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An investigation of T lymphocyte migration induced by CD44 activation

A Thesis submitted for the degree of

Doctor of Philosophy

by

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at

University of Dublin, Trinity College.

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Trinity Centre for Health Sciences,
St. James’s Hospital,
Declaration

I declare that, except where otherwise acknowledged, this thesis is entirely my own work. It has not been submitted previously for a higher degree at this or any other university. The library may lend or make copies of this thesis upon request.

Áine Fanning.
This thesis is dedicated to my parents,
Sean and Sheila Fanning

To dream anything that you want to dream.
That's the beauty of the human mind.
To do anything that you want to do.
That is the strength of the human will.
To trust yourself to test your limits.
That is the courage to succeed.

Bernard Edmonds
Summary

This thesis investigates the role of CD44 in signalling active migration in T lymphocytes. A number of murine studies have demonstrated that loss of CD44 expression by T lymphocytes resulted in a delayed migration to sites of inflammation. However, migration to lymph nodes was normal (Camp et al., 1993; Stoop et al., 2002), suggesting that this adhesion receptor is required for T cell migration under inflammatory conditions.

In this study, the responses of human T cells following cross-linking of CD44 were examined. Engagement of the CD44 receptor using immobilised monoclonal antibody induced cytoskeletal rearrangement and cell polarisation. Further analysis demonstrated that these changes in morphology were accompanied by active migration. Further investigation was carried out using pharmacological inhibitors to determine the signal-transduction pathways switched on following engagement of CD44. In particular, the roles of protein kinase C (PKC) isoforms and the lipid kinase, phosphoinositide 3-kinase (PI3-kinase) were examined. Go6976, a selective inhibitor of classical PKC isoymes, suppressed lymphocyte polarisation and migration following CD44 ligation. However, selective inhibition of PKCδ, using rottlerin, reduced CD44-activated migration but did not completely ablate it. Two inhibitors of PI3-kinase were used to examine the role of this lipid kinase in CD44-induced migration. The competitive inhibitor, Ly294,002, suppressed lymphocyte polarisation. However, wortmannin did not significantly alter lymphocyte morphology in response to CD44 engagement.

The subcellular localisation of PKC isoymes was examined using immunofluorescent microscopy. This revealed that in response to CD44 ligation, redistribution of the PKC isoymes α, β, δ, ε and ζ occurs. In particular, PKCβ was associated with the microtubule-organising centre (MTOC) and the microtubule-rich tail of the polarised cell and PKCδ was predominantly located about the region of the MTOC. Studies using a PKCβ-deficient cell line, K4, demonstrated that PKCβ expression was absolutely necessary for CD44 stimulated migration. PI3-kinase did not associate with the microtubule cytoskeleton and was found in discrete patches within the cell body. Engagement of the CD44 receptor induced phenotypic changes, cytoskeletal rearrangements and redistribution of PKC isoymes β and δ, that was strikingly similar
to those seen for the integrin, LFA-1. This suggests a potential convergence of
signalling pathways induced via CD44 and LFA-1. Preliminary studies using an in situ
immunoprecipitation technique, suggests that there may be an association of the CD44
receptor in the LFA-1 signalling complex of the migrating T cell.

During this study, work was also carried out to try to identify a potentially unique
protein, p45. This protein was identified by the anti-CD44 monoclonal antibody, D2.1.
D2.1 detected expression of this 45kDa protein, in addition to CD44, on the surface of
the epithelial-associated T lymphoma cell line, HUT-78 and interepithelial lymphocytes,
however, it was not expressed by normal peripheral blood lymphocytes. Antibodies
against a number of candidate proteins were tested for reactivity with p45, however
none of these reacted positively. In addition, p45 was purified from the complex
mixture of membrane proteins using a number of different techniques, such as
immunoaffinity purification, preparative SDS PAGE and two-dimensional
electrophoresis. Specific proteins were excised from these p45-enriched samples for
protein sequencing using Edman degradation and mass spectrometry. Mass
spectrometry identified a number of protein species present in the p45-enriched
samples, namely beta-actin, nucleolar phosphoprotein B23 and heterogeneous nuclear
ribonucleoprotein F.

In summary, these studies have characterised a novel model of T cell migration
stimulated by anti-CD44. It is interesting to note that there is a striking resemblance
between T cell responses to either CD44 or LFA-1 cross-linking. The response of T
cells following LFA-1 ligation has been well characterised and a critical role for the
enzyme PKCβ in cell migration has described. Further characterisation of this would
have potential use in the treatment of inflammatory disease such as rheumatoid
arthritis and colitis.
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Associated Publications

Papers

Presentations
“Cross-linking of CD44 induces PKC-mediated signalling events in lymphocyte migration”

“PKC-dependent intracellular signalling in migrating T lymphocytes induced via CD44”

“Cross-linking of CD44 induces PKC-mediated signalling events in lymphocyte migration”
Poster presentation, Cell and Tissue Morphogenesis, Brighton, 2001
## ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
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<tbody>
<tr>
<td>aa</td>
<td>amino acid</td>
</tr>
<tr>
<td>APC</td>
<td>antigen presenting cell</td>
</tr>
<tr>
<td>APS</td>
<td>ammonium persulfate</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
</tr>
<tr>
<td>CD44s</td>
<td>standard form CD44</td>
</tr>
<tr>
<td>CnBr</td>
<td>cyanogen Bromide</td>
</tr>
<tr>
<td>DMSO</td>
<td>dimethylsulfoxide</td>
</tr>
<tr>
<td>ECL</td>
<td>enhanced chemiluminescence</td>
</tr>
<tr>
<td>ECM</td>
<td>extracellular matrix</td>
</tr>
<tr>
<td>ECMRIII</td>
<td>extracellular matrix III</td>
</tr>
<tr>
<td>EGF</td>
<td>epidermal growth factor</td>
</tr>
<tr>
<td>ERM</td>
<td>ezrin, radixin and moesin</td>
</tr>
<tr>
<td>FCS</td>
<td>foetal calf serum</td>
</tr>
<tr>
<td>FITC</td>
<td>fluorescein isothiocyanate</td>
</tr>
<tr>
<td>GAG</td>
<td>glycosaminoglycan</td>
</tr>
<tr>
<td>GlyCAM-1</td>
<td>glycosylation-dependent cell adhesion molecule-1</td>
</tr>
<tr>
<td>HA</td>
<td>hyaluronan</td>
</tr>
<tr>
<td>HABD</td>
<td>hyaluronan-binding domain</td>
</tr>
<tr>
<td>HBSS</td>
<td>hank’s balanced salt solution</td>
</tr>
<tr>
<td>HEV</td>
<td>high endothelial venule</td>
</tr>
<tr>
<td>HRP</td>
<td>horse radish peroxidase</td>
</tr>
<tr>
<td>ICAM</td>
<td>intracellular cell adhesion molecule</td>
</tr>
<tr>
<td>IEL</td>
<td>intraepithelial lymphocyte</td>
</tr>
<tr>
<td>IEF</td>
<td>isoelectric focusing</td>
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<tr>
<td>IFN-γ</td>
<td>interferon-γ</td>
</tr>
<tr>
<td>Ig</td>
<td>immunoglobulin</td>
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<tr>
<td>Acronym</td>
<td>Abbreviation</td>
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</tr>
<tr>
<td>IL-1</td>
<td>interleukin-1</td>
</tr>
<tr>
<td>IPG</td>
<td>immobilised pH gradient</td>
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<tr>
<td>LFA-1</td>
<td>lymphocyte function-associated antigen-1</td>
</tr>
<tr>
<td>LPAM-1</td>
<td>lymphocyte-Peyer's patch adhesion molecule-1</td>
</tr>
<tr>
<td>LPS</td>
<td>lipopolysaccharide</td>
</tr>
<tr>
<td>mAb</td>
<td>monoclonal antibody</td>
</tr>
<tr>
<td>MHC</td>
<td>major histocompatibility complex</td>
</tr>
<tr>
<td>MS</td>
<td>mass spectrometry</td>
</tr>
<tr>
<td>MTOC</td>
<td>microtubule organising centre</td>
</tr>
<tr>
<td>Np40</td>
<td>nonidet p40 (Igepal CA-630)</td>
</tr>
<tr>
<td>NK</td>
<td>natural killer</td>
</tr>
<tr>
<td>NMR</td>
<td>nuclear magnetic resonance</td>
</tr>
<tr>
<td>PAS</td>
<td>protein A sepharose</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate buffered saline</td>
</tr>
<tr>
<td>Pgp-1</td>
<td>phagocyte glycoprotein-1</td>
</tr>
<tr>
<td>PI3-kinase</td>
<td>phosphoinositide 3-kinase</td>
</tr>
<tr>
<td>PKC</td>
<td>protein kinase C</td>
</tr>
<tr>
<td>PGS</td>
<td>protein G sepharose</td>
</tr>
<tr>
<td>PMA</td>
<td>phorbol myristate acetate</td>
</tr>
<tr>
<td>PNAd</td>
<td>peripheral-node addressins</td>
</tr>
<tr>
<td>PVDF</td>
<td>polyvinylidene difluoride</td>
</tr>
<tr>
<td>SCR</td>
<td>short consensus repeats</td>
</tr>
<tr>
<td>SDS</td>
<td>sodium dodecyl sulfate</td>
</tr>
<tr>
<td>SDS–PAGE</td>
<td>sodium dodecyl sulfate polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>SLC</td>
<td>secondary lymphoid-tissue chemokine</td>
</tr>
<tr>
<td>TCR</td>
<td>T cell receptor</td>
</tr>
<tr>
<td>TEMED</td>
<td>N,N,N',N'-tetramethyl ethylene-diamine</td>
</tr>
<tr>
<td>Th</td>
<td>T helper</td>
</tr>
<tr>
<td>TNFα</td>
<td>tumour necrosis factor-α</td>
</tr>
<tr>
<td>VLA-4</td>
<td>very late Antigen-4</td>
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CHAPTER 1

GENERAL INTRODUCTION
1.1 INTRODUCTION

Within the context of a normally functioning adaptive immune system, T lymphocyte migration plays an important role. Naïve T lymphocytes survey the immune system, constantly recirculating from the blood to the lymph nodes and returning to the blood again. Naïve T cells migrate across specialized endothelial cells into the lymph nodes via the afferent vessels where they come into close contact with antigen-presenting cells. Following recognition of bound microbial peptide in the context of the MHC receptor, lymphocyte activation begins. These effector T lymphocytes leave the lymph node primed to respond to the antigenic insult. Indeed, without an antigenic signal, lymphocytes return to the circulation and continue their surveillance of the immune system. Activated lymphocytes migrate across inflamed endothelium and travel towards the source of antigenic insult where they are critically involved in the resolution of infection. However, defects in T lymphocyte migration may result in susceptibility to infection, highlighting the importance of this process.

This thesis has set out to investigate the role of CD44 in human lymphocyte migration, with an examination of physical changes that take place in the cell following CD44 ligation and the signalling pathways that are key to that event. This work has been set in context with work carried out with the integrin, LFA-1, an adhesion molecule whose role in lymphocyte migration has been well documented. This project also carried out an investigation into the identity of p45. An unidentified protein, p45 is expressed on certain lymphocyte subpopulations and is recognised by a novel anti-CD44 monoclonal antibody, D2.1. This general introduction aims to examine features of T lymphocyte migration in the context of the immune response. This requires a study of the role of adhesion molecules in mediating this response. Some of the features of cell migration will also be introduced in terms of the morphologic changes and mechanical properties of this process. This was followed by an examination of some of the signal transduction pathways implicated in cell migration.

1.2 T LYMPHOCYTE HOMING AND MIGRATION

The immune system has evolved to protect the organism from a wide variety of infectious microbes, allowing their elimination but providing a balance against excessive damage to self (Chaplin, 2003). This protection is made up of two elements, the innate immune system which provides the first line of defence against many
common microorganisms by the recognition of molecular patterns shared by many microbes. But for pathogens and altered-self cells (i.e. tumours cells) that escape this checkpoint the adaptive immune system provides a specific and potent response to invading pathogen. The adaptive immune system also provides immune memory thus allowing a rapid response to subsequent re-infection. These elements of the immune system work in concert with each other. The innate immune system has an important role in initiating and directing the adaptive immune response and in turn the adaptive immune response requires the cells of the innate immune system for the clearance of the microorganism (Janeway and Travers, 1997). This review is primarily interested in the role played by T lymphocytes in the adaptive immune response and the importance of lymphocyte homing and migration in mediating this response.

1.2.1 T lymphocytes

T lymphocytes are critically involved with the recognition of foreign antigen and directing the elements of the immune system in the elimination of the pathogenic microbe. This requires the recognition by T lymphocytes of foreign peptides on the cell surface. Specific recognition by, and activation of the T cell results in the acquisition of an effector T lymphocyte phenotype that can coordinate the appropriate response required for the elimination of the pathogen. T lymphocytes originate from stem cells present in the bone marrow, with final maturation occurring within the thymus (Janeway and Travers, 1997). Within the thymus T cells undergo a series of selection steps. As the cell moves through the thymus it is first positively selected based on T cell receptor (TCR) affinity for self-major histocompatibility complex (MHC) molecules, and finally screened for potential autoreactivity with self-antigen. Cells that survive this negative selection are exported to the circulation (Chaplin, 2003).

These circulating naïve T cells will recognise pathogen-derived peptide in the context of self-MHC, expressed on the cell surface of an antigen presenting cell (APC), such as a dendritic cell. This allows the T cells of the immune response to identify and coordinate the destruction of infected host cells, i.e. host cells infected by microbes, as well as altered host cells (i.e. tumourigenic). In order for this encounter to be possible, T lymphocytes must be able to migrate to the sites where antigen is found (von Andrian and Mackay, 2000). Our immune system copes with the diversity of possible peptide antigens by generating a large repertoire of T cells, each with a unique T-cell receptor. This repertoire of T cells that have never encountered antigen, naïve T cells, in adults consist of 25-100x10⁶ distinct clones (Arstila et al., 1999).
Although the T cell population is defined by the cell surface expression of the TCR, a transmembrane heterodimeric protein that recognises antigen displayed in the context of an MHC receptor on antigen presenting cells (APC), its functionality is determined by costimulatory molecule expression (i.e. CD8 or CD4), which is also selected within the thymus. Intracellular infection, for example by a virus, results in the synthesis of foreign proteins within the cell. Cellular machinery proteolytically degrade these foreign proteins and the resulting non-host peptide fragments are expressed on the surface of the cell by a class I MHC molecules. These antigenic fragments are recognised by the TCR of cytotoxic T cells (CD8+) in the context of class I MHC receptor.

In the case of a bacterial infection, the proteins of the extracellular pathogens are ingested by the certain cell types and proteolytically processed. These resulting peptide fragments are expressed on the cell surface by class II MHC molecules, expressed constitutively on professional antigen presenting cells such as B cells, dendritic cells, monocytes and macrophages and whose expression can be induced on many other cell types, including epithelial and endothelial cells, after stimulation with interferon-γ (IFN-γ), interact with helper T cells (CD4+). Interaction of the TCR/CD3 complex with an antigenic peptide presented by an MHC receptor, as well as the required costimulatory signals results in T cell activation. Both CD4+ and CD8+ T cells differentiate into functionally distinct subsets after exposure to antigen. Resting naïve CD4+ T cells, release little detectable cytokine. Following stimulation by antigen and APC, the T helper (Th) cells begin to differentiate towards Th1 and Th2 depending on the nature of the cytokines present at the site of activation. Th1 cells support cell-mediated immune responses and Th2 cells support humoral, antihelminth and allergic responses (Chaplin, 2003).

1.2.2 Role of T lymphocyte migration in the immune system

The immune system has evolved to enable T cells to find a rare foreign antigen rapidly in the body. The adaptive immune response allows the elimination of pathogen by certain T cells, whereas others find pathogen specific B cells, which need the help of T cells for efficient antibody responses. The role of T lymphocyte migration is critical to all of these features of the adaptive immune response. In order to enable this important surveillance and defence against pathogen or tumour, T cells are present in various differentiation states, i.e. naïve, effector, and memory cells. These patrol the body in an organ-specific manner based on the interaction of T cell subset adhesion molecules with counter-receptors expressed on the surface of the target endothelial
cells (Shimizu et al., 1991). Naive T lymphocytes following maturation in the bone marrow and thymus prior to antigen encounter continuously circulate from the blood into the peripheral lymphoid tissues. They enter by means of adhesive interactions with the specialised endothelium, known as high endothelial venules, present in the blood vessels supplying these tissues that allow them to transmigrate between these cells. Naive T lymphocytes travel to the T cell areas of the lymph nodes in search of antigen presented by dendritic cells. The migrating T cells scan the surface of these professional APC for specific peptide-MHC complexes. If they do not recognise antigen presented by these cells, they eventually leave the node via an efferent lymphatic vessel, which returns them to the blood so that they can recirculate through other lymph nodes (Janeway and Travers, 1997).

Following recognition of the specific antigen-MHC complex activation begins, with proliferation and generation of effector cells that can migrate into B cell areas or to inflamed tissue. Once these specific lymphocytes have undergone a period of proliferation and differentiation, they leave the lymph nodes and return to the circulation as effector cells through the efferent lymphatic vessel (Janeway and Travers, 1997). Encounter with antigen induces T cell proliferation, yielding approximately 1000 times more descendants with identical antigenic specificity. Eventually these activated lymphocytes acquire effector functions capable of combating the infection and home to sites of inflammation (von Andrian and Mackay, 2000). In these peripheral tissues, at the site of pathogenic invasion, local inflammation takes place by the action of innate immune cells in the battle to clear the infection. Thus effector cells up-regulate the expression of receptors for inflammation-induced endothelial adhesion molecules and inflammatory chemokines.

1.2.3 Model of extravasation
Leukocyte migration, including the migration of lymphocytes, is thought to result from the sequential interactions of various adhesion receptors in a process known as extravasation (Figure 1.1). This process of extravasation enables both the homing of naive T lymphocytes to peripheral lymphoid organs and the subsequent delivery of effector T cells to sites of infection. Leukocyte homing and transmigration involves a series of consecutive steps. First leukocytes in the bloodstream become tethered and roll slowly downstream; a process described as primary adhesion. Tethering is largely mediated by the interaction of selectin molecules with their counter-receptors (expressing sialyl-Lewis^x-like oligosaccharides) (von Andrian and Mackay, 2000).
Figure 1.1 Extravasation of T lymphocytes, mediated by cooperative interactions between adhesion molecules.
The next step in the process of extravasation requires firm adhesion of the leukocytes to the endothelium, mediated by integrin interaction with their counter-receptors. Integrin activation first takes place, following exposure to a chemotactic stimulus provided by the interaction of chemokines with their G-protein coupled seven transmembrane receptors. Some of these chemokines are presented on the endothelial surface, whereas others are secreted or diffuse freely. This integrin activation allows binding to members of the endothelial immunoglobulin superfamily resulting in firm adhesion and finally transmigration of the leukocyte (von Andrian and Mackay, 2000). Each of these steps are necessary for lymphocytes to enter lymphoid tissues (except the spleen) and for the accumulation of leukocytes at sites of inflammation (Janeway and Travers, 1997). There are a large number of leukocyte-adhesion receptors, endothelial counter-receptors, chemokines, and chemokine receptors meaning that there are hundreds of possible three-step combinations. Several multi-step combinations occur only in specialised tissues, where only a distinct subgroup cells can participate in every step. Endothelial adhesion molecules with a dominant role in tissue-specific migration are called "vascular addressins"; their counter-receptors on lymphocytes are called "homing receptors" (von Andrian and Mackay, 2000).

Circulating naïve T cells access the lymph node by means of the high endothelial venules. The first step in this process is the loose tethering and rolling mediated by selectins. L-selectin on naïve T cells binds to sulfated carbohydrates on the vascular peripheral-node addressins (PNAd), a group of endothelial sialomucins, which include glycosylation-dependent cell adhesion molecule-1 (GlyCAM-1) and CD34. All of which express a sulphated sialyl-Lewis* (sLe*)-like motif (von Andrian and Mackay, 2000). CD34 is expressed on endothelial cells in many tissues but is properly glycosylated for L-selectin binding only on the high endothelial venule cells of lymph nodes. L-selectin binding promotes a rolling interaction. Although this interaction is too weak to promote extravasation, it is essential for the initiation of the stronger interactions that then follow between the T cell and the high endothelium, which is mediated by molecules with a relatively broad tissue distribution (Janeway and Travers, 1997).

High endothelial venules synthesise a large amount of secondary lymphoid-tissue chemokine (SLC), which is presented on the luminal surface. Exposure of chemokine receptor 7 (CCR7) on rolling T cells to SLC leads to the downstream activation of the integrin LFA-1 (von Andrian and Mackay, 2000). Thus increasing its affinity for ICAM-2, expressed constitutively on all endothelial cells, and ICAM-1, which, in the absence of
inflammation, is expressed only on the high endothelial venule cells of peripheral lymphoid tissues. The binding of lymphocyte function-associated antigen-1 (LFA-1) to its ligands ICAM-1 and ICAM-2 plays a major role in T-cell arrest and migration through the wall of the blood vessel into the lymph node. The increased integrin affinity as a result of cytokine activation allows the leukocyte to attach firmly to the endothelium and the rolling is arrested (Janeway and Travers, 1997). Jones et al., revealed that during extravasation at sites of inflammation lymphocyte adhesion to endothelial cells involves interactions between both ICAM-1/CD18 integrin and VCAM-1/α4β1 integrin. The adhesion molecules responsible for the primary adhesion of T cells to activated endothelial cells could not be defined and were probably independent of the three selectin receptors (Jones et al., 1994).

Finally the leukocyte extravasates, or crosses the endothelial wall. This step also involves the leukocyte integrins LFA-1 and Mac-1, as well as a further adhesive interaction. This involves an immunoglobulin-related molecule called PECAM or CD31, which is expressed both on the leukocyte and at the intercellular junctions of endothelial cells. These interactions enable the leukocytes to squeeze between the endothelial cells. They then penetrates the basement membrane (an extracellular matrix structure) with the aid of proteolytic enzymes that break down the proteins of the basement membrane. The movement through the vessel wall is known as diapedesis, and allows leukocytes to enter the site of infection (Janeway and Travers, 1997).

1.2.4 Features of specialised vasculature

T lymphocytes continuously recirculate from the blood though the lymphoid system and back into the blood. This provides a means of surveillance of the immune system. Lymphocyte adhesion and migration occurs at the high endothelial venules (HEVs), specialised postcapillary venules found in lymphoid tissues that support high levels of lymphocyte extravasation from the blood (Girard and Springer, 1995). HEVs were first described in 1898 by R. Thome, however, it was only in the 1960's their role in the immune system was first described when J. L. Gowans demonstrated that lymphocytes are able to leave the bloodstream via the HEVs, reviewed by (Krall and Mebius, 1997). It is estimated that approximately 26% of lymphocytes passing through the node in the blood stream will adhere and migrate into the node (Bjerknes et al., 1986). In 1976 lymphocytes were described as actively migrating across HEV walls by their initial attachment to HEV surfaces, followed by passing through extracellular spaces and enter the node (Anderson and Anderson, 1976).
HEVs are found in all secondary lymphoid organs (except the spleen) and are also found in chronically inflamed non-lymphoid tissues (Girard and Springer, 1995). The high endothelial venules are located in the T-cell dependent areas of the lymph nodes, such as the paracortical areas and the interfollicular regions (Krall and Mebius, 1997). The high endothelial cells of HEVs have a distinctive appearance, express specialised ligands for lymphocytes and are able to support high levels of lymphocyte extravasation (Girard and Springer, 1995). The endothelial cells of HEVs have a "plump", almost cuboidal, appearance, which is in contrast to the flat morphology of endothelial cells that line other vessels (Girard and Springer, 1995) (Figure 1.2). Large numbers of lymphocytes are detected within the walls of the HEVs. The cytosol of the high endothelial cells are characterised by a prominent Golgi complex, abundant ribosomes and rough endoplasmic reticulum. All of which is indicative of biosynthetic activity not present in flat endothelial cells. A number of vesicles are present suggesting a function in secretion. Another feature of HEVs is the presence of discontinuous junctions between the cells, which is probably to facilitate the passage of lymphocytes between high endothelial cells (Girard and Springer, 1995).

The specialised endothelial cells that line the HEVs in lymph nodes and Peyer's patches constitutively express so-called addressins, which support the homing of naïve lymphocytes. This is in contrast to endothelial cells elsewhere that permit little or no leukocyte binding unless they are exposed to inflammatory mediators (von Andrian and Mackay, 2000). Peripheral lymph node HEVs express the highly glycosylated and sulphated forms of sialomucins, such as the GlyCAM-1, CD34, podocalyxin, endoglycan and endomucin. These highly glycosylated and sulphated sialomucins express unique sugar structures, known as PNAds. Although these sialomucins are expressed in normal venules, they lack the unique PNAd epitopes. Thus lymphocytes expressing L-selectin preferentially home to peripheral lymph nodes through the recognition of the PNAds on HEVs (Miyasaka and Tanaka, 2004).

Effector T cells, which are poised to act in defence of the immune system, can exit the blood vessels at any site of injury or infection. This extravasation occurs preferentially in post-capillary venules (except for the spleen, lung and liver) and the series of events that initiate leukocyte-endothelial interactions and leukocyte migration and diapedesis are similar to those that occur at lymph node HEVs. However, unlike lymph node HEVs transmigration only occurs at these sites upon exposure to inflammatory mediators. The subsets of cell adhesion molecules required for this process depend on the type of tissue and the type of inflammatory stimulus (Vestweber, 2003). It is
Figure 1.2: The sequence of lymphocyte emigration across the walls of HEV.

This drawing shows the characteristic structure of HEV with their cuboidal endothelium, delicate basement membrane and multilaminated reticular cell sheath (RC) which is anchored to the lymph node reticulum by collagen bundles (C). Sequential stages of lymphocyte emigration are designated: (1) attachment to endothelial surfaces; (2) position after migrating around interendothelial junctions; (3) location of lymphocytes moving beneath endothelial cells; (4) migration into the reticular sheath (RC) after crossing the basement membrane; (5) movement across successive laminations of the sheath; and (6) emigration from the sheath into the region of the node (Anderson and Anderson, 1976).
noteworthy that the action of pro-inflammatory cytokines induces the expression of hyaluronan on endothelial cells which has been found to mediate CD44-dependent rolling during inflammation (Mohamadzadeh et al., 1998). Also the expression of CD44 on lymphocytes is required for their extravasation to sites of inflammation (Camp et al., 1993; DeGrendele et al., 1997a).

1.2.5 Role of chemokines in T lymphocyte migration

Chemokines are a large superfamily of low molecular weight proteins (8-15 kDa) that were identified because of the ability to recruit various cell types into sites of inflammation. These secreted heparin-binding molecules serve as powerful chemoattractants for cells of the immune system. More than 50 chemokines and 18 chemokine receptors have been identified to date (Ono et al., 2003). Chemokines are divided into four subfamilies based on the number and spacing of conserved cysteine residues near the N-terminal: CC, CXC, C and CX3C (Mackay, 2001). These chemokines interact with a family of heterotrimeric, 7-pass transmembrane, G protein-coupled receptor. These are expressed almost exclusively on leukocytes (Ono et al., 2003). Chemokines are secreted polypeptides that bind to specific surface receptors, which transmit signal through 7-pass transmembrane G protein receptors. Like adhesion molecules, chemokine receptors can be up-regulated or lost as cells differentiate, allowing leukocytes to coordinate their migratory routes with their immunological function. Since lymphocytes must be positioned correctly to interact with other cells, the patterns of chemokine receptors and the type and distribution of chemokines in tissues critically influence immune responses (von Andrian and Mackay, 2000).

Another means of classification of chemokines has examined the conditions and locations of chemokine productions as well as cellular distribution of chemokine receptors. This has provided a means of distinguishing between “inflammatory” or inducible chemokines and “homeostatic” or constitutive chemokines (see Table 1.1). Inflammatory chemokines are expressed within inflamed tissue, and act to recruit effector cells, including monocytes, granulocytes and effector T cells. Homeostatic chemokines are produced in discrete microenvironments within lymphoid or non-lymphoid tissue such as the skin or mucosa. These constitutive chemokines play a critical role in immune surveillance and the trafficking of lymphocytes to secondary lymphoid tissue (Moser and Loetscher, 2001). Only activated effector lymphocytes can
### Table 1.1 Human chemokines and chemokine receptors in lymphocyte traffic: a functional classification into inflammatory (pale blue) and homeostatic (blue) chemokines. Chemokines belonging to both subfamilies are shown in white.

The chemokines are denoted using the most common names in current use. I-TAC, interferon-inducible T cell α-chemoattractant; MIG, monokine-induced by γ interferon; IP10, interferon-inducible protein 10; RANTES, regulated on activation normal T cell expressed and secreted; MIP-1α, macrophage inflammatory protein 1α; MCP-1, monocyte chemoattractant protein 1; MEC, mucosae-associated epithelial chemokine; MDC, macrophage-derived chemokine; TARC, thymus- and activation-regulated chemokine; LARC, liver- and activation-regulated chemokine, CTACK, cutaneous T cell-attracting chemokine; SDF-1, stromal cell-derived factor-1; BCA-1, B cell-activating chemokine 1; SLC, secondary lymphoid tissue chemokine; ELC, Epstein Barr virus-induced receptor ligand chemokine; TECK, thymus-expressed chemokine; DC-CK1, dendritic cell chemokine 1; BRAK, breast and kidney chemokine (Moser and Loetscher, 2001).
usually respond to inflammatory chemokines, as naïve T cells do not typically express the receptors for these chemokines (Olson and Ley, 2002).

Most chemokines are secreted and in order to effectively induce chemotaxis in vivo, these proteins must be immobilised on cell or extracellular matrix surfaces by interacting with negatively charged glycosaminoglycans. It is interesting to note that specific chemokines bind different types of glycosaminoglycans with divergent affinities, offering another layer of regulation. Glycosaminoglycan expression can differ depending on cell type, location and inflammatory status. Oligomerisation of chemokines on glycosaminoglycan structures near their site of production, may provide an effective mechanism of establishing a chemokine gradient and enabling leukocyte migration toward the relevant site (Olson and Ley, 2002).

There is a variety of responses to specific chemokines; these include altered morphology, extension of lamellipodia through cytoskeletal changes, release of oxygen radicals, histamine and cytotoxic proteins from neutrophils, basophils and eosinophils, respectively as well as the promotion of angiogenesis or angiostasis (Mackay, 2001; Olson and Ley, 2002). Certain chemokines can trigger the integrin-dependent firm adhesion of rolling cells and it is this aspect of chemokine signalling that is of interest in this section. Selectins are constitutively activated and signal lymphocyte rolling in response to an encounter with their specific glycoprotein ligand, thus enabling the tethering of lymphocytes to endothelium. However the next step of lymphocyte extravasation involves the integrin family of adhesion molecules, which must be activated to mediate adhesion. Rapid conformational changes must take place in order to regulate integrin affinity for its ligand, leading to firm arrest and transendothelial migration (Mackay, 2001).

1.3 ADHESION RECEPTORS

Although originally thought to simply act as sticky molecules, adhesion receptors have also been found to function in cellular signalling (Ponta et al., 2003). Cell surface adhesion receptors not only anchor cells to their surroundings, but they also regulate cell mobility and provide cells with sensors of their environment. Cell adhesion molecules are subject to regulation at multiple levels, including transcription, alternative RNA splicing, and posttranslational modifications such as phosphorylation, glycosylation and sulfation. Proteolytic processing is emerging as a key mechanism
underlying the regulation of several cell surface adhesion molecules, including members of the cadherin and selectin families (Cichy and Pure, 2003). Here the key families of adhesion molecules are reviewed, as well as their functions within the cell. Of particular interest in this study were the integrin and CD44 adhesion receptors and therefore these topics were reviewed in more detail.

1.3.1 The immunoglobulin superfamily
This was the first class of adhesion receptor to be identified. The immunoglobulin superfamily is the most abundant family of cell surface molecules (Holness and Simmons, 1994). Immunoglobulin superfamily is characterised by the presence of multiple immunoglobulin domains (Figure 1.3). The basic immunoglobulin domain consists of 90-100 amino acids arranged in a sandwich of two sheets of anti-parallel β-strands, which are usually stabilised by a disulfide bond at its centre (Springer, 1990). This family consists of in excess of 100 molecules which serve many different functions including: receptors for growth factors (CSF-1 receptor, PDGF receptor, FGF receptors); cell surface proteins that participate in cellular recognition including TCR, MHC class I and II, and as adhesion molecules CD2, CD4, CD8, ICAM-1, -2, -3, VCAM-1, MAdCAM-1, NCAM and LFA-3 (Holness and Simmons, 1994).

Immunoglobulin receptors play roles in many processes such as embryonic development and are expressed on cells of the nervous system, epithelial and leukocytes. Of particular interest are the ICAM receptors, which help to localise leukocytes to areas of tissue injury and play important roles in immune-mediated cell-cell interactions.

The ICAMs are structurally related members of the immunoglobulin superfamily of adhesion receptors and are ligands for the β2 integrins expressed on leukocytes, emphasizing the importance of these molecules in immune interactions. Five ICAM molecules have been identified and these have been classified together because they contain two or more immunoglobulin domains in their extracellular domain. These molecules are transmembrane proteins with a short cytoplasmic tail. ICAM-1 (CD54) has five extracellular immunoglobulin-like domains with the first domain responsible for binding to LFA-1 (CD11a/CD18) and the third domain responsible for binding to MAC-1 (CD11b/CD18) (Figure 1.3). ICAM-3 (CD50) also has five immunoglobulin-like domains. The molecules ICAM-2 (CD102) and ICAM-4 have only two domains and ICAM-5 (telencephalin) has nine immunoglobulin-like domain (Hubbard and Rothlein, 2000).
Figure 1.3: A diagram of an ICAM-1 molecule.
The immunoglobulin domains are indicated D1-D5 with the approximate location of binding sites of LFA-1 and MAC-1 shown. The sites of glycosylation are also shown (indicated by lollipop-shaped structures) (Dietrich, 2002).
ICAM-1 is expressed constitutively at low levels on endothelial cells and on some lymphocytes and monocytes. ICAM-1 expression is increased in response to inflammatory cytokines, such as interleukin-1 (IL-1), tumour necrosis factor-α (TNFα) and IFNγ or with lipopolysaccharide (LPS) (Hubbard and Rothlein, 2000). It binds to LFA-1 and MAC-1 present on the membranes of neutrophils, T cells and macrophages (Elangbam et al., 1997). ICAM-1 is an important receptor for human rhinovirus, the most frequent cause of upper respiratory tract infections (Whiteman et al., 2003). The plasma protein, fibrinogen has also been shown to act as a ligand for ICAM-1 (Tsakadze et al., 2002). ICAM-1 participates in the recruitment of leukocytes to inflamed endothelium after injury or stress. ICAM-2 is constitutively expressed on endothelial cells and mononuclear leukocytes and binds to LFA-1 (Hubbard and Rothlein, 2000). ICAM-2 plays a role in the initial localisation of neutrophils to sites of tissue injury (Elangbam et al., 1997).

ICAM-3 is found highly expressed on resting leukocytes, which bind LFA-1 (de Fougerolles and Springer, 1992). This molecule is thought to play a key role in the initiation of immune responses and it may be important in the establishing dendritic cell interactions with T lymphocytes because of its constitutive expression on lymphocytes (Hubbard and Rothlein, 2000; van Kooyk and Geijtenbeek, 2002). ICAM-3 is potentially the most important ligand for LFA-1 in the initiation of an immune response because the expression of ICAM-1 on resting leukocytes is low (Elangbam et al., 1997). It has also been shown that ICAM-3 is important in scanning the surface of the antigen presenting cell by T cells and in initiating the adhesive interactions between these cells that are critical for generating an immune response (Montoya et al., 2002). ICAM-4 is restricted to erythrocytes and erythroid precursors, with an unknown function. ICAM-5 is strongly expressed in brain gray matter (Hubbard and Rothlein, 2000). These ICAM/integrin interactions (i.e., ICAM-1 with CD11a and CD11b; ICAM-2 with CD11a; ICAM-3 with CD11a) provide a mechanism for selective recruitment of leukocytes in different pathological situations (Elangbam et al., 1997).

1.3.2 Cadherins

Cadherins are an important family of cell-adhesion molecules that are responsible for strong cell-cell contacts through homophilic interaction (Ponta et al., 2003). The cadherins are a family of calcium-dependent adhesion molecules that form and maintain adhesive interactions between cells. Cadherins are single-pass transmembrane proteins, characterised by an extracellular domain consisting of tandemly repeated cadherin sequences. These repeat sequences required calcium for
their rigidity, adhesiveness and stability. This family of adhesion molecules can be classified into a number of subsets, the type I (classical) and type II cadherins, the desmosomal cadherins and the protocadherins (Patel et al., 2003). The classical members of the cadherin family have been the most widely studied, with considerable interest in the role of E-cadherin in epithelial tissue. In epithelial tissue, E-cadherin is thought to form anti-parallel dimers with identical molecules in the adjacent cell, creating a zipper-like structure between cells. On the cytoplasmic side, E-cadherin interacts with either β- or γ-catenin. α-Catenin then links the bound β- or γ-catenin to the actin cytoskeleton (Guilford, 1999). The interaction of cadherins with the intracellular proteins provides a link to the cytoskeleton.

E-cadherin is essential during development and in the maintenance of an intact epithelium with an established polarity. In addition during cell migration, there appears to be a down-regulation in the surface expression of E-cadherin, apparently facilitating the reduction in adhesion required for migration (Guilford, 1999). In order for tumour cells to metastasise from the primary site the malignant cell must first of all reduce intercellular adhesiveness. Thus E-Cadherin is thought to act as an important buffer against epithelial tumour cell invasion and metastasis. In a transgenic mouse model of pancreatic β-cell tumorigenesis it was shown that loss of E-cadherin results in the progression from adenoma to invasive carcinoma (Perl et al., 1998). Indeed germline mutations of the E-cadherin gene have been shown to result in an inherited susceptibility to gastric cancer (Guilford, 1999).

Cadherins are essential mechanical homeostasis of tissue, however cadherins do not play a significant role in the immune system and will not by discussed further in this context.

1.3.3 Integrins

The integrins are a family of heterodimeric, glycosylated, type 1 transmembrane receptors, composed of α- and β- subunits that are important for cell-cell and cell-extracellular matrix adhesion. Integrins and their ligands play key roles in development, immune responses, leukocyte traffic, haemostasis, and cancer. There are 24 members, consisting of one of 18 α and one of 8 β subunits that are non-covalently associated. Alternative splicing of mRNA of some α and β subunits and post-translational modifications of integrin subunits further increase the diversity of the integrin family (van der Flier and Sonnenberg, 2001). They were originally named as
integrins to reflect their function in linking the extracellular matrix (ECM) and the cytoskeleton.

<table>
<thead>
<tr>
<th>Integrin</th>
<th>Ligand</th>
<th>Cell-cell</th>
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<tbody>
<tr>
<td>α4β1 (VLA-4)</td>
<td>Fibronectin</td>
<td>VCAM-1</td>
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<tr>
<td></td>
<td>Osteopontin</td>
<td></td>
</tr>
<tr>
<td>α4β7 (LPAM-1)</td>
<td>Fibronectin</td>
<td>VCAM-1</td>
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<td></td>
<td></td>
<td>MAdCAM</td>
</tr>
<tr>
<td>αLβ2 (LFA-1)</td>
<td></td>
<td>ICAM-1-5</td>
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<tr>
<td>αMβ2 (Mac-1, CR3)</td>
<td></td>
<td>ICAM-1</td>
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<td></td>
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<td>VCAM-1</td>
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<td>αDβ2</td>
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<td></td>
<td>VCAM-1</td>
</tr>
<tr>
<td>αXβ2 (p150,95)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>αEβ7</td>
<td></td>
<td>E-cadherin</td>
</tr>
</tbody>
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Table 1.2: Integrins that participate in the immune response and their ligands

However, although the majority of integrins bind to ECM proteins, certain integrins interact with counter-receptors on other cells, soluble plasma proteins or microorganisms (Table 1.2) (Miranti and Brugge, 2002). Most integrins recognise relatively short peptide motifs and, in general, a key constituent residue is an acidic amino acid, such as aspartic acid or glutamic acid (Hynes, 2002). The combination of α and β subunits determines the ligand specificity of the integrin. Many integrins bind to the same ligands, however it is the combination of the integrin expression/activation pattern and the availability of ligand that specifies the interactions in vivo (van der Flier and Sonnenberg, 2001).

Following integrin-mediated adhesion and clustering, integrins recruit different cytoskeletal and cytoplasmic proteins, which anchor the newly formed complexes to the actin cytoskeleton. Downstream of this event is the local re-modeling of the actin cytoskeleton, with the formation of specialised adhesive structures, called focal adhesions. Focal adhesions not only act as a structural link between the ECM and the actin cytoskeleton, they are also important sites of signal transduction (van der Flier and Sonnenberg, 2001). An important feature of integrins is that they can signal through the cell membrane in either direction. The extracellular binding activity of
integrins is regulated from the inside of the cell (inside-out signalling), while the binding of the ECM elicits signals that are transmitted into the cell (outside-in signalling) (Giancotti and Ruoslahti, 1999).

The ligand-binding site of integrins is present in the globular head domain formed by the α and β subunits, while the rest of the extracellular domain forms the membrane-proximal stalk-like part of the molecule (Figure 1.4). The α subunits have seven repeat motifs, in their extracellular domain, that are thought to fold into a seven-bladed propeller structure that forms one globular domain with the ligand-binding site on the upper surfaces (Springer, 1997). The extracellular domains of several integrin α subunits contain an insertion of 200 amino acids, the I domain, which is critical for ligand binding. This domain exists in an “open” ligand binding or “closed” nonbinding conformation (Lu et al., 2001; Salas et al., 2002). Within the integrin I domain there is a characteristic, metal ion-dependent (Mg$^{2+}$) adhesion site motif (MIDAS), which is critical for ligand binding (Emsley et al., 2000).

The cytoplasmic tails of integrin subunits are generally short and always devoid of enzymatic features. Hence, integrins transduce signals by associating with adapter proteins that connect the integrin to the cytoskeleton, cytoplasmic kinases and transmembrane growth factor receptors. Integrin signalling and assembly of the cytoskeleton are intimately linked. As integrins bind to ECM, they become clustered in the plane of the cell membrane and associate with a cytoskeletal and signalling complex that promotes the assembly of actin filaments. Actin filaments reorganise into larger stress fibers, thus causing more integrin clustering, enhancing the matrix binding and organisation by integrins in a positive feedback system (Giancotti and Ruoslahti, 1999).

Integrin expression by leukocytes can change in a signal- and time-dependent manner. It is interesting to note that β2 heterodimers are restricted to cells of the leukocyte lineage (Harris et al., 2000). In leukocytes, integrins such as LFA-1 (αLβ) are usually in an inactive state to ensure that they do not bind inappropriately to their ligands. However, in the process of emigration from blood into tissues, the exposure of leukocytes to stimuli such as chemokines rapidly activates integrin through changes in affinity and/or avidity (Chan et al., 2003). Several genetic diseases have been found to be due to mutations in integrin subunits. For example, the human genetic disease, called leukocyte adhesion deficiency (LAD), is due to the lack of functional integrin β2-
Figure 1.4: Features of the β2 integrins.
The αβ heterodimeric structure is common to all integrins. The α chain includes seven extracellular N-terminal homologous repeats organised in a propeller structure. The α chain I domain (pink) with the embedded MIDAS motif (orange) and the β chain I-like domain with MIDAS domain. The GFFKR sequence (green) in the cytoplasmic tail of the α is involved in heterodimer assembly and regulation for ligand recognition. The heterodimer is in the "closed" or inactive state that undergoes tertiary and quaternary changes in response to inside-out signals (Harris, 2000).
chains (CD18), essential for neutrophil extravasation into sites of inflammation. As a result, these patients suffer from life threatening infections (Bunting et al., 2002).

1.3.4 Selectins

Selectins are a family of single pass transmembrane proteins that consist of three members, L-, E- and P-selectin (Figure 1.5). These names were designated according to the first cell type where they were identified: L-selectin is expressed on most leukocyte types, E-selectin is expressed on activated endothelium and P-selectin was first identified in the storage granules of platelets and is also expressed by endothelial cells. Their function is in the first stage of leukocyte extravasation where they act to capture leukocytes from the bloodstream to the blood vessel wall (Vestweber and Blanks, 1999). All three selectin genes have been mapped to a gene cluster of approximately 300kb on the long arm of chromosome 1 in the human genome. Structurally the selectins have an N-terminal domain with homologies to the Ca2+-dependent lectins, preceded by an epidermal growth factor (EGF)-like motif (Springer, 1990). This motif is then followed by a varying number of short consensus repeat (SCR) motifs similar to motifs found in many proteins involved in regulating complement activation. The number of SCR, which vary from two to nine in the three different selectins, may enable the positioning of their N-terminal lectin-like putative binding sites at varying distances from the plasma membrane. The selectins function as lectins and bind carbohydrate ligands. These cell adhesion molecules are anchored in the membrane by a single transmembrane region that is followed by a short cytoplasmic tail (Vestweber and Blanks, 1999).

L-Selectin

L-selectin was originally described as a lymphocyte “homing receptor” involved with the initial attachment of lymphocytes to lymph node HEV. L-selectin is expressed on all classes of leukocytes, except activated memory lymphocytes (Camerini et al., 1989). It participates in both lymphocyte and neutrophil extravasation at inflammatory sites (Kishimoto et al., 1989). L-selectin antibodies can block leukocyte migration to peripheral lymph nodes and to the inflamed peritoneum of mice (Watson et al., 1991).

P-Selectin

P-selectin with nine SCR motifs in its extracellular domain, was originally identified in activated platelets (McEver and Martin, 1984). It is now known that P-selectin is expressed by endothelial cells and platelets for the recruitment of neutrophils and macrophages from the circulation (Larsen et al., 1990). P-selectin is constitutively
Selectins are composed of an N-terminal lectin domain, a single epidermal growth factor (EGF)-type repeat, and various numbers of consensus repeats or complement binding domains. Proteins have a single transmembrane region and a short cytoplasmic tail (Vestweber and Blanks, 1999).
expressed and held in Weibel-Palade bodies, allowing the instant translocation to the plasma membrane upon activation. Stimuli such as, thrombin and TNF-α, can mobilise intracellular stores of P-selectin (Sugama et al., 1992; Weller et al., 1992). Activation results in the phosphorylation of P-selectin on cytoplasmic tyrosine, threonine and serine residues, with concomitant translocation to the cell membrane (Fujimoto and McEver, 1993; Green et al., 1994). Shortly after its initial appearance P-selectin can be internalised within vesicles from where it can either be recycled or degraded within lysosomes (Green et al., 1994). Two P-selectin ligands have been characterised to date: P-selectin glycoprotein ligand-1 (PSGL-1), a transmembrane sialomucin of 110 kDa which is associated into dimers by disulfide bonds and the 120 kDa P-selectin ligand which is a sialoprotein and binds P-selectin in a Ca2+ dependent fashion (Norgard et al., 1993). It has been shown that truncation of the number of SCR domains in P-selectin can impair its ability to support rolling of leukocytes (Vestweber and Blanks, 1999).

**E-Selectin**

E-selectin is perhaps the most specific inducible endothelial cell surface molecule supporting the binding of neutrophils, monocytes and a sub-population of lymphocytes to inflammatory tissues. In addition, eosinophils bind E-selectin and this appears to facilitate access to areas of allergic inflammation (Kyan-Aung et al., 1991). E-selectin has six SCR motifs and is expressed mainly by endothelial cells of post capillary venules and to a lesser degree by capillaries. The expression of E-selectin on endothelial cells requires *de novo* synthesis. IL-1, TNFα, lymphotoxin and LPS can stimulate the production of E-selectin (Shimizu et al., 1991; Weller et al., 1992).

A genetic disease, named LAD II (leukocyte adhesion deficiency), has been described where, there is a marked reduction in the ability of neutrophils to adhere to endothelium, recurrent bacterial infections, and localised cellulites without pus formation. The disease is thought to be due to a defect in fucose metabolism. These patients lack sialyl Lewis*, a fucose-containing tetrasaccharide known to bind all three selectins. Physiological selectin ligands contain α-(1,3)-fucose as an essential structural element. Neutrophils of these patients do not bind to E- or P-selectin-expressing endothelial cells *in vitro* (Philips et al., 1995) and do not roll in venules under shear force (von Andrian et al., 1993).
1.3.5 Link protein family

Many hyaluronan-binding proteins (also termed hyaladherins) contain a common structural domain of approximately 100 amino acids in length, termed a link module, that is involved in ligand binding. The diversity in responses to hyaluronan is mediated largely by the large number of hyaluronan-binding proteins; proteins that exhibit differences in their distribution, expression, affinity and regulation (Day and Prestwich, 2002). An example of this is the hyaluronan receptor, LYVE-1. The expression of this protein is restricted to lymph vessel endothelium and may play a role in hyaluronan degradation (Banerji et al., 1999).

The link module (also referred to as a proteoglycan tandem repeat (Watanabe et al., 1997)) was first identified in the link protein isolated from cartilage. Its structure consists of two α-helices and two triple-stranded anti-parallel β-sheets. The link protein is comprised of an immunoglobulin domain and two contiguous link modules. The proteoglycans aggrecan, versican, neurocan, and brevican all contain domains homologous to the link module (Day and Prestwich, 2002). These proteoglycans form huge, link protein-stabilised complexes with hyaluronan that provide the load-bearing function in articular cartilage, give elasticity to blood vessels and contribute to the structural integrity of many tissues such as skin and tissue (Day and Prestwich, 2002; Watanabe et al., 1997; Yamaguchi, 2000). The cell surface protein CD44 and the secreted tumour necrosis factor inducible protein (TSG-6) both possess a single copy of this link module (Goldstein et al., 1989; Lee et al., 1992). As the glycoprotein, CD44, is central to the work carried out in this study a more complete review of the protein and its functions follows.

1.4 CD44

CD44 refers to a collection of structural isoforms expressed on distinct cell types. CD44 existed in a number of different guises in much of the early literature such as; phagocyte glycoprotein-1 (Pgp-1) (Lesley and Trowbridge, 1982; Trowbridge et al., 1982), the blood group antigen In(Lu) p80 (Telen et al., 1983), hyaluronate receptor (Culty et al., 1990), extracellular matrix III (ECMRIII) (Carter and Wayner, 1988; Gallatin et al., 1989), Hermes antigen and H-CAM (Picker et al., 1989). Around the same time, a number of groups working in unrelated fields of research identified CD44 independently and attributed distinct functions to this receptor. This confusion was due
largely to the diversity in structure and function of this protein. When the International Workshop on Human Leukocyte Differentiation Antigens began to catalogue the growing number of cell surface molecules, it became clear that the assortment of proteins belonged to the CD44 family of proteins.

Jalkanen et al (1986) isolated a 90kDa glycoprotein expressed on the surface of human lymphocytes, a protein recognised by the monoclonal antibody, Hermes-1 and found to be important in the endothelial cell recognition and lymphocyte trafficking in humans (Jalkanen et al., 1986). Monoclonal antibodies recognising the Hermes lymphocyte adhesion antigen, the polymorphic glycoprotein Pgp-1 antigen, and the human CD44 antigen were shown to recognise the same molecule (Picker et al., 1989). Indeed, mouse Pgp-1 was identified as being homologous to human CD44 (Zhou et al., 1989). Many reviews have helped to clarify the diverse historical nomenclature and present the evidence that has brought this assortment of molecules and their proposed functions together under the designation CD44 (Lesley et al., 1993).

CD44 is a broadly distributed transmembrane glycoprotein that plays a role in a variety of cell functions, including adhesion, migration, invasion and survival. It is expressed on many cell types, such as most haematopoietic cells, keratinocytes, chondrocytes, many epithelial cell types and some endothelial and neural cells (Lesley and Hyman, 1998). CD44 is the principle cell surface receptor for hyaluronan, with loss of CD44 binding to tissue following predigestion with hyaluronidase (Aruffo et al., 1990).

1.4.1 Gene structure of CD44
A single, highly conserved gene encodes all CD44 proteins, but alternative splicing generates multiple isoforms. Mouse CD44 is located on chromosome 2 and human CD44 is located on the short arm of chromosome 11 (11p13) reviewed by (Naor et al., 1997). The heterogeneity of proteins expressed by the CD44 gene is generated by both alternative splicing of the CD44 gene and posttranslational modifications, such as glycosylation. Screaton et al first described the structure of the CD44 gene. It was described in both humans and mouse as containing 20 exons spanning 60 kilobases of human DNA, with 10 exons that can be alternatively spliced to produce an array of variant isoforms (Figure 1.6) (Screaton et al., 1993; Screaton et al., 1992).

The first 5 exons encode for the extracellular domain (exons 1-5), whereas the next 10 exons are subject to alternative splicing. The generation of variant isoforms is due to the use of internal splice donor and acceptor sites within the individual exons 5 and 16. Thus generating a variable region containing different exon combinations, with the
Figure 1.6: CD44 structure.
(a) The genomic organisation of CD44. The human gene encoding CD44 consists of 20 exons. Exons 1-5, 16-17 encode the extracellular domain of the standard form of CD44 (yellow), with exons 2 and 3 encoding the hyaluronan binding domain (cross-hatched). Exons 18 encodes the transmembrane domain (stippled), exons 19-20 encode the cytoplasmic domain (red), and V1-V10 encode the variant exons (orange).
(b) mRNA splicing pattern in CD44. The standard form of CD44, CD44s, comprises exons 1-5, 16-18 and 20. Most variant forms of CD44, CD44v, contain the variant exons 6-15 (v1-v10). The inclusion of exon 19, normally absent in most CD44 transcripts, results in a short tail variant due to the presence of an alternative stop codon (Thorne et al., 2004).
variable region exons designated V1 to V10. The ten alternatively spliced exons were identified in the extracellular domain. Exons 16 and 17 encode the membrane-proximal region of the extracellular domain, (together with part of exon 5 with optional inclusion of the variant exons). The hydrophobic transmembrane region is encoded by exon 18. The cytoplasmic domain is subject to alternative splicing, with exon 19 and 20 encoding the short tailed or long tailed version of the cytoplasmic domain, respectively (Figure 1.6) reviewed by (Naor et al., 1997; Ponta et al., 2003; Screaton et al., 1992).

1.5 PROTEIN STRUCTURE OF CD44

Antibodies raised against CD44 recognise a polymorphic group of proteins (80-200kDa in size) which, as discussed above, are derived from a single gene. Using alternative splicing and post-translational modification (which may depend on the cell type and growth conditions) allows this diverse group of proteins to be generated. The standard or haematopoietic isoform of CD44 (designated CD44s or CD44H respectively) is a single-pass (type 1) transmembrane molecule which does not carry any products of the alternatively spliced exons. CD44s is a single-chain molecule composed of a distal extracellular domain (containing the ligand-binding sites), a membrane proximal region, a transmembrane-spanning domain, and a cytoplasmic tail (Figure 1.7) (Naor et al., 1997). The membrane proximal region of the extracellular domain is relatively non-conserved, whereas the rest of the molecule is well conserved between species (Naor et al., 1997). The variable exons may be inserted by alternative splicing between the amino acids 202 and 203 as previously outlined in Figure 1.6 (Lesley and Hyman, 1998). The following review will examine the structural features of CD44 protein.

1.5.1 Extracellular domain

The extracellular domain of CD44 consists of 270 amino acids and can be divided between conserved and non-conserved regions (Goodison et al., 1999). The first five (non-variable) exons of CD44 encode the N-terminal ectodomain. The N-terminal 180 amino acids of CD44 are relatively conserved among mammalian species (~85% homology) (Lesley and Hyman, 1998). These are thought to fold into a globular tertiary structure by the formation of disulfide bonds between the six cysteine residues present in this section (Goodison et al., 1999). The N-terminal domain consists of a region of approximately 100 amino acids with homology to other hyaluronan binding proteins. This "link module" will be examined in greater detail in the next section (Section 1.5.2).
Figure 1.7: The protein structure of CD44. The protein structure of CD44 is compared with that of the largest variant isoform CD44v1-10, which shows that the sequences encoded by the variant exons are in the stem region (Ponta et al 2003)
Additionally there are five conserved N-glycosylation sites (at positions Asn25, Asn57, Asn100, Asn110 and Asn120 designated N1-N5 respectively) present within the N-terminal 150 residues of CD44 (Lesley and Hyman, 1998). The role of these glycosylation sites and post-translational modification in the function of CD44 will be reviewed in Section 1.5.3.

The amino-terminal globular domain of CD44s is separated from the plasma membrane by a short (46 residues) stem structure. This stem structure contains putative cleavage sites. A membrane-associated metalloproteinase has been described as cleaving CD44 at the membrane proximal domain, resulting in the promotion of CD44-mediated tumour cell migration (Okamoto et al., 1999). The importance of this domain in the rolling of lymphoid cells on hyaluronan has been demonstrated using CD44 mutants lacking 83 amino acids from the "non-conserved" membrane proximal region of the extracellular domain. These CD44 mutants firmly adhered to the hyaluronan substrate but were unable to support rolling at any of the shear forces tested (Gal et al., 2003). This is in agreement with previous findings that the membrane proximal domain of CD44 is not essential for hyaluronan binding (He et al., 1992).

1.5.2 The hyaluronan binding domain

Most hyaluronan binding proteins interact with hyaluronan via a link module, a domain of approximately 100 residues. The link module comprises two anti-parallel β sheets and two α helices, stabilised by two highly conserved disulfide bridges (Kohda et al., 1996). Also, this domain is structurally related to C-type lectins (Barjorath et al., 1998; Kohda et al., 1996). As reviewed in Section 1.3.5, link modules are found in extracellular matrix molecules, for example link protein, aggrecan, versican, and the protein product of tumour necrosis factor-stimulated gene-6 (TSG-6). It is this structure that is thought to be critically involved with binding of hyaluronan to CD44, although recent evidence has shown that other residues outside of this domain are also required for hyaluronan binding. Bajorath et al., 1998 developed a structural model of the link module of CD44 based on the solution structure of the homologous region of TSG-6 that had been previously determined by NMR (nuclear magnetic resonance) (Kohda et al., 1996).

However, the hyaluronan-binding domain (HABD) of CD44 involves the contribution of sequences outside the consensus link module. Constructs with only the link module expressed in E. coli fail to refold and are functionally inactive (Banerji et al., 1998).
Also, site-directed mutagenesis indicates that amino acids in the flanking sequences participate in hyaluronan binding in addition to those located in the link module (Barjorath et al., 1998). Mapping of the epitopes for monoclonal antibodies that block hyaluronan binding indicates that the cluster of basic residues located in the C-terminal extension is in close proximity to a region within the first 22 amino acids of the mature protein (Liao et al., 1995). All these suggest that CD44 contains an extended HABD that is specially adapted for the regulation of ligand binding.

Recently, Teriete et al. have elucidated the regulatory HABD structure in CD44 (namely amino acids 20-178). Using both NMR and X-ray crystallography the role of sequences flanking the link module in the structure of the HABD were described. Previously the secondary structure of the HABD of CD44 had been reported to comprise of two $\alpha$-helices and two $\beta$-sheets, with sheet I composed of five strands and sheet II described as having two-anti-parallel strands (Takeda et al., 2003). However, Teriete et al. determined that the HABD comprised of two orthogonally disposed $\alpha$ helices and ten $\beta$ strands organised in a single long, curving $\beta$ sheet (Figure 1.8). The regions flanking the Link module provide an additional four $\beta$ strands, extending the $\beta$ sheet (SI). The presence of key sites for N-glycosylation (Asn25 and Asn120) within this enlarged CD44 HABD is thought to provide a mechanism of regulation of ligand binding in response to extracellular factors (Teriete et al., 2004).

1.5.3 Posttranslational modification

Posttranslational modifications of CD44 regulate the affinity of this receptor for its ligands, such as by the addition of carbohydrate sidechains. Many of these modifications are outlined in Table 1.3, and their importance in mediating ligand binding is described in the relevant section.
Figure 1.8: The structure of CD44 hyaluronan binding domain determined by NMR and X-ray crystallography.
Cartoon representations of the energy minimised averaged structure determined by NMR (a) and the crystallographic structure (b). Cysteine residues participating in disulfide bonds are drawn in ball and stick representation (Teriete et al, 2004).
This section will review the role of glycosylation in CD44. Section 1.5.1 identified five conserved consensus sites for N-glycosylation, located in the N-terminal 120 amino acids of CD44. Using murine CD44s, English et al., 1998, found that there was no specific requirement for these five N-glycosylation sites. Indeed, the individual mutation of N1 and N5 was shown to convert CD44 from an inducible hyaluronan-binding state to a constitutively active state in murine lymphoma cell lines, having a positive effect on hyaluronan binding (English et al., 1998). However, mutation of any one of the five N-glycosylation sites of human CD44s resulted in the loss of wild type CD44-mediated adhesion to hyaluronan in human melanoma cell lines. Also, recognition of the CD44 structure by certain monoclonal antibodies was also lost, implying that N-glycosylation may be required to maintain the molecule in a conformation that allows ligand binding (Bartolazzi et al., 1996). Other evidence indicates that a minimal N-glycan structure is important for HA binding. Bartolazzi et al and Skelton et al, demonstrated that the complete absence of N-glycosylation inhibited HA binding, whereas truncated N-glycans enhanced HA binding. It was also reported that the first N-linked N-acetyl-glucosamine residue was sufficient to promote HA binding, and subsequent modification of the N-glycans, especially by alpha 2,3-linked sialic acid, reduced binding affinity (Skelton et al., 1998).

Table 1.3: Structural variations in CD44 domains (Kincade et al., 1997)

<table>
<thead>
<tr>
<th>CD44 Domain</th>
<th>Possible variability</th>
</tr>
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<tbody>
<tr>
<td>Amino terminal</td>
<td>Sequence polymorphism</td>
</tr>
<tr>
<td></td>
<td>N-glycan additions</td>
</tr>
<tr>
<td>HA binding</td>
<td>N-glycan additions</td>
</tr>
<tr>
<td>Membrane proximal</td>
<td>Alternate mRNA splicing</td>
</tr>
<tr>
<td></td>
<td>(producing variable sequences)</td>
</tr>
<tr>
<td></td>
<td>glycosylation, glycosaminoglycan attachment</td>
</tr>
<tr>
<td>Transmembrane</td>
<td>Dimerisation, acylation, association with other proteins</td>
</tr>
<tr>
<td>Cytoplasmic</td>
<td>Alternative mRNA splicing</td>
</tr>
<tr>
<td></td>
<td>(affecting lengths)</td>
</tr>
<tr>
<td></td>
<td>Phosphorylation, association with other proteins</td>
</tr>
</tbody>
</table>
Investigations into the role of N-glycosylation in hyaluronan binding in human and murine CD44 has yielded conflicting evidence. This may be due to differences between murine and human CD44 sequences around this region such that certain mutations affect the conformation of one CD44 differently than the other. Alternatively they may reflect differences in oligosaccharide structure between the cell lines in the two studies. There are also four serine-glycine (Ser-Gly) motifs in the membrane proximal domain, which provide potential sites for glycosaminoglycan side chain attachment. Mutation of any one of these sites resulted in a decrease in the hyaluronan binding ability (Bartolazzi et al., 1996). In the absence of N-glycosylation, O-glycans have a positive effect on hyaluronan binding of CD44 (Skelton et al., 1998).

There is considerable evidence highlighting the importance of glycosylation in regulating the activation state of CD44 in terms of hyaluronan binding: (a) inactive, (b) inducible and (c) constitutively active. CD44 from active cells had less N-glycosylation than that from inducible cells and CD44 from inducible cells was less N-glycosylated than that from inactive cells (based on relative migration of their CD44 in SDS-PAGE before and after N-glycanase treatment (Katoh et al., 1995; Lesley et al., 1995). Inhibition of N-glycosylation with tunicamycin activated hyaluronan binding in some cell lines that were inducible or inactive (Katoh et al., 1995; Lesley et al., 1995). A number of studies have shown that the removal of sialic acid (both from cell surface and from CD44-Ig fusion proteins) enhances hyaluronan binding by inducible or inactive CD44 (English et al., 1998; Katoh et al., 1995; Skelton et al., 1998; Zheng et al., 1997). Indeed, Zheng et al., 1997, showed that hyaluronan binding cells had less sialic acid on their CD44 than non-binding cells. When combined with the results that inhibition of N-glycosylation by tunicamycin, truncation of N-glycans and mutation of specific N-glycosylation sites can all enhance hyaluronan binding, it is likely that cell specific sialylation of N-glycans is involved in restricting hyaluronan recognition by CD44.

Although there is a large body of evidence that suggests that cell-specific modification at N-glycosylation sites have an important role in ligand recognition, until recently there was little understanding about how these modifications could alter CD44 binding to hyaluronan. Two of these N-glycosylation sites are present outside of the link domain (Asn25 and Asn 120), and the mutation of either one of these has been shown to convert CD44 from an inducible to a constitutively active state in murine lymphoma cell lines (English et al., 1998). Modelling of the HABD of CD44, established that Asn25 was located in the hyaluronan-docking site. Thus, the attachment of a negatively charged sugar chain to Asn25 would directly obstruct binding of the anionic
hyaluronan to its docking site. Asn120 was on the rear face of the HABD and it has been proposed that this site regulates hyaluronan binding by preventing the formation of CD44 dimers or higher oligomers, which may be required for optimal binding to adjacent sites on the polymeric hyaluronan (Teriete et al., 2004).

1.5.4 Transmembrane domain

The transmembrane domain of CD44 is well conserved among several mammalian species. A transmembrane domain and a minimal cytoplasmic domain are required for efficient ligand binding but it appears that this specific sequence is not absolutely required for hyaluronan binding. Either domain can be replaced with equivalent domains from unrelated receptors without hindering or enhancing the binding of soluble hyaluronan, adhesion of cells to a hyaluronan substrate or hyaluronan-dependent rolling (reviewed by (Lesley and Hyman, 1998; Thorne et al., 2004). As a result there has been no role defined for the transmembrane and cytoplasmic domain of CD44 in “inside-out” signalling.

CD44 has been described as existing in Triton X-100-insoluble extracts. This is thought to reflect the association of CD44 with lipid rafts in the cell membrane. The proportion of CD44 found in the Triton-X insoluble fractions appears to be cell type dependent, with a high proportion detected in fibroblast cells but little present in the detergent insoluble fractions of epithelial cells (Neame et al., 1995). The association of CD44 in detergent insoluble fractions was demonstrated to depend on the transmembrane domain of the protein. Mutants with the transmembrane domain replaced by equivalent domain of either CD3 ζ chain or CD45 were completely Triton X-100 soluble (Perschl et al., 1995). Lipid rafts have been described as lateral assemblies of glycosphingolipids and cholesterol and have been proposed to form platforms for a number of cellular events, such as membrane trafficking, signalling and cell adhesion. They function by a separation or concentration of specific membrane proteins or lipids in membrane microdomains. They may serve as a platform for the recruitment and concentration of signalling molecules at the plasma membrane (Harder et al., 1998).

Two possible sites for acylation (i.e. covalent modification of protein with lipid) are present in CD44. These are Cys286 in the transmembrane domain and Cys295 in the cytoplasmic domain (Figure 1.9) (Lesley and Hyman, 1998). CD44 has been demonstrated to be palmitoylated in mouse lymphoma cells and removal of this palmitic acid from CD44 reduces the binding to ankyrin (Bourguignon et al., 1991),
Figure 1.9: The transmembrane and cytoplasmic domain of CD44. The C-terminal cytoplasmic domain supports the binding of proteins with crucial functions in cytoskeletal organisation and signalling (Ponta et al 2003)
inferring that palmitoylation may act to regulate attachment of CD44 to the cytoskeleton via ankyrin (Lesley and Hyman, 1998). The presence of a cysteine residue in the transmembrane domain (Cys286) has also been implicated in the dimerisation of CD44 molecules, a function associated with cellular activation. Dimerisation of CD44s was seen after phorbol myristate acetate (PMA) treatment of Jurkats, with enhanced hyaluronan binding. This suggests disulfide bond formation through Cys286 may serve to stabilise aggregates of CD44 in the plasma membrane, thus potentiating ligand binding (Liu et al., 1998; Liu and Sy, 1997).

1.5.5 Cytoplasmic domain

The importance of the cytoplasmic domain (residues 290-362) is highlighted by the high degree of homology between species (approximately 80-90%) (Figure 1.9) (Naor et al., 1997). The main purpose of the cytoplasmic domain is to transduce signals from extracellular stimuli and it does that through interaction with a number of intracellular proteins, namely ankyrin and ezrin, radixin and moesin (ERM) family of proteins. These interactions are discussed in greater detail in the following section. The cytoplasmic domain of CD44 has a role in the subcellular localisation of this protein. CD44 expression is restricted to the basolateral surfaces of polarised epithelial, whereas the tailless protein is found apically and within the cell (Neame and Isacke, 1993).

Within the cytoplasmic domain the serine residues at positions 323 and 325 are subject to phosphorylation by a serine/threonine kinase. It has been shown that although CD44 phosphorylation at these sites is not required for hyaluronan binding, it is required for melanoma and fibroblast migration on a hyaluronan substratum (Peck and Isacke, 1996). It was later found that phosphorylation of Ser325 is critical for CD44-dependent cell migration (Peck and Isacke, 1998). A recent paper has demonstrated the importance of serine phosphorylation for CD44-dependent directional cell migration, by the modulation of CD44 interaction with the protein ezrin (Legg et al., 2002).

CD44 can mediate the uptake of hyaluronan and subsequently degrade it. The mechanism of CD44-hyaluronan uptake is unclear. CD44 appears to utilise a non-clathrin, non-caveolae-dependent mechanism of endocytosis, followed by CD44 recycling (Thorne et al., 2004). A recent study demonstrated that the cytoplasmic domain of CD44 was required for the hyaluronan binding and retention in the pericellular matrix, as well as CD44-mediated endocytosis. In contrast, the "short tail"
isoform CD44st, a CD44 variant generated by the presence of an early stop codon in
exon 19, did not support the ligand binding, the assembly of pericellular matrices or
mediate the internalisation of bound hyaluronan. Thus, leading to the hypothesis that
CD44st may act as a potential modulator of CD44s functions and highlighting the
importance of the cytoplasmic domain (Jiang et al., 2002).

1.5.6 Interaction with the cytoskeleton
Proteins that bind to the cytoplasmic domain of CD44 also mediate the association of
this protein with the cytoskeleton. Early work by Lacy and Underhill described the
association of CD44 with the actin cytoskeleton, but the precise nature of this
association was unknown (Lacy and Underhill, 1987). Around the same time CD44
was found to be associated with ankyrin in mouse T lymphoma cells (Bourguignon et
al., 1987; Kalomiris and Bourguignon, 1988). The ankyrin-binding domain of CD44 has
been mapped to a 15 amino acid sequence between Asn304 and Leu318. There was
a correlation found between the presence of the ankyrin-binding motif and hyaluronan
binding (Lokeshwar et al., 1994). This implicates the binding of CD44 to ankyrin as a
requirement for hyaluronan binding but there is evidence that conflicts with this finding.
Mouse lymphoma cells transfected with tailless CD44 (i.e. with the putative ankyrin
binding domain deleted) exhibited little change in CD44-mediated adhesion and rolling
interaction with immobilised hyaluronan with respect the wild type CD44 (Gal et al.,
2003). This leaves the role of ankyrin in mediating hyaluronan binding unclear.

Tsukita et al., 1994, first identified the association of CD44 with the cytoskeleton
membrane linker proteins, ezrin, radixin and moesin members of the band 4.1
superfamily (Tsukita et al., 1994). Merlin (a tumour suppressor protein) is related to the
ERM family, and was also found to bind to the cytoplasmic domain of CD44 (Sainio et
al., 1997). A cluster of basic amino acids (residues 292-300) next to the
transmembrane domain of CD44 interacts with these proteins (Thorne et al., 2004).
ERM proteins are thought to be important in the regulation of cell migration and cell
shape. Their secondary structure consists of a globular N-terminal domain, an α-
helical region and a charged C-terminal domain. ERM proteins interact with CD44
through their N-terminal domain and the C-terminal domain binds F-actin, thus acting
as linkers between cell surface and the actin cytoskeleton (Tsukita and Yonemura,
1999).

Activation of ERM proteins is mediated by phosphorylation and binding to membrane
phospholipids and this ability to switch between active and inactive conformation
provides a mechanism to make and break CD44-cytoskeletal associations (Thorne et al., 2004). A recent paper described the association of phosphorylated ezrin with the uropod of polarised lymphocytes. This study demonstrated the role of phosphorylated ERM proteins in uropod formation and cell polarisation in cooperation with the GTPase protein, Rho (Lee et al., 2004). Indeed CD44 phosphorylation also provides a mechanism for the regulation of CD44-ezrin association, an event that has important consequences for the directional cell migration. PKC-mediated phosphorylation of Ser 291, with accompanying dephosphorylation of Ser325, regulates a dynamic association and disassociation of CD44 and ezrin, allowing directional migration (Legg et al., 2002).

1.5.7 CD44 variants

CD44s, the standard form of CD44, is ubiquitously expressed on leukocytes as well as some other cell types such as fibroblasts. This is sometimes termed CD44H reflecting its expression on haematopoietic cells. Some larger CD44 molecules are found expressed on both normal and malignant cells. Indeed cells may simultaneously express a number of different CD44 isoforms. These alternative CD44 molecules result from the alternative splicing of the CD44 gene. The combination of alternative splicing as well as posttranslational modification help to enrich the repertoire of possible CD44 molecules (Naor et al., 1997). The variable region of the gene is inserted between amino acid 202 and 203 of the mature human CD44 sequence thus adding an additional stretch of 381 amino acids (mouse 423) (Screaton et al., 1993). With the CD44 variable region (exon V1 to exon V10) there are an additional four potential N-glycosylation sites and a large number of O-glycosylation sites (especially exons V2 and V8-10). There is also a potential site for the insertion of glycosaminoglycans (GAG) (Screaton et al., 1993). CD44 molecules carrying exon V3 can be decorated with heparin sulfate and those carrying exon V6 can be modified by an H blood group sugar. Also, additional variant exons may enhance phosphorylation of the CD44 cytoplasmic tail (Naor et al., 1997).

To date 20 different CD44 transcripts, out of a theoretical possible 768 CD44 isoforms, have been identified (Naor et al., 1997). Expression of specific variants has been associated with specific cell types and/or functions. Alternative splicing is regulated by tissue-specific factors, which either splice out these variant exons (exon skipping) or express a CD44 protein with variant-exon-encoded sequences (exon inclusion). Proliferating cells tend to produce larger CD44 proteins, suggesting that mitogenic signalling pathways may regulate alternative splicing. There is evidence that the Ras-MEK-ERK pathway triggers the inclusion of variant exons in the mature mRNA (Ponta
et al., 2003). The expression of two variants of CD44 was reported in haematopoietic cells (Dougherty et al., 1991). These had a molecular weight of 115 and 130kDa designated CD44R2 (containing exon v10 only) and CD44R1 (containing exon v8-10, also described as CD44E) respectively (Dougherty et al., 1991; Droll et al., 1995). These isoforms were detected in the mononuclear cells and granulocytes from normal donors as well as leukaemia patients and were found to have a role in mediating adhesive interactions (Dougherty et al., 1991; Droll et al., 1995). Activated human T cells transiently express variants carrying exon 6 (v6) or 9 (v9) products that are independently expressed on distinct sets of CD44 molecules. These molecules effectively binding hyaluronan and have a role in CD44-mediated signalling and effector function activation (Galluzzo et al., 1995). The importance of exon 6 in T cell function was demonstrated with the generation of transgenic mice with T cells constitutively expressing CD44v4-v7. These T cells respond faster to activating stimuli, implicating this CD44 variant in the process of lymphocyte activation (Moll et al., 1996).

Epithelial CD44 (CD44E) with a molecular weight of 130kDa, carries exons V8-10, and is preferentially expressed on epithelial cells (Naor et al., 1997). A panel of exon-specific monoclonal antibodies allowed the study of variant expression in both normal and neoplastic tissue. Expression of variant CD44 by normal tissue seems to be largely restricted to the epithelia, indicating that the alternative splicing of CD44 appears to be tightly regulated (Fox et al., 1994). Also, variant CD44 expression by tumour cells appears to be highly heterogenous (Fox et al., 1994). Other studies examining the expression of CD44 variants encoding exons v4, v6 and v9, found that these variant isoforms were widely expressed throughout the body. Most epithelial cells expressed high levels of CD44 exon v9-containing isoforms, with a few exceptions, for example the stomach and intestine were weakly positive (Mackay et al., 1994; Terpe et al., 1994b). Exon v6 specific isoforms were found predominantly in squamous epithelia, whereas exon v4-containing isoforms were only detected in the epidermal layer of the skin and the oesophageal epithelium. A similar profile of expression was seen in foetal tissue (Terpe et al., 1994b). Also CD44s and CD44 v3-v10, v4-v10 and v6-v10 are expressed at critical sites of epithelial morphogenesis in the developing mouse embryo (Yu and Toole, 1997). The highest level of variant isoforms expression was seen in cells with a high degree of turnover (Mackay et al., 1994).

In malignant tissue (mostly breast and colon cancer) there is overproduction of variant forms of CD44, whereas in normal tissue CD44s is predominantly expressed
(Matsumura and Tarin, 1992). Although normal gastric mucosa is CD44 negative, a correlation has been found between the expression of exon 9 containing variants and gastric malignancy (Mayer et al., 1993). An interesting observation is that cells that normally express variants of CD44 share many properties with metastasising tumour cells. Cells expressing CD44 variants in general have a high degree of turnover or a certain degree of mobility, suggesting that metastatic progression of tumour cells may reactivate the expression of gene segments restricted to certain stages of development and/or differentiation.

1.6 CD44 LIGANDS

In 1990, Culty et al confirmed that CD44 was identical to the hyaluronate receptor (Culty et al., 1990). However although CD44 has long been recognised as the principle receptor for hyaluronan, there are several other molecules that it interacts with. CD44 can interact with several ECM proteins, such as collagen types I and VI, fibronectin, laminin and chondroitin sulfate with varying degrees of affinity (Naor et al., 1997). Osteopontin (Eta-1), a chemotactic phosphoprotein secreted by activated T cells and osteoblasts is a CD44 ligand. Osteopontin was found to induce a chemoattractant response in CD44-transfected fibroblasts. There was a suggestion that osteopontin secretion coupled with CD44 expression could lead to metastasis of tumour cells to specific sites (Weber et al., 1996). E-selectin has also been described as a protein ligand for CD44 expressed on haematopoietic progenitor cells. In order to function as an E-selectin ligand, CD44 requires the appropriate post-translational glycosylation. This pattern of glycosylation is recognised by the monoclonal antibody HECA-452. Expression of this HECA-452 reactive epitope is restricted to haematopoietic progenitor cells and this interaction may direct the homing of these cells to E-selectin-expressing bone marrow (Dimitroff et al., 2001).

In addition to these, a number of chondroitin sulfate proteoglycans have been identified as CD44 ligands. The chondroitin sulfate-modified invariant chain has been suggested as a ligand for CD44 (Naujokas et al., 1993). CD44 also interacts with the chondroitin sulfate proteoglycans, serglycin (Toyama-Sorimachi et al., 1995), versican (Kawashima et al., 2000) and aggrecan (Fujimoto et al., 2001). The CD44-binding elements on these proteoglycans are the chondroitin sulfate side chains. These chondroitin sulfate proteoglycans may be involved in the adherence and activation of CD44-expressing cells. The multifunctional nature of CD44, as well as its ability to bind many ligands...
may be accounted for by the polymorphic properties of the protein due to alternative
splice as well as differential glycosylation and glycanation (Naor et al., 1997). However
compared with hyaluronan there is less known about the regulation of the interactions
of these ligands with CD44 and therefore most interest in CD44 function has focused
on its ability to bind hyaluronic acid, a major component of the extracellular matrix.

1.6.1 Hyaluronan
Hyaluronan is an extracellular matrix glycosaminoglycan that was originally thought to
act simply as a space-filling material. Now there is evidence to indicate that it
contributes to a number of physiological processes, including the development of an
immune response. Hyaluronan is a polysaccharide that shares a common structure of
linear repeating disaccharides of D-glucuronic acid and β-1,3-N-acetyl-D-glucosamine-
β1,4, which exists in vivo as a polyanion (Tammi et al., 2002). The molecular weight of
this polymer ranges in size from small oligosaccharides to macropolysaccharides of
several million daltons (Termeer and Sleeman, 2003). Hyaluronan has a large
hydrodynamic volume and forms solutions with high viscosity and elasticity, thus
enabling its space filling, lubricating and filtering functions (Tammi et al., 2002). As well
as acting to expand the extracellular space by binding salt and water, hyaluronan also
functions to interact with a variety of extracellular molecules to form the extracellular
matrix and to be recognised by a number of cell surface receptors leading to the
activation of intracellular signalling pathways or the internalisation of hyaluronan.
Hyaluronan is present in high amounts during embryonic development and at sites of
wound healing (Camenisch and McDonald, 2000).

Hyaluronan is synthesised by a family of plasma membrane associated
glycotransferases, Has1, -2 and -3. The expression of each of these hyaluronan
synthases is dependent on the stage of development and there are also tissue and
cell-specific differences in expression (Tammi et al., 2002). Has2 has been identified
as the major source of hyaluronan during embryonic development and mice lacking
Has2 die around day 9/10. Loss of Has1 and/or Has3 does not affect mice viability.
Most data suggests that Has1 and Has2 synthesise high molecular weight hyaluronan,
whereas Has3 makes shorter polysaccharide chains (Camenisch and McDonald,
2000). Evidence that dependent on the size of the hyaluronan molecule different
intracellular responses are activated, therefore differential regulation of hyaluronan
synthesis may be important in modulating cell behaviour (Tammi et al., 2002). Five
hyaluronidases have been identified and these enable the removal of about a third of
the hyaluronan present in humans each day (Termeer and Sleeman, 2003).
Hyaluronan removal occurs either within the tissue where it was made or in the lymphatics (Banerji et al., 1999) and the liver via specific endocytic receptors and catabolised (Weigel et al., 2003). Indeed there is a picture of the balanced regulation of hyaluronan synthesis and catabolism leading to tissue function (Tammi et al., 2002).

During embryonic development the extracellular matrix surrounding migrating and proliferating cells is enriched with hyaluronan and as differentiation occurs the level of hyaluronan in the matrix decreases (Toole, 1997). Many malignant tumours are associated with high levels of hyaluronan. The extent of stromal hyaluronan accumulation is a strong, negative predictor of patient prognosis. In tissues, such as breast and ovaries, where there is a low basal level of hyaluronan, enhanced expression predicts a poor prognosis (Toole et al., 2002). As well as having a role in morphogenesis, hyaluronan has been associated with wound healing and immune function. Under certain circumstances hyaluronan can act as immunomodulators by activating antigen-presenting cells, such as dendritic cells and macrophages (Termeer and Sleeman, 2003). Hyaluronan has also been shown to play a role in the antigen presentation and antigen-specific T cell activation by dendritic cells (Mummert et al., 2002). The response of cells of the immune system to hyaluronan is related to its molecular weight. Under normal, non-inflammatory conditions, this high molecular weight hyaluronan does not induce any direct signalling events in dendritic cells or macrophages. At sites of inflammation and tissue injury, low-MW hyaluronan species accumulate and have proinflammatory functions (McKee et al., 1996). These small molecular weight hyaluronan fragments induced dendritic cell maturation (Termeer et al., 2000) and macrophage activation (McKee et al., 1996).

1.6.2 CD44 and hyaluronan

Culty et al, 1990, confirmed that CD44 was identical to the hyaluronate receptor and at about the same time, CD44 was determined to be the principal cell surface receptor for hyaluronan (Aruffo et al., 1990; Culty et al., 1990). Binding of CD44 to hyaluronan has been implicated in both cell adhesion to the extracellular matrix components and cellular signalling cascades (Lesley et al., 1993; Naor et al., 1997; Ponta et al., 2003). The regulation of the affinity of CD44 for the ubiquitously expressed hyaluronan is required as promiscuous binding of hyaluronan to CD44 would be undesirable. There are three states of binding of CD44 to hyaluronan defined as constitutive active, activatable and inactivatable. Most primary cells express CD44 but in a low affinity state that does not exhibit a hyaluronan binding capacity (Cichy and Pure, 2003). Transition from the "inactive" low affinity state to the "active" high affinity state of CD44
on leukocytes can be induced by the ligation of antigen receptors, and on leukocytes and epithelial and other mesenchymal cells by soluble factors including cytokines (Brown et al., 2001; Cichy and Pure, 2000; Levesque and Haynes, 1997).

A number of mechanisms have been implicated in mediating the transition from inactive to active CD44, including variant exon usage, receptor oligomerisation, glycosylation and sulfation. Some confusion surrounds the role of glycosylation in mediating hyaluronan binding to CD44, which is due largely to differences between various cell types and these have been discussed in greater detail in Section 1.5.3. However, cell activation that results in the induction of hyaluronan-binding, by stimuli such as, PMA, anti-CD3, IL-5, requires several hours and probably requires new protein synthesis, thus allowing for the synthesis of new glycosylation patterns (Murakami et al., 1994). Zheng et al., 1997, showed that glucose deprivation can alter hyaluronan binding function of some cell line within hours, implicating synthesis of new CD44 with reduced glycosylation. It was found that in vivo growth of a tumour cell that bound hyaluronan poorly reversibly converted it to an active hyaluronan binding state, raising the possibility that local environmental conditions may influence CD44 function, perhaps through influencing glycosylation pathways (Zheng et al., 1997).

Induced hyaluronan binding seen following stimulation of lung derived epithelial cells with oncostatin M appears to be due modulation of the sulfation of CD44 (Cichy and Pure, 2000). Similarly sulfation of CD44 is one mechanism of inducing hyaluronan binding in CD14+ peripheral blood monocytes in response to the inflammatory cytokines, TNF-α and IFN-γ (Brown et al., 2001). The transfection of a melanoma cell line with either CD44s or CD44E allowed the investigation of exons v8-v10 in hyaluronan binding. CD44s bound hyaluronan more efficiently than CD44E, suggesting that the expression of exons v8-v10 and its associated O-linked glycosylation is responsible for the reduction in hyaluronan binding function of CD44E (Bennett et al., 1995b).

CD44 has been found to play a role in hyaluronan internalisation and catabolism. Culty et al, demonstrated that in fibroblasts and alveolar macrophages that the CD44 receptor participates in the uptake and degradation of hyaluronan, a process proposed to occur in the lysosomal vesicles (Culty et al., 1992). Similarly in chondrocytes, CD44 has been found to be required for hyaluronan internalisation and degradation (Hua et al., 1993), indicating a role played by this receptor in the homeostasis on cartilage tissue. Indeed, during lung development a role has been demonstrated for CD44 in
mediating hyaluronan uptake and clearance by macrophages (Underhill et al., 1993). The association of hyaluronan oligomers with CD44 has been examined and it has been determined that hexameric hyaluronan is the minimum size for efficient occupation of the hyaluronan-binding site of CD44. Increased binding avidity with hyaluronan ranging from 20 to 24 sugar residues, suggests that divalent binding occurs at this point (Lesley et al., 2000). However, recent work, using structural analysis of the HABD, has suggested that hyaluronan with eight sugar residues is likely to be the minimum size that fully occupies the hyaluronan binding site of CD44 (Teriete et al., 2004).

1.7 CD44 FUNCTION

CD44 is involved in many important tissue functions. These include the interaction between cells and extracellular tissues, the support of cell migration in blood vessels and inside tissues, the presentation of growth factors, cytokines, chemokines and enzymes to other cells or to the surrounding tissues, and signal transmission from the cell surface to its interior, leading to apoptosis or cell survival and proliferation (Lesley and Hyman, 1998; Lesley et al., 1993; Naor et al., 1997). CD44 participates in many cellular processes, including the regulation of growth, survival, differentiation and motility. The altered expression or dysfunction of CD44 proteins contributes to numerous pathological conditions (Ponta et al., 2003).

Additional functions include its capacity to mediate inflammatory cell function and tumour growth and metastasis. CD44 was initially thought to be a transmembrane adhesion molecule that also played a role in the metabolism of its principal ligand HA. Indeed CD44 has been shown to be important in the clearance of HA and mediates cell-matrix interactions involved in tumour formation, metastasis and T cell extravasation (Bartolazzi et al., 1994; DeGrendele et al., 1997a; Teder et al., 2002; Yu et al., 1997). Transmembrane CD44 serves multiple roles, including mediating the metabolism of HA, in the regulation of tumour invasiveness and in the modulation of inflammatory cell function. Most of the known functions of CD44 on cell adhesion, migration and metastasis are associated with its capacity to regulate cell attachment to hyaluronan. This review will focus on the importance of CD44 in the immune response and metastasis as these highlight the role of CD44 in cell migration.
1.7.1 CD44 and metastasis

Tumour metastasis is composed of a complex series of events: (1) a tumour cells exits the primary tumour; (2) invades the host tissue and progresses through the ECM basement membrane and stroma. (3) The cell penetrates (intravasation) through vascular endothelium, and thus has access to the entire vascular system. (4) Eventually the cell arrests at a distal site in the vasculature, (5) exits from circulation (extravasation) by traversing the endothelial basement membrane (6) and enters the target tissue. Then (7) proliferation and angiogenesis begin at the secondary site. Events, such as, deregulation of cell adhesion molecule expression lead to the separation of metastatic cells from the primary tumour and, the expression of degradative enzymes enables the dissolution of the surrounding matrix. CD44 has been found in some cases to facilitate the growth and dissemination of tumour cells.

An alteration in CD44 expression has been found in many types of cancer and sometimes correlates to a poor patient prognosis. Increased expression of CD44s has been detected in colorectal cancer tissues (Khousheed et al., 2002). It has also been noted that there are elevated levels of soluble CD44 present in the serum of patients with advanced gastric and colon cancer (Guo et al., 1994). The expression of certain CD44 variants correlates with poor disease prognosis, for example, CD44 isoforms containing variant exon 9 were associated with gastric tumour recurrence and mortality (Mayer et al., 1993). Also, the expression of variant exon 6-containing CD44 proteins was a negative prognostic marker in colorectal cancer (Mulder et al., 1994). Whereas many of these examples have suggested a role for CD44 in tumour promotion, a role for CD44 in tumour suppression is seen in prostate cancer. It is interesting to note that the expression of CD44 and variant isoforms such as, CD44v6, are downregulated in human prostatic carcinomas (De Marzo et al., 1998). It has been shown that hypermethylation of the promoter of CD44 is associated with the down-regulation of protein expression during progression of prostate cancer to a metastatic state (Lou et al., 1999).

It is not surprising that the same lymphocyte homing receptors that are important in the trafficking of normal lymphocytes, also participate in the metastasis of malignant lymphoid tissue. The expression of particular homing receptors by malignant tissue reflects the trafficking between lymph nodes and the blood seen with normal lymphocytes. There was a preferential expression of CD44 isoforms containing the variants exons 3, 6 and 9 in high-grade malignant non-Hodgkin’s lymphoma (Terpe et
al., 1994a). Also, the expression of CD44 by gastrointestinal lymphomas is associated with a poor prognosis (Joensuu et al., 1993).

Experimentally it has been demonstrated that the expression of certain CD44 isoforms confers a metastatic phenotype to certain cell types. Melanoma cells transfected with CD44s adhere and migrate on hyaluronate, whereas transfection with CD44E does not confer cells with this phenotype (Thomas et al., 1992). Similarly, increased tumourigenicity and metastatic potential is seen in human B cell lymphoma cells transfected with CD44s but not when transfected with CD44E (Sy et al., 1991). Transfection of melanoma cells with CD44s cytoplasmic domain deletion mutants results in a loss of hyaluronate dependent motility but they still have the ability to adhere (Thomas et al., 1992).

Another aspect of metastasis requires the progression of the tumour cells through the ECM. It is interesting to note that there is increased production of hyaluronan in the stroma of many tumours (Knudson and Knudson, 1995). A number of groups have described the cleavage of CD44 expressed in cancer cells at the membrane-proximal region, which is mediated by a membrane-associated metalloprotease, membrane-type 1 matrix metalloproteinase (MT1-MMP) (Kajita et al., 2001b; Okamoto et al., 1999). This cleavage of the CD44 extracellular domain enables efficient cell detachment from its hyaluronate substrate and tumour cell migration. This data suggests that CD44 may also be one of the targets of proteolysis involved in tumour invasion and metastasis.

1.7.2 CD44 and the immune response

One of the earliest descriptions of CD44, or Hermes-1, was defined by a monoclonal antibody produced following rat immunisation with human tonsil lymphocytes following selection on the basis of high expression on the surface of cells with high HEV binding capacity. Expression of Hermes-1 on the surface of lymphocytes correlated with their HEV binding capacity (Jalkanen et al., 1986). Subsequent studies demonstrated that CD44 defines a receptor family involved in HEV recognition at lymph node, mucosal and synovial sites (Jalkanen et al., 1987). Furthermore, high expression of homing receptors, such as CD44, predicts a poor prognosis in non-Hodgkin’s lymphoma indicating that molecules involved in normal lymphocyte recirculation may also be important in the migration of their malignant counterparts (Jalkanen et al., 1991).
In addition to a proposed role in the homing of lymphocytes to HEV sites, CD44 has also been implicated in the activation of T lymphocytes. Receptor activation using a CD44 monoclonal antibody demonstrated a role for this receptor in enhancing the CD3 or CD2 response, resulting in enhanced T cell proliferation, IL-2 receptor expression and IL-2 production, thus acting to co-stimulate T cell activation (Huet et al., 1989). In addition, the blockade of OKT3-mediated T cell receptor activation by a CD44-specific monoclonal antibody, suggests that there may be some cross-talk between the CD3-TCR complex and CD44 in the regulation of T cell activation (Rothman et al., 1991).

Following antigen encounter by T cells, CD44 has been shown to be upregulated and activated. This is thought to highlight the importance of this receptor in recruiting T cells to inflammatory sites, functioning to mediate primary adhesion at sites of inflammation (DeGrendele et al., 1997b; Estess et al., 1998). Conclusive evidence demonstrating the importance of CD44 in lymphocyte homing to sites of inflammation was seen in number of murine models. The antibody-induced loss of CD44 slowed the onset of a cutaneous delayed-type hypersensitivity response following antigen challenge, although migration to lymphoid sites was normal. The CD44-negative, unactivated lymphocytes migrate identically to cells from control mice, but following challenge the response is altered, with lymphocyte extravasation to inflammatory sites delayed (Camp et al., 1993). Similarly a study using murine CD44'' T cells confirmed that receptor expression was not required for normal lymph node homing but the migration of CD44-deficient cells into areas of inflammation was delayed (Stoop et al., 2002).

The principal ligand for CD44 is hyaluronan (HA), a broadly distributed glycosaminoglycan, whose expression on endothelial cells is inducible by proinflammatory cytokines and mediates CD44-dependent rolling during inflammation (Estess et al., 1999; Mohamadzadeh et al., 1998). Indeed, it has been shown that patients with active autoimmune disease, such as systemic lupus erythematosus and arthritis, express elevated levels of activated CD44 in circulating T cells (Estess et al., 1998). Administration of anti-CD44 antibodies inhibited inflammation in murine models of inflammatory bowel disease, collagen- and proteoglycan-induced arthritis, and cutaneous inflammation (Pure and Cuff, 2001), and has lead to interest in CD44 as a target for possible therapeutic intervention or as a marker of autoimmune disease activity.
A role has been described for CD44 in resolving lung inflammation. Following lung injury by the intratracheal administration of bleomycin, CD44-deficient mice develop an unremitting inflammation, characterised by the impaired clearance of apoptotic neutrophils, persistent accumulation of hyaluronan fragments at the site of tissue injury and impaired activation of TGF-β1. CD44-deficient mice had massive infiltration of inflammatory cells within alveolar interstitium until death by respiratory failure day 14. In these mice the hyaluronan content continued to rise until death and those that survived beyond day 14 showed a decline in hyaluronan content, indicating that there may be a relationship between hyaluronan clearance and survival. This suggests that CD44 plays an important role in hyaluronan homeostasis following lung injury, influencing recovery from pulmonary inflammation (Teder et al., 2002).

CD44 has also been found to play an important role in the host response to infection. An example of this is seen following murine infection with Toxoplasma gondii, which results in the up-regulation and activation of CD44. Host resistance to this parasite is dependent on the production of IL-12, thus stimulating a strong Th1-type response, but this response can also lead to a severe immunopathology. Oral infection of mice with T. gondii results in a lethal CD4+ T cell-mediated IFN-γ-dependent inflammatory response in the ileum. Treatment of these mice with anti-CD44 led to a reduced pathology and enhanced survival. In vitro studies indicate that stimulation of CD44, using low molecular weight hyaluronan, results in increased production of IFN-γ by CD4+ T cells from infected mice, which is dependent on endogenous IL-12 (Blass et al., 2001). The importance of CD44 for the recruitment of macrophages following Mycobacterium tuberculosis challenge also highlights the role of this receptor in the host response to infection. CD44 was required for the binding and uptake of M. tuberculosis by macrophages as well as protective immunity against lung tuberculosis. Following M. tuberculosis challenge, CD44/- mice displayed a decreased survival when compared to wildtype mice. The numbers of macrophages and T lymphocytes in the lungs were reduced, as was the concentration of IFN-γ (Leemans et al., 2003).

1.7.3 Role of CD44 shedding
CD44 shedding may occur following receptor activation, a process that appears to be dependent on the type of ligand. It is interesting to note that antibody cross-linking of CD44 can result in cleavage of this adhesion receptor. Cross-linking of CD44 using immobilised monoclonal antibody resulted in enhanced shedding of the extracellular domain of CD44 in murine fibroblasts and monocytes, due the action of a zinc-
dependent metalloprotease. Importantly this was not seen in cells grown on immobilised CD44 ligand, hyaluronan. Disruption of the actin cytoskeleton using cytochalasin B inhibited antibody-induced CD44 shedding (Shi et al., 2001). Strong cell adhesion can impede cell movement, shedding might be one of the mechanisms used by cells in an attempt to break up or prevent CD44-dependent strong adhesions, especially when a high-affinity antibody is the adhesion ligand (Shi et al., 2001). Also, shedding of CD44 could be induced in lymphocytes by antibody treatment (Camp et al., 1993).

Organized ECM–cell interaction is essential for cell migration. It appears critical to regulate detachment and attachment in an organized manner in order to accomplish cell migration. CD44 cleavage is reported to play a critical role in CD44-mediated tumour cell migration that occurs through a dynamic interaction between CD44 and the extracellular matrix, a phenomenon that may be mediated by the direct association of CD44 with the membrane-type 1 matrix metalloproteinases (MT1-MMP) (Kajita et al., 2001a; Okamoto et al., 1999). Indeed there are increased levels of soluble CD44 in the serum of patients with gastric and colon cancers which correlated with tumour burden and metastasis (Guo et al., 1994).

Interestingly, a recent study has described a novel activity for HA fragments. HA fragments (between 6-36mers) can induce CD44 cleavage, not seen with smaller fragments or high molecular weight HA and promoted the migration of tumour cells (Sugahara et al., 2003). This has developed the notion of CD44 cleavage in mediating a role in CD44-mediated tumour cell migration. Many cellular responses to HA are dependent on the size of the glycosylaminoglycan. High molecular weight HA, present under non-inflammatory conditions, has distinct signaling capability. In areas of inflammation, there is increased accumulation of HA fragments which, influence the response of cells of the immune system (Termeer and Sleeman, 2003).

1.8 CD44 SIGNALLING

In addition to the ability of CD44 to interact with the cytoskeleton, this adhesion receptor can also transduce intracellular signalling events in response to ligand binding or cross-linking with specific antibodies leading to changes in gene expression. In common with other adhesion receptors, CD44 lacks any intrinsic kinase activity and must associate with other proteins in order to modulate signalling.
The Rho GTPases, such as RhoA and Rac1 participate in the interaction between CD44 and cytoskeletal proteins. For example, following CD44-hyaluronan interaction in keratinocytes there is Rac1-mediated activation of protein kinase N-γ (PKNγ), which leads to the downstream upregulation of phospholipase C γ1 (PLCγ1) activity and modification of the actin cytoskeleton (Bourguignon et al., 2004). CD44 has also been found associated with Src, Fyn, Lck and Lyn, and in some cells types, antibody-induced activation of CD44 stimulates tyrosine phosphorylation of these kinases and their substrates (Bates et al., 2001; Illangumaran et al., 1998; Roscic-Mrkić et al., 2003; Taher et al., 1996). An example in human T cells, CD44-mediated signalling results in the activation of Lck and the subsequent phosphorylation of ZAP-70. However, there is no characteristic Lck binding motif present in the cytoplasmic domain of CD44. It was found that CD44 cross-linking results in the physical association of CD44 with CD4, whose cytoplasmic domain associates with Lck (Dianzani et al., 1999). Additionally, Src-family kinases are modified by acylation, a modification that facilitates targeting to lipid rafts. Therefore, the association of CD44 with these components may not be due to direct interaction but instead due to their co-localisation in lipid rafts (Illangumaran et al., 1998).

There is evidence that CD44 acts as a substrate for protein kinase C (PKC) and that this phosphorylation regulates the interaction of the receptor with the cytoskeleton. Phosphorylation of CD44 by PKC enhances the receptors affinity for ankyrin (Kalomiris and Bourguignon, 1989). CD44 is constitutively phosphorylated at Ser325 and Legg et al, 2002, have demonstrated that, following PKC activation, there is a carefully regulated dephosphorylation of Ser325 and phosphorylation of Ser291. This phosphorylation of Ser291 regulates the interaction between CD44 and ezrin, enabling CD44-dependent directional cell migration (Legg et al., 2002). PKC activation, using phorbol myristate acetate (PMA), induces CD44 clustering thereby enhancing hyaluronan binding by the receptor (Liu and Sy, 1997).

There is also strong evidence to support the signalling role of CD44 comes from its ability to act as a co-receptor. CD44 can bind growth factors and cytokines (Bennett et al., 1995a; Jones et al., 2000; Roscic-Mrkić et al., 2003; Sherman et al., 2000; Weber et al., 1996) or MMPs that can process growth factors to their active form. Thus, CD44 can indirectly promote signalling events by modulating the activity, affinity or local concentrations of signalling factors. Also, CD44 can associate with and modify the function of growth factor receptors and members of the MT-MMP family. For example,
CD44 acts as a co-receptor for the ErbB family of receptor tyrosine kinases and for the c-Met receptor, and these associations are essential for activation of receptor kinase activity and the regulation of diverse cellular processes, including cell survival, proliferation and differentiation (Orian-Rousseau et al., 2002; Sherman et al., 2000; van der Voort et al., 1999; Yu et al., 2002). It is not known whether the CD44 transmembrane and cytoplasmic domains play a role in this process, although recruitment of CD44 to lipid rafts and/or association of the cytoplasmic domains with the actin cytoskeleton may well be important for promoting heterologous receptor interactions.

Recent work has examined the role of the intracellular domain in regulating gene transcription. Cleavage of the extracellular domain of CD44 can be followed by proteolysis within the transmembrane domain. This process has been shown to be dependent on presenilin-1, a putative membrane aspartyl protease (Lammich et al., 2002; Okamoto et al., 2001). The liberated intracellular domain of CD44 plays a role in regulating gene transcription (Okamoto et al., 2001). It appears to translocate to the nucleus and promote gene transcription mediated through TPA-response elements, including transcription of CD44, acting as a positive feedback mechanism for regulating CD44 expression.

1.9 CELL MOTILITY

The crawling of single cells is of critical importance in many systems including the migration of ameboid cells as well as the movements of single cells within multicellular organisms. These include the programmed migration of cells within the developing embryo in order to shape the emerging organism, the movements of fibroblasts and epithelial cells to heal wounds, the formation of metastasis by crawling malignant tumour cells, the extension of dendrites by the developing neuron as well as the motions of lymphocytes and macrophages in the various processes of the immune system (reviewed in (Stossel, 1993). Many of the key features of the process of cellular motility which are common to all of these systems will be discussed here.

1.9.1 Model of cell migration

Present understanding of cell migration is based on studies from a variety of cell types in different environments. Cells usually initiate crawling in response to an extracellular cue, which may be a protein, peptide or other small molecule binding to a specific
receptor. Cell migration can be thought of as a cyclic process, which involves a cycle of four steps: protrusion of the leading edge, adhesion to the substratum, retraction of the rear, and de-adhesion (Pollard and Borisy, 2003). The initial response of a cell to a migration-promoting agent is to polarise, resulting in the extension of protrusions in the direction of migration. These protrusions can be either large, broad lamellipodia or spike-like filopodia, and are usually driven by actin polymerisation. Transmembrane receptors linked to the actin cytoskeleton stabilise these protrusions by adhering to the extracellular matrix or adjacent cells. These adhesions serve as traction sites for migration as the cell moves over them, and they are disassembled at the cell rear, allowing it to detach (Ridley et al., 2003). The disassembly of the cross-linked filaments into short fragments or monomeric subunits away from the leading edge supplies components for the actin assembly reactions that drive protrusion (Stossel, 1993).

1.9.2 The role of the cytoskeleton in the migrating cell

Actin filaments are double helical polymers of globular subunits arranged in head-to-tail fashion, which confer polarity on the filament (Pollard and Borisy, 2003). The polarity of the actin filaments is used to drive membrane protrusion, with fast-growing "barbed" ends and slow-growing "pointed" ends (Ridley et al., 2003). The organisation of filaments depends on the type of protrusion required. In lamellipodia, actin filaments form a branching "dendritic" network, whereas in filopodia they are organised into long parallel bundles (Welch and Mullins, 2002). The design of lamellipodia and filopodia provides enable them to perform distinct functions. The dendritic organisation of lamellipodia provides a tight brush-like structure that is able to push along a broad length of plasma membrane (Pollard and Borisy, 2003). Arp2/3 is a protein complex that is the only cellular factor known to nucleate new actin filaments with free barbed ends (Welch and Mullins, 2002). Localised activation of the Arp2/3 complex could induce lamellipodium to grow in a particular direction, providing the basis for directional migration (Ridley et al., 2003).

In lamellipodia, actin polymerisation is mediated by Arp2/3 complex, which binds to the sides or tip of a pre-existing actin filament and induces the formation of a new daughter filament that branches off the mother filament (Pollard and Borisy, 2003; Welch and Mullins, 2002). Activation of the Arp2/3 complex is localised by WASP/WAVE family members, which are activated at the cell membrane (Welch and Mullins, 2002). A large number of actin-binding proteins exist each with a role to play in the control of actin filament assembly and disassembly. The actin-binding protein, profilin can
prevent self-nucleation by binding to actin monomers and also serves to selectively target monomers to barbed ends. This regulates the rate and organisation of actin polymerisation in protrusions by affecting the pool of available monomers at the free ends (Pollard and Borisy, 2003). Capping proteins terminate filament elongation, restricting the polymerisation to new filaments close to the plasma membrane. Additionally the disassembly of older filaments, with the help of proteins of the ADF/cofilin family (which sever filaments and promote actin dissociation from the pointed end) help to generate actin monomers needed for polymerisation at the front end. Cortactin stabilises branches, whereas filamin A and α-actinin stabilise the entire network by cross-linking filaments (Welch and Mullins, 2002).

Filopodial protrusions are thought to be generated by a filament treadmilling mechanism. Actin filaments within a bundle elongate at their barbed ends and release actin monomers from their pointed ends (Welch and Mullins, 2002). The long, unbranched filament organisation is consistent with assembly occurring by elongation. Ena/VASP proteins, which are enriched at the filopodial tips, bind to the barbed ends of actin filaments and antagonise both capping and branching, allowing the continuous elongation of filaments. Fascin bundles actin filaments and may help to provide the stiffness needed to allow efficient pushing of the plasma membrane in filopodia (Welch and Mullins, 2002). Filopodia with a parallel bundle organisation, are well designed to act as sensors to explore the local environment but are not essential for chemotaxis (Ridley et al., 2003).

1.9.3 Polarisation
Polarisation is required for a cell to migrate. Therefore, the molecular processes at the front and back of a moving cell are different (Ridley et al., 2003). An initial signal instructs the cell in which direction to polarise. A signal transduction pathway then communicates the signal to the interior of the cell. This leads to the eventual redistribution of some of the cell contents and cell polarisation. Because the polarity of a single cell can dictate the polarity of an entire organism, an initial polarisation can cause a chain reaction of downstream consequences, causing many other structures to become distributed non-uniformly. The unequal distribution of a few molecules leads to the nonuniform distribution of many other molecules, thus enabling cells to perform a wide variety of processes including migration (Glotzer and Hyman, 1995). The establishment and maintenance of cell polarity in response to extracellular stimuli is mediated by a set of interlinked positive feedback loops involving Rho family GTPases,
phosphoinositides (PI3-kinases), integrins, microtubules and vesicular transport (Ridley et al., 2003).

Cdc42 acts as a master regulator of cell polarity (Ridley et al., 2003). It is active at the front of the migrating cell (Itoh et al., 2002). Inhibition and global activation of Cdc42 can disrupt the directionality of migration (Etienne-Manneville and Hall, 2002). Cdc42 can influence polarity by restricting where lamellipodia form (Rodriguez et al., 2003). Cdc42 has a role in localising the microtubule-organising centre (MTOC) and Golgi apparatus in front of the nucleus, oriented toward the leading edge. Cdc42-induced MTOC orientation may contribute to polarised migration by facilitating microtubule growth into the lamella and microtubule-mediated delivery of Golgi-derived vesicles to the leading edge, providing membrane and associated proteins needed for forward protrusion (Etienne-Manneville and Hall, 2002; Rodriguez et al., 2003). The role of Cdc42, the Ras family GTPase, in the induction of polarisation of T cells at the point of contact with the antigen presenting cell was described by Stowers et al. Cells expressing mutant Cdc42 failed to reorientate their MTOC towards the APC and to polymerise actin at the site of cell-cell contact. Another interesting finding describes the requirement of PI 3-kinase activity (known to bind to Cdc42) for the reorientation of the MTOC (Stowers et al., 1995).

1.10 SIGNAL-TRANSDUCTION PATHWAYS IMPLICATED IN ADHESION RECEPTOR SIGNALLING

"Signal-transduction pathways are no longer thought of as linear sequences of biochemical modification, but as networks with several levels of complexity" (Ponta et al., 2003).

Indeed this requires us to remember that each pathway examined must be considered in context. The response of a cell to a particular stimulus begins a series of cellular changes – starting with receptor activation, the amplification of the receptor signal and the downstream effector mechanism. This simplified sequence of signal transduction does not account for the cross-talk that may occur as a result of other receptor activation. This review examines some of the signal transduction pathways that are up-regulated during cellular migration.
1.10.1 Adhesion molecule signal transduction to the cytoplasm

Target cells respond and signal downstream signalling events in response to receptor activation. In most cases the receptors are transmembrane proteins and when they bind a specific ligand, they become activated so as to generate a cascade of intracellular signals that alter cell behaviour (Alberts et al., 1994). Unlike many receptor-types, adhesion molecules do not act using single ligand-receptor interactions. For the most part they do not carry a typical cytoplasmic domain with signalling properties, i.e. tyrosine kinase activity. Adhesion molecules act as sensors to enable the cell to detect its extracellular environment (Aplin et al., 1999). They detect the extracellular matrix via integrins and syndecans and neighbouring cells via cadherins, selectins and immunoglobulins (Aplin et al., 1999).

Many of these adhesion molecules have been found to act as signalling molecules, either by directly responding to ligation or by modulating the signals up-regulated by other receptors (Aplin et al., 1999). Integrins have been shown to play a key role in modulating signalling pathways up-regulated by receptor tyrosine kinases and G protein-coupled receptors (Short et al., 2000). An emerging paradigm is that cell adhesion molecules tether the actin cytoskeleton as well as modulating the signalling that impinges on cellular events (Aplin et al., 1999). Additionally the organisation of adhesion receptors, such as CD44, along with other signalling components is enabled by the presence of lipid rafts as part of the cell membrane. Signalling from these so-called membrane microdomains is thought to occur in response to multivalent ligand binding resulting in raft-associated protein redistribution thus allowing coordinated receptor activation and the initiation of the signalling process (Horejsi, 2003).

1.10.2 Small GTPase proteins

GTPases are a family of small guanosine triphosphate (GTP)-binding proteins that act as molecular switches. A simple biochemical process allows these proteins to control complex cellular processes, namely the hydrolysis of GTP to GDP (guanosine diphosphate) (Etienne-Manneville and Hall, 2002). These guanine nucleotide binding proteins rapidly cycle between GDP-bound and GTP-bound state, thus acting as molecular switches in response to environmental changes (Cantrell, 2003). Guanine nucleotide exchange factors (GEFs) catalyse the release of GDP, allowing GTP to bind as it is present intracellularly in high concentrations (Bar-Sagi and Hall, 2000). GTPases adopt different conformations dependent on whether in a GDP-bound (inactive) or GTP-bound (active) state. When in a GTP-bound state GTPases can interact with downstream effectors initiating downstream responses which include
protein kinases, lipid modifying enzymes and activators of the Arp2/3 complex, and thus regulate many important biological processes (Cantrell, 2003; Etienne-Manneville and Hall, 2002). An intrinsic GTPase activity, which is further catalysed by GTPase activating proteins (GAPs), leads to the hydrolysis of GTP and the GTPase returns to its inactive state (Bar-Sagi and Hall, 2000).

As many as 60 small GTPases have been identified in mammals (Etienne-Manneville and Hall, 2002). Many members of the Ras family of GTPases, such as Ras and Rap1A, and the Rho family GTPases, Cdc42Hs, Rac1, Rac2 and RhoA, are important in signalling from antigen receptors, costimulatory, cytokine and chemokine receptors in the regulation of the immune response (Cantrell, 2003). The importance of Ras signalling in T cell function is highlighted by the finding that loss of Ras function prevents the activation of proliferation and cytokine gene induction in response to antigen receptor activation (Genot and Cantrell, 2000). The Rho family of small GTPases have been found to be key regulators of actin and adhesion organisation and control formation of lamellipodia and filopodia. Rac, Cdc42 and RhoG are the Rho GTPases required for the protrusion of lamellipodia and filopodia (Ridley et al., 2003). Rac and Cdc42 mediate actin polymerisation in protrusions by targeting the WASP/WAVE family of Arp2/3 complex activators. Rac stimulates lamellipodial extension by activating WAVE proteins (Cory and Ridley, 2002). Interestingly, Cdc42, RhoA and Rac1, regulate stromal cell derived factor-1 (SDF-1)-induced lymphocyte polarisation and chemotaxis (Del Pozo et al., 1999).

1.10.3 PI3-Kinase
A family of lipid kinases, the phosphoinositide 3-kinases (PI3-kinases) phosphorylate the 3'-OH position of the inositol ring of inositol phospholipids, producing PtdIns(3)P, PtdIns(3,4)P2 and PtdIns(3,4,5)P3 (Figure 1.10). This enzyme family is divided into three main classes based on their structure and possible mechanism of regulation (Figure 1.11) (Domin and Waterfield, 1997):

Class I PI3-kinases
These enzymes form a heterodimeric complex with an adaptor protein, thus allowing them to respond to ligand stimulation which preferentially phosphorylate PtdIns(4,5)P2 in the cell (Domin and Waterfield, 1997). The class I PI3-kinases comprise a p110 catalytic subunit and a regulatory adaptor subunit. This class is further subdivided based on the form of the adaptor subunit with which the catalytic subunit associates, as outlined in Figure 1.11. P110α and p110β both have ubiquitous tissue distribution,
Figure 1.10: Reactions catalyzed by PI3-kinase and PTEN.
The class I PI3-kinase enzymes can phosphorylate the 3 position of PtdIns, PtdIns4P, or PtdIns(4,5)P2 to produce PtdIns3P, PtdIns(3,4)P, or PtdIns(3,4,5)P3, respectively. PtdIns(3,4)P2 can also be produced by dephosphorylating the 5 position of PtdIns(3,4,5)P3, and one enzyme that does this is an SH2-containing 5-phosphatase called SHIP. In addition, PtdIns(3,4)P2 can be produced by phosphorylating the 4 position of PtdIns3P by an unidentified PtdIns3P 4-kinase. PTEN has been shown to dephosphorylate the 3 position of both PtdIns(3,4,5)P3 and PtdIns(3,4)P to reverse the reactions catalyzed by PI3-kinase (Cantley and Neel, 1999).
Figure 1.11: The classification of PI3-kinase family members.
The assignment of catalytic subunits to a particular class is based on
sequence homology within the catalytic domain. To date, PI3K-C2 is
the only class II member which has been shown to phosphorylate
PtdIns(4,5)P2 (Domin and Waterfield, 1997).
whereas the expression of p110δ is restricted to leukocytes (Vanhaesebroeck et al., 1997). There is also some tissue specific distribution of these adaptor subunits (Domin and Waterfield, 1997). In addition to lipid kinase activity, class Ia PI3-kinases possess an intrinsic serine/threonine protein kinase activity.

Class II PI3-kinases
These catalytic subunits are the largest (170-210kDa) and the catalytic domain is 45-50% homologous to class I PI3-kinases. Class II PI3-kinases also have a C-terminal domain that has homology to the C2 domains that mediate calcium/lipid binding in classical protein kinase C isoforms (Cantrell, 2001). These enzymes are refractory to both wortmannin and Ly294,002 (Domin and Waterfield, 1997) and little is known about their in vivo biology (Cantrell, 2001).

Class III PI3-kinases
The substrate for Vps34p and its human homologue, p150 is PtdIns. Vps34p is the only PI3-kinase present in yeast and this suggests that members of this class may be the primordial form of PI3-kinase. This lipid kinase has been shown to have a role in vesicular trafficking, osmoregulation and endocytosis (Domin and Waterfield, 1997).

Phosphorylated lipids produced at cell membranes during signalling events contribute to the recruitment and activation of various signalling components. These phosphorylated lipids bind to the pleckstrin homology (PH) domains of signalling proteins, thus controlling the activation and subcellular localisation of these signal transduction molecules (Cantrell, 2001). Three major classes of signalling molecule have domains that specifically bind D-3 phosphoinositides to PH domains; guanine-nucleotide-binding proteins for Rho family GTPases, protein tyrosine kinases, and the AGC superfamily of serine/threonine protein kinases. These proteins are located in the cytosol of unstimulated cells but in response to lipid phosphorylation they accumulate at the plasma membrane because of their ability to associate with the newly formed D-3 phosphoinositides. At the membrane, these proteins become activated and initiate signalling responses, including actin polymerisation, assembly of signalling complexes, and priming of protein kinase cascades (Cantley, 2002).

In the context of this study two classes of PI3-kinase targets are of interest. The first targets of relevance to this study are the GTPases Rac and Rho. These GTPases coordinate the organisation of the actin cytoskeleton. They have been demonstrated to interact with PI3-kinase (Bokoch et al., 1996) and play a PI3-kinase dependent role in membrane ruffling of T cells (Arrieumerlou et al., 1998). It has been demonstrated that the activity of a Rho GTPase and the lipid products of PI3-kinase are required for the
establishment of neutrophil polarisation and chemotaxis in response to chemoattractant signal (Servant et al., 2000). The protein serine/threonine kinases, Akt (also called protein kinase B (PKB)) and phosphoinositide-dependent kinase 1 (PDK1) are also of particular interest. Association with PtdIns(3,4,5)P$_3$ at the membrane brings these proteins into proximity and facilitates the phosphorylation of the activation loop of Akt by PDK-1 (Stephens et al., 1998; Stokoe et al., 1997). This phosphorylation stimulates the catalytic activity of Akt, resulting in the phosphorylation of a range of other proteins that affect cell growth, cell cycle entry and cell survival (Cantley, 2002). Additionally, PDK1 regulates phosphorylation of the activation loop of the PKC isoforms, δ and ζ, in a PI3-kinase dependent manner (Le Good et al., 1998).

The termination of PI3-kinase signalling by degradation of PtdIns(3,4,5)P$_3$ can be mediated by at least two different types of phosphatases (Cantley, 2002). The Src-homology 2 (SH2)-containing phosphatases (SHIP1 and SHIP2) dephosphorylate the 5 position of the inositol ring to produce PtdIns(3,4)P$_2$. SHIP mediates the conversion of PtdIns(3,4,5)P$_3$ to PtdIns(3,4)P$_2$ and certain proteins have PH domains with higher affinity for PtdIns(3,4)P$_2$ (Figure 1.10). Therefore SHIP may be important in modulating different signalling pathways, rather than acting as an “off” switch (Seminario and Wange, 2003). The phosphatase PTEN dephosphorylates the 3 position of PtdIns(3,4,5)P$_3$ and PtdIns(3,4)P$_2$ to produce PtdIns(4,5)P$_2$ and PtdIns(4)P, respectively (Figure 1.10) (Cantley and Neel, 1999). PI3-kinase activation stimulates PTEN phosphorylation, indicating that a function of PI3-kinase activation may be to activate and stabilise PTEN, acting as a negative feedback loop (Seminario and Wange, 2003). Loss of PTEN protein or function has been found in a large number of cancers, highlighting the importance of this phosphatase (Cantley, 2002).

1.10.4 PKC
Protein Kinase C (PKC) enzymes are a family of kinases activated by many extracellular signals and are a subfamily of the protein kinase A, protein kinase G and protein kinase C (AGC) serine/threonine kinases (Spitaler and Cantrell, 2004). Within the cell, activation of PKCs is regulated by a number of lipid second messengers, in particular diacylglycerol and the acidic phospholipid, phosphatidyserine. Members of the PKC family are divided into three classes based on their domain composition and their cofactor requirements (i) classical PKC (α, β I, β II, γ) isozymes require calcium, diacylglycerol and phosphatidyserine, (ii) novel PKC (δ, ε, η, θ) isozymes are calcium
independent but still require diacylglycerol and phosphatidylserine and (iii) atypical PKC (ζ, η/λ) isozymes only require phosphatidylserine (Figure 1.12) (Toker, 1998).

PKCs have been implicated in diverse cell functions. These include but are not limited to, the regulation of transcription, mediating immune responses and the regulation of cell growth (Newton, 1995). Tumour-promoting phorbol esters mimic diacylglycerol and activate PKC. The availability of these reagents has enabled examination of the cellular role of PKC isoforms. Although this information must be considered with caution, because the metabolic stability of phorbol esters means the cellular responses caused by these agents differ somewhat from those caused by membrane-permeant diacylglycerols (Nishizuka, 1992). PKC typically phosphorylates serine or threonine residues in basic sequences. An additional regulator of PKC function in vivo is subcellular distribution of both the enzyme and substrate, thus conferring an additional level of enzyme specificity. PKC isoforms are distributed differentially throughout the cell and a number of targeting proteins have been described (Newton, 1995).

PKC family members are a single polypeptide, comprised of an N-terminal regulatory region and a C-terminal catalytic region (Figure 1.12). This consists of four conserved domain: C1-C4. The C1 domain contains a cysteine-rich motif, that forms the diacylglycerol/phorbol ester binding site, which is preceded by an autoinhibitory pseudosubstrate sequence (Newton, 1995). The pseudosubstrate domain at the N-terminus of PKC closely resembles that of the optimal substrate phosphorylation motif of the predicted serine-threonine phosphorylation site (Spitaler and Cantrell, 2004). The C2 domain contains the recognition site for acidic lipids and, in some isoforms, the Ca^{2+}-binding site (Newton, 1995). The regulatory domain serves to both target the kinase to its appropriate cellular location and to regulate its kinase activity by acting as an autoinhibitory module (Newton, 2003). The C3 and C4 domains form the ATP- and substrate-binding lobes of the kinase core (Newton, 1995).

PKC function is regulated by two mechanisms (i) phosphorylation of key residues is required to render the enzyme catalytically active and accomplish localisation and (ii) removal of the pseudosubstrate domain is required to allow substrate binding and enzyme activation. All PKCs have an N-terminal pseudosubstrate domain which acts as an auto-inhibitor of the enzyme. This domain resembles the optimal substrate recognition motif except that the serine/threonine has been replaced by an alanine and in the inactive molecule this binds to the substrate domain (Toker, 1998). Binding of
Figure 1.12: The classification and structural characteristics of PKC family members.

The catalytic domain of PKC is conserved but the three subgroups have different regulatory domains. The classical PKC isoforms (cPKC) share all typical regulatory features: the autoinhibitory pseudosubstrate motif, two DAG-binding C1 domains (C1a and C1b) and the calcium-binding C2 domain. Novel PKC isoforms (nPKC) lack a calcium-binding motif but are still regulated by DAG. The catalytic activity of atypical PKC isoforms (aPKC) is independent of DAG and calcium (Spitaler and Cantrell, 2004).
diacylglycerol results in conformational changes that unmask the active site thus acting to relieve autoinhibition (Newton, 2003).

Phosphorylation also plays a key role in the activation of PKC. There are three conserved phosphorylation motifs: the activation loop segment, the turn motif and the hydrophobic motif. Activation of PKC is dependent on a series of ordered phosphorylations, including activation loop phosphorylation. Phosphorylation of the threonine residue in the activation loop is required before the kinase can phosphorylate substrates. In the conventional PKC isoforms this residue corresponds to Thr500. This kinase that catalyses this phosphorylation is thought to be triggered by phosphoinositide-dependent kinase-1 (PDK-1). The other conserved phosphorylation motifs have a role in stabilisation of the enzyme structure (Newton, 2003).

PKC isozymes specificity is determined by their subcellular localisation. After activation, enzymes are translocated to their unique subcellular site where they are anchored by specific protein, termed RACKs (Dempsey et al., 2000). RACKs (receptor for activated C-kinase) are a group of anchoring proteins that act as neither a PKC substrate nor an inhibitor, but appear to increase PKC phosphorylation of substrates by stabilising the active form of PKC (Ron et al., 1995). RACKs bind activated PKC through a site distinct from the substrate binding site of PKC, suggesting that PKC binding to RACKs occurs after cell stimulation, to localise the active enzyme to the RACK site (Mochly-Rosen, 1995). It is likely that specificity is determined in part by differential localisation of isozyme-specific RACKs, providing anchorage close to its physiological substrates (Mochly-Rosen, 1995).

1.11 AIMS OF THESIS

The primary aim of this thesis was to make a detailed examination of T lymphocyte responses to CD44 ligation. This study examined both the morphological and physical changes that take place following CD44 cross-linking. CD44 receptor activation was examined both in terms of cross-linking anti-CD44 monoclonal antibodies, but also in terms of a physiological ligand, hyaluronan. One of the CD44 monoclonal antibodies, D2.1, had been previously described as recognised a 45kDa protein, named p45. Work was carried out in order to identify this unknown molecule. A series of pharmacological inhibitors were used in order to elaborate the signalling events that
take place following CD44 receptor ligation and to try to identify some of the key signalling proteins, such as PKC and PI3-kinase, involved in this migratory response.
CHAPTER 2

GENERAL METHODS
2.1 CELL TISSUE CULTURE

2.1.1 Source of cell lines
HUT78, a T lymphoma cell line was obtained from the American Tissue Culture Collection (ATCC). K4, a clone of HUT78 cells, deficient in PKC β, was generated by our research group (Kelleher and Long, 1992).

2.1.2 Maintenance of cell cultures
Cells were maintained in either complete RPMI media or complete CO2 Independent media, dependent on the type of experiment being carried out. CO2 Independent media enabled cell survival during time-lapsed video-microscopy studies, when cells were incubated on a heated stage in the absence of a controlled CO2 environment. Complete medium was supplemented with 10% foetal calf serum (FCS), 100units/ml penicillin and 100μg/ml streptomycin, 2mM L-glutamine and 50μM β-mercaptoethanol [Appendix 1].

2.1.3 Enumeration and determination of cell viability
Cell viability and number was assessed using ethidium bromide and acridine orange (EB/AO) [Appendix 1]. Viable cells fluoresce green due to the presence of EB and non-viable cells fluoresce red due to the presence of AO under ultra-violet (UV) light, thus allowing enumeration of viable cells. The number of cells present was estimated by the dilution of cell suspension 1:10 with the EB/AO working solution. Cells were vortexed and then pipetted under a coverslip on a Neubauer haemocytometer. The cells were visualised under microscope using an UV filter.

2.1.4 Isolation and activation of peripheral blood lymphocytes
Approximately 10mls of peripheral blood was collected by venesection in heparinised evacuated blood tubes from normal volunteers. This was diluted with HBSS (10ml) and layered onto a Ficoll Hypaque density gradient (5ml). The sample was then centrifuged at 400 g for 30 minutes. The peripheral blood lymphocytes (PBL) were contained in the 'buffy coat' layer present at the interface. This layer was carefully removed and washed twice using HBSS for 10 minutes at 750 g. Cell viability was determined and cells resuspended in complete media. Cells were resuspended at 10^6 cells/ml and placed in a tissue culture flask for an hour at 37°C before transferring into a fresh flask, to reduce the numbers of contaminating platelets. Cells were incubated for 72 hours in the presence or absence of phorbol myristate acetate (PMA), at a
concentration of 25ng/ml. Assessment of cell populations following PMA activation indicated that the resulting activated T cells were contaminated with B cells (3 ± 1%) and with macrophages (5.6 ± 1.6%) (Kelleher et al., 1995).

2.1.5 Cryopreservation of cell stocks
Long term stocks were maintained by the storage of an early cell passage number in cryopreservation media in liquid nitrogen. Cells were enumerated and resuspended in cryopreservation media (10% DMSO, 90% FCS) at a concentration of 1ml per 2 x 10^6 cells. 1ml of cell suspension was quickly dispensed in labelled vials and placed on ice. Stocks were maintained in -80°C freezer for 1-2 weeks, before transfer to liquid nitrogen storage.

2.1.6 Reconstitution of cell stocks
Vial was removed from liquid nitrogen storage and placed in water bath for 1 minute at 37°C. The defrosted cell suspension was transferred to warmed (37°C) media and centrifuged for 3 minutes at 420 g to remove traces of DMSO. Cells were gently resuspended in 5ml of media supplemented with 20% FCS (to encourage cell viability).

2.2 SUBCELLULAR FRACTIONATION

2.2.1 Preparation of total cell proteins
Prior to cell lysis, cells were enumerated and percentage viability estimated. In the case where viability was less than 90%, the dead cells were cleared using a Ficoll Hypaque density gradient. Cells were washed with ice cold PBS and centrifuged at 600 g for 5 minutes. Protease inhibitors were added to the lysis buffer (10mM PMSF, 10μg/ml leupeptin). The cell pellet was resuspended in ice cold PBS/0.5% Np40 (0.5ml per 10^7 cells) [Appendix 1]. Cellular proteins were solubilised by leaving suspension on ice for 30 minutes, vortexing every 5 minutes. Suspension was ultra-centrifuged at 100,000 g for 30 minutes. The supernatant was retained as the total cell proteins.

2.2.2 Preparation of cytosol and membrane proteins
As above, cells were enumerated before being washed in ice cold PBS. Cells were centrifuged at 600 g for 5 minutes. Protease inhibitors were added to the hypotonic lysis buffer (10mM PMSF, 10μg/ml leupeptin). The cell pellet was resuspended in ice
cold hypotonic lysis buffer (1ml per 5 x 10^7 cells) [Appendix 1]. The cell suspension was vortexed for 2 minutes, and sonicated at 15 second interval, on power output 3, for a total of 1 minute at 4°C. The sonicate was centrifuged at 400 g for 5 minutes to clear lysate of any unlysed cells and nuclei. The supernatant was ultracentrifuged at 100,000 g for 10 minutes at 4°C. The supernatant, namely the cytosolic fraction, was aliquoted and stored at -20°C.

The remaining pellet was resuspended, washed in ice cold PBS and centrifuged at 420 g for 10 minutes at 4°C. Protease inhibitors were added to the PBS/1% Np40 buffer (10mM PMSF, 10μg/ml leupeptin) [Appendix 1]. The cell pellet was resuspended in ice cold PBS/1% Np40 buffer (0.5 ml per 4 x 10^7 cells). The suspension was maintained on ice and vortexed at 5 minute intervals for 30 minutes. This was ultracentrifuged at 100,000 g for 30 minutes at 4°C. The supernatant, the membrane fraction, was aliquoted and stored at -20°C.

2.2.3 Preparation of cytosol, membrane and cytoskeletal proteins
Cells were enumerated, washed with ice cold PBS and centrifuged at 420 g for 5 minutes at 4°C. Protease inhibitors (10mM PMSF, 10μg/ml leupeptin) were added to lysis buffers just before use, as PMSF is inactive 30 minutes following its addition to aqueous solutions. Cells were lysed on ice in buffer A (1ml per 1x10^7 cells), sonicated for 5 seconds and centrifuged at 600g to remove the nuclei and unlyzed cells. The resulting supernatant after ultracentrifugation at 100,000 g for 10 minutes at 4°C was the cytosolic fraction. The pellet was resuspended in buffer B (0.5ml per 1x10^7 cells), and centrifuged at 15,000g for 30 minutes at 4°C. The supernatant was the detergent-soluble membrane fraction. The remaining pellet (the detergent-resistant cytoskeletal fraction) was dissolved in boiling buffer C (0.2ml per 1x10^7 cells).

2.3 ESTIMATION OF PROTEIN CONCENTRATION

2.3.1 Bradford assay
In order to standardise the amount of protein sample used in subsequent work, such as SDS-PAGE, the protein concentration of each subcellular lysate must be determined. The Bradford assay was routinely used to determine protein concentration. A standard curve was constructed using a stock solution of BSA [Appendix 1]. The standard solutions contained 0, 25, 50, 75, 100μg/100μl of BSA.
Table 2.1: Preparation of standard solutions for Bradford Assay

The standard curve was prepared in duplicate as described in Table 2.1 in labelled tubes. Sample concentration (also in duplicate) was tested using the following dilutions, 1:2, 1:5, 1:10, using sample buffer as diluent. 5ml of filtered Bradford reagent was added to each tube. Tubes were vortexed and colour allowed to develop for approximately 30 minutes. The absorbance at 595nm in a 1cm light path was measured against a reagent blank. The standard curve was plotted from BSA standards and the protein concentration of samples was determined using this.

2.3.2 Markwell-Lowry assay

This is the method of choice when estimating the protein concentration of a lysate containing SDS. A standard curve was constructed using a stock solution of BSA [Appendix 1]. The standard solutions contained 0, 25, 50, 100, 150, 200 or 250μg/μl of BSA.

The standard curve was prepared in duplicate as described in Table 2.2 in labelled tubes. Sample concentration (also in duplicate) was tested using the following dilutions, 1:5, 1:10, 1:25, using sample buffer as diluent. Protein standards and unknown samples were made up to a final volume of 100μl. 900μl of Reagent C
(prepared fresh using 1 volume of Reagent B and 100 volume of Reagent A) was added to protein solution. Tubes were mixed well and left at room temperature for 10 minutes. 90μl of Reagent D (Folins reagent diluted 1:3 in water) was added to each sample. Tubes were vortexed and left at room temperature for 45 minutes for colour to develop. The absorbance at 660nm in a 1cm light path was measured against a reagent blank. The standard curve was plotted from BSA standards and the protein concentration of samples was determined using this.

2.3.3 Acetone precipitation of proteins
Acetone precipitation of protein was used in circumstances when the sample concentration was dilute, in order to increase the amount of protein loaded per well. Having determined the concentration of protein in each sample, an appropriate volume was aliquoted so that there was, for example 25μg of protein per lane. To this 5 volumes of acetone (-20°C) was added, the samples vortexed and stored at -20°C for a minimum of 30 minutes. Samples were subsequently centrifuged at 15,000 g for 5 minutes, to pellet the precipitated protein. The acetone was aspirated from protein pellet and allowed to air dry for 15 minutes to remove remaining traces of acetone. The protein sample was completely resuspended in sample buffer (1x) [Appendix 1] so that 20μl of sample buffer is equivalent to 50μg of protein. Samples were boiled at 95-100°C for 5-10 minutes. Following boiling, samples were loaded directly onto SDS PAGE gel.

2.4 ELECTROPHORESIS AND WESTERN BLOTTING

2.4.1 Preparation of SDS-PAGE gels
Glass plates and gaskets were thoroughly washed. The plates were then cleaned with ethanol to remove residual traces of proteins that may interfere with the running of the gel and allowed to dry thoroughly. The depth of the resolving gel was marked (depth 6.5cm, width 9cm). The composition of the polyacrylamide gel was dependent on the molecular weight of the protein(s) of interest, but in most cases a 10% gel was used.
Component Resolving Gel (ml) Stacking Gel (ml)
Acryl/Bis 30% 6.66 1.33
1.5M Tris, pH 8.8 5.0 -
1.0M Tris, pH 6.8 - 3.05
Distilled H₂O 8.23 5.55
APS 100μl 50μl
TEMED 10μl 10μl

Table 2.3: Composition of 10% SDS-PAGE gel mix (sufficient for 2 mini-gels)

The ammonium persulfate (APS) solution was prepared on the day of casting [Appendix 1]. The APS and TEMED were added last with gentle mixing to avoid incorporation of oxygen which inhibits polymerisation. The mixture was then pipetted into the casting stand. Finally a layer of water-saturated butanol [Appendix 1] was carefully layered on top of the resolving gel to exclude oxygen. The gel was allowed to polymerise for 1 hour. The water-saturated butanol was completely removed using water. At this stage the gel can be overlaid with resolving gel buffer and stored at 4°C to allow complete polymerisation or the stacking gel can be prepared immediately. Each casting stand was filled until overflowing and clean combs were carefully inserted into the polymerising gel. The stacking gel was allowed to polymerise for 30 minutes. Prior to running the gel the casting stand, the gasket and comb were removed. Any unpolymerised gel was removed from the wells by gentle rinsing with distilled water and finally the wells were straightened using a gel-loading tip.

1x running buffer was prepared and the bottom of the gel rig was filled with running buffer. Gels were placed in the gel rig with the notched plate facing inwards, taking care to avoid any bubbles at the bottom of the gel and spacers were placed to seal unit. In the event of just one gel being run in a gel rig, an empty set of plates was placed in the opposite side with the plates in the orientated in reverse. Inner chamber of the rig was filled with running buffer, clearing any bubbles from the wells. Protein samples were prepared either by addition of 5x sample buffer [Appendix 1] to make 1x sample buffer the final concentration or when required samples were initially concentrated using acetone precipitation [See 2.3.3] before resuspension in 1x sample buffer. Samples were boiled at 100°C for 5-10 minutes before loading onto gel. Molecular weight markers and samples were loaded into wells using gel-loading tips.
taking care to avoid spillage of samples from top of wells (maximum volume was 20μl). The gel rig was connected up to the power supply; making sure anode and cathode were correctly hooked up and electrophoresed at a constant current of 25mA/gel for approximately 1.5 hours. When dye-front was approximately 0.5cm from the end of the gel the power supply was switched off and the gel rig disassembled. The glass plates were carefully pried apart using a plastic comb. The stacking gel was removed using a scalpel blade. At this stage the gel was either directly stained to visualise the protein in the sample or specific proteins were detected by western blot following electrotransfer.

2.4.2 Coomassie G staining
There are a number of methods that can be used to visualise proteins on an SDS PAGE gel. A number of factors need to be considered during the choice of protein stain, i.e. what level of sensitivity is required for detection, what is the final use of these proteins and will the protein need further analysis? Coomassie staining is frequently used because it is a relatively simple staining procedure and can detect protein at a sensitivity of approximately 0.2-0.4μg. Although this method is not highly sensitive, it is suitable for use when proteins need to be subsequently analysed (e.g. mass spectroscopy analysis). SDS PAGE gels were removed from between the glass plates and placed in clean glass container. Gels were submerged in Coomassie stain [Appendix 1] (approximately 50ml) and placed on shaking platform for 1hr to overnight at room temperature. The stain was then decanted and destain [Appendix 1] was added to the container. Gels were returned to the shaking platform and destained until background was cleared and protein bands were well contrasted (changes of the destain solution may be required).

2.4.3 Silver staining of protein or LPS
This method of silver staining provides a fast and sensitive method of protein and LPS detection in PAGE gels. Silver staining is 30-50 times more sensitive than Coomassie G stain and can detect protein with a sensitivity of 80-160ng. Care should be taken when handling the gel as this can result in distortion of the staining. It is also important that solutions are made up freshly using highest quality water and reagents available. Once removed from the glass plates the PAGE gel was transferred to a clean glass container and incubated with 50% methanol, 12% glacial acetic acid solution for 10 minutes on shaking platform at room temperature. Once the first solution was decanted the gel was incubated with 10% ethanol and 5% glacial acetic acid for 10 minutes. At this point the gel can be kept indefinitely at 4°C in this solution.
To complete staining the gel was incubated for 5 minutes with 0.06% potassium permanganate, followed by 5 minutes incubation with 0.1% potassium carbonate. The gel was washed with several changes of distilled water (3 x 10 minutes). Following this wash step the gel was incubated with 0.1% silver nitrate for 10 minutes and then rinsed twice in distilled water. 2% potassium permanganate, 0.015% formaldehyde was used to develop the gel and was incubated in this solution until bands are clearly visible or until background begins to stain yellow. Transferring the gel into 1% glacial acetic acid for 2-5 minutes stopped staining. In this solution bands became more contrasted and then started to fade. Finally the gel was transferred to water for at least 10 minutes. This can be used to store the gel although the bands will fade with prolonged exposure to light so it should be processed as soon as possible.

2.4.4 Transfer of proteins to PVDF membrane

Proteins were transferred from an SDS PAGE gel by semi-dry transfer onto PVDF. Ten pieces of Whatman filter paper and one PVDF membrane (6.5 x 9cm) were cut for each gel. The PVDF membrane was wet by placing in methanol for 30 seconds and then placed in transfer buffer [Appendix 1] for 20-30 minutes. Following removal of the resolving gel from the glass plates, this was carefully placed in plastic tray containing transfer buffer and allowed to equilibrate on shaking platform for 10 minutes. The electrode plates of the transfer unit were rinsed with distilled water and dried before use. Five sheets of filter papers were soaked in transfer buffer and applied to the bottom plate of the transfer apparatus (cathode), taking care to exclude air bubbles that may interfere with the transfer efficiency. The PVDF membrane was placed on top of these, followed by the equilibrated resolving gel and finally the remaining five sheets of filter paper were soaked in transfer buffer and layered over the gel. Care must be taken to ensure that any air bubbles are excluded and also that the paper does not overlap the edges of the gel, as this might result in current short-circuiting. Any residual transfer buffer was removed and the anode was carefully placed on top of the transfer sandwich. The transfer apparatus was connected to a power supply and run at 0.85mAmps/cm² for 60 minutes. Once transfer was complete the PVDF membrane and gel were processed separately. Coomassie staining of the gel post-transfer allows the efficiency of transfer to be assessed. Immediately following transfer the individual lanes were visible on the PVDF membrane and their position indicated. The membrane was placed in a plastic tray containing PBS. The lane containing the molecular weight markers was removed and stained with Coomassie Blue [Appendix
for 10 minutes followed by destaining using 50% methanol. The remaining membrane was processed for immunodetection.

2.4.5 Western blot analysis
Non-specific binding sites on the membrane were blocked by incubation with Blotto/Tween Blocking Solution [Appendix 1] for 60 minutes on a shaking platform. The blocked membrane was cut into individual lanes, as required by experimental conditions and incubated with the appropriate dilution of the primary antibody. This incubation step took place in a sealed bag on a shaking platform overnight at 4°C. The PVDF strips were washed 3 times, 5 minutes each time, in 0.05% Tween/PBS. A suitable HRP-linked secondary antibody was selected for detection of the primary antibody. The PVDF membrane was incubated with the secondary antibody with shaking for 60 minutes at room temperature. As before, the PVDF strips were washed in 0.05% Tween/PBS, with a final wash in PBS alone and were now ready for detection using ECL.

2.4.6 Enhanced chemiluminescence
Following incubation of the PVDF membrane with HRP-conjugated secondary antibody, the membrane was washed twice with PBS/Tween with a final wash with PBS alone. The membrane was then ready for detection by enhanced chemiluminescence (ECL). The antigen/antibody complexes were detected by incubating the membrane in a solution of iodophenol (400μM), luminol (1.25mM) and hydrogen peroxide (0.01% v/v) in 0.1M Tris-HCl (pH 8.8) for 1 minute [Appendix 1]. The membrane was carefully placed between two sheets of acetate (i.e. clear plastic), taking care to exclude any air bubbles. The blots were exposed to X-ray film for between 30 seconds to 5 minutes, depending on the strength of signal (weaker signals may require longer exposure). Exposed films were then developed, fixed and allowed to air dry.

2.5 IMMUNOPRECIPITATION TECHNIQUES

2.5.1 Immunoprecipitation
Immunoprecipitation was carried out using Protein-G-Sepharose (PGS), which specifically conjugates antibody via their Fc receptor. PGS beads were aliquoted such that there were 10μg of PGS per immuno-precipitate for pre-clearing and a 10μg
aliquot for conjugation to antibody. PGS beads were washed 3 times with 0.5% Np40 and once with PBS. Antibody was conjugated to PGS by adding either 3μl ascities, 20μl tissue culture supernatant or 5μl commercial antibody. 200μl of PBS was added to each vial and conjugation was allowed to proceed at 4°C for 2 hours with end-over-end mixing. Cell lysates were removed from −70°C and allowed to thaw. 10μg of washed PGS was resuspended with the lysate and the protease inhibitors (PMSF and leupeptin) were added to each tube. Lysates were precleared for 2 hours. Unbound antibody was removed from the preconjugated beads by washing twice with 0.5% Np40 and twice with Tris buffer. The precleared lysates were incubated with the washed conjugated beads at 4°C, end-over-end for a further 2 hours. After the incubation period the tubes were centrifuged. The immunoprecipitates were washed 4 times with 0.5% Np40, with protease inhibitors as before. Microfuge tubes were changed 3 times during the wash steps. The final 2 washes were with PBS alone. The immunoprecipitates were resuspended in 15μl of 1x sample buffer. The samples were incubated at 95°C for 5-10 minutes.

2.5.2 In situ immunoprecipitation
HUT-78 cells (1×10⁶/ml) were exposed to cross-linking antibodies immobilized on 6 well plates [See 2.6.1]. Cells were then incubated either at 37°C or 4°C (low temperature preserves cell attachment but blocks active locomotion). Following 3 hours incubation, cells were washed twice in warmed PBS (1ml per well) to remove non-attached cells prior to lysis. Adherent cells were lysed with buffer containing (PBS pH 7.4, 0.5% Np40, 5mM MgCl₂, 1mM EGTA, 1mM PMSF, 10μg/ml leupeptin and 0.2μM okadaic acid) at 25°C. After washing 3 times with the lysis buffer (1ml per well) all that remains attached to the plastic via receptor/ligand interactions are receptor-associated complexes. These are extracted with SDS-containing buffer C (200μl per well) [Appendix 1] and concentrated by acetone precipitation. Proteins obtained from equivalent cell numbers kept at 4°C or 37°C were loaded onto gels to enable quantitative comparative analysis.

2.6 MICROSCOPY TECHNIQUES

2.6.1 Preparation of cells for morphological analysis
Lymphocyte migration studies were performed using Labtek chambered slides (permanox plastic or borosilicate) or 96 well plates. The surfaces were pre-coated with
200µl goat anti-mouse immunoglobulin (diluted 1:100 in PBS) overnight at 4°C. The wells were washed twice with PBS to remove unbound antibody. The appropriate cross-linking monoclonal antibody was incubated (normally using a volume of 200µl, diluted 1:100 in PBS) either at 37°C for 2 hours or overnight at 4°C. The wells were washed as before with PBS. After this wash the cells were seeded into chambers at a density of 2x10^4 cells per well in 200µl of warmed CO₂-independent media. The cells were incubated at 37°C during which time cells became activated and developed polarised phenotype. Live imaging of cells was made during this time or cells were fixed 4 hours after incubation with immobilized antibody in pre-coated chambers (when activation was maximal). Cells were processed further depending on the analysis to be carried out.

As a control for cell activation, wells were coated with poly L-lysine or cells were seeded onto positively charged slides, in order to enable the fixation of cells to the slide without receptor engagement. During the inhibitor studies, cells were pre-treated with the inhibitor at 37°C for 30 minutes before addition to well.

2.6.2 Time-lapse video microscopy
Cell migration was monitored on a Nikon TE-300 inverted microscope equipped with a JVC TK-C1380 CCD camera. The microscope was also equipped with a heated stage which allowed live cell imaging over an extended observation period. Sequential image frames were digitised and cell migration was evaluated by the distance travelled by the cell centroid over the entire observation period (indicated in figure legends for each study), with between 40 and 50 cells were recorded per observation field.

2.6.3 Morphological studies using deformation index values
In our system, as cells migrate they develop a polarised, elongated phenotype (a migration-associated phenotype). The deformation index was developed as a quantitative indicator of cells morphology. Following activation cells were fixed using para-formaldehyde (3.7% PFA). Images were taken using a Nikon TE-300 inverted microscope equipped with Hoffman-modulation contrast (HMC) optics. Images from 5 randomly selected fields of view were recorded for analysis. For quantitative characterisation of cell morphology, cell borders were manually outlined on the HMC images and scored using the following formula for the deformation index (D.I.).
Deformation Index = 

Circularity Index

Where

Elongation Index = 

And

Circularity Index = 4 \times \pi \times \frac{\text{Area}}{\text{Perimeter}^2}

This index provides a strong measure of the degree of deformation of a cell (i.e. its deviation from circularity) and provides information about cell morphology. Typically values of 1-3 are assigned to cells that were non-migratory i.e. cell shape nearing circular. Those cells that display a polarized phenotype (cell body and trailing uropod) and therefore a higher degree of deformation, had higher D.I. values (>5) (Manuscript in preparation). On average > 40 cells were scored for each set of conditions. Analysis was performed using the National Institute of Health Image software (Scion Image for Windows).

2.6.4 Immunofluorescent staining

The surfaces of 8-well chambered slides were prepared by incubation with 200µl goat anti-mouse immunoglobulin (diluted 1:100 in PBS) overnight at 4°C. The wells were washed twice with PBS to remove unbound antibody. The appropriate cross-linking monoclonal antibody was incubated (normally using a volume of 200µl, diluted 1:100 in PBS) either at 37°C for 2 hours or overnight at 4°C. The wells were washed as before with PBS. After this wash the cells were seeded into chambers at a density of 2x10^4 cells per well in 200µl of warmed media. The cells were incubated at 37°C for 2-4 hours during which time cells became activated and developed polarised phenotype. The chambered wells and gasket were carefully removed and the unbound cells were
washed using warm PBS (37°C) for 5 minutes. Excess PBS was carefully removed and adherent cells were fixed by gentle immersion in acetone at -20°C for 10 minutes. The fixed cells were allowed to air dry and either stained immediately or stored at -80°C until required for staining.

Permeabilisation of fixed cells was carried out using 0.1% Triton X-100/PBS for 30 minutes. This was followed by a blocking step where 40μl of normal goat serum (or other appropriate blocking agent, as required) was pipetted onto the middle of the slide and then carefully overlaid with a clean coverslip. This was incubated in a humidified chamber for 30 minutes at room temperature. The slide was carefully washed with wash buffer (0.05% Tween/PBS) and excess wash buffer removed, before incubation with the primary antibody. For example, isoform specific PKC antibodies were used to determine their subcellular localisation. The antibodies were prepared as directed by manufacturers instructions (unless stated otherwise). The antibodies were aliquoted, either 40μl per slide or 20μl per well, and incubated in the humidified chamber for 2 hours at room temperature. The slides were washed in three changes of wash buffer and after final wash excess wash buffer carefully removed. In the experiments where a second primary antibody was used (i.e. for double staining), for example tubulin, this was aliquoted at this time and incubated for 30 minutes as before. After washing in three changes of wash buffer, both FITC and TRITC (1:50) conjugates were aliquoted and incubated for 30 minutes. The slides were washed twice with wash buffer and a final wash with PBS alone. The excess PBS was carefully removed before the slide was mounted beneath a clean coverslip with fluorescence-preserving mounting medium. Once the mounting media dried, the slides were ready for photography.

2.7 DEGLYCOSYLATION OF PROTEINS

Cell membrane fractions were prepared and protein concentration estimated. 50μg of protein was aliquoted per condition into a labelled eppendorf and either acetone precipitated immediately or boiled for 2-3 minutes to reduce the protein tertiary structure before acetone precipitation. Samples were made up to 90μl with the deglycosylation buffer (0.25M sodium acetate, pH 6.0-7.0, 20mM EDTA and 10mM 2-mercaptoethanol) or else as directed in experiment. To each tube 1μl leupeptin (2mg/ml) and 1μl PMSF (1M) was added. Buffer alone was added into control tubes and 4μl of Endoglycosidase-F (0.8U) was added to the tubes undergoing
deglycosylation. The total sample volume was approximately 100μl. In order to prevent evaporation samples were overlaid with toluene. The samples were placed in a 37°C incubator for 18 hours. Following this incubation step, the samples were acetone precipitated, boiled in 1x sample buffer and applied to a 10% SDS-PAGE gel. Following transfer onto PVDF membrane, samples were probed by western blot with specific antibodies.

2.8 PINOCYTIC LOADING OF CELLS IN SUSPENSION

Prior to treatment, cells were enumerated and percentage viability estimated. In the case where viability was less than 90% dead cells were cleared using a Ficoll Hypaque density gradient (lymphocytes). Cells were resuspended in a sterile eppendorf at a concentration of 2x10⁵ cells in 0.5ml of warm media. Peptide inhibitors were provided at a stock concentration of 250μM and were diluted to a working concentration of 10μM in hypertonic loading buffer. 20μl of hypertonic loading medium containing the peptide inhibitor was prewarmed, as well as 3ml of the hypotonic lysis medium and 2ml of the recovery medium to 37°C, per experimental condition. Cells were centrifuged for 1 minute at 750 g and the media was carefully decanted. 20μl of loading medium (+ peptide) was added and the cells gently resuspended. Cells were incubated at 37°C for 10 minutes. To each tube 1ml of hypotonic lysis medium was gently added to the cell suspension and transferred to a labelled universal tube with 2ml of hypotonic lysis medium. The cell suspension was aliquoted between 2 sterile eppendorfs and incubated for 1.5 minutes at 37°C. Cells were pelleted as before and supernatant removed. The cells were resuspended in 1ml of recovery medium and allowed to recover for 10 minutes before further manipulation.

2.9 PURIFICATION OF ANTIBODY

2.9.1 Ammonium sulfate precipitation

Tissue culture supernatant was defrosted and pooled. Total volume of supernatant being purified was determined. The antibody solution was centrifuged at 3000g for 30 minutes. Supernatant was transferred to a beaker with stirrer bar and placed on a magnetic stirrer. While antibody solution was being stirred enough saturated ammonium sulfate solution was added slowly to bring final volume concentration to
50% saturated solution. This was transferred to the cold room and stirred overnight. The precipitate was centrifuged at 3000 g for 30 minutes. The supernatant was removed carefully and the pellet well drained. The pellet was resuspended in PBS using 0.1 of the starting volume for tissue culture supernatant (for serum or ascites resuspend pellet in 0.3 - 0.5 of the starting volume). The antibody solution was then transferred to dialysis tubing [See 2.10.2] and dialysed against three changes of PBS overnight.

2.9.2 Dialysis
Dialysis tubing of a suitable diameter was selected and cut into convenient lengths, submerged in a solution of 2% sodium carbonate/0.5% EDTA and boiled for 10 minutes. The solution was discarded and the tubing was boiled in distilled water twice for 10 minutes each, cooled and stored at 4°C in 0.1% sodium azide. Before use the dialysis tubing was washed inside and out with distilled water. One end of the tubing was sealed with a dialysis clip. The solution to be dialysed was then added; air expelled from the tubing and the other end was sealed with a second dialysis clip. The tubing was then placed in a large volume of exchange buffer and agitated gently with a magnetic stirrer, at 4°C. The buffer was changed twice when equilibrium was reached, after about 3 hours.

2.9.3 Affinity purification of antibody using protein G sepharose
Dialysed antibodies were further purified using Protein G Sepharose (PGS). The capacity of the beads for mouse antibody was 6mg/ml. Sufficient beads were placed in a sintered glass column and prepared by washing with three changes of PBS. Before placing the antibody solution onto the column, it was filtered, using a 0.2μm filter, to remove any large particles. The antibody solution was carefully overlaid and the antibody solution was recirculated through the beads for 3 hours, using a peristaltic pump. PBS was run through the column (approximately 10 volumes) in order to wash any non-specific proteins present. The elution buffer 100mM glycine, pH 2.5) was placed on the column and 1ml fractions were collected in labelled eppendorfs containing 100μl of neutralisation buffer (1M tris-HCl, pH 8.0). The fractions were mixed carefully. The antibody content of each fraction was estimated by absorbance readings at 280nm, where 1 OD = 0.8mg/ml.
2.10 PREPARATION OF ANTIBODY LINKED TO PROTEIN G-SEPHAROSE

Equal volumes of antibody solution and protein G-sepharose (PGS) beads were mixed for 1 hour at room temperature. Mixing was carried out using gentle agitation. The slurry was centrifuged and the amount of unbound antibody was determined by measuring the absorbance at 280nm. Using the assumption that 1 OD$_{280}$ = 0.8mg/ml, the amount of bound antibody can be quantified. The PGS beads were washed twice with 10 volumes of 0.2M sodium borate, pH 9.0, followed by centrifugation at 3000g for 5 minutes. The beads were resuspended in 10 volumes of 0.2M sodium borate, pH 9.0. To this suspension enough dimethylpimelimidate (DMP) was added to bring the final concentration to 20mM. This slurry was mixed for 30 minutes at room temperature. The reaction was stopped by washing the beads once in 0.2M ethanolamine, pH 8.0, followed by gentle mixing in 0.2M ethanolamine, pH 8.0 for 2 hour at room temperature. The beads were resuspended with PBS after the final wash (if the beads are to be stored long term 0.01% merthiolate should be added). In order to test the efficiency of antibody coupling, samples taken before and after reaction were examined on a 10% SDS polyacrylamide gel and stained with Coomassie blue.

2.11 TWO-DIMENSIONAL ELECTROPHORESIS

2.11.1 Two-dimensional electrophoresis using carrier ampholytes in isoelectric focusing step

Glass tubes were used as a support for the isoelectric focusing (IEF) gel. One end of the tube was sealed using parafilm and the 8cm was marked from the end. The IEF gel mix (Table 2.5) was added carefully avoiding air bubbles and overlaid with distilled water to help polymerisation.
Table 2.5: Composition of carrier ampholyte isoelectric focusing gel mix

<table>
<thead>
<tr>
<th>Component</th>
<th>IEF Gel</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acryl/Bis 30%</td>
<td>0.532ml</td>
</tr>
<tr>
<td>Urea</td>
<td>2.2g</td>
</tr>
<tr>
<td>10% Np-40 (w/v)</td>
<td>0.8ml</td>
</tr>
<tr>
<td>Distilled H₂O</td>
<td>0.788ml</td>
</tr>
<tr>
<td>Ampholytes (pH 3-10)</td>
<td>0.3ml</td>
</tr>
<tr>
<td>10% APS</td>
<td>8µl</td>
</tr>
<tr>
<td>TEMED</td>
<td>4µl</td>
</tr>
</tbody>
</table>

The IEF gels were run vertically with 10mM H₃PO₄ used as running buffer in the lower chamber (anode) and 20mM NaOH used as running buffer in the upper chamber (cathode). Following polymerisation, the tubes were placed in the gel rig and overlaid with 30µl of sample buffer A [Appendix 1]. The appropriate running buffer was placed in the chambers of the gel rig, ensuring that both ends of the tubes were immersed in buffer and any air bubbles were excluded. Before sample application the following pre-run was carried out (15 minutes at 200V, 30 minutes at 300V).

Once the pre-run was completed, some of the upper running buffer was decanted and any liquid on top of the IEF gel was carefully removed. The samples were dissolved in 25µl of sample buffer A and overlaid on the gel, followed by 10µl of overlay buffer [Appendix 1]. Ensuring there were no air bubbles, the tube was covered with the upper chamber running buffer. The IEF gels were run for 16 hours at 300V, followed by 1 hour 15 minutes at 800V. Once run was completed, the gels were frozen to aid removal from tubes. The gels were carefully removed using distilled water and a syringe to loosen the gel from the glass tubing. The gels were equilibrated in sample buffer B [Appendix 1] for 30 minutes, before being carefully placed on the stacking gel of the SDS PAGE gel. The IEF gel was fixed in place using 1% agarose solution, carefully excluding air bubbles.

2.11.2 Two-dimensional electrophoresis using an immobilised pH gradient in the isoelectric focusing step

The ceramic strip holders (13cm) were carefully washed and dried before use. The samples were dissolved in 250µl of rehydration buffer [Appendix 1] and the
rehydration buffer (and sample) were pipetted slowly into the centre of the strip holder, taking care not to introduce air bubbles (Figure 2.1). The immobilised pH gradient (IPG) strip was carried using the acidic (pointed) end, avoiding any damage to the basic (square) end. Position the IPG strip with the gel side down and the pointed (anodic) end of the strip directed toward the pointed end of the strip holder. With the pointed end first, the IPG strip was carefully lowered into the solution, ensuring the strip is fully coated with the solution and that the gel is in contact with the strip holder electrodes at each end. The IPG cover fluid was carefully applied to minimise sample evaporation and the strip holder cover applied.

The strip holders were placed on the Ettan IPGphor platform, checking that the pointed end of the strip holder (up to 12 strip holders may be used) was placed over the anode and the blunt end was over the cathode. The safety lid was closed and the following settings were used for 13cm, linear IPG strips, pH 3-10 (Rehydration: 12 hour, step 1: 500V 1 hour, step 2: 1,000V 1 hour, step 3: 8,000V 2 hour). After IEF, the IPG strips were placed in screw-cap tube and can be stored at -70°C or processed immediately for second-dimension separation. The equilibration step saturates the IPG strip with the SDS buffer system required for the second-dimension separation. The equilibration buffer (10mls) [Appendix 1] was added the IPG strip in the screw-cap tube. With the tube firmly capped, it was placed on its side on a rocker and equilibrated for 15 minutes. Following equilibration the IPG strip was removed and carefully placed on a prepared SDS PAGE gel. The IPG strip was fixed in place using 1% agarose solution, carefully excluding air bubbles.
Figure 2.1: Sample loading of ceramic holders of immobilised pH gradient strips.
(a) Rehydration and sample application, (b-c) positioning of IPG strip and (d) application of IPG cover fluid (Ettan IPGphor II system, Amersham)
CHAPTER 3

CD44-INDUCED T LYMPHOCYTE MIGRATION

In part published:

CD44 cross-linking induces protein kinase C-regulated migration of human T lymphocytes: *International Immunology, Apr. 2005*
3.1 INTRODUCTION

Lymphocyte migration is critical to the operation of an effective adaptive immune response. It allows the homing of naïve T lymphocytes to peripheral lymphoid organs and the delivery of effector T cells to sites of infection. Here we examined the lymphocyte response to CD44 activation and the induction of a migratory phenotype.

The migration at peripheral lymph nodes is characterised by a series of receptor and counter-receptor interactions involving adhesion molecules, beginning with the tethering of the lymphocyte to the surface of specialized endothelial cells (primary adhesion) (Girard and Springer, 1995). This is thought to be mediated largely by selectin interaction with their carbohydrate ligands expressed on the endothelium surface, although other lymphocyte receptors have been implicated in this process (Drillenburg and Pals, 2000; Siegelman, 2001; von Andrian and Mackay, 2000). Similarly in areas of inflammation, the secretion of cytokines and chemokines locally induces ligand expression, such as the up-regulation of ICAM-1 expression on endothelial cells in response to pro-inflammatory cytokines TNF-α, IL-1 and IFN-γ (Hubbard and Rothlein, 2000). These endothelial cell ligands then interact with the specific receptors expressed on the surface of effector lymphocytes. The resulting slowing of lymphocyte flow and the rolling of cells along the surface of the endothelium allows chemokines to activate molecules of the integrin family on T cells, leading to lymphocyte arrest, firm adhesion and subsequent diapedesis across the endothelium.

A series of morphological and molecular changes occur within the migrating lymphocyte and many common themes in terms of signalling events have been recognised with other migratory cells. In general terms, the cell begins to polarise in response to the initiation of migration and extend small protrusions in the direction of migration. These are either broad lamellipodia or spike-like filopodia, which are usually driven by actin polymerisation and are stabilised by adhesion receptors, linking the actin cytoskeleton to the extracellular matrix or adjacent cells. These adhesions serve as traction sites for migration as the cell moves forward and are disassembled at the cell rear, allowing it to detach (Ridley et al., 2003). In response to activation, spherical resting lymphocytes become polarised and develop a clearly defined trailing cytoplasmic projection (sometimes referred to as a uropod). This lymphocyte polarisation is accompanied by the redistribution of adhesion receptors, such as ICAM-3, ICAM-1, CD43 and CD44, to the trailing structure (Del Pozo et al., 1995). This is accompanied by cytoskeletal rearrangement, with F-actin accumulation in the direction
of migration (Campanero et al., 1994; Porter et al., 2002) and microtubule redistribution to the trailing cytoplasmic projection (Volkov et al., 1998). T lymphocyte polarisation and migration in response to chemoattractant is regulated by the activities of Rho GTPases (Del Pozo et al., 1999).

Cross-linking antibodies have long been used as a tool to investigate the functional role of adhesion receptors. Kelleher et al., 1995, described cytoskeletal rearrangement leading to the induction of a locomotion-associated dendritic phenotype following LFA-1 cross-linking using the monoclonal antibody, SPV-L7. The use of cytoskeletal inhibitors, such as cytochalasin B completely inhibited the development of these processes indicating the importance of the cytoskeleton in acquiring this phenotype (Kelleher et al., 1995). Similarly, motility was induced in human peripheral T lymphocytes following cross-linking of LFA-1 or VLA-4 in a transmigration assay (Hauzenberger et al., 1997). This phenomenon is thought to describe a physiologically relevant event at the stage of cell extravasation.

A typical sequence of events resulting in successful cell movement following activation of the integrin LFA-1 is the formation of the leading lamella, translocation of the nucleus and extension of the trailing tail accompanied by rearward movement of the integrins. Contraction of the trailing tail with rear release of bound membrane integrin on the substrate and recondensation of integrin clusters precedes a new locomotion cycle. During this process, LFA-1 is redistributed away from the leading edge toward the rear of the nucleus over MTOC and subsequently redistributed (Volkov et al., 1998). Also this work has revealed that LFA-1 activation results in significant rearrangement of the microtubule cytoskeleton and the association of the PKC isoforms β1 and δ with the microtubule cytoskeleton (Volkov et al., 1998). Further work found PKCβ1 to be critical in the development of a polarised morphology and it has been demonstrated that transfection of a PKCβ1 deficient T cell clone with this enzyme restores the cells locomotory behaviour (Volkov et al., 2001). Active cell migration induced by LFA-1 ligation, using a motility inducing antibody or recombinant ICAM-1, was slow (2.0-0.5μm/min), compared to non-activated PBTLs rolling on endothelial cells (10μm/s) (Jones et al., 1994) and was accompanied by stronger adhesion and a large amount of cell polarisation (Volkov et al., 2001). Indeed this was comparable to the rate of migration measured for human skin and oral fibroblasts (0.3-0.2μm/min) (Lepekhin et al., 2002).
In this study, we were interested in examining the role of CD44 in lymphocyte migration. CD44-HA interaction has been implicated in the primary adhesion required for extravasation at inflammatory sites. For example, antigen stimulation of T lymphocytes results in the up-regulation of activated surface CD44 expression and an increase in the number of cells rolling on hyaluronan, under physiological shear stress (DeGrendele et al., 1997). The role of CD44 in lymphocyte homing to sites of inflammation has been clearly demonstrated with the slowed onset of a cutaneous delayed-type hypersensitivity response following antigen challenge following antibody-induced loss of surface CD44, although migration to lymphoid sites was normal. The CD44-negative, unactivated lymphocytes migrate in an identical fashion to cells from control mice, but following challenge the response is altered, with a delay in lymphocyte extravasation to inflammatory sites (Camp et al., 1993). Similarly a study using murine CD44^T^ T cells confirmed that receptor expression was not required for normal lymph node homing but the migration of CD44-deficient cells into areas of inflammation was delayed (Stoop et al., 2002).

A report investigating the preferential transendothelial migration of murine Th1 lymphocytes has described the inhibition of this cytokine independent migration using antibodies directed against CD44 and LFA-1. Cross-linking of CD44 on murine Th1 cells lead to the development of an elongated polarised phenotype. This transendothelial migration was also demonstrated to require the activity of Srk-tyrosine kinase, PI3-kinase and PLC-γ (Katakai et al., 2002). In the current study carried out here, lymphocyte responses to CD44 ligation were further investigated primarily using the monoclonal antibody, D2.1. D2.1 is a monoclonal antibody that was prepared by generating monoclonal antibody to the cutaneous lymphoma cell line HUT-78 lysate using standard hybridoma technology. The D2.1 molecule was identified by screening for antibodies that induced cytoskeletal rearrangement in HUT-78 cells (Kelleher et al., 1994). This monoclonal antibody was identified as specifically recognising CD44 with work carried out by McGrath et al (In preparation). CD44 cross-linking using L3D1 or D2.1 has previously been described as leading to the development of a polarised morphology in lymphocytes (Kelleher et al., 1995; McGrath, In preparation) and lymphocyte responses to ligation of CD44 were investigated in this study.
3.2 OBJECTIVES

The objective of this study is to characterise T cell responses following CD44 ligation particularly in the context of cell morphology and to investigate the signalling pathways involved in these morphological changes. Previous work has demonstrated a role for LFA-1 in signalling T lymphocyte migration and this study examined cell responses to LFA-1 ligation in parallel to investigation of the CD44 response.

1. The morphological changes that occur following cross-linking of CD44 in T cells, both HUT-78 cells (a T lymphoma cell line) and PBTLs, were examined.
2. The dramatic polarisation that takes place following CD44 cross-linking was examined in real-time to ascertain if it is accompanied by active cell migration (as seen in LFA-1 activated T cells).
3. The roles played by a number of signalling proteins in the development of a migratory phenotype were examined, including the classical and novel PKC isoforms, and PI3K.

3.3 RESULTS

3.3.1 Morphological changes in PBTLs induced by antibodies to CD44 and LFA-1 antibodies

The responses of T lymphocytes to CD44 and LFA-1 ligation were investigated using mouse monoclonal cross-linking antibodies immobilised on a plastic substrate (i.e. cell culture 96 well plate) (see Section 2.6.1), a system that has been well described in the study of lymphocyte responses to LFA-1 ligation (Kelleher et al., 1995; Volkov et al., 1998; Volkov et al., 2001). It has been previously described that the CD44 monoclonal antibody, L3D1, induces morphological rearrangement in PBTLs (Kelleher et al., 1995). The antibody D2.1 was selected on the basis of its ability to induce cytoskeletal rearrangement in HUT-78 (Kelleher et al., 1994), so the response of lymphocytes to CD44 ligation was investigated. In order to study the influence of ligation of the CD44 hyaluronan binding site the monoclonal antibodies, F10-44-2 and J173 were also tested. F10-44-2, has been reported to up-regulate HA binding ability of activated T cells whereas J173, was found to inhibit hyaluronan adhesion in these activated cells (Galandrini et al., 1994).
Peripheral blood lymphocytes (PBLs) were isolated from the blood of normal donors, using Ficoll-Hypaque density gradient (see Section 2.1.4). The isolated PBLs were activated by incubation with 25ng/ml PMA at 37°C for 72 hours. Following 72 hours incubation with PMA these cells have been reported to be greater than 90% T lymphocytes. Also, in response to anti-LFA-1 ligation, the majority of cells producing cytoplasmic projections are T cells (CD2 positive) and are referred to as peripheral blood T lymphocytes (PBTLs) (Kelleher et al., 1995). As a control PBTLs were incubated for 72 hours at 37°C without PMA (designated as resting). In the analysis of PBTL responses to the activation of CD44, these cells (both activated and resting) were incubated on immobilised CD44 monoclonal antibodies for 3 hours at 37°C. Resting PBTLs did not display any morphological changes in response to ligation of either CD44 or LFA-1, but required pre-activation with PKC inducers such as the phorbol ester, PMA. The activated cells developed a polarised phenotype in response to ligation of CD44 using D2.1 (IgG1) (Figure 3.1 (b)) characterised by a single elongated cytoplasmic projection whereas the resting PBTLs remained rounded and show no sign of polarisation (Figure 3.1 (a)). These cells were examined using time-lapsed video-microscopy (see Section 3.3.3), and a relationship was demonstrated between cell migration and the degree of cell polarisation following CD44 cross-linking by D2.1. When activated using J173 (IgG1) (Figure 3.1 (c)) the cells adhered and polarised but with shortened projections compared to D2.1. L3D1 (IgG1) activated PBTLs (Figure 3.1 (d)) showed some evidence of polarisation. Along with T lymphocyte polarisation, F10-44-2 (IgG2a) activated cells (Figure 3.1 (e)) sometimes produced multiple cytoplasmic projections and clumping of activated cells. With D2.1 a dramatic elongated polarised phenotype was seen, when other CD44 monoclonal antibodies were used they did not uniformly induce this elongated polarised phenotype and instead tended to have a shortened polarised phenotype with an irregular appearance.

PBTLs following the ligation of LFA-1 (Figure 3.1 (g)) developed a polarised phenotype in a similar manner to that seen in D2.1 activated cells i.e. the cells developed an elongated polarised morphology with a single projection. Resting T lymphocytes (Figure 3.1 (f)) when incubated with SPV-L7 (IgG1) (a motility-inducing LFA-1 monoclonal antibody) remained rounded and did not show signs of polarisation. To explore whether this was a non-specific phenomena, the response of two other transmembrane receptors were tested – CD3 and ICAM-1. Ligation of CD3, using OKT3 (IgG2a) (Figure 3.1 (h)), resulted in PBTLs with a irregular appearance. When
Figure 3.1: Phenotypic changes in PBTLs induced by CD44 antibodies. PBTLs cells (activated for 72 hours using 25ng/ml PMA) developed a polarised morphology following cross-linking of CD44 using the monoclonal antibody D2.1, b and to a lesser extent using the CD44 antibodies J173, c, L3D1, d, F10-44-2, e. Resting PBTLs (not activated via antibody or PMA pre-treatment) remained static and rounded when incubated with D2.1, a. Similar phenotypic changes were seen with resting and PMA pre-treated cells activated by cross-linking LFA-1, using SPV-L7, f and g, respectively. Cross-linking of CD3 using OKT3, h and ICAM-1 using Mem111, i, did not induce the same morphological changes.
ICAM-1 was cross-linked using Mem111 (IgG2a) (Figure 3.1 (i)) the PBTLs had an irregular appearance and did not adhere (indicated by the formation of clumps).

3.3.2 Morphological changes in HUT-78 and MUTU III induced by CD44 and LFA-1 antibodies

HUT-78 is a cutaneous T lymphoma cell line, which displays an activated T cell phenotype, which can be induced to secrete IL-2. In the analysis of HUT-78 responses to the activation of CD44, these lymphoma cells were incubated on immobilised CD44 monoclonal antibodies for 3 hours at 37°C (see Section 2.6.1). Cells developed a polarised phenotype in response to cross-linking of CD44 with all antibodies used (Figure 3.2). Ligation of CD44 using various monoclonal antibodies resulted in these lymphoma cells acquiring a migratory associated morphology, although some slight differences in the characteristics of this phenotype were seen. D2.1 activated HUT-78 lymphoma cells (Figure 3.2 (a)) tended to develop a polarised phenotype with an elongated cytoplasmic projection extended behind the cell body, with the trailing tail split at times. Cells activated by cross-linking CD44 using J173 (Figure 3.2 (b)) developed a polarised morphology, with a long tail that sometimes had a ruffled appearance. L3D1 induced some cells to develop an elongated morphology, with a ruffled tail. Other cells that did not acquire this phenotype remain polarised but with a short tail with multiple cytoplasmic projections and a somewhat spiked appearance. HUT-78 cells activated using F10-44-2 (Figure 3.2 (d)) developed a polarised morphology in most cells; some had a single projection, in addition to a sometimes-spiked cytoplasmic projection.

In a similar manner LFA-1 activation of HUT-78 resulted in the development of a polarised morphology. When the cells were incubated with immobilised SPV-L7 (Figure 3.2 (e)) they polarised with an elongated tail. Some cells extended multiple cytoplasmic projections. To confirm this was a specific effect and that cross-linking of LFA-1 and CD44 induced polarisation of HUT-78 cells, the response of these cells to cross-linking of other transmembrane receptors was examined. OKT3, which ligates CD3, resulted in the adhesion of HUT-78 to the substrate with the cells remaining rounded (figure 3.2 (f)). In some cells tiny membrane projections were visible from the cell body. Ligation of ICAM-1, using Mem111, did not induce HUT-78 polarisation (Figure 3.2 (g)). The cells adhered to the immobilised antibody and acquired an irregular shape. Examination of B lymphoma responses to CD44 or LFA-1 cross-linking were examined using the Epstein Barr virus infected B cell line, MUTU III. Cross-linking of CD44 using D2.1 resulted in the development of an elegant elongated
Figure 3.2: Phenotypic changes in HUT-78 T lymphoma cells and MUTU III B cell line induced by CD44 and LFA-1 antibodies.

HUT-78 cells developed a polarised morphology following cross-linking of CD44 using the monoclonal antibodies D2.1, a, J173, b, L3D1, c, F10-44-2, d. Similar phenotypic changes were seen with cells activated by cross-linking LFA-1, using SPV-L7, e. Cross-linking of CD3 using OKT3, f and ICAM-1 using Mem111, g, did not induce the same morphological changes. MUTU III also developed a polarised phenotype following CD44 cross-linking using D2.1, h but tended to form clumps when LFA-1 was cross-linked, i.
polarised morphology, typical of migrating cells (Figure 3.2 (h)). When MUTU III responses to LFA-1 ligation were examined using immobilised SPV-L7 the phenotype was quite different, with adhesion, a small degree of polarisation and formed a substantial amount of clumping (Figure 3.2 i).

3.3.3 PBTLs migrate actively following CD44 or LFA-1 ligation

In order to determine if these morphological changes were associated with cell locomotion, a study of cellular events in real-time was required. The use of time-lapsed video-microscopy allowed the real-time study of the series of morphological changes that take place following receptor ligation. PBTLs were isolated from the blood of a normal donor as described previously (see Section 2.1.4). These cells were activated by incubation with 25ng/ml PMA at 37°C for 72 hours. At this point activated PBTLs formed large clumps of cells. When seeded onto chambered slide for observation activated T lymphocytes rapidly adhered to the immobilised anti-CD44 mAb. The cells flattened and began to polarise. Membrane ruffles extended from the front of the cell and a long cytoplasmic process remained posterior to the cell body. Figure 3.3 highlights the morphological changes that take place in the migrating cell. Cells that are actively migrating also demonstrate a high degree of polarisation. Measuring the displacement of the cell centroid during an hour observation demonstrated active migration of PBTL when activated by cross-linking of CD44 (Figure 3.4) (see Section 2.6.2), which travelled at a mean velocity of 12.0μm per hour (mean value of peak velocity – 39.7μm per hour). LFA-1-activated PBTLs migrated at a similar velocity (15.0μm per hour, mean value of peak velocity – 56.7μm per hour), with a slow apparently random locomotion.

3.3.4 HUT-78 migrate actively following CD44 or LFA-1 ligation

In a similar manner the response of HUT-78 following cross-linking of CD44 was monitored using time-lapsed video-microscopy. HUT-78 cells before CD44 ligation had a rounded appearance, when activated through receptor cross-linking; the cells adhered and flattened to the substrate within 10 minutes. These lymphoma cells become polarised, with lamellipodia advancing from the leading edge of the cell and a trailing process. These features were accompanied by active T cell migration. Measuring the displacement of the cell centroid during an hour (see Section 2.6.2) demonstrated that HUT-78 cells activated through cross-linking of CD44 travelled at a mean velocity of 14.4μm per hour (mean value of peak velocity – 52.9μm per hour), which was similar to that of cells activated through cross-linking of LFA-1 (14.7μm per
Figure 3.3: A time course of PBTL migration in response to CD44 cross-linking using D2.1.
PBTLs cells (activated for 72 hours using 25ng/ml PMA) were incubated with immobilised D2.1. The above panels demonstrate cellular changes that take place following CD44 cross-linking. The arrow indicates the direction of migration of the cell *, which migrates approximately 50μm during the 60 minute observation period.
Figure 3.4: Active PBTL migration following ligation of CD44 or LFA-1.
The average distance travelled by PBTLs activated by ligation of CD44 or LFA-1 over 1 hour interval measured using time-lapsed video-microscopy. Cell locomotion was triggered via CD44 pathway using immobilised D2.1 (CD44) and by LFA-1 cross-linking with immobilised SPV-L7 (LFA-1). Bars reflect mean values +/- SEM obtained in three independent studies (dotted, grey and hatched bars, respectively). Each study represents the distance travelled by the cell centroid of 40 – 50 cells over 1 hour observation interval.
hour, mean value of peak velocity – 76.65μm per hour) (Figure 3.5), a slow and controlled locomotion.

3.3.5 The response of HUT-78 and PBTL to the physiological CD44 ligand, hyaluronan

The principle ligand for CD44 is the glycosaminoglycan, hyaluronan. Two forms of hyaluronan were tested in order to determine whether T cells responses to a native ligand of CD44 were similar to those seen following receptor activation using an immobilised cross-linking antibody (Figure 3.6 and Figure 3.7). Cellular responses to hyaluronan differ depending on the molecular weight of the glycosaminoglycan (Termeer and Sleeman, 2003). In the examination of lymphocyte responses to hyaluronan two different forms of the polymer were used; high molecular weight hyaluronan, peak molecular weight, 200 kDa (Sigma) and intermediate molecular weight hyaluronan, 50 kDa (ICN pharmaceutical) also described here as hyaluronan fragments. 96 well plates were coated with 2mg/ml hyaluronan in carbonate buffer, pH 9.3 overnight at 4°C. Plates were washed twice with PBS and blocked with 0.25% BSA, 0.05% Tween20, PBS for 1 hour at room temperature.

HUT-78 cells were either pre-treated for 30 minutes with or without PMA (25ng/ml) before incubation with hyaluronan-coated plates. Cells (both untreated and PMA pre-treated) plated on immobilised anti-CD44 (D2.1) developed a migratory phenotype with a polarised morphology (Figure 3.7). When HUT-78 cells were placed on the hyaluronan-coated plates there was no development of a polarised phenotype. The response was similar whether high molecular weight and intermediate weight hyaluronan was used. Additionally pre-treatment with PMA did not alter the cellular responses when placed on hyaluronan.

PBTLs were also tested to determine their response to hyaluronan. As before PBTLs were isolated from the blood of a normal donor. These cells were activated by incubation with 25ng/ml PMA at 37°C for 72 hours. After this activated PBTLs characteristically formed large clumps of cells. PBTLs were placed directly onto the D2.1 or hyaluronan-coated (high and intermediate molecular weight) plates. As can be seen in Figure 3.7 (a) PBTLs become polarised and develop a migratory phenotype in response to cross-linking of CD44 using D2.1. However this was not the case when PBTLs were placed in the hyaluronan-coated plates (Figure 3.7 (c) and (g)). When incubated with high molecular weight hyaluronan, cells did not adhere well, and over
Figure 3.5: Active HUT-78 migration following ligation of CD44 or LFA-1.

The average distance travelled by HUT-78 cells activated by ligation of CD44 or LFA-1 over 1 hour interval measured using time-lapsed videomicroscopy. Cell locomotion was triggered via the CD44 pathway using immobilised D2.1 (CD44) and by LFA-1 cross-linking with immobilised SPV-L7 (LFA-1). Bars reflect mean values +/- SEM obtained in 3 independent studies (dotted, grey and hatched bars, respectively). Each study represents the distance travelled by the cell centroid of 40 – 50 cells over 1 hour observation interval.
Figure 3.6: Phenotypic changes in HUT-78 T lymphoma cells following incubation with the physiological CD44 ligand, Hyaluronan.

HUT-78 cells were incubated either with immobilised D2.1, high molecular weight HA (High MW HA, Sigma) or intermediate molecular weight HA (Intermediate MW HA, ICN Pharmaceutical), directly or pretreated with PMA (pretreatment 30 minutes using 25ng/ml PMA) prior to incubation with the respective substrate.
Figure 3.7: PBTLs responses to hyaluronan.
PBTLs cells (activated for 72 hours using 25ng/ml PMA) were incubated with immobilised D2.1, a and b, high molecular weight hyaluronan (Sigma), c, d, e and f or intermediate molecular weight fragments of hyaluronan (ICN pharmaceuticals), g, h and i. Cells were either placed onto substrate directly a, c and g or pre-incubated with D2.1, e and h, J173, f and i, or intermediate molecular weight HA fragments, b and d. Control cells were incubated in uncoated tissue culture wells, panel j.
the 3 hours observation the cells had begun to clump again (Figure 3.7 (c)). Observed cells did not appear to actively migrate. In the case of intermediate molecular weight hyaluronan (fragments) there was a similar response. Cells did not adhere well and over time cellular aggregates began to develop again (Figure 3.7 (g)). Cells were also incubated in an uncoated well, as a control for hyaluronan (Figure 3.7 (j)). Few cells were found to adhere to this substrate.

An additional experiment examined whether preincubation with either HA fragments (50μg/ml), D2.1 or the monoclonal antibody, J173 would influence PBTLs response to immobilised hyaluronan. This experiment was carried to test whether either the hyaluronan fragments or the monoclonal antibodies in solution would influence the conformation of CD44 on the cell surface, and allow the development of migratory phenotype when in contact with immobilised hyaluronan. PBTLs pre-treated with hyaluronan fragments before placing on immobilised D2.1 still developed a migratory phenotype, although the trailing cytoplasmic tail did not develop to the same extent as that seen in cells placed directly on D2.1 (Figure 3.7 (b)). Also PBTLs pre-treated with either hyaluronan fragments, D2.1 or J173 did not develop a migratory phenotype when incubated with immobilised hyaluronan (Figure 3.7).

Also as part of this experiment an estimate of PBTL adhesion to hyaluronan was carried out. The primary purpose of this experiment had been to investigate whether PBTLs developed a migratory phenotype when incubated with immobilised hyaluronan. There was little evidence of migration under these conditions, although there was some evidence of adhesion. The results of this single adhesion experiment are presented here, but as this was not a primary objective of this study this was not repeated. After PBTL incubation for 3 hours on a hyaluronan-coated plate, as described above, the wells were washed with PBS once, twice or three times. The number of single cells remaining adherent were then counted and averaged from 5 randomly selected views in duplicate wells. As Figure 3.8 illustrates a large number of cells were loosely adherent to the high molecular weight hyaluronan, but after a second wash many of these weakly adherent cells were removed. Cells incubated with hyaluronan fragments were less sensitive to the wash steps. Pre-treatment of PBTLs with hyaluronan fragments or with J173 greatly reduced cell adhesion to high molecular weight hyaluronan, whereas D2.1 did not seem to alter PBTL adhesion when compared to the untreated cells. This data is supported by experiments to determine the blocking effect of various CD44 specific monoclonal antibodies on binding of CD44-Ig chimera to immobilised hyaluronan that were carried out by Dr. D. G. Jackson, University of
Figure 3.8: PBTL binding to hyaluronan substrate.

PBTLs cells (activated for 72 hours using 25ng/ml PMA) were incubated for 3 hours with immobilised high molecular weight hyaluronan (Sigma) or intermediate molecular weight fragments of hyaluronan (ICN Pharmaceuticals) (as indicated). Cells were either added onto substrate directly or pre-incubated with D2.1, J173, or intermediate molecular weight HA fragments. Cells were also incubated in uncoated tissue culture wells as control. Cells were washed with PBS (see legend) and counts of the single cells that remained adherent are illustrated here.
Oxford. D2.1 was found to slightly enhance hyaluronan binding of the chimera in a manner similar to that of the CD44 antibody, F10-44-2, which has previously been described (Galandrini et al., 1994). When J173 pre-treated PBTLs were placed on hyaluronan fragments cell adhesion was almost entirely lost. D2.1 pre-treatment inhibited PBTL adhesion to hyaluronan but not to the same extent as J173. This experiment was only carried out once, but it does indicate that there are some functional differences between the CD44 monoclonal antibodies, D2.1 and J173, even though as immobilised cross-linking antibodies they produce similar morphological changes.

3.3.6 The examination of signalling pathways involved with the development of a motile phenotype in T cells following CD44 ligation

In the investigation of the signal-transduction pathways involved with development of a phenotype associated with CD44-induced migration in T cells, i.e. an elongated, polarised morphology, a number of selective inhibitors of signalling proteins were tested using a range of effective concentrations for cell culture conditions. To provide information and a numerical measure of cell morphology a formula was used to score cell shape, the deformation index (D.I.) (see Section 2.6.3). This formula combines the elongation index and the cell’s circularity index and provides a powerful measure of the degree of deformation of a cell, thus providing considerable information about the cell shape in terms of polarisation and elongation. Figure 3.9 shows an example of typical values for the D.I. of lymphocytes. Typically values of 1-3 are assigned to cells that were non-migratory i.e. cell shape nearing circular (for example, cell no. 2). Those cells that display a polarized phenotype (cell body and trailing uropod) and therefore a higher degree of deformation had higher D.I. values (>5) (cell no. 1, 3 and 4). The D.I. was used as a tool to assess the impact of selective inhibitors on HUT-78 cell polarisation in response to activation of either CD44 or LFA-1. On average > 40 cells were scored for each set of conditions. The significance of differences observed in the various assays was evaluated by the Mann-Whitney test, an analysis of non-paired, non-parametric measurements. The availability of a tool such as the deformation index provided a quantitative descriptor in the examination of cell morphology following receptor ligation.

Previous data from our group had indicated roles for the PKC isoforms, β and δ, in the development of a motile phenotype in LFA-1 activated T lymphocytes (Volkov et al.,
Figure 3.9: Sample data of deformation index values. The deformation index values for a variety of cell shapes. HUT-78 cells were activated by ligation of CD44. The CD44-activated cells were fixed using PFA. The image taken using 40x lens on a Nikon Diaphot TE300 inverted microscope and analysis was performed using the National Institute of Health Image program.
and we were interested in testing the role of these enzymes in the morphological changes that take place following CD44 activation. There is substantial evidence to implicate the lipid kinase PI 3-kinase in lymphocyte migration. PI3-kinase has been found to play an important role in T lymphocyte polarisation and chemotaxis. Loss of PI3-kinase activity abrogated PBL polarisation and migration induced by the chemokine, stromal cell-derived factor-1α (SDF-1α) (Vicente-Manzanares et al., 1999). Also IL-2-induced formation of lamellipodia in T lymphocytes is blocked by wortmannin and Ly294,002, two inhibitors of PI3-kinase (Arrieumerlou et al., 1998). This provided a basis for the investigation of the role of PI3-kinase in CD44-induced lymphocyte migration. The role of the Map kinase, p38 was examined using the selective inhibitor, SB203,580 (IC₅₀ = 600nM) (Figure 3.10). This MAP kinase has been described as being stimulated in neutrophils in response to the chemoattractants, fMLP and C5a, with PI3-kinase being required for the chemotactic response of neutrophils to IL-8 and LTB₄ (Heit et al., 2002). HUT-78 cells were pre-treated with this inhibitor for 30 minutes at 37°C before activation in anti-CD44 coated plates. As can be seen in Figure 3.10 there is no significant alteration in the morphology acquired by the SB203,580 pre-treated cells. No further work was carried out in investigating the role of this protein kinase in T cell migration.

3.3.7 The impact of Go6976 on changes in T cell morphology induced following activation through CD44

Go6976 is a selective pharmacological inhibitor of classical PKC isozymes (α, βI, βII and γ) (IC₅₀ = 7.9nM). Pre-treatment of HUT-78 cells with Go6976 (30 minutes at 37°C) prior to incubation with immobilised anti-CD44 or anti-LFA-1 monoclonal antibodies allowed the role of classical PKC isoforms in T cell migration to be examined (see Section 2.6.1). As can be seen in Figure 3.11 control HUT-78 (incubated with DMSO) develop a polarised morphology when CD44 is cross-linked. There was a loss of this polarisation in a dose dependent manner (1, 5, 10μM) when cells are pre-treated with Go6976 before ligation of CD44. Cells adhered but no longer develop extended cytoplasmic projections. A similar dose dependent loss of polarisation was seen in Go6976 pre-treated cells when LFA-1 was cross-linked. Using the deformation index (see Section 2.6.3) to quantify the impact of Go6976 on cell morphology demonstrated that there was a significant decrease in cell deformation in a dose dependent manner (Figure 3.12). HUT-78 responses were similar following either CD44 or LFA-1 activation.
Figure 3.10: HUT-78 migration-associated morphology in response to CD44 ligation following inhibition of p38 MAP kinase.

Typical polarised morphology of HUT-78 cells following ligation of CD44 using D.21 (DMSO control). Cells pre-treated with SB203580 (selective inhibitor of p38 MAP kinase) at the indicated concentration prior to ligation of CD44 did not develop significant changes in morphology with respect to control during the 3 hour observation.
Figure 3.11: HUT-78 migration-associated morphology in response to CD44 ligation following inhibition of classical PKC isoforms.

Typical polarized morphology of HUT-78 cells following ligation of CD44 using D2.1 or LFA-1 using SPV-L7 (DMSO control). Cells pre-treated with Go6976 (selective inhibitor of classical PKC isoforms) at the indicated concentration lost this migration-associated morphology in a dose-dependent manner during 3 hour observation. Cells pre-treated with 10μM Go6976 display a rounded morphology when incubated with immobilised anti-CD44 or anti-LFA-1.
Figure 3.12: Dose dependent loss of HUT-78 migration-associated morphology following inhibition of classical PKC isozymes. Mean deformation index values for HUT-78 cells activated by ligation of CD44, using D2.1 or LFA-1, using SPV-L7. Cells were either pretreated with DMSO (NT) or the indicated Go6976 concentration. Images were acquired of 5 randomly selected fields of view, with the deformation index determined for >40 cells. Statistics were carried out using Mann-Whitney non-parametric analysis of independent samples (* indicated p<0.05). Representative result of n=3 is shown here.
3.3.8 The impact of Rottlerin on changes in T cell morphology induced following activation through CD44

Rottlerin is a selective inhibitor of the novel PKC isoform, δ (IC₅₀ = 3-6 μM). HUT-78 cells were pre-treated with Rottlerin for 30 minutes at 37°C prior to incubation with immobilised anti-CD44 or anti-LFA-1 monoclonal antibodies (see Section 2.6.1). This allowed the role of PKCδ in T lymphocyte morphology to be assessed. Control HUT-78 cells incubated in the inhibitor carrier, DMSO, typically developed a polarised morphology following CD44 cross-linking (Figure 3.13). HUT-78 cells were pre-treated with Rottlerin, at concentrations of 1, 5 and 10μM, for 30 minutes before CD44 ligation. On morphological examination cell adhesion was not influenced by suppression of PKCδ activity. Cells appeared flattened, and frequently produced multiple thin cytoplasmic projections. Although there was significant cytoskeletal rearrangement, HUT-78 cells did not acquire the typical elongated polarised morphology seen in migrating T cells. Using the deformation index to quantify CD44-activated T cell responses (see Section 2.6.3) following Rottlerin pre-treatment did not show significant alteration in morphology (Figure 3.14). The dose response curve was a representative result of n=3 with no clear or significant pattern apparent in these experiments. LFA-1-activated cells responded to Rottlerin pre-treatment in a similar manner.

3.3.9 The impact of peptide inhibitors of PKC isoforms on changes in T cell morphology induced following activation through LFA-1

The limitations of pharmacological inhibitors include the relatively low specificity of available antagonists for PKC versus other kinases, and the difficulty in finding antagonists to discriminate among multiple PKC isozymes (Davies et al., 2000). Following stimulation, protein kinase C isozymes associate with the insoluble cell fraction, and in addition to binding to lipids, activated PKC isozymes bind to specific anchoring proteins termed RACKs (receptors for activated C-kinase). Stimulation-induced translocation of a PKC isozyme to its appropriate target is required for its function. Peptides were developed that compete for binding of activated PKC isozymes to their RACKs and act as isozyme-selective inhibitors of PKC translocation and function (Gray et al., 1997; Johnson et al., 1996; Ron and Mochly-Rosen, 1994). Selective peptide inhibitors of the PKC isoforms β and δ and a control for the carrier peptide were obtained from Prof. D. Mochly-Rosen, Stanford, U.S.A. HUT-78 cells were loaded with PKC isozyme selective peptide antagonists using pinocytosis (see Section 2.8) and followed by seeding onto immobilised anti-LFA-1 (SPV-L7) coated
Figure 3.13: HUT-78 migration-associated morphology in response to CD44 ligation following inhibition of PKCδ.

Typical polarised morphology of HUT-78 cells following ligation of CD44, using D2.1 or LFA-1, using SPV-L7 (DMSO control). Cells were pre-treated with Rottlerin (selective inhibitor of PKCδ) at the indicated concentration prior to activation using immobilised antibodies to CD44 or LFA-1 and observed over the 3 hour incubation.
Figure 3.14: Dose response of HUT-78 migration-associated morphology following inhibition of PKCδ.

Mean deformation index values for HUT-78 cells activated by ligation of CD44, using D2.1 or LFA-1, using SPV-L7. Cells were either pre-treated with DMSO (NT) or the indicated concentration of Rottlerin (selective PKCδ inhibitor). Images were acquired of 5 randomly selected fields of view, with the deformation index determined for >40 cells. Statistics were carried out using Mann-Whitney non-parametric analysis of independent samples (* indicated p<0.05). Representative result of n=3 is shown here.
plates (see Section 2.6.1). Following 3 hours incubation at 37°C, there was no significant difference between the different treatments and treated cells had a similar appearance to untreated LFA-1-activated cells (Figure 3.15). HUT-78 cells acquired a polarised morphology, associated with lymphocyte migration.

(a) Comparision of non-treated HUT-78 with peptide inhibitor treatment (LFA-1 activation)

<table>
<thead>
<tr>
<th>Treatment</th>
<th>N</th>
<th>Mean</th>
<th>S.E.</th>
<th>N</th>
<th>Mean</th>
<th>S.E.</th>
<th>z</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Beta Inhibitor</td>
<td>90</td>
<td>6.751</td>
<td>0.7283</td>
<td>80</td>
<td>5.3326</td>
<td>0.449</td>
<td>-1.801</td>
<td>0.072</td>
</tr>
<tr>
<td>Delta Inhibitor</td>
<td>90</td>
<td>6.751</td>
<td>0.7283</td>
<td>90</td>
<td>7.7957</td>
<td>0.7692</td>
<td>-0.795</td>
<td>0.426</td>
</tr>
<tr>
<td>Carrier Peptide</td>
<td>85</td>
<td>6.751</td>
<td>0.7283</td>
<td>85</td>
<td>5.5869</td>
<td>0.6241</td>
<td>-2.357</td>
<td>0.018</td>
</tr>
</tbody>
</table>

(b) Comparision of carrier peptide treated HUT-78 with peptide inhibitor treatment (LFA-1 activation)

<table>
<thead>
<tr>
<th>Treatment</th>
<th>N</th>
<th>Mean</th>
<th>S.E.</th>
<th>N</th>
<th>Mean</th>
<th>S.E.</th>
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<tbody>
<tr>
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<td>85</td>
<td>5.5869</td>
<td>0.6241</td>
<td>80</td>
<td>5.3326</td>
<td>0.449</td>
<td>-0.357</td>
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</tr>
<tr>
<td>Delta Inhibitor</td>
<td>85</td>
<td>5.5869</td>
<td>0.6241</td>
<td>90</td>
<td>7.7957</td>
<td>0.7692</td>
<td>-2.87</td>
<td>0.004</td>
</tr>
</tbody>
</table>

Table 3.1: Deformation index data for LFA-1-activated HUT-78 cells following treatment with peptide inhibitors of PKC isozymes

Deformation index values provided information about the influence of the carrier peptide on the cellular response (see Section 2.6.3), i.e. there was a significant decrease in the deformation of carrier peptide treated cells with respect to untreated cells when incubated with immobilised anti-LFA-1 (Table 3.1 (a)). Visually there were fewer cells adherent and the carrier peptide may have had a somewhat toxic effect. The comparison of deformation index values of the PKCδ peptide inhibitor treated cells with the control carrier peptide treatment indicated a significant increase in cell deformation in HUT-78 cells in response to activation of LFA-1 (Table 3.1 (b)). These were the results of a single experiment and therefore inconclusive. Further work is needed to ensure that the peptide has been introduced to a large percentage of the cell population or that the cells that have acquired the peptide inhibitor can be specifically tracked. Previous methods to introduce these peptides have been microinjection (Ron and Mochly-Rosen, 1994), or transient permeabilisation using saponin (Gray et al., 1997; Ron et al., 1995) and a more stringent method of introducing the peptide inhibitors to the cells may be required.
Figure 3.15: HUT-78 migration-associated morphology in response to LFA-1 ligation following treatment with PKC peptide inhibitors. Typical polarised morphology of HUT-78 cells following ligation of LFA-1, using SPV-L7. Cells pre-treated with control carrier peptide, PKC beta peptide inhibitor or PKC delta peptide inhibitor developed a similar polarised morphology when activated using immobilised anti-LFA-1 cells.
3.3.10 The impact of Ly294,002 and Wortmannin on changes in T cell morphology induced following activation through CD44

In the investigation of the role of PI 3-kinase in CD44 and LFA-1 induced T cell migration a number of tools were available. The pharmacological inhibitor, Ly294,002 is a selective inhibitor of PI3-kinase inhibitor (IC$_{50}$ = 1.4μM), acting as a competitive inhibitor for the ATP binding site of PI3-kinase (Vlahos et al., 1994). Also available is Wortmannin, an inhibitor that binds covalently and irreversibly to the active site catalytic subunit of PI 3-kinase, preventing production of the D3 phosphorylated lipid product of the enzyme. Initial work was carried out using Ly294,002 and as before HUT-78 cells were pre-treated with the inhibitor at the indicated concentration for 30 minutes before incubation on the immobilised antibody substrate, either anti-CD44 (D2.1) or anti-LFA-1 (SPV-L7) (see Section 2.6.1). As can be seen in Figure 3.16 HUT-78 cells pre-treated with DMSO, the control for the inhibitor carrier developed a polarised, elongated morphology when incubated with anti-CD44. As the concentration of Ly294,002 was increased there was a gradual lose of this motility-associated morphology, becoming strikingly apparent at 25 and 50μM Ly294,002 (concentrations described as effective in cell-based assays). At these concentrations the cells were no longer polarised, were adherent, flattened and had a ruffled appearance to their cell edges. A similar response was seen when Ly294,002 pre-treated HUT-78 cells were incubated with anti-LFA-1. Pre-treatment with the DMSO control did not alter HUT-78 ability to acquire a polarised morphology when LFA-1 was ligated, but with increasing Ly294,002 concentrations there was a loss of this phenotype (Figure 3.17) with the acquisition of a flattened, ruffled appearance. This sensitivity of HUT-78 to Ly294,002 pre-treatment was quantified using deformation index values (see Section 2.6.3) and as can be seen in Figure 3.18 there is a dose dependent decrease in deformation with increasing inhibitor pre-treatment.

Wortmannin is an alternative PI 3-kinase inhibitor and evidence indicates that it has a more specific activity than Ly294,002 (Davies et al., 2000). The HUT-78 response to 100nM wortmannin pre-treatment was tested, a concentration that inhibits PI 3-kinase in cell-based assays (Davies et al., 2000). HUT-78 cells were pre-treated with 50μM Ly294,002, 100nM wortmannin or the DMSO control for inhibitor carrier for 30 minutes at 37°C before incubation with immobilised monoclonal antibodies to CD44 or LFA-1. Examination of cell morphology revealed an interesting finding (Figure 3.19). DMSO pre-treated cells developed a polarised morphology in response to CD44 cross-linking. This was lost when HUT-78 were pre-treated with Ly294,002, but wortmannin pre-
Figure 3.16: HUT-78 migration-associated morphology in response to CD44 ligation following inhibition of PI3-Kinase.

Typical polarised morphology of HUT-78 cells following ligation of CD44, using D2.1 (DMSO control). Cells pre-treated with Ly294,002 (selective inhibitor of PI3-Kinase) at the indicated concentration lost this migration-associated morphology in a dose dependent manner during 3 hour observation. Cells pre-treated with 50 μM Ly294,002 display a rounded morphology with an irregular edge when incubated with immobilised anti-CD44.
Figure 3.17: HUT-78 migration-associated morphology in response to LFA-1 ligation following inhibition of PI3-Kinase.

Typical polarised morphology of HUT-78 cells following ligation of LFA-1, using SPV-L7 (DMSO control). Cells pre-treated with Ly294,002 (selective inhibitor of PI3-Kinase) at the indicated concentration lost this migration-associated morphology in a dose dependent manner during 3 hour observation. Cells pre-treated with 50μM Ly294,002 display a rounded morphology with an irregular edge when incubated with immobilised anti-LFA-1.
Figure 3.18: Dose dependent loss of HUT-78 migration-associated morphology following inhibition of PI3-Kinase.

Mean deformation index values for HUT-78 cells activated by ligation of CD44, using D2.1 or LFA-1, using SPV-L7. Cells were either pre-treated with DMSO (NT) or the indicated Ly294,002 concentration. Images were acquired of 5 randomly selected fields of view, with the deformation index determined for >40 cells. Statistics were carried out using Mann-Whitney non-parametric analysis of independent samples (* indicated p<0.05). Representitative result of n=3 is shown here.
Figure 3.19: HUT-78 migration-associated morphology in response to CD44 or LFA-1 ligation following inhibition of PI3-Kinase using the selective pharmacological inhibitors, Ly294,002 or Wortmannin.

Typical polarised morphology of HUT-78 cells following ligation of CD44, using D2.1 or LFA-1, using SPV-L7 (DMSO control). Cells pre-treated with 50μM Ly294,002 display a rounded morphology when incubated with immobilised anti-CD44 or anti-LFA-1. When cells were pre-treated with Wortmannin LFA-1 activated HUT-78 had a rounded morphology, whereas Wortmannin did not inhibit the development of a migration-associated polarised morphology in CD44 activated HUT-78.
treatment did not inhibit the development of a polarised phenotype. LFA-1 activated HUT-78 responded in a similar manner when pre-treated with Ly294,002 but pre-treatment with wortmannin also resulted in a loss of motility-associated phenotype (Figure 3.19). These morphological responses were reflected in the deformation index values and as can be seen in Figure 3.20 Ly294,002 pre-treatment results in a significant decrease in the deformation of both CD44 and LFA-1 activated HUT-78. LFA-1-induced polarisation of HUT-78 cells is sensitive to wortmannin pre-treatment but morphology changes induced by CD44 ligation is not sensitive to wortmannin.

A differential response to Ly294,002 and wortmannin has been found in other cell systems. Wortmannin was found to inhibit the enhanced association of PI3-kinase with PKCδ in an erythroleukemia cell line, TF-1, following GM-CSF stimulation but Ly294,002 did not, highlighting the differences in the mechanism of action of these inhibitors (Ettinger et al., 1996). One reason for the sensitivity of LFA-1 induced polarisation to wortmannin and insensitivity of CD44 induced responses, may be related to the strength of the CD44 signal. Inhibition of Erk activation by wortmannin has been found to not only vary between cell lines, but also within a single cell type (Swiss 3T3 cells) depending on the kind and concentration of growth factor used. It was postulated that low concentrations of receptor activation required a PI3-kinase dependent pathway but with high numbers of receptors activated this enzyme becomes less important with the activation of a second redundant pathway (Duckworth and Cantley, 1997). This prompted the examination of HUT-78 cell responses to a range of immobilised anti-CD44 concentrations, to determine if high levels of CD44 ligation were activating a second redundant signalling pathway that was wortmannin insensitive. HUT-78 cells were pre-treated with 50μM Ly294,002 or 100nM wortmannin prior to incubation on immobilised anti-CD44 (D2.1) using the range of concentration described in Figure 3.21. Over the range of D2.1 concentrations used there was a small decrease in the deformation index of the non-treated HUT-78 cells, but there was no change in the HUT-78 response to either Ly294,002 or wortmannin inhibition. Cells remained sensitive to Ly294,002 pre-treatment but induction of a HUT-78 migration-associated morphology following activation of CD44 over a range of immobilised D2.1 concentrations was insensitive to wortmannin pre-treatment.

3.3.11 The impact of Go6976 on HUT-78 cell migration
As described before time-lapsed video microscopy was used to determine the distance travelled by HUT-78 cells during one hour recording. This allowed the influence of
Anti-CD44 activated HUT-78, pre-treated with Ly294,002 or Wortmannin

Anti-LFA-1 activated HUT-78, pre-treated with Ly294,002 or Wortmannin

Figure 3.20: HUT-78 migration-associated morphology following inhibition of PI3-Kinase, using Ly294,002 and Wortmannin. Mean deformation index values for HUT-78 cells activated by ligation of CD44, using D2.1 or LFA-1, using SPV-L7. Cells were either pre-treated with 50µM Ly294,002 (Ly) or 100nM Wortmannin (Wort). Images were acquired of 5 randomly selected fields of view, with the deformation index determined for >40 cells. Statistics were carried out using Mann-Whitney non-parametric analysis of independent samples (* indicated p<0.05). The results from 3 separate experiments are displayed here.
Figure 3.21: HUT-78 migration-associated morphology in response to CD44 ligation, using a range of immobilised antibody concentrations, following inhibition of PI3-Kinase using Ly294,002 or Wortmannin.

Mean deformation index values for HUT-78 cells activated by ligation of CD44 using a range of immobilised D2.1 concentrations (as indicated). Cells were pre-treated with DMSO (NT), Ly294002 or Wortmannin. Images were acquired of 5 randomly selected fields of view, with the deformation index determined for >40 cells. Statistics were carried out using Mann-Whitney non-parametric analysis of independent samples (* indicates p<0.05). Results of single experiment shown here.
Go6976 on T cell migration to be assessed. Cells were pre-treated with 10μM Go6976 or DMSO as control for 30 minutes at 37°C and then plated at density of 2 x 10^4 on immobilised anti-CD44 or anti-LFA-1 (see Section 2.6.1). Approximately 30 minutes after seeding cells onto immobilised antibody recording of a single field of cells was begun (see Section 2.6.2). At 20x magnification the responses of >40 cells could be monitored. In all cases there was a significant decrease in the ability of HUT-78 cells to migrate in response to CD44 ligation when pre-treated with Go6976 (Figure 3.22), with a decrease from a mean velocity of 10.67μm per hour under control conditions to 4.65μm per hour when cells where pre-treated with Go6976. A similar result was seen in LFA-1 activated HUT-78 cells, where untreated cells migrate at an average velocity of 12.42μm per hour and Go6976 treated cells migrate at approximately 4.46μm per hour. Taken together this implicates classical PKC isoforms in the cytoskeletal reorganisation and migration that occurs following CD44 cross-linking. Similarly these isoenzymes were demonstrated to play an important role in LFA-1 induced migration.

3.3.12 The impact of Rottlerin on HUT-78 cell migration

A similar approach was taken to examine the impact of suppression of PKCδ on T cell migration. Using time-lapsed video-microscopy CD44 induced cell motility could be monitored in the presence of Rottlerin or DMSO control (see Section 2.6.2). Cells were pre-treated with 5μM Go6976 or DMSO as control for 30 minutes at 37°C and then plated at density of 2 x 10^4 on immobilised anti-CD44 or anti-LFA-1 (see Section 2.6.1). Approximately 30 minutes after seeding cells onto immobilised antibody recording of a single field of cells was begun. At 20x magnification the responses of >40 cells could be monitored. As described in Figure 3.23 a decrease in cell velocity was seen. There was a decrease from a mean velocity of CD44-activated cells from 14.50μm per hour under control conditions to 11.56μm per hour (p = 0.0478) when cells where pre-treated with Rottlerin. A similar result was seen in LFA-1 activated HUT-78 cells, where untreated cells migrate at an average velocity of 20.38μm per hour and Rottlerin treated cells migrate at approximately 13.94μm per hour (p = 0.0086). The precise role played by PKCδ in cell migration is unclear and an alternative approach may be required to define the importance of this enzyme in CD44 induced T cell migration.
Figure 3.22: HUT-78 migration in response to CD44 ligation following inhibition of classical PKC isozymes.

The average distance travelled by HUT-78 cells activated by ligation of CD44, having been pre-treated with 10μM Go6976 or DMSO control, over 1 hour interval measured using time-lapsed video-microscopy. Cell locomotion was triggered via CD44 pathway using immobilised D2.1 and by LFA-1 cross-linking with immobilised SPV-L7. Bars reflect mean values +/- SEM obtained in three independent studies (dotted, grey and hatched bars, respectively). Also shown are typical values for HUT-78 cells under same conditions activated through LFA-1 using SPV-L7. Statistics were carried out using Mann-Whitney non-parametric analysis of 2 independent samples (a, b, c, d, e, f indicates p<0.05). Each study represents the distance travelled by the cell centroid of 40 – 50 cells over 1 hour observation interval.
T lymphocyte migration during 1 hour analysis using time-lapsed video microscopy

![Graph showing T lymphocyte migration](image)

Figure 3.23: HUT-78 migration in response to CD44 ligation following inhibition of PKCδ.

The average distance travelled by HUT-78 cells activated by ligation of CD44, having been pre-treated with 5μM Rottlerin or DMSO control, over 1 hour interval measured using time-lapsed video-microscopy. Cell locomotion was triggered via CD44 pathway using immobilised D2.1 and by LFA-1 cross-linking with immobilised SPV-L7. Bars reflect mean values +/- SEM obtained in three independent studies. Statistics were carried out on the grouped data using a paired t test (*, p = 0.0478 and **, p = 0.0086). Each study represents the distance travelled by the cell centroid of 40 – 50 cells over 1 hour observation interval.
3.3.13 Examination of K4 migration and induced morphological changes following CD44 ligation

The use of a HUT-78 clone deficient in PKCβ, K4, allowed closer examination of the role of this enzyme in T cell migration. Cells were incubated on immobilised D2.1 and any morphological changes were monitored using time-lapsed video-microscopy (see Section 2.6.1). As can be seen in Figure 3.24, K4 cells adhered to the immobilised anti-CD44 substrate but did not develop the elongated, polarised morphology evident in HUT-78 cells following CD44 cross-linking. When compared with HUT-78 cells during 1-hour incubation with immobilised D2.1, cells had clearly developed a migratory phenotype with a defined leading cell body and an elongated trailing cytoplasmic projection. When time-lapsed video-microscopy was used to examine cell motility, there was little active migration evident in these cells (see Section 2.6.2). The mean velocity of K4 cells following CD44 cross-linking was 6.38 µm per hour (mean of three experiments) (see Figure 3.25) whereas anti-CD44 activated HUT-78 had an average velocity of 14.43 µm per hour. Cell movement was primarily due to rolling and non-specific migration, unlike that seen in the case of anti-CD44 activated HUT-78 cells where cell movement was deliberate and required the coordinate activities of the cell. This provides further evidence for the critical role of PKCβ in CD44 induced T cell migration.

3.5 DISCUSSION

A functional link has been described between CD44 expression and cell migration in a number of different environments. The first reports of CD44 function described its role in mediating lymphocyte adhesion to high endothelial venules (Jalkanen et al., 1986). The retardation of metastatic growth following the administration of anti-CD44 monoclonal antibodies was one of the first indications of a role for CD44 in metastasis formation. An early report described retardation of metastasis growth by immunisation of mice with a monoclonal antibody directed against a new variant of CD44, although the functional significance of this receptor in tumour growth was unknown (Reber et al., 1990). Melanoma cells variants that were high-expressers of CD44 were more motile than low-expressers in a wound-healing assay. They also adhered better to a hyaluronate substrate and when injected into athymic nude BALB/c mice there were significantly more lung nodules, although there was no obvious difference between the
Figure 3.24: Phenotypic changes in K4 cells induced by CD44 cross-linking.
K4 cells adhered but did not develop a polarised morphology as seen with HUT-78 cells following cross-linking of CD44 using the monoclonal antibody D2.1. Panel (a) describes K4 cells 5 minutes after incubation with D2.1, panel (b) shows the same field after 60 minutes. Panel (c) describes HUT-78 cells 5 minutes after incubation with D2.1, panel (d) shows the same field after 60 minutes.
Figure 3.25: K4 migration in response to CD44 activation.
The average distance travelled by K4 cells (PKCβ deficient) activated by ligation of CD44 over 1 hour interval measured using time-lapsed video-microscopy. Also shown are typical values for HUT-78 (expressing PKCβ) activated by CD44 ligation. Cell locomotion was triggered via CD44 pathway using immobilised D2.1. Bars reflect mean values +/- SEM obtained in three independent studies (dotted, grey and hatched bars, respectively). Each study represents the distance travelled by the cell centroid of 40 – 50 cells over 1 hour observation interval.
two variants in the growth rate of the primary subcutaneous tumours (Birch et al., 1991).

The transfection of tumour cells with CD44 constructs has been described as conferring a metastatic phenotype to non-migratory cells. These include melanoma cells transfected with CD44s which adhere and migrate on hyaluronate, whereas transfection with CD44E does not confer cells with this phenotype (Thomas et al., 1992). Similarly increased tumourigenicity and metastatic potential is seen with human B lymphoma cells transfected with CD44s but not CD44E when transferred to nude mice (Sy et al., 1991). Transfection of melanoma cells with CD44s cytoplasmic domain deletion mutants results in a loss of hyaluronate dependent motility but these cells still have the ability to adhere (Thomas et al., 1992).

Here the T lymphocyte response to CD44 activation was investigated using a panel of cross-linking antibodies directed against this adhesion receptor. D2.1 has been characterised by McGrath et al (In preparation) and is thought to recognise an epitope in the hyaluronan binding region of the CD44 protein. The antibody, D2.1 was selected on the basis of its ability to induce cytoskeletal rearrangement in HUT-78 (Kelleher et al., 1994). Additional CD44 antibodies tested include, F10-44-2, which has been reported to up-regulate HA binding ability of activated T cells and J173, which was found to inhibit HA adhesion in these activated cells (Galandrini et al., 1994). Interestingly D2.1 has been found to enhance CD44 binding of hyaluronan in a manner similar to that of F10-44-2 (McGrath, In preparation). L3D1 has been described as inducing a migratory-associated phenotype in PBTLs (Kelleher et al., 1995).

Each of these immobilised anti-CD44 antibodies induced a polarised, elongated, migration-associated morphology in PMA-activated PBTLs (Figure 3.1). This morphology was similar to that seen following ligation of LFA-1 in these activated PBTLs. PMA, an analogue of diacylglycerol, acts to up-regulate PKC activity. This in turn begins a signalling cascade resulting in the activation of T lymphocytes in a manner similar to those that take place following CD3 stimulation (Manger et al., 1987). Indeed, T cells pre-exposed to the tuberculin antigen (purified protein derivative), also developed a locomotory phenotype following CD44 cross-linking in the antigen-responsive cells (Fanning et al., 2005). A similar polarised, elongated morphology was induced in HUT-78 cells following activation of the CD44 using this panel of immobilised antibodies (Figure 3.2). Consideration should also be given to possible cellular responses that occur as a result of Fc ligation. Fc receptors are expressed on
the surface of most effector cells of the immune system including B lymphocytes, however they are absent from T lymphocytes (Ravetch and Bolland, 2001). These receptors ligate the Fc portion of an IgG, triggering transmembrane signalling such as PKC activation (Dasgupta et al., 2000), or modulating the immune response (Boruchov et al., 2005). As T lymphocytes, which constitute >90% of the cell population studied here, do not express an Fc receptor, the role of Fc ligation is not likely to be significant. Any possible role for Fc ligation could be investigated by assessing cellular responses to immobilised F(ab')2 fragments (Jakus et al., 2004).

Using time-lapsed video-microscopy, active migration was demonstrated in the case of PMA-activated PBTLs and HUT-78 cells (Figure 3.4 and 3.5) following ligation of CD44, using immobilised D2.1. This rate of migration (0.2μm/min for PBTLs and 0.24μm/min for HUT-78) following CD44 ligation was similar to that seen following LFA-1 ligation (0.25μm/min for PBTLs and 0.245μm/min for HUT-78). These CD44-activated lymphocytes are migrating with slow deliberate action similar to that seen subsequent to leukocyte arrest, firm adhesion and activation. As the lymphocyte travels across the surface of the substrate, the cells polarise with the F-actin accumulating and pushing forward at the leading edge and formation of a trailing cytoplasmic tail. This tail is retracted in order to allow for the forward movement of the cell. This highlights the importance of the formation of adhesion interactions in the direction of migration with the disassembly of these interactions at the rear of the cell. This velocity was comparable to previous measured velocities when activated T cells are triggered via LFA-1 using immobilised recombinant ICAM-1-Fc protein, where it was proposed that these moderate cell velocities correspond to the physiological rates of recruitment of T cells into inflammatory sites. High affinity ligand-receptor interactions are likely to be important for successful site-specific T cell extravasation in situations of high flow (Volkov et al., 2001). These velocities were also comparable with that measured for human fibroblasts (0.3-0.2μm/min) (Lepekhin et al., 2002).

Here we were interested in examining the role of CD44 in lymphocyte migration. CD44-HA interaction has been implicated in the primary adhesion required for extravasation at inflammatory sites, for example, antigen stimulation of T lymphocytes results in the up-regulation of activated CD44 expression on the surface of these cells and an increase in the number of cells rolling on HA, under physiological shear stress (DeGrendele et al., 1997). The role of CD44 in lymphocyte homing to sites of inflammation was clearly demonstrated by Camp et al, who showed that antibody-
induced loss of CD44 slowed the onset of a cutaneous delayed-type hypersensitivity response following antigen challenge in a murine system, although migration to lymphoid sites was normal. The CD44-negative, unactivated lymphocytes migrate identically to cells from control mice, but following challenge the response is altered, with lymphocyte extravasation to inflammatory sites delayed (Camp et al., 1993). Similarly a study using murine CD44− T cells confirmed that receptor expression was not required for normal lymph node homing but that the migration of CD44-deficient cells into areas of inflammation was delayed (Stoop et al., 2002).

The principal ligand for CD44 is hyaluronan (HA), a broadly distributed glycosaminoglycan, whose expression on endothelial cells is inducible by proinflammatory cytokines and mediates CD44-dependent rolling during inflammation (Estess et al., 1999; Mohamadzadeh et al., 1998). Although not all ligands are identified, CD44 is known to interact with the extracellular matrix components, collagen, laminin and fibronectin, as well as osteopontin and serglycin (Borland et al., 1998). We did not observe a clear polarization response of T cells to purified HA immobilized on a planar substrate (Figure 3.6 and 3.7). Both high molecular weight (peak molecular weight 200kDa) and intermediate molecular weight hyaluronan were used in the examination of lymphocyte responses. No morphological changes were observed when HUT-78 cells were incubated with these forms of hyaluronan, some adherence occurred but no polarisation was apparent. When PMA-activated PBTLs were incubated with these two different types of hyaluronan there were few morphological changes. A small number of cells became polarised but the majority of cells did not form strong adhesions with the substrate.

PBTLs were pre-incubated with CD44 antibodies in order to test whether they could increase receptor binding to hyaluronan. There is evidence that certain antibodies can enhance CD44 binding to hyaluronan, for example F10-44-2 (Galandrini et al., 1994) and D2.1 (McGrath, In preparation), whereas others can inhibit CD44 binding to hyaluronan, such as J173, (Galandrini et al., 1994). This had little impact on cell morphology and counting the number of single cells that remain following a number of wash steps tested cell adhesion. The greatest adhesion was seen to high molecular weight hyaluronan. This adhesion was greatly reduced when cells were pre-incubated with HA fragments or with J173, but not as greatly as with D2.1. This was an interesting observation but as this was not the primary focus of the study this experiment was not repeated.
HA is abundantly expressed in many tissues and was formally thought to simply act as a filling material for the extracellular matrix, although it is now known to play a role in the regulation of cell motility, invasion and proliferation (Tammi et al., 2002). Although HA is the principle ligand for CD44, the failure of HA to elicit the same response in T cells as cross-linking antibodies may be due to a number of reasons. It may be because another CD44 ligand is involved in triggering this response or because under physiological conditions HA might be presented to the lymphocyte CD44 receptor in a more complex spatially organized form, for example, already immobilized by a counter-receptor on the endothelial cell surface (Nandi et al., 2000). Recently, it has been demonstrated that there was significantly enhanced T cell migration into an extracellular matrix gel enriched with high-molecular weight hyaluronan, when compared to the gel alone or the gel enriched with hyaluronan fragments (Fanning et al., 2005).

More recent data has examined the impact receptor shedding may have on the cells ability to migrate. Cell migration requires the continuous breakage and formation of attachments between cell adhesion receptors and the surrounding extracellular matrix. Findings describe the association of a matrix metalloproteinase with CD44, which may direct cleavage of the extracellular domain of CD44, thus mediating the release of cells and allowing migration (Kajita et al., 2001; Seiki, 2003; Sugahara et al., 2003). As a result of its presence at the leading edge of the migrating cells, this cleavage of the extracellular domain of CD44 may be crucial in allowing cell migration.

However, it is interesting to note that antibody cross-linking of CD44 can result in cleavage of this adhesion receptor. In mouse fibroblast cells CD44 ligation using immobilised antibodies results in the shedding of the extracellular domain of the receptor but this response is not seen when exposed to HA coated surface. It was hypothesized that this difference in response is due to a difference in affinity for CD44 (i.e. high affinity antibody versus lower affinity HA) (Shi et al., 2001). Also, shedding of CD44 could be induced in lymphocytes by antibody treatment (Camp et al., 1993). CD44 cleavage is reported to play a critical role in CD44-mediated tumour cell migration that occurs through a dynamic interaction between CD44 and extracellular matrix, a phenomenon that may be mediated by the direct association of CD44 with matrix metalloproteinases (Kajita et al., 2001). Organized ECM–cell interaction is essential for cell migration. It appears critical to regulate detachment and attachment in an organized manner in order to accomplish cell migration.
Interestingly, a recent study has described a novel activity for HA fragments. HA fragments (between 6-36mers) can induce CD44 cleavage, not seen with smaller fragments or high molecular weight HA and promote the migration of tumour cells (Sugahara et al., 2003). This has developed the notion of a role for CD44 cleavage in mediating CD44-mediated tumour cell migration. Many cellular responses to HA are dependent on the size of the glycosaminoglycan. High molecular weight HA, present under non-inflammatory conditions, has little signaling capability. In areas of inflammation, there is increased accumulation of HA fragments which, influence the response of cells of the immune system (Termeer and Sleeman, 2003). This may offer an alternative explanation for why no migration response was seen when we used intermediate and high molecular weight HA to activate T cells. This leaves a question of how would these cells respond to smaller HA fragments and whether cleavage of CD44 is important in the migration of T cells to inflammatory sites?

The migrating lymphocyte develops a polarised, elongated morphology and this was used to investigate some of the signalling pathways that are up-regulated by CD44 cross-linking. The availability of selective kinase inhibitors provided a means of examining the role of a number of proteins in the acquisition of a motile phenotype. The deformation index was used as a quantitative index for the cell shape. The evidence provided here demonstrates that there is good correlation between increased deformation index and the ability for cells to migrate. Go6976 was used as a selective inhibitor of classical PKC isozymes. Pre-treatment with Go6976 resulted in a dose dependent loss in the motility-associated phenotype in both CD44- and LFA-1-activated HUT-78 (Figure 3.11 and 3.12). This correlated well with the loss in CD44- and LFA-induced migration in 10μM Go6976 pre-treated HUT-78 cells (Figure 3.22).

The critical role for PKCβ was further demonstrated by observations in CD44-activated K4 cells. K4 is a HUT-78 clone that is deficient in the PKCβ isozyme (Kelleher and Long, 1992). Following CD44 ligation K4 cells adhered to the immobilised antibody substrate but the cells did not polarise nor did they produce any cytoplasmic projections (Figure 3.24). When time-lapsed video-microscopy was used to examine the ability of K4 cells to migrate following CD44-ligation, this was reduced with respect to CD44-induced migration in HUT-78 cells. Likewise it has been shown that K4 cells do not migrate in response to LFA-1 ligation. Transfection of these cells with a construct for PKCβ returns the migratory phenotype to these LFA-1-activated cells (Volkov et al., 2001). It would be interesting to test whether reconstitution of PKCβ
expression to K4 cells would return the ability of these to migrate in response to CD44 activation.

The role of PKCδ was investigated using the selective inhibitor, Rottlerin. A lot of criticism has been directed towards this pharmacological inhibitor. This includes data indicating that even at 20µM concentrations, Rottlerin had no effect on PKCδ activity but inhibited other protein kinases, for example MAPKAP-K2, at concentrations lower than this (Davies et al., 2000). Rottlerin has also been demonstrated to act as a mitochondrial uncoupler, resulting in reduced levels of ATP in parotid acinar cells (Soltoff, 2001) or sensitising colon carcinoma cells to TNF-related apoptosis-inducing ligand (TRAIL)-induced apoptosis (Tillman et al., 2003). This all suggests that caution should be used in drawing any conclusions from the use of Rottlerin as a PKCδ-specific inhibitor. In this study, there was no dose dependent loss of CD44-induced migratory phenotype. Rottlerin pre-treated HUT-78 cells produced many cytoplasmic projections in response to CD44-activation, which was unlike the typical single cytoplasmic extension in untreated cells (Figure 3.13 and 3.14). HUT-78 cells pre-treated with 5µM Rottlerin had a reduced ability to migrate following CD44-ligation. Similarly Rottlerin pre-treated HUT-78 cells had a decreased rate of migration when activated by LFA-1 ligation (Figure 3.23), although this was not always significant. Some recent work, using the PKCδ-specific peptide inhibitor, demonstrated that there was reduced migration of the treated T cells into an extracellular matrix incorporating high molecular weight hyaluronan when compared to either the control peptide or the peptide blocking the PKCe isoform (Fanning et al., 2005).

This data indicates that there may be a role for PKCδ in both CD44- and LFA-1-induced T lymphocyte migration but an alternative method of investigation is required. PKCδ isotype specific peptide inhibitors offer a useful tool for investigating the role of this enzyme in T lymphocyte migration (Johnson et al., 1996), as does the possibility of HUT-78 transfection with dominant negative PKCδ construct (Jain et al., 1999). This requires an efficient means of delivering the peptide/plasmid across the plasma membrane.

There is strong evidence for the role of PI3-kinase in T lymphocyte migration and chemotaxis. IL-2 induces membrane ruffling in T cells in a PI3-kinase dependent manner (Arrieumerlou et al., 1998). Lymphocyte polarisation and chemotaxis in response to SDF-1α results in PI3-kinase activation and loss of this enzyme activity.
results in inhibited polarisation (Vicente-Manzanares et al., 1999). Also the importance of PI3-kinase is highlighted by the translocation of PKB to the leading edge of migrating neutrophils. PKB binds with high affinity to the 3'-phophorylated products of PI3-kinase and this translocation is inhibited by a specific PI3-kinase inhibitor, Ly294,002 (Servant et al., 2000). Indeed PI3-kinase and its lipid products appear to be a common method of amplifying the cellular response to a chemoattractant, including leukocyte chemotaxis and Dictyostelium amoebas migration towards nutrient sources (Comer and Parent, 2002; Rickert et al., 2000).

Does PI3-kinase plays a role in the development of the migration-associated morphology in HUT-78 cells in response to CD44 or LFA-1 ligation? The selective pharmacological inhibitors of PI3-kinase, Ly294,002 and wortmannin were used to examine the role of this enzyme. Pre-treatment with Ly294,002 resulted in a dose dependent loss of migration-associated morphology in response to both CD44 and LFA-1 ligation (Figure 3.16, 3.17 and 3.18). This implicated PI3-kinase in the migratory response of HUT-78 following activation of either receptor. Inhibition of PI3-kinase, using Ly294,002 resulted in the loss of motility-associated morphology in both CD44- and LFA-1-activated T lymphocytes. When HUT-78 cells were pre-treated with wortmannin there was a differential response to ligation of these receptors. CD44-induced morphological changes were not inhibited by 100nM wortmannin, whereas LFA-1-induced polarisation was inhibited by wortmannin (Figure 3.19 and 3.20).

A differential response to these inhibitors has been described in other cell systems and may reflect differences in the mechanisms of action of these agents. The specificity of Wortmannin in the inhibition of PI3-kinase was found to be greater than that of Ly294,002. Ly294,002 was found to inhibit CK2 with a similar potency to PI3-kinase (Davies et al., 2000). Also GM-CSF activation of an erythroleukemic cell line results in the enhanced association of PKCδ with PI3-kinase. This association was inhibited by wortmannin, which covalently binds to the catalytic domain of PI3-kinase. Ly294,002 did not inhibit the association of these proteins, which is a competitive inhibitor (Ettinger et al., 1996). This may help to explain the differences in cellular responses. Perhaps Ly294,002 is non-specifically inhibiting another enzyme that may be critical for lymphocyte migration. The more PI3-kinase specific wortmannin may be demonstrating the importance of this enzyme in LFA-1-induced HUT-78 migration.

Another possible explanation for the differential response of CD44- and LFA-1-activated HUT-78 cells to wortmannin pre-treatment is related to the strength of the
receptor signal. With a large number of receptors activated a redundant pathway may
be stimulated in order to signal downstream of PI3-kinase. The possibility of a
redundant, non-PI3-kinase dependent pathway, that was up-regulated in response
to a large number of growth factor receptor activation was investigated by Duckworth and
Cantley. Wortmannin inhibited the PDGF activation of Erk MAP kinase in fibroblast
cells at lower PGDF concentrations but not at higher concentrations (Duckworth and
Cantley, 1997). The possibility that the strength of CD44 activation was upregulating a
PI3-kinase independent signalling pathway was investigated by diluting the
concentration of immobilised D2.1 used to activate HUT-78 cells. Over the range of
concentrations used there were few changes. The migration-associated morphology in
response to CD44-igation was still insensitive to wortmannin pre-treatment even at the
lowest D2.1 concentration used (Figure 3.21).

This study demonstrates the induction of active migration in T lymphocytes in response
to CD44-igation. The polarised, elongated morphology and the rate of migration that
these cells acquire is similar to that acquired by LFA-1-activated cells. The classical
PKC isozymes, in particular PKCβ, are critically required for this CD44-induced T cell
migration. Inhibition of classical PKC isozyme activity results in the loss of the
migration-associated morphology following CD44 activation, as well as the cell’s ability
to migrate.

3.6 SUMMARY OF FINDINGS

1. Cross-linking of CD44 using a panel of antibodies led to the development of a
polarised, elongated motility-associated morphology in T lymphocytes.

2. In response to CD44 activation T lymphocytes actively migrated in a slow, and
deliberate manner.

3. T lymphocytes did not produce the same response when incubated with
hyaluronan immobilised on a planar structure. Recent findings have
demonstrated that hyaluronan in a matrix gel induced HUT-78 migration and
polarisation.

4. Inhibition of classical PKC isozymes resulted in a loss of the typical morphology
associated with the activation of CD44 on T lymphocytes and a loss in ability to
migrate. Further investigation, using K4 cells (a HUT-78 clone deficient in PKCβ) demonstrated the importance of the classical PKCβ isozyme in CD44-induced T lymphocyte migration.

5. Inhibition of PKCδ did not result in a loss of motility-associated morphology following activation of CD44 but there was a reduction in the ability of cells to migrate.
CHAPTER 4

ANALYSIS OF INTRACELLULAR SIGNALLING EVENTS DURING CD44-INDUCED MIGRATION

_in part published:_

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4.1 INTRODUCTION

Of primary interest in this study was the investigation of the downstream signalling events that occur following CD44 activation. Chapter 3 described the central role played by PKC isozymes and PI 3-kinase in mediating the morphological changes in response to CD44 (and LFA-1) ligation. The use of selective pharmacological inhibitors provided an insight into the role of these enzymes in the development of a polarised morphology and the motile phenotype. However this did not provide any information about intracellular signalling events that occur following receptor ligation. This prompted the further investigation, in this chapter, of intracellular changes that are taking place within the cell.

Previous data has described the association of the PKC isoforms, β and δ, with the microtubule cytoskeleton and the MTOC in the motile T cell following LFA-1 ligation (Volkov et al., 1998), which led to the question of where PKC isoforms were located in the CD44-activated T cell. A role for PKC in mediating various CD44 activities has been reported by a number of groups. For example, the binding of CD44 to its native ligand hyaluronan is altered following PMA treatment, an activator of classical and novel PKCs (Liao et al., 1993). Additionally there are sites within the cytoplasmic domain that suggest a role for CD44 as a substrate for PKC phosphorylation (serine 323 and 325). Evidence has demonstrated that phosphorylation of Ser325 is required for CD44-dependent migration (Peck and Isacke, 1998). More recently it has been shown that the complex regulation of phosphorylation and dephosphorylation of the cytosolic domain of CD44 by PKC activation controls directional migration, a process that allows the association and dissociation of the CD44-ezrin complex (Legg et al., 2002). In the migrating T cell, PKCβ I (identified here as PKCβ) has been shown to be crucially important for LFA-1-mediated locomotion (Volkov et al., 2001). Therefore the role of PKC isoforms, within the cell, in mediating the migratory response of T cells following CD44 ligation was investigated.

The products of PI3-kinase govern many cellular events, such as cell growth, cytoskeletal remodelling and the trafficking of intracellular organelles (Koyasu, 2003). Distinct members of the PI3-kinase family are activated in the immune system according to the type of cell and/or receptor. Cytokines activate class Ia PI3-kinases in many immune cells, including T cells and dendritic cells, through the action of Janus kinase and tyrosine phosphorylation of various proteins (Koyasu, 2003). Phosphoinositides are phosphorylated on the 3-position of the inositol ring by PI3-
kinases in response to extracellular stimuli. Phosphoinositides are ubiquitous in the cellular membranes of many types of cell and tissue and are important second messengers for intracellular signalling in several processes. Lipid products of PI3-kinase provide an anchor for assembling signalling proteins at specific location in the membrane in response to cell stimulation. These signalling proteins coordinate complex events leading to changes in cell metabolism, cell growth, cell movement and cell survival (Cantley, 2002). Various signalling proteins have domains that specifically bind D-3 phosphorylated phosphoinositides. These proteins are located in the cytosol of unstimulated cells but in response to lipid phosphorylation accumulate at the plasma membrane because of their ability to associate with the newly formed phosphoinositides. At the membrane these proteins become activated and initiate various local responses, including polymerisation of actin, assembly of signalling complexes and priming of protein kinase cascades.

Signalling proteins with pleckstrin-homology (PH) domains accumulate at sites of PI3-kinase activation by directly binding to PtdIns(3,4,5)P₃. These include the protein serine-threonine kinases Akt (also known as protein kinase B (PKB)) and phosphoinositide-dependent kinase 1 (PDK1). D-3 phosphorylated phosphoinositides bind to the PH domain of Akt, and recruit this enzyme to the plasma membrane. At the membrane PKB is phosphorylated on Thr308 in the kinase activation loop and on Ser473 in a hydrophobic region of the C-terminus. Phosphorylation of Thr308 is carried out by phosphoinositide-dependent protein kinase 1 (PDK1) (Stephens et al., 1998). This kinase was also found to be important in the regulation of many PKC superfamily members (Williams et al, 2000) and phosphorylates key residues in the activation loop of PKC isoforms (Le Good et al., 1998).

PI3-kinase has also been shown to have a role in the detection of the direction and intensity of an extracellular chemical gradient and the directed migration toward the source of the chemical (chemotaxis). These mechanisms are sensitive enough to detect gradients that differ by as little as 2% between the front and the back of the cell. This external gradient of chemoattractant is translated into a steep intracellular signalling gradient by the rapid and transient translocation of PH domains from the cytosol to the activated plasma membrane, leading to cell polarisation and directional migration. This describes a model of gradient sensing in which localised 3-phosphoinositides production directs the anterior accumulation of the signalling components and machinery required for chemotaxis (Comer and Parent, 2002).
In addition to examining the roles of individual signalling proteins, we were interested in examining the relationship between the adhesion receptors CD44 and LFA-1. Evidence has demonstrated the presence of CD44 in detergent insoluble lipid rafts (Neame et al., 1995; Perschl et al., 1995). These are thought to exist as platforms for the assembly and concentration of signalling molecules at the plasma membrane. They function to separate or concentrate specific membrane proteins or lipids in membrane microdomains. Lipid rafts have been implicated in a number of cellular processes, in particular the signalling of cell adhesion receptors where avidity changes are critical to signal transduction (Harder et al., 1998).

The emerging paradigm is that the compartmentalisation of the proteins is required for signalling events. Additionally the role of anchoring proteins, such as CG-NAP (centrosome and Golgi localized PKN-associated protein) (Takahashi et al., 1999), enables the association of signalling proteins in close proximity to each other. The modular combination of proteins allows the more efficient and specific association of enzymes. The formation of a "signalling complex" has been widely examined in response to integrin-ligand interaction, with the recruitment of cytoskeletal and cytoplasmic proteins, which become anchored to the actin cytoskeleton (focal adhesions). This signalling complex gathers together proteins in response to receptor ligation, which in addition to their role linking the extracellular domain and the actin cytoskeleton, are also important sites of signal transduction (van der Flier and Sonnenberg, 2001).

4.2 OBJECTIVES

The objective of this study was to investigate the intracellular signalling events that take place in the T cell following ligation of CD44. Chapter 3 highlighted the role of PKC isozymes and the lipid kinase PI3-kinase in the migrational response of T cells following CD44 ligation. The role of these signalling proteins was further examined in this study. Also the relationship of CD44 and LFA-1 as the T cell migrates was examined, to investigate if these receptors were associated within a signalling complex.

1. The intracellular localisation of PKC isozymes was examined in HUT-78 cells and any alteration in subcellular localisation was investigated following cross-linking of CD44.
2. The intracellular localisation of PI3-kinase was examined in HUT-78 cells and alterations in subcellular localisation as a result of CD44 cross-linking was assessed.

3. The association of CD44 and LFA-1 in a ligand-activated signalling complex was investigated in the migrating HUT-78 cells.

4.3 RESULTS

4.3.1 PKC translocation events associated with anti-CD44 activation

Observations in Chapter 3 indicated a role for the PKC serine/threonine kinases in signalling following CD44 ligation leading to T cell migration. This led to the question of whether CD44 ligation influenced the localisation of PKC isoforms within the cell. This had been examined in terms of the localisation of PKCβ and δ in the LFA-1 activated T cell and there was an association of these isoforms with the microtubule cytoskeleton. The location of a panel of PKC isoforms in CD44-activated HUT-78 cells was detected using immunofluorescence. The panel of antibodies used where mouse monoclonal anti-PKCα (Upstate Biotechnology), rabbit polyclonal anti-PKCβ1 (Sigma), rabbit polyclonal anti-PKCδ (R&D antibodies), rabbit polyclonal anti-PKCε (R&D antibodies) and rabbit polyclonal anti-PKCζ (R&D antibodies). Other isoforms such as PKCβII (no specific staining) and PKCγ (only present in brain tissue) were not examined.

As can be seen in Figure 4.1, the distribution of these isoforms within the resting HUT-78 and the CD44-activated (motile) HUT-78 differed. The data illustrated here is a typical example of results obtained during microscopy studies. Immunofluorescence staining was performed on three different occasions, with protein localisation examined in a minimum of 50 cells. In the resting cell PKCα had a perinuclear distribution, with some indication of capping at particular areas of the cell. Following CD44-activation using immobilised D2.1 the distribution of this isoform was altered with a concentration at an area posterior to the leading cell body, which may correlate with the area of the MTOC and a particulate distribution at the cell body and along the length of the trailing process. PKCβ in the resting HUT-78 cell was found throughout the cytoplasm with a particulate distribution and it was also associated in a discrete spot at what may be the MTOC. In response to anti-CD44 activation of HUT-78 cells, there was a redistribution of PKCβ to the trailing cytoplasmic projection of the migrating cell (Figure 4.1). The novel PKC isoform, δ, was diffusely associated about an area which may also relate to
Figure 4.1: Localization of PKC isoforms in CD44-activated HUT-78 cells.
Resting (left vertical panel), HUT-78 on poly L-lysine coated slides. CD44 (right panel), migrating HUT-78 activated by CD44 cross-linking, using immobilized D2.1. PKC alpha staining using mouse monoclonal anti-PKC alpha (Upstate Biotechnology), PKC beta staining using rabbit polyclonal anti-PKC beta (SIGMA), PKC delta staining using rabbit polyclonal anti-PKC delta (R&D antibodies), PKC epsilon staining using rabbit polyclonal anti-PKC epsilon (R&D antibodies) and PKC zeta staining using rabbit polyclonal anti-PKC zeta (R&D antibodies).
Figure 4.2: PKC beta localisation in CD44- and LFA-1-activated HUT-78 cells.
Resting (left vertical panel), HUT-78 on poly L-lysine coated slides. CD44 (middle vertical panel), Migrating HUT-78 activated by CD44 cross-linking, using immobilised D2.1. LFA-1 (right panel), Migrating HUT-78 activated by LFA-1 cross-linking, using immobilised SPV-L7. PKC beta (green) staining in resting cells and tubulin (red) staining for the same field. In the CD44-activated HUT-78, PKC beta association with the microtubule rich tail of the migrating cell is seen. A similar association of PKC beta with the microtubule rich tail is seen in LFA-1-activated HUT-78. Images acquired using UltraView Live Cell imager.
Examination of PKCδ localization in resting HUT-78 cells showed a diffuse cytoplasmic staining with an association with the MTOC. In the migrating CD44-activated T cell PKCδ was found localized to a position adjacent to the MTOC (Figure 4.3) and also was present as uninvolved cytoplasmic pool in the cell body. These translocation events were strikingly similar to that seen in the LFA-1 activated HUT-78 (Figure 4.2 & 4.3)

4.3.3 Localisation of PKC isoforms in subcellular fractions of CD44 activated HUT-78

To determine if CD44 ligation altered the subcellular localization of the PKC isoforms, β and δ, as was apparent from the immunofluorescent studies, western blot analysis of HUT-78 cellular fractions was conducted. PKCβ was detected in high amounts in the membrane of resting cells and was also detected in the cytosol. When HUT-78 cells were activated by cross-linking of the CD44 receptor there was a marked association of PKCβ with the cytoskeletal fraction. There was a reduction in the amount of enzyme detected in the membrane fraction (Figure 4.4) and PKCβ appeared present at a similar level in cytosolic fraction in resting and D2.1 activated cells. Examination of PKCδ subcellular distribution revealed that in the resting cell there was an association of PKCδ with the membrane fraction with some enzyme detected in the cytosolic fraction. When HUT-78 cells were activated using D2.1 the enzyme becomes associated with the cytoskeletal fraction of the migrating cells and the level of enzyme detected in the cytosolic and membrane fractions remained similar (Figure 4.4). Of note was the presence of a 165kDa species detected by the anti-PKCδ antibody. This was detected in high levels in the membrane fraction of resting, PMA and CD44-activated HUT-78. Following activation of HUT-78 cells, either by PMA or by CD44 ligation there was an enrichment of this protein in the cytosolic and cytoskeletal fractions. In the resting cell this species was not detected in the cytosolic fraction but following activation was present in the cytosol. Interestingly there was an enrichment of this species in the cytoskeletal fraction of activated HUT-78. To date this species has not been identified but there is a possibility that this may be a dimer of PKCδ. Analysis of the tubulin content of HUT-78 cells showed its enrichment in the cytoskeletal fraction of CD44 activated cells (Figure 4.4). In resting HUT-78 there was a reduced amount of tubulin detected in the cytoskeleton, demonstrating the importance of the microtubule cytoskeleton in the motile cell. This data confirms the association of the PKC isoenzymes with the microtubule cytoskeleton in the migrating cell.
Figure 4.3: PKC delta localisation in CD44- and LFA-1-activated HUT-78 cells.

Resting (left vertical panel), HUT-78 on poly L-lysine coated slides. CD44 (middle vertical panel), migrating HUT-78 activated by CD44 cross-linking, using immobilised D2.1. LFA-1 (right panel), migrating HUT-78 activated by LFA-1 cross-linking, using immobilised SPV-L7. PKC delta (green) staining in HUT-78 cells and tubulin (red) staining for the same field. Merged image, identical microscope field illustrating PKC delta and tubulin co-localisation (yellow/orange overlay). Images acquired using the UltraView Live Cell Imager.
Figure 4.4: Western blot of HUT-78 subcellular fractions. Cytosolic (C), membrane (M) and cytoskeletal (S) cell fractions, as well as total cell extracts (T) were probed for PKC delta (a), PKC betal (b) and tubulin (c) expression. The lanes represent resting HUT-78, cells activated by PMA (10ng/ml) alone for 30 minutes and CD44 activated HUT-78 incubated on immobilized D2.1 for 3 hours.
4.3.4 Impact of Go6976 on PKC translocation

A role for classical PKC isoforms in the morphological changes that occur following CD44 and LFA-1 ligation had been shown using the pharmacological inhibitor Go6976. Go6976 pre-treatment selectively inhibited enzyme activity leading to a loss of polarisation and migration (Chapter 3). The importance of PKCβ in particular was further highlighted by the inability of K4 cells, a PKCβ-deficient HUT-78 clone, to polarise and migrate when CD44 or LFA-1 is activated (Chapter 3). Having determined that loss of PKCβ activity resulted in the loss of HUT-78 polarisation and migration in response to CD44 and LFA-1 ligation, we were interested in determining the impact enzyme inhibition had on the subcellular localisation of PKCβ and δ. As described before microscopy studies were performed up to five different occasions, with typical data illustrated here. As Go6976 had been previously shown to inhibit both CD44- and LFA-1-stimulated morphological changes, it was not surprising to find that cells did not form microtubule rich tails. There was no significant development of a polarized phenotype but the MTOC was located in the position adjacent to the nucleus typical of motile cells. In these CD44-activated cells there was a clear association with PKCβ at the area of the MTOC but not with the microtubules. Rather this isoform remained in a diffuse cytosolic location (Figure 4.5). A similar distribution was seen in HUT-78 cells pre-treated with Go6976 before cross-linking of LFA-1 using immobilised SPV-L7. In Go6976 treated cells CD44 cross-linking did not induce redistribution of PKCδ and it remained concentrated in discrete spots in the region of the MTOC similarly to the resting cells (Figure 4.6). Again Go6976 pre-treatment did not induce significant PKCδ redistribution following LFA-1 ligation and remained localised about the MTOC.

4.3.5 Impact of Rottlerin on PKC translocation

In Chapter 3, the role of the novel PKC isoform δ in the development of a polarised phenotype and motility in HUT-78 cells following ligation of CD44 was investigated. This did not give a clear answer as to the role of this enzyme in signalling cell polarisation or motility. Using the selective PKCδ inhibitor, Rottlerin, it was clear that cells remained able to adhere and extend elongated cytoplasmic projections when activated using a cross-linking antibody to CD44 or LFA-1. Interestingly, these cells frequently extended multiple processes, which may point to a role played by this enzyme in regulating the number and direction of these projections. The impact of Rottlerin pre-treatment on HUT-78 motility following CD44 and LFA-1 ligation was also
Figure 4.5: Localization of the PKC isoform beta in Go6976 pre-treated HUT-78 cells following CD44 or LFA-1 cross-linking.

Resting (left vertical panel), HUT-78 on negatively charged slides. CD44 + Go6976 (middle vertical panel), 10μM Go6976 pre-treated HUT-78 cells incubated with immobilized D2.1. LFA-1 + Go6976 (right panel), 10μM Go6976 pre-treated HUT-78 cells incubated with immobilized SPV-L7. PKC beta (green) staining of HUT-78 cells and tubulin (red) staining of the same field. Arrows indicate the positions of the MTOC.
Figure 4.6: Localization of the PKC isoform delta in Go6976 pre-treated HUT-78 cells following CD44 or LFA-1 cross-linking. Resting (left vertical panel), HUT-78 on negatively charged slides. CD44 + Go6976 (middle vertical panel), 10μM Go6976 pre-treated HUT-78 cells incubated with immobilized D2.1. LFA-1 + Go6976 (right panel), 10μM Go6976 pre-treated HUT-78 cells incubated with immobilized SPV-L7. PKC delta (green) staining of HUT-78 cells and tubulin (red) staining of the same field. Arrows indicate position of MTOC.
investigated and there was a reduction in cell velocity without a complete loss of migration. The question remains of whether loss of PKCδ activity impacted on the localisation of the PKC isoforms, β and δ. HUT-78 cells treated with Rottlerin prior to cross-linking of CD44 or LFA-1 were examined using immunofluorescence to determine the localisation of PKCβ and PKCδ. Immunofluorescent analysis was carried out up to five different occasions, with typical data illustrated here. As can be seen in Figure 4.7 and 4.8 the extended processes are tubulin rich and are supported by well-defined microtubule structures. While PKCβ remained associated about the area of the MTOC, it was not fully associated with the microtubule-rich projections in CD44-activated HUT-78 (Figure 4.7). This distribution of PKCβ was also seen in LFA-1-activated HUT-78, with the enzyme located at area of the MTOC and an incomplete association with the microtubule processes. In the resting cell localisation of PKCδ at the MTOC appears diffuse. As can be seen in Figure 4.8, Rottlerin-treated cells PKCδ displayed a granular pattern and not as tightly associated with the MTOC as commonly seen in the untreated CD44-activated T cell (Figure 4.3). A similar distribution of this enzyme was seen in Rottlerin pre-treated cells following LFA-1 activation.

4.3.6 Localisation of PI3-kinase in HUT-78

The role of PI3-kinase in signalling morphological changes in T cells following CD44 or LFA-1 activation was investigated using the pharmacological inhibitors, Ly294,002 and Wortmannin. The findings in Chapter 3 implicate PI3-kinase in signalling the development of a motile phenotype in both CD44 and LFA-1-activated HUT-78 cells when enzyme activity was suppressed using Ly294,002. When Wortmannin was used to inhibit PI3-kinase activity there was a loss of LFA-1-induced polarisation but there was no alteration in HUT-78 response to CD44 ligation. Immunofluorescence was used to examine the intracellular localisation of PI3-kinase in both resting HUT-78 and CD44- or LFA-1-activated HUT-78. Following optimisation of the PI3-kinase staining, studies were performed on three different occasions and the following data illustrates typical results obtained. Cells were fixed by first treating with 3.7% para-formaldehyde at room temperature for 4 minutes followed by fixation using methanol at −20°C for 5 minutes. This method of fixation gave the clearest visualisation of PI3-kinase.

As can be seen in both Figure 4.9 and 4.10 in the resting HUT-78, PI3-kinase was present in the cytosol in discrete patches and is perhaps located in a vesicle. Following CD44 activation, using immobilised D2.1, there was a development of a polarised phenotype and rearrangement of the microtubule cytoskeleton. PI3-kinase
Figure 4.7: Localisation of the PKC isoform beta in Rottlerin pre-treated HUT-78 cells following CD44 or LFA-1 cross-linking.

Resting (left vertical panel), HUT-78 on negatively charged slides. CD44 + Rottlerin (middle vertical panel), 5μM Rottlerin pre-treated HUT-78 cells incubated with immobilized D2.1. LFA-1 + Rottlerin, 5μM Rottlerin pre-treated HUT-78 cells incubated with immobilized SPV-L7. PKC beta (green) staining of HUT-78 cells and tubulin (red) staining of the same field. Arrows indicate position of the MTOC.
Figure 4.8: Localisation of the PKC isoform delta in Rottlerin pre-treated HUT-78 cells following CD44 or LFA-1 cross-linking.

Resting (left vertical panel), HUT-78 on negatively charged slides. CD44 + Rottlerin (middle vertical panel), 5μM Rottlerin pre-treated HUT-78 cells incubated with immobilized D2.1. LFA-1 + Rottlerin (right panel), 5μM Rottlerin pre-treated HUT-78 cells incubated with immobilized SPV-L7. PKC delta (green) staining of HUT-78 cells and tubulin (red) staining of the same field. Arrows indicate position of MTOC.
Figure 4.9: PI3-Kinase localisation in CD44-activated HUT-78 cells. Resting (top panel), HUT-78 on poly L-lysine coated slides. CD44 (lower panels), migrating HUT-78 activated by CD44 cross-linking using immobilised D2.1. Cells were fixed 10 minutes (middle panel) or 3 hours (bottom panel) after CD44 ligation. PI3-Kinase, p85 (green) staining and tubulin (red) staining for the same field. Merged image, overlay of identical microscope field. Arrows indicate location of PI3-Kinase in migrating cells.
Figure 4.10: PI3-Kinase localisation in LFA-1-activated HUT-78 cells.
Resting (top panel), HUT-78 on poly L-lysine coated slides. LFA-1 (lower panels), migrating HUT-78 activated by LFA-1 cross-linking using immobilised SPV-L7. Cells were fixed 10 minutes (middle panel) or 3 hours (bottom panel) after LFA-1 ligation. PI3-Kinase, p85 (green) staining and tubulin (red) staining for the same field. Merged image, overlay of identical microscope field. Arrows indicate location of PI3-Kinase in migrating cells.
localisation was examined in HUT-78 cells at 10 minutes and 3 hours post-incubation with the immobilised monoclonal antibody, in order to examine early and late events that may take place following receptor activation. Many PI3-kinase signalling events occur at an early time point and are transient. As can be seen in Figure 4.9 there was a similar staining pattern apparent in the migrating cell 10 minutes post-activation, i.e. a small number of discrete patches, however there was some degree of association of these patches with the anterior of the cell. The staining for PI3-kinase in HUT-78 cells following 3 hours incubation with D2.1 was more diffuse and less intense. Similarly in response to LFA-1 activation, HUT-78 cells began to polarise and developed microtubule rich projections. 10 minutes after receptor ligation, PI3-kinase was found in discrete patches within the cytosol, with some degree of association with the leading edge of the cell. At the later time point there is a similar staining pattern seen with a number of discrete spots of PI3-kinase detected in the cell body of the migrating cell (Figure 4.10).

4.3.7 Localisation of CD44 in \textit{in situ} immunoprecipitates of LFA-1 activated HUT-78

This study has demonstrated a striking similarity in the responses of T cells following ligation of both CD44 and LFA-1. Firstly, receptor activation results in the development of a migration-associated morphology. Additionally, receptor ligation results in the activation of some common signalling proteins that are required for migration, for example, PKC\(\beta\). In order to investigate the relationship between CD44 and LFA-1 in signalling migration in T cells a technique called \textit{in situ} immunoprecipitation was used. This provides a method of testing if there was any association between LFA-1 and CD44 in the signalling complex of the migrating cells. \textit{In situ} immunoprecipitation has been developed as a technique to examine the intracellular signalling complex formed following integrin receptor engagement (Volkov et al., 2001). Cells migrate on the immobilised antibody substrate as a result of the specific interaction between the target receptor (antigen) and the cross-linking antibody. Signalling events following the formation of the antibody-antigen complex lead to formation of an intracellular signalling complex. This complex includes components of the cytoskeleton, which are critical for the movement of proteins within the cell, but also key signalling molecules.

Cells were incubated with the immobilised antibody either at 4\(^\circ\)C or at 37\(^\circ\)C for 3 hours before lysis. Incubation of the cells at 4\(^\circ\)C acts to prevent the formation of the signalling complex (tubulin does not polymerise at 4\(^\circ\)C) and acts as a control for cell

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activation. Cells lysates were prepared as described in Section 2.5.2 and following acetone precipitation, the in situ immunoprecipitates were probed for the presence of CD44 by western blot using Bric 238. CD44 was detected in anti-CD44 (D2.1) in situ immunoprecipitates generated from cells at both 4 and 37°C. As can be seen in Figure 4.11 at 4°C there appears to be some constitutive association of CD44 in lysates from anti-LFA-1 (SPV-L7) activated HUT-78. However this association is enhanced in HUT-78 cells incubated with anti-LFA-1 at 37°C, i.e. migrating HUT-78 cells, suggesting that LFA-1 may be associated with CD44 in the migrating HUT-78 cell.

4.4 DISCUSSION

Chapter 3 investigated the physical and morphological changes that take place within the T cell following ligation of the CD44 receptor. In response to CD44 activation, cells adhere, flatten and polarise. Active migration is evident within 10 minutes, a phenomenon very similar to that seen following LFA-1 cross-linking in T cells (Volkov et al., 1998). Pharmacological inhibitors provided a tool to investigate the role of a number of signalling proteins in this CD44-induced T cell migration. This highlighted the importance of the PKC family in the migratory response of T cells. Also a role for the lipid kinase, PI3-kinase was indicated. Chapter 4 has attempted to better define the role played by these kinases, in particular, in the signalling events that take place following CD44 receptor ligation. Evidence is provided here for the redistribution of PKC isoforms and PI3-kinase in response to CD44 ligation, in a manner that bears striking similarity to that seen following LFA-1 cross-linking, indicating that there may be some redundancy in the pathways up-regulated during T lymphocyte migration.

Having established the importance of PKC isoforms in signalling T cell migration in response to cross-linking of CD44, Chapter 4 investigated the role of these serine/threonine kinases in the intracellular changes taking place in the migrating cell. Immunofluorescence staining was used to examine PKC isozyme localisation within the cell and to investigate changes that may occur following CD44 ligation. However, better definition of PKC isoform localisation could be determined using electron microscopy. Chapter 3 demonstrated the critical role of the classical subfamily of PKC (cPKC) isoforms in anti-CD44 induced T cell migration. In particular, data from K4 cells (PKCβ deficient clone of HUT-78) indicates that PKCβ is required for establishment of a polarised, elongated phenotype in response to CD44 cross-linking. The members of the classical PKC subfamily are α, β1, βII and γ and require calcium,
Figure 4.11: *In situ* immunoprecipitate of HUT-78 cells activated with anti-CD44 or anti-LFA-1 tested for the presence of CD44. HUT-78 cells were incubated on anti-CD44 (D2.1) (lanes 2, 3, 7 and 8) or anti-LFA-1 (SPV-L7) (lanes 4, 5, 9 and 10) (referred to as IP) for 2 hours at either 4°C or 37°C. Total HUT-78 cell lysates were loaded into lanes 1 and 6 (T). These were blotted using Bric238 (CD44) or anti-IE (−ive control). Specific bands are indicated by arrows.
diacylglycerol and phosphatidylserine for their function. PKCγ is only found in brain tissue (Wetsel et al., 1992) and was not relevant to this study. Also preliminary examination of the distribution of PKCβII displayed a non-specific distribution of this splice variant of PKCβI and was therefore not investigated further.

PKCα had a perinuclear distribution in the resting cell (Figure 4.1). Although some cells displayed a non-uniform distribution of this kinase, with some evidence of a capped distribution in certain areas. Following the cross-linking of CD44, this isoform redistributed to an area posterior to the leading cell body, which may correlate with the area of the MTOC and a particulate distribution at the cell body and along the length of the trailing process. Previous studies have demonstrated a role for PKCα in carcinoma cell migration. PKCα has been shown to play a role in the mobilization of α6β4 from hemidesmosomes and its redistribution to cell protrusions in A431 squamous carcinoma cells (Rabinovitz et al., 1999). In addition PKCα is associated with β1 integrin and is required for directional tumour cell migration in human breast carcinoma cells (Parsons et al., 2002). The particulate distribution of PKCα following cross-linking of CD44 may relate to enzyme association with integrin receptors, although this was not examined in these cells.

The association of PKCβI with the MTOC and the microtubule cytoskeleton in T cell migrating in response to LFA-1 ligation has been described (Volkov et al., 1998). Therefore, we were interested in investigating the relationship between PKCβI and the microtubule cytoskeleton, in anti-CD44 activated T cells. In resting HUT-78, PKCβI was found to be diffusely stained with evidence of localisation to a granular cytoplasmic pattern and partial association with the microtubule cytoskeleton. Following CD44 cross-linking, the microtubule cytoskeleton becomes reorganized with the MTOC located to the rear of the migrating cell body with a microtubule rich trailing process. There was also a redistribution of PKCβ to the region of the MTOC and along the microtubule based trailing process in the CD44-activated HUT-78 (Figure 4.2). The importance of PKC association with the microtubule cytoskeleton has been highlighted by a number of reports. Evidence in the Aplysia has described the association of the PKC isoforms Apl I (Ca^{2+}-dependent with homology to PKCα, β and γ) and Apl II (Ca^{2+}-independent with homology to PKCe and η) with the microtubule cytoskeleton of the neuronal growth cone. A role for PKC in the regulation of microtubule advance in neuronal growth cones has been suggested (Kabir et al., 2001; Nakhost et al., 2002).
The novel PKC isozymes (δ, ε, η, θ) require diacylglycerol and phosphatidylserine for activation but are calcium independent. In this study to localisation of the isozymes, PKCδ and ε were investigated. PKCε showed a discrete localisation in the resting HUT-78 (Figure 4.1). This novel PKC isozyme was located in spots at a single location within the cell, which may correlate with the MTOC. Following cross-linking of CD44, PKCε was found posterior to the nucleus and the direction of cell migration. This may reflect an association of this PKC isozyme with the centrosomal-anchoring protein, CG-NAP. Immature, hypophosphorylated PKCε has previously been described as anchored to CG-NAP, and may act to facilitate the complete phosphorylation and activation of this enzyme (Takahashi et al., 2000).

PKCδ was found localised to a diffuse cytoplasmic staining about the region of the MTOC in resting HUT-78 cells. In the polarised anti-CD44-activated HUT-78 cell, PKCδ was found localized to a position adjacent to the MTOC (Figure 4.3) and also was present as uninvolved cytoplasmic pool in the cell body. This distribution of PKCδ bears striking similarity to that seen in LFA-1-activated HUT-78 cells (Figure 4.3) (Volkov et al., 1998). γ-Tubulin is part of a larger complex that functions at the MTOC (Pereira and Schiebel, 1997). Co-staining of PKCδ with γ-tubulin would enable clarification of the relationship of this PKC isoform with the MTOC. A role for PKCδ in cell migration has previously been demonstrated. PKCδ levels are increased in highly metastatic mammary tumour cells when compared with less metastatic cell lines. Expression of an inhibitory regulatory domain of PKCδ (RDδ) in highly metastatic cells inhibited anchorage-independent growth and significantly reduced lung metastasis formation (Kiley et al., 1999). Additionally, epidermal growth factor receptor signalling induces fibroblast migration as a result of myosin activation, in a PKCδ-dependent manner (Iwabu et al., 2004).

The atypical PKC subfamily (ζ and λ) does not require diacylglycerol and calcium but still requires phosphatidylserine for activity. Examination of PKCζ localisation in the resting HUT-78 cell showed a thread-like localisation of the isozyme, which was concentrated in one area of the resting cell. CD44 cross-linking resulted in HUT-78 polarisation and redistribution of PKCζ along fibres the length of the trailing cytoplasmic projection. A role for PKCζ as a mediator of cytoskeletal reorganisation is response to regulation by small GTPase proteins has been reported by a number of groups. The atypical PKCλ and PKCζ are required for the Ras-mediated rearrangement of actin cytoskeleton in fibroblasts (Hellbert et al., 2000; Uberall et al., 1999). Also in a
separate study, a wound-healing assay was used to study the signal transduction pathway involved with the establishment of cell polarity. Following the wound, the cells at the leading edge polarise, with the MTOC, the microtubule cytoskeleton and the golgi reorganised to face the area of the damage. PKCζ activity is required, through its action on the microtubule protein dynein for all aspects of induced polarity in these cells (Etienne-Manneville and Hall, 2001). A role for PKCζ as a Ras effector has been proposed based on the interaction of GTP-loaded Ras and PKCζ in vitro (Diaz-Meco et al., 1994). All this suggests a model where Ras.GTP localises PKCζ to the membrane where it is regulated by PtdIns-3,4,5-P3 and PDK-1 (Toker, 1998). There is also evidence to contradict the link between PKCζ and Ras. For example, activation of mitogen-activated protein kinase (MAPK) takes place following the addition of exogenous phosphatidylycholine-specific phospholipase C (PC-PLC) in a PKCζ-dependent but Ras-independent manner (van Dijk et al., 1997).

Further investigation was carried out using western blot to determine subcellular localisation of the PKC isozymes, β and δ. This was carried out to try to determine whether there was an association of these isozymes with the cytoskeleton following HUT-78 cell activation by CD44 cross-linking. As can be seen in Figure 4.4 (b) there was little or no PKCβ detectable in the cytoskeletal fraction of resting HUT-78 cells and was predominantly membrane and cytosol associated. In the anti-CD44-activated HUT-78 there was in marked association of PKCβ with the cytoskeletal fraction. This is in agreement with data reported by Volkov et al., 1998, where there was also an enhanced association of PKCβ with the cytoskeletal fraction of anti-LFA-1 activated HUT-78 cells. Immunofluorescence studies suggested an enhanced association of PKCβ with the microtubule cytoskeleton (Figure 4.2). The data from examination of HUT-78 subcellular fractions also provides evidence for an enhanced cytoskeletal association of PKCβ following CD44 cross-linking. An increase in the level of cytoskeleton-associated tubulin (i.e. microtubule fibres) was also seen in anti-CD44 activated HUT-78. This correlates well with the development of microtubule-rich cytoplasmic projections in the migrating HUT-78.

PKC δ was predominantly associated with the membrane fraction in the resting HUT-78 cell with some enzyme present in the cytosolic fraction. Following cross-linking of CD44, PKC δ became associated with the cytoskeletal fraction of the migrating cells, with similar levels of enzyme detected in the cytosolic and membrane fractions (Figure 4.4). The high molecular weight species (~165kDa) detected by the anti-PKCδ
antibody is worthy of discussion. This was detected in high levels in the membrane fraction of resting, PMA- and CD44-activated HUT-78. Following HUT-78 activation, using PMA or CD44 cross-linking, led to an enrichment of this species in the cytosolic and cytoskeletal fractions. This species has not been identified yet but it will be interesting to determine if this is a dimer of PKCδ or PKCδ associated with another unidentified protein.

In order to examine the functional significance of PKC isoforms in anti-CD44-induced T cell migration the pharmacological inhibitors, Go6976 and Rottlerin were used (Chapter 3). Go6976 acts as a selective inhibitor of classical PKC isoforms and HUT-78 cells pre-treated with Go6976 failed to develop a motile phenotype when CD44 was cross-linked. Loss of classical PKC isoform activity resulted in the loss of HUT-78 polarisation and migration in response to CD44 and LFA-1 ligation. As Figure 4.5 and 4.6 demonstrate, there is no formation of microtubule-rich tails in response to either CD44- or LFA-ligation on the surface of Go6976 pre-treated HUT-78. There was a clear association with PKCβ at the area of the MTOC but not with the microtubules. There was a clear association of PKCβ at the area of the MTOC but not with the microtubules following anti-CD44-activation of Go6976 pre-treated cells (Figure 4.5). In Go6976 treated cells CD44 cross-linking PKCδ remained concentrated in discrete spots in the region of the MTOC similarly to resting cells (Figure 4.6). A similar distribution of these PKC isozymes was seen in anti-LFA-1 activated HUT-78 that had been pre-treated with Go6976.

The critical role of PKCβ association with the microtubule cytoskeleton in LFA-1-induced HUT-78 migration has previously been demonstrated (Volkov et al., 2001). K4 cells, i.e. a PKCβ-deficient HUT-78, do not migrate in response to LFA-1 cross-linking, but when transfected with functional PKCβ the ability for these cells to migrate is restored. Similarly it was demonstrated in Chapter 3, that K4 cells did not migrate in response to anti-CD44 ligation. This all indicates that the association of PKCβ with the microtubule cytoskeleton may play a critical role in anti-CD44-induced T cell migration. This data supports the idea that that microtubule-directed intracellular signaling in T cells may be based on phosphorylation of tubulin or associated proteins by specific PKC isoenzymes (Volkov et al., 2001).

Using Rottlerin as a selective inhibitor of PKCδ allowed the role of this isozyme in HUT-78 migration to be investigated (Chapter 3). This did not give a clear answer as to the
role of this enzyme in signalling cell polarisation or motility. Pre-treatment with Rottlerin did not inhibit HUT-78 ability to adhere and extend elongated cytoplasmic projections when activated by cross-linking CD44 or LFA-1. These cells frequently extended multiple processes, which may point to a role played by this enzyme in regulating the number and direction of these projections. There was a slowing in cell velocity without a complete inhibition of migration. HUT-78 cells pre-treated with Rottlerin prior to receptor cross-linking (either CD44 or LFA-1) extended microtubule-rich cytoplasmic projections (Figure 4.7 and 4.8). Examination of PKCβ and δ localization in Rottlerin pre-treated cells indicated that the association of these enzymes with the microtubule cytoskeleton and the MTOC remained intact following CD44 cross-linking, although PKCβ association with the microtubules of the trailing cell projection was notably reduced. A similar distribution of PKC isozymes was seen in Rottlerin pre-treated cells in response to LFA-1 cross-linking.

The importance of PKC association with the microtubule cytoskeleton has been highlighted by a number of reports. Evidence in the Aplysia describes the association of PKC isoforms (Apl I and Apl II) with the microtubule cytoskeleton of the neuronal growth cone and Nakhost et al have suggested a role for PKC in regulating microtubule advance (Nakhost et al., 2002). In lymphocytes, Volkov et al reported a decrease in the association of PKCβ with the trailing projection preceding contraction of this tail and the start of a new locomotory cycle (Volkov et al., 2001). The incomplete association of PKCβ with the microtubule structure in the cell extensions of the Rottlerin pre-treated anti-CD44 and anti-LFA-1 activated cells suggests a potential role for PKCβ in the retraction of the microtubule cytoskeleton and that these cytoplasmic projections may no longer be involved in the process of cell migration.

It is interesting to note that a similar response to G66976 and Rottlerin pre-treatment evident in both CD44 and LFA-1-activated HUT-78 cells indicating that both receptors may share common signal transduction pathways in the migratory response. Our data demonstrate that cross-linking of CD44 induces phenotypic changes, cytoskeletal rearrangements and redistribution of the PKC isoforms β and δ, resulting in cell migration. The induction of T cell migration and redistribution of PKC isoforms by CD44 was apparently identical to that induced by LFA-1, suggesting potential convergence of intracellular signaling pathways induced via CD44 and LFA-1 integrin. These findings underscore the importance of CD44 in the inflammatory response and may have significant implications for the understanding of the mechanisms underlying cell

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migration in inflammatory processes and might contribute towards the development of novel approaches for its targeted regulation.

In Chapter 3, a role for PI3-kinase was suggested by the loss of a migration-associated phenotype in both anti-CD44 and anti-LFA-1 activated HUT-78 cells as a result of pre-treatment with Ly294,002. Further studies using an alternative PI3-kinase inhibitor, wortmannin, somewhat contradicted this result. There was no loss of anti-CD44-induced HUT-78 polarisation following wortmannin pre-treatment, whereas cells did not polarise in response to LFA-1 cross-linking. Immunofluorescence was used in order to determine if PI3-kinase was involved in signal transduction pathways up-regulated in response to CD44 and/or LFA-1 cross-linking. The detection of PI3-kinase is made somewhat difficult by the lack of good antibodies against the kinase (Stein and Waterfield, 2000). The antibody used here was a rabbit polyclonal against the p85 regulatory subunit (Santa Cruz). Some optimisation of the fixation step was required in order to most clearly visualise the structure detected by this antibody.

In the resting HUT-78, PI3-kinase was present in the cytosol in discrete patches and is perhaps located in a vesicle. Many PI3-kinase events occur rapidly and are transient (within 30 seconds to 20 minutes) (Al-Aoukaty et al., 1999; Servant et al., 2000; Vicente-Manzanares et al., 1999). For this reason the cells were examined at 10 minutes, as well as a later time point (3 hour), the time point used for the analysis of other signalling events. Following CD44 cross-linking (10 minutes), PI3-kinase appeared to remain distributed in a small number of discrete patches, with some degree of association with the leading edge of the polarising cell. PI3-kinase staining in anti-CD44-activated HUT-78 cells was more diffuse and less intense when examined at a later time point (3 hour). There was a similar distribution of PI3-kinase seen in anti-LFA-1-activated HUT-78 cells. Subcellular fractionation by density gradient centrifugation of cell homogenates would enable separation of different organelles. This would help locating PI3-kinase within the cell body and discerning if PI3-kinase is present in a vesicle structure. This study did not provide any conclusive evidence about the role of this lipid kinase in the development of a migration-associated phenotype in HUT-78 cells.

Previously the localisation of the lipid products of PI3-kinase has been used as an indirect method of investigating the role of this enzyme in the cellular response to a chemoattractant. In particular GFP-labelling of the pleckstrin-homology (PH) domain of the protein kinase AKT/PKB (PHAKT-GFP) in HL-60 cells enabled the neutrophil
chemotactic response to be examined. PHAKT-GFP binds selectively to PI3-kinase lipid products and this demonstrated the importance of this enzyme in the establishment of polarisation during chemotaxis (Ricl<ert et al., 2000; Servant et al., 2000). A similar approach could be used here to examine the role of PI3-kinase in the T cell response to cross-linking of CD44 or LFA-1.

4.5 SUMMARY OF FINDINGS

1. Cross-linking of CD44 resulted in specific subcellular localisation of PKC isozymes in HUT-78 cells.

2. The trailing cytoplasmic extension typical of the migration-associated phenotype seen in HUT-78 cells following cross-linking of CD44 results was microtubule-rich.

3. PKCβ in anti-CD44 activated HUT-78 was associated with this trailing microtubule-rich tail and PKCδ was found posterior to leading cell body at the MTOC. CD44 cross-linking resulted in an enhanced association of these PKC isozymes with the cytoskeleton.

4. Inhibition of classical PKC isozymes, using Go6976, resulted in a loss of the migration-associated morphology and the microtubule-rich tail, seen following CD44 cross-linking on HUT-78 cells. The association of PKCβ with the microtubule cytoskeleton typically seen following CD44 ligation was absent in cells pre-treated with Go6976.

5. Inhibition of PKCδ did not significantly alter PKCβ and δ translocation events seen following CD44-ligation, however there was a reduction in the extent of PKCβ association with the microtubule-rich cytoplasmic projections.

6. PI3-kinase was found in discrete patches (vesicles) in resting HUT-78 cells. Following CD44 cross-linking (10 minutes), these structures associated to a small extent with the leading edge of the polarised cell. These structures were less distinct at a later time point.

7. There was a striking degree of similarity in the localisation of PKCβ and δ and PI3-kinase seen in both CD44- and LFA-1-activated HUT-78 cells.
8. *In situ* immunoprecipitates of anti-LFA-1 activated HUT-78 cells suggests that there is some association of CD44 with LFA-1 in the migrating cell.
CHAPTER 5

CHARACTERISATION OF p45
5.1 INTRODUCTION

Earlier chapters have looked at the role of CD44 in signal transduction from the external environment and the changes this induces in the cytosol, in particular, highlighting the importance of this receptor in signalling migration in T lymphocytes. Not only does CD44 act as an adhesion molecule mediating cell-extracellular matrix interactions, it also plays a role in the transduction of cellular responses to the external environment and may alter adhesive properties in response to intracellular signalling events. Here the membrane-associated proteins recognised by the anti-CD44 monoclonal antibody, D2.1, were examined with particular interest in a 45kDa protein, p45. Kelleher et al, 1994, first described this 45 kDa protein during the characterisation of the monoclonal antibody, D2.1. D2.1 detected CD44 in PBLs, as well as in HUT-78 cells. However, an additional protein with a molecular weight of approximately 45kDa was detected in HUT-78 cells but not in PBLs. This protein was also detected in the membrane of intraepithelial lymphocytes (Kelleher et al., 1994). The characterisation of D2.1 as an anti-CD44 monoclonal antibody was completed by Dr. S. McGrath (manuscript in preparation). D2.1 was shown in Chapter 3 to induce migration, accompanied by morphological changes both in PBLs and HUT-78 cells. However, as PBLs only express CD44, but not p45, we can assume that the effects described are due to CD44 ligation and not as a result of p45 ligation. In this chapter the relationship of CD44 to other proteins and their interactions come into focus, and as well as some of the approaches taken to identify the unknown protein, p45.

This review highlights some features of protein interactions with CD44 to help identify potential candidates for p45. The cytoplasmic domain provides an important site of interaction particularly in the linkage of CD44 to the cytoskeleton. CD44 is known to interact with ankyrin thus linking this transmembrane receptor to the actin cytoskeleton (Bourguignon et al., 1987; Kalomiris and Bourguignon, 1988); further evidence indicates that the presence of the ankyrin binding motif in CD44 is required for hyaluronan binding (Lokeshwar et al., 1994). The cytoskeleton membrane linker proteins, ezrin, radixin and moesin (ERM), and the related protein, Merlin, have also been found to bind to the cytoplasmic domain of CD44 (Sainio et al., 1997; Tsukita et al., 1994). Binding CD44 with their N-terminal domains and F-actin with their C-terminal domains, ERM proteins act as linkers between the cell surface and the actin cytoskeleton (Tsukita and Yonemura, 1999).
The transmembrane domain of CD44 provides an additional source of protein-protein interaction. CD44 exists in detergent insoluble fractions (lipid rafts) and the CD44-transmembrane domain has been shown to be important for this property. When the transmembrane domain of CD44 was replaced with the transmembrane domain of either CD3 or CD45, the CD44 mutants were completely soluble in Triton X (Perschil et al., 1995). These lipid rafts serve as a platform for protein association, and post-translational modifications, such as acylation, act to direct proteins to this cholesterol-rich domain (Bourguignon et al., 1991; Melkonian et al., 1999). These lipid domains are required for the association of CD44 with a number of proteins, and down-stream signalling events (Bourguignon et al., 2004; Oliferenko et al., 1999; Singleton and Bourguignon, 2004).

The function of CD44 as a hyaluronan-binding protein is dependent on the presence of the Link Module in its extracellular domain and Link modules have been described in a variety of extracellular matrix molecules including Link Protein (Day and Prestwich, 2002). Link protein is able to enhance and stabilise the binding of proteoglycans to hyaluronan, thus allowing the formation of a stable complex between proteoglycans and hyaluronan. The ubiquitous presence of link protein is suggestive of a general function in the organisation of extracellular matrices of various tissues (Binette et al., 1994). Three link proteins have been identified in human cartilage tissue with a molecular weight of 48, 44 and 41kDa (Roughley et al., 1982). The recognition of p45 by a monoclonal antibody to CD44 suggests a common epitope shared by both proteins, making Link Protein a possible candidate for p45. A similar rationale was used in the investigation of other possible candidate proteins for p45. These included the testing of LYVE-1, a CD44 homologue, which has lymph vessel-specific distribution. This hyaluronan binding protein has a molecular weight of 60kDa (Banerji et al., 1999). The tissue specific distribution of LYVE-1, as well as the presence of a link module in the protein structure, merited further investigation.

The syndecans are a family of highly conserved type I transmembrane heparan sulfate proteoglycans, with functions that include cell adhesion, chemokine and growth factor binding and presentation (Gotte, 2003). Four syndecans have been identified in mammals. Syndecans-1, -2 and -3 are expressed in a cell type-specific manner, whereas syndecan-4 is ubiquitously expressed (Gotte, 2003). Syndecan-4 has been shown to be required for focal adhesion formation in fibroblasts and to modulate the integrin response to extracellular matrix changes (Greene et al., 2003). Focal adhesions bring together proteins that regulate cytoskeletal changes, transduce signals
Syndecan-4 within the focal adhesions has been found to directly interact with cytoskeletal components, and also interacts and activates signalling proteins such as PKCα (Greene et al., 2003; Lim et al., 2003). Syndecan-4 acts as a coreceptor with integrins in focal adhesion formation (Lim et al., 2003).

Syndecan proteins are composed of an ectodomain that contains Ser-Gly consensus sequences for glycosaminoglycan attachment, a highly conserved transmembrane domain and a short highly conserved cytoplasmic domain (Gotte, 2003). Syndecans are released from the cell surface by shedding as a result of cleavage by a family of enzymes known as sheddases or secretases, this releases their ectodomains relatively intact from the cell surface (Park et al., 2000). There is also evidence that PKC activation can accelerate syndecan-1 and syndecan-4 ectodomain shedding (Park et al., 2000). Previous work has suggested that the proposed association of CD44 and p45 is PKC-regulated, yielding the protein identified as p135 (McGrath, In preparation).

Interestingly, a paper by Suzuki et al, 2002, identified two novel cell-associated proteins that interacted with bikunin (bik). These proteins were the 40kDa Link Protein, and a 45kDa specific receptor for bik (bik-R), which is a membrane-associated unidentified molecule. Bik is a secreted glycoprotein that is found in normal human serum and constitutively produced and secreted by hepatocytes. Bik is proposed to act in the inhibition of tumour cell invasion and metastasis possibly through both the direct inhibition of cell-associated plasmin activity and the suppression of urokinase-type plasminogen activator (uPA) expression. Bik binding on the cell surface has been found to result in the inhibition of PKC translocation and activation. This suggests that membrane complexes formed by bik with Link Protein and bik-R initiate the modulation of signal transduction, resulting in bik-mediated suppression of cell invasiveness. Indeed, the hypothesis is that interaction of bik-R with CD44 is important in bik-dependent signal transduction and the suppression of co-stimulatory signals (i.e. uPA over-expression) delivered by CD44 clustering and activation. It is likely that bik-R is a candidate for a functional receptor of bik and may be an accessory receptor for CD44 proteins (Suzuki et al., 2002). The reactivity of p45 against the bik-binding proteins, Link Protein and bik-R was tested, based on the proposed interaction of bik-R, a 45 kDa protein, with CD44.

This candidate protein approach was one of the methods used to identify p45. This was based on the knowledge that p45 (currently unidentified) shares an epitope with...
CD44 (i.e. both protein species were detected by the same monoclonal antibody, D2.1) and also that it shows cell-specific expression. These candidate proteins have been reviewed above and some of the rationale for examining them has been discussed. However in addition to this, work was also carried out to purify p45, in order to allow the protein sequence to be determined. Protein purification occurs by exploiting the individual properties of various molecules, such as molecular weight, charge, glycosylation, subcellular localisation, etc. Details of the methods used to separate p45 from the complex mixture of proteins present in HUT-78 membrane fractions are outlined in Appendix 3. A number of different methods were used here in order to purify p45 to allow its protein sequence to be determined. These included using immunoaffinity purification, separation by molecular weight using the PrepCell and separation by isoelectric point and molecular weight using 2-dimensional electrophoresis.

Edman degradation is a method of protein and peptide sequencing developed by P. Edman in the 1950’s. It identifies proteins amino acid by amino acid from the N-terminal domain. This process developed by P. Edman, in the 1950’s, is based on the cyclic degradation of peptides based on the reaction of phenylisothiocyanate with the free amino group of the N-terminal residue such that amino acids are removed one at a time, allowing them to be identified as their phenylthiohydantoin derivatives. Proteins are electroblotted protein onto an immobilising membrane, such as PVDF. The protein band of interest is excised and analysed using Edman chemistry. There are a number of limitations to this method. One such limitation is that repeating cycles of N-terminal sequencing are limited to about 30 reactions. An additional problem encountered by this type of sequencing is that proteins are often blocked at their N-terminal in the course of their biosynthesis, thus preventing their sequencing by this method. Another source of N-terminal modification is thought to result from trace compounds in buffers or by reaction with free amino groups in incompletely polymerised gels (Gevaert and Vandekerckhove, 2000). Although this is a useful method of protein/peptide identification using their N-terminal sequence, mass spectrometry offers an alternative method of protein identification that overcomes the limitations of this method encountered as a result of N-terminal blockage.

Mass spectrometry (MS) consists of an ion source, a mass analyser, that measures the mass-to-charge ratio (m/z) of the ionised analytes, and a detector, that registers the number of ions at each m/z value (Aebersold and Mann, 2003). There are two main approaches to protein/peptide fingerprinting using MS; matrix-assisted laser desorption
ionisation/time of flight (MALDI-TOF) and electrospray ionisation (ESI) MS. These are primarily distinguished by their method of ionising samples. ESI ionises the analytes from solution and is therefore readily coupled to liquid-based separation. MALDI sublimates and ionises the samples out of a dry, crystalline matrix using laser pulses and is normally used to analyse relatively simple peptide mixtures. ESI, however, ionises the analytes from solution and is readily coupled to liquid-chromatography, making ESI ideally suited for the analysis of complex samples (Aebersold and Mann, 2003). Peptide mass fingerprint analysis is achieved by the cleavage of the protein of interest (either enzymatically or chemically cleavage) and the peptide mixture is analysed by mass spectrometric techniques. The obtained peptide mass fingerprint is compared to "virtual" fingerprints obtained by theoretical cleavage of protein sequences stored in databases and the top-scoring proteins are retrieved as possible candidate proteins (Gevaert and Vandekerckhove, 2000).

5.2 OBJECTIVES

The objective of this study was to identify the 45kDa protein recognised by the monoclonal anti-CD44 antibody, D2.1. This unknown protein, named p45, has previously been described as a membrane-associated protein, with a cell-specific distribution and a scheme to identify this protein took advantage of some of these properties.

1. A number of antibodies against candidate proteins were used to determine whether any cross-reactivity exists.
2. HUT-78 membrane proteins were collected and a number of separation techniques were used in order to purify p45.
3. Fractions enriched for p45 were analysed using a number of protein sequencing methods in order to identify the protein.

5.3 RESULTS

5.3.1 Detection of the novel protein, p45, by the monoclonal antibody, D2.1

The distribution of p45 in HUT-78 and K4 T lymphoma cells was determined by the examination of membrane and cytosol lysates (see Section 2.2.2). Bric238 is a CD44-
specific monoclonal antibody (Anstee et al., 1991). As can be seen in Figure 5.1, CD44s (with a molecular weight of 85-95kDa) was detected in the membrane lysates of HUT-78 and K4 cells. The diffuse band reflects the glycosylated nature of CD44. When these lysates were examined using D2.1, a specific band was seen with a molecular weight of 85-95kDa, corresponding to CD44s. This was only present in the membrane fraction of the cells examined. This protein was characterised and determined to be CD44 (McGrath, In preparation). A second protein was detected by D2.1 with a molecular weight of 45-55kDa. This was detected only in the membrane fraction of HUT-78 and K4 cells. There also appears to be a higher abundance of p45 in K4 cells relative to HUT-78, as Bric 238 detected similar levels of the non-specific 41kDa protein and CD44s in both cell types.

Under certain conditions an additional protein can be detected by D2.1. This protein was more abundant in K4 cells (Figure 5.2) and has an estimated molecular weight of 110kDa. This protein was named p135, reflecting the proposed hypothesis that p135 may be due to an unstable complex of p45 and CD44s. The presence of higher levels of p135 in K4 cells, a HUT-78 clone deficient in PKCβ, may implicate this enzyme in mediating this association. CAPS buffer was used in the transfer of this protein onto PVDF membrane for western analysis. CAPS buffer is more efficient at transferring high molecular weight proteins. As has been demonstrated here, different sources of D2.1 display different affinities for CD44s, p45 and p135. As the primary interest in this chapter was the study of p45, an antibody with high affinity for this protein was selected.

5.3.2 Determination of the isoelectric point of p45

The isoelectric point (pI) of p45 was crudely determined in order to further characterise this protein. Two-dimensional electrophoresis enables the separation of the proteins in the membrane lysate by isoelectric point in the first dimension and by molecular weight in the second dimension (see Section 2.11.1). Using 2-dimensional electrophoresis there was good separation of HUT-78 membrane proteins, which were detected in the gel using Coomassie blue staining (Figure 5.3 (a)). Western blotting using D2.1 detected two sets of proteins, these series of spots corresponded to CD44s and p45 (Figure 5.3 (b)).

An isoelectric focusing gel was run under the same conditions, removed from the glass tubing and cut into 1cm pieces. The pH of these 1cm pieces was determined, using both pH meter electrode and universal indicator paper, and graphed (Figure 5.4). This
Figure 5.1: Western blot of HUT-78 and K4 membrane and cytosol lysates using Bric238 and D2.1.

(a) HUT-78 membrane lysates (50μg) were loaded into lanes 1, 3, 5 and 7. HUT-78 cytosol lysates (50μg) were loaded into lanes 2, 4, 6 and 8. The lanes were blotted using Bric238 (1:100) (lanes 1 and 2), D2.1 supernatant (1:100) (lanes 3 and 4), D2.1 ascities (1:1000) (lanes 5 and 6) and IE (1:100) (lanes 7 and 8). A non-specific (NS) band with molecular weight 41kDa was detected.

(b) K4 membrane lysates (50μg) were loaded into lanes 1, 3, 5 and 7. K4 cytosol lysates (50μg) were loaded into lanes 2, 4, 6 and 8. The lanes were blotted using Bric238 (1:100) (lanes 1 and 2), D2.1 supernatant (1:100) (lanes 3 and 4), D2.1 ascities (1:1000) (lanes 5 and 6) and IE (1:100) (lanes 7 and 8). Non-specific (NS) bands with molecular weight 41 and 73kDa was detected.
Figure 5.2: Western blot of HUT-78 and K4 membrane lysates using panel of D2.1 antibodies, which had been electro-transferred using CAPS buffer, in order to optimise transfer of high molecular weight proteins.

HUT-78 membrane lysates (50μg) were loaded into lanes 1, 3, 5 and 7. K4 membrane lysates (50μg) were loaded into lanes 2, 4, 6 and 8. The samples were separated using 10% SDS PAGE and transferred using CAPS transfer buffer. The lanes were blotted using D2.1 supernatant (1:100), D2.1 ascites (1:1000), Bric238 (1:100) and IE (1:100). Specific bands are indicated by arrows.
Figure 5.3: Analysis of HUT-78 membrane lysates using 2-dimensional electrophoresis. HUT-78 membrane lysates (300μg) were separated in the first dimension by isoelectric focusing (ampholyte range pH 3-10) and in the second dimension by 10% SDS-PAGE.

(a) Separated proteins were visualised using coomassie blue staining.

(b) The transferred gel was blotted using D2.1 (1:100). CD44 and p45 are indicated by arrows.
pH range measured in IEF gel

Figure 5.4: Estimation of pH of isoelectric focusing gel. A IEF gel prepared and run under the same conditions as those used to examine HUT-78 membrane protein. The pH was measured for 1cm pieces of the gel using pH meter. The following pH range was determined for a gel prepared using pH 3-10 ampholytes.
allowed a rough estimation of the pi of p45 to be made (pi 6.16-6.73). This system of isoelectric focusing using carrier ampholytes has a number of problems, which make reproducible results difficult. As the isoelectric focusing gel is prepared on the day using carrier ampholytes; differences in gel preparation, height of the gel and running conditions can have an impact on the pH gradient in the gel. Another problem is that the pH gradients established using carrier ampholytes are unstable and have a tendency to drift, usually toward the cathode over time. Gradient drift can also lead to a flattening of the pH gradient at each end. Therefore, using the resources available it was difficult to make an accurate estimate of the isoelectric point of p45.

5.3.3 Deglycosylation of p45
In order to test whether p45 was glycosylated, as is the case for CD44, N-glycosidase F was used to catalyse the cleavage of N-glycosides. McGrath et al previously determined that O-glycosidase, chondroitinase ABC and neuraminidase had little effect on molecular weight of the proteins detected by D2.1. In order to complete deglycosylation, it is important to try to reduce the tertiary structure by denaturation of the protein structure. A number of conditions were tested to determine the conditions required for p45 deglycosylation. Conditions tested were a) no denaturation, b) boiling for 3 minutes and c) boiling with SDS (10mg/ml) for 3 minutes. In samples that were denatured by boiling with SDS, a further step is required. As SDS inhibits enzyme activity, it must be diluted 1:60 with 1% NP-40, as there is no inhibition in the presence of non-ionic detergents. Deglycosylation was completed as per Section 2.8 and the resulting samples were analysed using western blot. N-glycosidase F treatment led to a reduction in the molecular weight of p45 in all of the conditions tested of approximately 5kDa (Figure 5.5 (a)) and a reduction of approximately 7kDa in the molecular weight of CD44s (Figure 5.5 (b)). As can be seen in Figure 5.5 the deglycosylation of p45 is incomplete when samples have no denaturation step. Deglycosylation goes to almost completion when samples are denatured by either boiling alone or by boiling with SDS before incubation with the deglycosylation enzyme.

5.3.4 Comparison of p45 with link protein
The Link Module is a conserved motif that is required for hyaluronan binding and is present in other hyaluronan binding proteins (Day and Prestwich, 2002), a motif that is also present in CD44 (Goldstein et al., 1989). A series of monoclonal antibodies have been raised against Swarm rat chondrosarcoma Link Protein 2 (9/30/6-A-1 and 9/30/8-A-4). In particular, it was found that 9/30/8-A-4 recognised an epitope present in Link Protein 1, 2 or 3 from several animal species, including human (Caterson et al., 1986).
Figure 5.5: Incubation of HUT-78 membrane fractions with N-glycosidase F under a range of denaturation conditions.
Membrane fractions from HUT-78 cells were aliquoted (50μg protein per vial) and incubated with N-glycosidase F for 18 hours at 37°C (lanes 2, 4 and 6). Prior to incubation with N-glycosidase F samples were either untreated (lanes 2 and 3) or denatured by boiling (lanes 4 and 5) and boiling in 1% SDS (lanes 6 and 7). The corresponding controls were incubated with distilled water (lanes 3, 5 and 7). Untreated HUT-78 membrane proteins were run as a control (lane 1). Proteins were recoved by acetone precipitation, separated by 10% SDS-PAGE and transferred to PVDF membrane. Protein bands were visualised using ECL. A short exposure time revealed p45 (a), whereas longer exposure was required to visualise sCD44 (b).
This monoclonal antibody was used to test for cross-reactivity between our unknown protein, p45 and Link Protein. As described in Figure 5.6 (a) p45 can be detected by D2.1 in both HUT-78 and K4 membrane fractions. The antibody 9/30/8-A-4 appears to specifically detect a protein present in both HUT-78 and K4, with a molecular weight of approximately 10kDa greater than that of p45 i.e. approximately 55kDa. Preliminary data does not concur with Link Protein being the species recognised by D2.1.

5.3.5 Comparison of p45 with LYVE-1

This lymph-specific hyaluronan receptor is a member of the link protein superfamily, with a conserved link domain. It is a glycosylated protein with a molecular weight of approximately 60kDa, with expression restricted to lymph vessel walls (Banerji et al., 1999). The protein under investigation displays tissue specific expression, i.e. it is detected on epithelial-associated lymphocytes but not on peripheral blood lymphocytes and we were interested in testing whether p45 could be recognised by anti-LYVE-1. In Figure 5.6 (b) D2.1 detects a 45kDa protein in HUT-78 membrane lysate. When HUT-78 membrane lysate was probed using anti-LYVE-1 there was no specific band detected at 45kDa, suggesting that D2.1 is not recognising LYVE-1 in HUT-78 cells.

5.3.6 Comparison of p45 with syndecan-4

Syndecan-4 is a ubiquitously expressed transmembrane protein with a molecular weight of approximately 36kDa. Rabbit polyclonal antibodies against the ectodomain (32kDa) and the cytoplasmic domain (5-7kDa) of syndecan-4 were used to probe HUT-78 and Jurkat total cell lysates. Lysates were prepared using either 0.5% or 1% Np40/PBS to determine if detergent sensitivity would alter the distribution of epitopes. As can be seen in Figure 5.7 (a) p45 was detected by D2.1 in HUT-78 lysates, but there were no proteins detected using this monoclonal in Jurkat lysates (a CD44-negative T lymphoma cell line) Figure 5.7 b. In HUT-78 cells there was enrichment in the number of proteins and the concentrations of proteins detected by the antibody to the syndecan ectodomain (Figure 5.7 a, lanes 3 and 4). This may be due to an increased number of detergent soluble proteins in the lysate.

5.3.7 Comparison of p45 with the novel antibody, F99-496

The monoclonal antibody, F99-496, was received as a gift from Dr. G. David and was used to probe HUT-78 lysates. Characterisation of this antibody, by his research group, found that it detected CD44 in an unstable complex with an additional protein, in fibroblasts. The molecular weight of this unknown protein was approximately 45kDa.
Figure 5.6: Western blotting of HUT-78 and K4 T lymphoma cell line with anti-link protein, anti-LYVE-1 and D2.1.
(a) HUT-78 lysates were loaded into lanes 1, 2, 5 and 6 and K4 lysates were loaded into lanes 3, 4, 7 and 8. The lysates were loaded as either 50µg (lanes 1, 3, 5 and 7) or 100µg (lanes 2, 4, 6 and 8). The lanes were blotted using 9/30/8-A-4 (1:500) (lanes 1, 2, 3 and 4) or D2.1 (1:100) (lanes 5, 6, 7 and 8).

(b) HUT-78 lysates (50µg) were loaded into lanes 1 and 2. The lanes were blotted using the monoclonal D2.1 (1:100) or the rabbit polyclonal, anti-LYVE-1 (1:100).
Figure 5.7: Western blotting of HUT-78 and Jurkat T lymphoma cell lines with D2.1 and syndecan antibodies against ectodomain and cytoplasmic domain.

(a) HUT-78 lysates were loaded into all lanes (total cell lysate of $5 \times 10^5$ cells), with lysates in lanes 1, 3, 5, 7 and 9 prepared using 0.5% Np40/PBS and lanes 2, 4, 6, 8 and 10 prepared using 1% Np40/PBS.

(b) Jurkat lysates were loaded into all lanes (total cell lysate of $5 \times 10^5$ cells), with lysates in lanes 1, 3, 5, 7 and 9 prepared using 0.5% Np40/PBS and lanes 2, 4, 6, 8 and 10 prepared using 1% Np40/PBS.

The lanes were blotted using D2.1, anti-ectodomain (Ecto), anti-cytoplasmic domain (Cyto), normal rabbit serum control (NRS) and IE, monoclonal control (all diluted 1:100).
As both this monoclonal antibody and D2.1 detects CD44 and an associated 45kDa protein, we were interested in testing whether this new antibody could detect p45 in HUT-78 cells. We were unable to detect any proteins using the test antibody in both HUT-78 and fibroblast lysates, under a variety of conditions. As can be seen in Figure 5.8 no protein bands were detected by this test antibody, whereas D2.1 strongly detected both p45 and CD44s in these HUT-78 lysates. Also, when this novel antibody was used for immunoprecipitation from HUT-78 lysates there were no specific proteins detected (comparing lane 2 and 8 of Figure 5.8). However when D2.1 was used as an immunoprecipitating antibody, p45 and CD44s (weakly) were present when D2.1 was used as a detecting antibody (lane 6, Figure 5.8).

5.3.8 Comparison of p45 with bikunin-binding proteins
As described in the introduction (Section 5.1) two proteins have been identified that associate with bikunin. One of these has been determined to be the hyaluronan-binding Link protein. There was no cross-reactivity found between p45 and an antibody to a common motif found in human Link proteins (see Section 5.3.4). However, we were interested in testing whether p45 recognised the novel bikunin receptor (bik-R). Bik-R has not been fully identified but this 45kDa protein is known to be membrane-associated and to play a role in the modulation of CD44 signalling.

D2.1 detected the presence of p45 and CD44s in HUT-78 lysates (20 and 40μg respectively), but was unable to detect any specific proteins in the supplied positive control, hyaluronan-binding proteins (HABP) (Figure 5.9). The negative control (mouse ascities) for the monoclonal antibody, D2.1, detected a number of high molecular weight proteins but no specific proteins were detected at the molecular weights corresponding to p45 and CD44s. The polyclonal antibody raised against bikunin-binding proteins (bik BP) detected two specific proteins in the positive control, HABP. These did not correspond to p45, as in the D2.1 western blot. Likewise anti-bik BP, did not detect a protein corresponding to p45 in HUT-78 lysates. This provides evidence that p45 does not correspond to a bik BP, in particular the novel bik-R.

5.3.9 Purification of D2.1 from tissue culture supernatant for use in immunoaffinity chromatography
Tissue culture supernatant was collected and pooled from several flasks of D2.1 hybridoma. Before being used in immunoaffinity chromatography a number of purification steps were required in order to concentrate and improve the purity of the
Figure 5.8: Western blotting of HUT-78 T lymphoma cell line with D2.1 and the antibody, F99-496.

HUT-78 total cell lysates were loaded in lanes 1, 4 and 7. Total cell lysates were immunoprecipitated using the antibody, F99-496 (F) (lanes 2, 5 and 8) or D2.1 (D) (lanes 3, 6 and 9). The lanes were blotted using F99-496 (lanes 1, 2 and 3), D2.1 (lanes 4, 5 and 6), or control mouse ascites (lanes 7, 8 and 9). Specific bands are indicated by arrows.
Figure 5.9: Western blotting of HUT-78 T lymphoma cell line with D2.1 and anti-Bik binding proteins.

HUT-78 lysates were loaded into lanes 1, 2, 4, 6, 7, 9 (total cell lysate 20μg (lanes 1 and 6 only) or 40μg) with lanes 3, 5, 8 and 10 loaded with HABP. The lanes were blotted using D2.1 (lanes 1, 2 and 3), anti-Bik BP (lanes 6, 7 and 8), mouse control ascites (lanes 4, 5) and normal rabbit serum control (lanes 9, 10). Specific bands are indicated by arrows.
antibody. The initial step used for concentration and partial purification of the antibody solution was ammonium sulfate precipitation (see Section 2.10.1). With a starting volume of supernatant of 246.5ml, 77.15g of ammonium sulfate was added slowly, yielding a 50% saturated solution. This was allowed to stir overnight at 4°C and the resulting precipitate was resuspended in 0.1 volume of PBS. A dialysis step (see Section 2.10.2) was required in order to de-salt the antibody solution. Measuring the optical density at 280nm provides an estimate of the antibody concentration of this solution. This was estimated to be approximately 0.632mg/ml ($OD_{280} = 0.79$) [assuming that $1 \ OD_{280} = 0.8\ mg/ml$]. Thus in 24ml of solution there was approximately 15.168mg of antibody.

This provided a crude method for the concentration and purification of antibody but a second step was required in order to obtain antibody sufficiently pure antibody for use in immunoaffinity chromatography. Protein G is available immobilised to Sepharose beads. This protein G binds specifically and reversibly to the antibody Fc region thus providing the basis of the second stage of purification. The binding capacity of protein G sepharose (PGS) to mouse IgG is approximately 6mg/ml, therefore 15.168mg of antibody required 2.528ml of PGS suspension. 2.5ml of PGS suspension was placed on sintered glass column. The beads were equilibrated by washing with PBS and then carefully overlayed with the partially purified antibody solution. This was recirculated through the beads for 3 hours using a peristaltic pump. This ensured maximal contact between the PGS beads and the antibody solution. 10 volumes of PBS were run through the column in order to wash away any non-specific proteins. The presence of non-specific proteins was determined by reading the $OD_{280}$ of the eluate. The final $OD_{280} = 0.011$, indicating negligible proteins in the eluate.

Finally the antibodies were eluted using a low pH buffer, 100mM Glycine, pH 2.5. In order to ensure that there is no denaturation of the antibody this eluate was immediately neutralised using 1M Tris-HCl, pH 8.0. In order to prepare for antibody elution 100μl of the neutralisation buffer were added to labelled eppendorfs. The elution buffer was placed on the column and 1ml fractions collected in the prepared eppendorfs, ensuring careful mixing and neutralisation of the solution. The $OD_{280}$ of the collected fractions were measured (see Table 5.1) and antibody-containing fractions (fraction 3, 4 and 5) were pooled and stored at -20°C until use.
Table 5.1 $\text{OD}_{280}$ of eluted fractions from PGS column

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<th>Fraction</th>
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<tr>
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<tr>
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5.3.10 Purification of p45 using immunoaffinity chromatography

The availability of D2.1, a monoclonal antibody that recognises p45, provided a useful tool for the purification of this protein using immunoaffinity chromatography [see Appendix 3]. Protein G sepharose (PGS) beads were used to immobilise D2.1. PGS beads were incubated with the purified D2.1 antibody and were allowed to associate. In order to immobilise the antibody on the beads a chemical cross-linker was used; dimethylpimelimidate (DMP) (see Section 2.9). The efficiency of coupling was checked using samples taken before and after bead coupling (Figure 5.10 (a)). The PGS beads efficiently bound D2.1, as there is no antibody present in the sample after incubation with the beads. Before cross-linking with 20mM DMP both the heavy chain (55kDa) and the light chain (25kDa) of immunoglobulin G (IgG) was detected in the bead sample. Following the reaction with DMP only the light chain of the IgG is faintly detectable, indicating efficient cross-linking of the antibody to the PGS beads. The antibody-PGS beads were transferred onto a sintered glass filter and washed with 20 volumes of 100mM glycine, pH 2.5, followed by 20 volumes of PBS. HUT-78 membrane lysate (5ml) were pre-cleared using 50μl of unconjugated PGS beads (to prevent any non-specific protein binding to the antibody-PGS beads). The pre-cleared membrane proteins were applied onto the antibody-PGS column and allowed recirculate through the beads for 2 hours using a peristaltic pump. The beads were washed with PBS until the $\text{OD}_{280}$ of the eluate neared zero, indicating that any non-specific proteins had been washed through.
Figure 5.10: Immunoaffinity purification of p45 using D2.1 immobilised to protein G sepharose beads.

(a) Coomassie blue staining of D2.1 antibody solution before (lane 1) and after (lane 2) with PGS beads) and D2.1 antibody-PGS beads before (lane 3) and after (lane 4) incubation with 20mM DMP.

(b) The following samples were loaded; HUT-78 membrane lysate after incubation with D2.1 antibody-PGS beads (lane 1), D2.1 antibody-PGS beads (lane 2), pre-elution fractions (lane 3 and 4), elution fractions (lanes 5, 6, 7 and 8) and final equilibration fractions (lanes 9, 10 and 11). The lanes were blotted using D2.1.
Low pH conditions (i.e. 100mM glycine, pH 2.5) were used to elute the antigen. In order to ensure a rapid change in pH and elution from the D2.1-PGS matrix in a smaller volume, 10mM phosphate, pH 8.0, was used as a pre-elution buffer, thus removing the buffering capacity of PBS. As described in 5.3.7, to ensure no denaturation of the antigen occurs the eluate must be immediately neutralised using 1M Tris-HCl, pH 8.0. In preparation, 100μl of the neutralisation buffer was added to labelled eppendorfs. The elution buffer was placed on the column and 1ml fractions collected in the prepared eppendorfs, ensuring careful mixing following elution. The resulting fractions were acetone precipitated and examined by western blot. As can be seen from Figure 5.10 (b), p45 is still present in the HUT-78 membrane lysate, indicating that there has not been complete removal of p45 from the sample. p45 is detected in all fractions, both before and after the elution steps. However, there does seem to be a higher proportion of p45 in the first elution step (lane 5). When using these same elution conditions, the glycine eluates were pooled, acetone precipitated, resuspended in sample buffer for electrophoresis. A problem was encountered with the high level of salt in the sample and dialysis was carried out into 20mM Tris, pH 7.5. The sample was run out on a 10% SDS polyacrylamide gel. The gel was transferred onto ProBlott and stained using amido black. Although the protein molecular weight markers were stained well using amido black, no other proteins were detected.

5.3.11 Isolation of p45 using preparative SDS PAGE

Two attempts to isolate p45 from the membrane fractions of HUT-78 and K4 cells were carried out using preparative SDS PAGE [For experimental details see Appendix 3].

HUT-78 membrane proteins (Run 1)

Approximately 14mg of HUT-78 membrane fractions were pooled for this first PrepCell run. Following acetone precipitation, the resulting precipitate was resuspended in approximately 1.5ml of 1x sample buffer and denatured. The sample was carefully loaded on a 10% gel and the gel was run under the following settings; constant current 45mA, voltage 500mV and power 12W. Fractions were collected once the bromophenol blue dye front had reached the end of the gel. Every fifth fraction (10μl) was tested for the presence of p45 using western blotting with D2.1 and p45 could be detected in fraction 130-185 (Figure 5.11). To determine the level of purity of the proteins in these fractions, the samples were run out on a 10% SDS polyacrylamide gel and silver stained (Figure 5.12). As can be seen there appears to be a protein band appearing around fraction 135 that begins to disappear around fraction 175. These samples were pooled and sent for sequencing (see Section 5.3.11 and 5.3.12).
Figure 5.11: Western blot of fractions 105-190 from PrepCell separation of HUT-78 membrane lysates using D2.1
HUT-78 membrane fractions were separated using 10% SDS PAGE on the BioRad PrepCell. The above fractions (fraction number is indicated in each lane) were separated using 10% SDS PAGE and tested for the presence of p45 using D2.1. The ability of D2.1 to detect p45 was confirmed using HUT-78 membrane lysates (HUT).
HUT-78 membrane fractions were separated using 10% SDS PAGE on the BioRad PrepCell. The above fractions (fraction number is indicated in each lane) were separated using 10% SDS PAGE and proteins were visualised using silver staining. Also shown are molecular weight markers (first and final lane) with the 45kDa marker indicated.

Figure 5.12: Silver stain of fractions 100-195 from PrepCell separation of HUT-78 membrane lysates
K4 membrane proteins (Run 2)

K4 membrane fractions (approximately 20mg) were pooled. The acetone-precipitated proteins was resuspended in approximately 2ml of 5x sample buffer and denatured. The aim of this experiment was to isolate both p45 and p135. This was an attempt to test whether p135 would fractionate leaving CD44s and p45 (with no other contaminants with a similar molecular weight). The sample was loaded on a 7.5% gel and run under the following settings; constant current 50mA, voltage 500mV and power 12W. Fractions were collected once the bromophenol blue dye front had reached the end of the gel. The fractions (10μl) were tested for the presence of p45 and p135 using western blotting with D2.1. p135 was not detected in any of the fractions collected. This may be because of protein degradation due to the length of time taken for elution. p45 could be detected in fractions 11-30, as well as CD44s in fractions 17-21 (Figure 5.13). To determine the level of purity of the proteins in these fractions, the samples were run out on a 10% SDS polyacrylamide gel and silver stained (Figure 5.14). There was not sufficient separation and samples were too contaminated for sequencing. For this reason an additional separation step was required and 2-dimensional electrophoresis was used (see Section 5.3.12).

5.3.12 Separation of p45-containing fractions using two-dimensional electrophoresis

Proteins, initially separated using a PrepCell, were further separated using two-dimensional electrophoresis [see Appendix 3]. Fractions 14-18 and 22-24 were combined and dialysed into 5mM Tris, pH 7.5. This was done to reduce the concentration of SDS present in the samples, as this confers a uniform charge to the proteins, being incorporated in a ratio of 1.4g SDS/g protein. These SDS/protein complexes have a roughly constant net negative charge per unit mass and makes successful isoelectric focusing (IEF) difficult, as this is a separation technique based on the characteristic charge of individual proteins. Following the dialysis step and protein concentration using acetone precipitation, the sample was resuspended in IEF rehydration buffer [Appendix 1] and loaded onto a 13cm IPG strip, pH 3-10, linear, with a small amount of sample run on a separate IEF strip for use in a comparative western blot using D2.1 (see Section 2.11.2). Both were run overnight on the Ettan IPGphor IEF system. Following IEF both IPG strips were equilibrated in 20ml of equilibration buffer before being place on 10% SDS polyacrylamide gels, along with prestained markers. One gel was coomassie stained (Figure 5.15) as per protocol for
Figure 5.13: Western blot of fractions 11-30 from PrepCell separation of K4 membrane lysates
K4 membrane fractions were separated using 7.5% SDS PAGE on the BioRad PrepCell. The above fractions (fraction number is indicated in each lane) were separated using 10% SDS PAGE and following electrotransfer p45 and sCD44 were visualised by western blot using D2.1. K4 was loaded onto the final lane as a positive control. Also indicated are the molecular weight markers.
Figure 5.14: Silver stain of fractions 11-30 from PrepCell separation of K4 membrane lysates

K4 membrane fractions were separated using 7.5% SDS PAGE on the BioRad PrepCell. The above fractions (fraction number is indicated in each lane) were separated using 10% SDS PAGE and proteins were visualised using silver staining. Also shown are molecular weight markers (first lane).
Figure 5.15: Coomassie stain of fractions 14-18 and 22-24 from PrepCell separation of K4 membrane lysates
K4 membrane fractions were initially separated using 7.5% SDS PAGE on the BioRad PrepCell. These fractions were separated in the first dimension using isoelectric focusing and in the second dimension using 10% SDS PAGE. The separated proteins were visualised using coomassie staining.
MS sequencing [Appendix 1], whereas the other gel was electroblotted and probed using D2.1 (Figure 5.16). There were a number of discrete spots visualised using coomassie. However, smearing was also apparent and this is probably due to some of the difficulties that arise when carrying out two-dimensional electrophoresis of membrane proteins. Due to their hydrophobic nature they are not ideally suited to IEF and are not readily incorporating into the gel. D2.1 detected a series of vertically oriented spots with a molecular weight of approximately 45-47kDa. This probably reflects the glycosylated nature of the protein. The western blot did not match any of the discrete spots that were present in the coomassie stained gel. Bearing this in mind some of the discrete protein spots were excised and sent for MS sequencing.

5.3.13 N-terminal sequencing of proteins using Edman degradation
In the first attempt to determine the protein sequence of p45, fractions 155-165 were combined. These were concentrated to an approximately 1ml volume by placing fraction in dialysis tubing and covering with polyethylene glycol. This was then dialysed against 20mM Tris, pH 7.5 and acetone precipitated overnight. The resulting precipitant was resuspended in sample buffer. This protein sample was separated under electrophoresis using 10% SDS PAGE and transferred using CAPS buffer onto ProBlott PVDF membrane. Care was taken to avoid any chemical modification of the proteins to be sequenced, for example due to the presence of any unpolymerised acrylamide (free primary amines). Steps taken included; the use of ultra-pure water, the preparation of the acrylamide gel a day in advance, to ensure complete polymerisation had taken place, and the use of thioglycolate (2mM) in the running buffer, which can neutralise any primary amines. Following transfer onto ProBlott, the proteins were visualised using amido black. The strongest band was sent to the National Food Biotechnology Centre, University College Cork for N-terminal sequencing using Edman degradation. The results from this were inconclusive and a report was returned stating that the signal was too low to determine a reliable sequence. The protein sent for analysis was the most abundant protein but this may not actually reflect the status of p45 in the sample. D2.1 has a very high affinity for p45 and could therefore be giving a disproportionate view of the abundance of p45.

5.3.14 Protein identification using mass spectrometry
Following the separation of p45 from other protein components of the HUT-78 membrane, samples were sent to outside institutes for analysis and protein identification using ESI MS. The first sample MS analysis was prepared from the HUT-78 membrane PrepCell (Figure 5.17) and was sent to Dr. A. Wallace, Queens
K4 membrane fractions were initially separated using 7.5% SDS PAGE on the BioRad PrepCell. These fractions were separated in the first dimension using isoelectric focusing and in the second dimension using 10% SDS PAGE. After electroblotting, p45 was visualised by western blot using D2.1.
Figure 5.17: Coomassie blue staining of purified p45 fraction. HUT-78 membrane fractions were separated using 10% SDS PAGE on the BioRad PrepCell. The above fractions (fraction number is indicated in each lane) were separated using 10% SDS PAGE and tested for the presence of p45 using D2.1. The presence of p45 in these fractions was confirmed by western blot using D2.1 (not shown).
University Belfast. This identified beta-actin as the protein present in the sample. Actin is a ubiquitous protein with a molecular weight of 42kDa, which is known to be associated with the cytoplasmic domain of CD44 via the interaction of the actin binding proteins, ankyrin and ERM family proteins (Lokeshwar et al., 1994; Tsukita et al., 1994).

As this result was not fully satisfactory, p45 was separated from K4 membrane proteins using an alternative approach and new IEF technology available within our facility. K4 membrane proteins that were firstly separated using the PrepCell (see Section 5.3.11) and then using 2-dimensional electrophoresis (see Section 5.3.12). A number of spots were excised from this (Figure 5.18) and sent to Dr. Len Packman, the Protein and Nucleic Acid Chemistry facility, the University of Cambridge, for sequencing using ESI MS. The results from this are in Figure 5.19. The results identified the proteins as pairs with sample 1=2, 3=4 and 5=6 (an additional sample 7 was identified as a contaminant, i.e. sheep keratin).

The first protein identified, from samples 1 and 2, was the nucleolar protein B23. This protein acts as a molecular chaperone with a role in ribosome assembly. Substrate release by protein B23 is dependent on its phosphorylation by the protein kinase CK2 (Szebeni et al., 2003). Also, heterogeneous nuclear ribonucleoprotein F (hnRNP F) was identified in samples 3 and 4, and were present in a mixture of proteins identified in samples 5 and 6. hnRNPs are a family of ubiquitously expressed RNA binding proteins, that complex with heterogeneous nuclear RNA (hnRNA). These proteins are associated with pre-mRNAs in the nucleus and appear to influence pre-mRNA processing and other aspects of mRNA metabolism and transport. While all of the hnRNPs are present in the nucleus, some seem to shuttle between the nucleus and the cytoplasm. hnRNP F was found to be strongly expressed in the cytoplasm of tumour tissue, although there was no data about its expression in lymphocytes (Honore et al., 2004). Beta-actin was identified, yet again, in the mixture of proteins identified in samples 5 and 6. This reflects the role played by this cytoskeletal protein in the linkage of many transmembrane proteins, including CD44, to the cytoskeleton.

5.4 DISCUSSION

This chapter outlines attempts to identify the unknown protein, p45. A two-pronged approach was used in the task of identifying this novel protein. Firstly, a panel of
Figure 5.18: Protein spots excised for electrospray ionisation mass spectrometry
K4 membrane fractions 14-18 and 22-24 were initially separated using 7.5% SDS PAGE on the BioRad PrepCell. These fractions were separated in the first dimension using isoelectric focusing and in the second dimension using 10% SDS PAGE. The separated proteins were visualised using coomassie staining and the indicated spots were excised.
Fanning spot 1

1. gi|112079  Mass: 28482  Total score: 320  Peptides matched: 9
   nucleolar phosphoprotein B23.2 - rat
   which is the same as
   gi|10835063  Mass: 32726  Total score: 320  Peptides matched: 9
   nucleophosmin (nucleolar phosphoprotein B23, numatrin);
   Nucleophosmin 1 (nucleolar phosphoprotein B23, numatrin) [Homo sapiens]
   http://www.matrixscience.com/cgi/master_results.pl?file=../data/20030523/Ftota rua.dat

Fanning spot 2

1. gi|16876992  Mass: 32665  Total score: 251  Peptides matched: 5
   nucleophosmin (nucleolar phosphoprotein B23, numatrin) [Homo sapiens]
   http://www.matrixscience.com/cgi/master_results.pl?file=../data/20030523/Ftoxa e.dat

Fanning spot 3

1. gi|4826760  Mass: 45985  Total score: 329  Peptides matched: 8
   heterogeneous nuclear ribonucleoprotein F; nucleolin-like protein
   [Homo sapiens]
   http://www.matrixscience.com/cgi/master_results.pl?file=../data/20030523/Ftots mnsn.dat

Fanning spot 4

1. gi|16876910  Mass: 46013  Total score: 395  Peptides matched: 9
   heterogeneous nuclear ribonucleoprotein F [Homo sapiens]
   http://www.matrixscience.com/cgi/master_results.pl?file=../data/20030523/Ftotmr uT.dat

*Figure legend overleaf*
Figure 5.19: Mass spectroscopy results of spots excised from two-dimensional gel of K4 PrepCell fractions
antibodies from candidate proteins were screened against the monoclonal antibody, D2.1. In parallel with this, a number of protein separation methods were used to allow purification of p45 from HUT-78 membranes proteins thus enabling protein sequencing to be carried out. D2.1 has been used in previous chapters as a CD44-specific antibody. Using this monoclonal antibody and other anti-CD44 antibodies, it has been demonstrated that cross-linking of CD44 results in cellular polarisation and migration in both PBTLs and HUT-78 cells. Although CD44 was the only protein recognised by D2.1 in PBTLs, an additional protein, p45, was detected in HUT-78 cells. The identification of p45 has been the objective of this chapter.

As had been previously shown D2.1 detected a protein with a molecular weight of approximately 45-47kDa in the membrane fractions of HUT-78 (Figure 5.1). This was also present in K4 cells (somewhat enriched). Indeed there was also a weak band detected with a molecular weight of approximately 135kDa (Figure 5.2), which is suggestive of a possible complex taking place between CD44 and p45. The pI of this protein was found to be approximately 6.16-6.73 (Figure 5.3 and 5.4). A negative charge is typical of N-glycosylated proteins. Following deglycosylation using N-glycosidase F, which catalyses the cleavage of N-glycosides, the molecular weight of p45 was reduced by approximately 5kDa (Figure 5.5). It is noteworthy that D2.1 still recognised p45 and CD44 following deglycosylation, which suggests that the epitope recognised by D2.1 is not a carbohydrate motif. As glycosylation patterns on certain proteins, for example CD44, are cell type and activation state specific if D2.1 had recognised to a carbohydrate motif this may have complicated any further studies.

Initially work was carried to test if D2.1 recognised a protein known to share an epitope with CD44. The Link module is a critical hyaluronan-binding motif present in CD44 and Link protein. Link protein stabilises the interaction between extracellular matrix proteoglycans and hyaluronan (Binette et al., 1994). Three Link proteins have been identified in human tissue and we tested HUT-78 membrane extracts against the rat monoclonal antibody, 9/30/8-A-4, which has been shown to recognise a common epitope present in all three link proteins (Caterson et al., 1986). As can be seen in Figure 5.6, p45 did not correspond to a specific band detected by 9/30/8-A-4, suggesting that this unknown protein is not one of the link proteins. The presence of a link module in the lymph vessel specific hyaluronan binding protein, LVYE-1, with a molecular weight of 60kDa, suggested that this protein would be interesting to test against p45. However p45 was not recognised specifically by anti-LYVE-1 antibody.
LYVE-1 has been found to be only associated with lymph vessel cells and therefore this is not a surprising finding (Banerji et al., 1999).

The possibility of p45 corresponding to syndecan-4 was also tested. Syndecan-4 is known to interact with integrins and is shed in a PKC-regulated manner (Lim et al., 2003; Park et al., 2000). Syndecan-4 has been shown to interact with integrins in the formation of focal adhesions (Lim et al., 2003), and we examined here the possibility that p45 corresponded to syndecan-4 (both the shed ectodomain or the cytoplasmic domain). Western blot analysis using antibodies against the ectoplasmic domain and the cytoplasmic domain revealed that these did not recognise a specific protein in either HUT-78 or jurkat cells (Figure 5.7). The antibody, F99-496, was received for Dr. Guido David, which had been found to recognise CD44 in complex with an unidentified 45kDa protein in fibroblasts. This offered another possible candidate for p45, however the antibody did not detect any proteins in HUT-78 cells (Figure 5.8). We were unable to detect any proteins in a fibroblast cell line tested.

New research published regarding the protein, bikinin, described the identification of two proteins with which it interacts. These bik binding proteins were the previously described link protein and a membrane-associated bik receptor (Suzuki et al., 2002). As this work had described the role of bik R in the modulation of CD44 signalling events, we were interested in testing whether this protein corresponded to p45. However, when tested in HUT-78 cell lysates, no proteins were detected that corresponded to p45 by anti-bik BP (Figure 5.9). Anti-bik BP was able to detect two proteins in its positive control, HABP.

Although there was no success with the candidate proteins tested to determine if they corresponded to p45, this was not the only approach taken to identify this protein. Using a number of different separation techniques enabled the enrichment of the test sample with p45, thus providing an opportunity to attempt an identification of p45. The first method of p45 purification used the monoclonal antibody, D2.1, to prepare an immunoaffinity chromatography matrix. There were some difficulties experienced with this method of isolating p45. D2.1 was successfully linked to protein G sepharose beads and covalently fixed (Figure 5.10). Following incubation of HUT-78 membrane fractions with the immunoaffinity matrix, p45 remained bound to the antibody matrix. However, p45 was present in the pre-elution, the elution and equilibration fractions, suggesting that the elution conditions may not be optimal.
Preparative SDS PAGE was used as a method of isolating p45-containing fractions from HUT-78 and K4 membrane proteins. The first run using HUT-78 membrane proteins enabled the isolation of p45 with few contaminants (Figure 5.17). It was assumed, probably incorrectly that the pre-dominant protein in this coomassie stained gel was p45. This band was excised and sent for protein identification. Initially, Edman degradation was used in order to obtain N-terminal protein sequence. Fractions containing p45 were separated using 10% SDS PAGE and electrotransferred onto a ProBlott (a high quality PVDF membrane). Following amido black staining the predominant band was carefully excised and sent for sequencing. The results were inconclusive and which was probably due to chemical modification of the N-terminus. N-terminal modification can take place during post-translational events but it can also occur during the separation procedures carried out prior to sequencing. Steps were taken to avoid introducing any chemical modifications, such as 1) using the highest-grade water and reagents available, 2) allowing polyacrylamide gels to set overnight to ensure complete polymerisation, 3) adding 2mM thioglycolate to the running buffer to mop up any primary amines present in the gel. Despite the steps taken to prevent the introduction of chemical modification to the protein to be sequenced, N-terminal modification made Edman degradation an unsuitable technique for the identification of p45.

Mass spectrometry, unlike Edman degradation, does not rely on chemical reaction with the N-terminal, and instead matches the measured m/z ratio of cleaved peptide fragments with a database of predicted fragments from known proteins. It provides an ideal alternative for the identification of p45. As described above, fractions containing p45 from the HUT-78 PrepCell run were combined and separated using 10% SDS PAGE. Following coomassie staining the most prominent band was sent for MS analysis. This first attempt to characterise p45, identified the presence of β-actin in the sample, with a predicted molecular weight of 42kDa. The presence of this cytoskeletal protein in the membrane fraction of HUT-78 cells lysates is not entirely unexpected. The association of many membrane proteins, including CD44 with the actin cytoskeleton is well documented (Lacy and Underhill, 1987), as are many of the linker proteins involved with mediating this interaction, for example, ankyrin (Kalomiris and Bourguignon, 1988) and the ERM family of proteins (Tsukita et al., 1994). Indeed, integrin association with the actin cytoskeleton is induced upon the binding of extracellular matrix ligand, leading to the formation of focal adhesions (Schoenwaelder and Burridge, 1999).
Preparative electrophoresis was used again to isolate p45 from K4 membrane proteins. Separation was carried out using a 7.5% SDS gel, as the principle objective was to isolate p135. Previous observations have suggested that p135 is an unstable complex of CD44s and p45. It was anticipated that by isolating p135-containing fractions, that the complex would disassociate at low protein concentrations. This would provide p45 with few contaminants, as the only proteins present would be CD44s (molecular weight 85-90kDa) and other high molecular weight proteins. A polyacrylamide gel of 7.5% provided the optimal separation of p135 but all fractions were negative for p135 when tested using D2.1. The loss of p135 during the PrepCell run may be due protein degradation as a result of time (24-36 hours) required to obtain the high molecular weight containing fractions.

In order to make best use of the PrepCell run, the lower molecular weight fractions were tested for the presence of p45 (i.e. fractions 11-30). Both p45 and CD44s could be detected by D2.1 (Figure 5.11). However, when the proteins present in these fractions were examined by silver staining (Figure 5.12) there were a high number of contaminating proteins present. In order to isolate p45 from the other proteins an additional separation step was required. Two-dimensional electrophoresis separates proteins according to their pi (first dimension) and their molecular weight (second dimension). There was some success in separating and detecting p45 following electrotransfer of two-dimensional gels using D2.1 (Figure 5.13). However, there were problems with reproducibility.

The separation of the PrepCell fractions by two-dimensional electrophoresis had a number of difficulties associated with it. These included the presence of SDS in the samples (i.e. they were eluted in running buffer), which conferred a relatively constant negative charge on all proteins. Also, acetone precipitation was carried out in order to reduce the sample volume, leading to high salt concentrations in the final sample. Dialysis, into 5mM Tris, was used to reduce the both the level of salt and SDS present in the final sample, although it is difficult to completely remove SDS from solution. In addition, there are some technical issues related to the separation of membrane proteins by IEF. It is difficult to obtain a high degree of resolution for hydrophobic proteins and membrane proteins are largely hydrophobic, by their nature. Many membrane proteins are inefficiently solubilised in the standard IEF sample buffers used here. Although, there are some newer reagents available which enhance the solubilisation of hydrophobic proteins (Herbert, 1999; Kashino, 2003), these were not tested using our samples. These include the use of thiourea as a chaotrope,
amidosulphobetain 14 as a surfactant, and tributyl phosphine instead of DTT as a reducing agent in the sample buffer (Kashino, 2003).

A number of p45-containing fractions were combined and separated using two-dimensional electrophoresis. This provided sufficient protein for visualisation using a coomassie blue stain suitable for MS analysis. When a portion of this was visualised on a western blot using D2.1, there was not a clean p45 spot (Figure 5.16). Although it was not possible to detect any discrete spots about the predicted region of the p45, some others spots excised and sent for MS fingerprinting. When the results were returned, the samples corresponded to pairs of identical proteins that were identified as the nucleolar phosphoprotein B23 (B23), heterogeneous nuclear ribonucleoprotein F (hnRNP F) and a mixture of proteins, which included beta-actin and hnRNP F (Figure 5.18 & 5.19).

This study did not successfully identify the protein species, p45. Our initial approach, which attempted to test if p45 corresponded to a series of previously identified protein, was unsuccessful. Additionally, attempts were made to purify the protein from the membrane proteins of HUT-78 and K4 cells. As has been outlined in this chapter there was some difficulty in obtaining p45 that could be detected using standard protein staining protocols. This was further compounded by the requirement that certain staining protocols (which offer poor sample resolution) are recommended for use during the preparation of protein samples for sequencing. Therefore, although using western blot analysis we were able to confirm that the samples were enriched with the p45 species, this was not evident following protein staining. D2.1 appears to be a very high affinity antibody and our work to date indicates that p45 is a low abundance protein. A future approach may be to isolate p45 using D2.1 conjugated to PGS beads and then to directly try to identify the components of the immunoprecipitate using ESI, thereby circumventing the need for protein detection.

5.5 SUMMARY OF FINDINGS

1. D2.1 detects an approximately 45kDa glycosylated protein in the membrane fractions of HUT-78 and K4 cells.
2. This protein did not correspond to any of the human link proteins (molecular weight 48, 44 and 41kDa), nor the lymph-specific, hyaluronan-binding protein, LYVE-1 (molecular weight 60kDa).

3. In addition, the protein did not correspond to syndecan-4, a bikunin binding protein, or the 45kDa protein detected by the antibody, F99-496.

4. HUT-78 and K4 membrane samples were successfully enriched for p45 using preparative SDS PAGE. K4 samples required an additional two-dimensional electrophoresis in order to distinguish individual protein species.

5. Protein sequencing of p45 using Edman degradation was unsuccessful as the sample was N-terminally blocked.

6. Protein identification using mass spectrometry enabled the identification of a number of protein species, such as beta-actin, nucleolar phosphoprotein B23 and heterogeneous nuclear ribonucleoprotein F, in the p45-enriched K4 membrane fractions, however these are not likely candidates for p45.
CHAPTER 6

GENERAL DISCUSSION
An understanding of the key elements involved in the control and regulation of leukocyte migration may yield novel strategies in the treatment of a variety of inflammatory disorders. For example, many autoimmune-mediated diseases are characterised by an influx of inflammatory cells, which is inappropriate. The aim of this thesis was to examine the role of CD44 in T lymphocyte migration, in order to gain a better insight into this adhesion molecule and its role in inflammation. Administration of anti-CD44 antibodies inhibited inflammation in many murine models of inflammation, including cutaneous delayed-type hypersensitivity response (Camp et al., 1993) and experimental autoimmune encephalomyelitis (Brocke et al., 1999).

By exploiting the capacity of monoclonal antibodies to cross-link and increase receptor avidity, thereby activating receptors, we were able to model lymphocyte responses to CD44 activation. In previous studies, cross-linking anti-CD44 antibodies up-regulate T cell proliferation and effector function (Galandrini et al., 1993). Immobilisation of the cross-linking antibodies provided an activating matrix for the surface expressed CD44. This allowed a careful examination of both PMA-activated PBTLs and HUT-78 responses using a number of microscopy based techniques.

This model has been previously been used to demonstrate that LFA-1 activation, using either an anti-LFA-1 monoclonal or a native LFA-1 ligand, ICAM-1, induced active T cell migration (Volkov et al., 1998; Volkov et al., 2001). Migration was accompanied by the development of a polarised elongated structure; with a leading cell body and a trailing cytoplasmic tail. This polarised morphology has been described previously in response to chemokines and was accompanied by the redistribution of several adhesion receptors to the trailing tail, including CD44 (Del Pozo et al., 1995). Here, following the cross-linking of CD44 using immobilised anti-CD44 monoclonal antibodies, a similar series of morphological changes take place (as seen with LFA-1 ligation). Initially, a flattening and spreading of the cell is observed following receptor ligation, with a subsequent polarisation and protrusion of lamellipodia in a particular direction. A forward rolling/reaching with the broad front of the leading edge and cell body occurs in the direction of cell migration, with the remainder of the cell trailing behind, until retraction.
Pharmacological inhibitors allowed the role of certain signalling proteins in the development of this morphology to be investigated. Functional classical PKC isozymes, in particular PKC\(\beta\), were found to be critical for cell migration. PKC\(\beta\) was also required for LFA-1-induced T cell migration (Volkov et al., 2001). The role of the protein, PKC\(\delta\), was also examined, but the results were not as definitive. A number of questions regarding its role in T cell migration remain unanswered. It remains to be determined whether PKC\(\delta\) is involved in the control of directional chemotaxis (perhaps it is not required for chemokinesis, as is the case with PKC\(\beta\)). Perhaps this enzyme is required for effective migration under a chemotactic gradient and it would be interesting to examine how loss of PKC\(\delta\) activity would influence cell migration in this scenario. Indeed, the subcellular localisation of these PKC isozymes and their association with microtubule cytoskeleton suggests a role in signals carried by the dynamic microtubule network.

Studies using an extracellular matrix gel enriched with high-molecular weight hyaluronan demonstrated that there was enhanced T cell migration through the gel, when compared with the extracellular matrix gel alone or with the gel enriched with hyaluronan fragments (Fanning et al., 2005). This provided evidence of T lymphocyte migration through a three-dimensional hyaluronan matrix and it now will be interesting to examine the signalling pathways upregulated during this migratory event. This will allow a comparison to be made between the signalling events that are critical for both antibody-induced and hyaluronan matrix-induced migration, with particular interest in the role of PKC activity. It will also be interesting to determine whether preincubation with anti-CD44 antibodies will influence T cell migration through the hyaluronan matrix. F10-44-2 has been described as enhancing CD44 binding to hyaluronan, whereas J173 has been shown to inhibit CD44 binding to hyaluronan (Galandrini et al., 1994). This would help determine whether enhanced hyaluronan binding can influence T cell ability to migration through the matrix.

The role of the lipid kinase, PI3-kinase, was examined using the inhibitors, Ly294,002 and wortmannin. T cells were unable to polarise and develop a migratory phenotype in response to CD44 cross-linking following Ly294,002 pre-treatment. However, the HUT-78 response to CD44 ligation was not greatly altered following pre-treatment with wortmannin, highlighting differences in the mechanism of action of these inhibitors. As before, HUT-78 pre-treatment with either Ly294,002 or wortmannin prevented the development of a migratory phenotype in response to activation of LFA-1. When
visualised using immunofluorescent microscopy, PI3-kinase was detected in discrete granules within the cytoplasm. A recent publication has examined the localisation of phosphatidylinositol-3-phosphate (PtdIns-3-P) in a model of cell migration. PtdIns-3-P was present in discrete patches throughout the cell, with rapid translocation to the membrane upon lysophosphatidic acid stimulation (Maffucci et al., 2005). This pattern of staining somewhat resembles our staining pattern for PI3-kinase and suggests that we may be detecting co-localisation of PI3-kinase with the phosphorylated lipid pools in the migrating cells.

However, a more thorough investigation is needed in order to determine whether PI3-kinase is activated following CD44 ligation. A number of approaches could be taken in examining the role of this lipid kinase in signalling from the CD44 receptor. Firstly by measuring the generation of 3' phosphorylated lipid phosphatidylinositol products, generated downstream of receptor activation, using methods such as thin layer chromatography. GFP-labelling of the pleckstrin-homology (PH) domain of the protein AKT provides a means of analysing the distribution of the 3' phosphorylated lipid products of PI3-kinase in real-time (Servant et al., 2000).

These studies were carried out using pharmacological inhibitors as a means of investigating the role of various signalling proteins. An important consideration in the use of these chemical agents is that they often lack complete specificity (Davies et al., 2000). A better approach to the study of protein activity within a cell environment is the knockout of protein expression using a number of molecular methods. Constructs are available that express dominant negative forms of signalling proteins and the availability of small inhibitory RNA (siRNA) provide a more specific method of inhibiting kinase activity. These would allow more specific examination of the role of signalling kinases in T cell migration. Also the use of GFP-linked constructs allows the visualisation of proteins in real-time and is an important tool in the study of protein function within the cell.

In most circumstances, the response to CD44 cross-linking of HUT-78 was strikingly similar to the response seen following LFA-1 cross-linking. This highlights the question of why the cell would have both systems in place. Firstly, the ligands are different and hence both may be used in different cellular environments. A second question is whether these are interdependent. Recently the relationship between CD44 and the β1 integrin, VLA-4 during T cell extravasation has been the subject of investigation. Earlier studies suggested that extravasation of activated T cells that is initiated by
CD44/hyaluronan-mediated primary adhesion, requires VLA-4 for firm adhesion (and not LFA-1) (Seigelman et al., 2000). Indeed, the in situ immunoprecipitation, indicated that CD44 was associated with the LFA-1-signalling complex in the migrating cell. However, further work is needed to firmly identify interdependence of LFA-1 and CD44 signalling pathways. Such research could include, for example, co-immunoprecipitation studies, sequential signalling studies and studies in which both proteins are sequentially examined.

Recent work has demonstrated that although CD44/hyaluronan mediate the initial capture and primary adhesion of T cells, a physical association between the cytoplasmic tail of CD44 and VLA-4 is required in order for VLA-4-mediated arrest and firm adhesion to proceed (Nandi et al., 2004). LFA-1 is not found to be associated with CD44 in this situation. It would now be interesting to test the role of VLA-4 in our system; for example, are VLA-4 and CD44 associated and is this association critical to the observed migration following CD44 cross-linking. An interesting twist to this story is brought to light by the observation in a breast cancer cell line, where CD44 cross-linking induces the increased expression of LFA-1 and VLA-4 by exocytosis. This induced integrin expression results in the increased integrin-mediated adhesion to endothelial cells and transendothelial migration (Wang et al., 2005). Although this study was carried out in a breast cancer cell line, and not in T lymphocytes, it further emphasizes the relationship between CD44 activation and integrin receptor function.

The importance of cholesterol-rich lipid rafts in their role, as a platform for signalling interaction has been the subject of much research. Unlike the bulk of cell membranes, which are rich in phospholipids and in a disordered state, the rafts have a high glycosphingolipid and cholesterol content and are packed in an ordered structure. CD44 is fatty acylated (Bourguignon et al., 1991) and up to 40% of total cellular CD44 is accumulated in lipid rafts (Ilangumaran et al., 1998). The integrity of these lipid rafts has been demonstrated to be important for hyaluronan-CD44 mediated signalling (Bourguignon et al., 2004; Singleton and Bourguignon, 2004). Indeed, it has been described that the polarisation and development of a uropod in T lymphocytes is dependent on the presence of lipid rafts (Millan et al., 2002). It would be interesting to see what role lipid rafts play in CD44-induced T lymphocyte migration and also which proteins interact to form a signalling complex within this ordered lipid domain. Preliminary studies in our group have demonstrated that some signals, which induce lipid raft formation inhibit LFA-1 induced migration. It would be of interest to test if CD44 is functional for migration when occupied in lipid rafts.
In addition to the role of lipid rafts in collecting together proteins required for signal integration, linkage to the cytoskeleton is important in the intracellular transduction of signals from CD44. A number of proteins have been found to be involved in the linkage of the CD44 cytoplasmic domain to the cytoskeleton; namely ankyrin (Kalomiris and Bourguignon, 1988) and the ERM family of proteins (Tsukita et al., 1994). More importantly, ezrin has been described as being important in CD44-mediated migration; mediated by dephosphorylation of serine 325 and the phosphorylation of serine 291 by PKC. This phosphorylation controls the interaction of CD44 with ezrin and CD44-dependent directional migration (Legg et al., 2002). In our study following CD44 cross-linking on the surface of T cells, there is a dramatic rearrangement of the microtubule cytoskeleton, but how do changes in the extracellular domain of CD44 lead to changes in the microtubule cytoskeleton? It is not if the linker proteins previously described are required for signalling changes to the tubulin cytoskeleton. It would be interesting to determine the role of the actin cytoskeleton during the morphological changes that take place during CD44-induced T cell migration. The study by Legg et al., 2002, has highlighted that phosphorylation may take place at one site with dephosphorylation at another site. This may be a critical control point and it will be interesting to examine the phosphorylation status of CD44 following receptor activation. Furthermore, it would be important to determine whether a site-specific phosphorylation deficient mutant would inhibit T cell migration.

It has been demonstrated that small hyaluronan oligosaccharides could induce CD44 ectodomain cleavage and promote tumour migration in a CD44-dependent manner (Sugahara et al., 2003). It would be interesting to test whether CD44 cleavage is important in the migratory response of T cell migration following receptor engagement. It has been suggested that the activity of membrane-type 1 matrix metalloproteinase catalyses the cleavage of the extracellular domain of CD44 enabling cell migration (Kajita et al., 2001). Adhesions formed between the cell and the substrates are critical for the traction required to enable forward movement. However without a mechanism for breaking these adhesions there would be no release of the trailing cell. Previous work has demonstrated CD44 cleavage following antibody cross-linking (Shi et al., 2001), however the response to the native ligand, hyaluronan, is more complex. The size of the hyaluronan oligosaccharides is can influence whether or not CD44 cleavage takes place (Sugahara et al., 2003). In our system, it will be important to determine whether T cell migration, following CD44 receptor engagement, depends on cleavage of the extracellular domain.
The final section of this thesis attempted to identify the protein, p45. This protein was identified using the CD44 monoclonal antibody, D2.1. Our interest in this protein was because of its apparent cell-specific expression; it has been detected on IELs and HUT-78 cells. HUT-78 is a T lymphoma cell line isolated from the peripheral blood of an individual suffering from Sezary Syndrome, which is a CD4+ T cell lymphoma derived from a subcutaneous site (Diamandidou et al., 1996). The absence of p45 on the surface of PBTLs suggests that this protein may be solely expressed by lymphocytes with an affinity for the epithelium. Although to date there has been no success in identifying this protein, it would be interesting to determine the protein sequence of p45. This information could be used to determine the genetic sequence of this protein, which in turn could be inserted into an expression vector. These tools would help with the identification of possible ligands for p45 and in attributing a function to the p45-ligand interaction.

In this thesis, I have demonstrated that CD44 is a signalling molecule for lymphocyte migration. We have identified a number of the downstream signalling events involved in the process. Further characterisation of ligand interaction with CD44 and more precise analysis of the nature of the CD44 signalling complex could permit development of an understanding of the role of CD44-induced migration in normal, physiological disease and potentially in inflammatory disease. Ultimately, if we can understand the position of CD44 in the signalling hierarchy for cell migration, we may be able to design specific inhibitors, blocking a single process in migration. Such inhibition would have potential for use as therapies in inflammatory disease such as rheumatoid arthritis and colitis.
Reagents and Buffers

Cell Tissue Culture

Culture Cocktail

- L-Glutamine 100ml
- Penicillin/Streptomycin 100ml
- 2-mercaptoethanol 37μl

Stored in 10ml aliquots at -20°C.

Hanks Balanced Salts Solution (HBSS) Washing Buffer

- HBSS 500ml
- 1M Hepes Buffer 10ml

RPMI Complete Media

- RPMI 1640 (GibcoBRL) 500ml
- Heat inactivated FCS 50ml
- Culture Cocktail 10ml

CO₂ Independent Complete Media

- CO₂ Independent Media (GibcoBRL) 500ml
- Heat inactivated FCS 50ml
- Culture Cocktail 10ml

Ethidium Bromide (EB) Stock

- EB 100mg
- PBS 20ml

Acridine Orange (AO) Stock

- AO 20mg
- PBS 20ml

EB/AO Working Solution

- EB Stock 4ml
- AO Stock 4ml
Distilled water 1000ml

**Cryogenic Buffer**
- FCS 9ml
- Dimethylsulphoxide (DMSO) 1ml

**Cell Protein Preparation**

**10x Phosphate Buffered Saline (PBS)**
- Na₂HPO₄.2H₂O (8mM) 14.24g
- KH₂PO₄ (1.5mM) 2.04g
- NaCl (137mM) 80.0g
- KCl (2.7mM) 2.0g

pH 7.5 and make up to 1 litre with distilled H₂O.

**Protease Inhibitors Stocks**
- Leupeptin 2mg/ml
- PMSF 1M

**1% Nonidet p40 (NP40)**
- PBS 100ml
- EGTA 38mg
- Nonidet p40 (Igepal CA-630) 946μl

**Hypotonic Cell Lysis Buffer**
- 1mM NaHCO₃ 0.017g
- 5mM MgCl₂ 0.203g

Make up to 200ml with distilled H₂O.

**Buffer A**
- 20mM Tris-HCl, pH 7.5
- 0.25M Sucrose
- 2mM EGTA
- 2mM EDTA

**Buffer B**
- 20mM Tris-HCl, pH 7.5
- 1% (w/v) NP40
150mM NaCl
1mM EGTA
1mM EDTA

**Buffer C**
20mM Tris-HCl, pH 7.5
1% SDS
150mM NaCl
1mM EGTA
1mM EDTA

**BSA Stock Solution (for Bradford Assay)**

BSA 50mg
Make up solution to 50ml with buffer of unknown protein sample to be assayed.

**Bradford Reagent**

Coomassie Blue G 100mg
95% Ethanol 50ml
0.85% Phosphoric Acid 100ml
Make up to 1 litre with distilled water.

**BSA Stock Solution (for Markwell-Lowry Assay)**

BSA 25mg
Make up solution to 50ml with buffer of unknown protein sample to be assayed.

**Markwell-Lowry Reagent A**

Na₂CO₃ (2% w/v) 4g
NaOH (0.4% w/v) 0.8g
K/Na tartrate (0.16% w/v) 0.32g
SDS (1% w/v) 2g
Make up to 200ml with distilled H₂O and store at RT.

**Markwell-Lowry Reagent B**

CuSO₄.5H₂O (4% w/v) 0.4g
Make up to 10ml with distilled H₂O and store at RT.
**Carbonate Buffer**
15mM sodium carbonate
34mM sodium bicarbonate
pH to 9.3 and make up to 100ml. Filter before use.

**SDS-polyacrylamide gel electrophoresis**

**5x Sample Buffer**
- Glycerol 5ml
- 2-mercaptoethanol 5ml
- SDS 2g
- Distilled water 4.5ml
- Stacking gel buffer 5ml
- 1% Bromophenol Blue 0.5ml

**Stock Resolving Gel Buffer**
- Tris base (1.5M) 18.165g
- Distilled water 100ml
- Adjust to pH 8.8 with conc. HCl

**Stock Stacking Gel Buffer**
- Tris base (1M) 12.11g
- Distilled water 100ml
- Adjust to pH 6.8 with conc. HCl

**10% Ammonium Persulphate (APS)**
- APS 100mg
- Distilled water 1ml

**Water Saturated Butanol**
- Butanol 10ml
- Distilled water 10ml
- Mix solution well and allow to settle. Pipette aliquots from the top layer.

**10x Electrode Buffer Stock**
- Tris base 30.3g
- Glycine 144g
- SDS 10g
Distilled water 1000ml
Dilute 1 in 10 with distilled water before use.

**Semi-dry Transfer**

**Transfer Buffer**

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tris base</td>
<td>2.9g</td>
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<tr>
<td>Glycine</td>
<td>1.45g</td>
</tr>
<tr>
<td>SDS</td>
<td>0.185g</td>
</tr>
<tr>
<td>Methanol</td>
<td>100ml</td>
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</tbody>
</table>

Make up to 500ml with distilled water.

**CAPS Transfer Buffer**

3-[cyclohexylamino]-1-sulfonic acid (10mM)  
10% Methanol

Prepare stock of 100mM CAPS. Titrate to pH 11 with 4M NaOH. Store at 4°C.

**Coomassie Blue Gel Stain**

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Quantity</th>
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<tbody>
<tr>
<td>Coomassie Brilliant Blue R</td>
<td>0.5g</td>
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<tr>
<td>Methanol</td>
<td>200ml</td>
</tr>
<tr>
<td>Glacial Acetic Acid</td>
<td>35ml</td>
</tr>
<tr>
<td>Distilled water</td>
<td>265ml</td>
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**Coomassie Blue Gel Stain (for mass spectroscopy analysis)**

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<thead>
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</tr>
</thead>
<tbody>
<tr>
<td>Coomassie Brilliant Blue R</td>
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</tr>
<tr>
<td>Methanol</td>
<td>90.8ml</td>
</tr>
<tr>
<td>Glacial Acetic Acid</td>
<td>9.2ml</td>
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</table>

Make up to 200ml with ultra pure water.

**Gel Destain**

<table>
<thead>
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<th>Ingredient</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Methanol</td>
<td>25ml</td>
</tr>
<tr>
<td>Glacial Acetic Acid</td>
<td>37.5ml</td>
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</tbody>
</table>

Make up to 500ml with distilled water

**Western Blotting**

**Blotto/Tween Blocking Solution**

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Low fat skimmed milk</td>
<td>5g</td>
</tr>
<tr>
<td>PBS</td>
<td>100ml</td>
</tr>
</tbody>
</table>
Tween 20

Make up fresh when required.

Detection of antigen by Enhanced Chemiluminescence

Developing Solution

- Luminol: 12mg
- Iodophenol: 4mg
- DMSO: 0.5ml
- \( \text{H}_2\text{O}_2 \) (30% solution v/v): 18\( \mu \)l
- 0.1M Tris-HCl (pH 8.8): 50ml

Isoelectric focusing gels

Sample Buffer A

- Urea: 0.57g
- Mercaptoethanol: 50\( \mu \)l
- 10% NP-40: 0.2ml
- Ampholytes (pH 3-10): 50\( \mu \)l

Make up to 1ml with distilled water.

Overlay Buffer

- Urea: 0.54g
- Ampholytes (pH 3-10): 25\( \mu \)l

Make up to 1ml with distilled water.

Sample Buffer B (1x)

- SDS: 2.0g
- 1M Tris (pH 6.8): 2.5ml
- Glycerol: 10ml
- 0.4% Bromophenol Blue in ethanol: 0.3ml
- Mercaptoethanol: 5ml

Make up to 100ml with distilled water.

Agarose (1%)

- Agarose: 0.1g
- Distilled water: 10ml

Heat until melted, reheat as necessary.
Rehydration Solution

8M Urea 12g
CHAPS (2% w/v) 0.5g
Pharmalyte 3-10 (0.5% v/v) 125μl
Bromophenol blue (0.002%) 50μl of 1% solution

Make up to 25ml with distilled water. This stock solution can be stored frozen in 2.5ml aliquots. Before use the rehydration solution is thawed and 7mg of DTT added.

SDS Equilibration Buffer

50mM Tris, pH 8.8 10ml of Resolving Gel Buffer
6M Urea 72.07g
Glycerol (30% v/v) 69ml of 87% v/v stock
SDS (2% w/v) 4.0g
Bromophenol blue (0.002%) 400μl of 1% solution

Make up to 200ml with distilled water. Store at −20°C in 10ml aliquots. Before use thaw equilibration solution and add 100mg DTT per 10ml of buffer.
APPENDIX 2

Suppliers

Peptide Inhibitors
Control peptide, carrier/carrier  Prof. D. Mochly-Rosen, Stanford, U.S.A.
PKCβ antagonist, βC2-4/carrier  Prof. D. Mochly-Rosen, Stanford, U.S.A.
PKCδ antagonist, δV1-1/carrier  Prof. D. Mochly-Rosen, Stanford, U.S.A.

Antibodies
Anti-CD3 (OKT3)  ATCC, Manassas, VA, U.S.A.
Anti-CD44 (D2.1)  Prof. M. B. Omary, San Diego, U.S.A.
Anti-CD44 (J173)  Immunotech, Marseille, France
Anti-CD44 (L3D1)  Prof. M. B. Omary, San Diego, U.S.A.
Anti-CD44 (F10-44-2)  Serotech, Oxford, U.K.
Anti-ICAM-1 (Mem111)  Sanbio, Uden, The Netherlands
Anti-LFA-1 (SPV-L7)  Sanbio, Uden, The Netherlands.
Mouse anti-PKCβ  Zymed Laboratories, San Francisco, U.S.A.
Rabbit anti-PKCβ  Sigma
Mouse anti-PKCδ  Transduction Laboratories, Lexington, U.S.A.
Rabbit anti-PKCδ  Research and Diagnostic Antibodies, U.S.A.
Mouse anti-tubulin beta  Sigma
APPENDIX 3

Protein Purification Techniques

Immunoaffinity chromatography

The recognition of an antigen by an antibody and the formation of a non-covalently bonded immunological complex is the basis of immunoaffinity chromatography. Properties of the antigen-antibody interaction include the formation of a stable but reversible complex, with highly selective recognition of a single molecule from a complex mixture of components. Usually this process does not lead to irreversible damage of the component of interest, which may retain biological activity. Another advantage of this method is that frequently the immunoaffinity matrix can be reused. The availability of D2.1, a monoclonal antibody that recognises p45 provided a useful tool for the purification of this protein. The process of immunoaffinity chromatography requires as a first step the chemically bonding of the monoclonal antibody to an inert polymer support, which acts as an immunoadsorbant. The crude antigen is then passed through the immunosorbant column and the unabsorbed materials are washed away. The specifically bound antigen is then eluted with mild desorbing agents.

Protein G sepharose (PGS) beads were used to immobilise D2.1. Protein G binds to the Fc region of IgG, leaving the bound antibody orientated so the Fab region is available for antigen binding. PGS beads were incubated with the purified D2.1 antibody and were allowed to associate. In order to immobilise the antibody on the beads a chemical cross-linker was used; dimethylpimelimidate (DMP). The efficiency of coupling can be checked using samples taken before and after coupling bead coupling. Before cross-linking with DMP both the heavy chain (55kDa) and the light chain (25kDa) of the antibody IgG can be detected in the beads sample when run on a SDS polyacrylamide gel. However, following incubation with DMP only the light chain of the IgG should be detectable, as the heavy chain is now covalently linked to the PGS beads. With the antibody now covalently linked to the PGS beads, this can now be used as an immunoaffinity matrix.

Low pH conditions (i.e. 100mM glycine, pH 2.5) were used to elute the antigen. In order to ensure a rapid change in pH, 10mM phosphate, pH 8.0, was used as a pre-elution buffer, thus removing the buffering capacity of PBS. As described in 5.3.7, to ensure no denaturation of the antigen occurs the eluate must be immediately
neutralised using 1M Tris-HCl, pH 8.0. In preparation, 100μl of the neutralisation buffer was added to labelled eppendorfs. The elution buffer was placed on the column and 1ml fractions collected in the prepared eppendorfs, ensuring careful mixing of the solution. The resulting fractions were acetone precipitated and examined by western blot. As can be seen from figure 5.10 b p45 is still present in the HUT-78 membrane lysate, indicating that there has not been complete removal of p45 from the sample. p45 is being detected in all fractions, both before and after the elution conditions. There does seem to be a higher proportion of p45 in the first step of the elution (lane 5).

**Preparative SDS PAGE**

Preparative SDS PAGE was used to isolate p45 from pooled membrane fractions. This provides a method of separating protein on the basis of molecular weight from a complex protein mixture. The Model 491 Prep Cell is designed to purify proteins or nucleic acids form complex mixtures by continuous-elution electrophoresis (Figure A). During a run the samples are electrophoresed through a cylindrical gel. As the molecules migrate through the gel matrix they are separated into ring shaped bands. When individual bands migrate off the bottom of the gel they pass directly into the elution chamber for collection. Purified proteins are drawn up through the elution collection tube by a peristaltic pump, which in turn is connected to a fraction collector.

Individual fractions were then analysed by western blotting for the presence of the antigen. The variables to be considered for optimum resolution in preparative SDS-PAGE are; gel pore size, gel length and gel tube diameter. A balance between degree of separation (i.e. length of gel) and sharpness of protein bands (i.e. estimated time for elution of protein) is required to determine the parameters of the gel poured.

Membrane proteins were collected from HUT-78 and K4 cells. When sufficient protein was collected these were separated using the Prep Cell.

**Pouring of the Resolving Gel**

Before pouring the resolving gel, ice-cold water was recirculated through the cooling core of the rig (to ensure slow polymerisation). It is important to ensure that the gel tube apparatus on the casting stand is level, by using the spirit level to help with adjustments to the legs of the casting stand. The monomer mixture was carefully added to ensure there were no bubbles trapped within the tube and tapping against the bench top dislodged any bubbles present. The resolving gel was carefully overlaid with water-saturated butanol. The cylindrical SDS polyacrylamide gel was set overnight in the cold-room, exchanging the water-saturated butanol with resolving buffer. Cooling
Figure A: Model 491 Prep Cell Components.
This figure taken from BioRad website illustrates the operational features of the Model 491 Prep Cell. This preparative electrophoresis separates a complex protein mixture by molecular weight, with separated proteins collected by a fraction collector.
of the gel as it polymerises slows the process and ensures a more uniform gel. The gel was prepared the day before use to ensure complete polymerisation and to reduce the presence of free primary amines in the gel, which may lead to N-terminal modification of the proteins. Also, the 4 litres of running buffer required for the PrepCell run was cooled overnight.

HUT-78 membrane proteins (Run 1)
The membrane fractions were pooled (approximately 15ml) from subcellular fractionation experiments carried out on HUT-78 cells. These were dialysed against 50mM Tris, pH 7.5, 0.1% Np40, to reduce the salt content of the sample. The protein concentration was estimated to be approximately 14mg using a Markwell assay [See 2.3.2]. This was acetone precipitated overnight at -20°C, centrifuged at 14,000 rpm for 15 minutes, the acetone aspirated and the pellet allowed to air dry. The resulting precipitate was resuspended in approximately 1.5ml of 1x sample buffer and boiled for 5 minutes.

For the isolation of p45, the optimal gel pore size/polyacrylamide concentration was a 10% gel mix. The 10% resolving gel was poured to a height of 8.3cm a day in advance of the experiment and the stacking gel was made up to a final height of 10cm. Once the gel is complete and the apparatus set up, the cooled running buffer (with 2mM thioglycolate) was placed in the PrepCell. The sample was carefully loaded and gel was run under the following settings (constant current 45mA, voltage 500mV and power 12W). Fractions were collected once the bromophenol blue dye front had reached the end of the gel.

K4 membrane proteins (Run 2)
As described above, K4 membrane fractions were pooled (approximately 20ml) from subcellular fractionation experiments. These were dialysed against 50mM Tris, pH 7.5, 0.1% Np40, to reduce the salt content of the sample. The protein concentration was estimated to be approximately 20mg using a Markwell assay [See 2.3.2]. This was acetone precipitated overnight at -20°C, centrifuged at 14,000 rpm for 15 minutes, the acetone aspirated and the pellet allowed to air dry. The resulting precipitate was resuspended in approximately 2ml of 5x sample buffer and boiled for 5 minutes.

The aim of this experiment was to isolate both p45 and p135. This was an attempt to test whether p135 would disassociate leaving sCD44 and p45, with no other contaminants with a similar molecular weight. For the isolation of p135, the optimal gel
pore size/polyacrylamide concentration was a 7.5% gel mix. The 7.5% resolving gel was poured to a height of 9.8cm a day in advance of the experiment and the stacking gel was made up to a final height of 11cm. Once the gel is complete and the apparatus set up, the cooled running buffer (with 2mM thioglycolate) was placed in the PrepCell. The sample was carefully loaded and gel was run under the following settings (constant current 50mA, voltage 500mV and power 12W). Fractions were collected once the bromophenol blue dye front had reached the end of the gel.

**Two-dimensional electrophoresis**

Two-Dimensional electrophoresis separates proteins using isoelectric focusing in the first dimension and by molecular weight using SDS PAGE in the second dimension. The principle of isoelectric focusing is that in a pH gradient proteins migrate towards the anode or the cathode to the pH value, where their net charge is zero: their isoelectric point (pI). If the protein were to diffuse away from its pI, it would gain a charge and migrate back. The proteins are driven to their isoelectric point by an electric field.

There are two principle methods for establishing a pH gradient for isoelectric focusing. The first method uses carrier ampholytes. These are mixtures of small molecular weight amphoteric compounds with a spectrum of isoelectric points between the defined pH range and these form a pH gradient under the influence of the electric field. This was the type of pH gradient was initially used (see section 5.3.2) and reproducibility is made difficult because differences in gel preparation, height of the gel and running conditions can have an impact on the pH gradient in the gel. However many of these problems are overcome by the use of immobilised pH gradients (IPG), as these have fixed gradients. IPG DryStrips when used with the Ettan IPGphor Isoelectric Focusing System (Amersham) allow for highly reproducible isoelectric focusing.
CD44 cross-linking induces protein kinase C-regulated migration of human T lymphocytes

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Keywords: microtubules, protein kinase C, T cell

Abstract

The cell surface receptor CD44 is widely implicated in leukocyte migration to inflammatory sites. In this study, the responses of human T cells following cross-linking of CD44 were examined. We demonstrate that engagement of CD44 using immobilized mAbs or hyaluronan-enriched extracellular matrix lattices induces active migration in T lymphocytes accompanied by cycles of cytoskeletal rearrangement and cell polarization. We have investigated the functional impact and subcellular localization of protein kinase C (PKC) isoenzymes, β and δ, previously shown by our group to be involved in active T cell locomotion induced by leukocyte function-associated antigen-1 (LFA-1) integrin receptors. PKCδ was associated with the centrosome and the microtubule-rich tail of the polarized cell and PKCδ was predominantly located about the region of the microtubule organizing center. A selective pharmacological inhibitor of classical PKC isoforms, G66976, suppressed lymphocyte polarization and migration following CD44 ligation. Selective targeting of PKCδ using the pharmacological inhibitor rottlerin or a pseudosubstrate-blocking peptide reduced CD44-activated cell migration but did not completely ablate it. Our data demonstrate that ligation of CD44 induces phenotypic changes, cytoskeletal rearrangements and redistribution of PKC isoforms β and δ, resulting in cell migration, as previously described for the cell surface receptor, LFA-1. This suggests potential convergence of intracellular signaling pathways induced via CD44 and LFA-1 integrin.

Introduction

Efficient operation of the adaptive immune system requires the continuous migration of T lymphocytes from circulation and across the endothelium into tissue and back to blood again, either at lymphoid organs or at sites of inflammation. This process is characterized by initial tethering of the lymphocyte to the surface of specialized endothelial cells (1), which is thought to be mediated largely by selectin interaction with their carbohydrate ligands expressed on the endothelium surface, although other lymphocyte receptors have been implicated in this process (2–4). This slows lymphocyte flow and cells roll along the surface of the endothelium until chemokines activate molecules of the integrin family on T cells, leading to lymphocyte arrest and subsequent diapedesis across the endothelium. Here, we examine T cell migration induced by cross-linking of the CD44 receptor, a glycoprotein previously ascribed a role in lymphocyte homing and inflammation (2, 5–7).

CD44 is a broadly distributed transmembrane glycoprotein that is involved in a number of normal processes, such as regulation of growth, survival, differentiation and motility, whereas its altered expression or dysfunction may contribute to malignancy; for example, expression of CD44 variants can mean a poor prognosis in colorectal carcinomas [reviewed in Ponta et al. (8)]. This receptor has also been associated with several functions of the normal immune system. Expression is increased following antigen stimulation of the TCR (9), and CD44 is reported to be co-stimulatory for T cell activation (10). The role of CD44 in lymphocyte homing to sites of inflammation was clearly demonstrated by Camp et al. (5), who showed that antibody-induced loss of CD44 slowed the onset of a cutaneous delayed-type hypersensitivity response following antigen challenge in a murine model, although migration to lymphoid sites was normal. The CD44-negative, unactivated

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lymphocytes migrate identically to cells from control mice, but following challenge, the response is altered, with lymphocyte extravasation to inflammatory sites delayed. Similarly, a study using murine CD44−/− T cells confirmed that receptor expression was not required for normal lymph node homing but the migration of CD44-deficient cells into areas of inflammation was delayed (11). A role for CD44 in the pathogenesis of inflammatory disease has been implicated by a number of studies. The level of circulating T cells expressing activated CD44 was found to be elevated in patients with autoimmune diseases, such as systemic lupus erythematosus and arthritis (12). Administration of anti-CD44 antibodies inhibited inflammation in murine models of inflammatory bowel disease, collagen (CL)- and proteoglycan-induced arthritis and cutaneous inflammation (13), and has led to interest in CD44 as a target for possible therapeutic intervention or as a marker of autoimmune disease activity. The principal ligand for CD44 is hyaluronan (HA), a broadly distributed glycosaminoglycan, whose expression on endothelial cells is inducible by pro-inflammatory cytokines and mediates CD44-dependent rolling during inflammation (7, 14).

Although not all ligands are identified, CD44 is known to interact with the extracellular matrix (ECM) components, CL, laminin and fibronectin, as well as osteopontin and serglycin (15). Protein kinase C (PKC) enzymes are a family of serine-threonine kinases that are divided into three classes (i) classical (α, β I, β II, γ), (ii) novel (δ, ε, η, θ) and (iii) atypical (ζ, τ)/ and are responsible for signal transduction in many systems (16). PKC signaling has been implicated in CD44 function. For example, the binding of CD44 to its native ligand HA is altered following phosphor myristate acetate (PMA) treatment (17) and CD44 acts as a substrate for PKC phosphorylation (18).

Importantly, a recent study describes the complex regulation of phosphorylation and dephosphorylation of the cytosolic domain of CD44 by PKC activation, a process that allows the dissociation of the CD44–ezrin complex and controls directional migration (19). In addition, PKCβ I (identified here as PKCβ) has been shown to be crucially important for leukocyte function-associated antigen-1 (LFA-1)-mediated locomotion (20). Therefore, we investigated the role of PKC isoforms in mediating the migratory response of T cells following CD44 ligation. Using HUT-78 cells, a T lymphoma cell line and peripheral blood T lymphocytes (PBTLs), we were able to demonstrate active migration following CD44 cross-linking. The PKC isoforms, β and δ, were found to be associated with the microtubule cytoskeleton in cells displaying a migratory, polarized phenotype. The use of pharmacological inhibitors and pseudosubstrate-blocking peptides allowed the function of these PKC isoforms in CD44-induced migration to be investigated and established a critical role for PKCβ in this event.

Methods

Cells and cell culture

In most experiments, the human T lymphoma cell line HUT-78 (American Type Culture Collection, Manassas, VA, USA) was used. Where indicated, normal human PBTLs were isolated using a Ficoll-Hypaque density gradient. Before PBTLs were used for experiments they were activated (21) by pre-treatment with 25 ng ml−1 PMA (Sigma, St. Louis, MO, USA) or 500 U ml−1 of purified protein derivative (PPD) prepared from Mycobacterium tuberculosis (Medeva Pharma Ltd, Leatherhead, UK) for 72 h at 37°C. Cells were maintained using CO2-independent Media (Gibco BRL, Rockville, MD, USA) supplemented with 10% FCS, 1% penicillin and 1% streptomycin at 37°C.

T lymphocyte migration

Lymphocyte migration studies for 2-D analysis and video recording were performed using Lab-Tek eight-well chambered slides (permanox plastic or borosilicate) or 96-well plates (Nalge Nunc Intl., Naperville, IL, USA). The surfaces were pre-coated with goat anti-mouse Ig (Dako, Bucks, UK) and subsequently incubated with cross-linking mAbs. Antibodies to CD44 used were D2.1 (22), J173 (Immunotech, Marseille, France), L3D1 (a gift from M. B. Omary) or F10-44-2 (Serotech, Oxford, UK). Other antibodies used were an anti-LFA-1 mAb, SPV-L7, an anti-intracellular adhesion molecule-1 (ICAM-1) mAb, Mem111 (Sanbio, Uden, The Netherlands) and an anti-CD3 mAb, OKT3 (American Type Culture Collection). As a control, wells were coated with poly-L-lysine or cells were seeded onto positively charged slides (cells adhered to this surface without activation). Cells were seeded into chambers at a density of 2 × 10⁴ cells per well in 200 μl of warmed media. Studies were carried out using the selective PKCδ inhibitor, rottlerin (inhibitory concentration 50% (IC50) = 3–6 μM) (Alexis Corp., San Diego, CA, USA) and a selective classical PKC inhibitor G66976 (IC50 = 7.9 nM) (Calbiochem, San Diego, CA, USA). The PKCδ and ε-specific inhibitory peptides and control peptide were a kind gift of Daria Mochly-Rosen (Stanford University, CA, USA). Cells were pre-treated with inhibitors at the indicated concentration for 30 min at 37°C before being seeded onto immobilized cross-linking antibodies. Cells were fixed 4 h after incubation with immobilized antibody in pre-coated chambers (when activation was maximal).

The assays utilizing T cell migration in 3-D matrices were performed in the wells of flat-bottomed 96-well plates pre-filled with ECM gel (Sigma) alone or enriched with either of two distinctive molecular weight hyaluronic acid variants mixed with the ECM gel at the final concentration of 1 mg ml−1. High-molecular weight (1.76 × 10⁶ Da) HA was from LifeOmic Biomedical, Inc., Chaska, MN, whereas HA fragments (200 kDa) were purchased from ICN (Valeant) Pharmaceuticals (Costa Mesa, CA, USA). T cells were seeded on the surface of polymerized gels and allowed to migrate into the gel in the presence or absence of PKC inhibitors. The assays were stopped after 60 min by addition in the wells of 4% formaldehyde at ambient temperature for 30 min. The wells were then washed in PBS, cells stained with acridine orange solution and the number of cells migrated into the gels analyzed on the confocal UltraVIEW Live Cell Imager (Perkin Elmer, Cambridge, UK) using ×20 dry long working distance lens. At least five randomly chosen microscopic fields taken at the equivalently positioned Z-plane for experiment and control wells were included in the analysis. For the high-resolution analysis of the morphology of the migrating cells,
the assays were set up in a similar manner, but using eight-well chambered borosilicate coverslips (Nunc) instead of 96-well plates.

**Deformation index studies**

In order to study cell morphology, the activated lymphocytes were fixed using 3.7% PFA. Images from five randomly selected fields of view were recorded for analysis. The fixed cells were scored using the following formula for the deformation index (DI):

\[
\text{deformation index} = \frac{\text{elongation index}}{\text{circularity index}}
\]

where

\[
\text{elongation index} = \frac{\text{major ellipse diameter}}{\text{minor ellipse diameter}}
\]

and

\[
\text{circularity index} = 4 \times \pi \times \left(\frac{\text{area}}{\text{perimeter}^2}\right)
\]

This index provides a stronger measure of the degree of cell polarization in comparison to elongation index and circularity alone. Typically values of 1-3 are assigned to cells that were non-migratory, i.e. cell shape nearing circular. Those cells that displayed a polarized phenotype (cell body and trailing uropod) and therefore a higher degree of deformation had higher DI values (>5) (Volkov et al., manuscript in preparation). On average, >40 randomly chosen cells were scored for each set of conditions. Analysis was performed using the NIH Image software (Scion Corporation, Frederick, MD, USA).

**Time-lapse video microscopy**

Cell migration was monitored on a Nikon TE-300 inverted microscope equipped with a JVC TK-C1380 CCD camera. Sequential image frames were digitized and cell migration was evaluated by the distance traveled by the cell centroid over the entire observation period (indicated in the figure legends for each study). Between 40 and 50 cells were recorded and analyzed per observation field.

**Immunofluorescence staining**

Prior to fixation for immunofluorescence analysis, the unbound cells were washed from slides with warm PBS, and attached cells were fixed in acetone at -20°C. Isoform-specific PKC antibodies were used to determine the subcellular localization of PKCα (Research and Diagnostic Antibodies, Berkeley, CA, USA) and PKCβ (I) (Sigma). These were visualized using a FITC-labeled secondary antibody (Dako). The microtubule cytoskeleton was examined using a mAb raised against α-tubulin and detected using a tetramethylrhodamine isothiocyanate (TRITC)-labeled secondary antibody (Sigma). Fluorescently labeled cells were examined using an oil immersion ×100 lens on a Nikon TE3000 inverted microscope attached to UltraVIEW Live Cell Imager (Perkin Elmer) confocal workstation.

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**Statistics**

The significance of differences observed in the various assays was evaluated by the Mann-Whitney test, an analysis of non-paired, non-parametric measurements.

**Results**

Cross-linking CD44 induces a polarized, locomotion-associated phenotype in T lymphocytes

In this study the response of both HUT-78, a T lymphoma cell line, and PBTLs following ligation of their CD44 receptor was examined using a series of immobilized CD44 mAbs. The antibodies used were D2.1, J173, L3D1 and F10-44-2. F10-44-2 has been reported to up-regulate HA-binding ability of activated T cells whereas J173 was found to inhibit HA adhesion in these activated cells (23). When the CD44 receptor was cross-linked in HUT-78, the cells developed a polarized morphology (Fig. 1), characterized by a leading cell body and a trailing process, in agreement with data previously reported by Kelleher et al. (21). All antibodies used induced a migratory phenotype. A similar polarized phenotype was seen in LFA-1-activated lymphocytes (Fig. 1) and these morphological changes were demonstrated to correlate with active lymphocyte locomotion (20). Cross-linking of CD3, ICAM-1, and other adhesion and signaling receptors expressed on the surface of T cells did not induce these morphological changes (Fig. 1). Cells adhered to the substrate following cross-linking of CD3, but they did not develop a polarized morphology. A similar response was seen with the ligation of ICAM-1. Resting PBTLs did not display any morphological changes in response to ligation of either CD44 or LFA-1, but required pre-activation with a PKC activator such as the phorbol ester PMA or antigen stimulation for 72 h. Pre-exposure of T cells to the tuberculin antigen, PPD, resulted in the formation of a locomotory phenotype following CD44 cross-linking in a discrete significant population of antigen-responsive cells, similar to PKC-activating agents.

Using time-lapse video microscopy, we verified that the locomotory phenotype displayed by CD44-activated cells was accompanied by active migration. Measuring the displacement of the cell centroids over a 60-min observation period gave clear evidence that both HUT-78 cells and PBTLs triggered through cross-linking of CD44 traveled at a similar rate to that of cells activated through cross-linking of LFA-1. Mean velocity traveled by both LFA-1 and CD44-activated HUT-78 cells were similar at 14.7 μm h^-1 (mean value of peak velocity -76.65 μm h^-1) and 14.4 μm h^-1 (mean value of peak velocity -52.94 μm h^-1), respectively, while PBTLs were migrating at 15.0 μm h^-1 (mean value of peak velocity -56.7 μm h^-1) and 12.0 μm h^-1 (mean value of peak velocity -39.7 μm h^-1) following ligation of LFA-1 and CD44, respectively (Fig. 2), a slow and apparently random mode of locomotion.

**CD44-dependent T cell migration in 3-D ECM lattices**

Following cross-linking of CD44 in the activated PBTLs, the cells developed a polarized morphology, with the response most pronounced in D2.1 activated cells (Fig. 1). However,
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Fig. 1. Phenotypic changes in HUT-78 T lymphoma cells and PBTLs induced by CD44 antibodies. HUT-78 cells and PBTLs (activated for 72 h using PMA or antigen-stimulated with PPD as described in Methods) developed a polarized morphology following cross-linking of CD44 using the mAbs D2.1, J173, L3D1 and F10-44-2. Panel PPD + D2.1 shows antigen-specific T cells acquiring a locomotory behavior on anti-CD44 D2.1 mAbs. Similar phenotypic changes were seen with cells activated by cross-linking LFA-1, using SPV-L7. Cross-linking of CD3 using OKT3 and ICAM-1 using Mem111 did not induce the same morphological changes. Resting PBTLs (not activated via antibody or PMA pre-treatment) remain static and non-polarized (data not shown).

Fig. 2. Active T lymphocyte migration following ligation of CD44. The average distance traveled by HUT-78 cells and PBTLs activated by ligation of either CD44 or LFA-1 over 1-h intervals measured using time-lapse video microscopy. Cell locomotion was triggered via the CD44 pathway using immobilized D2.1 and by LFA-1 cross-linking with immobilized SPV-L7. Bars reflect mean values ± SEM obtained in three independent studies (light gray, dark gray and hatched bars, respectively).

Translocation of PKC isoforms to the microtubule cytoskeleton in response to CD44-induced migration

We next examined the distribution of the PKC isoforms following ligation of CD44. Resting HUT-78 stained for PKCε showed a diffuse staining pattern with discrete cytoplasmic localization and partial association with the microtubule cytoskeleton. Following activation of CD44 by receptor cross-linking, the microtubule cytoskeleton becomes reorganized with the microtubule organizing center (MTOC) located to the rear of the migrating cell body with a microtubule-rich trailing process. There was a redistribution of PKCβ to the region of the MTOC and along the microtubule-based trailing process in the CD44-activated HUT-78 (Fig. 4). Examination of PKCδ localization in resting HUT-78 cells showed a diffuse cytoplasmic staining with an association with the MTOC. In the migrating T cells, PKCδ was found localized to a position adjacent to the MTOC (Fig. 5) and also present as uninvolved cytoplasmic pool in the cell body.

Suppression of PKC activity attenuates CD44-induced T cell motility

In order to further investigate the role of the above-indicated PKC isoforms, selective inhibitory strategies were implicated. As PKCβ translocation was observed following CD44 ligation, we used G66976 to examine the functional consequences of classical PKC isoform inhibition. HUT-78 cells were pre-treated with G66976 for 30 min before activation on immobilized cross-linking antibody. Untreated CD44-activated cells...
developed a polarized and elongated morphology (Fig. 6a), but when pre-incubated with G66976, HUT-78 cells on immobilized anti-CD44 remained adherent, but no longer acquired this morphology (Fig. 6b). Similar results were seen with anti-LFA-1 (Fig. 6d and e). Using the DI as a quantitative descriptor of cell morphology, the development of a motile phenotype was inhibited by G66976 in a dose-dependent manner (Fig. 6g) when cells were activated by CD44 cross-linking. LFA-1-activated cells respond to G66976 pre-treatment in a comparable manner (Fig. 6h).

Further work was carried out utilizing time-lapse video microscopy to determine if the failure of cells to develop a migratory phenotype was also accompanied by the loss of active locomotion. Following G66976 pre-treatment, HUT-78 cells showed significantly decreased migration in response to either LFA-1 or CD44 activation (Fig. 6i). Additional evidence for the role of PKCβ in CD44-induced migration was obtained by analyzing the response of K4 cells to CD44 ligation. K4 cells are a HUT-78 clone deficient in PKCβ (20, 24). These cells failed to migrate successfully following cross-linking of CD44 (Fig. 6c, f and i).

G66976 inhibited CD44-stimulated cytoskeletal reorganization and cells did not form microtubule-rich tails. There was no significant development of a polarized phenotype, but the MTOC was located in the position adjacent to the nucleus typical of motile cells. In these cells, there was a clear association of PKCβ at the area of the MTOC, but not with the microtubules. Rather, this isoform remained in a diffuse cytosolic location (Fig. 7). In G66976-treated cells, CD44 cross-linking did not induce redistribution of PKCβ and it remained concentrated in discrete spots in the region of the MTOC similar to the resting cells (Fig. 8).
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We subsequently used an isomform-specific PKC inhibitor rottlerin to examine the role of PKCδ in T cell migration. HUT-78 cells were pre-treated with rottlerin, at concentrations of 1, 5 and 10 μM, for 30 min before activation through either CD44 or LFA-1. On morphological examination cells appeared flattened, and frequently produced multiple thin cytoplasmic projections reflecting that the adhesion per se was not distorted (Fig. 9b and d). The DI did not show significant alteration in response to rottlerin pre-treatment (Fig. 9e and f) and time-lapse video revealed migration to be reduced but not completely ablated (Fig. 9g).

HUT-78 cells treated with rottlerin prior to cross-linking of CD44 were subsequently examined to determine the localization of PKCβ and PKCδ. As can be seen in Figs 7 and 8, the extended processes are tubulin-rich and are supported by well-defined microtubule structures. While PKCβ remained associated with the area of the MTOC, it was not fully associated with the microtubule-rich projections (Fig. 7). In the resting cell, localization of PKCδ at the MTOC appears diffuse. As can be seen in Fig. 8, in rottlerin-treated cells PKCδ displayed a granular pattern and were not as tightly associated with the MTOC as commonly seen in the untreated migrating T cell (Fig. 5).

In order to more clearly delineate the impact of PKCδ on T cell locomotory properties, we further extended the studies in a 3-D model described above utilizing HA-enriched ECM in the presence of cell-permeable isomform-specific pseudosubstrate PKC inhibitory peptides (Fig. 10). The PKCδ-specific peptide significantly decreased T cell migration into the 3-D ECM lattices incorporating high-molecular weight HA in comparison to the ECM–HA alone and control peptide. The peptide blocking another functionally distinctive novel PKC isoform did not significantly affect cell motility. These findings clearly demonstrate the requirement of intact PKCδ function for T cell locomotion in a physiological microenvironment.

Discussion

This study was designed to examine the response of human T cells to ligation of the adhesion receptor, CD44. Following activation of CD44, these cells acquired a polarized migratory phenotype, which we subsequently confirmed to be associated with active T cell migration. Further investigation revealed a role for the PKC isoforms δ and ε in the migratory response following receptor triggering. Using a series of immobilized mAbs to cross-link CD44, we observed in human lymphocytes and HUT-78 cells a human T lymphoma cell line, the development of an elongated morphology with leading edge, cell body and posterior trailing cytoplasmic projections (Fig. 1). We did not observe an equivalent percentage of polarized T cells in response to purified HA immobilized on a planar substrate. However, incorporation of HA into 3-D ECM lattices significantly enhanced T cell polarization and migration. HA is abundantly expressed in many tissues and is now known to play a role in the regulation of cell motility, invasion and proliferation (25). Although HA is the primary ligand for CD44, the failure of HA to elicit the same magnitude of response in T cells as cross-linking antibodies in a 2-D model may be due to a number of reasons. The activation state, glycosylation and density of CD44 will influence its response to HA ligation (9, 17, 26, 27). In addition, under near-physiological conditions mimicked by a 3-D ECM lattice, HA is presented to the lymphocyte CD44 receptor in a more complex spatially organized form. In a real in vivo situation HA might be also immobilized and spatially oriented by a counter-receptor on the endothelial cell surface (28).

Using time-lapsed video microscopy, we have demonstrated active HUT-78 and human T lymphocyte migration in response to cross-linking of CD44 using immobilized mAbs. These CD44-activated T cells develop a similar morphology and migrate at a similar velocity as those cells activated by ligation of LFA-1 (Fig. 2). The relevance of this slow cell migration to the in vivo microenvironment is discussed by Volkov et al. (20). A recent study by Katakai et al. (29) demonstrated that the transendothelial migration of Thy1 cells across a murine endothelial cell line could be blocked using antibodies to CD44 (as well as LFA-1 and ICAM-1). Cross-linking of CD44 on the surface of these murine Thy1 cells resulted in the development of an elongated polarized morphology, which was similar to the phenotype seen in this study (29). T cells develop a similar polarized morphology following cross-linking of LFA-1 (using both recombinant ICAM-1 and a motility-inducing anti-LFA-1 antibody), which was earlier demonstrated to be associated with active migration (20). During our study of T cell migration, Jurkat, a CD44-negative T leukemic cell line, did not produce
a migratory response to LFA-1 cross-linking. This finding may further underline the co-operative interaction between CD44 and LFA-1 in the induction of a T cell migration.

Several sets of evidence have stressed the importance of CD44 in T lymphocyte migration to sites of inflammation. There is increased expression of functionally active CD44 on T lymphocytes in the peripheral blood of human patients with active autoimmune diseases (12). Expression of HA on endothelial cells is up-regulated in response to stimulation by pro-inflammatory cytokines (7,14). This has served to build up a model for the role of CD44 in inflammatory conditions, with the initial activation of CD44 on antigen-specific T cells within the lymph node, subsequent mobilization of these cells into the periphery and finally CD44-dependent migration to inflammatory sites. Here, we have evidence that CD44 ligation signals the firm adhesion, polarization and repeated cycles of cytoskeletal reorganization required for the controlled migration of human T cells during extravasation to inflammatory sites.

Our data demonstrate both the dependence of CD44-induced T cell migration on the functional PKC status and isoform-specific intracellular redistribution of PKC enzymes accompanying active cell locomotion. A number of studies have implicated PKC signaling in the migratory response. The importance of PKCβ in mediating LFA-1-induced T cell migration was demonstrated by Volkov et al. (20) and highlighted the critical role of PKCβ association with the microtubule cytoskeleton. Inhibition of classical PKC isoforms significantly decreased HUT-78 migration in response to CD44 ligation (Fig. 61). When localization of PKCβ and δ was examined these PKC isoforms remained associated about the area of the MTOC when pre-treated with 10 μM G66976 or dimethyl sulfoxide (DMSO) control followed by receptor cross-linking using SPV-L7 (anti-LFA-1) or D2.1 (anti-CD44) (* indicates P<0.05 reduction in migration relative to control). Also shown is distance traveled by K4 cells following cross-linking of CD44.

Fig. 6. HUT-78 migration-associated characteristics in response to CD44 ligation following inhibition of classical PKC isozymes. Typical polarized morphology of HUT-78 cells following ligation of CD44 (a) and LFA-1 (d). Cells pre-treated with 10 μM G66976 display a rounded morphology when incubated with immobilized anti-CD44 (b) and anti-LFA-1 (e). Also shown is the typical morphology of CD44-activated K4 cells at t = 0 and t = 60 min following incubation with immobilized D2.1 (c) and (f), respectively. Dose-response migration inhibition by G66976 in cells activated via immobilized anti-CD44 (g) and anti-LFA-1 (h) (as determined by measurement of the deformation index). (i) Distance traveled during the 1-h recording interval was measured for cells pre-treated with 10 μM G66976 or dimethyl sulfoxide (DMSO) control followed by receptor cross-linking using SPV-L7 (anti-LFA-1) or D2.1 (anti-CD44) (* indicates P<0.05 reduction in migration relative to control). Also shown is distance traveled by K4 cells following cross-linking of CD44.
Fig. 7. Localization of the PKCβ isoform in G6976 and rottlerin pre-treated HUT-78 cells following CD44 cross-linking. Resting (left panel), HUT-78 on negatively charged slides. CD44 + G6976 (middle panel), 10 μM G6976 pre-treated HUT-78 cells incubated on immobilized D2.1. CD44 + Rottlerin (right panel), 5 nM rottlerin pre-treated HUT-78 cells incubated with immobilized D2.1. PKCβ (green) and α-tubulin (red) staining of the same cells. Merged image, identical microscopic fields illustrating PKCβ and tubulin co-localization (yellow/orange overlay).

Fig. 8. Localization of the PKCδ isoform in G6976 and rottlerin pre-treated HUT-78 cells following CD44 cross-linking. Resting (left panel), HUT-78 on negatively charged slides. CD44 + G6976 (middle panel), 10 μM G6976 pre-treated HUT-78 cells incubated on immobilized D2.1. CD44 + Rottlerin (right panel), 5 nM rottlerin pre-treated HUT-78 cells incubated with immobilized D2.1. PKCδ (green) and α-tubulin (red) staining of the same cells. Merged image, identical microscopic fields illustrating PKCδ and tubulin co-localization (yellow-orange overlay).
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Fig. 9. HUT-78 migration-associated characteristics in response to CD44 ligation following inhibition of PKCδ. Typical polarized morphology of HUT-78 cells following ligation of CD44 (a) and LFA-1 (c). Cells pre-treated with 5 μM rottlerin followed by incubation with immobilized anti-CD44 (b) and anti-LFA-1 (d). Rottlerin dose-response curve followed by incubation with immobilized anti-CD44 (e) and anti-LFA-1 (f) (as determined by measurement of the deformation index). Distance traveled during the 1-h recording interval was measured for cells pre-treated with 5 μM rottlerin or dimethyl sulfoxide (DMSO) control followed by receptor cross-linking using SPV-L7 (anti-LFA-1) or D2.1 (anti-CD44) (a,b indicates P < 0.05 reduction in migration relative to control).

relevant model of cell migration in HA-enriched 3-D ECM lattices, PKC-δ-specific pseudosubstrate inhibitory peptide clearly reduced T cell migratory capacity thereby highlighting the involvement of PKC-δ in this process.

The finding that T cells activated via CD44–HA interaction or by CD44 antibody cross-linking acquired active motility perfectly correlates to the unique positioning of this receptor, spanning the extracellular environment with subcellular structures and underlying cytoskeleton via its cytoplasmic domain. Receptor association with the cytoskeleton is important in the transduction of the "outside in" signals and altering the phosphorylation status of the cytoplasmic domain is often a feature of cell migration and adhesion (19, 30, 31). Phosphorylation of the cytoplasmic domain of CD44 was required for migration in fibroblast and melanoma cells on a HA substrate, and phosphorylation mutants could no longer migrate on HA but could still bind HA (30). Also, PKC activation alters CD44 phosphorylation and its association with the cytoskeletal linker protein, ezrin. This dynamic association and dissociation of CD44 and ezrin influences directional migration (19).

It is interesting to note that the phenotypic changes accompanying T cell migration, cytoskeletal rearrangements and the response to PKCδ and δ inhibition are evidently similar in both CD44 and LFA-1-activated (32) HUT-78 cells, indicating that these receptors may operate through converging or shared signal transduction pathways in the migratory response. These findings underscore the importance of CD44 in the inflammatory response and may have significant implications for the understanding of the mechanisms underlying cell migration in inflammatory processes.
and might contribute towards the development of novel approaches for its targeted regulation.

Supplementary data

High-resolution 3-D reconstruction of the HUT-78 T lymphoma cell migrating into the ECM enriched with high-molecular weight HA. Cells display a characteristic polarized phenotype typical of locomotory cells with a clearly defined leading edge and trailing tail projection (uropod). The image is rotated to provide a 360° view around the /-axis.

Supplementary data are available at International Immunology Online.

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Abbreviations

- CL: collagen
- DI: deformation index
- ECM: extracellular matrix
- HA: hyaluronan
- IC50: inhibitory concentration 50%
- LFA-1: leukocyte function-associated antigen-1
- MT2C: microtubule organizing center
- PBTLs: peripheral blood T lymphocytes
- PKC: protein kinase C
- PMA: phorbol myristate acetate
- PPD: purified protein derivative
- PMA: phorbol myristate acetate
- PKC: protein kinase C
- PBTLs: peripheral blood T lymphocytes
- PMA: phorbol myristate acetate
- PPD: purified protein derivative

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