approximately 30 - 50% of the highest FL-HA binding and 50% of the lowest FL-HA binding KG1a cells were differentially selected using a FACS Vantage SE Turbo Sort® cell sorter (Becton Dickinson). Cells were cultured in RPMI / 10% FCS, and sorting was repeated (2 - 3 times) until stable cell lines with high or low HA binding were established.

# 2.2.4 Isolation, culture, and stimulation of Peripheral Blood Mononuclear Cells (PBMC)

# **2.2.4i** Isolation of PBMC from whole blood

Venous blood (40 - 100 ml) was collected from healthy volunteers in accordance with ethical guidelines in vials containing heparin as an anticoagulant (Becton Dickinson). Blood was diluted three-fold in Hanks Balanced Salt Solution (HBSS, Invitrogen Life Technologies) and separated by centrifugation over a Ficoll-Paque® Plus (Amersham Pharmacia Biotech, Baie d'Urfé, PQ) density gradient. White blood cells were isolated from the buffy coat and contaminating red blood cells were lysed with 10 mM Tris pH 7, 0.83% NH<sub>4</sub>Cl for 3 to 5 mins on ice. The total number of PBMC isolated was determined by trypan blue exclusion. Cells were seeded at densities described below in complete RPMI / 10% FCS and cultured at 37°C in 5% CO<sub>2</sub>.

# **2.2.4ii** *Positive Selection of CD14<sup>+</sup> peripheral blood cells*

Following culture with [ $^{35}$ S]-sodium sulfate in the presence or absence of stimulation or inhibitors, PBMC were washed twice into PBS containing 5 mM EDTA and 2% FCS to a final concentration of ~1 x 10<sup>7</sup> cells/ml. Cells were rotated end over end at 4°C for 1 hr with 25 µl of anti-CD14 conjugated magnetic beads (M450; Dynal Inc., Lake Success, NY) per ml of cells. Beads (with CD14<sup>+</sup> PBMC attached) were immobilized on a magnet (Dynal Inc.) and washed 3 x 1 ml in PBS, pooling washes to collect CD14<sup>-</sup> cells. Purity and yield of the separated populations was determined by flow cytometry analysis (labeling with anti-CD14 mAb) on total cells prior to selection and non-immobilized cells following selection. CD14<sup>+</sup> PBMC (on the magnetic beads) and CD14<sup>-</sup> PBMC (in the supernatant) were lysed immediately in the manner described in section 2.4.2.

# **2.2.4iii** Negative Selection of CD14<sup>+</sup> peripheral blood cells

Peripheral blood mononuclear cells were isolated from whole blood (described in section 2.2.4i) and then enriched for CD14<sup>+</sup> monocytes by magnetic removal of CD14<sup>-</sup> PBMC according to manufacturer's directions (Dynal Inc.). The negative isolation kit contains a

cocktail of antibodies against CD2, CD7, CD16a, CD16b, CD19 and CD56 for removal of T cells, granulocytes, B cells and NK cells. Purity and yield were determined by flow cytometry analysis of selected cells (labeling with anti-CD14) prior to and following selection.

# 2.2.4iv Stimulation of Peripheral Blood Mononuclear Cells (PBMC)

PBMC (5 x 10<sup>6</sup>) were seeded into 6-well tissue culture dishes (Nunc; Invitrogen BRL Life Technologies, Burlington, ON) at 2.5 x 10<sup>6</sup> cells/ml for 72 hr at 37°C in 5% CO<sub>2</sub> with or without stimulation with 1 µg/ml LPS (Sigma-Aldrich Canada) or various human recombinant cytokines or chemokines as follows: 20 ng/ml TNF- $\alpha$ , 20 ng/ml IL-1 $\beta$ , 50 ng/ml MIP-1 $\alpha$ , 50 ng/ml of MCP-1, 200 U/ml of IFN- $\gamma$  (all from R&D Systems). Alternatively, PBMC (3 x 10<sup>6</sup>) were seeded into 6-well tissue culture dishes at 1.5 x 10<sup>6</sup> cells/ml in the presence or absence of 5 µg/ml PHA (Sigma-Aldrich Canada) and incubated for 12 - 48 hr at 37°C in 5% CO<sub>2</sub>.

# 2.2.4v Stimulation of Peripheral Blood Monocytes (PBM)

After negative selection of PBM, total cell count was determined by trypan blue exclusion. 5 x  $10^6$  PBM were seeded into 6-well tissue culture dishes at 2.5 x  $10^6$  cells/ml. Cells were incubated for 72 hr at 37°C in 5% CO<sub>2</sub> with or without 20 ng/ml of human recombinant TNF- $\alpha$  or 200 U/ml of human recombinant IFN- $\gamma$ .

### 2.2.5 Isolation and differentiation of mouse bone marrow

Following CO<sub>2</sub> asphyxiation in accordance with animal care regulations, organs and bone marrow were isolated from mice under sterile conditions. Wild type mouse strains (C57BL6, DBA-2, Balb/c) were purchased from Charles River Laboratories (USA), whereas CD44- (277) and CD45-deficient (278) C57BL6 strains were received from J. Penninger (formerly at U. of Toronto, Toronto, ON). Sulfurylase kinase-2 (SK2; aka PAPS synthetase) -deficient mice (84) were supplied by F. Jirik (U. Calgary, Calgary, AB). Femurs were isolated from deceased mice, and the head of the bone was punctured with a 26 gauge, 1/2-inch needle attached to a 10 ml syringe containing PBS. The bones were flushed with PBS, the marrow was pooled then combined with DMEM containing 10% FCS (~25% of final volume) and centrifuged at 500 g for 5 min. The marrow was washed twice more with media then cultured on 2 - 100 mm<sup>2</sup> petri dishes per femur in 10 ml of differentiation media (DMEM containing 1 mM sodium pyruvate, 2 mM L-glutamine, 20% FCS and no less than 2.5% L-cell conditioned media (LCCM) or 1 ug/ml of recombinant murine M-CSF (R&D systems). Adherent cells arising from the bone

marrow culture are grown to confluence, approximately 5 - 7 days, at  $37^{\circ}$ C in 5% CO<sub>2</sub>. Adherent cells are collected, tested by flow cytometry for expression of the macrophage marker F4/80, used immediately for analysis, or frozen for future use.

# 2.2.5i Generation of L cell conditioned media (LCCM)

LCCM is generated from the culture of mouse L929 fibroblasts (ATCC #CCL-1) and is a source of M-CSF. L929 cells were grown in complete RPMI / 10% FCS on tissue culture flasks, expanding the culture as necessary and reducing the serum concentration to 2.5 - 5% during the last sub-culturing of cells. After several weeks of growth, the culture was centrifuged at 1000 g for 10 min to pellet cells. The cleared, conditioned media was concentrated ten-fold under nitrogen gas at 4°C, filter sterilized, aliquotted, and stored at -80°C. Due to batch variability, LCCM concentration was titrated against 1 µg/ml of M-CSF to determine the concentration of LCCM to induce bone marrow cell differentiation, typically 2.5 to 10% LCCM.

2.2.5ii Culture and stimulation of mouse bone marrow-derived macrophages (BMDM) Frozen aliquots of BMDM were thawed in differentiation media, seeded into petri dishes at ~50% confluence, and cultured at 37°C in 5% CO<sub>2</sub> for 2 - 24 hr prior to use. Cells were stimulated in DMEM containing 1 mM sodium pyruvate, 2 mM L-glutamine, 20% FCS, and no less than 1% LCCM for 72 hr in the presence or absence of 20 ng/ml of murine recombinant TNF- $\alpha$  (R&D Systems).

### 2.2.6 Use of inhibitors in cell culture

**2.2.6i** Treatment of cells with the sulfation inhibitor, sodium chlorate  $(NaClO_3)$ SR91 cells, PBM, and BMDM were seeded under the same conditions described for stimulation, and 5 x 10<sup>5</sup> KG1a cells/ml were seeded into 6-well tissue culture dishes. Cells were incubated for 24 - 72 hr in cell media in the presence of 0 - 50 mM sodium chlorate (NaClO<sub>3</sub>; Sigma-Aldrich Canada) for the entire course of the stimulation where applicable.

## 2.2.6ii Treatment of cells with cytoskeletal destabilizers

A maximum of  $1 \times 10^6$  cells/ml were treated with 20 - 40 ng/ml of the microtubule inhibitor, nocadazole (Sigma-Aldrich Canada), 10 - 20 mM of 2,3-butanedione 2-monoxime, a myosin ATPase inhibitor (Calbiochem-Novabiochem Corporation, La Jolla, CA), or 1 - 50  $\mu$ M of actin filament destabilizing agents, cytochalasin D or latrunculin A (Calbiochem-Novabiochem Corp.), for up to 1 hr at  $37^{\circ}$ C in 5% CO<sub>2</sub>. In the case where cells were pretreated with stimulation or sodium chlorate, inhibitors were added for the last hour of the 24 hr incubation period for SR91 cells or for the last hour of the 72 hr incubation period for BMDM, PBM, and KG1a cells.

**2.2.6iii** Inhibition of O-glycosylation, GAG addition, and removal of sialic acid residues Cells were incubated in the presence or absence of stimulation and in the presence or absence of 1 mM xyloside (p-nitrophenyl  $\beta$ -D-xylopyranoside; Sigma-Aldrich Canada), an inhibitor of GAG assembly or 1 mM BADGal (benzyl-2 acetamido 2-deoxy- $\alpha$ -D- galactopyranoside; Sigma-Aldrich Canada), an inhibitor of O-glycan addition, or both inhibitors together over the entire course of the stimulation. Alternatively, after incubation in the presence or absence of stimulation, cells were washed into RPMI-1640:PBS (1:1 pH 6.7) at 1 x 10<sup>7</sup> cells/ml and treated with 0.05 units/ml of neuraminidase (from *Vibrio cholerae*, Roche Diagnostics, Laval PQ) for 1 hr at 37°C, to remove terminal sialic acid residues.

#### **2.2.6iv** Treatment of cells with the protein synthesis inhibitor, cycloheximide

Cycloheximide (CHX; Sigma Aldrich Canada) was used at 0.05 - 1 ug/ml to inhibit protein synthesis in SR91 cells over 24 hr culture in the presence or absence of TNF- $\alpha$  (as previously described in section 2.2.2).

#### **2.3 CELLULAR ANALYSIS**

#### 2.3.1 Flow cytometry

Cells intended for analysis by flow cytometry were removed from their culture media and washed into PBS containing 5 mM EDTA and 2% FCS (buffer).  $F_c$  receptors on PBMC and BMDM were blocked with 100 µl mouse antibodies and/or human plasma for 20 min on ice then washed twice in buffer. Cells were incubated with primary antibodies for 30 min on ice as follows: 100 µl of anti-CD44 (3G12 or IM7) TCS, anti-CD45 (I3/2) TCS, anti-F4/80 TCS, neat or 1/8 dilution of TCS containing IgM antibodies against sulfo-epitopes, 3 µg/ml anti-CD40 Ab, 2 µg/ml PE-anti-MHC II Ab, 2 µg/ml PE-anti-CD86 (B7.2) Ab, or 3 µg/ml biotinylated anti-CD3 Ab. Cells were washed twice then incubated with secondary antibody for 20 min on ice as follows: 100 µl of 1/100 FITC-goat anti-mouse (IgG or IgM specific) or FITC-goat anti-rat Abs, 1/1000 PE-anti-CD14 Ab, 1/300 PE-SA, or 10 µg/ml of FL-HA. PBMC were double-

labeled with FL-HA and anti-CD14 or anti-CD3 Abs. FL-HA binding was blocked by preincubation for 10 min with 1/100 rat anti-human CD44 mAb, Hermes-1, followed by a 20 min incubation with 10  $\mu$ g/ml FL-HA. Alternatively, cells were incubated with buffer, isotype controls or secondary antibody alone. Cells were washed twice and re-suspended in buffer containing 5  $\mu$ g/ml of propidium iodide (Sigma-Aldrich). A minimum of 2000 live SR91 or KG1a cells, ten thousand live PBMC, 5000 live CD3<sup>+</sup> PBMC, or 2000 size-gated (large, dense PBMC selected on forward and side scatter profiles) events were collected on a FACScan® flow cytometer (Becton Dickinson) and analyzed using CellQuest® software.

#### 2.3.2 Microscopy

# 2.3.2i Phase contrast

Cells in suspension were photographed under phase contrast using a Zeiss Axiophot microscope or Nikon Elipse TS100 inverted microscope with a Nikon Coolpix 990 digital camera.

# 2.3.2ii Confocal Microscopy

Before preparing cells for microscopic analysis, control cells were analyzed by flow cytometry analysis to confirm that normal levels of FL-HA were bound and that the various inhibitors had the desired effect.

# 2.3.2iia Fixing cells

In culture,  $1 \times 10^6$  KG1a cells or PBM were incubated in a final concentration of 4% paraformaldehyde for 10 min at 4°C to fix cells.  $1 \times 10^5$  cells were washed with 200 µl of PBS containing protease and phosphatase inhibitors (PBS/inh) at concentrations described in the CD44 co-immunoprecipitation procedure (section 2.4.3ii). All subsequent washes and antibody dilutions were conducted using PBS/inh. Prior to intracellular protein staining, fixed cells were permeabilized with 100 µl of 0.1% Triton X-100 or 0.2% saponin (Sigma-Aldrich Canada) in PBS for 10 min at 4°C, then washed twice.

# 2.3.2iib Staining

KG1a cells or PBM were incubated with 100  $\mu$ l of buffer containing 1  $\mu$ g of anti-CD44 (BU52) Ab, 1  $\mu$ g of anti-human CD45 Ab, or 4  $\mu$ g of anti-CD34 Ab for 30 min at 4°C, or 100  $\mu$ l of 1/50 anti-ERM Ab or 1/100 anti-phospho-ERM Ab for 1 hr at 4°C, or cells were double-labeled with anti-CD44 and anti-ERM or anti-phospho-ERM for 1 hr at 4°C. Cells were washed twice then incubated for 30 - 60 min at 4°C with 100  $\mu$ l of 1/100 Alexa 488-conjugated goat antimouse or anti-rabbit Abs, 1/100 Alexa 594-conjugated goat anti-mouse Abs, 1/25 Texas Red phalloidin, or 1  $\mu$ g of FL-HA. Alternatively, cells were incubated with isotype controls, secondary Ab, or buffer alone. Cells were washed twice then transferred to an 8-well glass chamber slide (Lab-Tek; Nalge Nunc Inernational, Naperville, IL) and incubated for 10 min at 4°C. To adhere cells to the slide, 4% glutaraldehyde was added to each chamber for 10 min at 4°C. Chambers were washed twice with 450  $\mu$ l PBS/inh (see above section) then allowed to dry. Approximately 10  $\mu$ l of mounting media (90% glycerol with 2.5% DABCO (1,4-Diazabicyclo [2.2.2] octane); Sigma-Aldrich Canada) was added per section, and a cover slip was sealed to the slide with clear nail polish. Slides were stored in the dark at 4°C until microscopic visualization.

# 2.3.2iic Data collection and imaging

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Confocal microscopy analysis was performed using the confocal laser scanning system (Bio-Rad MRC-600 with a Nikon Optiphot-2 upright light microscope or Bio-Rad Radiance Plus with an inverted Zeiss Axiovert microscope, Bio-Rad Labs Ltd., Hercules, CA). Digital images of serial cellular sections were compiled to a three-dimensional image using NIH image software or left as single slices and merged using Adobe Photoshop® Image Software.

#### 2.3.3 Cell adhesion and migration on three-dimensional gel matrices

# 2.3.3i Preparation of matrigel<sup>TM</sup> matrix and incorporation of HA

Matrigel<sup>TM</sup> basement membrane matrix (Becton Dickenson) is made from the Engelbroth-Holm-Swarm (EHS) mouse sarcoma, a rich source of ECM proteins. The matrix is in the liquid phase at 4°C but solidifies at room temperature. Aliquots stored at -80°C were thawed and kept on ice at 4°C for no more than 7 days. Matrigel<sup>TM</sup> was diluted in ice cold media with or without HA, then 50 µl per well was spread evenly in 12-well transwell filters (8 um pore size, Becton Dickinson), and allowed to solidify at 37°C in 5% CO<sub>2</sub> for 1 - 2 hr. In general, matrigel<sup>TM</sup> was diluted 2 - 3 fold in DMEM or RPMI with or without HA such that the final concentration of HA in the gel is 0.5 mg/ml.

### 2.3.3ii Transwell adhesion, migration, and invasion assays

For all assays, cells were incubated in the presence or absence of stimulation or inhibitors under the normal culture conditions previously described. Following the incubation period, cells were counted, washed into PBS / 0.1% BSA then rotated for 1 hr at 4°C in the presence or

absence of 2.5 mg/ml of the HA blocking anti-CD44 mAb, Hermes-1, or isotype control antibody.  $4 - 10 \ge 10^5$  cells/ml were washed into DMEM or RPMI containing reduced serum, either 2% FCS for human cells, or 1% FCS / 0.1% LCCM for mouse BMDM. 500 µls of media containing 2 - 5  $\ge 10^5$  cells were added per matrix-coated transwell insert mounted in a 12-well plate (Becton Dickinson) containing 1.5 ml of complete media (RPMI 10% FCS or DMEM / 10% FCS / 1% LCCM) per well, establishing a 5- or 10-fold serum gradient, between the top and bottom of the transwell insert.

# 2.3.3iia Quantitation of cell polarization

To investigate initial adhesion and polarization events, cells were incubated for 2 - 6 hrs at  $37^{\circ}$ C in 5% CO<sub>2</sub>. The cells were photographed prior to and after the transwells were washed twice to remove non-adherent cells. The number of attached cells and the number of polarized cells were counted and averaged over three fields of view.

# **2.3.3iib** *Quantitation of cell migration by KG1a cells*

Cell migration assays were set up in the same manner as described in section 2.3.3ii, and cells were incubated for an extended period of time at 37°C in 5% CO<sub>2</sub>. KG1a cell migration was analyzed after 12 hrs. Transmigrated cells present in the lower chamber were transferred and centrifuged at 500 g for 3 min at room temperature. Cells were washed into a final volume of 100  $\mu$ l of PBS. The concentration of cells was determined using trypan blue exclusion, and the total number of transmigrated cells was calculated. The percentage of migrated cells ((transmigrated cells / input cells) x 100) was reported.

# **2.3.3iic** *Quantitation of invasion by BMDM*

BMDM were incubated for 24 hr in the transwell system. To determine the number of cells embedded in the matrigel, the transwells were washed gently with PBS then soaked in ice cold methanol for 20 mins on ice to fix the cells. To stain the cells, methanol was aspirated and replaced with ~150 µls per insert of 0.2% crystal violet / 10% phosphate-buffered formalin pH 7.0 for 10 min at room temperature. Excess dye was removed by 4 x 1 ml washes with ddH<sub>2</sub>O. Transwells were inverted and allowed to dry for 30 mins or overnight at room temperature. Using a razor blade, the filter (with matrigel and cells) was excised from the transwell insert and mounted bottom side down on a drop of glycerol on a glass microscope slide. Another drop of glycerol was added to the top of the filter, and a cover slip was secured around the edges with clear nail polish. Embedded cells were viewed at  $100 \times 000 \times 0000$  magnification, photographed, enumerated, and averaged over 2 - 3 fields of view. For each experiment, the parameter that yielded the least number of invasive cells was assigned a relative migratory value equal to one to which the relative migration of cells under different conditions was calculated.

### **2.4 MOLECULAR ANALYSIS**

Where appropriated in the methods described in this section, a parallel experiment using nonradiolabeled control cells were analyzed by flow cytometry analysis to confirm that normal levels of FL-HA were bound and that the various inhibitors had the desired effect.

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# 2.4.1 [<sup>35</sup>S]-sodium sulfate labeling of cells

PBMC (2.5 x  $10^6$  cells/ml) were cultured in complete RPMI / 10% FCS in 6-well tissue culture plates in the presence or absence of stimuli and 0 - 50 mM NaClO<sub>3</sub> at 37°C in 5% CO<sub>2</sub> for 72 hr. Cells were supplemented with 200 µCi/ml of [<sup>35</sup>S]-sulfate as Na<sub>2</sub><sup>35</sup>SO<sub>4</sub> (specific activity ~43 Ci/mg; ICN Biomedicals Inc., St. Laurent, PQ) for the entire incubation period or pulsed for 2 hr with label at 0, 24, 48, and 72 hr. BMDM were cultured under the same conditions described for stimulation, that is, seeded at 50% confluence in the presence or absence of 20 ng/ml of recombinant mouse TNF- $\alpha$ . KG1a cells were seeded at 5 x 10<sup>5</sup> cells/ml in 6-well TC dishes. KG1a cells or BMDM were labeled for 72 hr at 37°C in 5% CO<sub>2</sub> with 150 - 200 µCi/ml of [<sup>35</sup>S]-sodium sulfate in the presence or absence of stimuli and 50 mM NaClO<sub>3</sub>.

# 2.4.1i Autoradiography

Immunoprecipitated CD44 (see section 2.4.3) was resolved by electrophoresis on a 7.5% SDSpolyacrylamide gel (SDS-PAGE) and transferred to polyvinylidene difluoride (PVDF) Immobilon-P membranes (Millipore Canada Ltd., Mississauga, ON). Membranes were exposed to Kodak BioMax MR film (InterScience, Markham, ON) with an intensifying screen at -80°C for 7 - 21 days. [<sup>35</sup>S]-sulfate incorporation was normalized with respect to the amount of CD44 protein (determined by western blotting) by spot densitometry using NIH image software. Where appropriate, a fraction of the sample was loaded on a separate gel for spot densitometry analysis of CD44 protein levels at sub-saturating concentrations following western blotting.

#### 2.4.2 Cell lysis

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5 x  $10^6$  - 1 x  $10^7$  CD14<sup>+</sup> cells (obtained by positive selection on magnetic beads), CD14<sup>-</sup> cells (remainder of cells after positive selection of CD14<sup>+</sup> cells), BMDM, or 1 x  $10^6$  KG1a cells were re-suspended in 1 ml of lysis buffer (1% Triton X-100, 10 mM Tris pH 7.5, 150 mM NaCl, 2 mM EDTA) containing 200 µM PMSF, 1 µg/ml leupeptin, 1 µg/ml aprotinin, and 1 µg/ml pepstatin for 10 min on ice then centrifuged at 16,000 g for 10 min. The supernatant was transferred and the cell lysates were analyzed by SDS-PAGE or subject to immunoprecipitation of CD44. When analyzing potentially phosphorylated proteins, cells were lysed in the Triton X-100 buffer described above with the addition of protein tyrosine phosphatase and serine/threonine phosphatase inhibitors at concentrations described in section 2.4.3ii. Note that to co-immunoprecipitate CD44, the cells were lysed in a different buffer described in section 2.4.3ii (CD44-ERM co-immunoprecipitation).

### 2.4.3 CD44 immunoprecipitation

# 2.4.3i from CD14<sup>+</sup>PBMC, CD14<sup>-</sup>PBMC, BMDM, and KG1a cells

Cell lysates (section 2.4.2) were pre-cleared with 15  $\mu$ l of sepharose CL-4B beads (Sigma-Aldrich Canada) for 1 hr at 4°C, rotating end over end. CD44 was immunoprecipitated from pre-cleared lysates with 15  $\mu$ l of IM7.8.1 mAb-conjugated, CNBr-activated, sepharose beads (at 4 mg/ml in a 50% slurry) for 2 hr at 4°C, rotating end over end. Immunoprecipitates were washed with 3 x 1 ml of lysis buffer containing 500 mM NaCl, 2 x 1 ml of lysis buffer, then boiled for 2 min in reducing sample buffer.

# 2.4.3ii CD44-ERM co-immunoprecipitation

5 x  $10^6$  - 1 x  $10^7$  KG1a cells were washed once in PBS then re-suspended at 1 x  $10^7$  cells/ml in lysis buffer (10 mM Tris pH 7.5, 2 mM EDTA, 1% Triton X-100) containing protease inhibitors (200 µM PMSF, 1 µg/ml leupeptin, 1 µg/ml aprotinin, 1 µg/ml pepstatin), protein tyrosine phosphatase inhibitors (200 µM sodium molybdate, 500 µM sodium orthovanadate) and serine/threonine protein phosphatase inhibitors (1 nM microcystin-LR; Calbiochem-Novabiochem Corporation, and 300 nM okadaic acid: Sigma-Aldrich Canada) for 10 min on ice. Lysates were centrifuged at 16,000 g at 4°C for 20 mins then pre-cleared with 25 - 50 µl of sepharose CL-4B beads (Sigma-Aldrich Canada) for 1 hr at 4°C. CD44 was immunoprecipitated from cleared lysates with 25 - 50 µl of IM7.8.1 mAb-conjugated, CNBractivated, sepharose beads for 2 hr at 4°C rotating end over end. Immunoprecipitates were washed 3 x 1 ml in lysis buffer, then boiled for 2 min in reducing sample buffer.

#### 2.4.4 Immunoblotting

#### 2.4.4i Western blot analysis

PVDF membranes were incubated with a 1/4 dilution of anti-CD44 (3G12) TCS or 1/1000 dilution of anti-ERM or anti-Phospho-ERM Ab in TBST (20 mM Tris pH 7.5, 150 mM NaCl, 0.1% Tween 20) containing 5% skimmed milk powder (TBST/milk) for 1 hr. Membranes were washed for 1 hour in TBST then incubated with 1/5000 HRP-conjugated goat anti-mouse or goat anti-rabbit mAb (in TBST/milk) for 30 min. Membranes were washed 30 - 60 min in TBST and developed with the enhanced, or enhanced plus chemiluminescence assay (ECL or ECL Plus<sup>™</sup>; Amersham Pharmacia Biotech), according to manufacturer's instructions. ERM proteins resolve to 2 or 3 bands on SDS-PAGE but were treated as one band for the purpose of densitometric analysis using NIH image software.

## 2.4.4ii Far western blot analysis

CD44 immunoprecipitated from ~5 x  $10^5$  CD14<sup>+</sup> PBMC was resolved on a 7.5% SDSpolyacrylamide gel under non-reducing conditions and transferred to a PVDF membrane. Membranes were incubated with 2 µg/ml of FL-HA diluted in TBST/milk for 1 hr at room temperature with agitation. Membranes were washed 6 x 10 min in TBST then incubated with 1 µg/ml of HRP-conjugated goat anti-fluorescein Abs (in TBST/milk) for 30 min at room temperature with agitation. Membranes were washed 6 x 5 min in TBST and developed with ECL Plus<sup>TM</sup> (Amersham Pharmacia Biotech) according to manufacturer's instructions.

#### 2.4.5 Enzymatic digestion of CD44

CD44 was immunoprecipitated with anti-CD44-conjugated sepharose beads as described above (section 2.4.3i). CD44 was removed from the beads by boiling in 150  $\mu$ l of 10 mM Tris-HCl pH 7.5 containing 1% SDS for 2 min. Buffer (containing CD44) was transferred, diluted in 8 volumes of ice cold acetone, incubated for 2 hr at -20°C, and centrifuged at 16, 000 g for 30 min at 4°C to precipitate CD44. CD44 was re-suspended in an appropriate buffer (according to manufacturer's instructions) for incubated over 16 hr at 37°C with or without 500 units of PNGaseF (New England Biolabs, Beverly, MA) or 0 - 8 units/ml of arylsulfatase (Calbiochem Novabiochem Corp.) to remove N-linked glycans or sulfate from tyrosine residues, respectively.

### 2.4.6 RT-PCR on CD44 mRNA

Total cytoplasmic mRNA was isolated from  $10^6 - 10^7$  PBM or KG1a cells according to manufacturers directions (RNeasy Mini kit, Qiagen, Mississauga, ON). Briefly, cells were lysed in 175 ul of cold buffer (10 mM Tris-Cl pH 8.0, 140 mM NaCl, 1.5 mM MgSO4, 0.5% (v/v) Nonidet P-40 containing 1000 U/ml ribonuclease inhibitor (Invitrogen Life Technologies) for 5 min. Isolated cytoplasmic RNA was used immediately or stored at -80°C. Contaminating DNA was digested with 1 U/ $\mu$ l of deoxyribonuclease I for 15 min. cDNA was obtained by reverse transcription of mRNA for 50 min at 42°C with 200 units of Superscript II RNase H reverse transcriptase (Invitrogen), appropriate dNTPs (dATP, dGTP, dCTP and dTTP), and oligo (dT)<sub>18</sub> according to manufacturer's instructions. The first PCR used primers designed to flank either side of the entire CD44 transcript (designed by A. Maiti, synthesized by I. Sadowski) with 1.25 units per reaction of Pfx DNA polymerase (Invitrogen) in appropriate buffer with dNTPs as described by the manufacturer. One tenth of the reaction mixture was subject to a second PCR using nested primers (designed by A. Maiti, synthesized by I. Sadowski) to amplify regions encoding the variant forms of CD44. Primers to amplify β-actin (synthesized by I. Sadowski) were incorporated in the first PCR as a quantitative control. Templates were denatured at 95°C for 5 min, then cycled at [95°C for 45 sec, 56°C for 45 sec, 68°C for 90 sec] and extended at 68°C for 10 min to complete the reaction. Cycling was repeated 40 times with primer set #1 and 20 times with primer set #2 and  $\beta$ -actin primers. The first PCR yields a transcript of ~675 bp corresponding to CD44H. The second reaction yields a CD44H transcript of ~369 bp and ~550 bp for  $\beta$ -actin. For all reactions, a negative control without template was included and ddH<sub>2</sub>0 containing 0.1% DEPC (diethyl pyrocarbonate, Sigma) was used. Products were separated by agarose gel electrophoresis and visualized with ethidium bromide according to standard molecular biology techniques.

Forward (Fwd) and Reverse (Rev) Primers (1) and Nested Primers (2) for human CD44: Fwd 1: exon 4 (extracellular domain) 5' CTC-CAC-CTG-AAG-AAG-ATT-GTA-C 3' Rev 1: exon 19 (cytoplasmic domain) 5' CTG-TCA-TAA-ACT-GGT-CTG-GAG-T 3' Fwd 2: exon 5 (extracellular domain) 5' ATG-TCC-AGA-AAG-GAG-AAT-ACA-G 3' Rev 2: exon 16 (extracellular domain) 5' TAT-AGG-ACC-AGA-GGT-TGT-GTT-T 3' Forward and Reverse Primers for mouse and human β-actin Fwd: 5' GAC-TAC-CTC-ATG-AAG-ATC-CT 3'

Rev: 5' ATC-CAC-ATC-TGC-TGG-AAG-GT 3'

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**CHAPTER THREE** 

Identification of factors that influence HA-binding by myeloid cells

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#### **3.1** INTRODUCTION AND RATIONALE

A number of studies have shed light on the importance of CD44 and HA in lymphohemopoeisis (102), myelopoeisis (125), and in the pathogenesis of multiple inflammatory disorders (reviewed in 123). Anti-CD44 antibodies that block HA-binding inhibit inflammation in murine models of cutaneous inflammation (137), inflammatory bowel disease (IBD, 135), collagen-induced arthritis (279), proteoglycan-induced arthritis (135), vascular leak syndrome (280), and experimental autoimmune encephalomyelitis, a murine disease reminiscent of multiple sclerosis (MS) in humans (136). Conversely, anti-CD44 antibodies that induce binding to HA exacerbated inflammatory symptoms in proteoglycan-induced arthritis (138). In vivo studies in CD44<sup>-/-</sup> mice report that CD44 plays a role in inflammatory colitis, specifically CD44v7 (143), in collagen-induced arthritis (281), bacterial superantigen-induced peritonitis (282), E. coli-induced pneumonia (144), and bleomycin-induced lung inflammation (145). Currently, inflammatory diseases are treated with broad-range immunosuppressants that down regulate the function of all cells in the immune system. Identification of a specific drug target to treat inflammatory disorders or to block the onset of disease is appealing. The trafficking of naïve cells to lymph nodes was unaffected in experiments that implicate CD44 in inflammatory disorders (137, 282, 283). This suggests that the function of CD44 is specific to cell trafficking during an inflammatory response, not in the normal re-circulation of leukocytes, thereby making CD44 an attractive, specific therapeutic target to treat inflammatory diseases.

CD44 has been reported to bind multiple ligands however, an interaction between CD44 and HA has been most frequently implicated in disease. At present there is an insufficient understanding of the complexities of CD44 activation and the regulation of HA-binding. CD44 is constitutively expressed on virtually all cells, and HA is ubiquitously expressed in virtually all tissues. However, most cells that express CD44 do not bind to HA. Under normal immune conditions, CD44-positive leukocytes do not interact with HA, or do so weakly. Tight regulatory mechanisms control the conversion of CD44 between forms possessing variable aptitudes for binding to HA. It has been reported that inflammatory stimuli activate CD44 and induce T cells, monocytes, and macrophages to engage high levels of HA (204, 210, 212, 215, 224). Molecular mechanisms that control the conversion of CD44 from a low to a high HA-binding form have been proposed, but have not been linked to specific cell types or have not been proven to occur in response to particular stimuli.

Cytokine-activated monocytes quickly infiltrate damaged or infected tissue to control the initial spread of infection and to alert other immune cells to the problem. Monocytes are implicated in inflammatory diseases such as arthritis, where monocytes have been found in arthritic synovial joints, and in atherosclerosis where monocytes propagate plaque formation. The ligand-binding function of CD44 is activated in monocytes by inflammatory stimuli, and CD44-HA interactions also have been implicated in these diseases. Therefore, the factors that induce CD44-mediated HA-binding by monocytes, in particular inflammatory agent-activated monocytes, were investigated. The CD34<sup>+</sup> KG1a progenitor cell line, the CD33<sup>+</sup> human leukemic SR91 cell line, and human peripheral blood monocytes were chosen for analysis in order to encompass a range of human myeloid cells and cell lines. The induction of murine monocyte binding to HA is a challenge since significant numbers of peripheral blood monocytes are difficult to obtain and identify. The mouse system has many advantages over the human system, the most obvious being the convenience of analyzing cells from a plethora of knock out or transgenic mice both in vitro and in vivo. A population of murine myeloid cells that could easily be obtained, cultured, and induced to bind to HA was sought, such that factors required for HA-binding in murine macrophages could be analyzed in parallel with the human studies. The importance of various transcriptional, posttranslational, and cytoskeletal changes that influence the HA-binding capacity of CD44 were assessed. HA-binding by myeloid cells was measured by flow cytometry analysis using soluble fluoresceinated hyaluronan (FL-HA).

### **3.2 RESULTS**

### 3.2.1 Factors that induce CD44-mediated HA-binding in myeloid cells

**3.2.1i** CD44-mediated HA-binding is induced in human CD14<sup>+</sup> PBMC by TNF- $\alpha$ , LPS, IL-1 $\beta$ , or IFN- $\gamma$ 

Previous to this study, it was reported that HA-binding could be induced in PBM following culture of PBMC for 72 hr in RPMI medium containing autologous human serum (215). For the experiments described here, PBMC were isolated from blood that was donated by healthy volunteers then incubated from 0 - 72 hr in RPMI medium containing 10% FCS. Under these conditions, approximately 20% of PBMC bound low levels of FL-HA immediately following isolation (Figure 3.1A left panel). PBMC incubated for 72 hr did not bind to FL-HA (Figure 3.1A middle panel), thus, for subsequent experiments, PBMC were incubated in medium containing FCS in which CD44 is not activated to bind to HA, as was reported to occur by culture of PBMC in the presence of human serum. Incubation of PBMC with TNF- $\alpha$  for 72 hr
induced a population of cells (~15% of PBMC) to bind high levels of FL-HA (Figure 3.1A right panel). In Figure 3.1A, there is a small population of cells with background fluorescence that is higher than the negative control for cultured or stimulated PBMC. This peak corresponds to the autofluorescence of large, dense PBMC (primarily monocytes and granulocytes), a population that varies in number based on sample variation. If the subpopulation of dense cells is sufficiently large, this background fluorescence is also observed upon analysis of PBMC immediately following isolation. The large, dense PBMC were selectively gated during flow cytometry analysis (dot plot, Figure 3.1B) to determine the ability of this population to bind to FL-HA. TNF- $\alpha$  increased both the percentage of CD14<sup>+</sup> cells in this PBMC population and significantly up regulated CD44 expression (~4-fold, Figure 3.1B). Further, all of the induced FL-HA-binding shown in Figure 3.1A resided within this population of larger cells. Double staining showed that >85% of the cells that were positive for the monocyte marker, CD14 (Figure 3.1C, dot plot of CD14 expression) were responsible for the FL-HA-binding induced by TNF- $\alpha$  (Figure 3.1C, FL-HA-binding by CD14<sup>+</sup> PBMC). FL-HAbinding in the CD14<sup>+</sup> population was observed after 24 hr with TNF- $\alpha$  (data not shown) and reached a maximum after 72 hr when approximately 60% of the CD14<sup>+</sup> cells bound to FL-HA. Likewise, approximately 50 to 70% of CD14<sup>+</sup> cells were induced to bind to HA by LPS, IL-1β, or IFN-y (Figure 3.2 and summarized in Table 3.3). In all cases, the induced HA-binding was inhibited by an anti-CD44 mAb, Hermes-1 (Figure 3.2), demonstrating that CD44 is solely responsible for the induced binding to HA. To reiterate, HA-binding by CD14<sup>+</sup> PMBC is not induced by FCS over 72 hr, but CD44-mediated HA-binding is induced by pro-inflammatory stimuli, namely TNF- $\alpha$ , IL-1 $\beta$ , LPS, or IFN- $\gamma$ .

### 3.2.1ii Induction of HA-binding in PBM is independent of CD14<sup>-</sup> PBMC

It has been suggested that TNF- $\alpha$ -, IL-1 $\beta$ -, and LPS-induced HA-binding by peripheral blood monocytes requires monocyte secretion of TNF- $\alpha$  (204), meaning that the exogenous stimuli may not directly or completely activate monocytes. Figure 3.1 demonstrates that although all PBMC were exposed to the inflammatory stimuli, FL-HA-binding was induced only in the CD14<sup>+</sup> population. Experiments were conducted to address if CD14<sup>-</sup> PBMC respond to inflammatory stimuli to participate in monocyte activation. To address the influence of CD14<sup>-</sup> PBMC on the induction of HA-binding in monocytes, PBMC were stimulated as described in

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A. PBMC



FIGURE 3.1 FL-HA-binding by PBMC following incubation in the presence or absence of TNF- $\alpha$ . A Fluoresceinated-hyaluronan (FL-HA) binding, CD44 and CD14 expression on PBMC after 0 hr (Fresh) or 72 hr culture in RPMI-1640/10% FCS (cultured) in the absence or presence of TNF- $\alpha$  (+TNF- $\alpha$ ). B After 72 hr culture of PBMC in the presence (+TNF- $\alpha$ ) or absence (-) of TNF- $\alpha$ , cells within the enclosed area on the dot plot to the left (large, dense PBMC) were selected in flow cytometry for analysis of FL-HA-binding, CD14 and CD44 expression. The results are shown in the histograms to the right. C CD14<sup>+</sup> monocytes (shown in the upper Y-axis quadrant of the dot plot) were selected from the size gated population shown in B and analyzed for FL-HA-binding, shown to the right in the histogram. Fluorescence intensity (log scale) is represented on the x-axis and cell number on the y-axis. These data are representative of results obtained from experiments repeated more than three times.



FIGURE 3.2 CD44-mediated FL-HA-binding by CD14<sup>+</sup> PBMC in response to inflammatory stimuli. The panel on the left represents FL-HA-binding of unstimulated CD14<sup>+</sup> cells after 72 hr in culture. The middle panel shows FL-HA-binding in response to various stimuli (indicated on the left). The right panel is FL-HA-binding of stimulated CD14<sup>+</sup> cells in the presence of anti-CD44 mAb (+ anti-CD44 mAb). Fluorescence intensity (log scale) is represented on the x-axis and cell number on the y-axis. These data are representative of results obtained from three repeat experiments.

the previous section or CD14<sup>+</sup> PBMC (PBM) were enriched (~70 - 90%) by positive removal of CD14<sup>+</sup> PBMC, and then PBM were exposed to the inflammatory stimuli. The percent of CD14<sup>+</sup> PBMC that bind high levels of FL-HA following stimulation of PBMC or enriched PBM was calculated (Figure 3.3). Table 3.1 shows that equivalent percentages of CD14<sup>+</sup> PBMC were induced by TNF- $\alpha$  or IFN- $\gamma$  to bind to high levels of FL- HA regardless of whether the monocytes were enriched prior to stimulation, or cultured in the presence of physiological levels of CD14<sup>+</sup> PBMC. CD14<sup>-</sup> PBMC did not influence the induction of HA-binding in CD14<sup>+</sup> PBMC, and the inflammatory cytokines acted directly on PBM to induce HA-binding. This does not eliminate the possibility that the mode of action requires the secretion of monokines, in particular TNF- $\alpha$ , within the CD14<sup>+</sup> PBMC population.

# **3.2.1iii** TNF- $\alpha$ or IFN- $\gamma$ stimulate the expression of monocyte differentiation markers on PBM

Given that the induction of maximal HA-binding by PBM requires 72 hr culture *in vitro*, the maturation state of cultured monocytes may change over this time period in the presence or absence of stimulation, implying what impact, if any, the maturation status of monocytes has on HA-binding ability. Thus, if the differentiation of peripheral blood monocytes corresponds with the acquisition of HA-binding activity, then PBM capable of binding high levels of HA, following incubation with inflammatory agents, would express markers of differentiation. Thus, the expression of monocyte maturation markers was analyzed by flow cytometry. CD40, CD86 (B7.2), and MHC II expression were enhanced on TNF- $\alpha$ - or IFN- $\gamma$ -stimulated PBM (Figure 3.4). Neither marker expression nor FL-HA-binding was enhanced by 72 hr culture of PBM in the absence of stimuli. IFN- $\gamma$  is a potent activator of monocytes and macrophages, and IFN- $\gamma$  consistently induced HA-binding in a higher percentage of PBM than other stimuli used in this study (indicated in Table 3.3, page 70). This data shows that IFN- $\gamma$  also induced monocytes to express higher levels of CD40, CD86 (B7.2), and MHC II than TNF- $\alpha$ . These data show that inflammatory agents induce monocytes to express markers of differentiation that correlate with an acquired binding capacity for HA.



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FIGURE 3.3 FL-HA-binding by CD14<sup>+</sup> PBMC following stimulation of total PBMC or purified PBM. FL-HA binding of CD14<sup>+</sup> cells within the population of total PBMC (left panel) or purified PBM (right panel) that were stimulated with TNF- $\alpha$  for 72hr in RPMI-1640/10% FCS. Negative control is cells alone. Fluorescence intensity (log scale) is represented on the x-axis and cell number on the y-axis. These results are summarized in Table 3.1.

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TABLE 3.1 Effect of CD14 PBMC on TNF- $\alpha$ - or IFN- $\gamma$ -induced FL-HA binding in CD14<sup>+</sup> PBMC

	% stimulated CD14 <sup>+</sup> PBMC that bind to FL-HA				
	+ CD14 <sup>-</sup> PBMC <sup>a</sup>	n	- CD14 <sup>°</sup> PBMC <sup>b</sup>	n <sup>d</sup>	
TNF-α	63 <u>+</u> 15 °	34	62 <u>+</u> 20	14	
IFN-γ	73 <u>+</u> 23	23	72 <u>+</u> 20	7	

<sup>a</sup> stimulation of total PBMC <sup>b</sup> stimulation of purified PBM in the absence of CD14<sup>-</sup> PBMC <sup>c</sup> average number of CD14<sup>+</sup> cells that bind to FL-HA <u>+</u> standard deviation <sup>d</sup> number of experiments performed

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FIGURE 3.4 Expression of CD40, B7.2, and MHC II on PBM following culture in the presence or absence of TNF- $\alpha$  or IFN- $\gamma$ . Flow cytometry analysis of B7.2, MHC II and CD40 expression on CD14<sup>+</sup> PBMC immediately following isolation (fresh) or following 72 hr in culture (unstimulated) with TNF- $\alpha$  (+ TNF- $\alpha$ ) or IFN- $\gamma$  (+ IFN- $\gamma$ ). The negative control is cells alone. Fluorescence intensity (log scale) is represented on the x-axis and cell number on the y-axis. These data are representative of results obtained from three repeat experiments.

# **3.2.1iv** *HA*-binding is induced by TNF- $\alpha$ in murine bone marrow-derived macrophages (BMDM) but not in CD44<sup>-/-</sup> BMDM

Thymocytes, splenocytes, lymph node, and bone marrow cells were isolated from C57BL6, Balb/c, and DBA-2 mice, and then cultured in DMEM/10% FCS in the presence or absence of varying concentrations of murine recombinant TNF- $\alpha$ . As analyzed by flow cytometry at intervals over a 96 hr period, FL-HA-binding was not induced in any of the aforementioned populations (data not shown). To test the ability of inflammatory stimuli to stimulate HAbinding in mature myeloid cells, M-CSF was used to differentiate mouse bone marrow. A population of F4/80<sup>+</sup> bone marrow-derived-macrophages (BMDM) was generated which bound low levels of FL-HA. FL-HA-binding increased modestly with culture alone, but was significantly augmented by TNF- $\alpha$  within 24 hr, reaching a plateau by 72 - 96 hr (Figure 3.5 at 72 hr). Furthermore, FL-HA-binding was induced in CD45<sup>-/-</sup> BMDM but not in CD44<sup>-/-</sup> BMDM confirming that the induction of HA-binding is dependent on CD44 expression (Figure 3.5). There was a small but consistent increase in FL-HA-binding in the CD44-deficient cells after TNF- $\alpha$  stimulation. This is possibly due to the expression of other HA-receptors on the cell surface or non-specific binding. Unlike PBM, all BMDM bind to FL-HA, and FL-HAbinding capacity increases in all cells under normal culture conditions, reinforcing the notion that HA-binding may be induced more consistently in mature myeloid cells.

To summarize, inflammatory agent-induced HA-binding has been established in two cell systems, one in human monocytes, and the other in mouse macrophages. Other factors that alter inflammatory cytokine-stimulated HA-binding will be discussed in the remainder of this chapter.

# **3.2.3** The effect of modifications to the cell surface and cytoskeletal architecture of myeloid cells on HA-binding

### 3.2.2i Relationship between CD44 expression and HA-binding

3.2.2ia CD44 expression is enhanced on high HA-binding cells

Expression levels of CD44 have been shown to affect HA-binding in many cell lines (209). TNF- $\alpha$  increased the expression of CD44 in the human CD33<sup>+</sup> leukemic SR91 cell line and induced sulfation-dependent, CD44-mediated binding to soluble HA and an endothelial cell line (116). Data presented in the above section shows that CD44 expression is increased in human PBM also following stimulation with TNF- $\alpha$  (Figure 3.1).



FIGURE 3.5 FL-HA-binding by F4/80<sup>+</sup> BMDM following culture in the presence or absence of TNF- $\alpha$ . FL-HA-binding by BMDM (left panel), CD45<sup>-/-</sup> BMDM (middle panel) and CD44<sup>-/-</sup> BMDM (right panel), after 72 hr culture in DMEM/10% FCS in the absence (-) or presence of 20 ng/ml of TNF- $\alpha$  (+; as indicated on the left). Cells alone (negative) and secondary antibody (Ab control) are the negative controls for the staining. Fluorescence intensity (log scale) is represented on the x-axis and cell number on the y-axis. These data are representative of the results obtained from three experiments.

To determine if the level of cell surface CD44 is important for constitutive, CD44-mediated HA-binding by myeloid progenitors, the human  $CD34^+$  KG1a cell line was analyzed (276). KG1a cells constitutively but heterogeneously bind to HA (262). As indicated in Figure 3.6, approximately 50 - 70% of KG1a cells bind large quantities of FL-HA. This is similar to the percentage of PBM that bind HA following stimulation with inflammatory cytokines. HAbinding by KG1a cells was mediated by CD44 since two anti-CD44 antibodies, IM7.8.1 and Hermes-1, could each prevent FL-HA-binding (data not shown). To compare the characteristics of cells with high or low engagement of HA, the KG1a cell line was sorted into two populations based on FL-HA-binding capacity of the cells. After 2 - 3 rounds of sorting, a population of cells that bound high levels of HA (HA<sup>high</sup>) and a population of cells with low HA-binding (HA<sup>low</sup>) were obtained. FACS analysis revealed that approximately 90% of the HA<sup>high</sup> population bound high levels of HA, and approximately 80% of the HA<sup>low</sup> population bound low amounts of HA. Further, the HA<sup>high</sup> cells expressed  $2.2 \pm 0.9$  (n = 3) fold more CD44 per cell than HA<sup>low</sup> cells (Figure 3.6). The increase in expression of CD44 on high HAbinding KG1a cells is consistent with the TNF- $\alpha$ -induced expression of CD44 in PBM and BMDM (summarized in Table 3.2). Therefore, CD44 expression is increased on KG1a cells selected for high HA-binding and on TNF- $\alpha$ -stimulated PBM and BMDM. Together, these data emphasize the importance of CD44 surface density for high HA-binding capacity.

# **3.2.2ib** Cycloheximide inhibits the TNF- $\alpha$ -induced expression of CD44 and HA-binding in SR91 cells

Acquisition of ligand binding ability by cell adhesion molecules can occur quickly when receptors expressed on the cell surface are modified or new, pre-formed receptors are expressed (reviewed in 284). Neither change requires *de novo* protein synthesis, yet the change in ligand reactivity could be accompanied by an increase in cell surface density of the receptor. TNF- $\alpha$  can induce the expression of CD44 (207), but it is not known if this requires new protein synthesis. To address this question, SR91 cells were incubated in the presence or absence of both TNF- $\alpha$  and cycloheximide, a chemical inhibitor of eukaryotic protein synthesis. In the presence of a minimum of 0.1 ug/ml of cycloheximide, TNF- $\alpha$  neither enhanced the expression of CD44 nor induced HA-binding (Figure 3.7). An increase in CD44 expression that is detectable by FACS requires a minimum of 18 hours of stimulation (data not shown), thus,



Fluorescence intensity

#### FIGURE 3.6 Expression of CD44 on KG1a cells with high or low constitutive HA-binding.

Flow cytometry analysis of FL-HA-binding (right panel) and CD44 expression (middle panel) by unsorted KG1a cells (bottom row), and cells sorted for low (middle row, HA<sup>low</sup>) or high (top row, HA<sup>high</sup>) HA-binding. The negative control is cells alone. Fluorescence intensity (log scale) is represented on the x-axis and cell number on the y-axis. These results are representative of one experiment repeated more than seven times.

	Relative expression of CD44 <sup>a</sup>	nf
۴KGla	2.2 <u>+</u> 0.9 <sup>b</sup>	7
<sup>d</sup> CD14 <sup>+</sup> PBM	3.7 <u>+</u> 2.0	9
<sup>e</sup> BMDM	$1.3 \pm 0.2$	4

TABLE 3.2 Relative expression of CD44 on high and low HA-binding cells

<sup>a</sup> mean fluorescence intensity (MFI) of CD44 staining on high HA-binding cells divided by the MFI of CD44 staining on cells with low HA-binding determined by flow cytometry <sup>b</sup> average of mean fluorescence intensity (MFI)  $\pm$  standard deviation <sup>c</sup> CD44 expression on high compared to low HA-binding cells (1.0  $\pm$  0.0) KG1a <sup>d,e</sup> CD44 expression on TNF- $\alpha$ -stimulated PBM or BMDM normalized to CD44 expression on

unstimulated cells  $(1.0 \pm 0.0)$ 

<sup>f</sup> number of experiments

2



FIGURE 3.7 Cycloheximide blocks TNF- $\alpha$ -induced expression of CD44 and FL-HA-binding by SR91 cells. Flow cytometry analysis of CD44 expression and FL-HA-binding of SR91 cells incubated for 24 hr in the presence (+ TNF- $\alpha$ ) or absence (unstimulated) of TNF- $\alpha$ , or co-incubated with TNF- $\alpha$  and the protein synthesis inhibitor, cycloheximide (+ TNF- $\alpha$  + CHX). Fluorescence intensity (log scale) is represented on the x-axis and cell number on the y-axis. These data are representative of results obtained three repeat experiments.

*de novo* synthesis of CD44 is likely to occur. These results indicate that the induction of HAbinding and an increase in CD44 expression on SR91 cells in response to TNF- $\alpha$  requires *de novo* protein synthesis.

# **3.2.2ic** Alternatively spliced isoforms of CD44 are expressed on myeloid cells, but the expression does not correlate with HA-binding

It has been shown that CD44 isoform expression on PBM can change and correlate with HAbinding when cells are cultured with human serum (215). Figure 3.1 demonstrates that PBM cultured in the absence of human serum or stimulation do not enhance CD44 expression or activate CD44 function as an HA receptor. An increase in CD44 expression and binding to HA was observed on both PBM stimulated with TNF- $\alpha$  and on HA<sup>high</sup> KG1a cells, but it is not known if the expression of isoforms of CD44 correlate with high HA-binding in these cells. Cytoplasmic mRNA was isolated from low HA-binding, unstimulated PBM as well as from the high HA-binding, TNF- $\alpha$ -stimulated PBM. Reverse transcription and successive PCR with flanking and nested primers (see Chapter Two: Materials and Methods, section 2.4.6) was predicted to yield at least two transcripts (one from each primer set) that correspond to CD44H, the standard form lacking alternatively spliced exons. In addition to the standard form, additional transcripts were detected following RT-PCR on mRNA isolated from PBM cultured for 72 hr (Figure 3.8, lanes without stimulation at 0 and 72 hr). Unstimulated or TNF- $\alpha$ activated PBM, with low or high HA-binding capacity respectively, had identical CD44 isoform message (Figure 3.8, with stimulation at 72 hr). These data indicate that CD44 isoform expression is induced by the culture of monocytes and potentially correlates with cell maturation, but are not induced by TNF- $\alpha$ . RT-PCR on mRNA transcripts isolated from KG1a cells showed identical splicing of isoforms to that observed from mRNA that was isolated from cultured PBM (Figure 3.8). Further, the message from KG1a cells with either high or low HAbinding capacity also yielded the same banding pattern. This analysis revealed no major differences in CD44 isoform message expression in low or high HA-binding PBM or in KG1a cells. This data demonstrates that CD44 is alternatively spliced in KG1a cells and in PBM, but CD44 isoforms are neither induced by TNF- $\alpha$ , nor do the mRNA transcripts of specific isoforms correlate with HA-binding by monocytes.



FIGURE 3.8 Detection of CD44 splicing in PBM and KG1a cells by RT-PCR. Detection of CD44H and CD44v mRNA expressed in CD14<sup>+</sup> PBMC (left panel) immediately following isolation (left lane at 0 hr), after 72 hr culture (72 hr, no stimulation), and after 72 hr in the presence of TNF- $\alpha$  (72 hr, + stimulation). Detection of CD44H and CD44v mRNA constitutively expressed in KG1a cells (right panel, wt), in HA<sup>low</sup> KG1a cells (low), and in HA<sup>high</sup> KG1a cells (high). Loading control is  $\beta$ -actin and the negative controls (neg) are from the RT and PCR reactions without template. Base pair markers are indicated between the two panels. These data are representative of the results obtained in three repeat experiments.

# **3.2.2ii** Variation in cell surface GAGs, glycosylation, or sialylation differentially regulate binding to HA

Previous reports suggest that changes in sialylation or glycosylation on CD44 regulate HAbinding to PBM (217, 219). To determine the effect of these posttranslational modifications on FL-HA-binding ability, PBMC were negatively selected for CD14<sup>+</sup> PBM then incubated in the presence or absence of TNF- $\alpha$  and inhibitors of GAG assembly [1 mM xyloside (p-nitrophenyl β-D-xylopyranoside)] or O-linked glycosylation [1 mM BADGal (benzyl-2 acetamido 2-deoxy- $\alpha$ -D-galactopyranoside)] for 72 hr. Likewise, BMDM were cultured in the presence or absence of stimulation and inhibitors for 72 hr. Lack of an effective inhibitor of N-glycosylation prevented an analysis of the effect of cell surface N-glycosylation on binding to HA. Following the incubation period, cultured and TNF- $\alpha$ -stimulated PBM or BMDM were tranferred into RPMI-1640:PBS (1:1, pH 6.7) at 10<sup>7</sup> cells/ml and treated with 0.05 units/ml of neuraminidase for 1 hr at 37°C to remove terminal sialic acid residues. FL-HA-binding by PBM or BMDM was determined by flow cytometry as illustrated in Figure 3.9. The presence of an inhibitor of O-linked glycan addition had no significant effect on TNF-α-induced FL-HAbinding by BMDM or PBM. These data suggests that O-linked glycans do not affect CD44mediated HA-binding; however, the negative result is not conclusive since there is no information to support that the inhibitor is functioning efficiently. These data demonstrate that BMDM or PBM with or without TNF- $\alpha$  stimulation have enhanced binding to FL-HA following treatment with neuraminidase. Therefore, the removal of sialic acid residues promotes unstimulated and activated monocyte binding to HA. Like sialylation and glycosylation, the glycosaminoglycans (GAG) chondroitin sulfate and heparan sulfate decorate the surface of many cell receptors including CD44. Inhibition of GAG assembly also enhanced binding to FL-HA by cultured and stimulated BMDM or PBM. In summary, the effect of Nglycan addition on monocyte binding to HA is not known, and O-glycan addition is inconclusive. GAG addition or sialylation on PBM or BMDM have a negative effect on HAbinding, yet it is not known if the changes in GAG addition or sialylation are induced by TNF- $\alpha$  or if the changes occur on CD44.

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### 3.2.2iii An inhibitor of sulfation reduces HA-binding by myeloid cells

In the CD33<sup>+</sup> leukemic SR91 cell line, TNF- $\alpha$  induced sulfate incorporation on CD44 and HAbinding ability by SR91 cells, which promoted an interaction between the SR91 cells and an

#### A. Human PBM





FIGURE 3.9 FL-HA-binding by PBM or BMDM altered in GAG, O-glycan, or sialic acid content. Flow cytometry analysis of FL-HA-binding by A PBM or B BMDM after incubation for 72 hr in the presence (+ TNF- $\alpha$ ) or absence (-) of TNF- $\alpha$  and inhibitors of GAG addition (xyloside), O-glycans (BADGal), or following treatement with neuraminidase to remove sialic acid residues (N'ase). (+) on the left hand side indicates the presence of treatment with the indicted chemical or enzyme, thus, (+) indicates FL-HA-binding under conditions in which O-glycans, GAGs, or sialic acid residues are absent. Fluorescence intensity (log scale) is represented on the x-axis and cell number on the y-axis. These data are representative of results from three repeat experiments. endothelial cell monolayer (116). To determine if sulfation played a role in the regulation of HA-binding by primary monocytes induced by TNF- $\alpha$  or other inflammatory stimuli, PBMC were treated with stimuli for 72 hr in the presence or absence of 50 mM sodium chlorate, a compound that inhibits sulfation by blocking the transfer of sulfate to the universal sulfate donor (PAPS) in the cell (85). FL-HA-binding by CD14<sup>+</sup> PBMC was analyzed by flow cvtometry. Figure 3.10 demonstrates that TNF- $\alpha$ -, LPS-, IL-1 $\beta$ -, or IFN- $\gamma$ -induced FL-HAbinding by CD14<sup>+</sup> PBMC is significantly inhibited by the presence of sodium chlorate. On average, the induction of FL-HA-binding was inhibited in approximately 40% of stimulated and sodium chlorate-treated CD14<sup>+</sup> PBMC (Table 3.3). These data suggest that sulfate incorporation contributes to induced HA-binding in CD14<sup>+</sup> PMBC as was observed in the SR91 cell line. In similar experiments, TNF- $\alpha$ -induced FL-HA-binding by BMDM was significantly reduced by the presence of sodium chlorate  $(76 \pm 21\%)$  reduction in mean fluorescence intensity (MFI) of FL-HA on BMDM treated with sodium chlorate, n = 4, Figure 3.11). Neither PBM nor BMDM treated with sodium chlorate in the absence of stimulation acquired a capacity to bind to HA, thus, sulfation appears to be a TNF- $\alpha$ -inducible mechanism to regulate HAbinding by primary monocytes and macrophages as well as in the SR91 cell line. To determine whether sulfation played a role in regulating constitutive HA-binding in myeloid progenitors, KG1a cells were incubated for 72 hr in the presence or absence of sodium chlorate, then analyzed for FL-HA-binding by flow cytometry. The MFI of FL-HA-binding by KG1a cells treated with sodium chlorate was consistently reduced by approximately  $33 \pm 24\%$ , n = 4 (Figure 3. 11). These results had a substantial degree of variability to suggest that sulfation is not a consistently significant factor that contributes to HA-binding in this cell line. Contrary to this, sulfation was a significant factor induced by TNF- $\alpha$  to regulate HA-binding by human PBM and mouse BMDM. In all experiments using 50 mM sodium chlorate on cells, the inhibitor did not alter the expression of CD44 or affect FL-HA binding by unstimulated cells (data not shown).

### **3.2.2iv** Cell shape and HA-binding are sensitive to cytochalasin D treatment

**3.2.2iv,a** Membrane protrusions on KG1a cells are rich in F-actin and unstable in the presence of cytochalasin D that also abrogates HA-binding

KG1a cells, like primary human CD34<sup>+</sup> cells, project extensions or pseudopods (285 and Fig. 2A). To determine the cytoskeletal components responsible for maintenance of the pseudopod, KG1a cells were treated with cytoskeletal inhibitors as follows: cells were incubated



**FIGURE 3.10 FL-HA-binding by stimulated CD14<sup>+</sup> PBMC in the presence of the sulfation inhibitor, sodium chlorate.** The middle panel shows CD44-mediated FL-HA-binding of CD14<sup>+</sup> PBMC after 72 hr in response to various inflammatory stimulants (indicated on the left) and in the presence of the sulfation inhibitor, sodium chlorate (+ NaClO<sub>3</sub>, right panel) as determined by flow cytometry. The first panel represents FL-HA-binding of unstimulated CD14<sup>+</sup> PBMC. Fluorescence intensity (log scale) is represented on the x-axis and cell number on the y-axis. These results are summarized in Table 3.3.

Table 3.3 FL-HA-binding by CD14<sup>+</sup> PBMC induced by inflammatory mediators and reduced by sodium chlorate (NaClO<sub>3</sub>)

	% CD14 <sup>+</sup> PBMC that bind FL-HA		% loss in Fl	L-HA	
			binding + N	laClO <sub>3</sub>	
TNF-α	$63 \pm 15^{a,b}$	34 °	47 <u>+</u> 17	26	
LPS	69 <u>+</u> 18	7	42 <u>+</u> 7	4	
IL-1β	51 <u>+</u> 17	7	36 <u>+</u> 23	3	
IFN-γ	73 + 23	23	41 + 18	19	

<sup>a</sup> percentages are reported <u>+</u> the standard deviation.
<sup>b</sup> values were subtracted for background FL-HA-binding by unstimulated CD14<sup>+</sup> PBMC incubated in the presence or absence of sodium chlorate (NaClO<sub>3</sub>).
<sup>c</sup> number of times the experiment was performed.



FIGURE 3.11 FL-HA-binding of KG1a cells and TNF- $\alpha$ -stimulated BMDM following culture with sodium chlorate. FL-HA-binding of KG1a cells (left) and TNF- $\alpha$ -stimulated BMDM (right) in the presence of the sulfation inhibitor, sodium chlorate (+ NaClO<sub>3</sub>), as determined by flow cytometry. Unstimulated BMDM were treated with sodium chlorate that had no effect on low FL-HA-binding (data not shown). The negative controls are cells alone. Fluorescence intensity (log scale) is represented on the x-axis and cell number on the y-axis. These data are representative results from three repeat experiments.

for 1 hr with 0 - 50  $\mu$ M of cytochalasin D or 10 - 50  $\mu$ M of latrunculin A, both of which disrupt actin polymerization (286), or cells were incubated with the microtubule inhibitor, nocadazole, at 10 - 20 ng/ml or with 10 - 20 mM of the myosin ATPase inhibitor, 2.3-butanedione 2monoxime. The pseudopod was completely abolished in the presence of either 25  $\mu$ M cytochalasin D or 10 µM latrunculin A (Figure 3.12A), but the pseudopod was not profoundly affected by nocadazole or 2,3-butanedione 2-monoxime (data not shown). This suggests that the pseudopod is comprised of an F-actin scaffold, not a network of microtubules. Labeling KG1a cells with fluorescent-phalloidin confirmed that F-actin is concentrated in the pseudopod, and the localized distribution of F-actin is abolished when the cells are treated with cytochalasin D (Figure 3.12B). It was previously reported that CD44 is expressed in KG1a pseudopods (263) thus, CD44 may be linked to the actin cytoskeleton in this region of the cell. To determine if the actin cytoskeleton influences CD44-mediated FL-HA-binding, the cells were treated with cytochalasin D or latrunculin A for 1 hr. Either cytochalasin D or latrunculin A substantially diminished FL-HA-binding by KG1a cells, as demonstrated in Figure 3.12C with cytochalasin D. Thus, a filamentous actin network is essential for maintenance of the pseudopod structure in KG1a cells and constitutive FL-HA-binding.

## **3.2.2iv,** $\mbox{W} NF-\alpha$ induces cytoskeletal changes and cytochalasin D-sensitive HAbinding in SR91cells, PBM, and BMDM

It was observed that human SR91 cells, human CD14<sup>+</sup> PBM, and mouse BMDM experienced changes in cell morphology in response to TNF- $\alpha$  (Figure 3.13), suggesting that TNF- $\alpha$  can induce changes to the cell cytoskeleton. BMDM are normally polarized and adherent in culture but extend multiple projections in response to TNF- $\alpha$  (Figure 3.13). TNF- $\alpha$  induced PBM to adhere and spread in culture (Figure 3.13) whereas TNF- $\alpha$ -stimulated SR91 cells lose cell-cell clusters and extend small protrusions reminiscent of the KG1a pseudopods. Higher magnification of TNF- $\alpha$ -treated SR91 cells and primary monocytes indicates that these cells also extended spikes or filopodia (see inserts in Figure 3.13). Clearly, changes to the cytoskeleton in SR91 cells, BMDM, and PBM occur in response to TNF- $\alpha$  that correlate with the acquisition of HA-binding. To determine if de-stabilization of the actin cytoskeleton affects the HA-binding induced in SR91 cells, PBM, or BMDM as it did in KG1a cells that constitutively bind to HA, the cells were treated with 25  $\mu$ M cytochalasin D or 10  $\mu$ M latrunculin A for 1 hr. Figure 3.14 shows that the presence of cytochalasin D significantly







C + cyto. D FL-HA CD44 negative  $10^{1}$   $10^{2}$   $10^{3}$   $10^{1}$   $10^{2}$   $10^{3}$ Fluorescence intensity

FIGURE 3.12 HA-binding and F-actin rich KG1a pseudopods are abolished by cytochalasin D. A Phase contrast image of KG1a cells in suspension under normal growth conditions. The arrow indicates a pseudopod and the scale bar represents 10  $\mu$ m. B Distribution of F-actin after 1 hr in the presence (+ cyto. D) or absence of 50  $\mu$ M cytochalasin D. See materials and methods for details. Scale bar is 4  $\mu$ m. C Flow cytometric analysis of FL-HA-binding and CD44 expression by KG1a cells that were pre-treated for 1 hr in the presence (+ cyto. D) or absence (-) of 25  $\mu$ M cytochalasin D. The negative control is secondary antibody alone. Fluorescence intensity (log scale) is represented on the x-axis and cell number on the y-axis. A-C These results are representative of results from experiments repeated more than three times.

Α

В



FIGURE 3.13 TNF- $\alpha$  induces changes in SR91, PBM, and BMDM cell morphology. Phase contrast images of SR91 cells (left panel) after 24 hr incubation in the presence (+ TNF- $\alpha$ ) or absence (-) of 10 ng/ml of TNF- $\alpha$  (scale bar represents 4 µm). Insert (lower panel) shows a compiled fluorescent image of CD44 distribution on TNF- $\alpha$ -stimulated SR91 cells. Phase contrast images of PBM (middle panel) were taken after 72 hr incubation in the presence or absence of 20 ng/ml of TNF- $\alpha$  (scale bar represents 10 µm). Insert (lower panel) shows a slice of a fluorescent image of CD44 distribution on a single unattached PBM. Right panels are phase contrast images of BMDM after 96 hr incubation in the presence or absence of 20 ng/ml of TNF- $\alpha$  (scale bar represents 10 µm). These data are representative of the results of three experiments.



FIGURE 3.14 TNF- $\alpha$  induces cytochalasin D-sensitive HA-binding. FL-HA-binding of SR91 cells after stimulation with TNF- $\alpha$  (left panel) for 24 hr and incubation with 25  $\mu$ M cytochalasin D for the last hour (+ cyto. D). FL-HA-binding of CD14<sup>+</sup> PBM (middle panel) or BMDM (right panel) after 72 hr of stimulation with 20 ng/ml of TNF-  $\alpha$  and 50  $\mu$ M cytochalasin D for the last hour of the incubation. In each case, the negative control are cells alone. Fluorescence intensity (log scale) is represented on the x-axis and cell number on the y-axis. The results are summarized in Table 3.4.

reduced TNF- $\alpha$ -induced FL-HA-binding by SR91 cells and reduced FL-HA-binding in ~50% of TNF- $\alpha$ -stimulated PBM or BMDM. Cytochalasin D has no effect on FL-HA-binding by unstimulated myeloid cells. These data imply that changes to the F-actin cytoskeleton are induced by TNF- $\alpha$ , are reflected in the observed morphological changes, and facilitate HA-binding.

### 3.2.3 Primary factors associated with high HA-binding capacity in myeloid cells

**3.2.3i** HA-binding in myeloid cells positively correlates with enhanced cell surface expression of CD44, posttranslational sulfation, and an intact, filamentous actin network From the analysis of factors with the potential to affect HA-binding in myeloid cells, CD44 expression, sulfation, and changes in the F-actin cytoskeleton were induced by inflammatory stimuli, in particular by TNF- $\alpha$ , and had a positive correlation with HA-binding by PBM or BMDM. The impact of each factor varied in the different cell types. Cytochalasin D has the most significant impact on reducing FL-HA-binding by the cell lines, SR91 and KG1a, abolishing greater than 80% of the FL-HA-binding. In primary cells, neither cytochalasin D nor sodium chlorate had such a dominant effect on FL-HA-binding thus neither are solely responsible for the TNF- $\alpha$ -induced binding to HA. PBM and BMDM were cultured in the presence of the sulfation inhibitor (50 mM sodium chlorate) for 72 hr then subsequently treated for the final hr of the incubation period with 25  $\mu$ M cytochalasin D. In this experiment, FL-HA-binding was decreased an additional 20 - 30% in PBM or BMDM by the combination of inhibitors compared to either inhibitor alone. The effect of both inhibitors was additive and together, prevented the acquisition and maintenance of elevated binding to HA by the vast majority of cells (Figure 3.15). These data demonstrate that both sulfation and the actin cytoskeleton contribute to HA-binding in myeloid cells, the contribution of each factor varying between the different myeloid cells studied (Table 3.4), but clearly establishes two inducible mechanisms involving sulfation and the F-actin cytoskeleton as the major regulatory factors contributing to inflammatory agent-induced binding to HA by monocytes.



FIGURE 3.15 FL-HA-binding by TNF- $\alpha$ -stimulated PBM or BMDM in the presence of sodium chlorate and cytochalasin D. FL-HA-binding of CD14<sup>+</sup> PBM (left panel) or BMDM (right panel) after 72 hr stimulation with TNF- $\alpha$  in the presence (+) or absence (-) of 50  $\mu$ M cytochalasin D (cyto. D) or 50 mM sodium chlorate (NaClO<sub>3</sub>) or a combination of both inhibitors. See materials and methods for details. The negative control is cells alone. Fluorescence intensity (log scale) is represented on the x-axis and cell number on the y-axis. Theses results are summarized in Table 3.4.

	+ NaClO <sub>3</sub> <sup>a</sup>	n	+ cyto D <sup>b</sup>	n	+ cyto D °	n
_					$+ NaClO_3$	
KG1a <sup>g</sup>	33 <u>+</u> 24	4	89 <u>+</u> 3	3	nd <sup>e</sup>	•
SR91 <sup>h</sup>	$nd^d$		80 <u>+</u> 2	3	$nd^{f}$	
PBM <sup>i</sup>	57 <u>+</u> 18	8	45 <u>+</u> 18	6	81 <u>+</u> 8	3
BMDM <sup>j</sup>	76 <u>+</u> 21	4	61 <u>+</u> 26	6	85 <u>+</u> 15	5

**TABLE 3.4** Percent loss of FL-HA-binding by KG1a cells or TNF-α-stimulated PBM, SR91 cells, or BMDM in the presence of sodium chlorate and/or cytochalasin D

<sup>a</sup> relative loss in FL-HA binding by cells incubated in the presence of 50 mM sodium chlorate over the incubation period

 $^{\rm b}$  relative loss in FL-HA binding by cells incubated in the presence of 25  $\mu M$  cytochalasin D for the last hour of the incubation period

<sup>c</sup> relative loss in FL-HA binding by cells incubated in the presence of sodium chlorate and cytochalasin D

<sup>d</sup> no data; percent loss in TNF- $\alpha$ -induced HA binding by SR91 cells treated with sodium chlorate according to Maiti *et al* 1998.

<sup>e,f</sup> no data; combinatorial inhibition was not performed due to the decisive reduction in FL-HA binding by cytochalasin D

 $^{g,h,j}$  relative loss in FL-HA binding determined by the percent reduction in mean fluorescence intensity (MFI)  $\pm$  standard deviation of FL-HA

<sup>i</sup> relative loss in the percentage of PBM  $\pm$  standard deviation that bound to FL-HA following 72 hr culture with TNF- $\alpha$ 

#### **3.3 DISCUSSION**

### 3.3.1 Inflammatory cytokines induce HA-binding by myeloid cells

Like other cell adhesion molecules, ligand binding by CD44 is tightly regulated. It has been documented that newly explanted human peripheral blood monocytes (PBM) do not bind significant levels of HA. However, PBM cultured *in vitro* in the presence of autologous human serum acquire the ability to bind HA (205, 215) which was further enhanced by the inflammatory cytokines TNF- $\alpha$ , IL-1, and LPS (204). In contrast, data presented here demonstrates that HA-binding is not induced in PBM by culture in the presence of fetal calf serum, implying that factors specific to human serum promote HA-binding in monocytes. For these studies, PBM were cultured in the absence of human serum to prevent CD44 activation prior to the addition of exogenous stimuli. Under these conditions, HA-binding by PBM was augmented by the presence of the inflammatory agents TNF- $\alpha$ , IFN- $\gamma$ , IL-1 $\beta$ , or LPS. MIP-1 $\alpha$  and MCP-1 are chemokines present at sites of inflammation and are potent inducers of monocyte and macrophage chemotaxis. Neither MIP-1 $\alpha$  nor MCP-1 induced HA-binding, nor did the chemokines augment cytokine-induced HA-binding in PBM (data not shown), emphasizing that inflammatory cytokines, not chemokines, induce HA-binding in monocytes.

Induction of CD44-mediated HA-binding by cytokines concurs with previous reports (204-206) that also suggested that TNF- $\alpha$  is secreted following stimulation of PBMC with LPS, IL-1, IL-3, GM-CSF, or IFN- $\gamma$ , and that TNF- $\alpha$  is primarily responsible for inducing HA-binding (204). Data presented here suggests that TNF- $\alpha$  is not solely responsible for the induction of HA-binding since IFN- $\gamma$ , not TNF- $\alpha$ , induced binding to HA by the greatest percentage of PBM. The difference cannot be accounted for by TNF- $\alpha$  alone. Further, CD14 PBMC are not required for TNF- $\alpha$ -, LPS-, IL-1 $\beta$ -, or IFN- $\gamma$ -induced HA-binding, thus, monocytes independently regulate binding to HA. These data do not dismiss the fact that TNF- $\alpha$  is a critical cytokine, but do clarify two points. First, exogenous stimuli can act directly on monocytes in the absence of serum factors or CD14 PBMC to stimulate HA-binding. Second, additional signals, potentially resulting in secretion of TNF- $\alpha$ , are generated within the monocyte population.

The induction of HA-binding in PBMC by IFN- $\gamma$  has been observed by others (206), but contrasts with one study in PBMC (204) and another in the leukemic cell line, SR91 (116). In

addition to the induction of HA-binding, IFN- $\gamma$  or TNF- $\alpha$  enhance the expression of CD40, CD86 (B7.2), and MHC II, markers of differentiation. Notably, IFN-y-stimulated cells express each marker to a greater degree than that expressed on cells stimulated with TNF- $\alpha$ . This correlates with the ability of IFN-y to induce HA-binding in a higher percentage of PBM. These markers are expressed to a lesser, but equal extent on PBM both prior to and following *in vitro* culture, correlating with the inability of these populations to bind significant levels of HA. The markers are not induced by culture in medium containing 10% fetal calf serum. These results show that HA-binding coincides with monocyte maturation and suggest that factors in human serum may promote the differentiation of monocytes to macrophages and induce the observed HA-binding in some PBM. It follows that monocytes cultured in human serum would express markers of monocyte differentiation as the population progressively acquires HAbinding, but this was not tested in this investigation. CD40, CD86 (B7.2), and MHC II are associated with antigen presentation and with co-stimulation of an immune response, in addition to activating the phagocytic and anti-bacterial function of macrophages (287). CD44 activation has also been reported to coincide with macrophage activation (288). Taken together, these observations imply that the CD44-HA interaction is functionally significant in activated monocytes and macrophages under inflammatory conditions.

HA-binding was not induced by inflammatory stimuli in total or F4/80<sup>+</sup> murine cells harvested from the spleen, thymus, lymph nodes, or bone marrow (data not shown) but HA-binding was observed in TNF- $\alpha$ -stimulated F4/80<sup>+</sup> macrophages derived from the bone marrow of wild type, but not CD44-deficient mice. As mentioned in section 3.2.1iv, TNF- $\alpha$ -simulated CD44<sup>-/-</sup> BMDM emit fluorescence slightly above background when incubated with FL-HA. It has been suggested that toll-like receptors (TLRs) can bind to small molecular mass forms of HA (P. Noble and P. Johnson, personal communication). The expression of receptors for HA other than CD44 can be determined by flow cytometry analysis. Similar to the human system, chemokines MCP-1 and MIP-1 $\alpha$  did not induce HA-binding or augment HA-binding induced by cytokines (data not shown). Therefore, CD44 activation in macrophages, like in PBM, occurs in response to inflammatory cytokines, not chemokines. HA-binding was induced to an equal extent in C57BL6 or Balb/c BMDM that express CD44.2 or CD44.1 alleles respectively, demonstrating that the allelic differences in CD44 are irrelevant with respect to TNF- $\alpha$ -induced HA-binding (data not shown). CD44 expression was modestly enhanced (1.5x, Table 3.2) in BMDM from both mouse strains following TNF- $\alpha$  stimulation (data not shown), an observation consistent with the increase in CD44 expression on stimulated human monocytes and HA<sup>high</sup> KG1a progenitor cells.

#### 3.3.2 HA-binding requires a threshold level of CD44

In general, HA-binding requires a threshold level of CD44 on the cell surface (reviewed in 118, 148, 289) above which, further increases in CD44 expression contribute to HA-binding. thereby implying an avidity effect. CD44-mediated HA-binding was induced in a significant population of stimulated CD14<sup>+</sup> PBMC (on average, in 50 - 70% of cells) but not in all CD14<sup>+</sup> cells. Heterogeneous binding to HA is also seen in the KG1a cell line. The majority of cells bind either high or low, but not intermediate, levels of HA. A threshold level of 'activated' CD44 required for HA-binding may explain why two discrete populations of CD14<sup>+</sup> cells are observed, those that bind to HA and those that do not. This is consistent over the time course of the stimulation where an increase in the number of FL-HA-binding cells, not an increase in FL-HA-binding per cell was observed (data not shown). This type of HA-binding profile suggests that a threshold level of activated CD44 must be present on the cell surface to engage considerable amounts of HA. One way to surmount the activation threshold may be through the enhanced expression of CD44 that, on average, increased four fold on PBM following 72 hr stimulation with TNF- $\alpha$ , and was elevated, albeit to a lesser extent, on TNF- $\alpha$  stimulated BMDM and HA<sup>high</sup> KG1a cells. The induction of HA-binding and enhanced expression of CD44 required *de novo* protein synthesis. The flow cytometry profile shows that upregulation of CD44 surface expression reached a plateau by 48 hr in all cells (data not shown), yet all cells did not engage high levels of HA. Instead, the number of cells overcoming the threshold for HA-binding steadily increased over the entire 72 hr time period, implying that factors in addition to CD44 expression must be required to surpass the threshold and achieve high HAbinding capacity.

*In vitro* culture of PBM in human serum, under adherent conditions, induced the expression of alternatively spliced forms of CD44 (205, 206, 215) including the CD44 isoform containing variant exon 3 (v3) which is modified by heparan sulfate (218). CD44 isoform expression and HA-binding was also observed in infiltrating monocytes isolated from cutaneous inflammatory sites and in alveolar macrophages (206, 215). These data imply that the activation of monocytes or macrophages correlates with HA-binding and the alternative splicing of CD44

isoforms. In addition to the standard form of CD44 (CD44H), KG1a cells express variant isoforms CD44R1, CD44R2, and CD44RC (290, 291). In addition to the standard form of CD44, three alternative CD44 mRNA transcripts were detected by RT-PCR on mRNA isolated from KG1a cells and cultured PBM. An increase in CD44 isoform and standard CD44H message was observed in PBM after 72 hr in culture as previously reported (215). Note that CD44H and CD44v message was neither altered in PBM following stimulation with TNF- $\alpha$  nor was it different between high and low HA-binding KG1a cells (Figure 3.8). Therefore, CD44 isoforms are expressed on monocytes regardless of HA-binding potential. Alternative splicing is supported at the protein level as several bands of varying molecular mass are detected by western blots on CD44 immunoprecipitated from KG1a and PBM cell lysates. RT-PCR was not performed on murine macrophages as higher or lower molecular mass bands were not detected by western blots for CD44 from these cell lysates.

The data presented here support previous reports stating that monocytes express alternative isoforms of CD44. The possibility that CD44 isoforms participate in HA-binding cannot be excluded, yet it can be concluded that the induction of CD44v isoforms does not occur in response to TNF- $\alpha$  and contrary to the prediction, isoform expression is alone insufficient to confer HA-binding ability to monocytes.

#### 3.3.3 Posttranslational modifications in monocytes influence HA-binding

Although activated monocytes can upregulate and alternatively splice variant forms of CD44, other changes must occur upon monocyte activation to induce HA-binding. CD44 is heavily glycosylated, accounting for an approximate 50 kDa increase in actual molecular mass of CD44H and more than an 150 kDa increase in molecular mass of certain alternatively spliced isoforms compared to the mass predicted from the amino acid sequence of CD44H. Extensive posttranslational modifications can alter the conformation, charge, and location of cell surface molecules, thereby regulating the biological function of the molecule. Several reports implicate glycosylation in regulating HA-binding, yet once again the regulation differs with cell type, maturation state, and activation status.

With respect to monocytes, LPS-induced HA-binding and increased sialidase activity in a human monocyte cell line (THP-1) and incubation of LPS-stimulated monocytes with a sialidase inhibitor reduced HA-binding (217). HA-binding induced by the activation of PBM

with human serum or TNF- $\alpha$  has been shown to involve protein synthesis and possible glycosylation changes that can be inhibited by IL-4 (219). Activation of CD44 in the SR91 cell line induced sulfate incorporation on CD44 which was required for HA-binding and adhesion to an endothelial cell monolayer (292). Differences in N-linked glycosylation have been observed for CD44 between resident and elicited mouse macrophages (220) and after TNF- $\alpha$  stimulation of human PBM (219). These data indicate that cellular activation results in glycosylation changes to CD44, as well as alterations in sulfation (230, 293).

In this study, inhibition of GAGs or enzymatic removal of sialic acid residues enhanced HAbinding by unstimulated PBM, suggesting a negative regulatory role for cell surface glycosaminoglycans and sialic acid residues in binding HA. The inhibitors also enhanced TNF- $\alpha$ -stimulated HA-binding, but had little effect on the very high levels of HA-binding induced by IFN- $\gamma$  (data not shown). These data implies that if GAGs and sialylation are negative regulators of HA-binding, not all stimuli can eliminate their effect to completely activate HA-binding. Since HA-binding is mediated solely by CD44, it infers that the modifications controlling HA-binding capacity are specific to CD44 on the cell surface. However, these results do not exclude the impact of other GAG-modified and/or sialylated cell surface molecules on HA-binding. Broad range inhibitors, like those used in this work, can significantly alter the distribution, conformation, and net charge of individual molecules and the architecture of the entire cell surface that may indirectly impact HA-binding. Although results should be interpreted with caution, these data do support the notion that GAG addition and sialylation have a negative impact on HA-binding by monocytes.

It has been suggested that a reduction in the total negative charge on the cell surface by removal of sialic acid residues and GAGs could dampen charge repulsion and allow greater binding to HA which also carries a large negative charge. This type of mechanism is speculative and directly contradicts data reported in the SR91 cell line, where TNF- $\alpha$ -induced HA-binding correlated with the incorporation of negatively charged sulfate on to CD44. Data in this report show that cytokine-induced HA-binding in primary monocytes was also dependent on sulfation as TNF- $\alpha$ -, IL-1 $\beta$ -, LPS-, or IFN- $\gamma$ -induced HA-binding was significantly inhibited by sodium chlorate, a potent inhibitor of sulfation. Sulfation appears to play a role in TNF- $\alpha$ -induced HA-binding by PBM, BMDM, and to a lesser extent, in constitutive HA-binding by the myeloid progenitor KG1a cell line. These results imply that sodium chlorate reduces HA-binding by inhibiting sulfate incorporation to CD44. This conclusion relies on the fact that HA-binding is mediated by CD44 and that sodium chlorate is a specific inhibitor of sulfation, preventing the transfer of sulfate to APS (85). Treatment of cells with up to 50 mM sodium chlorate did not affect cell viability or CD44 expression suggesting that the cells can survive with reduced sulfate incorporation, and that 50 mM sodium chlorate is not toxic to the cells. Treatment with sodium chlorate did not inhibit the induction of HA-binding in all cells (50 mM sodium chlorate abolished HA-binding in approximately 40% of the HA-binding population). Since threshold levels of CD44 are required for HA-binding, it is inviting to propose that a threshold level of sulfation is also required. Perhaps some CD14<sup>+</sup> cells do not reach this threshold and cannot bind elevated levels of HA. Likewise, in cells that surmount the threshold necessary for HA-binding, sodium chlorate would have to suppress sulfation to levels below the threshold value in order to prevent HA-binding. This is supported by the observation that a titration of sodium chlorate does not yield a significant intermediate HA-binding population, just a decrease in the high HA-binding population and a corresponding increase in the low HA-binding population (see data in chapter four). In the presence of complete media, treatment of cells with 50 mM sodium chlorate reduced the incorporation of  $[^{35}S]$  sulfate by ~70%, but did not abolish it. These results will be discussed further in chapter four.

In contrast to previous reports, CD44 isoform expression did not correlate with TNF- $\alpha$ -induced HA-binding by PBM or constitutive HA-binding by KG1a cells. Further, GAG addition and cell surface sialylation had a negative influence on FL-HA-binding by unstimulated and TNF- $\alpha$ -stimulated PBM and BMDM. At present, there is no evidence to suggest that TNF- $\alpha$  induces alterations in GAG addition or the presence of terminal sialic acid residues, or sialylated cell surface proteins. The data presented here suggest that posttranslational sulfation correlates with inflammatory cytokine-induced HA-binding in PBM and BMDM.

### 3.3.4 HA-binding requires an intact actin cytoskeleton

Since sodium chlorate could not completely inhibit the induction of HA-binding in TNF- $\alpha$ stimulated PBM or BMDM, other as yet unidentified factors must have contributed to the induction of HA-binding in monocytes. Despite heterogeneity in morphology and binding to HA, the majority of KG1a cells spontaneously project a pseudopod. Pseudopods have been detected on primary human CD34<sup>+</sup> cells (285), and CD44 has previously been reported to reside in pseudopods (263). Generation of membrane protrusions requires rearrangement of the cytoskeleton. A precedent for such rearrangement to correlate with HA-binding has been established in human PBM as LPS activated PBM to bind to HA (204), and LPS has been shown to induce actin reorganization (294). TNF- $\alpha$  activated PBM to bind to HA (204, 293) and like LPS, TNF- $\alpha$  also induced changes in cell morphology in human PBM (Figure 3.13). Resident and elicited macrophages have been shown to differ in their detergent solubility of CD44, suggesting that macrophage activation results in changes in association with the cytoskeleton (220).

Data presented in this chapter illustrated TNF- $\alpha$  induced cytoskeletal changes in SR91 cells, PBM, and BMDM that correlated with the induction of HA-binding. F-actin destabilizing agents significantly disrupted TNF- $\alpha$ -induced or constitutive FL-HA-binding. Cytochalasin D destroyed the pseudopod in KG1a cells and HA-binding, both of which were reversible following overnight culture (data not shown). The inhibitors cytochalasin D or latrunculin A decreased HA-binding in a concentration and time dependent manner (data not shown). The reduction in HA-binding due to disruption of actin polymerization by cytochalasin D or latrunculin A varied between cell lines and PBM. This cautions extrapolation of results between cell lines and primary cells. In primary PBM and BMDM, approximately 15% of TNF- $\alpha$ -induced HA-binding was not regulated by sulfation or the F-actin cytoskeleton. Obviously there are minor mechanisms responsible for HA-binding likely to involve one or a combination of factors tested in this study, for example, sialylation or GAG addition.

This data illustrates that sulfation and an intact F-actin cytoskeleton are TNF- $\alpha$ -inducible affects required for acquisition of HA-binding by activated human monoyctes, mouse macrophages, and constitutive HA-binding by a human myeloid progenitor cell line. These two mechanisms are investigated at the molecular level in subsequent experiments, the results of which are presented in the following chapters.

**CHAPTER FOUR** 

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## Regulation of HA-binding through the sulfation of CD44

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### 4.1 INTRODUCTION AND RATIONALE

Carbohydrates impart a large degree of structural diversity to glycoproteins and glycolipids by the inherent branched structures and stereospecific linkages in sugars. Covalent posttranslational modifications such as sulfation, acetylation, and phosphorylation impart further structural variety to molecules. Of these, sulfation is a prevalent posttranslational modification particularly on carbohydrates.

Sulfated cell surface glycoproteins and proteoglycans have been implicated in a variety of cell functions including cell adhesion, the presentation of growth factors, cell signaling and embryonic development (reviewed in 295). Animal cells take sulfate in from the extracellular fluid through carrier-mediated transport (296). Carbohydrate sulfate esters are synthesized along the secretory pathway and then are found exclusively in the extracellular milieu (review by 296). The charge density of polysaccharides is determined by the content and the precise arrangement of sulfate groups that can alter the chemical and physical properties of individual cell surface molecules and the entire glycocalyx. In this manner, the addition of sulfate can create specialized niches or epitopes to regulate cellular interactions and sense the extracellular environment.

The enzymatic placement of sulfate esters onto carbohydrate and protein has been recognized as a mechanism for mediating highly specific molecular-recognition events in a range of cells from bacteria to leukocytes (88). Sulfated moieties have been actively investigated for their role in the immune system as key modulators of leukocyte-endothelial cell interactions (69, 90). The sulfation of L- and P-selectin ligands is strictly required for functional L- and P-selectin binding, therefore, sulfation during glycan biosynthesis must create a critical recognition motif (297-299). The P-selectin-PSGL-1 interaction required for leukocyte rolling on activated endothelium is dependent on the sulfation of tyrosine residues on PSGL-1 (93, 300, 301). The endothelial-derived carbohydrate ligands for L-selectin that mediate lymphocyte adherence in lymph nodes bear GlcNAc-6-sulfate within sialyl Lewis x-capped structures attached to O-glycans (49, 98-100). The mucin-like glycoprotein, GlyCAM-1, was identified as an L-selectin ligand expressed by HEV (50) whose recognition component lies within a 6-sulfo GlcNAc moiety within the core of a sulfated sialyl Lewis x structure (98). To generate the sulfated L-selectin ligands, GlcNAc-6-o-sulfotransferase must be activated. This

sulfotransferase was identified in porcine peripheral lymph nodes with restricted expression to endothelial cells within the lymphoid tissue (302), and later it was identified in humans and mice with the same distribution pattern and substrate specificity (303).

The involvement of sulfation in selectin-independent cell adhesion by immune cells was reported in the regulation of CD44-HA binding. The pro-inflammatory cytokine, TNF- $\alpha$ , induced sulfate incorporation into CD44 in a human CD33<sup>+</sup> leukemic cell line that correlated with induced HA-binding and adhesion to an endothelial cell monolayer (116). Data presented in chapter three established that an inhibitor of sulfation significantly dampened inflammatory cytokine-induced HA-binding by primary monocytes. Given the importance of sulfation for mediating the interaction between selectins and their ligands as well as the evidence that sulfation is required for CD44-mediated HA-binding in a human leukemic cell line and PBM, this chapter explores the manner by which sulfation regulates HA-binding by monocytes in response to inflammatory agents.

The relationship between cellular sulfation and HA-binding was addressed in leukocytes, and specifically in myeloid cells in response to stimulation with inflammatory agents. FL-HA-binding by PBM, BMDM, and KG1a cells was measured at a cellular level by flow cytometry analysis. Flow cytometry was also used to evaluate the expression of novel, sulfated Lewis x, or sulfated LacNAc epitopes on PBM and the effect of inflammatory stimuli on their expression pattern. CD44-HA binding was analyzed on a molecular level by far western blot analysis on immunoprecipitated CD44. HA-binding was analyzed under conditions in which the incorporation of sulfate in CD44 was suppressed, typically by incubation of cells with sodium chlorate. Alternatively, the sulfation status of CD44 in sulfurlyase kinase-deficient (SK2<sup>-/-</sup>, also known as PAPS synthase) BMDM was investigated to determine if cells derived from the bone marrow of this mouse are naturally defective in sulfate incorporation thereby eliminating the need to use a chemical inhibitor of sulfation.

After a correlation between HA-binding and the sulfation of CD44 was reached, the next objective was to determine if the content and distribution of sulfate on CD44 was altered following stimulation with inflammatory agents such as TNF- $\alpha$ . Sulfate incorporation by CD44 was detected by autoradiography after immunoprecipitated of CD44 from [<sup>35</sup>S]-sulfate radiolabeled cells. One report indicated that labeling in cysteine-methionine-free, low sulfate

medium increased the resultant radioactive material incorporated into molecules without affecting protein synthesis or the proportion of the sulfate label that in this case was measured by PNGaseF digestion (304). Another group found that the concentration of exogenous sulfate can alter both the composition and the degree of sulfation of some macromolecules (305, 306). To guard against qualitative alterations in the distribution of sulfated moieties, radiolabeling of cells was conducted in complete medium. According to flow cytometry results, the significant increase in CD44 expression was maximal by 48 hr, yet from 48 - 72 hr there was a significant increase in the number of PBM or BMDM with high HA-binding. Cells were radiolabeled for the initial 2 hr of incubation or for the final 2 to 24 hr of the culture time (typically 72 hr) to capture the sulfate distribution on CD44 when cellular HA-binding is lowest and highest. A change in the molecular mass of CD44 immunoprecipitated from cells inhibited for GAG or Oglycan addition, or following digestion of N-glycans or sulfate from tyrosine residues indicates that CD44 was modified by the particular structures. Under the same conditions, a corresponding loss of sulfate on CD44 suggests that the structure was sulfated. The contribution of sulfate associated with each modification was calculated as the relative difference in sulfate incorporation per CD44H in the presence or absence of inhibitors of GAG or O-glycan addition or enzymatic digestion of N-glycans or sulfate from tyrosine.

Data presented in this chapter describe the relationship of cellular sulfation and sulfated epitopes with HA-binding by myeloid cells. The sulfation status of CD44 is described in terms of the quantity and locale of sulfate incorporation into CD44 in myeloid cells in the presence or absence of inflammatory stimuli. These results form the foundation for a model, presented at the end of the chapter, by which the sulfation of CD44 may regulate HA-binding by monocytes.

### 4.2 RESULTS

**4.2.1 Transient binding to HA by PHA-stimulated CD3<sup>+</sup> T cells is not dependent on sulfation** In the previous chapter, the data showed that a sulfation inhibitor reduced inflammatory agentinduced, CD44-mediated HA-binding in monocytes and macrophages. Inflammatory cytokines induced HA-binding in CD14<sup>+</sup> monocytes in a sulfate-dependent manner (Figure 3.10) but did not induce HA-binding in other PBMC.

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The CD44-HA interaction has been reported in vitro to mediate T cell rolling on immobilized HA (101, 224). Sulfation is required for selectin-mediated lymphocyte homing and in vivo extravasation of leukocytes, setting a precedent for sulfation-mediated adhesive events in activated, non-monocytic cells. To determine if sulfation is a general requirement for induced HA-binding by immune cells, a series of murine T cell lines and macrophage cell lines were treated with or without sodium chlorate then assessed for HA-binding ability by flow cytometry analysis. All of the cell lines tested were highly positive for CD44 expression and bound FL-HA either constitutively, after stimulation with PMA, or after crosslinking with an anti-CD44 Ab, IRAWB14.4 (115). The results are summarized in Table 4.1. Like primary BMDM, HAbinding by one macrophage cell line was reduced by sodium chlorate however the HA-binding induced by an anti-CD44 Ab antibody in two other macrophage cell lines was not affected by sodium chlorate. The latter finding seems to contradict previous results. Recall that cytoskeletal rearrangement and sulfation are independent regulatory mechanisms for HAbinding, and that sodium chlorate only reduced HA-binding that resulted from the stimulation of PBM and BMDM. The use of anti-CD44 antibodies may artificially enhance avidity for HA by aggregating CD44 molecules on the cell surface. The resultant increase in HA-binding may solely reflect the cytoskeletal contribution to HA-binding in the absence of induced sulfation. This would explain the different effects of sodium chlorate on HA-binding by macrophages, and supports the hypothesis that sulfation events are a stimuli-responsive mechanism to promote HA-binding. The discrepancy may also be compounded by differences between cell lines and primary cells, as well as the source and maturation state of the macrophages. By the same arguments, the reduced HA-binding in one of three murine T lymphocyte cell lines treated with sodium chlorate may not be an accurate depiction of the potential regulation of HA-binding by sulfation in primary cells. Thus, the effect of sodium chlorate on the induction of HA-binding in activated, primary human T cells was tested. PBMC were activated with phytohemagglutinin (PHA), and HA-binding by CD3<sup>+</sup> T lymphocytes was monitored over time by flow cytometry. Maximum FL-HA-binding was observed in approximately 20% of CD3<sup>+</sup> cells after 18 - 24 hr in culture with PHA. The FL-HA-binding was transient and significantly reduced by 48 hr (Figure 4.1). Co-incubation of PBMC with PHA and 50 mM sodium chlorate for 24 hr had no effect on the induction of HA-binding (Figure 4.1) inferring that sulfation does not regulate HA-binding in PHA-activated human T cells.

Hest	Cell Type	Cell Line	Source	<sup>c</sup> HA <sup>d</sup> mAb-		eInduced	fReduced
Πυδί			binding		induced	Induced	by NaClO <sub>3</sub>
Mouse	T lymphocyte	<sup>a</sup> BW5147	Donated by		<sup>j</sup> nd	nd	
		CD45 <sup>-</sup>	B. Mallisen	+			-
		<sup>b</sup> BW5147	Donated by		nd	nd	
		$CD45^+$	B. Mallisen	+			-
		EL-4	Donated by		+	+	g .
			H. Ziltener	-			в <del>.</del> †
		T28	Donated by		+	+	-
			F. Takei	+			
	Macrophage	P815	ATCC TIB 64 _ +		+	nd	-
		J774A.1	Donated by		+	nd	-
			B. Finlay	-			
		RAW 264	Donated by	1			<sup>h</sup> +
			R. Hancock	+	+	nd	
		Primary	C57BL mouse		nd	<sup>i</sup> TNF-α	+
		BMDM	bone marrow	na			
Human	Myeloid	KGla	ATCC CCL 246.1	+	nd	nd	+
		SR91	Donated by		nd	<sup>i</sup> TNF-α	4
			H. Klingemann				Τ
		PBM	By donation	nd	nd	'TNF-α	÷
	T lymphocyte	PB T cell	By donation	nd	nd	<sup>k</sup> PHA	-

Table 4.1 Effect of sodium chlorate on HA-binding by human and mouse cells

<sup>a,b</sup> BW5147 T cell line (a) defective in CD45 expression (b, as described in 307, 308)

<sup>c</sup> + indicates constitutive FL-HA-binding, or not (-) as measured by flow cytometry; + does not provide information regarding the relative fluorescence intensity

<sup>d</sup> + indicates that FL-HA-binding was induced by an anti-CD44 mAb, IRAWB14.4 (115)

<sup>e</sup> FL-HA-binding was induced with 20 ng/ml of PMA unless otherwise stated

<sup>f</sup> + indicates decreased FL-HA-binding after minimum of 24 hr culture with 50 mM sodium chlorate (NaClO<sub>3</sub>)

<sup>g</sup> PMA-induced FL-HA-binding was reduced by sodium chlorate

<sup>h</sup> constitutive FL-HA-binding was reduced by sodium chlorate

<sup>i,k</sup> FL-HA-binding was induced after 24 hr with 20 ng/ml of murine / human recombinant

TNF- $\alpha$  (i) or 5 µg/ml of human PHA (k) (not PMA)

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<sup>j</sup> not determined

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FL-HA binding

FIGURE 4.1 FL-HA-binding by PHA-stimulated CD3<sup>+</sup> PBMC in the presence or absence of sodium chlorate. CD3<sup>+</sup> PBMC transiently bind to FL-HA following stimulation with 5  $\mu$ g/ml of PHA (left panel). FL-HA-binding peaks by 24 hr (time indicated on the left) but reverts by 48 hr. The right panel shows FL-HA-binding of PBMC cultured with PHA in the presence or absence of 50 mM sodium chlorate (+ NaClO<sub>3</sub>) for 24 hr. Unlabeled cells are shown as the negative control. Fluorescence intensity (log scale) is represented on the x-axis and cell number on the y-axis. These data are representative of results obtained from three experiments.

## 4.2.2 Characterization of CD44 sulfation on myeloid cells

## **4.2.2i** TNF- $\alpha$ enhances sulfate incorporation in CD44H in CD14<sup>+</sup>PBMC but not in BMDM

Data presented in chapter three strongly suggested that sulfation was required for the induction of HA-binding in PBM by TNF- $\alpha$  or various inflammatory agents (Figure 3.10). Antibodies against the HA-binding domain of CD44 blocked inflammatory agent-induced HA-binding in PBM, proving that the interaction was solely mediated by CD44 (Figure 3.2). To determine if CD44 in CD14<sup>+</sup> cells is sulfated, if inflammatory agents induce the incorporation of sulfate in CD44, and to establish that sulfation is inhibited by sodium chlorate, unstimulated and stimulated PBMC were labeled with [<sup>35</sup>S]-sodium sulfate for 72 hr in the presence or absence of 50 mM sodium chlorate. After the incubation period, CD44 was immunoprecipitated from either CD14<sup>+</sup> or CD14<sup>-</sup> PBMC, subjected to SDS-PAGE and analyzed for [<sup>35</sup>S]-sulfate incorporation by autoradiography and for CD44 protein level by western blot analysis. Figure 4.2A demonstrates that low levels of [<sup>35</sup>S]-sulfate were incorporated into CD44 in unstimulated CD14<sup>-</sup> and CD14<sup>+</sup> PBMC, and [<sup>35</sup>S]-sulfate incorporation was significantly reduced in cells treated with 50 mM sodium chlorate. Neither sulfate incorporation on CD44 nor CD44 expression was increased in CD14<sup>-</sup> PBMC after stimulation with TNF- $\alpha$  (1.1 ± 0.1 fold increase in [<sup>35</sup>S]-sulfate incorporation per unit CD44H; n = 3). In contrast, stimulation of PBMC with TNF- $\alpha$  increased the quantity and sulfation of all molecular mass forms of CD44 in CD14<sup>+</sup> PBMC as observed on western blot and on autoradiographs. The 85kDa band is CD44H, the lower molecular mass forms are potential degradation products of CD44 or a soluble form, denoted CD44RC (291), and the higher molecular mass bands represent glycosaminoglycan-modified or alternatively spliced isoforms of CD44. Treatment of CD14<sup>+</sup> cells with TNF- $\alpha$  caused a significant increase in  $[^{35}S]$ -sulfate incorporation into CD44H (2.8 + 1.0 fold increase in sulfation per unit CD44H; n = 9) in addition to an increase ( $\sim$ 3-fold) in CD44 expression. To further illustrate the increase in CD44 sulfation in TNF- $\alpha$  stimulated CD14<sup>+</sup> cells, equivalent amounts of CD44 were loaded on the gel after incubation with TNF- $\alpha$  over a time course (Figure 4.2C). In this experiment, [<sup>35</sup>S]sodium sulfate was added for the final 2 hr of the incubation. Figure 4.2C shows an approximate 3-fold increase in [<sup>35</sup>S]-sulfate incorporation on CD44H at both the 48 hr and 72 hr time points. The addition of 50 mM sodium chlorate significantly inhibited [<sup>35</sup>S]-sulfate incorporation as illustrated at the 72 hr time point.



**FIGURE 4.2 Sulfate labeling of CD44 in PBMC in the presence or absence of TNF-α or IFN-γ.** CD44 was immunoprecipitated from 1 - 5 x 10<sup>6</sup> CD14<sup>+</sup> or CD14<sup>-</sup> PBMC after 72 hr incubation with [<sup>35</sup>S]sulfate and TNF-α (**A**) or IFN-γ (**B**). The left panels are autoradiographs showing the incorporation of [<sup>35</sup>S]-sulfate in CD14<sup>-</sup> cells (top of **A**) and CD14<sup>+</sup> cells (bottom of **A** and panel **B**). The right panels are western blots of the same membrane using an anti-CD44 mAb (see material and methods for details). **C** CD44 immunoprecipitated from CD14<sup>+</sup> cells that were incubated in the presence or absence of TNF-α at various time points. [<sup>35</sup>S]-sulfate was added for the last 2 hr of each time point and stimulation for 72 hr was performed in the presence or absence of sodium chlorate. The top panel is an autoradiograph showing [<sup>35</sup>S]-sulfate incorporation and the lower panel is a CD44 western blot of the same membrane. Prestained molecular mass standards are indicated on the right in kDa. The results are summarized in Table 4.2. These results support those obtained from experiments on the SR91 cells (116) indicating that TNF- $\alpha$  increased the expression and sulfation of CD44H. Unlike the SR91 cells, CD14<sup>+</sup> PBM increased CD44 expression and sulfate incorporation in response to IFN- $\gamma$  (2.3 ± 0.6 fold increase in sulfation per unit CD44H; n = 3, Figure 4.2B). The increase in CD44 expression supports findings from flow cytometry analysis. TNF- $\alpha$  or IFN- $\gamma$  increased the expression and the incorporation of [<sup>35</sup>S]-sulfate on CD44 in CD14<sup>+</sup>, but not CD14<sup>-</sup> PBMC. These data established a correlation between the induction of CD44 sulfation and the induction of HA-binding by TNF- $\alpha$  or IFN- $\gamma$  in human PBM.

A similar analysis of sulfate incorporation in CD44H was performed in murine BMDM. As seen with PBM, CD44H was sulfated on resting cells. Unlike CD44H in PBM, sulfate incorporation in CD44H on BMDM stimulated for 72 hr with TNF- $\alpha$  was not enhanced (Figure 4.3 and Table 4.2). Therefore, the effect of TNF- $\alpha$  on total sulfate incorporation in CD44H appears to differ between human monocytes and murine macrophages. It is not known if the discrepancy is related to cell type or species specificity.

## **4.2.2ii** CD44H is preferentially sulfated on carbohydrates to promote HA-binding

An inhibitor of posttranslational sulfation, sodium chlorate, reduces HA-binding by inflammatory agent-activated CD14<sup>+</sup> PBMC (Figure 3.10) and TNF- $\alpha$ -simulated BMDM (Figure 3.11). Figure 4.2 demonstrated that CD44H in PBM and BMDM was sulfated, and the sulfate incorporation could be prevented by sodium chlorate. Posttranslational sulfation may occur on glycosaminoglycans (GAGs), O- or N-linked oligosaccharides, or on tyrosine residues in the protein backbone. It is important to establish the precise nature and location of the sulfated moiety on CD44H that contributes to HA-binding ability. A preliminary analysis was conducted to address the extent of sulfate incorporation into carbohydrates or protein on CD44H in unstimulated and stimulated primary cells.

To determine if sulfate on CD44H is associated with tyrosine residues, CD44 was immunoprecipitated from 1 - 5 x  $10^6$  [<sup>35</sup>S]-sulfate labeled PBM or BMDM that had been incubated for 72 hr in the presence or absence of TNF- $\alpha$ . Immunoprecipitated CD44 was incubated for 16 hr in the presence or absence of 8 mU/ml of arylsulfatase to remove sulfate from tyrosine residues.



FIGURE 4.3 Sulfate labeling of CD44 in BMDM with or without TNF- $\alpha$  stimulation. CD44 was immunoprecipitated from 1 x 10<sup>6</sup> BMDM after 72 hr incubation with TNF- $\alpha$  and [<sup>35</sup>S]-sulfate and the presence or absence of 50 mM sodium chlorate. The top panel is an autoradiograph showing [<sup>35</sup>S]-sulfate incorporation and the lower panel is a western blot of the same membrane using an anti-CD44 mAb (see material and methods for details). Prestained molecular mass standards are indicated on the right in kDa. Stimulation of HA-binding by TNF- $\alpha$  was determined by flow cytometry in a parallel experiment with non-radiolabeled samples. The results are summarized in Table 4.2.

<sup>35</sup> SO <sub>4</sub> per CD44H	n <sup>c</sup>	
unstim = $1.0^{a}$		
$1.1 \pm 0.1^{b}$	3	
2.8 <u>+</u> 1.0	9	
$0.8 \pm 0.4$	3	
	$^{35}SO_4 \text{ per CD44H}$ unstim = 1.0 <sup>a</sup> $1.1 \pm 0.1^{b}$ $2.8 \pm 1.0$ $0.8 \pm 0.4$	$n^{c}$ unstim = 1.0 <sup>a</sup> $1.1 \pm 0.1^{b}$ $2.8 \pm 1.0$ $0.8 \pm 0.4$

TABLE 4.2 Relative incorporation of sulfate on CD44H in PBM or BMDM

<sup>a</sup> values are reported as <sup>35</sup>SO<sub>4</sub> per CD44H and relative to the ratio of sulfate incorporation per CD44H from unstimulated cells, set to  $1.0 \pm 0.0$ <sup>b</sup> <sup>35</sup>SO<sub>4</sub> per CD44H on stimulated cells reported  $\pm$  the standard deviation. <sup>c</sup> number of times the experiment was performed.

Figure 4.4 illustrates that the sulfate incorporation into CD44H was not altered by treatment with arylsulfatase. In all cases, sulfate incorporation into CD44H was within the standard deviation of the sulfate incorporation on CD44H incubated with arylsulfatase. The ExPASy Sulfinator data bank (<u>http://kr.expasy.org/tools/sulfinator/</u>) predicts possible sites of tyrosine sulfation on proteins. Using this analysis, 2 of 14 tyrosine residues (Y161 and Y169) in the amino terminal region of human CD44, and 3 of 15 tyrosine residues (Y117, Y120 and Y410) in mouse CD44, two of which are in the amino terminus, have a predicted sequence for tyrosine sulfation. Despite the potential for tyrosine sulfation on these few sites, no evidence was found to support tyrosine sulfation on CD44H as determined by arylsulfatase treatment of CD44 isolated from [<sup>35</sup>S]-sulfate labeled-PBM or -BMDM incubated in the presence or absence of TNF- $\alpha$  (Figure 4.4). Likewise, there was no evidence for tyrosine sulfation of CD44H in SR91 cells determined by a similar analysis (A. Maiti and P. Johnson, unpublished data). As a result, attention was directed toward the sulfate distribution of carbohydrates of CD44H.

PBM and BMDM were cultured for 72 hr with  $[^{35}S]$ -sodium sulfate in the presence or absence of TNF- $\alpha$  and inhibitors of either GAG assembly (1 mM xyloside (p-nitrophenyl  $\beta$ -Dxylopyranoside)) or O-linked glycosylation (1 mM BADGal (benzyl-2 acetamido 2-deoxy-α-Dgalactopyranoside)). CD44 was immunoprecipitated from  $1 - 5 \times 10^6$  cells and subsequently treated with 0.05 U/ml of PNGaseF to remove N-linked glycans. [<sup>35</sup>S]-sulfate incorporation (determined by autoradiography) relative to CD44H protein (determined by western blotting) was calculated to determine the percent sulfation associated with carbohydrates (Figure 4.5). The analysis of sulfate incorporation was performed on the standard 85kDa form of CD44, CD44H, not CD44v forms. In unstimulated monocytes, sulfate was primarily associated with GAGs and O-glycans of CD44H ( $\sim$ 34 ± 16% contribution of sulfate on GAGs; n = 3 and  $\sim$ 50 ± 30% contribution of sulfate on O-glycans; n = 3) with a lesser contribution from N-linked glycans (~16 + 13%; n = 3). Following stimulation of PBM with TNF- $\alpha$  or IFN- $\gamma$ , the percent of sulfate incorporation on GAGs was slightly enhanced ( $\sim 46 + 10\%$  (n = 4) and  $\sim 52 + 31\%$  (n = 3) contribution of sulfation on GAGs of CD44H in cells stimulated with TNF- $\alpha$  or IFN- $\gamma$ respectively). Sulfate incorporation on O-glycans was substantially, but not reproducibly, decreased (to less than 20%; n = 3) after TNF- $\alpha$  or IFN-y stimulation. The sulfate associated



#### FIGURE 4.4 Sulfation on tyrosine residues of CD44 in PBM or BMDM. CD44 was

immunoprecipitated from human PBM (left panel) or mouse BMDM (right panel) after 72 hr incubation in the presence of [ $^{35}$ S]-sulfate and the presence (+) or absence (-) of TNF- $\alpha$ . Immunoprecipitated CD44 was incubated with 8 U/ml of arylsulfatase (A. S'ase) for 16 hr to remove sulfate specifically incorporated on tyrosine residues (see material and methods for details). A (+) indicates that the enzyme was present, thus, sulfate on tyrosine residues was removed from CD44. The top panels are autoradiographs showing [ $^{35}$ S]-sulfate incorporation and the lower panels are western blots for CD44 on the same membrane. Pre-stained molecular mass standards are indicated on the right in kDa. These results are representative of three repeat experiments. with N-glycans of CD44H was significantly increased in response to TNF- $\alpha$  (to ~41 ± 23%; n = 4) or IFN- $\gamma$  (to ~48 ± 19%; n = 3) compared to that on CD44 from unstimulated PBM (~16 ± 13%; n = 3, Figure 4.5A).

These data show that sulfate incorporation in PBM is complex, occurring on GAGs, O- and Nlinked glycans. Further, sulfate incorporation on N-glycans of CD44H in PBM is less pronounced than that on GAGs and O-glycans, however, after stimulation of PBM with inflammatory cytokines for 72 hr, the sulfate associated with N-glycans of CD44H doubled. Therefore, pro-inflammatory cytokines not only induce sulfate incorporation on CD44H, but sulfate is preferentially associated with N-glycans. Although total sulfate incorporation on CD44H in BMDM did not increase following stimulation of BMDM with TNF- $\alpha$  (Figure 4.3), as in PBM, the sulfate distribution on CD44H in BMDM is complex, occurring on GAGs, Oand N-glycans. A difference was observed in the distribution of sulfate on CD44H from BMDM prior to and following activation. GAG sulfation accounted for ~30 - 40% of the sulfated moieties on CD44H in BMDM in the presence or absence of TNF- $\alpha$  [~39 + 24% (n = 3) and ~33  $\pm$  30% (n = 3) sulfation on GAGs of CD44H from BMDM or TNF- $\alpha$ -stimulated BMDM respectively, Figure 4.5B]. Sulfate incorporation on O-glycans of CD44H increased in BMDM stimulated with TNF- $\alpha$  [from ~27 ± 19% (n = 3) to ~42 ± 2% (n = 3)]. On unstimulated cells, the sulfation of CD44H-associated N-glycans ( $\sim 33 \pm 27\%$ ; n = 3) remains unchanged or was slightly decreased following TNF- $\alpha$  stimulation of cells (~25 ± 11%; n = 3, Figure 4.5B).

In summary, these data show that while the sulfation of GAGs remains relatively unchanged in response to stimuli, TNF- $\alpha$  stimulated an increase (~2-fold) in sulfate associated with O-glycans on CD44H in BMDM, and an increase (~2- to 3-fold) in the contribution of sulfation on N-glycans of CD44H in PBM after stimulation with TNF- $\alpha$  or IFN- $\gamma$ . These data demonstrates that a shift in sulfate distribution from GAGs to N- or O-linked carbohydrates of CD44 in PBM or BMDM occurs in response to inflammatory stimuli. Such a shift in sulfate distribution may be the key to ligand reactivity as opposed to a total increase of identical sulfated determinants on CD44.

Human PBM

Α



FIGURE 4.5 Sulfate distribution on GAGs, N- or O-glycans on CD44 in PBM or BMDM. CD44 was immunoprecipitated from human PBM (A) or mouse BMDM (B) after 72 hr incubation in the presence of [ $^{35}$ S]-sulfate and the presence (right panels) or absence (left panels) of TNF- $\alpha$ . Cells were incubated in the presence or absence of inhibitors of GAG addition (xyloside) or O-linked glycosylation (BADGal) and immunoprecipitated CD44 was incubated with PNGaseF to digest N-linked glycans (see material and methods for details). In A and B, the top panel is an autoradiograph showing [ $^{35}$ S]-sulfate incorporation and the lower panel is a western blot of the same membrane using an anti-CD44 mAb. These data representative of three repeat experiments and summarized in the text on the flanking pages. Prestained molecular mass standards are indicated on the right in kDa.

### **4.2.2iii** TNF- $\alpha$ induces the expression of sulfated epitopes on PBM

A panel of antibodies that recognize various sulfated epitopes were screened for reactivity with PBM by flow cytometry analysis. The antibodies recognize the following epitopes; 6,6'-disulfo LacNAc (mAb L4L4-8), sialyl 6-sulfo LacNAc/Lewis x (mAb G72), 6-sulfo LacNAc/Lewis x (mAb AG105 and mAb AG107) (271), sialyl 6,6'-disulfo LacNAc/Lewis x (mAb G270-16), 3-sulfo Lewis x (mAb SU59) and sialyl 6-sulfo Lewis x/sialyl Lewis x (mAb 2F3) (100, 269, 270, 272, 273). Prior to this analysis, mAb G72 (against a 6-sulfo Lewis x determinant) reacted with human HEV (100), establishing a potential use for these reagents in the identification of sLex determinants *in vivo*.

Two antibodies, AG105 and AG107, recognize 6-sulfo LacNAc/Lewis x determinants and reacted with CD14<sup>+</sup> PBM (Figure 4.6A). These antibodies bound to PBM only following TNF- $\alpha$ or IFN-y stimulation and subsequent removal of terminal sialic acid residues with neuraminidase (N'ase). Thus, the epitopes are induced on PBM by inflammatory stimuli, and are normally masked by sialylation. Figure 4.6B confirms that sulfate is required for epitope recognition by mAbs AG105 and AG107 as incubation of PBM with TNF- $\alpha$  and increasing concentrations of sodium chlorate progressively decreased antibody reactivity. Both mAbs are reactive against epitopes containing 6-sulfo LacNAc or 6-sulfo Lewis x (271). The mAb DD2 detects 6-sulfo LacNAc but not 6-sulfo Lewis x (provided by Reiber, 274), thus was used to identify which sulfated structure was induced on PBM. Illustrated by Figure 4.7C, mAb DD2 bound to neuraminidase-treated PBM following culture in the presence or absence of TNF- $\alpha$  for 72 hr. Unlike the increase in mAb AG105 and mAb AG107 reactivity observed on PBM following TNF- $\alpha$  stimulation, mAb DD2 recognized a 6-sulfo LacNAc epitope with similar intensities on both unstimulated and stimulated PBM. These results from flow cytometry suggests that both 6sulfo LacNAc and 6-sulfo Lewis x structures are present on PBM, and the increase in AG105 or AG107 mAb reactivity on TNF- $\alpha$ -stimulated PBM reflects an increase in 6-sulfo Lewis x containing motifs. Although 6-sulfo LacNAc epitope expression is constant on the surface of non-activated and TNF- $\alpha$ -stimulated PBM, there is no information regarding the molecular distribution of this determinant or the 6-sulfo Lewis x epitope. Experiments to address the reactivity of mAb AG105, AG107, and DD2 on CD44 immunoprecipitated from PBM are being pursued in the laboratory.



**FIGURE 4.6 Expression of sulfated epitopes on human PBM.** A, B, C Flow cytometry analysis of  $CD14^+$  PBMC following culture for 72 hr and subsequent treatment with or without neuraminidase. A Binding of mAbs AG105 and AG107 to  $CD14^+$  PBMC incubated for 72 hr (-) with TNF- $\alpha$  (+ TNF- $\alpha$ ) or IFN- $\gamma$  (+ IFN- $\gamma$ ). B AG105 and AG107 reactivity with TNF- $\alpha$  stimulated CD14<sup>+</sup> PBMC incubated in the presence of increasing concentration of sodium chlorate. C Binding of mAb DD2 to CD14<sup>+</sup> PBMC following incubation with (+ TNF- $\alpha$ ) or without (-) TNF- $\alpha$ . Secondary antibody alone is the negative control in A and C whereas in B, cells alone are the negative control. Fluorescence intensity (log scale) is represented on the x-axis and cell number on the y-axis. These results are representative of three repeat experiments.

**4.2.2iv** *HA*-binding capacity correlates with sulfated epitope expression in KG1a cells To determine if CD44 is sulfated on myeloid progenitors with constitutive HA-binding, CD44 was immunoprecipitated from KG1a cells labeled with  $[^{35}S]$ -sodium sulfate. In KG1a cells, the standard 85 kDa form of CD44, CD44H, was the major sulfated species, yet other sulfated forms of higher and lower molecular mass than CD44H were detected. Sulfate incorporation to CD44H was significantly inhibited (by  $73 \pm 21\%$ , n = 5) in cells treated for 72 hr with 50 mM sodium chlorate (Figure 4.7). To determine if total sulfate incorporation in CD44 correlated with HA-binding capacity, CD44 was immunoprecipitated from  $\int_{1}^{35}$ Sl-sulfate labeled HA<sup>low</sup> and HA<sup>high</sup> KG1a cells. Despite the difference in HA-binding capacity of the KG1a subpopulations, equivalent levels of [<sup>35</sup>S]-sulfate were incorporated into CD44H immunoprecipitated from HA<sup>low</sup> or HA<sup>high</sup> cells (Figure 4.7). To confirm that sulfation was required for HA-binding by the sorted populations, cells were incubated with increasing concentrations of sodium chlorate then FL-HA-binding capacity was measured by flow cytometry. As with wild type KG1a cells, the presence of 50 mM sodium chlorate reduced HA-binding in the HA<sup>high</sup> KG1a population (Figure 4.8A).

This data shows that sulfate is constitutively incorporated into CD44H on KG1a cells and that the total incorporation of sulfate on CD44H did not differ between the KG1a cells with high or low HA-binding strength. Nonetheless, mAb DD2 had increased reactivity with a greater percentage of HA<sup>high</sup> than HA<sup>low</sup> KG1a cells (Figure 4.8B). These results demonstrates that quantitatively, CD44H sulfation does not correlate with HA-binding capacity, yet the expression of a specific sulfated epitope, 6-sulfo LacNAc (recognized by the DD2 antibody), does coincide with HA-binding. The differential binding of mAb DD2 to KG1a cells with different HA-binding ability does not agree with the results obtained with these antibodies on PBM (Figure 4.6). This discrepancy in mAb DD2 binding may be reconciled if the antibodies have different specificities or if the antibodies preferentially detect one form of sulfated LacNAc over another.

These results illustrate that sulfated moieties can fluctuate on the cell surface without an observable shift in total sulfate levels. Similar to KG1a cells, specific sulfated epitopes may also be induced on BMDM and specifically, on CD44H in response to TNF- $\alpha$  despite no detectable increase in total sulfate incorporation on CD44H. The sulfated epitope-specific antibodies used to analyze KG1a cells and PBM were not reactive on mouse BMDM (data not shown), thus, the presence of these sulfated epitopes remains unknown in murine macrophages.



**FIGURE 4.7 Sulfation of CD44 in KG1a cells** Autoradiograph (top panel) of [<sup>35</sup>S]-sulfate incorporation into CD44H immunoprecipitated from KG1a cells in the presence or absence of sodium chlorate (left panel) and HA<sup>low</sup> or HA<sup>high</sup> KG1a cells (right panel). CD44 levels as detected by western blots are shown in the lower panel. Prestained molecular mass markers are indicated to the right of the blots. These data are representative of results from three repeat experiments.



**FIGURE 4.8 Expression of sulfated epitopes in high and low HA-binding KG1a cells A** FL-HAbinding of HA<sup>low</sup> (left panel) or HA<sup>high</sup> (right panel) KG1a cells following 72 hr incubation in the presence of increasing concentrations of sodium chlorate. The negative control is cells alone. **B** Binding of mAb DD2 to HA<sup>low</sup> (left panel) or HA<sup>high</sup> (right panel) KG1a cells. The negative control is secondary antibody alone. The x-axis is fluorescence intensity, and the y-axis is cell number. These data are representative of results from three repeat experiments.

#### 4.2.3 Relationship between sulfate incorporation in CD44H and HA-binding ability

**4.2.3i** Decreasing levels of sulfate incorporation in CD44H correlates with decreased FL-HAbinding by TNF- $\alpha$ - or IFN- $\gamma$ -stimulated CD14<sup>+</sup> PBMC

To strengthen the correlation between CD44 sulfation and FL-HA-binding ability, PBMC were stimulated with TNF- $\alpha$  or IFN- $\gamma$  for 72 hr in the presence of [<sup>35</sup>S]-sodium sulfate and increasing concentrations of sodium chlorate. Unlabeled CD14<sup>+</sup> PBMC were analyzed for FL-HA-binding by flow cytometry (Figure 4.9A) and CD44 sulfation was analyzed by autoradiography on CD44 immunoprecipitated from radiolabeled cells (Figure 4.9B). The percentage of FL-HA-binding cells and the incorporation of sulfate in CD44H decreased in TNF- $\alpha$ -activated PBM incubated with increasing concentrations of sodium chlorate (Tables 4.3 and 4.4 and Figures 4.9 and 4.10). Titration with increasing concentrations of sodium chlorate decreased cellular FL-HA-binding that was paralleled by a decrease in sulfate incorporation on CD44H. The decline in PBM that bound to FL-HA and the decrease in CD44H sulfation in response to increasing concentrations of sodium chlorate were not linear, decreasing rapidly then reaching a plateau between 30 and 50 mM sodium chlorate. At this concentration, approximately 30% of CD44H sulfation and 50 -60% of HA-binding monocytes remained. These data show that the reduction in CD44H sulfation correlates with reduced HA-binding by both TNF- $\alpha$ - and IFN- $\gamma$ -induced CD14<sup>+</sup> PBMC. Therefore, TNF- $\alpha$  or IFN- $\gamma$  induce sulfate incorporation on CD44H in CD14<sup>+</sup> peripheral blood monocytes that positively correlates with the HA-binding capacity of PBM.

### 4.2.3ii Sulfation on CD44H directly contributes to FL-HA-binding

TNF- $\alpha$  or IFN- $\gamma$  induced the sulfation of CD44 and CD44-dependent HA-binding in CD14<sup>+</sup> PBMC. Treatment of PBMC with sodium chlorate decreased the percentage of cells that bound to FL-HA, which correlated with the decrease in CD44H sulfation. To demonstrate that the sulfation on CD44 directly affects HA-binding, CD44 was immunoprecipitated from TNF- $\alpha$ - and IFN- $\gamma$ -treated CD14<sup>+</sup> PBMC cultured for 72 hr in the presence or absence of 50 mM sodium chlorate. CD44 was immunoprecipitated from equivalent numbers of cells, resolved under non-reducing conditions then analyzed for FL-HA-binding by far western blot analysis. The results indicated that CD44H isolated from TNF- $\alpha$ - or IFN- $\gamma$ -stimulated CD14<sup>+</sup> PBMC bound to FL-HA, but CD44H from unstimulated cells did not (Figure 4.11). CD44H from TNF- $\alpha$ -stimulated CD14<sup>+</sup> PBMC bound 2.7 ± 0.7 (n = 4) times more FL-HA than CD44H immunoprecipitated



FIGURE 4.9 Sulfate incorporation on CD44H correlates with FL-HA-binding by CD14<sup>+</sup> PBMC. In A and **B**, CD14<sup>+</sup> PMBC were stimulated with 20 ng/ml TNF-α (top panels) or 500 U/ml IFN-γ (bottom panels) for 72 hr. A FL-HA-binding by stimulated CD14<sup>+</sup> PBMC in the presence of increasing concentrations of NaClO<sub>3</sub> (mM concentration noted on the left hand side) as analyzed by flow cytometry. The x-axis is fluorescence intensity (log scale) and the y-axis is cell number. **B** CD44 was immunoprecipitated from stimulated CD14<sup>+</sup> PBMC in the presence of 0 to 50 mM NaClO<sub>3</sub> and [<sup>35</sup>S]sulfate. Sulfation was analyzed by autoradiography (left panels) and CD44 expression by western blotting (right panels). Prestained molecular mass markers are on the right in kDa. The results are summarized in Table 4.3, Table 4.4, and Figure 4.10.

[NaClO <sub>3</sub> ] (mM)	0	5	10	20	30	40	50	n <sup>e</sup>	
	% CD14 <sup>+</sup> PBMC that bind FL-HA <sup>a</sup>								
	100 <u>+</u> 0 <sup>b</sup>	79 <u>+</u> 9	66 <u>+</u> 9	61 <u>+</u> 15	42 <u>+</u> 10	44 <u>+</u> 10	45 <u>+</u> 11	3	
	% <sup>35</sup> SO <sub>4</sub> /CD44H <sup>c</sup>								
	$98\pm4$	<u>.99 ± 2</u>	82 <u>+</u> 11	51 <u>+</u> 3	46 <u>+</u> 0	33 <u>+</u> 0	<u>33 + 10</u>	3	

Table 4.3 Effect of sodium chlorate (NaClO<sub>3</sub>) concentration on FL-HA-binding and CD44H sulfation induced by  $TNF-\alpha$  in CD14<sup>+</sup> PBMC

**Table 4.4** Effect of sodium chlorate (NaClO<sub>3</sub>) concentration on FL-HA-binding and CD44H sulfation induced by IFN- $\gamma$  in CD14<sup>+</sup> PBMC

[NaClO <sub>3</sub> ] (mM)	0	5	10	20	30	40	50	n e	
•	% CD14 <sup>+</sup> PBMC that bind FL-HA <sup>a</sup>								
	100 <u>+</u> 0 <sup>b</sup>	84 <u>+</u> 12	74 <u>+</u> 12	64 <u>+</u> 18	70 <u>+</u> 5	58 <u>+</u> 10	59 <u>+</u> 9	3	
	% <sup>35</sup> SO <sub>4</sub> /CD44H <sup>c</sup>								
	$100\pm0^{d}$	92 <u>+</u> 2	84 <u>+</u> 4	54 <u>+</u> 4	42 <u>+</u> 6	4 <u>3</u> <u>+</u> 1	36 <u>+</u> 1	. 3	

<sup>a</sup>values are calculated as the percent of stimulated CD14<sup>+</sup> PBMC that bind to FL-HA then reported as a relative percentage to the highest number of stimulated CD14<sup>+</sup> PBMC that bind to FL-HA (set to 100%), typically PBM stimulated in the absence of sodium chlorate

<sup>b</sup> % of CD14<sup>+</sup> PBMC that bind to FL-HA  $\pm$  the % standard deviation.

<sup>c</sup>values are calculated as  ${}^{35}SO_4$  per CD44H then reported as a relative percentage compared to the highest incorporation of sulfate per CD44H (set to 100%), typically on CD44 isolated from PBM stimulated in the absence of sodium chlorate

<sup>d</sup> %  $^{35}SO_4$  per CD44H  $\pm$  the % standard deviation.

<sup>e</sup> number of times the experiment was performed.

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Figure 4.10 Effect of sodium chlorate concentration on FL-HA-binding and CD44H sulfation in PBM. PBM were incubated for 72 hr in the presence of TNF- $\alpha$  (left graph) or IFN- $\gamma$  (right graph) with increasing concentrations of sodium chlorate (NaClO<sub>3</sub>, x-axis). Spot densitometry was used to determine the sulfate incorporation per CD44H. Sulfate incorporation in CD44 isolated from cells incubated in the presence of sodium chlorate is expressed relative to sulfate incorporation on CD44 from cells incubated in the absence of sodium chlorate (100% sulfate incorporation) as represented on the y-axis with grey bars. The percentage of CD14<sup>+</sup> PBMC that bind to FL-HA in the presence of increasing concentrations of sodium chlorate (100%) as represented on the y-axis with black bars. These data are shown in Figure 4.9 and summarized in Table 4.3 and Table 4.4.



FIGURE 4.11 FL-HA-binding by CD44 isolated from PBM treated with or without sodium chlorate. A CD44 was immunoprecipitated from unstimulated (left lane), TNF- $\alpha$  stimulated (20 ng/ml; top panel), or IFN- $\gamma$  stimulated (500 U/ml; bottom panel) CD14<sup>+</sup> PBMC incubated in the presence or absence of 50 mM sodium chlorate (NaClO<sub>3</sub>). FL-HA-binding was analyzed by far western blotting (left panels) and CD44 expression determined by western blotting (right panels) on the same membrane. The pre-stained markers indicate the relative molecular mass on the right. B Spot densitometry was used to determine the amount of FL-HA bound relative to CD44 protein levels, as illustrated in the graph. These data are representative of results obtained from four experiments. The average binding of FL-HA  $\pm$  the standard deviation are reported in the text.

from TNF- $\alpha$ -stimulated CD14<sup>+</sup> PBMC treated with 50 mM sodium chlorate. Similar, 3.0 ± 1.5 (n = 4) times more FL-HA bound to CD44H isolated from IFN- $\gamma$ -stimulated CD14<sup>+</sup> PBMC compared to CD44H from stimulated cells treated with sodium chlorate. This demonstrates that the sulfation of CD44H that is induced by TNF- $\alpha$  or IFN- $\gamma$  has a direct and a positive effect on FL-HA-binding by CD14<sup>+</sup> PBMC.

# **4.2.3iii** SK2<sup>-/-</sup> BMDM have reduced sulfate incorporation on CD44H and reduced FL-HA-binding capacity

PAPS is the cellular substrate for the enzymatic transfer of sulfate to glycoproteins, glycolipids, and proteoglycans that is catalyzed by golgi-resident sulfotransferases. PAPS is generated from APS by a bifunctional sulfurylase kinase (SK) polypeptide, encoded by one of two genes, SK1 or SK2, thought to have non-redundant, tissue specific expression in mice (296). A missense mutation in the SK2 gene has led to a natural defect in the enzymatic formation of activated sulfate, PAPS (309), and diminished sulfation in cells dependent on the SK2 gene product to generate PAPS. Mice that are deficient in SK2 have undersulfated proteoglycans, abnormal bone development, and disproportionate dwarfism defined as brachymorphism (310). To analyze the importance of sulfation for HA-binding without the use of a chemical inhibitor, BMDM were generated from the bone marrow of three mice that are deficient for the PAPS synthetase gene product (SK2<sup>-/-</sup> mice donated by F. Jirik). Femurs from the SK2<sup>-/-</sup> mice were reduced in size compared to femurs from wild type C57BL6 mice, and fewer cells were present in the marrow obtained from the SK2<sup>-/-</sup> femurs. Nonetheless, a sufficient number of F4/80<sup>+</sup>

SK2<sup>-/-</sup> macrophages were successfully derived under identical conditions used for differentiation of wild type bone marrow. Prior to this investigation, the activity of SK1 or SK2 in BMDM was not known thus, sulfate labeling was performed to determine if SK2<sup>-/-</sup> BMDM are defective in their ability to sulfate CD44. Wild type and SK2<sup>-/-</sup> BMDM were incubated for 72 hr in the presence of [<sup>35</sup>S]-sulfate and the presence or absence of TNF- $\alpha$ . The incorporation of sulfate in CD44H immunoprecipitated from SK2<sup>-/-</sup> BMDM was reduced (by 20 - 30%) in comparison to the sulfate incorporated in CD44H from wild type BMDM (Figure 4.12A). FL-HA-binding of SK2<sup>-/-</sup> BMDM was analyzed by flow cytometry following 72 hr stimulation in the presence or absence of TNF- $\alpha$ . As illustrated by Figure 4.12B, the expression of CD44 increased in both the wild type and SK2<sup>-/-</sup> BMDM following stimulation with TNF- $\alpha$ . TNF- $\alpha$ -induced FL-HAbinding by SK2<sup>-/-</sup> BMDM, however, FL-HA-binding was consistently reduced in comparison to





FIGURE 4.12 CD44 sulfation on, and FL-HA-binding by, SK2<sup>-/-</sup> BMDM. A Sulfate incorporation in CD44 immunoprecipitated from wild type (C57BL6, left panel) or SK2<sup>-/-</sup> (right panel) BMDM following 72 hr culture of BMDM in the presence or absence of TNF- $\alpha$ . The upper panels are autoradiographs depicting sulfate incorporation in CD44 and the lower panels show CD44 levels by western blots of the same membrane. **B** Flow cytometry analysis of CD44 expression (middle panel) and FL-HA-binding (right panel) of wild type (C57BL6, lower rows) and SK2<sup>-/-</sup> BMDM (upper rows) following 72 hr culture in the presence of TNF- $\alpha$ . The negative control is cells only. The x-axis is fluorescence intensity (log scale) and the y-axis is cell number. These data are representative of results from six experiments.

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FL-HA-binding by wild type BMDM. In 4 of 6 experiments, FL-HA-binding was reduced (mean fluorescence intensity (MFI) of FL-HA-binding by  $SK2^{-/-}$  BMDM is  $0.73 \pm 0.23$  (n = 4) relative to wild type BMDM, set to  $1.0 \pm 0.0$  MFI), but never greater than FL-HA-binding by wild type BMDM. Sulfate levels on CD44H were reduced in BMDM derived from  $SK2^{-/-}$  mice potentially due to a smaller pool of PAPS. Using cells that are naturally suppressed in sulfate incorporation on CD44, diminished sulfation of CD44H correlates with a decrease in HA-binding, a result that supports data obtained with the chemical inhibitor, sodium chlorate. These data provide additional evidence that sulfation is an important factor required for TNF- $\alpha$ -induced HA-binding in myeloid cells.

### **4.3 DISCUSSION**

## 4.3.1 Sulfation in immune cells regulates ligand binding

There is increasing evidence that sulfation mediates cell-cell and cell-ligand interactions in a variety of systems (reviewed in 87, 295). The best example is in the immune system, where sulfation on selectin ligands is necessary for mediating leukocyte tethering and rolling during lymphocyte homing and leukocyte extravasation at inflammatory sites (reviewed in 43, 311, 312). In addition to sulfation on selectin ligands, CD34 and CD45 were sulfated on PHA-stimulated peripheral blood lymphocytes and implicated in homotypic aggregation initiated by anti-CD34 mAb (313). The C5a anaphylatoxin receptor is tyrosine sulfated (314) as well as the chemokine receptors, CXCR4 and CCR5, where sulfation assisted in MIP-1 $\alpha/\beta$  chemokine binding as well as the binding and entry of HIV through CCR5 (315). Chrondroitin sulfate (CS) regulates CD44-mediated cell adhesion in fibroblasts, keratinocytes, lung epithelial derived cancer cells and colon carcinoma cells (222, 227-229). In SR91 cells (292) and PBM (Tables 3.1), sodium chlorate did not inhibit the increase in CD44 surface expression. These results suggested that an increase in CD44 receptor levels is not sufficient to engage high levels of HA, and that sulfate incorporation on CD44H is required for HA-binding.

In this chapter, the results show that CD44 is sulfated on CD14<sup>+</sup> PBMC and to a lesser extent on CD14<sup>-</sup> PBMC. In response to TNF- $\alpha$  or IFN- $\gamma$ , only CD14<sup>+</sup> PBMC showed enhanced sulfate incorporation into CD44H and increased binding to HA. Sodium chlorate treatment of PBM decreased inflammatory agent-induced binding to HA on a cellular level and reduced CD44H engagement of HA in a direct binding assay (far western blot analysis). CD44 isolated from

unstimulated PBM bound very low levels of HA, thereby mimicking cellular HA-binding observed by flow cytometry. CD44H isolated from PBM that were stimulated in the presence of sodium chlorate bound approximately 3-fold less HA than sulfated CD44H isolated from agonist-stimulated CD14<sup>+</sup> control cells. These data suggest that the induced sulfation on CD44, not the presence of other sulfated cell surface molecules or the total sulfation state on the cell surface, is required for engagement of HA by PBM stimulated with inflammatory agents.

## 4.3.2 Sulfation as a regulatory mechanism of HA-binding

### **4.3.2i** *TNF*- $\alpha$ alters sulfate distribution on CD44H

An analysis of sulfate distribution on CD44 in PBM and BMDM revealed a complex distribution pattern of sulfation that could be altered in cells stimulated with TNF- $\alpha$ . The major, 85 kDa form of CD44, CD44H, is the primary sulfated form of CD44 expressed on PBM prior to and following stimulation with TNF- $\alpha$  or IFN- $\gamma$ . Several sulfated molecular mass forms ranging from 85 - 175 kDa were identified following cellular sulfate-labeling and immunoprecipitation of CD44 from PBM lysates. All CD44 forms may be modified by GAGs with supplementary sites for addition in the variant forms, CD44v3 and CD44v10 (148, 316) thus, the higher molecular mass forms could potentially represent GAG-modified forms of CD44 isolated from PBM incubated in the presence of an inhibitor of GAG addition, implying that these forms are not the product of GAG addition to CD44H, but are likely alternatively spliced isoforms.

Sulfate incorporation, but not molecular mass, was significantly diminished on CD44H from PBM and BMDM incubated in the presence of an inhibitor of GAG addition. Without a detectable shift in the molecular mass of CD44H, these data suggest that the GAG side chains may be relatively short therefore do not alter the molecular mass of CD44H when resolved by SDS-PAGE. As reported by Delcommenne *et al* (230), sulfation of CD44H on unstimulated SR91 cells was primarily attached to GAGs (~85% of total sulfate was present as chondroitin sulfate). However, TNF- $\alpha$  induced a 2- to 3-fold increase in sulfate that is associated with N-and O-glycans (to ~35% and 25% of CD44H sulfation respectively) that reduced the relative amount of total sulfate associated with GAGs on CD44H to 50%. Sulfation of CD44H in PBM and BMDM decreased with inhibition of GAG assembly in PBM and BMDM prior to or following TNF- $\alpha$  stimulation suggesting that TNF- $\alpha$  does not significantly change the incorporation of sulfate on GAGs or the addition of sulfated GAGs to CD44H. However, the

relative contribution of sulfation from GAG addition to CD44H in PBM and BMDM decreased after the cells were stimulated with TNF- $\alpha$ . Sulfate incorporation increased on N-glycans of CD44H from PBM or O-glycans on CD44H from BMDM in response to TNF- $\alpha$ . Although sulfate addition on GAGs was not altered in response to the inflammatory cytokines, the total sulfation associated with N- or O-glycans was enhanced, such that the overall contribution to sulfation on CD44H by GAGs was reduced after TNF- $\alpha$  stimulation.

The variability in sulfate incorporation to GAGs, N- and O-glycans of CD44H may reflect the normal cellular variations in glycosylation, sulfation, and GAG addition that can be influenced by culture conditions themselves. It has been reported that glucose levels effect CD44 glycosylation (317) and glutamine degradation can down regulate sialylation (318). Despite the combined use of inhibitors for GAG addition, O-glycan assembly, and enzymatic digest of Nlinked glycans from CD44, [<sup>35</sup>S]-sulfate was still incorporated in CD44. Sulfate does not appear to be incorporated on tyrosine residues, thus, the inability to completely inhibit sulfate incorporation suggests that the inhibition or removal of certain oligosaccharides is incomplete or that the radiolabel is incorporated into the protein backbone on other sulfur-containing amino acids. The oxidation of sulfur containing amino acids can release inorganic sulfate (296) that in CHO cells is required for GAG assembly (319). Esko et al propose that sulfur-containing amino acids can provide all the sulfate needed for growth and development, and the under-sulfation of GAGs observed on chondrocytes (320, 321), fibroblasts (322), and endothelial cells (305) is due to low amino acid turnover in these tissues or an insufficient supply of sulfur-containing amino acids. In another report, addition of sulfur-containing amino acids to lung fibroblasts partially inhibited incorporation of [<sup>35</sup>S]-sulfate into macromolecules, yet supplementation of smooth muscle cells in the same way had no effect (309). These reports suggest that tissues may differentially coordinate the uptake and metabolism of inorganic sulfate and sulfur-containing amino acids, perhaps in response to other metabolic requirement, such as protein synthesis or sulfation of glycoproteins or glycolipids (319). At the present time, there is no direct evidence that a pool of sulfate can contribute to the synthesis of the essential sulfur-containing amino acids, cysteine and methionine. Thus, the residual sulfation observed on CD44H is likely attributable to the suboptimal action of the inhibitors or enzymes used in this analysis, not to [<sup>35</sup>S]-sulfate incorporation into amino acids on the protein backbone.

The impact of changes in sulfation and glycosylation on the stability of CD44 on the cell surface is not understood. The rate of CD44 turnover was reported as  $t_{1/2} = 48$  hr in normal melanocytes and was double that in aggressive, highly invasive melanoma cells (323). The rate in primary hematopoietic cells has not been determined. The influence of TNF- $\alpha$  on shedding CD44 from the cell surface is also not clear. Determination of the proportion of newly synthesized, sulfated CD44 that must be expressed to facilitate HA-binding is also not known.

Overall, the data suggests that a shift in sulfate distribution on the carbohydrates of CD44H occurs in response to TNF- $\alpha$ , favouring O- or N-glycans and indirectly diminishing the contribution of GAGs.

## **4.3.2ii** Sulfation of carbohydrates is a potential positive and negative regulator of HAbinding

From the above section, it is clear that the sulfation on CD44H is complex. GAGs, namely chondroitin sulfate, heparan sulfate, or keratan sulfate attached to CD44, have previously been shown to play a role in regulating CD44-mediated cell adhesion in non-hematopoeitic cells (222, 227-229). However, the results from these studies conflict, reporting that GAGs can both enhance and inhibit CD44-HA adhesion. TNF- $\alpha$ -induced HA-binding was enhanced in PBM and BMDM incubated with an inhibitor of glycosaminoglycan addition (Figure 3.9), an observation also made in the SR91 cell line (230). This agrees with the lesser contribution of sulfated GAGs on CD44H in TNF-a-stimulated PBM and BMDM and indicates that GAG addition is a constitutive, negative regulator of HA-binding in SR91 cells, PBM, and BMDM. This is supported in K562 cells, a human cell line originated from a chronic myeloid leukemia. These cells are positive for CD44 expression, not reactive to HA, and heavily sulfated on GAGs, with ~75% of sulfation associated with HS and ~5% associated with CS (304). GAG addition occurs at Ser-Gly motifs present on all CD44 forms in the membrane proximal region (outside the HAbinding domain) as well at additional sites in CD44v3 and CD44v10 (reviewed in 316), but it is not known how GAG addition on CD44 negatively regulates HA-binding in BMDM and PBM. The interaction between CD44 and HA likely requires appropriate spacing of CD44 molecules such that key residues on individual CD44 molecules are poised to interact with a complementary site on HA, and that a sufficient number of molecules are able to act in concert to generate strong avidity for large polymers of HA. There is evidence that chondroitin sulfate is required for intercellular adhesion mediated by a CD44-CD44 interaction (182). GAGs may also

be able to mediate an interaction between adjacent CD44 molecules on the cell surface of monocytes. In this manner, GAGs could determine the spacing of both standard and variant forms of CD44 in flexible or rigid positions that discourage adaptation to an optimal HA-binding conformation.

Sites for O-glycan addition are also clustered in the membrane proximal region and may facilitate HA-binding through a conformational change in individual CD44 molecules or through the aggregation of neighbouring molecules. Sulfation of O-glycans did increase on CD44H in stimulated BMDM, but the glycosylation inhibitor did not affect HA-binding by flow cytometry. Therefore, it is difficult to determine the exact role of this sulfated carbohydrate moiety in producing a functional CD44 receptor. Six sites for N-glycan addition reside in the aminoterminal, HA-binding region of CD44. Although the effect of N-glycosylation on HA-binding was not determined, sulfation of N-glycans increased on CD44H in PBM after stimulation with TNF- $\alpha$ , correlating with the TNF- $\alpha$  induced HA-binding. An increase in sulfation on N-linked glycosylation sites could encourage binding to HA through a shift in conformation of CD44H.

These results suggest that an increase in the total sulfate incorporation on CD44H is not required for HA-binding, but a shift in the location of sulfate on CD44H is necessary. These data suggest that inflammatory agents may release CD44H in human SR91 cells, PBM, and mouse BMDM from the negative regulatory effects of GAGs by inducing the expression of new cell surface molecules of CD44H with a more significant incorporation of sulfate on N- or O-glycans. The analysis of sulfate distribution on CD44H has proven to be a useful starting point for identification of the precise sulfated site(s) that confer HA-binding reactivity.

It has been speculated by others that the functionality imparted by posttranslational sulfate addition occurs by one of two mechanisms (87); either by the biosynthesis of multiple carbohydrate sulfoforms within a GAG chain to produce a versatile, adaptive, and interactive scaffold or by the conversion of a common oligosaccharide into a functional ligand (or receptor) through sulfate addition (87). Heparan sulfate (HS) is a prime example of the first tactic to achieve functionality through sulfation as HS has an array of unique sulfated motifs with functionally diverse potential. The second strategy is exemplified by the metabolism of hormones. Sulfation can regulate the circulatory half-life of hormones by preventing their premature degradation by glycosidases or by promoting their removal by sulfate-dependent receptors (88). Based on data presented in this chapter, the HA-reactivity in monocytes that is attributed to the sulfation of CD44 would be a mechanism consistent with the first theme. Figure 4.13 illustrates a model for sulfate incorporation on CD44H in PBM following exposure to inflammatory stimuli that promotes a functional, HA-reactive form of CD44. The sulfation of CD44 may enhance the affinity as well as the avidity of CD44 molecules on the cell surface for HA, possibly by facilitating CD44 aggregation or a conformational change.

## **4.3.2iii** Sulfated epitopes are induced on CD44 by TNF- $\alpha$

TNF- $\alpha$  induced the expression of 6-sulfo LacNAc / Lewis x epitopes on TNF- $\alpha$  stimulated SR91 cells (230) as recognized by the AG107 mAb (271). The epitope was expressed on CD44 immunoprecipitated from stimulated cells following neuraminidase treatment and western blotting with the carbohydrate specific AG107 mAb (230). Here, TNF- $\alpha$  or IFN- $\gamma$  induced the expression of this same epitope, 6-sulfo LacNAc/Lewis x, on primary monocytes as both mAbs AG105 and AG107 reacted with stimulated PBM after neuraminidase treatment. Fresh PBM did not express 6-sulfo LacNAc epitopes (data not shown) recognized by the DD2 mAb, although PBM cultured for 72 hr in the presence or absence of TNF- $\alpha$  did. The results from flow cytometry analysis suggest that monocytes express 6-sulfo LacNAc-containing structures when cultured and can be induced by TNF- $\alpha$  to express 6-sulfo Lewis x determinants.

In the absence of stimulation, KG1a cells constitutively bind high or low levels of HA. Although total sulfate incorporation on CD44H did not correlate with HA-binding potential, 6-sulfo LacNAc, detected by the DD2 mAb in flow cytometry analysis, was enhanced on high HA-binding cells. The increased expression of mAb DD2-reactive determinants in HA<sup>high</sup> KG1a cells contrasts with results from PBM. The shift in the expression of a sulfated epitope on KG1a cells with no change in the sulfation state of the predominant sulfated surface receptor, CD44, indicates that either the epitope is not expressed on CD44 or if it is, these data re-emphasize the importance of dissecting the precise nature, location, and cell surface levels of individual sulfated moieties. The presence and location of determinants on CD44 from KG1a cells and PBM is yet to be determined. There is no evidence at this point to support the presence of a 6-sulfo LacNAc or 6-sulfo Lewis x determinant on CD44 in KG1a cells or PBM, as the antibodies have weak affinity for their antigen and failed to react with lysate proteins or immunoprecipitated CD44 by western blots.

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**FIGURE 4.13 Proposed regulation of HA-binding by sulfation on CD44 in PBM.** In myeloid cells with low capacity for HA-binding, CD44H is sulfated on short chain GAGs in the membrane proximal region (a). Inflammatory stimuli (b) induce sulfate incorporation on CD44H but more importantly, promote sulfation of N-glycans in the amino terminal, HA-binding region of CD44H. The shift in sulfate distribution may affect both affinity and avidity of CD44 for HA through a conformational change (c) and/or receptor clustering.

Monocytes were analyzed for the binding of sulfo-epitope specific mAbs only after 72 hr of culture in the presence or absence of stimulation when induced HA-binding was maximal in the majority of cells. Sulfated epitopes recognized by the antibodies AG105, AG107, DD2 or mAbs that were non-reactive on cells at the 72 hr time point may be expressed prior to this time. This analysis may provide temporal insight to the expression and functional significance of these sulfated epitopes and the relation, if any, to HA-binding potential of monocytes. Given the nature of the sulfated epitopes, it can be speculated that sulfated LacNAc on N-or O-glycans of CD44 may positively regulate HA-binding. However, the role of sulfated Lewis x epitopes on CD44 may also suggest a function other than promoting HA-binding. A 98 kDa form of CD44, HCELL (hematopoeitic cell, E- and L- selectin ligand) in KG1a cells has been reported to act as a O-sialofucosylated N-glycan dependent, but sulfation independent, L-selectin ligand that can support L-selectin mediated rolling (324, 325). The MECA-79 mAb recognizes a sulfated terminal 6-sulfo LacNAc epitope on core 1 mucin type O- or N-glycans on HEV. This antibody was not reactive against PBM (data not shown), suggesting that the determinant on monocytes is distinct from the L-selectin ligands expressed on HEV. CD44 on CD34<sup>+</sup> progenitor cells has also been reported to be an E-selectin ligand, with engagement to E-selectin being mediated by sialylated and fucosylated determinants on N-glycans of CD44, but again, the interaction was independent of sulfation (326). It is inviting to suggest that CD44 may engage selectins and HA simultaneously or sequentially during the initial tethering steps of monocytes on the blood vessel lining in response to inflammatory stimuli. The expression of sulfated Lewis x epitopes on CD44 may encourage cell-cell rather than cell-ECM interactions by blood borne leukocytes during trafficking or in myeloid progenitor cell mobilization from the bone marrow, previously shown to be regulated by sulfated fucans (327). At this point, the function of induced sulfated epitopes is purely speculative. These data provide evidence that sulfated epitopes are present on monocytes and are induced on PBM in response to TNF- $\alpha$ , but the expression pattern of these determinants on CD44 and their contribution to HA-binding is unknown.

## 4.3.3 Regulation of CD44 sulfation by sulfotransferases

Cystic fibrosis (CF) cells in culture showed altered sulfation and glycosylation of glycoproteins compared to wild type cells (328, 329), and the sulfate content on bronchial mucins secreted by CF patients was increased (330-332). In a comparison of sialic acid, sulfate, and sialyl Lewis x content in either chronic bronchitis or CF in patients with or without infection, the sulfate content on mucins was higher in infected patients than that on mucins from non-infected patients. Thus,

a link between inflammation and glycosylation (333) exists, and more specifically, these datasuggest that severe inflammation controls sulfation of bronchial mucosa (334). This raises the question of how inflammation affects posttranslational modifications such as glycosylation and sulfation. To answer this question, the signaling machinery that regulates glycosyltransferases and sulfotransferases in response to inflammatory agents such as TNF- $\alpha$  must be unraveled. Certainly neutrophils, T lymphocytes, and endothelial cells involved in inflammation and several epithelial tumor cells have the machinery to synthesize sialyl Lewis x determinants (335, 336) as well as change the expression of Lewis x epitopes and their derivatives on glycoproteins in response to TNF- $\alpha$  (337-339). To date, PKC and TGF- $\alpha$  were shown to regulate the activity of a sulfotransferase in renal carcinoma cells (340), whereas sulfotransferase activity in rat salivary glands was stimulated by phosphatidylinositol and sphingosine (341), and sulfation of hematopoietic tumor cell proteins appeared to be stimulated by differentiation-inducing agents (342).

Carbohydrate sulfotransferases are transmembrane residents in the golgi network that recognize and modify glycans attached to lipids and proteins along the secretory pathway (reviewed in 343, The first carbohydrate sulfotransferase, a chick chondroitin-6-sulfotransferase, was 344). reported in 1995 (345). Specific data concerning the expression of sulfotransferases in mammalian cells is limited at this point. In Drosophila, sulfateless (sfl) is a sulfotransferase mutant with impaired signaling (346), and *pipe* is a sulfotransferase mutant that blocks dorsoventral polarity (347). Carbohydrate sulfotransferases likely regulate extracellular signaling and adhesion by generating unique ligands on a sugar scaffold similar to the function of tyrosine sulfotransferases also present in the Golgi network. The expression pattern of these sulfotransferases (STs) is restricted, for example, GlcNAc6ST-3 has been shown to be important in generating L-selectin ligands specifically in HEV (reviewed in 348). The site for sulfate incorporation on the 6-sulfo LacNAc / Lewis x determinant that was identified on stimulated PBM occurs on the 6-N-acetyl glucosamine. Six sulfotransferases are capable of catalyzing sulfate addition to this site have been identified in humans. GlcNAc6ST-1 (CHST-2 or GST-2, (349) and GlcNAc6ST-4 are more widely expressed and are potential candidates for sulfation of CD44 sugars in monocytes. Identification of the ST responsible for the sulfation of CD44 is currently under investigation in the laboratory. The sulfotransferase obviously determines the generation of CD44 sulfotopes and thus, regulates any biological functions dependent on this sulfated moiety.
#### 4.3.4 Summary

These data presented in this chapter demonstrate that sulfation is induced on CD44 by proinflammatory cytokines, which directly assists in HA-binding, a regulatory mechanism not seen in antigen-activated T lymphocytes. The work reveals the complexity of sulfate incorporation, residing on three different oligosaccharide modifications to CD44H. Sulfate distribution shifts on CD44H following inflammatory agent activation of PBM or BMDM, correlating with enhanced HA-binding. These data suggests that the sulfation of CD44H occurs on glycans and GAGs but is induced by inflammatory agents only on glycans. Furthermore, using an array of monoclonal antibodies against sulfated epitopes, determinants containing 6-sulfo LacNAc and 6sulfo Lewis x structures were identified on high HA-binding KG1a cells and TNF- $\alpha$ -stimulated PBM, potentially representing a novel sulfated epitope on CD44 that is required for ligand reactivity. The induced expression of CD44 with an altered sulfate distribution is an independent regulatory mechanism that promotes HA-binding in myeloid cells.

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### CHAPTER FIVE

HA-binding requires an F-actin dependent localization of CD44 and phosphorylated ezrin/radixin/moesin (ERM) proteins to membrane protrusions

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#### **5.1 INTRODUCTION AND RATIONALE**

Prior to this investigation, reports indicated that the cytoplasmic domain of CD44 (CD44cyt) is required for binding high levels of HA (115, 350, 351). A truncated form of CD44 lacking all but the first 6 amino acids of the cytoplasmic domain was unable to bind to HA when expressed in AKR T cells (350). The first 16 amino acids of CD44cyt were critical for anti-CD3 mAb-, PMA- or anti-CD44 mAb-induced HA-binding by Jurkat cells transfected with CD44H or the deletion mutant (352). Further, melanoma cells transfected with CD44 lacking the cytoplasmic tail failed to migrate on HA-coated plates (169). The regulation of HA-binding by the cytoplasmic domain of CD44 may be an inducible mechanism tightly linked to changes to the cytoskeleton given that TNF- $\alpha$  and LPS can induce actin-reorganization (Figure 3.12 and 200) and HA-binding in PBM ((215), Figure 3.1, and Figure 3.2). Further, CD44 in resident and elicited macrophages differs in detergent solubility, suggesting that macrophage activation results in changes in the association of CD44 with the cytoskeleton (220). Data presented in chapter three illustrated that TNF- $\alpha$  can induce HA-binding and morphological changes in murine macrophages. Furthermore, HA-binding by KG1a cells, or HA-binding induced in SR91 cells, PBM, or BMDM was reduced in cells treated with cytochalasin D or latrunculin A, reagents that destabilize actin filaments (286). The effect of cytochalasin D varied between the cells studied, suggesting that the actin cytoskeleton regulates HA-binding, but to varying degrees in cells of the myeloid lineage. These reports offer increasing evidence that myeloid cell activation, HA-binding, and cytoskeletal rearrangement are coordinated events. Questions remained unanswered regarding how the cytoplasmic domain of CD44 and the cytoskeleton interact, what regulates the interaction, and by what mechanism does this intracellular association regulate extracellular adhesion to HA.

The human myeloid progenitor cell line, KG1a, was used to investigate mechanisms by which the actin cytoskeleton may regulate CD44-mediated HA-binding. The majority of KG1a cells in culture extended F-actin-rich pseudopods (Figure 3.12), and the majority of cells constitutively bound high levels of HA (262). The heterogeneous HA-binding displayed in the KG1a cell population was used to establish two stable subpopulations of KG1a cells based on selection for high or low HA-binding (Figure 3.6, HA<sup>high</sup> and HA<sup>low</sup> KG1a cells). The populations were generated to establish differences that justify their differential binding of HA. Sulfation was established as a major mechanism to regulate HA-binding in SR91 cells and PBM (discussed in chapter four). HA-binding by KG1a cells was moderately reduced after the cells were cultured in the presence of sodium chlorate. HA<sup>high</sup> and HA<sup>low</sup> KG1a cells were labeled with radioisotope to determine the sulfation status of CD44. Sulfate incorporation to CD44H was equivalent in KG1a subpopulations with differential HA-binding ability (Figure 4.8). Due to the complexity of CD44 sulfation revealed in chapter four, the contribution of unique sulfated epitopes to the difference in HA-binding by the HA<sup>high</sup> and HA<sup>low</sup> cell populations in the absence of an overall difference in total sulfate incorporation on CD44H cannot be excluded. However, destabilization of F-actin reduced HA-binding to a more significant extent than did sodium chlorate (Table 3.4) suggesting that F-actin may play a substantial role in regulating HA-binding in KG1a cells. This provided support that the HA<sup>high</sup> and HA<sup>low</sup> KG1a cells are a useful system to study sulfation-independent factors, particularly the relationship between CD44 and the actin cytoskeleton, that cause the ligand binding function of CD44 to differ between the subpopulations. Confocal microscopy was used to visualize the distribution of CD44, F-actin, and HA-binding on KG1a cells. The expression of potential actin-binding proteins in cell lysates and in association with immunoprecipitated CD44 was determined by western blotting. Actin-binding proteins associated with CD44 were identified and their distribution in HA<sup>low</sup> and HA<sup>high</sup> KG1a cells was studied by confocal microscopy. The study was extended to primary cells, studying the same actin-binding proteins identified in the KG1a cell system in terms of their expression, localization, and interaction with CD44 in peripheral blood monocytes prior to and following TNF- $\alpha$  stimulation.

#### **5.2 RESULTS**

#### 5.2.1 HA-binding and CD44 are localized to the pseudopod of KG1a cells

The majority of KG1a cells extended a pseudopod (Figure 3.12 and 285) that was rich in Factin and destroyed by cytochalasin D treatment (Figure 3.12). Figure 5.1A demonstrates that CD44 was markedly localized to the pseudopod. The localization was specific to CD44 as two other cell surface molecules, CD34 and CD45, showed an even distribution between the pseudopod and the cell body (Figure 5.1B and C). HA-binding was dependent on the presence of actin filaments as agents that disrupted these fibers, cytochalasin D and latrunculin A, abolished FL-HA-binding in flow cytometry analysis (Figure 3.12). Examination of FL-HAbinding by confocal microscopy showed that like CD44, HA-binding was restricted to pseudopods, and in particular, to the tips of these structures (Figure 5.1D). HA-binding was



**FIGURE 5.1 CD44 and FL-HA-binding are specifically localized to KG1a cell pseudopods.** A-E Fluorescent micrographs of KG1a cells compiled from a series of confocal images using NIH Image software (see material and methods for details). Cells were labeled with (A) anti-CD44, (B) anti-CD34, (C) anti-CD45, (D) FL-HA, and (E) secondary antibody alone. The scale bar represents 4 μm. These confocal images are representative of results obtained in a minimum of 3 separate experiments. mediated by CD44 (261), and since CD44 was expressed exclusively in the pseudopod, the question arose as to what influence the distribution of CD44 has on binding HA.

#### 5.2.2 CD44 localization in the pseudopod is not affected by sodium chlorate

KG1a cells incubated with sodium chlorate had reduced HA-binding (Figure 3.11). The mechanism by which sulfation affects HA-binding in KG1a cells is not known. In PBM, the data presented in the previous chapter (far western blot, Figure 4.11) suggested that sulfation can directly contribute to the affinity between CD44 and HA. CD44 on KG1a cells was sulfated (Figure 4.7). To determine if inhibition of sulfation effects the distribution of CD44 to the pseudopod, confocal microscopy was performed on KG1a cells after treatment with 0 or 50 mM sodium chlorate for 72 hr. Figure 5.2 demonstrates that pseudopod formation was not affected by treated with sodium chlorate. Some staining for CD44 was observed in the cell body, but the majority of CD44 remained highly restricted to the pseudopod following incubation of KG1a cells in the presence of the sulfation inhibitor. Although posttranslational sulfation affects HA-binding without altering the localization of CD44 in the pseudopod.

#### 5.2.3 CD44 localization in the pseudopod is disrupted by cytochalasin D

Figure 3.12 demonstrated that the KG1a pseudopods are comprised of F-actin. It also demonstrated that in KG1a cells treated with cytochalasin D, a reagent that destabilizes actin polymers, the pseudopod collapsed, actin polymers were dispersed, and FL-HA-binding was significantly reduced (Figure 3.12). To determine if CD44 localization in the pseudopod was disrupted when coalesced actin filaments were perturbed, KG1a cells were treated with or without cytochalasin D or latrunculin A then the distribution of CD44 was examined by confocal microscopy. Figure 5.3 shows a loss in the localized distribution of CD44 in KG1a cells treated with cytochalasin D over a time course (1 - 60 min), the loss in CD44 localization was synchronized with the destruction of the pseudopod and decreased FL-HA-binding (data not shown). These findings suggested that a filamentous actin network is required for maintenance of the pseudopod, localization of CD44, and HA-binding by KG1a cells.



FIGURE 5.2 Sulfation is not required for CD44 localization to the pseudopod. Compiled confocal images of KG1a cells labeled with anti-CD44 antibody following 72 hr incubation in the presence (+ NaClO<sub>3</sub>, right panel) or absence (left panel) of 50 mM sodium chlorate. Scale bar is 4  $\mu$ m. These images are representative of the results obtained in five repeat experiments.



FIGURE 5.3 Cytochalasin D destroys CD44 localization in pseudopods. Distribution of CD44 in KG1a cells following incubation in the presence (+ cyto. D, right panel) or absence (left panel) of 50  $\mu$ M cytochalasin D for 1 hr. See materials and methods for details. Scale bar is 4  $\mu$ m. These data are representative of results from five repeat experiments.

# 5.2.4 ERM proteins co-immunoprecipitate with CD44, an interaction that is reduced in KG1a cells treated with cytochalasin D

A concentrated assembly of F-actin in the pseudopod was required for the structural integrity of the pseudopod and CD44 localization. This observation suggested that CD44 and F-actin may interact. CD44 cannot bind to F-actin directly, but it has been shown that CD44 can associate with actin-linker proteins such as ankyrin in murine T lymphoma cells (353) and the ezrin, radixin and moesin (ERM) protein family in baby hamster kidney (BHK) cells and mouse L cells fibroblasts (159). The identification of an actin-linker protein that associates with CD44 would provide evidence that an interaction between CD44 and F-actin can occur in the pseudopod. A yeast-2-hybrid screen using the cytoplasmic domain of CD44 was performed prior to this investigation (R. Li and P. Johnson, unpublished data), but by this method there was no evidence to suggest that actin-binding proteins interact with the cytoplasmic tail of CD44. In this study, western blot analysis was performed first to identify the presence of actinbinding proteins in the lysate of KG1a cells and second, to determine if these linker proteins associate with CD44 following immunoprecipitation from KG1a cells. Ankyrin, WASP, annexin I and VI were detected in KG1a lysates, but these proteins did not coimmunoprecipitate with CD44 (Figure 5.4A shows results for Annexin I). In contrast, ERM proteins were expressed in KG1a cell lysates and co-immunoprecipitated with CD44. Furthermore, the disruption of actin polymers in KG1a cells treated with 50 µM cytochalasin D or 10 µM latrunculin A caused a decrease in the quantity of ERM proteins that associated with CD44 (Figure 5.4B, first observed by R. Li). These data provide evidence that in KG1a cells, CD44 can interact with ERM proteins. Second, the interaction was diminished in cells treated with cytoskeletal-destabilizing agents, inferring that a CD44-ERM-actin complex exists. Third, the decreased association between ERM proteins and CD44 in cells treated with cytochalasin D or latrunculin A correlated with the collapse of the pseudopod and loss of CD44 localization to this protrusion in cells exposed to the F-actin destabilizing agents. Together, these data provided evidence that the CD44-ERM-actin complex occured in the pseudopod. Fourth, a decrease in CD44-ERM interaction correlated with a decrease in FL-HA-binding by KG1a cells, implicating the CD44-ERM association in the regulation of ligand binding by CD44 in F-



FIGURE 5.4 Co-immunoprecipitation of actin-linker proteins with CD44 from KG1a cells. A Western blot of Annexin I (upper panel) and CD44 (lower panel) expression in KG1a lysates (middle lane) or following immunoprecipitation of CD44 (right lane). + indicates the positive control for the Annexin I western blot. **B** Immunoprecipitation of CD44 from KG1a cells (-) or KG1a cells pre-treated for 1 hr with 50  $\mu$ M cytochalasin D (cyto. D), or 10  $\mu$ M latrunculin A (lat. A), or an equivalent amount of DMSO (DMSO). Immunoprecipitates were resolved by SDS-PAGE and probed with anti-ERM antibody (upper panel) and anti-CD44 antibody (lower panel). In **A** and **B**, the prestained molecular mass markers are indicated on the right in kDa. These data are representative of the results from three repeat experiments.

actin-rich KG1a cell pseudopods. To summarize, ERM proteins interacted with the cytoplasmic domain of CD44 creating a link between CD44 and the actin cytoskeleton, an interaction that potentially regulated both CD44 localization and HA-binding.

### 5.2.5 High HA-binding KG1a cells project single, distinct pseudopods

To further support the role for a CD44-ERM-actin complex in the regulation of HA-binding, the high and low HA-binding KG1a cells (described in chapter three, Figure 3.6) were examined for differences in morphology, CD44 localization (as described below) and ERM protein expression and localization (section 5.2.6). Figure 5.5A shows that HA<sup>low</sup> and HA<sup>high</sup> KG1a cells were morphologically distinct. The majority of the HA<sup>high</sup> cells (approximately 80%) had a single pseudopod. Unlike the HA<sup>high</sup> cells, only  $\sim 16\%$  of the HA<sup>low</sup> cells extend a single pseudopod whereas ~60% of cells had multiple projections (Figure 5.5A). Moreover, in the HA<sup>low</sup> cell population, the pseudopods were less extended than the pseudopods observed on HA<sup>high</sup> cells. Confocal microscopy was used to visualize the expression pattern of CD44 and sites of FL-HA-binding on the cell surface of HA<sup>low</sup> and HA<sup>high</sup> KG1a cells. The staining of CD44 on the HA<sup>high</sup> cells was more intense than that on the HA<sup>low</sup> cells (Figure 5.5A vs C). This result supports the finding from flow cytometry analysis (Figure 3.6) that CD44 expression is elevated on HA<sup>high</sup> cells. Figure 5.5Bc demonstrates that CD44 was concentrated in the pseudopod on the majority of HA<sup>high</sup> cells (CD44 was localized to the pseudopod in ~87% of cells). In contrast, CD44 expression on the HA<sup>low</sup> cells was decreased, and CD44 was not exclusively localized to the pseudopod. Approximately 50% of the HA<sup>low</sup> cells have a global distribution of CD44 (Figure 5.5Ba). Similar to FL-HA-binding in the unsorted KG1a population (Figure 5.1D), FL-HA bound to the tips of HA<sup>high</sup> cell pseudopods (Figure 5.5Bd). On the HA<sup>low</sup> cells, very little FL-HA-binding was observed (Figure 5.5Bb). This result infers that insufficient levels of CD44 were localized on HA<sup>low</sup> cells to support a detectable level of FL-HA-binding by immunofluorescence microscopy. A single extended F-actin-rich pseudopod on HA<sup>high</sup> KG1a cells illustrates that the pseudopod structure is necessary for the presentation of a high density of CD44, a key factor in promoting HA-binding in KG1a progenitor cells. These data re-emphasizes that CD44 localization correlates with HA-binding ability and raise the question of how CD44 sequestration in the pseudopod of HA<sup>high</sup> KG1a cells differs from that in HA<sup>low</sup> KG1a cells.

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HAlow

HAhigh



FIGURE 5.5A KG1a cells with high HA-binding capacity (HA<sup>high</sup>) concentrate CD44 in a single, prominent pseudopod. Phase contrast image of HA<sup>high</sup> and HA<sup>low</sup> KG1a cells. Scale bar represents 4  $\mu$ m. Graph illustrates the percentage of cells with zero (0), one (1), or greater than one (>1) pseudopod on more than 200 high (white bars) or low (black bars) HA-binding KG1a cells.

Α



FIGURE 5.5B KG1a cells with high HA-binding capacity (HA<sup>high</sup>) concentrate CD44 in a single, prominent pseudopod. Fluorescent micrographs of HA<sup>high</sup> (right panel) and HA<sup>low</sup> (left panel) KG1a cells compiled from a series of confocal images using NIH Image software (see material and methods for details). Cells were labeled with (**a**, **c**) anti-CD44 or (**b**, **d**) FL-HA. The scale bar is 4  $\mu$ m. These images are representative of the results obtained from three repeat experiments.

В

# 5.2.6 ERM proteins are elevated in HA<sup>high</sup> cells, and phosphorylated-ERM proteins are localized in the pseudopod

The co-immunoprecipitation of ERM proteins with CD44 from KG1a cells (Figure 5.4) suggests that ERM proteins link CD44 to F-actin polymers that are concentrated in the pseudopod of KG1a cells. Since CD44 is frequently localized to the pseudopod in HA<sup>high</sup> but not HA<sup>low</sup> cells, the expression and distribution of ERM proteins with CD44 was compared between the high and low HA-binding cells. HA<sup>high</sup> and HA<sup>low</sup> cell lysates were resolved by SDS-PAGE and western blot analysis revealed that, like CD44, the level of ERM proteins was elevated in the HA<sup>high</sup> cells (approximately 1.5 times the expression of ERM proteins in the HA<sup>low</sup> cells, Figure 5.6A). ERM proteins can be resolved to 2 to 3 bands by SDS-PAGE. The lower band represents moesin, which appears to be the most abundant family member expressed in KG1a cells, and the upper band (or two) is ezrin and radixin. The phosphorylation of ERM proteins at conserved threonine residues is indicative of an open, 'active' ERM protein conformation that can facilitate the linkage of membrane proteins to the actin cytoskeleton. In the open form, the C-terminal domain of ERM may interact with actin, and the N-terminal domain may bind to the cytoplasmic region of a membrane protein (354). Using a phosphothreonine specific antisera against threonine residues 567, 564, and 558 on ezrin, radixin, and moesin respectively, Figure 5.6A also illustrates that HA<sup>high</sup> KG1a cell lysates had elevated levels of phosphorylated-ERM proteins.

To investigate the distribution of ERM and phospho-ERM proteins in the HA<sup>high</sup> and HA<sup>low</sup> KG1a populations, cells were labeled with anti-ERM or anti-phospho-ERM antibodies then analyzed by confocal microscopy. Figure 5.6B shows that ERM staining was faint, yet ERM proteins were consistently distributed throughout the cytoplasm in both HA<sup>high</sup> and HA<sup>low</sup> cells. Staining with antisera specific for moesin (355) also resulted in a diffuse staining pattern throughout the KG1a cells (data not shown). In contrast, the phospho-ERM proteins had a restricted pattern of expression. In the majority of the HA<sup>high</sup> cells (~79% based on 50 cells), phospho-ERM proteins were specifically localized to the pseudopod (Figure 5.6Be). In the HA<sup>low</sup> cells, phospho-ERM proteins were present in clusters or patches throughout the cell (Figure 5.6Bb). Single pseudopods occur with less frequency in HA<sup>low</sup> cells, yet when distinct pseudopods were observed, phospho-ERM proteins were localized to this region only 50% of the time (n = 3, 50 cells per experiment).

136



Α

83

HAlow

HA<sup>high</sup> HA<sup>low</sup> HA<sup>high</sup>

FIGURE 5.6 Phosphorylated-ERM proteins are enhanced in HA<sup>high</sup> KG1a cell pseudopods. A Western blot of ERM (left panel) and phosphorylated-ERM (right panel) expression in  $1 \times 10^6$  HA<sup>high</sup> and HA<sup>low</sup> total KG1a cell lysates. Results are from one experiment repeated 3 times. B Fluorescent micrographs of HA<sup>high</sup> (right panel) and HA<sup>low</sup> (left panel) KG1a cells compiled from a series of confocal images using NIH Image software (see material and methods for details). Cells were labeled with (a, d) anti-ERM, (b, e) anti-phospho-ERM, and (c, f) secondary antibody alone. The scale bar is 4  $\mu$ m. These images are representative of the data obtained from four repeat experiments in which CD44, ERM, and phospho-ERM staining to pseudopods was determined by analyzing 50 compiled images. These data show that the expression of ERM and phospho-ERM proteins, like CD44, is increased in HA<sup>high</sup> cells. Both phospho-ERM and CD44 specifically localize to the pseudopod in high HA-binding cells. This coordinated expression pattern of CD44 and phospho-ERM proteins strongly suggests that phospho-ERM proteins are responsible for linking CD44 to F-actin within the pseudopod structure. Failure to co-localize results in a decreased ability to bind to HA as demonstrated in the HA<sup>low</sup> KG1a cells.

# 5.2.7 TNF-α induces re-localization of CD44 and phosphorylated ERM proteins in CD14<sup>+</sup> peripheral blood monocytes.

Cultured PBM did not bind or bound low levels of HA (Figure 3.1), had ruffled membranes (Figure 3.13), multiple extensions, and in these ways, resembled the HA<sup>low</sup> KG1a cells. TNF- $\alpha$ -stimulated PBM increased the expression of CD44 (Figure 3.1 and Table 3.1), had increased adherence to plastic, elongated (Figure 3.13), distributed CD44 in membrane extensions (Figure 3.13 insert), and acquired cytochalasin D-sensitive binding to HA-binding (Figure 3.13). In these ways, stimulated PBM were similar to HA<sup>high</sup> KG1a cells. Experiments were conducted to determine if the morphological similarities between low HA-binding KG1a cells and unstimulated PBM or high HA-binding KG1a cells and TNF- $\alpha$ -stimulated PBM could be extended to CD44 and phospho-ERM distribution. Although no significant difference was observed in ERM protein expression, TNF- $\alpha$  induced a small but consistent increase in phospho-ERM protein expression in PBM ( $1.3 \pm 0.2$  fold increase in phospho-ERM in lysates from TNF- $\alpha$ -stimulated PBM compared to lysates from unstimulated PBM, Figure 5.7A). Note that high HA-binding KG1a cells elevated ERM and phospho-ERM levels, but not phosphorylation per ERM. TNF- $\alpha$  stimulation of PBM caused an increase in phosphorylation events, not total protein expression, illustrating that the same effect (elevation of 'active' ERM proteins) may be achieved in more than one way, but in either case, an increase in phospho-ERM proteins correlates with HA-binding.

Phosphorylated ERM proteins co-immunoprecipitated with CD44 from  $HA^{high}$  and  $HA^{low}$  KG1a cells and from unstimulated and TNF- $\alpha$ -stimulated PBM (data not shown). These data indicate that the interaction between CD44 and phosphorylated ERM proteins can occur, yet there was no significant difference in the quantity of ERM proteins or phosphorylated ERM



B

A



**FIGURE 5.7 TNF-\alpha induces CD44 and phospho-ERM co-localization in PBM.** A Western blot of ERM (left panel) and phospho-ERM (right panel) expression in 2.5 x 10<sup>6</sup> PBM detergent soluble lysates following induction in the presence (+) or absence (-) of TNF-a for 72 hr. **Ba-f** Fluorescent micrographs represent a single confocal image (see material and methods for details) of PBM cultured in the presence (lower panel) or absence (upper panel) of TNF- $\alpha$  then double stained with (a, d) anti-CD44 with Alexa 594 labeled goat anti-mouse antibody and (b, e) anti-phosphorylated ERM with Alexa 488 labeled goat anti-rabbit antibody. Single slices are merged in the right column (c, f). The scale bar is 4  $\mu$ m.

proteins that associated with equal amounts of CD44 immunoprecipitated from HA<sup>high</sup> versus  $HA^{low}$  KG1a cells or between unstimulated and TNF- $\alpha$ -stimulated PBM. Coimmunoprecipitation of ERM proteins was performed under low physiological salt conditions (discussed in chapter one). Under these conditions, the CD44-ERM association could occur post-lysis, thus, the ability of phospho-ERM proteins to interact with CD44 in a coimmunoprecipitation experiment may not reflect the true association between the molecules under physiological conditions in the cell. For these reasons, the localization of CD44 and phospho-ERM proteins in PBM was investigated in situ by confocal microscopy. Figure 5.7Bb shows that like the HA<sup>low</sup> KG1a cells, unstimulated PBM had a patchy distribution of phospho-ERM in the cell. CD44 was distributed in a similar manner (Figure 5.7Ba) in unstimulated PBM and when the two labels (phospho-ERM and CD44) were merged, there was no significant co-localization of CD44 and phospho-ERM in unstimulated PBM (Figure 5.7Bc). Following TNF- $\alpha$  stimulation, CD44 and phospho-ERM proteins were localized at the cell periphery (Figure 5.7Bd,e). When the images were merged, there was a significant overlap between CD44 and phospho-ERM (Figure 5.7Bf). This indicates that TNF- $\alpha$  stimulates the relocalization of phospho-ERM and CD44 that results in their co-localization in PBM.

#### **5.3 DISCUSSSION**

#### 5.3.1 Regulation of HA-binding by the cytoplasmic domain of CD44

Several molecules have been identified that interact with the cytoplasmic domain of CD44 in T cells, PBMC, endothelial, and epithelial cells. Included are the Src-family tyrosine kinases Lck, Fyn, and Lyn (165, 188, 189), Rho-GDP dissociation inhibitor (Rho-GDI, 259), ankyrin (149, 167, 235, 353, 356), ERM (159), and Merlin (357), the protein product of neurofibromatosis type 2 (NF2) gene and a distant relative of the ERM protein family (reviewed in 358). An association between ERM proteins and CD44 was first described for a specific 140 kDa form of CD44 in baby hamster kidney (BHK) cells and then for the standard form of CD44 (CD44H) in a fibroblast cell line (159). The N-terminal domain of ERM interacts with a region of basic residues on CD44cyt that is very close to the transmembrane domain indicating that the interaction is ionic and occurs close to the membrane (359, 360). As stated previously, the phosphorylation of a conserved threonine residue in ezrin, radixin, or moesin, or PIP<sub>2</sub> binding to a consensus sequence also near the C-terminus is believed to maintain ERM proteins in an open conformation capable of interacting with F-actin and

membrane proteins. Co-immunoprecipitation of ERM proteins with CD44 requires the presence of  $PIP_2$  (259) or a sub-physiological concentration of salt (159 and this study). This is not specific to the CD44-ERM interaction as a low concentration of salt was also required for an *in vitro* interaction between ERM proteins and L-selectin (361) and  $PIP_2$  was reported to enhance ezrin binding to ICAM-1 and ICAM-2 (362).

Results presented in this chapter indicate that ERM proteins co-immunoprecipitated with CD44 from KG1a cells (Figure 5.4), an interaction that was reduced in KG1a cells treated with reagents that abolished the pseudopod and CD44 localization (Figure 5.3). The expression of CD44 was elevated in high HA-binding KG1a cells and TNF- $\alpha$ -stimulated PBM (Table 3.2). Phosphorylated-ERM proteins were also enhanced in the lysates of HA<sup>high</sup> KG1a cells (Figure 5.6A) and TNF- $\alpha$ -stimulated PBM (Figure 5.7A). The phosphorylated form of ERM was shown to specifically co-localize with CD44 in high HA-binding KG1a cell pseudopods and on PBM following stimulation with TNF- $\alpha$ . This is the first time that an association and co-distribution of CD44 and phospho-ERM proteins has been shown in activated monocytes and in myeloid progenitor cells. Furthermore, the co-localization of CD44 and phospho-ERM was induced in PBM by TNF- $\alpha$ . This also is the first time that an association between CD44 and phospho-ERM monocytes and in myeloid proteins has been linked to the induction of HA-binding. Overall, these data indicate that HA-binding in myeloid cells and TNF- $\alpha$ -activated primary monocytes is mediated by actin-dependent clustering of CD44 and phosphorylated-ERM proteins to extended membrane structures.

The current model by which the cytoskeleton regulates HA-binding is based on results that show that anti-CD44 mAbs can induce HA-binding, but Fab fragments cannot (363), that CD44 dimerization via cysteine residues in CD44cyt promote HA-binding in PMA-stimulated Jurkat T cells (163), and the-self association of CD44 molecules via the transmembrane domain promoted HA-binding in transfected BW5147 T cells (162). The mode of action for binding HA in these examples is embedded in the notion that the affinity of CD44 for HA is relatively low, yet a stable interaction can be achieved through co-operative binding by CD44 molecules. Results presented in this chapter support this general mode of action for HA-binding by CD44 on progenitor cells and PBM. A model (Figure 5.8) based on the data presented in this chapter proposes that the regulation of CD44 binding to HA is dependent on an association of the



**FIGURE 5.8 Proposed regulation of HA-binding via CD44 and phospho-ERM associations in Factin membrane protrusions.** (a) In a low HA-binding conformation, CD44 molecules on the cell surface and cytoplasmic ERM proteins are expressed in different patches around the cell (b) until inflammatory stimuli (c) enhances CD44 and ERM expression, F-actin assembly, and a CD44-phospho-ERM-F-actin complex (d) that is concentrated into multiple membrane extensions as seen in PBM (e) or a single projection as seen in KG1a cells (f). This creates extended membrane protrusions rich in CD44 to mediate HA-binding. cytoplasmic domain of CD44 with phospho-ERM proteins and actin. The elevated expression and clustering of CD44 and phosphorylated-ERM proteins in extended F-actin-rich membrane structures augments the local density of CD44 to favour co-operative HA-binding, particularly at the tips of the pseudopods.

#### 5.3.2 Monocyte membrane protrusions are distinct from T lymphocyte uropods

Chemokines induce T cell polarization that is characterized by the formation of an F-actindependent leading edge (lamellipodia) and a tubulin-dependent rear pseudopod-like structure, or uropod (364). Cellular polarization results in the sequestration of several adhesion molecules to the front or rear of the cell. ICAMs and CD44 in chemokine-stimulated T cells are expressed in the uropod and co-localize with moesin at the tip (365). Membrane protuberances have been detected on primary human CD34<sup>+</sup> cells (285). The inflammatory agents LPS and TNF- $\alpha$  can induce the reorganization of actin in human PBM [(204, 294) and Figure 3.13], and TNF- $\alpha$  supports the presence of membrane protrusions containing CD44 (Figure 3.12). Although the T cell uropods have some similarities with the KG1a pseudopods and extensions on myeloid cells, there are also several distinctions. A major difference is that pseudopods in myeloid cells are actin-based extensions (Figure 3.12). Both microtubule and myosin ATPase inhibitors known to disrupt T cell-uropod formation had little effect on the stability of the KG1a pseudopod (data not shown). In addition, ICAM-3 is normally localized to uropods, but it was not restricted to the pseudopod (data not shown). With respect to the biological purpose of such structures, the uropod is required for cell migration, a role that will be investigated in chapter seven for pseudopod-containing myeloid cells. Finally, the pseudopod is required for CD44-mediated HA-binding whereas the HA-binding ability of CD44 in the uropod is not known. In conclusion, the T cell uropods implicated in cell migration are distinct from F-actin-based pseudopods and protrusions observed on the KG1a progenitor cells and activated PBM.

#### 5.3.3 Activated ERM proteins are linked to F-actin based membrane structures

Several lines of evidence suggest that ezrin proteins, particularly multimers of ezrin, are present in, and involved with the generation of actin-rich structures, primarily microvilli (366-368). In *Drosophila*, moesin is the only ERM protein expressed, and it is required for anchoring microfilaments to the oocyte cortex, without which, severe defects in antero-posterior polarity develop (369). The distribution of ERM proteins is responsive to extracellular stimuli (355, 370, 371). For example, activation of platelets with thrombin induced the localization of moesin to newly formed filopodial and lamellipodial projections (370). Epithelial growth factor (EGF) enhanced the elongation of microvilli and membrane ruffles (372) by the recruitment and oligomerization of ezrin proteins in A431 cells (371). Antisense oligonucleotides for ERMs were injected into epithelial MTD-1A cells and thymoma L5178Y cells and suppressed microvilli formation (373), providing more evidence that ERM proteins have membrane-organizing properties. COS-1 fibroblasts transfected with the PIP<sub>2</sub>-containing domain of ezrin form long filopodia (374), but this was not found in CV1 cells (375). Mutants of ezrin that lack the PIP<sub>2</sub> binding region were capable of forming multimers, yet they were not able to interact with actin (255). These data suggests that the  $PIP_2$  binding site of ezrin may act as a target for ERM localization, for docking to membrane proteins, and as a nucleation site of F-actin-rich structures. Alternatively, the over-expression of a phosphomimetic form of moesin (T559D) in Drosophila imaginal discs resulted in a marked up-regulation of cortical F-actin, complementary to the loss in apical actin seen in moesin-deficient cells (376). A similar mutation was introduced in human moesin that activated F-actin binding in vitro (256), implying that moesin controls actin assembly.

Actin polymerization may be nucleated by the expression and activation of ERM proteins but also by an increase in cytoskeletal-binding protein complexes. For example, over expression of cytoskeletal or lipid-binding proteins results in the elongation of microvilli (377, 378). In fibroblasts, the over-expression of CD44 is thought to be an organizing center that together with activated ERM proteins can recruit actin filaments and promote microvillar elongation (379). In these experiments, chimeras containing the extracellular domain of E-cadherin with a CD44 transmembrane and cytoplasmic domain induced elongation of microvilli in L and CV-1 fibroblasts (359). However, in epithelial MTD-1A and A431 cells, the E-cadherin/CD44 chimera was excluded from preexisting microvilli, clearly indicating that the induction of membrane extensions may be cell type dependent. In other studies on a melanoma cell line, RPM-MC, the localization of ezrin to microvilli did not depend on CD44 expression (360) but instead, may be regulated by cytoskeletal associations (375). Without the formation of proper membrane structures, CD44 and ERM proteins do not co-localize in EGF-stimulated A431 cells (380). At present, it is not known whether CD44 or ERM localization or actin polymerization in KG1a cells triggers pseudopod formation, only that F-actin is essential for the integrity of the pseudopod.

### 5.3.4 Phosphorylation of ERM proteins and CD44

In HA<sup>high</sup> KG1a cells, phospho-ERM, but not total ERM, proteins were localized to the pseudopod (Figure 5.6). A similar distribution pattern was observed in MTD-1A and A431 cells where phospho-ERM proteins were associated with the plasma membrane, but total ERM proteins were distributed in both the cytoplasm and plasma membrane (381). Further, EGFtreated A431 cells preferentially concentrate phosphorylated-ERM proteins in elongated microvilli (379). Site-directed mutants that maintain ezrin in the open conformation, the predicted form of phospho-ERM proteins, were recruited to the plasma membrane and to elongated microvilli in A431 cells (379). The same effect was reported when a similar 'open' mutant of moesin was tranfected into COS7 cells (382). In RAW264.7 macrophages, Thr 558phosphorylated moesin co-localized with F-actin in filopodial protrusions (383). These data support the findings presented here regarding restricted phospho-ERM distribution and further suggest that phosphorylated ERM proteins are required for the formation of, and localization into, membrane protrusions. The phosphorylation of ERM proteins must be an inducible event as non-phosphorylated-ERM proteins have been detected in capillaries, intestinal villi, smooth muscle cells, epidermal cells of stratified epithelium and granulose cells surrounding oocytes (381). ROCK, PKC- $\alpha$ , and PKC- $\theta$  kinases have been implicated in the phosphorylation of ERM family proteins (234, 259, 384). In one study, ezrin was reported to bind to active PKC (385). Alternatively, in NIH 3T3 cells, the phosphorylation on Thr 567 of ezrin was dependent on RhoA and subsequent activation of ROCK, without which, ezrin failed to localize to membrane protrusions (386).

The fraction of CD44 molecules that is phosphorylated on a cell at any given time is not known. Phosphorylation on threonine residues or tyrosine residues has not been observed (351, 387, 388). It has been suggested that CaMKII may phosphorylate Ser 323 and Ser 325 (389) on the cytoplasmic tail of CD44, whereas Ser 291 has a consensus sequence for PKC (390). Through a mutational analysis, it was concluded that the serine residue 325 on CD44cyt is phosphorylated *in vivo* (388). The phosphorylation of Ser 325 was shown to be important for melanoma cell migration on immobilized HA but not adhesion to soluble HA (391). After phorbol ester treatment of a melanoma cell line, the phosphorylation of Ser 291 and the dephosphorylation of Ser 325 were observed. In this case, the association of CD44 with ezrin was reduced, but an association between CD44 and phosphorylated-merlin (392), another actin

binding protein, increased. These reports indicate that regulation of the phosphorylation status of ERM proteins and CD44 will have a direct impact on their interaction, distribution, and association with other molecules.

#### 5.3.5 Signaling to the actin cytoskelton

#### 5.3.5i Activation of PKC

The results of a previous report determined that approximately 20% of the KG1a cell population spontaneously bound to immobilized HA in a CD44-dependent manner (261). This interaction was prevented by staurosporine, an inhibitor of PKC and PKA. Conversely, treatment with TPA, an agonist of PKC, stimulated 80% of cells to adhere to HA, suggesting that PKC-signaling regulates HA-binding in KG1a cells. Activation of PKC was also required for moesin, but not ezrin or radixin, binding to L-selectin in mouse lymphocytes (361). A dependence on PKC for the induction of HA-binding may be related to the PKC-dependent activation and localization of ERM proteins. However, in EGF-stimulated A431 cells PKC-theta activation led to the disruption of the CD44-ezrin interaction and in this case, promoted cell motility (380).

#### **5.3.5ii** Activation of Rho GTPases

Rho family proteins are regulatory molecules for the organization of the actin cytoskeleton (393-395). In human umbilical vein endothelial cells attached to collagen, but not fibronectin, RhoA was translocated to F-actin-rich focal contact domains of lamellipodia (396). It has been demonstrated that Rho directly interacts with the N-terminus of ERM proteins *in vitro* (397). The regulation of ERM family proteins by Rho GTPase activation has been reported in NIH 3T3 cells where the activation of RhoA and ROCK induced the re-localization of phosphorylated-ezrin to actin-containing cell surface protrusions, that along with focal adhesion formation, was prevented by inhibition of ROCK (386). In *Drosophila* epithelial cells lacking moesin, Rho pathway activity was increased and epithelial cell migration was induced, whereas the presence of a phosphorylated-moesin mimic promoted Rho-dependent actin assembly (376). This was supported in mammalian LLC-PK1 epithelial cells that experienced an increase in Rho GTPase activation following transfection with a dominant negative form of ERM proteins, suggesting that ERM proteins negatively regulate Rho activity, rather than being a downstream target of this pathway (376).

It has been reported that in BHK cells, Rho-GDI co-immunoprecipitated with CD44-ERM complexes and the CD44-ERM interaction was regulated by Rho GTPase activation (259). In a subsequent report, Rho-GDI was shown to interact directly with ERM and contributed to Rho activation and actin reorganization (397).

Signal transduction pathways leading to the activation of Rho may also be triggered by TNF- $\alpha$ and facilitate a CD44-ERM interaction, clustering of receptors, and HA-binding. In Rat2 fibroblasts, TNF- $\alpha$  increased sphingomyelinase activity, ceramide formation, and Rho family GTPase activity. This pathway was held responsible for the formation of the majority of stress fibers in these cells and the induction of cytoskeletal reorganization (398). It can be envisaged TNF- $\alpha$ -induces signal transduction pathways through ceramide in myeloid cells that instigates Rho activation of ROCK and PI4K5 kinase. The action of these kinases to phosphorylate ERM proteins and produce PIP<sub>2</sub> would allow multimer formation by ERM proteins, an association with actin and CD44, and nucleation of actin-based structures. This pathway has the potential to generate the CD44-phospho-ERM-F-actin complex required for HA-binding by myeloid cells and PBM. Moreover, the transduction pathways that signal to the actin cytoskeleton converge through all Rho family GTPases. Another GTPase, Rac, was activated in L929 cells after TNF- $\alpha$  stimulation (399). Rho or Rac can be initiated by extracellular signals such as thrombin, EGF, PMA, LPA, FCS, or LPS (reviewed in 400) and may be responsible for actin, ERM, or CD44 reorganization and membrane ruffling documented in other reports that were initiated through different extracellular stimuli, but converge on the same signal transduction pathway to produce similar morphological outcomes.

#### 5.3.6 Biological function of adhesion molecule interactions with ERM proteins

A few reports have attributed an inducible biological function to the reorganization of ezrin, actin, and adhesion molecules. For example, ERM proteins are anchored in the immunological synapse (401), and are responsible for the localization of CD43, a necessary event for T cell activation (402-405). Non-adherent thymoma cells transfected with ezrin formed uropod-like extensions, re-localized ICAM-2 to the uropod, and became sensitive to NK killing (406). The re-localization of ezrin and F-actin has also been reported to be important for producing sites for Shigella entry in HeLa cells (407). Anti-CD44 antibodies reduced foci formation, ezrin recruitment, and Shigella entry. In a melanoma cell line, 'outside-in' signaling, initiated from

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engagement of CD44, resulted in co-immunoprecipitation of de-phosphorylated Merlin with CD44 and inhibition of cell growth. Phosphorylated-ERM proteins associated with nonengaged CD44 when the cells were at low densities to maintain growth (408). It has been demonstrated that the cytoplasmic domain of CD44 (CD44cyt) is required for CD44-mediated melanoma cell migration (115, 350, 351) and melanoma cell movement on HA coated plates, thus implicating the cytoplasmic tail in cell migration events (169). However, studies in a human melanoma cell line implied that CD44-ERM association was neither required for HAbinding in these cells nor was it required for cell motility on laminin (392).

In this chapter, evidence demonstrates that on  $CD34^+$  hematopoietic progenitor cells and TNF-  $\alpha$  activated PBM, the elevated expression and clustering of CD44 and phosphorylated-ERM proteins to cytochalasin D-sensitive membrane protrusions is a mechanism to regulate HAbinding. These data indicate that an association and co-localization of CD44 and phospho-ERM occurs in myeloid cells to promote HA-binding and potentially, cell adhesion to the ECM. This raises the possibility that the association of CD44 with phospho-ERM in myeloid cells occurs to promote cell adhesion, and events that disrupt this interaction may promote cell migration.

### CHAPTER SIX

## HA-binding capability determines myeloid cell motility

#### **6.1 INTRODUCTION AND RATIONALE**

The results presented within the previous chapters addressed where, when, and how HAbinding is regulated in monocytes. What remains to be determined is why the interaction occurs, that is, to what advantage does cytokine-induced activation of CD44 and the engagement of HA confer to monocytes? Although CD44 could be involved in many aspects of monocyte function, findings from experiments presented in earlier chapters of this thesis focused attention on the migratory ability of monocytes.

It is well understood that leukocytes exit the blood at positions along the vascular endothelium adjacent to sites of inflammation. The process of leukocyte transmigration to damaged tissues is delineated in the cell adhesion cascade that was discussed in chapter one. To reiterate, the initial contact between leukocytes and endothelial cells is mediated by an interaction between selectins and their ligands. Subsequent firm adhesion results from engagement of chemokineactivated integrins. Leukocyte transmigration across the endothelial layer is less well defined, as is the migration within the inflamed tissue. Inflammatory cytokines produced during an immune response can stimulate an increase in HA expression on endothelial cells (189) and HA-binding by some activated T cells (101, 224, 409) and monocytes (204, 294). CD44-HA interactions have been shown to be important in T cell rolling in vitro on immobilized HA (101), suggesting that a CD44-HA interaction could promote leukocyte-endothelial cell interactions. However, the time required for the induction of HA-binding in monocytes places a CD44-HA interaction downstream of the initial rolling steps in the cascade. The induction of HA-binding in a small percentage of PBM required a minimum of 24 hr to occur (data not shown). Monocytes infiltrate damaged tissue within 24 hr, at which time, the vast majority of cytokine-activated monocytes in vitro bound to low levels of HA (data not shown). HAbinding was highest in the majority of PBM after 72 hr of activation. If CD44 is involved in the initial tethering and rolling of PBM in the cascade, as implied to occur in T lymphocytes, it is likely through a low avidity interaction with HA. A strong, stable CD44-HA interaction induced during an inflammatory response may regulate the motility of maturing monocytes within the inflamed tissue, downstream from the initial tethering and rolling steps of the extravasation cascade.

To determine if CD44-HA binding regulates the migratory ability of monocytes as they mature and migrate to the inflamed site, the content of this chapter describes preliminary data using a three-dimensional (3D) gel matrix system to monitor myeloid cell adhesion and migration. A 3D gel system is more complex, but potentially more informative and physiologically relevant than the more common two-dimensional (2D) migration assays. Two-dimensional analysis of migration has yielded significant findings with respect to the chemotaxis and transmigration of cells however, the physiological relevance is controversial, as similar experiments performed in 2D and 3D yielded different results (discussed in 410, 411). This suggests that cells are phenotypically and behaviourally different when two- versus three-dimensional contact is made with the surrounding environment. Even within 3D matrices, two modes of tumour-cell motility have recently been described that are controlled by Rho signaling pathways and are associated with different cell morphologies (412).

Matrigel<sup>TM</sup> basement membrane is rich in ECM components and was chosen to create a threedimensional matrix *in vitro* to simulate the ECM of tissues. HA was incorporated into the gels at a final concentration similar to that in tissues (0.5 mg/ml). HA<sup>high</sup> and HA<sup>low</sup> KG1a cells were chosen to study the effect of HA-binding ability on cell adhesion and migration in the gel matrices. The KG1a subpopulations have constitutive, homogeneous, high or low binding to HA, unlike the primary PBM that require stimulation to induce bimodal binding to HA. The invasive properties of BMDM were analyzed as HA-binding tends to be homogeneous yet different prior to and after TNF- $\alpha$  stimulation of BMDM.

#### 6.2 RESULTS

# 6.2.1 HA<sup>low</sup> KG1a cells elongate on the Matrigel<sup>™</sup> ECM, which is augmented by the presence of HA

For adhesion and migration experiments, matrices were constructed on filter inserts onto which cells were seeded in culture medium containing 2% or 10% serum. Culture medium with equivalent or 5-fold more serum (final concentration of 10%) was placed below the insert. Cells were allowed 2 hr to adhere to the matrices, then washed and photographed to determine the number of adherent cells. Both KG1a cell subpopulations formed adherent contacts with the matrix within 30 min in the presence or absence of a 5-fold serum gradient beneath the Matrigel<sup>TM</sup>. Figure 6.1 demonstrates that a small number of HA<sup>low</sup> KG1a cells elongated on the matrix alone. The percent of elongated cells increased when HA was incorporated into the gel (Figure 6.1, Matrigel<sup>TM</sup>-HA). Recall that HA<sup>high</sup> KG1a cells are highly polarized in suspension,



**FIGURE 6.1 HA**<sup>low</sup> **KG1a cell elongation is enhanced on Matrigel<sup>TM</sup> matrices containing HA.** Phase contrast images of HA<sup>low</sup> (left panels) and HA<sup>high</sup> (right panels) KG1a cells 2 hr after cells were seeded on Matrigel<sup>TM</sup> basement membrane matrix (upper panel) or matrix containing HA (lower panels). These images are representative of results obtained from three repeat experiments. projecting a distinct pseudopod. Although a few cells deviated from this morphology and elongated in a manner similar to HA<sup>low</sup> cells on Matrigel<sup>TM</sup>-HA, the pseudopod on HA<sup>high</sup> cells was primarily retained after the cells were seeded on the matrices.

Cell adhesion to matrices devoid of HA suggests that HA-independent adhesion and elongation can occur however, the incorporation of HA into the matrix enhanced both the number of elongated cells and the degree to which the cells elongated, particularly in HA<sup>low</sup> KG1a cells. The random, seemingly erratic membrane protrusions on low HA-binding KG1a cells in culture may be indicative of a dynamic cytoskeleton that can be rapidly reorganized in response to extracellular signals associated with engagement with the matrix. This reorganization of the cytoskeleton from matrix adhesion, suggests an outside-in signaling pathway from CD44 to the cytoskeleton. The rigidity of the pseudopod on high HA-binding cells does not suppress binding to the matrix, an interaction that occurred on matrices without HA, but may yield the cell less responsive to signaling or incapable of cytoskeletal rearrangement and cell elongation.

#### 6.2.2 Unstimulated, but not TNF-α-stimulated, BMDM elongate on Matrigel<sup>™</sup> ECM

BMDM were incubated in the presence or absence of TNF- $\alpha$  for 72 hr prior to incubation on Matrigel<sup>TM</sup> or Matrigel<sup>TM</sup>-HA. In the absence of a serum gradient, BMDM did not adhere or elongate on matrices, even with extended incubation time suggesting that chemotactic serum components are required for adhesion (Figure 6.2). In the presence of a ten-fold serum gradient, BMDM elongate in a manner similar to that of BMDM on tissue culture dishes. Unstimulated BMDM, with low HA-binding capacity, adhered and elongated after incubation for approximately 6 hr on the matrix (Figure 6.2). In the presence of HA, more BMDM are inclined to extend short, multiple projections (Figure 3.12) reminiscent of the phenotype of TNF- $\alpha$ -stimulated BMDM when cultured on tissue culture dishes. Although adherent to tissue culture plastic, TNF- $\alpha$ -stimulated BMDM, having enhanced HA-binding capacity, were reluctant to adhere to the Matrigel<sup>TM</sup> even in the presence of a serum gradient (Figure 6.3). TNF- $\alpha$  stimulated BMDM were particularly reluctant to adhere to matrices with HA (Figure 6.3). As with KG1a cells, these data using BMDM suggest an outside-in CD44 signaling



**FIGURE 6.2 Unstimulated BMDM elongate on Matrigel<sup>TM</sup> ECM in the presence of a serum gradient.** Phase contrast images 6 hr after BMDM were seeded on Matrigel<sup>TM</sup> basement membrane matrix (left panels) or matrix containing HA (right panels) without (1:1, upper panels) or with a ten-fold serum gradient below the matrix (1:10, lower panels). These images are representative of results obtained from three repeat experiments.



Figure 6.3 TNF- $\alpha$ -stimulated BMDM adhere poorly to ECM matrices. Phase contrast images of unstimulated BMDM (C57BL, upper panels) or BMDM stimulated with TNF- $\alpha$  for 72 hr (+ TNF- $\alpha$ , lower panels). Photos were imaged 6 hr after seeding cells on Matrigel<sup>TM</sup> basement membrane matrix (left panels) or matrix containing HA (right panels) in a ten-fold serum gradient. These images are representative of results obtained from three repeat experiments.

Pathway initiated on immobilized HA that can result in cytoskeletal rearrangement in low, but not high, HA-binding cells.

#### 6.2.3 Low HA-binding KG1a cells transmigrate faster than high HA-binding cells

In transwell migration experiments, matrices were formed in the same manner described for adhesion assays. Cells were seeded in media containing 5 to 10-fold less serum than that below the filter insert. HA<sup>low</sup> and HA<sup>high</sup> KG1a cellls were allowed 24 hr to transverse the matrices. Transmigrated cells that navigated through the Matrigel<sup>™</sup> into the lower chamber were quantified as a percent of input cells and averaged over a number of experiments. Both high and low HA-binding KG1a cells migrated in the absence of a serum gradient, suggesting that the cells are motile by nature. In fact, the matrix promoted the migration of cells compared to the filter alone (data not shown). Migration was enhanced when cells were placed in a 5-fold serum gradient favouring the lower chamber. The results are presented graphically in Figure 6.4, illustrating the percent of input cells that migrated through the matrix under each condition. Table 6.1 shows the relative range of transmigrated cells in the experiments conducted. On average, twice as many low HA-binding cells transmigrated through the Matrigel<sup>™</sup> containing HA than high HA-binding cells under the same conditions. Although cells can traverse the matrix in an HA-independent fashion, the presence of HA enhances the motility of cells, particularly those possessing a low capacity for HA. High HA-binding cells are capable of transmigration, and do so in a way that is augmented by HA, but migration occurs at a reduced rate compared to HA<sup>low</sup> cells.

The results suggest that a low strength CD44-HA interaction promotes elongation (demonstrated in Figure 6.1) and encourages cell movement (Figure 6.4). Although HA incorporated into the matrix favours cell migration, strong cellular binding to HA impedes cell movement.

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Cells	Gel Matrix	Serum Gradient	
		1:1 <sup>b</sup>	5:1 <sup>°</sup>
HA <sup>low</sup> KG1a	Matrigel <sup>™</sup>	0 - 2%	2 - 10%
( <sup>d</sup> n=4)	Matrigel <sup>™</sup> /HA <sup>a</sup>	0.5 - 3.5%	5 - 18%
HA <sup>high</sup> KG1a	Matrigel™	0 - 2%	2 - 4%
(n=4)	Matrigel™ /HA	0.5 - 2.5%	4 - 7%

Table 6.1 Percent range of HA <sup>low</sup> or l	HA <sup>high</sup> KG1a cells that transmigrate
through the gel matrix in the	presence or absence of a serum gradent

<sup>a</sup> Matrigel containing 0.5 mg/ml of HA
<sup>b</sup> 10% FCS above and below the gel matrix
<sup>c</sup> 2% FCS above and 10% FCS below the gel matrix, yielding a 5:1 gradient
<sup>d</sup> number of experiments

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**Figure 6.4 HA**<sup>low</sup> and HA<sup>high</sup> KG1a cell transmigration. The bar graph represents the percentage of input KG1a cells (Y axis) with low (left bars) or high (right bars) HA-binding that migrated through the 3D matrix with (black bars) or without (white bars) HA towards a 5-fold serum gradient. The percentages were averaged over four experiments and the standard deviation is indicated by error bars.

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#### 6.2.4 Involvement of CD44 in KG1a cell transmigration

To assess the involvement of CD44 in KG1a cell migration, KG1a cells were incubated with anti-CD44 neutralizing antibodies (that block HA-binding) immediately prior to transwell migration assays. The results are summarized in Figure 6.5. The migration of HA<sup>low</sup> cells through the Matrigel<sup>TM</sup> was not affected by the blocking antibodies, however, the migration of HA<sup>low</sup> cells through Matrigel<sup>TM</sup> containing HA was significantly reduced by anti-CD44 mAbs (to ~40 ± 3% of cell migration in the absence of blocking antibodies). The number of migratory cells was reduced to levels similar to the percent of migratory cells through Matrigel<sup>TM</sup>. This suggests that a degree of HA<sup>low</sup> KG1a cell migration was independent of CD44. Blocking antibodies inhibited the migration of high HA-binding cells to a lesser and similar degree in the presence or absence of HA in the matrix (the migration of cells incubated with anti-CD44 mAb was reduced to ~65 ± 15% of cell migration in the absence of blocking antibodies), suggesting that a portion of migration in these cells also occurs through CD44-HA independent mechanisms. Clearly, KG1a cell motility through 3D matrices is facilitated by a low-strength CD44-HA interaction.

#### 6.2.5 Unstimulated, but not TNF-α-activated, BMDM invade matrices

Unlike suspension cells, BMDM tether very strongly to surfaces, including the Matrigel<sup>TM</sup>. Compared to KG1a cells that transit the matrix in as little as 2 hr, BMDM require a minimum of 48 hr to traverse the entire matrix. Using a time course shorter than 48 hr, the invasion of BMDM into the Matrigel<sup>TM</sup> after 12 hr was measured in lieu of transmigration. The embedded cells were fixed in the matrix, stained, photographed over multiple fields, counted, and the number of cells was assigned a relative value for invasion. Shown in Figure 6.6, unstimulated BMDM migrated into the gel matrices. In these experiments, only unstimulated BMDM infiltrated the gel, leaving the majority of stimulated BMDM on the surface of the matrix. The presence of HA in the matrix appears to slow, not accelerate, the migration of unstimulated BMDM. Yet, similar to the results obtained in the transmigration assays with the KG1a cells, these data measuring the invasion of BMDM imply that low HA-binding promotes the migration of cells, and that strong HA-binding in hinders elongation and migration.



**Figure 6.5 Effect of anti-CD44 mAb on HA**<sup>low</sup> and HA<sup>high</sup> KG1a cell transmigration. The bar graph represents the percent of input KG1a cells (Y axis) with low (left bars) or high (right bars) HA-binding that migrated through the 3D matrix towards a 5-fold serum gradient. Cell migration through Matrigel<sup>TM</sup> is indicated by black bars or through Matrigel<sup>TM</sup> containing HA by grey bars. The migration of KG1a cells that were pre-incubated with anti-CD44 mAb are indicated by white bars. The percentages were averaged over three experiments and the standard deviation is indicated with error bars.



Figure 6.6 BMDM are slower to invade the basement membrane matrices with HA. Phase contrast images of C57BL wild type BMDM embedded in the Matrigel<sup>TM</sup> basement membrane matrix (left panel) or matrix containing HA (right panel) 12 hr after seeding cells in a ten-fold serum gradient. These images are representative of results obtained from three repeat experiments. Note that cells are dark and elongated structures (indicated by  $-\bullet$ ) whereas pores in the matrix are white circles (indicated by  $-\bullet$ ).

#### 6.3 DISCUSSION

# 6.3.1 Mechanisms of leukocyte migration

Sub-endothelial cell migration results from the direct and coordinated interaction of cells with the ECM. Until recently, the mechanisms of directional cell migration have been based on the original model of metazoan or fibroblast migration across planar surfaces (413-415) and that of cell attachment via focal adhesions (416). The model proposes that cells establish integrinbased clusters along the cell surface that act as a treadmill over which the cell body advances. pulled by lamellopodia at the leading edge and released by retraction of the trailing edge, or uropod, defined as the tethering 'foot' in T lymphocytes (reviewed by 417). The structure of the rear membrane protrusion has been well described for migratory T cells (264, 364, 418). It was concluded in chapter five that the KG1a cell pseudopods are not analogous to the T cell uropod. This chapter shows another distinction between the two structures; the distinct pseudopod extended on high HA-binding KG1a cells promoted an adhesive phenotype in contrast to the migratory phenotype attributed to the T cell uropod. By videomicroscopy, KG1a cells moved randomly on surfaces of fibronectin and offered no clear indication if the pseudopod led or trailed the direction of movement or participated at all in motility on fibronectin (Genetic Circuits Research Group, UCSD, Migration Mechanisms of Immature Hematopoietic Cells,

<u>http://gcrg.ucsd.edu/presentations/migratory/migratory.htm</u>). Instead of pseudopods, the dynamic extension of long tenupodia and magnupodia on KG1a cells have been implicated in cell adhesion to fibronectin, collagen IV and laminin as well as migration and homing after bone marrow transplants (285, 419).

Monocytes have been shown to slip beneath an activated endothelial layer in a fraction of the time that the cells spent moving on top of the endothelium (420, 421). When matrices were stained over a range of time points within the 24 hr assay, few cells were found embedded in the gel (data not shown), indicating that KG1a cells move through the matrix quickly, and that the rate-limiting step during the process of transmigration must lie in the initiation steps. The slow, methodical, integrin-, uropod- and focal adhesion-dependent migration of fibroblasts proposed in the original 2D model may not reflect the mechanisms of 3D migration in small, fast leukocytes such as neutrophils and monocytes. It was suggested by Lee *et al* that the locomotion of certain cells, particularly cells capable of rapid movement, may utilize diffuse, highly labile contact regions of unknown molecular compositions to move (422). More

recently, different modes of tumour cell invasion in 3D Matrigel<sup>™</sup> matrices were reported (412). This report described a rounded, or 'amoeboid' mode of cell migration that was dependent on Rho signaling and ROCK activation. A second, ROCK-independent mode of cell migration was associated with cell polarization and asymmetric accumulation of phosphatidylinositol-3,4,5-triphosphate and F-actin at one pole of the cell. The less polarized cells that utilized an 'amoeboid' mode of cell migration were also correlated with a higher metastatic potential. Results in this chapter provide evidence that the lack of a rigid pseudopod structure correlated with contact-induced elongation, and CD44-dependent migration of low HA-binding KG1a cells on matrices containing HA. The polarization and distribution of CD44 in KG1a pseudopods may be a unique regulatory mechanism for cell adhesion, contrary to the uropod-dependent polarization that was characterized for migration in 2D and for T lymphocyte motility. Further, CD44 expressed on monocytes in a low HA-binding conformation may be one of the 'unknown' components suggested by Lee *et al* to promote cell migration, utilizing a Rho-dependent, 'amoeboid'-like mechanism similar to that described in tumour cells. To test this mechanism for KG1a cell migration, an inhibitor of ROCK could used to determine the dependence of HA<sup>low</sup> and HA<sup>high</sup> KG1a cell migration on Rho pathway activation.

## 6.3.2 Potential outside-in-signaling from CD44-HA engagement

Outside-in signaling with anti-CD44 mAbs is thought to mimic the activation of CD44 that occurs upon engagement of HA and induces cytoskeletal changes in T cells (423, 424) and B cells (425, 426) to promote cell spreading. Antibody-mediated cross-linking of CD44 can induce homotypic aggregation of hematopoietic cell lines (113, 427) and integrin-mediated cell-cell adhesion in T cells and in colon cancer cells (428, 429). These reports suggest that CD44 and integrin signaling pathways can cross-talk and an intact actin cytoskeleton is necessary for signaling (428). The evidence that CD44 is a co-stimulatory molecule in T cell activation (430) provides support that CD44 can signal inwards.

It is commonly reported in the literature that polymers and fragments of HA produce intrinsically different results upon binding to CD44. These results suggest that only the fragments of HA can mediate outside-in signal transduction through CD44 (288, 431, 432). HA fragments induce the secretion of cytokines and chemokines (288, 433), induce nitric oxide

synthase (193, 434) and activate NF-κB (190, 195) in macrophages. Cell spreading studies in T cells suggest that fragmented HA activates the GTPase, Rac1, to cause rearrangement of the cytoskeleton in a Src-family kinase-dependent manner (233, 435). In human chrondrosarcoma cells, HA fragments upregulated urokinase-type plasminogen receptor as well as the upregulation and tyrosine phosphorylation of the c-Met receptor (197, 198). Contrary to this, CD44 ligation by antibodies or its natural (high molecular mass) ligand, HA, induced cytokine secretion in dendritic cells (436) and induced Rac1 and Ras activation through CD44v3 during ovarian tumor cell migration and growth (437). B cell activation, IgM production and elevated CD44 expression *in vitro* and splenic B cell proliferation *in vivo* were also reported to occur via a CD44-HA interaction (438). The data presented in this chapter suggests that outside-in signaling occurs through CD44 after engagement of large polymers of immobilized HA incorporated in a matrix. The evidence of signaling is present as a change in morphology and motility of BMDM or KG1a cells after engagement with the matrix and in particular, this resultant phenotype is enhanced in cells known to have a weak association with HA.

It is not known if signal transduction through CD44 that engages low or high levels of HA is different, or if identical pathways are induced with comparable intensity and duration, yet the outcome (elongation) differs because the rigid cell structure and strong CD44-HA interaction in the HA<sup>high</sup> cells inhibits restructuring of the cytoskeleton. Further, BMDM adhesion and elongation required the presence of a serum gradient, suggesting that outside-in signal transduction by serum components can promote adhesion and cytoskeletal rearrangement, but presently it is not known if this signaling overlaps with that mediated by a CD44-HA interaction. The transduction pathways leading to and from CD44 are beginning to be unraveled. What is presently clear is that CD44 must be associated with cytoplasmic signaling components in order to induce dynamic elongation seen in the HA<sup>low</sup> KG1a cells. Associations of this nature are believed to occur in detergent-insoluble membrane divisions such as lipid rafts that are membrane fractions enriched in molecules for receptor-mediated signaling (reviewed in 439, 440).

Data presented in the previous chapter showed that CD44 localized with phosphorylated ERM proteins into F-actin membrane protrusions to favour engagement with HA. In concert with these data presented in this chapter, such a CD44-HA interaction has been interpreted as a mechanism to reinforce adhesive contacts and prevent migration. Adhesive cells did not favour

cytoskeletal rearrangement, thus, it is unlikely that the engagement of HA in this case is for the purposes of signal transduction unless a message is relayed to maintain the rigid architecture and prevent alterations to the cell membrane. It is possible that the phosphorylated ERM proteins anchor CD44 to F-actin in membrane protrusions not for the purposes of signal transduction but to facilitate adhesion and suppress migration. The presence of HA in the Matrigel<sup>TM</sup> also appears to inhibit BMDM elongation and migration. This is quite striking since BMDM naturally elongate and form strong adhesive contacts. The phenotype of BMDM on HA is similar to that of TNF- $\alpha$ -stimulated BMDM, failing to elongate bi-directionally. Conversely, matrix-induced cell elongation was coincident with enhanced migratory ability of KG1a cells and BMDM. Thus, two scenarios are possible to promote migration; through removal of signals that suppress membrane rearrangement and coalescing of the normal signaling machinery or via direct signaling from a low intensity interaction between CD44 and immobilized HA. The latter scenario would suggest that CD44 talks to the cytoskeleton through an as yet unidentified mediator, not via ERM proteins in low HA-binding cells. This is likely given that CD44 and phospho-ERM do not co-localize in low HA-binding KG1a cells or in PBM.

## 6.3.3 CD44-mediated motility in myeloid cells

In KG1a cells, anti-CD44, antibodies that prevent binding to HA inhibited the migration of  $HA^{low}$  KG1a cells on matrices containing HA, providing evidence that migration through this medium was mediated by a CD44-HA interaction. However, the results indicate that either some KG1a cell migration was CD44-independent or that the antibody blocking experiments may not be representative of the contribution of CD44 to migration. Several possibilities may account for this: the antibodies may induce receptor internalization, the antibodies may bind insufficiently at 37°C to completely inhibit the rapid transmigration of KG1a cells, and other CAMs, such as  $\beta$ 2 integrins expressed on KG1a cells may be activated in the presence of the blocking antibodies to promote migration. Therefore, the absence of an effect with the antibodies does not mean that these processes are independent of CD44.

Complications associated with using blocking antibodies were avoided in BMDM invasion assays where cells derived from the CD44<sup>-/-</sup> mouse were used (data not shown). Preliminary results indicate that BMDM derived from the CD44-knock out mouse invaded the matrix 3 - 5 fold faster than wild type cells (data not shown). This effect was independent of the presence

of HA in the matrigel suggesting that BMDM migration is independent of HA and likely to involve CD44 engagement of another ECM component present in the Matrigel<sup>™</sup>. There are also caveats associated with these experiments. Given that myeloid progenitors in the knock out mouse have defective egress from the bone marrow, macrophages derived from the CD44deficient bone marrow may be defective in ways that have not been identified. Given the degeneracy among cell adhesion receptors, compensation for the lack of a gene may result in unforeseen effects since a change in context (of the receptor) may create altered, functionally different interactions downstream. Further, the Matrigel<sup>™</sup> basement membrane matrix is composed of laminin, collagen IV, enactin, proteoglycans and other ECM components, potentially elastin, thrombospondin, vitronectin and fibronectin. Thus CD44, in particular CSmodified forms of CD44 are capable of non-HA-dependent interactions such as with laminin, fibronectin, collagen IV and heparin sulfate proteoglycans (reviewed in 208, 441). To investigate the effect of CD44 on BMDM migration *in vivo*, studies have been initiated (with P. Kubes, U. of Calgary, Calgary, AB) to compare the infiltration of wild type and CD44-deficient bone marrow-derived macrophages introduced into wild type mice with a localized inflammatory response.

The discrepancy in results between the human mAb blocking studies and the CD44-deficient BMDM is the same conundrum expressed in the current literature using mouse models of pulmonary edema (139, 145). That is, CD44-neutralizing antibodies dampen the response in allergic respiratory inflammation (experimental asthma) by preventing lymphocyte and eosinophil accumulation in the lung (139), yet a lack of CD44 allows persistent inflammation in a murine model of non-infectious lung injury and a failure to clear apoptotic neutrophils, suggesting that CD44 promotes resolution of lung inflammation (145). Data presented in this chapter can potentially explain both results, implicating a role for CD44 in leukocyte migration to the inflamed site as well as in the resolution of the response through induced adhesive interactions with HA in the ECM.

## 6.3.4 Contribution of sulfation to myeloid motility

Based on findings in chapter four, sulfate incorporation promotes high levels of HA-binding. This can be extrapolated to suggest that sulfation also promotes an adhesive phenotype in this system. Data presented in chapter four demonstrated that sodium chlorate inhibited the incorporation of sulfate on CD44 (Figure 4.7) and FL-HA-binding by HA<sup>high</sup> KG1a cells

(Figure 4.8). These data imply that in cells with strong, sulfation-dependent HA-binding, that sulfation on CD44H would impede migration. Although sulfation is a less significant mechanism that regulates HA-binding in KG1a cells, initial experiments were conducted to address the role of sulfation in KG1a cells and BMDM migration and adhesion (data not shown). The migratory capability of KG1a cells was assessed in a transwell migration assay following pre-treatment with increasing concentrations of the sulfation inhibitor, sodium chlorate, for 72 hr. Migration by high HA-binding KG1a cells was significantly enhanced by pre-treatment with the sulfation inhibitor at 10 - 25 mM. This result correlates a decrease in the incorporation of sulfate on CD44 and FL-HA-binding with an enhanced rate of cell migration. Thus, high HA-binding correlates with CD44 sulfation and myeloid cell adhesion. Incubation of HA<sup>low</sup> cells with sodium chlorate had no effect on cell migration through Matrigel<sup>™</sup> devoid of HA. However, these cells migrated faster on matrices with HA, suggesting that a decrease in sulfation further liberates KG1a cells from adhesive contacts to promote migration. Thus, high HA-binding correlates with CD44 sulfation and myeloid cell adhesion. As suggested in chapter four, the complicated role of sulfation cannot be interpreted as an all-or-none event to regulate HA-binding, but as a continuum of sulfated sites on carbohydrates or GAGs that differentially regulate binding affinities to soluble HA that dictate cellular adhesion and migration events (see Figure 6.6). This contrasts with the absolute requirement for sulfation at a specific site on selectin ligands that is essential for selectin-selectin ligand binding and rolling of leukocytes.

## 6.3.5 Working model of CD44-HA-binding in cell migration and adhesion

This chapter aimed to evaluate the effect of CD44-HA interactions in myeloid cell migration. Figure 6.6 illustrates the relationship between myeloid cell binding to FL-HA, adhesive properties, and the sulfation status of CD44. Results suggest that non-activated CD34<sup>+</sup> myeloid progenitors or BMDM that engage low levels of HA have a highly flexible, re-organizable cytoskeleton that favours a migratory phenotype in three-dimensional *in vitro* assays. This phenotype is also associated with low sulfate incorporation on sites of CD44 with a negative consequence for HA-binding and loose, non-restricted localization of CD44 on the cell surface. These characteristics impart propitious properties to CD44 such that engagement with immobilized ligand may transduce signals that promote cell elongation and HA-dependent motility. Inflammatory agent-induced re-shaping of CD44 surface distribution (discussed in



**FIGURE 6.7 Proposed relationship between HA-binding, sulfation, and myeloid cell motility.** Based on results with KG1a myeloid progenitor cells, a migratory phenotype is predominant in nonactivated cells with low CD44 reactivity to the ligand, HA. With appropriate cell activation or maturation such that CD44 reactivity to HA increases, a phenomenon often associated with a redistribution of sulfate, myeloid cells are more sessile, and adhesive rather than motile. Thus, low CD44-HA-binding promotes cell migration over adhesion. Activation, sulfation and redistribution of CD44 promotes high HA-binding and cell adhesion. chapter five) promotes stronger HA-binding. In parallel, the expression of CD44 that is sulfated on positive regulatory sites for HA-binding further encourages CD44-HA interactions that promote cell motility. Beyond a threshold for CD44-HA-binding, the interaction no longer promotes migration, but promotes adhesion. The cells show characteristics of cell rearrangement, sequestration of CD44 into membrane protrusions, sulfate incorporation and an enhanced capacity for HA. This type of CD44-HA interaction has the potential to stop myeloid cell motility thereby supporting adhesive contacts in the ECM at inflammatory sites, a concept that will be discussed further in chapter seven.

# CHAPTER SEVEN

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# **Summary and Perspectives**

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#### 7.1 RESULTS AND FUTURE DIRECTIONS

#### 7.1.1 Synopsis of results

At the onset of this work, the activation and conversion of CD44 to a ligand-binding form in response to specific physiological stimuli was largely unknown despite a generous number of reports describing alterations in CD44 expression, gene splicing, glycosylation, sulfation, and interaction with the cytoskeleton. To understand the stimuli and corresponding cellular mechanisms that regulate CD44-mediated HA-binding in myeloid cells, the first objective was to establish factors that affect HA-binding in primary ex vivo human monocytes, cells previously reported to engage HA following incubation in the presence of pro-inflammatory cytokines. The analysis was extended to murine macrophages as well as a human myeloid progenitor cell line. Results presented in the third chapter of this manuscript indicate that proinflammatory cytokines, in particular TNF- $\alpha$ , induce CD44-mediated FL-HA-binding in primary human monocytes and bone marrow-derived murine macrophages. The elevated expression of CD44, posttranslational sulfation as well as cytochalasin D-sensitive cytoskeletal changes all contributed to the induction of HA-binding. No change in CD44 isoform expression was supported in this investigation. Other groups have shown an increase in sialidase activity in the THP-1 monocytic cell line and PBM following TNF- $\alpha$  and LPS stimulation (217). It was not determined in our system if sialidase activity increased in response to TNF- $\alpha$ , only that an increase in HA-binding was observed following neuraminidase digestion of PBM prior to and following cellular activation.

After identification of the cytokine-induced factors that affect HA-binding, primarily sulfation and cytoskeletal rearrangement, the second aim was to understand the mechanism through which an effect on HA-binding was accomplished. The results shown in chapter four demonstrate that the extent of CD44H sulfation directly correlated with FL-HA-binding capacity by PBM at the cellular level and sulfated CD44H had enhanced reactivity with HA at a molecular level in a direct binding assay. Further analysis determined that a shift in sulfate distribution on CD44H occurred following cellular activation of PBM or BMDM. Based on the molecular and cellular data, both the quantity and location of sulfate incorporation determines HA-binding in monocytes. The results indicated that GAG addition to CD44H is a negative factor for HA-binding, the influence of which can be subdued by TNF- $\alpha$ -induced Low HA-binding

**High HA-binding** 



FIGURE 7.1 Proposed regulation of HA-binding in myeloid cells via the extracellular and cytoplasmic domains of CD44. In a low HA-binding conformation, CD44 on the cell surface is primarily sulfated on short chain GAGs in the membrane proximal region (a) and expressed at different sites on the cell (b) than cytoplasmic ERM proteins (c) until inflammatory stimuli (d) induces CD44 and ERM expression, a shift in sulfation favouring N-glycans in the amino terminal, HA-binding end of CD44H in PBM (e), F-actin assembly, and a CD44-phospho-ERM-F-actin complex (f) that is concentrated into multiple membrane extensions as seen in PBM (g) or a single projection as seen in KG1a cells (h). This creates an extended membrane protrusion with a dense populous of sulfated, clustered CD44 molecules with enhanced capacity for HA.

sulfation of O- or N-glycans on CD44H. With respect to the regulation of HA-binding by the intracellular domain of CD44, a CD44-phospho-ERM complex co-localizes to F-actin rich membrane protrusions in a myeloid progenitor cell line and activated PBM, cells that bind high levels of HA. Destruction of the actin rich membrane structure decreases the CD44-ERM interaction and significantly reduces HA-binding (by ~80%). The mechanisms by which CD44 sulfation and phospho-ERM-CD44 localization in F-actin protrusions enhance HA-binding capacity are summarized in models in chapters four and five (Figure 4.13 and Figure 5.8). A combined model in Figure 7.1 illustrates the co-operative action of sulfation and the cytoskeleton to regulate the ligand binding function of CD44.

Preliminary results were presented in chapter six to address the role for a CD44-HA interaction in myeloid cell motility. These data provide evidence that high HA-binding capacity can promote cell adhesion in mouse BMDM and a human myeloid progenitor cell line. Conversely, low levels of HA-binding promote cellular elongation and migration through a three-dimensional matrix system, particularly for the progenitor cells when HA is incorporated into the matrices at a concentration similar to that found in tissues.

In summary, this work contributes to the understanding of HA-binding regulation in myeloid cells specifically in response to inflammatory mediators. Two primary mechanisms regulate CD44 ligand binding capacity; one mechanism involves sulfation of the extracellular domain of CD44, and the other is based on an association of the cytoplasmic domain of CD44 with the actin cytoskeleton. Further, the CD44-HA interaction by myeloid cells is sufficient to support both cell-ECM migration and adhesion.

# 7.1.2 Regulation of receptor clustering and HA-binding by thecytoplasmic domain of CD44

CAMs expressed on naïve or non-activated cells normally engage their respective ligands with low affinity until a high avidity interaction is triggered by cellular stimulation. Clustering of CAM receptors is a fast, relatively simple, and readily reversible mechanism to boost affinity or avidity for CAM ligands. Receptor clustering has been described as a regulatory mechanism for ligand-binding by integrins and Ig superfamily molecules (442-444), and the clustering of integrins is regulated by the actin cytoskeleton (445, 446). Dimers or multimers of LFA-3 and Mac-1 enhance binding affinity for their ligands, CD2 and C3bi respectively (447, 448), and homodimers of ICAM-facilitate binding to LFA-1 (449).

The most characterized ligand for CD44 is HA, a non-sulfated, secreted GAG of such numerous repeating disaccharide units that HA is normally produced as a large polymer (Figure 1.5). Tight receptor binding to such an expansive molecule would require multivalent interactions. Although the enhanced surface expression of CD44 could generate the potential for multivalency, and has been shown as a mechanism to enhance HA-binding (209), an increase in CD44 expression is not sufficient to support HA-binding in myeloid cells. An increase in CD44 expression may be secondary to an increase in receptor density, or clustering, similar to the regulation of ligand-binding by integrins and Ig superfamily molecules. Since the amino-terminal link domain on CD44 must engage a corresponding, repeating epitope on HA, receptor clustering may result in the appropriate spacing of CD44 molecules proximal to the repetitive binding domain on HA. The membrane-proximal, transmembrane, and cytoplasmic domains of CD44 have the potential to cluster molecules of CD44 and regulate HA-binding. As shown by Chiu et al (182), CS-modified isoforms of CD44 can interact with CD44H, but not CS-modified forms of CD44, on an opposing cell surface that is dependent on CS. GAGs may mediate intermolecular interactions and spacing between CD44 neighbours on the same cell surface, where the length of the GAG chains determines the spacing of CD44 molecules. A loss of GAGs, shown in chapter three to promote HA-binding, may allow CD44 molecules to adopt a more appropriate spacing that facilitates HA-binding. The presence of alternatively spliced forms of CD44, with additional sequence in the membrane proximal region that carries sites for glycosylation and GAG addition may be involved in HA-binding through a similar mechanism that determines CD44H placement on the cell surface with respect to neighbouring molecules of CD44. The results presented in this thesis do not provide evidence to support or refute this theory of regulation by the membrane proximal region of CD44 or by GAG addition. Instead, the clustering of CD44 necessary to regulate HA binding is based on a novel, inducible interaction between the cytoplasmic domain of CD44 and phospho-ERM proteins concentrated into F-actin rich membrane protrusions.

To further understand the regulation of CD44 by the ERM protein family and F-actin, it will be useful to elucidate the sequence of events by which TNF- $\alpha$  induces changes to the cytoskeleton and molecular distribution of CD44, ERM proteins, and actin. The kinetics of CD44-phospho-

ERM localization and complex formation in F-actin membrane protrusions is not known. Confocal microscopy analysis revealed that phosphorylated ERM proteins are present in PBM prior to and following stimulation. In non-activated cells, the phosphorylated-ERM proteins may interact with membrane proteins other than CD44. It is not known if the phosphorylated ERM proteins observed prior to stimulation remain phosphorylated after TNF- $\alpha$  treatment and are simply sequestered to the pseudopod, or if new phosphorylation events occur following TNF- $\alpha$  stimulation, or both. The phosphorylation and redistribution of ERM proteins to F-actin-containing microvilli was reported to occur in epithelial and melanoma cell lines stimulated with growth factors such as EGF (379, 381). It is not known if the localization of ERM proteins, and CD44 redistribute independently and the subsequent assembly of new complexes occurs in the pseudopod. The intramolecular head-to-tail conformation of ERM proteins has been shown to block two membrane protein binding sites but not CD44 (reviewed in 251). CD44 may interact with non-phosphorylated ERM proteins and phosphorylation may be a secondary event to tether the CD44-ERM complex to F-actin.

# 7.1.3 Regulation of HA-binding by sulfation of CD44

An appreciation and understanding of glycobiology is necessary to explain aspects of normal cellular function, intercellular communication, cell-cell interactions, and signaling in the immune system (450). Sulfation is restricted to the extracellular domain of glycolipids, glycoproteins, and proteoglycans and is gaining acceptance as a mechanism to regulate cell surface interactions analogous to the regulation of intracellular communication by phosphorylation. Previous examples of sulfation conferring biological function to cells are in selectin-selectin ligand interactions (90), a report that sulfation regulates HA-binding by CD44 on a human CD33<sup>+</sup> leukemic cell line (292), and evidence that sulfated receptors engage viruses, bacteria and chemokines (reviewed in 295).

Data generated in this investigation demonstrates that the sulfation of CD44 in primary monocytes imparts HA-binding function at both a cellular and a molecular level. Delving into the details of CD44 sulfation revealed a more complicated picture than first imagined. The presence of GAGs had a negative effect on HA-binding, but the addition of sulfate to O- or N-glycans on CD44 conveyed a positive effect on HA-binding. Extensively glycosylated molecules such as CD44 possess a plethora of sites for sulfate addition leading to the

generation of 'sulfoforms', isoforms of a carbohydrate moiety that are sulfated on different sites, each with the potential to serve a different biological purpose. CD44 likely has multiple sulfoforms of the standard CD44H form alone. Antibodies against specific sulfated moieties were used as a starting point to evaluate the expression of particular sulfated epitopes on the cell surface and on individual molecules prior to and following cellular activation. Using this method, potentially sialylated, 6-sulfo Lewis x and 6-sulfo LacNAc structures were identified on activated PBM. Further analysis into the location and biological function of these sulfated epitopes is necessary. The induction of Lewis x determinants implies that CD44 could be an inducible selectin ligand that may facilitate cell-cell adhesion with leukocytes, or leukocyte rolling on activated endothelium in an HA-independent manner. However, the evidence to date suggests that sulfation contributes to CD44 function as a receptor for HA. Identification of the responsible sulfotransferases and inhibition of these will allow an initial screening of the effect of particular sites of sulfate addition on biological function.

#### 7.2 SIGNAL TRANSDUCTION

It is not known which signal transduction pathways are activated by inflammatory cytokines that result in the expression of functional CD44. Both TNF- $\alpha$  R1 and R2 are expressed on peripheral blood monocytes prior to and following TNF- $\alpha$  stimulation (data not shown). Induction of HA-binding was prevented only in the presence of blocking antibodies for both receptors (data not shown) suggesting that signaling components initiated by both TNF receptors may participate in monocyte activation and the regulation of HA-binding. It was speculated in chapter five that a Rho-dependent signaling pathway in monocytes results from stimulation with TNF- $\alpha$  to promote cytoskeletal rearrangement, CD44-phospho-ERM complex formation, and the regulation of HA-binding. It would be useful to test the activation of Rhopathway component and to determine the signal transduction pathways that result in changes to sulfation. Which sulfotransferases are activated or downregulated? Is the enzyme activity regulated at the protein level? Such information will offer insight into appropriate sites for intervention into the regulation of CD44 that may affect CD44 function in general or in specific cells that utilize particular mechanisms to regulate CD44.

# 7.3 A PROPOSED PHYSIOLOGICAL ROLE FOR CD44-HA INTERACTIONS IN MYELOID CELL MOTILITY

Together, monocytes and macrophages secrete a variety of inflammatory mediators that dictate the recruitment of different cell types, activation of these cells, and initiation of both humoral and cell mediated facets of the adaptive immune response. Note that while the presence of cells of the mononuclear phagocytic system at the inflammatory site is required for healing, their continued presence in chronic inflammatory sites can also be pathogenic. Thus, both initiation and cessation of cell migration events must be tightly controlled for a short-lived, effective response. Since infiltrating monocytes are a key source of macrophages, the migration of monocytes is directly linked to macrophage function in the tissue. The myeloid cell system is particularly interesting to study in terms of cell migration as small, rapid, low integrin expressing monocytes mature into large, sessile, macrophages with clustered integrins. Yet, the details of cell migration in the immune system have tended to focus on chemoattractant-driven neutrophil migration or T lymphocyte homing as discussed in chapter one.

The results presented in chapter six show that low HA-binding BMDM and KG1a progenitor cells were adept at elongating and migrating through a three dimensional matrix, especially when matrices were embedded with HA. In contrast, high HA-binding by cells correlated with an adhesive, non-migratory phenotype. Figure 7.2 illustrates the role of CD44-HA interactions in a model of cell adhesion and migration based on the leukocyte cell adhesion cascade presented in chapter one (Figure 1.1). In the context of an inflammatory response, the CD44-HA interaction may facilitate leukocyte rolling on the vascular endothelium through a low affinity CD44-HA engagement, and given that there is cross-talk between CD44 and integrin signal transduction pathways, CD44 may be indirectly involved in firm adhesion through HAinduced signal transduction that results in integrin activation. The dynamic membrane and loose adhesive contact with HA may encourage monocytes to elongate, exit the blood stream, and migrate into the tissue, where HA is abundant. As monocytes extravasate, a process of CD44-receptor remodeling may be initiated upon exposure to pro-inflammatory cytokines, leading to enhanced receptor expression, induction of CD44 sulfation, alteration to the actin cytoskeleton, as well as co-localization of CD44 and phospho-ERM proteins. The result is a highly differentiated monocyte (macrophage) that engages HA, is retarded in migration, and is



**FIGURE 7.2 Implication of CD44-HA interactions in mediating myeloid adhesion and migration during an inflammatory response.** In the initial steps of monocyte extravasation to inflamed tissue, CD44 has a low intensity interaction with HA, a phenotype that promotes cytoskeletal rearrangement and cell migration. Monocyte maturation and activation causes CD44 remodeling such that HA-binding capacity increases and CD44 is anchored in membrane protrusions with phosphorylated ERM proteins to promote cell adhesion within the inflamed tissue.

retained in the inflamed tissue. This temporary adhesion and residence in the tissue may be necessary to allow macrophages to phagocytose debris and assist in damage control at the site of assault. In the study by Teder *et al*, inflammation was exacerbated when macrophages failed to phagocytose apoptotic cells at the inflammatory site, due to a deficiency in cell retention rather than a defect in phagocytosis (145). In this model, it can be speculated that the degradation of HA that occurs at sites of inflammation (reviewed in 199, 451) may release CD44 from strong adhesive contacts and promote the migration of cells to the lymphatic system. This motility may also be mediated by an interaction of CD44 with low molecular mass HA (fHA) that either stimulates migration directly or indirectly through signaling to integrins or other CAMs. Thus, this model attributes the intensity of the CD44-HA interaction to the migration or adhesion of myeloid cells in a sulfation and cytoskeletal dependent manner in an inflammatory response.

#### 7.4 Universal theme for CD44-ha-mediated cell adhesion and migration

It is not surprising that CD44 is regulated by more than one mechanism. Partial regulation by multiple factors is an ingenious way to add diversity and a level of compensation within each individual molecule. In this way, the loss of regulation by one mechanism does not ablate receptor function entirely. Further, activation of ligand-binding through one mechanism or another, or all mechanisms in concert may generate ligand-binding of differential affinity and confer different biological functionality. Although stimuli, timing, and the location of CD44 activation may differ, the hypothesis is that the induction of strong HA-binding will promote adhesion and sub-optimal HA-binding will facilitate a migratory phenotype. In this study, a role for CD44-HA interactions under inflammatory conditions was investigated, the results of which contributed to the development of the model proposed in Figure 7.2. Suppose that the underlying theme of CD44 function is both a 'stop' and 'go' signal. CD44 could assist in the immune function of cells other than those of a myeloid lineage. The difference in regulation between cell types may be reflected in the altered timing, longevity, and intensity of the CD44-HA interaction that is controlled by favouring particular mechanisms over others, as guided by secondary signals. Although the studies here were designed to link a particular stimulus to CD44 activation, secondary stimulus of a different nature would ideally give information, such as an environmental cue, that could alter the outcome of the chemical cues. As stated earlier, factors in serum affect CD44 function in PBM, the differentiation of monocytes correlates with

CD44 activation, the presence or absence of CD45 in BMDM and a murine T cell line, BW5147<sup>-/+</sup>, affects HA-binding (Figure 3.5 and data not shown), and the engagement of matrices by HA<sup>low</sup> KG1a cells induces elongation and migration. Such secondary cues could streamline the function of CD44 to a particular cell type, state of activation, or stage of maturation while still embracing the notion that high intensity CD44-HA interactions promote adhesion. For example, factors in human serum promote low but increasing HA-binding capacity *in vitro* in PBM, which may play a role in the turnover rate of blood monocytes, the lifespan of which is approximately 3 days in the blood. This is similar to the sulfation of hormones to facilitate their removal from the blood by engagement of sulfation-dependent receptors. PHA activates T lymphocytes to transiently bind to HA. As implied by DeGrendele *et al* (101), CD44 is important for T lymphocyte rolling along the endothelium, which according to this model, would be attributed to a low engagement of HA. Perhaps in peripheral lymph nodes, an elevated and stable CD44-HA interaction may assist in T cell adhesion and residency in the node during T cell activation and proliferation.

#### 7.5 CONCLUDING REMARKS

This work has identified specific factors that are altered in response to inflammatory stimuli that regulate HA-binding in primary *ex vivo* human monoyctes, murine macrophages, and a human myeloid progenitor cell line. The manners by which sulfation and an interaction with the cytoskeleton enhance reactivity of CD44 with HA were investigated in detail, and mechanisms of action were proposed. Further, a CD44-HA interaction was shown to facilitate both the migration and adhesion of the progenitor cell line and macrophages, also implicating sulfation as a regulatory mechanism that controls this biological function depending on the intensity of the CD44-HA interaction. Experiments carried out using mouse bone marrow-derived macrophages laid the foundation for understanding the physiological role of CD44 through investigations with the CD44-deficient mice. Overall, this body of research aids our understanding of the regulation of HA-binding by CD44. It offers insight into the complexity of molecular regulation that may be applied to other molecules or to CD44 on other cell types. Further, an appreciation of the regulatory mechanisms of inflammatory disorders attributed to a failure in the regulation of CD44-HA interactions and opens avenues for novel points of intervention.

# CHAPTER EIGHT References

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