CONTROL OF LEUKOCYTE INTEGRIN ACTIVITY ON T LYMPHOCYTES

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For my husband, Jon
and my parents, Hugh and Elizabeth
with love, and with thanks
ABSTRACT

The control of lymphocyte extravasation from the circulation into sites of inflammation is critical for the co-ordination of an appropriate and effective immune response. Much previous work has focused on how leukocytes cross the endothelial barrier, but there has been less emphasis on subsequent events. This thesis aims to investigate aspects of adhesion involved in the migration of extravasated leukocytes across the extracellular matrix. The integrin family of cell surface receptors are a major family of lymphocyte adhesion molecules involved in these events. It is already well established that lymphocyte integrins are not constitutively active and require activation before being able to bind to their ligands. Such activation can be achieved by signals received by the T lymphocyte through its cell surface receptor, the T cell receptor (TCR/CD3) complex, and also by direct effects of cations on the integrin ectodomain in the absence of an intracellular signal. In this thesis, I describe experiments examining the interaction of human T lymphocytes with respiratory epithelial cells and with components of the extracellular matrix, such as collagen and fibronectin, following various methods of integrin activation. In particular, I present the first direct evidence for cross-talk between β2 and β1 integrins on T lymphocytes. A model of lymphocyte extravasation is proposed in which cross-talk co-ordinates sequential integrin activation and successful transmigration. In addition, this thesis also includes work aimed at defining signalling pathways involved in the activation of the β2 and β1 integrins following TCR/CD3 engagement, and the role of the lymphocyte cytoskeleton in such integrin activation. In summary, I have described aspects of the control of leukocyte integrins on T lymphocytes following various different stimuli, and the role of this controlled activation in successful T lymphocyte migration.
First and foremost I would like to acknowledge the encouragement, guidance and inspiration that I have received from my supervisor, Nancy Hogg, over the years. Thank you, Nancy, for being always so generous with your time, help and ideas. Next, of course, many many thanks to all my fellow MacLab members who have made the past years so enjoyable and memorable.

I should also like to thank Peter Beverley, my supervisor at University College, London for invaluable help and advice.

Thank you to Peter Jordan and Andrew Edwards for initial help in setting up confocal imaging and to all those who contributed to this work with generous gifts of mAbs, cell lines and other reagents.

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Finally, an enormous thank you to my husband Jon, whose unwavering support and infectious enthusiasm makes everything so much more fun.
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**ABBREVIATIONS AND DEFINITIONS**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
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<tbody>
<tr>
<td>AA</td>
<td>arachidonic acid</td>
</tr>
<tr>
<td>ADP</td>
<td>adenosine diphosphate</td>
</tr>
<tr>
<td>APC</td>
<td>antigen presenting cell</td>
</tr>
<tr>
<td>APS</td>
<td>ammonium persulphate</td>
</tr>
<tr>
<td>ATCC</td>
<td>American Type Culture Collection</td>
</tr>
<tr>
<td>BAL</td>
<td>broncho-alveolar lavage</td>
</tr>
<tr>
<td>BAPTA</td>
<td>bis-(o-aminophenoxy)-ethane-N,N,N',N'-tetraacetoxy methyl ester</td>
</tr>
<tr>
<td>BCECF/AM</td>
<td>2',7-bis (carboxyethyl)-5(6')-carboxyfluorescein pentaacetoxymethyl ester</td>
</tr>
<tr>
<td>BDM</td>
<td>2,3 butanedione 2-monoxime</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
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<td>C3</td>
<td>C3 exoenzyme of <em>Clostridium botulinum</em></td>
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<tr>
<td>[Ca^{2+}]_i</td>
<td>cytoplasmic Ca^{2+}</td>
</tr>
<tr>
<td>CaMKII</td>
<td>Ca^{2+}/calmodulin kinase II</td>
</tr>
<tr>
<td>CD</td>
<td>cluster of differentiation</td>
</tr>
<tr>
<td>CD3XL</td>
<td>T Cell Receptor/CD3 complex cross-linking</td>
</tr>
<tr>
<td>CHO</td>
<td>Chinese hamster ovary</td>
</tr>
<tr>
<td>CLA</td>
<td>cutaneous lymphocyte-associated antigen</td>
</tr>
<tr>
<td>Coll</td>
<td>collagen</td>
</tr>
<tr>
<td>CR</td>
<td>complement receptor</td>
</tr>
<tr>
<td>CS-1</td>
<td>connecting segment 1</td>
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<td>CTL</td>
<td>cytotoxic T lymphocyte</td>
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<td>DMEM</td>
<td>Dulbecco’s modified Eagle’s medium</td>
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<td>DMSO</td>
<td>dimethyl sulphoxide</td>
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<tr>
<td>DTT</td>
<td>dithiothreitol</td>
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<tr>
<td>ECM</td>
<td>extracellular matrix</td>
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<tr>
<td>EDTA</td>
<td>ethylene diaminetetraacetic acid disodium salt</td>
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<tr>
<td>EGTA</td>
<td>ethylene glycol-bis (b-aminoethyl ether) N,N,N',N'-tetra-acetic acid</td>
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<tr>
<td>ELISA</td>
<td>enzyme-linked immunosorbent assay</td>
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<tr>
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<td>extracellular signal-related kinase</td>
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<tr>
<td>ERM</td>
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<tr>
<td>F-actin</td>
<td>filamentous actin</td>
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<td>FACS</td>
<td>fluorescence-activated cell sorter</td>
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<tr>
<td>FAK</td>
<td>focal adhesion kinase</td>
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<tr>
<td>FcγR</td>
<td>receptor for the Fc portion of IgG</td>
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<td>granulocyte-macrophage colony-stimulating factor</td>
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<td>GPI</td>
<td>glycosyl-phosphatidylinositol</td>
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<tr>
<td>GTP</td>
<td>guanine nucleotide triphosphate</td>
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<tr>
<td>HEPES</td>
<td>N-[2-hydroxyethyl] piperazine-N’-[2-ethanesulphonic acid]</td>
</tr>
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<td>HEV</td>
<td>high endothelial venule</td>
</tr>
<tr>
<td>hr</td>
<td>hour</td>
</tr>
<tr>
<td>IAP</td>
<td>integrin-associated protein</td>
</tr>
<tr>
<td>IC₅₀</td>
<td>concentration giving half maximal inhibition</td>
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<td>intraepithelial lymphocytes</td>
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<td>IFN</td>
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<td>immunoglobulin</td>
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<td>IL</td>
<td>interleukin</td>
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### Abbreviations & Definitions

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<td>ILK</td>
<td>integrin-linked kinase</td>
</tr>
<tr>
<td>kDa</td>
<td>kilodaltons</td>
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<tr>
<td>KLH</td>
<td>keyhole limpet haemocyanin</td>
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<tr>
<td>LAT</td>
<td>linker for activation of T cells</td>
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<tr>
<td>LFA</td>
<td>lymphocyte function-associated antigen</td>
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<td>nm</td>
<td>nanometre</td>
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<tr>
<td>OD</td>
<td>optical density</td>
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<tr>
<td>OPD</td>
<td>o-phenylenediamine dihydrochloride</td>
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<td>PAGE</td>
<td>polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>PBMC</td>
<td>peripheral blood mononuclear cell</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate buffered saline</td>
</tr>
<tr>
<td>PdBu</td>
<td>phorbol-12,13-dibutyrate</td>
</tr>
<tr>
<td>PECAM-1</td>
<td>platelet-endothelial cell adhesion molecule-1</td>
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<tr>
<td>PHA</td>
<td>phytohaemagglutinin</td>
</tr>
<tr>
<td>PI</td>
<td>propidium iodide</td>
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## Abbreviations & Definitions

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
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<tr>
<td>PI 3-kinase</td>
<td>phosphatidylinositol 3-kinase</td>
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<tr>
<td>4,5 PIP₂</td>
<td>phosphatidylinositol-4,5-bisphosphate</td>
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<tr>
<td>PIP₃</td>
<td>phosphatidylinositol trisphosphate</td>
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<td>PKA</td>
<td>protein kinase A</td>
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<td>protein kinase C</td>
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<td>PLC</td>
<td>phospholipase C</td>
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<td>PLL</td>
<td>poly-L-lysine</td>
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<td>PMA</td>
<td>phorbol-12-myristate, 13-acetate</td>
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<td>PMSF</td>
<td>phenyl-methyl-sulphonyl-fluoride</td>
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<td>PP1</td>
<td>protein phosphatase type 1</td>
</tr>
<tr>
<td>PP2A</td>
<td>protein phosphatase type 2A</td>
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<td>PSGL-1</td>
<td>P-selectin glycoprotein ligand-1</td>
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<td>PVDF</td>
<td>polyvinylidene difluoride</td>
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<tr>
<td>RANTES</td>
<td>regulated on activation, normal T cell expressed and secreted</td>
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<td>RαMIg</td>
<td>rabbit antibody anti-mouse Ig</td>
</tr>
<tr>
<td>ROCK</td>
<td>Rho-associated coiled-coil forming kinase</td>
</tr>
<tr>
<td>ROKα</td>
<td>Rho-associated kinase (Rho-kinase)</td>
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<tr>
<td>RSV</td>
<td>respiratory syncitial virus</td>
</tr>
<tr>
<td>RT</td>
<td>room temperature</td>
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<td>SD</td>
<td>standard deviation</td>
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<td>sodium dodecyl sulphate</td>
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<td>SEM</td>
<td>standard error of the mean</td>
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<td>SDF</td>
<td>stromal cell derived factor</td>
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<tr>
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<td>secondary lymphoid-tissue chemokine</td>
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<tr>
<td>sLeᵃ</td>
<td>sialyl Lewisᵃ antigen</td>
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<tr>
<td>sLeᵇ</td>
<td>sialyl Lewisᵇ antigen</td>
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<td>sVCAM-1</td>
<td>soluble vascular cell adhesion molecule-1</td>
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<tr>
<td>TCR</td>
<td>T cell receptor</td>
</tr>
<tr>
<td>TCR/CD3</td>
<td>T cell receptor/CD3 complex</td>
</tr>
<tr>
<td>T&lt;sub&gt;H₁&lt;/sub&gt;</td>
<td>T helper-1 T cells</td>
</tr>
<tr>
<td>T&lt;sub&gt;H₂&lt;/sub&gt;</td>
<td>T helper-2 T cells</td>
</tr>
<tr>
<td>TEMED</td>
<td>N,N,N',N'-tetramethylethylenediamine</td>
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<tr>
<td>Abbreviation</td>
<td>Definition</td>
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<td>--------------</td>
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<tr>
<td>TIAM</td>
<td>T cell-invasion-associated molecule</td>
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<tr>
<td>TM4SF</td>
<td>transmembrane-4 superfamily</td>
</tr>
<tr>
<td>TNF</td>
<td>tumour necrosis factor</td>
</tr>
<tr>
<td>TRITC</td>
<td>tetramethyl-rhodamine isothiocyanate</td>
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<tr>
<td>uPA</td>
<td>urokinase type plasminogen activator/urokinase</td>
</tr>
<tr>
<td>uPAR</td>
<td>urokinase receptor</td>
</tr>
<tr>
<td>UDP</td>
<td>uridyl diphosphate</td>
</tr>
<tr>
<td>V</td>
<td>volts</td>
</tr>
<tr>
<td>VCAM-1</td>
<td>vascular cell adhesion molecule-1</td>
</tr>
<tr>
<td>VLA</td>
<td>very late antigen</td>
</tr>
<tr>
<td>VSMC</td>
<td>vascular smooth muscle cell</td>
</tr>
<tr>
<td>w/v</td>
<td>weight/volume</td>
</tr>
<tr>
<td>WAS</td>
<td>Wiskott Aldrich immunodeficiency syndrome</td>
</tr>
<tr>
<td>WASp</td>
<td>Wiskott Aldrich immunodeficiency syndrome protein</td>
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</tbody>
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CHAPTER ONE
INTRODUCTION

1.1 Cells of the immune system

The immune system provides constant host surveillance, searching out potential pathogens and destroying them. Innate immunity is present at all times, unlike the adaptive immune response which is induced by antigen and gives rise to long lasting protection against disease. The cells of the immune system are the leukocytes (white blood cells) comprised of the myeloid cells and the lymphoid cells.

1.1.1 Myeloid cells

The myeloid cells include the polymorphonuclear leukocytes (neutrophils, basophils and eosinophils), monocytes, macrophages and mast cells which are all derived from the same myeloid stem cells in the bone marrow. In general, the myeloid cells are part of the innate immune system and can directly attack and kill foreign micro-organisms by phagocytosis aided by complement-mediated opsonisation or antibody-dependent cellular cytotoxicity. On myeloid cells complement receptor (CR)1, CR3 and CR4 are responsible for binding pathogens which have been opsonised by the complement products C3b and iC3b, and immunoglobulin (Ig) receptors bind the Fc portion of Ig bound to antigen (Ag). Synergistic activation of these receptors results in successful endocytosis, and lysis of pathogens. The complement receptors CR3 and CR4 are members of the integrin family of adhesion molecules (section 1.5) and are also known as Mac-1 (αMβ2) and p150,95 (αXβ2) respectively.

1.1.2 Lymphocytes

The lymphocytes are the cells responsible for adaptive immune responses. Most lymphocytes are small, rather featureless cells with little cytoplasm and as late as the 1960s they had no known function. Much of their chromatin is in the condensed state and the cytoplasm contains few organelles, both
features of an inactive cell. A great step forward came in the early 1960s when James Gowans described the continual recirculation of lymphocytes from blood to lymph by way of the lymph nodes. In addition he found that when he depleted rats of the small lymphocyte all known adaptive immune responses were also lost. Immune function was, however, totally restored when the lymphocytes were replenished (Gowans, 1996). These experiments demonstrated for the first time the role of lymphocytes as the professional immune effector cells responsible for immunological memory.

Two functional types of antigen-specific lymphocytes have been distinguished that are similar by light- or electron-microscopy but differ by the molecules that they bear on their surfaces. B lymphocytes, or B cells, develop into plasma cells and secrete antibodies. T lymphocytes, or T cells, develop into effector cells able to kill infected host cells and to activate other cells of the immune system, including macrophages and B cells. Lymphocytes derive from specialised stem cells in the bone marrow, and are found in four main sites, bone-marrow, thymus, peripheral lymph organs, and mucosal surfaces as well as in blood and lymph. Prospective T lymphocytes migrate to the thymus to undergo maturation and acquire antigen specificity, while B lymphocytes undergo maturation in the bone marrow. Mature antigen-specific lymphocytes migrate from these tissues, via the blood, to the organised secondary lymphoid organs including lymph nodes, spleen and gut-associated lymphoid tissues. Lymphocytes leave the circulation and enter these lymphoid organs across specialised endothelial cells in the postcapillary venules. These cells are cuboidal and called high endothelial cells, and the vessels that they line are known as high endothelial venules (HEVs). The B and T cells that have not yet encountered antigen are referred to as naïve lymphocytes. Naïve lymphocytes continually circulate from the blood to the peripheral lymphoid tissues, where they encounter antigen, after which they are returned to the blood via the lymphatics. They traffic continuously until they die or respond to the antigen presented to them in the secondary lymphoid environment. This recirculation is essential to allow the rare
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interception of antigen by the specific lymphocyte. Following antigen stimulation, with the appropriate co-stimulatory secondary signals, naïve T cells (CD45RA⁺) transform into effector/activated T cells, and these cells then transform into memory cells (CD45RO⁺) becoming smaller and losing some of the makers associated with acute activation. Memory and effector cells are then transported back to the blood and display migratory properties different from naïve cells, migrating now to non-lymphoid tissue and continuously recirculating via secondary lymphoid tissue and the thoracic duct back to the blood. Once activated at a specific site of the body, memory T cells migrate remarkably selectively to the tissues which were originally involved in the foreign antigen exposure. In the normal situation most recirculating lymphocytes selectively bind to the endothelium of HEV but almost ignore normal vascular endothelium. In contrast, inflamed or damaged tissue triggers the adjacent endothelium to express new surface molecules, resulting in the ordered local extravasation of leucocytes including many lymphocytes. Inflammation, therefore, augments the influx of lymphocytes into tissues and alters the selectivity that normally governs homing.

1.2 T lymphocyte activation

T lymphocyte maturation is characterised by the acquisition of clonally distributed antigen receptors, known as the T cell receptor (TCR), which exists in association with other surface molecules as the TCR/CD3 complex (TCR/CD3). Activation of naïve T cells into competent effector cells requires two signals, an antigen-specific signal mediated by the interaction of TCR/CD3 with major histocompatibility complex (MHC)-Ag complexes and a second co-stimulatory signal delivered by a B cell, macrophage or dendritic cell. These co-stimulatory cells are also the cells that process and present Ag on their cell surface in the form of MHC-Ag complex and are therefore known as Ag-presenting cells (APCs). CD28 is the primary T cell receptor that delivers co-stimulatory signals upon interaction with its ligands B7.1 (CD80) and B7.2 (CD86) that are expressed on APCs (for reviews see Lenschow et al., 1996; and Chambers and Allison, 1997). When Ag is presented to naïve T cells in the
absence of a co-stimulatory signal the T cells are rendered anergic. When fully differentiated effector T cells encounter specific antigen, they are activated without the need for co-stimulation. T cells can be further divided into two subtypes based on their expression of the CD4 or CD8 surface molecules. CD4 binds to invariant parts of the MHC class II molecule, and CD8 to invariant parts of MHC class I molecule. During antigen recognition CD4 and CD8 associate on the cell surface with components of the TCR/CD3 complex and are therefore known as co-receptors. CD4⁺ (helper) T cells recognise exogenous Ag processed as peptides and presented in the context of MHC class II on APCs. Signals from TCR/CD3 and CD4 lead to T cell proliferation and cytokine production. CD8⁺ (cytotoxic) T cells recognise viral and other foreign endogenous Ag products in the context of MHC class I molecules. Co-stimulatory signals generated from ligation of TCR/CD3 and CD8 result in the production of cytotoxic granules by the T cell which when discharged are able to lyse the infected host cell. In addition cloned CD4⁺ T cells from mice and humans can be further divided into functional subpopulations based on their patterns of differential cytokine secretion. T-helper-1 (Tₜ1) are defined as those that produce the pro-inflammatory cytokines interleukin (IL)-2, interferon (IFN)-γ, tumour necrosis factor (TNF)-α, and TNF-β in response to an antigenic challenge. In contrast T-helper-2 (Tₜ2) secrete a distinct pattern of cytokines (IL-4, IL-5, IL-6 and IL-10) in response to Ag (for review see Jordan and Fredrich, 1997).

1.3 Lymphocyte adhesion molecules

The key characteristic of the cells of the immune system is their ability to migrate freely around the body but able to respond promptly and appropriately to danger signals by making use of transient, controlled and reversible adhesive events. The result is a group of cells that circulate in the blood but have the capacity to adhere to endothelium under shear-stress. These cells can regulate their adhesion molecules to make contact with other cells or extracellular matrix (ECM), and are able to cross endothelial and epithelial barriers. The most well studied examples of adhesion are leukocyte
extravasation across inflamed endothelium or into secondary lymphoid tissues and the adhesion of T cells to APCs or target host cells in an immune response. There are four main classes of adhesion receptor involved in these interactions: selectins, integrins, immunoglobulin super family (IgSF) members and proteoglycans which will be discussed in some detail below.

1.4 Lymphocyte rolling and firm adhesion

1.4.1 Attachment and rolling

Under flow conditions, circulating leukocytes can attach and roll on vascular endothelial cells. This rolling requires the reversible interaction of leukocyte receptors with their ligands on recently stimulated endothelium. Selectins are specialised cell surface receptors that mediate adhesion under, and even initiated by, shear-stress. The interaction of leukocyte selectins with their endothelial ligands is characterised by rapid on/off rates. The result is rapidly formed, and rapidly broken, cell-cell contacts that allow the rolling of leukocytes along the endothelium in the direction of blood flow.

1.4.1.1 Selectins

There are three members of the selectin family: L-(leukocyte; CD62L), E-(endothelial; CD62E) and P-(platelet; CD62P) selectin, named after the cell types in which they were originally discovered (for review see Kansas, 1996). P-selectin is also expressed on endothelial cells. L-selectin is constitutively expressed on all circulating leukocytes, except for a subpopulation of memory T cells, but is shed upon cell activation (for review see Kansas, 1996). In contrast, the endothelial selectins, P- and E-selectin, are mainly expressed at sites of inflammation rather than unactivated endothelium and their presence offers the lymphocyte the earliest clue that homeostasis has been disrupted. Each of the selectins has an N-terminal Ca\(^{2+}\)-dependent lectin domain that binds to specific glycoprotein ligands. Like other lectins, the selectins all bind selectively, but with low affinity, to particular oligosaccharides, so that all selectins bind to the tetrasaccharide sialyl Lewis x (sLe\(^x\)) and its isomer sialyl Lewis a (sLe\(^a\)). However, selectins bind with higher

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affinity to only a few glycoproteins which appear to be recognised as a "discontiguous carbohydrate epitope" in the context of a specific protein or carbohydrate core (for review see Kansas, 1996). In addition, cell specific differences in the expression of glycosyl-transferases, that may reflect cellular activation, can affect the ability of a cell to correctly modify a core protein to act as a high affinity selectin ligand.

Candidate glycoprotein ligands for each of the selectins have been described although it is uncertain if these are true ligands in vivo. Peripheral lymph node (PLN)-specific vascular addressins defined by the MECA-79 mAb were the first characterised counter receptors for lymphocyte L-selectin (Streeter et al., 1988). Although normally expressed only in PLN, during inflammation these addressins can be expressed at non-PLN sites (Salmi and Jalkanen, 1997). Known collectively as peripheral node addressin (PNAd), these ligands include glycosylation-dependent cell adhesion molecule-1 (GlyCAM-1; sgp50) that appears to be released from HEV as a soluble ligand, a glycoform of CD34 (sgp 90) and sgp 200 (Salmi and Jalkanen, 1997). Another ligand for L-selectin is the mucosal addressin cell adhesion molecule-1 (MAdCAM-1) (Berg et al., 1993) expressed in Peyer's patch and mesenteric lymph node HEV, and of particular interest for being a ligand not only for L-selectin, but also for the integrin α4β7 (Butcher and Picker, 1996). In addition there are several other ligands that bind L-selectin (Salmi and Jalkanen, 1997) but it remains unclear which, if any, acts as the L-selectin ligand expressed on activated human non-lymphoid endothelium (Varki, 1997). P- and E-selectin have both shared and unique ligands. The most convincing of all the selectin ligands is the P-selectin glycoprotein ligand (PSGL)-1, which can also bind E- and L-selectins with a lower affinity (Varki, 1997). The polypeptide backbone for PSGL-1 although widely expressed on all cells, including T cells, only acts as a P-selectin ligand on a subset of activated/memory T cells because of cell-specific differences in carbohydrate modifications (Kansas, 1996). On T cells the ability to bind E-selectin correlates with the expression of cutaneous lymphocyte-associated antigen (CLA; HECA-452), which has recently been identified as an
inducible carbohydrate modification of PSGL-1, expressed on a small subset of memory T cells that home to the skin (Fuhlbrigge et al., 1997). The picture has been further confused by the excellent ability of selectins to recognise ligands across species. So that although the carbohydrate-epitope may be similar between species it may be presented on very distinct protein scaffolds. For example, the human homologue of GlyCAM-1 has yet to be described. In very general and simplified terms therefore it appears that lymphocyte CLA on PSGL-1 interacts with E-selectin, lymphocyte PSGL-1 with P-selectin, and endothelial PNAd and MAdCAM-1 with lymphocyte L-selectin. However, it is worth bearing in mind that polypeptides, such as PSGL-1 only act as ligands if they are correctly modified (i.e. glycosylated/sulphated) and that despite many potential ligands one of the most controversial areas of selectin biology remains the identity of the true, biologically relevant ligands for these receptors.

Some insight into the role of the selectins in leukocyte extravasation has come from the study of 'knock-out' mice. Taken together the data from deficient mice suggest that P- and L-selectins are required for early leukocyte rolling (Arbonés et al., 1994; Mayadas et al., 1993) with an overlapping involvement of E-selectin at later time points (Labow et al., 1994). This is in keeping with the time course of selectin expression, with P-selectin rapidly mobilised to the plasma membrane from Weibel-Palade bodies and E-selectin expression occurring later and requiring mRNA and protein synthesis. However, the roles of the different selectins may be partially overlapping and even synergistic as suggested by the P- and E-selectin double knock-out (Bullard et al., 1996). Although expressed widely on leukocytes, the selectins have a specialised role in some aspects of lymphocyte recirculation. For example, L-selectin is essential for the naïve T cells both to enter PLN through HEV (Bradley et al., 1994) and to roll on MadCAM-1 as a prelude to entering mucosal lymph nodes (Bargatze et al., 1995). These requirements are consistent with the preferential expression of L-selectin on the naïve subset of T lymphocytes.
Figure 1.1: The multistep model of lymphocyte-endothelial cell recognition. T cells roll along stimulated endothelium, on selectins and $\alpha 4$ integrins, presented on the tips of microvilli. T cells undergo an activation step and firmly adhere using LFA-1 and $\alpha 4\beta 1$, then transmigrate using LFA-1.
1.4.1.2 Other rolling receptors on lymphocytes

Unlike other leukocytes, lymphocytes can also use α4 integrins, as well as the selectins, and probably also vascular adhesion protein (VAP)-1 (Salmi et al., 1997) for rolling on endothelium (Alon et al., 1995; Berlin et al., 1995; Luscinskas et al., 1994). Rolling mediated by α4 integrins, is jerkier and slower than that mediated by selectins and may act as a bridge between fast selectin-mediated rolling and firm adhesion. Constitutively active α4β7 appears particularly important in the rolling of memory T lymphocytes on MAdCAM-1 of mucosal lymphoid organs (Bargatze et al., 1995). Interestingly these tethering molecules, L-selectin, α4β7 and α4β1 are all highly concentrated at the tips of lymphocyte microvilli an effect which dramatically increases the efficiency of initial endothelial contact under flow (Berlin et al., 1995; Picker et al., 1991; von Andrian et al., 1995).

1.4.2 Activation-dependent firm adhesion

These rolling adhesion receptors are able to slow the transit of leukocytes and expose them to stimuli causing activation-dependent firm adhesion. The integrins α4β1, α4β7 and leukocyte function-associated antigen (LFA)-1 (αLβ2) have been implicated in activation-dependent stable arrest of lymphocytes under flow (Bargatze et al., 1995; Luscinskas et al., 1995). LFA-1 however, unlike the α4 integrins, cannot initiate adhesion under these conditions without L-selectin and/or α4-integrins first tethering the lymphocyte to the vessel wall (Bargatze et al., 1995; Luscinskas et al., 1995), presumably because the formation of bonds between integrins and their ligands occurs too slowly to achieve a stable interaction under shear stress. In addition, ICAM-1 is needed for optimal P-and L-selectin-mediated rolling of leukocytes (Steeber et al., 1998) arguing against sequential independent pathways for the different adhesion receptors. Following arrest, LFA-1 is the principle integrin involved in transendothelial migration (Luscinskas et al., 1995; Oppenheimer-Marks et al., 1991; Smith et al., 1989; van Epps et al., 1989) although the stimulus that
induces this β2 integrin-dependent movement across the endothelial layer is unclear. Interaction with, and migration across, fibronectin and other ECM components is then necessary for the successful completion of lymphocyte migration into the tissues. Transmigration of any one T cell is, therefore, a multistep process dependent on the tight regulation of the sequential, and often overlapping, activities of the expressed integrins (Butcher and Picker, 1996) (Figure 1.1).

1.5 Integrins

The term integrin was first used to describe cell surface molecules that could integrate the inner cell cytoskeleton with molecules present in the ECM. The integrins are heterodimeric glycoproteins present on all nucleated cells. As well as their involvement in lymphocyte migration they are also involved in interactions with other cells of the immune system. The integrins consist of noncovalently associated α- and β- subunits each with a single hydrophobic transmembrane segment. Data from electron-microscopic images suggest that the integrins are asymmetric molecules comprised of an extracellular ligand binding globular head with contributions from both α- and β-subunits and two stalks extending to the lipid bilayer (Weisel et al., 1992). At present 16α (~150-210 kDa) and 8β (~95-110 kDa; 64 205 kDa) subunits have been described, some of which can also exist as alternative spliced variants. Although in theory these subunits could give rise to over 100 unique heterodimers, in fact the actual diversity appears more restricted and to date there are only 22 recognised αβ combinations (Figure 1.2). Thirteen integrins are found on leukocytes. These belong to the β1, β2, β3 and β7 subfamilies, with β2 and β7 integrins exclusive to leukocytes. The majority of integrins recognise ECM molecules and soluble ligands although others such as LFA-1 recognise surface receptors on other cells (Table 1.1). Many of these counter-receptors belong to the immunoglobulin superfamily (section 1.6.1). The very large numbers of different integrins, their overlapping specificities and
Figure 1.2: The integrin family of adhesion molecules: The pairs marked with red lines have been found on leukocytes. Boxes indicate $\alpha$-subunits that contain I-domains. * indicates subunits that undergo alternative splicing.
<table>
<thead>
<tr>
<th>Integrin subunits</th>
<th>Ligands and counterreceptors</th>
<th>Recognition site</th>
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</thead>
<tbody>
<tr>
<td><strong>β1</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>α1</td>
<td>Collagen (I, IV and VI), Laminin</td>
<td></td>
</tr>
<tr>
<td>α2</td>
<td>Tenascin, Collagen (I, IV and VI), Laminin, echivirus 1</td>
<td>DGEA (Collagen)</td>
</tr>
<tr>
<td>α3</td>
<td>Laminin, Epiligrin, Collagen I, Fibronectin, Entactin, α2β1</td>
<td>RGD</td>
</tr>
<tr>
<td>α4</td>
<td>Fibronectin (CS1), VCAM-1, Invasin</td>
<td>LDV (CS-1) IDSP (D 1&amp;4 VCAM-1)</td>
</tr>
<tr>
<td>α5</td>
<td>Fibronectin, Denatured Collagen</td>
<td>RGD</td>
</tr>
<tr>
<td>α6</td>
<td>Laminin, PH30</td>
<td></td>
</tr>
<tr>
<td>α7</td>
<td>Laminin</td>
<td></td>
</tr>
<tr>
<td>α8</td>
<td>Fibronectin, Vitronectin, Tenascin C</td>
<td></td>
</tr>
<tr>
<td>α9</td>
<td>Tenascin</td>
<td></td>
</tr>
<tr>
<td>αV</td>
<td>Fibronectin, Vitronectin,</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>RGD</td>
</tr>
<tr>
<td><strong>β2</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>αL</td>
<td>ICAM 1, ICAM-2, ICAM-3, ICAM-4 and ICAM-5</td>
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<td>C3bi, Factor X, Fibrinogen, ICAM 1, ICAM-2.</td>
<td>30kD fragment (Fg)</td>
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<td>C3bi, Fibrinogen</td>
<td>GPRP (Fg)</td>
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<td>αD</td>
<td>ICAM-3</td>
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</tr>
<tr>
<td><strong>β3</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>αIib</td>
<td>Vitronectin, Thrombospondin, von Willebrand factor Fibronectin, Fibrinogen,</td>
<td>RGD, KQAGDV (Fg)</td>
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<tr>
<td>αV</td>
<td>Vitronectin, Thrombospondin, von Willebrand factor Fibronectin, Fibrinogen, Collagen, Osteopontin, Tenascin.</td>
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</tr>
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<td><strong>β4</strong></td>
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<td>Laminin</td>
</tr>
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</tr>
<tr>
<td><strong>β7</strong></td>
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</tr>
<tr>
<td>α4</td>
<td>Fibronectin (CS1), VCAM-1, MadCAM-1</td>
<td>LDV (CS-1) IDSP (D 1&amp;4 VCAM-1) LDT (MadCAM-1)</td>
</tr>
<tr>
<td>αE</td>
<td>E-cadherin</td>
<td></td>
</tr>
<tr>
<td>β8</td>
<td>αV</td>
<td>Vitronectin</td>
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Table 1.1: Integrins and their ligands
redundancies raise questions about their functions and differences. One answer may lie with the integrin cytoplasmic domains, which are different among the individual α-subunits and show highly conserved regions among several of the β-subunits. From such diversity different cellular responses might arise in response to a single ligand (see Chan et al., 1992).

Divalent cations are essential for integrin function, and the nature of the cation can affect the affinity and specificity for ligands. Divalent cations may also been necessary for αβ associations of some integrins (Loftus et al., 1994). This has provoked a continuing search for both cation- and ligand-binding domains within the integrin subunits, using function blocking monoclonal antibodies (mAbs), ligand cross-linking studies and mutational analysis. From these studies it is clear that high affinity ligand-binding requires both α- and β-subunits. In addition, integrins contain several ligand-contact points with essential regions and specific residues defined in both α- and β-subunits. The three major regions essential for ligand binding are the α-subunit I-domain, the amino-terminal repeats of the α-subunit and a conserved region of the β-subunit that may have some functional homology to the α-subunit I-domain. There is also accumulating evidence that these regions show various affinities and specificities for the binding of divalent cations. Taken together these findings have reinforced the idea that cation- and ligand-binding are intimately linked. All three of these regions will be discussed in more detail below.

1.5.1 Integrin α-subunits

At the amino terminus of the α-subunits are seven extracellular tandemly repeated domains (numbered I-VII or W1-7) of about 60 amino acids each with weak sequence homology to one another (Figure 1.3). The homologies include FG and GAP consensus sequences (single letter amino acid code). The last three of these repeats (or four in non-I-domain containing integrins) are
Figure 1.3: Schematic structure of a leukocyte integrin: Both I-domain and non I-domain containing α-subunits are shown. The α chains are formed by similar repeats (I-VII or W1-7) including four divalent cation-binding sequences (or three in an I-domain containing α chain) shown in purple. The α-subunit is non-covalently linked to the β-subunit. Important motifs in the α- and β- subunit cytoplasmic tails are represented as single letter amino acid codes.
thought to contain domains that bind the divalent cations Ca" or Mg^2+. Isolated domains from these regions of the α-subunit of LFA-1 (Stanley et al., 1994) and αIIb (D'Souza et al., 1990) have ligand-binding sites, and a potential ligand-contact site has mapped to this region of the α4-subunit (Irie et al., 1997; Kamata et al., 1995). Although these domains have putative cation-binding sites it is still unclear how ligand- and cation-binding are related, if at all (Stanley et al., 1994). It has recently been suggested that the FG-GAP repeats (I-VII or W1-7), previously thought of as independent domains, may fold cooperatively, around a central axis, into a single β-propeller domain related to that of a G protein β-subunit (Springer, 1997). The upper surface of the propeller, to which each FG-GAP repeat contributes, would be the predicted ligand-binding site (Irie et al., 1997; Springer, 1997). This would place the Ca^2+ binding motifs of the α-subunits on the lower surface of the propeller away from interactions with ligand (Springer, 1997).

Seven α-subunits (αL, αM, αX, αD, α1, α2 and αE) have an inserted domain, or I-domain between repeats II/W2 and III/W3 (Figure 1.3). This domain is homologous to the A-domain of von Willebrand factor (vWF). The I-domain plays a very important role in ligand binding and several groups have shown that invariably the isolated α-subunit I-domain retains a major component of ligand-binding activity of the parent integrin. This has been demonstrated for the isolated α-subunit I-domains of LFA-1 (Randi and Hogg, 1994), Mac-1(Ueda et al., 1994; Zhou et al., 1994) and α2β1 (Kamata and Takada, 1994; Tuckwell et al., 1995). The I-domains also have divalent cation co-ordination sites that are essential for ligand-binding (Michishita et al., 1993). Crystal structures of the I-domains from Mac-1 (Lee et al., 1995) and LFA-1 (Qu and Leahy, 1995) dramatically altered ideas about this domain. These crystal structures showed the bound cation (Mg^2+ or Mn^2+) co-ordinated to a DxSxSx amino acid sequence (single letter amino acid code where x is any amino acid) and to two sequentially distant oxygenated amino acids, a threonine and an
aspartate (Figure 1.3). This new cation binding motif is called the metal ion-dependent adhesion site (MIDAS) motif. This motif is absolutely conserved in integrin α-subunit I-domains (Lee et al., 1995) and there is strong evidence that it plays a role in ligand-binding by integrins. Divalent cations are critical for most integrin-ligand interactions and mutagenesis of amino-acids that form the MIDAS motif abolish ligand binding (Kamata and Takada, 1994; Lee et al., 1995; Michishita et al., 1993; Ueda et al., 1994). The role of bound cation in promoting the binding of ligand to the I-domain has provoked much interest. Recent studies have demonstrated that the binding of cation to the metal free I-domain of Mac-1 does not induce a conformational change of the I-domain (Baldwin et al., 1998). This suggests that rather than inducing a ligand-competent I-domain, the I-domain bound cation might directly bind ligand. In the β propeller model, the I-domain, with homology to the α-subunit of G proteins, is predicted to insert itself on the top of the β propeller domain (Huang and Springer, 1997; Springer, 1997).

1.5.2 Integrin β-subunits

The most highly conserved region of the integrin β-subunits is an extracellular ~250 amino acid stretch located near the amino-terminus (Figure 1.3). This region has been implicated in ligand and cation binding. Surprisingly, this region also has an absolutely conserved DxSxSx sequence which is found in all eight of the known integrin β-subunits (Lee et al., 1995). For β3 integrins, cross-linking studies using RGD as ligand, and terbium luminescence experiments have directly implicated the MIDAS motif in both ligand and cation binding (Cierniewski et al., 1994; Smith and Cheresh, 1988). Ligand and cation binding are closely inter-linked, and ligand appears to cause an unstable ternary intermediate complex of β3, ligand and cation from which cation can be displaced (D'Souza et al., 1994). Mutations of the DxSxSx sequence abolish ligand-binding not only of β3 integrins (Bajt and Loftus, 1994; Loftus et al., 1990) but also of α4β1 (Kamata et al., 1995), α5β1 (Takada et
al., 1992) and even integrins with α-subunit I-domains, such as LFA-1 and Mac-1 (Bajt et al., 1995). Although there is no sequence homology between the α-subunit I-domain and the β-subunit using standard algorithms, their consensus hydrophy plots can be favourably superimposed when aligned around the conserved DxSxS sequences (Lee et al., 1995). This has led to the proposal that the ligand-binding region of the integrin β-subunit might consist of a MIDAS motif within a three dimensional structure similar to the α-subunit I-domain (Lee et al., 1995). Mutagenesis of candidate oxygenated residues in the β1, β2, β3 and β5 subunits suggest that the ligand binding region in these β-subunits adopt a similar but not identical fold to the α-subunit I-domain (Lin et al., 1997; Puzon-McLaughlin and Takada, 1996; Tozer et al., 1996). If a MIDAS-type motif was involved in ligand-binding by the integrin β-subunit this would suggest that perhaps all integrins, and not just those containing an α-subunit I-domain, might be regulated in a similar way by divalent cations.

1.6 Integrin ligands

1.6.1 Immunoglobulin super family members

The immunoglobulin super family (IgSF) encompasses a large group of molecules with multiple Ig-like domains. Some of the IgSF members expressed on endothelium and leukocytes are counter-receptors for integrins. These integrin receptors include intercellular adhesion molecule (ICAM)-1 (CD54), ICAM-2 (CD102), ICAM-3 (CD50), vascular cell adhesion molecule-1 (VCAM-1; CD108) and MAdCAM-1 (Figure 1.4). Expression of different IgSF members can determine the specificity of the interactions between leukocytes and the endothelium. For example, as resting neutrophils have no α4 integrins, they can only interact with endothelium expressing ICAM-1, and not VCAM-1. In addition, whilst lymphocytes expressing high L-selectin can
Figure 1.4 The immunoglobulin supergene family that are involved in leukocyte-endothelial interactions: Schematic structures of IgSF members involved in leukocyte interactions. The specific integrins that bind to these structures, and the domains to which the integrins bind are shown. L-sel, L-selectin.
interact with HEV of PLN, only lymphocytes expressing α4β7 can interact with MadCAM-1 of mucosal lymph node HEV, so that specificity is regulated by both leucocytes and endothelium.

1.6.1.1 ICAMs
ICAM-1, the most widely distributed ICAM, is weakly expressed on resting leukocytes and endothelium with expression greatly increased on cytokine stimulation. ICAM-2 is broadly expressed on leukocytes (with the exception of neutrophils) and is constitutively expressed at a high level on resting endothelial cells but its expression is down-regulated by activation (McLaughlin et al., 1998). ICAM-3 is found exclusively on leukocytes and most highly expressed on T cells and neutrophils where it is the main ligand for LFA-1 during the early phase of antigen presentation (de Fougerolles and Springer, 1992). As LFA-1 has greater affinity for ICAM-1 than for ICAM-2 (de Fougerolles and Springer, 1992) it is possible that ICAM-2 may be responsible for low level constitutive transendothelial traffic with de novo expression of ICAM-1 regulating the main inflammatory traffic, as demonstrated in ICAM-1 deficient mice (Sligh et al., 1993). ICAM-1, unlike ICAM-2, exists primarily as a dimer at the cell surface (Miller et al., 1995) which may orientate ICAM-1 molecules to interact most efficiently with LFA-1 and prevent interactions in cis between LFA-1 and ICAM-1 on the same cell (Cassanovas et al., 1998). ICAM-4 is restricted to erythrocytes and erythroid precursors, and telencephalin, now called ICAM-5, is strongly expressed in the grey matter of the telencephalon. Although all the ICAMs are thought to be ligands for LFA-1 the functions of ICAM-4 and -5 are, as yet, unknown (Gahmberg et al., 1997).

1.6.1.2 VCAM-1
VCAM-1 is a cytokine-inducible adhesion molecule on human endothelial cells. A single VCAM-1 gene gives rise through alternative splicing to distinct isoforms, but the major form of VCAM-1 in humans contains seven Ig domains containing two homologous sections spanning domains (D) 1-3 and
Integrins α4β1 and α4β7 both function as leukocyte receptors for VCAM-1 (Chan et al., 1992; Elices et al., 1990; Springer, 1994). There is a binding site for both integrins in D1 with a contribution from a synergy site, unique for each integrin, in D2 (Newham et al., 1997). In addition, there is a second and independent binding site for both integrins in D4 (Kilger et al., 1995; Osborn et al., 1992). Although α4β1 binds D1 and D4 via a common mechanism mediated by the IDSP motif there appear to be different integrin activation requirements for binding to these two sites, with binding to D1 occurring at 4°C (Kilger et al., 1995; Lobb et al., 1995; Needham et al., 1994). VCAM-1 is also expressed in several non-vascular cell types, such as dendritic cells (Lobb and Hemler, 1994), and VCAM-1-α4β1 interactions are involved in co-stimulation for T cell proliferation (Burkly et al., 1991; Damle and Aruffo, 1991). Mice deficient in VCAM-1 have illustrated the importance of this molecule in development, as they suffer from defects in the development of the placenta and heart and die during embryogenesis (for review see Hynes, 1996).

1.6.1.3 CD31 (PECAM-1)

One other member of the IgSF that may not act as an integrin ligand but which can influence integrin function is CD31 or Platelet endothelial cell adhesion molecule-1 (PECAM-1). CD31 is expressed on platelets, monocytes, neutrophils and selected T cell subsets, biased towards CD8+, CD45RA+ (Tanaka et al., 1992). CD31 mediates the adhesion of leukocytes and platelets to endothelial cells and is also a major constituent of the endothelial intercellular junction (For review see Newman, 1997). The extracellular domain of CD31 is organized into six Ig-like homology domains (D1-D6), followed by a single-pass transmembrane domain and an 118 amino-acid cytoplasmic tail that contains specific sites for the assembly of cytosolic signalling molecules (Jackson et al., 1997). Its amino-terminal Ig homology domains D1 and D2 mediate homophilic cell-cell adhesion (Sun et al., 1996). When endothelial cells come into contact with each other CD31 redistributes
to the cell border and is thought to participate in cation-independent homophilic interactions with CD31 on the neighbouring endothelial cell. There is good evidence for an involvement of CD31 in leukocyte extravasation both in vitro (Muller et al., 1993) and in vivo (Bogen et al., 1994), and blocking CD31 mAbs have been shown to reduce infarct size in models of ischaemia-reperfusion injury (Newman, 1997). Functions mediated by CD31 in other cells are less well worked out although binding of ligand to CD31 activates α4β1, α5β1, the β2 integrins LFA-1 and Mac-1 and αIIbβ3 in a number of leukocytes (Berman et al., 1996; Newman, 1997; Tanaka et al., 1992) and can modulate migration supported by β2 integrins in neutrophils (Rainger et al., 1997). In addition mAbs against D6 of CD31 can enhance αIIbβ3-mediated platelet aggregation and adhesion (Varon et al., 1998). It is thought that all of these effects are mediated by a signal transduced by homophilic CD31-CD31 interactions. It is fascinating to speculate that interaction of CD31 on leukocytes with CD31 on endothelial cells might activate integrins in preparation for transendothelial migration and tissue infiltration. In addition to homophilic interactions, several studies have shown that CD31 can also undergo cation-dependent heterophilic interactions with glycosaminoglycans and αvβ3 (Buckley et al., 1996; DeLisser et al., 1993; Piali et al., 1995) although these observations require further confirmation.

1.6.1.4 Mucosal addressin cell adhesion molecule (MAdCAM)-1

MAdCAM-1 is a member of the IgSF, and also a mucin family member (Briskin et al., 1993) that is selectively expressed by venules involved in the lymphocyte trafficking to mucosal tissues. MAdCAM-1 is a ligand for α4β7 (Berlin et al., 1993) and this interaction plays a critical role in the targeting of lymphocytes to mucosal Peyer's patches and lamina propria. In addition, carbohydrates attached to the mucin-like domain of MAdCAM-1 bind L-selectin and mediate lymphocyte rolling (Berg et al., 1993). MAdCAM-1 is unique in this dual role as both an integrin and selectin ligand. MAdCAM-1 has also been reported to bind weakly to α4β1 in vitro following Mn$^{2+}$
treatment of lymphocytes, but the in vivo relevance of this interaction is uncertain (Berlin et al., 1993; Strauch et al., 1994)

1.7 Integrin activation

1.7.1 Affinity versus avidity

Leucocyte integrins are normally inactive and require an activating stimulus before they can bind to ligand. It is now well established there are at least two mechanisms of activation. These involve either an increase in affinity or an increase in avidity of the integrin. These two forms of activation are not necessarily mutually exclusive and may be complementary (Hato et al., 1998). Higher affinity forms of α4β1 and LFA-1 on T cells can be recognised by their ability to bind soluble ligand independent of integrin-cytoskeletal connections (Jakubowski et al., 1995; Stewart et al., 1996). This increase in affinity is thought to reflect a change in the conformation of the integrin extracellular domains. There is some evidence that naturally occurring agonists can cause such an increase in integrin affinity (Faull and Ginsberg, 1995). In addition, integrin affinity can be increased in vitro with specific combinations of divalent cations (section 1.7.8), or the use of activating mAbs (section 1.7.2), both of which are thought to directly alter the conformation of the integrin without a requirement for intracellular signalling. In contrast, a change in integrin avidity appears to involve an increase in number or clustering of integrins in the cell membrane often in response to signals received through another cell surface receptor such as the TCR/CD3 (section 1.7.4). Connections between integrin and cytoskeleton are essential for this clustering and avidity modulation (Stewart et al., 1996).

1.7.2 Monoclonal antibodies

In keeping with the idea that integrin activation involves a conformational change, a large number of anti-integrin mAbs have been reported that either perturb or enhance function. Certain mAbs, so called ‘activation reporters’ recognise epitopes that are regulated by cations and only expressed on
integrins that are in a high affinity state. Such antibodies include mAb 24, that reacts with high affinity β2 integrins (Cabañas and Hogg, 1993; Dransfield et al., 1992; Stewart et al., 1996) and two β1 integrin mAbs, HUTS-21 and 15/7 (Gomez et al., 1997; Luque et al., 1996; Yednock et al., 1995). Other mAbs can induce and stabilise a high affinity form of the integrin. For example, mAbs 8A2 and TS2/16 are able to induce a 4-20 fold increase in the affinity of α5β1 for a soluble form of its ligand, fibronectin (Arroyo et al., 1993; Faull et al., 1993). Activating mAbs for the β2 integrins, for example mAbs KIM185 (Andrew et al., 1993) and KIM127 (Robinson et al., 1992) have also been described. In addition some mAbs such as mAb HUTS-21 (Gomez et al., 1997), and the β1 activating mAb 12G10 (Mould et al., 1995) appear to recognise a true ligand-induced binding site (LIBS) epitope so that their binding to integrin, at saturating concentrations of mAb, is increased in the presence of ligand. There is also some evidence that mAb 24 may recognise a ligand-dependent epitope under certain conditions (Cabañas and Hogg, 1993).

1.7.3 Inside-out signalling

Inside-out signalling is an energy-dependent process in which intracellular signals induce changes in ligand-binding by alterations either in the integrin extracellular domain, or in the integrin-cytoskeletal associations. There is increasing evidence for a complex role of both α- and β-subunit cytoplasmic domains in this process. Although, in general, there is little homology between the different integrin cytoplasmic domains the membrane-proximal regions of both domains are highly conserved across integrin families (Williams et al., 1994). These conserved sequences for α- and β-subunits are xGFFKR and LLviD respectively (with less conserved amino acids in the lower case, and x indicates a non conserved amino acid). Deletion or mutation (Hughes et al., 1996) of these sequences in either the αIIb (O'Toole et al., 1994), αL (Peter and O'Toole, 1995) or β3 (Hughes et al., 1996) subunit will produce high affinity integrin independent of intracellular signalling (Hughes
et al., 1996). It is proposed that in the normal resting cell, a salt bridge forms between the integrin α- and β- subunits at the "integrin hinge", and this bridge holds the integrin in an inactive conformation (Hughes et al., 1996). Other residues in the β-subunit have also been shown to be important in integrin affinity. For example, point mutations in the membrane proximal NPxY/F motif (common to all integrin β-subunits except β4 and β8) can prevent activation of β1 and β3 integrins as measured by the expression of an activation epitope (O'Toole et al., 1995). A distal, less well conserved NPxY/F motif, only exactly present in β1 and β2 integrins, is probably also important (Hibbs et al., 1991; O'Toole et al., 1995). Interestingly, a TTT motif, that lies between the two NPxY motifs of the β2 subunit, is essential for cytoskeleton-association and efficient ligand-binding of LFA-1 even when the integrin is locked in a high affinity state (Hibbs et al., 1991; Peter and O'Toole, 1995). Taken together these findings suggest that the inside-out signalling pathway probably acts upon both α- and β- subunit cytoplasmic domains. The result may be a change in integrin affinity by altering integrin conformation, or a change in integrin avidity by altering integrin-cytoskeletal connections or both.

1.7.4 Cell surface receptors that generate an inside-out signal

Many cell surface receptors have been described that are capable of delivering a intracellular signal that upregulates integrin activity. Such activation occurs rapidly, typically within minutes, with no alteration in cell surface integrin expression. On T cells the activators of LFA-1 and the β1 integrins, α4β1 and α5β1, include the antigen specific TCR/CD3 complex, CD7, CD28 and CD31 (Dustin and Springer, 1989; Shimizu et al., 1992; Shimizu et al., 1990; Tanaka et al., 1992; van Kooyk et al., 1989). In general the signals transduced enhance integrin avidity rather than increasing integrin affinity (Jakubowski et al., 1995; Stewart et al., 1996; Tanaka et al., 1992). In contrast, ligation of L-selectin, either with its physiological HEV ligand GlyCAM-1 or with mAbs, stimulates
the adhesion of naïve but not memory T cells to ICAM-1 and fibronectin. This appears to come about by an induction of high affinity forms of β2 and β1 integrins that express mAb 24 (Hwang et al., 1996) and bind soluble fibronectin (Giblin et al., 1997) respectively. Cross-linking of the IgSF-members CD2 (Shimizu et al., 1990; van Kooyk et al., 1989) and CD50 (ICAM-3) (Cid et al., 1994) also increases T cell adhesion through LFA-1 and β1 integrins, although, as yet, there are no suggestions as to whether this reflects changes in affinity or avidity of the integrins.

Other receptors implicated in integrin activation are receptors for the small peptide chemoattractants, chemokines. The rapid activation of lymphocyte binding to HEV in vivo is pertussis-toxin-sensitive, implicating a role for a Gα1-linked chemokine receptor in integrin activation (Murphy, 1994) (Bargatze and Butcher, 1993). There have been several reports of chemokines inducing binding of T cells to endothelial cells or purified ligands (for example: (Lloyd et al., 1996; Taub et al., 1996)). However these assays have often involved long incubation times, arguing against a role for these chemokines in rapid adhesion. Recently it has become apparent that higher cell surface levels of chemokine receptors are needed to trigger firm adhesion, than are needed to mediate chemotaxis (Campbell et al., 1996; MacKay, 1996). The levels of known chemokine receptors are low on T cells (10^3/cell), but can be increased by stimuli, such as IL-2 (Loetscher et al., 1996). In addition, T cells respond rapidly to chemokines when the appropriate receptor is transfected at high levels (10^4-10^5/ cell) (Campbell et al., 1996). This suggests that the T cell has the right intracellular machinery to transduce rapid signals from chemokine receptors if these receptors are present and stimulated in sufficient number. These recent results have added impetus to the search for chemokines that can produce rapid, within minutes, integrin-dependent arrest of lymphocytes under physiological shear. Using these strict criteria there is good evidence that LFA-1 activity on T cells can be rapidly upregulated by secondary lymphoid-tissue chemokine (SLC) (Gunn et al.,
stromal cell derived factor (SDF)-1α, 6-C-kine, macrophage inflammatory protein (MIP)-3β and MIP-3α (Campbell et al., 1998). In addition, monocyte chemotactic protein-1 (MCP-1), RANTES (regulated on activation, normal T cell expressed and secreted), MIP-1α and MIP-1β can selectively activate the β1 integrins, α4β1 and α5β1 on T cells with no effect on LFA-1 (Campbell et al., 1998; Carr et al., 1996). These results have provided an important confirmation of the role of chemokines in T cell integrin activation and suggest that other chemokines and their receptors remain to be discovered. One speculation is that these, perhaps as yet undiscovered, chemokine receptors will be expressed at high levels of T cells, and might display differential effects on the integrins that they regulate.

1.7.5 Signalling pathways involved in inside-out signalling

The signals involved in inside-out activation of integrins are far from elucidated. Engagement of TCR/CD3 initiates the activation of membrane proximal tyrosine kinases such as Fyn, Lck and Zap 70 which lead to tyrosine phosphorylation of LAT (linker for activation of T cells) (Zhang et al., 1998) and couple the TCR to phospholipase C (PLC) γ and Ras related signalling pathways (Figure 1.5). PLCγ acts on phosphatidylinositol bisphosphate (PIP₂) to generate diacylglycerol (DAG) and inositol triphosphate (IP₃) leading to the mobilisation of [Ca²⁺] and activation of protein kinase C (PKC). However, unlike the adhesion induced by phorbol esters, that stimulated by TCR/CD3 cross-linking has varying sensitivity to the non-specific PKC inhibitor, staurosporine suggesting that other pathways may be involved (Dustin and Springer, 1989; Shimizu et al., 1992). In addition cell adhesion to fibronectin following CD2 cross-linking is only partially inhibited by the selective PKC inhibitor, bisindolylmaleimide (Toullec et al., 1991) at concentrations that completely inhibit PdBu-induced adhesion (Shimizu et al., 1995).

Another pathway for integrin activation might involve PI 3-kinase (Figure 1.5). In T cells a variety of cell surface receptors, including the TCR, CD2 and
CD28, have been shown to regulate PI 3-kinase activity by recruiting the p85/110 isoform via SH2-phosphotyrosine interactions. Cross-linking of CD2, CD7 or CD28 leads to PI 3-kinase activation and β1 integrin mediated adhesion (Chan et al., 1997; Shimizu et al., 1992; Zell et al., 1996) and treatment of Jurkat cells with a PI 3-kinase inhibitor blocks their adhesion to ICAM-1 following TCR/CD3XL (Nagel et al., 1998). How PI 3-kinase might activate integrins is unclear. A constitutively active form of PI 3-kinase will activate LFA-1-mediated adhesion of Jurkat cells potentially through the recruitment of cytohesin-1 to the Jurkat cell membrane (Nagel et al., 1998). Cytohesin-1 has previously been described as a cytoplasmic regulator of LFA-1 function (section 1.7.6) suggesting at least one possible effector downstream of PI 3-kinase. PI 3-kinase has also been identified as an upstream regulator of the Rho-subfamily of small GTPases (section 1.13.1) in T cells (Arrieumerlou et al., 1998; Henning and Cantrell, 1998). Rho, itself, has recently been implicated in inside-out activation of lymphocyte integrins in response to chemokine stimulation (Laudanna et al., 1996) suggesting another potential pathway linking PI 3-kinase to integrin activation (Figure 1.5).

The small GTPase, Ras is also activated on TCR/CD3 cross-linking. Constitutively active R-ras can increase the binding affinity of the integrins αvβ3, α4β1 and α5β1 (Zhang et al., 1996). In contrast activated H-ras or its downstream kinase Raf-1 inhibits the ability of αIIbβ3 to become active (Hughes et al., 1997). Together these findings suggest a complex feed-back modulation of integrin activity mediated through the distal effectors of the mitogen activated protein kinase (MAPK) cascade. In a further twist, there is indirect evidence that in fibroblasts PI 3-kinase links Ras to the Rho-subfamily member Rac (Rodriguez-Viciiana et al., 1997). In T cells Ras is also linked to Rho and Rac signalling cascades but apparently independent of PI 3-kinase (for review see Henning and Cantrell, 1998).
Figure 1.5: Selected pathways of signal transduction from integrins and the TCR/CD3 complex: Mediators implicated in outside-in signalling and increases in integrin affinity are shown in red.
1.7.6 Cytoplasmic molecules that bind to integrins

As it became increasingly apparent that increases in integrin avidity were dependent on clustering of integrins and an intact cytoskeleton (Stewart et al., 1998; Stewart et al., 1996), the search for potential adapter proteins that might link integrins with the actin cytoskeleton increased in momentum. Integrins have very short cytoplasmic tails and two strategies have been used to identify the cytoplasmic associations that they form. These strategies are firstly, in vitro binding assays with candidate molecules that co-localise with integrins, and secondly, affinity columns using synthetic peptides based on sequences in the cytoplasmic domains of integrins. The problem is that the cytoplasmic molecules involved only bind to integrins with low affinity and conditions have to be right to allow co-purification but also to distinguish specific from non-specific binding. In addition the full interaction of integrins with cytoplasmic proteins appears critically dependent on both occupancy and clustering of the integrins involved (Miyamoto et al., 1995). One potential way of overcoming these problems has been the recent development of recombinant structural mimics of the cytoplasmic face of occupied and clustered integrins for use in affinity columns (Pfaff et al., 1998). To date, the most convincing cytoskeletal associations are of integrin β-subunits with talin (Horwitz et al., 1986; Pfaff et al., 1998), α-actinin (Otey et al., 1990) and filamin (Pfaff et al., 1998). Interestingly several other non-cytoskeletal proteins have also been shown to specifically bind certain integrin cytoplasmic domains. These include the calcium binding protein calreticulin which binds to the conserved GFFKR motif on the integrin α-subunit (Rojiani et al., 1991); the serine/threonine kinase, integrin-linked kinase (ILK) that associates with several integrin β-subunits and may be a negative regulator of integrin function (Hannigan et al., 1996); cytohesin-1, which co-precipitates with the β2 integrin cytoplasmic subunit and appears to be a positive regulator of LFA-1-mediated adhesion and overexpression of its pleckstrin-homology domain inhibits LFA-1-mediated binding to ICAM-1 (Kolanus et al., 1996); endonexin,
which interacts specifically with the β3 integrin cytoplasmic subunit and may promote β3 integrin activation (Kashiwagi et al., 1997); focal adhesion kinase (FAK) which has been shown to bind in vitro to peptides derived from several β-subunit cytoplasmic domains (Schaller et al., 1995); finally, a protein named integrin cytoplasmic domain-associated protein (ICAP)-1, has recently been identified that binds specifically to the β1-subunit NPXY motif and is phosphorylated following β1-integrin engagement (Chang et al., 1997). Together these observations suggest that a complex interaction of cytoplasmic subunits with regulatory and cytoskeletal molecules might influence both the affinity and avidity of integrin and more cytoplasmic integrin binding proteins probably remain to be discovered.

1.7.7 The role of cations

Divalent cations are critical for ligand-binding by integrins and can directly modulate the adhesive state. For many integrins a similar, though not exclusive, pattern of cation dependence of ligand binding can be seen. For example, physiological (0.4 mM) levels of extracellular Ca$^{2+}$ and Mg$^{2+}$ produce only low levels of cell adhesion in the absence of an additional stimulus. However, the same levels of cations are necessary for, and able to support, the upregulation of integrin avidity seen with inside-out signalling. In contrast extracellular Mn$^{2+}$, and to a lesser extent Mg$^{2+}$, can stimulate integrin-mediated adhesion in the absence of an intracellular signal and this adhesion is inhibited by extracellular Ca$^{2+}$. This led to a model in which integrins are regulated in a complex manner through separate binding sites for Mn$^{2+}$, Mg$^{2+}$ and Ca$^{2+}$ (Mould et al., 1995). In this model, occupancy of either a high affinity Mn$^{2+}$ binding site (site 1) or a low affinity Mg$^{2+}$ binding site (site 2) could support ligand-binding. Although Ca$^{2+}$ competes with Mg$^{2+}$ at site 2, it is also able to bind to a separate intermediate affinity Ca$^{2+}$ effector site (site 3). Binding of Ca$^{2+}$ to site 3 increases the affinity of Mg$^{2+}$ for site 2, but binding of Ca$^{2+}$ to either site 2 or 3 appears to non-competitively inhibit the binding of Mn$^{2+}$ to site 1. This model, developed for α5β1, appears broadly applicable to
other integrins including αvβ3 (Smith et al., 1994), α1β1 (Luque et al., 1994), α2β1 (Staatz et al., 1989) and LFA-1 (Dransfield et al., 1992; Stewart et al., 1996). It is unlikely that the cation-binding sites are all part of the ligand-binding site, and they may act in other ways to promote ligand binding such as enhancing the association of the integrin α and β subunits, the clustering of individual integrins or the interaction of integrin with other molecules in cis. Mn$^{2+}$ is able to induce high affinity integrins on its own, as shown for LFA-1, α4β1 (Jakubowski et al., 1995) and α5β1 (Mould et al., 1995). Supraphysiological Mg$^{2+}$ with EGTA to remove extracellular Ca$^{2+}$ can also induce high affinity LFA-1 (Stewart et al., 1996). It is not clear whether Mg$^{2+}$/EGTA can induce high affinity of other, in particular non-αI-domain-containing, integrins. It is also unclear whether Ca$^{2+}$ alone can support adhesion although this has been reported for αIIbβ3 (Smith et al., 1994), and α4β1-mediated binding to VCAM-1 but not to fibronectin (Masumoto and Hemler, 1993). Extracellular Ca$^{2+}$ certainly plays a role in LFA-1 clustering and avidity changes of LFA-1 (van Kooyk et al., 1994).

1.8 Cross talk between integrins

As well as regulation of integrins by other cell surface receptors (section 1.7.4) there is increasing evidence that, on a given cell, one subset of integrins may be regulated by ligation of another. For example α5β1 ligation activates α2β1-mediated collagen binding in monocytes, an effect that requires the α5 cytoplasmic tail and is mediated by PKC (Pacifici et al., 1994). Transfection of αvβ3 into K562 cells that endogenously express α5β1, provides a system in which ligation of β3 inhibits the phagocytic, but not adhesive, function of α5β1 (Blystone et al., 1994). Similarly, ligation of transfected αIIbβ3 will inhibit the function of co-transfected α2β1 or endogenous α5β1 in Chinese hamster ovary (CHO) cells expressing these integrins (Diáz-González et al., 1996). Such effects are dependent on an intact β3 cytoplasmic tail, and are
Chapter One-Introduction

considered to involve signal transduction (Blystone et al., 1995; Diáz-González et al., 1996). In a further example, ligand binding by α4β1 on fibroblasts is able to suppress the ability of α5β1 to induce metalloproteinase expression (Huhtala et al., 1995).

1.9 Integrin associations with other receptors
Integrins can also form cis associations with other receptors on the same cell to form multi-receptor complexes in which receptors co-operate to influence a variety of signalling pathways. These complexes recruit signalling molecules to sites of cell-cell or cell-matrix adhesion, such as focal complexes or focal adhesions. Some of the key molecules involved in such integrin partnerships are discussed below.

1.9.1 Integrin associated protein (IAP; CD47)
Integrin associated protein (IAP) is a 50 kDa single chain protein comprising an extracellular IgSF domain, five membrane spanning sequences and a short cytoplasmic tail. A ligand for IAP is the cell binding domain of thrombospondin which regulates motility and proliferation in many cell types (Gao et al., 1996). IAP has also been found in associations with αvβ3, αIIbβ3 (Lindberg et al., 1996), α2β1 (Wang and Frazier, 1998) and potentially with αvβ5 (Lindberg et al., 1996). Although expressed on all cell types IAP appears particularly important in myeloid cell activation (Brown, 1997) and migration across endothelial and epithelial monolayers (Brown, 1997; Cooper et al., 1995; Parkos et al., 1996). IAP<sup>−/−</sup> mice fatally fail to recruit activated neutrophils to sites of infection further emphasising the central role of this protein in leukocyte migration and host defence (Lindberg et al., 1996). Human cells that lack IAP are deficient in αvβ3-mediated ligand binding (Lindberg et al., 1996) but transfection of such cells with the extracellular IgSF-like domain of IAP can reconstitute αv integrin function (Lindberg et al., 1996). IAP appears to modulate the activity of the β3 by forming a functional
membrane unit that signals through heterotrimeric G proteins (Gao et al., 1996) rather than by a direct physical interaction.

1.9.2 Tetraspans (TM4SF proteins)

The transmembrane-4 superfamily (TM4SF) includes at least 21 different proteins with 20-30% sequence homology. The members of the TM4SF, so called tetraspans, are presumed to have four membrane spanning domains, resulting in two extracellular loops, and two intracellular tails (Hemler et al., 1996). Like IAP, they are expressed on all cells tested to date, usually with more than one tetraspan per cell type. Most of the tetraspan/integrin associations have been demonstrated by reciprocal co-immunoprecipitation studies which have shown that CD9, CD53, CD63, CD81, CD82, CD151/PETA-3 and NAG-2 can be physically associated in separate complexes with certain integrins (Hemler et al., 1996; Tachibana et al., 1997; Yáñez-Mó et al., 1998). The integrins that have been identified in complexes with tetraspans are α3β1, α4β1, α6β1, α4β7 and αIbβ3, but so far no association of tetraspans with α2β1, α5β1, α6β4, αv or the β2 integrins has been shown (Hemler et al., 1996). Only a small fraction of each receptor type (5-10%) is committed to the tetraspan/integrin complex (Mannion et al., 1996). Although the precise function of the tetraspan family is not known, its members have been implicated in the control of cell motility, metastasis and growth (for review see Hemler et al., 1996). As integrins are also involved in these functions, their associations with tetraspans may be highly relevant. There have been suggestions that association of integrins with tetraspans affects cell adhesion (Behr and Schriever, 1995; Masselis-Smith and Shaw, 1994; Yáñez-Mó et al., 1998), although others have found no consistent effects (Hemler et al., 1996; Mannion et al., 1996) (M. Hemler-personal communication). It is now becoming clear that the tetraspan/integrin association is involved in the control of more complex integrin-mediated events such as cell motility (Shaw et al., 1995) and the control of integrin recycling at the leading edge of the cell (Berditchevski et al., 1995; Berditchevski et al., 1997; Berditchevski et al., 1996).
1.9.3 CD98, an affinity modulator

CD98 is an αβ heterodimer expressed early in T cell activation. Recently, the CD98 α-subunit has been found to release β1 integrins from dominant suppression, caused by overexpression of β1 cytoplasmic tails (Fenczik et al., 1997), by promoting a high affinity form of the integrin (Fenczik et al., 1997). The speculation is that this rescue from suppression, depends on an intracellular interaction between the cytoplasmic sequences of integrin and the α subunit of CD98 (Fenczik et al., 1997).

1.9.4 GPI-linked proteins

Glycosyl-phosphatidylinositol (GPI)-linked proteins lack intracellular domains and must signal through membrane spanning partners. The most well studied GPI-linked receptor that functions by this means is the urokinase type plasminogen activator receptor (uPAR). There is a physical association between uPAR and the β2 integrins Mac-1 and LFA-1 in monocytes (Bohuslav et al., 1995) and uPAR has been co-immunoprecipitated with β1 integrins (Wei et al., 1994; Xue et al., 1997). Again, only a small proportion of either uPAR or integrin is committed to the uPAR/integrin complex (Wei et al., 1996). On human monocytes uPAR is a bonafide vitronectin receptor (Chapman, 1997; Kanse et al., 1996; Wei et al., 1994) and activation or ligation of Mac-1 promotes uPAR-mediated binding of vitronectin (Simon et al., 1996; Wong et al., 1996), which synergistically increases Mac-1 binding of fibrinogen (Simon et al., 1996; Sitiri et al., 1996). In contrast, soluble uPAR (Wei et al., 1996) or binding of uPA to cell-surface uPAR, inhibits Mac-1 and α5β1 function (Simon et al., 1996). This differential effect of uPAR on integrin function depending on the form of uPAR (soluble or cell surface expressed) and the ligand it has bound (uPA, or vitronectin) is in apparent contrast to the homogeneous effects of the other integrin-receptor pairings. Other GPI-linked receptors shown to interact with integrins are FcγRIIIB (CD16) which binds Mac-1 to promote antibody-dependent phagocytosis; and
CD14 which binds the bacterial lipopolysaccharide and binding protein complex (LPS/LBP) in association with Mac-1 causing the generation of proinflammatory mediators (for review see Petty and Todd, 1996).

1.10 Integrin-mediated signalling

As well as mediating adhesion the integrins are involved in various other biological processes. They play a role in co-stimulation of T lymphocytes and the highly specific interactions between cells responsible for an adaptive immune response (Bachmann et al., 1997). Integrins also co-operate with growth factors to promote cell proliferation. In addition, adhesion is necessary for cell survival and differentiation. In all these areas integrin-associated signalling is thought to be involved and these pathways are beginning to be defined (Juliano, 1996). Integrins have no intrinsic kinase and phosphatase activity themselves but are able to recruit signalling molecules to sites of cell-matrix and cell-cell adhesion. In adherent cells such as fibroblasts and endothelial cells these are known as focal contacts or focal adhesions. The proteins in focal adhesions have been extensively reviewed (for review see Burridge and Chrzanowska-Wodnicka, 1996), and include a number of structural proteins such as actin, vinculin, talin and paxillin. In addition there are a number of signalling molecules including tyrosine kinases, such as FAK; serine/threonine kinases such as PKC; adapter proteins which include paxillin, p130 Cas, Grb2; proteases such as calpain II and other proteins such as PI 3-kinase and the small GTP-binding proteins. The two most well studied examples of direct integrin mediated signalling events are the activation of FAK and the MAPK cascade (Howe et al., 1998). T lymphocytes express both FAK and the related PYK2 which are auto-phosphorylated in response to activation of β1 (Maguire et al., 1995) and β3 integrins (Ma et al., 1997). Intriguingly, TCR/CD3 cross-linking and ligand-binding by integrin synergise to cause increased FAK (Ma et al., 1997; Maguire et al., 1995) and PYK2 (Ma et al., 1997) phosphorylation and MAPK activation (Figure 1.5) (Maguire et al., 1995) expanding the list of FAK regulators to include the TCR/CD3 complex.
1.11 Cell migration

Adhesion molecules are involved not just in the mechanics of static adhesion, but also in the dynamic events of cell movement. Cell migration is central to many biological processes including embryonic development, wound healing and the immune response. Despite this, our understanding of the molecular events by which adhesions form, release and generate traction is still relatively limited. Initiation of cell migration involves a protrusion of the cell's leading edge, presumably via actin polymerisation, to form a lamellipodium which subsequently attaches to the substrate. Adhesion receptors then connect the cytoskeleton to the substratum and the cell can generate traction and force required for moving the cell body forward. The final step is to release the adhesions, now at the rear of the cell, with detachment and retraction of the rear end. Cell migration requires a co-ordinated response to multiple environmental cues, and receptor cross talk may play a role in integrating these signals. The focal adhesion has been used as the model for the adhesive complex that forms between the cell and its surrounding substratum. In general, cells that form strong focal adhesions are less migratory, and more motile cells form less organised structures. In lymphocytes these structures are smaller and less organised than focal adhesions and are called focal contacts. Theory and experiment suggest that an intermediate level of adhesive strength allows maximal migration. At high adhesive strength cells cannot release their rear ends, and at low adhesive strength they are unable to generate sufficient traction for migration (DiMilla et al., 1993; Huttenlocher et al., 1996; Palecek et al., 1997). At intermediate adhesiveness cytoskeletal forces are roughly in balance with adhesion so that traction can be maintained at the cell front while it can be disrupted at the cell rear, allowing the cell body to move. Concentrations of substrate and receptor are, therefore, critical determinants of cell migration as are integrin-cytoskeletal linkages (Huttenlocher et al., 1996). Changes in integrin affinity are also central in controlling cell migration so that although a high affinity integrin state supports adhesion it must be transient to allow migration (Gomez et al., 1997; Huttenlocher et al., 1996; Kuijpers et al., 1993;
Palecek et al., 1997). The cytoplasmic regions of the integrin receptors can therefore potentially affect migration in a number of ways by affecting ligand affinity, and by altering integrin-cytoskeletal linkages. Optimal migration also depends on the formation of new adhesions at the front of the cells and their subsequent release at the cell rear, suggesting there is an adhesive asymmetry in the cell which is essential for cell progression. Intracellular Ca$^{2+}$-transients play a role in maintaining this adhesive asymmetry. Ca$^{2+}$-depleted or -buffered neutrophils are unable to migrate to a chemotactic stimulus due to a failure to detach the rear end of the cell rather than failure to protrude a lamellipodium. In neutrophils migrating on vitronectin these Ca$^{2+}$-transients are thought to work through the Ca$^{2+}$-calmodulin-regulated phosphatase, calcineurin, to regulate detachment and αvβ3 recycling to the front of the neutrophil (Lawson and Maxfield, 1995). In another model the Ca$^{2+}$-dependent protease, calpain regulates cell locomotion and rear retraction in CHO cells migrating on fibronectin or fibrinogen by destabilising cytoskeletal linkages (Huttenlocher et al., 1997).

1.12 Actin-myosin interactions for adhesion & migration

Ultimately, the driving force for traction and propulsion of the cell comes from the actin-myosin cytoskeleton. Myosins are actin activated ATPases that generate force by translational movement along actin cables. Members of the myosin family consist of two heavy chains (200 kDa) and two sets of light chains (myosin light chains; MLC; 16-20 kDa). Myosin II is the best characterised for its ability to promote cell migration and contraction in non-muscle cells. Myosin II function is regulated by phosphorylation of the regulatory light chains by the Ca$^{2+}$-calmodulin-regulated enzyme myosin light chain kinase (MLCK). Phosphorylation of MLC by MLCK promotes ATPase activity and polymerisation of actin cables. This results in a fully functional contractile unit for cell motility, and MLCK had been shown to be essential for migration in both haematopoietic (Walker et al., 1998) and non haematopoietic cells (Klemke et al., 1997). Recently, MAPK has been demonstrated to directly phosphorylate, and therefore activate, MLCK in
carcinoma cells leading to the phosphorylation of MLC. This activation of myosin II is essential for integrin-mediated cell motility (Figure 1.5) (Klemke et al., 1997).

1.13 The Rho-subfamily in adhesion & migration

1.13.1 Rho, Rac and Cdc42 in adhesion and migration

In addition to an obvious role in cell migration, the actin cytoskeleton has been implicated in the clustering of integrins to allow high avidity binding although it is unclear how these cytoskeletal changes are brought about (Stewart et al., 1996). The Rho-subfamily of the Ras superfamily of GTP-binding proteins have recently become major candidates implicated in the changes in actin polymerisation that contribute to the regulation of cell adhesion and migration. The Rho-subfamily consists of several members including Rho, Rac and Cdc42 which are active in the GTP-bound state and inactive in the GDP-bound state. Their activation state is controlled by two main classes of regulatory proteins: guanine nucleotide exchange factors (GEFs) which promote the transition from the inactive GDP-bound to the active GTP-bound conformation and the GTPase activating proteins (GAPs) which stimulate GTP inactivation. In fibroblasts members of the Rho family have specific functions and comprise a hierarchical cascade so that Cdc42 activates Rac which in turn activates Rho. This cascade co-ordinates the dynamic organisation of the actin cytoskeleton and the assembly of associated integrin structures (Tapon and Hall, 1997). There is strong evidence that the same happens in T cells. Cdc42 regulates T cell polarisation towards APCs, an event that is critical for efficient T cell/APC contact and for the directed release of cytokines (Stowers et al., 1995). In addition, the genetic defect in patients with the Wiskott Aldrich immunodeficiency syndrome (WAS) maps to a Cdc42 effector protein called WASp (Symons et al., 1996). WAS is an X-linked recessive disorder characterised by recurrent infections due to defects in T and B cell function, thrombocytopenia and eczema. The cellular defects are limited to haematopoietic cells and include cytoskeletal abnormalities of
lymphocytes. Similarly, although Rho and Rac are essential for the mechanics of macrophage migration, it is Cdc42 that is necessary for the polarisation and directed migration of these cells in a chemotactic gradient (Allen et al., 1998). Rho appears to be directly involved in macrophage migration due to its ability to stimulate contractility (Allen et al., 1998). In lymphocytes Rho has been implicated in β1 and β2 integrin mediated adhesion following PMA (Tominaga et al., 1993) and chemokine (Laudanna et al., 1996) activation, and may also be a target for protein kinase A (PKA)-mediated feedback inhibition of integrin-dependent adhesion (Laudanna et al., 1997; Rovere et al., 1996). It is not clear whether the effects of Rho on integrin-mediated lymphocyte adhesion are due to changes in integrin affinity or effects on avidity following integrin clustering, or a quite unrelated mechanism. To date, there is no established role for Rho in mediating cytoskeletal changes in lymphocytes although this may change when our rudimentary knowledge of the effectors of Rho in lymphocytes becomes more extensive (for review see Reif and Cantrell, 1998). By contrast Rac, but not Rho or Cdc42, has been shown to regulate integrin-mediated cell spreading and adhesion of T cells. This adhesion is dependent on an increase in integrin clustering and avidity, with no change in integrin affinity (D'Souza-Schorey et al., 1998). In addition to a role in adhesion, a role for Rac in lymphocyte migration is supported by the demonstration that TIAM (T cell-invasion-associated molecule)-1, a molecule that promotes T cell migration is also a specific exchange factor for Rac (Michiels et al., 1995).

1.13.2 Cellular targets of the Rho-subfamily

Like all GTPases, the cellular effects of the Rho-subfamily are mediated through their GTP-dependent interaction with cellular targets. Although the mechanisms involved in cytoskeletal organisation by members of the Rho-subfamily are not fully understood, numerous candidate effectors have been described (for review see van Aelst and D'Souza-Schorey, 1997). In the case of Rho, the Rho-associated kinases ROCK and ROKα have attracted much attention. These kinases phosphorylate myosin light chains (MLCs) (Amano
et al., 1996) and also phosphorylate and inhibit MLC phosphatase (Kimura et al., 1996). The result is to activate myosin by a net increase in MLC phosphorylation by Rho-associated kinases and other protein kinases, such as MLC kinase (MLCK) (Figure 1.5). Phosphorylation of MLCs leads to a conformational change in myosin and allows it to interact with actin and to promote the bundling of actin and myosin filaments into polarised contractile fibres (Chrzanowska-Wodnicka and Burridge, 1996). There have also been several reports that Rho and Rac control the production of phosphatidylinositol-4,5-bisphosphate (4,5 PIP₂) by regulating the activity of phosphatidylinositol 4-phosphate 5-kinase (PIP 5-kinase) (Chong et al., 1994; Hartwig et al., 1995). PIP₂ is known to interact with a number of actin binding proteins and to be a key regulator of actin polymerisation so that it is likely that PIP 5-kinase is an important target of Rho and Rac in mediating cytoskeletal reorganisations (Figure 1.5) (Tapon and Hall, 1997). Numerous other candidate targets for Cdc42 and Rac have also been identified and of these p65 PAK, IQGAP, WASp, and POR1 have been suggested to play a role in actin filament assembly (van Aelst and D'Souza-Schorey, 1997). One recent development is the finding that Rac activates LIM-kinase-1 which phosphorylates cofilin, and prevents cofilin from depolymerising actin-filaments (Arber et al., 1998; Yang et al., 1998). Other molecules that may be common to all three GTPase dependent pathways are the ezrin/radixin/moesin (ERM) family of proteins. The ERM proteins bind F-actin, act as membrane cytoskeleton linkers, and have been shown to play an essential role in both Rho- and Rac-dependent actin rearrangements (Mackay et al., 1997). In addition three new proteins have been characterised in fibroblasts and epithelial cells that form a distinct branch of the Rho family: Rnd 1, Rnd 2 and Rnd 3/RhoE that appear to act as negative regulators of actin assembly and of cell adhesion (Nobes et al., 1998). Their expression in leukocytes remains to be established.
1.14 Transendothelial migration of lymphocytes

The greatest barriers facing leukocytes in their migration are the endothelial and epithelial tight junctions, and negotiating these junctions is one of the least understood parts of the whole migratory process. Following arrest of the lymphocyte on the endothelium, LFA-1 plays an essential role in the transendothelial migration of lymphocytes (Luscinskas et al., 1995; Oppenheimer-Marks et al., 1991; van Epps et al., 1989), but this does not preclude a role for additional molecules (Carlos and Harlan, 1994). In monocyte and neutrophil models, CD31 on both the leukocyte and the endothelial surface is required for successful transendothelial migration in vitro and in vivo (Bogen et al., 1994; Muller et al., 1993). However, CD31 is only expressed on a subset of T lymphocytes and so may not be involved in T cell extravasation (Tanaka et al., 1992). Similarly, although mAb blocking studies have implicated IAP in neutrophil transendothelial migration (Cooper et al., 1995), a role for this molecule in the migration of other leukocytes has not been demonstrated. A role for lymphocyte CD31 in transmigration is attractive because of the potential effects that ligation of this molecule has on subsequent integrin-activation (section 1.6.1.3). One might speculate that such cross talk could prime the migrating lymphocyte in preparation for interaction with the sub-endothelial ECM. Once across the endothelium, proteolytic activity is required to aid leukocyte migration through the ECM. Integrin engagement can synergise with TCR/CD3 activation to induce uPAR expression (Bianchi et al., 1996). Upon binding to uPAR, the serine protease urokinase type plasminogen activator (uPA) is activated to convert plasminogen into plasmin. Plasmin is then able to degrade fibrin and other ECM proteins, and acts as the major pericellular fibrinolytic pathway. In support of the importance of this pathway is a report that uPA-deficient mice are unable to mount an immune response against lung pathogens due to a failure of T cells, neutrophils and macrophages to migrate into the lung interstitium (Gyetko et al., 1996)
1.15 Lymphocyte trafficking into the airway epithelium

Although T cell trafficking into the airway epithelium may be critical for mucosal immunity, the mechanism of this trafficking is even more poorly defined than for endothelial transmigration. In some way analogous to endothelial transmigration the T cell must successfully interact with the epithelial cell, pass between the tight junctions of neighbouring cells and into the alveolar air space (Figure 1.6). Once in the alveolar space the T cell interacts not only with allergens and pathogens but also the luminal surface of the epithelial cell. Just as the endothelial cells play an important role in transendothelial migration, the pulmonary epithelial cells are not silent bystander in these events. As well as providing a surface for gas exchange these epithelial cells are also involved in modulating inflammatory reactions within the lung and maintaining a fine balance between combating infection whilst keeping detrimental inflammation to a minimum. They achieve this by the synthesis of a variety of pro-inflammatory mediators such as platelet activating factor (Holtzman et al., 1991), prostaglandins (Churchill et al., 1989) and several cytokines including granulocyte-macrophage colony-stimulating factor (GM-CSF) and IL-6 (Churchill et al., 1992; Marini et al., 1992) and chemokines such as IL-8 (Kwon et al., 1994; Nakamura et al., 1991), MCP-1 (Sousa et al., 1994), MCP-4 (Stellato et al., 1997) and RANTES (Stellato et al., 1994; Taguchi et al., 1998). Many of these mediators are known to be chemoattractant for T cells (Carr et al., 1994; Larsen et al., 1989; Schall et al., 1990) suggesting that their production in response to pathogens or allergens could be involved in the epithelial recruitment of T cells.

As much is known of the initial interaction of T cells with, and adhesion to, the inflamed endothelium it is tempting to propose similar mechanisms in the interaction of T cells with the epithelium. However, there are critical differences, the most obvious being that leukocyte recruitment through the endothelium occurs in a apical-to-basal direction but in the airway epithelium leukocyte recruitment is from basal-to-apical. The first stages of transendothelial T cell migration depend on the co-ordinated regulation of
Figure 1.6: Transepithelial migration of T cells into alveolar space. Schematic cross-section of the lung to show the migration of T lymphocytes (yellow) from the vascular space, across the vascular endothelium and extracellular matrix (ECM) to reach the pulmonary epithelial cells. After interacting with the basal surface of these airway cells the T lymphocytes cross into the pulmonary air-space and interact with their luminal surface.
selectins, integrins and possibly chemokines. In contrast the driving force and molecular interactions that govern T cell migration across the airway epithelium remain to be discovered.

1.16 Major aim of this thesis

The major aim of this thesis was to investigate the regulation of \( \beta_1 \) and \( \beta_2 \) integrin activity on T cells. Various integrin activating stimuli were assessed for differential effects on \( \beta_1 \) and \( \beta_2 \) integrins on T cells. In addition cross influences between the \( \beta_1 \) and \( \beta_2 \) integrins were examined. This involved investigating the role of integrin activation in T cell adhesion and migration using in vitro assay systems.
CHAPTER TWO

MATERIALS AND METHODS

2.1 Materials

2.1.1 Monoclonal antibodies

Monoclonal antibody (mAb) G25.5 (CD11a; LFA-1 α subunit non-function blocking; IgG2a) was purchased from Becton Dickinson (Oxford, UK), MAbs 38 (CD11a; LFA-1 α subunit function blocking; IgG2a), 15.2 (CD54; ICAM-1 function blocking; IgG1), 24 (CD11/CD18; β2 integrin activation reporter; IgG1) (Dransfield et al., 1992; Dransfield and Hogg, 1989), 3.9 (CD11c; p150,95 α-subunit function blocking; IgG1), ICRF-44 (CD11b; Mac-1 α-subunit non-function blocking; IgG1) 4U (control antibody; IgG2a) and 52U (control antibody; IgG1) were prepared in this laboratory, and purified from ascites or tissue culture supenmatant by Protein A Sepharose chromatography (Ey et al., 1978) as described in section 2.2.1. MAbs HUTS-21 (CD29; β1 subunit activation reporter; IgG2b), and 5E8D9 (CD49a; α1 subunit, function blocking; IgG2a) were gifts from Dr Carlos Cabañas, Madrid. MAb 5E8 (CD49b; α2 subunit, function blocking; IgG1) was a gift from Dr R. B. Bankert, Buffalo, NY. MAbs HP1/2 (CD49d; α4 subunit, function-blocking; IgG1) and TS2/16 (CD29; β1 subunit activating; IgG1) were gifts from Roy Lobb, Biogen Inc., Cambridge, MA. The hybridoma producing mAb TS2/9 (CD58; LFA-3 function blocking; IgG1) was purchased from the American Type Culture Collection (ATCC), Rockville, MD. MAb SAM-1 (CD49e; α5 subunit function blocking; IgG2b) was purchased from Eurogenetics, Hampton, UK. MAb 7.2 (CD49d; α4 subunit, non-blocking; IgG1) was a gift from Dr John Marshall, ICRF, London. MAb P5D2 (CD29; β1 subunit function blocking; IgG1) was obtained from Developmental Studies, Hybridoma Bank, Iowa. MAb MEM 56
MAbs UCHL1 (CD45RO; IgG1), UCHL4 (CD8; IgG1), UCHT1 (CD3; IgG1) and UCHT2 (CD5; IgG1) were gifts from Prof. Peter Beverley, University College, London. MAb QS4120 (CD4; IgG1) was prepared by ICRF Hybridoma Service. MAb G19.4 (CD3; IgG1) was a gift from Bristol-Myers Squibb Pharmaceuticals Ltd., Princeton, NJ. MAb K1M127 (CD18; β2 subunit activating; IgG1) and K1M185 (CD18; β2 subunit activating; IgG1) were kindly given by Dr. Martyn Robinson (Celltech Ltd., Slough, UK). The murine IgM mAb against human myosin light chains (MLC; 20 kDa) was purchased from Sigma. The FITC conjugated mAb 44H6 (CD49d; α4 subunit, non function-blocking; IgG1) was purchased from AMS Biotechnology, Witney, UK. Unconjugated rabbit anti-mouse IgG Fc, FITC-conjugated rabbit anti-mouse IgG Fc, and peroxidase conjugated goat anti-human IgG Fc were purchased from Sigma, Poole, UK. FITC-conjugated goat anti-human IgG Fc was purchased from Jackson Immunoresearch Labs., West Grove, PA and FITC-conjugated rabbit anti-mouse IgG2b from AMS Biotechnology.

2.1.2 Cells

The BEAS-2B cell line, immortalised by infection of normal human bronchial epithelial cells with an adenovirus 12-simian virus 40 hybrid virus preparation (Reddel et al., 1988), was purchased from ATCC. The BEAS-2B cell line has been cultured continuously for >100 passages with a stable phenotype. The cells were cultured in serum-free LHC-9 medium. The cells were grown on plastic culture flasks that had been coated for 2 hr at 37°C with a solution of 30 μg/ml collagen, 10 μg/ml fibronectin and 10 μg/ml BSA. LFA-1 transfected (K562/LFA-1; KL/4) and untransfected K562 erythroleukaemic cells were a gift from Dr Martyn Robinson, Celltech Ltd., and were cultured in Dulbecco’s modified eagle medium (DMEM; ICRF Central Cell Services) plus 10% foetal calf serum (FCS; Gibco BRL, Paisley, UK) (Ortlepp et al., 1995). All other cell lines were obtained from ICRF Central Cell Services and were cultured in RPMI 1640 plus 5% FCS. All cells were cultured at 37°C in an humidified atmosphere of 5% CO₂ in air.
2.1.3 Buffers

PBS-A and RPMI-1640 buffers were supplied autoclaved from the ICRF Central Cell Services and their constituents are described in Appendix 1. LHC-9 medium was purchased from Clonetics, San Diego, California.

2.1.4 Recombinant proteins

Tumour necrosis factor (TNF)-α (15 μg/ml; Sigma); Monocyte chemoattractant protein (MCP)-1 (50 μg/ml; Sigma) and Interleukin (IL)-1β (100 μg/ml; PeproTech E.C. Ltd., London UK.) were purchased as powders and reconstituted in low endotoxin water to the concentrations stated. All proteins were stored at -20°C for the short term or -70°C for the long term.

2.1.5 Inhibitors, stimuli and other reagents

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<th>Supplier</th>
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</thead>
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<td>Calbiochem</td>
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</table>

### 2.2 Methods

#### 2.2.1 Antibody purification on Protein A sepharose

MAbs were purified from ascites and tissue culture supernatant (TCS) by affinity chromatography on Protein A sepharose (Pharmacia, Uppsala, Sweden). Approximately 10 ml of ascites or TCS was microfuged and then diluted with an equal volume of 0.1 M phosphate buffer, pH 8, and filtered through a 0.4 µm filter (Millipore, Bedford, MA). This was then loaded onto a Protein A sepharose column that had been pre-equilibrated with phosphate.
buffer. The column was washed with phosphate buffer to remove unbound material. IgG subclasses were then sequentially eluted with 0.1 M citrate buffers of varying pH: IgG1, pH 6; IgG2a, pH 4; IgG2b, pH 3; Eluted fractions were rapidly neutralised with an appropriate volume of 1 M Tris, pH 9. Fractions containing mAb were pooled, and protein concentration estimated (section 2.2.2) and then concentrated and stored. The column was regenerated with at least 10 volumes of 0.1 M Tris pH 8.5 containing 0.5 M NaCl, followed by at least 10 volumes of 0.1 M sodium acetate pH 4.5, containing 0.5 M NaCl then re-equilibrated with phosphate buffer prior to re-using.

2.2.2 Estimation of protein concentration

Protein concentrations were estimated using the Bio-Rad Protein Assay dye reagent (Bio-Rad Laboratories, Hemel Hempstead, UK) in 96 well microtitre plates. Gamma-globulin solutions of known concentrations (0-500 μg/ml) were used as standards to allow a calibration curve to be drawn. Briefly, 5 μl of either standard or test protein was added to wells along with 250 μl of dye reagent concentrate diluted 1:4. Wells were performed in triplicates. The plate was incubated at room temperature (RT) for 5-15 min. Absorbance at 595 nm was measured using a Titertek Multiskan plate reader. Test protein concentrations were calculated from the standard curve of protein concentration versus absorbance readings of gamma-globulin standards.

2.2.3 Preparation of ICAM-1 Fc

A construct encoding a chimeric form of ICAM-1 consisting of the five domains of ICAM-1 fused to the hinge and Fc regions of human IgG1 (Berendt et al., 1992) was a kind gift of Drs A. Berendt and A. Craig (Institute of Molecular Medicine, Oxford, UK). This ICAM-1 Fc construct was transfected stably into Chinese hamster ovary (CHO)-K1 cells by Paula Stanley (Leukocyte Adhesion Laboratory, ICRF) using CaPO₄ precipitation. These cells were then grown in GMEM-S medium (Appendix I) in the presence of 100 mM methionine sulfoxammine (Sigma; stock solution 100 mM in GMEM-S) to select high ICAM-1 Fc producers. Cells were passaged and expanded forty-fold.
until approximately 1.5 litres of cell culture supernatant was obtained. Cells were then washed into serum free GMEM-S supplemented with 5 mM sodium butyrate (Sigma, stock solution 1M in GMEM-S) to enhance ICAM-1 Fc production. After ten days the supernatant was harvested. Cell debris was removed by centrifugation at 2000 g for 5 min and filtration through a 0.22 μm filter (Milipore). Sodium azide (Sigma; 0.02%) was added to the supernatant which was then loaded onto a prewashed Protein A Sepharose column at a rate of 1 ml/min. The column was washed thoroughly with PBS-A/0.02% sodium azide to remove unbound material. The ICAM-1 Fc was eluted from the column in 0.1 M citrate buffer, pH 3 and collected in 2 ml fractions. High protein containing fractions, as judged by reading absorbance at 268 nm, were pooled and concentrated. The identity of the protein was confirmed on sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE; section 2.2.18) and enzyme-linked immunosorbent assay (ELISA; section 2.2.4), and its concentration measured using the Bio-Rad protein assay (section 2.2.2)

2.2.4 ICAM-1 Fc ELISA assay

ICAM-1 Fc was detected in the supernatant and final product using an ELISA. A 96-well Immulon 3 plate (Dynatech, Chantilly, VA) was coated with 50 μl/well of mAb 15.2 (anti-ICAM-1) or mAb 52U (isotype matched control), both at 0.25 μg/ml in 50 mM Tris, pH 9, and incubated overnight at 4°C. The plate was then washed 4 times with PBS-A and blocked with 10% FCS in PBS-A for 2 hr at RT. The plate was washed twice in PBS-A prior to the addition of 50 μl/well of sample material, and incubation at RT for 30 min. The plate was again washed 4 times prior to the addition of 50 μl/well of peroxidase conjugated goat anti-human IgG Fc (1:500; Sigma). After a further 30 min at RT wells were washed 4 times in PBS-A before the addition of 75 μl of OPD (0-phenylene diamine dihydrochloride; Sigma) substrate (one OPD tablet added to 10 μl of H₂O₂ and 25 ml of a mixture of 2 parts 0.1M NaHPO₄: 1 part 0.1 M citric acid by volume). The colour reaction was allowed to develop over 5
min and then stopped with 50 μl/well of 3 M H₂SO₄. Absorbance at 492 nm was measured using a Titertek Multiskan plate reader.

2.2.5 Preparation of T lymphoblasts (T cells)

Peripheral blood mononuclear cells were prepared from single donor leukocyte buffy coats by centrifugation at 600g through Lymphoprep® (Sodium metrizoate/ Ficoll solution; Pharmacia). T lymphoblasts were expanded from this population by culturing in RPMI 1640 (ICRF, Media Supplies) plus 10% FCS in the presence of phytohaemagglutinin (PHA; Murex Diagnostics, Dartford, UK) at 1 μg/ml for 72 hours as previously described (Dransfield et al., 1992). Cells were then washed and maintained for 1-2 weeks in medium supplemented with 20 ng/ml of recombinant interleukin (IL)-2 (Euro Cetus UK Ltd., Harefield, UK). The cells were used between days 10 and 14.

2.2.6 Production of mAbs against BEAS-2B cell surface epitopes

Antibody producing clones were produced by fusing spleen cells from a BALB/c mouse immunised with BEAS-2B cells, with the SP2 mouse myeloma cell line using standard procedures (ICRF, Hybridoma Services). Supernatant from these clones was screened for mAb with the ability to recognise surface epitopes on BEAS-2B cells by flow cytometry (section 2.2.7). Of over 700 clones screened one, 7C2/10, was positive.

2.2.7 Flow cytometry for detection of antibody binding

Flow cytometry for detection of mAb binding was carried out using direct or indirect immunofluorescence as described below. Where necessary before labelling with mAb, adherent cells were released from cell culture plates by washing once in PBS-A, followed by addition of trypsin (0.05%; ICRF cell services) and EDTA (0.02%; ICRF cell services) for 5 min at 37°C. After this time an equal volume of FCS was added and released cells were removed and washed. Cells were then washed three twice into 20 mM HEPES, 140 mM NaCl, 2 mg/ml glucose, pH 7.4 (assay buffer) and 2 x 10⁵ cells in 50 μl of assay buffer were transferred to each well of a Flexiwell® plate (Dynatech). For direct
immunofluorescence, cells were incubated with the FITC-conjugated mAb of interest, together with appropriate concentrations of cations, stimulants, inhibitors and other antibodies, on ice or at 37°C for 30 min. Cells were then washed three times in ice cold assay buffer and samples were kept on ice prior to analysis. For indirect immunofluorescence, the unconjugated mAbs of interest were added to the wells with other reagents on ice or at 37°C as above. Cells were then washed 3 times in ice cold assay buffer prior to incubation with FITC-conjugated rabbit anti-mouse-Ig (Sigma) for 30 min on ice. Cells were then washed three times in ice cold assay buffer and kept on ice prior to analysis as described for direct immunofluorescence. Fluorescence (FL) of live cells was measured on a FACScan® flow cytometer (Becton Dickinson, Mountainview, USA), after counterstaining with 50 μl of 50 μg/ml propidium iodide (PI; Sigma). PI is taken up by dead cells and causes fluorescence in the FL2 channel allowing them to be distinguished from live cells in the same population. Data was acquired and analysed by the Cell Quest® Program (Becton Dickinson, UK). Data is presented as relative fluorescence intensity on a logarithmic axis (labelled with the name of the mAb or the surface antigen recognised by that mAb; X axis) plotted against cell number (Y axis).

2.2.8 Adhesion to respiratory epithelium

BEAS-2B cells were grown as monolayers in tissue culture flasks. The cells from the monolayers were harvested with trypsin/EDTA and resuspended in PBS-A, centrifuged at low speed (250 g, 5 min) and resuspended in fresh medium before seeding in 96 well tissue culture plates. The cells were grown to confluence in these plates over 5-7 days using the same conditions as for cell culture. At 3-6 days post-confluence the culture medium was changed and replaced with new medium and cytokine treatment where necessary. After 24 hr the plates were washed three times in 20 mM HEPES, 140 mM NaCl, 2 mg/ml glucose, pH 7.4 (assay buffer). T cells were washed 3 times in assay buffer and labelled with 2.5 μM BCECF-AM (Calbiochem) in the same buffer for 30 min at 37°C followed by two further washes. T cells (2 x 10⁵ cells) were added to each well in assay buffer with blocking mAbs. Ca²⁺ and Mg²⁺
were included at 0.4 mM in some wells. In addition some wells were treated with 5 mM EDTA, and in others the BEAS-2B cells were removed with trypsin/EDTA to investigate the interaction of T cells with the underlying matrix components. Plates were incubated for 15 min on ice prior to 40 min incubation at 37°C. Non-adherent cells were removed by washing 4 times in warmed assay buffer (150 μl/well). Adhesion was quantified by recording emission at 530 nm, following excitation at 485 nm, using a Fluoroskan® II (Lab Systems, Basingstoke, UK), and expressing the reading for each well as a percentage of the total emission prior to incubation.

2.2.9 Transmigration across respiratory epithelium

BEAS-2B cells were harvested as above and seeded on 6.5 mm diameter, 8.0 μM pore-size Transwell® polycarbonate cell culture inserts (Costar, Cambridge, MA) that had been coated with a sterile mixture of 30 μg/ml collagen (Vitrogen-100, Celtrix, Santa Clara, CA), 10 μg/ml human fibronectin (FN; Sigma), and 10 μg/ml BSA (Sigma) in PBS-A. The inserts were then housed in a 24 well container with 300 μl medium in the insert and 800 μl in the outer chamber. The cells were allowed to grow to confluence over a 5-7 day period. Monolayer integrity was assessed by the ability of a representative monolayer to exclude passage of 4.4 kDa FITC-dextran (Sigma). Moreover, we have found that confluent monolayers have negligible water permeability and can maintain a fluid level gradient between insert and outer chamber for a minimum 24 hr period. To assess transmigration the inserts with adherent BEAS-2B monolayers were washed three times in 20 mM Hepes, 140 mM NaCl, 2 mg/ml glucose, pH 7.4, 0.25% BSA, 0.4 mM Mg^{2+}/0.4 mM Ca^{2+} (transmigration buffer) and then washed. T cells were then added to the insert at a concentration of 5 x 10^5 in 100 μl transmigration buffer with appropriate mAbs. At 6 hr, migrated T cells were removed from the outer well and counted on a haemocytometer. The total number of migrated T cells was calculated as a % of the number added.
2.2.10 T cell attachment assay

Flat-bottomed Immulon-1® 96-well plates (Dynatech) were precoated with 50 μl of either FN (20 μg/ml), VCAM-1 Fc (7 μg/ml; gifts from Roy Lobb, Biogen Inc.), Collagen type I (40 μg/ml) gift of Dr Joanna Brayshaw, EISAI, London) or ICAM-1 Fc (4.8 μg/ml) in PBS-A overnight at 4°C. The plates were blocked with 2.5% BSA (Sigma) in PBS-A for 2 hr at RT, then washed 4 times in assay buffer (section 2.2.8) at 4°C. T cells were washed 3 times in assay buffer and labelled with 2.5 μM BCECF-AM (Calbiochem) in the same buffer for 30 min at 37°C followed by two further washes. T cells (2 x 10^6 cells) were treated with 3 mM Mg^{2+}/2 mM EGTA, 20 nM PdBu or CD3 mAb G19.4 at indicated levels as well as inhibitors, and other mAbs in 100 μl of assay buffer. Ca^{2+} and Mg^{2+} were included at 0.4 mM for experiments involving PdBu or TCR/CD3 (T cell receptor/CD3 complex) crosslinking (CD3XL). Blocking mAbs were titrated on T cells by FACS® analysis and used at saturating concentrations to block T cell function. For FN and VCAM-1 binding assays, all wells contained anti-LFA-1 mAb 38 at function blocking concentrations of 10 μg/ml. This prevents cells aggregating via LFA-1/ICAM-1 interactions which would cause spuriously high binding to β1 ligands through the ‘piggy-back’ interaction of non-adherent cells with truly adherent cells. Plates were incubated for 15 min on ice prior to 40 min incubation at 37°C. Non-adherent cells were removed by washing 4 times in warmed assay buffer (150 μl/well). Adhesion was quantified by recording emission at 530 nm, following excitation at 485 nm, using a Fluoroskan® II (Lab Systems, Basingstoke, UK), and expressing the reading for each well as a percentage of the total emission prior to incubation.

2.2.11 T cell transmigration assay

Assays were performed in 20 mM Hepes, 140 mM NaCl, 2 mg/ml glucose, pH 7.4, 0.25% BSA, 3 mM Mg^{2+}/2 mM EGTA (transmigration buffer) using 6.5 mm diameter, 3.0 μm pore size Transwell® plates (Costar Corp., Cambridge, MA). The upper and lower surfaces of the inserts were coated with FN or
ICAM-1 at concentrations ranging from 0-100 μg/ml in PBS overnight at 4°C. The inserts were positioned in wells containing 600 μl of transmigration buffer. Cells were then plated in the insert at a concentration of 5 x 10^6 cells in 100 μl of transmigration buffer with appropriate mAbs. The mAbs were used at the following final concentrations: mAb HP1/2 at 1.0 μg/ml, mAb 7.2 at 11 μg/ml, mAb SAM-1 at 0.3 μg/ml, mAb 24 at 11 μg/ml and mAb 52U at 11 μg/ml. The anti-LFA-1 mAb 38 was added to all FN coated inserts at function blocking concentrations of 10 μg/ml to prevent a spurious decrease in migration on substrates other that ICAM-1, due to cell aggregation when LFA-1 is activated. The plates were then incubated for 6 hr at 37°C. After this time the bottom surface of the insert was scraped to release migrated but adherent cells into the bottom well, and the migrated cells were counted either using a haemocytometer or using a flow cytometer (Becton Dickinson), after counterstaining with 50 μl of 50 μg/ml PI to exclude dead cells and cell debris. Three samples were counted per well, and readings were averaged from duplicate samples.

2.2.12 Ligand coated beads

A modified protocol (Pyszniak et al., 1994) was developed in which 200 μl (1 x 10^6) of 3.2 μm polystyrene beads (Sigma) were washed twice in distilled water, followed by 2 further washes and resuspension in 0.1 M bicarbonate buffer pH 9. FN, ICAM-1 VCAM-1 or BSA, as control, were added to these beads to a final concentration of 10 μg/ml. The beads were rotated at RT for 1 hr, washed once in PBS and blocked with 0.1% heat denatured BSA for 2 hr at RT whilst being rotated. The beads were then washed twice in 20 mM HEPES, 140 mM NaCl, 2 mg/ml glucose, pH 7.4 (assay buffer) containing 3 mM Mg^{2+}/2 mM EGTA, for use in the adhesion experiments as described.
2.2.13 T cell bead attachment assay

Multiwell Lab-Tek® Chamber Slides (Nunc, Naperville, Illinois) were left as uncoated controls or coated with either ICAM-1 Fc (10 μg/ml in PBS) or rabbit anti-mouse Ig (1:100 dilution in PBS) overnight at 4°C. The following day mAb tissue culture supernatant was added to wells precoated with anti-mouse Ig and left on ice for 1 hr. Wells were washed twice with PBS and non-specific binding sites were blocked with 0.1% denatured BSA for 2h at RT. Cells (150 μl of 2 x 10^6/ml) in assay buffer (section 2.2.8) containing 3 mM Mg^{2+}/2 mM EGTA, were added to the wells and allowed to settle on ice for 15 min. Freshly prepared ligand-coated beads (section 2.2.12) were added to the wells at 100:1 bead to cell ratio in 50 μl. After 30 min at 37°C the unbound beads and cells were removed by 4 washes in warmed assay buffer. Bound cells were fixed with 1% formaldehyde in PBS for 20 min at RT. Cells were then stained with haematoxylin for 10 min. Beads and cells were counted per high power field (Zeiss, x40 oil immersion objective), and the number of beads per hundred cells was determined (attachment index).

2.2.14 Biotinylation of fibronectin

Prior to biotinylation, fibronectin (Sigma) was dialysed into PBS-A to a concentration of 0.4 mg/ml. To 3 ml of fibronectin was added 3 mg of sulfosuccinimidyl-6-(biotinamideo)hexanoate (EZ-Link™ Sulfo-NHS-LC-Biotin, Pierce, Rockford, IL) and the reaction incubated for 2 h at RT whilst rotating. The reaction was stopped by the addition of 175 μl of 1 M Tris buffer, pH 8. Unbound biotin was removed by extensive dialysis into PBS-A. The biotinylated fibronectin (FN-bt) was stored at 4°C with addition of 0.02% sodium azide.

2.2.15 Flow cytometry for detection of soluble ligand binding

T cells were washed three times into assay buffer (section 2.2.8), and then resuspended at 4x10^6 cells/ml into assay buffer containing 1% BSA and appropriate concentrations of cations, PdBu, and EGTA. T cells were then
added to Flexiwell® plates (Dynatech) in 50 µl aliquots and the appropriate concentration of ICAM-1 Fc, VCAM-1 Fc or biotinylated-fibronectin (FN-bt) was added together with the relevant mAbs. After a 30 min incubation at 37°C, cells were washed twice in ice-cold assay buffer/0.1% BSA and then incubated with 10 µg/ml FITC-conjugated goat anti-human IgG Fc specific Ab (Jackson Immunoresearch Labs.) for ICAM-1 and VCAM binding, or streptavidin-FITC (1:200; Amersham International plc, Little Chalfont, UK) to detect FN-bt binding, for 20 min on ice. Cells were washed twice in ice cold assay buffer/0.1% BSA to remove excess unbound mAb and FL1 of live cells was detected using a (Becton Dickinson) flow cytometer. Dead cells were distinguished by their ability to take up PI and were therefore excluded from analysis. Data is presented as relative fluorescence intensity on a logarithmic axis (labelled with the name of the ligand of interest; X axis) plotted against cell number (Y axis).

2.2.16 Immunofluorescence and confocal microscopy

For immunofluorescence analysis, 13 mm round glass coverslips were precoated with 300 µl of either FN (20 µg/ml), VCAM-1 Fc (7 µg/ml) or ICAM-1 Fc (2.4 µg/ml) in PBS overnight at 4°C. Coverslips were blocked with 2.5% BSA in PBS-A for 2 h at RT and then washed three times with PBS-A and once with assay buffer (section 2.2.8) at 4°C. For some experiments additional coverslips were coated with poly-L-lysine (0.01%; Sigma) for 5 min at RT and then washed 3 times with RPMI. T cells were washed three times in assay buffer before being added to the coverslips (5x10^5 cells/coverslip in) 100 µl of assay buffer in the presence of appropriate concentrations of divalent cations, primary and stimulating mAbs, EGTA or PdBu where appropriate. Cells were incubated for 10 min on ice and then a further 30 min at 37°C. Unbound cells were removed with 4 gentle washes with assay buffer (containing the appropriate cations). Adherent cells were then fixed with 1% formaldehyde in PBS-A for 20 min at RT. The coverslips were again washed 3 times in ice-cold assay buffer before addition of the appropriate secondary FITC-conjugated mAb. In order to stain for intracellular actin, cells were first permeabilised
with 0.2% Triton-X-100 in assay buffer for 10 min on ice, and then incubated
with 0.25 μg/ml TRITC-conjugated phalloidin (Sigma) for 20 min on ice
before washing twice and mounting on slides for immunofluorescence.
Confocal microscopy was performed using a Leica TCS NT microscope
equipped with a 60 x oil immersion objective (Leica, Wetzler, Germany)

2.2.17 Flow cytometry and assessment of intracellular actin
Polymerised actin (filamentous (F)-actin) was determined by staining with
FITC-conjugated phalloidin (Sigma). T cells were washed three times into
assay buffer (section 2.2.8), and pre-treated with or without cytochalasin D (10
μg/ml) for 30 min at RT. T cells at 4 x 10^6 cells/ ml were then added to
Flexiwell® plates (Dynatech) in 50 μl aliquots of assay buffer containing 1%
BSA and appropriate concentrations of cations, mAbs, PdBu, and EGTA. T
cells were then warmed to 37°C, and at various time points the content of F-
actin was assessed. At these points cells were washed in ice-cold assay buffer
/0.1% BSA before fixing with 1% formaldehyde in PBS-A for 20 min at RT.
Cells were then permeabilised with 0.2% Triton-X-100 in assay buffer for 10
min on ice, before addition of 0.25 μg/ml TRITC-conjugated phalloidin for 20
min on ice. Cells were washed twice in ice cold assay buffer/0.1% BSA and
fluorescence was detected using a flow cytometer (Becton Dickinson).
Fluorescence was expressed either as % of relevant control fluorescence or as
mean relative fluorescence intensity per cell.

2.2.18 SDS-PAGE
A 7.5% (for high molecular weight proteins) or 15% (for low molecular
weight proteins) polyacrylamide gel (stock 30% w/v acrylamide/ 0.8% w/v
bis-acrylamide; Anachem Ltd., Luton, UK) in 375 mM Tris, pH 8.8. 0.1% SDS,
0.04% ammonium persulphate (APS; Sigma) and 1/500 N,N,N',N'-
tetramethyl-ethylenediamine (TEMED; Sigma) was used to separate the
proteins. If a wide range of proteins were to be separated then a gel with a 7.5-
15% linear gradient of acrylamide in the separation slab gel was prepared. The
separating gel was overlaid with a 3% polyacrylamide stacking gel made up in
125 mM Tris, pH 6.8, 0.1% SDS, and polymerised as above with APS and TEMED. Sample buffer (125 mM Tris, pH 6.8, 4% SDS, 27.5% glycerol, 0.002% bromophenol blue) with 1% β2-mercaptoethanol (Sigma) for samples that required reducing conditions, was added to protein samples. The samples were then boiled for 5 min in a water bath. Molecular weight rainbow markers (Amersham) covering a range of molecular weights 2.5-220 kDa were prepared in the same way as the protein samples. Samples were then loaded onto the gel and underwent electrophoresis in 25 mM Tris/192 mM glycine, pH 8.3, 0.1% SDS at 100 V until the sample dye front reached the stacking/separating gel interface at which time the voltage was increased to 180 V. Proteins were either visualised by staining with Coomassie Blue (0.5% w/v Coomassie Blue, 40% ethanol, 10% glacial acetic acid) and destained with 20% ethanol/ 10% glacial acetic acid, or gels were prepared for immunoblot analysis.

2.2.19 Glycerol/urea PAGE

The method for glycerol/urea PAGE was adapted from previous descriptions (Perrie and Perry, 1970; Taylor and Stull, 1988) as follows. T cells (5 x 10⁵-5 x 10⁷) were resuspended in 9 M urea, 22 mM glycine, 20 mM Tris, 2 mM dithiothreitol (DTT), 20 mM sucrose and 1 mM EGTA, pH 8.8 with addition of 0.04% w/v bromophenol blue in ethanol and then heated to 90°C for 5 min, or left overnight at 4°C. Samples were electrophoresed on a separating gel containing 10% acrylamide, 40% glycerol, 22 mM glycine, 20 mM Tris, pH 8.8 and polymerised with APS and TEMED, after a stacking gel consisting of 4% acrylamide, 6 M urea, 40% glycerol, 22 mM glycine, 20 mM Tris, pH 8.8, APS and TEMED. Running buffer was 22 mM glycine and 20 mM Tris, pH 8.8. Gels were pre-electrophoresed for 1 h at 350 V with 1 mM DTT added to the upper chamber. The samples were then loaded and electrophoresed for 1.5 h at 450 V. The gels were then prepared for immunoblot analysis.
2.2.20 Immunoblot analysis (western blotting)

Approximately $4 \times 10^5$ T cells were disrupted by addition of 40 μl of SDS sample buffer (section 2.2.18), and boiled for 5 min. SDS-PAGE was carried out as above (section 2.2.18); the samples were then electrophoretically transferred (0.2 A current for 1 h) onto Hybond® ECL nitrocellulose membranes (Amersham). Protein transfer was confirmed by staining the membrane in Ponceau S solution (0.1% w/v in 5% acetic acid; Sigma, UK). The membrane was then incubated with blocking solution (5% w/v skim milk or 5% w/v BSA in PBS-A/0.1% v/v Tween 20) overnight at 4°C, and reacted with primary mAb in the blocking solution for 2 h at RT. The membrane was washed 3 times in PBS-A/0.1% v/v Tween 20, and incubated with the appropriate horseradish peroxidase (HRP)-conjugated secondary mAb in the blocking solution for 2 h at RT, before washing 4 times as before and reacting with Supersignal® substrate for Western blotting (Pierce) for 1 min at RT. The image was then detected in a dark-room using Hybond® ECL® film (Amersham).

2.2.21 Statistics

Results are presented as mean ± SD (or SEM for samples of 6 or more readings) and confidence intervals of 95% were deemed to approximate to mean ± 2 SDs. Further statistical analysis could be performed on this data to calculate the level of significance between two data sets. As numbers of samples are small it may not be possible to determine normality in all cases. To compare serial sets of data parametric analysis of variance (ANOVA), or the non-parametric equivalent, Kruskal-Wallis would be performed. For sample comparisons data should be analysed either using the Students t-test or Mann-Whitney U-test.
CHAPTER THREE
LYMPHOCYTE ADHESION TO EXTRACELLULAR MATRIX AND TO THE RESPIRATORY EPITHELIUM

3.1 Introduction

The transmigration of T lymphocytes from the blood stream and into inflamed epithelium is essential for successful immune surveillance and resolution of infection. In healthy non-smokers continuous leukocyte trafficking into the lung results in a large number of intraluminal lymphocytes (calculated as 4x10³/μl of broncho-alveolar fluid; Saltini et al., 1988). The majority of these are memory T cells with 70-100% CD45RO⁺ (Becker et al., 1990) and approximately 20% expressing the very late activation (VLA) markers α1β1 (VLA-1) (Saltini et al., 1988) and α4β1 (VLA-4) (Garlepp et al., 1992). Although only 1.7% of peripheral T cells are positive for αEβ7 (E = epithelial), this integrin is expressed on over 40% of T cells found in broncho-alveolar lavage (BAL) of healthy non-smokers with αEβ7 found most frequently on the CD8⁺ subset of T cells (Erle et al., 1994). It was an interesting surprise when the ligand for αEβ7 was discovered to be E-cadherin suggesting a mechanism for retention of intra-epithelial lymphocytes (IEL) within the epithelium (Cepek et al., 1993; Cepek et al., 1994). The ratio of CD4 to CD8 cells in BAL is highly variable (0.3-6.6; mean 2.1) (Becker et al., 1990). T cell numbers are increased even further in BAL in response to various infectious and inflammatory stimuli including Mycobacterium tuberculosis, respiratory syncitial virus (RSV) and inhaled allergens. Although undoubtedly important in specific immunity, T cells in excessive numbers and activation-states are implicated in the pathogenesis of many chronic inflammatory lung conditions such as asthma, pulmonary fibrosis and rejection of transplanted lung, with increased numbers of activated T cells found in BAL from patients with these conditions. In particular CD4⁺ T cells with a cytokine profile corresponding to that of T_{H2} type are known to play a
central role in maintaining pulmonary inflammation and airway obstruction in asthma (for review see Corrigan and Kay, 1992). A striking recent study demonstrates that even in the absence of identified environmental allergens or gross airway inflammation, genetically determined airway responsiveness in mice is also regulated by T cells. Although the T cells are present at only normal levels in the lung the animals hyperresponsive airways improve on depletion of CD4+ T cells (De Sanctis et al., 1997). One speculation is that it is the cytokine profile produced by T cells in response to low levels of antigen that determines airway reactivity. As baseline airway responsiveness is a recognised risk factor for subsequent lung disease (for example asthma or chronic obstructive pulmonary disease) these T cell dependent mechanisms warrant study in humans. In the mouse there is also a distinct subpopulation of T cells that instead of an αβ TCR express a single non-variant γδ T cell receptor, and most of these cells are CD4 and CD8 negative (Augustin et al., 1989). There is recent evidence that these γδ T cells may be responsible for driving the TH2 response that leads to the production of IgG1 and IgE and the symptoms of airway inflammation (Zuany-Amorim et al., 1998). However, it is not clear whether these findings can be extrapolated to human subjects (Erle, 1995).

Much previous work has focused on how leukocytes interact with the endothelial barrier but there has been less emphasis on subsequent events including adhesion to, and migrate across, the subendothelial matrix which contains fibronectin and collagen (Figure 1.6). It has been shown that T lymphocytes bind to fibronectin using the β1 integrins α4β1 and α5β1 (Shimizu et al., 1990) and to collagen using the β1 integrin α2β1 (Goldman et al., 1992). Such adhesion to components of the extracellular matrix serves not only to localise leukocytes but may directly alter their function. For example both collagen and fibronectin may provide a costimulus for T cell receptor mediated proliferation (Brunmark and O'Rourke, 1997; Dang et al., 1990; Nojima et al., 1990). Having negotiated the extracellular matrix, those T cells trafficking to the lungs come into contact with the basolateral surface of the
pulmonary epithelial cells. The T cells must then continue their migration between the pulmonary epithelial cells and into the alveolar space. Little is known of the presence and regulation of adhesion molecules on airway epithelium. An important role for ICAM-1 was demonstrated in a non human primate model of asthma in which antigen inhalation resulted in increased ICAM-1 expression on both endothelial and epithelial cells in the airways (Wegner et al., 1990). An increase in ICAM-1 expression can also be seen in vitro on lung epithelial cell cultures after incubation with cytokines (Colgan et al., 1993; Cunningham and Kirby, 1995) and after viral infection (Tosi et al., 1992). The relevance of this increased ICAM-1 expression has been suggested by the inhibition of T cell adhesion to airway epithelium with an anti-ICAM-1 mAb (Cunningham and Kirby, 1995); however inhibition was not complete (54% to 77%) indicating that other cell adhesion molecules must be involved. The aims of these experiments were to establish the mechanism of interaction of T cells with the respiratory epithelium and components of the extracellular matrix in an attempt to understand more about normal and pathological trafficking of T cells into the lungs.

3.2 Results

3.2.1 Phenotype of T cells cultured in interleukin-2

T lymphocytes were expanded from peripheral blood mononuclear cells (PBMCs) and stimulated with phytohaemagglutinin (PHA) for 4 days, followed by incubation in interleukin (IL)-2 for 10 days as described in section 2.2.5. All experiments were performed with T cells at day 8-14. These cells were phenotyped using flow cytometry and shown to be a homogeneous population of CD3⁺ lymphocytes with a CD8⁺:CD4⁺ ratio of 2:1 (Figure 3.1). In addition, the cells were double positive for CD45RO and CD45RA and negative for the natural killer (NK) cell marker CD16 (FcγRIII) (Figure 3.2). The cells were uniformly positive for integrin LFA-1 (αLβ2), but had only very low levels of Mac-1 (αMβ2) and p150,95 (αXβ2) (Figure 3.3).
Figure 3.1: Phenotypic analysis of cultured T cells. PBMCs were stimulated with PHA for 3 days, and then cultured in the presence of IL-2. After 10 days in culture, flow cytometric analysis using mAbs showed the cells to be (A) 100% CD3 positive; (B) 67% CD8 positive; (C) 33% CD4 positive. FITC-conjugated rabbit antibody anti-mouse Ig (FITC-RaMlg) is shown in black, and cells incubated with primary and secondary mAbs in green. The data shown are from one representative experiment of three.
Figure 3.2: Phenotypic analysis of cultured T cells. PBMCs were stimulated with PHA for 3 days, and then cultured in the presence of IL-2. After 10 days in culture, flow cytometric analysis using mAbs showed the cells to be (A) 100% CD45RO positive; (B) 100% CD45RA positive; (C) 100% CD16 negative. FITC-μM1g alone is shown in black, and cells incubated with primary and secondary mAbs in blue. The data shown are from one representative experiment of three.
Figure 3.3: Phenotypic analysis of cultured T cells. PBMCs were stimulated with PHA for 3 days, and then cultured in the presence of IL-2. After 10 days in culture, flow cytometric analysis using mAbs showed the cells to be 100% LFA-1 (αLβ2) positive (A); but with only low levels of Mac-1 (αMβ2) (B); and p150,95 (αXβ2) (C). FITC-RαMlg alone is shown in black, and cells incubated with primary and secondary mAbs in red. The data shown are from one representative experiment of three.
3.2.2 Phenotype of the bronchial epithelial cell line BEAS-2B

The human bronchial epithelial cell line BEAS-2B was cultured and phenotypically characterised. These cells express MHC class I, ICAM-1 (CD54), LFA-3 (CD58), CD44, E-cadherin and β1 integrins, but were negative for LFA-1, ICAM-3, and CD31 (data not shown).

3.2.3 T cell adhesion to the BEAS-2B cell line has integrin-dependent and integrin-independent components

The functional activity of alveolar epithelial adhesion molecules was then studied by determining their ability to bind T cells. Adhesion of T cells to the luminal surface of confluent monolayers of BEAS-2B cells peaked at 15 minutes (data not shown) and took place even in the absence of additional cations (Figure 3.4). The addition of physiological amounts of Ca\(^{2+}\) (0.4 mM) and Mg\(^{2+}\) (0.4 mM) enhanced adhesion by ~ 60% (Figure 3.4). Blocking mAbs showed adhesion between LFA-1 on the T cells and ICAM-1 on the epithelial cells to be the most important interaction with a small and less consistent contribution from CD2 on the T cell binding to LFA-3 on the epithelial cell (Figure 3.4).

3.2.4 Effect of BEAS-2B cell activation on T cell adhesion

In view of the dependence of adhesion on LFA-1 and previous reports that cytokine treatment can increase the levels of ICAM-1 on respiratory epithelium, the effect of preincubation of the respiratory epithelium for 24 h with either TNF-α or IL-1 was examined. Neither cytokine affected the level of integrin-dependent or integrin-independent T cell binding (Figure 3.5). Under both stimulated and unstimulated conditions approximately 50% of the adhesion was not be blocked by 5 mM EDTA (Figure 3.5) and was also temperature independent, occurring at 4°C (data not shown), both features indicate an integrin-independent adhesion process.

*"Physiological" in this work is defined as the concentration of Mg\(^{2+}\) and Ca\(^{2+}\) in ICRF produced RPMI. Serum levels of these cations in man are, in fact, Mg\(^{2+}\), 0.75-1.05 mM and ionized Ca\(^{2+}\), 1.0-1.25 mM.
Figure 3.4: T cell adhesion to a respiratory epithelial (BEAS-2B) cell monolayer has a cation-dependent LFA-1 mediated component. T cells bind confluent BEAS-2B monolayers in a cation-independent manner. This binding is enhanced by 0.4 mM Mg$^{2+}$ and 0.4 mM Ca$^{2+}$. The additional adhesion is blocked by LFA-1 and, to a lesser extent, LFA-3 blocking mAbs. Results are expressed as mean ± standard deviation (S.D.) of triplicate wells. One representative experiment of five is shown.
Figure 3.5: T cell adhesion to a BEAS-2B cell monolayer is not enhanced by stimulation of the epithelial monolayer with TNF-α or IL-1β. Adhesion is inhibited (approx. 50%) by blocking LFA-1 (mAb 38) or ICAM-1 (mAb 15.2), and by cation chelation (5 mM EDTA). Adhesion is not enhanced by 24 hr pre-incubation of the monolayer with TNF-α (10 ng/ml) or IL-1β (0.1 ng/ml). Data are mean ± S.D. of triplicate wells. One representative experiment of two is shown.
3.2.5 Activation of T cell integrins is not due to a soluble factor

The activity of LFA-1 in the absence of an activating stimulus is surprising and suggests that an interaction with BEAS-2B cells in some way activates the T lymphocytes. The inability of culture medium from BEAS-2B cell cultures to enhance the binding of T cells to purified ICAM-1 or fibronectin (data not shown) suggested that the activating event was not the result of a diffusible soluble substance, and might depend on a physical interaction between the T cell and the epithelial cell. The sensitivity of the integrin-mediated binding to Cytochalasin D suggests that the LFA-1 is of low affinity and binding is dependent on receptor clustering and cell spreading (Figure 3.6.A).

3.2.6 Transmigration of T cells across BEAS-2B monolayers

Having shown that LFA-1 is involved in the adhesion of T cells to BEAS-2B cells, it was of interest to look at the role of integrins in T cell migration across an epithelial monolayer. BEAS-2B cells were grown on 8.0 μm pore size Transwell® inserts, and unstimulated T cells were allowed to migrate across the monolayer over a 4 hr period. Because of the difficulties in examining migration in the physiological direction, migration was measured in the less physiological apical-to-basal direction (Figure 3.6.B). Migration across the uncoated filters was greater than that across the intact monolayer. Surprisingly, the migration across the monolayer could not be reduced by mAbs to either LFA-1 or LFA-3 suggesting that adhesion through these receptors was not a prerequisite for transmigration and that other methods of T cell adhesion to the monolayer are sufficient for initiation of migration. Cytochalasin D abolished migration emphasising that the migration of T cells as measured in this assay involves an active process that is dependent on an intact cytoskeleton.
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Figure 3.6: T cell adhesion to a confluent BEAS-2B cell monolayer, and migration across the monolayer are inhibited by cytochalasin D. (A) The cation-dependent component of T cell adhesion to BEAS-2B cells is inhibited by 2 μM cytochalasin D; (B) Migration of T cells across the BEAS-2B monolayer is also inhibited by 2 μM cytochalasin D, but not by blocking mAbs to LFA-1 or LFA-3. Results are expressed as mean ± S.D. of triplicate wells. One of two similar experiments is shown.
3.2.7 Immunisation of mice with BEAS-2B cells and screening of reactive mAbs led to the isolation of the mAb-producing clone, 7C2/10

To aid the identification of other adhesion molecules on bronchial epithelial cells that might mediate T cell adhesion, BALB/c mice were immunised with the BEAS-2B cell line and mAb producing hybridomas were produced (section 2.2.6). The resultant hybridomas were screened for the production of mAbs that recognised cell surface molecules expressed on BEAS-2B cells. Over 700 samples were screened and one clone, 7C2/10, was shown to stain BEAS-2B cells when used in flow cytometry. The 7C2/10 clone was grown up and the mAb 7C2/10 (IgG1) was purified. However, when used in adhesion and transmigration assays the mAb 7C2/10 had no functional effect on adhesion of T cells to, or their migration across, the bronchial epithelial cell line.

3.2.8 Expression of the 7C2/10 epitope

The distribution of the 7C2/10 epitope was characterised on a variety of cells and cell lines using flow cytometry. 7C2/10 is strongly expressed on a variety of haematopoietic cells including T cells, monocytes and neutrophils (data not shown). Biochemical characterisation of the 7C2/10 antigen from peripheral T cells has not been possible. The mAb 7C2/10 does not immunoprecipitate any proteins from T cell lysates (Dr Lisa Williams, ICRF), and does not react convincingly with any protein bands on a Western blot of reduced or non-reduced T cell lysates (data not shown).

3.2.9. Adhesion of T cells to collagen (type 1) and fibronectin

One of the characteristics of chronically inflamed asthmatic lung is a thickening of the basement membrane and in particular increased collagen deposition (for review see Corrigan and Kay, 1992). For optimal growth the BEAS-2B cell line requires plating on a mixture of collagen type I, fibronectin and bovine serum albumin (BSA) (section 2.1.2). Initial experiments showed that while T cells could bind to BEAS-2B cells in the absence of an activating stimulus, there was no binding of unstimulated T cells either to the matrix on
which the BEAS-2B cells are normally grown or to the matrix that they secrete when confluent (data not shown). Experiments were therefore aimed at looking at the interaction of T cells with each of the individual matrix components. The T lymphocytes used in these experiments express very low levels of Mac-1 and can not be induced to bind to the Mac-1 ligands, bovine serum albumin (BSA) or fibrinogen using any of the activating stimuli described in this thesis (data not shown; Newton, 1998). For this reason BSA is an ideal agent for blocking non-specific binding sites in our T cell binding assays. T cells can bind both collagen (Figure 3.7) and fibronectin (Figure 3.8), when stimulated with phorbol ester (PdBu; 50 nM) (Figure 3.7) or by cross-linking the TCR/CD3 complex (CD3XL) (Figure 3.8) in agreement with previous findings (Goldman et al., 1992; Shimizu et al., 1990). The binding to collagen is mediated by α2β1 (Figure 3.7.B). Both α4β1 and α5β1 can be involved in T cell binding to fibronectin (Figure 3.8.A) and if one is blocked the other can partially compensate at all concentrations of fibronectin (Figure 3.8.B) as previously described (Wayner et al., 1989).

3.2.10 MCP-1 differentially affects β1 and β2 integrins

Previous work in this and other laboratories had established that T cells can firmly adhere to purified ICAM-1, through the integrin LFA-1, and it was of interest to see if T cells could be induced to migrate across Costar filters coated with fibronectin or ICAM-1. Monocyte chemoattractant protein (MCP)-1 has been recently shown to act as a T cell chemoattractant (Carr et al., 1994). Experiments were therefore performed to assess the chemotactic migration of T cells across ligand-coated filters, in response to 50 ng/ml of MCP-1 in the lower well. The results of such experiments demonstrated a differential effect of MCP-1 on β1 and β2 integrins, so that while migration across fibronectin-coated filters was significantly increased by MCP-1 (Figure 3.9.B), migration across ICAM-coated filters was not affected (Figure 3.9.A). This was the first suggestion of differential activation of β1 and β2 integrins on these T cells.
Figure 3.7: T cell adhesion to collagen is mediated by α2β1, not α1β1
A. T cells stimulated with PdBu (50 nM) in hepes with 0.4 mM Mg\(^{2+}\)/Ca\(^{2+}\) bind collagen type I; B. This adhesion is inhibited by mAb 5E8 (α2-blocking; 10 μg/ml), but not by mAb 5E8D9 (α1-blocking; 10 μg/ml). Results are expressed as mean ± S.D. of triplicate wells. One representative experiment of five is shown.
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Figure 3.8: T cell adhesion to fibronectin is mediated by α4β1 and α5β1, and when one is blocked the other can compensate.

(A) T cells stimulated by TCR/CD3 cross-linking (CD3XL; mAb UCHT1; 10 μg/ml) in hepes with 0.4 mM Mg2+/Ca2+ bind fibronectin; Adhesion is inhibited by a combination of mAb HP1/2 (α4-blocking), and mAb SAM-1 (α5-blocking); (B) Adhesion is mediated by α4β1 and α5β1 at all concentrations of fibronectin. Results are expressed as mean ± S.D. of triplicates. One representative experiment of four is shown.

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Figure 3.9: MCP-1 promotes migration of T cells across fibronectin, but not across ICAM-1. (A) Migration of T cells across ICAM-1-coated filters (—□—) is not enhanced by monocyte chemoattractant protein (MCP)-1 (50 ng/ml) in the lower chamber (—---■---); (B) Migration of T cells across fibronectin-coated filters (—□—) is increased by MCP-1 in the lower chamber (—■---); Results are expressed as % of added cells migrated/well ± S.D. of triplicates. One representative experiment of three is shown.
3.3 Discussion

This chapter describes initial experiments to characterise the interaction of human T cells with the respiratory epithelium. Initial investigations characterised the phenotype of the cultured T lymphocytes as memory-like (CD45RO+) and therefore able to respond to CD3XL without the requirement for a second signal. Subsequently the expression of adhesion molecules on the bronchial epithelial cell line, BEAS-2B, was studied. The findings were similar to other studies of BEAS-2B cells (Bloeman et al., 1993) and of primary human bronchial epithelial cells (Bloeman et al., 1993; Cunningham and Kirby, 1995).

The ability of human alveolar epithelial cells to bind T lymphocytes through an LFA-1/ICAM-1 interaction was demonstrated. This extends previous murine findings (Christensen et al., 1993) to human lung, and has subsequently been shown by another group (Cunningham et al., 1994). It is however clear that not all T cell/BEAS-2B interactions could be explained by these molecules. In most instances ~50% of binding was not inhibited by anti-LFA-1 mAbs. There was a small and variable contribution to adhesion from LFA-3 which is known to bind CD2 on lymphocytes (Shaw et al., 1986) and has been implicated in T cell adhesion to the IFN-γ stimulated bronchial epithelial cell line, H292 (Bruinier et al., 1994). Over 40% of lung lymphocytes express αEβ7 (Erle et al., 1994) and these cells can bind epithelial cells by interacting with their ligand E-cadherin (Cepek et al., 1993; Cepek et al., 1994). However, the residual T cell binding after blocking LFA-1 and LFA-3 was neither cation-dependent nor temperature-sensitive and therefore unlikely to be mediated by integrins. There are, of course, other plasma molecules that are important in adhesion. For example, CD44, a transmembrane proteoglycan that is a hyaluronate receptor, is expressed on cultured T cells, and CD45, one of the most abundantly expressed leukocyte surface membrane molecules has also been implicated in adhesion and migration. It is an
experimental priority to define the role of these and other non-integrin
adhesion receptors in the systems described in this chapter.

It is interesting that T cells appear to bind epithelial cells through LFA-
1/ICAM-1 interactions without prior activation of LFA-1. The levels of LFA-
1-dependent adhesion were 20-40% which is what would normally be seen
when activated T cells bind purified ICAM-1. This suggests that LFA-1 on the
T cells is being activated by the interaction of T cells with the respiratory
epithelium, and that epithelial cells may play a role in activating T cells at
mucosal surfaces. The activation was not due to a soluble factor, suggesting a
role for a physical interaction between the T cells and the epithelium. Recent
studies have demonstrated CD2-dependent T cell activation (measured by
increased expression of the IL-2 and transferrin receptors) occurring 24 hr after
the adhesion of clonal lung T cells to IFN-γ-activated airway epithelial cells
(Bruinier et al., 1994). CD2 expression is upregulated on alveolar T
lymphocytes (Yamaguchi et al., 1991) and ligation of CD2 has been shown to
activate both β1 (Shimizu et al., 1995) and β2 (van Kooyk et al., 1989) integrins
on T cells. However, in this system the upregulation of LFA-1-mediated
adhesion was not blocked by an LFA-3 blocking mAb suggesting that ligation
of CD2 with LFA-3 was not involved.

There is increasing evidence that cytokines, like the proinflammatory
cytokines IL-1β and TNF-α may participate in the inflammatory response in
asthma. However we found that there was no change in T cell adhesion to
BEAS-2B monolayers after stimulation of the monolayers with either IL-1β or
TNF-α for 24 hr. Recent studies have shown either no change in ICAM-1
expression of primary epithelial cells (Cunningham and Kirby, 1995) or
increased expression in both primary epithelial cells and BEAS-2B cells
(Bloeman et al., 1993) after treatment with IL-1β or TNF-α. The results
described here do not necessarily support the former finding as there are
examples of ICAM-1 expression being upregulated on primary bronchial
epithelial cells without increasing ICAM-1-dependent T cell adhesion presumably because in these situations the levels of ICAM-1 may already have been optimal (Cunningham et al., 1994).

The BEAS-2B cells are able to form fluid impermeable junctions in culture as evidenced by their ability to exclude 4.4 kDa dextran-FITC and inhibit lymphocyte transmigration. Interestingly migration across the confluent monolayer did not depend on integrin-mediated adhesion. In endothelial migration assays it has been hard to dissect a role for the β2 integrins because of their fundamental role in firm adhesion which is a prerequisite for migration across the tight junction. It was surprising to find that the migration across this epithelial monolayer is apparently quite different. There is only one other report in the literature of T cell trafficking across lung epithelial monolayers in vitro (Taguchi et al., 1998). In that report PdBu-stimulated primary T cells migrate across IFN-γ-stimulated primary airway epithelium in both directions, but with slightly enhanced migration in the physiological basal-to-apical direction. The level of migration when neither T cells or epithelium were stimulated was ~4%, rising to ~9% with stimulation of both T cells and epithelium. This extra migration, but not the background 4%, could be blocked with LFA-1 or ICAM-1 blocking mAbs. These levels of migration are in agreement with those shown here, and suggest that in both systems there is residual migration in the unstimulated situation that is integrin-independent (Taguchi et al., 1998). Recent work from this laboratory has shown that stimulation of cultured T cells with the S100 protein migration inhibitory factor related protein (MRP)-14 can induce the expression and activation of Mac-1 on these cells (Newton, 1998). It would therefore be of interest to examine the potential role of Mac-1 on T-cell adhesion to and migration across the respiratory epithelium.

Experiments were also designed to look at the ability of T cells to transmigrate across ECM substrate in response to MCP-1. MCP-1 has recently been shown to be chemotactic for a subset of activated memory T cells (Carr et al., 1994).
The finding that MCP-1 differentially increased T cell migration across fibronectin as compared to ICAM-1 was surprisingly and for the first time suggested differential regulation of β1 and β2 integrins on these T cells. Recently MCP-1 has been shown to differentially modulate the adhesion of T cells under shear stress by increasing T cell adhesion to fibronectin (α4β1 and α5β1) but not to VCAM-1 (α4β1) or to ICAM-1 (LFA-1) (Carr et al., 1996). Taken together these findings were the first examples of an integrin-activating stimulus, MCP-1, differentially regulating integrin activity on the same cell. This strongly suggested that there may be a general ability for T cell integrins to be differentially regulated and attention was directed into looking at this further.
CHAPTER FOUR

THE DIVALENT CATION Mg\textsuperscript{2+} DIFFERENTIALLY AFFECTS T CELL ADHESION MEDIATED BY THE INTEGRINS LFA-1 & \( \alpha 4\beta 1 \)

4.1 Introduction

In Chapter 3 it was demonstrated that the treatment of T cells with phorbol ester (PdBu) or CD3/TCR cross-linking (CD3XL) leads to \( \beta 1 \) integrin activation. The same stimuli are also able to activate LFA-1 on T cells (Stewart et al., 1996). These methods of activation are thought to generate intracellular signals which switch the integrins into active conformations, so called 'inside-out' signalling. In addition, LFA-1 can be directly converted to a high affinity form using the divalent cations Mn\textsuperscript{2+} or Mg\textsuperscript{2+} (Dransfield et al., 1992; Dransfield and Hogg, 1989). It is thought that these extracellular cations cause direct changes to the integrin ectodomain and bypass the need for intracellular signalling. This is supported by the finding that divalent cations can induce integrin-mediated adhesion in cell free systems. For example, Mn\textsuperscript{2+} can induce fibrinogen binding by isolated Mac-1 (Altieri, 1991) and Mg\textsuperscript{2+} can induce isolated \( \alpha 2\beta 1 \)-mediated adhesion to collagen (Staatz et al., 1989). In contrast to PdBu and CD3XL, Mg\textsuperscript{2+} is able to induce a high affinity form of LFA-1 that expresses the activation epitope 24, and is able to bind soluble ICAM-1 (Stewart et al., 1996). In addition, this high affinity LFA-1 mediates cell adhesion to ICAM-1 in the absence of actin rearrangements or intracellular mediators such as protein kinase C (PKC) and intracellular Ca\textsuperscript{2+} ([Ca\textsuperscript{2+}]i) (Stewart et al., 1996). In order for Mg\textsuperscript{2+} to induce such a high affinity form of LFA-1, it is essential to remove extracellular Ca\textsuperscript{2+} using EGTA (Dransfield et al., 1992)\textsuperscript{†} (p124)

The \( \beta 1 \) integrin \( \alpha 4\beta 1 \) mediates adhesion both to VCAM-1 and the alternatively spliced (CS-1) domain within the Hep II region of fibronectin.
Antibody inhibition data suggests that there are distinct but overlapping sites on \( \alpha 4 \beta 1 \) for recognition of the two ligands (Elices et al., 1990). In some studies \( \alpha 4 \beta 1 \)-mediated adhesion to either VCAM-1 or to fibronectin appear to be regulated quite distinctly, and with different cation dependencies. For example, although \( Ca^{2+} \) is less effective than \( Mg^{2+} \) at supporting binding to Fn (CS-1), \( Ca^{2+} \) alone is able to support binding to VCAM-1 (Masumoto and Hemler, 1993; Masumoto and Hemler, 1993). It appears therefore that \( \alpha 4 \beta 1 \) can assume a number of different functional states which appear to be regulated by extracellular cation concentrations.

Under normal conditions \( Mg^{2+} \) is at a 15 fold excess over \( Ca^{2+} \) intracellularly, but \( Ca^{2+} \) predominates extracellularly. However, various conditions of tissue injury could result in the release of intracellular \( Mg^{2+} \) and the chelation of extracellular \( Ca^{2+} \), and a change in this balance (discussed in Grzesiak et al., 1992). It would be fascinating if this was yet another way in which a differential regulation of integrin function on T cells could be rapidly achieved.

There have been both positive and negative reports of the ability of \( Mg^{2+}/EGTA \) to induce \( \beta 1 \) integrin-mediated adhesion (Luque et al., 1996; Shimizu and Mobley, 1993). Based on these observations, the effect of \( Mg^{2+} \) stimulation on the \( \beta 1 \)- and \( \beta 2 \)-mediated adhesion of human T cells was investigated. A potential artefact was highlighted when looking at the binding of stimulated T cells to ligands other than ICAM-1, in that the activation of the cells can cause them to form homotypic aggregates through LFA-1/ICAM-1 interactions (Figure 4.1.A). These clusters can spuriously increase the binding of T cells to a ligand other than ICAM-1. The result is that an LFA-1 or ICAM-1 blocking antibody, by breaking up the clusters, may inhibit cell binding by preventing cells piggy-backing to other bound cells (Figure 4.1.B). This may give the erroneous impression that some of the cell
binding to β1 substrates is dependent on β2 integrins. For this reason all our experiments on β1 integrin substrates contain LFA-1 blocking mAb.

4.2 Results

4.2.1 Adhesion of T cells to fibronectin can be induced with Mg2+/EGTA

Initial experiments showed that 3 mM Mg2+/1 mM EGTA can induce T cell adhesion to fibronectin through α4β1 and α5β1 (Figure 4.2). Under the same conditions T cells could also be stimulated to bind to VCAM-1 through α4β1, and to collagen through α2β1 (data not shown). Treatment with 3 mM Mg2+/1 mM EGTA induced LFA-1-mediated T cell adhesion to ICAM-1, and this adhesion was resistant to treatment with the [Ca2+]i chelator BAPTA (Figure 4.3.A). In contrast, under the same conditions T cells were induced to bind fibronectin, but this adhesion was sensitive to treatment of T cells with BAPTA (Figure 4.3.B). This suggests that [Ca2+]i plays a role in β1, but not β2, integrin-mediated T cell adhesion following treatment with Mg2+/EGTA. This was the first indication that treatment with Mg2+/EGTA might differentially affect β1 and β2 integrins on T cells. To investigate the role of Mg2+ concentration in this differential effect on β1 and β2 integrins, a titration of Mg2+ was performed (Figure 4.4). The α5β1-mediated component of T cell adhesion to fibronectin was blocked throughout (mAb SAM-1; α5-blocking), allowing the α4β1-mediated component to be looked at in isolation (Figure 4.4.B). LFA-1-mediated cell binding was supported by lower levels of Mg2+ (Figure 4.4.A) than α4β1-mediated cell binding (Figure 4.4.A). Mg2+-stimulated binding by both integrins was reduced to a similar extent on addition of 1 mM Ca2+ (Figures 4.4.A & B). Mn2+ was able to support binding by both integrins, and this was inhibited by 1 mM Ca2+ (Figures 4.5.A & B).
Figure 4.1: T cell adhesion to β1 integrin ligands, can be artefactually enhanced by the formation of LFA-1/ICAM-1-mediated T cell aggregates which can be blocked with an LFA-1 blocking mAb. (A) T cells stimulated to adhere to fibronectin form LFA-1/ICAM-1 clusters which increase the apparent level of T cell binding; (B) These clusters can be inhibited with blocking mAbs to LFA-1 to unmask the level of β1 mediated adhesion. One representative experiment of two is shown.
Figure 4.2: Mg\(^{2+}\)/EGTA stimulates \(\alpha_4\beta_1\) & \(\alpha_5\beta_1\)-mediated T cell adhesion
(A) T cells only bind fibronectin at low levels in Hepes buffer (---), even with addition of 0.4 mM Mg\(^{2+}\)/Ca\(^{2+}\) (-----) but binding can be stimulated with 3 mM Mg\(^{2+}\)/1 mM EGTA (-----); (B) Adhesion is inhibited by a combination of mAb HP1/2 (\(\alpha_4\)-blocking), and mAb SAM-1 (\(\alpha_5\)-blocking); Results are expressed as mean ± S.D. of triplicates. One representative experiment of four is shown.
Figure 4.3: Mg\(^{2+}\)/EGTA stimulated adhesion of T cells to fibronectin, but not to ICAM-1, is inhibited by the intracellular Ca\(^{2+}\) chelator BAPTA. (A) T cells stimulated with 1 mM EGTA and various concentrations of Mg\(^{2+}\) adhere to ICAM-1 (■). This adhesion is not inhibited by pretreatment of T cells with BAPTA (50 μM) (□); (B) Adhesion of T cells under the same conditions to fibronectin (■) is inhibited by BAPTA (50 μM) (□); Results are expressed as mean ± S.D. of triplicates. One representative experiment of four is shown.
Figure 4.4: Mg$^{2+}$-stimulated adhesion of T cells to ICAM-1 and fibronectin requires EGTA, and is inhibited by addition of extracellular Ca$^{2+}$. Mg$^{2+}$-stimulated T cells adhere to (A) ICAM-1 and (B) Fibronectin in the presence of 1 mM EGTA to remove extracellular Ca$^{2+}$ (red symbols). Addition of 1 mM Ca$^{2+}$, in the absence of EGTA, (blue symbols) inhibits adhesion to both ICAM-1 (A) and Fibronectin (B); Results are expressed as mean ± S.D. of triplicates. One representative experiment of four is shown.
Figure 4.5: Mn$^{2+}$-stimulated adhesion of T cells to ICAM-1 and fibronectin is inhibited by addition of extracellular Ca$^{2+}$. Mn$^{2+}$-stimulated T cells adhere to (A) ICAM-1 and (B) Fibronectin (red symbols). Addition of 1 mM Ca$^{2+}$ (blue symbols) inhibits adhesion to both (A) ICAM-1 and (B) Fibronectin; Results are expressed as mean ± S.D. of triplicates. One representative experiment of four is shown.
4.2.2 Cytochalasin D has no effect on the binding of Mg\(^{2+}\)-stimulated T lymphoblasts to ICAM-1, but inhibits the binding of these cells to fibronectin and VCAM-1

The binding of Mg\(^{2+}\)/EGTA stimulated T cells to ICAM-1 is resistant to treatment of T cells with cytochalasin D, an inhibitor of actin polymerisation as previously reported from this laboratory (Stewart et al., 1996) and shown in Figure 4.6.A. In contrast the binding to fibronectin under the same conditions is inhibited by cytochalasin D even at high levels of Mg\(^{2+}\) (Figure 4.6.B). When T cells are stimulated with PdBu or CD3XL to bind ICAM-1 the binding, although quantitatively similar to that induced by Mg\(^{2+}\)/EGTA, is now susceptible to treatment with cytochalasin D as shown in Figure 4.7.A. When T cells bind fibronectin under the same conditions, adhesion promoted by all three stimuli, Mg\(^{2+}\)/EGTA, PdBu or CD3XL show almost identical susceptibility to treatment with cytochalasin D, and dependence on an intact cytoskeleton (Figure 4.7.B). In addition, the binding of Mg\(^{2+}\)/EGTA-stimulated T cells to fibronectin through $\alpha_5\beta_1$ and to VCAM-1 through $\alpha_4\beta_1$ is also sensitive to cytochalasin D (Figure 4.8). One possibility is that the sensitivity to cytochalasin D of $\alpha_4\beta_1$-mediated adhesion compared to LFA-1-mediated adhesion, reflects the relative levels of the integrins on the cells. There is approximately 3-fold more LFA-1 than $\alpha_4\beta_1$ on our T cells (Figure 4.9). However, surprisingly the IC\(_{50}\) of cytochalasin D to block $\alpha_4\beta_1$-mediated adhesion is not further reduced by titrating in increasing amounts of the $\alpha_4$-blocking mAb HP1/2 and therefore reducing the amount of functional $\alpha_4$ (data not shown). In addition a careful titration of suboptimal amounts of the LFA-1 blocking mAb 38 does not render Mg\(^{2+}\)/EGTA-stimulated adhesion to ICAM-1 susceptible to cytochalasin D, even at very low levels of residual binding (data not shown). This suggests that there may be qualitative, rather than just quantitative differences between $\alpha_4\beta_1$ and LFA-1 when stimulated by Mg\(^{2+}\)/EGTA in this assay system.
**Figure 4.6:** Mg$^{2+}$-stimulated adhesion of T cells to fibronectin, but not to ICAM-1, requires an intact cytoskeleton and is inhibited by cytochalasin D. Mg$^{2+}$-stimulated T cells adhere to (A.) ICAM-1 and (B.) Fibronectin in the presence of 1 mM EGTA to remove extracellular Ca$^{2+}$ (red symbols). Addition of 2 μM cytochalasin D (blue symbols) inhibits binding to (B) Fibronectin, but not to (A) ICAM-1; Results are expressed as mean ± S.D. of triplicates. One representative experiment of four is shown.
Figure 4.7: Mg\(^{2+}\)-stimulated adhesion of T cells to fibronectin through α4β1, but not to ICAM-1 through LFA-1, requires an intact cytoskeleton and is inhibited by cytochalasin D. Following stimulation of T cells with 5 mM Mg\(^{2+}\)/2 mM EGTA, binding to ICAM-1 (A; — — — —), but not to fibronectin through α4β1 (B; — — — —; α5 blocked throughout with mAb SAM-1) is insensitive to cytochalasin D. In contrast, following stimulation of T cells with PdBu (——) or CD3XL (····················), the binding to both ICAM-1 (A) and fibronectin (B; α4β1) is blocked with cytochalasin D. Specificity of binding is shown by complete blocking with the appropriate mAb (●). Results are expressed as mean ± S.D. of triplicates. One representative experiment of four is shown.
Figure 4.8: Mg\textsuperscript{2+} stimulated activation of α4β1- and α5β1-mediated adhesion requires an intact cytoskeleton and is inhibited by cytochalasin D. T cells induced to adhere to various ligands with 3 mM Mg\textsuperscript{2+} /1 mM EGTA show varying susceptibility to treatment with cytochalasin D; LFA-1-mediated adhesion to ICAM-1 (□) is unaffected; binding to fibronectin through α4β1 (●) or α5β1(○) or a combination of the two (■) is inhibited as is α4β1-mediated binding to VCAM-1 (▲); Results are expressed as mean ± S.D. of triplicates. One representative experiment of four is shown.
Figure 4.9: Phenotypic analysis of cultured T cells. Flow cytometric analysis of cultured T cells (days 10-14) showed the cells to be (A) 100% LFA-1 positive; mean FL1 894.09; (B) 100% α4β1 positive; mean FL1 297; (C) 100% α5β1 positive; mean FL1 79. (A-C) FITC-αMlg alone is shown in black. The data shown are from one representative experiment of three.
4.2.3 Mg+2-stimulated T lymphoblasts are not able to bind soluble VCAM-1 Fc or soluble fibronectin.

These findings suggested that Mg+2/EGTA did not induce high affinity forms of the α4β1 and α5β1 integrins. Previous work in this laboratory has shown that Mg+2/EGTA can promote the binding of soluble ICAM-1 to T cell LFA-1 (Stewart and Hogg, 1995). The ability of Mg+2/EGTA to induce T cell binding of soluble fibronectin or soluble VCAM-1 was therefore examined. Soluble biotinylated-fibronectin (FN-bt) (section 2.2.14) and a soluble VCAM-1 Fc fusion protein (sVCAM-1) were used as ligands. Soluble VCAM-1 consists of the two N-terminal Ig-like domains of human VCAM-1 (containing an α4β1 binding site) fused to the Fc part of human IgG1 (Jakubowski et al., 1995). Binding of either FN-bt or sVCAM-1 to the T cell surface integrins can be recorded by flow cytometry (section 2.2.15). Titration of sVCAM-1 binding to T cells in the presence of Mn+2 revealed that saturation binding was achieved at 6 μg/ml, so this concentration was used throughout. For comparison the binding of soluble ligands to the Jurkat T cell line was also examined. Jurkat cells have approximately 2-fold higher levels of α4β1 and lower levels of LFA-1 than cultured primary T cells, so that the levels of LFA-1 and α4β1 on Jurkat cells are approximately equal (data not shown). FN-bt was used at the highest final concentration possible, 1.25 mg/ml. Mn+2 was able to induce soluble fibronectin and VCAM-1 binding to both T cells (FN-bt, Figure 4.10.A; VCAM-1, Figure 4.12.D) and Jurkat cells (FN-bt, Figure 4.11.A; VCAM-1, Figure 4.12.B). This binding was specific and could be blocked by the appropriate combination of α4- and α5-blocking mAbs (Figures 4.10.B, 4.11.B, 4.12.B & D). Binding was consistently greater and saturated at lower ligand concentrations to Jurkat cells as compared to T cells (data not shown). By comparison even at high levels of soluble ligand (up to 1 mg/ml) in the presence of Mg+2/EGTA there was no soluble FN-bt or VCAM-1 binding to either T cells (FN-bt, Figure 4.10.C; VCAM-1, Figure 4.12.C) or Jurkat cells (FN-bt, Figure 4.11.A; VCAM-1, Figure 4.12.A).
Figure 4.10: Binding of soluble fibronectin by T cells is stimulated by Mn$^{2+}$ but not by Mg$^{2+}$/EGTA. (A) Mn$^{2+}$ (0.5 mM) stimulates T cell binding of biotinylated fibronectin (FN-bt); (B) This binding is specific and can be inhibited by blocking mAbs to $\alpha_4$ (HP1/2) and $\alpha_5$ (SAM-1); C. 3 mM Mg$^{2+}$/1 mM EGTA is unable to stimulate T cell binding of FN-bt; Binding of streptavidin-FITC alone is shown in black, and cells incubated with FN-bt and streptavidin-FITC in red. The data shown are from one representative experiment of three.
Figure 4.11: Binding of soluble fibronectin by Jurkat cells is stimulated by Mn$^{2+}$ but not by Mg$^{2+}$/EGTA. (A) Mn$^{2+}$ (0.5 mM) stimulates Jurkat cell binding of biotinylated fibronectin (FN-bt); (B) This binding is specific and can be inhibited by blocking mAbs to \(\alpha 4\) (HP1/2) and \(\alpha 5\) (SAM-1); C. 3 mM Mg$^{2+}$/1 mM EGTA is unable to stimulate Jurkat cell binding of FN-bt; Binding of streptavidin-FITC alone is shown in black, and cells incubated with FN-bt and streptavidin-FITC in blue. The data shown are from one representative experiment of three.
Figure 4.12: Soluble VCAM-1 Fc binding to Jurkat and T cells is stimulated by Mn^{2+} but not by Mg^{2+}/EGTA. 3 mM Mg^{2+}/1 mM EGTA is unable to stimulate (A) Jurkat cell or (C) T cell binding of soluble VCAM-1 Fc (sVCAM-1). Mn^{2+} (0.5 mM) stimulates binding of sVCAM-1 to both (B) Jurkat cells and (D) T cells and this binding can be blocked with an α4-blocking mAb (HP1/2; 2.5 μg/ml); sVCAM-1 binding was detected with 10 μg/ml FITC-conjugated goat anti-human Fc mAb (FITC-2ndry). Binding of FITC-2ndry alone is shown as control (A-D). The data shown are from one representative experiment of three.
4.2.4 Mg$^{2+}$-stimulated T lymphoblasts express the LFA-1 activation epitope 24, but not HUTS-21.

There have been previous reports that Mg$^{2+}$/EGTA and Mn$^{2+}$ can induce the \( \beta_1 \) activation reporter epitope HUTS-21 on T cells (Luque et al., 1996). Both the HUTS-21 and 15/7 epitopes have been taken as markers of high affinity \( \beta_1 \) integrins (Luque et al., 1996; Yednock et al., 1995), so it was of interest to see if they could be induced on these T cells by Mg$^{2+}$. Treatment of T cells with Mn$^{2+}$ induced low levels of HUTS-21, but treatment with Mg$^{2+}$/EGTA had no effect (Figure 4.13.A). Similar results were found for 15/7 (data not shown). By comparison both Mn$^{2+}$ and Mg$^{2+}$/EGTA were able to induce expression of the 24 epitope on the same T cells (Figure 4.13.B). This again suggested indirectly that Mg$^{2+}$/EGTA is able to induce high affinity LFA-1, but not high affinity \( \beta_1 \) integrins on T cells.

4.2.5 Increased actin polymerisation in PdBu-stimulated, but not in Mg$^{2+}$/EGTA-stimulated cells

The finding that Mg$^{2+}$-stimulated T cells require both [Ca$^{2+}$], and an intact cytoskeleton for \( \alpha 4\beta_1 \)-mediated adhesion suggested a role for intracellular signalling and actin polymerisation. This led to the development of an assay system to examine whether Mg$^{2+}$/EGTA treatment of T cells could induce the polymerisation of intracellular actin. Phalloidin is a bicyclic heptapeptide from poisonous mushrooms which specifically binds F-actin but not monomeric actin (Cooper, 1987). Phalloidin binds with high affinity and specificity to F-actin in intact cells, after the cells have been fixed and permeabilised. Under these conditions, the amount of fluorochrome-labelled phalloidin bound to a cell, at saturating concentrations of phalloidin, is directly proportional to the total amount of F-actin in the cell (Peppelenbosch et al., 1993). T cells were stimulated at RT for 30 min with either Mg$^{2+}$/EGTA or PdBu with 0.4 mM Ca$^{2+}$/ 0.4 mM Mg$^{2+}$, before being fixed in suspension, and then permeabilised for staining of actin with TRITC-labelled phalloidin.
Figure 4.13: Mg$^{2+}$/EGTA stimulates expression of the $\beta$2 activation epitope 24, but not the $\beta$1 activation epitope HUTS-21. (A) HUTS-21 expression is induced by 0.5 mM Mn$^{2+}$ (green), but not by 3 mM Mg$^{2+}$/1 mM EGTA (red); (B) Both Mn$^{2+}$ (green) and Mg$^{2+}$/EGTA (red) can induce expression of 24; (A-B). Neither epitope is expressed in the absence of cations (blue); binding of FITC-2ndry alone is shown as control (black). The data shown are from one representative experiment of three.
The content of polymerised actin could then be read using flow cytometry (section 2.2.17). In the absence of cations (Figure 4.14; control; white bars) or with Mg$^{2+}$/EGTA (Figure 4.14; control; light grey bars) levels of actin polymerisation were similar and low. However on treatment of the cells with 50 nM PdBu in the presence of physiological amounts of Mg$^{2+}$ and Ca$^{2+}$ (0.4 mM of each) there was a dramatic increase in polymerised actin (Figure 4.14; control; dark grey bars). This increase could be brought down to the level seen with Mg$^{2+}$/EGTA or no cations by treatment with either 2 μM cytochalasin D. or with a 50-fold excess of unlabelled phalloidin, showing that the binding was specific. This demonstrated that although actin polymerisation is a prerequisite for β1 integrin-mediated adhesion of Mg$^{2+}$-stimulated T cells, Mg$^{2+}$-stimulation alone could not promote actin polymerisation.

4.2.6 Ligand induced expression of β1 activation reporters in Mg$^{2+}$-stimulated T cells adherent to fibronectin

The lack of actin polymerisation in Mg$^{2+}$-stimulated T cells in suspension suggested that actin polymerisation might require the interaction of integrins with immobilised ligand. Confocal experiments were therefore performed to examine the actin cytoskeleton during cell binding to ICAM-1 and fibronectin. Mg$^{2+}$-stimulated T cells adherent to the non-integrin ligand poly-L-lysine (PLL) show strong actin staining at the cell periphery with relatively little punctate staining at cell-substrate contact zones (Figures 4.15.A & 4.16.A). In comparison Mg$^{2+}$-stimulated T cells adherent to ICAM-1 (Figures 4.15.B & 4.16.B) or fibronectin (Figures 4.15.C & 4.16.C) show increased overall staining and in particular an increase in the punctate staining at cell-substrate attachment sites. There were no obvious differences in actin staining between ICAM-1- and fibronectin-adherent T cells. The T cells were also stained with mAb 24 (Figures 4.15.D-F) and mAb 15/7 (Figures 4.16.D-F). MAb 24 staining was bright even when cells were adherent to PLL (Figure 4.15.D). However,
Figure 4.14: Stimulation of T cells with PdBu, but not with Mg^{2+}/EGTA, causes increased levels of actin polymerisation. T cells stimulated with PdBu + 0.4 mM Mg^{2+}/Ca^{2+} have high levels of F-actin (purple), that can be reduced by cytochalasin D; T cells stimulated with 3 mM Mg^{2+}/1 mM EGTA (blue) have low levels of F-actin, not further reduced by cytochalasin D. F-actin was measured by TRITC-phalloidin binding to permeabilised T cells. Specificity is shown by the ability of excess unlabelled phalloidin to block this binding. One of three similar experiments is shown. Unstimulated cells are shown as control only (pink) and not following treatment.
Figure 4.15: Mg^{2+}-stimulated T cells express the 24 (β2) activation epitope at sites of LFA-1 interaction with ligand. Confocal microscopy of actin staining (A-C), and epitope 24 expression (D-F) of T cells. T cells were stimulated with 3 mM Mg^{2+}/1 mM EGTA, and adhered to poly-L lysine (PLL: A & D), ICAM-1 (ICAM-1; B & E), or fibronectin (Fn; C & F). Expression of the 24 epitope is greatly enhanced at the cell-substrate interface on ICAM-1 (E), and at sites of cell-cell interaction (D-F). The data shown are from one representative experiment of three.
Figure 4.16: Mg²⁺-stimulated T cells express the 15/7 (β1) activation epitope at sites of β1 integrin interaction with ligand. Confocal microscopy of actin staining (A-C), and 15/7 expression (D-F) of T cells. T cells were stimulated with 3 mM Mg²⁺/1 mM EGTA, and adhered to poly-L lysine (PLL: A & D), ICAM-1 (ICAM-1: B & E), or fibronectin (Fn; C & F). Expression of the 15/7 epitope is weak when cells are adherent to ICAM-1 but is enhanced at sites of cell-cell interaction on PLL (D); and at the cell-substrate interface on FN (F). The data shown are from one representative experiment of three.
staining was increased at the cell-substrate interface when cells were adherent to ICAM-1 (Figure 4.15.E) as compared to fibronectin (Figure 4.15.F). This might represent either modulation of active integrin to the binding interface or induction of the mAb 24 epitope by interaction with ligand. Although Mg\(^{2+}\)/EGTA was unable to induce 15/7 expression on flow cytometry analysis (data not shown), some expression of 15/7 was seen at cell-cell junctions (Figs. 4.16.D-F) and at cell-substrate contact areas when T cells bound to fibronectin (Figure 4.16.F). There was no 15/7 expressed at cell-substrate contact areas when T cells bound to PLL (Figure 4.16.D) or ICAM-1 (Figure 4.16.E). Because flow cytometry is more sensitive than confocal it is unlikely that even a low level of 15/7 would be missed on flow cytometry and seen on confocal. It is therefore unlikely that the expression of the 15/7 epitope seen when T cells are coated onto fibronectin is due to integrin modulation and is more likely to represent induction of the 15/7 epitope by ligand. These results demonstrate that the actin polymerisation, essential for the \(\beta1\) integrin-mediated adhesion of Mg\(^{2+}\)-stimulated T cells is dependent on integrin-substrate interactions and/or cell spreading.

4.3 Discussion

These results show that Mg\(^{2+}\)/EGTA can support adhesion of T cells to fibronectin, but the levels of Mg\(^{2+}\) needed (3-10 mM) are higher than those needed for T cell adhesion to ICAM-1 (1-10 mM). In agreement with other studies Ca\(^{2+}\) is shown to inhibit both Mg\(^{2+}\)- and Mn\(^{2+}\)-stimulated adhesion to ICAM-1 (LFA-1) and fibronectin (\(\alpha 4\beta 1\)). There are obvious differences between adhesion by LFA-1 and that by \(\alpha 4\beta 1\) following Mg\(^{2+}\) stimulation. Mg\(^{2+}\)-stimulated adhesion by \(\alpha 4\beta 1\) requires \([Ca^{2+}]\) and immobilised ligand, whereas that mediated by LFA-1 is independent of post receptor occupancy events. This does not appear to be explained by the quantitative difference in expression of these integrins. It is tempting to speculate that the difference between these integrins in response to Mg\(^{2+}\)/EGTA may reflect the fact that LFA-1 contains an \(\alpha\)-subunit I-domain, and \(\alpha 4\beta 1\) does not. The I-domain appears to play a role in Mg\(^{2+}\)/EGTA-stimulated adhesion of T cells to ICAM-1.
Chapter Four - Mg\(^{2+}\) Differentially Regulates \(\beta_1\) & \(\beta_2\) Integrins

1, and the isolated I-domain is able to inhibit the binding of T cells to ICAM-1 following stimulation with Mg\(^{2+}\)/EGTA, but not following other stimuli such as CD3XL or PdBu (McDowall et al., 1998). Such differential regulation of integrin function may be vitally important in the regulation of T cell trafficking to areas of inflammation where tissue damage has led to alterations in local cation concentrations.

The use of activation reporters has proved valuable in dissecting out the role of conformational changes in integrin activation. MAb HUTS-21 is an activation reporter for the \(\beta_1\) integrins, and the HUTS-21 epitope maps to a segment (residues 207-218) close to the cysteine rich region of the \(\beta_1\) subunit (Luque et al., 1996). The HUTS-21 epitope had been reported to be induced by Mn\(^{2+}\) and TS2/16 and also by Mg\(^{2+}\)/EGTA, albeit to lesser extent. The epitope can also be induced by treatment of cells with PdBu and Ca\(^{2+}\) ionophores such as A23187 (Luque et al., 1996). The \(\beta_1\) activation epitope 15/7 is induced in a similar way by treatment of cells with Mn\(^{2+}\), activating \(\beta_1\) mAbs and PMA (Yednock et al., 1995). This is in marked contrast to the 24 epitope of LFA-1 which is only induced by stimulators of high affinity LFA-1 such as \(\beta_2\) stimulating mAbs, Mn\(^{2+}\) and Mg\(^{2+}\)/EGTA and not by 'inside-out' stimulators such as PdBu and CD3XL (Dransfield et al., 1992; Dransfield and Hogg, 1989; Stewart et al., 1996). These differences suggest that the \(\beta_1\) activation reporters might report integrin clustering rather than, or together with, conformational changes of individual integrins. All three reporter antibodies, mAb 24, mAb HUTS-21 and mAb 15/7 can stimulate integrin mediated adhesion (Dransfield et al., 1992; Luque et al., 1996; Yednock et al., 1995). Presumably they do this by stabilising integrin-ligand interactions. Interestingly the adhesion induced by mAb HUTS-21 is inhibited by cytochalasin D suggesting, again, that clustered, rather than high affinity, integrin might be involved (Gomez et al., 1997). In addition, both HUTS-21, 15/7 and possibly 24 are LIBS epitopes (i.e. induced by ligand) (Cabañas and Hogg, 1993; Gomez et al., 1997; Yednock et al., 1995). This might explain the paradox that although no expression of the HUTS-21 or
15/7 epitopes is seen on Mg\(^{2+}\)-stimulated T cells on flow cytometry, some expression of these epitopes can be seen on confocal. On confocal expression of the HUTS-21 and 15/7 epitopes is restricted to sites of cell-fibronectin or cell-cell interaction, emphasising a potential role for ligand in the expression of these epitopes.

The use of TRITC-phalloidin allows a quantification of actin polymerisation in non-adherent T cells in solution. The finding that treatment of T cells with Mg\(^{2+}\)/EGTA, unlike treatment with PdBu does not promote actin polymerisation is intriguing. In addition, cytochalasin D can inhibit binding mediated by Mg\(^{2+}\)/EGTA to fibronectin but does not further reduce the low level of polymerised actin seen in these T cell in solution. The degree of actin staining seen on confocal on Mg\(^{2+}\)-stimulated T cells was low when cells were adherent to PLL. This staining was increased and became punctate when the T cells bound to either ICAM-1 or to fibronectin, and to the same extent on either ligand. Taken together these findings suggest, albeit indirectly, that actin polymerisation occurs as a result of integrin interaction with immobilised ligand. This suggests that there might be an initial \(\beta1\) integrin-ligand interaction but that subsequently actin polymerisation is required to establish adhesion. These results do not exclude the possibility that Mg\(^{2+}\) does induce a higher affinity form of \(\alpha4\beta1\) able to initiate an interaction with ligand, and that the increased binding affinity is still too low or too transient or too dependent on ligand to be detected by the means available. An alternative, but speculative, possibility is that the \(\beta1\) integrins might possess a second Mg\(^{2+}\) binding site that is involved in a role other than affinity modulation, such as integrin clustering.

Although it is now well established that integrin clustering and the actin cytoskeleton play a vital role in integrin-mediated adhesion (Hato et al., 1998; Stewart et al., 1998), the driving force for these events remains one of the key questions in the field of integrin biology. In particular the question of the relative roles of ligand and/or intracellular signals in integrin clustering still
remains unanswered. The interaction of integrins with ECM alone is not sufficient to induce integrin clustering and focal complex formation in fibroblasts and other signalling events are involved (Hotchin and Hall, 1995). In T cells, the clustering of LFA-1 following CD3XL occurs in the presence of LFA-1 blocking mAbs (Stewart et al., 1998) and is driven by the T cell cytoskeleton (Wülfing et al., 1998) which might argue against a direct role for ligand in the clustering process. However, there is some evidence that ligand induced 'outside-in' signals can affect integrin activation. For example, a peptide from ICAM-2, which binds to purified LFA-1, can induce LFA-1-ICAM-1-dependent T cell aggregation that can be blocked by the serine-threonine kinase inhibitor staurosporine (Li et al., 1993). In addition, under certain situations ICAM-1 may be necessary for full activation of LFA-1 (Cabañas and Hogg, 1993), and ICAM-1 on an APC may sustain the increase in 

$[Ca^{2+}]_i$ of an attached T cell, presumably acting through LFA-1 (Wülfing et al., 1998). It is well established that integrin occupancy and aggregation synergise to recruit the full complement of signalling and cytoskeletal proteins to focal complexes (Miyamoto et al., 1995; Miyamoto et al., 1995). Taken together the evidence points to an active cellular process from within the cell that clusters integrin into high avidity states. It may be that interaction with multi-valent ligand serves to either stabilise the integrin clusters mechanically or to induce an 'outside-in' signal to reinforce actin-rearrangements. The signalling pathways involved in integrin clustering and outside-in signalling are not well defined, but given the role of actin rearrangements one candidate family of signalling molecules is the Rho-subfamily of small GTPases (Chrzanowska-Wodnicka and Burridge, 1996; D'Souza-Schorey et al., 1998; Hotchin and Hall, 1995). In Chapter Seven the role of cell signalling and actin-myosin interactions in integrin activation will be addressed.

Although EGTA is able to chelate both Mg$^{2+}$ and Ca$^{2+}$, the stability constant (K$^\circ$: [M-EGTA]/[M][EGTA], where M is the metal ion and M-EGTA is the metal ion-EGTA complex) is different for the two ions. Log $K^\circ$: Mg$^{2+}$, 5.2; Ca$^{2+}$, 11.0. The result is that low level EGTA can be used to chelate Ca$^{2+}$ in the presence of Mg$^{2+}$. 

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5.1 Introduction

The work detailed in this thesis so far has provided evidence that activating agents such as chemokines and cations are able to differentially affect the activation of β1 and β2 integrins on T cells. Another potential way in which integrins may be differentially regulated on the same cell involves integrin "cross talk". Integrin cross talk describes a phenomenon in which activation or occupancy of one set of integrins directly affects the ability of other integrins, on the same cell, to interact with ligand. Most examples of integrin cross talk have come from studies of transfected cell lines and it is hard to know how biologically relevant such effects really are. However, there are indirect suggestions that integrin cross talk may be important for regulation of T cell adhesion. For example, resting lymphocytes use both LFA-1 and α4β1 to bind endothelial cells, but when T cells become activated adhesion is mediated through LFA-1 with little or no contribution from α4β1 (van Kooyk et al., 1993). Additionally, in some leukaemic T cell lines, functional α4β1 is found only when LFA-1 is either not expressed or inactive (van Kooyk et al., 1993). There appears, therefore, to be a T cell integrin hierarchy in which α4β1 is inactive if LFA-1 is active.

An experimental system was designed to investigate cross influences on function between the β1 integrins and LFA-1 on T cells. This system examined the ability of T cells, adherent to various ligands, to bind fibronectin (FN)-coated beads. The advantage of this system is that a variety of substrates and mAbs can be used to immobilise T cells via different T cell surface receptors. Activation of the leukocyte integrins was achieved by treating T cells with 3 mM Mg^{2+}/2 mM EGTA (Dransfield et al., 1992). For the β2
integrin, LFA-1, the advantage of such treatment is that it directly alters the integrin ectodomain, bypassing the requirement for an intracellular stimulus and inducing minimal cell spreading (Stewart et al., 1996). This was important as changes in shape would affect cell surface area available for FN bead binding. The form of LFA-1 induced by Mg\textsuperscript{2+}/EGTA is considered to be of high affinity because it has increased ability to bind soluble ICAM-1 (Stewart et al., 1996). To further examine the generality of cross-influences between these integrins, we also investigated T cells stimulated with phorbol ester (PdBu) or by TCR/CD3 crosslinking (CD3XL). Both these stimuli act from within the cell to activate integrins, so called 'inside-out' signalling and may be considered more representative of the in vivo situation.

5.2 Results

5.2.1 Adhesion of T cells to ICAM-1 decreases binding of fibronectin-coated beads

T cells were adhered to immobilised ICAM-1 via LFA-1, or to a control substrate, and their ability to bind FN-coated beads through the β1 integrins, α4β1 and α5β1, was then investigated. Anti-CD5 mAb was used as a control substrate after establishing that CD5 cross-linking with this mAb had no effect on β1 or β2 integrin activation on the same T cell. FN-coated beads were bound by T cells adherent to the control substrate, anti-CD5 mAb, immobilised on plastic (Figure 5.1.A) and the specificity of adhesion was demonstrated by blocking bead-binding with a combination of α4 and α5 blocking mAbs (Figure 5.1.B). However, when T cells were adherent to ICAM-1 as substrate they bound fewer FN-coated beads (Figure 5.1.C). When binding of the FN-coated beads was quantified there was a decreased level of FN bead binding when T cells were adherent to ICAM-1 (Figure 5.2) (inhibition: 65.0 ± 23.4% = mean ± SD; n=6).
Figure 5.1: The binding of fibronectin-coated beads to T cells is reduced when the T cells are adherent to ICAM-1. Cultured human T cells treated with 3 mM Mg$^{2+}$/2 mM EGTA and adhered to plastic coated with CD5 mAb (UCHT2) (A & B) or ICAM-1 (C) were incubated with fibronectin-coated beads. Bead-binding was blocked using a combination of the $\alpha_4$ and $\alpha_5$ blocking mAbs HP1/2 (0.5 $\mu$g/ml) and SAM-1 (0.5 $\mu$g/ml) (B). Bar is 20 $\mu$m
Figure 5.2: Inhibition of fibronectin binding by T cells bound to ICAM-1.
T cells stimulated with Mg²⁺/EGTA were adhered to either (A) CD5 mAb (UCHT2), LFA-1 mAb (38) or ICAM-1 and incubated with FN-coated beads (dark bars). Bead attachment was blocked with α4 and α5 mAbs HP1/2 (0.5 μg/ml) and mAb SAM-1 (0.5 μg/ml) (open bars); or (B) CD5 (mAb UCHT2), FN or ICAM-1 and incubated with ICAM-1-coated beads (stippled bars) or BSA-coated control beads (open bars). Data is expressed as Binding Index (beads bound/hundred cells), and represent the mean of six high power fields ± SEM. One representative experiment of three is show
This result demonstrated that on human T cells the interaction of LFA-1 with its ligand ICAM-1 could downregulate the function of the β1 integrins. In contrast, T cells adhered to anti-LFA-1 mAb bound beads at a similar level as T cells adherent to control mAb. This indicated that the LFA-1 inhibitory effect could not be mimicked by cross-linking LFA-1 with immobilised CD11a mAb 38 (Figure 5.2.A). Conversely there was no difference between the ability of T cells adherent to anti-CD5 mAb, FN or ICAM-1 to bind ICAM-1-coated beads (Figure 5.2.B), indicating that adhesion to immobilised FN did not alter the extent of ICAM-1 bead binding by LFA-1. This is the first evidence that LFA-1 could dominate the activity of the FN binding receptors but that the reverse situation did not hold.

5.2.2 Adhesion of T cells to ICAM-1 down-regulates α4β1 activity

In order to resolve the effects of LFA-1 ligand binding on each of the T cell FN receptors, α4β1 and α5β1, it was essential to look at each receptor individually. Figure 5.3.A shows the binding of FN-coated beads by T cells adherent to anti-CD5 mAb. This binding can be partially blocked with either an α4-blocking mAb, an α5-blocking mAb or completely with a combination of both blocking mAbs, showing that T cells bind these beads through a mixture of α4β1 and α5β1 integrins. However, when T cells are adherent to ICAM-1 the binding index for FN-coated beads is lower, and reduced only by an α5-blocking mAb (Figure 5.3). Binding in this situation is therefore mediated mainly through α5β1 with little contribution from α4β1, demonstrating that the binding activity of α4β1 has been compromised. The binding by T cells of VCAM-1-coated beads, which is exclusively mediated by α4β1, reveals a similar downregulation when T cells are adherent to ICAM-1 as compared to anti-LFA-1 mAb (Figure 5.4).
Figure 5.3: Binding by α4β1 is differentially inhibited when T cells are adherent to ICAM-1. T cells stimulated with Mg$^{2+}$/EGTA were adhered to plastic coated with CD5 mAb (UCHT2; black) (A) or ICAM-1 (blue) (B) and incubated with FN-coated beads. Bead attachment was assessed in the presence of α4- (HP1/2; 0.5 μg/ml) and α5- (SAM-1; 0.5 μg/ml) blocking mAbs, alone or in combination. Data are the mean of six high power fields ± SEM. One representative experiment of three is shown.
Figure 5.4: Inhibition of VCAM-1 binding when T cells adhere to ICAM-1. T cells treated with Mg²⁺/EGTA were adhered to plastic coated with CD5 mAb (UCHT2), LFA-1 mAb (38) or ICAM-1 and incubated with VCAM-1-coated beads (hatched bars). Bead attachment was blocked (open bars) by an α4 blocking mAb (HP1/2; 0.5 μg/ml). Data represent mean of six high power fields ± SEM. One representative experiment of three is shown.
5.2.3 Activation of the β2 integrin LFA-1 on T cells inhibits their β1-mediated binding to fibronectin.

The inhibitory effect of LFA-1 on β1-mediated ligand binding could not be demonstrated by cross-linking LFA-1 with an anti-LFA-1 mAb but required LFA-1 to bind to its ligand, ICAM-1. This suggested that high affinity or ligand occupied LFA-1, rather than receptor crosslinking, was necessary for cross talk. This led to the development of an assay in which T cells were first stimulated with Mg\(^{2+}\)/EGTA, CD3XL or PdBu, and then exposed to the activation reporter mAb 24. MAb 24 binds to an epitope only expressed on those active forms of LFA-1 that are able to interact with ligand. Once bound to the active integrin, mAb 24 holds LFA-1 in an active conformation as if occupied by ligand (Dransfield et al., 1992). MAb 24 causes increased T cell binding to ICAM-1 following titration of Mg\(^{2+}\) (Figure 5.5.A), CD3 mAb G19.4 (Figure 5.5.B) and PdBu (not shown). In contrast mAb 24 caused inhibition of T cell binding to FN following Mg\(^{2+}\)/EGTA (Figure 5.6.A) or TCR/CD3 crosslinking (Figure 5.7.A).

5.2.4 The effect of LFA-1 activation on T cells is mediated predominantly through α4β1

Because T cells bind to FN through both α4β1 and α5β1, we analysed the effects of LFA-1 activation individually on these integrins using function blocking mAbs and either Mg\(^{2+}\)/EGTA (Figures 5.6.B & C) or CD3XL (Figures 5.7.B & C) to stimulate adhesion. Following either stimuli, prolonged activation of LFA-1 with mAb 24 had a small effect on total FN binding (Figures 5.6.A & 5.7.A) and on α5β1-mediated adhesion (Figures 5.6.B & 5.7.B), but a much greater effect on α4β1-mediated adhesion (Figures 5.6.C & 5.7.C). By comparison higher levels of α4β1 inhibition by mAb 24 were seen when the integrins were activated with CD3XL or PdBu than with Mg\(^{2+}\)/EGTA (Figure 5.8).
Figure 5.5: MAb 24 increases T cell adhesion to ICAM-1. Activation of LFA-1 by mAb 24 augments T cell adhesion to ICAM-1 following (A) Mg²⁺/EGTA or (B) CD3XL (mAb G19.4 with 0.4 mM Ca²⁺/Mg²⁺). MAb 24 (LFA-1 activation) or mAb 52U (IgG1 control) were used at 20 µg/ml. Adhesion was blocked with mAb 38 (LFA-1 blocking, 10 µg/ml). Data are means of triplicates ± SD. One representative experiment of three is shown.
3 mM Mg²⁺/ 2 mM EGTA

Figure 5.6: Prolonged activation of LFA-1 blocks α4β1 and to a lesser extent α5β1 mediated binding to fibronectin. Adhesion to FN was induced by Mg²⁺/EGTA; (A) adhesion mediated by α4β1 & α5β1; (B) α5β1-mediated adhesion after α4β1 had been blocked (mAb HPl/2; 0.5 µg/ml); (C) α4β1-mediated adhesion after α5β1 had been blocked (mAb SAM-1; 0.5 µg/ml). Adhesion after LFA-1 activation (mAb 24; open symbols), or isotype control (mAb 52U; closed symbols) used at 20 µg/ml. Data are means of triplicates ± SD. One representative experiment of three is shown.
Figure 5.7: Prolonged activation of LFA-1 blocks α4β1 and to a lesser extent α5β1 mediated binding to fibronectin. Adhesion to FN was induced by CD3XL (mAb G19.4 in hepes with 0.4mM Ca²⁺/Mg²⁺); (A) adhesion mediated by α4β1 & α5β1; (B) α5β1-mediated adhesion after α4β1 had been blocked (mAb HP1/2; 0.5 µg/ml); (C) α4β1-mediated adhesion after α5β1 had been blocked (mAb SAM-1; 0.5 µg/ml). Adhesion after LFA-1 activation (mAb 24; open symbols), or isotype control (mAb 52U; closed symbols) used at 20 µg/ml. Data are means of triplicates ± SD. One representative experiment of three is shown.
Figure 5.8: Prolonged activation of LFA-1 blocks α4β1-mediated fibronectin binding following various stimuli. Adhesion to FN was induced by CD3XL (mAb G19.4; 2.5 μM) with 0.4 mM Ca^{2+}/Mg^{2+} (stippled bars); PdBu 50 nM with 0.4 mM Ca^{2+}/Mg^{2+} (dark bars); or 3 mM Mg^{2+}/2 mM EGTA (cross-hatched bars). α5β1 was blocked with mAb SAM-1 (0.5 μg/ml). Adhesion was assessed in the presence of 20 μg/ml of either mAb G25.2 (an LFA-1 non-activating & non-blocking mAb), mAb 24 (LFA-1 activation) or mAb 52U (isotype control). Data are means of triplicates ± SD. One experiment of three is shown. The specificity of the adhesion is shown with an α4- blocking mAb.
In addition there was no effect of the non-function altering anti-LFA-1 mAb G25.5 which again emphasised the requirement for LFA-1 activation (Figure 5.8). Under equivalent activating conditions, mAb 24 also decreased α4β1-mediated adhesion to VCAM-1 to the same extent as to FN (Figure 5.9.A). Together these results reinforced the findings that α4β1 function is particularly sensitive to the state of LFA-1 activation. Monovalent Fab' fragments of mAb 24 produced the same degree of inhibition as bivalent mAb 24 (Figure 5.9.B). This demonstrates that activation or ligand occupancy of LFA-1 in the absence of clustering is sufficient to alter α4β1-mediated adhesion.

5.2.5 Activation of β1 integrins on T cells has no effect on LFA-1 binding to ICAM-1.

It was of interest to reverse the situation to investigate the effect on β2 integrin-mediated adhesion of maintaining β1 integrins in an active state, using the β1 integrin-stimulating mAb TS2/16. This mAb increased binding to FN following the three activating treatments (Figure 5.10.A) but had no effect on β2-mediated binding to ICAM-1 (Figure 5.10.B), confirming the results from bead-binding assays that the β1 integrins were unable to influence the ligand binding activity of LFA-1.

5.2.6 Inhibition of α4β1 increases α5β1 mediated migration.

The effects of LFA-1-mediated cross talk on α4β1- and α5β1- mediated T cell migration on FN were then investigated. Using the Transwell® system it was established that T cells undergo random migration on FN using α4β1 and α5β1 (Figures 5.11.A & B), and this migration is completely inhibited when T cells are activated with CD3XL (Figure 5.11.A), or PdBu (data not shown). Even attempts to titrate down the activating stimulus to very low levels did not yield a concentration that enhanced migration above control.
Figure 5.9: Prolonged activation of LFA-1 with mAb 24 of fab' fragments of mAb 24 blocks α4β1 mediated binding to VCAM-1 and fibronectin. (A) Adhesion to VCAM-1, induced by Mg\(^{2+}/\)EGTA (□) was also inhibited by LFA-1 activation (mAb 24; 20 μg/ml; ■); (B) α4β1-mediated adhesion to fibronectin (□) was inhibited equally well by mAb 24 (■) or fab' fragments (●). Data are means of triplicates ± SD. One representative experiment of three is shown; (B) The specificity of the adhesion is shown by the block achieved using α4- and α5- blocking mAbs together (●).
Figure 5.10. MAb TS2/16 (β1 integrin activator) increases binding to fibronectin but has no effect on β2 integrin function. T cell adhesion to fibronectin (A) and ICAM-1 (B) was induced by CD3XL (mAb G19.4; 2.5 μg/ml; 0.4 mM Ca²⁺/Mg²⁺); or PdBu (50 nM; 0.4 mM Ca²⁺/Mg²⁺); or 3 mM Mg²⁺/2mM EGTA in the presence or absence of β1 activation (mAb TS2/16; 10 μg/ml) or isotype control (mAb 52U; 10μg/ml). Specificity of adhesion was shown by blocking of fibronectin binding with a combination of α4- and α5- blocking mAbs (A), and ICAM-1 binding with the LFA-1 blocking antibody, mAb 38 (B). Data represent means of triplicates ± SD. One representative experiment of three is shown.
Figure 5.11. Migration on fibronectin of T cells in 0.4 mM Ca\(^{2+}\)/Mg\(^{2+}\) is mediated by \(\alpha 4\beta 1\) and \(\alpha 5\beta 1\), and inhibited by CD3XL. (A) Migration of unstimulated T cells on FN (10\(\mu\)g/ml) is mediated by both \(\alpha 4\beta 1\) & \(\alpha 5\beta 1\) and if one is blocked the other can compensate. Migration is inhibited by CD3XL. (B) Treatment of T cells with Mg\(^{2+}\)/EGTA reduced migration (white bars) when compared to unstimulated T cells (black). In addition migration was mediated only through \(\alpha 5\beta 1\). Migrated cells are expressed as % input cells, and represent the mean of triplicate wells \(\pm\) S.D. Data are representative of 4 similar experiments.
This is in agreement with recently published studies that show that migration depends on both adhesion and deadhesion events in a critical balance, and that overactivation of integrins causes firm adhesion and inhibition of migration (Dustin et al., 1997; Gomez et al., 1997; Huttenlocher et al., 1996; Huttenlocher et al., 1995; Kuijpers et al., 1993; Palecek et al., 1997). One possibility was that by using titrations of function blocking mAbs against \( \alpha 4 \beta 1 \) and \( \alpha 5 \beta 1 \) it might be possible to relieve the inhibition of migration caused by overactivation of these integrins following CD3XL or PdBu treatment. However this was not the case and migration could not be restored (data not shown). Intriguingly, in contrast to other activating protocols when T cells were treated with 3 mM Mg\(^{2+} \)/2 mM EGTA they were able to migrate on FN using \( \alpha 5 \beta 1 \) exclusively. Further experiments showed that this migration was enhanced by an \( \alpha 4 \) blocking mAb HP1/2, but not affected by an \( \alpha 4 \) non-blocking mAb 7.2 (Figure 5.11.B). In addition, migration on differing concentrations of FN remained solely dependent on \( \alpha 5 \beta 1 \) (Figures 5.12.A & B), and could be enhanced either with \( \alpha 4 \) blocking mAb HP1/2 (Figure 5.12.C) or by maintaining LFA-1 activation with mAb 24 (Figure 5.12.D). Such increased migration was blocked with an \( \alpha 5 \) blocking mAb. Therefore, by decreasing the \( \alpha 4 \beta 1 \) contribution to FN adhesion with either an \( \alpha 4 \) blocking mAb or an LFA-1 activation mAb, the ability of \( \alpha 5 \beta 1 \) to mediate migration was increased.

5.3 Discussion

These experiments were undertaken to examine the functional interaction on T cells between LFA-1 and the \( \beta 1 \) integrin FN receptors \( \alpha 4 \beta 1 \) and \( \alpha 5 \beta 1 \). The main finding is that the occupation of T cell LFA-1 by its ligand ICAM-1 decreases the binding of \( \alpha 4 \beta 1 \) to ligands FN and VCAM-1. This inhibitory cross talk also results from the prolonged activation of LFA-1 induced by the activation reporter mAb 24 in combination with several T cell adhesion inducing protocols. The adhesive activity of \( \alpha 5 \beta 1 \) is affected to a lesser extent.
Figure 5.12. Migration of Mg²⁺/EGTA treated T cells on fibronectin is mediated by α5β1, and promoted by blocking α4β1 function. (A) Migration of T cells on FN (10 μg/ml) is mediated by α5β1 and enhanced when α4β1 is blocked with mAb HPl/2. The non-blocking α4 mAb 7.2 has no effect. (B) Migration of T cells on FN (0-50 μg/ml) is α5β1-dependent; (C) The α4 blocking mAb HPl/2 or (D) the LFA-1 activation mAb 24 increase α5-dependent migration above control mAb. Data are total number of migrated cells, and represent the mean of four readings from duplicate wells. Bars indicate the range of readings. Data are representative of 4 similar experiments.
Although active LFA-1 downregulates the avidity of α4β1, the reverse does not occur as neither β1 integrin activating mAb TS2/16 nor β1-mediated binding to FN affected the avidity of LFA-1. Under certain conditions, such as seen with Mg²⁺-stimulated T cells, the downregulation of α4β1 activity increases the efficiency of α5β1-mediated migration on FN. These experiments therefore demonstrated differential regulation of two integrin subclasses and a hierarchy of integrin usage in which the β2 integrin LFA-1 will suppress the function of β1 integrins, particularly α4β1.

Previous studies have demonstrated the pre-eminent role of α4β1 in the rolling and arrest of leukocytes on the vascular endothelium. In particular α4β1 appears to bridge the gap between fast selectin-mediated rolling and LFA-1-mediated firm adhesion, by its ability to act both as a rolling and as an adhesive receptor. However, once arrest has occurred LFA-1, and not α4β1, is the chief integrin involved in transendothelial migration (Moser et al., 1992; Oppenheimer-Marks et al., 1991; van Epps et al., 1989). Furthermore, in vitro experiments during flow have emphasised the requirement for an integrin hierarchy to allow co-ordinated migration of lymphocytes across the endothelium into the tissues (Butcher and Picker, 1996). The finding that active LFA-1 is able to decrease the ligand binding activity of α4β1 has direct implications for the sequential activity of these integrins in such an adhesion cascade. LFA-1 may function optimally in the absence of α4β1 adhesion, allowing the T cell to deadhere from the apical surface of the endothelium and transmigrate. These findings also argue against a redundancy amongst integrin-ligand pairs in leukocyte transmigration and imply specific roles for each integrin.

This study had also demonstrated that, in contrast to adhesion, the migration of activated T cells on FN can vary greatly depending on the activating stimulus. In particular T cell activation with 3 mM Mg²⁺/2 mM EGTA is
unique in promoting both adhesion and migration. This suggests that in vivo there may be several different stimuli able to activate integrins with very different effects. It has been considered that Mg\(^{2+}\)/EGTA is a less physiological stimulus than other methods of activation, although this may prove to be a simplification. Migration following treatment with Mg\(^{2+}\)/EGTA is mediated by α5β1 with no contribution from α4β1. In addition, suppressing α4β1 activity on T cells, either by mAb 24 or α4 function blocking mAbs, enhanced the level of α5β1 migration, particularly at low FN levels. This may reflect the compensatory increase in α5β1-mediated adhesion, with its migratory potential, when binding through the non-migratory α4β1 is blocked. Another possibility is that the enhanced migration by α5β1 is due to removal of a restraint imposed by α4β1. The importance of strength of adhesion in regulating cell migration is well documented (Huttenlocher et al., 1996; Palecek et al., 1997) suggesting that firm adhesion by both α4β1 and α5β1 may make conditions suboptimal for migration. Alternatively, α4β1 may be involved in a more specific inhibition of α5β1 function as has been described in the control of metalloproteinase expression in fibroblasts (Huhtala et al., 1995). The promotion of migratory behaviour by α5β1 through loss of α4β1 binding activity is in keeping with the more prominent role of α5β1 within the tissues following successful negotiation of T cells across the endothelium (Miyake et al., 1992). A hierarchy of integrin activity may feature at this later stage of the adhesion cascade with LFA-1 providing a link between α4β1 and α5β1, and acting to uncouple the former in order to enhance migration by the latter. The result, phenotypically, is a less adhesive, more migratory T cell.
Chapter Six
Investigation of the Mechanism of LFA-1 Cross Talk

6.1 Introduction

Having observed the phenomenon of integrin cross talk between LFA-1 and \( \alpha_4\beta_1 \) in T cells it was of interest to establish the mechanism of this inhibition. Although several examples of one integrin exerting a suppressive effect on another integrin have been described, the majority involve a \( \beta_3 \) integrin in the suppressive role. In addition, dissections of the pathways involved in the inhibitory pathway have come entirely from studies involving suppressive \( \beta_3 \) integrins. In all these examples of cross talk the mechanistic pathways involved have been hard to define and remain unclear. Unfortunately there are also no obviously unifying concepts inherent in the different models. In K562 cells, ligation of transfected \( \alpha_\nu\beta_3 \), with either monoclonal antibody (mAb) or ligand, inhibits endogenous \( \alpha_5\beta_1 \)-mediated phagocytosis, with no effect on \( \alpha_5\beta_1 \)-mediated adhesion. This inhibition requires the cytoplasmic tail of \( \alpha_\nu\beta_3 \) (Blystone et al., 1995) and involves a transduced signal. The inhibition is reversed by the serine-threonine kinase inhibitor H7, and the protein kinase C (PKC) inhibitor calphostin C, but not by more specific tyrosine kinase inhibitors, making PKC a possible candidate in the signalling pathway (Blystone et al., 1994). Although \( \alpha_\nu\beta_3 \) ligation only regulates those functions, such as phagocytosis, that require a high affinity form of \( \alpha_5\beta_1 \), the inhibition does not prevent expression of high affinity \( \alpha_5\beta_1 \) but appears to interfere with an event down-stream of ligand binding. In another model of integrin cross talk the ability of transfected \( \alpha_{IIb}\beta_3 \) to inhibit the function of endogenous \( \alpha_5\beta_1 \) in Chinese hamster ovary (CHO) cells depended on the induction of a conformational change in \( \alpha_{IIb}\beta_3 \) which was brought about by
ligand-binding or certain, but not all, mAbs directed against the integrin. Again, the cytoplasmic tail of the suppressive integrin was essential. However, inhibition involved an effect both on the affinity of α5β1 and on post receptor occupancy events such as cell spreading. The inhibition in this model could not be reversed with the protein kinase inhibitors, H7, staurosporine or calphostin C (Diáz-González et al., 1996). In a third model ligation of αvβ3 inhibits the migration of human umbilical vein endothelial cells (HUVECs) on fibronectin (FN). The transfection of human embryonic kidney (HEK) cells that endogenously express α5β1, with wild type and mutant forms of αvβ3 revealed that this inhibition required not the β3 cytoplasmic tail but the β3 transmembrane domain. The effects of H7 could not be assessed as even at low concentrations it completely inhibited even background levels of migration (Simon et al., 1997). In a further example of cross talk, occupancy of αvβ3 inhibits chemotaxis of rat and human vascular smooth muscle cells (VSMCs) on a FN substrate in response to platelet-derived growth factor (PDGF), but has no effect on adhesion to FN. In this model occupancy of αvβ3 appears to exert a suppressive effect by preventing the stimulation of the serine/threonine kinase, Ca^{2+}/calmodulin kinase II (CaMKII) by PDGF. The suppressive effect of αvβ3 can be reversed by expression of a constitutively active form of CaMKII or by increasing cytoplasmic Ca^{2+} ([Ca^{2+}]) which itself activates CaMKII (Bilato et al., 1997). The down stream effectors of CaMKII in this system have not been elucidated. A role for CaMKII in migration on FN is interesting and in apparent conflict with the effects of this kinase in CHO cells that express α5β1. In these cells CaMKII maintains α5β1 in a low affinity state, and the CaMKII inhibitor, KN62, can increase the affinity of α5β1, and CHO cell adhesion to FN (Bouvard et al., 1998). Taken together the accumulating evidence suggests that there may not be a unifying mechanism involved in each example of integrin cross talk, and that the signalling pathways involved may depend not
only on the integrins involved but also on the cells in which they are expressed.

The aim of the work presented in this chapter was to find a way to reverse the inhibition imposed on \( \alpha 4 \beta 1 \) by activation or ligand occupancy of LFA-1 on T cells. It was hoped that if the inhibition could be reversed then some insight might be gained into the pathways involved in the original inhibition.

6.2 Results

6.2.1 Comparison of other LFA-1 activating mAbs and the activation reporter mAb 24.

Previous results presented in Chapter Five have demonstrated that the activation reporter mAb 24 not only binds to active forms of LFA-1, but can also act to stabilise these forms. The result is that mAb 24 can increase LFA-1-mediated adhesion of T cells to ICAM-1. In addition, this ability to hold LFA-1 in an active conformation mimics the conformational change induced in LFA-1 on ligand-binding, and inhibits the function of \( \alpha 4 \beta 1 \). It was of interest to see if activating mAbs to LFA-1 had the same effect. One such mAb is KIM185 (CD18; \( \beta 2 \) activating), which is able to induce adhesion through LFA-1 (Andrew, 1993). MAb KIM185 differs from mAb 24 in recognising an epitope that is expressed on a proportion of LFA-1 molecules at an intensity that appears independent of temperature (data not shown; Andrew, 1993) and divalent cations (Figure 6.1; Andrew, 1993). However, binding of such activating mAbs is thought to induce a conformational change in the integrin which favours ligand binding. Both mAb KIM185 and mAb 24 induce binding to ICAM-1 in the presence of 0.4 mM \( \text{Ca}^{2+} / 0.4 \text{mM Mg}^{2+} \). Although maximal adhesion induced by the two mAbs is the same, the concentration of ICAM-1 required for maximal adhesion is ~10 fold higher for mAb 24-stimulated adhesion than for mAb KIM185 (Figure 6.2.A). This may reflect further induction of mAb 24 epitope expression by ligand, when mAb 24 expression is suboptimal (Cabañas and Hogg, 1993). MAb 24 expression is low
Figure 6.1: Expression of the mAb KIM185 (β2 activating) epitope on LFA-1 is constitutive, but the mAb24 (β2 activation reporter) epitope is expressed only on active LFA-1. MAb KIM185 (green) binds to T cell LFA-1 to the same extent (A) in the absence of cations or (B) following LFA-1-activation with 3 mM Mg²⁺/2 mM EGTA; MAb 24 (red) shows (B) increased binding to active LFA-1. MAb binding was detected with 10 μg/ml FITC-conjugated goat anti-mouse Fc mAb (FITC-2ndry). Binding of FITC-2ndry (black) and mAb 38 (total LFA-1; active=inactive; blue) are shown as controls. The data are from one similar experiment of three.
Figure 6.2: MAbs KIM185 (β2 activating) and 24 (β2 activation reporter), both induce T cell adhesion to ICAM-1. (A) Adhesion to ICAM-1 does not occur in 0.4mM Ca^{2+}/0.4mM Mg^{2+} in the absence of stimulation (□), but can be induced by mAbs KIM185 (red; 10 μg/ml) or 24 (blue; 10 μg/ml). All adhesion is blocked by LFA-1 blocking (mAb 38, 10 μg/ml; not shown); (B) Both mechanisms of activation (mAb KIM185, red; mAb 24, blue) show a similar time course for adhesion of cells to ICAM-1 (10 μg/ml). Data are means of triplicates ± SD. One of three similar experiments is shown.
in only physiological levels of cations as compared to the high levels seen with Mn$^{2+}$ or Mg$^{2+}$/EGTA. The time course of adhesion to ICAM-1 induced by the two mAbs, KIM185 and 24, is similar although mAb KIM185 has a shorter lag between stimulation and the beginning of adhesion (Figure 6.2.B). Results with the mAb KIM127 (CD18; β2 activating; Robinson, 1992) were identical to those for KIM185 in these assays (data not shown).

It was also of interest to look at the role of the cytoskeleton in these examples of integrin activation. An intermediate level of Mg$^{2+}$ (3 mM) was selected to stimulate high affinity adhesion in the presence of 1 mM EGTA. Cytochalasin D was then titrated in and used at a level (6 μM) that inhibited adhesion in normal T cells. Addition of either mAb KIM185 or mAb 24 had no effect on background adhesion but prevented the inhibition caused by cytochalasin D (Figure 6.3.A). This demonstrates the ability of both mAbs to stabilise a high affinity integrin. In contrast when the experiment was repeated using physiological levels of cations (0.4 mM Ca$^{2+}$/0.4 mM Mg$^{2+}$) although mAb KIM185 and mAb 24 both slightly increased the background level of T cell adhesion, only mAb KIM185 can protect against cytochalasin D (Figure 6.3.B). This suggests that although mAb KIM185 can induce high affinity LFA-1 under a variety of different cation conditions, mAb 24 induces adhesion that is variably dependent on cell spreading, and this depends on the underlying affinity of the integrins as determined by the cation conditions.

6.2.2 Other LFA-1 activating mAbs also induce cross talk

T cells were stimulated by CD3/TCR cross-linking (CD3XL) to bind FN. Under these conditions both of the β2 activating mAbs, mAb KIM185 and mAb KIM127 inhibited the α4β1-mediated binding of T cells to FN (Figure 6.4). This inhibition of α4β1 was of the same extent as that seen on stimulation of the T cells with the LFA-1 activation reporter, mAb 24. In contrast, there was no effect of mAb G25.2 (CD11a; non-function altering). This demonstrated that either activation or occupancy of LFA-1 could initiate inhibition of α4β1.
Chapter Six - The Mechanism of LFA-1-Mediated Cross-Talk

Figure 6.3: Under certain conditions LFA-1-mediated adhesion induced by mAb 24 (β2 activation reporter) requires an intact cytoskeleton. Adhesion to ICAM-1 in the presence of (A) 3mM Mg²⁺/1mM EGTA; or (B) 0.4 mM Ca²+/Mg²⁺ can be increased by 10 µg/ml of either mAb KIM185 or mAb 24 (■); KIM185 induced adhesion is resistant to cytochalasin D under both conditions; mAb 24 induced adhesion is only resistant to cytochalasin D in presence of Mg²⁺/EGTA (B). In all cases adhesion was blocked by LFA-1 block with mAb 38 (10 µg/ml; not shown); Data are means of triplicates ± SD. One representative experiment of three is shown.
T cell adhesion to fibronectin (\(\alpha 4\beta 1\)) stimulated by CD3/TCR cross-linking is inhibited by all LFA-1 activating mAbs.

Figure 6.4: T cell adhesion to fibronectin (\(\alpha 4\beta 1\)) is inhibited by mAb 24 and other LFA-1 activating mAbs. The \(\alpha 4\beta 1\)-mediated component (all wells \(\alpha 5\)-block; mAb SAM-1; 0.5 \(\mu g/ml\)) of T cell binding to FN following CD3XL (mAb UCHT1; 10 \(\mu g/ml\)) is shown; Adhesion is inhibited by LFA-1 activation mAbs (24, KIM127 and KIM185; all at 10 \(\mu g/ml\)), but not by an LFA-1 control mAb (G25.2; 10 \(\mu g/ml\)); Adhesion was blocked by \(\alpha 4\)-blocking mAb HP1/2 (not shown); Data are means of triplicates \(\pm\) SD. One of three similar experiments is shown.
6.2.3 Cross talk does not decrease integrin expression

In an attempt to define the mechanism of integrin cross talk, the levels of \( \alpha 4\beta 1 \) and \( \alpha 5\beta 1 \) were measured on T cells before and after treatment with mAb 24. There was no change in the levels of either integrin showing that activation of LFA-1 does not decrease surface expression of these integrins (Figure 6.5).

6.2.4 Cross talk acts upstream of the actin cytoskeleton.

The possibility that LFA-1 activation might be influencing a cytoskeletal event was then tested. Although mAb 24 decreased the overall level of \( \alpha 4\beta 1 \)-mediated adhesion to FN there was no change in the sensitivity of binding to cytochalasin D (Figure 6.6). Therefore LFA-1 cross talk affects an event in cell adhesion following receptor occupancy but preceding changes in the actin cytoskeleton and is independent of both.

6.2.5 Cross talk involves an intracellular signalling event, independent of PKA, PKC and MAPKinase

In view of previous examples of cross talk it was of interest to look at the effect of various inhibitors on the cross talk phenomenon. Both mAb KIM185 and mAb 24 inhibited \( \alpha 4\beta 1 \)-mediated adhesion of T cells to FN (Figure 6.7.A). Preincubation of T cells with H7 (50 nM) reduced the background levels of CD3XL-stimulated adhesion by \(-50\%\). However, there was no effect on the inhibition induced by LFA-1 activation/occupancy (Figure 6.7.B). In addition the protein kinase A (PKA) inhibitor, H89 reduced the \( \alpha 4\beta 1 \)-mediated adhesion of both control and mAb 24-treated T cells to the same extent (Figure 6.8). It was then of interest to look at a potential role for the mitogen-activated protein kinase (MAPK) cascade in the cross talk phenomenon. After pre-treatment with the MAPK kinase (MEK) inhibitor PD98059, T cells were stimulated with CD3XL and allowed to adhere to ICAM-1 or FN. The inhibitor had no effect on T cell binding to ICAM-1 stimulated with CD3XL or CD3XL together with mAb KIM185 (Figure 6.9.A). In addition there was a
Figure 6.5: Activation of LFA-1 on T cells does not alter cell surface levels of α4β1 or α5β1. Flow cytometric analysis of T cells using mAbs to (A) α4β1 (mAb FITC-HP1/2; 5 μg/ml) and (B) α5β1 (mAb SAM-1; 0.5 μg/ml) showed no change in relative fluorescence levels for either integrin before or after activation of LFA-1 using mAb 24 (10 μg/ml). Cells (A); or secondary FITC-conjugated rabbit anti-mouse IgG2b (B) alone are shown as control. The data shown are from one representative experiment of three.
Figure 6.6: Cross talk acts upstream of the actin cytoskeleton and does not affect sensitivity of α4β1-mediated adhesion to cytochalasin D
Integrin α5β1 was blocked with mAb SAM-1 (0.5 µg/ml) allowing α4β1 adhesion to be looked at in isolation. T cells were treated with mAb 24 (LFA-1 activating) or control mAb 52U at 20 µg/ml. Cytochalasin D was used at 0-10 µg/ml (0-20 µM). Specificity of adhesion was shown by block with mAb HP1/2 (anti-α4). Data represent means of triplicates ± SD. One representative experiment of six is shown.
Figure 6.7: Cross talk is not reversed by the serine/threonine kinase inhibitor H7. T cells were (A) untreated or (B) preincubated with H7 (50 nM) for 30 min at 37°C before stimulation with CD3XL (mAb UCHT1; 0.10 µg/ml) to allow α4β1-mediated adhesion to fibronectin (α5β1-block; mAb SAM-1; 0.5 µg/ml). T cells were treated with 20 µg/ml of mAbs; 24 (■■■), KIM 185 (■ ■■), or isotype control mAb 52U (O—). Adhesion was blocked by 10 µg/ml mAb HP1/2 (Q). Data are means of triplicates ± SD. One representative experiment of three is shown.
Figure 6.8: Cross talk is not reversed by the protein kinase A (PKA) inhibitor H89. T cells were preincubated with various concentrations of the PKA inhibitor H89 (0-100 μM) for 30 min at 37°C before being stimulated by CD3XL (mAb UCHT1; 10 μg/ml) to adhere to FN through α4β1 (α5β1-block; mAb SAM-1; 0.5 μg/ml). T cells were treated with 20 μg/ml of either mAb 24 (□, LFA-1 activation) or mAb 52U (■, control). All adhesion was blocked by α4-block (not shown). Data are means of triplicates ± SD. One representative experiment of three is shown.
Figure 6.9: Cross talk is not reversed by the MAPKinase inhibitor PD98059. T cells were preincubated with various concentrations of the MAPKinase inhibitor PD98059 for 30 min at 37°C and allowed to adhere to (A) ICAM-1 through LFA-1 or (B) FN through α4β1 (α5β1-block; mAb SAM-1; 0.5μg/ml) in the presence (---) or absence of (-----) CD3XL (mAb UCHT1; 10μg/ml). T cells were treated with 20 μg/ml of either mAb KIM185 (○, LFA-1 activating) or mAb 52U (■, isotype control). All adhesion was blocked by appropriate mAb (A&B;△). Data are means of triplicates ± SD. One representative experiment of three is shown.
slight effect on α4β1-mediated binding to FN stimulated by CD3XL, but no effect on the inhibition of this binding induced by mAb KIM185 (Figure 6.9.B). Taken together these results show no evidence for a role for PKC, PKA or the MAPK pathway in our model of cross talk.

6.2.6 A subset of receptors that are sensitive to cross talk are also sensitive to inhibition by KT5926

Another protein kinase that might be involved in cross talk is CaMKII, which has been implicated in one model of cross talk (Bilato et al., 1997). KT5926 is a specific inhibitor of CaMKII, but also inhibits MLCK at higher concentrations (IC₅₀: CaMKII, 4.4 nM; MLCK, 18 nM). When used at concentrations up to 10 nM, KT5926 inhibited ~30% of the binding of CD3XL-stimulated T cells to FN. This was similar to the inhibition of binding seen on LFA-1 activation (Figure 6.10). In addition the effects of LFA-1-activation and KT5926 were not additive at up to 10 nM KT5926. This suggested that a subpopulation of α4β1 integrins were susceptible both to low dose (<10 nM) KT5926 and to integrin cross talk following activation of LFA-1. At higher levels of KT5926 (1 μM) all adhesion was inhibited (Figure 6.10). This suggested that CaMKII might be involved in cross talk, whilst another kinase, perhaps MLCK, might be involved in integrin adhesion following CD3XL.

6.2.7 The serine/threonine PP1 and PP2A phosphatase inhibitor, okadaic acid inhibits low affinity binding to ICAM-1 and fibronectin with no effect on integrin cross talk.

The potential role of protein phosphorylation in cross talk and integrin activation suggested that dephosphorylation events might also be important. Although a positive correlation between cell adhesion and CD18 phosphorylation has been seen, the role of phosphorylation in integrin-mediated adhesion has remained unsettled (Valmu and Gahmberg, 1995). Okadaic acid (OA) is a specific and potent inhibitor of protein phosphatases type 1 (PP1) and 2A (PP2A). Previous studies have also shown that T cells incubated with 1.5 μM OA show increased threonine phosphorylation of
Figure 6.10: Subset of αβ1 receptors sensitive to cross talk are also sensitive to the myosin light chain kinase (MLCK) inhibitor KT5926. T cells were stimulated by CD3XL (mAb UCHT1; 10 μg/ml) to adhere to FN through αβ1 (α5β1-block; mAb SAM-1; 0.5 μg/ml), in the presence of the MLCK inhibitor KT5926, and 20 μg/ml of either mAb KIM185 (--; LFA-1 activation) or 52U (--; isotype control). All adhesion was blocked by α4-block (■). Data are means of triplicates ± SD. One representative experiment of three is shown.
CD18 after PdBu or CD3XL-stimulation of T cells (Valmu and Gahmberg, 1995), and in these cells OA inhibited CD18-dependent T cell aggregation (Valmu and Gahmberg, 1995). Okadaic acid inhibited binding of CD3XL-stimulated T cells to ICAM-1, although binding could be rescued by inducing high affinity LFA-1 with mAb KIM185 (Figure 6.11). In addition, OA also inhibited CD3XL-stimulated T cells to FN (α4β1), and was unable to rescue T cells from the mAb KIM185-mediated inhibition of α4β1 (Figure 6.11). It therefore appears that phosphatase activity is essential for CD3XL-stimulated adhesion but does not appear to play a role in cross talk.

### 6.2.8 Cross talk is not seen with K562/LFA-1 or Jurkat T cells

Several attempts were made to find cell lines that reproducibly demonstrated the cross talk phenomenon as it is often easier to dissect out the signalling pathway in such cells by transfection or inhibitor treatment. K562 is a human erythroleukaemic cell line that does not normally express CD18, but can be stably transfected with cDNA encoding CD18 and CD11a, to express LFA-1 (Ortlepp et al., 1995). The K562/LFA-1 cells unlike their parent cell line can be induced to bind to ICAM-1 in an LFA-1-dependent manner when stimulated jointly with PMA (100 ng/ml) and KIM127 (20 μg/ml). In contrast both the parent cell line and K562/LFA-1 bind to FN when stimulated with PMA, and in either case this is unaffected by mAb KIM127 (Figure 6.12). The failure of the K562/LFA-1 cells to demonstrate cross talk may be due to the fact that their binding to FN is mediated by α5β1. Previous results have shown that most of the cross talk effect is on α4β1, with only a small effect on α5β1. Another cell line that expresses both α4β1, α5β1 and LFA-1 is the human T cell line, Jurkat. When stimulated with Mg²⁺/EGTA these cells bind FN through α4β1 and α5β1, and when one is blocked the other compensates. However, there was no effect on adhesion to FN through either integrin when LFA-1 was activated (Figure 6.13). Jurkat T cells are known to have a defect in LFA-1 mediated signalling and cannot be induced to bind to ICAM-1 by inside out signals (data not shown; Mobley et al., 1994). This suggests that
Figure 6.11: The serine/threonine PP1 and PP2A phosphatase inhibitor, okadaic acid inhibits binding to ICAM-1 and FN with no effect on integrin cross talk. T cell adhesion to (A) ICAM-1; and (B) FN (α4β1 only) in 0.4 mM Ca^{2+} and Mg^{2+} (●) is increased by CD3XL (mAb UCHT1 10 μg/ml; ■). This adhesion is inhibited by preincubation (30 min at 37°C) of cells with okadaic acid (OA; 0-3.4 μM). OA did not affect adhesion to ICAM-1 stimulated by mAb KIM185 (10 μg/ml) (A; ○); or inhibition of FN binding by mAb KIM185 (B; ○). Data are means of triplicates ± SD. One representative experiment of three is shown.
Figure 6.12: Activation of transfected LFA-1 allows K562 cells to adhere to ICAM-1, but does not affect α5β1-mediated adhesion to fibronectin. (A) K562 cells (▪) do not express LFA-1 and are unable to bind ICAM-1 even after stimulation with PMA (100 ng/ml). LFA-1 transfected K562 cells (■; K562/LFA-1; KL/4) do not spontaneously bind ICAM-1, but LFA-1-mediated binding can be induced by treatment of K562/LFA-1 cells with PMA and KIM127. (B) K562 (▪), and K562/LFA-1 (■) bind FN through α5β1, and in neither cell type is this affected by LFA-1 activation. Data are means of triplicates ± SD. One representative experiment of three is shown.
Figure 6.13: No cross-talk between LFA-1 and α4β1 on Jurkat T cell line. Jurkat cells bind FN (A & B; -○-) through α4β1 and α5β1 and when one integrin is blocked the other can compensate. This allowed α4- (A; squares; α5-blocking mAb Sam-1 0.5 μg/ml) and α5- (B; triangles; α4-blocking mAb HP1/2 0.5 μg/ml) mediated adhesion to be examined separately. Adhesion was stimulated (A & B) with Mg^{2+} (0-5 mM)/2 mM EGTA in the presence (closed symbols) or absence (open symbols) of mAb 24 (LFA-1 activation). Data are means of triplicates ± SD. One representative experiment of three is shown.
an inherent signalling problem in the Jurkat cells makes them unable to cross talk.

6.3 Discussion

The results shown here demonstrate that mAbs that activate LFA-1 can also produce inhibition of \( \alpha 4\beta 1 \) on T cells through cross talk, with an effect that is strikingly similar to that of mAb 24. The mechanism for LFA-1 downregulation of \( \alpha 4\beta 1 \) was explored in several ways. It was first established that there was no alteration in expression of either \( \alpha 4\beta 1 \) or \( \alpha 5\beta 1 \) during the experimental period. Furthermore, results from experiments presented earlier in this thesis using confocal microscopy and mAbs specific for \( \alpha 4\beta 1 \), \( \alpha 5\beta 1 \) and the \( \beta 1 \) activation reporter mAb 15/7 allow some comments to be made about integrin modulation. In particular, when T cells adhere to ICAM-1, LFA-1 is relocated to the integrin-substrate interface. This redistribution of occupied LFA-1 did not cause ligand-competent \( \beta 1 \) integrin (i.e. epitope 15/7 expressing) redistribution on the T cell membrane (Figure 4.16.E). In addition, although stimulation of T cells with \( \text{Mg}^{2+} / \text{EGTA} \) induces high affinity LFA-1 (Stewart et al., 1996), the three stimulating protocols \( \text{Mg}^{2+} / \text{EGTA}, \text{PdBu} \) or CD3XL do not appear to induce high affinity \( \alpha 4\beta 1 \) or \( \alpha 5\beta 1 \) (sections 4.2.2 & 4.2.3). Although LFA-1 cross talk inhibits \( \beta 1 \)-mediated adhesion stimulated by these three protocols, there is no inhibition of \( \beta 1 \)-mediated adhesion stimulated by \( \text{Mn}^{2+} \) or with the \( \beta 1 \)-activating mAb TS2/16 (data not shown). Together these findings suggest that LFA-1 cross talk does not alter the affinity of the \( \beta 1 \) integrins, nor cause their redistribution or shedding from the cell surface, but appears to target events following ligand binding. This result is in keeping with other studies in which cross talk is ultimately dependent on the presence of the \( \beta \) subunit cytoplasmic tail and steps subsequent to modulation of integrin affinity (Blystone et al., 1994; Diáz-González et al., 1996).
It seemed possible that the cytoskeleton was a target of LFA-1-mediated cross talk because both α4β1 and α5β1-mediated adhesion were more sensitive to changes in actin, such as treatment with cytochalasin D than was adhesion through LFA-1 (section 4.2.2). However for β1 integrin-mediated adhesion, the similarity in cytochalasin D sensitivity of mAb 24 treated and untreated cells supported the evidence that inhibition occurs up-stream of cytoskeletal changes. These results implied that cross-talk affects an event in cell adhesion occurring after receptor occupancy but preceding actin-mediated cytoskeletal changes, but independent of both. In addition PKA associated with LFA-1 signalling and de-adhesion (Rovere et al., 1996), and PKC implicated in some previous cross talk studies (Blystone et al., 1994; Pacifici et al., 1994) were not involved in this phenomenon.

LFA-1 cross talk was evident following several different adhesion inducing protocols showing that the phenomenon was not stimulus specific. The fact that cross talk was dependent on ICAM-1, the LFA-1 activation reporter mAb 24 or the LFA-1 activating mAb KIM185 indicated that an extracellular conformational change in LFA-1 was a prerequisite. Although the signalling pathways that are activated upon engagement of the β2 integrins are not well understood, certain observations suggested that cross talk did activate specific intracellular signalling pathways. Cross talk was not observed using the Jurkat T cell line which is known to have a defect in LFA-1 signalling (Mobley et al., 1994). In addition cross talk was induced by mAb 24 Fab' fragments (section 5.2.4), but not by immobilised anti-LFA-1 mAb (section 5.2.1) emphasising the requirement for a mechanism beyond LFA-1 clustering. For α5β1 on human fibroblasts, although clustering of integrin by mAbs on beads induced phosphorylation and accumulation of focal adhesion kinase (FAK) and tensin, ligand occupancy recruited further cytoskeletal proteins to the signalling complex (Miyamoto et al., 1995; Miyamoto et al., 1995).
One potential player in cross talk is CaMKII. Low levels (<10 nM) of KT5926 are thought to selectively inhibit this kinase, and such levels produce a similar effect on the α4β1 integrins, as LFA-1 activation. This suggests that LFA-1 activation may exert its effects by inhibition of CaMKII. However, it is essential to repeat these experiments with other specific inhibitors of CaMKII such as KN62 (Tokumitsu et al., 1990). In addition it would be of interest to look at the effect of a constitutively active CaMKII on cross talk. Initial experiments show that ionomycin is unable to relieve cross talk (data not shown) arguing against a simple inhibition of CaMKII.

One speculation is that the targets of LFA-1 cross talk may be the proteins providing the link between integrins and actin. However, several observations suggested that cross talk does not represent a simple sequestering of such intracellular proteins. Firstly, integrin activity operates in one direction only, so that prolonged activation of the β1 integrins using mAb TS2/16 does not alter LFA-1 binding to ICAM-1 (section 5.2.5). Secondly, LFA-1 predominantly affects the activity of α4β1, despite a three-fold abundance of α4β1 over α5β1 (Figure 4.9). Future work will address the role of potential integrator molecules in the cross talk phenomenon. Higher doses of KT5926 are thought to be reasonably specific for MLCK, and it was interesting that inhibition of this enzyme inhibited α4β1-mediated adhesion to FN completely. It was hoped that understanding the mechanism by which CD3XL-stimulated integrin adhesion might shed some light on the potential interactions of integrin and cytoskeleton that might be disrupted by LFA-1 activation. In an effort to appropriately direct investigations into the mechanisms of cross talk it was decided to look at the role of the cytoskeleton in integrin mediated adhesion following CD3XL.
CHAPTER SEVEN

A POTENTIAL ROLE FOR ACTIN-MYOSIN INTERACTIONS IN INTEGRIN ACTIVATION FOLLOWING CD3/TCR CROSSLINKING

7.1 Introduction

It is essential for an effective immune response that circulating T lymphocytes interact appropriately with other cells, in particular antigen presenting cells (APCs), as well as with the extracellular matrix (ECM). The receptors responsible for this cell-cell adhesion include the integrins. In particular LFA-1 has been found to enhance the contact between T cells and APCs and facilitate cytotoxic T lymphocyte (CTL)-target cell interactions (Davignon et al., 1981). Involvement of α4β1 in T cell-mediated killing (Takada et al., 1989) and homotypic adhesion (Bednarczyk and McIntyre, 1990; Sánchez-Mateos et al., 1993) implicate α4β1 in cell-cell adhesion, although its cellular counter-receptor is not fully defined (Altevogt et al., 1995). Stimulation of the T cell by T Cell Receptor/CD3 complex (TCR/CD3) cross-linking (CD3XL) has been shown to transiently increase the binding of several integrins, including LFA-1 (Dustin and Springer, 1989) and α4β1 (Shimizu et al., 1990) to their ligands. In addition, adhesion through both LFA-1 and α4β1 can synergise with signals transduced by CD3XL to promote T cell proliferation (Damle and Aruffo, 1991; Springer, 1990; van Seventer et al., 1991). It is therefore possible that both LFA-1 and α4β1 might be involved not only in establishing the initial T cell-APC contact, but also, after an activation step, to strengthen the contact between T cells and APCs. Prior to activation T cells recirculate throughout the blood and lymph in a non adhesive state on a search for presented antigen. T cell recognition of an APC bearing the appropriate antigen-MHC complex follows a well described series of molecular and cellular events. The T cell flattens against the APC, T cell migration stops (Dustin et al., 1997) and a tight interface, known as the contact cap, forms at one pole of the T cell (Shaw
and Dustin, 1997; Stowers et al., 1995; Wülfing et al., 1998). Within the contact cap the high concentration of TCRs might promote their serial engagement with antigen by forcing antigen-MHC complexes to percolate through a dense field of TCRs (Valentine et al., 1995). Although CD3XL is sufficient for the formation of a cap (Lowin-Kropf et al., 1998) the stability of the cap may depend on other molecules. For example, LFA-1 is thought to act as a co-stimulatory molecule for T cell proliferation by promoting the adhesion of T cells to APCs. This results in increased stability of the contact cap and a reduction in the amount of antigen necessary for T cell triggering (Bachmann et al., 1997). Further contact cap stability may result from lateral interactions between ligated TCR/antigen-MHC complexes (Reich et al., 1997). Both cap formation and T cell polarisation are active processes orchestrated by Vav, a guanine-nucleotide exchange factor (GEF) for the Rho family of small GTPases such as Cdc42 (Stowers et al., 1995), and the T cell cytoskeleton (Fischer et al., 1998; Holsinger et al., 1998). The formation of the contact cap is reminiscent of the phenomenon of immunological capping which was described over 25 years ago, in which cross-linking of membrane proteins results in clustering and aggregation of proteins at one pole of the cell (Unanue et al., 1972).

The mechanism by which CD3XL induces integrin-mediated adhesion is not fully understood, but might involve Ca^{2+} fluxes. Increases in cytoplasmic Ca^{2+} ([Ca^{2+}],) accompany triggering through the TCR and integrin activation can be mimicked by agents that increase [Ca^{2+}], (Hartfield et al., 1993; Stewart et al., 1998). Ca^{2+} is known to activate many mediators of T cell signalling pathways such as PKC, calcineurin, calmodulin, calreticulin, myosin light chain kinase and calpain which may be involved in integrin regulation.

The increased LFA-1-mediated adhesion following CD3XL is dependent on clustering of low affinity LFA-1 (Stewart and Hogg, 1995; Stewart et al., 1996). Prior to CD3XL, LFA-1 and α4β1 are constrained by cytoskeletal connections and prevented from moving into or out of clusters. Activation of the T cell
with PMA or low levels of cytochalasin D releases LFA-1, putatively from the
cortical cytoskeleton, so that it is able to freely diffuse in the plasma
membrane (Kucik et al., 1996). Implicit is the assumption that this increased
mobility is proadhesive because it leads to increased integrin accumulation at
the adhesive site and therefore greater adhesion strengthening, as has been
shown for LFA-3 and CD2 (Chan et al., 1991). This explains the paradox that
low doses of the cytoskeletal disrupting agent cytochalasin D can facilitate
adhesion by LFA-1 (Kucik et al., 1996; Lub et al., 1997) and α4 tail-deletion-
mutants (Yauch et al., 1997) by increasing integrin lateral-mobility and
clustering, with no effect on receptor affinity (Yauch et al., 1997). In contrast
agents such as jasplakinolide that prevent actin disassembly, prevent LFA-1
clustering and T cell adhesion (Stewart et al., 1998). There is good, but
indirect, evidence that the physiological increase in lateral mobility following
CD3XL is mediated by the Ca\(^{2+}\)-induced activation of a calpain-like enzyme
that cleaves LFA-1 from an unidentified cytoskeletal protein (Stewart et al.,
1998). It is not clear whether integrin clustering is driven by, or independent
of, ligand. In at least one study the clustering of LFA-1 is ligand-independent
(Stewart et al., 1998) suggesting an active process from within the T cell.

Myosin light chain kinase (MLCK) is another T cell enzyme that might play a
role in integrin clustering. Myosins are actin-activated ATPases capable of
generating force by promoting translational movement along actin cables
(Figure 7.1). Several classes of myosin have been described and myosin II is
the best characterised for its ability to promote cell contraction and migration
in non-muscle cells including lymphocytes. Members of the myosin family
consist of two heavy chains (200 kDa) and two sets of light chains (16-20 kDa).
Myosin II function is regulated by phosphorylation of the regulatory light
chains on serine-19 by the Ca\(^{2+}\)/calmodulin-dependent enzyme MLCK.
Phosphorylation of myosin light chains (MLC) by MLCK is a critical regulatory
step in myosin function since it catalyses the interaction of the myosin head
with actin and allows the myosin ATPase activity to produce sliding force.
There is indirect evidence that MLCK provides the contractile force for
surface-receptor capping in lymphocytes, based on the accumulation of phosphorylated MLC (Bourguignon et al., 1981) and MLCK (Bourguignon et al., 1982) beneath capped structures and the inhibition of capping by inhibitors of calmodulin (Bourguignon et al., 1981). The phosphorylation state of MLC is also regulated by the small GTPase Rho, known to be involved in cytoskeletal rearrangements. Rho stimulates a Rho-associated coiled-coil forming kinase (p160 ROCK) that works down stream of Rho to induce focal adhesions and stress fibers (Ishizaki et al., 1997), and Rho also stimulates a homologue of p160 ROCK called Rho-kinase (ROKα). There is some suggestion that binding of GTP-Rho to these effectors may facilitate their translocation to the cell membrane or cytoskeleton in a manner that may, in some cases, be integrin-dependent (Narumiya et al., 1997). ROKα is able to phosphorylate the p130 regulatory subunit of MLC phosphatase (mPP; an isoform of PP1), the myosin binding subunit (MBS) and inactivate this phosphatase in vitro (Kimura et al., 1996). In addition ROKα and MLCK phosphorylate MLC on the same serine residue (Amano et al., 1996) suggesting that ROKα, like MLCK, can increase myosin contractile activity. Although phosphorylation of MLC on serine-19 by MLCK and ROKα is thought to regulate contractility, MLCK is also able to phosphorylate threonine-18 of MLC. In addition, MLCK can be phosphorylated by CaMKII, PKA and PKG at sites which result in a decreased affinity of MLCK for Ca^{2+}/calmodulin and the inhibition of MLCK activity (Figure 7.1; for review see Burridge and Chrzanowska-Wodnicka, 1996). In a similar way, PKC can catalyse inhibitory phosphorylation of MLC at serine-1, serine-2 and threonine-9 (Figure 7.1; for review see Tan et al., 1992). Based on these observations the role of MLCK and actin-myosin interactions in integrin-activation in T cells following CD3XL was investigated.
Chapter Seven—TCR/CD3 cross-linking and integrin activation

Figure 7.1: Regulation of contractility in smooth muscle and nonmuscle cells. The interaction of myosin with actin is regulated by phosphorylation of myosin light chains (MLC). Active proteins are red, inactive are blue. MLC phosphorylation of Ser-19 and Thr-18 is catalysed by MLCK. The same sites can be phosphorylated by CaMKII. MLCK is inhibited by phosphorylation by CaMKII, PKA or PKG. MLC are dephosphorylated by myosin phosphatase (mPP) which is inhibited by active Rho and also by arachidonic acid (AA). Rho stimulates Rho-kinase which inactivates mPP, and also phosphorylates MLC. Inhibitory phosphorylation of MLC is catalysed by PKC and the mitotic kinase p34<sup>cdc2</sup>. 
7.2 Results

7.2.1 CD3/TCR crosslinking activates adhesion through LFA-1 and α4β1 that is inhibited by the MLCK inhibitors KT5926 or wortmannin.

Results in section 6.2.6 have shown that the α4β1-mediated adhesion of T cells to fibronectin following CD3XL is sensitive to the MLCK inhibitor, KT5926. It was of interest to see if other MLCK inhibitors had the same effect. Wortmannin also inhibits MLCK (IC$_{50}$ 200 nM), but not other protein kinases. T cells were preincubated with wortmannin for 30 min at 37°C and then allowed to adhere to fibronectin. Wortmannin was able to inhibit the binding to fibronectin of CD3XL-stimulated T cells but not of those T cells stimulated by Mn$^{2+}$ (Figure 7.2). To establish whether LFA-1-mediated adhesion was susceptible to the same inhibitors, T cells were treated with wortmannin or KT5926 in the same way as previously and were then stimulated to adhere to ICAM-1. LFA-1-mediated adhesion following CD3XL was inhibited by both wortmannin (Figure 7.3.A) and KT5926 (Figure 7.3.B). However, high affinity LFA-1-mediated binding, as induced by Mn$^{2+}$ (Figure 7.3.A) or mAb KIM185 (Figure 7.3.B) was not affected. As well as inhibiting MLCK, wortmannin is also known as a specific inhibitor of phosphatidylinositol 3-kinase (PI 3-kinase; IC$_{50}$ 5 nM). It was therefore important to exclude a role for this enzyme in LFA-1-mediated adhesion. Prior to CD3XL, T cells were treated with LY 294002, a cell permeable potent and specific inhibitor of PI 3-kinase (IC$_{50}$ 1.4 μM). Preincubation of T cells with LY 294002 for 30 min at 37°C had no effect on LFA-1 mediated adhesion following stimulation of T cells with CD3XL, Phorbol ester (PdBu; 50 nM) or Mn$^{2+}$ (0.5 mM) (Figure 7.4). These results strongly suggest that MLCK-mediated phosphorylation of myosin and subsequent myosin-actin interactions are involved in integrin-mediated adhesion following TCR activation.
Figure 7.2: Wortmannin inhibits low affinity T cell binding to fibronectin, but not high affinity T cell binding stimulated by Mn$^{2+}$. (A) Adhesion to FN through $\alpha 4\beta 1$ ($\alpha 5$ blocked as previously) does not occur in 0.4 mM Ca$^{2+}$/0.4 mM Mg$^{2+}$ in the absence of stimulation (□). Adhesion induced by CD3XL (●; mAb UCHT1 10 μg/ml) is inhibited by wortmannin; in contrast adhesion induced by Mn$^{2+}$ (○) is not affected by up to 5 μM wortmannin. Adhesion is blocked by mAb HP1/2 ($\alpha 4$ block; 0.5 μg/ml; ■). Data are means of triplicates ± SD. One representative experiment of three is shown.
Figure 7.3: Wortmannin and the myosin light chain kinase (MLCK) inhibitor KT5926 inhibit low affinity T cell binding to ICAM-1, but not high affinity T cell binding induced by Mn^{2+} or mAb KIM185. T cell adhesion to ICAM-1 does not occur in 0.4 mM Ca^{2+}/0.4 mM Mg^{2+} in the absence of stimulation (A & B; □). Adhesion induced by CD3XL (●; mAb UCHT1 10 μg/ml) is inhibited by (A) wortmannin and (B) KT5926; in contrast adhesion induced by (A) Mn^{2+} (○); or (B) 10 μg/ml mAb KIM185 (○) is not affected by either (A) 0.5 μM wortmannin; or (B) 0.5 μM KT5926. Adhesion is blocked by mAb 38 (10μg/ml; ■). Data are means of triplicates ± SD. One representative experiment of three is shown.
Figure 7.4: The phosphatidylinositol 3-kinase inhibitor LY294002 does not inhibit T cell binding to ICAM-1 induced by a variety of stimuli. Only low levels of T cell adhesion to ICAM-1 are supported by 0.4 mM Ca^{2+} / 0.4 mM Mg^{2+} in the absence of stimulation (□). Adhesion induced by either CD3XL (●; mAb UCHT1 10 μg/ml), PdBu (■), or Mn^{2+} (▲) is unaffected by preincubation of T cells for 30 min at 37°C with various concentrations of LY294002 (0-0.5 μM). Adhesion is blocked by mAb 38 (10 μg/ml; ▼). Data are means of triplicates ± SD. One representative experiment of three is shown.
7.2.2 Adhesion promoting stimuli that increase \([Ca^{2+}]_i\) are susceptible to inhibition of MLCK.

Increases in cytoplasmic \(Ca^{2+}\) ([\(Ca^{2+}\)]) accompany triggering through the TCR/CD3 complex and are also an important component of other adhesion inducing stimuli. PdBu-induced T cell adhesion to ICAM-1 is \(Ca^{2+}\)-dependent (Stewart et al., 1996), and calcium-mobilising agents cause integrin-mediated adhesion (Hartfield et al., 1993; Stewart et al., 1998). The ability of MLCK inhibitors to block LFA-1-mediated cell adhesion to ICAM-1 following CD3XL suggested that other stimuli that cause a rise in \([Ca^{2+}]_i\) might also be susceptible. To test this possibility, \([Ca^{2+}]_i\) increases were triggered in T cells using ionomycin (0.7 \(\mu\)M) or thapsigargin (5 \(\mu\)M). Thapsigargin specifically inhibits the \(Ca^{2+}\)-ATPase activity of the intracellular \(Ca^{2+}\) stores, resulting in the depletion of intracellular \(Ca^{2+}\) stores, and the triggering of an influx of \(Ca^{2+}\) through the plasma membrane (Randriamampita and Tsien, 1993; Thastrup et al., 1990; Zweifach and Lewis, 1993). Other stimuli were also investigated, such as PdBu (50 nM), \(Mn^{2+}\) (0.5 mM) and \(Mg^{2+}\) (3 mM)/EGTA (2 mM), as well as the mAbs, KIM185 (LFA-1 activating) and HUTS-21 (\(\beta_1\), LIBS/activation reporter). All stimuli, except \(Mn^{2+}\) and \(Mg^{2+}/EGTA\) were in the presence of 0.4 mM \(Mg^{2+}/0.4\) mM \(Ca^{2+}\). T cells were untreated, or treated with K252a (500 nM) which inhibits MLCK (\(IC_{50}\) 17 nM), but also PKA (\(IC_{50}\) 18 nM) and PKC (\(IC_{50}\) 25 nM), and were then stimulated to adhere to ICAM-1 or fibronectin (through \(\alpha 4\beta 1\)). For T cell adhesion to ICAM-1 (Figure 7.5.A) treatment with K252a inhibited binding stimulated by CD3XL and the calcium-mobilising agents back to background levels. There was less effect of K252a on adhesion stimulated by PdBu (Figure 7.5.A), and no effect on adhesion mediated by divalent cation treatments or mAb KIM185 (Figure 7.5.A) showing that high affinity LFA-1 was not affected. Binding to fibronectin mediated by \(\alpha 4\beta 1\) (the \(\alpha 5\beta 1\)-mediated component of binding was blocked throughout) was similar.
Figure 7.5: The MLCK inhibitor K252a inhibits low affinity binding to ICAM-1 and FN. T cells were stimulated to bind (A) ICAM-1; or (B) FN (α4β1; α5 component blocked with mAb SAM-1) in the presence (■) or absence (□) of K252a (500 nM). The stimuli were: 0.4 mM Ca\(^{2+}\) / 0.4 mM Mg\(^{2+}\) alone (control), or with thapsigargin (5 μM), ionomycin (0.7 μM), mAb UCHT1 10 μg/ml (CD3XL) or PdBu (50 nM). Additional stimuli were 3 mM Mg\(^{2+}\) /2 mM EGTA or Mn\(^{2+}\) (0.5 mM) or 10 μg/ml of the stimulating mAbs (A) KIM 185; or (B) HUTS-21. Mn\(^{2+}\)-induced adhesion was blocked by (A) mAb 38 (10 μg/ml; (B) mAb HP1/2 (0.5 μg/ml). Data are means of triplicates ± SD. One representative experiment of three is shown.
(Figure 7.5.B) but differed in that binding stimulated by 3 mM Mg$^{2+}$/2 mM EGTA was inhibited. This is in agreement with earlier findings that although treatment with 3 mM Mg$^{2+}$/2 mM EGTA induces high affinity LFA-1-mediated adhesion, this same treatment only induces a low affinity form of α4β1 (section 4.2.2). In addition the binding stimulated by the mAb HUTS-21, which is also of low affinity (Gomez et al., 1997), was also inhibited. The same results were seen with KT5926 and another MLCK inhibitor, ML-7 (IC$_{50}$: MLCK, 0.3 µM; PKA, 21 µM; PKC, 42 µM). There was consistently more effect of all of these inhibitors on PdBu-stimulated adhesion to fibronectin than on PdBu-stimulated adhesion to ICAM-1. These results show that additional stimuli that increase [Ca$^{2+}$], also act through MLC phosphorylation to increase integrin-mediated adhesion.

7.2.3 The myosin disrupting agent butanedione monoxime inhibits integrin-mediated adhesion following CD3XL, but not following the β2-stimulating mAb KIM185.

To confirm a role for actin-myosin interactions in integrin-mediated adhesion following CD3XL, the effect of another inhibitor, the myosin-disrupting agent 2,3 butanedione 2-monoxime (BDM) was examined. BDM inhibits myosin-ATPase activity by slowing the release of phosphate from myosin after ATP hydrolysis (McKillop et al., 1994). BDM was able to block the background adhesion of T cells to ICAM-1, and their stimulated adhesion following CD3XL (Figure 7.6). However, BDM had no effect on the LFA-1-mediated adhesion stimulated by mAb KIM185 (Figure 7.6), although this adhesion could be blocked with mAb 38, an LFA-1 blocking mAb (Figure 7.6). A titration showed that the optimal concentration of BDM for blocking CD3XL stimulated adhesion was at 20 mM. BDM was also able to inhibit the binding to T cells to fibronectin through α4β1 following stimulation with CD3XL but had no effect on binding following Mn$^{2+}$ (data not shown). These results confirm a role for actin-myosin interactions in integrin-mediated adhesion stimulated by TCR activation.
Figure 7.6: The myosin disrupting agent butanedione monoxime (BDM) inhibits low affinity T cell binding to ICAM-1. T cell adhesion to ICAM-1 in 0.4 mM Ca²⁺/0.4 mM Mg²⁺ (●) is increased by TCR/CD3 cross linking (□; CD3XL; mAb UCHT1 10 μg/ml), and both are inhibited by butanedione monoxime (BDM; 0-30 μM). Adhesion induced by 10 μg/ml mAb KIM185 (○) is not affected by BDM. Adhesion with all three stimuli is blocked by mAb 38 (10 μg/ml; ▲). Data are means of triplicates ± SD. One representative experiment of three is shown.
7.2.4 TCR/CD3XL-mediated adhesion is only partially inhibited by PKC inhibitors, but is abolished by inhibition of MLCK.

A comparison of the effects of various inhibitors on T cell binding to ICAM-1 following stimulation of T cells with PdBu (50 nM), CD3XL and Mn^{2+} (0.5 mM) shows striking similarities between the inhibition caused by the actin disrupting agent, cytochalasin D; the myosin disrupting agent BDM; and the MLCK inhibitors wortmannin and ML-7 (Figure 7.7). In particular these agents inhibited adhesion stimulated by PdBu or by CD3XL to the same extent, with no effect on binding stimulated by Mn^{2+}. By contrast the PKC inhibitor Ro-31-8220 (IC_{50}: PKA, 0.9 μM; PKC, 0.01 μM) inhibited PdBu stimulated adhesion but had much less effect on binding stimulated by CD3XL (Figure 7.7). This suggests that although actin-myosin interactions are involved in the adhesion induced by both PdBu and TCR activation, the pathway leading to myosin phosphorylation differs between the two stimuli. It appears that PKC lies upstream of these cytoskeletal events in PdBu-stimulated adhesion, but may not be essential in the signalling pathway stimulated by CD3XL.

7.2.5 CD3XL causes polymerisation of actin which is inhibited by cytochalasin D or the MLCK inhibitor, KT5926

Much previous work has suggested a role for actin rearrangements following T cell activation. In particular when the anti-CD3 mAbs used for T cell stimulation are immobilised, they induce marked morphological changes characterised by T cell spreading and the formation of pseudopodia (Pardi et al., 1992; Parsey and Lewis, 1993). This suggested that the activation of MLCK induced by TCR activation might result in actin bundling and increased levels of bundled or filamentous actin. It was therefore of interest to look at the effects of CD3XL on the reorganisation of the filamentous actin (F-actin) based cytoskeleton in T cells and the effects of MLCK inhibitors. F-actin was quantified by using fluorescently labelled phalloidin in cytofluorometric assays (section 4.2.5). Results from such experiments demonstrate a dramatic increase in the level of F-actin in T cells following CD3XL, when compared to
Figure 7.7: Inhibitors of actin-myosin abolish both PdBu and CD3XL induced T cell adhesion to ICAM-1, while inhibition of PKC preferentially affects adhesion induced by PdBu. T cells were stimulated to bind ICAM-1 following various stimuli: 0.4 mM Ca\(^{2+}\)/0.4 mM Mg\(^{2+}\) alone (control), with PdBu or with mAb UCHT1 (CD3XL); or Mn\(^{2+}\). Various inhibiting agents were present: cytochalasin D (Cyto. D); wortmannin (Wortmn.); the MLCK inhibitor, ML-7; butanedione monoxime (BDM); the PKC inhibitor RO-318220 or mAb 38 (10 μg/ml; LFA-1 block). Data are means of triplicates ± SD. One representative experiment of three is shown.
unstimulated T cells (Figure 7.8). As expected the increase in F-actin can be completely abrogated by treating T cells with cytochalasin D (Figure 7.8) and in addition the increased F-actin can be inhibited by the MLCK inhibitor KT5926 (Figure 7.8). This demonstrated that the increase in F-actin associated with CD3XL on T cells is brought about by the activity of MLCK.

7.2.6 Biochemical attempts to show increased MLC phosphorylation following TCR/CD3 cross-linking

Although it is an attractive hypothesis that MLCK is involved in integrin adhesion following TCR activation, it is a suggestion that relies heavily on the use of ‘specific’ kinase and myosin inhibitors. To confirm these findings it will be essential to show some of these effects biochemically. In particular it is important to assess the phosphorylation of MLC following CD3XL and the effects of MLCK inhibitors on this phosphorylation. It would also be of interest to look at the MLC phosphorylation following T cell stimulation with agents such as Mn²⁺ that activate integrin-mediated adhesion that is not susceptible to actin-myosin disruption. Several approaches have, therefore, been tried to quantify the phosphorylation of MLC in T cells following stimulation. Many previous authors have used glycerol/urea PAGE (based on the original method see Perrie and Perry, 1970) to separate out non-, mono- and di-phosphorylated forms of MLC from whole cell lysates followed by quantification of the relative amounts of each form of MLC using an immunoblot, or radiographic procedure. The basis of a phosphorylation-dependent shift in MLC mobility is not clear, but is thought not to reflect the additional negative charge associated with a phosphate group, but probably a phosphorylation dependent change in the tertiary and/or quaternary structure of the molecule (Persechini et al., 1986). Most of these experiments have been performed in smooth muscle cells (Cande et al., 1983; Taylor and Stull, 1988) that contain high levels of MLC. Attempts to use such procedures with T cells were unsuccessful because only a small fraction of the total T cell lysate proteins entered the glycerol/urea gel even using 6 M urea gels, and after denaturing the cell lysates in 9 M urea over night (data not shown).
Figure 7.8: Stimulation of T cells with PdBu and CD3XL causes actin polymerisation, that is inhibited by the MLCK inhibitor, KT5926. The F-actin content of T cells in cation free buffer (control), is increased by 0.4 mM Ca\textsuperscript{2+}/0.4 mM Mg\textsuperscript{2+}, and further increased by mAb CD3XL (UCHT1; 10 μg/ml). This increase is inhibited by cytochalasin D (cyto. D.; 2 μM) or KT5926 (1 μM). F-actin was quantified by FACS analysis of FITC-phalloidin binding by permeabilised T cells. One of two similar experiments is shown.
These findings are in agreement with the original description of the method in which it was believed that only 10-15% of the total protein applied entered the glycerol/urea gel (Perrie and Perry, 1970). This does not pose such a problem when dealing with muscle cells in which a high proportion of intracellular protein is myosin, but is much more of a problem when dealing with the already relatively low levels of myosin in T cells. For these reasons, this was not thought to be an accurate method to quantify potentially small changes in phosphorylated MLC in leukocytes. Attempts were then made to immunoprecipitate MLC from T cells. Although an anti-MLC mAb gave a clear single band on an immunoblot of T cell lysates it was not able to immunoprecipitate MLC from the T cells (data not shown). Current attempts are aimed at immunoprecipitating T cells with an anti-myosin II mAb, to bring down both heavy and light chains of myosin which can be identified on PAGE. This would allow the unambiguous identification of MLC and quantification of MLC phosphorylation would then be possible. Quantification of MLC phosphorylation could be achieved by using either $^{32}$P-labelled T cells and autoradiography of the PAGE gel, or by use of unlabelled T cells and immunoblot analysis using either a mAb against phosphoserine, or a mAb specific for the phosphoserine-19 of MLC (Fukuda et al., 1997; Matsumura et al., 1998). Given the possible different forms of phosphorylated MLC (section 7.1; Figure 7.1) the use of a mAb specific for phosphoserine-19 of MLC would give the most accurate method of quantification of regulatory MLC phosphorylation. In order to produce specific mAbs to phosphorylated MLC, a phosphopeptide (RPQRATzNVFAC; z=phosphoserine) was synthesised by the ICRF Peptide Synthesis Laboratory. 20 mg of this peptide was conjugated to keyhole limpet haemocyanin (KLH; Calbiochem) and a rabbit is currently being immunised with this peptide.
7.2.7 Blocking isoprenylation with lovastatin inhibits CD3XL stimulated T cell adhesion to ICAM-1, which can be rescued by addition of mevalonate.

Having established a role for actin-myosin interactions in integrin-mediated adhesion, it was of interest to investigate potential pathways that lead from TCR activation to MLC phosphorylation. Recently one pathway for the stimulation of MLCK was demonstrated to be the MAPK pathway (Klemke et al., 1997) which is well documented to be activated on TCR activation (Cantrell, 1998; Henning and Cantrell, 1998). The results presented above (section 6.2.5) show no effect of the MAPK kinase (MEK) inhibitor, PD098059, on integrin-mediated cell adhesion following CD3XL and implicate an alternative pathway upstream of MLC phosphorylation. It has recently been suggested that some of the cytoskeletal changes induced by Rho are due to Rho-mediated regulation of MLC phosphorylation (Chrzanowska-Wodnicka and Burridge, 1996). Interactions of small GTP-binding proteins with mAbs, target proteins and regulatory proteins generally require isoprenylation of CAAX sequences (single letter amino-acid code) at their C-termini (Fenton et al., 1992). Blocking isoprenyl moieties with drugs such as lovastatin and compactin has been used, therefore, to block this class of signalling molecules (Chong et al., 1994; Fenton et al., 1992). When T cells were cultured in 40 μM lovastatin for 4 h they were unable to bind to ICAM-1 following CD3XL (Figure 7.9). However, addition of 1 mM mevalonate for 24 h to bypass the blockade of isoprenyl-group synthesis completely reversed this effect (Figure 7.9). These results therefore suggest that a member of the small GTP-binding protein family or some other protein that requires isoprenylation for its function is critical for integrin-mediated T cell binding following CD3XL. Attempts were made to look at more specific inhibitors of the Rho-sub family of GTPases. All Rho-subfamily proteins (e.g. Rho, Rac and Cdc42) are targets for Clostridium difficile toxins A and B (Just et al., 1994). These toxins specifically monoglucosylate Rho proteins using UDP-glucose as co-substrate (Just et al., 1995). Compared to toxin B, toxin A is about 3 orders of magnitude less potent. Both toxins are intracellularly acting and have to be internalised
Figure 7.9: Blocking isoprenylation with lovastatin inhibits CD3XL stimulated T cell adhesion to ICAM-1, which can be rescued by addition of mevalonate. T cells were maintained in RPMI/5% FCS with no addition (control; □; 40 μM lovastatin; ●); or addition of 40 μM lovastatin and 1 mM mevalonate (○); for 24 h at 37°C. Data are means of triplicates ± SD. One representative experiment of three is shown.
by receptor-mediated endocytosis, or microinjected into target cells, to exert their cytotoxicity (Just et al., 1994). Modification of Rho occurs at threonine 37 (and on the equivalent residue, Thr-35, of Rac and Cdc42). This highly conserved threonine binds the nucleotide through the co-ordination of Mg\textsuperscript{2+} and is located in the effector region of Rho where coupling with the effector protein takes place. It is probable that glucosylation blocks this interaction (Aktories, 1997). Toxin B was obtained from Dr Klaus Aktories (Mainz, Germany) and its activity was confirmed by its effect on cultured fibroblasts. Swiss 3T3 cells were seeded at a density of 10\textsuperscript{6}/well in 6 well culture dishes and were cultured in Dulbecco’s modified eagle medium (DMEM) supplemented with 10% FCS for 24 hr. The cells were then treated with 0.1 ng/ml of toxin B. After 24 hr the cells showed marked cell rounding with beaded dendritic processes (data not shown). The cells were still viable as assessed by their ability to exclude trypan blue. However, T cells cultured in various concentrations of toxin B (0.1-100 ng/ml) for 24 hr underwent normal LFA-1/ICAM-1 dependent aggregation when stimulated with 50 nM PMA (data not shown). As such aggregation has been shown to be Rho A dependent in the B cell line JY (Tominaga et al., 1993), the results with T cells suggests that toxin B was not entering the cells.

7.3 Discussion

In this study, we demonstrate a role for actin-myosin interactions in the regulation of LFA-1 and \(\alpha_4\beta_1\) integrin activity following CD3XL. Inhibitors of actin-myosin and MLCK inhibited both the increase in intracellular F-actin, and the increased integrin-mediated T cell adhesion following CD3XL. These inhibitors also reduced adhesion promoted by other integrin activating stimuli, such as PdBu and Ca\textsuperscript{2+}-mobilising agents, but not the adhesion mediated by high affinity forms of the integrins. In addition, the data also implicate the small GTP-binding proteins in these events.

How might MLCK and actin-myosin interactions be involved in the integrin-mediated adhesion? It is well established that adhesion can be induced either
by an increase in integrin affinity or by the clustering of low affinity receptors (Stewart et al., 1996). CD3XL promotes adhesion through low affinity LFA-1 and α4β1. These forms of integrins, although unable to bind soluble ligands (Jakubowski et al., 1995; Stewart et al., 1996), can support cell adhesion to immobilised ligands through a combination of integrin activation and cell spreading (Jakubowski et al., 1995; Stewart et al., 1996). MLCK inhibitors do not inhibit the high affinity integrin-mediated cell adhesion promoted by Mn^{2+} and activating mAbs such as mAb KIM 185, suggesting a role for MLCK in post-ligand binding events, such as integrin clustering. The clustering of integrins is reminiscent of receptor capping an event thought to be dependent on MLCK (Bourguignon et al., 1982; Bourguignon et al., 1981; Bourguignon et al., 1988).

The inhibitory effect of MLCK inhibitors on CD3XL-stimulated adhesion suggests, albeit indirectly, that CD3XL acts to increase MLC phosphorylation. Future efforts will be directed at investigating this directly. The pathways by which CD3XL might stimulate MLC phosphorylation are of interest. One potential pathway would be the MAPKinase pathway which is activated on TCR stimulation and is implicated in the activation of MLCK (Klemke et al., 1997). However, inhibition of MAPK kinase (MEK) with the inhibitor PD098059 had no effect on integrin mediated adhesion, although the same batch of inhibitor was known to be working in other systems (Dr Rebecca Newton, person. commun.).

Other potential upstream regulators of MLC phosphorylation are the members of the Rho family of small GTPases that have been implicated in cytoskeletal rearrangements although it is still not clear how they bring about these changes (Narumiya et al., 1997). One suggested mechanism is that cytoskeletal changes come about as a result of Rho-stimulated contractility. The effects of activated Rho to produce stress fibers and focal adhesions are accompanied by MLC phosphorylation and can blocked by inhibitors of MLCK (Chrzanowska-Wodnicka and Burridge, 1996). Rho has been shown to
regulate MLC phosphorylation by at least two different pathways. Rho activates ROKα, which phosphorylates MLC on serine 19, the same residue as is phosphorylated by MLCK (Amano et al., 1996). In addition, in vitro, ROKα is able to phosphorylate and deactivate a MLC phosphatase subunit to allow increased MLC phosphorylation (Kimura et al., 1996). A preliminary experiment demonstrating the inhibition of adhesion following treatment of T cells with lovastatin suggested that a member of the Rho family of small GTPases might be involved in integrin activation following CD3XL. It was hoped that the use of the more specific inhibitor toxin B would resolve this issue but it seemed unlikely that toxin B was taken up by the T cells. As well as being susceptible to toxin B, the Rho proteins are also targets for the ADP-ribosyltransferase, C3 exoenzyme (C3) which is produced by Clostridium botulinum type C and D strains. Several isoforms of C3 exist and all ADP-ribosylate Rho (Rho A, B and C) but not Rac or Cdc42 at asparagine 41 in the effector region of the GTPase, and inhibit the interaction of Rho with its effector(s) (Aktories, 1997). Because toxin B is also able to inhibit C3-induced ADP-ribosylation of Rho proteins (Just et al., 1994; Just et al., 1995), the ability of C3 to [32P]ADP-ribosylate substrate in lysates of untreated T cells and of T cells treated with toxin B could resolve the question of toxin B penetration into T cells. In addition C3 could be used directly to inhibit Rho in T cells. However, C3-like exoenzymes contain no cell binding and membrane translocation unit and therefore cell accessibility is poor, and high concentrations (>10 μg/ml) are needed for non-specific uptake into cultured cells. Previous groups have used electroporation to introduce C3 into T cells (Lang et al., 1992), as spontaneous C3 uptake is lower for T cells than for other leukocytes, such as B cells (Laudanna et al., 1996; Toda et al., 1993; Tominaga et al., 1993) and neutrophils (Laudanna et al., 1996). One potential approach is to produce a fluorochrome tagged form of C3 so that T cells that had taken up the exoenzyme could be distinguished from those that had not, in standard adhesion assays. This would provide a relatively straight forward assay to quantify the effect of C3 on the integrin-mediated T cell adhesion induced by various stimuli.
The ability to inhibit Rho function using inhibitors of MLCK (Chrzanowska-Wodnicka and Burridge, 1996), suggests either that Rho directly activates MLCK, for which there is no evidence, or that the actions of both MLCK and Rho are necessary for myosin function. This involvement of both MLCK and a small GTPase in these events may seem unnecessary but this overlap in function may play an important regulatory role. For instance MLCK could work synergistically with ROKα. In this scenario, MLCK-stimulated phosphorylation of MLC might be increased by ROKα-mediated inhibition of myosin phosphatase. This implies that the primary effect of ROKα is the inhibition of myosin phosphatase and that MLC phosphorylation by ROKα might be a secondary effect. Cells may need this two pronged approach to sustain the uninterrupted increase in cellular contractility that characterises cell adhesion. Another factor is that unlike MLCK which needs Ca²⁺/calmodulin for activation, ROKα is not Ca²⁺-dependent, so that ROKα phosphorylation of MLC might provide a Ca²⁺-independent mechanism for activation of myosin II. Interestingly there is evidence in endothelial cells that the influx of [Ca²⁺] from the extracellular space in response to thapsigargin and bradykinin is MLCK-dependent (Takahashi et al., 1997). This suggest a potential amplification method for MLCK-mediated contractility induced by various agonists.

It is of interest that the integrin-mediated adhesion stimulated by CD3XL differs from that induced by PdBu in its sensitivity to PKC inhibition. PdBu binds to the DAG site of PKC and is thought to activate integrins in a PKC dependent way. Previous studies have also implicated PKC in the integrin activation induced by CD3XL (Dustin and Springer, 1989; Shimizu et al., 1990; van Kooyk et al., 1989). However, these studies have used staurosporine, an inhibitor that lacks specificity and inhibits a number of other enzymes (e.g. Ki: PKC, 0.7 nM; MLCK, 1.3 nM; PKA, 7 nM). In at least one study staurosporine completely inhibited PdBu-stimulated adhesion but only partially inhibited
CD3XL-stimulated adhesion of T cells to ICAM-1 (Dustin and Springer, 1989). Ro-31-8220 is a highly selective inhibitor of PKC and was found to inhibit PdBu-stimulated integrin-mediated adhesion completely, but only partially inhibit that stimulated by CD3XL while the opposite is true for the MLCK inhibitor K252a. Taken together the inhibitor data suggests that PdBu and TCR stimulation activate integrins through different signalling pathways which may both result in MLC phosphorylation.

This is the first report to define a potential mechanism and role for myosin II mediated contractility in the integrin-mediated adhesion of T cells that follows CD3XL. One possible model for the events that follow TCR stimulation is as follows. Prior to activation the integrins are not clustered but are dispersed over the surface, and the filaments of the actin cytoskeleton are organised as a loose network. On CD3XL, MLCs undergo phosphorylation which promotes the productive interaction of myosin heads with actin filaments, generating contractility and isometric tension. In addition, calpain activation might release integrins from cytoskeletal restraint and allow their free lateral mobility in the cell membrane. At this stage LFA-1 and α4β1 might re-associate with the cytoskeleton (Pardi et al., 1992), which now consists of F-actin bundles aligned by the tension generated by actin-myosin contraction and the ability of myosin to bundle actin. At the membrane the bundled actin filaments would aggregate the integrins to which they are attached, either directly or through linker molecules. It may be that this clustering from the inside is also important for signal transduction pathways. Indeed integrin clustering, rather that ligand occupancy, is important for some signal transduction pathways such as the activation of FAK (Miyamoto et al., 1995) and clustering of integrins from the outside has been shown to stimulate FAK activity (Schaller et al., 1995). In support of this model inhibition of MLCK has been shown to prevent the redistribution of β1 integrins to focal adhesions when fibroblasts adherent to fibronectin are stimulated with lysosphosphatidic acid (LPA) and to prevent the Rho-induced tyrosine phosphorylation of focal adhesion proteins (Chrzanoswska-Wodnicka
and Burridge, 1996). However, this model assumes that integrin clustering is enough for activation of ligand binding and this point is not fully settled.

Future experiments will be aimed at biochemically establishing a role for MLC phosphorylation in these events. If such a role is confirmed it will then be important to establish a link between actin-myosin contractility, induced by MLC phosphorylation, and integrin clustering. In addition the role of Rho and other members of the family of Rho GTPases in these events should prove fascinating.
CHAPTER EIGHT

SUMMARY AND FUTURE DIRECTIONS

8.1 Outline summary

8.1.1 T cell adhesion to the extracellular matrix and the respiratory epithelium

(1) T cells expanded from PBMCs are double positive for CD45RA and CD45RO. The cells are CD3+ and with a CD8+:CD4+ ratio of 2:1. The ratio of LFA-1: α4β1: α5β1 is ~9:3:1

(2) Cultured T cells will bind to the respiratory epithelial cell line, BEAS-2B, without additional activation of either the T cell or the epithelial cells. There are both integrin-dependent and integrin-independent components of this adhesion. The interaction between LFA-1 on the T cells and ICAM-1 on the epithelial cell is quantitatively the most important, with a small less consistent contribution between CD2 on the T cell binding to LFA-3 on the epithelial cell. At least 60% of the adhesion is temperature and cation independent and the receptors involved remain to be characterised.

(3) The interaction of T cells with respiratory epithelium is able to activate LFA-1. This does not appear to be due to a soluble activating factor and may depend on cell-cell interactions.

(4) T cell migration across a confluent monolayer of epithelial cells is not inhibited by an LFA-1 blocking mAb.

(5) T cells can be stimulated following treatment with PdBu or by CD3XL to adhere to collagen (type 1) or fibronectin. The binding to collagen is mediated by α2β1. Binding to fibronectin is mediated through both α4β1 and α5β1 and when one is blocked the other can compensate.

(6) MCP-1 enhances T cell migration across fibronectin (α4β1 and α5β1), but not ICAM-1 (LFA-1). This was the first suggestion of differential regulation of β1 and β2 integrins on these T cells
8.1.2. Differential regulation of LFA-1 and α4β1 by Mg²⁺/EGTA

(1) T cells can be induced to bind fibronectin with Mg²⁺/EGTA. This requires higher levels of Mg (~5 mM) than are needed for LFA-1 binding (~3 mM). Mg²⁺-stimulated adhesion to both ICAM-1 and fibronectin is inhibited by extracellular Ca²⁺.

(2) Mg²⁺/EGTA-stimulated adhesion of T cells to fibronectin, unlike that of Mg²⁺/EGTA-stimulated T cells to ICAM-1, requires [Ca²⁺], and an intact cytoskeleton. In addition Mg²⁺/EGTA is unable to stimulate binding of soluble fibronectin or soluble VCAM-1 to T cells, suggesting that the β1 integrins are not in a higher affinity state following Mg stimulation. Furthermore, although Mg²⁺-stimulated T cells express the β2 activation reporter epitope 24 they do not express the β1 activation reporter epitopes HUTS-21 or 15/7. These results suggest that there is a qualitative difference, as well as a quantitative difference, between the β1 and β2 integrins on T cells following stimulation with Mg²⁺/EGTA.

8.1.3 Cross talk between LFA-1 and α4β1 on T cells.

(1) Occupation of T cell LFA-1 by its ligand ICAM-1 decreases the binding of α4β1 to ligands fibronectin and VCAM-1, so called “Cross talk”. The adhesive activity of α5β1 is affected to a lesser extent. Such cross talk is also seen with the LFA-1 activating mAbs KIM185 and KIM127 and the LFA-1 activation reporter mAb 24.

(2) Cross talk inhibits the adhesion of α4β1 induced by each of the following stimuli: PdBu, CD3XL and Mg²⁺/EGTA. Cross-talk is not seen following β1 integrin stimulation with Mn²⁺ or with the β1 activating mAb TS2/16.

(3) Cross talk appears unidirectional and there is no effect of activated or occupied β1 integrins on the activity of LFA-1 on these T cells.

(4) The effect of cross talk is to decrease T cell adhesion to the α4β1 ligand VCAM-1 and to enhance α5β1-mediated migration across fibronectin, perhaps by releasing α5β1 from inhibition by active α4β1.
8.1.4 Mechanism of cross-talk

(1) Cross talk results neither from a decrease in number or affinity of the β1 integrins, nor from modulation of their position on the cell surface, but appears to target a post-ligand binding event upstream of the actin cytoskeleton.

(2) Cross talk appears to result from an LFA-1-transduced signalling which does not involve PKA or PKC, but can be mimicked by the inhibition of CaMKII.

8.1.5 The role of actin myosin interactions in integrin activation.

(1) Various inhibitors of actin-myosin contractility for example: inhibitors of MLCK (wortmannin, KT5926, ML-7, K252a); the myosin disrupting agent, BDM; and cytochalasin D all inhibit α4β1- and LFA-1-mediated T cell adhesion following CD3XL. There is a less dramatic inhibition following treatment with the PKC inhibitor, Ro-31-8220. This suggests that actin-myosin interactions and contractility are important in the signalling pathway from the T cell receptor to integrin activation.

(2) There was no effect of the MAPKinase inhibitor PD098059 on this adhesion suggesting that MAPK does not lie upstream of the actin-myosin interactions.

(3) Integrin-mediated adhesion following CD3XL can also be inhibited with lovastatin, an inhibitor of isoprenylation, and this inhibition is reversed by treatment with mevalonate which bypasses the blockade of isoprenyl group synthesis. This suggests that a small GTP-binding protein might be involved in the signalling pathway that leads from CD3XL to integrin activation.
8.2 Future Directions

(1) To further define the receptors, integrin and non-integrin, that are involved in the adhesion of T cells to the respiratory epithelium. To define the mechanism by which respiratory epithelial cells are involved in activation of T cell integrins.

(2) To define the signalling pathways involved in the inhibition of α4β1 by active LFA-1. Recent observations suggest an involvement of CaMKII and it is hoped to take these observations further. Having defined the signalling pathways it would be of interest to look at the effects on T cell transendothelial migration when cross talk is blocked. The model proposed here might predict that blocking cross talk would inhibit such migration.

(3) To establish the role of actin-myosin based contractility in integrin activation following T cell receptor activation. In particular to look at the effects of inhibitors of actin-myosin contractility on integrin clustering. It would also be of interest to look at the role of the Rho-subfamily of small GTPases in the signalling that leads from CD3XL to the actin cytoskeleton.


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subunits contain a metal ion-dependent adhesion site-like motif but lack an I doman. J. Biol. Chem 272, 14236-14243.


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APPENDIX I- BUFFERS AND MEDIA

A.1.1 PBS-A

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A.1.5 50 x nucleosides (Sigma)

35 mg of each of adenosine, guanosine, cytidine, uridine; 12 mg thymidine
Added to 100 ml distilled water, filter sterilised and store at 4°C
PUBLICATIONS ARISING FROM THIS WORK

Porter JC, Hogg N.
Integrin Cross-Talk: Activation of LFA-1 on human T cells alters α4β1- and α5β1-mediated function.

Alexandroff AB, McIntyre CA, Porter JC, Zeuthen J, Vile RG, Taub DD
Sticky and smelly issues: lessons on tumour cell and leucocyte trafficking, gene and immunotherapy of cancer

Porter JC, Hogg N.
Integrins take partners: Cross-talk between integrins and other membrane receptors

Porter JC, Hogg N.
Myosin light chain kinase (MLCK) is involved in the regulation of LFA-1 and β1 integrin activity following TCR/CD3 crosslinking.
Manuscript in preparation

Porter JC, Hogg N.
The divalent cation Mg^{2+} differentially affects T cell adhesion mediated by the integrins LFA-1 & α4β1
Manuscript in preparation

Thiel M* and Porter JC*, Newton R and Hogg N.
Chemokines and the activation of β2 integrins on T cells and monocytes.
Manuscript in preparation.
Integrin Cross Talk: Activation of Lymphocyte Function-associated Antigen-1 on Human T Cells Alters α4β1- and α5β1-mediated Function

Joanna C. Porter and Nancy Hogg
Leukocyte Adhesion Laboratory, Imperial Cancer Research Fund, Lincoln's Inn Fields, London WC2A 3PX, United Kingdom

Abstract. A regulated order of adhesion events directs leukocytes from the vascular compartment into injured tissues in response to inflammatory stimuli. We show that on human T cells, the interaction of the β2 integrin leucocyte function-associated antigen-1 (LFA-1) with its ligand intercellular adhesion molecule-1 (ICAM-1) will decrease adhesion mediated by α4β1 and, to a lesser extent, α5β1. Similar inhibition is also seen when T cells are exposed to mAb 24, which stabilizes LFA-1 in an active state after triggering integrin function through divalent cation Mg²⁺, PdBu, or T cell receptor/CD3 complex (TCR/CD3) cross-linking. Such cross talk decreases α4β1 integrin-mediated binding of T cells to fibronectin and vascular cell adhesion molecule-1 (VCAM-1). In contrast, ligand occupancy or prolonged activation of β1 integrin has no effect on LFA-1 adhesion to ICAM-1. We also show that T cell migration across fibronectin, unlike adhesion, is mediated solely by α5β1, and is increased when the α4β1-mediated component of fibronectin adhesion is decreased either by cross talk or the use of α4-blocking mAb. The ability of mAb 24 Fab’ fragments to induce cross talk without cross-linking LFA-1 suggests signal transduction through the active integrin. These data provide the first direct evidence for cross talk between LFA-1 and β1 integrins on T cells. Together, these findings imply that activation of LFA-1 on the extravasating T cell will decrease the binding to VCAM-1 while enhancing the subsequent migration on fibronectin. This sequence of events provides a further level of complexity to the coordination of T cell integrins, whose sequential but overlapping roles are essential for transmigration.

The regulation of lymphocyte extravasation from the circulation into sites of inflammation is critical in coordinating an appropriate and effective immune response. There is careful control not only of the particular tissues into which migration occurs, but also of the various subtypes of leukocytes involved (Butcher and Picker, 1996). Under flow conditions, circulating lymphocytes can attach and roll on vascular endothelium using the selectins and α4 integrins (Alon et al., 1995; Berlin et al., 1995; Luscinskas et al., 1995). These adhesion receptors are able to slow the transit of lymphocytes and expose them to stimuli causing activation-dependent firm adhesion. The integrins αβ1, αβ7, and leucocyte function-associated antigen-1 (LFA-1) have been implicated in activation-dependent stable arrest of lymphocytes under flow (Bargatze et al., 1995). LFA-1, however, unlike the α4 integrins, cannot initiate adhesion under these conditions without L-selectin and/or α4β7 first tethering the lymphocyte to the vessel wall (Bargatze et al., 1995). Subsequently, LFA-1 is the principal integrin involved in transendothelial migration (van Epps et al., 1989; Smith et al., 1989; Oppenheimer-Marks et al., 1991), although the stimulus that induces this β2 integrin–dependent movement across the endothelial layer is unclear. Interaction with and migration across fibronectin and other extracellular matrix components are then necessary for the successful completion of migration into the tissues. Therefore, transmigration of any one T cell is a multistep process dependent on the tight regulation of the sequential and often overlapping activities of the expressed integrins (Bargatze et al., 1995).

There is increasing evidence that, on a given cell, one subset of integrins may be negatively regulated by ligation of another. Transfection of αβ3 into K562 cells that endogenously express α5β1 provides a system in which ligation of β3 inhibits the phagocytic but not adhesive function of α5β1 (Blystone et al., 1994). Similarly, ligation of transfected αβ1β3 will inhibit the function of cotransfected αβ1 or endogenous α5β1 in CHO cells expressing these integrins (Díaz-González et al, 1996). Such effects are dependent on an intact β3 cytoplasmic tail, and are considered to involve signal transduction (Blystone et al., 1994).
Materials and Methods

Preparation of T Lymphoblasts

Peripheral blood mononuclear cells were prepared from single donor leukocyte buffy coats by centrifugation through Lymphoprep® (Pharmacia Diagnostics AB, Uppsala, Sweden). T cells were expanded from this population by culturing in RPMI 1640 plus 10% FCS (GIBCO BRL, Paisley, UK) in the presence of phytohaemagglutinin (Murex Diagnostics, Dartford, UK) at 1 μg/ml for 72 h as previously described (Dransfield et al., 1992a). Cells were then washed and maintained for 1–2 wk in medium supplemented with 20 ng/ml recombinant IL-2 (Euro Cetus UK Ltd., Harefield, UK). The cells, which were used between days 10 and 14, were a 99% CD3+ population, containing 65% CD8+ and 35% CD4+ cells. The population was negative for the natural killer cell marker CD56.

mAbs and Other Reagents

mAb 925.2 (CD11a; LFA-1a subunit, nonblock) was purchased from Becton Dickinson (Oxford, UK). mAbs 38 (CD11a; LFA-1a subunit-blocking), and 24 (CD11/CD18; β2 integrin activation reporter) (Dransfield et al., 1992b; Dransfield and Hogg, 1989) and 52U (control antibody) were prepared in this laboratory, and purified from ascites or tissue culture supernatant by protein A-Sepharose chromatography (Ey et al., 1978). mAbs HIP/2 (CD49d; α4 subunit-function-blocking) and TS2/16 (CD29; β1 subunit-activating) were gifts from R. Lobb (Biogen, Inc., Cambridge, MA). mAb SAM-1 (CD49e; α4 subunit-blocking) was purchased from Sigma Chemical Co. (Poole, UK).

ICAM-1Fc was produced as a chimeric protein, consisting of the five extracellular domains of ICAM-1 fused to the Fc fragment of human IgG1 (Berendt et al., 1992). ICAM-1Fc, produced as a chimera consisting of the two amino-terminal domains of human VCAM-1 fused to the Fc fragment of human IgG1 (Jakubowski et al., 1995), was a gift from R. Lobb. Fibronectin (0.1% solution from human plasma) was purchased from Eurogenetics (Hampton, UK). mAb UCHT2 (CD49d; α4 subunit-function-blocking) and TS2/16 (CD29; β1 subunit-activating) were gifts from R. Lobb (Biogen, Inc., Cambridge, MA). mAb SAM-1 (CD49e; α4 subunit-blocking) was a gift from P. Beverley (University College, London, UK). mAb G19.4 was a gift from Bristol My- labe. Cells (2 x 106 cells) were treated with 3 mM Mg2+/2 mM EGTA, 50 nM phorbol-12,13-dibutyrate (PdBu), or CD3 mAb at indicated levels, as well as inhibitors and mAbs in 100 μl assay buffer. mAbs included at 0.4 mM for experiments involving PdBu or T cell receptor/CD3 complex (TCR/CD3) cross-linking with mAb G19.4. Blocking mAbs were titrated on T cells by FACS® analysis (Becton Dickinson, Mountain View, CA) and used at saturating concentrations to block T cell function. For fibronectin- and VCAM-1-binding assays, all wells contained anti-LFA-1 mAb 38 at function-blocking concentrations of 10 μg/ml. This prevents cells aggregating via LFA-1/ICAM-1 interactions, which would cause spurious high binding to β1 ligands through the piggy-back interaction of nonadherent cells with truly adherent cells. Plates were incubated for 15 min on ice, followed by centrifugation at 40 g for 1 min, before 40-min incubation at 37°C. Nonadherent cells were removed by washing four times in warmed assay buffer (150 μl/well). Adhesion was quantified by recording emission at 530 nm, after excitation at 485 nm, using a Fluoroskan® II (Labsystems, Inc., Basingstoke, UK), and by expressing the reading for each well as a percentage of the total emission before incubation.

Transmigration Assays

Assays were performed in 20 μl Heps, 140 mM NaCl, 2 mg/ml glucose, pH 7.4, 0.25% BSA, 3 mM Mg2+/2 mM EGTA (transmigration buffer) using 6.5-mm-diam Transwell® plates (Costar Corp., Cambridge, MA). The upper and lower surfaces of the inserts were coated with fibronectin at concentrations ranging from 0 to 50 μg/ml in PBS overnight at 4°C. The inserts were positioned in wells containing 600 μl transmigration buffer. Cells were then placed in the insert at a concentration of 5 x 105 cells in 100 μl transmigration buffer with appropriate mAbs. The mAbs were used at the following final concentrations: mAb HP1/2 at 0.7 μg/ml, mAb 7.2 at 5 μg/ml, mAb SAM-1 at 5 μg/ml, mAb 24 at 5 μg/ml, and mAb 52U at 5 μg/ml. The anti-LFA-1 mAb 38 was added to all inserts at function-blocking concentrations of 10 μg/ml to prevent a spurious decrease in migration due to cell aggregation when LFA-1 is activated. The plates were then incubated for 6 h at 37°C. The bottom surface of the insert was then
scraped to release migrated but adherent cells into the bottom well, and the migrated cells were counted in a hemocytometer. Nine grids (0.1 mm$^2$ per grid) were counted per well, and readings were averaged from duplicate samples. All assays were performed in duplicate, and each experiment was repeated a minimum of four times.

**Results**

In this study, we have investigated cross influences on function between the $\beta_1$ integrins and LFA-1 on T cells. As one method of activating these leukocyte integrins, we treated T cells with 3 mM Mg$^{2+}$/2 mM EGTA (Dransfield et al., 1992a). For the $\beta_2$ integrin, LFA-1, the advantage of such treatment is that it directly alters the integrin ectodomain, bypassing the requirement for an intracellular stimulus (Stewart et al., 1996). This form of LFA-1 is considered to be of high affinity because it is able to bind soluble ICAM-1 (Stewart et al., 1996). There have been both positive and negative reports of the ability of Mg$^{2+}$/EGTA to induce fibronectin receptor-mediated adhesion (Shimizu and Mobley, 1993; Luque et al., 1996). In this study, we show that T cells do bind fibronectin, immobilized either on plates or on beads, in an Mg$^{2+}$-dependent manner. To examine further the generality of cross-influences between these integrins, we also investigated T cells stimulated with phorbol ester or by TCR/CD3 cross-linking. Both of these stimuli act from within the cell to activate integrins, so called inside-out signaling, and may be considered more representative of the in vivo situation.

**Adhesion of T Cells to ICAM-1 Will Decrease Binding of Fibronectin-coated Beads**

To determine the effect of LFA-1 ligation on the function of the $\beta_1$ integrins on the same T cell, we developed a ligand-coated bead-binding system. T cells were adhered to immobilized ICAM-1 via LFA-1, or to a control substrate, and their ability to bind beads coated with ligand for the $\beta_1$ integrins, $\alpha_4\beta_1$ and $\alpha_5\beta_1$, was then investigated. Fibronectin-coated beads were bound by T cells adherent to the control substrate, anti-CD5 mAb, immobilized on plastic (Fig. 1A), and the specificity of adhesion was demonstrated by blocking bead-binding with a combination of $\alpha_4$ and $\alpha_5$ function-blocking mAbs (Fig. 1B). However, when T cells were adherent to ICAM-1 as substrate, they bound fewer fibronectin-coated beads (Fig. 1C). When binding of the fibronectin-coated beads was quantified, there was a decreased level of fibronectin bead-binding when T cells were adherent to ICAM-1 (Fig. 2A) (inhibition: 65.0 $\pm$ 23.4% = mean $\pm$ SD; $n = 6$). This result demonstrated that, on human T cells, the interaction of LFA-1 with its ligand ICAM-1 could downregulate the function of the $\beta_1$ integrins. In contrast, T cells adhered to anti-LFA-1 mAb bound beads at a similar level as T cells adherent to control mAb. This indicated that the LFA-1 inhibitory effect could not be mimicked by cross-linking LFA-1 with immobilized CD11a mAb 38 (Fig. 2A). Conversely, there was no difference between the ability of T cells adherent to anti-CD5 mAb, fibronectin, or ICAM-1 to bind ICAM-1-coated beads (Fig. 2B), indicating that adhesion to immobilized fibronectin did not alter the extent of ICAM-1 bead binding by LFA-1. This is the first evidence that LFA-1 could dominate the activity of the fibronectin-binding receptors and that the reverse situation did not hold.

**Adhesion of T Cells to ICAM-1 Decreases Binding of Fibronectin- and VCAM-1-coated Beads by Downregulating $\alpha_4\beta_1$ Activity**

We then looked at the effects of LFA-1 ligand-binding on each of the T cell fibronectin receptors, $\alpha_4\beta_1$ and $\alpha_5\beta_1$. Both of these integrins can be involved in T cell binding to fibronectin, and, if one is blocked, the other can partially
The inhibitory effect of LFA-1 on β1-mediated ligand-binding could not be demonstrated by cross-linking LFA-1 with an anti-LFA-1 mAb, but required binding to Igand ICAM-1. This suggested that high affinity LFA-1 rather than receptor cross-linking was necessary for cross talk. This led to the development of an assay in which T cells were first stimulated with Mg^{2+}/EGTA, TCR/CD3 cross-linking, or PdBu and then exposed to mAb 24, which holds LFA-1 in an active conformation as if occupied by Igand (Dransfield et al., 1992b). mAb 24 caused increased T cell binding to fibronectin after titration of Mg^{2+} (Fig. 5 A), CD3 mAb G19.4 (Fig. 5 B), and PdBu (not shown). In contrast, mAb 24 caused inhibition of T cell binding to fibronectin after TCR/CD3 cross-linking (Fig. 6 A) or Mg^{2+}/EGTA (data not shown). Monovalent Fab' fragments of mAb 24 produced the same degree of inhibition as bivalent mAb 24 (data not shown). This demonstrated that activation or ligand occupancy of LFA-1 in the absence of clustering is sufficient to alter fibronectin-mediated adhesion. mAb KIM185 (CD18; β2-activating) behaved similarly to mAb 24, depressing binding of T cells to fibronectin while enhancing adhesion to ICAM-1 (data not shown).

The Effect of LFA-1 Activation on T Cells Is Mediated Predominantly through α4β1

Because T cells bind to fibronectin through both α4β1 and α5β1, we analyzed the effects of LFA-1 activation individually on these integrins using function-blocking mAbs and either TCR/CD3 cross-linking (Fig. 6 B and C) or Mg^{2+}/EGTA (data not shown) to stimulate adhesion. Prolonged activation of LFA-1 with mAb 24 had only a small effect on total fibronectin-binding (Fig. 6 A) and on α5β1-mediated adhesion (Fig. 6 B), but had a much greater effect on α4β1-mediated adhesion (Fig. 6 C). Similar levels of α4β1 inhibition by mAb 24 were seen when the integrins were activated with PdBu or Mg^{2+}/EGTA (Fig. 7). In addition, there was no effect of the nonfunction-altering anti-LFA-1 mAb G25.5, which again emphasized the requirement for LFA-1 activation (Fig. 7). Under equivalent activating conditions, mAb 24 and the β2 integrin–activating mAb KIM185 decreased α4β1-mediated adhesion to VCAM-1 to the same extent as to fibronectin (data not shown). Together, these results reinforced the findings that α4β1 function is particularly sensitive to the state of LFA-1 activation.

Activation of β1 Integrins on T Cells Has No Effect on LFA-1 Binding to ICAM-1

We then reversed the situation to investigate the effect on

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**Figure 2.** Inhibition of fibronectin bead–binding when T cells adhere to ICAM-1. (A) Cultured human T cells treated with 3 mM Mg^{2+}/2 mM EGTA and adhered to plastic coated with anti-CD5 (mAb UCHT2), anti-LFA-1 (mAb 38), or ICAM-1 and incubated with fibronectin-coated beads (dark bars). Bead attachment was blocked with a combination of α4 and α5 function-blocking mAbs HI1/2 and SAM-1 as previously (open bars). (B) Cultured human T cells treated with 3 mM Mg^{2+}/2 mM EGTA and adhered to plastic coated with anti-CD5 (mAb UCHT2), fibronectin, or ICAM-1 were incubated either with ICAM-1-coated beads (stippled bars) or with BSA-coated control beads (open bars). The bead-binding assays were performed as described in Materials and Methods, and data are expressed as binding index (beads bound/100 cells). Data represent the mean of six high-power fields ± SEM. One representative experiment of three is shown.
β2 integrin-mediated adhesion of maintaining β1 integrins in an active state, using the β1 integrin-stimulating mAb TS2/16. This mAb increased binding to fibronectin after the three activating treatments (Fig. 8 A), but had no effect on β2-mediated binding to ICAM-1 (Fig. 8 B), confirming that the β1 integrins were unable to influence the ligand-binding activity of LFA-1.

Inhibition of α4β1 with Blocking mAbs or by LFA-1 Activation Increases α5β1-Mediated Migration

The effects of LFA-1-mediated cross talk on α4β1- and α5β1-mediated T cell migration on fibronectin were then investigated. Using the Transwell® system, we established that T cells undergo random migration on fibronectin us-

Figure 3. Fibronectin bead-binding mediated by α4β1 is differentially inhibited when T cells are adherent to ICAM-1. Cultured human T cells treated with 3 mM MgCl₂/2 mM EGTA were adhered to plastic coated with anti-CD5 (mAb UCHT2) (A) or ICAM-1 (B) and incubated with fibronectin-coated beads. Bead attachment was assessed in the presence or absence of the α4 and α5 function-blocking mAb HPI/2 and SAM-1 either alone or in combination, as previously. The data are expressed as binding index (beads bound/100 cells), and are the mean of six high-power fields ± SEM. One representative experiment of three is shown.

Figure 4. Inhibition of VCAM-1 bead-binding when T cells adhere to ICAM-1. Cultured human T cells treated with 3 mM MgCl₂/2 mM EGTA were adhered to plastic coated with anti-CD5 (mAb UCHT2), anti-LFA-1 (mAb 38), or ICAM-1 and incubated with VCAM-1-coated beads (hatched bars). Bead attachment was prevented by the α4-blocking mAb HPI/2 (open bars). Data are expressed as binding index (beads bound/100 cells) and represent the mean of six high-power fields ± SEM. One representative experiment of three is shown.
Figure 5. Prolonged activation of LFA-1 by mAb 24 increases T cell adhesion to ICAM-1 induced either by Mg^{2+} and 2 mM EGTA (A) or by TCR/CD3 cross-linking through CD3 mAb G19.4 in the presence of Ca^{2+} and Mg^{2+} at 0.4 mM (B). The LFA-1 activation antibody, mAb 24 (○) or mAb 52U (IgG1 isotype control) (●) were used at 20 μg/ml. Specificity of adhesion was shown by block of ICAM-1 binding with mAb 38 (LFA-1–function blocking, 10 μg/ml) (▲). Data represent means of triplicates ± SD. One representative experiment of three is shown.

Figure 6. Prolonged activation of LFA-1 blocks α4β1- and to a lesser extent α5β1-mediated binding to fibronectin. Adhesion to fibronectin was induced by TCR/CD3 cross-linking through CD3 mAb G19.4 in the presence of Ca^{2+} and Mg^{2+} at 0.4 mM. (A) Adhesion mediated by α4β1 and α5β1 together. (B) α5β1-mediated adhesion after the α4β1 component had been blocked with mAb HP1/2 (0.5 μg/ml). (C) α4β1-mediated adhesion after the α5β1 component had been blocked with mAb SAM-1 (0.5 μg/ml). Adhesion was assessed in the presence of the LFA-1 activation antibody, mAb 24 (open symbols), or the isotype-matched control antibody, mAb 52U (closed symbols), used at 20 μg/ml. Data represent means of triplicates ± SD. One representative experiment of three is shown. The specificity of the adhesion is shown by the block achieved using α4- and α5-blocking mAbs as indicated.
Investigation of the Mechanism of LFA-1 Cross Talk

Treatment of T cell LFA-1 with Mg$^{2+}$/EGTA directly induces a high affinity form of the integrin that is able to bind soluble ICAM-1 (Stewart et al., 1996). Therefore, we looked at the ability of B1 integrins to adopt a high affinity state. However, treatment with Mg$^{2+}$/EGTA yielded no detectable binding of soluble fibronectin or VCAM-1, even when these ligands were used at concentrations up to 1.2 mg/ml. In contrast, 0.5 mM Mn$^{2+}$ was able to induce fibronectin ($\alpha$B1 and $\alpha$5B1) - and VCAM-1 ($\alpha$4B1) - binding to both T cells and Jurkat cells (data not shown), as has been reported by others (Jakubowski et al., 1995; Gomez et al., 1997). Similarly, while 0.5 mM Mn$^{2+}$ was able to induce expression of the $\beta$1 activation reporter epitopes recognized by mAb 15/7 (Yednock et al., 1995) or mAb HUTS-21 (Luque et al., 1996), no expression of these epitopes was observed with Mg$^{2+}$/EGTA treatment (data not shown). These findings imply that the fibronectin-binding integrins are in a low affinity state after all three methods of stimulation, and that LFA-1 cross talk causes inhibition of postreceptor occupancy events, rather than direct modulation of receptor affinity.

We next tested the possibility that LFA-1 activation might be influencing a cytoskeletal event. Although mAb 24 decreased the overall level of $\alpha$4B1-mediated adhesion to fibronectin, there was no change in the sensitivity of binding to cytochalasin D (Fig. 10). Therefore, LFA-1 cross talk affects an event in cell adhesion after receptor occupancy but before changes in the actin cytoskeleton, and is independent of both.

Discussion

This study was undertaken to examine the functional interaction on T cells between LFA-1 and the $\beta$1 integrin fibronectin receptors $\alpha$B1 and $\alpha$5B1. The main findings are that (a) the occupation of T cell LFA-1 by its ligand ICAM-1 decreases the binding of $\alpha$4B1 to ligands fibronectin and VCAM-1; (b) this inhibitory cross talk also results from the prolonged activation of LFA-1 induced by the activation reporter mAb 24 in combination with several T cell adhesion-inducing protocols; (c) the adhesive activity of $\alpha$5B1 is affected to a lesser extent; (d) while active LFA-1 downregulates the avidity of $\alpha$4B1, the reverse does not occur, as neither $\beta$1 integrin-activating mAb TS2/16 nor $\beta$1-mediated binding to fibronectin affected the avidity of LFA-1; and (e) downregulation of $\alpha$4B1 activity increases the efficiency of $\alpha$5B1-mediated migration on fibronectin. Therefore, we have demonstrated differential regulation of two integrin subclasses and a hierarchy of

![Figure 7. Prolonged activation of LFA-1 blocks $\alpha$4B1-mediated fibronectin-binding after various stimuli. Adhesion to fibronectin was induced by TCR/CD3 cross-linking using mAb G19.4 (2.5 $\mu$M) with 0.4 mM Ca$^{2+}$ and Mg$^{2+}$ (white bars), PdBu 50 nM with 0.4 mM Ca$^{2+}$ and Mg$^{2+}$ (dark bars), or 3 mM Mg$^{2+}$/2 mM EGTA (cross-hatched bars). Integrin $\alpha$5B1 was blocked with SAM-1 (0.5 $\mu$g/ml), allowing $\alpha$4B1 adhesion to be investigated in isolation. Adhesion was assessed in the presence of mAb G25.2 (an LFA-1 nonactivating and nonblocking mAb), mAb 24, or mAb 52L (the IgG1 isotype-matched control antibody), each used at 20 $\mu$g/ml. Data represent means of triplicates ± SD. One representative experiment of three is shown. The specificity of the adhesion is shown with an $\alpha$4-blocking mAb.](image-url)
Figure 8. β2 integrin activation decreases β1-mediated binding of T cells, but the reverse is not true. T cell adhesion to fibronectin (A) and ICAM-1 (B) was induced by TCR/CD3 cross-linking using mAb G19.4 (2.5 μg/ml) with 0.4 mM Ca2+ and Mg2+, PdBu 50 nM with 0.4 mM Ca2+ and Mg2+, or 3 mM Mg2+/2 mM EGTA, and the adhesion assay was performed in the presence or absence of the β1-activating mAb TS2/16 (10 μg/ml) or the isotype-matched control, mAb 52U (10 μg/ml). Specificity of adhesion was shown by blocking of fibronectin-binding with a combination of α4- and α5-blocking mAbs, and ICAM-1-binding with the LFA-1-blocking antibody, mAb 38. Adherent cells are expressed as percentage of total cells added, and data represent means of triplicates ± SD. One representative experiment of three is shown.

integran usage in which the β2 integrin LFA-1 will suppress the function of β1 integrins, particularly α4β1.

Previous studies have demonstrated the involvement of α4β1 in leukocyte adhesion to but not migration across endothelium, and of LFA-1 as the chief integrin in transendothelial migration (van Epps et al., 1989; Oppenheimer-Marks et al., 1991; Moser et al., 1992). Furthermore, in vitro experiments during flow have emphasized the requirement that an integrin hierarchy allow coordinated migration of lymphocytes across the endothelium into the tissues (Butcher and Picker, 1996). Our finding that active LFA-1 is able to decrease the ligand-binding activity of α4β1 has direct implications for the sequential activity of these integrins in such an adhesion cascade; LFA-1 may function optimally in the absence of α4β1 adhesion, allowing the T cell to deadhere from the apical surface of the endothelium and transmigrating. Our findings also argue against a redundancy among integrin–ligand pairs in leukocyte transmigration, and imply specific roles for each integrin.

In this study, we have demonstrated that, in contrast to adhesion, the migration of activated T cells on fibronectin is mediated by α5β1 with no contribution from α4β1. In addition, suppressing α4β1 activity on T cells either by mAb 24 or α4 function-blocking mAbs enhanced the level of α5β1 migration, particularly at low fibronectin levels. This may reflect the compensatory increase in α5β1 adhesion, with its migratory potential, when binding through
Figure 9. Migration of T cells on fibronectin is mediated by α5β1 and promoted by blocking α4β1 function. (A) Migration of T cells on fibronectin (10 μg/ml) is mediated by α5β1 and enhanced when α4β1-mediated adhesion to fibronectin is blocked by the function-blocking α4 antibody mAb HPI/2. The non-function-blocking α4 antibody mAb 7.2 had no effect. (B) Migration of T cells across membranes coated with fibronectin at various concentrations (0-50 μg/ml) is dependent on α5β1. (C) Addition of the α4-blocking mAb HPI/2 increases migration above control mAb 52U. This increased migration can be blocked with the α5-blocking mAb SAM-1 (5 μg/ml). (D) Addition of the LFA-1-activation mAb 24 increases migration above control mAb 52U. This increased migration can be blocked with the α5-blocking mAb SAM-1. The assay was performed as described in Materials and Methods, and data are expressed as the total number of migrated cells. Data represent the mean of two readings from each well. All assays were performed in duplicate, with bars indicating the range of readings. Experiments are representative of four similar experiments.

the nonmigratory α4β1 is blocked. Another possibility is that the enhanced migration by α5β1 is due to removal of a restraint imposed by α4β1. The importance of strength of adhesion in regulating cell migration is well documented (Huttenlocher et al., 1996; Palecek et al., 1997), suggesting that firm adhesion by both α4β1 and α5β1 may make conditions suboptimal for migration. Alternatively, α4β1 may be involved in a more specific inhibition of α5β1 function, as has been described in the control of metalloproteinase expression in fibroblasts (Huhtala et al., 1995). The promotion of migratory behavior by α5β1 through loss of α4β1-binding activity is in keeping with its more prominent role within the tissues after successful negotiation of T cells across the endothelium (Miyake et al., 1992). Therefore, a hierarchy of integrin activity may feature at this later stage of the adhesion cascade, with LFA-1 providing a link between α4β1 and α5β1.

The mechanism for LFA-1 downregulation of α4β1 was explored in several ways. We first established that there was no alteration in expression of either α4β1 or α5β1 during the experimental period (data not shown). Furthermore, confocal microscopy using mAbs specific for α4β1, α5β1, and the β1-activation reporter mAb 15/7 indicated that avid LFA-1 did not cause β1 integrin redistribution on the T cell membrane (data not shown). In addition, although stimulation of T cells with Mg²⁺/EGTA induces high affinity LFA-1 (Stewart et al., 1996), none of the three stimulating protocols induced high affinity α4β1 or α5β1. This implied that cross talk was not affecting high affinity β1 integrins. Together, these findings suggested
Figure 10. Inhibition of \( \alpha 4 \beta 1 \)-mediated adhesion by mAb 24 does not alter the sensitivity of adherent T cells to cytochalasin D. Integrin \( \alpha 5 \beta 1 \) was blocked with SAM-1, as previously, allowing \( \alpha 4 \beta 1 \) adhesion to be investigated in isolation. Adhesion was stimulated with 3 mM MgCl\( _2 \)/2 mM EGTA in the presence of the LFA-1–activation antibody, mAb 24 (○), or the isotype-matched control antibody, mAb 529 (●), as previously. Cytochalasin D was used at 0–10 \( \mu \)g/ml (0–20 \( \mu \)M). Specificity of the adhesion was shown by blocking with mAb HP1/2 (anti-\( \alpha 4 \) ) (■). Data represent means of triplicates ± SD. One representative experiment of four is shown.

that the \( \beta 1 \) integrins had not undergone a detectable alteration in affinity nor been redistributed or shed from the cell surface, and that LFA-1 cross talk was targeting events after ligand-binding. This result is in keeping with other studies in which cross talk is ultimately dependent on the presence of the \( \beta \) subunit cytoplasmic tail and steps subsequent to modulation of integrin affinity (Blystone et al., 1994; Díaz-González et al., 1996).

It seemed possible that the cytoskeleton was a target of LFA-1–mediated cross talk because both \( \alpha 4 \beta 1 \)- and \( \alpha 5 \beta 1 \)-mediated adhesion were more sensitive to changes in actin than was adhesion through LFA-1 (data not shown). However, for \( \beta 1 \) integrin–mediated adhesion, the similarity in cytochalasin D sensitivity of mAb 24–treated and untreated cells and the synergism between suboptimal doses of cytochalasin D and mAb 24 in the inhibition of \( \alpha 4 \beta 1 \)-mediated binding to fibronectin (data not shown) supported the evidence that inhibition occurs upstream of cytoskeletal changes. These results implied that cross talk affects an event in cell adhesion occurring after receptor occupancy but before actin-mediated cytoskeletal changes, and independent of both. In addition, protein kinase A, associated with LFA-1 signaling and deadhesion (Rovere et al., 1996), and protein kinase C, implicated in some previous cross talk studies (Blystone et al., 1994; Pacifici et al., 1994), were not involved in this phenomenon (data not shown).

LFA-1 cross talk was evident after several different adhesion-inducing protocols, showing that the phenomenon was not stimulus specific. The fact that cross talk was dependent on ICAM-1 or mAb 24 indicated that occupancy of LFA-1 was a prerequisite. Although the signaling pathways activated upon engagement of the \( \beta 2 \) integrins are not well understood, certain observations suggested that cross talk did activate specific intracellular signaling pathways. Cross talk was not observed using the Jurkat T cell line, which is known to have a defect in LFA-1 signaling (Mobley et al., 1994). In addition, cross talk was induced by mAb 24 Fab' fragments but not by immobilized anti-LFA-1 mAb, emphasizing the requirement for a mechanism beyond LFA-1 clustering. For \( \alpha 5 \beta 1 \) on human fibroblasts, although clustering by mAbs of integrin on beads induced phosphorylation and accumulation of p125 focal adhesion kinase and tensin, ligand occupancy recruited further cytoskeletal proteins to the signaling complex (Miyamoto et al., 1995a,b). One speculation is that the targets of LFA-1 cross talk may be the proteins providing the link between integrins and actin. However, several observations suggested that cross talk does not represent a simple sequestering of such intracellular proteins. First, integrin activation operates in one direction only, so prolonged activation of the \( \beta 1 \) integrins using mAb TS2/16 does not alter LFA-1 binding to ICAM-1. Second, LFA-1 predominantly affects the activity of \( \alpha 4 \beta 1 \), despite a sixfold abundance of \( \alpha 4 \beta 1 \) over \( \alpha 5 \beta 1 \) (data not shown). Future work will address the role of potential integrator molecules in the cross talk phenomenon.

In summary, we describe inhibition of \( \alpha 4 \beta 1 \)-binding activity in T cells as a consequence of LFA-1 activation. A speculation is that deadhesion of \( \alpha 4 \beta 1 \) from the apical surface of the endothelium is required for LFA-1–mediated migration across endothelium to proceed. Another observation is that T cell migration on fibronectin is mediated by \( \alpha 5 \beta 1 \), and that this migration is enhanced by interfering with \( \alpha 4 \beta 1 \) adhesion. LFA-1 might provide a link between \( \alpha 4 \beta 1 \) and \( \alpha 5 \beta 1 \) by uncoupling the former in order to enhance migration by the latter. While the actual mechanism by which cross talk is achieved is unclear, our findings implicate a downstream signaling event brought about by maintaining LFA-1 in a highly avid state.

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References


Integrins take partners

RNA localization mechanisms

Functions of scatter factor

Proteasome inhibitors

Cellular microbiology

Signal sequences
Integrins take partners: cross-talk between integrins and other membrane receptors

Joanna C. Porter and Nancy Hogg

This review discusses an emerging theme in the understanding of how an integrin contributes to the life of a cell. Previously, integrins have been thought to ‘go it alone’, but it is now appreciated that their duties extend beyond that of being ‘sticky’ receptors. By interacting in cis with other receptors on the cell membrane, integrins and their partner receptors interact to form distinct membrane complexes that recruit signalling molecules to each receptor’s mutual benefit. Here, Joanna Porter and Nancy Hogg discuss a few of the best characterized of these specialist integrin partnerships.

Over the past decade, there has been an increasing appreciation of the role of integrins in cell biology. Earlier efforts were directed at describing new members of the integrin family. The emphasis now is on characterization of the functions of individual integrins, with the focus on five main areas. Integrins are key effectors of cell migration. They play a role in co-stimulation of T lymphocytes and the highly specific interactions between cells responsible for an adaptive immune response. Integrins also cooperate with growth factors to promote cell proliferation, a mechanism explained, in part, by the anchorage dependence of the G1-to-S-phase transition in cell-cycle progression. In addition, adhesion is necessary for cells to exit from the cell cycle and to differentiate. Finally, when adherent tissue cells are released from their surrounding extracellular matrix (ECM), they forfeit survival signals and undergo apoptosis. Integrin-associated signalling might be involved in all these areas, and the reader is referred to a selection of recent reviews.

As well as interacting with molecules on neighbouring cells or ECM, integrins can also form cis associations with other receptors on the same cell to form multireceptor complexes. These complexes recruit signalling molecules to sites of cell–cell or cell–matrix adhesion, such as focal complexes or focal adhesions. As yet, there is little evidence that integrins can signal through exclusive, integrin-specific pathways. Instead, they appear to cooperate with other cell-surface receptors to influence a variety of signalling pathways. In one example, integrins are even able to utilize the platelet-derived growth factor (PDGF) receptor and its signalling pathway in the absence of PDGF7.

These complexes of integrins and partner receptors can be formed either in response to or independent of integrin activation and ligation. Either way, once assembled, they then allow ‘on the spot’ modulation of integrin function. There are several ways in which this might be achieved: the cis partner might alter integrin conformation directly, thereby altering the affinity of the integrin for ligand; clustering of other receptors in the complex might lead indirectly to clustering of associated integrins, with an increase in strength of integrin-mediated ligand binding and a resultant increase in transduced signal; or the complex might present a functional package to facilitate migration through tissues, such as occurs upon the association of an integrin with protease-containing complexes.

This review discusses examples of cis interactions in which an integrin is associated physically with another transmembrane protein and elaborates the possible biological consequences. The classes of receptors known to be associated both physically and functionally with integrins are shown in Table 1. Most of the associations involve extracellular domains. However, the way in which the membrane proteins caveolin and CD98 interact with integrins has not yet been determined fully and might involve the cytoplasmic domains of both the integrin and its partner.

Integrin-associated protein

Integrin-associated protein (IAP; CD47) is a 50-kDa single-chain protein composed of an extracellular immunoglobulin superfamily (IgSF) domain, five membrane-spanning sequences and a short cytoplasmic tail. IAP was first isolated by Eric Brown and colleagues as a protein associated with the two β3 integrins, the ubiquitous αvβ3 (Fig. 1a) and the platelet-restricted αIIbβ3 (Fig. 1b), as well as having a potential association with αvβ5. More recently, IAP has been shown to associate with α2β1 in human smooth muscle cells. Although one of four splice variants of IAP is expressed on all cell types, IAP appears to be particularly important in myeloid cell activation and migration across endothelial and epithelial monolayers. One model for this is that leukocyte PECAM-1 (CD31) engages IAP-αvβ3 on the endothelium and causes a Ca2+ influx. This influx of Ca2+ then leads to endothelial retraction and loosening of the tight junctions to allow leukocyte migration. Consistent with this model, there is evidence that, on binding of ligand, leukocyte IAP-αvβ3 transduces an intracellular signal to inhibit α4β1-mediated adhesion to the endothelial ligand VCAM-1 and thereby increases the speed of cell motility. The
<table>
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<td><strong>Integrin-associated protein</strong></td>
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<td>(αβ3)</td>
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<td><strong>TM4SF</strong></td>
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<td>At least 21 members</td>
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<td>including:</td>
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<td>CD9, CD37, CD53, CD63, CD81, CD82, CD151/PETA, NAG-2</td>
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<td>LFA-1 (αβ2)</td>
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<td>p150,95 (αxβ2)</td>
<td>FRET on cell surface</td>
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<td>Tripartite complex of caveolin, integrin and uPAR</td>
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<td>LFA-1 (αβ2)</td>
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<td><strong>Others</strong></td>
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<tr>
<td>EMMPRIN/BASIGIN/OX47/M6/CD147</td>
<td>αβ1, αβ3</td>
<td>Unknown, but do not contain TM4SF complexes</td>
<td>56</td>
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*Abbreviations: FRET, fluorescence resonance energy transfer; GPI, glycosylphosphatidylinositol; IAP, integrin-associated protein; IgSF, immunoglobulin superfamily; IR, insulin receptor; LPS/LBP, lipopolysaccharide and binding protein complex; NADG, N-acetyl-D-glucosamine; PDGFβR, platelet-derived growth factor β-receptor; TM, transmembrane; TM4SF, transmembrane-4 superfamily; uPAR, urokinase-type plasminogen activator receptor.

The production of IAP-null mice has further emphasized the central role of this protein in leukocyte migration and host defence. These mice rapidly succumb to peritoneal challenge with *Escherichia coli* owing to a failure of neutrophil recruitment and activation.

Human cells that lack IAP are deficient in αβ3-mediated ligand binding, and transfection of such cells with deletion mutants and chimeric constructs of IAP has shown that the extracellular IgSF domain of IAP is sufficient for its association with integrins and reconstitution of αβ integrin function. Co-immunoprecipitation experiments show that, at any one time, only a small proportion of total IAP and β3 integrins are associated together. IAP has its own role as a T-cell co-mitogen, acting by an adhesion-dependent CD28-independent pathway, apparently independent of an IAP-integrin association. Indeed, additional integrin-independent function is a recurring theme of all the receptors found associated with integrins that are discussed in this review.

IAP binds to the cell-binding domain (CBD) of thrombospondin (TS-1) and the 4NIK peptide (KRFYVVKK) from that domain, increasing αβ3-mediated spreading but not adhesion of human melanoma cells, and increasing α2β1-mediated chemotaxis of smooth muscle cells. This effect is sensitive to pertussis toxin, acting upstream of protein kinase C (PKC) and phosphoinositide 3-kinase (PI 3-K). In addition, a detergent-stable complex of αlβββ3, IAP, c-Src and focal-adhesion kinase (FAK) has been isolated, with increased phosphorylation of FAK on platelet stimulation. This suggests that IAP does not modulate the activity of the β1 and β3 integrins by direct physical interaction but actually forms a functional membrane unit that signals through heterotrimeric G-proteins.
The tetraspans (TM4SF proteins)

The transmembrane-4 superfamily (TM4SF) includes at least 21 different proteins, which have 20-30% sequence homology. The members of the TM4SF, so-called tetraspans, are presumed to have four membrane-spanning domains, resulting in two extracellular loops and intracellular N- and C-termini. Similar to IAP, they are expressed on all cells tested to date, usually with more than one tetraspan per cell type. Most of the tetraspan-integrin associations have been demonstrated by reciprocal co-immunoprecipitation studies, which have shown that CD9, CD53, CD63, CD81, CD82, CD151, PETA-3 and NAG-2 can associate physically in separate complexes with certain integrins. These tetraspan-integrin complexes vary depending on cell type, and one integrin can associate with more than one tetraspan (Fig. 2). The integrins that have been identified in complexes with tetraspans are α3β1, α4β1, α6β1, α4β7 and α1β3, but so far no association of tetraspans with α2β1, α5β1, α6β4, αv or the β2 integrins has been shown. Only a small fraction of the total pool of either integrins or tetraspans (5-10%) is committed to the tetraspan-integrin complex. Tetraspans can also associate with themselves and with other cell-surface proteins, including CD4, CD8, CD19, CD21 and major histocompatibility complex (MHC) class II. No clear pattern has yet emerged as to the composition or stoichiometry of these surface-molecule clusters, although the complexes might contain several types of tetraspans. The ability to associate with each other and with other membrane proteins could be a general feature of all tetraspans. Unlike the more promiscuous tetraspans, CD151 associates with α3β1 in a stable complex, uniquely resistant to detergent disruption (M. Hemler, pers. commun.). Whether the associations maintained during mild detergent extraction reflect the situation on the intact cell membrane is not known. However, it is reassuring that some tetraspan-integrin associations seen in mild detergent lysis have been verified by immunofluorescence microscopy of cell footprints and cell-surface clusters.

The association of integrins with tetraspans is not dependent on the integrin α subunit cytoplasmic tail, integrin activation or divalent cations. However, both the α and β integrin subunits confer specificity on this interaction, and adhesion-disrupting mutations of the extracellular, putative divalent-cation-binding region of the α4 subunit (D346E and D408E) can prevent the association of α4β1 with CD81. As these mutations are not part of the recognized α4β1 ligand-binding site, they might represent the region of integrin that makes physical contact with CD81 and potentially other tetraspans. This finding implies either that the association of integrin with CD81 is necessary for adhesion or, alternatively, that the mutations that disrupt adhesion have an indirect effect on the...
integrin–tetraspan association, for example by affecting integrin clustering.

Although the precise function of the tetraspan family is not known, its members have been implicated in the control of cell motility, metastasis and growth. As integrins are also involved in these functions, their associations with tetraspans might be highly relevant. There have been suggestions that pairing of integrins with tetraspans affects cell adhesion, and β1-integrin-mediated cell binding can be increased with the use of monoclonal antibodies (mAbs) against tetraspans—although others have found no consistent effect with the same antibodies.

These variable effects that mAbs against tetraspans have on cell adhesion suggest that the tetraspan–integrin association might be involved in the control of more complex integrin-mediated events such as cell motility and metastatic spread. Recently, there has been a direct investigation into these mechanisms by Hemler, Berditchevski and colleagues. Using confocal microscopy, they observed tetraspan–integrin complexes in unique membrane islands associated with lamellipodia at the leading edge of migrating cells where recycling of integrin occurs. In cells that have reduced levels of tetraspans compared with controls, the rate of integrin recycling is substantially decreased. Certain tetraspans, including CD63, can be found intracellularly in trafficking vesicles, and both CD63 and CD81, along with α3β1, are linked physically and functionally to phosphatidylinositol 4-kinase, an enzyme associated with transport of these vesicles. The model proposed is that at least some tetraspans could have a role in lamellipodial extension and cell movement by controlling integrin recycling at the leading edge of the cell.

Together with this potentially important role in integrin recycling, the tetraspans might also be involved in directing integrin localization on the cell surface. For example, CD151, CD9 and CD81 are expressed by confluent endothelium exclusively at adherens junctions where they are physically linked to α3β1 but not to other integrins or molecules such as CD31, VE-cadherin and catenins. Although signals from the matrix are strongly implicated in the prevention of endothelial cell anoikis, those from cell–cell contacts might be similarly influential, and it is possible that the tetraspans, by directing integrins to the adherens junctions, have the dual function of preventing anoikis and preserving endothelial monolayer integrity.

The tetraspans are found on cellular microvilli, rather than on the planar cell surface, and, by physically interacting with the integrins, they could direct them onto the microvilli, thereby allowing greater accessibility for adhesive interactions. This might also explain the importance of the integrin extracellular domain in integrin localization as this domain would determine the ability of the integrin to interact with, and be relocated by, partner molecules. Interestingly, IAP is also found at high levels on microvilli and might play a similar role in directing integrins. Finally, two tetraspans, CD9 and CD82, have been identified, like IAP, as T-cell costimulatory molecules, suggesting that cellular localization might also have importance for their functioning in immune phenomena.

Growth-factor receptors (GFRs)

Adhesive interactions are essential to both cell-cycle progression and proliferative responses initiated by growth factor receptors (GFRs). This functional synergism and the localization of several GFRs within focal-adhesion complexes first suggested a possible association of GFRs with integrins. It is not known whether all GFRs are in direct physical interaction with integrins, but there are three clear examples where such an arrangement affects integrin function. In mouse, rat and human fibroblasts and rat microvascular endothelial cells, a 190-kDa protein associates with αβ3 but not αβ1, in response to PDGF, and is independent of integrin engagement of ligand. The 190-kDa protein is tyrosine phosphorylated in response to PDGF, and this is independent of integrin engagement of ligand. The 190-kDa protein is tyrosine phosphorylated in response to PDGF, and this can be further enhanced when αβ3 interacts with vitronectin. The identity of this 190-kDa protein is unclear, with evidence for it being the receptor for PDGF. This synergism between integrins and growth factors has a positive effect on αβ3-mediated cell migration, which is Arg-Gly-Asp (RGD)-dependent and can be blocked with the tyrosine kinase inhibitor herbimycin A. The effects of this receptor synergism on cell proliferation have, however, been variable, with reports of both an increase and no effect. In another study, clustering of αβ integrins or engagement with ligand caused direct phosphorylation of the receptor for PDGF in the absence of PDGF.

Insulin stimulation causes αβ3 to coprecipitate with ~5–8% of total insulin receptor (IR) β subunits together with a small amount of highly phosphorylated insulin receptor substrate 1 (IRS-1), a cytoplasmic signal-transduction mediator of.
integrin-IR- (3-subunit-IRS-l complex. Such molecules, which can be identified in the same insulin. After insulin has bound to its receptor, IRS-1 by cell adhesion. One speculation is that the association of GFR with integrins could cluster the GFRs to allow more efficient signalling or to inhibit early dephosphorylation of the GFRs that have innate tyrosine kinase activity.

CD98: the affinity modulator

Another intriguing protein, CD98, appears to regulate the affinity of (3 integrins directly. CD98 is an (a-8) heterodimer expressed early in T-cell activation. Like caveolin (discussed below), the CD98 (a subunit is a type II membrane protein with the N-terminus inside the cell. The finding that mAbs against CD98 induce integrin (a381-dependent cell fusion by several viruses, and also homotypic aggregation, suggested a role in integrin function. More recently, the CD98 (a subunit has been found to convert (3 integrins into a high-affinity state, shown by its ability to overcome the suppression of (3 integrin function caused by overexpression of (3 cytoplasmic tails. Overexpression of other integrin partners such as IAP or the tetraspan protein CD9 did not bring about a similar increase in affinity. It is speculated that this rescue from suppression depends on an intracellular interaction between the cytoplasmic sequences of integrin and the (a subunit of CD98. This might be the first example of affinity regulation of an integrin by a membrane partner, and further molecular details are eagerly awaited.

Caveolin

Caveolae are plasma membrane invaginations involved in transcytosis and are present in most cells, except fresh monocytes and neutrophils and certain endothelia. They are specialized microdomains that have many attributed functions, including concentration of glycosylphosphatidylinositol (GPI) -linked receptors and signalling proteins. These receptors and proteins are recruited to the caveolae upon clustering or activation. The main structural component of caveolae is caveolin, a 22-kDa protein. Caveolin has a putative 30-40 amino acid membrane-spanning sequence, which may pass the membrane once or twice. A 20 amino acid residue N-terminal cytosolic domain, the ‘caveolin scaffolding domain’, associates with an assortment of signalling molecules, such as the Ga subunits of heterotrimeric G proteins, Ha-Ras and Src-family tyrosine kinases. Caveolin interacts preferentially with the inactive forms of these molecules, and a peptide from the caveolin scaffolding domain converts kinases from active to inactive states, suggesting that caveolin acts as a general inhibitor of associated kinases. This had led to the idea that caveolin plays a central role in the orchestration of signalling events in caveolae.

Caveolin and caveolae might also play a role in ECM-mediated promotion of cell proliferation or terminal differentiation, depending on the cell type and matrix composition. The signalling pathways involved in these processes are incompletely understood but are likely to be mediated by integrins. A major advance in this area came with the finding that mAb-mediated ligation of certain (3 integrins, as well as (a383, caused recruitment of the adaptor protein Shc, leading to activation of the mitogen-activated protein kinase (MAPK) pathway. This led to the compelling idea that integrin-dependent cell survival results in one of two outcomes depending on the ability of the engaged integrins to activate Shc. Adhesion mediated by integrins that activate Shc promotes cell-cycle entry and cell proliferation, whereas adhesion mediated by integrins that do not activate Shc results in cell-cycle exit and differentiation. The ability to recruit Shc is specified by the same membrane-proximal extracellular and transmembrane domains of the integrin (a subunit that interact with caveolin, implicating caveolin, and potentially caveolae, in this integrin-Shc interaction. One possibility is that caveolin recruits a local pool of inactive Ha-Ras. This Ha-Ras pool would then be available for activation by those integrins recruited to the same caveolae.

Another fascinating functional interaction between integrins and caveolin and a third partner, urokinase-type plasminogen activator receptor (uPAR/urokinase receptor/CD87; see below) has been described recently. In a model system of uPAR-transfected human kidney cells, (3 integrins have been found in association with caveolin and uPAR. Interestingly, although only a small pool of the total (3 integrins is involved, the formation of these complexes switches the ligand specificity of cell binding from integrin-mediated fibronectin binding to uPAR-mediated vitronectin binding. This again emphasizes the significance of even a small number of integrin-partner complexes.

Mac-1 and its many partners

A slightly different example of cis interactions is the association of integrins, such as the leukocyte-specific (8 integrin Mac-1 (CD11b/CD18), with GPI-linked membrane glycoproteins. GPI-linked proteins lack intracellular domains and must signal through membrane-spanning partners. Examples of GPI-linked receptors that function this way are uPAR (see below); FcγRIIB (CD16), which binds to Mac-1 to promote antibody-dependent phagocytosis; and CD14, which binds to the bacterial lipopolysaccharide and binding protein complex (LPS/LBP) in association with Mac-1, causing the generation of proinflammatory mediators. Fluorescence resonance energy transfer (FRET) microscopy suggests that, during neutrophil chemotaxis, uPAR dissociates from Mac-1 and polarizes to the leading edge of the migrating cell, where it associates with another (82 integrin, p150,95 (CD11c/CD18; Fig. 3). The advantage of this...
shuttling effect for myeloid cell migration is presently unknown. Interestingly, this association of uPAR with β2 integrins, unlike the association with β1 integrins, does not involve caveolin (see above), which is not expressed in these cells.

It appears that GPI-linked receptors form cis interactions with Mac-1 by binding to a lectin site on the integrin α subunit. This idea has come from the observations of Gordon Ross and colleagues that mannose, glucose and polysaccharides rich in N-acetyl-

N-linked carbohydrates (NADG), such as β-glucan or its derivative SZP, interact with a binding site for N-linked carbohydrates on Mac-1. This site is C-terminal to the I domain on the α subunit. When polysaccharides bind to the lectin site, the neutrophil is primed for catalytic activity, respiratory burst and other functions. Both FcγRIII and uPAR activities are blocked by small sugars, such as NADG, suggesting that these GPI-linked receptors also bind to the carbohydrate-recognition site on Mac-1. In addition, the mAb VIM12 uncouples FcγRIII from Mac-1 and might recognize an epitope within this lectin site. The interaction at the lectin site also causes tyrosine phosphorylation of several cytoplasmic proteins and increased expression of the CBRI/M activation epitope. This CBRI/M epitope is located within the Mac-1 I domain, and the induction of this epitope by polysaccharides suggests that an integrin-associated molecule might actually alter the conformation of an integrin.

Cells on the move: the uPAR connection

We have already introduced uPAR in the context of its association with β1 integrins in caveolae and its association with the lectin site of Mac-1 in myeloid cells (see above). However, uPAR has been best known as the cell-surface receptor for the serine protease urokinase type plasminogen activator (uPA). Upon binding to uPAR, uPA is activated to convert plasminogen into plasmin. Plasmin is then able to degrade fibrin and other extracellular membrane proteins and acts as the major pericellular fibrinolytic pathway. It is becoming increasingly apparent, however, that, together with plasminogen activator inhibitor 1 (PAI-1), uPAR and uPA form a cell-membrane-based package that not only regulates pericellular proteolysis but might also affect integrin-mediated function. The suggestion that αvβ3 provides a membrane site for activation of other proteases, such as matrix metalloprotease 2 (MMP-2), offers another example of reciprocal regulation of protease and integrin. A combination of integrin and protease activities might be crucial for cell migration through tissues and is notably upregulated in many metastatic tumour cells.

A number of other studies have demonstrated various degrees of colocalization between uPAR and αvβ5, β146, and β348 integrins as well as Mac-1. Interestingly, the colocalization of β1 and β3 integrins with uPAR, like that between uPAR and Mac-1, can be decreased by the sugar molecule NADG, suggesting the formation of a complex. Although many integrins colocalize with uPAR, there are relatively few demonstrations of an actual physical association of uPAR with integrins. However, uPAR has been immunoprecipitated with β1 integrins. There is also a physical association between uPAR and the β2 integrins Mac-1 and LFA-1 (CD11a/CD18) in monocytes, and a uPAR complex of uPAR, LFA-1, Mac-1 and four tyrosine kinases, lyn, lyn, hck and fgr, but no other monocyte GPI-linked proteins, can be isolated from monocytes following mild detergent extraction. Coimmunoprecipitation of β1 integrins and uPAR again reveals that only a small proportion of either uPAR or integrin is committed to the uPAR-integrin complex.

Purified Mac-1 interacts with soluble uPAR, and this interaction increases on integrin activation with Mn+ (Ref. 39), suggesting that formation of the integrin–uPAR complex is dependent on integrin activity. On human monocytes, uPAR is a receptor for vitronectin, and activation or ligation of Mac-1 promotes uPAR-mediated binding of vitronectin, which synergistically increases Mac-1 binding of fibrinogen. By contrast, soluble uPAR or binding of uPA to cell-surface uPAR inhibits Mac-1 and α5β1 function. As yet, there is no known functional effect of soluble uPAR on LFA-1 and αv integrins.

PAI-1 binds to and inactivates the uPA–uPAR complexes. This not only limits proteolysis but also releases Mac-1 from uPA–uPAR-mediated inhibition. PAI-1 can also competitively block both uPAR- and αv-mediated binding of vitronectin, suggesting that all three receptors must bind to vitronectin in a similar manner. This differential effect of uPAR on integrin function depending on the form of uPAR (soluble or cell-surface expressed) and the ligand it has bound (uPA or vitronectin) is in apparent contrast to the homogeneous effects of the other receptor pairings that we have discussed, although there might still be details to learn about the fine regulation of other interactions.

Are there any unifying principles?

Although these are early days in understanding the mechanistic details of the cis interactions of integrins, a few trends are emerging.

• Integrins can co-cluster with other types of receptors and with signalling molecules to form functional membrane units.
• Integrin–protease complexes promote migration, defining another type of functional package.
• Only a low percentage of the integrins and cis partners of a cell participate in complex formation at one time.
• Integrins can be directed to micro-domains of the cell membrane by their associations with other receptors. Alternatively, the concentration of integrins in focal adhesions or contacts can co-cluster other types of receptors, allowing more efficient signalling. Despite apparent similarities, differences are emerging between the different types of integrin complexes.
• Although TM4SF integrin complexes are found to be quite stable (F. Berditchevski, pers. commun.), FRET studies with uPAR suggest that this might not apply to all integrin partnerships.
• Some complexes depend on the activation state of the integrin, but others might not.

It is attractive to speculate that the multimeric-spanning feature of some partners (IAP, five-pass; TM4SF, four-pass) could be key to the firm anchorage of the integrin-cis partner complexes in the membrane, allowing docking of integrins above and of signalling molecules below the membrane bilayer. This has not yet been firmly established for caeovolin and CD98, but, as both are type II membrane proteins, they might be two-pass proteins. It is important to note that the identification of membrane-spanning domains has come from protein domain prediction programmes rather than from rigorous biochemistry.

As well as those integrin–membrane-protein complexes mentioned in this review, others have also been identified. For example, CD147/EMMPRIN complexes with αβ1 and αβ156, and a novel 150-kDa α5β1-associated protein has a sequence suggestive of three membrane passes (R. Juliano, pers. commun.). In the next few years, many more integrin-associated proteins will probably be discovered, and this newly recognized aspect of integrin function will bring us closer to an understanding of how integrins integrate the responses of cells to their microenvironment.

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Sticky and Smelly Issues: Lessons on Tumour Cell and Leucocyte Trafficking, Gene and Immunotherapy of Cancer

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Summary The Second Meeting of the British Society for Immunology Tumour Immunology Affinity Group (TIAG) took place at King’s College (London, UK) on 17–18 June 1997 and brought together over 100 tumour immunologists from the UK and abroad. In contrast to previous meetings the focus of the meeting was on the role of adhesion in immunosurveillance and tumour dissemination. In addition, recent achievements in the areas of chemokines, cytotoxic T-lymphocyte (CTL) and natural killer (NK) cells, co-stimulation, gene and adoptive immunotherapy were also addressed. The purpose of this report is to outline current trends in tumour immunology.

Keywords: gene and adoptive cancer immunotherapy; cell adhesion; chemotaxis; apoptosis; natural killer cells

ADHESION AND CHEMOTAXIS

The conference began with an overview of cell adhesion molecules in the interaction of leucocyte and tumour cells with the endothelium (Dr N Hogg, London, UK). Well-known adhesive ligands were described including PSGL-1 (a ligand for all three selectins, especially relevant for neutrophil adhesion), CD44 (as a rolling receptor for haematopoietic (DeGrendele et al, 1996) and potentially non-haematopoietic cells, such as pancreatic carcinoma and melanoma) and α4β1 (adherence receptor for leucocytes and tumour cells) (Figure 1). The role of cell adhesion in leucocyte activation ('in/out' signalling) was also highlighted. Furthermore, chemokines, with few exceptions (Szabo et al, 1997), appear unable to activate adhesion molecules (such as LFA-1) on non-primed T lymphocytes, as 'naïve' T cells do not express corresponding chemokine receptors. However, the adhesion molecules on activated leucocytes can be 'switched on' if chemokine receptors are cross-linked (e.g. the cross-linking of IL-8 receptors by IL-8 bound to extracellular matrix proteins). N Hogg also discussed the ability of the α4β1 integrin complex to either facilitate (Matsuura et al, 1996) or inhibit (Qian et al, 1994) the metastasis of neoplastic cells by decreasing the invasive potential of melanoma and even by inducing apoptosis of lymphoma cells (if expressed in situ). Furthermore, additional studies described the ability of the metalloproteinase MMP2 to form a complex with αVβ3 integrin (vitronectin R) (Brooks et al, 1996) and facilitate melanoma cell migration through its ability to digest collagen, unveiling its hidden RGD sequence and thus permitting integrin-mediated adhesion. In another model, the urokinase-type plasminogen activator receptor (uPAR) forms a complex with active integrins, which has the effect of destabilizing integrin adhesion and promoting adhesion and migration on vitronectin, thus potentially enhancing the invasive properties of tumour cells (Wei et al, 1996).

The adhesion and trafficking mechanisms of lymphocyte trans-migration in both normal and adjacent tumour endothelium remain poorly understood. In an elegant in vivo model using a mouse cremaster muscle implanted with a tumour and fluorescent confocal microscopy, murine lymphocytes were examined for their ability to traffic into tumour-bearing tissues (Dr N Brown, Sheffield, UK). Quite unexpectedly, IL-2-activated T cells and lymphocytes from previously immunized but not naive animals appeared to be 'trapped' by endothelium adjacent to tumour. This local arrest/ extravasation could be partly blocked by the use of antibodies specific for VLA-4 and VCAM, confirming an involvement of these integrin molecules in the trafficking event rather than a purely mechanical non-specific homing mechanism.

An elegant in vitro model of lymphocyte migration from blood to lymph node (involving transmigration of lymphocytes through high endothelium cells) also demonstrated the role of VLA-4/VCAM-1 in the lymphocyte trafficking process (Dr A Ager, London, UK). Although blocking of LFA-1/ICAM-1 interactions alone did not affect the T-cell transmigration, when combined with block of VLA-4/VCAM-1 interactions, lymphocyte movement was almost totally abolished. Freshly isolated CD4+ and CD8+ T cells as well as B lymphocytes but not lymphoma cells (WEHL, Jurkat) behaved similarly in these assays. Interestingly, the inhibition of L-selectin shedding through the use of the zinc-dependent matrix metalloproteinase inhibitor Ro 31-9790 also prevented the lymphocyte migration. Additional studies also revealed the existence of a novel, phosphol ester inducible, metalloproteinase (L-selectin shed­dase) responsible for the L-selectin shedding (Preece et al, 1996).

The biology of the recently discovered CD31 molecule was outlined by D Simmons (Oxford, UK). CD31 can be detected on a...
trans-endothelial migration of any one metastasizing cell type will depend on similarities with leucocytes, the complete sequence of events leading to extravasation of metastasizing tumour cells. However, studies of various surrounding tissues are well characterized, much less is known of the mechanisms by which leucocytes migrate from the vascular space into its unique adhesive repertoire, and is yet to be determined. 'Giavazzi et al mimic at least some of the leucocyte adhesion mechanisms. Despite such Figure 1 Possible mechanisms of tumour cell extravasation. While the mechanisms by which leucocytes migrate from the vascular space into surrounding tissues are well characterized, much less is known of the transmigration of leucocytes.

Table 1 Members of the C-X-C, C-C and C chemokine superfamily

<table>
<thead>
<tr>
<th>C-X-C*</th>
<th>C-C</th>
<th>C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human</td>
<td>Mouse*</td>
<td>Human</td>
</tr>
<tr>
<td>IL-8</td>
<td>MCP-1</td>
<td>JE</td>
</tr>
<tr>
<td>GCP-1</td>
<td>MCAF</td>
<td>MIP-2</td>
</tr>
<tr>
<td>GRD1a</td>
<td>KC</td>
<td>MIP-2</td>
</tr>
<tr>
<td>GRO1c</td>
<td>MCP-3</td>
<td>MIP-3</td>
</tr>
<tr>
<td>GRO2</td>
<td>MCP-4</td>
<td>-</td>
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<tr>
<td>GRO3</td>
<td>MCP-5</td>
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<tr>
<td>GRO4</td>
<td>ENA78</td>
<td>RANTES</td>
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<tr>
<td>NAP1-4</td>
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<td>-</td>
</tr>
<tr>
<td>NAP-2</td>
<td>-</td>
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<td>GCP2</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>PF-4</td>
<td>pAT44</td>
<td>-</td>
</tr>
<tr>
<td>IP-10</td>
<td>I309</td>
<td>TCA-3</td>
</tr>
<tr>
<td>Mig</td>
<td>-</td>
<td>C10</td>
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number of cell populations, including endothelium, T lymphocytes and monocytes, and is capable of facilitating both homophilic (when it functions as a gatekeeper and provides tissue integrity) or heterophilic adhesion interactions (through α4β1). Further studies revealed that homophilic adhesion of CD31 molecules constitutes a diimer; however, in contrast to the ICAMs, all domains of CD31 are required for this interaction. Once endothelium is activated, CD31 can also act as a heterophilic adhesion molecule facilitating the transmigration of leucocytes.

Like CD31, the junction adhesion molecule (JAM) is also a member of the Ig superfamily but contains only two domains and no glycosylation sites (Dr D Simmons). JAM was initially identified by its unique pattern of expression in endothelial and epithelial cell junctions and was found to have splice variants. Similar to CD31, it can also support the migration of neutrophils and monocytes. Despite the fact that JAM-mediated adhesion is thought to be cation and cytoskeleton dependent, the ligand for JAM has not yet been found. Little if anything is presently known about the expression of CD31 and JAM within tumour sites. Similar to cadherins, which are also involved in the maintenance of tissue integrity, these new adhesion molecules are expected to play an important role in tumour biology (Charpin et al, 1997).

In addition to adhesive interactions, chemokines also play an important role in the trafficking (Taub and Oppenheim, 1994; Taub, 1996) of a variety of immunocompetent cells (Table 1; Dr DD Taub, Baltimore, MD, USA). Chemokines are a family of low-molecular-weight peptides that have from 20% to 70% amino acid homology and are related by a conserved motif containing four cysteine residues. The chemokine superfamily is separated into four distinct families (C-X-C, C-C, C, C-X-C) based on their chromosomal localization, primary and secondary structures, and the placement and spacing of conserved cysteine motifs. Each of these chemokines has been shown to induce the directional migration of selected cell types, including granulocytes, monocytes and lymphocytes (Table 2). In addition, many tumours can also constitutively secrete or be induced to express these chemotactic molecules. While the biological relevance of tumour-produced chemokines remains obscure, studies have revealed that several chemokines can stimulate tumour cell growth in an autocrine manner (e.g. IL-8 and melanoma). In addition, several C-X-C chemokines have been shown to possess either angiogenic (IL-8) or angiostatic (IP-10) activities, which appear to modulate tumour growth in vivo. Alternatively, constitutive chemokine production by certain tumour cells has been suggested to break down the local chemokin gradient and, by this means, inactivate chemotaxis of immune cells into various tissues or tumour sites (Dr DD Taub).

In general, it is worth noting that the detection of a chemokine by immunassay most likely reflects its biological activity as no natural antagonists or neutralizing autoantibodies have yet been clearly identified. Furthermore, it has also been shown that the mere presence of chemokine receptors on the surface of a cell population does not necessarily render these cells responsive to soluble chemokine ligands (e.g. IL-8 and NK cells) (Taub and Oppenheim, 1994, Taub, 1996). In addition, extensive examination of chemokine subfamily effects on a wide panel of human and murine T-cell clones has demonstrated a differential ability to migrate and adhere to chemokines in response to various chemokines, despite the fact that many of these clones were derived from the same donor under identical conditions. Finally, chemokines appear to not only modulate leucocyte trafficking and adhesion but also contribute to T-cell co-stimulation and IL-2 production, cytotoxic T-lymphocyte (CTL) degranulation and cytosis, induce B cell-stimulatory molecule (β-chemokines) on antigen-presenting cells (Taub et al, 1996a) and, in certain cases, may even direct T-cell responses towards either Th2 (MCP-1) or Th1 responses (MIP-1α).

An in vivo correlation between MCP-1 expression and the infiltration of ovarian tumours by CD8 lymphocytes (accompanied by CD45+ cells and monocyes) was reported by RPM Negus (London, UK). Local fluctuations of oxygen tension have been suggested to affect MCP-1 production by carcinoma cells and the
Table 2  In vitro effects of chemokine family members on leukocytes

<table>
<thead>
<tr>
<th>Target cell</th>
<th>Biological effects on various target cells</th>
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<tbody>
<tr>
<td>Neutrophils</td>
<td>Chemotaxis (CXC)</td>
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<tr>
<td></td>
<td>Shape change</td>
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<tr>
<td></td>
<td>Increased degranulation</td>
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<tr>
<td></td>
<td>Increased respiratory burst</td>
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<tr>
<td></td>
<td>Increased cytosolic Ca++</td>
</tr>
<tr>
<td></td>
<td>Increased adhesion to endothelial cells, fibrinogen and ECM</td>
</tr>
<tr>
<td></td>
<td>Increased killing of micro-organisms</td>
</tr>
<tr>
<td></td>
<td>Increased expression of CD11a, CD11b, CD11c and CD18</td>
</tr>
<tr>
<td></td>
<td>Increased lysosomal enzyme release</td>
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<tr>
<td>T lymphocytes</td>
<td>Chemotaxis (CC, C and CXC)</td>
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<tr>
<td></td>
<td>Stimulated polyphosphoinositol hydrolysis</td>
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<td></td>
<td>Increased adhesion to endothelial cell monolayers and ECM</td>
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<td></td>
<td>Increased metalloproteinase release</td>
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<td></td>
<td>Increased CTL killing of tumour cell targets</td>
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<td></td>
<td>Increased degranulation</td>
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<td></td>
<td>T-cell activation</td>
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<td></td>
<td>Activation of PI-3-kinase</td>
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<tr>
<td>NK cells</td>
<td>Chemotaxis (CC and CXC)</td>
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<td></td>
<td>Increased adhesion to endothelial cell monolayers and ECM</td>
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<td></td>
<td>Increased killing of tumour targets</td>
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<td></td>
<td>Increased degranulation</td>
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<tr>
<td></td>
<td>Increased adhesion to extracellular matrix proteins</td>
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<tr>
<td>B lymphocytes</td>
<td>Chemotaxis (CXC and C)</td>
</tr>
<tr>
<td></td>
<td>Inhibits IL-4-induced IgE production</td>
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<tr>
<td></td>
<td>Increases B7 expression</td>
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<td></td>
<td>Increased immunoglobulin secretion</td>
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<tr>
<td></td>
<td>Increased B cell proliferation</td>
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<tr>
<td>Monocytes</td>
<td>Chemotaxis (CC and CXC)</td>
</tr>
<tr>
<td>Basophils</td>
<td>Chemotaxis (CXC and C)</td>
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<tr>
<td></td>
<td>Increased histamine release</td>
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<td></td>
<td>Increased intracellular calcium</td>
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<td>Increased leukotriene release</td>
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<td>Eosinophils</td>
<td>Increased superoxide anion release (CC and CXC)</td>
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<td>Increased cytosolic Ca++</td>
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<tr>
<td>Mast cells</td>
<td>Chemotaxis (CC and CXC)</td>
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<td></td>
<td>Increased histamine release</td>
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See also Taub (1996).

have been shown to use a repertoire of killer-cell inhibitory receptors (KIR or NKIR) to sense loss of MHC class I molecules (Lanier and Phillips, 1996; Lopez-Botet et al, 1996). Examination of NK cell clones has demonstrated that the NK cell populations are quite heterogeneous and that every cell expresses, at least, one of KIR (though a coexpression may also occur). The mechanism of KIR recognition is so sophisticated that even a single allele loss (with a single amino acid residue substituted) can be recognized. This is very convenient because of the ability of many tumours (e.g. carcinomas) to lose HLA expression, including losses of single HLA alleles. Furthermore, IL-15 has been shown to play an important role in the maturation of CD94+ NK cells (Mingari et al, 1997) and induction of functionally active CD94/NKG2A receptors on CD8+ T lymphocytes (Mingari et al, submitted). Indeed, a small proportion of T cells (both αβ and γδ, but mostly CD8+) have been shown to express KIR and lyse targets in NK-like fashion. These T lymphocytes express memory cell phenotypes and are represented by oligo- and monoclonal populations (Mingari et al, 1996). These populations are believed to expand after prolonged antigen stimulation to prevent an autoimmune response. In support of this theory, CTL lysis of human immunodeficiency virus (HIV)-expressing targets has been improved by masking KIR interactions with monoclonal antibodies. Following an earlier observed correlation between the expression of B7- and NK-mediated killing (Azuma et al, 1992), J Galea-Lauri (London, UK) convincingly demonstrated CD28 but not CTLA-4 or B7-1 expression on peripheral blood CD3-CD56+CD16+ lymphocytes. This CD28 expression varied from minimal to abundant depending on the human donor used. In addition, C-C chemokines have been shown to bind to and chemotact a human NK cells and NK cell clones (Dr DD Taub) (Taub et al, 1996b). Several of these chemokines also promoted NK cell killing and cellular degranulation. However, the biological significance of chemokines on NK cells activities has not yet been described.

Using a highly sensitive reverse transcription polymerase chain reaction (RT-PCR) method, it has been possible to quantify tumour infiltrating lymphocytes (TILs) expressing different T-cell receptors (TCR) V regions in tumour biopsies (Dr J Zeuken, Denmark). The TCR repertoire of TILs appeared to be skewed (indicating a clonal or oligo-clonal T-cell expansion) and reproducibly differed between primary and metastatic melanoma lesions (Scholler et al, 1994). Furthermore, similar changes have been observed in progressing and non-progressing parts of the same primary melanomas, suggesting the presence of different T-cell repertoires (Thor Straten et al, 1996). In an effort to understand why specific parts of the same tumour behave differently, it would be interesting to analyse the in situ cytokine production of primary and metastatic lesions. Surprisingly, the induction of HLA-DR on melanomas by IFN-γ has correlated with their escape from CTL surveillance (Kirkin et al, 1996). A similar adverse effect has been also reported in one clinical trial; however, in another trial, the systemic administration of IFN-γ was found to restore lost HLA class I expression (Dr A Knuth, Frankfurt, Germany). In the former case, HLA-DR may play a 'reporter' role of unrelated phenotypical changes in melanoma (e.g. FAS ligand (FAS-L) induction). Notably, the CTL-mediated killing of an immunogenic melanoma could be improved by the antibody-mediated neutralization of FAS-L (Dr J Zeuhen).

A prolonged vaccination with HLA-A2-restricted peptides derived from tumour-associated antigens (Melan A, tyrosinase, gp100) produced encouraging results in patients with progressive
stage 4 melanoma. Seven out of 12 patients whose melanomas were progressing achieved stable disease post vaccination (Table 3; Dr A Knuth). All patients developed delayed type hypersensitivity (DTH) and CTL responses to at least one peptide (tyrosinase, Melan A and influenza). Improved DTH responses were observed with the addition of intradermal granulocyte–macrophage colony-stimulating factor (GM-CSF) administration. Furthermore, immunohistochernical analysis showed an intensive infiltration of biopsies (CD4+, CD8+, CD1a+, HLA DR+) and a TH1 type immune response (IFNγ, TNFα+, IL-4−, IL-10−). At the same time progressive disease was associated with a local loss of antigen (Melan A, tyrosinase) and/or HLA class I expression.

The use of polyvalent vaccines (to avoid a selection of antigen-loss variants) combined with certain cytokines (similar to IFN-γ in their ability to up-regulate MHC class I expression) as well as an optimization of the dose and route of delivery of these specific therapies has been suggested for future protocols.

M Adams (Cardiff) reported on the successful propagation of dendritic cells (DCs) from peripheral blood of healthy donors and patients by IL-4 and GM-CSF. The DCs obtained could be loaded with peptides (HPV 16 E7, HER-2-Neu) and subsequently generate specific CTLs. Unfortunately, DCs expanded from four patients with cervical cancer did not express CD1a, even though they expressed similar levels of MHC class II, CD80 (B7-1) and CD54 (ICAM-1). Interestingly, when patients’ DCs were used to generate CTLs in vitro the resulting CTL response was defective compared with that achieved with DCs obtained from normal healthy volunteers. Further studies are required to unravel the basis of this defective response and how it might be corrected. E M-L Evans (Cardiff, UK) identified HPV 16 E7-specific CTLs in peripheral blood (four out of five), draining lymph nodes (two out of three) and tumours (one out of three) of cervical cancer patients but not healthy donors. Notably, in all patients tested, the frequency of HPV-specific CTLs was higher in lymph nodes and tumour tissue compared with peripheral blood.

J Saba (Sheffield, UK) showed that it is possible to generate CTLs to the HLA-A2-restricted peptides for the melanoma antigens (Melan A, tyrosinase and gp100) from the peripheral blood of ocular melanoma patients using a bulk culture method that uses a maximum of 60 ml of blood. CTL were generated to all peptides in a donor-dependent fashion and, if an response to tyrosinase was generated, these CTLs were always the most potent cytolytic cells compared with those generated to other antigens.

LG Durrant (Nottingham, UK) reported on studies mapping the helper T-cell epitope within the 105D7 vaccine, an anti-idiotypic antibody that mimics the 79Tgp72 antigen, which is overexpressed on colon carcinomas. The corresponding CDRH3 peptide (HLA DR1, -3 and -7 binding motifs) stimulated T-cell proliferation from naive donors (eight out of eight of permissve haplotype) and could prime them to respond to both 105AD7 or 79Tgp72 positive cells, although the response to the whole Ag (105AD7) was much stronger. In addition, a DNA vaccine incorporating heavy- and light-chain variables of 105AD7 antibody has been produced and shown in preliminary experiments to induce antibody production in a mouse model (Dr V Potter, Nottingham, UK).

**GENE AND ADOPTIVE IMMUNOTHERAPY**

Cancer gene therapy is intended to improve host immunosurveillance pathways that a tumour may evade through a number of mechanisms. These include decreased immunogenicity (e.g.

down-modulation of HLA class I molecule or antigen expression); suppression of the immune system (e.g. up-regulating FASL on tumour and FAS molecules on effector cells); the loss of the TCR zeta chain or DNA binding molecules in T cells obtained from tumour-bearing hosts; and the stimulation of tumour growth and increased angiogenesis (Dr R Vile, London, UK). A fine equilibrium between tumour growth and suppression can be shifted (we all hope) by improving/restoring a number of links in the chains of the immune response (Chong and Vile, 1996). The successes initially achieved with transfer of IL-2, IL-4 or, later, B7 genes could be explained by bypassing 'the helper arm' and directly activating CD8 and possibly NK cell responses. In contrast, recent success with GM-CSF gene transfer to tumour cells is probably a result of the recruitment and activation of antigen-presenting cells. Several of the approaches tested to date have yielded tumour rejections and, on some occasions, systemic and, most importantly, long-lasting immunity (Chen et al, 1997). While there is a worryingly long list of successes achieved using many cytokine or costimulatory genes (Forni and Foa, 1996), this does not mean that any cytokine transfection can cure cancer but rather reflects the situation that, for any given tumour (model), a particular co-stimulatory combination can be found (or adjusted). However, this does not necessarily echo the situation in patients. Perhaps it is time to stop and think about the mechanisms involved in the generation of an anti-tumour response rather than plunge straight into clinical trials.

Gene therapy offers a number of approaches to fighting various tumours. We can attempt to convert tumour cells to antigen-presenting cells by making them more immunogenic (B7 co-stimulation); restore lost MHC molecules; transflect with cytokines capable of a direct stimulation of lymphocytes (IL-2) or even enhance tumour antigen release and its subsequent up-take by professional antigen-presenting cells (presuming that antigen-specific T cells are still in the circulation or can be generated from naive precursors). The last approach also conforms with a recently suggested 'Danger Model' (Ridge et al, 1996) theory according to which induced necrosis may attract antigen-presenting cells (macrophages, dendritic cells), increase their up-take and the subsequent delivery of antigen in an appropriate fashion to T lymphocytes. The recruitment of antigen-presenting cells can be further potentiated by the local production of GM-CSF or IL-12. Based on this rationale and supporting data obtained from animal models, this group hopes to progress to clinical trial in patients with melanoma using retrovirus incorporating the HSV-TK and GM-CSF genes and a tumour-specific promoter/enhancer (e.g. tyrosinase).

A successful effect (50% complete remission) of locally delivered co-stimulatory IL-12 on the growth of otherwise fatal mesothelioma appeared to correlate with the infiltration of tumour

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**Table 3 Response of stage 4 melanoma patients immunized with polyvalent peptide vaccine**

<table>
<thead>
<tr>
<th>Clinical response</th>
<th>Treatment with peptides</th>
<th>Treatment with peptides and intradermal GM-CSF</th>
</tr>
</thead>
<tbody>
<tr>
<td>Complete response</td>
<td>1/12</td>
<td>2/16</td>
</tr>
<tr>
<td>Partial response</td>
<td>1/12</td>
<td>2/16</td>
</tr>
<tr>
<td>Stabilization of disease</td>
<td>7/12</td>
<td>7/16</td>
</tr>
<tr>
<td>Progression of disease</td>
<td>3/12</td>
<td>5/16</td>
</tr>
</tbody>
</table>
with CD4+ and CD8+ lymphocytes (Dr RA Lake, Australia). However, it remains to be established whether there is a connection between IL-12 secretion and the induction of heat shock proteins, as the latter also had a beneficial effect in this tumour (Alexandroff and Dalgleish, 1997). Furthermore, a phase I clinical trial was conducted in patients with mesothelioma using a replication-defective Vaccinia virus containing cDNA for IL-2 (Dr RA Lake). This trial demonstrated that intravesical administration of the viral construct is safe and non-toxic and results in intratumoral expression of IL-2 for up to 1 week.

AL Barnard (London, UK) reported on the development of a semi-synthetic melanoma vaccine that may be convenient for clinical use. Also, possible differences in the expansion of tumour from a cancer patient or finding a completely HLA-matched tumour and encouraging results recently reported about the use of allogeneic melanoma vaccines should be kept in mind when pondering a potential vaccine (Knight et al, 1996). In the developed hybrid model, the combined transfection with B7.1 and IL-2 demonstrated a beneficial anti-tumour effect. In this respect, a stimulatory effect of bladder cancer cells on the proliferation of allogeneic peripheral blood lymphocytes was observed (Dr AB Alexandroff, Edinburgh, UK). The observed stimulatory effect correlated with tumour cell expression of CD40, ICAM-1, -2 and cytokine production (IL-6) but not B7 or CD40L expression. Of note, addition of recombinant CD40L to bladder or pancreatic carcinoma cell lines markedly up-regulated expression of ICAM-1 and FAS as well as stimulated production of IL-6 but not IL-4, IL-10 or IL-1. Furthermore, FAS expression could also be enhanced by treatment with IFN-γ and TNF-α. These findings may shed some light on the recently reported role of CD40L in the protective immunity induced by tumour vaccines (Mackey et al, 1997). FAS and FAS-L coexpression was also observed on normal breast epithelial cells, while many breast carcinoma cells appear to lose FAS but continued to express FAS-L (Dr CB Ragnarsson, Reykjavik, Iceland). Overall, the expression of FAS-L has been reported on a number of neoplasms constitutively or after chemotherapy and has been suggested to play an important role in evasion of immune surveillance (Strand et al, 1997; Walker et al, 1997).

Because of a high rate of relapse and an acquired resistance to chemo- and radiotherapy, bone marrow transplantation (BMT) remains a therapy of choice for a number of haematological malignancies, including chronic and acute myeloid leukaemia (CML, AML), myelodysplastic syndrome, etc. Although allogeneic BMT has been shown to have adverse effects (e.g. liver and FAS-L-mediated skin damage), in studies on a large number of patients in Europe and North America, these transplants have been shown to have a lower relapse rate compared with syngeneic BMT. While the precise mechanism of this advantage is not totally clear, the immune response of donor T lymphocytes against residual malignant cells is believed to play an important role in these transplants (Dr E Fuchs, USA). These results have been confirmed, on the one hand, by an inverse correlation between graft vs host (GvH) disease and relapse rate, and, on the other hand, by an increased relapse after T-cell grafting of donor BMT (up to fivefold in CMLs). Similarly, positive effects have been observed in an animal BMT model. CML cells are notorious for their resistance to both chemotherapy and radiotherapy as a result of the induction of the p210 fusion protein (a product of c-abl) and a frequent p53 mutation. However, it appears that, while a pre-B cell line may acquire resistance to chemo- and/or radiotherapy after transfection with p210 fusion protein, it still remained sensitive to CTL-mediated lysis. Moreover, thymocytes derived from p53-deficient mice remain readily susceptible to both Fas- and CTL-mediated apoptosis, but are no longer sensitive to radio- and chemotherapy-induced apoptosis. These findings give clear incentives for an immunological approach towards improving allogeneic BMT. Indeed, the infusion of donor T cells to CML patients during blast crisis induced a long-lasting remission (2–5 years) in 62% of the patients. Additional experiments have demonstrated that, in the CML model, purified B cells provided a 'survival' (possibly bcI2-associated) rather than a 'proliferative' co-stimulatory signal to T lymphocytes. However, this effect can be reversed using spleen cells, containing dendritic cells. Based on these data, a clinical adoptive immunotherapy trial involving co-transfusion of donor T cells and recipient dendritic cells has been envisaged. The recently reported fusion of dendritic and tumour cells (Gong et al, 1997) as well as the immortalization of dendritic and follicular dendritic cells open up further perspective of their use in immunotherapy.

CONCLUSIONS

This short meeting has highlighted some important new developments in areas related to tumour biology as well as pointing to new directions for the future. Progress has been made in the characterization of some of the molecules involved in cell attachment and migration (of both immune cells and tumour cells), yet further work is required to fully elucidate the mechanisms involved, together with the role of cytokines and chemokines in these processes. NK cells and CTLs are also involved in tumour cell destruction but the recognition and cytolytic mechanisms mediating these responses are unclear. Nevertheless, tumour antigen-based vaccines, in the form of peptides, have shown promising results with future studies being planned incorporating different combinations of peptides or whole antigen molecules in combination with cytokines or immunological adjuvants. Clearly, while advances have been made in understanding the factors involved in leucocyte trafficking and in gene and immunotherapy of cancer, efforts are still needed in all these areas and, until we have a more thorough understanding of all the processes involved in these arenas, no appropriate and effective prevention and treatment of cancer will be available.

CONTRIBUTORS

The following invited speakers contributed to the meeting: A Ager (London), N Brown (Sheffield), E Fuchs (USA), N Hogg (London), A Knuth (Frankfurt), J Galea-Lauri (London), L Moretta (Italy), D Simmons (Oxford), D Taub (USA), R Vile (London) and J Zeuthen (Denmark).

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