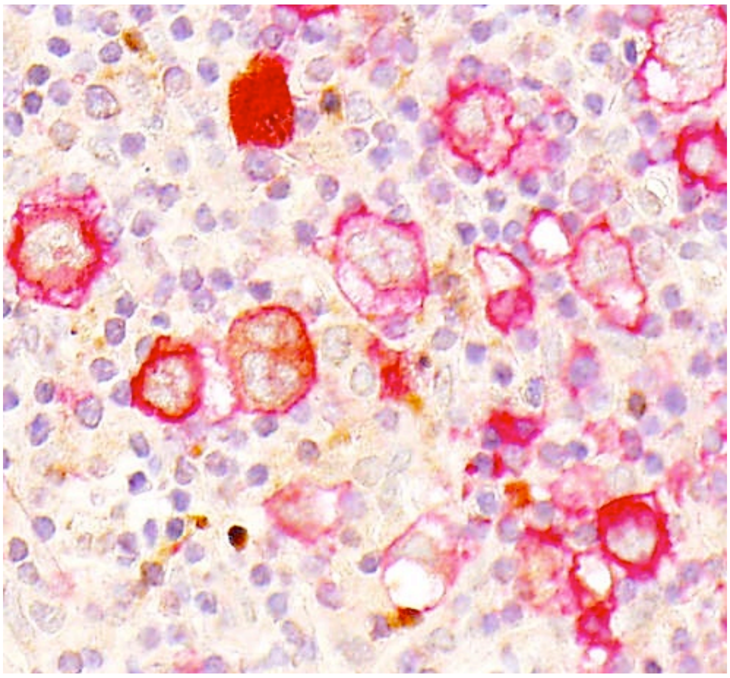
Classic Hodgkin Lymphoma: the malignant cells and tumour microenvironment in adults of different ages



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Declaration

In accordance with the postgraduate degree regulations of the University of Edinburgh, I declare that this thesis was composed solely by myself, that the work described is my own and that it has not been submitted for any other degree or professional qualification.

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Abstract

Classic Hodgkin Lymphoma (cHL) has an annual incidence of 2.4 cases per 100 000 population in the UK, and is one of the most common malignancies diagnosed in young adults aged 15 to 34. The majority of younger patients have a good long-term outcome with between 80 and 90% disease-specific survival but cHL also affects older adults in whom the prognosis is significantly poorer. The role of tumour-associated macrophages (TAM) in cHL has gained much interest, with several studies reporting an association between high numbers of CD68-positive TAM and poor prognosis. There is also a question over the prognostic significance of Epstein-Barr Virus (EBV) infection which is implicated in up to 50% of cHL cases in developed countries. Published data suggests that EBV positivity in elderly patients may be associated with a poorer outcome, whereas in younger adults may be of prognostic benefit. Differences related to age are of interest particularly as an age-related decline in immunity has been linked with the development of certain subtypes of Non-Hodgkin Lymphoma in older patients.

In a retrospective study, two separate cohorts of patients with cHL were examined with the aim of identifying:

- Differences in the cellular composition of the tumour microenvironment in cHL which has arisen in young and elderly adult patients;
- Differences in the cellular composition of the tumour microenvironment in cHL associated with or without EBV infection;
- Factors within the tumour microenvironment which may influence prognosis and may be targeted for novel treatments.

One group consisted of patients aged between 15 and 34 years at diagnosis and the other, of those aged 60 or over at presentation. Tissue obtained at the time of diagnosis was examined with regard to a number of factors related to the malignant cells and the surrounding microenvironment, including the number and phenotype of macrophages, the number of plasmacytoid dendritic cells and the number of malignant Hodgkin Reed-Sternberg (HRS) cells and non-

malignant ‘background’ cells undergoing apoptosis. Comparisons were made between the two age groups, also taking into account the EBV-status of tumours, cHL subtype and gender.

Results confirmed the current understanding that EBV-positive cHL is more common in older patients and has a strong, but not exclusive, association with the MCHL subtype. In addition, a strong link between young males and EBV-positive disease was shown. Macrophages were found to vary between the two age groups, in number and phenotype and there were clear differences associated with the presence or absence of EBV infection. While no definite link with outcome and macrophages was identified it was apparent that the implications of macrophages in the tumour microenvironment may differ between the two age groups. The number of apoptotic cells correlated closely with the number of macrophages and in the young the number of HRS cells was associated with prognosis.

Investigation of the tumour microenvironment is complex and caution is needed in interpreting studies which do not differentiate between patients according to age, as tumour characteristics may have variable implications in different age groups.

In this thesis a number of clinicopathological differences were identified between the two age groups. These point to the need for further larger studies to delineate how such age-related differences may or may not be associated with immune function and how this information could be translated into treatments to improve outcomes.

Lay Summary

Classic Hodgkin Lymphoma is a form of cancer diagnosed in patients of all ages but is particularly common in young adults. The majority of patients do well with current treatments but a significant minority relapse and die of the disease and older patients tend to do less well than young adults. A number of other factors are known to be associated with a poor outcome and are used to predict which patients may benefit from more intensive treatments but it is not clearly understood why some patients do so well and others succumb. Age appears to be an important factor and there is much interest in the influence of the 'tumour microenvironment'. In cHL, the malignant cells (Hodgkin Reed-Sternberg cells) make up only a small proportion of the tumour, the majority of which, is composed of non-cancerous, reactive cells of the patient's immune system. How these cells may influence the way the tumour behaves and how it may respond to treatment is of great importance as if this is understood, there is the potential for new treatments to be developed to target these cells and improve outcome.

There are different subtypes of cHL and up to 50% of cases are associated with a viral infection (Epstein-Barr Virus). It is also crucial to understand how these factors might relate to outcome.

This study looked at two different groups of adults diagnosed with cHL. One consisted of young adults, aged 15 to 34 years at diagnosis and the other of older adults, aged 60 years or over at presentation. Tumour tissue, taken at the time of diagnosis was examined for each patient and compared between the two groups for a number of different factors which included:

- presence or absence of the Epstein Barr-Virus
- the number and type of macrophages and plasmacytoid dendritic cells (non-malignant cells of the immune system) in the tumour microenvironment

- the number of cells undergoing ‘programmed cell death’ (apoptosis) in each tumour
- proteins expressed by the malignant Hodgkin Reed-Sternberg cells

From this it was evident (1) that tumour characteristics varied according to the patient’s age; (2) non-malignant cells in the tumour microenvironment had different properties and behaved differently in older and younger patients; and (3) Epstein-Barr virus infection was associated with some tumour characteristics but these differed between the two age groups. Further larger studies are required to delineate these findings further.

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Abbreviations

ABVD	Doxorubicin, Bleomycin, Vinblastine, Dacarbazine
ACAMP	Apoptotic-cell associated molecular pattern
ALCL	Anaplastic Large Cell Lymphoma
ALK	Anaplastic Lymphoma Kinase
AP	Alkaline Phosphatase
AP-1	Activator Protein 1
APC	Antigen Presenting Cell
ATP	Adenosine Triphosphate
BCG	Bacille Calmette Guerrin
BCR	B-cell Receptor
BCSH	British Committee for Standards in Haematology
BDCA2	Blood Dendritic Cell Antigen 2
BSAP	B-cell lineage Specific Activator Protein
c-PARP	Cleaved poly-ADP-ribose polymerase 1
CCL	Chemokine (C-C motif) ligand
cHL	Classic Hodgkin Lymphoma
cHL NOS	Classic Hodgkin Lymphoma not otherwise specified
ChlVPP	Chlorambucil, Vinblastine, Procarbazine, Prednisolone
CLL	Chronic Lymphocytic Leukaemia
CM	Cytotoxic Molecule
CMneg	Cytotoxic Molecule-negative
CMpos	Cytotoxic Molecule-positive
CMV	Cytomegalovirus
CSF-1	Colony Stimulating Factor-1 (=M-CSF)
CTL	Cytotoxic T-lymphocyte
CXCL	Chemokine (C-X-C motif) Ligand
DAB	Diaminobenzidine
DC	Dendritic Cell
DLBCL	Diffuse Large B-cell Lymphoma
DNA	Deoxyribonucleic acid
ds	Double-stranded
DSS	Disease Specific Survival
EBERs	Epstein-Barr virus Encoded RNAs
EBNA	Epstein-Barr virus Nuclear Antigen

EBV	Epstein-Barr Virus
EDTA	Ethylenediaminetetraacetic acid
EMAP II	Endothelial-monocyte-activating Polypeptide II
ER	Endoplasmic Reticulum
EU	European Union
FDC	Follicular Dendritic Cell
FFPE	Formalin-Fixed Paraffin-wax Embedded
FFS	Failure Free Survival
FKN	Fractalkine
FL	Follicular Lymphoma
GEP	Gene Expression Profiling
GM-CSF	Granulocyte-macrophage Colony Stimulating Factor
H&E	Haematoxylin and Eosin
HCl	Hydrochloric acid
HD	Hodgkin Disease
HHV8	Human-Herpes Virus 8
HIV	Human Immunodeficiency Virus
HL	Hodgkin Lymphoma
HLA	Human Leucocyte Antigen
HRP	Horseradish Peroxidase
HRS	Hodgkin Reed-Sternberg cells
HSP	Heat Shock Protein
IARC	International Agency for Research on Cancer
IFN	Interferon
IFN-a	Interferon-alpha
IFN-G	Interferon-gamma
Ig	Immunoglobulin
IgH	Immunoglobulin heavy chain
IHC	Immunohistochemistry
Il	Interleukin
ILT-2	Immunoglobulin-like Transcript 2
ILT-7	Immunoglobulin-like Transcript 7
IM	Infectious Mononucleosis
IMS	Industrial Methylated Spirits
iNOS	Inducible Nitric Oxide Synthase
IPS	International Prognostic Score
ISH	In-situ Hybridisation

JAK2	Janus Kinase 2
LAG-3	Lymphocyte Activation Gene-3
LCM	Laser Capture Microdissection
LDcHL	Lymphocyte-depleted classic Hodgkin Lymphoma
LMP	Latent Membrane Protein
LPC	Lysophosphatidylcholine
LPS	Lipopolysaccharide
LRcHL	Lymphocyte-rich classic Hodgkin Lymphoma
M-CSF	Macrophage Colony Stimulating Factor (=CSF-1)
MCHL	Mixed-Cellularity classic Hodgkin Lymphoma
MCP-1	Macrophage Chemotactic Protein-1
mDC	Myeloid Dendritic Cell
MGUS	Monoclonal Gammopathy of Uncertain Significance
MHC	Major Histocompatibility Complex
mM	Millimolar
MSC	Myeloid Suppressor Cell
MYD88	Myeloid Differentiation Primary Response 88
NF-KB	Nuclear Factor-Kappa B
NHL	Non-Hodgkin Lymphoma
NK	Natural Killer cells
NLPHL	Nodular Lymphocyte Predominant Hodgkin Lymphoma
NO	Nitric Oxide
NOD-SCID	Non-obese Diabetic-Severe Combined Immunodeficiency
NOS	Not otherwise specified
NSCP	Cellular Phase of Nodular Sclerosing Hodgkin Lymphoma
NSHL	Nodular Sclerosing classic Hodgkin Lymphoma
OAS	O-Acetylserine
OS	Overall Survival
p=NS	p-value = Non-significant
PAF	Platelet Activating Factor
PAMP	Pathogen-Associated Molecular Pattern
PAX5	Gene encoding the B-cell lineage specific activator protein
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PD-1	Programmed Death-1
PD-L	Programmed Death-1 Ligand
PD-L1	Programmed Death-1 Ligand-1

PD-L2	Programmed Death-1 Ligand-2
pDC	Plasmacytoid Dendritic Cell
PECC	Prednisolone, Etoposide, Chlorambucil, Lomustine
PEN	Polyethylene Naphthalate
PFS	Progression Free Survival
PGE_2	Prostaglandin-E_2
PRP	Pattern Recognition Receptor
PTCL	Peripheral T-cell Lymphoma
PTLD	Post-transplant Lymphoproliferative Disorder
PVACE-BOP	Vinblastine, Etoposide, Chlorambucil, Procarbazine, Doxorubicin, Vincristine, Prednisolone, Bleomycin
RFS	Relapse-Free Survival
RNA	Ribonucleic Acid
RT-PCR	Reverse Transcription Polymerase Chain Reaction
SCID	Severe Combined Immunodeficiency
STAT	Signal Transducers and Activators of Transcription
T-reg	T-regulatory Cell
TAP	Transporter Associated with Antigen Processing
TBS	Tris Buffered Saline
TCR	T-cell receptor
TGF-a	Transforming Growth Factor-alpha
TGF-B	Transforming Growth Factor-Beta
T_H	T-helper cell
T_{H1}	T-helper cell type 1
TIA-1	T-cell-restricted Intracellular Antigen-1
TLR	Toll-like Receptor
TMA	Tissue Microarray
TNF	Tumour Necrosis Factor
TNF-a	Tumour Necrosis Factor-alpha
TRAIL	TNF-related Apoptosis Inducing Ligand
TSP-1	Thrombospondin-1
TUNEL	Terminal transferase-mediated dUTP Nick-end Labelling
UTP	Uridine triphosphate
VEGF	Vasoactive Endothelial Growth Factor
VEPEMB	Vinblastine, Cyclophosphamide, Procarbazine, Prednisolone, Etoposide, Mitoxantrone, Bleomycin
WGH	Western General Hospital, Edinburgh

WHO	World Health Organisation
µl	Microlitre
µm	Micrometre

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Chapter 1

Introduction

Hodgkin Lymphoma

Descriptions of primary lymph node tumours date back as far as 1666 (Ortiz-Hidalgo 1994) but Thomas Hodgkin, in his writings, 'Morbid appearances of the absorbent glands and spleen', first described in 1832 what later became known as Hodgkin's Disease (HD) (Mani & Jaffe 2010; Ortiz-Hidalgo 1994). There was debate for many years over its nature, with many people believing it to be an infectious condition rather than a malignant one. In some ways this controversy continued until it was re-termed Hodgkin lymphoma (HL) after the cell of origin was finally found to be a post-germinal centre B-cell (Enblad et al. 2007; Grur et al. 1997; Küppers et al. 1994; Weiss et al. 1986; Hummel et al. 1996). The malignant cells, or 'Hodgkin Reed-Sternberg' cells (HRS), are named after Dorothy Reed and Carl Sternberg who separately described them in 1902 and 1898 respectively. At this stage Sternberg asserted, as did many, that HD was a chronic inflammatory process triggered by infection with the tubercle bacillus of Mycobacterium Tuberculosis. Dorothy Reed, on the other hand, argued that although TB was often found in lymph nodes affected by HD, this was a secondary process. She too, however, thought HD represented a chronic inflammatory condition although the pathological agent had yet to be discovered (Zwitter et al. 2002). It is easy to see why it was not immediately evident that HD is a malignancy when histologically the malignant cells make up only a minority of the tumour mass (Skinnider & Mak 2002; Küppers 2009b; Chetaille et al. 2009). The majority of cells (95-99%) are non-clonal, non-malignant inflammatory cells including lymphocytes, plasma cells, eosinophils, neutrophils and macrophages. The role these play in the pathogenesis and progression of HL is the focus of much research as are the 'microenvironmental' cells of tumours in general. It is anticipated that a greater understanding of these non-malignant elements will lead to novel treatments for diseases, based on undermining those factors that nurture the tumour and enabling the benefits of the immune system to be harnessed to counteract them.

Early understanding of infection and the immune response

In terms of human history, knowledge of the infectious causes of many diseases and the immune response to such conditions is very recent. Louis Pasteur, born in 1822, was the first to demonstrate that organisms from the surrounding environment were responsible for 'souring' wine, beer and milk and thus described the basis of the 'germ theory' of infectious disease, going on to identify an infectious cause for diseases including anthrax, cholera and tuberculosis (Kaufmann 2008). The 'invasion' of white blood cells by infectious agents was first observed microscopically by Robert Koch (1843-1910), who identified anthrax bacilli within white blood cells and tubercle bacilli in granulomatous giant cells. However, he interpreted the host as a passive agent in this process and it was Elie Metchnikoff (1845-1916) who first described phagocytes and phagocytosis as the fundamental basis of the innate immune system. Metchnikoff's initial studies identified a process by which cells took in nutrients but later experiments, involving starfish larvae, showed these same nutrient-engulfing cells responding to attack by a foreign body. Taking this, and Robert Koch's earlier observations, led him to the conclusion that phagocytosis was an active host defence against invading microbes (Kaufmann 2008). He then went on to provide an early description of macrophages engulfing apoptotic cells through his examinations of tadpoles developing into frogs and thus identified another function of phagocytes in addition to host defences. Whilst Metchnikoff is credited with identifying the basis of the innate immune system, Paul Ehrlich (1854-1915) was the first to describe the fundamentals of humoral immunity with his 'side-chain' theory. He proposed that all cells expressed specific receptors for the nutrients they required to sustain them and that nutrients were taken up by cells once they had interacted with the corresponding receptor. He hypothesised that harmful substances could mimic nutrients but once these bound to the specific cell surface receptor an excess of the same receptor was produced to enable neutralisation of the toxic agent. He also described the process of bacteriolysis and haemolysis based on a similar mechanism, recognising and naming 'complement' (although this had already been described and named as 'alexins' by others; Eduard Buchner and Jules

Bordet) as a group of molecules causing cell destruction once activated by cell surface 'side-chains' that had bound specifically to other cells or bacteria (Kaufmann 2008). His hypothesis that the immune system could suppress the development of malignancy was one of the earliest on the subject but this concept was not widely accepted and little was done to examine it further (Dunn et al. 2002). Similarly, Metchnikoff's work on phagocytosis was laid aside until many years later (Kaufmann 2008).

A historical perspective on the recognition of the immune response to malignancy

We now have a good understanding of the immune response to infectious pathogens but the role of the immune system in malignancy is still not well delineated. Rudolf Virchow in 1863, and before Paul Ehrlich's observations, is credited with noting the presence of white cells in human tumours and speculating on their interaction with malignant cells (Farinha et al. 2005; Dunn et al. 2002). Hardley wrote of tumour regression in melanoma associated with high numbers of white cells in the early 1900's (Mantovani et al. 1992) but it was the 1950s before the hypothesis of 'cancer immuno-surveillance' was proposed by Burnet and Thomas; theorising that the immune system could recognise and destroy cells which had undergone malignant transformation i.e. introducing the concept of specific tumour antigens (Burnet 1957). Much of this work came from mouse experiments into allograft rejection, which showed that cellular components of the immune system were largely responsible for this phenomenon. Subsequent studies then suggested the presence of tumour specific antigens. Tumours transplanted between genetically identical mice were destroyed if the recipient mouse had been 'vaccinated' against the tumour beforehand and this too was mediated by 'cellular' immunity (Dunn et al. 2002). However, the theory of cancer immuno-surveillance fell out of favour in the 1970's. There was data showing immunosuppressed mice were more prone to virally induced tumours and spontaneous lymphomas but this was not taken forward after a large body of work using athymic nude mice showed no increase in spontaneous or carcinogen-induced tumours. It wasn't until much later that the flaws in these experiments were understood. It had been assumed that athymic mice were profoundly immunosuppressed when in fact they have fewer, rather than absent T-cells, with persistence of Natural Killer (NK) and gamma-delta T-cells which are not made in the thymus (Dunn et al. 2002). After the concept of immuno-surveillance had been rejected, opposing theories suggesting a role for the immune system in tumour promotion arose, and in reality both are probably correct. The demonstration that interferon-gamma (IFN-G) protected mice against tumour development as did Perforin, a key component of cytolytic granules in both cytotoxic T-cells (CTL) and NK cells, raised interest once more in immuno-surveillance and carcinogenesis in the late 1990's. Greater understanding allowed the development of truly immuno-deficient mice with evidence of increased tumorigenesis. Human studies also showed an increased incidence of malignancy in immunosuppressed patients and although many of these tumours were induced by viral infections, others were not and it was clear that non-virus-triggered malignancies, including melanoma, lung cancer and gastrointestinal malignancies were also more common (Dunn et al. 2002).

Early understanding of phagocytosis and macrophages

Interest in phagocytes was revived in the 1970's, once it became apparent that phagocytosis and antigen presentation were an important part of the adaptive immune response with the realisation that T-lymphocytes could not recognise antigens directly (Kaufmann 2008). Following on from Metchnikoff's observations we now know that macrophages are derived from peripheral blood monocytes that have migrated into tissues in response to antigenic stimuli (Van Ginderachter et al. 2006). Their physiological role is to initiate the innate immune response against pathogens, which they do by first recognising Pathogen-Associated Molecular Patterns (PAMP) via their Pattern Recognition Receptors (PRP). This allows them to phagocytose the potentially harmful agent, destroying it in the process and subsequently presenting its antigens to T-cells which augment the innate immune response and lead to a humoral response (Gordon & Taylor 2005). This process of pathogen destruction involves an inflammatory response which, if left unchecked, would become harmful to the host through tissue and DNA damage. Macrophages are not only key in triggering this process but also play a fundamental role in limiting subsequent inflammation to allow healing and restoration of normality. Therefore, even whilst inflammation is being triggered, processes are put in motion to limit it and alter the function of macrophages to allow wound healing. The same macrophage can both initiate inflammation and be key in its resolution, moving apparently seamlessly between the two roles and if necessary back again (Van Ginderachter et al. 2006).

Macrophages in malignancy

It is therefore now well established that macrophages function differently in different circumstances and their study is, as a result, fraught with difficulties. Until the late 1970's it was assumed that white cells within tumours were combatting the tumour and that 'activated' macrophages in particular would kill any malignant cells (Nathan et al. 1975; Evans 1978). Exposing macrophages to Bacille Calmette Guerrin (BCG) was a recognised method of 'activating' macrophages and such cells were therefore expected to inhibit the growth of malignant cells. Indeed, it had been shown in mouse studies, that BCG treated mice exhibited slower growth of lymphocytic leukaemias and lymphomas (Nathan et al. 1975). However, questions arose after a comparison was made between 'activated' macrophages from BCG exposed mice with peritoneal, 'normal' macrophages from non-exposed mice with regard to their effect on a number of murine lymphoma cell lines. Rather unexpectedly, the 'normal' macrophages, which were expected to have little or no effect appeared to stimulate cell growth whereas the BCG 'activated' macrophages had much less of an effect (Nathan et al. 1975). The authors concluded that macrophages could produce 'profound cytotoxicity' but could also cause 'equally intense stimulation' of cells and that this was dependent on the stimulus applied to the macrophages, the target cells and the assays used to assess the activity; which today still seems a fair appraisal. Other groups took things further and were able to show that murine fibrosarcoma growth was dependent on the presence of non-malignant white cells including macrophages i.e. not only were the tumour white cells not causing tumour regression but were critical to its progression. This was demonstrated using whole body irradiation in mice with fibrosarcoma, causing leucodepletion that resulted in reduced tumour growth. Several groups found the same for a number of different tumours but it was not universal and certain tumour types appeared to grow equally well if not better without the presence of white cells (Evans 1978; Prehn & Outzen 1977; Tyan 1974; Kleiweg & Rijswijk 1966; Gillette & Wunderlich 1977). By the 1990s techniques had moved on with the development of immunohistochemistry on frozen tissue sections that allowed inflammatory infiltrates to be better defined and in many tumour types these were found to consist largely of T-cells and macrophages (Mantovani et al. 1992). New ways of measuring macrophage 'activation' states were developed once certain molecules could be recognised such as TNF-alpha (TNF-a), interleukin-1 (Il-1), TGF-beta (TGF-B). Not only this, but the concept of macrophage 'plasticity' was mooted when the gain, loss and subsequent further gain of surface molecules such as MHC class II was noted (Mantovani et al. 1992; Elgert et al. 1998). It was proposed that perhaps the tumour cells themselves were producing factors that attracted white cells; chemotactic factors such as Macrophage Chemotactic Protein-1 (MCP-1), which had levels correlating with macrophage numbers in many tumours. 'Tumour-associated Macrophages' (TAM) as a distinct entity were referred to, and specific mechanisms for the varying effects of white cells, and macrophages in particular, were suggested including reduced cytotoxicity of macrophages in response to GM-CSF; tissue invasion via the production of plasminogen activator; and the promotion of angiogenesis (Mantovani et al. 1992; Elgert et al. 1998; Mills et al. 2000; Mantovani et al. 2002). One particular pathway, still of interest today, was described by a group in 1992 who hypothesised that arginine metabolism by macrophages was key in tumour promotion or regression (Mills et al. 1992). They proposed that if arginine was metabolised by Nitric Oxide synthase, this would produce Nitric Oxide (NO) which is cytotoxic and therefore would cause tumour regression. If, on the other hand, arginine was metabolised by arginase to produce ornithine and urea, this would support tumour growth as ornithine is a precursor of polyamines needed for cell replication. They provided evidence for this hypothesis using a mouse model where they could demonstrate increased NO-synthase activity and NO production, with an associated increase in IFN-G, in mice rejecting tumours and a reduction in NO with increased ornithine production and arginase activity in those mice with tumour progression. They therefore concluded that this macrophage pathway of arginine metabolism could influence host immune responses to a tumour (Mills et al. 1992).

Knowledge has continued to accrue and although macrophages can be demonstrated to present tumour antigens to the immune system and exhibit direct anti-tumour cytotoxicity, in other circumstances they can be shown to promote tumour cell growth and to inhibit both T- and NK-cell anti-tumour responses (Elgert et al. 1998). Malignant cells have been shown to actively recruit macrophages to tumours via the production of chemotactic factors such as TGF-B and MCP-1. Macrophages themselves produce growth factors and cytokines with widespread actions, including GM-CSF, TGF-alpha and -beta, many interleukins and prostaglandins. Some of these can promote angiogenesis, whilst the inhibition of other factors such as TNF-a, also allow angiogenesis (Elgert et al. 1998). Other substances seem to affect macrophages differently according to the location of the macrophage within the tumour. For example increased NF-KB might be stimulated in macrophages distant to the tumour, resulting in increased levels of cytotoxic molecules, whereas the same stimulants would have a different effect on macrophages within the tumour mass itself (Elgert et al. 1998). The theory behind this is that more remote macrophages are allowed to produce factors that might be cytotoxic but are also immunosuppressant and therefore of benefit to the tumour, whilst being too distant from it for the cytotoxic factors to be harmful, and at the same time those cytotoxic elements are inhibited in TAM (Elgert et al. 1998). This was shown to be true for a number of factors including TGF-B, Il-4, Il-6, M-CSF and GM-CSF, which all seem to activate macrophages at the periphery of a tumour while down-regulating the actions of macrophages within the body of the tumour itself (Elgert et al. 1998). Other studies have shown that cytokines, like

TNF-a, also do not have uniform activities in different macrophage groups. TNF-a would normally be expected to stimulate macrophages to produce cytokines that enhance T-cell proliferation and the immune response. This was shown to occur in macrophages from the spleen but macrophages obtained from the peritoneum and exposed to TNF-a were found to be immunosuppressive, probably due to increased Prostaglandin-E_2 (PGE_2) production which blocks the actions of TNF-a and suppresses lymphocyte proliferation (Elgert et al. 1998). Tumour cells and certain macrophages can produce PGE_2 which could act in this way. Another cytokine of interest is Il-10 which also may be produced by tumour cells and macrophages alike. Il-10 blocks macrophage cytotoxicity within tumours by, amongst other things, inhibiting the production of NO and TNF-a and directly suppressing the proliferation of stimulated T-helper cells (T_H). T_{H1} cells are affected more than T_{H2}, resulting in overall inhibition of macrophage activation as this is normally stimulated by IFN-G from T_{H1} cells and suppressed by T_{H2} cells (Elgert et al. 1998; Mills et al. 2000; Mantovani et al. 2002).

Attempts to delineate macrophages by function

In an attempt to rationalise the description of macrophage functions, the terms M1/M2 macrophage were proposed in 2000, based on the similarities and connections with T_{H1}/T_{H2} responses. Cytokines produced by T-lymphocytes were shown to affect the functions of macrophages e.g. IFN-G leads to macrophage 'activation' or 'M1' actions including the production of NO and the stimulation of T_{H1} responses that would normally be cytotoxic and lead to the destruction of pathogens. These 'classically activated' macrophages produce many pro-inflammatory cytokines'. In turn 'anti-inflammatory' cytokines such as Il-4 and Il-10 lead to so called macrophage 'inhibition' or 'M2' actions with increased arginase activity and ornithine production rather than NO, with stimulation of a T_{H2} and subsequent humoral immune response rather than a cytotoxic one (Mills et al. 2000). Macrophage inhibition was evidently an inappropriate term for this and rather an 'alternative activation' state was being described which physiologically plays a role in the resolution of inflammatory responses, adaptive immunity, tissue remodelling and repair. M1/M2 macrophages were seen to respond to T-lymphocytes and their cytokines but could also influence T-cells and their subsequent functions and it became less clear which came first; T_{H1} cells causing M1 macrophages or M1 macrophages stimulating a T_{H1} response (Mills et al. 2000; Mantovani et al. 2002). Groups started to define the differences between M1 and M2 cells, showing that they varied by receptor expression, effector function and cytokine/chemokine production. M1 cells were high producers of Il-12 and TNF with high levels of iNOS activity and NO production. On the other hand, M2 cells were low in Il-12, high in Il-10, with high levels of arginase activity and ornithine production as already described (Mantovani et al. 2002). M1 cells produce CXCL9 and 10, attracting T_{H1} cells, with inhibition of CCL18, 22 and 24, exactly opposed by M2 cells which produce CCL18, 22 and 24 in response to Il-4 which in turn attract T_{H2} and T-regulatory cells (T-reg) (Mantovani et al. 2002; Mantovani et al. 2005).

Potential cell surface markers of M1/M2 cells were identified. CD163, a member of the 'scavenger receptor cysteine rich superfamily', was found to be expressed on human peripheral blood monocytes and on most tissue macrophages but was not found on dendritic cells. Such cells appeared to be found during the healing phase after acute inflammation and produced both anti-inflammatory and pro-angiogenic factors. CD163 expression was not seen in macrophages stimulated by pro-inflammatory factors such as IFN-G, lipopolysaccharide (LPS) or TNF-a but was associated with stimulation by Il-10 and Il-6 which were often produced subsequent to LPS and TNF-a activation, after the acute phases of an inflammatory reaction (Buechler et al. 2000). CD163 has therefore been proposed as a marker of M2 type macrophages involved in the healing process after acute inflammation.

Despite these clearer descriptions of different macrophage activation states it is apparent that this is a considerable oversimplification of the process and that M1/M2 macrophages at best represent the extremes of a complicated continuous spectrum of actions. The M1/M2, T_{H1}/T_{H2} analogy is useful and logical but unlike T-lymphocytes, macrophages do not develop into lineage-defined subsets with inherent differences and permanent molecular changes but rather respond and fluctuate according to their surroundings and influences (Mantovani et al. 2002; Mantovani et al. 2005). As a result, there have been numerous attempts to further define functional subtypes of macrophages using terms such as 'host defence', 'wound healing' and 'immune regulation' or further dividing M2 macrophages into M2a, M2b and M2c whilst continuing to appreciate that every form of intermediary cell may exist (Mosser & Edwards 2008; Van Ginderachter et al. 2006). Fundamentally however, the problems in defining, identifying and therefore studying these cells remain. Not only do these cells have the ability to carry out any number of functions but their 'plasticity' means they can move back and forward between roles whilst still expressing or exhibiting the remnants of their previous functional status having already moved on to carry out an opposing task (Mosser & Edwards 2008).

Tumour-associated macrophages

Tumour-associated macrophages have been proposed as yet another distinct entity. It has been shown that CCL2, a chemokine commonly produced by malignant cells, is chemotactic for macrophages. In melanoma, one study showed low levels of CCL2 were associated with lower or more 'physiological' numbers of macrophages within the tumour; something which appeared to be associated with tumour progression. However, in those melanomas with very high levels of CCL2, massive numbers of macrophages were evident and these TAM appeared able to combat the tumour, causing regression (Mantovani et al. 2002). This was contrary to the findings of many other studies into different tumour types where TAM appeared to be linked to a poorer prognosis and again illustrates the complexity of studying these cells (Mantovani et al. 2002). TAM have been likened to M2 macrophages, highlighting similar functions such as poor cytotoxicity, lack of NO production, poor antigen presenting capacity, promotion of angiogenesis and cell proliferation with high Il-10 and low Il-12 expression (Mantovani et al. 2002; Mills et al. 2000) but others have highlighted that TAM have features of both M1 and M2 cells with tumours benefitting from the pro-tumour effects of each whilst avoiding the detrimental actions (Van Ginderachter et al. 2006). At different stages of tumour development, macrophages also may play different roles. At the site of previous pulmonary TB, M1 macrophages are found and play a pro-inflammatory role. This may increase the risk of DNA damage and malignant change via the release of factors such as reactive oxygen species but these cells also retain the ability to remove malignant cells. However, later if a tumour does develop at the site, the macrophages take on a different phenotype with a more M2-like pattern including high levels of Il-10 which contributes to the inhibition of the immune response to tumour antigens and promotion of tumour survival alongside increased angiogenesis allowing tumour growth (Mosser & Edwards 2008). The trigger which leads to this switch in macrophage function has yet to be ascertained but it is likely that hypoxia, prostaglandins, immune complexes and apoptotic cells all play a role. Specific pathways of importance have been suggested including the activation of the NF-KB transcription factor through MyD88 activation, but there is much to be clarified (Mosser & Edwards 2008). NF-KB activation and the subsequent production of pro-inflammatory cytokines looks to be a key pathway, not just in malignancy. IFN-G stimulation of macrophages, itself stimulates the expression of TLR-4 and other key components of the MYD88 signalling path leading to NF-KB activation. Interestingly Il-10, a stimulant of the M2 phenotype, inhibits all of these actions (Van Ginderachter et al. 2006). It is hoped that gene expression profiling (GEP) techniques may come to clarify these issues and work is already being done to identify particular genetic markers of different functional macrophages.

Factors which influence macrophage function

There are many influences on macrophage function. Viral infections are generally thought to stimulate an M1 macrophage phenotype via IFN-G but some intracellular organisms appear able to alter this process to their own advantage. Leishmania species' and Mycobacterium tuberculosis can both interfere with IFN-G signalling to avoid destruction via M1 processes (Mosser &

Edwards 2008). There is also evidence that viral infections, such as Human Herpes Virus 8 (HHV8) in Kaposi's sarcoma play a direct role in promoting tumour progression. Viral encoded chemokines appear to favour T_{H2}/M2 responses with the effect of reducing anti-viral and therefore also anti-tumour immune responses (Mantovani et al. 2002). Parasitic, bacterial and viral pathogens are all thought capable of exploiting macrophage function by mimicking some of the stimuli of more 'regulatory' macrophages through, for example, the production of 'chitinase-like' proteins, which lead to defective antigen presentation and immune stimulation and ultimately defective pathogen killing (Mosser & Edwards 2008; Van Ginderachter et al. 2006).

The nature of cells being phagocytosed by macrophages also may influence the subsequent actions of the latter. Apoptotic cells are frequent within tumours and an important normal physiological role of macrophages is to clear debris including apoptotic cells. Phagocytosis of apoptotic cells results in the release of anti-inflammatory factors such as TGF-B, PGE and platelet activating factor (PAF), with suppression of pro-inflammatory mediators such as TNF-a (Turner et al. 2003; Mosser & Edwards 2008). Given the prominence of apoptotic cells in many malignancies, the further study of these factors and their role in inflammation and tumour progression would be of value.

CSF-1 (also known as M-CSF) is key in controlling the proliferation, differentiation and survival of macrophages and their precursors (Van Ginderachter et al. 2006). It is known that CSF-1-null mice show markedly reduced tumour growth and delayed metastatic disease. Myeloid Suppressor Cells (MSCs) are also of interest. These immature myeloid cells are increased in those with progressive tumour growth of almost any kind. They are the result of increased myelopoiesis stimulated by growth factors and inflammatory cytokines from both tumour and host cells such as GM-CSF, VEGF, Il-1beta (Van Ginderachter et al. 2006). They accumulate in the bone marrow, blood and secondary lymphoid tissue where they can act as antigen presenting cells (APC), but ones that have been inadequately activated and are therefore able to tolerise the host to antigens rather than elicit the expected immune response. They therefore may play a role in suppressing the immune response to tumour antigens (Van Ginderachter et al. 2006).

Macrophage activation and function and the influence of macrophages over other cells is therefore very complex. The difficulties in studying macrophages, deciphering their role in individual tumour types and, more specifically, in individual patients becomes evident.

Epstein-Barr virus and Hodgkin Lymphoma

Another key area of interest in HL is that of infection and, as discussed above, this has been a point of debate since the disease was first described. Initially thought to be an infectious disorder due to the predominance of inflammatory cells within tumours, it was eventually accepted to be a malignant disease, although the question of an infectious trigger remains. The wide epidemiological variations in HL are what led to the 'three disease hypothesis' first proposed by MacMahon in 1966 (MacMahon, 1966) which essentially speculated that an infective agent (then unspecified and now possibly thought to be EBV) accounted for the disease in young children when they developed the primary infection; re-activation of this infective agent could cause HL in elderly adults and perhaps late exposure and infection with this agent could cause HL in young adults (Jarrett, Armstrong, & Alexander, 1996; MacMahon, 1966).

The Epstein-Barr virus has infected >95% of humans by adulthood (Snow & Martinez 2007) and is therefore considered ubiquitous, even having reached geographically remote populations that have avoided infection by other common viruses (WHO 1996b). The age at which infection is acquired varies according to socio-economic status with those in the lowest socio-economic groups tending to be infected in early childhood. As spread is mostly via the oral route, closer living quarters in poorer societies may play a role in this. In the developed world, infection is often delayed until adolescence and at that age, half of all infections will be clinically apparent, manifesting as Infectious

Mononucleosis (IM). In general, infection at a younger age correlates with a lower risk of clinically evident symptoms (WHO 1996b).

EBV was the first oncogenic virus to be identified through its association with endemic Burkitt's Lymphoma. The role of EBV in the pathogenesis of Post-transplant Lymphoproliferative Disorders (PTLD) and in the majority of HIV-associated lymphomas is now also well established (Graig et al. 1993; Epstein et al. 1964; Hamilton-Dutoit et al. 1991; Lenoir & Bornkamm 1987; WHO 1996b; Snow & Martinez 2007; Bellas et al. 1996). In 1997 the International Agency for Research on Cancer (IARC) reviewed the role of EBV in human malignancies and concluded that there was sufficient data to support the theory that EBV is a causal factor in EBV-positive cHL (WHO 1996a). The reasons quoted for reaching this conclusion are several:

- EBV is found in up to 50% of cHL cases in Western countries and up to 100% in some populations.
- Anti-EBV antibody titres have been found to be increased at presentation in cHL patients with EBV-positive disease and for some years prior to diagnosis (WHO 1996a; Glaser et al. 1997).
- The incidence curve for Infectious Mononucleosis according to age lies parallel to that for the incidence of HL in different populations (Place et al. 2000).
- Virtually all the malignant cells in EBV affected cases are positive for the virus (WHO 1996a).
- In affected cases, EBV is evident in all sites of disease and remains evident when disease recurs (WHO 1996a).
- Monoclonal EBV episomes are identifiable in tumour cells indicating that infection has taken place prior to the malignant transformation of cells (Weiss et al. 1986; WHO 1996a).
- RT-PCR transcriptional studies have shown evidence of transcription of some viral genes (EBNA-1, LMP-1, 2A, 2B, EBERs) in HRS cells and immunohistochemistry has confirmed expression of the corresponding

proteins, all of which suggests that the viral genome is actively expressed in these cells and is not a 'silent passenger' (WHO 1996a).

- EBV gene expression in immortalized rodent cell lines results in malignant transformation of infected cells both in-vitro and in-vivo (WHO 1996a).
- EBV has been shown to immortalize human B-lymphocytes and make them tumorigenic in immunodeficient mice (Glaser et al. 1997; WHO 1996a).

The incidence of EBV-positive HL varies according to age, gender, ethnicity and geographical location. In North America and the EU, EBV positivity is demonstrated in 26-50% of cHL (Jarrett et al. 1996; Armstrong et al. 1998; WHO 1996a). This percentage is much higher in Asia and higher still in Latin America. There is a strong association between EBV and Mixed Cellularity disease and also a strong age relationship. Young children and older adults are significantly more likely to have EBV-positive disease than young adults. Those with the lowest incidence of EBV-positive disease are young adults with NSHL, with only 14% of cases EBV-positive in one series. This contrasts with children less than five years old who almost invariably have EBV-related disease (Jarrett et al. 1996; Armstrong et al. 1998) and older adults, in whom up to 64% are EBV-positive in some studies (Armstrong et al. 1998). Even when accounting for subtype and gender, age is an independent risk factor for EBV-related cHL (Jarrett et al. 1996).

The tumour microenvironment in Hodgkin Lymphoma

Beyond all the specific, theoretical reasons as to why non-malignant cells in cHL may influence HRS cells, there are three basic observations which demonstrate the importance of the tumour microenvironment in cHL and why it should be studied. 1. HRS cells are very rarely found circulating in peripheral blood; 2. HRS cell lines are difficult to establish and maintain in culture and 3. HRS cells are unable to survive in immunodeficient mice (Enblad et al. 2007). Other fundamental observations regarding variations in the behaviour, of what otherwise appears to be the same histological disease, in different epidemiological groups, illustrates how important it is not to make generalisations about the presence of factors, such as macrophages, within tumours when we have very little knowledge of what they are doing there. This is why studies simply enumerating macrophages are inadequate and why it is vital to tightly control the groups being studied to limit the number of confounding factors as far as is possible. We know that the actions of macrophages are influenced by the factors that stimulate them but that they may also be altered by the presence of infections, by treatments being used (Taskinen et al. 2007), by genetics and by other factors such as age, which is known to fundamentally affect our immune function.

Aims of the study

A study was therefore designed to examine the following:

- Differences in the cellular composition of the tumour microenvironment in cHL which has arisen in young and elderly adult patients;
- Differences in the cellular composition of the tumour microenvironment in cHL associated with or without EBV infection;
- Factors within the tumour microenvironment which may influence prognosis and may be targeted for novel treatments.

Age is a significant prognostic factor in cHL. The immune system changes with ageing and there is a recognised immunodeficiency in the elderly (Asano et al. 2009; Said 2007; Garibaldi & Nurse 1986; Jones 1990; Lakatta et al. 1990; Ferguson 1994; Rukavina et al. 1998; Sansoni et al. 1993; Utsuyama et al. 1992; Erkeller-Yuksel et al. 1992; Bloom 1991; Bloom et al. 1990; Horvath et al. 1992; Paganelli et al. 1992; Ademokun et al. 2010; Espino et al. 2012; Grubeck-loebenstein et al. 2009; Kaszubowska 2008). We also see that the number of macrophages in the cHL microenvironment varies with age, again making this an area of interest. In the published studies to date, patients of all ages have been considered together. Because cHL has a significant peak incidence in young adults the majority of studies contain a predominance of patients under

45 years of age but include a broad range of individuals spanning from childhood to the ninth decade. This could conceivably mask any differences that may be seen according to age and therefore two separate cohorts of patients; one representing young adults aged 15-34 years and the other, more elderly patients, aged 60 years and over, were chosen. Fifty years is used as the cut-off for the diagnosis of EBV-positive diffuse large B-cell lymphoma (DLBCL) of the elderly (Nakamura et al. 2008) and therefore 60 years was thought to be a reasonable differentiating age to use. Within these two groups EBV-positive cases were separated from EBV-negative cases given that the Epstein-Barr Virus is thought to play a key pathogenic role in 40% of cHL and the incidence of EBV-associated disease is known to vary according to age. Macrophages and plasmacytoid dendritic cells (pDC), as antigen presenting cells, are likely to be important in the immune response to malignant cells as well as the role they have in responding to viral infections, such as EBV. Added to this is the evidence that various infections can influence the function of immune cells including macrophages and therefore both macrophages and pDC were examined. Apoptosis is a key feature of malignancy and apoptotic cells also appear to influence the function of macrophages. As HRS cells are thought to be resistant to apoptosis, a mechanism seen as key in allowing tumour progression, apoptosis was investigated in both the non-malignant micro-environment and HRS cells.

Chapter 2

Materials and Methods

Cohort Selection

Cohort one, young adults: a cohort of patients with cHL, aged between 15 and 34 years at diagnosis, was identified from the histopathology database of the Western General Hospital (WGH), Edinburgh. 137 patients of the desired age, presented with cHL in the South East of Scotland between the first of January 1993 and the 31st of December 2005. All patients, presenting during this period had given consent at the time of diagnosis, for tissue and data to be used for research purposes. Presentation, management and outcome data were obtained from case notes (see appendix A, data collection sheet) and original diagnostic biopsies retrieved. Cases of relapsed disease (numbering two) were excluded as were those of patients HIV-positive at presentation (numbering three) or for whom there was insufficient clinical data or tissue for further analysis (50 in total). This left 82 cases. For 46 of the cases excluded due to a lack of clinical data, permission was sought through the Caldicott guardian to access data from the National Records of Scotland regarding whether these patients were currently alive or deceased.

Cohort two, older adults: data and tissue from patients aged over 60 years was made available from the SHIELD study (Proctor et al. 2012). This was a UK National Cancer Research Network study that recruited between 2007 and August 2009. The study comprised a treatment arm, designed to look at the use of VEPEMB chemotherapy (Vinblastine, Cyclophosphamide, Procarbazine, Prednisolone, Etoposide, Mitoxantrone, Bleomycin), and a registration-only arm, to collect data on all patients presenting over 60 years of age with cHL. This provided the opportunity to examine a group of elderly patients with cHL for whom there was clear and consistent clinical data. After all diagnoses were confirmed by a specialist haematopathologist, formalin-fixed paraffin wax-embedded (FFPE) diagnostic tissue, from patients in the VEPEMB arm, was made available for further study. Any cases with insufficient tissue had to be disregarded although no other exclusions were made. In total material from 77 of the 111 registered patients was available.

When specifically looking at T-cell antigen expression in cHL, six additional tumours were examined. One was from a young patient, identified in the original cohort but excluded from other analyses as the tumour was HIV-associated. Five other tumours, were identified through routine diagnostic work at the WGH between 2010 and 2013. Two of these were over 60 years at diagnosis and would therefore have been eligible for the elderly cohort had they presented during the study period and a third met criteria for the young cohort. The fourth and fifth cases were from patients aged 38 and 51 years at presentation who would therefore not have been eligible for either the elderly or young cohorts.

Clinical Data

The clinical stage of disease was defined according to the Cotswold modification of the Ann Arbor Staging system (Lister et al. 1989) which is detailed in table 2.1. Cases in each cohort were grouped into 'early stage' (stage IA-IIA) or 'advanced stage' (IIB-IV) for some analyses, as these are the clinical groupings of prognostic significance according to current treatment regimens (Eghbali et al. 2005).

Table 2.1
Ann Arbor staging of HL, Cotswold modification

Stage	Definition
I	Involvement of single lymph node region or lymphoid structure eg. Spleen, thymus, Waldeyer's ring
II	Two/more lymph node regions or lymph node structures on the same side of the diaphragm
III	Involvement of lymph node regions or lymph node structures on both sides of the diaphragm
IV	Multiple extranodal sites or lymph node sites and extranodal disease
A/B	'B' denotes presence of any B-symptoms: unexplained weight loss of >10% body weight in preceeding 6 months; unexplained persistent/recurrent fever with temperature >38^{o}C during the preceeding month; recurrent drenching night sweats during the preceeding month 'A' denotes the absence of any B-symptoms
E	Single isolated site of extranodal disease or extranodal extension
X	Bulk disease with a single lymph node or conglomerate lymph node mass measuring >/= 10cm in maximal dimension

(Lister et al. 1989)

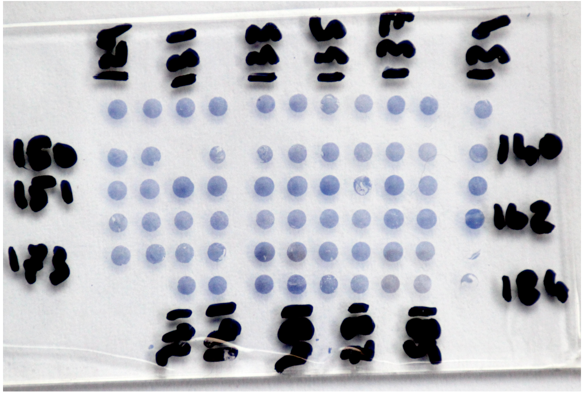
Outcome was measured by four different parameters in the two cohorts, both separately and with the cohorts combined:

- 'Relapse': the number of patients with primary progressive cHL or who were subsequently diagnosed with relapsed cHL during the follow-up period.
- 'Relapse or escalation of treatment': the number of patients with primary progressive cHL, subsequent relapse of cHL, or who required an intensification in their treatment due to a poor initial response to therapy.
- 'Died': the number of patients who died of any cause during the follow-up period.
- 'Died of cHL': the number of patients who died directly due to cHL during the follow-up period.

Tissue Microarray Construction

The use of Tissue Microarrays (TMA) is an efficient way of evaluating a large number of tumours using techniques such as immunohistochemistry (IHC) and has been validated in cHL giving results which correlate well with those from whole tissue sections (García et al. 2003; Glimelius et al. 2012; Hedvat et al. 2002). Approval was obtained from the Tissue Committee of the Edinburgh Experimental Cancer Medicine Centre to build a TMA of cHL tumours from the younger cohort. Similar approval was obtained through the SHIELD study for the older group. Suitable sites, from which to take cores of tissue from each case, were identified by examining and marking Haematoxylin and Eosin (H&E) stained sections for the presence of HRS or mononuclear Hodgkin cells. Four, one millimetre cores were taken from each tumour to build a TMA (figure 2.1) using a Beecher instrument tissue arrayer (Beecher Instruments, Wisconsin, USA). For some small biopsies, fewer than four cores were taken and in other cases, if tissue was insufficient for extracting cores, whole sections were examined.

Figure 2.1
Tissue Microarray



TMA C, young cohort, CD163 IHC

Immunohistochemistry (IHC)

Four-μm sections of the TMA were first stained with H&E to ensure appropriate cores of tissue, containing malignant cells, had been obtained. In addition to those used in the routine diagnostic panel, subsequent sections were examined by immunohistochemistry with the antibodies listed in table 2.2.

The 'Shandon Sequenza' manual system (ThermoFisher Scientific, Waltham, Massachusetts, USA) as described below, was used to label all sections with anti-CD163, anti-LAG-3 along with TMA sections from the young cohort with anti-CD68 (using CD68 'sequenza' antibody). The Leica Bondmax automated system (Leica Microsystems, Wetzlar, Germany) was used to label sections with all other antibodies with the process carried out according to manufacturers instructions for each antibody. Antigen retrieval in the automated system was achieved using Ethylenediaminetetraacetic Acid (EDTA) for all antibodies with the exception of CD20 for which sodium citrate was used. Both techniques used appropriate positive and negative controls.

Table 2.2
Antibodies used to examine cHL tumours

Antibody (*supplier)	'Animal' + clonality	Dilution	clone	Ig class	Expression
Anti-activated caspase-3*1	Rabbit polyclonal	1:100	Polyclonal (PC679)	IgG	Cytoplasm
BDCA2*2 (anti-CD303)	Mouse monoclonal	1:100	124B3.13	IgG1	Membrane
CD2*3	Mouse monoclonal	1:75	AB75	IgG1	Membrane
CD3*3	Mouse monoclonal	1:200	LN10	IgG1	Membrane ± cytoplasm
CD4*3	Mouse monoclonal	1:50	4B12	IgG1	Membrane
CD5*3	Mouse monoclonal	1:100	4C7	IgG1	Membrane
CD7*3	Mouse monoclonal	1:50	LP15	IgG2b	Membrane
CD8*3	Mouse monoclonal	1:100	4B11	IgG2b	Membrane
CD15*4	Mouse monoclonal	1:100	Carb-3	IgM	Membrane ± cytoplasm
CD20*4	Mouse monoclonal	1:200	L26	IgG2a	Cytoplasm
CD30*4	Mouse monoclonal	1:50	Ber-H2	IgG1	Membrane
CD68*5 (sequenza)	Mouse monoclonal	1:50	PG-M1	IgG3	Cytoplasm
CD68*4 (Bondmax)	Mouse monoclonal	1:200	PG-M1	IgG3	Cytoplasm
CD163*3	Mouse monoclonal	1:100	10D6	IgG1	Membrane
Granzyme B*4	Mouse monoclonal	1:50	GrB-7	IgG2a	Cytoplasm, granular
LAG-3*6	Rabbit polyclonal	1:100	Polyclonal	'purified Ig'	Cytoplasm
LMP-1*4	Mouse monoclonal	1:2000	Mix of 4 clones: CS.1-4	IgG1	Membrane ± cytoplasm
PAX5*7	Mouse monoclonal	1:50	24/Pax-5	IgG1	Nuclear
Perforin*3	Mouse monoclonal	1:20	5B10	IgG1	Cytoplasm, granular
TIA-1*8	Mouse monoclonal	1:500	2G9A10F5	IgG1	Cytoplasm, granular

Suppliers: *1 - Merck Millipore, Billerica, Massachusetts, USA; *2- Dendritics, Lyon, France; *3 - Novocastra, Newcastle upon Tyne, UK; *4 - Dako, Ely, UK; *5 - Abcam, Cambridge, UK; *6 - Sigma-Aldrich, Gillingham, UK; *7 - BD Bioscience, New Jersey, USA; *8 - Beckman Coulter, Marseille, France

IHC, Manual Technique

De-waxing

The following steps were undertaken to label sections with antibodies to CD68, CD163 and LAG-3. Slide mounted sections were de-waxed by sequential immersion in two changes of xylene for five minutes each followed by rehydration with reducing concentrations (100%, 90%, 70%) of Industrial Methylated Spirits (IMS) diluted with deionized water for five minutes each and finally in deionized water alone.

Antigen Retrieval

This was achieved using a heat induced antigen retrieval technique. For CD68 an alkaline Tris-EDTA buffer, consisting of 10mM Tris Base with 1mM EDTA solution and 0.05% Tween 20 (Sigma-Aldrich, Gillingham, UK) at pH9 was used; for CD163 an acidic, citrate based 'Vector antigen unmasking solution' (Vector Laboratories, Burlingame, California, USA) was used diluted 1:100 with deionized water and for LAG-3, a 10mM sodium citrate buffer with 1mM EDTA, pH6. Each buffer was pre-heated in a microwave pressure cooker at full power (850 watts) for two minutes before slides were added and the pressure cooker further heated at full power, for 30 minutes in the case of CD68 and 20 minutes for CD163 and LAG-3. The pressure cooker and slides were then allowed to cool for ten minutes. CD68 slides were further cooled with running water and CD163 and LAG-3 with phosphate buffered saline (PBS).

Slides were then loaded into individual 'coverplates' as per the 'Shandon sequenza' system, which uses capillary attraction to hold liquid reagents in contact with tissue sections.

Endogenous Peroxidase Block

400µl of 3% Hydrogen Peroxide solution in PBS, was added to each section and incubated at room temperature for five minutes before being washed for five minutes with a Tris buffered saline and Tween solution, TBS-Tween (100mM Tris, 150mM sodium chloride, 0.05% Tween 20, pH7.9).

Primary Antibodies

To each section 120µl of the appropriate primary antibody as listed above, was applied. CD68 was diluted 1:50 with antibody diluent (Dako, Ely, UK) and incubated at room temperature for two hours. CD163 and LAG-3 were both diluted 1:100 with antibody diluent and CD163 incubated for one hour and LAG-3 for 30 minutes at room temperature. Sections were then washed for five minutes with TBS-Tween.

Secondary Antibodies

All secondary antibodies were diluted 1:100 with antibody diluent and 150µl applied to each section. For CD68 and CD163, polyclonal sheep anti-mouse IgG-Horseradish Peroxidase (HRP) (GE Healthcare Life Sciences, Little Chalfont, UK) was used and for LAG-3, polyclonal goat anti-rabbit Ig-HRP (P0448, Dako, Ely, UK). Following a one-hour incubation at room temperature, sections were washed for five minutes with TBS-Tween.

Immunodetection

A streptavidin-biotin HRP immunodetection kit with a diaminobenzidine (DAB) chromogen (DAB vector kit, Dako, Ely, UK) was used as per the manufacturer's instructions to visualise specific binding of antibody. Briefly, sections were incubated for three minutes with 3,3'-diaminobenzidine tetrahydrochloride, 0.035%, in Tris buffered saline containing 0.1% H_2O_2. After rinsing with deionized water, a Haematoxylin counterstain was applied for 30 seconds. Sections were then rinsed with tap water before incubation with a 0.1% sodium bicarbonate solution for one minute. After washing with water, sections were dehydrated with increasing concentrations of IMS (70%, 90%, 95%, 100%) for three minutes each and then two changes of Histo-clear (National diagnostics, Hessle, UK), for five minutes each. Sections were dried and mounted under DPX mountant (Sigma-Aldrich, Dorset, UK).

Dual-label Immunohistochemistry

Dual-labelling of TMA sections with the following combinations of antibodies was undertaken using the Leica Bondmax automated system (Leica Microsystems, Wetzlar, Germany) with antibodies diluted as in table 2.2:

- anti-CD30 + anti-activated caspase-3
- anti-PAX5 + anti-TIA-1
- anti-PAX5 + anti-Granzyme B
- anti-PAX5 + anti-Perforin

In each case, the two primary antibodies were applied sequentially. Anti-active caspase-3 and anti-TIA-1, anti-Granzyme B or anti-Perforin were applied first, with immunodetection using streptavidin-biotin HRP and DAB chromogen to give a brown colour. All excess DAB was removed before the second primary antibody; anti-CD30 or anti-PAX5 for each respective combination, was added. These were detected using streptavidin-biotin alkaline phosphatase (AP) and the red, New Fucsin chromogen. Sections were mounted in water-soluble mountant (Ultramount, Dako, Ely, UK). Cell membrane CD30 expression was therefore evident by a red label and cytoplasmic activated caspase-3 expression by a brown label, allowing identification of CD30 expressing HRS cells undergoing apoptosis. For each of the PAX5 and cytotoxic molecule combinations, PAX5 expression was evidenced by a red nuclear label and cytotoxic molecule expression by a brown cytoplasmic label.

EBV Identification

In the majority of cases, evidence of EBV in the malignant HRS cells was determined using both IHC for the EBV-encoded protein, LMP-1 and in-situ hybridisation (ISH) for the detection of Epstein-Barr Virus encoded RNAs (EBERs). All LMP-1 negative cases were further examined by EBER-ISH.

IHC for LMP-1 was performed as described above. Positive cells showed evidence of LMP-1 expression in the cytoplasm and/or cell membrane.

EBER-ISH was carried out according to the manufacturer's instructions, on FFPE sections using a kit complete with anti-fluoroscein antibody, RNA negative control probe and EBER probe (Leica Biosystems, Newcastle upon Tyne, UK). This involved the following steps:

1) sections were de-waxed in xylene and hydrated with reducing concentrations of ethanol followed by water. They were incubated at 37^{o}C for 15 minutes with a combination of Proteinase K and 2-methyl-4-isothiazolin-3-one solution before being washed in water, dehydrated in increasing concentrations of ethanol and air-dried. Endogenous peroxidases were blocked by incubating in 1% H_2O_2 and methanol for 30 minutes.

2) The fluorescein-labelled oligonucleotide probe, in hybridization solution, was applied to sections which were incubated at 37^{o}C for two hours then washed in 0.1% TBS.

3) The anti-fluoroscein antibody (a purified IgG fraction of a mouse monoclonal antibody) was applied to sections and incubated for 30 minutes. After washing, antibody binding was detected using streptavidin-biotin HRP with DAB chromogen. Sections were counterstained with Haematoxylin and mounted in a solvent-based mountant (DPX, Sigma-Aldrich, Dorset, UK).

Positive cells showed nuclear EBER expression evidenced by a brown label (Figure 2.2). Only those cases with evidence of EBERs +/- LMP-1 expression, within the malignant HRS cells themselves, were deemed EBV-positive.

Demonstration of B- and T-cell clones

Eleven cases of those cHL that were found to express cytotoxic molecules, had adequate remaining tissue to allow further examination for evidence of B- or T-cell clones. This included five of the six 'additional' cases of cHL examined for T-cell antigen expression.

Extraction and purification of DNA from whole tissue sections

Ten-μm sections of formalin-fixed paraffin wax-embedded tissue from each case were incubated overnight (18 hours) with 180μl of Qiagen lysis buffer, ATL (Qiagen, Dusseldorf, Germany) and 20μl of proteinase K, in a waterbath at 56^{o}C until tissue had completely lysed. They were then incubated for a further one hour in a waterbath at 90^{o}C. Two hundred-μl of the lysate, lying beneath the wax layer after centrifugation, underwent DNA purification using the automated 12GC magtration system (Precision System Science, California, USA) and subsequent amplification using a HotStar Taq Master Mix kit (Qiagen, Dusseldorf, Germany), according to the manufacturer's instructions, on a Peltier thermal cycler (Genetic Technologies, Miami, USA).

Identification of B- and T-cell clones

These reactions were carried out in duplicate for each case. The following Identiclone primers (Invivoscribe, San Diego, USA) were used; for B-cell clones: IgH, tubes A-E targeting framework one, two and three regions and the diversity and joining regions of the immunoglobulin heavy chain gene; IgK, tubes A and B targeting the variable and joining regions, JK-CK intron and Kde regions of the immunoglobulin Kappa light chain gene and IgL targeting the V lambda and J lambda regions of the immunoglobulin lambda light chain gene; for T-cell clones: T-cell receptor (TCR)–gamma A, TCR-gamma B, TCR-beta-A, -B and –C.

One-μl of purified DNA from each case was mixed with each of the primers. Twenty-four μl of HotStar Taq Master Mix kit (Qiagen, Dusseldorf, Germany) providing polymerase and nucleotides required for DNA replication, was added to each reaction. A negative control was provided by using 24μl of HotStar Taq Master Mix without the addition of DNA. A 50 to 1000 base pair DNA Ladder (Qiagen, Dusseldorf, Germany), was added to DNA from each case as a positive control. After centrifugation, DNA was amplified, according to the manufacturer's instructions, on a Peltier thermal cycler (Genetic Technologies, Miami, USA). One-μl of the amplified product from each reaction was added to 12.5μl of HiDi Formamide (Applied Biosystems, California, USA) to allow analysis using a capillary electrophoresis system with laser detection of fluorescently labelled nucleic acids (3130 Genetic Analyser), and results processed using GeneMapper software (both Applied Biosystems, California, USA).

Microdissection

Twelve cytotoxic molecule positive (CMpos) cases, including seven from the original young cohort and five of the six 'additional' tumours, underwent Laser Capture Microdissection (LCM) to remove individual HRS cells from which DNA was extracted, with a view to identifying whether these cells were of B- or T-cell origin. A substantial part of this work was carried out by Dr John Goodlad, using the following method:

From each case, freshly cut 12µm sections of FFPE tissue were mounted onto glass, 2µm polyethylene naphthalate-membrane coated slides (PEN-membrane slides) (Leica Microsystems UK Ltd, Milton Keynes, UK). These were stained with H&E, and slides allowed to dry thoroughly in a dessicator for two hours before being mounted, tissue face down, on the LMD6500 laser microdissector (Leica Microsystems UK Ltd, Milton Keynes, UK). This is a non-contact laser microdissection system which uses gravity in conjunction with the laser to remove dissected tissue from slides directly into a collection vessel, in this case a 0.5ml eppendorf tube. HRS cells were identified based on their morphological appearance and individual HRS cells drawn around using the supplied software (figure 2.3). The computer-guided laser then cut around each cell, according to the line drawn, and individual HRS cells were transferred into an empty eppendorf tube below (figures 2.4, 2.5). For each case an average of 278 (range, 15-457) HRS cells were removed. The Arcturus PicoPure DNA extraction kit (Applied Biosystems, California USA) was then used according to the manufacturer's instructions to extract, purify and amplify DNA. Primers for TCR-gamma and IgH FR3-JH (Invivoscribe, San Diego, USA) were applied with appropriate positive and negative controls and run in duplicate, to identify any B- or T-cell clones. Two rounds of amplification were carried out using a Peltier thermal cycler (Genetic Technologies, Miami, USA). The first amplification was of 35 cycles before a second round of 25 cycles was undertaken on a one-μl extract of PCR product from the first round. As described above PCR products were analysed using the 3130 Genetic Analyser and GeneMapper software (Applied Biosystems, California, USA).

IHC Cell Counts

To quantify the different cell types, sections labelled with the appropriate corresponding antibody were examined and the number of positive cells counted with the aid of a graticule. Using an Olympus BX50 microscope (Olympus, Southend-on-Sea, UK), with x40 objective lens the standard area of the graticule was calculated and from this the median number of positive cells per mm^2 in each tumour ascertained. For cases processed as whole sections, to minimise variation, four areas were marked prior to counting and a maximum of 40 graticule areas were examined which matched as closely the number available for each tumour in the TMA. If an area of less than $0.5mm^2$ was available for examination in any one tumour (equating to fewer than eight graticules), it was excluded from analysis. This figure was chosen as it was greater than the minimum area quoted in studies validating the use of TMAs in cHL (García et al. 2003; Glimelius et al. 2012; Hedvat et al. 2002).

When counting macrophages identified by CD68 or CD163 antibodies, only clearly identifiable mononuclear cells with definite cytoplasmic or membrane positivity were included. Extracellular debris or cellular fragments were not counted. The ratio of CD163- to CD68-positive cells was determined by dividing the median number of CD163 cells per mm^2, by the median number of CD68 cells per mm^2, for the same tumour.

Apoptotic cells were identified using the activated caspase-3 antibody. Only those cases included in the TMAs were examined using this antibody. Both apoptotic HRS cells and non-malignant, microenvironment cells were of interest. Apoptotic HRS cells were identified by dual-labelling with anti-CD30.

CD30-positive HRS cells were identified by a red cell membrane label and expression of activated caspase-3 was identified by a brown cytoplasmic label within the same cell. ‘Background’ cells undergoing apoptosis were identified and counted by their lack of CD30 positivity, morphological appearances and positivity for activated caspase-3. When closely associated clusters of small, apoptotic bodies, positive for activated caspase-3 were identified, these were counted as one event to avoid over-estimating the extent of apoptosis. Dividing the median number of apoptotic HRS cells per mm^2 by the median number of all CD30-positive HRS cells per mm^2 and multiplying by 100 determined the percentage of HRS cells undergoing apoptosis in any one tumour. The median number of ‘background’, non-malignant cells undergoing apoptosis was ascertained by subtracting the median number of apoptotic HRS cells per mm^2 from the median number of all apoptotic cells per mm^2 for any one tumour.

When evaluating the expression of cytotoxic T-cell antigens (Perforin, Granzyme B, TIA-1) by HRS cells, these were identified by their morphological appearance and the percentage of positive HRS cells assessed for each antibody. Cases were marked as negative if fewer than ten percent of HRS cells expressed the antigen, with any higher percentage considered as positive. Cases expressing any of the three cytotoxic molecules were divided into two groups for evaluation; those with HRS cells co-expressing PAX5 and those that were PAX5-negative. HRS cell expression of other T-cell antigens was carefully assessed using criteria described by Jaffe and colleagues (Venkataraman et al. 2013). As HRS cells are often surrounded by a ‘rosette’ of benign T-lymphocytes it is important to recognise the potential for nonspecific adsorption of T-cell antigens onto HRS cells which may lead to the false impression of T-cell antigen expression. Therefore, HRS cells were only scored as positive for each antigen if >10% of all HRS cells present showed partial or complete membrane expression, readily distinguishable from adjacent T-cells. If >5% of HRS cells present showed cytoplasmic expression of T-cell antigens, usually seen as a cytoplasmic dot in the golgi region, they were also scored as positive (Venkataraman et al. 2013).

Statistics

Dr Rob Elton, statistician from the Centre for Population Health Sciences, University of Edinburgh, provided guidance regarding statistical analysis. The small number of cases in many subgroups, and consequent limited statistical power, precluded the use of multivariate analyses and therefore univariate methods were used.

Statistics were analysed using Minitab 17 software (Minitab Inc. State College, Pennsylvania, USA). A combination of Mann-Whitney and Fisher's exact tests were used for non-parametric data. Pearson's correlation was used to examine continuous data, specifically looking for evidence of an association between two cell types. 'P-values' of greater than 0.05 were deemed non-significant (p=NS). Positive numbers using Pearson's correlation denoted a positive relationship between two cell types, whereas a negative number denoted an inverse relationship. The closer the value was to one for each comparison, the stronger the relationship between the two cell types.

The following categories were used for Pearson correlation values ±:

a) < 0.2 = no evidence of a significant relationship

a) > 0.2 = weak relationship

b) > 0.3 = moderately strong relationship

c) > 0.4 = strong relationship

d) > 0.7 = very strong relationship

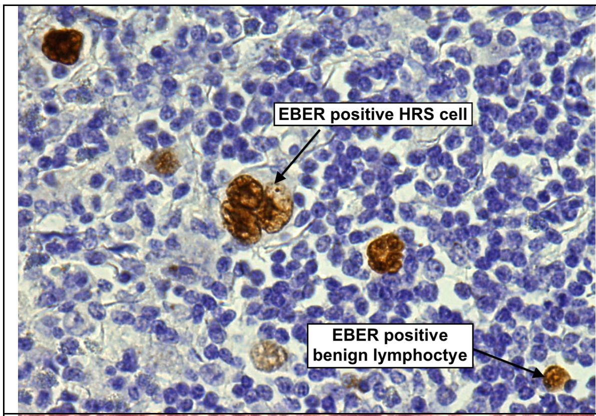


Figure 2.2
EBER-ISH
Tumour 12, Young cohort, MCHL (x40)
EBV-positive HRS cells and benign surrounding EBV-positive lymphocytes.

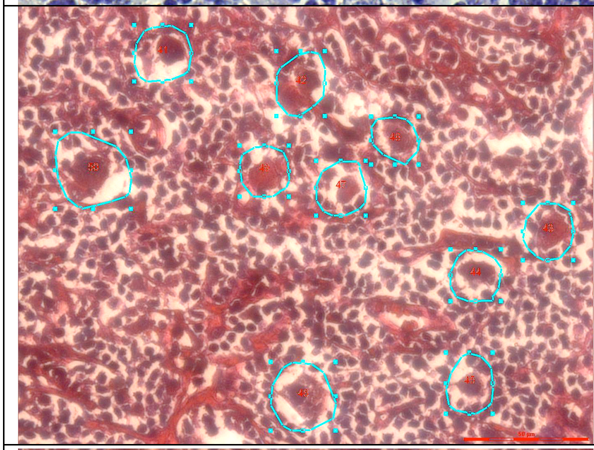


Figure 2.3
Laser-capture Microdissection – section before
Tumour 60, Young cohort
HRS-cells identified prior to laser microdissection.

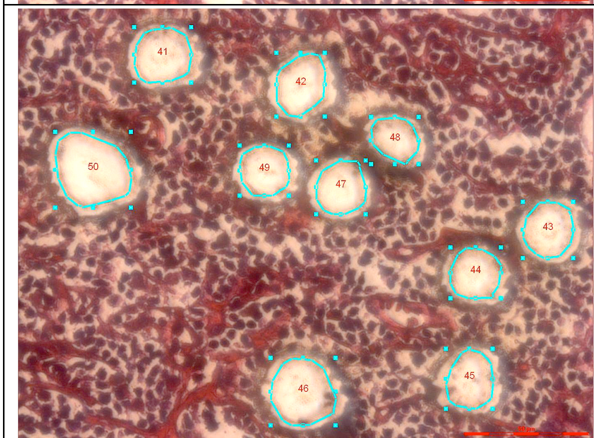


Figure 2.4
Laser-capture Microdissection – section after
Tumour 60, Young cohort
Section after HRS-cells removed by laser microdissection.

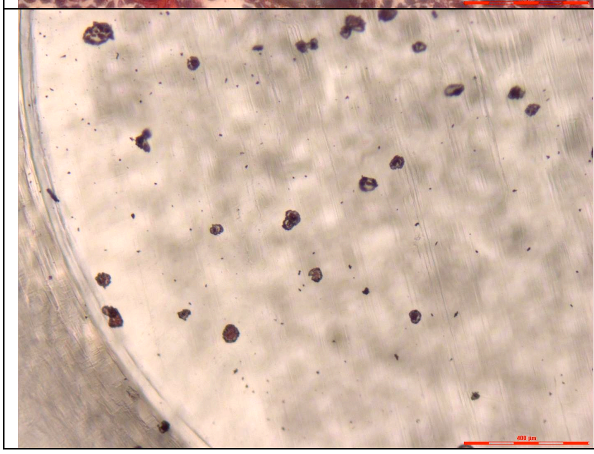


Figure 2.5
Laser-capture Microdissection – single cells
Tumour 60, Young cohort
Single HRS-cells lying in the eppendorf cap after removal from the tissue section.

Chapter 3

Classic Hodgkin Lymphoma - morphology

Introduction

Over the last 180 years the diagnosis of HL has evolved, with the current diagnostic criteria being agreed by the World Health Organisation (WHO) in 2008 (Swerdlow et al. 2008; Parker et al. 2010; Eichenauer et al. 2011). These are based on the morphological appearances of H&E stained lymph node sections and the immunohistochemical features of malignant HRS cells. Hodgkin Lymphoma comprises Classic Hodgkin Lymphoma (cHL), which accounts for 95% of all HL and is the focus of this thesis, and Nodular Lymphocyte Predominant Hodgkin Lymphoma (NLPHL), now accepted to be a separate, rarer entity which makes up 5% of all HL cases and is not discussed further. cHL is divided on morphological grounds into four subtypes: Nodular Sclerosing (NSHL), Mixed-cellularity (MCHL), Lymphocyte-rich (LRcHL) and Lymphocyte-depleted (LDcHL) (Parker et al. 2010; Swerdlow et al. 2008).

Morphology

cHL is characterised by the presence of distinctive malignant cells, Hodgkin Reed-Sternberg cells (HRS), and these are evident in all four subtypes, although the number and appearances do vary. HRS cells are derived from post-germinal centre B-cells (Mani & Jaffe 2010; Enblad et al. 2007; Grur et al. 1997; Hummel et al. 1996; Weiss et al. 1986) but unusually these make up only a minority of the tumour mass (Skinnider & Mak 2002; Küppers 2009b; Chetaille et al. 2009). The majority of cells (95-99%) are non-clonal, non-malignant inflammatory cells including lymphocytes, plasma cells, eosinophils, neutrophils and macrophages (figure 3.1). Classically, HRS cells are large (15-45 micrometers), binucleate cells with each nucleus appearing as a mirror image of the other and containing a large, eosinophilic nucleolus often surrounded by a clear halo (figure 3.2). However, the binucleate appearance is in fact artefactual and the result of deep folds in a single nucleus. As a result there are many variants including both mononuclear and those of multinucleate appearance. In NSHL, HRS cells are often described as "lacunar cells" due to retraction of the cytoplasmic membrane, commonly occurring in areas adjacent to fibrosis, leaving the nucleus apparently sitting in an empty space, as a result of fixation artefact (figure 3.3). 'Mummified' HRS cells have also long been recognised with distinctive condensed cytoplasm and a dark pyknotic nucleus. These are widely thought to represent apoptotic HRS cells (Lynny Yung & Linch 2005; Eichenauer et al. 2011; Stein 2008). Apoptotic bodies are also clearly evident within the surrounding non-malignant cell component and apoptosis is further discussed in chapter seven.

The presence of HRS cells is an absolute requirement for the diagnosis of cHL but these must be seen within the typical background of non-malignant inflammatory cells and have the typical immunohistochemical features as similar cells can be seen in other, both benign and malignant, conditions including IM, melanoma, sarcoma, ALK-negative anaplastic large cell lymphomas (ALCL) and other Non-Hodgkin Lymphomas (NHL) (Lynny Yung & Linch 2005; Listinsky 2002; Parker et al. 2010; Stein 2008).

Nodular Sclerosing classic Hodgkin Lymphoma (NSHL)

This is characterised by cellular nodules separated by dense fibrotic bands of birefringent collagen and a thickened lymph node capsule (figure 3.4). Classical HRS cells are often infrequent but HRS cells with a lacunar appearance can be prominent. Surrounding inflammatory cells may form 'microabscesses' of neutrophils and eosinophils. There can be some preservation of the normal nodal architecture with the occasional presence of residual follicular dendritic cell (FDC) meshworks. In comparison to other subtypes there are often a greater proportion of B- rather than T-lymphocytes in the surrounding microenvironment. (Listinsky 2002; Mani & Jaffe 2010; Martin et al. 2005; Parker et al. 2010; Stein 2008).

Mixed-Cellularity classic Hodgkin Lymphoma (MCHL)

Classical HRS cells are seen distributed within a diffuse background of inflammatory cells with no capsular thickening or fibrotic bands (figures 3.1, 3.5). Most commonly the normal nodal architecture is completely effaced but occasionally there can be evidence of residual non-neoplastic follicles.

(Listinsky 2002; Mani & Jaffe 2010; Martin et al. 2005; Parker et al. 2010; Stein 2008).

Lymphocyte-Rich classic Hodgkin Lymphoma (LRcHL)

This is an uncommon variant of cHL, accounting for around 6% of cases (Fend 2010; Listinsky 2002; LRF 2015), which was first described in 1995 but only recognised as a distinct subtype in the 2008 WHO classification. Morphologically it can closely resemble NLPHL but HRS cells have the distinct immunophenotypical features of cHL. HRS cells are typically few in number and smaller than in other subtypes (figure 3.6). They are often noted within the mantle and marginal zones of residual, reactive germinal centres. (Listinsky 2002; Mani & Jaffe 2010; Martin et al. 2005; Parker et al. 2010; Pileri et al. 2002; Stein 2008).

Lymphocyte-Depleted classic Hodgkin Lymphoma (LDcHL)

LDcHL is the rarest subtype (fewer than 1% of cHL) (Fend 2010; Listinsky 2002; LRF 2015) and is diagnosed with caution based on its typical immunophenotypical appearance, as morphologically, Non-Hodgkin Lymphomas may mimic it. There is a diffuse infiltrate of HRS cells with very few surrounding inflammatory cells (Listinsky 2002; Mani & Jaffe 2010; Martin et al. 2005; Parker et al. 2010; Pileri et al. 2002; Stein 2008).

Immunohistochemistry

The further definition of HL requires immunohistochemistry. The WHO have produced a recommended panel of immunohistochemical antibodies required for the diagnosis of cHL and this has been accepted and reproduced by different bodies including the British Committee for Standards in Haematology (Parker et al. 2010; Stein 2008). These are outlined in table 3.1 below.

Table 3.1
Recommended diagnostic panel of antibodies for cHL

Antigen & normal expression	Expression in cHL
CD2 / CD3 Pan-T-cell antigens.	**Negative**
CD20 Common B-cell antigen, expressed by B-cell precursors and mature B-cells, not plasma cells.	**Negative / weak Positive**
CD79a Pan-B-cell antigen.	**Negative / weak Positive**
CD45 Leucocyte common antigen expressed by haematopoietic cells (not erythrocytes or megakaryocytes).	**Negative**
CD30 Lymphocyte activation antigen.	**Positive**
CD15 Normally expressed by granulocytes.	**Variable, may be Postive / Negative**
ALK1 (CD246) Expressed by some Anaplastic Large Cell Lymphomas.	**Negative**
EMA Common epithelial antigen, also expressed by some ALCL.	**Negative / weak Positive**
LMP1/EBER-ISH LMP1=EBV encoded protein, EBER=EBV-encoded RNAs.	**Variable, may be Positive/Negative**
OCT2 Expressed by Germinal centre B-cells.	**Negative**
BOB1 Expressed by Germinal centre B-cells.	**Negative**
J Chain Component part of IgA and IgM, therefore marker of B-cells producing these immunoglobulins.	**Negative**
MUM1/IRF4 Expressed by Germinal centre B-cells and plasma cells.	**Positive**
PAX5/BSAP Transcription factor expressed by early but not late-stage B-cells.	**Positive**
CD10 Expressed by haematopoietic cells.	**Negative**
Bcl6 Expressed by Germinal centre B-cells and some $CD4^+$ T-cells.	**Negative**
CD75 Expressed by mature B-cells. Expressed by malignant cells in NLPHL.	**Negative**
CD57 Expressed by NK and some T-cells in addition to a number of non-haematopoietic tissues.	**Negative**

[Adapted from 'Best practice in Lymphoma diagnosis and reporting' (Parker et al. 2010) and from 'Pathologyoutlines.com' (Pernick et al. 2014)]

In 75-85% of cHL cases, HRS cells express CD15, but in any one tumour only a small number of these cells may be positive. CD15 expression is seen in the golgi zone and cell membrane. This is a normal granulocyte and monocyte antigen but is not normally expressed by lymphocytes (Listinsky 2002). HRS cells in the majority of cHL cases will express CD30 (figure 3.2) but it should be noted that CD30 expression is not specific to cHL and therefore the cellular morphological appearance is also extremely important. Benign lymphocytes reacting to a stimulus, such as EBV infection, will be CD30-positive, a marker also expressed by some NHL such as ALK-positive ALCL, some Diffuse Large B-cell Lymphoma (DLBCL) and Peripheral T-cell Lymphomas (PTCL) (Listinsky 2002; Mani & Jaffe 2010; Pileri et al. 2002; Barry et al. 2003). Although of B-cell origin, B-cell lineage antigens are not consistently expressed by HRS cells. CD20 is reported in anything up to 50% of cHL but expression is patchy and may only be seen weakly in a few HRS cells. Other markers, such as CD79a are expressed even less consistently (Foss, Reusch, Demel, Lenz & Anagnostopoulos 1999; García-Cosío et al. 2004; Venkataraman et al. 2013; Seitz et al. 2000; Tzankov et al. 2003; Mani & Jaffe 2010). PAX5 however, is detectable in between 75 and 100% of cHL cases, probably varying due to different antibodies/epitopes and antigen retrieval techniques (Foss, Reusch, Demel, Lenz & Anagnostopoulos 1999; García-Cosío et al. 2004; Venkataraman et al. 2013; Seitz et al. 2000; Torlakovic et al. 2002).

Other than HRS cells, the tumour mass is composed largely of benign inflammatory cells. A large proportion of these are lymphocytes, which are predominantly polyclonal T-cells, with $CD4^+$ T-cells in the majority in most cases. In contrast to this, NLPHL contains more B-lymphocytes in the tumour microenvironment and the malignant cells are negative for CD15 and CD30. LRcHL can closely resemble NLPHL morphologically, including the background B-cells, but immunophenotypically the malignant cells have the characteristics of cHL (Listinsky 2002).

Results

Unless stated otherwise, p-values refer to Fisher's exact test.

Table 3.2
Demographics

	Elderly + Young Combined	Young	Elderly	Published Data (all age groups)
Total number studied	159	82	77	
Median follow up, months (range)	56 (0.5-208)	113 (6-208)	24 (0.5-64)	
Mean age, yrs (age range)	47 (15-85)	26 (15-34)	72 (60-85)	
Male/Female (%) ratio	74/79 (48/52) 0.95:1 *6 missing	37/45 (45/55) 0.82:1	37/34 (52/48) 1.09:1 *6 missing	All ages, 1.1-1.5 : 1*1 young adults 1:1*2

*1 ((Fend 2010; Roman & Smith 2011); *2 ((Fend 2010)

Table 3.3
Immunohistochemical features

	Elderly + Young Combined	Young	Elderly	Published Data (all age groups)
CD30 pos HRS (%)	126 (100) *33 missing	76 (100) *6 missing	50 (100) *27 missing	
CD15 pos HRS (%)	75 (59) *32 missing	52 (68) *5 missing	23 (46) *27 missing	75-85%*3
CD20 pos HRS (%)	35 (29) *37 missing	23 (31) *8 missing	12 (25) *29 missing	<20-50%*4
PAX 5 pos HRS (%)	119 (94) *33 missing	71 (93) *6 missing	48 (96) *27 missing	75-100%*5

*3 (Listinsky 2002); *4 (Foss, Reusch, Demel, Lenz & Anagnostopoulos 1999; García-Cosío et al. 2004; Venkataraman et al. 2013; Seitz et al. 2000; Tzankov et al. 2003; Mani & Jaffe 2010); *5 (Foss, Reusch, Demel, Lenz & Anagnostopoulos 1999; García-Cosío et al. 2004; Venkataraman et al. 2013; Seitz et al. 2000; Torlakovic et al. 2002)

Presenting features – young cohort

After morphological and immunohistochemical examination described above, cases from each cohort were classified according to the WHO diagnostic criteria for NSHL, MCHL, LRcHL, LDcHL or cHL-not otherwise specified (cHL-NOS). Both cohorts, showed the expected mix of cHL subtypes according to age (table 3.4). In the young, 137 patients aged 15 to 34 years were identified. Of these, clinical data and tissue were available for 82 with a median follow-up of 113 months (range 6-208 months) and mean age at diagnosis of 26 years (table 3.2). 74% (61/82) were NSHL, 16% (13/82) MCHL and 10% (8/82) cHL-NOS, LRcHL or LDcHL. This young cohort included slightly more women than men (55% and 45% respectively). 46% presented with stage I or IIA disease and the remainder with stage IIB to IV disease. Of the 82 cases, 20 (24%) were EBV-positive, with the rest EBV-negative (figure 3.7, table 3.4).

Presenting features – elderly cohort

Of the elderly cohort, identified through the VEPEMB arm of the SHIELD study (Proctor et al. 2012), 111 patients registered and there were sufficient data and tissue for 77 to be studied further. Median follow up was shorter for this cohort, at 24 months (range 0.5-64 months). Mean age at diagnosis was 72 years (table 3.2). 51% (39/77) were NSHL subtype, 36% (28/77) MCHL and 13% (10/77) LRcHL, LDcHL or cHL-NOS. In this group, there was a slight predominance of men (52% versus 48%); 35% had stage I to IIA disease at presentation, and the remainder stage IIB to IV disease. Forty-two of the 77 elderly (55%) were EBV-positive and the others EBV-negative (figure 3.7, table 3.4).

Therefore, as seen in other studies, although NSHL predominates in both age groups (74% and 51%), MCHL is significantly more frequent in the older population (36% MCHL in elderly versus 16% in young adults, $p=<0.01$). The young cohort studied here, contained a slight excess of women in comparison to men (55% and 45% respectively), whereas there were more men in the elderly cohort. This is also in keeping with expectation. Also as described elsewhere, EBV-positive disease is significantly more common in elderly patients than the young (55% compared to 24%, $p=<0.001$).

Figure 3.7
cHL subtype per cohort

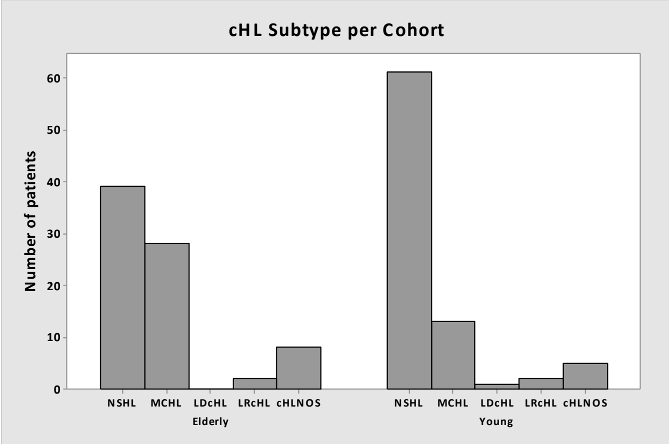


Table 3.4
Presenting features

	Elderly + Young Combined	Young	Elderly	Young v Elderly	Published Data (all age groups)
Stage IA-IIA/Stage IIB-IV (%)	60/89 (40/60) *10missing	35/42 (46/55) *5 missing	25/47 (35/65) *5 missing	p=NS	
B symptoms (%)	69 (47) *11missing	38 (46) *6 missing	31 (40) *5 missing	p=NS	
NSHL (%)	100 (63)	61 (74)	39 (51)	p=<0.01	60-80*6
MCHL (%)	41 (26)	13 (16)	28 (36)	p=<0.01	20-30*7
LRcHL (%)	4 (3)	2 (2)	2 (3)		6*8
LDcHL (%)	1 (0.6)	1 (1.2)	0		<1*8
cHL-NOS (%)	13 (8)	5 (6)	8 (10)		6+*9
EBV-pos (%)	62 (39)	20 (24)	42 (55)	p=<0.001	26-50*10
EBV-neg (%)	97 (61)	62 (76)	35 (46)	p=<0.001	60*11
EBV-pos NSHL (%)	24 (24)	8 (13)	16 (41)	P=<0.01	10-25*12
EBV-pos MCHL (%)	30 (73)	9 (69)	21 (75)	p=NS	75*12
EBV-pos CHL, other (%)	8 (44)	3 (38)	5 (50)	p=NS	

*6 (LRF 2015); *7 (LRF 2015; Fend 2010); *8 (Listinsky 2002; LRF 2015; Fend 2010); *9 (Listinsky 2002; LRF 2015; Fend 2010); *10 (Jarrett et al. 1996; Armstrong et al. 1998; WHO 1996a); *11 (Jarrett et al. 1996; Stark et al. 2002; Armstrong et al. 1998; Shimoyama et al. 2008; Chetaille et al. 2009); *12 (Fend 2010; Jarrett et al. 1996; Armstrong et al. 1998; WHO 1996a)

Treatment and outcome – young cohort

Fifty-five patients received ABVD (doxorubicin, bleomycin, vinblastine, dacarbazine) or ABVD-like chemotherapy, 11 received PVACE-BOP (vinblastine, etoposide, chlorambucil, procarbazine, doxorubicin, vincristine, prednisolone, bleomycin), 10 had ChlVPP (chlorambucil, vinblastine, procarbazine, prednisolone), three had radiotherapy alone and three had unknown treatment. Thirty-seven patients received additional radiotherapy and seven were treated with autologous stem cell transplant in addition to initial chemotherapy.

In this young group, 15 patients relapsed (19%) and an additional four required an escalation in their treatment because of a poor initial response (total 24%). Six patients (7%) died during the follow-up period. Three died of relapsed or progressive cHL; one suffered a cardiac arrest six months after presentation, at a time when disease status was not clear; one patient died of metastatic adenocarcinoma of lung 138 months after presentation with cHL and another patient died of secondary acute myeloid leukaemia 160 months after initial cHL diagnosis. Both of these patients were in remission from cHL at the time of their deaths.

Of the total 137 young patients presenting with cHL during the study period, 55 (40%) were excluded, the majority of which (50), due to a lack of clinical data and/or tissue. Through the National Records of Scotland, data on 46 of these patients was found and it was ascertained that of the 50 excluded patients, 52% (26 of 50) were male, 32% (16 of 50) female and 16% (8 of 50) of unknown gender. 62% (31 of 50) remain alive, 28% (14 of 50) have died and for five patients this information was not available.

Treatment and outcome – elderly cohort

The majority (61) in this group were treated with VEPEMB chemotherapy (vinblastine, cyclophosphamide, procarbazine, prednisolone, etoposide, mitoxantrone, bleomycin), three had ABVD, four ChlVPP, one received PECC (prednislone, etoposide, chlorambucil, lomustine), one had radiotherapy alone and for seven, treatment was not known. Additional radiotherapy was given to 21 patients and none went on to have an autologous stem cell transplant.

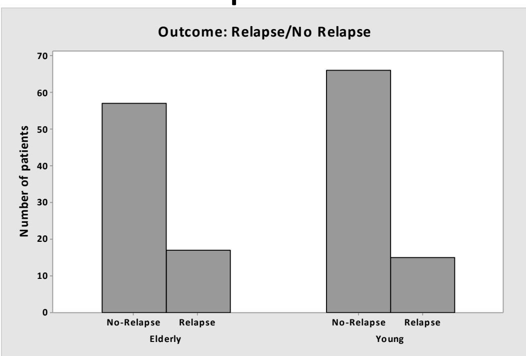
Relapse disease occurred in 17 patients (23%) and a further five (22, 32% in total) required an escalation in treatment due to an initial poor response. These figures were therefore not significantly different from those of the young cohort (figure 3.8, tables 3.5 & 3.6). In total 31 patients died during follow-up (40%), which was significantly greater than in the young group (p=<0.0001). When those cases from the young cohort, excluded from other analyses were also considered, in total 20 of 127 (16%) young patients died in comparison with 31 of 77 (40%) elderly patients (p=<0.001). The difference therefore remained significant. Of the elderly cases, 15 died of cHL; seven died of other malignancies which included prostate carcinoma, myelodysplasia, secondary acute myeloid leukaemia, lung and colonic carcinoma; two died of ischaemic heart disease; one of an ischaemic stroke; two of infection, one of which was related to chemotherapy; and four died with no recorded cause.

There was no significant difference in outcome (relapse [table 3.5], relapse or intensification of treatment [table 3.6], death any cause [table 3.7] or death due to cHL [table 3.8]) between men and women in either group, individually or combined. When comparing elderly to young patients, 14 of 37 (38%) elderly men died compared to five of 37 (14%) young men (p=<0.05) and a significant result was also seen between elderly and young women; 13 of 34 (38%) elderly women died and one of 44 (2%) young women (p=<0.0001) (table3.7). When only deaths directly due to cHL were considered this difference persisted for women; six of 33 (18%) elderly women died of cHL versus one of 44 (2%) young women (p=<0.05), but was no longer significant for men; six of 36 (17%) elderly males died of cHL versus two of 37 (5%) young males (table 3.8).

Within each individual group, there was no significant difference in outcome between those with NSHL and those with MCHL. This was the case when considering relapsed disease, relapse or intensification of treatment, death from any cause or death due to cHL, (tables 3.5 to 3.8). When the elderly and young cohorts were compared, those elderly with NSHL did less well than the young with NSHL. Of 39 elderly NSHL, 15 died of any cause (39%) and of the 60 young with NSHL, four (7%) died (p=<0.0001) (table 3.7). If only those deaths due to cHL were examined, the difference was no longer significant with six (16%)

elderly and three (5%) young with NSHL dying directly of the disease (table 3.8). Of those with cHL other than NSHL or MCHL, significantly more elderly patients in this category died of any cause; five of 10 (50%) elderly compared to zero of 8 (0%) young, (p=<0.05) (table 3.7). Again, if only deaths due to cHL were considered, the difference between old and young was not significant (table 3.7). When examining patients with MCHL, there was no difference between the number of elderly or young patients who died of cHL or died of any cause (tables 3.7 & 3.8). No difference was seen between elderly and young patients with NSHL, MCHL or cHL other, regarding the number relapsing or requiring intensification of treatment (tables 3.5 & 3.6).

Figure 3.8
Patient outcome per cohort



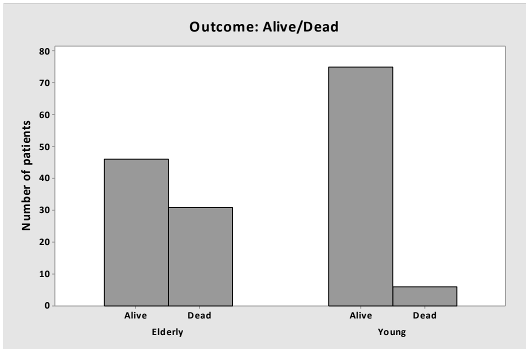


Table 3.5
Outcome: relapse

	Elderly + Young Combined	Young	Elderly	Young v Elderly
Total relapse (%)	32 (21) *4 missing	15 (19) *1 missing	17 (23) *3 missing	p=NS
Male relapse (% all male)	15 (21) *2 missing	8 (22) *1 missing	7 (19) *1 missing	p=NS
Female relapse (% all female)	14 (18) *2 missing	7 (16)	7 (22) *2 missing	p=NS
Relapse male v female	p=NS	p=NS	p=NS	
NSHL relapse (% all NSHL)	18 (18) *2 missing	11 (18)	7 (19) *2 missing	p=NS
MCHL relapse (% all MCHL)	10 (25) *1 missing	3 (25) *1 missing	7 (25)	p=NS
Relapse cHL other (% all cHL other)	4 (24) *1 missing	1 (13)	3 (33) *1 missing	p=NS
Relapse NSHL v MCHL	p=NS	p=NS	p=NS	

Table 3.6
Outcome: relapse or escalation in treatment

	Elderly + Young Combined	Young	Elderly	Young v Elderly
Total relapse or escalation in Rx (%)	41 (28) *10 missing	19 (24) *1 missing	22 (32) *9 missing	p=NS
Male relapse or escalation in Rx (% all male)	20 (29) *6 missing	9 (25) *1 missing	11 (34) *5 missing	p=NS
Female relapse or escalation in Rx (% all female)	18 (24) *4 missing	10 (22)	8 (27) *4 missing	p=NS
Relapse or escalation in Rx male v female	p=NS	p=NS	p=NS	
NSHL relapse or escalation in Rx (% all NSHL)	25 (26) *5 missing	15 (25)	10 (29) *5 missing	p=NS
MCHL relapse or escalation in Rx (% all MCHL)	12 (32) *3 missing	3 (25) *1 missing	9 (35) *2 missing	p=NS
cHL other, relapse or escalation in Rx (% all cHL other)	4 (25) *2 missing	1 (13)	3 (38) *2 missing	p=NS
Relapse or escalation in Rx NSHL v MCHL	p=NS	p=NS	p=NS	

Table 3.7
Outcome: death, any cause

	Elderly + Young Combined	Young	Elderly	Young v Elderly
Total died, any cause (%)	37 (23) *1 missing	6 (7) *1 missing	31 (40)	p=<0.0001
Died male (% all male)	19 (25.7)	5 (13.5)	14 (37.8)	p=<0.05
Died female (% all female)	14 (18) *1 missing	1 (2)	13 (38)	P=0.0001
Male v female	p=NS	p=NS	p=NS	
NSHL, died (% all NSHL)	19 (19) *1 missing	4 (7) *1 missing	15 (39)	p=0.001
MCHL, died (% all MCHL)	13 (32)	2 (15)	11 (39)	p=NS
cHL other, died (% all cHL other)	5 (28)	0 (0)	5 (50)	p=<0.05
Died, NSHL v MCHL	p=NS	p=NS	p=NS	

Table 3.8
Outcome: death due to cHL

	Elderly + Young Combined	Young	Elderly	Young v Elderly
Total died of cHL (%)	18 (12) *3 missing	3 (4) *1 missing	15 (20) *2 missing	p=<0.01
Died cHL male (% all male)	8 (11) *1 missing	2 (5)	6 (17) *1 missing	p=NS
Died cHL female (% all female)	7 (9) *2 missing	1 (2) *1 missing	6 (18) *1 missing	p=<0.05
Male v female	p=NS	p=NS	p=NS	
NSHL, died cHL (% all NSHL)	9 (9) *2 missing	3 (5) *1 missing	6 (16) *1 missing	p=NS
MCHL, died cHL (% all MCHL)	6 (15) *1 missing	0	6 (22) *1 missing	p=NS
cHL other, died cHL (% all cHL other)	3 (17)	0 (0)	3 (30)	p=NS
Died cHL NSHL v MCHL	p=NS	p=NS	p=NS	

Discussion

The epidemiology of cHL is complex. There are variations noted on geographical grounds, socio-economic status and age. In all age groups NSHL predominates and overall accounts for 60-80% of cases (LRF 2015). MCHL, in developed countries, accounts for around 20-30% of all cHL (LRF 2015; Fend 2010). LRcHL is much less common; only around 5-6% of cases, and 1% or fewer cases are LDcHL (Listinsky 2002; LRF 2015; Fend 2010). Although NSHL is the most common subtype in all age groups, it is particularly prominent in young adults, whereas the percentage of MCHL is higher in young children and older adults (Fend 2010). The median age at diagnosis for NSHL is only 37 years. For MCHL it is 60 years and for LRcHL 58 years (Roman & Smith 2011). For most age groups there is a male preponderance which is particularly notable in developing countries and becomes more pronounced in older adults (Fend 2010; Roman & Smith 2011). Interestingly, however, in young adults there is an equal balance of male to female if not a slight excess of women affected (Fend 2010). Overall, 39% of cases in the present study were EBV-positive, which is consistent with studies of cHL in developed countries (Jarrett et al. 1996; Armstrong et al. 1998; WHO 1996a; Chetaille et al. 2009; Shimoyama et al. 2008; Stark et al. 2002). When the cohorts were examined individually it was seen that EBV-positive disease was significantly more common in the elderly group. EBV in cHL is examined further in chapter five.

Considering the above data, both the young and elderly cohorts in the present study appear representative, with the expected distribution of cHL subtypes, male:female split and incidence of EBV-positive disease. Figures of around 33% (Montalbán et al. 2004) are quoted regarding the number of cHL patients who go on to relapse or have primary progressive disease, which is similar to the numbers in the combined cohort who relapsed or required an escalation in treatment. However, of all the young patients presenting with cHL during the study period, a disproportionately high percentage of those who subsequently died were excluded due to a lack of clinical data and/or tissue. This had been a concern, due to the NHS policy of destroying notes if not used for a set period of time (often five years) and inevitably may have introduced a degree of bias into the analysis of the young cohort and if not, at least limited the ability to discriminate between factors of prognostic significance. Despite this, the overall numbers of patients who died during the study period was in keeping with the figures widely reported which state that in the region of 10-30% of all patients die from cHL (David et al. 2007; Montalbán et al. 2004).

Although there is a consensus that cHL is a B-cell malignancy, in a minority of cases, HRS cells are seen to express T-cell antigens. This has led some to question whether the B-cell origin of cHL is universal and also to question the role of these T-cell molecules. To address this observation further, analysis of both cohorts was undertaken and is described in chapter four.

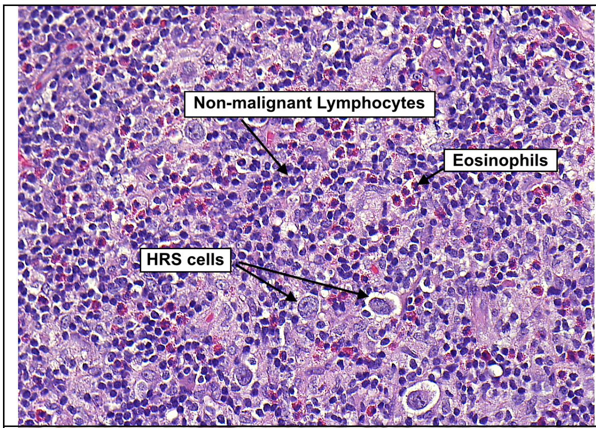


Figure 3.1
MCHL
H&E, Tumour 12, Young cohort (x10)
HRS cells surrounded by a diffuse infiltrate of non-malignant inflammatory cells.

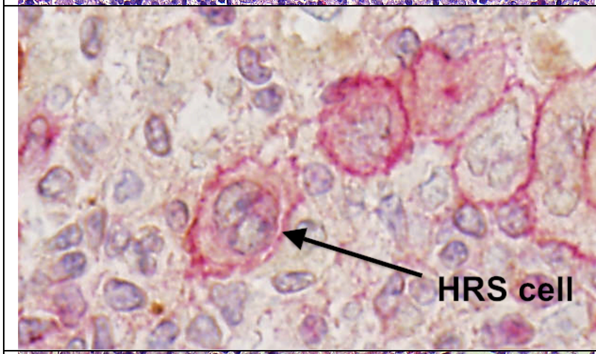


Figure 3.2
Binucleate HRS-cells
CD30 IHC. Tumour 4, Young cohort (x40)
Binucleate HRS cell with cell membrane CD30 expression, red New Fucsin label.

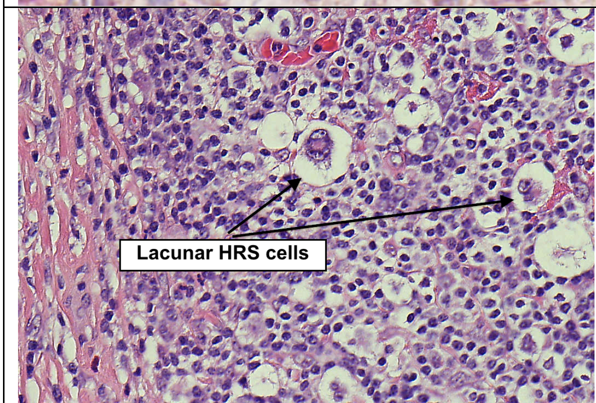


Figure 3.3
NSHL, Lacunar HRS-cells
H&E, Tumour 61, Young cohort (x20)
Lacunar HRS cells, artefactual retraction of cytoplasmic membrane.

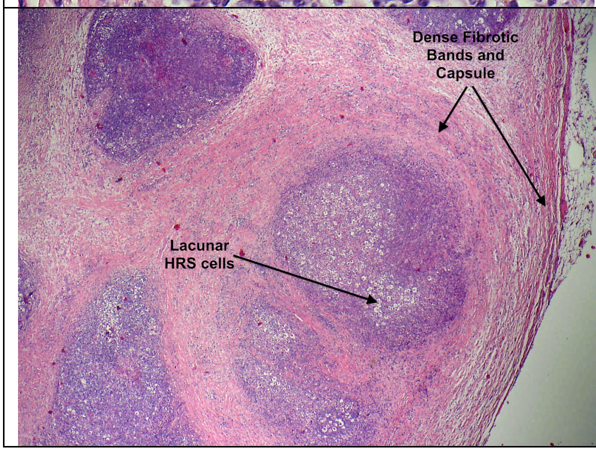


Figure 3.4
NSHL
H&E, Tumour 61, Young cohort (x2)
NSHL, cellular nodules separated by dense fibrotic bands + thickened capsule.

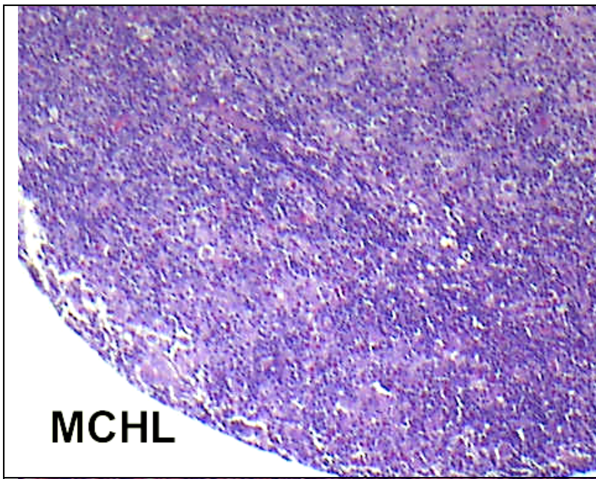


Figure 3.5
MCHL
H&E, Tumour 12, Young cohort (x4)
Diffuse background of inflammatory cells and interspersed HRS cells.

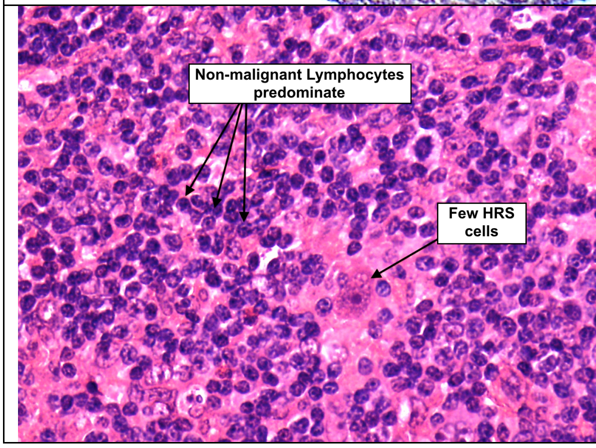


Figure 3.6
LRcHL
H&E, Tumour 20, Young cohort (x40)
Sparse HRS cells, smaller than in other subtypes, surrounded predominantly by lymphocytes.

Chapter 4

Expression of T-cell antigens in classic Hodgkin Lymphoma

Introduction

The majority of HRS cells have lost expression of lineage specific antigens and are negative or only weakly positive for CD20. However, it is now well established that HRS cells are derived from post-germinal centre B-cells (Kanzler et al. 1996; Weiss et al. 1986; Küppers 2009b; Kuppers et al. 1999; Marafioti et al. 2000; Seitz et al. 2000; Küppers 2009a) and since this discovery, the pan-B-cell antigen, B-cell lineage specific activator protein (BSAP, the protein product of the PAX5 gene), has been identified and can be detected in the majority of cHL using IHC (figure 4.1) (Foss, Reusch, Demel, Lenz & Anagnostopoulos 1999; García-Cosío et al. 2004; Venkataraman et al. 2013; Seitz et al. 2000; Torlakovic et al. 2002). Therefore, as per the WHO diagnostic criteria previously discussed, HRS cells should be negative or only weakly positive for CD20, positive for PAX5 and negative for the pan-T-cell antigens, CD2 and CD3. The cytotoxic T-cell markers, T-cell-restricted intracellular antigen (TIA-1), Granzyme B and Perforin, along with other T-cell markers, CD4, 5, 7 and 8 are not routinely examined in cHL but HRS cells would not be expected to express these antigens (Parker et al. 2010; Stein 2008). Published data however, suggests that between 2.5 and 18% of cHL tumours express at least one of the cytotoxic molecules, TIA-1, Granzyme B or Perforin, with or without the co-expression of other pan-T-cell antigens (Steidl et al. 2012; Asano et al. 2006; Asano et al. 2011; Krenacs et al. 1997; Foss, Reusch, Demel, Lenz & Anagnostopoulos 1999; Müschen et al. 2000; Seitz et al. 2000; Oudejans et al. 1996; Felgar et al. 1997; Kanavaros et al. 1999; Willenbrock et al. 2002).

Cytotoxic molecules are normally found within the cytoplasmic granules of CD8+ cytotoxic T-lymphocytes (CTL) and are directly responsible for many of these cells' cytotoxic actions. Perforin is a protein that forms pores in the cell membrane of target cells. Both Granzymes, which are serine proteases, and TIA-1 induce apoptosis of target cells, triggering DNA fragmentation (Asano et al. 2011; Krenacs et al. 1997). All three proteins are detectable in FFPE and frozen tissue by IHC. The PAX5 gene product, BSAP, is a transcription factor important for B-lymphocyte development, and expression appears to be specific to B-cells, only rarely being seen in T-cell disorders (Foss, Reusch, Demel, Lenz, Anagnostopoulos, et al. 1999; Asano et al. 2011).

To investigate the incidence of cytotoxic molecule expression and other pan-T-cell antigens in both the elderly and young cohorts, a series of further IHC studies were carried out using the following antibodies in addition to the previous diagnostic panel.

Table 4.1
T-cell and cytotoxic T-cell antibodies

Antibody	Significance of target protein
TIA-1	Cytotoxic granule-associated RNA binding protein. Nucleolytic activity against target cells. May be involved in induction of apoptosis.
Granzyme B	Cytotoxic T-lymphocyte associated serine esterase. Enzyme which activates caspases leading to apoptosis of target cells and therefore target cell lysis in a cell-mediated immune response.
Perforin	Pore forming protein. Key effector molecule for T-cell and NK cell mediated cytolysis. Creates transmembrane tubules leading to the lysis of target cells.
CD2	Pan-T-cell antigen expressed by all peripheral blood T-cells and NK cells but not B-cells. Mediates adhesion between T-cells and antigen presenting cells, inhibits apoptosis of activated peripheral T-cells.
CD3	Pan-T-cell antigen. Required for T-cell receptor cell surface expression and signal transduction.
CD4	Antigen expressed by T-helper cells.
CD5	General T-cell antigen, also expressed by some B-cells.
CD7	Pan-T-cell antigen, expressed by early T-cells, prior to TCR rearrangement and persisting until the terminal stages of T-cell development.
CD8	Marker of cytotoxic T-cells and T-cell suppressor cells.

(Pernick et al. 2014)

Materials and Methods

IHC labelling for the above antibodies was performed as previously described (chapter two), and TMA sections were dual-labelled with the PAX5 antibody plus each of the following antibodies in turn: TIA-1, Granzyme B and Perforin (figures 4.2 to 4.4). In each case, HRS cells were assessed as positive only if 10% or more of them were seen to express the antibody in question. For all other T-cell markers (CD2,3,4,5,7,8), HRS cells were scored as positive if >10% showed partial or complete membrane labelling or if >5% showed cytoplasmic expression of the T-cell antigen in question (Venkataraman et al. 2013). In addition to tumours from the original young and elderly cohorts, six other cases of cHL were assessed for cytotoxic molecule and T-cell antigen expression. One was a young patient, identified in the original cohort but excluded as their disease was HIV-related. The five other cases were identified through routine diagnostic work at the WGH between 2010 and 2013, and consisted of two elderly patients and one young patient, who would have met the criteria for respective cohorts if they had presented during the study period. The other two cases were aged 38 and 51 years and would not therefore have met the criteria for either the young or elderly cohorts.

Those cytotoxic molecule-positive (CMpos) cases with sufficient remaining tissue were investigated further using PCR techniques on whole sections of tissue as previously detailed (chapter two), to look for evidence of B- or T-cell clonality.

Eight cases from the young cohort (including the one excluded from other analyses due to the association with HIV) and four of the five additional cases identified between 2010 and 2013 (including the patient aged 51 years at diagnosis), also underwent Laser Capture Microdissection (LCM) to remove HRS cells from which DNA was extracted for analysis (chapter two) (figures 2.3 to 2.5).

The CMpos cases were divided into three groups. These were: CMpos cases that were PAX5-negative (n=three); CMpos cases that were PAX5-positive (n=20) and lastly, all CMpos cases regardless of PAX5 status (n=23).

Results

Cytotoxic Molecule expression

Of the original 159 cases of cHL, 128 were assessed for the expression of cytotoxic molecules; 51 and 77 from the elderly and young cohorts respectively. Of these, 17 (13%) were found to express at least one of the three cytotoxic molecules, eight of 51 (16%) from the elderly cohort and nine of 77 (12%) from the young group (table 4.2).

Table 4.2
All cases assessed for cytotoxic molecule expression

	Young cohort	Elderly cohort	Others	Combined
Assessed for CM expression	77	51	6*	134
Age range (years)	17-30	60-73	29-85	17-85
Mean follow-up period, months from diagnosis (range)	85 (6-163)	41 (0-64)	29 (0-128)	55 (0-163)
CMpos PAX5-neg (%)	1 (1)	1 (2)	1	3
CMpos PAX5-pos (%)	8 (10)	7 (14)	1	20
Total CMpos (%)	9 (12)	8 (16)	6	23

*included 2 young, 2 elderly and 2 aged outwith either cohort

Of these 17, nine (53%) expressed only Perforin; four (24%) only Granzyme B; three (18%) only TIA-1; and one tumour (5%) expressed all three cytotoxic molecules. Fifteen of these 17 (88%) co-expressed PAX5. The two PAX5-negative cases were both positive for Perforin and consisted of one young and one elderly patient. Two of the PAX5-positive cases also expressed CD20. All of the other 15 cases were CD20 negative. The tumour of the additional young patient, who's case was excluded from other analyses due to the association with HIV, was Perforin, PAX5 and CD20-positive.

Of the additional five cases, the tumour of the young patient had PAX5-positive HRS cells which co-expressed TIA-1, Granzyme B and Perforin. This patient was on an immunosuppressant drug (azathioprine) at the time of presentation. The two older patients also had PAX5-positive tumours, one with HRS cells co-expressing Perforin and TIA-1 and the other co-expressing Granzyme B. The tumour from the 38-year-old patient was PAX5-negative but CD20-positive and expressed all three cytotoxic molecules and that of the 51-year-old was PAX5 and Perforin-positive but CD20-negative.

Therefore in total there were 23 CMpos cases. In one of these, HRS cells were also shown to express CD3. No other T-cell antigens (CD2, CD3, CD4, CD5, CD7 or CD8) were expressed by any of the 22 other CMpos cases. Of the four cases that were CD20-positive, one was PAX5-negative and three PAX5-positive. HRS cells in all 23 cases were CD30-positive but 12 of 23 (52%) were CD15-negative (table 4.3).

Whole section PCR

In 11 of the CMpos cases, adequate tissue was available to attempt DNA extraction and PCR analysis. Of these cases, DNA of sufficient quality was successfully extracted from six cases. In four of these, both immunoglobulin (Ig) and TCR genes were polyclonal. Case '19' however, showed a clonal Ig gene rearrangement with polyclonal TCR genes. This EBV-negative tumour expressed all three cytotoxic molecules, was CD20-positive but negative for PAX5. Case '21' also showed evidence of a clone but this time a clonal TCR-gene rearrangement was found with polyclonal Ig genes. This tumour was EBV-negative, expressed TIA-1 and Perforin, was CD20-negative but expressed PAX5 (table 4.3).

Laser-Capture Microdissection (LCM)

LCM of HRS cells was carried out on 12 of the 23 CMpos cases. In Nine out of 12 cases, PCR reactions failed. In two cases (20 and 23), polyclonal Ig genes were identified but in both, the TCR gene reactions failed. In case '3', an EBV-negative, PAX5, Perforin-positive tumour from the young cohort, a clonal IgH gene rearrangement was evident (figure 4.5). Again in this case the TCR, PCR reactions failed. Case '19' which showed evidence of a B-cell clone on whole section PCR did not undergo LCM. Case '21', with evidence of a T-cell clone on whole section PCR did undergo LCM but the PCR reactions failed. Results for all CMpos cases are detailed in table 4.3.

Table 4.3
IHC and PCR results for all cases expressing cytotoxic molecules

Tumour	Cohort	CD30	CD15	CD2	CD3	CD4	CD5	CD7	CD8	Granzyme B	Perforin	TIA-1	Pax-5	CD20	Whole Section PCR	Microdissected HRS-cell PCR
1	Y	+	-	-	+s	-	-	-	-	+	+	+	+	-	F	F
2	Y	+	-	-	-	-	-	-	-	+	-	-	+	-	F	F
3	Y	+	-	-	-	-	-	-	-	-	+	-	+	-	F	§B+
4	Y	+	+	-	-	-	-	-	-	-	+	-	+	-	P	F
5	Y	+	+	-	-	-	-	-	-	-	+	-	+	-	ND	ND
6	*Y	+	-	-	-	-	-	-	-	-	+	-	+	+	P	F
7	Y	+	+	-	-	-	-	-	-	-	+	-	+	-	ND	F
8	Y	+	+	-	-	-	-	-	-	-	-	+	+	-	ND	ND
9	Y	+	-	-	-	-	-	-	-	-	+	-	-	-	F	F
10	Y	+	+	-	-	-	-	-	-	-	-	+	+	+	P	F
11	E	+	-	-	-	-	-	-	-	-	+	-	+	-	ND	ND
12	E	+	-	-	-	-	-	-	-	-	+	-	+	-	ND	ND
13	E	+	-	-	-	-	-	-	-	+	-	-	+	-	ND	ND
14	E	+	+	-	-	-	-	-	-	+	-	-	+	-	ND	ND
15	E	+	-	-	-	-	-	-	-	-	+	-	+	-	ND	ND
16	E	+	+	-	-	-	-	-	-	+	-	-	+	+	ND	ND
17	E	+	+	-	-	-	-	-	-	-	-	+	+	-	ND	ND
18	E	+	-	-	-	-	-	-	-	-	+	-	-	-	ND	ND
19	X	+	-	-	-	-	-	-	-	+	+	+	-	+	B+	ND
20	**Y-a	+	+	-	-	-	-	-	-	+	+	+	+	-	ND	^P
21	E-a	+	+	-	-	-	-	-	-	-	+	+	+	-	T+	F
22	E-a	+	+	-	-	-	-	-	-	+	-	-	+	-	F	F
23	X	+	-	-	-	-	-	-	-	-	+	-	+	-	P	^P

Y=Young cohort; Y-a=additional case, age as per young cohort ; E=Elderly cohort; E-a=additional case, age as per elderly cohort; X=age outwith parameters for either young or elderly cohort; *HIV-associated, excluded from other analyses; **Patient on immunosuppressant drugs at presentation; +s=surface expression; +c=cytoplasmic expression ; ND=not done; F=PCR failed; P=Polyclonal TCR and Ig genes; T+=clonal TCR gene rearrangement; B+=clonal Ig gene rearrangement; §=clonal IgH gene rearrangement, TCR PCR failed; ^=Polyclonal IgH genes, TCR PCR failed

T-cell antigen expression

Of the 111 cases found not to express any of the cytotoxic molecules, 102 (42 elderly, 60 young) had sufficient remaining tissue to be examined for all of the other T-cell markers. Sixteen of 102 (16%) were found to express at least one T-cell antigen, either with surface or cytoplasmic expression, when assessed according to the criteria outlined in chapter two. There were three from the elderly (3 of 42, 7%) and 13 from the young cohort (13 of 60, 22%). Seven (7%) expressed a single T-cell antigen and nine (9%) expressed two or more. All of these cases were PAX5-positive and nine of 16 (56%) expressed CD20. All 16 were CD30-positive and 14 (88%) expressed CD15 (table 4.4).

Table 4.4
IHC results for all CMneg cases expressing T-cell antigens

Tumour	Cohort	CD30	CD15	CD2	CD3	CD4	CD5	CD7	CD8	Pax-5	CD20
A	Y	+	+	-	-	+s	-	-	-	+	-
B	Y	+	+	-	-	+s	+s	+s	-	+	-
C	Y	+	+	-	+c	+c	-	-	-	+	+
D	Y	+	+	-	-	+s	+s	-	-	+	+
E	Y	+	+	-	+c	-	-	-	-	+	+
F	Y	+	+	+s/c	+s	+s	-	-	-	+	-
G	Y	+	+	-	+s	+s	-	-	-	+	+
H	Y	+	-	-	-	+s	+s	-	-	+	+
I	Y	+	+	-	-	+s	-	-	-	+	+
J	Y	+	+	-	-	+s	-	-	-	+	-
K	Y	+	+	-	-	-	-	-	+c	+	-
L	Y	+	+	+s	+c	+c	+c	-	-	+	+
M	Y	+	+	+s	+s	+s	-	+s	-	+	+
N	E	+	+	-	-	+s	-	-	-	+	-
O	E	+	+	-	-	+s	-	-	-	+	
P	E	+	-	+s	-	+s	-	-	-	+	+

Y=Young cohort; E=Elderly cohort; +s=surface expression; +c=cytoplasmic expression

Cytotoxic Molecule-positive versus Cytotoxic Molecule-negative

All CMpos cases were compared with all cytotoxic molecule-negative (CMneg) cases. In addition, CMpos and CMneg tumours in the elderly and young cohorts were examined separately. There was no difference in the immune profile of CMpos or CMneg tumours (table 4.5).

Table 4.5
IHC profile of cases assessed for cytotoxic molecule expression

	Combined: young, elderly + others		Young		Elderly	
	CMpos	CMneg	CMpos	CMneg	CMpos	CMneg
No. cases (% of total)	23 (17)	111 (83)	11 (14)	68 (86)	10 (19)	43 (81)
% CD30-positive	100	100	100	100	100	100
	p=NS		p=NS		p=NS	
% CD15-positive	48	61	55	69	50	48
	p=NS		p=NS		p=NS	
% PAX5-positive	87	95	91	94	90	98
	p=NS		p=NS		p=NS	
% CD20-positive	17	32	18	34	10	27
	p=NS		p=NS		p=NS	

There was no significant difference in the incidence of CMpos cHL in the elderly compared with the young (16% and 12% CMpos in each respective cohort) (table 4.2). In none of the comparisons, young, elderly or combined, were there significant differences in the proportion of males/females or NSHL/MCHL in the CMpos compared with the CMneg cases. However, in each of the three comparisons, a greater proportion of patients in the CMpos group presented with advanced stage disease, (stage IIB-IV) than in the CMneg subgroup. In the young and combined groups this difference was statistically significant (young, p=<0.05; combined, p=0.05). In the young, elderly and combined cohorts, significantly more patients in the CMpos group presented with B-symptoms than was the case in the CMneg group (young, p=<0.01; elderly, p=0.05; combined, p=0.001) (table 4.6). For all four measures of outcome, relapse, relapse or escalation in treatment, death from any cause and death due to cHL, there was no significant difference between those with CMpos and CMneg disease in any cohort.

Table 4.6
Presenting features of cases assessed for cytotoxic molecule expression

	Combined: young, elderly + others		Young		Elderly	
	CMpos	CMneg	CMpos	CMneg	CMpos	CMneg
% stage I-IIA/IIB-IV	19/81	42/58	9/91	49/51	25/75	32/68
	p=0.05		p=<0.05		p=NS	
% B symptoms	80	39	90	42	75	34
	P=0.001		P=<0.01		P=0.05	

CMpos PAX5-positive versus CMpos PAX5-negative

The three CMpos PAX5-negative cases were compared with the 20 CMpos PAX5-positive cases. No statistically significant differences were evident for any of the following comparisons: proportion from elderly and young cohorts; gender; cHL subtype; those presenting with early or advanced stage disease; those presenting with or without B-symptoms; outcome by any of the four measures as above.

T-cell antigen-positive versus T-cell antigen-negative

Significantly more cases from the young cohort were found to express T-cell antigens (20% of young cohort, 6% of elderly cohort, p=<0.05) (table 4.7). A greater proportion of the T-cell antigen-positive tumours expressed CD15 and CD20 when compared with the T-cell antigen-negative cases. This difference reached statistical significance in the combined cohort (p=<0.05 for both CD15 and CD20) (table 4.8).

Table 4.7
Cases assessed for T-cell antigen expression

	Combined: young + elderly		Young		Elderly		Young v Elderly
T-cell antigen	**Pos**	**Neg**	**Pos**	**Neg**	**Pos**	**Neg**	
No. cases (% of total)	17 (14)	108 (86)	14 (20)	57 (80)	3 (6)	49 (94)	P=<0.05

Table 4.8
IHC profile of cases assessed for T-cell antigen expression

	Combined: young + elderly		Young		Elderly	
T-cell antigen	**Pos**	**Neg**	**Pos**	**Neg**	**Pos**	**Neg**
% CD30-positive	100	100	100	100	100	100
	p=NS		p=NS		p=NS	
% CD15-positive	82	54	86	61	67	47
	p=<0.05		p=NS		p=NS	
% PAX5-positive	100	94	100	95	100	96
	p=NS		p=NS		p=NS	
% CD20-positive	56	26	57	28	50	23
	P=<0.05		P=0.06		p=NS	

Comparisons were also made in the young, elderly and combined cohorts for the following factors, none of which showed a statistical difference between those tumours with or without T-cell antigen expression: gender; cHL subtype; clinical stage at presentation; presence or absence of B-symptoms at presentation; outcome according to any of the four measures.

Discussion

All cHL cases in this study were diagnosed according to the WHO 2008 diagnostic criteria. In 17 cases (13%) HRS cells were shown to express at least one cytotoxic molecule. This is consistent with the 2.5 to 18% quoted in published data (Steidl et al. 2012; Asano et al. 2006; Asano et al. 2011; Krenacs et al. 1997; Foss, Reusch, Demel, Lenz & Anagnostopoulos 1999; Müschen et al. 2000; Seitz et al. 2000; Oudejans et al. 1996; Felgar et al. 1997; Kanavaros et al. 1999; Willenbrock et al. 2002). Of these 17 cases, HRS cells in 15 (88%) were also positive for PAX5 using double-labelling IHC, and two were PAX5-negative. This did not differ significantly from the percentage of PAX5-positive cases in the CMneg tumours (95%). As expected, fewer tumours expressed CD20 than PAX5 but again there was no significant difference between the CMpos and CMneg cases. The literature does however suggest that significantly fewer cases of CMpos cHL express PAX5. In published data on 61 CMpos cases of cHL, only two of 37 cases (5%) examined for PAX5 expression were positive and none of 55 cases were CD20-positive (Asano et al. 2011; Müschen et al. 2000; Asano et al. 2006; Foss, Reusch, Demel, Lenz, Anagnostopoulos, et al. 1999; Seitz et al. 2000). This much lower figure for PAX5 expression may be influenced by studies specifically selecting PAX5-negative CMpos cases and in part may also be due to the use in previous studies, of earlier PAX5 antibodies with different target epitopes (Asano et al. 2011).

The proportion of PAX5-positive cases did not differ between the T-cell antigen-positive and –negative subgroups but in those T-cell antigen-positive tumours, significantly more cases were also CD15 and CD20-positive. Why this should be so is not known but it allows the suggestion that those cases expressing T-cell antigens may be suffering a more general failure to control normally the expression of genes associated with the different stages of lymphoid differentiation and maturation.

Cytotoxic molecule and/or T-cell antigen expression by what appear to be HRS cells is of importance as it raises several possibilities: (1) the ectopic expression of T-cell markers by HRS cells which may cause diagnostic difficulties and/or be of prognostic significance; (2) the possibility of a subgroup of cHL derived from T-cells; (3) the misclassification of T-cell lymphoproliferative disorders, with or without the ectopic expression of B-cell antigens, which may lead to inappropriate treatment choices. Defining whether the malignant cells in such tumours are of B- or T-cell origin may help resolve such questions.

Of the 23 CMpos cases discussed in this thesis, evidence for the presence of a B-cell clone was found in two, (one by whole section PCR and the other using PCR on microdissected HRS cells) and in a third, evidence for a T-cell clone was shown using whole section PCR. The T-cell clone was found in a PAX5-positive, CD20-negative tumour which expressed Perforin and TIA-1. The B-cell clone found using whole section PCR was in a PAX5-negative, CD20-positive tumour that expressed all three cytotoxic molecules. The B-cell clone found using PCR on microdissected HRS cells was in a PAX5-positive, CD20-negative tumour which co-expressed Perforin.

In only a small number of cases of cHL have clonal gene rearrangements been identified using PCR on 'whole sections' of tissue and when it has been successful the tumours have often contained unusually high numbers of HRS cells. The significance of a B- or T-cell clone found in this way is difficult to interpret. Of the 37 published CMpos cases of cHL mentioned previously, there were two PAX-5 positive cases, one of which was examined by single-cell PCR for TCR gene rearrangements. No evidence of a clone was found (Seitz et al. 2000; Foss, Reusch, Demel, Lenz, Anagnostopoulos, et al. 1999). A total of 35 cases were CMpos, PAX5 negative and two of these were also examined by single-cell PCR and on this occasion both were found to harbour clonal TCR-gamma gene rearrangements with no evidence of IgH clonal rearrangements. Thirty-two of the other cases were examined by PCR on whole sections of FFPE tissue and none were shown to have clonal IgH or TCR gene rearrangements (Asano et al. 2011; Foss, Reusch, Demel, Lenz, Anagnostopoulos, et al. 1999; Seitz et al. 2000).

Overall in the literature to date, only eight cases of cHL (including the two described above) have been found to contain HRS cells with clonally rearranged TCR genes. Of these eight cases, six were examined by IHC for one or more cytotoxic molecules and three were positive for at least one of TIA-1, Granzyme B or Perforin. Two of the three remaining cases were only examined for TIA-1, the third was only examined for Perforin and all were negative (Aguilera et al. 2006; Tzankov et al. 2005; Willenbrock et al. 2002; Seitz et al. 2000; Müschen et al. 2000). All clonal TCR-gene rearrangements were identified by the microdissection of HRS cells and subsequent PCR. Three were taken from frozen tissue sections and five from FFPE tissue (Müschen et al. 2000; Seitz et al. 2000; Willenbrock et al. 2002; Tzankov et al. 2005; Aguilera et al. 2006). Of all eight cases, three expressed PAX5 by IHC, four were PAX5-negative (including two of the three CMpos cases) and in one it was not looked for (the third CMpos case). One PAX5-positive case was also positive for CD20. The case not examined for PAX5 was CD20-negative, one PAX5-negative case was positive for CD20 and in two cases CD20 IHC was not done (Aguilera et al. 2006; Tzankov et al. 2005; Willenbrock et al. 2002; Seitz et al. 2000; Müschen et al. 2000). All eight cases were EBV-negative.

The importance of defining these differences depends on whether or not there are clinical distinctions related to the expression of certain tumour antigens and ultimately whether these may influence treatment decisions and patient outcome.

In this thesis, when comparing all CMpos cases with all CMneg, those expressing cytotoxic molecules were associated with tumours presenting at a more advanced stage and with B-symptoms. These are both poor prognostic features in cHL but the outcome for patients with CMpos tumours did not differ significantly from those with CMneg disease. The number of CMpos, PAX5-negative tumours was small which limited interpretation but no significant variations were found between CMpos, PAX5-negative and CMpos, PAX5-positive tumours. Comparisons were also made between those tumours that expressed T-cell antigens and those that did not. Only one T-cell antigen-positive case was also CMpos and this was excluded from these analyses. T-cell antigen-positive tumours were found significantly more frequently in the young cohort. There was no difference in the clinical stage or presence of B-symptoms in those patients with or without tumours expressing T-cell antigens, nor was there any difference in outcome.

The literature regarding the prognostic significance of cytotoxic molecule and/or T-cell antigen expression in cHL is mixed. One large study, published in 2006, looked at 324 cases of cHL. Twenty of these expressed TIA-1, Granzyme-B or both and an additional seven expressed other T-cell antigens; CD3, CD4 or CD8. They found that this group of 27 patients had a significantly poorer prognosis on both uni- and multi-variate analysis. Indeed, on multivariate analysis only T-cell or cytotoxic T-cell antigen expression and age over 45 years were significant independent prognostic factors (Asano et al. 2006). Asano and colleagues in 2011, similarly found a significantly poorer disease specific survival (DSS) in their group of 32 CMpos, PAX5-negative cHL in comparison to 439 CMneg cases (Asano et al. 2011). Numbers in many studies are too small for meaningful statistical analyses but certainly other groups have also noted a poor prognosis in some of their CMpos cases of cHL. For example, Tzankov noted that one of two cHL cases with clonal TCR-gamma gene rearrangements (also PAX5-negative) had a markedly poorer outcome than expected using other prognostic markers (Tzankov et al. 2005). In contrast, other groups have found no difference in progression free survival (PFS) or overall survival (OS) between 11 Granzyme-B positive and 50 Granzyme-B negative cases of cHL (Oudejans et al. 1996). In one small study of four $CD3^+$ cases, all in young adult women with NSHL, a 'good outcome' was noted for each and this was used as one argument against classifying these as cases of PTCL which would usually be expected to have a significantly poorer prognosis (Aguilera et al. 2006). Ideally a much larger study, examining T-cell antigen expression and distinguishing CMpos, PAX5-negative and CMpos, PAX-5-positive cases from CMneg cHL, would be required to ascertain more reliably the prognostic significance of T-cell and cytotoxic molecule expression in cHL.

Whether or not cytotoxic molecule expression, with or without PAX5 expression is indicative of a different disease process is also not clear. The conclusion reached in the published literature to date appears to be that HRS cells, derived from B-cells, may aberrantly express T-cell markers including cytotoxic molecules. However, in a very small number of what, by all other parameters, appear to be cases of cHL, 'HRS' cells, are of T-cell rather than B-cell origin and to further complicate matters, a proportion (three of seven, 43%) of these cells of T-cell origin, may aberrantly express the B-cell marker, PAX5. Overall the majority of CMpos cHL cases can probably be considered of B-cell origin if they also express PAX5. However, this is less certain for those that are PAX5-negative. Whether the presence of a T-cell clone in the eight published cases truly reflects a rare form of cHL originating from a T-lymphocyte or whether these are better considered unusual T-cell lymphomas very much depends on whether lineage specificity is believed to be key to the classification of lymphomas and cHL in particular.

As described, the diagnosis of cHL requires a detailed morphological examination of the tumour, including analysis of the immunohistochemical features of the malignant cells. However, this does not give sufficient information to understand the pathogenesis of cHL. In 1997 the IARC (chapter one) concluded that EBV has a causal role in some cases of cHL. In the following chapter, the presence and potential significance of EBV in both the young and elderly cohorts, is examined and discussed.

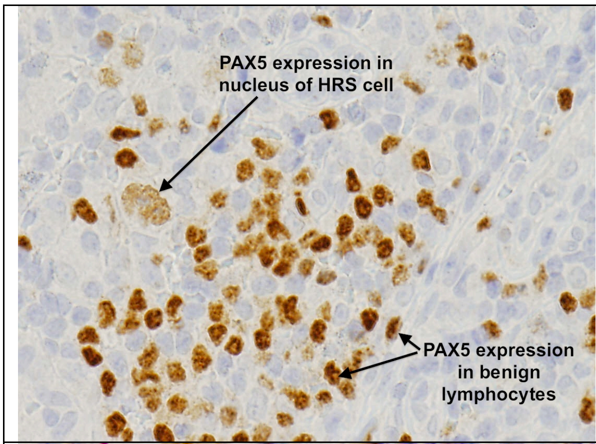


Figure 4.1
PAX5 IHC
Tumour 16, Young cohort, MCHL (x20)

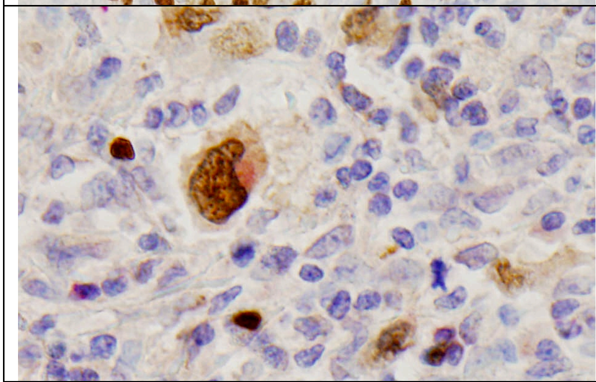


Figure 4.2
TIA-1 & PAX5 dual-labelling
Tumour 10 of cytotoxic positive cases (x40)
HRS cell with nuclear PAX5 expression (brown DAB label) and cytoplasmic TIA-1 expression (red New Fucsin label). Small benign PAX5-positive lymphocytes in the background.

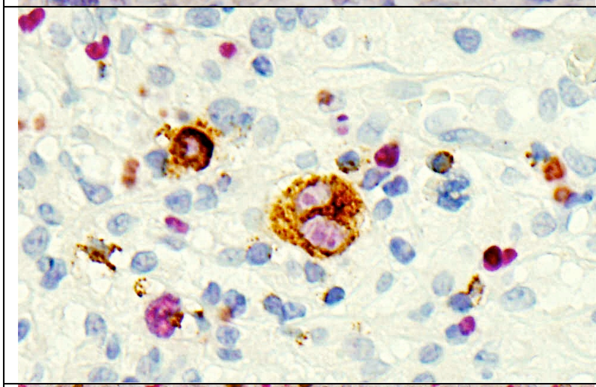


Figure 4.3
Granzyme B & PAX5 dual-labelling
Tumour 2 of cytotoxic positive cases (x40)
Binucleate HRS cell with nuclear PAX5 expression (red New Fucsin label) and cytoplasmic Granzyme B expression (brown DAB chromogen). Benign PAX5-positive and Granzyme B-positive lymphocytes in the background.

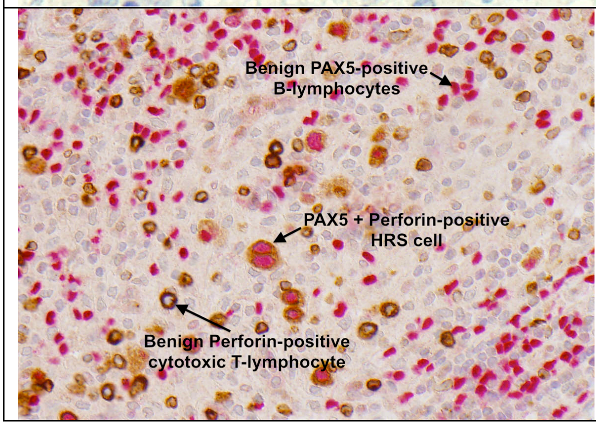


Figure 4.4
Perforin & PAX5 dual-labelling
Tumour 23 of cytotoxic positive cases (x20)
Binucleate HRS cell with nuclear PAX5 expression (red New Fucsin label) and cytoplasmic Perforin expression (brown DAB chromogen). Benign PAX5-positive and Perforin-positive lymphocytes in the surrounding microenvironment.

Figure 4.5
PCR trace.
Tumour 3 of cytotoxic positive cases, evidence of B-cell clone

1st + 2nd panels: Immunoglobulin Heavy chain gene primers, run in duplicate. Clonal peak at 100-101 base pairs of Framework 3 region
3rd + 4th panels: T-cell receptor gene primers, run in duplicate. 4th panel shows no PCR amplification in duplicate run

Chapter 5

Epstein-Barr Virus and classic Hodgkin Lymphoma

Introduction

EBV was the first identified human oncogenic virus (chapter one) and is now recognised as an important aetiological factor in around 40% of cHL (Jarrett et al. 1996; Stark et al. 2002; Armstrong et al. 1998; Shimoyama et al. 2008; Chetaille et al. 2009). It is more common in certain subtypes however, with 75% of MCHL cases EBV-positive contrasting with only 10-25% of NSHL (Fend 2010; Jarrett et al. 1996; Armstrong et al. 1998; WHO 1996a). As discussed (chapter one), the highest incidence of EBV-positive cHL occurs in the poorest communities of the developing world (Armstrong et al. 1998; Jarrett et al. 1996; WHO 1996a; Barros et al. 2011). The incidence also varies according to age and there is some evidence to suggest that EBV is of varying prognostic significance in different age groups with cHL. Although young adults tend to present with EBV-negative NSHL (Armstrong et al. 1998; Jarrett et al. 2005), those with EBV-positive disease, show a trend towards, or even in some studies, a significant improvement in survival (Armstrong et al. 1994; Naresh et al. 2000; Vassallo et al. 2001; Capkun et al. 2001; Keegan et al. 2005; Kwon et al. 2006; Barros, Vera-Lozada, et al. 2012). This is contrary to the findings in EBV-positive elderly patients who may have an inferior outcome (Armstrong et al. 1994; Naresh et al. 2000; Vassallo et al. 2001; Capkun et al. 2001; Keegan et al. 2005; Kwon et al. 2006; Enblad et al. 1999; Diepstra et al. 2009; Jarrett et al. 2005). Gender also appears to be important, with the risk of developing EBV-positive cHL significantly greater in men than women (Tan et al. 2012; Barros et al. 2011; Gandhi et al. 2004; Huang et al. 2010; Küppers 2009b; Asano et al. 2009; Horstman et al. 1996). In addition, those 20-40% of the population who present with Infectious Mononucleosis (IM) in response to primary EBV infection, have an increased risk of developing EBV-positive cHL (Hjalgrim et al. 2010).

The incidence of all lymphoproliferative disorders, including cHL, is increased in the context of immunosuppression of any cause, whether congenital or acquired (Nador et al. 2003; Tran et al. 2008; Said 2007). The majority of lymphomas occurring in this scenario are EBV-positive, with around 80% of PTLD, 60% of all HIV-associated lymphomas and 100% of HIV-associated cHL being EBV-related (Tran et al. 2008; Lee et al. 1998). Most are B-cell lymphomas, fitting with the concept of immunosuppression 'allowing' uncontrolled EBV-driven lymphoproliferation, although other factors such as chronic antigen stimulation from HIV or Cytomegalovirus (CMV) infection, or from persistent low-level allograft rejection may also be important (Tran et al. 2008). The WHO classification of lymphoid neoplasms (Raphael et al. 2008), describes three categories of lymphoproliferative disorders in which immunosuppression is recognised to play a role. The third, and most recent of these, EBV-positive DLBCL of the Elderly, is still a provisional entity. This form of DLBCL occurs in patients with no known immunodeficiency disorder. Histologically, there is a marked inflammatory background, malignant cells are EBV-positive and frequently exhibit type III latency (see appendix B, EBV and its interaction with the immune system), suggesting immunodeficiency of the host (Raphael et al. 2008; Campo et al. 2014). This is significant as it provides evidence of immune dysfunction associated with age, sufficient to result in lymphoma. Plasmablastic Lymphoma and HHV8-related Primary Effusion Lymphoma are generally considered to be HIV-specific disorders which are EBV-positive, with tumour cells expressing type I latency (see appendix B). However, these lymphomas also occur rarely in patients with no recognisable immunodeficiency and again, those affected tend to be elderly (Campo et al. 2014). This would also seem to suggest that there are acquired immunodeficiency states, more common with ageing, sufficient to result in an increased risk of lymphoproliferative disorders. Although currently this is only recognised for some forms of NHL, it would be reasonable to propose that a similar process may be involved in the pathogenesis of some cases of cHL.

In view of this, the EBV status of the tumours in both cohorts was ascertained and analysed. The following factors were examined:

1. The number of EBV-positive cases in each age group.
2. The distribution of EBV-positive cases between male and female patients.
3. The proportion of EBV-positive cases according to histological subtype.

4. T-cell antigen expression in EBV-positive and –negative cases.
5. The outcome of treatment for EBV-positive and -negative groups.

Materials and Methods

IHC for the EBV-encoded protein, LMP-1 and EBER-ISH (figure 2.2) was undertaken as described in chapter two, to identify those tumours which were EBV-related. All p-values were calculated using the Fisher's exact test unless stated otherwise.

Results

EBV-positive cases of cHL in each cohort

Of the 82 cases in the young cohort, 20 (24%) were EBV-positive and 62 (76%) EBV-negative. Of the 77 elderly patients, 42 (55%) were EBV-positive and 35 (45%) EBV-negative. The incidence of EBV-positive cHL was significantly greater in the elderly cohort when compared to the young (p=<0.001) (table 5.1, figure 5.1).

Figure 5.1
EBV related cases in each cohort

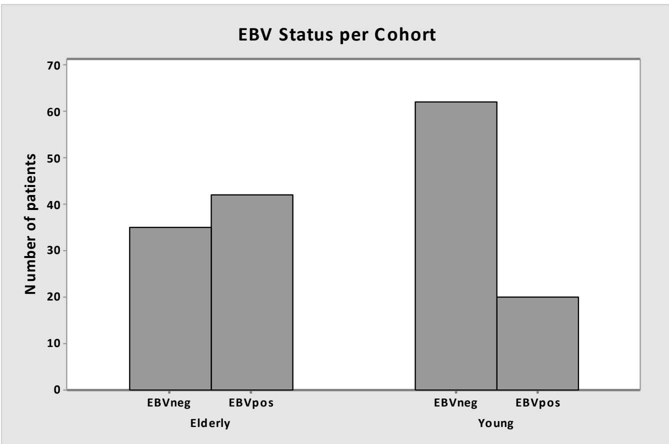


Table 5.1
EBV related cases in each cohort

	Elderly + Young combined	Young	Elderly	Young v Elderly
Total	159	82	77	
EBV pos (%)	62 (39)	20 (24)	42 (55)	P=<0.001
EBV neg (%)	97 (61)	62 (76)	35 (46)	

EBV-positive cases of cHL according to gender

When both young and elderly groups were examined together, 36 of the 74 males (49%) were EBV-positive, while 21 of the 71 women (27%) had EBV-positive disease (p=<0.01). When the cohorts were examined separately, it became evident that the young cohort was responsible for this difference. In the elderly group there was no significant difference in EBV-related disease between the genders (table 5.2). The younger cohort included slightly more women than men (55% v 45%). Five of the 45 (11%) women had EBV-positive disease, which was significantly fewer than the 15 of 37 (41%) young men with EBV-positive cHL (p=<0.01). Of the elderly, overall there were more men than women, (52% v 48%) but there was no significant difference between the proportion of men (21 of 37) or women (16 of 34) with EBV-positive cHL.

Table 5.2
EBV and gender

	Elderly + Young combined	Young	Elderly	Young v Elderly
EBV pos Male (% all male)	36 (49)	15 (41)	21 (57)	p=NS
EBV pos Female (% all female)	21 (27)	5 (11)	16 (47)	P=0.001
EBV pos Male v Female	p=<0.01	p=<0.01	p=NS	

EBV-positive cases of cHL according to histological subtype

Table 5.3
cHL subtypes per cohort

	Elderly + Young Combined	Young	Elderly	Young v Elderly
NSHL (%)	100 (63)	61 (74)	39 (51)	p=<0.01
MCHL (%)	41 (26)	13 (16)	28 (36)	p=<0.01

Data also presented chapter 3, table 3.4

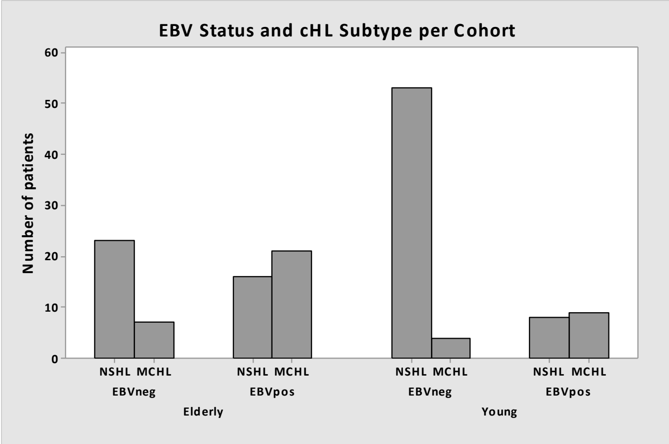
As previously described (chapter three), in total there were 100 cases of NSHL and this subtype predominated in all age groups (63% of both cohorts combined; 74% of the young cohort; 51% of the elderly cohort). Of the 100 cases, 24 were EBV-positive; eight of 61 (13%) young and 16 of 39 (41%) elderly (p=<0.01). MCHL accounted for 41 cases altogether and was significantly more frequent in the older age group (16% young v 36% elderly, p=<0.01). In this subtype, nine of 13 (69%) young and 21 of 28 (75%) elderly were EBV-positive, so while there was a significant association between MCHL and EBV-positive disease there was no significant difference between the two age groups (table 5.3).

Of the 74% with NSHL in the young cohort, eight of 61 (13%) were EBV-positive, compared with the 16% of the young with MCHL, of which, nine of 13 (69%) were EBV-positive (p=<0.001). In the elderly group, 51% had NSHL, of which 16 of 39 (41%) were EBV-positive. This compared with 36% of the elderly cohort who had MCHL, of which 21 of 28 (75%) were EBV-positive (p=<0.01) (table 5.4, figure 5.2).

Table 5.4
EBV and cHL subtype

	Elderly + Young combined	Young	Elderly	Young v Elderly
EBV-pos NSHL (% all NSHL)	24/100 (24)	8/61 (13)	16/39(41)	p=<0.01
EBV-pos MCHL (% all MCHL)	30/41 (73)	9/13 (69)	21/28(75)	p=NS
EBV-pos cHL other (% all cHL other)	8/18 (44)	3/8 (38)	5/10(50)	p=NS
NSHL v MCHL	p=<0.0001	p=<0.0001	p=<0.01	

Figure 5.2
EBV and cHL subtype



Thus the proportion of NSHL that was EBV-related, was significantly greater in the elderly compared to the young (41% v 13%, p=<0.01) while there was no significant difference, between the two cohorts, of EBV-positive MCHL (table 5.4).

EBV-positive cases of cHL and T-cell antigen expression

When CMpos were compared with CMneg and CMpos, PAX5-positive compared with CMpos, PAX5-negative tumours, there was no difference in the proportion that were EBV-positive/-negative in any of the three cohorts; young, elderly or combined.

There was evidence of an association between T-cell antigen expression and EBV-negative disease. This was apparent in all three cohorts but statistically significant in the combined cohort (p=<0.01) (table 5.5).

Table 5.5
EBV and T-cell antigen expression

	Combined, young + elderly		Young		Elderly	
T-cell antigen	**Pos**	**Neg**	**Pos**	**Neg**	**Pos**	**Neg**
Total no. cases	17	108	14	57	3	49
% EBV pos	6	42	7	28	0	57
% EBV neg	94	58	93	72	100	43
	p=<0.01		p=NS		p=NS	

Outcome of treatment for EBV-positive and -negative groups

Outcome was measured in the two cohorts, both separately and combined, according to the four parameters as described in chapter two. In brief these were: 1) relapse of disease; 2) relapse of disease or escalation in treatment; 3) those who died; 4) those who died of cHL.

The proportion of patients in each cohort that suffered a relapse of disease or required an intensification of treatment did not differ significantly. In the young, 15 of 81 (19%) relapsed compared to 17 of 74 (23%) of the elderly. When those that required an intensification of treatment were included, these figures rose to 19 of 81 (24%) of the young and 22 of 68 (32)% of the elderly. However, significantly more elderly patients than young died during follow-up (40% v 7%, p=<0.0001) and this difference was still evident when only deaths due to cHL were examined (20% v 4%, p=<0.01) (table 5.6).

Table 5.6
Summary of outcome, all patients

	Elderly + Young Combined	Young	Elderly	Young v Elderly
Relapse (%)	32 (21) *4 missing	15 (19) *1 missing	17 (23) *3 missing	p=NS
Relapse or escalation in Rx (%)	41 (28) *10 missing	19 (24) *1 missing	22 (32) *9 missing	p=NS
Death all causes (%)	37 (23) *1 missing	6 (7) *1 missing	31 (40)	p=<0.0001
Death due to cHL (%)	18 (12) *3 missing	3 (4) *1 missing	15 (20) *2 missing	p=<0.01

Data also presented chapter 3, tables 3.5 to 3.8

Considering EBV status and comparing elderly with young cohorts, there was a trend suggesting EBV-positive elderly patients may be more likely to relapse or require intensification of treatment than young EBV-positive patients, but this was not statistically significant (tables 5.7 & 5.8). Elderly EBV-positive patients were also more likely to die of any cause, (19 of 42, 45%) than EBV-positive young patients, (3 of 20, 15%), (p=<0.05), with a similar, but not statistically significant, trend in deaths due to cHL (27% of EBV-positive elderly v 5% of

EBV-positive young). EBV-negative elderly patients were significantly more likely to die of any cause (12 of 35, 34%) than EBV-negative young patients (3 of 61, 5%), (p=<0.001) (tables 5.9 & 5.10).

Within the elderly group, the data shows a poorer outcome for EBV-positive disease when compared to EBV-negative disease regarding relapse, relapse/intensification of treatment, and death due to cHL, however the difference was not significant (tables 5.7, 5.8 & 5.10). Overall there was no statistically significant difference in outcome related to EBV status, in either group when examined separately.

When both cohorts were combined, those with EBV-positive disease were more likely to die of any cause or specifically of cHL, than those who were EBV-negative. (Death all causes, 36% of all EBV-positive v 16% of all EBV-negative, p=<0.01. Death due to cHL, 20% of all EBV-positive v 6% of all EBV-negative, p=<0.05) (tables 5.9 & 5.10).

Table 5.7
EBV and outcome: relapse

	Elderly + Young Combined	Young	Elderly	Young v Elderly
EBV pos relapse (% all EBV pos)	15 (25) *3 missing	3 (16) *1 missing	12 (30) *2 missing	p=NS
EBV neg relapse (% all EBV neg)	17 (18) *1 missing	12 (19)	5 (15) *1 missing	p=NS
Relapse, EBV pos v neg	p=NS	p=NS	p=NS	

Table 5.8
EBV and outcome: relapse or escalation in treatment (Rx)

	Elderly + Young Combined	Young	Elderly	Young v Elderly
EBV pos relapse or escalation in Rx (% all EBV pos)	18 (32) *5 missing	3 (16) *1 missing	15 (40) *4 missing	p=0.08
EBV neg relapse or escalation in Rx (% all EBV neg)	23 (25) *5 missing	16 (26)	7 (23) *5 missing	p=NS
Relapse or escalation in Rx, EBV pos v neg	p=NS	p=NS	p=NS	

Table 5.9
EBV and outcome: death, any cause

	Elderly + Young Combined	Young	Elderly	Young v Elderly
EBV pos death any cause (% all EBV pos)	22 (36)	3 (15)	19 (45)	p=<0.05
EBV neg death any cause (% all EBV neg)	15 (16) *1 missing	3 (5) *1 missing	12 (34)	p=<0.001
Death any cause, EBV pos v neg	p=<0.01	p=NS	p=NS	

Table 5.10
EBV and outcome: death due to cHL

	Elderly + Young Combined	Young	Elderly	Young v Elderly
EBV pos death cHL (% all EBV pos)	12 (20) *2 missing	1 (5)	11 (27) *2 missing	p=0.08
EBV neg death cHL (% all EBV neg)	6 (6) *1 missing	2 (3) *1 missing	4 (11)	p=NS
Death cHL, EBV pos v neg	p=<0.05	p=NS	p=NS	

Discussion

Significantly more cases of cHL were EBV-positive in the elderly than the young cohort. This association, of EBV-related disease with cHL in older patients, is well established (Armstrong et al. 1994; Jarrett et al. 1996).

In the younger patients, significantly more EBV-positive cases were found in men than women but in the older group this was not the case. Again, there is a recognised association between males and EBV-positive cHL, but the reasons for this have not been adequately explained (Asano et al. 2009; Barros et al. 2011; Gandhi et al. 2004; Horstman et al. 1996; Huang et al. 2010; Tan et al. 2012).

As expected, in all groups, there was a strong association between the MCHL subtype and EBV infection, and those young patients with MCHL were equally likely to have EBV-positive tumours as those diagnosed with this subtype in the elderly. However, cases of NSHL were significantly more likely to be EBV-related if the patient was from the elderly cohort rather than the young. (Armstrong et al. 1994; Fend 2010; Jarrett et al. 1996; WHO 1996a).

These findings, within the two cohorts studied, suggest that these groups were representative of patients more generally with cHL and were consistent with other groups of cHL patients studied over the years.

The percentage of EBV-positive and –negative tumours did not differ between cases which did and did not express cytotoxic molecules, but there was a significant association between T-cell antigen-positive tumours and EBV-negative disease. There was also a statistically significant association between young patients with cHL and T-cell antigen-positive tumours (chapter four). These two are confounding factors as young patients with cHL are more likely to have EBV-negative disease and therefore the significance of each is not clear.

Although findings were not statistically significant, the elderly patients with EBV-positive disease appeared to do less well than the young with EBV-positive tumours. Whether this difference may become significant with larger group sizes is not clear. Overall, the number of patients who relapsed or required an escalation in treatment due to a poor initial response, did not differ between the elderly and young but there was a suggestion that those elderly with EBV-positive disease relapsed/required an escalation in treatment, more than the equivalent group of young patients. The number of patients with EBV-positive disease who had a poor outcome, especially in the young cohort, was very small however, and was insufficient to permit meaningful statistical analysis.

This again is in keeping with the findings of many cHL studies. Those that have shown EBV to be of prognostic significance have tended to examine patients within defined age groups and have found that the young may do better if they have EBV-positive disease (Armstrong et al. 1994; Naresh et al. 2000; Vassallo et al. 2001; Capkun et al. 2001; Keegan et al. 2005; Kwon et al. 2006; Barros, Vera-Lozada, et al. 2012), whereas older patients with EBV-related disease may do less well (Armstrong et al. 1994; Naresh et al. 2000; Vassallo et al. 2001; Capkun et al. 2001; Keegan et al. 2005; Kwon et al. 2006; Enblad et al. 1999; Diepstra et al. 2009; Jarrett et al. 2005). No single study has been able to provide definitive information however, which may suggest that any association between the virus and outcome is not strong, and is open to any number of interacting factors, of which age is only one.

Examining those patients who died of cHL or of any cause, both EBV-positive and EBV-negative elderly patients were significantly more likely to die of any cause than their younger counterparts. However, this simply reflects the observation that more elderly than young died overall and does not provide information about any associations with EBV infection.

When both cohorts were combined, those with EBV-positive disease were significantly more likely to die of cHL or of any cause, than those that were EBV-negative. However, rather than this reflecting a true difference due to EBV, this is likely related to the finding that significantly more elderly than young patients died of cHL or of any cause. It has already been demonstrated that the elderly were significantly more likely to have EBV-positive disease and therefore a greater proportion of those who died, being elderly, were EBV-positive. When the cohorts were examined separately there was no significant difference between those EBV-positive and –negative who died.

Although there were no significant differences in any of the four outcome measures between the EBV-positive and –negative cases in each cohort, a greater proportion of the elderly with EBV-positive disease suffered relapse, relapse/escalation of treatment, or died of cHL than did from the EBV-negative elderly group. The opposite appeared to be true in the young cohort. These differences were not statistically significant but the number of patients who did less well in each cohort was small and therefore a true difference cannot be excluded.

Review of Literature

It has long been recognised that those with cHL exhibit signs of immune dysfunction, regardless of whether they have EBV-positive or -negative disease. Dorothy Reed commented on this in 1902 when she noted that those with HL did not react to tuberculin (Zwitter et al. 2002; Poppema 1996). Many have commented generally on the increased susceptibility to infection in cHL patients, and more specifically there are studies showing reduced hypersensitivity skin reactions to certain agents (e.g. Dinitrochlorbenzene) and reduced rejection of skin grafts in patients with cHL (Poppema 1996; Grur et al. 1997). There is also an increased incidence of shingles in patients, old and young, in the year prior to them presenting with EBV-positive cHL (Jarrett et al. 2005). Even in the apparently immunocompetent patient who develops EBV-positive cHL, there must be an impairment of normal antiviral responses because healthy individuals do have CTL specific to the more limited viral proteins expressed by EBV-positive HRS cells (type II latency) and should be able to control their proliferation (Asano et al. 2009; Merlo et al. 2010). How EBV and its interaction with the immune system may be linked with these observations i.e. the epidemiological variations in cHL and the others discussed above including the association between EBV and cHL in elderly patients; certain subtypes of cHL; and differing outcomes in those older and younger with EBV-related disease, is of importance. (For a summary of EBV and its interaction with the immune system see Appendix B).

Immune compromise and EBV-positive cHL

An enormous number of factors influence the immune system. Not only may they affect the way an individual responds to aetiological factors for cHL, such as EBV, but once malignant transformation has occurred they will influence the way in which the disease is manifest, including its phenotype, its progression and prognosis with treatment. The various influences on the immune system in cHL may be thought of under different categories: (1) genetic factors affecting the immune response; (2) acquired factors, for example related to co-morbid disease, ageing and the effect of immunosenescene; (3) the effects of the

Epstein-Barr virus itself. There are considerable overlaps and interconnections between these arbitrary groups but the divisions are useful for examining processes and each will be discussed in turn.

(1) Genetic factors and the immune response

It is known that CTL require EBV-associated antigens to be expressed via HLA class I molecules to allow killing of infected cells but that the effectiveness of antigen presentation depends in part on how efficiently individual HLA alleles bind those antigens. It is thought that only certain class I molecules can effectively present, for example LMP-1 and -2, to elicit a CTL response (Huang et al. 2010; Diepstra et al. 2005). HLA class II antigen presentation is also important in the eradication of EBV by $CD4^+$ T-cells (Huang et al. 2010) and therefore HRS cell expression of both HLA class I and II molecules is relevant to the immune response and it is thought that the down-regulation of one or both HLA classes, by HRS cells may be one mechanism of immune escape for these tumours (Huang et al. 2010). An individual's HLA-type is genetically determined and may be one rationale for why family studies strongly suggest an inherited predisposition to cHL. The monozygotic twin of a patient with cHL is ninety nine times more likely to also develop the disease than the dizygotic twin of such a patient. In addition, around 4.5% of cHL patients have a history of other affected family members (Diepstra et al. 2005). A personal or family history of autoimmune disorders, which are associated with immune dysfunction and known to have a hereditary component, are also associated with an increased risk of lymphoma; both non-Hodgkin and Hodgkin, even in the absence of immunosuppressive drug use (Said 2007). This again supports a genetic link to the immune system and predisposition to cHL.

From epidemiological investigations it is recognised that cHL is seen less frequently in Asian than Caucasian populations, regardless of where individuals live (Diepstra et al. 2005). In one such study, an association between EBV-positive cHL and HLA class II expression by HRS cells was seen in Dutch patients, whereas in Chinese patients, HLA class II expression did not vary between EBV-positive and -negative cases and was less frequent in EBV-related disease than in the Dutch cohort (Diepstra et al. 2005). The authors concluded that this may be due to ethnic differences in the frequency of different HLA alleles, suggesting that in the Chinese population, HLA class II alleles, better at presenting EBV antigens to the immune system, may be more common and therefore these alleles are selected out for 'non-expression' by tumour cells. If such alleles are less frequent in the Dutch population, the selection pressure will be less and therefore more EBV-positive tumours will express HLA class II (Huang et al. 2010). In the Dutch population, lack of class II expression in EBV-positive disease was a poor prognostic marker but this did not seem to be the case in the Chinese group (Huang et al. 2010), again perhaps suggesting ethnic differences in the common HLA class II types, relevant to the immune handling of EBV (Diepstra et al. 2005).

Some HLA class I alleles are more common in those with cHL than in those who do not develop the disease and vice versa, again providing a rationale for ethnic variations and familial cases. There are also specific associations between certain HLA alleles and EBV-positive cHL (Huang et al. 2010; Diepstra et al. 2005; Tran et al. 2008; Hjalgrim et al. 2010). As above, it is thought that only some HLA class I alleles are able to present EBV antigens, such as LMP-1 and -2, to elicit a CTL response, and it has therefore been suggested that if an individual lacks any of these then he/she will be predisposed to develop EBV-positive cHL (Diepstra et al. 2005). Studies such as that of Diepstra in 2005 provide evidence to support this, having found an association between two HLA class I alleles and EBV-positive cHL. Fifty percent of those with EBV-positive disease expressed these two alleles as opposed to only 27% of those with EBV-negative cHL. The techniques used were limited to showing that these alleles were situated close to the HLA-A gene on chromosome six but could not conclusively show that the HLA-A gene and not a neighbouring one were responsible for the variations. The same study also identified a link between variations in the HLA class III subregion of chromosome six and a predisposition to cHL, both EBV-positive and -negative (Diepstra et al. 2005). Theoretically, differences in HLA-A could readily influence the immune handling of EBV, and therefore the risk of EBV-related cHL, as HLA-A in particular is known to play a key role in presenting viral antigens to the immune system. This would also support the hypothesis that the immune system's handling of EBV is an important factor in the pathogenesis of some cases of cHL. Other studies have also consistently shown a predisposition to EBV-positive cHL with certain HLA-A alleles. HLA-A*01 has an association with an increased risk of developing EBV-positive cHL and HLA-A*02 with a reduced risk (Hjalgrim et al. 2010). Also of interest is that these same HLA-A alleles are linked to the incidence of IM. IM occurs in the minority of people at the time of primary infection with EBV and there is an increased risk of developing EBV-related cHL in those who have had IM. The question is whether this is because of an altered immune response to primary EBV infection, which then predisposes to cHL, or whether it simply reflects an underlying genetic susceptibility to both disorders (Hjalgrim et al. 2010).

(2) Acquired factors and the immune response – co-morbid disease and ageing

Co-morbid disease can affect the immune system in many ways. For example, the presence of autoimmune disorders may reflect a fundamental defect in the immune system, but treatments, including immunosuppressants, may contribute further to the development of cHL. Other conditions, such as HIV, more obviously influence the immune system and increase the risk of developing malignancies but many other disorders and their treatments also have an effect (Jones 1990; Said 2007). This is exemplified by the finding that the frail elderly are more at risk of serious infections than the fit elderly (Jarrett et al. 2005; Jones 1990; Garibaldi & Nurse 1986). There is also significant epidemiological data to support the idea of immunosenescene, or an age-related decline in immune function as is seen in EBV-positive DLBCL of the elderly. Reactivation of infections like Tuberculosis and the Varicella Zoster Virus causing shingles, are more common with increasing age (Garibaldi & Nurse 1986) and ageing is also associated with an increased risk of autoimmune disorders and malignancy in general (Ademokun et al. 2010; Jarrett et al. 2005; Espino et al. 2012). Vaccinations are known to be less effective in the elderly who appear unable to mount the same immunological response (Ademokun et al. 2010), all of which suggests clinically significant changes in the immune system associated with ageing.

This epidemiological data is backed up by studies on a cellular and molecular level, not all of which agree on all aspects but there is consensus that the overall number of circulating B- and T-lymphocytes declines with age (Lakatta et al. 1990; Ferguson 1994; Rukavina et al. 1998; Sansoni et al. 1993; Utsuyama et al. 1992; Erkeller-Yuksel et al. 1992; Paganelli et al. 1992; Ademokun et al. 2010; Espino et al. 2012; Grubeck-loebenstein et al. 2009). The proportions of B- and T-cell subsets also change. $CD4^+$ cells decline less than $CD8^+$ T-cells (Utsuyama et al. 1992; Grubeck-loebenstein et al. 2009); naïve B- and T-cells reduce while memory and effector cells of both subtypes increase, with an increase in activated T-cells, T-reg and T-suppressor cells (Rukavina et al. 1998; Sansoni et al. 1993; Utsuyama et al. 1992; Erkeller-Yuksel et al. 1992; Espino et al. 2012; Grubeck-loebenstein et al. 2009). Functional studies suggest that T- and B-cell function is also affected detrimentally with age. Although activated T-cells are present, T-cell proliferation is reduced, perhaps due in part to lower Il-2 production with age (Lakatta et al. 1990; Ferguson 1994; Rukavina et al. 1998; Bloom 1991). Perforin and Granzymes, required for T- and NK-cell cytotoxicity, are also reduced with age and may contribute to poorer immune function (Rukavina et al. 1998; Bloom et al. 1990; Horvath et al. 1992; Kägi et al. 1994). Impaired CTL function may also be contributed to by reduced $CD4^+$ T_H cell function (Bloom 1991; Grubeck-loebenstein et al. 2009). In large part, the involution of the thymus, which starts after adolescence, is thought to be responsible for the reduction in absolute T-cell numbers (Lakatta et al. 1990; Sansoni et al. 1993; Utsuyama et al. 1992; Espino et al. 2012; Grubeck-loebenstein et al. 2009). In addition, oxidative damage and telomere shortening leave cells of all subtypes more prone to apoptosis, (Espino et al. 2012; Kaszubowska 2008) and both are well recognised processes in ageing.

The increased incidence of autoimmune disorders, monoclonal gammopathies (MGUS) and Chronic Lymphocytic Leukaemia (CLL) with age, particularly support the concept of reduced B-cell function (Paganelli et al. 1992). Although B-lymphocyte numbers fall with ageing, an increase in some immunoglobulin subtypes is seen, namely IgA and IgG (Rukavina et al. 1998; Sansoni et al. 1993; Paganelli et al. 1992). This may be linked to the increased number of memory B-cells but other factors may be important such as an altered sensitivity to T-cell signals (Paganelli et al. 1992) or poorer B-cell selection and increased production of low-affinity antibodies (Ademokun et al. 2010). Somatic recombination of immunoglobulin genes and an individual B-cell's ability to switch production between different sub-classes of immunoglobulin is important for the maintenance of the full repertoire of antigen-antibody responses and when this is impaired, as may occur in ageing, the affinity of antibodies produced is impaired (Grubeck-loebenstein et al. 2009). B-cell function and antibody production, is also inter-dependent on T-cell help and FDC, in particular. The effects of ageing on these cells will also therefore have consequences for the humoral immune response (Ademokun et al. 2010). For example, normally FDC present antigen to selected B-cells within the germinal centre and are important for selecting out those B-cells with the highest affinity B-cell receptors (BCR) for the antigen in question. Mouse studies, show that with increasing age, FDC provide a poorer proliferative stimulus to B-cells, fewer germinal centres are formed and they bind fewer immune complexes (Ademokun et al. 2010). Again, in mouse studies, there is evidence that some B-cell specific genes produce proteins with reduced function as age increases. The PAX5 gene and its corresponding protein product, BSAP have been studied. In ageing mice, although levels of PAX5 RNA are maintained, the BSAP protein is less efficient at binding target DNA and triggering transcription, resulting in impaired B-cell differentiation (Ademokun et al. 2010).

Ageing also has an effect on the innate immune response. There is conflicting data but NK-cells may be present in higher numbers in the elderly. Despite this, functional NK activity is reduced (Rukavina et al. 1998; Sansoni et al. 1993;

Utsuyama et al. 1992; Espino et al. 2012; Grubeck-loebenstein et al. 2009; Kaszubowska 2008), perhaps in part due to poorer Perforin and/or Granzyme production (Rukavina et al. 1998). Some suggest neutrophil numbers are reduced with age and others show no change but studies agree that neutrophil function is impaired in the elderly (Espino et al. 2012; Grubeck-loebenstein et al. 2009; Kaszubowska 2008). Macrophage, monocyte and dendritic cells (DC) may all be reduced in number and function with increasing age (Ademokun et al. 2010; Grubeck-loebenstein et al. 2009; Kaszubowska 2008).

With regard to cHL itself, young children tend to show a T_{H1}/CTL profile within the tumour microenvironment, whereas older children (ten years and above), have a T-reg/T_{H2} profile (Barros, Vera-Lozada, et al. 2012). Other data suggests that older cHL patients have an increased number of $CD4^+$ and fewer $CD20^+$ cells within tumours (Barros et al. 2011). Whether this simply reflects the changing pattern of peripheral blood lymphocytes with age is unclear as it is recognised that children have higher numbers of circulating $CD20^+$ and fewer $CD4^+$ cells than adults (Barros et al. 2011).

One hypothesis regarding the differing rates of NSHL and MCHL according to age, and varying rates of EBV-positive and -negative subtypes in different communities, is that these subtypes reflect different immune responses to EBV, or other causative agents of cHL (Barros et al. 2011).

(3) Effects of EBV on the immune system

EBV is a highly immunogenic virus, illustrated by the clinical syndrome of IM seen in some patients. Symptoms of IM are largely due to the number of cytokines released by activated CTL combatting the virus, and after exposure an individual retains virus-specific T-cells lifelong. However, these are not sufficient to eliminate EBV which employs a number of mechanisms to evade the immune system (see appendix B).

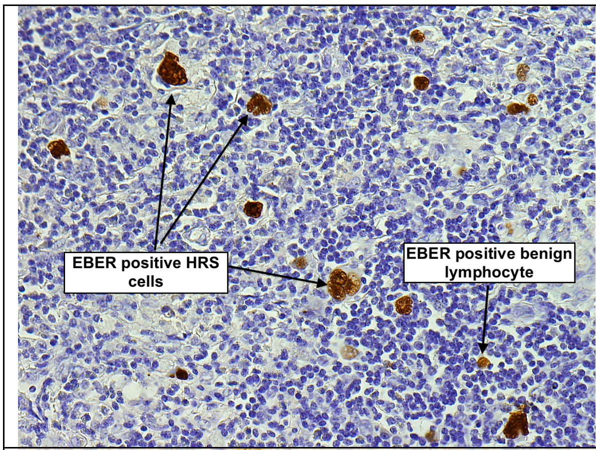
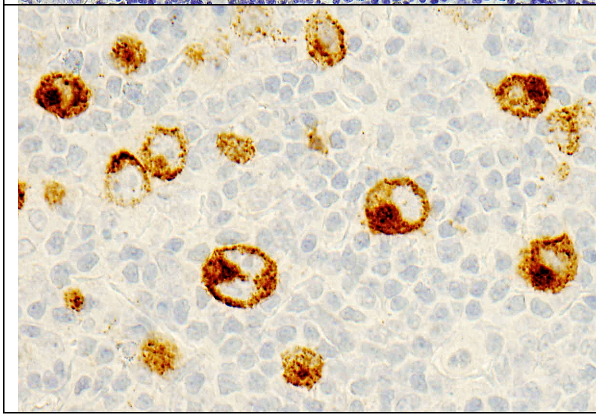
In addition to this, there is evidence of local immunosuppression within the tumour microenvironment of EBV-positive cHL. Tumour infiltrating lymphocytes in EBV-positive disease do not exhibit an EBV-specific response, whereas those from EBV-negative tumours do (Merlo et al. 2010), presumably because the majority of patients will have been exposed to EBV even if they have EBV-negative cHL. Although the presentation of antigens by MHC class I and II molecules is impaired by EBV, a number of studies have shown that the expression of MHC class I and II molecules is increased in EBV-positive HRS cells when compared to EBV-negative cHL (Huang et al. 2010; Lee et al. 1998). One hypothesis is that LMP 1 may up-regulate MHC class I and 'Transporter associated with Antigen Processing' (TAP) genes (Lee et al. 1998). In theory this should increase the vulnerability of EBV-positive HRS cells to both class I restricted CTL and class II restricted T_H cell responses, but this does not evidently occur. It is possible that HLA class I expression could be advantageous to the tumour by inhibiting NK-cell mediated cytotoxicity (Huang et al. 2010; Van Den Broek et al. 1995; Campoli & Ferrone 2008). If this is the case then NK-cell cytotoxicity might be expected to be more pronounced in cHL tumours lacking HLA class I expression (primarily EBV-negative cases), but this does not appear to be so and there may be an explanation for this. HLA-G expression, a non-classical MHC class I molecule, is strongly associated with EBV-negative and otherwise HLA class I non-expressing cHL. HLA-G activates inhibitory receptors on NK-cells as well as some T-cells, DCs and macrophages and may therefore be an alternative mechanism for these tumours to evade the innate immune system (Diepstra et al. 2008; Campoli & Ferrone 2008).

Also suggestive of an altered immune response in EBV-related disease is the finding that there are different immune markers of prognosis in EBV-positive and -negative cHL. Although not a universal finding (see below) and perhaps varying with age, in some studies EBV-positive disease with an increased number of T-reg cells has been found to carry a poor prognosis, whereas this is not the case in EBV-negative disease. Conversely, increased CTL are a poor prognostic marker in EBV-negative but not in EBV-positive disease (Barros, Vera-Lozada, et al. 2012).

Gene expression profiling studies have found a number of differences between EBV-positive and -negative cHL. Many more genes are up-regulated in EBV-positive compared to EBV-negative disease, including IFN-G and IFN-G inducible genes such as CXCL10 and 9, as well as TLR-1, TLR-8 and OAS which are all important in mounting an antiviral response. Activated T-cell genes are also overexpressed, as are macrophage-associated genes including CD68, CD163 and STAT 1 (Chetaille et al. 2009). Previously it was thought that the microenvironment in cHL is predominantly a T_{H2} one (Skinnider & Mak 2002) but this study suggests that in EBV-related cHL there is a predominantly T_{H1} environment which theoretically reflects an antiviral response. This might therefore lead to expectations of an improved outcome for patients with EBV-positive disease, but in this study of children and adults this is not borne out clinically (Chetaille et al. 2009). Using IHC, it can be seen that EBV-positive cHL tumours contain more $CD4^+$ cells than EBV-negative (Tran et al. 2008). CXCL10 and CCL20, increased in EBV-positive cHL, are chemotactic for T-reg cells but CXCL10 is also chemotactic for activated T-cells (Baumforth et al. 2008; Kamper et al. 2011). Although higher numbers of T-reg cells might be expected to suppress the immune response against tumour cells, resulting in a poorer outcome as has been seen in some studies of EBV-positive cHL (Barros, Vera-Lozada, et al. 2012), a larger number of studies looking at both EBV-positive and-negative disease have not found this. This may be due to the use of different antibodies identifying different groups of T-reg cells, or may reflect differences between EBV-positive and -negative cHL or variations according to age, but in these IHC studies, the combination of fewer T-reg cells (FOXP3 positive) and higher numbers of TIA-1 positive cells was linked to a poorer outcome (Chetaille et al. 2009; Álvaro et al. 2005; Alvaro-Naranjo et al. 2005; Kelley et al. 2007; Tzankov et al. 2008).

The role of EBV in cHL is therefore of great interest and may show us much about how the immune system is implicated in the disease itself and potentially how it may be manipulated to improve outcome. Clear changes in the immune system with age provide a rationale for why EBV-positive cHL may be more common in different age groups and why it may carry a different prognostic significance for those patients.

In order to investigate the immune system in cHL further, and how it may vary according to age, a number of different cell-types were examined in the tumours of each cohort. The first of these were macrophages.

	**Figure 5.3** **EBER-ISH** **Tumour 12, Young cohort, MCHL (x20)** **EBV-positive HRS cells and benign surrounding EBV-positive lymphocytes**
	**Figure 5.4** **LMP-1 IHC** **Tumour 12, Young cohort, MCHL (x40)** **EBV-positive HRS cells expressing LMP-1**

Chapter 6

Macrophages in classic Hodgkin Lymphoma

Introduction

As discussed in chapter one, macrophages in malignancy have been of particular interest since the 1970's and this includes their role in Hodgkin's Lymphoma. This study was designed to examine the following factors in relation to macrophage numbers in cHL:

- Age

  A number of studies have identified a variation in macrophage numbers in cHL, according to age (Colby et al. 1981; Ree & Kadin 1985; Steidl, Lee, et al. 2010; Tzankov et al. 2010; Tan et al. 2012). To clarify the nature of any variation, tissues from the two cohorts described earlier, (patients aged between 15 and 34 years, and patients aged over 60 years) were examined by IHC.

- EBV

  EBV is an important aetiological factor in around 40% of cHL (Jarrett et al. 1996; Stark et al. 2002; Armstrong et al. 1998; Shimoyama et al. 2008; Chetaille et al. 2009). The number of tumour infiltrating macrophages differs between EBV-positive and -negative cases (Azambuja et al. 2012; Tan et al. 2012). In addition, the incidence of EBV-related cHL varies according to age (Tan et al. 2012; Horstman et al. 1996).

- cHL Histological Subtype

  The histological subtype of cHL with current treatments, is not evidently of prognostic significance but there are variations in macrophage numbers according to these classifications (Colby et al. 1981; Ree & Kadin 1985; Tzankov et al. 2010; Tan et al. 2012). There are also variations in the frequency of different subtypes according to age.

- Gender

  Being male is a poor prognostic marker in cHL (Hasenclever & Diehl 1998) and there also appears to be a difference in the number of patients with EBV-related disease, according to gender (Tan et al. 2012; Huang et al. 2010; Küppers 2009b; Gandhi et al. 2004; Barros et al. 2011).

Materials and Methods

Two different antibodies were selected to identify potentially different functional groups of macrophages; CD163 and CD68. CD68 is thought to be a generic marker of all macrophages and CD163 labels a subtype of macrophages thought to be of an 'alternatively activated' (M2) phenotype. (Figures 6.1 to 6.3).

Immunohistochemistry and cell enumeration were carried out as described in chapter two. P-values were calculated using the Mann-Whitney test unless stated otherwise.

Results

All 'cell numbers' quoted in the following sections relate to the number of cells, CD68-positive or CD163-positive, measured as the median number of cells per mm^2 of tumour. The ratio of CD163:CD68 refers to the median number of CD163-positive cells per mm^2, divided by the median number of CD68-positive cells per mm^2. The term 'ratio' is used as a shortened version referring to the ratio of CD163:CD68 cells as described above. The phrases 'CD68 cells' and 'CD68-positive cells' are used interchangeably as are the same phrases for CD163.

CD68 by age and gender

The median number of CD68-positive cells per mm^2 was greater in the elderly than the young ($p=<0.0001$). This remained true when elderly males were compared to young males ($p=<0.001$) and elderly females compared with young females ($p=<0.0001$). When the two genders were compared within each cohort, there was no difference between the median cell count between elderly males and females but young males had significantly more CD68 cells than young females ($p=<0.05$) (table 6.1).

Table 6.1
CD68-positive macrophages and gender

	Elderly + Young Combined Median cell count per mm^2	**Young** Median cell count per mm^2	**Elderly** Median cell count per mm^2	**Young v Elderly**
All cases (n) (range)	688 (155) (48-1968)	544 (79) (192-1336)	824 (76) (48-1968)	p=<0.0001
Male (n)	752 (72)	612 (36)	856 (36)	p=<0.001
Female (n)	608 (77)	456 (43)	796 (34)	p=<0.0001
Male v Female	p=<0.05	p=<0.05	p=NS	

CD163 by age and gender

The median number of CD163-positive cells per mm^2, was not significantly different when all elderly were compared with all young. Similarly, no difference was evident between elderly males and young males, but elderly women did have significantly higher numbers of CD163 cells in comparison to young women (p=<0.05). When the two genders were compared within each cohort, as for CD68, young males had significantly higher numbers of CD163-positive cells compared to young females (p=<0.01) but no difference was seen between the genders in the elderly group (table 6.2).

Table 6.2
CD163-positive macrophages and gender

	Elderly + Young cohorts combined Median cell count per mm^2	**Young cohort** Median cell count per mm^2	**Elderly cohort** Median cell count per mm^2	**Young v Elderly**
All cases (n) (range)	32 (150) (0-1216)	32 (76) (0-1216)	68 (74) (0-808)	p=NS
Male (n)	64 (70)	56 (34)	68 (36)	p=NS
Female (n)	16 (75)	16 (42)	48 (33)	p=<0.05
Male v Female	p=<0.05	p=<0.01	p=NS	

CD163:CD68 ratio by age and gender

The ratio of CD163:CD68 was significantly higher in young men than in young women (p=<0.05), but with all other comparisons there were no significant differences (table 6.3).

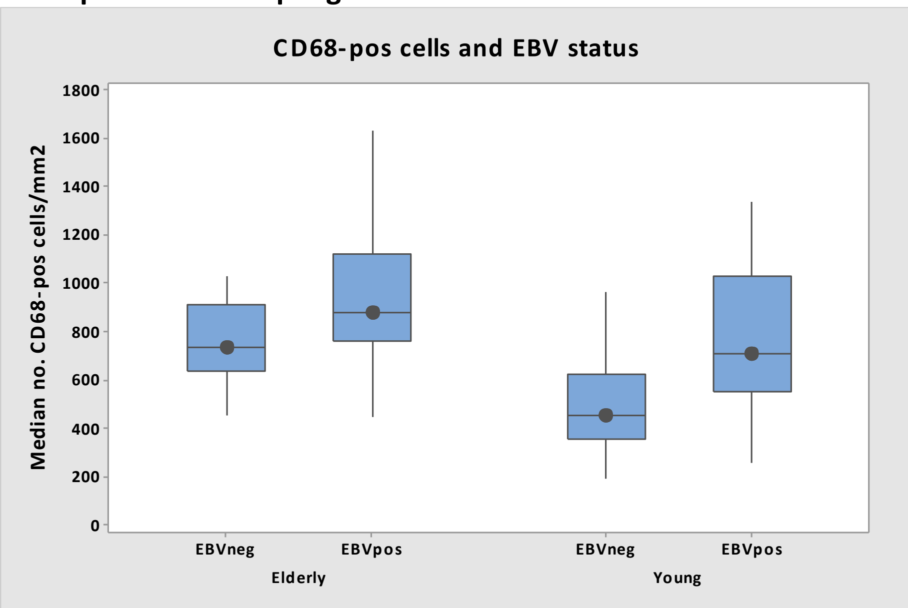
Table 6.3
CD163:CD68 ratio and gender

	Elderly + Young cohorts combined Ratio of cell counts	**Young cohort** Ratio of cell counts	**Elderly cohort** Ratio of cell counts	**Young v Elderly**
All cases (n) (range)	0.070 (146) (0.00-1.143)	0.053 (73) (0.00-1.143)	0.089 (73) (0.00-1.031)	p=NS
Male (n)	0.085 (68)	0.100 (33)	0.070 (35)	p=NS
Female (n)	0.028 (73)	0.013 (40)	0.097 (33)	p=NS
Male v Female	p=NS	p=<0.05	p=NS	

CD68 by EBV status

In both cohorts, the EBV-positive tumours contained significantly more CD68 cells than the EBV-negative tumours (elderly: p=<0.05; young: p=<0.01) (figure 6.4).

Figure 6.4
CD68-positive macrophages and EBV



(Elderly p=<0.05, Young p=<0.01)

The EBV-negative tumours from elderly (both male and female) patients contained more CD68-positive cells than the EBV-negative tumours from young patients (p=<0.001) but there was no significant difference in the number of CD68 cells between EBV-positive elderly and young cases (table 6.4).

Table 6.4

CD68-positive macrophages and EBV

	Elderly + Young combined Median cell count per mm^2	**Young** Median cell count per mm^2	**Elderly** Median cell count per mm^2	**Young v Elderly**
EBV pos (n)	872 (61)	712 (20)	880 (41)	p=NS
EBV neg (n)	608 (94)	456 (59)	736 (35)	p=<0.0001
EBV pos v neg	p=<0.0001	p=<0.01	p=<0.05	

The number of CD68 cells was greater in tumours from young males with EBV-positive disease when compared to young males with EBV-negative disease (p=<0.01) but there was no significant difference in the number of CD68 cells when a similar comparison was made for young females. Other comparisons were not significant (table 6.5).

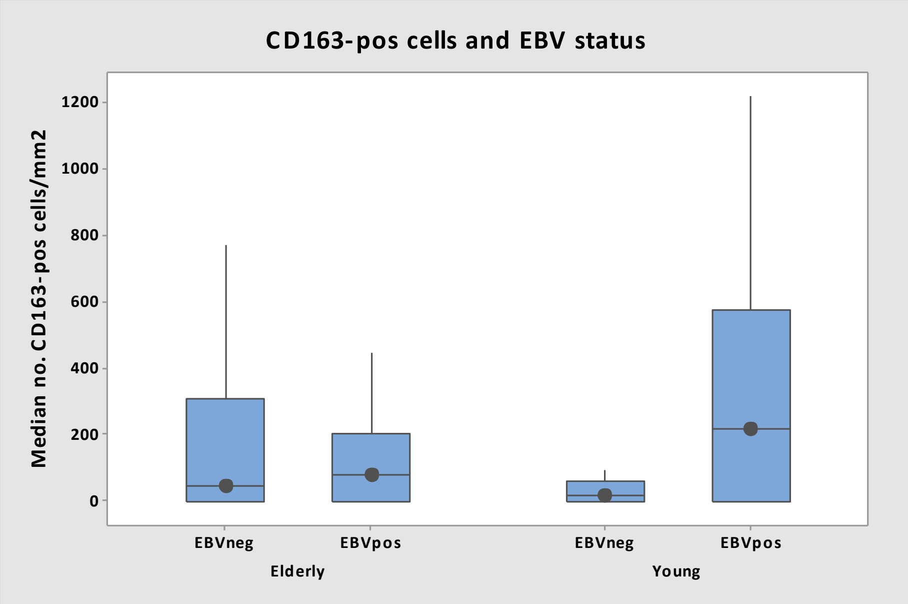
Table 6.5
EBV, gender and CD68-positive macrophages

	Elderly + Young combined Median cell count per mm^2		**Young** Median cell count per mm^2		**Elderly** Median cell count per mm^2		**Young v Elderly**	
EBV status	**Pos**	**Neg**	**Pos**	**Neg**	**Pos**	**Neg**	**Pos**	**Neg**
Male (n)	872 (35)	640 (37)	736 (15)	496 (21)	888 (20)	808 (16)	p=NS	p=<0.001
	p=<0.01		p=<0.01		p=NS			
Female (n)	880 (21)	596 (56)	512 (5)	456 (38)	892 (16)	696 (18)	p=NS	p=0.0001
	p=<0.001		p=NS		p=0.065			
Male v Female	p=NS	p=NS	p=NS	p=NS	p=NS	p=NS		

CD163 by EBV status

Only in the young and combined cohorts, did EBV-positive tumours contain significantly more CD163-positive cells than EBV-negative tumours (young, p=<0.01; combined, p=<0.05). No significant difference was seen between EBV-positive and -negative tumours in the elderly cohort (figure 6.5, table 6.6).

Figure 6.5
CD163-positive macrophages and EBV



(Elderly p=NS, Young p= <0.01)

There was a trend suggesting EBV-negative tumours of the elderly contained more CD163 cells than EBV-negative tumours of the young, but this was not statistically significant (table 6.6).

Table 6.6
CD163-positive macrophages and EBV

	Elderly + Young combined Median cell count per mm^2	**Young** Median cell count per mm^2	**Elderly** Median cell count per mm^2	**Young v Elderly**
EBV pos (n)	92 (60)	216 (19)	80 (41)	p=NS
EBV neg (n)	32 (90)	16 (57)	48 (33)	p=0.07
EBV pos v neg	p=<0.05	p=<0.01	p=NS	

The tumours of young males with EBV-positive disease, contained a greater median number of C163-positive cells than those of young males with EBV-negative disease (p=<0.05). CD163 cells were also more numerous in the tumours of EBV-positive young males compared with those of EBV-positive elderly males (P=<0.05). No other significant differences were evident between the genders or age groups (table 6.7).

Table 6.7
EBV, gender and CD163-positive macrophages

	Elderly + Young combined Median cell count per mm²		**Young** Median cell count per mm²		**Elderly** Median cell count per mm²		**Young v Elderly**	
EBV status	**Pos**	**Neg**	**Pos**	**Neg**	**Pos**	**Neg**	**Pos**	**Neg**
Male (n)	88(35)	40(35)	320(14)	36(20)	80(21)	56(15)	p=<0.05	p=NS
	p=NS		p=<0.05		p=NS			
Female (n)	48(21)	16(54)	0 (5)	16(37)	72(16)	48(17)	p=NS	p=0.06
	p=NS		p=NS		p=NS			
Male v Female	p=NS	p=NS	p=NS	p=NS	p=NS	p=NS		

CD163:CD68 ratio by EBV status

In the young, EBV-positive tumours had a significantly higher ratio than EBV-negative tumours (p=<0.05) but there was no evident significant difference in ratio when comparing EBV-positive to EBV-negative tumours in the elderly (table 6.8).

Table 6.8
CD163:CD68 ratio and EBV

	Elderly + Young cohorts combined Ratio of cell counts	**Young cohort** Ratio of cell counts	**Elderly cohort** Ratio of cell counts	**Young v Elderly**
EBV pos (n)	0.107 (59)	0.338 (19)	0.097 (40)	P=0.06
EBV neg (n)	0.047 (87)	0.041 (54)	0.070 (33)	P=NS
EBV pos v neg	P=NS	P=<0.05	P=NS	

When EBV-positive/-negative tumours were examined by gender, it was shown that only the ratio in EBV-positive tumours of young males was significantly greater than that in EBV-negative tumours of young males (p=<0.05). Other comparisons were not significant (table 6.9).

When EBV-positive tumours in all elderly were compared to those in the young, the ratio was higher in the young EBV-positive, but not significantly so (p=0.06) (table 6.8). When the EBV-positive tumours of young males were compared to the EBV-positive tumours of elderly males, the difference in ratio was significantly higher in the young group (p=<0.05) (table 6.9).

Table 6.9
EBV, gender and CD163:CD68 ratio

	Elderly + Young combined Ratio of cell counts		Young Ratio of cell counts		Elderly Ratio of cell counts		Young v Elderly	
EBV status	**Pos**	**Neg**	**Pos**	**Neg**	**Pos**	**Neg**	**Pos**	**Neg**
Male (n)	0.103 (34)	0.075 (34)	0.364 (14)	0.079 (19)	0.079 (20)	0.070 (15)	p=<0.05	p=NS
	p=NS		p=<0.05		p=NS			
Female (n)	0.063 (21)	0.027 (52)	0.000 (5)	0.026 (35)	0.093 (16)	0.097 (17)	p=NS	p=NS
	p=NS		p=NS		p=NS			
Male v Female	p=NS	p=NS	p=NS	p=NS	p=NS	p=NS		

CD68 by cHL subtype

In elderly patients the number of CD68 cells per mm^2 was significantly greater when compared to the young, in both NSHL and MCHL subtypes (MCHL, p=0.05; NSHL, p=<0.0001), but no significant difference was seen between the elderly and young when only 'cHL other' tumours were considered. In the young and combined cohorts, MCHL tumours contained a significantly greater number of CD68 cells than NSHL tumours (p=<0.01) but in the elderly cohort there was no significant difference between the two subtypes (table 6.10).

Table 6.10
CD68-positive macrophages and cHL subtype

	Elderly + Young combined Median cell count per mm^2	Young Median cell count per mm^2	Elderly Median cell count per mm^2	Young v Elderly
NSHL (n)	624 (97)	460 (58)	768 (39)	p=<0.0001
MCHL (n)	840 (41)	656 (13)	892 (28)	p=0.05
cHL, other (n)	608 (17)	572 (8)	800 (9)	p=NS
NSHL v MCHL	p=0.0001	p=<0.01	p=NS	

Looking at the possible implications of EBV in the old and young cohorts, numbers of CD68-positive cells were greater in EBV-positive NSHL tumours when compared with EBV-negative NSHL but this only reached significance when both cohorts were combined (p=<0.001). The number of CD68 cells did not differ between EBV-positive MCHL and EBV-negative MCHL in any group. When 'cHL other' tumours were considered, numbers of CD68-positive cells were greater in those that were EBV-positive when compared to those that were EBV-negative. This was seen in all groups but only reached significant levels when both cohorts were combined (p=<0.05) (table 6.11).

In all groups, the number of CD68-positive cells was higher in EBV-negative MCHL cases than it was in EBV-negative NSHL but only in the elderly and combined groups was this difference statistically significant (elderly, p=<0.05; combined, p=<0.01) (table 6.11).

When the EBV-negative NSHL tumours from the elderly were compared with the young EBV-negative cases of NSHL, and the same comparison made for EBV-negative MCHL, CD68-positive cells were more frequent in the elderly (NSHL, p=<0.0001; MCHL, p=0.01). For 'cHL other', EBV-positive cases in the elderly had significantly more CD68 cells than EBV-positive tumours in the young (p=0.05). Other comparisons were not significant (table 6.11).

Table 6.11
EBV, cHL subtype and CD68-positive macrophages

	Elderly + Young combined Median cell count per mm^2		**Young** Median cell count per mm^2		**Elderly** Median cell count per mm^2		**Young v Elderly**	
EBV status	**Pos**	**Neg**	**Pos**	**Neg**	**Pos**	**Neg**	**Pos**	**Neg**
NSHL (n)	928(24)	608(73)	968(8)	456(50)	888(16)	704(23)	p=NS	p=<0.0001
	p=<0.001		p=NS		p=NS			
MCHL(n)	856(30)	704(11)	736(9)	616 (4)	872(21)	944 (7)	p=NS	p=0.01
	p=NS		p=NS		p=NS			
cHL other(n)	776 (7)	476(10)	600(3)	384(5)	1160(4)	496 (5)	p=0.05	p=NS
	p=<0.05		p=NS		p=0.066			
NSHL v MCHL	p=NS	p=<0.01	p=NS	p=NS	p=NS	p=<0.05		

CD163 by cHL subtype

NSHL tumours from the elderly cohort had greater numbers of CD163-positive cells when compared to those from the young (p=<0.05) but no significant difference was seen between the age groups regarding MCHL or 'cHL other' subtypes. In the young cohort, MCHL tumours had a greater number of CD163 cells than NSHL tumours (p=<0.05) but in the elderly cohort there was no significant difference between the two subtypes (table 6.12).

Table 6.12
CD163-positive macrophages and cHL subtype

	Elderly + Young combined Median cell count per mm^2	**Young** Median cell count per mm^2	**Elderly** Median cell count per mm^2	**Young v Elderly**
NSHL (n)	32 (95)	16 (57)	84 (38)	p=<0.05
MCHL (n)	80 (41)	200 (13)	80 (28)	p=NS
cHL, other (n)	0 (14)	148 (6)	0 (8)	p=0.06
NSHL v MCHL	p=<0.05	p=<0.05	p=NS	

The EBV-positive NSHL tumours in the old and young combined, were seen to contain significantly more CD163 cells than EBV-negative cases (p=<0.05). No significant differences between EBV-positive and –negative MCHL were found in any cohort but EBV-negative MCHL compared to EBV-negative NSHL appeared to demonstrate, in all cohorts, a greater number of CD163 cells. This however, did not reach statistical significance (combined goup, p=0.06) (table 6.13).

In the EBV-positive MCHL tumours of the young there was a greater number of CD163-positive cells than in the equivalent elderly group (p=0.05) (table 6.13).

Table 6.13
EBV, cHL subtype and CD163-positive macrophages

	Elderly + Young combined Median cell count per mm^2		**Young** Median cell count per mm^2		**Elderly** Median cell count per mm^2		**Young v Elderly**	
EBV status	**Pos**	**Neg**	**Pos**	**Neg**	**Pos**	**Neg**	**Pos**	**neg**
NSHL (n)	104(24)	16(71)	64 (8)	16(49)	112(16)	32(22)	p=NS	p=NS
	p=<0.05		p=NS		p=NS			
MCHL(n)	108(30)	56(11)	256(9)	40 (4)	80 (21)	64 (7)	p=0.05	p=NS
	p=NS		p=NS		p=NS			
cHL other (n)	0 (6)	72 (8)	308(2)	148(4)	0 (4)	24 (4)	p=NS	p=NS
	p=NS		p=NS		p=NS			
NSHL v MCHL	p=NS	p=0.06	p=NS	p=NS	p=NS	p=NS		

CD163:CD68 ratio by cHL subtype

NSHL and MCHL subtypes were compared in both the elderly and young cohorts, but only in the young was the ratio significantly higher in the MCHL than NSHL tumours (p=<0.05) (table 6.14).

Table 6.14
CD163:CD68 ratio and cHL subtype

	Elderly + Young combined Ratio of cell counts	**Young** Ratio of cell counts	**Elderly** Ratio of cell counts	**Young v Elderly**
NSHL (n)	0.054 (92)	0.038 (54)	0.101 (38)	p=NS
MCHL (n)	0.103 (41)	0.305 (13)	0.091 (28)	p=NS
cHL, other (n)	0 (13)	0.266 (6)	0.000 (7)	p=NS
NSHL v MCHL	p=NS	p=<0.05	p=NS	

When EBV-positive MCHL tumours in the young were compared with the same tumour group in the elderly, the young were found to have a significantly higher ratio of CD163:CD68-positive cells (p=<0.05). All other comparisons were not significant (table 6.15).

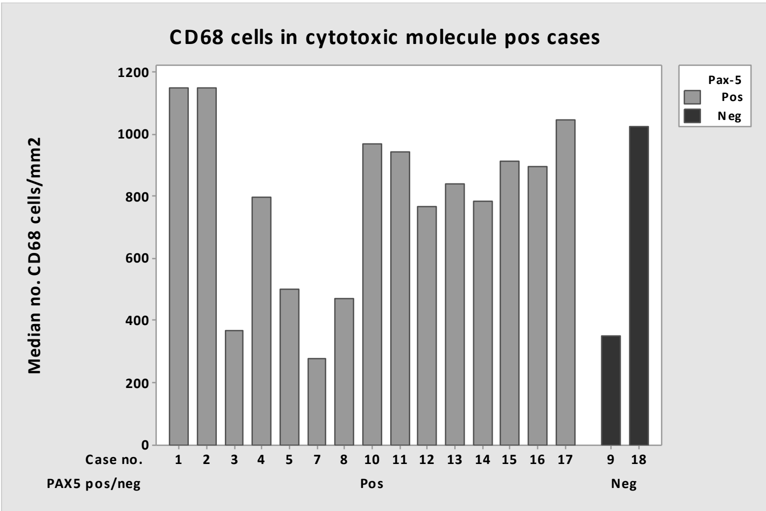
Table 6.15
EBV, cHL subtype and CD163:CD68 ratio

	Elderly + Young combined Ratio of cell counts		**Young** Ratio of cell counts		**Elderly** Ratio of cell counts		**Young v Elderly**	
EBV status	**Pos**	**Neg**	**Pos**	**Neg**	**Pos**	**Neg**	**Pos**	**Neg**
NSHL (n)	0.104 (24)	0.038 (68)	0.125 (8)	0.031 (46)	0.104 (16)	0.053 (22)	p=NS	p=NS
	p=NS		p=NS		p=NS			
MCHL (n)	0.131 (30)	0.079 (11)	0.390 (9)	0.063 (4)	0.092 (21)	0.080 (7)	p=<0.05	p=NS
	p=NS		p=NS		p=NS			
cHL other (n)	0.000 (5)	0.108 (8)	0.448 (2)	0.265 (4)	0.000 (3)	0.048 (4)	p=NS	p=NS
	p=NS		p=NS		p=NS			
NSHL v MCHL	p=NS	p=NS	p=NS	p=NS	p=NS	p=NS		

CD68, CD163 and cytotoxic molecule expression

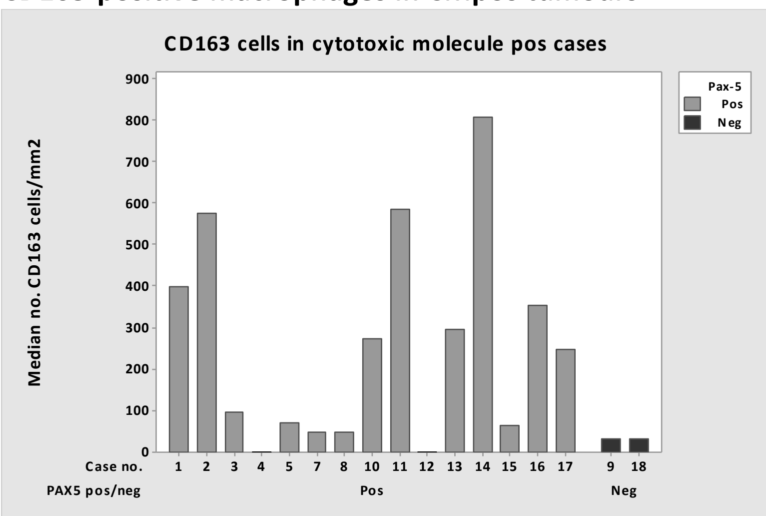
Macrophage numbers were compared between cases that did and did not express any of the three cytotoxic molecules, TIA-1, Granzyme B or Perforin. Of the 23 CMpos cases, data was available for the 17 tumours from the original cohorts (eight elderly, nine young), of which 15 co-expressed PAX5 and two did not. When the PAX5-positive and -negative CMpos cases were compared, there was no statistically significant difference in the median number of CD68 or CD163 cells (figures 6.6 & 6.7).

Figure 6.6
CD68-positive macrophages in CMpos tumours



CMpos PAX5 pos, median CD68=840; CMpos PAX5 neg, median CD68=688 (p=NS)

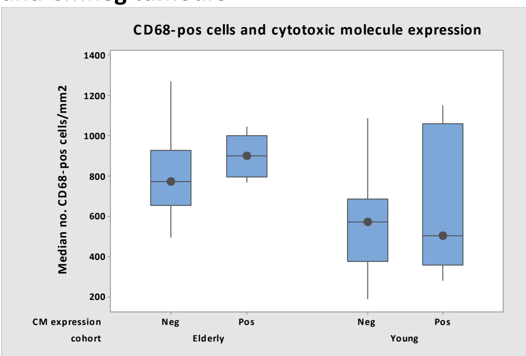
Figure 6.7
CD163-positive macrophages in CMpos tumours



CMpos PAX5 pos, median CD163=248; CMpos PAX5 neg, median CD163=32 (p=NS)

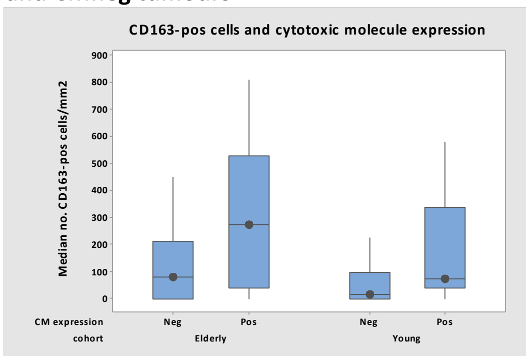
When all of the CMpos cases were compared with all of the CMneg cases, positive tumours had greater numbers of CD163 cells in all cohorts; young, elderly and combined. This difference was statistically significant for the young and combined cohorts (combined and young, p=<0.05). The median number of CD68 cells did not vary significantly between CMpos and CMneg tumours (figures 6.8 & 6.9, table 6.16).

Figure 6.8
CD68-positive macrophages in CMpos and CMneg tumours



(Elderly p=NS, Young p=NS)

Figure 6.9
CD163-positive macrophages in CMpos and CMneg tumours



(Elderly p=NS, Young p=<0.05)

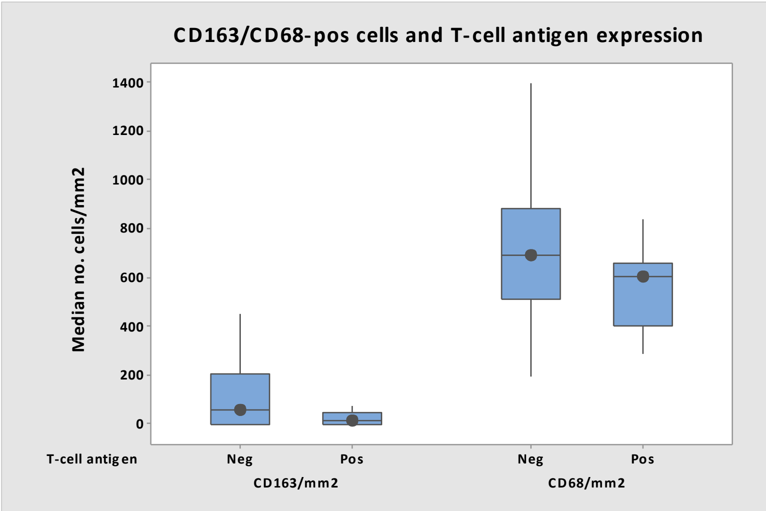
Table 6.16
CD68-cells, CD163-cells, CD163:CD68 ratio and cytotoxic molecule expression

	Elderly + Young Combined Median cell count per mm²/ratio of cell counts		**Young** Median cell count per mm²/ratio of cell counts		**Elderly** Median cell count per mm²/ratio of cell counts	
	CMpos	**CMneg**	**CMpos**	**CMneg**	**CMpos**	**CMneg**
CD68	840 (17)	636 (108)	504 (9)	576 (65)	904 (8)	776 (43)
	p=NS		p=NS		p=NS	
CD163	96 (17)	32 (104)	72 (9)	16 (63)	272 (8)	80 (41)
	p=<0.05		p=<0.05		p=NS	
CD163:CD68	0.237 (17)	0.064(101)	0.171 (9)	0.041 (60)	0.295 (8)	0.100 (41)
	p=<0.05		p=<0.05		p=NS	

CD68, CD163 and T-cell antigen expression

Comparisons were made between the 16 cases in which HRS cells expressed any of the T-cell antigens (CD2,3,4,5,7,8) and those that did not express T-cell antigens or any of the cytotoxic molecules. One case expressed CD3 in addition to all three cytotoxic molecules but this was excluded from these analyses. All other T-cell antigen-positive cases were CMneg. In each of the three groups; young, elderly and combined, the median number of both CD163- and CD68-positive cells was greater in those tumours that did not express T-cell antigens but this only reached statistically significant levels for CD68 in the combined group (p=<0.05) (figure 6.10).

Figure 6.10
CD68, CD163-positive cells and T-cell antigen expression, combined cohorts



(Combined cohorts: CD163 p=NS; CD68 p=<0.05)
[Median cell no's: CD163, T-cell pos=16 (n=16), T-cell neg=56 (n=81); CD68, T-cell pos=604 (n=16), T-cell neg=688 (n=83)]

CD68 and outcome

The four measures of outcome are detailed in chapter two, but briefly they are; 1) relapse of disease; 2) relapse of disease or escalation in treatment; 3) those who died; 4) those who died of cHL.

More CD68-positive cells were evident in the tumours of those who died of any cause than of those who lived. This was the case in all groups but only reached significance when both groups were combined (p=<0.01) (table 6.17).

Table 6.17
CD68-positive macrophages and outcome: death, any cause

	Elderly + Young combined Median cell count per mm^2	**Young** Median cell count per mm^2	**Elderly** Median cell count per mm^2	**Young v Elderly**
All dead (n)	840 (37)	616 (6)	848 (31)	p=NS
All not dead (n)	624 (117)	528 (72)	784 (45)	p=<0.0001
Dead v not dead	p=<0.01	p=NS	p=NS	

When those who died of cHL were considered, in the combined group, again CD68-positive cells were more frequent in those who died of cHL compared to those who did not (p=<0.05) (table 6.18).

Table 6.18
CD68-positive macrophages and outcome: death due to cHL

	Elderly + Young combined Median cell count per mm^2	**Young** Median cell count per mm^2	**Elderly** Median cell count per mm^2	**Young v Elderly**
All dead of cHL (n)	868 (18)	496 (3)	872 (15)	p=NS
All not dead of cHL (n)	656 (134)	544 (75)	816 (59)	p=<0.0001
Dead of cHL v not dead of cHL	p=<0.05	p=NS	p=NS	

In none of the cohorts, elderly, young or combined, did the number of CD68-positive cells differ between those who relapsed or did not, or between those who relapsed or required an escalation in treatment and those who did not.

The number of CD68 cells was greater in the elderly than the young, regardless of outcome, but only reached statistical significance in those with a better outcome (not dead, any cause and not dead from cHL, p=<0.0001). This remained so when only EBV-negative cases were included but was not so for the EBV-positive cases when examined separately (data not shown).

CD68 cell numbers did not differ according to stage of disease at presentation (I-IIA v IIB-IV), in any of the cohorts.

CD163 and outcome

When only EBV-negative tumours were considered, the number of CD163-positive cells was significantly higher in the elderly who did not relapse, compared to the elderly who did relapse (p=<0.05) (table 6.19). A similar trend was seen in the elderly when looking at those who relapsed or required an escalation in treatment compared to those who did not but this only reached significance when both the elderly and young groups were combined (p=<0.05) (table 6.20). In all of the comparisons between elderly and young cases of EBV-negative cHL, those elderly with a better outcome, according to all four measures, had a significantly higher number of CD163 cells than the EBV-negative young with the same outcome (tables 6.19 & 6.20).

Table 6.19
EBV-negative cases. CD163-positive macrophages and outcome: relapse

EBV-negative	Elderly + Young combined Median cell count per mm^2	Young Median cell count per mm^2	Elderly Median cell count per mm^2	Young v Elderly
EBV neg relapse (n)	16 (15)	16 (11)	0 (4)	p=0.058
EBV neg no relapse (n)	32 (74)	16 (46)	68 (28)	p=<0.01
Relapse v no relapse	p=NS	p=NS	p=<0.05	

Table 6.20
EBV-negative cases. CD163-positive macrophages and outcome: relapse or escalation in treatment (Rx)

EBV-negative	Elderly + Young combined Median cell count per mm^2	Young Median cell count per mm^2	Elderly Median cell count per mm^2	Young v Elderly
EBV neg relapse or escalation in Rx (n)	8 (20)	16 (15)	0 (5)	p=NS
EBV neg no relapse or escalation in Rx (n)	32 (65)	32 (42)	64 (23)	p=<0.05
Relapse or escalation in Rx v no relapse or escalation in Rx	p=<0.05	p=NS	p=NS	

CD163 cell counts did not differ significantly between those who died or did not (of any cause or of cHL), in any of the groups of EBV-negative cases.

In the EBV-positive tumours, CD163-positive cell numbers did not differ significantly according to outcome in any of the groups, elderly, young or combined. This was the case for all measures of outcome, except for the young regarding deaths due to cHL as the number in this last group was too small to allow any meaningful comparisons. However, in the EBV-positive cases it was the young with a good outcome who had a greater number of CD163 cells than the elderly with the same outcome, and in the relapse or escalation in treatment comparison this reached statistical significance (p=0.05) (table 6.21).

Table 6.21
EBV-positive cases. CD163-positive macrophages and outcome: relapse or escalation in treatment (Rx)

EBV-positive	**Elderly + Young combined** Median cell count per mm^2	**Young** Median cell count per mm^2	**Elderly** Median cell count per mm^2	**Young v Elderly**
EBV pos relapse or escalation Rx (n)	112 (18)	0 (3)	112 (15)	p=NS
EBV pos no relapse or escalation Rx (n)	80 (37)	256 (15)	32 (22)	p=0.05
Relapse or escalation in Rx v no relapse or escalation in Rx	p=NS	p=NS	p=NS	

CD163 cell numbers did not differ significantly according to stage of disease (I-IIA v IIB-IV) at presentation, in any of the cohorts.

CD163:CD68 ratio and outcome

When looking at the four outcome measures, there was no significant difference in ratio between those with a good or poor outcome in any of the cohorts, by any of the measures, (when EBV-positive and-negative cases were considered together) except for the young with relapsed disease/requiring an escalation in treatment. In this latter group there was a significantly higher ratio of cells in those who did not relapse or require an escalation in treatment (p=0.05), a difference not seen in the elderly (table 6.22).

Table 6.22
CD163:CD68 ratio and outcome: relapse or escalation in treatment (Rx)

	Elderly + Young combined Ratio of cell counts	**Young** Ratio of cell counts	**Elderly** Ratio of cell counts	**Young v Elderly**
All relapse/escalation Rx (n)	0.047 (36)	0.013(16)	0.104(20)	p=<0.05
All no relapse/escalation Rx (n)	0.079 (100)	0.079(56)	0.075(44)	p=NS
Relapse or escalation in Rx v no relapse or escalation in Rx	p=NS	p=0.05	p=NS	

When elderly were compared with young, the elderly with a poor outcome (by any measure) had a higher ratio than the young with a poor outcome but this only reached statistical significance for the relapse/escalation in treatment comparison (table 6.22).

Examining only EBV-negative tumours for outcome, the elderly without relapsed disease had a significantly higher ratio than those elderly who did relapse (p=0.01) (table 6.23). Similar trends were evident in the elderly with regard to those who did not relapse/require an escalation in treatment and in those elderly who did not die of cHL, but these did not reach statistical significance. No significant differences in ratio in relation to outcome were evident in the EBV-negative young cohort (table 6.23).

Table 6.23
EBV-negative cases. CD163:CD68 ratio and outcome: relapse

EBV-negative	**Elderly + Young combined** Ratio of cell counts	**Young** Ratio of cell counts	**Elderly** Ratio of cell counts	**Young v Elderly**
EBV neg relapse (n)	0.000 (13)	0.035 (9)	0.000 (4)	p=NS
EBV neg no relapse (n)	0.064 (73)	0.044 (45)	0.107 (28)	p=<0.05
Relapse v no relapse	p=<0.05	p=NS	p=0.01	

In the EBV-negative tumours, those elderly patients with a good outcome, had a higher ratio than those young with a good outcome, but this was only statistically significant in the relapse/no relapse comparison (p=<0.05) (table 6.23).

Looking at the same comparisons in outcome, but in EBV-positive tumours only, with all four outcome measures, the ratio of CD163:CD68 cells was higher in those elderly who did badly i.e. those who relapsed, relapsed/required an escalation in treatment, died of any cause or died of cHL (table 6.24). The number of patients with a poor outcome in each subgroup was small, and none of these differences in the elderly were statistically significant. In the young cohort, too few patients died to make comparisons meaningful. In the EBV-positive cases, the elderly with a good outcome had a significantly lower ratio than the comparable young group (p=<0.05). This was evident for all measures of outcome except for death due to any cause, where the difference was not significant (table 6.24).

Table 6.24
EBV-positive cases. CD613:CD68 ratio and outcome: relapse or escalation in treatment (Rx)

EBV-positive	**Elderly + Young combined** Ratio of cell counts	**Young** Ratio of cell counts	**Elderly** Ratio of cell counts	**Young v Elderly**
EBV pos relapse or escalation in Rx (n)	0.115 (18)	0.000 (3)	0.124 (15)	p=NS
EBV pos no relapse or escalation in Rx (n)	0.111 (36)	0.347(15)	0.063 (21)	p=<0.05
Relapse or escalation in Rx v no relapse or escalation in Rx	p=NS	p=NS	p=NS	

With regard to stage of disease at presentation, there was no significant difference in ratio between those presenting with stage I-IIA or IIB-IV disease in any of the cohorts or between the elderly and young.

Discussion

CD68-positive macrophages

Tumours from elderly patients had a greater number of CD68-positive cells per mm^2 than tumours from young patients. The elderly EBV-negative cases also had a greater number of CD68-positive cells than the young EBV-negative. However, the number of these cells in EBV-positive tumours did not differ significantly between the two age groups as the median number of CD68-cells in the young EBV-positive approached that seen in the elderly.

When the presence of EBV infection in the young was examined further, an apparent gender difference was seen. CD68 cells were more numerous in tumours from young males than young females, but significantly more young men than women had EBV-positive tumours (chapter five), which could account for this difference. To look at this, tumours of young EBV-positive men and women were compared, as were EBV-negative tumours, and it was seen that, in relation to EBV, CD68-positive cells were present in greater numbers in the tumours of young males but not so in young females. This therefore allows the suggestion that EBV-infection has an influence on the tumours of young males but not the same influence on those of young women, nor on the tumours of older patients of either gender. Accepting the limitations of group size, the data appears to show that not only are young males more likely to have EBV-positive disease than young women, but that EBV infection is associated with an increase in CD68 cell numbers in these young men but not in young women.

In the young cohort, MCHL tumours contained greater numbers of CD68-positive cells compared to NSHL tumours but this difference was not seen in the elderly cohort. This would be consistent with the finding that a greater proportion of cases of NSHL in the elderly were EBV-positive, whereas in the young, the majority of NSHL cases were EBV-negative and in both groups there was a strong association between EBV positivity and the MCHL subtype. Therefore any difference between MCHL and NSHL may simply reflect the differences in CD68 cell numbers already described in relation to EBV. Indeed, when EBV-positive NSHL was compared with EBV-positive MCHL, there was no difference between the two in any of the cohorts.

When each of the MCHL and NSHL subtypes were examined separately, and compared between the elderly and young cohorts, the differences between the age groups also largely appeared to reflect, as noted, the higher number of CD68 cells seen in the elderly and the association between the MCHL subtype and EBV positivity. However, when EBV-negative MCHL was compared with EBV-negative NSHL in all groups, there was a greater number of CD68-positive cells in the MCHL tumours and this difference reached statistical significance in the elderly and combined groups, although not in the young in whom there were very few EBV-negative MCHL tumours. This suggests that there is a tendency for MCHL tumours to contain greater numbers of CD68-positive cells than NSHL tumours, but only in EBV-negative cases.

CD163-positive macrophages

Unlike CD68, CD163-positive cell numbers were not significantly different in the elderly compared to the young although the tumours of elderly women contained more than those of young women. As with the CD68 cells, tumours of young males had greater numbers of CD163 cells than those of young women but no gender difference was seen in the elderly.

With EBV infection there was no evident difference in CD163 cell numbers between the elderly EBV-positive and -negative but, in the young, the EBV-positive tumours, compared to the EBV-negative, contained significantly more CD163-positive cells. When comparing young males with EBV-positive and EBV-negative disease, CD163-positive cell numbers were greater in the former, but with the same comparison between young females no difference was seen (although very few young women had EBV-positive disease). Equally in the elderly there was no difference in CD163 cell numbers between the EBV-positive and -negative tumours in either the men or the women.

As with CD68, there was a stronger association between EBV positivity and a greater number of CD163 cells, in young men than there was in any other group. This may therefore lie behind the gender difference in CD163 cell numbers noted between young men and women.

This observation may also explain some of the other differences noted between groups, including the finding that tumours of elderly patients with EBV-positive MCHL had fewer CD163-positive cells than the comparative young group. This may simply reflect that whilst EBV infection is associated with a microenvironment rich in CD163-positive cells in young men, no such association is seen in tumours of the elderly.

Again, differences between cHL subtypes, with regard to CD163 cell numbers, appear to be due to the association of the different subtypes with EBV-related disease more than the result of the subtypes themselves. The one exception to this may be MCHL, which, although not significantly so, did appear to have greater numbers of CD163-positive cells, even in EBV-negative cases. This was evidenced as a weak trend in the combined cohort but mirrored the significant differences seen in CD68 cell numbers in this group of tumours.

It appears that EBV-negative elderly cases who did well with regard to the four measures of outcome, had greater numbers of CD163-positive cells than the young EBV-negative cases classified in the same way. This also appears to reflect the earlier observation that suggested that elderly EBV-negative tumours might have greater numbers of CD163-positive cells than the young EBV-negative cases. This could also explain the observation that elderly NSHL tumours had greater numbers of CD163 cells than young NSHL tumours, as NSHL was more likely than other subtypes, to be EBV-negative.

CD163:CD68 ratio

The ratio of CD163:CD68-positive cells was also used to examine the cohorts, as these two immunohistochemical labels identify different subgroups of macrophages, the proportions of which may be as important as the absolute numbers of each. An increased ratio suggested a disproportionately increased number of CD163-positive cells compared to CD68, a reduction in CD68-positive cells or both. A reduced ratio suggested the opposite.

The lack of difference in ratio of CD163:CD68-positive cells, between the elderly and young cohorts shows that although CD68 cell numbers were greater in the elderly compared to the young, while CD163-positive cells were not significantly different, overall the proportion of CD163 and CD68-positive cells remained similar in both the elderly and young cohorts.

With age and gender, although it was shown that the number of CD68-positive cells was higher in the elderly than the young of either gender, and that CD163-positive cells were only significantly higher in elderly women, with no difference between elderly and young men, the ratio between old and young of either gender did not differ significantly. Proportionately, therefore, changes in CD68 and CD163-positive cells were not significant.

CD163 and CD68 cell numbers did not vary significantly between the elderly and young EBV-positive but the ratio did vary, with a trend towards a higher ratio in the young compared to the elderly. This reflects the finding that in the young, EBV positivity was associated with numbers of CD68 cells similar to those seen in the elderly but that CD163 cell numbers were only significantly higher in the young EBV-positive tumours and did not differ in the EBV-positive or-negative elderly cases. This would seem to be a likely explanation for the increased ratio seen in young EBV-positive males compared to elderly EBV-positive males and would explain the lack of difference for the same elderly and young comparison in EBV-negative tumours.

In young men the ratio was significantly higher than that in young women. Both CD68 and CD163 cell numbers were greater in the tumours of young men but this difference in ratio tells us that the increase in CD163 cells was proportionately greater than that of CD68-positive cells. This was not evident in the elderly, in whom there was also no difference in ratio between the EBV-positive and -negative so that although CD68 cells were more frequent in the EBV-positive tumours and C163 were not, this increase was insufficient to significantly alter the ratio. In the young however, the ratio in EBV-positive tumours was significantly higher than that in the EBV-negative cases. Therefore, although both CD68 and CD163 cell numbers were greater in young EBV-positive tumours, the apparent increase in CD163 was more marked. When this analysis also included gender, it was seen that the difference in ratios between EBV-positive and –negative cases was only evident in young men and was not apparent in young women. Again this illustrates that the link between

EBV and CD163 cells is greatest in young men, suggesting that the microenvironment associated with EBV-infection, in this group more than any other, favours the presence of CD163-positive macrophages.

Also as discussed, most of the differences evident between the subtypes of cHL appear to be linked to EBV infection which was found significantly more commonly in association with MCHL than with NSHL.

CD68, CD163, cytotoxic molecule and T-cell antigen expression

Cases of cHL in which HRS cells expressed any of the three cytotoxic molecules, TIA-1, Granzyme B or Perforin, had a significantly greater number of CD163 cells than those tumours that did not. Eight of the CMpos cases were from the elderly cohort and nine from the young and there was no evidence of an association between CM expression and the presence or absence of EBV infection. Therefore there were no obvious confounding factors to explain this association. When CMpos PAX5-negative cases were compared with the CMpos PAX5-positive, no significant difference in the number of CD68 or CD163 cells was found. However, the number of PAX5-negative cases was small and it is therefore not possible to exclude any associations.

When the T-cell antigen-positive cases were examined, they were found to have significantly fewer CD68 cells than the T-cell antigen-negative tumours. The number of cases was insufficient to look at this in more detail but as previously described (chapters four and five), there was a significant association between T-cell antigen expression and EBV-negative disease in young patients. Both of these factors are associated with the presence of fewer CD68 cells and the significance of each independently is not apparent on the basis of this study.

CD68, CD163-positive macrophages and outcome

In the combined group of elderly and young patients, the number of CD68-positive cells was greater in those who died, either of cHL or of any cause, when compared with those who did not. When outcome was measured looking at those who relapsed including or excluding those who required an escalation in treatment, compared to those who did not, no significant difference in the number of CD68-positive cells was evident in any group. Of those who died, a significantly greater proportion were from the elderly cohort, who would be expected to have greater numbers of CD68-positive cells when compared to the young. Of those who relapsed and/or required an escalation in treatment, the proportion from the elderly and young cohorts was very similar, in keeping with the lack of difference seen in CD68 cell numbers. These apparent differences between those who died or did not die may therefore be a reflection of the previously described age differences associated with CD68 cell numbers rather than a true difference according to outcome. This raises questions about any studies which link macrophage numbers to outcome and highlights the importance of recognising and accounting for confounding factors.

When EBV-negative and -positive tumours within each age group, were examined separately for each of the outcome measures, there was no convincing evidence that the number of CD68 or CD163 cells was linked to outcome. However, the small number of cases, particularly in the poor outcome groups, means it is not possible to exclude an association.

Comparisons were made between the elderly and young with regard to outcome and EBV infection. These appeared to show significantly more CD68 cells in elderly EBV-negative tumours of patients who did not relapse, require increased treatment or die (of any cause or cHL), when compared to the young and no difference between age groups and outcome in the EBV-positive patients or those patients who did less well. This underscores the differences in CD68 cell numbers already described in relation to age. EBV-negative tumours of the elderly had greater numbers of CD68-positive cells than EBV-negative tumours of the young and while the number of cases is probably too small to show this in the poor outcome groups, it is evident in the larger groups of patients who did well.

Similarly, when all EBV-negative elderly with a good outcome (by any measure) were compared with all EBV-negative young with a good outcome, the elderly consistently had significantly greater numbers of CD163 cells than the young. When EBV-positive cases were analysed, the young patients with a good outcome had greater numbers of CD163 cells than the elderly with a good outcome but this was only significant with regard to the relapse or no relapse comparison. These observations may simply be a further reflection of the young mounting a more marked CD163 response to EBV than the elderly.

Although the number of CD163 cells was not evidently linked to outcome, there were some findings to suggest that CD163-positive cells may play a different role in the two age groups and in EBV-positive compared to EBV-negative disease. An examination of EBV-negative cases appeared to show that in the elderly, those who did better tended to have a greater number of CD163 cells than those with a poor outcome. This was statistically significant with regard to relapse or no relapse. The same was not evident in the EBV-positive cases in the elderly.

These possible differences were perhaps more clearly seen when the CD163:CD68 ratio was examined. In the EBV-negative elderly who did not relapse, the ratio of CD163:CD68 was significantly higher than in the tumours of those elderly with a poor outcome. In the young a similar finding was made with a significantly higher ratio in those who did not relapse or require an escalation in treatment, with the same but non-significant trends in both the young EBV-positive and –negative. In the EBV-positive elderly, however, results were the opposite way round with a higher ratio (although not statistically significantly so) in those who did less well. This therefore raises the question of whether an increased proportion of CD163 cells in EBV-negative elderly patients, and in the young, reflects a good prognosis whereas in the elderly EBV-positive a greater proportion of CD163 reflects a poorer prognosis.

Conclusions

Thus the number of CD68-positive cells was influenced by age, by the presence or absence of EBV infection (more so in young males than young females or the elderly) and to some extent by cHL subtype, (apparently only in EBV-negative cases). With CD163 cells, there were fewer within each tumour than CD68 cells; in keeping with the premise that CD163 cells represent a subset of macrophages. CD163 cell numbers also varied according to EBV status and this association was significant for young males. As with the CD68 analysis, CD163-positive cells showed a degree of variation with subtype, being more frequent in EBV-negative MCHL than EBV-negative NSHL but this did not reach statistical significance. In the elderly EBV-negative, those who did not relapse had a significantly higher ratio of CD163 to CD68 cells than those who did relapse. Similarly, in the young, those who did not relapse/require an escalation in treatment, had a significantly higher ratio than those young who did. Does this mean that in the young and in the EBV-negative elderly, an increased proportion of CD163-positive cells is beneficial, or acts as a marker of other beneficial factors for patient survival? This did not appear to be so in the tumours of EBV-positive elderly patients. Consistently, although not statistically significant, the ratio of CD163:CD68 was higher in those elderly EBV-positive patients with a poor outcome. This raises the question as to whether CD163 cells may "behave" differently in the elderly with EBV-positive disease. This too permits the suggestion that CD163-positive cells in EBV-negative elderly and young cases, may reflect favourable factors for patient survival, whereas those in EBV-positive elderly tumours may indicate factors detrimental to survival. A greater number of cases would need to be examined to investigate this further and amongst other things, to allow multivariate analysis of potential confounding factors.

Review of Literature

The concept of macrophages functioning differently under different circumstances is not a new one. In 1973 a paper entitled '*Analysis of the Rye Classification of Hodgkin's Disease. The Prognostic Significance of Cellular*

Composition' was published looking at a variety of histological features in HD (Coppleson et al. 1973). This concluded that increased numbers of 'benign' macrophages, particularly when associated with low numbers of 'malignant' macrophages carried an especially poor prognosis. The distinction between these two cell types was based entirely on the histopathologists' assessment of H&E stained sections; something which limited these studies. However, it does illustrate a fundamental and longstanding recognition of the importance of the microenvironment in Hodgkin's Lymphoma as well as an early concept of macrophages with variable functions. Similarly, in 1981 Colby and colleagues also recognised that macrophages may behave differently according to their environment (Colby et al. 1981). This paper looked at 659 cases of Hodgkin's disease and scored them for a large number of histological parameters including histiocytes and 'atypical' histiocytes, again based only on morphological features. They found that numbers of histiocytes varied according to the subtype of HL and that there was a link with outcome but that the association between macrophages and outcome differed depending on subtype and age.

Again, a link between morphological subtype, age and macrophage numbers was seen in studies which started to use more objective techniques for identifying macrophages. Specific immunohistochemical labelling with peanut agglutinin, a lectin originating from *Arachis hypogaea,* was used by Ree and colleagues in their 1985 paper (Ree & Kadin 1985). Results showed a wide variation between subtypes of cHL with LDcHL having the largest number of macrophages followed by MCHL, while NSHL contained the fewest. Older patients were shown to have more macrophages and this seemed to correlate with other unfavourable clinical and pathological parameters leading to a poor outcome. In contrast, reactive lymph nodes used as controls, did not show any variation in macrophage numbers according to age.

The advent of molecular techniques has also provided evidence that macrophages may play different roles under different circumstances and that simple enumeration does not allow a complete understanding of this. A number of studies in Follicular Lymphoma (FL), have shown an association between CD68-positive macrophages and poor outcome (Dave et al. 2004; Farinha et al. 2005), but interestingly different host genes linked with macrophages were found in both good and bad outcome groups (Dave et al. 2004). Immunohistochemical studies have also been able to identify what appear to be functionally different groups of macrophages akin to what may be suggested by the CD163-positive cells in this study. Again in FL, a study using double-labelling with CD68 and STAT1, showed that patients with low numbers of CD68-positive cells but in whom those cells also expressed STAT1, had a shorter OS and PFS (García & Piris 2006), giving weight to the theory that not all macrophages are the same and that they may be linked with both poor and good prognosis depending on their function.

Studies into cHL have also identified 'macrophage genes' and a link with poor prognosis. The first of these was published in 2006 (Sanchez-Aguilera et al. 2006) at a time when there was a growing interest in the variable functional roles of macrophages (Biswas et al. 2006; Zhen et al. 2008). A subsequent study of 52 cases of cHL, using a gene array technique, identified a 'macrophage activation' signature with increased expression of 'macrophage' genes within whole tissue sections of cHL lymph nodes, concluding that these genes were expressed by macrophages in the microenvironment (Sánchez-Espiridión et al. 2009). In what many see as a landmark study, Steidl and colleagues published data on a larger cohort of cHL patients, heavily weighted for those with treatment failure, and almost exclusively of the NSHL subtype (Steidl, Lee, et al. 2010). They described a signature of genes expressed by tumour-associated macrophages, monocytes, adipocytes, angiogenic cells and RS cells in a treatment failure group and then demonstrated, in a separate cohort, equally weighted for poor outcome, that the number of macrophages positive for CD68 by IHC was linked with Disease Specific Survival (DSS) and PFS. Higher numbers of CD68-positive cells were associated, on univariate analysis, with poorer PFS, but not on multivariate analysis when factors from the International Prognostic Score (IPS), which includes age, were accounted for.

An association between increased CD68 cell numbers and a reduced ten-year DSS was found on both uni- and multivariate analysis. The factors considered on multivariate analysis were not specified but likely included age. However, other very important confounding factors such as EBV were not examined. They did describe an association between increased age and an increased expression of 'macrophage' genes but this was not reproduced in the cohort examined by IHC and the low median age of participants would have limited this analysis (Steidl, Lee, et al. 2010). There were other limitations, not least that the EBV status of tumours was not accounted for, but also that the majority of patients had NSHL, the heavy weighting for poor outcome and, what could be argued as, the artificial cut-off values for 'high' and 'low' CD68-positive cell numbers which is why in this thesis, CD68-cells were analysed as a continuous variable without introducing arbitrary thresholds.

Also providing evidence of the heterogeneous nature of macrophages is a study which used the expression of a number of genes, categorised by function, as the basis of a prognostic score for cHL (Sánchez-Espiridión et al. 2010). Genes were quantified using quantitative reverse transcription PCR (RT-PCR) on formalin-fixed whole sections of tumour. Eleven genes with the greatest discriminatory powers were identified and these fell into four functional groups:

1 – those expressed by 'activated' macrophages (LYZ and STAT1)

2 – those involved in apoptosis (BCL2, BCL2L1, Caspase 3)

3 – cell cycle genes (HMMR, CENPF, CCNA2, CCNE2, CDC2)

4 – 'others', a group of one, IRF4 (or MUM1) which is induced by NF-KB and is known to control B-cell proliferation and differentiation.

These results differed from those of Steidl's group in that increased expression of the macrophage genes was associated with a better failure free survival (FFS) suggesting that some macrophages in cHL are 'good'. Again this was a young cohort, with 72% of patients aged less than 45years. The authors hypothesised that STAT1 and LYZ delineated a different functional group of macrophages than had been described in Steidl's work and also questioned the different techniques used i.e. gene expression profiling by microarray versus quantitative RT-PCR (Sánchez-Espiridión et al. 2010).

Macrophages alongside various other parameters in cHL have now formed the basis of numerous papers. In a study of 105 cases of cHL diagnosed between 1974 and 2000, CD68 expression was examined along with that of FOXP3 (T-regulatory cell marker), Granzyme B (cytotoxic T-cell marker) and PD-1 (expressed by activated peripheral B and T-lymphocytes) (Okazaki & Tasuku 2007). The majority were NSHL (57%), with 30% MCHL and of the total, 19% were EBV-positive tumours. Only 28% of patients received what would now be standard treatment with ABVD ± radiotherapy. There was no weighting of groups for treatment failure. An evidently arbitrary cut-off of 0.82% was used to delineate high from low numbers of CD68-positive cells and with this, an association between older age and higher numbers of CD68 cells plus the MCHL subtype and higher macrophage counts was found. Older age was also linked to higher numbers of Granzyme B positive cells and fewer FOXP3 positive cells. There was a correlation between increased macrophage numbers and poorer OS but no link with DSS or Relapse Free Survival (RFS). Although the association with poorer outcome was seen, macrophage numbers were not an independent prognosticator when age, disease stage and numbers of other surrounding cells (granzyme B, FoxP3 and PD-1-positive cells) were considered. Increased numbers of CD68, Granzyme B and PD-1-positive cells were all associated with poorer OS and if all three were increased in any one tumour there was a particularly poor prognosis. The contrary was also true with a very good prognosis for those with reduced numbers of all three. It was noted that there were no cases where >9% of cells were CD68-positive whereas Steidl's group included bandings of 0-5% CD68-positive cells, 5-25% and >25% suggesting they found cases with many more CD68 expressing cells. This study used a different CD68 clone (PGM1) than Steidl's group (KP1) and it was argued that this may account for some differences, as PGM1 is thought to be more specific for macrophages and overall fewer cells are positive than with KP1 (Tzankov et al. 2010).

Thus while the majority of researchers suggest macrophages are linked to a poorer outcome in cHL (Civili & Matteo 2011; Touati et al. 2011; Tan et al. 2012; Tzankov et al. 2010), this is not a universal finding. A large Brazilian study published in 2012 failed to reproduce Steidl's results (Azambuja et al. 2012). Of the 265 cHL patients treated uniformly between 1997 and 2004, both CD68 and CD163 expressing cells were studied as well as the EBV status of the tumours. It was concluded that CD163 is a more specific marker of macrophages but that there was no association between either CD68- or CD163-positive cell numbers and PFS or DSS. It was however noted, as in our study, that EBV-positive tumours had greater numbers of both CD68- and CD163-positive cells and although EBV status did not affect PFS there was a significant correlation between EBV-positive disease and reduced five year DSS. This cohort differed from Steidl's in that it was a single centre study of consecutive cases with no weighting for poor outcome but it was again a young group (median age 29, range 15-82 years) and used the same scoring cut-offs for macrophage numbers as Steidl and the same CD68 antibody clone (Azambuja et al. 2012). A much smaller study, looking at 44 cases of cHL from one centre in New York, also found no association between OS or disease recurrence and numbers of CD68 or CD163 expressing macrophages (Harris et al. 2012). Again patients were on the whole young with a mean age of 36 years but as with most other studies the range of ages was broad (14-94 years). They concluded that CD68 is a less specific macrophage marker than CD163 as the CD68 KP1 clone picks out cells of myeloid lineage, DC, fibroblasts and endothelial cells. They also hypothesised that there may be a rationale for their finding of reduced numbers of CD163 cells in comparison to CD68-positive cells if CD163 expression is down-regulated by the T_H cell immune response evident within the microenvironment (Harris et al. 2012). The group that published the molecular risk score for cHL (see above), in a separate large study, also failed to reproduce Steidl's association between macrophage numbers and outcome (Sánchez-Espiridión et al. 2012; Sánchez-Espiridión et al. 2010). This time using immunohistochemistry on tissue microarrays, analysed largely by automated scanner, and looking at the expression of CD68 (KP1 and PGM1 clones), CD163, LYZ and STAT1, two separate patient cohorts were studied. One consisted of 266 cHL patients from the Spanish Hodgkin Lymphoma Study Group and the other, 103 patients identified through the MD Anderson (single centre, tertiary referral hospital, Texas, USA). Their previous molecular study had found that increased expression of LYZ and STAT1 was linked to a better outcome. On this occasion they found no association between the immunohistochemical expression of CD68, CD163, LYZ or STAT1 with FFS. There was also no association with the ratio of CD163:CD68 and FFS. Only in the Spanish cohort was there any link between higher numbers of CD68-positive cells and poorer DSS and this was only evident for a particularly high cut-off value if the PGM1 clone was used ('high CD68' as 4th quartile when CD68 expression for whole group considered). Again they concluded that not all CD68 antibodies are equal and that differences may occur when different quantification techniques are used and varying cut-off values for high and low expression (Sánchez-Espiridión et al. 2012).

Many more numerous are the publications broadly in support of Steidl's findings. There is published data linking higher macrophage numbers to a poorer outcome, with added information from PET-CT. One described higher numbers of CD163-positive cells (and Bcl 11a expression in HRS [normally down-regulated in haematopoietic cell differentiation, thought to potentially act as a B-cell oncogene]) as a predictor of poor outcome in those patients with a negative interim PET-CT, something which would normally be associated with a good prognosis (Civili & Matteo 2011). The other showed an association between higher numbers of CD68-positive cells and PET positivity at interim scanning (Touati et al. 2011). In 2012 Tan et al, published data from patients enrolled in a phase III randomised controlled trial of ABVD versus the Stanford V chemo regime in cHL (Tan et al. 2012). Two cohorts were obtained from 287 patients to form 'training' and 'validation' TMAs. Computer imaging analysis was used to quantify CD68 and CD163 cell expression by immunohistochemistry with the aim of establishing optimal thresholds for CD68/CD163 regarding prediction of OS before applying this to the 'validation' cohort looking for reproducibility. They concluded that higher numbers of CD68 and CD163 expressing cells were independent risk factors for reduced FFS and OS. They also found an association between higher numbers, older age, EBV positivity and MCHL subtype. Of note, during analysis, it was also evident that EBV positivity varied according to gender with significantly more EBV-positive cases in men. The cohorts were again largely young (84% <45yrs), 80% NSHL and 83% EBV-negative. Similar results were seen for both CD68 and CD163 antibodies. Cut-off values of 12.7% for high/low CD68 expression and 16.8% for CD163 were obtained, giving the most significant associations with failure free survival (FFS) and OS. High numbers of CD163-positive cells were associated with a poorer outcome in both EBV-positive and -negative tumours, which contrasts with our findings. For CD68 there was only a significantly poorer outcome in the EBV-negative cases and the association was lost for those with EBV-positive disease. Although higher numbers of CD68 and/or CD163 expressing cells were associated with poorer OS and with EBV positivity, no survival difference was found between EBV-negative and -positive cases. It is difficult to put too much weight on this finding as the numbers of EBV-positive cases were small but it would suggest there are other factors, over and above the viral infection in EBV-positive cases, that results in increased macrophage numbers and poorer outcome (Tan et al. 2012).

Thus the research summarised above, highlights how histology, immunohistochemistry and molecular techniques have been used to characterise cHL. From this, factors such as age, EBV status, histological subtype, gender and the examination of non-malignant cells within tumours, can all be seen to inform on disease progression and prognosis. The combination of molecular techniques, especially those such as the Fluidigm dynamic array, which allow the simultaneous analysis of multiple genes in multiple cases, alongside immunohistochemistry are attractive. However, there remain a number of fundamental issues with all of these studies. How do we know that the genes identified as 'macrophage' genes are being expressed by macrophages and only macrophages, especially in studies utilising whole tissue sections? How can we then expect to substitute one, or perhaps two protein markers of these cells and use IHC to identify different functional groups in such a complex and evolving situation as the tumour microenvironment? When we do decide on an appropriate IHC label, how can we be certain that one antibody clone identifies the same group of cells as another? By necessity the techniques available over-simplify the data and therefore there is the very real possibility of applying what may be useful prognostic information in one group, too widely, drawing inappropriate conclusions for other groups of patients. Thus the importance of identifying and accounting for confounding factors cannot be overstated.

The presence of greater numbers and different phenotypes of macrophages in some subgroups of cHL is of interest and leads to the question as to what their roles may be. One very important function of macrophages is phagocytosis which may or may not then be linked with subsequent antigen presentation. Dead or dying cells in any benign or malignant cell group must be cleared, a process which involves phagocytosis, and this leads on to the examination of apoptosis and how this may be associated with changes in the tumour microenvironment.

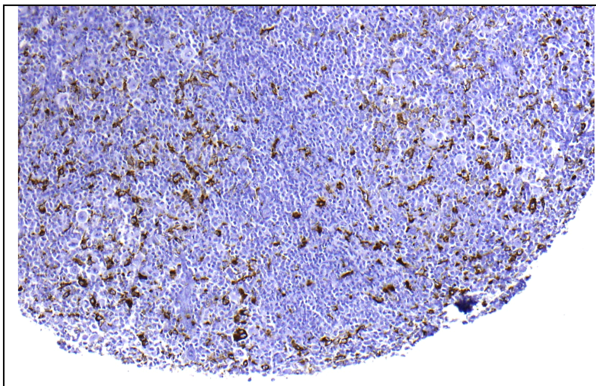


Figure 6.1
CD68 IHC
Tumour 55, Young cohort, NSHL (x10)
Scattered CD68-positive macrophages within the tumour microenvironment.

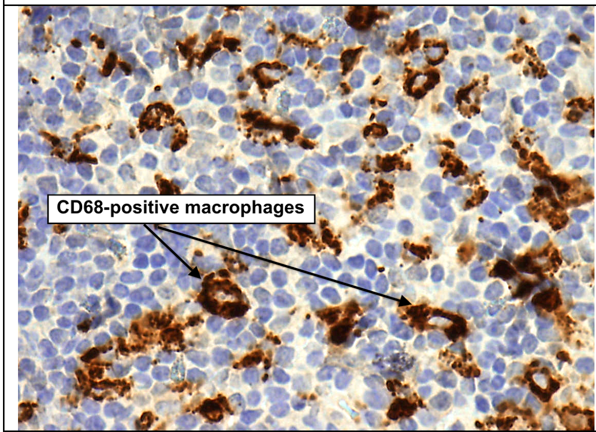


Figure 6.2
CD68 IHC
Tumour 20, Young cohort, LRcHL (x40)
High power view of macrophages with cytoplasmic expression of CD68.

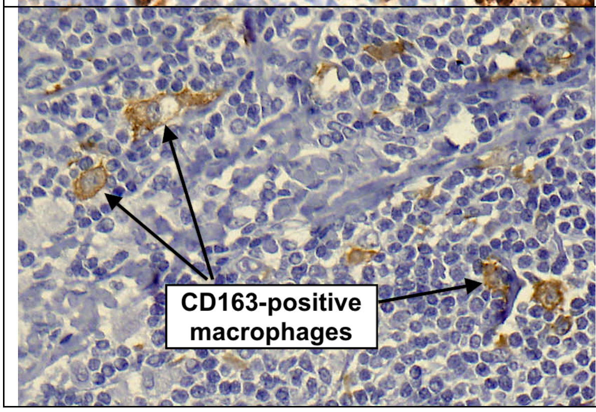


Figure 6.3
CD163 IHC
Tumour 31, Young cohort, NSHL (x40)
Macrophages with cytoplasmic expression of CD163.

Chapter 7

Apoptosis in classic Hodgkin Lymphoma

Introduction

Hodgkin Reed-Sternberg cell resistance to apoptosis is considered to be a key principle in the pathogenesis of HL (Rassidakis et al. 2002; Küppers 2009a). Post-germinal centre B-cells that have lost their surface B-cell receptor, should undergo apoptosis and failure to do so is thought to be an important step in malignant transformation (Rassidakis et al. 2002; Marafioti et al. 2000; Benharroch et al. 2010). Despite this however, it is clear that HRS cells are not completely resistant to apoptosis, with morphological evidence that it occurs in the form of 'mummified' HRS cells and immunohistochemical studies showing the expression, by HRS cells, of a number of different markers of programmed cell death. These include activated caspase-3, with or without the co-expression of PARP-1 (a cleavage product of caspase-3 indicating its function) and the TUNEL assay which is based on the principle of identifying DNA fragmentation resulting from apotosis (Eichenauer et al. 2011; Lynny Yung & Linch 2005; Dukers 2002; Benharroch et al. 2010; Benharroch et al. 1999). Programmed cell death has been recognised for many years and the term 'apoptosis', with a comprehensive description of the process, was first proposed by Kerr, Wyllie and Currie in 1972 (Kerr et al. 1972). Apoptosis, a word originating from Greek meaning 'dropping off' or 'falling off', describes a form of planned cell death which Kerr, Wyllie and Currie recognised as of key importance in tissue homeostasis. Without it, cell division would result in the inexorable growth of tissues. The importance of apoptosis in embryology, physiological involution of organs such as the thymus and also as one mechanism of tumour regression was recognised. Their electron-microscopic studies of tissues undergoing apoptosis describe a controlled, active process, which occurs in individual cells, distributed throughout tissues, as opposed to necrosis which tends to affect large numbers of cells within one focal area. Shrinkage/condensation of the nucleus and cytoplasm occurs first, before the nucleus fragments. Small 'blebs' then develop on the surface of the cell before these separate from the cell completely. These are membrane-bound particles of varying size and only the larger forms are visible by light microscopy. There are many more microparticles, only visible by electron microscopy, which tend to disperse widely. Intact organelles and nuclear remnants are contained within these apoptotic bodies, which are then phagocytosed either by neighbouring cells or by specialist phagocytic cells such as DC or macrophages. Once internalised, the apoptotic bodies, now within phagosomes, undergo a process more like autolysis where membranes breakdown. Lysosomes then fuse with the phagosomes and hydrolases further reduce the apoptotic bodies to residual lysosomal bodies (Kerr et al. 1972). All of this occurs rapidly, possibly within minutes to just a few hours (Gregory & Pound 2011; Kerr et al. 1972) which is one reason why it is a difficult process to study and why even the presence of a small number of apoptotic bodies in histological sections suggest the process is occurring extensively (Kerr et al. 1972; Gregory & Pound 2011).

Previously, apoptosis was considered to be a process which caused minimal disruption to surrounding cells, unlike its counterpart necrosis. However, it is now clear that apoptosis has an active, often anti-inflammatory, role to play with wide-reaching and varying effects on the immune response, including the ability to induce both immune tolerance and to be immunostimulatory. In other circumstances, it can also be pro-inflammatory (Mosser & Edwards 2008; Savill et al. 2002; Truman et al. 2004; Gregory & Devitt 2004; Truman et al. 2008; Kim et al. 2003; Albert 2004; Maderna & Godson 2003; Tesniere et al. 2008; Bournazou et al. 2009; Gregory & Pound 2011; Revesz 1956; Chew et al. 2010; Westhoff et al. 2014) and if apoptotic cells persist, without being phagocytosed, secondary necrosis intervenes and is linked to many pathological processes including autoimmune disorders and chronic inflammatory conditions (Gregory & Devitt 2004; Gregory & Pound 2011).

The significance of apoptosis in cHL has been studied largely by looking for markers of apoptosis in HRS cells themselves, or using whole section gene expression profiling techniques. Results are contradictory with some studies showing a poor outcome in relation to the increased expression by HRS cells, of activated caspase-3, a marker of apoptosis (Sánchez-Espiridión et al. 2009; Sánchez-Espiridión et al. 2010; Benharroch et al. 1999), whereas others suggest that the same finding is linked to a better prognosis (Dukers 2002). The expression of anti-apoptotic genes has also been associated with a poor outcome and by inference the converse is good (Rassidakis et al. 2002; Devilard et al. 2002; Sánchez-Espiridión et al. 2010). Equally there are studies where, although genes associated with the regulation of apoptosis are linked to outcome, it is not clear whether the overall effect of these genes is to increase or reduce the incidence of apoptosis (Sanchez-Aguilera et al. 2006; Chetaille et al. 2009). Immunohistochemical studies have concentrated on apoptosis occurring within HRS cells, and GEP studies, extracting genetic material from whole tissue sections, cannot distinguish between the apoptosis of malignant cells and that of microenvironmental cells. Both therefore have their limitations. As apoptosis appears to influence the immune system regardless of the cell type undergoing programmed cell death, it is important to examine not just apoptosis of malignant HRS cells but also of the surrounding non-malignant cells.

There are three known routes to apoptosis. The extrinsic pathway involves the activation of 'death receptors' on the surface of cells; the intrinsic pathway is triggered by DNA damage and involves activation of mitochondrial death signals resulting in the release of cytochrome-c into the cytosol; and thirdly the immune system can induce apoptosis via cytotoxic T-cells. This involves perforin producing a tunnel into the target cell through which granzymes enter the cell and activate apoptosis. All three of these pathways culminate in the activation of caspases-3 and -7, which ultimately result in the breakdown of cytosolic and nuclear proteins, thereby causing apoptosis (Westhoff et al. 2014). Activated caspase-3, which is detectable immunohistochemically, is therefore a useful marker of apoptosis, regardless of the trigger and even in cells that may still appear morphologically normal.

Materials and Methods

Tissue Microarrays of both the young and elderly cohorts were examined by IHC for the expression of activated caspase-3 (figure 7.1). Double-labelling of sections for activated caspase-3 and the cell surface marker CD30 was also undertaken to allow the identification of apoptotic HRS cells (figures 7.2 & 7.3). These cells and apoptotic non-HRS cells were counted as previously described (chapter two). Results were obtained for the total number of apoptotic cells per area counted ('total apoptosis'), the total number of HRS cells per area, the number of apoptotic HRS cells per area and the number of 'background' apoptotic cells per area (equal to the total number of apoptotic cells minus the number of apoptotic HRS cells per area), as described in chapter two.

Results

In total 120 cases with sections in the TMAs, (72 of the 82 young cohort and 48 of the 77 elderly cohort) were assessed regarding apoptosis. Of the young cohort examined, 33 were male and 39 female. Of the elderly, 23 were male, 22 female and for three cases data regarding gender was unavailable.

Results for the 'total apoptosis' and 'background apoptosis' cell counts did not differ substantially in the majority of areas. These two groups are therefore discussed together, with any significant differences highlighted.

Unless stated otherwise, p-values relate to the Mann-Whitney test.

Total apoptosis by age and gender

The elderly cohort as a whole compared with the young cohort, had a greater median number of apoptotic cells per mm^2 ($p=0.01$). When the groups were divided by gender, this remained the case for females, as elderly women had more apoptotic cells than young women ($p=0.01$). However, there was no significant difference between elderly and young men. Within the young cohort, men had a greater total number of apoptotic cells than women ($p=<0.05$), but no gender difference was evident in the elderly group (table 7.1).

Table 7.1
Total apoptosis and gender

	Elderly + Young combined Median cell count per mm^2	**Young** Median cell count per mm^2	**Elderly** Median cell count per mm^2	**Young v Elderly**
All cases (n) (range)	264 (120) (80–1056)	232 (72) (80-1056)	320 (48) (80-944)	p=0.01
Male (n)	292 (56)	272 (33)	320 (23)	p=NS
Female (n)	240 (61)	208 (39)	328 (22)	p=0.01
Male v Female	p=<0.05	p=<0.05	p=NS	

HRS-cells by age and gender

The number of HRS cells was greater in the tumours of young patients than the elderly (p=<0.0001) and this was true for both males and females. There was no evident difference in the number of HRS cells between the genders in either cohort (table 7.2).

Table 7.2
HRS-cells and gender

	Elderly + Young combined Median cell count per mm^2	**Young** Median cell count per mm^2	**Elderly** Median cell count per mm^2	**Young v Elderly**
All cases (n) (range)	112 (119) (0-960)	160 (70) (16-960)	64 (49) (0-624)	p=<0.0001
Male (n)	116 (56)	192 (33)	48 (23)	p=<0.001
Female (n)	120 (60)	144 (37)	80 (23)	p=<0.05
Male v Female	p=NS	p=NS	p=NS	

Apoptotic HRS-cells by age and gender

Apoptotic HRS cells were more numerous in the tumours of young patients than those of the elderly (p=<0.001) and the percentage of HRS cells undergoing apoptosis was also greater (p=<0.05). No significant difference was evident between genders (table 7.3).

Table 7.3
Apoptotic HRS-cells and gender

	Elderly + Young combined		Young		Elderly		Young v Elderly	
Apoptotic HRS-cells	Median	%	Median	%	Median	%	Median	%
All cases	16	12.9	32	16.2	0	4.7	p=<0.001	p=<0.05
Male	32	15.4	32	16.7	0	2.9	p=<0.01	p=NS
Female	16	12.5	16	15.4	16	10	p=0.066	p=NS
Male v Female	p=NS	p=NS	p=NS	p=NS	p=NS	p=NS		

Median=median cell count per mm^2; %=apoptotic HRS-cells as % of all HRS-cells

Total apoptosis by EBV status

With regard to EBV, in positive cases in the young there appeared to be a trend towards a greater number of apoptotic cells but this was not seen in the elderly. The difference, between EBV-positive and -negative young, did reach statistical significance when only background apoptosis was counted (p=<0.01). As discussed above, overall tumours of the elderly cohort contained more apoptotic cells than the young and this remained true when EBV-negative tumours in the elderly were compared with those in the young (p=<0.01). However, when only EBV-positive cases were examined this difference was no longer apparent (table 7.4).

Table 7.4
Total apoptosis and EBV

	Elderly + Young combined Median cell count per mm^2	Young Median cell count per mm^2	Elderly Median cell count per mm^2	Young v Elderly
EBV pos (n)	336 (41)	300 (16)	352 (25)	p=NS
EBV neg(n)	248 (79)	220 (56)	312 (23)	p=<0.01
EBV pos v neg	p=NS	p=0.06	p=NS	

When gender was also considered, only the elderly female EBV-negative compared with the young female EBV-negative differed significantly (p=<0.05), with no significant differences found between the other groupings (table 7.5).

Table 7.5
Total apoptosis, EBV and gender

	Elderly + Young combined Median cell count per mm^2		Young Median cell count per mm^2		Elderly Median cell count per mm^2		Young v Elderly	
EBV status	**Pos**	**Neg**	**Pos**	**Neg**	**Pos**	**Neg**	**Pos**	**Neg**
Male (n)	368 (26)	272 (30)	384 (13)	264 (20)	352 (13)	316 (10)	p=NS	p=NS
	p=NS		p=NS		p=NS			
Female (n)	304 (13)	220 (48)	240 (3)	200 (36)	344 (10)	304 (12)	p=NS	p=<0.05
	p=NS		p=NS		p=NS			
Male v Female	p=NS	p=NS	p=NS	p=NS	p=NS	p=NS		

Comparing EBV-positive and –negative cases within each gender, there appeared to be a trend for young EBV-positive males to have a greater number of apoptotic cells than their EBV-negative counterparts. This was most apparent with background apoptotic cells, although it did not reach statistical significance (p=0.06).

HRS-cells by EBV status

In both the EBV-positive and EBV-negative, the young had significantly more HRS cells than the elderly (p=0.01). Also, the EBV-negative tumours had greater numbers of HRS cells than the EBV-positive (p=<0.05 in the young and p=<0.001 in the combined cohorts) (table 7.6).

Table 7.6
HRS-cells and EBV

	Elderly + Young combined Median cell count per mm^2	Young Median cell count per mm^2	Elderly Median cell count per mm^2	Young v Elderly
EBV pos (n)	80 (43)	112 (17)	40 (26)	p=0.01
EBV neg (n)	144 (76)	192 (53)	80 (23)	p=0.01
EBV pos v neg	p=<0.001	p=<0.05	p=NS	

However, when those with EBV-positive or –negative disease were divided by gender, the difference was only statistically significant in young males (p=<0.01) and the tumours of young EBV-negative males contained significantly more HRS cells than those of young EBV-negative females (p=<0.01) (table 7.7).

Table 7.7
HRS-cells, EBV and gender

	Elderly + Young combined Median cell count per mm^2		**Young** Median cell count per mm^2		**Elderly** Median cell count per mm^2	
EBV status	**Pos**	**Neg**	**Pos**	**Neg**	**Pos**	**Neg**
Male (n)	80 (26)	200 (30)	96 (13)	244 (20)	32 (13)	68 (10)
	p=<0.01		p=<0.01		p=NS	
Female (n)	88 (15)	128 (45)	200 (4)	136 (33)	48 (11)	88 (12)
	p=NS		p=NS		p=NS	
Male v Female	p=NS	p=NS	p=NS	p=<0.01	p=NS	p=NS

Apoptotic HRS-cells by EBV status

Those elderly with EBV-negative disease had a greater number (p=0.001) and a greater percentage (p=<0.001) of apoptotic HRS cells than the elderly with EBV-positive tumours but this difference was not apparent in the young. Those young, with EBV-positive disease had a significantly greater number and percentage of apoptotic HRS cells than their elderly counterparts (both, p=<0.01) but there was no difference between the cohorts when only EBV-negative disease was considered (table 7.8).

Table 7.8
Apoptotic HRS-cells and EBV

	Elderly + Young combined		**Young**		**Elderly**		**Young v Elderly**	
Apoptotic HRS-cells	Median	%	Median	%	Median	%	Median	%
EBV pos (n)	0 (43)	0 (40)	32 (17)	17 (17)	0 (26)	0 (23)	p=<0.01	p=<0.01
EBV neg (n)	32 (76)	17 (74)	32 (53)	16 (53)	32 (23)	20 (21)	p=NS	p=NS
EBV pos v neg	p=<0.001	p=<0.01	p=NS	p=NS	p=0.001	p=<0.001		

Median=median cell count per mm^2; %=apoptotic HRS-cells as % of all HRS-cells

When gender was also taken into consideration, only young males with EBV-positive disease compared with their elderly counterparts, had a significantly greater number of apoptotic HRS cells (p=0.01) (table 7.9).

Table 7.9
Apoptotic HRS-cells, EBV and gender

	Elderly + Young combined		Young		Elderly		Young v Elderly	
Apoptotic HRS-cells	Median	%	Median	%	Median	%	Median	%
Male EBV pos (n)	0 (26)	0 (25)	32 (13)	25 (13)	0 (13)	0 (12)	p=0.01	p=<0.01
Male EBV neg (n)	32 (30)	17 (28)	32 (20)	16 (20)	24 (10)	23 (8)	p=NS	p=<0.05
Female EBV pos (n)	0 (15)	7 (13)	16 (4)	8 (4)	0 (11)	0 (9)	p=NS	p=NS
Female EBV neg (n)	16 (45)	17 (45)	16 (33)	17 (33)	32 (12)	14 (12)	p=NS	p=NS

Median=median cell count per mm²; %=apoptotic HRS-cells as % of all HRS-cells

Total apoptosis by cHL subtype

In each cohort, cases of MCHL had a greater number of apoptotic cells than NSHL cases but this only reached statistical significance when both cohorts were combined (p=<0.01) (table 7.10).

Table 7.10
Total apoptosis and cHL subtype

	Elderly + Young combined Median cell count per mm²	**Young** Median cell count per mm²	**Elderly** Median cell count per mm²
NSHL (n)	228 (80)	208 (55)	288 (25)
MCHL (n)	368 (30)	272 (11)	392 (19)
cHL, other (n)	248 (10)	340 (6)	224 (4)
NSHL v MCHL	p=<0.01	p=0.064	p=NS

EBV-positive MCHL and NSHL tumours did not differ significantly in their number of apoptotic cells but in EBV-negative MCHL tumours there were significantly greater numbers than in EBV-negative NSHL, both in the combined and elderly cohorts (combined, p=<0.01; elderly, p=0.01) (table 7.11).

Table 7.11
Total apoptosis, cHL subtype and EBV

	Elderly + Young combined Median cell count per mm^2		**Young** Median cell count per mm^2		**Elderly** Median Cell count per mm^2	
EBV status	**Pos**	**Neg**	**Pos**	**Neg**	**Pos**	**Neg**
NSHL (n)	304 (15)	224 (65)	304 (7)	196 (48)	272 (8)	288 (17)
	p=NS		p=NS		p=NS	
MCHL (n)	344 (22)	552 (8)	296 (7)	272 (4)	352 (15)	848 (4)
	p=NS		p=NS		p=<0.05	
cHL, other (n)	284 (4)	248 (6)	304 (2)	352 (4)	284 (2)	160 (2)
	p=NS		p=NS		p=NS	
NSHL v MCHL	p=NS	p=<0.01	p=NS	p=NS	p=NS	p=0.01

Total apoptosis was greater in the EBV-negative than the EBV-positive MCHL of the elderly (p=<0.05). The opposite was observed with the young EBV-positive and-negative cases of NSHL, with greater numbers of apoptotic cells in the EBV-positive cases. This difference appeared to be so for both total and background apoptosis but was only statistically significant for background apoptosis (p=<0.05).

HRS-cells by cHL subtype

There was no difference in the number of HRS cells between MCHL and NSHL subtypes of cHL overall (data not shown). However, when young EBV-negative cases of MCHL were compared to young EBV-negative NSHL cases, the former contained significantly more HRS cells (p=<0.01). No such difference was seen for the EBV-positive cases. Also in the young and combined groups, cases of MCHL that were EBV-negative had significantly more HRS cells than those that were EBV-positive (young, p=<0.05; combined p=<0.01) (table 7.12).

Table 7.12
HRS-cells, cHL subtype and EBV

	Elderly + Young combined Median cell count per mm^2		**Young** Median cell count per mm^2		**Elderly** Median Cell count per mm^2	
EBV status	**Pos**	**Neg**	**Pos**	**Neg**	**Pos**	**Neg**
NSHL (n)	88 (17)	144 (62)	116 (8)	176 (45)	80 (9)	80 (17)
	p=NS		p=NS		p=NS	
MCHL (n)	48 (22)	336 (8)	96 (7)	636 (4)	32 (15)	100 (4)
	p=<0.01		p=<0.05		p=NS	
cHL, other (n)	92 (4)	40 (6)	92 (2)	112 (4)	104 (2)	NP
	p=NS		p=NS		NP	
NSHL v MCHL	p=NS	p=NS	p=NS	p=<0.01	p=NS	p=NS

NP=calculation not possible

Apoptotic HRS-cells by cHL subtype

In the young MCHL cases, apoptotic HRS cells were significantly more frequent than in the NSHL cases ($p<0.05$) but the difference in percentage values was not significant (table 7.13).

Table 7.13
Apoptotic HRS-cells and cHL subtype

	Elderly + Young combined		**Young**		**Elderly**	
Apoptotic HRS-cells	Median	%	Median	%	Median	%
NSHL (n)	16 (79)	13 (77)	32 (53)	15 (53)	12 (26)	6 (24)
MCHL (n)	16 (30)	16 (27)	48 (11)	24 (11)	0 (19)	5 (16)
cHL, other (n)	0 (10)	0 (10)	8 (6)	8 (6)	0 (4)	0 (4)
NSHL v MCHL	p=NS	p=NS	p=<0.05	p=NS	p=NS	p=NS

Median=median cell count per mm^2; %=apoptotic HRS-cells as % of all HRS-cells

When EBV status was also included, then this significant difference between the subtypes only occurred when EBV-negative MCHL was compared with EBV-negative NSHL as EBV-positive cases of each subtype differed little. In EBV-negative cases of both NSHL and MCHL in the combined cohort, there were significantly more apoptotic HRS cells, (also with a significantly greater percentage of apoptotic HRS cells) than in their EBV-positive equivalents (tables 7.14 & 7.15).

Table 7.14
Number of apoptotic HRS-cells, cHL subtype and EBV

	Elderly + Young combined Median cell count per mm^2		**Young** Median cell count per mm^2		**Elderly** Median Cell count per mm^2	
EBV status	**Pos**	**Neg**	**Pos**	**Neg**	**Pos**	**Neg**
NSHL (n)	0 (17)	32 (62)	16 (8)	32 (45)	0 (9)	32 (17)
	p=0.05		p=NS		p=NS	
MCHL (n)	0 (22)	64 (8)	32 (7)	100 (4)	0 (15)	60 (4)
	p=<0.001		p=NS		p=<0.01	
NSHL v MCHL	p=NS	p=<0.01	p=NS	p=0.01	p=NS	p=<0.05

Table 7.15
% Apoptotic HRS-cells, cHL subtype and EBV

	Elderly + Young Combined %		**Young** %		**Elderly** %	
EBV status	**Pos**	**Neg**	**Pos**	**Neg**	**Pos**	**Neg**
NSHL (n)	0 (17)	16 (60)	8 (8)	17 (45)	0 (9)	16 (15)
	p=<0.05		p=NS		p=NS	
MCHL (n)	10 (19)	25 (8)	25 (7)	20 (4)	0 (12)	43 (4)
	p=<0.05		p=NS		p=<0.01	
NSHL v MCHL	p=NS	p=NS	p=NS	p=NS	p=NS	p=NS

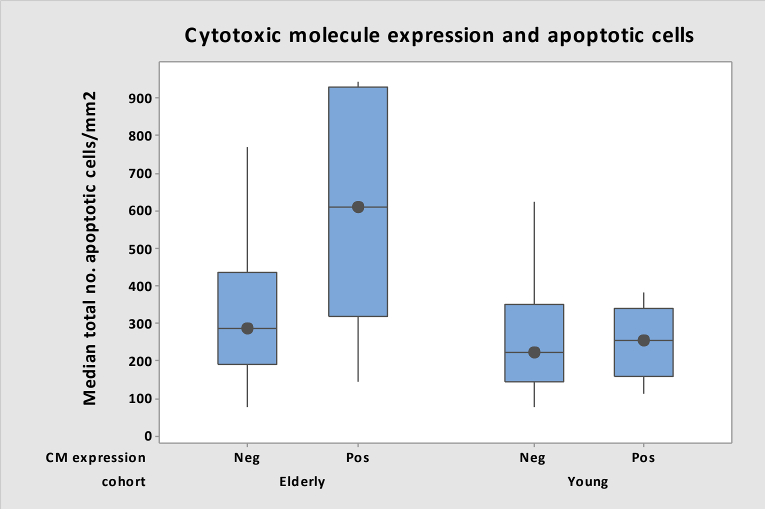
Apoptosis and cytotoxic molecule expression

The total and background number of apoptotic cells was significantly increased in the CMpos compared with the CMneg tumours in the elderly cohort (total and background p=<0.05) (figure 7.4).

HRS-cells and cytotoxic molecule expression

The median number of HRS cells did not differ significantly between CMpos and CMneg cases in any of the cohorts; combined, young or elderly.

Figure 7.4
Total apoptosis and cytotoxic molecule expression



(Elderly: CMpos, median = 608, CMneg, median = 288, p=<0.05
Young: CMpos, median = 256, CMneg, median = 224, p=NS)

Apoptotic HRS-cells and cytotoxic molecule expression

The number and percentage of apoptotic HRS cells was lower in the CMpos compared with the CMneg cases in the young cohort but the opposite appeared to be true in the elderly with a greater number and percentage of apoptotic HRS cells in the CMpos tumours (table 7.16).

Table 7.16
HRS-cells, apoptotic HRS-cells and cytotoxic molecule expression

	Elderly + Young Combined Median cell count per mm^2/%		**Young** Median cell count per mm^2/%		**Elderly** Median cell count per mm^2/%	
CM status	**Pos**	**Neg**	**Pos**	**Neg**	**Pos**	**Neg**
HRS cells (n)	88 (16)	120(103)	136 (8)	160(62)	76 (8)	64 (41)
	p=NS		p=NS		p=NS	
Apoptotic HRS cells (n)	16 (16)	16 (103)	8 (8)	32 (62)	32 (8)	0 (41)
	p=NS		p=0.01		p=<0.05	
% Apoptotic HRS cells (n)	12 (16)	13 (98)	2 (8)	17 (62)	31 (8)	0 (36)
	p=NS		p=<0.05		p=<0.05	

There were no evident differences regarding the total number of apoptotic cells; the number of HRS cells; the number or proportion of apoptotic HRS cells, between CMpos, PAX5-positive and CMpos, PAX5-negative cases.

Apoptosis and T-cell antigen expression

In the combined cohort, the total and the background number of apoptotic cells was greater in the T-cell antigen-negative tumours compared with those that expressed T-cell antigens. The difference was statistically significant for the number of background apoptotic cells (p=<0.05) but did not reach statistical significance in any other group (data not shown).

HRS-cells and T-cell antigen expression

In each of the three cohorts, the number of HRS cells was greater in the tumours that expressed T-cell antigens. This reached statistical significance in the young (p=<0.01) and combined groups (p=<0.0001) (table 7.17).

Table 7.17
HRS-cells, apoptotic HRS-cells and T-cell antigen expression

	Elderly + Young Combined Median cell count per mm^2		**Young** Median cell count per mm^2		**Elderly** Median cell count per mm^2	
T-cell antigen expression	**Pos**	**Neg**	**Pos**	**Neg**	**Pos**	**Neg**
HRS cells (n)	248(16)	96 (82)	264(13)	132(44)	200 (3)	48 (38)
	p=<0.001		p=<0.01		p=NS	
Apoptotic HRS cells (n)	32 (16)	16 (82)	32 (13)	32 (44)	32 (3)	0 (38)
	p=<0.01		p=NS		p=<0.01	
% Apoptotic HRS cells (n)	15 (16)	13 (77)	13 (13)	19 (44)	21 (3)	0 (33)
	p=NS		p=NS		p=<0.05	

Apoptotic HRS-cells and T-cell antigen expression

In the elderly, the number and percentage of apoptotic HRS cells was geater in those T-cell antigen-positive tumours (number apoptotic HRS-cells, p=<0.01; percentage apoptotic HRS-cells, p=<0.05). This was not seen in the young (table 7.17).

Total apoptosis and outcome

The four measures of outcome are described in chapter two; 1) relapse, 2) relapse and escalation, 3) those who died, 4) those who died of cHL. No significant difference in the number of apoptotic cells was evident between any of the good or poor outcome measures in any of the cohorts, young, elderly or combined.

Similarly, the number of apoptotic cells did not vary according to the stage of disease (I-IIA v IIB-IV) at presentation.

HRS-cells and outcome

As shown in table 7.2, the young had significantly more HRS cells than the elderly regardless of outcome. In the young, unlike the elderly, there appeared to be a trend towards greater numbers of HRS cells in the tumours of those patients who did less well (tables 7.18 to 7.20).

Table 7.18
HRS-cells and outcome: relapse

	Elderly + Young combined Median cell count per mm^2	**Young** Median cell count per mm^2	**Elderly** Median cell count per mm^2	**Young v Elderly**
All relapse (n)	168 (22)	272 (13)	96 (9)	p=<0.05
All no relapse (n)	108 (94)	144 (56)	64 (38)	p=0.0001
Relapse v no relapse	p=NS	p=NS	p=NS	

This finding appeared to be stronger in the EBV-negative young, as those in this group that went on to relapse, had tumours with significantly more HRS cells than those who did not (p=<0.05) (data not shown). If those who relapsed or required an escalation in treatment were included, the difference became statistically significant for all young patients as well as just those EBV-negative cases (all young, p=0.05; EBV-negative young, p=<0.05) (table 7.19).

Table 7.19
HRS-cells and outcome: relapse or escalation in treatment (Rx)

	Elderly + Young combined Median cell count per mm^2	**Young** Median cell count per mm^2	**Elderly** Median cell count per mm^2	**Young v Elderly**
All Relapse/increase Rx (n)	128 (31)	272 (17)	64 (14)	p=<0.01
All – No relapse/increase Rx (n)	112 (83)	144 (52)	72 (31)	p=<0.01
Relapse/increase Rx v No relapse/increase Rx	p=NS	p=0.05	p=NS	

However, when death due to any cause was used as an outcome measure, in the combined cohort there were a significantly greater number of HRS cells in the group that had a better outcome (p=<0.05). Again, as noted above, significantly more HRS cells were present in the young compared with the elderly, irrespective of outcome (table 7.20).

Table 7.20
HRS-cells and outcome: death, any cause

	Elderly + Young combined Median cell count per mm^2	**Young** Median cell count per mm^2	**Elderly** Median cell count per mm^2	**Young v Elderly**
All Dead (n)	76 (24)	232 (5)	32 (19)	p=<0.05
All – not dead (n)	120 (94)	144 (64)	72 (30)	p=0.001
Dead v not dead, any cause	p=<0.05	p=NS	p=NS	

The number of HRS cells did not vary according to the stage of disease (I-IIA v IIB-IV) at presentation.

Apoptotic HRS-cells and outcome

Only when EBV-negative disease was considered separately, was any difference in the number of apoptotic HRS cells evident according to outcome. In the combined cohort (EBV-negative), those who went on to relapse had significantly more apoptotic HRS cells than those who did not (p=<0.05), but no difference was seen in the percentage of apoptotic HRS-cells (table 7.21). The table below shows that when relapse/requirement for an escalation in treatment was the outcome measured, in both the young and combined EBV-negative cohorts, the number of apoptotic HRS cells (but not the percentage) was significantly greater in those with a poorer outcome ($p=<0.05$ combined cohort; $p=0.05$ young cohort) (table 7.21).

Table 7.21
EBV-negative cases.
Apoptotic HRS-cells and outcome: relapse or escalation in treatment (Rx)

EBV-negative	**Elderly + Young combined**		**Young**		**Elderly**	
Apoptotic HRS-cells	Median	%	Median	%	Median	%
Relapse/increase Rx (n)	48 (18)	21 (17)	48 (14)	18 (14)	40 (4)	33 (3)
No relapse/increase Rx(n)	16 (57)	16 (56)	16 (39)	16 (39)	24 (18)	19 (17)
Relapse/increase Rx v No relapse/increase Rx	p=<0.05	p=NS	p=0.05	p=NS	p=NS	p=NS

Median=median cell count per mm^2; %=apoptotic HRS-cells as % of all HRS-cells

However, when death due to any cause was the outcome, the number of apoptotic HRS cells in the combined group was significantly greater in those who did not die and therefore had a better outcome ($p=<0.05$) but again, the percentage of apoptotic HRS cells did not vary (table 7.22).

Table 7.22
Apoptotic HRS-cells and outcome: death, any cause

	Elderly + Young combined		**Young**		**Elderly**	
Apoptotic HRS-cells	Median	%	Median	%	Median	%
All dead (n)	0 (24)	3 (20)	48 (5)	21 (5)	0 (19)	0 (15)
All not dead (n)	16 (94)	15 (93)	32 (64)	16 (64)	16 (30)	10 (29)
Dead v not dead, any cause	p=<0.05	p=NS	p=NS	p=NS	p=NS	p=NS

Median=median cell count per mm^2; %=apoptotic HRS-cells as % of all HRS-cells

Correlation of different cell types

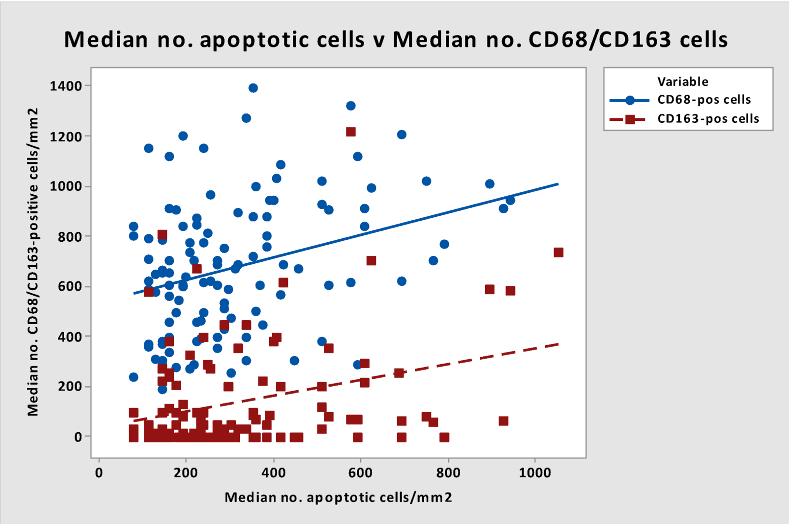
The number of apoptotic cells within each case was then compared with a number of other cell types present within each tumour and the Pearson correlation was used to calculate significance as described in chapter two.

Total number apoptotic cells v CD68-positive and CD163-positive macrophages

A statistically significant positive relationship between the total number of apoptotic cells and the number of both CD68- and CD163-positive cells was found when tumours from both the young and elderly were considered together (figure 7.5).

Figure 7.5

Combined cohorts: total apoptotic cells versus CD68 and CD163-positive cells



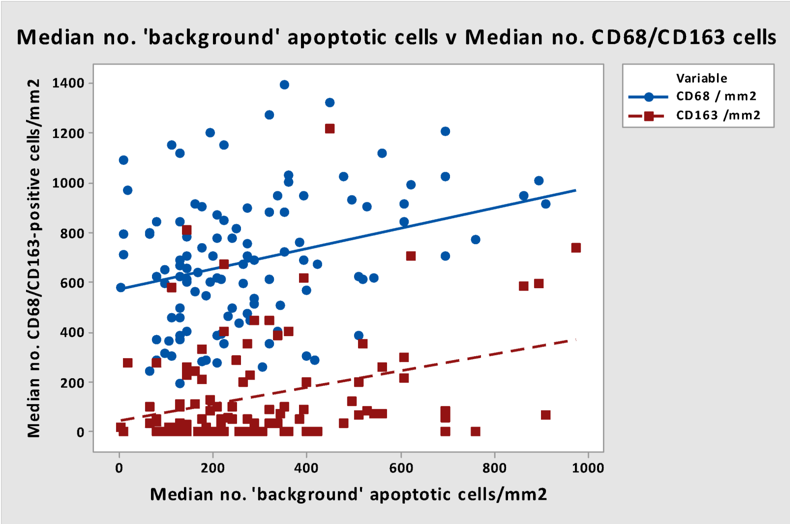
Pearson correlation: CD68 = 0.34 (p=<0.001), CD163 = 0.31 (p=0.001)

A similarly significant positive relationship was also evident between the total number of apoptotic cells and both the CD68 (Pearson correlation 0.31, p=0.01) and CD163 (Pearson correlation 0.45, p=<0.001) cells in the young, but no significant correlation was evident when the elderly group was considered independently.

Background apoptotic cells v CD68-positive and CD163-positive macrophages

The results for background apoptotic cells were broadly similar to those described for the total number of apoptotic cells in each case (figure 7.6).

Figure 7.6
Combined cohorts: background apoptotic cells versus CD68 and CD163-positive cells



Pearson correlation: CD68 = 0.32 (p=0.001), CD163 = 0.32 (p=0.001)

Apoptotic HRS-cells v CD68-positive and CD163-positive macrophages

When all cases were considered together, in each of the three cohorts, no apparent significant associations between the number or percentage of apoptotic HRS cells, with either CD68- or CD163-positive cells were seen (data not shown). The groups were then subdivided according to EBV status and cHL subtype (tables 7.23 & 7.24).

Table 7.23
Correlation between number of apoptotic HRS-cells and number of macrophages

	Elderly + Young combined Pearson correlation (p value)		**Young** Pearson correlation (p value)		**Elderly** Pearson correlation (p value)	
	CD68	**CD163**	**CD68**	**CD163**	**CD68**	**CD163**
EBV pos	0.19 (p=NS)	0.50 (p=0.001)	0.42 (p=NS)	0.59 (p=0.01)	0.29 (p=NS)	-0.02 (p=NS)
EBV neg	0.01 (p=NS)	-0.04 (p=NS)	0.08 (p=NS)	-0.01 (p=NS)	0.35 (p=NS)	0.12 (p=NS)
MCHL EBV pos	0.22 (p=NS)	0.63 (p=<0.01)	0.44 (p=NS)	0.76 (p=<0.05)	0.12 (p=NS)	0.02 (p=NS)
MCHL EBV neg	-0.23 (p=NS)	-0.13 (p=NS)	0.66 (p=NS)	0.64 (p=NS)	-0.30 (p=NS)	0.51 (p=NS)

Table 7.24
Correlation between % apoptotic HRS-cells and number of macrophages

	Elderly + Young combined Pearson correlation (p value)		**Young** Pearson correlation (p value)		**Elderly** Pearson correlation (p value)	
	CD68	**CD163**	**CD68**	**CD163**	**CD68**	**CD163**
EBV pos	0.14 (p=NS)	0.22 (p=NS)	0.35 (p=NS)	0.15 (p=NS)	0.19 (p=NS)	0.05 (p=NS)
EBV neg	0.27 (p=<0.05)	0.13 (p=NS)	-0.03 (p=NS)	-0.04 (p=NS)	0.60 (p=<0.01)	0.12 (p=NS)
MCHL EBV pos	0.09 (p=NS)	0.20 (p=NS)	0.15 (p=NS)	-0.06 (p=NS)	0.17 (p=NS)	-0.01 (p=NS)
MCHL EBV neg	0.77 (p=<0.05)	0.94 (p=0.001)	0.35 (p=NS)	0.08 (p=NS)	0.77 (p=NS)	0.98 (p=<0.05)

In the young and combined cohorts there was a positive relationship between the number of apoptotic HRS cells and the number of CD163 (but not the number of CD68) cells. However, this did not apply to the percentage of apoptotic HRS cells (tables 7.23 & 7.24).

In the elderly and combined cohorts, there was a strongly positive relationship between the percentage of apoptotic HRS cells in EBV-negative MCHL cases and the number of CD163-positive cells. A similar association between CD68 cells and the percentage of apoptotic HRS cells was also seen in the combined cohort but did not reach statistical significance in the elderly. However, for EBV-negative cases as a whole, a positive relationship between the number of CD68 cells and the percentage of apoptotic HRS cells was seen and was statistically significant (table 7.24).

HRS-cells v CD68-positive and CD163-positive macrophages

No significant associations between the number of HRS cells and the number of CD68- or CD163-positive cells were seen in any of the three cohorts or subgroups thereof (table 7.25).

Table 7.25

Correlation between the number of HRS-cells and macrophages

	Elderly + Young combined Pearson correlation (p)	Young Pearson correlation (p)	Elderly Pearson correlation (p)
CD68 v HRS	-0.14 (p=NS)	0.01 (p=<0.05)	-0.02 (p=NS)
CD163 v HRS	-0.15 (p=NS)	-0.11 (p=NS)	-0.18 (p=NS)

HRS-cells v apoptotic cells

No correlation was found between the number of HRS cells and the total number of apoptotic cells.

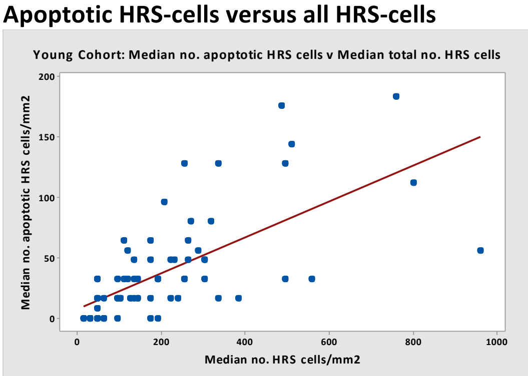
Other cell combinations

There was a strong correlation between the number of HRS cells and the number of apoptotic HRS cells in all cases (figures 7.7 & 7.8). CD68- and CD163-positive cell numbers were closely associated in the young cohort but not in the elderly (figures 7.9 & 7.10).

Figure 7.7

Young cohort:

Apoptotic HRS-cells versus all HRS-cells

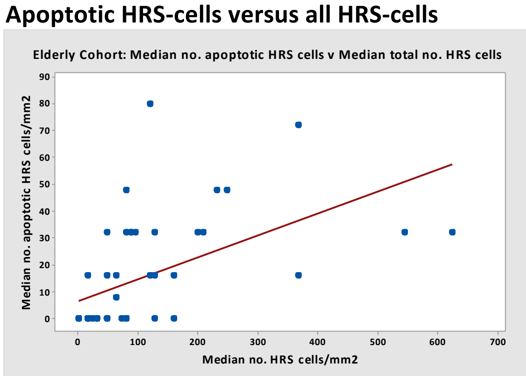


Pearson correlation=0.66 (p=<0.001)

Figure 7.8

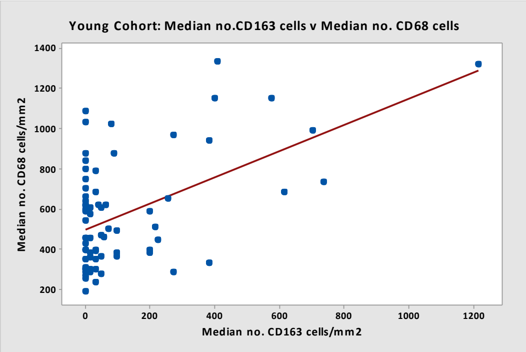
Elderly cohort:

Apoptotic HRS-cells versus all HRS-cells



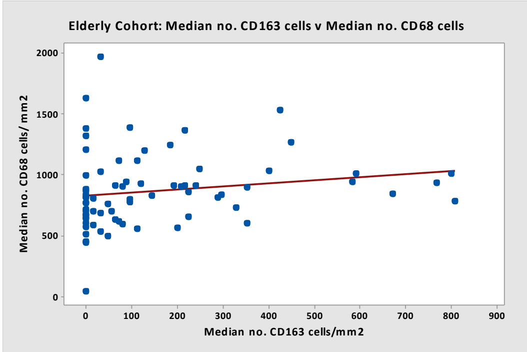
Pearson correlation=0.53 (p=<0.001)

Figure 7.9
Young cohort:
CD163 versus CD68-positive cells



Pearson correlation=0.52 (p=<0.001)

Figure 7.10
Elderly cohort:
CD163 versus CD68-positive cells



Pearson correlation=0.18 (p=NS)

Discussion

Recording apoptosis

There are a number of different ways of identifying apoptotic cells within histological sections. These include identification of the morphological features of apoptosis by light microscopy; electron-microscopy; the identification of DNA fragmentation using, for example, in-situ hybridisation for DNA strand breaks or terminal transferase-mediated dUTP nick-end labelling (TUNEL) assays; and IHC for apoptosis associated proteins such as activated caspase-3, caspase-7 or the cleaved products of CK18 or Poly-ADP-ribose polymerase 1 (c-PARP). These all have their limitations but IHC for activated caspase-3 was chosen as caspase-3 is activated in all three apoptosis pathways and is one of the main 'executioner' proteins of the process. It has been shown to be a sensitive, reliable and relatively simple technique for the identification of apoptotic cells, even before any of the typical morphological features of apoptosis develop. IHC labelling for activated caspase-3 does not appear to be affected by differences in fixation, unlike the antibody for CK18. The TUNEL assay has been widely used as a sensitive method of detecting DNA fragmentation associated with apoptosis but reliable results are heavily dependent on technique and it is also influenced by fixation, which in a retrospective study, such as this, cannot be controlled. c-PARP is an attractive target for IHC as it represents the cleaved product of PARP, a substrate of both caspase-3 and -7, but results have been less consistent than with activated caspase-3 antibodies. Caspase-7 is also an 'executioner' caspase but its role is less well understood than that of caspase-3. It is likely that some apoptotic cells are missed if activated caspase-3 is used as this will not identify cells expressing caspase-7 or other potential end-stage caspases but overall the interpretation of caspase-7 expression is less clear (Duan et al. 2003; Bressenot et al. 2009).

Total apoptosis

An increased number of apoptotic cells, both HRS and microenvironmental, was associated with an increased infiltration of macrophages in the cohorts studied. All of the results for the total number of apoptotic cells in each tumour, considering age, gender, EBV status and cHL subtype, essentially mirrored those seen for the number of CD68- and CD163-positive cells. This was confirmed when a consistent, statistically significant correlation was found between the number of apoptotic cells and the number of CD68-positive and CD163-positive cells. One explanation is that chemotactic factors produced by apoptotic cells are responsible for this. It is thought that specialised phagocytic cells only become involved in clearing apoptotic cells when the rate of apoptosis is high and therefore beyond the capacity of adjacent, non-specialist, cells (Savill et al. 2002; Truman et al. 2004; Gregory & Devitt 2004). If apoptotic cells are not cleared rapidly they undergo secondary necrosis which can be damaging to tissues and is implicated in the development of chronic inflammatory and autoimmune disorders (Gregory & Pound 2011; Savill et al. 2002; Gregory & Devitt 2004; Maderna & Godson 2003). A number of factors produced by cells undergoing apoptosis are chemotactic for monocytes and macrophages, including lysophosphatidylcholine (LPC), fractalkine (FKN), nucleotides ATP and UTP, S19, S1P, Macrophage Chemotactic Protein-1 (MCP-1), Endothelial-monocyte-activating polypeptide II (EMAP II), thrombospondin-1 (TSP-1), TGF-B, annexin-1, lactoferrin and heat shock proteins (HSP) (Truman et al. 2004; Truman et al. 2008; Bournazou et al. 2009; Gregory & Pound 2011), which may be in keeping with the findings in both cohorts. However, this does not explain why apoptosis is more frequent in some cases than in others.

EBV and apoptosis

In the young cohort, EBV-positive in comparison to EBV-negative cases, were found to have a greater number of apoptotic cells overall but there was no difference in the number or proportion of HRS cells undergoing apoptosis. The EBV status of cHL cases refers to whether the HRS cells show evidence of infection by EBV and therefore it might be expected that any effects of the virus on apoptosis would be within HRS cells themselves. However, GEP studies on whole sections of cHL tumours have found an increased expression of caspase-1 in EBV-positive cases and this may suggest a more general increase in apoptosis in EBV-positive cases, through as yet unknown mechanisms (Chetaille et al. 2009).

Evidence about the effects of EBV on apoptosis however, is contradictory. It has been proposed that HRS cells may avoid apoptosis in EBV-positive cases, through the actions of EBV-encoded proteins including LMP-1 (Benharroch et al. 2010; Benharroch et al. 1999; Snow & Martinez 2007). Others however, have shown that the percentage of HRS cells undergoing apoptosis, measured using the TUNEL assay, was greater in EBV-positive cases than EBV-negative cHL (Benharroch et al. 1999). Infection by other viruses such as the measles virus has also been associated with increased apoptosis, including a study which looked at the number of apoptotic HRS cells in cHL but did not distinguish according to EBV status (Benharroch et al. 2010). Viruses are thought to be able to trigger 'apoptosis-like' cell death leading to NF-KB activation and pro-inflammatory effects (Savill et al. 2002) but apoptosis is also one protective mechanism used by hosts to eradicate virally infected cells (Cvetanovic & Ucker 2004). One mechanism of viral-induced apoptosis may be through endoplasmic reticulum (ER) stress which if not rectified by a cell leads to its apoptosis (Verfaillie et al. 2013). EBV infection has also been shown to increase activation of both STAT 1 and STAT 3 signalling pathways which have opposing effects on apoptosis (Alvaro et al. 2008) and it is therefore possible that EBV may be associated with the promotion or inhibition of apoptosis according to circumstance.

As well as an increase in apoptosis, as discussed in chapter five, tissues from EBV-positive cases showed an increased number of macrophages. At least in part this may be a response to the increase in apoptosis and the resultant macrophage chemotactic factors produced but it is not possible to exclude the possibility that increased numbers of macrophages induce more apoptosis. An association is also described between gender and apoptosis, with cHL in men showing increased apoptotic rates. As previously discussed, there is a recognised link between EBV-positive cHL and male gender, which may also explain the link with increased apoptosis.

Apoptosis and prognosis

In none of the three cohorts, young, elderly or combined, was any association between the total number of apoptotic cells and outcome demonstrated. This included comparisons of those who relapsed and those who did not; those who relapsed or required an escalation in treatment and those who did not; plus those who died of any cause and those who lived. Data regarding those who died of cHL however was not included, as the number of patients in this category, especially in the young cohort, was too few. Similarly, it is not possible to exclude an association between apoptosis and prognosis because the number of patients doing badly by any of the outcome measures, was small.

HRS-cells

HRS cells were more numerous in the tumours of young than elderly cHL patients. HRS cells were also more frequent in EBV-negative than in EBV-positive cases. While this did not reach statistical significance in all subgroups, it should be noted that the number of cases in each subgroup could be quite small and there were especially few cases of EBV-positive disease in young women.

HRS cell numbers in young EBV-negative men were greater than in young EBV-negative women. The question therefore arises as to whether this could be related to the number of CD163-positive cells, which was also greater in EBV-negative young males than females. However this would not appear to be the case as a greater number of CD163 cells was also evident in EBV-positive young men compared to their EBV-negative counterparts and yet these EBV-positive young men had fewer HRS cells. Similarly, when a direct comparison of the number of HRS cells to CD163 or CD68 cells was made, no evident relationship was found in any group.

When comparing MCHL and NSHL subtypes of cHL, no difference overall was seen in the number of HRS cells in either. However when EBV-negative cases were compared with each other, EBV-negative MCHL cases had a greater number of HRS cells than EBV-negative cases of NSHL and this was statistically significant in the young cohort. In this group, EBV-negative MCHL also was shown to have more HRS cells than EBV-positive MCHL. It is difficult to explain this in the terms of the other cells examined within the tumours in this study, but it does add weight to the argument raised in chapter six, that there is something intrinsically different about MCHL tumours over and above their evident association with, and the effects of EBV infection.

In the young, but not the elderly, there was some evidence that the number of HRS cells was relevant to outcome as those young who relapsed or required an escalation in treatment had significantly more HRS cells than those who did not. Other studies, including patients of all ages, from children to the elderly, have shown no effect on OS related to increased HRS cells (Benharroch et al. 1999), but not distinguishing between the elderly and young could result in a subtle association, evident in one age group and not another, being missed. In the two cohorts examined here there were too few cases to conclude whether the number of HRS cells was related to other factors such as cHL subtype or EBV infection but the association with outcome was more marked in the EBV-negative cases. It should also be noted that EBV-negative cases as a whole had a greater number of HRS cells.

Despite the above findings regarding relapsed disease, opposing results were noted for those patients who died of all causes compared to those who did not. The data appeared to show that a greater number of HRS-cells was associated with a better outcome, i.e. associated with being alive at the end of follow-up. However, an explanation for this apparently contrary result may be that significantly more elderly than young patients died. The results may therefore be distorted as all elderly patients had significantly fewer HRS-cells than the young.

Apoptotic HRS-cells

The number of apoptotic HRS cells was greater in tumours of the young cohort and while this fits with the previous observation that the young had more HRS cells overall than the elderly did, it does not explain why the young had a greater percentage of HRS cells undergoing apoptosis.

Similarly, it was shown that EBV-negative tumours contained more HRS cells than EBV-positive cases, and in the elderly EBV-negative, there were a greater number and percentage of apoptotic HRS cells than in the EBV-positive. However, this was not so for the young in whom EBV-positive and –negative cases did not differ significantly in their number of apoptotic HRS cells and in whom EBV-positive cases had a greater number and percentage of apoptotic HRS cells than the elderly EBV-positive. EBV-negative cases did not vary, in the number or percentage of apoptotic HRS cells, according to age. In other words, although both age and EBV infection appear to be relevant to the number of apoptotic HRS cells, the association with EBV appears to differ between the age groups.

Although differences were evident between elderly and young males with EBV-positive disease, no differences were seen between old and young EBV-positive females. This may in large part however, be due to the small number of young women with EBV-positive disease and so the evidence for a variation according to gender is not convincing.

As with the observations of variations in the nature of EBV-negative MCHL (in relation to macrophage numbers, total apoptosis and total number of HRS cells), this subgroup also appeared to have a greater number of apoptotic HRS cells than its EBV-negative NSHL counterpart. However, the percentage of apoptotic HRS cells did not differ. This may therefore be a consequence of the greater total number of HRS cells in these tumours but is a further example of MCHL exhibiting differences not explained by any association with EBV infection.

The evident association between EBV-negative tumours and an increased number and percentage of apoptotic HRS cells has already been described above, and the finding that EBV-negative NSHL and MCHL both had a significantly greater number and percentage of apoptotic HRS cells is in accord with this.

It was found that of the EBV-negative cases in the young, those with increased numbers of HRS cells and increased numbers of apoptotic HRS cells, were more liable to relapse and had a poorer outcome. This did not hold true when considering apoptotic HRS cells as a proportion of the total and it appears that the absolute number of apoptotic HRS cells was more important. The total number of apoptotic cells was not linked to outcome. As was the case with HRS cells, those patients who did badly when death was used as the outcome, had fewer apoptotic HRS cells (although the percentage apoptotic HRS cells was unchanged). The disproportionate number of elderly patients in the group who died may offer an explanation, as the elderly would all be expected to have fewer apoptotic HRS cells.

There are theoretical reasons why apoptotic cells, HRS or otherwise, may be associated with both tumour growth and regression:

1. Increased numbers of HRS cells and apoptotic HRS cells may simply act as a marker of rapid cell turnover and indicate an ‘aggressive’ tumour which carries a poorer prognosis (Benharroch et al. 1999). However, one study has shown that an increased proportion of apoptotic HRS cells

(>5%) carries with it a favourable outcome (Dukers 2002), although this study did not make any reference to actual numbers of apoptotic cells.

2. Apoptotic cells themselves may be anti-inflammatory, producing a number of factors such as Il-10 and TGF-B which may be seen as 'pro-tumour' (Savill et al. 2002). However, in other circumstances apoptotic cells may release pro-inflammatory factors including Il-8, FAS-Ligand, TNF, Il-1B and Il-18 (Savill et al. 2002) with opposing effects on tumour progression.
3. As discussed above, apoptotic cells may attract macrophages and also inhibit the chemotaxis of neutrophils, with potential implications for tumour growth (Truman et al. 2004). Macrophages, having phagocytosed apoptotic cells, exhibit an anti-inflammatory response which suppresses both the innate and adapative immune responses and as such has a pro-tumour effect. Factors released, including TGF-B_1, PGE_2, PAF all reduce TNF levels. Anti-inflammatory Il-10 is also produced as well as factors such as lactoferrin and FKN (Mosser & Edwards 2008; Savill et al. 2002; Cvetanovic & Ucker 2004; Maderna & Godson 2003; Tesniere et al. 2008; Gregory & Pound 2011; Bournazou et al. 2009). Even if macrophages have previously been stimulated by, for example, bacterial LPS to produce an inflammatory response, phagocytosis of an apoptotic cell will significantly diminish this via inhibition of NF-KB and AP-1 induced transcription (Cvetanovic & Ucker 2004), resulting in suppression of such pro-inflammatory factors as TNF-a, Il-1B, Il-8 and Il-12 (Cvetanovic & Ucker 2004; Gregory & Pound 2011). However, the type of macrophage receptor engaged by an apoptotic cell is thought to be crucial to the response. Macrophages may behave in a pro-inflammatory, and therefore anti-tumour way if stimulated via the Fc receptor. This may occur when apoptotic cells are opsonised by the binding of antibody (Gregory & Devitt 2004). If, on the other hand, macrophages are stimulated via receptors for apoptotic-cell associated molecular patterns (ACAMP), anti-inflammatory Il-10 and TGF-B_1 are produced (Gregory & Devitt 2004). ACAMP are thought to be akin to

PAMP but unlike the pathogen related receptors that produce pro-inflammatory factors, they have an anti-inflammatory, pro-tumour effect.

4. Similarly to macrophages, DC that phagocytose apoptotic cells can inhibit or promote the immune response. In general apoptotic cells are thought to delay the maturation of DC. Il-12 production is inhibited resulting in reduced IFN-G from T-cells and suppression of a cytotoxic T-cell response in favour of developing immune tolerance to antigens (Kim et al. 2003; Maderna & Godson 2003).
5. The cell type undergoing apoptosis is also likely to influence the immune response and this may explain why an increased number of apoptotic HRS cells appears to be of importance to the outcome when the total number of all apoptotic cells is not. Again, which macrophage receptor is activated is of importance and tumour cells may ectopically express proteins on their cell surface, which are normally only intracellular and this is likely to influence apoptotis. HSP and calreticulin are two such examples (Albert 2004).
6. It is also suggested that when the number of apoptotic cells in any one area increases, the proportion of these engulfed by professional phagocytic cells rather than neighbouring cells is likely to increase and it is very likely that this has downstream effects on the immune response (Savill et al. 2002; Truman et al. 2004; Albert 2004). Therefore, in tumours with fewer apoptotic HRS cells, it is conceivable that a larger proportion of these are phagocytosed by neighbouring, non-professional cells, the effects of which, very little is known (Albert 2004).
7. Aside from the anti-inflammatory effects of apoptotic cells, they also appear to promote cell proliferation and differentiation of surrounding cells resulting in an overall expansion of tumour cells in some cases. Neighbouring cells are stimulated to produce a number of growth and pro-angiogenic factors such as vasoactive endothelial growth factor (VEGF). Microparticles, originating from apoptotic cells are thought to travel significant distances and may aid metastasis by promoting a 'tumour-friendly' environment through interactions with stromal cells.

In addition, caspases-3 and -7 may increase cell motility as well as stimulate cell differentiation and proliferation. Apoptotic cells may directly promote new vessel formation through electrostatic membrane changes which influence endothelial directional sprouting (Gregory & Pound 2011; Westhoff et al. 2014). Studies dating back as far as 1956 have suggested that apoptotic cells can accelerate tumour growth. A very neat illustration comes from a murine study, which involved inoculating three groups of mice with combinations of viable and, what are now recognised to be, apoptotic tumour cells. Those mice injected with both cell types exhibited a much more rapid tumour growth than those only given viable tumour cells and, as would be expected, those only given apoptotic cells (the result of previous treatment with irradiation) did not develop tumours at all (Revesz 1956). Again however, it should be noted that these findings are not universal and in Hepatocellular Carcinoma for example, an increased number of apoptotic tumour cells has also been linked with a more favourable outcome (Chew et al. 2010).

Within this general discussion there is therefore a rationale for why the absolute number of apoptotic HRS-cells in a tumour, rather than just the percentage, may affect outcome and why this should be distinguished from the effects of apopototic cells of other types.

Of the young cohort, those cHL tumours with increased numbers of HRS-cells were associated with an increased risk of relapse or requirement to escalate treatment, even without considering the number of apoptotic HRS-cells. In this group, EBV-negative tumours had significantly more HRS-cells than EBV-positive. EBV-negative tumours are more likely to be of NSHL subtype and there is thought to be a link between NSHL and the expression of the anti-apoptotic protein Bcl-2 (Rassidakis et al. 2002). Although increased apoptosis of HRS cells may be one mechanism of disease progression, it is unlikely to be the only factor and it may be that in some cases, added resistance to apoptosis allows tumour cells to proliferate and/or evade treatment-related apoptosis leading to a less favourable outcome. A link with reduced HRS-cell numbers and less resistance to apoptosis may be one reason why, in the young cohort, those with EBV-positive disease showed improved outcome. It is argued that the EBV protein, LMP-1, theoretically inhibits apoptosis but the data does not appear to support this (Benharroch et al. 2010; Benharroch et al. 1999).

Expression of cytotoxic molecules and apoptosis

Apoptosis in relation to the cytotoxic molecule status (i.e. expression by HRS-cells of any of the cytotoxic molecules TIA-1, Granzyme B, or Perforin) of each case was also examined as was the expression of pan-T-cell antigens. In the elderly but not in the young, the CMpos tumours had significantly greater numbers of apoptotic cells. The number of cases was small but no difference was seen between those that were CMpos, PAX5-positive and CMpos, PAX5-negative. Although there was no difference between the CMpos and CMneg tumours with regard to the absolute number of HRS-cells, there was a difference in the number and percentage of apoptotic HRS-cells which also varied between the two age groups. In the young, those CMpos rather than CMneg tumours had fewer apoptotic HRS-cells, but in the elderly the contrary appeared to be true. Similarly, when comparisons were made between those tumours that did or did not express T-cell antigens, in the elderly those that were T-cell antigen-positive had a significantly greater number and percentage of apoptotic HRS-cells and this was not seen in the young. This latter result does however need to be interpreted with caution as there were very few T-cell antigen-positive tumours altogether and especially few in the elderly cohort. When looking further at the T-cell antigen expressing tumours, in the combined cohort these had significantly fewer background apoptotic cells than their T-cell antigen-negative counterparts. This however also needs to be interpreted in context. The majority of T-cell antigen expressing tumours were EBV-negative and in young patients both of which factors, as described previously, have an association with smaller numbers of apoptotic cells and therefore the significance of each cannot be determined by this study. Finally when looking at T-cell antigen expression, those positive tumours had greater numbers of HRS cells. Again, it has been shown that tumours from young patients tend to have more HRS cells but in this case, the difference was also evident in the young cohort when examined separately, i.e. the T-cell antigen-positive tumours from young patients had greater numbers of HRS cells than the T-cell antigen-negative tumours from the young.

Interpretation of these findings is limited by the small number of cases and there is no published data looking at this. There is limited data examining general rates of apoptosis in association with numbers of CTL in the microenvironment of cHL. There appears to be an association between reduced numbers of CTL and fewer apoptotic HRS-cells and it is suggested that this may be the result of increased anti-apoptotic factors. On the contrary, increased numbers of activated CTL, along with other cells of the immune system, is linked to an increased proliferation of tumour cells and with EBV-positive disease (Alvaro et al. 2008). One hypothesis is that HRS-cells, resistant to apoptosis, may prevent CTL mounting an effective response to EBV within HRS-cells and may be why these tumours are seen to proliferate (Barros, Vera-Lozada, et al. 2012). CTL normally induce apoptosis via either FAS/FAS-L or via Granzyme B and Perforin directly, both of which culminate in the activation of caspase-3 (Dukers 2002). It might therefore be supposed that CMpos cases of cHL would be associated with increased apoptosis but of key importance is that the expression of these molecules by HRS-cells is ectopic and Granzymes and Perforin are unlikely to function as they would in a true CTL. Why expression of such molecules by HRS-cells should be associated with reduced or increased levels of apoptosis and what implications this may have is yet to be discovered.

Cell-cell relationships

As noted above, the total number of apoptotic cells in each tumour, mirrored the results for CD68 in both cohorts, as well as for CD163 in the young, with a statistically significant positive correlation between both CD68- and CD163-positive cell numbers and the number of apoptotic cells.

When subgroups of each cohort were examined, the small number of cases in some subgroups limited the statistical power of the test. Considering the number and percentage of apoptotic HRS-cells, no statistically significant associations were seen with the number of CD68- or CD163-positive cells in most subgroups. However, with the number of cases available it is not possible to exclude significant relationships. In other comparisons, positive correlations were evident. For example, in EBV-positive cases, the number of CD163 cells was positively associated with the number of apoptotic HRS-cells. This is perhaps not unexpected given the correlation between apoptosis in general, and CD163 cells, and the strong association with CD163 in EBV-positive young cases. The lack of any association with the percentage of HRS-cells undergoing apoptosis is probably more relevant. In a similar way, the relationship between an increased percentage (although not absolute number) of apoptotic HRS-cells and the number of macrophages, in EBV-negative elderly patients, and especially in those EBV-negative MCHL cases, is of interest. This is yet a further example of MCHL tumours exhibiting differences from other tumour subtypes, not apparently related to their association with EBV infection and its effects.

No association was evident between the number of macrophages in each tumour and the number of HRS-cells and there was no significant association between the number of HRS-cells and the total number of apoptotic cells per tumour. However, the number of HRS-cells undergoing apoptosis, strongly correlated with the total number of HRS-cells in each tumour and also the number of CD68- and CD163-positive cells correlated with each other in the young (although not in the elderly).

Conclusions

The total number and number of 'background' apoptotic cells correlated with the number of CD68 and CD163 cells in each cohort and all subgroups. The literature would suggest that this may be because apoptotic cells 'attract' macrophages but whether macrophages are also responsible for inducing cells to undergo apoptosis cannot be answered by this study.

Once more, EBV-negative MCHL exhibited features different to those of other subgroups, but as before, the small number of cases in this subgroup limits interpretation.

HRS-cells and apoptotic HRS-cells were more frequent in the young, in whom there appeared to be a link between greater numbers of HRS-cells and a poor prognosis. This differed from the elderly in whom no such association was seen. In both groups, EBV negativity was associated with greater numbers of HRS-cells but the elderly also differed from the young with regard to the association between EBV infection and the number of apoptotic HRS-cells. In the elderly, the EBV-positive had fewer apoptotic HRS-cells but in the young, this was not apparent. This raises the question as to whether EBV-infection and the apoptosis of HRS-cells in the elderly influence each other differently than in the young. Although, not reaching statistical significance, there was also a trend towards greater total numbers of apoptotic cells in the EBV-positive young but no difference between EBV-positive and –negative cases in the elderly, which also highlights apparent variations between EBV infection in the young and elderly.

Of uncertain significance, but also suggesting a difference between the elderly and young was that the number and percentage of apoptotic HRS-cells varied between CMpos and CMneg cases in both groups but in opposing ways. In the young with CMpos compared with CMneg disease, there were fewer apoptotic HRS-cells, but in the elderly the contrary appeared to be true.

Thus, apoptosis, previously seen as an inert process, merely present to keep tissue expansion in check and important in embryogenesis, is seen to have potentially wide-reaching effects on tumour progression as well as being one of the key ways in which anti-cancer treatments take effect. While this process can have directly opposing effects, in most malignancies, apoptosis is thought to play an anti-inflammatory/pro-tumour role, attracting macrophages but programming them to be tumour promoting, while suppressing the innate and adaptive anti-tumour immune responses and stimulating angiogenesis and cell proliferation as well as enabling tumour metastasis. The varying response to the apoptosis of malignant cells compared to their surrounding non-malignant counterparts is also of great interest and merits further study.

Plasmacytoid dendritic cells are key antigen presenting cells and as such may be involved in the response to apoptotic cells within a tumour. These cells can be identified immunophenotypically and were also examined in tumours of both the young and elderly cHL cohorts.

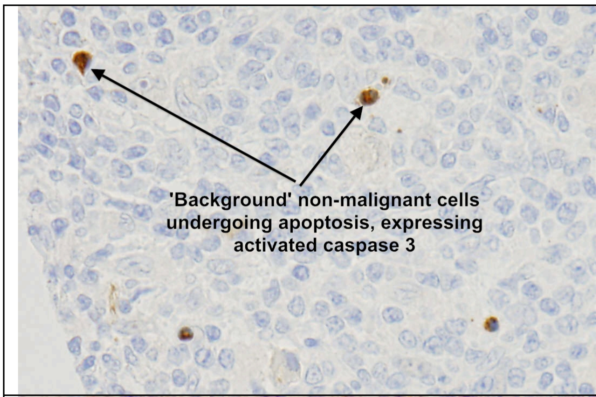


Figure 7.1
Activated caspase-3 IHC
Tumour 26, Young cohort, MCHL (x40)
Apoptotic benign cells in the tumour microenvironment.

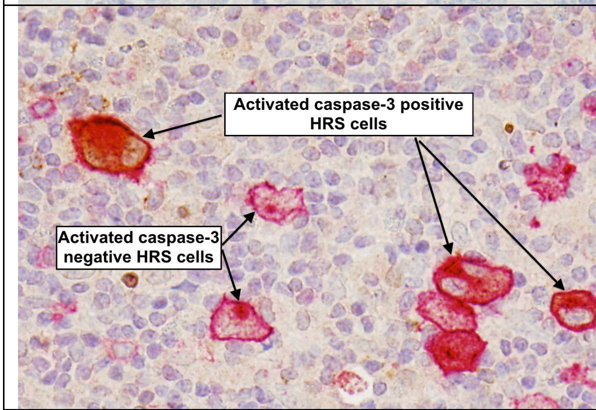


Figure 7.2
CD30 & activated caspase-3 dual-labelling
Tumour 13, Young cohort, NSHL (x40)
HRS-cells expressing cytoplasmic activated caspase-3 and thus evidence of apoptosis (brown DAB chromogen). HRS-cell membrane expression of CD30 (red New Fucsin label).

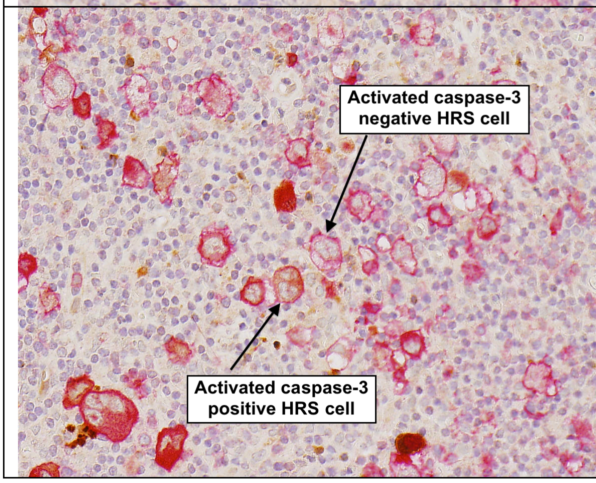


Figure 7.3
CD30 & activated caspase-3 dual-labelling
Tumour 13, Young cohort, NSHL (x20)

Chapter 8

Plasmacytoid Dendritic Cells in classic Hodgkin Lymphoma

Introduction

Dendritic cells are haematopoietic cells which, as part of the innate immune system, have an important role in the first line of defence against pathogens but are also antigen presenting cells, and therefore key in linking innate and adaptive immune responses (Merad & Manz 2009; Enblad et al. 2007).

Plasmacytoid dendritic cells are thought to develop from a progenitor cell in the bone marrow and enter the peripheral circulation once fully mature, from where they migrate into T-cell rich areas of lymphoid organs (Lande & Gilliet 2010). Although few in number, pDC are mainly found in the blood (0.2 – 0.8% of peripheral blood cells), bone marrow and lymphoid tissue (Lande & Gilliet 2010; Reizis et al. 2011). In response to viral infections or inflammation they migrate into affected tissues but other than the intestine and kidney, are rarely found in peripheral tissues outwith these circumstances (Lande & Gilliet 2010; Merad & Manz 2009).

Immunophenotypically pDC express CD4, CD56 and CD68 but lack most of the B-cell, T-cell, NK and myeloid lineage markers. CD123 (interleukin-3 receptor), BDCA4 (neuronal receptor, neurolipin-1), BDCA2 (Blood Dendritic Cell antigen) and ILT-7 (Immunoglobulin-like transcript 7) are all thought to be relatively specific markers of pDC (Lande & Gilliet 2010; Reizis et al. 2011; Marafioti et al. 2008; Merad & Manz 2009).

The main distinguishing feature of these cells is their ability to produce extremely large quantities of Interferon-alpha (IFN-a) in response to viruses. In comparison to other haematopoietic cells they produce up to 1000 times more interferon, accounting for over 95% of all type-1 interferons produced in the blood. Interferon production is mediated via the Toll-like receptors-7 and -9 (TLR-7, TLR-9) (Lande & Gilliet 2010; Reizis et al. 2011). TLR-7 and -9 are located intracellularly. TLR-7 recognises single-stranded RNA, specifically guanosine and uridine-rich single-stranded RNA, and therefore recognises genomes such as those of the influenza virus. TLR-9 recognises unmethylated

CpG oligonucleotides within DNA and therefore responds to double-stranded DNA viruses such as Herpes Simplex Virus. Because of their intracellular location, TLR-7 and -9 can only respond to a virus if there is endocytosis of the virion, bringing the RNA or DNA genome into contact with the receptors. On recognition of their respective ligands multiple steps ultimately lead to the transcription of type-1 IFN genes and the production of large quantities of IFN-a (Severa et al. 2013; Lande & Gilliet 2010; Reizis et al. 2011). Type-1 IFN induces the transcription of a number of genes which inhibit viral replication and the spread of viruses, it primes CTL and NK cell responses and stimulates the humoral immune response from B-cells (Lim et al. 2007). In addition to IFN, TLR-7/-9 stimulation results in the production of other cytokines, such as TNF-a, and chemokines that attract other effector cells of the immune system. It induces more effective antigen presentation and stimulates T-cell activation, proliferation, and polarisation to, for example, a T_{H1} phenotype (Lande & Gilliet 2010; Reizis et al. 2011). Simultaneously however, activated DC, use negative feedback mechanisms to induce T-reg cells and limit the T-cell response (Lande & Gilliet 2010). pDC may also be activated via alternative TLR-7/-9 signalling pathways or via other receptors and in these circumstances pro-inflammatory cytokines may be produced or T-cells primed to a T_{H2} or T-reg cell phenotype. Non-activated pDC also play an important role, particularly in immune/self tolerance. These cells present antigens but usually endogenous/self-antigens and instead of stimulating destructive T-cell responses, antigen-specific T-reg cells proliferate preventing auto-immune reactions (Darrasse-Jèze et al. 2009).

pDC are found in many solid tumours, including head and neck, breast, ovarian, lung and skin cancers. On the whole, these cells appear to be present in a non-activated state and are thought to contribute to an immunosuppressive environment within the tumour (Sawant et al. 2012; Conrad et al. 2012; Lande & Gilliet 2010; Steinman & Banchereau 2007). In breast and ovarian tumours, higher numbers of pDC are a poor prognostic marker, associated with tumour metastasis and relapse (Conrad et al. 2012; Lande & Gilliet 2010).

There is very little published data about pDC in HL. Recognising their evident prognostic significance in other tumours and, in addition, their importance in responding to viruses such as EBV, their role in cHL is of great interest.

Materials and Methods

BDCA2 was chosen as an appropriate, specific marker of pDC and identified using IHC, with cells expressing BDCA2 enumerated as previously described (chapter two). Only cases included in the TMAs were examined. P-values relate to the Mann-Whitney test unless stated otherwise.

Results

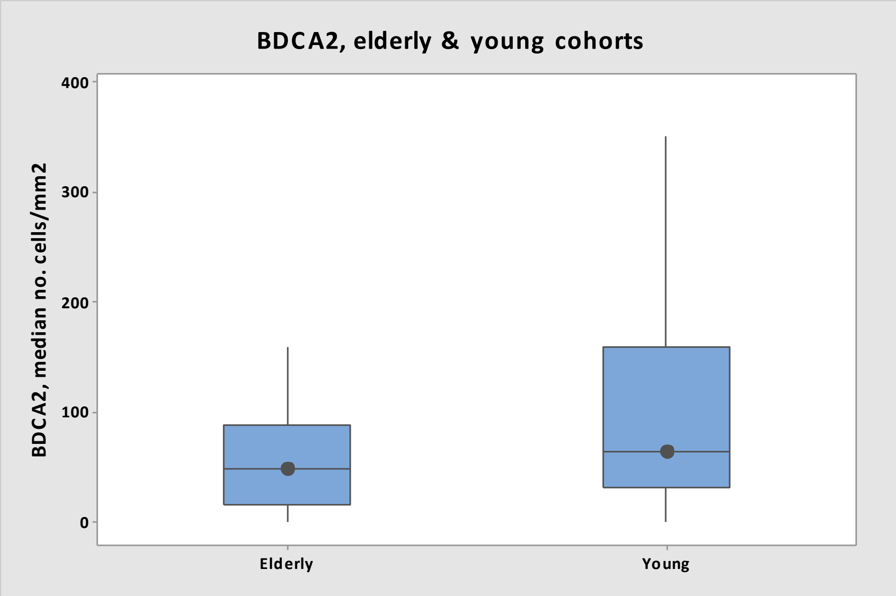
BDCA2 by age and gender

The median number of BDCA2-positive cells was greater in the young cohort than the elderly (p=<0.05). There was no difference in the number of BDCA2 cells according to gender (table 8.1 & figure 8.1).

Table 8.1
BDCA2-positive cells and gender

	Elderly + Young combined Median cell count per mm^2	**Young** Median cell count per mm^2	**Elderly** Median cell count per mm^2	**Young v Elderly**
Total no. cases	118 *41 missing	71 *11 missing	47 *30 missing	
All cases (n) (range)	48 (118) (0-848)	64 (71) (0-848)	48 (47) (0-432)	p=<0.05
Male (n)	52(56)	64 (33)	48 (23)	p=NS
Female (n)	48(59)	60 (38)	32 (21) *3 missing	p=<0.05
Male v Female	p=NS	p=NS	p=NS	

Figure 8.1
BDCA2-cells per cohort



(Elderly median = 48, Young median = 64, p=<0.05)

BDCA2 by EBV status

The median number of BDCA2 cells did not differ between EBV-positive and -negative cases in either cohort or in either gender.

BDCA2 by cHL subtype

In cases of NSHL, the young had a greater median number of BDCA2 cells than the elderly although this did not reach statistical significance (64 v 32, p=0.06). In MCHL cases, the median number of BDCA2 cells varied less between the young and elderly (40 and 44 respectively, p=0.34). No statistical difference was evident between NSHL and MCHL tumours in either cohort (data not shown).

When EBV-positive and –negative cases of each subtype were compared, EBV-negative NSHL had greater numbers of BDCA2 cells than EBV-negative MCHL. This was evident in all cohorts but reached statistical significance in the young and combined groups (young, p=0.05; combined, p=<0.01). In the combined cohort, EBV-positive cases of MCHL had significantly greater numbers of BDCA2 cells than EBV-negative MCHL (p=0.05). No such difference was evident between EBV-positive and –negative cases of NSHL (table 8.2).

Table 8.2
BDCA2-cells, cHL subtype and EBV

	Elderly + Young combined Median cell count per mm²		**Young** Median cell count per mm²		**Elderly** Median cell count per mm²	
EBV status	**Pos**	**Neg**	**Pos**	**Neg**	**Pos**	**Neg**
NSHL (n)	32 (16)	48 (64)	32 (7)	64 (47)	32 (9)	32(17)
	p=NS		p=NS		p=NS	
MCHL (n)	80 (21)	16 (8)	96 (7)	24 (4)	60(14)	0 (4)
	p=0.05		p=NS		p=NS	
cHL other (n)	216 (3)	80 (6)	228(2)	40 (4)	NP	NP
	p=NS		p=NS		NA	
NSHL v MCHL	P=NS	P=<0.01	P=NS	P=0.05	P=NS	P=NS

NP=calculation not possible

BDCA2 and cytotoxic molecule expression

The median number of BDCA2 cells did not differ significantly between those tumours that did or did not express cytotoxic molecules in any of the three cohorts (table 8.3). There was also no evident difference between those CMpos cases that were PAX5-positive or -negative.

Table 8.3
BDCA2-cells and cytotoxic molecule expression

	Elderly + Young combined Median cell count per mm²		**Young** Median cell count per mm²		**Elderly** Median cell count per mm²	
	CMpos	**CMneg**	**CMpos**	**CMneg**	**CMpos**	**CMneg**
BDCA2 (n)	80 (17)	48 (101)	96 (9)	60 (62)	80 (8)	32 (39)
	p=NS		p=NS		p=NS	

BDCA2 and T-cell antigen expression

In all three cohorts, the T-cell antigen-positive tumours had fewer BDCA2 cells than those that did not express T-cell antigens. This reached statistical significance in the combined and young cohorts (p=0.05 and p=<0.05 respectively) (table 8.4).

Table 8.4

BDCA2-cells and T-cell antigen expression

	Elderly + Young combined Median cell count per mm^2		**Young** Median cell count per mm^2		**Elderly** Median cell count per mm^2	
T-cell antigen	**Pos**	**Neg**	**Pos**	**Neg**	**Pos**	**Neg**
BDCA2 (n)	32 (16)	48 (81)	48 (13)	80 (45)	16 (3)	32 (36)
	p=0.05		p=<0.05		p=NS	

BDCA2 and outcome: relapse

When all cases, EBV-positive and –negative, were considered together, no difference was seen between those that did and did not relapse. However, in EBV-negative cases, those who relapsed had tumours containing fewer BDCA2 cells than those who did not relapse (young and combined cohorts, p=<0.05) (table 8.5).

Table 8.5

BDCA2-cells, EBV and outcome: relapse

	Elderly + Young combined Median cell count per mm^2		**Young** Median cell count per mm^2		**Elderly** Median cell count per mm^2	
EBV status	**Pos**	**Neg**	**Pos**	**Neg**	**Pos**	**Neg**
Relapse (n)	160 (7)	32 (12)	256 (2)	32 (10)	160 (5)	36 (2)
	p=0.01		p=0.06		p=NS	
No relapse (n)	48 (31)	56 (65)	80 (13)	64 (45)	40 (18)	40 (20)
	p=NS		p=NS		p=NS	
Relapse v no relapse	p=<0.05	p=<0.05	p=NS	p=<0.05	p=<0.05	p=NS

This contrasted with the EBV-positive cases in which it was the tumours of those who did not relapse that contained significantly fewer BDCA2 cells when compared to those who subsequently relapsed (elderly and combined cohorts, p=<0.05) (table 8.5).

When EBV-positive cases of patients who did not subsequently suffer relapse were compared with EBV-negative equivalent cases, there was no significant difference in the number of tumour infiltrating BDCA2 cells. However, in keeping with the above results, EBV-positive cases of relapse contained significantly greater numbers of BDCA2 cells than EBV-negative cases of relapse (combined cohort, p=0.01) (table 8.5).

BDCA2 and outcome: relapse or escalation in treatment

When cases of patients who required an escalation in treatment were included with those who developed relapsed disease, results were very similar to those described above. In the young cohort, those patients who relapsed or required an escalation in treatment had tumours containing significantly fewer BDCA2-positive cells (p=<0.01) (table 8.6).

Table 8.6
BDCA2-cells and outcome: relapse or escalation in treatment (Rx)

	Elderly + Young combined Median cell count per mm^2	**Young** Median cell count per mm^2	**Elderly** Median cell count per mm^2
Relapse or escalation Rx (n)	36 (28)	32 (16)	48 (12)
No relapse or escalation Rx (n)	64 (85)	80 (54)	48 (31)
Relapse or escalation v no relapse or escalation	p=NS	p=<0.01	p=NS

Again, when EBV-negative and –positive cases were examined separately it was seen that EBV-negative cases, in which the patient went on to relapse or require an escalation in treatment, had significantly fewer BDCA2 cells (p=<0.001 in young and combined cohorts). This contrasted with the EBV-positive cases in whom, although not reaching statistically significant levels, it was those who did not go on to relapse or require an escalation in treatment that had tumours with fewer BDCA2-positive cells (table 8.7).

The difference between EBV-positive and –negative cases was confirmed when EBV-positive and –negative tumours of those patients who went on to relapse or require an escalation in treatment were compared. In all cohorts EBV-positive cases that relapsed or had an escalation in treatment contained greater numbers of BDCA2 cells than the equivalent EBV-negative cases. In the young and combined cohorts this reached statistical significance (P=<0.05 and p=<0.01 respectively). There was no evident difference in the median number of BDCA2 cells when comparing EBV-positive and –negative cases of those who did not go on to relapse or require an escalation in treatment (table 8.7).

Table 8.7
BDCA2-cells, EBV and outcome: relapse or escalation in treatment (Rx)

	Elderly + Young combined Median cell count per mm²		**Young** Median cell count per mm²		**Elderly** Median cell count per mm²	
EBV status	**Pos**	**Neg**	**Pos**	**Neg**	**Pos**	**Neg**
Relapse or escalation Rx (n)	104(10)	32 (18)	256 (2)	32 (14)	84 (8)	28 (4)
	p=<0.01		p=<0.05		p=NS	
No relapse or escalation Rx (n)	56 (26)	64 (59)	80 (13)	88 (41)	32 (13)	48 (18)
	p=NS		p=NS		p=NS	
Relapse or escalation v no relapse or escalation	p=NS	p=<0.001	p=NS	p=<0.001	p=NS	p=NS

BDCA2 and outcome: death, any cause and death due to cHL

There was no evident difference in the median number of BDCA2 cells in tumours of those patients who did or did not die, of any cause or as a result of cHL. This was so when all cases were examined together and when EBV-negative and –positive subgroups were analysed separately (data not shown).

BDCA2 and stage at presentation

In all cohorts, those presenting with 'early stage' disease, ie stage I-IIA, had tumours containing significantly greater numbers of BDCA2-positive cells than those who presented with more advanced stage disease (table 8.8).

Table 8.8
BDCA2-cells and stage at presentation

	Elderly + Young combined Median cell count per mm²	**Young** Median cell count per mm²	**Elderly** Median cell count per mm²
Stage I-IIA (n)	112 (43)	120 (30)	80 (13)
Stage IIB-IV (n)	44 (70)	48 (38)	32 (32)
Stage I-IIA v Stage IIB-IV	p=<0.01	p=<0.05	p=<0.05

Early-stage=stage I-IIA, Advanced-stage=stage IIB-IV

When EBV-positive and –negative subgroups were examined, it was evident that the difference in BDCA2 cell numbers was more marked in the EBV-positive tumours. In both EBV-positive and –negative cases, there were greater numbers of BDCA2-positive cells in the tumours of those presenting with stage I-IIA disease but this was only statistically significant in the EBV-positive young and combined cohorts (p=<0.01 and p=0.001 respectively). In the young, those EBV-positive tumours, in patients presenting with low-stage disease had significantly greater numbers of BDCA2 cells than the equivalent EBV-negative cases (p=0.01). This difference was not evident in the elderly cohort or in those presenting with high-stage disease (table 8.9).

Table 8.9
BDCA2-cells, EBV and stage at presentation

	Elderly + Young combined Median cell count per mm^2		**Young** Median cell count per mm^2		**Elderly** Median cell count per mm^2	
EBV status	**Pos**	**Neg**	**Pos**	**Neg**	**Pos**	**Neg**
Stage I-IIA (n)	188 (12)	88 (32)	224 (7)	80 (24)	80 (5)	96 (8)
	p=NS		p=0.01		p=NS	
Stage IIB-IV (n)	32 (26)	48 (43)	32 (9)	48 (28)	32 (17)	32 (15)
	p=NS		p=NS		p=NS	
Stage I-IIA v Stage IIB-IV	p=0.001	p=NS	p=<0.01	p=NS	p=NS	p=NS

Early-stage=stage I-IIA, Advanced-stage=stage IIB-IV

In the young cohort, those patients with early-stage disease but who subsequently went on to relapse or require an escalation in treatment, had significantly fewer BDCA2 cells than those who did not relapse or require increased treatment (p=<0.05). No such difference was evident between patients presenting with advanced-stage disease (table 8.10). Above, it is shown that those with early-stage disease had greater numbers of BDCA2 cells than those with advanced-stage disease (table 8.8) but it can be seen that this was only true in the tumours of those patients who did not go on to relapse/increase treatment, and in those who did there was no significant difference in the median number of BDCA2 cells (table 8.10).

Table 8.10
BDCA2-cells, stage at presentation and outcome: relapse or increase in treatment

	Elderly + Young combined Median cell count per mm²		Young Median cell count per mm²		Elderly Median cell count per mm²	
Relapse or increase Rx	**Yes**	**No**	**Yes**	**No**	**Yes**	**No**
Stage I-IIA (n)	48 (11)	120 (33)	32 (8)	160 (23)	160 (3)	80 (10)
	p=NS		p=<0.05		p=NS	
Stage IIB-IV (n)	32 (14	48 (50)	36 (6)	48 (30)	32 (8)	32 (20)
	p=NS		p=NS		p=NS	
Stage I-IIA v Stage IIB-IV	p=NS	p=<0.01	p=NS	p=<0.05	p=0.06	p=NS

Early-stage=stage I-IIA, Advanced-stage=stage IIB-IV

There were too few cases to look at EBV-positive and -negative subgroups of early- and advanced-stage disease who did and did not subsequently relapse or require an escalation in treatment.

Correlation of different cell types

The number of BDCA2-positive cells within each case was then compared with a number of other cell types present within each tumour and the Pearson correlation was used to calculate significance as described in chapter two.

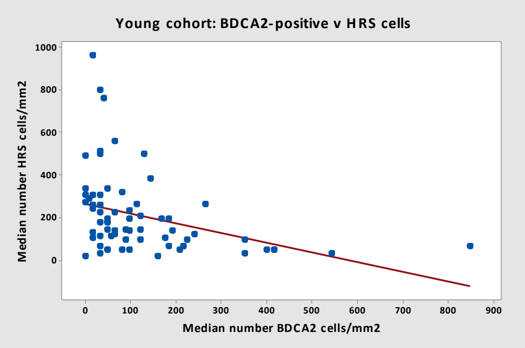
BDCA2-cells versus CD68-positive and CD163-positive macrophages

There was no evident correlation between the median number of BDCA2 cells, CD68 or CD163-positive cells in any cohort. This was when all cases were examined together, when tumours from males and females were examined individually and when EBV-positive and –negative cases were analysed separately (data not shown).

BDCA2-cells versus HRS-cells

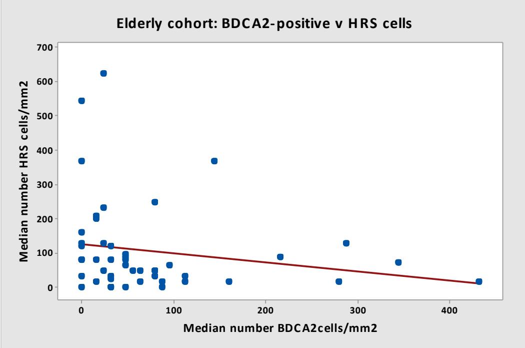
In the young and combined cohorts, but not in the elderly, there was a significant negative correlation between the median number of BDCA2 cells and HRS cells. This was true regardless of gender and was evident in EBV-negative tumours but was not statistically significant in EBV-positive cases, although it may be noteworthy that the number of EBV-positive cases in the young was small (figures 8.2 & 8.3, table 8.11).

Figure 8.2
Young cohort: BDCA2-cells versus HRS-cells



Pearson correlation -0.35 (p=<0.01)

Figure 8.3
Elderly cohort: BDCA2-cells versus HRS-cells



Pearson correlation -0.19 (p=NS)

Cases of NSHL also showed a negative correlation between BDCA2 and HRS-cell numbers but no statistically significant correlation was evident in cases of MCHL (table 8.11).

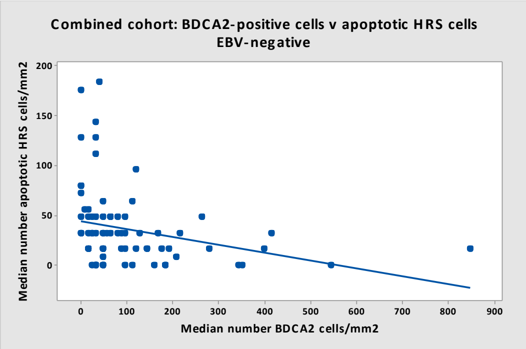
Table 8.11
BDCA2-cells versus HRS-cells

	Elderly + Young combined Pearson correlation (p value)	Young Pearson correlation (p value)	Elderly Pearson correlation (p value)
All	-0.25 (p=<0.01)	-0.35 (p=<0.01)	-0.19 (p=NS)
EBV pos	-0.18 (p=NS)	-0.33 (p=NS)	-0.21 (p=NS)
EBV neg	-0.29 (p=0.01)	-0.37 (p=<0.01)	-0.18 (p=NS)
NSHL	-0.24 (p=<0.05)	-0.32 (p=<0.05)	-0.18 (p=NS)
MCHL	-0.22 (p=NS)	-0.51 (p=NS)	-0.19 (p=NS)

BDCA2-cells versus apoptotic HRS-cells

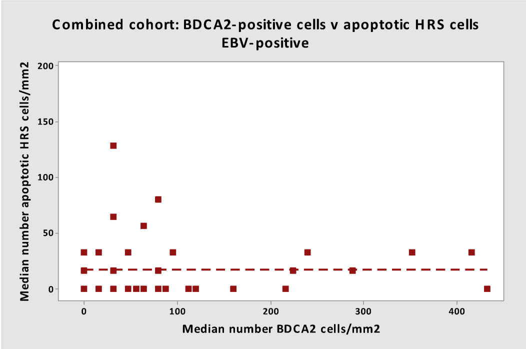
A negative correlation was seen between the number of BDCA2-positive cells and apoptotic HRS-cells in the young and combined cohorts but not in the elderly. This was only statistically significant when the whole cohort was examined together (combined, Pearson correlation -0.20, p=<0.05; young, Pearson correlation -0.29, p=<0.05) and in the EBV-negative subgroup (combined, Pearson correlation -0.28, p=0.01; young, Pearson correlation -0.31, p=<0.05). In male/female subgroups the negative correlation was not statistically significant. In EBV-positive cases there was no evident correlation between apoptotic HRS and BDCA2-positive cells (figures 8.4 & 8.5).

Figure 8.4
EBV-negative:
BDCA2-cells versus apoptotic HRS-cells



Pearson correlation -0.28 (p=0.01)

Figure 8.5
EBV-positive:
BDCA2-cells versus apoptotic HRS-cells



Pearson correlation 0.002 (p=NS)

BDCA2-cells versus total and background apoptotic cells

No correlation was evident between the number of BDCA2 cells and the total number of apoptotic cells or number of 'background' apoptotic cells (as defined in chapter two) in any cohort, male/female or EBV-positive/-negative subgroups (data not shown).

Discussion

IHC for BDCA2 was selected to identify pDC as BDCA2 is thought to be a specific marker of these cells. Amongst other antigens, pDC may also express CD68 (Marafioti et al. 2008; Lande & Gilliet 2010; Merad & Manz 2009; Reizis et al. 2011) and it could therefore be argued that CD68 was not an appropriate marker of macrophages to use (chapter six) as it may not distinguish between the two cell types. However, no correlation was found between the number of CD68-positive and BDCA2-positive infiltrating cells in the cases of cHL studied, whereas there was a strong correlation between the number of CD68 cells and the other 'macrophage marker' CD163. This suggests that BDCA2 identified a group of cells distinct from those expressing CD68. Dual-labelling IHC for BDCA2 and CD68 may be useful but with single-labelling IHC it would not be possible to conclude that the CD68-positive cells, assumed to be macrophages, did not include pDC. However, even if this were the case, BDCA2 appears to have identified a phenotypically distinct group of pDC.

Greater numbers of BDCA2-positive cells were seen in tumours from the young cohort when compared with the elderly. DC may be reduced in number and function with increasing age (Ademokun et al. 2010; Grubeck-loebenstein et al. 2009; Kaszubowska 2008) and it is not clear whether these findings reflect this or a more specific change related to the tumour microenvironment in cHL. In cases of MCHL, no significant difference in BDCA2 cell numbers was evident according to age, whereas a difference (although not statistically significant) was seen in cases of NSHL. This inconsistency may suggest that any age-related difference in BDCA2 cell numbers is not simply a reflection of normal physiology.

Patients who are immunocompromised through various different mechanisms, exhibit impaired cell-mediated immunity, which may in part be due to reduced numbers of pDC. Renal and cardiac transplant patients have been shown to have fewer circulating pDC with an associated reduction in type-1 IFN production in response to viral infections. One theory is that this poor pDC response may contribute to EBV-associated lymphoproliferative disorders in these patients. Studies in mice would appear to support this with reduced numbers of pDC in NOD-SCID mice associated with an increased risk of EBV-related infections and lymphoproliferative disorders (Lim et al. 2007). With fewer pDC in elderly patients, this may be one explanation for the higher incidence of EBV-related cHL in older adults.

When comparing EBV-positive and –negative cases, no significant overall difference in the number of pDC was found but EBV-positive cases of MCHL had significantly greater numbers of pDC than EBV-negative MCHL tumours. EBV-negative NSHL also had greater numbers of BDCA2 cells than EBV-negative MCHL. Once more, EBV-negative MCHL exhibits unique features; in this case fewer pDC than was evident in other subgroups.

There appeared to be no association between CMpos or CMneg disease and the number of tumour infiltrating pDC. However, those tumours in which HRS cells expressed T-cell antigens, had significantly fewer BDCA2 cells; although the rationale for this is not clear.

In the young cohort, but not the elderly, there was a statistically significant negative correlation between the number of HRS-cells in a tumour and the number of BDCA2-positive cells. This was seen in EBV-positive and –negative subgroups of young cHL, as well as in NSHL and MCHL but did not reach statistical significance in either the EBV-positive or MCHL cases (the majority of which were EBV-positive), which may have been due to the small number of tumours in each of these subgroups. A similar negative correlation was also evident between the number of apoptotic HRS-cells and BDCA2-cells in the young. Interestingly, HRS-cells were evident in greater numbers in the tumours of the young cohort than the elderly, so despite the inverse relationship, both cell types were more frequent in tumours of the young than the elderly. The negative correlation was reflected in a number of other observations discussed previously (chapter seven). In the young, EBV-negative cases of NSHL had fewer HRS-cells than EBV-negative cases of MCHL which fits with the observation that in the same cohort, EBV-negative NSHL had greater numbers of BDCA2 cells than EBV-negative MCHL. Similarly, EBV-negative MCHL in the young had greater numbers of HRS-cells than EBV-positive MCHL whilst the number of BDCA2-cells was fewer in the EBV-negative MCHL tumours. When considering outcome, those young patients who went on to relapse or require an escalation in treatment had tumours with greater numbers of HRS-cells than those who did not whereas the number of BDCA2-cells was fewer in the same group.

It is not clear why the number of HRS-cells should be inversely proportional to the number of pDC and why this should vary between the elderly and young cohorts. It could be that in the young, pDC are more effective at presenting tumour antigens to elicit a specific $CD4^+$ T_{H1} response which in turn enables a more effective anti-tumour CTL response resulting in fewer HRS-cells (de Jong & Enblad 2008). Alternatively, it is possible that HRS-cells produce an inhibitory factor against pDC and that this is more active in the young. For example, it is thought that BST-2 is expressed by a number of tumours and may, by binding to ILT-7 receptors on pDC, inhibit their activation. Similarly, viruses such as HIV and Hepatitis B, are thought to inhibit pDC actions by binding to BDCA2 and it is possible that HRS-cells could act through similar mechanisms (Lande & Gilliet 2010; Reizis et al. 2011).

With regard to outcome, greater numbers of BDCA2-positive cells were associated with both a good and bad prognosis depending on the EBV-status of the tumour. In EBV-negative cases, in all cohorts, (statistically significant in the young and combined groups) greater numbers of pDC were found in the tumours of those who did not relapse or require an escalation in treatment. However in EBV-positive cases (although not reaching statistical significance), the opposite appeared to be true, with greater numbers of BDCA2-cells, in all cohorts, found in the tumours of those who subsequently relapsed or require an escalation of treatment.

No variation was found in the number of pDC between those who did and did not die, either of any cause or as a direct result of cHL. However, the number of patients who died was small and therefore it is not possible to exclude significant differences between these subgroups.

The clinical stage of disease at the time of presentation is of prognostic importance and is included in the International Prognostic Score for cHL (Hasenclever & Diehl 1998). Presenting with 'early-stage' disease is normally seen as an indication of a good prognosis but this is not absolute and there are patients with early-stage disease who subsequently go on to relapse. In each of the three cohorts, those patients with 'early-stage' disease (stage I-IIA) had tumours with significantly greater numbers of pDC than those presenting with more advanced disease. In those with 'advanced-stage' disease (stage IIB-IV), there was no difference in the number of BDCA2 cells between those with EBV-positive or –negative tumours but in the young cohort, those with EBV-positive, early-stage disease had significantly greater numbers of pDC than their EBV-negative counterparts, something which was not mirrored in the elderly cohort. It would therefore appear that in the young, EBV-positive disease associated with greater numbers of BDCA2-positive cells was associated with a poor outcome, i.e. relapse or requirement for an escalation in treatment as described above, but there was also an association between greater numbers of pDC in young patients with EBV-positive disease who presented at an early clinical stage; a combination which would normally be recognised as a marker of a good prognosis. In view of this, the number of BDCA2-positive cells in those with early-stage disease who did and did not go on to relapse or require an escalation in treatment was examined. There were too few cases to examine EBV-positive and –negative subgroups with early- and advanced-stage disease who relapsed or not but when overall numbers in each cohort were examined it was shown that in the young, those with early-stage disease who went on to relapse/increase treatment, had significantly fewer BDCA2-positive cells than those young with early-stage disease who did not. No difference was seen between those with advanced-stage disease at presentation who did and did not relapse/increase treatment. In the elderly, no statistically significant differences were evident in either those with early-or advanced-stage disease, although it should be noted that the number of elderly cases in each subgroup was small, limiting interpretation.

The prognostic significance of pDC in cHL therefore appears to vary according to circumstance. Akin to macrophages, pDC exhibit 'plasticity' with the ability to switch between different functional states without necessarily altering their phenotype, or at least BDCA2 expression, (Reizis et al. 2011; Lande & Gilliet 2010) which makes their study more complex. In some malignancies, such as breast and ovarian, greater numbers of pDC are associated with poorer survival. This is thought perhaps to be due to non-activated pDC which stimulate T-reg cells but cannot activate CTL and therefore contribute to tumour immune tolerance, permitting tumour growth (Conrad et al. 2012; Lande & Gilliet 2010; Darrasse-Jèze et al. 2009). Another mechanism may be related to CXCL10 levels which are increased when there are fewer pDC. CXCL10 inhibits angiogenesis and thus tumour growth therefore potentially contributing to a better outcome for those with tumours containing fewer pDC (Sawant et al. 2012).

Other malignancies, such as Follicular NHL however, are associated with a better outcome when greater numbers of pDC are present, which is consistent with the previous practice of using IFN-a as a treatment for Follicular NHL (Butsch et al. 2011). pDC, activated via TLR, stimulate CTL and NK-cell anti-tumour responses. For an effective anti-tumour CTL response, $CD8^+$ T-cells must recognise tumour antigens presented by tumour cells, on their cell surface, in association with MHC class I molecules. In addition $CD8^+$ cells require stimulation by cytokines produced by specific $CD4^+$ T_{H1} cells. These T_{H1} cells must first have been primed to the tumour antigens in association with MHC class II molecules by APC, which include pDC (de Jong & Enblad 2008). Activated pDC also express TNF-related apoptosis inducing ligand (TRAIL) and may directly induce tumour cell death by apoptosis (Lande & Gilliet 2010).

The influence of EBV on pDC function may also be of importance. As a double-stranded (ds) DNA virus, EBV is able to stimulate pDC via TLR-9, to produce IFN-a, activate NK- and T-cells responses (resulting in IFN-G production) and stimulate adaptive B-cell responses. However, these responses appear attenuated when compared to other viral infections. The small, non-encoding ds-RNA EBERs have single-stranded RNA motifs and as such can also stimulate pDC via TLR-7 (Severa et al. 2013; Lim et al. 2007). In addition to IFN-a, pDC activated by EBV, produce Il-10. This appears to be specific to EBV stimulation and is probably due to intrinsic Il-10 production by the viral genome itself. Il-10 is an immunosuppressive cytokine and may be part of the virus' strategy for immune evasion. This may explain why EBV-stimulated pDC produce less IFN-a than those activated by other herpes viruses and it is proposed that these reduced levels may be insufficient to activate CTL responses in the usual way

(Lim et al. 2007). EBV-activated pDC show an impaired capacity to induce T-cell proliferation and polarisation to T_{H1}. Co-stimulatory molecule expression is also reduced as is IFN-G production, all of which suggests less effective CTL activation (Severa et al. 2013). EBV is also known to directly infect pDC which then express Programmed Death-1 ligand (PD-L). This binds to PD-1 on tumour-infiltrating T-cells resulting in the inhibition of T-cell proliferation and impaired IFN-G production (Yamamoto et al. 2008). These observations could be relevant in explaining why greater numbers of pDC in EBV-positive cHL are associated with an increased risk of relapse whereas in EBV-negative cases the association is with a better prognosis. IFN-a has been used effectively to treat EBV-positive PTLD, also suggesting a rationale for a poorer outcome if IFN-a production is reduced (Lim et al. 2007).

Conclusions

The examination of pDC in the two cohorts has highlighted once more that it is not possible to apply universal conclusions regarding the tumour microenvironment in cHL to all patients. Greater numbers of pDC appear in most scenarios to be a marker of a good prognosis but in EBV-positive disease are associated with an increased incidence of relapse or requirement for an escalation in treatment. However, the young with EBV-positive disease, presenting at an early-stage have greater numbers of pDC than the equivalent patients with EBV-negative cHL and its not clear whether this is associated with a better or worse outcome as there were too few patients in each subgroup to allow a meaningful analysis. No such difference between EBV-positive and –negative cases of early-stage disease was evident in the elderly cohort and this raises many questions including whether this finding could be implicated in why young patients with EBV-positive disease do better than the elderly with EBV-related disease? It at least highlights possible differences between old and young in the tumour microenvironment, and therefore perhaps the immune response to cHL.

Chapter 9

Discussion

Classic Hodgkin Lymphoma has a UK annual incidence of 2.4 per 100 000 population (Jarrett et al. 1996) with an annual mortality of 0.7 per 100 000 (Jarrett et al. 1996). It is the second most common cancer in men aged 15-34 years and the fourth most common in women of that age group (L Yung & Linch 2005). Although less common in the elderly 10-20% of cases do occur in those over 60 years of age (20-44% in those >60 years in some series) and overall it accounts for about one quarter of all lymphomas (Stark et al. 2002). More than 90% of adolescents and young adults, presenting with early-stage disease, survive long-term and are essentially 'cured' but this figure is closer to 80% for those with advanced-stage disease at diagnosis (Jarrett et al. 1996; David et al. 2007). In elderly patients, outcome is vastly different with disease specific survival quoted at 36% for those with early-stage disease and as low as 14% for those presenting with advanced-stage disease (Stark et al. 2002). The debate as to what accounts for this huge prognostic variation is ongoing. Undoubtedly there is evidence that co-morbidities and generally poorer tolerance of intensive treatments in the elderly limits the effectiveness of therapy but whether Hodgkin Lymphoma is pathologically different in older patients is less clear (chapter one).

It has become increasingly evident that the non-malignant cells within a tumour are as important as the malignant cells in allowing that tumour to thrive and progress. HL exemplifies this more than many in that the vast majority of tumour cells are not malignant and HRS-cell dependence on these 'inflammatory' cells is illustrated clearly by the difficulty in maintaining HRS cell lines in culture and establishing cHL in immunodeficient mice (Enblad et al. 2007). A number of studies have concentrated on the significance of macrophages in cHL but a more comprehensive study of the tumour microenvironment, focusing on potential differences related to age and the presence or absence of EBV was desirable. Macrophages remain of interest as do other APC such as pDC, plus the role of apoptosis, not only in HRS-cells but also in the surrounding non-malignant cells, and the interaction of apoptotic cells with the immune response. In addition, further defining the different subtypes of cHL, including the uncommon cases in which T-cell and cytotoxic T-cell antigens are expressed, and examining whether these findings are of clinical significance was considered to be important with the potential to inform on prognosis.

Each of the two cohorts, elderly and young, were intended to be representative of cHL as a whole in their respective populations, only excluding those tumours from relapsed disease and those arising on the background of HIV infection (chapters two & three). The same specialist Haematopathologist reviewed the diagnoses according to the 2008 WHO diagnostic criteria in all cases. The young cohort was formed from patients presenting in the South East of Scotland and the elderly from patients registered with the population based, UK SHIELD study and therefore would be expected to contain patients of a similar ethnic and socio-economic mix. In terms of cHL subtype, gender and EBV-status each cohort appeared comparable with the published literature on cHL in a Western population (chapter three). However, from National Registry data it was apparent that those cases excluded from the young cohort due simply to a lack of tissue and/or clinical data included a disproportionately high number of patients who had died. This was not unexpected but did limit the power of the study and in particular the ability to distinguish factors affecting prognosis.

A number of differences between the elderly and young were identified, many of which involved variations between the two groups and their associations with EBV. Some of these were well-established differences including the increased incidence of MCHL and EBV-positive disease in the elderly. An increased proportion of the elderly died but interestingly no difference was seen in the percentage of young or elderly patients who relapsed or required an escalation in their treatment due to a poor initial response (chapter three). The number of CD68-positive macrophages was greater in the tumours of the elderly than those of the young and in both groups was greater in EBV-positive than EBV-negative tumours. However, this was not so for CD163-positive cells, which were not more frequent in tumours of the elderly and only appeared to vary with EBV in the young (chapter six). Results for the total number of apoptotic cells mirrored those of the macrophages, with tumours in the elderly containing greater numbers of apoptotic cells as a whole than the young. As was the case for CD163, the number of background apoptotic cells was greater in the tumours of the young with EBV-positive disease than in the EBV-negative tumours of that group; something which was not evident in the elderly (chapter seven). In keeping with these observations, it is recognised that a number of factors produced by apoptotic cells are chemotactic for macrophages but what is less clear is how much influence macrophages themselves have on inducing apoptosis of surrounding cells and what, if any, influence this has on outcome.

The number of pDC also varied between the age groups with tumours of the young containing greater numbers. In general, greater numbers of pDC were associated with markers of good prognosis, namely early-stage disease. Of particular interest, was the finding that those young with early-stage disease (and therefore normally expected to do well) but who did go on to relapse had tumours containing significantly fewer BDCA2-positive pDC. This area also highlighted further differences according to EBV-status. EBV-negative tumours with greater numbers of pDC were associated with patients who did not relapse or require an escalation in treatment whereas those EBV-positive tumours with greater numbers of pDC were associated with an increased incidence of relapse/escalation in treatment (chapter eight). These findings lead on to the question of whether there might be a difference in pDC numbers, between those elderly and young with early-stage disease, who have EBV-positive or –negative tumours and who do or do not go on to relapse, but there were insufficient cases to look at this. However, what was evident in the young was a negative correlation between the number of pDC cells and the number of HRS-cells. In keeping with this and mirroring the generally poorer prognosis associated with a tumour having fewer pDC was the finding that in the young, greater numbers of HRS cells were associated with a poorer outcome (chapters seven & eight).

There were also other areas in which there were differences between the elderly, young, EBV-status and their associations with prognosis. There is a significant quantity of published data suggesting that EBV-positive disease is associated with a better outcome in the young but a poorer prognosis in the elderly (Armstrong et al. 1994; Barros, Hassan, et al. 2012; Capkun et al. 2001; Keegan et al. 2005; Kwon et al. 2006; Naresh et al. 2000; Vassallo et al. 2001; Diepstra et al. 2009; Enblad et al. 1999; Jarrett et al. 2005). In the relatively small cohorts studied for this thesis, this was also suggested by a non-significant trend towards an increase in relapsed disease in the elderly with EBV-positive tumours but a trend towards fewer cases of relapsed disease in the young with EBV-related tumours (chapter five). Similarly of interest, was the observation that the elderly with EBV-negative disease and the young who did not relapse had tumours with increased numbers of CD163-positive cells and an increased ratio of CD163:CD68 cells. In the elderly with EBV-positive disease the opposite appeared to be true, with a greater proportion of CD163 cells linked to an increased incidence of relapsed disease (chapter six). This raises the question as to whether macrophages in the elderly with EBV-infection, function differently to those in the young with EBV-positive disease and whether this may be part of the explanation for the variation in prognosis associated with EBV in the two age groups. Another difference between the elderly with and without EBV-related disease was the observation that those elderly with EBV-positive disease had fewer apoptotic HRS cells; both fewer in absolute number and as a proportion of all HRS cells. Those with EBV-negative disease appeared to have a greater number and percentage of apoptotic HRS cells (chapter seven). Again these findings lead on to the question as to whether this could also be relevant to the poorer outcome seen in the elderly with EBV-positive disease.

As a whole, tumours in the young had greater numbers of HRS cells than the elderly and within the young cohort, a greater number of HRS cells was evident in those with EBV-negative disease. There was also an association between increased numbers of HRS cells and a poorer outcome in young patients, plus in particular an association between increased numbers of apoptotic HRS cells in the EBV-negative young who did poorly and again the question is raised as to whether this is relevant to the variation in prognosis for the young with and without EBV-related disease (chapter seven).

One consistent finding throughout was that the small number of MCHL tumours that were not EBV-related were different, both from EBV-negative cases of NSHL and from EBV-positive MCHL. These tumours had greater numbers of CD68 and CD163 cells than their EBV-negative NSHL counterparts; a difference which was not evident between EBV-positive cases of MCHL and NSHL (chapter six). In keeping with this and the correlation between macrophages and apoptotic cells, was the increased total number of apoptotic cells in EBV-negative MCHL. These tumours in the young also had comparatively greater numbers of HRS cells and apoptotic HRS cells with a corresponding smaller number of pDC than either EBV-negative NSHL or EBV-positive MCHL (chapters seven, eight). These results indicate that it would be of value to examine these tumours within a larger cohort to see if any of these histological variations are associated with prognosis.

T-cell antigens were expressed significantly more frequently by HRS cells in cHL of young patients and there was also an association with EBV-negative tumours in this group. Those T-cell antigen-positive cases also appeared to contain greater numbers of HRS cells and fewer pDC. Although, in the young these two features had been linked with a poorer prognosis, the expression of T-cell antigens themselves did not seem to be associated with clinical outcome (chapters four, seven & eight). Cytotoxic molecule expression by HRS cells was seen in a minority of cases, in keeping with published figures. These cases were as likely to express the B-cell antigen PAX5 as any CMneg cases and there did not appear to be any association between the expression of cytotoxic molecules and the other T-cell antigens. In only one CMpos case (which was also PAX5-positive), were PCR studies successfully completed using microdissected HRS cells to show a clonal IgH gene rearrangement and therefore evidence that the CMpos cells were of B-cell origin. Two further cases were found to contain clonal gene rearrangements using PCR on whole tissue sections. One was a CMpos, PAX5-negative case and this was found to contain a B-cell clone and the second was a CMpos, PAX5-positive case that was found to contain a T-cell clone (chapter four). It is difficult, using whole section PCR, to know whether these clonal cells are the malignant CM-expressing HRS cells or otherwise. In the small number of published CMpos cases, two which were also PAX5-negative, were found to contain clonal TCR rearrangements and it is not known whether a third case that also had evidence of a T-cell clone on HRS single-cell PCR, expressed PAX5 or not. This is an area that merits further study as, although no significant differences in outcome between CMpos and negative cases was observed, those with CMpos tumours presented more frequently with B-symptoms and advanced-stage disease, both of which are considered markers of a poor prognosis. The importance of defining these differences and eliciting whether some cases of what appear to be cHL by all other parameters are in fact T-cell disorders depends on whether this influences patient outcome and therefore treatment decisions. With the information currently available, it would seem reasonable to conclude that the majority of CMpos cases of cHL which are also PAX5-positive, are B-cell disorders. However, in CMpos, PAX5-negative tumours the possibility of a T-cell lymphoma should be especially carefully considered, even though there is no definite evidence that these patients fare less well with cHL targeted treatment. The expression of T-cell antigens in the absence of cytotoxic molecules does not appear to be of clinical significance.

Immunohistochemistry as a technique has advantages over gene expression profiling, namely that it identifies not just gene expression but gene translation into proteins, therefore also accounting for post-transcriptional modifications. However, the sensitivity of protein detection is not clear for each antigen/antibody reaction and it is possible that protein expression below the level of IHC detection may still be clinically significant and equally that detectable levels are not significant. Making comparisons between different IHC studies is also not straightforward as different antibody clones may not identify identical proteins plus there may be differences in the enumeration of positive-cells. Many of the published studies examining macrophages have used what appear to be arbitrary values for 'high' and 'low' macrophage numbers, which is why it is important, as in this study, to deal with cell quantification as a continuous variable. The identification of individual cells expressing some proteins is also more difficult than others. For example, enumerating apoptotic cells, which have started to disperse into numerous apoptotic bodies, is difficult and inevitably introduces a degree of subjectivity. More fundamentally, we also should ask how likely it is, particularly for cells such as macrophages and pDC that are known to exhibit 'plasticity', that a single protein will be unique to a single cell-type undertaking unique functions. A combination of targeted gene expression analysis and IHC would seem likely to give a more comprehensive assessment of important proteins and pathways in the tumour microenvironment. The use of laser-capture microdissection to remove particular cells of interest, not just HRS cells but microenvironmental cells such as macrophages, also holds significant promise.

The importance of delineating the role of the immune system in cHL is to inform us as to how these complex interactions may be modulated thus leading to new ways of combatting the disease. While this is a long-term goal there are already promising results in animal models, such as a mouse study in which metastatic renal carcinomas were seen to regress after treatment with Il-2 and a stimulating anti-CD40 antibody (Weiss et al. 2009). More pertinent to cHL however, is the recent interest in 'immune checkpoint pathways' which include the Programmed Death-1 pathway. PD-1 is expressed by cells of the immune system, and binding of the PD-1 receptor by either of its ligands, PD-L1 or PD-L2, results in a reversible inhibition of T-cell activation and proliferation with reduced T-cell cytotoxicity, reduced production of T-cell cytokines and an increased susceptibility of immune cells to apoptosis (Armand 2015; Ansell et al. 2015). The PD-1 pathway is implicated as one mechanism of immune evasion in many tumour types including cHL (Paydas et al. 2015; Armand 2015; Ansell et al. 2015). HRS cells have been shown to express PD-L1 and PD-L2 with evidence of amplification of chromosome 9p24.1, which contains the gene loci, not just for these proteins but also for Janus Kinase 2 (JAK2). Increased JAK-STAT activity induces further PD-L1 transcription, thus demonstrating more than one mechanism by which HRS cells 'overexpress' PD-1 ligands. In addition some studies suggest that EBV also increases PD-L1 expression in EBV-positive cases of cHL. It is therefore thought that signalling through the PD-1 pathway may be one mechanism by which HRS cells evade the immune response (Armand 2015; Ansell et al. 2015). This has already been taken into clinical trials and in phase I studies, a human monoclonal antibody which blocks PD-1 (Nivolumab), and therefore prevents the inhibition of T-cell activity by interfering with PD-1 ligand binding, has demonstrated activity in cases of multiply relapsed HL with promising initial results (Ansell et al. 2015).

Conclusions

What this current study has been able to do is highlight the complex number of inter-dependent factors of the immune system and the cHL tumour itself that vary with age and/or EBV-status. Tumours of the elderly contained more macrophages than those of the young, as did EBV-positive tumours compared with EBV-negative, but those macrophages varied phenotypically with CD163 expression seen much less frequently in the elderly. The question raised is whether the altered phenotype corresponds with an altered function? In the young and EBV-negative elderly, a greater proportion of CD163-positive macrophages is a marker of a better prognosis, whereas in the EBV-positive elderly the same observation is a marker of a poorer prognosis suggesting that CD163, although a useful marker of a subgroup of macrophages, does not adequately reflect the function of those cells.

There is evidence to suggest that both macrophages and pDC may play a different role in EBV-positive and –negative tumours as increased numbers of both were linked with good prognostic markers except in those EBV-positive cases. The number of macrophages corresponded with the total number of apoptotic cells within a tumour and this has not previously been demonstrated in cHL. pDC show promise in helping to identify those patients with early-stage disease (who would therefore normally be expected to do well) but who subsequently relapse, at least in the young. cHL tumours may express any of the three cytotoxic molecules and although this does not preclude the diagnosis of cHL in the majority of cases, these tumours do show some features suggestive of a poorer prognosis and merit further study.

The above observations allow the suggestion that the host's immune response to EBV affects the likelihood of cHL developing and once cHL has developed, the host's immune response to EBV (in EBV-positive cases) and the tumour itself (in all cases) affects how the disease progresses and responds to treatment. The immune system alters with age and is less effective in mounting a response to EBV in older patients. Therefore EBV is more likely to 'result' in cHL in the elderly and when older patients develop EBV-positive cHL, their immune system is less effective at combatting the tumour. In the young, on the other hand, if an EBV-positive tumour does develop, the immune system is better able to combat the tumour with the added 'target' of EBV. These age-related changes in the immune system may be exemplified by an altered number and phenotype of macrophages, and an altered number of pDC, in the different age groups.

Future research should use larger cohorts of cHL, differentiated by age and EBV-status and use a combination of quantitative PCR gene expression and IHC techniques to examine apoptosis, EBV-related proteins, macrophage and pDC functions in each of them. Techniques such as the Fluidigm Dynamic Array Integrated Fluidics Circuit could be used to quantitate the expression of up to 96 different genes in up to 96 cHL tumours at a time using RNA extracted from FFPE tissue of cHL tumours (a number of potential genes of interest to take this forward are identified and detailed in appendix D).

This thesis illustrates the potential to employ a combination of clinical, histopathological and molecular techniques, in assessing the prognosis of individual patients with cHL, adding to what can be predicted using the established prognostic factors. It highlights the importance of determining how microenvironmental factors vary according to circumstance and the need to account for this whilst also opening the door to the development of novel treatments aimed at manipulating the tumour microenvironment in cHL with the ultimate aim of improving patient survival.

References

Ademokun, A., Wu, Y.-C. & Dunn-Walters, D., 2010. The ageing B cell population: composition and function. *Biogerontology*, 11, pp.125–137.

Aguilera, N.S.I., Chen, J., Bijwaard, K.E., et al., 2006. Gene rearrangement and comparative genomic hybridization studies of classic Hodgkin lymphoma expressing T-cell antigens. *Archives of Pathology and Laboratory Medicine*, 130(12), pp.1772–1779.

Albert, M.L., 2004. Death-defying immunity: do apoptotic cells influence antigen processing and presentation? *Nature Reviews. Immunology*, 4, pp.223–231.

Alvaro, T., Lejeune, M., García, J.F., et al., 2008. Tumor-infiltrated immune response correlates with alterations in the apoptotic and cell cycle pathways in Hodgkin and Reed-Sternberg cells. *Clinical Cancer Research*, 14(3), pp.685–91.

Álvaro, T., Lejeune, M., Salvadó, M.T., et al., 2005. Outcome in Hodgkin ' s Lymphoma Can Be Predicted from the Presence of Accompanying Cytotoxic and Regulatory T Cells. *Clinical Cancer Research*, 11(4), pp.1467–1473.

Alvaro-Naranjo, T., Lejeune, M., Salvadó-Usach, M.T., et al., 2005. Tumor-infiltrating cells as a prognostic factor in Hodgkin's lymphoma: a quantitative tissue microarray study in a large retrospective cohort of 267 patients. *Leukemia & Lymphoma*, 46(11), pp.1581–91.

Ansell, S.M., Lisokhin, A.M., I, B., et al., 2015. PD-1 Blockade with Nivolumab in Relapsed or Refractory Hodgkin's Lymphoma. *New England Journal of Medicine*, 372(4), pp.311–319.

Armand, P., 2015. Immune checkpoint blockade in hematologic malignancies. *Blood*, 125(22), pp.3393–3400.

Armstrong, A., Lennard, A. & Alexander, F., 1994. Prognostic significance of Epstein-barr virus association in Hodgkin's Disease. *European Journal of Cancer*, 30A, pp.1045–1046.

Armstrong, A.A., Alexander, F.E., Cartwright, R., et al., 1998. Epstein–Barr virus and Hodgkin ' s disease: further evidence for the three disease hypothesis. *Leukemia*, 12, pp.1272–1276.

Asano, N., Kinoshita, T., Tamaru, J.-I., et al., 2011. Cytotoxic molecule-positive classical Hodgkin's lymphoma: a clinicopathological comparison with cytotoxic molecule-positive peripheral T-cell lymphoma of not otherwise specified type. *Haematologica*, 96(11), pp.1636–43.

Asano, N., Oshiro, A., Matsuo, K., et al., 2006. Prognostic Significance of T-Cell or Cytotoxic Molecules Phenotype in Classical Hodgkin's Lymphoma: A Clinicopathologic Study. *Journal of Clinical Oncology*, 24(28), pp.4626–4633.

Asano, N., Yamamoto, K., Tamaru, J., et al., 2009. Age-related Epstein-Barr virus (EBV)– associated B-cell lymphoproliferative disorders: comparison with EBV-positive classic Hodgkin lymphoma in elderly patients. *Blood*, 113(12), pp.2629–2636.

Azambuja, D., Natkunam, Y., Biasoli, I., et al., 2012. Lack of association of tumor-associated macrophages with clinical outcome in patients with classical Hodgkin's lymphoma. *Annals of Oncology*, 23(3), pp.736–42.

Banchereau, J. & Steinman, R.M., 1998. Dendritic cells and the control of immunity. *Nature*, 392(6673), pp.245–52.

Barros, H.M., Hassan, R. & Niedobitek, G., 2011. Disease patterns in pediatric classical Hodgkin lymphoma : a report from a developing area in Brazil. *Hematological Oncology*, 29, pp.190–195.

Barros, M.H.M., Hassan, R. & Niedobitek, G., 2012. Tumor-associated macrophages in pediatric classical Hodgkin lymphoma: association with Epstein-Barr virus, lymphocyte subsets, and prognostic impact. *Clinical Cancer Research*, 18(14), pp.3762–71.

Barros, M.H.M., Vera-Lozada, G., Soares, F. a, et al., 2012. Tumor microenvironment composition in pediatric classical Hodgkin lymphoma is modulated by age and Epstein-Barr virus infection. *International Journal of Cancer*, 131(5), pp.1142–52.

Barry, T.S., Jaffe, E.S., Sorbara, L., et al., 2003. Peripheral T-cell lymphomas expressing CD30 and CD15. *The American Journal of Surgical Pathology*, 27(12), pp.1513–22.

Baumforth, K.R.N., Birgersdotter, A., Reynolds, G.M., et al., 2008. Expression of the Epstein-Barr virus-encoded Epstein-Barr virus nuclear antigen 1 in Hodgkin's lymphoma cells mediates Up-regulation of CCL20 and the migration of regulatory T cells. *The American Journal of Pathology*, 173(1), pp.195–204.

Bellas, C., Santon, A., Manzanal, A., et al., 1996. Pathological, immunological and molecular features of Hodgkin's Disease associated with HIV infection-comparison with ordinary Hodgkin's Disease. *American Journal of Surgical Pathology*, 20(12), pp.1520–1524.

Benharroch, D., Einav, I., Feldman, A., et al., 2010. Apoptosis of Hodgkin-Reed-Sternberg cells in classical Hodgkin lymphoma revisited. *APMIS : Acta Pathologica, Microbiologica, et Immunologica Scandinavica*, 118(5), pp.339–45.

Benharroch, D., Levy, A., Prinsloo, I., et al., 1999. Apoptotic Index as a Prognostic Factor in Hodgkin's Disease. *Leukemia and Lymphoma*, 33, pp.351–359.

Biswas, S.K., Gangi, L., Paul, S., et al., 2006. A distinct and unique transcriptional program expressed by tumor-associated macrophages (defective NF-kappaB and enhanced IRF-3/STAT1 activation). *Blood*, 107(5), pp.2112–22.

Bloom, E.T., 1991. Functional importance of CD4+ and CD8+ cells in cytotoxic lymphocytes activity and associated gene expression. Impact on the age-related decline in lytic activity. *European Journal of Immunology*, 21, pp.1013–1017.

Bloom, E.T., Umehara, H., Bleackley, R.C., et al., 1990. Age-related decrement in cytotoxic T lymphocyte (CTL) activity is associated with decreased levels of mRNA encoded by two CTL-associated serine esterase genes and the perforin gene in mice. *European Journal of Immunology*, 20, pp.2309–2316.

Bournazou, I., Pound, J.D., Duffin, R., et al., 2009. Apoptotic human cells inhibit migration of granulocytes via release of lactoferrin. *The Journal of Clinical Investigation*, 119(1), pp.20–32.

Bressenot, A., Marchal, S., Bezdetnaya, L., et al., 2009. Assessment of apoptosis by immunohistochemistry to active caspase-3, active caspase-7, or cleaved PARP in monolayer cells and spheroid and subcutaneous xenografts of human carcinoma. *The Journal of Histochemistry and Cytochemistry*, 57(4), pp.289–300.

Van Den Broek, M.F., Kägi, D., Zinkernagel, R.M., et al., 1995. Perforin dependence of natural killer cell-mediated tumor control in vivo. *European Journal of Immunology*, 25, pp.3514–3516.

Buechler, C., Ritter, M., Orso, E., et al., 2000. Regulation of scavenger receptor CD163 expression in human monocytes and macrophages by pro- and antiinflammatory stimuli. *Journal of Leukocyte Biology*, 67, pp.97–103.

Burnet, M., 1957. Cancer - A Biological Approach. *British Medical Journal*, 1, pp.841–847.

Butsch, R., Lukas Waelti, S., Schaerer, S., et al., 2011. Intratumoral plasmacytoid dendritic cells associate with increased survival in patients with follicular lymphoma. *Leukemia & Lymphoma*, 52(7), pp.1230–8.

Campo, E., Swerdlow, S.H., Harris, N.L., et al., 2014. The 2008 WHO classification of lymphoid neoplasms and beyond : evolving concepts and practical applications The 2008 WHO classification of lymphoid neoplasms and beyond : evolving concepts and practical applications. *Blood*, 117(19), pp.5019–5033.

Campoli, M. & Ferrone, S., 2008. HLA antigen changes in malignant cells: epigenetic mechanisms and biologic significance. *Oncogene*, 27(45), pp.5869–85.

Capkun, V., Murray, P., Glavina-Durdov, M., et al., 2001. Assessment of the prognostic impact of the Epstein – Barr virus-encoded latent membrane protein-1 expression in Hodgkin ' s disease. *British Journal of Cancer*, 84(9), pp.1227–1234.

Chetaille, B., Bertucci, F., Finetti, P., et al., 2009. Molecular profiling of classical Hodgkin lymphoma tissues uncovers variations in the tumor microenvironment and correlations with EBV infection and outcome. *Blood*, 113(12), pp.2765–3775.

Chew, V., Tow, C., Teo, M., et al., 2010. Inflammatory tumour microenvironment is associated with superior survival in hepatocellular carcinoma patients. *Journal of Hepatology*, 52(3), pp.370–379.

Civili, S. & Matteo, I.P.S., 2011. The prognostic value of biologic markers in classical Hodgkin's Lymphoma patients. *Annals of Oncology*, 22(suppl 4), pp.153–154 Abstract 206.

Colby, T. V, Warnke, R.A. & Utility, H.E.C., 1981. Hodgkin Disease: A Clinicopathological study of 659 cases. *Cancer*, 49(9), pp.1848–1858.

Conrad, C., Gregorio, J., Wang, Y.-H., et al., 2012. Plasmacytoid dendritic cells promote immunosuppression in ovarian cancer via ICOS costimulation of Foxp3(+) T-regulatory cells. *Cancer Research*, 72(20), pp.5240–9.

Coppleson, L.W., Rappaport, H., Strum, S.B., et al., 1973. Analysis of the Rye Classification of Hodgkins Disease- The prognostic significance of cellular composition. *Journal of the National Cancer Institute*, 51, pp.379–390.

Corthay, a, 2009. How do regulatory T cells work? *Scandinavian Journal of Immunology*, 70(4), pp.326–36.

Cvetanovic, M. & Ucker, D.S., 2004. Innate immune discrimination of apoptotic cells: repression of proinflammatory macrophage transcription is coupled directly to specific recognition. *Journal of Immunology*, 172(2), pp.880–9.

Darrasse-Jèze, G., Deroubaix, S., Mouquet, H., et al., 2009. Feedback control of regulatory T cell homeostasis by dendritic cells in vivo. *The Journal of Experimental Medicine*, 206(9), pp.1853–62.

Dave, S.S., Wright, G., Ph, D., et al., 2004. Prediction of survival in Follicular Lymphoma based on molecular features of tumor-infiltrating immune cells. *New England Journal of Medicine*, 351, pp.2159–2169.

David, K. a, Mauro, L. & Evens, A.M., 2007. Relapsed and refractory Hodgkin lymphoma: transplantation strategies and novel therapeutic options. *Current Treatment Options in Oncology*, 8(5), pp.352–74.

Devilard, E., Bertucci, F., Trempat, P., et al., 2002. Gene expression profiling defines molecular subtypes of classical Hodgkins disease. *Oncogene*, 21, pp.3095–3102.

Diepstra, A., van Imhoff, G.W., Schaapveld, M., et al., 2009. Latent Epstein-Barr virus infection of tumor cells in classical Hodgkin's lymphoma predicts adverse outcome in older adult patients. *Journal of Clinical Oncology*, 27(23), pp.3815–21.

Diepstra, A., Niens, M., Vellenga, E., et al., 2005. Association with HLA class I in Epstein-Barr-virus-positive and with HLA class III in Epstein-Barr-virus-negative Hodgkin ' s lymphoma. *Lancet*, 365, pp.2216–2224.

Diepstra, A., Poppema, S., Boot, M., et al., 2008. HLA-G protein expression as a potential immune escape mechanism in classical Hodgkin's lymphoma. *Tissue Antigens*, 71(3), pp.219–26.

Duan, W.R., Gamer, D.S., Williams, S.D., et al., 2003. Comparison of immunohistochemistry for activated caspase-3 and cleaved cytokeratin 18 with the TUNEL method for quantification of apoptosis in histological sections of PC-3 subcutaneous xenografts. *Journal of Pathology*, 199(2), pp.221–228.

Dukers, D.F., 2002. High numbers of active caspase 3-positive Reed-Sternberg cells in pretreatment biopsy specimens of patients with Hodgkin disease predict favorable clinical outcome. *Blood*, 100(1), pp.36–42.

Dunn, G.P., Bruce, A.T., Ikeda, H., et al., 2002. Cancer immunoediting : from immuno- surveillance to tumor escape. *Nature Immunology*, 3(11), pp.991–998.

Eghbali, H., Raemaekers, J. & Carde, P., 2005. The EORTC strategy in the treatment of Hodgkin's lymphoma. *European Journal of Haematology*, 75(Suppl 66), pp.135–140.

Eichenauer, D. a, Engert, A. & Dreyling, M., 2011. Hodgkin's lymphoma: ESMO Clinical Practice Guidelines for diagnosis, treatment and follow-up. *Annals of Oncology*, 22 Suppl 6(Supplement 6), pp.vi55–8.

Elgert, K.D., Alleva, D.G. & Mullins, D.W., 1998. Tumor-induced immune dysfunction : the macrophage connection. *Journal of Leukocyte Biology*, 64, pp.275–290.

Enblad, G., Molin, D., Glimelius, I., et al., 2007. The potential role of innate immunity in the pathogenesis of Hodgkin's lymphoma. *Hematology/Oncology Clinics of North America*, 21(5), pp.805–23.

Enblad, G., Sandvej, K., Sundstro, C., et al., 1999. Epstein – Barr Virus Distribution in Hodgkin's Disease in an Unselected Swedish Population. *Haematologica*, 38, pp.425–429.

Epstein, M., Achong, B. & Barr, Y., 1964. Virus particles in cultured lymphoblasts from Burkitt's Lymphoma. *Lancet*, pp.702–703.

Erkeller-Yuksel, F.M., Deneys, V., Yuksel, B., et al., 1992. Age-related changes in human blood lymphocyte subpopulations. *The Journal of Pediatrics*, 120, pp.216–222.

Espino, J., Pariente, J.A. & Rodríguez, A.B., 2012. Oxidative stress and immunosenescence: Therapeutic effects of melatonin. *Oxidative Medicine and Cellular Longevity*, 2012(670294), pp.1–9.

Evans, R., 1978. Macrophage Requirement for Growth of a Murine Fibrosarcoma. *British Journal of Cancer*, 37, pp.1086–1089.

Farinha, P., Masoudi, H., Skinnider, B.F., et al., 2005. Analysis of multiple biomarkers shows that lymphoma-associated macrophage (LAM) content is an independent predictor of survival in follicular lymphoma (FL). *Blood*, 106(6), pp.2169–74.

Felgar, R.E., Macon, W.R., Kinney, M.C., et al., 1997. TIA-1 Expression in Lymphoid Neoplasms. Identification of Subsets with Cytotoxic T Lymphocyte or Natural Killer Cell Differentiation. *American Journal of Pathology*, 150(6), pp.1893–1900.

Fend, F., 2010. Hematopathology. In E. S. Jaffe et al., eds. *Hematopathology*. Saint Louis, Missouri: Saunders, Elsevier, pp. 454 –473.

Ferguson, F., 1994. Age-related changes in immune parameters in a very old population of Swedish people: A longitudinal study. *Experimental Gerontology*, 29(5), pp.531–541.

Foss, H., Reusch, R., Demel, G., Lenz, G., Anagnostopoulos, I., et al., 1999. Frequent Expression of the B-Cell-Specific Activator Protein in Reed-Sternberg Cells of Classical Hodgkin's Disease Provides Further Evidence for Its B-Cell Origin. *Blood*, 94(9), pp.3108–3113.

Foss, H., Reusch, R., Demel, G., Lenz, G. & Anagnostopoulos, I., 1999. Frequent Expression of the B-Cell– Specific Activator Protein in Reed-Sternberg Cells of Classical Hodgkin's Disease Provides Further Evidence for Its B-Cell Origin. *Blood*, 94(9), pp.3108–3113.

Gandhi, M.K., Lambley, E., Duraiswamy, J., et al., 2006. Expression of LAG-3 by tumor-infiltrating lymphocytes is coincident with the suppression of latent membrane antigen-specific CD8+ T-cell function in Hodgkin lymphoma patients. *Blood*, 108(7), pp.2280–9.

Gandhi, M.K., Tellam, J.T. & Khanna, R., 2004. Epstein-Barr virus-associated Hodgkin's lymphoma. *British Journal of Haematology*, 125(3), pp.267–81.

García, J.F., Camacho, F.I., Morente, M., et al., 2003. Hodgkin and Reed-Sternberg cells harbor alterations in the major tumor suppressor pathways and cell-cycle checkpoints: analyses using tissue microarrays. *Blood*, 101(2), pp.681–9.

García, J.F. & Piris, M.A., 2006. The presence of STAT-1 positive tumour associated macrophages and their relation to outcome in patients with Follicular NHL. *Haematologica*, 91(12), pp.1605–1612.

García-Cosío, M., Santón, A., Martín, P., et al., 2004. Analysis of transcription factor OCT.1, OCT.2 and BOB.1 expression using tissue arrays in classical Hodgkin's lymphoma. *Modern Pathology*, 17(12), pp.1531–8.

Garibaldi, R.A. & Nurse, B.A., 1986. Infections in the elderly. *American Journal of Medicine*, 81, pp.53–58.

Gillette, R.W. & Wunderlich, D.A., 1977. Retarded Growth of Lymphoma in Immunodepressed Mice. *Journal of the National Cancer Institute*, 58(4), pp.1131–1133.

Van Ginderachter, J. a, Movahedi, K., Hassanzadeh Ghassabeh, G., et al., 2006. Classical and alternative activation of mononuclear phagocytes: picking the best of both worlds for tumor promotion. *Immunobiology*, 211(6-8), pp.487–501.

Glaser, S.L., Lin, R.J., Stewart, S.L., et al., 1997. Epstein-Barr Virus-associated Hodgkin's Disease: Epidemiologic Characteristics in International Data. *International Journal of Cancer*, 70, pp.375–382.

Glimelius, I., Qvarnström, F., Simonsson, M., et al., 2012. Tissue microarray and digital image analysis: a methodological study with special reference to the microenvironment in Hodgkin lymphoma. *Histopathology*, 61(1), pp.26–32.

Gordon, S. & Taylor, P.R., 2005. Monocyte and macrophage heterogeneity. *Nature reviews. Immunology*, 5(12), pp.953–64.

Graig, F., Gulley, M. & PM, B., 1993. Posttransplantation lymphoproliferative disorders. *American Journal of Clinical Pathology*, 99, pp.265–276.

Gregory, C.D. & Devitt, A., 2004. The macrophage and the apoptotic cell: an innate immune interaction viewed simplistically? *Immunology*, 113(1), pp.1–14.

Gregory, C.D. & Pound, J.D., 2011. Cell death in the neighbourhood: Direct microenvironmental effects of apoptosis in normal and neoplastic tissues. *Journal of Pathology*, 223, pp.177–194.

Grubeck-loebenstein, B., Bella, S. Della, Iorio, A.M., et al., 2009. Immunosenescence and vaccine failure in the elderly. *Aging Clinical and Experimental Research*, 21(3), pp.201–209.

Grur, H., Pinto, A., Duyster, J., et al., 1997. Hodgkin’s disease: a tumor with disturbed immunological pathways. *Immunology Today*.

Hamilton-Dutoit, S., Pallesen, G., Franzmann, M., et al., 1991. AIDS-related lymphoma. Histopathology, immunophenotype and association with Epstein-Barr virus as demonstrated by in situ nucleic acid hybridisation. *American Journal of Pathology*, 138, pp.149–163.

Harris, J.A., Jain, S., Ren, Q., et al., 2012. CD163 versus CD68 in tumor associated macrophages of classical Hodgkin lymphoma. *Diagnostic Pathology*, 7(1), p.12.

Hasenclever, D. & Diehl, V., 1998. A Prognostic Score for Advanced Hodgkin’s Disease. *The New England Journal of Medicine*, 339, pp.1506–1514.

Hedvat, C. V., Hegde, A., Chaganti, R.S.K., et al., 2002. Application of tissue microarray technology to the study of non-Hodgkin’s and Hodgkin's lymphoma. *Human Pathology*, 33(10), pp.968–974.

Hjalgrim, H., Rostgaard, K., Johnson, P.C.D., et al., 2010. HLA-A alleles and infectious mononucleosis suggest a critical role for cytotoxic T-cell response in EBV-related Hodgkin lymphoma. *Proceedings of the National Academy of Sciences*, 107(14), pp.6400–6405.

Horstman, A., Vos, W. & Valk, P. Van Der, 1996. Analysis of major histocompatibility complex class I expression on Reed- Sternberg cells in relation to the cytotoxic T-cell response in Epstein- Barr virus-positive and -negative Hodgkin's disease. *Blood*, 87(9), pp.3844–3851.

Horvath, J. a, Mostowski, H.S., Okumura, K., et al., 1992. Pore-forming protein in individual cytotoxic T lymphocytes: the effect of senescence provides a probe for understanding the lytic mechanism. *European Journal of Immunology*, 22, pp.2649–2654.

Huang, C.T., Workman, C.J., Flies, D., et al., 2004. Role of LAG-3 in regulatory T cells. *Immunity*, 21(4), pp.503–13.

Huang, X., van den Berg, A., Gao, Z., et al., 2010. Expression of HLA class I and HLA class II by tumor cells in Chinese classical Hodgkin lymphoma patients. *PloS ONE*, 5(5), p.e10865.

Hummel, M., Marafioti, T., Ziemann, K., et al., 1996. Ig rearrangements in isolated Reed-Steinberg cells : Conclusions from four different studies. *Annals of Oncology*, 7(SuppL 4), pp.31–33.

Janeway, C.J., Travers, P. & Walport, M., 2001. Immunobiology: The Immune System in Health and Disease. In C. J. Janeway, P. Travers, & M. Walport, eds. *Immunobiology: The Immune System in Health and Disease. Principles of Innate and Adaptive Immunity.* New York: Garland Science.

Jarrett, A.F., Armstrong, A.A. & Alexander, E., 1996. Epidemiology of EBV and Hodgkin's lymphoma. *Annals of Oncology*, 7(SuppL 4), pp.5–10.

Jarrett, R.F., Stark, G.L., White, J., et al., 2005. Impact of tumor Epstein-Barr virus status on presenting features and outcome in age-defined subgroups of patients with classic Hodgkin lymphoma : a population-based study. *Blood*, 106(7), pp.2444–2451.

Jegalian, A.G., Facchetti, F. & Jaffe, E.S., 2009. Plasmacytoid dendritic cells: physiologic roles and pathologic states. *Advances in Anatomic Pathology*, 16(6), pp.392–404.

Jones, S.R., 1990. Infections in frail and vulnerable elderly patients. *The American Journal of Medicine*, 88(suppl 3C), p.30S–33S.

De Jong, D. & Enblad, G., 2008. Inflammatory cells and immune microenvironment in malignant lymphoma. *Journal of Internal Medicine*, 264(6), pp.528–36.

Kägi, D., Ledermann, B., Bürki, K., et al., 1994. Cytotoxicity mediated by T cells and natural killer cells is greatly impaired in perforin-deficient mice. *Nature*, 369, pp.31–37.

Kamper, P., Bendix, K., Hamilton-Dutoit, S., et al., 2011. Tumor-infiltrating macrophages correlate with adverse prognosis and Epstein-Barr virus status in classical Hodgkin's lymphoma. *Haematologica*, 96(2), pp.269–76.

Kanavaros, P., Viychou, M. & Stefanaki, K., 1999. Cytotoxic protein expression in Non-Hodgkin's Lymphoma and Hodgkin's Disease. *Anticancer Research*, 19(2A), pp.1209–1216.

Kanzler, B.H., Hansmann, M. & Klaus, P., 1996. Hodgkin and Reed-Sternberg Cells in Hodgkin's Disease Represent the Outgrowth of a Dominant Tumor Clone Derived from (crippled) Germinal Center B Cells. *Journal of Experimental Medicine*, 184, pp.1495–1505.

Kaszubowska, L., 2008. Telomere shortening and ageing of the immune system. *Journal of Physiology and Pharmacology*, 59(suppl 9), pp.169–186.

Kaufmann, S.H.E., 2008. Immunology's foundation : the 100-year anniversary of the Nobel Prize to Paul Ehrlich and Elie Metchnikoff. *Nature Immunology*, 9(7), pp.705–712.

Keegan, T.H.M., Glaser, S.L., Clarke, C. a, et al., 2005. Epstein-Barr virus as a marker of survival after Hodgkin's lymphoma: a population-based study. *Journal of Clinical Oncology*, 23(30), pp.7604–13.

Kelley, T.W., Pohlman, B., Elson, P., et al., 2007. The ratio of FOXP3+ regulatory T cells to granzyme B+ cytotoxic T/NK cells predicts prognosis in classical Hodgkin lymphoma and is independent of bcl-2 and MAL expression. *American Journal of Clinical Pathology*, 128(6), pp.958–65.

Kerr, J.F.R., Wyllie, A.H. & Currie, A.R., 1972. Apoptosis: A basic biological phenomenon with wide-ranging implications in tissue kinetics. *British Journal of Cancer*, 26, pp.239–257.

Kim, S.J., Gershov, D., Ma, X., et al., 2003. Opsonization of apoptotic cells and its effect on macrophage and T cell immune responses. *Annals of the New York Academy of Sciences*, 987, pp.68–78.

Kleiweg, L. & Rijswijk, Z.H., 1966. Neonatal Thymectomy and Tumor Induction With Methylcholanthrene in Mice. *Journal of the National Cancer Institute*, 36, pp.513–521.

Krenacs, L., Wellmann, A., Sorbara, L., et al., 1997. Cytotoxic Cell Antigen Expression in Anaplastic Large Cell Lymphomas of T- and Null-Cell Type and Hodgkin's Disease: Evidence for Distinct Cellular Origin. *Blood*, 89, pp.980–989.

Küppers, R., 2009a. Molecular biology of Hodgkin lymphoma. *Hematology*, pp.491–6.

Küppers, R., 2009b. The biology of Hodgkin's lymphoma. *Nature Reviews. Cancer*, 9(1), pp.15–27.

Küppers, R., Rajewsky, K., Zhao, M., et al., 1994. Hodgkin disease: Hodgkin and Reed-Sternberg cells picked from histological sections show clonal immunoglobulin gene rearrangements and appear to be derived from B cells at various stages of development. *Proceedings of the National Academy of Sciences of the United States of America*, 91(23), pp.10962–6.

Kuppers, R., U, K. & Hansmann, M., 1999. cellular origin of human B-cell lymphomas. *New England Journal of Medicine*, 341(20), pp.1520–1529.

Kwon, J.M., Park, Y.H., Kang, J.H., et al., 2006. The effect of Epstein-Barr virus status on clinical outcome in Hodgkin's lymphoma. *Annals of Hematology*, 85(7), pp.463–468.

Lakatta, E.G., Makinodan, T. & Timiras, P., 1990. The aging process. *Annals of Internal Medicine*, 113, pp.455–466.

Lande, R. & Gilliet, M., 2010. Plasmacytoid dendritic cells: key players in the initiation and regulation of immune responses. *Annals of the New York Academy of Sciences*, 1183, pp.89–103.

Lee, S.P., Constandinou, C.M., Thomas, W.A., et al., 1998. Antigen Presenting Phenotype of Hodgkin Reed-Sternberg Cells: Analysis of the HLA Class I Processing Pathway and the Effects of Interleukin-10 on Epstein-Barr Virus-Specific Cytotoxic T-Cell Recognition. *Blood*, 92(3), pp.1020–1030.

Lenoir, G. & Bornkamm, G., 1987. Burkitt's Lymphoma, a human cancer model for the study of the multistep development of cancer. Proposal for a new scenario. *Advances in Viral Oncology*, 7, pp.173–206.

Lim, W.H., Kireta, S., Russ, G.R., et al., 2007. Human plasmacytoid dendritic cells regulate immune responses to Epstein-Barr virus (EBV) infection and delay EBV-related mortality in humanized NOD-SCID mice. *Blood*, 109(3), pp.1043–1050.

Lister, T., Crowther, D., Sutcliffe, S., et al., 1989. Report of a committee convened to discuss the evaluation and staging of patients with Hodgkin's disease: Cotswolds meeting [published erratum appears in J Clin Oncol 1990 Sep;8(9):1602]. *Journal of Clinical Oncology*, 7(11), pp.1630–1636.

Listinsky, C.M., 2002. A practical approach to the diagnosis of Hodgkin lymphoma. *American Journal of Clinical Pathology*, 117(Suppl 1), pp.S76–94.

LRF, 2015. Lymphoma research foundation. *http://www.lymphoma.org/site/pp.asp?c=bkLTKaOQLmK8E&b=6300137*.

MacMahon, B., 1966. Epidemiology of Hodgkin's Disease. *Cancer Research*, 26, pp.1189–1200.

Maderna, P. & Godson, C., 2003. Phagocytosis of apoptotic cells and the resolution of inflammation. *Biochimica et Biophysica Acta*, 1639, pp.141–151.

Mani, H. & Jaffe, E.S., 2010. Hodgkin Lymphoma: an update on its biology with newer insights into classification. *Clinical Lymphoma Myeloma*, 9(3), pp.206–216.

Mantovani, A., Bottazzi, B., Colotta, F., et al., 1992. The origin and function of tumor-associated macrophages. *Immunology Today*, 13(7), pp.265–70.

Mantovani, A., Sica, A. & Locati, M., 2005. Macrophage polarization comes of age. *Immunity*, 23(4), pp.344–6.

Mantovani, A., Sozzani, S., Locati, M., et al., 2002. Macrophage polarization : tumor-associated macrophages as a paradigm for polarized M2 mononuclear phagocytes. *Trends in Immunology*, 23(11), pp.549–555.

Marafioti, T., Hummel, M., Foss, H., et al., 2000. Hodgkin and Reed-Sternberg cells represent an expansion of a single clone originating from a germinal center B-cell with functional immunoglobulin gene rearrangements but defective immunoglobulin transcription. *Blood*, 95(4), pp.1443–1450.

Marafioti, T., Paterson, J.C., Ballabio, E., et al., 2008. Novel markers of normal and neoplastic human plasmacytoid dendritic cells. *Blood*, 111(7), pp.3778–3792.

Martin, S.I., Zukerberg, L. & Robbins, G.K., 2005. Reactive Epstein-Barr virus-related polyclonal lymphoproliferative disorder in a patient with AIDS. *Clinical Infectious Diseases*, 41(8), pp.e76–9.

Merad, M. & Manz, M.G., 2009. Dendritic cell homeostasis. *Blood*, 113(15), pp.3418–27.

Merlo, A., Turrini, R., Dolcetti, R., et al., 2010. The interplay between Epstein-Barr virus and the immune system: A rationale for adoptive cell therapy of EBV-related disorders. *Haematologica*, 95, pp.1769–1777.

Mills, C.D., Kincaid, K., Alt, J.M., et al., 2000. M-1/M-2 Macrophages and the Th1/Th2 Paradigm. *The Journal of Immunology*, 164(12), pp.6166–6173.

Mills, C.D., Shearer, J., Evans, R., et al., 1992. Macrophage Arginine Metabolism and the Inhibition or Stimulation of Cancer. *The Journal of Immunology*, 149(8), pp.2709–2714.

Montalbán, C., García, J.F., Abraira, V., et al., 2004. Influence of biologic markers on the outcome of Hodgkin's lymphoma: a study by the Spanish Hodgkin's Lymphoma Study Group. *Journal of Clinical Oncology*, 22(9), pp.1664–73.

Mosser, D.M. & Edwards, J.P., 2008. Exploring the full spectrum of macrophage activation. *Nature reviews. Immunology*, 8(12), pp.958–69.

Müschen, B.M., Rajewsky, K., Bräuninger, A., et al., 2000. Rare Occurrence of Classical Hodgkin's Disease as a T Cell Lymphoma. *Journal of Experimental Medicine*, 191(2), pp.387–394.

Nador, R.G., Chadburn, A., Gundappa, G., et al., 2003. Human immunodeficiency virus (HIV)-associated polymorphic lymphoproliferative disorders. *The American Journal of Surgical Pathology*, 27(3), pp.293–302.

Nakamura, S., Jaffe, E.S. & Swerdlow, S.H., 2008. EBV positive diffuse large B-cell lymphoma of the elderly. In S. H. Swerdlow et al., eds. *WHO Classification of Tumours of Haematopoietic and Lymphoid Tissues*. Lyon: International Agency for Research on Cancer, pp. 243–244.

Naresh, K.N., Johnson, J., Srinivas, V., et al., 2000. Epstein-Barr virus association in classical Hodgkin's disease provides survival advantage to patients and correlates with higher expression of proliferation markers in Reed-Sternberg cells. *Annals of Oncology*, 11, pp.91–96.

Nathan, B.Y.C.F., Terry, W.D., Dba, M.F., et al., 1975. Differential stimulation of murine lymphoma growth in vitro by normal and BCG-activated macrophages. *Journal of Experimental Medicine*, 142, pp.887–902.

Okazaki, T. & Tasuku, H., 2007. PD-1 and PD-1 Ligands: from discovery to clinical application. *International Immunology*, 19(7), pp.813–824.

Ortiz-Hidalgo, C., 1994. A Short History of Hodgkin's Disease and Burkitt's Lymphoma. *American Journal of Clinical Pathology*, 101(4), pp.S27–S33.

Oudejans, J.J., Kummer, J.A., Jiwa, M., et al., 1996. Granzyme B Expression in Reed-Sternberg Cells of Hodgkin ' s Disease. *American Journal of Pathology*, 148(1), pp.233–240.

Paganelli, R., Quinti, I., Fagiolo, U., et al., 1992. Changes in circulating B cells and Immunoglobulin classes and subclasses in a healthy aged population. *Clinical and Experimental Immunology*, 90, pp.351–354.

Parker, A., Bain, B., Devereux, S., et al., 2010. Best Practice in Lymphoma Diagnosis and Reporting. Specific disease appendix. *British Committee for Standards in Haematology*.

Paydas, S., Bagir, E., Seydaoglu, G., et al., 2015. Programmed death-1 (PD-1), programmed death-ligand 1 (PD-L1), and EBV-encoded RNA (EBER) expression in Hodgkin lymphoma. *Annals of Hematology*, 94(9), pp.1545–1552.

Pernick, N., Johnson, G. & Stuart, L.N., 2014. http://www.pathologyoutlines.com/. *http://www.pathologyoutlines.com/*. Available at: http://www.pathologyoutlines.com/ [Accessed February 14, 2015].

Pileri, S. a, Ascani, S., Leoncini, L., et al., 2002. Hodgkin's lymphoma: the pathologist's viewpoint. *Journal of Clinical Pathology*, 55(3), pp.162–176.

Place, T., Virus, F., Road, B., et al., 2000. Risk factors for Hodgkin's disease by Epstein-Barr virus (EBV) status: prior infection by EBV and other agents. *British Journal of Cancer*, 82, pp.1117–1121.

Poppema, S., 1996. Immunology of Hodgkin 's disease. *Bailliere's Clinical Haematology*, 9(3), pp.447–457.

Prehn, L.M. & Outzen, H.C., 1977. Primary tumor immunity in nude mice. *International Journal of Cancer*, 19, pp.688–691.

Proctor, S.J., Wilkinson, J., Jones, G., et al., 2012. Evaluation of treatment outcome in 175 patients with Hodgkin lymphoma aged 60 years or over: the SHIELD study. *Blood*, 119(25), pp.6005–15.

Raphael, M., Said, J. & Borisch, B., 2008. Immunodeficiency-associated lymphoproliferative disorders. In S. H. Swerdlow et al., eds. *WHO Classification of Tumours of Haematopoietic and Lymphoid Tissues.* Lyon: International Agency for Research on Cancer, pp. 335–352.

Rassidakis, G.Z., Medeiros, L.J., Vassilakopoulos, T.P., et al., 2002. BCL-2 expression in Hodgkin and Reed-Sternberg cells of classical Hodgkin disease predicts a poorer prognosis in patients treated with ABVD or equivalent regimens. *Blood*, 100(12), pp.3935–3941.

Ree, H. & Kadin, M., 1985. Macrophage-Histiocytes in Hodgkin's Disease. The Relation of Peanut-Agglutinin-Binding Macrophage-Histiocytes to

Clinicopathologic Presentation and Course of Disease. *Cancer*, 56, pp.333–338.

Reizis, B., Bunin, A., Ghosh, H.S., et al., 2011. Plasmacytoid dendritic cells: recent progress and open questions. *Annual Review of Immunology*, 29, pp.163–83.

Ressing, M.E., Horst, D., Griffin, B.D., et al., 2008. Epstein-Barr virus evasion of CD8+ and CD4+ T cell immunity via concerted actions of multiple gene products. *Seminars in Cancer Biology*, 18, pp.397–408.

Revesz, L., 1956. Effect of Tumour Cells killed by X-rays upon the Growth of Admixed Viable Cells. *Nature*, 178, pp.1391–1392.

Roman, E. & Smith, A.G., 2011. Epidemiology of lymphomas. *Histopathology*, 58(1), pp.4–14.

Rukavina, D., Laskarin, G., Rubesa, G., et al., 1998. Age-related decline of perforin expression in human cytotoxic T lymphocytes and natural killer cells. *Blood*, 92(7), pp.2410–2420.

Said, J.W., 2007. Immunodeficiency-related Hodgkin lymphoma and its mimics. *Advances in Anatomic Pathology*, 14(3), pp.189–94.

Sanchez-Aguilera, A., Montalban, C., Cueva, P. De, et al., 2006. Tumor microenvironment and mitotic checkpoint are key factors in the outcome of classic Hodgkin lymphoma. *Blood*, 108(2), pp.662–668.

Sánchez-Espiridión, B., Martin-Moreno, A.M., Montalbán, C., et al., 2012. Immunohistochemical markers for tumor associated macrophages and survival in advanced classical Hodgkin's lymphoma. *Haematologica*, 97(7), pp.1080–4.

Sánchez-Espiridión, B., Montalbán, C., López, A., et al., 2010. A molecular risk score based on 4 functional pathways for advanced classical Hodgkin lymphoma. *Blood*, 116(8), pp.e12–7.

Sánchez-Espiridión, B., Sánchez-Aguilera, A., Montalbán, C., et al., 2009. A TAQMAN low-density array to predict outcome in advanced Hodgkin's Lymphoma using paraffin-embedded samples. *Clinical Cancer Research*, 15(4), pp.1367–1375.

Sansoni, P., Cossarizza, a, Brianti, V., et al., 1993. Lymphocyte subsets and natural killer cell activity in healthy old people and centenarians. *Blood*, 82, pp.2767–2773.

Savill, J., Dransfield, I., Gregory, C., et al., 2002. A blast from the past: clearance of apoptotic cells regulates immune responses. *Nature Reviews. Immunology*, 2, pp.965–975.

Sawant, A., Hensel, J. a, Chanda, D., et al., 2012. Depletion of plasmacytoid dendritic cells inhibits tumor growth and prevents bone metastasis of breast cancer cells. *Journal of Immunology*, 189(9), pp.4258–65.

Seitz, V., Hummel, M., Marafioti, T., et al., 2000. Detection of clonal T-cell receptor gamma-chain gene rearrangements in Reed-Sternberg cells of classic Hodgkin disease. *Blood*, 95(10), pp.3020–3024.

Severa, M., Giacomini, E., Gafa, V., et al., 2013. EBV stimulates TLR- and autophagy-dependent pathways and impairs maturation in plasmacytoid dendritic cells: implications for viral immune escape. *European Journal of Immunology*, 43(1), pp.147–58.

Shimoyama, Y., Yamamoto, K., Asano, N., et al., 2008. Age-related Epstein-Barr virus-associated B-cell lymphoproliferative disorders: special references to lymphomas surrounding this newly recognized clinicopathologic disease. *Cancer Science*, 99(6), pp.1085–91.

Skinnider, B.F. & Mak, T.W., 2002. The role of cytokines in classical Hodgkin lymphoma. *Blood*, 99(12), pp.4283–97.

Snow, A.L. & Martinez, O.M., 2007. Epstein-Barr virus: evasive maneuvers in the development of PTLD. *American Journal of Transplantation*, 7(2), pp.271–7.

Stark, G.L., Wood, K.M., Jack, F., et al., 2002. Hodgkin's disease in the elderly: a population-based study. *British Journal of Haematology*, 119(2), pp.432–440.

Steidl, C., Diepstra, A., Lee, T., et al., 2012. Gene expression profiling of microdissected Hodgkin Reed-Sternberg cells correlates with treatment outcome in classical Hodgkin lymphoma. *Blood*, 120(17), pp.3530–40.

Steidl, C., Lee, T., Shah, S., et al., 2010. Tumor-Associated Macrophages and Survival in Classic Hodgkin's Lymphoma. *The New England Journal of Medicine*, 362(10), pp.875–885.

Steidl, C., Telenius, A., Shah, S.P., et al., 2010. Genome-wide copy number analysis of Hodgkin Reed-Sternberg cells identifies recurrent imbalances with correlations to treatment outcome. *Blood*, 116(3), pp.418–427.

Stein, H., 2008. Hodgkin Lymphoma. In S. H. Swerdlow et al., eds. *WHO classification of tumours of haematopoietic and lymphoid tissues*. Lyon: International Agency for Research on Cancer, pp. 321–334.

Steinman, R.M. & Banchereau, J., 2007. Taking dendritic cells into medicine. *Nature*, 449(7161), pp.419–26.

Swerdlow, S.H., Campo, E., Harris, N.L., et al., 2008. *WHO classification of tumours of haematopoietic and lymphoid tissues* 4th ed. S. Swerdlow et al., eds., Lyon: International Agency for Research on Cancer.

Tan, K.L., Scott, D.W., Hong, F., et al., 2012. Tumor-associated macrophages predict inferior outcomes in classic Hodgkin lymphoma: a correlative study from the E2496 Intergroup trial. *Blood*, 120(16), pp.3280–7.

Taskinen, M., Karjalainen-Lindsberg, M.-L., Nyman, H., et al., 2007. A high tumor-associated macrophage content predicts favorable outcome in follicular lymphoma patients treated with rituximab and cyclophosphamide-doxorubicin-vincristine-prednisone. *Clinical Cancer Research*, 13(19), pp.5784–9.

Tesniere, a, Panaretakis, T., Kepp, O., et al., 2008. Molecular characteristics of immunogenic cancer cell death. *Cell Death and Differentiation*, 15, pp.3–12.

Torlakovic, E., Torlakovic, G., Nguyen, P.L., et al., 2002. The Value of Anti-Pax-5 Immunostaining in Routinely Fixed and Paraffin-Embedded Sections. A Novel Pan Pre-B and B-cell Marker. *The American Journal of Surgical Pathology*, 26(10), pp.1343–1350.

Touati, M., Delage-Corre, M., Monteil, J., et al., 2011. CD68+ tumor-associated macrophages predict unfavorable treatment outcomes in classic Hodgkin's Lymphoma in correlation with early FDG-PET assessment results. *Blood (ASH Annual Meeting Abstracts)*, 118(21), p.1558.

Tran, H., Nourse, J., Hall, S., et al., 2008. Immunodeficiency-associated lymphomas. *Blood Reviews*, 22(5), pp.261–81.

Truman, L. a, Ford, C. a, Pasikowska, M., et al., 2008. CX3CL1/fractalkine is released from apoptotic lymphocytes to stimulate macrophage chemotaxis. *Blood*, 112(13), pp.5026–36.

Truman, L. a, Ogden, C.A., Howie, S.E.M., et al., 2004. Macrophage chemotaxis to apoptotic Burkitt's lymphoma cells in vitro: role of CD14 and CD36. *Immunobiology*, 209(1-2), pp.21–30.

Turner, C., Devitt, A., Parker, K., et al., 2003. Macrophage-mediated clearance of cells undergoing caspase-3-independent death. *Cell Death and Differentiation*, 10(3), pp.302–12.

Tyan, M.L., 1974. In vivo and in vitro responses to tumor-associated antigens: apparent absence of T cell participation. *European Journal of Immunology*, 4, pp.727–731.

Tzankov, A., Bourgau, C., Kaiser, A., et al., 2005. Rare expression of T-cell markers in classical Hodgkin's lymphoma. *Modern Pathology*, 18(12), pp.1542–9.

Tzankov, A., Matter, M.S. & Dirnhofer, S., 2010. Refined prognostic role of CD68-positive tumor macrophages in the context of the cellular micromilieu of classical Hodgkin lymphoma. *Pathobiology*, 77(6), pp.301–8.

Tzankov, A., Meier, C., Hirschmann, P., et al., 2008. Correlation of high numbers of intratumoral FOXP3+ regulatory T cells with improved survival in germinal center-like diffuse large B-cell lymphoma, follicular lymphoma and classical Hodgkin's lymphoma. *Haematologica*, 93(2), pp.193–200.

Tzankov, A., Zimpfer, A., Pehrs, A.-C., et al., 2003. Expression of B-cell markers in classical hodgkin lymphoma: a tissue microarray analysis of 330 cases. *Modern Pathology*, 16(11), pp.1141–7.

Utsuyama, M., Kirokawa, K., Kurashima, C., et al., 1992. Differential age-change in the numbers of CD4+CD45RA+ and CD4+CD29+ T cell subsets in human peripheral blood. *Mechanisms of Ageing and Development*, 63, pp.57–68.

Vassallo, J., Metze, K., Traina, F., et al., 2001. Expression of Epstein-Barr virus in classical Hodgkion's Lymphomas in Brazilian adult patients. *Haematologica*, 86, pp.1227–1228.

Venkataraman, G., Song, J.Y., Tzankov, A., et al., 2013. Aberrant T-cell antigen expression in classical Hodgkin lymphoma is associated with decreased event-free survival and overall survival. *Blood*, 121(10), pp.1795–804.

Verfaillie, T., Garg, A.D. & Agostinis, P., 2013. Targeting ER stress induced apoptosis and inflammation in cancer. *Cancer Letters*, 332(2), pp.249–264.

Vetsika, E.-K. & Callan, M., 2004. Infectious mononucleosis and Epstein-Barr virus. *Expert Reviews in Molecular Medicine*, 6(23), pp.1–16.

Weiss, J.M., Back, T.C., Scarzello, A.J., et al., 2009. Successful immunotherapy with IL-2/anti-CD40 induces the chemokine-mediated mitigation of an immunosuppressive tumor microenvironment. *Proceedings of the National Academy of Sciences of the United States of America*, 106(46), pp.19455–60.

Weiss, L.M., Strickler, J.G., Hu, E., et al., 1986. Immunoglobulin gene rearrangements in Hodgkin's disease. *Human Pathology*, 17(10), pp.1009–1014.

Westhoff, M.A., Brühl, O., Nonnenmacher, L., et al., 2014. Killing me softly-future challenges in apoptosis research. *International Journal of Molecular Sciences*, 15, pp.3746–3767.

WHO, 1996a. *IARC Monographs on the Evaluation of Carcinogenic Risks to Humans. Epstein-Barr virus and Kaposi's sarcoma Herpes virus/Human Herpesvirus 8*, Lyon.

WHO, 1996b. *IARC Monographs on the Evaluation of Carcinogenic Risks to Humans. Epstein-Barr virus, virus-host interactions*, Lyon.

Wikipedia, 2015. Human Leucocyte Antigen. *Wikipedia, The Free Encyclopedia*, p.640531822.

Willenbrock, K., Ichinohasama, R., Kadin, M.E., et al., 2002. T-Cell Variant of Classical Hodgkin's Lymphoma with Nodal and Cutaneous Manifestations Demonstrated by Single-Cell Polymerase Chain Reaction. *Laboratory Investigation*, 82(9), pp.1103–1109.

Yamamoto, R., Nishikori, M., Kitawaki, T., et al., 2008. Brief report PD-1 – PD-1 ligand interaction contributes to immunosuppressive microenvironment of Hodgkin lymphoma. *Blood*, 111(6), pp.3220–3224.

Yung, L. & Linch, D., 2005. Hodgkin's Lymphoma. In A. V. Hoffbrand, D. Catovsky, & E. C. Tuddenham, eds. *Postgraduate Haematology*. Massachusetts: Blackwell Publishing, pp. 722–734.

Yung, L. & Linch, D., 2005. Hodgkin's Lymphoma. In A. Hoffbrand, D. Catovsky, & E. Tuddenham, eds. *Postgraduate Haematology*. Massachusetts: Blackwells, pp. 722–734.

Zhen, Y., Zheng, J. & Zhao, Y., 2008. Regulatory CD4+CD25+ T cells and macrophages: communication between two regulators of effector T cells. *Inflammation Research*, 57(12), pp.564–70.

Zwitter, M., Cohen, J.R., Barrett, A., et al., 2002. Dorothy Reed and Hodgkin's Disease: a reflection after a century. *International Journal of Radiation Oncology, Biology and Physics*, 53(2), pp.366–375.

Appendix A
cHL Data Collection Sheet

Specimen No.	
Name	
DOB / Age at diagnosis (yrs)	
Male / Female	MALE / FEMALE
Date of Diagnosis	
Site of Diagnostic Biopsy	
Subtype of cHL	NSHL / MCHL / LDHL / LRHL / NOS
ECOG Performance Status **0 = no limits, 1= minor sx, near normal activity, 2=mod sx, ltd function, 3=severe ltd function, 4=bedridden**	0 / 1 / 2 / 3 / 4
Hasenclever Score >/=45 Male stageIV Alb<40 Hb<10.5 Lymph <0.6	0-2 / 3-4 / >5
Staging Investigations	CT / MRI / CXR / Abdo USS / Bone Marrow / other -
Stage at Diagnosis (Ann-Arbor IA-IVB)	
Number of nodal sites involved	
Sites of involved nodes	Cervical / Supraclav / Mediastinal / Axillary / Intra-abdo / Inguinal / Iliac Chain / other intrathoracic / spleen
Sites of Extranodal Involvement	Bone Marrow / Liver / other -
Bulk Disease: >33% CTR on AP CXR	Yes / No
>5cm nodal mass	Yes / No
>10cm nodal mass	Yes / No
EBV	IgG pos / IgG Neg
HIV	Pos / Neg
Haematological Markers	Hb WCC Neut Lymph Plt ESR
Biochemical Markers	Ur Tot Prot Cr Alb Na+ ALk P K+ AST/ALT Urate LDH CRP B2 micro
Planned Treatment	

Actual treatment received	
Mid-treatment response	Stable/progressive Partial Response Complete Remission
End of treatment response	Stable/progressive Partial response Complete remission
Date of CR (if achieved)	
Date of end of treatment	
Date of Relapse / Time since Rx	
Site of relapse Number of nodal sites involved/extranodal	
Treatment given/planned post relapse	
Response to 2nd line treatment	
Date last assessed/time since dx, mths	
Outcome	Death HL related Death other Loss to F/U Ongoing F/U Discharge
SNLG Prognostic Index	

Appendix B
Epstein-Barr Virus and its Interaction with the Immune System

The Normal Immune System

The innate immune response is the first defence against invading pathogens, protecting the body during the several days taken to form a specific adaptive immune response. Macrophages play a key role in this by recognising Pathogen-Associated Molecular Patterns (PAMP), i.e. common constituents of, in particular, many bacterial surfaces, via their Pattern Recognition Receptors (PRP). This allows them to phagocytose the potentially harmful agent, destroying it in the process and subsequently presenting its antigens to T-cells, via MHC class II molecules, which augment the innate immune response and lead to a humoral response (Gordon & Taylor 2005; Janeway et al. 2001). In addition, macrophages recruit other components of the innate immune response, such as neutrophils, to the site of 'invasion'. Macrophages however, are not equally effective against all pathogens. Encapsulated bacteria evade recognition by hiding the recognisable components of their cell surface (PAMP), and viruses carry no common molecules recognisable by PRP. Dendritic cells are another important component of the innate immune system. These highly specialised antigen presenting cells, derived from the same bone marrow precursors as macrophages, do not rely solely on pattern recognition and instead continually take in extracellular matter, including virus particles, through a process called macropinocytosis (Janeway et al. 2001; Steinman & Banchereau 2007). There are different subtypes of dendritic cells; myeloid DC (mDC) and plasmacytoid DC (pDC). Relatively few circulate in peripheral blood and most are resident within tissues (Steinman & Banchereau 2007; Jegalian et al. 2009; Banchereau & Steinman 1998).

Proteins of the Major Histocompatibility Complex (MHC), also known as Human Leucocyte Antigens (HLA), play a crucial role in linking innate and adaptive immune responses. In humans there are more than 200 MHC genes and many other associated genes, mostly found on chromosome six (Janeway et al. 2001).

MHC genes are divided into class I (classical and non-classical), class II and class III, all of which are highly polymorphic. Classical MHC class I proteins are HLA-A, -B and –C. These are expressed on the cell-surface of all nucleated cells and present antigens to cytotoxic T-cells. These $CD8^+$ T-cells bind to HLA class I molecules via their 'CD8-receptor'. If the T-cell's adjacent T-cell receptor recognises the antigen presented by the HLA class I molecule, then it triggers the HLA class I bearing cell to undergo apoptosis i.e. induces cell cytotoxicity (Wikipedia 2015). The non-classical MHC class I molecules include HLA-E, -F and –G, although there are numerous others. These are not expressed on the cell surface but tend to have intracellular functions, often aiding MHC class II molecules to present antigens. HLA-E and -G are both thought to play a role in inhibiting NK-cell cytotoxicity. Foetus-derived placental cells lack expression of classical MHC class I proteins but express HLA-G. The lack of classical MHC class I makes them unrecognisable to $CD8^+$ CTL but normally, would result in the cells being killed by NK-cell cytotoxicity. The presence of HLA-G prevents this happening. An ILT-2 receptor on NK-cells recognises HLA-G causing an inhibitory signal (Janeway et al. 2001; Campoli & Ferrone 2008; A. Diepstra et al. 2008).

MHC class II molecules (HLA-DR, -DP and -DQ) are only expressed on the cell surface of 'professional' APC, for example, macrophages, B-lymphocytes and DC. They are recognised and bound by $CD4^+$ T-helper cells. This time, if the T_H cell's adjacent TCR recognises the antigen being presented, an adaptive immune response is triggered involving expansion of specific CTL and ultimately antibody production by B-cells (Wikipedia 2015; Janeway et al. 2001). MHC class III genes encode proteins, for example components of the complement cascade and cytokines such as TNF-a, which are important for the immune response but not involved in antigen processing or presentation (Wikipedia 2015).

The Normal Immune Response to EBV

EBV preferentially infects B-lymphocytes and while latent EBV infection is largely confined to these cells, in healthy carriers it has also been found in epithelial cells and T-lymphocytes. This is consistent with the fact that EBV is detectable in some non-B-cell malignancies including T-cell lymphomas and some carcinomas, especially of nasopharyngeal origin. Because the main mode of transmission is oral, initial infection often occurs via tonsillar B-cells (WHO 1996b; Vetsika & Callan 2004). Glycoprotein gp350 on the viral envelope interacts with CD21 expressed on the lymphocyte cell surface. This results in endocytosis of the virus particle and subsequent fusion of the viral envelope with the lymphocyte cell membrane via three other viral glycoproteins; gp85, gp25 and gp42. EBV infection of non-CD21 expressing cells can also occur but the mechanisms of this are less clear. Contrary to this, some CD21-positive cells, both in-vitro and in-vivo, appear resistant to EBV infection. Once the viral particle has entered the cell it usually remains in its circular episomal form within the nucleus but it can integrate with the host chromosomal DNA (WHO 1996b). There is an initial lytic phase of infection in which tonsillar B-cells act as a site for reproduction of virus particles. This is asymptomatic in many, but largely due to the quantities of cytokines released by activated $CD8^+$ cells, is the time when some patients manifest the clinical symptoms of IM (Snow & Martinez 2007; Merlo et al. 2010; Vetsika & Callan 2004). This reaction is eventually brought under control by the production of specific CTL after which, EBV largely exists as a latent infection within memory B-lymphocytes (Snow & Martinez 2007; Merlo et al. 2010; Ressing et al. 2008; Vetsika & Callan 2004) and viral replication only occurs intermittently in a small percentage of infected cells. Virions are still detectable within the asymptomatic host's saliva but these are kept to a minimum by the ongoing specific CTL response to EBV antigens of the replicative cycle (Merlo et al. 2010; Vetsika & Callan 2004).

In order to persist lifelong in the host, EBV has established mechanisms to evade the immune system including entering a state of 'latency' where expression of the most immunogenic viral proteins is suppressed. There are three different latency states, each aiding virus survival in a different way. In type III latency, all of the EBV latent genes (EBNA 1, 2, 3A, 3B, 3C, LP, LMP 1, 2A, 2B, EBERS 1, 2) are expressed, allowing survival of infected naïve B-cells but also providing strong immunogenic targets for the host. These cells would exhibit uncontrolled growth and expansion if not prevented, as normally occurs, by a specific CTL response. Type II latency describes naïve infected B-lymphocytes, resembling B-lymphoblasts, which differentiate in the absence of any stimulation from an antigen reaction associated with FDC or T_H cells (i.e. without activation of the B-cell receptor) as would normally be required. In this state EBNA2 is not expressed and LMP-1 and LMP-2A have key roles in promoting cell survival whilst inhibiting growth. Although less immunogenic, healthy carriers of EBV do have specific CTL against these viral proteins, which presumably keep them in check, but this is the latency pattern exhibited by EBV-positive HRS-cells in cHL. Type I latency is a stage in which none of the EBV-encoded genes are expressed with the exception of intermittent EBNA1 expression which appears to have a role in ensuring the viral episome is transmitted during cell division. EBNA1, however, is very poorly recognised by $CD8^+$ cells, in part because it inhibits its own processing into antigenic peptides (Ressing et al. 2008). This lack of viral gene expression and antigen presentation allows infected host cells to evade EBV-specific CTL and survive in the host (Snow & Martinez 2007; WHO 1996b; Ressing et al. 2008; Merlo et al. 2010).

Although limited, replication however, does occur and requires expression of the full spectrum of viral proteins. To prevent elimination of the virus during this stage, other processes are in place. Some viral proteins inhibit different stages of MHC class I and II antigen processing and presentation. For example, BNLF2a inhibits the Transporter associated with Antigen Processing (TAP) thereby suppressing the loading of antigens onto HLA class I molecules and preventing the class I presentation of viral antigens; BILF1 down-regulates cell surface HLA class I presentation and a number of gene products interfere with the antigen presenting capacity of HLA class II molecules. The virus also produces homologues of some human cytokines, such as IL-10, which again interfere with antigen processing (Merlo et al. 2010; Ressing et al. 2008). Therefore, although there is a CTL response to the virus whilst replicating, it is limited and insufficient to eradicate the infection completely.

For EBV to cause malignancy, it would seem that two criteria must be met: (1) there must be a local or systemic defect in the host immune response to allow sufficient EBV-positive lymphoblasts to proliferate; (2) EBV infected cells must remain in cell cycle and continue to replicate.

Appendix C
T-Regulatory Cells in the Young Cohort

Introduction

T-regulatory cells are $CD4^+$ T-cells that have suppressive actions on T_H cell activities. Their normal role in health is thought to include the prevention of autoimmune reactions, the suppression of atopy and in feto-maternal tolerance (Corthay 2009). The identification of T-reg cells is difficult and it is not clear whether they are a true T-cell subset. There is some evidence to suggest that other T-cell subtypes, including T_H cells may also have suppressive actions in some circumstances (Corthay 2009).

A number of cell-surface proteins have been proposed as T-reg cell markers but none have been shown to be truly specific. These include CD25, CTLA-4, GITR and CD127 which are also expressed on effector/memory T-cells after antigen exposure (Huang et al. 2004; Corthay 2009). FOXP3 is a transcription factor, expressed in the nucleus, which appears to be selectively expressed by T-reg cells derived from the thymus. However, it is less clear whether peripherally derived T-reg cells also express FOXP3 and there is evidence that T-reg cells may subsequently differentiate into T_H cells whilst maintaining FOXP3 expression, making it a less specific marker (Huang et al. 2004; Corthay 2009; Gandhi et al. 2006).

Lymphocyte Activation Gene-3 (LAG-3), in a murine model, was shown to be up-regulated in T-cells with regulatory actions (Huang et al. 2004). The gene is expressed as a cell-surface protein which binds MHC class II. It appears to be directly involved in suppressive actions and is only expressed after specific antigen exposure (Huang et al. 2004; Gandhi et al. 2006). It is not clear however, whether LAG-3 is a universal marker of T-reg cells or whether it may only be expressed by a subgroup. Its expression in cHL has been examined in humans using IHC for LAG-3 and it is expressed by tumour infiltrating lymphocytes in cHL (Gandhi et al. 2006). This study also looked at FOXP3 expression in the same group of tumours. LAG-3 was expressed more frequently by lymphocytes in EBV-positive cHL, MCHL and LRcHL than in other subgroups and was more frequently expressed than FOXP3 for which no association was found with EBV or cHL subtype. This same study, using human tissue showed that LAG-3 and FOXP3 expression correlated with a loss in LMP1 and 2 specific T-cell activity and that depleting LAG-3-positive cells enhanced T-cell activity against EBV-related proteins (Gandhi et al. 2006). For these reasons, LAG-3 was chosen as a marker of T-reg cells and its expression was examined in the young cohort.

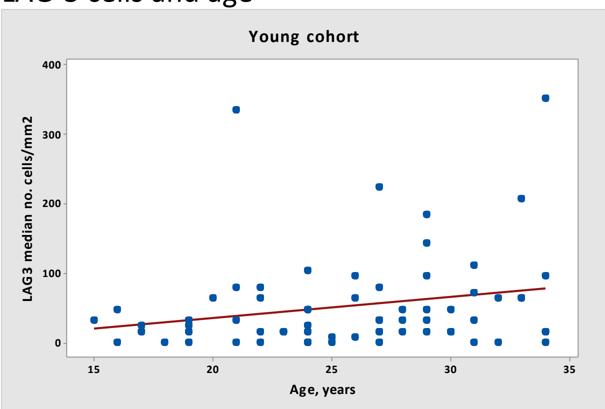
Results

P-values relate to the Mann-Whitney test unless stated otherwise.

LAG-3 cells and age

The number of LAG-3 cells in each tumour was only assessed in the young cohort, with patients ranging in age from 15 to 34 years. Within this group there was a weak positive association between increased age and an increased number of tumour-infiltrating LAG-3-positive cells, but this did not reach statistical significance (Pearson correlation 0.22, p=0.07) (figure A.1).

Figure A.1
LAG-3 cells and age



Pearson correlation = 0.22, p=0.07

LAG-3 cells and gender

The median number of LAG-3 cells did not vary between males and females (table A.1).

Table A.1
LAG-3 cells and gender

	Young Median cell count per mm^2
All cases (n)	32 (67)
Male (n)	32 (33)
Female (n)	28 (34)
Male v Female	p=NS

LAG-3 cells and clinical stage

The median number of LAG-3 cells did not vary according to the stage of disease at presentation (table A.2).

Table A.2
LAG-3 cells and stage at presentation

	Young Median cell count per mm^2
Stage I-IIA (n)	24 (28)
Stage IIB-IV (n)	32 (38)
Stage I-IIA v Stage IIB-IV (n)	p=NS

LAG-3 cells and EBV

The median number of LAG-3 cells did not vary between EBV-positive and –negative cases (table A.3).

Table A.3
LAG-3 cells and EBV

	Young Median cell count per mm^2
EBV pos (n)	16 (16)
EBV neg (n)	32 (51)
EBV pos v EBV neg	p=NS

The median number of LAG-3 cells did not vary between EBV-positive and –negative cases within either gender or between genders with EBV-positive or –negative disease (table A.4).

Table A.4
LAG-3 cells, EBV and gender

	Young Median cell count per mm^2	
	EBV Pos	**EBV Neg**
Male (n)	16 (13)	48 (20)
	p=NS	
Female (n)	16 (3)	32 (31)
	p=NS	
Male v Female	p=NS	p=NS

LAG-3 cells and cHL subtype

The median number of LAG-3 cells did not vary according to cHL subtype (table A.5).

Table A.5
LAG-3 cells and cHL subtype

	Young Median cell count per mm^2
NSHL (n)	32 (50)
MCHL (n)	64 (11)
cHL, other (n)	16 (6)
NSHL v MCHL	p=NS

The median number of LAG-3 cells did not vary between EBV-positive and –negative cases of each cHL subtype and did not vary between subtypes if EBV-positive or –negative (table A.6).

Table A.6
LAG-3 cells, cHL subtype and EBV

	Young Median cell count per mm²	
	EBV Pos	**EBV Neg**
NSHL (n)	32 (7)	32 (43)
	p=NS	
MCHL (n)	16 (7)	64 (4)
	p=NS	
cHL, other (n)	16 (2)	48 (4)
	p=NS	
NSHL v MCHL	p=NS	p=NS

LAG-3 cells, cytotoxic molecule and T-cell antigen expression

The median number of LAG-3 cells did not differ significantly between those tumours that did or did not express any of the cytotoxic molecules or T-cell antigens (table A.7).

Table A.7
LAG-3 cells, cytotoxic molecule and T-cell antigen expression

	Young CM expression Median cell count per mm²		**Young CMpos, PAX5 pos/neg** Median cell count per mm²		**Young T-cell Ag expression** Median cell count per mm²	
	CMpos	**CMneg**	**PAX5pos**	**PAX5neg**	**T-cell pos**	**T-cell neg**
LAG-3 (n)	16 (9)	32 (58)	16 (8)	48 (1)	24 (13)	32 (42)
	p=NS		NP		p=NS	

NP=calculation not possible

LAG-3 cells and outcome

The median number of LAG-3 cells did not vary between the tumours of those who relapsed and did not relapse in any group of patients; EBV-positive, -negative or combined (tables A.8 & A.9).

Table A.8
LAG-3 cells and outcome: relapse

	Young Median cell count per mm^2
All relapse (n)	36 (10)
All no relapse (n)	32 (56)
Relapse v no relapse	p=NS

Table A.9
LAG-3 cells, EBV and outcome: relapse

	Young Median cell count per mm^2	
	EBV Pos	**EBV Neg**
Relapse (n)	112 (2)	36 (8)
	p=NS	
No relapse (n)	16 (13)	32 (43)
	p=NS	
Relapse v no relapse	p=NS	p=NS

The median number of LAG-3 cells did not vary between the tumours of those who relapsed or required an escalation of treatment and those who did not in any group of patients; EBV-positive, -negative or combined (tables A.10 & A.11).

Table A.10
LAG-3 cells and outcome: relapse or escalation in treatment (Rx)

	Young Median cell count per mm^2
All Relapse or escalation Rx (n)	32 (14)
All no relapse or escalation Rx (n)	32 (52)
Relapse or escalation v no relapse or escalation Rx	p=NS

Table A.11
LAG-3 cells, EBV and outcome: relapse or escalation in treatment (Rx)

	Young Median cell count per mm^2	
	EBV Pos	**EBV Neg**
Relapse or escalation Rx (n)	112 (2)	32 (12)
	p=NS	
No relapse or escalation Rx (n)	16 (13)	32 (39)
	p=NS	
Relapse or escalation v no relapse or escalation Rx	p=NS	p=NS

The median number of LAG-3 cells did not vary between the tumours of those who died and those who did not die, of any cause, in any group of patients; EBV-positive, -negative or combined (tables A.12 & A.13).

Table A.12
LAG-3 cells and outcome: death, any cause

	Young Median cell count per mm^2
All dead (n)	56 (4)
All not dead(n)	32 (62)
Dead v not dead	p=NS

Table A.13
LAG-3 cells, EBV and outcome: death, any cause

	Young Median cell count per mm^2	
	EBV Pos	**EBV Neg**
Dead (n)	32 (2)	56 (2)
	p=NS	
Not dead (n)	16 (14)	32 (48)
	p=NS	
Dead v not dead	p=NS	p=NS

In the young cohort, very few patients died of cHL and it was not possible to make any meaningful comparisons regarding the number of LAG-3 cells in the tumours of those who did and did not die of cHL.

By using the Pearson correlation, the young cohort was examined for any evidence of an association between the median number of LAG-3 cells and various other cell types.

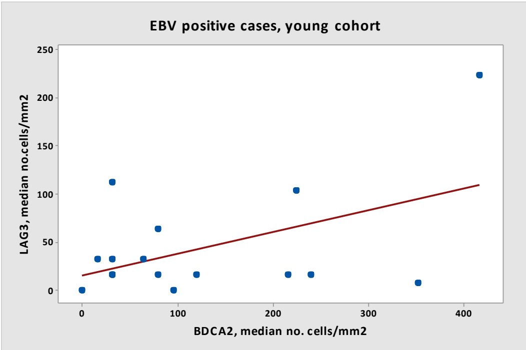
LAG-3 cells and plasmacytoid dendritic cells (BDCA2-positive)

In EBV-positive cases in the young cohort, although not reaching statistical significance, there was a positive correlation between the number of T-reg and pDC (Pearson correlation 0.49, p=0.06). This was not seen in EBV-negative cases (figures A.2 & A.3).

A similar positive correlation was seen in MCHL cases (Pearson correlation 0.56), but not NSHL tumours, which may be expected given the strong link between MCHL and EBV positivity. However, again the association did not reach statistical significance (figures A.4 & A.5).

Figure A.2

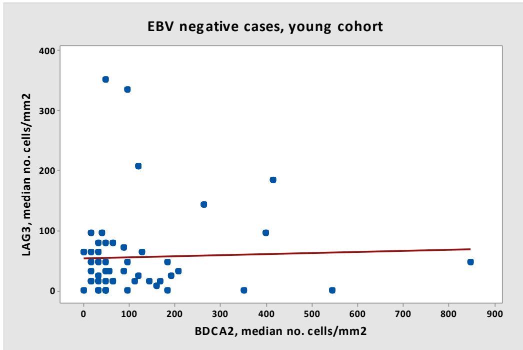
EBV-positive: LAG-3 and BDCA2-cells



Pearson correlation=0.49, p=0.06

Figure A.3

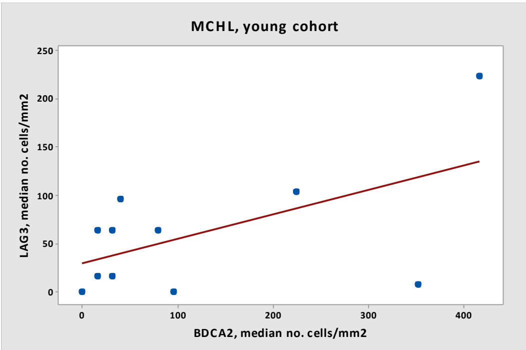
EBV-negative: LAG-3 and BDCA2-cells



Pearson correlation=0.04, p=NS

Figure A.4

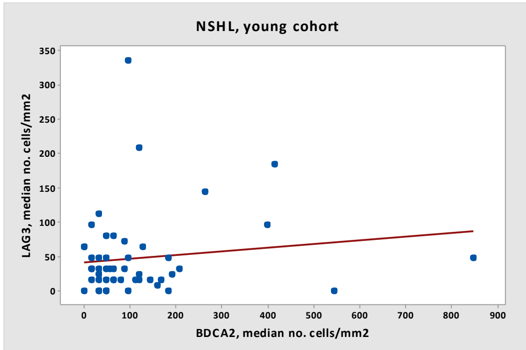
MCHL: LAG-3 and BDCA2-cells



Pearson correlation=0.56, p=0.07

Figure A.5

NSHL: LAG-3 and BDCA2-cells



Pearson correlation=0.13, p=NS

LAG-3 cells and macrophages

There was no evidence that the median number of LAG-3 cells was associated with either the median number of CD68 or CD163 cells in the young cohort. This remained the case when EBV-positive and –negative cases were examined individually and when the tumours of males and females were examined separately.

LAG-3 cells and HRS-cells

There was no evidence that the median number of LAG-3 cells was associated with the median number of HRS-cells or the number of apoptotic HRS-cells in the young cohort or any subgroup; EBV-positive, -negative, male or female.

LAG-3 cells and apoptosis

There was no evidence that the median number of LAG-3 cells was associated with the median total number of apoptotic cells or background apoptotic cells in the young cohort or any subgroup; EBV-positive, -negative, male or female.

Appendix D

Suggested Gene List

FAS-L*	http://www.genecards.org/cgi-bin/carddisp.pl?gene=FASLG http://www.uniprot.org/uniprot/P48023 Cytokine that binds to TNFRSF6/FAS, a receptor that transduces the apoptotic signal into cells. May be involved in cytotoxic T-cell mediated apoptosis and in T-cell development. TNFRSF6/FAS-mediated apoptosis may have a role in the induction of peripheral tolerance, in the antigen-stimulated suicide of mature T-cells, or both. Binding to the decoy receptor TNFRSF6B/DcR3 modulates its effects. The FasL intracellular domain (FasL ICD) cytoplasmic form induces gene transcription inhibition.	Expression by tumour cells and microenvironmental lymphocytes associated with apoptosis (data not specific to HL) Both CD95 and CD95L expressed by HRS cells. Only CD95 expressed by surrounding lymphocytes ?suggests RS cells can induce apoptosis of lymphs but not vice versa
P53*	http://www.uniprot.org/uniprot/P04637 Acts as a tumor suppressor in many tumor types; induces growth arrest or apoptosis depending on the physiological circumstances and cell type. Involved in cell cycle regulation as a trans-activator that acts to negatively regulate cell division by controlling a set of genes required for this process. One of the activated genes is an inhibitor of cyclin-dependent kinases. Apoptosis induction seems to be mediated either by stimulation of BAX and FAS antigen expression, or by repression of Bcl-2 expression. In cooperation with mitochondrial PPIF is involved in activating oxidative stress-induced necrosis; the function is largely independent of transcription. Induces the transcription of long intergenic non-coding RNA p21 (lincRNA-p21) and lincRNA-Mkln1. LincRNA-p21 participates in TP53-dependent transcriptional repression leading to apoptosis and seem to have to effect on cell-cycle regulation. Implicated in Notch signaling cross-over. Prevents CDK7 kinase activity when associated to CAK complex in response to DNA damage, thus stopping cell cycle progression. Isoform 2 enhances the transactivation activity of isoform 1 from some but not all TP53-inducible promoters. Isoform 4 suppresses transactivation activity and impairs growth suppression mediated by isoform 1. Isoform 7 inhibits isoform 1-mediated apoptosis.	?no as in apoptosis get increased phosphorylation of p53 rather than increased transcription. ?yes because of association with PIDD = mediator of p53 dependent apoptosis + basal level of PIDD mRNA depends on p53 gene status
PIDD*	http://www.uniprot.org/uniprot/Q9HB75 Promotes apoptosis downstream of the tumor	Mediator of p53 dependent apoptosis. PIDD mRNA is induced by gamma-

	suppressor as component of the DNA damage/stress response pathway that connects p53/TP53 to apoptosis. Associates with NEMO/IKBKG and RIP1 and enhances sumoylation and ubiquitination of NEMO/IKBKG which is important for activation of the transcription factor NF-kappa-B. Associates with CASP2/caspase-2 and CRADD/RAIDD, and induces activation of CASP2 which an important regulator in apoptotic pathways.	irradiation in a p53 dependent manner. Overexpression of PIDD inhibits cell growth in a p53 like manner by inducing apoptosis. PIDD involved in the activation of Caspase 2
GADD 45B*	http://www.uniprot.org/uniprot/O75293 Involved in the regulation of growth and apoptosis. Mediates activation of stress-responsive MTK1/MEKK4 MAPKKK.	Suppresses apoptosis but ?interesting given link with CD40. In B-lymphs, induction of apoptosis by FAS is suppressed by triggering of CD40 – activates protective genes mediated by NF-KB transcription factors. GADD 45B induced by CD40 via mechanism requiring NF-KB and induction suppresses Fas mediated killing. CD40 expressed by HRS cells, LMP-1 mimics function of CD40.
HOX B1*	http://www.uniprot.org/uniprot/P14653 Sequence-specific transcription factor which is part of a developmental regulatory system that provides cells with specific positional identities on the anterior-posterior axis. Acts on the anterior body structures.	Expressed normally in normal terminally differentiated peripheral blood cells. Enforced expression of HOX B1 in an APML cell line (HL60) causes decreased cell proliferation, increased apoptosis, increased cell differentiation along monocyte and granulocytic lineages.
TIA 1*	http://www.uniprot.org/uniprot/P31483 Involved in alternative pre-RNA splicing and regulation of mRNA translation by binding to AU-rich elements (AREs) located in mRNA 3' untranslated regions (3' UTRs). Possesses nucleolytic activity against cytotoxic lymphocyte target cells. May be involved in apoptosis.	Has nucleolytic activity against cytotoxic T lymphocyte target cells. May induce apoptosis via DNA fragmentation. Regulates the human apoptotic gene FAS.
TNF a*	http://www.uniprot.org/uniprot/P01375 cytokine that binds to TNFRSF1A/TNFR1 and TNFRSF1B/TNFBR. It is mainly secreted by macrophages and can induce cell death of certain tumor cell lines. It is potent pyrogen causing fever by direct action or by stimulation of interleukin-1 secretion and is implicated in the induction of cachexia, Under certain conditions it can stimulate cell proliferation and induce cell differentiation. The TNF intracellular domain (ICD) form	Cytokine produced by large no. of cell types – mediates lots on inflame and immunologic responses. 'antiviral agent' – cells infected with viruses are susceptible to inhibition by TNF. TNF mediated killing resembles apoptosis. Exerts its effects via 2 cell surface receptors TNFRI and TNFRII. T cells from older humans

	induces IL12 production in dendritic cells.	have increased susceptibility to Fas mediated apoptosis. TNFa levels are increased in old people. Downstream signaling of Fas mediated apoptosis and TNF induced apoptosis are similar. Increased TNF induced apoptosis in Tcells from old people assoc with increased TNFFI and TRADD but decreased TNFRII and TRAF2.
IFN G*	http://www.uniprot.org/uniprot/P01579 http://www.genecards.org/cgi-bin/carddisp.pl?gene=IFNG&search=interferon+gamma Produced by lymphocytes activated by specific antigens or mitogens. IFN-gamma, in addition to having antiviral activity, has important immunoregulatory functions. It is a potent activator of macrophages, it has antiproliferative effects on transformed cells and it can potentiate the antiviral and antitumor effects of the type I interferons.	Produced by % of CD8+ Tcells in IM patients in response to Ag recognition. ?also produced by CD4+ cells specific to EBNA1 Ag.
CCR7*	http://www.genecards.org/cgi-bin/carddisp.pl?gene=CCR7&search=CCR7 http://www.uniprot.org/uniprot/P32248 The protein encoded by this gene is a member of the G protein-coupled receptor family. This receptor was identified as a gene induced by the Epstein-Barr virus (EBV), and is thought to be a mediator of EBV effects on B lymphocytes. This receptor is expressed in various lymphoid tissues and activates B and T lymphocytes. It has been shown to control the migration of memory T cells to inflamed tissues, as well as stimulate dendritic cell maturation. The chemokine (C-C motif) ligand 19 (CCL19/ECL) has been reported to be a specific ligand of this receptor. (provided by RefSeq, Jul 2008)	As immune response to acute EBV infection proceeds the phenotype of responding lymphocytes changes. Initially express CD38, CD45RO, CD27 with only minority expressing CD62L or CCR7. Later get loss of CD38, increased CD62L and CCR7 with reversion from CD45Ro to CD45RA. If cells express CCR7, fail to express IFN-G (Steidl et al. 2012), increased in HRS cells cf germinal centre profiles (54.4 fold increase)
CD38*	http://www.uniprot.org/uniprot/P28907 http://www.genecards.org/cgi-bin/carddisp.pl?gene=CD38&search=CD38 CD38 is a novel multifunctional ectoenzyme widely expressed in cells and tissues especially in leukocytes. CD38 also functions in cell adhesion,signal transduction and calcium signaling. (provided by RefSeq, Jul 2008) Synthesizes cyclic ADP-ribose, a second	Expressed by activated, Ag experienced CD8+T cells, therefore expressed in acute phase of IM but expression downregulated as infection evolves. (Steidl et al. 2012), 6.6 fold decrease expression in HRS cf germinal centre profiles

	messenger for glucose-induced insulin secretion. Also has cADPr hydrolase activity. Also moonlights as a receptor in cells of the immune system.	
CD27**	http://www.uniprot.org/uniprot/P26842 http://www.genecards.org/cgi-bin/carddisp.pl?gene=CD27&search=CD27 The protein encoded by this gene is a member of the TNF-receptor superfamily. This receptor is required for generation and long-term maintenance of T cell immunity. It binds to ligand CD70, and plays a key role in regulating B-cell activation and immunoglobulin synthesis. This receptor transduces signals that lead to the activation of NF-kappaB and MAPK8/JNK. Adaptor proteins TRAF2 and TRAF5 have been shown to mediate the signaling process of this receptor. CD27-binding protein (SIVA), a proapoptotic protein, can bind to this receptor and is thought to play an important role in the apoptosis induced by this receptor. (provided by RefSeq, Jul 2008)	Majority of the CD8+ Tcells in IM express CD27 'costimulatory' molecule. (Steidl et al. 2012), 8.5 fold decrease expression in HRS cf germinal centre profiles
IL-10**	http://www.uniprot.org/uniprot/P22301 http://www.genecards.org/cgi-bin/carddisp.pl?gene=IL10&search=Il10 The protein encoded by this gene is a cytokine produced primarily by monocytes and to a lesser extent by lymphocytes. This cytokine has pleiotropic effects in immunoregulation and inflammation. It down-regulates the expression of Th1 cytokines, MHC class II Ags, and costimulatory molecules on macrophages. It also enhances B cell survival, proliferation, and antibody production. This cytokine can block NF-kappa B activity, and is involved in the regulation of the JAK-STAT signaling pathway. Knockout studies in mice suggested the function of this cytokine as an essential immunoregulator in the intestinal tract. Mutations in this gene are associated with an increased susceptibility to HIV-1 infection and rheumatoid arthritis.(provided by RefSeq, May 2011) Inhibits the synthesis of a number of cytokines, including IFN-gamma, IL-2, IL-3, TNF and GM-CSF produced by activated macrophages and by helper T-cells. Produced by a variety of cell lines, including T-cells, macrophages, mast cells and other cell types.	The response to LMP1 ?dominated by Treg cells producing Il10. HRS cells produce Il10. When EBV pos this is both viral and human Il10. Il10 has many actions inc: Downregulation of HLA-I (by inhibiting transcription / expression of TAP1) but may also upregulate antiviral Tcell responses. Modifies immune response from Th1 to Th2

IL-10RA**		
IL-5**	http://www.uniprot.org/uniprot/P05113 http://www.genecards.org/cgi-bin/carddisp.pl?gene=IL5&search=Il5 This gene encodes a cytokine that acts as a growth and differentiation factor for both B cells and eosinophils. The encoded cytokine plays a major role in the regulation of eosinophil formation, maturation, recruitment and survival. The increased production of this cytokine may be related to pathogenesis of eosinophil-dependent inflammatory diseases. This cytokine functions by binding to its receptor, which is a heterodimer, whose beta subunit is shared with the receptors for interleukine 3 (IL3) and colony stimulating factor 2 (CSF2/GM-CSF). This gene is located on chromosome 5 within a cytokine gene cluster which includes interleukin 4 (IL4), interleukin 13 (IL13), and CSF2 . This gene, IL4, and IL13 may be regulated coordinately by long-range regulatory elements spread over 120 kilobases on chromosome 5q31. (provided by RefSeq, Jul 2013)	Less known about CD4 response to EBV. CD4+ Tcells expand relatively little during primary EBV infection but do get small pop's of activated CD4+ cells reactive to EBV and response persists during lifelong persistent phase. Majority with IM have CD4 responsive to lytic cycle antigens and EBNA1 but much fewer to other latent Ags. Some say EBNA1 is dominant target for CD4. CD4+ cells expressing Il5 and Il13 dominated response to EBNA1 in 1 study. Others suggest IFN-G producing CD4+ cells important. (Steidl et al. 2012), IL5RA (receptor alpha) 5.5 fold increase in HRS cf germinal centre.
IL-13**	http://www.uniprot.org/uniprot/P35225 http://www.genecards.org/cgi-bin/carddisp.pl?gene=IL13&search=Il13 This gene encodes an immunoregulatory cytokine produced primarily by activated Th2 cells. This cytokine is involved in several stages of B-cell maturation and differentiation. It up-regulates CD23 and MHC class II expression, and promotes IgE isotype switching of B cells. This cytokine down-regulates macrophage activity, thereby inhibits the production of pro-inflammatory cytokines and chemokines. This cytokine is found to be critical to the pathogenesis of allergen-induced asthma but operates through mechanisms independent of IgE and eosinophils. This gene, IL3, IL5, IL4, and CSF2 form a cytokine gene cluster on chromosome 5q, with this gene particularly close to IL4. (provided by RefSeq, Jul 2008)	As above, for Il5, produced by CD4+ cells specific to EBNA1
TNF**	http://www.genecards.org/cgi-bin/carddisp.pl?gene=TNF&search=TNF http://www.uniprot.org/uniprot/P01375 This gene encodes a multifunctional proinflammatory cytokine that belongs to the	Produced by CD8+ Tcells responding to Ag. ?more marked during response to immediate early / early EBV Ags though. Also produced by CD4+ cells

	tumor necrosis factor superfamily. This cytokine is mainly secreted by macrophages. It can bind to, and thus functions through its receptors TNFRSF1A/TNFR1 and TNFRSF1B/TNFBR. This cytokine is involved in the regulation of a wide spectrum of biological processes including cell proliferation, differentiation, apoptosis, lipid metabolism, and coagulation. This cytokine has been implicated in a variety of diseases, including autoimmune diseases, insulin resistance, and cancer. Knockout studies in mice also suggested the neuroprotective function of this cytokine. (provided by RefSeq, Jul 2008)	reacting to Ags (?likely to be ltd in EBV malignancies – although most Tcells in HL LNs are CD4+)
HLA-A ***		
HLA-F ***		
HFE ***	http://www.genecards.org/cgi-bin/carddisp.pl?gene=HFE&search=HFE The protein encoded by this gene is a membrane protein that is similar to MHC class I-type proteins and associates with beta2-microglobulin (beta2M). It is thought that this protein functions to regulate iron absorption by regulating the interaction of the transferrin receptor with transferrin. The iron storage disorder, hereditary haemochromatosis, is a recessive genetic disorder that results from defects in this gene. At least nine alternatively spliced variants have been described for this gene. Additional variants have been found but their full-length nature has not been determined. (provided by RefSeq, Jul 2008)	Reduction associated with increased EBNA1 expression. (Therefore ?in viral infected cells).
TAP2 ***	http://www.uniprot.org/uniprot/Q03519 Involved in the transport of antigens from the cytoplasm to the endoplasmic reticulum for association with MHC class I molecules. Also acts as a molecular scaffold for the final stage of MHC class I folding, namely the binding of peptide. Nascent MHC class I molecules associate with TAP via tapasin. Inhibited by the covalent attachment of herpes simplex virus ICP47 protein, which blocks the peptide-binding site of TAP. Inhibited by human cytomegalovirus US6 glycoprotein, which binds to the lumenal side of the TAP complex and inhibits peptide translocation by specifically blocking ATP-binding to TAP1 and prevents the conformational rearrangement of TAP induced by peptide binding. Inhibited by human adenovirus E3-19K glycoprotein, which binds the TAP complex and acts as a tapasin	Reduction associated with increased EBNA1 expression. (Therefore ?in viral infected cells).

	inhibitor, preventing MHC class I/TAP association. Induced by IFN-G	
IFN G receptor **	BZLF1 gene product Zta is an immediate early lytic phase protein produced by EBV. It inhibits the expression of IFN-G receptor in infected cells.	In virus infected cells.
CCL8**	http://www.genecards.org/cgi-bin/carddisp.pl?gene=CCL8&search=CCL8 This gene is one of several chemokine genes clustered on the q-arm of chromosome 17. Chemokines form a superfamily of secreted proteins involved in immunoregulatory and inflammatory processes. The superfamily is divided into four subfamilies based on the arrangement of N-terminal cysteine residues of the mature peptide. This chemokine is a member of the CC subfamily which is characterized by two adjacent cysteine residues. This cytokine displays chemotactic activity for monocytes, lymphocytes, basophils and eosinophils. By recruiting leukocytes to sites of inflammation this cytokine may contribute to tumor-associated leukocyte infiltration and to the antiviral state against HIV infection. (provided by RefSeq, Jul 2013)	(Steidl et al. 2012) Fold increase 3.5
LILRB1**	http://www.genecards.org/cgi-bin/carddisp.pl?gene=LILRB1&search=LILRB1 This gene is a member of the leukocyte immunoglobulin-like receptor (LIR) family, which is found in a gene cluster at chromosomal region 19q13.4. The encoded protein belongs to the subfamily B class of LIR receptors which contain two or four extracellular immunoglobulin domains, a transmembrane domain, and two to four cytoplasmic immunoreceptor tyrosine-based inhibitory motifs (ITIMs). The receptor is expressed on immune cells where it binds to MHC class I molecules on antigen-presenting cells and transduces a negative signal that inhibits stimulation of an immune response. It is thought to control inflammatory responses and cytotoxicity to help focus the immune response and limit autoreactivity. Multiple transcript variants encoding different isoforms have been found for this gene. (provided by RefSeq, Jul 2008) Receptor for class I MHC antigens. Recognizes	(Steidl et al. 2012) Fold increase 3.4

	a broad spectrum of HLA-A, HLA-B, HLA-C and HLA-G alleles. Receptor for H301/UL18, a human cytomegalovirus class I MHC homolog. Ligand binding results in inhibitory signals and down-regulation of the immune response. Engagement of LILRB1 present on natural killer cells or T-cells by class I MHC molecules protects the target cells from lysis. Interaction with HLA-B or HLA-E leads to inhibition of the signal triggered by FCER1A and inhibits serotonin release. Inhibits FCGR1A-mediated phosphorylation of cellular proteins and mobilization of intracellular calcium ions	
PERP**	http://www.genecards.org/cgi-bin/carddisp.pl?gene=PERP&search=PERP Component of intercellular desmosome junctions. Plays a role in stratified epithelial integrity and cell-cell adhesion by promoting desmosome assembly. Plays a role as an effector in the TP53-dependent apoptotic pathway (By similarity)	(Steidl et al. 2012) Fold increase 3.2
IL-12B§	http://www.uniprot.org/uniprot/P29460 http://www.genecards.org/cgi-bin/carddisp.pl?gene=IL12B&search=Interleukin+12 This gene encodes a subunit of interleukin 12, a cytokine that acts on T and natural killer cells, and has a broad array of biological activities. Interleukin 12 is a disulfide-linked heterodimer composed of the 40 kD cytokine receptor like subunit encoded by this gene, and a 35 kD subunit encoded by IL12A. This cytokine is expressed by activated macrophages that serve as an essential inducer of Th1 cells development. This cytokine has been found to be important for sustaining a sufficient number of memory/effector Th1 cells to mediate long-term protection to an intracellular pathogen. Overexpression of this gene was observed in the central nervous system of patients with multiple sclerosis (MS), suggesting a role of this cytokine in the pathogenesis of the disease. The promoter polymorphism of this gene has been reported to be associated with the severity of atopic and non-atopic asthma in children. (provided by RefSeq, Jul 2008)	M1 macrophages induced by IFN-G with or without LPS (microbial stimuli) or cytokines (TNF-a, GM-CSF) have an Il-12 ^{high}, Il-23 ^{high} and Il-10 ^{low} phenotype. M1:M2 =10

	Cytokine that can act as a growth factor for activated T and NK cells, enhance the lytic activity of NK/lymphokine-activated killer cells, and stimulate the production of IFN-gamma by resting PBMC. Associates with IL23A to form the IL-23 interleukin, a heterodimeric cytokine which functions in innate and adaptive immunity. IL-23 may constitute with IL-17 an acute response to infection in peripheral tissues. IL-23 binds to a heterodimeric receptor complex composed of IL12RB1 and IL23R, activates the Jak-Stat signaling cascade, stimulates memory rather than naive T-cells and promotes production of proinflammatory cytokines. IL-23 induces autoimmune inflammation and thus may be responsible for autoimmune inflammatory diseases and may be important for tumorigenesis.	
***IL-12A*§**	http://www.uniprot.org/uniprot/P29459 http://www.genecards.org/cgi-bin/carddisp.pl?gene=IL12A&search=Interleukin+12A Cytokine that can act as a growth factor for activated T and NK cells, enhance the lytic activity of NK/lymphokine-activated Killer cells, and stimulate the production of IFN-gamma by resting PBMC. This gene encodes a subunit of a cytokine that acts on T and natural killer cells, and has a broad array of biological activities. The cytokine is a disulfide-linked heterodimer composed of the 35-kD subunit encoded by this gene, and a 40-kD subunit that is a member of the cytokine receptor family. This cytokine is required for the T-cell-independent induction of interferon (IFN)-gamma, and is important for the differentiation of both Th1 and Th2 cells. The responses of lymphocytes to this cytokine are mediated by the activator of transcription protein STAT4. Nitric oxide synthase 2A (NOS2A/NOS2) is found to be required for the signaling process of this cytokine in innate immunity. (provided by RefSeq, Jul 2008)	M1 macrophages induced by IFN-Y with or without LPS (microbial stimuli) or cytokines (TNF-a, GM-CSF) have an Il-12 ^{high}, Il-23 ^{high} and Il-10 ^{low} phenotype.
***IL-23*§**	http://www.uniprot.org/uniprot/Q9NPF7 http://www.genecards.org/cgi-bin/carddisp.pl?gene=IL23A&search=Interleukin+23 Associates with IL12B to form the IL-23 interleukin, a heterodimeric cytokine which	M1 macrophages induced by IFN-Y with or without LPS (microbial stimuli) or cytokines (TNF-a, GM-CSF) have an Il-12 ^{high}, Il-23 ^{high} and Il-10 ^{low} phenotype.

	functions in innate and adaptive immunity. IL-23 may constitute with IL-17 an acute response to infection in peripheral tissues. IL-23 binds to a heterodimeric receptor complex composed of IL12RB1 and IL23R, activates the Jak-Stat signaling cascade, stimulates memory rather than naive T-cells and promotes production of proinflammatory cytokines. IL-23 induces autoimmune inflammation and thus may be responsible for autoimmune inflammatory diseases and may be important for tumorigenesis. This gene encodes a subunit of the heterodimeric cytokine interleukin 23 (IL23). IL23 is composed of this protein and the p40 subunit of interleukin 12 (IL12B). The receptor of IL23 is formed by the beta 1 subunit of IL12 (IL12RB1) and an IL23 specific subunit, IL23R. Both IL23 and IL12 can activate the transcription activator STAT4, and stimulate the production of interferon-gamma (IFNG). In contrast to IL12, which acts mainly on naive CD4(+) T cells, IL23 preferentially acts on memory CD4(+) T cells. (provided by RefSeq, Jul 2008)	
IL-15 [§]	http://www.genecards.org/cgi-bin/carddisp.pl?gene=IL15&search=interleukin+15 The protein encoded by this gene is a cytokine that regulates T and natural killer cell activation and proliferation. This cytokine and interleukine 2 share many biological activities. They are found to bind common hematopoietin receptor subunits, and may compete for the same receptor, and thus negatively regulate each other's activity. The number of CD8+ memory cells is shown to be controlled by a balance between this cytokine and IL2. This cytokine induces the activation of JAK kinases, as well as the phosphorylation and activation of transcription activators STAT3, STAT5, and STAT6. Studies of the mouse counterpart suggested that this cytokine may increase the expression of apoptosis inhibitor BCL2L1/BCL-x(L), possibly through the transcription activation activity of STAT6, and thus prevent apoptosis. Alternatively spliced transcript variants of this gene have been reported.	M1:M2 = 9 (Steidl et al. 2012) 1 of 21 most influential overexpressed genes in primary treatment failure with macrophage signature.

	Function: Cytokine that stimulates the proliferation of T-lymphocytes. Stimulation by IL-15 requires interaction of IL-15 with components of IL-2R, including IL-2R beta and probably IL-2R gamma but not IL-2R alpha	
***TNF α*§**	http://www.genecards.org/cgi-bin/carddisp.pl?gene=TNF&search=TNFa http://www.uniprot.org/uniprot/P01375 Cytokine that binds to TNFRSF1A/TNFR1 and TNFRSF1B/TNFBR. It is mainly secreted by macrophages and can induce cell death of certain tumor cell lines. It is potent pyrogen causing fever by direct action or by stimulation of interleukin-1 secretion and is implicated in the induction of cachexia, Under certain conditions it can stimulate cell proliferation and induce cell differentiation. The TNF intracellular domain (ICD) form induces IL12 production in dendritic cells. The soluble form derives from the membrane form by proteolytic processing. The membrane-bound form is further proteolytically processed by SPPL2A or SPPL2B through regulated intramembrane proteolysis producing TNF intracellular domains (ICD1 and ICD2) released in the cytosol and TNF C-domain 1 and C-domain 2 secreted into the extracellular space. This gene encodes a multifunctional proinflammatory cytokine that belongs to the tumor necrosis factor (TNF) superfamily. This cytokine is mainly secreted by macrophages. It can bind to, and thus functions through its receptors TNFRSF1A/TNFR1 and TNFRSF1B/TNFBR. This cytokine is involved in the regulation of a wide spectrum of biological processes including cell proliferation, differentiation, apoptosis, lipid metabolism, and coagulation. This cytokine has been implicated in a variety of diseases, including autoimmune diseases, insulin resistance, and cancer. Knockout studies in mice also suggested the neuroprotective function of this cytokine. (provided by RefSeq, Jul 2008)	Inflammatory cytokine secreted by M1 macrophages and also 'induces' M1 activation of macrophages. In M2c macrophages, TNF-a and Il-1B proinflammatory cytokines are suppressed. Heme-oxygenase 1 (Ho-1) suppresses both of these and is upregulated in M2 macrophages. M1:M2 = 21
***IL-6*§**	http://www.genecards.org/cgi-bin/carddisp.pl?gene=IL6&search=Interleukin+6 http://www.uniprot.org/uniprot/P05231 Cytokine with a wide variety of biological functions. It is a potent inducer of the acute	Pro-inflammatory cytokine secreted by M1 macrophages M1:M2 = 7 (Steidl et al. 2012) 1 of 21 most influential

	phase response. Plays an essential role in the final differentiation of B-cells into Ig-secreting cells Involved in lymphocyte and monocyte differentiation. It induces myeloma and plasmacytoma growth and induces nerve cells differentiation Acts on B-cells, T-cells, hepatocytes, hematopoietic progenitor cells and cells of the CNS. Also acts as a myokine. It is discharged into the bloodstream after muscle contraction and acts to increase the breakdown of fats and to improve insulin resistance. This gene encodes a cytokine that functions in inflammation and the maturation of B cells. In addition, the encoded protein has been shown to be an endogenous pyrogen capable of inducing fever in people with autoimmune diseases or infections. The protein is primarily produced at sites of acute and chronic inflammation, where it is secreted into the serum and induces a transcriptional inflammatory response through interleukin 6 receptor, alpha. The functioning of this gene is implicated in a wide variety of inflammation-associated disease states, including suspectibility to diabetes mellitus and systemic juvenile rheumatoid arthritis.	overexpressed genes in primary treatment failure with macrophage signature.
***CD86*§**	http://www.genecards.org/cgi-bin/carddisp.pl?gene=CD86&search=CD86 This gene encodes a type I membrane protein that is a member of the immunoglobulin superfamily. This protein is expressed by antigen-presenting cells, and it is the ligand for two proteins at the cell surface of T cells, CD28 antigen and cytotoxic T-lymphocyte-associated protein 4. Binding of this protein with CD28 antigen is a costimulatory signal for activation of the T-cell. Binding of this protein with cytotoxic T-lymphocyte-associated protein 4 negatively regulates T-cell activation and diminishes the immune response. Alternative splicing results in several transcript variants encoding different isoforms. Receptor involved in the costimulatory signal essential for T-lymphocyte proliferation and interleukin-2 production, by binding CD28 or CTLA-4. May play a critical role in the early events of T-cell activation and	Surface expression by M1 macrophages (evidently only if GM-CSF activated/stimulated) ?Look also at CTLA4 expression in HL as this appears to result in diminishing the T-cell immune response +/- look at CD28 antigen expression

	costimulation of naive T-cells, such as deciding between immunity and anergy that is made by T-cells within 24 hours after activation. Isoform 2 interferes with the formation of CD86 clusters, and thus acts as a negative regulator of T-cell activation	
CD120b TNFRFS1B§	http://www.genecards.org/cgi-bin/carddisp.pl?gene=TNFRSF1B&search=CD120b The protein encoded by this gene is a member of the TNF-receptor superfamily. This protein and TNF-receptor 1 form a heterocomplex that mediates the recruitment of two anti-apoptotic proteins, c-IAP1 and c-IAP2, which possess E3 ubiquitin ligase activity. The function of IAPs in TNF-receptor signalling is unknown, however, c-IAP1 is thought to potentiate TNF-induced apoptosis by the ubiquitination and degradation of TNF-receptor-associated factor 2, which mediates anti-apoptotic signals. Knockout studies in mice also suggest a role of this protein in protecting neurons from apoptosis by stimulating antioxidative pathways. (provided by RefSeq, Jul 2008) Receptor with high affinity for TNFSF2/TNF-alpha and approximately 5-fold lower affinity for homotrimeric TNFSF1/lymphotoxin-alpha. The TRAF1/TRAF2 complex recruits the apoptotic suppressors BIRC2 and BIRC3 to TNFRSF1B/TNFR2. This receptor mediates most of the metabolic effects of TNF-alpha. Isoform 2 blocks TNF-alpha-induced apoptosis, which suggests that it regulates TNF-alpha function by antagonizing its biological activity	M1 macrophage cell surface expression (Steidl, Telenius, et al. 2010)genes overexpressed in treatment failure.
LILRB1§	http://www.genecards.org/cgi-bin/carddisp.pl?gene=LILRB1&search=LILRB1 Receptor for class I MHC antigens. Recognizes a broad spectrum of HLA-A, HLA-B, HLA-C and HLA-G alleles. Receptor for H301/UL18, a human cytomegalovirus class I MHC homolog. Ligand binding results in inhibitory signals and down-regulation of the immune response. Engagement of LILRB1 present on natural killer cells or T-cells by class I MHC molecules protects the target cells from lysis. Interaction with HLA-B or HLA-E	Part of a gene cluster of immune-modulatory proteins expressed in M1 macrophages.

	leads to inhibition of the signal triggered by FCER1A and inhibits serotonin release. Inhibits FCGR1A-mediated phosphorylation of cellular proteins and mobilization of intracellular calcium ions This gene is a member of the leukocyte immunoglobulin-like receptor (LIR) family, which is found in a gene cluster at chromosomal region 19q13.4. The encoded protein belongs to the subfamily B class of LIR receptors which contain two or four extracellular immunoglobulin domains, a transmembrane domain, and two to four cytoplasmic immunoreceptor tyrosine-based inhibitory motifs (ITIMs). The receptor is expressed on immune cells where it binds to MHC class I molecules on antigen-presenting cells and transduces a negative signal that inhibits stimulation of an immune response. It is thought to control inflammatory responses and cytotoxicity to help focus the immune response and limit autoreactivity. Multiple transcript variants encoding different isoforms have been found for this gene.	
CXCL9§	http://www.genecards.org/cgi-bin/carddisp.pl?gene=CXCL9&search=CXCL9 Function: Cytokine that affects the growth, movement, or activation state of cells that participate in immune and inflammatory response. Chemotactic for activated T-cells. Binds to CXCR3	M1:M2 = 58 (Steidl et al. 2012) 1 of 21 most influential overexpressed genes in primary treatment failure with macrophage signature.
CXCL10§	http://www.uniprot.org/uniprot/P02778 http://www.genecards.org/cgi-bin/carddisp.pl?gene=CXCL10&search=CXCL Chemotactic for monocytes and T-lymphocytes. Binds to CXCR3. (secreted protein) Induced by IFNG/IFN-gamma. A diverse population of cell types rapidly increases transcription of mRNA encoding this protein. This suggests that gamma-induced protein may be a key mediator of the IFNG/IFN-gamma response. This gene encodes a chemokine of the CXC subfamily and ligand for the receptor CXCR3. Binding of this protein to CXCR3 results in pleiotropic effects, including stimulation of monocytes, natural killer and T-cell migration,	Secreted by M1 macrophages. ?also look at CXCR3 expression M1:M2 = 59 (Steidl et al. 2012) 1 of 21 most influential overexpressed genes in primary treatment failure with macrophage signature.

	and modulation of adhesion molecule expression.	
CXCL11§	http://www.genecards.org/cgi-bin/carddisp.pl?gene=CXCL11&search=CXCL Chemokines are a group of small (approximately 8 to 14 kD), mostly basic, structurally related molecules that regulate cell trafficking of various types of leukocytes through interactions with a subset of 7-transmembrane, G protein-coupled receptors. Chemokines also play fundamental roles in the development, homeostasis, and function of the immune system, and they have effects on cells of the central nervous system as well as on endothelial cells involved in angiogenesis or angiostasis. Chemokines are divided into 2 major subfamilies, CXC and CC. This gene is a CXC member of the chemokine superfamily. Its encoded protein induces a chemotactic response in activated T-cells and is the dominant ligand for CXC receptor-3. The gene encoding this protein contains 4 exons and at least three polyadenylation signals which might reflect cell-specific regulation of expression. IFN-gamma is a potent inducer of transcription of this gene. Chemotactic for interleukin-activated T-cells but not unstimulated T-cells, neutrophils or monocytes. Induces calcium release in activated T-cells. Binds to CXCR3. May play an important role in CNS diseases which involve T-cell recruitment. May play a role in skin immune responses	Secreted by M1 macrophages. ?also look at CXCR3 expression M1:M2 = 212
GBP1§	http://www.genecards.org/cgi-bin/carddisp.pl?gene=GBP1&search=GBP1 Function: Hydrolyzes GTP to GMP in two consecutive cleavage reactions. Exhibits antiviral activity against influenza virus. Promote oxidative killing and deliver antimicrobial peptides to autophagolysosomes, providing broad host protection against different pathogen classes Guanylate binding protein expression is induced by interferon. Guanylate binding proteins are characterized by their ability to specifically bind guanine nucleotides (GMP, GDP, and GTP) and are distinguished from the	Increased expression in M1 macrophages. ?antiviral activity ?interesting to look at in EBV pos and neg HL

	GTP-binding proteins by the presence of 2 binding motifs rather than 3.	
NOS 2§	http://www.genecards.org/cgi-bin/carddisp.pl?gene=NOS2&search=NOS2 Function: Produces nitric oxide (NO) which is a messenger molecule with diverse functions throughout the body. In macrophages, NO mediates tumoricidal and bactericidal actions. Also has nitrosylase activity and mediates cysteine S-nitrosylation of cytoplasmic target proteins such COX2 Inducible nitric oxide synthase (iNOS), along with neuronal nitric oxide synthase (nNOS) and endothelial nitric oxide synthase (eNOS), catalyze the generation of nitric oxide and L-citrulline from L-arginine and molecular oxygen. It displays high affinity for Ca2+/calmodulin, thus appears to be constitutively active. Tetrahydrobiopterin, flavin adenine dinucleotide, flavin mononucleotide and NADPH co-factors are required for catalytic activity. iNOS gene expression is regulated by various pathways including NF-kappaB, IFN-gamma (signaling through the JAK-STAT cascade) and HIF-1, which induce expression, and TGF-beta and interleukins -4, -10 and -13, which inhibit expression. Post-translational regulation occurs by prevention of iNOS homodimerization by NAP110. iNOS has roles in the innate immune system and displays antibacterial, antiviral and antifungal activity. The human gene encoding iNOS is localized to chromosome 17q11.2-q12.	Increased expression in M1 macrophages. Expression inhibited by Il-10. Regulated via NF-KB and IFN-Y etc
CCR7§	http://www.genecards.org/cgi-bin/carddisp.pl?gene=CCR7&search=CCR7 The protein encoded by this gene is a member of the G protein-coupled receptor family. This receptor was identified as a gene induced by the Epstein-Barr virus (EBV), and is thought to be a mediator of EBV effects on B lymphocytes. This receptor is expressed in various lymphoid tissues and activates B and T lymphocytes. It has been shown to control the migration of memory T cells to inflamed tissues, as well as stimulate dendritic cell maturation. The chemokine (C-C motif) ligand	CCR7 is a membrane receptor expressed by M1 macrophages. NB also under EBV induced list as thought to be induced by EBV and a mediator of EBV effects on lymphocytes. Links in with CCL19 it's ligand which is also increased in M1 macrophages. M1:M2 = 107

	19 (CCL19/ECL) has been reported to be a specific ligand of this receptor. (provided by RefSeq, Jul 2008)	
CCL19§	http://www.genecards.org/cgi-bin/carddisp.pl?gene=CCL19&search=CCL19 This gene is one of several CC cytokine genes clustered on the p-arm of chromosome 9. Cytokines are a family of secreted proteins involved in immunoregulatory and inflammatory processes. The CC cytokines are proteins characterized by two adjacent cysteines Function: May play a role not only in inflammatory and immunological responses but also in normal lymphocyte recirculation and homing. May play an important role in trafficking of T-cells in thymus, and T-cell and B-cell migration to secondary lymphoid organs. Specifically binds to chemokine receptor CCR7. Recombinant CCL19 shows potent chemotactic activity for T-cells and B-cells but not for granulocytes and monocytes	Increased expression in M1 macrophages. Ligand for CCR7 which is also increased in M1 macrophages and recognized as part of the immune response to EBV. M1:M2 = 130 (Steidl et al. 2012) 1 of 21 most influential overexpressed genes in primary treatment failure with macrophage signature.
CCL8§	http://www.genecards.org/cgi-bin/carddisp.pl?gene=CCL8&search=CCL8 This gene is one of several chemokine genes clustered on the q-arm of chromosome 17. Chemokines form a superfamily of secreted proteins involved in immunoregulatory and inflammatory processes. The superfamily is divided into four subfamilies based on the arrangement of N-terminal cysteine residues of the mature peptide. This chemokine is a member of the CC subfamily which is characterized by two adjacent cysteine residues. This cytokine displays chemotactic activity for monocytes, lymphocytes, basophils and eosinophils. By recruiting leukocytes to sites of inflammation this cytokine may contribute to tumor-associated leukocyte infiltration and to the antiviral state against HIV infection	
IL-15RA§	http://www.genecards.org/cgi-bin/carddisp.pl?gene=IL15RA&search=interleukin+15RA This gene encodes a cytokine receptor that specifically binds interleukin 15 (IL15) with high affinity. The receptors of IL15 and IL2 share two subunits, IL2R beta and IL2R gamma. This forms the basis of many overlapping	M1:M2 ratio 16

	biological activities of IL15 and IL2. The protein encoded by this gene is structurally related to IL2R alpha, an additional IL2-specific alpha subunit necessary for high affinity IL2 binding. Unlike IL2RA, IL15RA is capable of binding IL15 with high affinity independent of other subunits, which suggests distinct roles between IL15 and IL2. This receptor is reported to enhance cell proliferation and expression of apoptosis inhibitor BCL2L1/BCL2-XL and BCL2. Multiple alternatively spliced transcript variants of this gene have been reported.(provided by RefSeq, Apr 2010)	
BCL2A1[§]	http://www.genecards.org/cgi-bin/carddisp.pl?gene=BCL2A1&search=BCL2A1 This gene encodes a member of the BCL-2 protein family. The proteins of this family form hetero- or homodimers and act as anti- and pro-apoptotic regulators that are involved in a wide variety of cellular activities such as embryonic development, homeostasis and tumorigenesis. The protein encoded by this gene is able to reduce the release of pro-apoptotic cytochrome c from mitochondria and block caspase activation. This gene is a direct transcription target of NF-kappa B in response to inflammatory mediators, and is up-regulated by different extracellular signals, such as granulocyte-macrophage colony-stimulating factor (GM-CSF), CD40, phorbol ester and inflammatory cytokine TNF and IL-1, which suggests a cytoprotective function that is essential for lymphocyte activation as well as cell survival. Alternatively spliced transcript variants encoding different isoforms have been found for this gene. Function: Retards apoptosis induced by IL-3 deprivation. May function in the response of hemopoietic cells to external signals and in maintaining endothelial survival during infection (By similarity)	Increased in M1 macrophages but seems to link in with inhibiting apoptosis M1:M2 = 34
FAS[§]	http://www.genecards.org/cgi-bin/carddisp.pl?gene=FAS&search=Fas Function: Receptor for TNFSF6/FASLG. The adapter molecule FADD recruits caspase-8 to the activated receptor. The	Increased in M1 macrophages. Associated with induction of apoptosis but note isoforms may inhibit apoptosis.

	resulting death-inducing signaling complex (DISC) performs caspase-8 proteolytic activation which initiates the subsequent cascade of caspases (aspartate-specific cysteine proteases) mediating apoptosis. FAS-mediated apoptosis may have a role in the induction of peripheral tolerance, in the antigen-stimulated suicide of mature T-cells, or both. The secreted isoforms 2 to 6 block apoptosis (in vitro) The protein encoded by this gene is a member of the TNF-receptor superfamily. This receptor contains a death domain. It has been shown to play a central role in the physiological regulation of programmed cell death, and has been implicated in the pathogenesis of various malignancies and diseases of the immune system. The interaction of this receptor with its ligand allows the formation of a death-inducing signaling complex that includes Fas-associated death domain protein (FADD), caspase 8, and caspase 10. The autoproteolytic processing of the caspases in the complex triggers a downstream caspase cascade, and leads to apoptosis. This receptor has been also shown to activate NF-kappaB, MAPK3/ERK1, and MAPK8/JNK, and is found to be involved in transducing the proliferating signals in normal diploid fibroblast and T cells. Several alternatively spliced transcript variants have been described, some of which are candidates for nonsense-mediated mRNA decay (NMD). The isoforms lacking the transmembrane domain may negatively regulate the apoptosis mediated by the full length isoform. (provided by RefSeq, Mar 2011)	M1:M2 = 14 (Steidl et al. 2012) 1 of 21 most influential overexpressed genes in primary treatment failure with macrophage signature.
IDO-1[§]	http://www.genecards.org/cgi-bin/carddisp.pl?gene=IDO1&search=INDO This gene encodes indoleamine 2,3-dioxygenase (IDO) - a heme enzyme that catalyzes the first and rate-limiting step in tryptophan catabolism to N-formyl-kynurenine. This enzyme acts on multiple tryptophan substrates including D-tryptophan, L-tryptophan, 5-hydroxy-tryptophan, tryptamine, and serotonin. This enzyme is thought to play a role in a variety of pathophysiological processes such as antimicrobial and antitumor	Enzyme M1:M2 = 117

	defense, neuropathology, immunoregulation, and antioxidant activity. Through its expression in dendritic cells, monocytes, and macrophages this enzyme modulates T-cell behavior by its peri-cellular catabolization of the essential amino acid tryptophan.	
APOL1§	Apolipoprotein Part of a gene cluster APOL (also 1,2,3,6 increased in M1) APOL1, APOL2 in macro cluster APOL 6 IFN cluster	
APOL2§		
APOL3§	http://www.genecards.org/cgi-bin/carddisp.pl?gene=APOL3&search=Apolipoprotein+L3 This gene is a member of the apolipoprotein L gene family, and it is present in a cluster with other family members on chromosome 6. The encoded protein is found in the cytoplasm, where it may affect the movement of lipids, including cholesterol, and/or allow the binding of lipids to organelles. In addition, expression of this gene is up-regulated by tumor necrosis factor-alpha in endothelial cells lining the normal and atherosclerotic iliac artery and aorta. Alternative splicing results in multiple transcript variants. (provided by RefSeq, Jun 2009)	"Extracellular mediator" M1:M2 = 12 (APOL L1 = 7 APOL L2 = 6 APOL L6 = 5) (Steidl et al. 2012) APOL2, APOL6 = 1 of 21 most influential overexpressed genes in primary treatment failure with macrophage signature
APOL6§		
CD163§§	http://www.genecards.org/cgi-bin/carddisp.pl?gene=CD163&search=CD163 The protein encoded by this gene is a member of the scavenger receptor cysteine-rich (SRCR) superfamily, and is exclusively expressed in monocytes and macrophages. Function: Acute phase-regulated receptor involved in clearance and endocytosis of hemoglobin/haptoglobin complexes by macrophages and may thereby protect tissues from free hemoglobin-mediated oxidative damage. May play a role in	Surface expression M2 macrophages ?M2c forms

	the uptake and recycling of iron, via endocytosis of hemoglobin/haptoglobin and subsequent breakdown of heme. Binds hemoglobin/haptoglobin complexes in a calcium-dependent and pH-dependent manner. Exhibits a higher affinity for complexes of hemoglobin and multimeric haptoglobin of HP*1F phenotype than for complexes of hemoglobin and dimeric haptoglobin of HP*1S phenotype. Induces a cascade of intracellular signals that involves tyrosine kinase-dependent calcium mobilization, inositol triphosphate production and secretion of IL6 and CSF1. Isoform 3 exhibits the higher capacity for ligand endocytosis and the more pronounced surface expression when expressed in cells Function: After shedding, the soluble form (sCD163) may play an anti-inflammatory role, and may be a valuable diagnostic parameter for monitoring macrophage activation in inflammatory conditions	
CLEC4A§§	http://www.genecards.org/cgi-bin/carddisp.pl?gene=CLEC4A&search=CLEC+4 Function: May be involved in regulating immune reactivity. May play a role in modulating dendritic cells (DC) differentiation and/or maturation. May be involved via its ITIM motif (immunoreceptor tyrosine-based inhibitory motifs) in the inhibition of B-cell-receptor-mediated calcium mobilization and protein tyrosine phosphorylation	
CCL13§§	http://www.genecards.org/cgi-bin/carddisp.pl?gene=CCL13&search=CCL13 Function: Chemotactic factor that attracts monocytes, lymphocytes, basophils and eosinophils, but not neutrophils. Signals through CCR2B and CCR3 receptors. Plays a role in the accumulation of leukocytes at both sides of allergic and non-allergic inflammation. May be involved in the recruitment of monocytes into the arterial wall during the disease process of atherosclerosis. May play a role in the monocyte attraction in tissues chronically exposed to exogenous pathogens	M1:M2 -38
CD226§§	http://www.genecards.org/cgi-bin/carddisp.pl?gene=CD226&search=CD226	M2 cell surface marker

	This gene encodes a glycoprotein expressed on the surface of NK cells, platelets, monocytes and a subset of T cells. It is a member of the Ig-superfamily containing 2 Ig-like domains of the V-set. The protein mediates cellular adhesion of platelets and megakaryocytic cells to vascular endothelial cells. The protein also plays a role in megakaryocytic cell maturation. Function: Receptor involved in intercellular adhesion, lymphocyte signaling, cytotoxicity and lymphokine secretion mediated by cytotoxic T-lymphocyte (CTL) and NK cell	
CD1a§§	http://www.genecards.org/cgi-bin/carddisp.pl?gene=CD1A&search=CD1a Function: Antigen-presenting protein that binds self and non-self lipid and glycolipid antigens and presents them to T-cell receptors on natural killer T-cells	M2 cell surface marker
CD1b§§	http://www.genecards.org/cgi-bin/carddisp.pl?gene=CD1B&search=CD1b This gene encodes a member of the CD1 family of transmembrane glycoproteins, which are structurally related to the major histocompatibility complex (MHC) proteins and form heterodimers with beta-2-microglobulin. The CD1 proteins mediate the presentation of primarily lipid and glycolipid antigens of self or microbial origin to T cells. The human genome contains five CD1 family genes organized in a cluster on chromosome 1. The CD1 family members are thought to differ in their cellular localization and specificity for particular lipid ligands. The protein encoded by this gene localizes to late endosomes and lysosomes via a tyrosine-based motif in the cytoplasmic tail, and requires vesicular acidification to bind lipid antigens Function: Antigen-presenting protein that binds self and non-self lipid and glycolipid antigens and presents them to T-cell receptors on natural killer T-cells	M2 cell surface marker
CD93§§	http://www.genecards.org/cgi-bin/carddisp.pl?gene=CD93&search=CD93 The protein encoded by this gene is a cell-surface glycoprotein and type I membrane protein that was originally	M2 cell surface marker

	identified as a myeloid cell-specific marker. The encoded protein was once thought to be a receptor for C1q, but now is thought to instead be involved in intercellular adhesion and in the clearance of apoptotic cells. The intracellular cytoplasmic tail of this protein has been found to interact with moesin, a protein known to play a role in linking transmembrane proteins to the cytoskeleton and in the remodelling of the cytoskeleton. (provided by RefSeq, Jul 2008)	
***IL-4*§§**		Useful to look at as stimulate polarisation of macrophages to M2 phenotype. IL-4 described as enhancing pro-tumour effects of TAMs
***IL-10*§§**		Useful to look at as stimulate polarisation of macrophages to M2 phenotype. IL-4 described as enhancing pro-tumour effects of TAMs
***IL-13*§§**	http://www.genecards.org/cgi-bin/carddisp.pl?gene=IL13&search=IL13 This gene encodes an immunoregulatory cytokine produced primarily by activated Th2 cells. This cytokine is involved in several stages of B-cell maturation and differentiation. It up-regulates CD23 and MHC class II expression, and promotes IgE isotype switching of B cells. This cytokine down-regulates macrophage activity, thereby inhibits the production of pro-inflammatory cytokines and chemokines. This cytokine is found to be critical to the pathogenesis of allergen-induced asthma but operates through mechanisms independent of IgE and eosinophils. This gene, IL3, IL5, IL4, and CSF2 form a cytokine gene cluster on chromosome 5q, with this gene particularly close to IL4.	Useful to look at as stimulate polarisation of macrophages to M2 phenotype. IL-4 described as enhancing pro-tumour effects of TAMs
***MRC 1*§§**	http://www.genecards.org/cgi-bin/carddisp.pl?gene=MRC1&search=MRC1 The protein encoded by this gene is a type I membrane receptor that mediates the endocytosis of glycoproteins by macrophages. The protein has been shown to bind high-mannose structures on the surface of potentially pathogenic viruses, bacteria, and fungi so that they can be neutralized by phagocytic engulfment.	M2 surface membrane receptor M1:M2 -43 (Steidl et al. 2012) 1 of 21 most influential overexpressed genes in primary treatment failure with macrophage signature.

	Function: Mediates the endocytosis of glycoproteins by macrophages. Binds both sulfated and non-sulfated polysaccharide chains. Acts as phagocytic receptor for bacteria, fungi and other pathogens	
***MS4A4A*§§**	http://www.genecards.org/cgi-bin/carddisp.pl?gene=MS4A4A&search=MS4A4A This gene encodes a member of the membrane-spanning 4A gene family. Members of this nascent protein family are characterized by common structural features, similar intron/exon splice boundaries, and display unique expression patterns in hematopoietic cells and nonlymphoid tissues. Alternatively spliced transcript variants encoding different isoforms have been found for this gene. Function: May be involved in signal transduction as a component of a multimeric receptor complex	M2 surface membrane receptor M1:M2 -43
***CD36*§§**	http://www.genecards.org/cgi-bin/carddisp.pl?gene=CD36&search=CD36 Function: Seems to have numerous potential physiological functions. Binds to collagen, thrombospondin, anionic phospholipids and oxidized LDL. May function as a cell adhesion molecule. Directly mediates cytoadherence of Plasmodium falciparum parasitized erythrocytes. Binds long chain fatty acids and may function in the transport and/or as a regulator of fatty acid transport. Receptor for thombospondins, THBS1 AND THBS2, mediating their antiangiogenic effects	M2 surface membrane receptor M1:M2 -37
***CD209*§§**	http://www.genecards.org/cgi-bin/carddisp.pl?gene=CD209&search=CD209 Function: Pathogen-recognition receptor expressed on the surface of immature dendritic cells (DCs) and involved in initiation of primary immune response. Thought to mediate the endocytosis of pathogens which are subsequently degraded in lysosomal compartments. The receptor returns to the cell membrane surface and the pathogen-derived antigens are presented to resting T-cells via MHC class II proteins to initiate the adaptive immune response. Probably recognizes in a calcium-dependent	M2 surface membrane receptor M1:M2 -27

	manner high mannose N-linked oligosaccharides in a variety of pathogen antigens, including HIV-1 gp120, HIV-2 gp120, SIV gp120, ebolavirus glycoproteins, cytomegalovirus gB, HCV E2, dengue virus gE, Leishmania pifanoi LPG, Lewis-x antigen in Helicobacter pylori LPS, mannose in Klebsiella pneumonae LPS, di-mannose and tri-mannose in Mycobacterium tuberculosis ManLAM and Lewis-x antigen in Schistosoma mansoni SEA Function: On DCs it is a high affinity receptor for ICAM2 and ICAM3 by binding to mannose-like carbohydrates. May act as a DC rolling receptor that mediates transendothelial migration of DC presursors from blood to tissues by binding endothelial ICAM2. Seems to regulate DC-induced T-cell proliferation by binding to ICAM3 on T-cells in the immunological synapse formed between DC and T-cells	
HS3ST2§§		
ALOX5AP §§		
FIBRONECTIN 1§§	http://www.genecards.org/cgi-bin/carddisp.pl?gene=FN1&search=Fibronectin+1 This gene encodes fibronectin, a glycoprotein present in a soluble dimeric form in plasma, and in a dimeric or multimeric form at the cell surface and in extracellular matrix. Fibronectin is involved in cell adhesion and migration processes including embryogenesis, wound healing, blood coagulation, host defense, and metastasis. The gene has three regions subject to alternative splicing, with the potential to produce 20 different transcript variants. However, the full-length nature of some variants has not been determined. Function: Fibronectins bind cell surfaces and various compounds including collagen, fibrin, heparin, DNA, and actin. Fibronectins are involved in cell adhesion, cell motility, opsonization, wound healing, and maintenance of cell shape. Involved in osteoblast compaction through the fibronectin fibrillogenesis cell-mediated matrix assembly process, essential for osteoblast mineralization. Participates in the regulation of type I collagen	Extracellular mediator increased in M2 M1:M2 -25 (Steidl et al. 2012) 1 of 21 most influential overexpressed genes in primary treatment failure with macrophage signature.

	deposition by osteoblasts Function: Anastellin binds fibronectin and induces fibril formation. This fibronectin polymer, named superfibronectin, exhibits enhanced adhesive properties. Both anastellin and superfibronectin inhibit tumor growth, angiogenesis and metastasis. Anastellin activates p38 MAPK and inhibits lysophospholipid signaling	
FIBRINOGEN-LIKE 2§§	http://www.genecards.org/cgi-bin/carddisp.pl?gene=FGL2&search=FGL2 The protein encoded by this gene is a secreted protein that is similar to the beta- and gamma-chains of fibrinogen. The carboxyl-terminus of the encoded protein consists of the fibrinogen-related domains (FRED). The encoded protein forms a tetrameric complex which is stabilized by interchain disulfide bonds. This protein may play a role in physiologic functions at mucosal sites. Function: May play a role in physiologic lymphocyte functions at mucosal sites	Extracellular mediator increased in M2 M1:M2 -39 (Steidl et al. 2012) 1 of 21 most influential overexpressed genes in primary treatment failure with macrophage signature.
MAF§§	http://www.genecards.org/cgi-bin/carddisp.pl?gene=MAF&search=MAF Function: Acts as a transcriptional activator or repressor. Involved in embryonic lens fiber cell development. Recruits the transcriptional coactivators CREBBP and/or EP300 to crystallin promoters leading to up-regulation of crystallin gene during lens fiber cell differentiation. Activates the expression of IL4 in T helper 2 (Th2) cells. Increases T-cell susceptibility to apoptosis by interacting with MYB and decreasing BCL2 expression. Together with PAX6, transactivates strongly the glucagon gene promoter through the G1 element. Activates transcription of the CD13 proximal promoter in endothelial cells. Represses transcription of the CD13 promoter in early stages of myelopoiesis by affecting the ETS1 and MYB cooperative interaction. Involved in the initial chondrocyte terminal differentiation and the disappearance of hypertrophic chondrocytes during endochondral bone development. Binds to the sequence 5'-[GT]G[GC]N[GT]NCTCAGNN-3' in the L7 promoter. Binds to the T-MARE (Maf response	DNA binding factor M1:M2 -46

	element) sites of lens-specific alpha- and beta-crystallin gene promoters. Binds element G1 on the glucagon promoter. Binds an AT-rich region adjacent to the TGC motif (atypical Maf response element) in the CD13 proximal promoter in endothelial cells (By similarity). When overexpressed, represses anti-oxidant response element (ARE)-mediated transcription. Involved either as an oncogene or as a tumor suppressor, depending on the cell context. Binds to the ARE sites of detoxifying enzyme gene promoters	
MAFB §§		
MS4A6A §§	http://www.genecards.org/cgi-bin/carddisp.pl?gene=MS4A6A&search=MS4A6A This gene encodes a member of the membrane-spanning 4A gene family. Members of this nascent protein family are characterized by common structural features and similar intron/exon splice boundaries and display unique expression patterns among hematopoietic cells and nonlymphoid tissues. The gene encoding this protein is localized to 11q12.1, among a cluster of family members. Alternative splicing of this gene results in several transcript variants that encode different protein isoforms. Function: May be involved in signal transduction as a component of a multimeric receptor complex	M1:M2 -33 (Steidl et al. 2012) 1 of 21 most influential overexpressed genes in primary treatment failure with macrophage signature.
CLEC4E §§		
CLEC7A §§	http://www.uniprot.org/uniprot/Q9BXN2 http://www.genecards.org/cgi-bin/carddisp.pl?gene=CLEC7A&search=CLEC7A Function: Lectin that functions as pattern receptor specific for beta-1,3-linked and beta-1,6-linked glucans, such as cell wall constituents from pathogenic bacteria and fungi. Necessary for the TLR2-mediated inflammatory response and for TLR2-mediated activation of NF-kappa-B. Enhances cytokine production in macrophages and dendritic cells. Mediates production of reactive oxygen species in the cell. Mediates phagocytosis of C.albicans conidia. Binds T-cells in a way that does not involve their surface glycans and plays a role in T-cell	Membrane receptor M1:M2 -19

	activation. Stimulates T-cell proliferation (By similarity)	
P2RY13§§	P2RY13 is in macro cluster	
P2RY10§§	P2RY10 is in T-cell cluster	
TLR1§§	TLR 1,2,7,8 in macro cluster	
TLR2§§		
TLR7§§		
TLR8§§		
TGFBR2§§	http://www.genecards.org/cgi-bin/carddisp.pl?gene=TGFBR2&search=TGFBR2 This gene encodes a member of the Ser/Thr protein kinase family and the TGFB receptor subfamily. The encoded protein is a transmembrane protein that has a protein kinase domain, forms a heterodimeric complex with another receptor protein, and binds TGF-beta. This receptor/ligand complex phosphorylates proteins, which then enter the nucleus and regulate the transcription of a subset of genes related to cell proliferation. Mutations in this gene have been associated with Marfan Syndrome, Loeys-Deitz Aortic Aneurysm Syndrome, and the development of various types of tumors. Function: Transmembrane serine/threonine kinase forming with the TGF-beta type I serine/threonine kinase receptor, TGFBR1, the non-promiscuous receptor for the TGF-beta cytokines TGFB1, TGFB2 and TGFB3. Transduces the TGFB1, TGFB2 and TGFB3 signal from the cell surface to the cytoplasm and is thus regulating a plethora of physiological and pathological processes including cell cycle arrest in epithelial and hematopoietic cells, control of mesenchymal cell proliferation and differentiation, wound healing, extracellular matrix production, immunosuppression and carcinogenesis.	(Steidl et al. 2012) 1 of 21 most influential overexpressed genes in primary treatment failure with macrophage signature.
CATHEPSIN D¶	http://www.genecards.org/cgi-bin/carddisp.pl?gene=CTSD&search=cathepsin+D Function: Acid protease active in intracellular protein breakdown. Involved in the pathogenesis of several diseases such as breast cancer and possibly Alzheimer disease	Labelled as M2 like. 6.4 fold increase
CATHEPSIN B¶	http://www.genecards.org/cgi-	'M2 like'

	bin/carddisp.pl?gene=CTSB&search=cathepsin+B Function: Thiol protease which is believed to participate in intracellular degradation and turnover of proteins. Has also been implicated in tumor invasion and metastasis	5.8 fold increase
CATHEPSIN L	http://www.genecards.org/cgi-bin/carddisp.pl?gene=CTSL&search=cathepsin+L Function: Important for the overall degradation of proteins in lysosomes The main function of cathepsins is protein recycling within the lysosome but they are also known to be involved in a range of other physiological, as well as pathological processes, including maturation of the MHC class II complex, bone remodeling, keratinocyte differentiation, tumor progression and metastasis, rheumatoid arthritis, osteoarthritis and atherosclerosis.	'M2 like' 5.4 fold increase
CATHEPSIN S	http://www.genecards.org/cgi-bin/carddisp.pl?gene=CTSS&search=Cathepsin+S The protein encoded by this gene, a member of the peptidase C1 family, is a lysosomal cysteine proteinase that may participate in the degradation of antigenic proteins to peptides for presentation on MHC class II molecules. The encoded protein can function as an elastase over a broad pH range in alveolar macrophages. Alternatively spliced transcript variants encoding distinct isoforms have been found for this gene.	'M2 like' 2.1 fold increase
MSR 1	http://www.genecards.org/cgi-bin/carddisp.pl?gene=MSR1&search=MSR1 This gene encodes the class A macrophage scavenger receptors, which include three different types (1, 2, 3) generated by alternative splicing of this gene. These receptors or isoforms are macrophage-specific trimeric integral membrane glycoproteins and have been implicated in many macrophage-associated physiological and pathological processes including atherosclerosis, Alzheimer's disease, and host defense. The isoforms type 1 and	'apoptotic cell clearance / M1' 17 fold increase

	type 2 are functional receptors and are able to mediate the endocytosis of modified low density lipoproteins (LDLs). The isoform type 3 does not internalize modified LDL (acetyl-LDL) despite having the domain shown to mediate this function in the types 1 and 2 isoforms. It has an altered intracellular processing and is trapped within the endoplasmic reticulum, making it unable to perform endocytosis. The isoform type 3 can inhibit the function of isoforms type 1 and type 2 when co-expressed, indicating a dominant negative effect and suggesting a mechanism for regulation of scavenger receptor activity in macrophages.	
***LRP1*¶**	http://www.genecards.org/cgi-bin/carddisp.pl?gene=LRP1&search=CD91 The protein encoded by this gene is an endocytic receptor involved in several cellular processes, including intracellular signaling, lipid homeostasis, and clearance of apoptotic cells. In addition, the encoded protein is necessary for the A2M-mediated clearance of secreted amyloid precursor protein and beta-amyloid, the main component of amyloid plaques found in Alzheimer patients. Expression of this gene decreases with age and has been found to be lower than controls in brain tissue from Alzheimer patients.	'apoptotic /M1' 8.4 fold increase ?increased in older age
***MERTK*¶**	http://www.genecards.org/cgi-bin/carddisp.pl?gene=MERTK&search=MERTK Function: Receptor tyrosine kinase that transduces signals from the extracellular matrix into the cytoplasm by binding to several ligands including LGALS3, TUB, TULP1 or GAS6. Regulates many physiological processes including cell survival, migration, differentiation, and phagocytosis of apoptotic cells (efferocytosis). Ligand binding at the cell surface induces autophosphorylation of MERTK on its intracellular domain that provides docking sites for downstream signaling molecules. Following activation by ligand, interacts with GRB2 or PLCG2 and induces phosphorylation of MAPK1, MAPK2, FAK/PTK2 or RAC1. MERTK signaling plays a role in various processes such as macrophage clearance of apoptotic cells, platelet aggregation, cytoskeleton	Apoptotic / M1 7.5 fold increase NB binds to LGALS3 see below

	reorganization and engulfment. Functions in the retinal pigment epithelium (RPE) as a regulator of rod outer segments fragments phagocytosis. Plays also an important role in inhibition of Toll-like receptors (TLRs)-mediated innate immune response by activating STAT1, which selectively induces production of suppressors of cytokine signaling SOCS1 and SOCS3	
LGALS3 GALECTIN 3¶	http://www.genecards.org/cgi-bin/carddisp.pl?gene=LGALS3&search=LGALS3 This gene encodes a member of the galectin family of carbohydrate binding proteins. Members of this protein family have an affinity for beta-galactosides. The encoded protein is characterized by an N-terminal proline-rich tandem repeat domain and a single C-terminal carbohydrate recognition domain. This protein can self-associate through the N-terminal domain allowing it to bind to multivalent saccharide ligands. This protein localizes to the extracellular matrix, the cytoplasm and the nucleus. This protein plays a role in numerous cellular functions including apoptosis, innate immunity, cell adhesion and T-cell regulation. Alternate splicing results in multiple transcript variants. Function: Galactose-specific lectin which binds IgE. May mediate with the alpha-3, beta-1 integrin the stimulation by CSPG4 of endothelial cells migration. Together with DMBT1, required for terminal differentiation of columnar epithelial cells during early embryogenesis (By similarity). In the nucleus: acts as a pre-mRNA splicing factor. Involved in acute inflammatory responses including neutrophil activation and adhesion, chemoattraction of monocytes macrophages, opsonization of apoptotic neutrophils, and activation of mast cells	Apoptosis/M1 5.2 fold increase
TREM2¶	http://www.genecards.org/cgi-bin/carddisp.pl?gene=TREM2&search=TREM2 Function: May have a role in chronic inflammations and may stimulate production of constitutive rather than inflammatory chemokines and cytokines. Forms a receptor signaling complex with	Apoptosis / M1 3.5 fold increase

	TYROBP and triggers activation of the immune responses in macrophages and dendritic cells	
TGFB1	http://www.genecards.org/cgi-bin/carddisp.pl?gene=TGFB1&search=TGF+B1 Function: Multifunctional protein that controls proliferation, differentiation and other functions in many cell types. Many cells synthesize TGFB1 and have specific receptors for it. It positively and negatively regulates many other growth factors. It plays an important role in bone remodeling as it is a potent stimulator of osteoblastic bone formation, causing chemotaxis, proliferation and differentiation in committed osteoblasts	Apoptosis/M1 2.3 fold increase
GPNMB	http://www.genecards.org/cgi-bin/carddisp.pl?gene=GPNMB&search=GPNMB The protein encoded by this gene is a type I transmembrane glycoprotein which shows homology to the pMEL17 precursor, a melanocyte-specific protein. GPNMB shows expression in the lowly metastatic human melanoma cell lines and xenografts but does not show expression in the highly metastatic cell lines. GPNMB may be involved in growth delay and reduction of metastatic potential. Two transcript variants encoding different isoforms have been found for this gene.	Trophic, angiogenic, matrix remodelling 8.8 fold increase
PLAU	http://www.genecards.org/cgi-bin/carddisp.pl?gene=PLAU&search=PLAU This gene encodes a serine protease involved in degradation of the extracellular matrix and possibly tumor cell migration and proliferation. A specific polymorphism in this gene may be associated with late-onset Alzheimer's disease and also with decreased affinity for fibrin-binding. This protein converts plasminogen to plasmin by specific cleavage of an Arg-Val bond in plasminogen. Plasmin in turn cleaves this protein at a Lys-Ile bond to form a two-chain derivative in which a single disulfide bond connects the amino-terminal A-chain to the catalytically active, carboxy-terminal B-chain. This two-chain derivative is also called HMW-uPA (high molecular weight uPA). HMW-uPA can be further	Trophic, angiogenic, matrix remodelling 6.7 fold increase

	processed into LMW-uPA (low molecular weight uPA) by cleavage of chain A into a short chain A (A1) and an amino-terminal fragment. LMW-uPA is proteolytically active but does not bind to the uPA receptor. Alternatively spliced transcript variants encoding different isoforms have been found for this gene.	
TIMP2¶	http://www.genecards.org/cgi-bin/carddisp.pl?gene=TIMP2&search=TIMP2 This gene is a member of the TIMP gene family. The proteins encoded by this gene family are natural inhibitors of the matrix metalloproteinases, a group of peptidases involved in degradation of the extracellular matrix. In addition to an inhibitory role against metalloproteinases, the encoded protein has a unique role among TIMP family members in its ability to directly suppress the proliferation of endothelial cells. As a result, the encoded protein may be critical to the maintenance of tissue homeostasis by suppressing the proliferation of quiescent tissues in response to angiogenic factors, and by inhibiting protease activity in tissues undergoing remodelling of the extracellular matrix. Function: Complexes with metalloproteinases (such as collagenases) and irreversibly inactivates them by binding to their catalytic zinc cofactor. Known to act on MMP-1, MMP-2, MMP-3, MMP-7, MMP-8, MMP-9, MMP-10, MMP-13, MMP-14, MMP-15, MMP-16 and MMP-19	Trophic, angiogenic, matrix remodelling 4 fold increase
CD68¶	http://www.genecards.org/cgi-bin/carddisp.pl?gene=CD68&search=cd68 This gene encodes a 110-kD transmembrane glycoprotein that is highly expressed by human monocytes and tissue macrophages. It is a member of the lysosomal/endosomal-associated membrane glycoprotein (LAMP) family. The protein primarily localizes to lysosomes and endosomes with a smaller fraction circulating to the cell surface. It is a type I integral membrane protein with a heavily glycosylated extracellular domain and binds to tissue- and organ-specific lectins or selectins. The protein is also a member of the scavenger receptor family. Scavenger	Include because has been quoted in many different papers as macrophage marker and quantified by immunophenotyping.

	receptors typically function to clear cellular debris, promote phagocytosis, and mediate the recruitment and activation of macrophages. Alternative splicing results in multiple transcripts encoding different isoforms. (provided by RefSeq, Jul 2008) Could play a role in phagocytic activities of tissue macrophages, both in intracellular lysosomal metabolism and extracellular cell-cell and cell-pathogen interactions. Binds to tissue- and organ-specific lectins or selectins, allowing homing of macrophage subsets to particular sites. Rapid recirculation of CD68 from endosomes and lysosomes to the plasma membrane may allow macrophages to crawl over selectin-bearing substrates or other cells	
INHBA¶¶	http://www.genecards.org/cgi-bin/carddisp.pl?gene=INHBA&search=INHBA The inhibin beta A subunit joins the alpha subunit to form a pituitary FSH secretion inhibitor. Inhibin has been shown to regulate gonadal stromal cell proliferation negatively and to have tumor-suppressor activity. In addition, serum levels of inhibin have been shown to reflect the size of granulosa-cell tumors and can therefore be used as a marker for primary as well as recurrent disease. Because expression in gonadal and various extragonadal tissues may vary severalfold in a tissue-specific fashion, it is proposed that inhibin may be both a growth/differentiation factor and a hormone. Furthermore, the beta A subunit forms a homodimer, activin A, and also joins with a beta B subunit to form a heterodimer, activin AB, both of which stimulate FSH secretion. Finally, it has been shown that the beta A subunit mRNA is identical to the erythroid differentiation factor subunit mRNA and that only one gene for this mRNA exists in the human genome. (provided by RefSeq, Jul 2008) Inhibins and activins inhibit and activate, respectively, the secretion of follitropin by the pituitary gland. Inhibins/activins are involved in regulating a number of diverse functions such	(Steidl et al. 2012) 1 of 21 most influential overexpressed genes in primary treatment failure with macrophage signature.

	as hypothalamic and pituitary hormone secretion, gonadal hormone secretion, germ cell development and maturation, erythroid differentiation, insulin secretion, nerve cell survival, embryonic axial development or bone growth, depending on their subunit composition. Inhibins appear to oppose the functions of activins	
TGFBI¶¶	http://www.genecards.org/cgi-bin/carddisp.pl?gene=TGFBI&search=TGFBI This gene encodes an RGD-containing protein that binds to type I, II and IV collagens. The RGD motif is found in many extracellular matrix proteins modulating cell adhesion and serves as a ligand recognition sequence for several integrins. This protein plays a role in cell-collagen interactions and may be involved in endochondrial bone formation in cartilage. The protein is induced by transforming growth factor-beta and acts to inhibit cell adhesion. Mutations in this gene are associated with multiple types of corneal dystrophy. (provided by RefSeq, Jul 2008)	(Steidl et al. 2012) 1 of 21 most influential overexpressed genes in primary treatment failure with macrophage signature.
APOL2¶¶	APOL2 Macro' + HK clusters	
APOL6¶¶	APOL6 'IFN' cluster	
****IRF7***	http://www.genecards.org/cgi-bin/carddisp.pl?gene=IRF7&search=IRF7 Function: Key transcriptional regulator of type I interferon (IFN)-dependent immune responses and plays a critical role in the innate immune response against DNA and RNA viruses. Regulates the transcription of type I IFN genes (IFN-alpha and IFN-beta) and IFN-stimulated genes (ISG) by binding to an interferon-stimulated response element (ISRE) in their promoters. Can efficiently activate both the IFN-beta (IFNB) and the IFN-alpha (IFNA) genes and mediate their induction via both the virus-activated, MyD88-independent pathway and the TLR-activated, MyD88-dependent pathway. Required during both the early and late phases of the IFN gene induction but is more critical for the late than for the early phase. Exists in an inactive form in the cytoplasm of uninfected cells and following viral infection, double-stranded	(Steidl et al. 2012) 1 of 21 most influential overexpressed genes in primary treatment failure with macrophage signature.

	RNA (dsRNA), or toll-like receptor (TLR) signaling, becomes phosphorylated by IKBKE and TBK1 kinases. This induces a conformational change, leading to its dimerization and nuclear localization where along with other coactivators it can activate transcription of the type I IFN and ISG genes. Can also play a role in regulating adaptive immune responses by inducing PSMB9/LMP2 expression, either directly or through induction of IRF1. Binds to the Q promoter (Qp) of EBV nuclear antigen 1 a (EBNA1) and may play a role in the regulation of EBV latency. Can activate distinct gene expression programs in macrophages and regulate the anti-tumor properties of primary macrophages	
IRF8¶¶	http://www.genecards.org/cgi-bin/carddisp.pl?gene=IRF8&search=IRF8 Interferon consensus sequence-binding protein (ICSBP) is a transcription factor of the interferon (IFN) regulatory factor (IRF) family. Proteins of this family are composed of a conserved DNA-binding domain in the N-terminal region and a divergent C-terminal region that serves as the regulatory domain. The IRF family proteins bind to the IFN-stimulated response element (ISRE) and regulate expression of genes stimulated by type I IFNs, namely IFN-alpha and IFN-beta. IRF family proteins also control expression of IFN-alpha and IFN-beta-regulated genes that are induced by viral infection. (provided by RefSeq, Jul 2008) Specifically binds to the upstream regulatory region of type I IFN and IFN-inducible MHC class I genes (the interferon consensus sequence (ICS)). Plays a negative regulatory role in cells of the immune system. Involved in CD8(+) dendritic cell differentiation by forming a complex with the BATF-JUNB heterodimer in immune cells, leading to recognition of AICE sequence (5'-TGAnTCA/GAAA-3'), an immune-specific regulatory element, followed by cooperative binding of BATF and IRF8 and activation of genes (By similarity)	(Steidl, Lee, et al. 2010) genes overexpressed in treatment failure.
TLR2¶¶	http://www.genecards.org/index.php?path=/Search/keyword/TLR2	(Steidl, Lee, et al. 2010) genes overexpressed in treatment failure.

	The protein encoded by this gene is a member of the Toll-like receptor family which plays a fundamental role in pathogen recognition and activation of innate immunity. TLRs are highly conserved from Drosophila to humans and share structural and functional similarities. They recognize pathogen-associated molecular patterns (PAMPs) that are expressed on infectious agents, and mediate the production of cytokines necessary for the development of effective immunity. The various TLRs exhibit different patterns of expression. This gene is expressed most abundantly in peripheral blood leukocytes, and mediates host response to Gram-positive bacteria and yeast via stimulation of NF-kappaB. (provided by RefSeq, Jul 2008) Cooperates with LY96 to mediate the innate immune response to bacterial lipoproteins and other microbial cell wall components. Cooperates with TLR1 or TLR6 to mediate the innate immune response to bacterial lipoproteins or lipopeptides. Acts via MYD88 and TRAF6, leading to NF-kappa-B activation, cytokine secretion and the inflammatory response. May also promote apoptosis in response to lipoproteins. Recognizes mycoplasmal macrophage-activating lipopeptide-2kD (MALP-2), soluble tuberculosis factor (STF), phenol-soluble modulin (PSM) and B.burgdorferi outer surface protein A lipoprotein (OspA-L) cooperatively with TLR6	TLR5 is in the M2 list. Link with CLEC7A in M2 list
CSF1R¶¶	http://www.genecards.org/cgi-bin/carddisp.pl?gene=CSF1R&search=CSF1R The protein encoded by this gene is the receptor for colony stimulating factor 1, a cytokine which controls the production, differentiation, and function of macrophages. This receptor mediates most if not all of the biological effects of this cytokine. Ligand binding activates the receptor kinase through a process of oligomerization and transphosphorylation. The encoded protein is a tyrosine kinase transmembrane receptor and member of the CSF1/PDGF receptor family of tyrosine-protein kinases. Mutations in this gene have been associated with a predisposition to myeloid malignancy.	(Steidl, Lee, et al. 2010) genes overexpressed in treatment failure. (Steidl et al. 2012) Key gene in Steidl 2012 paper as well. Part of score with CD68 expression by IHC to predict clinical outcome PFS / OS.

	The first intron of this gene contains a transcriptionally inactive ribosomal protein L7 processed pseudogene oriented in the opposite direction. (provided by RefSeq, Jul 2008)	
ITGA4¶¶	http://www.genecards.org/cgi-bin/carddisp.pl?gene=ITGA4&search=ITGA4 Integrins alpha-4/beta-1 (VLA-4) and alpha-4/beta-7 are receptors for fibronectin. They recognize one or more domains within the alternatively spliced CS-1 and CS-5 regions of fibronectin. They are also receptors for VCAM1. Integrin alpha-4/beta-1 recognizes the sequence Q-I-D-S in VCAM1. Integrin alpha-4/beta-7 is also a receptor for MADCAM1. It recognizes the sequence L-D-T in MADCAM1. On activated endothelial cells integrin VLA-4 triggers homotypic aggregation for most VLA-4-positive leukocyte cell lines. It may also participate in cytolytic T-cell interactions with target cells	TAM and survival in cHL paper NEJM Steidl 2010 – genes overexpressed in treatment failure
ITGB1¶¶	http://www.genecards.org/cgi-bin/carddisp.pl?gene=ITGB1&search=ITGB1 Integrins are heterodimeric proteins made up of alpha and beta subunits. At least 18 alpha and 8 beta subunits have been described in mammals. Integrin family members are membrane receptors involved in cell adhesion and recognition in a variety of processes including embryogenesis, hemostasis, tissue repair, immune response and metastatic diffusion of tumor cells. This gene encodes a beta subunit. Multiple alternatively spliced transcript variants which encode different protein isoforms have been found for this gene. (provided by RefSeq, Jul 2008)	(Steidl, Lee, et al. 2010) genes overexpressed in treatment failure.

* **Apoptosis associated genes**
** **EBV associated genes**
§ **M1 genes**
§§ **M2 genes**
¶ **TAM C Gregory genes**
¶¶ **cHL treatment failure genes**