MOLECULAR MECHANISMS OF T CELL HOMING IN HODGKIN'S LYMPHOMA: IMPLICATIONS FOR T-CELL-BASED THERAPIES.

BY

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Abstract

Recent years have seen important advances in the area of T cell-based therapy for human malignancies. Epstein Barr virus-associated tumours like Hodgkin’s lymphoma provide important models in this field. If a T cell-based therapy is to be effective however, T cells must be capable of trafficking to the tumour. To address this, the molecular mechanisms of T cell homing to Hodgkin’s Lymphoma were explored.

Chemokine and adhesion receptors were examined on infiltrating T cells. CXCR3, CXCR4, and CCR7 were expressed on major T cell populations with CXCR5, CXCR6, CCR4, and CCR5 on minor populations. Tumour cells expressed CXCL10, CXCL12 and CCL21. Vessels expressed ICAM-1, CXCL12, CCL17 and CCL21. Tumour cell lines secreted factors that mediated chemotaxis of lymphoblasts in vitro and TIL demonstrated chemotaxis to CXCL12 but not CCL17. VAP-1 was expressed on vessels and a tissue-binding assay was evaluated to examine VAP-1 function.

T cell clones generated as part of an existing clinical trial of adoptive T cell therapy were found to express a polarised Tc1 phenotype (CXCR3, CXCR6 and CCR5), which was typically independent of target antigen specificity, CD4/CD8 and donor status. However, lack of CCR7 expression and an inability to capture to VCAM-1 in a chemokine dependent manner suggested that clones expanded in vitro using existing protocols may be inefficient at trafficking to tumour tissue and thus may require modification of their homing phenotype.
Acknowledgments

I wish to thank many people for their help and friendship over the last few years. I have benefited considerably from having two mentors, Steve Lee and David Adams, who have generously provided their time, effort and experience in advising me with my investigations of T cell homing in Hodgkin’s lymphoma and introducing me to translational research.

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Abbreviations.

aa Amino acid
ACT Adoptive cellular therapy
ADP Adenosine diphosphate
ANOVA Analysis of variance
AP1 Activator protein 1
APAAP Alkaline phosphatase-anti alkaline phosphatase
APC Antigen presenting cell
ATCC American type culture collection
B cell Bone marrow derived cell
BCA-1 B cell associated 1
bcl-2 B-cell lymphoma/leukemia-2
BCR B cell receptor
BSA Bovine serum albumin
CCL CC chemokine ligand
CCR CC chemokine receptor
CD Cluster of differentiation
cDNA Complementary deoxyribonucleic acid
c-FLIP FLICE-inhibitory protein
CLA Cutaneous leukocyte antigen
CMP Cytidine monophosphate
CRC Colorectal carcinoma
CTL Cytotoxic T lymphocyte
CXCL CXC chemokine ligand
CXCR CXC chemokine receptor
DAB Diamino benzidine
DC Dendritic cell
DMSO Dimethylsulfoxide
DTH Delayed type hypersensitivity reaction
EBNA Epstein Barr associated nuclear antigen
EBV Epstein Barr virus
ECM Extracellular matrix
EDTA Ethylene-diamine-tetra-acetic acid
EGF Epidermal growth factor
ELC EBI1-ligand chemokine
ELISA Enzyme linked immunosorbant assay
ELIspot Enzyme linked immunospot
EST Expressed sequence tag
F-actin Filamentous actin
Fc Fragment crystallisable
FCS Foetal calf serum
FucTVII Fucosyl transferase VII
GAG Glycosaminoglycan
GalNAc N-acetyl galactosamine
GATA3 GATA binding protein 3
GDP Guanine dinucleotide phosphate
GFP Green fluorescent protein
GlyCAM-1 Glycosylation dependent cell adhesion molecule 1
GM-3 Monosialoganglioside 3
<table>
<thead>
<tr>
<th>Acronym</th>
<th>Description</th>
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<tr>
<td>GM-CSF</td>
<td>Granulocyte/Macrophage colony stimulating factor</td>
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<tr>
<td>GST-3</td>
<td>Glutathione S-transferase 3</td>
</tr>
<tr>
<td>GTPase</td>
<td>Guanosine triphosphatase</td>
</tr>
<tr>
<td>HEV</td>
<td>High endothelial venule</td>
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<tr>
<td>HL</td>
<td>Hodgkin's lymphoma</td>
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<tr>
<td>HLA</td>
<td>Human leukocyte antigen</td>
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<td>HRP</td>
<td>Horseradish peroxidase</td>
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<td>H-RS</td>
<td>Hodgkin and Reed Sternberg</td>
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<td>HUVEC</td>
<td>Human umbilical vein endothelium</td>
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<tr>
<td>ICAM</td>
<td>Intracellular adhesion molecule</td>
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<tr>
<td>IF</td>
<td>Immunofluorescence</td>
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<td>Ig</td>
<td>Immunoglobulin</td>
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<td>IHC</td>
<td>Immunohistochemistry</td>
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<td>IκBα</td>
<td>Inhibitor of NFκBα</td>
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<td>IL</td>
<td>Interleukin</td>
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<td>Infectious mononucleosis</td>
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<td>IFN</td>
<td>Interferon</td>
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<td>γ-IFN Inducible Protein-10</td>
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<td>ISH</td>
<td>In situ hybridisation</td>
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<td>ITAC</td>
<td>Interferon-inducible T cell a chemoattractant</td>
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<td>ITAM</td>
<td>Immunoreceptor Tyrosine-based activation motif</td>
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<td>JAK</td>
<td>Janus activated kinase</td>
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<tr>
<td>JAM</td>
<td>Junctional adhesion molecule</td>
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<tr>
<td>LAD</td>
<td>Leukocyte adhesion deficiency</td>
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<tr>
<td>LAK cell</td>
<td>Lymphokine activated killer cell</td>
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<tr>
<td>LCL</td>
<td>Lymphoblastoid cell line</td>
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<tr>
<td>LDHL</td>
<td>Lymphocyte depleted HL</td>
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<tr>
<td>LFA</td>
<td>Leukocyte functional antigen</td>
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<tr>
<td>LMP</td>
<td>Latent membrane protein</td>
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<tr>
<td>LN</td>
<td>Lymph node</td>
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<tr>
<td>(n)LPHL</td>
<td>Nodular lymphocyte predominant Hodgkin lymphoma</td>
</tr>
<tr>
<td>LRHL</td>
<td>Lymphocyte rich HL</td>
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<tr>
<td>mAb</td>
<td>Monoclonal antibody</td>
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<tr>
<td>Mac-1</td>
<td>Macrophage differentiation antigen 1</td>
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<tr>
<td>MACS</td>
<td>Magnetic associated cell sorting</td>
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<tr>
<td>MadCAM-1</td>
<td>Mucosal addressin cell adhesion molecule-1</td>
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<tr>
<td>MAGE</td>
<td>Melanoma-associated antigen gene E</td>
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<tr>
<td>MART</td>
<td>Melanoma Antigen Recognized by T cells</td>
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<tr>
<td>MCHL</td>
<td>Mixed cellularity HL</td>
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<tr>
<td>MCP</td>
<td>Monocyte chemotactic protein</td>
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<tr>
<td>MDC</td>
<td>Macrophage-derived chemokine</td>
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<tr>
<td>MFI</td>
<td>Mean channel fluorescence</td>
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<tr>
<td>MIDAS</td>
<td>The metal ion-dependent adhesion site</td>
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<tr>
<td>MIG</td>
<td>Monokine induced by γ-IFN</td>
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<tr>
<td>MIP</td>
<td>Macrophage Inflammatory Protein</td>
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<tr>
<td>MMP</td>
<td>Matrix metalloproteinase</td>
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<tr>
<td>mRNA</td>
<td>Messenger ribonucleic acid</td>
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<tr>
<td>NFκB</td>
<td>Nuclear factor κB</td>
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<tr>
<td>NK cell</td>
<td>Natural killer cell</td>
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<tr>
<td>NPC</td>
<td>Nasopharyngeal carcinoma</td>
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<tr>
<td>Abbreviation</td>
<td>Definition</td>
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<tr>
<td>NSHL</td>
<td>Nodular sclerosing HL</td>
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<tr>
<td>PBL</td>
<td>Peripheral blood leukocytes</td>
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<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
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<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
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<tr>
<td>PGE2</td>
<td>Prostaglandin E2</td>
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<tr>
<td>PHA</td>
<td>Phytohaemagglutinin</td>
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<tr>
<td>PI3K</td>
<td>Phosphatidylinositol 3-kinase</td>
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<tr>
<td>PKC</td>
<td>Protein kinase C</td>
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<tr>
<td>plt</td>
<td>Paucity of lymph node T cells</td>
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<tr>
<td>PNAld</td>
<td>Peripheral node addressin</td>
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<tr>
<td>PSGL-1</td>
<td>P-selectin glycoprotein ligand 1</td>
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<tr>
<td>PTLD</td>
<td>Post transplant lymphoproliferative disease</td>
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<tr>
<td>RACE</td>
<td>Rapid amplification of cDNA ends</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>Rap-1</td>
<td>Ras-associated protein</td>
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<td>RAPL</td>
<td>RAP-1 ligand</td>
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<tr>
<td>RCC</td>
<td>Renal cell carcinoma</td>
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<tr>
<td>REAL</td>
<td>Revised European American Lymphoma</td>
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<tr>
<td>REP</td>
<td>Rapid expansion protocol</td>
</tr>
<tr>
<td>RPMI</td>
<td>Roswell Park Memorial Institute</td>
</tr>
<tr>
<td>S1P</td>
<td>Sphingosine-1-phosphate</td>
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<tr>
<td>SAGE</td>
<td>Serial analysis of gene expression</td>
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<td>SCID</td>
<td>Severe combined Immunodeficiency disease</td>
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<tr>
<td>SD</td>
<td>Standard deviation</td>
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<tr>
<td>SDF-1</td>
<td>Stromal derived factor 1</td>
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<tr>
<td>SEM</td>
<td>Standard error of the mean</td>
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<td>SLC</td>
<td>Secondary lymphoid chemokine</td>
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<td>ST3Gal-III</td>
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<td>STAT</td>
<td>Signal transducers and activators of transcription</td>
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<td>Thymus derived cell</td>
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<td>Thymus associated receptor chemokine</td>
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<td>Tris buffered saline</td>
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<tr>
<td>TCR</td>
<td>T cell receptor</td>
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<td>Transendothelial migration</td>
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<tr>
<td>TGF</td>
<td>T cell growth factor</td>
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<tr>
<td>TIL</td>
<td>Tumour infiltrating leukocyte</td>
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<tr>
<td>TL</td>
<td>Tonsil</td>
</tr>
<tr>
<td>TNF</td>
<td>Tumour necrosis factor</td>
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<td>VAP-1</td>
<td>Vascular adhesion protein 1</td>
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<tr>
<td>VCAM</td>
<td>Vascular cell adhesion molecule</td>
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<tr>
<td>VE-cadherin</td>
<td>Vascular endothelial cadherin</td>
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<tr>
<td>VLA</td>
<td>Very late antigen</td>
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<td>WHO</td>
<td>World health organisation</td>
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Chapter 1. Introduction

1.1 An overview of lymphocyte trafficking in the immune system

Within the human body it has been estimated that there are some 500 billion lymphocytes, of which about half are T cells. 90% of those are αβ T cells and in a young person some 70% are naïve T cells. The repertoire of T cell receptor (TCR) specificities has been estimated at being between 25-100 million different receptors (Arstila et al., 1999). A conservative estimate therefore suggests only 6000 T cells in the human body share the same T cell receptor specificity (TCR). A fundamental question is how these 6000 cells ever encounter their cognate antigen in such a short space of time and confer protection? In the context of this thesis the question is refined to how such T cells ever locate antigen in association with tumours and how might this be exploited therapeutically. To begin to answer such questions it must be recognised that activated immune effector cells are not free to randomly roam the body but instead are programmed, channelled or compartmentalised in terms of their migratory capacity. Naïve T cells continuously circulate between the systemic circulation and secondary lymphoid tissue as part of immune surveillance for foreign antigen. (Butcher, 1991; Picker and Butcher, 1992). Naïve T cells encounter antigens in lymph nodes when antigen from peripheral sites such as the skin or the gut is taken up by tissue resident professional antigen presenting cells (APCs). In their tissue-resident, immature state dendritic cells (DC) display enhanced antigen uptake and can process exogenous antigen by phagocytosis or Fc receptor mediated endocytosis and become activated. This activated DC then upregulates cell surface homing receptors that allow it to respond to a gradient of chemoattractant signals in lymphatics thereby promoting trafficking into local draining lymphatics. Within the confines of the draining lymph node the rare antigen specific T cell scans T zone interdigitating DCs
for its cognate antigen. If recognition does not occur the T cell eventually drains from
the node and enters back into the systemic circulation via the thoracic duct. If the T
cell engages its target antigen presented by the appropriate HLA allele then activation
and clonal expansion ensue. Resulting effector lymphocytes are reprogrammed in
their migratory phenotype and selectively traffic to the site in which the antigen was
encountered. In addition to an effector response, a long lasting memory response may
be generated with the differentiation of long-lasting memory T cells which promote
enhanced responses to subsequent rechallenge with the same antigen.

Lymphocyte trafficking into tissue is dependent on a multistep adhesion cascade
(Figure 1.1) that allows lymphocytes that are rapidly flowing in the systemic
circulation to adhere to the vascular endothelium (Butcher, 1991). Transient tethering
interactions allow lymphocytes to roll along the endothelium, sampling the
endothelial glycocalyx for factors that trigger signalling events rapidly promoting an
increase in both affinity and avidity of integrin-mediated adhesion to integrin ligands
on the endothelium. The cell is then able to transmigrate through the endothelial
barrier and penetrate the basement membrane. Within the tissue interstitium
lymphocytes respond to a complex chemoattractant network that promotes directed
migration. If infiltrating T lymphocytes encounter cells expressing the appropriate
target antigen, then the T cell can interact via its TCR and associated adhesion and
costimulatory molecules to mediate effector functions such as target cell lysis.
Lymphocyte trafficking into tissue is dependent on a multistep adhesion cascade that allows lymphocytes that are rapidly flowing in the systemic circulation to adhere to the vascular endothelium (Butcher 1991). Transient tethering interactions allow capture and rolling of the lymphocyte. This promotes lymphocyte sampling of the endothelial glycocalyx for chemokines that trigger an increase in both affinity and avidity of integrin-mediated adhesion to Ig superfamily ligands expressed by the endothelium. The cell is then poised to transmigrate through the endothelial barrier and penetrate the basement membrane. Within the tissue interstitium lymphocytes respond to a complex chemoattractant network that promotes directed migration to discrete microenvironments. T cells interact with target cells expressing appropriate target antigens via the T cell receptor (TCR) and associated adhesion and costimulatory molecules to mediate target cell lysis.
1.2 Lymphocyte trafficking mechanisms.

1.2.1 The adhesion cascade.

1.2.1.1 Tethering/rolling adhesion.

Lymphocytes overcome the pressures imposed by high shear stress in the systemic circulation (40 µm/s) by interacting with the vascular endothelium via a series of transient adhesive interactions that allow the lymphocyte to roll rapidly along the vessel wall, but slow enough (40 µm/s) to sample the luminal surface for factors displayed by the endothelial proteoglycan (Tanaka et al., 1995). The selectins family and their ligands classically mediate this initial tethering interaction (Figure 1.2).
1.2 Lymphocyte trafficking mechanisms.

1.2.1 The adhesion cascade.

1.2.1.1 Tethering/rolling adhesion.

Lymphocytes overcome the problem imposed by high shear stress in the systemic circulation (0.05 Pa) by interacting with the vascular endothelium via a series of transient tethering interactions that allow the lymphocyte to roll rapidly along the vessel wall, but slow enough (40 µm/s) to sample the luminal surface for factors displayed by the endothelial proteoglycan (Tanaka et al., 1993). The selectin family and their ligands classically mediate this initial tethering interaction (Figure 1.2).

The selectin family consists of three members: L(leukocyte)-selectin (CD62L), P(platelet)-selectin and E(endothelial)-selectin. L-selectin was defined by the antibody MEL14 that prevented lymphocyte binding to high endothelial venules in mouse secondary lymphoid organs (Gallatin et al., 1983). E-selectin was identified by raising the antibody H18/7 to cytokine-or endotoxin-activated human umbilical vein endothelial cells (HUVEC) and demonstrating blocking of neutrophil adhesion (Bevilacqua et al., 1987). P-selectin was detected as a plasma membrane component of α granules in platelets and later shown to be expressed in Weibel-palade bodies in endothelial cells (Bonfanti et al., 1989; McEver et al., 1989).

1.2.1.1.1 Structural features of selectins.

Selectins are calcium dependent C-type lectins that contain a plant C-type lectin domain of approximately 120 residues (Bevilacqua et al., 1989). Experiments with chimeric selectins demonstrate the lectin domain is critical for ligand binding (Erbe et al., 1992). Selectins contain an epidermal growth factor (EGF) like domain of 35-40
Figure 1.2 Selectins and their ligands.

The selectin family consists of L(leukocyte)-selectin, P(platelet)-selectin and E(endothelial)-selectin (A). Selectins contain a plant C-type lectin domain of approximately 120 residues, an epidermal growth factor (EGF) like domain of 35-40 residues containing 6 cysteines that are conserved with the EGF family of proteins. There are between 2-9 consensus repeats (~60 residues) with homology to complement binding proteins. Selectins have a transmembrane and a cytoplasmic C-terminal domain (B). Selectins bind to o-glycan decorated core proteins (C). Figures reprinted from (A and B) http://bme.virginia.edu/ley/ and (C) http://www.med.osaka-u.ac.jp/pub/orgctl/www/member/hirata.html
A

E selectin $\text{NH}_2$ - Lectin - EGF $1 2 3 4 5 6$ - COOH

L selectin $\text{NH}_2$ - Lectin - EGF $1 2$ - COOH

P selectin $\text{NH}_2$ - Lectin - EGF $1 2 3 4 5 6 7 8$ - COOH

B

Lectin - EGF - 2-9 CR domains

\begin{itemize}
  \item \sim 20 \text{ aa}
  \item \sim 35 \text{ aa}
  \item \sim 60 \text{ aa}
\end{itemize}

C

Leukocyte

\begin{itemize}
  \item L-selectin
  \item ESL-1
  \item PSGL-1
  \item CD24
  \item E-selectin
  \item P-selectin
\end{itemize}

GlyCAM-1, CD34, MaCdAM-1, Sgp200, PCLP

Lymphoid High Endothelial Venule

Non-Lymphoid Endothelium
residues containing 6 cysteines that are conserved with the EGF family of proteins. Furthermore, there are between 2-9 consensus repeats (~60 residues) with homology to complement binding proteins (Bevilacqua et al., 1989). The number of these domains determines the length of the selectin. Deleting these domains in P-selectin abolishes function (Patel et al., 1995). Finally, selectins have a transmembrane and a cytoplasmic C-terminal domain.

1.2.1.1.2 Molecular interactions between Selectins and their ligands.

Attempts to identify and define selectin ligands have been hampered by the fact that they bind a range of O-glycan decorated protein scaffolds. This includes CD34 (Puri et al., 1995), podocalyxin (Sassetti et al., 1998), and endomucin (Samulowitz et al., 2002) in human tonsil and GlyCAM-1 (Lasky et al., 1992), CD34 (Baumheter et al., 1993), Sgp 200 (Hemmerich et al., 1994a) and endomucin (Samulowitz et al., 2002) in murine lymph nodes. These O-glycan structures are extensively modified by glycosyltransferases which can use several sugar nucleotide substrates to form either α or β glycosidic bonds. Posttranslational modifications include sialylation (use of CMP-sialic acid), and fucosylation (use of GDP-fucose). For example GlyCAM-1 has four associated sulphated monosaccharides that constitute part of the GlyCAM-1 recognition site (Hemmerich et al., 1994a) and form a core of Sialyl Lewis X and its analogues, which bind selectins (Foxall et al., 1992). Recent gene knockout studies have more accurately defined L-selectin ligands expressed on high endothelial venules (HEV) within secondary lymphoid organs. L-selectin binding to its ligands is dependent on at least two fucosyl transferases (Homeister et al., 2001) and one sulfotransferase (van Zante et al., 2003) in synthesising the L-selectin recognising carbohydrate chains.
1.2.1.3 Enzymatic modification of selectin ligands.

1.2.1.3.1 Core O-glycan synthesis.

Initiation of O-glycan synthesis (Figure 1.3) is mediated by addition of N-acetyl galactosamine (GlcNAc) to serine/threonine residues within the protein scaffold by a number of GlcNAc transferases (GlcNAc-Ts) (Schachter and Brockhausen, 1989). Chain initiation proceeds with branch specific modification. Core 1 glycans are sialylated, or elongated and then sialylated. Core 1 O-glycans are also modified to Core 2 O-glycans. Other glycosyltransferases and sulphotransferases then provide terminal modifications.

Three core 2 GlcNAc transferases have been identified and targeted deletion of GlcNAcT-I has been characterised (Ellies et al., 1998). Lymphocyte homing is essentially unaffected in these mice, however neutrophil P-selectin ligand activity is deficient. The role in E-selectin ligand activity is unclear. Scaffold proteins were purified from GlcNAcT-I deficient mice. Analysis of the purified glycans identified novel core 1 extended glycans implying the existence of a relevant core 1 β3-GlcNAcT. Sequence alignment of EST databases enabled the characterisation of a core 1 β3-GlcNAcT expressed within high endothelial venules (HEV) (Yeh et al., 2001). Reconstitution experiments with this transferase, GST-3, FucTVII and a scaffold protein demonstrated enhanced L-selectin dependent tethering under conditions of shear flow.
Figure 1.3 Enzymatic modifications of selectin ligands

Initiation of O-glycan synthesis is typically mediated by addition of N-acetyl galactosamine (GalNAc) to serine/threonine residues within the protein scaffold by a number of GalNAc transferases (GalNAc-Ts) (A). Chain initiation proceeds with branch specific modification. Core 1 glycans are sialylated (B), or elongated and then sialylated (C). Core 1 O-glycans are also modified to Core 2 O-glycans. Other glycosyltransferases and sulphotransferases then provide terminal modifications (D). The sialyl lewis x motif is circled. Figure is adapted from Annu Rev Biochem. 2003;72:643-91. Epub 2003 Mar 27. A genetic approach to Mammalian glycan function.
Protein core (e.g., CD34, sgp200, GlyCAM-1)

- Fucose
- Galactose
- N-acetylgalactosamine
- N-acetylglucosamine
- Sialic acid

**Sialyl Lewis x**

- Sialyl (α1-3GalNAc)β1-4Glt
- GalT10
- ST3Gal
- GIcNAcT
- GnaCT
- Core 2
- Core 1

- ST3Gal
- Ser/Thr/ hr

- Protein core (e.g., CD34, sgp200, GlyCAM-1)

- Sialyl (α1-3GalNAc)β1-4Glt
- β1-3GlcNAcT
- α1-2FUT
- α1-3FUT

- ST3Gal
- Ser/hr
1.2.1.1.3.2 Sialylation.

Three sialyl transferases (ST3Gal-III, ST3Gal-IV, and ST3Gal-VI) form 2-3 sialic acid linkages and sialylate oligosaccharides in vitro that are likely to be important in selectin mediated adhesion. Targeted deletion of ST3 Gal-IV has resulted in a defect in E and P-selectin mediated adhesion in vitro, whereas only E-selectin dependent adhesion was defective in an in vivo model of neutrophil rolling (Ellies et al., 2002). It is possible that ST3Gal-VI is important and gene-targeting experiments are warranted.

1.2.1.1.3.3 Sulphation.

The importance of sulphation in selectin mediated adhesion was originally demonstrated by employing the metabolic inhibitor chlorate (Hemmerich et al., 1994b). Sulphotransferases transfer sulphate from the donor phosphadenosine phosphosulphate and probing sequence databases identified candidate 6-O sulphotransferases. The cDNA for GST-3 (LSST) was subsequently identified and its expression was restricted to HEV in mouse lymph nodes (Hiraoka et al., 1999; Kimura et al., 1999). In addition, reconstitution experiments transfecting chinese hamster ovary (CHO) cells with CD34 (or GlyCAM-1), FucT-VII (discussed below) and GST-3 provided greater L-selectin activity than with FucT-VII alone (Bistrup et al., 1999).

The recent analysis of a GST-3 deficient mouse demonstrated increased rolling and reduced adherence to HEV (van Zante et al., 2003). There was a reduction in the amount of incorporated sulphate from CD3.4 and GlyCAM-1 isolated from these animals and there were reduced levels of the MECA-79 epitope (which constitutes part of HEV expressed peripheral node addressin [PNAD]). Furthermore, GST-3 and
core 2 GlcNacT deficient mice help to define the MECA-79 epitope and it is this combination that cooperatively regulates L-selectin rolling (Hiraoka et al., 2004).

1.2.1.3.4 Fucosylation.

Evidence for the role of fucosylation in the synthesis of selectin ligands has been definitively demonstrated by the characterisation of targeted gene deletions of two fucosyltransferases. FucTIV and FucTVII are both expressed in leukocytes and are required for α-1,3 fucosylation of O-glycans. FucTVII deletion was first characterised and demonstrated to produce a LAD II (discussed in section 1.2.1.2.4) like deficiency in mice including blood leukocytosis, a deficit in E and P-selectin counter-receptor activity and compromised neutrophil homing (Maly et al., 1996). This was recapitulated in vitro with reconstitution studies transfecting this transferase with a suitable protein core (i.e. CD34), GST-3, and a core 2 transferase. Although FucTVII accounted for a considerable defect in leukocyte homing, there was still residual homing. Upon generation of a double gene knockout of both fucosyltransferases a more severe phenotype was observed, this time with a paucity of lymph node lymphocytes as they were unable to traffic to secondary lymphoid organs (Homeister et al., 2001).

1.2.1.2 Firm adhesion.

1.2.1.2.1 Integrin family members and their endothelial counter-receptors.

To convert leukocyte rolling along the vascular endothelium into firm and stable arrest, activation of cell surface integrins must occur. Integrins are activated and bind adhesion molecules belonging to the immunoglobulin superfamily expressed by the
core 2 GlcNacT deficient mice help to define the MECA-79 epitope and it is this combination that cooperatively regulates L-selectin rolling (Hiraoka et al., 2004).

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To convert leukocyte rolling along the vascular endothelium into firm and stable arrest, activation of cell surface integrins must occur. Integrins are activated and bind adhesion molecules belonging to the immunoglobulin superfamily expressed by the
vascular endothelium (Figure 1.4). Integrins are a family of cell surface type I heterodimers consisting of an α and β chain (Pribila et al., 2004).

β1, β2 and β7 integrins are the predominant integrins for lymphocyte adhesion and β2 integrins are exclusively expressed on leukocytes. These integrins are expressed on the majority of leukocytes populations but have weak interactions with their counter receptors unless they are triggered into a high affinity conformational state or/and have increased lateral mobility in the plasma membrane.

1.2.1.2.1.1 β1 integrins.

The β1 integrins were discovered in the context of T cell activation and have a delayed increase in expression upon mitogen simulation of cells (Hemler et al., 1985). They predominantly bind to the extracellular matrix (ECM) and this interaction occurs through recognition of the residues Arg-Gly-Asp (RGD) contained within ECM proteins like fibronectin and vitronectin (Shimizu et al., 1990). α4β1 (also known as VLA-4) is the exception in that it binds fibronectin via an alternative amino acid sequence, EILDV (Mould et al., 1990). α4β1 is distinctive as it binds ECM fibronectin, and a cell surface receptor, vascular cell adhesion molecule-1 (VCAM-1) (Elices et al., 1990). VCAM-1 is a member of the Ig superfamily and has alternate spliced forms, including a seven Ig-like domain form, and an alternatively spliced six Ig-like domain form (Osborn et al., 1989). Binding to VCAM-1 is mediated by recognition of the first and fourth amino-terminal Ig-like domains (Vonderheide and Springer, 1992). The five other α chains that pair with β1 subunits are expressed on lymphocytes and are important in lymphocyte-stromal interactions with ECM.
Figure 1.4 Integrins and their ligands.

Members of this family have a β2 chain in association with αL, αM, αD or αE. αL β2 (LFA-1) is perhaps the most crucial integrin heterodimer in this class, binding predominantly to the 90-115 kDa counter-receptor ICAM-1 (Martin and Springer, 1987). ICAM-1 is a member of the immunoglobulin superfamily and contains 5 Ig domains in which the first two α5 α4 α2 α1 Ig-Yce domains mediate LFA-1 binding.

### CELL SURFACE EXPRESSED INTEGRIN LIGANDS

<table>
<thead>
<tr>
<th>Transmembrane</th>
<th>Cytoplasmic</th>
<th>Extracellular</th>
</tr>
</thead>
</table>

**Ig-like domains**

- **α5 α4 α3 α2 α1**
- **α2 α1**
- **α5 α4 α3 α2 α1**
- **α9 α8 α7 α6 α5 α4 α3 α2 α1**

**ICAM-1 (CD54)**

**ICAM-2 (CD102)**

**ICAM-3 (CD50)**

**Telencephalin**

**7 D isoform**

- **α7 α6 α5 α4 α3 α2 α1**

**VCAM-1 (CD106)**

**6 D isoform**

- **α7 α6 α5 α4 α3 α2 α1**

**3 D isoform**

- **GPI α3 α2 α1**

**GPI-linked**

- **Mucin**

**MadCAM-1**

**PECAM-1 (CD31)**

**Ca-binding repeats**

- **5 4 3 2 1**

**E-Cadherin**

<table>
<thead>
<tr>
<th>RECEPTORS</th>
</tr>
</thead>
</table>

- **αL β2 Integrin (CD11a/CD18)**
- **αM β2 Integrin (CD11b/CD18)**
- **αL β2 Integrin (CD11a/CD18)**
- **αL β2 Integrin (CD11a/CD18)**
- **αL β2 Integrin (CD11a/CD18)**
- **αL β2 Integrin (CD11a/CD18)**
- **αL β2 Integrin (CD11a/CD18)**
- **α4 β1 Integrin (CD49d/CD29)**
- **α4 β1 Integrin (CD49d/CD29)**
- **α4 β1 Integrin (CD49d/CD29)**
- **α4 β1 Integrin (CD49d/CD29)**
- **α4 β7 Integrin (CD49d/CD7)**
- **α4 β1 Integrin (CD49d/CD29)**
- **L-Selectin (CD62L)**
- **α7 β3 Integrin (CD51/CD61)**
- **CD31 (Homophilic interaction)**
- **αE β7 Integrin (CD103/CD11a)**

The β7 chain can only pair with two known α-chains, namely α4 and αE. Both are expressed at low levels on lymphocytes but are induced with activation. α4β7 is important in mucosal housing and binds to the ligand MadCAM-1 (Berlin et al., 1995). MadCAM is heavily glycosylated and has three extracellular Ig domains (the N-terminal first two domains are homologous with ICAM-1 and VCAM-1) (Orthakio, 1996).
1.2.1.2.1 β2 integrins.

Members of this family have a β2 chain in association with αL, αM, αD or αX. αLβ2 (LFA-1) is perhaps the most crucial integrin heterodimer in this class, binding predominantly to the 90-115 kDa counter-receptor ICAM-1 (Marlin and Springer, 1987). ICAM-1 is a member of the immunoglobulin superfamily and contains 5 Ig domains to which the first two amino terminal domains mediate LFA-1 binding (Stanley et al., 1994). ICAM-1 is extensively glycosylated and expression on the vasculature is dependent upon activation. IL-1β, TNF-α, IFN-γ (Pober et al., 1986), endotoxin (Munro et al., 1991) and phorbol esters (Dustin et al., 1986) induce ICAM-1 expression. In addition, LFA-1 also binds ICAM-2, which contains only two extracellular Ig domains homologous to the first two Ig domains of ICAM-1 (Staunton et al., 1989). ICAM-2 is constitutively expressed on resting endothelium, as well as inflamed vessels (Nortamo et al., 1991). αMβ2 (Mac-1) is expressed on a subset of T cells and binds ICAM-1 through interactions with the third Ig domain (Diamond et al., 1991). αDβ2 can bind ICAM-3 (Van der Vieren et al., 1995), whereas αXβ2 binds ICAM-1 (Blackford et al., 1996) although their role in T cell adhesion in vivo is less clear.

1.2.1.2.1.3 β7 integrins

The β7 chain can only pair with two known α chains, namely α4 and αE. Both are expressed at low levels on lymphocytes but are increased upon activation. α4β7 is important in mucosal homing and binds to the ligand MadCAM-1 (Berlin et al., 1993). MadCAM is heavily glycosylated and has three extracellular Ig domains (the N-terminal first two domains are homologous with ICAM-1 and VCAM-1) (Briskin
et al., 1993). On HEVs (Briskin et al., 1997) in Peyers patches it mediates L-selectin mediated tethering because MadCAM-1 in the context of L-selectin activity acts as a scaffold protein for O-glycans (Berg et al., 1993). MadCAM-1 is differentially spliced and this can alter the glycosylation status of this core scaffold protein. Indeed, the unglycosylated form of MadCAM-1 is deficient in L-selectin mediated activity (Berg et al., 1993). α4β7 is able to bind VCAM-1 and fibronectin although the affinity of this receptor for these ligands is lower (Andrew et al., 1994). αEβ7 is expressed on intra-epithelial lymphocytes and mediates T-cell-epithelial interactions by binding epithelial expressed E-cadherin.

1.2.1.2 Structural features of integrins

The crystallisation of integrin heterodimers and isolated integrin binding domains has enabled a structural analysis of integrin function (Randi and Hogg, 1994; Xiong et al., 2002). Integrin heterodimers are non-covalently associated and contain an extracellular domain that has an α chain of ~940 residues and a β chain of ~640 residues. Integrins consist of a globular headpiece that binds ligand. The headpiece is attached to a 500-residue stalk region that consists of three β-sandwich domains including a so-called thigh, calf 1 and calf 2 domain (Figure 1.5).

The α subunit consists of seven β propeller domain repeats of approximately 60 residues each, of which in half of α chain integrins lies an inserted I-domain between domains two and three. This I-domain containing subset of α chains comprises a conserved region of approximately 200 residues that contain a dinucleotide binding (or “Rossmann fold”) fold (Lee et al., 1995). The I-domain contains six major α helices surrounded by a single β sheet (containing one anti-parallel and five parallel
Figure 1.5 Integrin structural features.

A number of α subunits contain an I-domain insertion (dotted line). Asterisks show Mg\(^{2+}\) (blue) and Ca\(^{2+}\) (red) binding sites. Lines below the stick diagrams depict disulfide bonds. (B) Organisation of the domains is based on three-dimensional crystal structure data. Reprinted from Immunol Rev. 2002 Aug; 186:141-63. Integrin activation and structural rearrangement. Takagi J, Springer TA.
divalent cations are located in the top of this domain and co-ordinate five side chains from three different loops. This functional unit comprises the metal ion dependent associated site (MIDAS), which comprises the metal coordinating residues and the surrounding metal binding site. Homologues containing this motif require Mg\(^{2+}\) (or Mg\(^{2+}\)) for function, and experiments defining the 1-domain of the α chain of integrin α5β1 demonstrated this domain has extensive contacts with the peptide (Qing et al., 2002). The domain has a characteristic PXXP motif analogous to the MIDAS motif in the α chain. The β-propeller domain repeats are arranged in a sandwich structure around an 1-domain in the α chain and indirectly influence the 1-like domain. This region is a hotspot for mutations that abrogate cell adhesion. In both domains, a hybrid domain comprising a β-propeller domain, a 1-like domain, and an I domain. The β-propeller domain is a conserved structural module consisting of cysteine rich repeats that provide structural rigidity and facilitate structural motion. Activation antibodies to integrin heterodimers were shown to bind this region. Finally, there is a β-tail domain at the C terminus that is cysteine rich and disulfide flavoured the α helix with a β-sheet to confer structural rigidity.

**A**

- **I domain**
- **β-propeller domain repeats**
- **PSI domain**
- **Hybrid domain**
- **I-EGF domains domain**
- **β-Tail**

**B**

- **β-Propeller**
- **Thigh**
- **PSI**
- **E1+2**
- **Calf-1**
- **I-like**
- **E3**
- **E4**
- **βTD**
- **Calf-2**
strands). Divalent cations are located in the top of this domain and co-ordinate five side chains from three different loops. This functional unit comprises the metal ion dependent associated site (MIDAS), which comprises the metal coordinating residues and the surrounding metal binding site. Heterodimers containing this motif require Mg\(^{2+}\) (or Mn\(^{2+}\)) for function and experiments deleting the I-domain of the α chain of integrins result in an inactive heterodimer (Michishita et al., 1993). Conversely, isolation of the I-domain alone demonstrated complete ligand binding activity (Randi and Hogg, 1994). The β subunit has fifty N-terminal residues that contain seven cysteines that restrain the integrin in the inactive conformer.

All β chains contain an I-like region that has structural homology with the I domain of α chains. Structural studies of αVβ3 in complex with ligand mimetic peptide demonstrate this domain has extensive contacts with the peptide (Xiong et al., 2002). The domain has a characteristic DXSXS motif analogous to the MIDAS motif in the α subunit and has weak homology to the I-domain. Functionally, this I-like domain binds directly to ligand in heterodimers that do not contain an I-domain in the α chain and indirectly influence I-domain containing integrins. This region is a hotspot for mutations that abrogate the association of β2 chains with α chains in LAD (discussed below). Flanking either side of this domain is a hybrid domain comprising a β sandwich structure that contains two covalent links between the I-like and hybrid domains. Contiguous with this region is an I-EGF domain consisting of cysteine rich repeats that provides structural rigidity and transmits structural motion. Activation antibodies to integrin heterodimers tend to bind this region. Finally, there is a β tail domain at the C-terminus that is cysteine rich and disulphide links the α helix with a β sheet to confer structural rigidity.
1.2.1.2.3 Affinity modulation versus clustering.

Two modes of regulating integrin binding to their counter receptors have been recognised. It was thought that increased binding of integrins was due to an increase in the affinity of the heterodimers due to a shift in its conformation. More recently it has been recognised that heterodimers, upon activation with chemokine or TCR triggering (for T cells), cluster into patches on the cell surface membrane. Numerous signalling pathways have been implicated in each modality of integrin activation and a further discussion of this will be detailed later (section 1.2.1.3.2). A number of observations implicated clustering as an important mechanism in integrin activation. This included the observation that active LFA-1 clustered on cells that expressed this integrin and that treatment of cells with phorbol esters allowed LFA-1 to become more mobile in the plasma membrane (Kucik et al., 1996).

1.2.1.2.4 Genetic disorders of integrin mediated adhesion.

Analyses of rare genetic disorders resulting in impaired leukocyte adhesion have aided the elucidation of the pathways important in integrin function in vivo. A number of related disorders have been described. The leukocyte adhesion deficiency (LAD-1), described over twenty years ago, results in an increased susceptibility to bacterial infection. The defect is caused by a number of point mutations in the gene of the β2 chain that impairs expression (Kishimoto et al., 1987). LAD-2 patients have defective biosynthesis of fucosylated glycans, which contain the carbohydrate ligands for both endothelial and leukocyte selectins (Etzioni et al., 1992). More recently a LAD-3 disorder has been identified that demonstrates defects in G-protein coupled signalling,
specifically a defect in Rap1 activation and impaired stabilisation of integrins (Kinashi et al., 2004).

1.2.1.3 Triggering.

1.2.1.3.1 The role of chemokines and their receptors in integrin activation.

Integrin activation is crucial for firm adhesion of leukocytes to the vasculature and subsequent entry into the tissue compartment. It has become increasingly apparent that chemokines are potent mediators of this event. Chemokines comprise a large family of at least 43 members and are small chemotactic cytokines (10-14 kDa) defined by their amino acid composition (Rot and Von Andrian, 2004). There are two major families, the CC and CXC family members denoted by a tetra-cysteine motif, where either the first two cysteines are separated from each other by a non-conserved amino acid or lie adjacent to each other. In addition, the molecules CX3CL1 (separated by three non-conserved residues), XCL1 and XCL2 (both lack two of the cysteines that would normally constitute the tetra-cysteine motif) are included in the family (Bazan et al., 1997). The NH2 terminus of some CXC chemokines contains a Glu-Leu-Arg (ELR) motif, preceding the first cysteine residue. Members that contain this motif are potent promoters of angiogenesis. The chemokine family possess a range of biological functions including proliferation, apoptosis, maturation and differentiation but focus will be restricted to their role in adhesion and migration.

Chemokines mediate their effects by binding seven spanning G-protein coupled receptors (binding affinity $10^{-9}$/10$^{-9}$ M). A family of 19 chemokine receptor family members have been described (Figure 1.6). Original studies of chemokine dependent integrin activation in leukocytes were performed with neutrophils (Buyon et al., 1988)
Figure 1.6 Chemokines and their receptors.

Solid (agonist) and dashed (antagonist) chemokines bind to their corresponding receptors. The dashed-dotted line that connects CXCR3 with CCL11 represents a nonagonist-nonantagonist binding interaction. The bars next to individual chemokine numbers reflect the colours assigned to their apposite receptors (A). Rot A, von Andrian UH. Reprinted from Chemokines in innate and adaptive host defence: basic chemokinese grammar for immune cells. Annu Rev Immunol. 2004; 22: 891-928.

There are two major families, the CC and CXC family members denoted by a tetra-cysteine motif. The first two cysteines are separated from each other by a non-conserved amino acid or lie adjacent to each other. In addition, the molecules CX3CL1/Fractalkine (separated by three non-conserved residues), XCL1/Lymphotactin and XCL2 (both lack two of the cysteines that would normally constitute the tetra-cysteine motif) are included in the family (Bazan et al. 1997) (B). Reprinted from Prog Retin Eye Res. 2004 Jul; 23(4): 435-48. The role of chemokines and their receptors in ocular disease. Wallace GR, John Curnow S, Wloka K, Salmon M, Murray PI.
and subsequently, studies with lymphocytes demonstrated a role for chemokines in triggering events on vascular endothelial in secondary lymphoid organs (Haugland and Buschmann, 1995). The receptors are coupled to G-protein, and their activation proceeds via a transient increase in intracellular calcium (Vaux et al., 1992). They can serve as chemoattractants for mononuclear phagocytes and lymphocytes and their expression may be regulated by TNF family cytokines (Merrifield et al., 2001). A transient increase in intracellular calcium was seen for CCL7 does not (Merrifield et al., 2001). Extrapolation of such results, however, must be carefully considered with respect to the expansion of chemokines in vivo. Vascular localization of CCL22 has not been detected at sites where this pathway could play a role in the characterization of endothelial-expressed chemokines. CCL22 promotes the maturation of memory T cells to a Th2 phenotype. The CXCR3 receptor is suggested to be a potential mediator of T cells to VCAM-1; however, CXCL12 in media expose CXCR3 receptor antagonists. This occurs as CXCL2 receptor antagonists in static conditions.
and subsequently, studies with lymphocytes demonstrated a role for chemokines in triggering arrest on vascular endothelium in secondary lymphoid organs (Bargatze and Butcher, 1993). Chemokine receptors are coupled to Gαi proteins, and their effects can be inhibited by pertussis toxin due to ADP ribosylation of the α subunit preventing association with the receptor. It was later demonstrated that chemokines were differentially expressed in a range of tissues and often presented in the context of glycosaminoglycans (GAGs) (Tanaka et al., 1993).

Recent studies highlight the importance of studying chemokine mediated integrin adhesion in the context of physiological shear stresses. Chemokines under static conditions can mediate adhesion that does not occur under flow where inadequate cell surface receptor levels, receptor affinity or coupling to signalling pathways exist. Flow based adhesion assays have demonstrated the differential ability of CCR4 ligands CCL17 and CCL22 to mediate VLA-4 dependent adhesion of T cells to VCAM-1 (D'Ambrosio et al., 2002). The increased on-off rate of CCL22 receptor engagement appears to be important in this process and is not appreciable in static adhesion assays. In addition, CCL22 rapidly mediates CCR4 receptor desensitisation whereas CCL17 does not (Mariani et al., 2004). Extrapolation of such results, however, must be carefully considered with respect to the expression of chemokines in situ. Vascular localisation of CCL22 has not been detected at sites where this pathway would be important (i.e. the skin) and a more detailed characterisation of endothelial-expressed chemokines in situ is required. The expression of apical chemokines in the context of GAGs promotes the induction of firm adhesion. However, more recently chemokines have been implicated in tethering and diapedesis as well as the triggering step of lymphocyte homing (Cinamon et al., 2001).
Potential signalling mechanisms.

The mechanisms underlying inside-out signalling for the triggering of integrins by chemokine receptors has until recently been poorly characterised. A number of putative pathways are emerging.

The GTPase RhoA becomes activated upon CXCL8 treatment of neutrophils and has been implicated in experiments pre-treating neutrophils and lymphocytes with a C3 transferase exoenzyme that specifically inhibits RhoA (Laudanna et al., 1996). The resulting loss of adhesion to VCAM-1 (and fibrinogen) implicates RhoA in VLA-4 and Mac-1 dependent adhesion of lymphocytes and neutrophils respectively. Supporting these findings, experiments employing inhibitory peptides to block the effector domains of RhoA highlight a role for this protein in activation of integrin high affinity states and cell surface lateral mobility. (Giagulli et al., 2004).

PKC ζ is an atypical PKC family member and in studies pretreating neutrophils with CXCL8, a rapid translocation to the cell membrane was observed (Laudanna et al., 1998). This was inhibited either with inhibitory peptides to PKC ζ (abolishing neutrophil binding to fibrinogen) or treatment with C3 exoenzyme that abolished recruitment of the kinase to the membrane. Inhibitory peptides were also employed in studies of lymphocyte adhesion to ICAM-1 demonstrating a critical role in modulating the lateral mobility but not affinity of LFA-1 (Giagulli et al., 2004).

PI3 kinase (PI3K) is important in lymphocyte adhesion as indicated by inhibition studies which abrogate chemokine induced adhesion to ICAM-1, but importantly only
at low density of ICAM-1 [500-1500 sites/\(\mu m^2\)] (Constantin et al., 2000). PI3K modulates lateral mobility of integrin heterodimers rather than modulating integrin affinity.

The involvement of Rap-1 in LFA-1 dependent adhesion has recently been highlighted. In T cells, chemokine or antigen stimulated activation of Rap-1 resulted in co-localisation with LFA-1. Expression of a constitutively active Rap-1 increased the affinity and avidity of LFA-1 for ICAM-1. This interaction was dependent on the GTP bound form of RAP-1. A yeast two-hybrid screen identified an association with a protein RAPL and the resulting complex co-immunoprecipitated with LFA-1. Furthermore binding was abolished upon mutation of specific lysines within the \(\alpha\) chain of LFA-1 (Katagiri et al., 2003).

1.2.1.4 Transendothelial migration/Diapedesis.

The final and least well understood stage of the adhesion cascade involves transendothelial migration (TEM) (or diapedesis) of leukocytes across the vascular endothelium and penetration of the basement membrane into the tissue interstitium. In most circumstances leukocytes pass between adjacent endothelial cells via interactions with endothelial junctions, however it has also been suggested that under some circumstances they can directly traverse endothelial cells by a transcellular route or preferential entry sites at tricellular junctions (Burns et al., 2000). A number of molecules have been implicated in transmigration of leukocytes through endothelial junctions.
CD31 has been implicated for a number of years in the transmigration of leukocyte subsets. Blocking antibodies to this receptor have demonstrated significant blocking of transmigration (approximately 90 %) (Bogen et al., 1994). However, TEM is never completely abrogated and targeted deletion of this receptor in mice does not significantly affect inflammatory recruitment (Duncan et al., 1999). CD31 is an alternatively spliced member of the Ig superfamily and is expressed on a number of cell types including leukocytes and endothelial cells (Wong et al., 2000). Homophilic interactions between endothelial and leukocyte expressed CD31 (through Ig domains 1 and 2) mediate TEM (Sun et al., 1996). Intravital microscopy demonstrated this homophilic interaction subsequently upregulates α6β1 (Dangerfield et al., 2002), which in association with matrix metalloproteinases (MMPs), mediates penetration of the basement membrane into the tissue parenchyma.

Another member of the Ig superfamily of proteins has also recently been implicated in TEM. The junctional adhesion molecule (JAM) family of proteins comprises three family members. JAM-A is expressed on epithelia and to a lesser extent on endothelia (Martin-Padura et al., 1998). It is concentrated at tight junctions but is expressed on the apical surface with cytokine treatment. In addition, it is also expressed by leukocytes and can form homophilic interactions either between adjacent endothelial cells or between leukocytes and endothelium. Interestingly, JAM-A also binds LFA-1 and has been implicated in adhesion as well as TEM (Ostermann et al., 2002). JAM-B has a more restricted expression pattern principally on HEVs of secondary lymph nodes (Palmeri et al., 2000). JAM-B mediates homophilic interactions like JAM-A but in addition binds two leukocyte ligands, namely JAM-C (Arrate et al., 2001) and VLA-4 (Cunningham et al., 2002). JAM-C does not make homotypic interactions but
binds JAM-B (Cunningham et al., 2002) and Mac-1 (Santoso et al., 2002). Its role in adhesion and TEM requires clarification.

CD99 is a cell surface receptor that is O-glycosylated and expressed on T cells and endothelium. Like CD31 it forms homotypic interactions (demonstrated in studies of monocytes) and antibody blockade impairs TEM (Schenkel et al., 2002). Blockade results in partial TEM across the junction and CD31 combined with CD99 blockade completely abolishes TEM.

At adherens junctions, the cadherin VE-cadherin has been implicated in TEM. This cadherin associates with cytosolic catenins (through its cytoplasmic tail) for functional activity and deletion of the cytoplasmic tail results in reduced adhesion (Navarro et al., 1995). Over-expression of this mutant acts as a dominant negative. Antibodies to VE-cadherin increase vascular permeability and advance homing in vitro and in vivo (Gotsch et al., 1997). Studies employing adenoviral-mediated infection of HUVEC with a VE-cadherin-GFP fusion protein have demonstrated removal of this molecule from junctions during neutrophil TEM (Shaw et al., 2001).

Vascular adhesion protein (VAP-1) (170 kDa) is expressed on endothelial cells. It is upregulated at sites of inflammation in vivo but is largely unresponsive to proinflammatory cytokine treatment in vitro. It was detected by a monoclonal antibody (clone 1B2) raised against human synovial endothelium and was subsequently shown to mediate lymphocyte binding to HEVs in secondary lymph nodes (Salmi and Jalkanen, 1992). VAP-1 is glycosylated and neuraminidase treatment reduces binding. VAP-1 was implicated in tethering, however recent studies
employing in vitro flow assays of T cell adhesion to primary liver endothelial monolayers implicates VAP-1 in both adhesion (with ICAM-1 and VCAM-1) and TEM (Lalor et al., 2002). In addition, neutrophil TEM in an in vivo inflammatory model was abolished by inhibition of VAP-1 (Koskinen et al., 2004). The mechanism of VAP-1 dependent TEM is unclear but tantalising hints to a mechanism are suggested by the observation that this receptor has enzymatic activity (Smith et al., 1998). Specifically, semicarbazide inhibitors abrogate the monoamine oxidase activity of the receptor. The ligand for this molecule is unknown although it has been suggested that VAP-1 catalyses the deamination of primary amines on the surface of lymphocytes.

Other molecules including occludins (Oshima et al., 2003), claudins (Nitta et al., 2003), CD146 (Solovey et al., 2001) and integrins like α2β1 and α5β1 (Lampugnani et al., 1991) have been associated with TEM (based on expression) however the significance of these results requires further investigation.

1.2.2 Tissue specific homing.

Tissue specific homing of lymphocytes was implied from the observation that lymphocytes isolated from a particular tissue or draining lymph node when transferred into the peripheral blood, tend to traffic back to the same location. Indeed, it has been demonstrated that dendritic cells from Peyer’s patches (but not other secondary lymphoid) imprint on T cells the ability to selectively traffic to the small intestine (Mora et al., 2003).
Homing in secondary lymphoid organs has been extensively characterised and confirms a number of features of the multistep adhesion cascade. Lymphocytes have two main access routes into secondary lymphoid organs, via the afferent lymphatics or High endothelial venules (HEVs). The bulk of lymphocytes gain efficient access through HEVs, however activated lymphocytes and mature dendritic cells can exit tissues via lymphatics and enter into local draining LN. The mechanism of lymphocyte specific trafficking through HEVs is well characterised. There is a requirement for L-selectin expression for tethering (Gallatin et al., 1983) (as blocking antibodies to L-selectin or peripheral node addressin (PNAd) block adhesion), CCL19 and CCL21 (and to a lesser extent CXCL12/SDF-1) for activation (Stein et al., 2000) and LFA-1-ICAM-1 interactions for firm adhesion (Warnock et al., 1998) This is exemplified by the fact that other leukocyte subsets are deficient in trafficking to secondary lymphoid organs. Granulocytes express L-selectin and LFA-1 but not CCR7. Dendritic cells express CCR7 and LFA-1 but not L-selectin consistent with their requirement to enter via lymphatics rather than HEV. Effector T cells show low levels of both CCR7 and L-selectin together with high expression of LFA-1, CXCR3 and CCR5 which targets their homing to sites of inflammation. However, so-called central memory T cells which lack immediate effector function express CCR7 and L-selectin enabling them to recirculate between the blood and the lymphatics (Sallusto et al., 1999).

The importance of the CCR7-CCL19/CCL21 pathway was demonstrated in the plt/plt (paucity of LN T cells) mouse (Nakano et al., 1997). This mouse fails to develop normal lymph nodes and was subsequently found to lack the genes for CCL19 and the
CCL21 ser form. Subsequently, CCR7−/− deficient mice were generated confirming the importance of this pathway (Forster et al., 1999).

Tissue specific homing to other organs has been best documented for skin and gut. Lymphocytes that traffic to the skin are characterised by high expression of CLA (Picker et al., 1990). This O-glycan linked receptor mediates tethering interactions with dermal venular expressed E-selectin, which can be blocked with the HECA-452 mAb (Berg et al., 1991). Activation of integrins is mediated by CCR4 which binds to CCL17/TARC and in flow based models CLA+ T cells undergo CCR4 dependent arrest on ICAM-1 (Campbell et al., 1999). Recent studies support a role for CCR8 in physiological trafficking to the skin (Schaerli et al., 2004).

Lymphocytic infiltration of the small intestine is critically dependent on lymphocyte binding to endothelial expressed MAdCAM-1 (Berlin et al., 1993). Whereas naïve T cell use L-selectin to bind MAdCAM-1 in mesenteric lymph nodes, the recruitment of memory T cells to the gut is dependent on an integrin, α4β7 expressed by mucosal lymphocytes, which can also bind to MAdCAM-1 to mediate arrest. Furthermore, CCR9 is expressed on these lymphocytes allowing them to respond to the chemokine TECK/CCL25 (Zabel et al., 1999) the expression of which is restricted to the small bowel epithelium and mucosal vessels.

1.2.3 Microenvironmental homing.

1.2.3.1 T cell polarisation.

Upon penetration of the basement membrane and access to the tissue interstitium, lymphocytes are poised to respond to an array of chemotactic cues that guide them to
specific microenvironments. They migrate through matrix by rearranging cytoskeletal actin and polarising their structure upon exposure to chemoattractants (Vicente-Manzanares et al., 1998). This polarisation of structure allows attachment of the leading edge to the substratum with concomitant detachment of the uropod (rear end). This interaction is combined with myosin-based contraction that generates traction (Rey et al., 2002). Moreover, These events allow the cell to move forward with renewed extension of the leading edge in an F-actin dependent manner.

There is a marked difference between non-polarised lymphocytes that maintain a homogenous distribution of cell surface receptors and polarised lymphocytes that demonstrate an asymmetric plane consisting of a leading edge and a uropod. Both cell surface and intracellular components are localised to these distinct regions of the lymphocyte. The leading edge consists of an enriched source of cytoskeletal components including F-actin, myosin light chain and talin (Vicente-Manzanares et al., 2002). The leading edge is also enriched in GM-3-marked lipid rafts (Gomez-Mouton et al., 2001), chemokine receptors including (CXCR4, CCR5, and CCR2) (Nieto et al., 1997) and have clusters of β1 integrins. The uropod is a slender region that extends from the substratum and consists of receptors like CD43, CD44, ICAM-1 (del Pozo et al., 1995) and PSGL-1 (Bruehl et al., 1997) as well as ezrin/radixin/moesin cytoskeletal components (Serrador et al., 1997). The polarized lymphocyte is then able to migrate into a specific subcompartment of the tissue responding to this chemoattractant network. In the context of lymphoid organs, lymphocytes are densely packed within a largely cellular environment with little interaction with the ECM. Indeed, collagen fibrils are sheathed in a reticular network.
Conversely, T cells within peripheral tissues are extensively in contact with collagen and other ECM proteins.

1.2.3.2 Microenvironmental homing.

The most comprehensively characterised organ with respect to microenvironmental positioning is the lymph node. The importance of CXCR5 and CCR7 has been shown in several murine models. CXCR5+ B cells were able to enter the spleen and Peyer’s patches but were unable to exit the interfollicular T-zone as they could not respond to follicular expressed BCA-1/CXCL13 (Forster et al., 1996). In addition, CXCR5 in collaboration with CCR7, positions B cells at the T-B interface. B cell activation results in upregulation of CCR7 and movement to this boundary responding to T zone expressed CCL19/CCL21. Retroviral mediated gene transfer (and overexpression) of B cells with CXCR5 prevented this (Reif et al., 2002). The importance of CXCR5-CCR7 axis is also true for a subset of follicular homing CD4+ T cells that express the marker CD57 and migrate to this T-B interface upon antigen stimulation to mediate T-dependent B cell responses (Kim et al., 2001).

1.2.3.3 Leukocyte emigration.

Much is known regarding leukocyte infiltration of tissues with respect to adhesion to the vascular endothelium. However, the mechanism of exit of leukocytes from tissue has remained elusive. Investigations with the drug FTY720 have revealed the importance of the sphingosine-1-phosphate (S1P) pathway in mediating lymphocyte emigration from lymphoid organs as treatment of mice with this drug severely reduces lymphocyte egress and results in marked lymphopenia (Mandala et al., 2002). The phosphorylated form of the drug binds 4 of the 5 sphingosine-1 phosphate receptors
(Brinkmann et al., 2002). Targeted disruption of sphingosine-1 phosphate receptor 1 in mice haematopoietic cells severely impairs their exit from the thymus and other lymphoid organs and FTY720 downregulates these receptors and impairs S1P dependent exit (Matloubian et al., 2004).

1.2.4 Leukocyte infiltration of human tumours.

Many tumours consist of leucocyte infiltrates often comprising macrophages, T cells, and in some tumours additionally eosinophils, B cells, NK cells and granulocytes. The presence of these cells in tumours can correlate with prognosis. Macrophage infiltration of tumours is often associated with poor prognosis (Bingle et al., 2002). Lymphocytic infiltration of tumours has been associated with positive clinical prognosis in ovarian cancer (Zhang et al., 2003) and colorectal carcinoma (Diederichsen et al., 2003). Conversely, in HL tumours, the presence of granzyme expressing CTLs correlates with a poor clinical outcome (Oudejans et al., 1997; ten Berge et al., 2001). In spite of a complex association between leukocyte infiltrates and cancer prognosis, the presence of such cells in tumours like HL, allows us to determine the mechanisms these cells employ to enter tumours, and to exploit these pathways for immunotherapy.

1.3 Cellular based immunotherapies for the treatment of human cancer.

Currently one in three people in their lifetime will be diagnosed with invasive cancer. Conventional therapy consists of surgery, radiotherapy or chemotherapy. These treatments only cure half of all individuals treated, and thus a fourth modality of cancer therapy, immunotherapy, is being devised. It aims to alter the body’s immune system such that it recognises and destroys tumour cells.
Immunotherapy in recent years has had a resurgence of interest with the success of monoclonal antibody therapy (i.e. Rituximab), in the treatment of particular cancers. A disadvantage of this kind of therapy, however, is the inability of antibody therapy to penetrate solid tumours. In addition, the therapy is of a passive nature and does not confer long lasting immunity to the tumour. In the last two decades, investigations into the use of cellular based therapies for the treatment of cancers refractive to conventional treatment have been sought. Clearly a complete account of immunotherapies is beyond the scope of this thesis, however, a discussion of strategies, both active and passive in the generation of T cell responses to tumours will be considered.

13.1 Active strategies.

To confer long lasting immunity to tumours, various vaccination strategies have been explored. A brief description of some of the active cellular strategies that may induce anti-tumour T cell responses will be discussed.

13.1.1 Tumour cells.

Classically autologous or allogeneic tumour cells (or cell extracts) were employed for immunisation purposes. A caveat to this approach is the inability of these cells to efficiently induce immune responses. To circumvent this patients have been vaccinated with genetically modified tumour cells. Strategies examined include transduction of tumour cells with cytokines and growth factors including GM-CSF, IL-2, TNFα, IL-12 and IFN-γ (Colombo and Rodolfo, 1995) as well as immune co-stimulatory molecules including CD80 and CD86 (Loskog et al., 2002).
1.3.1.2 Protein/peptide based vaccines

With the identification of novel tumour antigens there exists the possibility of vaccinating patients with specific proteins or peptides derived thereof. An advantage of a peptide strategy is the ease of manufacture but it is dependent on prior knowledge of the epitope and its restricting allele. High frequencies of CD8+ T cells were detected in five HLA A2 patients following immunisation with peptide derived from the melanoma tumour antigen gp100 (Powell and Rosenberg, 2004; Rosenberg et al., 1998). Alternative approaches employed recombinant virus to deliver short MAGE-1 and MAGE-3 sequences that coded for peptides presented by HLA class I. In four patients with regression, three demonstrated specific CTL responses (Karanikas et al., 2003). In addition to existing epitopes, known epitopes can also be mutated to produce a peptide that binds HLA with higher affinity and is more immunogenic (Linard et al., 2002).

An advantage of whole protein vaccines is the ability of the cell to degrade and present a range of peptides on HLA molecules including ones that are uncharacterised. This is particularly pertinent for putative epitopes targeted by CD4+ T cells, that until recently have been largely ignored. The ability to manufacture recombinant protein presents considerable financial and regulatory issues, however an example of such an approach includes vaccination with a fusion protein containing the C-terminal CD4 epitope rich region of EBNA-1 fused to full length LMP2. This is currently being evaluated in a phase I clinical trial for Nasopharyngeal carcinoma (Taylor et al., 2004).
13.1.3 Antigen presenting cell based vaccines.

Of particular interest recently has been the potential therapeutic vaccination of cancer patients with a professional antigen-presenting cell (APC) (exemplified by the dendritic cell) that has enhanced ability to prime T cell responses. The ability to purify these APCs from blood (or bone marrow) and pulse with antigen has re-ignited the possibility of a successful therapeutic vaccination approach for treating cancer (Marten et al., 2002). Antigen can be delivered to APCs exogenously in the form of peptide, whole protein or tumour cell lysate. Alternatively, tumour antigens can be expressed endogenously following infection of APC with recombinant virus or transfection with purified RNA or DNA.

In melanoma, sixteen patients were vaccinated with blood derived DCs pulsed with tumour lysate or a peptide cocktail. Specific CTL were recruited to the DTH challenge site with objective responses in five patients (Nestle et al., 1998). Another melanoma trial vaccinated eleven patients (stage IV melanoma) with a MAGE-3A1 peptide. This induced specific CTL in 8/11 patients and regression of metastasis in six patients (Thurner et al., 1999). A major advantage of this approach is that DCs in principle can prime naïve T cell responses and can induce immunological memory to the tumour. A limitation is the current requirement for ex vivo loading of DCs with tumour antigen. The ability to vaccinate DCs in vivo would greatly enhance the applicability of this strategy.

13.2 Passive strategies.

The holy grail of cancer immunotherapy is to therapeutically (or even prophylactically) vaccinate cancer patients and induce potent and sustained immunity
to the tumour. In many ways studies of adoptive cellular transfer (ACT) of large numbers of high avidity tumour specific T cells is the benchmark by which vaccination based approaches must be compared. To this end, a number of studies have investigated the use of ACT in the treatment of cancer. In this treatment regime, anti-tumour cells are isolated and expanded to high frequencies ($10^{10}$) \textit{ex vivo}. These cells can be optimally activated and reinfused back into the patient.

Early studies of ACT employed lymphokine activated killer (LAK) cells to eradicate tumours (Grimm et al., 1982) and anti-tumour activity was demonstrated with the infusion of LAK cells with IL-2 (Rosenberg et al., 1987). It was later recognised that IL-2 alone was as efficacious (Rosenberg et al., 1993). A second generation of ACT involved isolation and expansion of tumour-infiltrating lymphocytes (TIL) in high dose IL-2 (~ 1000 IU/ml). These cells provided anti-tumour activity in the treatment of melanoma and renal cell carcinoma (Figlin et al., 1997; Topalian et al., 1988). In these early studies, the antigen specificity of these cells was uncertain and concomitant administration of high dose IL-2 had side effects including vascular leak syndrome (Rosenstein et al., 1986). More recently, thirteen patients were treated with autologous T cells and high dose IL-2 with nonmyeloablative conditioning. Oligoclonal populations of MART-1 or gp100 TIL were generated, and following infusion these cells persisted \textit{in vivo} and trafficked to tumour deposits. Objective partial responses were observed in six patients (Dudley et al., 2002).

EBV is associated with several human tumours (including HL). These tumours provide an important model system in which to evaluate T cell therapies as the T cell response to this virus has been extensively characterised. Studies of EBV associated
post transplant lymphoma have demonstrated both the safety and efficacy of treating patients with EBV specific T cells (Papadopoulos et al., 1994; Rooney et al., 1995; Rooney et al., 1998b). 39 patients received prophylactic infusions of polyclonal CTL lines. No patients developed lymphoma following stem cell transplantation. This compared with 12% from the control group. In two patients who declined prophylactic treatment, cells were infused after diagnosis of lymphoma. In both cases there was resolution of the tumour. Interestingly, Infused T cells remained in the circulation for more than three months (several years if measured by PCR).

In PTLD, EBV expresses a full latent gene program including immunodominant antigens and therefore is a best case for adoptive T cell therapy (Figure 1.7). Other EBV malignancies express a more restricted latency program that excludes the immunodominant antigens (Figure 1.7). Indeed, EBV positive HL and NPC tumours express subdominant antigens (Deacon et al., 1993; Fahraeus et al., 1988) although promisingly T cell responses to epitopes from these proteins have been detected in the peripheral blood of healthy donors and weak responses detected in a minority of HL and NPC patients (Chapman et al., 2001; Lee et al., 2000) (Figure 1.8). As yet there is no evidence of relevant antigen specific cells at the site of HL and NPC tumours. However, the ability to generate the relevant cells for ACT provides a suitable rationale for the selective expansion of T cell clones directed against epitopes from these proteins. CTL lines from 11/15 HL patients were successfully produced by LCL reactivation (Rooney et al., 1998a). Interestingly, levels of TCR ζ chain were low in peripheral T cells but increased in established CTL lines. Three patients were infused (with gene marked cells) with minimal clinical responses. One patient had pleural effusions, which contained enrichment of the marker gene compared with peripheral
The latent infection of primary human B lymphocytes in vitro (LCL) is classed as a latency III pattern with the expression of the full complement of latent proteins including EBNA 1, EBNA 2, EBNA 3A, EBNA 3B, EBNA 3C, EBNA –LP (leader protein), LMP1, LMP2A and LMP2B as well as BARTs (spliced Bam A rightward transcripts) and two EBERs (nonpolyadenylated RNAs). This latent program is detected in post transplant lymphoproliferative disease (PTLD). Other EBV associated malignancies demonstrate the existence of two other latency programs. A latency I phenotype is present in Burkitt’s lymphoma with the expression of EBNA 1 protein, EBERs and BARTs. A Latency II pattern exhibits expression of EBNA 1, LMP1 and LMP2 as well as EBERs and BARTs and is typical of Hodgkin’s lymphoma and NPC.
Latency I

EBNA 1

BL

Latency II

NPC

HL

EBNA 1

LMPs 1, 2

Latency III (LCL)

EBNAs 1, 2

3A, 3B, 3C, LP

LMPs 1, 2

PTLD
Figure 1.8 T cell epitopes defined in HL-associated proteins.

CD4 and CD8 T cell target proteins expressed in HL tumours are represented with the corresponding mapped epitopes and restricting alleles. nd=not done. Adapted from Lee 2002.
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<th>Target protein</th>
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<th>Amino acid residues</th>
<th>HLA restriction</th>
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blood. Two patients had an initial reduction in peripheral blood EBV load. These initial investigations highlight some of the challenges of generating high avidity, tumour specific T cells from patients with HL.

1.3.3 Monitoring immune responses to tumours.

Regardless of the therapeutic strategy employed, it is vital to have an accurate and sensitive measure of the immune response. Previous readouts of this included the observation of delayed type hypersensitivity responses or measuring cytolytic activity. These methods are of low sensitivity or require in vitro manipulation and correlate poorly with clinical outcome. Recently, a number of sensitive assays that require little in vitro manipulation have been employed.

For studies of adoptive cellular therapy (ACT) infused cells can be tracked by infecting them with a retrovirus expressing a marker gene like neomycin. The marker gene is integrated into the genome and marked cells can be tracked by PCR (Economou et al., 1996). Safety issues include a lack of knowledge of where the marker gene will integrate within the host genome. However, incorporation of a suicide gene like thymidine kinase (from herpes simplex virus) enables destruction of the marked cells by treating patients with gancyclovir if the infused cells become abnormal (Junker et al., 2003). An obstacle to employing thymidine kinase is the possible immunogenicity of transgene-induced cells as well as the requirement for drug selection (Riddell et al., 1996). An alternative approach involved transducing T cells with a nerve growth factor receptor, and a Fas based suicide construct. Transduced cells were then isolated by immunomagnetic selection (Berger et al., 2003).
Alternatively, if knowledge of the TCR β chain sequence is known, then sensitive real time PCR can be employed to track clonally expanded antigen specific populations expressing a specific TCR clonotype, by using a 5' primer specific for the Vβ subtype and a constant region specific reverse primer. Indeed, the skewing of clonotypic responses can be assessed by analysing TCR Vβ cDNA clones using 5’rapid amplification of cDNA ends (RACE) (Boudinot et al., 2002). An alternative to realtime PCR is the use of Vβ specific antibodies, which provides multiparametric characterisation of antigen specific T cell populations (Khan et al., 2002).

HLA- peptide tetramers have revolutionised the analysis of antigen specific immune responses and such reagents can be employed where the antigen specificity and the restricting allele are known (Altman et al., 1996). The use of tetramer or multimer reagents in the in situ analysis of specific T cells in tissues is currently being pursued with some success (Haanen et al., 2000).

The immune response can be monitored functionally and this has been aided in particular with the use of the sensitive ELISpot assay for release of functional cytokines like IFN-γ from individual cells (Czerkinsky et al., 1988) and intracellular cytokine staining (Jung et al., 1993). Typically the monitoring of these responses has been performed on peripheral blood samples. Although technically challenging it is also important to characterise the nature of the response at the site of the tumour, as tumour specific T cells will have ideally trafficked to tumour deposits rather than remain in the peripheral circulation.
1.3.4 Caveats and future challenges for cell-based therapies.

1.3.4.1 Are T-cells of sufficient avidity and specificity?

The recent ability to transfer functional TCR from one T cell to another clone and express functional TCR α and β chains of T cells has greatly enhanced the feasibility of generating T cells of a required specificity and avidity. A library of cloned α and β chains could be generated and retrovirally transduced into T cells programming the appropriate specificity (Clay et al., 1999). Safety issues must be taken into consideration including the possible misspairing of transduced α and β chains with endogenous chains resulting in a chimeric TCR. Other options include the production of allorestricted T cells specific for an allo-HLA/peptide complex. For example, T cells from a HLA A2 negative donor stimulated with a HLA A2 target expressing appropriate peptide can produce allorestricted clones that are of high avidity and specificity that were not deleted during ontogeny (Munz et al., 1999).

1.3.4.2 Do T cells traffic to the tumour site?

The ability of T cells to traffic to the tumour site is critical for an anti-tumour response. Gene-marking studies and labelling of cells with indium-111 indicate that tumour reactive T cells can be detected at melanoma deposits after infusion (Fisher et al., 1989). In addition to tracking T cells it may also be desirable to modify their homing. For example, Induced expression of CXCR4 on T cells in leukaemia or bone marrow metastatic tumours should facilitate their recruitment in bone marrow where the CXCR4 ligand CXCL12 is highly expressed (Aiuti et al., 1997). Furthermore, expression of CXCR5 might facilitate localisation of T cells to BCA-1 expressing follicles in the treatment of follicular lymphoma (Husson et al., 2002). Finally, expression of CXCL1 by melanoma cells may allow them to recruit CXCR2
transduced T cells (Kershaw et al., 2002). Vaccination based approaches do not allow for the ex vivo modification of T cells and thus the route and mode of vaccination is likely to be critical in the nature (e.g. specificity of homing) of immune effectors generated.

1.3.4.3 Do T-cells retain function at the site of the tumour?

There is evidence that if anti tumour T cells gain entry to the tumour milieu they may encounter an environment that is hostile to an effective anti-tumour response including the presence of the anti-inflammatory cytokine IL-10 (de Waal Malefyt et al., 1991). This may be due to the direct impairment of T cell function. For example, in colorectal carcinoma (Yoong and Adams, 1998) and HL (Rooney et al., 1998b), isolated TIL demonstrated reduced expression of the TCR signalling chain ζ compared with controls. Alternatively, altered T cell function might occur at the level of the dendritic cell with impaired ability to prime T cell responses. Infiltrating macrophages are able to produce TGFβ and PGE2 that can suppress DC function (Fadok et al., 1998) and the expression of IL-10 by the tumour (at least in murine models) can tolerise the resident DC population (Yang and Lattime, 2003). Furthermore, it has been suggested that regulatory T cells are present at the site of tumours including HL (Marshall et al., 2004) and are a potent source of immunosuppression in the tumour.

1.4 The aetiology and pathology of Hodgkin’s lymphoma.

1.4.1 Pathology of Hodgkin’s lymphoma.

Hodgkin’s lymphoma (HL) is an unusual cancer that is characterised by a paucity of neoplastic tumour cells, namely the Hodgkin and Reed-Sternberg cells (H-RS) which
comprise less than 1% of the tumour mass. These cells have an abundant cytoplasm typically with 2-3 nuclei, each with a prominent nucleolus. There is a non-neoplastic infiltrate that consists of lymphocytes, plasma cells, histiocytes, and eosinophils. The tumour often has extensive areas of fibrosis. The presence of H-RS cells per se is not diagnostic of HL, as they have been observed in infectious mononucleosis, non-Hodgkin’s lymphomas as well as some carcinomas and sarcomas (Iacobuzio-Donahue et al., 2002; Lukes et al., 1969). Rather, it is the presence of these cells in the context of the background inflammatory infiltrate that provides the diagnosis of HL.

**1.4.1.1 HL subtypes**

The REAL/WHO classification (Harris et al., 1994) subdivides HL into classical HL and nodular lymphocyte predominant HL (a distinct clinical and aetiological disease). Classical HL is subdivided into four types including Nodular sclerosing (NSHL), mixed cellularity (MCHL), lymphocyte rich (LRHL) and lymphocyte depleted HL (LDHL).

MCHL comprises approximately 25% of HL cases and is the subtype most frequently associated with EBV (60-70% of cases) (Glaser et al., 1997). It consists of abundant normal inflammatory cells with comparatively frequent numbers of H-RS cells (5-15 H-RS cells per high-powered field). MCHL patients are typically elderly and present with symptoms including fever, sweats, weight loss and often have abdominal involvement and advanced disease. This subtype is more common in the developing world.

LDHL is rare (less than 1%) and recently some cases have been reclassified as non-Hodgkin’s lymphomas. It typically presents as advanced disease and histopathologically is characterised by tumour cells that outnumber normal cells.
NSHL is the most common subtype (60-70% of HL cases) although it is less common in the developing world. It is equally represented amongst men and women and tends to present in young adults and adolescents. It has a good prognosis although this subtype can be subdivided into two variants, NSI (low grade) and NSII (high grade) defined in terms of the degree of fibrosis and number of H-RS cells. NSHL has two defining features, namely the presence of collagen bands that divide the node into nodules and the presence of H-RS variants called lacunar cells that stand out from the reactive infiltrate when fixed in formalin based fixatives.

A recent subtype has been identified and included in the REAL classification scheme. Lymphocyte rich classical HL has H-RS cells that have the immunophenotype of other classical HL subtypes (CD30+, CD15+, CD20-, CD45-) but has an infiltrate similar to non-classical LPHL (i.e. B cells rather than T cells) and a clinical presentation resembling LPHL. This subtype can have a nodular or diffuse pathology and resemble NSHL, MCHL or LPHL. H-RS cells are rare and eosinophilia is typically not observed.

Non-classical nodular lymphocyte predominant Hodgkin lymphoma (nLPHL) is distinct both clinically and aetiologically. It comprises 5-10% of HL cases and is typically observed in children or the elderly. It is more common in men (3:1). The pathology consists of effacement of the Lymph node architecture with an abundance of normal polyclonal B cells. Interspersed within this background are H-RS variants called lymphocytic and histiocytic (or L+H/popcorn) cells. These cells have multilobed nuclei and there is little fibrosis within the tissue.
1.4.2 Aetiology of HL and its association with EBV

Hodgkin’s lymphoma is considered a disease derived from germinal centre B cells. When B cells enter the germinal centre they are selected for productive non-self reacting B cells and are rescued from apoptosis. Normally, B cells enter the dark zone of the germinal centre and undergo the germinal centre reaction characterised by somatic hypermutation of Ig V genes. This reaction increases antibody affinity and those cells that express low affinity or self-reactive B cell receptors (BCR) are deleted by apoptosis. H-RS cells are rescued from apoptosis in spite of crippling mutations that allow Ig V gene rearrangement but abolish functional BCR gene expression (Kuppers et al., 1994). The surviving cell is defective in expression of the normal B cell program including the lineage markers BCR, CD20 or CD79a. In addition, mutations in the octamer region of the Ig promotor may result in an inability of transcription factors to bind to this region (Theil et al., 2001). Complimenting this is the lack of Ig specific transcription factors detected in HL cells (both cultured and primary) (Stein et al., 2001). Somewhat paradoxically, although H-RS cells are typically germinal centre B cell derived, the B lineage developmental expression of Pax5 is absent (Hertel et al., 2002). The T lineage marker Notch1 (and GATA3) is expressed and counteracts Pax5 possibly halting the B cell program (Jundt et al., 2002).

Apoptosis of B cells produced in the germinal centre reaction is mediated by a Fas dependent mechanism. Studies on two H-RS cell lines indicate that they may be resistant to Fas mediated killing in spite of functional Fas expression (Re et al., 2000). H-RS cells however express inappropriate levels of c-FLIP, which is anti-apoptotic
and prevents expression of the apoptotic program (Dutton et al., 2004; Thomas et al., 2002).

H-RS cells maintain deregulated expression of transcription factors including NFκB which is constitutively active in H-RS cell lines (Bargou et al., 1996). This activity enhances proliferation and reduces sensitivity to apoptosis that can be relieved experimentally by introducing a dominant negative IκBα that prevents nuclear translocation of NFκB (Bargou et al., 1997). In addition, it was demonstrated that a proportion of cases have mutations in IκBα or gene amplification of the NFκB/Rel locus (Cabannes et al., 1999; Joos et al., 2002). These observations implicate the NFκB pathway in deregulated H-RS cell growth and survival.

Additional transcriptional deregulation has also been described in H-RS cells and includes members of the STAT family of which STAT3 is constitutively expressed, independently of IL-6 dependent JAK activation (Kube et al., 2001). Furthermore, STAT6 is constitutively expressed and is dependent on IL-13 expression, which is expressed with its receptor on these cells (Skinnider et al., 2002).

AP1 is another transcription factor that is constitutively expressed and regulates the cell cycle regulator cyclin D2 (Mathas et al., 2002). In summary, a number of putative pathways have been disrupted in HL to facilitate escape from apoptosis and allow clonal proliferation.
EBV has been implicated in 40% of HL cases as an aetiological agent in the development of HL (Glaser et al., 1997). Primary infection with EBV occasionally results in infectious mononucleosis (IM), IM patients are 2-3 times more likely to develop HL. Furthermore, antibody titres to EBV are elevated in HL patients (Mueller et al., 1989). At the molecular level, EBV gene or protein expression (detected by ISH or IHC) is detected in the tumour cells (Herbst et al., 1991; Wu et al., 1990). EBV positive HL cells express a restricted latency program including the Qp promoter driven nuclear antigen EBNA1 (required for maintenance of the viral episome) and the latent membrane proteins LMP1, LMP2a and LMP2b, as well as BamH1A transcripts (Deacon et al., 1993). LMP-1 behaves as a constitutively active CD40 driving NFκb pathways and upregulating bcl-2 and A20 (Gires et al., 1997). Furthermore, transgenic expression of LMP1 in mouse models confirms the oncogenic nature of this protein with the development of B cell lymphomas (Kulwichit et al., 1998). LMP-2a shuts down signalling through the B cell receptor (BCR) by competing for tyrosine kinases that bind ITAM regions of the BCR signalling chain. This blocks B cell activation and entry of EBV into the lytic cycle and concomitantly substitutes for the BCR by constitutively activating pro-survival downstream signalling components (Caldwell et al., 1998).
1.5 Rationale and plan of investigation.

Recent years have seen important advances in the area of T cell therapy for human malignancies. EBV-associated tumours like HL occupy a key position as models in this field. This thesis addresses the possibility of a T cell-based therapy for HL, by exploring the molecular mechanisms of T cell homing to this tumour. The work has focused on two main areas of research:

1) Identification of chemokines, chemokine receptors and adhesion molecules that mediate T cell recruitment into HL.

a) Immunohistochemical and flow cytometric studies.

Using frozen HL tumour biopsies, tumour cell suspensions and available cell lines, flow cytometry and immunohistochemistry was used to study the expression of (i) a panel of chemokines and their receptors, and (ii) integrins, selectins and their corresponding ligands to determine which might contribute to the cellular infiltrate. Staining of HL biopsies was compared with reactive lymph nodes and TILs from other tumours. For chemokine receptors expressed on infiltrating T cells, immunohistochemical studies were performed to determine whether the relevant chemokines were present on the tumour cells and the vascular endothelium.

Endothelial tissue within HL biopsies was examined by immunohistochemistry for expression of adhesion receptors. In complimentary studies, expression of the ligands for these receptors was determined on the tumour infiltrating lymphocytes (TILs). Adhesion receptor expression on the tumour was also assessed as this may determine whether a T cell is retained at the tumour site.
b) Functional studies.

Using available cell lines derived from HL tumour biopsies, the ability of individual chemokines to mediate T cell migration in an in vitro chemotaxis assay was studied. These studies used activated T cells derived from healthy laboratory donors expanded in vitro with interleukin-2.

Following the immunohistochemical studies outlined above, adhesion molecules of interest were tested functionally in an in vitro tumour tissue-binding assay using activated T cells and HL biopsy sections in the presence and absence of relevant blocking antibodies.

2) Analysing the expression and function of these molecules on T cells expanded in vitro for adoptive T cell therapy.

Having identified molecules that appear to mediate T cell recruitment into HL tumours, it is important to determine whether these molecules are expressed and function correctly on clones prepared for adoptive T cell therapy. EBV or melanoma tumour antigen specific T cell clones were isolated for this purpose and expanded to large numbers using a Rapid Expansion Protocol (REP). These clones were studied both by flow cytometry and using the functional assays for chemotaxis and cell adhesion. T cells were sampled at different time points during the REP and under different culture conditions to determine the optimal stage at which cells could be infused for an adoptive therapy.
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Chapter 2: Materials and Methods

2.1 Materials.

2.1.1 Clinical material.
Snap frozen tissue sections from 10 HL cases were kindly provided by Prof. John Crocker (Heartlands hospital, Birmingham, UK) (Table 2.1). Paraffin-embedded sections (10 HL cases) were obtained from Dr. Paul Murray, (University of Birmingham, Birmingham, UK) (Table 2.1). For flow cytometric analysis of tissue infiltrating lymphocytes, cell suspensions from HL cases were kindly provided by Prof. Ruth Jarrett (University of Glasgow, Glasgow, UK) (Table 2.1). Tonsil explants were obtained from Mr. Adrian Drake Lee (Queen Elizabeth hospital, Birmingham, UK). Clinical material from patients with colorectal carcinoma was kindly obtained by Dr. Beatrix Hunter (University of Birmingham, Birmingham, UK). Clinical material from patients with Renal cell carcinoma was provided by Dr. Ciaran Lynch (University of Birmingham, Birmingham, UK). T cells clones generated for a clinical trial of adoptive T cell therapy were provided by Dr. Steve Lee (University of Birmingham, Birmingham, UK) (Table 2.2). All clinical material was obtained after having obtained prior informed consent and ethical approval (South Birmingham research ethics committee LREC 5493, 5673 and MREC01/0/58).

2.1.2 Buffers, media and solutions.
Phosphate-buffered saline (pH 7.4) : (Oxoid, Basingstoke, UK) 160 mM NaCl, 3 mM KCl, 8 mM disodium hydrogen phosphate, 1 mM potassium dihydrogen phosphate in H2O. The pH was adjusted to pH 7.4 with HCl (Fisher chemicals, Loughborough, UK).
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* EBV status of tumour determined by *in situ* hybridization for EBER1 expression.

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<td>A2</td>
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<td>DR11</td>
<td>CD4</td>
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Table 2.2 Details of T cell clones generated for infusion therapy.
0.05 M Tris buffered saline (pH 7.4): 50 mM tris(hydroxymethyl)aminomethane (Sigma-Aldrich, Gillingham, UK), 139 mM NaCl (Sigma-Aldrich, Gillingham, UK) in H₂O. The pH was adjusted to pH 7.4 with HCl (Fisher chemicals, Loughborough, UK).

0.01 M Citrate buffer (pH 5.8): 10 mM citric acid (Sigma-Aldrich, Gillingham, UK) in H₂O. The pH was adjusted to pH 5.8 with NaOH (Fisher chemicals, Loughborough, UK).

Complete medium: RPMI 1640, 10 % foetal calf serum (heat inactivated, Invitrogen, Renfrew, UK), 2 mM glutamine (Sigma-Aldrich, Gillingham, UK), 100 µg/ml streptomycin (Sigma-Aldrich, Gillingham, UK), 100 IU/ml penicillin (Sigma-Aldrich, Gillingham, UK).

PHA blast media: Complete medium supplemented with 30 % MLA 144 supernatant, 50 IU/ml rhIL-2 (Chiron, Middlesex, UK).

MACS buffers:
Running buffer: PBS, 2mM EDTA, 0.5% BSA (Miltenyi biotech, Bisley, UK).
Rinsing solution: PBS, 2mM EDTA (Miltenyi biotech, Bisley, UK).
Cleaning solution: 70 % v/v ethanol.

Naphthol AS-MX substrate: 0.2 µg/ml naphthol AS-MX phosphate (Sigma-Aldrich, Gillingham, UK), 1/50 dimethylformamide (Sigma-Aldrich, Gillingham, UK),
1mg/ml fast red salt (Sigma-Aldrich, Gillingham, UK), 1 mM Levamisole (Sigma-Aldrich, Gillingham, UK).

2.2 Methods.

2.2.1 Tissue manipulation.

2.2.1.1 Tissue collection and preparation.
Fresh tumour or control biopsy material was collected within 1 hour of explantation from patients. Explants were cut into 1-cm³ blocks, snap-frozen in embedding media (Cryo-M-Bed, Bright, Huntingdon) in liquid nitrogen and stored at -70 °C until further use. Paraffin embedded explants were processed in the department of pathology. This was kindly done by Gary Reynolds (University Hospital Birmingham, Birmingham, UK).

2.2.1.2 Isolation of lymphocytes from tissue
Tissue explants were collected, rinsed with RPMI 1640 to remove any traces of blood and then disaggregated using a scalpel or Medimachine (Dako, Ely, UK). Samples were then either used immediately for phenotypic investigations or cell suspensions were centrifuged on Ficoll density gradients to purify mononuclear cells and stored frozen at -180 °C (described below).

2.2.2 Cell culture and manipulation.

2.2.2.1 Cryopreservation of cells
Cells suspensions were chilled on ice for 20 minutes and collected by centrifugation (538 g/ 5 minutes). The cell pellet was resuspended in 1 ml ice-cold media (RPMI 1640, 10 % DMSO (Fisher scientific, Horsham, UK), 30 % FCS (Invitrogen,
Renfrew, UK) and added to a pre chilled cryovial. The vial was stored overnight at -80 °C. The vial was transferred to long-term storage over liquid nitrogen at -180 °C.

Cells were recovered by rapid thawing of the vial at 37 °C. The cell suspension was transferred to a 15 ml tube and cold RPMI was added drop wise to cells to a volume of 5 ml. The cells were collected by centrifugation. The pellet was washed a second time in complete media.

### 2.2.2.2 Isolation of PBL from whole blood

Heparinised venous blood was taken from healthy adults and patients. This was diluted 1:1 with RPMI 1640 and layered 2:1 on lymphoprep density gradient solution (Robbins scientific, Wilmslow, UK) in 50 ml sterile universals, and centrifuged at 758 g for 30 minutes. (no brake) at room temperature. The mononuclear cells were removed from the density gradient and washed at 538 g for 10 min, 355 g for 5 minutes and 254 g for 5 minutes with RPMI 1640. A cell count was performed using a haemocytometer and lymphocyte viability was determined by Trypan-blue exclusion: equal volumes of 0.4 % Trypan-blue solution (Sigma-Aldrich, Gillingham, UK) and lymphocyte suspension were mixed, put into the haemocytometer chamber and examined using light microscopy. In all experiments the cell viability was 97-99%.

### 2.2.2.3 Immunomagnetic cell sorting

Cells were washed in RPMI 1640 and collected by centrifugation (255 g, 5 minutes). Cells were resuspended at 70 x 10⁶ cells/ml in buffer (PBS, 2 % FCS, 1 mM EDTA). 100 µl/ml of CD8 enrichment cocktail (Stemcell technologies, London, UK) was added to the suspension and incubated for 15 minutes at room temperature. 60 µl/ml
of magnetic colloid (Stemcell technologies, London, UK) was added to the suspension and incubated for 15 minutes at room temperature. The CD8+ fraction was collected by passing the cell suspension through an AutoMACS (Milteny biotech, Bisley, UK) machine (running depleteS program).

2.2.2.4 Generation and expansion of T lymphoblasts.
Peripheral blood lymphocytes were resuspended at 1x10^6 cells/ml in PHA blast media (see above) supplemented with 10 µg/ml PHA (Bio-stat, Stockport, UK). Cells were subsequently maintained and expanded in PHA blast media (without PHA) by feeding every 3-4 days.

2.2.2.5 Rapid expansion of T cells clones (REP)
T cell clones demonstrating specific recognition of antigen-positive targets in an ELISpot assay were selected. 5x10^4 Clones were expanded in 14-day cycles by using anti-CD3 antibody (OKT3, Orthoclone; Janssen-Cilag, High Wycombe, UK) at 30 ng/ml, irradiated (4000 rads) mixed allogeneic PBL (10^6 cells/ml), irradiated (4000 rads) allogeneic lymphoblastoid cell lines (2 x 10^5 cells/ml), and IL-2 (Chiron, Middlesex, UK) at 50 IU/ml every 3 days. The anti CD3 antibody was only added to the culture on day 0 of the REP cycle (and was washed off on day 4-5 of the cycle).
For studies of resting and activation of T cell clones the following conditions were employed:

1. Resting experiments were performed on day 14. Cells were washed thoroughly and resuspended in complete medium. After 24 hours, cells were collected and analysed by flow cytometry.
2. Activation experiments were performed on day 14. Cells were collected, resuspended in complete medium supplemented with 200 ng/ml of anti CD3 and anti CD28 (Pharmingen, Oxford, UK). After 24 hours, cells were collected and analysed by flow cytometry.

3. Day 14 cells were immediately analysed by flow cytometry.

2.2.2.6 Cell lines.
The H-RS cell lines used in this study were obtained from J. Wolf, V. Diehl, and H. Kamesaki. HD-LM2, L540, and L428 derive from advanced cases of nodular sclerosis subtype, whereas KM-H2 and L1236 were derived from advanced cases of mixed cellularity subtype; all of the lines are EBV genome negative. L591 is an EBV positive cell line and was obtained from the ATCC. This cell line was established from a pleural effusion of a female patient with nodular sclerosing HL. All HL cell lines were cultured in complete medium at densities between 0.2-1 x 10^6 cells/ml. Lymphoblastoid cell lines (LCL) were kindly provided by healthy laboratory donors and were generated in vitro by transformation of B cells using the standard EBV isolate B95.8. LCLs were maintained in complete medium.

2.2.3 Phenotypic analysis of homing markers.
2.2.3.1 Antibodies and related reagents.
Details of primary antibodies employed are in table 2.3.

2.2.3.2 Flow cytometry.
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<td>Mouse IgG2b</td>
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<tr>
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<td>R&amp;D</td>
<td>48607.121</td>
<td>Mouse IgG2b</td>
</tr>
<tr>
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<td>R&amp;D</td>
<td>61828.111</td>
<td>Rat IgG2a, κ</td>
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<tr>
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<td>Pharmingen</td>
<td>1G1</td>
<td>Mouse IgGl</td>
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<td>2D7/CCR5</td>
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<td>P4G9</td>
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<td>Pharmingen</td>
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Table 2.3 Primary monoclonal antibodies employed in study.

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<td>Alexa Fluor® 350 goat anti-mouse IgG(H+L)</td>
<td>Molecular Probes</td>
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<tr>
<td>Alexa Fluor® 350 goat anti-rabbit IgG(H+L)</td>
<td>Molecular Probes</td>
</tr>
<tr>
<td>Alexa Fluor® 594 goat anti-mouse IgM (µ chain)</td>
<td>Molecular Probes</td>
</tr>
<tr>
<td>Alexa Fluor® 594 goat anti-rabbit IgG(H+L)</td>
<td>Molecular Probes</td>
</tr>
<tr>
<td>Anti-fluorescein/Oregon Green® goat IgG fraction Alexa Fluor® 488 conjugate</td>
<td>Molecular Probes</td>
</tr>
<tr>
<td>Anti-Goat IgG(whole molecule)-TRITC antibody produced in rabbit</td>
<td>Sigma-Aldrich</td>
</tr>
<tr>
<td>Anti-Mouse IgG(whole molecule)-TRITC antibody produced in goat</td>
<td>Sigma-Aldrich</td>
</tr>
<tr>
<td>Goat Anti-Mouse IgG human ads.-BIOT</td>
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</tr>
<tr>
<td>Streptavidin Alexa Fluor® 594 conjugate</td>
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</table>

Table 2.4 Secondary reagents employed in study.
Cells were washed twice (PBS /2 % FCS), collected (538 g /5 minutes) and resuspended at 1-5×10⁵ cells/ml in PBS/2 % FCS. Cells were incubated with pre-titred concentrations of antibody in a final volume of 50 µl. The reaction was incubated on ice for 30 minutes. The cells were washed twice in PBS / 2 % FCS. Second and third step antibodies were also incubated on ice for 30 minutes. Cells were either analysed immediately or fixed in 2 % paraformaldehyde (Fisher scientific, Horsham, UK). Flow cytometry was performed on a Coulter Epics XL flow cytometer (Beckman Coulter) using System II software (Beckman Coulter). Analysis was performed using the software package WinMDI (Written by Joe Trotter, 1993-1998; trotter@scripps.edu).

2.2.3.3 Immunohistochemistry and Immunofluorescence.

2.2.3.3.1 Cryosectioning.

4-10 µm thick sections were cut by placing tissue blocks in a cryostat (-20 °C) for 20 minutes prior to sectioning (Clinicut 4000-001, Bright instruments, Huntingdon, UK). Sections were cut and thaw mounted onto vector bond coated slides (Vectorbond reagent, Vector Laboratories, Peterborough, UK), and dried for an hour at room temperature. Sections were wrapped in foil and stored at -80 °C for up to 6 months.

2.2.3.3.2 Fixation.

Snap frozen tissue sections were fixed for 10 minutes in 100 % acetone (at room temperature) (Fisher scientific, Horsham, UK).

2.2.3.3.3 Antigen retrieval.

Paraffin-embedded sections were deparaffinised in two changes of xylene (Fisher scientific, Horsham, UK), and rehydrated in two changes of 95 % ethanol. Slides (Surgipath, Peterborough, UK) were then washed in running tap water and endogenous peroxidase blocked with 3 % H₂O₂ (Sigma-Aldrich, Gillingham, UK) in
methanol for 10 minutes. Slides were briefly washed in tap water and immersed in 0.01 M citrate buffer (see above). Slides were heated in a conventional microwave oven for 20-50 minutes at 800 watts. Buffer was allowed to cool for 10 minutes and slides were washed in tap water briefly.

2.2.3.3.4 Blocking.
Optional blocking steps were performed to reduce background staining. Non-specific binding of secondary reagents was reduced by diluting secondary reagents in buffer (TBS or PBS) + 20 % normal serum (Dako) from the same species as the secondary antibody. Endogenous avidin/biotin activity was reduced with a blocking kit (Dako). Endogenous alkaline phosphatase activity was reduced with 1 mM levamasole. (Sigma-Aldrich, Gillingham, UK)

2.2.3.3.5 Incubation with primary antibodies.
Sections were rehydrated in PBS (or TBS for alkaline phosphatase based detection) and incubated overnight at 4 °C (or room temperature for 1h.) with the primary antibody in an appropriate dilution in PBS (or TBS). All incubations of tissue with antibody were carried out in a moisture box to prevent sections from drying out. Polyclonal antibodies to chemokines were purchased from R&D systems.

2.2.3.3.6 Indirect two and three-step staining /substrate visualisation.
Alkaline phosphatase based staining:
After washing off unbound primary antibodies in TBS, sections were incubated for 45 minutes at room temperature with rabbit anti-mouse immunoglobulins (Dako) diluted 1:25 with TBS. After washing in TBS, the sections were incubated for 45 minutes at room temperature with alkaline phosphatase-anti-alkaline phosphatase complex
(APAAP) (Dako) diluted 1:50 in TBS. Sections were washed and the monoclonal antibody was detected using the alkaline phosphatase substrate solution containing fast red substrate. This consisted of 10 mg Naphthol-Phosphate AS-MX, free acid (Sigma-Aldrich, Gillingham, UK) dissolved in 1 ml of N,N-Dimethlyformamide (Sigma-Aldrich, Gillingham, UK), 50 µl of 1M Levamisole (Sigma-Aldrich, Gillingham, UK) and 49 ml TBS, pH 8.2. 50 mg Fast red salt (Sigma-Aldrich, Gillingham, UK) was added to the buffer, which was filtered immediately before incubating with the sections for 15 minutes. The sections were rinsed with distilled water.

Peroxidase based staining:
After washing off unbound primary antibodies in PBS, Bound antibody was detected using a standard ABC immunoperoxidase method (Universal vectastain Elite ABC kit, Vector Laboratories, Peterborough, UK) (for mouse IgG primary antibodies) or HRP-conjugated anti goat and anti rabbit Ig (to detect rabbit and goat IgG secondaries respectively) and visualized with the 3,3 diaminobenzidine-based detection reaction (Sigma-Aldrich, Gillingham, UK).

Fluorescent based staining:
After washing off unbound primary antibodies in PBS, bound antibody was detected employing appropriate fluorophore conjugated secondary reagents at pre-titred concentrations (Table 2.4).

\section*{2.2.3.3.7 Nuclear staining.}
Nuclei were counterstained with Mayers’ Hematoxylin (BDH, Poole, UK).
2.2.3.8 Mounting.

For Immunohistochemistry, Sections were mounted with a coverslip and aqueous based mounting media (Immunomount, Shandon, Runcorn, UK) and air-dried before microscopic examination. For Immunofluorescence, sections were mounted with Prolong antifade mounting media (Cambridge biosciences, Cambridge, UK).

2.2.3.9 Controls.

Tonsil was used as a positive control. Negative controls consisted of consecutive test sections in which primary antibody was replaced with non-immune serum of the same subclass (for polyclonal antibodies) (R&D systems) or concentration- and isotype-matched negative control antibodies (for monoclonal antibodies) (Dako).

2.2.3.4 Microscopy.

Immunohistochemical images were recorded by brightfield microscopy (Zeiss-Axiovert 25, Welwyn Garden City, UK) with SPOT software v2.1(Diagnostic instruments, Sterling Heights, USA) Immunofluorescent images were recorded by confocal microscopy (Zeiss LSM510 ) employing laser lines at 351, 488 and 543 nm. Individual images were merged to provide a composite.
2.2.4 Functional Assays.

2.2.4.1 Flow-based adhesion Assay.

To determine the effects of physiological blood flow on lymphocyte adhesion, glass capillary tubes (Camlab Ltd, Cambridge, UK) were coated with 5µg/ml purified VCAM-1 (R&D systems, Abingdon, UK) (1h/37°C) and co-immobilised with CXCL9, 10, and 11 (0-1000 ng/ml) (1h/37°C) (PeproTech EC Ltd, London, UK). This was connected to the flow system as previously described (Lalor et al, 1997). Lymphocytes (10⁶ cells/ml) were perfused through the microslide at a shear stress of 0.05 Pa. Adherent lymphocytes were observed by phase-contrast microscopy using an Olympus IX50 inverted microscope (Olympus Ltd., Southall, Middlesex, UK). Adhesion was converted to cells per square millimeter and corrected for the number of lymphocytes perfused (i.e. adherent cells per square millimeter per 10⁶ perfused). Phase contrast video recordings made during lymphocyte perfusion were analysed off-line to determine the percentage of rolling cells, statically adherent cells, and total adhesion. Rolling cells moved slowly over the microslide surface during 5-10 s of observation, while stationary adherent cells made no discernable movement over the same period.

2.2.4.2 Tissue binding assay.

Cryostat sections (10 µm) of control or tumour tissue were cut onto vectorbond coated glass slides and fixed in acetone for 10 minutes. IL-2 expanded lymphoblasts were adjusted to a concentration of 10⁷ cells/ml in medium (RPMI 1640 and 0.5 % BSA (Sigma-Aldrich, Gillingham, UK)). Sections were preincubated with control mAb or blocking mAb for 30 minutes at room temperature before addition of 100 µl of cell suspension to each section with constant rotation (60 rpm) for 30 minutes at 4 °C.
Sections were gently washed with cold PBS to remove nonadherent lymphocytes and fixed in acetone for 10 minutes. Vascular endothelium and adherent lymphocytes were identified by anti-CD31 immunostaining, and developed with APAAP and fast red. The sections were counterstained with Mayer’s Hematoxylin (BDH, Poole, UK). The assays were done in the presence of a concentration and isotype matched control mAb or blocking concentrations (50 μg/ml) of mAb. Experiments were performed in triplicate and the number of lymphocytes adherent to vascular endothelium (detected with anti-CD31 or VAP-1) was counted using an ocular grid on every section. 100 vessels were randomly selected and counted on every tissue section. The number of lymphocytes adherent to endothelium in the presence of control mAb defined 100 % binding, and the number binding to vascular endothelium in the presence of blocking mAb was expressed as a percentage of this.

2.2.4.3 Transwell Chemotaxis assays.

Cells were washed twice in RPMI 1640 and enumerated. The pellet was incubated with 100 μCi Na$_2$^{51}$CrO$_4$ for 1 to 2 hours (Amersham, Little Chalfont, UK) at 37 °C/ 5 % CO$_2$. The cells were washed twice with RPMI 1640 (to remove any unincorporated chromium). Cells were resuspended at 5 X 10$^6$ cells/ml in RPMI 1640/0.5 % BSA and 100 μl of cell suspension was added to the upper chamber of a 3-μm pore size transwell filter (Appleton woods, Birmingham, UK). The transwell was then added to the well of 24 well Tissue culture plate containing 600 μl stimulus. The transwell chamber was incubated at 37°C/ 5% CO$_2$ for 1-6 hours and the migrated cell population was quantitated using a gamma counter (Packard Cobra Gamma counter, GMI, Albertville, USA). The assay was performed in triplicate with the mean and standard deviation calculated. Background migration was assessed by using
chemotaxis media alone as a stimulus and 100% migration was defined by adding 100 µl of cell suspension directly to the bottom of the chamber. Recombinant human chemokine was purchased from R&D systems (Abingdon, UK).

2.2.5 Statistical Analysis.

Statistical analyses were performed using Graphpad InStat (San Diego, CA, USA). HL patient flow cytometry data were analysed using One Way Analysis of Variance or Students t test. Patient clones were analysed by Mann-Whitney rank sum or Kruskal-Wallis tests. Statistical advice was provided by Dr Cindy Billingham from the clinical trials unit at the University of Birmingham.
Chapter 3: A phenotypic and functional characterisation of T cells infiltrating Hodgkin’s lymphoma.

3.1 Introduction.

T cells infiltrate tumours such as HL, and knowledge of the mechanisms employed by T cells to gain access to the tumour environment is important if novel cell based therapies are to be rationally generated and evaluated. A plethora of chemokines have been implicated in the pathogenesis of HL, including CXCL10/IP10, CXCL9/MIG, CCL5/RANTES, CCL3/MIP1α and CCL11/eotaxin based on increased transcript expression in tumour biopsies compared to LN controls (Teruya-Feldstein et al., 1999). Of particular interest was the finding that TGF-β produced by H-RS cell lines can induce secretion of eotaxin by resident fibroblasts which may account for the influx of eosinophils and TH2 cells expressing the receptor CCR3 (Jundt et al., 1999). CCL17/TARC expression was reported on the H-RS cells themselves and might directly recruit CCR4+ TH2 cells to the tumour (van den Berg et al., 1999).

To accurately define the relevant pathways in T cell recruitment to HL tumours, a comprehensive analysis of chemokine receptor expression on freshly isolated T cells was undertaken. To analyse the microenvironmental location of chemokine receptor expressing T cells, a triple immunofluorescence staining protocol was developed. With identification of highly or differentially expressed receptors, expression of the corresponding ligands was examined in situ. To dissect which receptor-ligand pathways were physiologically relevant, functional migration assays were developed.
3.2 Results.

3.2.1 The homing phenotype of intratumoural T cells.

3.2.1.1 CD3+/CD4+ T cells comprise the major T cell sub-population within HL tumours.

To analyse T cell populations in tumour infiltrates, flow cytometry was employed to analyse staining for the T cell markers CD3 and CD4 (Figure 3.1A). This was compared with isotype-matched controls to determine the proportion of CD3+/CD4+ cells (Figure 3.1B). The cell suspensions were kindly provided by Prof. Ruth Jarrett for this study and suspensions were produced as described in the materials and methods section (section 2.2.1.2).

Several comparative groups were studied to look for HL specific markers including inflamed tonsil (TL) as a reactive LN control and TIL isolated from two solid tumours (Colorectal (CRC) and renal cell carcinoma (RCC)). T cells from HL tumours (n=9) were predominantly CD4+ (82 ± 10 % SD) (Figure 3.1C). TL T cells (n=3) expressed a similar proportion of CD4 (78 ± 12 % SD), as did CRC TIL (76 ± 16 % SD) (Figure 3.1C). No significant difference was observed in the expression of CD4 between HL, TL, and CRC tumours (Figure 3.1C).

In RCC, T cell expression of CD4 from TIL was reduced (56 ± 3 % SD) compared with HL tumours although this did not reach statistical significance due to the small sample size (Figure 3.1C). The CD3+/CD4- populations within these tumours likely comprise CD3+/CD8+ T-cells and have been classified as such during this investigation.
Figure 3.1 Phenotypic analysis of CD4 expression on freshly isolated tumour infiltrating T cells.

Flow cytometry was employed to identify CD3+ and CD4+ populations (A) and to determine the expression of the CD4 antigen on the CD3+ T cell population compared with isotype controls (B). The proportion of CD3+/CD4+ T cells within the CD3+ population from different tissues was determined (C).

Cells were washed twice (PBS/2 % FCS), collected (538 g /5 minutes), and resuspended at 1-5x10^5 cells/ml in PBS/2 % FCS. Cells were incubated with saturating concentrations of anti CD3-PC5 (clone UCHT1) and anti CD4-PE (clone 13B8.2) conjugated antibodies in a final volume of 50 µl. The reaction was incubated on ice for 30 minutes. The cells were then washed twice in PBS/2 % FCS. Cells were either analysed immediately or fixed in 2 % paraformaldehyde. Cell analysis was done by flow cytometry. Lymphocytes were gated based on forward and side scatter profiles (A). Results were analysed with the software package WinMDI. Data represent mean ± SD percentage positive cells gated on the CD3+ population. Tissue infiltrating lymphocytes were isolated from HL (n=9), TL (n=3), CRC (n=5), and RCC (n=2) tissue.
CXCR3 was expressed on 47 ± 17 % SD of HL-derived CD3+ T cells with increased expression on CD8+ (74 ± 15 % SD) versus CD4+ (60 ± 20 % SD) subpopulations (p<0.01 ANOVA) (Figure 3.2). In TL, CXCR3 was also expressed predominantly on CD3+ cells (p<0.01 ANOVA) (Figure 3.2). In M1, tissue CXCR3 expression was lower than in TL-derived T cells (p<0.05 t-test) (Figure 3.2). Similarly, expression of CXCR3 was lower in TL-derived T cells than in HL.

CXCR4 was expressed on the majority of CD4+ T cells in HL (64 ± 15 % SD) and TL (74 ± 10 % SD) (Figure 3.3). Tissue levels of expression were higher on HL-derived T cells (p<0.05 t-test) (Figure 3.3, 3.4). CXCR3 expression varied between individual HL cases (Figure 3.4) but was lower than in CD4+ (45 ± 2 % SD) or CD8+ T cells (18 ± 10 % SD) (Figure 3.4). Similar results were obtained with TL.

Expression = 65.45%

% of CD3 T cells expressing CD4

<table>
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<th>TL</th>
<th>CRC</th>
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<td>%</td>
<td>80</td>
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3.2.1.2 A comparison of Chemokine receptor expression between tumour derived T cells and control T-cells implicate specific pathways of T cell recruitment.

Cells gated in the R2 and R3 region were further employed to look at subset specific expression of chemokine receptors (Figure 3.1A) on freshly isolated HL T cell populations. For comparison, HL T cells were compared with T cells from TL tissue.

3.2.1.2.1 CXC chemokine receptors.

CXCR3 was expressed on 47 ± 17 % SD of HL-derived CD3+ T cells with increased expression on CD8+ (74 ± 15 % SD) versus CD4+ (40 ± 20 % SD) subpopulations (p < 0.01 ANOVA) (Figure 3.2). In TL, CXCR3 was also expressed predominantly on CD8+ cells (p < 0.01 ANOVA) (Figure 3.2) but overall fewer TL-derived T cells expressed this receptor (23 ± 8 % SD CD3+ T cells) (p = < 0.05 t test) (Figure 3.2). Equally, levels of expression of CXCR3 were lower in TL-derived T cells than in HL TIL (Figure 3.4).

CXCR4 was expressed on the majority of CD4+ and CD8+ T cells in HL (84 ± 15 % SD) and TL (74 ± 10 % SD) (Figure 3.2). However, levels of expression were higher on HL-derived T cells (p<0.05 t test) (Figure 3.3,3.4).

CXCR5 expression varied between individual HL cases (Figure 3.4) but was predominantly expressed on CD4+ (42 ± 21 % SD) rather than CD8+ T cells (18 ± 10 % SD) (p< 0.05, ANOVA) (Figure 3.2). Similar results were obtained with TL-derived T cells (Figure 3.2).
Figure 3.2 Phenotypic analysis of chemokine receptor expression on freshly isolated tumour infiltrating T cells.

Flow cytometry was employed to determine the proportion of chemokine receptor positive cells on T cell subsets from HL tumours. The mean percentage positive cells of the total population were determined for CXC (left panels) and CC (right panels) chemokine receptors. HL T cells were compared with TL, CRC, and RCC T cells.

Cells were washed twice (PBS/2 % FCS), collected (538 g /5 minutes) and resuspended at 1-5x10^5 cells/ml in PBS/2 % FCS. Cells were incubated with pre-titred concentrations of chemokine receptor antibodies or isotype controls (mIgG1, CXCR3, CCR4; mIgG2a, CCR5; mIgG2b, CXCR4, CXCR5, CXCR6, CCR1, CCR2, CCR6; rIgG2a, CCR3, CCR7) in a final volume of 50 µl. The reaction was incubated on ice for 30 minutes. The cells were washed twice in PBS/2 % FCS. Visualisation was achieved with either anti-mouse or anti-rat IgG (biotin conjugated) followed by streptavidin Alexa 488. Cells were washed and incubated with normal mouse serum for 20 minutes. Finally, cells were incubated with saturating concentrations of anti CD3-PC5 (clone UCHT1) and anti CD4-PE (clone 13B8.2) conjugated antibodies. The reaction was incubated on ice for 30 minutes. The cells were then washed twice in PBS/2 % FCS. Cells were either analysed immediately or fixed in 2 % paraformaldehyde. Cell analysis was done by flow cytometry. Results were analysed with the software package WinMDI. Data represent mean ± SD. Significant differences between T cell subsets were determined by one-way ANOVA with post-test. Tissue infiltrating lymphocytes were isolated from HL (n=9), TL (n=3), CRC (n=5), and RCC (n=2) tissue.
CXCR6 was expressed on 21 ± 14 % SD of HL-derived CD8+ T cells (Figure 3.2). TL-derived T cells also expressed this marker on a minority of CD8+ T cells, which was significantly greater than the CD4+ population (p < 0.05, ANOVA) (Figure 3.2). However, in both HL and TL, levels of expression were relatively low (Figure 3.3).

3.2.1.2.2 CC chemokine receptor expression.

Few if any T cells in HL or TL expressed CCR1, CCR2 or CCR3 (Figure 3.2).

CCR4 was detectable on CD4+ T cells in HL although numbers of positive cells varied between cases (22 ± 16 % SD). In contrast, only a minority (8 ± 3 % SD) of CD3+ T cells in TL expressed this receptor (Figures 3.2 and 3.5).

The frequencies of CCR5-expressing T cells were similar between HL and TL cases (~ 20 % T cells) (Figure 3.2). In HL, equal proportions of CD4+ and CD8+ T cells expressed this receptor, whereas in TL more CD8+ T cells expressed CCR5 compared with CD4+ cells. (p< 0.05, ANOVA). This corresponded with higher cell surface levels of CCR5 on TL-derived CD8+ T cells (Figure 3.3).

Few if any T cells in HL or TL expressed CCR6 (Figure 3.2).

CCR7 expression was detectable on CD3+ T cells from both HL (59 ± 28 % SD) and TL (36 ± 13 %) with no difference between CD4+ and CD8+ subsets (Figure 3.2). Note that the frequency of CCR7+ T cells varied significantly between individual HL cases (20-80 %), as did levels of expression of this receptor (Figure 3.5).
Figure 3.3 Phenotypic analysis of chemokine receptor expression on freshly isolated tumour infiltrating T cells.

Flow cytometry was employed to determine the MFI (total cell population) of chemokine receptor positive cells on T cell subsets from HL tumours. MFI was determined for CXC (left panels) and CC (right panels) chemokine receptors. HL T cells were compared with TL, CRC, and RCC T cells.

Cells were washed twice (PBS/2 % FCS) and collected (538 g /5 minutes), resuspended at 1-5x10⁵ cells/ml in PBS/2 % FCS. Cells were incubated with pre-titred concentrations of chemokine receptor antibodies or isotype controls (mIgG1, CXCR3, CCR4; mIgG2a, CCR5; mIgG2b CXCR4, CXCR5, CCR6, CCR1, CCR2, CCR6; rIgG2a, CCR3, CCR7) as described in the legend to figure 3.2. Cell analysis was done by flow cytometry. Results were analysed with the software package WinMDI. Data represent mean ± SD. Significant differences between T cell subsets were determined by one-way ANOVA with post-test. Tissue infiltrating lymphocytes were isolated from HL (n=9), TL (n=3), CRC (n=5), and RCC (n=2) tissue.
Figure 3.4 Analysis of variation amongst individual HL and control samples for positively expressed CXC chemokine receptors.

Flow cytometry was employed to determine the percentage positive cells of the total population and MFI (total cell population) of CXC chemokine receptors on T cell subsets from HL and TL cases (Figure 3.4A). Representative histograms from patient 6766 denote percentage positive cells and MFI (total cell population) (Figure 3.4B).

Cells were stained as in the legend to Figure 3.2. Cell analysis was done by flow cytometry. Results were analysed with the software package WinMDI. Tissue infiltrating lymphocytes were isolated from HL TIL (n=9), and TL (n=3). Values were corrected for background staining by subtracting the isotype control values. (+) Represent TL cases and (X) represent HL cases.
Percentage positive cells of total population

+ Tonsil T cells
x HL T cells
B

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Figure 3.5 Analysis of variation amongst individual HL and control samples for positively expressed CC chemokine receptors.

Flow cytometry was employed to determine the percentage positive cells of the total population and MFI (total cell population) of CC chemokine receptors on T cell subsets from HL and TL cases. (Figure 3.5A) Representative histograms from patient 6766 denote percentage positive cells and MFI (total cell population) (Figure 3.5B)

Cells were stained as in the legend to Figure 3.2. Cell analysis was done by flow cytometry. Results were analysed with the software package WinMDI. Tissue infiltrating lymphocytes were isolated from HL TIL (n=9), and TL (n=3). Values were corrected for background staining by subtracting the isotype control values. (+) Represent TL cases and (X) represent HL cases.
Percentage positive cells of total population

+ Tonsil T cells
x HL T cells
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3.2.1.3 HL T cells have a distinct expression profile compared with T cells isolated from different tumours.

Few studies have examined chemokine receptor expression on tumour infiltrating T cells. To determine whether the homing phenotype identified on HL-derived T cells is unique to this tumour or represents a phenotype generally expressed on tumour-infiltrating T cells, the analysis was extended to include TIL from CRC (n=5), and RCC (n=2).

3.2.1.3.1 CXC chemokine receptors.

CXCR3 expression on CRC TIL (39 ± 25 % SD) was similar to that seen with HL-derived TIL (Figure 3.2). However, CXCR3 was not detected on RCC TIL.

CXCR4 was expressed on most CRC (82 ± 12 % SD) and RCC (97 ± 0.3 % SD) CD3+ T cells which is comparable with HL-derived TIL, (Figure 3.2). Cell surface levels of CXCR4 were particularly high on RCC T cells compared with T cells from all other tissues (Figure 3.3).

CXCR5 expression on CRC-derived TIL (21 ± 17 % SD) was similar to that seen in HL and TL, although CD4+ T cells did not predominantly carry it. In contrast, few T cells in RCC expressed CXCR5 (12 ± 1 % SD) (Figure 3.2).

In contrast to HL-derived TIL, CRC T cells expressed CXCR6 on a large proportion (67 ± 23 % SD) of T cells, including both CD4+ and CD8+ subsets (Figure 3.2), with high levels of cell surface expression (Figure 3.3). RCC T cells were similar to HL T cells with few cells expressing CXCR6 (Figure 3.2).
3.2.1.3.2 CC chemokine receptors

As in HL, few if any T cells in CRC and RCC expressed CCR1 or CCR3 (Figure 3.2). CCR2 was expressed on RCC-derived T cells (26 ± 4 % SD), with expression mainly restricted to CD4+ rather than CD8+ T cells (p<0.05, ANOVA) (Figure 3.2). CCR2 was also expressed on a minor population of CRC TIL (13 ± 7 % SD) (Figure 3.2) although levels of expression were low in both tumour types (Figure 3.3).

CCR4 was expressed on 22 ± 19 % of CD3+ T cells in CRC, which is similar to HL-derived TIL. In contrast, this receptor was expressed on few if any T cells in RCC (Figure 3.2).

In HL, only 23 ± 7 % SD of T cells carried CCR5. In contrast, this receptor was expressed at high levels on the majority of T cells infiltrating CRC (74 ± 25 % SD) and RCC (88 ± 2 % SD) with similar results for both CD4+ and CD8+ subsets (Figure 3.2 and 3.3).

CCR6 was poorly represented on HL- and RCC-derived T cells, but was expressed on 47 ± 25 % SD of CD3+ T cells in CRC (Figure 3.2). Expression in CRC was predominantly restricted to CD4+ rather than CD8+ T cells although this difference was not statistically significant due to the small sample size.

CCR7 was highly expressed on HL-derived TIL but few if any CRC nor RCC-derived T cells carried this receptor.
3.2.1.4 Chemokine receptor expressing T cells are found in association with H-RS cells and the vasculature within HL microenvironments.

Having identified chemokine receptors expressed on HL T cells by flow cytometry, it was important to confirm expression in situ and ascertain the relationship between T cells, tumour cells, and the vascular endothelium with respect to the expression of candidate chemokine receptors.

The expression of CXCR3 in situ, by single IHC staining of paraffin biopsies of HL cases (n=10) was first evaluated (Figure 3.6). This revealed strong staining of the non-neoplastic infiltrate, with cells that resembled lymphoid cells, whereas, cells with the morphology of H-RS cells were negative (Figure 3.6A). TL was employed as a positive control (Figure 3.6B) and positive staining was compared with a concentration and isotype matched negative control (Figure 3.6C).

To phenotype the CXCR3+ cells in detail, a triple fluorescent staining protocol was developed that worked on frozen tissue allowing the detection of multiple markers. This approach maximised the expression data that could be obtained from the clinical material available. For example, chemokine receptor expression could be determined on T cells, non-T cells and tumour cells, and the distribution of receptor positive cells within the tumour ascertained. This was particularly pertinent, as tissue morphology of some clinical material was poor, and ascertaining the cell type by morphology alone was problematic and observer dependent. An antibody to CD30 was employed to detect the rare H-RS cells and an antibody to CD3 allowed the detection of T cells. The appropriate chemokine receptor mAb was then stained in the third colour.
CXCR3 expression was examined by IHC (n=10) and IF (n=8) of paraffin and snap frozen biopsies. By IHC, CXCR3+ lymphoid cells were detected in the vicinity of cells having H-RS morphology (A). Staining was compared with positive control staining of hyperplastic TL (B) and concentration and isotype control staining from the same case (C).

5 µm paraffin sections were dewaxed, rehydrated and endogenous peroxidase activity blocked with 0.5 % H2O2 in methanol for 10 minutes, followed by a wash in tap water. Microwave pretreatment in 0.01 M citrate buffer, pH 5.8 (30 minutes) was performed. Anti-CXCR3 (clone 1C6) (20 µg/ml) was incubated overnight (4 °C). Reactivity was visualised with the Vector Elite secondary detection kit. Colour development was achieved with the addition of DAB substrate. Images were recorded by brightfield microscopy. Original magnification x600 (A-C).

CXCR3 expression was further investigated with triple IF staining for CD3 (blue), CD30 (green) and CXCR3 (red) (D). Individual images were merged to provide a composite with cells in pink representing CXCR3+ T cells. CXCR3+ T cells (pink) were also intimately associated with the vascular endothelium (green) (E)

5µm cryostat sections were fixed in 100 % acetone. Sections were blocked in PBS/20 % normal goat sera for 30 minutes. Staining was performed employing a 2-step protocol. Anti-CXCR3 (clone 1C6) (20 µg/ml) was incubated overnight (4 °C). Reactivity was visualised by incubation with anti-mouse IgG1 (biotin conjugated) followed by streptavidin Alexa 594 (A). Sections were subsequently blocked with PBS+20 % normal mouse sera. Anti-CD30 FITC (clone Ber-H2) and anti-CD3 (rabbit Ig) was incubated at room temperature for 45 minutes. Visualisation was achieved with the addition of anti-FITC Alexa 488 (B) and anti-rabbit Alexa 350 (C) secondary reagents for the CD30 and CD3 antigens respectively. Images were recorded by confocal microscopy (Zeiss LSM510) employing laser lines at 351, 488 and 543 nm.. Original magnification x630 (D, E). To examine the localisation of CXCR3 expressing cells with the vasculature (E), anti-CD30 FITC was replaced with anti-CD31 (clone B-B38) (1/20).
Triple immunofluorescence and confocal microscopy was employed to analyze the expression of CXCR3 in 5 μm snap frozen sections (Ki67) provided by Prof. Mike Crocker, Heartlands hospital, Birmingham. Control experiments were initially performed to ensure cross reactivity of secondary antihistone and antibodies. In HL-cocoon (n=8), CXCR3 reactivity could be detected on a high proportion of infiltrating T cells.
Triple immunofluorescence and confocal microscopy was employed to analyse the expression of CXCR3 in 5 µm snap frozen sections (kindly provided by Prof. John Crocker, Heartlands hospital, Birmingham). Control experiments were initially performed to ensure cross reactivity of secondary reagents did not occur. In HL cases (n=8), CXCR3 reactivity could be detected on a large proportion of infiltrating T cells as well as non-T cells, but was absent from the tumour cells (Figure 3.6D). CXCR3+ T cells were also intimately associated with the vascular endothelium (by staining for the endothelial marker CD31) (Figure 3.6E), however the endothelium was negative for CXCR3.

CXCR5 expression was detected by flow cytometry on freshly isolated T cells. Therefore, CXCR5 staining in situ was evaluated by single IHC staining (n=10) and was strongly expressed by the malignant H-RS cells (Figure 3.7A). Lymphoid cells were also reactive, and CXCR5 was observed on vascular endothelium including HEVs, which appeared to express higher levels than conventional vessels. This staining was compared with positive control staining in TL (Figure 3.7B), and isotype control staining from the same case (Figure 3.7C). Expression in situ was also assessed (n=8) with triple IF staining of snap frozen cases (Figure 3.7D). CXCR5 was detected on a proportion of T cells, as well as non-T cells, and strongly co-localised with CD30+ expressing H-RS cells. CXCR5 expressing T cells could be detected in the vicinity of H-RS cells.
Figure 3.7 *In situ* expression of CXCR5 in HL.

CXCR5 expression was examined by IHC (n=10) and IF (n=8) in paraffin and snap frozen biopsies respectively.

By IHC, CXCR5+ lymphoid cells were detected in the vicinity of cells having H-RS morphology (A). These H-RS cells strongly stained for CXCR5. Staining was compared with positive control staining of hyperplastic TL (B) and concentration and isotype negative control staining from the same case (C).

5 µm paraffin sections were dewaxed, rehydrated and endogenous peroxidase activity blocked with 0.5 % H₂O₂ in methanol for 10 minutes, followed by a wash in tap water. Microwave pre-treatment in 0.01 M citrate buffer, pH 5.8 (30 minutes) was performed. Anti-CXCR5 (clone 51505.111) (10 µg/ml) was incubated overnight (4 °C). Reactivity was visualised with the Vector Elite secondary detection kit. Colour development was achieved with the addition of DAB substrate. Images were recorded by brightfield microscopy. Original magnification x600 (A, C) x200 (B)

CXCR5 expression was further investigated with triple IF staining for CD3, CD30 and CXCR5 (D).

5µm cryostat sections were fixed in 100 % acetone. Sections were blocked in PBS/20 % normal goat sera for 30 minutes. Staining was performed employing a 2-step protocol. Anti-CXCR5 (clone 51505.111) (10 µg/ml) was incubated overnight (4 °C). Reactivity was visualised by incubation with anti mouse IgG2b (biotin conjugated) followed by streptavidin Alexa 594 (A). Sections were subsequently blocked with PBS+20 % normal mouse sera. Anti-CD30 FITC (clone Ber-H2) and anti-CD3 (Rabbit) was incubated at room temperature for 45 minutes. Visualisation was achieved with the addition of anti-FITC Alexa 488 (B) and anti-rabbit Alexa 350 (C) secondary reagents for the CD30 and CD3 antigens respectively. Images were recorded by confocal microscopy (Zeiss LSM510) employing laser lines at 351, 488 and 543 nm. Original magnification x630 (D). Individual images were merged to provide a composite.
3.2.1.5 The in situ expression of chemokine receptor ligands implicates specific receptor-ligand pathways for T cell homing in Hodgkin’s lymphoma.

Having identified chemokine receptors that are expressed on infiltrating T cells, immunohistochemical studies were performed to determine whether the relevant chemokines were present.

Antibodies specific for a number of chemokines were tested either on formalin fixed or snap frozen HL cases. In order to make appropriate use of clinical material, antibodies were first selected based on their ability to stain control tissue (tonsil).

Formalin fixed sections from 10 HL cases were examined for the expression of the CXCR3 ligand CXCL10/IP-10 (Figure 3.8). This ligand was detected at high levels on most neoplastic tumours cells (Figure 3.8A), with strong cytoplasmic and membranous staining (Figure 3.8B). The vascular endothelium expressed this marker weakly if at all. Positive staining was compared with controls (Figure 3.8C,D)

CXCL12/SDF-1 is the sole ligand for the receptor CXCR4 (Figure 3.9). In formalin fixed sections from HL cases (n=5), expression was strong in the tumour cells, with clear intracellular staining (Figure 3.9A). The receptor was also expressed on HEVs and vascular endothelia as well as macrophages, DCs and 30-40% of lymphocytes (Figure 3.9B). As a positive control, the same antibody was used to stain tonsil tissue. (Figure 3.9C) CXCL12/SDF-1 reactivity was observed on macrophages within B cell follicles, DCs within the mantle zone, as well as paracortical HEVs, whereas it was absent from the majority of lymphoid cells. Positive control staining was judged by comparing with isotype control staining of HL cases (Figure 3.9D)
Expression of CXCL10 chemokine was detected on H-RS cells (A). Immunoreactivity was detected in both the membrane and cytoplasm (B). This was compared with positive control staining in hyperplastic TL (C) and isotype negative control staining in HL tissue (D).

5µm paraffin sections were dewaxed, rehydrated and endogenous peroxidase activity blocked with 0.5 % H₂O₂ in methanol for 10 minutes, followed by a wash in tap water. Microwave pre-treatment in 0.01 M citrate buffer, pH 5.8 (30 minutes) was performed. Anti-CXCL10/IP10 (goat polyclonal) (5µg/ml) was incubated overnight (4°C). Reactivity was visualised by incubation with anti-goat IgG (peroxidase conjugated) followed by anti-rabbit IgG (peroxidase conjugated). Colour development was achieved with the addition of DAB substrate. Images were recorded by brightfield microscopy. Original magnification x600 (B, D) x400 (A, C).
Expression of CXCL10 chemokine was detected on H-RS cells (A). Immunoreactivity was detected in both the membrane and cytoplasm (B). This was compared with positive control staining in hyperplastic TL (C) and isotype negative control staining in HL tissue (D).

5µm paraffin sections were dewaxed, rehydrated and endogenous peroxidase activity blocked with 0.5 % H$_2$O$_2$ in methanol for 10 minutes, followed by a wash in tap water. Microwave pre-treatment in 0.01 M citrate buffer, pH 5.8 (30 minutes) was performed. Anti-CXCL10/IP10 (goat polyclonal) (5µg/ml) was incubated overnight (4°C). Reactivity was visualised by incubation with anti-goat IgG (peroxidase conjugated) followed by anti-rabbit IgG (peroxidase conjugated). Colour development was achieved with the addition of DAB substrate. Images were recorded by brightfield microscopy. Original magnification x600 (B, D) x400 (A, C).
Figure 3.9 In situ expression of CXCL12/SDF-1 in HL (n=5).

Expression of CXCL12/SDF-1 chemokine was detected on H-RS cells (A). CXCL12/SDF-1 was also expressed on the non-neoplastic lymphoid infiltrate and strongly expressed on HEVs (B). This was compared with positive control staining in hyperplastic TL (C) with staining of macrophages in the germinal centre and DC staining in the mantle zone regions. Isotype negative control staining of HL tissue was also performed (D).

5µm paraffin sections were dewaxed, rehydrated and endogenous peroxidase activity blocked with 0.5 % H2O2 in methanol for 10 minutes, followed by a wash in tap water. Microwave pre-treatment in 0.01 M citrate buffer, pH 5.8 (30 minutes) was performed. Anti SDF-1 (clone 79018) (10 µg/ml) was incubated overnight (4°C). Reactivity was visualised with the vector elite secondary detection kit. Colour development was achieved with the addition of DAB substrate. Images were recorded by brightfield microscopy. Original magnification x600 (A, B, D) x200 (C).
Expression of the CXCR3 ligand CCL13/BCA-1 was also examined in 10 formalin-fixed HL cases and localised in the non-neoplastic infiltrate (Figure 3.10A). Repopulation by the tumour cells and vascular endothelium was observed. In control cases, staining of DCs in the mantle zone was observed (Figure 3.10B). Positive staining was compared with negative controls (Figure 3.10C).
Expression of the CXCR5 ligand CXCL13/BCA-1 was also examined in 10 formalin fixed HL cases and localised to the non-neoplastic infiltrate (Figure 3.10A). Expression by the tumour cells and vascular endothelium was absent. In control cases, staining of DCs in the mantle zone was observed (Figure 3.10B). Positive staining was compared with negative controls (Figure 3.10C).

CCR4 was detected on HL T cells by flow cytometry, so we examined the expression of the ligand CCL17/TARC in tissue. Snap frozen sections (n=10) examined by IHC stained for CCL17/TARC on the vascular endothelium (Figure 3.11A) and in the malignant H-RS cells (Figure 3.11B). There was also reactivity within the lymphoid stroma (Figure 3.11A,B). In control cases, CCL17/TARC was detected on T zone HEVs (3.11C) and was absent in negative controls (Figure 3.11D).

CCL21/SLC is one of two known ligands for CCR7. In HL cases (n=10) CCL21/SLC was detected on the vasculature (Figure 3.12A), DCs and some lymphoid cells, but was absent from tumour cells (Figure 3.12B). Positive control staining of tonsil tissues demonstrated strong expression by DCs (Figure 3.12C), as well as the vascular endothelia including HEVs, larger vessels and possibly on some lymphocytes. In addition, expression was confined to the paracortex with no expression in follicles. Positive control staining was compared with negative controls (Figure 3.12D).
Expression of CXCL13/BCA-1 chemokine was observed in the non-neoplastic lymphoid infiltrate but not the tumour cells (A). This was compared with positive control staining of lymphoid follicles in TL (B) and isotype negative control staining of HL tissue (C).

5µm paraffin sections were dewaxed, rehydrated and endogenous peroxidase activity blocked with 0.5 % H₂O₂ in methanol for 10 minutes, followed by a wash in tap water. Microwave pre-treatment in 0.01 M citrate buffer, pH 5.8 (30 minutes) was performed. Anti BCA-1 (goat polyclonal) (1 µg/ml) was incubated overnight (4°C). Reactivity was visualised by incubation with anti-goat IgG (peroxidase conjugated) followed by anti-rabbit IgG (peroxidase conjugated). Colour development was achieved with the addition of DAB substrate. Images were recorded by brightfield microscopy. Original magnification x600 (C) x400 (A) x200 (B).
Figure 3.11 *In situ* expression of CCL17/TARC in HL (n=10).

Expression of CCL17/TARC chemokine was observed on the vascular endothelium (A) and the non-neoplastic lymphoid infiltrate as well as the tumour cells (B). This was compared with positive control staining in hyperplastic TL (C) and isotype negative control staining of HL tissue (D).

5 µm snap frozen sections were thawed at room temperature and fixed in 100 % acetone. Endogenous peroxidase activity was blocked with 0.5 % H₂O₂ in methanol for 10 minutes, followed by a wash in tap water. Anti-TARC (goat polyclonal) (20 µg/ml) was incubated overnight (4°C). Reactivity was visualised by incubation with anti-goat (peroxidase conjugated) followed by anti rabbit IgG (peroxidase conjugated). Colour development was achieved with the addition of DAB substrate. Images were recorded by brightfield microscopy. Original magnification x400 (A, B) x100 (C, D).
Expression of CCL21/SLC chemokine was observed on the vascular endothelium (A) but was absent on H-RS cells (B). This was compared with positive control staining of hyperplastic TL, with strong DC staining (C) and isotype negative control staining of HL tissue (D).

5µm paraffin sections were dewaxed, rehydrated and endogenous peroxidase activity blocked with 0.5 % H₂O₂ in methanol for 10 minutes, followed by a wash in tap water. Microwave pre-treatment in 0.01 M citrate buffer, pH 5.8 (30 minutes) was performed. Anti-SLC (goat polyclonal) (1 µg/ml) was incubated overnight (4°C). Reactivity was visualised by incubation with anti-goat IgG (peroxidase conjugated) followed by anti-rabbit IgG (peroxidase conjugated). Colour development was achieved with the addition of DAB substrate. Images were recorded by brightfield microscopy. Original magnification x600 (A-D).
3.2.2 Functional studies of T cell chemotaxis in Hodgkin's lymphoma.

3.2.2.1 Activated T cells migrate to factors produced by HL tumor cell lines in vitro.

The identification of the relevant migration pathways in HL tumors was addressed by developing an in vitro chemotaxis assay. Ideally, freshly isolated tumor cells and T cells would be isolated from biopsies and T cell migration to the tumor cells would

(A) HL

(Vessel)

(B) HL

(Vessel)

(C) Tonsil

(D) HL (Isotype control)

H-RS

DC

that were observed independent (as described in section 3.2.4.2). Peak migration responses (~40-50%) were typically observed at 200 ng/ml for CXCL12/SDP-1.

Recombinant human CXCL12/SDP-1 (200 ng/ml) was employed as a positive control in this tumor cell assay because CXCR4 is expressed on a majority of lymphoblasts...
3.2.2 Functional studies of T cell chemotaxis in Hodgkin's lymphoma.

3.2.2.1 Activated T cells migrate to factors produced by HL tumour cell lines in vitro.

The identification of the relevant migration pathways in HL tumours was addressed by developing an in vitro chemotaxis assay. Ideally, freshly isolated tumour cells and T cells would be isolated from biopsies and T cell migration to the tumour cells would be ascertained by blocking putative pathways. This is technically difficult for a number of reasons. 1) It is difficult to isolate sufficient cells of high enough purity from small biopsies. 2) Primary cultures of H-RS cells are difficult to work with, as the cells are prone to cell death. To overcome these problems, a panel of HL-derived cell lines were used. They were initially tested for their ability to secrete factors that mediated chemotaxis of activated lymphoblasts (derived from peripheral blood lymphocytes of healthy donors after activation with rIL-2 (50 IU/ml) and phytohemagglutinin (10 ng/ml) for 5 days (Figure 3.13)).

The migration assay was validated in a series of experiments using healthy donor derived T cells to recombinant CXCL12/SDF-1 (Figure 3.13A). These initial pilot experiments were important to establish parameters such as transwell pore filter size, the optimal number of cells to be loaded into the top chamber, the incubation time for chemotaxis, and the best methods of counting migrating cell numbers (in a manner that was observer independent) (Described in section 2.2.4.3). Peak migration responses (~ 40-50 %) were typically observed at 200-ng/ml for CXCL12/SDF-1.

Recombinant human CXCL12/SDF-1 (200 ng/ml) was employed as a positive control in this tumour cell assay because CXCR4 is expressed on a majority of lymphoblsta
Figure 3.13 HL cells secrete chemotactic factors for activated lymphocytes in vitro.

Optimization of assay conditions was performed using recombinant human CXCL12/SDF-1 (A). HL cells were cultured for 3 days in complete medium and culture supernatants were removed and tested for their ability to induce lymphoblast chemotaxis from healthy donors (B).

Donor derived lymphoblasts were washed twice in RPMI 1640 and enumerated. The pellet was incubated with 100 µCi [51Cr]O₄ for 1 to 2 hours at 37 °C/5 % CO₂. The cells were washed twice with RPMI 1640 to remove any unincorporated chromium. Cells were resuspended at 5 X 10⁶ cells/ml in RPMI 1640/0.5 % BSA and 100 µl of cell suspension were added to the upper chamber of a transwell filter (3 µm pore size). The transwell was added to the well of 24 well Tissue culture plate containing 600 µl supernatant or diluted recombinant chemokine. The transwell chamber was incubated at 37 °C/ 5 % CO₂ for 3 hours and the migrated cell population was enumerated with a γ-counter.

Data represent mean ± SD of triplicate determinations. Background migration was assessed; with chemotaxis media alone as a stimulus and 100 % migration was defined by adding 100 µl of cell suspension directly to the bottom of the chamber.
A

Recombinant human CXCL12/SDF-1 ng/ml

% Migration Of Input

0 10 20 30 40 50 60 70 80 90 100

0 100 200 300 400

B

Concentration of conditioned supernatant

% Migration Of Input

0 1 2 3 4 5 6 7 8 9 10

SDF FCS L591 N L591 1/2 L591 1/4 KMH2 N KMH2 1/2 KMH2 1/4 L1236 N L1236 1/2 L1236 1/4 DEV N DEV 1/2 DEV 1/4

L591 KMH2 L1236 DEV

40.72
(Figure 3.13B). 40% of lymphoblasts migrated into the bottom chamber. Migration to conditioned HL cell supernatants was weaker than the positive control but greater than background migration to media alone. The EBV positive cell line L591 induced the highest levels of migration of lymphoblasts with a 6-7-fold increase in migration over background. Both KMH2 and L1236 supernatants also stimulated chemotactic responses from lymphoblasts. Responses to DEV were not observed. In summary, activated T cells showed dose-dependent chemotactic responses to supernatants from HL lines in vitro, providing evidence to support the hypothesis that HL cells secrete lymphocyte chemotactic factors.

To begin to identify the chemotactic factors that mediated T cell migration in these studies, activated lymphoblasts were subsequently tested for migration to HL cell lines in the presence of specific blocking antibody. Work focused on CXCR3 as this receptor is highly expressed on HL-derived TIL and the corresponding ligands CXCL10/IP10 (Figure 3.8) and CXCL9/MIG (Ohshima et al., 2002) are expressed by H-RS cells in tumour tissue. Saturating concentrations (50 μg/ml) of anti-CXCR3 antibody (IC6) were used to block CXCR3-mediated T cell migration but a consistent blocking effect could not be demonstrated (data not shown).

3.2.2.2 Chemotaxis of TIL to purified chemokines.

Freshly isolated TIL from HL biopsies (n=5) were analysed for chemotaxis to purified chemokine to identify functional chemokine receptors. CXCL12/SDF-1 was tested as the receptor CXCR4 was expressed on a majority of TIL (as determined by flow cytometry). 4/5 biopsies provided TIL that migrated to recombinant CXCL12/SDF-1 over background levels (Figure 3.14A). Approximately 10% of TIL migrated to
Figure 3.14 Chemotaxis of TIL to purified CXCL12 and CCL17.

Freshly isolated TIL from HL biopsies (n=5) were tested for chemotaxis to recombinant human CXCL12/SDF-1 (200ng/ml) (A) and CCL17/TARC (100ng/ml) (B) using PBL and a CCR4+ T cell clone (T29 c73) as positive controls respectively.

TIL were washed twice in RPMI 1640 and enumerated. Chemotaxis assays were done according to the legend for Figure 3.13. Data represent mean ± SD of triplicate determinations. Background migration was assessed; with chemotaxis media alone as a stimulus and 100 % migration was defined by adding 100 µl of cell suspension directly to the bottom of the chamber.
A

% Migration Of Input

- No chemokine
- CXCL12/SDF-1

EBH20  EBH25  HD3051  HD5783  HD6452  PBL

43.64

B

% Migration Of Input

- no chemokine
- CCL17/TARC

EBH20  EBH25  HD3051  HD5783  HD6452  T29 c73

30
CXCL12/SDF-1 although levels reaching 18% of the total input population were observed for case HD6452. This was compared with PBL from a healthy donor where 44% of cells migrated to CXCL12/SDF-1.

Migration of HL TIL to CCL17/TARC was also examined as the receptor CCR4 was detected on a minor population of HL T cells (Figure 3.14B). Compared with a CCR4 expressing T cell clone (T29 c73), where 24% of cells migrated, there was no detectable migration of TIL to CCL17/TARC over background levels.
3.3 Discussion.

The premise for this investigation was that select T cell populations are recruited to HL tumours in response to a specific pattern of chemokine expression by the tumour. Chemokine receptor expression was studied on freshly isolated T cells from HL tumours and compared with tonsil T cells, and T cells from two solid organ tumours. In this way, the complex chemokine network implicated in the recruitment of the non-neoplastic infiltrate could be restricted to those pathways relevant to T cell recruitment.

The infiltrate was comprised predominantly of CD4+ T cells and this was similar to control T cells from tonsil and TIL from colorectal and renal tumours. Previous studies have reported that the HL infiltrates consist mainly of CD4+ T cells typically at a ratio of 4:1 with the CD8+ population (Kandil et al., 2001), but in immunocompromised patients, this can increase to 6:1 (Skinnider and Mak, 2002). H-RS cells often have a rosette of CD4+ T cells intimately associated with them, typically of a Th2 phenotype. Laser capture microdissected CD4+ T cells in association with H-RS cells have been analysed with respect to TCR repertoire and consist of a polyclonal population arguing against a tumour specific response (Roers et al., 1998). CD8+ T cells are a minority and do not directly associate with the tumour cells. Although CD8+ T cell responses to tumour antigens have been detected in the circulation of HL patients, such responses at the site of the tumour have never been detected (Chapman et al., 2001).

A comparatively restricted pattern of chemokine receptor expression was observed on HL TIL. In HL, members of the CXC family of chemokine receptors that were
expressed include CXCR3, CXCR4, CXCR5 and CXCR6. For the CC chemokine family, CCR4 and CCR5 were expressed on small populations of T cells. In contrast, CCR7 was expressed on a majority of T cells.

CXCR3 is typically expressed on activated T cells and T\textsubscript{II} effector cells. This receptor is implicated in recruitment of effector cells to many if not all inflammatory sites and it is likely that this receptor licenses T cells to gain entry into these sites (Kunkel et al., 2002). We detected CXCR3+ T cells by confocal microscopy intimately associated with both the tumour cells and the vascular endothelium. Expression of the CXCR3 ligand CXCL10/IP10 was strongly detected by the neoplastic tumour cells (although expression on the vasculature was absent in the cases examined). CXCR3 has two other ligands, CXCL9/MIG and the more recently cloned CXCL11/ITAC.

Expression of CXCL9/MIG has been reported in HL tumours (Teruya-Feldstein et al., 1999). This ligand was upregulated in HL tissue compared with control tissue, and had the highest level of expression in the mixed cellularity subtype. The tumour cells and some of the surrounding inflammatory cells were positive. The authors reported that CXCL9/MIG positive H-RS cells had a higher frequency of CXCR3 positive cells associated with them compared with CXCL10/IP10 positive cases. Furthermore, EBV positive cases had more intense staining of tumour cells. Vascular endothelial expression of CXCL9/MIG has not been reported.

CXCL11/ITAC expression has not been reported in HL and warrants investigation. Thus, either of these two chemokines might mediate activation-induced arrest on the
vascular endothelium, considering the association of CXCR3 high T cells with the vasculature.

A transwell chemotaxis assay was developed, employing conditioned supernatants from HL tumour cell lines as a stimulus and lymphoblasts as the responding cell population. The rationale was to demonstrate lymphocyte migration to Hodgkin's tumour cells, and dissect the relevant pathways \textit{in vitro}, particularly whether CXCR3 mediated T cell homing to H-RS cells. Direct studies of primary T cell migration to primary H-RS cells were not feasible for the reasons highlighted earlier. However, one published study investigated the migration of primary HL cell suspensions to recombinant human chemokine (Buri et al., 2001), and reported strong chemotactic responses to the CXCR3 ligand CXCL10/IP-10, and the CCR5 ligand CCL5/RANTES, with weak responses to CCL4/MIP1\(\beta\), and no response to CCL2/MCP-1.

Our work demonstrates the ability of HL cell lines to secrete factors that stimulate migration of lymphoblasts. Similar studies in a hepatoma model system successfully demonstrated migration responses to HepG2 supernatants of TIL (Yoong et al., 1999). Migration responses to supernatants from this cell line were sufficient for blocking studies to be undertaken. This study revealed CXCR3 and CCR5 dependent homing mechanisms to the HepG2 tumour cell line in keeping with the phenotype of primary \textit{ex vivo} T cells isolated from this hepatic neoplasm. In our system however, the levels of migration were relatively weak.
CXCR4 was expressed on most T cells from all tumours and was functional \textit{in vitro}. CXCR4 has been implicated in bone marrow trafficking (Petit et al., 2002), in retaining T cells in inflammatory sites including the synovium of patients with Rheumatoid arthritis (Buckley et al., 2000). Of particular interest in the context of HL, is a recent report describing a role for CXCR4 in central memory T cell homing to LNs (Scimone et al., 2004). CXCR4 is expressed on virtually all haematopoietic cells however levels of this receptor are regulated with T cell activation (Colantonio et al., 2002). We detected CXCL12/SDF-1 expression in HL tumours with strong expression by the H-RS cells, endothelium, macrophages and DCs. These results are in contrast to one report demonstrating CXCL12/SDF-1 transcript expression by the reactive cells but not by the tumour cells (Hopken et al., 2002). This difference in transcript and protein expression might be explained by the observation that chemokines are known to be transcytosed (Middleton et al., 1997), and perhaps the reactive cells secrete chemokines that are later transported and presented by other cell types.

CXCR5 is expressed on a subset of CD4+ T cells and regulates their positioning in secondary lymphoid organs at the T-B zone interface, facilitating B cell survival and function (Kim et al., 2001b). In HL tumours, this receptor was restricted to the CD4+ subset and showed variation between cases. It will be important to determine the functional significance of CXCR5+ T cells in HL tumours with respect not only to homing but also to their influence on tumour cell survival and function. Do CXCR5 T cells regulate H-RS cell function as they do B cell function in normal lymphoid sites? Although HL tumours did not express CXCL13/BCA-1 themselves, the surrounding infiltrate did. Could this be a mechanism by which the tumour evades the immune
response? Namely, by recruiting T cells that facilitate tumour cell survival and proliferation rather than target cell lysis.

CXCR6, although weakly expressed on HL T cells, was nevertheless enriched in the CD8+ population. This receptor is associated with a $T_{H1}$ or $T_{C1}$ phenotype and T cells expressing CXCR6 are enriched at tissue sites of inflammation (Kim et al., 2001a). Expression of this receptor mirrors that of CCR5 (Unutmaz et al., 2000) in that they are co-ordinately regulated. The ligand for CXCR6 is the recently cloned CXCL16. CXCL16 is similar to fractalkine in that it is a membrane-anchored chemokine. It is expressed on antigen presenting cells including cells of the B cell and macrophage lineage (Matloubian et al., 2000). To date, the expression of this ligand has not been investigated in HL, but with CXCR6 expression on a fraction of infiltrating CD8+ T cells, it will be important to discern the importance of this pathway.

At the beginning of this study CCR4 had been proposed to be an important pathway in the recruitment of T cells to HL tumours based on the observation that the ligand for CCR4, TARC, was detected by serial analysis of gene expression (SAGE) on the L1236 cell line (van den Berg et al., 1999). Subsequent in situ hybridisation of HL sections demonstrated expression of the mRNA and later the protein by H-RS tumour cells of all subtypes except non-classical cases. Furthermore, hybridisation of a CCR4 probe demonstrated expression of CCR4 transcript in the surrounding non-neoplastic infiltrate. Convincing antibody staining for CCR4 is lacking with some reports identifying CCR4 staining on only a minority of cells (Ohshima et al., 2002) Thus, the assumption has been that CCR4+ $T_{H2}$ T cells surround H-RS cells in response to a gradient of CCL17/TARC (or CCL22/MDC). This is based on the assumption that
T_{H2} cells are associated with CCR4 expression *in vivo*. Our studies demonstrated that CCR4+ was expressed on a minority of T cells in HL by flow cytometry, although we failed to demonstrate chemotaxis of HL TIL *in vitro* compared with a CCR4+ T cell clone as a positive control. An antibody to CCR4 was tested in HL tissue (data not shown). Positive staining was not detected. However, the lack of a suitable positive control tissue (i.e. inflamed skin) precluded determination of the CCR4 status in HL. Expression of the ligand CCL17/TARC in H-RS cells however, was demonstrated in agreement with previously published reports (Ohshima et al., 2003; van den Berg et al., 1999). This implies that CCR4 pathways are unlikely to dominate bulk T cell recruitment to HL tumours. However, within the tumour microenvironment, CCL17/TARC expressing H-RS cells may promote chemotaxis of a small population of CCR4+ T cells.

CCR5 like CCR4 was expressed on a minority of cells. CCR5 has a number of ligands including CCL5/RANTES, which is expressed by T cells, macrophages and endothelial cells in both hyperplastic TL and HL cases (Maggio et al., 2002). The tissue distribution of CCR5+ T cells in HL tumours requires clarification but one study has reported CCR5 expression on approximately half of non-neoplastic leukocytes (Buri et al., 2001). In addition, flow cytometry of CD3+ T cells from cell suspensions identified CCR5 on 30 % of cells with more on CD4+ (44 %) than CD8+ (10 %) cell. (Buri et al., 2001) This is similar to our findings where CCR5 was expressed on 20 % of cells although there was no difference in the proportion expressed on CD4+ or CD8+ T cells. The authors also reported CCR3 expression on 15 % of CD3+ T cells, which was upregulated, compared with their controls. In
contrast, our study revealed no expression of CCR3 on T cells when correcting for isotype control staining.

In the context of homeostatic secondary lymphoid homing, CCR7 mediates recruitment of naïve and central memory T cell populations (Stein et al., 2000) CCR7 binds endothelial expressed CCL21/SLC triggering activation dependent arrest. CCR7 T cells then responds to CCL19/ELC directing them through the tissue. In HL, the expression of CCR7 on T cell populations is second only to CXCR4 and is expressed on both CD4+ and CD8+ populations. Whether these CCR7+ populations represent cells with effector function is unclear and requires investigation, as there was no differential expression of CCR7 between tumour and control T cells. The CCR7 ligand CCL21/SLC was strongly expressed on the vascular endothelium, interdigitating DCs, and some lymphoid cells but not by the tumour cells. This agrees with a previous report looking at transcript expression of CCL21/SLC by ISH, where the authors detected expression by the reactive cells but not the tumour cells (Hopken et al., 2002). In a similar manner, CCL19/ELC was also reported to be absent from the tumour cells but present in the reactive infiltrate (Hopken et al., 2002). The expression of these CCR7 ligands by the reactive cells and vessels but not the H-RS cells implies that H-RS cells do not directly recruit CCR7+ T cells. However, CCR7 dependent vascular adhesion is likely to be required for adhesion from the circulation.

We compared HL TIL with TIL from solid organ tumours and this allowed us to make a number of preliminary observations regarding the expression of homing markers on T cells from colorectal and renal tumours. Colorectal T cells express CXCR3, CXCR4, CXCR5, CXCR6, CCR5 and CCR6 and a minority express low
levels of CCR4. It is surprising that CCR4 was detected in CRC as it has classically been considered a skin homing marker (Campbell et al., 1999). However, the levels of CCR4 were low and unlikely to be functionally important as CCR4 function depends on cell surface levels (Soler et al., 2003). The combination of CCR5 and CXCR3, likely represent a T_{TH} population of cells. In RCC, only CXCR4 and CCR5 were detected on a majority of T cells. However, it should be noted that these cells were isolated by collagenase digestion of tumour tissue, which is known to affect the expression of chemokine receptors including CXCR3 (Prof. David Adams, personal communication). It will be important to confirm these observations by examining more cases and without collagenase treatment of tissue.
Chapter 4: T cell adhesion pathways in Hodgkin’s lymphoma.

4.1 Introduction.

For T cells to enter HL tumours, they must recognise and bind to the appropriate adhesion molecules at the endothelial surface. Initial T cell-endothelial interactions are classically mediated by selectins, which capture flowing cells and induce them to roll on the luminal surface. The cell interacts with chemokines immobilised on the glycocalyx (Tanaka et al., 1993) which signals an increase in both the affinity of the α/β integrin heterodimer and the lateral mobility of receptors throughout the cell membrane (Kucik et al., 1996). Understanding the expression of adhesion molecules and their counter-receptors is vital in elucidating the pathways responsible for adhesion of T lymphocytes from the systemic circulation. In addition, adhesion molecules are likely to be important in the interaction of infiltrating T cells with stromal cells (i.e fibroblasts), and H-RS tumour cells.

HL arises within a lymph node. Therefore, this tumour can be considered both an inflammatory site as well as a secondary lymphoid organ (albeit with disrupted tissue architecture). In normal lymph nodes, L-selectin-PNAD interactions promote capture and rolling of naïve and central memory T cells on the endothelial surface (Gallatin et al., 1983). Activation of CCR7 by CCL21/SLC and CXCR4 by CXCL12/SDF-1 triggers stable adhesion of specific T cell populations via LFA-1-ICAM-1 interactions (Stein et al., 2000). The extent to which this well characterised LN adhesion pathway operates in the context of HL is unclear.

To address which pathways are important in T cell adhesion to the HL tumour vasculature, an investigation of the expression of cell adhesion molecules on tumour...
infiltrating T cells by flow cytometry was undertaken. The corresponding ligands were identified by IHC. To address the functional significance of these pathways, a rotary tissue-binding assay was explored to examine T cell adhesion to HL vascular endothelium.
4.2 Results.

4.2.1 Adhesion molecule expression on tumour infiltrating lymphocytes.

Flow cytometry was employed to examine the expression of adhesion molecules on CD3+, CD4+ and CD8+ T cell subsets isolated from HL tumours. Cells were gated as before to identify these T cell subsets (see Figure 3.1). Most HL CD3+ T cells (n=9) expressed LFA-1 (99 ± 1% SD), PSGL-1 (70 ± 17% SD), VLA-4 (92 ± 6% SD) and β7 (67 ± 29% SD) with no significant difference between CD4+ and CD8+ T cell subsets (Figure 4.1A). CD62L was expressed on 41 ± 16% SD of CD3+ cells whereas CLA expression was very weak (7 ± 4% SD) (Figure 4.1A). Cell surface levels as detected by MFI did not differ between T cell subsets (Figure 4.1B).

For comparison, T cells were isolated from tonsil (n=3) and examined for the same markers (Figure 4.1C). LFA-1 expression was consistently high (94 ± 11% SD of CD3+ T cells) and equally represented on CD4+ and CD8+ subsets. PSGL-1 (54 ± 13% SD), VLA-4 (74 ± 9% SD) and β7 (40 ± 15% SD) were also expressed on TL-derived T cells (Figure 4.1C). Each of these adhesion molecules was expressed at higher levels and on a greater proportion of CD8+ rather than CD4+ T cells (Figure 4.1C and 4.1D). The only significant difference between HL and tonsil T cells was an increased frequency of VLA-4+/CD3+ and CD4+ expressing T cells in HL (P<0.05 CD3; p<0.05 CD4 t test) (Figure 4.1A,C).

HL-derived T cells were also compared with T cells from CRC (n=5) and RCC (n=2) (Figure 4.2). CRC-derived T cells exhibited a similar pattern of adhesion molecule expression to HL-derived T cells with high-level expression of all the adhesion markers except CLA, which was very weak (5 ± 3% SD), and CD62L which was
Flow cytometry was employed to determine the expression of adhesion receptors on CD3, CD4 and CD8 T cell subsets. Mean percent expression was determined on HL (A) and tonsil (C) T cells. Mean channel fluorescence was compared on HL (B) and tonsil (D) T cells.

Cells were washed twice (PBS/2 % FCS), collected (538 g /5 minutes), and resuspended at 1-5x10^5 cells/ml in PBS/2 % FCS. Cells were incubated with pre-titred concentrations of adhesion receptor antibodies (CLA, (rat IgM), CD62L (mlgG1), LFA-1 (mlgG1), PSGL-1 (mlgG1), VLA-4 (mlgG3), β7 (rat IgG2a)) in a final volume of 50 µl. The reaction was incubated on ice for 30 minutes. The cells were washed twice in PBS/2 % FCS. Visualisation was achieved with either anti-mouse or anti-rat IgG (biotin conjugated) followed by streptavidin Alexa 488. Cells were washed and incubated with normal mouse serum for 20 minutes. Finally, cells were incubated with saturating concentrations of anti CD3-PC5 (clone UCHT1) and anti-CD4-PE (clone 13B8.2) conjugated antibodies. The reaction was incubated on ice for 30 minutes. The cells were washed twice in PBS/2 % FCS. Cells were either analysed immediately or fixed in 2 % paraformaldehyde. Cell analysis was performed by flow cytometry. Results were analysed using the software package WinMDI (written by Joe Trotter). Data represent mean +/- SD. Tissue infiltrating lymphocytes were isolated from HL (n=9) and tonsil (n=3) cases.
expressed on 21 ± 16 % SD of CD3+ T cells (Figure 4.2A and 4.2B). This pattern of expression was also observed for T cells from RCC except PSGL-1 was expressed on only 12 ± 4 % SD T cells (Figure 4.2C). MFI values for adhesion molecules were not significantly different between subsets from either tissue (Figure 4.2B and 4.2D) except for LFA-1 in RCC with higher cell surface levels on CD8+ T cells (Figure 4.2D).

4.2.2 Expression of endothelial cell adhesion molecules in HL tumours.

Having characterised adhesion molecule expression on T cells from HL tumours, expression of the corresponding ligands in tumour tissue was examined by IHC. As LFA-1 was expressed on virtually all HL T cells, the expression of the ligand ICAM-1 was examined in tissue sections from HL cases (n=10). ICAM-1 was expressed at high levels on the vascular endothelium as well as on H-RS cells (Figure 4.3A). In addition, there was strong reactivity throughout the lymphoid stroma. Staining was compared with isotype controls (Figure 4.3B) and positive control staining in tonsil (Figure 4.3C).

High-level expression of VLA-4 on HL-derived T cells was the rationale for examining VCAM-1 in HL tumours (n=10). The expression pattern of VCAM-1 was similar to ICAM-1 except H-RS staining appeared negative or weak (Figure 4.4A). There was strong staining of stromal cells (Figure 4.4A) and vessels (Figure 4.4B) compared with isotype (Figure 4.4C) and positive controls (Figure 4.4D).
Figure 4.2 Phenotypic analysis of adhesion receptor expression on CRC and RCC T cells.

Flow cytometry was employed to determine the expression of adhesion receptors on CD3, CD4 and CD8 T cell subsets. Mean percent expression was determined on CRC (a) and RCC (c) T cells. Mean channel fluorescence was compared on CRC (b) and RCC (d) T cells.

Cells were washed twice (PBS/2 % FCS), collected (538 g /5 minutes), and resuspended at 1-5x10^5 cells/ml in PBS/2 % FCS. Cells were incubated with pre-titred concentrations of adhesion receptor antibodies (CLA, (rat IgM), CD62L (mIgG1), LFA-1 (mIgG1), PSGL-1 (mIgG1), VLA-4 (mIgG3), β7 (rat IgG2a)) as described in the legend to figure 4.1. Cell analysis was performed by flow cytometry. Results were analysed using the software package WinMDI (written by Joe Trotter). Data represent mean +/- SD. Tissue infiltrating lymphocytes were isolated from CRC (n=5) and RCC (n=2) cases.
Figure 4.3 In situ expression of ICAM-1 in HL.

5µm paraffin sections from HL cases (n=10) were stained with an antibody to ICAM-1 (clone 84H10) (15 µg/ml) demonstrating vascular and H-RS reactivity (A). This staining was compared with isotype negative (B) and hyperplastic tonsil positive (C) controls.

For secondary detection sections were stained according to an indirect peroxidase based protocol (section 2.2.3.3.6). Immunohistochemical images were recorded by brightfield microscopy. Original magnification x600 (A, B, C).
Figure 4.4 *In situ* expression of VCAM-1 in HL.

5μm paraffin sections from HL cases (n=10) were stained with an antibody to VCAM-1 (2G7) (20 μg/ml) to determine H-RS (A) and vascular reactivity (B). This was compared with isotype negative (C) and hyperplastic tonsil positive (D) controls.

For secondary detection sections were stained according to an indirect peroxidase based protocol (section 2.2.3.3.6). Immunohistochemical images were recorded by brightfield microscopy. Original magnification x600 (A, B, C) x100 (D).
As well as examining the expression of ligands for receptors identified on HL T cells, expression of the orphan receptor VAP-1 in HL was also studied. VAP-1 has no known ligand on T cells, yet functionally is important in T cell trafficking to LN and chronic inflammatory sites (Salmi et al., 1997).

IF was employed to examine VAP-1 expression in frozen HL tissue (Figure 4.5) (n=10). Triple labelling experiments detected expression on the vascular endothelium (including HEVs), but not on tumour cells or tissue infiltrating T cells (Figure 4.5A). Vascular expression was confirmed by co-staining with the endothelial marker CD31 (Figure 4.5B). Note that VAP-1 expression did not always colocalise with CD31. Rather CD31 expression was luminal whereas VAP-1 expression was often subendothelial (Figure 4.5C). This was observed in HL tissue as well as in tonsil and liver (data not shown). Furthermore, VAP-1 was only expressed on CD31+ vessels, but not all CD31+ vessels expressed VAP-1 (Figure 4.5D).

4.2.3 Functional analysis of receptor-ligand pathways in HL.

To determine the functional relevance of specific receptor ligand pathways in HL, a modified Stamper-woodruff ex vivo tissue binding assay was explored. This assay is designed to study the ability of lymphocytes to bind vessels in frozen tissue sections. T cells are added to snap frozen tissue sections and allowed to adhere under rotary conditions. The number of cells binding to vascular endothelium in the presence of blocking antibodies can then be analysed by microscopy.

Given the limited availability of clinical material from HL patients, initial studies aimed at setting up this tissue-binding assay using tissue from tonsil or liver, and IL-2
Figure 4.5 *In situ* expression of VAP-1 in HL.

A) HL sections (n=10) were stained with an anti VAP-1 antibody (1B2) to determine expression.

5µm cryostat sections were fixed in 100 % acetone. Sections were blocked in PBS/20 % Normal goat sera for 30 minutes. Staining was performed employing a 2-step protocol. Anti–VAP-1 (clone 1B2) (5µg/ml) was incubated overnight (4°C) Reactivity was visualised by incubation with anti-mouse IgG1 (biotin conjugated) followed by streptavidin Alexa 350. Sections were subsequently blocked with PBS/20 % normal mouse sera. Anti-CD30-FITC (clone Ber-H2) and anti-CD3 (Rabbit) was incubated at room temperature for 45 minutes. Visualisation was achieved with the addition of anti-FITC Alexa 488 (B) and anti-rabbit Alexa 594 (C) secondary reagents for the CD30 and CD3 antigens respectively. Images were recorded by confocal microscopy (Zeiss LSM510) employing laser lines at 351, 488 and 543 nm. Original magnification x630 (A,) x400 (B, C) x100 (D).

B-D) Expression by vessels was confirmed by co-staining with anti CD31 (B). Vessels did not always co-localise with CD31 (C), and not all CD31 positive vessels were VAP-1 positive (D).

Dual staining was achieved as above, except CD31-FITC (clone B-B38) was employed instead of CD30, and VAP-1 was visualised with streptavidin Alexa 594.
expanded lymphoblasts from the peripheral blood of healthy donors. The role of VAP-1 in tissue binding was of particular interest because not only is this molecule expressed on vessels in HL, but the ability to block T cell adhesion to vessels in tonsil and liver using the anti-VAP-1 antibody 12C10 had been reported previously. Therefore, any blocking effect would not be underestimated by looking at VAP-1 vessels (which would be, if vessels were highlighted with anti-CD31 antibody).
expanded lymphoblasts from the peripheral blood of healthy donors. The role of VAP-1 in tissue binding was of particular interest because not only is this molecule expressed on vessels in HL, but the ability to block T cell adhesion to vessels in tonsil and liver using the anti VAP-1 antibody 1B2 had been reported previously.

VAP-1 is known to mediate CD8+ T cell adhesion. Therefore CD8+ T cells were enriched from peripheral blood by negative magnetic selection (see section 2.2.2.3) and activated with PHA (10ug/ml). These cells were then expanded in vitro in medium containing IL-2 (50 IU/ml). Note that negative selection obtained cell populations that were typically >90% CD8+.

Initial studies on T cell adhesion to liver tissue were possible with $10^5$-cells/tissue section. However, with tonsil tissue, more cells ($3 \times 10^6$) were required to achieve sufficient numbers of bound cells. The temperature of the assay is important because the role of tethering interactions in cellular adhesion may be masked at 37°C where integrin interactions are likely to dominate. This can be reduced by incubation at a lower temperature. Therefore when examining VAP-1 function the assay was conducted at 7°C (Stamper and Woodruff, 1976). To further reduce the influence of non-specific interactions, the assay was incubated on a rotating shaker (60 rpm).

To enhance the ability to detect VAP-1 dependent adhesion, sections were blocked with the 1B2 antibody, which in addition to blocking would identify VAP-1+ vessels. Therefore, any blocking effect would not be underestimated by looking at VAP- vessels (which would be, if vessels were highlighted with anti-CD31 antibody).
Using these conditions it was possible on a single occasion to partially block lymphocyte binding to VAP-1+ vessels in tonsil using the anti VAP-1 antibody 1B2 (Figure 4.6), with a reduction in lymphoblast binding of 24 ± 5% SD compared with isotype control antibody (p<0.01 t test) (Figure 4.7A). However, these results were not reproducible and consequently attempts to use this assay on HL tissue were unreliable (possibly due to the poor condition of tissue sections available for this work) (Figure 4.7B). Therefore, it was not possible to explore the functional role of VAP-1 and other identified adhesion molecules in HL.
Figure 4.6 Lymphoblast binding to tonsil endothelium under rotary conditions.

Fewer T cells bound to the vascular endothelium following pre-treatment of sections with blocking concentrations (50 µg/ml) of anti-VAP-1 mAb (clone 1B2) (A) compared with control mAb (B). The adherent T cells were detected by enumerating numbers of cells bound to VAP-1 stained endothelium. Micrographs have been focused at the level of adherent lymphoblasts.

Cryostat sections (10 µm) of control or tumour tissue were cut onto vectorbond coated glass slides and fixed in acetone for 10 minutes. IL-2 expanded lymphoblasts were adjusted to a concentration of 3 x 10^7 cells/ml of medium (RPMI 1640 and 0.5 % BSA). Sections were preincubated with isotype negative control mAb or blocking anti-VAP-1 mAb (clone 1B2) (50 µg/ml) for 30 minutes at room temperature. 100 µl of cell suspension was added to each section with constant rotation (60 rpm) for 30 minutes at 4 °C. Sections were gently washed with cold PBS to remove nonadherent lymphocytes and fixed in acetone for 10 minutes. Vascular endothelium and adherent lymphocytes were identified by anti-VAP-1 immunostaining, and developed with APAAP and fast red. The sections were counterstained with Mayer’s Hematoxylin. Data represent mean ± SD of triplicate sections. Lymphocytes adherent to vascular endothelium was counted using an ocular grid on every section. Original magnification x200 (A, B). One hundred vessels were randomly selected and counted on every tissue section. The number of lymphocytes adherent to endothelium in the presence of control mAb defined 100 % binding, and the number binding to vascular endothelium in the presence of blocking mAb was expressed as a percentage of this.
Adherent lymphocyte
Figure 4.7 Evaluating the role of VAP-1 in T cell adhesion to HL tumours employing an *in vitro* adhesion assay.

Assay conditions were optimised in tonsil with demonstration (inconsistent) of blocking activity of VAP-1 (Figure 4.7A). VAP-1 function was evaluated in HL tissue (Figure 4.7B). Data represent mean ± SD of triplicate sections. The assay was performed as described in the legend to Figure 4.6.
A

P< 0.01

B

Blocking antibody

Adhesion of lymphoblasts as % of isotype

Control

VAP-1

0 20 40 60 80 100 120

100

200

300

0

50

100

150

200

250

300

50

100

150

200

250

300

Tonsil Isotype

Tonsil VAP-1

HL Isotype

HL VAP-1
4.3 Discussion.

The role of adhesion molecules in T cell homing to normal lymphoid organs and sites of chronic inflammation have been well characterised. However, their role in T cell recruitment into human tumours is poorly understood. Indeed, in a number of tumours including melanoma, the vasculature may have downregulated expression of adhesion molecules (Piali et al., 1995). Furthermore, these tumour vessels may be unresponsive to pro-inflammatory cytokines that upregulate adhesion receptor expression (Carlos, 2001).

We examined the expression of adhesion receptors on freshly isolated T cells from HL tumours and compared them with T cells isolated from tonsil and two solid tumours. LFA-1 was expressed on virtually all infiltrating T cells and the corresponding ligand ICAM-1 was detected on HL vessels. Therefore, this pathway is likely to be important in mediating T cell adhesion to tumour vascular endothelium. Likewise, VLA-4 was detected on HL TIL and the corresponding ligand VCAM-1 was highly expressed in HL tissue. This suggests that VLA-4-VCAM-1 interactions may also be functionally important in adhesion. The detection of VCAM-1 on stromal cells implicates this pathway in recruitment and retention of T cells within the tumour. It will be important to investigate the functional contributions of pathways like LFA-1-ICAM and VLA-4-VCAM-1 in HL, considering the important homeostatic and inflammatory roles played by these receptor-ligand pairs. The availability of reporter antibodies like mAb 24 (specific to the activated form of LFA-1) (Dransfield and Hogg 1989) and 12G10 (VLA-4 specific) (Mould et al. 1995), aid in determining the proportion of T cells that express the conformationally active form of these integrins. Having defined the relevant chemokine receptors expressed on HL T cells (section 3),
it will be interesting to investigate how these receptors influence integrin function. Knowledge of the inside-out signalling events during integrin activation is the subject of considerable interest (reviewed in section 1.2.1.3.2).

PSGL-1 was expressed on a majority of T cells and it will be important to determine whether ligands like P-selectin are expressed within HL tissue. CLA was absent on T cells from all tissues examined in agreement with a tissue specific role for CLA in skin homing (Picker et al., 1990). CD62L is known to play an important role in recruiting T cells to LN via HEVs (Gallatin et al., 1983). Consistent with this role, it is significant that CD62L was expressed on HL and TL-derived T cells but only weakly expressed on a fraction of T cells from CRC and RCC tumours. In addition, CD62L may facilitate LN exit by binding the mannose receptor on lymphatic endothelium allowing entry into the efferent lymphatics (Irjala et al., 2001a). It will be important to examine the expression of CD62L ligands in situ. Recent evidence has shown that CCR7/CD62L expressing cells are also present at sites of organised lymphoid neogenesis. These can develop during chronic inflammation with infiltrates that consist of follicular structures and specialised HEV-like vessels (Hjelmstrom, 2001). Thus, CD62L plays a complex role by mediating entry of cells into lymphoid sites.

The observation that VAP-1 is not expressed by all CD31+ vessels in HL and is differentially expressed on both the luminal and/or subendothelial surfaces may reflect an important regulatory mechanism for T cell recruitment. VAP-1 was originally implicated in the tethering step of adhesion, however flow based experiments highlight a role for this molecule in TEM (Lalor et al. 2002). The ligand
for VAP-1 is unknown although the monamine oxidase activity of this receptor implies a novel mode of adhesive function. It is thought that VAP-1 engages lymphocyte expressed primary amines (Smith et al. 1998). Treatment with semi-carbazide, a specific inhibitor of VAP-1 enzyme activity, inhibits adhesion and TEM to a similar extent as the 1B2 blocking mAb. More recently, an inhibitory peptide has been shown to block VAP-1 dependent lymphocyte-endothelial interaction under conditions of flow (Yegutkin et al. 2004).

The Stamper-Woodruff tissue binding assay was initially developed to study lymphocyte binding to HEV in rat LN (Stamper and Woodruff, 1976). Since then, numerous modifications of this assay have been adapted to define the molecular pathways important in T cell adhesion in contexts such as chronic inflammation and cancer. For example, T cells employ PSGL-1 and P-selectin for adhesion to bronchial endothelium (Ainslie et al., 2002). In chronic inflammatory liver disease, MAdCAM-1 is aberrantly expressed and mediates adhesion of α4β7 T cells (Grant et al., 2001). Furthermore in epithelial tumours such as hepatocellular carcinoma, T cells adhere to vascular endothelium in a VAP-1/ICAM-1 dependent manner (Yoong et al., 1998).

We attempted to define which receptor-ligand pathways dominated T cell adhesion to HL vascular endothelium by evaluating a modified tumour tissue-binding assay. We attempted to block VAP-1 mediated binding to evaluate its role in T cell adhesion. Initial findings confirmed reports that VAP-1 mediates functional T cell binding to tonsil HEVs (Salmi et al., 1997) However, the assay proved to be unreliable because of low reproducibility of results possibly owing to the poor condition of tissue available for this work. For these reasons it proved impossible to extensively address
T cell binding to HL vascular endothelium. Importantly, the role of VAP-1 in T cell adhesion has been examined in other tumours (Irjala et al., 2001b). In these studies, the binding of TIL, lymphokine activated killer and Natural killer cells to tumour vessels was established and shown to bind in a VAP-1 dependent manner.

In addition to binding vessels, adhesion molecules are also important in the direct interaction of T cells with stromal cells (including H-RS cells) and extracellular matrix components (Buckley et al., 2001). Thus, T cells interact with H-RS cells through their TCR but also through adhesion receptors like LFA-1 following redistribution of such integrins to the immunological synapse (Sanders et al., 1988; Sims and Dustin, 2002). We have observed in situ expression of the adhesion molecule ICAM-1 by the malignant H-RS cells. ICAM-2, LFA-1 and LFA-3 expression has also been reported (Ellis et al., 1992). Furthermore, in vitro studies with H-RS cell lines implicated LFA-3/ICAM-1 interactions (and to a lesser extent LFA-1/ICAM-1 interactions) in functionally mediating T cell-H-RS adhesive interactions (Sanders et al., 1988).
Chapter 5: Characterising T cell clones generated for adoptive cell therapy of Hodgkin’s lymphoma.

5.1 Introduction.

Defining pathways of T cell trafficking in HL should increase the ability to deliver therapeutic effector T cells to tumour deposits for cellular based therapies. The rationale for developing adoptive cell therapy for cancers like HL is that T cells can be optimally activated and selected for antigenic specificity ex vivo and large numbers of T cells may be infused. Furthermore, characterisation of clones before infusion provides a more comprehensive understanding of the reasons for the success or failure of this type of approach.

EBV-associated tumours like HL provide an important model system for the evaluation of cellular based therapies. T cell responses to this virus have been extensively characterised in healthy virus carriers and patients with EBV-associated malignancy. In recent years a number of studies have emerged demonstrating both the safety and efficacy of infusing cancer patients with in vitro expanded T cells. In the EBV setting, the potential of adoptive immunotherapy for EBV malignancies has been elegantly demonstrated in the context of EBV associated immunoblastic lymphoma (Rooney et al., 1998).

Immunoblastic lymphoma has the complete latent viral program of gene expression (Latency III), including the immunodominant family of EBNA3 proteins (see Figure 1.7). However, in the setting of HL and NPC, both tumours demonstrate a restricted pattern of viral gene expression (latency type II) in the neoplastic cells. Therefore, only T cell responses to the viral proteins EBNA1, LMP1 and LMP2 will target the tumour. EBNA1 was considered an immunologically silent protein because an
internal glycine-alanine repeat domain within this molecule prevented processing through the HLA class I pathway and presentation to CD8+ T cells (Levitskaya et al., 1995). Recently however, CD8+ T cell clones, specific for EBNA-1 were shown to recognise EBV infected B cells (Lee et al., 2004; Tellam et al., 2004; Voo et al., 2004). Therefore, CD8+ as well as CD4+ T cell responses to EBNA1 ((Leen et al. 2001) may yet be exploitable in the control of HL. Responses to LMP2 (Chapman et al., 2001) and to a lesser extent LMP1 have also been detected at low frequencies in the peripheral blood of healthy donors as well as some HL patients.

With an interest in T cell targeting of EBV associated tumours, a phase I clinical trial was undertaken within our institute to generate clones for adoptive therapy for several human malignancies (including HL). This chapter details the characterisation of the homing phenotype and function of clones generated for adoptive T cell therapy of HL, and compares this phenotype with that of TILs isolated from HL tumours. T cell clones generated with the same protocol but targeting melanoma antigens were also examined for comparison.
5.2 Results.

5.2.1 Phenotypic characterisation of T cell clones.

PBL were isolated from patients and stimulated with autologous DC pulsed with peptides (50 µg/ml) representing defined epitopes in tumour-associated antigens. The culture media contained IL-2 (10 IU/ml) and IL-7 (5 ng/ml). 7 days later the polyclonal T cell culture was restimulated with DC + peptide and fed with medium containing 50 IU/ml IL-2 and 5ng/ml IL-7. On day 14, cultures were cloned and screened for antigen specific responses by IFN-γ ELISA and positively identified clones were grown to large cell numbers employing a rapid expansion protocol (REP). Dr. Steve Lee kindly provided these clones for phenotypic analysis of homing markers.

5.2.1.1 T cell clones generated in vitro express different homing receptors compared with TIL freshly isolated from HL tumours.

Due to the lack of HL patients available for recruitment to the trial, a limited number of EBV specific clones were available for analysis. Five LMP2 specific CD8+ clones were generated from a HLA-matched sibling donor (Donor T27) and were expanded to sufficient cell numbers to analyse homing marker expression. As T cell clones generated were all CD8+ T cells, the mean expression of homing markers between the CD8 T cell subset from HL tumours (n=9) and T27 clones (n=5) were directly compared.

A high proportion of CD8+ HL-derived TIL and CD8+ T27 clones expressed CXCR3 (60-70 %) and CXCR4 (65-85 %) (Figure 5.1A). However, levels of expression of
Flow cytometry was employed to determine the percent expression (A) and mean channel fluorescence (B) of CXC chemokine receptors on T cell clones and HL CD8+ TIL (described in Chapter 3).

Cells were washed twice (PBS/2% FCS), collected (538 g /5 minutes) and resuspended at 1-5x10^5 cells/ml in PBS/2 % FCS. Cells were incubated with pre-titred concentrations of CXC chemokine receptor antibodies in a final volume of 50 µl ((CXCR3 (mIgG1), CXCR4 (mIgG2b), CXCR5 (mIgG2b), CXCR6 (mIgG2b)). The reaction was incubated on ice for 30 minutes. The cells were washed twice in PBS/2 % FCS. Visualisation was achieved with either anti-mouse or anti-rat IgG (biotin conjugated) followed by streptavidin Alexa 488. Cells were washed and incubated with normal mouse serum for 20 minutes. Cells were incubated with saturating concentrations of anti-CD8-TRICOLOR (clone MHCD0806) conjugated antibody. The reaction was incubated on ice for 30 minutes. The cells were washed twice in PBS/2 % FCS. Cells were either analysed immediately or fixed in 2 % paraformaldehyde. Cell analysis was done by flow cytometry. Results were analysed with the software package WinMDI. Data represent mean ± SD.

Tissue infiltrating lymphocytes were isolated from HL cell suspensions (n=9), and clones from patient T27 (n=5). Values were corrected for background staining by subtracting the isotype control value.
**A**

Percentage positive cells of total population

- **CXCR3**
- **CXCR4**
- **CXCR5**
- **CXCR6**

Chemokine receptor

- T27 clones
- HL CD8 TIL

**B**

MFI

- **CXCR3**
- **CXCR4**
- **CXCR5**
- **CXCR6**

Chemokine receptor

- T27 clones
- HL CD8 TIL
CXCR4 were significantly higher on clones than TIL (Figure 5.1B) (p<0.05, Mann Whitney test). CXCR5 and CXCR6 were expressed on few cells in TIL or T27 clones (Figure 5.1A).

CC chemokine receptors were also compared, with few cells in HL-derived TIL or T27 clones expressing CCR1, CCR2, CCR3 or CCR6 (Figure 5.2A). CCR4 was virtually absent on CD8+ TIL but showed variable expression on T27 clones (Figure 5.2A and 5.2B). Thus, fewer than 20 % of cells from three T27 clones expressed the CCR4 receptor but 48 % of c41 cells and 83 % of c174 cells expressed CCR4. CCR5 was detected on 36 ± 24 % SD of clones but was present on 15 ± 10 % SD of TIL (Figure 5.2A). This corresponded with higher cell surface levels on clones (Figure 5.2B). However the only statistically significant difference between T27 clones and HL-derived TIL was the expression of CCR7, which was absent on T27 clones but expressed on 57 ± 20 % SD of CD8+ TIL (p<0.001, Mann Witney test).

For adhesion molecule expression, between 70-100 % of HL-derived CD8+ TIL and T27 clones expressed LFA-1, PSGL-1, VLA-4 and β7 (Figure 5.3A) with no significant difference between clones and TIL in the percent of cells expressing these receptors. However, T27 clones had higher cell surface levels of all four of these receptors than TIL (Figure 5.3B). CD62L expression was detected on T27 clones (41 ± 7 % SD) and TIL (27 ± 15 % SD) (Figure 5.3A) whereas neither of these cell populations expressed significant levels of CLA (Figure 5.3A and 5.3B).
Flow cytometry was employed to determine the expression (A) and mean channel fluorescence (B) of CC chemokine receptors on T cell clones and HL CD8+ TIL (described in Chapter 3).

Cells were washed twice (PBS/2% FCS), collected (538 g /5 minutes) and resuspended at 1-5x10⁵ cells/ml in PBS/2 % FCS. Cells were incubated with pre-titred concentrations of CC chemokine receptor antibodies in a final volume of 50 µl ((CCR1 (mIgG2b), CCR2 (mIgG2b), CCR3 (rIgG2a), CCR4 (mIgG1) CCR5 (mIgG2a), CCR6 (mIgG2b), and CCR7 (rIgG2a)). The reaction was incubated on ice for 30 minutes. The cells were washed twice in PBS/2 % FCS. Visualisation was achieved as described in legend for Figure 5.1. Cell analysis was performed by flow cytometry. Results were analysed with the software package WinMDI. Data represent mean ± SD.

Tissue infiltrating lymphocytes were isolated from HL cell suspensions (n=9), and clones from patient T27 (n=5). Values were corrected for background staining by subtracting the isotype control value.
Flow cytometry was employed to determine the percent expression (A) and mean channel fluorescence (B) of adhesion receptors on T cell clones and HL CD8+ TIL (described in Chapter 3).

Cells were washed twice (PBS/2% FCS) and collected (538 g /5 minutes), resuspended at 1-5x10^5 cells/ml in PBS/2 % FCS. Cells were incubated with pre-titrated concentrations of adhesion receptor antibodies in a final volume of 50 µl ((CLA (rIgM), CD62L (mIgG1), LFA-1 (mIgG1), PSGL-1 (mIgG1), VLA-4 (mIgG1), β7 (rIgG2a)). The reaction was incubated on ice for 30 minutes. The cells were washed twice in PBS/2 % FCS. Visualisation was achieved as described in legend for Figure 5.1. Cell analysis was done by flow cytometry. Results were analysed with the software package WinMDI. Data represent mean ± SD.

Tissue infiltrating lymphocytes were isolated from HL cell suspensions (n=9), and clones from patient T27 (n=5). Values were corrected for background staining by subtracting the isotype control value.
**A**

Percentage positive cells of total population

- **CLA**
- **CD62L**
- **LFA-1**
- **PSGL-1**
- **VLA-4**
- **Beta 7**

Adhesion receptor

- T27 clones
- HL CD8 TIL

**B**

MFI

- **CLA**
- **CD62L**
- **LFA-1**
- **PSGL-1**
- **VLA-4**
- **Beta 7**

Adhesion receptor

- T27 clones
- HL CD8 TIL
5.2.1.2 Comparison of CD4/CD8 clones from HL, NPC and melanoma patients.

To analyse the homing phenotype of clones generated by this protocol, the study was extended to include clones from other EBV (NPC) and non-EBV (melanoma) associated cancer patients (see table 2.2). The rationale was to address whether there was a general homing phenotype for clones generated by this protocol, or whether there were differences based on factors like CD4/CD8 status, antigen specificity, or donor specific differences.

CD4 (n=12) and CD8 (n=7) clones were studied and there was considerable heterogeneity between individual clones of both subsets. CXCR3 was expressed on a majority of both CD4+ (71 ± 18 % SD) and CD8+ clones (56 ± 29 % SD) (Figure 5.4A). CXCR4 was expressed on more CD8+ clones (64 ± 19 % SD) than CD4+ clones (25 ± 15 % SD) (p<0.01, Mann Whitney test) (Figure 5.4A). CXCR5 and CXCR6 were typically expressed on a small proportion of clones examined, in both the CD4+ and CD8+ subset (Figure 5.4A).

CCR1, CCR2, CCR6 and CCR7 were weakly (< 10 %) represented on all clones (Figure 5.4B). CCR3 and CCR4 were expressed on a variable proportion of cells depending on the individual clone examined (Figure 5.4B). CCR5 was expressed on the highest percent of cells (from the CC chemokine receptor family) for both CD4+ (52 ± 33 % SD) and CD8+ (32 ± 22 % SD) T cell clones (Figure 5.4B). The interclonal heterogeneity of expression of CC chemokine receptors meant that there were no significant differences in expression between the CD4+ and CD8+ populations.
Figure 5.4 Comparison of CD4+ and CD8+ clones from HL, NPC and melanoma patients.

Flow cytometry was employed to determine the percent expression of CXC (A) CC (B) and adhesion receptor expression (C) on CD4 (n=12) and CD8 (n=7) T cell clones from HL, melanoma and NPC patients.

Cells were washed twice (PBS/2% FCS), collected (538 g /5 minutes), and resuspended at 1-5x10^5 cells/ml in PBS/2 % FCS. Cells were incubated with pre-titred concentrations of CXC, CC, and adhesion receptor antibodies as described in the legends for Figures 5.1-3. Cell analysis was done by flow cytometry. Results were analysed with the software package WinMDI. Values were corrected for background staining by subtracting the isotype control value.
Percentage positive cells of total population

A

B

C

P< 0.01

P< 0.01

P< 0.01

CXCR3

CXCR4

CXCR5

CXCR6

CCR1

CCR2

CCR3

CCR4

CCR5

CCR6

CCR7

CD4

CD8

CLA

CD62L

LFA-1

PSGL-1

VLA-4

β7
Most adhesion receptors examined were expressed on the majority (65-100 %) of the cell population for all clones (Figure 5.4C). However, CLA expression was absent from virtually all clones except c73 from patient T29 that expressed CLA on 28 % of the cell population (Figure 5.4C). There was no difference in adhesion receptor expression between CD4+ and CD8+ subsets except for CD62L expression, which was on few CD4+ clones, but was expressed on all CD8+ clones tested (46 ± 20 %) (p< 0.01 Mann Witney test) (Figure 5.4C).

5.2.1.3 Phenotypic heterogeneity between individual donors.

The same panel of clones were analysed with respect to individual donors to determine inter-patient variation in expression of homing markers.

Although there was intra-patient clonal heterogeneity, there were significant inter-patient differences in mean expression of receptors on cells. In the CXC chemokine receptor family, this was observed with CXCR4 and CXCR5 (Figure 5.5A). Clones from donor T27 had more CXCR4+ cells (68 ± 19 % SD) than T19 (P< 0.05, Kruskall-Wallis test). T30 clones (59 ± 24 % SD) had more CXCR5+ cells than T27 (P< 0.05, Kruskall-Wallis test).

In a similar manner, differential expression of CC chemokine receptors on clones from individual donors was observed for CCR3, CCR5 and CCR7 (Figure 5.5B). T18 clones expressed more CCR3 (30 ± 15 % SD) on cells than T30 clones, which were negative (P< 0.05, Kruskall-Wallis test). CCR5 was expressed on a majority of cells from patient T19 (81 ± 6 % SD). This contrasted with clones generated from the other patients where CCR5+ expression was present but on no more than 40 % of the
Figure 5.5 Comparison of homing expression clones generated from individual donors.

Flow cytometry was employed to determine the percent expression of CXC (A) CC (B) and adhesion receptor expression (C) on donor (n=6) derived T cell clones from HL, melanoma and NPC patients.

Cells were washed twice (PBS/2% FCS), collected (538 g /5 minutes) and resuspended at 1-5x10^5 cells/ml in PBS/2 % FCS. Cells were incubated with pre-titred concentrations of CXC, CC, and adhesion receptor antibodies as described in the legends for Figures 5.1-3. Cell analysis was done by flow cytometry. Results were analysed with the software package WinMDI. Values were corrected for background staining by subtracting the isotype control value. (*) represents significant differences between donors.
population, with the exception of T27 c15 (77 %). CCR7 was expressed on few cells and differences in expression are unlikely to be functionally relevant.

Adhesion receptor expression tended not to vary between donors. Only VLA expression significantly differed between donors T18 and T30 (Figure 5.5C).

5.2.1.4 The role of antigenic specificity on the phenotype of T cell clones.

T cell clones were analysed based on their antigenic specificity. Clones (n=7) specific for epitopes from EBV proteins were compared with clones (n=12) specific to melanoma antigens. Expression was compared for CXC, CC, and adhesion receptors (Figure 5.6). For most receptors there was considerable heterogeneity in their expression even amongst clones of the same antigenic specificity. This meant that there were no significant differences between the two antigenic specificities. The notable exception was CXCR4 (Figure 5.6A), where clones specific for EBV antigens expressed CXCR4 on a greater number of cells (61 ± 20 % SD) than clones specific for melanoma antigens (27 ± 18 % SD) (p<0.01 Mann Witney test). There was also a significant difference between groups for CCR7 (Figure 5.6B), however the proportion of cells expressing this receptor was low (<15 %) and are unlikely to be physiologically relevant.

5.2.1.5 Activation of T cell clones alters their expression of homing receptors.

To enhance T cell recruitment to tumours clones can either be selected based on their homing phenotype or cultured to alter it. To test whether the homing phenotype could be altered during culture, the influence of resting or activation of T cell clones from
Figure 5.6 Analysis of the influence of antigenic specificity on the homing phenotype of T cell clones.

Flow cytometry was employed to determine the percent expression of CXC (A) CC (B) and adhesion receptor expression (C) on T cell clones from EBV (n=7), melanoma (n=12).

Cells were washed twice (PBS/2% FCS), collected (538 g /5 minutes) and resuspended at 1-5x10^5 cells/ml in PBS/2 % FCS. Cells were incubated with pre-titred concentrations of CXC, CC, and adhesion receptor antibodies as described in the legends for Figures 5.1-3. Cell analysis was done by flow cytometry. Results were analysed with the software package WinMDI. Values were corrected for background staining by subtracting the isotype control value.
melanoma patients T29 and T30 was examined. Clones (n=3) were treated as follows and examined for mean percent expression of homing markers.

On day 14 of the REP cycle, a sample of cells was analysed (day 14) for receptor expression. The remainder were split and treated in three ways (see section 2.2.2.5). Clones were maintained in culture containing IL-2, washed extensively and resuspended in media without IL-2, or activated with 100 ng/ml anti-CD3/CD28. 24 hours later clones were analysed for homing marker expression.

The influence of continued culture with or without IL-2 had little influence on homing marker expression (Figure 5.7-5.9). In contrast, activation with 100-ng/ml anti-CD3/CD28 had a considerable influence on levels of CXCR3 receptor expression with downregulation to virtually undetectable levels for all three clones (Figure 5.7B). A similar decrease although less dramatic was observed for CXCR6 (Figure 5.7A,B). An activation induced increase in expression for CXCR4 and CXCR5 was only observed for clone 19 (Figure 5.7B).

Although there were few changes in expression of CC chemokine receptors between day 14, IL-2 and resting conditions (Figure 5.8A), activation promoted a decrease in the numbers of CCR2 (only T30 c19) and CCR4 expressing clones (Figure 5.8A,B). T30 c19 exhibited a moderate increase in expression of CCR3 and CCR7 although it was limited to this one clone (Figure 5.8B). There was no difference in mean levels of CCR1, CCR5 and CCR6 between treatments (Figure 5.8A).
Figure 5.7 The influence of different culture conditions on CXC chemokine receptor expression

Flow cytometry was employed to determine the mean percent expression (A) and individual values (B) of CXC chemokine receptors on T29 c73, c121 and T30 c19 T cell clones from melanoma patients after altering in vitro culture conditions (see 2.2.2.5 for details).

Cells were washed twice (PBS/2% FCS), collected (538 g /5 minutes) and resuspended at 1-5x10^5 cells/ml in PBS/2 % FCS. Cells were incubated with pre-titred concentrations of CXC chemokine receptor antibodies in a final volume of 50 μl ((CXCR3 (mIgG1), CXCR4 (mIgG2b), CXCR5 (mIgG2b), CXCR6 (mIgG2b)). The reaction was incubated on ice for 30 minutes. The cells were washed twice in PBS/2 % FCS. Visualisation was achieved as described in the legend for Figure 5.1. Cell analysis was done by flow cytometry. Results were analysed with the software package WinMDI. Data represent mean ± SD. Values were corrected for background staining by subtracting the isotype control value.
A

Chemokine Receptor

Percentage positive cells of total population

Day 14
IL-2
Rest
Act.

CXCR3
CXCR4
CXCR5
CXCR6

B

MFI

Percentage positive cells of total population

Δ T29 c121
□ T29 c73
○ T30 c19
Flow cytometry was employed to determine the mean percent expression (A) and individual values (B) of CC chemokine receptors on T29 c73, c121 and T30 c19 T cell clones from melanoma patients after altering *in vitro* culture conditions (see 2.2.2.5 for details).

Cells were washed twice (PBS/2% FCS), collected (538 g /5 minutes) and resuspended at 1-5x10^5 cells/ml in PBS/2 % FCS. Cells were incubated with pre-titrated concentrations of CC chemokine receptor antibodies in a final volume of 50 µl (CCR1 (mIgG2b), CCR2 (mIgG2b), CCR3 (rIgG2a), CCR4 (mIgG2a) CCR5 (mIgG2a), CCR6 (mIgG2b), and CCR7 (rIgG2a)). The reaction was incubated on ice for 30 minutes. The cells were washed twice in PBS/2 % FCS. Visualisation was achieved as described in the legend for Figure 5.1. Cell analysis was performed by flow cytometry. Results were analysed using the software package WinMDI (written by Joe Trotter). Data represent mean ± SD. Values were corrected for background staining by subtracting the isotype control value.
Figure 5.9 The influence of different culture conditions on adhesion receptor expression.

Flow cytometry was employed to determine the mean percent expression (A) and individual values (B) of adhesion receptors on T29 c73, c121, and T30 c19 T cell clones from melanoma patients after altering in vitro culture conditions (see section 2.2.2.5 for details).

Cells were washed twice (PBS/2% FCS) and collected (538 g /5 minutes), resuspended at 1-5x10^5 cells/ml in PBS/2 % FCS. Cells were incubated with pre titred concentrations of adhesion receptor antibodies in a final volume of 50 µl ((CLA (rIgM), CD62L (mlgG1), LFA-1 (mlgG1), PSGL-1 (mlG1) VLA-4 (mlgG1), beta 7 (rlgG2a)). The reaction was incubated on ice for 30 minutes. The cells were washed twice in PBS/2 % FCS. Visualisation was achieved as described in the legend for Figure 5.1. Cell analysis was performed by flow cytometry. Results were analysed with the software package WinMDI. Data represent mean ± SD. Values were corrected for background staining by subtracting the isotype control value.
A

Expression of LFA-1, VLA-4 and β7 integrin adhesion receptors with IL-2 restimulation in primary Th2 naïve cells (Figure 5.10A). CIA had no discernible change in expression with IL-2 restimulation compared to rest (Rest). CLA had no discernible change in expression with IL-2 restimulation compared to rest (Rest).

B

5.2.1.6 The influence of innate immune cell infiltration on Th2 naïve cell lines and the expression of membrane adhesion receptors on Clonal expansion in primary Th2 naïve cell lines (Figure 5.10B). T27, T29 and T30 were expanded and reconstituted for 10 days. The expression of membrane adhesion receptors (Figure 5.10B). T30 c19 exhibited a decrease in the percentage positive cells of total population (80-50%) in the mitogen-stimulated condition (Act).
Expression of LFA-1, VLA-4 and β7 (2/3 clones) decreased upon activation (Figure 5.9A,B). CD62L was expressed on few cells but for c73 where CD62L was expressed on most cells, activation strongly downregulated expression (Figure 5.9B). The number of PSGL-1+ cells was also down regulated with activation for T30 c19 (Figure 5.9A,B). However, T29 c73 and 121 significantly upregulated PSGL-1 expression with IL-2 or resting conditions and slightly with activation. (Figure 5.9B). CLA had no discernible change in expression with treatment (Figure 5.9A,B).

5.2.1.6 The influence of successive REP cycles on phenotype.

To obtain sufficient cell numbers for infusion of patients, clones often require multiple cycles of expansion to generate enough cells. To examine whether the homing phenotype altered between cycles of expansion, clones were analysed at the end of each cycle (day 14) for two cycles of expansion. Four clones from patients T27, T29 and T30 were examined for expression of CXC, CC chemokine and adhesion receptors (Figure 5.10-12).

Expression of most markers remained consistent with little difference in percent expression (<20 %) and MFI on the cells analysed. Exceptions included T27 c15, which had a moderate increase (20-30 %) in the number of cells expressing CXCR4 (Figure 5.10), CCR5 (Figure 5.11) and CLA between the first and second REP. Conversely, CD62L expression decreased on a similar number of cells from c15. (Figure 5.12). T30 c19 exhibited a dramatic decrease in the number of CXCR5+ cells (Figure 5.10) and increase in the number of CCR2 (Figure 5.11) expressing cells between the two cycles of expansion (~40 %). T29 c121 also displayed differences (~20-30 %) in the number of expressing cells for CXCR3, CXCR4 (Figure 5.10) and
Flow cytometry was employed to determine the percent expression and MFI of CXC chemokine receptors on T27 c15, T29 c73, c121, and T30 c19 T cell clones from two cycles (14 days) of expansion.

Cells were washed twice (PBS/2% FCS), collected (538 g /5 minutes) and resuspended at 1-5x10⁵ cells/ml in PBS/2 % FCS. Cells were incubated with pre-titrated concentrations of CXC chemokine receptor antibodies in a final volume of 50 µl ((CXCR3 (mIgG1), CXCR4 (mIgG2b), CXCR5 (mIgG2b), CXCR6 (mIgG2b)). The reaction was incubated on ice for 30 minutes. The cells were washed twice in PBS/2 % FCS. Visualisation was achieved as described in the legend for Figure 5.1. Cell analysis was performed by flow cytometry. Results were analysed with the software package WinMDI.

Values were corrected for background staining by subtracting the isotype control value. Open symbols represent the first expansion cycle. Filled symbols denote the second REP cycle.
Percentage positive cells of total population

△ T27 c15
◇ T29 c73
□ T29 c121
○ T30 c19
Figure 5.11 The influence of cycles of rapid expansion on CC chemokine receptor expression.

Flow cytometry was employed to determine the percent expression and MFI of CC chemokine receptors on T27 c15, T29 c73, c121, and T30 c19 T cell clones from two cycles (14 days) of expansion.

Cells were washed twice (PBS/2% FCS), collected (538 g /5 minutes) and resuspended at 1-5x10^5 cells/ml in PBS/2 % FCS. Cells were incubated with pre-titred concentrations of CC chemokine receptor antibodies in a final volume of 50 µl ((CCR1 (mlgG2b), CCR2 (mlgG2b), CCR3 (rlgG2a), CCR4 (mlgG1) CCR5 (mlgG2a), CCR6 (mlgG2b), and CCR7 (rlgG2a)). The reaction was incubated on ice for 30 minutes. The cells were washed twice in PBS/2 % FCS. Visualisation was achieved as described in the legend for Figure 5.1. Cell analysis was done by flow cytometry. Results were analysed with the software package WinMDI.

Values were corrected for background staining by subtracting the isotype control values. Open symbols represent the first expansion cycle. Filled symbols denote the second REP cycle.
Percentage positive cells of total population
Flow cytometry was employed to determine the percent expression and MFI of adhesion receptors on T27 c15, T29 c73, c121, and T30 c19 T cell clones from two cycles (14 days) of expansion.

Cells were washed twice (PBS/2% FCS), collected (538 g /5 minutes) and resuspended at 1-5x10⁵ cells/ml in PBS/2 % FCS. Cells were incubated with pre-titred concentrations of adhesion receptor antibodies in a final volume of 50 µl ((CLA (rIgM), CD62L (mlgG1), LFA-1 (mlgG1), PSGL-1 (mlgG1) VLA-4 (mlgG1), beta 7 (rIgG2a)). The reaction was incubated on ice for 30 minutes. The cells were washed twice in PBS/2 % FCS. Visualisation was achieved as described in the legend for Figure 5.1. Cell analysis was done by flow cytometry. Results were analysed with the software package WinMDI.

Values were corrected for background staining by subtracting the isotype control value. Open symbols represent the first expansion cycle. Filled symbols denote the second REP cycle.
Percentage positive cells of total population

- △ T27 c15
- ◇ T29 c73
- □ T29 c121
- ○ T30 c19
CD62L (Figure 5.12). For CXCR3 and CXCR4 this was more obvious when considering MFI values, however CD62L had weak levels of expression (Figure 5.10). A more striking difference in the proportion of CCR4 expressing cells (~45 %) (Figure 5.11), and a large difference in proportion of PSGL-1 expressing cells (~70 %) was observed for c121 (Figure 5.12). T29 c73 had moderate differences in cell numbers expressing CXCR3, CXCR4 (Figure 5.10) and CLA (Figure 5.12) although CLA levels were very weak. A large difference in PSGL-1+ expressing cells was observed (Figure 5.12). In summary, there does not appear to be (in this limited panel of clones) a consistent pattern of altered expression of homing markers between cycles of cell expansion. Nevertheless, some markers can vary dramatically on individual clones.

5.2.2 Functional analysis.

5.2.2.1 Migration of clones to purified chemokine.

In order to determine whether the identified receptors were functional, Transwell chemotaxis assays were employed to determine the ability of clones to migrate to recombinant chemokine. Sufficient cell numbers from T29 c73 and c121 were available to study chemotaxis to recombinant CXCL10/IP-10, CXCL12/SDF-1 and CCL17/TARC, as these clones expressed detectable levels of CXCR3, CXCR4 and CCR4 respectively.

The strongest response was to the chemokine CCL17/TARC with migration levels peaking at 100 ng/ml for both c121 (39 %) and c73 (75 %) (Figure 5.13A). Weaker dose dependent responses were observed to CXCL10/IP10 (Figure 5.13B) with migration peaking at 400 ng/ml for c73 (15 %). The response to c121 does not peak in
Figure 5.13 Migration of melanoma clones to purified chemokine.

Clones 73 and 121 from patient T29 were analysed for chemotaxis to purified CCL17/TARC (A), CXCL10/IP-10 (B), and CXCL12/SDF-1 (C). Cells were washed twice in RPMI 1640 and enumerated. The pellet was incubated with 100μCi [51Cr]O₄ for 1 to 2 hours at 37 °C/5 % CO₂. The cells were washed twice with RPMI 1640 (to remove any unincorporated chromium). Cells were resuspended at 5 x 10⁶ cells/ml in RPMI 1640/0.5 % BSA and 100 μl of cell suspension was added to the upper chamber of a 3-μm pore size transwell filter. The transwell was then added to the well of 24 well Tissue culture plate containing 600 μl stimulus. The transwell chamber was incubated at 37°C/5% CO₂ for 3 hours and the migrated cell population were quantitated using a gamma counter. Background migration was assessed by using chemotaxis media alone as a stimulus and 100 % migration was defined by adding 100 μl of cell suspension directly to the bottom of the chamber. Data represents results of triplicate determinations ± SD.
the dose range tested but levelled out at ~ 9%. The weakest response tested was to the chemokine CXCL12/SDF-1, although peak levels of migration did not occur in the range tested. When 400-ng/ml chemokine was tested, migration was measured at 10% (c73) and 4% (c121) of the total input.

5.2.2.2 The adhesion of therapeutic EBV specific T cell clones under physiological shear.

To examine the ability of clones to bind endothelial adhesion molecules in response to chemokines, two T cell clones from donor T27 were examined for their ability to bind purified VCAM-1 under conditions of flow. The rationale for choosing VCAM-1 was that expression had been detected in HL tissue and VLA-4 was expressed on T cell clones (and HL TIL). In addition, CXCR3 (CXCL9/CXCL10/CXCL11) and CXCR4 ligands (CXCL12/SDF-1) were tested for their ability to modulate T cell adhesion to VCAM-1, as these chemokine receptors were expressed on both clones and tumour-infiltrating T cells.

PBL were used as a control cell population and showed a greater number of cells that adhered/million cells perfused/mm² of a VCAM-1 coated microslide compared with T27 c41 and c174 (p< 0.001, ANOVA) (Figure 5.14A). Co-immobilisation of CXCR3L or CXCL12/SDF-1 ligands with VCAM-1 reduced the number of adherent PBL compared with VCAM-1 alone. In contrast, clones exhibited no significant difference in total adhesion with co-immobilisation of CXCR3L or CXCL12/SDF-1. When analysing the proportion of cells that rolled, (Figure 5.14B) PBL exhibited no significant change upon addition of CXCR3 ligands. However, there was a dramatic decrease in rolling with the addition of CXCL12/SDF-1 (p< 0.001, ANOVA). This
Figure 5.14 Adhesion of donor T27 clones to VCAM-1 under physiological flow.

T27 c41 and c174 were examined for adhesion to purified VCAM-1 under physiological shear. Total adhesion (A), rolling (B), and static shape change (C) were determined. PBL were included as a control population.

Glass capillary tubes were coated with 5 µg/ml purified VCAM-1 (1h/37 °C) and co-immobilised with CXCR3L (CXCL9, 10, and 11 (0-1000 ng/ml for 1h/37 °C) or CXCL12/SDF-1 (1000 ng/ml for 1h/37 °C). This was connected to the flow system as previously described (Lalor et al., 1997). Lymphocytes (10^6 cells/ml) were perfused through the microslide at a shear stress of 0.05 Pa. Adherent lymphocytes were observed by phase-contrast microscopy using an Olympus IX50 inverted microscope. Adhesion was converted to cells per square millimeter and corrected for the number of lymphocytes perfused (i.e., adherent cells per square millimeter per 10^6 perfused). Phase contrast video recordings made during lymphocyte perfusion were analysed off-line to determine the percentage of rolling cells, statically adherent cells, and total adhesion. Rolling cells moved slowly over the microslide surface during 5-10 s of observation, while stationary adherent cells made no discernable movement over the same period.
corresponded with an increase in the proportion of cells that had undergone static shape change with the addition of CXCL12/SDF-1 ($p < 0.001$, ANOVA) (Figure 5.14C). In assessing rolling and static shape change for c41 and c174, the addition of CXCR3 or CXCR4 ligands had no significant effect. Notably, clones exhibited similar proportions of cells that had undergone static shape change (Figure 5.14C) as PBL, potently induced by binding CXCL12/SDF-1.
5.3 Discussion.

With the ability in recent years to clone and expand large numbers of antigen specific T cells, interest in cellular based therapies has increased. For T cell based therapies to be successful, clones must be specific for their target antigen, be optimally activated, persist *in vivo* and traffic to tumour deposits. At present, the optimal culture conditions that provide these ideal properties have not been determined. This may be influenced by many factors including the presence of IL-2, IL-15, and CD4+ clones to provide T cell help.

This study has evaluated homing receptor expression and function on T cell clones generated for a phase I clinical trial of adoptive T cell therapy. We have compared clones generated for therapy of HL, with freshly isolated TIL from HL tumours. HL clones were also compared with clones produced for treating another EBV associated malignancy (NPC) and a non-EBV related tumour (melanoma). Furthermore, we were interested in whether the homing phenotype of clones was influenced by their target antigen specificity, CD4/CD8 status or the donor.

5.3.1 Exploiting HL T cell homing pathways for therapy.

A comparison of CD8+ clones from donor T27 with CD8+ TIL revealed that many receptors showed comparable expression on the two cell types although there were notable differences that are likely to have important implications for T cell homing to HL tumour deposits.

Most notably the chemokine receptor CCR7 was absent from clones but highly expressed on infiltrating T cells in keeping with its role in recruiting T cells to
secondary LNs (Stein et al., 2000). The absence of CCR7 on clones implies that other chemokine receptor ligand pathways must to be exploited to activate integrins on HEV bound lymphocytes. CCR7 is not always expressed on T cells found in LN sites and recently a role for CXCR4 has been implicated in central memory T cell homing in a murine model (Scimone et al., 2004). Since CXCR4 is expressed on T27 clones, this may be sufficient for recruitment to HL via endothelial expressed CXCL12/SDF-1. However, the efficiency of this pathway in specific recruitment is likely to be low as mice deficient in CCR7 dependent trafficking have a paucity of LN T cells. In addition, high-level expression of CXCR4 on clones compared with TIL may retain them at sites of CXCL12/SDF-1 expression within the tumour similar to that observed in inflammatory sites (Buckley et al., 2000).

Alternatively, CXCR3 (like HL TIL) was expressed on HL clones and may provide a general passport for entry into HL since this tumour is essentially an inflammatory site (Kunkel et al., 2002). The expression of CXCR3 ligands on the tumour vasculature is unclear and requires further examination. We have analysed CXCL10/IP-10 expression, which was absent from vessels but present on tumour cells. However, this does not discount CXCR3 dependent adhesion via CXCL9/MIG or CXCL11/ITAC. High expression of CXCL10/IP10 by H-RS cells implies this receptor-ligand pathway may be important in chemotaxis to tumour cells.

There were differences between clones and TIL in CC chemokine receptor expression. CCR4 was absent from intra-tumoural CD8+ T cells and functionally; chemotaxis experiments (Chapter 3) suggest HL TIL are unable to migrate to purified CCL17/TARC. In contrast, CCR4 was expressed on some T27 CD8+ clones. This is
potentially important as H-RS cells secrete high levels of CCR4 ligands (Ohshima et al., 2003; van den Berg et al., 1999). The ability to generate CCR4+ CTL clones that can functionally migrate to purified CCR4 ligands or expressing H-RS cell lines, will lend support to the hypothesis that CCR4+ CTL clones (upon entering HL tumours) would migrate to H-RS cells in a CCR4 dependent manner. CD8 TIL do not directly rosette around H-RS cells in situ (Poppema et al., 1982) and perhaps the absence of CCR4 on CD8+ TIL explains this. CCR5 was expressed on a high proportion of cells from T27 clones but was expressed on a minority of TIL. Expression of a CCR5 ligand CCL5/RANTES was reported in HL (Maggio et al., 2002). The authors reported IHC staining of macrophages, T cells and endothelial cells. Therefore, CCR5 expression on clones might be exploited for both T cell adhesion and chemotaxis within the tumour.

This study has allowed a comparison of CD8+ HL clones with freshly isolated CD8+ TIL. It is recognised that CD4 “help” is a likely requirement for clones to persist in vivo (Shedlock and Shen, 2003) and it will be important to compare the homing phenotype of tumour specific CD4+ clones with CD4+ TIL.

5.3.2 Heterogeneity of clonal homing phenotype.

The selection of antigen specific clones (both EBV and non-EBV specific) based on – IFN-γ expression allowed us to test the hypothesis that clones would express a polarized type 1 homing phenotype including CXCR3, CCR5 and CXCR6 (Sallusto et al., 1998). Although a number of clones did express a type 1 homing phenotype, there was considerable variation in the proportion of the cell population expressing
these receptors. In addition, some clones expressed CCR3 and CCR4, a marker characteristic of a type 2 homing profile (Sallusto et al., 1998).

This heterogeneity in the number of receptor expressing cells in a given clonal population provides a rationale for phenotyping clones prior to infusion to select those expressing high levels of the appropriate homing receptors. The reasons for this heterogeneity are interesting considering they arise from one site (i.e. the blood rather than different tissue sites) and are cultured under identical conditions. These differences perhaps reflect the ability of chemokine receptor expression to vary with CD4/CD8, target antigen specificity or donor status. For example CXCR4 was expressed predominantly on CD8+ clones from donor T27 and on EBV specific effectors. However, given the limited number of clones examined it was not possible to determine which of these associations was most important. However, Inter-clonal variability in the number of expressing cells meant that overall there were few significant differences in expression based on these three parameters.

5.3.3 The Function of T cell clones generated for adoptive cellular therapy.

For effective ACT, T cells require not only the correct homing phenotype, but they must persist and function correctly in vivo. Of the clones phenotyped in this study, six clones from three patients have so far been successfully infused in this trial. These clones have been analysed for persistence in vivo by ELISPOT (by Dr Steve Lee) with blood samples collected pre-infusion, then 15 minutes, 1 day, and 7 days post infusion. This was then repeated for subsequent infusions with collection of patient samples extending out to 14 days, 6 weeks and 8 weeks where possible after the final infusion.
To date, we have demonstrated that T cell therapy is feasible, with the successful infusion of CD4+ and/or CD8+ T cells. The treatment was well tolerated, and for 5/6 patients, cells expanded in vivo, persisted (1-14 days; one case >2 months) and were functional immediately ex vivo. Homing in vivo was not monitored in this initial trial but in future will be of interest.

To analyse the function of T cell clones, we first confirmed that CD8+ T27 clones and CD8+ TIL expressed adhesion molecules on a similar proportion of cells for all of the receptors examined. Clones expressed higher cell surface levels and this is likely to be functionally important. Furthermore, with the exception of CD62L and CLA, most adhesion receptors were expressed on a majority of the cell populations. Although not examined in this study, other factors such as integrin clustering at the membrane and the proportion of integrins in the active conformer (Hogg et al., 2002) will be relevant in functional adhesion and cell-cell/matrix interactions within the tumour microenvironment. Having confirmed expression of VLA-4 on clones (and HL TIL), and expression of the corresponding ligand (VCAM-1) in HL tissue, we tested the homing function of two LMP2 specific clones (c41 and c174) generated for our current trial of adoptive cellular therapy. We tested the ability of these two clones to adhere to purified VCAM-1 (co-immobilised with chemokine) under physiological shear. Compared with PBL, clones 41 and 174 arrested rapidly from flow without the need for rolling. This is likely to be a consequence of the activated states of their integrins as reflected by a lack of requirement for chemokine to induce shape change (note both clones expressed CXCR3 and CXCR4). Total adhesion of PBL to VCAM-1 (without chemokine), was greater than both clones 41 and 174 and may reflect the
concentration of VCAM-1 used. Titration of VCAM-1 may allow clones to better adhere to lower concentrations of VCAM-1 than PBL (Lalor et al., 1997). Although these results are preliminary, they suggest that clones expanded in vitro for therapeutic use may have a loss of tissue tropism because adhesion is independent of chemokine triggering. Consequently, they will tend to adhere non-specifically to endothelium under conditions of low shear stress such as occur in the lung, liver and spleen. This is consistent with observations in humans where infused T cells rapidly become trapped in such tissues (Chin et al., 1993). These results suggested exploring the ability to manipulate adhesion molecule expression and function on T cells prepared for infusion.

5.3.4 Altering the phenotype of T cell clones.

The ability to alter homing expression was evaluated by resting or activating clones. Short-term culture of clones in the absence of IL-2 appeared to have no effect on their homing phenotype although extending the resting period may address this. However there is an increased likelihood of cell death, which may be prevented by culturing cells in type 1 interferons (Pilling et al., 1999) In contrast, stimulation with anti-CD3/CD28 resulted in downregulation of a number of adhesion receptors after 24 hours. It will be important to monitor the kinetics of re-acquisition of these integrins. Therapeutically, short-term downregulation of adhesion receptors may allow a greater degree of systemic recirculation before reacquiring receptor expression. Activation also has a profound effect on chemokine receptor expression. This was more complex, with upregulation of some receptors and downregulation of others. The ability to strongly downregulate expression of CXCR3 on clones is interesting as CXCR3 is typically associated with activation (Qin et al., 1998). Although less unambiguous,
other markers associated with an inflamed phenotype, include CCR2, CCR4 and CXCR6, which were also downregulated (on clones expressing these markers). There was a concomitant increase in expression of homeostatic receptors including CXCR4, CXCR5, and CCR7. It will be important to expand this study to include more clones.

One study has examined polyclonal CTL lines for expression following TCR stimulation with anti-CD3/CD28 (Sallusto et al., 1999a). The authors describe a similar short-term shift in chemokine receptor expression with downregulation of "inflammatory" chemokine receptors like CCR1, CCR2, CCR3, CCR5, CCR6 and CXCR3 within six hours. There was a concomitant increase in homeostatic receptors including CCR4, CCR7, CCR8 and CXCR5 over 2-3 days. IL-2 reversed these trends for CCR3, CCR5, CCR7, CCR8 and CXCR5. The ability to upregulate CCR7 expression on clones may provide a phenotype conducive to directing them into HL tumour deposits. However, the ability CCR7 positive clones to display immediate effector function (if necessary?) will have to be examined (Sallusto et al., 1999b). Our study has demonstrated the ability to modulate homing receptor expression upon activation. However, it will be important to confirm these results with functional assays of migration and adhesion. Furthermore, the importance of cytokines like IL-2, IL-15, and IFNs in modulating homing expression as well as enhancing cellular expansion and survival of therapeutic T cell clones in vitro will be an important area of investigation to improve T cell based therapies.
Chapter 6: Concluding Discussion.

This thesis addresses the possibility of a T cell-based therapy for HL, by exploring the molecular mechanisms of T cell homing to this tumour. HL is an attractive target for immunotherapy as the tumour is heavily infiltrated with T cells in contrast to other tumours like colorectal hepatic metastases (Yoong et al., 1998). Furthermore, the malignant H-RS cells lie within a background of reactive cells including T cells. In contrast, other solid tumours may have infiltrates that are located at peritumoural margins rather than infiltrating the tumour parenchyma.

The work has focused on two main areas of research. Firstly, we have exploited the observation that T cells infiltrate HL tumours and have attempted to elucidate the relevant homing pathways. This involved examining the homing phenotype of TIL isolated from HL tumours by analysing the expression of chemokine (chapter 3) and adhesion receptors (chapter 4). With identification of the relevant receptors, expression of the corresponding ligands was determined by IHC. Where possible this work was extended to include functional studies. Chemotaxis of healthy donor derived lymphoblasts to HL cell lines was demonstrated to define pathways of T cell homing in vitro. Complementing this, it was important to determine whether freshly isolated T cells from HL are able to migrate to the purified chemokine (known to be expressed in situ). Preliminary investigations demonstrated migration of non-expanded TIL to CXCL12/SDF-1 but not CCL17/TARC. Secondly, we have examined the phenotype and function of these molecules on T cell clones prepared for a clinical trial of adoptive T cell therapy for HL and other human malignancies (chapter 5).
6.1 Do HL-derived TIL have distinct homing properties?

The distinctive phenotype of TIL from HL compared with TIL from other solid tumours suggests that different tumours recruit T cells employing dissimilar homing pathways. This is unsurprising considering the requirements for recruitment to a LN in HL compared with recruitment to peripheral tissue. However, this is the first detailed study to show such differences and the results have implications for therapy.

Based on these results, we can propose a model of T cell homing into Hodgkin's tumours (Figure 6.1). Firstly, T cells expressing CD62L capture and roll on o-glycan linked core proteins (including PNAd) expressed on tumour vessels. VAP-1 expressed on the endothelium may also promote subset specific recruitment of CD8+ T cells by binding surface exposed primary amines. T cell expression of CCR7 and CXCR4 then allows engagement of CCL21/SLC and CXCL12/SDF-1 respectively, presented on the luminal surface by the glycocalyx. This activates Integrins such as LFA-1 and VLA-4, which causes cell arrest, and TEM into the tumour parenchyma. T cell expressing VLA-4 might also be directly captured from the circulation onto vessels expressing VCAM-1. T cells can then respond to CXCR3, CXCR4, and CCR7 ligands within the tumour promoting directed migration, with CXCR3+ T cells migrating to H-RS cells in response to CXCL10/IP10 expression. In addition, small populations of CXCR6 (CD8), CCR4 (CD4) and CCR5 (CD4 and CD8) expressing T cells may localise in proximity to H-RS tumour cells.
A model for T cell homing is proposed where T cells expressing L-selectin capture and roll on o-glycan linked core proteins (including PNAd) expressed on tumour vessels. Endothelial expressed VAP-1 may also promote subset specific recruitment of CD8+ T cells by binding surface exposed primary amines. T cell expression of CCR7 and CXCR4 allows engagement of CCL21/SLC and CXCL12/SDF-1 respectively, posted on the luminal surface by the glycocalyx. Integrins including LFA-1 and VLA-4 are activated and facilitate firm arrest. These LFA-1/ICAM-1 and VLA-4/VCAM-1 adhesive events promote TEM into the tumour parenchyma. T cells then respond to CXCR3, CXCR4, and CCR7 ligands within the tumour promoting directed migration. In addition, small populations of CXCR6 (CD8), CCR4 (CD4) and CCR5 (CD4 and CD8) T cells may respond to local chemokine gradients positioning them in proximity to H-RS tumour cells.
CD62L (VAP-1?)
Mediated capture
LFA-1/ICAM-1
VLA-4/VCAM-1
Firm adhesion

Triggering via
CCL21/CXCL12

Chemotaxis

CXCL9/10/11?
CXCL12
CXCL16?
CCL17/CCL22
CCL3,4,5,8,11,14,16?
CCL19?

H-RS cell

CXCL13

LFA-1
VLA-4
PSGL-1
Beta 7

CXCR3
CXCR4
CCR7

CXCR3
CXCR4
(CXCR5)
(CXCR6)
(CCR4)
(CCR5)
CCR7

CXCL13

CXCR4
6.2 What is the nature of the H-RS microenvironment?

HL is a complex model in which to evaluate T cell homing as the distinction between those cells that are recruited in response to a target antigen or simply migrate, as part of routine homeostatic trafficking is unclear. An inflammatory versus a homeostatic response is likely to be determined by the degree to which the nodal architecture has been effaced with tumour. This is further complicated by the classification of HL into distinct subtypes and an incomplete association of EBV with HL (40 % of cases). Too few samples were studied in this work but it will be interesting to determine if different homing phenotypes exist based on HL subtype or EBV status.

The ability of rare H-RS cells to survive in the face of an overwhelming reactive infiltrate remains enigmatic, as H-RS cells appear capable of processing and presenting antigen to T cells (Lee et al., 1998). This includes viral antigens from EBV but may also include other target antigens including MAGE A4 which is expressed in ~ 30 % of HL cases (mainly EBV- cases) (Chambost et al., 2000). Furthermore, H-RS cells express high levels of adhesion receptors and co-stimulatory molecules (Ellis et al., 1992). The homing phenotype of TIL suggests a complex infiltrate that may be composed of T\textsubscript{H1} (CXCR3, CCR5, CXCR6) and T\textsubscript{H2} (CCR3, CCR4) like T cells. T cells immediately surrounding H-RS cells are CD45RA- (Poppema, 1989) suggesting a memory phenotype and express activation markers including CD38 and CD69. In addition, they are predominantly CD4+/CD57+, which is normally ascribed to a germinal centre population of helper T cells (Bowen et al., 1991). Furthermore, polyclonal populations of CD4+ T cells rosetting H-RS cells were microdissected from tissue, arguing against an antigen specific T cell response (Roers et al., 1998). Taken together it has been proposed that a non-specific population of T\textsubscript{H2} T cells
locally interacts with H-RS cells aiding in their survival and proliferation in much the same way follicular homing CD4+ T cells rescue B cells from apoptosis (Choe et al., 1996). H-RS cells secrete cytokines and chemokines that were thought to promote the recruitment of this TH2 population of T cells. H-RS cells can induce fibroblasts to secrete eotaxin in an in vitro co-culture model (Jundt et al., 1999) promoting recruitment of CCR3 expressing TH2 cells. We report expression of CCR3 on few infiltrating T cells suggesting this may not be a major recruitment pathway. Chemokines like CCL17/TARC and MDC/CCL22 are directly produced by H-RS cells and have been implicated as an important mechanism for recruiting TH2 cells (van den Berg et al., 1999). We detected this receptor on a minor population of CD4+ TIL, which may play a role in microenvironmental homing. However, these pathways are unlikely to be a major pathway for bulk T cell recruitment. An alternative hypothesis reinterpreting this published data argues that the infiltrate comprises a predominantly regulatory T cell population (Marshall et al., 2004). This was supported by the observation that TIL were anergic to mitogen, poorly proliferates, and had few cells expressing a TH1 or TH2 cytokines compared with autologous PBL. Furthermore, TIL contained large populations of both IL-10 secreting and CD4+CD25+ regulatory T cells. Thus, the presence of regulatory T cells in HL tumours may explain H-RS cell survival.

6.3 Analysis of T cell clones and implications for therapy.

A phase I clinical trial of adoptive T cell therapy was initiated. This allowed us to explore an EBV-associated tumour as a suitable model for T cell immunotherapy. The ability to isolate and expand large numbers of antigen specific T cell clones from cancer patients in vitro precludes a number of uncertainties that arise with vaccination
strategies. Specificity, function, and the numbers of CTL can be defined in vitro before infusion. Infusion of antigen specific T cell clones into patients represents the “gold standard” regarding target antigen choice and aids the rational development of therapeutic vaccines. However, there are important considerations regarding the wide scale applicability of adoptive T cell therapy, which is technically demanding (requiring extensive safety and toxicity screening), expensive and tailored to the individual patient. In addition, in spite of knowledge of specificity, function and cell numbers, there remain important questions regarding the ability of such cells to persist in vivo and traffic to tumour deposits.

To evaluate the homing phenotype of cells generated using this protocol, and to determine the ability of infused clones to traffic to HL tumour deposits, we analysed homing receptor expression and function on EBV specific clones and compared them with clones specific to melanoma antigens. In general, clones expressed a polarised chemokine receptor phenotype with expression of CXCR3, CCR5 and CXCR6 (characteristic of type 1 response). This is consistent with the fact that they were selected in vitro based on IFN-γ production. However, some clones expressed markers indicative of a type 2 response including CCR3 and CCR4 implying clones may need to be phenotyped before infusion to ensure trafficking to the tumour. Furthermore, there was considerable heterogeneity in the proportion of clonal populations expressing these receptors. This meant there were few significant differences in expression based on antigen specificity, CD4/CD8 or donor status.

The homing phenotype of CD8+ T cell clones from donor T27 was compared with HL CD8+ TIL. There were few differences in expression of receptors with the
exception of CCR4 and CCR7. The presence of CCR4 expression on T27 clones may allow microenvironmental positioning within HL tumours based on high expression of CCR4 ligands detected *in situ* by us and others (van den Berg et al., 1999). However, the lack of expression of CCR7 on these cells indicates that clones may need to exploit alternative pathways to enter HL tumours. VLA-4 is highly expressed on HL TIL and clones, and may be activated by chemokine receptors like CXCR3 and CXCR4, suggesting these clones may still be able to enter HL tumours binding vessels by exploiting these alternative adhesion pathways.

Flow based adhesion studies of T cell clones adhering to purified VCAM-1 demonstrated the clones were unaffected by co-immobilising VCAM-1 with chemokine. The implication is that clones have highly activated integrins that can bind counter receptors independently of chemokine signalling. However, the concern is that clones have lost tissue tropism and will not efficiently traffic to tumour deposits.

The implication from these functional studies is that strategies are needed to alter the homing phenotype of T cell clones to restore tissue tropism. Activation of clones with anti-CD3/CD28 was examined as a possible way to downregulate integrin receptors in the short term and upregulate receptors like CCR7 that confer specificity for LN. Although limited in the numbers of clones analysed, activation appeared to switch expression from an inflammatory to a homeostatic homing phenotype. For therapy of HL, it may be desirable to have a population of clones that display a more central memory phenotype. Namely, they express high levels of CCR7 and CD62L and are able to enter LN. Furthermore, they would posses enhanced proliferative capacity and
persist in vivo (Sallusto et al., 1999). The ability to derive central memory cells in vitro for cellular therapy remains a significant challenge considering that clones produced by this protocol tend to be virtually all effector T cells (personal communication S. Riddell). Furthermore, the capacity of central memory clones to mediate efficient target cell killing will need to be examined although studies in animal models suggest central memory rather than effector memory cells are more effective in clearing viral and bacterial model infections (Wherry et al., 2003). Other approaches to achieve efficient homing include retroviral transduction of antigen specific clones with the appropriate chemokine receptor (i.e. CCR7). This approach has been successfully demonstrated with retroviral transduction of CXCR2 into T lymphoblasts, which then could migrate to a melanoma cell line in a CXCL1/Gro-α dependent manner (Kershaw et al., 2002). Alternatively, cells of the correct homing specificity (i.e. TIL) could be selected and transduced with cloned TCRs specific to the required target antigen (Kessels et al., 2001). It will be important to ensure modified T cells retain their homing phenotype after such manipulation in vitro.

6.4 Animal models of T cell homing.

Upon identification of T cell homing function in vitro, it will be important to confirm the in vivo relevance of these pathways. Previous studies have demonstrated that human chemokine function can be analysed in vivo in a SCID mouse model (Taub et al., 1996). SCID mice received an intraperitoneal injection of human PBL. They were then injected subcutaneously with recombinant human chemokine. CD3+ T cells were shown to accumulate at the injection site. It will be interesting to assess chemokine-mediated infiltration of HL tumours in vivo. This could be done by injecting SCID mice subcutaneously with chemokines known to be expressed by H-RS cells in situ or
alternatively with chemokine expressing H-RS cell lines (Borchmann et al., 2003). Human TIL are then adoptively transferred into these mice and stained for appropriate human T cell surface markers to assess the extent of T cell infiltration of the injection site. Blocking studies with neutralising antibodies to the relevant chemokine-receptor pathway would then confirm the role of that interaction in mediating infiltration. Xenograft models have been developed for investigating T cell trafficking including a SCID-hu Skin mouse model to investigate selective chemokine mediated homing of human T-lymphocytes to the skin (Carballido et al., 2003). Engraftment of human synovium onto SCID mice was used to examine the resulting effects of TNF injection in modulating leukocyte migration (Wahid et al., 2000). Promisingly for the study of migration in HL, SCID mice were transplanted with human peripheral LN to investigate CXCL12/SDF-1 dependent migration of human lymphocytes and a CXCL12/SDF-1 responsive cell line U937 (Blades et al., 2002). This model could be adapted transplanting HL LN to evaluate human T cell homing. Whether grafted tissue retains expression of human endothelial markers is something that would have to be validated but nevertheless would provide a useful in vivo model of T cell homing.

6.5 Tracking infused T cells in humans.

The ability to track infused cells in vivo has greatly aided in the elucidation of homing mechanisms. The availability of intravital microscopy and 2-photon imaging has provided a comprehensive understanding of trafficking in animal models (Iparraguirre and Weninger, 2003). Ultimately though, analysis of trafficking in humans requires alternative approaches. At present, infused cells are monitored by taking blood samples and looking for evidence of immune responses by ELISpot and chromium
release assays. Evidence of trafficking to, and function at, the site of the tumour are hard to determine. To address this problem, alternative approaches including genetic marking of infused cells (Heslop et al., 1994), as well as short term indium 111 labelling has been employed (Dummer et al., 1993). Indium 111 labelling of cells provides real time monitoring of labelled cells. The advantages are that the half-life of indium-111 is short enough to be safe, yet long enough for in vivo circulation, however the resolution of this technique is low. Early studies of indium labelled TIL from patients with metastatic melanoma revealed trafficking of cells to tumour deposits (Fisher et al., 1989). More recently, labelling of Melan-A specific CTL lines allowed the identification of these cells at metastatic sites as early as 48 hours after infusion (Meidenbauer et al., 2003).

To summarise, we have attempted to better understand the mechanisms of T cell recruitment to HL tumours. This information can then be applied to improve future T cell therapies by ensuring the appropriate homing receptors are expressed on antigen specific T cell effectors such that they efficiently traffic to HL tumour sites.
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